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Original Article

Efflux Pump Inhibitor Carbonyl Cyanide-m-chlorophenylhydrazone (CCCP) Enhances Bacteriostatic Activity of Trimethoprim-sulfamethoxazole Against Clinical *Stenotrophomonas maltophilia* Isolates from Korea

Ji-Young Rhee[1], Ji Young Choi[2] and Kwan Soo Ko[2*]

[1]Division of Infectious Diseases, Department of Medicine, Dankook University, Cheonan; [2]Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, Korea

Although trimethoprim-sulfamethoxazole (TMP-SXT) is considered the first-line therapy for *Stenotrophomonas maltophilia* infections, there is debate on the use of the bacteriostatic drug in serious infections, and recently, there has been an increasing occurrence of acquired resistance to TMP-SXT. In the present study, the effect of efflux pump inhibitors on the susceptibility of TMP-SXT and other antibiotics were investigated in *S. maltophilia* complex. The *sul* and/or *dfr*A genes were identified in only up to 27.8% of all 36 TMP-SXT-resistant *S. maltophilia* complex isolates. Thus, TMP-SXT resistance in *S. maltophilia* was not explained completely by the presence of *sul* and *dfr*A genes. Carbonyl cyanide-m-chlorophenylhydrazone (CCCP) decreased the minimum inhibitory concentration (MIC) of TMP-SXT by eight to 128 folds in all 14 isolates. In contrast, 2,4-dinitrophenol (DNP), phenyl-arginine-β-naphthylamide (PAβN), and reserpine did not reduce the MIC of TMP-SXT. In addition to TMP-SXT, slight decrease in MICs was observed for tigecycline and piperacillin/tazobactam by CCCP (by two folds) in one isolate. Although efflux pump may play a role in TMP-SXT resistance in *S. maltophilia*, inhibition of the efflux pump could be done by active proton pore.

Key Words: *Stenotrophomonas maltophilia*, Co-trimoxazole, Efflux pump

INTRODUCTION

Stenotrophomonas maltophilia is generally associated with septicemia and respiratory infections, especially in immunocompromised patients and cystic fibrosis patients (1). It exhibits resistance to many commonly used broad-spectrum antibiotics and treatment recommendations are controversial, partly due to a lack of consistency in susceptibility testing methods and poor correlation between *in vitro* studies and clinical outcome (1). *S. maltophilia* isolates are often highly resistant to most of the currently used antimicrobial agents, including carbapenems, aminoglycosides, and fluoroquinolones, because *S. maltophilia* has high intrinsic resistance to a variety of structurally unrelated antimicrobial agents, including β-lactams, aminoglycosides, and quinolones. A variety of antimicrobial resistance mechanisms, including efflux pumps and integrons, have been reported in *S. maltophilia* (2).

Although trimethoprim-sulfamethoxazole (TMP-SXT) is considered the first-line therapy for *S. maltophilia* infections, there is debate on the use of the bacteriostatic drug in serious

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*Corresponding author: Kwan Soo Ko, Ph.D. Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, 2066 Seobu-ro, Jangan-gu, Suwon 16419, Korea.
Phone: +82-31-299-6223, Fax: +82-31-299-6229, e-mail: ksko@skku.edu

infections, and more recently, there has been an increasing occurrence of acquired resistance to TMP-SXT (1). During recent years, there have been several reports of the emergence of TMP-SXT-resistant *S. maltophilia*, with a prevalence ranging from 3.8% in Latin America, North America, and Europe to 28.3% in Turkey (3~5). In our study, the rate of TMP-SXT-resistant *S. maltophilia* was 30.5% (6). In addition, it was shown *S. maltophilia* identified in microbiology labs includes multiple species, forming *S. maltophilia* complex (6).

Although only a few studies showed the mechanisms of TMP-SXT resistance-three main mechanisms have been suggested to be involved in bacterial resistance to the antibiotics-modification of the target and, enzymatic inactivation of the antibiotic, or default of its accumulation within the cell (lack of entry or efflux systems). Some bacteria have a decreased permeability for TMP-SXT or have a target enzyme with decreased affinity for the drugs. As examples, resistance of *Pneumocystis jirovecii* to SXT after the administration of TMP-SXT has been correlated with dihydropteroate synthase gene mutations (7), while dihydrofolate reductase gene mutations can account for high level resistance in other organisms such as *Enterococcus faecalis* and *Campylobacter jejuni* (8). Several studies performed on *S. maltophilia* isolates have shown that *sul*1 genes associated with class 1 integrons are the major mechanism of TMP-SXT resistance (7). A study indicated that the *sul*1 gene, in combination with *dfr*A17 and *dfr*A12 gene cassettes and *sul*2 genes lead to a high rate of SXT resistance (9). These genes are known to be mechanism of trimethoprim-sulfamethoxazole resistance. In *E. faecalis*, intrinsic resistance to SXT has been found, which are typically auxotrophic for folic acid (10). In addition, some bacteria species (e.g., including *Pseudomonas aeruginosa*) have an active efflux mechanism to eliminate the drug from the cell (11). Although the intrinsic resistance to multiple unrelated antibiotics and disinfectants of Gram-negative bacteria is mediated by innate mechanisms and is principally defined by expression of efflux pump systems and porins (12), only one study showed that SmeABC pumps were related to increase the MIC of TMP-SXT, ticarcillin-clavulanate, and ciprofloxacin (1).

