Human simulated studies of Aztreonam and Aztreonam-Avibactam to evaluate activity against Challenging Gram-negative Organisms, including Metallo-Beta-lactamase Producers.

Jared L. Crandon and David P. Nicolau

aCenter for Anti-Infective Research and Development, Hartford Hospital, Hartford, Connecticut, USA; bDivision of Infectious Diseases, Hartford Hospital, Hartford, Connecticut, USA.

Running title: Efficacy of Aztreonam and Aztreonam-avibactam

Key words: Pseudomonas aeruginosa, NDM-1, Extended spectrum beta-lactamase

*Corresponding author:

David P. Nicolau, Pharm.D., FCCP, FIDSA

Center for Anti-Infective Research and Development, Hartford Hospital

80 Seymour Street, Hartford, CT 06102

Tel: 860-545-3941; Fax: 860-545-3992; Email: dnicola@harthosp.org
ABSTRACT (234/250 words)

Secondary to the stability of aztreonam against metallo-beta-lactamases, coupled with avibatam’s neutralizing activity against often co-produced extended-spectrum beta-lactamases (ESBL) or AmpC enzymes, the combination of aztreonam and avibactam has been proposed as a principle candidate for the treatment of metallo-beta-lactamase producing Gram-negative organisms. Using the neutropenic thigh infection model we evaluated the efficacy of human simulated doses of aztreonam-avibactam and aztreonam against 14 Enterobacteriaceae and 13 *Pseudomonas aeruginosa*, of which 25 produced metallo-beta-lactamases. Additionally, 6 *P. aeruginosa* isolates were also evaluated in immunocompetent animals. A humanized aztreonam dose of 2g every 6h (1h infusion) was evaluated alone and in combination with avibactam 375mg or 600mg every 6h (1h infusion), targeting the percentage of the dosing interval in which free drug concentrations remained above the MIC ($f_{T>MIC}$). Efficacy was evaluated as the change in bacterial density after 24h as compared with bacterial density at the initiation of dosing. Aztreonam monotherapy resulted in bacterial reductions against 2 of the Enterobacteriaceae isolates (aztreonam MIC ≤ 32 μg/mL; $f_{T>MIC}$ ≥ 38%) and minimal activity to the remaining isolates (aztreonam MIC ≥ 128 μg/mL; $f_{T>MIC}$ = 0%). Alternatively, aztreonam-avibactam therapy resulted in bacterial reduction against all 14 Enterobacteriaceae (aztreonam-avibactam MIC ≤ 16 μg/mL; $f_{T>MIC}$ ≥ 65%) and no difference was noted between 375 and 600mg doses of avibactam. Similar pharmacodynamically predictable activity was noted in neutropenic and immunocompetent studies against *P. aeruginosa* with activity occurring when MIC ≤ 16μg/mL and variable efficacy noted when MICs ≥ 32μg/mL. Again, no differences in efficacy were observed between 375 and 600mg doses of avibactam. Aztreonam-avibactam represents an attractive treatment option for metallo-beta-lactamase producing Gram-negative pathogens.
co-produce ESBLs or AmpC.
INTRODUCTION

While the mainstay of therapy for a whole host of infection types is beta-lactams, production of beta-lactamases represents one of the most common mechanisms organisms have developed to threaten the viability of these agents. (9, 15, 16) Gram-negative organisms in particular have exhibited a variety of these enzymes and continually emerge with novel forms.

An example of such an enzyme is the New Delhi metallo-beta-lactamase (NDM) which was first isolated in 2008 (19) and has now been identified in many countries worldwide. NDM is only one of many types of metallo-beta-lactamases; other notable examples including both IMP and VIM. (5, 14) Collectively, these enzymes are of particular concern because they are potent hydrolyzers of not only the penicillins and cephalosporins, but also the carbapenems. (14) This coupled with other enzymatic and non-enzymatic resistant mechanisms typically leave the number of treatment options severely limited.

