

The Immunosuppressive and Toxic Effects of FK-506 are Mechanistically Related: Pharmacology of a Novel Antagonist of FK-506 and Rapamycin

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Summary

FK-506 inhibits Ca^{2+} -dependent transcription of lymphokine genes in T cells, and thereby acts as a powerful immunosuppressant. However, its potential therapeutic applications may be seriously limited by several side effects, including nephrotoxicity and neurotoxicity. At present, it is unclear whether these immunosuppressive and toxic effects result from interference with related biochemical processes. FK-506 is known to interact with FK-binding protein-12 (FKBP-12), an abundant cytosolic protein with *cis-trans* peptidyl-prolyl isomerase activity (PPIase) activity. Because rapamycin (RAP) similarly binds to FKBP-12, although it acts in a manner different from FK-506, by inhibiting T cell responses to lymphokines, such an interaction with FKBP-12 is not sufficient to mediate immunosuppression. Recently, it was found that the complex of FKBP-12 with FK-506, but not with RAP, inhibits the phosphatase activity of calcineurin. Here, we used L-685,818, the C18-hydroxy, C21-ethyl derivative of FK-506, to explore further the role of FKBP-12 in the immunosuppressive and toxic actions of FK-506. Although L-685,818 bound with high affinity to FKBP-12 and inhibited its PPIase activity, it did not suppress T cell activation, and, when complexed with FKBP-12, did not affect calcineurin phosphatase activity. However, L-685,818 was a potent antagonist of the immunosuppressive activity of both FK-506 and RAP. Moreover, L-685,818 did not induce any toxicity in dogs and rats or in a mouse model of acute FK-506 nephrotoxicity, but it blocked the effect of FK-506 in this model. Therefore, FK-506 toxicity involves the disruption of biochemical mechanisms related to those implicated in T cell activation. Like immunosuppression, this toxicity is not due to the inhibition of the PPIase activity of FKBP-12, but may be linked to the inhibition of the phosphatase activity of calcineurin by the drug FKBP-12 complex.

FK-506, rapamycin (RAP)¹, and cyclosporin A (CsA), define a class of immunosuppressive natural products that have in common the ability to inhibit T lymphocyte activation by interfering with intracellular signaling mechanisms (1, 2). However, these agents affect different biochemical pathways within the cascade of events that culminate in the expression of T cell effector functions. Both FK-506 and CsA disrupt T cell signaling pathways associated with a rise in the concentration of intracellular Ca^{2+} (3, 4), and this results in the inhibition of the transcription of a family of early activation genes, including IL-2 and IL-4 (5-7). In con-

trast, RAP does not inhibit Ca^{2+} -dependent lymphokine gene expression (5, 3), but impairs the response of T cells to growth-promoting lymphokines (3). Furthermore, FK-506 and RAP potentiate the immunosuppression induced by CsA (8, 9), whereas, under certain conditions, they antagonize each other's immunosuppressive action (9).

Despite their exquisite specificity for certain steps in T signal transduction, these immunosuppressants have significant *in vivo* toxicity. The adverse effects of FK-506 and CsA are very similar and include neurotoxicity and nephrotoxicity (10, 11), whereas RAP exerts little nephrotoxicity, but induces vasculitis (12) and myocardial necrosis (13). These toxic manifestations are of particular concern as they impose severe limitations in the potential therapeutic application of these immunosuppressants. At present, it is unclear whether im-

¹ Abbreviations used in this paper: BUN, blood urea nitrogen; CsA, cyclosporin A; Cyp, cyclophilin; FKBP-12, FK-binding protein-12; PPIase, peptidyl-prolyl isomerase activity; and RAP, rapamycin.

munosuppression and toxicity are mediated through the alteration by the drugs of similar biochemical processes in different cell types.

At the biochemical level, FK-506 and CsA interact with two distinct families of intracellular proteins, termed FK-binding proteins (FKBPs) (14) and cyclophilins (Cyps) (15), respectively. Despite its contrasting biological activity profile, RAP binds to the same FKBP protein family as FK-506 (16, 17). The two major forms of each of these receptor types, FKBP-12 and Cyp A, are peptidyl-prolyl *cis-trans* isomerases (PPIases), and catalyze the interconversion of the *cis* and *trans* rotamers of the peptidyl-prolyl amide bonds of peptide and protein substrates (16, 18, 19). FK-506, RAP, and CsA inhibit the PPIase activity of their respective binding proteins (16, 18–20). However, inhibition of this enzymatic activity is not sufficient to explain the biological activities of these immunosuppressants. We demonstrated that a CsA analog that inhibited Cyp A PPIase activity was neither immunosuppressive nor nephrotoxic, whereas another analog, which had little or no activity as a PPIase inhibitor, exhibited immunosuppressive activity (21). The similar potencies of FK-506 and RAP in inhibiting the PPIase activity of FKBP-12 also suggested a dissociation between this inhibition and the mode of immunosuppressive action of the macrolides (22). Moreover, a synthetic compound (506BD), corresponding to the portion of the FK-506 molecule that binds to FKBP-12 (23), was found to block the PPIase activity of this protein without exerting significant immunosuppression (22).

The discovery that the molecular complexes formed between FK-506 and FKBP-12 or between CsA and Cyp A bind to the Ca^{2+} - and calmodulin-dependent serine/threonine phosphatase, calcineurin (24, 25), and inhibit its enzymatic activity (26), may explain how these compounds exert their immunosuppressive effects. Since a RAP:FKBP-12 complex does not inhibit calcineurin phosphatase activity, the observations also provide an explanation for the mechanistic similarity between FK-506 and CsA. However, because the nonimmunosuppressive compound 506BD was found capable of inhibiting calcineurin phosphatase activity by up to 40% (26), additional studies with other macrolide analogs appear necessary to establish the relevance of these *in vitro* effects to immunosuppression.

