NOTE

In vitro evaluation of ciclopirox as an adjuvant for polymyxin B against gram-negative bacteria

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Polymyxin B is an antibiotic used to treat resistant gram-negative bacterial infections; it was originally isolated from the bacterium *Bacillus polymyxa*.¹ Polymyxin B is comprised of related compounds of polymyxins B1, B1-I, B2, B3 and B6; this mixture composition improves the permeability of the compound thus enhancing its bactericidal effects. Polymyxins, including polymyxin B, have been used extensively in topical otic and ophthalmic solutions for decades.^{2,3} Although polymyxins exhibit promising activity against gram-negative bacteria, the nephrotoxicities associated with these compounds have prevented their widespread use in favor of less toxic compounds. However, polymyxins have recently become a more common therapy in the treatment of infections exhibiting extensive multidrug resistance (MDR).

Antibiotic adjuvants or repurposed safe materials may be used to enhance the activity of polymyxins. For example, polymyxin E combined with azithromycin, ceftazidime, imipenem, minocycline, rifampicin or vancomycin shows *in vitro* synergy against multidrug-resistant *Acinetobacter baumannii*.^{4,5} Polymyxin B has also been combined with several agents to yield effective combined therapies,⁶ although such combinations may exacerbate the problem of MDR bacteria. Therefore, identification of new synergistic drug combinations may improve treatment efficacy.

Ciclopirox is a synthetic, broad-spectrum antifungal that inhibits dermatophyte growth and has an excellent safety profile.⁷ In over 20 years of clinical use, no fungal resistance has been identified. Therefore, ciclopirox may be repurposed as an anti-human immunodeficiency virus drug,⁸ a protective agent against mitochondrial cell damage,⁹ or a promoter of diabetic wound healing.¹⁰ Very recently, ciclopirox was also suggested as a repurposed bacteriostatic antibiotic for gram-negative bacteria.¹¹ Ciclopirox affects galactose metabolism and alters the composition of lipopolysaccharide (LPS),¹¹ a target of polymyxin B.¹² Thus, ciclopirox may activate polymyxin B function, possibly allowing the reduced use of polymyxin B in treating infections by gram-negative pathogens.

To test our hypothesis, the MICs for ciclopirox and polymyxin B were determined by broth microdilution in a 96-well plate that

blocked the visible bacterial growth and the checkerboard assay¹³ was performed to assess the synergy of the two-drug combination against bacterial strains.

The fractional inhibitory concentration index/indices (FICI) were determined from the results of checkerboard assay¹³ after the MICs of each drug for each strain were determined and calculated as follows:

$$FICI = MIC(A2)/MIC(A1) + MIC(B2)/MIC(B1),$$

Where MIC (A2) represented the MIC value of polymyxin B combined with ciclopirox or oxytetracycline, whereas MIC (A1) represented the MIC value of polymyxin B as monotherapy, with the same for MIC (B2) and MIC (B1) in the case of ciclopirox and oxytetracycline. The FICI values were interpreted as follows¹⁴ ≤ 0.5 , synergy; > 0.5 to < 1 , partial synergy; 1, addition; > 1 , indifference. The values indicated in Table 1 were isoeffective MIC ranges from five replicated experiments.

The MICs of ciclopirox and polymyxin B for both drug-sensitive *Escherichia coli* (*E. coli*) ATCC 25922 and BW25113 strains were 25 $\mu\text{g ml}^{-1}$ and 0.8 $\mu\text{g ml}^{-1}$, respectively. The checkerboard assay with two-drug combinations was performed against those strains and resulted in the reduction of polymyxin B susceptibility from 0.8 $\mu\text{g ml}^{-1}$ to the range of 0.2–0.05 $\mu\text{g ml}^{-1}$ (a 4- to 16-fold decrease in the polymyxin B MIC; Figure 1a and Table 1) by increasing concentrations of ciclopirox (3.12–12.5 $\mu\text{g ml}^{-1}$). Synergy was detected at 3.12–6.25 $\mu\text{g ml}^{-1}$ ciclopirox (FICI = 0.312–0.5). Synergy was also detected by growth curve and viability analysis of BW25113 cells treated with ciclopirox and/or polymyxin B (Figures 1b and d). Ciclopirox $\geq 12.5 \mu\text{g ml}^{-1}$ severely delayed or completely inhibited the growth, but did not result in cell death, thus confirming ciclopirox as a bacteriostatic.¹¹ However, the addition of ciclopirox to low-dose polymyxin B conferred bactericidal activity. Thus, ciclopirox may potentiate the bactericidal nature of polymyxin B.

