The Crisis of Resistance: Identifying Drug Exposures to Suppress Amplification of Resistant Mutant Subpopulations

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Antibiotic resistance is seen in both the hospital and community settings. Approaches are required to minimize the increase in resistant strains, such as good antibiotic stewardship and the limiting of antibiotic use to appropriate circumstances. There are instances when drug dose and/or schedule can be used to minimize the probability that mutants will take over the bacterial population. Over the past several years, significant advances have been made in understanding the relationship between drug concentrations and amplification of resistant mutant subpopulations. In this review, we examine the use of preclinical models for facilitating this understanding. We also use mathematical techniques, including Monte Carlo simulation, to bridge between the identification of exposures to minimize resistance and the examination of candidate drug doses to achieve this end. Examples are provided for *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis*. In each instance, quinolone antimicrobials were examined. More investigations with other pathogens and drug classes are required.

The worldwide population is aging rapidly, especially in Europe and the United States. As it has aged, the use of medical resources, including hospital care, has markedly increased. This has resulted in a number of unanticipated consequences. Indwelling medical devices breach naturally occurring mucosal barriers. Therapeutic interventions for cancer, immunological diseases, and rheumatological diseases and organ transplantation have created a large population of immunosuppressed hospitalized patients. Serious infection, especially with *Staphylococcus aureus* and gram-negative bacilli such as *Pseudomonas aeruginosa*, occurs frequently in this population. This results in the use of broad-spectrum anti-infective agents, often for prolonged durations. The frequent use of these agents, sometimes without identification of limitations on the duration of therapy, has resulted in the creation of a population of multidrug-resistant bacteria.

This nosocomial disaster has been recapitulated in the community setting. Here, the most common and deadly respiratory pathogen, *Streptococcus pneumoniae*, has become increasingly resistant to β-lactam agents, as well as to many macrolides. We have also seen the emergence of community-acquired methicillin-resistant *S. aureus* infection, which promises to markedly change community practice. The problem of drug resistance in this setting mirrors a worldwide rapid increase in drug resistance among one of humankind’s most common causes of death: *Mycobacterium tuberculosis*. The median prevalence of drug resistance among new cases of tuberculosis is now 10% (with prevalence as high as 57%), whereas the median prevalence among relapsed cases is 18% (highest country prevalence, 82%) [1]. This global problem is even more alarming given that New Drug Applications to the US Food and Drug Administration (FDA) for antimicrobials are at an all-time low. Because the time from new-drug target identification and validation to drug approval exceeds a decade, it is imperative that we identify ways of preserving the activity of available agents and of protecting new agents as they make their way into the physician’s therapeutic armamentarium. A potential way to achieve resistance suppression when decreased antimicrobial susceptibility is due to a mutational event is to employ pharmacodynamic modeling and determine the exposures to clinical doses that have the greatest probability of achieving this goal. Doses thus derived can be viewed as an affordable “technology transfer” between world communities.
THE BIOLOGY OF RESISTANCE

Clinically meaningful resistance may occur by multiple mechanisms. One path to resistance is the induction or stable overexpression of efflux pumps. The organisms pump out the antibiotic before it achieves effective intracellular concentrations. Resistance may also occur through DNA transfer from resistant organisms, as in the creation of mosaic chromosomes associated with β-lactam resistance, or through plasmid or transposon transfer in the case of extended-spectrum β-lactamases and aminoglycoside-modifying enzymes. In these circumstances, the judicious use of antimicrobial agents and appropriate infection control will have the largest impact on controlling the creation and dissemination of resistance-bearing organisms. There are circumstances, however, in which resistance may occur through spontaneous mutation. An example is when mutations occur in the topoisomerases II and/or IV genes that render organisms more resistant to quinolone antimicrobials.

