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Structure–activity profiles of macrolactam immunosuppressant FK-506 analogues

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The immunosuppressive agent FK-506 has received much attention due to its efficacy and potency in the areas of transplant rejection and autoimmune disease. Calcineurin, a Ca^{2+}-calmodulin activated phosphatase, was recently implicated in the immunosuppressive mechanism of FK-506. In our ongoing search for superior immunosuppressive agents, we have synthesized several analogues of FK-506 and tested their mechanistic and immunosuppressive actions. It was found that C-18 hydroxyl analogues of ascomycin, an analogue of FK-506 also called FR900520, bound tightly to immunophilin FKBP-12, but do not show any immunosuppressive activity in vitro or in vivo despite good bioavailability. Further, they reverse the inhibition of calcineurin caused by FK-506/FKBP-12 complex.

FK-506; Immunosuppressant; C-18 hydroxyl analogue; Antagonist; Calcineurin; Ascomycin

1. INTRODUCTION

The report by Fujisawa researchers in 1987 that the macrolactam antifungal FK-506, **1**, isolated from the fermentation broth of *Streptomyces tsukubaensis*, possessed powerful immunosuppressive activity set off a detailed investigation for its mechanism of action [1]. FK-506, which is 10–100 times more potent than the known immunosuppressive agent, cyclosporin A (CsA) **4**, has inhibitory effects on the production of various cytokines (IL-2, IL-3, IFN-γ) and on the expression of alloantigen-induced human lymphocyte IL-2 receptors (IL-2R, Tac antigen) and transferrin receptors [2,3]. Although FK-506 is structurally distinct from CsA, it possessed similar biological and immunological properties.

Efforts to ascertain the mechanism of action of FK-506 have narrowed the site of action to the calcium-dependent signal transduction pathway in the T-cell activation cascade [4]. This ultimately led to the characterization of FK-506 binding proteins (FKBP) that are present in relatively high concentrations (5 nanomolar) in cells of a variety of organisms [5] and that possess *cis–trans* peptidyl-prolyl isomerase activity [6–11]. Recent efforts by Schreiber and co-workers have led to the identification of calcineurin which is believed to play a critical role in the immunosuppressive mechanism of FK-506 [12–14]. FKBP is an essential component for inhibition of T-cell activation by FK-506, and the FKBP-FK506 complex, but not FK-506 alone, binds to and inhibits calcineurin commonly found in cells [12,13].

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Another structurally related immunosuppressant, rapamycin **3** that forms a complex with FKBP but inhibits T-cell activation through a different mechanism, has recently been highlighted due to its impressive and distinct biological activity [15–17]. These two structurally similar, but mechanistically distant molecules (**1** and **3**), along with a synthetic FK-506 analogue (506BD) [18] are thought to possess two domains, i.e. a binding domain (the 'left side' of the molecule as drawn in **1**–**3**, a domain that binds to FKBP) and an effector domain (the 'right side' of the molecules as drawn in **1**–**3**, a domain thought to be necessary for calcineurin interaction [19].

We have initiated synthetic efforts to elucidate the structural features in ascomycin **2** [20,21] a homologue of FK-506 also known as FR-900520, that determine its biological activity. As observed by others [18,22] modification of the effector domain can cause notable changes in biological and pharmacological activity. We report here the synthesis and biological characterization of a series of synthetic analogues. Ascomycin **2**, which differs from FK-506 by only one carbon, possesses the same potent biological effects as FK-506, and has been used exclusively herein for the chemical modifications.

1. EXPERIMENTAL

2.1. *Medium and reagents*

Ascomycin **2** was produced by fermentation with a method similar to that described in the literature [23]. Unless otherwise indicated, reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Penicillin-streptomycin was purchased from Gibco Laboratories (Grand Island, NY). Complete RPMI 1640 contained 10% heat-inactivated fetal bovine serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. FK-506 binding protein (FKBP) and the fusion protein composed of FKBP

and CMP-KDO synthetase (FKBP/CKS) were prepared at Abbott Laboratories (Abbott Park, IL) as previously described [24].

2.2. *Peripheral blood mononuclear cells*

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized blood from healthy donors. Stimulator PBMC were treated with 25 μg/ml of mitomycin C for 30 min and washed before being used.

