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ORIGINAL RESEARCH

In vitro Antibacterial Activity and Resistance Prevention of Antimicrobial Combinations for Dihydropteroate Synthase-Carrying *Stenotrophomonas maltophilia*

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Background: *Stenotrophomonas maltophilia* (*S. maltophilia*) is a multidrug-resistant gram-negative bacillus that is known to be an opportunistic pathogen, particularly in a hospital environment. The infection has a high morbidity and mortality. Sulfamethoxazole-trimethoprim (SXT) is the first-line agent recommended for its treatment. The global spread of dihydropteroate synthase (*sul*) genes has resulted in an increased resistance rate. However, the appropriate therapy for infections caused by *sul*-carrying *S. maltophilia* has not yet been established.

Objective: Our study aimed to identify the optimal antibiotic combinations that could both show high antibacterial activity against *sul*-carrying *S. maltophilia* and the ability to prevent the emergence of resistance at clinical dosage regimens.

Methods: Time-killing experiments and mutant prevention concentration (MPC) experiments were conducted to evaluate the antibacterial effect and ability to prevent resistance to minocycline, tigecycline, moxifloxacin, and ticarcillin/clavulanic acid (T/K), both alone and in combination, at clinically relevant antimicrobial concentrations.

Results: Minocycline, tigecycline, and T/K all exhibited bacteriostatic activity to *sul*-carrying *S. maltophilia*. The combination of minocycline plus T/K and tigecycline plus T/K neither enhanced the bactericidal ability nor prevented drug-resistant mutations. Moxifloxacin, at 2 mg/L, showed good bactericidal activity to most *S. maltophilia*, but bacterial regrowth at 24 h was observed in two strains. When combined with T/K, moxifloxacin showed good bactericidal activity in all moxifloxacin-sensitive strains. The concentrations of moxifloxacin alone were lower than most MPCs of the tested *sul*-carrying strains. When combined with T/K, the mean steady-state concentrations (MSC) of moxifloxacin could prevent 70% of resistance, and the peak concentration (C$_{max}$) prevented 95% of resistance.

Conclusion: The combination of moxifloxacin and T/K can achieve a good in vitro bactericidal effect and prevent the emergence of resistance at clinical dosage regimens, and may be an optimal therapeutic strategy for *S. maltophilia* infections, especially for vulnerable immunocompromised and critically ill patients.

Keywords: sulfamethoxazole-trimethoprim, dihydropteroate synthase, moxifloxacin, mutant prevention concentration, pharmacokinetic, C$_{max}$

Introduction

Stenotrophomonas maltophilia (*S. maltophilia*) is a multidrug-resistant organism found in hospital settings that can cause respiratory, bloodstream, abdominal, and other severe hospital-acquired infections, with a high incidence and mortality

rate.[1–4] *S. maltophilia* exhibits intrinsic resistance to most commonly-used antimicrobial agents, and sulfamethoxazole-trimethoprim (SXT) is the first-line agent recommended for treatment.[2,5,6] However, with the global spread of dihydropteroate synthase (*sul*) genes, SXT resistance has emerged, and there has been a rapid increase in its rates.[7,8] Our previous epidemiological survey confirmed that the presence of *sul* genes was the predominant resistance mechanism for SXT in clinically-isolated *S. maltophilia* in China.[9] *S. maltophilia* shows high susceptibility to tetracycline derivatives, including minocycline, doxycycline, and tigecycline, and these antimicrobials can be used as alternatives for the treatment of *S. maltophilia* infection, even for SXT-resistant strains.[1,2,9] However, an in vitro study by Wei et al found that tetracycline derivatives exhibited bacteriostatic activity against *S. maltophilia*, which can only inhibit the proliferation of *S. maltophilia* instead of effectively killing the organisms.[10] *S. maltophilia* infections typically occur in vulnerable, immunocompromised, and critically ill patients, whose immune system is usually impaired and cannot effectively kill pathogens.[2,3,11] Therefore, the use of a sole antimicrobial agent may not provide adequate treatment. Moreover, *S. maltophilia* possesses a variety of intrinsic drug resistance mechanisms, such as efflux pumps. Long-term use of improper antimicrobial agents may result in the development of drug resistance, and combination therapy should be recommended for infections caused by *sul*-carrying *S. maltophilia*.[1,6,10,12] However, there is limited data regarding which antibiotic combinations are the most effective.

