

Rapid and Prolonged Distribution of Tulathromycin into Lung Homogenate and Pulmonary Epithelial Lining Fluid of Holstein Calves Following a Single Subcutaneous Administration of 2.5 mg/kg Body Weight

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ABSTRACT

Twenty-four healthy Holstein calves received single subcutaneous injections of 2.5 mg/kg bw tulathromycin. At each of 8 time points ranging from 1 – 360 hr post-dose, three animals were randomly selected for euthanasia. At the time of euthanasia, whole blood, lung tissue, and broncho-alveolar lavage (BAL) samples were collected and subsequently assayed for tulathromycin with an LC MS/MS procedure. Concentrations of tulathromycin in pulmonary epithelial lining fluid (PELF) were determined from

the BAL supernatant concentrations using the urea dilution method. Concentrations of tulathromycin in the cells of the pulmonary epithelial lining fluid (PELF cells, predominantly macrophages) were also determined. Tulathromycin distributed rapidly and extensively into PELF, PELF cells, and lung homogenate, with areas under the concentration-time curve through 360 hr (AUC_{0-360}) that were 53 – 565 times the plasma AUC_{0-360} . Tulathromycin concentrations exceeded 1,000 ng/mL in PELF and 1,000 ng/g in lung homogenate by 3 hr post-dose, and the peak concentration in BAL cells was 19,500 ng/mL at 72 hr. The drug was slowly eliminated from lung homogenate, PELF, and PELF cells, with apparent $t_{1/2}$ values

>200 hr. These data demonstrate rapid achievement and prolonged maintenance of therapeutic tulathromycin concentrations at sites of respiratory infections in cattle following a single-dose administration.

INTRODUCTION

Tulathromycin is a triamilide antibiotic commonly used to treat respiratory infections in livestock. Typical MIC₉₀ values for tulathromycin against isolates of *M. haemolytica*, *P. multocida*, *H. somni* and *M. bovis* range from 1,000 – 4,000 ng/mL (Evans, 2005; Godinho, 2008). The drug is rapidly and almost completely absorbed (>90%) following subcutaneous administration to cattle at 2.5 mg/kg bw, with maximum plasma concentrations generally occurring within an hour and with mean peak concentrations of approximately 300 – 500 ng/mL (Galer, 2004; Nowakowski, 2004). The drug distributes extensively into lung tissue, with peak concentrations in lung homogenate of cattle that are approximately 8 times the peak plasma concentrations, and with an area under the concentration-time curve (AUC) that is more than 50-times the plasma AUC (Nowakowski, 2004).

The high lung homogenate concentrations in cattle, when coupled with the prolonged t_{1/2} (approximately 4 days in plasma and longer in lung homogenate) have been used to rationalize the single-dose efficacy of the drug for the treatment of bovine respiratory disease (Nowakowski, 2004; Evans, 2005). In the previous pharmacokinetic studies, tulathromycin was not measured in lung homogenate at any time before 12 hr post-dose (Galer, 2004; Nowakowski, 2004). The present study was performed to assess in cattle the distribution of tulathromycin into lung homogenate at earlier time points post-dose and to characterize the time-course of tulathromycin at sites in the lung that may be colonized with pathogenic bacteria. In this study, we measured concentrations of tulathromycin in pulmonary epithelial lining fluid (PELF) and cells, sites associated with infections. With the early sampling time points of these physiological-

ly relevant matrices, we provide a rationale for the rapid clinical response to tulathromycin seen in the treatment of respiratory infections.

