

Pulmonary Pharmacokinetics of Tulathromycin in Mice and Pigs

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Doctor of Philosophy Degree
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DEDICATION

I dedicate this dissertation to my wife, Fernanda, and my daughters Agustina, Victoria and Ana. Also, I want to extend the dedication to my parents and brothers.

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ABSTRACT

Tulathromycin is an antimicrobial approved for use in the treatment and control of bovine respiratory disease and treatment of swine respiratory disease in United States and European Union. This macrolide is a unique drug which has an extraordinary capacity to accumulate in lung tissue and shows distinctive pharmacokinetic features in animals.

The pulmonary pharmacokinetics of tulathromycin in mice and pigs were evaluated in this dissertation. In mice, the pulmonary pharmacokinetics of tulathromycin was evaluated in healthy animals and animals challenged with lipopolysaccharide of *Escherichia coli* (LPS-Ec). A group of challenged animals were also rendered neutropenic. In pigs, the pulmonary pharmacokinetics of tulathromycin was assessed in healthy animals and animals challenged intra-tracheally with LPS-Ec. The pulmonary pharmacokinetics of tulathromycin was assessed using lung tissue (middle and caudal lobes), pulmonary and bronchial epithelial lining fluid (BELF) samples.

We introduce a novel technique for the assessment of the concentration vs. time profile of tulathromycin in BELF. This technique is based in the use of fiberscopically guided bronchial micro-sampling probes (BMSp). The BMSp were first evaluated in a pilot trial. Then, the BMSp were used as sampling tool for harvesting BELF in a larger study. The use of BMSp represents a cost effective technique. This technique can be introduced in discovery and preclinical pharmacokinetics studies for screening of candidate drugs that will be indicated for the treatment of respiratory conditions. This technique might enhance the experimental design and maximize the experimental resources (number of animals, time, and cost).

This dissertation expands the understanding about the distribution of tulathromycin into the lungs. It evaluates the kinetics of tulathromycin in middle and caudal lobes and intra-airways compartment from healthy and animals with an acute pulmonary inflammatory

response. This work provides the first evidence of the pharmacokinetics of tulathromycin in lung tissue administered to neutropenic, normopenic, and neutrophilic mice. Finally, this dissertation introduces and evaluates an innovative technique for the study of pulmonary pharmacokinetics of drugs in veterinary species.

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Chapter 1:

PHARMACOKINETICS, PHARMACODYNAMICS AND CLINICAL APPLICATIONS OF TULATHROMYCIN IN VETERINARY MEDICINE

Introduction

Tulathromycin is indicated as both metaphylactic and therapeutic in cattle and therapeutic in swine for respiratory conditions. Its antimicrobial efficacy has been addressed in several studies in cattle, swine, horses, and goats. Studies with clinical applications available in the literature are summarized in Table 1.1.

The withdrawal time periods in cattle and swine intended for human consumption are relatively short when compared with other approved drugs for treatment of similar conditions. According to the United States Food and Drug Administration (FDA), when tulathromycin is used following label indications, treated animals must not be slaughtered within 18 (cattle) and 5 (swine) days after the last treatment administration (129)

Tulathromycin can be used safely according to label directions in a variety of species (98). In cattle and swine, there was a local tissue reaction from the injection that resolved over time (98). Studies of toxicity in bovine, swine and caprine revealed minor clinical and injection site histopathologic changes that resolved over time (26, 98). Hyper-salivation, head shaking and pawing at the ground has also been reported at therapeutic doses (98). In foals, the use of tulathromycin has been associated with self-limiting diarrhea; elevated temperature, and swelling at the injection site (140).

Chemical structure

Tulathromycin is a semi-synthetic macrolide antimicrobial, consisting of a regioisomeric, equilibrated mixture of a 13- membered ring azalide (10%) and a 15-membered ring azalide (90%), (10, 87). Its chemical core is built of two sugar moieties attached to the macrolactone ring (molecular weight of 806.08) (38). The drug molecule has 3 nitrogen/amine functional groups representing the first member of a novel sub-class of macrolides known as triamilides (71). Each amino group can be positively charged at the appropriate pH. All macrolides are basic molecules with pKa higher than 7. The molecule of tulathromycin is more basic than other

macrolides. Depending upon the basic amino group in the molecule, pKas range from 8.6 to 9.6. The constant of dissociation of tulathromycin may favor the accumulation of the drug in acidic environments such as in airways of pneumonic patients and alveolar epithelial lining fluid (13, 84, 85). Tulathromycin is approximately fifty times more soluble in hydrophilic than hydrophobic media (readily soluble in water at pH 8.0 or below) (38).

Indications

Tulathromycin has been approved in the US for the treatment of bovine respiratory disease (BRD) associated with *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni* (*Haemophilus somnus*) and *Mycoplasma bovis*, and for the control of respiratory disease in cattle at high risk of developing BRD associated with *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* (*Haemophilus somnus*) and *Mycoplasma hyopneumoniae*. Infectious bovine keratoconjunctivitis associated with *Moraxella bovis* and interdigital necrobacillosis associated with *Fusobacterium necrophorum* and *Porphyromonas levii* have been included in the list of approved indications. The FDA approved label indications include only beef and non-lactating dairy cattle. In swine, tulathromycin can be used effectively and safely to treat respiratory condition associated with *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Bordetella bronchiseptica*, and *Haemophilus parasuis* (100).

Mechanism of action

Tulathromycin is a potent protein synthesis inhibitor (38). Macrolides bind reversibly to the peptidyl transferase region of the 23s ribosomal subunit (125, 146). A study measuring the displacement of ¹⁴C-erythromycin from the ribosome by tulathromycin indicated that tulathromycin shares a common binding site on the ribosome with erythromycin (100).

Pharmacodynamics

Both the macrolide ring and sugar moieties account for the antimicrobial activity of the drug (97). A remarkable feature of the chemical structure of tulathromycin is that the molecule is highly positively charged (87). This electrostatic charge has been associated with a markedly enhancement of the drug penetration into cells by displacing magnesium ions at the outer lipopolysaccharide layer of gram negative bacteria (87).

Tulathromycin, like other macrolides, is considered a bacteriostatic agent when tested against *Staphylococcus aureus* and *Escherichia coli* (100). However, tulathromycin demonstrated that it is bactericidal against *M. haemolytica*, *A. pleuropneumoniae* and *Pasteurella multocida* revealed that tulathromycin is bactericidal (at 4x and 8x MIC) for some isolates (49, 87).

The bacteriostatic or bactericidal effect of an antimicrobial is dictated by the microorganism involved in the infection and the concentration time profile of the drug at the site of infection (33, 34, 79). Tulathromycin concentrates markedly in some compartments including pulmonary epithelial lining fluid, macrophages and neutrophils (29, 118). In these compartments, the distribution and accumulation occurs at different rates and extents (29).

For tulathromycin, some local conditions may impact the activity of the drug. The antimicrobial activity of tulathromycin against *Histophilus somni* and other fastidious microorganisms is dependent on the pH and pCO₂ of the surrounding environment (103). This study reported that MIC values increased as the pH decreased and pCO₂ increased. Similar observations have been reported for erythromycin, azithromycin and clarithromycin (36). These findings have not been observed *in vivo* nor shown to be consistent for other microorganisms. This local biochemical changes might be relevant in clinical situations that include abscesses and/or necrotic tissue and intracellular infections. Under this scenario, the pH might be lower and the pCO₂ higher than in plasma (110, 111). Two studies involving tulathromycin and abscessing conditions suggested that *in vitro* findings may not be fully correlated to all the *in*

vivo conditions and may warrant further *in vivo* research. Both studies assessed microorganisms that are intracellular pathogens and were responsible for triggering the formation of abscesses (138, 142). The first study reported an acceptable clinical response of goats with caseous lymphadenitis abscesses caused by *Corynebacterium pseudotuberculosis* when treated with tulathromycin (142). The other study was done in foals with lung abscesses (138). In the latter, abscesses were resolved after 53 days of therapy (2.5mg/Kg/once a week). The authors attributed the lung abscesses to *Rhodococcus equi* (*R. equi*) but in fact the causal microorganism(s) of the lesions was not identified. Under *in vitro* conditions, tulathromycin showed poor activity against *R. equi* (24), indicating that further research is necessary in this area before recommending the use of tulathromycin for the treatment of *R. equi* in foals.

As indicated earlier, tulathromycin concentrates largely in inflammatory cells, which does not imply that all the intracellular drug is readily available and active (134). Some fraction of the intracellular drug content may be bound to the membrane, cellular proteins or trapped in intracellular organelles (23, 131). Macrolides and flouroquinolones exhibit intracellular antimicrobial activity as indicated by their capacity to treat intracellular organisms located in phagosomes (*Brucella sp* in macrophages) (65), cytosol (*Listeria monocytogenes* in macrophages) (102), endosomes (*Mycobacterium tuberculosis* in macrophages) (101) and phagolysosomes (*Staphylococcus aureus* in macrophages and neutrophils). Nevertheless, changing intracellular conditions may also render the molecule not fully active (eg. intracellular pH)(134).

The exact intracellular location of tulathromycin has not been reported. The acidic environment of lysosomes and the constant of dissociation ($pK_a > 8$) of tulathromycin suggest that the drug may tend to be trapped within lysosomes, as reported for other macrolides (91, 130). The intracellular unbound and the unionized fraction of tulathromycin was no reported. Considering that the MIC of some microorganisms is inversely related to pH, it is possible to

hypothesize that the intracellular antimicrobial activity of tulathromycin is limited. On the other hand, the intracellular concentrations are higher than plasma concentrations (AUC_{0-360} cell:plasma ratio ~500) [17], which might overcome potential changes of MICs. There are many unanswered questions and based on the evidence available, it is not possible to predict the intracellular antimicrobial activity of tulathromycin.

The bactericidal action of macrolides has been described as time-dependent for erythromycin and concentration dependent for clarithromycin and azithromycin (86). Studies *in vitro* with *Haemophilus somnus* indicated that tulathromycin has a concentration dependent bactericidal activity particularly in the presence of plasma (103). The AUC/MIC ratio has been proposed as the PK/PD variable that best predicts the antimicrobial activity of tulathromycin (38). However, clinical PK/PD breakpoints for tulathromycin have not been reported yet. Microbiological breakpoints have been established for susceptibility testing of bovine and swine respiratory pathogens. Microbiological breakpoints have been defined based on bacterial isolates obtained from field studies conducted in 10 European countries and from library collections (48).

Non antimicrobial effects

Macrolide antibiotics affect the immune system by multiple mechanisms including recruitment of inflammatory cells (58, 76), secretion of pro-inflammatory cytokines (124) and pro-apoptotic activities (25, 69, 70, 145).

Tulathromycin significantly increased leukocyte apoptosis and reduced levels of pro-inflammatory leukotriene B₄ in *M. haemolytica*-challenged calves (39). *In vitro*, tulathromycin induces apoptosis in bovine neutrophils in a time and concentration dependent manner when evaluated at concentrations that may be aimed intracellularly (50 µg/ml to 2 mg/ml). It was also demonstrated *in vivo* that tulathromycin induces apoptosis of BAL fluid leukocytes 3 h after its administration. Tulathromycin did not significantly alter bronchoalveolar neutrophil numbers at

24 h of infection. The authors also reported that tulathromycin significantly reduced levels of phosphorylated inhibitor kappa beta, nuclear factor kappa-light-chain-enhancer of activated B cells, p65, and mRNA levels of pro-inflammatory interleukin-8 in lipopolysaccharide stimulated bovine neutrophils (39). The clinical significance of all these findings may warrant further research.

Pharmacokinetics

The pharmacokinetics of tulathromycin has been evaluated in multiple species including cattle, pigs, goats and foals. This macrolide has unique pharmacokinetic features.

Pharmacokinetic parameters available in the literature for multiple species are summarized in Table 1.2. Tulathromycin is characterized by a rapid rate of absorption and large systemic availability (~90%) after i.m and s.c. administration. The oral administration had a lower systemic availability (~ 50 %) (141). In plasma, it has a long terminal half-life, ranging across species from 60 to 110 h (Table 1.2). In cattle, linear pharmacokinetics is observed with subcutaneous doses ranging from 1.27 mg/kg BW to 5.0 mg/kg BW (98). No pharmacokinetic differences have been observed in castrated male versus female calves (36). Tulathromycin is eliminated by biliary and renal excretion (98). In cattle and pigs, one-half and two-thirds of the dose, respectively, are eliminated in the feces (100). Most of the dose is eliminated as unchanged drug (141). Protein binding studies indicated that the unbound fraction of tulathromycin ranges from 0.53 to 0.68 (99).

Lung pharmacokinetics

Lung pharmacokinetic studies in cattle, pigs and horses revealed an extraordinary capacity of tulathromycin for accumulate in lung tissue (10, 29, 89, 139, 147). The magnitude of the local accumulation and long persistence of the drug in the target tissue (lung) results in a

convenient treatment regimen (single administration) and positive clinical outcome rates for respiratory conditions (29).

Unbound plasma concentration is commonly accepted as a surrogate of interstitial fluid concentration. Interstitial fluid is considered the site where most extracellular bacteria are located during infection. For lung infections, pulmonary epithelial lining fluid (PELF) might be considered as a potential site of contamination/infection where interaction between drug and microorganisms might take place (32, 35). Thus, concentration of drug at this level might be truly relevant from a pharmacodynamics and pharmacokinetics (PK/PD) stand point. However, the ELF at the bronchial level does not represent a “pure” interstitial fluid or a plasma dialysate fluid (5, 17, 18, 28, 63, 106). This limits the value of plasma concentrations as surrogate for interstitial lung tissue concentrations (33, 80).

Multiples techniques have been applied to study pulmonary drug distribution including, mini-bronchial alveolar lavage, sputum, positron emission tomography and microdialysis (9, 59, 67, 72, 92, 102, 126, 127, 144). However, factors like cost and availability of the technique, experimental design limitations, accuracy, precision and informative value of the results limit their applicability in veterinary species (52, 77, 128). There is not a totally convenient technique available to study the drug kinetics in intra-airways compartments (unbound, interstitial, epithelial lining fluid and secretions) in all the veterinary species and scenarios (different animal sizes, multiple sampling, etc.). Some techniques may be applicable for some species and circumstances but not for all possible experimental conditions.

For most of the drugs, the study of their disposition in lung of veterinary species has been accomplished using lung homogenate samples. Consistent with this, the lung pharmacokinetic of tulathromycin has been mainly described by quantifying the drug in lung homogenate samples. Recently, the pharmacokinetics of tulathromycin has also been described in pulmonary epithelial lining fluid (PELF) from cattle (29, 140) and foals (29, 140).

Lung homogenate

Tulathromycin distributes into lung and persists in lung tissue for a long period of time after a single administration (Table 1.2). In pigs, lung tissue concentrations were 24-181 times those in plasma over a period of 0-360 h (10), while similar results were reported in cattle (Table 1.2) (29, 88).

Blood flow is one of the most relevant factors dictating the organ kinetics of a drug like tulathromycin. The blood flow in lung is not homogenous across the organ (19). This may affect the distribution of the drug within the organ (109). Lung homogenate samples are representative of the whole lung. Therefore this technique does not allow determining if the distribution of the drug within the lung is heterogeneous, which might have some relevance in terms of antimicrobial effect.

Multiple factors usually change during infection/inflammation processes which clearly might impact on the local accumulation and concentration time profile of a drug (34, 79). Reports about drug concentrations in pneumonic tissue are inconsistent. Apley and Upson (1993) reported lower concentrations of danofloxacin in pneumonic craneo-ventral tissue than in normal caudo-dorsal tissue from cattle (3). In contrast, higher concentrations of oxytetracycline in pneumonic calves have been reported (1, 3). In the case of tulathromycin, the concentration time profiles in healthy and diseased lungs from cattle have been reported (99). No pharmacokinetic results are presented or statistical comparisons reported.

The potential impact of a pulmonary inflammatory response on the concentration time profile of tulathromycin in lung tissue has not been evaluated yet. The assessment of the concentration time profile of tulathromycin in lung tissue from animals facing a pulmonary inflammatory response and estimation of pharmacokinetic parameters would provide an important information about the drug accumulation in lung tissue.

Lung homogenate samples do not represent a homogeneous sample. Lung homogenate samples are processed in such a way that measured concentrations represent a composite of

intracellular, interstitial and blood concentrations from both the alveoli and intrapulmonary airways. For most of the infections, concentrations in the interstitial fluid and PELF are of primary interest. Consequently, tissue:plasma ratios lack antimicrobial effect predictive value for these drugs and possibly most others. As mentioned (*vide supra*), the pharmacokinetics of tulathromycin in lung tissue from pigs was evaluated, using lung homogenate. The use of additional techniques to sample the lungs and the assessment of the pharmacokinetics of the drug in different lung compartments would expand the understanding of the drug accumulation in lung tissue.

Pulmonary epithelial lining fluid (PELF)

PELF is the fluid that distributes continuously throughout the respiratory tract including alveoli (82). The composition and function of the lining fluid is heterogeneous across the respiratory tract and the alveoli (82, 112).

It is accepted that biophase drug kinetics and accumulation are the primary factors controlling the antimicrobial response (34, 79). PELF is a potential site of bacterial contamination and colonization and as well as a site of bacteria to drug interaction. Therefore, the kinetics of the drug at the PELF may be relevant for the antimicrobial effect of a drug. The pharmacokinetic of tulathromycin in PELF was reported samples in clinically healthy bovines. Results indicate that tulathromycin rapidly distribute into the intra-airways compartment and persists there during a long period of time (terminal half-life 330 h). Tulathromycin also distributes extensively into PELF from where drug concentrations decrease at a very slow rate (29). Table 1.2 shows the pharmacokinetic parameters obtained in this study. The pharmacokinetics of tulathromycin in PELF from pigs has not been evaluated yet.

Pulmonary epithelial lining fluid is commonly harvested by broncho-alveolar lavage (BAL). The volume of PELF harvested in the BALF is usually estimated using serum and BALF urea as dilution factor (29). Therefore, the concentrations reported in PELF represent composite

drug concentrations from the trachea, lower airways and alveolar epithelial lining fluid. Each pulmonary compartment is physiologically different and structurally heterogeneous, which may affect the distribution of the drug. Consequently, the true informative value of the concentration of the drug in PELF harvested by BAL is limited and cannot be accurately linked to a particular anatomical site in the lung. It is important to stress that the use of BAL to harvest PELF does not allow for an evaluation of the relative concentrations of free versus bound drug. In addition, drug concentrations in PELF may be biased due to potential contamination of the PELF with drug released from cells of the BALF sample (60). This technique has practical limitations that affect the experimental design. The use of BAL to harvest PELF for quantitation of drugs should not be performed more than once per animal without *a priori* validation as it may confound the results. This is due to the changes that the lavage generates in the intra-airways and the inevitable residual fluid remaining in the airways following the procedure.

Bronchial epithelial lining fluid (BELF)

Bronchial epithelial lining fluid represents the bronchial surface liquid. There are not studies describing the concentration time profile of tulathromycin in BELF. The uses of bronchial micro-sampling (BMS) probes have been used experimentally in human settings and may provide a valuable alternative for pigs.

Alveolar macrophages and bronchial alveolar lavage cells

Macrolide antimicrobials accumulate to a high extent in different cell types (9, 59, 67, 72, 92, 102, 126, 127, 144). An *in vitro* study demonstrated that tulathromycin accumulates in neutrophils and blood macrophages from bovine and porcine (118).

The accumulation of tulathromycin in bronchoalveolar cells (BAC) was reported in foals (140). The concentration of tulathromycin in BAC was evaluated 24 and 192 h following a single drug administration and following the last dose administration at steady state plasma

concentrations. Results after a single administration indicates that tulathromycin accumulates over time and drug concentration in BAC is not in equilibrium with plasma concentrations (0.20 ± 0.08 and 0.30 ± 0.17 $\mu\text{g/ml}$ for $\text{BAC}_{24\text{h}}$ and $\text{BAC}_{192\text{h}}$, respectively and 0.14 ± 0.05 and 0.02 ± 0.01 $\mu\text{g/ml}$ for plasma 24h and plasma 192h, respectively). After multiple drug administrations, the concentration of tulathromycin was higher than after a single drug administration (about 7 and 4 times for $\text{BAC}_{24\text{h}}$ and $\text{BAC}_{192\text{h}}$, respectively) (140). These findings suggest that there was no saturation of the intracellular accumulation of the drug, after a single administration. Paradoxically, the co-administration of rifampicin, a potential efflux transporter blocker, was associated with lower accumulation of the drug in BAC cell ($p < 0.05$) (140).

Cox *et al.* (2010) described the concentration time profile of tulathromycin in pulmonary epithelial lining fluid cells (majority macrophages) from clinically healthy calves (29). This result demonstrates a notable accumulation of the drug in the PELF cells. The accumulation profile suggests that there were two different rates of intracellular accumulation. Initially, the accumulation profile is characterized by a fast phase of intracellular accumulation that goes from 0 to 11h post-dosing followed by a slower phase that is extended up to 72 h post-drug administration. After the maximal intracellular concentration (72 h) the drug tend do decline slowly over time (29).

It is important to highlight that most of the studies describing the drug concentration in alveolar macrophages are based on multiple assumptions. These assumptions are not always valid and may lead to misleading interpretations. The first assumption is that all the cells in the BALF sample represent alveolar macrophages. In fact, different cell populations (epithelial cells, alveolar epithelial cells, interstitial macrophages, lymphocytes, etc.) are usually harvested by BALF.

Second, the intracellular concentration is usually estimated using a reference intracellular volume reported in the literature rather than the actual intracellular volume of the sample (11, 29, 44, 114, 140). The use of a theoretical value requires one to assume that; (i)

the reference value is representative of intracellular volume of study samples, (ii) the intracellular volume is constant across all the cells (iii) the contamination of the cell pellet (10^6 cells) by non-macrophages cells does not impact on the results and (iv) all the alveolar macrophages obtained in the sample are structurally and functionally similar and with the same capacity to accumulate drug (43, 120, 122).

To obtain a cell pellet with 100 % alveolar macrophages, cell populations in the cell pellet of the BALF sample should be sorted. A pellet of cells obtained by centrifugation of the BALF sample always has a proportion of cells different to alveolar macrophages. Thus, it might not be accurate to express drug concentrations in alveolar macrophages when in fact the samples do not represent a pure cell population.

It is true that it may be difficult to set parallel experiments in order to rule out all those assumptions in the context of *in vivo* pharmacokinetic studies. Nevertheless, to assume that all this factors are not relevant in a study sample may lead to misinterpretation of the results.

The intracellular drug activity and its potential clinical significance was addressed in the pharmacodynamics section (*vide supra*).

Local penetration and accumulation mechanisms.

After drug administration, the drug distributes from the plasma to the intercellular compartments, airways secretions, epithelial lining fluid and cells (epithelial cells, fibroblast, alveolar macrophages, and bacteria, among others). The mechanism(s) implicated in the accumulation of tulathromycin in lung have not been elucidated yet. Many studies were done trying to unveil and explain the factors and mechanism (s) involved in the lung accumulation of macrolides and other drugs (Table 1.3). Several factors could have unequal impact on the penetration and accumulation of the drug at different levels of the airways and alveolar compartment (Table 1.3).

There is evidence that accumulation of macrolides in ELF and cells may be dependable on members of the organic anion transporting protein (OATP) family and efflux transporters (16, 64, 66, 115-117, 123). There is limited information about the potential interaction of tulathromycin, drug transporters and tissue accumulation. In a study done in horses, tulathromycin had no affinity to efflux carriers ABCB1 and ABCC2. Likewise, this study could not demonstrate a connection between transporters and the accumulation of tulathromycin in the PELF (140). More research is warranted in this area.

One of the most recurring hypothesis, but still conflicting, is the role of inflammatory cells in the extraordinary accumulation of macrolides in lung tissue (4, 6, 15, 40, 41, 46, 47, 73, 104, 133). As mentioned above, *in vitro* and *in vivo* studies demonstrate that tulathromycin concentrates in both alveolar and blood macrophages, neutrophils and bronchoalveolar lavage cells (29, 140). These results may explain, at least in part, the high concentrations of tulathromycin in lung tissue (60). In contrast, it is highly improbable that neutrophils have a relevant role in healthy animals since neutrophils do not represent, numerically, an important cell population in a normal lung. The potential role of neutrophils in drug accumulation might increase during the acute phase of inflammatory processes. During inflammation, there is an increased sequestration of neutrophils in the pulmonary capillaries as well as, accumulation of cells within the lung interstitial and in the bronchoalveolar space (20). The exposure of cells to antigens may leads to the accumulation of drug-loaded cells in the site of infection (5, 40, 41, 46, 47, 57). The antigen-cell interaction may trigger a rapid release of intracellular drug after migration toward the chemotactic foci (46, 133). Loaded cells can also phagocytose the microorganism(s) exposing it to high intracellular drug concentrations (46, 133). Clinically, this phenomenon could be relevant when treating neutropenic animals. If neutrophils are relevant to the accumulation of the drug in tissue, a lower drug exposure may be expected when neutropenic animals are treated. There is limited information regarding the impact of neutropenia on the accumulation of macrolides in the lung. A reduced tissue AUC of

azithromycin was reported in leucopenic mice (137). The potential role of neutrophils in the local lung concentration of tulathromycin over time has not been evaluated yet.

The paucity of conclusive studies explaining the lung accumulation of the macrolides may be based on (i) the complexity and number of processes that might affect drug accumulation (Table 1.3), (ii) the temporal inter-relationship among them and (iii) the difficulty to design and execute studies that provide conclusive information.

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Conflict of Interest

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Appendix

Table 1.1. Summary of key clinical studies

Species	Indication category	Objective of the study	Conclusions of the study	Ref
Caprine	Treatment efficacy	To compare the effectiveness of 3 treatment regimens for small ruminants with caseous lymphadenitis. Group A; draining and flushing the lesions and SC administration of PPG. Group B intra-lesional TUL, Group C TUL SC.	Proportions of lesions that had resolution of infection by 1 month after treatment did not differ significantly among the treatment groups	(142)
		To compare the efficacy and cost-effectiveness of TIL vs. TUL as a metaphylactic antimicrobial in feedlot calves at moderate risk for BRD	Calves TUL had lower initial BRD treatment rates compared with TIL calves ($p \leq 0.05$). There were no significant differences in the BRD relapse rate, railer rate, total mortality rate, mortality rate, ADG, and dry matter conversion between the two groups. TUL was less cost effective	(136)
Bovine	Cost effectiveness and metaphylaxis	The purpose of this study was to compare the efficacy and cost-effectiveness of FLR vs. TUL for initial treatment of UF in fall-placed steer calves that received metaphylactic TIL on arrival at the feedlot	No significant differences were observed in UF relapses or the crude case fatality rate. Calves treated with FLR had a lower case fatality rate ($p = 0.04$) for BRD and <i>Histophilus</i> disease than did calves treated with TUL. FLR was more cost-effective	(135)
		The purpose of this study was to determine the efficacy and cost-effectiveness of TUL vs. TIL or TET as a metaphylactic antimicrobial in feedlot calves	Calves that received TUL had significantly lower initial UF treatment and relapse rates; lower overall chronicity, overall mortality, and cause-specific mortality rates; higher ADG; and improved quality grades. However, calves that received TUL also had poorer ($p < 0.05$) yield grades compared with calves that received TIL or TET and worse feed conversion compared with calves that received TIL. TUL was more cost-effective	(14)

Table 1.1. Continued

Species	Indication category	Objective of the study	Conclusions of the study	Ref
Bovine	Cost effectiveness	The purpose of this study was to compare the relative cost-effectiveness of FLR with that of TUL for treatment of UF in feedlot calves at ultra-high risk of developing UF that receive metaphylactic TUL on arrival at the feedlot	Calves that received therapeutic FLR had lower overall mortality (P 0.45) and BRD mortality (P=0.05) compared with calves that received therapeutic TUL, but no significant differences were detected in feedlot performance, carcass characteristics, or other animal health variables. FLR was more cost-effective	(96)
	Metaphylaxis	Clinical effectiveness of TUL vs. TIL and saline for the control of respiratory disease in cattle at high risk for developing BRD	TUL was significantly more effective in reducing BRD morbidity when compared to the other groups	(61)
		To evaluate the efficacy of TUL compared with TIL and FLR for the BRD at high risk of developing BRD	TUL demonstrated superior efficacy compared with TIL and FLR	(107)
		To compare health and feed performance parameters of beef stocker calves at risk for bovine respiratory disease after metaphylactic administration of TUL or TIL	Animals treated with metaphylactic TUL displayed significant improvement in morbidity, mortality, first-treatment success rates, improved ADG and feed:gain ratio compared with TIL(p<0.05). Calves TUL were healthier through the growing phase compared with TIL.	(83)
	Treatment efficacy	To evaluate the efficacy of TUL in the treatment in commingled cattle with clinical BRD treated with TUL or FLR and prevention healthy animals in-contact cattle treated with TUL, TIL or saline	Similar percentages of animals showed sustained clinical improvement at day 14 (TUL 83.3% vs. FLR 81.0%) and had not relapsed by day 60 (TUL 63.3% vs. FLR 58.4%). Significantly more TUL-treated cattle remained healthy to day 14 (92.4%) than TIL-treated (83.7%) or saline-treated (63.7%) cattle, and this was maintained through day 60 (TUL 85.4% versus TIL 75.1% and saline 56.2%)	(51)
		To evaluate the clinical efficacy of a single injection of TUL, compared with saline solution-treated control calves, for treatment of induced infectious bovine KC in calves	A single dose of TUL was an effective treatment of calves with experimentally induced infectious bovine KC	(68)

Table 1.1. Continued

Species	Indication category	Objective of the study	Conclusions of the study	Ref
Bovine	Treatment Efficacy	The objective of this study was to evaluate the comparative efficacy of TUL vs. TIL at resolving BRD in North American feedlot cattle	TUL was associated with an approximately 50% reduction in the risk of re-treatment for BRD compared with treatment with TIL	(143)
		To evaluate the efficacy of three antimicrobial regimens CCFA followed by TUL 8 days later (Group 1) or a one-course regiment of CCFA (Group 2) or TIL (Group 3) as metaphylactic	Morbidity was significantly lower in Group 1 than in Group 2 and Group 3	(121)
		To evaluate the efficacy of TUL or ENR for initial treatment of naturally occurring BRD in feeder calves	TUL resulted in significantly higher therapeutic success than did ENR. Animals treated with TUL also had higher weight gains compared with ENR	(105)
		Comparative efficacy of TUL, TIL, and FLR in the treatment of BRD in stocker cattle	TUL proved to be significantly more effective than either FLR or TIL in the treatment of BRD	(90)
		Therapeutic efficacy of TUL and TIL against BRD in feeder calves	TUL administered as a single-dose treatment was efficacious in the treatment of undifferentiated BRD	(62)
		To evaluate the efficacy of TUL in the treatment of BRD associated with induced <i>Mycoplasma bovis</i> infections in young dairy calves	Compared with saline, BRD-related withdrawals, peak rectal temperatures, and lung lesion scores were significantly lower for TUL-treated calves than saline	(50)