The objectives of this study were to evaluate the in vitro antibacterial activity and ability to prevent drug-resistant mutations of different antimicrobial combinations against *sul*-carrying *S. maltophilia*. The concentrations of antimicrobial agents are chosen based on clinical pharmacokinetics to ensure that the therapeutic effects are evaluated in a clinically relevant manner.[10,13]

Materials and Methods

Bacterial Strains and Susceptibility Testing

Non-duplicated clinical *S. maltophilia* were collected from hospitalized patients at the Chinese PLA General Hospital and Air Force Medical Center from 2020 to 2021. These *S. maltophilia* strains were all isolated from respiratory tract specimens of patients with pulmonary infections. The identification of bacterial species was performed using a Vitek II bacterial identification system (bioMérieux, Marcy-l'Étoile, France) and further confirmed via a species-specific polymerase chain reaction.[9,14,15] The detection of the *sul* genes (including *sul1* and *sul2* genes) was presented as follows: SUL1-F(GCTATTGGTCTCGGTGTCGC) and SUL1-B(GCATGATCTAACCCTCGGTCT) for sul1; SUL2-F(TTTCGGCATCGTCAACATAA) and SUL2-B(CCACGCGACAAGGCATA) for sul2. The PCR reaction volume system and cycling parameters were the same as in our previously published literature.[15] Minimum inhibitory concentration (MIC) results for SXT, minocycline, tigecycline, ticarcillin/clavulanate (T/K), and moxifloxacin were obtained by the agar dilution method, and they were interpreted according to the breakpoints suggested by CLSI (2021), as previously described.[9,16] Moxifloxacin and tigecycline have no published breakpoint criteria for *S. maltophilia*, so they were interpreted with reference to those of Enterobacteriaceae (susceptibility at 2 ug/mL, intermediate at 4 ug/mL, and resistance at 8 ug/mL), as in the study by Wei et al.[10] Twenty *sul*-carrying bacterial strains susceptible to minocycline, tigecycline, moxifloxacin, and T/K were chosen for further in vitro experiments, and the carrying situation of *sul* genes and MICs are presented in Table S1.

Time-Kill Experiments

Six of the 20 candidate *sul*-carrying *S. maltophilia* were chosen randomly for the time-kill assays, and an overnight inoculum of approximately 10^6 colony-forming units (CFU)/mL was used. The drug concentrations used in the time-kill curves were lower than the mean steady-state concentrations (MSCs) of non-protein-bound drugs in humans to achieve a better simulation of the actual clinical conditions.[10,17] The MSCs for minocycline (200 mg po 24 h) and moxifloxacin (400 mg, q24 h) were calculated based on the area under the antibiotic concentration-time curve (AUC) in serum or plasma over 24 h divided by 24 h (AUC0-24/24 h). The MSC of tigecycline (50 mg, q12 h) was based on the AUC in serum or plasma over 12 h divided by 12 h, and the MSC of T/K (3 g/0.1g, q8 h) was based on the AUC in serum or plasma over 8 h divided by 8 h.[10,18] The pharmacokinetic parameters included in the time-kill experiments are shown in Table 1. The concentrations of each antimicrobial agent were as follows: minocycline, 2 mg/L; tigecycline, 0.25 mg/L;

Table 1 Summary of the Pharmacokinetic Parameters and the Experimental Concentrations of the Antimicrobial Agents Used in the Time-Kill Experiments