As the only available beta-lactam with inherent stability to metallo-beta-lactamases, aztreonam would theoretically present an attractive option for treating pathogens that produce these enzymes. Unfortunately, in most cases, these organisms come with an onslaught of other beta-lactamases (i.e. CTX-M-type, CMY-type, etc), against which aztreonam hydrolysis is imminent. (19) However, the availability of novel beta-lactamase inhibitors such as avibactam, which is active against a wide variety of these hydrolyzing enzymes but not protective against metallo-beta-lactamases, (11) may offer an excellent opportunity to marry these two compounds and effectively fill their respective “holes” in coverage. These suspicions were confirmed in a recent in vitro analysis evaluating the potency of aztreonam-avibactam against a number of metallo-beta-lactamase producing Enterobacteriaceae; (12) but no data are available describing the activity of this combination in vivo. Using the murine thigh infection model we sought to
evaluate human simulated doses of this combination against a variety of multi-drug resistant Gram-negative organisms, the majority of which producing metallo-beta-lactamases.
METHODS

Antimicrobial test agent

Commercially available aztreonam (Azactam®, Lot: 1G68718, Bristol-Myers Squibb, Princeton, NJ, USA) was obtained from the Hartford Hospital Pharmacy Department and utilized for all in vivo studies, while analytical grade aztreonam (Sigma-Aldrich, Lot: 031M0100V, St Louis, MO, USA) was utilized for ex-vivo protein binding studies and in vitro minimum inhibitory concentration (MIC) determination. Analytical grade avibactam was supplied by AstraZeneca Pharmaceuticals (Waltham, MA, USA). Clinical vials of aztreonam were reconstituted as described in the prescribing information and diluted as appropriate to achieve the desired concentrations; analytical aztreonam and avibactam powders were weighed in a quantity sufficient to achieve the required concentrations and reconstituted immediately prior to use.

Bacterial isolates

A total of 27 clinical, Gram-negative, isolates were utilized for these studies; included were: 13 P. aeruginosa, 12 E. coli, and 2 K. pneumoniae. Strains were provided by International Health Management Associates, Inc., Schaumburg, IL, USA. All isolates were maintained in double-strength skim milk (BD Biosciences, Sparks, MD) at –80°C. Each isolate was subcultured twice on trypticase soy agar with 5% sheep blood (BD Biosciences) prior to use in the experiments.

Susceptibility testing

The MICs of aztreonam and aztreonam-avibactam were determined for each isolate using the broth microdilution methodology as outlined by the Clinical and Laboratory Standards.
Institute (CLSI).(4) For aztreonam-avibactam, doubling dilutions of aztreonam were utilized in combination with a fixed 4 μg/mL concentration of avibactam. MIC studies were conducted in a minimum of 5 replicates and the modal MIC was reported.

Protein Binding Studies

Free drug was calculated for avibactam using previously described protein binding values of 18% and 10% for avibactam in man and mice, respectively.(7) The human protein binding of aztreonam was assumed to be 42% as previously reported in patients.(17) Given the lack of available data in the literature, aztreonam murine protein binding was determined over a range of concentrations herein.

Namely, aztreonam protein binding studies were conducted as three independent tests using Amicon Centrifree® Micropartition devices (Millipore, Bedford, MA) with 30,000 MW (molecular weight) cut-off filters according to the manufacturer’s package insert. Aztreonam concentrations of 425, 300, 200, 150, 140, 125, and 25 μg/mL were evaluated as was non-specific binding to the filter device at a concentration of 100 mg/L. Briefly, solutions were made in freshly collected mouse serum and heated at 37 ºC in a shaking water bath for 10 minutes, followed by centrifugation for 45 minutes at 10 ºC at 2000*g.

Percent protein binding (%PB) at each prepared concentration was calculated using the following equation: % PB = [(S – SUF) / S] x 100 where S is the aztreonam concentration in the initial serum solutions and SUF, the concentration in the ultrafiltrate.

Neutropenic thigh infection model
Pathogen-free, female ICR mice weighing approximately 25 g were acquired from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and utilized throughout these experiments. Animals were maintained and used in accordance to National Research Council recommendations, and provided food and water ad libitum. Mice were rendered neutropenic with 100 and 150 mg/kg intraperitoneal injections of cyclophosphamide (Cytoxan®; Bristol-Myers Squibb, Princeton, NJ) given 1 and 4 days prior to inoculation, respectively. Three days prior to inoculation, mice were also given a single 5 mg/kg intraperitoneal injection of uranyl nitrate which produces a predictable degree of renal impairment to aid in humanizing the drug regimens (2). Two hours prior to the initiation of antimicrobial therapy, each thigh was inoculated intramuscularly with a 0.1 ml solution containing approximately 10^7 colony forming units (CFU)/ml of the test isolate.

