Infection and Drug Resistance

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ORIGINAL RESEARCH

Synergistic Activity and Biofilm Formation Effect of Colistin Combined with PFK-158 Against Colistin-Resistant Gram-Negative Bacteria

Liqiong Chen[1]
Kaihang Yu[2]
Lijiang Chen[1]
Xiangkuo Zheng[1]
Na Huang[1]
Yishuai Lin[2]
Huaiyu Jia[1]
Wenli Liao[1]
Jianming Cao[2]
Tieli Zhou[1]

[1]Department of Clinical Laboratory, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, People's Republic of China; [2]Department of Medical Laboratory Science, School of Laboratory Medicine and Life Science, Wenzhou Medical University, Wenzhou, Zhejiang Province, People's Republic of China

Purpose: The emergence of colistin resistance among Gram-negative bacteria (GNB) poses a serious public health threat. Therefore, it is necessary to enhance the antibacterial activity of colistin through the combination with other drugs. In this study, we demonstrated the synergistic activity and the possible synergy mechanism of colistin with PFK-158 against colistin-resistant GNB, including non-fermenting bacteria and Enterobacteriaceae.

Patients and Methods: Thirty-one colistin-resistant GNB, including *Pseudomonas aeruginosa* (n = 9), *Acinetobacter baumannii* (n = 5), *Escherichia coli* (n = 8) and *Klebsiella pneumoniae* (n = 9), were collected as the experimental strains and the minimum inhibitory concentrations (MICs) of colistin, other routine antimicrobial agents and PFK-158 against all strains were determined by the broth microdilution method. The synergistic activity of colistin with PFK-158 was assessed by the checkerboard assay and time-kill assay. The biofilm formation assay and scanning electron microscopy were used to demonstrate the biofilm formation effect of colistin with PFK-158 against colistin-resistant GNB.

Results: The results of the checkerboard assay showed that when colistin was used in combination with PFK-158, synergistic activity was observed against the 31 colistin-resistant GNB. The time-kill assay presented a significant killing activity of colistin with PFK-158 against the 9 colistin-resistant GNB selected randomly, including *Pseudomonas aeruginosa* (n = 6), *Acinetobacter baumannii* (n = 1), *Escherichia coli* (n = 1), and *Klebsiella pneumoniae* (n = 1). The biofilm formation assay and scanning electron microscopjihy showed that colistin with PFK-158 can effectively suppress the formation of biofilm and reduce the cell arrangement density of biofilm against most experimental strains.

Conclusion: The results of the performed experiments suggest that the combination of colistin and PFK-158 may be a potential new choice as a new antibiofilm group for the treatment of infections caused by the colistin-resistant GNB.

Keywords: synergy, non-fermenting bacteria, enterobacteriaceae, bacterial biofilm, cytotoxicity

Introduction

Due to the rapid spread of antimicrobial resistance and the slow development of novel antimicrobials, Gram-negative bacteria (GNB) infections are becoming extremely challenging for clinicians and a real threat to international public health.[1,2] Rapid spread and dissemination of resistance-encoding genes among pathogens further exacerbate the problem, resulting in an ever-increasing rate of antimicrobial-resistant bacterial infections.[3–5] The traditional drug, colistin, is a defensive drug

Correspondence: Tieli Zhou
Tel +86-577-86689885
Email wyztli@163.com

Jianming Cao
Tel +86-577-88069595
Email wzcjming@163.com

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commonly used clinically to treat infections caused by multi-drug resistant and carbapenem-resistant GNB.[6] Unfortunately, the increased and inappropriate use of colistin has led inexorably to the worldwide emergence of colistin-resistant GNB in the last few years.[7–9] The emergence of colistin resistance among GNB poses a serious public health threat and warrants immediate action.[10–12] Therefore, it is necessary to enhance the antibacterial activity of colistin through the combination with other drugs.

Previous studies have shown that infections caused by GNB are often closely related to the formation of biofilm.[13,14] Bacterial biofilm is the organized bacterial populations formed by extracellular polymers, such as protein, polysaccharides and extracellular DNA.[15,16] The formation of biofilm can strengthen the ability of bacteria to adapt to the environment and make the penetration of conventional antibacterial drugs difficult; thus, it causes repeated chronic bacterial behavior infection.[17–19] In addition, the formation of biofilm is also conducive to the spread of drug resistance between strains.[20,21]

PFK-158 is a potent and selective 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 3 (PFKFB3) inhibitor, which shows extensive anti-tumor activity by reducing the uptake of glucose in cancer cells, the production of ATP, the release of lactic acid and inducing apoptosis and autophagy.[22,23] A Phase I clinical trial of PFK-158 was completed in July 2016.

At present, there is only one study having investigated the synergistic activity of colistin combined with PFK-158 against colistin-resistant Enterobacteriaceae.[24] However, there was no study exploring the synergistic activity of these two drugs against colistin-resistant *P. aeruginosa*and*A. baumannii*. Therefore, the main aim of this study was to determine the synergistic activity and biofilm formation effect of colistin in combination with PFK-158 against colistin-resistant GNB including *Pseudomonas aeruginosa* (*P. aeruginosa*) *and Acinetobacter baumannii* (*A. baumannii*) and Enterobacteriaceae, in order to provide new possible future therapeutic strategies to combat resistance to this antibiotic of last resort.

Patients and Methods

Bacterial Strains

A total of 31 non-duplicated Gram-negative clinical isolates were recovered from the First Affiliated Hospital of Wenzhou Medical University in China, including colistin-resistant *P. aeruginosa* (n = 9), *A. baumannii* (n = 5), *Escherichia coli* (*E. coli*) (n = 8), and *Klebsiella pneumoniae* (*K. pneumoniae*) (n = 9). These isolates were all identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS; bioMérieux, Lyons, France). All strains were stored in Luria Bertani (LB) broth medium (Oxoid, Britain) at −80 °C containing 30% v/v glycerol for later use. *P. aeruginosa* ATCC 27,853 and ATCC 25,922 were served as the quality controls, which were purchased from the National Center of Clinical Laboratory (NCCL).

