In vivo Pharmacodynamic Evaluation of an FtsZ Inhibitor, TXA-709 and its Active Metabolite TXA-707, in the Murine Neutropenic Thigh Infection Model

Alexander J. Lepak, Ajit Parhi, Michaela Madison, Karen Marchillo, Jamie VanHecker, David R. Andes

1Department of Medicine, University of Wisconsin, and William S. Middleton VA Hospital, Madison, Wisconsin, USA and 2TAXIS Pharmaceuticals, Inc., Tech Center of NJ, 675 US HWY 1, North Brunswick, NJ 08902

Running Title: PK/PD of FtsZ Inhibitor TXA-709 in a Murine Thigh Model

#Corresponding Author:
David Andes
1685 Highland Ave
Madison, WI 53705
University of Wisconsin
dra@medicine.wisc.edu
Abstract:

Antibiotics with novel mechanisms of action are urgently needed. Targeting 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 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 of the active metabolite TXA-707 were examined after oral administration of the prodrug TXA-709 at 10, 40, and 160 mg/kg. The half-life ranged from 3.2 - 4.4 h and the AUC and Cmax were relatively linear over the doses studied. All organisms exhibited an MIC of 1 mg/L. Dose-fractionation demonstrated AUC/MIC 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 PK/PD targets. Net stasis was achieved against all isolates and a 1-log kill in 4 isolates. PD targets included a total drug 24 h AUC/MIC of 122 for net stasis and 243 for 1-log kill. TXA-709/707 is a promising novel antibacterial class and compound for *S. aureus* infections. These results should prove useful for design of clinical dosing regimen trials.
INTRODUCTION:

Methicillin resistant *Staphylococcal aureus* (MRSA) infections are a major public health threat (1). In the United States, it is the most common cause of nosocomial infection and leads to more than eighty thousand illnesses and eleven thousand deaths yearly (2). Ambulatory visits for skin and soft tissue infection (SSTI) continue to increase and amount to more than 14 million in a 2005 survey (3). Methicillin-resistant *S. aureus* infections account for a disproportionate rise in incidence, need for hospitalization, and unfortunately has limited therapeutic options (3-6). Novel antimicrobial agents that target cellular functions distinctly different from current therapies and contain 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 while absent in eukaryotic cells.

We describe a pharmacodynamic 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 [1] determine the pharmacokinetic/pharmacodynamic (PK/PD) index (peak serum level over MIC [Cmax/MIC], 24 h area under the concentration-time curve over MIC [AUC/MIC], or duration of time serum levels exceed the MIC [Time above MIC]) associated with optimal drug efficacy and [2] identify the magnitude of the PK/PD index 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, 1 methicillin-susceptible) were studied (Table 1). The methicillin-resistant isolates included both hospital and community acquired isolates and three U.S. genotypes. Organisms were grown, subcultured, and quantified using Mueller-Hinton broth (MHB) and agar (Difco Laboratories, Detroit, MI). Prodrug TXA-709 and the active metabolite TXA-707 were supplied by the sponsor, 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-709/707(16). Animals were maintained in accordance with the American 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 weighing 23 to 27 g were used for all studies (Harlan Sprague-Dawley, Indianapolis, IN). Mice were rendered neutropenic (neutrophils <100/mm³) by injecting cyclophosphamide (Mead Johnson Pharmaceuticals, Evansville, IN) intraperitoneally 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 (18). Broth cultures of freshly plated bacteria were grown to the logarithmic phase overnight to an absorbance of 0.3 at 580 nm (Spectronic 88; Bausch and Lomb, Rochester, NY). After a 1:10 dilution into fresh MHB, bacterial counts of the inoculum ranged from $10^{6.5-6.7}$ 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. 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 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 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 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 ($C_{\text{max}}$),
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 measured protein binding in mice and humans
using equilibrium dialysis. The degree of binding was 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
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 Cmax/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: 

\[ E = \left( E_{\text{max}} \times D^N \right) / \left( ED_{50}^N - D^N \right), \]

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 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 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 oral route in 2-fold increasing doses 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, 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\textsubscript{10} kill over 24 h (i.e. static dose and 1-log\textsubscript{10} kill dose). The 24 h AUC/MIC values for the static and 1-log\textsubscript{10} kill doses were calculated using the sigmoid \( E_{\text{max}} \) model.
RESULTS

In vitro susceptibility testing. The MIC results were congruent between the two susceptibility experiments performed in duplicate for all 5 isolates and is shown in Table 1. The MIC range was narrow with all isolates demonstrating an MIC of 1 mg/L. 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 0.5-2 mg/L (unpublished data).

Drug pharmacokinetics. 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 Figure 1. TXA-709 concentrations were below the limit of detection in all serum samples. Maximum TXA-707 concentrations (Cmax) ranged from 0.5 to 13.7 mg/L. Area under the drug concentration curve from time zero to infinity (AUC0-∞) values ranged from 2.7 to 96.4 mg*h/L. The elimination half-life ranged from 3.2 to 4.4 hours. The pharmacokinetics were relatively linear over the dose range (AUC R² 0.99, Cmax R² 0.96).