In the present work, we describe L-685,818, a novel nonimmunosuppressive FK-506 relative, and use this molecule as a probe to further explore the mechanisms of action of FK-506 and RAP. L-685,818 is the C18-hydroxyl derivative of L-683,590, which differs from FK-506 by a single allyl to ethyl substitution at the C21 position of the macrolide ring (27). We found that L-685,818 binds to FKBP-12 and inhibits its PPIase activity. However, the FKBP-12:L-685,818 complex was unable to inhibit calcineurin phosphatase activity, in contrast to the FKBP-12:L-683,590 complex which was half as active as the FKBP-12:FK-506 complex in this assay. Moreover, L-685,818 behaved as a potent competitive antagonist of the inhibitory effects of both FK-506 and RAP, but not of CsA, in models of T cell activation *in vitro*. Although L-685,818 had no detectable toxicity *in vivo*, it proved to be an effective antagonist of FK-506, but not of CsA, in a murine model

of drug-induced nephrotoxicity. Altogether, these studies with L-685,818, clearly show that the inhibition of FKBP-12 PPIase activity is not involved in either the immunosuppressive or toxic effects of FK-506. Moreover, there exists a mechanistic relationship between these two types of biological effects of the drug, that may be linked to the inhibition of calcineurin phosphatase activity.

Materials and Methods

Mice. Female C57Bl/6 and BALB/c mice (6–12-wk-old) were obtained from the Jackson Laboratory (Bar Harbor, ME) or from Taconic Farms (Germantown, NY), and maintained under specific pathogen-free conditions until use.

Reagents. FK-506 and RAP were isolated by Dr. Robert Borris, at Merck Research Laboratories (MRL, Rahway, NJ) from *Streptomyces tsukubaensis* and *Streptomyces hygroscopicus*, respectively. L-683,590 (FK-520) was isolated from *S. hygroscopicus* var. *ascomycetus* by Drs. Kevin Byrne and Gino Salituro (MRL). L-685,818 was synthesized by chemical modification of L-683,590 as described elsewhere (Beattie, T., B. Arrison, and M. Wyvratt, manuscript in preparation). Tritiated dihydro-FK-506 was provided by Dr. Allen Jones (MRL). CsA was provided by Dr. Philippe Durette (MRL). PMA and ionomycin were from Sigma Chemical Co. (St. Louis, MO) and Calbiochem Corp. (La Jolla, CA), respectively. SKF-525A was obtained from Research Biochemicals Inc. (Natick, MA). Recombinant human IL-1 α was provided by Dr. Jack Schmidt (Merck Sharp & Dohme Research Laboratories). Recombinant human IL-2 was purchased from Amgen Biologicals (Thousand Oaks, CA). Inhibitor I peptide (28) was obtained from Dr. Philip Cohen (University of Dundee, UK). The murine IL-2 and IFN- γ cDNA probes were obtained from Dr. Bill Gauss (National Institutes of Health, Bethesda, MD) and from Dr. Gordon Freeman (Dana-Farber Cancer Institute, Boston, MA), respectively. The β -actin cDNA probe was obtained from Oncor Inc. (Gaithersburg, MD).

Cell Cultures. Splenic T cells from C57Bl/6 mice were isolated by nylon wool separation as previously described (3). The DO-11.10 T cell hybridoma (29) was provided by Dr. Philippa Marrack (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). The YAC-1 T cell lymphoma was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in culture medium made up of RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate, 2×10^{-5} M 2-ME, and 50 $\mu\text{g}/\text{ml}$ gentamycin. Microcultures of splenic T cells were set up in flat bottomed 96-well plates (Costar Corp., Cambridge, MA). Each well received 200 μl of cell suspension at 10^6 cells/ml. The T cell activators and various combinations of compounds were set up in triplicate wells. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 -95% air. Cultures were pulsed with [^3H]TdR (2 $\mu\text{Ci}/\text{well}$) for the last 4–5 h of a 48-h incubation period and harvested on glass fiber filters. Incorporated radioactivity was measured by using a Betaplate liquid scintillation spectrometer (Pharmacia-LKB, Piscataway, NJ). Cell cultures for analysis of mRNA expression were set up in 25-cm 2 tissue culture flasks (Corning Glass Works, Corning, NY) maintained in an upright position. DO-11.10 cells (2×10^5 cells/ml) were stimulated with ionomycin plus PMA in the presence or absence of drugs, and harvested for RNA extraction after 3 h of culture. YAC-1 cells (10^5 cells/ml) were incubated with IL-1 plus PMA in the presence or absence of drugs, and RNA was extracted after 20 h of culture.

RNA Extraction and Slot Blot Analysis. Total cellular RNA was isolated using RNeasy (Qiagen, Crawfordsville, IN). RNA samples were blotted onto nitrocellulose filters using a slot-blot Minifold II apparatus (Bio-Rad Laboratories, Richmond, CA). The cDNA probes were labeled with [³²P]dCTP to high specific activity using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). RNA blots were prehybridized and hybridized with the probes and autoradiographed using Hyperfilm-ECL (Amersham Corp., Arlington Heights, IL).

FKBP Binding Assay. Human FKBP-12 was purified as previously described (19). An LH-20 column binding assay (15) was used for competitive binding studies. Compounds were dissolved in ethanol and diluted in 20 mM sodium phosphate containing 0.5% BSA (binding buffer). The reaction was set up in siliconized round-bottomed 96-well plates. To 175 μ l of binding buffer, 25 μ l of the competitor compound solution, 25 μ l of FKBP-12 at 250 ng/ml, and 25 μ l of [³H]dihydro-FK-506 at 10 ng/ml, were added into each well. After mixing and 20 min incubation at 20°C, the bound [³H]dihydro-FK-506 was separated from free ligand by passing over a 2-ml LH-20 micro-column (Pharmacia-LKB). The column was eluted with 500 μ l of 20 mM sodium phosphate, and the void volume counted for radioactivity. Determinations were made in duplicate and the IC₅₀ calculated by linear regression on serial dilutions of competitor.

