Pharmacokinetics of tulathromycin in healthy and neutropenic mice challenged intra-nasally with lipopolysaccharide from *Escherichia coli*

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Abstract

Tulathromycin represents the first member of a novel sub-class of macrolides known as triamilide approved to treat bovine and swine respiratory disease. The objectives of the present study were to assess the concentration vs. time (C-T) profile of tulathromycin in the plasma and lung tissue in healthy and neutropenic mice challenged intra-nasally with lipopolysaccharide from *Escherichia coli* 0111:B4 (LPS-Ec). BALB/c mice were randomly allocated to four groups of 40 mice each: T-28, T-7, T7-LPS, and T7-LPS-CP. Mice in T-28 were treated with tulathromycin at 28 mg/kg S.C (time 0 h). The rest of the mice were treated with tulathromycin at 7 mg/kg S.C (time 0 h). Animals in dose group T-7-LPS and T7-LPS-CP received a single dose of LPS-Ec intra-nasally at -7 h. Mice in group T7-LPS-CP were also rendered neutropenic with cyclophosphamide (150 mg/Kg/intra-peritoneal) prior to the administration of tulathromycin. Blood and lung tissue samples were obtained from 5 mice from each dose group at each sampling time over 144 h post-tulathromycin administration.

There were not statistical differences in lung tissue concentrations among T-7, T-7-LPS and T7-LPS-CP. In all dose groups, the distribution of tulathromycin into the lungs was rapid and persisted at relatively high levels during 6 days post-administration. The C-T profile of tulathromycin in lung tissue was not influenced by the intra-nasal administration of LPS-Ec. Results suggest that, in mice, neutrophils may not have a positive influence on tulathromycin accumulation in lung tissue when the drug is administered during either a neutrophilic or neutropenic state.

Keywords: tulathromycin, antimicrobial, mice, lung, LPS
Tulathromycin is a semisynthetic, 15-membered ring macrolide derivative of erythromycin. It represents the first member of a novel sub-class of macrolides known as triamilide (15). This macrolide shows metaphylactic and therapeutic efficacy in bovine and swine respiratory disease after a single administration (20, 23). Tulathromycin has also been approved by the United States Food and Drug Administration (FDA) to treat infectious keratoconjunctivitis caused by Moraxella bovis and bovine inter-digital necrobacillosis associated with Fusobacterium necrophorum and Porphyromonas levii (22).

Tulathromycin is characterized by a rapid rate of absorption and a large volume of distribution (Vdss 13.2 L/kg in pig and 11 L/kg in cattle) (3, 19). Pharmacokinetic studies in cattle, swine, and foals revealed the capacity of tulathromycin to rapidly accumulate in lung tissue and persist for a long period of time (apparent elimination half-life is about 6 days in pigs and cattle) (3, 19). In one study, the area under the concentration-time curve in lung tissue homogenate to area under the plasma concentration-time curve (AUC0-360) ratio reported was 94 and 181 for bovine and swine, respectively (3, 8), and the pulmonary epithelial lining fluid apparent half-life was >100 h in Holstein calves (8). These features would explain to some extent the clinical outcome of tulathromycin when it is used within the label frame.

The mechanism by which tulathromycin accumulates in lung tissue remains unknown. As reported for other macrolides, tulathromycin is taken up by resident inflammatory cells (alveolar macrophages) (8). Tulathromycin also accumulates in blood macrophages and neutrophils (27). Increased amounts of azithromycin and in infected lungs have been reported (2, 29). It was suggested that the accumulation of azithromycin in tissues appears related to the rapid uptake and transport to the infection site by cells such as polymorphonuclear neutrophils, monocytes, alveolar macrophages, and fibroblasts (12, 13). Also, a reduced tissue AUC of azithromycin was seen in leucopenic mice (2, 29, 30). These results have been used to link neutrophil migration and pharmacokinetics of macrolides.
Neutropenia is a common cellular blood shift that occurs during an acute inflammatory process. Tulathromycin is indicated for both metaphylaxis and therapy of bacterial infections. Consequently, drug administration may take place during a neutropenic state. Under this scenario, our hypothesis is that the concentration vs. time profile of tulathromycin in lung tissue is influenced by neutropenia and acute pulmonary inflammatory response.

The present study has two objectives: to assess the concentration vs. time profile of tulathromycin at two different dose levels in mice and to assess the concentration time profile of tulathromycin in the plasma and lung tissue in healthy and neutropenic mice challenged intranasally with lipopolysaccharide from *Escherichia coli* 0111:B4.

**Material and methods**

**Animals and housing**

Female BALB/c mice (*n = 205*) (17.4–22.0 g) were purchased from a local commercial source (Charles River, Wilmington, MA, USA). Drinking water was available *ad libitum* throughout the study. Animals were acclimatized for a minimum of 7 days prior to administration of the treatments. Dose calculations were based on body weights determined the day of the drug administration previous to the dosing. Before initiation of the study, procedures involving the care or use of mice were reviewed and approved by the Pfizer Animal Health Institutional Animal Care and Use Committee (IACUC).

**Study design**

Mice were randomly allocated to four dose groups of 40 mice each: T-7, T-28, T7-LPS, and T7-LPS-CP (LPS, lipopolysaccharide; CP, cyclophosphamide). Within each dose group, animals were allocated randomly to eight sampling times (from 30 min to 144 h) with five animals in each. All animals within a treatment group (*n = 5*) were kept in a single cage. In addition, 45 mice were allocated into a control group. Animals within the control group were
assigned to one of three control groups: (i) CT-NT (no treatment), (ii) CT-LPS (intranasal administration of LPS), and (iii) CT-CP (cyclophosphamide).

