

# Restricting the Selection of Antibiotic-Resistant Mutants: A General Strategy Derived from Fluoroquinolone Studies

Xilin Zhao and Karl Drlica

Public Health Research Institute, New York

Studies with fluoroquinolones have led to a general method for restricting the selection of antibiotic-resistant mutants. The strategy is based on the use of antibiotic concentrations that require cells to obtain 2 concurrent resistance mutations for growth. That concentration has been called the “mutant prevention concentration” (MPC) because no resistant colony is recovered even when  $>10^{10}$  cells are plated. Resistant mutants are selected exclusively within a concentration range (mutant selection window) that extends from the point where growth inhibition begins, approximated by the minimal inhibitory concentration, up to the MPC. The dimensions of the mutant selection window can be reduced in a variety of ways, including adjustment of antibiotic structure and dosage regimens. The window can be closed to prevent mutant selection through combination therapy with  $\geq 2$  antimicrobial agents if their normalized pharmacokinetic profiles superimpose at concentrations that inhibit growth. Application of these principles could drastically restrict the selection of drug-resistant pathogens.

Antibiotic resistance among human pathogens now includes almost every bacterial species for which antibiotic therapies exist [1–3]. Among the more newsworthy are *Staphylococcus aureus* and *Mycobacterium tuberculosis*, the former due to its notorious role in hospital-acquired infections [4] and the latter from its worldwide prevalence and recent multidrug-resistant outbreaks [5–9]. The problem of antibiotic resistance is likely to get worse, in part because the therapeutic concentrations currently used, which block growth of the majority of the susceptible pathogens, are often the very concentrations required to selectively enrich the resistant mutant portion of the population. The problem is also worsening because the patient population having weakened immune systems is increasing from factors such as aging, radiotherapy, infection with HIV,

and treatment with immunosuppressive chemicals. The immune systems of such patients have reduced ability to eliminate microbial pathogens, and so outgrowth of resistant mutants during antibiotic treatment is more likely to occur. A third contribution to increased resistance is the inadequate control of antibiotic use in agriculture. For example, quinolone usage in the poultry industry has increased the frequency of quinolone-resistant *Escherichia coli* and *Campylobacter* species recovered from humans [10–13]. If therapeutic strategies are not developed to specifically prevent the outgrowth of resistant mutants, many bacterial diseases are likely to become untreatable [2].

For many forms of resistance, 2 events must occur: resistant mutants must be generated, and then those mutants must be selectively enriched (selected) in the bacterial population. Genetic alterations occur naturally, often as an intrinsic consequence of DNA synthesis or as horizontal transfer of resistance genes from one bacterium to another. Therefore, resistant cells can be present in a bacterial population prior to administration of an antibiotic. Some mutations can also be generated by antibacterial agents [14]. Unfortunately,

Financial support: National Institutes of Health (AI35257) and Bayer AG.

Reprints or correspondence: Dr. Karl Drlica, Public Health Research Institute, 455 First Ave., New York, NY 10016 (drlica@phri.nyu.edu).

**Clinical Infectious Diseases** 2001;33(Suppl 3):S147–56

© 2001 by the Infectious Diseases Society of America. All rights reserved.  
1058-4838/2001/3306S3-0009\$03.00

blocking the generation of mutations is currently beyond our reach. However, mutants usually arise at such a low frequency, 1 in  $10^6$  to 1 in  $10^8$  cells for spontaneous mutations, that they can be readily controlled by host defense systems if no selective amplification occurs. If the number of mutants can be kept at this low level through proper intervention at the enrichment step, it may be possible to preserve the usefulness of antibiotics.

In the present work, we discuss the idea that an antibiotic concentration window exists in which resistant mutants are selectively amplified. The upper boundary of the window is an antibiotic concentration that blocks growth of first-step resistant mutants and thereby requires wild-type cells to attain 2 resistance mutations for growth. Such events are expected to be so rare that mutants will not be recovered above the concentration that blocks mutant growth. The lower boundary is the drug concentration at which growth inhibition of the majority of susceptible cells begins, a concentration that can be approximated by the MIC for half of the cells in the population (MIC<sub>50</sub>). Because the upper and lower limits of the window are measurable, antibiotics can be compared to find compounds that have narrower windows and that are better suited for administration above the upper boundary.

Several points emerge from consideration of the mutant selection window. First, monodrug therapies that place drug concentrations in the window should lead to resistance. Although such concentrations usually clear infections in immunocompetent patients, repeated exposure in the selection window, either with a single patient or spread over many patients, will gradually enrich the mutant fraction of the population. Second, having drug concentrations drop below the lower boundary, as when therapy is not completed, may lead to treatment failure and the opportunity for more spontaneous mutants to arise during the expansion of the population; however, it should not select resistant mutants (subsequent antibiotic challenges at concentrations in the window are required for mutant selection). Third, compounds with lower MICs are not necessarily superior agents with respect to mutant selection (raising the lower limit can narrow the window). Fourth, compounds that kill wild-type cells, but not resistant mutants at concentrations below the upper limit of the window, may select resistant mutants faster than bacteriostatic compounds. Consequently, lethal compounds are not necessarily superior with respect to selection of resistant mutants—superior compounds block mutant growth and kill resistant mutants more avidly.

In the following discussion we rely heavily on fluoroquinolone action and resistance to support the selection window concept. However, we have been able to define the boundaries of the window for chloramphenicol, tobramycin, rifampicin, vancomycin, and penicillin by using *S. aureus*, for ampicillin and tetracycline by using *M. smegmatis*, for isoniazid by using *M. tuberculosis*, and for miconazole by using the yeast *Candida*

*glabrata* (X. Zhao, J. Zhou, Y. Dong, J. Wang, unpublished observations). Thus the selection window ideas are probably applicable to many species and to many antimicrobial agents.