Mutations occur spontaneously with a frequency that is generally in the range from 10^-6 to 10^-8. When no selective pressure is present, the occurrence of organisms bearing such resistance mutations is random. When the total population burden is substantially less than the inverse of the mutational frequency to resistance, there is a low probability that a resistant mutant is primarily resident in the microbial population. As the microbial population burden increases, the probability that it will harbor a resistant mutant increases. It should be noted that when the term “resistant mutant” is employed, it refers to a change in the genome that increases the MIC for a specific agent or class of drugs relative to that of the wild-type isolate. The resulting MIC may or may not exceed the susceptibility breakpoint promulgated by the National Committee for Clinical Laboratory Standards or the FDA. Furthermore, phenotypic changes (i.e., increased MIC) can result without mutation, as in the case of induction of efflux-pump expression. When antimicrobials are used to treat infections in which organisms bearing resistance mutations are present at baseline in the total population, the drug exposure represents selective pressure in favor of the resistant mutants in relation to the wild-type organisms. The rate of amplification of the drug-resistant mutant population will be governed by the degree of resistance conferred by the mutation to the drug being employed, the biofitness cost that bearing the mutation exacts, and the dose of the drug being employed. It is the dose of the drug employed over which we have the greatest control. There will be instances in which the degree of resistance produced by the mutation will be too great to be overcome by dosing and cases in which the drug dose that would suppress the mutant would be toxic to patients. In many instances, however, a dose of antibiotic can be chosen that will be sufficient to suppress resistance while causing little dose-related toxicity. Our intent in the present article is to discuss approaches to identifying drug concentrations, doses, and schedules that will help to suppress such mutants.

CLINICAL CIRCUMSTANCES THAT FAVOR RESISTANCE EMERGENCE DURING THERAPY

Clinically, we identify emergence of resistance during therapy most frequently when the organism population burden is large. This circumstance often occurs when patients with severe multilobar pneumonia or bacteremia are given treatment. This is because such patients have very large bacterial burdens, and often these burdens are large enough to substantially exceed the inverse of the mutational frequency to resistance, virtually guaranteeing that a resistant mutant subpopulation will be present at therapy initiation.

SUPPRESSION OF DRUG-RESISTANT MUTANTS THROUGH CONCENTRATION SELECTION

An early and important advancement with regard to this problem came from the laboratory of Drlica and colleagues [2–4]. These investigators had the important insight that the probability of attaining 2 resistance mutations simultaneously is vanishingly small. When organisms bear 2 mutations (e.g., quinolone-resistance mutations in topoisomerases II and IV genes), it is highly likely that acquisition occurred sequentially and not simultaneously. Any strategy that would prevent the first mutation would also prevent the subsequent event. This led to the development of the concept of the mutation prevention concentration (MPC) [4]. This approach involves a simple laboratory test in which a large population of organisms (≈10^10 colony-forming units) is placed on agar plates containing increasing concentrations of a drug [4, 5]. As the drug concentration increases, the number of colonies on the plates decreases, until a concentration of drug that prevents any organisms from growing on the plates is achieved. This concentration is the MPC, which prevents amplification of the most resistant first-step mutant [5, 6]. The concentration range between the MIC of the organism and the MPC, in which resistant mutants are selectively enriched, is defined as the mutant selection window (MSW) [6]. Drugs with a low ratio of MPC to MIC (termed “selection index” [6]) are therefore expected to be better at suppressing resistance than drugs with a higher selection index. One of the central tenets of this important hypothesis is “to minimize the time at which serum drug concentration is in the [mutant selection] window” [6, p. S150]. This concept is, in fact, similar to observations by Baquero and Negri [7] a few years earlier. Initially termed “mutant selection time” [6], this concept is now commonly termed the “time in mutant selection window (TMSW) hypothesis” [8, 9]. According
to this view, the best strategy to prevent resistance is to design drugs and drug regimens that minimize the $T_{MSW}$.

The MPC and $T_{MSW}$ have expanded our understanding of the prevention of resistance. However, several groups have pointed to some important limitations. Campion and colleagues [10, 11] published 2 investigations of ciprofloxacin and levofloxacin and the suppression of resistance in a hollow fiber infection model, along with a prospective validation. They demonstrated differences in amplification of different mutant populations when the average steady-state concentration (a transformation of the ratio of area under concentration-time curve [AUC] to MIC) was slightly above the MIC, was between the MIC and MPC, was near the MPC, and was significantly in excess of the MPC. They did not find any correlation between the amplification of mutant populations and the time spent in the MSW. Similarly, Allen et al. [12] also found no correlation between emergence of resistance and the $T_{MSW}$ hypothesis in pharmacodynamic in vitro models.

