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FR252921, a Novel Immunosuppressive Agent Isolated from *Pseudomonas fluorescens* No. 408813

III. *In Vivo* Activities

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A novel immunosuppressive agent, FR252921 was isolated from the cultured broth of a species of *Pseudomonas fluorescens*. We have shown that FR252921 inhibit activating protein-1 (AP-1) transcription activity and act dominantly against antigen presenting cells comparing to T cell. Possibility of FR252921 as concomitant drug of FK506, T-cell specific inhibitor was evaluated. FR252921 showed synergy with FK506 in immunosuppressive activity both in splenic proliferation and in murine skin transplantation.

Acute rejection after organ transplantation has been arrested in high success rate with treatment using calcineurin (CN) inhibitor, including FK506 or cyclosporin A (CsA)[1]. However, CN inhibitors are associated with side effects that can diminish quality of life and adversely affect long-term allograft and patient survival[2]. Therefore, the wide array of new drugs will be expected. They can offer the opportunity to use combinations that block different pathways of immune activation while selecting drug combinations with nonoverlapping toxicity profiles so that doses of each single drug can be reduced below toxicity levels[3]. To establish therapy with more efficacy and safety, we need another novel immunosuppressant that has different target from CN inhibitors.

We reported that structural novel compound, FR252921 (**1**) (Fig. 1) was isolated from the cultured broth of *Pseudomonas fluorescens*, using CN inhibitor-insensitive screening system[4]. This compound inhibits the lymphocyte proliferation stimulated not only with anti-CD3 mAb but also with LPS *in vitro*. On the other hand, FK506 and CsA, T cell specific immunosuppressants show strong inhibition of splenocyte proliferation stimulated with anti-CD3 mAb, in contrast to less effect on proliferation stimulated with LPS. Further studies on mode of action have revealed that **1** inhibits AP-1 transcriptional activity and is dominantly effective against antigen presenting cell rather than T cell[5].

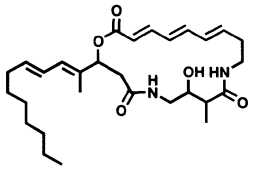
In this paper, we examined possibility of **1** as concomitant drug of CN inhibitor, FK506, *in vitro* and *in vivo*.

Materials and Methods

Drugs

FK506 and **1** were prepared in our Research Laboratories. Mycophenolate mofetil (MMF) was purchased from Roche (Basel, Switzerland). When we performed *in vitro* test, FK506, **1** and **2** were dissolved in acetonitrile and

Fig. 1. Structure of FR252921.



FR252921 (**1**)

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further diluted in RPMI-1640 medium and added to the culture. In the evaluation *in vivo*, **1** and **2**, suspended in 10% polyoxyethylated (60 mol) hydrogenated castor oil in saline (HCO60 solution), were administered intraperitonealy for 10 days starting from the day of transplantation. FK506 suspended in 0.5% methylcellulose was given for 10 days per oral. MMF dissolved in distilled water was administered for 10 days per oral.

Animals

Female mice of BALB/c (H-2^d) and C57BL/6 (H-2^b) strains were purchased from Charles River Japan Inc.

Immunosuppressive Activity *in Vitro*

Immunosuppressive activity was determined by lymphocyte growth inhibition assay. It was performed in 96-well U-bottomed microtiter plates with each well containing 1×10^5 splenocytes of C57BL/6 mice in 0.1 ml RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 mM 2-mercaptoethanol, penicillin (100 units/ml) and streptomycin (0.1 mg/ml), to which anti-mouse CD3 mAb (1 μg/ml) was added. The cells were incubated for 3 days at 37°C in a humidified atmosphere of 5% CO_2. Growth was measured by the colorimetric MTT (3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide) assay described by MOSMANN[6]. Briefly, 4 hours prior to termination of culture, 10 μg of MTT dissolved in RPMI-1640 was added to the each well. After removal of the medium from all wells, 2-propanol was added to each well and mixed thoroughly to dissolve the dark blue crystals. The plates were measured on a two-wavelength microplate photometer at 550 nm with a reference wavelength at 660 nm.

Murine Skin Transplantation

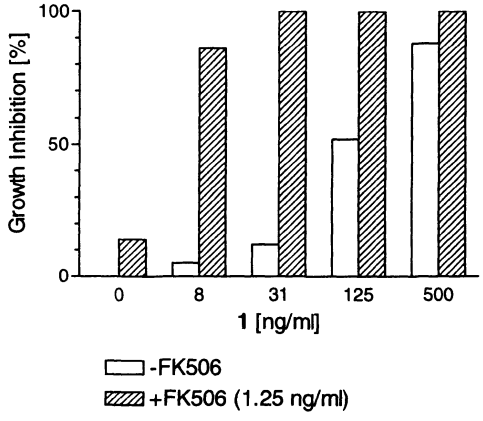
Full thickness tail skin from BALB/c donors was grafted onto the shaved back of the C57BL/6 recipients as previously described[7]. Grafts were covered with Band-Aid (Johnson & Johnson) for 6 days. Graft survival was followed by daily visual inspection, with rejection defined as necrosis occurred in a >70% portion of the graft.

Results

Synergy between **1** or **2** and FK506 in Immunosuppressive Activity *in Vitro*

In vitro immunosuppressive activities of **1** and FK506 were analyzed by inhibitory effect on splenocyte proliferation stimulated with soluble anti-CD3 mAb. FK506 and **1** showed inhibition in dose-dependent manner. Although neither 8 ng/ml of **1** nor 1.25 ng/ml of FK506 exerted inhibition, combination of **1** and FK506 at that concentration showed remarkable effect (Fig. 2). Therefore **1** showed strong inhibition against splenocyte proliferation in combination with sub-effective dose of FK506.