Pharmacokinetic/pharmacodynamic index determination. The relationship between the dose of TXA-709, dosing interval, and effect against S. aureus ATCC 25923 is shown in Figure 2. The dose response curves for each fractionated dosing regimen were very similar. The similarity of dose response curves among the dosing intervals suggests AUC/MIC would be the predictive pharmacodynamic index. The relationship between the log_{10} CFU/thigh and the PD indices AUC/MIC, Cmax/MIC, and the percentage of time serum concentrations exceed the MIC are illustrated in Figure 3A to 3C for S. aureus ATCC 25923. Analysis of this data suggests the importance of AUC/MIC as the predictive PK/PD index based on data fit and R² 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 effect were similar in multiple pathogens. The initial burden at the start of therapy was \(7.18 \pm 0.41 \log_{10} \text{CFU/thigh}\). The in vivo fitness of the isolates was relatively similar in untreated control mice based on an increase in burden of \(1.88 \pm 0.69 \log_{10} \text{CFU/thigh}\) over a 24-h period. The dose response data for each of the five S. aureus isolates is shown in Figure 4. The dose response relationships were quite similar, which would be expected given all isolates had the same MIC. Treatment of infection with all 5 isolates produced a net stasis results. 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 varied from 186 mg/kg/24h to 247 mg/kg/24h. The doses associated with a 1-log\(_{10}\) kill were 326 mg/kg/24h to 640 mg/kg/24h. 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 Figure 5. The relationship among the data for each of the five isolates studied was strong based upon both visual inspection and an \(R^2\) value of 0.74. The mean total drug 24h AUC/MIC associated with stasis was 122 (free drug 17.1) and that needed for a 1-log\(_{10}\) reduction was approximately 2-fold higher at 243 (free drug 34).

**DISCUSSION**

Methicillin-resistant S. aureus infections have steadily increased since first recognized in the 1960's (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 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 developed for MRSA infections, including the oxazolidinones (linezolid and tedizolid), lipopeptides (daptomycin), and beta-lactams (ceftaroline). A recent addition has been the approval of lipoglycopeptides (telavancin, dalbavancin and oritavancin). Importantly, only the oxazolidinones are orally bioavailable, representing a significant limitation to treatment of MRSA infections, especially skin and soft tissue infection. Additionally, drug resistance to these newer therapies, save lipoglycopeptides, 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 pre-clinical 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: [1] it is an essential bacterial protein for survival, [2] it is highly conserved across many bacterial species making it a potential broad target, [3] it is not present in eukaryotes and therefore toxicity would be expected to be low, and [4] it is a novel target currently unexploited by other therapeutic options and therefore would be expected to have low cross-resistance with other therapies. Previous in vitro studies have demonstrated 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 is 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 MSSA septicemia model, whereas a third study demonstrated
decrease bacterial burden for a single dose in a murine MSSA thigh model.

It is important to note that methoxybenzamide derivatives have been shown to be
equipotent against susceptible as well as beta-lactam resistant S. aureus isolates (53). Indeed,
we found a very narrow MIC range with similar potency for MSSA versus MRSA isolates similar
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 dataset
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. Importantly,
as suggested by the in vitro potency, the pharmacodynamic target was not influenced by beta-
lactam resistance or genotype.

In conclusion, these studies demonstrate 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 TXA709/707 is a
promising novel antibiotic class and compound against S. aureus. Further development and
study is warranted especially in light of the urgent need for novel drugs to address the rise of
drug-resistant infections.

ACKNOWLEDGEMENT

This study was funded by Taxis Pharmaceuticals, Inc.
Figure Legends

Figure 1. Single dose serum pharmacokinetics of TXA-707 in neutropenic mice. Three different doses of prodrug TXA-709 that varied by 4-fold concentration on a mg/kg basis were administered by oral route. Serum drug concentrations of the active metabolite TXA-707 were measured by the LC-MS/MS. Groups of three mice were sampled for each time point. Samples were collected at 1, 2, 4, 6, 12 and 24 hours after administration. Each symbol represents the mean value from three animals. The error bars represent the standard deviation. Shown for each dose is the maximum serum concentration (Cmax), the area under the drug concentration curve from 0-∞ (AUC), and the elimination half-life (T1/2).

Figure 2. In vivo dose fractionation with TXA-709 using a neutropenic mouse thigh model. Each symbol represents the mean and standard deviation from four thighs infected with S. aureus ATCC 25923. The error bars represent the standard deviation. Four total drug (mg/kg/24 h) dose levels were fractionated into one of four dosing regimens. The burden of organisms was measured at the start and end of therapy. The study period was 24 hours. The y-axis represents the change in organism burden from the start of therapy.

