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In Vivo Pharmacodynamic Evaluation of an FtsZ Inhibitor, TXA-709, and Its Active Metabolite, TXA-707, in a Murine Neutropenic Thigh Infection Model

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Antibiotics with novel mechanisms of action are urgently needed. Processes of cellular division are attractive targets for new drug development. FtsZ, an integral protein involved in cell cytokinesis, is a representative example. In the present study, the pharmacodynamic (PD) activity of an FtsZ inhibitor, TXA-709, and its active metabolite, TXA-707, was evaluated in the neutropenic murine thigh infection model against 5 *Staphylococcus aureus* isolates, including both methicillin-susceptible and methicillin-resistant isolates. The pharmacokinetics (PK) of the TXA-707 active metabolite were examined after oral administration of the TXA-709 prodrug at 10, 40, and 160 mg/kg of body weight. The half-life ranged from 3.2 to 4.4 h, and the area under the concentration-time curve (AUC) and maximum concentration of drug in serum (C_{max}) were relatively linear over the doses studied. All organisms exhibited an MIC of 1 mg/liter. Dose fractionation demonstrated the area under the concentration-time curve over 24 h in the steady state divided by the MIC (AUC/MIC ratio) to be the PD index most closely linked to efficacy ($R^2 = 0.72$). Dose-dependent activity was demonstrated against all 5 isolates, and the methicillin-resistance phenotype did not alter the pharmacokinetic/pharmacodynamic (PK/PD) targets. Net stasis was achieved against all isolates and a 1-$\log_{10}$ kill level against 4 isolates. PD targets included total drug 24-h AUC/MIC values of 122 for net stasis and 243 for 1-$\log_{10}$ killing. TXA-709 and TXA-707 are a promising novel antibacterial class and compound for *S. aureus* infections. These results should prove useful for design of clinical dosing regimen trials.

Methicillin-resistant *Staphylococcal aureus* (MRSA) infections are a major public health threat (1). In the United States, *S. aureus* is the most common cause of nosocomial infection and leads to more than 80 thousand illnesses and 11 thousand deaths yearly (2). Ambulatory visits for cases of skin and soft tissue infection (SSTI) continue to increase in number and amounted to more than 14 million in a 2005 survey (3). Methicillin-resistant *S. aureus* infections account for a disproportionate rise in the incidence of those cases and in the need for hospitalization and unfortunately have limited therapeutic options (3–6). Novel antimicrobial agents that target cellular functions distinctly different from those targeted by current therapies and that show activity against drug-resistant isolates are urgently needed (7–10). Bacterial cell division represents an attractive area for antibiotic research to meet these needs.

FtsZ is a major functional protein involved in cell division through formation of a Z-ring polymeric structure of FtsZ subunits (11–13). Disruption of this process leads to inhibition of cell division and eventual cell death (14). In addition to its novel mechanism of action, FtsZ is an attractive drug target because it is highly conserved in bacteria but absent in eukaryotic cells.

We describe a pharmacodynamic (PK) evaluation of a methoxybenzamide FtsZ inhibitor, TXA-709, and its active metabolite, TXA-707, in a murine neutropenic thigh infection model against *S. aureus*. The impact of dose and dosing regimen on the *in vivo* efficacy of this drug was assessed. Specifically, the studies included were designed to (i) determine the pharmacokinetic/pharmacodynamic (PK/PD) index (peak serum level divided by the MIC [C_{max}/MIC], area under the concentration-time curve over 24 h in the steady state divided by the MIC [AUC/MIC ratio], or duration of time serum levels exceed the MIC [time above MIC]) associated with optimal drug efficacy and (ii) identify the magnitude of the PK/PD index value required for efficacy among multiple *S. aureus* isolates, including those with beta-lactam resistance.

MATERIALS AND METHODS

Organisms, media, and antibiotics. Five isolates of *Staphylococcus aureus* (4 methicillin-resistant isolates and 1 methicillin-susceptible isolate) were studied (Table 1). The methicillin-resistant isolates included both hospital-acquired isolates and community-acquired isolates and three U.S. genotypes. Organisms were grown, subcultured, and quantified using Mueller-Hinton broth (MHB) and agar (Difo Laboratories, Detroit, MI). Prodrug TXA-709 and the active metabolite TXA-707 were supplied by the sponsor of the study, Taxis Pharmaceuticals, Inc.

