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EXPERIMENTAL THERAPEUTICS

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

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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 *Escherichia coli* isolates were utilized for the studies. MICs were determined using CLSI methods and ranged from 0.125 to 0.25 mg/liter. 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 of body weight. Pharmacokinetic studies exhibited maximum plasma concentration (C_{max}) values of 0.34 to 2.58 mg/liter, area under the concentration-time curve (AUC) from time zero to infinity ($AUC_{0-\infty}$) values of 2.44 to 57.6 mg · h/liter, and elimination half-lives of 3.9 to 17.6 h. Dose fractionation studies were performed using total drug doses of 6.25 mg/kg to 100 mg/kg fractionated into 6-, 8-, 12-, or 24-h regimens. Nonlinear regression analysis demonstrated that the 24-h free drug AUC/MIC (*f*AUC/MIC) was the PK/PD parameter that best correlated with efficacy ($R^2 = 0.80$). 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 2-fold increasing doses (range, 3.125 to 50 mg/kg) of eravacycline every 12 h. The mean *f*AUC/MIC magnitudes associated with the net stasis and the 1-log-kill endpoints were 27.97 ± 8.29 and 32.60 ± 10.85, respectively.

KEYWORDS eravacycline, pharmacodynamics, *Escherichia coli*

Received 5 February 2017 **Returned for modification** 21 March 2017 **Accepted** 11 April 2017

Accepted manuscript posted online 17 April 2017

Citation Zhao M, Lepak AJ, Marchillo K, VanHecker J, Andes DR. 2017. *In vivo* pharmacodynamic target assessment of eravacycline against *Escherichia coli* in a murine thigh infection model. Antimicrob Agents Chemother 61:e00250-17. https://doi.org/10.1128/AAC.00250-17.

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Diseases due to antibiotic-resistant 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 the treatment of 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 drug-resistant bacteria, including bacteria exhibiting tetracycline-specific efflux and ribosomal protection (2).

Escherichia coli organisms are the predominant pathogens in intra-abdominal infections (3) and urinary tract infections, accounting for 47 to 94% of isolates (4). Given this,

we sought to determine the pharmacokinetic (PK)/pharmacodynamic (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 neutropenic murine thigh infection model.

RESULTS

***In vitro* susceptibility testing.** The median MICs of eravacycline ranged from 0.125 to 0.25 μg/ml and are shown in Table 1.

Drug pharmacokinetics. The single-dose pharmacokinetics of eravacycline are shown in Fig. 1. At the doses studied, eravacycline drug concentrations increased in a dose-dependent manner across the dose range. The maximum plasma concentrations (C_{max}) ranged from 0.34 to 2.15 mg/liter. The area under the concentration-time curve (AUC) from time zero to infinity ($AUC_{0-\infty}$) values ranged from 2.44 to 57.6 mg · h/liter and were linear across the 2.5- to 80-mg dosing range ($R^2 = 0.99$). The elimination half-life ($t_{1/2}$) 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 are shown in Fig. 2. At the start of therapy, mice had 7.31 $\pm$ 0.21 $\log_{10}$ CFU/thigh of *E. coli* ATCC 25922, and the organism grew 2.15 $\pm$ 0.38 $\log_{10}$ 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 that 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 h is the same in each fractionation regimen. In contrast, C_{max} and the time above the MIC changed proportionally (and inversely) to each other on the basis of dose in each arm.

The relationships between microbiologic effect and each of the pharmacodynamic parameters 24-h free drug AUC/MIC (*f*AUC/MIC), 24-h free-drug C_{max}/MIC (fC_{max}/MIC), and the percentage of time that the free drug concentration exceeded the MIC (percent T_{MIC}) over 24 h against *E. coli* ATCC 25922 are shown in Fig. 3. As with other tetracyclines (5–8), the strongest relationship was seen when the results were correlated with the 24-h *f*AUC/MIC ratio, with an R^2 value of 0.80. Regression with both the percent T_{MIC} and C_{max}/MIC resulted 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 infection model are shown in Fig. 4. The burden at the start of therapy, growth in untreated controls (i.e., fitness), and drug effect were relatively similar for each isolate (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 the count of *E. coli* in eravacycline-treated mice compared to that in the untreated controls was -4.37 ± 0.48 $\log_{10}$ CFU/thigh, and the maximum kill from zero hour was -1.68 ± 0.50 $\log_{10}$ CFU/thigh. Net stasis was achieved against all strains, and a more than 1-log kill was achieved against five of six strains. The relationship between the organism burden in the thigh and the plasma 24-h *f*AUC/MIC ratio is shown in Fig. 5. The doses necessary to achieve a static and a 1-log-kill effect against multiple organisms are 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 24-h *f*AUC/MIC values associated with the 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 antibiotic class has been driven by semisynthetic approaches (10). Tigecycline, a glycylcycline derivative, was developed to