Figure 3. Impact of pharmacodynamic regression of the in vivo dose fractionation study with TXA-709 against S. aureus ATCC 25923. Each symbol represents the mean from four thighs. The dose data is expressed as AUC/MIC, Cmax/MIC, and the percentage of time serum concentrations exceed the MIC. The $R^2$ is the coefficient of determination. Also shown for each PD index is 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). The line drawn through the data points is the best fit line based upon the sigmoid Emax formula.
Figure 4. In vivo dose effect of TXA-709 against five select S. aureus strains using a neutropenic mouse thigh model. Each symbol represents the mean and standard deviation from four thighs. Six total drug dose levels were fractionated into an every 6 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 TXA-709 against five select S. aureus isolates using a neutropenic mouse thigh model. Each symbol represents the mean from four thighs. Six total drug dose levels were fractionated into an every 6 h regimen. The TXA-709 exposure is expressed as the total drug 24 h AUC/MIC. The burden of organisms was measured at the start and end of therapy. The study period was 24 hours. 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 R² represents the coefficient of determination. The ED50 represents the AUC:MIC associated with 50% of the maximal effect or 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 upon the sigmoid Emax formula.

REFERENCES

2. Report CT.
4. Fridkin SK, Hageman JC, Morrison M, Sanza LT, Como-Sabetti K, Jernigan JA, Harriman K, Harrison LH, Lynfield R, Farley MM, Active Bacterial Core Surveillance Program of the


19. National RC.


Table 1. *In vitro* antimicrobial susceptibility results of select *S. aureus* isolates to TXA-707. Also shown for each isolate is the presence or absence of beta-lactam (mexitilin) resistance. MSSA, methicillin-susceptible *S. aureus*. MRSA, methicillin-resistant *S. aureus*.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>TXA-707 MIC (mg/L)</th>
<th>Phenotype</th>
<th>Comment(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25923</td>
<td>1</td>
<td>MSSA</td>
<td></td>
</tr>
<tr>
<td>307109</td>
<td>1</td>
<td>MRSA</td>
<td></td>
</tr>
<tr>
<td>R-2527</td>
<td>1</td>
<td>MRSA</td>
<td>U.S. 300</td>
</tr>
<tr>
<td>ATCC 33591</td>
<td>1</td>
<td>MRSA</td>
<td>U.S. 200</td>
</tr>
<tr>
<td>MW2</td>
<td>1</td>
<td>MRSA</td>
<td>U.S. 400</td>
</tr>
</tbody>
</table>

\(^a\)Pulsed-field gel electrophoresis genetic lineage
Table 2. *In vitro* and *In vivo* Efficacy of TXA-707 Against Select *S. aureus* Isolates Using 24h AUC/MIC as Predictive Pharmacodynamic Index

<table>
<thead>
<tr>
<th>Isolate</th>
<th>TXA-707 MIC (mg/L)</th>
<th>24 h Static Dose (mg/kg)</th>
<th>24 h Static Dose tAUC/MIC</th>
<th>24 h Static Dose fAUC/MIC</th>
<th>24 h 1-log Kill Dose (mg/kg)</th>
<th>24 h 1-log Kill tAUC/MIC</th>
<th>24 h 1-log Kill fAUC/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25923</td>
<td>1</td>
<td>186.1</td>
<td>111.7</td>
<td>15.6</td>
<td>436.5</td>
<td>222.3</td>
<td>31.1</td>
</tr>
<tr>
<td>307109</td>
<td>1</td>
<td>157.5</td>
<td>98.3</td>
<td>13.8</td>
<td>325.5</td>
<td>173.3</td>
<td>24.3</td>
</tr>
<tr>
<td>R2527</td>
<td>1</td>
<td>235.6</td>
<td>133.5</td>
<td>18.7</td>
<td>527.3</td>
<td>262.4</td>
<td>36.7</td>
</tr>
<tr>
<td>ATCC 33591</td>
<td>1</td>
<td>222.7</td>
<td>127.9</td>
<td>17.9</td>
<td>NA*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MW2</td>
<td>1</td>
<td>247.0</td>
<td>138.6</td>
<td>19.4</td>
<td>640.0</td>
<td>312.2</td>
<td>43.7</td>
</tr>
<tr>
<td>Mean</td>
<td>1</td>
<td>209.8</td>
<td>122.0</td>
<td>17.1</td>
<td>482.3</td>
<td>242.6</td>
<td>34.0</td>
</tr>
<tr>
<td>Median</td>
<td>1</td>
<td>222.7</td>
<td>127.9</td>
<td>17.9</td>
<td>481.9</td>
<td>242.4</td>
<td>33.9</td>
</tr>
<tr>
<td>SD</td>
<td>0</td>
<td>37.1</td>
<td>16.7</td>
<td>2.3</td>
<td>133.6</td>
<td>59.0</td>
<td>8.3</td>
</tr>
</tbody>
</table>

*NA not achieved, t = total drug (free and protein bound), f = unbound fraction (not protein bound)*