In Vivo Pharmacodynamic Target Assessment of Eravacycline against Escherichia Coli in a Murine Thigh Infection Model

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Running Title: In vivo PK/PD of Eravacycline against Escherichia coli

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ABSTRACT

Eravacycline is a novel fluorocycline antibiotic with potent activity against a broad range of pathogens, including strains with tetracycline and other drug-resistance phenotypes. The goal of the studies was to determine which pharmacokinetic/pharmacodynamic (PK/PD) parameter and magnitude best correlated with efficacy in the murine thigh infection model. Six *E. coli* were utilized for the studies. MICs were determined using CLSI methods and ranged from 0.125 to 0.25 mg/L. A neutropenic murine thigh infection model was utilized for all treatment studies. Single-dose plasma pharmacokinetics were determined in mice after administration of 2.5, 5, 10, 20, 40, and 80 mg/kg. Pharmacokinetic studies exhibited peak concentration (C\text{max}) values of 0.34 to 2.58 mg/L, area under the concentration-time curve (AUC\text{0–∞}) values of 2.44 to 57.6 mg*h/L, and elimination half-lives of 3.9 to 17.6 h. Dose fractionation studies, were performed using total drug doses 6.25 mg/kg to 100 mg/kg fractionated into q6-, q8-, q12-, or q24-hourly regimens. Nonlinear regression analysis demonstrated 24h free drug AUC/MIC (fAUC/MIC) was the PK/PD parameter that best correlated with efficacy (R\text{2} 0.80). In subsequent studies, we used the neutropenic murine thigh infection model to determine if the magnitude of the AUC/MIC needed for the efficacy of eravacycline varied among pathogens. Mice were treated with two-fold increasing doses (range 3.125 to 50 mg/kg) of eravacycline every 12 hours. The mean fAUC/MIC magnitude associated with net stasis and 1-log kill endpoint were 27.97 ± 8.29
INTRODUCTION

Disease due to antibiotic-resistance bacteria are emerging at an alarming rate worldwide, warranting the development of new antimicrobial agents. Eravacycline is a novel synthetic fluorocycline that belongs to the tetracycline class of antibacterial agents and is currently in development for complicated intra-abdominal infection (cIAI) (1) and complicated urinary tract infection (cUTI) (2). Oral and intravenous formulations have been developed. As with other tetracyclines, eravacycline inhibits bacterial protein synthesis through binding to the 30S ribosomal subunit, and demonstrates potent and broad-spectrum antimicrobial activity. Importantly, the drug maintains activity against many multidrug-drug resistant bacteria, including bacteria exhibiting tetracycline-specific efflux and ribosomal protection (2).

E. coli organisms are the predominant pathogens in IAI (3) and UTI, accounting for 47-94% of isolates (4). Given this, we sought to determine the PK/PD index predictive of therapeutic success against E. coli and the magnitude of the PK/PD index associated with stasis and cidal outcomes in the murine neutropenic thigh model.

RESULTS

In vitro susceptibility testing. The median MICs of eravacycline ranged from 0.125-0.25 µg/ml and are shown in Table 1.
Drug pharmacokinetics. Single-dose pharmacokinetics of eravacycline are shown in Figure 1. At the doses studied, eravacycline drug concentrations increased in a dose-dependent manner across the dose range. $C_{\text{max}}$ concentrations ranged from 0.34 to 2.15 mg/liter. AUC$_{0-\infty}$ values ranged from 2.44 to 57.6 mg*h/liter and were linear across the 2.5- to 80-mg dosing range ($R^2$ of 0.99). The elimination half-life ranged from 3.9 to 17.6 h.

PK/PD parameter determination. The dose-response relationships for the four dosing intervals against *E. coli* ATCC 25922 is shown in Figure 2. At the start of therapy, mice had $7.31 \pm 0.21 \log_{10} \text{CFU/thigh}$ of *E. coli* ATCC 25922, and the organism grew $2.15 \pm 0.38 \log_{10} \text{CFU/thigh}$ after 24 h in untreated control mice. Each fractionation arm demonstrated relatively similar concentration-dependent activity as the dose was escalated with the highest doses studied resulting in net cidal activity. The similarity in the dose-response curves for each of the fractionated regimens suggests AUC/MIC is likely the predictive PK/PD index. This is because this parameter is held relatively constant as the total dose administered over 24 hours is the same in each fractionation regimen. In contrast, $C_{\text{max}}$ and time above MIC change proportionally (and inversely) to each other based on dose in each arm.

