FR901483, a Novel Immunosuppressant Isolated from *Cladobotryum* sp. No. 11231

Taxonomy of the Producing Organism, Fermentation, Isolation, Physico-chemical Properties and Biological Activities

KAZUTOSHI SAKAMOTO, EISAKU TSUJII, FUMIE ABE, TOMOKO NAKANISHI, MICHIO YAMASHITA†, NOBUHARU SHIGEMATSU, SHIZUE IZUMI†† and MASAKUNI OKUHARA

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Toyosato-machi, Tsukuba-shi, Ibaraki 300-26, Japan
†Fementation Development Laboratories, Fujisawa Pharmaceutical Co., Ltd., Nakagawara, Shinkawa, Nishikasugai-gun, Aichi 452, Japan
††Product Development Division, Fujisawa Pharmaceutical Co., Ltd., 2-1-6 Kashima, Yodogawa-ku, Osaka 532, Japan

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FR901483, a novel immunosuppressant, has been isolated from the fermentation broth of *Cladobotryum* sp. No. 11231. The molecular formula of FR901483 has been determined as $C_{20}H_{31}N_2O_6P$. FR901483 exerts a potent immunosuppressive activity *in vitro* and significantly prolongs graft survival time in the rat skin allograft model. This compound has an intriguing tricyclic structure possessing a phosphate ester in its molecule. The ester residue may play an important role in exerting immunosuppressive activity because the desphosphoryl compound is devoid of activity. It is thought that the primary target of immunosuppression by this compound is inhibition of purine nucleotide biosynthesis.

Since the early 1980s cyclosporin A (CsA) has revolutionized the field of organ transplantation. Almost a decade after its emergence, a more potent immunosuppressant tacrolimus (formerly designated as FK506) has been marketed clinically. Recently tacrolimus has been shown to have advantages over CsA in respect of lower rejection rates in human liver transplantation[1]. However, clinical immunosuppression by these agents is limited by drug-associated side effects such as nephrotoxicity, neurotoxicity and diabetogenicity. To improve therapeutic effects while reducing toxic side effects, combined drug therapies consisting of low-dose CsA or tacrolimus with other immunosuppressants such as azathioprine, predonizone and OKT3 are now in widespread use. Therefore, the search for safer immunosuppressants with different mechanisms of action from tacrolimus or CsA is of great importance.

Following the successful discovery of tacrolimus using an assay system involving murine mixed lymphocyte reaction, we have shown that the agent suppresses various T cell-related immune responses such as production of interleukin 2 (IL-2), interleukin 3 (IL-3) and interferon γ (IFN-γ), and expression of the IL-2 receptor (IL-2R)[2,3]. Following this, TOCCI and coworkers demonstrated that tacrolimus inhibits the transcription of mRNA for these lymphokines[4]. Moreover, DUMONT and coworkers have shown that the inhibitory action of tacrolimus on T-cell proliferation induced by ionomycin + phorbol myristate acetate (PMA) can be reversed by exogenously added IL-2[5]. This observation suggests that different pathways for T-cell proliferation which remain intact in the presence of tacrolimus could exist. Therefore, we constructed a screening system to search for immunosuppressants with mechanisms of action different from those of tacrolimus or CsA. This screening system tests the inhibitory activity of 12-O-tetradecanoylphorbol 13-acetate (TPA)-stimulated T-cell proliferation in the presence of exogenous IL-2. After screening a number of microbial culture broths, a potent immunosuppressant FR901483 was discovered. This paper describes the taxonomy of the producing microorganism, its fermentation, and the isolation and physico-chemical properties of FR901483, followed by studies of its immunosuppressive activity. By a combination of chemical and spectral evidence, the planar structure was elucidated. This structure (Fig. 1) was confirmed by X-ray crystallographic analysis which also revealed the relative stereochemistry as depicted in Fig. 2. The details of its determination will be published elsewhere.

Fig. 1. Structure of FR901483.

