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Ester prodrugs of levofloxacin to prevent chelate formation in presence of aluminium ion

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Abstract

**Background:** Intestinal absorption of levofloxacin (LFX) is decreased by the concomitant administration of antacids due to the formation of insoluble chelate complexes with various metal cations.

**Methods:** The following four ester prodrugs of LFX—cilexetil ester (LFX-CLX), medoxomil ester (LFX-MDX), ethoxycarbonyl 1-ethyl hemiacetal ester (LFX-EHE) and pivaloyloxymethyl ester (LFX-PVM) — were synthesized. Then, the lipophilicity, *in vitro* chelate formation with aluminum chloride (AlCl₃), chemical and enzymatic stability, minimum inhibitory concentrations (MICs) against some bacteria, and the efficacy in preventing chelate formation of prodrugs with aluminum hydroxide (Al(OH)₃) in rabbits were evaluated.

**Results:** The synthesized ester prodrugs of LFX exhibited high purity and higher lipophilicities than LFX depending on the ester moieties. MICs of the prodrugs against *S. aureus, E. coli*, and *P. aeruginosa* were more than 10 times higher than those of LFX. Prodrugs were stable chemically but unstable enzymatically and generated LFX in biological specimens. When AlCl₃ solution was mixed with LFX solution *in vitro*, insoluble chelate complex was formed immediately. In rabbits, co-administration of Al(OH)₃ with LFX reduced the oral bioavailability of LFX by approximately 40%. In contrast, no precipitation was observed when AlCl₃ solution was mixed with each prodrug solution *in vitro*, and co-administration of Al(OH)₃ exerted no significant effect on the oral bioavailability of LFX when each prodrug was administered in rabbits.

**Conclusion:** The ester prodrug approach of LFX could be a feasible strategy for avoiding chelate formation with aluminum ion *in vivo.*

**Keywords:** avoidance of chelate formation, interaction with antacids, levofloxacin, oral bioavailability, prodrug
Introduction

Fluoroquinolones (FQs), including new ones such as ciprofloxacin, ofloxacin (O FX), levofloxacin (L FX), and sparfloxacin, are an important group of synthetic antibacterial compounds with a fluorine atom at position 6 and a piperazine ring at position 7 of quinolone-3-carboxylic acid. FQs are prescribed widely as the first line of defense against various bacterial infections. New FQs exhibit the longer half-lives in vivo and an extended spectrum of antibacterial activity compared with the older-generation FQs. For example, LFX, a lev-isomer of D, L-racemate OFX, has improved activity against gram-positive bacteria and excellent activity against gram-negative bacteria and the atypical organisms compared to ciprofloxacin and OFX. The oral bioavailability of LFX is high, and LFX was classified as a Biopharmaceutics Classification System (BCS) Class I drug with high solubility and high permeability.

Orally administered FQs including LFX, however, form insoluble chelate complexes with various metal cations such as aluminum ion (Al$^{3+}$), magnesium ion, and calcium ion. Moreover, the oral bioavailability of FQs is greatly reduced when FQs are administered concomitantly with metal-containing drugs. It was reported that co-administration of 1g aluminum hydroxide (Al(OH)$_3$), a widely used antacid in clinical practice, with 100 mg LFX decreased the peak plasma concentration (Cmax) of LFX by 35.2%, the urinary excretion rate by 71.6%, and the value of the area under the concentration-time curve from 0 to 24 h (AUC$_{0-24}$h) by 56.3% in humans. Another study reported that co-administration of Al(OH)$_3$ reduced the oral bioavailability of LFX by approximately 60% in humans. In various experimental animals, antacids and metal cation-containing drugs also decreased the oral absorption of LFX. For example, the pretreatment of dogs with 1g sucralfate containing Al(OH)$_3$, which is used to treat and prevent gastrointestinal ulcers, decreased the Cmax by 70.8% and the oral bioavailability of orally administered LFX (5 mg/kg dose) by 55.6%. The binding of metal ions to the 4-keto- and 3-carboxyl-groups of FQs to form nonabsorbable chelates has been suggested as the possible mechanism responsible for the reduced absorption of FQs. To avoid chelate formation...
of LFX in clinical, staggered dosing was suggested, in which antacids taken 2 h after LFX ingestion.\textsuperscript{13,14} However, staggered dosing of antacids would be inconvenient in actual clinical and daily life situations. Previously, the efficacy of ester prodrugs in avoiding chelate formation was examined using the pivaloyloxymethyl (PVM) ester prodrug of OFX because the addition of an ester moiety at the 3-position carboxyl acid was thought to inhibit the binding of metal cations by steric hindrance.\textsuperscript{15} The PVM ester moiety has been used in prodrugs such as pivampicillin, pivmecillinam, and cefetamet pivoxil.\textsuperscript{16-18} In rabbits, co-administration of Al(OH)\textsubscript{3} with OFX decreased the AUC\textsubscript{0-24h} of plasma OFX by 47.6\% compared with the administration of OFX alone, but co-administration of Al(OH)\textsubscript{3} with OFX-PVM had no significant effect on the plasma OFX concentrations. The results suggested that the use of a certain ester prodrug can avoid the chelate formation of FQs in vivo.\textsuperscript{15} The usefulness of the prodrug approach in preventing chelate formation was also reported using the ethoxycarbonyl 1-ethyl hemiacetal ester prodrug of LFX (LFX-EHE) in rats. The co-administration of Al(OH)\textsubscript{3} with LFX-EHE had no significant effect on plasma LFX levels, although co-administration of Al(OH)\textsubscript{3} with LFX decreased the AUC\textsubscript{0-4 h} of plasma LFX by about 50\% of LFX alone. Based on these results, it was stated that LFX-EHE avoids insoluble chelate formation with metal-containing drugs in the intestinal tract and is rapidly hydrolyzed to the parent drug.\textsuperscript{19,20} Recently, the efficacy of the cilexetil ester prodrug of LFX (LFX-CLX) was examined.\textsuperscript{21} In in vitro binding study, LFX was precipitated by 76.1\% immediately after mixing with aluminum chloride (AlCl\textsubscript{3}) solution, but LFX-CLX was not. However, the co-administration of AlCl\textsubscript{3} with LFX-CLX decreased the AUC of plasma LFX by approximately 60\% compared with that after oral administration of LFX alone in rats,\textsuperscript{21} different from the cases of OFX-PVM\textsuperscript{15} and LFX-EHE.\textsuperscript{19,20} The significant difference in the chelate formation between AlCl\textsubscript{3} and Al(OH)\textsubscript{3} with ester prodrugs was thought to be derived from the cytotoxicity of AlCl\textsubscript{3}. AlCl\textsubscript{3}, an antiperspirant salt used to treat hyperhidrosis, may have induced barrier dysfunction and inflammation of the intestinal epithelium\textsuperscript{22,23}, which would induce enzymatic degradation of LFX-CLX and release
LFX in the intestinal lumen. Further study is necessary to clarify the efficacy of ester prodrugs of FQs in avoiding chelate formation in vivo using Al(OH)$_3$.