Antimicrobial Agents	Clinical Dosages	AUC (μg×h/mL)	MSC (ug/mL)	C_{max} (ug/mL)	Experimental Concentration (ug/mL)	Reference
Min	200 mg po 24h	48.3[a]	2.01	3.5	2	[10]
Tig	50 mg q 12h	3.07[b]	0.26	0.63	0.25	[18]
Mox	400 mg q 24h	47.97[a]	2.00	4.5	2	[19]
T/K	3 g/0.1 g q 8h	409.6[c]	51.2	100	32/2	[20–22]

Notes: [a]0–24 h AUC at steady state for multiple-dose studies; [b]0–12 h AUC at steady state for multiple-dose studies; [c]0–8 h AUC at steady state for multiple-dose studies.
Abbreviations: AUC, area under the curve; MSC, mean steady-state concentration of non-protein-bound drug; C_{max}, peak concentration; Min, minocycline; Tig, tigecycline; Mox, moxifloxacin; T/K, ticarcillin/clavulanic acid.

and moxifloxacin, 2 mg/L. As T/K is a compound preparation, of which ticarcillin is the antibacterial component and clavulanic acid is a β-lactamase inhibitor, 32/2 mg/L was used based on the MSC of ticarcillin. The antibacterial activities of minocycline, tigecycline, and moxifloxacin used alone and in combination with T/K were evaluated at 0, 3, 6, 12, and 24 h. Culture samples were serially diluted, spread on plates, and incubated at 35°C, and the resulting bacterial colonies were counted after 24 h. Bacteriostatic activity was defined as a decrease in bacterial concentration < 3 $\log_{10}$ compared to the bacterial concentration of the initial inoculum. Bactericidal activity was defined as a decrease in bacterial concentration ≥ 3 $\log_{10}$ compared with the initial inocula.[10,23]

Determination of MPCs

The mutant prevention concentrations (MPCs) of minocycline, tigecycline, and moxifloxacin alone and in combination with T/K were determined in 20 candidate *sul*-carrying isolates using a modified agar dilution method, as previously described.[24,25] In brief, approximately $0.3×10^{10}$ CFU/mL of bacterial cells were placed onto Mueller–Hinton Agar plates containing different concentrations of antimicrobial agents. Each drug concentration was included on at least four plates to ensure that the total cell number in the inoculum was > $1×10^{10}$. The plates were incubated at 35°C for 72 h. The MPC was defined as the lowest antibiotic concentration that prevented the visible growth of mutant colonies after 72 h.

Results
Time-Kill Experiments

The carrying situation of *sul* genes and MICs of the six strains chosen for the time-kill assays are presented in Table 2. Minocycline, tigecycline, and T/K exhibited bacteriostatic activity in all six *sul*-carrying *S. maltophilia* strains (Table 3). Minocycline plus T/K and tigecycline plus T/K combination therapies were not superior to minocycline or tigecycline monotherapy in terms of antimicrobial effects. Moxifloxacin at 2 mg/L showed good bactericidal activity in A1-A4

Table 2 The Carrying Situation of Dihydropteroate Synthase Genes and the Minimum Inhibitory Concentration of the Six Chosen *Stenotrophomonas maltophilia* Strains Used in the Time-Kill Experiment

Strain	MIC (ug/mL)					
	Sul	SXT	Mox	Min	Tig	T/K
A1	Sul 1	> 152/8	0.25	0.25	0.5	2/2
A2	Sul 2	152/8	0.5	0.5	1	8/2
A3	Sul 1	76/4	1	0.25	1	4/2
A4	Sul 2	> 152/8	1	1	2	16/2
A5	Sul 1	152/8	2	0.5	1	2/2
A6	Sul 1, Sul 2	> 152/8	2	2	2	8/2

Abbreviations: *Sul*, dihydropteroate synthase; MIC, minimum inhibitory concentration; SXT, sulfamethoxazole-trimethoprim; Mox, moxifloxacin; Min, minocycline; Tig, tigecycline; T/K, ticarcillin/clavulanic acid.

