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Penfluridol synergizes with colistin to reverse colistin resistance in Gram-negative bacilli

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The growing prevalence of antibiotic resistance in multidrug-resistant Gram-negative bacteria (MDR-GNB), exacerbated by the misuse of antibiotics, presents a critical global health challenge. Colistin, a last-resort antibiotic for severe MDR-GNB infections, has faced diminishing efficacy due to the emergence of colistin-resistant (COL-R) strains. This study evaluates the potential of penfluridol (PF), an antipsychotic drug with notable antibacterial and antibiofilm properties, to restore colistin activity against COL-R GNB *in vitro*. PF alone exhibited limited antibacterial activity against COL-R GNB; however, its combination with colistin demonstrated strong synergistic effects, significantly reducing colistin's minimum inhibitory concentrations (MICs) by 4-128 times. Time-kill assays confirmed the combination's superior bactericidal activity compared to either agent alone. Membrane permeability assays revealed that PF enhanced colistin's ability to disrupt bacterial membranes, likely by facilitating colistin binding to lipopolysaccharide. Furthermore, PF significantly inhibited the development of colistin resistance over a 30-day resistance development assay. In addition to its antibacterial effects, PF exhibited notable antibiofilm activity. The combination of PF and colistin effectively inhibited biofilm formation and eradicated mature biofilms in most of the tested COL-R GNB strains. These findings mark the first report of PF's synergistic interaction with colistin against GNB biofilms, offering a promising strategy to combat biofilm-associated infections. Overall, the colistin/PF combination holds potential as an effective therapeutic strategy to enhance colistin efficacy, delay resistance development, and manage biofilm-associated infections in MDR-GNB.

Keywords Gram-negative bacteria, Colistin resistance, Synergistic effect, Penfluridol, Antibiofilm, Drug repurposing

Global public health faces an escalating threat from antibiotic resistance[1], resulting in ineffective treatments, especially for MDR-GNB. Human behaviors like antibiotic overuse/misuse contribute, while microbial genomic plasticity and genetic exchange enable resistance[2–4]. The World Health Organization (WHO) published an updated list of bacterial priority pathogens in 2024, which includes carbapenem-resistant Enterobacterales and *Acinetobacter baumannii* in the critical group, and carbapenem-resistant *Pseudomonas aeruginosa* in the high-priority group[5]. These carbapenem-resistant pathogens exhibit significant resistance to many antibiotics. Colistin (polymyxin E) is among the few antibiotics that considered as a last resort drug for the treatment of infections caused by MDR-GNB[6]. Nevertheless, the rise of colistin-resistant (COL-R) strains has significantly restricted available antibiotic treatment options[7]. As the development and clinical implementation of new antibiotics lag behind the growing prevalence of resistant pathogens, there is an urgent need for alternative strategies that can reduce the emergence of resistant pathogens or improve the efficacy of colistin. One promising approach is drug repurposing, which involves exploring new uses for existing medications and has recently garnered renewed interest[8]. Apparently, drug repurposing holds significant potential for clinical use, and this approach can lead to the discovery of new antimicrobial agents. Several studies suggest that combining FDA-approved non-antibiotic drugs with colistin could offer an effective therapeutic strategy[9,10].

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Approximately 80% of bacterial infections are associated with biofilms, the most resilient form of bacterial aggregation[11]. Biofilms are complex, stationary communities of bacteria that adhere to surfaces or form clusters within an extracellular matrix. These biofilm-related pathogens are particularly challenging to treat because they can progress from acute to chronic stages, resulting in serious complications[12]. Intriguingly, the substantial evidence shows that biofilm formation is closely linked to antibiotic resistance, as it limits antibiotic penetration and alters the resistance phenotype and microenvironment[13]. GNB are a leading cause of biofilm-associated infections, especially those involving medical devices[14]. Of note, there has been a concerning increase in antibiotic-resistant GNB that are capable of forming biofilms, particularly among pathogens associated with healthcare settings[15]. Due to their inherent resistance, biofilms are often inadequately addressed by conventional antibiotic therapies[16]. Currently, there are no specific medications targeting bacterial biofilms in clinical trials. Therefore, developing effective antibiofilm treatments is essential for reducing and eliminating infections caused by biofilms.

Penfluridol (PF) is a first-generation, oral long-acting antipsychotic approved by the U.S. Food and Drug Administration for treating acute psychosis, schizophrenia, and Tourette syndrome[17]. It exerts its effects by blocking postsynaptic D2 dopamine receptors. Beyond its psychiatric property, a previous study showed that PF has significant antibacterial properties, effectively inhibiting biofilm formation and eradicating preformed biofilms of *Enterococcus faecalis*. PF also showed partial synergistic antimicrobial effects against *E. faecalis* in combination with penicillin G, gentamicin and amikacin[18]. Furthermore, PF demonstrated strong bactericidal activity inhibiting biofilm formation and eliminating preformed biofilms against methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* (MSSA and MRSA). Additionally, PF exhibited the synergistic activity when combined with tetracycline or polymyxin B nanopeptide (PMBN) against MRSA. Notably, no antibacterial activity was observed for PF against GNB, including *E. coli*, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa*[19]. Intriguingly, it remains unclear whether PF can be used in combination with colistin to treat Col-R GNB. To the best of our knowledge, the synergistic antibacterial and antibiofilm activities of the colistin and PF against colistin-resistant GNB have not been investigated previously.

In this study, a series of *in vitro* experiments designed to assess the synergistic antibacterial and antibiofilm effects of colistin and PF on clinical isolates of COL-R GNB with different colistin resistance mechanisms. A flow chart of all experiments is presented in Figure S1. Our results indicate that PF can potentiate the sensitivity of colistin against GNB *in vitro*. Compared to colistin alone, the combination of colistin and PF significantly enhanced colistin's antibacterial activity, reduced bacterial biofilm formation and effectively eradicated preformed mature biofilms. Additionally, synergistic mechanism between these two drugs was explored. The results revealed that PF enhanced the efficiency of colistin to alter bacterial outer and inner membrane permeability. Notably, the combination also suppressed the resistance development to colistin in mostly tested GNB strains. Overall, this study unveiled the great potential of PF as a colistin adjuvant, offering a promising new treatment strategy for tackling COL-R bacterial infections in the future.

Materials and methods

Ethical approval

The research was conducted in accordance with Biosafety guidelines and approved by the Mahidol University Institutional Biosafety Committee (MU-IBC), Mahidol University (Nakhon Pathom, Thailand) [MU 2022-016]. This study, involving anonymized clinical isolates, did not involve interaction with human subjects or access to identifiable private information. The Mahidol University Central Institutional Review Board (MU-CIRB), Mahidol University (Nakhon Pathom, Thailand), waived the requirement for IRB review.

Bacterial strains and reagents

In this study, a total of 16 non-duplicate COL-R GNB isolates were used. Specifically, 5 and 8 isolates of COL-R *E. coli* and *K. pneumoniae*, respectively were selected from clinical isolates in our previous studies[20,21]. Additionally, 1 and 2 isolates of COL-R *P. aeruginosa* and *A. baumannii*, respectively were obtained from the bacterial repository of the International Center for Medical and Radiological Technology, Faculty of Medical Technology, Golden Jubilee Medical Center, Mahidol University. *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, *P. aeruginosa* ATCC 27853, and *A. baumannii* ATCC 17978 served as control strains. PF and colistin sulfate were purchased from MedChemExpress (Monmouth Junction, NJ, USA) and Chem-Impex International (Wood Dale, IL, USA), respectively. Other antibiotics were acquired from Tokyo Chemical Industry (Tokyo, Japan). All culture media were obtained from Becton Dickinson (Frankin Lakes, NJ, USA). Other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Colistin solutions were prepared in sterile Milli-Q water before the experiments. PF stock solutions were freshly prepared in dimethyl sulfoxide (DMSO) at a concentration of 5.12 mg/mL and diluted in culture medium as needed prior to experimentation.