In this study, four ester prodrugs of LFX—LFX-CLX, LFX-EHE, medoxomil ester (LFX-MDX), and PVM ester (LFX-PVM)—were synthesized (Figure 1). CLX ester moiety has been used in prodrugs of sanfetrinem$^{24}$ and candesartan; the EHE ester moiety, in a prodrug of ampicillin;$^{26}$ and the MDX ester moiety, in prodrugs of azilsartan,$^{27}$ olmesartan,$^{28}$ and ertapenem.$^{29}$ We then evaluated the lipophilicity (the partition coefficient at pH 6.5), in vitro chelate formation with AlCl$_3$, chemical and enzymatic stability, minimum inhibitory concentrations (MICs) against some bacteria, and in vivo efficacy in preventing chelate formation with Al(OH)$_3$ in rabbits using each LFX prodrug.

Materials and Methods

Materials

The following materials were used: LFX (Apollo Scientific, UK); ciprofloxacin (LKT Laboratories, Inc., USA); 1-chloro ethyl cyclohexyl carbonate (Toronto Research Chemicals Inc., Canada); 1-chloroethyl ethyl carbonate (Tokyo Chemical Industry Co., Ltd., Japan); 4-(chloromethyl)-5-methyl-1,3-dioxol-2-one (BLDpharm, China); chloromethyl pivalate (FUJIFILM Wako Pure Chemical Corporation, Japan); dried Al(OH)$_3$ gel (Pfizer Japan Inc., Tokyo); and pancreatin (from porcine pancreas) and anhydrous AlCl$_3$ (Wako Pure Chemical Industries, Ltd., Japan). All other chemicals and solvents were of analytical grade.

Synthesis of levofloxacin prodrugs

The LFX ester prodrugs were synthesized according to the following modified method reported by Daehne et al.$^{30}$ To synthetize LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM, 1-chloro ethyl cyclohexyl carbonate (24 mmol); 1-chloroethyl ethyl carbonate (24 mmol); 4-(chloromethyl)-5-methyl-1,3-dioxol-2-one (24 mmol); or chloromethyl pivalate (24 mmol), respectively, added to an
N,N-dimethylformamide solution that contained anhydrous potassium carbonate (12 mmo1) and LFX (6 mmo1). The mixture solution was stirred for 2 h under argon atmosphere. In the case of LFX-CLX, LFX-EHE, and LFX-MDX, the product was extracted with ethyl acetate, washed with distilled water, dehydrated with anhydrous sodium sulfate, and evaporated until dry. Recrystallization of LFX-CLX, LFX-EHE, and LFX-MDX was made from chloroform and hexane, from diethyl ether, and from ethyl acetate, respectively. In the case of LFX-PVM, crushed ice and distilled water (360 mL) was slowly added to the mixture after it was stirred for 2 h and then cooled. Crystal precipitated was filtered with filter paper (ADVANTEC®, Toyo Roshi Kaisha, Ltd., Japan) and dried at 40°C for 5 h. The melting points were determined on Yanagimoto micro melting point apparatus. Proton nuclear magnetic resonance (¹H-NMR) spectra were recorded on a JEOL JNM-400S at 400 MHz (JEOL Ltd., Tokyo, Japan), and chemical shifts relative to Me₄Si (δ 0.00) were estimated. NMR spectra were measured in CDCl₃ (δ = 7.26 ppm). The carbon nuclear magnetic resonance (¹³C-NMR) spectroscopic data were recorded with a JEOL JNM-400S at 101 MHz (JEOL Ltd., Tokyo, Japan), and chemical shifts relative to CDCl₃ (δ = 77.0 ppm) were estimated. The mass spectra of the prodrugs were recorded on JEOL JMS-700 spectrometers (JEOL Ltd., Tokyo, Japan) through the direct inlet system.

