THE ROLE OF CHELATORS ON CLINICAL PHARMACOKINETICS AND PHARMACODYNAMICS OF TIGECYCLINE

By

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To my parents and Johannes
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<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium ion</td>
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<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>Peak concentration</td>
</tr>
<tr>
<td>$C_{\text{min}}$</td>
<td>Trough concentration</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>F</td>
<td>Bioavailability</td>
</tr>
<tr>
<td>fAUC</td>
<td>Free area under the curve</td>
</tr>
<tr>
<td>fC$_{\text{min}}$</td>
<td>Free trough concentration</td>
</tr>
<tr>
<td>fC$_{\text{max}}$</td>
<td>Free peak concentration</td>
</tr>
<tr>
<td>$f_u$</td>
<td>Fraction unbound</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
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<tr>
<td>MI</td>
<td>Metal ion</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>$P. \text{aeruginosa}$</td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>PD</td>
<td>Pharmacodynamics</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>PO</td>
<td>By mouth</td>
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<tr>
<td>PP</td>
<td>Plasma protein</td>
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<tr>
<td>$t_{1/2}$</td>
<td>Half life</td>
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<tr>
<td>TET</td>
<td>Tetracycline</td>
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<td>TIG</td>
<td>Tigecycline</td>
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<td>TMP</td>
<td>Trimethoprim</td>
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Tigecycline, a novel glycyclycline antibiotic and minocycline analog, exhibits atypical nonlinear plasma protein binding, defined as protein binding proportional to total concentration. At higher concentrations tigecycline exhibits typical nonlinear (saturable) protein binding. Given that this phenomenon was not previously observed, its impact on clinical pharmacokinetics and pharmacodynamics had not been assessed. This work overviews the potential impact on clinical pharmacokinetics, explores the role of metal ion chelators on in vitro activity, examines the mechanism by which calcium may mediate protein binding, characterizes the effects of tigecycline and tetracycline in combination against Pseudomonas aeruginosa, and evaluates its potential for clinical combination use. Static time kill curve experiments were performed for tigecycline and tetracycline alone and in combination. Up to a 4-log increase in bacterial kill was observed with the addition of tetracycline. A competitive inhibition PK/PD model with a general Pharmacodynamic interaction term was developed to describe the time-kill data and a statistically significant interaction was characterized by the model. Population PK models of tigecycline and tetracycline were implemented from literature or developed based on published studies. Various combination dosing regimens were simulated, but only the dosing regimen of tigecycline 2000 mg IV once, with 1000 mg administered every 12 hours thereafter, with
tetracycline 1500 mg every 6 hours, both for 14 days, demonstrated adequate killing and clinical synergy. Based on current penetration data in healthy subjects these doses would likely be unsafe for clinical use. There is some evidence in animals that penetration may be higher in infected tissues. With better knowledge of clinical tissue penetration in patients, tigecycline and tetracycline may be a promising combination to treat *P. aeruginosa* infections. Overall, metal ion chelation has surprising impacts on clinical protein binding, clinical pharmacokinetics and pharmacodynamics. The use of two drugs of the same class in combination is counterintuitive and the underlying mechanisms may provide new interesting avenues for future drug development and combinations.
CHAPTER 1
NONLINEAR PROTEIN BINDING: NOT WHAT YOU THINK

Introduction

For nearly a century, the protein binding of drugs has been depicted by a simple saturable binding of drug to protein binding sites. Very often, due to the large binding capacities of proteins or low binding affinities of drugs for proteins, saturation does not occur clinically and the unbound drug fraction is therefore independent of its total concentration. This is referred to as linear protein binding. Saturation of proteins results in concentration dependency and can occur and have clinical relevance for some drugs in general, or in specific clinical scenarios or populations. Until recently the saturation behavior was the only nonlinear phenomenon widely known. The recent development of several tetracycline derivatives (i.e. tigecycline, eravacycline) has revealed a new phenotype of atypical nonlinear protein binding. The counterintuitive trend of increased binding to protein as total drug concentrations increase was not previously observed and its implications on clinical efficacy or safety were not assessed.

This review aims to describe the concepts surrounding protein binding, while giving a more in depth view into nonlinear protein binding than presented previously. The theoretical pharmacokinetic (PK) impacts of nonlinear protein binding including typical and atypical will be discussed. And finally, tigecycline is used as an example to explore potential effects of atypical nonlinear plasma protein binding on clinical PK.

Basics of Protein Binding

A Brief History

The techniques by which protein binding of drugs are characterized were first developed in the early 1900s. The idea of blood as transport organ was described by Bennhold in the late 1930s. Langmuir’s isotherm established the chemical interaction basis for these protein-drug
interactions, which is still used today. Over the years there has been intense discussion regarding the clinical significance of protein binding and corresponding alterations. One could argue that for the majority of drugs, alterations in PK caused by changes in protein binding are clinically insignificant. Even if true only for a particular population, it should be part of the clinical reasoning to consider the effects of altered drug-protein binding in different disease states, age groups, acute inflammatory conditions, and polypharmacy.

If the efficacy of a drug is known to be potentially impacted by changes in plasma protein binding, it is the clinical and scientific communities’ duty to perform due diligence to investigate the potential for such alterations. Protein binding is an important part of drug development from preclinical to clinical to post-market phases. As lower protein binding is typically a more favorable attribute to avoid protein saturation, molecules may be chemically altered to decrease protein binding, while maintaining efficacy. Protein binding is evaluated in in vitro and in vivo pre-clinical settings and ideally is used to predict unbound (active) concentrations in clinical studies, allowing for lead optimization. For accurate extrapolation or prediction of free clinical drug exposure, differences in interspecies physiological, metabolic, and elimination processes should be considered. Furthermore, any expected pharmacodynamic effect, be it drug efficacy or toxicity, should be predicted based on free exposure. Given the relatively recent arrival of the use of pharmacometrics to predict clinical dosing, mathematical models may be developed to predict the effects of such alterations in a clinical setting. In the future, clinical models may be developed and implemented at the bedside to deliver optimized therapy to patients for drugs requiring adjustment in the presence of more complex PK phenomena, such as nonlinear protein binding.
**Definitions**

The expected binding behavior for almost all drugs is that the fraction unbound \( (f_u) \) is constant over a concentration range (i.e. linear protein binding) until a point at which protein binding sites begin to be saturated, leading to increasing unbound fraction with increasing total concentrations, typically referred to as nonlinear protein binding. This behavior is described as part of the Langmuir model\(^1,2\) (illustrated in Equation 1-1 and Figure 1-1), where \( A_{\text{max}} \) is the maximum binding capacity for substrate, \( K_d \) is the dissociation constant, \( C_u \) is the unbound drug concentration, and \( C_b \) is the bound drug concentration.

\[
C_b = \frac{A_{\text{max}} \times C_u}{K_d + C_u} \tag{1-1}
\]

Technically speaking, nonlinear protein binding is defined as any pattern of protein binding which is not linear (or at a concentration range at which the \( f_u \) is not constant). Until recently, nonlinear behaviors other than the Langmuir model had not been well described. During the development of tigecycline, a unique protein binding behavior was observed and not pursued further until recent investigations linked the nature of the behavior to divalent metal ion chelation\(^7-9\). Other tetracyclines (i.e. eravacycline\(^10,11\), TP-271\(^12\), minocycline and doxycycline\(^13\)) have displayed similar behavior, but further mechanistic investigations have not been performed. The nonlinear protein binding of tigecycline has been described as “U-shaped” with a counterintuitive decrease in \( f_u \) with increasing total concentrations and an eventual return to the appearance of the more typical saturation behavior (Figure 1-2).

Any behavior deviating from a typical Langmuir, saturation-related, nonlinear behavior, has been defined as “atypical nonlinear protein binding”\(^8\), while the former will be referred to as “typical nonlinear protein binding”. These definitions have been listed in Table 1-1 for quick reference and clarity.
Practical Matters

Various methods for plasma protein binding determination are available, including equilibrium dialysis, ultrafiltration, ultracentrifugation, charcoal adsorption, chromatographic methods, and solid phase microextraction. Equilibrium dialysis is often considered the gold standard but the ease of use and fast processing makes other methods like ultrafiltration attractive in many settings. Protein binding should be determined under appropriate physiological conditions, over a clinically meaningful concentration range, and with great methodological care for experimental factors such as pH, buffers and solvents used, device, temperature, animal species, protein concentration, proteins or endogenous substances present, and sample volume. Regardless of the method used, each method has its own caveats and considerations that have been reviewed and investigated extensively elsewhere.

Theoretical Implications and Examples

Theoretical Implications

The impacts of changing $f_a$ in linear scenarios have been previously discussed. In summary, any changes in protein binding may affect the clearance (CL) and/or volume of distribution ($V_d$) of a drug, which may or may not significantly impact PK parameters (i.e. half-life, and free and total steady state, minimum and maximum concentrations), PK in the tissues of interest, or bioavailability. These changes may be significant based on route of administration (oral versus parenteral), low versus high extraction, and original tissue distribution (or the magnitude of $V_d$). Given that changes may affect elimination processes, for orally administered drugs subject to first pass metabolism, bioavailability may be impacted for high extraction drugs but not for low extraction drugs. Conversely total clearance would be significantly affected for low extraction drugs for either route of administration. In the case of drugs with low $V_d$, 
changing $f_u$ may not significantly impact $V_d$, while for high $V_d$, changes in $f_u$ are more likely to impact distribution.

**Typical Nonlinear**

Similar to linear protein binding, effects of changing protein binding can be anticipated based on $V_d$, extraction ratio, and administration route. Given the time- and concentration-dependent nature of changing PK parameters in the presence of nonlinear binding, predicting PK is complicated. As concentration-time profiles may not explain much about changes in distribution, it is important to assess the implications of these alterations at clinically relevant doses/concentrations and be aware of underlying mechanisms. Several simulation and modelling exercises have been performed to investigate the effects of nonlinear binding behavior on clinical PK.

Martin evaluated dissociation constants ($K_d$) a main driver of the potential for albumin saturation stating that $K_d$ less than $1 \times 10^{-4}$ was conducive with potential saturation especially at higher doses\(^{18}\). He noted that calculating elimination rate constants ($k_e$) from apparently linear total concentration-time curves would underestimate elimination\(^{18,19}\). More drug is present in plasma as concentrations decline due to a decrease in saturation of proteins and decrease in $f_u$. As time goes on, this decline in $f_u$ leads to a continued decrease in $k_e$ and an increase in half life ($t_{1/2}$). $k_e$ as related to free drug concentration over time remains linear. Given the expected increase in protein saturation at high doses, $k_e$ at high concentrations is especially underestimated, while at later time points, when concentrations are lower and saturation decreases, $k_e$ is overestimated. Log(concentration)-time nonlinearities may be mistaken for slower elimination at higher doses, rather than nonlinear protein binding.

Øie and colleagues recognized that earlier simulations assumed linear tissue binding and thus explored saturable plasma and/or tissue binding in a one compartment model after
intravenous (IV) administration for drugs with various intrinsic clearance (CL_{int}) and V_d values. Their model also assumed binding in the extracellular space when saturable plasma protein binding was present and therefore defined tissue binding as that intracellular binding. Table 1-2 summarizes their simulated results for eight scenarios: Typical nonlinear plasma protein binding with low or high V_d, and low or high CL_{int}, linear plasma protein binding with typical nonlinear tissue binding, with low or high CL_{int}, and finally, typical nonlinear plasma and tissue protein binding, with low or high CL_{int}. After a single IV administration, when typical nonlinear binding was present in either plasma or tissues, as expected, unbound fraction in plasma and/or tissue (f_{uP} and f_{uT}) decreased with total concentration. Decreases in f_{uP} results in decreased V_d over time, while decreases in f_{uT} led to increased V_d over time. When both f_{uP} and f_{uT} change (in the case of typical nonlinear binding in plasma and tissue), V_d may increase, decrease or remain similar, depending on the relative magnitude of the unbound fraction changes. In this simulation, f_{uP} and f_{uT} changed proportionally and the subsequent changes in k_e, t_{1/2}, and PK profile reflect no change in V_d. As per theory, only changes in f_{uP} affect low extraction (low CL_{int}) drugs, while high extraction drugs are unaffected. In the case of low V_d and low extraction with typical nonlinear plasma binding only, since the V_d decreases with concentration, the concavity of the log-concentration time curve is less striking. For high V_d, low extraction drugs with only saturable plasma binding, the approximate changes in V_d and CL were similar, leading to no apparent change in k_e. This is interesting as the log-total concentration versus time curve exhibits convexity, which would lead one to assume nonlinear elimination. By examining the log-free concentration versus time profile, linear elimination is revealed. While these explorations by Øie and colleagues did not address all possible scenarios, especially those where simultaneous changes are not proportional, they serve as a solid starting point for predicting expected
alterations in PK resulting from changes in $f_{uP}$ and $f_{uT}$ under various PK scenarios. It should not go without emphasis that without considering saturable binding these curves may be misinterpreted as nonlinearities in drug metabolism or multiple compartmental distribution.

McNamara and colleagues concurred with Øie and colleagues model and derived new $V_d$ terms to describe the changing PK terms and applied this to ceftriaxone$^{21,22}$. They assumed no intracellular/tissue binding given the properties of ceftriaxone and included typical nonlinear binding of proteins in the extracellular space based on the expected concentration of albumin in the interstitium as compared to plasma. In their simulations they observed concave concentration-time profiles for ceftriaxone at higher doses.

**Atypical Nonlinear**

With these models of typical nonlinear protein binding, one could predict PK alterations for atypical nonlinear protein binding. The complexity arises from when the drug potentially reverses back to the typical nonlinear phenomenon at higher concentrations, which is the case for tigecycline. In these situations, use of pharmacometric modelling to simulate complex changes, is the ideal approach.