Suppression of resistance in an animal model. The mouse thigh infection model was described by Eagle and colleagues [18, 19]. The laboratory of Craig et al. [20, 21] has successfully used this model over the past several decades to elucidate pharmacodynamic principles. Our laboratory employed this model to examine the issue of suppression of resistance in $P. aeruginosa$, using the fluoroquinolone levofloxacin [22].

We first examined the impact of bacterial inoculum ($10^7$ vs. $10^8$ bacteria) on the AUC/MIC value that would be required to kill the total population of organisms (figure 1). The mutational frequency to resistance was $\approx 1/(2 \times 10^8)$, indicating that the larger inoculum would have a high probability of harboring a resistant population a priori. The results demonstrated that the drug exposure required for a specific microbiological effect increased by 2–5 fold when the inoculum increased. This increase in exposure was because of a differential effect of the drug on the 2 different bacterial populations: susceptible and mutant. The larger bacterial challenge had a larger population of resistant organisms. To test the hypothesis, we examined 4

**IDENTIFYING A DOSE TO SUPPRESS RESISTANCE BY USE OF PHARMACODYNAMIC EXPOSURES DERIVED IN PRECLINICAL DISEASE MODELS**

An alternative view has been to use pharmacodynamic principles that were developed to link drug exposure to the change in total organism numbers or to the probability of a good clinical or microbiological outcome [16]. In these studies, drug exposure measures, such as the ratio of AUC to MIC, the ratio of peak concentration to MIC, and the time that the drug concentration is above MIC, are related to microbial kill. The data presented below apply many of these same principles to the problem of resistance suppression. For an excellent overall review of the mathematics of resistance suppression, the interested reader should examine the work of Lipsitch and Levin [17].

**Figure 1.** Levofloxacin doses and effect on $Pseudomonas aeruginosa$ populations. Normal mice were inoculated with $\sim 10^7$ (a) or $10^8$ (b) bacteria per thigh. After 2 h, therapy was initiated at different levofloxacin doses. After 24 h, the number of bacteria was determined in each thigh. The small change in bacterial challenge resulted in a large change in the amount of drug exposure (ratio of area under concentration-time curve [AUC] to MIC) required to stop the organism from growing or to kill the population by 1, 2, or 3 log$_{10}$ colony-forming units (CFUs) [22].
different doses of levofloxacin and estimated the size of the total and resistant bacterial populations by plating samples on regular agar plates and on levofloxacin-containing plates (concentration, $3 \times$ MIC) to estimate the size of the resistant population. The results are displayed in figure 2. The dose of 90 mg/kg demonstrated a markedly discordant effect on the 2 subpopulations, with the susceptible subpopulation decreasing by 99% ($2 \log_{10}$) and the resistant subpopulation being amplified by approximately the same amount.

A large mathematical model was applied to all the data (changes in drug concentration, total population number, and resistant population number over time). For the interested reader, the system of equations employed can be seen at the Web page containing supplemental data for the article by Jumbe et al. [22] (http://www.jci.org/cgi/content/full/112/2/275/DC1). The model fit the data well, explaining >92% of the variance for the change in total organism counts over time and >93% of the variance for the change in resistant subpopulation over time. The results of the model were used to calculate an exposure that would prevent the mutant population from amplifying over a period of time (48 h) longer than the period over which the initial observations were developed (24 h). This exposure had an AUC/MIC of 157 (free-drug AUC/MIC, 110). Another exposure was identified that would result in near-maximal resistant-population amplification (total AUC/MIC, 52; free-drug AUC/MIC, 36). Results of this prospective validation are shown in figure 3.

The doses chosen to maximally amplify the pre-existent resistant bacterial population and to suppress it performed as predicted. This was, to our knowledge, the first prospective validation of a dose choice to suppress amplification of a resistant subpopulation. In the same study, we also examined ciprofloxacin dosing and employed Monte Carlo simulations to determine the likelihood that clinical doses would achieve the AUC/MIC associated with suppression of resistance. A ciprofloxacin dosage of 200 mg iv every 12 h would achieve this with a likelihood of 25% (likelihood of resistance, 75%), whereas a dosage of 400 mg iv every 8 h would achieve this with a likelihood of 62% (likelihood of resistance, 38%).