**Partition coefficients**

Partition coefficients of LFX and the synthesized prodrugs (LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM) were determined in a partition system of chloroform and pH 6.5, 0.1 M Tris-HCl buffer at 25°C, in which the initial concentration of each compound in a buffer solution was approximately 100 μg/mL. Concentrations of each compound in both solvents after vigorous shaking were determined by high-pressure liquid chromatography (HPLC). Calculated log P was estimated using ChemDraw, a chemical drawing tool.

**Chelation with Al³⁺ ion in vitro**
LFX and the LFX ester prodrugs were dissolved in pH 6.5 Tris-HCl buffer solutions at an initial concentration of 20 μg/mL. Separately, AlCl₃ was dissolved in the same buffer at concentrations of 100 and 200 μg/mL. Each drug solution and AlCl₃ solution were mixed at equal volumes, and the mixture was left to stand for 30 min at 25°C. In the chelate formation in vitro study, AlCl₃ was used instead of Al(OH)₃, because AlCl₃ is a water-soluble compound, but Al(OH)₃ is not. Also, Al(OH)₃ is converted to AlCl₃ in the acidic condition like gastric juice. The mixture solution (or suspension) was centrifuged at 3,000 x g for 10 min, and the supernatant was filtered through a syringe filter with a 0.22 μm pore size (Millipore, Tokyo, Japan). The concentration of LFX in each filtrate was determined by HPLC after the prodrug was hydrolyzed with 1 mol/L of sodium hydroxide (NaOH).

**Chemical and enzymatic stability**

To evaluate the chemical (non-enzymatic) stability of prodrugs, pH 6.5 and pH 7.4 phosphate buffer solutions (PBS) used. As preliminary experiments of enzymatic stability, LFX ester prodrugs (initial concentration: 0.1 mM) was incubated for 2 h at 37°C in the following specimens: plasma (male Sprague-Dawley rats), small intestinal mucosal homogenates, liver homogenates, pancreatin solution (1.0 mg/mL), and luminal fluid with intestinal contents of rats. Each biological specimen including pancreatin solution was prepared using pH 7.4, 25 mM Tris-HCl buffer. Based on the results of preliminary study, the enzymatic stability of the LFX ester prodrugs in rat 10% plasma and 2% small intestinal mucosal homogenates was determined for 15 min at 37°C to compare the enzymatic stability among ester prodrugs. Concentrations of generated LFX from each LFX ester prodrug in the reaction mixture were determined periodically, in which the further metabolic reaction after the sampling was stopped by adding methanol at a volume ratio of 1:2.

**Determination of the minimum inhibitory concentration (MIC) values**
MIC values of LFX and LFX prodrugs were determined by the agar plate dilution method using Mueller-Hinton broth (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan) in the same manner as reported previously.\textsuperscript{15} Briefly, LFX (10 mg) or each LFX ester prodrug (corresponding to 10 mg LFX) was dissolved in 1 mL of 0.1 mol/L hydrochloric acid, and 4 mL of 0.1 mol/L PBS (pH 7.4) was added to the solution. The final concentrations of LFX and each LFX ester prodrug in the incubation medium were adjusted to 62.50, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.49, 0.24, 0.12, 0.06, and 0.03 μg/mL. The bacterial numbers of \textit{Staphylococcus aureus} (ATCC29213), \textit{Escherichia coli} (ATCC25922), and \textit{Pseudomonas aeruginosa} (ATCC27853) were adjusted to McFarland 0.5 (1.5×10\textsuperscript{6} cells/mL) with a sterilized normal saline. These bacterial suspensions were inoculated into Mueller-Hinton broth that contained LFX or LFX ester prodrugs. Facultative anaerobic and aerobic bacteria were cultured under aerobic conditions at 37\textdegree C for 20 h.

\textit{The absorption study in rabbits}

Male albino rabbits purchased from Japan SLC, Inc. (Hamamatsu, Japan) weighing 2.0–3.0 kg were fasted overnight with free access to water before each experiment. The following drug was suspended in 3 mL water containing 0.2 mL of 1.0 mol/L HCl and was administered orally to rabbits using gastric intubation: LFX (20 mg/kg, 0.055 mmol/kg); LFX-CLX (29.4 mg/kg, 0.055 mmol/kg); LFX-EHE (26.4 mg/kg, 0.055 mmol/kg); LFX-MDX (26.2 mg/kg, 0.055 mmol/kg); and LFX-PVM (26.4 mg/kg, 0.055 mmol/kg) with or without Al(OH)\textsubscript{3} (100 mg/kg, 1.282 mmol/kg). Then, blood (100 μL each) samples were periodically collected from the ear veins of rabbits. The following pharmacokinetic parameters estimated: Cmax of LFX, the time to reach the Cmax (Tmax), and the AUC\textsubscript{0-6h} of LFX in plasma. The AUC\textsubscript{0-6h} value of plasma LFX was estimated by the trapezoidal rule.\textsuperscript{32}

\textit{Analysis of LFX}
The concentrations of LFX in various biological samples were determined using ciprofloxacin as an internal standard by HPLC in the same manner as reported previously\textsuperscript{21} according to the reported modified method.\textsuperscript{33} Briefly, to 0.1 mL sample containing LFX prodrug, 0.05 mL of 1.0 mol/L NaOH was added and incubated for 30 min and then neutralized with 0.05 mL of 1.0 mol/L HCl solution. The HPLC system (Shimazu, Kyoto, Japan) used consisted of a model LC-20AD pump, a 20μL fixed injection loop, and a model SPD-10Avp UV detector. Data were acquired with the Sepu3000’s processor (Hang Zhou, China). The column used was an L-column 2 ODS (150 mm × 4.6 mm, i.d., 5 μm, CERI Co., Ltd., Saitama, Japan). The mobile phase consisted of acetonitrile and water that contained 0.3% triethylamine (pH 3.3 adjusted with phosphoric acid) at a volume ratio of 16 : 84. The flow rate of the mobile phase was 1 mL/min. The detection was made at 295 nm.

