Physicochemical, Pharmacokinetic and Pharmacodynamic Evaluation of Liposomal Tacrolimus (FK 506) in Rats

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Purpose. Tacrolimus (FK 506) is a new potent immunosuppressant. Because of poor water solubility, the conventional intravenous dosage forms of FK 506 (C-FK 506) contain surfactants such as HCO-60 which may cause adverse effects. We sought a liposomal formulation of FK 506 (L-FK 506) containing endogenous phospholipids to target drug to the spleen, a major organ controlling the immune system. ***Methods.*** L-FK 506, consisting of 0.1 μm diameter vesicles of phosphatidylcholine and phosphatidylglycerol (molar ratio 9:1) and 7.5 mole% drug, was evaluated for in vitro stability. The intravenous disposition profile, spleen distribution, and immunosuppression of L-FK 506 was compared with that of C-FK 506 in the rat after single doses of 0.3 mg/kg. ***Results.*** The L-FK 506 showed good in vitro stability. L-FK 506 exhibited an increased volume of distribution at steady-state (V_{SS}) (from 3.41 to 14.71 L/kg) and increased mean residence time (MRT) (from 2.83 to 16.07 hr). FK 506 concentrations in spleen were increased by 40% at 10 hr after administration of the liposomal formulation. The pharmacodynamics of L-FK 506, evaluated by the extent of inhibition of splenocyte proliferation, was comparable to that of C-FK 506. ***Conclusions.*** Liposomal FK 506 may be an improved dosage form for parenteral use.

KEY WORDS: tacrolimus (FK 506); immunosuppressant; HCO-60 (castor oil derivatives); liposomes; formulation; pharmacokinetics; targeting; pharmacodynamics; splenocyte proliferation.

INTRODUCTION

Tacrolimus (FK 506) is a new macrolide immunosuppressant which is more potent than cyclosporine (CyA) and is being tested for organ transplantation (1). In patients, intravenous injection or infusion is necessary for immediate immunosuppression. Since FK 506 is poorly soluble in water (less than 100 ng/ml) (2), preclinical and clinical studies have used vehicles containing alcohol and castor oil derivatives such as Cremophor EL (BASF Wyandotte Co.) or HCO-60 for intravenous administration of FK 506.

However, Cremophor EL itself may partly account for the nephrotoxicity of Sandimmune (3,4), and causes anaphylactoid reactions (5–7), adverse effects on hemodynamics (8) and cholestasis by transitory hepatotoxicity (9). HCO-60 also may cause allergic symptoms such as leukocytosis, hyperpyrexia, eruption (10,11) and immunological suppression of the growth of erythroid progenitors in humans (12). FK 506 is given by prolonged infusion in the clinic to avoid the possible anaphylactoid reactions. Thus, an intravenous formulation of FK 506 without castor oil derivatives could be of value.

Liposomes have been studied as vehicles for formulation of lipophilic drugs such as antimicrobial, antifungal (13,14), and chemotherapeutic agents (15,16). Liposomes are much less immunogenic and toxic than vehicles containing castor oil derivatives and can be targeted preferentially to the reticuloendothelial system (RES) in the liver and spleen. This may reduce the delivery of drug to other potential sites of drug-induced toxicity such as kidney and heart. Moreover, immunosuppressant targeting to the spleen would be advantageous because spleen is a major organ for trafficking of T-cells involved in the immune system. Recently, a liposomal formulation of FK 506 was reported for ocular delivery (17). However, the formulation was developed for topical use, so FK 506 was not detectable in serum.

The purposes of this study were to develop a stable liposomal formulation of FK 506 (L-FK 506) for systemic use and to evaluate the pharmacokinetics and pharmacodynamics of the liposomal formulation, compared with an ethanolic FK 506 solution (C-FK 506) in rats.