MATERIALS AND METHODS

Study Design

Twenty-four healthy Holstein male calves with bw ranging from 198 to 262 kg were treated with tulathromycin in this randomized parallel group study. Although the tulathromycin treatments were part of a larger study, only the tulathromycin data are reported herein. Each animal was administered a single subcutaneous injection of tulathromycin (Draxxin® Injectable Solution) at 2.5 mg/kg bw. Three animals were randomly selected to be euthanized at each of the following times post-dose: 1, 3, 11, 24, 72, 168, 264, and 360 hours. Whole blood, lung tissue, and broncho-alveolar lavage (BAL) samples were obtained from each animal at the time of euthanasia. The blood samples were taken by venipuncture into K2 EDTA and serum tubes. After centrifugation, the plasma was stored at -20°C until assayed for tulathromycin, and the serum was stored at approximately 4 to 6°C until assayed for urea. One-half of each caudal lung lobe was removed, and the two lung samples were placed together in a plastic bag on ice until they were ground. Aliquots of the ground lungs (0.20 g) were homogenized (using a Polytron, Kinematica Inc., Bohemia, NY, USA) with 5 mL of 0.04 M phosphoric acid, and then frozen for later analysis.

A bronchus to a cranial lung lobe was identified, opened, and lavaged using a pipette, with two 100-mL volumes of phosphate buffered saline (PBS) at approximately 20°C. Dwell time of the lavage fluid in the lung was minimal, ie, the fluid was immediately aspirated back into the pipette with assistance of massage of the lung lobe. The recovered BAL samples were centrifuged, and the supernatant saved and frozen for later analysis. The PELF cells were washed twice with PBS and resuspended in 5 mL of PBS. The cells were counted using a Coulter counter (Z1 Dual

Threshold Model, Beckman Coulter, Brea, CA, USA), and the concentration of cells in each sample was adjusted to 10^6 cells/mL using PBS. The BAL supernatant samples were analyzed for urea in order to calculate the dilution of pulmonary endothelial lining fluid (PELF) during lavage. A colorimetric assay for hemoglobin was used to assay the BAL supernatant samples for blood contamination.

Handling of the cattle was done according to the regulations outlined in the USDA Animal Welfare Act (9 CFR Parts 1, 2, and 3) and the conditions specified in Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies, Champaign, IL, USA., First Revised Edition, 1999). Before the initiation of the study, the procedures involving the care or use of the cattle were reviewed and approved by an Institutional Animal Care and Use Committee.

Tulathromycin Assays

The plasma, lung homogenate, BAL supernatant, and PELF cell samples were analyzed for tulathromycin content by high performance liquid chromatography (HPLC) with tandem mass spectrometry detection (LC MS/MS). The assays for these matrices employed different extraction procedures, but used the same internal standard (roxithromycin) as well as the same chromatography and detection procedures. The chromatography and detection procedures are therefore described only for the plasma assay, but are relevant to the other assays as well.

Although no control samples (ie, pre-dose) were obtained during this study, control samples for each matrix were obtained prior to the study and used in the development and validation of the assays. For each of the assays, calibration curves were constructed with at least eight standards spanning the calibration range, ie, the lower limit of quantification to the upper limit of quantification. The R-square values for the calibration curves were at least 0.994.

Plasma: A 50- μ L plasma sample was

mixed with 200 μ L of acetonitrile containing roxithromycin, the internal standard, and centrifuged. A 160- μ L aliquot of the plasma supernatant was diluted with 40 μ L of 20 mM ammonium acetate, pH 4. A 10- μ L aliquot of the prepared samples was injected onto the HPLC system consisting of a BDS Hypersil C8 30 x 2.1 mm column (Thermo Fisher Scientific Inc., Waltham, MA, USA), with a mobile phase of acetonitrile and 20 mM pH 4 ammonium acetate (linear gradient from 20% to 80% acetonitrile over 0.5 minutes). Detection was accomplished with MS/MS using a Sciex 4000 triple-quadrupole mass spectrometer with an electrospray ionization source (MDS Sciex, Foster City, CA, USA). Transition ions of m/z 806 \rightarrow 577 were used for tulathromycin and 837 \rightarrow 679 used for roxithromycin. Calibration occurred over the range of 2.5 to 500 ng/mL, and three quality control samples (7.5, 20 and 300 ng/mL) were included in each analytical run. For the quality control samples, the inter- and intra-run coefficients of variation ranged from 4.6% to 13.2% and the inter- and intra-run biases ranged from -8% to +5.7 %.