**Statistical analysis**

The measurement data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using the unpaired Student’s $t$-test. P-values less than 0.05 were statistically significant.

**Results**

**Synthesis of LFX-ester prodrugs: LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM**

The purity of each prodrug that was synthesized in this study was high, since the $^1$H-NMR spectral data displayed only specific signals of each prodrug. The purity of each prodrug was also evaluated by thin layer chromatography (TLC) using chloroform-methanol-acetic acid-distilled water (15:5:2:1, v/v/v/v) as a developing solvent. Each spot was detected using shortwave ultraviolet light (254 nm). The Rf values of LFX, LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM in TLC were 0.45, 0.78, 0.67, 0.60, and 0.75, respectively. The chemical structures of LFX and its ester prodrugs that were synthesized in this study are shown in Fig. 1. The $^1$H NMR chart, $^{13}$C NMR chart, and mass spectrum of each prodrug are shown as supplementary data in Fig. 3 - Fig. 6.
**LFX-CLX**

The synthetic yield of LFX-CLX was 89.3% (2,845 mg). The melting point was 124–126°C (dec.). The chemical shifts relative to Me₄Si (δ 0.00) were as follows: ¹H-NMR (CDCl₃) δ: 1.22-1.47 (6H, m), 1.51 (3H, q, J = 3.4 Hz), 1.64-1.65 (3H, m), 1.73 (2H, m), 1.92-1.95 (2H, m), 2.54 (3H, s), 2.80 (4H, m), 3.48 (4H, m), 4.36 (2H, d, J = 9.6 Hz), 4.44-4.47 (1H, m), 4.64 (1H, td, J = 4.5, 8.9 Hz), 6.95-7.01 (1H, m), 7.46 (1H, dd, J = 12.1, 15.3 Hz), 8.22 (1H, d, J = 10.5 Hz). ¹³C-NMR (CDCl₃) δ: 18.3, 19.8, 23.6, 25.1, 31.4, 31.5, 35.5, 45.4, 49.3, 54.7, 55.1, 68.1, 91.4, 104.8, 104.9, 105.0, 105.2, 107.9, 108.2, 123.4, 123.5, 130.7, 130.8, 130.9, 139.7, 139.8, 139.9, 145.4, 145.8, 152.4, 152.5, 154.2, 154.3, 156.7, 156.8, 162.2, 162.9, 172.3, 172.5. and mass spectra m/z, 531 (M⁺). Calculated for C₂₇H₃₄FN₃O₇, molecular weight 531.58.

**LFX-EHE**

The synthetic yield of LFX-EHE was 85.5% (2,447 mg). The melting point was 167–173°C (dec.). The chemical shifts relative to Me₄Si (δ 0.00) were as follows: ¹H-NMR (CDCl₃) δ: 1.32 (3H, t, J = 7.3 Hz), 1.48 (3H, dd, J = 3.4, 6.6 Hz), 1.64 (3H, t, J = 5.5 Hz), 2.36 (3H, s), 2.55 (4H, m), 3.34 (4H, m), 4.20-4.27 (2H, m), 4.32-4.40 (2H, m), 4.52 (1H, t, J = 8.9 Hz), 6.94-6.99 (1H, m), 7.35 (1H, dd, J = 12.8, 22.0 Hz), 8.19 (1H, d, J = 11.4 Hz). ¹³C-NMR (CDCl₃) δ: 14.1, 18.4, 19.7, 46.3, 50.4, 50.5, 54.7, 55.7, 64.4, 68.0, 91.5, 91.6, 105.0, 105.1, 105.2, 105.4, 107.8, 108.0, 123.0, 123.4, 123.5, 131.7, 131.9, 131.9, 139.6, 145.4, 145.8, 153.0, 153.1, 154.4, 154.6, 156.9, 157.0, 162.4, 163.2, 172.4, 172.6. and mass spectra m/z, 477 (M⁺). Calculated for C₂₃H₂₈FN₃O₇, molecular weight 477.4894.

**LFX-MDX**
The synthetic yield of LFX-MDX was 75.4% (2,140 mg). The melting point was 185–187°C (dec.). The chemical shifts relative to Me₄Si (δ 0.00) were as follows: ¹H-NMR (CDCl₃) δ: 1.56 (3H, d, J = 6.4 Hz), 2.24 (3H, s), 2.35 (3H, s), 2.54 (4H, m), 3.30-3.38 (4H, m), 4.35-4.41 (3H, m), 5.05 (2H, s), 7.59 (1H, d, J = 12.3 Hz), 8.26 (1H, s). ¹³C-NMR (CDCl₃) δ: 9.5, 18.2, 46.4, 50.5, 50.6, 53.7, 55.0, 55.7, 68.1, 105.7, 105.9, 108.3, 123.2, 123.6, 131.9, 132.0, 133.7, 139.5, 140.2, 145.4, 152.3, 154.6, 157.0, 164.2, 172.7. and mass spectra m/z, 473 (M⁺). Calculated for C₂₃H₂₄FN₃O₇, molecular weight 473.1598.

