Mutant Selection Window Hypothesis Updated

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The mutant selection window hypothesis postulates that, for each antimicrobial-pathogen combination, an antimicrobial concentration range exists in which selective amplification of single-step, drug-resistant mutants occurs. This hypothesis suggests an antimutant dosing strategy that is keyed to the upper boundary of the selection window: the mutant prevention concentration. Correlations are described between the mutant prevention concentration—a static parameter that is measured with agar plates—and fluctuating drug concentrations that restrict mutant amplification in vitro and in animals. When drug resistance is acquired stepwise, the mutant selection window increases, making the suppression of each successive mutant increasingly more difficult. For agents that kill drug-resistant mutants in a drug concentration–dependent manner, the use of the area under the 24-h time–drug concentration curve value divided by the value of the mutant prevention concentration is suggested as an index for designing antimutant dosing regimens. The need for such regimens is emphasized by a clinical example in which acquisition of drug resistance occurs concurrently with eradication of susceptible bacterial cells. These data support using the mutant selection window to optimize antimicrobial dosing regimens.

Antimicrobial resistance is a complex problem that is likely to require attention at many levels [1]. Issues concerning dosing are addressed by the mutant selection window hypothesis [2, 3]. This hypothesis maintains that drug-resistant mutant subpopulations present prior to initiation of antimicrobial treatment are enriched and amplified during therapy when antimicrobial concentrations fall within a specific range (the mutant selection window). The upper boundary of the mutant selection window is the MIC of the least drug-susceptible mutant subpopulation, a value called the mutant prevention concentration (MPC) [4]. The lower boundary of the mutant selection window is the lowest concentration that exerts selective pressure, often approximated by the minimal concentration that inhibits colony formation by 99% (MIC_{99}). Two principles emerge from the hypothesis. First, traditional dosing strategies, which seek to block drug resistance by killing susceptible cells [5, 6], allow enrichment of drug-resistant pathogens when they place drug concentrations inside the window. Second, maintaining drug concentrations above the selection window throughout therapy should severely restrict the acquisition of drug resistance, just as maintaining concentrations above the MIC blocks the growth of drug-susceptible cells. These 2 ideas are central to the design of antimutant dosing strategies for bacterial populations that are not already fully drug resistant.

Although the hypothesis is straightforward, application is not, largely because doses required to restrict drug resistance are higher than are generally needed to cure patients. From the perspective of most patients, using the selection window to guide dosing constitutes a form of altruism: an individual accepts an increased risk of adverse toxic effects in exchange for slower acquisition of drug resistance within the community. However, as drug-resistant mutant subpopulations grow, the selection window will become increasingly important for all patients, because drug-resistance can be acquired during therapy [7–9]. Herein, we review recent tests and refinements of the window hypothesis.

Received 3 August 2006; accepted 13 November 2006; electronically published 24 January 2007.

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Clinical Infectious Diseases 2007; 44:681–8

1058-4838/2007/4405-0009$15.00
DOI: 10.1086/511642

MEASURING THE MUTANT SELECTION WINDOW

The selection window is easily observed when mycobacteria are applied to fluoroquinolone-containing agar (schematically shown in figure 1*A*). When drug concentration increases, the fraction of cells that are recovered as colonies decreases, levels to a broad plateau, and then decreases a second time [4, 10, 11]. The initial decrease arises from the inhibition of wild-type growth and occurs at concentrations approximated by the MIC. The plateau is the result of mutant subpopulations that are present at low frequencies (e.g., 10^{-6} to 10^{-8}). Colonies that are recovered at low fluoroquinolone concentrations are predominantly caused by nontarget (nongyrase) mutants [10, 12, 13], whereas at moderate quinolone concentrations, several different *gyrA* (gyrase) mutants are recovered [10]. When drug concentration increases, only the more protective *gyrA* mutations are observed; when drug concentration is high enough to block the growth of the least susceptible single-step mutant, colony recovery decreases sharply a second time. The antimicrobial concentration at the second sharp decrease corresponds to the MIC of the least susceptible single-step mutant [4, 11]. This value is called the MPC because, above that concentration, bacterial growth is expected to require $\geqslant$2 concurrent mutations, which are unlikely to occur. Single mutations can still occur in bacterial cells, but most—if not all—mutants fail to amplify when drug concentrations are maintained above the MPC.