EXPERIMENTAL

Materials

FK 506 was provided in powder form by the Fujisawa Pharmaceutical Co Ltd. (Osaka, Japan). Phosphatidylcholine (PC) and phosphatidylglycerol (PG) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). RPMI 1640 media and fetal calf serum (FCS) were purchased from Gibco (Grand Island, NY). Phytohemagglutinin (PHA) was purchased from ICN Biochemicals Inc. (Cleveland, OH) and Ficoll-Paque was purchased from Pharmacia (Uppsala, Sweden). ^{3}H-thymidine (6.7 Ci/mmol) was obtained from Amersham (Arlington Height, IL).

Liposomal Formulation

FK 506 liposomes were prepared as follows: PC and PG were combined in chloroform at a molar ratio of 9:1, and FK 506 was added as a chloroform solution. To find the optimal drug/lipid ratio, various formulations were prepared having drug:lipid ratios of 2.5, 5, 7.5, 10, and 15 mole%. The organic solvent was evaporated under argon gas at a reduced pressure using a rotary evaporator. The dried lipid-drug film was suspended in pH 7.4 HEPES buffer by vortex mixing for 5 min. Liposomes were sized to a uniform diameter by repeated passage through 0.1 μm polycarbonate filters using a high pressure extruder. Because extensive previous studies have shown that a wide range of liposome formulations extruded sequentially have mean diameters approaching the pore diameter of the polycarbonate membrane through which they were extruded (18), the liposomes used here had an estimated mean diameter of 0.1 μm, relatively homogeneous size distribution, and were below the resolution of optical microscopy. The extruded liposome suspensions were centrifuged for 30 min at 15000 rpm to remove crystals of unincorporated drug. Formulations were examined by op-

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tical microscopy for the presence of precipitated drug or aggregated liposomes. A phosphate assay was used to quantify phospholipid (19) and an enzyme immunoassay was used to quantify drug. Both drug and phosphate assays were done before extrusion, after extrusion, and after centrifugation.

For stability testing, the liposome-containing supernatant of each formulation was stored at 4°C. After 2, 5, and 8 days, each suspension was centrifuged for 30 min at 15,000 rpm immediately before taking samples that were subjected to both drug and phosphate assays to assess any further drug loss by crystallization.

The most stable liposomal formulation was selected based on the stability results, and was subjected to dialysis in pH 7.4 HEPES buffer at 4°C for 2 days to check for retention of drug in the liposomal membrane. Dialysis tubing which has a molecular weight cut-off of 12,000–15,000 was used, and the buffer solution outside the dialysis bag was changed frequently to maintain sink conditions. FK 506 concentrations and phosphate amounts inside and outside the dialysis tubing were determined whenever the buffer solution was changed.

Animals

Male Lewis rats weighing 230–270 g were purchased from Harlan Inc (Indianapolis, IN). Animals were housed in a 12 hr light-dark, constant temperature (22°C) environment for 1 week prior to study, with free access to drinking water and rat chow. On the day prior to study, the rats were anesthesized with the mixture of ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (5 mg/kg) and a polypropylene cannula was inserted into the right jugular vein. Cannula patency was maintained with sterile saline. Rats were allowed to recover for 24 hr and fasted for 12 hr prior to study, but were allowed to drink water. All procedures were approved by the Institutional Animal Care and Use Committee.

Pharmacokinetics

An ethanol solution of FK 506 (2.5 mg/ml) was mixed with water (1:1; C-FK 506) immediately before injection of a dose of 0.3 mg/kg via the jugular vein cannula. The L-FK 506 also was administered via cannula at the same dose. The amount of lipid required to give 0.3 mg/kg FK 506 was less than 3 μmole per 250 g rat.

Blood samples were collected in heparinized polypropylene tubes at 0.08, 0.25, 0.5, 1, 2, 4, 6, 10, 24, and 48 hr post-dose. At each sampling time, the removed blood volume was replaced with a citrate-dextran anticoagulant solution (20). Aliquots of collected blood samples were taken, and the remaining sample of the blood was centrifuged immediately to harvest plasma. Blood and plasma samples were stored at −80°C and assayed within 2 weeks.

