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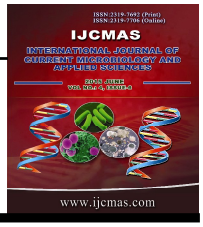

Original Research Article

Retention of Glutathione-Specific Acidity and Disruption of Intracellular Glutathione-Redox Homeostasis are Associated with Antibacterial Activity in *Pseudomonas aeruginosa*

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Keywords

Glutathione, Antibacterial activity, *Pseudomonas aeruginosa*

A B S T R A C T

Glutathione, a tri-peptide (γ-L-glutamyl-L-cysteinyl-glycine), is an intracellular antioxidant possessing one sulfhydryl and two free α-carboxyl groups. The sulfhydryl group plays a key role in optimizing intracellular redox potential with a variety of cellular functions. Antibacterial activity of exogenous glutathione was reported in *Pseudomonas aeruginosa*, a notorious multidrug-resistant nosocomial pathogen. However, molecular details of this antibacterial activity are currently unclear. Here, we tried to understand molecular details of the antibacterial activity in *P. aeruginosa*. Exogenous glutathione, in a dosage-dependent manner, acidified pure water and culture media. Cellular growth was significantly inhibited by pH 5.0 produced by glutathione (11 mM) but unaffected by the same pH produced by HCl. Bacterial cells were killed 100% by pH 4.5 produced by glutathione (14 mM) but grew (>10^3 cells/mL) at the same pH produced by HCl. The level of pH was retained during the cellular growth with glutathione but the same cellular growth with HCl was neutralized within 5 hours. Intracellular levels of glutathione were 70±16, 234±40, and 382±101 nmol in cells grown with glutathione at 0, 10, and 11 mM, respectively. Multidrug resistant clinical isolates of *P. aeruginosa* were all sensitized by all tested-antibiotics in combination with sub-inhibitory concentrations of glutathione. These results suggest that retention of the glutathione-mediated acidity along with the excess amount of intracellular glutathione is critical for the antibacterial activity. The results also suggest that exogenous glutathione alone or in combination with existing antibiotics may be useful to treat multidrug resistant *P. aeruginosa* infections.

Introduction

Pseudomonas aeruginosa is a Gram-negative human pathogen that accounts for 11 to 14% of all nosocomial infections (Pittet *et al.*, 1999; Kim *et al.*, 2000;

Wisplinghoff *et al.,* 2004). Treatment of *P. aeruginosa* infections however is difficult mostly due to intrinsic resistance to a variety of antibiotics and its capability of acquiring resistance to almost all commercially available antibiotics (Falagas and Bliziotis 2007; Falagas *et al.,* 2008; Perez *et al.,* 2014). Clinical isolates of *P. aeruginosa* are commonly resistance to multiple anti-pseudomonas antibiotics and often untreatable. Successful therapy will require development of a new anti-pseudomonas drug or a novel strategy using currently available antibiotics (Falagas and Bliziotis, 2007; Lee and Ko, 2012; Pena *et al.,* 2012; Koutsogiannou *et al.,* 2013).

Glutathione is an antioxidant composed of three amino acids (L-glutamate, L-cysteine, and L-glycine) synthesized in all eukaryotic cells and in many bacteria (Smirnova and Oktyabrsky, 2005). In *E. coli*, glutathione is synthesized by two sequential ATP-dependent reactions catalyzed by γ-glutamyl-cysteine synthetase and glutathionesynthetase. The molecular structure of glutathione consists of a γ-peptide linkage between the carboxyl group of the glutamate side-chain and the amino group of cysteine which is attached by normal peptide linkage to the glycine. A sulfhydryl (thiol) from cysteine is a major functional group for the glutathione (Huang *et al.,* 1988, Yamaguchi *et al.,* 1993).Glutathione also possesses two free α-carboxyl groups but their biological functions are currently unclear.

Intracellular glutathionein *E. coli* exists predominantly (>99%) in the thiol-reduced form. The remaining amount undergosthiol oxidation to form glutathione-disulfide and mixed-disulfides with target proteins. Glutathione is a more important intracellular redox buffer compared to NAD(P)H and other intracellular redox systems (Fahey *et al.,* 1978, Smirnova *et al.,* 2000). A central role of glutathione is maintenance of the particular reduction potential in the cytoplasm required for normal cellular metabolism (Schafer and Buettner, 2001). Alterations of the glutathione redox potential can impair glutathione function and levels of intracellular glutathione are strictly regulated by synthesis, degradation, and transport (Smirnova and Oktyabrsky, 2005).

