In vitro and in vivo activity of olamufloxacin (HSR-903) against Legionella spp.

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Received 6 May 2003; returned 12 July 2003; revised 24 September 2003; accepted 1 October 2003

The activity of the fluoroquinolone olamufloxacin (HSR-903) against Legionella spp. was studied in vitro and in vivo. The olamufloxacin MIC at which 50% of isolates are inhibited (MIC50) for 81 different Legionella spp. strains (59 type strains and 22 clinical isolates) was 0.008 mg/L, which was identical to sparfloxacin, whereas the MIC50s for erythromycin, levofloxacin and ciprofloxacin were 0.25, 0.032 and 0.032 mg/L, respectively. Olamufloxacin and sparfloxacin (at 0.008 mg/L) inhibited intracellular growth and subsequent cytotoxicity of L. pneumophila 80-045 in J774.1 macrophages, whereas levofloxacin and ciprofloxacin did not, at the same concentration. When olamufloxacin was given to the infected guinea pigs orally (5 mg/kg of body weight), peak levels in the lung were 3.02 mg/kg at 2 h post-administration, with a half-life of 3.41 h and an AUC0–12 of 12.31 mg·h/kg. The 2 day post-infection bacterial burden of the lung in the animals treated with olamufloxacin (5 and 1.25 mg/kg given orally twice a day) was much lower than in those treated with levofloxacin (same dose as olamufloxacin) or erythromycin (10 mg/kg given orally twice a day). When treated with olamufloxacin (5 mg/kg given orally twice a day) for 7 days, 11 of 12 L. pneumophila-infected guinea pigs survived for 14 days post-infection, as did all 12 guinea pigs treated with levofloxacin (5 mg/kg given orally twice a day) for 7 days. In contrast, only two of 12 animals treated with erythromycin survived and 10 of 11 died in the physiological saline group. Olamufloxacin was as effective as levofloxacin in a guinea pig model of Legionnaires’ disease. These data warrant further study of whether olamufloxacin is an option for the treatment of Legionella infections.

Keywords: fluoroquinolones, Legionnaires’ disease, Legionella pneumophila

Introduction

Several newly developed fluoroquinolones show broad-spectrum antimicrobial and potent bactericidal activity against both Gram-positive and -negative bacteria, and have been used for the treatment of respiratory infections.1 Olamufloxacin (HSR-903), (S)-(−)-5-amino-7-[(7-azaspiro[2,4]hept-5-yl)-1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-4-oxoquinoline-3-carboxylic acid methanesulphonate, is a new quinolone, synthesized by Hokuriku Seiyaku Co. Ltd (Fukui, Japan). Olamufloxacin has good in vitro and in vivo activity against a broad range of bacteria, including respiratory pathogens,2,3 such as Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Klebsiella pneumoniae, Staphylococcus aureus and Chlamydia spp.4 Legionella spp. are important pathogens causing both community-acquired5 and hospital-acquired6 pneumonia. The drugs of choice have been erythromycin and rifampicin.7 Recently, fluoroquinolones such as levofloxacin8 and ciprofloxacin9 have been reported to be more effective against Legionella infections, and these drugs are recommended for treatment of the infection.10 Several studies have shown that sparfloxacin has good activity against Legionella spp. in vitro and in vivo.11,12 In this study, the in vitro and in vivo activity of olamufloxacin against Legionella spp. was evaluated, and compared with those of erythromycin, levofloxacin, ciprofloxacin and sparfloxacin.

Materials and methods

Animals

The experimental protocol was approved by the Animal Care Ethics Committee of the Faculty of Medicine, University of the Ryukyus. Hartley

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Activity of olamufloxacin (HSR-903) against Legionella spp.

strain male guinea pigs weighing ~250 g (Japan SLC, Inc., Shizuoka, Japan) were quarantined for several days. The animals were housed in separate cages in the Animal Facility of the Faculty of Medicine, University of the Ryukyus.

Bacterial strains

Bacterial strains used in the study included 59 standard strains of different Legionella spp. and 22 clinical isolates. These strains were stocked in our laboratory, and were used for MIC determination. Clinical isolates of Legionella were identified on the basis of biochemical profile, slide agglutination test (Denka Seiken, Osaka, Japan) and a DNA hybridization technique using photobiotin-labelled bacterial DNA. The first Japanese clinical isolate of Legionella pneumophila SG1, strain 80-045, was used in the in vitro macrophage infection model and in vivo model of infection. The bacteria were cultured on buffered charcoal yeast extract supplemented with α-ketoglutarate (BCYE-α; Difco Laboratories, Detroit, MI, USA) plates at 35°C for 2–3 days for use in each experiment.

