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ORIGINAL ARTICLE

Efficient drug delivery to lung epithelial lining fluid by aerosolization of ciprofloxacin incorporated into PEGylated liposomes for treatment of respiratory infections

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Abstract

Purpose: The efficacy of aerosolization of ciprofloxacin (CPFX) incorporated into PEGylated liposomes (PEGylated CPFX-liposomes) for the treatment of respiratory infections was evaluated. *Method*: PEGylated CPFX-liposomes with 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000] (particle size: 100 nm) were prepared, and the drug distribution characteristics in lung epithelial lining fluid (ELF) following aerosolization of PEGylated CPFX-liposomes were examined in rats. Furthermore, the antibacterial effects of PEGylated CPFX-liposomes in ELF were evaluated by pharmacokinetic/pharmacodynamic analysis. *Results*: The elimination rate of CPFX from ELF following aerosolization of PEGylated CPFX-liposomes was significantly slower than that of CPFX incorporated into unmodified liposomes (unmodified CPFX-liposomes; particle size: 100 nm). According to pharmacokinetic/pharmacodynamic analysis, the PEGylated CPFX-liposomes exhibited potent antibacterial effects against pathogenic microorganisms in ELF. *Conclusion*: This study shows that PEGylated CPFX-liposomes are a useful aerosol-based pulmonary drug delivery system for the treatment of respiratory infections.

Key words: Aerosol-based pulmonary drug delivery system, ciprofloxacin, lung epithelial lining fluid, PEGylated liposomes, respiratory infections

Introduction

In the alveolus, the epithelial lining fluid (ELF) is a mucous layer with a pulmonary surfactant covering the apical surface of alveolar epithelial cells and alveolar macrophages (AMs) associated with biophylaxis in the ELF[1,2]. Several pathogenic microorganisms, such as *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*, avoid uptake and digestion by AMs, and survive or multiply in the ELF[3–7]. Thus, respiratory infections are frequently induced by these pathogenic microorganisms. For sterilization of these pathogenic microorganisms in ELF, the antibiotic concentration in ELF must be higher than the minimum inhibitory concentration for as long as possible. Consequently, direct and sustained delivery of antibiotics to the ELF is required to produce a significant antimicrobial effect. Clinically, antibiotics are generally given orally for the treatment of respiratory infections induced by pathogenic microorganisms as described above. However, because antibiotics distribute to many different tissues through the blood after oral administration, systemic side effects are frequently induced[8]. In contrast, an aerosol-based pulmonary drug delivery system (pDDS) is an efficient method for delivering antibiotics directly to the ELF. Thus, a reduction in the dose and avoidance of systemic side effects could be achieved by aerosol-based pDDS. Again, the use of drug carriers in pDDS may produce sustained distribution of antibiotics in the ELF.

PEGylated liposomes, which are able to circulate for a long time in the blood, have been widely used as a drug carrier to improve the blood circulation lifetime of entrapped therapeutic agents[9]. It is believed that the PEG on the surface of liposomes, providing a steric barrier against the attachment of plasma proteins such as

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opsonins and recognition by the cells of the mononuclear phagocyte system, in turn, results in a decrease in the rate of clearance of the liposomes from the blood circulation[10]. Surfactant proteins in ELF opsonize foreign substances, and these are recognized and digested by AMs[11–14]. Thus, aerosol-based PEGylated liposomes may deliver antibiotics efficiently to the ELF by avoiding opsonization by surfactant proteins and reducing recognition by AMs.

We and other groups have reported that inhalation of antibiotics solution compared with oral administration is more useful for treatment of respiratory infections[15–17]. However, antibiotics generally give rise to resistant bacteria depending on the concentration–time pattern[18]. Thus, if the mutant prevention effect is considered as well as the antimicrobial effect, a sustained high concentration for a long time must be obtained by designing pulmonary administration systems using drug carriers. We have also reported that aerosolized surface-nonmodified liposomes efficiently deliver antibiotic to ELF[19]. However, a sustained high concentration of antibiotic in ELF was not obtained because liposomes encapsulating antibiotic was phagocytosed by AMs. Hence, the development of aerosolized liposomes that produce sustained distribution of antibiotics in the ELF is expected.