***In vitro* susceptibility testing.** MICs were determined in MHB using standard CLSI microdilution techniques (15). All MIC tests were performed in duplicate and on two separate occasions.

Murine thigh infection model. The neutropenic-mouse thigh infection model was used for *in vivo* study of TXA-709 and TXA-707 (TXA-709/707) (16). Animals were maintained in accordance with the American

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TABLE 1 *In vitro* antimicrobial susceptibility of selected *S. aureus* isolates to TXA-707[a]

Isolate	TXA-707 MIC (mg/liter)	Phenotype	Pulsed-field gel electrophoresis genetic lineage
ATCC 25923	1	MSSA	
307109	1	MRSA	
R-2527	1	MRSA	USA 300
ATCC 33591	1	MRSA	USA 200
MW2	1	MRSA	USA 400

[a] In addition to the antimicrobial susceptibility data, the presence or absence of beta-lactam (methicillin) resistance is shown for each isolate. MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*.

Association for Accreditation of Laboratory Animal Care (AAALAC) criteria (17). All animal studies were approved by the Animal Research Committees of the William S. Middleton Memorial VA Hospital and the University of Wisconsin. Six-week-old, specific-pathogen-free, female ICR/Swiss mice (Harlan Sprague-Dawley, Indianapolis, IN) weighing 23 to 27 g were used for all studies. Mice were rendered neutropenic (neutrophils = $<100/mm^3$) by injecting cyclophosphamide (Mead Johnson Pharmaceuticals, Evansville, IN) intraperitoneally 4 days (150 mg/kg of body weight) and 1 day (100 mg/kg) before thigh infection. Previous studies have shown that this regimen produces neutropenia in this model for 5 days (18). Broth cultures of freshly plated bacteria were grown to the logarithmic phase overnight to an absorbance of 0.3 at 580 nm using a Spectronic 88 spectrophotometer (Bausch and Lomb, Rochester, NY). After a 1:10 dilution into fresh MHB, bacterial counts of the inoculum ranged from $10^{6.5}$ to $10^{6.7}$ 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 2 h before therapy with TXA-709.

Drug pharmacokinetics. Analysis of the single-dose serum pharmacokinetics of the active drug, TXA-707, was performed in the same animal model. Dose levels of the prodrug, TXA-709, included 10, 40, and 160 mg/kg administered by the oral route. Groups of three mice were sampled at each time point (6 total time points) for each dose level. Sampling times ranged from 1 to 24 h. Serum concentrations of the active metabolite (TXA-707) and prodrug (TXA-709) were determined by the sponsor of the study using liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques. The assay limit of quantification was 2.5 ng/ml, and the coefficient of variation was less than 10%. The values corresponding to the pharmacokinetic parameters, including the elimination half-life ($t_{1/2}$), 24-h area under the drug concentration-time curve (AUC), and maximum concentration of drug in serum (C_{max}), were calculated using a noncompartmental model. The half-life was determined by linear least-squares regression. The AUC was calculated from the mean concentrations using the trapezoidal rule. The pharmacokinetic estimates for dose levels that were not measured were calculated using linear interpolation for dose levels between those with measured kinetics (e.g., between 10 and 40 mg/kg) and linear extrapolation for dose levels greater than or less than the highest and lowest dose levels with kinetic measurements (i.e., 10 and 160 mg/kg). Previous (unpublished) investigations by the sponsor of the study measured protein binding in mice and humans using equilibrium dialysis. The degrees of binding were 86% and 91% in mice and humans, respectively. Both total and free drug fractions were considered in the PK/PD target analyses.

Pharmacokinetic/pharmacodynamic index determination. A dose fractionation experiment was performed using neutropenic mice infected with *S. aureus* ATCC 25923 as described above. Treatment with prodrug TXA-709 was initiated 2 h after infection and administered by the oral-gastric route. A total of 16 dosing regimens were studied over a 24-h period using 6-, 8-, 12-, and 24-h dosing intervals. Four thigh infections were included in each dosing group. The four total doses of TXA-709 ranged from 20 to 160 mg/kg/24 h. After 24 h, the mice were euthanized and the thighs were removed and processed for CFU determination. 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 C_{max}/MIC ratio, (ii) the 24-hour AUC/MIC ratio, and (iii) the percentage of the dosing interval during which serum levels exceeded the MIC for each of the dosage regimens studied. The correlation between efficacy and each of the three PK/PD indices was determined by nonlinear least-squares multivariate regression (SigmaPlot version 12.3; Systat Software, San Jose, CA). The model is derived from the Hill equation $E = (E_{max} \times D^N)/(ED_{50}{}^N - D^N)$, 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_{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 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 PD parameters.