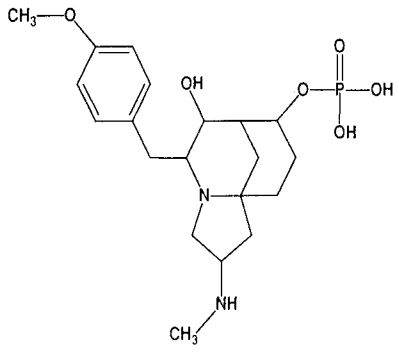
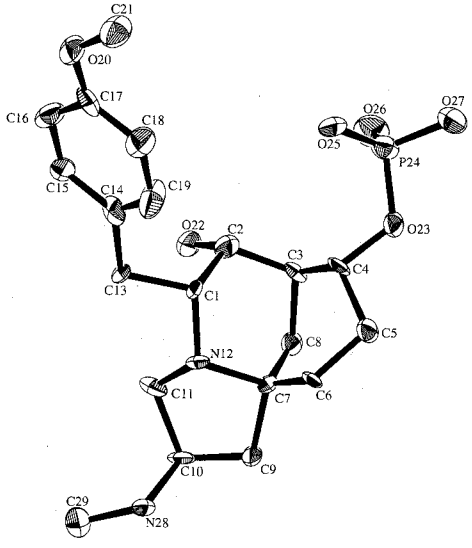


Fig. 2. X-ray structure of FR901483.



Materials and Methods

Taxonomic Studies

The fungal strain No. 11231 was isolated from litter collected at Iwaki city, Fukushima Prefecture. Mycological observations were made from cultures grown at 25°C for 14 days on malt extract agar (2.0% malt extract, 0.1% peptone, 2.0% dextrose, and 1.5% agar), potato dextrose agar (NISSUI) and mushroom agar. The mushroom agar was made from plain agar with the addition of shredded and sterilized carpophores of mushroom[6]. Morphological data were determined using this medium.

Fermentation

The strain No. 11231 growing as a mature slant culture was inoculated into 500 ml-Erlenmeyer flasks each containing 160 ml of seed medium consisting of sucrose 4%, Pharmamedia (Traders Protein Co., Ltd.) 2%, Molatein 1%, peptone 1%, KH_2PO_4 0.2%, $CaCO_3$ 0.2% and Tween 80 0.1% which had been sterilized at 120°C for 30 minutes. The seed flasks were incubated at 25°C for 3 days on a rotary shaker (5.1 cm-throw) at 250 rpm. The resultant seed cultures (3 liters) were transferred to a 200-liter jar fermenter containing 150 liters of production medium which had been sterilized at 125°C for 30 minutes. The production medium for strain No. 11231 consisting of glucose 1%, modified starch 4%, Pharmamedia 2%, soybean flour 2%, KH_2PO_4 1%, $Na_2HPO_4 \cdot 12H_2O$ 0.7%, $ZnSO_4 \cdot 7H_2O$ 0.001%, Adecanol LG-109 (antifoam, Asahi Denka Co. Ltd.) 0.025% and Silicon KM-70 (antifoam, Shinetsu Chemical Co. Ltd.) 0.025%. Fermentation was carried out at 25°C for 4 days with an air flow rate of 100 liters/minute and an agitation rate of 200 rpm.

FR901483 was quantified by HPLC using a YMC ODS-AM column (4.6 mm i.d. × 250 mm length, YMC Co. Ltd.), monitored at 230 nm, with a mobile phase of 8% CH_3CN-92% H_2O containing trifluoroacetic acid 0.1% and a flow rate of 1 ml/minute. To prepare the test samples for HPLC, a broth was vigorously mixed with an equal volume of methanol and then centrifuged at 2,500 rpm for 10 minutes to give a debris-free supernatant. Ten microliter of the supernatant was injected into a Hitachi Model L-6000 HPLC. The retention time of FR901483 was about 7.0 minutes.

T Cell Proliferation Assay

One-way mixed lymphocyte reaction (MLR) and concanavalin A (Con A) induced mitogenesis were conducted according to the previous report[7,8]. A third system for T cell proliferation induced by TPA plus IL-2 was performed as follows. Balb/c spleen cells (7×10^4 cells) suspended in 100 μl RPMI1640 complete medium containing 0.25 ng TPA (Sigma) and 0.005 U/ml IL-2 were cultured in round-bottomed microtiter plates at 37°C for 72 hours in a CO_2 incubator with and without the test compound. Incorporation of tritiated thymidine during the final 4 hours of culture was assessed according to the previous report[4].