**Immunocompetent thigh infection model**

Mice utilized in the immunocompetent studies underwent the same procedures as outlined above, except that cyclophosphamide was not given and an inoculum of 10^8 CFU/ml was used to produce thigh infection.

**Determination of the in vivo dosing regimen**

In these studies, we determined a dosing regimen, in mice, that simulated the percentage of the dosing interval for which free drug concentrations were above the MIC (fT>MIC) profile observed in man given 2000mg aztreonam every 6 hours as a 1 hour infusion as monotherapy or combined with avibactam at doses of 375mg or 600mg every 6 hours as a 1 hour infusion. Exposures of aztreonam in patients were derived from a previously published pharmacokinetic...
model, (17) while avibactam exposures were determined from a population pharmacokinetic
model (10).

First, single dose studies with aztreonam-avibactam and aztreonam alone were undertaken in
thigh infected neutropenic mice; for these analyses, animals were dosed with a single weight- 
based, 0.2ml, subcutaneous injection of the study drug(s) and groups of six mice were euthanized
at 8 time points over the following 12 hours. Blood samples were taken via cardiac puncture and
serum was stored at -80°C until analysis. Aztreonam concentrations were analyzed at the Center
for Anti-Infective Research and Development (Hartford, CT) using a high performance liquid
chromatography assay, (13) while avibactam concentrations were determined by Eurofins
Medinet, Inc (Chantilly, VA) using a liquid chromatography-tandem mass spectrometry (LC-
MS-MS) assay. (18) Intra-day and inter-day coefficient of variation for high and low check
samples for each assay was ≤5.4%.

Pharmacokinetic parameters for single doses of aztreonam-avibactam and aztreonam alone were
calculated using first-order input and elimination, by nonlinear least-squares techniques
(WinNonlin version 5.0.1, Pharsight, Mountain View, CA). Compartment model selection and
weighting schemes were based on visual inspection of the fit and use of the correlation between
the observed and calculated concentrations.

Using pharmacokinetic parameters derived in single dose studies, regimens in mice that
simulated the free drug exposure profile for patients given aztreonam-avibactam and aztreonam
alone were constructed. Confirmatory pharmacokinetic studies were undertaken in infected mice
prior to the use of these regimens in the pharmacodynamic analyses and an assessment of
$\text{T>MIC}$ was made from the resulting concentration-time profiles. For these studies, infected
neutropenic mice were dosed with the above calculated regimens and groups of six mice were
euthanized at 5 time points throughout the first dosing interval (i.e. 6 hours) to confirm target exposures.

In vivo efficacy

For each of the 14 Enterobacteriaceae (12 *E. coli* and 2 *K. pneumoniae*) and 13 *P. aeruginosa* isolates, groups of 3 mice were administered human simulated regimens of aztreonam or aztreonam-avibactam beginning 2 h after inoculation. All doses were administered as 0.2 ml subcutaneous injections and consisted of four 6h dosing intervals (i.e. 24h). To serve as control animals, an additional group of mice were administered normal saline at the same volume, route, and frequency as the treatment regimen. Thighs from all animals were harvested 24 hours after the initiation of therapy; mice that failed to survive for 24 hours were harvested at the time of expiration. The harvesting procedure for all study mice began with euthanization by CO2 exposure followed by cervical dislocation. After sacrifice, thighs were removed and individually homogenized in normal saline. Serial dilutions of the thigh homogenates were plated on trypticase soy agar with 5% sheep blood using spiral plating techniques for CFU determination. Given the narrow window between the final treatment dose and tissue harvesting, plates were observed for the presence of antibiotic carry-over. In addition to the above mentioned treatment and control groups, another group of 3 infected, untreated mice were harvested at the initiation of dosing and served as 0h controls. Efficacy, designated as the change in bacterial density, was calculated as the change in log_{10} bacterial CFU/mL obtained for treated mice after 24h from that of that starting densities observed in 0h control animals. Given that both stasis and 1 log reductions in bacterial density have been used to predict clinical
efficacy in humans, this target range was used throughout as a benchmark to evaluate therapies. 

(1, 8)
RESULTS

Bacterial isolates

The genotypic and phenotypic profiles of the 27 isolates included in the efficacy studies are shown in Table 1. Of note, all the Enterobacteriaceae isolates produced NDM beta-lactamases, as well as, various extended spectrum beta-lactamases. Moreover, nearly all the P. aeruginosa isolates were metallo-beta-lactamase producers.

