Characterization of Liposomal Tacrolimus in Lung Surfactant-like Phospholipids and Evaluation of Its Immunosuppressive Activity[†]

Olga Cañadas,[‡] Rosa Guerrero,[‡] Rafael García-Cañero,[§] Guillermo Orellana,[‖] Margarita Menéndez,[⊥] and Cristina Casals*,[‡]

Departments of Biochemistry and Molecular Biology I and Organic Chemistry, Complutense University of Madrid, 28040 Madrid, Spain, Department of Experimental Biochemistry, Hospital Puerta de Hierro, 28035 Madrid, Spain, and Instituto de Química-Física Rocasolano, Consejo Superior de Investigaciones Científicas, 28006 Madrid, Spain

ABSTRACT: Tacrolimus (FK506) is a hydrophobic immunosuppressive agent that rapidly penetrates the plasmatic membrane and inhibits the signal transduction cascade of T lymphocytes. The objective of this study was the characterization of liposomal FK506 with surfactant-like phospholipids to be administered intratracheally after lung transplantation or in inflammatory lung diseases. We evaluated the optimal incorporation of FK506 in dipalmitoylphosphatidylcholine (DPPC) and DPPC/1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) monolayers and bilayers and the effects of FK506 on the physical properties of DPPC and DPPC/POPG (8:2 w/w) vesicles. In addition, we assessed the immunosuppressive effects of surfactant-like phospholipid vesicles containing different amounts of FK506 on T-cell proliferation and interleukin 2 production. From surface pressure measurements of FK506/DPPC and FK506/DPPC/POPG mixed monolayers, we determined that FK506 was embedded into these monolayers up to an FK506 concentration of about 0.4 mol %. Beyond this concentration, FK506 was not quantitatively incorporated into the monolayer, suggesting possible concentration-dependent aggregation of tacrolimus. The incorporation of FK506 into DPPC monolayers, at concentrations ≤ 5 μM, occurred with a partition coefficient of $(3.9 \pm 0.3) \times 10^3$ at the bilayer equivalence pressure. FK506 was incorporated in DPPC bilayers up to an FK506 concentration of about 0.7–1 mol %, which was about double that obtained via the monolayer technique. FK506 hardly affected the transition enthalpy, the T_m, and cooperativity of the phase transition of DPPC and DPPC/POPG vesicles as determined by differential scanning calorimetry and steady-state 1,6-diphenyl-1,3,5-hexatriene anisotropy. Finally, this study provides evidence that liposomal FK506 retains the immunosuppressive efficacy of the drug.

Tacrolimus (FK506) is a hydrophobic macrolide lactone (Figure 1) produced by *Streptomyces tsukubaensis* (*1*) that acts as a powerful and clinically useful immunosuppressant through disruption of signaling events mediated by calcineurin in T lymphocytes (*2*). Tacrolimus is 50–100 times more potent than the immunosuppressant cyclosporin A (CsA)[1] in inhibiting T-cell activation (*1*). In addition to its immunosuppressive effect, FK506 can act as an antiinflammatory or antiallergic agent (*2*–*4*). Clinical trials have shown tacrolimus to be an effective alternative to cyclosporin for both primary immunosuppression after solid-organ transplantation and as rescue therapy for acute rejection recipients (*5*). On the basis of favorable results in kidney and liver transplantation, tacrolimus has been used as primary pro-
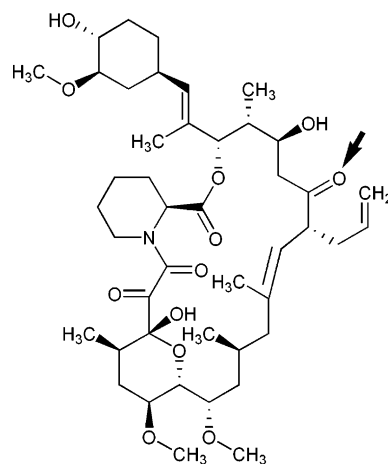

FIGURE 1: Chemical structure of tacrolimus (FK506). An arrow signals the tethering site of dansylcadaverine in the fluorescent-labeled FK506 (DNS−FK506).

phylaxis and conversion therapy following lung transplantation (*6*).

FK506 is hardly soluble in water (<0.003 mg/mL at 25 °C) but is soluble in some organic solvents such as chloroform, methanol, acetone, or dimethyl sulfoxide (*7*). The commercially available intravenous dosage form of

* Correspondence should be addressed to this author: Tel 34-91-3944261; fax 34-91-3944672; e-mail ccasalsc@bio.ucm.es.
‡ Department of Biochemistry and Molecular Biology I, Complutense University of Madrid.
§ Hospital Puerta de Hierro.
‖ Department of Organic Chemistry, Complutense University of Madrid.
⊥ Instituto de Química-Física Rocasolano, CSIC.

tacrolimus contains a solubilizing agent, polyoxyethylated castor oil, which has been reported to be toxic. Systemic side effects such as neurotoxicity and nephrotoxicity complicate the use of FK506 in the clinical setting, restricting the permissible dosage (*8*). Local immunosuppression is a potential approach to reduce the adverse effects of systemic immunosuppression. Inhaled tacrolimus administration after lung transplantation could be an alternative to its systemic or oral administration, provided that drug levels reached by this route were enough to inhibit the inflammatory process due to the ischemic-reperfusion injury, as well as T-cell activation. Tacrolimus liposolubility complicates its use by this route of administration. Studies on aerosolized cyclosporin dissolved in solvents such as propylene glycol or ethanol showed effectiveness in attenuating acute lung rejection (*9*). However, organic solvents such as ethanol or propylene glycol induce cough and airway irritability and are poorly tolerated in humans. Our hypothesis is that use of lung surfactant-like liposomes as a vehicle for intratracheal tacrolimus administration could ensure drug contact with the alveolar spaces of the lung. Liposomal FK506 would be delivered directly to the lung and need not pass through the general circulation.

Lung surfactant is a macromolecular complex composed of 90% lipids and 10% proteins that create a lipid-rich phase separating alveolar gas and liquid at the surfaces of alveolar epithelial cells (*10−12*). Pulmonary surfactant phospholipids form monolayers and multilayers that reduce surface tension in the alveolus to negligible levels, thereby stabilizing the alveoli and maintaining lung volumes at end-expiration. Two phospholipids, DPPC (dipalmitoylphosphatidylcholine) and unsaturated phosphatidylglycerol (PG), are markedly rich in surfactant relative to other mammalian membranes and are of special importance for surfactant function (*13*). Lung surfactant deficiency, dysfunction, or inactivation causes respiratory failure.

The first objective of this study was to determine the optimal amount of FK506 incorporated in surfactant-like monolayers and vesicles and the effect of FK506 on the biophysical properties of these vesicles. The second objective was to determine whether the incorporation of FK506 in lung surfactant-like vesicles affects the immunosuppressive effectiveness of this drug.

While reports are available concerning the potential clinical use of liposomal FK506 formulations for intravenous administration (*14−16*), none have dealt either with the physical characterization of liposomal FK506 or with the interaction of this hydrophobic compound with phospholipid monolayers or bilayers. In this study we report for the first time the incorporation of FK506 in DPPC or DPPC/POPG monolayers and determine the FK506 partition coefficient between the aqueous and lipidic phases. We also study the

effect of FK506 on the physical state of DPPC and DPPC/POPG (8:2 w/w) vesicles by using fluorescence intensity and anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), differential scanning calorimetry, and vesicle aggregation assays. In addition, we evaluate the immunosuppressive effects of DPPC and DPPC/POPG (8:2 w/w) vesicles containing different amounts of FK506 on Jurkat T-cell proliferation and IL-2 production.

EXPERIMENTAL PROCEDURES

Materials. FK506 was provided by Fujisawa GmbH. A fluorescently labeled dansyl-FK506 conjugate (DNS−FK506) was prepared by aminodehydroxylation with dansylcadaverine of the carboxymethoxyl oxime derivative of FK506 at C-22 (Figure 1). The solid is identified as the dansyl−FK506 conjugate by ^{1}H NMR (200 MHz), ^{13}C NMR (50 MHz), and electrospray mass spectrometry. DNS−FK506 shows an absorption maximum at 333 nm in methanol solution. Dansylcadaverine as well as DPH was purchased from Molecular Probes (Eugene, OR). POPG and DPPC were purchased from Avanti Polar Lipids (Alabaster, AL). Phospholipids were stored in chloroform at −20 °C. PMA and PHA were obtained from Sigma Pharmaceutical Co (St. Louis, MO). RPMI 1640 medium and heat-inactivated fetal calf serum (FCS) were obtained from Biowhittaker, Belgium. Cell proliferation reagent WST-1 was purchased from Roche (Germany). ELISA kits for IL-2 immunoassays were obtained from BD PharMingen (San Diego, CA). Lymphocyte-like cell line (Jurkat T cells) was supplied by the American Type Culture Collection. Chloroform and methanol were of spectroscopy grade, purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade, obtained also from Merck.

FK506/Phospholipid Mixed Monolayers. Mixtures of DPPC or DPPC/POPG (8:2 w/w) were spread from chloroform/methanol (3:1 v/v) solutions onto a 5 mM Tris/HCl (pH 7.4) and 150 mM NaCl subphase, in a thermostated Langmuir−Blodgett trough (302RB ribbon barrier film balance, NIMA Technologies, Coventry, U.K.) as previously described (*17, 18*). For FK506/phospholipid mixed monolayers, the drug was dissolved in methanol and the desired amount was added to the chloroform/methanol−lipid solution. Once spread, the monolayer was allowed to stand for at least 10 min in order to ensure complete evaporation of the solvent. Monolayers were compressed at 50 cm^2/min. For all the isotherms, an equal number of phospholipid molecules was spread at the interface (typically 13.6 nmol). These experiments were performed at 25 °C.

Determination of the FK506 Partition Coefficient. If a drug is incorporated in a monolayer, an increase of the area (ΔA) of the film maintained at constant surface pressure will be observed with increasing amounts of drug incorporated (*19, 20*). At low drug concentration, one can accept the addition of the areas is given by

$$A_0 + \Delta A = A_L N_L + A_D N_D \qquad (1)$$

where A_0 is the initial area of the lipid monolayer in the absence of the drug ($A_0 = A_L N_L$), A_L and A_D are the lipid and drug areas, and N_L and N_D are the number of lipid and drug molecules in the monolayer after drug incorporation. The measured lipid area, A_L, at 32 mN m^{-1} was 51 ± 1 Å^2.