Although a broad plateau in mutant recovery is observed for fluoroquinolones with mycobacteria [4, 10], for erythromycin and rifampicin with *Staphylococcus aureus* [3, 14], and for miconazole with *Candida* yeast [12], many other antimicrobial-pathogen combinations exhibit only a small inflection in the selection curve, rather than a distinct plateau [4, 9, 15]. A small inflection is expected if 2 independent targets having similar drug affinity are present (e.g., gyrase and topoisomerase for fluoroquinolones): drug concentrations that trap 1 enzyme on DNA (MICs) would be close to those that trap both (MPCs). As expected, mutations that eliminate 1 of the 2 gene products as a target create a broad plateau in the selection curve [15]. The presence of multiple drug targets with similar susceptibilities [16–18] explains why little difference is observed between MIC and approximations of MPC for β-lactams with pneumococci [19].

The absence of a distinct plateau makes it difficult to determine MPC from the shape of bacterial population analysis profiles; consequently, MPC is approximated as the drug concentration at which no colony is recovered when $>10^{10}$ cells are applied to agar plates [4]. Because bacterial infections generally contain $<10^{10}$ organisms, blocking the growth of 10^{10} bacterial cells should also block the growth of all single-step, drug-resistant subpopulations that are likely to be present spontaneously.

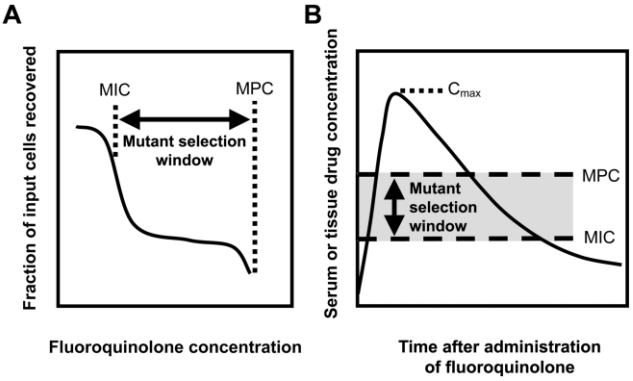

Figure 1. Schematic representation of the selection window. *A,* selection window determined with agar plate data. Mycobacterial cells were applied to agar that contained various concentrations of fluoroquinolone (*x-axis,* log scale), and colonies were scored (*y-axis,* log scale) to generate the solid curve. *Dashed lines,* MIC and mutant prevention concentration (MPC); *double-headed arrow,* the mutant selection window. *B,* selection window boundaries (MIC and MPC) from *A* are layered on hypothetical pharmacokinetic curve *(solid line). Double-headed arrow,* the mutant selection window. C_{max}, the peak concentration. Figure adapted from [2].

Situations have been observed in which a broad plateau occurs without a second decrease in colony recovery (that is, in which the selection window is infinitely wide) [3, 20]. Such cases, exemplified by rifampicin treatment of *Escherichia coli,* may reflect our inability to achieve drug concentrations that are high enough to block mutant growth (single-step mutants are highly protective). A wide selection window explains why rifampicin, a very active agent with *S. aureus*, is often unsuitable for monotherapy with this pathogen [21, 22].

The possibility has been raised that MPC might be a fixed multiple of MIC. If so, MPC could be determined from MIC measurements [23]. However, the correlation observed between MIC and MPC was low ($r^2 = 0.39$) for several closely related fluoroquinolones with *Mycobacterium smegmatis* [11], and recent comparison of ciprofloxacin MIC and MPC with clinical isolates of *E. coli* yielded an r^2 value of only 0.58 [24]. Poor correlations are also observed for a variety of quinolones with *S. aureus, Streptococcus pneumoniae,* and *Pseudomonas aeruginosa* [25], consistent with clinical isolates containing a variety of mutations—some having more effect on MPC and others having more effect on MIC. Thus, using MIC to predict MPC is likely to be inaccurate.