Lung Homogenate: Lung samples (0.200 g) were homogenized with 0.050 mL of 50 mM phosphate buffer, pH 6, and 4.95 mL of 0.04 M phosphoric acid. The homogenate was centrifuged, 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 Oasis MCX 1cc (30 mg) SPE cartridge. The cartridge was washed with 1 mL of 50 mM pH 6 phosphate buffer, 1 mL of distilled deionized water, and 1 mL of acetonitrile. Drug was then eluted from the cartridge with two 0.5-mL volumes of 5% ammonium hydroxide/95% acetonitrile. After evaporation under nitrogen gas, the sample was reconstituted with 0.25 mL of 20 mM pH 4 ammonium acetate and acetonitrile (80:20 v:v) and then diluted 1:10 with additional ammonium acetate:acetonitrile solution. A 10- μ L aliquot was analyzed by LC-MS/MS. Calibration occurred over the range of 100

to 25,000 ng/g lung tissue, and quality control samples of 200, 10,000 and 20,000 µg/g lung tissue were included in each analytical run. The inter- and intra-run coefficients of variation for the quality control samples ranged from 6.3 to 15.0%, and inter- and intra-run biases for these samples ranged from -12.2% to +3%.

PELF Cells and BAL Supernatant: For both of these matrices, a 100-µL volume was mixed with 400 µL of acetonitrile containing roxithromycin and centrifuged. A 390-µL aliquot of supernatant was evaporated under nitrogen gas and reconstituted with 100 µL of 20 mM pH 4 ammonium acetate: acetonitrile (80: 20 v:v). A 10-µL aliquot was analyzed by LC-MS/MS. For the PELF cells, the calibration range was 1 to 100 ng/106 cells. All of the PELF cell samples were analyzed in one run, and the intra-run coefficients of variation for the quality control samples at 3, 30, and 100 ng/106 cells ranged from 3.0 to 4.3%. The bias in the assays of the quality control cell samples ranged from -4.4 to 11.7%. The calibration range for the BAL supernatant was 1 to 500 ng/mL. As with the PELF cell samples, all of the BAL supernatant samples were analyzed in one run and the intra-run coefficients of variation for quality controls at 3, 30 and 300 ng/mL ranged from 10.2 – 16.7%. The bias in the assays of the quality control supernatant samples ranged from -6.7 to 11.7%.

Urea Assays

Serum urea concentrations were measured colorimetrically (BioChain Urea Assay Kit, Hayward, CA, USA). The colorimetric assay did not have the necessary sensitivity to assay urea in the BAL supernatant, however, and an LC-MS/MS procedure was used for these samples. A 2-µL aliquot of BAL supernatant was injected onto the HPLC system consisting of an Aquity UPLC BEH C18, 1.7 µm, 2.1 x 50 mm column (Waters Corp., Milford, MA, USA), with a mobile phase of methanol and 0.1% formic acid (linear gradient from 0% to 80% methanol over 4 minutes). Detection was accomplished us-

ing MS/MS with a Sciex 4000 triple-quadrupole mass spectrometer with an electrospray ionization source. Transition ions of m/z 61 → 44. were used for urea. Calibration occurred over the range of 0.005 to 0.5 mg/dL, and three quality control samples (0.015, 0.03 and 0.3 mg/dL) were included in each analytical run. All of the assays for urea in BAL supernatant were performed in one analytical run, and the mean accuracy for the quality control samples ranged from 96.1 to 110%, with coefficients of variation ranging from 0.9 to 9.8%.

