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Distribution Characteristics of Telithromycin, a Novel Ketolide Antimicrobial Agent Applied for Treatment of Respiratory Infection, in Lung Epithelial Lining Fluid and Alveolar Macrophages

Kohei Togami, Sumio Chono, Toshinobu Seki** and Kazuhiro Morimoto*

Department of Pharmaceutics, Graduate School of Pharmaceutical Sciences, Hokkaido Pharmaceutical University, Hokkaido, Japan

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Summary: The distribution characteristics of telithromycin (TEL), a novel ketolide antimicrobial agent, in lung epithelial fluid (ELF) and alveolar macrophages (AMs) were evaluated. In vivo animal experiments, the time-courses of the concentrations of TEL in ELF and AMs following oral administration of TEL solution (50 mg/4 mL/kg) to rats were markedly higher than in plasma, and areas under drug concentration-time curve (AUC) ratios of ELF/plasma and AMs/plasma were 2.4 and 65.3, respectively. In vitro transport experiments, the basolateral-to-apical transport of TEL through model lung epithelial cell (Calu-3) monolayers was greater than apical-to-basolateral transport. Rhodamine123 and verapamil, MDR1 substrates, reduced the basolateral-to-apical transport of TEL. In vitro uptake experiments, the intracellular equilibrated concentration of TEL in cultured AMs (NR8383) was approximately 40 times the extracellular concentration. The uptake of TEL by NR8383 was inhibited by rotenone and FCCP, ATP depleters and was temperature-dependent. These data suggest that the high distribution of TEL to AMs is due to the sustained distribution to ELF via MDR1 as well as the high uptake by AMs themselves via active transport mechanisms.

Keywords: telithromycin; ketolide; lung epithelial lining fluid; Calu-3; MDR1; alveolar macrophages; NR8383

Introduction

Telithromycin (TEL, Fig. 1) is a novel ketolide class antimicrobial agent1 which has an antibacterial spectrum that covers most major respiratory pathogens, including *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*.2,3 Furthermore, TEL has antibacterial activity against erm (B)- and mef (A)-mediated macrolide-resistant strains of *Streptococcus pneumoniae*4 and intracellular pathogens such as *Chlamydia pneumoniae* and *Legionella pneumophila*.5,6 Because TEL, unlike macrolide antimicrobial agents, has not been shown to modify MLSB resistance,7-9 the risk of appearance of resistant bacteria by TEL is low. TEL is given by the oral route for treatment of respiratory infections and it distributes to a variety of tissues, particularly the lung.10 Such a unique lung distribution of TEL is very consistent with its antibacterial therapeutic efficacy toward respiratory infection.
In the alveolus, the epithelial lining fluid (ELF) is a mucous layer having a pulmonary surfactant covering the apical surface of alveolar epithelial cells and alveolar macrophages (AMs) associated with biophylaxis reside in the ELF. Several pathogens, such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, avoid phagocytosis and digestion by AMs and propagate in ELF. Intracellular pathogens such as *Chlamydia pneumoniae* and *Legionella pneumophila* are taken up by AMs via phagocytosis and are resistant to the biocidal mechanisms of AMs and survive or multiply intracellularly in AMs. Thus, the concentrations of antimicrobial agents in ELF and AMs must be higher than the effective concentration for treatment of respiratory infections induced by these pathogens. Interestingly, TEL appears to be concentrated in ELF and AMs in the alveolus. However, the high distribution mechanisms of TEL in ELF and AMs are not well known. Recently, there has been increasing interest in the relationship between the pharmacokinetics (PK) and pharmacodynamics (PD) of antimicrobial agents. If the distribution characteristics of TEL in ELF and AMs and their mechanisms are fully understood, this should help in selecting dose and optimizing the treatment of individual patients based on PK/PD relationships.

In the present study, the distribution characteristics of TEL in ELF and AMs are evaluated. In addition, we discuss the distribution mechanisms of TEL in ELF and AMs according to transport characteristics through model lung epithelial cell (Calu-3) monolayers and uptake characteristics by cultured AMs (NR8383).