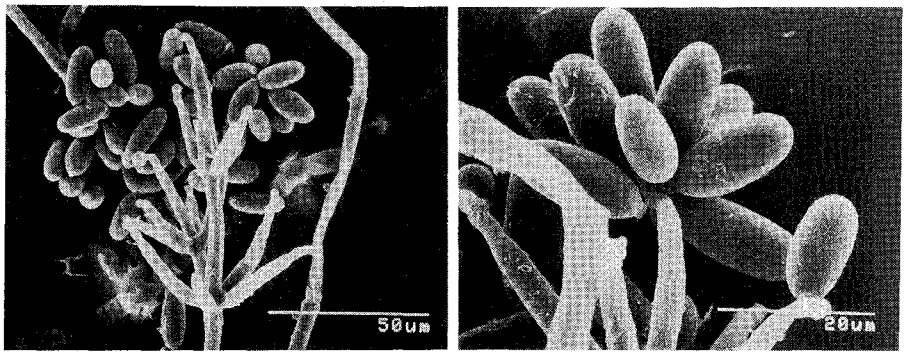
Delayed-type Hypersensitivity (DTH) Reaction

DTH was conducted according to the previous report[8].

Skin Grafting

Skin grafting was performed as follows, using allogeneic F344 (RT-1^{LV1}) and LEW (RT-1^{l}) rats as recipients and donors, respectively. F344 and LEW strains of rats, 6 to 7 weeks old, were obtained from Charles River Japan Inc. and were kept under specific pathogen-free conditions until used. Full-thickness ear skin from a LEW rat was engrafted onto the left side of the lateral thorax of a F344 rat. The graft was covered with sterile bactericidal gauze (Sofratulle, Roussel Laboratories, England) and dressing which were removed 5 days after transplantation. Each graft was inspected daily until rejection (defined as >90% necrosis of the graft epithelium). The test compound was dissolved in

Fig. 3. Scanning electron micrograph of *Cladobotryum* sp. No. 11231.
A: Conidial structure (scale: 50 μm), B: ridged conidia (scale: 20 μm).



saline and intraperitoneally injected for 14 consecutive days, beginning at the day of transplantation.

Results

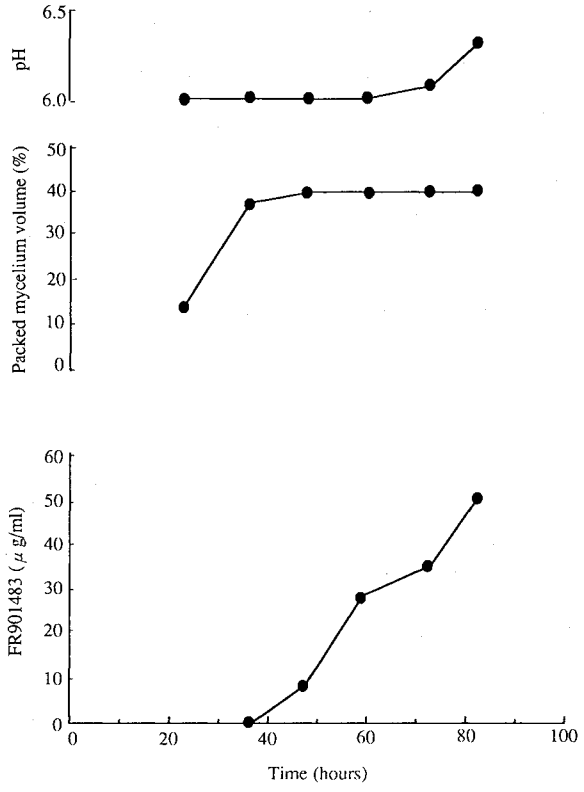
Taxonomy of the Producing Strain

The strain No. 11231 grew very rapidly attaining more than 8.5 cm in diameter, and formed felty and reddish yellow colonies on malt extract agar. Colonies on mushroom agar also grew very rapidly attaining more than 8.5 cm in diameter and the surface was felty and pale pink.

This organism formed anamorphs, consisting of conidiophores branching subverticillately and holoblastic conidia formed retrogressively. The conidiophores were fragile, hyaline, smooth, and branched subverticillately. Whorls on main stalks contained 2~3 branches, and ultimate branches bore 2~7 fertile cells verticillately. Conidiogeneous cells were straight, subulate to cylindrical, 20~45 μm long, widest (3.0~6.0 μm) just above the base, tapering slightly towards the 1.0~3.0 μm wide apex, and formed conidia retrogressively. The conidia were 1~3 septated, hyaline, straight, smooth, obclavate to cylindrical, and 18~29 × 7~11 μm, with rounded apices and acuminate bases (Fig. 3). This strain did not produce telemorph structures. The strain No. 11231 grew from 4 to 32°C on PDA.