Treatment dose group T-7, T7-LPS, and T7-LPS-CP were administered one S.C. injection of tulathromycin at 7 mg/kg in the inter-scapular space at time 0. The final concentration of tulathromycin was adjusted using the commercial formulation without tulathromycin provided by Pfizer Animal Health. Animals in dose group T-28 were administered one S.C. injection of tulathromycin (Draxxin Injectable Solution) at 28 mg/kg in the inter-scapular space at time 0. Animals in dose group T-7-LPS and T7-LPS-CP received a single LPS (E. coli 0111:B4) dose (30 µg of LPS diluted in 30 µL of sterile saline solution) at -7 h by intranasal administration under general anesthesia as described below. Additionally, mice from group T7-LPS-CP were treated with cyclophosphamide (Cytoxan cyclophosphamide for injection, USP, Bristol-Myers Squibb Company, Princeton, NJ) at 150 mg/kg by intra-peritoneal route at -4, -1, and + 2 days (when applicable).

Animals within the control group (CT-NT, not treated with tulathromycin), received no treatment. Mice in the CT-LPS group received an intranasal dose of LPS as described above. Animals in the CT-LPS-CP were treated with two doses of cyclophosphamide at -4 and -1 days before sampling time of these animals (day 0). Additionally, these animals were treated with LPS at -7 h as described above.

Preparation of inoculum

Lipopolysaccharide from E. coli 0111:B4 (LPS-Ec) purified by phenol extraction was purchased from Sigma Aldrich (lot number 118K4052) (Saint Louis, Missouri, USA). The LPS-Ec powder was re-suspended in saline solution (NaCl 0.9%) to obtain a final concentration of 1 µg/mL. The same inoculum size of LPS solution (30 µL) was used for all the LPS-Ec-treated animals.
Administration of inoculum 
A single LPS dose was administered intra-nasally under general anesthesia. Mice were placed on their back on a solid surface with an inclination of 35 degrees. The LPS solution was gently dropped in one of the nostrils. After LPS administration, mice were kept on the same inclined board until recovery from anesthesia. After recovery, mice were placed back in their corresponding cages.

Sampling
Five mice from each dose group per sampling time were anesthetized to obtain blood and lung tissue samples at 30 min, 1, 24, 48, 72, 96, 120 and 144 h post-tulathromycin administration.
Mice in the control group were euthanized and lungs excised as following: CT-NT (n = 3) and CT-LPS at 7 h (n = 3) and 24 h (n = 3) post-LPS administration. In the group CT-LPS-CP, lung tissue samples were taken at time 0 (7 h post LPS and after two doses of CP). Lungs were filled with formalin 10% (0.7 mL) and placed individually in a container with formalin 10% until histology evaluation. Also, blood samples were withdrawn from the CT-NT (n = 10), CT-LPS (n = 7), and CT-LPS-CP (n = 7) groups at time 0 for white cell counts. White cell count was performed using the automat cell counter (Advia® 120 hematology system, Deerfiel, IL, USA). rest of the animals and tissues were used for analytical purposes.

Plasma sampling
Blood samples were obtained by terminal cardiac puncture under general anesthesia. Briefly, once mice were under an appropriate anesthetic level, animals were placed on their back on a solid surface. The xiphoid process was palpated at the caudal aspect of the animal's sternum. A 22-gauge needle attached to a 1 mL syringe was inserted toward the heart as determined by palpating for the heartbeat. Immediately after blood samples were withdrawn, animals were euthanized using an overdose of pentobarbital.
Blood samples were placed into tubes with EDTA as an anticoagulant and kept in ice until centrifugation. Blood samples were centrifuged for 15 min at 1000 RPMs at 5°C. Plasma samples were placed into cryotubes and kept at -20°C until analysis.

Lung tissue sampling

Following euthanasia, lungs from each mouse were removed from the thorax cavity. Lung tissue samples were placed into cryotubes and kept at -20°C until analysis.

Wet-to-dry ratio determination

An approximate 0.5 cm x 0.5 cm subsection of lung tissue sampled for lung tissue homogenate was taken for wet-to-dry ratio determination. Samples were placed in an aluminum container and weighed. The wet weight was recorded. Then samples were dried in an oven at 60°C for 15 days. Samples were then weighed again for wet-to-dry ratio determination using the following equation:

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\text{Wet-to-dry ratio} = \frac{\text{wet weight (g)}}{\text{dry weight (g)}}
\]

Anesthesia and euthanasia

Both LPS administration and sampling general anesthesia were performed with intraperitoneal administration of ketamine (100 mg/kg) combined with xylazine (10 mg/kg). All experimental animals were euthanized after blood sampling and before recovery from anesthesia using an overdose of pentobarbital.

Analytical methods

All the matrices samples were analyzed for tulathromycin content by a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system with tandem mass spectrometry detection (LC-MS/MS) (Waters Corp., Milford, Massachusetts, USA) following procedures described (8, 11). Roxithromycin was used as internal standard (25). It was not evaluated.
stability of the analyte in the matrices. We considered previously evaluated stability data generated in a full validation study using lung samples from cattle and pig (11) as valid. We have used the same analytical method validated by Galer et al, 2004 for the analysis of our study samples. In this validation study, stability was demonstrated in different conditions. In this study, samples were evaluated within a month after collection. Samples were thaw only once for the analysis without any thawing and freezing cycles. All the samples were evaluated in one batch in a period no longer than 36 hs after thawing.

Plasma

Study plasma samples (100 µl) were spiked with 100 µL of internal standard (roxithromycin 25 ng/mL) and then extracted using solid phase extraction (SPE) cartridges (Oasis MCX 1mL, Waters). The SPE cartridges were preconditioned with 1.0 mL of acetonitrile followed by 1.0 mL of pH 6.8 and 50 mM K2HPO4. A weak vacuum was applied to the SPE manifold. Cartridges were loaded with quality control, blank plasma, and test samples (200 µL). The SPE tubes were then rinsed sequentially with 1.0 mL of pH 6.8, 50 mM K2HPO4, and 1.0 mL of H2O. Finally, analytes were eluted with 2 × 0.5 mL of freshly prepared 5% NH4OH/95% acetonitrile into labeled 13- × 100-mm polypropylene tubes. Collected extracts were evaporated to dryness under a stream of nitrogen at 50–55 °C. Extract residues were reconstituted with 200 µL of 20 mM ammonium acetate (pH 4.0) by vortex mixing and transferred into autosampler vials.