Clinical Data Collection

The medical records were reviewed to integrally collect the data of the patients with 31 colistin-resistant GNB. The study data included the following variables: gender, age of the patients and isolation date, isolation sample, isolation ward of the strains.

Antimicrobial Agents

PFK-158 was purchased from MedChemExpress (MCE) Co., Ltd (New Jersey, USA) and dissolved in 2.5% v/v dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Saint Louis, USA). All antibiotics, including colistin, aztreonam, ceftazidime, cefepime, imipenem, ciprofloxacin, levofloxacin, gentamicin, and tobramycin were purchased from Wenzhou Kangtai Biological Technology Co., Ltd (Zhejiang, China).

Antimicrobial Susceptibility Assay

The MICs of all antibiotics and PFK-158 were determined by the broth microdilution in cation-adjusted Mueller-Hinton Broth (CAMHB). The CAMHB (Biofroxx Biological Technology Co., Ltd., Germany) was prepared by adding an appropriate amount of Mg^{2+} and Ca^{2+} into Mueller-Hinton Broth, with final concentrations of 10.0–12.5 mg/L and 20–25 mg/L, respectively. The final bacteria concentration of each well was approximately at 7.5×10^5 colony forming units (CFU)/mL. Serial two-fold dilutions for colistin and PFK-158 were prepared in CAMHB 96-well microtiter plates (Flat bottom with lid, Sterile; Corning, USA). The results were observed after incubation at 37 °C for 16–18 h. The MICs of colistin determination for all strains were interpreted by the recommendation of CLSI. Multi-drug resistant (MDR) strains were defined as non-susceptible to three or more different antimicrobial categories.[25] All experiments were performed in triplicate.

Checkerboard Assay

The in-vitro synergistic activity of the combination against the colistin-resistant GNB was examined by using the checkerboard assay on a 96-well plate (Flat bottom with lid, Sterile; Corning, USA) as previously described. Colistin is 2-fold serially diluted along the x-axis, while PFK-158 is 2-fold serially diluted along the y-axis to form a matrix.[26] The final bacteria concentration of each well was also approximately at 7.5×10^5 CFU/mL. Wells containing CAMHB with or without bacterial cells were used as positive or negative controls, respectively. The 96-well plates were then incubated at 37 °C for 16–18 h and record the MICs of the single drug and the MIC_s of the combination of colistin with PFK-158.

The fractional inhibitory concentration index (FICI) was used to evaluate the potential synergistic activity of the two drugs in combination. The FICI was calculated with the formula: FICI = (MIC of drug A in the combination/MIC of drug A alone) + (MIC of drug B in the combination/MIC of drug B alone). FICI≤0.5, 0.5<FICI≤1.0, 1.0<FICI≤2.0, FICI>2.0 was expected as synergistic activity, additive activity, irrelevant activity, and antagonistic activity, respectively. All experiments were performed in triplicate.

Time-Kill Assay

The time-kill assay was conducted according to a published protocol with several modifications.[27,28] In brief, we randomly selected 9 strains as experimental strains, including colistin-resistant *P. aeruginosa* (n = 6), *A. baumannii* (n = 1), *E. coli* (n = 1), and *K. pneumoniae* (n = 1). The synergistic activity of these two drugs was achieved with combinations of colistin and PFK-158 used at the lowest concentration when it showed synergistic activity. At the same time, we set up the control group adding colistin and PFK-158 alone. Viable cells were counted by spreading 100 μL of samples after appropriate dilutions with saline and plating on antibiotic-free Mueller-Hinton agar plates at 0, 2, 4, 6, 12, and 24 h after antibiotic addition. Bacterial colonies were counted from the plates after incubation for 16 −18 h at 37 °C. The assay was performed in triplicate for these strains; then, we calculated the average value of viable CFU and plotted it on a semi-logarithmic graph. Antimicrobial carryover was controlled by the inhibition of colonial growth at the site of the initial streak according to NCCLS guidelines.[27] Bactericidal activity was defined as a ≥ 3 log10 decrease in CFU/mL by 24 h. Synergistic activity was defined as a ≥ 2 log10 decrease in CFU/mL at 24 h by the two drugs combination compared with the single drug.[27]

Biofilm Formation and Eradication Assay

The biofilm biomass was detected by crystal violet staining as previously described.[29] As for the biofilm formation assay, after inoculation for 18 h, strains were diluted 1:100 in fresh LB broth. 100 μL LB broth and an aliquot of each sample was then transferred to a 96-well microtiter plate (Flat bottom with lid, Sterile; Corning, USA) and incubated at 37 °C for 24 h. The biofilm was stained with 150 μL 1% w/v crystal violet staining solution (lot number: NO.20190324, Beijing Solarbio Biotechnology Co., Ltd., China) for 15 minutes at 37 °C, and each well was washed twice with normal saline. Add 150 μL of 95% v/v ethanol (95% absolute ethanol and 5% glacial acetic acid) for 10 minutes at 37 °C to solubilized the stained biofilms, and the OD_{595} were measured with a microplate reader (Multiskan FC). The difference between the biofilm formation and eradication experiment step was that the former added the drug before the biofilm was formed, and the latter added the drug after the mature biofilm was formed.

Scanning Electron Microscope

The biofilm of TL2314 was treated with or without colistin (at 2 μg/mL), PFK-158 (at the lowest concentrations that can show synergistic effects when combined with colistin) alone and in combination for 24 h as described above on sterile cover slips (lot number: NO.10211818C, CITOGLAS Co., Ltd., China) in a 6-well plate (NEST Biotechnology Co., Ltd., China).[30] After treatments, the cover slips were rinsed by sterile PBS for three times, and then fixed by 2.5% (v/v) glutaraldehyde overnight. After drying, the samples were dehydrated by increasing concentrations of ethanol (20%, 40%, 70%, 90%, 95% and 100%, v/v, 2 min each). Finally, the samples were processed by gold sputtering and observed by SEM (S-3000N, Japan).