FKBP-12 PPIase Assay. The PPIase activity of FKBP-12 was assayed as previously described (20) by measuring the *cis* to *trans* isomerization of the proline-phenylalanine peptide bond in the peptide, Suc-Ala-Leu-Pro-Phe-pNA. Under equilibrium conditions, ~88% of the peptide is present as the *trans* form and is readily cleaved by chymotrypsin. The remaining 12% of the peptide present in the *cis* form is cleaved upon enzymatic conversion to the *trans* form. The size of conversion was monitored by the change in absorbance at 390 nm because of the release of *p*-nitroaniline. The compounds were tested in 4-ml cuvettes containing 2.8 ml of a 70 mM solution of the substrate in assay buffer (35 mM Hepes, pH 7.8, 3% DMSO) to which were added 30 μ l of a 1.2-mM solution of FKBP-12. Various dilutions of compound in 10 μ l ethanol were added, and the resulting solution was left to reach thermal equilibrium at 10°C for 10 min. The reaction was initiated by addition of 100 μ l of a 2.2-mM solution of chymotrypsin in 1 mM HCl, and absorbance was recorded. The first order portion of the resulting progress curve was fit to a simple first order rate equation. The rate constants determined from the enzymatic isomerase activity was first corrected for the rate of nonenzymatic isomerization. This value, k_{inhib} , divided by the rate constant for the reaction in the absence of inhibitor, k_{control} , was plotted as a function of inhibitor concentration. The resulting plot was fit to a model for tight binding inhibition to obtain K_i and $[E_0]$.

Phosphatase Assay of Calcineurin. The assay was modified from the procedures described by Manalan and Klee (30) and Liu et al. (26). Inhibitor I peptide (28), which was phosphorylated with ³²P-labeled ATP (450 cpm/pmol) with the catalytic subunit of cAMP-dependent protein kinase, was used as a substrate. The assay buffer consisted of 40 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 6 mM MgCl₂, 0.1 mM CaCl₂, 0.1 mg/ml BSA, and 0.5 mM dithiothreitol. The assay mixture (60 μ l) contained 3 nM bovine brain calcineurin (Sigma), 190 nM calmodulin (Sigma), 420 nM recombinant human FKBP-12 and 7 μ M of phosphopeptide. The drug solutions were prepared in DMSO at concentrations of DMSO <0.04% in the final assay mixture. The incubation was carried out at 30°C for 10 min before the reaction was stopped and loaded onto 0.5-ml Dowex AG 50W-X8 (200–800 mesh; Bio-Rad Laboratories) columns. The level of [³²P] eluted from the columns was determined by liquid scintillation radioactivity measurement.

Toxicological Evaluation of L-685,818 in Dogs and Rats. Beagle dogs (two males and two females per group) or Sprague-Dawley rats (10 males and 10 females/group) were treated daily for 14 d with L-685,818 or the vehicle only. L-685,818 was administered by oral gavage as a suspension in 0.5% methylcellulose, at a dose of 25 mg/kg/d. The animals were observed daily for mortality and clinical signs of drug effect. Body weight and food consumption were also monitored. During the second week of treatment, hematologic and serum biochemical examinations and urinalysis were conducted. For the dog study, plasma drug level determinations were made on the first day of treatment and at study determination. At necropsy, gastrointestinal tract, liver, kidneys, spleen, lymph nodes, and thymus were examined for any gross lesions. Specimen of these tissues were preserved in 10% neutral buffered formalin and examined microscopically using paraffin-embedded and hematoxylin/eosin-stained sections.

Murine Acute Nephrotoxicity Assay. Treatment with the cytochrome P450 inhibitor, SKF-525A (31), was used to enhance the toxicity of FK-506 in BALB/c mice (Koprak, S., and F. Dumont, manuscript in preparation). SKF-525A was dissolved in saline, and a dose of 75 mg/kg was injected intraperitoneally 2 h before intravenous injection of 0.5 ml of the compound or vehicle control solutions. Stock solutions of compounds were made up in ethanol at 65 mg/ml, further diluted in ethanol, and mixed with Cremophor® (BASF, Parsippany, NJ) followed by saline, to give final solutions containing 10% (wt/vol) Cremophor® and 6% ethanol. The vehicle control solution was made up of saline with the same concentrations of Cremophor® and ethanol. The mice received these injections for 2 consecutive d and were killed the following day for autopsy and collection of serum and tissue (kidney, liver) samples. Measurement of blood urea nitrogen (BUN) levels in the serum was done on an autoanalyzer (model 203; Gilford Instrument Laboratories, Inc., Oberlin, OH).

Results

L-685,818 Binds to FKBP-12 and Inhibits its PPIase Activity but Does Not Inhibit T Cell Activation. As shown in Fig. 1, the chemical structure of L-685,818 is closely related to that of FK-506. L-685,818 was synthesized from L-683,590, which differs from FK-506 by an allyl to ethyl substitution at the C21 position (27). L-685,818 also contains a hydroxyl group at the C18 position of the macrolide ring. Consistent with their structural similarity to FK-506, both L-685,818 and L-683,590 bind to FKBP-12 with a high affinity (Fig. 2 a). Furthermore, L-685,818 is a potent inhibitor of the PPIase activity of this protein, comparable with FK-506 or RAP (Fig. 2 b).

In contrast to its activity in these biochemical assays, L-685,818 did not inhibit the proliferation of T cells activated by ionomycin plus PMA, even when tested at concentrations up to five orders of magnitude >FK-506 (Fig. 2 c). Under the same conditions, L-683,590 was only threefold less potent than FK-506, in agreement with earlier studies (27). L-685,818, at concentrations as high as 25 μ M, also had little effect on IL-2 plus PMA-induced T cell proliferation, whereas, as previously reported (3), such a response was suppressed by subnanomolar concentrations of RAP (Fig. 2 d).