2.3. *Human mixed leukocyte response*

One way allogeneic mixed leukocyte response assays were performed as described by Kino et al. [26] with slight modification. Briefly, 1×10^5 responder PBMC were mixed with 4×10^5 stimulator PBMC, composed of 1×10^5 PBMC from each of four different donors, in 0.2 ml of complete RPMI 1640 and cultured for 96 h at 37°C. During the final 6 h, the cells were labeled with 0.5 μCi per well of tritiated thymidine [^{3}H]TdR; DuPont NEN Research Products, Boston, MA). The cells were harvested by vacuum filtration onto glass fiber filters and the radioactivity measured by liquid scintillation spectrometry. Test compounds, dissolved at 10 mM in dimethylsulfoxide, were diluted in complete RPMI 1640 and added to the responder PBMC before addition of the stimulator PBMC.

2.4. *IL-2 Production*

The proliferation of an IL-2-dependent murine T cell line was used as a bioassay [27] for the production of IL-2 bioactivity in the MLR cultures and to determine the ability of compounds to affect IL-2-dependent cell proliferation. To test for IL-2 bioactivity, samples of the MLR cultures were removed after 48 h and frozen at −80°C. Twenty five μl of the culture supernatant was diluted to 100 μl with complete RPMI 1640 that contained 2.5×10^3 CTLL-2 cells and incubated for 24 h at 37°C. The proliferation of the CTLL-2 cells was determined by the incorporation of [^{3}H]TdR as described in the MLR assay. The amount of IL-2 in the samples was estimated by comparison to a standard curve relating CTLL-2 proliferation to recombinant human IL-2 concentration. When testing the affect of compounds on IL-2-dependent proliferation, dilutions of the compounds in complete RPMI 1640 were mixed with 1×10^4 CTLL-2 cells and 5 U/ml recombinant human IL-2 in a final volume of 100 μl. Proliferation was determined by the incorporation of [^{3}H]TdR as described above.

2.5. *FKBP binding assay*

A competition binding assay employing a recombinant FKBP-12 fusion protein was used to determine the affinity of compounds for FKBP. Human FKBP-12 was cloned as a fusion partner with CMP-KDO synthetase (CKS) and purified as described by Edalji et al. [24]. An ascomycin conjugate of alkaline phosphatase was prepared by active ester coupling of an ascomycin C-22 derivative (carboxymethyl oxime) with alkaline phosphatase (ascomycin-AP; Abbott Laboratories, Abbott Park, IL). In the assay, FKBP-CKS fusion protein was dissolved at 10–35 μg/ml in 20 mM sodium phosphate buffer, pH 7.4, and adsorbed to the wells of a Immuno Plate Maxisorp (Nunc, Naperville, IL) by incubation at ambient temperature for 2 h. A solution of phosphate-buffered saline (PBS), pH 7.4, containing 2% bovine serum albumin (BSA) and 0.2% Tween 20 was added to the wells to reduce nonspecific binding of the ascomycin-AP. After rinsing the wells with 0.2% Tween 20 in PBS, the test compound in the PBS/BSA/Tween 20 buffer, or buffer alone, was added to the wells. An equal volume of ascomycin-AP ligand at 1 μg/ml in PBS/BSA/Tween 20 was added to the wells and incubated 2 h at ambient temperature. After rinsing with 0.2% Tween 20 in PBS, paranitrophenyl phosphate at 1 mg/ml in 0.1 M aminomethyl propanol was added to the wells and the temporal change in 405 nm absorbance recorded.

2.6. *Calcineurin phosphatase assay*

Calcineurin activity was measured by the release of [^{32}P]PO_4 from a 19 amino acid residue phosphopeptide corresponding to the RII subunit of cAMP-dependent protein kinase [28] using procedures similar to those described by Hubbard and Klee [29]. The assay buffer consisted of 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mg/ml BSA, 0.5 mM dithiothreitol, and 0.2% Tween 20. A typical assay mixture contained 3 nM calcineurin, 30 nM calmodulin, 300–1000 nM FKBP-12, test compound, and 1 μM phosphopeptide substrate [DLDVPIPGRFDRRV[^{32}P]SVAAE] in a final volume of 50 μl. Compounds were tested at concentrations between 0.1 to 10,000 nM. Prior to initiation of the phosphatase reaction by addition of the substrate, the assay mixtures were preincubated for 10 min at 32°C. After addition of the phosphopeptide, the assay mixtures were incubated for 90 min at 32°C and stopped by the addition of 150 μl of 0.1 M potassium phosphate (pH 7) in 5% trichloroacetic acid. The stopped assay mixture was applied to a 150 μl column of Dowex AG 50W-X8 (200–400 mesh) equilibrated in the Cl^- form and the free [^{32}P]PO_4 was recovered in the eluate after a wash with 100 μl of H_2O. Radioactivity was measured by liquid scintillation spectrometry. To calculate the compound concentration which caused 50% inhibition (IC_{50}) in an assay, the inhibition data was fit to a log-logistic linear function.