In bacteria, key functions of glutathione, as reviewed by Smirnova and Oktyabrsky (Smirnova and Oktyabrsky, 2005) and Masip *et al.* (Masip *et al.,* 2006), include activation or inactivation of redox-sensitive proteins to regulate cellular process (e.g., transcriptional regulator OxyR); regulation of intracellular potassium concentration which plays a role in maintenance of cell turgor and intracellular pH; deactivation of toxic substances by glutathione-conjugate formation followed by degradation of the glutathione-conjugate for eventual transport out from cells; and finally, glutathione is involved in adaptation to various stresses (e.g., oxidative stress, temperature stress, or osmotic stress). Glutathione is also associated with antibacterial activity and antimicrobial susceptibility (Zhang and Duan, 2009; Schairer *et al.,* 2013).

However, molecular details of the antibacterial activity of glutathione remain unclear. In this study, we elucidated the role of exogenous glutathione in antibacterial activity. Our results suggest that retention of glutathione-mediated acidity and excess amount of intracellular glutathione are critical for the antibacterial activity in *P. aeruginosa*. The antibacterial activity of exogenous glutathione significantly enhanced antibiotic susceptibility in *P. aeruginosa*.

Materials and Methods

Bacterial strains, growth conditions, and chemicals

P. aeruginosa PAO1 and clinical isolates of *P. aeruginosa* from the previous studies (Barrow and Kwon, 2009) were used. All bacterial strains were routinely grown on Luria-Bertani (LB; Becton, Dickinson and Company, Sparks, MD) agar plates or broth at 37°C. All antibiotics, L-glutathione (reduced-glutathione), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Antibiotics or glutathione were added in the growth media when needed.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of glutathione, HCl (as a pH), and antibiotics

MIC and MBC levels of glutathione, HCl, and antibiotics were determined as guided by the Clinical and Laboratory Standards Institute (CLSI) (Kwon and Lu, 2006) with a minor modification. Briefly, Mueller-Hinton broth (MHB; Oxoid, Ogdensburg, NY) or LB broth containing glutathione (0, 5, 10, 11, 12, 13, 14, 15, and 20 mM), HCl, and/or antibiotics were dispensed into sterile 17- by 100-mm snap-cap Falcon culture tubes (1 mL/tube; Fisher Scientific). Stability of the pH levels in the broth media produced by glutathione or by HCl was confirmed by overnight culture of the media before MIC measurement. Fresh overnight cultures of *P. aeruginosa* PAO1 and clinical isolates were diluted in saline to an optical density at 600 nm of 0.1 to 0.12 (approximately 1 to 5 × 10^8 viable cells per mL; the viable cell numbers were determined by culturing the inoculum on plain LB agar plates). A portion of the adjusted cell suspension (5 μL) was inoculated to each of the Falcon culture tubes. The cells were incubated overnight (18 h) without shaking at 37°C. MIC was defined as the lowest concentration of glutathione, antibiotics, or the lowest pH that completely inhibited the cellular growth of the inoculum. To determine MBC, the cells incubated overnight for MIC measurement were spread on plain LB agar plates (10 plates for 1 mL) and incubated 24 hours at 37°C. MBC was defined as the lowest concentration of glutathione, antibiotics, or the lowest pH that killed 100% of the inoculum. Determination of MIC and MBC was repeated three times to confirm the results.

Growth curve determination

Bacterial growth curve was determined as described previously (Kwon and Lu, 2007). Briefly, *P. aeruginosa* PAO1 was cultured overnight in LB broth (3 mL) and the cells were diluted 100-fold in 100 mL of prewarmed MHB containing glutathione (11 mM; pH 5.0), HCl (pH 5.0) or MHB alone. The cells were incubated in a rotary shaker (250 rpm) (Excella E24; New Brunswick Scientific, Edison, NJ) at 37°C. Growth of the cells was monitored by optical density at 600 nm. The growth curve determination was repeated three times to confirm the results.

Killing assay of *P. aeruginosa*

Bacterial killing assay of *P. aeruginosa* PAO1 was performed as previously described (Kwon and Lu, 2007) with a minor modification. Briefly, the cells from MIC measurement for glutathione and pH levels were spread on plain LB agar plates with appropriate dilution. The LB agar plates were incubated for 24 hours at 37°C and colony forming units were counted for each concentration of glutathione or pH levels. The bacterial killing assay was repeated three times to confirm the results.

