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Efficient Drug Delivery to Alveolar Macrophages and Lung Epithelial Lining Fluid Following Pulmonary Administration of Liposomal Ciprofloxacin in Rats with Pneumonia and Estimation of its Antibacterial Effects

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The efficacy of pulmonary administration of liposomal ciprofloxacin (CPFX) in pneumonia was evaluated. In brief, the pharmacokinetics following pulmonary administration of liposomal CPFX (particle size, 1,000 nm; dose, 200 μg/kg) were examined in rats with lipopolysaccharide-induced pneumonia as an experimental pneumonia model. Furthermore, the antibacterial effects of liposomal CPFX against the pneumonic causative organisms were estimated by pharmacokinetic/pharmacodynamic (PK/PD) analysis. The time-courses of the concentration of CPFX in alveolar macrophages (AMs) and lung epithelial lining fluid (ELF) following pulmonary administration of liposomal CPFX to rats with pneumonia were markedly higher than that following the administration of free CPFX (200 μg/kg). The time course of the concentrations of CPFX in plasma following pulmonary administration of liposomal CPFX was markedly lower than that in AMs and ELF. These results indicate that pulmonary administration of liposomal CPFX was more effective in delivering CPFX to AMs and ELF compared with free CPFX, and it avoids distribution of CPFX to the blood. According to PK/PD analysis, the liposomal CPFX exhibited potent antibacterial effects against the causative organisms of pneumonia. This study indicates that pulmonary administration of CPFX could be an effective technique for the treatment of pneumonia.

Keywords liposomal ciprofloxacin; pulmonary administration; pneumonia; alveolar macrophages and lung epithelial lining fluid; PK/PD

INTRODUCTION

Infectious diseases are the greatest cause of death in the world and the most common is pneumonia (World Health Organization, 2003). *Chlamydia pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* frequently induce infectious pneumonia (Ishida, Hashimoto, Arita, Ito, & Osawa, 1998). *C. pneumoniae* and *L. pneumophila* are taken up by alveolar macrophages (AMs) via phagocytosis in the alveolus; however, they are resistant to the biocidal mechanisms of AMs and survive and multiply intracellularly in AMs (Ferrari, Langen, Naito, & Pieters, 1999; Greub & Raoult, 2004; Harb, Gao, & Abu Kwaik, 2000; McKinney et al., 2000). In contrast, *P. aeruginosa*, *H. influenzae*, and *S. pneumoniae* avoid uptake and digestion by AMs and survive and multiply in lung epithelial lining fluid (ELF) (Densen & Mandell, 1980; Horwitz & Silverstein, 1980; Krieg, Helmke, German, & Mangos, 1988; Peterson, Verhoef, Sabath, & Quie, 1977; Stiver, Zachidniak, & Speert, 1988). For sterilization of these pneumonic causative organisms in AMs and ELF, the antibiotic concentration in AMs and ELF must be higher than the minimum inhibitory concentration (MIC). Thus, efficient delivery of antibiotics to AMs and ELF is required in order to produce an antimicrobial effect. Ciprofloxacin (CPFX), a fluoroquinolone antibiotic, produces its antibacterial effects by inhibition of DNA gyrase and topoisomerase IV (Pan & Fisher, 1997; Smith, Nichol, Hoban, & Zhanel, 2003); it also has a wide antibacterial spectrum and is effective against the pneumonic causative organisms as described above (Andes & Craig, 2003, Dubois & St-Pierre, 2000; Harnett et al., 2004; Ikaheimo, Syrjala, Karhukorpi, Schildt, & Koskela, 2000; Onodera, Tanaka, & Sato, 2001; Otani et al., 2003; Otsu et al., 2003; Safdar & Armstrong, 2003; Takahata et al., 1999). At present, in clinical situations, CPFX is given orally, but the development of a pulmonary administration system for CPFX would be an important advance if it were possible to enhance its antibacterial effect, reduce the dose, and avoid systemic side effects, such as hypoglycemia, QT interval prolongation, and convulsions (Mohr et al., 2005; Patmore, Fraser, Mair, & Templeton, 2000; Tattevin, Messiaen, Pras, Ronco, & Biour, 1998). We have reported that pulmonary administration of CPFX is more effective in the