The drug area, A_D, was considered to be 860 $Å^2$ (*21*). The analysis of the partitioning of FK506 into lipid monolayers assumes that the incorporation of the drug does not change the lipid cross-sectional area. Measurements of ΔA at constant pressure will allow for the determination of the drug−lipid association constants as described below.

One can write, from $\Delta A = A_D N_D$ and $A_0 = A_L N_L$ (in eq 1)

$$\delta = \frac{N_D}{N_L} = \frac{(\Delta A)A_L}{A_0 A_D} \tag{2}$$

where δ is the number of moles of absorbed drug divided by the number of moles of lipid in the layer. Thus, the experimental determination of ΔA and A_0 at constant pressure will allow us to estimate δ.

The partition coefficient of tacrolimus between the lipid and aqueous phases, P, can be described by

$$P = \frac{x_{D/L}}{x_{D/W}} \tag{3}$$

in which $x_{D/L}$ and $x_{D/W}$ are the molar fractions of the drug in the lipidic and aqueous phases, respectively. $x_{D/L}$ can be expressed as a function of the drug-to-phospholipid ratio in the lipidic phase, δ:

$$x_{D/L} = \frac{\delta}{1 + \delta} \tag{4}$$

Therefore, δ is directly related to the partition coefficient, P, by

$$\delta = \frac{(\Delta A)A_L}{A_0 A_D} = \frac{P[D]_0}{1 + P[L]_0} \tag{5}$$

where $[D]_0$ and $[L]_0$ are the total drug and lipid concentrations, respectively. Equation 5 can be rewritten as a function of the ratio between the increase of the area, ΔA, and the initial area, A_0:

$$\frac{\Delta A}{A_0} = \frac{A_D}{A_L} \frac{P[D]_0}{1 + P[L]_0} \tag{6}$$

Preparation of Liposomal FK506. Unilamellar vesicles were used for cell assays and for spectroscopic measurements to reduce light scattering artifacts, whereas multilamellar vesicles (MLV) were used for differential scanning calorimetry. DPPC and DPPC/POPG (8:2 w/w) vesicles with or without different amounts of FK506 were prepared as follows: The required amounts of DPPC, POPG, and FK506 were dissolved in chloroform/methanol (3:1 v/v). The resulting mixtures were then evaporated to dryness under a gentle stream of nitrogen, and traces of solvent were subsequently removed by evacuation under reduced pressure overnight. When the dry residue was not used immediately, it was stored at −20 °C. In cases where DPH was incorporated, the probe was dissolved in methanol and the desired amounts were added to the chloroform/methanol− lipid solution before solvent removal (probe/phospholipid molar ratio of 1:200). The different lipid vesicles were prepared at a phospholipid concentration of 0.08, 1, or 3 mg/ mL by hydrating dry lipid films in a buffer containing 150 mM NaCl, 0.1 mM EDTA, and 5 mM Tris/HCl, pH 7.4, and allowing them to swell for 1 h at a temperature above the gel-to-liquid-phase transition temperature (T_m) of the corresponding phospholipid vesicles [50 °C for DPPC vesicles and 41 °C for DPPC/POPG (8:2 w/w) vesicles]. After vortexing, the resulting MLV were sonicated at the same temperature during 4 min at 390 W/cm² (burst of 0.6 s, with 0.4 s between bursts) in a UP 200S sonifier with a 2 mm microtip. Unilamellar vesicles were prepared freshly each day, just before the experiment was started. The final lipid concentration of both multilamellar and unilamellar vesicles was assessed by phosphorus determination.

For vesicle-size analysis in solution, quasielastic light-scattering was used as previously described (*22*). Light-scattering measurements were performed at 25 °C in a Zetasizer photon correlation spectrometer (Malvern 4700 instruments) with 256 channels. The light source was a Coherent Innova 300 Ar^+ laser operating at 514.5 nm. We studied the particle size of unilamellar vesicles containing various FK506 amounts. The diameter values of the tacrolimus-doped vesicles were similar to those of control vesicles without significant modifications linked to tacrolimus. DPPC/ POPG vesicles prepared by sonication were unilamellar vesicles. The mean diameters of the two major populations of DPPC/POPG vesicles prepared by sonication were 38 ± 4 nm (47% ± 4%) and 103 ± 13 nm (41% ± 5%). DPPC vesicles prepared by sonication consisted of a major population (70%) of unilamellar vesicles (mean diameter of 105 ± 6 nm) and a minor population (30%) of multilamellar vesicles (mean diameter about 2 μm) that were removed by centrifugation.

Fluorescence Measurements. Steady-state fluorescence measurements were carried out on an AB2 spectrofluorometer with a thermostated cuvette holder (±0.1 °C) with 2 × 10 mm path-length quartz cuvettes. Unilamellar vesicles containing DPH were prepared as reported above at a probe/ phospholipid molar ratio of 1:200. In cases where tacrolimus was incorporated, the desired amount of FK506 (dissolved in methanol) was added to the chloroform−lipid solution before solvent removal and vesicle preparation. Exposure to light was minimized throughout the liposome preparation process. DPH concentration was determined spectrophotometrically by absorbance at 350 nm, using a molar extinction coefficient in methanol of 88000 M^{-1} cm^{-1}. Absorbance spectra were recorded at 25 °C on a Beckman DU-640 spectrophotometer.

For DPH intensity measurements, the emission spectra were recorded with the emission polarizer set at the magic angle (m = 54.7°) relative to the vertically polarized excitation beam to reduce contributions from vesicle scattering and to avoid intensity artifacts due to molecular rotation during the lifetime of the excited state (*23*). Moreover, background intensities in DPH-free samples due to the vesicles were subtracted from each recording of fluorescence intensity. For fluorescence intensity measurements of dansylcadaverine−FK506 (DNS−FK506) in buffer and incorporated in phospholipid vesicles, excitation was at 340 nm. The fluorescence spectra were corrected for the scatter contribution due to the vesicles.

Fluorescence Emission Anisotropy. DPH fluorescence emission anisotropy measurements were obtained with Glan Prism polarizers. Excitation and emission wavelengths were

set at 359 and 427 nm, respectively. For each sample, prepared as described above, fluorescence emission intensity data in parallel and perpendicular orientations with respect to the exciting beam were collected 10 times each and then averaged. All measurements were performed at 25 °C. Anisotropy, r, was calculated as

$$r = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}} \tag{7}$$

where $I_{\parallel}$ and $I_{\perp}$ are the parallel and perpendicular polarized intensities measured with the vertically polarized excitation light, and G is the monochromator grating correction factor.

Differential Scanning Calorimetry. Calorimetric measurements were performed in a Microcal MCS differential scanning calorimeter (Microcal Inc., Northampton, MA) at a heating rate of 0.5 °C/min and under an extra constant pressure of 2 atm. DPPC and DPPC/POPG (8:2 w/w) multilamellar vesicles (4 mM) in the absence or presence of either 0.2 or 1 mol % FK506 were loaded in the sample cell of the microcalorimeter with buffer (150 mM NaCl and 5 mM Tris/HCl, pH 7.4) in the reference cell. Three calorimetric scans were collected from each sample between 25 and 60 °C. The standard Microcal Origin software was used for data acquisition and analysis. The excess heat capacity functions were obtained after subtraction of the buffer−buffer baseline.

Vesicle Aggregation Assay. The effect of free FK506 or FK506 incorporated in DPPC/POPG (8:2 w/w) vesicles on Ca^{2+}-dependent aggregation of these vesicles was determined by measuring the change in absorbance at 400 nm in a Beckman DU-640 spectrophotometer as previously reported (*24, 25*). On the other hand, DPPC/POPG (8:2 w/w) vesicle aggregation induced by human SP-A was monitored in the absence or presence of free FK506 as well as with FK506-containing liposomes. Briefly, DPPC/POPG (8:2 w/w) vesicles with or without increasing mole percentages of FK506 incorporated in the vesicle were added to both the sample and the reference cuvettes (80 μg/mL final concentration) in 5 mM Tris/HCl and 150 mM NaCl buffer, pH 7.4. After a 10-min equilibration at 37 °C, human SP-A (8 μg/mL) was added to the sample cuvette, and the change in optical density at 400 nm was monitored. Next, Ca^{2+} (2.5 mM) was added to both the sample and reference cuvettes, and the change in absorbance was monitored again. Experiments were also performed by adding SP-A (8 μg/mL) preincubated with or without 0.1 μM free-FK506 to 80 μg/mL DPPC/POPG (8:2 w/w) vesicles.

Human SP-A was purified from surfactant isolated from alveolar proteinosis patients by sequential butanol and *n*-octyl glucoside extractions as described elsewhere (*22, 24, 25*). The purity of SP-A was checked by one-dimensional SDS−PAGE in 12% polyacrylamide gel under reducing conditions (50 mM dithiothreitol). Quantification of SP-A was carried out by amino acid analysis in a Beckman System 6300 high performance analyzer.

Jurkat T-Cell Proliferation Assay. Jurkat T cells (ATCC, CRL-8163), a $CD4^+$ human lymphoblastoid cell line, were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, and penicillium G sodium (100 units/mL)/streptomycin sulfate (100 μg/mL), and 0.25 μg/mL amphotericin B at 37 °C in

95% air−5% CO_2 humidified atmosphere. The medium was changed every 48 h.

Jurkat T cells (seeded at 5×10^5 cells/mL) were stimulated with PMA (10 nM) and PHA (1 μg/mL) and cultured in 96-well microtiter plates for 24, 48, and 72 h in the presence of either DPPC or DPPC/POPG (8:2 w/w) vesicles with or without different amounts of FK506 (from 2.7×10^{-9} to 2.7×10^{-2} mg of FK506/mg of phospholipid, which would correspond to FK506 concentrations in the range of 10^{-12} to 10^{-5} M). Parallel experiments were performed in the absence or presence of free FK506 (from 10^{-12} to 10^{-5} M). Free FK506 was added to the culture medium from a stock solution in DMSO. The maximal final DMSO concentration (0.1%) achieved in the cultures did not affect cell proliferation. Surfactant-like phospholipid vesicles with or without different amounts of FK506 were prepared as described above but at the phospholipid concentration of 0.3 mg/mL. We found that DPPC/POPG (8:2 w/w) vesicles with or without FK506 prepared at phospholipid concentrations less than 0.5 mg/mL were not cytotoxic for the cells. All vesicles were prepared freshly each day just before the experiment was started.