### Materials and Methods

**Materials and animals:** TEL was supplied from Sanofi-Aventis (Paris, France). Rhodamine123, rotenone and carbonyl cyanide p-(trifluoromethoxy) phenyl hydrazone (FCCP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Verapamil hydrochloride and clarithromycin were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). All other regents were commercially available and of analytical grade. Male SD rats (200–230 g) were purchased from Japan SLC (Shizuoka, Japan). The animal experimental plan used was approved by the Committee of Laboratory Animal Center (No. 07–011) and conformed to the Guiding Principles for the Care and Use of Experimental Animals in Hokkaido Pharmaceutical University.

**In vivo animal experiments:** TEL dissolved in 0.1 M HCl was administered orally to rats at 50 mg/kg and dosage volume was 4 mL/kg. At each designated time point, rats were anesthetized i.p. with pentobarbital sodium at a dose of 40 mg/kg and blood was collected from the jugular vein. The trachea was immediately cannulated and the lungs were lavaged three times with 5 mL ice-cold phosphate buffered saline solution (pH 7.4). The bronchoalveolar lavage fluid was immediately centrifuged at 4°C (650 × g, 5 min) to separate AMs from ELF. AMs were extracted with 1 mL 0.1 M NaOH for HPLC analysis. To calculate the concentrations of TEL in ELF, the apparent volume of ELF was estimated using urea as an endogenous marker of ELF dilution. The mean value estimated in the present study was 393 μL/215 g rat. To calculate the concentrations of TEL in AMs, the intracellular volume in AMs was determined by velocity-gradient centrifugation using 3H-water and the mean value was 4.2 μL/mg cell protein. The concentration of TEL in each sample was measured by HPLC as described below. Protein concentration in AMs extracts was determined using Coomassie protein Assay reagent (Pierce Chemical Company, Rockford, IL, USA) with bovine serum albumin as the standard. For pharmacokinetic analysis, the area under the TEL concentration-time curve from time 0 to time 8 hr (AUC) was calculated by the trapezoidal rule.

**In vitro transport experiments:** Calu-3 cells (American Type Culture Collection, Manassas, VA, USA) were used as model lung epithelial cells. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal calf serum, 40 μg/mL gentamicin and 1% non-essential amino acid, in a humidified atmosphere of 5% CO2 at 37°C. Cells from passage numbers 53–62 were seeded (4.5 × 10^5 cells/cm²) on polyester filter inserts (pore size 0.4 μm, area 0.33 cm², Transwell, Coning, NY, USA) and cultivated in the medium for 10–14 days before the transport experiments. The quality of monolayers grown on the permeable membrane was assessed by measuring the transepithelial electrical resistance (TEER) using Millicell-ERS (Millipore, MA, USA) before the transport experiments and monolayers exhibiting 500–700 Ωcm² TEER were used for the experiments. The transport experiments were performed in Hank’s balanced salt solution (HBSS). TEL (50 μM) was added to either the apical (100 μL) or basolateral (600 μL) side of Calu-3 cell monolayers and drug-free HBSS was added to the opposite side. In the inhibition experiments, rhodamine123 (10 μM) or verapamil (100 μM) was added to the apical side. Aliquots of samples were taken from the apical or the basolateral side every 30 min for 2 hr and replaced with equal volumes of drug-free HBSS.

**In vitro uptake experiments:** NR8383 cells (American Type Culture Collection, Manassas, VA, USA) were used as cultured SD rat AMs. The cells were suspended at a concentration of 1.25 × 10^6 cells/mL in RPMI1640 medium (Sigma Chemical Co.) containing 1% fetal bovine serum (Sigma Chemical Co.). Aliquots (200 μL) of the cell suspension were then transferred to 96-well culture plates (Becton Dickinson, Lincoln Park, NJ, USA) which were then incubated for 90 min at 37°C with 5% CO2. After incubation, non-adhering cells were removed and then SFM medium (Gibco BRL, Life Tech-
nologies, Rockville, MD) was added to cells. TEL (50 μM) was added to the NR8383 cells which were incubated at 37°C or 4°C. At designated times, the medium was removed by aspiration and washed three times with ice-cold PBS. The cells were then extracted with 400 μL 0.1 M NaOH and the concentration of TEL in the cell extracts was measured by HPLC as described below. Protein concentration in the cell extracts was determined as described above. In the uptake inhibition experiments, NR8383 were pre-treated with rotenone (30 μM) or FCCP (2 μM) as ATP depletors or clarithromycin (50 μM) at 37°C for 30 min and then treated with TEL (50 μM) at 37°C for 2 hr without removing the pretreatment agents.