LFX-PVM

The synthetic yield of LFX-PVM was 89.0% (2,538 mg). The melting point was 214–216°C (dec.). The chemical shifts relative to Me₄Si (δ 0.00) were as follows: ¹H-NMR (CDCl₃) δ: 1.22 (9H, s), 1.55 (3H, d, J = 6.9 Hz), 2.36 (3H, s), 2.55 (4H, m), 3.33-3.39 (4H, m), 4.33-4.40 (3H, m), 5.96 (2H, s), 7.65 (1H, d, J = 12.8 Hz), 8.30 (1H, s). ¹³C-NMR (CDCl₃) δ: 18.2, 26.9, 38.8, 46.4, 50.6, 54.9, 55.7, 68.1, 79.8, 105.7, 105.9, 108.1, 123.2, 123.3, 123.5, 131.8, 132.0, 139.5, 145.5, 154.6, 157.0, 163.2, 172.7, 177.5. and mass spectra m/z, 475 (M⁺). Calculated for C₂₄H₃₀FN₃O₆, molecular weight 475.5174.

Partition coefficients

Calculated log P values and the apparent partition coefficients (PCs) of LFX and LFX ester prodrugs determined at pH 6.5 using chloroform as an organic phase are listed in Table 1. The PCs of LFX and the LFX ester prodrugs at pH 6.5 increased in the following order: LFX < LFX-CLX < LFX-MDX < LFX-EHE < LFX-PVM, in which the PCs of the ester prodrugs were approximately 3.0, 4.3, 13.4, and 22.6 times higher, respectively, than that of LFX (Table 1).

The chelate formation with aluminum ion (Al³⁺) in vitro
When the LFX (20 μg/mL) solution was mixed with AlCl₃ solution (100 μg/mL and 200 μg/mL), a cloudy precipitate was observed immediately after mixing, in which the precipitation percentage of LFX within 30 min was 68.0% and 76.1% of the initial LFX concentration, respectively. In contrast, the ester prodrugs examined showed no cloudy precipitation after mixing them with the AlCl₃ solution, and LFX was recovered at a concentration of 100% of the initial concentration from the mixture solution (Table 2). These results indicated that the examined ester prodrugs of LFX are stable chemically and can avoid the chelate formation with Al³⁺ ion.

**Chemical and enzymatic stability of the prodrugs**

To evaluate the chemical stability of ester prodrugs of LFX, the effect of pH was examined using pH 6.5 and pH 7.4 PBS. As shown in Table 3, examined prodrugs were stable chemically. In a preliminary study of enzymatic stability of prodrugs in biological specimens, prodrugs were hydrolyzed by almost 100% within 15 min and generated LFX in the intestinal mucosal homogenates, liver homogenates, and plasma, in which prodrugs were stable in the pancreatin solution (1.0 mg/mL). These results indicated that the synthesized ester prodrugs of LFX are rapidly hydrolyzed enzymatically. To compare the effects of different ester moieties on enzymatic stability, the stability of the prodrugs was determined periodically for 15 min using diluted plasma (10%) and intestinal mucosa homogenates (2%).

The enzymatic stability of different ester prodrugs was evaluated by determining the hydrolyzed amounts of LFX during 1-min incubation. The increasing order of enzymatic stability of ester prodrugs was in the following order: LFX-CLX < LFX-EHE < LFX-MDX, LFX-PVM in the 2% intestinal homogenates, and LFX-EHE, LFX-PVM < LFX-CLX, LFX-MDX in the 10% plasma (Table 3).

These results indicated that some differences exist in the enzymatic stability among different prodrugs, and between intestinal homogenates and plasma. However, these results also suggested that all four prodrugs examined are hydrolyzed rapidly in the intestinal membrane without showing any difference
in their enzymatic stability in \textit{in vivo} conditions (intact tissues), different from the cases in \textit{in vitro} condition.

\textbf{Determination of MIC values}

The MIC values of the LFX ester prodrugs against \textit{S. aureus}, \textit{E. coli}, and \textit{P. aeruginosa} were more than 10 times higher than that of LFX (Table 4). These data indicated that the LFX ester prodrugs themselves are pharmacologically inactive or have low activity levels as antibiotics, which agrees well with the prodrug concept.\textsuperscript{34}

\textbf{Effect of Al(OH)\textsubscript{3} on the oral bioavailability of LFX after administration of LFX and LFX ester prodrugs in rabbits}

The concentration-time profiles of LFX in plasma following oral administration of LFX, LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM (20mg/kg as LFX) with or without Al(OH)\textsubscript{3} (100 mg/kg) in rabbits are shown in Fig. 2(A–E), respectively. Intact LFX ester prodrugs were not detected in plasma even at 0.25 h after oral administration of prodrugs. Pharmacokinetic parameters such as Cmax, Tmax, and AUC\textsubscript{0-6h} of LFX in plasma are summarized in Table 5. The co-administration of Al(OH)\textsubscript{3} with LFX significantly reduced the Cmax and AUC\textsubscript{0-6h} of LFX by approximately 47\% and 40\% of LFX alone, respectively. The administration of prodrugs, except for LFX-CLX, showed a significantly higher AUC\textsubscript{0-6h} of LFX than the administration of LFX alone, and co-administration of Al(OH)\textsubscript{3} with prodrugs had no significant effect on AUC\textsubscript{0-6h} of LFX in plasma. In the case of LFX-CLX, AUC\textsubscript{0-6h} of the plasma LFX was comparable with that of LFX alone. However, the co-administration of Al(OH)\textsubscript{3} with LFX-CLX did not reduce AUC\textsubscript{0-6h} of plasma LFX, different from the case of LFX. Among all prodrugs, LFX-EHE showed a significantly higher Cmax and AUC\textsubscript{0-6h} and a shorter Tmax of plasma LFX even after co-administration with Al(OH)\textsubscript{3} compared with the LFX alone (Table 5).
Discussion