Inoculum size can dramatically affect the recovery of drug-resistant mutants. For example, if the inoculum size is small, mutants may be absent, thereby explaining failure to selectively enrich them [26]. At an intermediate inoculum size, nontarget mutants can appear to be the least drug-susceptible species [27,

Table 1. Values of the area under the 24-h time-concentration curve (AUC_{24}) divided by the MPC (AUC_{24}/MPC) that restrict the recovery of fluoroquinolone-resistant mutants.

Bacterial species	Fluoroquinolone used	Experimental model	Restrictive AUC_{24}/MPC (AUC_{24}/MIC), in h	Reference(s)
Staphylococcus aureus	Levofloxacin	In vitro	69 (200)[a]	[31, 65]
S. aureus	Moxifloxacin	In vitro	59 (220)	[31]
S. aureus	Gatifloxacin	In vitro	60 (240), 89 (310)	[31, 66]
S. aureus	Ciprofloxacin	In vitro	69 (240)[b]	[31]
S. aureus	ABT452	In vitro	69 (240)	[65]
S. aureus	Levofloxacin	Rabbit tissue cage	18 (107)[c]	[43]
Streptococcus pneumoniae	Moxifloxacin	In vitro	20 (100)	[32]
Escherichia coli	Ciprofloxacin	In vitro	22 (350)	[39]

NOTE. MPC, mutant prevention concentration.

[a] In another study [33], AUC_{24}/MIC and AUC_{24}/MPC values were lower (125 h and 36 h, respectively), probably as a result of a small inoculum size (10^8 colony-forming units).

[b] In another study [34], AUC_{24}/MPC and AUC_{24}/MIC values were higher (87 h and 580 h, respectively), probably as a result of a large dose gap between acquisition and restriction of drug-resistant mutants.

[c] Calculated from free-drug concentration.

28], because recovery of rare target mutants may require higher inocula [13]. A large inoculum size can also facilitate wild-type growth on agar plates, making it necessary to use many plates to reduce cell density when testing a total of 10^{10} cells. Because using many plates may not be logistically feasible when surveying large numbers of clinical isolates, an approximation is used in which 10^9 colony-forming units are applied to each agar plate in a series and drug concentration is varied by 2-fold increments [29, 30]. The large inoculum size and the large increment of increase in drug concentration cause the readout to be the transition from bacterial lawn to no growth.

DYNAMIC MODELS AND THE WINDOW HYPOTHESIS

Because drug concentrations are static in agar plates but fluctuate in vivo, it is necessary to relate the 2 measurements to one another. A general relationship is sketched in figure 1*B*. Testing involving *S. aureus* and using in vitro dynamic models reveals that drug-resistant mutants fail to amplify when fluctuating fluoroquinolone concentrations are maintained above or below the selection window that has been determined using agar plates; as expected, mutants are selectively amplified when concentrations are between the MIC and the MPC [31]. Similar observations were obtained with *S. pneumoniae* [32] and by additional experments with *S. aureus* [26, 33, 34]. The principle has also been extended to *S. aureus* for vancomycin and daptomycin [35].

Longer exposure inside the selection window should allow greater mutant amplification [36]. However, position in the window may be important for determining the effect of exposure time, because many more mutants—both in amount and type—are found in the lower portion of the window than in the upper portion, and because drug concentrations near the top of the window may be more effective at killing some mutant types [10]. The evolution of mutants may be a complex process that sometimes exhibits a correlation between time inside the window and outgrowth of mutants [35] and sometimes does not [26, 33, 34].