2.7. *Pharmacokinetic evaluation in the rat*

Lewis rats (obtained from Charles River, Wilmington, MA), weighing 225–250 g, were acclimated for one week in a AAALAC approved facility. All animals were fasted overnight prior to dosing and throughout the study period but were permitted free access to water. Groups of animals were given ascomycin **2** or its C-18-hydroxy analogue **5** at a dose of 5 mg/kg. Drugs were given in a 2 ml/kg volume of 10% ethanol, 40% propylene glycol and 2% cremophore in 5% dextrose solution for both oral (p.o.) and intraperitoneal (i.p.) administration, and in a 1 ml/kg volume of the same vehicle without cremophor for intravenous (i.v.) dosing. Blood samples were collected from the tail vein in heparinized tubes at selected time points 0.1, 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 hr after dosing.

Drugs were separated from the hemolyzed whole blood contaminants utilizing liquid-liquid extraction with ethylacetate/hexane (1:1 by volume). Samples were centrifuged at $1200 \times g$ for 10 min (4°C) and a constant volume of the organic layer was transferred to a conical centrifuge tube and evaporated to dryness with a gentle stream of dry air over low heat (~ 35°C). The samples were reconstituted with 40% (v/v) acetonitrile in water with vortexing. The compounds of interest were separated from the co-extracted components by a 5 cm × 4.6 mm, 3 μm Spherisorb ODS-2 column (Regis, Morton Grove, IL) with an acetonitrile/methanol/0.1% trifluoroacetic acid–0.01 M tetramethylammonium perchlorate mixture (45:5:50 by volume for ascomycin **2**, 40:5:55 for C-18-hydroxy analogue **5**) at a flow rate of 1.0 ml/min with UV detection at 205 nm. The temperature of the HPLC column was maintained at 70°C.

The concentration of each sample was calculated by a least squares linear regression analysis of the peak area compared to spiked rat blood standards. The following pharmacokinetic parameters were determined. Maximum whole blood concentrations (C_{max}) and the time of their occurrence (t_{max}) were compiled from the raw data. Terminal half-life ($t_{1/2}$) was calculated from the elimination rate constant. The volume of distribution (V_d) of i.v. drugs was estimated by standard techniques. Area under the blood drug concentration–time curve (AUC) up to the last measured blood concentration over the time course was also analyzed. Bioavailability was evaluated as the fraction derived by dividing the oral AUC by the AUC after i.v. administration.

2.8. *Popliteal lymph node (PLN) hyperplasia assay*

Inbred male rats 125–150 g were purchased from Harlan Sprague–Dawley, Inc. (Indianapolis, IN) and housed for one week before use. Spleen cells from Brown Norway rats (RT-1^n) were subjected to ammonium chloride to lyse red cells, washed in phosphate-buffered saline (PBS), X-irradiated (2000 rads), washed again and injected subcutaneously with a 27-gauge needle in the right hind foot of Lewis strain rats (RT-1^l) using 5×10^6/100 μl. Test compound was dissolved in a vehicle consisting of 10% ethanol, 40% propylene glycol, and 2% cremophore with the balance being sterile 5% dextrose in water (Abbott Laborato-

ries, Abbott Park, IL). Two ml/kg, i.p. was given to groups of 8 animals once daily, on days 0–3. On day four, the popliteal lymph nodes were removed and weighed on a microbalance. The mean weight of nodes from a group of uninjected control animals was subtracted to determine the net increase, and percent inhibition in reference to a vehicle control group was calculated.