The Complex FKBP12:L-685,818 Does Not Inhibit Calcineurin Phosphatase Activity. It was reported recently that the molecular complex formed between FKBP-12 and FK-506 is able to inhibit the Ca²⁺/calmodulin-dependent phosphatase ac-

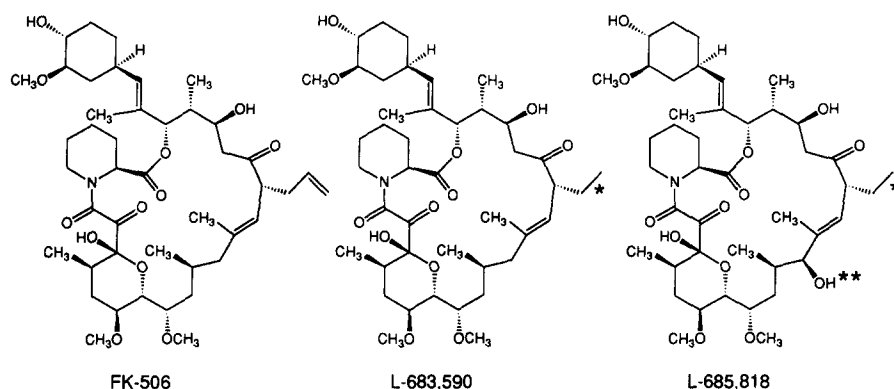


Figure 1. Comparison of the chemical structures of FK-506, L-683,590 (FK-520), and L-685,818. Note the C21 ethyl substitution in L-683,590 (*) and the C18 hydroxyl substitution in L-685,818 (**).

tivity of calcineurin in vitro (26). We wished to address the possibility that the lack of biological activity of L-685,818 was due to its inability to inhibit calcineurin phosphatase in the presence of FKBP-12. As shown in Fig. 3, when tested under experimental conditions where FK-506 and L-683,590 could block calcineurin function with ED₅₀s of 57 and 108 nM, respectively, L-685,818 was totally inactive at concentrations up to 2 μ M. Consistent with the data of Liu et al. (26), RAP also failed to suppress the phosphatase activity of calcineurin, even at concentrations 40-fold higher than FK-506.

L685,818 Antagonizes the Suppressive Effects of FK-506 and RAP on T Cell Proliferative Responses. These observations, together with our earlier finding of a mutual antagonism between FK-506 and RAP (9), prompted us to examine the possibility that L-685,818 might be a functional antagonist of these macrolides. We first tested whether L-685,818 would block the inhibitory effect of FK-506 in ionomycin plus PMA-stimulated T cells. FK-506 was used at 1.2 nM, a concentration that suppressed the proliferation by 93–97%, and var-

ious concentrations of L-685,818 were added simultaneously. As shown in Fig. 4 a, concentrations of L-685,818 >100 nM completely reversed the suppressive effect of FK-506. Therefore, L-685,818 is a potent antagonist of FK-506 ($ED_{50} = 63.1 \pm 7.2$ nM, mean \pm SEM of 13 experiments). In contrast, L-685,818 failed to reverse the inhibitory effect of CsA in T cell cultures similarly stimulated with ionomycin plus PMA (Table 1).

The Schild analysis method is used to determine the affinity of a pharmacological antagonist in a biological system (32), and was employed here to further evaluate the antagonist activity of L-685,818. T cells stimulated with ionomycin plus PMA were cultured in the presence of various concentrations of FK-506 either alone or together with increasing concentrations of L-685,818. Fig. 4 *b* demonstrates that addition of L-685,818 concentrations ranging from 19 nM to 2.5 μ M progressively shifted the dose-response inhibition curve of FK-506 to the right, indicating a decreased sensitivity of the cells to the immunosuppressant. Calculation of the ED₅₀s of the inhibitory effect of FK-506 in the absence or presence of var-

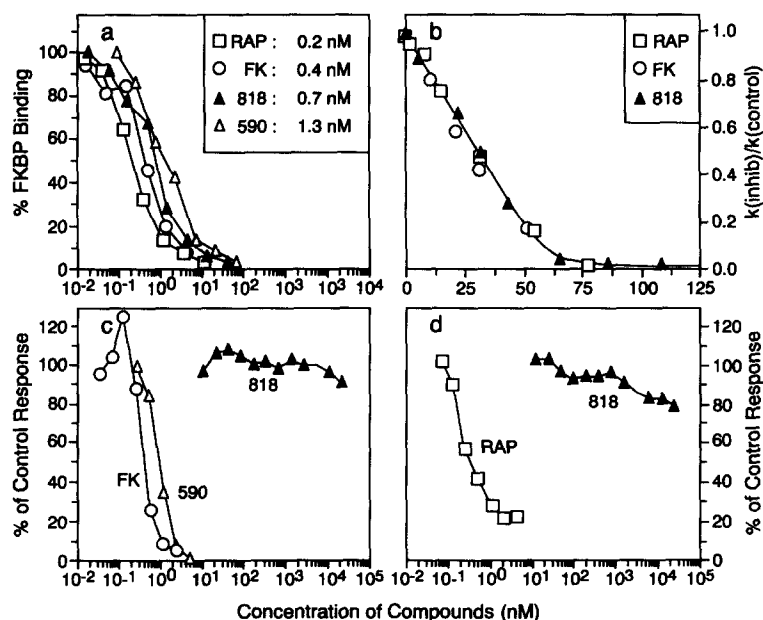


Figure 2. L-685,818 (\blacktriangle) binds to FKBP-12 (*a*) and inhibits its PPIase activity (*b*), but does not inhibit the proliferative responses of T cells stimulated, either with ionomycin plus PMA (*c*), a mode of activation sensitive to FK-506 (\circ) and L-683,590 (\triangle), or with IL-2 plus PMA (*d*), a mode of activation sensitive to RAP (\square). The binding to FKBP-12 was determined in a competitive assay using [^3H]dihydro-FK-506. The ED_{50} s for each compound are indicated on the figure. The inhibition of PPIase activity of FKBP-12 was very similar for the three compounds examined. The line corresponds to the best fit calculated by a nonlinear regression analysis. Mouse splenic T cells were cultured with ionomycin (250 ng/ml) plus PMA (10 ng/ml) or with IL-2 (100 U/ml) plus PMA (10 ng/ml) in the presence of various concentrations of compounds, and the cell proliferation was measured by [^3H]TdR uptake after 2 d of culture. The results were expressed as percent of the control [^3H]TdR incorporation in cultures that received the stimuli only (ionomycin + PMA = 467×10^3 cpm, IL-2 + PMA = 550×10^3 cpm).