In this study, ciprofloxacin (CPFX), used as a model fluoroquinolone antibiotic, was incorporated into PEGylated liposomes (PEGylated CPFX-liposomes; particle sizes: 100 nm), and then the drug distribution characteristics to the ELF following aerosolization were investigated in rats. Furthermore, the antibacterial effects against pathogenic microorganisms in ELF following aerosolization of PEGylated CPFX-liposomes (dose: 200 μg/kg as CPFX) were evaluated using pharmacokinetic/pharmacodynamic (PK/PD) analysis.

Materials and methods

Materials and animals

CPFX was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogenated egg phosphatidylcholine and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000] were purchased from NOF Co. (Tokyo, Japan), cholesterol was obtained from Wako Pure Chemicals Co., Ltd. (Osaka, Japan), and dicetylphosphate was obtained from Sigma Chemical Co. [^{3}H]Cholesterylhexadecylether ([^{3}H]CHE) was purchased from NEN Life Science Products, Inc. (Boston, MA, USA) and all other regents were commercially available and of analytical grade. Male SD rats (190–220 g) were used for the in vivo animal experiments. The animal experimental plan was approved by the Committee of Laboratory Animal Center (No. 09-009), and conforms to the Guiding Principles for the Care and Use of Experimental Animals in Hokkaido Pharmaceutical University.

Preparation of unmodified and PEGylated CPFX-liposomes

Preparation of unmodified and PEGylated CPFX-liposomes was performed by the lipid thin film hydration method[20,21]. Briefly, hydrogenated egg phosphatidylcholine, cholesterol, dicetylphosphate, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol)-2000] for PEG modification in a lipid molar ratio of 7/2/1/0 or 7/2/1/1 were dissolved in chloroform/methanol (9/1), followed by evaporation to obtain a thin film. The film was completely hydrated using CPFX solution (pH 5.5) to obtain the liposomes. CPFX solution was prepared by dissolution of CPFX in 50 mM phosphate buffer (pH 5.5). The liposomes were then extruded through polycarbonate filters with a pore size of 1000, 400, 200, and 100 nm (Nuclepore, Whatman, Piscataway, NJ, USA) five times. The particle sizes of the liposomes 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 unmodified and PEGylated CPFX-liposomes after extrusion (means ± SD) was 101.5 ± 11.4 and 98.5 ± 10.1 nm, respectively. [^{3}H]CHE was used as a non-exchangeable lipid phase marker[22,23] to label the liposomes. The concentration of CPFX was measured using high-performance liquid chromatography (HPLC) as reported previously[19]. The CPFX concentration and CPFX/lipid molar ratio in both types of liposomes were 2.07 μmol CPFX/mL and 0.114 mol CPFX/mol total lipids, respectively.

Animal experiments and analysis

For the in vivo pharmacokinetic analysis and evaluation of antibacterial effects, unmodified or PEGylated CPFX-liposomes labeled with or without [^{3}H]CHE (200 μg CPFX/250 μL/kg) were aerosolized into the rat lungs using a Liquid MicroSprayer™ (Model IA-1C, PennCentury, Inc., Wyndmoor, PA, USA) under pentobarbital anesthesia. The dosage volume was 250 μL/kg with a dose corresponding to 0.518 μmol CPFX/kg (200 μg CPFX/kg) and 4.55 μmol total lipid/kg. The dose of CPFX (200 μg/kg) used in this study was approximately 1/50th of the clinical oral dose. At the indicated time-points after aerosolization, the trachea was immediately cannulated under pentobarbital anesthesia and the lungs were lavaged three times with 5 mL ice-cold phosphate buffered saline (PBS, pH 7.4)[24]. The bronchoalveolar lavage fluid was immediately centrifuged at 4°C (650×*g* for 10 minutes) to separate AMs from the diluted ELF. Then, the AMs were extracted with 1 mL 0.1 M NaOH solution for scintillation analysis. To calculate the concentrations of CPFX in ELF, the apparent volume of ELF was estimated using urea as an endogenous marker of ELF dilution[25]. The mean value estimated in this study was 395 μL/225 g rat. The concentrations of CPFX in the diluted ELF were measured using HPLC as reported previously[19]. The concentrations of CPFX in ELF (μg/mL)

