J Infect Chemother 25 (2019) 28–33

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original Article

An α-Lipoic acid derivative, and anti-ROS agent, prevents the acquisition of multi-drug resistance in clinical isolates of *Pseudomonas aeruginosa*☆

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ARTICLE INFO

Article history:
Received 13 August 2018
Received in revised form
27 September 2018
Accepted 5 October 2018
Available online 3 November 2018

Keywords:
Multi-drug resistance
Reactive oxygen species
Pseudomonas aeruginosa
Efflux pump
Sodium zinc histidine dithiooctamide

ABSTRACT

Pseudomonas aeruginosa is one of the most common causes of nosocomial infections, and its multi-drug resistance has been a serious problem worldwide. The aim of this study was to evaluate whether exposure to piperacillin and reactive oxygen species (ROS) could lead to multi-drug resistance for clinical isolates of *P. aeruginosa.* The inhibition of this acquired resistance by the anti-ROS agent was also examined.

In vitro inducement of multi-drug resistance was performed against 20 clinical isolates. These strains were incubated for 24 h and transferred 5 times after being exposed to 1 mM H_2O_2 (ROS) in addition to a sub-MIC of piperacillin by the agar dilution method. Each MIC of piperacillin and levofloxacin was determined.

As the mechanism of levofloxacin resistance, mutation of QRDR was investigated. The expression level of genes encoding efflux pumps; *mexA, mexY, mexC*, and D2 porin; *oprD* were determined by real-time PCR. Multi-resistance to both piperacillin and levofloxacin was induced with 4 of 20 strains (20%). No amino acid change was confirmed in QRDR. These strains showed overexpression of *mexA, mexY, mexC,* and another one showed decrease of *oprD* expression. Resistance development in 4 strains was inhibited by the same method including the anti-ROS agent, sodium zinc histidine dithiooctanamide (DHL-His-Zn).

In conclusion, stimulation by ROS promoted acquisition of multi-drug resistance in 20% of isolates of *P. aeruginosa*, and DHL-His-Zn completely inhibited this acquisition of resistance. Therefore, this anti-ROS agent may be useful to assist antimicrobial chemotherapy by preventing multi-drug resistance.

1. Introduction

Pseudomonas aeruginosa is one of the organisms most responsible for healthcare-associated infection. Immunosuppressed patients often suffer diseases such as sepsis, pneumonia, or urinary tract infection due to these bacteria. Recently, drug resistance causing these treatment failures has become a serious problem [1]. According to antimicrobial resistance surveillance reported by the European Center for Disease Prevention and Control (ECDC), the resistance rates in *P. aeruginosa* were 16.3% for piperacillin/tazobactam, and 15.0% for fluoroquinolones. Also, the combined resistance rate to at least three antimicrobial categories out of fluoroquinolones, aminoglycosides and carbapenems was 1.5% [2].

It is known that one of the causes of the appearance of drug-resistant bacteria is exposure to an antimicrobial agent. It was reported that *Escherichia coli* showed drug-resistant mutation when exposed to reactive oxygen species (ROS) [3]. And it is known that oxidative stress modulate porin expression for other gram-negative

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https://doi.org/10.1016/j.jiac.2018.10.003
1341-321X/

rod, *Serratia marcescens* [4]. The mechanism of multi-drug resistance for *P. aeruginosa* related to oxidative stress is oxidization of MexR (regulator of *mexAB-oprM*), and results to overexpression of efflux pump [5,6]. As a clinical case, it was reported that *P. aeruginosa* from patients with cystic fibrosis was exposed to an oxygen radical derived from white blood cell or respiratory air. And occurrence ratio of drug resistance and/or mutation of these strains were higher [7].

We reported that *P. aeruginosa* PAO1 acquired multi-drug (piperacillin and levofloxacin) resistance by exposure to both piperacillin and ROS [8]. However, there were few reports about the effect of ROS on the acquisition of drug-resistance in *P. aeruginosa*. In this study, the tendency to acquire multi-drug resistance in clinical isolates and the mechanism of resistance were investigated. Furthermore, the inhibitory effect on multi-drug resistance by the anti-ROS agent was confirmed.

