Pharmacodynamic studies of moxifloxacin and erythromycin against intracellular *Legionella pneumophila* in an *in vitro* kinetic model

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Received 22 December 2004; returned 17 February 2005; revised 12 April 2005; accepted 15 April 2005

**Background:** Newer quinolones are highly active against *Legionella pneumophila*. Since this pathogen is intracellular, standard *in vitro* susceptibility tests may not accurately predict clinical efficacy. Few models for studies of intracellular *Legionella* have been described. In this study, we determined the pharmacodynamic activity of moxifloxacin against intracellular *L. pneumophila* in comparison with erythromycin.

**Methods:** A kinetic model for intracellular studies was constructed in which human pharmacokinetics could be simulated. The model consisted of a glass chamber with two exits and a metal rack fitting cell culture inserts. The inserts had a bottom membrane where cells could be cultured while nutrients and antibiotics passed through. The inserts were prepared with a monolayer of HEp-2 cells, which were exposed to a culture of *L. pneumophila*. At regular intervals cells were harvested and lysed, viable intracellular bacteria counted and compared with untreated controls.

**Results:** The MICs were 0.0156 mg/L for moxifloxacin and 0.5 mg/L for erythromycin. The human pharmacokinetics were simulated in the model with a mean initial antibiotic concentration of 2.4 mg/L for moxifloxacin and 8.4 mg/L for erythromycin. The mean half-life was 9 h for moxifloxacin and 3.4 h for erythromycin. At 12 h, a 2 log₁₀ reduction in bacterial counts was seen in cells treated with moxifloxacin and no regrowth was detected at 24 h. Cells treated with erythromycin showed no reduction in intracellular *L. pneumophila* at 12 h or 24 h. In experiments using static concentrations of 9 mg/L of erythromycin, similar results were obtained.

**Conclusions:** In this model, moxifloxacin exerts a significantly better antibacterial effect against intracellular *L. pneumophila* compared with erythromycin.

Keywords: HEp-2 cells, quinolones, pharmacokinetics

**Introduction**

Newer quinolones have been shown to be highly active against *Legionella pneumophila*. Few models have been described for studies of intracellular *Legionella* and they have usually been performed using static drug concentrations. A kinetic model for studies of intracellular pathogens where antibiotic concentrations were fluctuating during the experiments was developed at the Antibiotic Research Unit in Uppsala. In this model, the pathogens could be exposed to antibiotic concentration profiles simulating those obtained *in vivo*. The aim of this study was to develop this method to make it suitable for studies with *L. pneumophila* and to compare the efficacy of erythromycin and moxifloxacin against intracellular *L. pneumophila*. The bacteria were challenged with concentrations corresponding to the (non-protein bound) plasma levels of erythromycin obtained in humans after a dose of 1 g (intravenous) and 400 mg of moxifloxacin (orally).

**Materials and methods**

**Antimicrobial agents**

Moxifloxacin was supplied from Bayer AG, Wuppertal, Germany. Erythromycin and gentamicin were obtained from Apoteket AB,

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Sweden. The stock solutions were made in sterile distilled water and the following dilutions in cell culture medium.

**Bacterial culture**

*Legionella pneumophila* CCUG 29853 (Culture Collection University of Gothenburg) serogroup 1 was used. Forty-eight hours before the experiment, the strain was subcultured on buffered charcoal yeast extract (BCYE) agar, containing legionella CYE agar base CM655 (Oxoid Ltd, Basingstoke, Hampshire, UK) and 10% Supplement BCYE SR110C pH 6.9 ± 0.05 (Oxoid Ltd), and incubated at 35°C. Before the experiment, a bacterial suspension with ~10^8 cfu/mL was made in RPMI 1640 (Gibco BRL, Life Technology™, Paisley, UK), with 5% fetal calf serum, 2 mM glutamine, 20 mM HEPES and 0.05% sodium bicarbonate.

**Determination of MIC**

MICs were determined in fluid media on three different occasions. Two-fold serial dilutions of the antibiotic in yeast extract, phosphate and haemin medium (YPH) broth were made and inoculated with ~5 x 10^5 cfu/mL of the test strain and incubated at 35°C for 48 h. The MIC was defined as the lowest concentration of the antibiotic allowing no visible growth.

**Determination of antibiotic concentration**

A microbiological agar diffusion method was used. For moxifloxacin, a standardized inoculum of *Escherichia coli* MB3804 was mixed with Iso-Sensitest agar (Oxoid Ltd), and poured into plates. The standards were diluted in cell culture medium. Samples and standards were applied to agar wells. For determination of erythromycin, *Sarcina lutea* ATCC 9341 was used on Iso-Sensitest agar that was adjusted to pH 8.0.

**Cell culture**

A human epithelial cell line HEP-2 (ATCC CCL 23) was cultured without antibiotics for two passages in medium consisting of RPMI 1640 (Gibco BRL), with 5% fetal calf serum, 2 mM L-glutamine, 20 mM HEPES and 0.05% sodium bicarbonate. The cells were treated with trypsin and diluted to a concentration of 1-2 x 10^5 cells/mL. A volume of 0.75 mL of the cell suspension was seeded in Falcon cell culture inserts (Becton-Dickinson, Franklin Lake, NJ, USA) and allowed to grow at 35°C in 5% CO2 for 18 h to a monolayer.