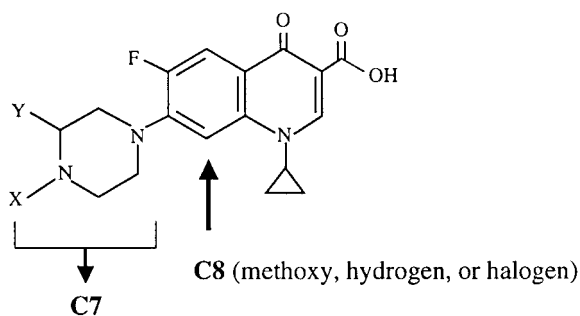
For some of the agents listed earlier, the upper boundaries of the window are too high to be therapeutically useful for monotherapy. Other compound-microbe combinations, such as rifampicin treatment of *E. coli*, may even lack an upper boundary (X. Zhao, unpublished observation). For such situations, selection of resistant mutants can be avoided by dual-drug therapy, because it also requires 2 mutations for pathogen growth. In a subsequent section we discuss how to match the pharmacokinetic profiles of 2 compounds to restrict the selection of resistant mutants even when patients fail to comply fully with therapy protocols.

It is important to emphasize that blocking the selection of resistant mutants is equivalent to halting the growth of mutants. Lethal antibiotic action constitutes a separate concept, which is considered after we discuss fluoroquinolone experiments that support the idea of the mutant selection window. We also stress that none of the ideas have been tested clinically or in animal models.

## FLUOROQUINOLONE RESISTANCE INCREASES STEPWISE

Stepwise resistance to fluoroquinolones arises when bacteria are sequentially challenged with increasing concentrations of drug. For gram-negative organisms, gyrase is usually the primary quinolone target, and first-step resistance alleles often map in *gyrA*. DNA topoisomerase IV is attacked only at higher concentrations than those required for gyrase, and so topoisomerase IV is considered to be a secondary target. Resistance mutations in *parC*, a gene encoding a subunit of topoisomerase IV, arise as second-step mutations after gyrase mutations have been established (in some bacteria 2 mutations occur in *gyrA* before 1 is found in *parC*, and cases have been found in which resistance alleles map in *gyrB* and *parE* [15, 16]). With gram-positive organisms, *parC* mutations generally arise before those in *gyrA*, although C8-substituted fluoroquinolones can have gyrase as a primary target [17, 18]. *M. tuberculosis*, and perhaps other mycobacteria, appear to have only gyrase as a target, and so both first-step and second-step resistance mutations map in gyrase [19]. Consequently, distinct patterns of susceptibility occur that are usually explained by the constellation and relative sensitivity of the drug targets. Adding to the complexity is the observation that within a given gene many different mutant alleles can occur. For example, among mycobacteria 12 different *gyrA* and 10 different *gyrB* mutations have been detected in collections of mutants selected by growth on fluoroquinolone-containing agar plates [20].

The availability of first-step resistance mutants encouraged



**Figure 1.** Fluoroquinolone structure. The C8 position and C7 ring are indicated by arrows.

us to test a variety of fluoroquinolones for the ability to attack mutants. Compounds having a C8-methoxy group (figure 1) were particularly effective [19, 21–23]. We noticed that the C8-methoxy group also enabled the fluoroquinolone to severely restrict the selection of resistant mutants when large numbers of wild-type cells (about  $10^{10}$ ) were applied to fluoroquinolone-containing agar [19, 21]. This was the result expected if the methoxy compound, through its attack of first-step mutants, required cells to have 2 resistance alleles for growth. When *E. coli* cells having a *parC* resistance allele but wild-type susceptibility were tested, the methoxy group had no effect on the recovery of mutants—for these cells only 1 additional mutation was needed for resistance rather than the 2 required with the wild-type strain [21]. Collectively these experiments showed that selection of resistant mutants could be restricted by requiring cells to have 2 resistance mutations for growth in the presence of drug.

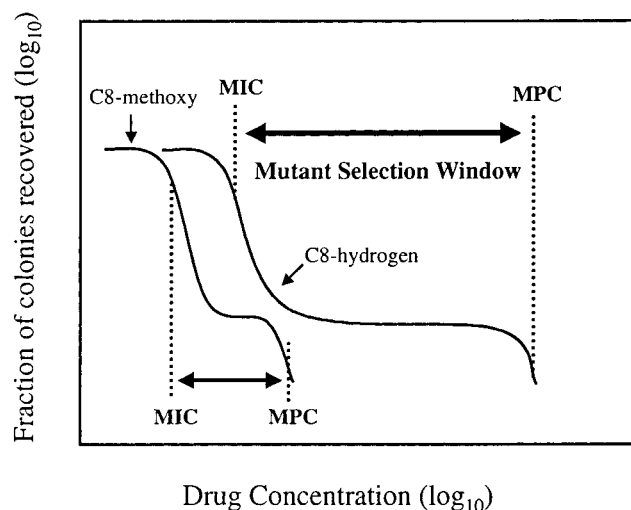
### FLUOROQUINOLONE CONCENTRATION AFFECTS SELECTION OF RESISTANT MUTANTS

The initial mutant selection experiments with the C8-methoxy fluoroquinolones were performed only at a few high drug concentrations. To further characterize selection we incubated large numbers of *M. bovis* BCG cells on agar plates containing different concentrations of fluoroquinolone and determined the number of colonies that arose [24]. As concentrations exceeded that required to block wild-type growth, the fraction of cells recovered as colonies dropped sharply. Further increases in drug concentration generated a plateau in mutant recovery, followed by a second sharp drop (figure 2). The plateau was caused by the growth of resistant mutants present in the population: DNA extracted from mutants collected from the plateau region contained quinolone-resistant *gyrA* alleles, as revealed by nucleotide sequence analysis [24]. The second sharp drop occurred at a concentration that blocked the growth of those mutants (the MIC of the most resistant, first-step mutant correlated with the concentration required to cause the second drop in mutant

recovery [25]). The same principles apply to *S. aureus*, a gram-positive organism that contains 2 topoisomerase targets for the fluoroquinolones, although the plateau is reduced to an inflection point [24]. In this case mutants recovered from the inflection region had mutations in *parC*.

