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Research Paper

Intracellular pharmacokinetics of telithromycin, a ketolide antibiotic, in alveolar macrophages

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Abstract

Objectives Telithromycin, a ketolide antibiotic, has an antibacterial range that covers intracellular parasitic pathogens that survive or multiply intracellularly in alveolar macrophages. The intracellular pharmacokinetics of TEL in alveolar macrophages was evaluated *in vitro*.

Methods Telithromycin (50 μM) was applied to NR8383 as cultured alveolar macrophages, followed by incubation at 37°C or 4°C. After incubation, the amount of telithromycin in cells was determined.

Key findings Telithromycin exhibited high accumulation in NR8383 and its intracellular accumulation was temperature dependent. Also, telithromycin distributed to the organelles and cytosol in NR8383 and, in particular, it accumulated in the acidic organelle compartments.

Conclusions This study suggests that the high accumulation of telithromycin in NR8383 is due to its high influx via active transport systems and trapping in acidic organelles, such as lysosomes. Moreover, this study provides important information for optimizing the treatment of respiratory intracellular parasitic infections based on the intracellular pharmacokinetics of antibiotics and parasitic sites.

Keywords acidic organelle; active transport; alveolar macrophages; intracellular pharmacokinetics; telithromycin

Introduction

The alveolar macrophages are located in the alveolus, and serve as a first barrier against inhaled pathogens.[1] Alveolar macrophages, through phagocytosis and sterilization, play an important role in defending the body from respiratory infections.[2] However, intracellular parasites, such as *Chlamydia pneumoniae* and *Legionella pneumophila*, are taken up by alveolar macrophages via phagocytosis, are resistant to the biocidal mechanisms of alveolar macrophages and survive or multiply intracellularly in alveolar macrophages.[3–5] Thus, severe and refractory respiratory infections are frequently induced by these intracellular parasites.[6–8]

To treat infections caused by intracellular parasites, the ability of antibiotics to accumulate in phagocytic cells is essential.[9] Recently, it has been reported that several antibiotics, including rifampicin, quinolones and macrolides, are concentrated and sterilize the intracellular parasites in phagocytic cells.[10–12] In particular, macrolide antibiotics exhibit a high level of accumulation in phagocytes. Thus, macrolide antibiotics are widely used in the treatment of respiratory infections caused by intracellular parasites. Intracellular parasites are categorized as cytosolic parasitic bacteria, such as *Listeria monocytogenes*, and lysosomal parasitic bacteria, such as *L. pneumophila*.[13] Thus, detailed information about the intracellular pharmacokinetics of antibiotics in alveolar macrophages is required to optimize the treatment of infections caused by intracellular parasites.

Telithromycin (TEL) is a ketolide antibiotic and is a semisynthetic derivative of erythromycin, a macrolide antibiotic.[14] TEL has an antibacterial spectrum that covers most major respiratory pathogens, including intracellular parasites such as *C. pneumoniae*, *L. pneumophila* and *Mycoplasma pneumoniae*.[15–17] Also, TEL exhibits antibacterial activity against erm(B) and mef(A) mediated macrolide-resistant pathogens.[18,19] TEL is given by the oral route for treatment of respiratory infections and it distributes to a variety

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of tissues, particularly the lung.[20] We have reported that TEL is highly distributed in alveolar macrophages.[21] This distribution of TEL to alveolar macrophages was due to high distribution from the blood to the alveolus through vascular endothelial cells and alveolar epithelial cells, and the high uptake characteristics of alveolar macrophages.[21] However, the detailed intracellular pharmacokinetics of TEL in alveolar macrophages has not been reported yet. In this study, the intracellular pharmacokinetics of TEL in NR8383 as cultured alveolar macrophages was evaluated *in vitro*.

Materials and Methods

Materials

TEL, ammonium chloride, propranolol hydrochloride and *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide were purchased from Wako Pure Chemicals Co., Ltd (Osaka, Japan). All other reagents were commercially available and were of analytical grade.