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treatment of respiratory intracellular parasitic infections, compared with oral administration, despite a low dose, and it avoids distribution of CPFX to the blood (Chono, Tanino, Seki, & Morimoto, 2007). We have also shown that pulmonary administration of liposomal CPFX at a particle size of 1,000 nm to rats is more efficient than free CPFX for drug targeting to AMs and sustained drug distribution in ELF (Chono, Tanino, Seki, & Morimoto, 2006). However, the efficacy of pulmonary administration of liposomal CPFX for the treatment of pneumonia is unknown.

In this study, to evaluate the efficacy of pulmonary administration of liposomal CPFX in pneumonia, the drug delivery to AMs and ELF following pulmonary administration of liposomal CPFX was examined in rats with lipopolysaccharide-induced pneumonia. Furthermore, the antibacterial effects of liposomal CPFX against the pneumonic causative organisms were estimated by pharmacokinetic/pharmacodynamic (PK/PD) analysis.

MATERIALS AND METHODS

Materials and Animals

CPFX was purchased from Sigma Chemical Co. (St Louis, MO, USA). Hydrogenated soybean phosphatidylcholine (HSPC) was purchased from NOF Co. (Tokyo, Japan), cholesterol (CH) from Wako Pure Chemicals Co., Ltd. (Osaka, Japan), and dicetylphosphate (DCP) from Sigma Chemical Co. Lipopolysaccharide from *Pseudomonas aeruginosa* was purchased from Sigma Chemical Co. All other regents were commercially available and of analytical grade. Male SD rats (200–250 g) were purchased from Japan SLC (Shizuoka, Japan). The animal experimental plan used was approved by the Committee of the Laboratory Animal Center and conforms to the Guiding Principles for the Care and Use of Experimental Animals in Hokkaido Pharmaceutical University.

Preparation of Liposomal CPFX

Liposomal CPFX was prepared by the lipid thin film hydration method (Chono et al., 2006). Briefly, HSPC, CH, and DCP in a lipid molar ratio of 7/2/1 were dissolved in chloroform/methanol (9/1), followed by evaporation to obtain a thin film. The film was completely hydrated using CPFX solution to obtain liposomal CPFX. CPFX solution using hydration was prepared by dissolution of CPFX in 50 mM phosphate buffer (pH 5.5). The liposomal CPFX was extruded three times through polycarbonate filters with pore sizes of 1,000 nm (Whatman, Florham Park, NJ, USA). The particle sizes were determined by photon correlation spectroscopy using a Coulter N4 plus a submicron particle analyzer (Coulter Co., Miami, FL, USA). The particle size distribution of the resulting liposomal CPFX ($M \pm SD$) was 989.1 ± 94.4. The zeta potential was determined by a laser Doppler method using a zeta potential analyzer (Zeta Plus, Nikkiso Co., Ltd., Tokyo, Japan). The zeta potential of the resulting liposomal CPFX was approximately –70 mV. The concentration of CPFX was measured by high-performance liquid chromatography (HPLC) as reported previously (Chono et al., 2006). The concentrations of CPFX and CPFX/lipid molar ratio in the resulting liposomal CPFX were 2 μmol CPFX/mL and 0.1 mol CPFX/mol total lipids, respectively.