Prodrugs are designed to overcome the limitations and enhance their pharmacokinetics and/or pharmacological profiles, such as low water solubility, disagreeable taste, poor lipophilicity, low absorption or distribution, and bacterial resistance of parent drugs.\textsuperscript{35-38} In this study, four ester prodrugs of LFX, LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM, were synthesized to avoid chelate formation by masking the binding site of metal cations on quinolone-3-carboxylic acid, because staggered dosing is required when both LFX and antacids or metal cation-containing drugs are ingested in clinicals.\textsuperscript{1,3,13,14} Ester moieties such as CLX, EHE, MDX, and PVM have been used in clinically available prodrugs of antibiotics, as described in the introduction.\textsuperscript{6,24-29} LFX is a zwitterionic compound with a carboxylate (pKa = 5.7) and an amine (pKa = 7.9)\textsuperscript{39} that has been reported to be freely soluble in glacial acetic acid and chloroform but sparingly soluble in water. As for the membrane permeability of LFX, its oral bioavailability has been reported to be almost 100\% in humans.\textsuperscript{5,6} Thus, it is classified as a BCS Class 1 drug with high solubility and high permeability.\textsuperscript{7} However, FQs, including LFX, form an insoluble chelate complex, and their oral bioavailability is greatly reduced when they are administered with drugs that contain metal cations.\textsuperscript{2,8,40}

This study compared four LFX ester prodrugs (LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM) in terms of their PCs, in vitro chelate formation with Al\textsuperscript{3+} ion, enzymatic stability in various biological specimens, MIC values for some bacteria, and the effect of Al(OH)\textsubscript{3} on the oral bioavailability of LFX from LFX ester prodrugs in rabbits. The prodrugs had higher PCs than LFX at pH 6.5 (Fig. 1, Table 1), and all of them avoided chelate formation with Al\textsuperscript{3+} ion completely in vitro (Table 2), possibly due to the masking of the metal cation-binding site on quinolone 3-carboxylic acid.\textsuperscript{1} All prodrugs examined were stable chemically, and rapidly hydrolyzed and generated LFX in the intestinal mucosal homogenates, liver homogenates, and plasma (Table 3). Some difference was observed in the enzymatic stability among four different ester moieties when the stability was evaluated at the relatively small number of enzymes and between the intestinal mucosal homogenates.
and plasma. However, all the four ester prodrugs examined would be hydrolyzed rapidly mostly in the small intestinal membrane during the absorption process. The MIC values of the ester prodrugs against some bacteria were much higher than that of LFX, and there was no difference between the MIC values of the four examined ester prodrugs (Table 4). Based on these in vitro studies, it was speculated that LFX ester prodrugs can avoid hydrolysis and chelate formation with metal cations in the gastrointestinal lumen at least partly and that the absorbed ester prodrugs are rapidly converted into the parent drug, LFX, in the intestinal membrane.

Next, we examined the oral bioavailability of LFX from the ester prodrugs after their administration with and without Al(OH)₃ in rabbits (Fig. 2 and Table 5). When prodrugs alone were administered orally, the AUC₀₋₆h values of plasma LFX after the oral administration of prodrugs, except of LFX-CLX, were significantly higher than that of LFX. The higher plasma AUC of LFX after prodrug administration would be due to the higher lipophilicity in the luminal fluid of the prodrugs (Table 1). In humans, however, the oral bioavailability of LFX in the absence of metal cations is reportedly almost 100%.³,⁶ However, the bioavailability of LFX in rabbits might be lower compared to that in humans because the prodrugs increased the Cmax and the AUC₀₋₆h values of LFX. The lower oral bioavailability of LFX in rabbits than in humans may be at least partly due to the coprophagy of rabbits. Rabbits perform coprophagy to obtain many essential nutrients given the structure of their digestive system,⁴¹ and the stomach contents of rabbits are not emptied completely even in untreated normal conditions.⁴² Orally administered LFX may bind to metal cations in gastric contents, and the binding, or chelate formation, of LFX in the stomach may reduce the intestinal absorption of LFX at least partly in rabbits. In contrast, different from the case in rabbits, there was no difference in AUC₀₋₄h value of plasma between LFX and LFX-EHE in rats.¹⁹ Further study will be necessary regarding the interspecies difference in oral bioavailability of FQs, including LFX, by focusing on the effect of coprophagy. Co-administration of Al(OH)₃ with LFX significantly reduced the Cmax and the AUC₀₋₆h values of the plasma LFX. In contrast, co-administration of Al(OH)₃ with the LFX ester prodrugs
had no significant effect on the intestinal absorption of LFX (Table 5). There are marked interspecies differences between various metabolic enzymes, including carboxylesterases (CESs). CESs play a critical role in catalyzing hydrolysis of esters, amides, carbamates, and thioesters, as well as in bioconverting prodrugs and soft drugs, and CES1 was found to be most abundant in the liver while CES2 was primarily expressed in the intestine in the species, with notable species differences.\textsuperscript{43,44}

Similarly, it was reported that, in preclinical animal models, CES2 isozymes were also major intestinal enzymes, but they have different substrate specificities to human CES2. Additionally, although Caco-2 cells are frequently used in absorption studies, they mainly express the human CES1 isozyme, hCE1, with a quite different substrate specificity from hCE2. Caco-2 cells are reportedly unsuitable for predicting human intestinal absorption of prodrugs.\textsuperscript{45} All these show that it is important to consider the contribution of interspecies’ substrate specificity and expression sites, and the extent of activity of CESs, to the pharmacokinetics and pharmacology of ester prodrugs in preclinical studies.