Examination of allelic diversity, using *M. smegmatis* as a test organism, led to a scenario in which selection at low fluoroquinolone concentration produces large numbers of low-level, multidrug-resistant mutants [20]. Target (gyrase) mutants are not recovered, apparently because they are present at a much lower frequency than the multidrug-resistant mutants. But as selection pressure increases, target (*gyrA*) alleles are recovered while the number of multidrug-resistant mutants declines sharply. So far 10 different amino acid alterations have been observed in *gyrA* with *M. smegmatis*. High concentrations of antibiotic reduce the number of allelic types to just a few, and eventually a concentration is reached at which no mutant is recovered. This concentration defines the upper limit of the mutant selection window.

The *gyrA* mutations are unlikely to be caused (induced) by exposure to the fluoroquinolone because they are recessive mutations and because the selecting drug concentrations are sufficient to kill a newly mutated cell before resistance can be



**Figure 2.** Effect of fluoroquinolone concentration on selection of resistant mutants. *Mycobacterium bovis* BCG was applied to agar plates containing various concentrations of fluoroquinolone, and, after suitable incubation, colonies were counted. The figure is stylized from data in [24], and the fraction of cells recovered at the plateaus was about  $1 \times 10^9$ . MIC and mutant prevention concentration (MPC) are indicated by dotted lines, and the mutant selection windows are indicated by the double-headed arrows. In principle, the lower boundary of the window occurs at the drug concentration where growth inhibition of susceptible cells begins, a concentration that is difficult to determine. We use MIC for inhibition of 99% of the cells in a population to approximate this limit. MICs determined according to the NCCLS standards can also be used for approximation of the lower limit.

expressed. Moreover, in *E. coli* a *lexA* mutation, which blocks the SOS response, has little effect on the plateau level observed when mutant recovery is measured (X. Zhao, unpublished observation). Therefore, high-level, gyrase-mediated resistance is probably due to mutants present in the population prior to drug treatment.

The spectrum of alleles selected depends on drug structure. For example, moving an ethyl group from the C7-ring nitrogen (position X in figure 1) to an adjacent carbon (position Y in figure 1) changes the nature of the mutation conferring the most resistance [20, 25]. Changing the type of mutation could be important because the properties of the most resistant, first-step mutant determine the antibiotic concentration required to cause the second sharp drop in mutant recovery (figure 2), which, as mentioned earlier, is the upper boundary of the mutant selection window.

## MUTANT PREVENTION CONCENTRATION

Concentrations of C8-methoxy fluoroquinolones were readily attained at which no mutant was recovered even when  $>10^{11}$  cells were plated [21, 24]. This is the result expected when a rare double mutation is required for cell growth at that concentration. Because a double mutation is expected to occur rarely, on the order of 1 per  $10^{12}$  to 1 per  $10^{16}$  cells, resistant mutants will arise rarely. If the number of pathogens present in an infection is substantially lower than the number required to observe a double mutant, as appears to be the case [26–28], no mutant will be selected. We designate the antibiotic concentration that allows no mutant to grow as the mutant prevention concentration (MPC) [24]. In principle, the MPC provides a numerical threshold that might be used to severely restrict, if not prevent, the selection of resistance during therapy. For technical convenience and comparative purposes, MPC is defined as no colony recovery when  $>10^{10}$  cells are applied to agar plates. With both mycobacteria and *S. aureus*, the MPC is lower for C8-methoxy fluoroquinolones than for C8-hydrogen derivatives [24, 25].

MPC is measured by the same agar dilution strategy customary for MIC determinations; however, many more cells ( $10^{10}$ ) must be applied to plates for the MPC assay. As an example, MPC has been used to examine fluoroquinolone potency against clinical isolates of *Streptococcus pneumoniae* [29]. To keep the number of agar plates manageable, we applied  $10^{10}$  cells to each plate. Under these conditions of high cell density it was necessary to measure a provisional MPC ( $MPC_{pr}$ ), which overestimated MPC by about 2-fold. For several fluoroquinolones, 90% of about 90 clinical isolates lacking a known resistance mutation had a value of  $MPC_{pr}$  that was close to or below serum levels attainable with a dosing regimen considered to be safe. Thus it is possible to administer some fluoroquin-

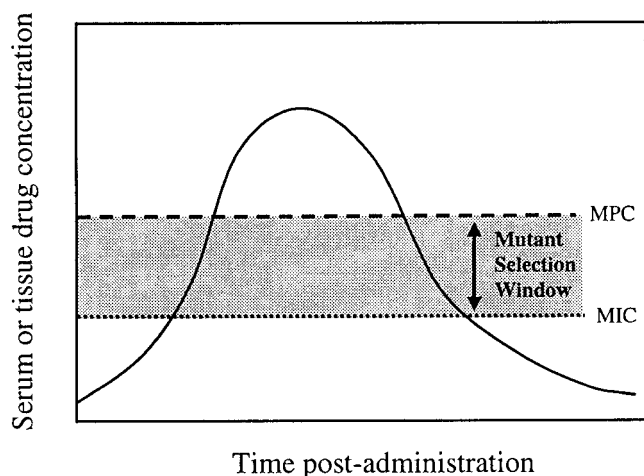
olones such that serum drug concentrations exceed the MPC of clinical isolates. That makes restricting selection of resistant mutants a realistic expectation if dosing protocols can be adjusted to maintain high concentrations. To be useful, MPC need not be measured routinely by clinical laboratories if for a given compound and given pathogen the ratio of MPC to MIC is first determined for many clinical isolates. Then clinicians need only know the ratio and the value of MIC to estimate MPC.

Because MPC is determined with wild-type cultures (figure 2), it is unnecessary to know the resistance mechanism or to have resistant mutants in hand to measure MPC. This feature should make the test applicable for many pathogens and for new compounds at early stages of development. In addition, resistant and susceptible populations can be more accurately discriminated by MPC than by MIC assays. For example, standard MIC determinations test  $<10^5$  cells for growth, while the mutation frequency may be 1 in  $10^7$  to  $10^8$  cells. Therefore, a population of  $10^8$  cells could be enriched for mutants by up to 1000-fold over the background level without being observed by MIC measurements. Such enrichment would be readily detected by the MPC assays because many more cells are applied to agar plates.