Pharmacokinetic/pharmacodynamic index target for efficacy. Thigh infections in groups of neutropenic mice as described above were performed with a total of 5 *S. aureus* isolates (1 methicillin-susceptible and 4 methicillin-resistant isolates). TXA-709 was administered by the oral route in doses increasing 2-fold from 2.5 mg/kg to 160 mg/kg every 6 h. Four thigh infections were included in each dosing regimen group. Therapy was initiated 2 h after infection. The study period was 24 h, after which animals were euthanized and thighs were immediately removed and processed for CFU determination. A sigmoid dose-response model derived from the Hill equation was used to calculate the dose of TXA-709 that produced a net bacteriostatic effect and a 1-$\log_{10}$ kill level over 24 h (i.e., the static dose and the 1-$\log_{10}$ kill dose). The 24-h AUC/MIC values for the static and 1-$\log_{10}$ kill doses were calculated using the sigmoid E_{max} model.

RESULTS

***In vitro* susceptibility testing.** The MIC results were congruent between the two susceptibility experiments performed in duplicate for all 5 isolates and are shown in Table 1. All isolates demonstrated an MIC of 1 mg/liter. Beta-lactam resistance did not impact TXA-707 potency. The results are in agreement with a previous study that utilized a large and heterogeneous population of more than 60 clinical *S. aureus* isolates, including those with beta-lactam resistance, where the MIC range was 0.5 to 2 mg/liter (unpublished data).

Drug pharmacokinetics. The single-dose serum pharmacokinetics of TXA-707 after oral administration of the prodrug TXA-709 at 10, 40, and 160 mg/kg are shown in Fig. 1. TXA-709 concentrations were below the limit of detection in all serum samples. Maximum TXA-707 concentrations (C_{max}) ranged from 0.5 to 13.7 mg/liter. Values corresponding to the area under the drug concentration curve from time zero to infinity ($AUC_{0-\infty}$) ranged from 2.7 to 96.4 mg · h/liter. The elimination half-life ranged from 3.2 to 4.4 h. The pharmacokinetics were relatively linear over the dose range (AUC $R^2 = 0.99$, C_{max} $R^2 = 0.96$).

Pharmacokinetic/pharmacodynamic index determination. The relationship between the dose of TXA-709, the dosing interval, and the effect against S. *aureus* ATCC 25923 is shown in Fig. 2. The dose-response curves for each fractionated dosing regimen were very similar. The similarity of dose-response curves among the dosing intervals suggests that AUC/MIC would be the predictive pharmacodynamic index. The relationships between the $\log_{10}$ CFU/thigh and the PD indices AUC/MIC, C_{max}/MIC, and the percentage of time during which serum concentrations exceeded the MIC are illustrated in Fig. 3A, B, and C, respectively, for *S. aureus* ATCC 25923. Analysis of these data suggests the impor-