2. Materials and methods

2.1. Bacterial strain and antibiotics

A total 413 clinical isolates of *P. aeruginosa* were collected from 16 general hospitals in the Tohoku district, Japan. Twenty clinical isolates which showed susceptibility to six anti-pseudomonal agents; piperacillin, levofloxacin, tazobactam/piperacillin, meropenem, ceftazidime and amikacin, and also their viability to survive against 1 mM H_2O_2, used as a ROS, were selected. The specimen origins of these strains are as follows: sputum (35%), urine (30%), pharynx (10%), and others (25%) (Table 1).

Powders of piperacillin (Taisho-Toyama Pharmaceutical Co., Ltd., Tokyo), levofloxacin (Wako, Osaka), amikacin (Wako), ceftazidime (Wako), meropenem (Sumitomo Dainippon Pharma Co. Ltd., Osaka), and tazobactam (Sigma-aldrich, Darmstadt, Germany) were used in this study. An α-lipoic acid derivative, sodium zinc histidine dithiooctamide (DHL-His-Zn) (Fig. 1) was used as anti-ROS agent [9–12]. Efflux pump inhibitors, carbonyl cyanide m-chlorophenyl hydrazone (CCCP: Wako) and phenylalanine-arginine-β-naphthylamide (PaβN: Sigma-aldrich) were also prepared.

Table 1
Antimicrobial susceptibilities and specimens against 20 clinical isolates of *Pseudomonas aeruginosa*.

Strain no.	origin	MIC (μg/mL)					
		PIPC	TAZ/PIPC	CAZ	MEPM	LVFX	AMK
Pa-1	sputum	2	2	1	0.125	0.125	1
Pa-2	pharynx	4	4	2	0.25	0.25	2
Pa-3	discharge of drain	2	2	1	0.125	0.25	1
Pa-4	pharynx	2	2	1	0.25	0.25	2
Pa-5	secretion	0.5	0.25	1	0.016	0.063	0.5
Pa-6	urine	1	1	0.5	0.125	0.25	1
Pa-7	urine	0.25	0.25	0.125	0.016	0.031	0.25
Pa-8	sputum	0.125	0.125	0.25	0.063	0.125	0.5
Pa-9	sputum	2	2	1	0.25	0.125	1
Pa-10	sputum	1	4	2	0.125	0.25	1
Pa-11	urine	1	1	0.5	0.25	0.5	4
Pa-12	urine	2	8	2	1	0.25	1
Pa-13	pus	4	4	4	0.25	0.5	2
Pa-14	sputum	0.5	0.5	2	0.016	0.063	4
Pa-15	sputum	4	8	2	0.25	0.25	2
Pa-16	urine	4	4	2	1	0.5	2
Pa-17	blood	4	4	4	0.5	0.25	2
Pa-18	urine	4	4	2	0.5	0.5	2
Pa-19	ascites	4	8	2	0.125	0.25	0.5
Pa-20	sputum	4	4	2	0.25	0.5	2

PIPC: piperacillin, TAZ/PIPC: tazobactam/piperacillin, CAZ: ceftazidime, LVFX: levofloxacin, MEPM: meropenem, AMK: amikacin.

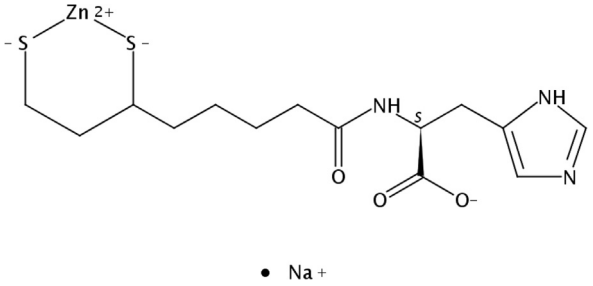


Fig. 1. The structure of sodium zinc histidine dithiooctamide.

2.2. Susceptibility testing

The MIC of each anti-pseudomonas agent was determined by the broth microdilution method. *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control strains. The break point MICs were interpreted according to EUCAST ver. 7.1 recommendations [13]. This was defined as the resistance when each MIC of piperacillin and levofloxacin showed >16 μg/mL and >1 μg/mL, respectively.