## MUTANT SELECTION WINDOW

Resistant mutants are selectively enriched only in the concentration range between the MIC of wild-type cells and the MPC (figure 2; MIC is an approximation, as noted in the legend to figure 2). Below MIC no mutant will be enriched because selective pressure is absent; above MPC no mutant will be selected because a double mutation is required for growth. We designate the concentration range between MIC and MPC as the mutant selection window (the term “selective window” has been used elsewhere [30, 31], but with a narrower meaning, without a precise upper limit, and with a different definition for the lower boundary). The mutant selection window can be easily visualized in an idealized pharmacokinetic plot (figure 3). If the window can be closed, no mutant will be selected. Therefore, closing or eliminating the window is a key to preventing the selection of antibiotic-resistant mutants.

Two strategies exist for narrowing the mutant selection window. One is to minimize the time at which the serum drug concentration is in the window (we use the term serum drug concentration for convenience; for a particular infection, the concentration in a specific tissue or organ may be a more appropriate parameter, and it may be necessary to consider the possible inactivating effect binding of drug to host protein may have). In principle, minimizing the mutant selection time can be accomplished by using compounds that quickly pass through the window after administration of the first dose and remain above the MPC throughout the treatment period. At the end



**Figure 3.** Idealized sketch of serum or tissue drug concentration after administration of a single dose of antibiotic to a patient. MIC and mutant prevention concentration (MPC), determined in laboratory studies, are indicated. The area between MPC and MIC (*shaded*) represents the mutant selection window.

of treatment, the concentration of the antibiotic should also return through the window quickly, although that may be less important than at the beginning of therapy because the number of pathogens, both wild-type cells and resistant mutants, may have been dramatically reduced (see subsequent discussion of lethal action). Reducing the duration of therapy by a regimen of high doses and appropriate drug formulation/administration may help minimize the length of time that the concentration is in the mutant selection window. Conversely, treatment protocols that utilize compounds having a long, gradual absorption by the body, gradual decay, and a recommended dose that places drug concentrations in the mutant selection window are the most likely to select resistant mutants [30].

The second way to narrow the window is to reduce the difference between MPC and MIC. For example, the C8-methoxy group of fluoroquinolones improves bacteriostatic action against resistant mutants [19, 21] and thereby lowers MPC [24]. With some organisms, such as *E. coli*, the methoxy group also raises wild-type MIC, further narrowing the selection window [21]. From this point of view the most effective compound may not always have the lowest MIC.

The relationship between MIC and MPC, expressed as a ratio (MPC:MIC), can be used to compare antibacterial agents for their ability to select resistant mutants. When measured with *E. coli* and *S. aureus*, the ratio, which we term the selection index, tends to be lower for C8-methoxy fluoroquinolones than for many other antibiotics, including rifampicin, tobramycin, and penicillin (the index is at least 4 orders of magnitude lower for C8-methoxy fluoroquinolones than for rifampicin when tested against *S. aureus*, X. Zhao, unpublished observation). The selection index could serve pharmaceutical companies as

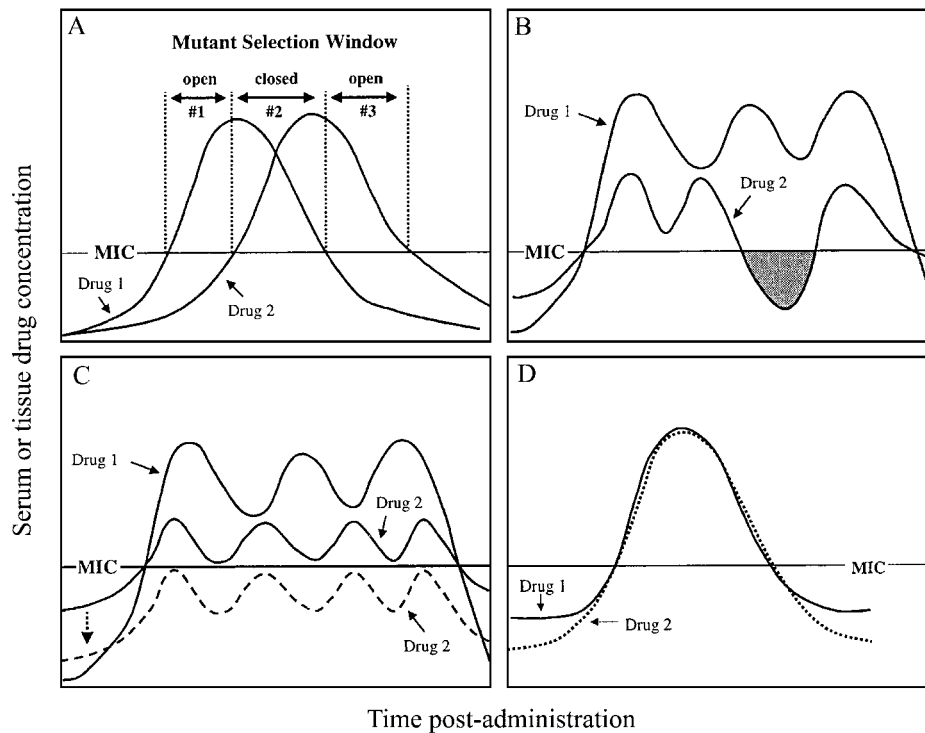
an additional criterion for identifying new therapeutic compounds, because smaller indices mean that a compound is less likely to select resistant mutants.

### CLOSING THE MUTANT SELECTION WINDOW WITH 2 ANTIMICROBIAL AGENTS

For some organisms it is difficult to find antibiotics whose MPC is below the attainable serum/tissue drug concentration or the concentration that causes host toxicity. Such is the case for most of the first-line agents used against *M. tuberculosis* [32], which would make single-drug treatment unsuccessful for tuberculosis even with good compliance [2, 33, 34]. Instead, 2- to 4-drug regimens are usually used with this disease. Successful treatment then occurs much more often because 2 concurrent mutations are required for bacterial growth when 2 antibiotics of different classes are each maintained at levels above their respective MICs. Dual-drug therapy provides a way to close the mutant selection window with moderate antibiotic concentrations, even with compounds that individually have very high MPCs.