Calculation of Tulathromycin Concentrations in PELF

Concentrations of tulathromycin and urea in BAL supernatant were corrected for blood contamination using Equation 1.

$$BAL_{corr} = BAL_{uncorr} - F_{Blood} \cdot BP_{ratio} \cdot C_{p/s} \quad (\text{Eq. 1})$$

In Equation 1, BAL_{corr} and BAL_{uncorr} are the concentrations corrected and uncorrected for blood contamination, respectively. F_{Blood} is the fractional blood contamination of the sample based on the hemoglobin assay; BP_{ratio} is the blood to plasma ratio for the analyte; and $C_{p/s}$ is the concentration of the analyte in either plasma or serum. Values of F_{Blood} ranged from <0.0001 to 0.0012, and the BP_{ratio} for urea was estimated to be 0.93 from previously published data (Ralls, 1943). The mean blood to plasma concentration ratio for tulathromycin was determined to be 1.4 in a study in which plasma was assayed for tulathromycin after fortification of bovine whole blood with drug at concentrations ranging from 2.5 to 500 ng/mL. The tulathromycin concentrations in PELF (C_{PELF}) were then calculated by the urea dilution method (Rennard, 1986) using Equation 2.

$$C_{PELF} = CBAL_{corr} \cdot Urea_{Serum} / Urea_{BAL,corr} \quad (\text{Eq. 2})$$

In Equation 2, $C_{BAL,corr}$ and $Urea_{BAL,corr}$ are the corrected BAL supernatant concentrations of tulathromycin and urea, respectively, and $Urea_{Serum}$ is the serum urea concentration. Microscopic observations of the PELF cells indicated that the majority of

the cells were alveolar macrophages (AM), and the concentrations of tulathromycin in the AM were estimated by dividing the mass of tulathromycin per 10^6 PELF cells by 1.28 $\mu\text{L}/10^6$ cells, the mean volume of bovine AM (Scoreaux, 1999). Given the large cell volume of AMs relative to other white cells in PELF (Kiem, 2008), the use of this cell volume is likely to result in a conservative estimate of PELF cell concentrations.

Pharmacokinetic Calculations

The tulathromycin concentration data for each sample matrix were log transformed and modeled statistically in SAS (SAS Institute, Cary, NC, USA) using a mixed linear model that accounted for the fixed effects of treatment, sample time and their interaction, and the random effects of pen and between and within animal error. Back-transformed least squares mean (LSMean) concentrations were reported for each sample time. Non-compartmental pharmacokinetic analysis of the LSMean concentration data was performed for each matrix. The observed peak concentration (C_{max}) and time of its occurrence (t_{max}) were obtained from inspection of the LSMean concentration data. WinNonlin v.5.2 (Pharsight Corp., Mountain View, CA, USA) was used to calculate area under the concentration-time curve through 360 hours post-dose (AUC_{0-360}), the terminal elimination rate constant (λ_z), and the terminal

elimination half-life ($t_{1/2}$). The AUC_{0-360} was calculated by log-linear trapezoidal rule, and λ_z was estimated from the concentration data at times from 168 – 360 hr and with inverse concentration as the weighting function.¹

RESULTS

Tulathromycin was rapidly absorbed, with peak plasma concentrations occurring by 3 hr. Distribution occurred rapidly into lung homogenate and PELF, with LSMean concentrations exceeding 1,000 ng/g or ng/mL by 1 and 3 hr respectively. Distribution into PELF cells was not quite as rapid, with LSMean concentrations increasing to above 1,000 ng/mL by 11 hr and continuing to increase to 19,500 ng/mL at 72 hr. These high tissue concentrations were maintained for a prolonged time, with concentrations at 360 hr in lung homogenate and PELF cells of 1,310 ng/g and 10,600 ng/mL, respectively.