Preparation of Rats with Pneumonia

An pneumonia model was used involving rats with lipopolysaccharide-induced pneumonia by modifying the method of Alba-Loureiro et al. (2006). Briefly, lipopolysaccharide solution was administered to rat lungs at a dose of 1.25 mg/250 μL/kg via the nasal cavity using a Liquid MicroSprayer™ (Model IA-1C; PennCentury, Inc., Philadelphia, PA, USA) under pentobarbital anesthesia. The development of pneumonia was checked by monitoring the nitric oxide levels in the ELF fraction. At 24 h after administration of lipopolysaccharide, the trachea were cannulated and the lungs were lavaged with 5 mL ice-cold phosphate-buffered saline (PBS, pH 7.4) (Antonini & Reasor, 1991). The bronchoalveolar lavage fluid was collected and centrifuged at 4°C (650 × g for 10 min) to separate the ELF fraction and AMs. The nitric oxide levels in the ELF fraction were determined using a NO_2/NO_3 Assay Kit-CII (DOJINDO Laboratories, Kumamoto, Japan). The nitric oxide levels in the ELF fraction from untreated normal rats and rats with pneumonia were 0.101 ± 0.003 μM and 9.1 ± 1.0, respectively ($p < 0.05$). Typical pulmonary images of untreated normal rat and rat with pneumonia at 24 h after administration of lipopolysaccharide are shown in Figure 1.

Experiment Involving Pulmonary Administration

Liposomal CPFX or free CPFX, as a comparison, was administered to rat lungs at a dose of 200 μg CPFX/250 μL/kg via the nasal cavity using a Liquid MicroSprayer™ (Model IA-1C; PennCentury, Inc.) under pentobarbital anesthesia. Free CPFX solution was prepared by dissolution of CPFX in 50 mM phosphate buffer (pH 5.5). The dose of CPFX (200 μg/kg) used in this study was approximately 1/150th of the clinical dose.

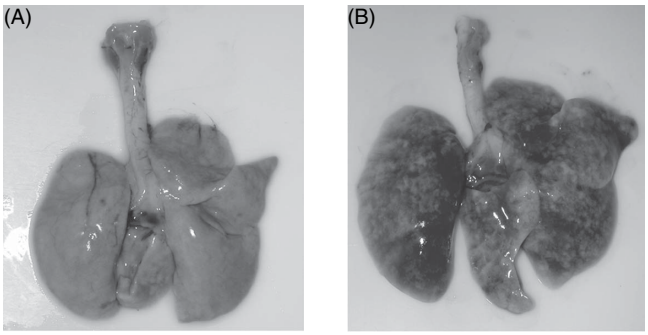

FIGURE 1. The pulmonary images on an untreated normal rat (A) and a rat with lipopolysaccharide-induced pneumonia (B).

At the indicated time points after administration, blood was collected from the jugular vein under pentobarbital anesthesia. The trachea were immediately cannulated and the lungs were lavaged three times with 5 mL ice-cold PBS, pH7.4 (Antonini & Reasor, 1991). The bronchoalveolar lavage fluid was immediately centrifuged at 4°C (650 × *g* for 10 min) to separate the ELF fraction and AMs. Then, the AMs were extracted with 1 mL of 0.1 M NaOH solution for quantitative analysis. The apparent volume of ELF was estimated using urea, an endogenous marker of ELF dilution (Rennard et al., 1986). The mean value estimated in this study was 395 μL/225 g rat. The intracellular volume of the AMs was determined by a velocity-gradient centrifugation technique using ^{3}H-water (Kohno, Yoshida, Suwa, & Suga, 1990) and was estimated to have a mean value of 4.2 μL/mg cell protein. The protein concentration in the cell extracts was determined using Coomassie Protein Assay reagent (Pierce Chemical Company, Rockford, IL, USA) with bovine serum albumin as a standard (Branford, 1976). The concentrations of CPFX in AM extracts, the ELF fraction, and plasma were measured by HPLC as reported previously (Chono et al., 2006). The concentrations of CPFX in AMs and ELF were calculated by normalization with the intracellular volume of the AMs and apparent volume of ELF, respectively.