Accumulation experiments

NR8383 cells (American Type Culture Collection, Manassas, VA, USA) were used as cultured Sprague-Dawley rat alveolar macrophages. The cells were suspended at a concentration of 1.25×10^6 cells/ml in RPMI1640 medium (Sigma Chemical Co., St Louis, MO, USA) 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) and the plates were incubated for 90 min at 37°C with 5% CO_2. After incubation, non-adherent cells were removed and then SFM medium (Gibco BRL, Life Technologies, Rockville, MD, USA) was added to the cells. TEL (50 μM) was applied to the NR8383 cells, and then the cells were incubated at 37°C or 4°C. At designated times (0.25, 0.5, 1, 2, 4, 8 and 24 h), 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. In order to calculate the concentration of TEL in NR8383 cells, the intracellular volume in NR8383 cells was determined by a velocity gradient centrifugation technique using ^{3}H-water and this was estimated to have a mean value of 4.2 μL/mg cell protein.[22] The concentration of TEL in each sample was measured by high-performance liquid chromatography as reported previously.[21] The protein concentration in the NR8383 cell extracts was determined using Coomassie protein assay reagent (Pierce Chemical Company, Rockford, IL, USA) with bovine serum albumin as a standard.[23] In the accumulation inhibition experiments, NR8383 cells were pretreated with ammonium chloride (0.1, 1, 10 mM) and propranolol (0.1, 1, 10 mM) as basic compounds at 37°C for 30 min for neutralization in lysosomes. After pretreatment, the medium was removed by aspiration and washed twice with medium. Then, the cells were treated with TEL (50 μM) at 37°C for 2 h.

Subcellular distribution experiments

NR8383 cells were treated with TEL (50 μM) at 37°C or 4°C for 2 h and then collected and homogenized in 250 mM sucrose, 1 mM ethylene glycol-bis (2-amino-ethylether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid and 3 mM imidazole (pH 7.4). In the group treated with ammonium chloride, NR8383 cells were pretreated with 1 mM ammonium chloride at 37°C for 30 min for neutralization in the lysosomes, and then treated with TEL (50 μM) at 37°C for 2 h. The homogenate was separated into a nuclear fraction (including nuclei and cell bulk) and another extract by low speed centrifugation (1600*g*, 10 min, 4°C). This extract was separated into a granules fraction (including intracellular organelles) and a soluble fraction (including cytosol) by high speed centrifugation (145 000*g*, 30 min, 4°C). In each fraction, the amount of TEL was measured by high-performance liquid chromatography as reported previously.[21] The activity of lactate dehydrogenase (LDH) as a marker enzyme of the soluble fractions (cytosol) was assayed by the LDH-Cytotoxic Test (Wako Pure Chemicals Co., Ltd) using LDH from chicken heart as a standard.[24] The activity of *N*-acetyl-β-glucosaminidase as a marker enzyme of the granules fractions (lysosome) was assayed by cleavage of nitrophenol from *p*-nitrophenyl-β-acetyl-D-glucosamide. Briefly, the soluble fractions (500 μl) were mixed with 10 mM *p*-nitrophenyl-β-acetyl-D-glucosamide dissolved in sodium citrate buffer (20 mM, pH 4.4), and then the mixture was incubated at 37°C for 15 min. The reaction was stopped with sodium carbonate buffer (10 mM, pH 10.7), and the reaction product was measured by spectrophotometry at 405 nm.[25]

Statistics

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

Results

Accumulation of TEL in NR8383 cells

The accumulation characteristics of TEL in NR8383 cells are shown in Figure 1. The accumulation of TEL was temperature dependent. The accumulation of TEL reached saturation after 2 h of incubation at 37°C and the equilibrium intracellular to extracellular concentration ratio (I/E) was approximately 40. The rate of accumulation at 4°C was considerably slower than that at 37°C, and the maximum I/E was 28.6.

Effects of basic compounds on the accumulation of TEL in NR8383 cells

The effects of basic compounds on the accumulation of TEL in NR8383 are shown in Figure 2. Both ammonium chloride and propranolol significantly inhibited the accumulation of TEL.

Subcellular distribution of TEL in NR8383 cells

The subcellular distribution of TEL and marker enzymes between the granules fraction and the soluble fraction is shown in Figure 3. *N*-Acetyl-β-glucosaminidase as a marker of lysosomes was found mainly in the granules fraction, whereas LDH as a marker of cytosol was found mainly in the soluble fraction. In the control group, approximately 57% of TEL was in the granules fraction, and 36% was in the soluble fraction. In the group treated at a temperature of 4°C, the