Currently the only class of drugs which appears to have atypical nonlinear PPB is the tetracyclines. While it is important to note that this behavior has not been observed or characterized for all tetracyclines, it has been recently well-described for minocycline$^{13}$, doxycycline$^{13}$, tigecycline$^{9,23}$, eravacycline$^{10,11}$, and TP-271$^{12}$. Of note, the plasma protein binding of omadacycline, a new tetracycline derivative in clinical development, was found to be linear over the investigated concentration range$^{24}$. This may be due to the overall decreased binding of omadacycline as compared to other tetracyclines exhibiting atypical nonlinear binding (21.3%$^{24}$ versus 73-93%$^{25}$). Clinical PK effects of these atypical phenomena have not been studied in clinical trials or modeled with the changing $f_u$ in mind. One study has investigated the
use of the $f_a$ in clinical breakpoint determination for tigecycline\textsuperscript{26}, which has also been adopted by others for eravacycline\textsuperscript{27}. There is a clear gap in work examining the expected PK alterations due to increase binding with total concentration.

**Potential Implications on Tigecycline**

**Pharmacokinetic Effects**

Current pharmacokinetic models do no account for the atypical nonlinear protein binding of tigecycline\textsuperscript{28–31}, eravacycline\textsuperscript{32}, doxycycline\textsuperscript{33} or minocycline\textsuperscript{34}. Based on what we know from drugs with typical nonlinear protein binding, the free drug will follow linear PK, while total drug will have nonlinear PK. Since most population PK models assume linear processes, the modelling of potentially nonlinear processes using linear models may mischaracterize the existing nonlinearity as additional compartments or multiple elimination processes\textsuperscript{35}. Use of these models in predictions may lead to error in PK profiles and expected exposures. Our group modified a population PK model of tigecycline in healthy volunteers\textsuperscript{28} to incorporate nonlinear protein binding based on a previously developed model\textsuperscript{26}. Fourteen-day dosage regimens were simulated in NONMEM (Version 7.3) for linear and nonlinear scenarios for doses ranging from 25 to 200 mg every 12 hours. As shown in Figure 1-3, CL and $V_d$ varied widely for nonlinear binding as compared to linear binding scenarios with predicted CL of 4.98–26.5 L versus 16.8 L/h and predicted $V_d$ of 14.8–48 L versus 27.8 L for nonlinear versus linear binding, respectively. The resulting fluctuation in $t_{1/2}$ was less substantial. This change could be naively overlooked as parameter variability. Simulated total exposures (AUC) were higher for the nonlinear scenario (up to 202% of linear binding) and free exposures (fAUC) were up to 30% lower for higher doses (Figure 1-4). Trends were maintained when CL\textsubscript{int} or tissue binding were changed. Overall, these simulations suggest that at higher doses free drug exposure may be overestimated by current models when atypical nonlinear binding is unaccounted for, which could lead to
subtherapeutic dosing and possibly clinical failure. In addition, PK variability across doses may result from nonlinear binding.

This analysis has some limitations that should be noted: Lacking the original data, a new model was not constructed, instead original model parameters were used as reference points in implementing alterations. There is little known about the tissue binding and nonlinear binding was only incorporated into the central compartment, a point also brought up by Øie and colleagues. With this these simulations should only be regarded as hypothesis generating and as motivation to examine free exposure of tigecycline at the site of action (infected tissue) to truly understand and develop an accurate model of the active concentration-time profiles.

**Calculating Penetration**

When determining pharmacological activity, free concentrations at the site of action need to be considered. Often predicted plasma concentrations are extrapolated to the tissues with the use of penetration ratios (i.e. \( \text{AUC}_{\text{tissue}}:\text{AUC}_{\text{plasma}} \)). In our analysis of free tigecycline penetration, we found that penetration ratios may also be overestimated if linear binding is assumed. Two studies, examining the penetration of tigecycline into ELF fluid utilized quantified total plasma concentrations. If the \( f\text{AUC} \) in plasma or serum are calculated by simply multiplying AUC by a constant \( f_u \) of 0.21, then the ratio of \( f\text{AUC}_{\text{ELF}} \) to \( f\text{AUC}_{\text{central}} \) were 5.04 and 7.94 for each study. If the clinical protein binding model developed by Bulik and colleagues is used to calculate free concentrations and subsequently \( f\text{AUCs} \), the resulting penetration ratios are much lower (1.86 and 2.32). In a subcutaneous microdialysis study, \( f\text{AUC}_{\text{tissue}}:f\text{AUC}_{\text{plasma}} \) ratios were 0.99 and 1.00 in the thigh and wound, respectively, closer to these values than those calculated based on linear binding.

The gross overestimation of drug penetration calculated based on linear binding for drugs exhibiting atypical nonlinear binding could lead to overestimation of pharmacodynamic effects
and subsequent underdosing. In the case of tigecycline, inadequate treatment of an infection could lead to prolonged and progressive illness, the need for intubation, additional antibiotics, or surgical intervention, or death. Subtherapeutic concentrations from underdosing may also result in increased antibiotic resistance, eventually leading to further development of superbugs against which few antibiotics are effective.

**Conclusion**

Assessment of protein binding in determining pharmacologically active drug concentrations at the site of action is critical in drug development and clinical practice. The PK effects of linear and typical nonlinear binding have been well studied for a variety of compounds. Given the discovery of the novel atypical nonlinear phenomenon, it is important to revisit the impacts of nonlinearity in protein binding on clinical pharmacokinetics and pharmacodynamics, and ultimately, clinical efficacy. The presented analyses using tigecycline as an example to demonstrate the need for further studies into this phenomenon, its underlying mechanism and impact on dosing across patient populations. The discovery of the atypical protein binding phenomenon also presents the promise of potential opportunity to exploit underlying mechanisms to develop new therapies and better understand molecular interactions of xenobiotics within the physiological system.
Table 1-1. Pertinent definitions and examples of different binding phenomenon.

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical concentration range</td>
<td>A concentration range which is clinically observable or expected for a given regimen</td>
<td>When X mg of drug A is given to a patient of interest, observable in vivo concentrations range from 0 to XX mg/L at the site where protein binding is being assessed (typically, the plasma)</td>
</tr>
<tr>
<td>Linear protein binding</td>
<td>- $f_u$ remains constant over a clinical concentration range</td>
<td>- Most drugs</td>
</tr>
<tr>
<td>Nonlinear protein binding</td>
<td>- $f_u$ does not remain constant over a clinical concentration range</td>
<td></td>
</tr>
</tbody>
</table>
| Typical Nonlinear protein binding     | - A subclassification of nonlinear binding  
- Protein is saturated and $f_u$ increases with total concentration (Langmuir model)  
- Often what is being referred as “nonlinear protein binding” or “concentration-dependent protein binding”                                                                                                                                                                                                                                           | - Most drugs exhibit this behavior at some concentration range (may or may not be clinical)  
- disopyramide$^{39}$  
- ceftriaxone$^{40}$  
- valproic acid$^{41}$  
- eplerenone$^{42}$  
- linagliptan$^{43}$  
- trandolaprilat$^{44}$                                                                                                        |
| Atypical Nonlinear protein binding    | - A subclassification of nonlinear binding  
- Any nonlinear behavior which does not follow typical saturation/Langmuir model                                                                                                                                                                                                                                                                                                              | - doxycycline$^{13}$  
- minocycline$^{13}$  
- tigecycline$^{7,23}$  
- eravacycline$^{10,11}$  
- TP-271$^{12}$                                                                                                                      |
Table 1-2. Simulated PK changes caused by nonlinear plasma and tissue protein binding. *During the simulations of Øie and colleagues\textsuperscript{20} changes in influential parameters impacted subsequent parameters differently depending on the relative changes in other parameters. For those with an asterisks, simulated results are listed but theoretically under different conditions the results may differ.

<table>
<thead>
<tr>
<th></th>
<th>f\textsubscript{uP}</th>
<th>f\textsubscript{uT}</th>
<th>CL\textsubscript{int}</th>
<th>V\textsubscript{d}</th>
<th>f\textsubscript{uP}</th>
<th>f\textsubscript{uT}</th>
<th>V\textsubscript{d}</th>
<th>CL</th>
<th>k\textsubscript{e}</th>
<th>t\textsubscript{1/2}</th>
<th>C\textsubscript{p}</th>
<th>C\textsubscript{u}</th>
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<tr>
<td><strong>Typical Nonlinear</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear Nonlinear</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>↓</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
<td>↓*</td>
<td>↑*</td>
<td>Convex*</td>
<td>Concave*</td>
<td>Concave*</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>↓</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
<td>-*</td>
<td>-*</td>
<td>Convex*</td>
<td>Linear*</td>
<td>Linear*</td>
</tr>
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<td></td>
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<td>-</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>-*</td>
<td>↑*</td>
<td>Concave*</td>
<td>Concave</td>
<td>Concave</td>
</tr>
<tr>
<td>High</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td>-</td>
<td>↓</td>
<td>↑*</td>
<td>-*</td>
<td>Concave*</td>
<td>Concave*</td>
<td>Concave*</td>
</tr>
</tbody>
</table>

26
Figure 1-1. Illustration of different types of nonlinear protein binding.
Figure 1-2. Observed “U-shaped” protein binding behavior of tigecycline (Mean±SD). Data from Mukker et al.\textsuperscript{23} and Dorn et al.\textsuperscript{9} HSA: human serum albumin
Figure 1-3. Effects of changing plasma protein binding on different PK parameters over a total concentration range with atypical (blue) and linear (orange) plasma protein binding.
Figure 1-4. Cumulative total and free exposure (CAUC and fCAUC) for each dosing regimen under atypical (blue) and linear (orange) plasma protein binding scenarios.
CHAPTER 2
ENHANCED IN VITRO TIGECYCLINE ACTIVITY IN THE PRESENCE OF CHELATING AGENTS¹

Introduction

Given the paucity of new antibiotic approvals and increase in antibiotic resistance, clinicians repeatedly turn to the use of antibiotic combinations to expand antibiotic spectrums or enhance activity of existing agents through novel mechanisms. Often the interactions with another agent have been exploited to improve the efficacy of therapeutic agents. These interactions could be pharmacokinetic or pharmacodynamic in nature and should be characterized appropriately to determine the true potential clinical impacts. A pharmacokinetic interaction would affect the active drug concentration available to affect a target. Conversely, a pharmacodynamic interaction would impact the ability of the drug to kill or inhibit growth of bacteria. A pharmacodynamic interaction may also impact the nature of the interaction between the bacteria and the drug. Separate from this, pharmacodynamic effects of other drugs or chemicals may, independent of the drug of interest, impact the overall effect.

Tetracyclines are known to chelate metal ions but our group has recently investigated the interaction of metal ions with the extent of plasma protein binding of tigecycline (TIG) which exhibits atypical nonlinear plasma protein binding (i.e. decrease in free fraction as total concentrations increase followed by an increase in free fraction at highest concentrations)²³, a phenomenon also observed with other tetracyclines¹¹,¹³. Thinking of what potential interaction TIG may have in vitro with chelators, a pharmacokinetic interaction may occur due to the competition of chelators for metal ions, allowing for more unchelated drug, which may lead to enhanced activity. On the other hand, the addition of chelators may also lead to a

¹ Chapter 2 is currently under review by the International Journal of Antimicrobial Agents.
pharmacodynamic interaction in which the drug behaves differently against one or more target sites, exhibits different maximal antibacterial activity, or a different concentration-effect relationship as it pertains to the killing or inhibition of bacterial proliferation. Independent of these interaction aspects, a separate pharmacodynamic effect may also be exhibited by the addition of a chelator, for example, the chelation of metal ions may inhibit bacterial proliferation independent of drug effect.

Our investigation explores the interactions of chelating agents with the in vitro activity of tigecycline.

**Materials and Methods**

**Chemicals, Bacteria, Equipment and Stock Solutions**

Ethylendiaminetetraacetic acid (EDTA) and sodium chloride were purchased from Fisher Scientific (Pittsburgh, PA). Tigecycline was obtained from TSZ CHEM (Framingham, MA). Tetracycline, and trimethoprim were obtained from Medisca Incorporated (Plattsburgh, New York). Oxytetracycline, chlortetracycline, and magnesium chloride were obtained from Sigma-Aldrich Corporation (St. Louis, MO). Calcium chloride was obtained from Allied Chemical Corporation (Morristown, NJ) and Fisher Scientific (Pittsburgh, PA). Bacterial dispersions in normal saline were prepared using the A-JUST Turbidimeter (Abbott Laboratories) along with McFarland Turbidity Standards (Remel Microbiology Products, Lenexa, KS). Cation-adjusted Mueller Hinton Broth was obtained from Becton, Dickinson and Company (Sparks, MD). *P. aeruginosa* ATCC® 27853 and a tetracycline-resistant methicillin-resistant *S. aureus* isolate were provided by UF Health Microbiology Laboratory (Gainesville, FL), and clinical isolates *E. coli* ARC3600 (NDM-1, CMY-6, OXA-1) and *K. pneumoniae* ARC3802 (NDM-1, SHV-2a, SHV-11, CTX-M-15, TEM-1) were provide by JMI Laboratories (North Liberty, IA).
**MIC Determination**

MICs were determined by serial macrodilution method (total volume = 1 mL) in culture media (prepared fresh weekly). Various chemical components in the following experiments were incubated with the bacteria (1.5 x 10⁶ CFU/mL) of interest. The 24-well plates were incubated at 37°C for 16-24 h prior to visual MIC determination where the lowest concentration with no visible growth was considered the MIC. Negative controls without bacteria were included to confirm absence of bacterial contamination in broth and stock solutions. Positive controls with bacteria were also included with and without inactive components.