Determination of colistin resistance mechanisms by gene amplification and sequencing

The possible contribution of *pmrAB*, *mgrB*, *phoPQ*, *lpxACD*, and *mcr-1* to the colistin resistance in *E. coli*, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* clinical isolates was investigated by PCR and sequencing analyses. DNA samples were extracted from the isolates using the TIANamp Bacteria DNA Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. The genes were amplified using the primers listed in Table 1. The amplified DNA fragments were purified with the Nucleospin™ gel and PCR clean-up kit (Macherey-Nagel, Duren, Germany) and subjected for sequencing (ATGC Co., Ltd., Bangkok, Thailand). The obtained DNA sequences of genes were translated to amino acid sequences using Expasy translate tool[22]. The nucleotide and deduced protein sequences were analyzed using Clustal Omega tools[23]. The amino acid substitutions in proteins associated with the colistin resistance phenotype of isolates were defined by comparing the query sequences against *E. coli* K-12 MG1655 (accession number NZ_CP010445.1), *K. pneumoniae* Kp52.145 (accession number

Species	Gene	Primer	Sequence (5′ to 3′)	Amplicon size (bp)	References
All species	*mcr-1*	mcr-1-F	GTG TGG TAC CGA CGC TCG G	460	[34]
		mcr-1-R	CAA GCC CAA TCG GCG CAT C		
E. coli	*pmrA* (*basR*)	basR-EC-F	GAG ACG AAG TAT TAC CAG GC	790	This study
		basR-EC-R	CGA TGG TCA ATA TCA GCC GT		
	pmrB	pmrB-EC-F	CTG GAA GTG CAT ATC CAC AA	1,277	
		pmrB-EC-R	GAG AGT GCA ATG AAA AAC CG		
	phoP	phoP-EC-F	CAC ATA ATC GCG TTA CAC TA	803	
		phoP-EC-R	GTT GCC AAC AGA AAA CGT AC		
	phoQ	phoQ-EC-F	CAA GAA GTG ATT ACC ACC GT	1,566	
		phoQ-EC-R	CTG CAA CCG ATT ATA ACG GA		
	mgrB	mgrB-EC-F	GTG CTA TCA GCA TAA CTG TG	466	
		mgrB-EC-R	GAT ACA ACC AAA GAC GCA AT		
K. pneumoniae	*pmrA*	pmrA-KP-F	TAT TTC CGC GCA CTG TCT GC	851	
		pmrA-KP-R	CAG CTT TCA GTT GCA AAC AG		
	pmrB	pmrB-KP-F	ACC TAC GCG AAA AGA TTG GC	1,214	[35]
		pmrB-KP-R	GAT GAG GAT AGC GCC CAT GC		
	phoP	phoP-KP-F	GAG CGT CAG ACT ACT ATC GA	740	This study
		phoP-KP-R	GGG AAG ATA TGC CGC AAC AG		[35]
	phoQ	phoQ-KP-F	ATA CCC ACA GGA CGT CAT CA	1,597	
		phoQ-KP-R	CAG GTG TCT GAC AGG GAT TA		
	mgrB	mgrB-KP-F	TTA AGA AGG CCG TGC TAT CC	253	[36]
		mgrB-KP-R	AAG GCG TTC ATT CTA CCA CC		
A. baumannii	*pmrA*	pmrA-AB-F	CCT CAA CTG GAC ATG TTG	806	This study
		pmrA-AB-R	CTG AAA ATT GAG GTG CCC		
	pmrB	pmrB-AB-F	TTT TAT CCG AAC CAT CCG	1,410	
		pmrB-AB-R	TCA CGC TCT TGT TTC ATT		
	lpxA	lpxA-AB-F	CTG AGT TGG TAA TGC AGA AG	1,038	
		lpxA-AB-R	CTG TGT CAG CAA ATC AAT AC		
	lpxC	lpxC-AB-F	GCG TTC TTC GCC AAT GAG TA	1,217	[37]
		lpxC-AB-R	CGACCCTAAGCTTAGCCAA		
	lpxD	lpxD-AB-F	GAC TGT TGC CTA TGA CGC TA	1,215	
		lpxD-AB-R	CGA ATC GTT TGA ATC TGC AT		This study
P. aeruginosa	*pmrA*	pmrA-PA-F	CTG AGC CTG TGG GAA TTG TG	734	
		pmrA-PA-R	GAC TCC GGT AGG CAG TTT TC		
	pmrB	pmrB-PA-F	CAA CCA GTT GAT CCG CAC CG	1,565	
		pmrB-PA-R	GTT GCG AAG CCG CTA GCC TA		
	phoP	phoP-PA-F	CGC CAA CTA CAA GTT CTA AA	1,139	
		phoP-PA-R	GCC ATT CTG GTC GTA GAT AT		
	phoQ	phoQ-PA-F	CTT CAA GCC GAT CGA TAC GG	1,441	
		phoQ-PA-R	CGG AAA CAC CTC AGA CGT AG		
	lpxA	lpxA-PA-F	GGT ATG CTC GGC TGA AAT CA	841	
		lpxA-PA-R	CTA CGC GTA ATC CGT CAG CC		
	lpxC	lpxC-PA-F	GAG GTG GCC TCC AAA TAT GG	987	
		lpxC-PA-R	GTT GAA AAC AGT TCG CAG TC		
	lpxD	lpxD-PA-F	CAA GCC GCA ATA CGA CAT CA	1,182	
		lpxD-PA-R	CTC GTT GAT GTC CAT CAT GT		

Table 1. List of primers used in this study.

FO834906.1), *A. baumannii* ATCC 19606 (accession number HM149345.1) and *P. aeruginosa* PAO1 (accession number AE004091.2).

Minimal inhibitory concentration (MIC) assay

Antibacterial activity of drugs was determined by the broth microdilution method according to the CLSI 2021 guidelines[24]. Briefly, compounds were two-fold serial dilution with cation-adjusted Mueller-Hinton II broth (CAMHB). Next, log-phase bacterial cultures were adjusted to 0.5 McFarland standard and diluted 1:100 in CAMHB to achieve a final concentration of 10^6 CFU/mL, then mixed with compounds in a 96-well U-bottom

microplate (Wuxi NEST Biotechnology, Jiangsu, China). Plates were incubated at 37 °C for 20–24 h, and the MIC values were detected as the lowest concentration of drugs with no visible bacterial growth.