were calculated by normalization with the apparent volume of ELF. The area under the concentration of CPFX–time curve in ELF (AUC) and the mean residence time (MRT) of CPFX in ELF were obtained using the moment analysis program, MOMENT[26]. The protein concentration in the AMs extract was determined using Coomassie Protein Assay reagent (Pierce Chemical Company, Rockford, IL, USA) with bovine serum albumin as a standard[27]. The [^{3}H]CHE content of the AM extract was assayed as follows: About 700 μL of each AMs extract and 6.3 mL Hionic-Fluor (Packard BioSci. Co., Meriden, CT, USA) were mixed and stored overnight. The [^{3}H]CHE radioactivity was determined using scintillation counting. The uptake of liposomes by AMs was expressed as an uptake percentage versus the total administered radioactivity per milligram cellular protein (% uptake/mg cell protein). The antibacterial effects in ELF following aerosolization of unmodified and PEGylated CPFX-liposomes were evaluated by PK/PD analysis. The ratio of the AUC of CPFX in ELF/minimum inhibitory concentration of CPFX at which 90% of isolates (MIC_{90}) was calculated as the PK/PD parameter of an antibacterial effect[28,29]. The effective values of AUC/MIC_{90} were greater than 125 (h)[30–35]. The MIC values against pathogenic microorganisms were taken from the literature. Also, the required dose (RD) of CPFX for effective therapy (a dose required for winning 125 (h) that is the effective AUC/MIC_{90} value) was calculated by the following Equation (1).

$$\mathrm{RD}\,(\mu g/kg) = \frac{200\,(\mu g/kg)}{(\mathrm{AUC}/\mathrm{MIC})/125} \qquad (1)$$

For the cytotoxic testing, aerosol-based unmodified or PEGylated CPFX-liposomes (200 μg CPFX/250 μL/kg) were aerosolized into the rat lungs as described above. PBS and 0.25% Triton-X 100 solution were also used as negative and positive controls, respectively: the dosage volume was 250 μL/kg in each case. At 24 hours after aerosolization, the diluted ELF was collected as described above. The lactate dehydrogenase (LDH) level in the diluted ELF was determined by the LDH-Cytotoxic Test wako (Wako Pure Chemicals Co., Ltd., Osaka, Japan) using LDH from chicken heart as a standard[36]. The LDH levels in ELF (IU/mL) were calculated by normalization with the apparent volume of ELF as described above.

Statistics

Statistical analysis was performed by the Mann–Whitney *U* test and Dunnett's test using Stat View software (Abacus Concepts Inc., Berkeley, CA, USA). *P*-values of <0.05 were considered statistically significant.

Results and discussion

In this study, the drug delivery to the ELF following aerosolization of PEGylated CPFX-liposomes was investigated in rats and their antibacterial effects against the pathogenic microorganisms in ELF were estimated.

The time-courses of the concentration of CPFX in ELF after aerosolization of CPFX-liposomes in rats are shown in Figure 1 and the pharmacokinetic parameters are summarized in Table 1. The elimination rate of CPFX from ELF following aerosolization of PEGylated CPFX-liposomes was significantly slower than that of unmodified CPFX-liposomes (Figure 1). The AUC and MRT of CPFX in ELF following aerosolization of PEGylated CPFX-liposomes were 5.3- and 3.5-fold greater than those of unmodified CPFX-liposomes, respectively (Table 1). These results indicate that PEGylated CPFX-liposomes induce sustained distribution of CPFX in the ELF. The uptake of liposomes by AMs after aerosolization of CPFX-liposomes is shown in Figure 2. The uptake of liposomes by AMs following aerosolization of PEGylated CPFX-liposomes was significantly lower than that of unmodified CPFX-liposomes (Figure 2). This suggests that PEGylated CPFX-liposomes deliver CPFX efficiently to ELF by avoiding opsonization by surfactant proteins and reduced recognition by AMs.