Measurement of intracellular glutathione

Intracellular levels of glutathione (reduced and oxidized) were measured as described by Akerboom and Sies (Akerboom and Sies, 1981) using glutathione assay reagents (Sigma-Aldrich, St. Louis, MO). Cells cultured overnight were diluted (1/100) into fresh MHB (50 mL) supplemented with 0, 10, and 11 mM glutathione. The diluted cells were grown in the rotary shaker (250 rpm) at 37^{o}C. The cells (1 mL) were collected at optical density (OD_{600}) of 1.0 and washed three times with phosphate buffer (pH 7.0). The washed cells were re-suspended in 1 mL of the phosphate buffer and broken by sonication (Misonix Sonicators, Newtown, CT; 37% amplitude [15W], 10 seconds, 10 times with 30 second cooling between bursts, until the cell extract was clear). The crude extract of the cells was spun down at 15,000 rpm for 5 min and the supernatant was used to measure levels of intracellular glutathione. The levels of glutathione were measured using a kinetic assay in which catalytic amounts of glutathione cause a continuous reduction of 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) to TNB, and the product (TNB) was assayed calorimetrically at 412 nm using a DU® 730UV/Visible spectrophotometer (Beckman Coulter, Inc; Indianapolis, IN). Glutathione standard solutions were used to calculate amount of glutathione and the calculation (nmoles glutathione per mL of sample) was (ΔA_{412}/min [sample] x dilution factor of original sample)/(ΔA_{412}/min [1 nmol] x volume of sample in the reaction in mL).

Results and Discussion

Acidity of glutathione

Acidity of glutathione was determined in pure water (ddH_2O) and in culture media. Glutathione at concentrations of 10, 100, and 250 mM dissolved in pure water exhibited pH levels of 3.0, 2.8, and 2.7, respectively. Solubility of glutathione in pure water is very poor at concentrations greater than 250 mM. The acidity of glutathione was also determined in broth culture media (MHB and LB). pH levels of MHB alone and LB broth alone, prepared by vendors' formula, was 7.1 and 6.7, respectively. MHB containing glutathione at concentrations of 5, 10, 11, 12, 13, 14, 15, and 20 mM exhibited pH levels of 5.9, 5.2, 5.0, 4.8, 4.6, 4.5, 4.4, and 4.1, respectively. LB broth containing glutathione at concentrations of 5, 10, 11, 12, 13, 14, 15, and 20 mM exhibited pH levels of 5.9, 5.0, 4.7, 4.6, 4.5, 4.4, 4.3, and 4.0, respectively. These results indicate that exogenous glutathione significantly acidifies the media in a dosage-dependent manner. Glutathione is composed of three amino acids with two free α-carboxyl groups from glutamate and glycine (Smirnova and Oktyabrsky, 2005). The free α-carboxyl groups may be involved in the acidification.

Antibacterial activity of exogenous glutathione

MIC and MBC of exogenous glutathione were 12 mM (pH 4.8) and 14 mM (pH 4.5) in *P. aeruginosa* PAO1, respectively. Similar results were also obtained from 3 clinical isolates of *P. aeruginosa* (Table 1). To understand the role of glutathione-mediated acidity in antibacterial activity, growth rate was determined in MHB supplemented with a sub-inhibitory concentration of glutathione (11 mM; pH 5.0) and the same medium adjusted to the same pH 5.0 with HCl. Results revealed that the sub-inhibitory concentration of glutathione significantly inhibited cellular growth. However, the growth rate in the same medium was similar as that in MHB

alone at the same pH 5.0 with HCl (Fig. 1). To clarify these results bacterial killing-effects of glutathione and HCl were compared. As shown in Fig. 2, 100% of cells were killed at 14 mM glutathione (pH 4.5); in contrast, a significant number of cells ($>10^3$ per mL) grew in the same medium at pH 4.5 with HCl. To further understand the role of glutathione-mediated acidity in the antibacterial activity pH levels were monitored during the cell culture supplemented with glutathione (11 mM; pH 5.0) or HCl (pH 5.0).

As shown in Table 2, acidity produced by glutathione was retained for 5 hours with significantly inhibiting the cellular growth while the acidity produced by HCl was neutralized during the same time period with a normal cellular growth as shown in the same medium without any supplement. Importantly, the antibacterial activity of glutathione however was almost all abolished when the culture medium for the growth curve and the bacterial killing-effect was adjusted to a neutral pH (7.0) (data not shown). These results suggest that retention of the acidity is critical for the antibacterial activity. Although molecular details of the antibacterial mechanism of exogenous glutathione is still need to clarify, one possible mechanism is that glutathione itself may be acting as a buffer that resists cellular functions counteracting the acidity.