Chromatography was accomplished with a BDS Hypersil C8 30 x 2.1 mm column (Thermo Fisher Scientific Inc., Waltham, MA, USA) at a flow rate of 0.300 mL/min with a linear gradient chromatography. The mobile phase initially consisting of 80% A (20 mM pH 4 ammonium acetate) and 20% B (acetonitrile) and was changed to at 0.5 min of 20% A and 80% B. Retention times were approximately 1 min for tulathromycin and 1.2 min for roxithromycin.
The mass spectrometer (AB Sciex Linear Ion Trap 4000 QTRAP equipped with a turbo spray ion source, Foster City, CA, USA) was used in the positive ion mode using an electrospray ionization source, and positive ions were monitored with precursor → product ion pairs of 806 → 577 for tulathromycin and 837 → 679 for roxithromycin. Calibration curves were generated automatically using Analyst 1.0 software (Perkin-Elmer, Waltham, MA, USA).

The calibration curve (R²>0.99) occurred over the range of 1.95 to 500 ng/mL using nine calibration standards (by duplicating each concentration), and five quality control samples (15.63, 62.5, 125, 250, 500 ng/mL) (by quadruplicating each concentration level). Standard curve and quality controls were run at the beginning and the end of the analytical run. The lower limit of quantification (LLOQ), defined as the standard concentration where analysis of 3 replicate samples did not exceed a coefficient of variation of 20%. The LLOQ was 3.90 ng/mL.

For the quality control samples, the intra-run coefficients of variation ranged from 12.9 to 19.0%, and the intra-run biases ranged from -14.0% to 10.0%. For the calibration curve samples, the intra-run coefficients of variation ranged from 2.0 to 19.7%, and intra-run biases for these samples ranged from -10.6% to 8.38%. All the study samples were analyzed in a single analytical run. The recovery ranges for tulathromycin and IS in spiked plasma was 60.1-66.2% and 51.1-62.4% respectively.

Lung tissue homogenate samples
Samples were processed as described previously by Galer et al. (2004) (11). Briefly, a lung tissue samples (0.100 g) were mixed with 0.050 mL of 50 mM phosphate buffer, pH 6, and 2.5 mL of 0.04 M phosphoric acid in polypropylene tubes. Samples were homogenized using a Polytron probe. The homogenate was centrifuged during 10 minutes at ~4500g, and 0.25 mL of the supernatant was then mixed with 0.25 mL of 50 mM pH 6 phosphate buffer and 0.25 mL of internal standard solution, and the entire sample was loaded onto an pre-conditioned Oasis
MCX 1cc (30 mg) SPE cartridge. Then the SPE cartridges was washed with 1 mL of 50 mM pH 6 phosphate buffer, 1 mL of distilled deionized water, and 1 mL of acetonitrile. The drug was then eluted from the cartridge with two 0.5 mL volumes of 5% ammonium hydroxide/95% acetonitrile. After evaporation with nitrogen gas at 50 °C, the sample was reconstituted with 0.95 mL of 20 mM pH 4 ammonium acetate and acetonitrile (80:20 v:v). A 10 µL aliquot was then injected into the chromatographic system. The calibration curve (R² > 0.99) occurred over the range of 3.90 to 1000 ng/g lung tissue using 10 calibration standards, and three quality control samples of lung tissue (7.80, 125, and 1000 ng/g) were included in each analytical run. The lower limit of quantification (LLOQ), defined as the standard concentration where analysis of 3 replicate samples did not exceeded a coefficient of variation of 20%. The LLOQ was 3.90 ng/g. For the quality control samples, the intra-run coefficients of variation ranged from 4.9 to 8.1%, and the intra-run biases ranged from -4.0% to 9.4%. For the calibration curve samples, the intra-run coefficients of variation ranged from 1.85 to 8.1%, and the intra-run biases ranged from -7.5% to 13.6%. All the study samples were analyzed in a single analytical run. The recovery ranges for tulathromycin and IS in incurred lung tissue samples was 87.1-93.2% and 71.1-76.4%, respectively.

Pharmacokinetic and biometrical analyses

Plasma and lung tissue homogenate drug concentrations were analyzed using an ANOVA linear mixed model as implemented by the Statistical Analysis System (SAS, version 9.2, Cary, NC, 2011). A natural log transformation (ln) was applied to the concentration data for both matrices prior to analysis. Least squared means (geometric mean) and 95% confidence bounds were calculated with natural logarithm transformed data and the final results back transformed representing geometric means (8). Standard errors of LS means were estimated and 95% confidence interval (CI) was constructed. Back-transformed LS means and CIs for each treatment and dose group were reported. Back transformed least squares mean (LS...
mean) concentration was used as an estimate of treatment means. The dose groups were
tested for significant differences for fixed effects at the $\alpha = 0.05$ level, indicating that $p$ values $\leq$
0.05 showed a significant difference for the effect. The wet-to-dry ratio was summarized as
arithmetic means, standard deviations (SDs), and coefficient variations (CVs).

Wet-to-dry ratios were compared statistically by the same linear mixed model. Linear
regression analysis was performed to test the linear association between wet-to-dry ratio and
raw drug concentration data.