Tulathromycin concentration data from the entire 360 hr duration of the study are shown in Figure 1, and data from only the first 24 hr post-dose are plotted in Figure 2. Pharmacokinetic variables from each matrix are listed in Table 1. The drug was slowly eliminated from lung homogenate, PELF, and PELF cells, with apparent $t_{1/2}$ values >200 hr. The $t_{1/2}$ of tulathromycin in plasma was 64 hr and numerically smaller than the $t_{1/2}$ values in the other matrices. The plasma concentrations of tulathromycin were con-

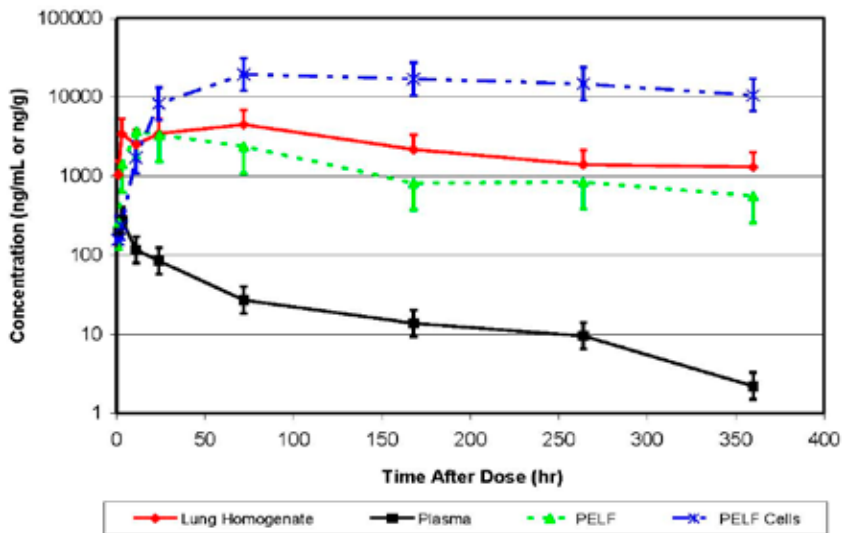
Table 1. Pharmacokinetic parameters for tulathromycin concentrations in lung homogenate, PELF cells, PELF and plasma.

| Matrix | $t_{1/2}$ (hr) | C_{max} (ng/mL or ng/g)* | t_{max} (hr) | AUC_{0-360} ($\mu\text{g hr/mL}$ or $\mu\text{g hr/g}$)* |
|-----------------|----------------|-----------------------------------|-----------------------|---|
| Lung Homogenate | 279 | 4510 | 72 | 867 |
| PELF Cells | 270 | 19500 | 72 | 5230 |
| PELF | 330 | 3730 | 11 | 492 |
| Plasma | 64 | 277 | 3 | 9.26 |

*Concentration units of $\mu\text{g/mL}$ for PELF cells, PELF and plasma but $\mu\text{g/g}$ for lung homogenate

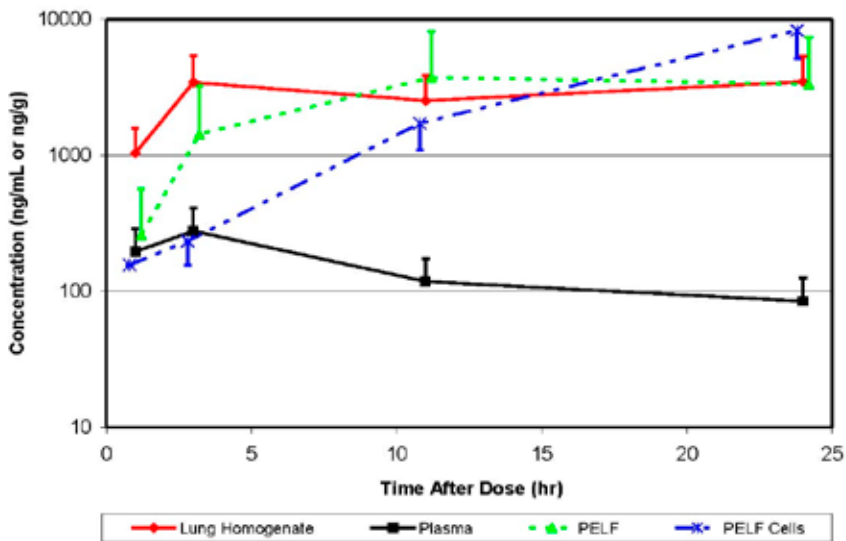
¹The final three time points were used to estimate λ_z of tulathromycin in all four matrices

Figure 1. LS Mean tulathromycin concentrations and 95% confidence intervals* for the 360 hr following a single 2.5 mg/kg IM dose to cattle.