**Effect of Metal Ion Chelation on in Vitro MICs of TIG and TET**

The impacts of metal ion chelation on *in vitro* activity was determined by the addition of Mg²⁺ or Ca²⁺ and/or EDTA to MIC assays for TIG and TET against various bacteria. TIG MIC assays were performed for *E. coli*, *P. aeruginosa*, and *K. pneumoniae*. MICs were determined with and without divalent metal ions, 4.2 mM Mg²⁺ and 10.4 mM Ca²⁺, and/or 1.5 mg/mL EDTA. *P. aeruginosa* and *E. coli* TIG MICs were also assessed in the presence of 0.65, 1.3, 2.6, and 5.2 mM Ca²⁺. *P. aeruginosa* TIG MICs were performed in the presence of additional EDTA concentrations (0.094, 0.19, 0.38, and 0.75 mg/mL) and additional Ca²⁺/EDTA combinations (0.65 and 2.6 mM Ca²⁺, with 1.5 mg/mL EDTA). TET MICs assays were performed for *P. aeruginosa* with and without the additions of varying levels of Ca²⁺ (0.65, 1.3, 2.6, 5.2, and 10.4 mM) and/or EDTA (0.094, 0.19, 0.38, 0.75, 1.5 mg/mL). Trimethoprim (TMP) was selected as a negative control drug to test against *P. aeruginosa*, as it is not subject to metal ion chelation. TMP MICs were determined in the presence and absence of 1.5 mg/mL EDTA and/or varying levels of Ca²⁺ (0.65, 1.3, 2.6, 5.2, and 10.4 mM).
TIG Interactions with Tetracyclines

Oxytetracycline (OXY), Chlortetracycline (CHL) and TET

Tigecycline MIC was tested in combination with several other tetracyclines (CHL, OXY, and TET) at concentrations below their determined MICs against *P. aeruginosa*, *E. coli*, and *K. pneumoniae* to determine if tetracyclines, which also act as chelators exhibit similar effects to EDTA.

Impact of TET on TIG MICs

The relationship between TIG and TET against *P. aeruginosa*, *E. coli*, and MRSA (clinical TET-resistant isolate) were examined further. Varying levels of TET (4, 8, and 12 mg/L) below the MIC (16-32 mg/L) were added to TIG MIC assays. When TET led to a decrease in TIG MIC, the effects of adding 10.4 mM Ca$^{2+}$ in combination with TET to TIG MIC assays were also assessed.

Results

Effect of Calcium Chelation on *in Vitro* MICs of TIG and TET

The effects on TIG MIC for all three bacterial pathogens are summarized in Table 1. While increased MICs are observed for all bacteria when Ca$^{2+}$ or Mg$^{2+}$ are added, the increase is most drastic with Ca$^{2+}$ addition. The addition of EDTA alone led to a decrease in MICs for all bacteria. While this effect could be reversed by the addition of divalent metal ions, the reversal was most potent when adding Ca$^{2+}$. The MICs with varying Ca$^{2+}$ levels for *P. aeruginosa* and *E. coli* are described in Table 2, and results for MICs in combination with varying EDTA with or without Ca$^{2+}$ for *P. aeruginosa* are shown in Table 3. Dose-related increases in MIC with increasing levels of Ca$^{2+}$ added were observed for both *E. coli* and *P. aeruginosa*. Dose-related decreases in MIC with increasing levels of EDTA added were observed for *P. aeruginosa*. A
dose-dependent MIC increase, reversing the effects of EDTA by adding increasing levels of Ca\(^{2+}\), was also observed for *P. aeruginosa*.

Results for TET MIC experiments are displayed in Table 3. Tetracycline exhibits a similar dose-dependent increase in MIC with increasing Ca\(^{2+}\) concentrations and decrease in MIC with increasing EDTA concentrations. The MIC lowering effect of EDTA can also be reversed with the addition of Ca\(^{2+}\).

Results of TMP MICs are shown in Table 3. The addition of EDTA led to a drop in MIC, while the addition of increasing levels of Ca\(^{2+}\) reversed the effects of EDTA, similar to those trends seen for TIG and TET.

**Interactions with Tetracyclines**

**Impact of OXY, CHL, and TET on TIG MICs**

MIC were first determined for OXY, CHL and TET against *P. aeruginosa*, *K. pneumoniae*, and *E. coli*. For *P. aeruginosa* MICs for OXY, CHL, and TET were determined to be 8, 16, and 16-32 mg/L, respectively. For *K. pneumoniae* MICs were determined to be 2-4, 4, and 2-4 mg/L for OXY, CHL, and TET, respectively, while for *E. coli*, MICs for all three tetracyclines were greater than 32 mg/L. When TIG MIC was examined in combination with concentrations of each tetracycline below its MIC, no significant changes in MICs were observed for *K. pneumoniae* or *E. coli*, with the exception of a drop in MIC from 1-2 (TIG alone) to 0.25-0.5 mg/L when TET 1 mg/L or CHL 2 mg/L was added against *K. pneumoniae*. Conversely decreases in TIG MIC were observed for all added tetracyclines against *P. aeruginosa* with decreases from 8-16 mg/L (TIG alone) to 2-4, 4, and 2-8 mg/L in the presence of 4 mg/L OXY, 8 mg/L CHL, and 8 mg/L TET, respectively.
Impact of TET on TIG MICs

Table 4 shows the resulting changes in TIG MICs in the presence of TET with or without Ca\(^{2+}\). There was a TET dose-related decrease in TIG MIC for \(P.\ aeruginosa\) but not for \(E.\ coli\) or MRSA. The addition of Ca\(^{2+}\) to the TIG MICs in the presence of TET led to an increase in the TIG MIC, reversing the effect of TET.

Discussion

These results show how a metal ion chelator, such as EDTA, leads to a decrease in TIG MIC which can be reversed by the addition of divalent metal ions, with Ca\(^{2+}\) having a particularly potent effect for \(E.\ coli, K.\ pneumoniae,\) and \(P.\ aeruginosa\). For \(E.\ coli\) and \(P.\ aeruginosa\) the dose-dependent effects of calcium on TIG MIC were demonstrated. The EDTA-dose dependent decrease in TIG MIC and Ca\(^{2+}\)-dependent reversal of this decrease were characterized for \(P.\ aeruginosa\). While there are no physiological levels of EDTA, total serum calcium is typically 2.2-2.6 mM, which was covered by the range of calcium used (only 0.5-0.62 mM Ca\(^{2+}\) is present in the media). Similar effects were observed for a similar set of experiments for TET against \(P.\ aeruginosa\), another calcium chelator.

Since EDTA is not a chelator that can be administered clinically, other tetracyclines, also with metal ion chelating properties, were tested, including TET, OXY and CHL. Interestingly, significant decreases in the MIC of TIG in the presence of other tetracyclines were mainly observed for \(P.\ aeruginosa\). The effects of TET with TIG were investigated further. An effect was observed for \(P.\ aeruginosa\) but not for \(E.\ coli\) or MRSA. Dose-dependent effects of TET on TIG \(P.\ aeruginosa\) MIC were evaluated at concentrations below the TET MIC (16-32 mg/L) and the reversal of these effects were demonstrated by adding Ca\(^{2+}\). These results exhibit the same trends as that seen with EDTA.
One hypothesis is that TIG and TET may compete for calcium, which may grant enhanced effects in combination. As to why these effects of TET may not be observed for other bacteria tested, there are many possibilities, some of which are discussed:

- One possibility is that TIG may be approaching its maximum effect without the addition of a calcium chelator. As TIG already has activity against *E. coli* and *K. pneumoniae*, the window for which an increase in microbiological activity could occur may be smaller than for *P. aeruginosa*.

- The other bacteria tested may not be as dependent on calcium for bacterial proliferation. In this case, the addition of a chelator may not have a profound effect, except with a more complete calcium chelation, like the one provided by the addition of EDTA.

- The effects on MIC caused by a chelating agent may not be attributable only to the competitive chelation of TIG. For example, competition for efflux pumps (both TIG and TET are subject to efflux pumps\(^n\)), and/or the ribosomal target site may exist.

The second point was partially confirmed by the series of TMP experiments, where a drug with some activity against *P. aeruginosa*, not known to be subject to chelation, was selected. Similar trends observed for TMP, TIG, and TET when calcium was added and removed supports that a pharmacodynamic effect independent of a drug being subject to calcium chelation exists. These effects were more drastic for TIG as compared to TMP, which may be attributed to a greater maximum effect of TIG, or a concurrent interaction (i.e. a pharmacokinetic interaction) not present with TMP.

Metals such as Ca\(^{2+}\) and Mg\(^{2+}\) are important in biological processes. The impacts of EDTA on bacterial inhibition and enhancing antimicrobial activity have been previously observed\(^{46-48}\). The effects on Ca\(^{2+}\) and other cations on biofilm formation and attachment for *Pseudomonas spp.* have been extensively studied\(^{49-51}\). Mg\(^{2+}\) binding by extracellular DNA and Ca\(^{2+}\) binding alginate (a biofilm component) have been associated with induced resistance genes in *P. aeruginosa*\(^{52,53}\). Interestingly for *P. aeruginosa* Ca\(^{2+}\) seems to have a more substantial
impact on binding to alginate than Mg$^{2+}$. Perhaps the planktonic effects of chelation by EDTA also occur in the case of TET and other Ca$^{2+}$ chelators.

These results merit further investigations into the impact of metal ion chelators on antibacterial activity specifically as it pertains to TIG and *P. aeruginosa*. The combination of TIG with other tetracyclines is unique and surprising in that drug combinations often consist of two drugs of different mechanisms and of different therapeutic classes. Full characterization of the TIG-TET interaction linked with clinical pharmacokinetic models could provide valuable insights into the design of physiologically viable chelators as candidates for future combination regimens and underlying mechanisms could be exploited to develop novel antibacterial agents.

**Conclusion**

This work has shown the potential for enhanced *in vitro* activity of TIG in the presence of metal ion chelation. This enhancement was reversible by the addition of metal ions, in particular Ca$^{2+}$ for which the dose-dependent reversal was observed. A similar phenomenon was also observed for TMP for *P. aeruginosa*, supporting a separate pharmacodynamic mechanism. Using other tetracyclines in place of EDTA, TIG’s activity was increased primarily against *P. aeruginosa*. These experiments support further exploration of the underlying mechanisms and the use of chelators in combination with antimicrobials to improve the efficacy.
Table 2-1. Tigecycline MIC results with and without divalent metal ions and/or EDTA.

<table>
<thead>
<tr>
<th>Added Component</th>
<th>P. aeruginosa</th>
<th>E. coli</th>
<th>K. pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8-16</td>
<td>0.5</td>
<td>0.25-0.5</td>
</tr>
<tr>
<td>4.2 mM Mg$^{2+}$</td>
<td>&gt;32</td>
<td>2-4</td>
<td>1-2</td>
</tr>
<tr>
<td>10.4 mM Ca$^{2+}$</td>
<td>&gt;64</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>1.5 mg/mL EDTA</td>
<td>0.0313-0.0625</td>
<td>&lt;0.015</td>
<td>0.125</td>
</tr>
<tr>
<td>1.5 mg/mL EDTA + 4.2 mM Mg$^{2+}$</td>
<td>0.5</td>
<td>2-4</td>
<td>1-2</td>
</tr>
<tr>
<td>1.5 mg/mL EDTA + 10.4 mM Ca$^{2+}$</td>
<td>&gt;=64</td>
<td>4</td>
<td>4-8</td>
</tr>
</tbody>
</table>
Table 2-2. TIG MIC results for varying levels of Ca\(^{2+}\).

<table>
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<tr>
<th>mM Ca(^{2+}) Added</th>
<th>P. aeruginosa</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.65</td>
<td>16</td>
<td>0.5</td>
</tr>
<tr>
<td>1.3</td>
<td>16-32</td>
<td>2</td>
</tr>
<tr>
<td>2.6</td>
<td>&gt;=64</td>
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</tr>
<tr>
<td>5.2</td>
<td>&gt;64</td>
<td>&gt;32</td>
</tr>
</tbody>
</table>
Table 2-3. TIG, TET, and TMP MIC results for varying levels of EDTA with or without Ca\(^{2+}\).