Cytotoxicity Assay by MTT Method

RAW 264.7 macrophage cell lines were purchased commercially from Procell Life Science and Technology Co., Ltd., China. Cytotoxicity of PFK-158 with different concentrations (1, 2, 4, 8, 16, 32, 64, and 128 μg/mL) and 2.5% DMSO was performed with RAW 264.7 macrophage cell lines with minor modifications. 2×10^5 macrophage cell lines were seeded into each well of 96-well tissue culture plates (Flat bottom with lid, Sterile; Corning, USA) and

cultured in Roswell Park Memorial Institute (RPMI-1640) supplemented with 10% v/v fetal calf serum (FCS). And then cell lines were treated with PFK-158, 2.5% v/v DMSO and incubated in the presence of 5% v/v CO_2 at 37 °C for 24 h. After that, the medium was replaced with a fresh RPMI containing 5 mg/mL 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated at 37 °C for 4 h. Followed by that, formazan crystals were dissolved in DMSO by 10 min incubation at room temperature and absorbance was measured using a microplate reader (SpectraMax 190) at test wavelength of "λ"= 490 nm.[31]

Statistical Analysis

Each experiment was performed in triplicate. Significance was determined by using two-sample *t*-test and mentioned as P value < 0.01 (noted with*), P value < 0.001 (noted with**) and P value < 0.0001 (noted with***). Statistical analyses were performed using Graph Pad Prism 7.03 statistical software.

Results

Bacterial Strains and Antimicrobial Agents and Susceptibility Test

Table 1 summarizes the patients characteristics and species distribution. Overall, the colistin-resistant *P. aeruginosa* strains were mainly from the sputum sample (88.9%, 8/9). The colistin-resistant *A. baumannii* strains were all from the sputum sample (100.0%, 5/5). Most of the colistin-resistant *K. pneumoniae* strains were

Table 1 Patient's Clinical Data and Characteristics of Analyzed Strains

Species	Strains	Isolation Date	Age	Gender	Sample	Ward
P. aeruginosa	TL1671	11/04/2015	74	M	Wound	Endocrinology
	TL1722	16/05/2015	63	M	Sputum	Neurosurgery
	TL1736	20/05/2015	44	M	Sputum	Neurosurgery
	TL1744	23/05/2015	63	M	Sputum	ICU
	TL2314	08/03/2016	66	M	Sputum	ICU
	TL2917	09/02/2017	58	M	Sputum	ICU
	TL2967	13/03/2017	26	M	Sputum	Emergency
	TL3008	05/04/2017	67	M	Sputum	Neurosurgery
	TL3086	24/05/2017	65	M	Sputum	Neurosurgery
A. baumannii	BM2370	25/02/2015	59	M	Sputum	ICU
	BM2431	19/03/2015	89	M	Sputum	ICU
	BM1539	11/02/2014	47	M	Sputum	ICU
	BM2412	13/03/2015	63	F	Sputum	ICU
	BM1595	28/02/2014	57	F	Sputum	Neurosurgery
E. coli	DC90	27/03/2012	76	M	Wound	Gastrointestinal Surgery
	DC3411	26/02/2015	75	M	Urine	Urology
	DC3539	26/03/2015	82	M	Drainage	Gastrointestinal Surgery
	DC3599	07/04/2015	72	M	Sputum	Respiratory Medicine
	DC3737	05/05/2015	52	M	Wound	Orthopedics
	DC3806	19/05/2015	17	F	Sputum	Hematology
	DC4887	23/02/2016	63	M	Urine	Urology
	DC5262	18/05/2016	32	F	Urine	Emergency
K. pneumoniae	FK1342	04/06/2014	21	F	Sputum	Anorectal Surgery
	FK26	15/03/2012	65	M	Sputum	Neurosurgery
	FK6556	04/04/2019	41	F	Sputum	Respiratory Medicine
	FK20	08/03/2012	88	F	Sputum	Neurology
	FK1913	19/01/2015	74	M	Sputum	Neurosurgery
	FK610	13/05/2013	83	M	Sputum	Trauma surgery
	FK591	02/05/2013	65	F	Urine	Neurology
	FK169	02/07/2012	79	F	Sputum	General
	FK150	19/06/2012	60	M	Pus	Emergency

Abbreviations: M, male; F, female.

from the sputum sample (77.8%, 7/9). But the separation site of colistin-resistant *E. coli* was relatively scattered, and the proportion of urine, sputum, wound and drainage were 37.5% (3/8), 25.0% (2/8), 25.0% (2/8), 12.5% (1/8), respectively. There were more strains from males than females (71.0% vs 29.0%, respectively). Strains were cultured from patients aged 17 to 89 years (average age 60.8 years). The colistin-resistant *P. aeruginosa* strains were mainly from patients in neurosurgery (44.4%, 4/9), the intensive care unit (ICU) (33.3%, 3/9). Most of the colistin-resistant *A. baumannii* strains were from patients in the intensive care unit (ICU) (80.0%, 4/5). However, the isolation wards of the remaining two strain species were relatively scattered.

As shown in Table 2, all the 31 GNB were all resistant to colistin (>2 μg/mL). In addition, MICs of PFK-158 were 512 μg/mL for all tested strains, showing the lack of any intrinsic antimicrobial activity of PFK-158 alone against these Gram-negative strains. And all of the colistin-resistant *A. baumannii* (n = 5), *E. coli* (n = 8), *K. pneumoniae* (n =9) and 4 of 9 colistin-resistant *P. aeruginosa* exhibited multidrug-resistance.