FK 506 Concentrations in the Spleen

Groups of three rats were sacrificed at 10 and 24 hr after a single 0.3 mg/kg iv dose of C-FK 506 or L-FK 506. The spleen was aseptically excised and cut in half. Part was weighed and frozen at −80°C for drug assay and the remainder was placed immediately in RPMI 1640 medium for measurement of splenocyte proliferation. Spleen samples for drug assay were homogenized in three volumes of saline and analyzed for FK 506 concentration.

Splenocyte Proliferation

All procedures were done by the method previously described (21). Briefly, about 0.4 g of spleen was homogenized in the growth medium (RPMI 1640 supplemented with 2 mM L-glutamine, 20 mM HEPES, 10% FCS, 1% penicillin/streptomycin and 5 mM 2-mercaptoethanol) by hand. The resulting cell suspensions were layered slowly over 5 ml Ficoll-Paque 400 solution and centrifuged for 30 min at 12,000 rpm. The interphase containing splenocytes was taken and washed with RPMI 1640 three times. The viability of the cells was more than 95%, as determined by counting in the presence of 0.2% trypan blue. Splenocyte suspension (5×10^5 cells/well) was cultured with stimulation by 150 μg/ml PHA, incubated for 66 hr and pulsed with 2 μCi/well of ^{3}H-thymidine 18 hr before harvesting. The percent inhibition of proliferation was calculated by the equation:

$$\text{Inhibition (\%)} = [1 - \{(\text{cpm}_{\text{FK506}} - \text{cpm}_{\text{blank}})/(\text{cpm}_0 - \text{cpm}_{\text{blank}})\}] \cdot 100$$

where cpm_0 represents the values corresponding to PHA-stimulated cells from drug-free rats, $\text{cpm}_{\text{blank}}$ represents the values for cells without PHA from drug-free rats, and $\text{cpm}_{\text{FK506}}$ represents the values for PHA-stimulated cells from the rats administered FK 506.

Drug Assay

FK 506 concentrations in liposome suspensions, rat plasma, whole blood and rat spleen were assayed by the enzyme immunoassay of Tamura et al (22) as modified by Jusko and D'Ambrosio (23).

Liposome suspensions were dissolved in methanol and diluted 10,000 times with human whole blood. To check the possible effect of liposomes on the assay results, blank liposomes were dissolved in methanol and diluted with human whole blood standards (L-STD) to a final concentration range of 0.5–60.0 ng/ml. L-STD were compared with liposome-free standards (STD) containing FK 506 in the same concentration range. As shown in Fig. 1, there was no significant effect of liposomes on the enzyme immunoassay for FK 506.

In assays of FK 506 in rat plasma, human plasma standards in the range of 0–10 ng/ml were used since the results from standards in rat plasma were essentially identical.

Rat whole blood standards and spleen standards in the range of 0–120 ng/ml were prepared using blank rat blood and spleen homogenates.

Pharmacokinetic Analysis

Plasma and whole blood FK 506 concentration (C_p) profiles were described by polyexponential equations:

$$C_p = \Sigma\, C_i\, e^{-\lambda i \cdot t}$$

where i = 3 for C-FK 506 in plasma or for either formulation in whole blood, and i = 2 for L-FK 506 in plasma. C_i and λ_i denote intercept coefficients and slopes. Least-squares regression analysis was performed using the PCNONLIN pro-

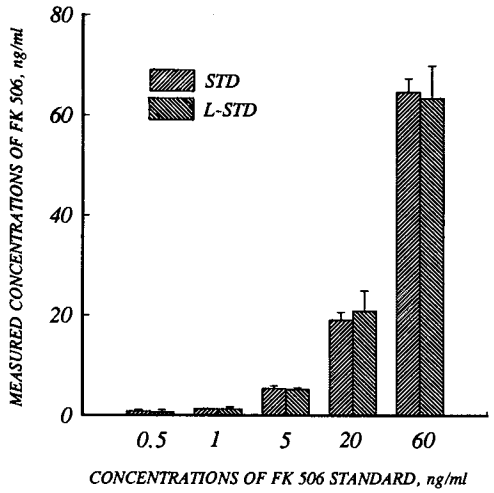

Fig. 1. Comparison of FK 506 concentrations (mean ± SD) of standards without liposomes (STD) and with liposomes (L-STD).