2.3. In vitro induction test of multi-drug resistance

The method for the *in vitro* induction test of multi-drug resistance previously described [8] was performed with some modifications. A bacterial suspension of McFarland No.2 was inoculated into Muller Hinton agar (MHA) to which was added sub-MIC of piperacillin and 1 mM H_2O_2, and was cultured at 37 °C for 24 h. The strain which had been growing on the agar was inoculated onto new component agar, and was cultured similarly. Such exposure incubation was repeated for five days. After five serial passages (5 days), MICs of piperacillin and levofloxacin were confirmed. It was further investigated whether the appearance of multi-drug resistance was inhibited due to the addition of 1 mM DHL-His-Zn, an anti-ROS agent [8]. As control experiments, each MIC was determined for isolates exposed to sub-MIC of piperacillin, 1 mM DHL-His-Zn or 1 mM H_2O_2.

2.4. Comparison of inhibitory effect to acquisition of multi-drug resistance about anti-ROS agent

Comparison test about inhibitory effect to acquisition of multi-drug resistance was performed, and *P. aeruginosa* PAO1 was used. As anti-ROS agent, ascorbic acid (Wako), glutathione (Wako) and DHL-His-Zn were prepared. It was investigated whether the acquisition of multi-drug resistance by stimulation of sub-MIC of piperacillin and 1 mM H_2O_2, was inhibited due to the addition of 1 mM each anti-ROS agent. Each MIC was determined by E-test method [8].

2.5. Multi-locus sequence typing

Multi-locus sequence typing (MLST) was performed and four wild type strains which showed multi-drug resistance were examined. Genomic DNA from each isolate was extracted by using InstaGene ™ Matrix (BIO-RAD, Hercules, CA). The resulted genomic DNA was used as template to amplify seven housekeeping genes: *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE* by the PCR method (T100™ Thermal Cycler, BIO-RAD). The PCR products were purified with Fast Gene™ Gel/PCR Extraction Kit (Nippon Genetics Co., Ltd., Tokyo) and analyzed according to Dye-terminator cycle sequencing method using the Genomelab™ GeXP GeXP ICEQ Genetic Analysis

System (Beckman Coulter, Brea, CA). Gene sequences were used to query the PubMLST database (http://pubmlst.org/paeruginosa/) to identify matches to known (numbered) alleles and ST types [14,15].

2.6. PCR-based ORF typing method (POT)

Four wild type strains used for MLST analysis were investigated about genotype correlation by PCR-based ORF typing method (POT) [16]. Cica geneus® Pseudo POT kit (KANTO KAGAKU, Tokyo) was used and PCR performed according to the instruction (T100™ Thermal Cycler, BIO-RAD), 25 cycles of amplification; 15s at 94 °C, 30s + 8s (8s was added per each cycle) at 65 °C. Then band patterns of genes were observed by 4% agarose gel electrophoresis.

2.7. DNA sequencing for the quinolone-resistance-determining region (QRDR)

The mutation in the gene of QRDR (*gyrA, gyrB, parC, parE*) of the strains which acquired resistance to both piperacillin and levofloxacin was confirmed by the Dye-terminator cycle sequencing method. Each PCR primer pair was used as described in the previous report [8]. The amplification procedure for *gyrA* and *parC* comprised denaturation at 95 °C for 3 min followed by 30 cycles of denaturation for 30s at 94 °C, annealing for 30s at 63 °C, and polymerization for 1 min at 72 °C, prior to final elongation at 72 °C. The protocol for *gyrB* was as follows: denaturation at 94 °C for 2 min, 25 cycles of amplification; 1 min at 94 °C, 30s at 62 °C, and 45s at 72 °C. For *parE,* 2-step PCR was conducted for 25 cycles with the following parameters: 30s at 94 °C, 1 min at 72 °C. The PCR products were purified by the procedure previously described, and the sequences were analyzed employing the Genomelab™ GeXP GeXP ICEQ Genetic Analysis System (Beckman Coulter).