TABLE 1 *In vitro* and *in vivo* efficacies of eravacycline against select *E. coli* isolates[a]

E. coli isolate	Comment	MIC (mg/liter)	Bacterial burden at start of therapy (log_{10} no. of CFU/thigh)	Growth in controls at 24 h (log_{10} no. of CFU/thigh)	No. of CFU/thigh for maximum kill at 24 h from 0 h (Δlog_{10} no. of CFU/thigh)	Stasis			1-Log kill		
						24-h dose (mg/kg)	24-h *t*AUC/MIC	24-h *f*AUC/MIC	24-h dose (mg/kg)	24-h *t*AUC/MIC	24-h *f*AUC/MIC
1-894-1	Tet^r	0.125	7.12	2.975	−1.74	15.66	92.88	30.91	26.73	119.84	37.90
1135	Tet(M), ESBL	0.25	7.37	3.13	−1.65	27.81	60.55	19.11	49.19	88.74	25.34
355	Tet (B), ESBL	0.125	7.33	2.975	−0.78	16.85	98.16	32.27	NA		
14714-1	ESBL	0.125	7.45	2.1875	−2.13	14.82	89.18	29.95	17.13	99.39	32.59
102-94090	ESBL	0.25	7.35	2.615	−1.61	18.34	52.35	16.98	31.44	62.67	19.67
ATCC 25922	ATCC	0.125	7.31	2.26	−2.17	29.08	122.58	38.61	45.87	162.23	47.52
Mean			7.32	2.69	−1.68	20.43	85.95	27.97	34.07	106.58	32.60
Median			7.34	2.80	−1.70	17.60	91.03	30.43	31.44	99.39	32.59
SD			0.11	0.40	0.50	6.34	25.78	8.29	13.37	37.32	10.85

[a]Δlog_{10} no. of CFU/thigh, change in the log_{10} number of CFU per thigh; *t*AUC, total drug AUC; NA, not achieved.