Levels of intracellular glutathione causing the antibacterial activity

Intracellular levels of glutathione causing the antibacterial activity were measured in *P. aeruginosa* PAO1 grown with or without exogenous glutathione. Results revealed that an intracellular level of glutathione was 70 ± 16 nmol in cells (OD_{600} 1.0) grown in MHB alone. The same cells grown with the sub-inhibitory concentrations of exogenous glutathione (10 mM and 11 mM) exhibit 234 ± 40 and 382 ± 101 nmol of intracellular glutathione, respectively (Fig. 3). These results suggest that *P. aeruginosa* synthesizes substantial levels of glutathione and an intracellular level of glutathione causing the antibacterial activity is at least over 5-fold higher than that present in normal cellular growth. An optimum level of intracellular glutathione is essential for the normal cellular growth, which is correlated to a variety of physiological functions such as redox-based cellular regulation, deactivation of toxic substances, and adaptation to various stresses (Smirnova and Oktyabrsky, 2005; Masip *et al.*, 2006).

In *E. coli*, a reduced-form of glutathione is predominant (>99%) and the others are an oxidized-form of glutathione (glutathione disulfide or GSSG) and glutathionylated proteins. Since the GSSG and glutathionylated proteins can react with free sulfhydryl groups of any cellular components, they are toxic to cells and must be recycled to the reduced form of glutathione(Fahey *et al.*, 1978; Alonso-Moraga *et al.*, 1987; Smirnova *et al.*, 2000). The excess levels of intracellular glutathione may increase levels of the GSSG and glutathionylated proteins as well as disrupt an optimum redox potential, which may lead to the antibacterial activity of exogenous glutathione.

Antibacterial activity of exogenous glutathione on antibiotic susceptibility

MIC measurement of antibiotics in combination with glutathione or HCl was performed to understand the antibacterial activity of exogenous glutathione on antibiotic susceptibility. In *P. aeruginosa* PAO1, MIC levels of all tested antibiotics in combination with 11 mM glutathione were significantly decreased when compared to those in MHB alone.

Table.1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of glutathione in *P. aeruginosa*

Isolate	MIC and MBCof glutathione (mM) [a]	
	MIC (pH)	MBC (pH)
PAO1	12 (4.8)	14 (4.5)
NY214[b]	10 (5.2)	12 (4.8)
NY215	10 (5.2)	13 (4.6)
NY220	11 (5.0)	14 (4.5)

[a]MIC and MBClevels were measured in Muller-Hinton broth and repeated three independent measurementswith identical results.
[b]Clinical isolates (NY214, NY215, NY220) were obtained from the previous report (Barrow and Kwon, 2009)

Table.2 Acidity levels during cellular growth with glutathione or HCl in *P. aeruginosa* PAO1

Culture condition	pH (cell density at OD_{600}) in a culture medium [a]					
	0	1hr	2hr	3hr	4hr	5hr
None	6.7 (0.051)	6.7 (0.061)	6.7 (0.181)	6.7 (0.725)	6.8 (1.548)	7.0 (1.880)
GSH (11 mM; pH 5.0)	5.0 (0.045)	5.0 (0.048)	5.0 (0.041)	5.0 (0.055)	5.1 (0.140)	5.2 (0.281)
HCl (pH 5.0)	5.0 (0.042)	5.1 (0.058)	5.5 (0.155)	6.0 (0.709)	6.5 (1.518)	6.7 (1.727)

[a]Muller Hinton broth was used for the culture medium. Three independent experiments were repeated with similar results and one representative result was shown here

Table.3 Effect of exogenous glutathione on antibiotic susceptibility of *P. aeruginosa* PAO1

Antibiotics	MICs (μg/mL)[a] in the presence of glutathione (GSH) or HCl					
	None (pH 7.1)[b]	GSH (10 mM) (pH 5.2)	GSH (11 mM) (pH 5.0)	GSH (11 mM) (pH 7.1)	HCl (pH 5.0)	HCl (pH 4.8)
Aztreonam	8	32	0.25	32	32	16
Carbenicillin	64	128	≤0.25	128	128	128
Ceftazidime	1	4	≤0.25	4	4	2
Meropenem	1	4	≤0.25	16	1	1
Chloramphenicol	128	16	≤0.25	64	16	16
Ciprofloxacin	0.25	4	≤0.25	0.25	4	2
Gentamicin	0.5	8	≤0.25	2	8	8
Tetracycline	4	1	≤0.25	32	2	2
None[c]	+++	+++	+	++	+++	+++