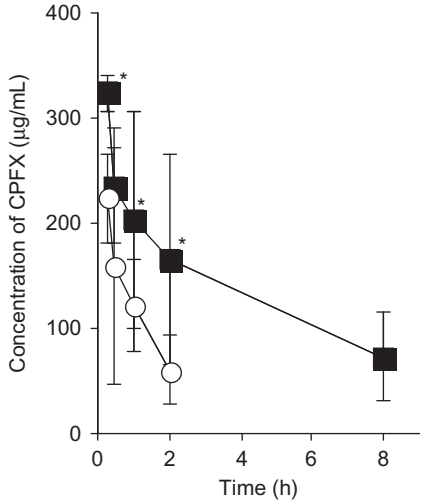

Figure 1. Time-courses of the concentration of CPFX in ELF after aerosolization of CPFX-liposomes. Unmodified (○) or PEGylated (■) CPFX-liposomes (200 μg CPFX/250 μL/kg) were aerosolized into rat lungs. At each time-point after aerosolization, diluted ELF was collected and concentrations of CPFX in ELF were determined. A significant difference (*$P < 0.05$) was found using the Mann–Whitney *U* test. Each value represents the mean ± SD ($n = 3$–5).

Table 1. The pharmacokinetic parameters of CPFX in ELF following aerosolization of CPFX-liposomes in rats.

CPFX-liposomes	AUC[a] (μg h/mL)	MRT[b] (hours)
Unmodified	264	0.79
PEGylated	1401	2.80

Pharmacokinetic parameters were obtained from data shown in Figure 1. [a]AUC from time 0 to final time. [b]MRT from time 0 to final time. AUC and MRT are represented as mean value.

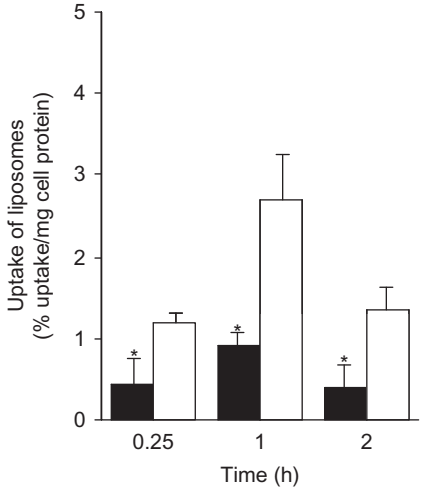

Figure 2. Uptake of liposomes by AMs after aerosolization of CPFX-liposomes. Unmodified (open column) or PEGylated (closed column) CPFX-liposomes labeled with [^{3}H]CHE (200 μg CPFX/250 μL/kg) were aerosolized into rat lungs. At each time-point after administration, AMs were collected and radioactivity in AMs was determined. A significant difference (*$P < 0.05$) was found using the Mann–Whitney U test. Each value represents the mean ± SD ($n = 3$).