Pharmacokinetic parameters were estimated based on the back-transformed LS means
(8). Pharmacokinetic parameters were estimated using WinNonlin software (Standard ed.,
version 5.1, Pharsight Corporation, Mountain View, CA, USA). The WinNonlin 200 model was
used for the non-compartmental analysis. The AUC through 144 h post-dose ($\text{AUC}_{0-144}$), area
under the concentration-first moment time curve through 144 h post-dose ($\text{AUMC}_{0-144}$), mean
residence time (MRT), and the terminal elimination half-life ($t_{1/2}$) were estimated. The $\text{AUC}_{0-144}$
was calculated by the log-linear trapezoidal rule, and $\lambda_z$ was estimated with uniform weighting.
The observed peak LS mean concentration ($C_{\text{max}}$) and time of its occurrence ($t_{\text{max}}$) was obtained
from inspection of the back-transformed LS mean concentration.

Results

All experimental animals remained in good health throughout the acclimatization period.
One animal (#149) of dose group T-7-LPS-CP (72 h) was euthanized due to animal welfare
concerns. Animal 145 (48 h) from the same dose group was found dead. These animals were
not replaced. The rest of the experimental animals remained in good health throughout the
study period. No adverse reactions or detrimental health effects were observed following the
administration of tulathromycin. Animals in dose groups T-7, T-7-LPS, T-7-LPS-CP, and T-28
received an actual averaged dose of 6.95, 6.90, 6.99, and 27.57 mg tulathromycin/kg,
respectively. In one animal from dose group T-7 (animal 4); there was leakage of the treatment
solution from the administration site. Therefore this animal was considered under-dosed and was excluded from the study.

Plasma

Tulathromycin was rapidly absorbed in both dose groups. The maximal LS mean concentration of tulathromycin was observed 30 min after tulathromycin administration for dose group T-7, T-28, and T-7-LPS and 1 h for T-7-LPS-CP. The $C_{\text{max}}$ LS mean ($\pm$ SE) of tulathromycin in plasma was $6790 \pm 2450$ ($t_{\text{max}}$ 30 min) and $6920 \pm 2520$ ng/mL ($t_{\text{max}}$ 1 h) in dose groups T-7-LPS and T-LPS-CP, respectively. Plasma concentrations of tulathromycin results are included in Table 1 and shown in Figures 1 and 2. The pharmacokinetic parameter results are included in Table 3.

Lung tissue homogenate

Tulathromycin rapidly and extensively distributed into lung tissue (Table 2, Figure 2 and 3). In dose groups T-7 and T-28, the tulathromycin $C_{\text{max}}$ was 4410 and 27800 ng/g (observed) at 30 min and 1 h post-tulathromycin, respectively (Figure 2). The $C_{\text{max}}$ in dose groups T-7-LPS and T-7-LPS-CP was 3.93 and 5.49 ng/g occurring at 30 min and 1 h post-dose tulathromycin, respectively (Figure 3). There were no statistically significant differences in tissue concentrations among the two dose groups treated with 7 mg/kg of tulathromycin. At all the sample times the lung tissue LS mean concentrations far exceeded the plasma concentrations in the four treatment groups. In all the dose groups, tulathromycin persisted for a long period of time with an MRT that ranged between 138 to 287 h. Summary of pharmacokinetic parameters are presented in Table 3. The lung-to-plasma AUC$_{0-144}$ ratio was 1.3 times higher in dose group T-7 than in group T-28 (AUC$_{0-144}$ ratio 7.95 vs. 5.77, respectively).
Wet-to-dry ratios for lung tissue

Wet and dry ratio weights were determined in all the dose groups. Summarized data are presented in Table 4. There were statistically significant differences between dose group T7 vs. T-7-LPS (p<0.01) and T7 vs. T-7-LPS-CP (p<0.001) between 72 and 144 h. No significant differences were observed between the T-7-LPS and T-7-LPS-CP dose groups (p > 0.05). Likewise, there were no statistically significant differences among the three dose groups during the first 48 h post-tulathromycin administration. There was a negative linear relationship (p<0.01) between raw data concentration and the wet-to-dry ratio for the T-7-LPS and T7-LPS-CP dose groups (Figure 4 and 5). However, the fraction of variation of the drug concentration explained by the wet-to-dry ratio (72 to 144 h) was low (R² <5%) for both dose groups.

Lung tissue histology

Neutrophil accumulations were observed in the lumen of bronchioles (minimal to mild), interstitial around bronchioles (minimal to mild), in alveolar spaces (minimal), and in alveolar septa (minimal to moderate) of animals treated with LPS alone (LPS–7 h and LPS – 24 h), but not in non treated animals (CT-NT) nor in animals pre-treated with cyclophosphamide and then treated with LPS.

White blood cell count

A white blood cell (WBC) count was performed in all control animals: CT-NT, CT-LPS, and CT-LPS-CP. The WBC results are summarized in Table 5 and neutrophil count results are presented graphically in Figure 6.

Discussion

The concentration vs. time profile of tulathromycin in lung tissue homogenate and plasma was evaluated in all dose groups. Also, pharmacokinetic parameters of tulathromycin were estimated in both matrices.
In dose groups T-7 and T-28, tulathromycin was rapidly absorbed (Figure 2). Tulathromycin was distributed extensively and rapidly into lung tissue in both of these dose groups, as previously reported in other species (8, 19). Tulathromycin was slowly eliminated from lung tissue of both dose groups T-7 and T-28 with an MRT of about ~6 days (Table 3).

A similar pharmacokinetic trend in mice has been reported for other macrolides including erythromycin, spiramycin, clarithromycin, and azithromycin (30). Furthermore, results suggest that tulathromycin has a larger accumulation and persists in lung tissue longer than other macrolides in mice (30). The high affinity of the drug for lung tissue and the long persistence of tulathromycin was also reported in cattle (8, 19), pigs (3, 19), and foals (31). Multiple factors have been hypothesized to explain the accumulation of macrolides in lung tissue, including interactions with phospholipids (17), intracellular accumulation (bronchial and alveolar epithelium and bronchoalveolar cells) due to ion trapping (6), active transport (carriers such as members of the organic anion transporting protein) (26), and endocytosis (5). However, the identification and description of the mechanism(s) involved in tulathromycin lung tissue accumulation still remains unknown.

