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Mutant Prevention Concentrations of Levofloxacin Alone and in Combination with Azithromycin, Ceftazidime, Colistin (Polymyxin E), Meropenem, Piperacillin-Tazobactam, and Tobramycin against *Pseudomonas aeruginosa*

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The mutant prevention concentrations (MPCs) of levofloxacin alone and in combination with ceftazidime, colistin (polymyxin E), meropenem, piperacillin-tazobactam, and tobramycin were established against *Pseudomonas aeruginosa*. Antibiotic combinations using levofloxacin with any antibiotic with individual activity against *P. aeruginosa* resulted in synergistic lowering (an at-least-fourfold reduction) of the isolate's MPC.

With a limited pool of available antibiotics capable of treating *Pseudomonas aeruginosa* infections, the suppression of the further emergence of resistance is important. Fluoroquinolones, such as ciprofloxacin and levofloxacin, are routinely used to treat patients with *P. aeruginosa* infections. Fluoroquinolone resistance can be selected for upon exposure to the fluoroquinolones, leading to dramatic increases in MICs and subsequent treatment failure (3, 4).

The manipulation of fluoroquinolone-dosing strategies has been suggested as a way to limit the selection for fluoroquinolone-resistant mutants and preserve this antimicrobial class (1–3, 9, 11). A range of fluoroquinolone concentrations, known as the mutant selection window (MSW), exists in which point mutations are more likely to be selected for. The MSW is bound by the MIC at its lower end and the organism's mutant prevention concentration (MPC) at its upper end. The MPC is the drug concentration required to prevent the emergence of all single-step-mutation, fluoroquinolone-resistant mutants in a population of approximately 10^{10} bacterial cells (2, 11). If fluoroquinolone-dosing strategies maintain concentrations above the MPC for a long enough duration, a reduced risk of selecting for single-step-mutation, resistant mutants may result.

The addition of a second antibiotic to a fluoroquinolone treatment regime has been shown to lower an organism's MPC (1, 2, 11). In order to survive treatment with two antimicrobials, an organism has to develop spontaneous mutations causing resistance to both drugs, assuming that the two antimicrobials act via different modes of action and that the organism is initially susceptible to both agents (1, 2, 11).

We examined this dual-drug MPC hypothesis by determining the MPCs of levofloxacin alone and in combination with other drugs against *P. aeruginosa.* Additionally, MPCs for the nonfluoroquinolone antimicrobials were established individually to examine whether any MPC changes observed with the dual-drug therapy were due to synergistic (an at-least-fourfold reduction) or additive (a less-than-fourfold reduction) effects. The working hypothesis was that two antimicrobials that possessed different mechanisms of action and that both displayed individual activity against *P. aeruginosa* would display a reduced MPC in combination relative to the individual antimicrobial MPCs.

The *P. aeruginosa* isolates (46139, 49674, and 36375) were collected as part of the North American Urinary Tract Infection Collaborative Alliance (10). The identity of each isolate was confirmed by the central reference laboratory (Health Sciences Centre, Winnipeg, Manitoba), and then the isolate was stored in skim milk and frozen at −80°C (10). The MICs were determined by broth dilution using cation-adjusted Mueller-Hinton broth in accordance with CLSI (formerly NCCLS) recommendations (6) and recorded as the lowest antibiotic concentrations required to inhibit visible growth (6). All MIC determinations were conducted at least in triplicate on separate days. The antimicrobials used in this study (and their respective suppliers) were levofloxacin (LVX; Biochemika, Buchs, Switzerland), tobramycin (TOB; Sigma, St. Louis, MO); ceftazidime (CAZ; Sigma, St. Louis, MO); piperacillin (Sigma, St. Louis, MO)-tazobactam (Wyeth-Ayerst, Monmouth Junction, NJ) (TZP), meropenem (MEM; Astra Zeneca, Mt. Prospect, IL), colistin (COL [polymyxin E]; Sigma, St. Louis, MO), and azithromycin (AZM; Pfizer, Inc., Groton, CT). The MICs (μg/ml) for *P. aeruginosa* isolates 46139, 49674, and 36375 were 0.5, 1, and 1 (levofloxacin); 1, 1, and 2 (tobramycin); 2, 1, and 4 (ceftazidime); 4-4, 2-4, and 32-4 (piperacillin-tazobactam); 0.25, 0.25, and 0.25 (meropenem); 1, 1, and 1 (colistin); and 256, 256, and 256 (azithromycin), respectively.