To assess cell proliferation, the cell proliferation reagent (WST-1) was added to the cell cultures 4 h before the end of treatment. This assay is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase. Absorbance of the samples was measured at 450 nm. Results were expressed as percentages of the control response of cells in the absence of either free or liposomal FK506 (percent of the control). Four different T-cell cultures were used ($n = 4$). The assays from each Jurkat T-cell culture were performed in quadruplicate, the quadruplicate values were averaged, and their mean was treated as a single point. The results are presented as the means ($\pm$ SEMs), obtained by combining the results from each cell preparation.

The potential cytotoxicity of phospholipid vesicles and free and liposomal FK506 at the concentrations assayed was assessed as the loss of exclusion of propidium iodide (PI) after application of PI directly to the cell culture medium at 5 μg/mL final concentration. After 1 h of incubation at 37 °C, red fluorescent (PI-positive) cells ($\lambda_x = 540$ nm, $\lambda_m = 590$ nm) were scored as a percentage of the total cells in any given field. Two separate culture vessels were measured per experiment condition; all experiments were performed at least twice, and the data were compiled.

IL-2 Assay. Jurkat T cells (5×10^5 cells/mL) stimulated with PMA (10 nM) and PHA (1 μg/mL) were incubated in 24-well plates in the presence of either DPPC or DPPC/POPG (8:2 w/w) vesicles with or without different amounts of FK506 as described above. Parallel experiments were also performed in the absence or presence of free FK506 (from 10^{-12} to 10^{-5} M). Free FK506 was added to the culture medium from a stock solution in DMSO. The maximal final DMSO concentration (0.1%) achieved in the cultures did not affect IL-2 secretion by stimulated Jurkat cells.

For measurement of cytokine production, supernatants from culture plates were collected after 24 and 48 h of culture, centrifuged, and stored frozen at −70 °C until analysis. Human IL-2 levels were determined by ELISA. Results were expressed as percentages of the control level of cytokine production by cells stimulated with PHA and PMA in the absence of either free or liposomal FK506. Four
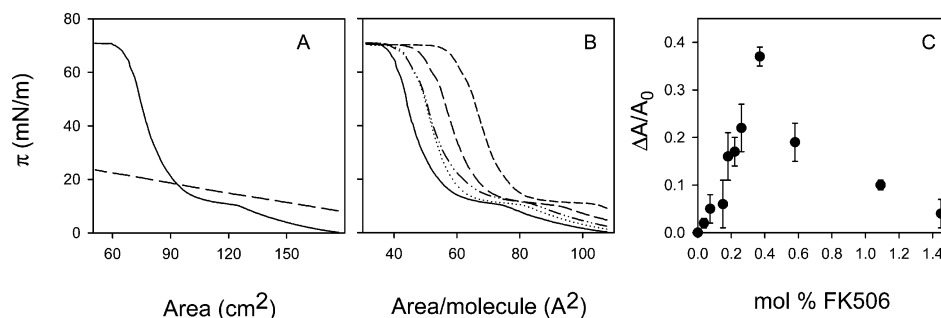
FIGURE 2: (A) Surface pressure (π)−area isotherms of DPPC (——) and FK506 (− − −) monolayers spread on 5 mM Tris/HCl (pH 7.4) and 150 mM NaCl at 25 °C. (B) Surface pressure−area per phospholipid molecule isotherms of mixed monolayers of DPPC containing variable mole percentages of FK506 at 25 °C. For clarity of presentation only selected isotherms are shown: pure DPPC (——), DPPC + 0.074 mol % FK506 (•••), DPPC + 0.26 mol % FK506 (− − −), DPPC + 0.4 mol % FK506 (- - -), and DPPC + 1.09 mol % FK506 (− •• −). For all the isotherms, an equal number of phospholipid molecules was spread at the interface (typically 13.6 nmol). (C) Change in the mean molecular area (ΔA) with respect to the initial molecular area of DPPC in the absence of FK506 (A_0) at constant surface pressure ($\pi = 32$ mN m^{-1}) as a function of mole percentage of FK506 in FK506/DPPC mixed monolayers at 25 °C.

different T-cell cultures were used ($n = 4$). The assays from each Jurkat T-cell culture were performed in triplicate, the triplicate values were averaged, and their mean was treated as a single point. The results are presented as the means ($\pm$ SEMs), obtained by combining the results from each cell preparation. For statistical analysis, mean comparison between free and liposomal FK506 was done by Mann−Whitney's test; a confidence level of 95% or greater ($p < 0.05$) was considered significant.

RESULTS

Mixed Monolayers of FK506 and DPPC or DPPC/POPG (8:2 w/w). Figure 2A shows the surface pressure−area isotherms for pure DPPC and FK506 monolayers. DPPC gave monolayers that exhibited a transition region between the expanded liquid state (LE) and the condensed liquid state (LC) at surface pressures in the range of 10−12 mN m^{-1} at 25 °C and on a subphase containing 150 mM NaCl and 5 mM Tris/HCl (pH 7.4). The collapse pressure for this phospholipid was about 70−72 mN m^{-1}. Unlike cyclosporin A (CsA) (*26*), FK506 did not form stable monolayers when deposited at the air−water interface. Figure 2B shows surface pressure versus area per molecule (π−A) isotherms of mixed monolayers of DPPC containing variable mole percentages of FK506. Isotherms of FK506/DPPC closely resembled the isotherm of pure DPPC but shifted along the x-axis; thus, the increase in mole percentage of FK506 in DPPC mono-layers caused a progressive expansion of the interfacial DPPC film, suggesting that the drug is occupying some space in the interface. The collapse of all FK506/DPPC mixed monolayers took place at a surface pressure of about 70 mN m^{-1}, which corresponds to that of the pure DPPC monolayer. Thus, the DPPC component in FK506/DPPC mixed mono-layers collapsed at the same surface pressure as in its pure form but at a surface area that increased with increasing FK506 content in the initial mixture. These results indicate that FK506 is not removed from the monolayer as the mixed film is compressed. Figure 2C plots the change in the mean molecular area (ΔA) with respect to the initial molecular area of DPPC in the absence of FK506 (A_0) at constant surface pressure ($\pi = 32$ mN m^{-1}) as a function of the mole percentage of FK506 in FK506/DPPC mixed monolayers. Figure 2C clearly shows that the maximum $\Delta A/A_0$ increase was observed at 0.4 mol % FK506. Beyond this mole

percentage, $\Delta A/A_0$ decreased. Similar results were observed at different surface pressures (data not shown). A representative isotherm of a DPPC monolayer containing an 1.09 mol % FK506 is shown in Figure 2B. This π−A isotherm was shifted to the left with respect to that corresponding to FK506/DPPC mixed monolayers containing an 0.4 mol % FK506. This indicates that at this mole percentage of FK506 (1.09) some drug might not have been incorporated in the monolayer, which suggests possible concentration-dependent aggregation of tacrolimus. Otherwise, a saturation effect would be observed.

On the other hand, while the incorporation of CsA into DPPC monolayers causes the LE−LC transition region to diminish (*26*), all the FK506/DPPC mixed monolayers showed LE−LC transition regions as deduced from the plateau regions of the isotherms at surface pressures in the range of 10−12 mN m^{-1} (Figure 2B). FK506/DPPC mixed monolayers showed the same onset pressure (π_s) of the plateau region as the pure DPPC monolayer but at an onset surface area that increased with increasing FK506 content in the initial mixture, causing an expansion of the plateau region in which there is phase coexistence. This occurred up to an FK506 concentration of 0.4 mol % (Figure 2B). A further increase in the number of FK506 molecules (beyond 0.4 mol % FK506) led to a narrowing of the LE−LC transition region with respect to that observed at 0.4 mol % FK506. This supports the idea that, at greater concentrations of FK506, concentration-dependent aggregation of tacrolimus might occur, resulting in decreased incorporation of the drug in the monolayer.

Figure 3A shows π−A isotherms of mixed monolayers of DPPC/POPG (8:2 w/w) in the absence and presence of 0.3 mol % FK506. In DPPC/POPG mixed films, a LE−LC transition occurs, although no plateau is observed in the π−A isotherm (*27*). The LC phase is highly enriched in DPPC and the LE phase consists of DPPC and POPG (*27*). The maximum surface pressure that can be achieved with this system is 68−69 mN m^{-1}. As has been described above for DPPC/FK506 mixed films, isotherms of DPPC/POPG/FK506 mixed monolayers resembled that of the mixed phospholipids alone but shifted to larger areas, although this shifting was not as significant as that obtained for DPPC monolayers. Figure 3B shows that the maximum $\Delta A/A_0$ increase was observed at 0.3 mol % FK506. Beyond this concentration, a
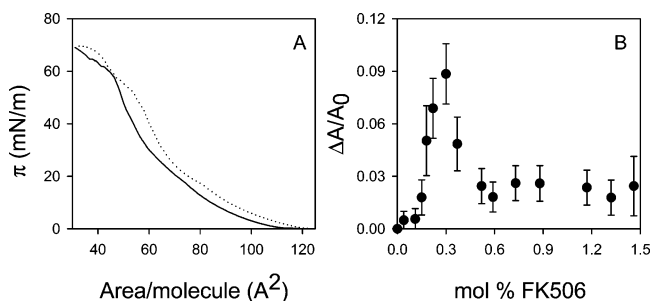
FIGURE 3: (A) Surface pressure−area per phospholipid molecule isotherms of mixed monolayers of DPPC/POPG 8:2 (w/w) in the absence (——) and presence (•••) of 0.3 mol % FK506 at 25 °C. (B) Change in the mean molecular area (ΔA) with respect to the initial molecular area of DPPC/POPG in the absence of FK506 (A_0) at constant surface pressure ($\pi = 32$ mN m^{-1}) as a function of mole percentage of FK506 in FK506/DPPC/POPG mixed monolayers at 25 °C.

shift to smaller areas is observed, probably indicative of a decreased incorporation of the drug in the monolayer as a consequence of the inability of the DPPC/POPG monolayer to incorporate higher amounts of FK506 or FK506 aggregates.