Table 1.1. Continued

Species	Indication category	Objective of the study	Conclusions of the study	Ref
Bovine Treatment Efficacy		To evaluate the relative efficacy of TUL vs. FLR for the treatment of UF in feedlot calves in Nebraska	The first UF relapse, overall mortality and BRD mortality rates in the TUL group were significantly lower than in the FLR group. A higher proportion of carcasses in the TUL group graded yield grade USDA-4 as compared with the FLR group. There were no significant differences between the TUL and FLR groups for the other animal health variables measured, in ADG, the overall distributions of quality grade and yield grade. TUL was more cost-effective	(113)
		Comparative efficacy of TUL vs. FLR and TIL against undifferentiated bovine respiratory disease in feedlot cattle	TUL was more efficacious in the treatment of undifferentiated BRD compared with FLR and, in one study, compared with TIL	(119)
		Evaluate the efficacy of TUL in interdigital necrobacillosis associated with <i>Fusobacterium necrophorum</i> and <i>Porphyromonas levii</i> in beef and non-lactating dairy cattle	The percentage of animals classified as a treatment success was significantly higher ($p < 0.0001$) in the TUL treated group (30/50, 60%) compared to the control group (4/50, 8%)	(99)
		To evaluate the effectiveness of TIL versus TUL for treatment of clinical vesiculitis in bulls	The recovery rate was higher for bulls treated with TUL (22/25=88%) than for TIL (11/23=48%) and both antibiotics resulted in a higher recovery rates than occurred the untreated control group (0/17; $p < 0.01$)	(108)
		This study demonstrated the ability of the antimicrobials TUL and ceftiofur to clear the spirochete <i>Leptospira borgpetersenii</i> serovar hardjo type hardjo-bovis (L. hardjo-bovis) from experimentally infected cattle	TUL resulted higher rate of clearance of L. hardjo-bovis organisms from the urine and kidney tissue than ceftiofur and control animals	(27)

Table 1.1. Continued

Species	Indication category	Objective of the study	Conclusions of the study	Ref
Equine	Treatment Efficacy	Evaluation of TUL in the treatment of pulmonary abscesses in foals	TUL was well tolerated and appears promising for the treatment of pulmonary abscesses in foals.	(138)
Porcine	Treatment Efficacy	To evaluate TUL vs. ENR and saline in the treatment of pneumonia in weaned pigs inoculated intra-nasally with <i>Mycoplasma hyopneumoniae</i>	Weight gain was significantly greater for TUL-treated pigs vs. saline and ENR. Compared with saline. Compared with ENR, there were no significant differences in proportional lung weight or weight gains, but coughing and lung lesion scores were greater for TUL-treated pigs ($p < 0.05$)	(75)
		Evaluation of the clinical efficacy of TUL vs. TIA and FLR in the treatment of natural outbreaks of SRD	For all the treatments there were significant reductions in mean rectal temperature and the severity of abnormal clinical signs on days 2 and 10 compared with day 0. There were no significant differences ($p > 0.05$) between treatments in average daily weight gain. Tulathromycin was found to be safe and highly effective in the treatment of natural outbreaks of SRD.	(81)
		Efficacy of TUL vs. ceftiofur and saline injectable solution for the treatment of naturally occurring SRD	The overall cure rate was 46.4% for saline-treated pigs, 71.1% for tulathromycin-treated pigs, and 63.1% for ceftiofur-treated pigs. The cure rate for tulathromycin-treated pigs was significantly higher than for saline-treated pigs ($p = 0.0116$). The mortality rate was significantly lower for both the tulathromycin- and ceftiofur-treated pigs compared with those treated with saline ($p = 0.0148$ and $p = 0.0195$, respectively).	(90)

Table 1.1. Continued

Species	Indication category	Objective of the study	Conclusions of the study	Ref
Porcine	Treatment Efficacy	Efficacy of TUL vs. ceftiofur in the treatment SRD caused by <i>Actinobacillus pleuropneumoniae</i>	The LSM bodyweight gains of the pigs treated with the TUL and ceftiofur were significantly higher than that of the saline-treated group, and the LSM % of the total lung involvement and incidence of respiratory disease associated with <i>A. pleuropneumoniae</i> were significantly lower, but there were no significant differences between the three groups of pigs treated with TUL and ceftiofur	(54)
		To evaluate the effect of a single or double dose of TUL vs. no treated in pigs carrying <i>Actinobacillus pleuropneumoniae</i> serotype 2 in their tonsils	There were no significant differences between the numbers of PCR-positive animals in the treated vs. control groups on any of the sampling dates	(2)
		To evaluate the effect of a single or double dose of TUL vs. no treated in pigs carrying <i>Actinobacillus pleuropneumoniae</i> serotype 2 in their tonsils	There were no significant differences between the numbers of PCR-positive animals in the treated vs. control groups on any of the sampling dates	(2)
		To evaluate the effect of a single or double dose of TUL vs. no treated in pigs carrying <i>Actinobacillus pleuropneumoniae</i> serotype 2 in their tonsils	There were no significant differences between the numbers of PCR-positive animals in the treated vs. control groups on any of the sampling dates	(2)

Table 1.2. Pharmacokinetics parameters (mean $\pm$ SD) of tulathromycin in multiple matrixes from cattle, pigs, horses, and goats

Species	Matrix	n	Terminal half-life (h)	Ke/ (1/h)	T _{max} (h)	C _{max} (ng/mL)	T _{last} (h)	AUC _{last} (hr* ng/mL or hr*ng/g)	AUC _{INF} (hr*ng/mL or hr*ng/g)	V _{ss} (L/Kg)	Vz_F_obs	Cl (mL/kg*hr)	MRT (h)	Reference
Beef calves	Plasma	36	90	0.007 $\pm$ 0.001	1.8 $\pm$ 3	500 $\pm$ 400	360	17,900 $\pm$ 1,700	18,700 $\pm$ 1,800	-	-	-	-	(88)
	LT	6	184 (158–220)	0.00377 (0.0043 – 0.0031)	24	4,100 (3320–5060)	360	903,600 (666000 – 1230000)	1,230,000 (846000 – 1820000)	-	-	-	-	
	Plasma ^{&}	3	77	0.009 $\pm$ 0.003	-	-	360	13,700 $\pm$ 1,000	14,100 $\pm$ 1,200	11 $\pm$ 2	-	178 $\pm$ 14	65 $\pm$ 9	
	Plasma	4	87	0.0080 $\pm$ 0.0011	2 $\pm$ 4	590 $\pm$ 170	360	13,000 $\pm$ 3,000	14,000 $\pm$ 3,000	-	-	-	-	
Holstein male calves ²	LT	3	279	-	72	4510	360	867	-	-	-	-	-	(29)
	Plasma	24	64	-	3	277	360	9.26	-	-	-	-	-	
	PELF	3	330	-	11	3730	360	492	-	-	-	-	-	
	PELF cells	3	270	-	72	19500	360	5230	-	-	-	-	-	

Table 1.2. Continued

Species	Matrix	n	Terminal half-life (h)	Ke/ (1/h)	T _{max} (h)	C _{max} (ng/mL)	T _{last} (h)	AUC _{last} (hr* ng/mL or hr*ng/g)	AUC _{INF} (hr*ng/mL or hr*ng/g)	Vss (L/Kg)	Vz_F_obs	Cl (mL/kg*hr)	MRT (h)	Reference
Horses ¹ 2-3 Mo old	Plasma	10	105 ± 25	-	2.47 ± 4.09	464 ± 178	192	-	-	-	-	-	89.1 ± 22.8	(140)
Horses ¹ 2-3 Mo old (at steady state)	Plasma	10	140 ± 51	-	0.36 ± 0.11	675 ± 174	192	-	-	-	-	-	58.6 ± 4.3	(140)
Boer goats ¹ 6 W old	Plasma	6	59.1 ± 7.6	0.012 ± 0.002	-	987.8 ± 226.9	360	-	10580 ± 2743	-	26.6 ± 16.4	250.3 ± 67.9 [#]	70.6 ± 3.0	(26)
Boer goats ¹ 6 Mo old	Plasma	5	60.9 ± 8.9	0.011 ± 0.001	-	1185 ± 481.8	360	-	7918 ± 1594	-	28.8 ± 7.1	328.0 ± 68.1 [#]	68.3 ± 7.1	(26)
Boer goats ¹ 5-7 Mo old	Plasma	10	110 ± 19.9	-	0.4 ± 0.26	633 ± 300	600	-	12500 ± 2020	-	32.6 ± 6.09	0.208 ± 0.37 [#]	80.8 ± 20	(147)

Table 1.2. Continued

Species	Matrix	n	Terminal half-life (h)	Ke/ (1/h)	T _{max} (h)	C _{max} (ng/mL)	T _{last} (h)	AUC _{last} (hr*ng/mL or hr*ng/g)	AUC _{INF} (hr*ng/mL or hr*ng/g)	V _{ss} (L/Kg)	Vz_F_obs	Cl (mL/kg*hr)	MRT (h)	Reference
Pigs ¹ 2-3 Mo old	Plasma	6	75.6 ± 13.7	0.009 ± 0.001	0.25	637 ± 207	360	15200 ± 3160	15600 ± 3150	-	-	-	-	(10)
	Plasma ^{&}	6	67.5 ± 13.5	0.010 ± 0.001	-	-	360	13700 ± 1860	14000 ± 1880	13.2 ± 1.86		181 ± 25.5	73.6 ± 12.4	
	Plasma	6	91.0 ± 38.9	0.007 ± 0.003	0.5	868 ± 350	360	12200 ± 3880	12600 ± 3710	-	-	-	-	
	LT	6	142	-	24	3470 (2740–4400) ⁺	360	615000	749,000	-	-	-	-	
Pigs 2 Mo old	Plasma ^{&}	8	76.5 ± 3.4	0.0093 ± 0.0017	0.080	5.43 ± 2.71	216	4.19 ± 1.43	4.67 ± 1.58	44.6 ± 18.3	64.3 ± 21.2	580 ± 170	78.8 ± 16.7	(141)
	Plasma [†]	8	78.7 ± 6.75	0.0089 ± 0.0008	3.75 ± 0.71	0.200 ± 0.05	216	1.93 ± 0.50	2.30 ± 0.540	-	130 ± 32.3	1140 ± 280	104 ± 8.61	
Dose regimen: Bovine and goats 2.5mg/kg subcutaneously. Pigs and foals 2.5mg/kg intramuscularly (& Intravenous administration oral administration). LT: lung tissue homogenate. PELF: Pulmonary epithelial lining fluid; mean. 1: mean ± SD; 2: PK parameters based on least squared means; # Cl/F (mL/kg*hr), +:values within parenthesis correspond to 99% confidence Interval.														

Table 1.3. Factors that may influence on the concentration time profile of the drug in lung tissue

Factors that might influence the penetration of the drug into lung tissue	
(i)	Epithelial permeability and absorptive capacity ^{1,2} a. the tightness of cell junctions decrease from a maximum in the trachea to a minimum in the distal airways before returning to a high value in the alveoli) b. permeability of barriers might change during inflammation
(i)	Organic anion transporting protein family and efflux transporters ^{3, 4, 5, 6, 7,}
(ii)	Differential histological features ^{8,9} a. the respiratory epithelium become gradually thinner with the number of bifurcations
(iii)	Tissue directed PK ^{8,9,10,11,12,13,14,15, 16, 17, 18} a. kinetics of inflammatory cells b. cell type^{13, 19,} c. cell status (eg. activated vs. inactive) d. type of chemotactic stimulus
(iv)	Differences and or changes of the surface area/tissue volume ^{21,} a. a low SA/V reduces the velocity of penetration (airways vs. alveoli)
(v)	Drug features ²² a. molecular weight b. plasma ionized vs. no ionized fraction c. liposolubility
(vi)	Intrapulmonary blood and lymph flow differences ^{22, 23} a. the alveolar region receives the entire cardiac output by the pulmonary circulation. Contrary, the airways receive approximately 1% of the cardiac output^{24, 25} b. intrapulmonary changes in blood and lymph flow due to disease c. surface areas and volume might change during infection lung
Factors that might influence the removal of the drug from lung tissue	
(i)	Retention of the drug in the mucus blanket over the ciliated epithelium of the airway
(ii)	Drug features ²² a. molecular weight²⁶ b. tissue ionized vs. no ionized fraction
(iii)	Extracellular tissue binding a. fibrin³² b. debris^{32, 33}
(vii)	Cellular uptake (immune, epithelial, and/or fibroblast cells) ^{13,14,15,16,17,18,34}
(iv)	Binding to phospholipids ³⁵
(v)	Intrapulmonary metabolism ³⁶
(vi)	Amount of respiratory secretions
Factors that might influence excretion and intra-airways distribution	
(i)	Mechanical excretion a. Cough and sputum excretion³⁷

Table 1.3. Continued

Factors that might influence excretion and intra-airways distribution

- b. metachronous beating of cilia (faster beating of the cilia in the trachea than in the small airways) (impaired vs. increased ciliary function)^{26, 27}
 - (ii) type of inflammatory response (chronic vs. acute)^{12, 31}
 - (iii) type of drug physicochemical features²²

1 (17); **2** (95); **3** (16), **4** (66), **5** (115), **6** (116), **7** (140), **8** (5), **9** (57), **10** (46), **11** (47), **12** (133), **13** (40), **14** (104), **15** (41), **16** (73), **17** (6), **18** (15), **19** (12), **20** (37), **21** (7), **22** (109), **23** (93), **24** (30), **25** (42), **26** (55), **27** (132), **28** (56), **28** (22), **30** (94), **31** (45), **32** (8), **33** (21), **34** (74), **35** (78), **36** (31), **37** (53)

Chapter 2:

PHARMACOKINETICS OF TULATHROMYCIN IN HEALTHY AND NEUTROPENIC MICE CHALLENGED INTRA-NASALLY WITH LIPOPOLYSACCHARIDE FROM *ESCHERICHIA COLI*

Abstract

Tulathromycin is a macrolide approved to treat bovine and swine respiratory disease. In this species, tulathromycin shows unique pharmacokinetic features including a remarkable accumulation in lung tissue. The objectives of the present study were to assess the concentration vs. time profile of tulathromycin at two different dose levels in mice and to assess the plasma and lung tissue pharmacokinetics of tulathromycin 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 lung tissue, the AUC_{0-144} of tulathromycin was 246000 h*ng/mL for group T-7, and 199000 h*ng/mL for both T7-LPS, and T7-LPS-CP. The lung to plasma AUC_{0-144} ratios were 7.95, 3.79 and 2.26 h for groups T-7, T7-LPS and T7-LPS-CP, respectively. The terminal half-life in lung was 82.5, 213 and 144 h for groups T-7, T7-LPS and T7-LPS-CP, respectively.

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 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 lung tissue when the drug is administered during either a neutrophilic or neutropenic state.

Keywords: tulathromycin, antimicrobial, mice, lung, LPS

Introduction

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 (28). This macrolide shows outstanding metaphylactic and therapeutic efficacy in bovine and swine respiratory disease after a single S.C. administration (1, 5, 7, 20, 22, 26, 31, 33, 37, 41, 49, 54, 55). 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* (39).

Tulathromycin has unique pharmacokinetic features. It is characterized by a rapid rate of absorption and a large volume of distribution ($V_{d_{ss}}$ 13.2 L/kg in pigs and 33 L/kg in goats) (5, 58). Pharmacokinetic studies in cattle, swine, and foals revealed an extraordinary 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) (5, 35). In one study, the area under the concentration-time curve in lung tissue homogenate to area under the plasma concentration-time curve (AUC_{0-360}) ratio reported was 94 and 181 for bovine and swine, respectively (5, 11), and the pulmonary epithelial lining fluid apparent half-life was >100 h in Holstein calves (11). These features would explain to some extent the excellent 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) (11). Tulathromycin also accumulates in blood macrophages and neutrophils (48). This might be relevant during the inflammatory process if tissue-directed pharmacokinetics hold true for tulathromycin (3, 4, 8, 14, 15, 18, 19, 30, 40, 53). Increased amounts of azithromycin and erythromycin in infected lungs have been reported (8, 17, 53). 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 (18, 19). Also, a reduced tissue AUC of azithromycin was seen in leucopenic mice (56). 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 g) were purchased from a local commercial source. 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 previous to 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 Draxxin 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 (Cytosan cyclophosphamide for injection, USP, Bristol-Myers Squibb Company, Princeton, NJ) at 150 mg/kg by intra-peritoneal (Figure 2.1) 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 (Figure 2.2.1 and 2.2.2). 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, Deerfield, IL, USA) the 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 3000 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:

$$\text{Wet-to-dry ratio} = \text{wet weight (g)} / \text{dry weight (g)}$$

Anesthesia and euthanasia

Both LPS administration and sampling general anesthesia were performed with intra-peritoneal 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 (11, 16). Roxithromycin was used as internal standard (43).

Plasma

Plasma samples were diluted (1:100 and 1:50) with 50 mM K_2HPO_4 . Samples (100 μ L) were spiked with 100 μ L internal standard (roxithromycin 25 ng/mL). Solid phase extraction (SPE) cartridges (Oasis MCX 1mL, Waters) were preconditioned with 1.0 mL of acetonitrile followed by 1.0 mL of pH 6.8 and 50 mM K_2HPO_4 . A weak vacuum was applied to the SPE manifold. All extracts were decanted onto the SPE cartridges. The SPE tubes were rinsed sequentially with 1.0 mL of pH 6.8, 50 mM K_2HPO_4 , and 1.0 mL of H_2O . Cartridges were loaded with quality control, blank plasma, and test samples (200 μ L). Analytes were eluted with 2×0.5 mL of freshly prepared 5% NH_4OH /95% acetonitrile into labeled 13- $\times$ 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 μ L of 20 mM ammonium acetate (pH 4.0) by vortex mixing. Reconstituted samples were transferred into autosampler vials. Chromatography was accomplished with a BDS Hypersil C_8 30 $\times$ 2.1 mm column (Thermo Fisher Scientific Inc., Waltham, MA) 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) was used in the positive ion mode using an electrospray ionization source, and positive ions were monitored with precursor $\rightarrow$ product ion pairs of 806 $\rightarrow$ 577 for tulathromycin and 837 $\rightarrow$ 679 for roxithromycin. Prior to the analysis of the study samples the potential interaction of LPS and cyclophosphamide was evaluated using quality control. There was no interference between those substances with tulathromycin. Calibration curves were generated automatically using Analyst 1.0 software (Perkin-Elmer). The calibration curve ($R^2 > 0.99$) occurred over the range of 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. 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.

Lung tissue homogenate samples

Samples were processed as described previously by Galer et al. (2004) (16). Briefly, a lung tissue sample (0.100 g) was homogenized with 0.050 mL of 50 mM phosphate buffer, pH 6, and 2.5 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. 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 ($R^2 > 0.99$) 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 exceed a coefficient of variation of 20%. The LLOQ was 3.90. 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.

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 (11). 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 ≤ 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 (11). 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 (AUC_{0-144}), area under the concentration-first moment time curve through 144 h post-dose ($AUMC_{0-144}$), mean

residence time (MRT), and the terminal elimination half-life ($t_{1/2}$) were estimated. The AUC_{0-144} was calculated by the log-linear trapezoidal rule, and λ_z was estimated with uniform weighting. The observed peak LS mean concentration (C_{max}) and time of its occurrence (t_{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_{max} LS mean ($\pm$ SE) of tulathromycin in plasma was 6790 ± 2450 (t_{max} 30 min) and 6920 ± 2520 ng/mL (t_{max} 1 h) in dose groups T-7-LPS and T-LPS-CP, respectively. Plasma concentrations of tulathromycin results are included in Table 2.1 and shown in Figures 2.3 and 2.4. The pharmacokinetic parameter results are included in Table 2.3.

Lung tissue homogenate

Tulathromycin rapidly and extensively distributed into lung tissue. Results are presented in Table 2.2. In dose groups T-7 and T-28, the tulathromycin C_{max} was 4410 and 27800 ng/g (observed) at 30 min and 1 h post-tulathromycin, respectively (Figure 2.5). The C_{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 2.5). 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 2.3. 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 2.4. 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 2.6 and 2.7). However, the fraction of variation of the drug concentration explained by the wet-to-dry ratio (72 to 144 h) was low ($R^2 < 5\%$) 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 2.5 and neutrophil count results are presented graphically in Figure. 2.8.

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 2.4). Tulathromycin was distributed extensively and rapidly into lung tissue in both of these dose groups, as previously reported in other species (11, 35). Tulathromycin was slowly eliminated from lung tissue of both dose groups T-7 and T-28 with an MRT of about ~6 days (Table 2. 3).

A similar pharmacokinetic trend in mice has been reported for other macrolides including erythromycin, spiramycin, clarithromycin, and azithromycin (56). Furthermore, results suggest that tulathromycin has a larger accumulation and persists in lung tissue longer than other macrolides in mice (56). The high affinity of the drug for lung tissue and the long persistence of tulathromycin was also reported in cattle (11, 35), pigs (5, 36), and foals (57). Multiple factors have been hypothesized to explain the remarkable accumulation of macrolides in lung tissue, including interactions with phospholipids, intracellular accumulation (bronchial and alveolar

epithelium and bronchoalveolar cells) due to ion trapping, active transport (carriers such as members of the organic anion transporting protein), and endocytosis (9, 11, 27, 32, 45-47, 50). However, the identification and description of the mechanism(s) involved in tulathromycin lung tissue accumulation still remains unknown.

Tulathromycin is a lipid-soluble triamilide (28). 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 AUC_{0-144} ratio adjusted by dose between dose groups T-7 and T-28 was 1.22. In lung tissue, the AUC_{0-144} ratio adjusted by dose between dose groups T-7 and T-28 was 0.89 (mean actual doses 6.95 vs. 27.57 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 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 (10). The administration of LPS in the respiratory system has been extensively used to trigger an acute inflammatory response (2). The administration of LPS in the respiratory system prompts an acute infiltration of polymorphonuclear neutrophils and the release of a myriad of inflammatory mediators (2, 13, 23, 25, 44, 52). The model implemented in the present study has been fully described elsewhere (51). The neutropenic mouse model has been

extensively used to unravel the pharmacokinetics and pharmacodynamics of antimicrobials (24, 29, 42).

In a pilot trial, we evaluated the accumulation of inflammatory cells in lung tissue from mice treated intra-nasally with LPS and with and without cyclophosphamide. Administration of LPS induced accumulation of neutrophils in the lung. In contrast, when cyclophosphamide was included in the protocol, there was no neutrophilic accumulation (Villarino, et al. data unpublished). In the present study, we evaluated the LPS response in lung tissue only in a subset of control 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 $7.24 \pm 1.64 \times 10^3/\mu\text{l}$) and with reference values for anesthetized BALB/c mice (Table 2.5) (34).

The WBC count in the animals treated with LPS and CP was lower than in the other groups ($0.93 \pm 0.27 \times 10^3/\mu\text{l}$). These changes were also reflected in the neutrophil count. Previous studies considered animals neutropenic when the cell count was $<100 \text{ cell}/\mu\text{l}$ (12, 42). In the present study, the mice were severely neutropenic regardless of the administration of LPS (0.04 ± 0.01 , 3.17 ± 1.02 , and $0.69 \pm 0.37 \times 10^3/\mu\text{l}$ for the CT-LPS-CP, CT-LPS, and CT-NT group, respectively) (2.5 and Figure 2.8) (34). 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 were neutropenic without recruitment of neutrophils into the lung.

The AUC_{0-144} 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 2.3). 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 2.6 and 2.7). Changes in the pharmacokinetic parameters estimated from plasma and lung tissue have also been reported for other macrolides in infected animals (56). 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 2.3 and Figure 2.3). 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 2.3).

Tissue-directed pharmacokinetics has been proposed as one of the mechanisms of local drug accumulation (3, 4, 8, 14, 15, 18, 19, 30, 40, 53). When azithromycin was administered to infected mice, the AUC in lung tissue was much lower in leucopenic animals (3). 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. 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 (3). 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 (9), cell type (6, 18, 30) cell status (38), extracellular conditions (pH) (21) and type of stimulus (18). Those factors should be taken into account when designing future studies.

Neutrophilia also did not affect drug accumulation in the lung in this study. 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.

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 lung tissue when the drug is administered during either a neutrophilic or neutropenic state. The microbiological and clinical impacts of these scenarios need to be evaluated.

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Conflict of Interest

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Appendix

Table 2.1. 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

Time point	Group	LS Mean [†]	95% CIs [†]	Time point	Group	LS Mean [†]	95% CIs [†]
30 min	T-28	11700	490-18500	72 h	T-28	109	20.2-199
	T-7-LPS	6790	184-11700		T-7-LPS	19.2	5.20-33.2
	T-7-LPS-CP	6700	1230-12200		T-7-LPS-CP	5.32	0.31-10.3
	T-7	3280	1500-5070		T-7	24.4	4.50-44.2
1 h	T-28	792	214-1370	96 h	T-28	26.2	7.10-45.4
	T-7-LPS	3870	713-7030		T-7-LPS	16.9	4.60-29.3
	T-7-LPS-CP	6920	1870-12000		T-7-LPS-CP	15.9	2.93-28.9
	T-7	2060	378-3700		T-7	9.28	2.50-16.0
24 h	T-28	145	26.7-260	120 h	T-28	19.2	3.50-34.9
	T-7-LPS	50.8	13.7-87.9		T-7-LPS	10.9	2.00-19.9
	T-7-LPS-CP	59.1	11.0-107		T-7-LPS-CP	6.89	1.27-12.5
	T-7	103	28.1-180		T-7	10.8	2.90-18.7
48 h	T-28	100	27.2-174	144 h	T-28	13.6	3.70-23.5
	T-7-LPS	51.1	13.8-88.4		T-7-LPS	7.82	0.50-15.2
	T-7-LPS-CP	57.0	15.4-98.6		T-7-LPS-CP	6.48	1.75-11.2
	T-7	60.0	16.2-103		T-7	15.4	4.20-26.7
† Back-transformed							

Table 2.2. 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

Time point	Group	LS Mean [†]	95% CIs [†]	Time point	Group	LS Mean [†]	95% CIs [†]
30 min	T-28	15700	4550-2600	72h	T-28	4800	1400-8210
	T-7-LPS	3940	1440-6430		T-7-LPS	889	325-1450
	T-7-LPS-CP	5240	1910-8560		T-7-LPS-CP	680	123-1240
	T-7	4410	2198-6621		T-7	1950	715-3196
1 h	T-28	27900	5050-50500	96 h	T-28	4760	1380-8130
	T-7-LPS	3250	1192-5320		T-7-LPS	824	301-1350
	T-7-LPS-CP	5500	2010-8980		T-7-LPS-CP	879	159-1600
	T-7	3150	1150-5140		T-7	1690	619-2770
24 h	T-28	1760	643-2870	120 h	T-28	4240	1550–6940
	T-7-LPS	1170	213-2140		T-7-LPS	516	189-844
	T-7-LPS-CP	1680	614-2740		T-7-LPS-CP	926	338-1510
	T-7	1170	426-1910		T-7	1230	359-2110
48 h	T-28	4770	1740-7790	144 h	T-28	3860	1120-6590
	T-7-LPS	1050	305-1800		T-7-LPS	1060	308-1810
	T-7-LPS-CP	1000	292-1720		T-7-LPS-CP	750	218-1280
	T-7	2070	603-3540		T-7	1020	375-1670
† Back-transformed for reporting							

Table 2.3. 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

Pharmacokinetic Parameter*	Group							
	T-28		T-7		T-7-LPS		T-7-LPS-CP	
	Plasma	LH	Plasma	LH	Plasma	LH	Plasma	LH
HL_{Lambda_z} (h)	18.1	112	13.5	82.0	16.5	213	17.6	144
T_{max} (h)	0.50	1.00	0.50	0.50	0.50	0.50	1.00	1.00
C_{max} (ng/mL)	11700	27900	3300	4410	6790	3940	6920	5500
C_{last} (ng/mL)	13.6	3860	15.4	1020	7.82	1060	6.48	750
AUC_{last} (hr*ng/mL)	152000	869000	31000	246000	52600	199000	88100	199000
AUC_{inf_obs} (hr*ng/mL)	152000	1500000	31200	378000	52800	487000	88300	356000
AUMC_{last} (hr*hr*ng/mL)	599000	45000000	274000	15700000	252000	8670000	259000	9330000
MRT_{INF_pred} (h)	4.16	148	8.91	138	4.99	287	3.03	177
AUC₍₀₋₁₄₄₎LH:Plasma ratio	5.77		7.95		3.79		2.26	

*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 2.4. Wet-to-dry weight ratios of lung tissue from BALB/c mice

Time point	Dose group	Mean	SD	%CV	Time point	Dose group	Mean	SD	%CV
30 min	T-7	3.18	0.65	20.3	72 h	T-7	3.95	0.28	7.05
	T-7-LPS	4.22	0.38	9.07		T-7-LP	4.87	0.22	4.70
	T-7-LPS-CP	4.69	0.14	3.16		T-7-LPS-CP	5.18	0.23	4.49
1 h	T-7	4.37	0.11	2.53	96 h	T-7	4.32	0.25	5.73
	T-7-LPS	4.26	0.15	3.53		T-7-LPS	4.96	0.62	12.6
	T-7-LPS-CP	4.75	0.17	3.65		T-7-LPS-CP	4.95	0.28	5.67
24 h	T-7	4.18	0.21	5.08	120 h	T-7	4.59	0.20	4.28
	T-7-LPS	4.47	0.12	2.85		T-7-LPS	4.71	0.32	6.81
	T-7-LPS-CP	5.05	0.10	2.02		T-7-LPS-CP	5.09	0.19	3.90
48 h	T-7	4.78	1.30	27.2	144 h	T-7	4.28	0.26	6.12
	T-7-LPS	4.64	0.15	3.24		T-7-LPS	4.97	0.77	6.45
	T-7-LPS-CP	4.81	0.46	9.60		T-7-LPS-CP	5.03	0.20	3.99

Table 2.5. Summary of white blood cell count in BALB/c-c mice

Dose Group	WBC ($\times 10^3/\mu\text{L}$)	N %	L %	M %	E %	B %	R %	N ($10^3\text{cells}/\mu\text{L}$)	L ($10^3\text{cells}/\mu\text{L}$)	M ($10^3\text{cells}/\mu\text{L}$)
CT-LPS-CP (n=7)										
Mean	0.93	5.93	89.1	0.37	2.79	0.26	0.08	0.04	0.84	0.00
SD	0.27	4.79	6.88	0.44	1.77	0.34	0.02	0.01	0.26	0.00
CV	0.29	0.81	0.08	1.18	0.64	1.33	0.32	0.29	0.32	2.65
CT-LPS (n=7)										
Mean	7.24	43.2	52.9	0.97	1.75	0.15	4.03	3.17	3.81	0.07
SD	1.64	7.00	6.92	0.32	0.98	0.05	0.64	1.02	0.83	0.03
CV	0.23	0.16	0.13	0.34	0.56	0.33	0.16	0.32	0.22	0.38
CT-NT (n=10)										
Mean	6.19	10.6	83.5	1.15	1.95	0.25	3.24	0.69	5.18	0.07
SD	2.94	2.05	1.33	0.28	0.51	0.11	0.48	0.37	2.45	0.04
CV	0.47	0.19	0.02	0.24	0.26	0.44	0.15	0.54	0.47	0.54
WBC white blood cell count; N:Neutrophils; L: Lymphocytes; M: Macrophages; E: Eosinophils; B: Basophils; R: Reticulocytes										

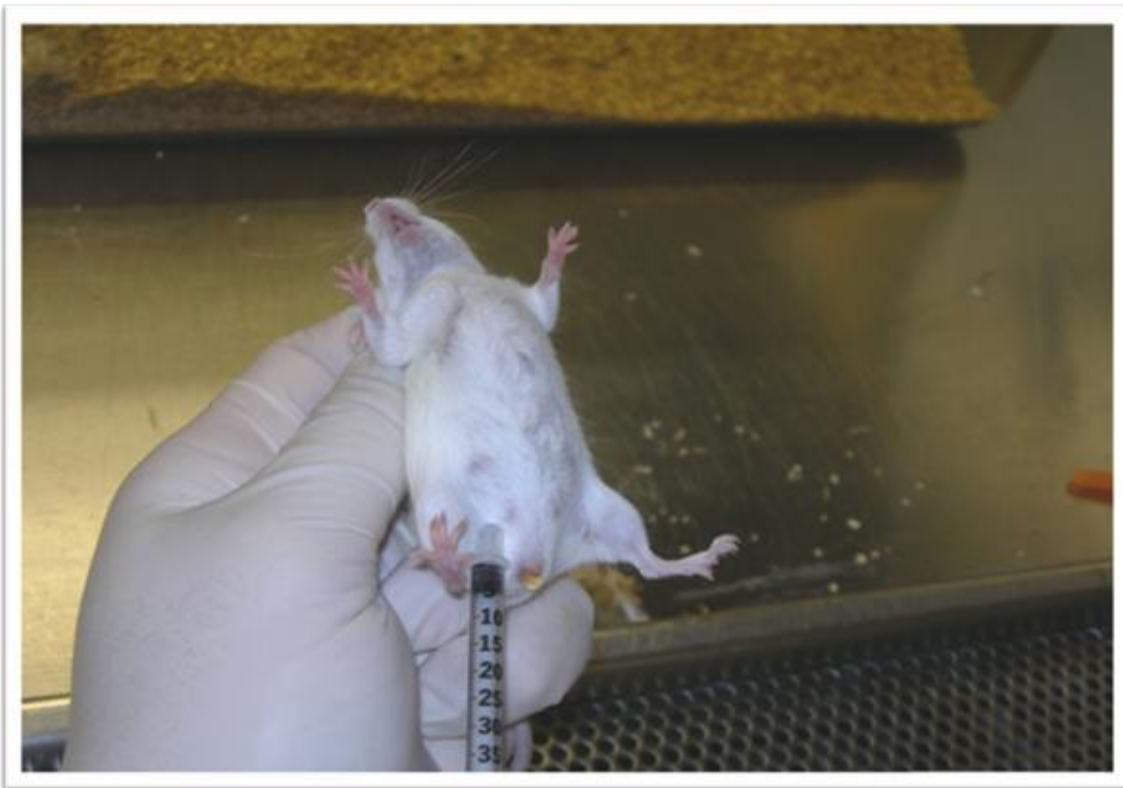


Figure 2.1. Intra-peritoneal administration of cyclophosphamide.



