Ocular Absorption of Topically Applied FK506 From Liposomal and Oil Formulations in the Rabbit Eye

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Purpose. To investigate the use of topically applied FK506, a new immunosuppressive compound, systemic and ocular absorption was determined in serum and various ocular tissues.

Methods. Two drops of 20 \( \mu l \) FK506 were applied using oil dissolved (OD-FK506) or liposome-bound (LIP-FK506) drug. FK506 concentrations were measured at intervals of 30, 60, and 120 minutes by immunoassay.

Results. After application of OD-FK506, the highest concentrations of FK506 were found in the cornea and the conjunctiva (200–1200 ng/g) with substantial drug also present in anterior and posterior sclera. Relatively low concentrations were measured in the aqueous and vitreous humors (0.2–1.0 ng/g) of these animals. Using the same treatment regimen, LIP-FK506 was effective in delivering significantly higher drug concentrations \( (P < 0.05) \) to all ocular tissues and particularly aqueous humor (5–28 ng/g) and vitreous humor (12–22 ng/g) at all time points. During the observation period drug concentrations produced by LIP-FK506 remained well above the therapeutic range. FK506 levels were not detectable in serum (<0.2 ng/ml) with either drug formulation.

Conclusion. These findings indicate that liposomes may be a promising formulation for topical use of FK506 in ocular immune-mediated diseases. Invest Ophthalmol Vis Sci. 1993;34: 2737–2742.

FK506 is a new immunosuppressant agent that has been isolated from the fermentation broth of \textit{Streptomyces tsukubaensis}. It has a mechanism of action similar to that of cyclosporine A, but is more potent. In vitro, FK506 inhibits the generation of cytotoxic T lymphocytes and the production of interleukin-2, interleukin-3 and gamma interferon at levels approximately 100 times lower than that of cyclosporine A.\textsuperscript{1–3} In vivo, FK506 showed a strong immunosuppressive effect in a variety of animal transplant models and in the treatment of experimental autoimmune uveoretinitis.\textsuperscript{4–8}

The use of FK506 is of special interest in ophthalmology because it may be effective in the treatment of immune-mediated diseases such as corneal graft rejection, keratitis, scleritis, ocular pemphigoid, and uveitis. To avoid systemic side effects, topical application of an agent is preferred to the oral and intravenous routes. However, topical application is often limited by the physical and chemical characteristics of the drug as well as the vehicle. Lipophilicity and molecular size are the major factors that determine corneal drug absorption.
A highly hydrophobic macrolide lactone (Figure 1), FK506 is almost insoluble in water and hexane but very soluble in methanol and slightly soluble in olive oil.\textsuperscript{9,10} In addition to its hydrophobic nature, the relatively high molecular weight of FK506 (822 dalton) may also limit corneal penetration. Because of its hydrophobic nature and relatively high molecular weight, FK506 is expected to penetrate the corneal epithelium with some difficulty and accumulate in the corneal stroma without resulting in effective intraocular drug levels.

The most commonly used vehicles for ocular drug preparations are based on aqueous solutions or, in the case of lipophilic substances, oily bases. For a variety of reasons, both vehicles are far from optimal in delivering topically applied drugs to the eye: aqueous solutions are subject to drainage into the nasolacrimal apparatus within 15–30 seconds after instillation, and oily bases cause blurring of vision and are often difficult for the patient to use.\textsuperscript{11} Liposomes, membrane-like vesicles consisting of one or more concentric bilayers alternating with aqueous compartments, have been studied as a way to enhance ocular drug absorption.\textsuperscript{12} They can be prepared easily from nontoxic materials that are not irritating to the ocular surface and do not blur vision. Liposomes have been studied as a drug delivery system for improvement in ocular drug bioavailability. In earlier studies we have shown that incorporation of lipophilic and high molecular weight agents into large unilamellar liposomes resulted in significantly improved intraocular penetration of these agents.\textsuperscript{13,14} These results and the lack of any studies about the ocular pharmacology of FK506 encouraged us to investigate ocular penetration and distribution of FK506 preparations in olive oil and bound to liposomes.