Data Analysis

For the PK analysis, the area under the CPFX concentration–time curve in AMs, ELF, and plasma from time 0 to 24 h (AUC) was calculated by the trapezoidal rule. The antibacterial effects of CPFX in AMs and ELF following pulmonary administration were evaluated by PK/PD analysis. The concentration of CPFX in AMs and the ELF–time curve (AUC)/MIC of CPFX at which 90% of isolates (MIC_{90}) ratio and the maximum concentration of CPFX in AMs and the ELF (C_{max})/MIC ratio were calculated as the PK/PD parameters reflecting the antibacterial effects. The MIC_{90} values against pathogenic microorganisms resisting sterilization systems of AMs were taken from the literature. The effective values of AUC/MIC_{90} and C_{max}/MIC_{90} were greater than 125 and 12, respectively (Blaser, Stone, Groner, & Zinner, 1987; Craig, 1995; Forrest et al., 1993; Hyatt, McKinnon, Zimmer, & Schentag, 1995; Moore, Lietman, & Smith, 2005; Toutain, del Castillo, & Bousquet-Melou, 2002).

RESULTS AND DISCUSSION

In this study, the drug delivery to AMs and ELF following pulmonary administration of liposomal CPFX was examined in rats with pneumonia. Furthermore, the antibacterial effects of liposomal CPFX against the pneumonic causative organisms were estimated.

The time courses of the concentrations of CPFX in AMs, ELF, and plasma after pulmonary administration of liposomal CPFX and free CPFX to rats are shown in Figure 2. The time-courses of the concentrations of CPFX in AMs and ELF following administration of liposomal CPFX to rats with pneumonia were markedly higher than those following administration of free CPFX (Figure 2A and B). These results indicate that liposomal encapsulation of CPFX is useful for enhancing the targeting efficiency of CPFX to AMs and providing sustained distribution of CPFX in ELF. These findings also suggest that the high targeting efficiency of CPFX to AMs by

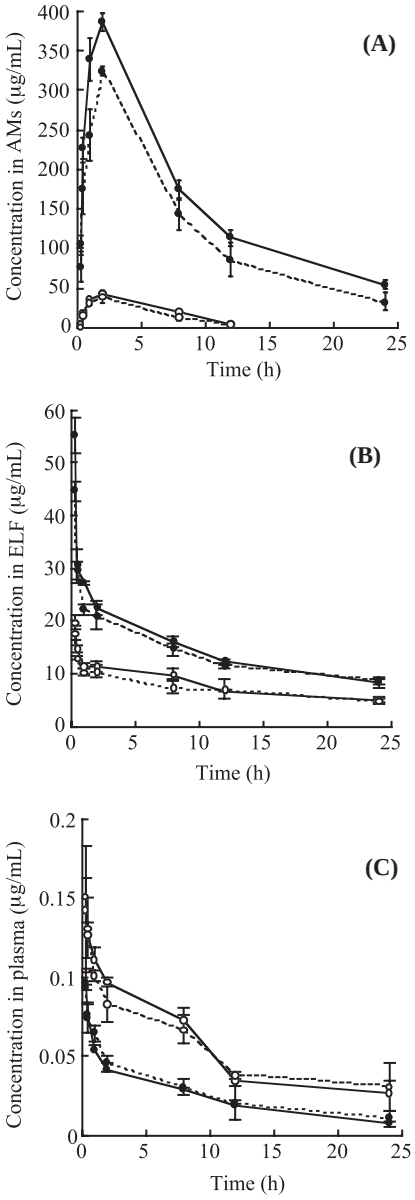

FIGURE 2. Time-courses of concentrations of ciprofloxacin (CPFX) in alveolar macrophages (AMs) (A), epithelial lining fluid (ELF) (B), and plasma (C) after pulmonary administration of liposomal CPFX (•) and free CPFX (○) in untreated normal rats (dotted line) and rats with pneumonia (solid line). The liposomal CPFX or free CPFX (200 μg/250 μL/kg as CPFX) was administered to rat lungs using a Liquid MicroSprayer™. At each time point after administration, AMs, ELF, and plasma were collected and the concentrations of CPFX were determined. Each value represents the $M \pm SE$ (n = 3–4).