A very simple method to measure membrane incorporation of drugs is monitoring the area increase in a lipid monolayer at constant surface pressure (*19, 20*). The surface pressure that produces monolayers that most closely resemble lipid bilayers is called the bilayer equivalence pressure and is about 32 mN m^{-1} (*28*). Using eq 6, we have experimentally calculated the partition coefficient value, P, for the incorporation of FK506 (concentrations between 0.5 and 5 μM) into DPPC monolayers, at pH 7.4 and at two different surface pressures, 32 and 20 mN m^{-1}. We assumed a drug area, A_D, of 860 Å^2 [according to Van Duyne et al. (*21*)] and a measured lipid area, A_L, of 51 $\pm$ 1 Å^2. The obtained P value was $(3.9 \pm 0.3) \times 10^3$. This value indicates that tacrolimus incorporates efficiently into DPPC monolayers and is compatible with its hydrophobic structure. The partition coefficient value obtained might be affected by the uncertainty in drug area. Because tacrolimus did not form monolayers (Figure 2A), drug area could not be fairly estimated, while DPPC area at different pressures could be measured. Octanol is commonly used to model solute partitioning into regions of bilayers. The experimental partition coefficient of FK506 in *n*-octanol/water was reported to be about 10^3 (*7*). In addition, we calculated the octanol/water partition coefficient value (log P) of FK506

using ACD/log P v 5.15 software (*29*), obtaining a log P value of 3.96 $\pm$ 0.83 (corresponding to a P value of 9.1 $\times$ 10^3). These experimental and calculated octanol/water partition coefficient values are consistent with the partition coefficient value for the incorporation of FK506 into DPPC monolayers reported above and indicate the hydrophobicity of this drug.

Effect of FK506 on the Fluorescence Anisotropy and Intensity of DPH Incorporated in Mixed FK506/Phospholipid Vesicles. The effect of increasing the amount of tacrolimus on DPPC and DPPC/POPG (8:2 w/w) vesicles was evaluated by fluorescence anisotropy of DPH at 25 °C. For DPPC at 25 °C, below its T_m, the steady-state anisotropy of DPH in the absence of tacrolimus was ∼0.31, in the range of the observable DPH anisotropy in phospholipid vesicles in the gel phase (0.30−0.34) (*30*). Increasing the FK506 concentration in DPPC vesicles resulted in a small, but significant, increase in anisotropy, showing a saturation effect at FK506 concentrations of about 0.7 mol % (Figure 4A).

To find out whether the increase in DPH steady-state anisotropy in DPPC vesicles caused by FK506 was due to greater molecular order of lipids surrounding DPH, and a consequent slowing in DPH rotational diffusion, or to changes in DPH fluorescence lifetime, and hence changes in DPH steady-state fluorescence intensity (*31*), we determined the effect of FK506 on the fluorescence emission spectra of DPH in DPPC vesicles at 25 °C (Figure 4B,C). Figure 4C shows that the fluorescence intensity of DPH in DPPC vesicles decreased with increasing mole percentage of FK506, showing a saturation effect at approximately 1 mol % FK506 with respect to DPPC. These results suggest that the changes observed in the fluorescence anisotropy of DPH in DPPC vesicles could be partially due to variations in DPH fluorescence intensity. The effect of FK506 on both DPH fluorescence intensity and anisotropy in DPPC vesicles showed saturation at 0.7−1 mol % FK506. Thus, in our system, fluorescence intensity and anisotropy of DPH serve as a measure of the maximum incorporation of FK506 in DPPC vesicles, which was about double that obtained with DPPC monolayers.

The tacrolimus concentration-dependent changes observed in DPH intensity might indicate different probe environments due to the incorporation of the drug in the bilayer. Changes in DPH lifetime distribution are indicative of environmental heterogeneity (*32*). Alternatively, a direct quenching of DPH fluorescence by FK506 could take place provided that a close
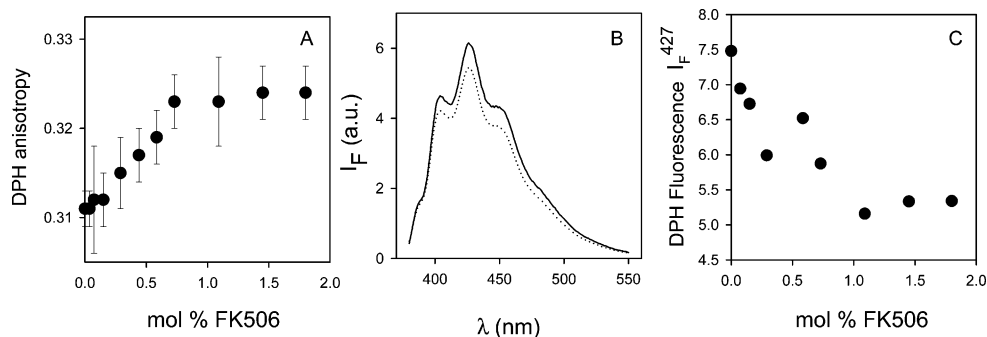

FIGURE 4: (A) Steady-state emission anisotropy of DPH incorporated into DPPC vesicles containing different concentrations of FK506 at 25 °C. ($\lambda_x = 359$ nm, $\lambda_m = 427$ nm). (B) Emission fluorescence spectra of DPH (I_{54}, $\lambda_x = 359$ nm) incorporated into DPPC vesicles in the absence (——) and presence (•••) of 0.6 mol % FK506 at 25 °C. (C) FK506 concentration-dependent effect on the fluorescence intensity of DPH at 427 nm.
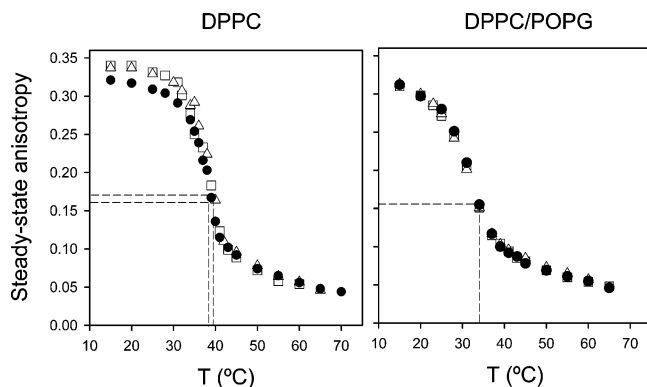
FIGURE 5: Temperature-dependent fluorescence anisotropy of DPH incorporated into DPPC and DPPC/POPG (8:2 w/w) vesicles in the absence (●) and presence of 0.6 (□) and 1.09 (△) mol % FK506. The standard deviation (SD) for each temperature was too small to be displayed by error bars. $\lambda_x = 359$ nm, $\lambda_m = 427$ nm.

approach of DPH and FK506 in the solid phase of the membrane occurs. However, an excess of FK506 in methanol did not affect the absorption spectrum of DPH in this solvent and slightly decreased the fluorescence intensity of DPH (data not shown). This indicates that reabsorption by FK506 was not responsible for the decrease in fluorescence intensity and that a direct quenching of DPH fluorescence by FK506 is reasonably improbable.

Results obtained with DPPC vesicles were qualitatively different from those obtained with DPPC/POPG. For DPPC/POPG at 25 °C, DPH anisotropy values in the absence of FK506 were lower than those for DPPC, consistently with slightly more freedom for wagging motion of DPH in DPPC/POPG (8:2 w/w) vesicles. Increasing the tacrolimus content resulted in small and not significant changes in DPH anisotropy (data not shown).

Effect of FK506 on the Gel to Liquid Crystalline Phase Transition of Surfactant-like Vesicles. We used steady-state DPH anisotropy to measure the effect of FK506 on the thermotropic behavior of surfactant-like vesicles. The fluorescence anisotropy of DPH decreases at temperatures higher than the T_m because the rotational freedom of the probe increases upon phospholipid acyl chain melting (*31*). The phase-transition temperature results from a condition of disorder due to the trans−gauche isomerization of phospholipid acyl chains. Figure 5 shows the effect of FK506 on the temperature dependence of the steady-state anisotropy of DPH embedded into DPPC and DPPC/POPG (8:2 w/w) vesicles. The fluorescence anisotropy of DPH significantly increased in the presence of FK506 in the gel phase, but it was altered neither in the liquid-crystalline phase nor in the transition region of DPPC vesicles. FK506 slightly shifts the T_m value of DPPC unilamellar vesicles from 39 °C in the absence of FK506 to 40 °C in its presence (Figure 5A). The T_m value obtained for DPPC vesicles was comparable to that obtained by other authors using polarization of DPH: 38.4 °C (*33*) and 39 °C (*34*). The incorporation of FK506 into DPPC/POPG (8:2 w/w) vesicles had no effect on DPH anisotropy in either the gel or the liquid-crystalline phase or on the T_m value of these vesicles (Figure 5B).

We also used the nonperturbing technique of differential scanning calorimetry (DSC) to probe the effect of FK506 on the thermotropic properties of phospholipid vesicles. DSC thermograms of FK506/DPPC and FK506/DPPC/POPG
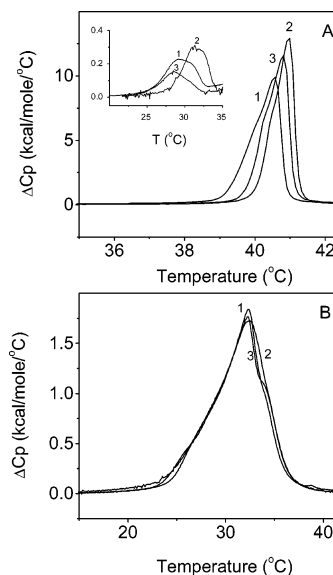

FIGURE 6: DSC heating scans of (A) DPPC and (B) DPPC/POPG (8:2 w/w) multilamellar vesicles (4 mM) in the absence or presence of variable mole percentages of FK506. The numbers on top of the peaks indicate (1) MLV without FK506, (2) MLV containing 0.2 mol % FK506, and (3) MLV containing 1 mol % FK506. The thermograms presented were the third after three consecutive scans from 25 to 60 °C were recorded from each sample, all of them being similar. Enlargement of thermograms around the main transition temperature are shown for both DPPC and DPPC/POPG vesicles. The inset of panel A shows amplification of DPPC thermograms around the pretransiton temperature. Calorimetric scans were performed at a rate of 0.5 °C/min.