Tulathromycin is a lipid-soluble triamilide (15). Therefore, the overall molecule contains three basic functional groups with pKas ranging from 8.6 to 9.9. These features may favor the penetration and intracellular accumulation of the drug in an acidic environment (ion trapping). Further research is necessary to elucidate the mechanism(s) and factor(s) involved in the intracellular accumulation of the drug.

We evaluated the pharmacokinetic profile of tulathromycin at two different dose levels in order to predict drug concentrations for future studies. In plasma, the AUC₀-₁₄₄ ratio adjusted by dose between dose groups T-7 and T-28 was 1.22. In lung tissue, the AUC₀-₁₄₄ ratio adjusted by dose between dose groups T-7 and T-28 was 0.89 (mean actual doses 6.95 vs. 27.6 mg/kg, respectively). The results obtained indicate that, at the two dose levels evaluated, the dose
response could be different in plasma and lung tissue. We cannot confirm dose proportionality or lack thereof because the data is insufficient. It is important to stress that both parallel design and destructive sampling are associated with increased imprecision of the data due to inter-individual variability. In plasma the concentration at the 1 hr time point for injection of 28 mg/kg of tulathromycin was much less than the concentrations achieved at 1 hr for the lower doses of 7 mg/kg. These data do not agree with the proportional differences in lung concentrations. We could not find a specific reason to explain this discrepancy. However, considering that we used terminal sampling (one data point per animal) and that the apparent discrepancy occurred during the early portion of the curve (1 h), when absorption, distribution and elimination processes are taking place concurrently, it may have been the case that some of the animals sampled at 1 h in the 28 mg/kg group exhibited a shorter distribution half-life, an increased clearance, and/or a decreased absorption rate that would have resulted in a later Tmax and lower concentrations in the early fraction of the curve.

The AUC last (to the last measured time point) and the AUC to infinity is exactly the same for plasma concentration AUC values in the 28 mg/kg group. The values for the other groups were almost exactly the same when comparing these two values. The reason for the similitude of the values is twofold. The area extrapolated for estimation of the AUC to infinity is really small. The other factor that determines the similitude of the values is that we are reporting the 3 significant figures. Therefore the numerical differences between the AUC last and AUC to infinity (in particular for the 28 mg/kg group) is lost in the process of rounding up the values.

In this study, two experimental models were implemented. It was used an experimentally-induced neutropenic mice and animals treated intra-tracheally with LPS-Ec. Lipopolysaccharides are structural and functional components of the gram negative bacterial outer membrane (7). The administration of LPS in the respiratory system has been extensively used to trigger an acute inflammatory response (1). The administration of LPS in the respiratory
system prompts an acute infiltration of polymorphonuclear neutrophils and the release of a
myriad of inflammatory mediators. The model implemented in the present study has been fully
described elsewhere (28). The neutropenic mouse model has been extensively used to unravel
the pharmacokinetics and pharmacodynamics of antimicrobials (9).

In the present study, we evaluated the LPS response in lung tissue only in a subset of
closeup mice (untreated and treated with LPS and LPS plus CP). At the time of tulathromycin
administration, neutrophils were present to a moderate extent in the alveolar septa. Mild
congestion in capillaries was noted only in the mice treated with LPS. Neither neutrophils nor
any other change was observed in the control and CT-LPS-CP animals. We also assessed the
WBC in a control group at the time of tulathromycin administration. The administration of LPS
induced a remarkable change in the average blood cell count when compared with control
animals (control 6.19 ± 2.94 vs. LPS-treated 7.24 ± 1.64 x 10³/µl) and with reference values for
anesthetized BALB/c mice (Table 5) (18).

The WBC count in the animals treated with LPS and CP was lower than in the other
groups (0.93 ± 0.27 x 10³/µl). These changes were also reflected in the neutrophil count.
Previous studies considered animals neutropenic when the cell count was <100 cell/µl (10, 24).
In the present study, the mice were severely neutropenic regardless of the administration of
LPS (0.04 ± 0.01, 3.17 ± 1.02, and 0.69 ± 0.37 x 10³/µl for the CT-LPS-CP, CT-LPS, and CT-NT
group, respectively) (Table 5 and Figure 6) (18). Therefore, at the time of tulathromycin
administration, animals treated with LPS were neutrophilic (abnormally higher number of
neutrophils in blood) and exhibiting accumulation of neutrophils in lung. In contrast, mice treated
with LPS plus CP where neutropenic without recruitment of neutrophils into the lung.

The AUC₀⁻¹₄₄ of tulathromycin in plasma of neutropenic mice was 1.7 and 2.8 times
higher than in healthy mice (T-7) for dose groups T-7-LPS and T-LPS-CP, respectively. The
reasons for this change in drug exposure are unknown.
In both LPS-treated groups, tulathromycin distributed extensively and rapidly into lung tissue, as previously observed for group T-7. In the LPS-treated animals, there is a trend to have lower AUC_{0-144} values than in non-LPS-treated (T-7) animals (Table 3). The reasons for lower AUC_{0-144} values in LPS-treated animals are unclear. This trend was not compared statistically because of the lack of dispersion parameters as a consequence of the destructive sampling used in this study.

An increase of the wet-to-dry ratio 48 h post-LPS administration could be caused by the edema associated with the inflammatory process. This could have a dilution effect on the drug in lung tissue homogenate explaining the lower LS mean concentrations observed in LPS-treated animals. However, the wet-to-dry ratio explained less than 5% of the change in the lung tissue drug concentration from LPS treated mice (Figure 4 and 5). Changes in the pharmacokinetic parameters estimated from plasma and lung tissue have also been reported for other macrolides in infected animals (30). The AUC of spiramycin, roxithromycin, and azithromycin in lung tissue increased; clarithromycin decreased, and erythromycin did not change when the drugs were administered 48 h post infection. Because the plasma AUC_{0-144} of tulathromycin in T-7 was 41 and 64% lower than in T-7-LPS and T-7-LPS-CP, respectively, the decrease of AUC_{0-144} in lung tissue accumulation (AUC_{0-144}) may not be attributable to changes in plasma concentration (Table 3 and Figure 1). Even though the plasma AUC was numerically higher in LPS treated animals, this was not reflected in the lung figures. The lung:plasma ratios of AUC_{0-144} were 3.79 and 2.26 for dose groups T-7-LPS and T-7-LPS-CP, respectively, and 7.95 for dose group T-7. Tulathromycin was slowly eliminated from lung tissue of both T-7-LPS and T-7-LPS-CP dose groups and resided somewhat longer in LPS-treated than non LPS-animals (Table 3).