2.8. Quantification of mRNA expression by real-time PCR for efflux pump and D2 porin

The acquired resistance of the multi-drug resistant strains to piperacillin and levofloxacin were investigated. The levels of expression of *mexA*, *mexY*, *mexC*, and *oprD* respectively encoding three efflux pumps, and D2 porin, were determined by real-time reverse transcription-PCR (real-time PCR; CFX Connect™ Real-Time System, BIO-RAD) as previously described [17] with some modifications. The primers were used for *mexA* and *oprD* as in the previous study [8], and the following pairs of primers were prepared: *mexY-1* (5′-CCGCTACAACGGCTATCCCT-3′) with *mexY-2* (5′-AGCGGGATCGACCAGCTTTC-3′), *mexC-1*(5′-AGCCAGCAGGACTTC-GATACC-3′) with *mexC-2*(5′-ACGTCGGCGAACTGCAAC-3′) [18,19]. Total RNA was eluted using the Aurum™ Total RNA mini kit (BIO-RAD). Then, 25 ng of purified total RNA was used for one-step reverse transcription and RT-PCR amplification, using the iTaq™ Universal SYBR® Green One-Step Kit (BIO-RAD). The reaction mix preparation and the thermal cycling protocol were as instructed by the manufacturer, and this experiment was performed in duplicate. Gene expression was normalized versus that of 16S ribosomal RNA (housekeeping gene) in each strain, and then calibrated relative to each wild type strain. In the normalized gene expression, ≥2 fold *mexA* expression and ≥5 fold expression for *mexY* and *mexC* were regarded as borderline overexpression [20,21]. Also, decreased *oprD* expression was considered relevant when the transcription level was ≤70% compared with that of *P. aeruginosa* wild type strains [18]. Additionally, the strains which showed efflux pump overexpression were investigated. The MICs of piperacillin and levofloxacin were tested either with or without an efflux pump inhibitor (EPI). 100 μM CCCP or 20 μg/mL PaβN were used as EPIs [22,23].

3. Results

3.1. Comparison of inhibitory effect to acquisition of multi-drug resistance about anti-ROS agent

Each MIC value of piperacillin and levofloxacin against *P. aeruginosa* PAO1 strain was increased to 32 μg/mL and 2 μg/mL by exposure of sub-MIC of piperacillin and H_2O_2, respectively. The addition of ascorbic acid or glutathione did not inhibit this multi-drug resistance, but DHL-His-Zn inhibited it dramatically (Table 2).

3.2. Change of MIC by exposure to sub-MIC of piperacillin and ROS, and the inhibitory effect of the anti-ROS agent

The MIC range of each anti-pseudomonas agent against the wild type strain was piperacillin: 0.125–4 μg/mL, piperacillin/tazobactam: 0.125–8 μg/mL, ceftazidime: 0.125–4 μg/mL, meropenem: 0.016–1 μg/mL, levofloxacin: 0.031–0.5 μg/mL, and amikacin: 0.25–4 μg/mL, respectively. Also, each MIC_{90} was piperacillin: 4 μg/mL, piperacillin/tazobactam: 8 μg/mL, ceftazidime: 2 μg/mL, meropenem: 0.5 μg/mL, levofloxacin: 0.5 μg/mL, and amikacin: 2 μg/mL. It was determined that these wild type isolates were all susceptible (Table 1).

A total 4 of 20 strains (20%; Pa-2, 12, 16, 18) acquired multi-drug resistance to both piperacillin and levofloxacin after exposure to sub-MIC of piperacillin and ROS. The acquisition of multi-drug resistance in these 4 strains was inhibited completely by the addition of DHL-His-Zn (Table 3).

3.3. Multi-locus sequence typing (MLST)

ST types were considered different of these 4 strains, but could not determine exact ST numbers (there were some novel combination of alleles). Approximate ST types were Pa-2 (109 or 317), Pa-12 (1161) and Pa-18 (1397 or 1470). The ST type of Pa-16 was predicted as some types (142, 183, 370, 419 and such like).

3.4. PCR-based ORF typing method (POT)

Agarose gel electrophoresis of POT for 4 wild types trains (Pa-2, 12, 16, 18) was shown in Fig. 2. It was confirmed that all 4 strains showed the different patterns of amplified band.

3.5. DNA sequencing for the quinolone-resistance-determining region (QRDR)

Of these 4 multi-drug resistant strains (Pa-2, 12, 16, 18), no strain showed mutations in *gyrA*, *gyrB*, *parC* and *ParE*.

Table 2
Inhibitory effect by each anti-ROS agent addition to *P. aeruginosa* PAO1strain that acquired multi-drug resistance by exposure to piperacillin plus ROS (H_2O_2).