<table>
<thead>
<tr>
<th>mg/mL EDTA Added</th>
<th>mM Ca(^{2+}) Added</th>
<th>TIG MIC (mg/L)</th>
<th>TET MIC (mg/L)</th>
<th>TMP MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>8-16</td>
<td>16-32</td>
<td>1024</td>
</tr>
<tr>
<td>0</td>
<td>0.65</td>
<td>16</td>
<td>32</td>
<td>ND</td>
</tr>
<tr>
<td>0</td>
<td>1.3</td>
<td>16-32</td>
<td>32</td>
<td>ND</td>
</tr>
<tr>
<td>0</td>
<td>2.6</td>
<td>&gt;64</td>
<td>32-64</td>
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<tr>
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<td>&gt;128</td>
<td>ND</td>
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<tr>
<td>0</td>
<td>10.4</td>
<td>&gt;64</td>
<td>&gt;128</td>
<td>1024-2048</td>
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<td>0</td>
<td>4-8</td>
<td>8-16</td>
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<td>0.5</td>
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<td>0</td>
<td>0.0625-0.125</td>
<td>0.5</td>
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<tr>
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<td>0.0313-0.0625</td>
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<td>64-128</td>
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<td>&lt;0.0313-0.0625</td>
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<td>64-128</td>
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<td>0.125</td>
<td>0.5</td>
<td>256</td>
</tr>
<tr>
<td>1.5</td>
<td>5.2</td>
<td>ND</td>
<td>ND</td>
<td>1024</td>
</tr>
<tr>
<td>1.5</td>
<td>10.4</td>
<td>&gt;=64</td>
<td>32-128</td>
<td>&gt;=2048</td>
</tr>
</tbody>
</table>
Table 2-4. TIG MIC results for varying sub-MIC levels of TET with or without Ca\textsuperscript{2+}. ND: not done

<table>
<thead>
<tr>
<th>mg/L TET Added</th>
<th>mM Ca\textsuperscript{2+} Added</th>
<th>P. aeruginosa</th>
<th>E. coli</th>
<th>MRSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>8-16</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>2-8</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0.5-2</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>10.4</td>
<td>&gt;32</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>10.4</td>
<td>&gt;32</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>10.4</td>
<td>32</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
CHAPTER 3
CHARACTERIZATION OF TIGECYCLINE AND TETRACYCLINE PLASMA PROTEIN BINDING

Introduction

The free, unbound drug is that which exerts pharmacological activity. While changes in plasma protein binding do not always have clinical implications (e.g. in cases of low protein binding)⁴,⁵, they are especially important to consider in cases of high protein binding, narrow therapeutic index drugs, and nonlinear total-free concentration relationships. To most accurately describe the true concentration-effect relationship the free concentration should be utilized whenever possible. Given the complex nature of tigecycline plasma protein binding⁸,⁹,²³, and similar observed phenomenon in other drugs in the tetracycline class¹⁰,¹³,²⁷,³⁴, it is imperative that the nature of tigecycline and tetracycline binding be fully characterized in all media of interest. In addition, such experiments may inform any binding interaction between the two drugs and calcium.

Tigecycline plasma protein binding has only been described at six concentrations between 0.1 and 100 mg/L. While this may be sufficient for typical nonlinear PPB, given the concentration-dependent increase, followed by a decrease in binding at higher concentrations (“U-shaped” phenomenon), shown in Figure 1-2⁹,²³, further characterization is desirable. The previous studies also showed that when divalent metal ions are removed via chelation, protein binding is linearized and unbound concentration increases⁸,⁹. The dose-dependent effect of calcium on the binding of tigecycline should also be further described. Together one could develop a model to predict free drug concentration given a known calcium concentration (e.g. in an experimental setting, at a site of infection, or in plasma).
Tetracycline binding has not been thoroughly investigated and it is not clear if it is subject to the same atypical phenomenon recently described for other drugs in its pharmacological class\textsuperscript{13,34}.

Overall, these experiments aim to develop an LC-MS/MS method for simultaneous quantification of tigecycline and tetracycline in saline and plasma, provide a more detailed description of tigecycline and tetracycline’s plasma protein binding over a wide range of concentrations.

**Methods**

**Materials**

Tigecycline was obtained from TSZ CHEM (Framingham, MA). Tetracycline hydrochloride was purchased from Medisca Incorporated (Plattsburgh, New York) and oxytetracycline was purchased from Sigma-Aldrich Corporation (St. Louis, MO). Ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), sodium chloride, acetonitrile (ACN), methanol (MeOH), and formic acid were obtained from Fisher Scientific (Pittsburgh, PA). Calcium chloride was obtained from Allied Chemical Corporation (Morristown, NJ) and Fisher Scientific (Pittsburgh, PA). Pooled (n>6 individuals from both genders) heparinized unfiltered human plasma was purchased from Bioreclamation LLC (Westbury, NY). Ultrafiltration cartridges were purchased from EMD Millipore (Billerica, MA).

**Bioanalytical Assay Development**

**Instrumentation and Chromatographic Conditions**

A gradient LCMS-MS method for tigecycline quantification in dialysate was previously developed\textsuperscript{23}. The assay developed and equipment used for simultaneous tigecycline and tetracycline quantification in saline and plasma was the same except for the use of a reverse-phase Waters Symmetry C18 Column (3.9 mm x 150 mm, 5 μm) without guard column and
absence of 1:1 mobile phase split before electrospray ionization (ESI). Briefly, the assay chromatographic separation was achieved using a gradient of 0.5% formic acid in water and 0.5% formic acid in 50:50 MeOH:ACN over 10 minutes (flow rate 0.8 mL/min).

**Stock and Standard Solutions Preparation**

Primary stock solutions were weighed separately for calibration curves and quality control samples. The primary stock solution of tigecycline (10 mg/mL) was prepared in 50:50 DMSO:ACN while the primary stock solution of tetracycline (1 mg/mL) was prepared in triple distilled water (TDW). A secondary stock of tigecycline (1 mg/mL) was prepared by diluting the primary stock with ACN. A combined stock of tigecycline and tetracycline together was prepared from the secondary tigecycline and primary tetracycline stocks to result in a stock of 100 µg/mL of tigecycline and tetracycline each. Combined tigecycline/tetracycline working stock solutions (0.1, 0.2, 0.5, 1, 2, 5, 7 and 10 µg/mL) were prepared using ACN. The calibration standards of tigecycline/tetracycline in ACN were in turn prepared from these working stocks. Quality control working stocks (lower limit-quantity control (LLOQ), low-quality control (LQC), middle-quality control (M1QC), 2nd middle-quality control (M2QC) and high-quality control (HQC)) were 0.1, 0.3, 2, 4, and 8 µg/mL, respectively. A quaternary stock solution of oxytetracycline (internal standard, IS) (0.5 µg/mL) was prepared in ACN by diluting the primary stock solution of oxytetracycline (1 mg/mL in ACN) several times. All stocks were stored at −80°C.

**Calibration and QC Standards Preparation**

Calibration standards and quality control standards were prepared by adding 10 µL combined tigecycline/tetracycline working stock solution to 90 µL of ACN. An addition 400 µL of IS working stock (0.5 µg/mL) was subsequently added to each sample prior to analysis. QC
samples (10, 30, 200, 400, and 800 ng/mL) were also prepared in a similar manner to measure for precision and accuracy.

**Validation Procedure**

Based on the FDA guidelines, a partial validation of the tigecycline and tetracycline bioanalytical assay was performed. Method specificity was evaluated in LLOQ samples in plasma and saline. The ratio of the peak area for tigecycline and tetracycline to the peak IS area was plotted versus the expected concentrations of tigecycline and tetracycline (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 0.7, and 1 µg/mL) and fitted using linear regression with 1/x² weighting. The coefficient of correlation should be at least 0.995. Six QC replicates on three different days were used to determine precision and accuracy across and within days with an accuracy acceptance criteria ±15% of the standard deviation from the expected value for the highest 4 QCs and ±20% for the LLOQ. Coefficients of variations should be less than 15% for all QCs except at LLOQ (< 20%).

**Plasma Protein Binding Determination**

Plasma protein binding experiments will be performed for total concentrations ranging from 100 to 200,000 ng/mL of tigecycline. Select concentrations will be evaluated in the presence of tetracycline 12 mg/L, EDTA (0.75 or 1.5 mg/mL), and/or calcium (2.6 or 10.4 mM). Plasma is thawed in an ice bath and centrifuged for 10 min at 3,800 x g, to remove precipitated fibrin. Plasma is then placed in a 37°C water bath. Prepare plasma samples and place in ultrafiltration cartridges. Centrifuge the cartridges in the pre-warmed centrifuge for 5-6 min at 1,000 g. Sample from the filtrate and original plasma sample for analysis.
Results

LC–MS/MS Method Development and Validation

Ideal gradient conditions for chromatographic separation were previously described. Tigecycline, tetracycline, and IS retention times were 3.65, 5.91, and 5.89 min, respectively. Manual tuning of mass spectrometry parameters for tigecycline, tetracycline and oxytetracycline was performed on an API 4000 with ESI source. Q1/Q3 were 586.3/569.2 product ions for the tigecycline, 445.4/410.3 for tetracycline, and 461.4/426.4 for oxytetracycline. Quadrupoles Q1 and Q3 were set on unit resolution. Optimal values of declustering potential (DP), entrance potential (EP), collision energy (CE), and collision exit potential (CXP) for highest signal intensity were determined by ramping in MRM mode. The optimum values for DP, EP, CE, and CXP were 112, 10, 29, and 15 V for tigecycline, 37, 9, 25, and 19 V for tetracycline, and 80, 10, 25, and 15 V for oxytetracycline, respectively. Nitrogen served as curtain and collision gas, and zero air was used as a source gas. Source temperature was 500°C and operated in ESI-positive ion mode, while ion spray voltage was 5500 V. Nebulizer gas (GS1), turbo heater gas (GS2), curtain gas (CUR), and collision-activated dissociation gas were 40, 40, 20, and 5 psi, respectively. Data was acquired and processed using the analyst software version 1.4.1 (AB SCIEX). Method validation is underway.

Plasma Protein Binding Determination

Protein binding experiments are pending method validation.
CHAPTER 4
AN INNOVATIVE APPROACH TO MECHANISTIC ANALYSIS: USE OF GLOBAL SENSITIVITY ANALYSIS TO INFORM HYPOTHESES

Introduction

In research, we often do not have the opportunity to know and understand an interaction on a molecular level, but rather, are informed by an indirect measurement of some outcome that results from an underlying mechanism. For instance, in the case of tigecycline’s atypical nonlinear plasma protein binding, the indirect measurement of this unusual phenomenon, is unbound fraction, $f_u$. Here we propose the application of global sensitivity analysis to physiologically-informed hypothesized mechanisms of tigecycline’s plasma protein binding to better understand potential drivers on $f_u$ and to guide future investigations.

A recent publication by Singh and colleagues used local sensitivity analysis to investigate whether experimental results can be observed with various hypothesized mechanisms. Sensitivity analyses are typically performed to determine if the outcome of interest (often a calculated value from a model) is sensitive to changes in a particular parameter. Often used in pharmacometrics to determine that the value of a parameter is meaningful to the predictions of the model, Singh and colleagues utilized the tool to determine if a set or sets of parameters for a given mechanism could describe experimental results of protein binding experiments for which the full mechanism is not fully understood.

This analysis is deemed “local” in that only one parameter is perturbed at a time. Local sensitivity analyses can help determine the effect of a parameter on the model outcome of interest, in that case, the apparent unbound fraction of tigecycline (TIG). It does not account for interactions between parameters since only one parameter is changed at a time in local sensitivity analysis. Global sensitivity analysis varies all parameter values simultaneously over their entire ranges and can evaluate parameter interactions. While changes in multiple parameters are more
difficult to visualize, statistical indices, known as sensitivity indices, can be calculated by
determining the proportion of the total variation in the outcome due to changes in a particular
parameter on its own or in combination with other parameters.

Sobol’s method is a tool that can be used in global sensitivity analysis to compute
sensitivity indices by a decomposition of variance, where the sensitivity of the model output is
the sum of the contribution of each parameter \((x_i, i=1,2,..,s)\) and its interaction with other
parameters (Equation 4-1)\(^{56,58}\).

\[
f(x) = f_0 + \sum_{i=1}^{s} f_i(x_i) + \sum_{i=1}^{s} \sum_{i \neq j}^{s} f_{ij}(x_ix_j) + \ldots f_{1\ldots s}(x_1,x_2,\ldots,x_s) \quad (4-1)
\]

This sensitivity can be decomposed into individual and interaction components
(Equations 4-2 and 4-3)\(^{56}\).

\[
f_i(x_i) = \int f(x) \prod_{k \neq i} dx_k - f_0 \quad (4-2)
\]

\[
f_{ij}(x_i,x_j) = \int f(x) \prod_{k \neq i,j} dx_k - f_0 - f_i(x_i) - f_j(x_j) \quad (4-3)
\]

and so on, for every additional factor.

This can then be transformed into Equation 4-4 and partial variances \(D_{1,\ldots,s}\) can be
computed.

\[
D = \sum_{i=1}^{k} D_i + \sum_{i<j} D_{ij} + \sum_{i<j<l} D_{ijl} + \ldots + D_{1,2,\ldots,k} \quad (4-4)
\]

From this, a Sobol sensitivity index, \(S\), can be calculated for a given set of parameters.

\[
S_{i_1 \ldots i_s} = \frac{D_{i_1\ldots i_s}}{D} \quad (4-5)
\]
This work aims to inform the analyses of Singh et al. by use of global sensitivity analysis as a tool that may account for interactions between terms and allow for variation of all parameters simultaneously, as well as incorporate new findings in a live progressing investigation of tigecycline’s complex plasma protein binding mechanism. This not only serves to narrow our investigation into this mechanism by informing hypotheses, but also, to the best of our knowledge, serves a first example of the novel application of global sensitivity analysis to be utilized in qualitative mechanistic investigations.

**Methods**

Sobol’s method using the Saltelli scheme\(^5\), specifically the “sensitivity” package (version 1.13.0) in R (version 3.3.2), was used to determine the decomposition of variance and calculate total and first order sensitivity indices. Simulations (N=10,000) were executed with a set seed and 100 bootstrap replicates.

Analyses were conducted in three phases: empiric models (I), mechanistic expansion (II), and data-informed models (III) (Figure 4-1). Empiric models are those with very little data about the mechanism. Based on a known interaction with calcium, several putative mechanisms were developed\(^8\). In Phase II, these putative mechanisms were then expanded integrating more mechanism components (e.g. the potential for multiple binding sites with different binding relationships, change in existing binding relationships). Based on Phases I and II, relationships including parameters identified as key drivers (based on a total sensitivity index confidence interval completely above a 0.05 threshold) were retained in Phase III mechanisms. In addition, Phase III implemented additional experimental data from published literature.