Figure 2.2. Intra-nasal administration of LPS Ec to anesthetized mice.

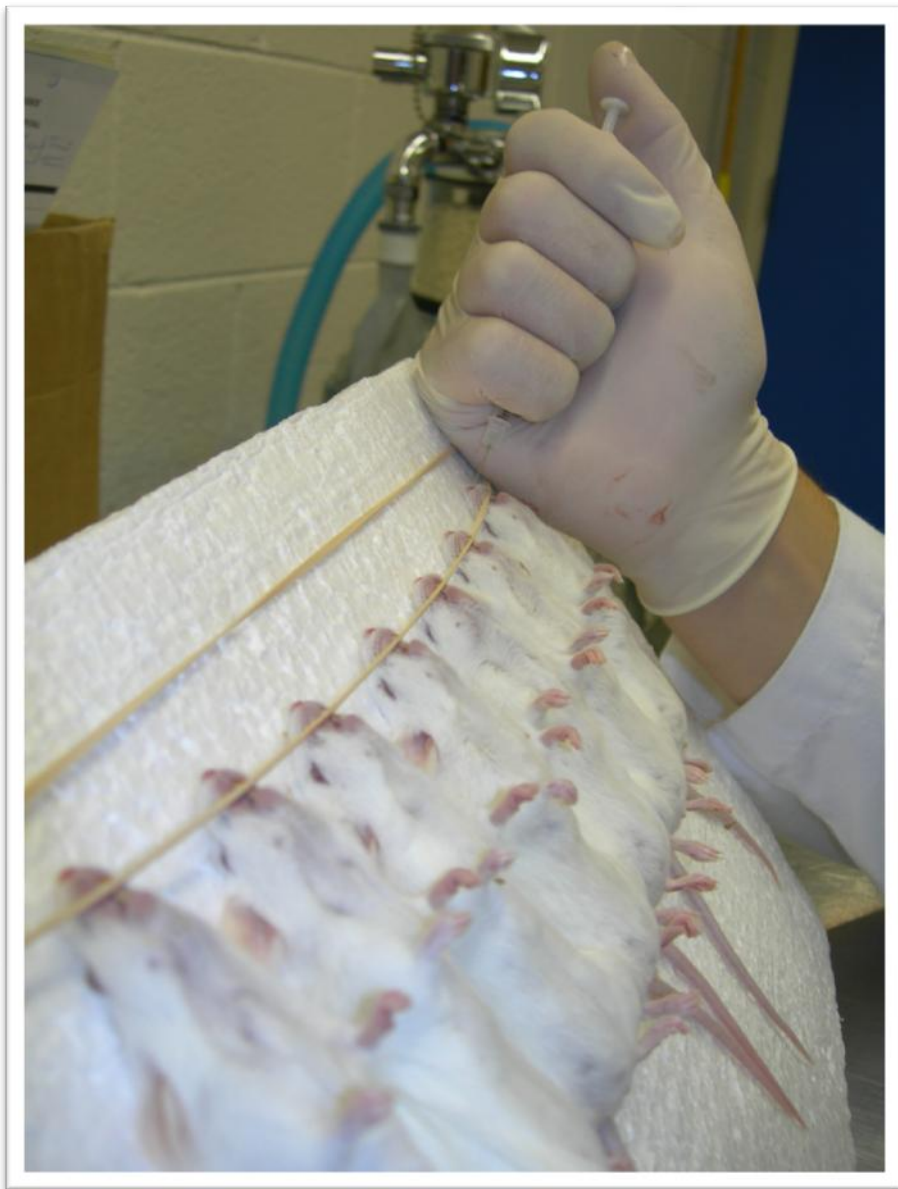


Figure 2.3. Intra-nasal administrations of LPS-*Ec* to anesthetized mice.

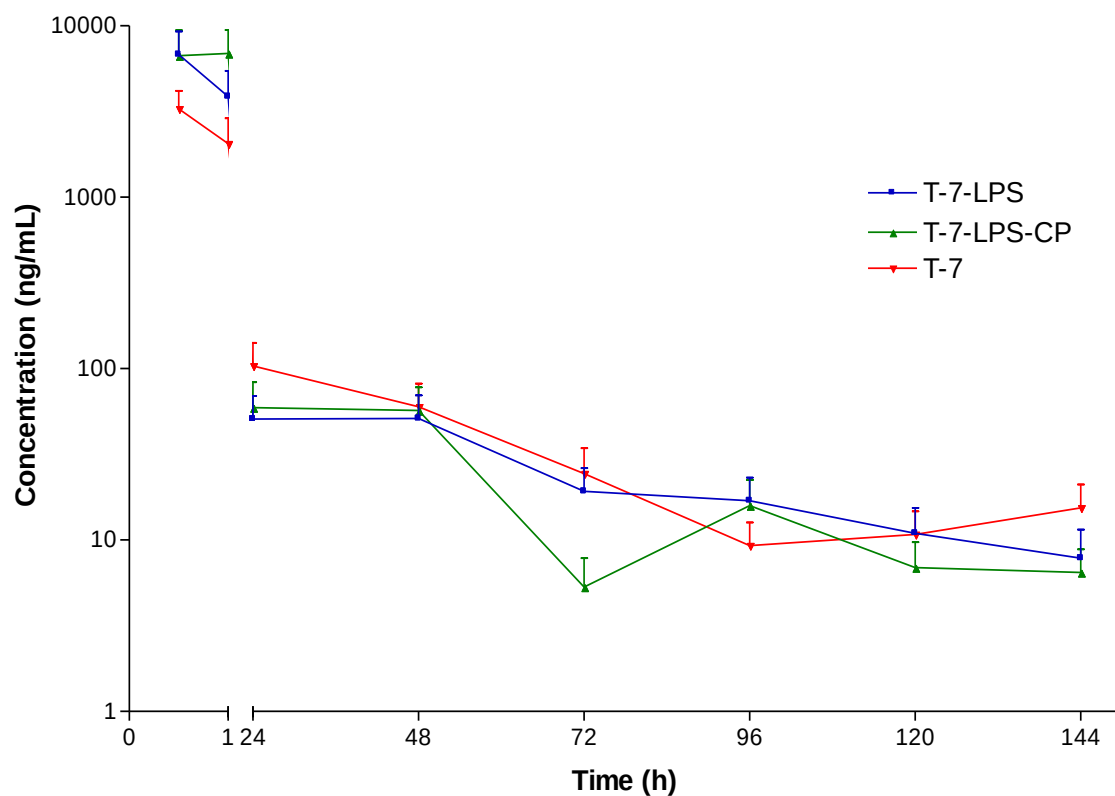


Figure 2.4. LS means concentration ($\pm$ SE) (ng/mL) of tulathromycin in plasma from mice treated s.c. with tulathromycin at 7 mg/kg. (For T-7-LPS at 30 min and 96 h n=4. For T7-LPS-CP at 30 min, 24 and 48 h, n=4 and at 72 h n=3. For T-7 30 min and 96 h n=4)

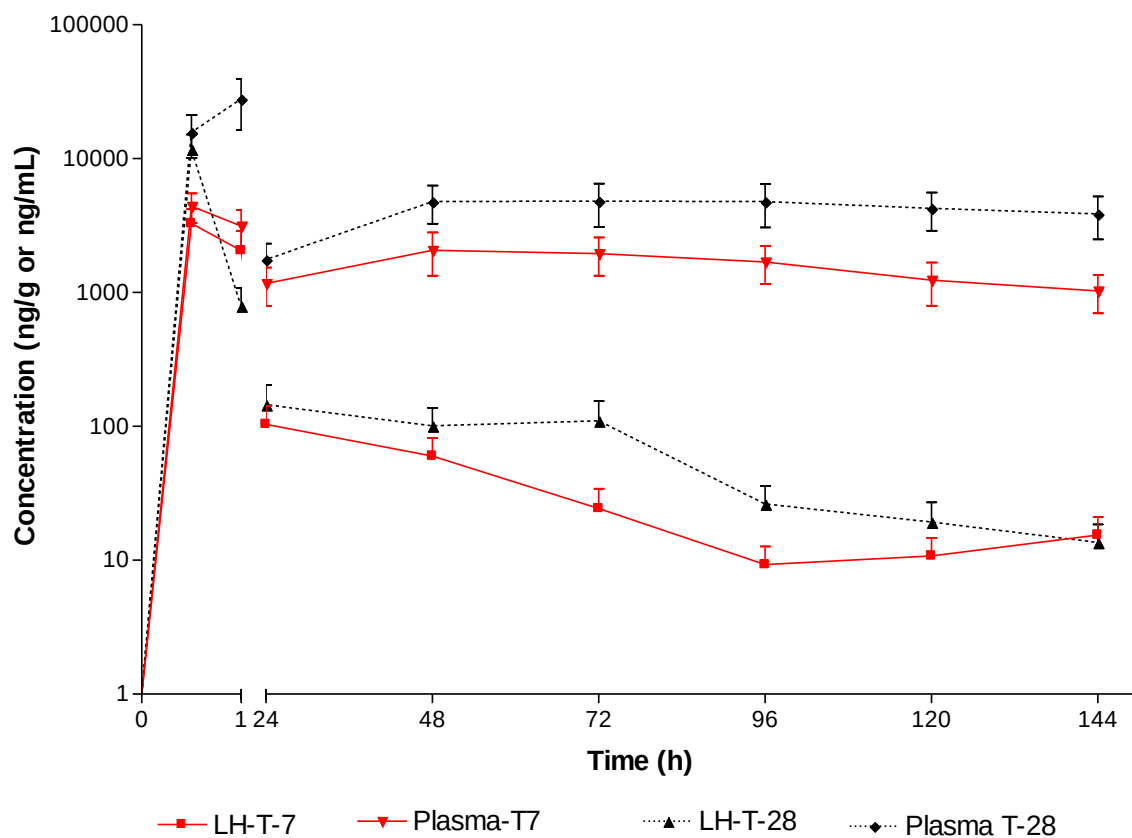


Figure 2.5. LS means concentration ($\pm$ SE) of tulathromycin in lung tissue homogenate (ng/g) and plasma (n/mL) from mice treated s.c. with tulathromycin at 7 and 28 mg/kg (For T7 at 48 h, n=4)

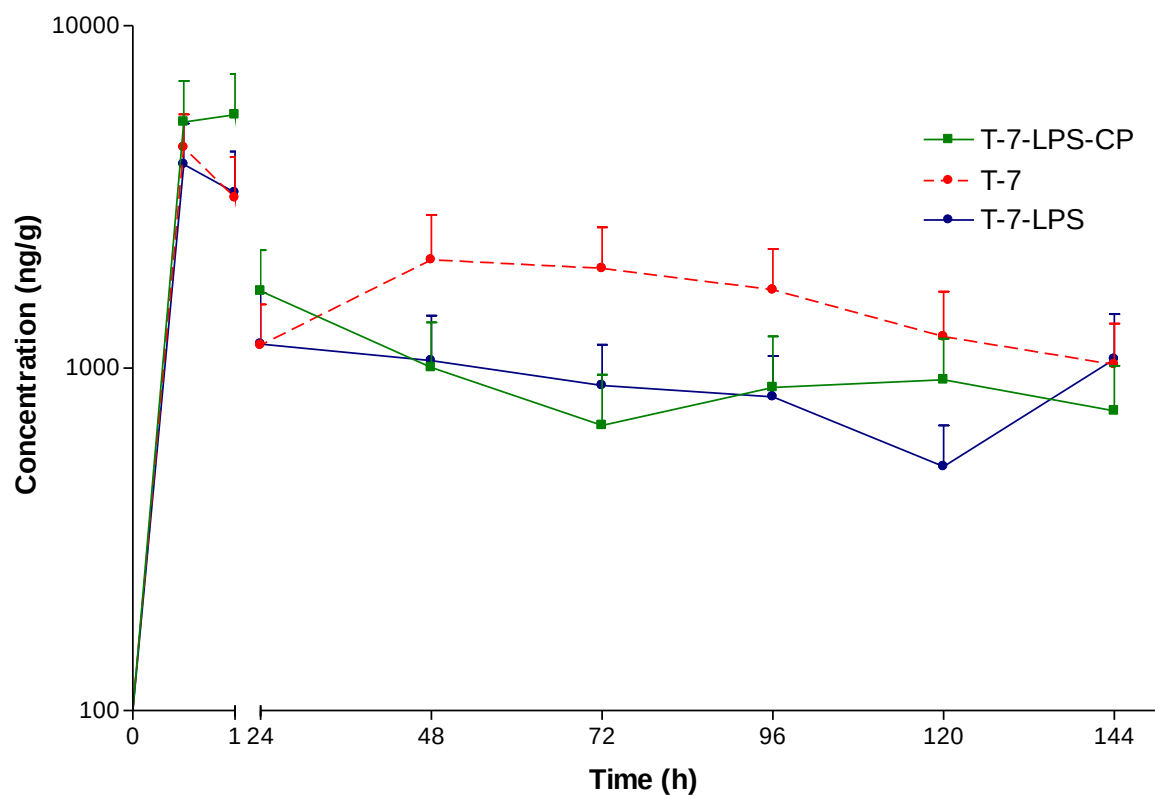


Figure 2.6. LS means concentration ($\pm$ SE) (ng/mL) of tulathromycin in lung tissue homogenate from mice treated s.c. with tulathromycin at 7 mg/kg. (For T7- LPS-CP at 48 h and 72 h, n=4, For T7 at 48 h, n=4)

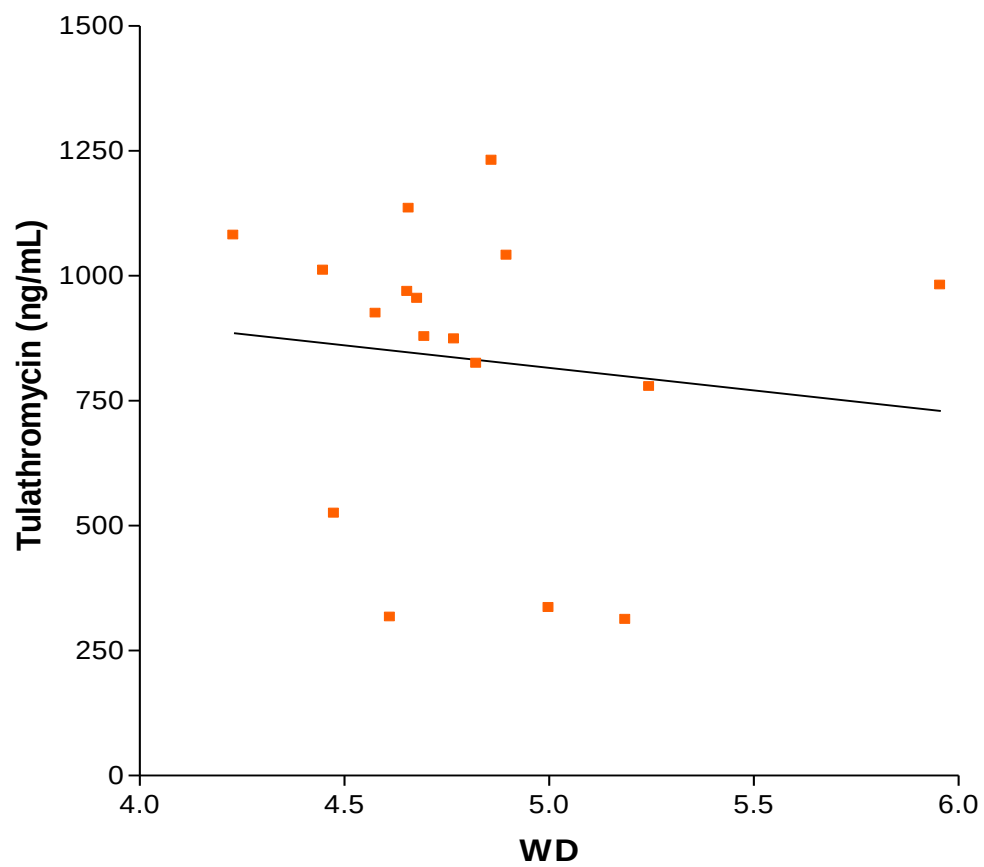


Figure 2.7. Linear regression concentration of tulathromycin in lung tissue vs. wet to dry ratio (from 72 to 144h) for T-7 LPS ($R^2= 0.01$).

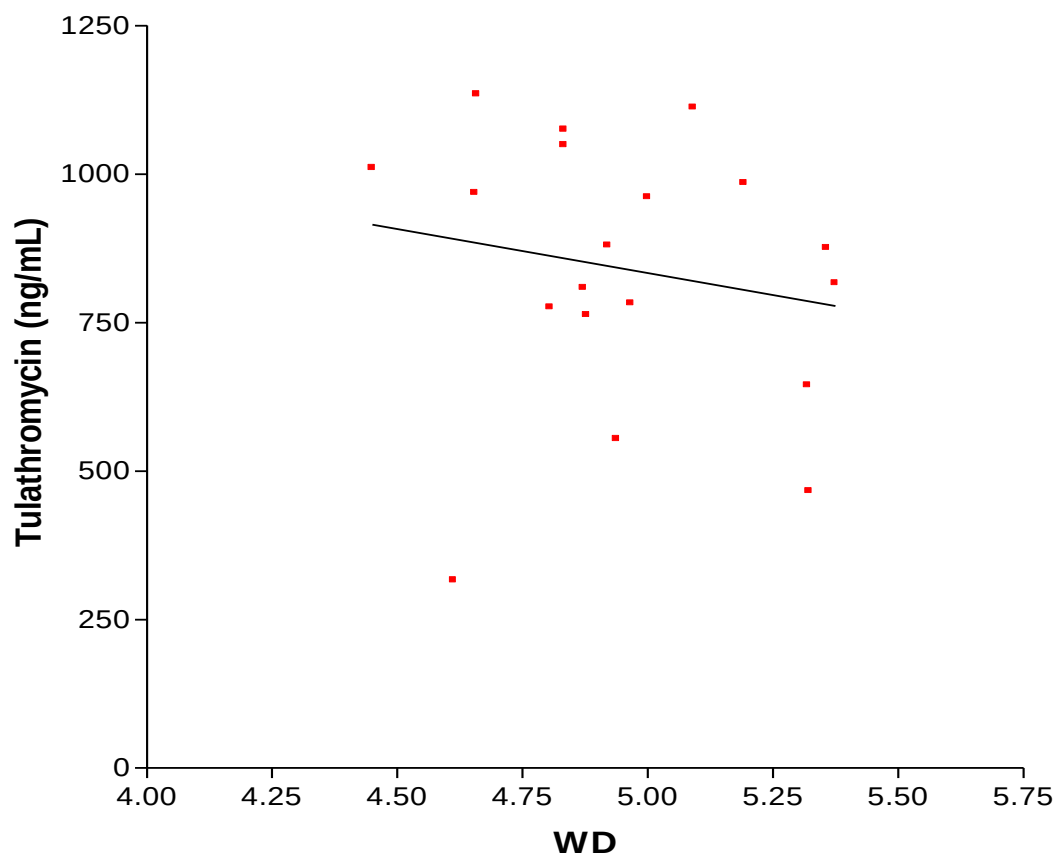


Figure 2.8. Linear regression concentration of tulathromycin in lung tissue vs. wet to dry ratio (from 72 to 144h) for T7-LPS-CP ($R^2 = 0.03$).

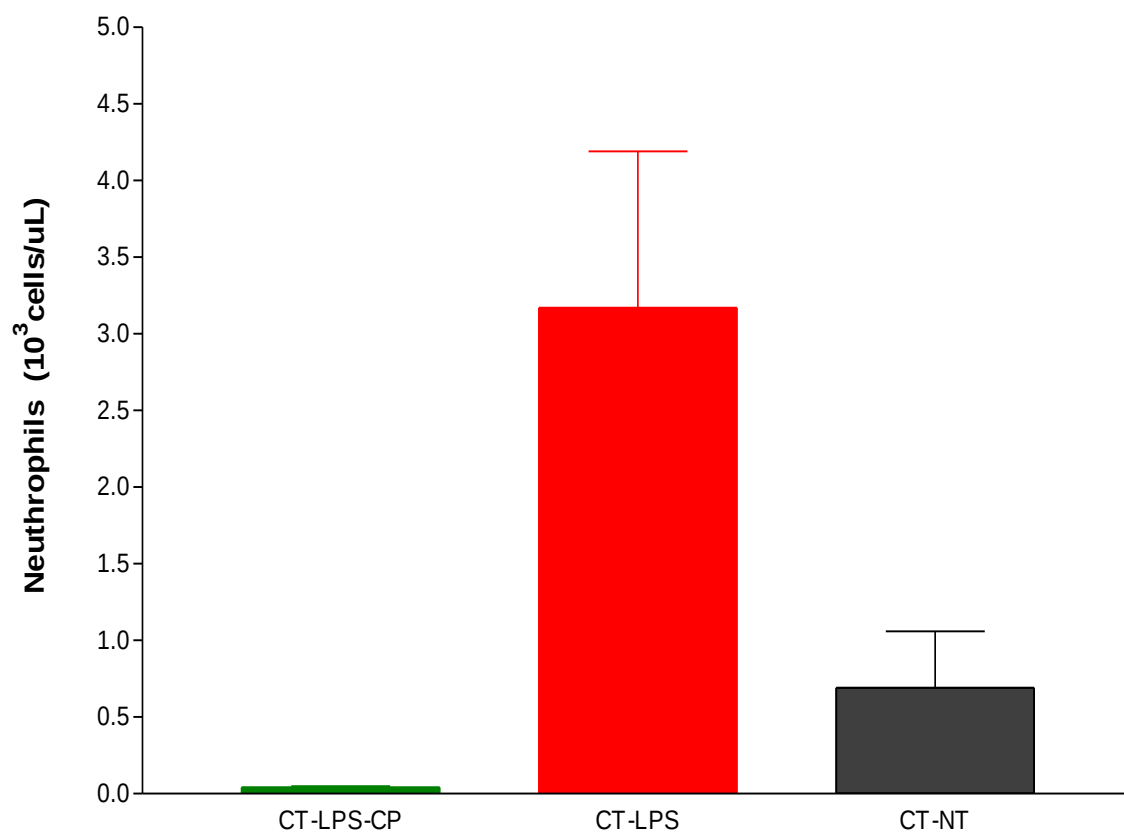


Figure 2.9. Number of neutrophils (mean $\pm$ SD) in blood from BALB/c mice.

Chapter 3:

AN ACUTE REVERSIBLE EXPERIMENTAL MODEL OF PNEUMONIA IN PIGS: TIME RELATED HISTOLOGICAL AND CLINICAL SIGNS CHANGES

Abstract

The objective of this study was to evaluate the long-term survival rates, clinical response, and lung gross and microscopic changes in pigs treated intra-tracheally with lipopolysaccharide of *Escherichia coli* 0111:B4 (LPS-*Ec*). Healthy pigs were randomly allocated to 3 groups; (i) no-LPS-*Ec* (n=1), (ii) LPS-*Ec*-T1 (1 mg/mL, 10 mL/pig) (n=7) and (iii) LPS-*Ec*-T2 (0.5 mg/mL, 10 mL/pig) (n=6). Two pigs from each dose group were euthanized at 24 (n=3 for T1), 48 and 144 h post-LPS-*Ec* challenge. LPS-*Ec*-treated animals showed macroscopic lesions in middle lobes of the lung. A reversible recruitment of macrophages and neutrophils was observed at 24, 48, and 144 h post-LPS-*Ec* challenge. The highest cellular infiltration level was observed at 24 h after challenge. The highest clinical scores were evident in both experimental dose levels within 3 and 5 h after LPS-*Ec* administration. The administration of LPS-*Ec*, under the conditions evaluated, can be used to induce a reproducible model of acute pulmonary inflammation in pigs.

Keywords: Lung, inflammatory model, pigs, lipopolysaccharide

Introduction

Pneumonia represents one of the most important pathological conditions both in veterinary and human medicine. Information about pathogenesis and treatment of this disease is generated from clinical cases. However, a considerable amount of the available data is based on the use of experimental animal models. Animal models of pneumonia have been used to (i) unravel the pathogenesis of pneumonia (17, 18), (ii) describe details about inflammatory response (11), and (iii) evaluate disposition and efficacy of drugs (7).

Pneumonia models have been induced using different types of challenges including; physical factors (7), microorganisms (*Actinobacillus pleuropneumoniae*, *Pseudomonas*, etc.) (2, 11, 15) and toxins (lipopolysaccharide of *Escherichia coli*, etc) (1, 8, 20). Lipopolysaccharide (LPS) is an extremely active immune stimulant substance from microbiological origin (4). Once recognized by cell membrane receptors (Toll like receptor type 4), LPS triggers a dose-dependent acute inflammatory response that can be used to study acute pneumonia (9, 10, 12).

Appropriate experimental models allow for controlled reproducible and predictable responses and for the design of experiments for particular objectives. However, a clear understanding of the model is necessary if it is to be used appropriately. Characteristics of the model will be dictated by factors involving the challenge (type, dose, route of administration, etc) and host (species, breed, age, immune status, etc.).

Even though LPS-induced pig pneumonia models have been reported (1, 8, 20), they have not been described in a manner that can be applied to a wide range of circumstances. Limited information is available regarding long-term survival rates (>3 days), dose dependent clinical and histological response, variability in the pulmonary response, and long term course of lesions. In addition, authors have used guinea pigs

(1) and piglets (8, 21) instead of growing pigs from commercial units, which would in fact represent the target population. All these elements need to be considered when designing acute pneumonia studies using the LPS model in pigs.

The objective of the present study was to evaluate the parameters of the intra-tracheal, two-dose LPS-Ec model of acute pneumonia in growing pigs, including the long-term survival rates, clinical response, and both macroscopic and microscopic changes in the middle lobe and caudal lobe of the lung.

Materials and methods

Animals and housing

Fourteen clinically healthy Duroc x Landrace pigs (mean bodyweight $\pm$ SD 25.6 $\pm$ 1.47 kg) (female n=7 and male n=7) were purchased from Bailey Terra Nova Farms (a local commercial source). The animals were housed together in a common pen adhering to standard procedures established at Pfizer Animal Health. Pigs had *ad-libitum* access to a standard swine grower ration and drinking water and were acclimatized to the building/pen for 7 days prior to dosing. Treatment of the pigs was in accordance with regulations (9 CFR Parts 1, 2 and 3) promulgated from the USDA Animal Welfare Act and the conditions specified in the 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 initiation of the study, procedures involving the care or use of the pigs were reviewed and approved by the Pfizer Animal Health Institutional Animal Care and Use Committee (IACUC).

Inclusion criteria

Animals with no signs of illness or infection as determined by a pre-dose physical inspection conducted by personnel trained in swine management were included in the

study. None of the animals had received antimicrobial therapy within 7 days of the start of the study. Likewise, none of the animals had been treated with glucocorticoids, immunotherapeutic, or other anti-inflammatory agents within 30 days prior to the scheduled start of the study.