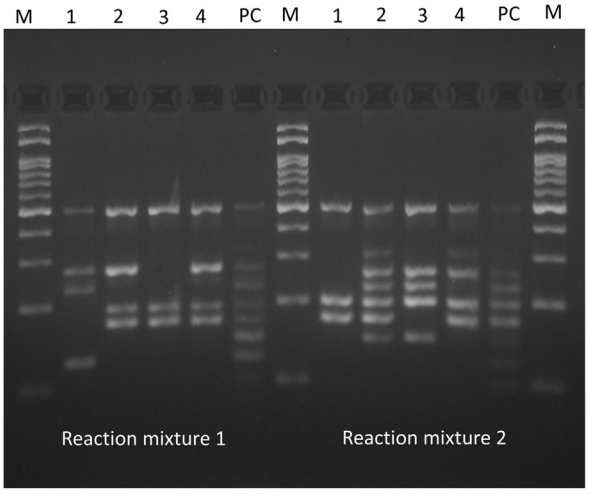
exposed by following agents (5 days)	MIC (μg/mL)	
	PIPC	LVFX
PAO1	2	0.25
H_2O_2 + PIPC	32	2
H_2O_2 + PIPC + DHL	2	0.5
H_2O_2 + PIPC + ASA	32	1
H_2O_2 + PIPC + GSH	64	1

PIPC: piperacillin, LVFX: levofloxacin, DHL: DHL-His-Zn, ASA: ascorbic acid, GSH: glutathione.

Table 3
Inhibitory effect by DHL-His-Zn addition to four strains that acquired multi-drug resistance by exposure to piperacillin plus ROS (H_2O_2).

Strain no.	MIC (μg/mL)											
	None (WT)		DHL		H_2O_2		PIPC		PIPC + H_2O_2		PIPC + H_2O_2 + DHL	
	PIPC	LVFX	PIPC	LVFX	PIPC	LVFX	PIPC	LVFX	PIPC	LVFX	PIPC	LVFX
Pa-2	4	0.25	4	0.5	4	1	32	1	32	2	8	0.5
Pa-12	2	0.25	4	0.25	4	0.5	8	0.25	128	2	16	0.5
Pa-16	4	0.5	4	0.5	4	0.5	64	2	128	2	64	1
Pa-18	4	0.5	4	0.5	4	0.5	64	2	64	2	32	0.5

PIPC: piperacillin, LVFX: levofloxacin, DHL: DHL-His-Zn.



M: maker, 1: Pa-2, 2: Pa-12, 3: Pa-16, 4: Pa-18, PC: positive control

Fig. 2. Agarose gel electrophoresis patterns of PCR-based ORF typing for 4 wild type strains.

3.6. Quantification of mRNA expression by real-time PCR for efflux pump and D2 porin

The transcription expression levels of *mexA*, *mexY*, *mexC* and *oprD* in the 4 strains which showed multi-drug resistance, are shown in Table 4. There were few changes mRNA level of control (16S ribosomal RNA) by treatment with piperacillin, H_2O_2, and DHL-His-Zn (Supplementary Fig. 1). Three (Pa-2, 12, 18) of these 4 strains showed overexpression of each efflux pump gene. Each expression of *mexA*, *mexY*, and *mexC* was increased by 4.59–10.84 fold, 5.08–9.85 fold and 5.00–11.84 fold, respectively. On the other hand, expression of *oprD* decreased by 0.55 fold in one other strain (Pa-16). It was confirmed that each gene expression recovered to a value close to the parent strain due to addition of DHL-His-Zn. However, the expression of *mexA* in 2 strains (Pa-2, -12) was inhibited only by 4.2 fold, 3.41 fold, respectively.

3.7. MIC changes of multi-drug resistant strains by efflux pump inhibitor

For three strains (Pa-2, -12, –18) which showed overexpression of each efflux pump gene, the MIC changes of piperacillin and levofloxacin after addition of 100 μM CCCP or 20 μg/mL of PaβN are shown in Table 5. By addition of CCCP, the MIC of piperacillin was reduced to 1/2–1/32 in these 3 strains. Of these, in two strains (Pa-12 and -18), the MIC of levofloxacin was less than 1/2. Similarly, for 20 μg/mL PaβN, the MIC changes were 1/4–1/16 for piperacillin, and 1/16–1/32 for levofloxacin.