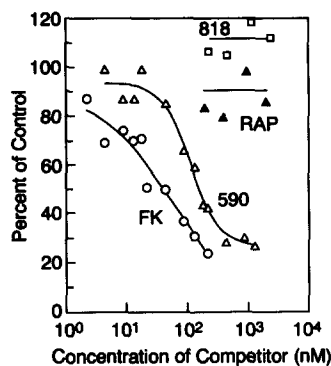


Figure 3. Effects of various concentrations of FK-506 (O), L-683,590 (Δ), L-685,818 (□) and RAP (▲) on the phosphatase activity of bovine brain calcineurin (3 nM) tested towards 32 P-inhibitor I peptide (7 μ M), in the presence of bovine calmodulin (190 nM) and of recombinant human FKBP-12 (420 nM). The results were expressed as percent of the control phosphatase activity obtained in the absence of any compound. The lines correspond to the four-parameter fits of individual experimental points. In this experiment, FK-506 gave an IC_{50} = 57 nM and L-683,590 gave an IC_{50} = 108 nM.

ious concentrations of L-685,818 allowed us to characterize the interaction of the two macrolides with their common receptor. The data plotted in Fig. 4 c indicate that L-685,818 competes with FK-506 for a single functional receptor in T cells (32). As calculated by this method, the K_d of the interaction of L-685,818 with this putative receptor is 8.7 ± 1.0 nM (mean \pm SEM of four experiments).

We next examined whether L-685,818 would reverse the immunosuppressive effect of RAP in T cells stimulated with IL-2 plus PMA. Using RAP at a concentration of 1.1 nM, which inhibited the proliferative response by 70–75%, the addition of L-685,818 resulted in a dose-dependent restoration of the proliferative response, with an ED_{50} = 45.5 ± 6.0 nM (mean \pm SEM of six experiments), as shown in Fig. 4 d. Schild analysis (Fig. 4, e and f) further demonstrated that L-685,818 acts as a competitive antagonist of RAP at the level of a single functional site with a K_d of 4.4 ± 0.5 nM (mean \pm SEM of three experiments). This value is close, but not identical to the K_d determined for FK-506 competition (Student's t test, p = 0.03).

L-685,818 Reverses the Immunosuppressive Effects of FK-506 or RAP Even When Added Late into the Cultures. Experiments were then conducted to define the temporal site of this antagonistic effect of L-685,818. Fig. 5 a demonstrates that, when added as late as 6 h after FK-506 in T cell cultures activated with ionomycin plus PMA, L-685,818 still fully restored the proliferative response measured at 48 h of culture. L-685,818 also reversed the inhibition of RAP by more than 50% even when added up to 12 h after the latter to cultures stimulated with IL-2 plus PMA (Fig. 5 b). Therefore, L-685,818 competes at the level of the functional receptors that mediate the pharmacologic action of the immunosuppressants, rather than at the level of a transport mechanism necessary for entry of the macrolides into the cell.

L-685,818 Antagonizes the Suppressive Effects of FK-506 and RAP on Lymphokine mRNA Induction. To confirm that L-685,818 acted as an antagonist at the level of transcriptional regulation, we studied its effect on IL-2 mRNA induction in DO-11.10 hybridoma T cells activated with ionomycin plus

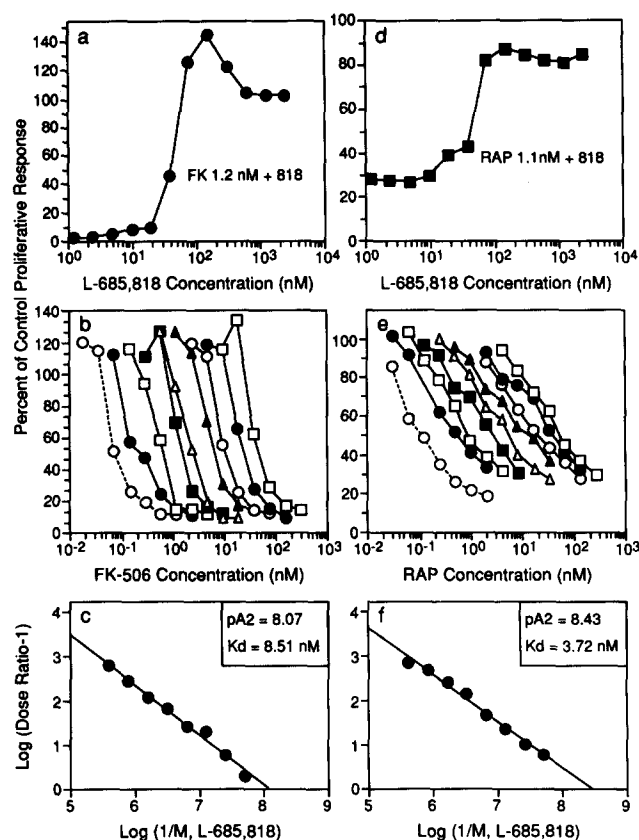


Figure 4. L-685,818 antagonizes the immunosuppressive effects of FK-506 (a–c) and RAP (d–f) in activated T cells. (a) Mouse splenic T cells were activated with ionomycin plus PMA in presence of 1.2 nM FK-506, and various concentrations of L-685,818 were added at the initiation of the cultures. (b) Mouse splenic T cells were activated with ionomycin plus PMA in presence of various concentrations of FK-506 either alone (O–O) or with L-685,818 at concentrations increasing in twofold increments from 19 nM to 2.5 μ M. (c) Schild analysis of the antagonism of FK-506 by L-685,818 in the experiment depicted in (b). (d) Mouse splenic T cells were activated with IL-2 plus PMA in presence of 1.1 nM RAP, and various concentrations of L-685,818 were added at the initiation of the cultures. (e) Mouse splenic T cells were activated with IL-2 plus PMA in presence of various concentrations of RAP, either alone (O–O) or with L-685,818 at concentrations increasing in twofold increments from 19 nM to 2.5 μ M. (f) Schild analysis of the antagonism of RAP by L-685,818 in the experiment depicted in (e).