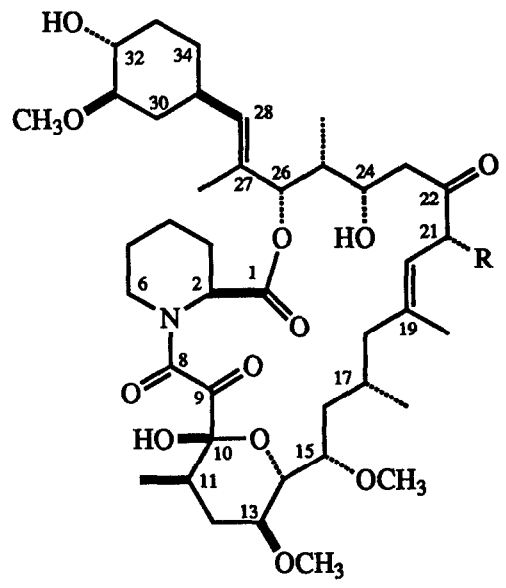
2.9. *Statistical analysis*

Unless indicated, all data are presented as the mean ± S.E.M. and were analyzed using Student's *t*-test of unpaired samples.

3. RESULTS

3.1. *Synthesis of ascomycin analogues*

The C-18-hydroxy-ascomycins were synthesized by two different routes, as shown in Scheme 1. A direct oxidation of ascomycin by selenium oxide-*tert*-butylhydrogen peroxide in methylene chloride gave three major products in a ratio of 1:3:4. Each of the products was isolated and characterized as C-23,24-dehydro **5a**, C-23,24-dehydro-C-18-hydroxy **5b**, and C-18-hydroxy derivative **5**, respectively. The C-18 hydroxyl derivative obtained was found to be a single isomer and the stereochemistry was assigned by NMR as the [*S*] configuration at C-18 composition [30]. Since the C-18[*R*]-isomer was not isolated in the direct oxidation of **2**, and dehydration at C-23 and C-24 was observed as a main side reaction, the hydroxyl groups at C-24 and C-32 were protected with *tert*-butyldimethylsilyl (TBDMS) groups



(1) FK-506: R= allyl

(2) Ascomycin: R= ethyl

Fig. 1. Structures of macrocyclic immunosuppressive agents: FK-506 and ascomycin.

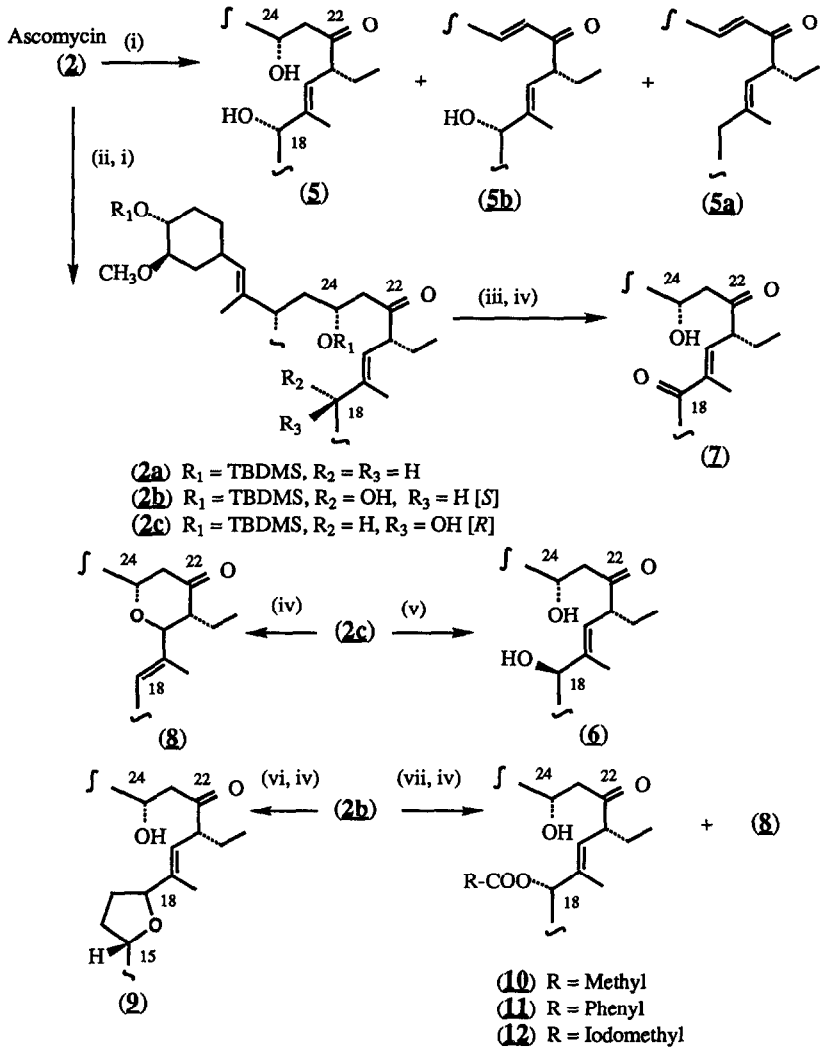
to yield **2a**. After the selenium oxide oxidation of **2a** was performed, two products in a ratio of 1:3 were isolated and purified. The minor compound was identified as C-24,32-bis-TBDMS-C-18[*R*]-hydroxy-ascomycin **2c** and the major one was determined as its C-18[*S*] isomer **2b**. Oxidation of the C-18 hydroxyl group to the corresponding ketone was performed by treatment with tetrapropylammonium perruthenate/*N*-methylmorpholine-*N*-oxide in methylene chloride. Deprotection with hydrogen fluoride in acetonitrile (HF-CH_3CN) provided the C-18 ketone, **7**, in moderate yield. Removal of TBDM.S groups from **2c** by HF-CH_3CN gave exclusively analogue **8e**, whereas treatment of **2c** with pyridinium *p*-toluenesulfonate in ethanol yielded the desired C-18[*R*]-hydroxy-ascomycin **6** in 60% yield. Activation of the C-18 hydroxyl group of **2b** with trifluoromethanesulfonic anhydride or methanesulfonyl chloride instantly yielded compound **9** The C-18 acylated analogues **10**–**12** were obtained from the deprotection of the corresponding bis-TBDMS analogues by HF-CH_3CN, respectively. Under these conditions, compound **8** was also isolated in each case, and the ratio of the desired acylated analogues versus **8** were 1:1.5 for **10**, 3.5:1 for **12**, and 1:1 for **11**, respectively. All compound gave satisfactory analytical data, and detailed synthetic procedures will be published elsewhere.