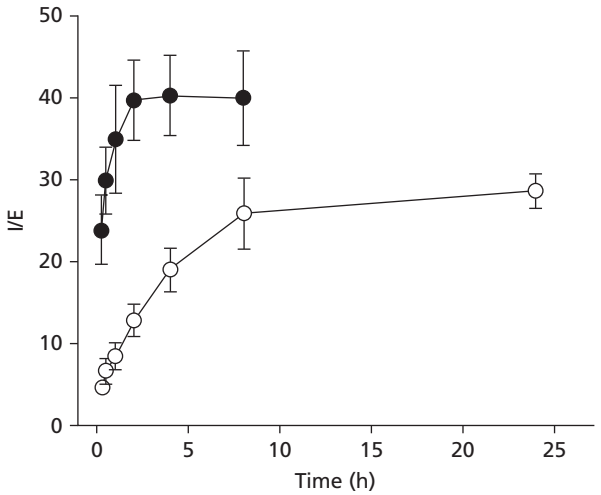

Figure 1 Cellular accumulation of telithromycin in NR8383 cells. Telithromycin (50 μM) was applied to NR8383 cells, followed by incubation at 37°C (●) or 4°C (○). The intracellular concentration of telithromycin was determined at 0.25, 0.5, 1, 2, 4, 8 and 24 h after incubation. The data is shown as the intracellular to extracellular concentration ratio (I/E). Each point represents the mean ± SD (n = 4–5).

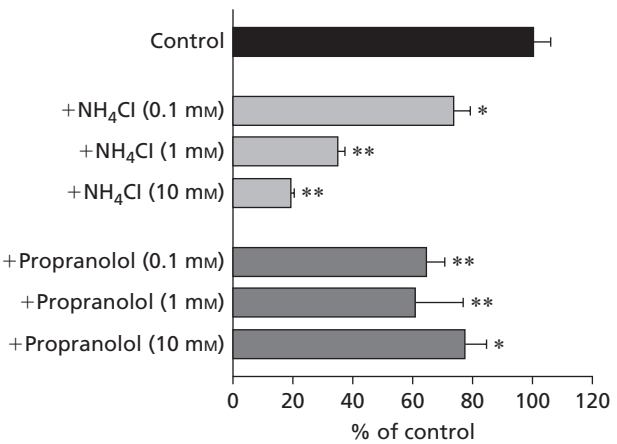

Figure 2 Effects of ammonium chloride and propranolol as basic compounds on the accumulation of telithromycin in NR8383 cells. NR8383 cells were pretreated with basic compounds at 37°C for 30 min for neutralization in lysosomes, and then treated with telithromycin (50 μM) at 37°C for 2 h. After incubation, the intracellular concentrations of telithromycin were determined. Each point represents the mean ± SD (n = 4–5) *P < 0.05, **P < 0.01, significantly different compared with the control.

distribution in the soluble fraction was 20% of the control, but no effect on accumulation in the granules fraction was observed. On the other hand, in the group treated with ammonium chloride, the accumulation in the granules fraction was 15% of that in the control.

Discussion

TEL exhibited high accumulation in NR8383 cells at 37°C, and the equilibrium I/E was approximately 40 (Figure 1).

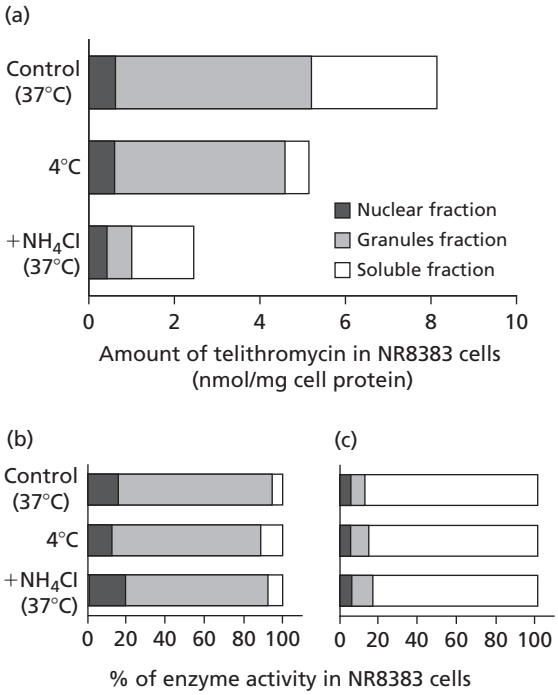

Figure 3 Subcellular distribution of telithromycin (a), *N*-acetyl-β-glucosaminidase (b) and lactate dehydrogenase (c) as marker enzymes. Telithromycin (50 μM) was applied to NR8383 cells, followed by incubation at 37°C or 4°C for 2 h. In the group treated with ammonium chloride, NR8383 cells were pretreated with 1 mM ammonium chloride at 37°C for 30 min for neutralization in lysosomes. After incubation, the cells were homogenized and the nuclear, granules and soluble fractions were collected. The amount of telithromycin and marker enzymes in each fraction was determined.