gram (SCI Software Inc, Lexington, KY). The area under the plasma (or whole blood) concentration versus time curve (AUC) and area under first moment curve (AUMC) were calculated from the slopes and coefficients (AUC = $\Sigma C_i/\lambda_i$, AUMC = $\Sigma C_i/\lambda_i^2$). The mean residence time (MRT) was determined as a ratio of AUMC/AUC. The apparent systemic clearance (CL) was calculated from Dose/AUC. The volume of distribution at steady-state (V_{SS}) was obtained from CL · MRT. The half-life of the terminal phase ($t_{1/2}$) was calculated from $0.693/\lambda_i$.

RESULTS

Stability of Liposome Formulations and Optimal Drug/Lipid Ratio

Electrostatically neutral PC was chosen as the principle phospholipid component in FK 506 liposomes, and 10 mole% of the negatively charged phospholipid PG was added to prevent aggregation. Stability data for PC:PG (9:1) formulations containing FK 506 in various molar ratios are shown in Fig. 2. It was found that the drug content could be increased up to 7.5 mole% without promoting drug crystallization. Based on this result, the 7.5 mole% formulation was

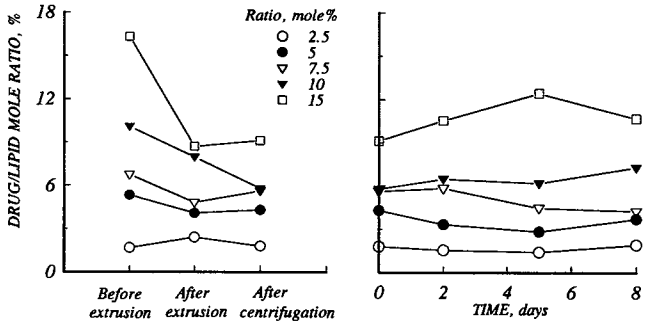

Fig. 2. Stability of FK 506 in PC:PG (9:1) formulations as a function of the initial drug/lipid molar ratios. Left panel shows the ratios at each of the indicated steps in preparing FK 506 liposomes and right panel shows the ratios at intervals after removal of precipitated drug by centrifugation.

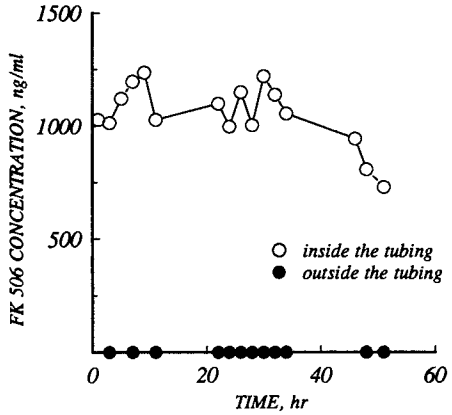

Fig. 3. FK 506 concentration-time profiles in the dialysis solution (4°C) under sink conditions.

selected for further evaluation. The final drug concentration of the liposome suspension was about 1.25 mg/ml and the phospholipid concentration was about 25 μmole/ml.

In order to assess the efficiency of drug incorporation and stability within the liposome membrane, the 7.5 mole% liposomal formulation was subjected to dialysis (Fig. 3). A comparison of the initial drug concentrations inside and outside the dialysis tubing demonstrate that the incorporation efficiency (= Drug amount incorporated in liposomes/Drug amount in the whole liposome suspension) was more than 95%. Drug concentrations inside the dialysis tubing did not change over 40 hr, and they were negligible outside the dialysis tubing. The amount of phospholipid inside the tubing was almost constant during dialysis and phospholipid was not detectable outside the dialysis tubing (data not shown).