3.2. *Inhibition of lymphocyte proliferation (MLR)*

As an in vitro model of allogenic T lymphocyte activation, human peripheral blood mononuclear leukocyte (PBL) are exposed to a pool of mitomycin C-treated PBL. After four days, the incorporation of [^{3}H]thymidine into cellular DNA is measured. FK-506 **1**, ascomycin **2** and rapamycin **3** showed complete inhibition of this response with IC_{50} values of subnanomolar to picomolar (Table I). In contrast, C-18-[*S*] and [*R*]-hydroxy-ascomycin analogues **5** and **6** as well as analogues **7**–**11** did not show any significant inhibitory activity, except compound **12**, which displayed a very weak inhibition (MLR = 0.17 μM).

3.3. *FKBP binding activity*

The discovery of *cis–trans* peptidylprolyl isomerase (PPIase or rotamase) activity for this cytoplasmic FKBP raised some very interesting questions whether this activity mediates the immunosuppressive action of FK-506. But, as has been pointed out earlier, the inhibition of PPIase activity of FKBP is not sufficient to mediate the immunosuppressive activity [18]. However, because FKBP binding appears to be necessary for immunosuppressive activity, we investigated the ability of synthetic analogues to bind. Data from a binding assay using cloned FKBP fused to CMP-KDO synthetase (CKS) as a receptor (Table I), showed that natural ligands **1**, **2**, and **3** as well as analogues **5** and **6** compete with an average IC_{50} of 0.9, 1.4, 2.9, 4.6 and 1.8 nM, respectively. Compounds **7**, **9** and **11** were shown to



Scheme 1. Synthetic routes of Ascoumycin analogues

possess weaker activities with IC_{50} values of 17, 25 and 41 nM, respectively. The data clearly suggest that, with the exception of **8**, all of the synthetic analogues bind tightly to FKBP.

3.4. *Inhibition of IL-2 production and CTLL-cell line*

Potent inhibition of IL-2 production (cyclosporin A and FK-506) and inhibition of cellular responses to IL-2 (rapamycin) appear to be critical, but perhaps divergent, mechanisms by which these immunosuppressants inhibit T lymphocyte proliferation [31]. Thus, inhibition of IL-2 production and inhibition of IL-2 induced proliferation of CTLL-2 cells was studied. Most of the synthetic analogues **5**–**11** caused no inhibition in either IL-2 or CTLL assays, as were expected based on their lack of activity in the MLR assay and in contrast to immunosuppressants **1**–**4** which inhibit these events (Table I). One exception is analogue **12**, which exhibited weak but detectable inhibition in the MLR, IL-2 and CTLL assays.