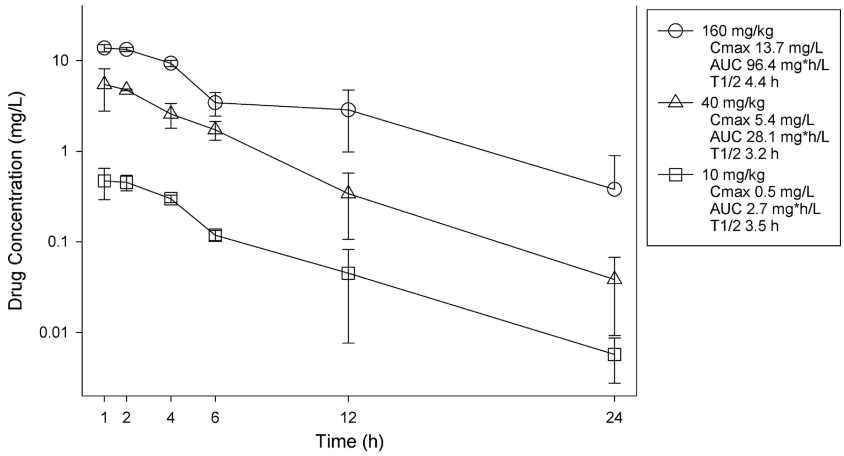

FIG 1 Single-dose serum pharmacokinetics of TXA-707 in neutropenic mice. Three different doses of prodrug TXA-709 that differed in concentration by 4-fold on a milligram-per-kilogram basis were administered by the oral route. Serum drug concentrations of active metabolite TXA-707 were measured by the use of LC-MS/MS. Groups of three mice were sampled for each time point. Samples were collected at 1, 2, 4, 6, 12, and 24 h after administration. Each symbol represents the mean value of data from three animals. The error bars represent the standard deviations. The maximum serum concentration (Cmax), the area under the drug concentration curve from h 0 to infinity (AUC), and the elimination half-life (T1/2) are shown for each dose.

tance of AUC/MIC as the predictive PK/PD index based on data fit and R^2 values.

Pharmacokinetic/pharmacodynamic index target for efficacy. A total of 5 *S. aureus* isolates were studied to determine if the AUC/MIC targets required for an effect were similar in multiple pathogens. The initial burden at the start of therapy was 7.18 ± 0.41 $\log_{10}$ CFU/thigh. The *in vivo* levels of fitness of the isolates were relatively similar in untreated control mice as determined on the basis of a burden increase of 1.88 ± 0.69 $\log_{10}$ CFU/thigh over a 24-h period. The dose-response data for each of the five *S. aureus* isolates are shown in Fig. 4. The dose-response relationships were quite similar, which would be expected given that all isolates had the same drug MIC. Treatment of infection with all 5 isolates produced results showing net stasis. For 4 of 5 isolates, treatment regimens achieved at least a 1-$\log_{10}$ kill endpoint. The doses necessary to produce a bacteriostatic effect and a 1-$\log_{10}$ reduction in organism burden as well as the corresponding total and free drug 24 h AUC/MIC values are shown in Table 2. The static doses ranged from 186 mg/kg/24 h to 247 mg/kg/24 h. The doses associated with a 1-$\log_{10}$ kill were 326 mg/kg/24 h to 640 mg/kg/24 h. The presence of beta-lactam resistance did not alter the pharmacodynamic target required to produce efficacy. The relationships between TXA-707 exposure (expressed as the AUC/MIC) and efficacy against all *S. aureus* isolates are shown in Fig. 5. The relationship among the data for each of the five isolates studied was strong based on both visual inspection and calculation of an R^2 value of 0.74. The mean total drug 24-h AUC/MIC value associated with stasis was 122 (free drug value, 17.1), and that needed for a 1-$\log_{10}$ reduction was approximately 2-fold higher at 243 (free drug value, 34).

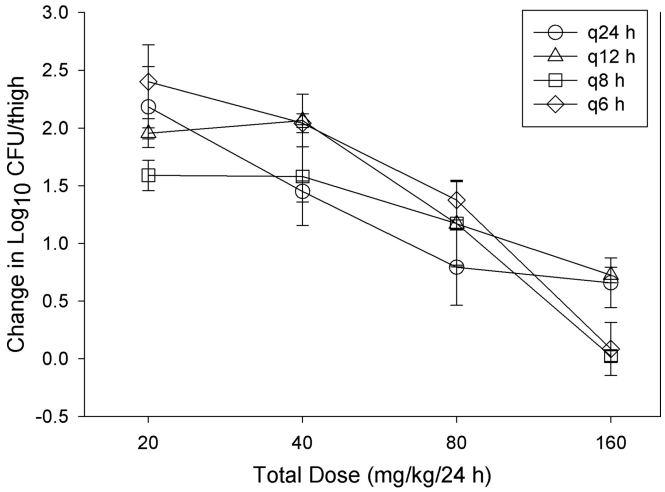

FIG 2 *In vivo* dose fractionation with TXA-709 using a neutropenic mouse thigh model. Each symbol represents the mean and standard deviation of data from four thighs infected with *S. aureus* ATCC 25923. The error bars represent the standard deviations. Four total drug dose levels (in milligrams per kilogram of body weight for 24 h) were fractionated into each of four dosing regimens. The burden of organisms was measured at the start and end of therapy. The study period was 24 h. The *y* axis data represent the changes in organism burden from that measured at the start of therapy. q6 h, every 6 h; q8 h, every 8 h; q12 h, every 12 h; q24 h, every 24 h.