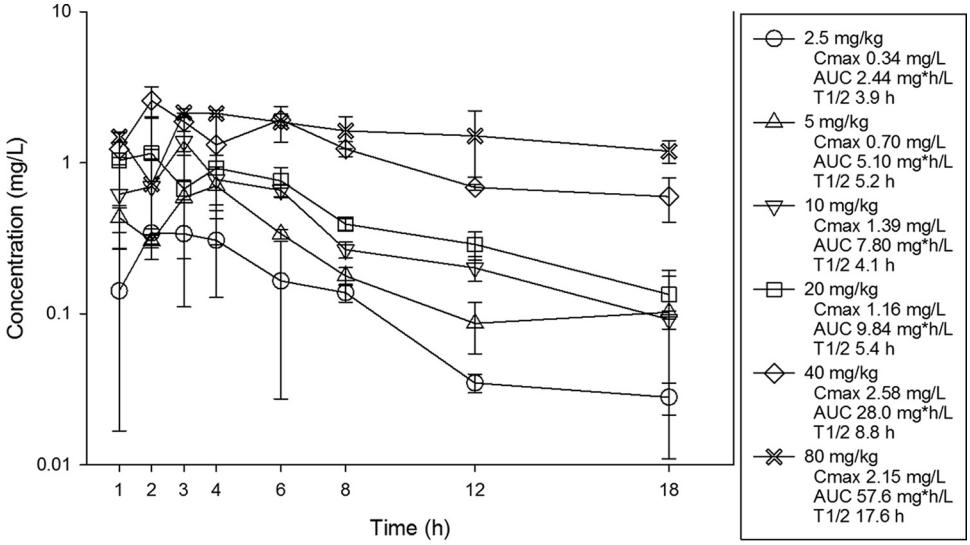

FIG 1 Single-dose plasma pharmacokinetics of eravacycline. Six different doses that varied by a 2-fold concentration on a milligram-per-kilogram basis were administered to mice by the i.p. route. Groups of three mice were sampled for each time point. Each symbol represents the mean ± SD for three animals. Shown in the legend is the maximum plasma concentration (C_{max}), the area under the concentration-time curve from time zero to infinity ($AUC_{0-\infty}$), and the elimination half-life ($t_{1/2}$).

enhance activity against tetracycline-resistant 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 the activity of eravacycline 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 methicillin-resistant *Staphylococcus aureus* (2, 12–14). We observed cidal activity against all isolates, and the shapes of the exposure-

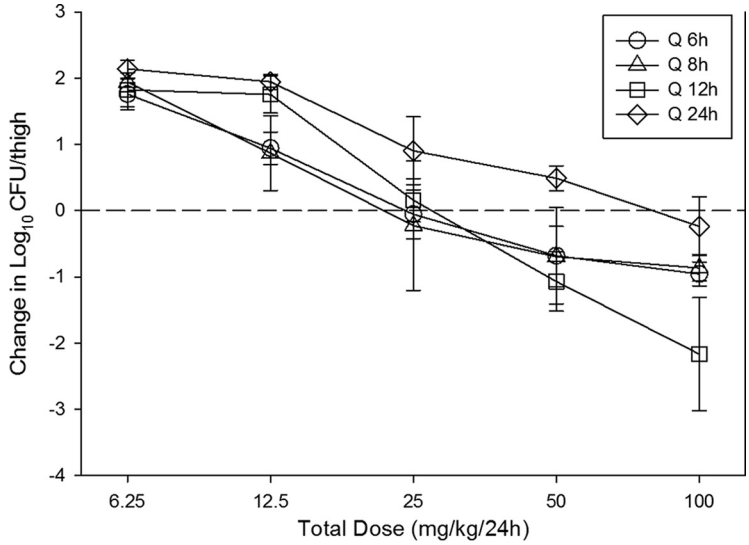

FIG 2 *In vivo* dose fractionation with eravacycline using a neutropenic murine thigh infection model. Each symbol represents the mean and standard deviation for four thighs infected with *E. coli* ATCC 25922. The error bars represent the standard deviations. The burden of organisms was measured at the start and end of therapy. Five total drug dose levels (in milligrams per kilogram every 24 h) were fractionated into one of four dosing regimens and are 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 the line represent net killing (cidal activity). Q 6h, Q 8h, Q 12h, and Q 24h, dosing every 6, 8, 12, and 24 h, respectively.