liposomal CPFX is due to the sustained distribution in ELF as well as the high uptake by the AMs themselves. Our previous study indicated that several liposomal formulations were taken up by AMs under conditions that incorporate drugs into the alveolus, and their formulations sustained distribution of drugs in ELF (Chono, Tanino, Seki, & Morimoto, 2008; Chono et al., 2006). In contrast, the time courses of the concentrations of CPFX in plasma following administration of liposomal CPFX to rats with pneumonia were lower than those following administration of free CPFX (Figure 2C). This result indicates that the distribution of CPFX to blood is avoided by liposomal encapsulation. Reduced distribution of CPFX to the blood will help avoid systemic side effects. The concentration of CPFX in AMs after administration of liposomal CPFX to rats with pneumonia was higher than that in untreated normal rats at each time point although the concentrations of CPFX in ELF and plasma in rats with pneumonia were similar to those in untreated normal rats (Figure 2). According to the nitric oxide levels in the ELF fraction, the activity of AMs in rats with pneumonia is higher than that in untreated normal rats. The uptake activity of CPFX by AMs in rats with pneumonia may have been higher than that in untreated normal rats. Thus, it is thought that the concentration of CPFX in AMs of rats with pneumonia was higher than that in untreated normal rats. The pharmacokinetic parameters of CPFX in AMs, ELF, and plasma following pulmonary administration to rats with pneumonia are summarized in Table 1. The AUC of CPFX in AMs and ELF following administration of liposomal CPFX were 4,289 and 352 μg h/mL, respectively, and the AUC ratios of AMs and ELF to plasma were 7,148 and 587, respectively (Table 1).

The estimated antibacterial effects of CPFX in AMs and ELF following pulmonary administration of liposomal CPFX and free CPFX to rats with pneumonia are summarized in Tables 2 and 3. Recently, there has been increasing interest in the relationship between the PK and the PD of antibiotics, and therefore, the use of PK/PD parameters is now widespread (Mouton, Dudley, Cars, Derendorf, & Drusano, 2002, 2005). It is now generally accepted that PK/PD analysis of antibiotic treatment is important for selecting a suitable dose and optimizing the treatment of individual patients. The effects of antibiotics are concentration- and/or time-dependent and the PK/PD parameters used generally are C_{max}/MIC, AUC/MIC, and the time above the MIC. Because the antibacterial effects of fluoroquinolones such as CPFX depend on the AUC/MIC or C_{max}/MIC (Forrest et al., 1993; Preston et al., 1998), the values of these in AMs and ELF following administration of CPFX were calculated in this study. The AUC/MIC and C_{max}/MIC of liposomal CPFX against the pneumonic causative organisms that survive and multiply in AMs and ELF were greater than the effective values (AUC/MIC, >125; C_{max}/MIC, >12) despite the use of one-fiftieth of the clinical oral dose (Tables 2 and 3). Because the values of AUC/MIC and C_{max}/MIC of liposomal CPFX against *L. pneumophila* and *H. influenzae* were so great, it may be possible to reduce the dose for sterilization of these pneumonic causative organisms (Tables 2 and 3). The AUC/MIC and C_{max}/MIC of free CPFX against organisms except for *S. pneumoniae* were also higher than the effective values (Tables 2 and 3). However, distribution of CPFX to blood after administration of free CPFX is higher than that produced by liposomal CPFX (Figure 2C). Thus, free CPFX may not be efficient, compared with liposomal CPFX, if a reduction in the dose and avoidance of systemic side effects are required. CPFX inhibits the production of cytokines, such as interleukin-1 (IL-1), IL-6, or tumor necrosis factor alpha (TNF-α), by lipopolysaccharide-stimulated human monocytes (Dalhoff &

TABLE 1
The Pharmacokinetic Parameters of CPFX in AMs, ELF, and Plasma Following Pulmonary Administration to Pneumonia Rats

Administration	Tissues	AUC (μg h/mL)[a]	C_{max} (μg/mL)[b]	T_{max} (h)[c]	AUC ratio[d]
Liposomal CPFX	AMs	4,289	387 ± 11	2	7,148
	ELF	352	55 ± 3	0.25	587
	Plasma	0.6	0.10 ± 0.01	0.25	1
Free CPFX	AMs	341[e]	44 ± 3	2	262
	ELF	194	20 ± 1	0.25	149
	Plasma	1.3	0.15 ± 0.01	0.25	1

