

CONCISE COMMUNICATION

Restricting the Selection of Antibiotic-Resistant Mutant Bacteria: Measurement and Potential Use of the Mutant Selection Window

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The selection of antibiotic-resistant mutant bacteria is proposed to occur in a drug concentration range (the mutant selection window) that extends from the minimum inhibitory concentration (MIC) of susceptible cells to the MIC of the least susceptible, single-step bacterial mutants (the mutant prevention concentration [MPC]). MPCs were estimated for tobramycin, chloramphenicol, rifampicin, penicillin, vancomycin, and several fluoroquinolones by use of *Escherichia coli* and *Staphylococcus aureus*. Comparisons among reported serum drug levels indicate that new fluoroquinolones are the least likely to enrich populations of resistant mutant bacteria during monotherapy. These data partly explain the selective enrichment of populations of resistant mutant bacteria in medical practice. The mutant selection window range (MPC:MIC) was narrowed for fluoroquinolones by structure modification, pointing to a new direction in antibiotic refinement. The mutant selection window and the MPC were determined for combinations of rifampicin and tobramycin, using *S. aureus*, as a guide for combination therapy with compounds that alone cannot block enrichment of mutant bacterial populations.

Antibiotic resistance is a growing problem that lacks a clear solution. Since spontaneous bacterial mutants usually arise at a low frequency (10^{-6} to 10^{-8}), preventing the selective enrichment of mutant bacterial populations may help restrict the development of antibiotic resistance. One way to achieve this may be to avoid prolonged therapy within the concentration range that selectively enriches the mutant fraction of bacterial populations [1, 2]. In the present study, we experimentally defined that concentration range, called the "mutant selection window," for a variety of antibacterial agents. We then used the window hypothesis to consider 3 approaches for suppressing the selection of resistant bacterial mutants. First, drug-pathogen combinations were identified for which previously reported serum drug concentrations exceeded the upper boundary of the window. Second, structural changes narrowed the window so that concentrations would be expected to be inside the window for shorter times. Third, conditions were defined for combinations of compounds that are outside the window. Collectively, these data show how the mutant selection window can serve as a microbiologic platform for selecting, determining the dose of, and refining compounds to help slow the development of resistance.

Materials and Methods

Bacterial strains, culture conditions, and antimicrobial agents. Strain DM4100, a derivative of *Escherichia coli* K-12, was grown in LB or on LB agar (Difeo). *Staphylococcus aureus* strain RN4220 was cultured in CY broth or on GL agar [3]. Growth temperature for both organisms was 37°C. Antimicrobial agents, except for some fluoroquinolones, were purchased from Sigma Chemical; ciprofloxacin, moxifloxacin, and Bay y3114 were obtained from Bayer; levofloxacin was obtained from Johnson-Ortho; and PD 135042 was a gift from Parke-Davis Pharmaceutical Research.

Measurement of MIC₉₉ and the mutant prevention concentration (MPC). The MIC₉₉ was measured by applying serial dilutions of stationary-phase cultures to agar plates containing various concentrations of antimicrobial agent. Bacterial colonies were counted after overnight incubation. Preliminary determinations using 2-fold dilutions of drug provided an approximate value of the MIC₉₉. This measurement was followed by a second determination, plus a replicate, that utilized linear drug concentration increments (~20% per sequential increase). The fraction of the colonies that were recovered was plotted against the drug concentration to determine MIC₉₉ by interpolation.

The MPC was defined as the lowest drug concentration that prevented bacterial colony formation from a culture containing $>10^{10}$ bacteria. The determination was similar to that for MIC₉₉, except that $>10^{10}$ cells were tested at high drug concentrations and interpolation was not used. Inoculated plates were incubated for 72 h, and colonies were counted at 24-h intervals until colony numbers became constant. The MPC was identical when exponentially growing bacterial cultures, rather than stationary-phase cells, were applied to agar plates.

Results

Definition of the mutant selection window. Selection of fluoroquinolone-resistant bacterial mutants depends strongly on

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drug concentration [4, 5]. Increasing concentration typically causes a sharp decline in the recovery of bacterial colonies, followed by a plateau, during which mutant bacteria present in the population are recovered. The plateau reflects mutation frequency. A second sharp decline occurs when the drug concentration blocks the growth of the least susceptible, single-step mutant bacteria [6]. This behavior is most clearly seen when only 1 intracellular target (gyrase) is present [4], as is the case for many mycobacteria. However, it can be observed with 2-target situations if target susceptibility differs sufficiently. Such is the case when *S. aureus* is treated with norfloxacin (figure 1A), a fluoroquinolone that strongly prefers topoisomerase IV to gyrase. The plateau is reduced, sometimes to an inflection point [4], when the compound attacks the 2 targets more equally. An example is seen when *S. aureus* is treated with ciprofloxacin (figure 1A). The drug concentration at which no bacterial mutant is recovered when $>10^{10}$ cells are applied to agar plates is operationally defined as the MPC [4]; above this concentration, 2 resistance mutations are expected to be necessary for growth.

