Mutant prevention concentration of tigecycline for clinical isolates of *Streptococcus pneumoniae* and *Staphylococcus aureus*

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Background: The mutant prevention concentration (MPC) reflects the antimicrobial susceptibility of the resistant mutant subpopulations present in large bacterial populations. In principle, combining the MPC with pharmacokinetic measurements can guide treatment to restrict the enrichment of resistant subpopulations, just as the MIC is used with pharmacokinetics to restrict the growth of bulk, susceptible populations. Little is known about the MPC of tigecycline, one of the more recently approved antimicrobials. Tigecycline is particularly interesting because it shows good activity against Gram-positive pathogens.

Methods: MPCs were determined using tigecycline-containing agar plates for clinical isolates of *Streptococcus pneumoniae* (n=47), MRSA (n=50) and MSSA (n=50).

Results: Trypticase soy agar containing sheep red blood cells, commonly used for the growth of *S. pneumoniae*, gave tigecycline MPC₉₀ values that were two orders of magnitude higher than expected. The addition of agar to Todd–Hewitt broth (solidified Todd–Hewitt broth) allowed the high-density growth of *S. pneumoniae* in the absence of red blood cells and lowered the MPC₉₀ of tigecycline by 100-fold to 0.5 mg/L. The addition of red blood cells to solidified Todd–Hewitt broth raised the MPC₉₀ by 100-fold. Thus, red blood cells reduce the efficacy of tigecycline against *S. pneumoniae*. The growth of *Staphylococcus aureus* was not sensitive to red blood cells; values of MPC₉₀ were 2 and 4 mg/L for MSSA and MRSA, respectively.

Conclusions: Values of MPC constitute a concentration threshold for restricting the emergence of tigecycline resistance that can now be used in animal studies to determine pharmacodynamic thresholds. The off-label treatment of *S. pneumoniae* blood infections with tigecycline may require caution due to blood-cell-mediated interference with the antimicrobial.

Keywords: mutant selection window, blood agar, resistance

Introduction

Historically, antimicrobial resistance has been addressed by developing new, more potent agents that bypass current resistance problems. A recent effort to apply this strategy resulted in tigecycline,¹ a glycycline antimicrobial that exhibits activity against a broad spectrum of aerobic and anaerobic bacteria. Susceptible pathogens include MRSA, penicillin-resistant *Streptococcus pneumoniae*, VRE, and ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*. Whether the doses chosen for tigecycline restrict or facilitate the selective enrichment of resistant mutant subpopulations of *Staphylococcus aureus* and *S. pneumoniae* is unknown.

Relationships between antimicrobial concentration and resistant mutant selection are described by the mutant selection window hypothesis.²⁻⁴ In essence, selective enrichment occurs at concentrations above the MIC for the bulk, susceptible population, but below the MIC for the least susceptible resistant subpopulation. The latter value has been termed the mutant prevention concentration (MPC) because it severely restricts the recovery of resistant mutants in laboratory settings.⁵ Keeping antimicrobial concentrations above the MPC throughout treatment is expected to restrict the emergence of resistance. The MPC threshold may be relaxed by finding suitable pharmacodynamic parameters that empirically restrict the emergence of resistance, since pharmacokinetic/pharmacodynamic measurements include bactericidal...
effects, while selection window determinations do not. As new compounds become available, the measurement of MPC provides a baseline for subsequent animal studies that estimate doses for restricting the emergence of resistance. The present work reports the MPCs of tigecycline for clinical isolates of *S. pneumoniae* and *S. aureus*, two sources of serious disease. Surprisingly, red blood cells, normally included in agar for plating *S. pneumoniae*, greatly reduced tigecycline susceptibility in high-density cultures.