**Conclusion**

The effectiveness of four ester prodrugs of LVFX in avoiding the chelate formation in the presence of aluminum ion were investigated. Without chelation, the oral bioavailability of LFX has been reported to be virtually 100% in humans. Thus, the target prodrugs should have high oral bioavailability and avoid chelate formation with metal cations \textit{in vivo}. The four examined ester prodrugs of LFX—LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM—exhibited higher lipophilicity than LFX, less antibacterial activity than LFX, chemically stable but enzymatically unstable, and avoidance of chelate formation \textit{in vitro}. In rabbits \textit{in vivo}, co-administration of Al(OH)$_3$ with LFX significantly reduced plasma LFX levels. However, co-administration of Al(OH)$_3$ exerted no significant effect on the intestinal absorption of LFX when administered as prodrugs. In conclusion, the ester prodrug approach of LFX could be a feasible strategy for avoiding chelate formation with Al$^{3+}$ \textit{in vivo}.
Author Contributions: Y. M. contributed to the research policy, experiment design, data analysis, and writing and editing of the manuscript. All the authors except for T. Mu. participated in the experiments. T. Mu. contributed to the experiment design, data analysis, and writing of the manuscript. All the authors have read and agreed to publish the final version of the manuscript.

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Ethical approval: The animal study was performed in compliance with the Care and Use of Laboratory Animals of the Committee for Animal Experiments of Fukuyama University. This study was approved on March 17, 2021 by the Ethics Committee of the Faculty of Pharmacy & Pharmaceutical Science of Fukuyama University. The license number of this animal study is 2021-A-12.

Informed Consent Statement: Not applicable.

Conflict of Interest: The authors declare no conflict of interest.

Acknowledgments: none

Appendix. Additional file

Supplementary material related to this article can be found, in the online version,
References


Pharmaceutical Sciences (Indexed in ISI and Scopus)
https://ps.tbzmed.ac.ir/


Table 1. Partition coefficients of LFX and the LFX ester prodrugs at pH 6.5

<table>
<thead>
<tr>
<th>Compound</th>
<th>Calculated log P</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFX</td>
<td>1.35</td>
<td>5.6</td>
</tr>
<tr>
<td>LFX-CLX</td>
<td>3.98</td>
<td>16.9</td>
</tr>
<tr>
<td>LFX-EHE</td>
<td>2.77</td>
<td>75.2</td>
</tr>
<tr>
<td>LFX-MDX</td>
<td>0.88</td>
<td>24.1</td>
</tr>
<tr>
<td>LFX-PVM</td>
<td>3.53</td>
<td>125.8</td>
</tr>
</tbody>
</table>

Calculated log P was estimated using ChemDraw, a chemical drawing tool.

PC: Partition coefficients were determined in a chloroform / pH 6.5, 0.1M Tris-HCL buffer partition system at 25°C.
Table 2. Precipitation percentages of LFX and the LFX ester prodrugs in the presence of 5- and 10-times higher concentrations of aluminum chloride

<table>
<thead>
<tr>
<th>Compound</th>
<th>Al(Cl)₃ concn.</th>
<th>50 µg/mL</th>
<th>100 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFX</td>
<td></td>
<td>68.0%</td>
<td>76.1%</td>
</tr>
<tr>
<td>LFX-CLX</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LFX-EHE</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LFX-MDX</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LFX-PVM</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Each sample was prepared with pH 6.5 Tris-HCl buffer. LFX and the LFX ester prodrugs solution (20 µg/mL) were mixed with Al(Cl)₃ solution (100 or 200 µg/mL) at a volume ratio of 1 : 1, respectively. The mixture solution was left to stand for 30 min at 24°C.
Table 3. Stability of LFX-CLX, LFX-EHE, LFX-MDX, and LFX-PVM in buffer solution (pH 6.5 0.1-M phosphate buffer; pH 7.4 0.1-M phosphate buffer), and enzymatic solution (2% intestinal mucosal homogenates; 10% plasma) at 37°C

<table>
<thead>
<tr>
<th></th>
<th>Extent of hydrolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1min</td>
</tr>
<tr>
<td>0.1M PBS (pH 6.5)</td>
<td></td>
</tr>
<tr>
<td>LFX-CLX</td>
<td>0</td>
</tr>
<tr>
<td>LFX-EHE</td>
<td>0</td>
</tr>
<tr>
<td>LFX-MDX</td>
<td>0</td>
</tr>
<tr>
<td>LFX-PVM</td>
<td>0</td>
</tr>
<tr>
<td>0.1M PBS (pH 7.4)</td>
<td></td>
</tr>
<tr>
<td>LFX-CLX</td>
<td>0</td>
</tr>
<tr>
<td>LFX-EHE</td>
<td>0</td>
</tr>
<tr>
<td>LFX-MDX</td>
<td>0</td>
</tr>
<tr>
<td>LFX-PVM</td>
<td>0</td>
</tr>
<tr>
<td>Mucosal homogenates</td>
<td></td>
</tr>
<tr>
<td>LFX-CLX</td>
<td>16.9±17.0</td>
</tr>
<tr>
<td>LFX-EHE</td>
<td>26.2±24.4</td>
</tr>
<tr>
<td>LFX-MDX</td>
<td>64.9±12.5</td>
</tr>
<tr>
<td>LFX-PVM</td>
<td>68.0±39.5</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
</tr>
<tr>
<td>LFX-CLX</td>
<td>10.1±4.1</td>
</tr>
<tr>
<td>Prodrug</td>
<td>Value 1 ± SD</td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
</tr>
<tr>
<td>LFX-EHE</td>
<td>4.8±0.8</td>
</tr>
<tr>
<td>LFX-MDX</td>
<td>37.6±14.7</td>
</tr>
<tr>
<td>LFX-PVM</td>
<td>7.9±1.4</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.D. (n=3).