Table 1: Phase Transition Parameters Determined for DPPC and DPPC/POPG (8:2 w/w) Multilamellar Vesicles with and without FK506[a]

sample	T_m (°C)	$T_{1/2}$ (°C)	ΔH (kcal/mol)
DPPC	40.6 ± 0.1	0.88 ± 0.06	10.7 ± 0.3
DPPC + 0.2 mol % FK506	41.0 ± 0.01	0.67 ± 0.01	10.5 ± 0.4
DPPC + 1.0 mol % FK506	40.8 ± 0.01	0.87 ± 0.05	10.4 ± 0.4
DPPC/POPG (8:2 w/w)	32.3 ± 0.04	5.2 ± 0.1	11.1 ± 0.3
DPPC/POPG + 0.2 mol % FK506	32.5 ± 0.08	6.1 ± 0.1	11.8 ± 0.4
DPPC/POPG + 1.0 mol % FK506	32.3 ± 0.02	6.0 ± 0.1	11.5 ± 0.1

[a] Data are taken from heating scan. Values are the mean ± SD of three experiments.

multilamellar vesicles are shown in Figure 6, panels A and B, respectively. DPPC alone (curve 1) showed endotherms composed of a pretransition (inset, Figure 6A), from a titled to rippled chain gel phase, and a main transition from the gel to the liquid crystalline phase. Two thermal transitions were also observed for DPPC liposomes in the presence of FK506. It can be observed in Figure 6A that, upon incorporation of FK506 at 0.2 mol %, both the main transition and the pretransition temperatures were shifted slightly upward (Figure 6A, curve 2), whereas with incorporation of higher amounts (1 mol %), variations in the main transition and pretransition temperatures were smaller than those observed at lower FK506 concentrations (Figure 6A, curve 3). In addition, the calorimetric transition enthalpy (ΔH) did not change in the presence of 0.2 or 1 mol % FK506 (Table 1). These data indicate that the thermodynamic properties of the lipid that participate in the phase transition were minimally affected by the incorporation of FK506 in DPPC membranes.
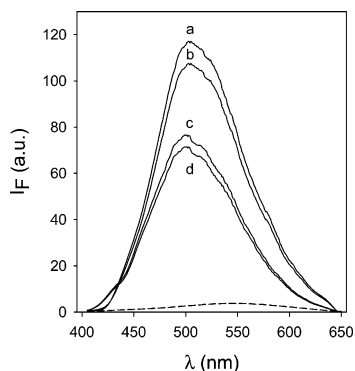
FIGURE 7: Fluorescence emission spectra of 0.5 μM DNS−FK506 in 150 mM NaCl and 5 mM Tris/HCl (pH 7.4) buffer (- - -) and incorporated in DPPC vesicles (—) at 25 °C. DPPC concentrations were (a) 1.36 mM, (b) 1.08 mM, (c) 0.27 mM, and (d) 0.136 mM. For all the assays, an equal concentration of DNS−FK506 was used (typically 0.5 μM). The emission maximum of DNS−FK506 in buffer was 545 nm. Incorporation of DNS−FK506 into DPPC vesicles led to a great increase in fluorescence intensity together with a blue shift of the emission maximum to 500 nm. At DNS−FK506/DPPC molar ratios higher than 0.04/100 (line a), the fluorescence intensity of DNS−FK506 decreased (lines b, c and d), indicating that DNS−FK506 was not quantitatively incorporated in DPPC vesicles. Similar results were found with DPPC/POPG vesicles. $\lambda_x = 340$ nm.

Figure 6B and Table 1 show that incorporation of either 0.2 or 1 mol % FK506 in DPPC/POPG (8:2 w/w) multilamellar vesicles did not cause any change in the transition temperature (32.5 ± 0.1 °C) and the calorimetric enthalpy change (11.5 ± 0.2 kcal/mol), which is consistent with previous results on the effect of FK506 on the temperature dependence of the steady-state anisotropy of DPH embedded into tacrolimus-doped DPPC/POPG (8:2 w/w) unilamellar vesicles. However, the main phase-transition width at half-peak height ($T_{1/2}$), which is a measure of destabilization of phospholipid assemblies, slightly increased in the presence of FK506 (Table 1).

Given the modest effect of FK506 in the thermotropic phase properties of DPPC and DPPC/POPG membranes, it can be argued that FK506 was excluded from intercalating into the bilayer because of the bulk of the FK506 ring. However, at the FK506 concentrations used in this study, incorporation of FK506 into the membrane was estimated quantitatively by calculating the partition coefficient value (P) for the incorporation of FK506 into DPPC monolayers. This P value was (3.9 ± 0.3) × 10^3, indicating that FK506 concentration in the lipid phase is 3.9 × 10^3 times its concentration in the aqueous phase.

Incorporation of DNS−FK506 in DPPC and DPPC/POPC Vesicles. To gain insight into the incorporation of tacrolimus in the phospholipid bilayer, we have used a dansylated analogue of tacrolimus. The interaction of dansylated tacrolimus with phospholipids could be followed by changes in fluorescence intensity and by spectral shifts. Figure 7 shows the fluorescence emission spectra of 0.5 μM DNS−FK506 in buffer and incorporated in DPPC vesicles at different phospholipid concentrations (from 1.36 to 0.136 mM). When the fluorescent derivative of tacrolimus is incorporated into phospholipid vesicles, the fluorescence emission spectrum of DNS−FK506 showed a great increase in fluorescence intensity accompanied by a notable blue shift of the emission maximum from 545 to 500 nm. This indicates a decrease of

the polarity of the environment around the fluorescent group upon DNS−FK506 incorporation into phospholipid vesicles. These data suggest that DNS−FK506 is located internally in the hydrophobic core of the membrane rather than being weakly partitioned near the polar, hydrated headgroups and the glycerol backbone. Figure 7 also shows that decreasing phospholipid vesicle concentration, which increased the DNS−FK506/phospholipid molar ratio, led to a diminution of the fluorescence emission intensity of DNS−FK506. This is probably a consequence of the incapability of DPPC membranes to quantitatively incorporate DNS−FK506 at a drug/phospholipid molar ratio higher than 0.04/100. Similar results were found with DPPC/POPG (8:2 w/w) vesicles (data not shown). The small amount of DNS−FK506 incorporated in the membrane in comparison with that of FK506 incorporated in phospholipid monolayers and bilayers can be explained by the greater hydrophobic nature of the dansylated analogue (the calculated log P value for DNS−FK506 was 6.90 ± 0.97).

Effect of FK506 on Ca^{2+}-Dependent Aggregation of DPPC/POPG Vesicles in the Presence and Absence of SP-A. We examined the effect of increasing amounts of FK506 (from 0.36 to 1 mol %) incorporated in DPPC/POPG (8:2 w/w) vesicles on lipid aggregation induced by the presence of millimolar concentrations of calcium in the medium. These assays were performed at temperatures below (25 °C) and above (42 °C) the T_m of these vesicles (32 °C). We found that DPPC/POPG (8:2 w/w) vesicles slightly aggregated in the presence of physiological concentrations of Ca^{2+} (2.5 mM), and the incorporation of FK506 in these vesicles did not affect vesicle aggregation (data not shown). Moreover, addition of free FK506 to the medium did not affect surfactant-like vesicle aggregation.

On the other hand, we found that free FK506 binds to surfactant protein A (SP-A), one of the major proteins present in the alveolar fluid. This was expected for the capability of SP-A to interact with a broad range of lipids (*11*). Figure 8A shows that when SP-A was incubated with 0.1 μM FK506 (FK506/SP-A molar ratio of 1/2), an enhancement of the vesicle aggregation induced by the protein was observed with respect to that obtained for the protein alone. Since addition of FK506 alone did not aggregate lipid vesicles (Figure 8A) or modify the thermotropic properties of these vesicles (data not shown), the effect of FK506 on SP-A-induced vesicle aggregation is likely due to the interaction of the drug with the protein. To find out whether SP-A can also interact with FK506 embedded in DPPC/POPG vesicles, we investigated SP-A-induced vesicle aggregation of FK506/DPPC/POPG (8:2 w/w) mixed bilayers containing different mole percentages of FK506 at temperatures below (25 °C) and above (42 °C) the T_m of these vesicles (32 °C) (Figure 8B). Within experimental error, no effect of the FK506 embodied into the bilayer was observed at different FK506 mole percentages and at different temperatures. These results indicate that SP-A does not interact with FK506 when the drug is embedded in the membrane. Since SP-A interacts primarily at bilayer surfaces (*11, 35*), these data strongly suggest an inward location for FK506 in the hydrophobic core of the membrane rather than an outward location at the bilayer surface.

Evaluation of Immunosuppressive Activity of Liposomal FK506. The data in Figure 9 (shaded bars) demonstrate that
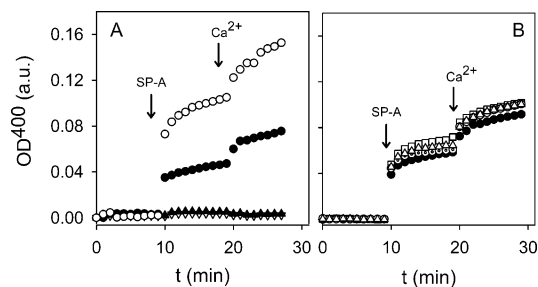
FIGURE 8: Aggregation of DPPC/POPC (8:2 w/w) vesicles induced by surfactant protein A. (A) Sample and reference cuvettes were first filled with DPPC/POPG (8:2 w/w) vesicles (80 μg/mL) in 5 mM Tris/HCl and 150 mM NaCl buffer at pH 7.4, with or without 0.1 μM free FK506. After a 10-min equilibration at 25 °C, SP-A (8 μg/mL) was added and the change in optical density was monitored. Next, Ca^{2+} (2.5 mM) was added and the change in absorbance was monitored again. Symbols indicate (▲) vesicles alone, (▽) vesicles + 0.1 μM free FK506, (●) vesicles + SP-A, and (○) vesicles + SP-A + 0.1 μM free FK506. (B) Experiments were performed as in panel A with DPPC/POPG (8:2 w/w) containing different amounts of FK506: (●) 0 mol % FK506, (☉) 0.36 mol % FK506, (□) 0.54 mol % FK506, (△) 0.72 mol % FK506, and (▽) 1.0 mol % FK506. Vesicles containing FK506 did not aggregate in the absence of SP-A (curves not shown). Similar results were found at 37 and 42 °C, but the extent of vesicle aggregation was lower as the SP-A-induced vesicle aggregation is strongly dependent on the physical state of the vesicles (*22*). Phospholipid/SP-A weight ratio was 10:1 and $[Ca^{2+}] = 2.5$ mM.