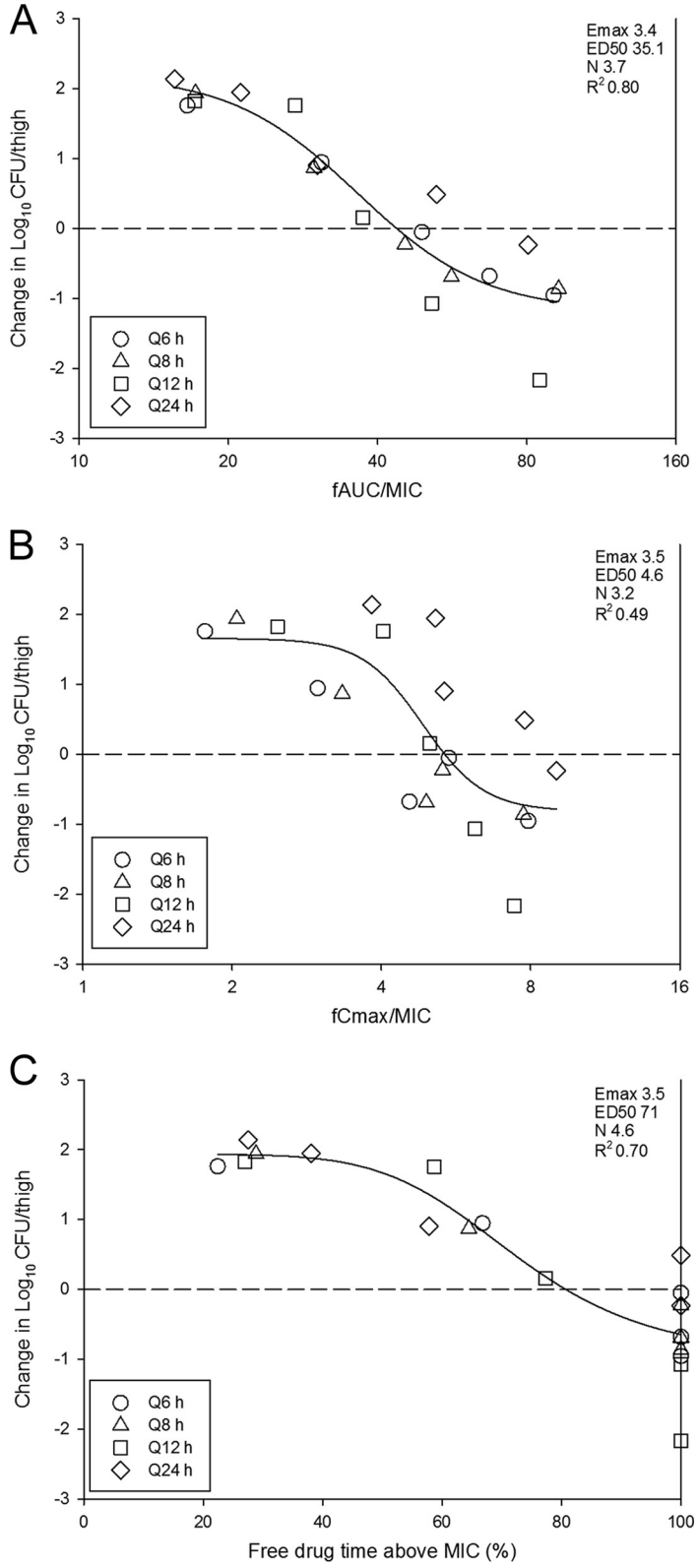

FIG 3 Impact of pharmacodynamic regression of the *in vivo* dose fractionation study with eravacycline against *E. coli* ATCC 25922. Each symbol represents the mean and standard deviation for four thighs. The dose data are expressed as *f*AUC/MIC (A), fC_{max}/MIC (B), and the percentage of time that the plasma free drug concentrations exceed the MIC (Free drug time above MIC) (C). 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

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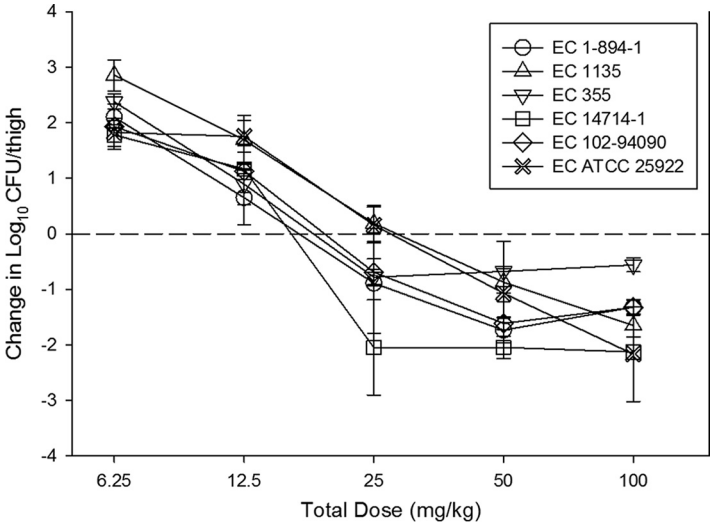