A wide variety of mutant bacteria, including both target and putative efflux variants [4, 5], are selectively amplified in the concentration gap between the MPC and the lowest concentration that inhibits the growth of the majority of susceptible bacteria (experimentally approximated by MIC_{99}). That gap, the mutant selection window, is denoted by double-headed arrows in figure 1A. Placement of the mutant selection window on the pharmacokinetic

profile of ciprofloxacin (figure 1A, inset) illustrates how drug concentration can fall inside the window for much of the dosing time.

Dosing such that concentrations exceed the MPC. If drug concentration is maintained above the MPC, populations of resistant bacterial mutants should not be selectively enriched. To identify situations in which this might be applicable, we determined the MPC for a variety of compounds, using laboratory strains of *E. coli* and *S. aureus* (table 1). For rifampicin, the MPC was extremely high with both species. With *S. aureus*, tobramycin, penicillin G, ciprofloxacin, levofloxacin, and moxifloxacin had MPC values that were lower than their respective maximum serum concentrations (C_{max}). With *E. coli*, tobramycin, chloramphenicol, and penicillin G met this standard (norfloxacin was the only fluoroquinolone tested).

The dosing interval that is necessary to maintain serum concentrations above the MPC can be calculated from pharmacokinetic parameters and the MPC. The dosing interval was >7 h for chloramphenicol with *E. coli* and for penicillin G, levofloxacin, and moxifloxacin with *S. aureus* (table 1). High-level resistance that enters the population horizontally (e.g., via plasmids), as is often the case for tobramycin, penicillin G, and chloramphenicol resistance [14], may further reduce the number of drug-pathogen combinations for which concentrations can exceed the MPC with monotherapy. Thus, few agents currently available are likely to be suitable for maintaining concentrations above the

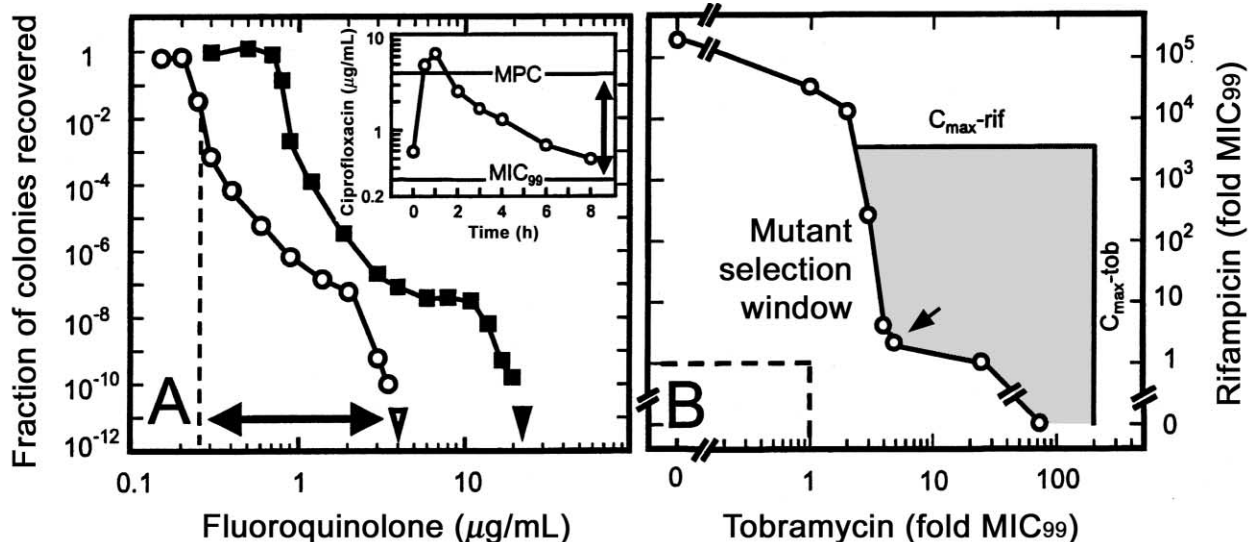


Figure 1. Mutant selection window. A, Treatment of *Staphylococcus aureus* cells with norfloxacin (squares) or ciprofloxacin (circles). The no. of colonies recovered after incubation is expressed as a fraction of input cells. The dashed line indicates the MIC_{99} of ciprofloxacin. Arrowheads indicate mutant prevention concentrations (MPCs) of ciprofloxacin or norfloxacin (i.e., concentrations that inhibited colony formation when $>10^{10}$ cells were applied to agar plates). Double-headed arrows indicate the mutant selection window. Inset, Pharmacokinetic profile of ciprofloxacin [7], with MIC_{99} and MPC values. B, Treatment of *S. aureus* cells ($>10^{10}$ cfu) with both rifampicin and tobramycin (expressed as multiples of MIC_{99}). Circles indicate combinations that prevented colony growth (MPC). " $C_{max-rif}$ " and " $C_{max-tob}$ " indicate maximum serum concentrations of rifampicin and tobramycin, respectively. The shaded area indicates achievable serum drug concentration combinations that exceeded the MPC. Dashed lines approximate the lower boundary of the mutant selection window.