Reagents

Yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was purchased from Research Organics (Cleveland, OH, USA). The following antimicrobial agents were used in this study: olamufloxacin (Hokuriku Seiyaku Co. Ltd), ciprofloxacin (Bayer Yakuhin Ltd, Osaka, Japan), sparfloxacin (Dainippon Pharmaceutical Co. Ltd, Osaka, Japan), levofloxacin (Daiichi Pharmaceutical Co. Ltd, Tokyo, Japan), erythromycin base (Dainippon Pharmaceutical Co. Ltd) and cefotizoxime (Fujiwasa Pharmaceutical Co. Ltd, Osaka, Japan).

MIC determination

The MIC of each antimicrobial agent for Legionella was determined by the microdilution method11 using buffered yeast extract supplemented with α-ketoglutarate (BYE-α) broth. The bacterial suspension was adjusted to 1×10^6 cfu/mL and bacteria were inoculated with a MIC-2000 inoculator (Dynatech Laboratories Inc., Alexandria, VA, USA) into 100 µL of BYE-α broth containing serial dilutions of the antibiotics in the wells of a microtitre plate (inoculum size ~10^5 cfu/well). The MIC was defined as the minimum concentration of the drug that inhibited visible bacterial growth after culture at 35°C for 2 days. L. pneumophila SG1 Philadelphia 1 strain (ATCC 33152) and Streptococcus pneumoniae ATCC 49619 were used as control strains.

Intracellular antimicrobial activity testing

A murine macrophage cell line, J774.1, was used as an in vitro infection model. The macrophages were maintained in RPMI 1640 (Nipro, Osaka, Japan) supplemented with 10% fetal calf serum (Whittaker, Walkersville, MA, USA). The cells were harvested at the logarithmic growth phase and were suspended in RPMI 1640 medium at 2×10^6 cells/mL. In the next step, 100 µL of the cell suspension was allowed to adhere to a 96-well flat-bottom tissue culture plate well in humidified air with 5% CO_2 at 37°C for 12 h. J774.1 macrophages were infected with L. pneumophila SG1 80-045 at 8×10^5 cfu/well for 12 h. The extracellular fluids and bacteria were removed by aspiration, then antimicrobials were added to each well at the indicated concentrations, and the cells were incubated for 3 days. Cefotizoxime is a cephalosporin which does not penetrate into the phagocytes, and we have used this drug as negative control for intracellular activity of drugs being tested against Legionella. Its conventional MIC against the strain tested is 0.125 mg/L. The cells were harvested in 9.8 mL of sterile distilled water, and vortexed for 20 s. The bacterial suspension was appropriately diluted, and an aliquot (50 µL) of the dilutions was inoculated onto BCYE-α agar. The number of viable Legionella in each well was determined by counting cfu after incubation at 35°C for 3 days.

Minimal extracellular concentrations (MIECs) causing inhibition of intracellular growth of L. pneumophila

Activity of drugs against intracellular L. pneumophila was also evaluated using a quantitative colorimetric assay system described previously.16 The activity of each antimicrobial was evaluated by its ability to inhibit the bacterial cytopathic effect. J774.1 macrophages were infected with L. pneumophila SG1 80-045 at 8×10^5 cfu/well for 12 h. After the extracellular bacteria were washed out, antibiotics were added to the wells at the indicated concentrations.

After 72 h of culture, viability of macrophages was quantified by a rapid colorimetric assay using the tetrazolium dye procedure. Viable macrophages can cleave the dye and develop colour. The supernatant and extracellular bacteria were removed after the culture with bacteria and antimicrobials indicated. MTT (0.5 mg/mL) in 100 µL of medium was added to each well. After incubation at 37°C for 90 min, the supernatant was removed and 100 µL of isopropyl alcohol containing 0.04 M HCl and 0.01% sodium dodecyl sulphate was added to each well. After vigorous shaking of the microplates to lyse the cell monolayers, the absorbance of each well was measured at 550 nm with an automatic plate reader. Treatment of Legionella-infected J774.1 cells with two-fold dilutions of the drug allowed measurement of the dose–response curve of each drug against the cytopathic effect of the bacteria and determination of the 50% inhibitory concentration of each drug for the bacterial cytopathic effect. The minimal concentration of drug resulting in >50% inhibition of the bacterial cytopathic effect was defined as the MIEC of each drug, representing a quantitative indicator of the intracellular activity of the antibiotic against Legionella.

Experimental L. pneumophila pneumonia in guinea pigs

An experimental Legionella infection was induced in guinea pigs according to the method described previously.17 Plate-grown bacteria were subcultured in BYE-α broth at 35°C for 24 h. Bacteria were harvested and re-suspended in sterile physiological saline. Guinea pigs were anaesthetized by injection of a solution containing xylazine sulphate (5 mg/kg) and ketamine sulphate (80 mg/kg), and the trachea was surgically exposed under an aseptic technique. A 0.2 mL volume of the bacterial suspension (8×10^5 cfu) was injected into the exposed trachea using a 1 mL disposable sterile syringe equipped with a 26-gauge needle. The incision was closed with steel clips, and the animals were allowed to recover.