Checkerboard assay

Synergistic activity of drugs was evaluated by checkerboard assay as previously described with slight modifications[25]. In brief, the antibiotic of interest was 2- fold serially diluted along the x-axis, whereas the PF was 2-fold serially diluted along the y-axis in a 96-well microliter plate to create a matrix where each well consisted of a combination of both agents at different concentrations. Bacterial suspensions from log-phase cultures were diluted to 0.5 McFarland turbidity, followed by 1:100 further dilution to 10^6 CFU/ mL in CAMHB, then inoculated on each well. After 20–24 h incubation at 37˚C, the MICs of each antibiotic or PF alone and in combination were determined. The fractional inhibitory concentration index (FICI) was calculated according to the formula: FICI = FIC(drug A) + FIC(drug B), where FIC = the MIC of the drug when in the combination/ MIC of drug tested individually. Synergy is defined as an FIC index 0.5. FICI ≤ 0.5 denotes synergy, FICI > 0.5-4 denotes no interaction and FICI > 4 denotes antagonism[26].

Time-kill assay

The time-kill assay was performed to further assess the synergistic effect of colistin and PF with minor modifications[27]. Briefly, 7 isolates were selected as the experimental strains: COL-R *E. coli* ($n = 2$), *K. pneumoniae* ($n = 2$), *A. baumannii* ($n = 2$), and *P. aeruginosa* ($n = 1$). Drug concentrations were selected based on checkerboard assay results. The log-phase cultures of bacteria were diluted to a final concentration of 10^5 CFU/mL in sets of tubes, then were incubated at 37˚C with either colistin, PF or combination and without any drug as the normal growth control. Aliquots were obtained at time intervals of 0, 1, 2, 4, 6, 8 and 24 h post-inoculation, and serially diluted in saline for determination of viable counts. Diluted samples were plated on MHA plates, and bacterial colonies were counted from the plates after incubation for 16–18 h at 37˚C. Killing curves were depicted by plotting viable counts against time. For the two-drug combination compared with either drug alone, a reduction in the number of colonies of 2 log10 by 24 h was considered as a synergistic action[28].

Outer membrane permeability assay

The outer membrane permeability of COL-R GNB treated with either colistin, PF or combination was investigated using the N-phenyl-1- Naphthylamine (NPN) uptake assay as previously described with a few modifications[29]. Initially, overnight cultures of selected COL-R strains were diluted 100-fold in CAMHB medium and grown to the mid-exponential phase. Cultures were treated with 0, 1/4 MIC colistin, 8 µg/mL PF and combination for 2 h. The cells were harvested by centrifugation, washed twice with assay buffer (5 mM HEPES, 5 mM glucose, pH 7.4) and resuspended in assay buffer to a final $OD_{600} = 0.2$. NPN was dissolved in acetone at a concentration of 50 mM and then diluted in HEPES buffer to make 50 µM working stock. Next, 40 µL of NPN and 160 µL of cells were mixed in a 96-well optical-bottom black plate (SPL Life Sciences, Pocheon-si, South Korea) to give a final NPN concentration of 10 µM and fluorescence was measured using a microplate reader (Ensight™, Multimode, Perkin Elmer, Waltham, MA, USA) at an excitation wavelength and emission wavelength, 350 and 420 nm respectively. Cell suspension without NPN served as a control for subtracting the background fluorescence.

Inner membrane permeability assay

The plasma membrane integrity of COL-R GNB treated with either colistin, PF or combination was evaluated by propidium iodide (PI) assay as describe previously with modifications[30]. Briefly, the mid-log phase cultures were treated with 0, 1/4 MIC colistin, 8 µg/mL PF and combination for 2 h. The cells were harvested by centrifugation, washed twice with assay buffer (10 mM PBS, pH 7.4) and resuspended in assay buffer to 0.2 at OD_{600}. The PI fluorescent dye was dissolved in PBS buffer at a concentration of 50 µg/mL and 40 µL of PI was added to 160 µL of cells in a 96-well optical-bottom black plate to give a final PI concentration of 10 µg/mL. The fluorescence intensity was measured using a microplate reader at an excitation wavelength of 488 nm and emission wavelength of 630 nm. The heat-treated (2 h at 80 °C) cells were used as the control for the inner membrane damaged cells.

Resistance development assay

The potential for resistance development of a combination of drugs was determined according to a reported protocol[31]. Concisely, the COL-S and COL-R strains were subjected to an MIC assay on the first day with either colistin or combination of colistin and PF. Subsequently, bacterial cells growing at 1/2 MIC were harvested after 24 h of incubation at 37˚C and another MIC assay was then conducted using this inoculum. The experiment was carried out consecutively for 30 days as described above. The MICs were recorded and plotted as the MIC (µg/ mL) compared to the first passage of the experiment.

Biofilm formation Inhibition assay

Crystal violet staining was employed to assess the inhibition of biofilm formation, as previously described with some modifications[32]. The 7 Col-R isolates (2 *E. coli*, 2 *K. pneumoniae*, 2 *A. baumannii* and 1 *P. aeruginosa*) were tested. Bacterial overnight cultures were adjusted to a 0.5 McFarland standard in CAMHB supplemented with 0.5% glucose before being dispensed in a 96-well U-bottom microplate with various concentrations of PF and 2 µg/mL colistin alone or in combination. After a 24-h incubation at 37 ˚C, free-floating planktonic cells were removed by washing twice with sterile deionized water. The biofilms were fixed with absolute methanol for 10 min, and a plate was left to air dry. Afterward, wells were stained with 1% (w/v) crystal violet for 30 min. The excess stain was then thoroughly rinsed away with deionized water, and the plate was allowed to dry. Once dry, 95% ethanol was added into wells to solubilize the stain. The optical density (OD) was measured by a microplate reader at 595 nm. The percentage of biofilm mass was calculated based on the growth control.

Mature biofilm eradication assay

The eradication effect of PF combined with colistin on mature biofilm was investigated using a previously described protocol with slight modifications[33]. Briefly, bacterial suspensions were prepared as described above and added to each well of a 96-well U-bottom plate. After 24 h of static incubation at 37 °C to allow mature biofilm formation, the supernatant was discarded, and the plate was washed three times with sterile deionized water to remove unattached cells. Then, 100 µL of fresh CAMHB supplemented with 0.5% glucose containing various concentrations of PF and either 2 µg/mL colistin alone or in combination, was added to each well. The broth medium without drugs was used as the control. Next, the plate was incubated for 24 h at 37 °C. The crystal violet staining and subsequent treatments were carried out as mentioned previously.

Statistical analysis

All experiments were carried out with at least three independent assays. All quantitative results were presented as mean ± standard deviation (SD). The statistical analysis was performed by GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA), and the data were analyzed by one-way analysis of variance (ANOVA) followed by multiple comparison using Tukey's test, and a p-value of < 0.05 was considered significant.