There are five families of multidrug resistance (MDR) efflux pumps: the resistance nodulation division (RND) family, the major facilitator superfamily (MFS), the staphylococcal multidrug resistance (SMR) family, the multidrug and toxic compound extrusion (MATE) family and the ATP-binding cassette (ABC) family (13). In addition, many strains of *S. maltophilia* possess efflux pumps, which confer further resistance to multiple antibacterial classes (14, 15).

In the present study, the effect of efflux pump inhibitors on the susceptibility of TMP-SXT and other antibiotics were investigated in *S. maltophilia* complex. The present study plans to correlate the TMP-SXT resistance patterns of the *S. maltophilia* complex with their antimicrobial efflux mechanisms.

MATERIALS AND METHODS

Bacterial isolates

All 36 TMP-SXT resistant *S. maltophilia* complex were investigated to detect *sul*1, *sul*2 and *dfr*A genes. Fourteen isolates (seven TMP-SXT resistant isolates and seven TMP-SXT susceptible isolates) among 118 clinical isolates tentatively identified as *S. maltophilia* complex were included in efflux pump inhibitors study. They were collected from seven tertiary-care hospitals in Korea from 2007 to 2011 and were identified conventionally using VITEK2 systems in the hospitals' clinical microbiology labs.

Antimicrobial susceptibility test

In vitro susceptibility testing was performed with isolates identified as *S. maltophilia* complex in this study using the broth microdilution method according to CLSI guidelines (16). Five antimicrobial agents were tested: ceftazidime, levofloxacin, TMP-SXT, piperacillin/tazobactam, and tigecycline. The interpretive criteria used were those established in CLSI standard M100-S21 (17). Regarding tigecycline, interpretive criteria were defined based on the USA-FDA breakpoint criteria for *Enterobacteriaceae* (susceptible ≤2 mg/l, intermediate 4 mg/l, and resistant ≥8 mg/l). *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as control strains. MDR isolate was defined as

Table 1. Oligonucleotide primers used in detecting *sul*1, *sul*2, and *dfr*A genes

Gene	PCR primers (5' to 3')	Ta	Product size (bp)
*sul*1	F : GCGATCGAAATGCTGCGAGT R : AACCCTCGGTCTCTGGCGGCGT	61℃	429
*sul*2	F : CCTGTTTCGTCCGACACAGA R : GAAGCGCAGCCGCAATTCAT	60℃	435
*dfr*A1	F : CTTGTTAACCCTTTTGCCAGA R : TTGTGAAACTATCACTAATGGTAG	58℃	480
*dfr*A5	F : ATCGTCGATATATGGAGCGTA R : TCCACACATACCCTGGTCCG	62℃	350
*dfr*A12/13	F : GTCGTTGTCATGGGGCGAAA R : TCGACGCGCATAAACGGAGT	60℃	381
*dfr*A17	F : GTTAGCCTTTTTTCCAAATCTGGTATG R : TTGAAAATATTATTGATTTCTGCAGTG	60℃	475

one showing resistance to two or more antimicrobial agents.

Polymerase chain reaction (PCR) for detecting *sul* and *dfr*A genes

The presence of *sul*1, *sul*2 and *dfr*A genes in 36 TMP-SXT resistant strains were assessed using the primers shown in Table 1 which were designed for this study using the Primer 3 online software http://frodo.wi.mit.edu/primer3/. Total DNA was extracted by suspending several overnight colonies in 0.5 ml of double-distilled water and heating the mixture at 100℃ for 10 min. The PCR cycling parameters were as follows: 95℃ for 5 min, followed by 35 cycles of 95℃ for 1 min, 55℃ for 1 min and 72℃ for 1~4 min depending on the sequence to be amplified, and ending with 10 min incubation at 72℃. PCR fragment length estimation was by reference to a 100 bp DNA size standard. Confirmation of the electrophoresis sizing observations was by DNA sequencing of selected PCR products.