4. Discussion

It is known that *in vivo* ROS are produced with ATP by mitochondria and with an immune response or killing of microorganisms by leukocytes. Generally, the resolution of *in vivo* ROS occurs rapidly by catalase and/or superoxide dismutase, but excessive ROS occasionally oxidize lipids, proteins, and enzymes, and cause DNA damage [24,25]. Although these ROS show bactericidal effect at high density against bacteria at the infection site, they may cause gene mutations resulting in drug resistance at low density [26]. In other words, pathogens are exposed to low concentrations of ROS from neutrophils during a period of infection *in vivo*. For example, it can be hypothesized that *P. aeruginosa* is exposed to anti-pseudomonas agents whose concentrations are lower than the MIC and ROS in the case of a pseudomonas infection. Therefore, an *in vitro* experimental model for induction of multi-drug resistance was designed with ROS added to anti-pseudomonas agents. Though H_2O_2 is released from white blood cells at the infection site before the antimicrobial agent administration, drug resistance of *P. aeruginosa* will not occur with that alone. H_2O_2 may function as a kind of accelerator of the resistance, and MIC did not increase by only H_2O_2 exposure in Table 3. H_2O_2 decomposed rapidly by catalase of *P. aeruginosa*, so it did not work as a mutagen enough to make drug-resistance in 5 days experiment. In this study, two of four strains acquired the resistance by exposure of piperacillin alone. The tendency to drug-resistance acquisition by exposure of antimicrobial agent was already known. However, it was found that

Table 4
Changes of mRNA expression encoding efflux pumps or D2 porin, and the recovery effect by DHL-His-Zn.

Strain no.	Relative gene expression (fold)											
	mexA			*mexY*			*mexC*			*oprD*		
	HP[a]		HPD[b]	HP[a]		HPD[b]	HP[a]		HPD[b]	HP[a]		HPD[b]
Pa-2	7.12	→	4.20	9.85	→	3.49	11.16	→	4.09	2.52	→	0.98
Pa-12	10.84	→	3.41	9.79	→	4.03	11.84	→	4.19	5.61	→	1.52
Pa-16	0.49	→	1.20	0.44	→	1.42	0.54	→	1.61	0.55	→	1.11
Pa-18	4.59	→	1.06	5.08	→	2.16	5.00	→	2.22	2.64	→	0.50

[a] Exposed to 1 mM H_2O_2 + sub-MIC of piperacillin.
[b] Exposed to 1 mM H_2O_2 + sub-MIC of piperacillin + 1 mM DHL-His-Zn.

Table 5
MIC change by each efflux pump inhibitor.

Strain no.	MIC (μg/mL)							
	piperacillin				levofloxacin			
	CCCP (-)	CCCP (+)	PAβN (-)	PAβN (+)	CCCP (-)	CCCP (+)	PAβN (-)	PAβN (+)
Pa-2	16	8	32	8	2	2	2	0.125
Pa-12	128	4	64	8	4	1	2	0.125
Pa-18	64	32	64	4	2	1	2	<0.063

CCCP: carbonyl cyanide m-chlorophenyl hydrazone.
PaβN: phenylalanine-arginine-β-naphthylamide.

MICs were more increased by exposure of H_2O_2 plus piperacillin (Table 3).

In the previous study, an exposure to various anti-pseudomonas agents and H_2O_2 as the ROS using the *P. aeruginosa* PAO1, was performed. Piperacillin induced cross-resistance against levofloxacin, and the PAO1 strain acquired multi-drug resistance [8]. The mechanism of multi-drug resistance was suppression of D2 porin. In this study using clinical isolates, one of four strains which acquired multi-drug resistance, similarly showed suppression of D2 porin. It is considered that both piperacillin and levofloxacin penetrate bacterial cells through the outer membrane pore called D2 porin [27]. The reduction of porin pores resulted in a decrease of antimicrobial diffusion into bacteria, and cross-resistance.

The other three strains showed overexpression of *mexA*, *mexY*, and *mexC*. MexAB-OprM, MexCD-OprJ and MexXY are known for RND efflux pumps, and these pumps extrude not only penicillin but also fluoroquinolone [27,28]. Exposure to ROS and piperacillin leads to overexpression of these efflux pumps, and fluoroquinolones were simultaneously expelled. This is therefore another multi-drug resistance mechanism.