Because MPC is a bacteriostatic parameter, it overestimates the threshold needed to restrict mutant subpopulation outgrowth for compounds that kill drug-resistant mutants. The effect of lethal activity is addressed by pharmacokinetic-pharmacodynamic considerations [37], which are commonly used to relate antimicrobial dosing regimens to efficacy (i.e., removal of drug-susceptible bacterial populations). For example, empirical evidence indicates that the area under the 24-hour time-concentration curve (AUC_{24}) value divided by the value of the MIC (AUC_{24}/MIC) predicts a favorable outcome for "concentration-dependent killers" [38]. Application of this relationship to drug-resistant mutant subpopulations leads to replacement of the MIC with the MPC (i.e., the MIC of the least drug-susceptible single-step mutant), making the AUC_{24} value divided by the MPC value (AUC_{24}/MPC) the upper limit of the selection window when lethal action is considered; the same logic would apply when the maximal concentration value divided by the MIC value predicts favorable outcome with drug-susceptible infections). The AUC_{24}:MPC value is expected to be more accurate than the AUC_{24}/MIC value, because MIC determinations generally ignore mutant subpopulations. Mutant-restrictive values of AUC_{24}/MPC for fluoroquinolones are 60–70 h with *S. aureus* and approximately one-third of that value with *S. pneumoniae* and *E. coli* (table 1). More work is required to understand species differences and to determine whether the AUC_{24}/MIC

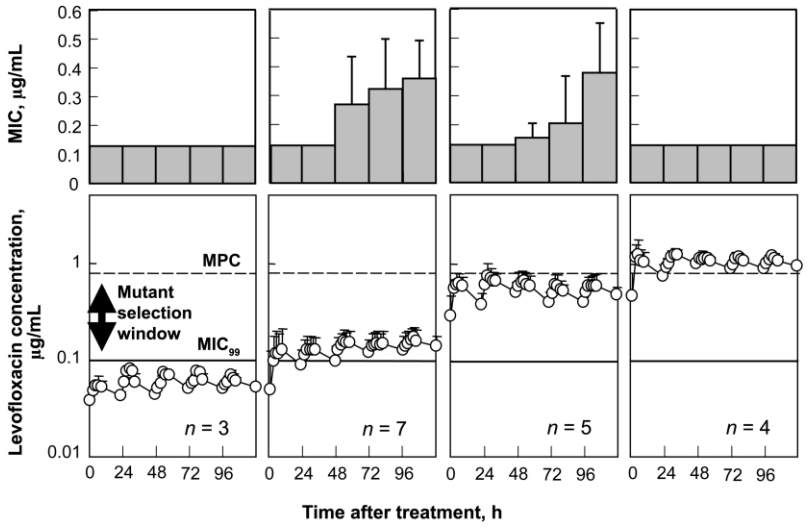

Figure 2. Mutant selection window in vivo. A tissue-cage infection model was established by surgical implantation under the skin of rabbits of hollow plastic balls with holes through their surface. After 4 weeks, *Staphylococcus aureus* was injected inside the ball. At the times indicated, oral levofloxacin was administered, and drug concentrations in the ball were determined. Bacterial samples were collected at 24-h intervals, and the MIC was determined. The MIC that blocks colony formation by 99% (MIC_{99}) and the mutant prevention concentration (MPC) were determined separately using levofloxacin-containing agar. The number of animals used is indicated in each panel. Data adapted from [43].

value or the AUC_{24}/MPC value is more predictive with individual isolates when measured in vitro [35, 39].

THE SELECTION WINDOW IN ANIMAL MODELS

Fitting in vitro determinations of window boundaries to animal systems requires 2 corrections. One concerns lethal action against mutants, as discussed above. Another involves the effect of host defense. When estimated with neutropenic and nonneutropenic mice, host defense has a 1.3- to 2-fold effect on pharmacodynamic indices that correlate with a good therapeutic outcome [40]. These corrections indicate that fluoroquinolone concentrations need not be maintained above the MPC throughout therapy to restrict mutant amplification.