Effect of efflux inhibitors on minimum inhibitory concentration (MIC) levels of Trimethoprim-sulfamethoxazole

To determine the extent of the efflux pump mediated TMP-SXT resistance in *S. maltophilia* isolates, MIC levels for TMP-SXT were determined using broth microdilution assay in the presence or absence of efflux pump inhibitors (CCCP [carbonyl cyanide-m-chlorophenylhydrazone] and PAβN [phenyl-arginine-β-naphthylamide]) in 14 isolates. For six isolates showing the MIC decrease in the presence of EPI, in addition, MIC levels for TMP-SXT were further determined using broth microdilution assay in the presence or absence of the other EPIs such as DNP (2,4-dinitrophenol) reserpine. For two isolates showing the prominent MIC changes of TMP-SXT in the presence of EPIs, MIC levels for ceftazidime, levofloxacin, piperacillin/tazobactam, and tigecycline were determined using broth microdilution assay in the presence or absence of EPIs. Stock solution of CCCP and DNP were prepared in DMSO while PAβN and reserpine were dissolved in distilled water. Final concentrations used in broth microdilution assay were 1 mg/l (CCCP), 20 mg/l (DNP), 25 mg/l (PAβN), and 5 mg/l (reserpine).

RESULTS

Detecting sul1, *sul*2 and *dfr*A genes

The *sul*1 gene was detected in five TMP-SXT-resistant *S. maltophilia* isolates. The *dfr*A1, *dfr*A5, and *dfr*A12/13 genes were positive in one, three, and seven, respectively. The *sul*2 and *dfr*A17 gene were not detected in all TMP-SXT resistant isolates. Three isolates harbored both sul1 and *dfr*A12/13 genes with high resistance to TMP-SXT (MIC, 16:304, >64:1216) and levofloxacin (MIC, 32 mg/l). Both *dfr*A5 and *dfr*A12/13 were detected in two isolates, which were also

Table 2. Distribution of *sul*1 and *dfrA* genes in TMP-SXT-resistant isolates

Isolate	MIC (mg/l)					Gene			
	TMP-SXT	LVX	CZM	P/T	TG	*sul*1	*dfr*A1	*dfr*A5	*dfr*A12/13
K01-OTH-06-119	4:76	16	2	16:4	8	−	−	+	+
K01-OTH-09-7	4:76	16	>64	64:4	2	−	−	+	+
K01-OTH-09-126	4:76	32	8	16:4	1	−	−	−	+
K01-OTH-06-3	8:152	0.5	64	32:4	2	+	+	−	−
K01-OTH-09-142	8:152	16	16	16:4	4	−	−	+	−
M11610766	16:304	32	>64	>256:4	16	+	−	−	+
M11733232	16:304	64	>64	256:4	8	−	−	−	+
K103-OTH-08-1	>64:1216	1	64	16:4	0.5	+	−	−	−
M11893222	>64:1216	32	64	32:4	0.5	+	−	−	+
M11893758	>64:1216	32	64	16:4	4	+	−	−	+

TMP-SXT, trimethoprim-sulfamethoxazole; LVX, levofloxacin, CZM, ceftazidime; P/T, piperacillin-tazobactam; TG, tigecycline.

Table 3. Trimethoprim-sulfamethoxazole MICs of *Stenotrophomonas maltophilia* complex in treating with efflux pump inhibitors including CCCP and PAβN

Isolate	MIC (mg/l)		
	TMP-SXT	TMP-SXT + CCCP (10 μM)	TMP-SXT + PAβN (25 mg/l)
K01-OTH-06-4	32:608	2:38	32:608
K01-OTH-06-116	4:76	0.125:2.375	8:152
K01-OTH-06-119	16:304	0.5:9.5	32:608
K01-OTH-06-167	16:304	0.125:2.375	32:608
K01-OTH-08-84	8:152	0.125:2.375	16:304
K01-OTH-09-7	64:1216	8:152	32:608
K103-OTH-08-1	32:608	4:76	32:608
K01-OTH-06-1	2:38	0.0625:1.1875	4:76
K01-OTH-06-6	2:38	0.0625:1.1875	2:38
K01-OTH-06-18	2:38	0.0625:1.1875	2:38
K01-OTH-06-20	2:38	0.0625:1.1875	2:38
K01-OTH-06-94	2:38	0.0625:1.1875	2:38
K01-OTH-06-122	2:38	0.0625:1.1875	2:38
K01-OTH-07-13	2:38	0.0625:1.1875	2:38

TMP-SXT, trimethoprim-sulfamethoxazole; CCCP, carbonyl cyanide-m-chlorophenylhydrazone; PAβN, phenyl-arginine-β-naphthylamide.

resistant to levofloxacin. One isolate showed positivity for *sul*1 and *dfr*A1, however it was not resistant to levofloxacin (Table 2).

