Mutant prevention concentration as a strategy to minimize antimicrobial resistance: a timely concept but will its acceptance be too late?

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Since the introduction of sulfonamide drugs and penicillin in the 1930s and 1940s, science and medicine has witnessed more than half a century of development and use of antimicrobial compounds which have clearly altered the course of medical history for both individuals and society and we continue to this day to define the antibiotic era. Unfortunately, we learned early about antimicrobial resistance as clinical isolates of penicillinase producing strains of *Staphylococcus aureus* rapidly appeared. Despite this early recognition of resistance and continual searches for natural or synthetic compounds with antimicrobial properties, past policies for dealing with resistance have at best been only partially effective and despite a greater understanding of the mechanisms of antimicrobial resistance, we continue to face the same challenges encountered in the beginning of the antibiotic era.

In 1960, Gould stated "we are as yet at an elementary stage in correlating the clinical administration of antibiotics with *in vitro* sensitivity determinations" [1].

Based on current knowledge, one can surmise four inherent principals involved with antimicrobial agents and antimicrobial resistance:

- Antibiotic resistance is an undeniable fact and will continue to be a problem for as long as we use (and misuse) antimicrobial agents
- Bacteria are remarkable adaptive and will continue to evolve and acquire new mechanism of resistance to antimicrobial agents
- Prior strategies for dealing with antibiotic resistance have failed to slow the progression of antimicrobial resistance and in some cases have clearly failed, therefore, novel approaches for understanding and dealing with resistance are required
- Changes to susceptibility breakpoints does not alter the fact that some bacteria are no longer as susceptible to antimicrobial agents as they once were and this observation is or will be clinically important

The mutant prevention concentration (MPC) is a novel concept coined by Dong and colleagues following the recognition of a two-stage decline in colony forming units when high density bacterial inocula were exposed to varying antimicrobial drug concentrations [2]. When $>10^9$ bacterial cells were exposed to fluoroquinolones, a sharp decline in colony recovery occurred at the MIC drug concentration. This was followed by a plateau, off of which, bacterial cells containing first step resistant mutations could be recovered. The second decline in colony recovery occurred at a drug concentration that was sufficiently high enough to block the growth of the first step resistant mutants. This drug concentration became know as the MPC. A second component of the MPC approach is the mutant selection window (MSW). The MSW defines the drug concentration between the MIC and MPC drug concentrations. When drug concentrations are below the MIC, neither susceptible nor first step resistant cells are inhibited and as such, there is no selective amplification of resistant subpopulations. For drug concentrations in excess of the MPC, both susceptible and first-step resistant cells are inhibited (killed?) and no selective amplification of resistant subpopulations occur. Unfortunately, when drug concentrations fall within the MSW, selective amplification of resistant subpopulations occurs as the drug concentration is above the MIC and inhibiting the susceptible cells in the population but not high enough to inhibit resistant subpopulations as the concentration is below the MPC. In an ideal world, drugs would be dosed to be above the MPC for as long as necessary to inhibit /kill all bacteria within the population.

For any given fluoroquinolone-pathogen combination the plateau in mutant recovery and MSW will be different. For example, for organisms which remain highly susceptible to fluoroquinolones, such as *S. pneumoniae*, the plateau in colony recovery will be relatively short, and the

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MPC may fall within the susceptible breakpoint for the drug. Conversely, *P. aeruginsoa* represents an organism where the differences between MIC and MPC measurements are large and mutant selection occurs over broad drug concentration ranges. However, despite these differences, the potential utility of the MPC measurement for restricting the selection of fluoroquinolone resistant mutants remain the same.