DISCUSSION

Methicillin-resistant *S. aureus* infections have steadily increased in number since they were first recognized in the 1960s (1, 19, 20) and continue to represent a significant cause of morbidity and mortality (2). For example, more people die of MRSA infection in the United States health care setting than of HIV infection and tuberculosis combined (21, 22). Unfortunately, new antibiotic development specifically targeting resistant Gram-positive infections has been sparse. Since the introduction of vancomycin in 1972, very few new classes of antibiotics, including the oxazolidinones (linezolid and tedizolid), lipopeptides (daptomycin), and beta-lactams (ceftaroline), have been developed for treatment of MRSA infections. A recent addition has been the result of approval of lipoglycopeptides (telavancin, dalbavancin, and oritavancin). Importantly, only the oxazolidinones are orally bioavailable, representing a significant limitation of treatment of MRSA infections, especially skin and soft tissue infection. Additionally, drug resistance to these newer therapies, save lipoglycopeptides,

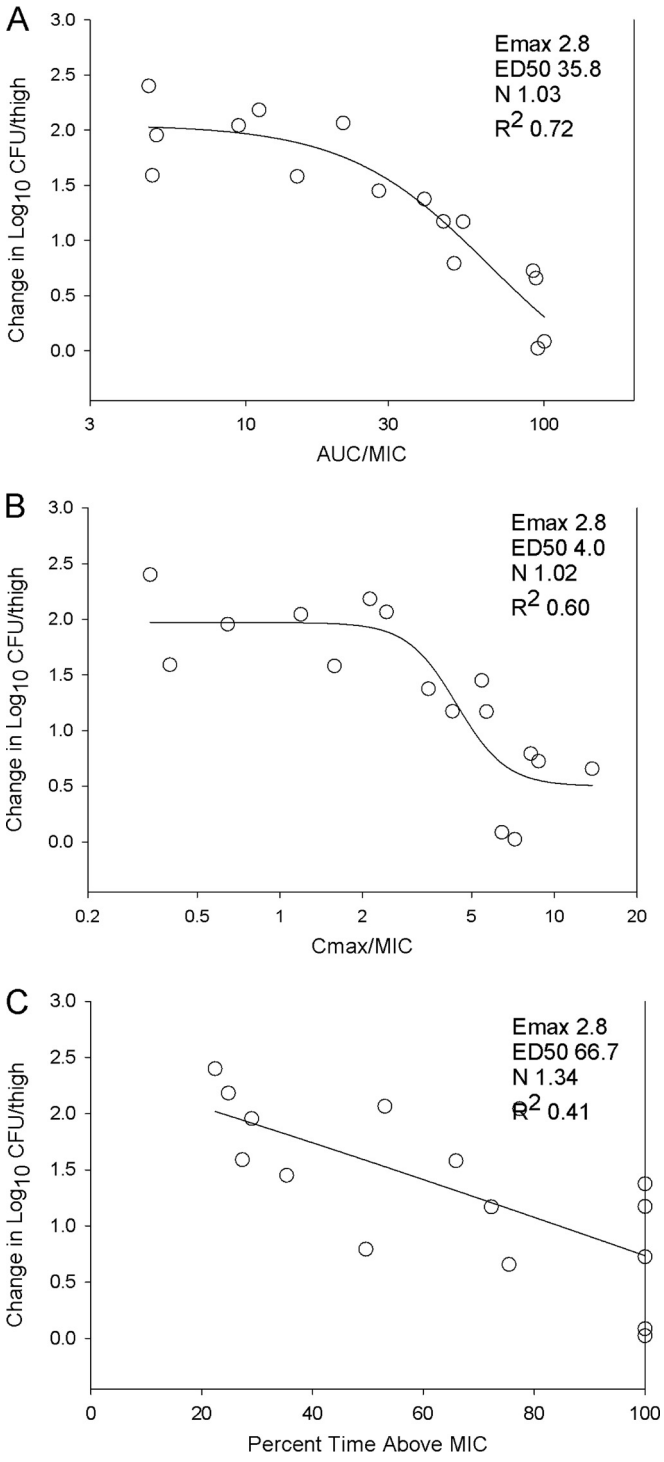

FIG 3 Impact of pharmacodynamic regression in the *in vivo* dose fractionation study with TXA-709 against *S. aureus* ATCC 25923. Each symbol represents the mean of data from four thighs. The dose data are expressed as AUC/MIC (A), C_{max}/MIC (B), and the percentage of time during which serum concentrations exceeded the MIC (C). The R^2 data represent the coefficient of determination. The maximal effect (Emax), the PD index value associated with 50% of the maximal effect (ED50), and the slope of the relationship or the Hill coefficient (N) are also shown for each PD index. The line drawn through the data points is the best-fit line based on the sigmoid E_{max} formula.

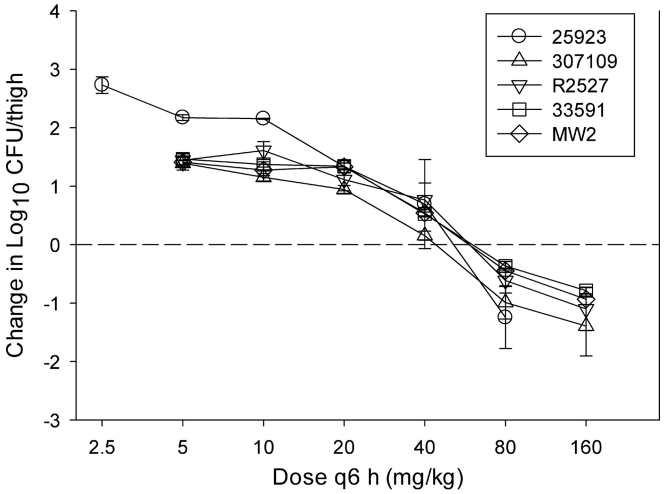

FIG 4 *In vivo* dose effect of TXA-709 against five selected *S. aureus* strains using a neutropenic mouse thigh model. Each symbol represents the mean and standard deviation of data from four thighs. Six total drug dose levels were fractionated into a regimen of administration every 6 h. The burden 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.