**Determination of TEL by HPLC:** The concentration of TEL in the samples was measured by HPLC as follows. The sample (40 μL), a diphenylamine solution (as an internal standard, 20 μL), methanol (40 μL) and 250 mM ammonium acetate solution (10 μL) were mixed and a 50 μL aliquot was subjected to HPLC using a system (Shimadzu Co., Kyoto, Japan) involving a Purospher RP-18e column (4.0 × 125 mm, Merck, Darmstadt, Germany). The mobile phase was 50 mM ammonium acetate/methanol/acetonitrile (5:4:2, v/v/v). The separation was performed at a flow rate of 1.0 mL/min at 50°C and the eluate from the column was monitored by fluorescence detection (excitation wavelength of 263 nm and emission wavelength of 460 nm).

**Statistics:** Statistical analysis was performed by Dunnett's test using Stat View software (Abacus Concepts Inc., CA, USA).

**Results**

**Pharmacokinetics of TEL in rats:** The systemic and pulmonary pharmacokinetics of TEL were examined in rats. Time-courses of the concentration of TEL in plasma, ELF and AMs after oral administration to rats are shown in Figure 2. The concentrations of TEL in ELF and AMs were markedly higher than in the plasma. The pharmacokinetic parameters of TEL in plasma, ELF and AMs are summarized in Table 1. AUC of TEL in plasma was 6.7 μg*hr/mL and AUC of TEL in ELF and AMs were 16.3 and 437 μg*hr/mL, respectively. AUC ratios of ELF and AMs to plasma were 2.4 and 65.3, respectively and AUC ratio of AMs to ELF was 26.8.

**Transport of TEL through Calu-3 cell monolayers:** The transport characteristics of TEL through model lung epithelial cell (Calu-3) monolayers were examined in vitro. The cumulative transport of TEL through Calu-3 cell monolayers is shown in Figure 3. The basolateral-to-apical transport of TEL was greater than apical-to-basolateral transport. The effects of rhodamine123 and verapamil, MDR1 substrates on the transport of TEL through Calu-3 cell monolayers are shown in Figure 4. Rhodamine123 and verapamil significantly reduced basolateral-to-apical transport and increased apical-to-basolateral transport.

**Uptake of TEL by NR8383:** The uptake characteristics of TEL by cultured AMs (NR8383) were examined in vitro. The time-profiles of the uptake and efflux of TEL by NR8383 are shown in Figure 5. The uptake of TEL equilibrated in a 2 hr incubation and the ratio of the intracellular to extracellular concentration (I/E ratio) was 39.7 ± 4.9. The effects of temperature, ATP depleters and clarithromycin, as a fourteen-membered ring macrolide, on the uptake of TEL by NR8383 are shown in Figure 6. The uptake of TEL at 4°C was significantly lower than at 37°C (Fig. 6A). Rotenone and FCCP, as ATP depleters, and clarithromycin significantly inhibited the uptake of TEL (Fig. 6B).

**Discussion**

This study examined the distribution characteristics of TEL in ELF and AMs to obtain important information for optimizing the treatment of respiratory infections induced by pathogens such as Chlamydia pneumoniae,
Fig. 3. Basolateral-to-apical (●) and apical-to-basolateral (○) transport of TEL through Calu-3 cell monolayers
TEL (50 μM) was added to apical or basolateral sides of cell monolayers, followed by incubation at 37°C. At each time point after incubation, samples were collected from the receiver side and concentrations of TEL in each sample were determined and the amount of TEL permeated was calculated. Each point represents the mean ± S.D. of four tests.