Table 2 Representative MICs Against 31 Colistin-Resistant GNB

Species	Strains	Antibiotics	ATM	CAZ	FEP	IPM	CIP	LVX	GEN	TOB	COL	PFK-158
		Breakpoints(S-R)	8–32	8–32	8–32	2–8	0.5–2	1–4	4–16	4–16	2–4	
P. aeruginosa	TL1671	MICs (μg/mL)	8	4	8	2	0.25	1	2	1	8	512
	TL1722		32	64	16	16	4	4	32	4	8	512
	TL1736		4	4	2	16	1	1	32	8	16	512
	TL1744		32	32	16	16	32	8	≥128	32	8	512
	TL2314		16	32	16	4	0.5	2	8	2	8	512
	TL2917		32	16	16	16	0.25	2	8	8	16	512
	TL2967		128	16	32	16	8	16	8	8	8	512
	TL3008		4	2	4	16	0.5	1	16	4	32	512
	TL3086		128	16	16	≥128	16	8	≥128	128	32	512
A. baumannii	**BM2370**	MICs (μg/mL)	8	32	128	8	128	8	4	1	16	512
	BM2431		64	64	64	16	4	8	1	1	32	512
	BM1539		16	8	64	16	4	2	1	1	16	512
	BM2412		16	64	64	16	4	8	4	1	8	512
	BM1595		2	32	8	4	64	8	≥128	≥128	4	512
E. coli	**DC90**	MICs (μg/mL)	≥128	32	64	≥128	64	32	≥128	128	8	512
	DC3411		64	32	16	1	64	32	4	2	8	512
	DC3539		64	128	≥128	0.5	64	16	128	128	32	512
	DC3599		64	16	8	1	4	8	16	8	4	512
	DC3737		64	64	64	16	4	8	16	16	4	512
	DC3806		64	64	16	1	4	8	16	16	4	512
	DC4887		1	4	32	1	4	16	16	8	8	512
	DC5262		≥128	16	16	0.25	≥128	128	32	16	32	512
K. pneumoniae	**FK1342**	MICs (μg/mL)	128	≥128	≥128	0.25	1	0.5	1	4	≥128	512
	FK26		≥128	64	16	0.5	2	0.5	≥128	≥128	≥128	512
	FK6556		64	64	64	16	4	8	16	16	32	512
	FK20		≥128	≥128	64	0.25	1	2	≥128	≥128	≥64	512
	FK1913		≥128	≥128	≥128	32	≥128	128	≥128	≥128	64	512
	FK610		≥128	≥128	≥128	32	128	32	128	64	8	512
	FK591		≥128	≥128	64	0.12	≥128	128	1	8	32	512
	FK169		≥128	32	32	0.25	32	16	2	2	8	512
	FK150		≥128	64	32	0.12	≥128	32	16	4	16	512

Notes: Bolded MICs values mean resistance. Bolded strain number indicates multidrug resistant (MDR) strain. S-R represents the susceptible (S) breakpoint to resistant (R) breakpoint, according to CLSI supplement M100 (30th edition) and EUCAST.

Abbreviations: GNB, Gram-negative bacteria; ATM, aztreonam; CAZ, ceftazidime; FEP, cefepime; IMP, imipenem; CIP, ciprofloxacin; LVX, levofloxacin; GEN, gentamicin; TOB, tobramycin, COL, colistin.

Synergistic Activity Testing by the Checkerboard Assay

The checkerboard assay demonstrated that the FICIs of all 31 colistin-resistant GNB were <0.5 (Table 3), which included *P. aeruginosa* (n = 9), *A. baumannii* (n = 5), *E. coli* (n = 8), and *K. pneumoniae* (n = 9), indicating significant synergistic activity of colistin combined with PFK-158 against colistin-resistant GNB. We observed that the combination of colistin and PFK-158 could reduce colistin MICs of the colistin-resistant strains by 4 to 32 fold. Therefore, the combination of colistin and PFK-158 was selected for further investigation.

Time–Kill Assay and Strains Activity

Time-kill curves against 9 colistin-resistant strains TL1671, TL1736, TL1744, TL2314, TL3008, TL3086,

Table 3 Summary of MICs and FICI of Colistin Combined with PFK-158 Against 31 Colistin-Resistant GNB and 2 Quality Control Strains

Colistin-Resistant GNB	Strains	Antimicrobial Susceptibility (MIC, μg/mL)		Antimicrobial Combination (MIC, μg/mL)			
		COL	PFK-158	COL + PFK-158	FICI	Potentiation	Interpretation
P. aeruginosa	TL1671	8	512	1+16	0.16	8-fold	Synergy
	TL1722	8	512	2+16	0.28	4-fold	Synergy
	TL1736	16	512	4+16	0.28	4-fold	Synergy
	TL1744	8	512	2+16	0.28	4-fold	Synergy
	TL2314	8	512	2+16	0.28	4-fold	Synergy
	TL2917	16	512	4+32	0.31	4-fold	Synergy
	TL2967	8	512	2+32	0.31	4-fold	Synergy
	TL3008	32	512	8+16	0.28	4-fold	Synergy
	TL3086	32	512	8+32	0.31	4-fold	Synergy
	ATCC 27,853	1	512	0.5+16	0.53	2-fold	Additivity
A. baumannii	BM2370	16	512	1+8	0.08	16-fold	Synergy
	BM2431	32	512	4+16	0.16	8-fold	Synergy
	BM1539	16	512	4+32	0.31	4-fold	Synergy
	BM2412	8	512	1+8	0.14	8-fold	Synergy
	BM1595	4	512	1+8	0.27	4-fold	Synergy
E. coli	DC90	8	512	2+16	0.28	4-fold	Synergy
	DC3411	8	512	1+32	0.19	8-fold	Synergy
	DC3539	32	512	4+16	0.16	8-fold	Synergy
	DC3599	4	512	1+8	0.27	4-fold	Synergy
	DC3737	4	512	1+8	0.27	4-fold	Synergy
	DC3806	4	512	1+16	0.28	4-fold	Synergy
	DC4887	8	512	1+8	0.14	8-fold	Synergy
	DC5262	32	512	4+8	0.14	8-fold	Synergy
	ATCC 25,922	0.5	512	0.25+16	0.53	2-fold	Additivity
K. pneumoniae	FK1342	≥128	512	8+32	0.13	16-fold	Synergy
	FK26	≥128	512	16+16	0.16	8-fold	Synergy
	FK6556	32	512	4+32	0.19	8-fold	Synergy
	FK20	≥64	512	2+4	0.04	32-fold	Synergy
	FK1913	64	512	2+4	0.04	32-fold	Synergy
	FK610	8	512	1+4	0.13	8-fold	Synergy
	FK591	32	512	4+4	0.13	8-fold	Synergy
	FK169	8	512	1+4	0.13	8-fold	Synergy
	FK150	16	512	1+16	0.09	16-fold	Synergy

Notes: Colistin MIC breakpoint for above strains: susceptible, ≤2 μg/mL; resistant, >2 μg/mL.
Abbreviations: GNB, Gram-negative bacteria; MIC, minimum inhibitory concentration; COL, colistin; FICI, fractional inhibitory concentrations index.