*For clarity, the upper 95% confidence intervals for PELF and the lower 95% confidence intervals for lung homogenate are not depicted in the graph.

Figure 2. LS Mean tulathromycin concentrations and 95% confidence intervals* for the 24 hr following a single 2.5 mg/kg IM dose to cattle.



*For clarity, only upper or lower 95% confidence intervals are depicted for each matrix and the plotted times for lung homogenate, PELF and PELF cells are offset slightly from the actual times.

siderably lower than the tissue concentrations, with AUC_{0-360} values for PELF, PELF cells and lung homogenate of 53, 565 and 94 times, respectively, the values for the plasma AUC_{0-360} .

DISCUSSION

Plasma concentration data have had limited utility in characterization of the pharmacodynamics of macrolide or macrolide-like antibiotics because this class of antibiotics achieves concentrations in lung tissues that are many times the plasma concentrations, and the plasma concentrations may never reach or be only transiently above minimum inhibitory concentrations. Predictions of efficacy based on lung homogenate concentrations have been questioned as the active, unbound concentrations of these drugs exhibit distinct concentration-time profiles within various lung tissues or fluids. Because of this, there has been an emphasis in pharmacodynamic studies with macrolides and macrolide-like drugs to measure drug concentrations at the site of the infection, eg, PELF for extracellular lung pathogens and AMs for intracellular pathogens, and to discourage the use of tissue homogenate concentrations (Anonymous, 2000; Joukhar, 2001; Cazzola, 2002; Toutain, 2002; Muller, 2004; Drusano, 2005). There have been several veterinary studies with these classes of drugs to evaluate pharmacokinetics in PELF or AM of foals (Jacks, 2001; Davis, 2002; Womble, 2006; Scheuch, 2007; Suarez-Mier, 2007; Venner, 2010), but to the authors' knowledge, none in cattle. This study was performed, in part, to provide information about the time-course for the distribution of tulathromycin to the site of respiratory infections in cattle. The tulathromycin concentrations in lung homogenate were intermediate between the concentrations in PELF and PELF cells, and provide yet another example of the limited value of lung homogenate concentrations in pharmacodynamic studies.

After rapid absorption, tulathromycin quickly distributed into lung homogenate and PELF, with LSMeans concentrations

exceeding 1,000 ng/mL by 1 and 3 hr respectively. Since protein binding of drugs in PELF is not usually considered clinically important (Kiem, 2008), these PELF data suggest that the efficacy of tulathromycin is due not only to high tissue concentrations, but early concentrations in the sites where bacterial pneumonia occurs in cattle.

The concentration data for tulathromycin in lung homogenate and plasma in this study are in reasonable accord with the data from two previous studies in cattle with the approved subcutaneous dose of 2.5 mg/kg bw (Galer, 2004; Nowakowski, 2004). The study by Nowakowski et al characterized the time course of tulathromycin in lung homogenate of cattle from 12 – 360 hr post-dose, and the study by Galer et al. provided lung homogenate concentration data at one time point, 7 days post-dose. Tulathromycin distributed extensively into lung in these previous studies, with lung homogenate AUC_{0-360} more than 50-times the plasma AUC_{0-360} (Nowakowski, 2004) and with the 7-day lung homogenate concentration of 80 times the plasma concentration (Galer, 2004). In the study by Nowakowski et al, geometric mean tulathromycin concentrations in lung homogenate were above 1,000 ng/g from 12 – 360 hr, with a C_{max} of 4,100 ng/g at 24 hr and with a concentration of 1,200 ng/g at 360 hr. In the current study, with additional sampling before 12 hr, tulathromycin distributed rapidly into lung, with LSMeans lung homogenate concentrations above 1000 ng/g from 1 – 360 hr post dose. Also in the current study, the lung homogenate C_{max} and the concentration at 360 hr were within 12% of the values by Nowakowski et al.