MPC measurement have been used to identify key differences in the antipneumococcal activity of fluoroquinolones deemed clinically equivalent [3]. When applied to drug pharmacokinetics profiles, MPC measurements were used to predict the potential of each agent to select for resistance. In order of descending activity, a hierarchy of potency based on the ability to inhibit first-step resistant mutants was determined with gemifloxacin > moxifloxacin > gatifloxacin = trovafloxacin > grepafloxacin > levofloxacin. Moxifloxacin and gemifloxacin were the only compounds tested whose serum/tissue concentrations are expected to remain in excess of the MPC for >12 hours of the dosing interval suggesting that they may be appropriate for once daily dosing [4]. Levofloxacin selected mutants at the highest concentration of any quinolone tested and serum concentrations of levofloxacin were projected to remain in excess of the MPC for approximately 3 h of the dosing (500 mg once daily) interval (the lowest of any quinolone tested). Thus, a higher (possibly 750 mg) dose of levofloxacin and/or more frequent dosing (even at the higher dosage) may be required to prevent the selection of fluoroquinolone resistant *S. pneumoniae*, which appears consistent with recent reports of levofloxacin associated clinical failures in the treatment of *S. pneumoniae* [5–8]. Sequence analysis of the quinolone resistance determining region (QRDR) of the *parC* and *gyrA* genes of selected clinical isolates revealed the presence of target mutants which raises concerns about the accumulation of fluoroquinolone resistance alleles among clinical isolates of *S. pneumoniae* that are not detected in traditional susceptibility testing procedures. Fluoroquinolone killing of *S. pneumonaie* at the MPC drug concentration resulted in increased killing and bacterial eradication by 24 h indicating that targeting the MPC may also impact on speed of clinical symptom resolution [9–11]. There is little doubt that fluoroquinolone resistance in strains of *S. pneumonaie* has increased during the past decade [6]. For example, a number of studies have shown that once the prevalence of resistance begins to increase noticeably, it can advance from below 5% to above 20% within a few years [13,14]. Currently, fluoroquinolone resistance among clinical isolates of *S. pneumoniae* remains relatively low in 2004 and new strategies/susceptibility testing procedures, such as the mutant selection window and the MPC, may dramatically impact on the degree of resistance and the rate at which it develops [15]. Past lessons, such as the escalation of penicillin resistance within *S. pneumoniae* has shown us that low-dose therapy most likely caused an increase in the carriage of resistant isolates and after mutant spread to fresh hosts, curing infections required higher doses of penicillin or new derivatives having greater potency or different binding targets. Prolonged and gradual step-wise selection with beta-lactams resulted in the selective enrichment of resistant isolates which has made penicillin less effective against a third of the *S. pneumoniae* isolates in some areas [15,19]. Dissemination of plasmid-borne factors has resulted in widespread resistance of many bacteria to beta-lactam compounds. Therefore, MPC testing for fluoroquinolones and *S. pneumoniae* represents a realistic approach for dealing with resistance before it becomes highly disseminated among clinical isolates and is of particular importance in light of the fact that fluoroquinolone treatment for *S. pneumoniae* is often administered prior to pathogen recovery and identification or in culture negative patients.

Drug development has inadvertently been associated with escalating antimicrobial resistance. Prior to convincing evidence linking pharmacokinetic/pharmacodynamic and microbiological observations and breakpoints with clinical success/failure and resistance development/prevention, drug development tended to favour the minimal dosages that were found to be clinically effective, with fewer adverse events and that could be conveniently administered so as to improve compliance and were cost effective. In today's environment, we now recognize that some of the same dosages that were shown to be clinically effective may, in fact, be the very dosages that escalate resistance. Several lines of evidence appear to suggest that this is the case. In addition to the *in vitro* MPC studies summarized above, Firsov and colleagues demonstrated that when fluoroquinolones were dosed to be above the MPC, below the MIC or within the MSW in an *in vitro* pharmacological model, changes to wild type MICs were only seen when dosing remained within the MSW [16,17]. Crossier and colleagues

reported in an *in vivo* model that when drug concentrations remained within the MSW for >45% of the dosing interval, it correlated with the selection of resistant mutants in 100% of the experiments [18]. Finally, for cases of levofloxacin treatment failure for community acquired pneumonia reported by Davidson and colleagues [7] comparison of organisms recovered prior to the start of therapy with matched isolates from those that failed therapy revealed an absence of any detectable mutations (in one case) in either of the *parC* or *gyrA* genes of the pretreatment organisms but a single mutation in each gene of the organism recovered after failure. From a second case, the pre-treatment organism has a single *parC* mutation and the organism recovered during therapeutic failure had a single mutation in each of the *parC* and *gyrA* genes. In both instances summarized, the pre-treatment and post-treatment organisms were identical to each other by pulsed field gel electrophoresis. These two cases clearly show that mutants are selected during antimicrobial therapy.