has been repeatedly exhibited very soon after clinical introduction (23–39).

There is an urgent need for the development of antibiotic compounds that exhibit novel mechanisms of action against drug-resistant pathogens (7, 9, 10). An encouraging area of drug development research over the previous decade has been the preclinical investigation of compounds that inhibit cell division. One of the more promising targets identified is the divisome, a macromolecular complex of cell division proteins (40, 41). FtsZ has been identified as a key component of the divisome and therefore is an attractive drug target (11, 12, 40–44). Its appeal is further supported for several reasons: (i) it is an essential bacterial protein for survival; (ii) it is highly conserved across many bacterial species, making it a potential broad target; (iii) it is not present in eukaryotes, and toxicity therefore would be expected to be low; and (iv) it is a novel target currently unexploited by other therapeutic options and therefore would be expected to present a low probability of cross-resistance with other therapies. Previous *in vitro* studies have demonstrated that a number of FtsZ inhibitor compounds contain antibacterial potency against Gram-positive pathogens, including isolates with phenotypic resistance to other antibiotics (14, 45–51).

These data represent the first preclinical animal model pharmacodynamic characterization of a novel, orally bioavailable methylbenzamide antibiotic compound, TXA-709, and its active metabolite, TXA-707, which targets bacterial cell division via FtsZ inhibition. Prior *in vivo* study of methoxybenzamide compounds which inhibit FtsZ was limited to a few proof-of-concept studies (14, 52, 53). In two of these studies, a single dose of the study compound led to improved survival in a murine methicillin-susceptible *S. aureus* (MSSA) septicemia model, whereas a third study demonstrated a decrease in bacterial burden for a single dose in a murine MSSA thigh model.