Three animal tests of the selection window hypothesis have been reported. The first involved treatment with levofloxacin of mouse thigh infected with *P. aeruginosa* [28]. Amplification of efflux mutants was restricted with the use of doses of levofloxacin that generated an AUC_{24}/MIC value of 110 h (free-drug concentration); the MPC was not measured. The second animal experiment focused on pneumococcal pneumonia in rabbits. When treated with moxifloxacin, the recovery of mutants was suppressed when serum drug concentrations exceeded the MPC for slightly less than one-half of the duration of the dosing period [41, 42]. Quantitative comparisons with other systems are difficult, because pharmacokinetics were not measured at the infection site. The third report examined tissue-cage infection of rabbits with *S. aureus* [43]. Hollow plastic balls with holes through their surface were implanted under the skin of rabbits. After 4 weeks, the interior of each ball formed a compartment, into which *S. aureus* was injected. When levofloxacin was administered orally, drug resistance was acquired only when drug concentrations inside the balls were within the selection window (figure 2). In this system, static agar-plate data accurately defined the selection window. The restrictive AUC_{24}/MPC value was 18 h for free drug—approximately one-third of that observed in vitro with *S. aureus* (table 1).

ACQUISITION OF DRUG RESISTANCE AND ERADICATION OF SUSCEPTIBLE CELLS

A central prediction of the selection window hypothesis is that acquired drug resistance can develop under conditions that eradicate susceptible cells. To test this idea, we observed a small number of patients who had tuberculosis and who were colonized by *S. aureus* in the interior nares. Exposure of *S. aureus* to rifampicin was an unintended clinical consequence of treatment for tuberculosis [44]. After 4 weeks of therapy, colonization was eradicated in 53 (92%) of 58 patients. At the same time, drug resistance was acquired by *S. aureus* in 5 patients (8%). DNA from each of the 5 drug-resistant isolates differed from each other in terms of PFGE banding pattern and *spa* type, which argues against dissemination as a source of resistance. Moreover, isolates obtained before and after therapy were identical according to the 2 genetic tests performed for each of the 5 patients carrying a resistant isolate. Thus, traditional treatment strategies aimed only at eradicating susceptible cells

are likely to be inadequate in blocking the acquisition of drug resistance [45].

STEPWISE ACCUMULATION OF DRUG-RESISTANCE MUTATIONS

Drug resistance can occur in a stepwise manner when bacteria have multiple ways to reduce antimicrobial susceptibility and when drug-resistance alleles have an additive effect [46, 47]. To determine the effect of stepwise accumulation of drug resistance on the mutant selection window, fluoroquinolone resistance of *Haemophilus influenzae* was examined [48]. When wild-type *H. influenzae* was applied to ciprofloxacin-containing agar, *gyrA* drug-resistance mutants were recovered. One of the mutants was then applied to agar containing higher concentrations of ciprofloxacin to obtain an additional mutation in *parC*. Subsequent challenges added mutations in *gyrA* and *parC*. At each step in the selection process, the values of MIC and MPC increased (i.e., the selection window increased as mutations were acquired) (figure 3).

With wild-type *H. influenzae*, the selection window is below the minimal serum concentration of ciprofloxacin measured in healthy volunteers (figure 3*A*); after the first-step *gyrA* mutation is acquired, the window partially overlaps serum concentration (figure 3*B*). Acquisition of the second-step *parC* mutation causes the window to fully overlap serum concentration (figure 3*C*). With the third-step *gyrA-parC-gyrA* mutant, the window exceeds serum concentration (figure 3*D*). These changes in window position relative to therapeutic drug concentrations should cause each successive mutation to be more readily fixed in the population as a result of the decreased time that mutant growth is restricted and of the diminished killing of mutant cells. The same principles should apply to the acquisition of nontarget mutations, such as those involved in drug efflux, and to the gradual loss of susceptibility observed when vancomycin-intermediate *S. aureus* subpopulations are enriched in patients [8, 9]. Thus, the window hypothesis provides a clear rationale for avoiding antimicrobial doses that place drug concentrations inside the window.