Administration of drugs started 24 h after the challenge of bacteria. A suspension of drugs in 0.2 mL of 0.5% tragacanth gum (Wako Pure Chemical Industries, Osaka, Japan) and 2.5% sucrose was administered orally to the infected animals. The therapeutic effect of olamufloxacin, levofloxacin and erythromycin in an experimental L. pneumophila pneumonia model were determined. Guinea pigs were infected intratracheally with Legionella. A drug (5 or 1.25 mg/kg for quinolones and 10 mg/kg for erythromycin17,18) was administered twice a day for 2 days, beginning 12 h after infection. The numbers of bacteria in the lungs were determined 12 h after the last administration. The MICs of olamufloxacin, levofloxacin and erythromycin were 0.008, 0.063 and 0.125 mg/L, respectively.

Concentrations of olamufloxacin in the lungs of infected guinea pigs were determined using a previously described bioassay19 with minor modification. The animals were anaesthetized as above, and the axillary artery was exposed and blood was drawn; the lungs and kidneys were then recovered aseptically. The tissue samples containing olamufloxacin were homogenized in distilled water, and stored at −80°C until analysis by thin-layer cup methods, with Escherichia coli kp used as an indicator.
organism. The homogenates were heated at 80°C for 10 min and mixed with an equal volume of 0.2 M HCl. After vigorous vortex mixing, the sample solutions were collected by centrifuging at 2000 g for 20 min at room temperature. Each sample solution was adjusted to neutral pH by adding 0.1 M NaOH, and the sample solutions were put into stainless cups.

Statistical analysis
Data were expressed as mean ± S.D. Differences between groups were tested for statistical significance using ANOVA and Schaffe’s test. A P value of <0.05 indicated a statistically significant difference.

Results
MICs
The MIC\textsubscript{50} of olamufloxacin against Legionella spp. bacteria was 0.008 mg/L, which was identical to that of sparfloxacin but lower than those of erythromycin, levofloxacin and ciprofloxacin (Table 1). No significant difference in MIC distribution of olamufloxacin between L. pneumophila and non-L. pneumophila was observed (data not shown). MICs for L. pneumophila SG1 80-045 of olamufloxacin, erythromycin, levofloxacin, ciprofloxacin and sparfloxacin were 0.032 (mg/L), 0.125, 0.063, 0.032 and 0.032, respectively.

Activity of drugs against intracellular L. pneumophila
L. pneumophila SG1 strain 80-045 grew well within J774.1 macrophages (data not shown). The activity of drugs was evaluated using this in vitro infection model. Serial four-fold dilutions of antimicrobials were added to each well, and the viable bacterial counts were determined (Figure 1). Olamufloxacin and sparfloxacin both inhibited intracellular growth of L. pneumophila at 0.008 and 0.032 mg/L, although olamufloxacin was more active at 0.032 mg/L than sparfloxacin. Levofloxacin and ciprofloxacin required more than 0.032 mg/L to inhibit the intracellular growth of bacteria. Intracellular activity of drugs was also evaluated using a colorimetric cytotoxic inhibition assay. Olamufloxacin inhibited the cytotoxicity of intracellularly multiplying L. pneumophila at much lower concentration (0.004 mg/L) than levofloxacin or ciprofloxacin (Figure 2).
Activity of olamufloxacin (HSR-903) against *Legionella* spp.

Figure 2. Inhibition of cytopathic effect of intracellular *L. pneumophila* by fluoroquinolones. J774.1 macrophages were infected with *L. pneumophila* SG1 80-045 for 12 h. After the extracellular bacteria were washed out, antibiotics were added to the wells at the indicated concentrations. After 72 h of culture the viability of macrophages was determined MTT assay. Each column represents the mean for five wells. The A\textsubscript{550} for the drug-free control was 0.001.

Figure 3. Therapeutic effects of olamufloxacin and other drugs in terms of pulmonary clearance of *L. pneumophila* SG1 strain 80-045. Guinea pigs were infected with *L. pneumophila*. Drugs were administered for 2 days. The numbers of bacteria in the lungs were determined 12 h after the last administration. The results are presented as mean ± S.D. log cfu in the lungs of five animals. *P < 0.01 versus results for control.