PMA. Fig. 6 a shows that IL-2 mRNA was detectable 3 h after activation and was inhibited by FK-506 in a dose-dependent manner. L-685,818 had no effect on this induction of IL-2 mRNA expression, but it blocked the inhibitory effect of FK-506. In a different model of lymphokine gene regulation, the induction of IFN- γ mRNA by IL-1 plus PMA in the YAC-1 T cell lymphoma (33), RAP was inhibitory at concentrations as low as 1 nM (Fig. 6 b). L-685,818, tested at 12 μ M, did not inhibit IFN- γ mRNA expression and totally restored this expression in RAP-treated cultures.

L-685,818 Is Not Toxic in Dogs and Rats. Based on the aforementioned data, L-685,818 appeared as an excellent tool to address the question of the mechanistic relationship between FK-506 immunosuppression and toxicity. First, we investigated

Table 1. *L-685,818 Does Not Block the Suppressive Effect of CsA in T Cells Stimulated with Ionomycin Plus PMA**

CsA concentration <i>nM</i>	T cell proliferation ($[^3\text{H}]\text{TdR}$ uptake) [†]			
	No L-685,818		+ L-685,818 (25 μM)	
	cpm $\times 10^{-35}$	Percent control [‡]	cpm $\times 10^{-3}$	Percent control
0	341.0	100.0	332.6	100.0
6	355.8	104.3	395.3	118.9
12	340.2	99.8	377.1	113.4
25	355.6	104.3	268.8	80.8
50	161.9	47.5	56.0	16.8
100	65.1	19.1	21.2	6.4
200	32.2	9.4	18.8	5.7

* Mouse splenic T cells were stimulated for 48 h with ionomycin (250 ng/ml) plus PMA (10 ng/ml).

[†] Cell proliferation was measured by pulsing the cultures with 2 μCi $[^3\text{H}]\text{TdR}$ for 4 h.

[‡] Each value is the mean of triplicate wells.

[§] Control proliferative response of cells stimulated with ionomycin plus PMA in the absence of drug.

whether L-685,818 would exert any of the side effects previously noted with FK-506 in animal models (10). Although highly sensitive to the toxic effects of FK-506 (34) or RAP (12), dogs were unaffected by a 2-wk oral treatment with

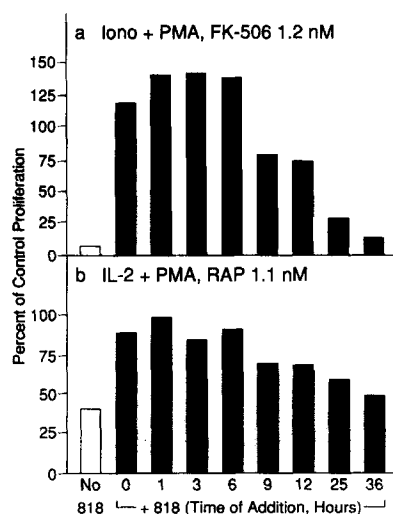


Figure 5. L-685,818 reverses the suppression of T cell proliferative responses by FK-506 (a) or RAP (b) even when added after either immunosuppressant to the cultures. (a) Mouse splenic T cells were activated with ionomycin plus PMA, and FK-506 (1.2 nM) was added at the initiation of the cultures. Replicate wells received L-685,818 (125 nM) either at the same time as FK-506 ($t = 0$) or at various times thereafter. (b) Mouse splenic T cells were activated with IL-2 plus PMA and RAP (1.1 nM) was added at the initiation of the cultures. Replicate wells received L-685,818 (125 nM) either at the same time as RAP ($t = 0$) or at various times thereafter. In both (a) and (b), the cultures were harvested after 48 h of incubation after a 6-h pulse with $[^3\text{H}]\text{TdR}$. The results were expressed as percent of the control response obtained with cells stimulated in the absence of compound (ionomycin + PMA = 425×10^3 cpm, IL-2 + PMA = 623×10^3 cpm).

L-685,818 at doses as high as 25 mg/kg/d, which gave plasma drug levels $>2 \mu\text{g/ml}$. Similarly, oral administration of L-685,818 in rats for 2 wk at 25 mg/kg/d, failed to cause the nephrotoxicity or behavioral changes that have been observed with FK-506 (10, 11). Therefore, L-685,818 is devoid of the toxicity known to be associated with FK-506.

L-685,818 Blocks the Nephrotoxic Action of FK-506. To examine the possibility that L-685,818 could antagonize FK-506-induced toxicity, we used an acute model in BALB/c mice, where the nephrotoxic potential of FK-506 was enhanced by cotreatment of the animals with the inhibitor of cytochrome P450, SKF-525A (31). Consistent with the recently demonstrated role of P450 enzymes in the metabolism of FK-506 (35), we found that SKF-525A augmented the serum level of FK-506 (not shown). Under these conditions, two daily intravenous injections of FK-506 in doses ranging from 3.1 to 12.5 mg/kg were sufficient to induce a significant elevation of BUN levels. L-683,590 (50 mg/kg) was also found to raise BUN concentration, whereas RAP (100 mg/kg) had no effect on this parameter, although it caused mortality (Table 2). In contrast, the same protocol of treatment with 100 mg/kg of L-685,818 did not produce any increase of BUN or mortality (Table 2 and Fig. 7). It is most interesting that administration of this high dose of L-685,818, together with toxic doses of FK-506, prevented almost completely the rise in BUN observed with FK-506 alone (Fig. 7 a). The dose-dependent nature of the antagonism is demonstrated in Fig. 7 b, where the animals were given a constant dose of FK-506 and varying doses of L-685,818. Therefore, L-685,818 is a potent antagonist of the acute nephrotoxic effect of FK-506 in this murine model. Additional experiments (not shown) demonstrated that L-685,818 does not block CsA-mediated nephrotoxicity.