Experience with tuberculosis shows that resistant mutants can arise even when  $\geq 2$  compounds are used [35–37]. Resistance is generally attributed to noncompliance with therapy regimens: antibiotic concentrations drop below MIC, the pathogen population, both mutant and wild-type, expands, and more spontaneous mutants are generated. When antibiotic treatment is reinstated, resistant mutants are selectively enriched. Treatment failure can occur. If treatment is stopped before the mutants reach a high number, they may not be detected until relapse occurs. For mutants to be selected, the concentration of one compound must occasionally fall below its MIC while that of the other remains above its MIC but below its MPC. Then the mutant selection window is open for the drug that remains above its MIC, and mutants resistant to that compound will be enriched (enrichment is expected to occur even when exposure time is short if it is repeated enough times). After a population resistant to one compound has grown out, the selection window opens for the other agent. Selection of resistance to the second compound soon follows when its concentration exceeds the MIC.

Several ways exist for dual-drug therapy to fail even if compliance is good. One hypothetical example is shown in figure 4A in which the concentration for 2 compounds exceeds the MIC at different times. Another example, shown in figure 4B, illustrates an effect of dosing that causes the serum drug concentration of one compound to drop temporarily below its MIC. This allows selection of resistance against drug 1 in the example. Avoiding resistance requires appropriate protocols and vigilance in dosing to keep the levels of *both* compounds above their MICs at all times.



**Figure 4.** Effect of pharmacokinetics on selection of resistance mutations during dual-drug therapy. Several scenarios illustrate opening and closing of the mutant selection window. In each case, pharmacokinetic profiles are normalized so the MIC of each compound can be represented by the same line. *A*, Mutant selection windows open due to insufficient overlap of pharmacokinetic profiles. Two antibiotics are shown rising above and dropping below the MIC at different times, and so situations exist in which only one compound is above its MIC (situations #1 and #3). Mutants are readily selected in these conditions. Only in situation #2 are both agents above their respective MICs. *B*, Mutant selection window open due to fluctuation in antibiotic concentration. An example is shown in which drug 2 temporarily drops below its MIC, leaving only drug 1 at a concentration above its MIC. This will lead to selection of resistance against drug 1 even though the concentrations of the 2 drugs were synchronized with respect to the time at which they exceeded MIC at the beginning of treatment and dropped below MIC at the end. *C*, Mutant selection window opened due to fluctuations in MIC or serum/tissue drug concentration. Variations among patients or subpopulations of a pathogen may raise the MIC of one compound to where it exceeds the serum concentration. In the case shown, the fluctuation leaves only drug 1 above the MIC, and that can lead to selection of resistance against drug 1. *D*, Mutant selection window always closed. When 2 compounds have identical pharmacokinetics above their MICs, no time exists at which one drug is at a concentration above its MIC while the other is below. Note that when both fall below MIC of wild-type cells, no selection of resistant mutants occurs. In practice, it may best to have the curves superimpose somewhat below the MIC as well as above it to guard against fluctuations in MIC, which can vary from strain to strain and person to person. Clinical experience will be necessary to determine how far below MIC the pharmacokinetic curves must superimpose to prevent selection of resistant mutants.

While vigilance in dosing can greatly reduce the selection of resistance, as evidenced by the directly observed therapy program for tuberculosis, it cannot eliminate failures arising from other types of fluctuation and human error. For example, the concentration of 2 drugs can be maintained above an MIC defined for most situations, but variation among patients or infecting organisms can cause the MIC to occasionally be above the serum/tissue concentration of one drug, as illustrated for drug 2 (dashed line in figure 4C). That is expected to lead to selection of resistance to drug 1, which has its concentration above its MIC but below its MPC.

In principle, the potential problems illustrated in figures 4A, 4B, and 4C cannot occur if 2 compounds have identical or very similar pharmacokinetic profiles (figure 4D): the concentra-

tions of the 2 antibiotics cross the MIC at the same time, and the absence of a gap between their normalized pharmacokinetic profiles prevents one compound from dropping below the MIC while the other remains above. Thus superimposition of  $\geq 2$  pharmacokinetic curves (figure 4D) should prevent selection of resistant mutants even when patients fail to adhere to dosing protocols, provided that all drugs are combined into a single carrier so patients cannot take one drug and not the other. Because these considerations involve comparison of  $\geq 2$  antibiotic pharmacokinetic responses that may occur at very different absolute drug concentrations, normalization is necessary. Normalization is most easily accomplished by expressing the drug concentration as multiples of MIC.

We do not yet know how closely 2 compounds must come

to the ideal situation of identical pharmacokinetics to prevent selection of resistance. However, it should be possible to manipulate dosing and formulations to achieve the desired effect once 2 compounds have been found that have similar pharmacokinetic profiles. Some flexibility can be tolerated if a third compound is added to the mix so that at least 2 are always at concentrations above their MICs. If the compounds are combined into a single delivery vehicle, a dosing error that leads to monotherapy and drug resistance is less likely to occur.

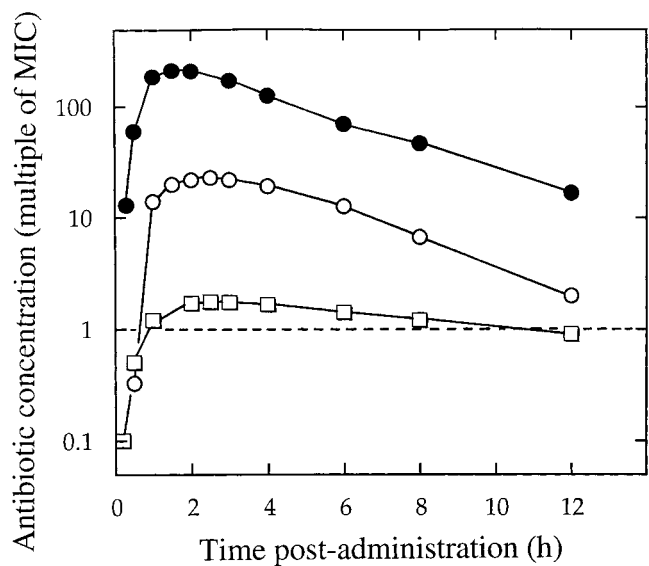
For dual-drug therapy to work, cross-resistance between the compounds must not occur, because then a single mutation would confer resistance to both compounds. In this respect drug efflux mutations can be a particular problem: they increase the probability of cross-resistance because many confer resistance to several classes of antibiotic simultaneously. Avoiding the use of low antibiotic doses may be the way to minimize selection of efflux mutants [20].