Results

MIC determination and colistin resistance mechanisms

The MICs of all isolates against either colistin and PF alone are showed in Table 2. All isolates were resistant to colistin (COL) with MICs ≥ 4 µg/mL. The result showed that PF exhibited weak or no antibacterial activity against all tested COL-R GNB (MIC > 64 µg/mL). The colistin resistance genes in the COL-R GNB isolates were amplified by PCR and determined their sequences by DNA sequencing. Analysis of the amino acid sequences showed that no amino acid alterations in PhoP and PhoQ were determined in all *K. pneumoniae* and *P. aeruginosa* isolates. One amino acid substitution in PhoP (I44L) was detected among most *E. coli* isolates. Notably, it has been previously reported that PhoPQ two-component system (TCS) is absent in *A. baumannii* genomes[38]. The substitutions in both PmrA and PmrB were detected in the majority of colistin-resistant *K. pneumoniae* isolates. The substitution in either PmrA (E57G) or PmrB (R256G) alone was determined in KP-07 and KP-04, respectively. The substitution in PmrA (E57G) and one nucleotide substitution causing a premature stop codon were found in the *mgrB* gene of KP-03. No mutation in all tested genes was determined in one isolate, KP-10, of COL-R *K. pneumoniae*. Additionally, the similar mutations in PmrB (A138T), LpxA (Y131H), LpxC (N287D), LpxD (E117K) were determined in both COL-R *A. baumannii* isolates. The substitutions in PmrA (T31I), PmrB (Y345H, A462T), LpxD (V20L) were detected in a COL-R *P. aeruginosa* isolate. Of note, *mcr-1*, the mobile colistin-resistance gene, was present in all COL-R *E. coli* strains, but no detection of this gene in other species. All amino acid substitutions in colistin-resistance genes of COL-R isolates are summarized and the mechanisms of colistin resistance were identified as shown in Table 2.

Synergistic activity of colistin and PF in checkerboard assay

The potential synergistic effect of PF in combination with colistin was evaluated against COL-R GNB. As presented in Table 3, the combination of PF with colistin showed synergistic effect against all tested isolates, with FICI < 0.5, and the synergistic activity was independent of the resistance mechanism. Evidently, the combination of colistin with PF caused a significant change in the sensitivity of most COL-R isolates toward colistin,

Species	Isolate number	Colistin resistance mechanism	MIC colistin (µg/mL)	MIC PF (µg/mL)
E. coli	EC-02	*mcr-1*, PmrA (S29G, G144S), PmrB (D283G, Y358N), PhoP (I44L)	4	> 64
	EC-04	*mcr-1*, PmrA (S29G, G144S), PmrB (D283G, Y358N), PhoP (I44L)	4	> 64
	EC-05	*mcr-1*, PmrA (S29G), PmrB (D283G, Y358N), PhoP (I44L)	4	> 64
	EC-07	*mcr-1*, PmrA (S29G)	8	> 64
	EC-09	*mcr-1*, PmrA (S29G), PmrB (D283G, Y358N), PhoP (I44L)	16	> 64
K. pneumoniae	KP-03	PmrA (E57G), MgrB (truncated protein, 29 aa)	32	> 64
	KP-04	PmrB (R256G)	16	> 64
	KP-07	PmrA (E57G)	64	> 64
	KP-08	PmrA (E57G) and deletion (452–461), PmrB (D150Y)	128	> 64
	KP-10	Unidentified	128	> 64
	KP-11	PmrA (E57G) and deletion (452–461), PmrB (D150Y)	128	> 64
	KP-13	PmrA (E57G) and deletion (452–461), PmrB (D150Y)	256	> 64
	KP-15	PmrA (E57G), PmrB (T157P)	32	> 64
A. baumannii	AB-01	PmrB (A138T), LpxA (Y131H), LpxC (N287D), LpxD (E117K)	16	> 64
	AB-02	PmrB (A138T), LpxA (Y131H), LpxC (N287D), LpxD (E117K)	8	> 64
P. aeruginosa	PA-01	PmrA (T31I), PmrB (Y345H, A462T), LpxD (V20L)	4	> 64

Table 2. Characteristics of GNB isolates used in this study.

Species	Isolate number	MIC of monotherapy (μg/mL)		MIC of combination (μg/mL)		FICI	Interpretation
		COL	PF	COL	PF		
E. coli	EC-02	4	>64	0.25	8	<0.125	Synergy
	EC-04	4	>64	0.25	4	<0.094	Synergy
	EC-05	4	>64	0.125	16	<0.156	Synergy
	EC-07	8	>64	0.25	8	<0.094	Synergy
	EC-09	16	>64	0.25	8	<0.078	Synergy
K. pneumoniae	KP-03	32	>64	0.5	4	<0.047	Synergy
	KP-04	16	>64	0.5	4	<0.063	Synergy
	KP-07	64	>64	0.5	4	<0.039	Synergy
	KP-08	128	>64	4	8	<0.094	Synergy
	KP-10	128	>64	2	4	<0.047	Synergy
	KP-11	128	>64	1	4	<0.039	Synergy
	KP-13	256	>64	4	4	<0.047	Synergy
	KP-15	32	>64	4	8	<0.188	Synergy
A. baumannii	AB-01	16	>64	0.5	2	<0.047	Synergy
	AB-02	8	>64	0.5	2	<0.078	Synergy
P. aeruginosa	PA-01	4	>64	1	8	<0.313	Synergy

Table 3. FICI values for colistin/PF combinations against COL-R GNB.

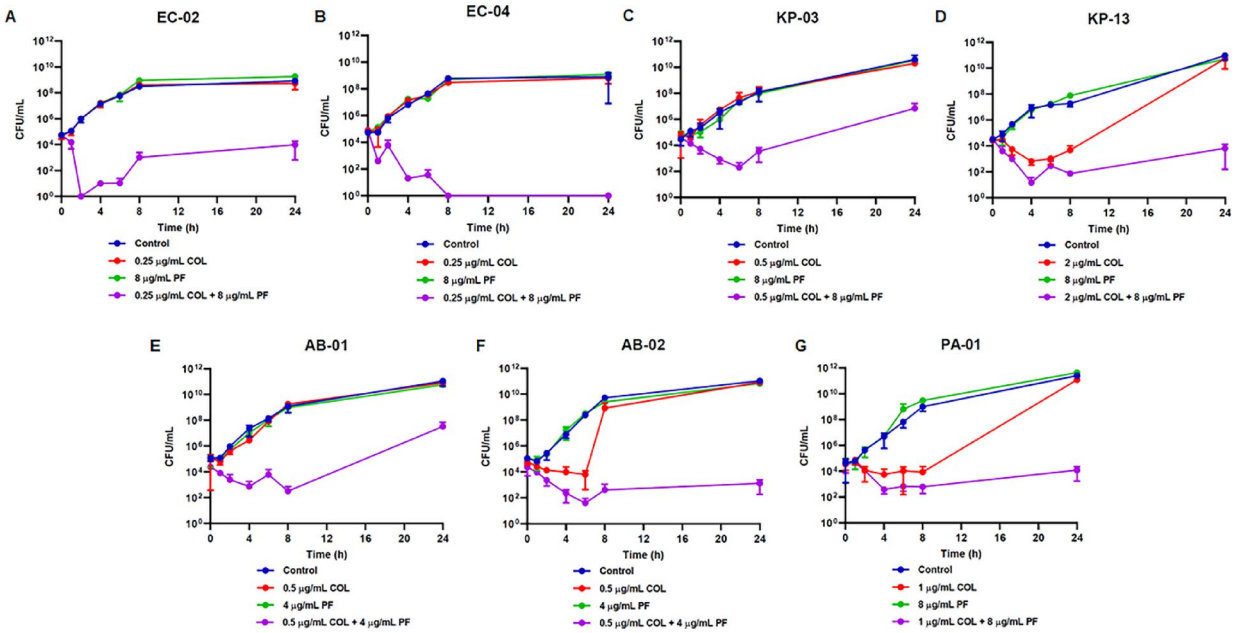

Fig. 1. Time-kill curves of colistin (COL) and PF alone or in combination against COL-R GNB. (**A**, **B**) COL-R *E. coli* strains EC-02 and EC-04; (**C**, **D**) COL-R *K. pneumoniae* strains KP-03 and KP-13; (**E**, **F**) COL-R *A. baumannii* strains AB-01 and AB-02; (**G**) COL-R *P. aeruginosa* strain PA-01.

resensitizing their drug-resistant phenotype to a sensitive phenotype. It is worth noting that all combinations of PF with other antibiotics, meropenem, ciprofloxacin, ceftazidime and chloramphenicol, showed no synergistic effect on all COL-R GNB strains as reported in Table S1-S4.