**Materials and methods**

**Bacterial cultures**

Isolates of *S. pneumoniae* and *S. aureus* from patients with various types of infection were from the Clinical Microbiology Laboratory, Royal University Hospital, Saskatoon, SK, Canada. Organisms were identified as described in the Manual of Clinical Microbiology; *S. aureus* was also identified by Vitek II (bioMerieux, St Laurent, QC, Canada). Methicillin resistance was detected by the incubation of *S. aureus* on Mueller–Hinton, oxacillin-containing screening plates; the results of preliminary tests were confirmed using an ‘in-house’ PCR assay to detect the mecA gene. No pre-selection criterion favoured the inclusion or exclusion of organisms having specific susceptibilities to tigecycline, although all organisms were susceptible based on initial screening using current susceptibility breakpoints. Duplicate isolates from the same patient were excluded. *S. pneumoniae* and *S. aureus* isolates were colony-purified on trypticase soy agar (TSA) plates containing 5% sheep red blood cells (SRBCs) (Oxoid, Ryegate, MT, USA) with incubation in 5% CO₂ (S. pneumoniae) or air (*S. aureus*) at 35–37°C for 18–24 h. Colonies were transferred to cryovials containing 0.5 mL of skimmed milk for storage at −70°C. *S. pneumoniae* ATCC 49619, MSSA (ATCC 29213 and ATCC 25923) and MRSA (ATCC 43300) were from ATCC (Rockville, MD, USA). ATCC strains were included in each susceptibility test.

**Susceptibility determinations**

MICs were determined by microbroth dilution as recommended by the CLSI. For *S. pneumoniae*, isolates were tested in Todd–Hewitt broth (THB) (Difco Laboratories, Detroit, MI, USA) with incubation for 18–24 h in 5% CO₂. MICs for *S. aureus* were determined using Mueller–Hinton broth with incubation in ambient air.

MPCs for *S. pneumoniae* were determined as previously described. Briefly, cultures of each isolate were grown on seven TSA/blood plates (TSA containing 5% SRBCs; PML, Richmond, BC, Canada) and then transferred with sterile swabs to 500 mL of THB for another overnight incubation to a concentration of $3 \times 10^8$ cfu/mL. Cultures were concentrated by centrifugation and resuspended in 3 mL of fresh THB. Aliquots of 0.2 mL, which contained more than $10^9$ cfu, were applied to each member of a series of agar plates (described in the Results section) containing a variety of tigecycline concentrations. Inoculated plates were incubated at 35–37°C in 5% CO₂ and screened for growth after 24 and 48 h. The MPC was recorded as the lowest drug concentration that prevented growth.

MPCs for *S. aureus* were determined as previously described. Briefly, each isolate was applied to two or three TSA plates that were incubated to produce confluent growth. The contents of the plates were transferred to 100 mL of Mueller–Hinton broth and incubated overnight. For each isolate, 0.1 mL containing $10^{10}$ cfu was applied to each member of a series of TSA plates containing various concentrations of tigecycline. Plates were examined for growth after incubation for 24 and 48 h. When a surface film formed on the plates, surface material from these plates was restreaked onto a fresh drug plate, incubated overnight and examined for colonies. This test confirmed the absence of *S. aureus* colonies.

**Results**

**Determination of tigecycline MPC for *S. pneumoniae***

The MICs and MPCs of tigecycline for *S. pneumoniae* showed typical bell-shaped distributions (Table 1), but the values of MPC were extraordinarily high with the standard medium, TSA containing 5% SRBCs. When the population values were calculated (Table 2), the ratio of MPC to MIC was also surprisingly high: MPC<sub>90</sub>/MIC<sub>90</sub> was more than 500 (Table 2). Previous work with tigecycline and *Acinetobacter baumannii* reported MPC<sub>90</sub>/MIC<sub>90</sub> to be 16–32.

To address the possibility that an interaction occurs between tigecycline, red blood cells and *S. pneumoniae* at the high cell density used for MPC determination, we prepared a new medium that allowed *S. pneumoniae* to grow without red blood cells. This medium, THB + 1.5% agar (solidified THB (sTHB)), yielded MPC values that were considerably lower than when measured using TSA + 5% SRBCs (Table 2). For example, MPC<sub>90</sub>/MIC<sub>90</sub> was only 16. The addition of red blood cells to sTHB elevated the MPC: MPC<sub>90</sub>/MIC<sub>90</sub> was greater than 250 (Table 2). Thus, tigecycline has unusually low activity against *S. pneumoniae* at a high cell density when red blood cells are present.

