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Report

Overcoming Native Macrolide and Acquired Multidrug-Resistant *Pseudomonas aeruginosa* with Azithromycin and Polymyxin B Nonapeptide

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Semi-synthetic antibiotic, azithromycin (AZM) does not show significant bactericidal activity against *Pseudomonas aeruginosa* (*P. aeruginosa*). We focused on potential for AZM as a multi targeting drug against *P. aeruginosa* and found combination of AZM and Polymyxin B nonapeptide (PMBN) to increase the permeability of the outer membrane. This combination is effective for *P. aeruginosa* including multidrug-resistance clinical isolates and shows 32-512-fold potentiation of the anti-*P. aeruginosa* activity of AZM. We found a great opportunity to create new anti-*P. aeruginosa* drug candidates based on AZM and PMBN.

Key words azithromycin (AZM), polymyxin B nonapeptide (PMBN), *Pseudomonas aeruginosa* (*P. aeruginosa*), multidrug-resistant clinical isolates

INTRODUCTION

Antimicrobial resistance contributes to the death of approximately 700,000 people each year globally, and the World Health Organization (WHO) predicts that by 2050, the number could reach to 10 million unless actions are made to curtail antimicrobial resistance and new antibiotics are developed.[1)] The WHO has recently released a list of the drug-resistant bacteria which pose the greatest threat to human health and for which new antibiotics are urgently needed.[2)] The list ranks 12 bacteria or bacterial families with three tiers of priority. Among them, *Pseudomonas aeruginosa* (*P. aeruginosa*) infections have become a substantial concern in hospital-acquired infections, especially in critically ill and immunocompromised patients. Studies have demonstrated that *P. aeruginosa* could trigger severe septic shock and multiple organ dysfunction, and result in high mortality rate.[3)] The emergence and proliferation of *P. aeruginosa* is becoming a serious problem due to the limited number of viable treatment options.[4)] Compared to Gram-positive bacteria, Gram-negative bacteria are more challenging to combat with antibiotics due to the presence of an additional barrier, the outer membrane.[5)] The outer membrane defends Gram-negative bacteria from many antibiotics that are effective to treat infections with Gram-positive bacteria. In this regard, combinational therapies with cationic peptides, such as polymyxins and colistin, have the ability to increase the permeability of the outer membrane and have been found to a promising strategy for overcoming intrinsic resistance to certain classes of antimicrobials (e.g., macrolides, lincosamides, and rifamycins) in Gram-negative pathogens.[6-15)] Azithromycin (AZM) is a semi-synthetic macrolide antibiotic from erythromycin A and the most prescribed antibiotic for many Gram-positive and some Gram-negative bacterial infections in the U.S.[16)] AZM does not show significant bactericidal activity against *P. aeruginosa* at a clinically reasonable level. However, AZM, as an anti-inflammatory drug, is administered to cystic fibrosis patients with chronic *P. aeruginosa* infection as a life-long therapy because of the ability to reduce tissue inflammation.[17-18)] Furthermore, AZM suppresses quorum sensing signal molecules related to the virulence in *P. aeruginosa* at sub-minimum inhibitory concentrations (sub-MIC) and possesses anti-biofilm activity.[19-21)] Considering the potential for AZM as a multi-targeting drug against Gram-negative bacteria, some groups have shown that cationic peptides enhance the activity of AZM against Gram-negative pathogens in a synergistic manner.[6-12)] In this paper, we revealed that just double combination of AZM and Polymyxin B nonapeptide (PMBN) is effective against *P. aeruginosa*, including multi-drug resistant clinical isolates.

MATERIALS AND METHODS

Chemicals AZM was purchased from TCI chemicals. PMBN was purchased from Sigma-Aldrich.