3.5. *Evaluation of antagonism*

Because the C18-[*S*]-hydroxy analogue **5** retained a high binding affinity for FKBP-12 but did not have demonstrable immunosuppressive activity, it was evaluated for its ability to reverse the inhibitory effects of

Table I

In vitro biological data for synthetic analogues of ascomycin 2

	Modifications[a]	MLR (nM)[b]	FKBP (nM)[c]	IL (nM)[d]	CTLL (nM)[e]
(1)	FK506	0.03	0.9	0.001	370
(2)	Ascomycin	0.08	1.4	0.001	350
(3)	Rapamycin	0.32	2.9	–	0.06
(4)	CsA	4.8	7% @ 10μM	–	–
(5)	C18-[*S*]-OH	n.a.[f]	4.6	n.a.	8%[g]
(6)	C18-[*R*]-OH	n.a.	1.8	n.a.	–
(7)	C18-Keto	n.a.	17.0	n.a.	11%
(8)	C18-ene-C20-oxa	n.a.	n.a.	n.a.	n.a.
(9)	C15-deMe- C15,18oxa	n.a.	25	n.a.	–
(10)	C18-[*S*]-O-Ac	n.a.	3.2	n.a.	–
(11)	C18-[*S*]-OBzo	n.a.	41	n.a.	–
(12)	C18-[*S*]-O-IAc	170	1.3	270	31

[a] See Scheme 1 for structures.
[b] Inhibition of allogeneic T lymphocyte proliferation.
[c] The binding activity of compound to FKBP.
[d] Inhibition of IL-2 production by allogeneic stimulated T lymphocytes.
[e] Inhibition of IL-2 induced proliferation of CTLL-2 cells.
[f] Not active at 0.1 μM inhibitor concentration.
[g] Activity at 0.1 μM inhibitor concentration.

FK-506 in the MLR and the calcineurin phosphatase assay. In data not shown, analogue 5 caused no inhibition of calcineurin phosphatase activity at concentrations up to 10 μM. As shown in Fig. 2, analogue 5 at a concentration 1000-fold higher than the FK-506 caused a significant reversal of FK-506's inhibition in the mixed leukocyte response assay. A ten-fold lower concentration of the analogue 5 was ineffective. Similarly, the inhibition of calcineurin phosphatase caused by a 10 nM concentration of FK506 1 was significantly reversed by 1 and 10 μM concentration of 5, as shown in Fig. 3.

3.6. *Pharmacokinetic studies*

Values of primary pharmacokinetic parameters in rats after a single dose of drugs with various administration routes are shown in Table II. Analogue 5 was found to have a shorter i.v. half-life than ascomycin 2, although its volume of distribution was comparable. After oral gavage, the peak blood level and AUC for 2 were two to threefold higher than with analogue 5, and no trace of the latter could be seen after four hours. Bioavailability comparable to ascomycin 2 has been reported for FK-506 in human [32] and rat [33,34] after oral dosing. In contrast to its low oral availability, analogue 5 was comparable in peak concentration and AUC to ascomycin 2 when given i.p. and had a better bioavailability.

3.7. *In vivo PLN assay*

The popliteal lymph node (PLN) hyperplasia response to alloimmune challenge with histoincompatible

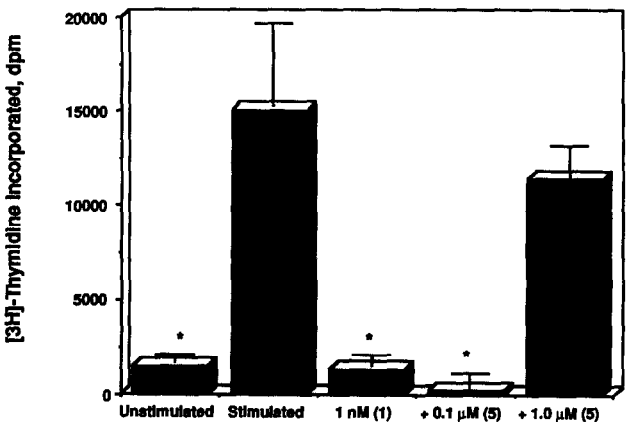


Fig. 2. Reversal by compound 5 of the inhibition by compound 1 of cell proliferation in the MLR. *$P < 0.001$, when compared to the control stimulated response.