Study design

In this parallel-designed study, pigs were randomly allocated to one of three groups: no treatment (NTX) (n=1), treatment 1 (T1), (n=7) and treatment 2 (T2), (n=6). The pig in NTX group was the non-treated control. Animals in T1 and T2 received a single intra-tracheal dose of LPS-*Ec* at 1 mg/mL (10 mL/pig) and 0.5 mg/mL (10 mL/pig), respectively.

Preparation of inoculums

Lipopolysaccharide from *Escherichia coli* 0111:B4 purified by phenol extraction was purchased from Sigma Aldrich, USA (Lot Number 129K4025). The LPS-*Ec* powder was re-suspended in saline solution (NaCl 0.9%) to obtain final concentrations; 0.5 mg of LPS-*Ec*/mL and 1 mg of LPS-*Ec*/mL. The same inoculum volume (10 mL) was used for all the LPS-*Ec* treated animals.

Administration of inoculum

Animals were physically restrained on standing position and a red rubber latex catheter (12 French) was placed intra-tracheally using a mouth opener device. The catheter placement was confirmed by air movement through the catheter. Afterwards, the inoculum was instilled into the mid-section of the trachea using a syringe. The syringe was flushed with air twice to ensure a complete administration of the inoculum.

Sampling design

Clinical signs were recorded by the blinded attending veterinarian and gross pathologic changes in the lungs were recorded by a blinded board certified veterinary pathologist. Clinical signs and gross pathologic changes were evaluated during a period of 0-144h post-LPS-*Ec* administration according to the schedule presented in Table 3.1. Clinical signs were assessed using a scoring system as presented in Table 3.2. After the last sample was collected, animals were anesthetized by an intramuscular injection of Telazol[®] injectable solution (tiletamine / zolazepam Fort Dodge Animal Health, Fort Dodge, IA, USA) with xylazine and then exanguinated.

Post-mortem gross pathologic and histopathologic observations

After euthanasia, lungs were removed from the animal and gross pathologic observations made by a pathologist. Briefly, lungs were inflated using approximately 600 mL of 10% buffered formalin instilled intra-tracheally allowing the lung to fix for at least 5 minutes. Then, the lungs were positioned with dorsal surface up on a cutting board. Lung tissue samples (approximately 2×1×0.5 cm) were obtained from the right (n=4) and left lobe (n=4). Samples captured portions of the cranial and middle lobe, primary bronchus and caudal lobe. Samples were placed individually into 10% buffered formalin for at least 24 h.

Samples for histopathology analysis from the right lung of the control animal (Animal number 5686), from both lungs of one animal in T1 (animal number 5684) at 24h post-LPS challenge and left lung of one animal of T1 (animal 5688) and T2 (animal 5681) at 48h post-LPS-*Ec* challenge were not taken since lung tissue was used for other proposes not directly related with the objective of this study. Sampled tissues from all animals were examined by the same pathologist using light microscopy. Morphological changes included but were not limited to: hemorrhage, interstitial edema, necrosis,

neutrophil and/or macrophage infiltration, and atelectasis. Morphologies (histopathology findings) were more fully described with topographical “locators” and “modifiers.” All prepared sections were scored by a blinded board certified pathologist (DGT) in terms of severity as follows: GRADE 1 = minimal / very few / very small, GRADE 2 = mild / few / small, GRADE 3 = moderate / moderate number / moderate size, GRADE 4 = marked / many / large, GRADE 5 = massive / extensive number / extensive size.

Results

The LPS-*Ec* inoculum was successfully instilled intra-tracheally in all the experimental animals. None of the animals coughed nor adversely reacted to the endotoxin challenge procedure, which ensured a consistent and complete inoculum administration. None of the animals died during the study. All the experimental animals were euthanized at the pre-assigned terminal sampling time meeting all the criteria established by the IACUC for maintaining the experimental animals alive during the study.

Clinical signs

All the experimental animals were considered clinically healthy at the pre- LPS-*Ec* administration. Following LPS-*Ec* administration, all of the animals experienced changes at their first clinical score observation (3 h post-LPS-*Ec* administration). Clinical observations scores are presented in Table 3.3. None of the clinical scores were higher than 2 (scale of 0-3) for any of the dose levels and minimal changes were observed in the general health score. Changes in respiratory score were evident at both dose levels at 3 h post-challenge. The highest scores were recorded in all the animals at 3 and 5 h post-LPS-*Ec* administration for the lower and higher dose levels, respectively. Afterwards, the score decreased towards pre-LPS-*Ec* administration (Score=0). All the

animals returned to score of 0 after 56 h post-LPS-*Ec* administration. Attitude change scores were comparable to respiratory scores. Maximum score changes were observed at 3 and 5 h post-LPS-*Ec* administration (Table 3.3).

The visual inspection of the clinical assessment scores indicated a tendency to revert to normal (score=0) after reaching maximal values.

Gross pathology changes

No macroscopic lesions were observed in the lungs from the non-LPS-*Ec*-treated animal. Gross abnormalities in the lung were observed in all the treated animals at 24 h post-challenge (Figure 3.1). Gross changes were mainly observed bilaterally at the cranial and middle lobes. The abnormalities were characterized by extensive areas of red to dark red discoloration and firm consistency. No evidence of differences was noticed between either of the LPS-*Ec* doses levels in terms of qualitative characteristics and magnitude of the lesions. Gross pulmonary changes were also found at 48 and 144 h post-treatment but gross pathology changes tended to be less severe with time.

Microscopic changes

Lungs from all the LPS-*Ec*-treated animals exhibited histopathological changes. The primary microscopic findings included alveolar and interstitial inflammation with neutrophils and macrophages. Also, there was an increased number of leukocytes (mainly macrophages and/or neutrophils) in the lumen of blood vessels (leukocytosis) (Figure 3.2, 3.3, 3.4, and 3.5). According with the histopathology findings, animals treated with 1.0 mg/mL of LPS-*Ec* (T1) exhibited higher severity scores than those treated with 0.5 mg/mL (T2) (Figure 3.3, 3.4, and 3.5). The greatest magnitude of changes was observed at 24 h post-treatment. Severity decreased at 48 and 144 h post-treatment observations in all the experimental animals at both dose levels. In all the

affected animals, the middle lobes presented more severe histological changes than the other lobes that were examined. No microscopic changes were seen in the caudal lobes at the last sample time. Most of these lesions correlated with the gross pathology findings at necropsy. Table 3.4 summarizes the scores on main microscopic changes in the pulmonary lobes at 24, 48 and 144 h after LPS-*Ec* administration.

Discussion and conclusion

Lipopolysaccharide (LPS) is a structural and functional component of the gram - negative bacterial outer membrane (5). Scientific evidence indicates that LPS acts as a potent pro-inflammatory toxin inducing the release and de novo synthesis of mediators responsible for the pathophysiologic changes associated with gram-negative infectious pneumonia (13, 16). In the present study, we evaluated the histological and clinical response of growing pigs from a commercial unit challenged intra-tracheally with LPS-*Ec*. The main goal of this study was to identify a LPS-*Ec* dose that would induce an acute pulmonary inflammatory response in pigs. For our model, we selected LPS from *Ec* due to (i) its high toxicity and consistency in triggering the inflammatory response in pigs (5, 8, 14, 19), (ii) the ease of handling the inoculum in terms of preparation and administration, (iii) the ease for dose adjustment/standardization, and (iv) the highly dose-dependent response (9).

Different studies have reported the use of LPS-*Ec* to induce an acute inflammatory response at several dose levels (8, 20). Halloy et al. (2004) used 100 µg/kg in piglets (8) and Arbibe et al. (1997) used 330 µg /kg in guinea pigs (1). In both studies experimental animals were euthanized within 24 h. Van Gucht et al. (2003) used a much lower dose (20 µg/kg in PBS) and kept the animals alive for a longer period of time (14 days) with no abnormal clinical changes (20). We have used the limited data published from experimental pigs to define a LPS dose and inoculum size for our study. Even

though LPS-*Ec* doses used in this study (~200 and 400µg/kg) were higher than those previously reported for pigs (100 µg/kg, 330 µg/kg and 20 µg/kg), none of the animals in the present study died during the observation period (0-144 h). This indicates that either dose could likely be used to induce an aggressive acute inflammatory response while keeping the pigs alive for at least 144 h.

Abnormal clinical signs, as reflected by health assessment scores, were evident acutely after the LPS-*Ec* administration at both dose levels (Table 3.2). Although doubling the LPS-*Ec* dose resulted in higher cell accumulation, health assessment scores only changed slightly.

The most evident gross pathology lesions were observed consistently in the middle lobe of both lungs (Figure 3.1). Lung areas with gross lesions had higher scores of microscopic changes. Even though microscopic changes were observed in all the experimental animals at the three sampling times, gross changes were not observed in the caudal lobes. By contrast, Halloy et al. (2004) reported macroscopic lesions in the caudal lobe, indicating a more dispersed distribution of the inoculums (8). It is possible that the inoculum volume plays a key role. In this study ~0.38mL/Kg of inoculum was used compared to 1mL/kg used by Halloy et al., (2004) (8). Another difference is that in the study by Halloy et al., (2004) the inoculum was instilled under general anesthesia (8). We instilled the inoculum in standing animals. The standing position and movement of the animals after the inoculation may have resulted in a pulmonary distribution of the inoculum, into the cranial and middle lower lung compartments. Thus, the relevance of inoculum volumes and methods of challenge administration may need to be further evaluated.

The evaluation of gross and microscopic changes as well as the clinical assessment indicates that LPS-*Ec* induced reversible pulmonary changes. Even though there was a clear tendency of inflammatory microscopic changes to move toward

normality, we have not evaluated the histopathology changes beyond 144 h post-LPS-*Ec* administration. Consequently, total reversibility of microscopic changes cannot be assured. We have not evaluated the effects of multiple LPS administrations but there is evidence that might indicate that a chronic inflammatory model can be induced by using multiple LPS administrations (3). On the other hand, some evidence suggests that induction of LPS-based chronic models may not be appropriate due to the decrease in the inflammatory response with time due to tolerance (6).

Based on these results we have successfully implemented this model (10mL/Pig with LPS-*Ec* at 10mg/mL) in a larger study involving 36 pigs (Chapter 5 and 6) and the findings have been consistent with those reported here, including normal clinical condition in pigs at 17 days LPS-*Ec* post-challenge.

In conclusion, an acute lung inflammatory response was induced by the intra-tracheal administration of LPS from *Ec* at two different dose levels. The observed response included a reversible and marked recruitment of macrophages and neutrophils. The magnitude of the inflammatory cell infiltration seemed to be dose dependent and decreased towards baseline levels with time after peak infiltration. Microscopic, gross pathology and clinical changes were well correlated. Even though an acute inflammatory response was induced at both dose levels, all the experimental animals survived for the duration of the study and did not require intervention due to health and welfare concerns. Therefore, the administration of LPS-*Ec*, under the conditions described in this study, can be used to induce a reproducible model of acute lung inflammatory response in growing pigs.

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Conflict of interest

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Appendix

Table 3.1. Sampling design

Treatment groups	Hours post-LPS-Ec challenge																
	3	5	8	12	18	24	28	34	40	48	56	72	80	96	104	120	144
NTX	X	X	X	X	X	X-N											
T1_{24h} & T2_{24h}	X	X	X	X	X	X-N											
T1_{48h} & T2_{48h}	X	X	X	X	X	X	X	X	X	X-N							
T1_{144h} & T2_{144h}	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X-N

X: Observation taken at this time (clinical evaluation); **N**: Necropsy performed at this time (Clinical and gross change evaluation of lung and lung sampling for histopathology); **NTX**: No LPS inoculation (n=1), **T1** treatment group challenged with LPS-Ec at 1mg/mL, total volume of inoculums: 10mL (n=2) (except for T1 at 24h (n=3)), **T2**: treatment group challenged with LPS-Ec at 0.5 mg/mL, total volume of inoculums: 10mL (n=2).

Table 3.2. Clinical signs scores

Observation	Score	Clinical Description
Attitude (depression)	0	Normal – Pig is up and active when entering the room or when approaching.
	1	Mild depression – Pig is slightly inactive, but gets up after being approached; pig moves slowly and appears lethargic; shows some interest in food and social interaction, but stays active only for a few minutes; animal may appear gaunt and have a rough coat.
	2	Moderate depression – Pig shows pronounced inactivity and gets up only after light back pressure; displays little or no interest in food and/or social interaction; tends to lie down almost immediately and/or stands with head down; noticeably gaunt and coat is rough; staggers and appears slightly uncoordinated at the walk.
	3	Severe depression/moribund – Pig is moribund, unresponsive to stimuli, unable to get up or does so with difficulty; shows no interest in surroundings, food, or social interaction; appears anxious; should be euthanized for humane reasons.
Respiratory Distress	0	None – Respiratory movements are subtle, superficial, and can be observed only when animal is quietly lying down.
	1	Mild – Respiratory movements are obvious and predominantly abdominal.
	2	Moderate – Respiratory movements are exaggerated, entirely abdominal, and labored.
	3	Severe – Respiration is very labored and entirely abdominal; pig breaths through mouth (continuously or intermittently); nose and/or ears are cyanotic; should be euthanized for humane reasons.
General Condition (includes appetite evaluation)	0	Clinically normal appearing animal
	1	Slightly abnormal condition
	2	Moderately abnormal condition
	3	Severely abnormal condition

Table 3.3. Clinical score changes in pigs treated intra-tracheally with LPS of *Ec* at two dose levels (n=14)

Treatment Group 5 mg/pig														Treatment Group 10 mg/pig														
ID	CS	Score												ID	CS	Score												
5683	A	2	2	0	0	0	0							5684	A	2	1	2	0	0	0							
	RD	2	1	0	2	0	0								RD	2	2	2	2	2	0							
	GH	0	0	0	0	0	0								GH	0	1	0	0	0	0							
5685	A	2	2	0	0	0	0							5687	A	2	1	0	0	0	0							
	RD	2	2	0	0	0	0								RD	2	2	0	0	0	0							
	GH	0	0	0	0	0	0								GH	0	0	0	0	0	0							
5679	A	1	2	0	0	1	2	1	0	0	1			5689	A	2	2	2	2	0	0							
	RD	2	2	0	0	1	1	1	0	1	0				RD	2	2	2	2	2	0							
	GH	1	0	0	0	0	0	0	0	0	0				GH	0	0	0	0	0	0							
5688	A	2	1	2	1	0	0	0	0	0	0			5681	A	2	2	2	1	2	1	1	1	1	1			
	RD	1	2	2	2	0	0	0	0	0	0				RD	2	2	2	2	1	1	1	1	1	1			
	GH	0	0	0	0	0	0	0	0	0	0				GH	0	0	0	1	0	0	0	0	0	0			
5680	A	1	1	0	1	2	1	0	0	0	0	0	0	5678	A	2	2	1	2	0	0	1	1	1	0			
	RD	1	2	2	1	1	0	0	0	0	0	0	0		RD	2	2	2	2	1	0	0	1	1	1			
	GH	0	0	0	0	0	0	0	0	0	0	0	0		GH	0	0	0	0	0	0	0	0	0	0			
5676	A	2	2	2	2	2	2	1	1	0	0	0	1	5682	A	2	2	2	1	2	0	0	0	0	0	0	0	
	RD	2	2	2	2	0	0	1	1	0	0	0	0		RD	2	2	2	2	0	0	0	0	0	0	0	0	
	GH	0	0	0	0	0	0	0	0	0	0	0	0		GH	0	0	0	0	0	0	0	0	0	0	0	0	
NTX	A	1	1	0	0	0	0							5677	A	2	2	2	2	1	1	0	0	0	0	1	0	
	RD	0	0	0	0	0	0								RD	2	2	2	2	0	0	0	0	0	0	0	0	
	GH	0	0	0	0	0	0								GH	0	0	0	0	0	0	0	0	0	0	1	0	
Time post LPS Administration (h)		3	5	8	12	17	24	28	34	40	48	56	72	80	Time post LPS Administration (h)	3	5	8	12	17	24	28	34	40	48	56	72	80
ID: identification; NTX; control animal; A: attitude; RD: respiratory distress; GH: general health. Results beyond 80h are not included. All the experimental animals had a clinical score of 0 between 96 and 144 h post-LPS- <i>Ec</i> administration for the three scores evaluated.																												

Table 3.4. Severity of Main Histological Findings in Pulmonary Samples from Pigs Treated Intra-tracheally with LPS of Ec at two dose levels (n=13)

Treatment	Time Post-dosing/ Group/ Animal ID												
	24 hr					48 hr				144 hr			
	NTX	LPS 5 mg/pig		LPS 10 mg/pig		LPS 5 mg/pig		LPS 10 mg/pig		LPS 5 mg/pig		LPS 10 mg/pig	
Animal ID (56-)	86	83	85	87	89	88	79	78	81	80	76	77	82
Left Cranial													
Acute alveolar Inflammation	0	1	1	1	4	-	2	2	-	2	2	1	1
Acute Interstitial Inflammation	0	0	1	0	1	-	1	1	-	1	2	0	0
Leukocytosis	0	1	1	0	2	-	0	1	-	0	1	1	1
Left Middle													
Acute alveolar Inflammation	1	3	4	5	5	-	1	2	-	2	2	1	1
Acute Interstitial Inflammation	1	1	1	1	3	-	0	2	-	2	2	0	0
Leukocytosis	1	2	1	1	3	-	0	1	-	1	1	1	1
Left Caudal													
Acute alveolar Inflammation	1	1	2	0	3	-	2	3	-	2	0	1	1
Acute Interstitial Inflammation	0	1	1	0	1	-	1	2	-	1	1	1	0
Leukocytosis	0	2	2	1	1	-	0	1	-	1	1	1	1
Left Primary Bronchi													
Acute alveolar Inflammation	0	2	2	2	3	-	2	2	-	1	0	1	0
Acute Interstitial Inflammation	0	1	1	1	1	-	2	1	-	1	0	0	0
Leukocytosis	0	2	1	1	2	-	1	1	-	1	1	0	1
Right Cranial													
Acute alveolar Inflammation	-	2	4	1	1	2	2	2	2	0	0	1	0
Acute Interstitial Inflammation	-	1	1	0	0	1	1	2	1	0	1	0	0
Leukocytosis	-	1	1	1	1	1	0	1	2	1	1	1	1
Right Middle													
Acute alveolar Inflammation	-	4	4	5	5	2	2	3	2	4	1	2	1
Acute Interstitial Inflammation	-	1	1	2	2	0	0	2	1	2	2	2	0
Leukocytosis	-	2	1	3	1	2	0	2	1	1	1	1	1
Right Caudal													
Acute alveolar Inflammation	-	2	3	1	4	2	2	2	1	4	1	1	1
Acute Interstitial Inflammation	-	0	1	0	2	1	2	1	1	2	0	2	0

Table 3.4. Continued

	Time Post-dosing/ Group/ Animal ID												
	24 hr					48 hr				144 hr			
Treatment	NTX	LPS 5 mg/pig		LPS 10 mg/pig		LPS 5 mg/pig		LPS 10 mg/pig		LPS 5 mg/pig		LPS 10 mg/pig	
Animal ID (56-)	86	83	85	87	89	88	79	78	81	80	76	77	82
Leukocytosis	-	1	1	1	1	1	1	0	1	1	1	1	2
Right Primary Bronchi													
Acute alveolar Inflammation	-	1	1	1	4	2	2	2	1	2	1	1	0
Acute Interstitial Inflammation	-	0	0	0	1	0	1	1	0	1	1	0	0
Leukocytosis	-	1	1	1	1	1	1	2	1	1	1	1	1

NTX; no LPS treatment. Samples from the caudal lobes taken at its cranial (**A**) and caudal levels (**B**). Numbers in columns indicate grade of severity: -= No lobe examined; 0= No lesion; 1=Minimal; 2=Mild; 3= Moderate; 4= Marked; 5= Severe. Acute alveolar inflammation was characterized by focal to diffuse infiltration of alveolar spaces with low to abundant numbers of macrophages and/or neutrophils. Acute interstitial inflammation was characterized by focal to diffuse thickening of the inter-alveolar septa by infiltration with variable numbers of macrophages and lesser neutrophils. Leukocytosis was characterized by increased numbers of leukocytes (mainly macrophages or neutrophils) in the lumen of large, medium, small blood vessels and capillaries. In some of the cases those leukocytes appear adhered to the endothelia. Congestion and/or hemorrhage were characterized by increased amount of blood in the lumen of blood vessels and capillaries (congestion) or by extravasation of red blood cells from blood vessels or capillaries into adjacent pulmonary tissue or alveolar spaces (hemorrhage). Atelectasis was characterized by focal or multifocal and coalescing areas where the alveolar spaces were markedly reduced in size and the tissue was collapsed. Commonly the alveoli in those areas were infiltrated with variable numbers of foamy macrophages and multifocal minimal to mild fibrin deposition. Peribronchial/peribronchiolar lymphoplasmacytic inflammation was characterized by nodular accumulation of variable numbers of lymphocytes and plasma cells in the periphery of the bronchi and/or bronchioles. Bronchial/bronchiolar inflammation was characterized by infiltration of bronchial or bronchiolar lumen with variable numbers of neutrophils. Chronic pleural inflammation (Pleuritis) was characterized by hypertrophy of the mesothelial cells, fibrosis, and inflammatory infiltration composed mainly of neutrophils, macrophages mixed with lesser lymphocytes, plasma cells, and eosinophils.



Figure 3.1. Lung gross changes induced by intra-tracheal administration of LPS-*Ec*. at 24 h post-challenge.

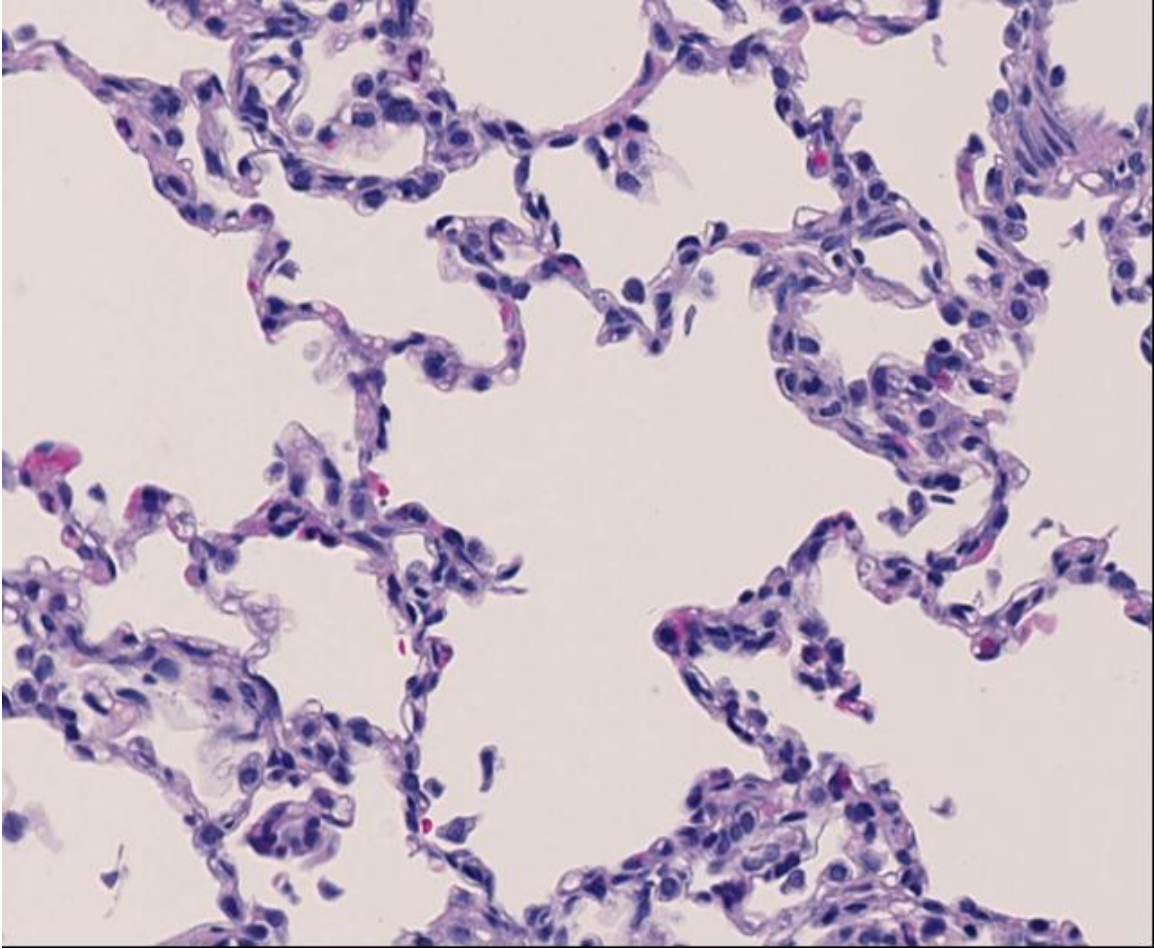


Figure 3.2. Lung histologic changes induced by intra-tracheal administration of *Ec*. Lung sections from saline (X 40). The pictures are representative of samples taken from areas with evident gross changes (LPS treated pigs). No gross changes were observed in the control animal; therefore picture is representative of samples taken from middle lobe.

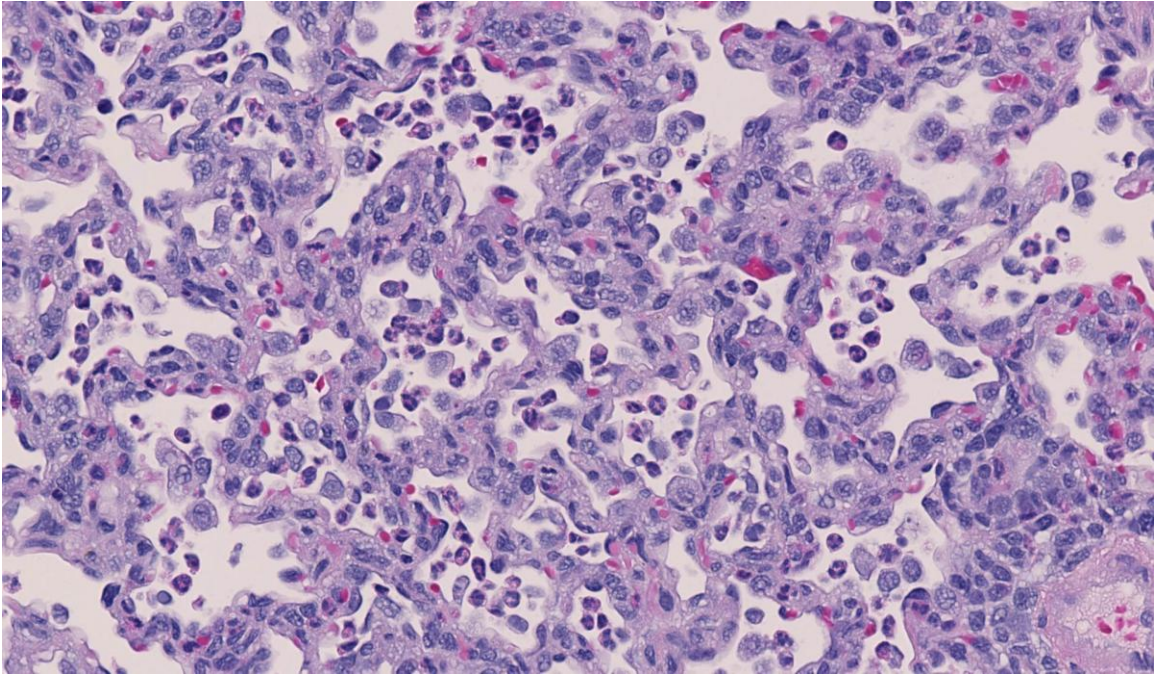


Figure 3.3. Lung histologic changes induced by intra-tracheal administration of *Ec*. Lung sections from LPS-*Ec*-treated pigs at 0.5 mg/mL (X40) at 24 h post-LPS *Ec* administration.

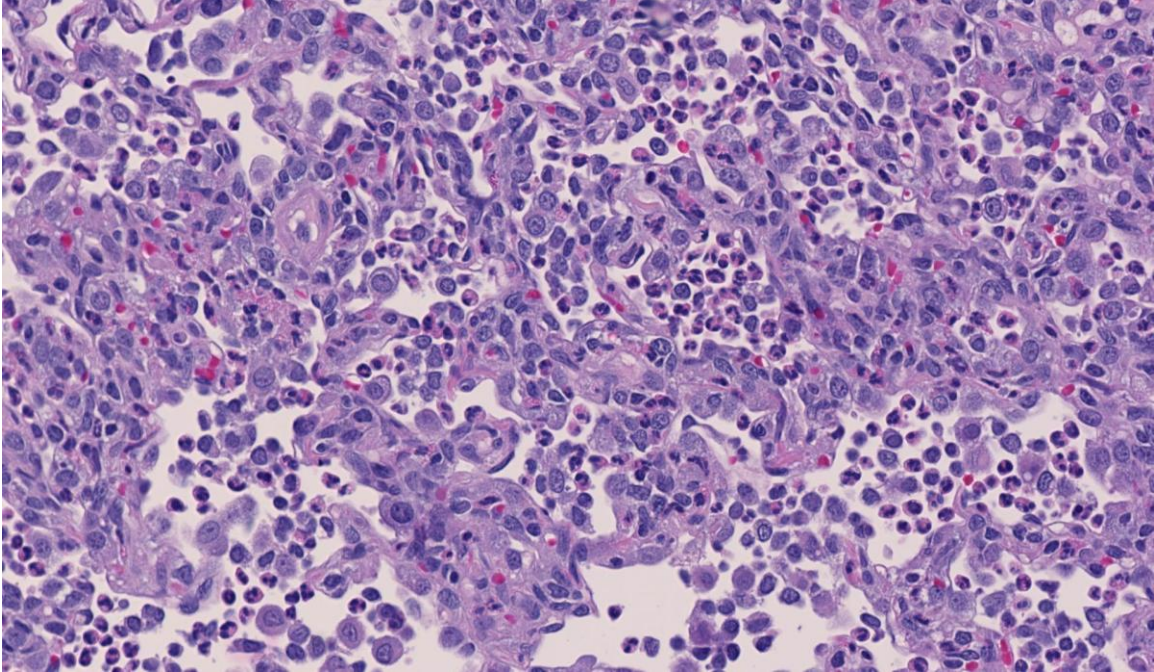


Figure 3.4. Lung histologic changes induced by intra-tracheal administration of *Ec*. Lung sections from LPS-*Ec*-treated pigs at 1 mg/mL (X 40) at 24 h post-LPS *Ec* administration. The pictures are representative of samples taken from areas with evident gross changes (LPS-treated pigs).