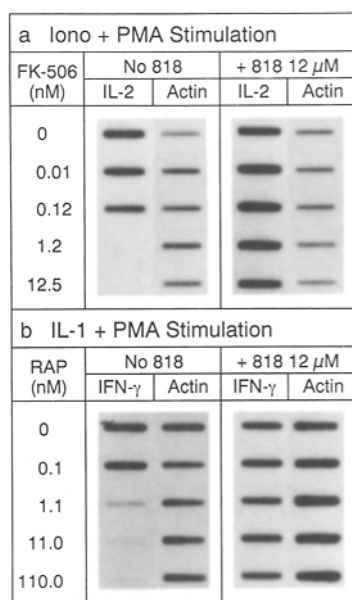


Figure 6. L-685,818 blocks the inhibitory effects of FK-506 (a) and RAP (b) on lymphokine mRNA induction. (a) DO-11.10 cells were activated with ionomycin plus PMA in presence or absence of various concentrations of FK-506, without or with L-685,818 (12 μM). The cells were harvested after 3 h of culture, and total RNA was extracted and blotted onto a nitrocellulose filter. The blot was hybridized with the IL-2 cDNA probe, stripped, and rehybridized with the actin cDNA probe. (b) YAC-1 cells were activated with IL-1 plus PMA in presence or absence of various concentrations of RAP, without or with L-685,818 (12 μM). The cells were harvested after 20 h of culture and total RNA was extracted and blotted onto a nitrocellulose filter. The blot was hybridized with the IFN-γ cDNA probe, stripped, and rehybridized with the actin cDNA probe.

Discussion

The present study exploits the unique pharmacological properties of L-685,818 to investigate the mechanism of action of FK-506 with respect to its biochemical target for immunosuppression, and the relationship between this target and toxicity. L-685,818 differs from FK-506 at two positions. Although the allyl to ethyl substitution at the C21 position results in a two to threefold loss in immunosuppressive potency, it is the addition of a hydroxyl group at the C18 position of the macrolide ring that converts the molecule to an antagonist. Recent x-ray crystallographic and nuclear magnetic resonance studies indicate that L-685,818 associates with FKBP-12 through its pipelicolic acid portion, in a manner identical to FK-506 (Becker, J., manuscript in preparation). Hence, it is not surprising that FK-506 and L-685,818 have equivalent activities in binding to FKBP-12 and in inhibiting its PPIase activity, since it is their common "face" that is responsible for these activities. Such observations are consistent with earlier studies using 506BD and RAP (22).

The observation that the physical interaction with FKBP-12 and the inhibition of PPIase may be necessary but not sufficient for immunosuppression has led to the hypothesis that the drugs must interact with other cellular components to disrupt the signaling processes of T cell activation (1, 2). This hypothesis is supported by the recent finding that the com-

Table 2. Effects of Two Daily Intravenous Injections of FK-506, L-683,590, RAP, or L-685,818 on BUN Levels in BALB/c Mice Cotreated with SKF-525A*

Expt. no.	Treatment (Dose, mg/kg)	BUN (mg/dl) Mean (SEM)	p†	Mortality
1	Vehicle	14.4 (0.5)		0/6
	FK-506 (12.5)	61.0 (16.2)	0.007	2/6
	L-683,590 (50)	50.2 (11.4)	0.007	1/6
2	Vehicle	20.7 (1.3)		0/6
	FK-506 (12.5)	34.8 (1.7)	0.0001	0/6
	RAP (100)	22.2 (1.7)	0.5	3/8
3	Vehicle	14.7 (0.9)		0/6
	FK-506 (12.5)	85.8 (15.6)	0.0007	1/6
	L-685,818 (100)	16.2 (0.7)	0.2	0/6

* Mice received an intraperitoneal injection of SKF-525A (75 mg/kg) 2 h before intravenous administration of the compounds. The animals were treated for 2 consecutive d and killed the next day.

† Statistical significance determined by Student's *t* test.

plex formed between FK-506 and FKBP-12 interacts with the Ca²⁺-calmodulin-dependent phosphatase, calcineurin, and inhibits its phosphatase activity in vitro (26). We found here that L-685,818 is unable to alter the activity of calcineurin in such a system, even when tested at concentrations up to 40-fold greater than FK-506, whereas L-683,590 did inhibit

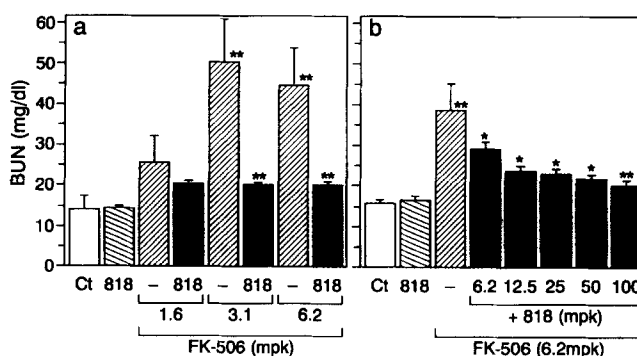


Figure 7. L-685,818 does not increase BUN but blocks the BUN elevating effect of FK-506 in BALB/c mice cotreated with SKF-525A. The mice received SKF-525A (75 mg/kg) intraperitoneally 2 h before intravenous injection of FK-506 or L-685,818 either alone or in combination. Control (Ct) mice received SKF-525A and the vehicle alone. All mice were treated on 2 consecutive d and were bled and killed the following day. (a) L-685,818 was administered at 100 mg/kg/d (SS). FK-506 was given at 1.6, 3.1, or 6.2 mg/kg/d either alone (□) or together with L-685,818 (100 mg/kg/d, ■). The results are the pooled data from three experiments, with each group including a total of 15–18 mice. (b) FK-506 was given at 6.2 mg/kg/d either alone (□) or together with various doses of L-685,818 (■). The results are the pooled data from two experiments, with each group including a total of 9–12 mice. The statistical significances (Student's *t* test) of the differences in BUN between the groups treated with FK-506 alone and the control group, and between the groups treated with FK-506 alone or with L-685,818 are indicated (*) *p* < 0.1, (**) *p* < 0.01.

this activity with a potency approximately twofold lower than FK-506. This demonstrates a clear-cut correlation between the ability of the drugs complexed to FKBP-12 to interfere with the phosphatase activity of calcineurin and their ability to suppress T cell activation induced in a Ca^{2+} -dependent manner. Such a correlation suggests that the inhibition of calcineurin enzymatic function by the FKBP-drug complex may be involved in the mechanism of immunosuppressive action of the drugs. Although a similar conclusion had been made based on studies with RAP (26), the strict concordance between the biochemical and cellular observations produced by a subtle structural change, such as the 18-hydroxy substitution, further solidifies this model.