In previous studies with serial blood sampling for PK, the tulathromycin plasma C_{max} values have generally ranged from 300 – 500 ng/mL, with t_{max} typically by 1 hr. (Galer, 2004; Nowakowski, 2004) The plasma t_{max} occurred somewhat later in this study (by 3 hr) but the C_{max} (277 ng/mL) was similar to the previously reported values. Tulathromycin exhibited a prolonged $t_{1/2}$ in this study in plasma, lung homogenate,

PELF, and PELF cells. The plasma $t_{1/2}$ in this study (64 hr) was somewhat shorter than in the previous studies (87 – 110 hr when blood sampling occurred for > 168 hr). However, within-animal serial blood sampling for PK was not used and the current study was not designed to provide as robust an estimate of plasma $t_{1/2}$ as the previous studies. The $t_{1/2}$ values for lung homogenate, PELF, and PELF cells may also be somewhat suspect, in part because of the modest decline in the concentrations during the apparent terminal disposition phase in these matrices. The data are nevertheless consistent with a much more prolonged $t_{1/2}$ of tulathromycin in lung homogenate, PELF, and PELF cells than in plasma.

Even more impressive than the distribution of tulathromycin into lung homogenate was the extensive distribution of the drug into PELF cells, with an AUC_{0-360} of more than 500 times the plasma value. Alveolar macrophages are a major constituent of PELF cells, and extensive uptake into phagocytic cells was noted in previous in-vitro studies with the drug. In a study with bovine alveolar macrophages and a 4-hr incubation time, the intracellular concentration of tulathromycin was 19-times the extracellular concentration (Siegel, 2004). Tulathromycin was also shown to concentrate into PELF cells of healthy foals 192 hr after a single 2.5 mg/kg bw intramuscular injection (Venner, 2010). The uptake into phagocytes may provide a mechanism for the sustained delivery of drug at the sites of infection (Drusano, 2005), and the tulathromycin concentrations in PELF, and PELF cells in this study with healthy animals may have underestimated concentrations in animals with pulmonary infections due to migration of white cells to the sites of infection. The extensive partitioning of tulathromycin into PELF cells may be related, in part, to the tri-basic chemical structure of the drug (Evans, 2005) and the trapping of ionized drug within acidic phagolysosomes (Toutain, 2002). Although the antibacterial activity of tulathromycin and other macrolides may be reduced within the phagolysos-

omes since activity is known to be reduced at acidic pH (Toutain, 2002; Evans, 2005), effective intracellular concentrations of tulathromycin are nevertheless maintained, as demonstrated by promising activity in the treatment of pulmonary lesions that were either positively cultured for, or presumed to be due to, intracellular pathogens (Venner, 2007; Washburn, 2009).

Tulathromycin concentrations in PELF were also much higher than in plasma, but interpretation of these data may be confounded by the high drug concentrations in PELF cells. Lysis of cells during the BAL procedure has been noted as a methodological problem for drugs with extensive intracellular distribution (Kiem, 2008), and a substantial bias in the PELF concentrations of tulathromycin could have occurred if even a small fraction of the PELF cells were lysed. Bias from lysed cells may not have been an important problem at the early time points in the study, eg, 1 – 11 hr, because PELF concentrations tended to be much higher than the PELF cell concentrations at these time points. At the later time points, eg, times greater than 72 hr, lysis of even 1% of the PELF cells could have produced a 20% bias in the measured PELF concentrations.

In summary, a single subcutaneous dose of 2.5 mg/kg bw tulathromycin resulted in rapid and extensive distribution of drug into lung homogenate, PELF, and PELF cells of cattle. Therapeutic concentrations of tulathromycin were achieved in PELF by 3 hr post-dose. The relatively high concentrations of tulathromycin in PELF and PELF cells soon after drug administration, coupled with prolonged $t_{1/2}$, is consistent with efficacy from a single dose for the treatment and control of bovine respiratory disease.

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