FK506 incorporated in DPPC/POPG (8:2 w/w) vesicles markedly reduced the IL-2 production (about 80%) by PMA/PHA-stimulated Jurkat T cells after 48 h of culture. This 80% inhibition of IL-2 production occurred at very low mole percentages of FK506 in the membrane (0.25×10^{-4} mol %), which corresponded to a concentration of free FK506 of 0.1 nM. Similar results were found with FK506 incorporated in DPPC vesicles. Importantly, cells treated with DPPC/POPG or DPPC vesicles without FK506 did not modify the IL-2 secretion levels observed in cells incubated in the absence of these vesicles. The effect of free FK506, added to the cell medium in DMSO at analogous concentrations, was similar but, as expected, occurred earlier. Figure 9 (solid bars) shows that concentrations of free FK506 equal to or higher than 0.1 nM inhibited IL-2 production by 80% after 24 h of culture. After 48 h of culture the inhibitory effect of similar amounts of free and liposomal FK506 on the production of IL-2 by PMA/PHA-stimulated Jurkat T cells was similar, without significant differences between them.

Figure 10 shows the effect of free FK506 and liposomal FK506 (incorporated in DPPC/POPG vesicles) on the proliferation of Jurkat cells induced by PHA (1 μg/mL) and PMA (10 nM), after 1, 2, and 3 days of culture. Free FK506 inhibited the proliferative response by more than 70% after 2 days of culture and 85% after 3 days of culture. In contrast, liposomal FK506 had little effect on the proliferation assessed on day 2 and led to a dose-dependent inhibition of the proliferative response after 3 days of culture. Similar results were found with FK506 incorporated in DPPC vesicles (data not shown). This inhibitory effect of liposomal FK506 on T-cell proliferation after 3 days of culture was not due to either nonspecific cytotoxicity of phospholipid vesicles or a direct effect of those vesicles on the proliferation of Jurkat T cells. We found that DPPC or DPPC/POPG (8:2 w/w) vesicles (prepared at phospholipid concentrations less than 0.5 mg/mL) with or without FK506 were not cytotoxic for
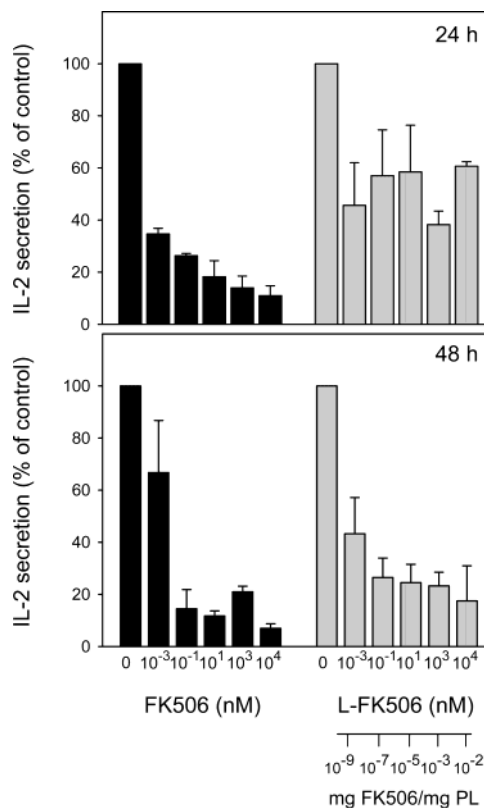

FIGURE 9: Effect of free and liposomal FK506 (L-FK506) on IL-2 production by Jurkat T cells stimulated with PMA (10 nM) and PHA (1 μg/mL). Solid bars represent cells incubated in the absence or presence of various concentrations of free FK506. Shaded bars represent cells incubated in the presence of DPPC/POPG (8:2 w/w) vesicles with or without different amounts of FK506. Incubations were performed for 24 or 48 h. Supernatants from triplicate wells were collected after 24 or 48 h of culture and the levels of IL-2 were measured by ELISA. Data presented are from four different T-cell cultures ($n = 4$). The results (mean ± SEMs from four cultures) were expressed as percentages of the control level of IL-2 production by stimulated cells in the absence of either free or liposomal FK506. FK506 to phospholipid weight ratio of L-FK506 preparations is also shown.

the cells (data not shown). In addition, we did not find significant differences in cell proliferation between cells treated with or without DPPC/POPG or DPPC unilamellar vesicles in the absence of FK506, although antiproliferative effects of surfactant lipid extracts on T cells have been reported (*36*). The inhibitory effect of liposomal FK506 on Jurkat proliferation may reflect an FK506-dependent inhibition in the production of IL-2 (*37*).

DISCUSSION

We have developed a new liposomal FK506 with surfactant-like phospholipids to be administered intratracheally after lung transplantation or in inflammatory diseases. Respiratory delivery of FK506 integrated in surfactant-like vesicles would facilitate FK506 influx to the alveolar spaces of the lung and intracellular delivery to alveolar macrophages and lymphocytes, without negatively affecting the lipid composition of pulmonary surfactant.

We investigated the optimal incorporation of FK506 in DPPC membranes, its effects on the physical properties of DPPC and DPPC/POPG vesicles, as well as the immunosuppressive effectiveness of liposomal FK506. Although the
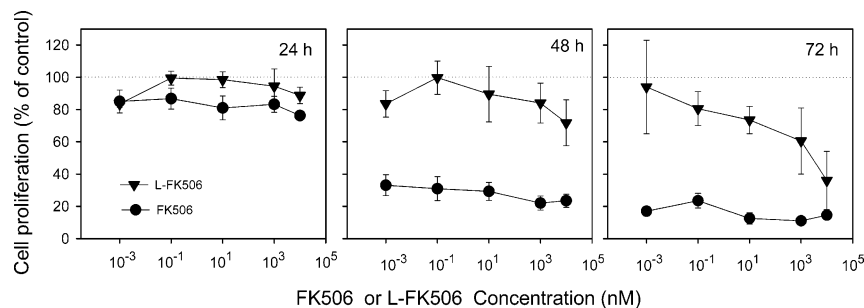
FIGURE 10: Effect of free and liposomal FK506 (L-FK506) on the proliferation of Jurkat T cells stimulated with PMA (10 nM) and PHA (1 μg/mL). Cells were incubated in the absence or presence of various concentrations of free FK506 (●) and in the presence of DPPC/POPG (8:2 w/w) vesicles with or without different amounts of FK506 (▼) for 24, 48, and 72 h. Four different T-cell cultures were used ($n = 4$). Medium alone or with DPPC/POPG vesicles (controls) and media with various concentrations of free or liposomal FK506 were distributed in quadruplicate wells at the initiation of the cultures. The results are presented as the means ($\pm$ SEMs) obtained by combining the results from each cell preparation. Results were expressed as percentages of the control response of stimulated cells in the absence of either free or liposomal FK506 (% of control).

effect of the incorporation of CsA, another immunosuppressant drug structurally dissimilar to FK506, into phospholipid monolayers and bilayers has been studied (*26*, *38−40*), we are not aware of studies on the effects of the interaction of FK506 with membranes. These studies have additional interest because FK506, as well as CsA, partitions into membranes. No receptors have been demonstrated for these drugs in cellular membranes, but they have been found in the cytosol. FK506 and CsA belong to the family of immunophilin-binding drugs, each drug binding to its respective cytosolic immunophilin: FK506 to FKBP and CsA to cyclophilin (*2*, *41*). These drug−immunophilin complexes have the same target, the Ca^{2+}/calmodulin-dependent phosphatase calcineurin, and thus have essentially identical biological effects (*2*, *41*). However, this notion was recently challenged by both clinical and experimental evidence (*42*). Partition of drugs into membranes can alter membrane organization. The study of the interaction of immunosuppressive drugs, such as FK506 or CsA, with membranes can help to understand molecular mechanisms of action or side effects of these drugs.

The superficial monolayer technique, which uses a phospholipid monolayer at the air−water interface, has been used as a membrane model to characterize drug or protein−membrane interactions (*43*). We investigated the interaction between FK506 and DPPC and DPPC/POPG membranes using the Langmuir film balance technique. While there are some studies on mixed monolayers of CsA and phospholipids (*26*, *40*), there are no references to the interaction of FK506 with phospholipid monolayers. We found that the presence of increasing amounts of FK506 in DPPC or DPPC/POPG monolayers caused a progressive expansion of the interfacial phospholipid film. FK506 is not removed from the monolayer as the mixed film is compressed. Thus, the DPPC component in FK506/DPPC mixed monolayers collapsed at the same surface pressure as in its pure form but at a surface area that increased with increasing FK506 content. The change in the mean molecular area (ΔA) with respect to the initial molecular area of DPPC film in the absence of FK506 (A_0), measured at different surface pressures, increased with increasing mole percentage of FK506 in the monolayer. The maximum $\Delta A/A_0$ increase was observed at an FK506 concentration of 0.4 mol %. Beyond this mole percentage, the $\Delta A/A_0$ ratio decreased and the DPPC/FK506 monolayer collapsed at lower surface areas than that found at 0.4 mol

% FK506. These results indicate that beyond 0.4 mol % FK506 was not quantitatively incorporated into the DPPC monolayer. A possible reason for these results is that, at higher concentrations, FK506 undergoes self-aggregation and FK506 aggregates are not incorporated in the monolayer. At large molecular areas (low surface pressure), the phospholipid molecules are loosely packed and the hydrophobic tails are tilted. Because the lipid tails are near the aqueous surface, FK506 would be in proximity with water and might aggregate at high FK506 concentrations (the solubility of FK506 in water is very low). Concentration-dependent aggregation of tacrolimus would explain the lack of a saturation effect. FK506 also incorporated in DPPC/POPG 8:2 (w/w) mixed monolayers up to 0.3 mol %. Beyond this concentration, the $\Delta A/A_0$ ratio decreased, indicating that some drug has not been partitioned in the monolayer and suggesting possible concentration-dependent aggregation of tacrolimus.