Synergistic activity of colistin and PF in time-kill assay

Time-kill assay against seven COL-R GNB isolates, including EC-02, EC-04, KP-03, KP-13, AB-01, AB-02, and PA-01 strains, was conducted to further verify the synergistic effect of colistin and PF. The drug concentrations used for time-kill analysis were obtained from the results of checkerboard assay, with FICI < 0.5. As shown in Fig. 1, bacteria exposed to each drug alone demonstrated killing curves comparable to the control cultures not exposed to drugs. In contrast, the colistin/PF combinations led to faster bacterial killing, with all strains showing

a > 2 log10 reduction in viable cells (CFU/ml) by 24 h, outperforming either agent alone. Taken together, the bactericidal activity of colistin was significantly enhanced when in combination with PF.

Effect of PF on the potentiation of colistin's membrane-damaging ability

It was hypothesized whether the addition of PF might increase colistin's ability to destroy the bacterial membrane. To validate this hypothesis, the effects of PF or colistin alone, and their combination, on the permeability of the outer and inner membranes of COL-R GNB (EC-04, KP-13, AB-02, and PA-01) were explored. The outer membrane permeabilization was determined using the NPN uptake assay, which reveals increased fluorescence when NPN, a neutral hydrophobic probe, integrates into the membrane. As shown in Fig. 2A-D, colistin at sub-inhibitory concentrations had no significant effect or slightly increased NPN uptake in COL-R strains. In addition, there was no significant increase in fluorescence among the COL-R strains exposed to PF alone. Notably, the fluorescence intensity of NPN increased dramatically when colistin was combined with PF comparing with colistin alone, suggesting that the combination clearly improved the permeability of the outer membrane. Subsequently, the effect of PF on the bacterial inner membrane of COL-R strains was studied using the PI uptake assay. PI is a membrane-impermeable fluorescent probe that binds to the nucleic acids of membrane-damaged bacteria, allowing for the detection of inner membrane integrity. Similarly, treatment with colistin or PF alone did not alter inner membrane permeability, as no significant difference was observed in fluorescence intensity when bacterial cells were exposed to each drug (Fig. 2E-H). Furthermore, the results demonstrated that combined treatment with colistin and PF caused inner membrane disruption, which increased fluorescence intensity. Altogether, these results indicate that PF potentiates colistin activity by enhancing both outer and inner membrane damage.

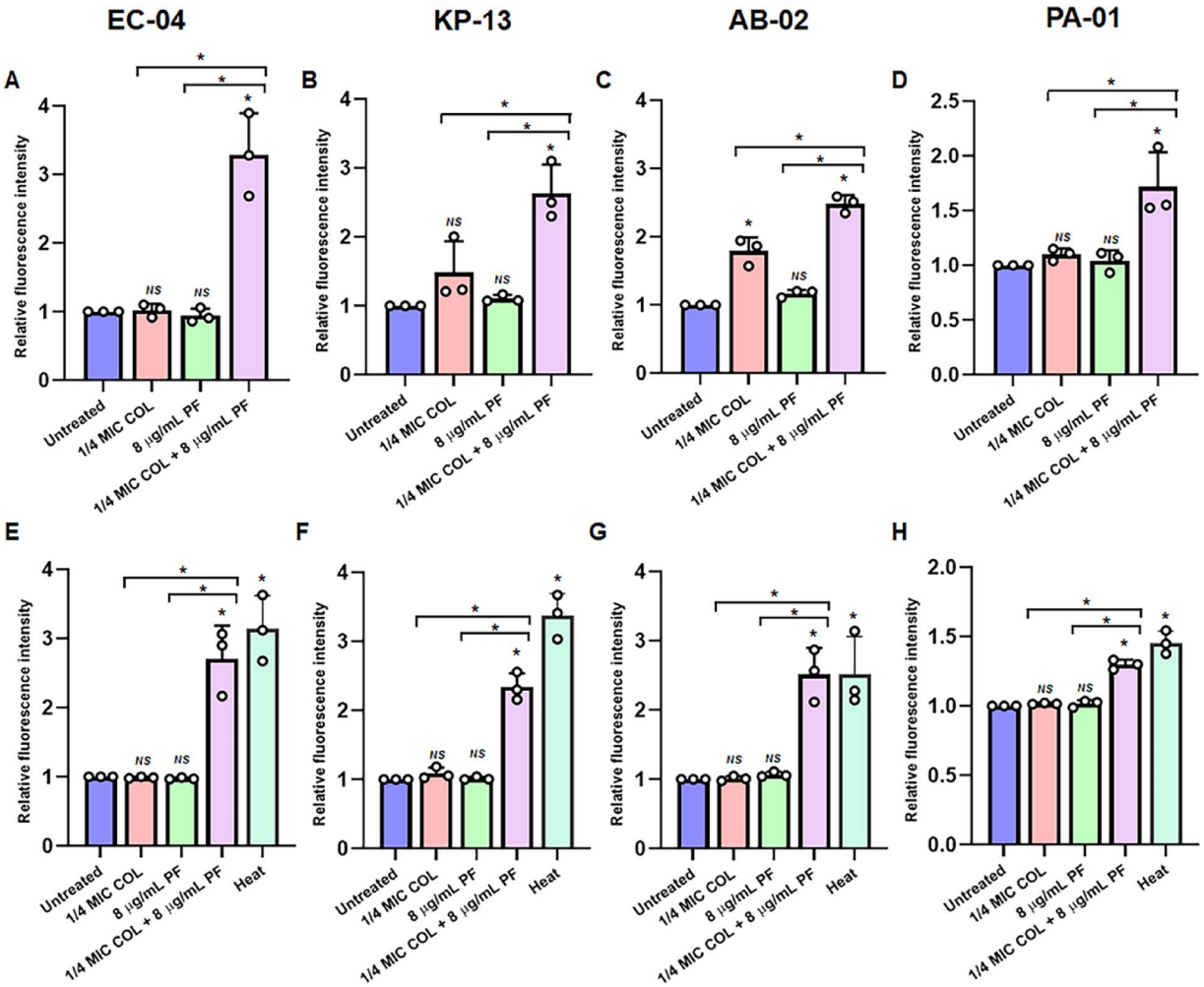

Fig. 2. Effects of COL, PF, or combination treatment on membrane permeability of COL-R GNB. (**A-D**) Permeability of the outer membrane probed with NPN. (**E-H**) Permeability of the inner membrane probed with PI. PF potentiated the damage of colistin to the bacterial membrane in COL-R GNB. The data were analyzed by one-way ANOVA; *p-value < 0.05.