For all models, the total concentration (\(C_T\)) is the sum of all bound (\(C_b\)) and unbound (\(C_u\)) TIG concentrations (Equation 4-6). PP, MI, and complex refer to plasma protein, metal ion and a
given complex of different components (i.e. PP-MI or MI-TIG binding to TIG or PP, respectively), respectively.

\[ C_T = C_u + C_{b,PP} + C_{b,MI} + C_{b,complex} \]  

(4-6)

The fraction unbound \( f_u \) can be calculated by dividing the apparent unbound concentration with the total concentration (Equation 4-7). Since chelated TIG will pass through filtration/dialysis membranes during protein binding experiments, when present the apparent unbound concentration will include concentrations of TIG bound to metal-ions \( (C_{b,MI}) \).

\[ f_u = \frac{C_u + C_{b,MI}}{C_T} \]  

(4-7)

\( A_{\text{max}} \) represents the maximum capacity of a binding site for a substrate (often TIG) and \( K_D \) represents the dissociation constant for a given binding relationship. Additional subscripts (i.e. PP, MI, PP-MI) denote the binding site. \( \gamma \) describes cooperatively factors for a given binding relationship.

Unbound fractions of tigecycline \( C_u \) were simulated in the range of 0.1 to 100 \( \mu \text{g/mL} \), where atypical nonlinear PPB can be observed\(^{23}\).

**Phase I: Empiric Models**

Empiric models were based on those developed by Singh et al, simplistically represented in Figure 4-2. Briefly, in Model I, TIG binds independently to plasma proteins (PP) (Equation 4-8), and forms chelating complexes with divalent MI in the plasma (Equation 4-9).

\[ C_{b,PP} = \frac{A_{\text{max},PP} \times C_u^{\gamma_{PP}}}{K_{D,PP}^{\gamma_{PP}} + C_u^{\gamma_{PP}}} \]  

(4-8)

\[ C_{b,MI} = \frac{A_{\text{max},MI} \times C_u}{K_{D,MI} + C_u} \]  

(4-9)
In Model II, TIG binds independently to PP (Equation 4-8), MI (Equation 4-9), and MI-PP complexes (Equation 4-10).

\[
C_{b,\text{complex}} = \frac{A_{\text{max,complex}} \times C_u^{\gamma_{\text{complex}}}}{K_{D,\text{complex}}^{\gamma_{\text{complex}}} + C_u^{\gamma_{\text{complex}}}}
\]  

(4-10)

In Model III, TIG independently binds to PP and MI, and TIG-MI complexes subsequently bind to PP (Equation 4-11).

\[
C_{b,\text{complex}} = \frac{A_{\text{max,complex}} \times C_{b,MI}^{\gamma_{\text{complex}}}}{K_{D,\text{complex}}^{\gamma_{\text{complex}}} + C_{b,MI}^{\gamma_{\text{complex}}}}
\]  

(4-11)

In Model IV, MI enhances binding of TIG to PP.

Given the little information available concerning the binding nature of TIG at the time of plasma protein binding experiments, potential ranges for parameter values were set based on literature values. For example the maximum binding capacity of PP for TIG was based on typical plasma protein concentrations\(^{60}\) (60-80 g/L) and the potential number of binding sites\(^{61}\) (1-8 sites/protein). Similarly a range for \(A_{\text{max,MI}}\) was set based on typical unbound physiological calcium concentrations\(^{62}\) as well as the number of potential binding sites for tetracyclines (3 sites/calcium ion). Dissociation constant (\(K_D\)) ranges (0.001-100 µM) were set based on several papers citing typical \(K_D\) for protein binding to drugs\(^{61,63}\). The cooperativity factor \(\gamma\) was set between 0.5 and 5.

**Phase II: Mechanistic Expansion**

Two possibilities were explored for both models I, II and III. First, two binding sites having different \(K_D\) values for MI binding of TIG were added to the models (Equations 4-12, 4-13, and 4-14). In a similar manner, a third binding site for this model was also explored.

\[
C_{b,MI} = C_{b,MI,1} + C_{b,MI,2}
\]  

(4-12)
\[
C_{b,MI,1} = \frac{A_{\text{max},MI,1} \times C_u}{K_{D,MI,1} + C_u} \quad (4-13)
\]

\[
C_{b,MI,2} = \frac{A_{\text{max},MI,2} \times C_u}{K_{D,MI,2} + C_u} \quad (4-14)
\]

Alternatively, rather than incorporating multiple binding sites for the MI-TIG binding relationship, a Hill coefficient \((\gamma_{MI})\) was added to the existing model to allow for a more flexible binding relationship (Equation 4-15).

\[
C_{b,MI} = \frac{A_{\text{max},MI} \times C_u\gamma_{MI}}{K_D^{\gamma_{MI}} + C_u^{\gamma_{MI}}} \quad (4-15)
\]

Expanding Model II, the nature of the formation of protein-MI (PP-MI) complex was incorporated. The maximum binding capacity of PP-MI to TIG is dependent on the concentration PP-MI \((C_{PP-MI})\) and the number of potential binding sites for TIG \((n_1)\) \(^{61}\) (Equations 4-16 and 4-17). Protein-MI binding parameters’ ranges were calculated based on unbound calcium levels\(^{62}\) and number of potential binding sites for protein binding of MI\(^{64}\).

\[
C_{PP-MI} = \frac{A_{\text{max},PP-MI} \times C_{MI}^{\gamma_{PP-MI}}}{K_{D,PP-MI}^{\gamma_{PP-MI}} + C_{MI}^{\gamma_{PP-MI}}} \quad (4-16)
\]

\[
A_{\text{max,complex}} = C_{PP-MI} \times n_1 \quad (4-17)
\]

Given the expansion of multiple binding sites for TIG binding MI, in Model III, the nature of how TIG-MI complexes can bind PP was redefined accordingly (Equations 4-18, 4-19, and 4-20).

\[
C_{b,complex} = C_{b,complex,1} + C_{b,complex,2} \quad (4-18)
\]

\[
C_{b,complex,1} = \frac{A_{\text{max,complex,1}} \times C_{b,MI,1}^{\gamma_{complex,1}}}{K_{D,complex,1}^{\gamma_{complex,1}} + C_{b,MI,1}^{\gamma_{complex,1}}} \quad (4-19)
\]

\[
C_{b,complex,2} = \frac{A_{\text{max,complex,2}} \times C_{b,MI,2}^{\gamma_{complex,2}}}{K_{D,complex,2}^{\gamma_{complex,2}} + C_{b,MI,2}^{\gamma_{complex,2}}} \quad (4-20)
\]
Model IV was also expanded to incorporate the presence of MI’s influence on the TIG-PP binding (eMI) (Equation 4-21). x and y are values between 0 and 1 and always sum to 1 to represent the varying concentrations of calcium present. For example when x equals 1, no calcium is present and y equals 0. When y equals 1, the maximum effective amount of calcium is present, x equals 0, and TIG-PP binding exhibits an enhanced interaction. Based on amount of calcium present, x and y can fall between 0 and 1, accounting for different proportions of the binding.

\[
C_{b,PP} = x \cdot \left( \frac{A_{max,PP} \times C_u \gamma_{PP}}{K_{D,PP} \gamma_{PP} + C_u \gamma_{PP}} \right) + y \cdot \left( \frac{A_{max,eMI} \times C_u \gamma_{eMI}}{K_{D,eMI} \gamma_{eMI} + C_u \gamma_{eMI}} \right) \tag{4-21}
\]

Sensitivity analysis ranges were revised for the different model variations based on the nature of the changes or expansion. For example, the Phase I range \(A_{max,MI}\) was based on 3 potential binding sites with same \(K_{D,MI}\) while a variation of this model in Phase II considers a maximum of 2 binding sites having different \(K_D\) values. In addition, since only a MI which is already bound to one TIG can bind a second TIG molecule, \(A_{max,MI,2}\) is directly related to \(C_{b,mi,1}\). Therefore the upper bound value of the range for \(A_{max,MI,1}\) should be recalculated for the number of potential binding sites, and subsequent MI-TIG binding relationships should be defined with the aforementioned relationships in mind.

**Phase III: Data-Informed Models**

Using results from Phases I and II as well as new publications, new models were developed and refined ranges (Table 4-1) were used to explore how more informed models impact results and guide further investigations.

In Phase II, we expanded the models in Phase I, increasing model complexity and characterization. GSA was used to evaluate whether the addition of a parameter or complexity significantly contributed to the outcome (\(f_u\)). For example, if a parameter simply replaced the
parameter it described in Phase I, which was a significant contributor in Phase I, the authors deemed that this new parameter did not add valuable contribution to the model’s ability to describe the $f_0$. Conversely if the parameter was found to be consistently significant across models and did not replace a significant parameter from Phase I, the authors deemed it was an important complexity to include in the model in Phase III.

During the course of our investigation, several papers were published describing relationships between PP binding, TIG, and MI. One publication described how TIG binds to HSA (typical values are about 30 to 50 g/L) with 1 binding site in the presence and absence of calcium. We used these results to set new ranges for $A_{\text{max,PP}}$, $A_{\text{max,complex}}$ and $A_{\text{max,eMI}}$ as well as fix several $K_D$ values. Another study by Arias and colleagues reported that 2 molecules of calcium bind to 3 molecules of TIG; $A_{\text{max,MI}}$ was recalculated considering that particular binding ratio.

**Results**

**Phase I: Empiric Models**

Total order indices by parameter appear in Figure 4-3 for each model. $A_{\text{max,PP}}$, $K_{D,PP}$, $A_{\text{max,MI}}$ and $A_{\text{max,complex}}$ were significant contributors for all models in which they were parameters. $\gamma_{PP}$ was significant for almost all models, except model III. $K_{D,MI}$ was only significant for Model III, whereas $K_{D,complex}$ and $\gamma_{complex}$ were only significant within Model II. All Model IV parameters were significant.

**Phase II: Mechanistic Expansion**

Table 4-2 displays parameters most often significant when present in models along with their instances for Phase II. Notably, PP-TIG parameters ($K_{D,PP}$, $\gamma_{PP}$, $A_{\text{max,PP}}$), TIG-MI parameters ($A_{\text{max,MI}}$, $A_{\text{max,MI,1}}$, $K_{D,MI}$, $\gamma_{MI}$) and complex parameters ($A_{\text{max,complex}}$, $K_{D,complex}$, $\gamma_{complex}$) were often significant contributors. $K_{D,MI}$ for the second and third TIG molecules binding MI-TIG were
never significant contributors (Figure 4-4). First order complex parameters were always significant for Models II and III (Figures 4-5, 4-6, 4-7). Similar to the addition of TIG-MI binding sites, second and third order binding parameters for TIG-MI-PP complexes were not always significant contributors. With flexible MI binding, MI-TIG parameters (A\text{max,MI}, K_{D,MI}, \gamma_{MI}) were significant, as shown in Figure 4-8, with the exception of \gamma_{MI} for Model I. For all model variations K_{D,PP-MI} and \gamma_{PP-MI} were not significant, whereas A\text{max,PP-MI} and number of binding sites (n1) did affect f_u. In the presence of flexible MI binding, complex parameters (complex and complex,1) were no longer universally significant. All Model IV parameters remained significant in Phase II (Figure 4-9).

**Phase III: Data-Informed Models**

From Phase II, we learned that incorporating a Hill coefficient in MI binding was important whereas adding multiple binding sites for either MI or complex interactions did not add value. For Model II, parameters for PP-MI complex replaced the complex parameters in Phase I, thus this relationship was not incorporated in Phase III.

Results from analyses for all models are shown in Figure 4-10. For all models, A\text{max} parameters were significant contributors, while no K_D parameters (K_{D,PP} and K_{D,MI}) included were significant. \gamma_{PP}, \gamma_{MI}, \gamma_{complex}, and \gamma_{eMI} only were considered influential in five of ten instances (2/4 for \gamma_{PP}, 1/3 for \gamma_{MI}, 1/2 for \gamma_{complex}, and 1/1 for \gamma_{eMI}). All Model IV parameters remained significant.

**Discussion**

In Phase I, the TIG-PP parameters were significant contributors to f_u for most of the proposed models. All A_{max} parameters were also significant contributors for all models. The general significance of A_{max} may be explained by the wide potential range of values for all mechanistic interactions. The lack of significance of K_{D,MI} may highlight its lack of influence on
the experimentally observed protein binding phenomenon. Complex parameters, besides $A_{\text{max}}$, were only significant contributors when the mechanism of TIG-MI-PP complexation occurred by TIG binding PP-MI complexes. With the same parameter ranges used for this complex interaction in Model II as the TIG-PP interaction, it is impossible to differentiate these contributions.

All parameters in Model IV were significant contributors to $f_u$. One might say that the results support the results of previous local sensitivity analysis\(^8\) in that all parameters for Model IV played a significant role in driving $f_u$. Model IV corresponds to Putative Mechanism IV, which satisfied all observed experimental conditions. It was also noted by Singh and colleagues\(^8\) that this mechanism was also described as a potential scenario by Stojanovic and colleagues\(^65\), who described the increased binding affinity of TIG in the presence of calcium with no change in the number of binding sites.

In Phase II, TIG-PP interaction parameters continued to play a major role, as well as capacity parameters for MI-TIG and complex interactions. The incorporation of second and third order binding of TIG to MI, or complexes, did not lead to identifying more drivers of $f_u$. The only exception was $A_{\text{max,complex,2}}$ which did significantly contribute. On the other hand, flexible MI binding did add value to the model as all MI-TIG parameters became significant when $\gamma$ is incorporated into the interaction. As $\gamma$ is always added as a power on substrate concentrations and $K_D$, it adds significant flexibility to the binding relationship, and mathematically slight changes could be expected to greatly influence output. Characterizing the PP-MI relationship did not produce additional drivers with the exception of binding capacity and number of sites. The influence of $A_{\text{max,PP-MI}}$ and $n_1$ makes logical sense as it serves as the maximum capacity of TIG for PP-MI complexes, replacing this parameter value from Phase I. Based on these results, the
tightness of PP-MI binding ($K_D$) or the shape of the binding relationship ($\gamma_{PP-MI}$) seem less important to variability in $f_u$. Focusing on Model IV, all parameters retained their influence in Phase II. With knowledge of the experimental data available, one might attribute this robustness to the ability of the model to describe these results and deem that Phase II further supports the previous local sensitivity analysis.