**Determination of tigecycline MPC for *S. aureus***

To determine whether the presence of blood in the test medium influences the MPC of tigecycline for other pathogens, we measured the susceptibility of clinical isolates of MSSA and MRSA. The MICs and MPCs of tigecycline for MSSA showed typical bell-shaped distributions, but for MRSA the distribution was bimodal (Tables 3 and 4); ~25% of the MRSA isolates exhibited reduced susceptibility. Nevertheless, the MPC<sub>90</sub>/MIC<sub>90</sub> was similar.

<table>
<thead>
<tr>
<th>Table 1. Distribution of MICs and MPCs of tigecycline for <em>S. pneumoniae</em></th>
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<tr>
<td><strong>Tigecycline MIC/MPC (mg/L)</strong></td>
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<tr>
<td>~&lt;0.008</td>
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<td>MIC determined by broth dilution using THB (number of isolates)</td>
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MPC (number of isolates)

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<tbody>
<tr>
<td>TSA+5% SRBCs</td>
<td>11</td>
</tr>
<tr>
<td>sTHB+5% SRBCs</td>
<td>2</td>
</tr>
<tr>
<td>sTHB</td>
<td>43</td>
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16 and 8 for MSSA and MRSA, respectively (Table 2). The addition of 5% red blood cells to the test medium lowered the MPC90 by one dilution for MSSA and had no effect for MRSA (Table 2). We conclude that the presence of red blood cells in agar has little effect on the MPCs of tigecycline for \textit{S. aureus}.

**Discussion**

In the present work, we measured the MPCs of tigecycline for \textit{S. pneumoniae} and \textit{S. aureus} to generate thresholds that might have predictive value for the emergence of resistance. With the recommended doses, the tigecycline concentration in the serum varies considerably among the test subjects,\textsuperscript{11} with about half having values that are above the \textit{S. pneumoniae} MPC90 for about 2 h; few have serum levels above the MPC90 for \textit{S. aureus}.

However, the tigecycline concentration can be higher in some tissues than in serum.\textsuperscript{15} For example, the AUC\textsubscript{0–24} is 2-fold greater in lungs than in serum,\textsuperscript{12} which may be important because pneumonia is one of the indications for tigecycline use. Since host defence systems reduce the bacterial burden, they relax the need to keep concentrations above the MPC throughout therapy to inhibit the enrichment of mutants. Mutant-restricting conditions can be estimated empirically using pharmacodynamic indices.\textsuperscript{13}

For tigecycline, the AUC\textsubscript{24}/MIC is considered to be a relevant pharmacodynamic index for a successful outcome,\textsuperscript{14} which would make the AUC\textsubscript{24}/MPC the empirical threshold for restricting the emergence of resistance. Animal studies can now be performed to identify those thresholds, which, along with the present MPC determinations, can be used to evaluate dosing protocols for their ability to restrict the emergence of resistance. Work of this type may be particularly important with Gram-negative bacteria as the emergence of tigecycline resistance among individual
patients clearly occurs.15–17 Indeed, the MPCs of tigecycline are very high for A. baumannii,10 a pathogen for which resistance is a major issue.15

To obtain the MPCs described above for S. pneumoniae, we omitted red blood cells from the test medium. When red blood cells were present, the MPC was hundreds of times higher than when they were absent, and the MPC/MIC ratio was greater than 500 (Table 2). This situation is unusual: the MPCs for many bacterium/antimicrobial combinations are generally 2- to 8-fold greater than the corresponding MIC values,18 and in the present work the MPCs of tigecycline for S. aureus were affected little by the presence of red blood cells. Additional work is required to understand the interaction between S. pneumoniae, tigecycline and red blood cells, which may involve the lysis of red blood cells by S. pneumoniae. Our observations illustrate the profound effect that the choice of growth medium can have on susceptibility testing and the importance of standardizing procedures for MPC determination. The striking effect of red blood cells suggests that caution may be required if tigecycline is used off-label for treating S. pneumoniae blood infections: elevated MPCs would make it very difficult to halt the growth of mutant subpopulations and the emergence of resistance in the absence of strong host defences.

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Transparency declarations
None to declare.

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