FIG 4 *In vivo* dose-effect of eravacycline against six *E. coli* (EC) strains using a neutropenic murine thigh infection model. Each symbol represents the mean and standard deviation for four thighs. Five total drug dose levels were fractionated into a regimen given every 12 h. The burden of organisms was measured at the start and end of therapy. The study period was 24 h. 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.

response curves were quite steep, with small increases in drug exposure resulting in large increases in cidal activity. Additionally, this *in vivo* efficacy was observed against multidrug-resistant strains expressing a variety of tetracycline and extended-spectrum β-lactamase (ESBL) genotypes and phenotypes. Similar to previous studies, we demonstrated that the PK/PD parameter 24-h AUC/MIC was the most predictive of efficacy (5–7). Specifically, the dose-response relationship against *E. coli* was not impacted by a change in the dosing interval, and pharmacodynamic regression was the strongest with the 24-h 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 an *f*AUC/MIC target value near 25 to be associated with net stasis (15). A similar study using doxycycline for *Streptococcus pneumoniae* found that an *f*AUC/MIC target of 24 was associated with net stasis (16). Eravacycline itself has been studied in a previous thigh infection 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 (*f*AUC/MIC, approximately 10). The total drug AUC/MIC target needed to achieve a 1-log kill was modestly higher than that needed to achieve stasis at 46.9. The pharmacodynamic characteristics of eravacycline against multiple *E. coli* isolates in the present study were quite similar. Importantly, the PK/PD target was similar across wild-type strains as well as those with distinct resistance mechanisms. Specifically, the *f*AUC/MIC numeric targets for the net stasis and 1-log-kill endpoints were noted at values of 28 and 33, respectively. The steep nature of the exposure-response relationship across these treatment endpoints was also congruent with the findings of the earlier investigations. These values are also comparable to the targets for the glycylcycline tigecycline in clinical trials. An exposure-response study of tigecycline in patients with community-acquired pneumonia found that a free drug AUC/MIC

FIG 3 Legend (Continued)
through the data points is the best-fit line based upon the sigmoid E_{max} formula. The dashed horizontal line represents net stasis over the treatment period. Points above the line represent net growth, and points below the line represent net killing (cidal activity).

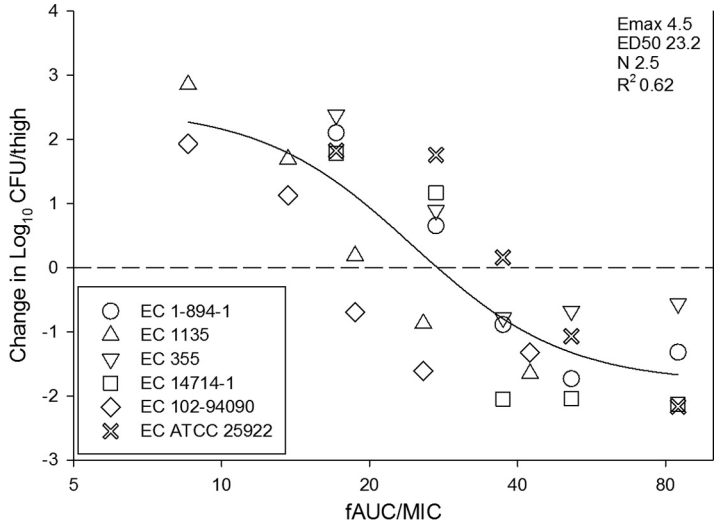

FIG 5 *In vivo* dose-effect of eravacycline against six *E. coli* isolates using a neutropenic murine thigh infection model. Eravacycline exposure is expressed as the free drug 24-h AUC/MIC (*f*AUC/MIC). R^2 represents the coefficient of determination. The ED_{50} represents the AUC/MIC associated with 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_{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.

of >12.5 increased the likelihood of cure (18). A pharmacodynamic AUC/MIC value near 25 is likely to be relevant in 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 the index that was the most strongly associated with efficacy. Free drug AUC/MIC targets were 28 and 33 for the 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 those with common tetracycline and beta-lactam resistance phenotypes. They were grown, subcultured, 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). The 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 from replicate assays is reported and was utilized in the PK/PD analyses.