Ultimately and unfortunately, science has nothing to do with cost and we must determine if we will be guided by cost or by science. The long term prospects for clinically effective antimicrobial agents may well depend on which decision we make. Regulatory agencies will ultimately play a key role in these decisions. Drug manufacturers design studies based on the various regulatory requirements of the different countries that they wish to market their drugs. Should such bodies ultimately require that endpoints other than clinical equivalency are necessary (i.e., PK/PD breakpoints), then drug dosages or dosing intervals that result in favorable clinical outcomes and minimize the potential for resistance may become a requirement and drug prescribers will prescribe based on these new standards. Perhaps a more challenging question for regulatory bodies is what to do with drugs that are currently approved and whose dosages and dosing frequencies may be inadequate. Can newly acquired knowledge be applied to existing agents that were approved based on a different set of standards? Hopefully the answer is yes. If not, new agents developed within a class that has less active compounds currently approved – while more potent – may be compromised by the use of less active agents or agents used suboptimally and thereby truncate their longevity and clinical utility.

The MPC measurement and concept of the mutant selection window provides a rational strategy designed to prevent the selection and amplification of *de novo* resistance, however, a number of limitations in MPC testing currently exist. As indicated by Allen, the most important difficulty encountered in determination of the MPC lies in achieving the targeted inoculums [20]. Bacterial organisms which do not readily achieve $\geq 10^{10}$ CFU/mL may require additional numbers of MPC plates, for each concentration tested, to ensure that 10^{10} CFU/mL are tested. This procedure does not lend itself to a high throughput clinical setting. If the MPC is to gain utility in a typical clinical setting, streamlining of the methodology may be necessary [20]. The application of MPC testing to the clinical microbiology laboratory may require batch studies to be preformed on a representative number of bacterial species which could serve as an indicator of activity for the entire species population. If a relationship between MIC and MPC results can be deciphered, then clinicians, microbiologist, pharmacologists and scientists may be able to extrapolate MPC activity directly from the MIC. Advances in molecular diagnostics may allow for the real-time detection of a single first-step mutant within a background of 10^8 cells [21–23]. Currently, MPC testing relies on an agar dilution method and some drug–pathogen combinations require broth dilution testing.

The application of MPC testing to nonquinolone antimicrobials is an area that requires further investigation. While the majority of experiments describing the MPC have been conducted with fluoroquinolones, additional studies on other antimicrobial agents have been preformed [24]. Zhao and colleagues published MPC data for chloramphenicol, penicillin G, rifampicin and tobramycin against *E.coli* [25] and recently, the effect of antimicrobial concentration on colony-forming ability of resistant mutant subpopulations of *M. smegmatis* and *S. aureus* for chloramphenicol, erythromycin, moxifloxacin, penicillin and tetracycline was described [26]. Ongoing investigations on MPC measurements with macrolides and *S. aureus* and *S. pneumoniae* suggest MPC studies are relevant to this class of agents [12,27]. MPC is most easily considered with organisms in which resistance arises as a function of a single point mutation. However, in the broadest sense, resistance to an antimicrobial agent means that a particular microorganism is able to reproduce in the presence of the agent under specified conditions. Resistance may be associated with a specific heritable alteration or induction of protective genes such as those

encoding β-lactamases. Heritability would seem to make resistance an absolute term since changes in DNA primary structure are unequivocal. However, in some cases resistance genes are not fully protective and some drug concentrations, perhaps used at higher concentrations, prove effective. Consequently, the term resistance must be qualified to take into account the antimicrobial concentration [28]. The MSW hypothesis (for which the MPC is the upper boundary) places no restriction on the type of resistance mechanism selected. As bacterial inoculums increase so too does the heterogeneity of bacterial culture. Increased inoculum may dilute intracellular targets or enzymatic activity of resistance mechanisms which serve to increase resistance beyond what is measured by the MIC. If under these conditions, higher concentrations of antimicrobial are required to inhibit bacterial growth, then an MPC measurement may apply. In a recent clarification of the MSW, Zhao suggests that once a small fraction of mutants are present in an infected individual or heterogeneous culture, the key idea in preventing the selection of resistance will be whether resistant mutants will be enriched; not how they came into being [29]. In this respect the MPC may apply to a number of different antimicrobial agents.