This value was higher compared with the values in other macrophages, such as THP-1 (human), RAW 264.7 (mouse) and J774 (mouse) cell lines.[26,27] Seral *et al*. reported that the I/E of TEL in J774 was increased in the presence of P-glycoprotein inhibitors.[28] P-glycoprotein is also expressed in RAW 264.7 and THP-1.[29,30] However, the accumulation of TEL in NR8383 was unaffected by verapamil and cyclosporin A as P-glycoprotein inhibitors (data not shown). P-glycoprotein might not be expressed or be poorly expressed in NR8383 cells. In contrast, at a low temperature (4°C), the rate of intracellular accumulation was slow (Figure 1). In addition, we have reported that the accumulation of TEL is inhibited by ATP depletors.[21] These findings suggest that the intracellular accumulation of TEL in alveolar macrophages is mediated by active influx systems. According to previous reports,[31,32] TEL may be transported to the intracellular region of alveolar macrophages via active transport systems that require Ca^{2+} and protein kinase A dependent phosphorylation, the same as in other phagocytes, such as human polymorphonuclear neutrophils and J774 murine macrophages.[30,31,33]

The I/E was 26.3 even when the accumulation experiments were performed at a low temperature (4°C) (Figure 1). This result suggests that energy-independent mechanisms, such as membrane-binding and pH-dependent distribution,

contribute to the high accumulation of TEL in alveolar macrophages. It is well known that the ionized forms of weak organic acids and bases diffuse much more slowly through biological membranes than their unionized forms. Therefore, basic compounds, such as macrolide antibiotics, ammonium chloride, propranolol and fluoroquinolone antibiotics, tend to become concentrated in acidic organelle compartments including lysosomes (pH 5).[34–37] The accumulation of TEL was markedly inhibited by ammonium chloride and propranolol (Figure 2). It has been reported that the uptake of propranolol by alveolar macrophages is inhibited in the presence of basic compounds (pKa > 7), such as procaine, lidocaine and nicotine.[38] Since TEL is also a basic compound (pKa = 2.4, 5.0 and 8.7),[39] it probably distributes to acidic organelle compartments. Basic compounds are included in many clinical medicines, such as imipramine, haloperidol and lidocaine. If these basic compounds are used with TEL, lysosomes in alveolar macrophages are neutralized and then the accumulation of TEL is reduced. Thus, the antimicrobial effects of TEL against intracellular parasites may be reduced by drug–drug interactions between TEL and these basic compounds. The combined medication of TEL and basic compounds should be done with caution.

The subcellular distribution of TEL in the soluble fraction, including cytosol and the granules fraction including lysosomes, was 36 and 57%, respectively, of the total amount of TEL accumulated in NR8383 cells (Figure 3). These subcellular distribution characteristics of TEL resemble that of several other weakly basic compounds.[34] These drugs also exhibit high accumulation in cells, and cell fractionation studies have demonstrated their accumulation, at least partially, in lysosomes.[40,41] The subcellular distribution of TEL in the granules fraction was markedly reduced in the presence of ammonium chloride (Figure 3). These results provide support for the hypothesis that TEL is distributed in lysosomes of alveolar macrophages. Although the antibacterial activity of TEL is weak at a low pH,[42] the volume of lysosomes represent only 2.5% of the cell volume.[43] Thus, it can be calculated that the concentration of TEL in lysosomes is 900-fold higher than the extracellular concentration and sufficient to treat parasitic infections in lysosomes. At a low temperature (4°C), the subcellular distribution of TEL in the soluble fraction is markedly reduced (Figure 3). It can be calculated that the concentration of TEL in cytosol is approximately 3-fold higher than the extracellular concentration. At a low temperature (4°C), accumulation of TEL in cytosol was based on passive diffusion and protein binding rather than active transport. This result suggests that TEL is taken up via active influx systems by alveolar macrophages and distributed in the cytosol in its protein unbound form. These findings indicate that both cytosolic parasitic bacteria and lysosomal parasitic bacteria in alveolar macrophages are exposed to TEL and are sterilized.

Conclusions

We have described the intracellular pharmacokinetics of TEL in alveolar macrophages. This study suggests that the high accumulation of TEL in NR8383 cells is due to the high influx via active transport systems and trapping in acidic organelles, such as lysosomes. Moreover, this study provides important information for optimizing the treatment of respiratory intracellular parasitic infections based on the intracellular pharmacokinetics of antibiotics in alveolar macrophages.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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