Note that methoxybenzamide derivatives have been shown to be equipotent against susceptible as well as beta-lactam-resistant

TABLE 2 *In vitro* and *in vivo* efficacy of TXA-707 against selected *S. aureus* isolates using the 24-h AUC/MIC ratio as a predictive pharmacodynamic index[a]

Isolate or parameter	TXA-707 MIC (mg/liter)	24-h static dose (mg/kg)	24-h static-dose tAUC/MIC	24-h static-dose fAUC/MIC	24-h 1-log kill dose (mg/kg)	24-h 1-log kill tAUC/MIC	24-h 1-log kill fAUC/MIC
Isolates							
ATCC 25923	1	186.1	111.7	15.6	436.5	222.3	31.1
307109	1	157.5	98.3	13.8	325.5	173.3	24.3
R2527	1	235.6	133.5	18.7	527.3	262.4	36.7
ATCC 33591	1	222.7	127.9	17.9	NA		
MW2	1	247.0	138.6	19.4	640.0	312.2	43.7
Parameters							
Mean	1	209.8	122.0	17.1	482.3	242.6	34.0
Median	1	222.7	127.9	17.9	481.9	242.4	33.9
SD	0	37.1	16.7	2.3	133.6	59.0	8.3

[a] tAUC, AUC for total drug (free and protein bound), fAUC, AUC for unbound fraction (not protein bound); NA, not achieved.

S. aureus isolates (53). Indeed, we found a very narrow MIC range with similar potencies for MSSA and MRSA isolates, similarly to previous reports. We demonstrated *in vivo* efficacy against a diverse group of 5 *S. aureus* isolates, including 4 MRSA isolates and three different U.S. genotypes, with the achievement of net stasis and 1-$\log_{10}$ kill endpoints. Pharmacodynamic evaluation of the dose fractionation experiments demonstrated the importance of AUC/MIC as the PD index that best predicts optimal efficacy. Modeling the AUC/MIC drug-response data for the entire organism data set also demonstrated a strong fit, with an R^2 of 0.74. A 24-h AUC/MIC target of approximately 120 was needed for net stasis, with a 2-fold increase in the target yielding a 1-$\log_{10}$ kill level. Importantly, as suggested by the *in vitro* potency, the pharmacodynamic target was not influenced by beta-lactam resistance or genotype.

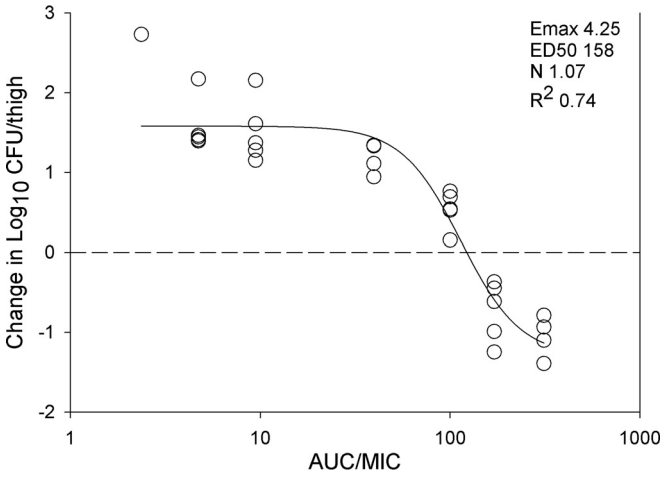

FIG 5 *In vivo* dose effect of TXA-709 against five selected *S. aureus* isolates using a neutropenic mouse thigh model. Each symbol represents the mean of data from four thighs. Six total drug dose levels were fractionated into a regimen of administration every 6 h. The TXA-709 exposure is expressed as the total drug 24-h AUC/MIC ratio. The burden of organisms was measured at the start and end of therapy. The study period was 24 h. The horizontal 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. The R^2 data represent the coefficient of determination. The ED_{50} data represent the AUC/MIC ratios associated with 50% of the maximal effect (Emax), 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 on the sigmoid Emax formula.

In conclusion, these studies demonstrated that TXA-709/707 exhibits dose-dependent *in vivo* activity against S. *aureus* isolates, including those with beta-lactam resistance. The AUC/MIC was the PK/PD index that best predicted efficacy, and we were able to demonstrate both net stasis and cidal endpoints in a clinically relevant animal infection model. The PD index and targets identified in this study, along with human PK data, will be useful in guiding appropriate dosing regimen design for future clinical studies. These findings suggest that TXA-709 and TXA-707 are a promising novel antibiotic class and compound against *S. aureus*. Further development and study are warranted, especially in light of the urgent need for novel drugs to address the rise of drug-resistant infections.

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