Are the molecular entities defined in the current *in vitro* calcineurin assay system the relevant members of the complex that mediate immunosuppressive activity *in vivo*? There are several reasons to consider this question. First, the *in vitro* system used in both the present and previous (26) studies is based on the interaction of heterologous proteins (bovine calcineurin and calmodulin with human FKBP-12). Second, the existence of several isoenzymes of calcineurin (25) should be noted. Third, a number of minor forms of FKBP have now been identified in mammalian cells (36–38).

Recent genetic experiments strongly implicate calcineurin as a component of a drug-sensitive signal transduction pathway in T cells. O'Keefe, et al. (38a) have demonstrated that transfection of a mutant, constitutively active, catalytic subunit of calcineurin into Jurkat cells synergizes with PMA alone to activate the IL-2 promoter in an FK-506- and CsA-sensitive manner. Although similar experiments have not been performed with FKBP-12, several results have called into question the assumption that the most abundant immunosuppressant receptors must mediate all the biological effects of FK-605 and CsA. In yeast, deletion of FKBP-12 does not confer resistance to the growth inhibitory effects of FK-506 (39), suggesting the existence of other FKBP family members. Pharmacological experiments with CsA analogs also suggested that Cyp A may not be the member of this family to mediate the immunosuppressive activity of CsA (21). Thus, whereas genetic studies clearly put calcineurin in the drug-sensitive signal transduction pathway, experiments performed to date do not permit us to identify which FKBP family member is the most relevant effector of calcineurin complex formation *in vivo*.

The identification of L-685,818 as the most potent antagonist of both FK-506 and RAP-mediated immunosuppression synthesized to date has allowed us to explore the nature of the functional receptor of these compounds from a different perspective. Schild analysis was used to demonstrate that L-685,818 interacted with a single receptor species when either FK-506 or RAP was used as the agonist. Furthermore, since L-685,818 could be added to the cultures several hours after FK-506, and since FK-506 is known to penetrate and reach equilibrium within the cells in <20 min (14), we concluded that the antagonism of L-685,818 does not reflect inhibition of a transport mechanism. The most straightforward conclusion from these data is that L-685,818 antagonizes FK-506 and RAP through their interaction with a common cellular

receptor, a notion already suggested by the fact that the two drugs antagonize each other's action with similar potencies (9). More thorough analysis suggests alternative possibilities, however. The affinities derived by Schild analysis (4–8 nM) differ significantly from those estimated from FKBP-12 competitive binding measurements (0.7 nM). This difference may be due to relative abundance of the relevant drug receptor within the cell. However, given the fact that the intracellular concentration of FKBP-12 is $\sim 5\text{--}10\ \mu\text{M}$ (Dumont, F. et al., manuscript in preparation), one might expect that even larger concentrations of L-685,818 would be required to observe antagonism and that the antagonism might be noncompetitive. Taken together, K_d estimates derived from the Schild analysis are not entirely consistent with the assumption that the antagonist and agonists are distributed uniformly within the FKBP-12 cytosolic receptor pool. Indeed, FK-506 and RAP may interact with receptors that are in lower abundance than FKBP-12, or may bind preferentially to a distinct subset of FKBP-12 molecules within the cell. Therefore, although FKBP-12 appears as the most plausible intracellular target for these compounds, we cannot exclude the possibility that another known, or as yet undiscovered FKBP may be involved.

The present study provides important insights into the relationship between the immunosuppressive and toxic effects of FK-506. Along with its lack of immunosuppressive activity, L-685,818 did not elicit any of the side effects known to occur after FK-506 treatment in several animal species, including the gastrointestinal pathology seen in dogs (34) and the nephrotoxicity observed in rats (11). Using a mouse model where the nephrotoxic effect of FK-506 was enhanced by coadministration of the P450 inhibitor SKF-525A (31), we found that L-685,818 produced little or no elevation of BUN, even when given at doses 5–10-fold greater than nephrotoxic doses of FK-506. Since interfering with FK-506 metabolism potentiates the toxicity of the drug, it is likely that the parent compound, rather than metabolites, may be primarily responsible for the induction of nephrotoxicity. Furthermore, the use of an inhibitor of macrolide metabolism also minimizes the possibility that L-685,818 could be modified *in vivo*. The most striking observation made with this model, however, is that L-685,818 reversed the nephrotoxic action of FK-506 in a dose-dependent manner.

The finding that the toxic effects of FK-506 can be reversed by the same antagonist that blocks FK-506 immunosuppression strongly suggests that the mechanisms of immunosuppression and toxicity are related. FK-506 may therefore affect biochemical processes that are part of signal transduction pathways shared in common by lymphoid cells and by non-lymphoid cells in the target toxicity organs. As for immunosuppression, it is unlikely that these processes involve inhibition of PPIase activity, which is similar to the conclusion reached in an analysis of CsA analogs (21). Instead, the toxicity of FK-506 may be linked to the ability of the drug-FKBP-12 complex to inhibit the phosphatase activity of calcineurin. This conclusion may also explain why FK-506 and CsA share such similar patterns of toxicity (11).

Since FKBP, Cyps, and calcineurins are widely distributed, it may be surprising that FK-506 and CsA do not produce

even more toxicity. There may be several reasons for the apparent selectivity of the drugs. Although calcineurin is present in a variety of tissues, it differs in abundance and isoenzyme distribution pattern (25), and may have diverse roles in the physiology of the cells. The substrates for calcineurin may also differ among cells. In addition, selectivity could be related to the tissue distribution of related FKBP that would confer

the appropriate affinity and functional ability of the drug complex to interfere with calcineurin. Nevertheless, the finding that immunosuppression and toxicity of FK-506 are mechanistically related will make it difficult to dissociate the immunosuppressive activity and toxic effects of molecules that use such common biochemical pathways.

We wish to thank Drs. Stella Vincent and Vernon Moore for critical reading of this manuscript, and Dr. Alan Williamson for encouragement and support.

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Received for publication 7 May 1992.

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