The isolates were grown overnight on Mueller-Hinton agar at 35°C in ambient air. The overnight growth was swabbed into Mueller-Hinton broth and incubated for 3 h at 35°C in ambient air in order to achieve inocula of ~10^{10} CFU (1, 8, 11). The inocula were quantified through the serial dilution and plating of 100-μl samples on drug-free medium. Simultaneously, *P. aeruginosa* mutants were selected by plating the inocula on

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TABLE 1. *P. aeruginosa* MPC/MIC ratios for antimicrobials alone or in combination with levofloxacin[a]

Isolate no.	MPC/MIC ratio for:												
	LVX	TOB	LVX + TOB	CAZ	LVX + CAZ	TZP	LVX + TZP	MEM	LVX + MEM	COL	LVX + COL[b]	LVX + COL[c]	LVX + AZM
46139	8	16	2	>32	1	>32	1	>32	1	>32	8	1	8
49674	8	8	1	>32	2	>32	2	>32	1	32	8	1	8
36375	4	4	2	>32	1	NT[d]	1	>32	1	>32	4	1	4

[a] MPC/MIC ratio is defined as the ratio of the MPC obtained to the original MIC.
[b] COL, 2 μg/ml.
[c] COL, 8 μg/ml.
[d] NT, not tested.

Mueller-Hinton agar containing 1×, 2×, 4×, 8×, or 16× their levofloxacin MIC alone and in combination with either tobramycin (6 μg/ml), ceftazidime (32 μg/ml), piperacillin and tazobactam (128 and 4 μg/ml, respectively), meropenem (16 μg/ml), colistin (2 μg/ml or 8 μg/ml), or azithromycin (0.4 μg/ml). *P. aeruginosa*-resistant mutants were not selected for at the MPC or at concentrations above the MPC. The selected concentrations of antimicrobials used in combination with levofloxacin reflect their average 24-h serum concentrations in healthy adults (5). While 2 μg/ml best approximates this concentration for colistin (5), 8 μg/ml was also tested, as this concentration may be attained with more aggressive dosing. Inocula were also plated on Mueller-Hinton agar containing 1×, 2×, 4×, 8×, or 16× the MIC of each nonfluoroquinolone antimicrobial except azithromycin. Azithromycin was not tested alone due to technical issues with preparing agar plates containing >256 μg/ml of the drug. For isolate 36375, this limitation also occurred with piperacillin-tazobactam. The antibiotic-containing plates were incubated in ambient air at 35°C for 48 h, and the antibiotic-free plates were incubated under the same conditions for 24 h. Following their respective incubation times, colonies were counted. All experiments were carried out in triplicate on separate days. All repetitions of MPC experiments resulted in MPC values within one dilution (a twofold difference).

The MPC for each drug-isolate combination was defined as the lowest antibiotic concentration that prevented the visible growth of mutant colonies (1, 8, 11). The MPC/MIC (μg/ml) ratio, defined as the ratio of the MPC obtained to the original MIC, was used to represent the data obtained (8). The MPC/MIC ratios are reported in Table 1. Mutational frequencies for each antibiotic MIC multiple were calculated by dividing the number of mutant colonies by the initial bacterial inoculum. As the units for both the mutant colonies selected for and the size of the initial bacterial inoculum were CFU/ml, there are no units for mutational frequency. The average mutational frequencies are reported in Table 2.