The relationships between microbiologic effect and each of the pharmacodynamic parameters, 24 hour fAUC/MIC, 24 hour f$C_{\text{max}}$/MIC, and the
percent of time free drug concentrations exceed the MIC over 24 hours against

*E. coli* ATCC 25922 is shown in Figure 3. As with other tetracycline (5-8), the
strongest relationship was seen when results were correlated with the 24h
fAUC/MIC ratio with an $R^2$ value of 0.80. Regression with both the %T>MIC
and $C_{\text{max}}$/MIC result in slightly less robust relationships. Consideration of total
or free drug levels did not appreciably impact the relationships between
efficacy and PK/PD parameters.

**PK/PD magnitude determination.** The dose-response relationships for
each of the six *E. coli* isolates in the neutropenic murine thigh model are
shown in Figure 4. The burden at the start of therapy, growth in untreated
controls (i.e. fitness), and drug effect were relatively similar for each isolate
(see Table 1.) At the start of therapy, mice had $7.32 \pm 0.11 \log_{10}$ CFU/thigh of

*E. coli*. The organisms grew $2.69 \pm 0.40 \log_{10}$ CFU/thigh in untreated control
mice. The maximal reduction in *E. coli* with eravacycline treated mice
compared to untreated controls was $-4.37 \pm 0.48 \log_{10}$ CFU/thigh and
maximum kill from zero hour was $-1.68 \pm 0.50 \log_{10}$ CFU/thigh. Net stasis
was achieved against all strains and a more than 1-log kill against five of six
strains. The relationship between organism burden in the thigh and the
plasma 24h fAUC/MIC ratio is shown in Figure 5. Calculation of the doses
necessary to achieve a static and 1-log kill effect against multiple organisms is
shown in Table 1. Also shown are the associated total and free-drug 24 h
AUC/MIC target ratios necessary to achieve these outcomes. The mean 24h fAUC/MIC values associated with net stasis and 1-log kill endpoints were 28 and 33, respectively.

**DISCUSSION**

Similar to other antibiotic classes, the widespread use of tetracyclines for over 60 years has resulted in an increase in the incidence of tetracycline-resistant infections (9, 10). Recently, the evolution of the tetracycline antibiotics class has been driven by semisynthetic approaches (10). Tigecycline, a glycyclcycline derivative, was developed to enhance activity against tetracycline-resistance bacteria (11). More recently, eravacycline, a novel fluorocycline antibiotic, was developed by a total synthetic method (12) and has been shown to be a potent translation inhibitor against strains expressing acquired tetracycline-specific resistance mechanisms (2).

The present studies demonstrated eravacycline activity against a diverse group of *E. coli* strains. This potent activity has been observed in previous in vitro and in vivo studies against both *E. coli* and MRSA (2, 12-14). We observed cidal activity against all isolates and the shape of the exposure-response curves were quite steep in which small increases in drug exposure resulted in large increases in cidal activity. Additionally, this in vivo efficacy was observed against multi-drug resistant strains expressing a variety of tetracycline and ESBL genotypes and phenotypes. Similar to previous
studies, we demonstrated the PK/PD parameter 24 h AUC/MIC was the most predictive PK/PD parameter for efficacy (5-7). Specifically, the dose-response relationship against E. coli was not impacted by a change in dosing interval and pharmacodynamic regression was strongest with the 24h AUC/MIC index.