Pharmacokinetics

Because FK 506 is reported to bind red blood cells in a concentration-dependent manner in rats (24), both plasma and whole blood concentrations were determined. Mean whole blood concentration profiles of drug after intravenous injection of C-FK 506 or L-FK 506 at a dose of 0.3 mg/kg are

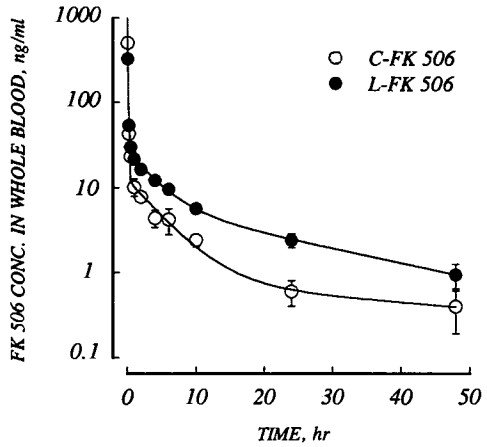

Fig. 4. Whole blood concentration profiles of C-FK 506 and L-FK 506 following single intravenous doses of 0.3 mg/kg to rats. Each point represents the mean ± SEM of three rats. Solid lines represent curve-fitting of data to polyexponential equations.

Table I. Pharmacokinetic Parameters after Single Intravenous Doses (0.3 mg/kg) of C-FK 506 or L-FK 506 in Rats (mean ± SEM)

	Plasma		Whole blood	
	C-FK 506	L-FK 506	C-FK 506	L-FK 506
AUC (μg · hr/L)	297.6 ± 31.8	341.3 ± 9.3	276.5 ± 74.2	314.8 ± 29.9
AUC_{blood}/AUC_{plasma}	0.91 ± 0.16	0.92 ± 0.06		
CL (L/hr/kg)	1.03 ± 0.10	0.88 ± 0.02	1.24 ± 0.29	0.97 ± 0.10
Vss (L/kg)	4.62 ± 2.11	6.28 ± 0.14	3.41 ± 1.08	14.71 ± 3.40[a]
$t_{1/2}$ (hr)	15.03 ± 6.96	8.17 ± 0.25	7.43 ± 1.24	20.08 ± 7.44
MRT (hr)	4.33 ± 1.93	7.15 ± 0.33	2.83 ± 0.74	16.07 ± 4.98[a]

[a] Significantly different by Student's t-test ($p < 0.05$).

shown in Fig. 4. Whole blood profiles were best fitted by triexponential equations for both C-FK 506 and L-FK 506. The plasma profile of C-FK 506 was also best fitted by a triexponential equation, but that of L-FK 506 was best described using a biexponential equation. The range of whole blood and plasma FK 506 concentrations are similar to those achieved after therapeutic doses in man (23).

Table I lists the pharmacokinetic parameters based on whole blood or plasma concentrations. There were no significant differences between the pharmacokinetic parameters of C-FK 506 and L-FK 506 based on plasma data, although the MRT of L-FK 506 showed a tendency to increase. In contrast, among the pharmacokinetic parameters based on whole blood data, V_{SS} and MRT of L-FK 506 were significantly different ($p < 0.05$) from those of C-FK 506, showing a marked increase from 3.4 to 14.7 L/kg for V_{SS}, and from 2.8 to 16.1 hr for MRT.

FK 506 Concentrations in the Spleen and Splenocyte Proliferation

The drug concentrations in the spleen and inhibition (%) of lymphocyte proliferation mediated by L-FK 506 were compared with those by C-FK 506 at 10 and 24 hr (Table II). It was reported in our previous work that blank liposomes which have the same composition (PC:PG = 9:1) did not alter the splenocyte proliferation results in the rat (21). At 10 hr after administration, the FK 506 concentrations in the spleen were significantly higher after L-FK 506 ($p < 0.05$). At 24 hr, there were no significant differences between formulations.

No significant differences between the FK 506 formulations in inhibition of splenocyte proliferation were found at 10 and 24 hr.