The initial concentration of LFX and its prodrugs were 0.1 mM in each incubation medium.

Intestinal mucosal homogenates (2%) and plasma (10%) were prepared using pH 7.4, 25 mM Tris-HCl buffer.
Table 4. Comparison of the antibacterial activity or minimum inhibitory concentrations (μg/mL) of LFX and the LFX prodrugs

<table>
<thead>
<tr>
<th></th>
<th>Staphylococcus aureus (ATCC29213)</th>
<th>Escherichia coli (ATCC25922)</th>
<th>Pseudomonas aeruginosa (ATCC27853)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFX</td>
<td>0.34±0.09</td>
<td>0.06±0.04</td>
<td>1.86±1.32</td>
</tr>
<tr>
<td>LFX-CLX</td>
<td>40.10±28.66</td>
<td>13.86±12.10</td>
<td>58.37±7.25</td>
</tr>
<tr>
<td>LFX-EHE</td>
<td>40.10±28.66</td>
<td>5.69±8.58</td>
<td>44.33±31.46</td>
</tr>
<tr>
<td>LFX-MDX</td>
<td>30.47±29.68</td>
<td>7.45±7.63</td>
<td>35.43±25.25</td>
</tr>
<tr>
<td>LFX-PVM</td>
<td>30.47±29.68</td>
<td>9.53±7.98</td>
<td>39.60±20.08</td>
</tr>
</tbody>
</table>

MICs (μg/mL), minimal inhibitory concentration. MIC was determined via the agar plate dilution method using Mueller-Hinton broth.
**Table 5.** Pharmacokinetic parameters of LFX following oral administration of LFX and the LFX ester prodrugs with or without Al(OH)$_3$ in rabbits

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cmax (μg/mL)</th>
<th>Tmax (h)</th>
<th>AUC$_{0-6h}$ (μg x h/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFX</td>
<td>6.0±1.1</td>
<td>1.3±0.5</td>
<td>19.1±0.5</td>
</tr>
<tr>
<td>+ Al(OH)$_3$</td>
<td>3.2±1.1*</td>
<td>0.9±0.8</td>
<td>11.2±3.5 **</td>
</tr>
<tr>
<td>LFX-CLX</td>
<td>7.0±2.9</td>
<td>0.6±0.4</td>
<td>19.5±2.7</td>
</tr>
<tr>
<td>+ Al(OH)$_3$</td>
<td>6.1±2.0</td>
<td>0.6±0.4</td>
<td>17.9±2.7</td>
</tr>
<tr>
<td>LFX-EHE</td>
<td>12.3±2.6**</td>
<td>0.4±0.1*</td>
<td>31.3±7.8*</td>
</tr>
<tr>
<td>+ Al(OH)$_3$</td>
<td>10.1±1.2</td>
<td>0.3±0.1*</td>
<td>25.5±2.4**</td>
</tr>
<tr>
<td>LFX-MDX</td>
<td>10.0±5.0</td>
<td>0.8±0.4</td>
<td>27.5±6.5*</td>
</tr>
<tr>
<td>+ Al(OH)$_3$</td>
<td>7.9±3.2</td>
<td>1.1±0.9</td>
<td>21.1±1.8</td>
</tr>
<tr>
<td>LFX-PVM</td>
<td>6.8±1.2</td>
<td>0.8±0.3</td>
<td>22.7±0.8*</td>
</tr>
<tr>
<td>+ Al(OH)$_3$</td>
<td>7.0±2.4</td>
<td>0.4±0.1</td>
<td>19.1±4.1</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD. The doses of LFX, LFX-CLX, LFX-EHE, LFX-MDX, LFX-PVM, and Al(OH)$_3$ were 20 mg/kg, 29.4 mg/kg, 26.4 mg/kg, 26.2 mg/kg, 26.4 mg/kg, and 100 mg/kg, respectively. *p < 0.05 and **p < 0.01 vs. LFX alone. In the case of the prodrugs, there was no significant difference between those with and without Al(OH)$_3$. 
Figure 1. Chemical structures of levofloxacin and its ester prodrugs. (A) LFX, levofloxacin; (B) LFX-CLX, levofloxacin-cilexetil ester; (C) LFX-EHE, levofloxacin-ethoxycarbonyl 1-ethyl hemiacetal ester; (D) LFX-MDX, levofloxacin-medoxomil ester; and (E) LFX-PVM, levofloxacin-pivaloyloxymethyl ester.
Figure 2. The concentration-time profiles of LFX in plasma following oral administration of LFX or its ester prodrug without (open circle) or with Al(OH)₃ (solid circle) in rabbits. Each value represents the mean ± SD. (A) LFX (trial number, n = 4); (B) LFX-CLX (n = 3); (C) LFX-EHE (n = 3); (D) LFX-MDX (n = 3); and (E) LVX-PVM (n = 3). The doses of LFX and the ester prodrugs were 20 mg/kg as LFX, and the dose of Al(OH)₃ was 100 mg/kg. *p < 0.05.