There is a paucity of pharmacodynamic target identification studies with the tetracycline class, especially with gram-negative pathogens. In vitro and in vivo studies with doxycycline against S. aureus identified the fAUC/MIC target associated with net stasis was a value near 25 (15). A similar study using doxycycline for Streptococcus pneumoniae found fAUC/MIC targets of 24 was associated with net stasis (16). Eravacycline itself has been studied in a previous thigh model exploring the PK/PD target against a single strain of methicillin-resistant S. aureus (17). The AUC/MIC value associated with stasis was relatively similar to the doxycycline target at a total drug AUC/MIC value of 38.4 (fAUC/MIC of approximately 10). The total drug AUC/MIC target needed to achieve a 1-log kill was modestly higher than stasis at 46.9. The pharmacodynamics characteristics of eravacycline against multiple E. coli isolates in the present study are quite similar. Importantly, the PK/PD target was similar across wild type strains as well as those with distinct resistance mechanism. Specifically, the fAUC/MIC numeric targets for net stasis and 1-log kill endpoints were noted at values of 27 and 33, respectively. The steep nature of the exposure response relationship across these treatment
endpoints was also congruent with the earlier investigations. These values are also comparable to the targets for the glycycycline, tigecycline, in clinical trials. In an exposure-response study of tigecycline in patients with community acquired pneumonia found a free drug AUC/MIC >12.5 increased likelihood of cure (18). The pharmacodynamic AUC/MIC value near 25 is likely to be relevant in for clinical studies with eravacycline and should be considered in the design of optimal dosing regimens.

In conclusion, eravacycline exhibited potent in vitro and in vivo efficacy against E. coli. The PK/PD index AUC/MIC was most strongly associated with efficacy. Free drug AUC/MIC targets were 28 and 33 for stasis and 1-log kill endpoints, respectively. These animal model PK/PD targets should be useful for dosing regimen design and the development of susceptibility breakpoints.

MATERIALS AND METHODS

Organisms, media, and antibiotic. Six E. coli strains were used for these studies (Table 1). The strains were chosen to include common tetracycline and beta-lactam resistance phenotypes. They were grown, sub-cultured, and quantified using Mueller-Hinton broth (MHB) and agar (Difco Laboratories, Detroit, MI). Eravacycline for in vitro and in vivo studies was supplied by the study sponsor (Tetraphase Pharmaceuticals, Inc., Watertown, MA). Compound was prepared by reconstitution in sterile water and subsequent
dilution in sterile 0.9% normal saline solution.

**In vitro susceptibility testing.** The MICs of eravacycline for the various isolates were determined using Clinical and Laboratory Standards Institute (CLSI) microdilution methods (19, 20). All MIC assays were performed in duplicate on three separate occasions. The median MIC of replicate assays is reported and utilized in PK/PD analyses.

**Murine thigh infection model.** Animals for the present studies were maintained in accordance with criteria of the American Association for Accreditation of Laboratory Animal Care criteria (21). All animal studies were approved by the Animal Research Committee of the William S. Middleton Memorial Veterans Hospital. Six-week-old, specific-pathogen-free, female ICR/Swiss mice weighing 23 to 27 g were used for all studies (Harlan Sprague-Dawley, Indianapolis, IN). Mice were rendered neutropenic (neutrophils, <100/mm$^3$) by injecting them with cyclophosphamide (Mead Johnson Pharmaceuticals, Evansville, IN) subcutaneously 4 days (150 mg/kg) and 1 day (100 mg/kg) before thigh infection. Previous studies have shown that this regimen produces neutropenia in this model for 5 days (22). Broth cultures of freshly plated bacteria were grown to logarithmic phase overnight to an absorbance of 0.3 at 580 nm...
After a 1:10 dilution into fresh Mueller-Hinton broth, bacterial counts of the inoculum ranged from $10^{7.0}$ to $10^{7.4}$ CFU/mL. Thigh infections with each of the isolates were produced by injection of 0.1 ml of inoculum into the thighs of isoflurane-anesthetized mice. Eravacycline therapy was initiated 2 h after the infection procedure. After 24 h, the animals were euthanized and thighs aseptically removed, homogenized, and plated for CFU determination. No treatment and zero-hour controls were included in all experiments.