The PK/PD parameters for estimating the antibacterial effects in ELF following aerosolization of CPFX-liposomes are summarized in Table 2. Recently, there has been increasing interest in the relationship between the PK and PD of antibiotics and, therefore, the use of PK/PD parameters is now widespread[28,29]. It is proposed that the PK/PD analysis of antibiotic treatments is important for selecting suitable doses and optimizing the treatment of individual patients. The antibacterial effects of antibiotics are concentration- and/or time-dependent and the PK/PD parameters used generally are the peak concentration/MIC_{90} ratio, AUC/MIC_{90} and the time above the MIC_{90}. Because the antibacterial effects of fluoroquinolones such as CPFX depend on the AUC/MIC_{90} or peak concentration/MIC ratio[32,38], the values of AUC/MIC_{90} in ELF following the aerosolization of CPFX-liposomes were calculated for several pathogenic microorganisms in this study. The AUC/MIC_{90} of PEGylated CPFX-liposomes was greater than the effective value (125 h) against all pathogenic microorganisms in spite of the use of 1/50th of the clinical oral dose (Table 2). Again, the values of RD indicate that it is possible to greatly reduce the dose of PEGylated CPFX-liposomes (Table 2). The AUC/MIC_{90} of unmodified CPFX-liposomes was also higher than the effective values (Table 2). However, unmodified CPFX-liposomes may not be efficient, compared with PEGylated CPFX-liposomes, if a reduction in the dose and antibacterial effects more potent than those of PEGylated CPFX-liposomes are required. This study indicates that efficient antibacterial effects of PEGylated CPFX-liposomes are obtained by aerosolization of a dose lower than that used clinically. These

Table 2. The estimated antibacterial effects of CPFX-liposomes against the pathogenic microorganisms in ELF.

CPFX-liposomes	Microorganisms (MIC_{90})[37]	AUC/MIC_{90} (hours)	RD (μg/kg)
Unmodified	*P. aeruginosa* (1 μg/mL)	264	75.8
	H. influenzae (0.03 μg/mL)	8800	2.3
	S. pneumoniae (2 μg/mL)	132	151.5
PEGylated	*P. aeruginosa* (1 μg/mL)	1401	14.3
	H. influenzae (0.03 μg/mL)	46,700	0.4
	S. pneumoniae (2 μg/mL)	701	28.6

AUC in ELF as described in Table 1 were used for calculation of PK/PD parameter. The MIC_{90} values were taken from the literature.

effects following aerosolization of PEGylated CPFX-liposomes in an animal model of respiratory infection should be investigated in more detail in future studies.

Also, cytotoxicity following aerosolization of PEGylated CPFX-liposomes was examined in rats. The LDH levels in ELF at 24 hours after aerosolization of CPFX-liposomes, PBS as a negative control or Triton-X100 solution as a positive control, are shown in Figure 3. The LDH level in ELF after aerosolization of PEGylated CPFX-liposomes was similar to that of PBS. This indicates that the aerosolization of PEGylated CPFX-liposomes does not injure lung tissues, at least at the dose used in this study.

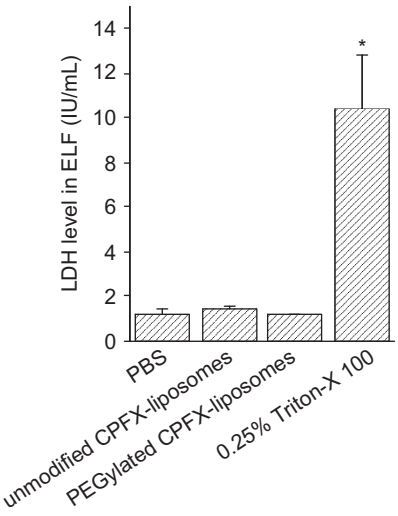

Figure 3. LDH levels in ELF after aerosolization of CPFX-liposomes. CPFX-liposomes, PBS, or 0.25% Triton-X 100 solution was aerosolized into rat lungs. At 24 hours after aerosolization, diluted ELF was collected and LDH levels in ELF were determined. *$P < 0.01$, significant difference compared with the treatment with PBS in Dunnett's test.

Conclusion

This study was carried out based on a hypothesis that sustained drug distribution in ELF is obtained by surface PEG modification of liposomes. We have shown that efficient drug delivery to ELF is possible by aerosolization of PEGylated CPFX-liposomes. Furthermore, it was shown that efficient antibacterial effects of PEGylated CPFX-liposomes against pathogenic microorganisms in ELF could be obtained at a lower dose than that used in clinical situations. This study indicates that the PEGylated CPFX-liposomes are an efficient aerosol-based pDDS for the treatment of respiratory infections.