BM2431, DC3539, and FK1342 are revealed in Figure 1. With colistin or PFK-158 alone, all colistin-resistant strains showed a regrowth after a reduction at 2 h. The combination of colistin and PFK-158 exhibited constant both synergistic and bactericidal activities for TL1736, TL2314, TL3008, TL3086, BM2431, DC3539, and FK1342 for 24 h. As for TL1671, synergistic activity was observed over 24 h for the combination of colistin and PFK-158, compared with colistin or PFK-158 alone. The combination showed a synergistic activity at 6 h against TL1744, but regrowth was observed at 24 h. In summary, this combination of colistin and PFK-158 enhanced their killing activity of drug exposure.

Influence of Colistin Combined with PFK-158 on Biofilm Formation and Eradication

Biofilm formation and eradication were determined using a crystal violet assay. Figure 2 shows that the combination of colistin and PFK-158 ($p < 0.05$) were found to effectively suppress the formation of biofilm on all the colistin-resistant *P. aeruginosa* (n = 9), *A. baumannii* (n = 5), *K. pneumoniae* (n = 9) and 4 of 8 colistin-resistant *E. coli* compared with the group of using colistin or PFK-158 alone and the control group as for 24-h preformed biofilm. However, as shown in Figure 3, the combination of colistin and PFK-158 had no obvious eradication effect on mature biofilm of all the experimental strain, compared to the control group ($p > 0.05$).

Images of Scanning Electron Microscopy

In scanning electron microscopy (SEM), the biofilm matrixes of TL2314 were almost removed and the biofilm cells were decreased significantly when using colistin in combination with PFK-158 compared with the drug-free control and colistin alone (Figure 4).

Evaluation of in-vitro Cytotoxicity of PFK-158 and DMSO

Figure 5 indicates that the PFK-158 (<32 μg/mL) and 2.5% dimethyl sulfoxide (DMSO) exerted negligible cytotoxicity on the RAW 264.7 macrophage cell lines compared with the control group treated with MTT ($P > 0.05$).

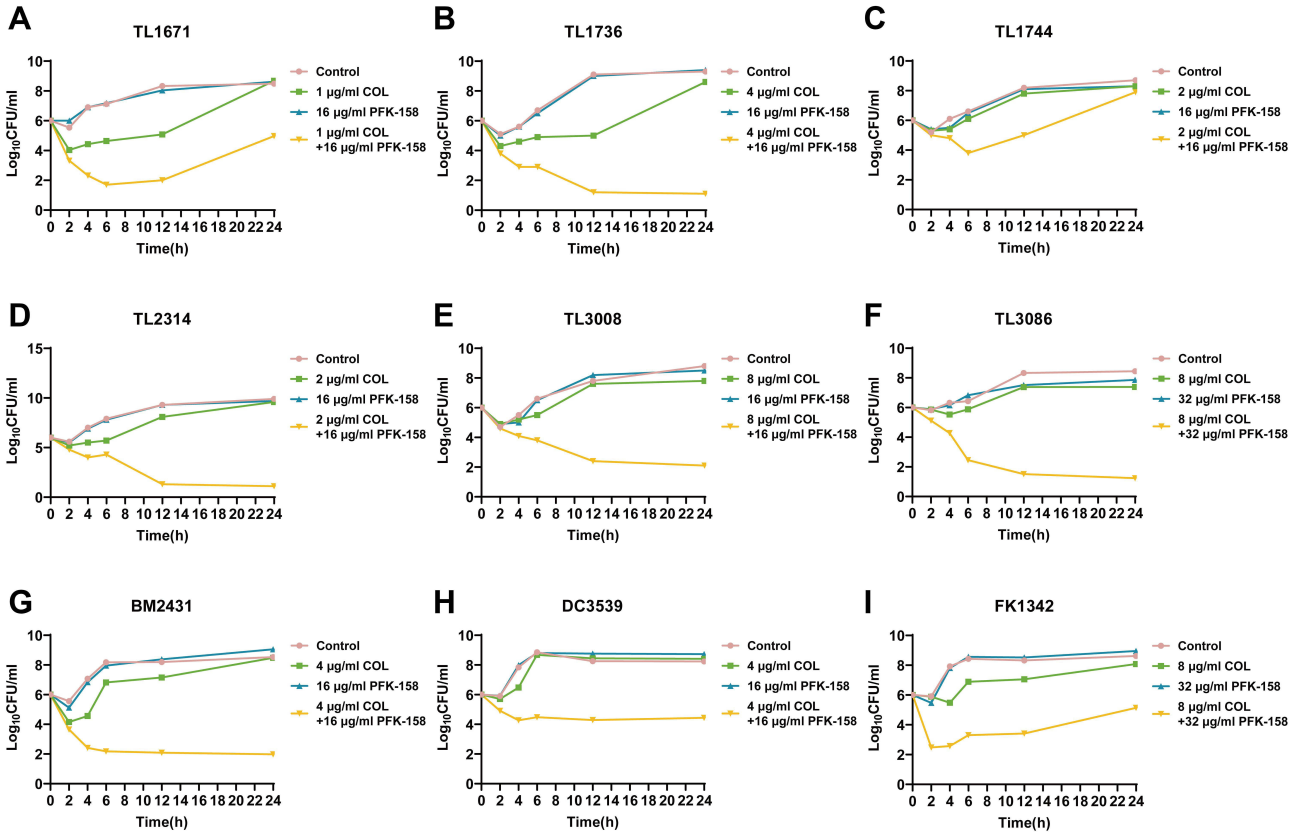

Figure 1 Time-killing curves of colistin and PFK-158 alone or in combination against 9 colistin-resistant GNB. (**A**) TL1671, (**B**) TL1736, (**C**) TL1744, (**D**) TL2314, (**E**) TL3008, (**F**) TL3086, (**G**) BM2431, (**H**) DC3539, (**I**) FK1342.
Abbreviation: GNB, Gram-negative bacteria.

