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# Communications to the Editor

## The C-32 Triacetyl-L-rhamnose Derivative of Ascomycin: A Potent, Orally Active Macrolactone Immunosuppressant

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Immunosuppressive drugs have been used for the prevention of organ transplant rejection and to some extent for the treatment of severe autoimmune disease for many years. A major advance was made with the introduction of cyclosporin A (CsA).[1] This drug acts primarily on thymus-derived lymphocytes or T cells, which are the major cells in the immune system responsible for mediating allograft rejection.[2] T cells also mediate the pathology of various autoimmune diseases,[3] and CsA has been used on an experimental basis to treat some of these conditions. Therapeutic effects have been observed in rheumatoid arthritis, psoriasis, Type I diabetes, and uveitis among others.[4]

Even though CsA has been used with remarkable success, it has many liabilities. Renal toxicity[5] is dose-limiting and includes an acute and reversible, as well as a chronic and irreversible, component. This toxicity results in histological damage to renal tubules in humans[6] and other species. Additional dose-limiting conditions include hypertension, hirsutism, and tremors.[7] The combination of a narrow therapeutic index coupled with variable pharmacokinetics has driven the search for better-tolerated immunosuppressants. One of the most promising new compounds is (tacrolimus, FK-506), a macrolactone immunosuppressant identified in 1987.[8] Although initially described as not only more potent but also less toxic than CsA, these safety claims have been revised substantially. Tacrolimus, while somewhat more efficacious in liver transplantation, is similarly nephrotoxic and also has significantly greater neurologic side effects ranging from tremor to coma.[9] Nevertheless, tacrolimus has been successfully utilized in patients who are unresponsive to CsA.[10] Given this background, a better-tolerated immune suppressant with equivalent efficacy should not only replace CsA and tacrolimus but could also be employed beyond transplantation to treat severe forms of autoimmune disease, thus satisfying an important medical need.

The mode of action of these potent immunosuppressive drugs has been the topic of intense study over the past few years.[11] Although the structure of tacrolimus and CsA are very different in nature (Chart 1), their pharmacology is in many ways comparable. Each compound inhibits lymphokine (IL-2, IL-3, IL-4, GM-CSF, TNFα, IFNγ) production through blockade of $Ca^{2+}$-dependent signal transduction pathways.[12] Initial work showed that these compounds interacted with specific *cis–trans*-prolyl isomerases (CsA with cyclophilin and tacrolimus with FKBP-12). Subsequently, it was observed that the complex of CsA–cyclophilin and the complex of tacrolimus–FKBP inhibited the function of the ubiquitous $Ca^{2+}$ calmodulin-dependent phosphatase, calcineurin A, in human T cells.[13] The current hypothesis is that a dephosphorylation event mediated by calcineurin is required for the translocation of a component of the IL-2 transcription factor, NF-AT, from the cytoplasm to the nucleus prior to gene transcription[14] and that these drug complexes inhibit this dephosphorylation event. This model explains some of the biological effects of these immunosuppressants, but differences exist between CsA and tacrolimus,[15] which may reflect different cellular targets (mast cells, macrophages,

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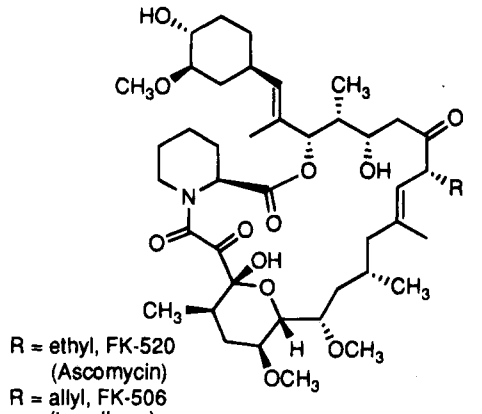
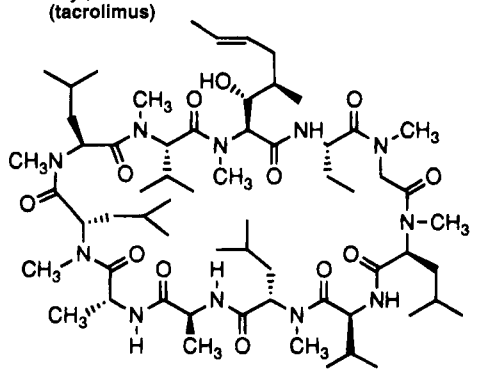
**Chart 1**