Table 3 Change in Bacterial Concentrations at 24 h of the Six *Sul*-Carrying *Stenotrophomonas maltophilia* Strains

Strain	Change in Concentration (log$_{10}$ CFU/mL) at 24 h							
	Min	**Mox**	**Tig**	**T/K**	**Min+T/K**	**Mox+T/K**	**Tig+T/K**	**Control**
A1	−0.40	−6.18	0.33	0.82	−1.00	−6.18	0.03	3.37
A2	−0.06	−5.90	0.88	1.16	−0.51	−5.90	−0.36	5.03
A3	0.15	−6.00	0.90	2.18	−0.07	−6.00	−0.05	4.28
A4	−0.03	−3.60	0.54	2.94	−0.20	−5.90	0.03	4.18
A5	−0.12	0.12	0.62	2.34	−0.08	−3.12	0.35	3.40
A6	0.09	1.02	0.77	−0.30	0.15	−3.54	−0.37	3.33

Abbreviations: *Sul*, dihydropteroate synthase; CFU, colony-forming units; Min, minocycline; Mox, moxifloxacin; Tig, tigecycline; T/K, ticarcillin/clavulanic acid.

strains (Table 3, Figure 1A), but bacterial regrowth at 24 h was observed in A5 and A6 strains (Table 3, Figure 1B). When combined with T/K, the bactericidal activity of moxifloxacin was observed against all six *S. maltophilia* strains (Table 3, Figure 1).

Effectiveness of Antimicrobial Combinations for Resistance Prevention

The MPCs of moxifloxacin, minocycline, and tigecycline alone and in combination with T/K are presented in Table 4. The MSCs and peak concentration (C_{max}) of the conventional clinical doses of moxifloxacin, minocycline, tigecycline, and T/K were all lower than the MPCs of all tested 20 strains of *sul*-carrying *S. maltophilia* and were within the mutant selection window (MSW). The ability to prevent resistance to minocycline and tigecycline was not significantly improved when these drugs were combined with T/K (Table 4). When moxifloxacin (400 mg q 24 h) was used, the MSC was 2 ug/mL, and the C_{max} was 4.5 ug/mL (Table 2). The MSC was within the MSW in all 20 strains, while the C_{max} could only prevent resistance of 10% of the strains. In combination with T/K, the MSC of moxifloxacin could prevent resistance of 70% of *sul*-carrying *S. maltophilia* strains, while the C_{max} could prevent resistance of 95% of strains (Table 4).

Discussion

SXT is the first-line agent recommended for the treatment of *S. maltophilia* infections.[2,5] The global spread of *sul* genes has increased the rates of resistance to SXT in recent years. However, there is no consensus regarding the treatment of *sul*-carrying *S. maltophilia*.[8,9] Therefore, we evaluated the in vitro antibacterial activity of different antimicrobial combinations against *sul*-carrying *S. maltophilia* by a time-kill experiment and investigated their ability to curb the emergence of resistance by an MPC experiment. We found that the commonly used alternative drugs, minocycline and
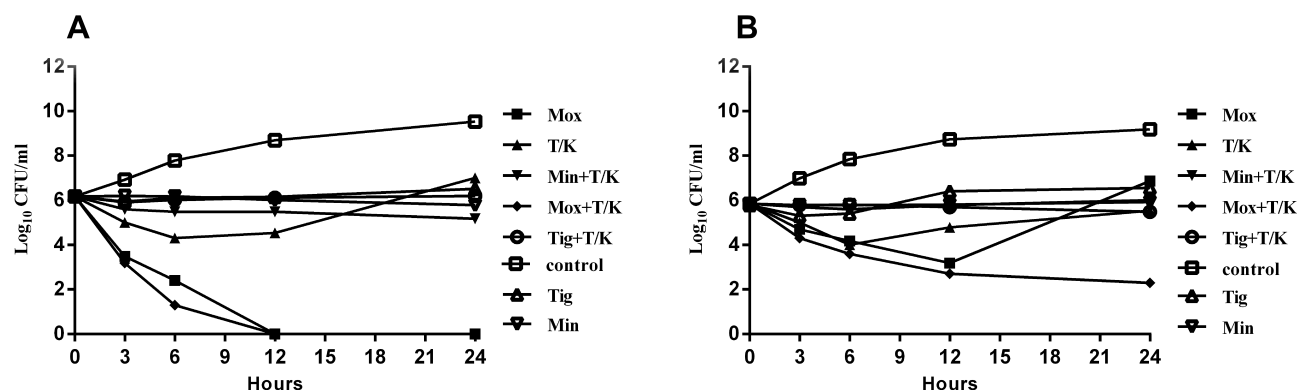