Declaration of interest

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References

1. Baldwin DR, Honeybourne D, Wise R. (1992). Pulmonary disposition of antimicrobial agents: Methodological considerations. Antimicrob Agents Chemother, 36:1171–75.
2. Wilson R, Dowling RB, Jackson AD. (1996). The biology of bacterial colonization and invasion of the respiratory mucosa. Eur Respir J, 9:1523–30.
3. Densen P, Mandell GL. (1980). Phagocyte strategy vs. microbial tactics. Rev Infect Dis, 2:817–38.
4. Horwitz MA, Silverstein SC. (1980). Influence of the Escherichia coli capsule on complement fixation and on phagocytosis and killing by human phagocytes. J Clin Invest, 65:82–94.
5. Krieg DP, Helmke RJ, German VF, Mangos JA. (1988). Resistance of mucoid *Pseudomonas aeruginosa* to nonopsonic phagocytosis by alveolar macrophages in vitro. Infect Immun, 56:3173–79.
6. Peterson PK, Verhoef J, Sabath LD, Quie PG. (1997). Effect of protein A on staphylococcal opsonization. Infect Immun, 15:760–64.
7. Stiver HG, Zachidniak K, Speert DP. (1988). Inhibition of polymorphonuclear leukocyte chemotaxis by the mucoid exopolysaccharide of *Pseudomonas aeruginosa*. Clin Invest Med, 11:247–52.
8. Yee D, Valiquette C, Pelletier M, Parisien I, Rocher I, Menzies D. (2003). Incidence of serious side effects from first-line antituberculosis drugs among patients treated for active tuberculosis. Am J Respir Crit Care Med, 167:1472–77.
9. Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A. (1991). Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. Biochim Biophys Acta, 1066:29–36.
10. Torchilin VP, Omelyanenko VG, Papisov MI, Bogdanov Jr AA, Trubetskoy VS, Herron JN, et al. (1994). Poly(ethylene glycol) on the liposome surface: On the mechanism of polymer-coated liposome longevity. Biochim Biophys Acta, 1195:11–20.
11. Benne CA, Benaissa-Trouw B, van Strijp JA, Kraaijeveld CA, van Iwaarden JF. (1997). Surfactant protein A, but not surfactant protein D, is an opsonin for influenza A virus phagocytosis: Binding and neutralization. Eur J Immunol, 27:886–90.
12. Holmskov U, Mollenhauer J, Madsen J, Vitved L, Gronlund J, Tornoe I, et al. (1999). Cloning of gp-340, a putative opsonin receptor for lung surfactant protein D. Proc Natl Acad Sci USA, 96:10794–99.
13. Johansson J, Curstedt T, Robertson B. (1994). The proteins of the surfactant system. Eur Respir J, 7:372–91.
14. Schagat TL, Wofford JA, Wright JR. (2001). Surfactant protein A enhances alveolar macrophage phagocytosis of apoptotic neutrophils. J Immunol, 166:2727–33.
15. Chono S, Tanino T, Seki T, Morimoto K. (2007). Pharmacokinetic and pharmacodynamic efficacy of intrapulmonary administration of ciprofloxacin for the treatment of respiratory infections. Drug Metab Pharmacokinet, 22:88–95.
16. Geller DE, Rosenfeld M, Waltz DA, Wilmott RW. (2003). Efficiency of pulmonary administration of tobramycin solution for inhalation in cystic fibrosis using an improved drug delivery system. Chest, 123:28–36.
17. Togami K, Chono S, Seki T, Morimoto K. (2010). Aerosol-based efficient delivery of telithromycin, a ketolide antimicrobial agent, to lung epithelial lining fluid and alveolar macrophages. Drug Dev Ind Pharm, 36:861–66.
18. Drlica K. (2003). The mutant selection window and antimicrobial resistance. J Antimicrob Chemother, 52:11–17.
19. Chono S, Tanino T, Seki T, Morimoto K. (2006). Influence of particle size on drug delivery to rat alveolar macrophages following pulmonary administration of ciprofloxacin incorporated into liposomes. J Drug Targ, 14:557–66.
20. Bangham AD, Standish MM, Watkins JC. (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol, 13:238–52.
21. Funato K, Yoda R, Kiwada H. (1992). Contribution of complement system on destabilization of liposomes composed of hydrogenated egg phosphatidylcholine in rat fresh plasma. Biochim Biophys Acta, 1103:198–204.
22. Derksen JT, Morselt HW, Scherphof GL. (1987). Processing of different liposomal markers after in vitro uptake of immunoglobulin-coated liposomes by rat liver macrophages. Biochim Biophys Acta, 931:33–40.
23. Pool GL, French ME, Edwards RA, Huang L, Lumb RH. (1982). Use of radiolabeled hexadecyl cholesterol ester as a liposome marker. Lipids, 17:445–52.
24. Antonini JM, Reasor MJ. (1991). Accumulation of amiodarone and desethylamiodarone by rat alveolar macrophages in cell culture. Biochem Pharmacol, 42:S151–S56.
25. Rennard SI, Basse Gt, Lecossier D, O'Donnell KM, Pinkston P, Martin PG, et al. (1986). Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. J Appl Physiol, 60:532–38.
26. Tabata K, Yamaoka K, Kaibara A, Suzuki S, Terakawa M, Hata T. (1999). Moment Analysis Program available on Microsoft Excel. Drug Metab Pharmacokinet, 14:286–93.
27. Branford MM. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 72:248–54.
28. Mouton JW, Dudley MN, Cars O, Derendorf H, Drusano GL. (2002). Standardization of pharmacokinetic/pharmacodynamic (PK/PD) terminology for anti-infective drugs. Int J Antimicrob Agents, 19:355–58.
29. Mouton JW, Dudley MN, Cars O, Derendorf H, Drusano GL. (2005). Standardization of pharmacokinetic/pharmacodynamic (PK/PD) terminology for anti-infective drugs: An update. J Antimicrob Chemother, 55:601–07.
30. Blaser J, Stone BB, Groner MC, Zinner SH. (1987). Comparative study with enoxacin and netilmicin in a pharmacodynamic model to determine importance of ratio of antibiotic peak concentration to MIC for bactericidal activity and emergence of resistance. Antimicrob Agents Chemother, 31:1054–60.
31. Craig WA. (1995). Interrelationship between pharmacokinetics and pharmacodynamics in determining dosage regimens for broad-spectrum cephalosporins. Diagn Microbiol Infect Dis, 22:89–96.