On the basis of its morphological characteristics, the strain No. 11231 closely resembled the *Cladobotryum*-state of *Hypomyces dectylarioides* G. Arnold 1971[6]. However, this strain did not produce telemorph structures. We named the producing strain *Cladobotryum* sp. No. 1123, and it was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-3665.

Fig. 4. Time course of FR901483 production in a 200-liter jar fermentor.



Production of FR901483

Fig. 4 shows the time course of FR901483 production by *Cladobotryum* sp. No. 11231 in a 200-liter fermentor, along with the pH and the packed mycelium volume. A maximal yield of 50 μg/ml was observed after 96 hours of cultivation.

Isolation and Purification

The purification scheme is shown in Fig. 5. The fermentation broth (160 liters) was filtered with an aid

of diatomaceous earth (1 kg) and the filtrate was discarded. Eighty liters of methanol was added to the mycelial cake with stirring. The mixture was allowed to stand for 1 hour and then filtered. The filtrate was concentrated to 20 liters *in vacuo* and the solution was passed through a column (6 liters) of polymeric adsorbent, SEPABEADS SP-207 (Mitsubishi Chemical Co., Ltd.). The column was washed with 18 liters of water and 18 liters of 50% aqueous methanol, and was then eluted with 18 liters of 50% aqueous methanol containing 0.14% NH_4OH. The eluate was concentrated to 10 liters *in vacuo*. After adjusting the pH to 3.0 with 1 N HCl, the aqueous solution was passed through a column (0.45 liter) of a cation exchange resin, Diaion SK1B (NH_4^+-type, Mitsubishi Chemical Co., Ltd.). The column was washed with 3.5 liters of water and was then eluted with 0.1 N NH_4OH. After the eluate was concentrated to 1.5 liter *in vacuo*, the solution was subjected to a column (0.12 liter) of an anion exchange resin, DEAE-Sephadex A-25 (OH^--type, Pharmacia Fine Chemicals). The column was washed with 3.5 liters of water and 0.6 liter of 0.025 N acetic acid, and was then eluted with 1 liter of 0.25 N acetic acid. The active fraction was evaporated to give a crude powder. The resultant crude powder was further purified on a YMC gel column (ODS-AM 120-S50, 1.5 cm i.d. × 50 cm length, made by

Fig. 5. Isolation procedure for FR901483.

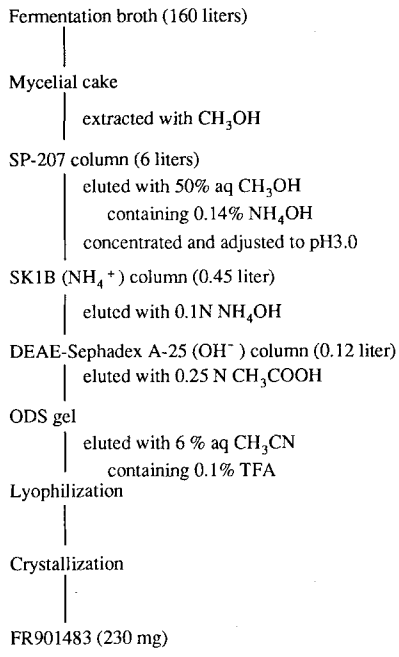


Table 1. Physico-chemical properties of FR901483.

Appearance	Colorless needles
Melting point	210 - 213°C
$[\alpha]_D^{25}$	-11° (c =0.74, $CHCl_3$)
UV λ_{max}^{MeOH} nm	225, 275, 285(sh)
IR ν_{max}^{KBr} cm^{-1}	3350, 2760, 1510, 1240, 1080, 1040
Molecular formula	$C_{20}H_{31}N_2O_6P$
Mass spectrum (FAB-MS)	427 $(M+H)^+$
Elemental analysis	
Calcd for $C_{20}H_{31}N_2O_6P \cdot HCl \cdot 2H_2O$:	C 48.14, H 7.27, N 5.61, Cl 7.11, P 6.21
Found:	C 47.65, H 7.31, N 5.75, Cl 6.94, P 6.41

Fig. 6. ^1H NMR spectrum of FR901483 in CD_3OD (400 MHz).