Existing combination therapies do not meet the criteria required to eliminate the mutant selection window. For example, a 3-drug cocktail called Rifater (Marion Merrell Dow) has been formulated in which isoniazid, rifampicin, and pyrazinamide are combined into a once-per-day treatment of tuberculosis. The pharmacokinetic profiles for these 3 compounds do not superimpose (figure 5). After half a day, both pyrazinamide and rifampicin drop below their MICs, and so isoniazid would act as monotherapy for about half of the treatment time. Isoniazid resistance should develop first; rifampicin resistance is expected to arise next because pyrazinamide drops below its MIC before rifampicin (figure 5).

It should be noted that the double-mutation idea does not apply for single regimen, dual drugs such as Augmentin (SmithKline Beecham), Timentin (SmithKline Beecham), Unasyn (Pfizer), and Zosyn (Wyeth-Ayerst Laboratories). These are combinations of  $\beta$ -lactams and  $\beta$ -lactamase inhibitors that constitute monotherapy. With these formulations a resistance mutation to either compound confers resistance.

## LETHAL ACTION AND SELECTION OF RESISTANCE

So far we have paid little attention to lethal (bactericidal) action, because MPC and the mutant selection window are based only on bacteriostatic considerations. Nevertheless, lethal effects are likely to be important for implementation of the MPC-based ideas and for special cases. For example, lethal action should narrow the temporal aspect of the mutant selection window, and so it should increase the ability to administer high antibiotic concentrations (shorter treatment times may reduce toxic side effects). For diseases caused by organisms that are not readily attacked by the immune system or for immunocompromised



**Figure 5.** Pharmacokinetic profiles of isoniazid, rifampicin, and pyrazinamide after treatment with Rifater (Marion Merrell Dow), a fixed-dose, triple-drug combination tablet. Serum antibiotic concentrations, taken from [38], were converted to multiples of MIC by using values of 0.03, 0.3, and 20  $\mu\text{g}/\text{mL}$  for isoniazid, rifampicin, and pyrazinamide, respectively, against *Mycobacterium tuberculosis*, as reported in [39–41]. Dashed line denotes MIC. ●, isoniazid; ○, rifampicin; □, pyrazinamide. Possible effects of protein binding are not considered.

patients, lethal action is required because blocking growth may by itself fail to clear infection. Lethal action should also restrict the outgrowth of cells acquiring a recessive, induced mutation caused by the drug.

Care needs to be taken in using lethal compounds, because killing susceptible bacteria while allowing resistant mutants to live could actually speed the selection process. Desirable compounds should kill resistant mutants at the same concentration required to block growth. In practical terms, this means that the MBC for a mutant, which is measured by using incubation periods comparable to those used to allow colony growth, should approximate mutant MIC. Then the underlying resistant population would be reduced, and treatment times could be shorter than for compounds that fail to kill mutants. With fluoroquinolones MBC and MIC are sometimes quite close for wild-type cells [42]. Whether this is true for mutants is not known.

It is important to note that bacteriostatic effects, reflected in the MIC, do not always predict lethal action, even for highly bactericidal compounds. In the case of fluoroquinolones, one structural derivative can be more effective than another at blocking growth but less effective at killing cells [21]. This difference arises because blocking growth is physiologically distinct from lethal action (reviewed in [43]). Therefore, finding

optimal compounds may require experimental testing with resistant mutants.

## IMPLEMENTATION OF THE MPC-BASED STRATEGY FOR RESTRICTING SELECTION OF RESISTANT MUTANTS

The most obvious conclusion from the ideas sketched earlier is the inadvisability of monotherapy with bacteriostatic antibiotics at doses that place the drug concentration in the mutant selection window for long periods of time. Such compounds might be best reserved for multidrug therapy.

The MPC-based strategy provides guidance for the development of new antimicrobial agents. The key will be obtaining compounds that have low selection indices and that can be administered safely above the MPC. Because the possibility of human error in dosing compromises even compounds having low MPCs, a major effort should be made to develop dual-drug and multidrug combination therapies. For that, extensive pharmacokinetic studies are needed to satisfy the requirements illustrated in figure 4D. Bioavailability, bioeffectiveness, and tissue compartmentalization of compounds must all be taken into account when trying to find 2 compounds whose pharmacokinetic profiles superimpose. Because the lower limit of the mutant selection window can only be approximated (legend to figure 2) and because the MIC can vary over a broad range with clinical isolates, it may be necessary to establish a safety zone around the modal MIC of clinical isolates to prevent fluctuations in MIC from opening the mutant selection window. The pharmacokinetic profiles for at least 2 compounds need to superimpose in this safety zone.

A problem with compounds in current use is that resistant mutants are already present in pathogen populations, especially in those associated with hospital infections. In some cases it may be possible to keep drug concentrations above the MPC of the mutants. This can be achieved for *E. coli* with a C8-methoxy fluoroquinolone [44]. However, most pathogens are unlikely to be this highly susceptible. For them it may be necessary to close the mutant selection window by finding 2 antibiotics suitable for dual-drug, combination therapy. If only one antibiotic is available, as is the case for vancomycin with many methicillin-resistant strains of *S. aureus*, it may be necessary to reserve the compound for life-threatening cases until a second drug is developed that will allow the criteria shown in figure 4D to be met. From a public health point of view it may be inadvisable to use new, highly potent members of an antibiotic class as monotherapy against pathogens that are already resistant to older, less potent members of the same class. Such a strategy may quickly render the new compound ineffective, and more importantly, it is expected to lower the susceptibility of the pathogen population. That will severely limit

the usefulness of future derivatives. Likewise, less potent derivatives should not be used at low doses in agriculture: resistant bacteria will be readily selected, they will be transferred to the human population, and then resistance to new, more potent derivatives will be readily selected.