This type of resistance (amplification of a pre-existent mutant subpopulation) is an important clinical problem. Peloquin et al. [23] examined patients with lower respiratory tract infections caused by $P$. aeruginosa that were treated with the fluoroquinolone ciprofloxacin and showed that, with a regimen of 200–300 mg iv every 12 h, there was an emergence of resistance rate of $\approx 70\%$ during therapy. This is similar to the 75% rate predicted by our pharmacodynamic study discussed above. Likewise, Fink et al. [24] performed a randomized com-

Figure 2. Effect of levofloxacin doses of 0 mg/kg (a), 90 mg/kg (b), 225 mg/kg (c), and 600 mg/kg (d) on the total population and the resistant bacterial subpopulation of $P$. aeruginosa. Drug dosing was at time zero only. The 90-mg/kg dose caused a decline of $2 \log_{10}$ colony-forming units (CFUs) in total bacterial burden. However, the resistant population amplified by almost the same amount, trading sensitive for resistant organisms in the population. The 225-mg/kg dose allowed minimal resistant subpopulation amplification, whereas the 600-mg/kg regimen prevented resistant bacterial subpopulation amplification [22].
Figure 3. Results of evaluation of the mathematical model. The model was prospectively validated by identifying the dose that near-maximally amplify the mutant subpopulation (a), with a ratio of area under concentration-time curve (AUC) to MIC of 52, and suppress its amplification (b), with an AUC/MIC of 157. Levofloxacin dosing was performed at 24 h and 48 h, a period longer than that over which the model data in figure 2 were developed (24 h). The continuous lines in the figures are not lines of best fit; rather, they represent prospective prediction lines, around which the observations are scattered [22]. CFUs, colony-forming units.

Suppression of resistance in an in vitro model of infection. An in vitro hollow fiber system, which allows fluctuating drug concentrations that can simulate the free-drug plasma concentration-time profile seen in humans, has been used in the past to relate drug exposure to organism kill [25]. Dudley et al. [26] have subsequently used this system to examine the effect of drug exposure on emergence of resistance. Our laboratory first employed this in vitro pharmacodynamic infection model in an examination of *M. tuberculosis*, and we fit a mathematical model to all the data to explore the relationship between drug exposure to the quinolone moxifloxacin and the ability to kill the total population burden of organisms while suppressing amplification of the resistant mutant subpopulation [27]. We examined a range of drug exposures. The organism growth seen for the 3 lowest drug exposures and for a no-treatment control is displayed in figure 4. There are 2 important aspects to note. First, effective microbial kill does not guarantee that resistance will not emerge. In fact, effective microbial kill should only be viewed as part of the response, because effective microbial kill may be complicated by emergence of resistance over time (figure 4b and 4c). The second important aspect is that there are exposures at which resistant mutants are suppressed—in this case, at AUC/MICs of >40.4 and ≤101.6 (figure 4c and 4d).

Application of a mathematical model similar to that employed above for *P. aeruginosa* allowed calculation of an exposure able to suppress resistant mutants, a nonprotein-bound drug AUC/MIC of 53. We recently performed a prospective validation of this exposure, in which we treated *M. tuberculosis* with a moxifloxacin AUC/MIC of 72—above the AUC/MIC of 53—which resulted in suppression of resistance during 8 weeks of therapy. We also put this exposure breakpoint (AUC/MIC, 53) into clinical context by using Monte Carlo simulation. Through delineation of the variability in drug exposure seen in humans when a fixed drug dose is administered to a large patient population, we examined the probability that moxifloxacin doses of 400 mg, 600 mg, and 800 mg would achieve a free-drug AUC/MIC of 53 against the range of moxifloxacin MIC values seen in a collection of *M. tuberculosis* isolates. When the range of AUC values seen in a 10,000-subject simulation is examined and the range of MIC values is also examined, the overall probability of attaining the free-drug AUC/MIC required for mutant subpopulation suppression was 59%, 86%, and 93% for the 400-mg, 600-mg, and 800-mg doses, respectively. The probability of target attainment for the 800-mg dose by MIC is displayed in figure 5. This result is interesting, in that the currently recommended moxifloxacin dosage for tuberculosis treatment in its role as a second-line agent is 400 mg/day [28]. Quinolone resistance is now a significant problem in resource-poor countries [29], where quinolones are central to the World Health Organization recommended treatment of multidrug-resistant tuberculosis [30]. Since quinolones are often the only effective drug in the multidrug regimen [28], moxifloxacin dosages of 400 mg/day, as currently recommended, may contribute to resistance.
Figure 4. Responses of the total (diamonds) and the drug-resistant (circles) Mycobacterium tuberculosis populations over the course of 10 days of moxifloxacin exposure (free drug). Please note that untreated controls contained some drug-resistant mutants, but they were few. When the colony-forming units per milliliter (cfu/mL) measurements were transformed to log₁₀ values, they tended toward 0, and a line in panel a (circles) appears to be flat. Panels a–d show responses of the total M. tuberculosis population and the drug-resistant subpopulation to different moxifloxacin exposures: moxifloxacin free-drug area under the concentration-time curve from 0 to 24 h (AUC₀–₂₄/MIC) ratios are 0 (control) (a), 24.3 (b), 40.4 (c), and 101.6 (d) [27].