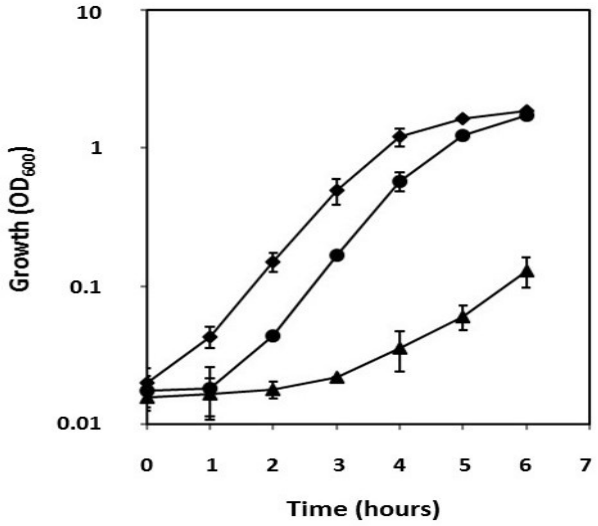
[a]MIC measurement was repeated three times with identical results in Muller-Hinton broth (MHB).MIC results were recorded after 18 hours' incubation without shaking at 37°C.
[b]pH levels of MHB containing GSH or HCl.
[c] Levels of cell density in MHB; visible to <1.0 (+), >1.0 to 2.0 (++), >2.0 (+++) at OD_{600}.

Table.4 Effect of sub-inhibitory concentrations of exogenous glutathione on antibiotic susceptibility in clinical isolates of *P. aeruginosa*

Antibiotics	Compound	MICs (μg/mL)[a] of *P. aeruginosa* clinical isolates		
		NY214 [c]	NY215	NY220
Aztreonam	none	8	4	32
	GSH [b]	≤0.25	≤0.25	1
Carbenicillin	none	128	256	>256
	GSH	≤0.25	≤0.25	≤0.25
Ceftazidime	none	8	4	16
	GSH	≤0.25	≤0.25	≤0.25
Meropenem	none	16	16	32
	GSH	0.5	0.5	≤0.25
Chloramphenicol	none	16	64	64
	GSH	≤0.25	≤0.25	≤0.25
Ciprofloxacin	none	16	2	32
	GSH	≤0.25	≤0.25	≤0.25
Gentamicin	none	256	2	4
	GSH	1	≤0.25	≤0.25
Novobiocin	none	>256	>256	>256
	GSH	≤0.25	≤0.25	≤0.25
Tetracycline	none	8	16	32
	GSH	0.25	≤0.25	≤0.25

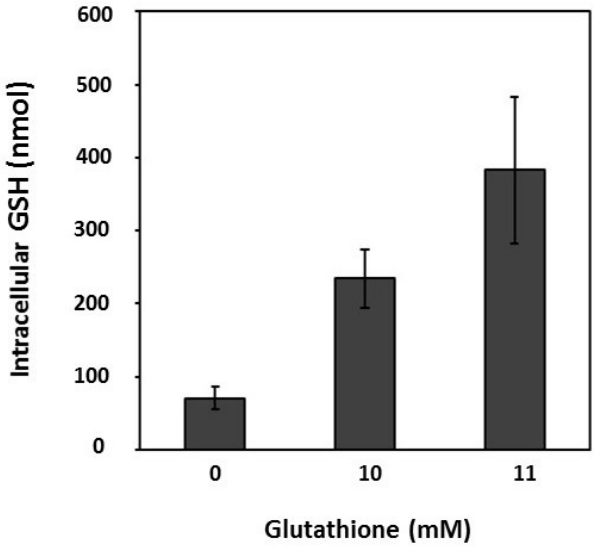
[a]MIC measurement was repeated three times with identical results in MHB (pH 7.1).
[b] Sub-inhibitory concentrations of GSH were used at 9 mM (NY214 and NY215) and 10 mM (NY220).
[c] Clinical isolates (NY214, NY215, NY220) were obtained from the previous report (Barrow and Kwon, 2009).