Figure 1 Time-kill curves for clinical A1 and A6 *sul*-carrying *Stenotrophomonas maltophilia*. (**A**) Time-kill curves for A1 *sul*-carrying *S.maltophilia*; (**B**) Time-kill curves for A6 *sul*-carrying *S.maltophilia*.
Abbreviations: *Sul*, dihydropteroate synthase; CFU, colony-forming units; Min, minocycline; Tig, tigecycline; Mox, moxifloxacin; T/K, ticarcillin/clavulanic acid.

Table 4 The Ability of Antimicrobial Agents to Prevent the Occurrence of Resistance at Clinical Dosage Regimens Against *Sul*-Carrying *S. maltophilia*

Antibiotic	MPC Range (ug/mL)	MPC$_{50}$/MPC$_{90}$ (ug/mL)	MSC Prevent Resistance (%)	C$_{max}$ Prevent Resistance (%)
T/K	> 256/2	> 256/2[a]	0	0
Min	4–16	8/16	0	0
Tig	8–16	16/16	0	0
Mox	4–32	16/32	0	10
Min+T/K	4–16	8/16	0	5
Tig+T/K	8–16	16/16	0	0
Mox+T/K	1–8	2/4	70	95

Note: [a]The MPC$_{50}$ and MPC$_{90}$ of T/K are both > 256/2 ug/mL.

Abbreviations: *Sul*, dihydropteroate synthase; MPC, mutant prevention concentration; MSC, mean steady-state concentrations; C$_{max}$, peak concentration; T/K, ticarcillin/clavulanic acid; Min, minocycline; Tig, tigecycline; Mox, moxifloxacin.

tigecycline, showed bacteriostatic effects and could not effectively prevent resistance, even after being combined with TK. Moxifloxacin showed bactericidal effects against most strains, but bacterial regrowth at 24 h was observed in several strains. When moxifloxacin was combined with TK at a clinically relevant concentration, it not only showed good bactericidal effects but also inhibited the occurrence of resistance.

S. maltophilia, even *sul*-carrying *S. maltophilia*, exhibits high sensitivity to tetracycline derivatives, such as minocycline and tigecycline.[1,2,9] However, both minocycline and tigecycline showed bacteriostatic activity instead of bactericidal activity towards *S. maltophilia*,[4,10,26] and there was no increase in antibacterial activity in combination with T/K. We further evaluated the ability of these drugs to prevent resistance using an MPC experiment. MPC is the concentration threshold above which no single-step drug-resistant mutant strains can be selected.[25] MSW is the concentration range from the MIC to the MPC. When bacteria grow within the MSW concentration for prolonged periods, there is an enrichment of drug-resistant strains.[24,27] The MSC and C$_{max}$ values of minocycline, tigecycline, and T/K at clinically relevant doses were lower than the MPC values and were within the MSW of all *sul*-carrying *S. maltophilia* strains, suggesting that long-term single-agent therapy with these drugs may result in enrichment of strains with resistant mutations. When used in combination with T/K, minocycline and tigecycline could still prevent the occurrence of resistance. Based on these results, even in combination with T/K, clinically utilized minocycline or tigecycline dosing regimens may fail to achieve the expected effect against *S. maltophilia* infection in patients with hematological malignancies, prolonged neutropenia, or receiving broad-spectrum antimicrobial therapy.[11,28]