**Drug pharmacokinetics.** Single-dose plasma pharmacokinetics of eravacycline were performed in mice uninfected mice. Animals were administered single intraperitoneal doses (0.2 mL/dose) of eravacycline at dose levels of 2.5, 5, 10, 20, 40 and 80 mg/kg. Groups of three mice were sampled at each time point (seven time points, consisting of 1, 2, 3, 4, 6, 8, 12, and 18 h) and dose level. Samples were then centrifuged for 5 min at 4,000 rpm, and plasma was removed and frozen at -20 ºC until assay. Plasma concentrations were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS) by the sponsor. Internal standards ranged from 10 – 5000 ng/mL and were linear ($R^2$ 0.99) over the measurement range. The lower limit of detection of was 10 ng/mL. Inter- and intra-assay coefficient of variation (CV) was <10%. Pharmacokinetic parameters, including elimination half-life ($T_{1/2}$), area under the concentration-time curve ($AUC_{0-\infty}$), and peak
concentrations (C\text{\text{max}}) were calculated using a non-compartmental model. The T_{1/2} was determined by linear least-squares regression. The AUC_{0-\infty} was calculated from the mean concentrations using the trapezoidal rule. Pharmacokinetic estimates for dose levels that were not directly measured were calculated using linear interpolation for dose levels between those with measured kinetics and linear extrapolation for dose levels above or below the highest and lowest dose levels with kinetic measurements. A previous study of eravacycline demonstrated a non-linear relationship between eravacycline concentration and level of protein binding (23). This relationship was well described by the formula: \( y = -0.085 \ln(x) + 0.2752 \), where \( x \) is eravacycline total drug concentration and \( y \) is the percent free drug. This equation was used in the current study to calculate free drug concentrations for analysis.

**PK/PD parameter determination.** A dose fractionation study was undertaken to determine the PK/PD index (AUC/MIC, C\text{\text{max}}/MIC or Time Above MIC) that was predictive of efficacy for eravacycline. Two-fold increasing doses (range 6.25 mg/kg to 100 mg/kg) of eravacycline were fractionated into q6, q8, q12, and q24 h dosing regimens. Mice were infected with isolate ATCC 25922 as described above and administered eravacycline by IP injection according to the dosing regimen prescribed in the fractionation design. After 24 h the mice were euthanized and CFU count determined in the thighs. To determine which PK/PD index was most closely linked with efficacy, the number of bacteria in...
the thigh at the end of 24 h of therapy was correlated with (i) the free $C_{\text{max}}$/MIC ratio ($fC_{\text{max}}$/MIC), (ii) the 24-hour free AUC/MIC ratio ($f\text{AUC}$/MIC), and (iii) the percentage of the dosing interval during which plasma free drug levels exceeded the MIC for each of the dosage regimens studied ($%T$/MIC). The correlation between efficacy and each of the three PK/PD indices was determined by nonlinear least-squares multivariate regression derived from the Hill equation $E = (E_{\text{max}} \times D^N)/(ED_{50}^N + D^N)$, where $E$ is the effector, in this case, the log change in CFU per thigh between treated mice and untreated controls after the 24-h period of study, $E_{\text{max}}$ is the maximum effect, $D$ is the 24-h total dose, $ED_{50}$ is the dose required to achieve 50% of the $E_{\text{max}}$, and $N$ is the slope of the dose-effect curve. The values for indices $E_{\text{max}}$, $ED_{50}$, and $N$ were calculated using nonlinear least-squares regression. The coefficient of determination ($R^2$) was used to estimate the variance that might be due to regression with each of the PK/PD indices. Given the prolonged half-life in the animals, drug accumulation was accounted for in multi-dosing regimens. The fraction of drug remaining prior to the next administration was calculated using the formula $f = e^{-k\tau}$, where $f$ is the fraction of drug remaining, $e$ is Euler’s number (2.71828), $k$ is the terminal elimination rate constant, and $\tau$ is the dosing interval (24).

**PK/PD parameter magnitude studies** Dose-response experiments using the thigh model were performed for six $E. coli$ isolates as described in methods.
above. The dose range consisted of two-fold increases (range 3.125 to 50 mg/kg/12 h) in drug concentration with administration by IP route. The dose-response relationships were quantified and the relationship between the PK/PD parameter AUC/MIC and treatment efficacy using the sigmoid E\textsubscript{max} (hill) model was performed using Sigma Plot version 12.3, Systat Software, San Jose, CA). These PK/PD relationships were examined utilizing the plasma total and free drug concentrations from pharmacokinetic studies. The coefficient of determination (R\textsuperscript{2}) from this model was used to numerically quantify the strength of this relationship. This coefficient represents the percentage of the variance in bacterial numbers that can be attributed to the PK/PD parameter. The doses required for a static effect (static dose) and 1-log kill (1-log kill dose) compared to the start of therapy for multiple E. coli pathogens in the thigh model were determined utilizing the plasma total and free drug concentrations using the following equation:

\[
\log_{10} D = \log_{10}\left[\frac{E/(E_{\text{max}} - E)}{N}\right] + \log ED_{50}
\]