Protein Binding Studies

The protein binding of aztreonam was concentration dependent over the concentration range of 25 to 200 mg/L (range: 90.8 to 40.3%); while protein binding remained constant at concentrations ≥ 200 μg/mL with a mean protein binding value of 43.5 ± 6.6%. Non-specific binding studies revealed no binding of aztreonam to the filter device. Given that the peak concentrations observed during human simulated studies fell within this static range, a set protein binding of 43.5% was utilized for free drug calculations.

Determination of dosing regimen for in vivo studies

The pharmacokinetics of aztreonam and avibactam were best described using a 1-compartment model with first-order input and elimination. Human simulated regimens consisted of 5 doses for each 6 hour dosing interval. The free drug pharmacokinetic profiles determined in vivo for aztreonam 2000mg-avibactam 375mg are shown in Figure 1, aztreonam 2000mg-avibactam 600mg in Figure 2, and aztreonam 2000mg alone in Figure 3. The comparative aztreonam /T>MIC attained for these regimens in mice and that anticipated in man are shown in Table 2 and highlight the similarities between exposures.
In vivo efficacy

Enterobacteriaceae studies were conducted in only neutropenic animals; during these evaluations, 0h control mice displayed a mean bacterial density of $5.87 \pm 0.19 \log_{10}\text{CFU}$, which increased to an average of $8.23 \pm 0.90$ logs in untreated mice after 24 hours. Infection related mortality was observed in control animals and a small number of aztreonam treated mice; all aztreonam-avibactam animals were alive at 24 hours. Antibiotic carry-over was not observed on any of the treatment plates. The results of these studies are shown in Figure 4. While aztreonam monotherapy reduced bacterial density against only the 2 isolates with aztreonam MICs $\leq 32\, \mu g/mL$ ($f_{T>MIC} \geq 38\%$), secondary to the increased in vitro potency, aztreonam-avibactam treatment resulted in maximal activity against all 14 isolates. Also of note, efficacy of combination therapy was similar between the two avibactam regimens.

Pseudomonal studies were conducted in both neutropenic (Figure 5) and immunocompetent (Figure 6) animals. The respective bacterial densities in control mice at the initiation of dosing were $4.98 \pm 0.24$ and $6.44 \pm 0.27 \log_{10}\text{CFU}$, increasing to $7.85 \pm 0.85$ and $7.69 \pm 1.14 \log_{10}\text{CFU}$ after 24 hours. A large number of untreated control animals succumbed to infection, as did aztreonam treated mice infected with PSA 1451 (ATM MIC $>128\, \mu g/mL$); all aztreonam-avibactam treated mice survived to 24 hours. Antibiotic carry-over was not observed on any of the treatment plates. In general, neutropenic studies showed the activity of aztreonam monotherapy and aztreonam-avibactam to be predictable based on the pharmacodynamic profile with maximal activity occurring when $f_{T>MIC} \geq 65\%$ (i.e. MIC $\leq 16\, \mu g/mL$) and variable efficacy noted when $f_{T>MIC}$ dropped to $\leq 38\%$ (i.e. MICs $\geq 32\, \mu g/mL$). Similar observations were made in immunocompetent animals with a slight enhancement in activity for both
aztreonam and aztreonam-avibactam against organisms in which minimal activity was seen in neutropenic studies. Regardless of immune status, no differences in efficacy were observed between 375 and 600mg doses of avibactam.
DISCUSSION

Secondary to the high rates of resistance to our current armamentarium coupled with the lack of novel agents in development, Gram-negative pathogens bring difficulties to present-day clinical practice and fear for days to come. While a number of resistance mechanisms contribute to the bleak phenotypic profiles displayed by many of these organisms, production of beta-lactamases, both new and old, play a vital role. It is for this reason that novel beta-lactamase inhibitors such as avibactam are of great interest in a number of development programs.\(^{(3)}\)

While avibactam has a broad spectrum of beta-lactamase inhibition, it is inactive against metallo-beta-lactamases. Owing to its inherent stability against metallo-beta-lactamases, aztreonam combined with avibactam represents a possible approach to treating these organisms. Using the murine thigh infection model we found that while aztreonam monotherapy was ineffective against a large percentage of metallo-beta-lactamase producers, aztreonam-avibactam yielded activity against a large proportion of these isolates.