RND efflux pump require energy dependent on protons (H^+) for active transport [29,30]. In this data, the MICs of 3 strains which showed overexpression of *mexA, mexY* and *mexC* were decreased due to addition of each EPI. The MICs of levofloxacin decreased to 1/16-1/32 with PAβN, and remained at 1-1/4 with CCCP. The effect of PAβN is mainly competitive inhibition of the efflux system of fluoroquinolones [31]. Therefore, the efflux pump expels PAβN instead of levofloxacin, and levofloxacin remains intracellularly. Hence, in this study, the MICs of levofloxacin were dramatically decreased as in previous reports [23,31,32]. In contrast, CCCP is also known as an uncoupler. Outside the outer membrane, the concentration of H^+ is kept higher, and antibiotics are expelled with exchange of H^+. CCCP captures H^+ and reduces the difference in the concentration gradient of H^+. By inhibition of proton motive force, the rate of elimination is slowed, and this results in an increase of intracellular antibiotic concentration [29,33]. The reason that the decrease of the MIC due to CCCP was low is because the effect is not selective for the antibiotic, and the activity of the efflux pump was indirectly reduced. For these 3 strains, the MICs of levofloxacin and piperacillin decreased due to addition of EPIs. This result suggested that overexpression of the efflux pump was related to multi-drug resistance.

The period of this *in vitro* induction of multi-drug resistance was 5 days, and the mutation of QRDR was not determined for isolates with acquired levofloxacin resistance. The resistance mechanism was considered to be that the functions of the outer membrane were modified. Because antibiotic exposure is prolonged for infections such as empyema, pyelonephritis and sepsis, the causative bacteria may be influenced by ROS, and QRDR mutations may occur. The MIC of levofloxacin in these mutants shows a higher value. Also, it was reported that susceptibility did not recover by consecutive subcultures [34]. Piperacillin is used as a common combination agent with tazobactam, and the dosing period is often prolonged. For the emergence inhibition of antimicrobial resistance, appropriate use of such antimicrobial agents is important.

Finally, these results suggested that the frequency of acquisition of multi-drug resistance for *P. aeruginosa* clinical isolates had been increased by stimulation of ROS, and oxidative stress is one of the important factors. All acquisitions of multi-drug resistance were suppressed by the addition of the ROS scavenger DHL-His-Zn. The result that each gene expression of *mexA, mexY, mexC* and *oprD* almost recovered to the value in each parent strain was confirmed. DHL-His-Zn used as the anti-ROS agent is an α-lipoic acid derivative, and has antioxidant effect due to inactivating free radicals directly [35]. The inhibitory effects on acquisition of multi-drug resistance were compared with other typical antioxidants, i.e., ascorbic acid and glutathione. As a result, DHL-His-Zn showed the most remarkable effect. In fact, this agent has already been practically used as a medicine for external use to obtain an inhibitory effect on melanin generation in the skin [36], or alopecia in cancer chemotherapy [10]. And Renal ischemia-Reperfusion models of rat were treated with DHL-His-Zn by continuous intravenous infusion *in vivo*, and it was reported that renal injury was reduced [11]. Therefore it was considered that DHL-His-Zn was distributed by blood stream, and could reach to infection site. However, for *mexA* overexpression of Pa-2 and Pa-12, the changes of mRNA transcription levels did not recover completely. It may be that the antioxidant effect of DHL-His-Zn is not sufficient. It was suggested that developing a stronger anti-ROS agent is important to suppress the appearance of multi-drug resistant strains.

In conclusion, we showed that 4 of 20 (20%) clinical isolates of *P. aeruginosa* acquired multi-drug resistance stimulated with a low dose of piperacillin and oxidative stress by ROS. Additionally, the anti-ROS agent may be useful as a compound inhibiting the multi-drug resistance of *P. aeruginosa* because the addition of DHL-His-Zn inhibited all multi-drug resistance.

Conflicts of interest

Akira Watanabe received speaker honoraria from MSD K.K., Kobayashi Pharmaceutical Co., Ltd., Shionogi & Co., Ltd., Daiichi Sankyo Co., Ltd., Taisho Toyama Pharmaceutical Co., Ltd., Sumitomo Dainippon Pharma Co., Ltd., Mitsubishi Tanabe Pharma Co., Chugai Pharmaceutical Co., Ltd., Pfizer Japan Inc.; donations from Astellas Pharma Co., Ltd., Daiichi Sankyo Co., Ltd., and Sumitomo Dainippon Pharma Co., Ltd., Shigeru Fujimura received speaker honoraria from MSD K.K., and Taisho Toyama Pharmaceutical Co., Ltd. Other authors report no conflicts of interest.

Funding

None.

Acknowledgement

We thank Emiko Furukawa as research assistant. This study won prize for encouragement in 66th Congress of the Japanese Society of Chemotherapy in 2018.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jiac.2018.10.003.

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