Tissue-directed pharmacokinetics has been proposed as one of the mechanisms of local drug accumulation (2, 12, 29). When azithromycin was administered to infected mice, the AUC in lung tissue was much lower in leucopenic animals (2). Based on these results the authors...
suggested that leucocytes may help to transport macrolides to the site of infection. However, results might be confounded by a lower plasma concentration in leucopenic animals. The results obtained in the present study do not allow delineating a relationship between neutrophils and lung tissue drug accumulation in mice. Several scenarios may explain this lack of relationship. First, neutrophils may be truly irrelevant in the pulmonary accumulation of tulathromycin in mice. Second, tulathromycin may not concentrate in neutrophils from mice or it may concentrate at low level at the given dose. Third, the accumulation of cells in lung tissue may not have been enough to generate an evident change in the lung tissue pharmacokinetics of the drug. Lastly, the relationship between time of drug administration after the challenge (and concentration time profile) and kinetics (movement of cell from blood to lung) of neutrophils did not match.

In this study, we administered the drug after the challenge based on the initial manifestation of abnormal clinical signs observed in preliminary studies simulating a field scenario. Azoulay-Dupuis et al. (1991) determined that the accumulation of azithromycin at the site of infection was influenced by the time of administration and progression of the disease (2). In the present study we evaluated drug pharmacokinetics after administration at a fixed time with regard to the challenge administration. The evaluation of multiple scenarios would provide valuable information about the potential relevance of inflammation and drug tissue accumulation or drug kinetics. It is important to highlight that the rate and extent of intracellular accumulation and release depend on multiple factors including the chemical structure of the drug (5), cell type (4, 12, 16) cell status (21), extracellular conditions (pH) (14) and type of stimulus (12). Those factors should be taken into account when designing future studies.

In mice, neutrophilia also did not affect drug accumulation in the lung. From a therapeutic standpoint, this may represent an advantage since the dose regimen may not need to be adjusted based on the cell count in order to reach target therapeutic concentrations. The prospective evaluation of the mentioned statement using multiple scenarios would certainly
provide valuable information. Ideally, this should be evaluated in target species such as pig and cattle, under field conditions.

In conclusion, we evaluated the pharmacokinetics of tulathromycin in mouse lung tissue homogenates and plasma in three different dose groups: healthy mice and LPS-challenged neutropenic and neutrophilic animals. In all dose groups, the distribution of the drug into the lungs was rapid and persisted at relatively high levels during 6 days post-administration. The concentration vs. time profile of tulathromycin in lung tissue was not influenced by the intra-nasal administration of LPS-Ec. Results suggest that neutrophils may not have a positive influence on tulathromycin accumulation in mouse lung tissue when the drug is administered during either a neutrophilic or neutropenic state. The potential role of neutrophils on the accumulation of tulathromycin in lung tissue should be evaluated in other species and probably for other macrolides. The microbiological and clinical impacts of these scenarios need to be evaluated.

Acknowledgements

The authors are grateful to the Metabolism and Safety Unit staff of Pfizer Animal Health, Kalamazoo, MI, USA, that assisted with this study. Also we want to thanks Misty Bailey (University of Tennessee) for her comments on this manuscript.

Conflict of Interest

The present study was funded by Pfizer AH, Kalamazoo, MI, USA
References


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Table 1. Summary of the LS mean concentration (ng/mL) of tulathromycin in plasma from mice treated with a single s.c. injection of tulathromycin at 7 and 28 mg/kg body weight.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Group</th>
<th>LS Mean†</th>
<th>95% CIs†</th>
<th>Time point</th>
<th>Group</th>
<th>LS Mean†</th>
<th>95% CIs†</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>T-28</td>
<td>11700</td>
<td>490-18500</td>
<td>72 h</td>
<td>T-28</td>
<td>109</td>
<td>20.2-199</td>
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<td></td>
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<td>6790</td>
<td>184-11700</td>
<td>T-7-LPS</td>
<td>19.2</td>
<td>5.20-33.2</td>
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</tr>
<tr>
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<td>6700</td>
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<td>0.31-10.3</td>
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</tr>
<tr>
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<td>T-7</td>
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<td>24.4</td>
<td>4.50-44.2</td>
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</tr>
<tr>
<td>1 h</td>
<td>T-28</td>
<td>792</td>
<td>214-1370</td>
<td>96 h</td>
<td>T-28</td>
<td>26.2</td>
<td>7.10-45.4</td>
</tr>
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<td>713-7030</td>
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<td>16.9</td>
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<td>2.50-16.0</td>
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<tr>
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<td>T-28</td>
<td>145</td>
<td>26.7-260</td>
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<td>T-28</td>
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<td>3.50-34.9</td>
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</tr>
<tr>
<td></td>
<td>T-7</td>
<td>103</td>
<td>28.1-180</td>
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<td>10.8</td>
<td>2.90-18.7</td>
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</tr>
<tr>
<td>48 h</td>
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<td>100</td>
<td>27.2-174</td>
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<td>T-28</td>
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<td>7.92</td>
<td>2.50-15.2</td>
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<tr>
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<td>T-7-LPS-CP</td>
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<td>1.75-11.2</td>
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</tr>
<tr>
<td></td>
<td>T-7</td>
<td>60.0</td>
<td>16.2-103</td>
<td>T-7</td>
<td>15.4</td>
<td>4.20-26.7</td>
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† Back-transformed
Table 2. Summary of the LS mean concentration (ng/mL) of tulathromycin in lung tissue homogenate from mice treated with a single s.c. injection of tulathromycin at 7 and 28 mg/kg body weight