Resistance that arises at high frequency requires slight refinement of the strategy. If the mutation frequency is higher than seen with the quinolones, then antibiotic concentrations may have to be adjusted so that more than 2 concurrent mutations are required for pathogen growth. For example, if the mutation frequency is  $10^{-5}$  and the number of organisms in the body is expected to be  $10^{10}$ , then requiring 3 mutations would mean that 1 mutant would occur per  $10^{15}$  cells. That would provide a buffer of 5 orders of magnitude for selection of resistance. How large this buffer needs to be must be determined with animal models in which resistance is readily selected.

Sometimes resistance arises when expression from protective genes is induced rather than from a change in the gene. This phenotypic resistance (also called induced resistance [45] and references therein) can be partially alleviated if the inducing antibiotic kills very rapidly. Therefore, bacteriostatic assays will not be particularly meaningful for agents that cause phenotypic resistance. Efforts to bypass this type of resistance with agents that do not induce it can still use the MPC ideas to restrict the selection of genetic resistance.

## CONCLUDING REMARKS

Our current antibacterial dosing strategies are directed at clearing infection. Drug concentrations that exceed MIC, or some multiple of MIC, have been adequate for this purpose. However, MIC-based strategies require only 1 resistance mutation for a cell to grow in the presence of antibiotic, and infections can easily contain enough bacterial cells for many first-step resistant mutants to be present. Consequently, resistant mutants are readily enriched. When millions of infections are considered, outgrowth of resistant mutants occurs often enough to eventually render the compounds ineffective. We propose that antibiotic concentrations be raised to require 2 resistance mutations for growth rather than 1. Compounds that cannot be administered such that relevant tissue concentrations exceed MPC should be protected from the development of resistance by being administered as combination therapies.

While the concept underlying the 2-mutation strategy is straightforward, clinical validation is required. Then a change in emphasis may be appropriate within the pharmaceutical industry. At present the industry is set up to find and produce new agents for monotherapy rather than to conserve existing compounds through combination therapy. If one assumes that a new class of agent will always be available to replace those

that have been neutralized by resistance, then little harm is done by keeping a weak compound on the market as monotherapy. If the assumption about a continuous supply of new antibacterial agents is invalid, we as a society will need to find a way to preserve what we have. Perhaps regulatory changes are required. Among patients and physicians, a shift in emphasis from individual health to public health may be necessary. Low doses usually control infection, and the chance is small that treatment failure in any given patient arises from the development of resistance. Consequently, the tendency to administer doses at low levels is strong, and switching to an MPC-based therapy takes on a higher risk of side effects with little benefit for the immediate patient. The benefit of MPC-based therapy is having the antibiotic available for future infections.

## Acknowledgments

We thank Arnold Bendich, Marila Gennaro, Serban Iordanescu, Samuel Kayman, Barry Kreiswirth, Don Low, Frank Lowy, David Perlin, Bo Shopsin, Carl Urban, and Lew Weinstein, for critical comments on the manuscript, and Glenn Tilotson, for encouragement.