Fig. 2. Compound **1** inhibited splenic proliferation stimulated with anti-CD3 mAb in synergy with sub-effective concentration of FK506.



Synergy between **1** and FK506 in Murine Skin Allograft Survival

We evaluated immunosuppressive effect of FK506 in the murine skin allograft model. Median survival time (MST) of skin allograft was 7 days posttransplantation in vehicle control group. Administration of 10 mg/kg/day of FK506 (p.o., day 0~9) showed slight prolongation effect (MST=9 days, Table 1). We considered 10 mg/kg/day as optimal dose of FK506 in this model, because increase of dose up to 32 mg/kg/day did not showed marked improvement (Table 1).

Next, we investigated whether **1** and mycophenolate mofetil (MMF), concomitant drug with FK506 in clinical, have additive efficacy on optimal treatment of FK506. Comparing to treatment of FK506 (10 mg/kg/day, p.o., day 0~9) alone, combination of **1** (0.1 mg/kg/day, i.p., day 0~9) with optimal dose (10 mg/kg/day) of FK506 prolonged MST by 4 days, though **1** alone did not showed obvious effect (Table 2). MMF also showed synergistic activity with FK506 in this model. Combination of MMF (100 mg/kg/day, p.o., day 0~9) with FK506 prolonged MST by 4 days in comparison to treatment of FK506 alone (Table 3). As

Table 1. The survival of BALB/c skin allograft on the back of C57BL/6 treated with FK506.

Drug	Graft survival (days)	Median Survival Time (days)
Vehicle	6,7,7,7,8,8,9	7
FK506 (3.2 mg/kg)	6,6,7,7,7,7,9	7
FK506 (10 mg/kg)	6,7,7,9,9,11,11	9
FK506 (32 mg/kg)	7,8,8,10,11,11,11	10

Table 2. The survival of BALB/c skin allograft on the back of C57BL/6 treated with **1** or combination of **1** with FK506.

Drug	Graft survival (days)	Median Survival Time (days)
Vehicle	7,7,7,7,7,8,8	7
1 (0.03 mg/kg)	7,7,7,7,9,10,11	7
1 (0.1 mg/kg)	7,7,7,7,8,9,11	7
FK506 (10 mg/kg)	7,7,8,8,8,8,9	8
FK506 (10 mg/kg) + **1** (0.03 mg/kg)	8,8,9,9,9,11,12	9
FK506 (10 mg/kg) + **1** (0.1 mg/kg)	12,12,12,12,12,13,13	12

Table 3. The survival of BALB/c skin allograft on the back of C57BL/6 treated with MMF or combination of MMF with FK506.

Drug	Graft survival (days)	Median Survival Time (days)
Vehicle	7,7,7,8,8,8	7.5
MMF (10 mg/kg)	6,7,7,8,8,9	7.5
MMF (32 mg/kg)	7,7,7,8,9,9,9	8
MMF (100 mg/kg)	6,9,10,11,11	10
FK506 (10 mg/kg)	6,6,8,8,10,13	8
FK506 (10 mg/kg) + MMF (10 mg/kg)	6,8,8	8
FK506 (10 mg/kg) + MMF (32 mg/kg)	8,9,9,10,10,13,14	10
FK506 (10 mg/kg) + MMF (100 mg/kg)	7,11,12,12,12,15	12

shown above, additive effect of **1** on optimal dose of FK506 was comparable to that of MMF.

Discussion

In this paper, we examined possibility of **1** as concomitant drug of FK506. In the lymphocyte proliferation assay *in vitro*, we found synergy of **1** with FK506. CD3-triggered splenocyte proliferation was sufficiently inhibited by the combination of low concentration of FK506 and **1**, respectively insufficient for inhibition alone (Fig. 2). This result suggests that combination of **1** and FK506 can reduce dose of FK506 with keeping immunosuppressive efficacy.

For assessment of potency as immunosuppressant *in vivo*, tail skin of BALB/c mice were grafted to C57BL/6 mice. FK506 postponed rejection but monotherapy with **1** did not. This difference may be attributed to inability of **1** to inhibit IL-2 production by T cell. However, **1** prolonged allograft survival in combination with FK506 (Table 2). Adding dose of optimal FK506 (10 mg/kg/day, p.o.) up to 32 mg/kg/day could not show notable efficacy (by 1 day in MST). Combination of **1** and FK506 (10 mg/kg/day, p.o.) prolonged graft survival by 4 days comparing to FK506 alone. Therefore, additive activity of **1** to optimal dose of FK506 was superior to that of FK506 itself. These results indicate that **1** can enhance immunosuppressive effect of optimal dose of FK506 without increasing FK506. This synergistic action was not associated with modulation of FK506 metabolism. Compound **1** did not inhibit metabolic enzyme, CYP3A4 that is known to degrade FK506 *in vitro* and administration of **1** did not cause increase of concentration FK506 in blood in pharmacokinetic study (data not shown).

In our study, MMF also exhibited synergistic activity with FK506 and the efficacy of MMF is comparable to that of **1** (Tables 2 and 3). Considering that MMF has been used as concomitant drug with FK506 in clinical[8,9], it is expected that **1** will also be a member of combination therapy for rejection control.

As described in preceding paper, **1** inhibits splenocyte proliferation stimulated with anti-CD3 antibody or LPS by blocking AP-1 pathway[5]. It has also been revealed that **1** acts dominantly against APC rather than T cell. As shown above, although **1** alone did not show strong efficacy in transplantation, combination FK506 and **1** prolong allograft survival. These results may indicate that AP-1 inhibitor such as **1** will be an excellent member of combination therapy for rejection control in clinical use.

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