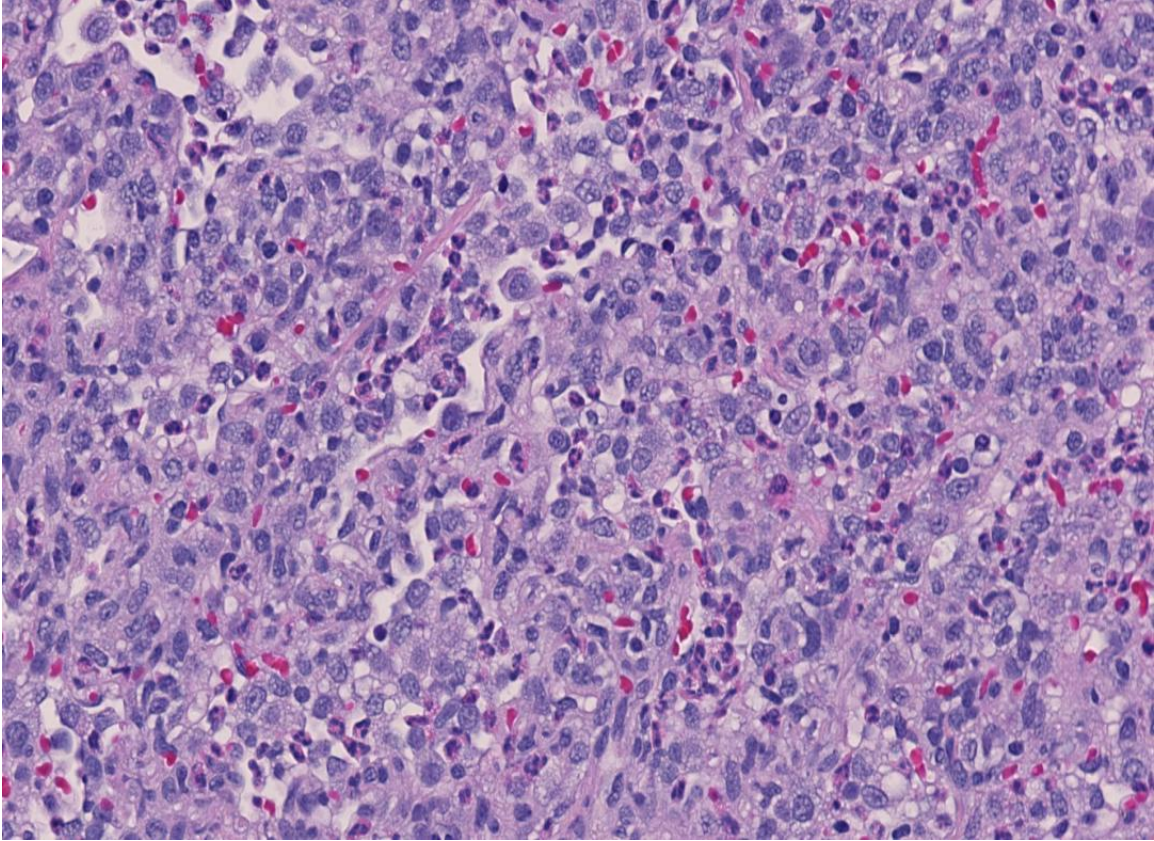


Figure 3.5. Lung histologic changes induced by intra-tracheal administration of *Ec*. Lung sections LPS-*Ec*-treated pigs at and 1 mg/mL at 24 h post-LPS *Ec* administration. The pictures are representative of samples taken from areas with evident gross changes (LPS treated pigs).

Chapter 4:

A PILOT ASSESSMENT OF TRACHEO-BRONCHIAL FIBERSCOPY AND BRONCHIAL MICRO-SAMPLING PROBES TO EVALUATE ANTIMICROBIAL CONCENTRATIONS IN LOWER RESPIRATORY AIRWAYS IN PIGS

Introduction

Infection site concentrations are linked to the efficacy of any antimicrobial treatment. Free drug plasma concentrations are considered surrogate for the concentrations at the site of infection (4, 10). However, the actual concentration-time profile at the site of infection can differ from that in plasma, questioning in some circumstances, its value as a surrogate of peripheral drug concentrations (4, 10). In the lung, determination of drug concentration is often accomplished by homogenizing lung tissue and bronchoalveolar lavage (BAL). However, these techniques often provide conflicting information and/or have many shortcomings from the experimental design standpoint (7). More sophisticated techniques have also been applied to study pulmonary drug distribution including positron emission tomography and microdialysis, but their applicability in veterinary medicine is limited to a small number of experimental circumstances and logistical concerns (2, 6, 13-15).

This study seeks to find an alternative method to sampling drug concentrations in lung tissue that is faster, minimizes labor, is appropriate in multiple sampling designs, and provides meaningful information. There are few convenient sampling procedures available to harvest bronchial epithelial lining fluid (BELF) in pigs. Bronchial micro-sampling (BMS) probes have been used experimentally in human setting and are likely to provide a valuable alternative for pigs, but are yet to be tested in veterinary species (8, 9, 16). The objective of this study was to evaluate the use of BMS probes (BC-402C; Olympus; Tokyo, Japan) for quantification of tulathromycin BELF in pigs.

Materials and methods

Animals and housing

Four healthy female Duroc x Landrace pigs (20-30 kg) were purchased from a local commercial source. Treatment and housing conditions of the pigs were in accordance with regulations outlined in the USDA Animal Welfare Act (9 CFR Parts 1, 2 and 3). Procedures involving the care or use of the pigs were approved by a Pfizer Institutional Animal Care and Use Committee.

Treatment and sampling design

Animals (n=3) were dosed with tulathromycin at 2.5 mg/kg (Draxxin[®] Injectable Solution, Pfizer Animal Health, New York, NY) at the supplier location and transferred to Pfizer Animal Health for procedural work approximately 23 h after dosing. The remaining animal was used as an untreated control. Blood samples were taken by venipuncture 24 hours after the administration of tulathromycin. Afterwards, animals were euthanized by captive bolt stunning followed by exsanguination. BELF was gathered from each animal using bronchial micro-sampling (BMS) probes (Model BC-402C Olympus; Tokyo, Japan) as described previously in humans (8, 9, 16) (Figure 4.1). Immediately after euthanasia, the trachea was exposed and a fiber-optic flexible endoscope (Olympus, model XP-10) and a BMS probe were inserted into the trachea and directed into the middle lobe following the bronchi until resistance was met. The sampling portion of the BMS probe was uncovered from the protective sheath and was placed in contact with the bronchus wall for approximately 20 seconds. This was visually verified in all animals. The sample portion of the probe was drawn back into the sheath to avoid contamination before removing the device from the respiratory airways. Three independent samples were taken from a secondary bronchus at the right and left middle lobes of each experimental animal (6 bronchial samples/animal). The cotton tip (sample portion) was removed

from the rest of the sampling device, collected into a polypropylene tube, transported on wet ice ($\sim 0^{\circ}\text{C}$) and stored refrigerated until analysis. The fluid volume gathered from each probe was estimated by weight as previously reported (8, 9, 16). After the BMS procedures, lung tissue was collected from each animal. The tissue was individually homogenized and placed into plastic containers. Samples were kept at -10°C until analysis.

Analytical methods

Tulathromycin assays

Probe extracts, lung homogenate and plasma samples were analyzed for tulathromycin content by high performance liquid chromatography (HPLC) with tandem mass spectrometry detection (LC-MS/MS) following procedures described by Galer et al. (2004) (5) and Cox et al. (2010) (3). Roxithromycin was used as internal standard (12).

Bronchial epithelial lining fluid assay

Drug extraction procedures from probes have been described previously for multiple compounds (8, 9, 16). Briefly, the method for extraction of tulathromycin from the probe starts with the introduction of 2 mL of 20 mM pH 4 ammonium acetate:acetonitrile (50:50 v:v) into the tube containing the sampling probe. The sample was vortexed for approximately 1 min and then sonicated for 2 min. A 50 μL sub-sample aliquot was spiked with 200 μL of roxithromycin (25 ng/mL) into a glass tube, vortexed for 1 min and centrifuged at ~ 1500 RCF (~ 3000 RPMs) for 10 min. the supernatant (~ 200 μL) was transferred into a glass vial and 5 μL were injected into the system for analysis. The calibration range was 1.0 to 500 ng/mL ($R^2 > 0.99$) using 7 calibration standards. The bias for the calibration standards ranged from -10.2 to 10.0%. Study samples were analyzed in one run. Three quality control samples (2.5, 25 and 100 ng/mL) were included in the analysis. The bias in the assays of the quality control samples ranged from -2.0 to 10.8%.

Recovery of tulathromycin from probes was assessed by spiking BMS probes in triplicate with tulathromycin at 100 ng/mL. Probes were processed as described above for the study samples. Recovery of tulathromycin from probes was determined to be >94%.

Results

Tulathromycin was detected in all the matrices studied from treated animals but not in matrixes from the control animal. The concentrations (mean $\pm$ SD) of tulathromycin in plasma and lung homogenate at 24 h after dosing were 41.2 ± 15.4 ng/mL and 3900 ± 431 ng/g, respectively and were consistent with results published in the literature (1).

Tulathromycin concentrations were notably high in the BELF (Table 4.1). Consistent drug concentrations were obtained from equivalent anatomical sites from the same animal (4. 2). The concentration of tulathromycin in BELF probes and the intra-site variability are provided in Table 4.1. Inter-site and inter-animal variability of tulathromycin concentration in BELF are shown in Table 4.2. This demonstrates that the drug accumulates in the airway compartment of the lungs from pigs. We did not evaluate whether the harvested BELF sample was a free-cell sample or whether the drug was unbound. Consequently, we cannot assure that the concentrations obtained are free extracellular concentrations.

Multiple studies performed in humans using BMS probes recommend the use of three probes per sample (8, 9, 16). In the present study, we used three probes per sampling site, however, the drug was quantified from individual probes to provide an objective understanding from each individual probe sampling performance and provide evidence for the design of future studies. Results obtained indicate that there is an important inter-probe variability (Table 4.1). which suggests that the use of multiple probes per sampling site improves the precision of the data (Table 4.2). Probes with larger contact surfaces might also improve their sampling performance.

Bronchoalveolar lavage (BAL) is a common technique used to harvest pulmonary epithelial lining fluid (PELF). Pulmonary epithelial lining fluid represents the cell-free fluid

obtained from BAL after normalization with urea (11). Thus, the drug concentration in PELF represents lower intra-airway and alveolar composite concentrations. This indicates that concentration results in PELF obtained by BAL have poor anatomical resolution. Although we have identified some limitations in terms of applicability, BMS probes provide many advantages when compared with traditional procedures to study pulmonary drug pharmacokinetics (Table 4.3). We have successfully conducted a larger study in 36 pigs using BMS probes as BELF sampling device (Villarino, chapter 5 and 6). Results were consistent with those obtained in the present study.

In the present study, some probes were contaminated by blood present in the airways. The presence of blood in the airways was probably consequence of the method of euthanasia used. In order to reduce the likelihood of extra-pulmonary blood contamination of airways it is recommended to use anesthesia followed by exsanguination.

Conclusion

The use of BMS probes represents a fast and simple technique for sampling BELF for quantitation of drugs in pigs, even in large scale studies. In addition, BMS probes allow studying drug kinetics in the epithelial lining fluid avoiding many of the shortcomings described for bronchoalveolar lavage (7) and increasing the interpretative value of the data when compared with lung homogenate and PELF.

In conclusion, in this study we have identified and described many of the advantages and some of the limitations of BMS probes to harvest BELF. These were not available in the literature and represent valuable information to be considered when designing future lung pharmacokinetic studies in veterinary species. Results obtained in the present study provide evidence to justify the use of BMS probes in a larger scale in order to gain a full understanding about their applicability of BMS probes on the study of pulmonary drug pharmacokinetics in animals.

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Appendix

Table 4.1. Concentration (ng/ml) and intra-site probe variability of tulathromycin in BELF in pigs dosed at 2.5 mg/kg i.m.

Animal ID	Anatomical Sampling Site											
	Left Bronchi						Right Bronchi					
	Probe			Mean	SD	%CV	Probe			Mean	SD	%CV
	1	2	3				1	2	3			
665	2970*	4680	1930	3190	1390	43.5	2920	2710	1400	2340	824	35.1
666	4060*	3640	2060	3250	1050	32.4	3690	3360	3390	3480	182	5.24
667	1280*	2830*	963	1690	999	59.1	1840*	1220*	2180*	1750	487	27.9
* Probe was visibly contaminated with blood												

Table 4.2. Inter-animal variability of tulathromycin concentration (ng/mL) in BELF in pigs dosed at 2.5 mg/kg i.m..

Animal	Anatomical Sampling Site	
	Left ^{&} Bronchus	Right ^{&} Bronchus
665	3190	2340
666	3250	3480
667	1690	1750
Mean	2710[#]	2520[#]
SD	885	881

[&]: Tulathromycin concentration from probes from each site (n=3) per animal were pooled considering three probes a unique sample (8, 9, 16)..

[#] Drug concentrations from the left and right bronchus were tested statistically using *un-paired* student t test (level of significance <0.05). Results indicated no statistical differences (p>0.8) between both anatomical sites.

Table 4.3. Advantages and limitations of bronchial microsampling probes to obtain bronchial epithelial lining fluid (BELF).

Advantages	Limitations
Applicable in multiple species	Some species/animals might require sedation or anesthesia (e.g. pigs)
Quick and minimally invasive procedure	Requires the use of a fiberscope and expertise in endoscopy
Since it does not modify the sample site environment, it can be applied in multiple sampling protocols. <i>This s decrease variance inflation thus increasing power of the design. In addition, the number of experimental animals can be considerably reduced.</i>	Animal size <i>The use of fiberscope and probes is limited by the larynx/trachea vs. fiberscope diameter.</i>
Extraction procedures are fairly simple <i>Minimize cost and time of the quantification</i>	Analytical technique <i>It would require highly sensitive analytical instruments such as mass spectrometry</i>
It permits sampling pre-defined anatomical sites within the organ <i>It allows independent sampling at different levels along the airways.</i>	The anatomical site to sample <i>The anatomical site to sample is limited by airway/fiberscope diameter and probe/fiberscope length.</i>

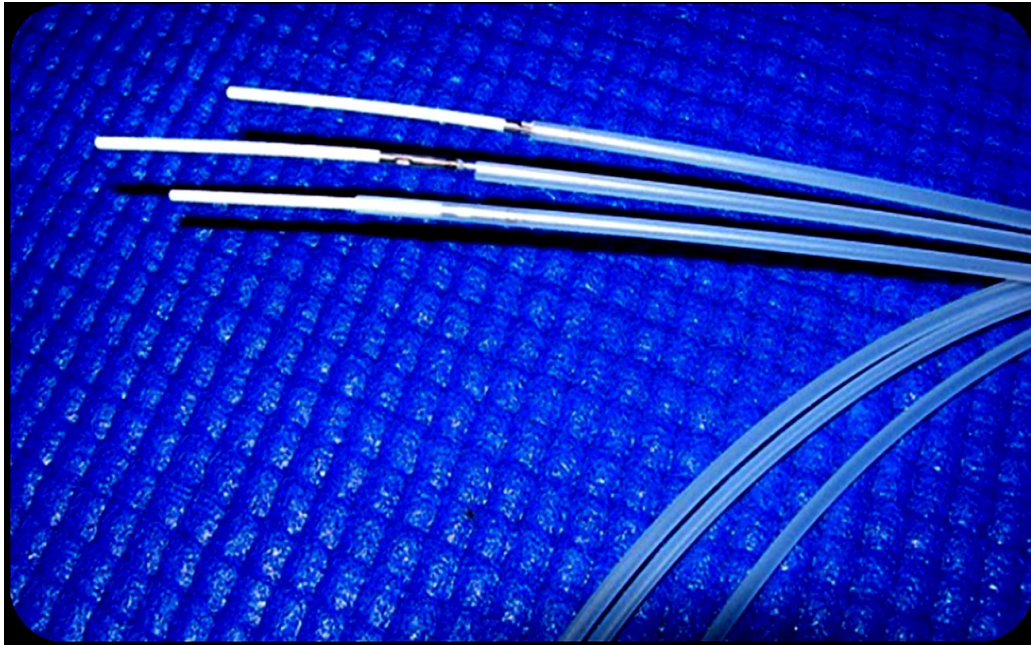


Figure 4.1. Bronchial micro-sampling probes (BC-402C; Olympus; Tokyo, Japan).

Chapter 5:

PULMONARY PHARMACOKINETICS OF TULATHROMYCIN IN SWINE: LUNG HOMOGENATE IN HEALTHY PIGS AND PIGS CHALLENGED INTRA-TRACHEALLY WITH LIPOPOLYSACCHARIDE OF *ESCHERICHIA COLI*

Abstract

Tulathromycin is a macrolide antimicrobial approved in the US for the treatment and control of bovine and swine respiratory disease. The objective of the study was to assess the pharmacokinetics of tulathromycin in lung tissue homogenate (LT) and plasma from healthy and lipopolysaccharide (LPS)-challenged pigs. Clinically healthy Duroc x Landrace pigs were allocated to two dosing groups of 36 animals each (group 1 and 2). All animals were treated with tulathromycin (2.5 mg/Kg). Animals in group 2 were also challenged intra-tracheally with LPS from *Escherichia coli* (LPS-Ec) 3 h prior to tulathromycin administration. Blood and LT samples were obtained from all animals during 17 days post-tulathromycin administration. For LT, one sample from the middle (ML) and caudal lobes (CL) were taken. Tulathromycin distributed rapidly and extensively into the lung. The concentration vs. time profile of the drug in the middle lobe in animals challenged with LPS-Ec (group 2) differs from the concentration vs. time of tulathromycin from animals from group 1 ($p < 0.02$). These differences are due to a change in the concentration time vs. of the drug between 0 and 72 h post-tulathromycin administration. For middle lobe there were statistical differences in drug concentration at 6 h ($p = 0.03$) and 72 h ($p < 0.01$). For the caudal lobe there were statistical differences at 24 ($p < 0.02$) and 72 h ($P = 0.04$). The area under the concentration-time curve through 408 h (AUC_{0-408}) for $LT_{Group-1-ML}$, $LT_{Group-2-ML}$, $LT_{Group-1-CL}$, and $LT_{Group-2-CL}$, were 506000, 450000, 504000, and 473000 ng*h/g, respectively. Tulathromycin (AUC_{0-480}) in the lung exceeded the plasma AUC_{0-408} by 91.3, 78.9, 83.9, and 88.8 fold for $LT_{Group-1-ML}$, $LT_{Group-2-ML}$, $LT_{Group-1-CL}$, and $LT_{Group-2-CL}$, respectively. The drug was slowly eliminated from the lung (terminal half-life > 135 h for all lobes) with a LT: plasma ratio > 300 at 17 days post-tulathromycin administration.

In conclusion, the drug distributed extensively and rapidly into the lungs in both groups. It also persisted in lung tissue at relatively high levels for 17 days post-administration. The concentration vs. time profile of the drug in the middle and caudal lobe in animals challenged

with LPS-*Ec* was influenced by the intra-tracheal administration of LPS-*Ec*. The clinical significance of these findings is unknown.

Keywords: tulathromycin, antimicrobial, pig, lung, lipopolysaccharide

Introduction

Respiratory disease represents one of the most common pathological conditions in livestock (1). Administration of antimicrobials to treat and control the course of respiratory disease is part of the health management practices for both pigs and cattle. Tulathromycin (Draxxin) is a triamilide antimicrobial effective in treating and controlling bovine and swine respiratory disease (15, 18, 20, 26)

When used as recommended, tulathromycin is a safe drug that requires a withdrawal time of 5 days. It is rapidly ($t_{\max} < 1\text{h}$) and nearly completely absorbed when administered following label indications. This drug also has many relevant pharmacokinetic features: tulathromycin not only distributes extensively into lung tissue but also persists in the lung, at high concentrations, for a long period of time (apparent elimination half-life ~6 days). The area under the concentration-time curve (AUC) in lung tissue is more than 60 times higher than the plasma AUC (5, 19).

Multiple factors affect the distribution and accumulation of a drug in a peripheral compartment (e.g. blood flow, tissue binding, cellular infiltration) (22). Some of those systemic and/or local conditions change as a consequence of local inflammation, which may influence the tissue kinetics of the drug (2). Inflammation is a multifactorial, physiological response to tissue injury that changes over time. Therefore, it is reasonable to expect that the influence of the inflammatory response on the tissue kinetics of a drug will also change with time (3, 12). Tulathromycin has been approved by the FDA to control and treat swine respiratory disease. Therefore, the drug may be administered to animals at risk of developing respiratory disease (metaphylactic administration) or sick animals at any of the stages of inflammation (acute, sub-acute, or chronic stage). In previous pharmacokinetic studies, lung disposition of tulathromycin was based on LT samples from healthy animals obtained between 12 and 360 h post-

administration (5, 19). However, no information is available about the pharmacokinetic profile of the drug in lung tissue or plasma from pigs with a pulmonary inflammatory condition.

The objective of the present study was to assess the pharmacokinetics of tulathromycin in lungs and plasma from healthy and lipopolysaccharide (LPS)-challenged pigs during a period between 0 to 408 h post-tulathromycin administration. In this study, we assessed the pulmonary concentration-time profile of tulathromycin using lung homogenate samples. In a companion paper, it is reported the concentration-time profile of tulathromycin in bronchial and pulmonary epithelial lining fluid and bronchoalveolar lavage cells.

Material and methods

Animals and housing

Seventy-eight clinically healthy male and female Duroc x Landrace pigs (20–30 kg) were purchased from a local commercial source. Drinking water and food was available *ad-libitum* throughout the study. Animals were acclimatized for a minimum of 7 days prior to administration of tulathromycin. Dose calculations were based on bodyweights determined day -1 and 0. Treatment and housing conditions of the pigs were in accordance with regulations (9 CFR Parts 1, 2 and 3) promulgated from the USDA Animal Welfare Act and the conditions specified in the 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 initiation of the study, procedures involving the care or use of the pigs were reviewed and approved by the Pfizer Animal Health Institutional Animal Care and Use Committee (IACUC).

Study design

Seventy-two animals were enrolled on the study (36 animals in each dosing group; group 1: 21 males, 15 females and group 2: 16 males, 20 females). Animals (n=6) were

allocated randomly to treatments (T01 to T06, representing the sampling time), group (n=2 non-LPS and LPS-treated animals) and pens (n=6) according to a split plot design (1 extra non-enrolled animal). Animals within a group were allocated into 3 pens. Each pen contained 12 animals. Thus, the group represents the whole plot factor, and treatment the split plot factor. The whole plot was a completely randomized design with a one-way treatment structure.

Treatment administration

Animals in group 1 were administered one intramuscular (i.m.) injection of tulathromycin (Draxxin Injectable Solution, Pfizer Animal Health, New York, NY) at 2.5 mg/Kg bodyweight in the neck at time 0. Animals in group 2 were administered 10 mL LPS/animal at -3 h followed by tulathromycin at time 0.

Administration of LPS-Ec

Preparation of the inoculum

Preparation of the inocula, final volume, and potency was performed as previously reported (Chapter 3). Briefly, LPS-Ec 0111:B4 purified by phenol extraction was purchased from Sigma Aldrich, USA (Lot Number 129K4025). The LPS-Ec powder was re-suspended in saline solution (NaCl 0.9%) to obtain a final concentration of 1 mg/mL. The same inoculum size (10 mL) was used for all LPS-Ec-treated animals.

Administration of the inoculum

Animals were physically restrained in standing position, and a red rubber latex catheter (12 French) was placed intra-tracheally using a mouth opener device. Catheter placement was confirmed by air movement through the catheter. Afterwards, the inoculum was instilled in the middle trachea using a syringe. The syringe was flushed with air twice to ensure complete administration of the inoculum.

Health Assessment- Post-Treatment Observations for LPS-treated Animals

As described previously, the intra-tracheal administration of LPS-*Ec* was expected to generate a reversible acute inflammatory response. Associated with this, animals were expected to show signs of respiratory distress and abnormal attitude at three hours after the LPS challenge. Therefore, we pre-defined the time of tulathromycin administration at three hours after the administration of the LPS-*Ec*. For the health assessment, the general condition, respiratory distress, and attitude scores of the animals were recorded. Table 5.1 presents the score assessment criteria implemented (Chapter 3). LPS-*Ec*-treated animals were evaluated at pre-LPS-*Ec* and post-LPS-*Ec*. administration between -3 to 408 h post-tulathromycin administration, as indicated in Table 5.2.

Blood Collection

Blood (for plasma) was collected during a period of 0–408 h from all animals, following the scheme presented in Table 5.3.

Euthanasia and Necropsy

After collection of the last blood sample from each treatment group, animals were anesthetized with Telazol® injectable solution (tiletamine / zolazepam Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine followed by exsanguination. Animals were euthanized within $\pm 5\%$ of the scheduled time. Necropsies of experimental animals from both groups were performed at 6 different times after the administration of tulathromycin as presented in 5. 2.

Lung homogenate

For animals enrolled in group 1 (tulathromycin only), 2 lung tissue samples were collected from the middle and caudal lobe of the lung.

For animals enrolled in group 2 (tulathromycin and LPS-*Ec*), lung tissue samples were collected from areas with macroscopic lesions in the middle lobe. If macroscopic lesions were not evident samples were taken from tissue that appeared healthy. A second lung sample was taken from the hemi lateral caudal lobe at a macroscopic normal area. Lung samples were individually homogenized and then frozen in a plastic container at -80°C until analysis.

Wet-to-dry ratio determination

Two cm x two cm sub-sections of the middle and caudal lobes were taken for wet/dry ratio determination. Whenever a lesion was present in the middle lobe, a subsample from the lesion area was collected. Samples were placed in sealed bags for transportation. Samples were weighed in aluminum container and the wet weight recorded. Then, samples were dried in an oven at approximately 60°C for 15 days. Samples were then weighed again for wet to dry ratio determination using the equation;

$$\text{Wet-to-dry ratio} = \text{wet weight (g)} / \text{dry weight (g)}$$

Analytical methods

All matrices samples were analyzed for tulathromycin concentration by high performance liquid chromatography with tandem mass spectrometry detection following procedures described (8, 11). Roxithromycin was used as an internal standard (23).

Plasma

A 50-μL plasma sample was mixed with 200 μL of acetonitrile containing roxithromycin, the internal standard, and centrifuged. A 160-μL aliquot of the plasma supernatant was diluted with 40 μL of 20 mM ammonium acetate, pH 4, and 10 μL was injected into the chromatographic system. Chromatography was accomplished with a BDS Hypersil C₈ 30 x 2.1 mm column (Thermo Fisher Scientific, Waltham, MA, USA), at a flow rate of 0.300 mL/min, and

with linear gradient chromatography with a 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 1.0 min for tulathromycin and 1.2 min for roxithromycin. The mass spectrometer (Sciex API 4000, 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.

The calibration curve ($R^2 > 0.99$) ranged from 0.5 to 500 (ng/mL), using 11 calibration standards, and 6 quality control samples (1.95, 3, 18, 30, 195, 300, and 500 ng/mL) were included. For the quality control samples, the intra-run coefficients of variation ranged from 6.6 to 11.0%, and the intra-run biases ranged from -11.0% to 9.0%. For the calibration curve samples, the intra-run coefficients of variation ranged from 1.2% to 18.8%, and intra-run biases for these samples ranged from -11.6% to 6.0%. The inter-run coefficients of variation ranged from 1.7% to 16.9%, and inter-run biases for these samples ranged from -11.0% to 9.0%.

Lung homogenate samples

Samples were processed as described previously by Galer et al. (2004) (11). Briefly, 0.200 g of the lung tissue sample was 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 uL aliquot was injected into the chromatographic system. The calibration curve ($R^2 > 0.99$) ranged from 100 to 25000 ng/g lung tissue using 11 calibration standards, and 3 quality control lung tissue samples (200, 2000 and 10000 ng/g) in each analytical run. For the quality control samples, the intra-run coefficients of variation ranged from 4.7 to 7.6%, and the intra-run biases ranged from -6.5% to 2.5%. For the calibration curve samples, the intra-run biases ranged from -6.4% to 9.0%.

Pharmacokinetic and statistical analyses

Plasma concentration data were summarized using descriptive statistics by treatment and time point. Means (raw means) and 95% CIs were calculated and reported along with the range of the data. For lung homogenate, data was log transformed and then analyzed using a mixed model as followed;

$$Y_{ijk} = \mu + \varphi_i + \beta_{j(i)} + t_k + \varphi t_{ik} + \varepsilon_{ijk}$$

Where;

Y_{ijk} =(Ln transformed) observation of the i^{th} group, the j^{th} block within the i^{th} group, and the k^{th} treatment

μ =overall constant

φ_i =fixed effect of the i^{th} group

$\beta_{j(i)}$ =random effect of the j^{th} block within the i^{th} group,

t_k =fixed effect of the k^{th} treatment

φt_{ik} =fixed interaction effect of the i^{th} group and the k^{th} treatment

ε_{ijk} =residual

$i = 1 \dots \text{number of group}$

$j = 1 \dots \text{number of blocks within group}$

$k = 1 \dots \text{number of treatments}$

Back-transformed LS means (geometric means), standard errors of LS means and 95% confidence intervals (CIs) were calculated. Least squares mean (LS mean) concentration was used as an estimate of treatment mean. The significance level for fixed effects was set at 0.05. The wet-to-dry ratios were summarized as arithmetic means, standard deviations (SDs), and coefficient variations (CVs). Wet-to-dry ratios from both groups were compared statistically by analysis of variance. Linear regression analysis was performed to test the linear association between wet-to-dry ratio and drug raw concentration. Score data for health assessments were summarized for animals in group 2 using frequency Tables.

Pharmacokinetic parameters were estimated using the WinNonlin software package (Standard edition version 5.1, Pharsight Corporation, CA, USA). WinNonlin model 200 was used for the noncompartmental analysis. Pharmacokinetic parameters were estimated using back-transformed LS means derived from the naïve pooling from each sample point (8, 11). Therefore, dispersion data of the PK parameters was not possible to be estimated. The area under the concentration-time curve through 408 h post-dose, AUC_{0-408} , area under the first moment curve through 408 hours post-dose $AUMC_{0-408}$, mean residence time (MRT), and the terminal elimination half-life ($t_{1/2}$) were estimated. The AUC_{0-408} was calculated by log-linear trapezoidal rule, and λ_z was estimated with uniform weighting. The observed back-transformed LS mean peak concentration (C_{max}) and time of its occurrence (t_{max}) was obtained from inspection of the back-transformed LSM concentration.