Strains *P. aeruginosa* PAO1[22)] was given to us by Prof. Taiji Nakae. (Department of Molecular Life Science, Tokai University School of Medicine, 143 Shimokasuya, Isehara 259–1193, Japan). ATCC number strains were obtained from American Type Culture Collection. Eight clinically isolated *P. aeruginosa* strains with added KUB numbers which were

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Table 1. Checkerboard Assay with Azithromycin and Polymyxin B Nonapeptide for *P. aeruginosa*

Strains		AZM MIC (μg/mL) of combination PMBN concentration				
		PMBN (μg/mL)				
		0	1	2	4	8
Pseudomonas aeruginosa	PAO1	128	128	128	0.5	0.125
Pseudomonas aeruginosa	ATCC27853	512	256	64	≤ 0.5	≤ 0.5

Table 2. Combination Effect with Azithromycin and Polymyxin B Nonapeptide for *P. aeruginosa*

Strains	MIC (μg/mL)			
	AZM alone	PMBN alone	AZM MIC of combination with PMBN	
			PMBN (μg/mL)	AZM MIC
KUB3182	128	8	2	4
KUB3183	128	256	8	0.25
KUB3184	>128	>256	8	2
KUB3185	128	>256	8	≤ 0.125
KUB3188	128	8	2	1
KUB3198	256	>256	8	1
KUB3200	>128	>256	8	0.5
KUB3207	256	>256	8	0.25
PAO1	128	>256	8	0.25

collected from national surveillance in Japan were purchased from Three Academic Societies Joint Antimicrobial Susceptibility Surveillance Program.[23-25]

Antimicrobial Activity Minimum inhibitory concentrations (MICs) of the antimicrobial agents were determined with the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guideline.[26] Susceptibility testing was performed with Eiken dry plates (Eiken Chemical Co., Ltd., Tokyo, Japan), and *P. aeruginosa* ATCC27853 was used as a quality control strain.

Combination Assay The combination assay was performed using microbroth dilution method according to the Clinical Microbiology Procedures Handbook.[27]

RESULTS AND DISCUSSION

The antibacterial activity of PMBN against a panel of both gram-negative and gram-positive bacteria was decreased 2-64-fold comparing to that of polymyxin B, while the cytotoxicity against K562 cells was approximately 100-fold lower[28]and PMBN retains the potentiation activity of several antibiotics such as linezolid against *Klebsiella pneumoniae*.[10, 29,30] In this way, PMBN has been found to synergistically increase the drug sensitivity of Gram-negative bacteria to several antibiotics, including AZM,[31] due to its ability to increase the permeability of the outer membrane. However, the potentiation activity against *P. aeruginosa* of AZM by PMBN has not been validated. From this advantage, Espada *et al*. reported that triple combination of PMBN, the efflux pump inhibitor PAβN, and certain antibiotics generates a synergistic effect on *P. aeruginosa*.[10] Especially, this combination dramatically reduced the MIC of AZM against a standard laboratory-adapted strain, PAO1.[10] Based on Espada *et al.*'s report,[10] we optimized the combination assay and found that PMBN only has good potentiation activity of AZM with higher concentration than Espada *et al.*'s report (Table 1). The result of checkerboard assays against the strains of *P. aeruginos*a, PAO1 and ATCC27853, revealed that 4 μg/mL concentration of PMBN potentiated the antibacterial activity of AZM to 0.5 μg/mL and ≤ 0.5 μg/mL, respectively. With hope for repurposing AZM against *P. aeruginosa* at a clinically reasonable level, we decided to conduct the combination assay against multi-drug resistant clinical isolates and selected eight different strains whose drug sensitivity was shown in Table S1. According to Table S1, eight clinical isolates are highly resistant to several drugs having different mode of actions. Based on the MIC value of PMBN against clinical isolates, we set the concentration of PMBN for each strain (Table 2). Intriguingly, multi drug resistant strains, KUB3182 and KUB3188 show much higher drug sensitivity to PMBN than the other stains. According to Table S1, these two strains are resistant to multiple antibiotics with different types of modes of action. Therefore, we assumed that there are several mutations in the strains and one of which might alter drug sensitivity to PMBN. As we expected, this combination strategy is effective against multi-drug resistant clinical isolates such as β-lactam, fluoroquinolone, and aminoglycoside antibiotics resistance strains.

We have focused on the potential antibacterial activity of macrolide antibiotics against *P. aeruginosa* and screened potential anti-*P. aeruginosa* active compounds from our in-house macrolide library and found a semisynthetic macrolide, OMT.[15,32] Therefore, this study also suggests that macrolide antibiotics induce strong antibacterial activity against *P. aeruginosa* in combination with PMBN, providing a major foothold toward new therapeutic approaches. We are currently evaluating the effectiveness of this combination against other Gram-negative bacteria.

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Conflict of interest The authors declare no competing financial interest. K. S, N. A, and K. A are employed by Kowa Company LTD. H. H and Y. A receives research funding from Kowa Company LTD.

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