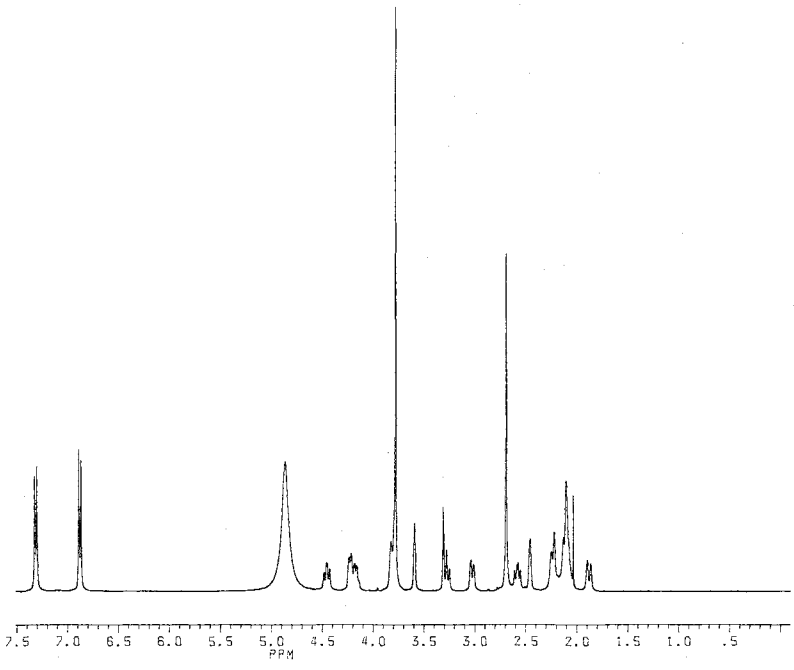


Fig. 7. ^{13}C NMR spectrum of FR901483 in CD_3OD (100 MHz).

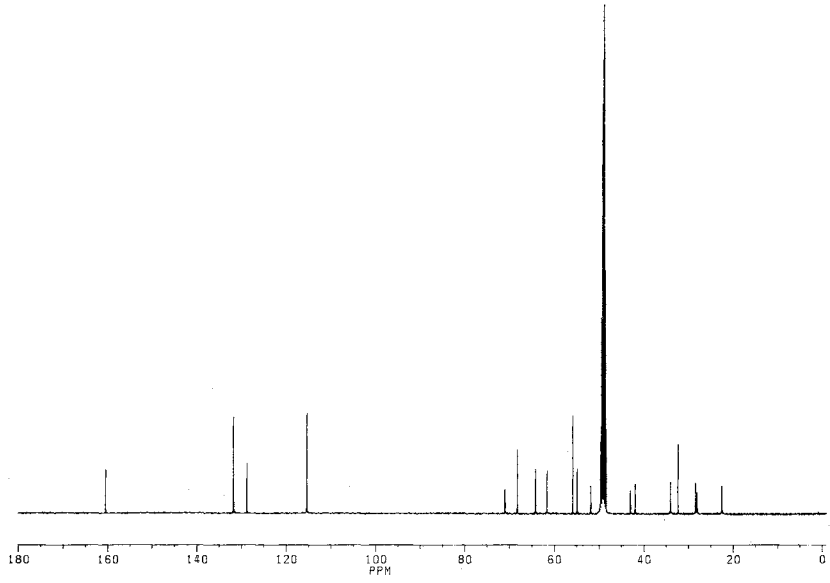
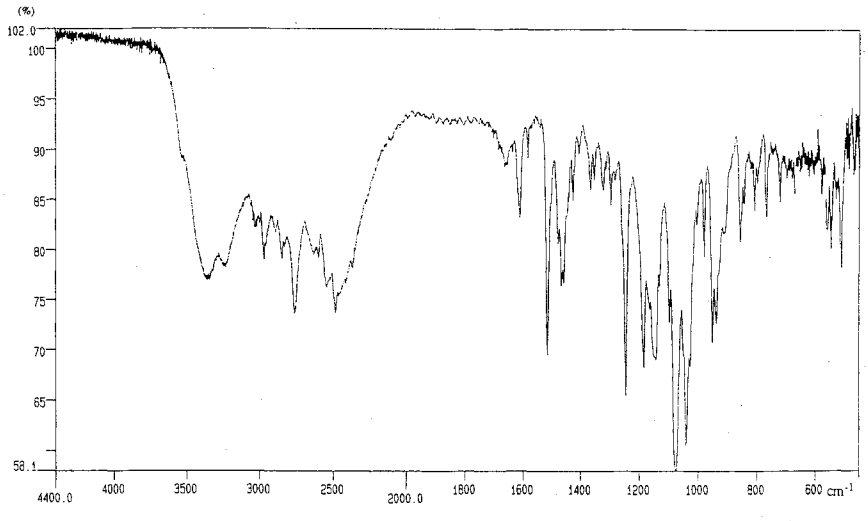