Effect of PF on the reduction of resistance development to colistin

The emergence of resistance to either colistin or the combination of colistin and PF was investigated in GNB. Both COL-S and COL-R strains of *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* were serially passaged in a medium containing colistin, with or without the addition of PF. As demonstrated in Fig. 3A-D, colistin alone significantly increased MICs in COL-R strains after 30 serial passages: 256-fold in EC-04, 32-fold in KP-03, 256-fold in AB-02, and 64-fold in PA-01. PF reduced resistance development in all COL-R GNB. Additionally, the resistance development was also performed in colistin-susceptible strains. The result showed that MICs of colistin increased by 2,048-fold in *E. coli* ATCC 25922 and 4,096-fold in *K. pneumoniae* ATCC 700603, while PF prevented further increases in both strains (Fig. 3E and F). Surprisingly, no significant MIC fold change was observed with colistin alone against the COL-S *A. baumannii* ATCC 17978 and *P. aeruginosa* ATCC 27853 strains over 30 passages, with the MICs not rising above 2-fold and no further increase in the MICs of colistin in the presence of PF (Fig. 3G and H). The MICs of colistin, whether alone or in combination with PF, remained below the CLSI susceptibility breakpoint (≥ 4 μg/mL). Taken together, our results suggest that the presence of PF is an effective approach to suppress and minimize the potential development of colistin resistance.

Impact of the combination of colistin with PF on bacterial biofilm

The effect of colistin, PF, and their combination on biofilm formation in COL-R GNB strains was evaluated. As illustrated in Figs. 4A-G, treatment with 2 μg/mL of colistin alone did not result in a significant reduction in biofilm formation for any of the COL-R strains when compared to untreated controls. Similarly, PF demonstrated no significant inhibitory effect on biofilm formation in COL-R *E. coli* and *A. baumannii* strains. However, PF alone significantly reduced biofilm formation in COL-R *K. pneumoniae* and *P. aeruginosa* strains, with a particularly pronounced effect at higher concentrations (≥ 4 μg/mL). Notably, the combination of colistin and PF was more effective than either agent alone in inhibiting biofilm formation across all tested strains, particularly at PF concentrations ≥ 2 μg/mL. Further investigations also explored the potential of colistin and PF in eradicating preformed mature biofilms. As depicted in Fig. 5A-G, the combination treatment significantly enhanced the eradication of mature biofilms in most strains, compared to untreated controls and monotherapy, in a dose-dependent manner. Nevertheless, the combined treatment did not significantly differ from PF alone in eradicating biofilms of the COL-R *P. aeruginosa* strain. In conclusion, these findings demonstrate that the combination of colistin and PF effectively inhibits biofilm formation and eradicates mature biofilms in COL-R GNB strains.

Discussion

Rising infectious disease burden and antibiotic resistance are predicted to significantly increase deaths from drug-resistant infections[39]. Specifically, antibiotic resistance in MDR-GNB, fueled by drug overuse and misuse, poses significant global health challenges. Colistin remains one of the few effective antibiotics against these pathogens and is considered a last-line treatment for severe infections associated with MDR-GNB[40]. However, its use has contributed to the emergence of Col-R strains[41]. In response to increasing drug resistance and recent treatment failures with colistin monotherapy, the exploration of combination therapies targeting colistin-resistant bacteria has intensified. A promising approach involves using non-antibiotic drugs as adjuvants to restore or enhance antibiotic efficacy, offering a viable solution to combat resistance[42,43]. Repurposing these non-antibiotic drugs as adjuvants provides a more time- and cost-effective alternative to developing new antibiotics, as their combination has a synergistic effect that lowers the MICs of antibiotics and reduces the required dose

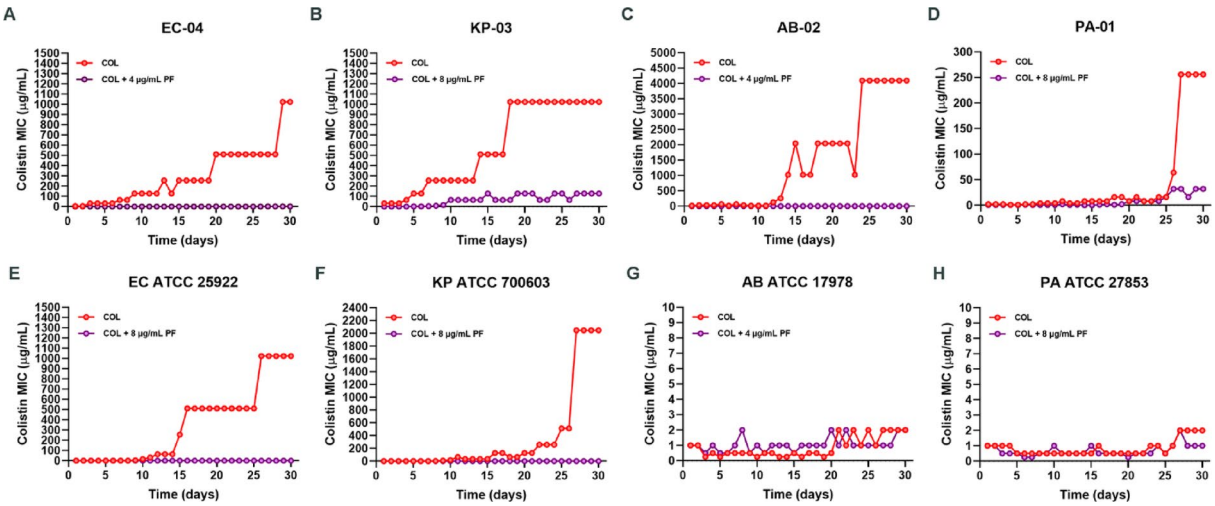

Fig. 3. Emergence of bacterial resistance in COL-R and COL-S GNB; (**A**, **E**) *E. coli* strains EC-04 and ATCC 25922, (**B**, **F**) *K. pneumoniae* strains KP-03 and ATCC 700603, (**C**, **G**) *A. baumannii* strains AB-02 and ATCC 17978, and (**D**, **H**) *P. aeruginosa* strains PA-01 and ATCC 27853 after 30 serial passages of 1/2 MIC COL with or without PF. MIC values shown on the y-axis are those obtained over 30 days.

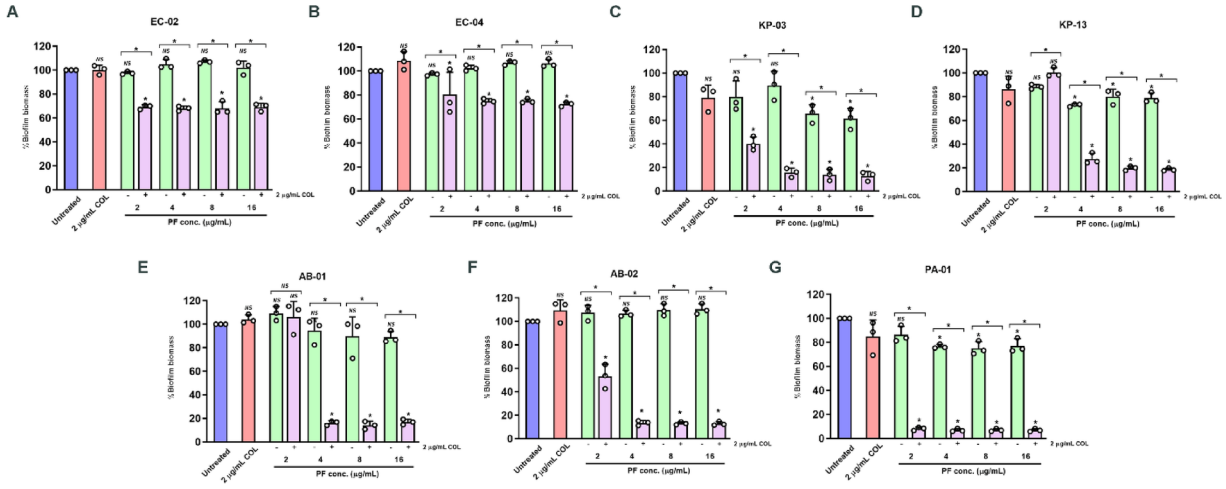

Fig. 4. Inhibitory effect of PF combined with COL on biofilm formation of COL-R GNB; (**A**, **B**) *E. coli* strains EC-02 and EC-04, (**C**, **D**) *K. pneumoniae* strains KP-03 and KP-13, (**E**, **F**) *A. baumannii* strains AB-01 and AB-02, and (**G**) P. *aeruginosa* strain PA-01. The data were analyzed by one-way ANOVA; **p*-value < 0.05.