The MPC/MIC ratio for levofloxacin tested alone was 4 to 8 for each of the three isolates (Table 1). When a second antimicrobial was used in combination with levofloxacin, the MPC/MIC ratio for the combination decreased four- to eightfold for all combinations with the exception of strain 36375 (the MPC/MIC ratio for levofloxacin and tobramycin decreased by only twofold) and strains for which levofloxacin combinations with colistin (at 2 μg/ml) and azithromycin were used, in which cases no decrease in MPC occurred (Table 1). Regarding mutational frequencies, for agents used alone, only with levofloxacin and tobramycin were actual MPCs attained (Tables 1 and 2). For ceftazidime, piperacillin-tazobactam, meropenem, and colistin, an actual MPC was not obtained at any concentration tested. Combinations of levofloxacin and meropenem resulted in the lowest mutational frequencies (the least-frequent mutation development) for all strains relative to their individual mutational frequencies, followed by piperacillin-tazobactam and ceftazidime (Table 2). Why mutational frequencies were higher than expected for strain 49674 with the levofloxacin in combination with piperacillin-tazobactam or ceftazidime is unclear but confirms the finding that higher doses of antimicrobials are more able to prevent resistance selection. For both strains 46139 and 36375, the combination of levofloxacin and tobramycin was expected to result in a mutational frequency of $\sim > 1 \times 10^{-13}$, and yet frequencies were higher, at 7.46×10^{-10} and 1.66×10^{-7}, respectively (Table 2). Why the levofloxacin-tobramycin combination mutational frequencies for strains 46139 and 36375 were higher than expected is unclear, but this again confirms the finding that higher doses of aminoglycosides are more able to prevent resistance selection. Combinations of levofloxacin and colistin (2 μg/ml) or azithromycin did not reduce the mutational frequencies relative to that for levofloxacin alone (Table 2).

Our results demonstrate that when individual antimicrobials are used alone, only levofloxacin and tobramycin at high doses prevent resistance in *P. aeruginosa*. This is consistent with the fact that these agents are concentration-dependent bacterial killers (8). Of ceftazidime, colistin, meropenem, and piperacillin-tazobactam, none was able to prevent resistance when used alone. These data provide a caution regarding using these agents alone instead of in combination for the treatment of infections caused by *P. aeruginosa.* Our results also demonstrate that the combination of levofloxacin and a second antimicrobial (with each antimicrobial possessing independent activity against *P. aeruginosa* and acting with a different mechanism of action) is more effective at preventing resistance selection in *P. aeruginosa* than are the two agents individually. This is demonstrated by lowered mutational frequencies in the presence of a second agent compared to that for levofloxacin alone (Table 2). This observation was substantiated by the finding that the antimicrobial combination MPC/MIC ratios were significantly lower than the MPC/MIC ratios for drugs administered individually (Table 1). The absence of a decrease in MPC/MIC ratios in the levofloxacin combination regimens utilizing low-dose colistin or azithromycin supports the hypothesis that for dual-drug therapy to be effective in preventing selection for resistant mutants, bacteria must be susceptible to both antimicrobials. It is unclear why the combination of levofloxacin with low-dose colistin resulted in high mutational frequencies. Perhaps

TABLE 2. Mutational frequencies of drug-isolate combinations at each increasing MIC multiple

