Testing the mutant selection window in rabbits infected with methicillin-resistant Staphylococcus aureus exposed to vancomycin

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Objectives: To test the mutant selection window (MSW) hypothesis with Staphylococcus aureus exposed to vancomycin in an animal model and to compare in vivo and in vitro exposures that restrict the enrichment of resistant mutants.

Methods: Local infection with S. aureus was established in rabbits, and the infected animals were treated with various doses of twice-daily vancomycin (half-life 6 h) for 3 consecutive days to provide antibiotic concentrations below the MIC, between the MIC and the mutant prevention concentration (MPC), and above the MPC. Changes in susceptibility and the numbers of surviving organisms were monitored daily on agar plates containing 2× and 4× MIC of vancomycin.

Results: S. aureus lost vancomycin susceptibility when drug concentrations at the site of infection fluctuated between the lower and upper boundaries of the MSW, defined in vitro as the MIC99 and the MPC, respectively. Both boundaries were determined in vitro, before starting animal studies. The value at which resistant mutants are not enriched in vivo was estimated as an AUC 24/MPC value of ≏15 h, where AUC 24 is the area under the drug concentration time curve in a 24 h interval. The estimated anti-mutant AUC/MIC ratio in vivo was ≥200 h.

Conclusions: These findings support the MSW hypothesis and the anti-mutant AUC/MIC ratio estimated in vivo is consistent with that reported in in vitro studies. Keeping vancomycin concentrations above the MPC or AUC24/MPC > 15 h is a straightforward way to restrict the acquisition of resistance.

Keywords: animal models, antimicrobial agents, pharmacokinetic variables

Introduction

Methicillin-resistant Staphylococcus aureus (MRSA) was first reported sporadically in Europe beginning in 1961 and over the span of the last 20 years has emerged as a major multidrug-resistant pathogen worldwide.1 Vancomycin has been largely used worldwide against MRSA infections since the mid-1980s.2 As a result of empirical and directed therapy, clinicians have relied on vancomycin alone for many years in the management of serious MRSA infections and have enjoyed a substantial period without vancomycin resistance appearing in S. aureus.3,4 However, clinical failures with vancomycin against MRSA infections have challenged vancomycin's standing as a first-line antimicrobial for these infections. Increasing vancomycin MIC values in MRSA clinical isolates make the optimization of vancomycin dosing pivotal to its continued use. Unfortunately, limited data exist regarding the optimal MIC- or MPC-related pharmacokinetic variables target to improve bacterial killing and clinical outcomes with vancomycin.

Appropriate antibiotic dosing is the key to eradication of infection-causing bacteria and an important factor in the emergence and proliferation of antibiotic-resistant strains. The mutant prevention concentration (MPC) is an interesting concept for use in trying to minimize the emergence of resistance by modifying antibiotic drug concentrations.5 The idea of an MPC is derived from the ‘mutant selection window’ (MSW) hypothesis, which postulates that a specific drug concentration zone exists where antibiotic exposure selects for mutant bacterial strains with reduced drug susceptibility.6 The upper boundary of the MSW is the MIC of the least drug-susceptible mutant subpopulation, a value called the MPC.7 The lower boundary of the MSW is the lowest concentration that exerts selective pressure,
often approximated by the minimal concentration that inhibits colony formation by 99% (MIC99). According to this hypothesis, the possible reason for clinical failures is that the concentration of drugs in vivo falls within the MSW so that resistant mutants are enriched, with a concomitant loss in susceptibility.

Pharmacokinetics may play an important role in the use of the MSW hypothesis to slow the development of resistance. There have been studies suggesting that an antibiotic- and bacterial strain-independent relationship exists between an integral index of the entire antimicrobial effect, intensity and the AUC24/MIC ratio as simulated in vitro, where AUC24 is the area under the drug concentration time curve in a 24 h interval. The AUC24/MIC ratio that protects against the selection of resistant mutants was predicted at ≥ 200 h. However, these conclusions have not been directly tested in vivo. So we chose the tissue-cage infection model to test the MSW hypothesis for vancomycin and investigated the appropriate MIC- or MPC-related pharmacokinetic variables to restrict the amplification and enrichment of resistant mutants.