When evaluating the \textit{in vitro} MIC data for the Enterobacteriaceae isolates included in this analysis, it is clear that while aztreonam may be stable against hydrolysis by the NDM enzymes produced by these organisms,\(^{(14)}\) the multitude of other beta-lactamases and/or potential non-enzyme-mediated mechanisms confirmed high levels of resistance to aztreonam. The addition of avibactam to aztreonam resulted in a potency shift of 3 to \(\geq 11\) doubling dilutions rendering all organisms quite responsive to combination therapy \textit{in vivo}. Similar \textit{in vitro} results were noted in a previous study conducted by Livermore \textit{et al}.\(^{(12)}\), in which 17 NDM-1 producing isolates revealed a median MIC of \(\geq 256\ \mu g/mL\) (range: 0.06 to \(\geq 256\ \mu g/mL\)) for aztreonam and 0.25 \(\mu g/mL\) for aztreonam-avibactam (range: \(\leq 0.03\) to 4 \(\mu g/mL\)). Of note, while the current analysis did not include Enterobacteriaceae isolates that produced other metallo-beta-lactamases of...
interest (i.e. IMP, VIM), the MIC distribution of the tested isolates was inclusive of that anticipated for these organisms based on the Livermore et al. data (≤4 μg/mL).(12) For P. aeruginosa, the majority of which producing metallo-beta-lactamases, MICs of most isolates were similar for aztreonam and aztreonam-avibactam suggesting that beta-lactamases within the inactivation profile of avibactam were playing a minor role in aztreonam resistance.

Using human simulated strategies, we found the activity of aztreonam monotherapy and aztreonam-avibactam to be quite predictive based on the aztreonam $f_{T>MIC}$ profile. Namely, using the 2g q6h (1h infusion) dose, maximal activity was noted when MICs were ≤ 16 μg/mL ($f_{T>MIC} \geq 65\%$) and became variable when MICs were ≥ 32 μg/mL ($f_{T>MIC} \leq 38\%$). While we did not conduct traditional dose ranging studies in this analysis, the break in activity noted based on $f_{T>MIC}$ was similar to targets reported in the literature (i.e. 50-60%) for aztreonam alone.(6) This observation is of particular interest for aztreonam-avibactam as it provides in vivo support for the use of a set avibactam concentration of 4 μg/mL during MIC testing and perhaps an early look into a potential clinical breakpoint.

Another important observation of this study was the similarity in activity between 375 and 600mg doses of avibactam when combined with aztreonam. This similarity was noted regardless of organism, aztreonam-avibactam MIC, genotype, or the MIC reduction provided by avibactam as compared with aztreonam alone. While previous in vitro hollow fiber studies of avibactam combined with ceftaroline suggested that avibactam free time above a threshold concentration was required for maximal cephalosporin activity, the specific threshold concentration and/or percentage of time has not been fully elucidated; further, no studies have been published for the combination of aztreonam-avibactam. Based on the data described
herein, clearly the pharmacodynamic profile of avibactam at doses of 375 and 600 mg q6h were sufficient to restore aztreonam activity against the isolates evaluated.

When comparing the activity of aztreonam and aztreonam-avibactam against isolates in which the addition of avibactam did not alter aztreonam potency, similar activity was noted for a majority of isolates. There were, however, isolates such as PSA 1447 (aztreonam and aztreonam-avibactam MIC=32 μg/mL) in which the activity of aztreonam-avibactam was greater than aztreonam monotherapy. (Figure 5) While the reason for this observation is not entirely clear, it is possible that the addition of avibactam may have reduced the actual MIC, but not in a quantity sufficient to fall below the next doubling dilution (i.e. 16 μg/mL). In this example, the theoretical reduction in actual MIC could therefore improve the observed fT>MIC to a value somewhere between 38 and 65%.