Fig. 8. IR spectrum of FR901483 in KBr.



Yamamura Chemical Institute), in acetonitrile-H_2O-trifluoroacetic acid (6:94:0.1, v/v) as a mobile phase. The eluate was monitored by UV at 230 nm and appropriate fractions were combined and lyophilized. The lyophilized powder was dissolved in 5 ml of 97.5% aqueous acetonitrile and 1 N HCl solution was added to the solution drop by drop to give colorless crystals of FR901483 (230 mg).

Physico-chemical Properties

The physico-chemical properties of FR901483 are summarized in Table 1. FR901483 is soluble in methanol and water, and is insoluble in acetone and ethyl acetate. Color reactions of FR901483 are as follows: Positive in iodine vapor, ceric sulfate and ninhydrin tests, negative in Molish and Ehrlich tests. The Rf values of FR901483 on silica gel TLC (silica gel 60 F_{254}, E. Merck) developed with *n*-butanol-ethanol-chloroform-ammonia (4:7:2:7) and *n*-butanol-acetic acid-water (4:1:2) were 0.50 and 0.14, respectively. The molecular formula was determined as $C_{20}H_{31}N_2O_6P \cdot HCl$ (molecular weight: 426) by FAB-MS and elementary analysis. The ^1H NMR, ^{13}C NMR and IR spectra of FR901483 are shown in Figs. 6, 7 and 8, respectively.

Biological Activities

Fig. 9A shows representative results demonstrating a suppressive effect of FR901483 on the proliferative response of murine spleen cells which were induced by the combined treatment of TPA and human rIL-2. The IC_{50} value of FR901483 was 21.7 ng/ml. In contrast, tacrolimus failed to inhibit proliferation in this assay system even at 10 ng/ml (Fig. 9B). As shown in Figs. 10A and 11A, FR901483 was able to inhibit lymphocyte proliferation in both murine MLR and Con A-induced mitogenesis with IC_{50} values of 7.5 and 8.1 ng/ml, respectively. The IC_{50} values of tacrolimus in these systems were 0.22 and 0.21 ng/ml, respectively (Fig. 10B and Fig. 11B).

In summary, FR901483 inhibits both types of lymphocyte proliferation, namely those which are independent and dependent on endogenous IL-2 production, whereas tacrolimus specifically inhibits lymphocyte proliferation where endogenous production of IL-2 is extensively involved. This finding suggests that FR901483 is not an inhibitor of IL-2 production. Moreover, we have found that FR901483 was incapable of suppressing IL-2R expression on T-cells (data not shown).

Next, we evaluated whether FR901483 would inhibit nucleic acid biosynthesis, as this mechanism might explain the nonspecific inhibitory effect of FR901483 on lymphocyte proliferation. We examined whether immunosuppression by FR901483 would be abrogated by the separate addition of an excessive amount of nucleosides or bases, which included adenosine, deoxyadenosine, deoxyguanosine, deoxycytidine, uridine, and thymidine. As shown in Table 2, addition of adenosine or deoxyadenosine resulted in the elimination of FR901483-induced immunosuppression. This finding suggests that FR901483-induced immunosuppression is closely linked to a decrease in adenylosuccinate or AMP and also indicates that FR901483 is an inhibitor of the purine nucleotide biosynthetic pathway.

The *in vivo* immunosuppressive activity of FR901483 was further evaluated using two animal models. First, we examined the effect of intraperitoneally injected of FR901483 on the DTH reactions in mice where sheep

Fig. 9. Effect of FR901483 (A) and FK506 (B) on the proliferative response in mitogenesis induced by TPA and IL-2.