Murine thigh infection model. Animals for the present studies were maintained in accordance with the criteria of the Association for Assessment and Accreditation of Laboratory Animal Care (21). All animal studies were approved by the Animal Research Committee of the William S. Middleton Memorial VA 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 (neutrophil count, $<100/mm^3$) 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 overnight to logarithmic phase to an absorbance at 580 nm of 0.3 (Spectronic 88; Bausch and Lomb, Rochester, NY). After 1:10 dilution into fresh Mueller-Hinton broth, the 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 the thighs were aseptically removed, homogenized, and plated for determination of the number of CFU. No-treatment and zero-hour controls were included in all experiments.

Drug pharmacokinetics. The single-dose plasma pharmacokinetics of eravacycline were determined in 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 of body weight. Groups of three mice were sampled at each time point (eight 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°C until assay. Plasma concentrations were determined by the sponsor using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Internal standards ranged from 10 to 5,000 ng/ml and were linear ($R^2 = 0.99$) over the measurement range. The lower limit of detection was 10 ng/ml. The inter- and intra-assay coefficient of variation (CV) was <10%. Pharmacokinetic parameters, including elimination half-life ($t_{1/2}$), the area under the concentration-time curve from time zero to infinity ($AUC_{0-\infty}$), and the maximum plasma concentration (C_{max}), were calculated using a noncompartmental model. $t_{1/2}$ was determined by linear least-squares regression. $AUC_{0-\infty}$ 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 nonlinear relationship between the eravacycline concentration and the level of protein binding (23). This relationship was well described by the formula $y = -0.085 \ln(x) + 0.2752$, where x is the 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_{max}/MIC, or time above the MIC) that was predictive of efficacy for eravacycline. Twofold increasing doses (range, 6.25 mg/kg to 100 mg/kg) of eravacycline were fractionated into regimens of dosing every 6, 8, 12, and 24 h. Mice were infected with isolate ATCC 25922 as described above and administered eravacycline by intraperitoneal (i.p.) injection according to the dosing regimen prescribed in the fractionation design. After 24 h the mice were euthanized and the numbers of CFU in the thighs were determined. To determine which PK/PD index was the 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 drug C_{max}/MIC ratio (fC_{max}/MIC), (ii) the 24-h free drug AUC/MIC ratio (*f*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 (percent 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, as follows: $E = (E_{max} \times D^N)/(ED_{50}^N + D^N)$, where E is the effector, in this case, the log change in the number of CFU per thigh between treated mice and untreated controls after the 24-h period of study, E_{max} is the maximum effect, D is the 24-h total dose, ED_{50} is the dose required to achieve 50% of the E_{max}, and N is the slope of the dose-effect curve. The values for the indices E_{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 multidosing regimens. The fraction of drug remaining prior to the next administration was calculated using the formula $f = e^{-k \cdot tau}$, 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 were performed for six *E. coli* isolates using the thigh infection model as described above. The dose range consisted of 2-fold increases (range, 3.125 to 50 mg/kg/12 h) in the drug concentration with administration by the i.p. route. The dose-response relationships were quantified and the relationship between the PK/PD parameter AUC/MIC and treatment efficacy was determined using the sigmoid E_{max} (Hill) model in SigmaPlot software (version 12.3; Systat Software, San Jose, CA). These PK/PD relationships were examined utilizing the plasma total and free drug concentrations from the pharmacokinetic studies. The coefficient of determination (R^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 number of bacteria at the start of therapy for multiple *E. coli* pathogens in the thigh infection model were determined utilizing the plasma total and free drug concentrations and the following equation: $\log_{10} D = \{\log_{10}[E/(E_{max} - E)]/N\} + \log_{10} ED_{50}$, where E is the growth from zero hour, E_{max} is the maximum effect, ED_{50} is the dose required to achieve 50% of the E_{max}, N is the slope of the dose-effect curve, and D is the dose required to achieve net stasis. For 1-log kill, E was set to growth from zero hour plus 1 in order to calculate the dose (D) for 1-log kill. The associated 24-h total and free drug AUC/MIC targets for each organism were calculated.

ACKNOWLEDGMENT

This study was funded by Tetraphase Pharmaceuticals, Inc.

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