It should also be noted that the current CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) aztreonam susceptibility breakpoints for Enterobacteriaceae are ≤ 4 and ≤ 1 μg/mL, respectively. However, using the humanized 2g q6h dose of aztreonam monotherapy, we saw activity against two isolates with respective aztreonam MICs of 16 and 32 μg/mL. While EUCAST did not make specific comments, CLSI states that this breakpoint recommendation is generated from a 1g q8 regimen, which would clearly result in a comparatively reduced pharmacodynamic profile. Similar comments could be made for P. aeruginosa where respective CLSI and EUCAST susceptibility breakpoints are ≤ 8 and ≤ 1 μg/ml. In these cases, CLSI comments that the breakpoint is based off doses of 1g q6 or 2g q8 and EUCAST denotes the use of “high dose therapy”. As noted previously, the efficacy seen in the current study for both aztreonam and aztreonam-avibactam against isolates with MICs ≤ 16 μg/ml were predictable based on fT>MIC profile for the 2g q6h regimen.
With the continued evolution and spread of beta-lactamases among Gram-negative pathogens, novel treatment approaches are in dire need. Therapeutic options for specifically metallo-beta-lactamase producers are of particular interest. Using the murine thigh infection model, we found that while human simulated regimens of aztreonam 2g q6 was relatively ineffective against metallo-beta-lactamase producing organisms due to co-production of ESBL or AmpC enzymes, the addition of avibactam at doses of 375 or 600mg restored aztreonam in vivo activity and yielded maximal activity against isolates with MICs ≤ 16 μg/ml. Based on these finding, aztreonam-avibactam represents an attractive treatment option for metallo-beta-lactamase producing Gram-negative pathogens that co-produce ESBL or AmpC enzymes.
ACKNOWLEDGMENTS

Thanks to Michael Huband and Linda Otterson (AstraZeneca Pharmaceuticals) for providing the phenotypic and genotypic profiles of the bacterial isolates. We would also like to thank Mary Anne Banevicius, Amira Bhalodi, Henry Christenson, Mao Hagihara, Seth Housman, Jennifer Hull, Philip Moore, Debora Santini, Pam Tessier, and Lindsey Tuttle (Center for Anti-Infective Research and Development) for their assistance in the in vivo studies and Christina Sutherland for aztreonam concentration determination. This study was sponsored by a grant from AstraZeneca Pharmaceuticals, Waltham, MA. and Hartford Hospital received a fee for service in relation to preparing the manuscript which was funded by AstraZeneca, Macclesfield, United Kingdom.
REFERENCES:


Table 1. Phenotypic and genotypic data for the *E. coli* (EC), *K. pneumoniae* (KP), and *P. aeruginosa* (PSA) isolates utilized during the in vivo efficacy studies

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*denotes isolates evaluated in neutropenic and immunocompetent studies; ATM-AVI, aztreonam-avibactam; ATM, aztreonam; ND, Not Determined
Table 2. Human simulated \( fT>MIC \) profile for aztreonam 2000mg q6h (1h infusion) in man as compared with that observed in mice. This profile is similar irrespective of avibactam administration.

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<th>Aztreonam ( fT&gt;MIC ) (%)</th>
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Figure 1. Free drug concentration-time profile of human simulated 2000mg aztreonam (ATM)-375mg avibactam (AVI) in thigh infected ICR mice. Circles represent mean ± standard deviation.
Figure 2. Free drug concentration-time profile of human simulated 2000mg aztreonam (ATM)-
600mg avibactam (AVI) in thigh infected ICR mice. Squares represent mean ± standard
deviation.
Figure 3. Free drug concentration-time profile of human simulated 2000mg aztreonam (ATM) in thigh infected ICR mice. Triangles represent mean ± standard deviation.
Figure 4. Comparative efficacy of human simulated doses of aztreonam 2000mg q6h (1h infusion) as monotherapy (ATM) or combined with avibactam 375mg q6h (ATM-AVI 375mg) or avibactam 600mg q6h (ATM-AVI 600mg) against Enterobacteriaceae in the neutropenic thigh infection model.
Figure 5. Comparative efficacy of human simulated doses of aztreonam 2000mg q6h (1h infusion) as monotherapy (ATM) or combined with avibactam 375mg q6h (ATM-AVI 375mg) or avibactam 600mg q6h (ATM-AVI 600mg) against *P. aeruginosa* in the neutropenic thigh infection model.
Figure 6. Comparative efficacy of human simulated doses of aztreonam 2000mg q6h (1h infusion) as monotherapy (ATM) or combined with avibactam 375mg q6h (ATM-AVI 375mg) or avibactam 600mg q6h (ATM-AVI 600mg) against *P. aeruginosa* in the immunocompetent thigh infection model.
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