The issue of "collateral damage", or the unwanted selection of resistant organisms due to high quinolone concentrations, was an issue raised in regard to the MPC by Livermore [30]. Prolonged low-dose therapy has unquestionably led to the development of resistance, however, dosing based on MPC measurements champions higher doses administered over a potentially shorter duration of therapy: a hypothesis supported in preliminary killing experiments [9]. The implication that quinolones may directly contribute to the selection of resistant mutants can be inferred based on evidence that quinolones are strong inducers of the S.O.S response [31,32]. However, a number of observations suggest that quinolones contribute minimally to resistance. For example, maximal induction of the S.O.S response by fluoroquinolones in various organisms typically occurs at concentrations 10–15 fold above the MIC values for a number of bacterial species [33]. The MPC results, for most bacterial species, are generally 2–8-fold greater than typical MIC results. Mutational studies on a LexA- strain of *E. coli* revealed no differences in the mutational frequency at which quinolone resistance developed when compared to LexA isogenic strains [Zhao *et al.*, Pers. Comm.].

Perhaps the most important question surrounding the MPC concerns the potential clinical impact. Although *in vitro* observations and retrospective clinical observations support the role of MPC in minimizing the selection of resistant mutants, no direct evidence from human trials is currently available to test the hypothesis that incorporation of MPC based testing in antimicrobial management will correlate with decreased clinical resistance and improved therapeutic outcomes. A recent report which used a rabbit model of pneumococcal infection to investigate the selection of resistant mutants of *S. pneumoniae* revealed that, after levofloxacin or moxifloxacin treatments, mutants could be recovered from strains with a pre-existing *parC* mutation [34]. The authors rationalized this finding by suggesting that strains with a pre-existing *parC* mutation caused drug concentrations to fall below the MPCs of these strains. Further *in vivo* (animal and human trials) are now required to test the principals of MPC.

Conventional wisdom suggests that resistant mutants are inherently less "fit" than wild-type cells and as a result, elicit reduced growth rates. However, recent data suggests that mutations in *parC* and *gyrA* genes may, on some occasions, not be associated with a physiological deficit [35]. Furthermore, in some cases resistance may be associated with an increase in fitness, as assessed by an increased growth rate [36]. The clinical consequence of mutant sub-populations is currently unknown. The theory behind the MPC measurement implies that one resistant mutant is as etiologically important as 100,000 mutants, however, from a clinical perspective, this argument may not hold true. The dissemination of penicillin resistant *S. pneumoniae* serotypes 3, 14, 19F and 23F demonstrate how the spread of individual resistant clones can impact on global resistance and demonstrates the necessity for minimizing resistance. In the context of *S. pneumoniae,* recent evidence indicates that failures are associated with resistant organisms which were not present at the start of therapy. In light of these observations, strategies designed to minimize the impact of resistance should incorporate ideas which make it difficult for organisms to select and/or acquire resistance mechanisms. In this context, the clinical application of the MPC is clear: maintaining serum/tissue concentrations in excess of the MPC (within tolerable doses) will require cells to obtain two concurrent

resistance mutations for growth and thereby severely restrict the likelihood that resistant mutants will be selected during therapy.

As we learn more about the accumulation of antibiotics in infected tissues, the types of resistance mutants selected by different agents within different concentration spectrums, and the concentrations required to inhibit their growth, the theory of the MPC will continue to develop. In a recent review of MPC, Blondeau and colleagues [4] noted that the development of MPC is a relatively new concept that continues to evolve with every report. Therefore, with such a new concept and relatively limited studies published to date, it may be premature to comment on overstretching the limits of MPC when we do not yet know if the limits have been defined [37]. Open debates and discussions will help to develop a greater understanding of fluoroquinolone resistance and the potential impact of the MPC. Based on the development of penicillin-resistant *S. pneumoniae*, clinical validation may come in the form of increased clinical failures and a rapid rise in the rates of resistance. Thus, perhaps the most meaningful clinical question regarding the MPC is whether or not we can afford to take a wait and see approach to fluoroquinolone (and other drug classes) resistance?

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