<table>
<thead>
<tr>
<th>Time point</th>
<th>Group</th>
<th>LS Mean†</th>
<th>95% CI†</th>
<th>Time point</th>
<th>Group</th>
<th>LS Mean†</th>
<th>95% CI†</th>
</tr>
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<tbody>
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<td>30 min</td>
<td>T-28</td>
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<td>4550-2600</td>
<td>72h</td>
<td>T-28</td>
<td>4800</td>
<td>1400-8210</td>
</tr>
<tr>
<td></td>
<td>T-7-LPS</td>
<td>3940</td>
<td>1440-6430</td>
<td>T-7-LPS</td>
<td>489</td>
<td>325-1450</td>
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<td>T-7-LPS-CP</td>
<td>5240</td>
<td>1910-8560</td>
<td>T-7-LPS-CP</td>
<td>680</td>
<td>123-1240</td>
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</tr>
<tr>
<td></td>
<td>T-7</td>
<td>4410</td>
<td>2198-6621</td>
<td>T-7</td>
<td>1950</td>
<td>715-3196</td>
<td></td>
</tr>
<tr>
<td>1 h</td>
<td>T-28</td>
<td>27900</td>
<td>5050-50500</td>
<td>96 h</td>
<td>T-28</td>
<td>4760</td>
<td>1380-8130</td>
</tr>
<tr>
<td></td>
<td>T-7-LPS</td>
<td>3260</td>
<td>1192-5320</td>
<td>T-7-LPS</td>
<td>824</td>
<td>301-1350</td>
<td></td>
</tr>
<tr>
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<td>3150</td>
<td>1150-5140</td>
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<td>1690</td>
<td>619-2770</td>
<td></td>
</tr>
<tr>
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<td>T-28</td>
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<td>1740-7790</td>
<td>144 h</td>
<td>T-28</td>
<td>3860</td>
<td>1120-6590</td>
</tr>
<tr>
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<td>305-1800</td>
<td>T-7-LPS</td>
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<td>308-1810</td>
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</tr>
<tr>
<td></td>
<td>T-7-LPS-CP</td>
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<td>292-1720</td>
<td>T-7-LPS-CP</td>
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<td>218-1280</td>
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</tr>
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<td>603-3540</td>
<td>T-7</td>
<td>1020</td>
<td>375-1670</td>
<td></td>
</tr>
</tbody>
</table>

† Back-transformed for reporting
Table 3. Summary of the pharmacokinetic parameters of tulathromycin in lung tissue homogenate and plasma in mice treated with a single s.c. injection of tulathromycin at 7 and 28 mg/kg body weight.

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter*</th>
<th>Group</th>
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<th>T-7</th>
<th>T-7-LPS</th>
<th>T-7-LPS-CP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma LH</td>
<td>Plasma LH</td>
<td>Plasma LH</td>
<td>Plasma LH</td>
<td>Plasma LH</td>
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<tr>
<td>HL_{tambda_z} (h)</td>
<td>18.1</td>
<td>112</td>
<td>13.5</td>
<td>82.0</td>
<td>16.5</td>
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<tr>
<td>T_{max} (h)</td>
<td>0.50</td>
<td>1.00</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>C_{max} (ng/mL)</td>
<td>11700</td>
<td>27900</td>
<td>3300</td>
<td>4410</td>
<td>6790</td>
</tr>
<tr>
<td>C_{last} (ng/mL)</td>
<td>13.6</td>
<td>3860</td>
<td>15.4</td>
<td>6790</td>
<td>7.82</td>
</tr>
<tr>
<td>AUC_{last} (hr*ng/mL)</td>
<td>152000</td>
<td>869000</td>
<td>3100</td>
<td>6790</td>
<td>246000</td>
</tr>
<tr>
<td>AUC_{inf obs} (hr*ng/mL)</td>
<td>152000</td>
<td>1500000</td>
<td>31200</td>
<td>378000</td>
<td>52800</td>
</tr>
<tr>
<td>AUMC_{last} (hr<em>hr</em>ng/mL)</td>
<td>599000</td>
<td>45000000</td>
<td>274000</td>
<td>15700000</td>
<td>252000</td>
</tr>
<tr>
<td>MRT_{INF pred} (h)</td>
<td>4.16</td>
<td>148</td>
<td>8.91</td>
<td>138</td>
<td>4.99</td>
</tr>
<tr>
<td>AUC_{LH:Plasma ratio}</td>
<td>5.77</td>
<td>7.95</td>
<td>3.79</td>
<td>2.26</td>
<td></td>
</tr>
</tbody>
</table>

*Dispersion data of the PK parameters are not presented because PK parameters were estimated based on least square means derived from the naïve pooling from each sample point.
Table 4. Wet-to-dry weight ratios of lung tissue from BALB/c mice