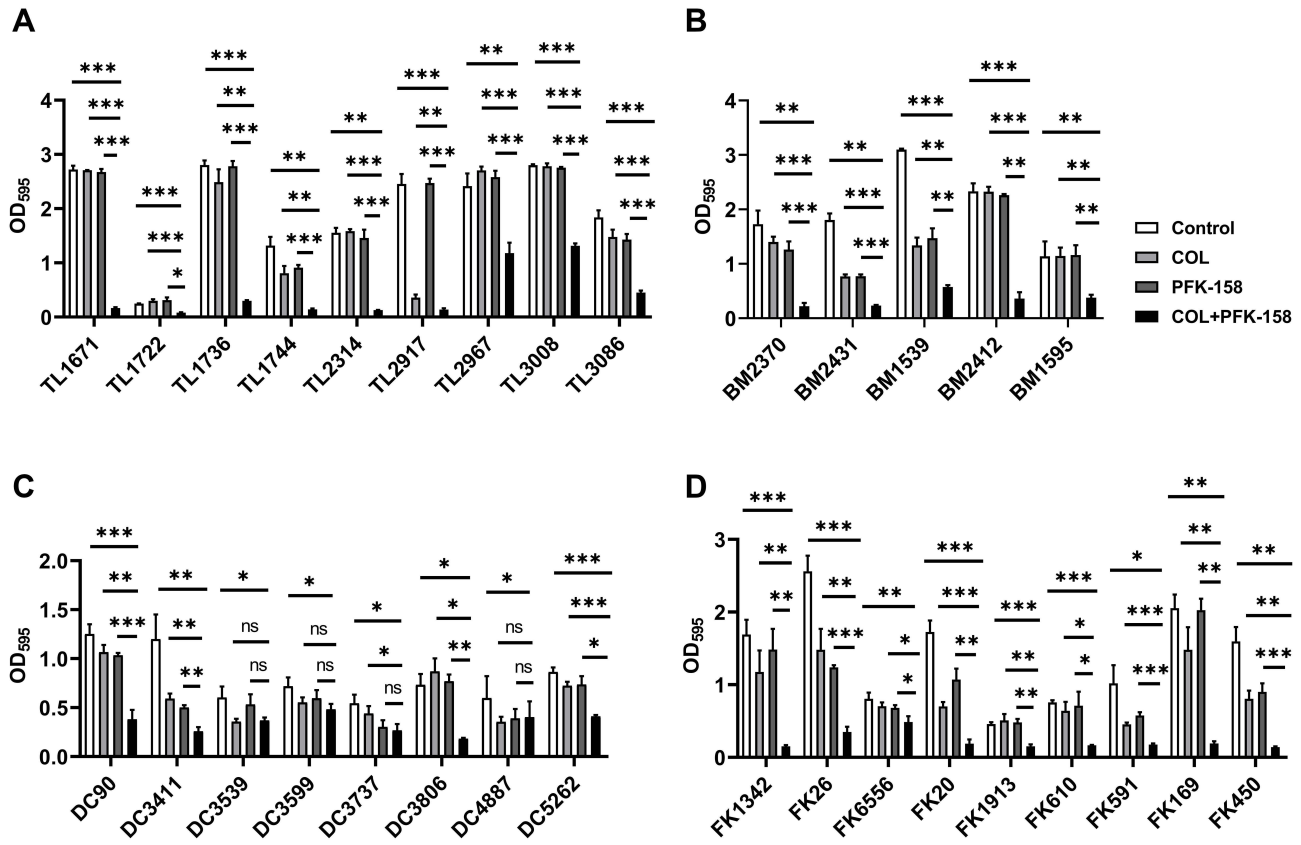

Figure 2 Biofilm inhibitory effects of colistin combined with PFK-158 on colistin-resistant GNB. (**A**) *P. aeruginosa*; (**B**) *A. baumannii*; (**C**) *E. coli*; (**D**) *K. pneumoniae*. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (determined by a two-sample *t*-test). The results are shown as the mean and standard deviation of three independent experiments.
Abbreviations: ns, not significant; GNB, Gram-negative bacteria.

Discussion

The current epidemiological situation is especially worrying with multidrug-resistant (MDR) GNB spreading worldwide and with a paucity of novel marketed antibiotics; thus, colistin is gaining increasing interest from many clinicians worldwide.[32] However, the increasing numbers of discoveries of colistin-resistant pathogens broke the last barrier between super-bacteria and humans, and new therapy strategies are urgently required.[33] A number of previous studies have reported the synergistic activity of colistin combined with other antibiotics against colistin-resistant or carbapenem-resistant strains.[34–37] Besides, a few studies based on colistin in combination with the nonantibiotics were also performed previously.[38–40] In this study, we evaluated the synergistic activity and biofilm formation effect of colistin combined with PFK-158 against colistin-resistant GNB, including non-fermenting bacteria (*P. aeruginosa, A. baumannii*) and Enterobacteriaceae (*E. coli* and *K. pneumoniae*).

Firstly, as shown in Table 2, it demonstrated that all the colistin-resistant *A. baumannii* (n = 5), *E. coli* (n = 8), *K. pneumoniae* (n = 9) and 4 of 9 colistin-resistant *P. aeruginosa* exhibited multi-drug resistance. In other words, the proportion of multidrug-resistant strains in colistin-resistant GNB was relatively high in this study. Therefore, finding a potential new drug group for the treatment of infections caused by the colistin-resistant GNB seems particularly important.