Ciclopirox inhibited the growth of antibiotic-resistant strains and was effective against some gram-negative pathogens;¹¹ this synergy might also be effective against MDR isolates and known ciclopirox-targeted strains. We performed checkerboard assays using MDR *E. coli*

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Table 1 MIC and FICI values of *E. coli*, *A. baumannii* and *P. aeruginosa*

Species	Strain	FICI _{polB/cic} range	MIC _{polB} ($\mu\text{g ml}^{-1}$)	MIC _{polB/cic} range ($\mu\text{g ml}^{-1}$)	MIC _{cic} ($\mu\text{g ml}^{-1}$)	MIC _{cic/polB} range ($\mu\text{g ml}^{-1}$)
<i>E. coli</i>	ATCC25922	0.312	0.8	0.025–0.2	25	3.12–12.5
	BW25113	0.375	0.8	0.05–0.2	25	3.12–12.5
	MG1655	0.312	0.8	0.05–0.2	25	3.12–12.5
	Keio- <i>galE</i>	0.375	0.4	0.05–0.1	25	3.12–12.5
	Keio- <i>galU</i>	0.5	0.2	0.025–0.1	25	3.12–12.5
	Keio- <i>rfaB</i>	0.375	0.4	0.025–0.1	25	3.12–12.5
	Keio- <i>rfaC</i>	0.312	0.2	0.012–0.1	25	3.12–12.5
	Keio- <i>rfaF</i>	0.375	0.2	0.025–0.1	25	3.12–12.5
	Keio- <i>rfaI</i>	0.375	0.2	0.025–0.1	25	3.12–12.5
	Keio- <i>rfaJ</i>	0.5	0.4	0.1–0.2	25	3.12–12.5
	Keio- <i>rfaL</i>	0.625	0.4	0.1–0.2	25	3.12–12.5
	Keio- <i>rfaP</i>	0.5	0.2	0.05–0.1	25	3.12–12.5
	Keio- <i>rfaQ</i>	0.375	0.8	0.05–0.2	25	3.12–12.5
	Keio- <i>rfaY</i>	0.375	0.8	0.05–0.2	25	3.12–12.5
MDR <i>E. coli</i>	CCARM 1A074	0.25–0.5	0.8	0.05–0.1	25	3.12–6.25
	CCARM 1A078	0.25–0.5	0.8	0.05–0.1	25	3.12–12.5
	CCARM 1A645	0.375–0.5	0.4	0.05–0.1	25	3.12–12.5
<i>A. baumannii</i>	ATCC 15150	0.375–0.5	1.6	0.012–0.4	25	3.12–12.5
MDR <i>A. baumannii</i>	CCARM 12001	0.375–0.5	1.6	0.4	25	3.12–12.5
	CCARM 12003	0.375–0.5	1.6	0.4	25	3.12–12.5
<i>P. aeruginosa</i>	PAO1	0.531–1.0	1.6	0.4–0.8	100	3.12–75.0

Abbreviations: Cic, ciclopirox; FICI, fractional inhibitory concentration index/indices; MDR, multidrug resistance; PolB, polymyxin B.

The FICI_{polB/cic} indicates the values at the most synergistic combination between polymyxin B and ciclopirox. MIC_{polB} and MIC_{cic} indicate the MIC values of polymyxin B and ciclopirox, respectively. MIC_{polB/cic} and MIC_{cic/polB} represent the MIC values of polymyxin B and ciclopirox in the presence of ciclopirox or polymyxin B except the point of MIC values of single compound, respectively. All FICI values were determined from Figure 1 and Supplementary Figures 1–3. All keio collections (<http://www.shigen.nig.ac.jp/ecoli/strain/>) were confirmed by PCR amplification with gene-specific primers (Supplementary Table 1) and DNA sequencing.