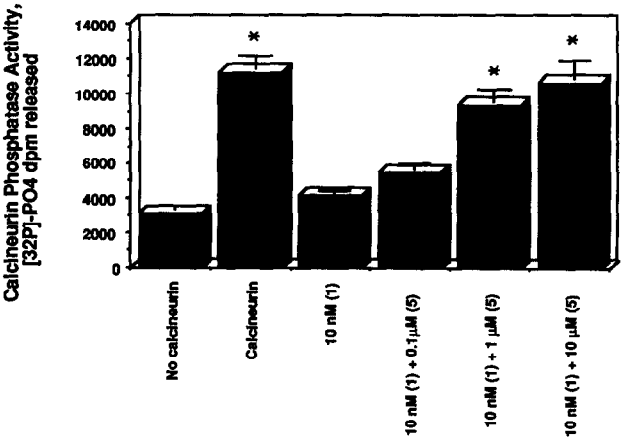


Fig. 3. Reversal by compound 5 of compound 1 inhibition of calcineurin phosphatase. *$P < 0.02$, when compared to the 10 nM 1 sample.

Table II

Pharmacokinetic parameters of ascomycin 2 and C18-OH analogue 5 in rats after single intravenous (i.v.), oral (p.o.), or intraperitoneal (i.p.) administration at a dosage of 5 mg/kg body weight

Compound (2)				Compound (5)		
i.v.	p.o.	i.p.		i.v.	p.o.	i.p.
–	0.5	0.5	t_{max} (h)	–	0.25	0.25
–	390	519	C_{max} (ng/ml)	–	182	636
2.05	–	–	$t_{½}$ (h)	1.29	–	–
2.07	–	–	V_d (l/kg)	2.28	–	–
2984	778	1878	AUC (ng·hr/ml)	1210	200	1650
–	26	62	Bioavailability (%)	–	16	137

splenocytes injected in the foot, was used as an in vivo model of lymphoproliferation. As shown in Fig. 4, the average control response to splenocyte injection (stimulated) was twofold higher than with buffer alone (unstimulated). With administration of 5 at a dose of 30 mg/kg/day, i.p., the average challenged node weight was not significantly different from the vehicle control. In contrast, ascomycin 2 was found to have an ED_{50} of 0.3 mg/kg/day, i.p., for inhibiting the node weight increase.

4. DISCUSSION

An earlier report identified protein phosphatase calcineurin as a common target for FKBP-FK506 and cyclophilin-CsA complexes [12]. These complexes, not immunosuppressant alone nor immunophilin alone, inhibit calcineurin phosphatase activity. The inhibition of this phosphatase activity may be an early step in a series of events that results in the inhibition of T cell cytokine gene transcription [19]. In this study, the biological aspects of the interactions between synthetic ligands and FKBP, and ligand/FKBP complexes and calcineurin were investigated. Data, summarized in Table I, show that various reference immunosuppressants 1–4 potently inhibit allogeneic T lymphocyte proliferation. In contrast, C-18-hydroxy analogues 5 and 6 as well as analogues 7–11 did not inhibit the human mixed lymphocyte reactions nor inhibit IL-2 production by allogeneically stimulated T lymphocytes. In fact, one of the C-18 hydroxyl analogues with unspecified stereochemistry has been reported to antagonize both FK-506 and rapamycin, but not cyclosporin A, inhibition of T-cell response to PMA or IL-2 plus PMA [22]. These observations raise many questions with respect to immunosuppressive activity. (1) Do the non-immunosuppressive analogues bind tightly to FKBP, similar to 1 and 2? (2) If so, are the FKBP-bound conformations of analogues 5 and 6 different from those of 1 or 2? (3) Are observed binding affinities to FKBP comparable to those of 1 and 2? (4) Are the complexes of FK-506 analogue/immunophilin capable of inhibiting calcineurin? (5) Can they antagonize the effects of FK-506? (6) Do the in vitro data predict in vivo properties?

First, we investigated the binding activity of compounds to FKBP (Table I). Although the functional role of the major intracellular binding protein, FKBP-12, remains poorly understood, one approach to address this issue is to study the relationship between immunosuppression and FKBP binding activity of a series of structurally related analogues. The significant similarity in structure resulted in similar FKBP binding affinity among 1, 2, 5 and 6. Other analogues, such as 7, 9, and 11 have comparatively weak binding affinities.