In Phase III, we pivoted the analysis and asked how might the results be affected by incorporating experiment knowledge along with findings from Phases I and II. The previously described model adjustments were made based on Phases I and II, and values or ranges, and structural model changes, were incorporated based on literature results regarding this specific interaction. Parameters in Model IV remained significant through Phase III, further supporting the robustness of the proposed mechanistic model. $K_{D,MI}$, which was a major driver in Phase II lost its impact on $f_u$ in Phase III, while $A_{max,MI}$ maintained its role as driver of $f_u$. This result may have been anticipated as $K_{D,MI}$ became more informed in Phase III. Other than the particular mathematical relationship of the parameter to the outcome of interest, a main driver of whether a parameter will flag as a significant contributor is the range used. This also applies to the effects of other interaction parameters. For example, as PP-TIG interactions become more informed, the relative ranges become smaller compared to the wider ranges for less informed values. In this sense while Phases I and II may give insight into which parameters are critical and which are superfluous in potential mechanistic models, Phase III may dictate more about which parameters we know the least about and whether that uncertainty relative to the knowledge we have about other parameters could influence the outcome. With that, the more informed the parameter ranges, the more confidence one might have in the output indices. This sort of information could guide future mechanistic investigations in the wet lab. For instance, if parameters for a particular
relationship were still not significant drivers after narrowing ranges, it may not be important to pursue them further experimentally. For example, some TIG-MI parameters lost influence with narrowed ranges and it may be less impactful to examine the chelation of TIG by MI in the wet lab space. On the other hand, for an interaction consistently identified as a driver, it may be worth a further look; For Model IV, all parameters remained significant throughout the different phases, justifying further validation of these models with addition protein binding data.

While these findings need to be confirmed on a molecular level, this proposed novel use of global sensitivity analysis could serve as a tool to refine mechanistic investigations by understanding major contributors to a particular outcome and guiding future experiments. It is a quick approach to better understand a proposed mechanism in a biological content that can be refined as more information becomes available.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Simulated Range/Value (μM)</th>
<th>Model(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{\text{max}, PP}$</td>
<td>$1 - 800^{60,65}$</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>$K_{D, PP}$</td>
<td>$19.608 - 55.556^{65}$</td>
<td>I</td>
</tr>
<tr>
<td>$A_{\text{max}, MI}$</td>
<td>$1 - 2250^{66}$</td>
<td>I, II, III</td>
</tr>
<tr>
<td>$K_{D, MI}$</td>
<td>$11 - 15^{66}$</td>
<td>I, II, III</td>
</tr>
<tr>
<td>$A_{\text{max}, \text{complex}}$</td>
<td>$1 - 800^{60,65}$</td>
<td>II, III</td>
</tr>
<tr>
<td>$K_{D, \text{complex}}$</td>
<td>$19.608^{65}$</td>
<td>II, III</td>
</tr>
<tr>
<td>$A_{\text{max}, \text{eMI}}$</td>
<td>$1 - 800^{60,65}$</td>
<td>IV</td>
</tr>
<tr>
<td>$K_{D, \text{eMI}}$</td>
<td>$19.608^{65}$</td>
<td>IV</td>
</tr>
</tbody>
</table>
Table 4-2. Common significant contributors in Phase II.

<table>
<thead>
<tr>
<th>Parameter</th>
<th># Significant/Total # Occurrences</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{D,PP}$</td>
<td>13/13</td>
</tr>
<tr>
<td>$\gamma_{PP}$</td>
<td>13/13</td>
</tr>
<tr>
<td>$A_{max,PP}$</td>
<td>12/13</td>
</tr>
<tr>
<td>$A_{max,MI,1}$</td>
<td>8/8</td>
</tr>
<tr>
<td>$\gamma_{complex}$</td>
<td>6/7</td>
</tr>
<tr>
<td>$K_{D,complex}$</td>
<td>6/7</td>
</tr>
<tr>
<td>$K_{D,MI}$</td>
<td>4/4</td>
</tr>
<tr>
<td>$A_{max,complex}$</td>
<td>4/4</td>
</tr>
<tr>
<td>$A_{max,MI}$</td>
<td>4/4</td>
</tr>
<tr>
<td>$\gamma_{MI}$</td>
<td>3/4</td>
</tr>
<tr>
<td>$A_{max,PP-MI}$</td>
<td>3/3</td>
</tr>
<tr>
<td>n1</td>
<td>3/3</td>
</tr>
<tr>
<td>$A_{max,complex,1}$</td>
<td>2/2</td>
</tr>
<tr>
<td>$\gamma_{complex,1}$</td>
<td>2/2</td>
</tr>
<tr>
<td>$K_{D,complex,1}$</td>
<td>2/2</td>
</tr>
<tr>
<td>$\gamma_{complex,2}$</td>
<td>1/2</td>
</tr>
<tr>
<td>$A_{max,complex,2}$</td>
<td>1/2</td>
</tr>
<tr>
<td>$A_{max,eMI}$</td>
<td>1/1</td>
</tr>
<tr>
<td>$K_{D,eMI}$</td>
<td>1/1</td>
</tr>
<tr>
<td>$\gamma_{eMI}$</td>
<td>1/1</td>
</tr>
<tr>
<td>x</td>
<td>1/1</td>
</tr>
</tbody>
</table>
Figure 4-1. Global sensitivity analysis workflow. Beginning with empiric models (Phase I), expanding those models to add complexity (Phase II), then combining the information from Phases I and II along with experimental data to refine hypotheses (Phase III). Model II is depicted here as an example.
Figure 4-2. Schematic of empiric models adapted from Singh et al\textsuperscript{8}. $C_u$: unbound TIG concentration, $C_b$: bound TIG to specified site, PP: plasma protein, MI: metal ion, PP-MI: plasma protein-metal ion complex, Complex: complex of TIG (Model II) or TIG-MI (Model III) with PP.
Figure 4-3. Total indices for empiric models in Phase I.
Figure 4-4. Total indices for MI parameters in Phase II when adding multiple binding sites to various models.
Figure 4-5. Total indices for $A_{\text{max,complex}}$ parameters in Phase II when adding multiple binding sites to various models.
Figure 4-6. Total indices for $K_{D,\text{complex}}$ parameters in Phase II when adding multiple binding sites to various models.
Figure 4-7. Total indices for $\gamma_{\text{complex}}$ parameters in Phase II when adding multiple binding sites to various models.
Figure 4-8. Select total indices for addition of flexible MI binding in Phase II.
Figure 4-9. Total order indices for Model IV for all phases. Data from Phase I is the same as Figure 4-3.
Figure 4-10. Total indices for data-informed models of Phase III. Model IV data also appears in Figure 4-8.
CHAPTER 5
QUANTIFICATION OF TIGECYCLINE-TETRACYCLINE INTERACTION IN STATIC TIME KILL CURVE EXPERIMENTS

Introduction

An estimated 700,000 people die each year from antimicrobial resistant infections, a number which may rise to over 10 million per year globally by 2050. Multidrug-resistant *Pseudomonas aeruginosa* with resistance to three or more drug classes is classified by the CDC as a serious threat and is part of the Infectious Diseases Society of America’s (IDSA) ESKAPE list of threatening pathogens. *P. aeruginosa* is commonly associated with hospital-associated infections including pneumonia, urinary tract infections, blood stream infections, and surgical site infections. Patients in intensive care units, in particular those on ventilators and those with cystic fibrosis, are especially at risk. *Pseudomonas* resistance has been growing over the past quarter century, with fluoroquinolone resistance at an incidence of greater than 30% in 2003.

Even in the presence of the growing threat of antimicrobial resistance, there has been a steady decline in antibiotic drug approval by the FDA since the 1980s, with eight new approvals from 2010 to 2015, only one possessing a novel mechanism. This drought of novel therapeutic options has driven research in the repurposing of older antibiotics and investigating efficacy of combination therapies to treat MDR infections. Expected and unexpected synergisms and antagonisms have been uncovered, allowing for more informed decision-making in practice.

The current paradigm of drug interactions in the field of antimicrobials is primarily based on the fractional inhibitory concentration index (FICI), which is calculated from results of checkerboard MIC experiments or E-tests, among other MIC-based susceptibility tests. Any MIC determination is a semi-quantitative measurement, taken at a single time point, with inherent 2-fold variability. With its use in determining presence or absence of an interaction, it fails to capture the changing nature of the interaction at different concentrations and at varying
time points\textsuperscript{73}. An alternative, more quantitative method is time kill curves to determine the effect
of a combination over time for a particular pathogen. Time-kill technique also has its own
shortcomings as it assumes no difference in effect for varying bacterial loads and that drug
concentrations remain constant over time. In addition, determining synergy using time-kill
curves, also requires that the additional agent has no activity on its own. The use of
pharmacometrics in antimicrobial dose optimization has allowed researchers to overcome such
barriers, by quantifying and accounting for these factors in semi-mechanistic mathematical
models of time-kill data\textsuperscript{74}.

Tigecycline, a novel glycylcycline antibiotic, a derivative of minocycline, approved in
2005, is used for the treatment of community-acquired pneumonia, complicated intraabdominal
and skin and skin structure infections. Tigecycline is already a broad-spectrum treatment option
for Gram- positive and negative organisms, including methicillin-resistant Staphylococcus
aureus (MRSA). The use of tetracycline in combination with tigecycline has shown enhanced
activity against \textit{P. aeruginosa}, for which tigecycline alone is not active against (Chapter 2). Here
we describe the drug effects of tigecycline and tetracycline alone and in combination over time
against \textit{P. aeruginosa}, including monitoring drug stability and the development of resistance.

\textbf{Materials and Methods}

\textbf{Chemicals, Media, Bacteria and Equipment}

Tigecycline and tetracycline hydrochloride was obtained from TSZ CHEM (Framingham, MA) and Medisca Incorporated (Plattsburgh, New York), respectively. Sodium chloride was
obtained from Fisher Scientific (Pittsburgh, PA). \textit{P. aeruginosa} ATCC\textsuperscript{®} 27853 was obtained
from UF Health Microbiology Laboratory. Bacterial suspensions in normal saline were prepared
using the A-JUST Turbidimeter (Abbott Laboratories) calibrated with McFarland Turbidity
Standards (Remel Microbiolgy Products, Lenexa, KS). Cation adjusted Mueller Hinton Broth,
Mueller Hinton Agar, and Sheep’s blood agar plates were obtained from Becton, Dickinson and Company (Sparks, MD) and Remel Microbiology Products (Lenexa, KS), respectively. Experiments were incubated in Labline Model 460 incubator.

**Static Time Kill Curve Experiments**

*In vitro* static time-kill curves were performed in triplicate to determine the effect over time at varying concentrations (0, 0.25, 0.5, 1, 2, 4, 8×MIC) of tigecycline (TIG) alone and in combination with varying concentrations of tetracycline (TET) (4, 8, and 12 µg/mL). Curves were also performed in triplicate for TET alone (0, 0.25, 0.5, 1, 2, 4, 8×MIC).

To set up, a sufficient number of bacterial colonies were suspended in sterile normal saline to a turbidity within the 0.5 McFarland standard range (1.5 x 10^8 CFU/mL). One hundred mcL of bacterial suspension were then added to 20 mL culture media for a final bacterial concentration of approximately 7.5 x 10^5 CFU/mL. Flasks were incubated for 2 hours before drug solutions were added. Flasks were then returned to the incubator for the remainder of the experiment (with the exception of sampling) and mixed on a rocker. Samples were taken from each flask at 0, 2, 4, 6, 8, 10, 12, 16, and 24 h and diluted in normal saline. Ten 10-mcL saline-diluted and undiluted aliquots were plated on Sheep’s blood agar and incubated for 16-24 hours. Bacterial colonies were then counted and bacterial concentrations were then calculated for each time point based on total volume and dilution (Limit of detection: 10 CFU/mL). Curves were performed in triplicate.

**Drug Degradation**

Drug degradation currently being performed by Wicha and colleagues.

**Resistance Testing**

The development of tigecycline and tetracycline resistance was also determined after 24 hours of 0.25, 1, and 8×MIC TIG or TET exposure, or 0.25, 1, and 8×MIC TIG with 12 mg/L
TET exposure (Table 5-1). Flasks were set up using the same procedure as time kill curve experiments. Ten 10-mcL aliquots were plated on 3×MIC TIG-containing and 3×MIC TET-containing Mueller Hinton agar plates post-drug exposure. Plates were then incubated for 24 h and subsequently read. A positive or negative result was determined by the presence or absence of visible growth. Experiments were performed in duplicate.

**Results and Discussion**

**Static Time Kill Curves**

Figures 5-1 and 5-2 depict results of the kill curve experiments. Curves of TIG and TET alone are visually similar at their relative MIC values. While exposures less than 1xMIC still grew well beyond starting inocula, there still was some slight effect on maximum bacterial load achieved. For TIG 8 mg/L and 16 mg/L, as well as TET 16 mg/L, there was some regrowth of bacteria after initial killing at earlier time points. Of note, for TIG 16 mg/L this regrowth was delayed as compared to TIG 8 mg/L. There are two possible explanations for this regrowth, emergence of bacterial resistance or drug degradation within the *in vitro* system. While tetracyclines are known to be thermosensitive and there is likely unquantified degradation, it does not rule the possibility that a significant portion of this regrowth may also be due to resistance development. At higher exposures for both TIG and TET, regrowth does not occur.