Effect of efflux inhibitors on minimum inhibitory concentration (MIC) levels of Trimethoprim-sulfamethoxazole

CCCP decreased the MIC of TMP-SXT by eight to 128 folds in all 14 isolates (Table 3). In contrast, DNP, PAβN and reserpine did not reduce the MIC of TMP-SXT (Table 4). Because DNP resolved in DMSO did not reduce the MIC, DMSO itself may not affect the bacterial death. In addition to TMP-SXT, slight decrease in MICs was observed for tigecycline and piperacillin/tazobactam by CCCP (two folds difference, Table 5) in one isolate, K01-OTH-06-119.

DISCUSSION

Overall, *sul* genes were rarely detected in TMP-SXT-resistant *S. maltophilia* isolates in Korea. In previous study, Song *et al*. have reported that no isolates carried *sul*2 gene and 13 of 19 TMP-SXT-resistant isolates were detected with

Table 4. Trimethoprim-sulfamethoxazole MICs of Trimethoprim-sulfamethoxazole resistant strains in treating with efflux pump inhibitors

Isolate	MIC (mg/l)				
	TMP-SXT	TMP-SXT + CCCP (10 mg/l)	TMP-SXT + DNP (1 mM/ml)	TMP-SXT + PAβN (25 mg/l)	TMP-SXT Reserpine (20 mg/l)
K01-OTH-06-4	32:608	2:38	32:608	32:608	32:608
K01-OTH-06-116	4:76	0.125:2.375	16:304	8:152	4:76
K01-OTH-06-119	16:304	0.5:9.5	32:608	32:608	16:304
K01-OTH-06-167	16:304	0.125:2.375	32:608	32:608	16:304
K01-OTH-08-84	8:152	0.125:2.375	16:304	16:304	8:152
K103-OTH-08-1	32:608	4:76	16:304	32:608	32:608

TMP-SXT, trimethoprim-sulfamethoxazole; CCCP, carbonyl cyanide-m-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; PAβN, phenyl-arginine-β-naphthylamide.

Table 5. MICs of a trimethoprim-sulfamethoxazole resistant strain K01-OTH-06-119 and K01-OTH-06-167 in treating with efflux pump inhibitors

Antimicrobial agent	Strain	MIC (mg/l)				
			+ CCCP (10 μM)	+ DNP (1 mM)	+ PAβN (25 mg/l)	Reserpine (20 mg/l)
TMP-SXT	K01-OTH-06-119	16:304	0.5:9.5	32:608	32:608	16:304
	K01-OTH-06-167	16:304	0.125:2.375	32:608	32:608	16:304
levofloxacin	K01-OTH-06-119	8	8	16	16	16
	K01-OTH-06-167	4	4	4	4	4
Piperacillin /tazobactam	K01-OTH-06-119	>256:4	128:4	>256:4	>256:4	>256:4
	K01-OTH-06-167	8:4	8:4	16:4	8:4	8:4
Ceftazidime	K01-OTH-06-119	2	2	2	2	2
	K01-OTH-06-167	2	2	2	2	2
Tigecycline	K01-OTH-06-119	4	2	4	8	4
	K01-OTH-06-167	0.5	0.5	0.5	2	0.5

TMP-SXT, trimethoprim-sulfamethoxazole; CCCP, carbonyl cyanide-m-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; PAβN, phenyl-arginine-β-naphthylamide.

*sul*1 gene (13). In this study, *sul* genes and/or *dfr*A genes were identified in only up to 27.8% of all 36 TMP-SXT-resistant *S. maltophilia* complex. Therefore, only the presence of these genes may not explain TMP-SXT resistance mechanisms in *S. maltophilia*. In addition, *sul* and *dfr* genes have been detected in TMP-SXT-susceptible *S. maltophilia* isolates (18).