Results

All animals remained in good health throughout the acclimatization period. Two animals in group 2 were euthanized 3 h after dosing with LPS. Animal 283 (from T04) had severe respiratory distress, and animal 278 (from T02) was moribund. These animals were not replaced. Consequently, the final number of animals in this group was 34. One animal in group

1 (242 from T06) was affected with bronchopneumonia, and no lung samples were taken from that animal. Animal 233 (from T02) had attitude score of 1 at the time of LPS administration. The rest of the pigs remained in good health throughout the study as pre-defined in the IACUC approved protocol. No adverse reactions or detrimental health effects were observed following the administration of tulathromycin. Macroscopic lesions in the middle lobe of the group 2 animals were seen in the first 72 h after tulathromycin administration (Figure 5.1)

Animals in group 1 received an average tulathromycin dose of 2.63 mg /kg while animals in group 2 received an average dose of 2.64 mg/kg.

Health assessment

Overall, the health assessment indicates that before starting the study all the animals were considered clinically normal. At the time of tulathromycin administration (3 hours post-LPS-Ec administration) all the animals were clinically sick. At six hours after the LPS administration two animals were severely affected, therefore they were euthanized as pre-established in the protocols.

Attitude

At the time of tulathromycin administration only 2/36 animals had a score of 0. The rest of the animals showed mild (13/36), moderate (19/36) and severe (2/36) changes. By six hours after the administration of LPS-Ec (three hours after receiving the tulathromycin dose), all animals were affected. Some were affected mildly (8/34) however most were moderately affected (26/34). The animals recovered quickly, with over 80% receiving normal scores at nine hours after receiving post LPS challenge (6 hours post-tulathromycin) and 100% of animals were considered normal at all observations after 24h post-LPS.

General condition

Three hours after receiving the LPS challenge (at the time of tulathromycin administration), 25 out of 36 had mild changes in the general condition score while 2 out of 36 had and severe changes. The rest of the animals (25/36) were still considered to be generally healthy. Six hours after LPS-*Ec* administration 6 out of 36 animals were mildly affected. All animals were normal at ≥ 27 h after receiving the LPS-*Ec* dose.

Respiratory distress

Within one hour of LPS challenge, over 50% of the animals had mild or moderate clinical signs of respiratory disease. Three hours after LPS challenge (at the time of tulathromycin administration) 2 out of 36 animals had a score of 0. The rest of the animals showed mild (17/36), moderate (16/36) and severe (1/36) changes. Six hours after LPS challenge (three hours after tulathromycin administration), animals had mild (5/34), moderate (11/34) and severe (18/34) respiratory distress. By 9 h after dosing LPS-*Ec*, >75% of the animals were clinically normal with the remainder having only mild respiratory distress. All subsequent time points showed all animals having normal respiration.

Health assessment scores are presented in Figure 5.2.

Plasma

Tulathromycin was rapidly absorbed in both groups. In group 1, tulathromycin plasma $C_{\max}$ was 630 ± 181 ng/mL (ranged from 272 to 1200 ng/mL) and occurred at 0.5 h ($t_{\max}$). In group 2, the plasma concentration vs. time (CT) profile was consistent with that of group 1 (AUC_{0-408} 5765 and 5542 for group 1 and 2, respectively). The plasma $C_{\max}$ was 286 ± 33 ng/mL (ranged from 162 to 363 ng/mL) and occurred at 1 h ($t_{\max}$) post administration of tulathromycin.

In this group, the drug concentration range at 0.5 h was from 213 to 1190. The apparent $t_{1/2}$ was 57.36 h.

The individual plasma concentrations of tulathromycin are included in Table 5.4 and shown in Figure 5.3 and 5.4. Pharmacokinetic parameters are included in Table 5.5.

Lung homogenate

Tulathromycin rapidly and extensively distributed into the lung tissue (Table 5.6). In group 1, the tulathromycin mean concentration (observed) in the middle lobe reached a maximum of 2590 (ng/g) at 72 h post-dose, while in group 2 the $C_{\max}$ was 2340 (ng/g) at 24 h post-dose (Figure 5.3 and 5.4). In the caudal lobe, the $C_{\max}$ for tulathromycin was 2590 ng/g at 24 h post-dose for group 1, while pigs from group 2 had a $C_{\max}$ of 2010 ng/g at 72 h post-dose. Overall, there were no statistically significant differences in caudal lobe concentrations between the two dose groups ($p=0.07$) (Figure 5.3 and 5.4) but there were statistical differences at 24 ($p<0.02$) and 72 h ($p=0.04$). The partial AUC_{0-144} was 305000 and 254000 for group 1 and 2, respectively. For the middle lobe, the concentration time profile was different ($p<0.02$) between both dose groups, in particular at 6 h ($p=0.03$) and 72 h ($p<0.01$) post-tulathromycin administration. The partial AUC_{0-144} was 300000 and 247000 for dose group 1 and 2, respectively. There was not an association between the severity of the clinical signs and the drug concentration in lung tissue. Tulathromycin was slowly eliminated from lung tissue and persisted for a long period of time in all lobes from both experimental groups. At 408 h post-tulathromycin administration, the lung:plasma ratio was > 300 for both lung lobes from both groups. A summary of pharmacokinetic parameters is presented in Table 5.7.

Wet to dry ratios for lung tissue

Wet and dry ratio weights were determined for both lobes. Summarized data for the wet to dry ratios are listed in Table 5.8. There were no statistical differences in the wet-to-dry ratios in lung tissues between the two groups and lobes ($p>0.05$). No apparent trend could be

observed over time. There was also no linear relationship ($p>0.05$) between LS mean concentration and the wet-to-dry ratio (Figure 5.5 and 5.6).

Discussion

The CT profile of tulathromycin in LT and plasma was evaluated in both groups and pharmacokinetic parameters were estimated in both matrices. The intra-tracheal administration of LPS-*Ec*. triggers an acute inflammatory response. The LPS-*Ec*. model implemented in the present study has been fully described elsewhere (Chapter 3). The intra-tracheal administration of LPS-*Ec*. causes acute and reversible macroscopic and microscopic changes with remarkable abnormal clinical signs. In the present study, consistent macroscopic lesions were observed in both middle lobes within 72 h post-tulathromycin administration (75 h post-LPS-*Ec*). At the last 3 sampling times, no macroscopic lesions were observed in the post-LPS-*Ec*-challenged animals. All animals received normal health assessment scores prior to the start of the study (except for one animal (233) at -3 h) (Figure 5.2). Changes in clinical signs became evident 3 h after the administration of the LPS- *Ec* challenge. Clinical scores and progression of clinical signs were consistent with those reported previously (Chapter 3). Within 3 h after LPS-*Ec*. administration, 34 of the 36 animals were at least mildly affected, with 2 animals receiving an attitude score of 3. Those two animals were euthanatized. In the present study, maximal score changes were recorded within 9 h post-LPS-*Ec*. administration. After that, the clinical score returned to normal. Tulathromycin was administered to all animals in group 2, 3 h after the administration of the challenge. The time of drug administration was selected because the manifestation of respiratory distress might represent a deciding factor to initiate treatment under field conditions.

The time course of tulathromycin has been characterized in LT and plasma of pigs from 12 to 360 h and 0.5 to 360 h post-dose, respectively (5, 19). In the present study, the sampling protocol was extended up to 408 h post-treatment administration. In addition, an earlier sample time was included at 6 h post-tulathromycin administration. Another relevant difference in the

experimental design compared to previous studies by Benchaoui et al. (2004) (5) and Nowakowski et al. (2004) (19) is that in the present study, two independent lung samples—one from the middle and another from the caudal lobe—were taken for quantification of tulathromycin. In previous studies, tulathromycin was quantified from a single representative lung tissue sample.

Consistent with results published by Benchaoui et al. (2004) (5), tulathromycin was rapidly absorbed ($t_{\max}$ 0.5 h) and had a prolonged elimination $t_{1/2}$. In group 2, a plasma CT profile consistent with that of group 1 was obtained (Table 5.5 and Figures 5.3 and 5.4). This indicates that the intra-tracheal administration of LPS-*Ec* did not impact on the CT profile of the drug in plasma.

Tulathromycin distributed extensively and rapidly into the lung, as previously reported in pigs (5, 19), cattle (8, 19) and foals (27). In healthy animals (group 1), the LS mean CT profile in both lung lobes was consistent (Table 5.6 and Figures 5.3 and 5.4). A similar observed LS mean $C_{\max}$ was observed at 24 h and 72 h post-administration for middle and caudal lobes, respectively. In both lung lobes, the drug exposure was about similar. Tulathromycin was slowly eliminated from both lung lobes with an apparent $t_{1/2}$ of around 5.5 days (Table 5.7)

In the LPS-treated animal (group 2) there was a statistically significant change of the CT in lung samples from the middle and caudal lobe of LPS-treated animals. In ML, lower drug concentrations were obtained at 6 h ($p=0.03$) and 72 h ($p<0.01$). For the caudal lobe there were statistical differences at 24 ($p<0.02$) and 72 h ($p=0.04$) post-tulathromycin administration. Changes in the CT profile in lung tissue of LPS-treated animals are reflected in the pharmacokinetic parameters, mainly in the AUC_{0-144} . The $LT_{\text{group1}}: LT_{\text{group2}}$ ratio was 1.20 and 1.21 for the middle and caudal lobes, respectively. Despite the lower concentrations obtained at some sampling times in LT of LPS-challenged animals, the overall exposure to tulathromycin in both lung lobes was relatively high ($AUC_{0-408} / AUC_{0-408 \text{ plasma}}$ was > 78 fold). Also, high drug concentrations persisted for a long period of time, with the LS mean concentration in LT >300

times the plasma concentration 17 days after drug administration. The clinical significance of changes in the CT profile and the pharmacokinetics parameters in LPS-challenged animals is unknown.

Reports about drug concentration in pneumonic tissue are inconsistent. Concentrations of danofloxacin in pneumonic craneo-ventral tissue were lower than those reported in normal caudo-dorsal tissue (2). On the other hand, higher concentrations of oxytetracycline in pneumonic than in normal calves have also been published (2). Many factors may determine the rate and extent of drug accumulation during an inflammatory process, including the (i) kinetics of the inflammatory cells in a particular tissue, (ii) chronic vs. acute inflammatory response (3, 12), (iii) site of infection (25), and (iv) type of drug (7, 17). The reason (s) for changes in the tulathromycin CT profile and PK parameters in LT from the LPS-challenged animals are unknown. Changes of the CT profile in LT cannot be attributed to changes in the plasma CT profile (Figures 5.3 and 5.4). The systemic response to the LPS-*Ec* challenge may trigger a hyper-dynamic state and local vascular changes, which may modify the disposition of drugs within tissues (16). Despite the fact that this phenomenon might represent a potential reason to explain the results obtained, this was not evaluated. Edema, as a consequence of the inflammatory response, might have diluted the drug. This could also explain the lower concentrations of the drug during the period of maximal tissue changes. Nevertheless, the wet-to-dry ratio results do not support such hypothesis (Figures 5.5 and 5.6). Interestingly, there were no statistical differences in the LS mean concentration value in pulmonary epithelial lining fluid (PELF) between groups (Chapter 6).

It was reported that there is a considerable uptake and accumulation of tulthromycin in neutrophils and macrophages (8, 24). There is published evidence indicating that some classes of antimicrobials accumulate in inflammatory cells. The exposure to antigens cause the accumulation of loaded cells at the site of infection/inflammation and triggers a rapid release of intracellular drug after migration of the cell (3, 4, 6, 9, 10, 13, 14, 17, 21, 25). The inflammatory

model used in the present study induces a remarkable influx of neutrophils and macrophages into the middle lobe of the lung (Chapter 3). Consequently, it was expected that the significant influx of neutrophils into the middle lobe would lead to a significantly larger local drug accumulation. However, this was not reflected in the lung homogenate LS mean concentration results. It could be possible that a more prolonged inflammatory response and/or multiple drug administration might be required to detect a positive relationship between cellular infiltration and the local drug accumulation as was reported for azithromycin (3). No significant differences were detected between lung concentrations measured in infected and non-infected mice when the dose was given at 48 or 72 h after delivery of the challenge or sterile broth. In contrast, a 4- to 7-fold increase in lung concentration was observed in lungs taken from infected animals dosed at 96 or 120 h after challenge (3).

In conclusion, in both healthy pigs and LPS-challenged animals, the distribution of the drug into the lungs was rapid and persisted at relatively high levels for 17 days post-administration. The distribution of the drug within the lung seems to be homogenous, at least between the middle and caudal lobes within dosing groups. The LS mean CT profile of the drug and pharmacokinetic parameters in 2 different lung areas (middle and caudal lobe) were consistent within the groups. The concentration of tulathromycin was significantly lower in the middle lobe after the intratracheal administration of LPS-*E. coli*. The clinical significance of the change in the CT profile is unknown.

Two additional pieces of information certainly may help to fully unravel the unique pharmacokinetic profile of tulathromycin in lung tissue, namely (i) the evaluation of the impact of chronic inflammation on the pulmonary pharmacokinetics of the drug, and (ii) the influence of the time of drug administration with respect to the pathological stage on the CT profile of the drug in lung tissue.

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Conflict of Interest

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Appendix

Table 5.1. Clinical score assessment used for evaluation of animals challenged intra-tracheally with LPS-*Ec*.

Observation	Score	Clinical Description
Attitude (depression)	0	Normal – Pig is up and active when entering the room or when approaching.
	1	Mild depression – Pig is slightly inactive, but gets up after being approached; pig moves slowly and appears lethargic; shows some interest in food and social interaction, but stays active only for a few minutes; animal may appear gaunt and have a rough coat.
	2	Moderate depression – Pig shows pronounced inactivity and gets up only after light back pressure; displays little or no interest in food and/or social interaction; tends to lie down almost immediately and/or stands with head down; noticeably gaunt and coat is rough; staggers and appears slightly uncoordinated at the walk.
	3	Severe depression/moribund – Pig is moribund, unresponsive to stimuli, unable to get up or does so with difficulty; shows no interest in surroundings, food, or social interaction; appears anxious; should be euthanized for humane reasons.
Respiratory Distress	0	None – Respiratory movements are subtle, superficial, and can be observed only when animal is quietly lying down.
	1	Mild – Respiratory movements are obvious and predominantly abdominal.
	2	Moderate – Respiratory movements are exaggerated, entirely abdominal, and labored.
	3	Severe – Respiration is very labored and entirely abdominal; pig breaths through mouth (continuously or intermittently); nose and/or ears are cyanotic; should be euthanized for humane reasons.
General Condition (includes appetite evaluation)	0	Clinically normal appearing animal
	1	Slightly abnormal condition
	2	Moderately abnormal condition
	3	Severely abnormal condition

Table 5.2. Sampling (tissue) and treatment design implemented for healthy pigs and animals challenged intra-tracheally with LPS-*Ec* 3 h prior to tulathromycin administration.

Days	-1	0					1	2	3	4–5	6	7–9	10	11–16	17
h	NA	-3	-2	0	3	6	24	48	72	96–120	144	168–216	240	264–384	408
T01	X	LPS	X	XT	X	XN									
T02	X	LPS	X	XT	X	X	XN								
T03	X	LPS	X	XT	X	X	X	X	XN						
T04	X	LPS	X	XT	X	X	X	X	X	X	XN				
T05	X	LPS	X	XT	X	X	X	X	X	X	X	X	XN		
T06	X	LPS	X	XT	X	X	X	X	X	X	X	X	X	X	XN

X–Clinical observation taken (evaluations from 96 h were performed daily); T–tulathromycin administration; LPS:LPS-*Ec* dose administration; N–Necropsy performed. For animals in dose group 1, evaluations were performed as with dose group 2 but starting at 0 h.

Table 5.3. Blood sampling design for pigs administered a single i.m. injection of tulathromycin at 2.5 mg/kg body weight.

Treatment group	T01	T02	T03	T04	T05	T06
	05	4	32	80	169	264
Times blood	1	8	48	96	192	312
sampled (h)	2	10	56	120	216	360
	6	24	72	144	240	408

Table 5.4. Summary of concentration (ng/mL) of tulathromycin in plasma from pigs treated with a single i.m. injection of tulathromycin at 2.5 mg/kg body weight.

Dose Group	Treatment Group (h)	n	h	Raw Mean	95% CIs	Range
1	T01 (6)	6	0.5	630	267-992	272–1200
			1	275	192-358	187–448
			2	203	161-244	153–273
			4	149	128-170	161–426
	T02 (24)	6	6	266	173-360	114–187
			8	149	121-176	115–210
			10	115	90.0-140	88.3–170
			24	61.9	45.2-78.6	42.5–99.3
	T03 (72)	6	32	37.6	30.9-44.3	29.6–48.1
			48	25.4	19.6-31.2	18.3–36.5
			56	22.6	17.7-27.5	19.2–34.6
			72	22.5	20.8-24.2	19.8–24.7
	T04 (144)	6	80	9.10	8.17-10.0	7.74–11.1
			96	10.8	9.77-11.8	9.16–12.6
			120	6.99	5.18-8.81	5.46–11.3
			144	6.25	5.13-7.38	4.47–8.48
	T05 (240)	6	168	3.65	2.70-4.60	2.29–5.75
			192	2.74	2.12-3.36	1.52–3.53
			216	2.14	1.53-2.74	1.16–2.99
			240	1.82	1.33-2.30	1.11–2.65
	T06 (408)	6	264	1.63	1.28-1.99	1.13–2.49
			312	1.74	1.39-2.09	1.04–2.53
			360	1.21	1.07-1.35	1–1.79
			408	1.36	0.90-1.82	0.8–2.27
2	T01 (6)	6	0.5	269	851-529	213–1190
			1	286	219-352	162–363
			2	212	137-288	117–375
			4	204	89.7-317	80.6–436
	T02 (24)	5	6	247	126-367	131–460
			8	85	64.0-106	49.1–108
			10	87.7	72.3-103	58–100
			24	86.8	67.8-106	61.4–109
	T03 (72)	6	32	35.6	30.1-41.2	23.4–42.6
			48	25.7	21.4-30.0	20.1–33.9
			56	17.5	15.7-19.3	14.5–20.2
			72	19.8	16.9-22.7	14.1–23.6

Table 5.4. Continued

Dose Group	Treatment Group (h)	n	h	Raw Mean	95% CIs	Range
	T04 (144)	5	80	11.2	8.06-14.2	7.95–16.3
			96	9.65	8.41-10.9	8.12–11.2
			120	6.70	5.49-7.91	4.96–8
			144	7.67	6.61-8.72	5.82–8.8
	T05 (240)	6	168	3.17	2.65-3.68	2.44–3.97
			192	2.47	2.01-2.93	1.9 –.23
			216	2.41	2.10-2.72	1.92–2.86
			240	2.19	1.77-2.61	1.51–2.75

Table 5.5. Summary of plasma pharmacokinetic parameters in pigs treated with a single i.m. injection of tulathromycin at 2.5 mg/kg body weight.

Pharmacokinetic parameter*	Treatment group	
	Group 1	Group 2
HL_{Lambda_z} (h)	54.4	57.3
T_{max} (h)	0.50	0.50
C_{max} (ng/mL)	630	286
C_{last} (ng/mL)	1.31	1.32
AUC_{last-pred} (h*ng/mL)	5760	5540
AUC_{inf ~obs} (h*ng/mL)	5780	5560
AUMC_{last} (h*h*ng/mL)	288000	282000
MRT_{INF_pred} (h)	51.4	52.5

* Dispersion data of the PK parameters are not presented because PK parameters were estimated based means derived from the naïve pooling from each sample point.

Table 5.6. Summary of the LS mean concentration (ng/g) of tulathromycin in LT from pigs treated with a single i.m. injection of tulathromycin at 2.5 mg/kg body weight.

Lung Section	Treatment (h)	Dose Group	n	LS Mean [†]	95% CIs [†]	Range [#]
Middle Lobe	T01 (6)	1	6	1660	1370-1950	1370–2530
		2	6	1200	992-1410	971–1840
	T02 (24)	1	6	2380	1960-2790	1980–3480
		2	5	2340	1900-2790	1740–2850
	T03 (72)	1	6	2590	2140-3040	1880–3330
		2	6	1750	1450-2060	1320–2360
	T04 (144)	1	6	1260	1040-1480	974–1650
		2	5	1380	1120-1640	1160–1830
	T05 (240)	1	6	884	730-1040	765–1000
		2	6	828	670-986	665–961
	T06 (408)	1	5	357	289-426	237–431
		2	6	330	273-388	215–469
Caudal Lobe	T01 (6)	1	6	1740	1470-2010	1390–2270
		2	6	1740	1470-2000	1140–2400
	T02 (24)	1	6	2590	2190-2990	2170–3570
		2	5	1670	1380-1950	1040–1990
	T03 (72)	1	6	2530	2140-2930	1950–2960
		2	6	2010	1700-2330	1660–2200
	T04 (144)	1	6	1310	1110-1510	1040–1610
		2	5	1580	1320-1860	1290–2090
	T05 (240)	1	6	735	621-849	491–890
		2	6	837	707-967	745–1040
	T06 (408)	1	5	464	385-543	399–552
		2	6	388	327-448	307–588

[†] Back-transformed data for reporting, [#] correspond to raw drug concentrations

Table 5.7. Summary of the pharmacokinetic parameters of tulathromycin in LT in pigs treated with a single i.m. injection of tulathromycin at 2.5 mg/kg body weight.

Pharmacokinetic parameter*	Treatment Group			
	Group 1		Group 2	
	Middle lobe	Caudal lobe	Middle lobe	Caudal lobe
HL_{Lambda_z} (h)	143	142	137	137
T_{max} (h)	72.0	24.0	24.0	72.0
C_{max} (ng/mL)	2590	2590	2340	2010
C_{last} (ng/mL)	357	464	330	388
AUC_{last}(h*ng/mL)	506000	504000	450000	473000
AUC_{inf_obs} (h*ng/mL)	580000	6000000	515000	550000
AUMC_{last}(h*hr*ug/mL)	68600000	68200000	63800000	69100000
MRT_{INF_pred} (h)	197	203	202	209
AUC₀₋₄₀₈ LT:Plasma ratio	87.4	90.9	78.1	85.3

*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 5.8. Wet-to-dry weight ratios of lung tissue from healthy pigs and animals challenged intra-tracheally with LPS-*Ec* 3 h prior to tulathromycin administration.

Lobe	Treatment (h)	Group	Mean	SD	%CV
Middle	T01 (6)	1	5.77	0.28	5.00
		2	5.58	0.11	2.06
	T02 (24)	1	5.64	1.63	28.9
		2	5.77	0.07	1.35
	T03 (72)	1	6.27	0.80	12.8
		2	5.89	0.35	6.01
	T04 (144)	1	6.31	1.07	16.9
		2	5.65	0.21	3.88
	T05 (240)	1	5.68	0.05	1.00
		2	5.75	0.12	2.11
	T06 (408)	1	5.91	1.02	17.2
		2	5.49	0.70	12.9
Caudal	T01 (6)	1	5.63	0.30	5.33
		2	5.59	0.10	1.89
	T02 (24)	1	6.34	1.56	24.6
		2	5.66	0.20	3.53
	T03 (72)	1	5.55	0.11	2.04
		2	5.78	0.14	2.43
	T04 (144)	1	5.53	0.14	2.57
		2	5.45	0.08	1.60
	T05 (240)	1	5.37	0.09	1.68
		2	5.44	0.09	1.66
	T06 (408)	1	5.59	0.58	10.5
		2	5.54	0.24	4.34
No statistical differences were found between lobes					

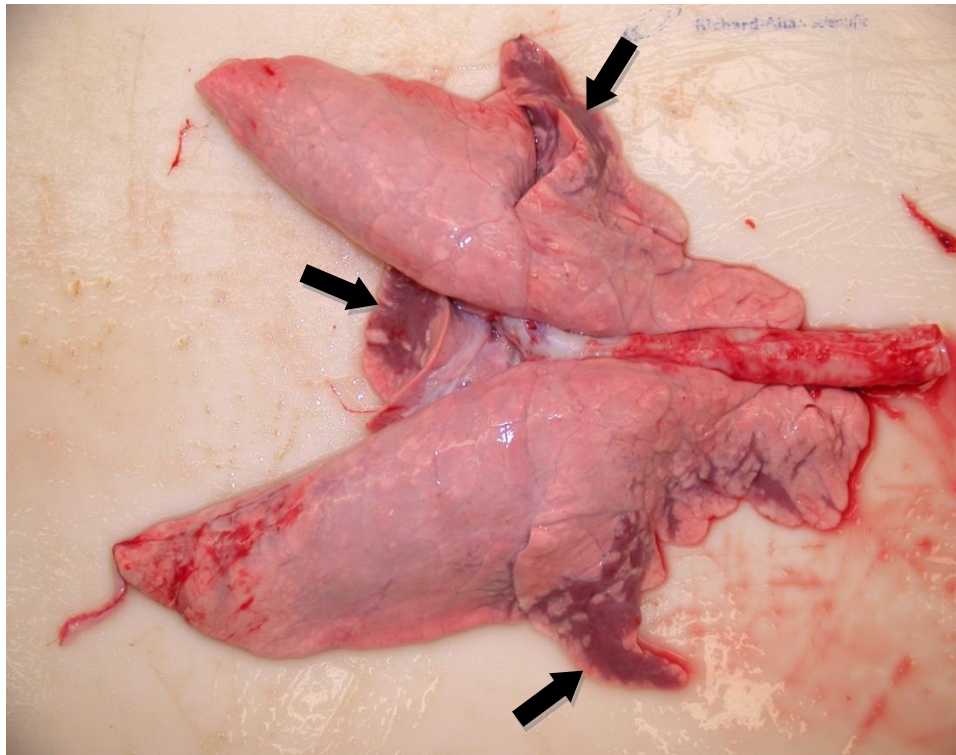


Figure 5-1 Lung gross changes induced by intra-tracheal administration of LPS *Ec.* at 24h post-challenge (arrows point at abnormal gross areas).

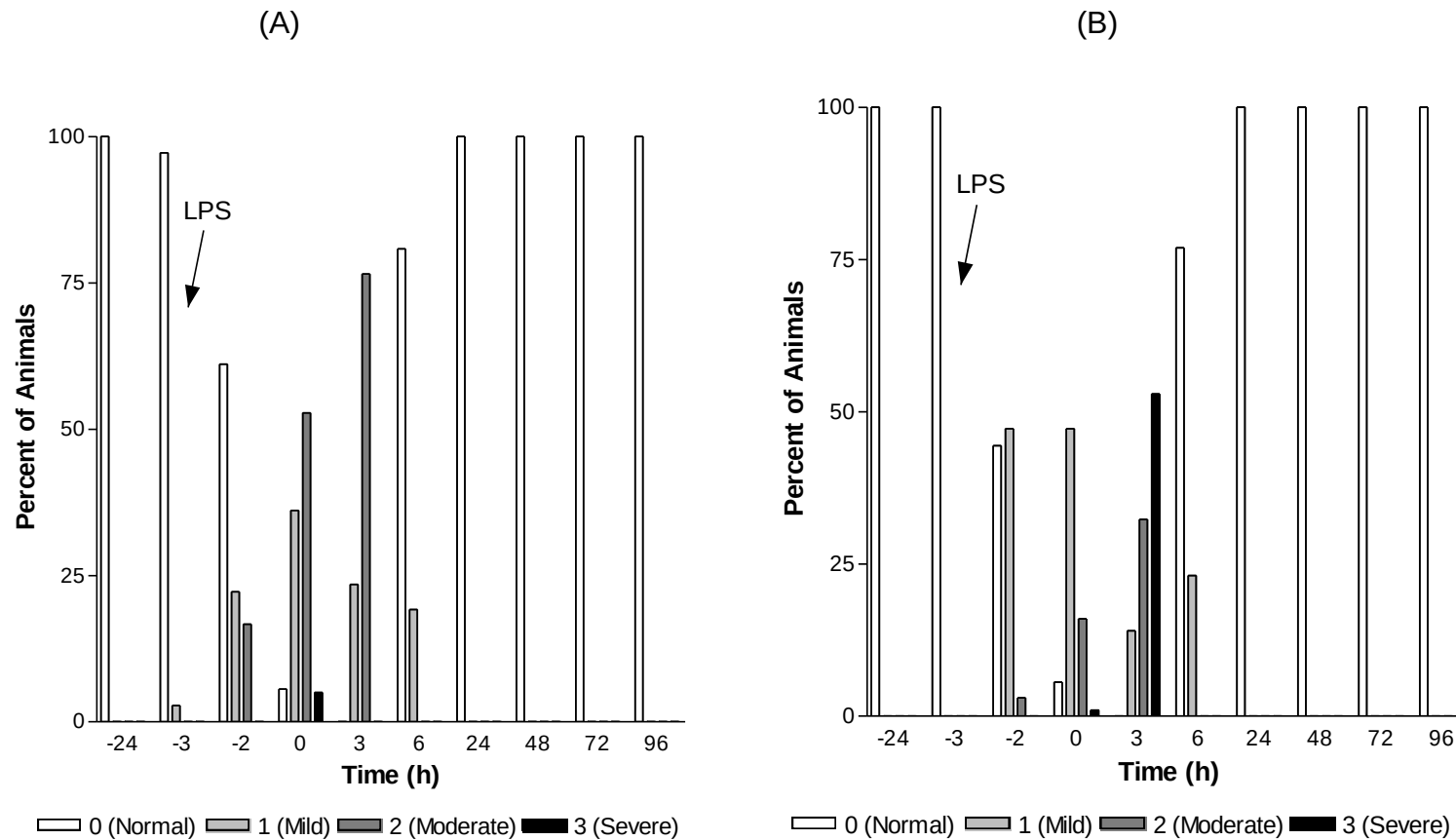


Figure 5.2. Clinical score assessment results from pigs challenged intra-tracheally with LPS-*Ec*. (A) Attitude score results. (B) Respiratory distress score results. For both scores, results beyond 96 h were not included because all animals had a normal score. Clinical assessment scores for general health are not graphically presented. For these scores, mild changes were recorded in ~25% of animals, only between -3 and 6 h post LPS.