Fig.1 Growth effect of acidity produced by exogenous glutathione or HClon *P. aeruginosa* PAO1



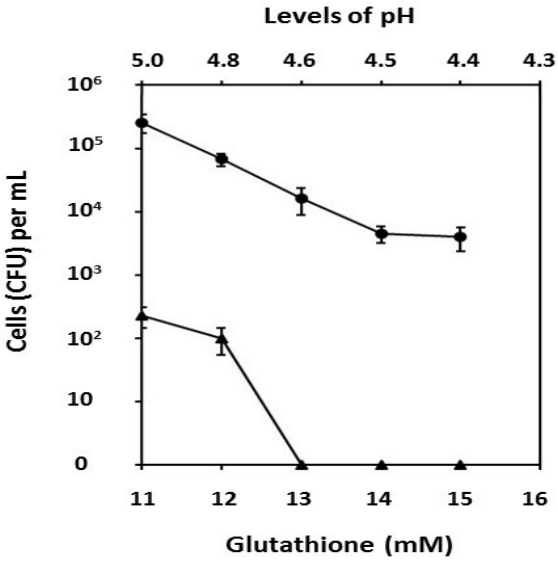
Cellular growth with or without glutathione (or HCl) was measured at OD_{600}. Culture medium (MHB) alone (diamond; pH 7.1), MHB with HCl (closed circle; pH 5.0), and MHB with exogenous glutathione (triangle; pH 5.0 produced by 11 mM). Three independent growth curve measurements were used to calculate the standard deviation

Fig.2 Killing effect of exogenous glutathione or HClon *P. aeruginosa* PAO1



Cells (~10^6) grown overnight were inoculated in 1 mL of MHB containing 11, 12, 13, 14, 15, and 16 mM of glutathione; in parallel, the same number of cells were inoculated in 1 mL of MHB adjusted pH levels by HCl: 5.0, 4.8, 4.6, 4.5, 4.4, and 4.3, which are equivalent pHsto the concentrations of glutathione, respectively. The cells in the presence of glutathione or HCl were incubated without shaking for 18 hours at 37°C and counted as a colony forming unit (CFU) on plain LB agar plates. Closed circles were for HCl-acidity and closed triangles were for exogenous glutathione. Three independent experiments were used to calculate the standard deviation.

Fig.3 Levels of intracellular glutathione in *P. aeruginosa* PAO1



Cells were grown with or without glutathione and harvested at OD_{600} 1.0. Levels of intracellular glutathione were measured as described in Materials and Methods. Three independent measurements were used to calculate the standard deviation.

However, MIC levels of all tested antibiotics in the same medium with the same amount of glutathione (11 mM), but the medium was adjusted to a neutral pH (pH 7.1), were similar or higher than those in MHB alone (Table 3). MIC levels of all tested antibiotics, except for chloramphenicol and tetracycline, in combination with 10 mM glutathione or pH levels of 5.0 or 4.8 with HCl were similar or higher than those in MHB alone. MIC levels of chloramphenicol and tetracycline in combination with 10 mM glutathione or pH levels of 5.0 or 4.8 with HCl were always decreased up to 8-fold (Table 3). These results indicate that antibiotic susceptibility is enhanced by the antibacterial activity of exogenous glutathione. The results also suggest that the retention of acidity is critical for the antibiotic susceptibility. Susceptibility of chloramphenicol and tetracycline was always increased in combination with exogenous glutathione as well as with acidity produced by HCl, suggesting the susceptibility mechanism of these antibiotics may be different from that of the other antibiotics tested here.

To understand availability of exogenous glutathione in clinical isolates three carbapenem-associated multidrug resistant clinical isolates of *P. aeruginosa* were used to determine MIC levels. Results revealed that MIC levels of all three isolates were sensitized (MICs <1 μg/mL) for all tested antibiotics in combination with sub-inhibitory concentrations of exogenous glutathione (Table 4).These results suggest that the exogenous glutathione at sub-inhibitory concentrations with any tested antibiotics can sensitize the multidrug resistant clinical isolates. Infection with carbapenem-associated multidrug resistant *P. aeruginosa* is currently a major therapeutic problem worldwide (Pena *et al.*, 2012; Lucena *et al.*, 2014; Perez *et al.*, 2014). Exogenous glutathione is not significantly cytotoxic at less than 50 mM (Schairer *et al.*, 2013), suggesting exogenous glutathione alone or in combination with currently available antibiotics may be applicable to treat multidrug resistant *P. aeruginosa* infections.

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