POTENTIAL APPLICATIONS

General principles are beginning to emerge from the window hypothesis. For example, allowing drug concentrations to remain in the bottom portion of the selection window should rapidly enrich mutant subpopulations, because more mutant types are able to grow at low drug concentrations than at high concentrations [10, 49]. Moreover, infrequent dosing with long-lived compounds that place concentrations inside the selection window for days at a time is expected to lead to drug resistance more rapidly than dosing that keeps concentrations above the window [50, 51]. Third, if drug–resistance mutations

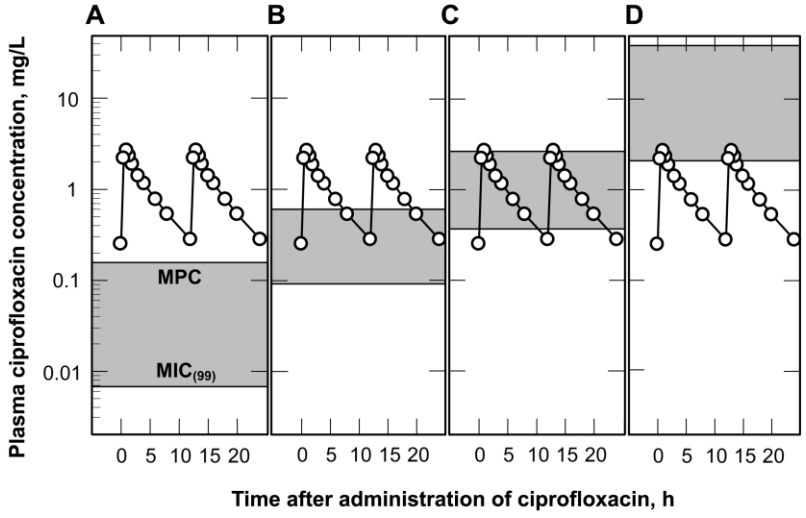

Figure 3. Effect of step-wise accumulation of fluoroquinolone-resistance mutations on the mutant selection window. *A,* Wild-type *Haemophilus influenzae* was applied to agar that contained various concentrations of ciprofloxacin, to determine the minimal concentration that blocks colony formation by 99% (MIC_{99}) and the mutant prevention concentration (MPC), which were taken as the lower and upper boundaries of the mutant selection window, respectively *(shaded area)* [48]. *B,* A first-step *gyrA* mutant was recovered and reapplied to agar that contained various concentrations of ciprofloxacin in order to determine MIC_{99} and MPC values for the first-step mutant *(shaded area). C,* A second-step *gyrA parC* double mutant was recovered, and its selection window *(shaded area)* was determined (as above). *D,* A third-step *gyrA parC gyrA* triple mutant was recovered and was used to determine its selection window *(shaded area).* To relate the selection window changes to drug pharmacokinetics, ciprofloxacin concentration in human plasma *(circles)* is shown. Samples were obtained from healthy volunteers who had received 500 mg oral doses twice daily for 7 days; data were recorded over the course of 24 h on day 7 of treatment [67].

confer such high levels of protection that antimicrobial concentrations cannot be maintained either above the MPC for bacteriostatic compounds or above a critical value of AUC_{24}/MPC for lethal agents, restriction of mutant amplification may require dual- or triple-drug therapy with good pharmacodynamic overlap among the antimicrobial agents being used [2]. Combination therapy may also be needed when drug-resistance genes enter a bacterial population at high frequency, such as through the horizontal transfer of integrons and plasmids [52, 53] or when mutation frequency is elevated [54, 55].

Another application is the identification of infections that are likely to become drug resistant. For example, population analysis of isolates acquired from *S. aureus* infections has revealed subpopulations that have reduced susceptibility to vancomycin [8, 9]. Isolates that were recovered after administration of further vancomycin therapy were increasingly dominated by the mutants. The presence of large mutant subpopulations can be readily established by population analysis or, more simply, by MPC determination.