<table>
<thead>
<tr>
<th>Time point</th>
<th>Dose group</th>
<th>Mean</th>
<th>SD</th>
<th>%CV</th>
<th>Time point</th>
<th>Dose group</th>
<th>Mean</th>
<th>SD</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>T-7</td>
<td>3.18</td>
<td>0.65</td>
<td>20.3</td>
<td>72 h</td>
<td>T-7</td>
<td>3.95</td>
<td>0.28</td>
<td>7.05</td>
</tr>
<tr>
<td></td>
<td>T-7-LPS</td>
<td>4.22</td>
<td>0.38</td>
<td>9.07</td>
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<td>T-7-LP</td>
<td>4.87</td>
<td>0.22</td>
<td>4.70</td>
</tr>
<tr>
<td></td>
<td>T-7-LPS-CP</td>
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<td>0.14</td>
<td>3.16</td>
<td></td>
<td>T-7-LPS-CP</td>
<td>5.18</td>
<td>0.23</td>
<td>4.49</td>
</tr>
<tr>
<td>1 h</td>
<td>T-7</td>
<td>4.37</td>
<td>0.11</td>
<td>2.53</td>
<td>96 h</td>
<td>T-7</td>
<td>4.32</td>
<td>0.25</td>
<td>5.73</td>
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<tr>
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<td>T-7-LPS</td>
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<td>0.15</td>
<td>3.53</td>
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<td>T-7-LPS</td>
<td>4.96</td>
<td>0.62</td>
<td>12.6</td>
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<td>T-7-LPS-CP</td>
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<td>0.28</td>
<td>5.67</td>
</tr>
<tr>
<td>24 h</td>
<td>T-7</td>
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<td>0.21</td>
<td>5.08</td>
<td>120 h</td>
<td>T-7</td>
<td>4.59</td>
<td>0.20</td>
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<td>1.30</td>
<td>27.2</td>
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<td>4.28</td>
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<td>0.15</td>
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<td>4.97</td>
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<td>6.45</td>
</tr>
<tr>
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<td>0.46</td>
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<td>0.20</td>
<td>3.99</td>
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</table>
Table 5. Summary of white blood cell count in BALB/c-c mice

<table>
<thead>
<tr>
<th>Dose Group</th>
<th>WBC (x10³/µL)</th>
<th>N</th>
<th>L</th>
<th>M</th>
<th>E</th>
<th>B</th>
<th>R</th>
<th>N  (10⁵ cells/µL)</th>
<th>L  (10⁵ cells/µL)</th>
<th>M  (10⁵ cells/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT-LPS-CP (n=7)</td>
<td></td>
<td>0.93</td>
<td>5.93</td>
<td>89.1</td>
<td>0.37</td>
<td>2.79</td>
<td>0.26</td>
<td>0.04</td>
<td>0.84</td>
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<tr>
<td>Mean</td>
<td>0.27</td>
<td>4.79</td>
<td>6.88</td>
<td>0.44</td>
<td>1.77</td>
<td>0.34</td>
<td>0.02</td>
<td>0.01</td>
<td>0.26</td>
<td>0.00</td>
</tr>
<tr>
<td>SD</td>
<td>0.29</td>
<td>0.81</td>
<td>0.08</td>
<td>1.18</td>
<td>0.64</td>
<td>1.33</td>
<td>0.32</td>
<td>0.29</td>
<td>0.32</td>
<td>2.65</td>
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<tr>
<td>CV</td>
<td>0.29</td>
<td>0.81</td>
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<td>1.18</td>
<td>0.64</td>
<td>1.33</td>
<td>0.32</td>
<td>0.29</td>
<td>0.32</td>
<td>2.65</td>
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<tr>
<td>CT-LPS (n=7)</td>
<td>7.24</td>
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<td>4.03</td>
<td>3.17</td>
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<tr>
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<td>1.64</td>
<td>7.00</td>
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<td>0.32</td>
<td>0.98</td>
<td>0.05</td>
<td>0.64</td>
<td>1.02</td>
<td>0.83</td>
<td>0.03</td>
</tr>
<tr>
<td>SD</td>
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<td>0.16</td>
<td>0.13</td>
<td>0.34</td>
<td>0.56</td>
<td>0.33</td>
<td>0.16</td>
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<tr>
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<td>0.23</td>
<td>0.16</td>
<td>0.13</td>
<td>0.34</td>
<td>0.56</td>
<td>0.33</td>
<td>0.16</td>
<td>0.32</td>
<td>0.22</td>
<td>0.38</td>
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<td>CT-NT (n=10)</td>
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<td>83.5</td>
<td>1.15</td>
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<td>0.69</td>
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<td>Mean</td>
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<td>2.05</td>
<td>1.33</td>
<td>0.28</td>
<td>0.51</td>
<td>0.11</td>
<td>0.48</td>
<td>0.37</td>
<td>2.45</td>
<td>0.04</td>
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<td>SD</td>
<td>0.47</td>
<td>0.19</td>
<td>0.02</td>
<td>0.24</td>
<td>0.26</td>
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<tr>
<td>CV</td>
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<td>0.19</td>
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<td>0.24</td>
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<td>0.54</td>
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WBC white blood cell count; N: Neutrophils; L: Lymphocytes; M: Macrophages; E: Eosinophils; B: Basophils; R: Reticulocytes
Figure 1. LS means concentration (±SE) (ng/mL) of tulathromycin in plasma from mice treated s.c. with tulathromycin at 7 mg/kg. (For T-7-LPS at 30 min and 96 h n=4, For T7-LPS-CP at 30 min, 24 and 48 h, n=4 and at 72 h n=3, For T-7 30 min and 96 h n=4)
Figure 2. LS means concentration (±SE) of tulathromycin in lung tissue homogenate (ng/g) and plasma (ng/mL) from mice treated s.c. with tulathromycin at 7 and 28 mg/kg (For T7 at 48 h, n=4)
Figure 3. LS means concentration (±SE) (ng/mL) of tulathromycin in lung tissue homogenate from mice treated s.c. with tulathromycin at 7 mg/kg. (For T7- LPS-CP at 48 h and 72 h, n=4, For T7 at 48 h, n=4)
Figure 4 Linear regression concentration of tulathromycin in lung tissue vs. wet to dry ratio (from 72 to 144h) for T-7 LPS ($R^2 = 0.01$).
Figure 5. Linear regression concentration of tulathromycin in lung tissue vs. wet to dry ratio (from 72 to 144h) for T7-LPS-CP ($R^2 = 0.03$)
Figure 6. Number of neutrophils (mean ± SD) in blood from BALB/c mice