Secondly, as shown in Table 3, we noticed that for all the 31 colistin-resistant GNB we tested, the addition of PFK-158 demonstrated the enhanced colistin susceptibility by reducing colistin MICs (4–32 fold) and fractional inhibitory concentration indices ($FICI_S$) of the combination of colistin and PFK-158 were all below 0.5, indicating that there was a synergistic activity of colistin combined with PFK-158. It should be emphasized that a synergistic activity was observed against all tested colistin-resistant P. aeruginosa, A. baumannii, E.coli, and *K. pneumoniae*, suggesting that the combination may be a potential strategy to treat the infections caused by these four kinds of colistin-resistant GNB. Recently, Zhang et al have reported on the synergistic activity of colistin combined

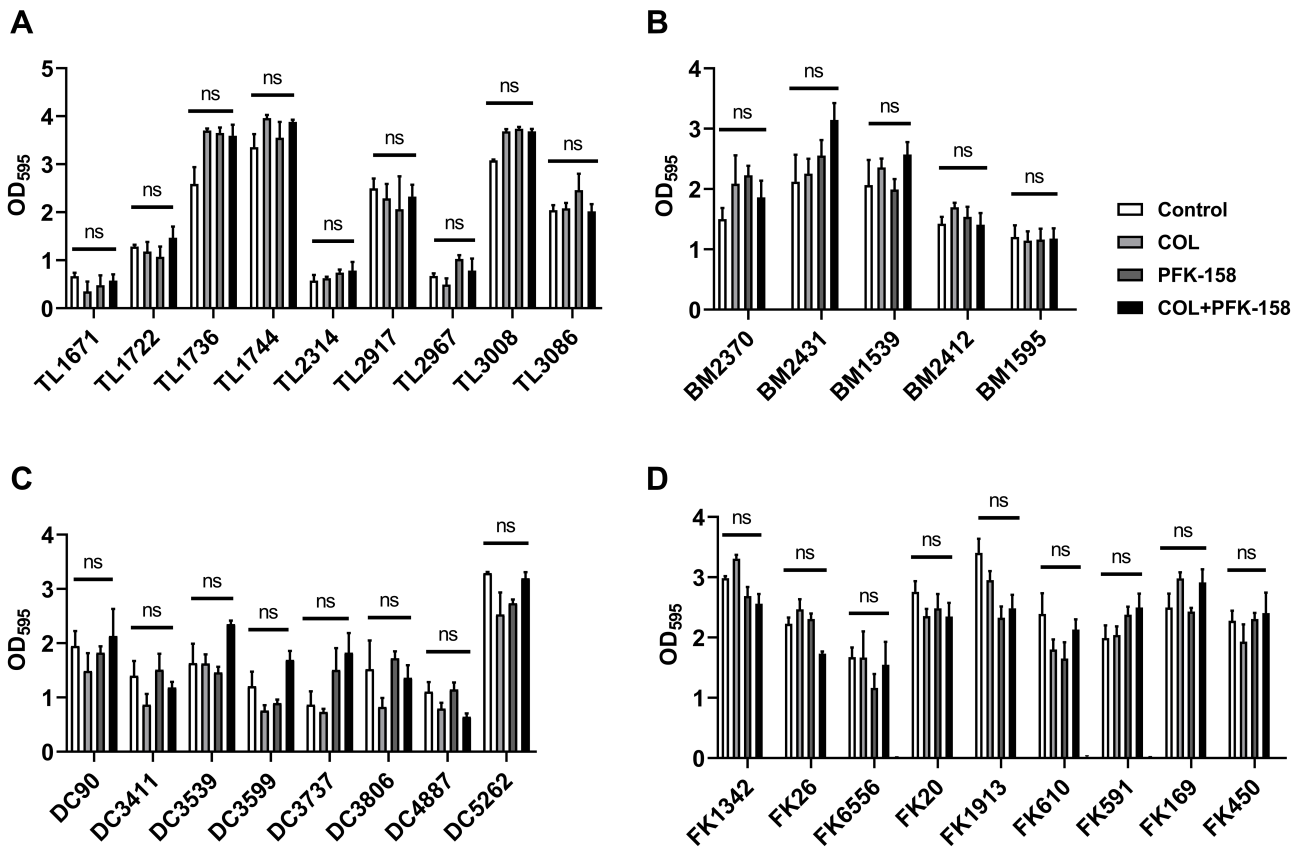

Figure 3 Biofilm eradication effects of colistin combined with PFK-158 on colistin-resistant GNB. (**A**) *P. aeruginosa*; (**B**) *A. baumannii*; (**C**) *E. coli*; (**D**) *K. pneumoniae*. (determined by a two-sample *t*-test). The results are shown as the mean and standard deviation of three independent experiments.
Abbreviations: ns, not significant; GNB, Gram-negative bacteria.