32. Forrest A, Nix DE, Ballow CH, Goss TF, Birmingham MC, Schentag JJ. (1993). Pharmacodynamics of intravenous ciprofloxacin in seriously ill patients. Antimicrob Agents Chemother, 37:1073–81.
33. Hyatt JM, McKinnon PS, Zimmer GS, Schentag JJ. (1995). The importance of pharmacokinetic/pharmacodynamic surrogate markers to outcome. Focus on antibacterial agents. Clin Pharmacokinet, 28:143–60.
34. Moore RD, Lietman PS, Smith CR. (2005). Clinical response to aminoglycoside therapy: Importance of the ratio of peak concentration to minimal inhibitory concentration. J Infect Dis, 155:93–99.
35. Toutain PL, del Castillo JR, Bousquet-Melou A. (2002). The pharmacokinetic-pharmacodynamic approach to a rational dosage regimen for antibiotics. Res Vet Sci, 73:105–14.
36. Decker T, Lohmann-Matthes ML. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods, 115:61–69.
37. Neu HC, Fang W, Gu JW, Chin NX. (1992). In vitro activity of OPC-17116. Antimicrob Agents Chemother, 36:1310–5.
38. Preston SL, Drusano GL, Berman AL, Fowler CL, Chow AT, Dornseif B, et al. (1998). Pharmacodynamics of levofloxacin: A new paradigm for early clinical trials. J Am Med Assoc, 279:125–29.