Measurements of MPC can also be used to evaluate potential applications for new compounds, such as garenoxacin. This quinolone exhibits potent activity against *S. aureus,* and some investigators have suggested that it might be useful with ciprofloxacin-resistant *S. aureus* [56–59]. However, determination of MPC with ciprofloxacin-resistant isolates indicates that garenoxacin resistance would be acquired as quickly as ciprofloxacin resistance by fully susceptible isolates [60, 61]; in both cases, serum drug concentrations are within the selection window for much of the dosing period [62]. These same in vitro measurements indicate that garenoxacin trials with ciprofloxacin-susceptible isolates might be successful if drug concentrations at the site of mutant amplification are at least as high as serum concentrations (i.e., serum drug concentrations are above MPC for the entire dosing period) [62, 63].

The most important application of the selection window hypothesis may ultimately involve the design and screening of new compounds [3]. Activity against drug-resistant mutant subpopulations can be monitored early in drug development, and compounds can be sought that have a very narrow selection window (i.e., values of MIC and MPC that are approximately equal) rather than just a low value of MIC. For such compounds, drug concentration would be inside the window only for short durations, thereby reducing drug-resistance problems caused by dosing errors and patient-to-patient variation in drug pharmacokinetics.

LIMITATIONS OF THE SELECTION WINDOW HYPOTHESIS

The drug concentration threshold that is required to restrict amplification of drug-resistant mutants depends on the size of treated pathogen populations. If that value is calculated by considering multiple patients (who can number in the millions with some bacterial infections), maintaining drug concentrations above the MPC or above an AUC_{24}/MPC threshold is expected to slow—but not prevent—the selective amplification of mutants: the occurrence of $\geqslant 2$ concurrent resistance mutations in the same bacterial cell is statistically likely, and double mutants would be enriched despite drug concentrations being maintained above the MPC. In such cases, we would rely solely on host defenses to eliminate drug-resistant subpopulations. Another limitation concerns the determination of drug pharmacokinetics, because drug concentration at the infection site is central to determining antimutant efficacy. With approved antimicrobials, serum pharmacokinetics in healthy volunteers are available. However, obtaining patient data may not be straightforward, especially if multiple sites of infection exist and if the data are not readily accessible. These issues, plus person-to-person pharmacokinetic variation, emphasize the importance of developing new compounds that have very narrow selection windows.

CONCLUSIONS

For situations in which the amplification of drug-resistant mutants can be described as hill climbing [68], the mutant selection window hypothesis provides a quantitative strategy for making dosing decisions: by requiring bacteria to obtain $\geqslant 2$ concurrent mutations for growth, the organisms are forced to climb a steep cliff. The use of the antimicrobial to block mutant growth constitutes an important distinction from traditional efforts that focus on killing susceptible cells and blocking susceptible cell outgrowth, because traditional strategies require cells to acquire only 1 mutation for growth. Thus, traditional approaches can eradicate susceptible portions of a bacterial population, while allowing the amplification of drug-resistant mutants [44].

Animal infection studies are beginning to show that antimutant thresholds can be predicted from agar-plate data. For antimicrobials that kill drug-resistant mutants in immunocompetent animals, mutant-restricting dosing regimens require an AUC_{24}/MPC value of only one-third of that observed in vitro. Thus, many more antimicrobial-pathogen combinations may be amenable to MPC-based dosing than are expected from in vitro data alone [64]. Applying the window hypothesis to compounds other than fluoroquinolones and conducting prospective clinical tests of the hypothesis are now priorities.

Acknowledgments

We thank Marila Gennaro and Richard Pine for their critical comments on the manuscript.

Financial support. Bayer, Bristol-Myers-Squibb, Mylan Pharmaceuticals, Cubist, and the National Institutes of Health (AI 35257).

Potential conflicts of interest. K.D. has served as a consultant to or received research support from Bayer, Bristol-Myers-Squibb, Mylan Phar-

maceuticals, and Cubist. X.Z. has received research support from Bayer, Bristol-Myers-Squibb, and Cubist.

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