**Materials and methods**

**Antimicrobials and chemicals**

Penicillin, as a sodium salt for injection, was purchased from Zhongnuo Pharmaceutical Group Corporation (Shijiazhuang, China). Vancomycin was provided by Eli Lilly Japan K. K., Seishin Laboratories (Nishi-Ku, Kobe, Japan). Vancomycin standard and norvancomycin standard were from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The perchloric acid and acetonitrile (chromatography grade) were from Fisher Scientific.

**Bacterial strain and susceptibility testing**

*S. aureus* ATCC 43300 was stored at the Anhui Center for Surveillance of Bacterial Resistance. MICs of antimicrobial agents were determined by means of the agar dilution method according to a standard procedure described by the CLSI in 2011. The measurement was followed by a second determination, plus a replicate that utilized linear drug concentration increments (10% per sequential increase). The fraction of the colonies that were recovered was plotted against the drug concentration to determine the MIC99 by interpolation.

The MPCs were determined as described elsewhere. The MPC was defined as the lowest drug concentration that prevented bacterial colony formation from a culture containing >10^10 bacteria. The inoculated plates were incubated for 72 h at 35°C and screened visually for growth. To estimate the exact MPC, logarithms of bacterial numbers were plotted against antibiotic concentrations. The MPC was taken as the lowest vancomycin concentration that completely inhibited growth.

**Tissue-cage infection model**

This study was approved by the Committee on the Ethics of Animal Experiments of Anhui Medical University (permit number 12-2866). Female New Zealand White rabbits weighing 2.5–3 kg each were supplied by the Anhui Medical University Experimental Animal Center. The rabbits were housed individually, in accordance with the Guide for the Care and Use of Laboratory Animals. Before implantation of the perforated plastic balls, rabbits were anaesthetized with intravenous sodium pentobarbital (30 mg/kg). Then a plastic ball (Wiffle ball, 43 mm in diameter, with a volume of 34 mL) was implanted subcutaneously into each rabbit through a dorsal midline incision under aseptic conditions. After surgery, the rabbits were treated with intramuscular penicillin (100,000 IU/kg) twice daily for 3 days to prevent infection. The rabbits were given a 4 to 6 week recovery period before the initiation of any studies. During this recovery period, the rabbits were observed daily, and the Wiffle ball became walled off and filled with sterile fluid. Approximately 3.0 × 10^10 cfu *S. aureus* ATCC 43300 culture was concentrated in 1 mL of 0.9% NaCl and injected into each pre-implanted plastic ball. Two days after infection, 0.5 mL of tissue-cage fluid was withdrawn from each plastic ball for a viable bacteria count. Rabbits having >1 × 10^8 cfu/mL viable bacterial cells were treated with various doses of vancomycin.

**Simulated pharmacokinetic profiles**

Rabbits were treated with vancomycin (as two 12 hourly doses) for 3 consecutive days, with a half-life of 6 h for the serum concentrations, in accordance with values reported in humans. According to the results of the prior experiment, rabbits were administered vancomycin at 0, 5, 10, 20, 40, 60 and 80 mg/kg of body weight daily by use of an intravenous drip beginning at 3 days after infection. These rabbits were randomly assigned to each treatment group in order to provide vancomycin pharmacokinetics at concentrations below the MICs, between the MICs and MPCs, and above the MPCs. Tissue-cage fluid (0.5 mL) was removed from the plastic ball 0, 1, 2, 2.5, 2.5, 2.75, 3, 4, 6, 8, 10 and 12 h after the each vancomycin dose. Fluid samples were clarified by centrifugation at 8900 g for 10 min and stored at −80°C.

**Pharmacokinetic measurements**

The total drug concentration of vancomycin was determined by HPLC. Briefly, a standard curve was constructed by adding a known amount of vancomycin to tissue-cage fluid over a concentration range of 0.43–112 mg/L. Norvancomycin was also added to each sample as an internal control. Both standard and experimental samples were then treated with the precipitation agent (the volume proportion for 10% perchloric acid to acetonitrile is six), clarified by centrifugation and applied to an HPLC column. Linear regression of the concentration of vancomycin standard (y) versus the ratio (x) of the vancomycin peak area to the peak area of norvancomycin (internal control) generated the formula y = 0.0776x − 0.0391 (R^2 = 0.9999). The vancomycin concentration in experimental samples was calculated from the standard-curve formula by use of the determined peak area ratio of vancomycin to norvancomycin. All vancomycin concentration determinations were performed in duplicate. Pharmacokinetic/pharmacodynamic indices were calculated according to a non-compartmental model by use of DAS software (version 2.0; Wuhu, China). All indices were determined after the sixth dose, at which time steady-state kinetics were reached.