Based on Figure 5-2, the effect of the combination was more apparent at intermediate TIG exposures. As TIG concentrations increased, the effect on bacterial killing of adding TET was amplified up to TIG 4 mg/L, where the effect was maximized. For TIG concentrations less than or equal to 2 mg/L at 24 h, increases in bacterial kill were not greater than 1-log_{10}(CFU/mL) for various TET concentrations. Differences in bacterial kill between TET-containing combinations and TIG monotherapy were most apparent at TIG 4 mg/L (Figure 5-3). Mean log_{10}CFU/mL decrease was 1.38, 3.42, and 4.21 at 12 h and approximately 0.31, 0.66, and 2.40
at 24 h, for TET 4, 8, and 12 mg/L, respectively. As expected at TIG concentrations above 8 µg/mL (TIG’s MIC), there is a saturation of effect against *P. aeruginosa* and no additional effect is elucidated by the addition of TET. Using this method, one can determine the true effect of tigecycline and tetracycline combinations in terms of the relationship of concentration and effectiveness as it varies over time, or the PK/PD relationship.

These studies confirm the augmented effects of TIG-TET combinations seen in MIC studies. While there appeared to be a loss of effect after 12 h for all concentrations, this may be explained by drug degradation in the experiment, or resistance development. Either of these aspects should be accounted for in future studies using pharmacokinetic/pharmacodynamic (PK/PD) modelling quantifying the full antimicrobial effect. This data may be used in conjunction with PK/PD modeling to determine an adequate dosing regimen for future trials. Granted the success of these future studies, this novel combination has the potential to broaden the spectrum of tigecycline activity when given with tetracycline, potentially allowing for lower doses of tigecycline to be administered.

**Resistance Testing**

Table 5-2 displays the results of resistance testing. After 24 h of 0.25 and 1×MIC TIG drug exposure resistance was observed. For 8×MIC TIG and for any TET exposure alone there was no observed resistance. Comparatively, after combination exposure, resistance to TIG was observed at all exposure levels and TET resistance remained absent. While these results suggest that there may be increased TIG resistance in the presence of TET based on relative MIC exposure, the actual TIG exposure for which resistance was observed after combination exposure for 8×MIC TIG was the same as the 1×MIC TIG exposure during single-drug experiments. Based on this point, it is not clear if TET truly increases the resistance development for TIG, or if
TET simply does not inhibit TIG resistance development even though MIC decreases are observed.

More extensive investigations are required to understand the interplay between TIG and TET in resistance development. In addition, drug degradation in agar plates should be evaluated. These results serve as a basis for future experiments and the incorporation of resistant bacterial populations in PK/PD models of time kill curves, especially in regards to TIG at lower concentrations.

All together these experiments serve as a pharmacodynamic quantification of the effects of tigecycline and tetracycline alone and in combination on *P. aeruginosa* and serves as a launching point for further experiments and dose exploration analyses. The observed effect of tigecycline-tetracycline shows promise given adequate exposure levels for enhanced activity.
Table 5-1. Concentrations of each drug added to each experimental flask (based on multiples of the MIC of TIG or TET alone or in combination).

<table>
<thead>
<tr>
<th>Concentration</th>
<th>TIG Only</th>
<th>TET Only</th>
<th>TIG + TET 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25X MIC</td>
<td>2</td>
<td>4</td>
<td>0.25</td>
</tr>
<tr>
<td>1X MIC</td>
<td>8</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>8X MIC</td>
<td>64</td>
<td>128</td>
<td>8</td>
</tr>
</tbody>
</table>
Table 5-2. Results of resistance testing after 24 hour exposure to antibiotic(s) added in Table 5-1.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>TIG Only</th>
<th>TET Only</th>
<th>TIG +/ TET 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25X MIC</td>
<td>+</td>
<td>-</td>
<td>TIG +/ TET -</td>
</tr>
<tr>
<td>1X MIC</td>
<td>+</td>
<td>-</td>
<td>TIG +/ TET -</td>
</tr>
<tr>
<td>8X MIC</td>
<td>-</td>
<td>-</td>
<td>TIG +/ TET -</td>
</tr>
</tbody>
</table>
Figure 5-1. Results from static time kill curve experiments for TIG and TET alone.
Figure 5-2. Results from time kill curve experiments. Each panel shows a tigecycline (TIG) concentration with each curve representing a different concentration of tetracycline (TET) added. Some curves are duplicated from Figure 5-1 to allow for visual comparison.
Figure 5-3. TIG 4 mg/L time kill curves. The same data appears in Figure 5-2.
CHAPTER 6
EXPLORING TIGECYCLINE AND TETRACYCLINE COMBINATION REGIMENS FOR TREATMENT OF P. AERUGINOSA INFECTIONS

Introduction

*In vitro* experiments of tigecycline and tetracycline against *Pseudomonas aeruginosa* have shown promise to enhance bacterial kill (Chapter 2 and Chapter 5). The addition of tetracycline at sub-MIC concentrations led to an up to 8-fold decrease in tigecycline MIC and demonstrated up to a 4-log$_{10}$ increase in bacterial kill in static time kill curve experiments. Some exposure combinations show more promise than others, and therefore it is imperative to assess whether the combination is advantageous at clinically observable concentrations. To examine the clinical utility of TIG-TET combinations, this work aims to describe existing time kill curve data using PK/PD modelling and link this with existing clinical pharmacokinetic data to simulate potential combination dosing regimens.

Pharmacometric modelling has allowed pharmaceutical scientists to more thoroughly characterize Drug-Exposure, Drug-Effect, and Exposure-Effect relationships through the development of empiric, semi-mechanistic, mechanistic, and systems models. These models may be used to simulate dosing regimens not previously studied in trials and predict the viability of regimens to treat disease and/or their potential to cause certain toxicity without putting patients at unnecessary risk. In antimicrobial development, simulations of pharmacokinetic data from established models may be linked to semi-mechanistic pharmacokinetic/pharmacodynamic models to predict the potential for bacterial kill or stasis in the absence of an immune response. Using literature models and pharmacokinetic models developed from published data, patient exposure for various doses will be predicted. These exposure profiles can then be used to predict effect on a simulated bacterial infection over time. By this, different combination regimens of tigecycline and tetracycline may be assessed for potential future trials in humans.
**Methods**

**Software and Utilization**

NONMEM (Version 7.3), Nonlinear Mixed Effects Modeling, was utilized to model pharmacokinetic and pharmacokinetic/pharmacodynamics time kill curve data, as well as implement models from published literature. Goodness of fit (GOF) plots, change in Objective Function Values (OFVs), and Visual Predictive Checks (VPCs) were used to determine the best models to move forward. PlotDigitizer (Version 2.6.8, plotdigitizer.sourceforge.net) was used to extract data from published literature when necessary. R (Version 3.3.2) and RStudio (Version 1.0.136) were utilized for plotting data and some minor statistical analyses where mentioned.

**Bacterial Pharmacokinetic/Pharmacodynamic Modelling**

PK/PD modeling of static time kill curve data for varying exposures of tigecycline and tetracycline, alone and in combination, against *P. aeruginosa*, was performed (Chapter 5). In summary, static time kill curve experiments were performed over 24 hours for 0.25, 0.5, 1, 2, 4, and 8 x minimum inhibitory concentration (MIC) of TIG (MIC 8 mg/L) or TET (MIC 16 mg/L) alone. Kill curves were also perform for combinations of 0.25, 0.5, 1, 2, 4, and 8 x MIC of TIG with 4, 8, or 12 mg/L of TET. In the presence of 4, 8, and 12 mg/L TET, corresponding TIG MICs were 4, 2, and 1 mg/L (Chapter 2).

Bacterial growth was first characterized by a logistic growth model, where $k_s$ is the growth rate constant, $S$ is the number of bacteria in the system, and $N_{\text{max}}$ is the maximum bacterial capacity of the system (Equation 6-1).

$$\frac{dS}{dt} = k_s \times \left(1 - \frac{S}{N_{\text{max}}} \right) \times S$$  \hspace{1cm} (6-1)

Subsequently, bacterial killing (described using an $E_{\text{max}}$ model) was characterized by the addition of a resting persistent (R) bacterial subpopulation, not susceptible to drug (Figure 6-1).
The conversion from the susceptible to the resting persistent state, $k_{SR}$, was dependent on bacterial load relative to $N_{max}$. A death rate constant, $k_d$, was also incorporated (Equations 6-2 and 6-3).

\[
\frac{dS}{dt} = \left( k_s - E_{MAX} - k_d - k_{SR} \times \frac{S + R}{N_{max}} \right) \times S \tag{6-2}
\]

\[
\frac{dR}{dt} = \left( k_{SR} \times \frac{S + R}{N_{max}} \right) \times S - k_d \times R \tag{6-3}
\]

The drug effect and interaction of TIG and TET was implemented using an $E_{max}$ model with a General Pharmacodynamic Interaction (GDPI) term$^{75}$ in a competitive inhibition model, where INT is the interaction term, C is the corresponding drug concentration, EC$_{50}$ is the concentration which exerts 50% of the maximal effect, $k_{max}$ is the maximal kill rate constant, and H is the Hill coefficient (Figure 6-2). When one drug is not present, this term collapses to a traditional sigmoidal $E_{max}$ model.

Drug degradation was also considered for both TIG and TET, and estimated individually using an exponential decay, where $k_{deg}$ is the drug degradation constant (Equation 6-4).

\[
C_{Time=t} = C_{Time=0} \times e^{-k_{deg} \times t} \tag{6-4}
\]

**Population Pharmacokinetic Models**

A population pharmacokinetic model for tigecycline in healthy subjects was obtained from the literature$^{28}$ and implemented in NONMEM. In brief, the model was developed from 203 tigecycline levels quantified from samples from 13 healthy volunteers who received 25-100 mg IV tigecycline infused over 1 h every 12 hours for up to 10 days in a study by Muralidharan and colleagues$^7$. The PK model consists of a 3-compartment structural model with first order elimination and zero order infusion$^{28}$. Although all the subjects recruited were male, another study showed that sex did not significantly influence pharmacokinetics in healthy volunteers$^{76}$. 
A published pharmacokinetic model for tetracycline in healthy subjects was not found, so an extensive literature search was performed for available pharmacokinetic data. All published data where tetracycline was administered orally was only available as average data. Of the studies, microbiological and other outdated quantification methods were excluded. Dose-exposure proportionality was considered in order to justify simulation within and beyond the range of doses previously studied. A rich pharmacokinetic data set was selected for pharmacokinetic modelling. A one-compartment model with first order absorption was fit to the average data.

**Pharmacokinetic/Pharmacodynamic Simulations**

Literature models or models developed from extracted pharmacokinetic data were used to simulate drug concentration-time profiles under various dosing regimens, including a standard dosing (Regimen A), high dosing (Regimen B), and very high dosing (Regimen C) regimens. The specifics of these regimens are noted in Table 6-1. Pharmacokinetic simulations were performed for each combination regimen and pharmacodynamic killing of bacteria was simultaneously simulated based on the developed PK/PD model from time kill experiments. Unbound concentrations of tigecycline and tetracycline were calculated based on free concentration in the plasma. For tigecycline, a clinical plasma protein binding model was used\(^{38}\) (Equation 6-5).

\[
f_u = \left( \frac{9.0896 + 15.339}{C_{TIG}} - \frac{0.999}{C_{TIG}^2} + \frac{0.0232}{C_{TIG}^3} \right) \times 100 \tag{6-5}
\]

For tetracycline the average of literature values were used (59.5% binding)\(^{25}\). Free concentrations of tetracycline in tissue was assumed to be 1:1 and penetration of tigecycline based on calculated free area under the curve (fAUC) for epithelial lining fluid (ELF) to fAUC
The AUCs for epithelial lining fluid (ELF) and serum concentrations were calculated using trapezoidal rule with data from a published multiple dose study in healthy volunteers\textsuperscript{36}. The fAUC\textsubscript{serum} was calculated after C\textsubscript{serum} were transformed to C\textsubscript{serum,free} using Equation 6-5. The resulting fAUC\textsubscript{ELF}:fAUC\textsubscript{serum} penetration constant, to be used in simulations, was 1.86.

**Results**

**Time Kill Curve Modelling**

The two-subpopulation bacterial model with GDPI term on K\textsubscript{max} of a competitive inhibition model adequately described the effects of tigecycline and tetracycline alone and in combination against *P. aeruginosa* in static time kill curve experiments. GOF plots, VPCs, and model estimates are shown in Figure 6-3, Figure 6-4, and Table 6-2, respectively. All estimates are reasonable with RSE below 30%. Based on the model, the interaction term (INT) estimate has a 95% confidence interval of (0.192, 0.638), indicating a true interaction.

**Tigecycline and Tetracycline Pharmacokinetic Models**

The tigecycline PK model was successfully implemented in NONMEM.

For tetracycline, over a dozen pharmacokinetic studies were identified in healthy volunteers. Of those with suitable plasma concentration data, four were excluded due to the use of microbiological or outdated quantification methods. Data from one study could not be extracted due to unclear axis scales used in graphical data representations. Seven oral single dose studies remained\textsuperscript{77-83}, including one with an additional multiple dose steady state arm\textsuperscript{83}. Doses ranged from 250 to 541 mg tetracycline hydrochloride. Data sets were extracted using PlotDigitizer (Version 2.6.8, plotdigitizer.sourceforge.net). Single dose data sets were plotted and visually compared (Figure 6-5). AUC\textsubscript{0-24} and AUC\textsubscript{0-inf} were calculated for each trial (Figure 6-6). 1/CL proportionality constant was calculated by dividing the AUCs by Dose (Figure 6-6).
Linear regression was performed in R and dose was not found to significantly impact any proportionality constant.