This in vitro model system was also used to examine the same strain of P. aeruginosa [31] that was studied in the mouse thigh infection model [22], but a different quinolone (garenoxacin) was used. Here, again, a range of exposures was examined. After a mathematical model was fit to all data simultaneously, we calculated that a free-drug AUC/MIC of 190 was required to prevent mutant subpopulation amplification. Again, a prospective validation was undertaken. This is displayed in figure 6. When a substantial exposure at a free-drug AUC/MIC of 137 was used, there was amplification of the mutant subpopulation, with replacement of the total population by mutants within 36 h, as predicted by the model. A measured free-drug AUC/MIC of 200 held the number of mutants steady at their baseline level, again as prospectively predicted. The breakpoint value in the in vitro system is ∼1.7-fold higher than that seen in the mouse thigh model (free-drug AUC/MIC, 110). This is likely because the mouse model employed mice with normal numbers of granulocytes, whereas the in vitro system completely lacks any immune function. Andes and Craig [32] have demonstrated, in their mouse thigh system, that removal of granulocytes causes a 1.5–2.0-fold increase in the exposure target required for a specific microbiological effect.

It should be noted that our model predictions were recapitulated by experiment, which validated both the microbiological methods and the mathematical model. It should also be noted that a quite substantial drug exposure caused early, extensive kill (almost 6 log₁₀) but allowed subsequent complete replacement of the total population with resistant mutants. This emphasizes the need to choose doses that will result in exposures that suppress mutants as well as produce good overall microbial kill.

Figure 5. Proportion of 10,000 simulated subjects that attained the desired exposure (free-drug ratio of area under the concentration-time curve to MIC of 53) to suppress amplification of Mycobacterium tuberculosis mutants with a moxifloxacin dose of 800 mg (solid line) and the distribution of moxifloxacin MIC values for 243 clinical isolates of M. tuberculosis (dashed line) [27].
CONCLUSIONS

Pharmacodynamics may be effectively employed to examine microbial kill, which has been shown to correlate with findings in patients. However, achieving microbial kill in the total bacterial population is only half the response. The other important half is to suppress the emergence of resistance, which is central to our efforts to deal with the threat that drug resistance poses to drug therapy. Pharmacodynamically derived drug exposures that achieve both optimal microbial kill and suppression of resistance can be determined, whether the mechanism of resistance is chromosomal mutation or induction of efflux pumps. Monte Carlo simulations can then be used to select doses that have the highest possibility of suppressing resistance in the clinical arena. Such doses are an important public health weapon in ensuring that resistance does not emerge at all in diverse clinical settings across the globe. Our brief review has focused on quinolone antimicrobials, the class of compounds with which we performed our studies. More investigations with other drug classes and other pathogens are required. Although we have provided an example in which drug doses identified in preclinical models correlate with those from studies of human patients in suppression of *P. aeruginosa* resistance by ciprofloxacin, it will be important to obtain clinical validation of many of our findings in prospective clinical trials. Doses identified to achieve optimal effects in the preclinical studies can be compared with standard doses in randomized controlled trials.

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