**Intracellular in vitro kinetic model**

The model consisted of a glass chamber with two exits and a metal rack fitting cell culture inserts. The inserts had a bottom membrane with 0.45 μm pores where cells could be cultured while nutrients and antibiotics passed through. The glass chamber was connected to a pump (C6-T; Alitea AB, Stockholm, Sweden) by Santoprene tubes (Alipren; Alitea AB) and placed on a magnetic stirrer to ensure homogeneous mixing. The device was placed in a thermostat at 35°C with 5% CO2. A bacterial suspension of 1 mL containing ~5 x 10^5 cfu/mL was added to HEP-2 cells previously cultured in the inserts. The cells were thereafter incubated for 2 h in 35°C with 5% CO2. To minimize the number of extracellular bacteria, serial washing with medium containing gentamicin at a concentration of 50 mg/L was performed, followed by incubation for 1.5 h with the same medium. Finally, gentamicin was removed by washing three times with PBS. Before addition of the test antibiotics, cells from two inserts were removed for determination of the initial intracellular inoculum. Thereafter 0.2 mL of cell culture medium, with a concentration of 2.5 mg/L for moxifloxacin and 9.0 mg/L for erythromycin, was added to the cell inserts. The same cell medium was also added to the glass chamber. The initial concentrations corresponded to the maximal free serum antibiotic levels obtained in humans after a dose of 400 mg (oral) for moxifloxacin and 1 g (intravenous) for erythromycin. The pump was adjusted to simulate the elimination half-life obtained in human plasma (10 h and 3 h, respectively). Since only one dose was given and erythromycin is normally dosed several times daily, we also performed experiments with constant erythromycin concentrations at 9 mg/L. At 3, 6, 9, 12 and 24 h, cells from two inserts were harvested. The cell culture medium from the inserts was pooled and transferred to an Eppendorf tube for analysis of the antibiotic concentration. The cells were washed three times with PBS to reduce bacteria that were released from the cells to the extracellular medium. Determination of extracellular bacteria was made at each time point by viable count. The cells were removed by scraping with a plastic Pasteur pipette, transferred to Eppendorf tubes and centrifuged for 5 min at 10000 g. The pellet was lysed for 10 min in 1 mL of sterile distilled water. Viable counts of intracellular bacteria were made on BCYE agar after incubation for 72 h at 35°C. Control experiments without antibiotics were performed in parallel. All experiments were made in triplicate. The limit of detection of the assay was 1 log10 cfu/mL.

**Results**

**MICs**

The MICs were 0.0156 mg/L of moxifloxacin, 0.5 mg/L of erythromycin and 0.5 mg/L of gentamicin.

**Antibiotic concentration**

The initial antibiotic concentration in the cell culture inserts was 2.4 ± 0.2 and 8.4 ± 0.3 mg/L for moxifloxacin and erythromycin, respectively (mean ± SD). The elimination half-life was 9 ± 1 h for moxifloxacin and 3.4 ± 0.6 h for erythromycin.

**Antibacterial activity**

In cells treated with moxifloxacin, a killing of intracellular *Legionella* of 2 log10 cfu/mL was noted during the first 12 h after which time the bacterial numbers stabilized (Figure 1). In cells treated with erythromycin, only a bacteriostatic effect against *L. pneumophila* was obtained (Figure 2). Extracellular bacterial levels were at least ≥2 log10 cfu/mL below the total count at each time point, except at 24 h for erythromycin where extracellular level numbers were almost identical with the total numbers. This data point was therefore excluded. Experiments where cells were treated with a static concentration of 9 mg/L erythromycin for 24 h confirmed a bacteriostatic effect against *L. pneumophila* (data not shown).

**Discussion**

*L. pneumophila* may cause severe pneumonia with a high mortality rate. This bacterium grows intracellularly and is therefore only treatable with antibiotics having an intracellular activity such as macrolides and quinolones. For many years, erythromycin has been considered the drug of choice in the treatment of legionnaires’ disease. Various in vitro models have been
developed for studies of the antimicrobial effect against intracellular *L. pneumophila*. Common for those models is that they are using static antibiotic concentrations. To our knowledge, there are no previous studies performed in kinetic models where the cells are exposed to concentrations simulating human kinetics.

*L. pneumophila* has special fastidious growth requirements. The most commonly used growth medium is BCYE agar, containing activated charcoal which binds a range of antimicrobials such as erythromycin and moxifloxacin. In this study, we used a YPH broth instead for determination of MIC. *L. pneumophila* grew well in this medium and was suitable for MIC determinations. The MIC value obtained was within the range that has been found in other studies for strains of *L. pneumophila* serogroup 1. Since *L. pneumophila* is an intracellular pathogen, animal models or *in vitro* cellular models are important when the effectiveness of antimicrobial agents is evaluated. In the latter, the possible influence of extracellular bacteria on the results seems not always to have been taken into account. Our goal was that the difference between the total bacterial count (after cell lysis) and the extracellular amount (before lysis) throughout the experiment should be at least 2 log_{10} cfu/mL, e.g. the extracellular amount should not exceed 1%.

In our study, the effect of moxifloxacin against *L. pneumophila* showed a rapid onset and was more pronounced than for erythromycin. Even in the experiments with a constant concentration at 9 mg/L of erythromycin, only a bacteriostatic effect was noted. Our results are in accordance with those of Jonas et al. who, in experiments with human monocytes, showed that *L. pneumophila* exposed to erythromycin at 10 × MIC were not significantly killed whereas levofloxacin produced a significant killing during 24 h. In another study using Mono Mac 6 cells, a marked reduction of intracellular *L. pneumophila* was shown for several fluoroquinolones, including moxifloxacin, already at static concentrations below MIC.

In conclusion, our model seems to be a useful tool for studies of the pharmacodynamics of antimicrobial agents against *L. pneumophila*.

Acknowledgements

This study was supported with a grant from Bayer AG, Wuppertal, Germany and The Swedish Fund for Research Without Animal Experiments.

References