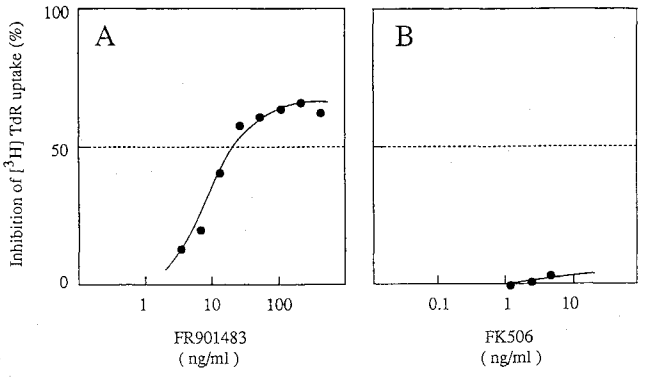


Fig. 10. Effect of FR901483 (A) and FK506 (B) on the proliferative response in mixed lymphocyte reaction.

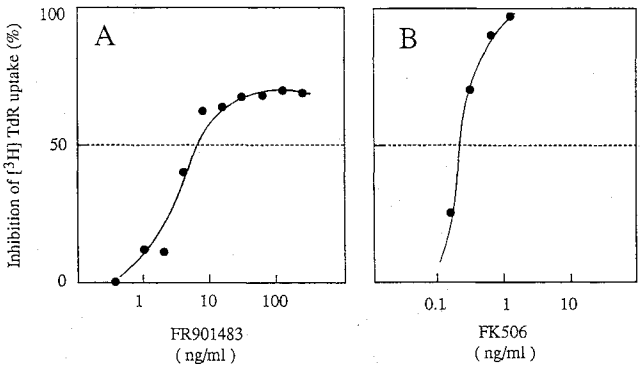


Fig. 11. Effect of FR901483 (A) and FK506 (B) on the proliferative response in concanavalin A-induced mitogenesis.

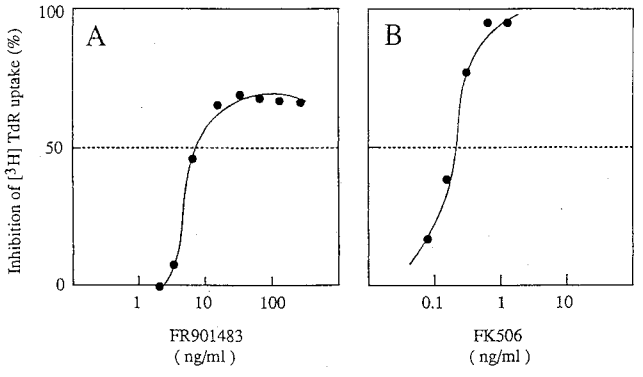


Table 2. Effect of nucleosides and bases on the inhibition of ConA-induced mitogenesis by FR901483.

Nucleoside [a)]	IC50 (ng/ml)
None	13.0
Adenosine	>500
Deoxyadenosine	>500
Deoxyguanosine	5.3
Deoxycytidine	10.3
Uridine	15.6
Thymidine	13.6

[a] Each nucleosides were added at 20 ng/ml.

Table 3. Effect of FR901483 on DTH reaction to SRBC in Balb/c mice.

Drug	Dose[a] (mg/kg)	n[b]	Footpad swelling (% Inhibition)	ED_{50} (mg/kg)
Vehicle		3		-
FR901483	0.32	3	24.5	
	1.0	3	99.6[c]	0.39
	3.2	3	102.2[c]	

[a] The test compound was dissolved in saline and administered intraperitoneally for 6 days.
[b] Number of rats per group.
[c] $P<0.001$ as compared with vehicle-treated group (MANN-WHITENEY's U-test).

Table 4. Effect of FR901483 on skin allograft survival in rats.

Drug	Dose[a] (mg/kg)	n[b]	Graft survival time (days)	Median survival time (days)
Vehicle		5	9, 10, 11, 11, 12	11.0
FR901483	0.32	5	13, 14, 14, 14, 16	14.0[c]
	0.56	5	14, (14)[d], 18, 18, 20	18.0[c]
	1.0	5	13, (14)[d], (14)[d], 14, 16	14.0

[a] The test compound was dissolved in saline and administered intraperitoneally for 14 consecutive days, beginning at the day of transplantation.
[b] Number of rats per group.
[c] $P<0.01$ as compared with vehicle-treated group (MANN-WHITENEY's U-test).
[d] Died with active graft.