The intrinsic resistance to multiple unrelated antibiotics and disinfectants of Gram-negative bacteria is mediated by innate mechanisms and is principally defined by expression of efflux pump systems and porins (12). Active efflux pumps have been identified as a common mechanism of multiple antibiotic resistance and cyclohexane tolerance in *Enterobacteriaceae* (12). The bacterial multidrug efflux transporters can be divided into five classes: (i) small multidrug resistance (SMR), (ii) major facilitator superfamily (MFS), (iii) resistance nodulation cell division (RND), (iv) multidrug and toxic compound extrusion (MATE), (v) ATP-binding cassette (ABC). Those five classes obtain energy required for the active transporting either from H^+ protons (RND, SMR, and MFS), Na^+ dependent (MATE), or by hydrolysis ATP (ABC) (19). Multiple antibiotic resistance in *S. maltophilia*, like in other gram-negative bacteria, is also attributable in part to limited outer membrane permeability and active antibiotic extrusion, although these mechanisms are poorly characterized to date (19). The outer membrane limits access of drugs to their bacterial targets, while multidrug efflux pumps actively remove drugs from the cell (20). One study revealed that *sme*ABC efflux genes were related to TMP-SXT resistance by using anti-efflux pump antibody (1).

The phenylalanine-arginine β-naphthylamide (PAβN) was first identified as an inhibitor of MDR pumps of *P. aeruginosa*. PAβN have been introduced as efflux pump inhibitors (EPIs); their mechanism of action is through competitive inhibition with antibiotics on the efflux pump resulting in increased intracellular concentration of antibiotic, hence, restoring its antibacterial activity (20). The activity of the PAβN is dipeptide against the RND efflux pump (21). The pump may have specific antibiotic-binding sites and that the degree of inhibition of the pump promoted by PAβN may be due to competition for these specific binding sites has similar activity against other Gram-negative bacteria, such as *Enterobacter aerogenes* (22). The multidrug resistance can also be eliminated by reserpine, and this result was taken to indicate the involvement of an active efflux system to block putative ABC efflux pumps (23). Interestingly, reserpine does not inhibit RND pumps (23).

CCCP is a chemical inhibitor of oxidative phosphorylation (24). It causes the gradual destruction of living cells and death of the organism. The chemical acts essentially as an ionophore and reduces the ability of ATP synthase to function optimally (24). CCCP is an energy uncoupler, which collapses the membrane energy involved in the efflux process. The CCCP inhibited the P55-determined drug resistance in *Mycobacterium tuberculosis*, suggesting the active export of the compounds by use of the transmembrane proton and electrochemical gradients as sources of energy (25). Cells that lacked the p55 gene displayed smaller colony sizes and had a growth defect in liquid culture (25). CCCP is an H^+ ionophore, which dissipates the H^+ gradient and thus uncouples electron transport from ATP synthesis (25). It can transport protons into the cell without the participation of ATP synthase (26). Therefore, the proton motive force (PMF) is cancelled out, and ATP can no longer be synthesized (26). CCCP is frequently used in the study of the active transport of substances (22). CCCP and DNP are the proton motive force inhibitors. DNP, $C_6H_4N_2O_5$, is an inhibitor of efficient energy (ATP) production in cells with mitochondria (27). DNP acts as a protonophore, allowing protons to leak across the inner mitochondrial membrane and thus bypass ATP synthase. This makes ATP energy production less efficient. DNP is probably the best known agent for uncoupling oxidative phosphorylation. DNP uncouples oxidative phosphorylation, causes release of calcium from mitochondrial stores and prevents calcium re-uptake.31 DNP has less steep uncoupler than CCCP.

In this study, only CCCP exhibited the effect on TMP-SXT MICs of *S. maltophilia* complex isolates. On the other hand, PAβN, reserpine, and DNP had no role in reducing MICs in *S. maltophilia* complex isolates. Despite DNP is also PMF inhibitor as CCCP, it did not reduce MICs of TMP-SXT. Thus, the effect CCCP on the TMP-SXT suscep-

tibility in *S. maltophilia* may not be directly related to the role as an uncoupler of oxidative phosphorylation itself. The CCCP affects the protein synthesis reactions and causes an uncoupling of the proton gradient that is established during the normal activity of electron carriers in the electron transport chain (24). Effect on the protein synthesis reactions by CCCP may take account of this study results. CCCP acts as an energy uncoupler by destroying the proton motive force of the membrane. Therefore, increased accumulation of the drug in clones when treated with CCCP is possibly due to the complete inhibition of active efflux in the deenergized cells (27). While CCCP is active proton pore, DNP just allows protons to leak. This difference may play a role in affecting the resistance of TMP-SXT in *S. maltophilia* complex isolates.

In short, *sul* and *dfr*A genes were identified only in some TMP-SXT-resistant *S. maltophilia* isolates, thus TMP-SXT resistance in *S. maltophilia* were not explained only by the genes. CCCP, one of EPIs, affected greatly the sensitivity of TMP-SXT in *S. maltophilia*, unlike the other EPIs. Thus, efflux pump may play a role in TMP-SXT resistance in S. *maltophilia*, but inhibition of the efflux pump could be done by active proton pore.

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