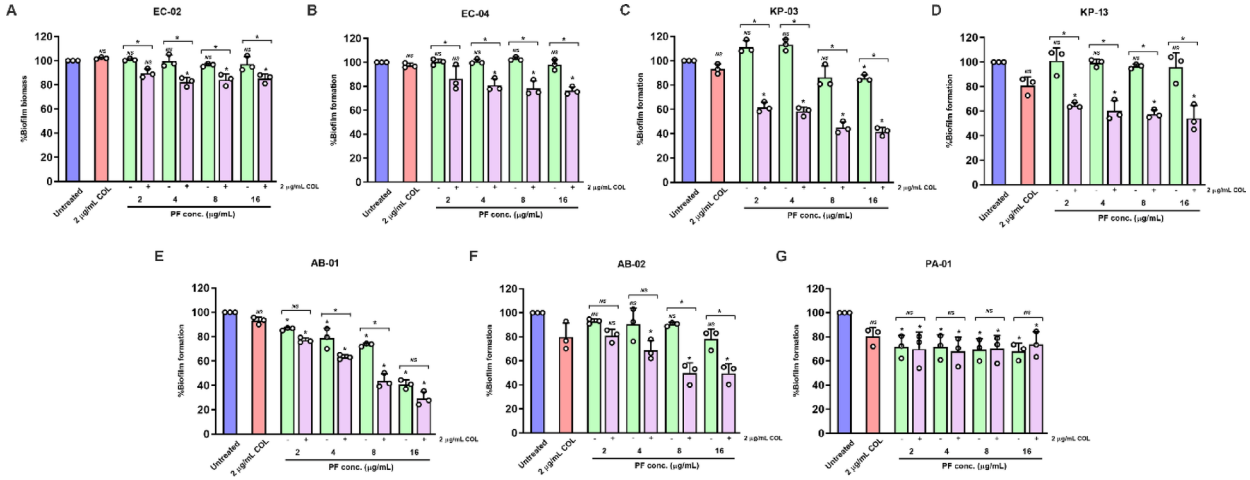

Fig. 5. Eradication effect of PF combined with COL on mature biofilm of COL-R GNB; (**A**, **B**) *E. coli* strains EC-02 and EC-04, (**C**, **D**) *K. pneumoniae* strains KP-03 and KP-13, (**E**, **F**) *A. baumannii* strains AB-01 and AB-02, and (**G**) P. *aeruginosa* strain PA-01. The data were analyzed by one-way ANOVA; **p*-value < 0.05.

for therapeutic success[44,45]. Herein, the potential of PF as an antibiotic adjuvant to restore colistin activity against COL-R GNB *in vitro* was evaluated.

PF, an antipsychotic drug, exhibited several biological functions, such as anticancer, anti-inflammatory, antioxidant, antibacterial and antibiofilm activities[18,19,46,47]. As antibacterial and antibiofilm agents, PF had significant antibacterial and antibiofilm effects against *E. faecalis* and *S. aureus*. Of note, it has been reported that PF had no antibacterial activity against GNB with MICs > 128 μg/mL. This result is in good agreement with our results that the MICs against all COL-R GNB were > 64 μg/mL. All COL-R GNB isolates included in our study exhibited a resistant phenotype toward major classes of antibiotics that were defined as MDR, and some of these were XDR according to international standard definitions for acquired resistance[48]. The colistin resistance mechanisms of these isolates have been determined. Intriguingly, all mutations in colistin resistance genes observed in this study have been previously reported, except for one novel mutation in LpxD (V20L) in PA-01, which was newly identified. Unexpectedly, no mutations were found in any of the tested genes in the COL-R *K. pneumoniae* isolate KP-10. This suggests that colistin resistance mechanisms may be complex and involve additional genes not yet fully identified. Furthermore, the plasmid-mediated *mcr-1* was detected in all COL-R *E. coli* isolates, but not in other species. Although *mcr-1* has been spreading among various Gram-negative species, the prevalence of *mcr-1* is the highest among *E. coli* isolates[49]. Our results of checkerboard assay demonstrated that the combination of PF and colistin had a synergistic effect on all tested strains, regardless of the different colistin resistance mechanisms. Previous studies indicated that PF exhibited additive antimicrobial effects against *E. faecalis* and MRSA when combined with several antibiotics, except for a synergistic effect

with tetracycline and PMBN against MRSA, with FICIs of 0.5 and < 0.375, respectively. Our results suggest that the colistin/PF combination showed a stronger synergistic effect against COL-GNB strains, with a lower FICI. Notably, when colistin was combined with PF, its MIC values of colistin were significantly reduced by 4-128 times[18,19]. The reduced dose of colistin may help minimize its adverse effects[50]. The synergistic activity of colistin/PF combination on all tested COL-R GNB was confirmed with time-kill assays, which provided precise measurements of the bactericidal effects of the combination over time. The results revealed that this combination reduced bacterial counts more effectively than either drug alone. In our study, the combination of PF with various other antibiotics, each representing different mechanisms of action, did not demonstrate any synergistic effect against COL-R GNB strains. Notably, a previous study identified a synergistic interaction between PF and the antibiotic tigecycline against *K. pneumoniae*[51]; however, tigecycline was not included in this study. Taken together, we report for the first time the synergistic activity of PF in combination with colistin against GNB.

The mechanism by which adjuvants enhance colistin activity has been documented to involve an increase in membrane permeability. Several drugs or compounds have demonstrated their own ability to increase the membrane permeability and synergized the membrane destructive activity of colistin[52–55]. On the other hand, some drugs or compounds themselves has minimal influence on the permeability of the outer membrane but they can facilitate the binding of colistin to the outer membrane LPS target, thereby enhancing the antibacterial activity of colistin[56–59]. In this study, we investigated the impact of PF alone and in combination with colistin on bacterial membrane disruption using membrane permeability assays with membrane-impermeable fluorescent probes (NPN and PI). Our results showed that colistin at concentration 1/4 MIC alone was unable to increase membrane permeability in COL-R bacterial strains. Similarly, PF at concentration 8 μg/mL alone also did not increase membrane permeability. However, the combination of PF and colistin caused substantial damage to both the outer and cytoplasmic membranes. This indicates that PF's mechanism of action does not directly affect the membranes of GNB, which contrasts with its action on Gram-positive bacteria (GPB)[19]. This difference might be attributed to variations in the cell membrane structures of GNB and GPB. Colistin's bactericidal action is mainly due to its binding to lipid A in the lipopolysaccharide (LPS) of the bacterial outer membrane, which also affects the permeability of the cytoplasmic membrane. This interaction disrupts both membranes, causing leakage of cellular contents and leading to cell death[60]. In contrast, colistin resistance generally results from alterations to lipid A that prevent colistin binding[61]. Our findings show that combining colistin with PF results in more effective membrane damage than either agent alone, which has minimal impact. We propose that PF may facilitate colistin binding to bacterial cells, thereby enhancing its effectiveness. PF, a highly lipophilic compound, distributes extensively in fatty tissues after oral administration[62]. Its lipid solubility may allow it to bind to the LPS target site on the bacterial outer membrane through hydrophobic interactions, increasing the negative charge on the LPS surface. This, in turn, strengthens the electrostatic interaction between colistin and LPS, enhancing outer membrane permeability and further damaging the plasma membrane.