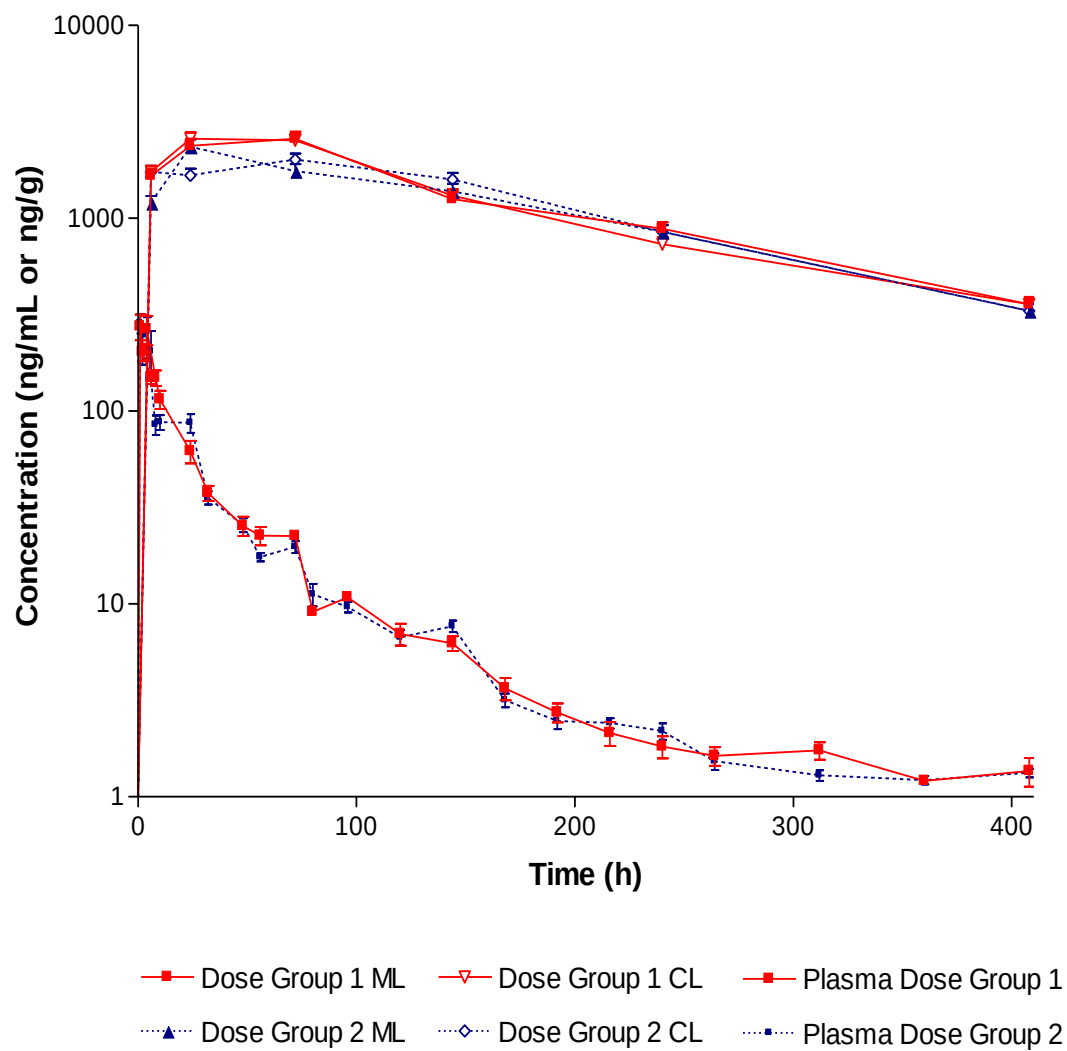


Figure 5.3. Least squares mean concentration ($\pm$ SE) of tulathromycin in LT (ng/g) and mean concentration ($\pm$ SE) in plasma (ng/mL) from pigs administered a single i.m. injection of tulathromycin at 2.5 mg/kg body weight. Overall, there were statistical differences between middle lobe drug concentrations from both dose groups ($p < 0.02$) but not for caudal lobe ($p = 0.07$).

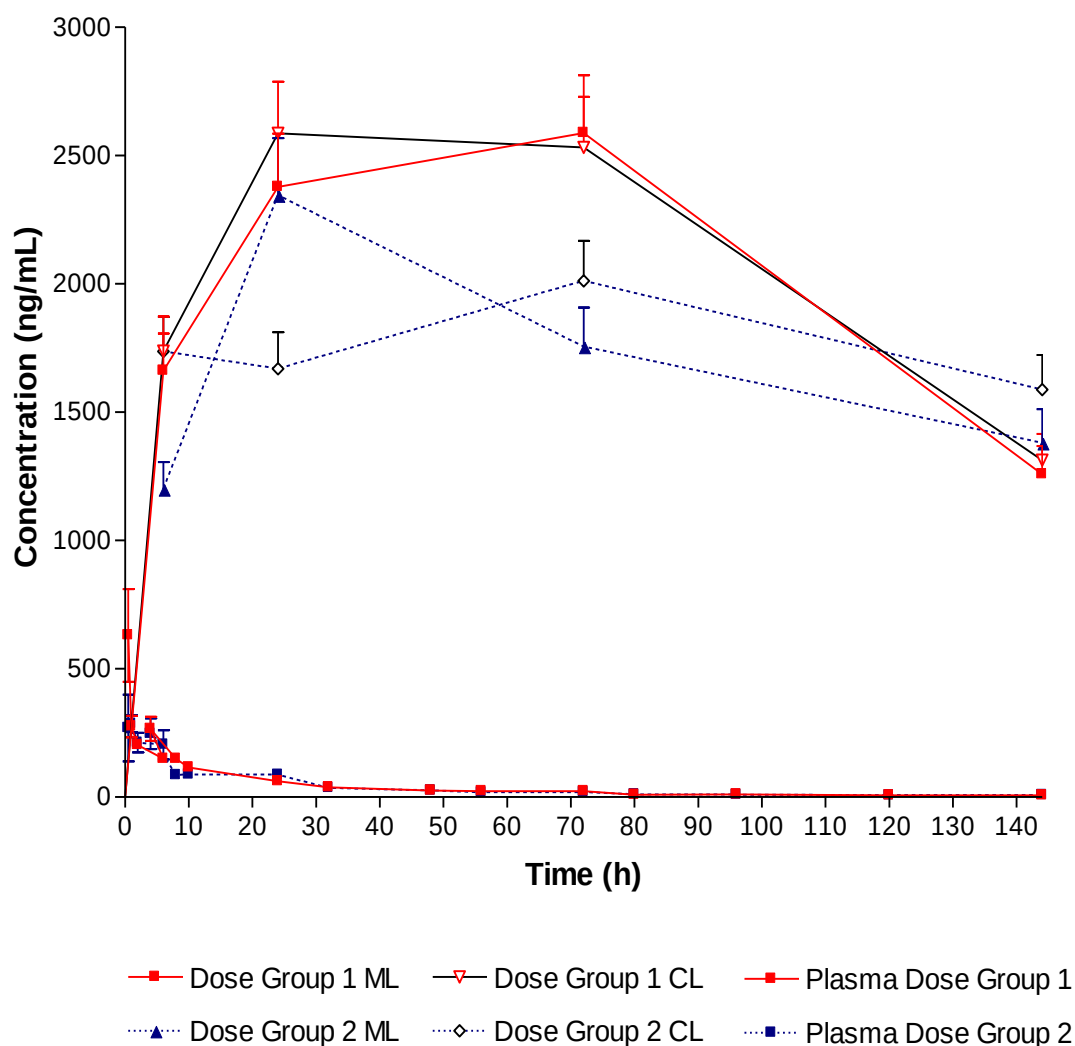


Figure 5.4. Least squares mean concentration ($\pm$ SE) of tulathromycin in LT (ng/g) and mean concentration ($\pm$ SE) in plasma (ng/mL) from pigs administered a single i.m. injection of tulathromycin at 2.5 mg/kg body weight. For clarity, the small graph depicts tulathromycin concentration 0–72 h post administration. ML: correspond to middle lobe and CL, caudal lobe. For middle lobe there were statistical differences at 6 h ($p=0.03$) and 72 h ($p<0.01$). For caudal lobe there are statistical differences at 24 ($p<0.02$) and 72 h ($p=0.04$).

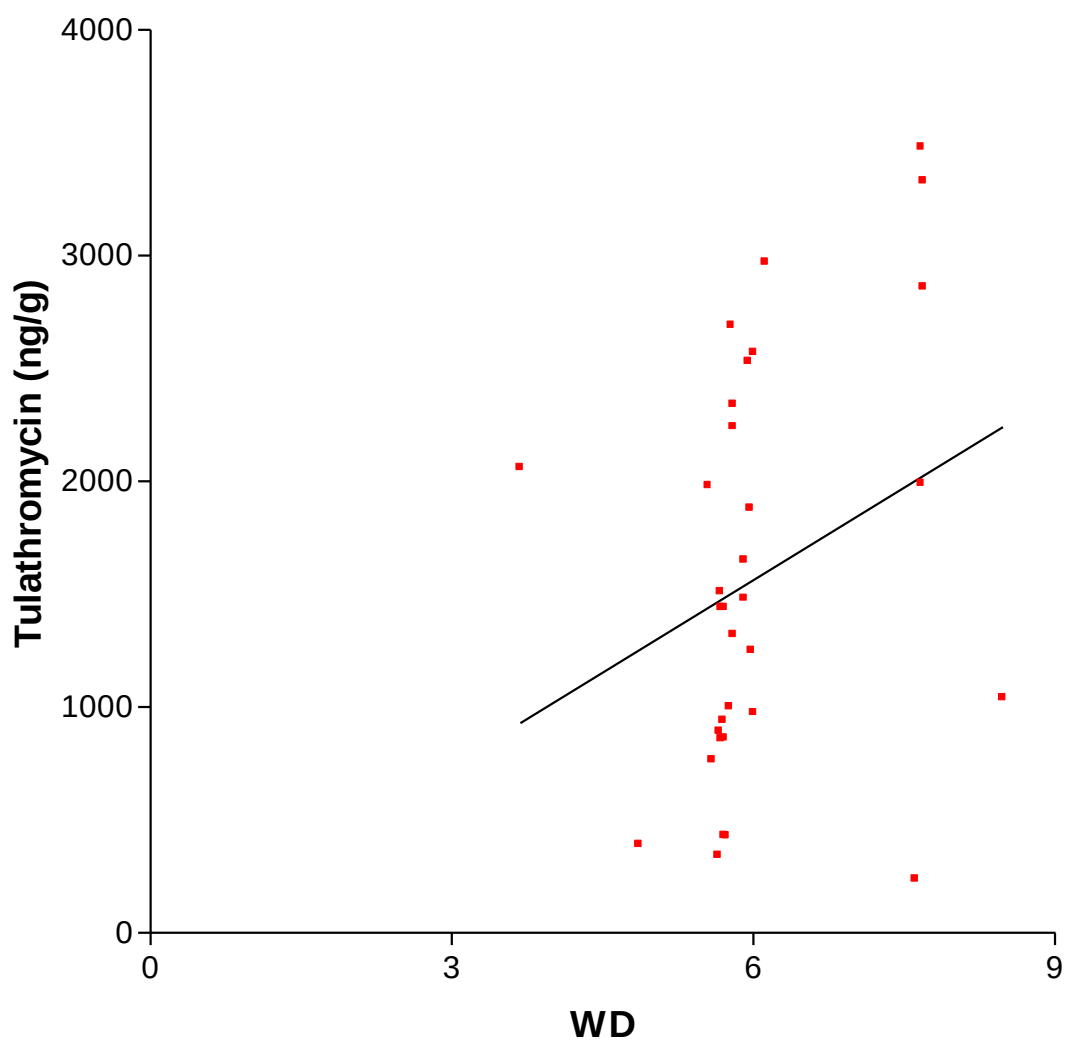


Figure 5.5. Linear regression concentration of tulathromycin in lung tissue vs. wet to dry ratio for middle lobe ($R^2 = 0.08$).

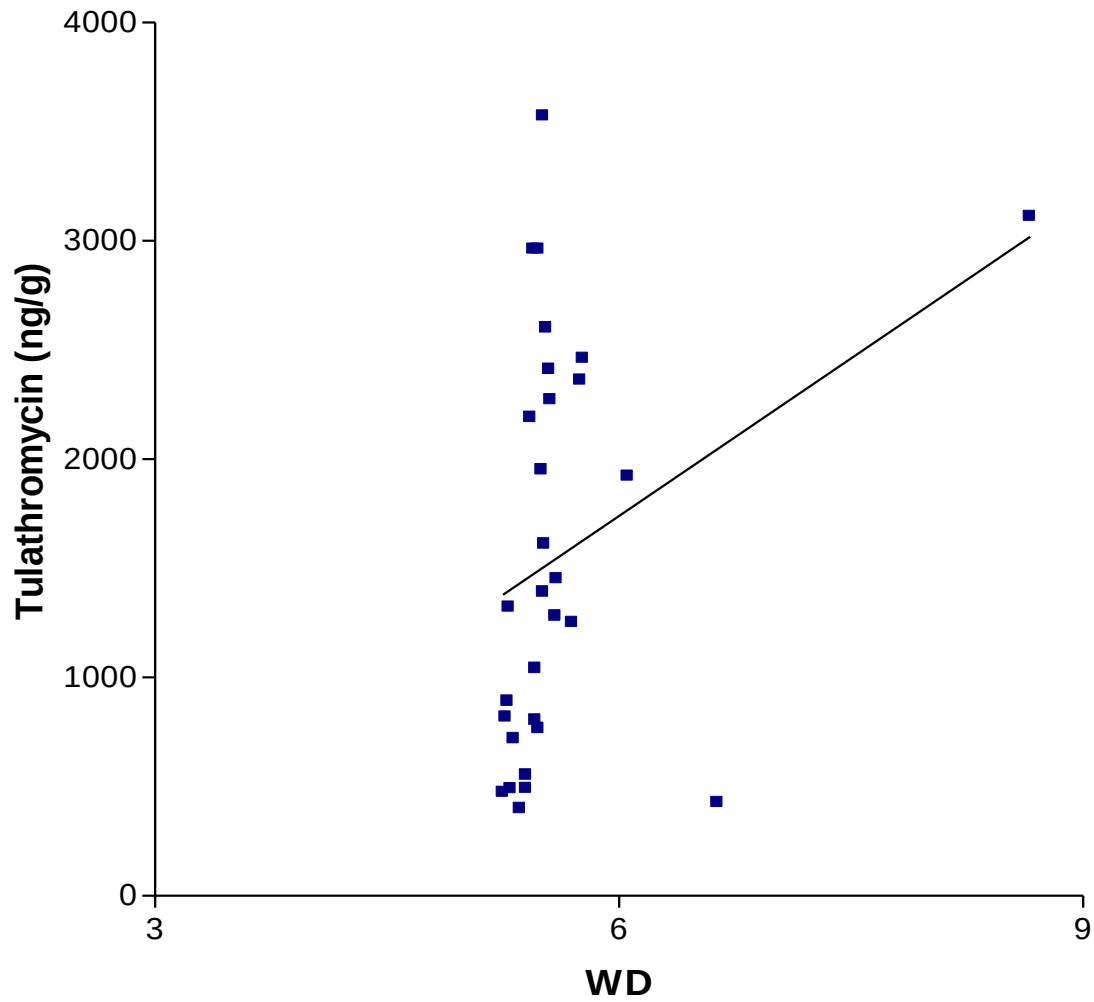


Figure 5-6. Linear regression concentration of tulathromycin in lung tissue vs. wet to dry ratio for caudal lobe ($R^2= 0.10$).

Chapter 6:

PULMONARY PHARMACOKINETICS OF TULATHROMYCIN IN SWINE: INTRA-AIRWAYS COMPARTMENTS

Abstract

Knowledge of drug concentration in the intra-airway compartments can provide valuable information for treatment. The objective of this study was to assess the pharmacokinetics of tulathromycin in pulmonary and bronchial epithelial lining fluid (PELF and BELF) from pigs. Clinically healthy Duroc x Landrace pigs were allocated to two groups of 36 animals each. All animals were treated with tulathromycin (2.5 mg/kg). Animals in group 2 were also challenged intra-tracheally with lipopolysaccharide from *Escherichia coli* 3 h prior to tulathromycin administration. Both PELF and BELF were harvested using bronchoalveolar lavage fluid and bronchial microsampling probes, respectively. Samples were taken for 17 days post-tulathromycin administration. No statistical differences in the concentration of tulathromycin were observed in PELF between groups. The concentration vs. time profile in BELF was evaluated only in Group 1. Tulathromycin distributed rapidly and extensively into the airway compartments of both groups. Area under the concentration-time curve through 408 h post-dose was 522000, 348000, and 1290000 for PELF_{Group-1}, PELF_{Group-2}, and BELF_{Group-1}, respectively. The PELF:plasma concentration ratio ranged from 29.6 to 271 and 6.39 to 194 for Group 1 and 2, respectively. The BELF:plasma concentration ratio range was 2.69–1615 for Group 1. The drug was slowly eliminated from PELF and BELF, with apparent terminal elimination half-life values of 87.3, 108, and 203 h for PELF_{Group-1}, PELF_{Group-2}, and BELF, respectively. This is the first study describing the disposition of tulathromycin in both BELF and PELF in pigs. Tulathromycin not only distributed rapidly into intra-airway compartments at relatively high concentrations but also resided in the airway lining fluid for a long time (>4 days).

Keywords: tulathromycin, antimicrobial, pig, bronchial epithelial lining fluid, lung

Introduction

Drug concentrations at the site of infection are clinically relevant (15), as it is broadly accepted that biophase drug kinetics is the primary controller in the antimicrobial response (15-17, 37). Tulathromycin is a macrolide antimicrobial that has shown an extraordinary efficacy to treat and prevent bovine and swine respiratory disease when used following labeled indications. The efficacy of this drug to treat the labeled conditions may be a consequence of an extensive, rapid, and prolonged accumulation of the drug in the lung tissue. Previous studies characterized the pharmacokinetics of tulathromycin using lung homogenate from pigs and cattle (4, 42). However, little is known about the accumulation of this drug in the different pulmonary compartments. In fact, there is no information about the intra-airway concentration vs. time profile of tulathromycin in pigs. Recently, Cox *et al.* (2010) described the concentration vs. time profile of tulathromycin in not only lung homogenate but also pulmonary epithelial lining fluid (PELF) and bronchoalveolar lavage (BAL) cells. Consistent with results observed in lung homogenate samples, tulathromycin also extensively accumulated in the intra-airway compartments both intra-and extra-cellularly.

The intra-airway compartment represents the primary niche for bacterial contamination and infection (14, 18). Consequently, information about target site drug penetration and kinetics in the airway epithelial lining fluid would provide valuable evidence to aid in understanding the lung accumulation and positive clinical outcomes of tulathromycin. Due to the anatomical location of lungs, its conformation, and function, veterinary scientists face a unique challenge in studying drug-lung pharmacokinetics. This may explain why most lung pharmacokinetic studies are based only on lung homogenate concentrations rather than in airways and alveolar surface fluid.

The objective of this study was to assess the pharmacokinetics of tulathromycin in the intra-airway compartments in healthy pigs and pigs challenged intra-tracheally with lipopolysaccharide *Escherichia coli* (LPS-Ec) during a period between 0 to 408 h.

Material and methods

Animals and housing

Animals and housing conditions were fully described in part 1 of the study (chapter 5).

Study design

The full study design was fully described in part 1 of the study (chapter 5). Briefly, 72 healthy animals were randomly allocated into six treatment groups with six experimental animals per treatment group according to a split plot design. Each group was scheduled for euthanasia at 6, 24, 72, 144, 240, or 408 h post-tulathromycin administration. Of the 72 experimental animals, 36 were treated with an intra-tracheal inoculum of LPS-Ec 3 h prior to the administration of tulathromycin. On study day 0, all animals were administered one i.m. injection of tulathromycin (Draxxin[®] Injectable Solution, Pfizer Animal Health, New York, NY) at 2.5 mg/kg in the neck.

Bronchial epithelial lining fluid and pulmonary epithelial lining fluid

Bronchial epithelial lining fluid.

Bronchial epithelial lining fluid (BELF) samples were taken from all animals in group 1 and animals scheduled to be euthanized at 24 h post-tulathromycin administration in group 2. Bronchial epithelial lining fluid was gathered from each animal using bronchial micro-sampling (BMS) probes (Model BC-402C Olympus, Tokyo, Japan) as described previously (29, 32, 33, 60). Briefly, immediately after euthanasia, the trachea was exposed and a flexible fiber-optic endoscope (Olympus, model XP-10) and a BMS probe were inserted into the trachea and

directed into the right middle lobe following the bronchi until resistance was met. The sampling portion of the BMS probe was uncovered from the protective sheath and was visually placed in contact with the bronchus wall for approximately 20 sec. The sample portion of the probe was pulled back into the sheath before removing the BMS probe from the bronchoscope. The cotton tip (sample portion) was trimmed off from the rest of the sampling device, collected into a polypropylene tube, transported on wet ice ($\sim 0^{\circ}\text{C}$) and stored at -20°C until analysis. After the first sampling procedure, the fiberscope was kept in place, and the sampling procedure was repeated two additional times using new BMS probes each time. The three sampling probes were pooled into a common polypropylene tube. Thus, samples harvested from the three sampling probes from each animal at each sampling time represented a single (pooled) BELF sample. The fluid volume gathered from each probe was estimated by weight as previously reported (29, 32, 33, 60).

Pulmonary epithelial lining fluid.

Following BMS procedures, PELF was collected from each animal by BAL. Briefly, one of the bronchi was obliterated using hemostatic forceps. Thus, PELF was harvested from the contralateral lung. Lung was kept in a vertical position, and approximately 50 mL of 0.9% saline was instigated through the trachea into the bronchus using a disposable pipette, assuring complete distribution across the lung. Recovery of BALF into 50 mL polypropylene tubes was accomplished by gentle aspiration with a disposable pipette. Immediately after recovery of BAL fluid, the sample was centrifuged at 450 RCF for 10 min at $\sim 4^{\circ}\text{C}$ for separation of the cell fraction, and then the cell-free BALF fraction was gently decanted into a polypropylene tube (2, 12, 31). Estimation of PELF volume was performed as previously described, quantifying urea as a dilution factor (47).

BAL fluid cell fractions

Cells isolated from BAL fluid were washed twice with 5 mL of 0.9% saline. Upon addition of the first 5 mL of wash solution, a 20 μ L aliquot of the re-suspended material was transferred to glass slides (in duplicate) and stained with Diff-Quick solution for cell differential counting. Following the second wash, the cell pellet was re-suspended in 5 mL of 0.9% saline for shipping and processing. Cells were processed as quickly as possible to preserve their integrity. Differential cell population count was performed by a pathologist (DGT) by microscopy. A predefined-cell population proportion criterion was set at > 90% of either neutrophils or macrophages. Thus, a minimum of > 90% of a single cell population was required in order to use a reference intracellular volume to express drug concentration in terms of intracellular concentration.

Intra-lower respiratory airways and alveolar inflammatory cells

The total number of cells harvested by the BAL fluid was counted using an automatic method as implemented by the ADVIA® 120 counter (Advia® 120 hematology system, Deerfield, IL, USA). Then, the samples were adjusted to 10^6 cells/mL with 0.9% saline. After normalization of the number of cells, the samples were sonicated. The resulting solution was analyzed for concentration of tulathromycin.

Serum samples

Blood (for serum) samples were collected from all animals at necropsy at 6, 24, 72, 144, 240, or 408 h post-tulathromycin administration.

Analytical methods

All the matrix samples were analyzed for tulathromycin content by high performance liquid chromatography with tandem mass spectrometry detection following procedures

described by Galer *et al.* (2004) (23) and Cox *et al.* (2010) (12) Roxithromycin was used as an internal standard (50).

Bronchial epithelial lining fluid

Briefly, the method for extraction of tulathromycin from the probe began with introduction of 2 mL of 20 mM pH 4 ammonium acetate:acetonitrile (50:50 v:v) into the tube containing the sampling probe. The sample was vortexed for approximately 1 min and then sonicated for 2 min. A 50 μ L sub-sample aliquot was spiked with 200 μ L of roxithromycin (25 ng/mL) into a glass tube, vortexed for 1 min and centrifuged at $\sim$ 1500 RCF for 10 min. A supernatant aliquot ($\sim$ 200 μ L) was transferred into a glass vial, and 5 μ L was injected into the system for analysis. The calibration range was 1.0 to 500 ng/mL ($R^2 > 0.99$) using nine calibration standards. The intra-run bias for the calibration standards ranged from -10.0 to 12.0%. Study samples were analyzed in one run. Three quality control samples (2.5, 25 and 100 ng/mL) were included in the analysis. The bias in the assays of the quality control samples ranged from -14.3 to 9.2%.

Cell-free BAL fluid

A 100 μ L volume of the BAL supernatant was mixed with 400 μ L of acetonitrile containing roxithromycin. After centrifugation, 390 μ L of supernatant was transferred to another tube and evaporated under nitrogen gas. The sample was then reconstituted with 100 μ L of 20 mM pH 4 ammonium acetate:acetonitrile (80:20 v:v), and a 10 μ L aliquot was injected into the chromatographic system. The calibration range was 1.0 to 500 ng/mL ($R^2 > 0.99$) using nine calibration standards. The intra-run coefficients of variation for standards ranged from 0.1 to 14.6% and the intra-run bias ranged from -5.6 to 11.0%. All 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 10.0 to 14.4%. The intra-run bias in the assays of the quality control samples ranged from -7.3 to -1.3%.

Urea assays

Serum and cell-free BAL urea concentrations were measured using an LC-MS/MS as previously described (12). Cell-free BAL fluid supernatant (2 μ L) was injected onto a chromatographic system. Chromatography was accomplished with a BEH C₁₈, 1.7 μ m, 2.1 x 50 mm column (Waters Corp., Milford, MA, USA) with gradient elution. For the gradient elution, mobile phase containing 100%, 0.1% formic acid in water was used initially (0–0.2 min post-injection). Then, a linear gradient elution from 0 to 80% methanol and 20% formic acid (0.1%) was implemented over 4 min. A flow rate of 0.250 μ L/min was applied. The retention time for urea was approximately 1 min. Detection occurred via a Sciex API 4000 mass spectrometer using an electrospray ionization source, and positive ions were monitored with precursor $\rightarrow$ product ion pairs of 61 $\rightarrow$ 44. For both matrices, calibration occurred over a range of 0.005 to 1 mg/dL ($R^2 > 0.99$) using eight calibration standards, and three quality control samples (0.015, 0.03, and 0.3 mg/dL) were included in each analytical run. All assays for urea in both matrices were performed in one analytical run. For the serum quality control samples, the intra-run coefficients of variation ranged from 2.6 to 10.1%, and the intra-run biases ranged from -2.0 to 4.7%. For the BAL fluid quality control samples, the intra-run coefficients of variation ranged from 1.9 to 7.6%, and the intra-run biases ranged from 0.3 to 6.0%.

Biometrical and pharmacokinetic analyses

To analyze BELF, PELF, and cellular concentrations, A log transformation was applied to the concentration data for all tissues prior to analysis. Least squares means were estimated using a mixed model as followed;

$$Y_{ijk} = \mu + \varphi_i + \beta_{j(i)} + t_k + \varphi t_{ik} + \varepsilon_{ijk}$$

Where;

Y_{ijk} =(Ln transformed) observation of the i^{th} group, the j^{th} block within the i^{th} Dose Group, and the k^{th} treatment

μ =overall constant

φ_i =fixed effect of the i^{th} group

$\beta_{j(i)}$ =random effect of the j^{th} block within the i^{th} group,

t_k =fixed effect of the k^{th} treatment

$\varphi_{t_{ik}}$ =fixed interaction effect of the i^{th} group and the k^{th} treatment

ε_{ijk} =residual

$i = 1 \dots \text{number of group}$

$j = 1 \dots \text{number of blocks within group}$

$k = 1 \dots \text{number of treatments}$

Least squared means and standard errors of LS means were estimated and 95% CIs were constructed. Back-transformed LS means (geometric means) and CIs for each treatment and group were reported. Least squares mean (LS mean) concentration was used as an estimate of treatment means. The groups were tested for significant differences for fixed effects at the $\alpha = 0.05$ level, indicating that p values ≤ 0.05 showed a significant difference for the effect. Drug concentrations were expressed in ng/mL unless stated differently.

Pharmacokinetic parameters were estimated using WinNonlin software (Standard edn. version 5.1, Pharsight Corporation, CA, USA). WinNonlin model 200 was used for the non-compartmental analysis. Pharmacokinetic parameters were estimated using LSM derived from the naïve pooling from each sample point [13]. Therefore, dispersion data of the PK parameters was not possible to be estimated. Area under the concentration-time curve through 408 h post-dose (AUC_{0-408}), area under the concentration-first moment time curve through 408 h post-dose ($AUMC_{0-408}$), mean residence time (MRT), and the terminal elimination half-life ($t_{1/2}$) were estimated. The AUC_{0-408} was calculated by the log-linear trapezoidal rule, and λ_z was estimated

with uniform weighting. The observed peak LS mean concentration ($C_{\max}$) and time of its occurrence ($t_{\max}$) were obtained from inspection of the LSM concentration.

Results

All animals remained in good health throughout the acclimatization and study periods. One animal (No. 242 from the 408 h group 1) was determined to be affected with bronchopneumonia and no samples were collected. Two animals in group 2 were euthanized. Animal 283 (from the 144 h treatment group) had severe respiratory distress and animal 278 (from the 24 h group) was moribund after the administration of LPS-*Ec*. No adverse reactions or detrimental health effects were observed following the administration of tulathromycin. The overall average dose of tulathromycin administered was 2.64 mg/kg which represents an over dose of 5.4%.

BELF

In group 1, tulathromycin was quantified during a period of 17 days post-administration. The maximal LS mean concentration was 5040 ± 801 at 72 h post-administration of the drug. After 72 h, the drug concentration declined slowly (apparent $t_{1/2}$ values 213 h). At 17 days (408 h) post administration, the concentration of the drug was still relatively high (1680 ± 291). LS mean concentration results are presented in Table 1. The concentration vs. time profile is shown in Figure 6-1. The concentration in BELF from LPS-*Ec*-challenged animals was evaluated only at one sampling point (24 h post-tulathromycin administration). The LS mean concentration obtained was consistent with concentrations obtained at the same sampling time in group 1 (LS mean $\pm$ SE, 3240 ± 561 in group 2 vs. 3570 ± 567 in group 1) ($p > 0.05$).

Cell-free fraction of BAL fluid (PELF)

Tulathromycin LS mean concentrations were evaluated in PELF from both groups (Fig. 1 and Table 1). A summary of the LS mean concentration results are presented in Table 1. The

summary of pharmacokinetic parameters is presented in Table 2. In group 1, the maximum LS mean concentration (LS mean $\pm$ SE, 7890 $\pm$ 2680) was observed at 6 h post-tulathromycin administration. In group 2, the maximal LS mean ($\pm$ SE) concentration was 2580 $\pm$ 951 at 24 h. After the peak tulathromycin concentration, it declined slowly with a terminal half-life of 87 and 108 h in PELF_{Group-1} and PELF_{Group-2} respectively.