The new fluoroquinolones, such as moxifloxacin and levofloxacin, have good in vitro activity and safety and are thus widely used in the treatment of pulmonary infections, hence being called respiratory quinolones. Based on observational evidence, levofloxacin and moxifloxacin are reasonable alternatives to SXT for the treatment of bloodstream and lower respiratory tract infections caused by *S maltophilia*.[10,29–31] It is reported that *sul*-carrying *S. maltophilia* were more susceptible to moxifloxacin than levofloxacin in in vitro experiments, and moxifloxacin can inhibit the biofilm they form on the surface of the respiratory tract or intubation tubes.[9,32–34] Therefore, moxifloxacin has a high clinical value as a therapeutic option for *sul*-carrying *S. maltophilia*. Based on clinical pharmacokinetics, the antibacterial activity and the ability to prevent resistance to moxifloxacin (400 mg q 24 h) in MSC (2 ug/mL) and C$_{max}$ (4.5 ug/mL) concentration were evaluated. Moxifloxacin, at 2 ug/mL, showed bactericidal effects against most strains, but bacterial regrowth at 24 h was also observed in several strains, as a previous study reported.[35] The MPCs of most tested strains were higher than 4.5 ug/mL, and long-term application of single-agent moxifloxacin may result in the selective enrichment of resistant mutants and lead to treatment failure. It is reported that overuse of fluoroquinolones worldwide has resulted in a higher resistance rate among many bacterial pathogens, including *S. maltophilia*[36,37] Moreover, overexpression of the *SmeDEF* efflux pump is the common drug resistance mechanism for *S. maltophilia* to quinolones, doxycycline, and tigecycline,[15] and improper long-term use of quinolones may lead to extensive resistance through hyperexpression of *SmeDEF* efflux pumps. In combination with T/K, moxifloxacin

exhibited bactericidal effects against all tested *sul*-carrying *S. maltophilia* strains and reduced the MPCs of most tested *S. maltophilia* to below 2 ug/mL, indicating that the combination can effectively prevent the emergence of resistance. Based on in vitro experiments, moxifloxacin (400 mg q 24h) combined with T/K (3 g/0.1 g q 8 h) may be an optimal therapeutic option for *sul*-carrying *S. maltophilia* infections. The serum concentration of moxifloxacin after intravenous administration was 31% higher than that after oral administration.[35,38] Therefore, intravenous administration of moxifloxacin is first recommended.

Our study had some limitations. First, although the pharmacokinetic parameters of antimicrobial agents in humans were taken into consideration, the antibiotic concentrations used in the experiments were constant, which cannot simulate the dynamic drug concentration changes in the human body. In addition, the immune system plays an important role in defending against bacterial infections, but its effect was not considered in our experiment. Further in vitro pharmaco-kinetic/pharmacodynamic and animal studies are still needed to fully evaluate the efficacy of this drug combination.

Conclusion

Minocycline, tigecycline, and T/K all exhibited bacteriostatic activity against *sul*-carrying *S. maltophilia*, and long-term single-agent therapy with these drugs may result in the enrichment of strains with resistant mutations. The combination of moxifloxacin and T/K can achieve good in vitro bactericidal effects and prevent the emergence of resistance at clinical dosage regimens and may be an optimal therapeutic strategy for *sul-carrying S. maltophilia* infection, especially for vulnerable immunocompromised and critically ill patients.

Abbreviations

S. maltophilia, Stenotrophomonas maltophilia; SXT, Sulfamethoxazole-trimethoprim; *sul*, dihydropteroate synthase; MSC, mean steady-state concentrations; C_{max}, peak concentration; CFU, colony-forming units; MIC, minimum inhibitory concentration; MPC, mutant prevention concentration; Min, minocycline; Tig, tigecycline; Mox, moxifloxacin; T/K, ticarcillin/clavulanic acid; AUC, area under the antibiotic concentration-time curve.

Data Sharing Statement

The data used to support the findings of this study are available from Dong Wang upon request through dongairforce@163.com.

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Disclosure

The authors report no conflicts of interest in this work.

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