In the ongoing challenge of antibiotic resistance, bacterial resistance typically increases over time through successive mutations[63]. To investigate this, a resistance development assay was performed to assess potential of PF in inhibiting the emergence and evolution of colistin resistance. After 30 days of continuous passages, we observed that PF significantly attenuated the rise in colistin MICs in all COL-R strains tested, as well as in COL-S *E. coli* and *K. pneumoniae*. However, the protective effect of PF against the evolution of colistin resistance in COL-S *A. baumannii* and *P. aeruginosa* remained unclear, likely due to the low propensity of these strains to develop resistance when exposed to sub-inhibitory concentrations of colistin. In this context, it appears that colistin did not induce drug resistance mutations in either of the COL-S strains after 30 days, suggesting that bacteria were unable to evolve resistance to the multiple deleterious effects of colistin without compromising their physiological fitness. This phenomenon may explain why the prevalence of colistin resistance in *A. baumannii* and *P. aeruginosa* is lower than in Enterobacterales, particularly *K. pneumoniae* and *E. coli*[64–67]. Overall, our study indicates that a drug regimen combining these two agents could effectively prevent the development of resistance in GNB.

Biofilm formation confers antibiotic resistance, rendering most antibiotics ineffective. Consequently, biofilm-associated bacterial infections are difficult to manage and a leading cause of persistent infections[68]. Therefore, this study also investigated the antibiofilm effects of PF and colistin. The biofilm formation inhibition and eradication tests demonstrated that the combination of PF and colistin effectively inhibited biofilm formation and eradicated mature biofilms in most of the tested COL-R GNB strains. The antibiofilm mechanism of the combination may warrant further exploration. Notably, the biofilm-forming ability of COL-R *K. pneumoniae* and *P. aeruginosa* strains was decreased in the presence of PF alone, suggesting that PF inhibited biofilm formation. Interestingly, the combined treatment did not show a significant enhancement in eradicating mature biofilms of the COL-R *P. aeruginosa* strain compared to PF treatment alone. However, the antibiofilm effect of PF on COL-R *P. aeruginosa* may be limited relative to other COL-R strains, as only one strain was tested. Previous studies reported that PF effectively inhibited biofilm formation and eradicated biofilms in *E. faecalis* and *S. aureus*, which are GPB[18,19]. However, there is no report of PF on the biofilm inhibition and eradication effects against GNB as well as when combined antibiotics with PF. This is the first study to report on the effect of colistin/PF on GNB biofilms. Our results are promising, suggesting that the colistin/PF combination can inhibit biofilm formation at an early stage and eradicate established biofilms.

PF has demonstrated low cytotoxicity across various mammalian cell lines. Specifically, human bronchial epithelial (HBE) and human lung adenocarcinoma (A549) cell lines showed negligible cytotoxicity at 32 μg/mL, a concentration significantly above the observed MIC against MRSA, which ranged from 4 to 8 μg/mL[19]. Furthermore, human brain microvascular endothelial cells (HBMEC) showed no significant death at the MIC. Additionally, *in vivo* toxicity studies have shown that PF did not cause mortality in mice after intraperitoneal injection of 20 mg/kg PF for 7 days. This dose regimen has also been shown to be effective in reducing bacterial load in the liver, kidney, and spleen in mice infected with MRSA[19], and in a separate study, with *E. faecalis*[18].

Clinically, a single weekly oral dose of 30 mg (~ 12.5 to 25 mg/kg for mouse model) has been used to treat acute psychoses in patients without reported side effects[69]. Long-term administration of 10 mg/kg PF intragastrically for 55 days in mice also did not result in significant behavioral side effects or toxicity[70]. Notably, PF has also been investigated as an anticancer agent, with doses of 50 mg/day being used in preclinical models[71]. While neurological side effects such as epilepsy, fatigue, dyskinesias, parkinsonism, akathisia, dystonia, and depression are more likely to occur at these higher doses, the doses used for antibacterial applications are significantly lower, minimizing this risk. Therefore, PF has low cytotoxicity as well as reasonable pharmacokinetic properties. However, we acknowledge that the administered dose of PF should be carefully considered, particularly when using higher doses. To overcome this limitation, the structure of PF could be optimized in the future to reduce its cytotoxicity at high doses before clinical application. Recent development of PF analogs as anticancer drugs has shown success in reducing antipsychotic effects[71].

Clinical research suggests that the optimal dosage of PF ranges from 40 to 80 mg/week[46] and the half-life is typically reported to be in the range of 66 h[69]. From a treatment perspective, PF's long-lasting efficacy and weekly dosing offer advantages in terms of enhanced patient compliance and reduced drug consumption[62]. Because of the lipophilic property of PF, a prolonged half-life allows for the maintenance of therapeutic drug concentrations for extended periods, maximizing the drug's effectiveness against target bacteria, particularly in infections within tissues. However, we also acknowledge that PF's long half-life raises concerns about potential drug accumulation and prolonged side effects[72]. This is a valid concern that requires further investigation.

Taken together, the combination of PF and colistin significantly enhanced the sensitivity of COL-R GNB to colistin and effectively disrupted biofilms. These findings highlight PF's potential to restore bacterial sensitivity to colistin, preserving its role as a last-resort antibiotic for treating MDR Gram-negative infections. However, our study has limitations. More thorough research is needed to validate the therapeutic potential of this combined regimen, and its clinical application warrants additional investigation. Furthermore, the study does not provide sufficient insight the specific molecular mechanism of PF. We recognize that elucidating the molecular mechanism of PF is essential for a comprehensive understanding of its mechanism of action. Therefore, future research should prioritize determining how PF potentiates colistin activity.

Conclusion

In summary, our study demonstrates that combining PF with colistin exhibits a strongly synergistic effect against COL-R GNB with various colistin resistance mechanisms. PF enhances both outer and inner membrane permeability, likely by increasing colistin binding to bacterial membranes. Additionally, colistin/PF synergy helps prevent the emergence of colistin resistance and effectively inhibits biofilm formation and eradicates established biofilms. This combination shows promise in repurposing colistin, restoring its antimicrobial activity while reducing its dosage and minimizing side effects like neurotoxicity and nephrotoxicity. To our knowledge, this is the first report on the synergistic efficacy of PF/colistin for eliminating COL-R GNB and associated biofilms, offering a promising strategy for future clinical applications.

Data availability

All data generated or analyzed during this study are included in this published article and its Supplementary Information files.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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