Fig. 4. Effects of MDR1 substrates on the basolateral-to-apical (A) and apical-to-basolateral (B) transport of TEL through Calu-3 cell monolayers
TEL (50 μM) was added to the apical or basolateral sides of monolayer and verapamil (100 μM) or rhodamine123 (10 μM) as MDR1 substrates were added to the apical side, followed by incubation at 37°C for 30 min. Other details are the same as those in Fig. 3. Each point represents the mean ± S.D. of four tests. *p < 0.01; significantly different from control.

Legionella pneumophila, Streptococcus pneumoniae and Haemophilus influenzae.
TEL was highly distributed in ELF from blood after oral administration and the TEL AUC ratio of ELF to plasma was 2.4 (Fig. 2 and Table 1). TEL given orally reaches the alveolar surface through alveolar epithelial cells from the blood. It is reported that the substance distribution from blood to ELF is based on permeation characteristics through alveolar epithelial cells. Alveolar epithelial cells that are tightly connected by numerous zonulae occludens are a significant alveolar barrier and, accordingly, the transport of substances between blood and ELF may be restricted by alveolar
epithelial cells.[12] We used Calu-3 to study the transport characteristics of TEL from blood to ELF through the alveolar barrier because the Calu-3 cell monolayer model is likely to represent tight-junctions[28,29] and drug transporters[30–33] of alveolar epithelial cells. The basolateral-to-apical transport of TEL through Calu-3 cell monolayers is greater than apical-to-basolateral transport (Fig. 3). This significant asymmetrical transport resulted from secretion of TEL by MDR1 transporter (Fig. 4). These phenomena are similar to the transport characteristics in human Caco-2 cell monolayers as a model of intestinal epithelia.[34] It is thought that TEL is secreted by efflux transporters, such as MDR1 transporter, expressed at the apical side of intestinal epithelia. MDR1 transporters are expressed at the apical side of alveolar epithelial type I cells.[35] TEL may thus undergo efficient secretion from blood to the ELF via MDR1 transporters on the alveolar epithelial type I cells.

TEL was concentrated highly in AMs via ELF from blood after oral administration and the AUC ratio of AMs to ELF was 26.8 (Fig. 2 and Table 1). The uptake of TEL by NR8383 (equilibrated I/E ratio: 39.7) was remarkably high (Fig. 5), as was also the uptake by phagocytes, such as human polymorphonuclear neutrophils,[36] human macrophages[37] and J774 cells as mouse macrophages.[38] These findings indicate that the uptake characteristics of TEL by NR8383 are similar to those by other phagocytes. This uptake was mediated by temperature- and ATP-dependent systems (Fig. 6). These results suggest that the high accumulation of TEL in AMs is mediated by active transport systems (Fig. 5 and 6). The uptake of TEL was inhibited by clarithromycin as an active influx process of macrolides and ketolides,38–40 the mechanisms are not well known. No carrier system for TEL is likely to represent tight-junctions28,29) and drug transporters30–33) of alveolar epithelial cells. Thus, TEL may be transported to the intracellular region of AMs via an active transport process which requires Ca2+ and protein kinase A-dependent phosphorylation as required for the uptake of macrolides and TEL by phagocytes.35,41 Thus, TEL may be transported to the intracellular region of AMs via an active transport process which requires Ca2+ and protein kinase A-dependent phosphorylation, as noted for phagocytes. The I/E ratio of TEL is more than 10–25 even when experiments are performed at 4°C and under coexistence of ATP depleting compounds (Fig. 6). These results suggest that TEL accumulation in AMs is also mediated by a temperature- and ATP- independent process. Macrolides such as erythromycin and roxithromycin (pKa = 8.8 and 9.2) basic compounds that accumulate in intracellular acidic lysosomes (pH 5)[42] as lysosomotropic agents.43,44 Like these macrolides, TEL (pKa = 2.4, 5.0 and 8.7)[43] may accumulate in the acidic lysosomes of AMs.

In conclusion, we show that TEL is efficiently distributed in ELF and AMs, two sites of respiratory infections. The present study suggests that the high distribution of TEL to AMs is due to sustained distribution in ELF via MDR1 as well as high uptake by AMs themselves via active transport mechanisms. This study provides important information for optimizing the treatment of respiratory infection based on PK/PD relationships in ELF and AMs.

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