We felt an understanding of the secondary and tertiary structure of FKBP/analogue complexes might be helpful in rationalizing their biological effects. We therefore determined the three-dimensional structure of two analogues 5 and 6, when bound to [U-^{13}C,^{15}N]FKBP by isotope-filtered 2D NMR experiments [30]. From a comparison of the 3D structure of the analogues 5, 6 with ascomycin 2 when bound to FKBP, it was concluded that the bound conformations of 5 and 6 are virtually identical to the bound conformation of 2 [30]. Thus, substitution of a hydroxyl group at the C-18 position has essentially no effect on the conformation of the bound ligands, and the lack of immunosuppressive activity, therefore cannot be explained by altered bound conformation.

When tested in the rat PLN assay, an in vivo model of lymphoproliferation, analogue 5 was devoid of immunosuppressive activity (Fig. 4) despite its good bioavailability relative to 2 and having been given at a dose (30 mg/kg/day) much higher than that required for immunosuppression with 2. This observation is consistent with the lack of in vitro inhibitory activity displayed by compound 5. The lack of immunosuppressive activity of 5 in vitro and in vivo led us to investigate the interaction of FKBP complex with calcineurin. The immunophilin complexes with either 5 or 6 do not inhibit calcineurin, whereas the complexes with 1 or 2 inhibit

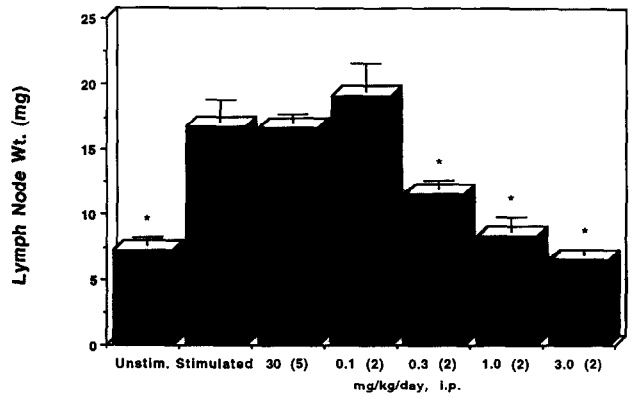


Fig. 4. Effect of compounds of 5 and 2 on popliteal lymph node hyperplasia in the rat. *$P < 0.05$ when compared to the control stimulated response.

calcineurin phosphatase activity with IC_{50} values of 4 and 10 nM, respectively. To date, there is no direct evidence that the immunophilin complexes with 5 or 6 do not bind calcineurin, but as has been pointed out earlier [30] introduction of a C-18 hydroxyl group to a hydrophobic, calcineurin binding site could disrupt the hydrophobic surface and inhibit binding of calcineurin to the complex. Because analogue 5 did not have demonstrable immunosuppressive activity, but showed a high affinity for FKBP-12, investigation of its ability to reverse inhibition of calcineurin caused by FK-506 is also very important to confirm the central role of calcineurin in the immunosuppressive action. As shown in Fig. 2, analogue 5 at a concentration 1000-fold higher than FK-506 caused a significant reversal of FK-506's inhibition in the mixed leukocyte response assay. Analogue 5 also blocked the inhibition of calcineurin phosphatase caused by FK-506 (Fig. 3), suggesting that these two compounds could bind to a common binding region of FKBP and interact with calcineurin in a competitive fashion.

5. CONCLUSION

In this investigation, we have systematically studied the biological and physiological activities of several *non*-immunosuppressive analogues of FK-506. Despite the fact that FK-506 analogues such as 5 and 6, bind to FKBP-12 with virtually identical affinity and bound conformation relative to 1 and 2, these compounds lack immunosuppressive activity both in vitro and in vivo. Competitive antagonism of FK-506 effects using 5 was demonstrated in MLR and calcineurin assays. The PLN assay results show that analogue 5 was devoid of in vivo immunosuppressive activity despite its comparable bioavailability relative to 2. Although no direct evidence has not yet been obtained regarding the ability of the immunophilin complexes with 5 or 6 to bind calcineurin, we suggest that the C-18 hydroxy group could disrupt the hydrophobic surface and inhibit binding of calcineurin to the complex.

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