**MATERIALS AND METHODS**

**Preparation of Liposome-Bound FK506**

Large unilamellar liposomes containing FK506 were prepared by fast and controlled dialysis of mixed detergent-lipid micelles.\textsuperscript{15} Phosphatidylcholine (egg-yolk, Sigma, St. Louis, MO) and phosphatidylserine (bovine brain, Sigma, St. Louis, MO) in a 7:3 molar ratio were mixed with FK506 (Fujisawa Pharmaceutical Co, Ltd, Osaka, Japan) dissolved in methanol. The nonentrapped FK506 was separated by ultracentrifugation, and n-octyl-β-D-glucopyranoside (0.2 mol/mol lipid) was used as a detergent. The solvent was removed completely under reduced pressure and the resulting dry mixture was redissolved in phosphate-buffered saline (10 mmol/l phosphate, 150 mmol/l NaCl, pH adjusted to 7.4) and dialyzed for 24 hours. The trapping efficiency was calculated as the ratio of the amount of FK506 in the supernatant after ultracentrifugation to the amount of FK506 in liposomes after lysis with Tween 80. FK506 concentrations were determined by enzyme immunoassay.\textsuperscript{16} The final drug concentration was 4.8 mg/ml in the liposome suspension. The calculated trapping efficiency was 96%.

**Preparation of Empty Liposomes**

Liposomes containing the same constituents with the exception of entrapped FK506 were prepared by fast and controlled dialysis.

**Characterization of the Resulting Liposomes**

Vesicles were homogeneous in size with a mean diameter of 700 nm for the FK506-liposome preparation and 145 nm for the empty liposome preparation as determined by laser autocorrelation spectrometry (Nanosizer, Coulter, UK) and by gel filtration (Sephrose CL-2B, Pharmacia/LKB, Bromma, Sweden) (Figure 2).

Although the preparations contain identical phospholipids FK506 binding to the liposomal membrane in the FK506-liposome preparation accounts for the larger size of these liposomes.

**Preparation of FK506 Olive Oil Drops**

FK506 0.48% in olive oil was prepared as a sterile ophthalmic solution. FK506 was mixed in a beaker with olive oil USP to yield a 0.48% solution of FK506. The solution was sterilized by passage through a 0.2 μm filter (Nalgene, Rochester, NY).
FIGURE 2. Electron micrograph of liposome preparation. Freeze fracture technique (bar = 100 nm).

Animals
Twenty-four female New Zealand albino rabbits weighing approximately 3–4 kg each were used. All investigations were carried out in accordance with the “Guiding Principles in the Care and Use of Animals” of the National Institutes of Health and the “ARVO Resolution on the Use of Animals in Research.”

There were two groups, each consisting of 12 rabbits, in the experiment. The right eye of each animal in the first group received two drops of 20 μl olive oil administered 5 minutes apart. The left eye of these animals received the same volume of olive oil dissolved FK506 (OD-FK506).

The right eye of the second group received two drops (20 μl) of topically applied empty liposomes. The left eye was given two drops (20 μl) of liposome-bound FK506 (LIP-FK506). In all eyes receiving drop treatment, the upper lid was slightly raised and the lower lid was slightly pulled away from the globe. The drug was instilled directly onto the cornea and the lids were returned to their normal position immediately after drop application. Application of the second drop was performed exactly 5 minutes after initial drop instillation.