The incorporation of FK506 into DPPC monolayers, at concentrations $\leq 5\ \mu$M, occurred with a partition coefficient of $(3.9 \pm 0.3) \times 10^3$, i.e., FK506 concentration in the lipid phase is 3.9×10^3 times its concentration in the aqueous phase. This indicates that FK506 is efficiently incorporated into DPPC membranes at low drug concentrations. This value is consistent with the calculated octanol/water partition coefficient value for FK506 and indicates the hydrophobicity of this drug.

On the other hand, from fluorescence intensity and anisotropy measurements of DPH embedded in DPPC vesicles containing increasing amounts of FK506, we determined that FK506 was incorporated in DPPC membranes up to an FK506 concentration of about 0.7−1 mol %, which was about double that obtained by the monolayer technique. These findings from both the phospholipid monolayer and the bilayer models are useful for the optimization of liposomal formulation of FK506 and for better understanding of FK506−membrane interaction.

DPH embedded in DPPC vesicles is located toward the bilayer center. The fact that the fluorescence intensity of DPH decreased with increasing FK506 mole percentage in the membrane suggests an inward location for FK506 in the hydrophobic core of the membrane rather than a weak distribution of the drug near the polar headgroups and the glycerol backbone. This inward location was demonstrated by analyzing the incorporation of DNS−FK506 in DPPC or

DPPC/POPG vesicles. Incorporation of DNS−FK506 in membranes led to a great increase in fluorescence intensity accompanied by a marked blue shift of the emission maximum, indicative of reduced polarity of the environment around the fluorescent group upon incorporation of DNS−FK506 into phospholipid vesicles. In addition, we found that surfactant protein A, one of the major lipid binding proteins present in the alveolar fluid that interacts primarily at bilayer surfaces (*11, 35*), was unable to interact with FK506 embedded in DPPC/POPG vesicles but not with free FK506. This strongly supports an inward location of FK506 in the membrane. Results on the incorporation of FK506 in DPPC and DPPC/POPG monolayers and of DNS−FK506 in DPPC and DPPC/POPG bilayers indicate that there is a limit value for the quantitative incorporation of FK506 into the membrane. This limited access of FK506 into DPPC and DPPC/POPG membranes might be due to its bulky ring, as was also shown for the hydrophobic anticancer drug Taxol (paclitaxel) (*44, 45*). Clinically, these findings suggest that concentrations of this type of drug in the blood or the alveolar fluid above a certain limiting value may not facilitate drug penetration into the cell membrane and might cause adverse side effects.

We used differential scanning calorimetry and steady-state DPH anisotropy to investigate the effect of FK506 on the thermotropic phase properties of DPPC and DPPC/POPG vesicles. We found that FK506 hardly affected the transition enthalpy, T_m, and cooperativity of the phase transition of DPPC and DPPC/POPG (8:2 w/w) vesicles. Jain and Wu (*46*) developed a model that associated changes in the lipid phase transition profiles induced by various solutes with the location of the solute inside the bilayer. Molecules that induce a simultaneous decrease of T_m and cooperativity are preferentially located in the upper methylene region (from C_1 to C_8 of the fatty acid chains), which is the cooperative region of the membrane. Molecules that do not change the cooperativity of the transition are preferentially located in the lower methylene area from C_9 to the end of the acyl chains. Applying this model to our DSC scans with FK506 suggests the preferential location of FK506 in the lower methylene region (C_9−C_{16}) of DPPC membranes instead of in the upper region. These results contrast with those found when the immunosuppressant CsA, which is a hydrophobic 11-residue cyclic peptide, is incorporated in saturated phospholipid vesicles (*38, 39*). CsA decreases the T_m and broadens the phase transition, indicating the presence of CsA in the cooperative region of these membranes. CsA decreases phospholipid acyl chain order at temperatures below the main phase transition and increases order at temperatures above the transition (*38, 39*). These effects are similar to those caused by cholesterol, and it has been suggested that the locations of CsA and cholesterol in membranes are similar (*39*). Taken together, these results indicate that the immunosuppressant FK506 and CsA have different effects on membrane structure.

The immunosuppressive effectiveness of increased amounts of FK506 incorporated in DPPC/POPG (8:2 w/w) or DPPC unilamellar vesicles was compared with those of similar amounts of free FK506 dissolved in DMSO. This study provides evidence that FK506 embedded in surfactant-like vesicles at an FK506-to-lipid ratio as low as 0.2×10^{-4} mol % inhibits certain events of Jurkat T-cell activation that lead to IL-2 production and proliferation. Although some authors have reported antiproliferative and immunosuppressive effects of phospholipids on T cells (*36*), in our study the treatment of Jurkat T cells with DPPC/POPG (8:2 w/w) or DPPC unilamellar vesicles, in the absence of FK506, did not influence the values of proliferation and IL-2 secretion observed in control cells stimulated with PMA and PHA in the absence of these vesicles. Liposomal FK506 induced 80% inhibition of IL-2 production at an FK506 concentration of 0.2×10^{-4} mol %, which corresponded to 0.1 nM free FK506. In addition, treatment of stimulated Jurkat T cells with liposomal FK506 led to a dose-dependent inhibition of T-cell proliferation, which emerged later (after 72 h culture) than that observed with free FK506. This delay in the immunosuppressive action of FK506 embedded in liposomes with respect to that of similar amounts of free FK506 suggests that the mechanism by which liposomal FK506 and free FK506 are delivered to the cells is different. Taken together, the results reported here indicate that FK506 embedded in surfactant-like phospholipid vesicles retains the immunosuppressive efficacy of the drug.

The immunosuppressive efficacy of other formulations of liposomal FK506, administered systemically, has been tested in animal models of liver (*14*) and heart (*15*) transplantation. Liposomal FK506 has also been tested in cell transplantation or xenotransplantation (*16, 47*) and in a model of immune-mediated skin disease (*48*). The liposomal FK506 formulations used in these studies were prepared by incorporation of FK506 in phosphatidylcholine/cholesterol vesicles at an FK506 mole percentage greater than the limit value estimated in this study for the quantitative incorporation of FK506 in surfactant-like vesicles. In all of these in vivo studies, it was concluded that encapsulation of FK506 in lipid vesicles increased the FK506 immunosuppressive efficacy in organ allotransplantation or in xenotransplantation models (*14−16, 47*). In addition, liposomal FK506 has the ability to penetrate skin and achieves higher skin concentrations than when FK506 is systemically administered, suggesting that liposomes can be an effective vehicle for FK506 treatment in skin disorders including psoriasis (*48*). In relation to lung transplantation, systemic FK506 has been used as primary prophylaxis therapy following lung transplantation (*6*), and it has been recently reported that preoperative systemic administration of FK506 decreases lung ischemia-reperfusion injury in rats (*49*). Liposomal FK506 could be intratracheally administered either before or after reperfusion to protect the lung against ischemia-reperfusion injury. According to our results, a dose of 0.2 mg of FK506/kg of body weight (which is the recommended dose for oral administration) could be intratracheally administered in 25 mg of phospholipid/kg of body weight (the established dose for lung surfactant therapy is about 50−100 mg of phospholipid/kg of body weight). Surfactant-like phospholipids would not only warrant tacrolimus influx to the alveolar spaces of the lung but would also contribute to the improvement of pulmonary function, since previous studies have shown that endogenous surfactant phospholipids are negatively affected by ischemic-reperfusion injury after lung transplantation (*50*). Further work in documenting the actual doses and effectiveness of FK506 incorporated in surfactant-like phospholipids needs to be done in animal models of lung transplantation. In addition, experiments are in progress to determine whether specific

interactions of free and liposomal FK506 with other surfactant components influence the immunosuppressive efficacy of the drug and its delivery to alveolar cells.

REFERENCES

1. Kino, T., Hatanaka, H., Hashimoto, M., Nishimaya, M., Goto T., Okuhara, M., Kohsaka, M., Aoki, H., and Imanaka, H. (1987) FK-506, a novel immunosuppressant isolated from a *Streptomyces*. I. Fermentation, isolation, and physicochemical and biological characteristics, *J. Antibiot. 40*, 1249−1255.
2. Clardy, J. (1995) The chemistry of signal transduction, *Proc. Natl. Acad. Sci. U.S.A. 92*, 56−61.
3. Keicho, N., Sawada, S., Kitamura, K., Yotsumoto, H., and Takaku, F. (1991) Effects of an immunosuppressant, FK506, on interleukin 1 alpha production by human macrophages and a macrophage cell line U937, *Cell Immunol. 132*, 285−294.
4. Sakr, M. F., McClain, C. J., Gavaler, J. S., Zetti, G. M., Starzl, T. E., and Van Thiel, D. H. (1993) FK 506 pretreatment is associated with reduced levels of tumor necrosis factor and interleukin 6 following hepatic ischemia/reperfusion, *J. Hepatol. 17*, 301−307.
5. Mayer, A. D., Dmitrewski, J., Squifflet, J. P., Besse, T., Grabensee, B., Klein, B., Eigler, F. W., Heemann, U., Pichlmayr, R., Behrend, M., Vanrenterghem, Y., Donck, J., van Hooff, J., Christiaans, M., Morales, J. M., Andres, A., Johnson, R. W., Short, C., Buchholz, B., Rehmert, N., Land, W., Schleibner, S., Forsythe, J. L., Talbot, D., Pohanka, E., et al. (1997) Multicenter randomized trial comparing tacrolimus (FK506) and cyclosporine in the prevention of renal allograft rejection: a report of the European Tacrolimus Multicentre Renal Study Group, *Transplantation 64*, 436−443.
6. Scott, L. J., McKeage, K., Keam, S. J., and Plosker, G. L. (2003) Tacrolimus, a further update of its use in the management of organ transplantation, *Drugs 63*, 1247−1297.
7. Hane, K., Fujioka, M., Namiki, Y., Kitagawa, T., Kihara, N., Shimatani, K., and Yasuda, T. (1992) Physicochemical properties of (−)-(1*R*,9*S*,12*S*,13*R*,14*S*,17*R*,18*E*,21*S*,23*S*,24*R*,25*S*,27*R*)-17-allyl-1,14-dihydroxy-12-[(*E*)-2-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-tetrone hydrate (FK-506), *Iyakuhin Kenkyu 23*, 33−43.
8. Platz, K. P., Mueller, A. R., Blumhardt, G., Bachmann, S., Bechstein, W. O., Kahl, A., and Neuhaus, P. (1994) Nephrotoxicity following orthotopic liver transplantation. A comparison between cyclosporin and FK506, *Transplantation 58*, 170−183.
9. Iacono, A. T., Smalldone, G. C., Keenan, R. J., Diot, P., Dauber, J. H., Zeevi, A., Burchart, G. J., and Griffith, B. P. (1997) Dose-related reversal of acute lung rejection by aerosolized cyclosporin, *Am. J. Respir. Crit. Care Med. 155*, 1690−1695.
10. Goerke, J. (1998) Pulmonary surfactant: functions and molecular composition, *Biochim. Biophys. Acta 1408*, 79−89.
11. Casals, C. (2001) Role of surfactant protein A (SP-A)/lipid interactions for SP-A functions in the lung, *Pediatr. Pathol. Mol. Med. 20*, 249−268.
12. Whitssett, J. A., and Weaver, T. E. (2002) Hydrophobic surfactant proteins in lung function and disease. *N. Engl. J. Med. 347*, 2141−2148.
13. Van Golde, L. M. G., and Casals, C. (1997) Metabolism of lipids, in *The Lung: Scientific Foundations* (Crystal, R. G., and West, J. B., Eds.) 2nd ed., pp 9−18, Lippincott−Raven Publishers, Philadelphia, PA.
14. Ko, S., Nakajima, Y., Kanehiro, H., Horikawa, M., Yoshimura, A., Taki, J., Aomatsu, Y., Kin, T., Yagura, K., and Nakano, H. (1995) The enhanced immunosuppressive efficacy of newly developed liposomal FK506 in canine liver transplantation, *Transplantation 59*, 1384−1388.
15. Moffatt, S. D., McAlister, V., Calne, R. Y., and Metcalfe, S. M. (1999) Potential for improved therapeutic index of FK506 in liposomal formulation demonstrated in a mouse cardiac allograft model, *Transplantation 67*, 1205−1208.
16. Alemdar, A. Y., Baker, K. A., Sadi, D., McAlister, V. C., and Mendez, I. (2001) Liposomal tacrolimus administered systemically and within the donor cell suspension improves xenograft survival in hemiparkinsonian rats, *Exp. Neurol. 172*, 416−424.
17. Ruano, M. L. F., Nag, K., Worthman, L. A., Casals, C., Pérez-Gil, J., and Keough, K. M. W. (1998) Differential partitioning of pulmonary surfactant protein SP-A into regions of monolayers of dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylcholine/dipalmitoylphosphatidylglycerol, *Biophys. J. 74*, 1101−1109.