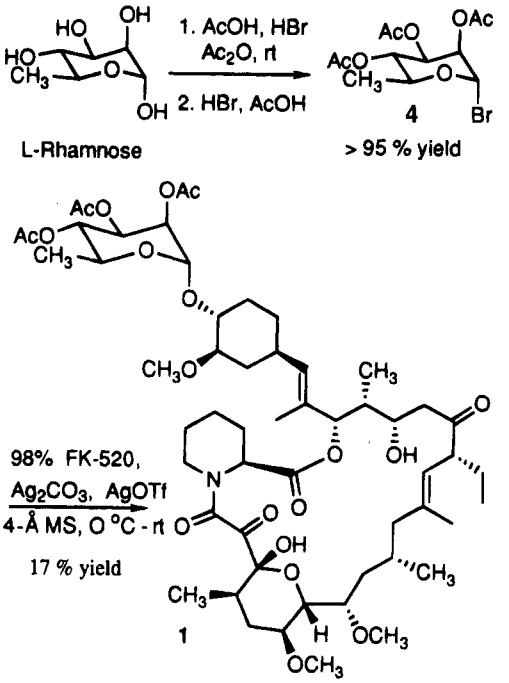



Cyclosporin A

endothelial cells), drug tissue distribution, or immunophilin subtypes with differential cellular distributions.[16]

The key issue regarding the evaluation of a CsA or a tacrolimus-like molecule is not the efficacy of the drug, since this mechanistic class of compound has proven to be highly successful in clinical transplantation but rather toleration of the drug.[17] With this thought in mind, we undertook a research program targeted at producing a potent, selective, and better-tolerated immunosuppressant based on the macrocyclic lactone, FK-520 (**3**, ascomycin), an analog of tacrolimus (Chart 1). Among the variety of approaches that were considered, one that could moderate the toxic effects of **3** would be to influence the tissue distribution of this class of compounds by introducing basic, acidic, lipophilic, or water-soluble moieties. Modifications at the cyclohexane unit of **3** were initially chosen because this group interacted with an external surface of the FK-binding protein, it was not directly involved with binding to calcineurin, and the C-32 hydroxyl group was readily functionalized with electrophilic agents. One novel method to obtain a diverse group of compounds at this position might be through glycosylation of the C-32 hydroxyl group with an appropriately substituted carbohydrate unit. Carbohydrates have a broad role in biological systems[18] and have recently been reported to be essential in cellular trafficking, viral infectivity, and immunological regulation.[19] These analogs would not only have different physical properties than **3** but might also distribute *in vivo* in a targetted manner and thereby show an increased therapeutic index. Carbohydrates were chosen on the basis of synthetic accessability and the potential for lectins on lymphocytes to recognize unique sugar residues. Of particular interest was the report that receptors on human lymphocytes existed which had specificity for L-rhamnose.[20] A

**Scheme 1**




number of laboratories have research efforts directed toward the identification of macrocyclic immunosuppressants based on the skeleton of tacrolimus.[21] In this communication we describe the novel, potent IL-2 biosynthesis inhibitor, **1** (CP-123,369) (Scheme 1), that contains a triacetyl-L-rhamnose sugar on the C-32 position of **3**. This compound is the first reported macrolactone-based molecule to possess a favorable therapeutic index relative to neurologic side effects (plasma drug concentration and dose) when compared to tacrolimus in 14–21 day rodent toxicology studies.