**Quantification of the antimicrobial effect and susceptibility changes**

In each experiment, multiple sampling of bacteria-containing medium from the central compartment was performed throughout the observation period. Samples of 100 μL were plated onto Mueller–Hinton agar plates. In order to account for antibiotic carryover, all samples were diluted sufficiently prior to plating, thus reducing the antibiotic concentration below the MIC of the drug. The lower limit of accurate detection was 2 × 10^5 cfu/mL. To detect changes in susceptibility during treatment, multiple samples from the model were determined every 12 h before the vancomycin intravenous drip, during vancomycin treatment, and 24, 48, 72 h after the termination of vancomycin treatment. Each sample was plated onto agar plates containing 2× and 4× MIC of vancomycin (detection limit 10 cfu/mL). In addition, the MICs of vancomycin for bacteria from each rabbit were determined prior to and after treatment.
Statistical analysis
Fisher's exact test was used for statistical analysis of the pharmacokinetic/pharmacodynamic data, with an infected but untreated set of rabbits as a control. P < 0.05 was considered to be statistically significant.

Results

MIC, MIC99 and MPC of vancomycin for S. aureus ATCC 43300

The MIC and MIC99 of vancomycin were estimated at 1 and 0.8 mg/L for S. aureus ATCC 43300. The MPC of vancomycin was estimated at 16 mg/L by the agar double-dilution method. The exact MPC of vancomycin was estimated at 14.4 mg/L (16 × 90%) for S. aureus ATCC 43300. All experiments were performed in duplicate.

Effect of vancomycin dose on bacterial survival in the tissue-cage model

No severe illness or distress occurred during the 9 day observation period for the infected rabbits. Bacterial concentrations remained constant at ~1 × 10^9 cfu/mL when rabbits were treated with saline twice daily for 3 days. In a preliminary experiment, intravenous vancomycin was administered every 12 h for 3 days, beginning at day 3 after infection. Treatment was followed by a 3 day untreated period to allow the outgrowth of residual bacteria (Figure 1). Doses of vancomycin at 5 mg/kg/day reduced bacterial numbers at first and bacteria grew quickly to the original level. At doses of vancomycin > 10 mg/kg/day but < 60 mg/kg/day, the extent of bacterial killing was concentration dependent: the greater the dose of vancomycin, the more pronounced the reduction of the starting inoculum. However, bacterial growth was observed during the late treatment and post-treatment for doses of vancomycin at 10 or 20 mg/kg/day. But bacterial numbers remained lower during the growth recovery phase for doses of vancomycin at 40 or 60 mg/kg/day. Vancomycin at 80 mg/kg/day caused bacterial numbers to decrease significantly, and bacterial growth was not observed later during treatment. Thus the bacterial response depended on the dose of vancomycin.

Effect of vancomycin concentration on loss of susceptibility and mutant enrichment

In vivo pharmacokinetics after the sixth dose, when steady-state kinetics were reached, are described in Figure 2(a). Samples of S. aureus in tissue-cage fluid were examined for susceptibility to vancomycin after treatment with various doses. Figures 2(b) and (c) show the time courses of numbers of surviving bacteria on vancomycin-containing agar plates (2× and 4× MIC) at antibiotic concentrations within or outside the MSWs over most of the dosing interval. When vancomycin concentrations were either below the MIC99 (group A) or above the MPC (group F), no mutant of S. aureus 43300 resistant to 2× and 4× the MIC of vancomycin was selected either during or after therapy. However, when antibiotic concentrations fell into the MSWs (groups B, C, D and E), the population was enriched with resistant mutants on the plates with 2× and 4× the MIC of vancomycin. Interestingly, the results were different when the S. aureus 43300 fell in a different portion of the MSW. The mutants of S. aureus 43300 were selected only to 2× the MIC of vancomycin for group B when exposed in the lower portion of the MSW. The viable counts of the mutants resistant to 2× the MIC of vancomycin were more than that to 4× the MIC of vancomycin for groups C and D when exposed in the middle portion of the MSW. However, the mutants of S. aureus 43300 selected with 2× the MIC of vancomycin were almost equal to that of 4× the MIC of vancomycin for group E when exposed in the upper portion of the MSW.