Table 1. Potency and pharmacokinetic parameters for various antimicrobial agents used against *Escherichia coli* and *Staphylococcus aureus*.

Organism, antimicrobial agent	MPC, $\mu\text{g/mL}$	C_{max} , $\mu\text{g/mL}$ ^a	$C_{\text{max}}/\text{MPC}$	$t_{1/2}$, h ^a	Dosing interval, h ^b	MIC ₉₉ , $\mu\text{g/mL}$	MPC:MIC
<i>E. coli</i>							
Tobramycin ^c	25	52.2	2.1	1.8	1.9	1.2	21
Chloramphenicol	12	26	2.2	6.5	7.4	1.9	6.3
Rifampicin	>4000	9.5	<0.002	2	ND	7	>571
Penicillin G ^d	300	512	1.7	0.9	0.69	2.4	125
Norfloxacin	1.6	1.3	0.81	5.1	ND	0.045	36
<i>S. aureus</i>							
Tobramycin ^c	20	52.2	2.6	1.8	2.5	0.27	74
Chloramphenicol	40	26	0.65	6.5	ND	1.9	21
Rifampicin	480	9.5	0.02	2	ND	0.003	160,000
Penicillin G ^d	1	512	512	0.9	8.1	0.015	67
Vancomycin	40	39	1	6.5	0	0.65	62
Norfloxacin	22	1.3	0.06	5.1	ND	0.85	26
Ciprofloxacin ^c	4	6	1.5	3.9	2.3	0.3	13
PD135042 ^e	0.45	NA	NA	NA	NA	0.076	6
Ofloxacin	4.5	4.5	1	4.6	0	0.28	16
Levofloxacin	2.5	5.2	2.1	7.4	7.9	0.18	14
Moxifloxacin ^c	0.6	2.5	4.2	13.1	27	0.05	12
Bay y3114 ^e	1.7	NA	NA	NA	NA	0.05	34

NOTE. C_{max} , maximum serum concentration; MPC, mutant prevention concentration; NA, not available; ND, not determined because the MPC was greater than the C_{max} and, thus, the dosing interval would be a negative no. when calculated.

^aReferences for pharmacokinetic and dosing information were as follows: tobramycin [8], chloramphenicol [9], rifampicin [10], penicillin G [11], vancomycin [12], ciprofloxacin [7], and other quinolones [13].

^bThe longest dosing interval was calculated by $\text{DI} = t_{1/2} \times \log_2(C_{\text{max}}/\text{MPC})$, where $t_{1/2}$ is the half-life of the plasma drug concentration.

^cPharmacokinetics listed for tobramycin are for a once-daily dose used for the treatment of cystic fibrosis.

^dSpecific activity: 1600 U/mg.

^eCiprofloxacin and PD135042 have a piperazinyl ring at position C-7; Bay y3114 and moxifloxacin have a larger diazo bicyclo structure at C-7.

MPC. Such a result is not surprising, since these agents were developed for achieving concentrations above the MIC rather than the MPC.

Narrowing the mutant selection window. Another approach for restricting the selection of resistance is to use compounds having a narrow mutant selection window (i.e., a low ratio of MPC to MIC [selection index]). Selection indices varied considerably among the different agents and from one bacterial species to another (table 1). Thus, the range of the mutant selection window is specific for each compound-bacterium combination.

Members of a family of agents can differ with respect to the selection index. For example, the selection index for moxifloxacin, a C-8 methoxy fluoroquinolone, was approximately one-third that observed for its C-8 hydrogen cognate, Bay y3114 (table 1). Thus, addition of a methoxy group narrows the mutant selection window. Comparison of 2 C-8 hydrogen compounds, ciprofloxacin and Bay y3114, indicated that the selection index is greater when the C-7 group is larger (table 1; ciprofloxacin contains a C-7 piperazinyl ring, whereas Bay y3114 and moxifloxacin have a larger C-7 diazo bicyclo structure). These two features can be combined by adding a C-8 methoxy group to ciprofloxacin. The resulting compound, PD135042, had the lowest selection index of the quinolones tested (table 1). Thus,

compounds can be refined to narrow the mutant selection window.