The synthesis of compound **1** is shown in Scheme 1. L-Rhamnose is treated with 48% hydrobromic acid and acetic anhydride in acetic acid to afford the triacetylglycosyl bromide **4** as a yellow oil which is used without purification.[22] To a solution of **3**,[23] in methylene chloride at 0 °C, was added silver carbonate, catalytic silver trifluoromethane sulfonate, and 4-Å molecular sieves. Bromide **4** was then added dropwise and the mixture stirred at 22 °C for 4–8 h to provided the crude glycosylated product. Purification was accomplished by chromatography over silica gel followed by reverse-phase chromatography using C18 silica gel and then recrystallization from cyclohexane:isopropyl ether to give a 17% overall yield of **1**. Assignment as the α anomer off the C-32 position of **3** was made by NMR analysis and confirmed by X-ray crystal structure.

Compound **1** is a potent inhibitor of human T-lymphocyte proliferation and lymphokine production. Treatment of human T cells with phorbol myristate acetate (PMA, 5 ng/mL) and ionomycin (125 ng/mL)[24] activates calcium-dependent T-cell proliferation as measured by [$^3$H]thymidine incorporation. In this system, **1** inhibits the proliferative response with an $IC_{50} = 10.4$ nM ($n = 3$). Compound **1** was also effective in blocking production of the T-cell growth factor, IL-2. T cell production of IL-2 using PMA (10 ng/mL) and phytohemaglutinin (PHA, 2 μg/mL) stimulation was blocked by **1** with an $IC_{50} = 11.0$ nM ($n = 10$).[24]

Two methods were used to assess the *in vivo* activity of this molecule. The rat adjuvant-induced arthritis

**Table 1.** Comparative Pharmacology of Immunosuppressive Agents

| | 1 | CsA | FK-506 |
|---|---|---|---|
| *in vitro* ($IC_{50}$)[a] | | | |
| human IL-2 (nM) | 11.0 | 8.1 | 0.9 |
| human T-cell proliferation (nM) | 10.4 | 13.7 | 0.5 |
| in vivo | | | |
| rat heart[b] transplantation: est. $ED_{100}$ (mg/kg) | 30 | 15 | 3–5 |
| rat adjuvant arthritis:[c] $ED_{50}$ (mg/kg) | | | |
| 1° lesion | 10 | 3.4 | 1.9 |
| 2° lesion | 2.3 | 0.7 | 0.6 |

[a] In vitro $IC_{50}$ determinations were performed as described in ref 24. [b] Brown Norway rat hearts into Lewis rats heterotopically ($n$ = 8). $ED_{100}$ = no rejection. [c] Arthritis was induced in Lewis rats ($n$ = 24) as described in ref 25.

model[25] was used to evaluate the oral activity of **1** in this model of autoimmune disease. In this study, Lewis rats were treated daily by the oral route (10:20:70 cremophor/ethanol/water vehicle) starting the day before injection of a small volume of mycobacteria in oil into the right rear footpad. After 16 days the volumes of the injected (primary lesion, acute inflammation) and uninjected (secondary lesion, chronic systemic inflammation) footpads were measured. $ED_{50}$ values for the primary and secondary lesions were 10.6 and 2.3 mg/kg, respectively. Plasma levels of **3** generated by the cleavage of the rhamnose sugar from **1** were less than 0.2 ng/mL as measured by HPLC/MS. Compound **1** was also efficacious in a rat heterotopic heart transplant model.[26] In this model, hearts from Brown Norway rats were surgically implanted into the peritoneal cavity of histoincompatible Lewis rats. Rejection was assessed by palipitating the transplanted heart and grading the strength of beating on a scale of 0–4, with 0 indicating no beat and therefore complete rejection and 4 indicating that a heart was beating as strongly as the host organ. Animals ($n$ = 8) treated orally from days 0 to 50 with 30 mg/kg of **1** did not reject their grafts after surgery and displayed scores of 3–4 at day 50.

Both CsA and tacrolimus were evaluated in similar experimental protocols. By comparison (Table 1), compound **1** has similar *in vitro* and *in vivo* potency as CsA and is about 10-fold less potent than tacrolimus. Since tacrolimus is currently administered at 0.05–0.15 mg/kg/day in humans, we believed that compound **1** was sufficiently potent for clinical evaluation if an advantage in therapeutic index could be demonstrated over tacrolimus.