AMs, alveolar macrophages; AUC, area under the curve; CPFX, ciprofloxacin; ELF, epithelial lining fluid.
Pharmacokinetic parameters were obtained from data shown in Figure 2. AUC are represented as mean value. C_{max} are represented as $M \pm SE$.
[a]AUC from time 0 to 24 h.
[b]The maximum concentration of CPFX.
[c]The time to reach C_{max} after administration.
[d]The ratio to AUC in plasma.
[e]AUC of free CPFX was calculated using 0 μg/mL as concentration of CPFX at 24 h after administration because concentration at 24 h was less than determination limit.

TABLE 2
The Estimated Antibacterial Effects Against the Pneumonic Causative Organisms Which Survive and Multiply Intracellularly in AMs

Administration	Organisms (MIC_{90})	AUC/MIC_{90} (h)	C_{max}/MIC_{90}
Liposomal CPFX	*C. pneumoniae* (2 μg/mL)[a]	2,145	194
	L. pneumophila (0.06 μg/mL)[b]	71,483	6,450
Free CPFX	*C. pneumoniae* (2 μg/mL)[a]	171	22
	L. pneumophila (0.06 μg/mL)[b]	5,683	733

AMs, alveolar macrophages; AUC, area under the curve; CPFX, ciprofloxacin; MIC, minimum inhibitory concentration; PK/PD, pharmacokinetic/pharmacodynamic.

AUC and C_{max} in AMs as described Table 1 were used for calcuration of PK/PD parameters. The MIC_{90} values were taken from the literature.

[a]Miyashita et al. (1997).

[b]Otani et al. (2003).

TABLE 3
The Estimated Antibacterial Effects Against the Pneumonic Causative Organisms Which Survive and Multiply in ELF

Administration	Organisms (MIC_{90})[a]	AUC/MIC_{90} (h)	C_{max}/MIC_{90}
Liposomal CPFX	*P. aeruginosa* (1 μg/mL)	352	55
	H. influenzae (0.03 μg/mL)	11,733	1,833
	S. pneumoniae (2 μg/mL)	176	28
Free CPFX	*P. aeruginosa* (1 μg/mL)	194	20
	H. influenzae (0.03 μg/mL)	6,467	667
	S. pneumoniae (2 μg/mL)	97 (↓)	10 (↓)

AUC, area under the curve; CPFX, ciprofloxacin; ELF, epithelial lining fluid; MIC, minimum inhibitory concentration; PK/PD, pharmacokinetic/pharmacodynamic.

AUC and C_{max} in ELF as described Table 1 were used for calcuration of PK/PD parameters. The MIC_{90} values were taken from the literature.

Arrows are shown less than effective value.

[a]Neu et al. (1992).

Shalit, 2003) and activation of IL-6 and IL-8 in a cystic fibrosis epithelial cell line (Blau, Klein, Shalit, Halperin, & Fabian, 2007). Therefore, the anti-inflammatory effects of liposomal CPFX may also be effective in addition to its antibacterial effects for the treatment of pneumonia. In this study, we focused on drug delivery to AMs and ELF following pulmonary administration of the liposomal CPFX and then antibacterial effects were estimated by PK/PD analysis. The antibacterial and anti-inflammatory effects following pulmonary administration of liposomal CPFX to animals with bacterial infections should be investigated in more detail in future studies.

CONCLUSION

We have shown that efficient targeting of CPFX to AMs and ELF in rats with pneumonia is possible following pulmonary administration of liposomal CPFX. Furthermore, it was estimated that the antibacterial effect against the pneumonic causative organisms following pulmonary administration of liposomal CPFX is exhibited at a dose lower than that used clinically. These findings indicate that pulmonary administration of liposomal CPFX could be an efficient method for the treatment of pneumonia.

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