isolates and a drug-sensitive *A. baumannii* (ATCC 15150) strain and observed synergy between polymyxin B and ciclopirox, with FICI values of 0.25–0.5 in *E. coli*-MDR (Figure 1e) and 0.375–0.5 for *A. baumannii* (Figure 1f). The synergy of combined polymyxin B and azithromycin, imipenem or rifampicin is highly strain dependent;⁵ however, we found that the synergy of polymyxin B and ciclopirox was the same in all the *A. baumannii* strains regardless of drug-resistant status (FICI=0.375–0.5; Figure 1f). Thus, the *in vitro* synergy of ciclopirox and polymyxin B seemed to be more generalized for gram-negative bacteria than other previously tested combinations.

To investigate whether efficient ciclopirox targeting is required for synergy, we selected *Pseudomonas aeruginosa* PAO1, an inefficient target for ciclopirox.¹¹ The MIC of ciclopirox in PAO1 was 100 $\mu\text{g ml}^{-1}$, much higher than in *E. coli* or *A. baumannii* (MICs: 25 $\mu\text{g ml}^{-1}$; Figure 1g and Table 1). Meanwhile, the combination of ciclopirox and polymyxin B reduced the MICs in PAO1 by two to fourfold (3.12–75 $\mu\text{g ml}^{-1}$ ciclopirox and 0.4 $\mu\text{g ml}^{-1}$ polymyxin B); however, partial synergy or addition (FICI=0.531 to 1.0) was observed at these concentrations (Figure 1g and Table 1). These data showed that effective ciclopirox targeting seems to be required for synergy with polymyxin B.

Because polymyxin B acts in synergy with multiple antibiotics rather than a single specific antibiotic against gram-negative bacteria^{6,15,16} it may questioned whether ciclopirox also shows the synergy with multiple antibiotics rather than specific to polymyxin B. Checkerboard assays were performed to determine the effect of ciclopirox in combination with five classes of antibiotics including cephalothin (cephalosporin), gentamicin (aminoglycosides), levofloxacin (quinolone), meropenem (carbapenem) and minocycline (tetracycline). None of the tested antibiotics showed synergy or adjuvant effects; an increase in ciclopirox concentration did not improve *E. coli*

susceptibility to the antibiotic mixtures (Supplementary Figure 1), suggesting the interaction of ciclopirox with polymyxin B is specific.

Polymyxin B-containing products such as Terramycin, an ointment that also contains oxytetracycline, are used worldwide. Such mixtures are used to eliminate gram-negative bacterial infections, but prolonged use of oxytetracycline greatly increases the frequency of tetracycline-resistant bacteria in humans and feed animals.¹⁷ Drug resistance leads to more serious bacterial infections. Therefore, we suggest polymyxin B could be alternately combined with ciclopirox and oxytetracycline for improved killing of gram-negative bacteria. To compare the efficacies of both mixtures, the synergy between oxytetracycline and polymyxin B was measured by checkerboard assay with *E. coli* ATCC 25922 (Figure 1h). The MIC of oxytetracycline was 0.25 $\mu\text{g ml}^{-1}$ and the MIC of polymyxin B was reproducibly decreased by twofold with 0.125 $\mu\text{g ml}^{-1}$ oxytetracycline. However, no synergy was detected between polymyxin B and oxytetracycline at this concentration (FICI=1.0; addition). Therefore, ciclopirox plus polymyxin B provided better synergy than oxytetracycline plus polymyxin B. Therefore, the addition of ciclopirox to oxytetracycline/polymyxin B or the combination of ciclopirox and polymyxin B might provide an alternate to Terramycin.

Ciclopirox modifies the composition of the O-antigen, the outermost layer of the LPS structure;¹¹ thus, we suspect that the absence of O-antigen may inhibit the synergistic effect. This possibility was tested by checkerboard assays for MG1655, a strain lacking O-antigen,¹⁸ and the O-antigen producing *E. coli* ATCC25922, which showed synergy was not eliminated by the absence of O-antigen (FICI=0.312–0.5; Supplementary Figure 2 and Table 1). Therefore, O-antigen modification by ciclopirox does not increase susceptibility to polymyxin B.