Table II. FK 506 Concentrations in the Spleen and Inhibition (%) of Lymphocyte Proliferation at 10 and 24 hr Post-Dose of C-FK 506 and L-FK 506 in Rats (mean ± SEM)

Time (hr)	C-FK 506	L-FK 506
	FK 506 Conc. in spleen (ng/g tissue)	
10	95.4 ± 8.5	134.4 ± 2.1[a]
24	51.7 ± 7.2	68.4 ± 9.1
	Inhibition (%)	
10	57.4 ± 8.1	53.8 ± 5.5
24	57.1 ± 17.0	75.6 ± 5.2

[a] Significantly different by Student's t-test ($p < 0.05$).

DISCUSSION

The feasibility of liposomes as an alternative to the FK 506 intravenous dosage form containing the surfactant, HCO-60, was assessed. The optimal liposomal formulation was sought based on retention of incorporated drug, manifested as the change in drug/lipid molar ratio after each step of the manufacturing process. For formulations containing 10 or 15 mole% FK 506, the drug/lipid ratios were considerably decreased after extrusion or centrifugation, suggesting that drug precipitates were formed and removed during the process. This was confirmed by microscopic observation of crystalline drug in liposomal formulations; precipitation is not desirable because it can accelerate drug release from the liposome during storage, and could be harmful to inject. Since accumulation of lipid in the RES can lead to impairment of RES function, the formulation containing 7.5 mole% FK 506 was selected to obtain the highest stable incorporation of drug and to minimize the amount of lipid administered. The dialysis results show that the formulation was stable enough to retain the drug in liposomes for at least 40 hr in HEPES buffer.

The pharmacokinetics of L-FK 506 was compared with that of C-FK 506 in rats. Because of the unusually high red blood cell binding of FK 506 in man, the pharmacokinetic parameters were calculated by both plasma and whole blood concentration. The pharmacokinetic parameters were similar based on plasma drug concentrations of C-FK 506 and L-FK 506. In contrast, whole blood V_{SS} and MRT were significantly increased ($p < 0.05$) by liposomal formulation, although the AUC and CL values were similar. These results imply that nonlinear red blood cell binding of FK 506 in rats affects the disposition of the liposomal formulation. However, the ratios of AUC_{blood}/AUC_{plasma} for each formulation were similar. Since the ratio of whole blood:plasma concentrations of FK 506 in rats (0.5 to 3) is much less than in humans (10 to 40, depending on the concentration) (25), the disposition of the liposomal formulation may be affected more greatly by nonlinear blood partitioning of FK 506 in humans. Thus, rabbits, reported to have blood binding similar to human (26), might be a better animal model than rats in future studies. The significant increase in MRT of L-FK 506 is due mainly to the increase in V_{SS}, suggesting that

L-FK 506 can remain in the body longer than C-FK 506 and is distributed more extensively into tissues. The 40% higher concentration of L-FK 506 in the spleen at 10 hr, and the marked increase of V_{SS}, imply that L-FK 506 may be taken up much greater by the RES of the liver and thereby much less by the tissues other than RES such as kidney, heart, and pancreas, which might be sites of adverse effects of FK 506 (27). Toxicity was not investigated in this study because single intravenous doses of FK 506 are not likely to evoke toxicity.

Pharmacodynamic effects of both formulations were evaluated by examining inhibition of splenocyte proliferation. The immunosuppressive effects of L-FK 506 were similar to that of C-FK 506, although the percent inhibition of proliferation by L-FK 506 after 24 hr showed a tendency to increase. The 40% greater drug concentration in the spleen was insufficient to produce a significant change in splenocyte proliferation.

In conclusion, our liposomal formulation of FK 506 demonstrates good in vitro stability, prolonged disposition, and immunosuppressive activity comparable to that of free drug. Liposomal formulations might be useful as an alternative intravenous dosage form for FK 506, and may abrogate the adverse effects of HCO-60 used in the conventional FK 506 intravenous solution.

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