Where E is the growth from zero hour, E\textsubscript{max} is the maximum effect, ED\textsubscript{50} is the dose required to achieve 50% of the E\textsubscript{max}, and N is the slope of the dose-effect curve, and D is the dose required to achieve net stasis. For 1-log kill, the E was set to growth from zero hour plus 1 in order to calculate dose (D) for 1-log kill. The associated 24 h total and free drug AUC/MIC targets for each organism were calculated.
This study was funded by Tetraphase Pharmaceuticals.
Table 1. In vitro and in vivo efficacy of Eravacycline against select *E. coli*.

<table>
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<tr>
<th>Isolate</th>
<th>Comment</th>
<th>MIC (mg/L)</th>
<th>Bacterial burden at start of therapy (log_{10} CFU/ thigh)</th>
<th>Growth in controls at 24 h (log_{10} CFU/ thigh)</th>
<th>Maximum kill CFU/ thigh at 24 h from 0 h (Δlog_{10} CFU/ thigh)</th>
<th>24 h dose IAUC/MIC (mg/kg)</th>
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*NA, not achieved*
FIGURE 1. Single dose plasma pharmacokinetics of eravacycline. Six different doses that varied by 2-fold concentration on a mg/kg basis were administered to mice by IP route. Groups of three mice were sampled for each time point. Each symbol represents the mean ± SD from three animals. Shown in the legend is the maximum plasma concentration (C_{max}), the area under the concentration curve from 0-∞ (AUC), and the elimination half-life (T_{1/2}).

FIGURE 2. In vivo dose fractionation with eravacycline using a neutropenic mouse thigh model. Each symbol represents the mean and standard deviation from four thighs infected with *E. coli* ATCC 25922. The error bars represent the standard deviation. The burden of organisms was measured at the start and end of therapy. Five total drug (mg/kg/24 h) dose levels were fractionated into one of four dosing regimens and is shown on the x-axis. The y-axis represents the change in organism burden from the start of therapy. The dashed horizontal line represents net stasis over the treatment period. Points above the line represent net growth and points below represent net killing (cidal activity).

Figure 3. Impact of pharmacodynamic regression of the in vivo dose fractionation study with eravacycline against *E. coli* ATCC 25922. Each symbol represents the mean and standard deviation from four thighs. The dose data is expressed as fAUC/MIC, fC_{max}/MIC, and the percentage of time plasma free drug concentrations exceed the MIC (Time above MIC). The R^2 is the coefficient of determination. Also shown for each PD index is the maximal effect (E_{max}), the PD index value associated with 50% of the maximal effect (ED_{50}), and the slope of the relationship or the Hill coefficient (N). The line drawn through the data points is the best fit line based upon the sigmoid E_{max} formula.

Figure 4. In vivo dose effect of eravacycline against six *E. coli* strains using a neutropenic mouse thigh model. Each symbol represents the mean and standard deviation from four thighs. Five total drug dose levels were fractionated into an every 12 h regimen. The burden of organisms was measured at the start and end of therapy. The study period was 24 hours. The horizontal dashed line at 0 represents the burden of organisms in the thighs of mice at the start of therapy. Data points below the line represent killing and points above the line represent growth.

Figure 5. In vivo dose effect of eravacycline against six *E. coli* isolates using a neutropenic mouse thigh model. Eravacycline exposure is expressed as the free drug 24 h AUC/MIC (fAUC/MIC). The R^2 represents the coefficient of determination. The ED_{50} represents the AUC/MIC associated with 50% of the maximal effect (E_{max}) and N is the slope of the relationship or the Hill coefficient. The line drawn through the data points is the best fit line based
upon the sigmoid $E_{\text{max}}$ formula. The dashed line represents the burden at the start of therapy. Points above the line represent net growth and those below the line represent killing.

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