Along with augmentation of the drug concentration in the MSW, the viable counts of the mutant increased for dose groups B, C and D. However, in comparison with dose group D, the viable counts of the mutant decreased for group E, although drug concentration increased. In addition, the time for the mutant selected was also different during therapy, when it fell in a different portion of the MSW. The mutants were selected after the second or third dose when exposed in the lower portion of the MSW. However, the mutant was selected after the fourth or fifth dose when exposed in the upper portion of the MSW. None of the regimens for dose groups A, E and F led to a loss in susceptibility of vancomycin-exposed staphylococci. However, loss of bacterial susceptibility occurred in 12 of 14 rabbits when exposed to the regimens for dose groups B, C and D. Compared with the MICs determined prior to treatment, the MICs of vancomycin determined after treatment for the 12
Figure 2. Effect of vancomycin concentration for the tissue-cage fluid on loss of susceptibility and mutant enrichment. A, B, C, D, E and F correspond to the six different treatment groups. In vivo simulated pharmacokinetics (a), and time courses of S. aureus 43300 that survived on antibiotic-containing plates with 2× MIC (b) and 4× MIC (c) of vancomycin. VAN, vancomycin; d, day.
rabbis were 2 mg/L by means of the agar dilution method according to a standard procedure described by the CLSI in 2011.

**Comparing in vivo and in vitro exposures that restrict the enrichment of resistant mutants**

Relationships between MIC- or MPC-related pharmacokinetic variables, determined as steady-state values after the sixth dose, and loss of susceptibility are shown in Table 1. For vancomycin, AUC₂₄/MIC is the most common index associated with restricting susceptible cell growth.® These data provide the first in vivo dynamic estimate of the value at which resistant mutants are not enriched. For no rabbit was loss of susceptibility seen when AUC₂₄/MIC₉₉ was <25 h. For only 2/10 rabbits was loss of susceptibility seen when AUC₂₄/MIC₉₉ was >200 h. However, loss of bacterial susceptibility occurred in 10 of 12 rabbits when AUC₂₄/MIC₉₉ was between 25 and 200 h. AUC₂₄/MPC, which is probably the appropriate index for the upper boundary of the MSW, restricted mutant selection when >15 h. Mutant selection was promoted when AUC₂₄/MPC fell between 3 and 15 h. The P-values were calculated by Fisher’s exact test as <0.05 for the two MIC- or MPC-related pharmacokinetic variables, with a set of three infected but untreated rabbits used as a control.

**Discussion**

MRSA is a common cause of bloodstream infection and is often associated with invasive infections and high rates of mortality. Although there are alternatives to vancomycin, such as linezolid, daptomycin and quinupristin/dalfopristin, they are expensive and not easy to access, especially in developing countries. So vancomycin is still the mainstay of chemotherapy for serious MRSA infections. With a limited pool of available antimicrobial agents capable of treating MRSA infections, the suppression of further emergence of resistance is of vital importance. MPC values, when considered with drug pharmacokinetics, may allow prediction of the probability of resistance selection when bacteria are exposed to antimicrobial agents during therapy for infectious diseases.® Traditionally, in vitro antimicrobial studies and animal infection models have been used to assess the reduction in the total bacterial population at an infectious site while often ignoring the impact of drug pressure on the amplification of the drug-resistant subpopulation.® In addition, in vitro measurements cannot account for the host’s immune response, which is necessary for successful recovery from infectious diseases. However, no animal or clinical studies are yet available to compare in vivo and in vitro exposures that restrict the enrichment of resistant mutants. So it is necessary to test the MSW hypothesis with S. aureus exposed to vancomycin in an animal model. Localized infections are particularly well suited for testing the MSW hypothesis, because both drug concentration and the selective amplification of resistant mutants can be directly measured at the site of infection. In this study, the tissue-cage infection model provided a clear demonstration of the MSW in vivo and supported arguments for how antimicrobial treatment regimens can be adjusted to severely restrict the amplification and enrichment of resistant mutants.