The results seen with sparfloxacin were identical to those seen with olamufloxacin. In a previous study,\textsuperscript{16} the MIEC for *Legionella* was defined as the minimum concentration inhibiting more than 50% of the cytotoxic effect of *L. pneumophila*. According to this definition, the MIECs of olamufloxacin, levofloxacin, ciprofloxacin and sparfloxacin for *L. pneumophila* SG1 strain 80-045 were 0.004, 0.032, 0.125 and 0.004 mg/L, respectively (Figure 2).

Therapeutic effect of drugs in the pulmonary *L. pneumophila* infection model

The protective effects of olamufloxacin, levofloxacin and erythromycin against pulmonary infection of *L. pneumophila* in guinea pigs were evaluated. When olamufloxacin was given to infected guinea pigs orally (5 mg/kg), the maximum concentrations in the lung were 3.02 mg/kg at 2 h post-administration, with a half-life of 3.41 h and AUC\textsubscript{0-12} of 12.31 mg h/kg.

**Discussion**

Olamufloxacin has a broad spectrum of activity against pathogens of pulmonary infections, and here we demonstrate that this drug has good activity against *Legionella* spp., as well. The MIC\textsubscript{50} of olamufloxacin for our laboratory collection of *Legionella* spp. was equal to that of sparfloxacin but much lower than those of erythromycin, levofloxacin and ciprofloxacin. The MIC of erythromycin, levofloxacin, ciprofloxacin for *L. pneumophila* SG1 Philadelphia-1 strain (ATCC 33152) was almost identical to that reported by Stout et al.\textsuperscript{20}

In this study, a J774.1 macrophage cell line was used to measure the antimicrobial activity of drugs against intracellular *L. pneumophila*. Walz et al.\textsuperscript{21} describe that J774 macrophages can be used for such a study and that newer quinolones have excellent intracellular activity against *L. pneumophila*. Our previous study\textsuperscript{16} showed that the results using J774.1 were almost compatible with other studies using an infection model *in vitro* and *in vivo*. In this study, olamufloxacin was more potent against intracellular *L. pneumophila* than levofloxacin and ciprofloxacin, but identical to sparfloxacin. In our previous study\textsuperscript{10} using same assay system, erythromycin required more than 1 mg/L to inhibit intracellular growth of *L. pneumophila*, and was less potent than levofloxacin. Olamufloxacin and sparfloxacin inhibited the cytotoxic effect of intracellularly multiplying *L. pneumophila* at a concentration four times lower than levofloxacin. The efficacy of erythromycin, levofloxacin,\textsuperscript{6} ciprofloxacin\textsuperscript{6} and sparfloxacin\textsuperscript{11,12} in the pulmonary infection model has been evaluated elsewhere, and all these drugs are effective. In this study, olamufloxacin was almost as effective as levofloxacin and more effective than erythromycin (20 mg/kg/day) in the pulmonary infection model. The same dosing
of erythromycin as a control has been used for experimental Legionella pneumonia in several studies.\textsuperscript{17,18}

In this pulmonary infection model, the maximum concentration ($C_{\text{max}}$) of olamufloxacin in the lung was 3.2 mg/L 2 h after a single oral dose of 5 mg/kg, with a half-life of 3.41 h and an AUC$_{0-12}$ of 12.31 mg·h/kg. The $C_{\text{max}}$ demonstrated here was much higher than the concentration of the drug that inhibited the extracellular and intracellular growth of $L.\,\text{pneumophila}$ (0.032 and 0.004 mg/mL, respectively). Reflecting these data, the bacterial burden in the lung decreased significantly after 2 day treatment of olamufloxacin, and most animals survived a 7 day treatment of the drug. It is reported that the ratio of the concentration of olamufloxacin in the lungs and plasma after a single oral 5 mg/kg dose in mice and rats is 7\textsuperscript{19} and 12.7,\textsuperscript{22} respectively. These data suggest that olamufloxacin concentrates well in the lung, while the plasma level of the drug is relatively low. In humans, olamufloxacin showed a plasma $C_{\text{max}}$ of 0.86 mg/L and a long elimination half-life (18 h) in a Phase I trial following a single dose of 200 mg daily.\textsuperscript{21} If the rodent pharmacokinetic data is extrapolated to man, the $C_{\text{max}}$ of the drug in the human lung would be 5–10 mg/L, which exceeds the concentration required to control the growth of $L.\,\text{pneumophila}$ in the lung, although the pharmacokinetic factor(s) responsible for the clinical effectiveness in Legionella pneumonia remain to be clarified.

Olamufloxacin is more potent against $L.\,\text{pneumophila}$ in vitro than erythromycin, ciprofloxacin or levofloxacin, but is similar to sparfloxacin. Oral administration of the drug was effective in a guinea pig model of Legionnaires’ disease. These data suggest olamufloxacin might be an option for the treatment of Legionella infections, and warrant further study.

Acknowledgements

We thank Tomoe Mullins for her excellent technical assistance.

References