Given a desire to characterize steady state, single and multiple dose data from Sjoelin-Forsberg and Hermannson\(^{83}\) was ultimately used to develop a population pharmacokinetic model for tetracycline in NONMEM using a First Order Conditional Estimation with Interaction (FOCE + I) estimation algorithm. Initial estimates were obtained or calculated from published literature values\(^{25,83}\) when available. While 1- and 2- compartment oral PK models provided satisfactory diagnostic and visual predict checks, ultimately, based on statistical superiority (change in objective function value, \(-2 \log \text{likelihood}\)), a 1-compartment oral PK model with absorption lag time was selected. Selected GOF plots and VPCs of the final model are provided in Figures 6-7 and 6-8 respectively. Parameter estimates are listed in Table 6-3.

**PK/PD Simulations**

No reasonable regimen was able to achieve optimal bacterial kill alone or in combination. The predicted unbound tissue concentrations for tigecycline (\(\text{fC}_{\text{TIG}}\)) and tetracycline (\(\text{fC}_{\text{TET}}\)) for each regimen are shown in Figure 6-9. For Regimens A and B, \(\text{fC}_{\text{TIG}}\) and \(\text{fC}_{\text{TET}}\) do not reach concentrations above 0.591 and 4.34 mg/L respectively at steady state. For regimen C, the tigecycline dose is 10 times that of regimen B and reaches peak steady state concentrations around 3.44 mg/L. Comparatively when 3x the dose of tetracycline is administered for regimen C versus B, peak steady state concentrations are 13.0 mg/L.

Predicted bacterial concentrations during each treatment regimen are shown in Figure 6-10. For all three regimens, these exposures of tigecycline when administered alone have little to no effect on bacterial concentrations. The dose of tetracycline for Regimen C, when administered alone confers some bacterial killing compared to no treatment (0.906-\(\log_{10}\) lower bacterial concentration), but does not lead to bacteria concentrations lower than the initial inoculum. Only
for Regimen C, does the combination of tigecycline and tetracycline show a synergistic effect with final bacterial concentrations of 6.93-\(\log_{10}\) below the initial inoculum and 6.03-\(\log_{10}\) below tetracycline alone (the most active monotherapy).

**Discussion**

Modelling of static time kill curve experiments demonstrated the use of the recently developed GDPI term\textsuperscript{75} to adequately describe the activity of tigecycline and tetracycline alone and in combination, as well as improve model fit compared to conventional interaction models. The estimate of the interaction term indicates a true interaction between TIG and TET, while standard measures of calculating a fractional inhibitory concentration indices showed no synergistic interaction. While the model adequately described all performed experiments, drug effects may have been potentially misspecified as drug degradation constants were estimated and not determined by experimental assay. While drug degradation seems to explain regrowth of bacteria, if degradation was overestimated by the model, it may lead to overestimating drug effect or masking resistant subpopulation development. On the contrary, if drug degradation is underestimated, drug effects may be underestimated. Accurate drug effects and the presence of resistant subpopulations are important aspects for assessing clinical viability.

PK models for simulation were drawn from a published literature model for tigecycline and developed based on published data for tetracycline. In the absence of available dose-exposure data for tetracycline, a brief analysis was performed and showed CL to remain constant across a given dose range (250-541 mg tetracycline HCl) for single dose studies. Both these models were based on pharmacokinetics in healthy volunteers. While pharmacokinetics in other populations has been shown to differ (i.e. in the case of hepatic impairment\textsuperscript{84}, and gender\textsuperscript{29} in some patient populations), most data thus far has indicated that pharmacokinetics observed in patients is similar to that observed in healthy volunteers.
These PK models were successfully linked with the developed PK/PD model to simulate the bacterial killing of several dosing regimens. These simulations, based on current models of pharmacokinetics and knowledge of drug penetration, dictate that tigecycline and tetracycline are not a viable combination for human use. Doses needed to reach optimum tissue concentrations would likely be intolerable or toxic to patients. Given the limited data on drug tissue penetration in patients as well as the impacts of nonlinear protein binding on pharmacokinetics, this result is not completely conclusive.

Free, active drug concentrations at the site of infection are directly related to pharmacological effects. While free exposure in the plasma or serum may serve as a surrogate for tissue concentrations when this relationship has been established, the unbound exposure in target site tissues is often overlooked and assumed to be equal to unbound central compartment exposure. In the case of tigecycline, which exhibits concentration dependent plasma protein binding\textsuperscript{23,38}, the assumption of linear pharmacokinetics and corresponding tissue exposure, could potentially lead to miscalculations in true unbound drug exposure at the site of action (Chapter 1). Only a handful of clinical studies have aimed to assess free drug concentrations at the site of action, including the lung (using epithelial lining fluid concentrations\textsuperscript{30,36,37,85}) and subcutaneous tissue of the thigh and wound (via microdialysis in diabetic patients\textsuperscript{38}). Using ELF and plasma data from Conte and colleagues\textsuperscript{36}, and accounting for nonlinear plasma protein binding in calculating $f\text{AUC}_{\text{Serum}}$ using a model developed by Bulik and colleagues\textsuperscript{38}, we arrived at a penetration ratio ($f\text{AUC}_{\text{lung}}:f\text{AUC}_{\text{serum}}$) of 1.86, as compared to the 6.28-6.59 calculated by others\textsuperscript{37,86} assuming linear plasma protein binding. A higher ratio would have led to higher free tissue concentrations, and potentially inaccurately predicted an increased activity of the combination regimens.
Another limitation of the available penetration data concerns the fact that drug penetration in the lung was not determined in subjects with active pneumonia. While plasma pharmacokinetics appear similar between patients and healthy subjects, distribution to tissues may also be higher in the case of infected tissues due to increased permeability due to inflammation and migration of polymorphonuclear cells to the infection site\textsuperscript{30,87}. This increased penetration has been noted in mice models\textsuperscript{86}, but not yet determined in humans.

Issues with nonlinearities and penetration aside, given the extensive binding of tigecycline to tissues and to calcium, especially in bone, how do we know what the true active concentration is? Is metal-ion chelated drug active? If one considers the answer to be “yes”, while current techniques do allow for quantification of chelated drug in extracellular fluid (as it will penetrate microdialysis membranes), these tools cannot account for chelated complexes in tissue or within the intracellular space. On the other hand, if one assumes chelated drug is inactive, then how does one account for adequate treatment of some cases of osteomyelitis\textsuperscript{88–90}?

To further complicate things, all of these considerations may also apply to tetracycline, for which there is even less data on protein binding and drug penetration. Recent investigations into minocycline’s\textsuperscript{13} and eravacycline’s\textsuperscript{10,11} plasma protein binding also described a similar concentration-dependent phenomenon.

In summary, based on current information on free exposure of tigecycline and tetracycline in tissues, and the PK and PK/PD models from literature and those developed here, while there is a significant \textit{in vitro} pharmacodynamic interaction, there does not seem to be a clinically feasible regimen for which adequate concentrations for efficacy against \textit{P. aeruginosa} may be reached at this time. Fortunately these analyses have characterized an interaction between two antibiotics of the same structural class, shed light on many important points for
consideration of pharmacokinetics in nonlinear protein binding, and questioned what concentrations of tigecycline are clinically active.
Table 6-1. Simulated dosing regimens. IV: intravenous, HCl: hydrochloride salt, PO: by mouth, Q: every

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Tigecycline Dose (IV)</th>
<th>Tetracycline (HCl) (PO)</th>
<th>Duration (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Standard Dosing</td>
<td>100 mg once, 50 mg Q12h</td>
<td>500 mg Q12h</td>
<td>14</td>
</tr>
<tr>
<td>B: High Dosing</td>
<td>200 mg once, 100 mg Q12h</td>
<td>500 mg Q6h</td>
<td>14</td>
</tr>
<tr>
<td>C: Very High Dosing</td>
<td>2000 mg once, 1000 mg Q12h</td>
<td>1500 mg Q6h</td>
<td>14</td>
</tr>
</tbody>
</table>
Table 6-2. Parameter estimates for PK/PD model of time kill curve data. RSE: relative standard error, ks: growth rate constant for susceptible bacteria, N_max: maximum capacity of bacterial system, CFU_start: starting concentration of bacteria, IIV: interindividual variability, k_d: natural death rate, k_SR: conversion rate from susceptible to resting persistent bacterial states, k_max: maximum kill rate constant for a particular drug, EC_50: concentration producing 50% of maximum effect, k_deg: drug degradation constant, H: Hill coefficient, INT: interaction term, a Exponential error model

<table>
<thead>
<tr>
<th>Parameter (Units)</th>
<th>Estimate (RSE, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ks (h⁻¹)</td>
<td>1.15 (4.1)</td>
</tr>
<tr>
<td>N_max (log_{10} \frac{CFU}{mL})</td>
<td>9.4 (0.7)</td>
</tr>
<tr>
<td>CFU_start (log_{10} \frac{CFU}{mL})</td>
<td>5.59 (0.8)</td>
</tr>
<tr>
<td>IIV</td>
<td>0.735 (16.2)</td>
</tr>
<tr>
<td>k_d (h⁻¹)</td>
<td>0.187 (Fixed)</td>
</tr>
<tr>
<td>k_SR (h⁻¹)</td>
<td>1.25 (5.8)</td>
</tr>
<tr>
<td>k_max,TIG (h⁻¹)</td>
<td>1.49 (9.4)</td>
</tr>
<tr>
<td>EC_{50,TIG} (mg/L)</td>
<td>2.7 (15.3)</td>
</tr>
<tr>
<td>k_max,TET (h⁻¹)</td>
<td>1.35 (5.5)</td>
</tr>
<tr>
<td>EC_{50,TET} (mg/L)</td>
<td>8.86 (9.1)</td>
</tr>
<tr>
<td>k_deg,TIG (h⁻¹)</td>
<td>0.0909 (11.7)</td>
</tr>
<tr>
<td>k_deg,TET (h⁻¹)</td>
<td>0.0539 (12.8)</td>
</tr>
<tr>
<td>H</td>
<td>1.88 (11.4)</td>
</tr>
<tr>
<td>INT</td>
<td>0.415 (27.5)</td>
</tr>
<tr>
<td>Residual variability^a</td>
<td>0.17 (9.9)</td>
</tr>
</tbody>
</table>
Table 6-3. Parameter estimates for tetracycline PK Model. RSE: relative standard error, $k_a$: absorption rate constant, $T_{lag}$: absorption lag time, $V$: volume of distribution, CL: clearance from central compartment, a Additive error model

<table>
<thead>
<tr>
<th>Parameter (Units)</th>
<th>Estimate (RSE, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_a$ (h$^{-1}$)</td>
<td>0.74 (9)</td>
</tr>
<tr>
<td>F</td>
<td>0.911 (2)</td>
</tr>
<tr>
<td>$T_{lag}$ (h)</td>
<td>0.268 (15)</td>
</tr>
<tr>
<td>V (L)</td>
<td>86.2 (3)</td>
</tr>
<tr>
<td>CL (L/h)</td>
<td>9.32 (1)</td>
</tr>
<tr>
<td>Residual variability$^a$</td>
<td>0.169 (6)</td>
</tr>
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</table>
Figure 6-1. Schematic diagram of final model developed to describe static time kill curve data. S: susceptible bacteria population, R: resting persistent bacteria population, $k_s$: growth rate constant for susceptible bacteria, $k_d$: natural death rate, $k_{SR}$: conversion rate from susceptible to resting persistent bacterial states, $k_{deg}$: drug degradation constant.
Figure 6-2. $E_{\text{max}}$ equation for model of static time kill curves.
Figure 6-3. GOF plots for final model of static time kill curve data.
Figure 6-4. VPCs for final model of static time kill curve data stratified by unique TIG and TET combination.
Figure 6-5. All single dose data separated in panels by dose (mg tetracycline HCl).
Figure 6-6. AUCs and dose proportionality constant (1/CL) by dose.
Figure 6-7. Selected diagnostic plots for the final tetracycline PK model.
Figure 6-8. Visual predictive checks (VPCs) of the final tetracycline PK model.
Figure 6-9. Predicted free tissue concentrations over time for each tested regimen for tigecycline and tetracycline.
Figure 6-10. Predicted bacterial kill over time for each tested regimen.
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BIOGRAPHICAL SKETCH

Amelia N. Deitchman was born in Jacksonville, Florida, and raised in Atlantic Beach, Florida. She is the youngest of three children. Amelia graduated valedictorian in 2008 from Duncan U. Fletcher High School and completed one year of pre-pharmacy coursework at the University of Florida where she was admitted to the pharmacy program. She obtained her Doctor of Pharmacy degree from the University of Florida in 2013. During pharmacy school, she starting working in the research group of Dr. Hartmut Derendorf and began pursuing her PhD after graduation. During her PhD, she completed an internship in the Clinical Pharmacology, Modelling and Simulation group at GlaxoSmithKline in King of Prussia, Pennsylvania. In addition to her own PhD studies, she mentored 19 undergraduate and visiting students, including 1 Master’s thesis and 1 Undergraduate Honors thesis, was involved in several organizations including the American College of Clinical Pharmacology, American Society of Clinical Pharmacology and Therapeutics, and the American Association for Pharmaceutical Sciences, published 6 papers, presented at 15 meetings and completed a Minor in Statistics. Amelia was awarded the Doctor of Philosophy in Pharmaceutical Sciences from the University of Florida in fall 2017.