red blood cell (SRBC) was used as an antigen. As shown in Table 3, FR901483 was able to prevent DTH reactions in a dose-dependent manner with an ED_{50} value of 0.39 mg/kg. Oral administration of FR901483 failed to exert a similar effect in this model (data not shown). We then examined the immunosuppressive activity of FR901483 in the rat skin allograft model. Table 4 demonstrates that the intraperitoneal administration of 0.32 and 0.56 mg/kg of FR901483 led to a significant prolonging of the median graft survival time compared to the vehicle treatment. Treatment with 1.0 mg/kg of FR901483 was found less effective than that with 0.56 mg/kg and this finding was presumed to be due to the toxicity of this compound.

Discussion

In this paper we have presented a novel immunosuppressant FR901483. This compound was discovered during a screening program for immunosuppressants with mechanisms of action different from that of tacrolimus. FR901438 is a secondary metabolite of the fungus *Cladobotryum* sp. No. 11231, and has a new type of tricyclic structure possessing a phosphate ester residue.

Immunosuppressive activity of FR901483 was found in an assay system where lymphocyte proliferative responses were induced by the combined stimulation of TPA, a potent activator of protein kinase C, and human rIL-2. In this system, resting T-cells are initially activated by TPA to express IL-2R on their cell surface and subsequently these activated T-cells are driven to proliferate in response to IL-2 which is exogenously added to the cell cultures. Due to less involvement of endogenous IL-2 production, higher concentrations of tacrolimus are required for suppressing T-cell growth in this system compared to other systems including MLR and Con A mitogenesis. In contrast to tacrolimus, FR901483 is able to inhibit T-cell growth induced by TPA plus IL-2 as potently as MLR and Con A-induced mitogenesis, suggesting that FR901483 is not an inhibitor of IL-2 production.

Immunosuppressive activity of FR901483 was also confirmed in animal models when applied ip but not po. Failure in immunosuppression by oral dosing of this compound can be explained by the decomposition of FR901483 mainly due to hydrolysis of the phosphate ester. This explanation is based on the *in vitro* finding which demonstrates that the dephosphorylated form of FR901483 is much inferior to FR901483 in immunosuppressive potency, suggesting that phosphate ester is essential for FR901483 to exert immunosuppression.

In order to elucidate the possible mode of FR901483-induced immunosuppression, a series of experiments were subsequently performed. In these studies, it was found that FR901483 was incapable of inhibiting expression of IL-2R on T-cells. Moreover, we investigated the possibility that FR901483 might exert immunosuppression through binding to either FK506 binding proteins (FKBPs) or cyclophilins which are the respective and specific binding proteins for FK506 or CsA. The fact that rapamycin, a microbial product with similar structure to FK506 and which also has an immunosuppressive function, specifically binds to FKBPs leading to competition with FK506 for FKBPs encouraged us to make this investigation[9]. For this purpose, both FK506 and CsA were tested for their capability in abrogating FR901483-induced inhibition of lymphocyte proliferation in the TPA plus IL-2 system. As a result, it was found that the immunosuppressive activity of FR901483 was not abrogated by the addition of larger amount of either FK506 or CsA (data not shown). This finding implies that it is unlikely for FKBPs and cyclophilins to be involved in immunosuppression by FR901483.

The non-specific immunosuppressive profile of FR901483 prompted us to examine the possibility that FR901483 is an anti-metabolite. Experimental results obtained indicate that FR901483 is likely to interfere with purine nucleotide biosynthesis by inhibiting the enzyme(s), adenylosuccinate synthetase and/or adenylosuccinase. These are the key enzymes catalyzing those

steps in the *de novo* purine nucleotide biosynthetic pathway where adenylosuccinate and AMP are respectively generated.

It is well known that some anti-metabolites have beneficial effects on excessive immune reactions such as graft rejection. The best example is azathiopurine[10], a representative inhibitor of purine biosynthesis, which is currently used as one of the standard immunosuppressants for the prophylactic treatment of transplant patients. In addition, two other immunosuppressants, RS61443[11] and brequinar sodium[12] are inhibitors of purine and pyrimidine biosynthesis, respectively. Clinical trials are now being carried out to investigate their immunosuppressive potential.

Taking the available data on FR901483 into consideration, we believe that its immunosuppressive action is mainly attributed to the anti-metabolic effect on immunocompetent cells.

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