The 12 rabbits in each group were divided equally into three subgroups for measurements at 30, 60, and 120 minutes. At predetermined time points, after sedation by intramuscular injection of 2 ml chlorpromazine hydrochloride (25 mg/ml, Schein Inc., Port Washington, NY), the rabbits were killed by marginal ear vein injection of 10–15 ml Equi-Theme (Chloralhydrate 42.5 mg/ml and sodium pentobarbital 10.5 mg/ml, UCLA Pharmaceutical Service, Los Angeles, CA). The eyes were carefully rinsed with normal saline and dried with filter paper to remove remaining drug. Corneal epithelium was carefully removed using a scalpel. Two milliliters of blood was collected by car-diac puncture. Approximately 200 μl of aqueous humor was obtained from each eye by paracentesis using a 30-gauge needle attached to a 1-ml syringe. Corneas were excised at the limbus with scissors. The central vitreous humor was aspirated using an 18-gauge needle attached to a 2-ml syringe before the lens and iris ciliary body were removed. The sclera was scraped to remove all adherent choroidal and retinal tissues before it was divided at the equator into anterior and posterior segments. All tissues were thoroughly minced with a scalpel and then transferred to pre-weighted tubes. All tubes were weighed again before samples were stored at −70°C before analysis.

All FK506 measurements were performed using the enzyme immunoassay procedure of Tamura modified as previously described. The assay employs a mouse monoclonal anti-FK506 antibody (Fujisawa Pharmaceutical Co Ltd, Osaka, Japan). Briefly, goat antimouse immunoglobulin G is adsorbed onto 96-well flat-bottomed microtiter plates (ICN Flow Inc, Costa Mesa, Ca) overnight at 4°C and the plates were blocked with 1% bovine serum albumin and 0.05% Tween-20 in phosphate buffer (BTPBS). FK506 standards (Fujisawa) were prepared in methanol before dilution into plasma to produce standards ranging from 0.05 to 30 ng/ml. Standards (0.3ml) and experimental samples (mixed or diluted to 0.5 ml with blank plasma) were added to 1 ml of pH 7.4 phosphate buffer and FK506 was extracted into 9 ml of methylene chloride. After centrifugation at 4°C and removal of the upper aqueous phase, 7.5 ml of methylene chloride was transferred to a glass tube and evaporated to dryness using N₂ gas. Samples were reconstituted with 450 μl of BTPBS and then 140 μl were placed in duplicate into microtiter wells. In each well, 40 μl of the FK506-peroxidase enzyme conjugate (Fujisawa) diluted in phosphate buffer and 50 μl of the diluted anti-FK506 antibody were added and incubated overnight at 4°C in a humidified chamber. The wells were aspirated, washed, and O-phenylenediamine HCL (Sigma, St. Louis, MO) solution was added to each well. After 15 minutes, the reaction was stopped with 50 μl of 4 N H₂SO₄. The absorbance was measured at 490 nm using a Biotek 2000 Kinetics Elisa Reader (Fisher Scientific, Rochester, NY). Controls and unknowns were calculated using the KineticCalc 2.026 software program (Fisher Scientific). Based on a four-paramater logistic standard curve and coefficient of variation range 4–27% over the standard range, the lower limit of the modified enzyme immunoassay was 0.2 ng/ml.

Statistical Analysis
Paired t-tests were used to analyze differences in the mean FK506 levels between right and left eyes within
each group of rabbits, whereas unpaired t-tests were used to analyze differences between groups. Statistical significance was limited to \( P \) values < 0.05.

**RESULTS**

FK506 concentrations were determined in various ocular tissues at 30, 60, and 120 minutes after topical administration. In all experiments no ocular side effects were observed in animals receiving any of the ocular agents. No measurable FK506 drug concentrations (< 0.2 ng/ml) were detected with either formulations in the undosed eyes or in serum.

Figure 3 illustrates the FK506 content of rabbit eyes treated with LIP-FK506. Peak concentrations were reached in the corneal stroma, conjunctiva, and anterior sclera within the first 30 minutes after application. Highest levels in iris ciliary body and posterior sclera were obtained 60 minutes after application. Strictly speaking, the corneal drug concentrations represented those in the corneal stroma. It was not possible to accurately assay for the drug concentrations in the small quantities of corneal epithelium collected (< 2 mg).

After local application of OD-FK506, a different pattern of distribution within the various tissues and a different time course of the drug concentrations were observed (Figure 4). The cornea and conjunctiva demonstrated the highest values. However, in both tissues, drug concentrations were significantly lower at all time points \( (P < 0.05) \) as compared to LIP-FK506 application. Drug concentrations in the iris ciliary body, anterior, and posterior sclera remained below therapeutic values at all time points and were also significantly lower compared to the liposome preparation \( (P < 0.05) \).