Traditionally, pharmacokinetic/pharmacodynamic parameters have been used to predict antibiotic efficacy, but there is now increasing interest in trying to use MIC- or MPC-related pharmacokinetic variables to minimize the development of resistance. The three MIC-related pharmacokinetic variables commonly used to predict antibiotic efficacy are: (i) the ratio of maximum serum concentration to the MIC (Cₘ₅₀/MIC); (ii) the ratio of the area under the plasma concentration versus time curve (AUC) versus MIC (AUC/MIC); and (iii) the duration of the dosing interval when plasma concentrations exceed the MIC (Tₚ/MIC).® Recent experiments suggest that the AUC/MIC ratio may be used to identify vancomycin exposures associated with the emergence of resistance in S. aureus.® AUC₂₄/MIC relationships of the final-to-initial MIC ratio and logarithm of the ratio of maximal-to-initial numbers of organisms resistant to 2x and 4x the MIC of vancomycin were bell-shaped and bacterial strain and antibiotic independent in an in vitro dynamic model. Based on these relationships, an AUC₂₄/MIC ratio that protects against the selection of resistant mutants was predicted at ≥200 h.® These findings support the MSW hypothesis and the conclusion has been directly tested in the tissue-cage infection model. As seen in Figure 2, when antibiotic concentrations fell into the MSWs (groups B, C, D and E), the population was enriched with resistant mutants on the 2x and 4x the MIC of vancomycin plates. However, when AUC₂₄/MIC₉₉ was ≥200 h, there were still 2/10 rabbits with loss of susceptibility in the study.

**Table 1. Correlation of pharmacokinetic/pharmacodynamic parameters with selection of resistance**

<table>
<thead>
<tr>
<th>Pharmacokinetic/pharmacodynamic index, value</th>
<th>Fraction of rabbits with resistant bacteria</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC₂₄/MIC₉₉ &gt;200 h</td>
<td>2/10</td>
<td>0.577</td>
</tr>
<tr>
<td>25–200 h</td>
<td>10/12</td>
<td>0.022</td>
</tr>
<tr>
<td>&lt;25 h</td>
<td>0/3</td>
<td>NA</td>
</tr>
<tr>
<td>AUC₂₄/MPC &gt;15 h</td>
<td>0/8</td>
<td>NA</td>
</tr>
<tr>
<td>3–15 h</td>
<td>12/14</td>
<td>0.015</td>
</tr>
<tr>
<td>&lt;3 h</td>
<td>0/3</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not applicable. 
Indeces were calculated using total drug concentrations. 
*p-values were calculated by Fisher’s exact test, with a set of three infected but untreated rabbits used as a control. High values indicate no difference from the control.
strategies do not. It was reported that the AUC/MPC ratio of ciprofloxacin was suggested to be a better predictor of the enrichment of resistant *Escherichia coli* than AUC/MIC. Although this statement was not entirely supported by the data from an in vitro dynamic model of vancomycin, it does not mean that the AUC24/MPC is not the appropriate parameter used to define the top of the selection window. The fact that AUC/MPC is more predictive of bacterial resistance than AUC/MIC has been reported in an in vitro study with ciprofloxacin-exposed staphylococci. In the present work, the mutant restrictive value of AUC24/MPC was 15 h, correlated with restricted outgrowth of resistant mutant subpopulations. This is the first reported AUC24/MPC correlated with restricted outgrowth of a resistant mutant of MRSA to vancomycin.

The development of staphylococcal resistance to vancomycin has been associated with prolonged exposure to low serum concentrations of the drug. Keeping vancomycin concentrations above the MPC or AUC24/MPC >15 h is a straightforward way to restrict the acquisition of resistance. However, this is not easy to implement in actual clinical work. Since pharmacokinetic fluctuations often place antibiotic concentrations in the MSW, populations of resistant bacterial mutants are likely to be enriched by antibiotic treatment.

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Transparency declarations

None to declare.

References