Products of *galE* gene rescued bacterial growth at restrictive ciclopirox concentration and gene knockouts in *galU*, *rfaI*, *rfaB*, *rfaC* or *rfaQ* make cells susceptible to ciclopirox.¹¹ These genes are

Figure 1 Synergy of ciclopirox and polymyxin B. (a) Synergistic effects of ciclopirox and polymyxin B in *E. coli* cells. MIC and synergistic patterns in ATCC25922 and BW25113 are shown. (b–d) Effect of ciclopirox, polymyxin B or ciclopirox/polymyxin B on the growth and cell viability of *E. coli* BW25113 cells. One of the representatives from three independent experiments. (e) Synergistic effects of ciclopirox and polymyxin B in MDR *E. coli* strains. Clot images of CCARM 1A074, CCARM 1A078 and CCARM 1A645 strains are shown. (f, g) Conservation of synergy. Clot images are shown for *A. Baumannii* strains ATCC 15150, CCARM 12001 and 12003, and *P. aeruginosa* PAO1. (h) Evaluation of oxytetracycline as a synergistic agent to polymyxin B. The checkerboard assays were performed using *E. coli* ATCC 25922 strain. Synergistic combination is indicated with black circle. Absence of a clot indicates the bactericidal effect of antibiotics in mono- or combination-therapy.

associated with galactose modification and LPS biosynthesis. Therefore, we evaluated the role of specific gene products and pathways in the synergy between ciclopirox and polymyxin B by checkerboard assays and the Keio collection of *E. coli* and its parental strain BW25113 (Supplementary Figure 3 and Table 1). The genes were

divided into groups on the basis of the FICI values at the strongest synergic combinations: (i) non-regulatory genes *galE*, *rfaB*, *rfaC*, *rfaF*, *rfaI*, *rfaQ* and *rfaY* (FICI=0.312–0.375); (ii) partially effective genes *galU*, *rfaJ* and *rfaP* (FICI=0.5); and (iii) a partial synergy gene *rfaL* (FICI=0.625), which is the first gene in the synthesis of the outer core

of the LPS structure. Thus, the *rfaL* product is likely the most effective modulator of synergy. In addition, knockout of *rfaL* affected the susceptibility to polymyxin B. Our data suggest that efficient synergy between polymyxin B and ciclopirox requires modified sugar utilization LPS structure between component change through ciclopirox and disruption of structure by polymyxin B (Supplementary Figure 4).

Both polymyxin B and ciclopirox have been also used as topical fungal agents. Therefore, the synergy of ciclopirox and polymyxin B might also be useful as a new antifungal formulation, similar to the current use of fluconazole to reduce polymyxin B toxicity.¹⁹ Fluconazole is a widely used topical drug for treating superficial and systemic fungal infections; it has been used to reduce the high MIC of polymyxin B to clinically relevant concentrations in the treatment of *Aspergillus fumigatus*,¹⁹ thus reducing the side effects of polymyxin B. The administration of ciclopirox, a compound with an excellent safety profile and no fungal resistance in more than 20 years of clinical use,¹¹ warranted treatment of phase I hematologic malignant patients.²⁰ Alone, it does not produce severe toxicity or adverse effects in humans. For instance, dose-limiting gastrointestinal toxicities in patients with hematologic malignancies were not observed with oral administration of 80 mg m⁻² ciclopirox olamine four times daily; 10–40 mg m⁻² once daily administration did not produce dose-limiting toxicity. The effective dose of ciclopirox for human fungal or bacterial infection in combination with polymyxin B is much lower than the dose used to treat hematologic malignancy. In addition, <5% of treated patients showed mild local reactions following skin and vaginal application of ciclopirox.⁷ Therefore, ciclopirox may be more beneficial in combination with polymyxin B for the treatment of fungal infections.

In conclusion, we are the first to report ciclopirox with polymyxin B as a new synergistic combination with the ability to modulate the resistance of MDR *E. coli* and *A. baumannii*. This provides a potential alternative to the current limited use of polymyxin B in treating gram-negative bacterial infection. Detailed mechanistic studies including identification of genes modulated by ciclopirox or the combination of ciclopirox and polymyxin B are required to understand the synergy between these drugs. Clinical trials are also needed to support the use of this drug combination in the practical setting.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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