Figures 5 and 6 show the concentrations of both FK506 formulations achieved in aqueous humor, lens, and vitreous humor. In all ocular tissues, significantly higher drug concentrations were obtained after application of LIP-FK506 at all time points \( (P < 0.05) \). Liposomal incorporation of FK506 increased the drug concentration by 5- to 20-fold in all these tissues, which were well above values considered to be therapeutically effective. High drug concentrations were even obtained in the vitreous humor after topical application of liposome-bound FK506.
FK506 Liposomes

FIGURE 6. Mean concentrations of FK506 (±SEM) observed in the rabbit eye. Two drops (20 μl) of 0.48% olive oil dissolved FK506 were applied topically 5 minutes apart.

DISCUSSION

FK506 is a potent immunomodulating agent that affects different stages of the immune response. The concentrations required to control immune-mediated diseases of the eye have not been established, however, the recommended minimal FK506 serum level in patients undergoing organ transplantation is 2–10 ng/ml.18 This drug concentration might be considered as the minimal effective concentration to control the immune response in immune-mediated eye diseases. Topical application of 0.48% FK506 given twice in olive oil solution to normal rabbit eyes resulted in drug concentrations well above this level in corneal stroma, conjunctiva, and anterior sclera, the most frequent target sites of extraocular manifestations of immune-mediated eye diseases. However, intraocular drug concentrations remained low. We were unable to detect any measurable FK506 in aqueous humor or vitreous after a single drop application of FK506 dissolved in olive oil (data not shown). The experimental design of the study was therefore modified to a two-drop application within 5 minutes.

Significantly higher drug concentrations were obtained with liposome-bound FK506 in all ocular structures compared to olive-oil-dissolved FK506. Drug concentrations in the corneal stroma, conjunctiva, and anterior sclera reached highest levels 30 minutes after topical application. At the same time, relatively high FK506 concentrations were detected in the aqueous and vitreous humors. In contrast to olive-oil-dissolved FK506, liposome-bound FK506 resulted in intraocular drug concentrations considered therapeutically effective. Intraocular drug levels were significantly higher at all time points as compared to olive-oil-dissolved FK506. The higher corneal concentrations are consistent with interactions of liposomes with the corneal epithelial surface thereby increasing the probability of drug penetration.19,20 This is especially important for an agent with relatively high molecular weight like FK506.

In addition to increasing corneal drug absorption, there is evidence that liposomes may enhance intraocular drug supply via the noncorneal route. Liposomes have, like other microparticulate systems, the tendency to accumulate in the conjunctival folds after drainage has subsided.21 Several studies suggest that conjunctival and scleral penetration are important in delivering poorly absorbed drugs to intraocular tissues.22–25 Absorption by the conjunctiva and diffusion through the underlying sclera was reported to contribute to intraocular drug supply of liposomal incorporated agents.23,24 The conjunctiva is 2–20 times more permeable for lipophilic and high molecular weight agents as compared with the cornea. Studies using high molecular weight inulin demonstrated that encapsulation in liposomes resulted in a sixfold increase in accumulation in the iris ciliary body.23

Both mechanisms, enhanced absorption at the cornea and conjunctiva, may have contributed to the relatively high drug levels in aqueous and vitreous humors.

The effect of systemic absorption of FK506 and recirculation after tear drainage is unlikely. We were not able to detect FK506 in the serum.

Although the bioavailability of topically applied LIP-FK506 in the human eye may differ from the rabbit eye because of higher blinking frequency and lower corneal permeability, the potential advantages of the liposome-bound preparation may apply for the human eye as well. The findings of substantial drug concentrations after topical application of LIP-FK506, especially intraocularly, may indicate that this formulation is useful in ocular immune-mediated diseases.

Key words
FK506, immunoassay, immunomodulating treatment, liposomes

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