Mutant selection window with 2 compounds in combination. Simultaneous administration of 2 compounds of different classes at concentrations above their respective MICs should require 2 concurrent resistance mutations for growth; therefore, no bacterial colony should be recovered when $>10^{10}$ cells are applied to drug-containing agar. To test this idea, combinations of tobramycin and rifampicin concentrations that blocked *S. aureus* colony formation were determined. Minimum drug concentrations represent values of combination MPC (figure 1B). The shaded portion of figure 1B represents combinations of rifampicin and tobramycin that were above the combination MPC and within achievable serum concentrations. In figure 1B, the mutant selection window extends from the data points down to the dashed lines. Below the dashed lines the concentration of neither compound is above its MIC₉₉; thus, those lines approximate the lower boundary of the mutant selection window.

The minimum concentration for restricting resistance is 2 and 4 times the MIC₉₉ for rifampicin and tobramycin, respectively (figure 1B). The time required for rifampicin to drop from its C_{max} to twice the MIC₉₉ was >22 h ($11 \times t_{1/2}$, where $t_{1/2}$ is the half-life of the plasma drug concentration); >13 h ($7 \times t_{1/2}$) were needed

for the tobramycin concentration to fall from C_{\max} to 4 times its MIC_{99} . If we assume that the total serum drug concentration represents the effective concentration at the site of infection, twice-daily dosing would maintain concentrations above the MPC for the combination regimen.

Discussion

The mutant selection window was experimentally defined by determination of MIC_{99} and MPC for a variety of antimicrobial agents. For some bacterium-drug combinations, such as *E. coli* treated with rifampicin, resistance appeared to be an all-or-none phenomenon, since no upper boundary of the window could be detected. Such compounds will be vulnerable to the selection of resistance when used as monotherapy, even if their MICs are very low. For bacterium-drug pairs that have measurable windows, the range of the mutant selection window, measured as the ratio of MPC to MIC, varied from ~ 6 (chloramphenicol with *E. coli* and PD135042 with *S. aureus*) to 160,000 (rifampicin with *S. aureus*) (table 1). Thus, window size is likely to be influenced by resistance mechanisms. It is also affected by small changes in drug structure, as was revealed by fluoroquinolone measurements (table 1).

Since pharmacokinetic fluctuations often place antibiotic concentrations in the mutant selection window, populations of resistant bacterial mutants are likely to be enriched by antibiotic treatment. Host defense systems usually eliminate mutant bacteria, especially after growth of susceptible pathogens is blocked by the antibiotic. Consequently, treatment failure due to the development of resistance in individual patients is not frequently observed [15]. However, when millions of antibiotic prescriptions are considered, resistant strains will accumulate, particularly among patients with weakened immune systems. Thus, suppression of the development of resistance requires use of antibiotic concentrations that directly attack resistant bacterial mutants, not just susceptible cells.

Pharmacokinetics are likely to play an important role in use of the mutant selection window hypothesis to slow the development of resistance. For example, when antibiotic concentrations at the site of infection exceed the MPC for a longer time, the antibiotic is expected to better restrict selection of resistance. Treatment times may be shortened for agents having a post-antibiotic effect against mutant bacteria. Since pharmacokinetic profiles are likely to vary among patients, concentrations are less likely to fall inside the window for compounds having a narrow gap between the MIC and MPC. Indeed, new compounds can be sought that have very narrow windows, and old compounds with broad windows, such as rifampicin, can be avoided for monotherapy. Even combination therapy is likely to be affected by pharmacokinetics because temporal mismatches (i.e., when the concentration of only 1 agent is above its MIC [2]) will create the equivalent of monotherapy. Plots like that shown in figure 1B help define the conditions (shaded area) for avoiding

periods of monotherapy that lead to enrichment of mutant bacterial populations. As data become available, such plots should be corrected for differences between effective tissue drug concentration, including those caused by protein-binding effects, and the more easily measured serum concentrations.

The mutant selection window forms the conceptual basis of a pharmacodynamic approach to slow the development of resistance by keeping 2 or more mutational steps ahead of bacterial populations. By use of drug concentrations greater than the mutant MPC or by use of combination therapy to stay beyond the mutant selection window, the agents directly attack mutant bacteria. The data presented here should help guide further animal and clinical tests. To date, none has been reported. The standard empirical approach, which relates antimicrobial exposure to the susceptibility of cells and to clinical outcome, relies on host defenses to remove mutant bacteria. This approach allows antimicrobial concentrations to fall inside the mutant selection window; thus, it is expected to restrict enrichment only of mutant bacterial populations that confer low to moderate levels of resistance. However, as pharmacodynamic parameters such as C_{\max}/MIC are raised, a point will be reached at which drug concentrations exceed the MPC throughout therapy. At that point, the standard pharmacodynamic approach will converge with the mutant selection window strategy.

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