## References

1. Tenover F, McGowan JE. The epidemiology of bacterial resistance to antimicrobial agents. In: Evans AS, Brachman PS, eds. *Bacterial infections of humans*. New York: Plenum Medical Book Company, 1998: 83–93.
2. Levy SB. Multidrug resistance: a sign of the times. *N Engl J Med* 1998; 338:1376–8.
3. Milatovic D, Braveny I. Development of resistance during antibiotic therapy. *Eur J Clin Microbiol* 1987; 6:234–44.
4. Archer G. *Staphylococcus aureus*: a well-armed pathogen. *Clin Infect Dis* 1998; 26:1179–81.
5. Frieden T, Sterling T, Pablos-Mendez A, Kilburn J, Cauthen G, Dooley S. The emergence of drug-resistant tuberculosis in New York City. *N Engl J Med* 1993; 328:521–6.
6. Sepkowitz KA, Telzak EE, Recalde S, Armstrong D. Trends in the susceptibility of tuberculosis in New York City, 1987–1991. New York City area tuberculosis working group. *Clin Infect Dis* 1994; 18:755–9.
7. Bifani P, Plikaytis BB, Kapur V, et al. Origin and interstate spread of a New York City multidrug resistant *Mycobacterium tuberculosis* clone family: adverse implications for tuberculosis control in the 21st century. *JAMA* 1996; 275:452–7.
8. Xu C, Kreiswirth BN, Sreevatsan S, Musser JM, Drlica K. Fluoroquinolone resistance associated with specific gyrase mutations in clinical isolates of multidrug resistant *Mycobacterium tuberculosis*. *J Infect Dis* 1996; 174:1127–30.
9. Coninx R, Mathieu C, Debacker M, et al. First-line tuberculosis therapy and drug-resistant *Mycobacterium tuberculosis* in prisons. *Lancet* 1999; 353:969–73.
10. Gaunt PN, Piddock LJ. Ciprofloxacin resistant *Campylobacter* spp. in humans: an epidemiological and laboratory study. *J Antimicrob Chemother* 1996; 37:747–57.
11. Smith KE, Besser JM, Hedberg CW, et al. Quinolone-resistant *Campylobacter jejuni* infections in Minnesota, 1992–1998. Investigation team. *N Engl J Med* 1999; 340:1525–32.
12. Endtz HP, Ruijs GJ, van Klingeren B, Jansen WH, van der Reyden T, Mouton RP. Quinolone resistance in *Campylobacter* isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. *J Antimicrob Chemother* 1991; 27:199–208.
13. Garau J, Xercavins M, Rodriguez-Carballerira M, et al. Emergence and dissemination of quinolone-resistant *Escherichia coli* in the community. *Antimicrob Agents Chemother* 1999; 43:2736–41.
14. Phillips I. Bacterial mutagenicity and the 4-quinolones. *J Antimicrob Chemother* 1987; 20: 771–3.
15. Belland RJ, Morrison SG, Ison C, Huang WM. *Neisseria gonorrhoeae* acquires mutations in analogous regions of *gyrA* and *parC* in fluoroquinolone-resistant isolates. *Mol Microbiol* 1994; 14:371–80.
16. Drlica K, Zhao X, Zhao X. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol Mol Biol Rev* 1997; 61:377–92.
17. Pan X, Fisher LM. Targeting of DNA gyrase in *Streptococcus pneumoniae* by sparfloxacin: selective targeting of gyrase or topoisomerase IV by quinolones. *Antimicrob Agents Chemother* 1997; 41:471–4.
18. Fukuda H, Hiramatsu K. Primary targets of fluoroquinolones in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1999; 43:410–2.
19. Dong Y, Xu C, Zhao X, Domagala J, Drlica K. Fluoroquinolone action against mycobacteria: effects of C8 substituents on bacterial growth, survival, and resistance. *Antimicrob Agents Chemother* 1998; 42:2978–84.
20. Zhou J-F, Dong Y, Zhao X, et al. Selection of antibiotic resistance: allelic diversity among fluoroquinolone-resistant mutations. *J Infect Dis* 2000; 182:517–25.
21. Zhao X, Xu C, Domagala J, Drlica K. DNA topoisomerase targets of the fluoroquinolones: a strategy for avoiding bacterial resistance. *Proc Natl Acad Sci USA* 1997; 94:13991–6.
22. Zhao X, Wang J-Y, Xu C, et al. Killing of *Staphylococcus aureus* by C-8-methoxy fluoroquinolones. *Antimicrob Agents Chemother* 1998; 42: 956–8.
23. Zhao B-Y, Pine R, Domagala J, Drlica K. Fluoroquinolone action against clinical isolates of *Mycobacterium tuberculosis*: effects of a C8-methoxyl group on survival in liquid media and in human macrophages. *Antimicrob Agents Chemother* 1999; 43:661–6.
24. Dong Y, Zhao X, Domagala J, Drlica K. Effect of fluoroquinolone concentration on selection of resistant mutants of *Mycobacterium bovis* BCG and *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1999; 43:1756–8.
25. Sindelar G, Zhao X, Liew A, et al. Mutant prevention concentration (MPC) as a measure of fluoroquinolone potency against mycobacteria. *Antimicrob Agents Chemother* 2000; 44:3337–43.
26. Bingen E, Lambert-Zechovsky N, Mariani-Kurkdjian P, et al. Bacterial counts in cerebrospinal fluid of children with meningitis. *Eur J Clin Microbiol Infect Dis* 1990; 9:278–81.
27. Feldman W. Concentrations of bacteria in cerebrospinal fluid of patients with bacterial meningitis. *J Pediatr* 1976; 88:549–52.
28. Mitchison DA. Drug resistance in mycobacteria. *Br Med Bull* 1984; 40:84–90.
29. Blondeau J, Zhao X, Hansen G, Drlica K. Mutant prevention concentration (MPC) as a guide for treating *Streptococcus pneumoniae* with fluoroquinolones. *Antimicrob Agents Chemother* 2001; 45:433–8.
30. Baquero F, Negri MC. Strategies to minimize the development of antibiotic resistance. *J Chemother* 1997; 9(Suppl 3):29–37.
31. Baquero F. Resistance to quinolones in Gram-negative microorganisms: mechanisms and prevention. *Eur Urol* 1990; 17(Suppl 1):3–12.
32. Dong Y, Zhao X, Kreiswirth B, Drlica K. Mutant prevention concentration as a measure of antibiotic potency: studies with clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2000; 44:2581–4.
33. Moulding T, Dutt A, Reichman LB. Fixed-dose combinations of anti-tuberculous medications to prevent drug resistance. *Ann Intern Med* 1995; 122:951–4.
34. East African Hospitals and British Medical Research Council. Comparative trial of isoniazid alone in low and high dosage and isoniazid plus PAS in the treatment of acute pulmonary tuberculosis in East Africa. *Tuber Lung Dis* 1960; 40:83–102.

35. Mason GR, Nitta A. Emergence of MDR TB during standard therapy in AIDS [abstract]. *Am J Respir Crit Care Med* **1997**;155:A221.
36. Second East African/British Medical Research Council Study. Controlled clinical trial of four short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis. *Lancet* **1974**;2:1100–6.
37. Vernon A, Burman W, Benator D, Khan A, Bozeman L. Acquired rifamycin monoresistance in patients with HIV-related tuberculosis treated with once-weekly rifapentine and isoniazid. *Lancet* **1999**;353:1843–7.
38. Acocella G, Nonis A, Perna G, Patane E, Gialdroni-Grassi G, Grassi C. Comparative bioavailability of isoniazid, rifampin, and pyrazinamide administered in free combination and in a fixed triple formulation designed for daily use in antituberculosis chemotherapy. II. Two-month, daily administration study. *Am Rev Respir Dis* **1988**;138:886–90.
39. Rastogi N, Labrousse V, Goh KS. In vitro activities of fourteen antimicrobial agents against drug susceptible and resistant clinical isolates of *Mycobacterium tuberculosis* and comparative intracellular activities against the virulent H37Rv strain in human macrophages. *Curr Microbiol* **1996**;33:167–75.
40. Stottmeier KD, Beam RE, Kubica GP. Determination of drug susceptibility of mycobacteria to pyrazinamide in 7H10 agar. *Am Rev Respir Dis* **1967**;96:1072–5.
41. Aranda CP. Pyrazinamide. In: Rom WN, Garay SM, eds. *Tuberculosis*. Boston: Little, Brown, and Co, **1996**:799–802.
42. Chow R, Dougherty T, Fraimow H, Bellin E, Miller M. Association between early inhibition of DNA synthesis and the MICs and MBCs of carboxyquinolone antimicrobial agents for wild-type and mutant [*gyrA nfxB(ompF) acrA*] *Escherichia coli* K-12. *Antimicrob Agents Chemother* **1988**;32:1113–8.
43. Drlica K. Mechanism of fluoroquinolone action. *Curr Opin Microbiol* **1999**;2:504–8.
44. Lu T, Zhao X, Drlica K. Gatifloxacin activity against quinolone-resistant gyrase: allele-specific enhancement of bacteriostatic and bactericidal activity by the C-8-methoxy group. *Antimicrob Agents Chemother* **1999**;43:2969–74.
45. Tenover FC, McGowan JE. Reasons for the emergence of antibiotic resistance. *Am J Med Sci* **1996**;311:9–16.