Isolate	MIC multiple	Mutational frequency for:												
		LVX	LVX with:						TOB	CAZ	TZP	MEM	COL
			TOB	CAZ	TZP	MEM	COL[a]	COL[b]	AZM					
46139	1×	1.31×10^{-6}	7.46×10^{-10}	—	—	—	$>2.65 \times 10^{-6}$	—	2.22×10^{-4}					
	2×	4.42×10^{-8}	—				1.75×10^{-7}		1.50×10^{-5}	7.00×10^{-7}	7.25×10^{-7}	1.31×10^{-5}	$>2.4 \times 10^{-4}$	$>1.06 \times 10^{-5}$
	4×	5.48×10^{-8}					4.13×10^{-8}		1.74×10^{-7}	1.97×10^{-7}	1.29×10^{-6}	1.34×10^{-6}	3.98×10^{-6}	6.40×10^{-6}
	8×	—					6.55×10^{-9}		—	1.08×10^{-7}	$>1.34 \times 10^{-7}$	$>9.45 \times 10^{-7}$	1.27×10^{-6}	1.61×10^{-6}
	16×						—			—	$>1.34 \times 10^{-7}$	$>8.99 \times 10^{-7}$	4.80×10^{-7}	$>4.15 \times 10^{-7}$
	32×										$>1.25 \times 10^{-8}$	$>5.10 \times 10^{-7}$	1.99×10^{-8}	$>9.27 \times 10^{-8}$
49674	1×	7.25×10^{-5}	—	6.43×10^{-7}	1.04×10^{-8}	—	$>3.75 \times 10^{-6}$	—	9.21×10^{-6}	1.53×10^{-6}	8.66×10^{-6}	$>1.15 \times 10^{-4}$	$>2.17 \times 10^{-4}$	4.66×10^{-6}
	2×	4.79×10^{-6}		—	—		6.69×10^{-7}		1.15×10^{-6}	1.97×10^{-7}	5.59×10^{-7}	7.80×10^{-6}	$>1.53 \times 10^{-4}$	3.34×10^{-6}
	4×	1.26×10^{-7}					8.90×10^{-8}		3.79×10^{-8}	3.56×10^{-8}	2.57×10^{-7}	1.02×10^{-7}	6.68×10^{-6}	2.82×10^{-6}
	8×	—					—		—	—	2.71×10^{-7}	5.79×10^{-7}	8.68×10^{-7}	$>2.28 \times 10^{-7}$
	16×										$>3.26 \times 10^{-7}$	$>5.76 \times 10^{-7}$	$>2.13 \times 10^{-7}$	$>4.65 \times 10^{-8}$
	32×										1.78×10^{-7}	$>5.76 \times 10^{-7}$	$>1.15 \times 10^{-7}$	—
36375	1×	8.61×10^{-5}	1.66×10^{-7}	—	—	—	$>3.78 \times 10^{-5}$	—	2.86×10^{-3}	8.01×10^{-7}	$>1.5 \times 10^{-5}$	Not tested	$>6.2 \times 10^{-4}$	$>1.81 \times 10^{-3}$
	2×	8.97×10^{-6}	—				2.83×10^{-6}		2.54×10^{-5}	1.06×10^{-7}	$>8.8 \times 10^{-6}$		$>6.2 \times 10^{-4}$	$>1.89 \times 10^{-5}$
	4×	—					—		—	—	$>5.5 \times 10^{-7}$		3.39×10^{-5}	$>4.23 \times 10^{-5}$
	8×										$>6.0 \times 10^{-8}$		5.30×10^{-6}	$>1.33 \times 10^{-5}$
	16×										$>7.0 \times 10^{-8}$		7.65×10^{-7}	$>3.02 \times 10^{-6}$
	32×										$>1.0 \times 10^{-7}$		8.55×10^{-7}	1.03×10^{-7}

[a] COL, 2 μg/ml.
[b] COL, 8 μg/ml.
[c] —, the MIC multiple was the MPC; frequency was less than inoculum.

levofloxacin selected for mutants that were cross resistant to colistin via some sort of permeation mechanism. It should be mentioned that combination therapy with two antimicrobials with different mechanisms of action is not a new idea, as it has been reported to result in reduced mortality in *P. aeruginosa* bacteremia (7). What is novel about combination MPC is the concept of using specific combinations of antimicrobials not to simply increase bacterial killing but to actually maximize resistance prevention. This study demonstrated that the levofloxacin-meropenem combination was most effective at preventing resistance selection, followed by levofloxacin combinations with high-dose colistin, followed by ceftazidime and piperacillin-tazobactam, followed by tobramycin (Table 2).

One potential mechanism to explain these results is the closing of the fluoroquinolone MSW by a second antimicrobial (1–3, 11). Because two different antibiotics with different bacterial targets are used, bacteria must obtain mutations causing resistance to both agents in order to survive. A similar effect was noted by Firsov et al. (3), who found that doxycycline in combination with moxifloxacin additively suppressed MIC increases in *Staphylococcus aureus* in a pharmacodynamic model. Our results show that in an in vitro environment with static antimicrobial concentrations, the MPC for levofloxacin-susceptible *P. aeruginosa* strains can be successfully lowered when levofloxacin is combined with a second antibiotic that has a different mechanism of action and is provided at doses exceeding the strain's MIC. Levofloxacin has a high MPC for *P. aeruginosa* that can be difficult to achieve clinically. By employing one or more antimicrobials in combination with levofloxacin, it might be possible to minimize selection for resistant bacterial cells.

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