Differential counts

Smear samples from BAL fluid from both groups were evaluated. In group 1, most of the cells counted belonged to the epithelial cell population. In 9/35 samples, macrophages were identified. However, none of the samples evaluated reached the minimum predefined cell population proportion criteria (> 90% of macrophages). In group 2, the cell population proportions differed from the trend observed in group 1. During the first 27 h after challenge administration (24 h post-tulathromycin administration), neutrophils were the most representative cell population observed. The cell population proportion observed was not consistent within the treatment groups nor across the 27 h post-LPS-*Ec* administration. In addition, none of the samples evaluated reached the minimum predefined cell population proportion criteria (> 90% of neutrophils). After 27 h post-LPS-*Ec* administration, the cell population proportion was consistent. However, the main cell population counted was based on epithelial cells.

BAL fluid cells concentration

The concentration vs. time profile of tulathromycin in 10⁶ cells is presented in Figure 6-2. There was a significant difference in tulathromycin concentrations between animals in group 1 and group 2. The concentration vs. time profile was similar in both groups during the first 72 h post dosing. The maximum LS mean concentration was 13.3 and 14.7 ng/mL for group 1 and 2, respectively. After the peak concentration (72 h), tulathromycin declined more slowly in group 2 than group 1.

Discussion

Several studies have shown that tulathromycin exerts a positive role in the treatment and control of bovine and swine respiratory disease (28, 40, 43, 56, 57). An important reason for this is the extraordinary lung selectivity and unique concentration vs. time profile of this triamylide in pulmonary tissue (4, 16, 42). Predictions about antibacterial efficacy are generally performed using serum/plasma drug concentrations as a surrogate for the drug concentration at the site of infection. However, the actual concentration-time profile at some sites of infection could differ from that in serum or plasma. This caveat puts into question the value of serum/plasma concentration as a surrogate for peripheral drug concentration for certain antimicrobials (15, 38). This holds true in particular for macrolides, including tulathromycin, for the treatment of pneumonia since plasma concentration does not mirror tissue concentration (4, 12, 41, 58). In respiratory conditions, the target tissue is the lung. In particular the extracellular fluid, like the epithelial lining fluid that covers the route of entry and colonization of microorganisms associated with pneumonia. Technical complications limit the use of most techniques to take appropriate samples from potential infection sites in the intra- and extra-pulmonary airways and lung tissue. Consequently, the characterization of the concentration vs. time profile of tulathromycin in lung tissue from pigs, and other antimicrobials in different species, has been accomplished mainly by homogenizing lung tissue. However, total antibiotic concentration measurements from tissue homogenate cannot be routinely considered as valid, and results or interpretations based on them may be misleading. The main reason for this argument is that lung homogenate is a hybrid heterogeneous sample, since it encompasses multiple compartments (e.g. intravascular, extracellular, and intracellular). For most microorganisms, the actual target biophase is the interstitial space fluid; therefore, effective concentrations might be under or overestimated (39).

Even though the concentration of tulathromycin in BELF from pigs has been previously reported (Chapter 4), this is the first study describing the concentration vs. time profile of

tulathromycin in BELF and PELF in pigs. Bronchial epithelial lining fluid was defined in this study and others as the sample obtained from the secondary bronchi at the middle lobe by the BMS probe (29, 32, 33, 60). Pulmonary epithelial lining fluid represented the cell-free fluid obtained from BALF after normalization with urea (47). Some studies available in the literature consider the concentrations of the drug as a result of this technique as drug concentration in alveolar epithelial lining fluid. However, it is important to recognize that the epithelial lining fluid, harvested by BAL fluid, represents a pooled lining fluid that covers the entire lower airway compartments and the alveolar lining fluid. Thus, the epithelial lining fluid obtained by BAL fluid is considered in the present study as PELF, and the concentration of tulathromycin in PELF represents lower intra-airway and alveolar composite concentrations.

As reported for lung tissue samples (Benchouai *et al.* 2004) (4), tulathromycin concentrations were notably high in the intra-airway compartment, BELF, and PELF in both groups (Table 1). The AUC₀₋₄₀₈ ratio of PELF-plasma and BELF-plasma was 90 and 223, respectively. The extraordinary clinical outcome of the drug after a single administration may be explained by not only the magnitude of drug concentration, but also the long persistence of the drug in the intra-airway compartments (5, 28, 40).

The concentration of tulathromycin in the intra-airway compartments far exceeded plasma concentrations (Table 1). The basic nature of the drug and a limited degree of ionization at physiological pH (tulathromycin with a pK_a range of 8.49–9.80) represent features that favor the distribution of the drug into extra-vascular compartments (Benchouai *et al.* 2004) (4). Nevertheless, the exact mechanism of drug tissue distribution is unknown. The drug penetrated rapidly into the airways, but concentration declined slowly. Also, the drug concentration ratio PELF:plasma and BELF:plasma change in a time and concentration dependent manner following drug administration (ratio range 29.6–271 and 2.69–1615 for PELF:plasma and BELF:plasma, respectively) (Table 1). This result might indicate that different factors intervene

in the process of movement of the drug *in* and *out* between the plasma and the airway compartments.

A common misconception is that the lung is a uniform matrix, but it is important to remark that there are relevant histological and physiological differences (regional blood flow, heterogeneous rate of formation, absorption, and clearance and chemical composition of the airway surface liquid, etc.) among lung sections. These differences may account for variability in the local pharmacokinetics of the drug at different intra-pulmonary levels (7). The equilibrium between compartments does not occur instantaneously, and the shape of the concentration-time profile in either compartment can therefore be markedly different (36). It is evident in Figure 6-1 that the shapes of the concentration-time profiles of tulathromycin in PELF and BELF in group 1 differ from each other. Tulathromycin in PELF reached the maximum concentration at 6 h post-administration of the drug, while the BELF $C_{\max}$ was later (72 h post-tulathromycin administration) and lower (LS mean $\pm$ SE 5040 ± 801.8 vs. 7890 ± 2680). The decline of the drug in BELF took longer than in PELF (half-life of terminal phase; 213 h for BELF vs. 87 h for PELF). The reasons for the unequal penetration and removal rate and extent of accumulation of tulathromycin from PELF and BELF are not clear. However, the differences observed in the rate of accumulation in PELF and BELF may be due to the combination of at least three factors: (i) unequal surface areas (larger for PELF) (ii) differential histological features, and (iii) differences in blood flow (44). The alveolar region receives nearly the entire proportion of the cardiac output by pulmonary circulation. On the contrary, the airways receive approximately 1% of the cardiac output (13, 22). Unequal removal of the drug from the intra-airways and alveolar compartment might be dictated by differences in epithelial permeability and absorptive capacity (8, 46), metachronous beating of cilia (10, 11, 45, 46), heterogeneous binding to different intrapulmonary components (mucus phospholipids), unequal release of the drug (19, 25) and drug structural modifications (34). Undoubtedly, further work is needed to identify the intervenient factor(s) accounting for differences in the intrapulmonary compartment.

The concentration of tulathromycin in BELF from animals challenged intra-tracheally was evaluated only at one sampling point (24 h post administration). The reason for this was that only preliminary reference information from this group was desired. Results, at 24 h post-tulathromycin administration, were consistent with concentrations obtained at the same sampling time in group 1 (Table 2).

The concentration vs. time profile of tulathromycin in PELF in group 2 differed from group 1. This was evident during the first 24 h post-tulathromycin administration. As stated earlier, the peak concentration of PELF from group 1 (LS mean $\pm$ SE, 7890 $\pm$ 2680) was at 6 h post-tulathromycin administration, while in group 2, it was lower (LS mean $\pm$ SE, 2580 $\pm$ 951) and later (24 h post-tulathromycin administration). However, these differences in tulathromycin concentrations were not statistically significant. The change in the concentration vs. time profile of tulathromycin observed in the middle lobe from animals challenged with LPS-*Ec* was not reflected in PELF concentration results obtained in the same group (Chapter 5). The reasons for this result are not clear.

During the last decade, the concept of tissue-directed pharmacokinetics has appeared consistently in the literature referring to macrolides and lung tissue (1, 3, 6, 20, 21, 24, 25, 35, 48, 55). Tulathromycin has been found to accumulate in inflammatory cells such as neutrophils and macrophages (12, 51), and it was demonstrated that the administration of LPS-*Ec* recruits inflammatory cells (Chapter 3). In the present study, recruitment of cells was reflected in the cell populations obtained in BAL fluid, mainly during the first 24 h post-dose administration (Fig.2). Despite the given context, the concentrations of tulathromycin in PELF in group 2 were not statistically different from those in group 1.

There were statistical differences in intracellular tulathromycin concentrations between samples from group 1 and group 2, but the concentration vs. time profile of tulathromycin in both groups is indistinguishable during the first 72 h post-dosing (Fig. 2). After that time, the tulathromycin concentrations in group 2 decline at a remarkably lower rate than in group 1.

Interestingly, evident differences in cell population proportions in BAL fluid samples between the two groups were observed during the first 27 h post-LPS-*Ec* administration. However, statistical differences in drug concentrations are determined by the concentration after 27 h post LPS-*Ec*. During this period, the cell population observed in both treatment groups was represented by epithelial cells. Contrary to what we were expecting, it was not possible to characterize the samples into a unique cell population of either macrophages or neutrophils. The reason for this result is unclear; the use of a fiberscope might explain these findings for group 1 but not for group 2 (except for the 24 h sampling time). We were expecting to obtain mostly macrophages (alveolar) in group 1 and neutrophils in the LPS-*Ec*-treated animals (group 2). Based on previous studies, the intracellular concentration is estimated using a reference intracellular volume calculated in a pellet of 10^6 macrophages or neutrophils. We set a minimum proportion criterion (> 90% of either macrophages or neutrophils) in order to consider the reference intracellular volume valid. None of the samples reached the minimum acceptance criterion. Consequently, the results obtained only allow expression of the concentration of tulathromycin in 10^6 cells rather than intracellular drug concentration in a specific cell population.

Despite wide acceptance that free drug concentrations dictate drug effects, we did not evaluate if tulathromycin was unbound in BELF and PELF samples. This was not estimated due to restrictions of techniques to harvest those samples. However, protein binding of tulathromycin in ELF is expected to be negligible, since total protein concentration in ELF is relative low (~10% of plasma concentration of protein) (26, 30, 49). Also, it is possible that the drug binds to other components such as mucus, cells, and phospholipids. Consequently, we cannot assure that the results obtained represent free extracellular drug concentrations.

Although the BAL fluid technique has been extensively used to describe drug concentrations in the lung, some limitations have been recognized regarding the validity of drug concentrations in PELF using this technique (12, 29, 59). One of the main drawbacks of this technique is that cell-free PELF drug concentrations might be “contaminated” by the release of

the intracellular drug, mainly from the highly loaded alveolar macrophages (31). An overestimation of drug concentrations in PELF is strongly associated with the BAL fluid technique and, at least for tulathromycin in cattle, might have a relevant impact on PELF concentrations after 11 h post-administration of the drug (12, 29, 31). The magnitude of the contamination in the samples is difficult to estimate; however, a short dwell time (~20s), the use of pipettes, and immediate separation of the cell fraction from the sample after harvest of the BAL fluid were implemented in order to minimize the potential confounding effect of the drug concentrations in PELF (2, 31).

Different techniques have been used to describe the pulmonary drug distribution in animals and humans, but their application is limited (9, 27, 52-54). Bronchoalveolar lavage fluid has been extensively used in multiple species; however, pouring fluid into the airways certainly modifies the sample site environment. This restricts the use of this technique to single or sparse sampling schemes. Therefore, characterization of drug concentration profiles in PELF by the use of BAL fluid may require a large number of animals to obtain acceptable and precise data. Advantages and limitations of BMS probes are described elsewhere (Chapter 4), but in the present study it was demonstrated that BMS probes can be used to describe the concentration vs. time profile of tulathromycin in the intra-airway compartments. In addition, one of the most remarkable advantages of this technique is that BMS probes can be introduced in multiple sampling protocols, reducing the number of study animals and improving the precision of the results. Additionally, it would eliminate and/or decrease the use of terminal studies to describe the concentration vs. time profile of drugs, as required when using lung homogenate. Bronchial micro-sampling probes represent a cost-effective technique that can be introduced in discovery and preclinical pharmacokinetic studies for screening of candidate drugs that could be indicated for treatment of respiratory conditions.

In conclusion, this is the first study that describes the disposition of tulathromycin in both BELF and PELF after a single intramuscular dose of tulathromycin in pigs. The concentration-

time profile of tulathromycin in airway fluid has been described using a novel technique. The use of BMS probes might enhance the experimental design and maximize experimental resources (number of animals, time, and cost). Pharmacokinetic parameters of tulathromycin were estimated for BELF and PELF in healthy and LPS-*Ec*-challenged animals, and it was demonstrated that tulathromycin not only distributed rapidly into the intra-airway compartments (BELF and PELF) but also persisted in the airway lining fluid (at least 408 h) at relative high concentrations in both healthy and LPS-*Ec*-challenged pigs.

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Conflict of Interest

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Appendix

Table 6.1. Summary of tulathromycin geometric mean drug concentrations (and 95% confidence limits) (ng/mL) in pulmonary and bronchial epithelial lining fluid from pigs following a single i.m. injection of tulathromycin at 2.5 mg/kg body weight.

Time (h)	Treatment	Group	Pulmonary epithelial lining fluid			Bronchial epithelial lining fluid			Plasma ^{&}	Ratio		
			LS Mean [†]	95% CIs [†]	Range	LS Mean [†]	95% CIs [†]	Range	Raw Mean	PELF/ Plasma	BELF/ Plasma	PELF/ BELF
6	T01	1	7890	3940-15800	2410-67800	717	514-1000	268-1430	149	29.6	2.69	11.0
		2	1580	789-3170	367- 5780	na	na	na	247	6.39	na	na
24	T02	1	4670	2330-9360	1550-28700	3570	2560-4970	2320-6420	61.9	75.4	57.7	1.30
		2	2580	1220-5460	1000- 4290	3240	2260-4640	1950-4500	86.8	29.7	37.2	0.79
72	T03	1	2270	1130-4540	1850- 3180	5040	3610-7030	3750-6380	22.5	101	224	0.45
		2	1630	811-3260	424- 2840	na	na	na	19.8	82.3	na	na
144	T04	1	566	282-1130	122-1610	3940	2830-5500	3060-4790	6.99	90.5	630	0.14
		2	1070	507-2270	531-2440	na	na	na	7.67	140	na	na
240	T05	1	495	247-994	256-1970	2940	2110-4100	2020-4250	1.82	271	1610	0.16
		2	261	123-554	71.8-423	na	na	na	2.19	119	na	na
408	T06	1	291	137-618	181-463	1680	1170-2410	1020-2610	1.36	213	1235	0.17
		2	258	128-519	132-617	na	na	na	1.33	194	na	na

[†]Back transformed; NA, not applicable. [&]Plasma results were fully described in chapter 5 of the dissertation.

Table 6.2. Summary of pharmacokinetic parameters for tulathromycin in PELF and BELF in pigs treated i.m. with tulathromycin at 2.5 mg/kg

Pharmacokinetic parameter*	Matrices		
	PELF		BELF
	Group 1	Group 2	Group 1
HL_{Lambda_z} (h)	87.3	108	213
T_{max} (h)	6.00	24.0	72.0
C_{max} (ng/mL)	7890	2580	5040
C_{last} (ng/mL)	291	258	1680
AUC_{last}(h x ng/mL)	522000	348000	1290000
AUC_{inf_obs} (h x ng/mL)	559000	388000	1810000
AUMC_{last}(h x h x ng/mL)	46600000	39300000	223000000
MRT_{INF_pred} (h)	107	146	328
AUC₀₋₄₀₈ BELF:Plasma ratio[†]	NA	NA	223
AUC₀₋₄₀₈ PELF:Plasma ratio[†]	90.6	62.8	NA

*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. NA, not applicable. [†]Plasma drug concentration and AUC₀₋₄₀₈ was described and estimated in chapter 5 of the study

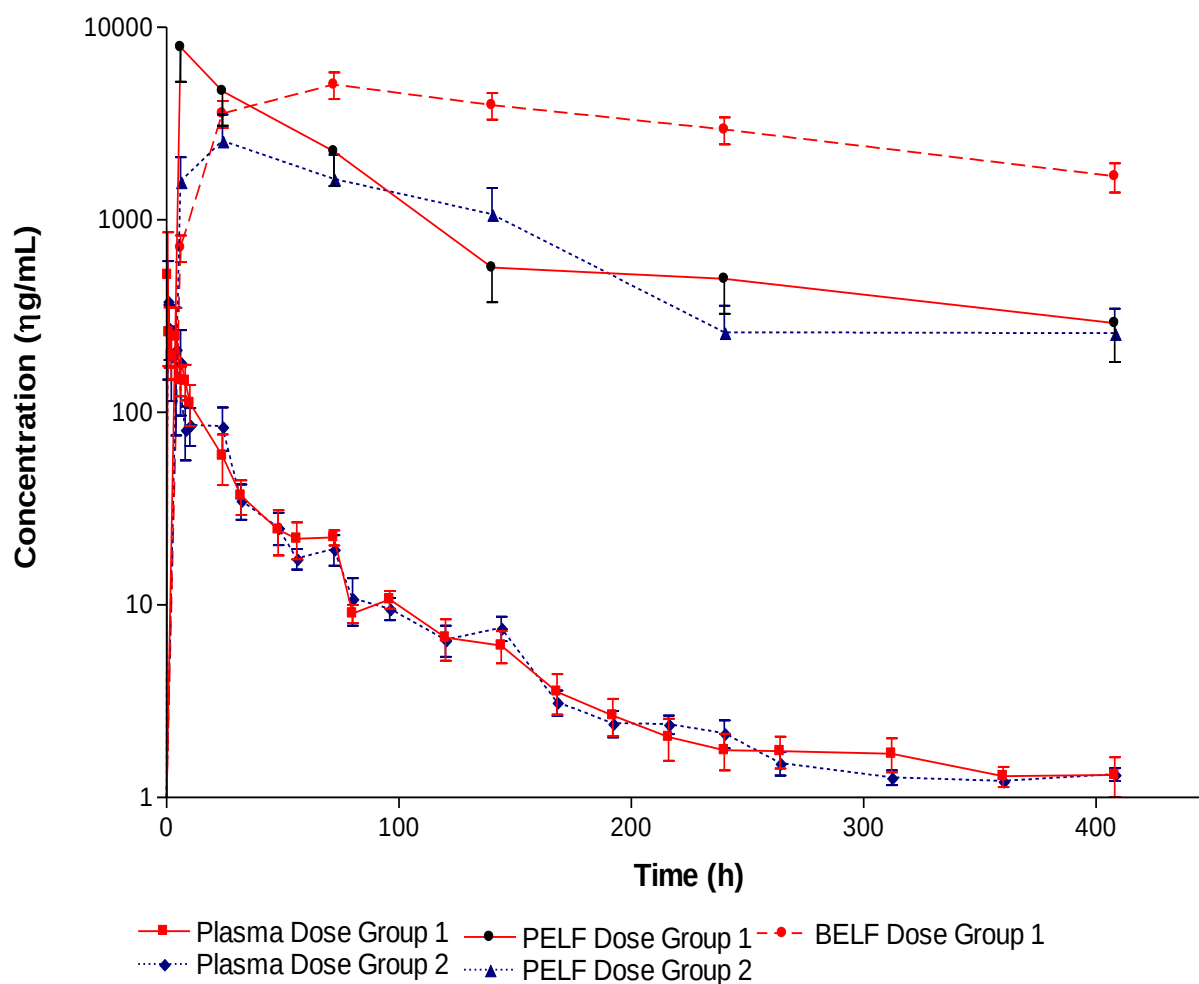


Figure 6.1. Least squared means concentration ($\pm$ SE) (ng/mL) of tulathromycin in PELF and BELF, and mean concentration ($\pm$ SE) in plasma from pigs treated i.m. with tulathromycin at 2.5 mg/kg((n=6) (except for group 1 in PEL F and BELF at 408h n=5 and group 2 in PEL F at 24 and 144 h (n=5)).

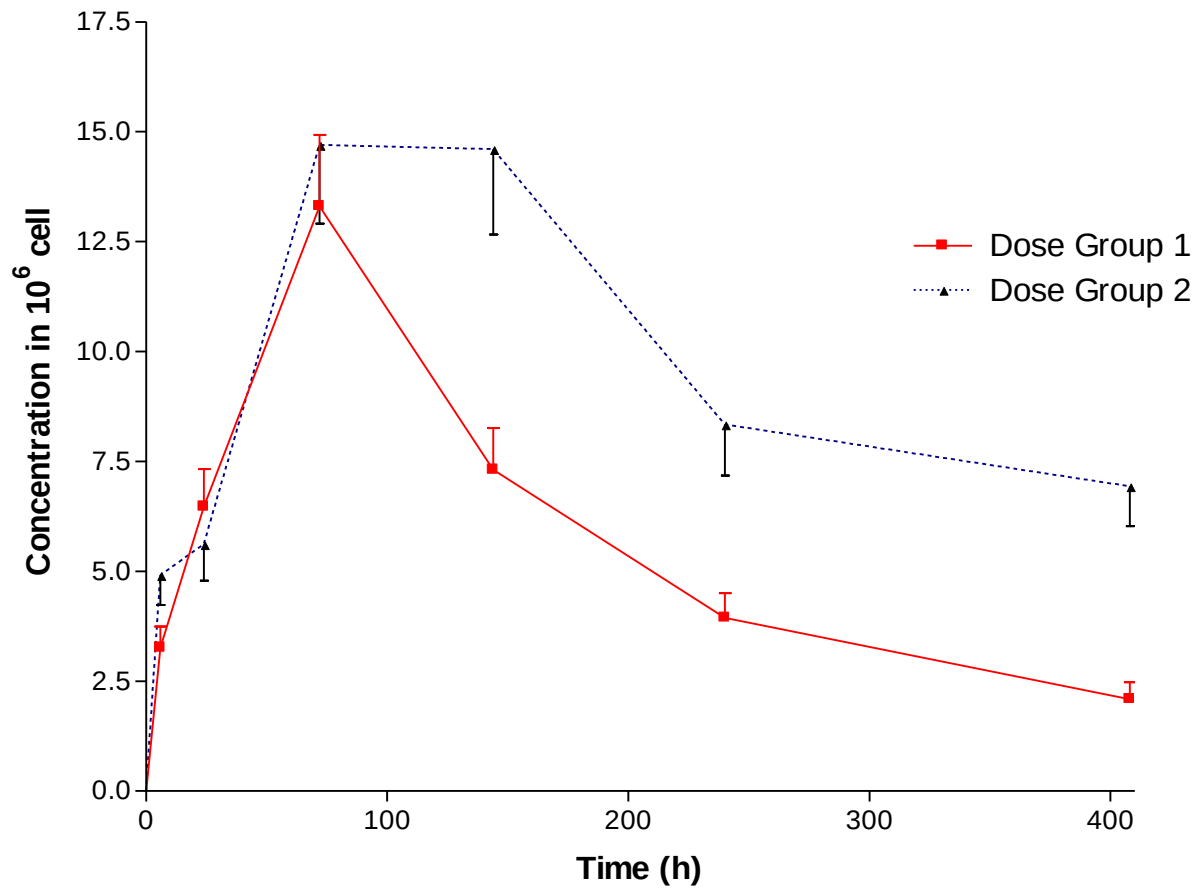


Figure 6.2. Least squared means concentration ($\pm$ SE) (ng/mL) of tulathromycin in 10^6 cells harvested by BAL fluid from pigs ((n=6) (except for group 1 at 408h, n=5 and group 2 at 24 and 144 h, n=5) treated i.m. with tulathromycin at 2.5 mg/kg.

Chapter 7:

CONCLUSION

This dissertation evaluates the pulmonary pharmacokinetics of tulathromycin in mice and pigs. Tulathromycin is widely used as part of the health management of swine and bovine practices as metaphylactic and therapeutic for respiratory disease. One of the most relevant pharmacokinetic features of tulathromycin is its capacity to accumulate extensively in lung tissue after a single dose. The kinetics of the drug in lung tissue is characterized by a rapid rate of accumulation but a slow rate of depletion from the organ. However, the amount of information available about the pharmacokinetics of tulathromycin in lung tissue is scarce. The mechanism (s) of accumulation of the drug in lung tissue is unknown and information about the kinetics of the drug in target anatomical areas is limited. In pigs, there was only one study that evaluated the pulmonary pharmacokinetics of tulathromycin. This study was based on a single representative lung homogenate sample per animal. However, there was no information available concerning the within organ distribution and pharmacokinetics of the drug in the intra-airways compartment. However, unequal drug distribution within an organ may have important therapeutic consequences.

Studies in this dissertation provide information about the pharmacokinetics of tulathromycin at two different lung levels, middle and caudal lobe. Results indicate that tulathromycin accumulates notably in lung tissue at different sections within the organ. The concentration vs. time profile of the drug and pharmacokinetic parameters in middle and caudal lobes were similar. This suggests that tulathromycin distributes homogeneously within the lungs, at least in the middle and caudal lobes.

In general, pulmonary pharmacokinetic studies are done in healthy animals. However, inflammation may change the disposition of drugs in tissues. Therefore, information generated from healthy animals limits the applicability of the results. Information from healthy animals may be valuable for drugs with metaphylactic claims but may be misleading for therapeutic claims.

In order to evaluate the potential impact of an inflammatory response on the concentration vs. time profile of tulathromycin, animals were challenged with lipopolysaccharide of *Escherichia coli* which triggers an acute inflammatory response. The first study was done in mice followed by the study in pigs.

Tulathromycin was administered to neutropenic and neutrophilic mice. There were no statistically significant differences in the concentration of tulathromycin in lung tissue and plasma among healthy mice, neutropenic mice and neutrophilic mice challenged with LPS-*Ec*. In addition, neutrophils may not have a positive influence on tulathromycin accumulation in lung tissue and an acute inflammatory response may not explain the fast and extensive accumulation and long persistence of tulathromycin in lung. Further research is necessary to elucidate if inflammatory cells have a relevant role in the accumulation of the drug in lung tissue of animals with respiratory disease. Multiple scenarios should be evaluated including acute and chronic respiratory disease.

In pigs challenged with LPS-*Ec*, statistically significant lower tulathromycin concentration were found at 6 and 72 h post-drug administration in the middle lobe and at 24 and 72 h post-drug administration in the caudal lobe. These lower concentrations influenced the concentration vs. time profile of the drug in lung tissue from the middle and caudal lobe. Even though the concentration vs. time profile of the drug in lung tissue changed, the drug still distributed into lung tissue to a large extent. The distribution of the drug was rapid and persisted at relatively high levels for 17 days post-tulathromycin administration (LT: plasma ratio > 300 at 17 days post-tulathromycin administration).

The clinical significance of the change in the concentration vs. time profile in lung tissue is unknown. The inflammatory model used in this dissertation was not compared to natural occurring respiratory disease caused by usual target microorganisms. Therefore, results obtained and conclusions made in this dissertation are not fully extrapolable to field conditions. Further research is necessary in this area. Evaluation of the potential impact of natural occurring respiratory disease on the concentration vs. time profile of the drug in lung compartments would provide valuable information for the understanding of the factors affecting the clinical efficacy of tulathromycin.

Drug concentration at the site of infection is linked to the efficacy profile of antimicrobials. In respiratory conditions, the target tissue is the lung, in particular the extracellular fluid, such as the epithelial lining fluid that covers the route of entry, and colonization of microorganisms associated with pneumonia. Therefore, the accumulation and concentration time profile of the drug in the intra-airways compartment represents relevant information to understand the antimicrobial effect of the drug and may help to delineate dosage regimes based on target microorganism susceptibilities.

As reported for lung tissue samples, results indicate that the tulathromycin concentrations were notably high in the intra-airway compartment. The concentration of tulathromycin in the pulmonary and bronchial epithelial lining fluid (PELF and BELF) far exceeded plasma concentrations (ratio range 29.6-271 and 2.69-1610 for PELF:plasma and BELF:plasma, respectively). Tulathromycin penetrated quickly into the airway compartment, but was slowly eliminated. Therefore, the extraordinary clinical outcome of the drug after a single administration may be explained not only by the magnitude of drug accumulation but also the long persistence of the drug in the intra-airway compartments. Interestingly, the lower concentrations observed in lung tissue at some sample points of LPS-challenged animals were not reflected in PELF concentration.

Fiberscopically guide bronchial microsampling probes (BMSp) were used for the assessment of the concentration-time profile of tulathromycin in BELF. This technique has not previously been evaluated in veterinary species. We identified the limitations and advantages for the use of this technique for the evaluation of the pharmacokinetics of drugs in BELF from veterinary species. The limitations of BMSp are (i) it may not be possible to harvest unbound drug and (ii) it is not possible to harvest BELF at all airways sections. In contrast to other techniques, one of the most important advantages of BMSp, is that it makes possible to improve the anatomical resolution of the samples. Bronchial micro-sampling probes permits to take samples from potential infection sites in the intra- and extra-pulmonary airways.

Due to the anatomical location and function of the lung, it is experimentally difficult to obtain relevant information to evaluate lung pharmacokinetic of the drugs. The studies done in this dissertation indicate that the BMSp may represent a valuable tool to study the pulmonary pharmacokinetics of drugs not only in pigs but also in other veterinary species. The use of BMSp represents a cost-effective technique that enhances the experimental design and maximizes experimental resources (number of animals, time, and cost).

This dissertation expands our understanding about the distribution of tulathromycin into lungs. Studies in this dissertation are the first studies that evaluate the kinetics of the drug in middle and caudal lobes and intra-airway compartments from healthy pigs and animals with an acute pulmonary inflammatory response. This work provides the first evidence of the pharmacokinetics of tulathromycin in lung tissue administered to neutropenic, normopenic and neutrophilic mice. Finally, this dissertation introduces and evaluates an innovative technique for the study of pulmonary pharmacokinetics of drugs in veterinary species.

Further research is necessary to define the mechanisms and factors involved in the kinetics of tulathromycin in the lungs. Two additional pieces of information certainly may help to unravel the unique pharmacokinetic profile of tulathromycin in lung tissue, namely (i) the evaluation of the impact of chronic inflammation on the pulmonary pharmacokinetics of the drug,

and (ii) the influence of the time of drug administration with respect to the pathological stage on the concentration vs. time profile of the drug in the intra-airways compartment. Finally, research emphasis should be put on the characterization of the unbound drug concentration at different levels in the intra-airways compartment.

VITA

Nicolás Francisco Villarino was born on June 14th, 1976 in La Plata, Provincia de Buenos Aires, Argentina. After obtaining the degree in veterinary medicine (2000) from the National University of La Plata, Argentina, he was exposed to multiple professional experiences in different countries. Those experiences involved research and clinical activities in several species. In 2006, he completed his first postgraduate program obtaining the degree in Doctor of Veterinary Sciences from the Faculty of Veterinary Medicine, National University of La Plata, Argentina. In 2011, he completed a residency program in Veterinary Clinical Pharmacology and obtained a Doctor of Philosophy degree in Comparative and Experimental Medicine from The University of Tennessee, USA.