In order to determine the therapeutic index of compound **1** in relation to tacrolimus, Sprague–Dawley rats were orally gavaged, daily with **1** (30/100/300 mg/kg) or tacrolimus (3/6/12/24 mg/kg) for a period of 14–21 days or until neurotoxicity was observed. Drug plasma levels were determined on the last day of dosing (steady state) at 1, 2, 4, 8, and 24 h. Compound **1** and tacrolimus are both extensively metabolized in rats; however, the major metabolites (monodeacetylation for **1** and O-demethylation for tacrolimus) are 10–100 times less potent than the parent drugs. The toxic doses and associated plasma $C_{max}$ values from the toleration studies were then compared to the doses and plasma $C_{max}$ values required to show $ED_{50}$ activity in the primary lesion of the rat adjuvant arthritis model (see Table 2). Compound **1** at an $ED_{50}$ dose of 10 mg/kg gave a $C_{max}$ plasma concentration of 254 ng/mL ($n$ = 6). At the highest dose tested of 300 mg/kg po, with the related plasma $C_{max}$ of 2908 ng/mL ($n$ = 10), no toxicologic side effects were observed. By comparison, tacrolimus showed overt neurotoxic side effects (tremors, convulsions, hyperactivity as measured by a digiscan monitor and death) at oral doses of 12 mg/kg (day 10–14) with an associated plasma drug $C_{max}$ of 52 ng/mL ($n$ = 3). In the adjuvant arthritis model, tacrolimus administered at an $ED_{50}$ dose of 2 mg/kg resulted in a plasma $C_{max}$ of 24 ng/mL ($n$ = 9). Calculation of the plasma $C_{max}$ ratio of the minimum neurotoxic dose over the $ED_{50}$ dose in rat adjuvant arthritis results in a therapeutic index of >10 for compound **1**, whereas tacrolimus has a therapeutic index of ~2. Since the pharmacokinetic properties of the two drugs are very similar, calculations of the therapeutic index using total exposure over 24 h (AUC) gives equivalent toleration ratios. In addition, these results are in accord with the clinical observations of tacrolimus in human transplantation patients.[27] A subsequent 30 day rat toxicology study with **1** has recently been conducted as described above. Neurologic toxicities, similar to those observed with tacrolimus were seen with **1** at the median 100 mg/kg dose resulting in a somewhat decreased therapeutic index relative to shorter term studies. As tacrolimus was not included in this study, and the neurotoxic effects of **1** were not dose responsive, a direct comparison of the relative therapeutic indices cannot be determined for the 30 day dosing regimen.

**Table 2.** Comparative Therapeutic Indexes of 1 and tacrolimus

| | 1 | tacrolimus |
|---|---|---|
| $ED_{50}$ dose (mg/kg) | 10 | 2 |
| $ED_{50}$ $C_{max}$ (ng/mL) | 254 | 24 |
| MND (14–21 day) dose (mg/kg) | >300 | 12 |
| MND (14–21 day) $C_{max}$ (ng/mL) | >2908 | 52 |
| Therapeutic index (14 day) MND/$ED_{50}$ $C_{max}$ | >10 | ~2 |

[a] Based on $ED_{50}$ primary lesion in rat adjuvant arthritis (Table 1) and minimum neurotoxic dose (MND)/plasma $C_{max}$ observed in Sprague–Dawley rats ($n$ = 10/dose).

Compound **1** is a macrolactone with efficacy in *in vitro* assays of immune suppression using human T cells and in relevant animal models including rat heart allograft rejection and rat adjuvant arthritis. Of greater importance is the observation that **1** may be less neurotoxic than tacrolimus. Specifically, in 14–21 day experiments in which tacrolimus administration resulted in neurotoxicity as well as mortality, compound **1** showed no toxicity at doses providing equal or greater immunosuppressive activity to that of tacrolimus. Future studies, including longer term experiments, are needed with the prototypic compound to examine this apparent toleration advantage and to help facilitate the search for improved immunosuppressive agents.

**Supplementary Material Available:** Spectral and physical data for compound **1** and experimental conditions for plasma $C_{max}$ determinations (4 pages). Ordering information is given on any current masthead page.

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