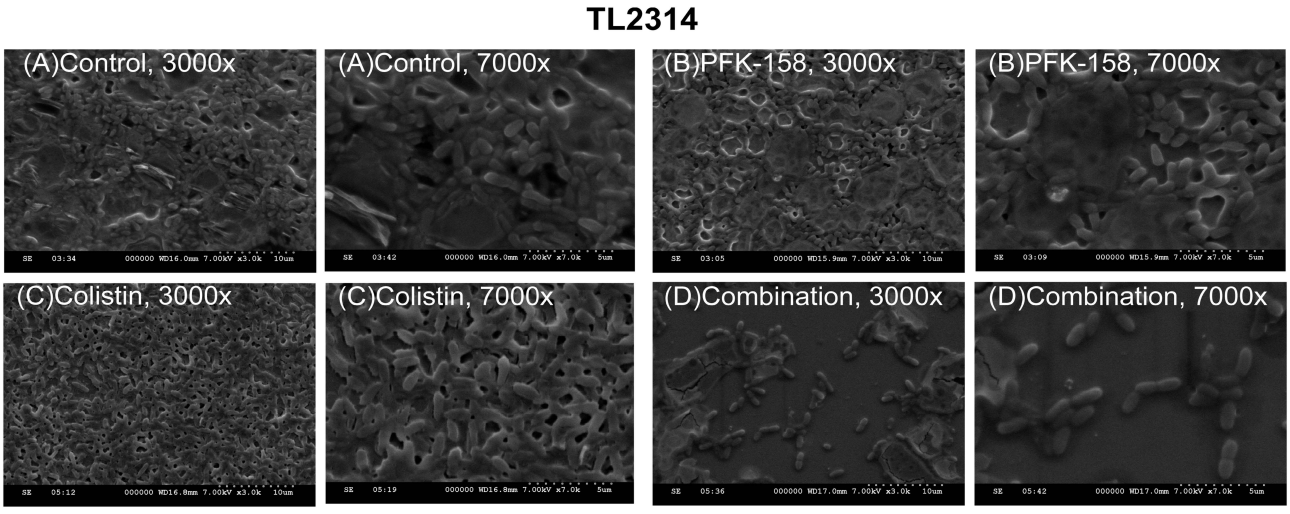

Figure 4 Images from SEM for colistin-resistant P. aeruginosa TL2314 exposed to different condition. (**A**) Control; (**B**) 16 μg/mL PFK-158 alone; (**C**) 2 μg/mL colistin alone; and (**D**) 2 μg/mL colistin and 16 μg/mL PFK-158 in combination.
Abbreviation: SEM, scanning electron microscopy.

with PFK-158 against colistin-resistant Enterobacteriaceae,[24] including *E.coli, K. pneumoniae,* and *E. cloacae*, the result of which is consistent with the result in our study. Compared to the study of Zhang et al, the advantage of our study is that we have explored not only the synergistic activity of colistin in combination with

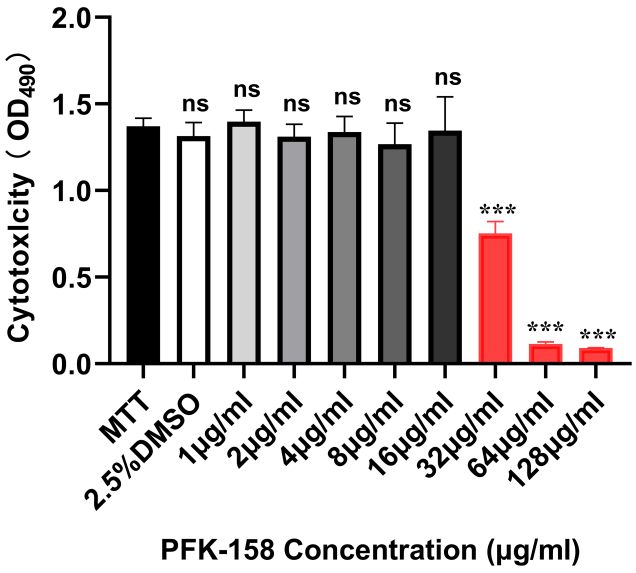

Figure 5 Cytotoxic effect of PFK-158 with different concentrations and 2.5% DMSO against RAW 264.7 murine macrophage cell line (absorbance values at 490 nm). ***$p < 0.001$. (determined by a two-sample *t*-test). The results are shown as the mean and standard deviation of three independent experiments.
Abbreviations: ns, not significant; DMSO, dimethyl sulfoxide.

PFK-158 against Enterobacteriaceae but also non-fermenting bacteria (*P. aeruginosa, A. baumannii*). In other words, the combination of colistin and PFK-158 showed a wide range of synergistic activity on colistin-resistant GNB. This suggests that the mechanism of the synergistic activity of the two drugs combination is probably not related to the difference of the strains property.

Moreover, in order to further explore the reasons for the synergistic effect of colistin and PFK-158, the biofilm matrices and the number of cell morphology were observed by crystal violet staining method and scanning electron microscopy. It showed that colistin combined with PFK-158 can effectively suppress the formation of biofilm and reduce the cell arrangement density of biofilm against all the colistin-resistant *P. aeruginosa, A. baumannii, K. pneumoniae,* and half of colistin-resistant *E. coli* that we tested. Based on the results as described above, we speculate that the synergistic effect of colistin and PFK-158 may be achieved by inhibiting the formation of biofilm. However, whether the combined application of colistin and PFK-158 will affect the expression of colistin resistance genes and lipid A modification is still worthy of further investigation by us.

Besides, PFK-158 shows extensive anti-tumor activity by reducing the uptake of glucose in cancer cells, the production of ATP, the release of lactic acid and inducing apoptosis and autophagy.[15,16] And in order to assess the safety of PFK-158, we conducted the cytotoxicity assay using the MTT method. The result illustrated that the PFK-158 (<32μg/mL) and 2.5% DMSO exerted negligible cytotoxicity on the RAW 264.7 macrophage cell lines, which indicates that PFK-158 has good safety and is worthy of further exploration and research.

Finally, it is crucial to evaluate the clinical significance of these observations and to better understand the dose–response relationships of a combination of colistin with PFK-158. As we all know, colistin has the nephrotoxicity and neurotoxicity[41] thus, if the combination therapy can lower the concentration and dosage of colistin, it will be extremely meaningful for the clinical treatment of colistin-resistant GNB infections, especially for the patients with cancer, who are a special group with the low immunity and are more prone to be infected. Therefore, further clinical study is recommended to assess the potential application of the combination of the two drugs in clinical practice.

Conclusion

In conclusion, our results strongly exhibited that the combination of colistin and PFK-158 displayed the significantly synergistic activity against all tested colistin-resistant GNB. PFK-158 was found to potentiate the antibacterial activity of colistin against a wide panel of colistin-resistant GNB no matter what species (including non-fermenting bacteria and Enterobacteriaceae). Besides, the study explored the antibiofilm potential of colistin combined with PFK-158 against colistin-resistant GNB, which may be the main mechanism of the synergistic effect of the combination of colistin and PFK-158. It may be a potential new alternative as a new antibiofilm group for the treatment of infections caused by the colistin-resistant clinical GNB.

Ethical Statement

Whole investigation protocols in this study were approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. There are no studies with humans or animals performed by any of the authors in this article. Informed consent was waived because this study, with its observational nature, mainly focused on bacteria and gave no interventions to patients.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval for the version to be published; have agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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