18. Cruz, A., Worthman, L. A., Serrano, A. G., Casals, C., Keough, K. M., and Pérez-Gil, J. (2000) Microstructure and dynamic surface properties of surfactant protein SP-B/dipalmitoylphosphatidylcholine interfacial films spread from lipid−protein bilayers, *Eur. Biophys. J. 29*, 204−213.
19. Banerjee, S., Bennouna, M., Ferreira-Marques, J., Ruysschaert, J. M., and Caspers, J. (1999) Lipid−drug interaction and colligative properties in phospholipid vesicles, *J. Colloid Interface Sci. 219*, 168−177.
20. Angelova, A., Ringard-Lefebvre, C., and Baszkin, A. (1999) Drug−cyclodextrin association constants determined by surface tension and surface pressure measurements, *J. Colloid Interface Sci. 212*, 280−285.
21. Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L., and Clardy, J. (1991) Atomic structure of FKBP−FK506, an immunophilin−immmunosuppressant complex, *Science 252*, 839−842.
22. Casals, C., Miguel, E., and Pérez-Gil, J. (1993) Tryptophan fluorescence study on the interaction of pulmonary surfactant protein A with phospholipid vesicles, *Biochem. J. 296*, 585−593.
23. Azumi, T., and McGlynn, S. P. (1962) Polarization of the luminescence of phenanthrene, *J. Chem. Phys. 37*, 2413−2420.
24. García-Verdugo, I., Guirong, G., Floros, J., and Casals, C. (2002) Structural analysis and lipid binding properties of recombinant human surfactant protein A (SP-A) derived from one (SP-A1 or SP-A2) or both genes, *Biochemistry 41*, 14041−14053.
25. Garcia-Verdugo, I., Sanchez-Barbero, F., Bosch, F. U., Steinhilber, W., and Casals, C. (2003) Effect of hydroxylation and N187-linked glycosylation on molecular and functional properties of recombinant human surfactant protein A, *Biochemistry 42*, 9532−9542.
26. Sández, M. I., Gil, A., and Suárez, A. (2001) Mixed monolayers of cyclosporin-A and phospholipids at the air−water interface, *J. Colloid Interface Sci. 235*, 241−246.
27. Ma, J., Koppenol, S., Yu, H., and Zografi, G. (1998) Effects of a cationic and hydrophobic peptide, KL4, on model lung surfactant lipid monolayers, *Biophys. J. 74*, 1899−1907.
28. Portlock, S. H., Lee, Y., Tomich, J., and Tamm, L. K. (1992) Insertion and folding of the N-terminal amphiphilic signal sequence of mannitol and glucitol permeases of *Escherichia coli*, *J. Biol. Chem. 267*, 11017−11022.
29. Petrauskas, A. A., and Kolovanov, E. A. (2000) ACD/Log P method description, *Perspect. Drug Discovery Des. 19*, 99−116.
30. Lentz, B. R. (1989) Membrane 'fluidity' as detected by diphenylhexatrien probes, *Chem. Phys. Lipids 50*, 171−190.
31. Lakowicz, J. R. (1999) *Principles of fluorescence spectroscopy*, 2nd ed., Kluwer Academic/Plenum Publishers, New York.
32. Fiorini, R., Valentino, M., Wang, S., Glaser, M., and Gratton, E. (1987) Fluorescence lifetime distributions of 1,6-diphenyl-1,3,5-hexatriene in phospholipid vesicles, *Biochemistry 26*, 3864−3870.
33. Antollini, S. S., and Aveldaño, M. I. (2002) PCs with long and very long chain PUFAs isolated from retinal rod outer segment membranes, *J. Lipid Res. 43*, 1440−1449.
34. Hazel, J. R., Williams, E. E., Livermore, R., and Mozingo, N. (1991) Thermal adaptation on biological membranes: functional significance in phospholipid molecular species composition, *Lipids 26*, 277−282.
35. Morrow, M. R., Abu-Libdeh, N., Stewart, J., and Keough, K. M. (2003) Interaction of pulmonary surfactant protein SP-A with DPPC/egg-PG bilayers, *Biophys. J. 85*, 2397−2405.
36. Kremlev, S. G., Umstead, T. M., and Phelps, D. S. (1994) Effects of surfactant protein A and surfactant lipids on lymphocyte proliferation in vitro, *Am. J. Physiol. 267* (4, Pt. 1), L357−L364.
37. Dumont, F. J., Staruch, M. J., Koprak, S. L., Melino, M. R., and Sigal, N. H. (1990) Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin, *J. Immunol. 144*, 251−258.
38. O'Leardy, T. J., Ross, P. D., Lieber, M. R., and Levin, I. W. (1986) Effects of cyclosporin A on biomembranes: Vibrational spectroscopy, calorimetric, and hemolysis studies, *Biophys. J. 49*, 795−801.
39. Wiedmann, T. S., Trouard, T., Shekar, S. C., Polikandritou, M., and Rahman, Y. E. (1990) Interaction of cyclosporin A with dipalmitoylphosphatidylcholine, *Biochim. Biophys. Acta 1023*, 12−18.
40. Söderlund, T., Lehtonen, J. Y. A., and Kinnunen, P. K. J. (1999) Interactions of cyclosporin A with phospholipid membranes: Effect of cholesterol, *Mol. Pharmacol. 55*, 32−38.
41. Huai, Q., Kim, H., Liu, Y., Zhao, Y., Mondragon, A., Liu, J. O., and Hengming, K. (2002) Crystal structure of calcineurin−

cyclophilin−cyclosporin shows common but distinct recognition of immunophilin−drug complexes, *Proc. Natl. Acad. Sci. U.S.A. 99*, 12037−12042.

42. Almawi, W. Y., and Melemedjian, O. K. (2000) Clinical and mechanistic differences between FK506 (tacrolimus) and cyclosporin A, *Nephrol. Dial. Transplant. 15*, 1916−1918.

43. Brockman, H. (1999) Lipid monolayers: why use half a membrane to characterize protein−membrane interactions? *Curr. Opin. Struct. Biol. 9*, 438−443.

44. Zhao, L., Feng, S., and Go, L. (2004) Investigation of molecular interactions between paclitaxel and DPPC by Langmuir film balance and differential scanning calorimetry, *J. Pharm. Sci. 93*, 86−98.

45. Ali, S., Minchey, S., Janoff, A., and Mayhew E. (2000) A differential scanning calorimetry study of phosphocholines mixed with paclitaxel and its bromoacylated taxanes, *Biophys. J. 78*, 246−256.

46. Jain, M. K., and Wu, N. M. (1977) Effect of small molecules on the dipalmitoyllecithin liposomal bilayer, *J. Membr. Biol. 34*, 157−201.

47. Yang, H., McAlister, V. C., Al-Jazaer, A., Wright, J. R. (2002) Liposomal encapsulation significantly enhances the immunosuppressive effect of tacrolimus in a discordant islet xenotransplant model, *Transplantation 73*, 710−713.

48. Erdogan, M., Wright, J. R., and McAlister, V. C. (2002) Liposomal tacrolimus lotion as a novel topical agent for treatment of immune-mediated skin disorders: experimental studies in a murine model, *Br. J. Dermatol. 146*, 964−967.

49. Krihnadasan, B., Naidu, B., Rosengart, M., Farr, A. L., Barnes, A., Terrier, E. D., and Mulligan, M. S. (2002) Decreased lung ischemia-reperfusion injury in rats after preoperative administration of cyclosporine and tacrolimus, *J. Thorac. Cardiovasc. Surg. 123*, 756−767.

50. Valiño, F., Casals, C., Guerrero, R., Álvarez, L., Santos, M., Varela, A., Sáenz, A., Claro, M. A., Tendillo, F., and Castillo-Olivares, J. L. (2004) Inhaled Nitric Oxide Affects Endogenous Surfactant in Experimental Lung Transplantation, *Transplantation 77*, 812−818.

BI036227Z