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SNF4435C and D, Novel Immunosuppressants Produced by a Strain of *Streptomyces spectabilis*

III. Immunosuppressive Efficacy

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Novel immunosuppressants, SNF4435C and D produced by a strain of *Streptomyces spectabilis*, were examined for their pharmacodynamical profiles. SNF4435C and D suppressed the responses of both murine splenocytes and human peripheral blood lymphocytes in the mixed lymphocyte reaction (MLR) with IC_{50} values of 0.5 μM and 0.2 μM, respectively. In the mouse MLR experiments, SNF4435C and D did not block the production of interleukin-2 (IL-2) and the compounds-induced suppression was not restored by the addition of exogeneous IL-2. In addition, the significant inhibitory action was still retained even when SNF4435C or D was added after 48 hours from the start of the culture. These results were distinct from the behaviors observed with FK-506. SNF4435C, the major component, suppressed mouse delayed type hypersensitivity (DTH) and prolonged rat skin allograft survival.

In the course of screening for bioactive compounds derived from microbial products, we found novel immunosuppressants, SNF4435C and D, in the culture broth of a strain of *Streptomyces spectabilis*[1,2]. The compounds are nitrophenyl pyrones having an intriguing tricyclic ring system and are diastereoisomers of each other. Interestingly, SNF4435C and D preferentially suppress B-cell proliferation induced by LPS compared with T-cell proliferation induced by Con A in non-cytotoxic concentrations. Several T-cell specific inhibitors such as cyclosporin A (CsA)[3] and tacrolimus (FK-506)[4] have been developed and put to clinical use. Although a few B-cell specific inhibitors[5,6] have been found, they have not been developed as pharmaceuticals yet. We therefore have been interested in the immunosuppressive action of SNF4435C and D. The present paper describes the immuno-phamacodynamical profiles of the new immunosuppressants, SNF4435C and D.

Materials and Methods

Reagents

SNF4435C and D were isolated from the culture broth of a strain of *Streptomyces spectabilis* SNF4435 in our laboratory, as described previously[1]. The structures of these compounds are illustrated in Fig. 1. FK-506 was purchased from Fujisawa Pharm. (Osaka). The recombinant mouse IL-2 was obtained from Genzyme (Cambridge).

Animals

BALB/c and C57BL/6 mice (male, 5 weeks old) were obtained from Charles River Japan (Kanagawa). WKAH and F344 rats (male, 6 weeks old) were also purchased

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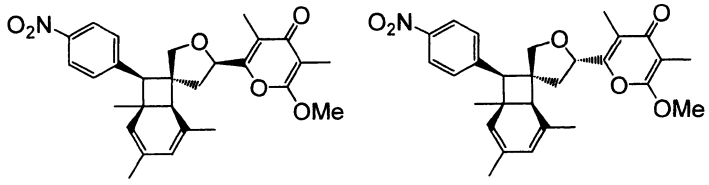
Fig. 1. Relative structures of SNF4435C (**1**) and D (**2**).



1 **2**

from Charles River Japan. They were maintained under specific pathogen-free conditions at 23±2°C and 50±10% relative humidity. Lighting was automatic under 12 hours light/dark cycle. The animals were allowed free access to commercially available standard diet and water.

Mouse Mixed Lymphocyte Reaction (MLR)

A one-way MLR was performed according to the procedure described by GIBB *et al.*[7]. Splenocytes from BALB/c and C57BL/6 mice (male, 6~8 weeks old) were used as responder and stimulator cells, respectively. The mice were sacrificed, and then spleens were excised and teased into a single cell suspension in phosphate-buffer saline (PBS) using a scalpel and forceps. The suspension was filtered through a nylon mesh to remove clumps. The cells were freed of red blood cells by the treatment with ammonium chloride buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$ and 0.1 mM Na_2EDTA, pH 7.4) and washed three times with PBS. The splenocytes from BALB/c mice were suspended in the culture medium, RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 100 IU/ml penicillin (Meiji Seika) and 100 μg/ml streptomycin (Meiji Seika). The splenocytes from C57BL/6 mice suspended in PBS were treated with 25 μg/ml mitomycin C (Wako) at 37°C for 30 minutes, washed three times with PBS, and then resuspended in the culture medium. The sample was dissolved in dimethyl sulfoxide and serially diluted with the culture medium. The 5×10^5 responder cells (50 μl), the 5×10^5 stimulator cells (50 μl), and the test sample solution (100 μl) were added into each well in a 96-well microtiter plate. The plate was incubated at 37°C for 72 hours in a humidified 5% CO_2 atmosphere. At the end of the culture period, the cells were pulse-labeled with 1 μCi/well of [^{3}H] thymidine (Amersham) for 4 hours and then harvested with a cell harvester (MICROMATE 196, PACKERD). The [^{3}H] thymidine incorporation into the cells was measured by a liquid scintillation counter (MATRIX 96, PACKERD). The inhibitory activity of the sample was calculated as percentage of [^{3}H] thymidine incorporated into the cells in the presence of the sample to that in the absence of the sample. The IC_{50} values (the concentration giving 50% inhibition) were determined from the dose-response curve. The mean of the counts of triplicate measurement for each sample was determined.

Human MLR

Human MLR was carried out using the methodology described by ITO[8]. Human peripheral blood lymphocytes (PBL) were separated from the peripheral blood of healthy volunteers by density gradient centrifugation on Lymphoprep (NYCOMED), washed with PBS and resuspended in RPMI-1640 medium containing 100 μg/ml streptomycin, 100 IU/ml penicillin, 25 mM HEPES (SIGMA) and 10% fetal bovine serum. PBL from one donor suspended in PBS were treated with 25 μg/ml mitomycin C at 37°C for 30 minutes, washed three times with PBS and then resuspended with the medium. The sample was dissolved in dimethyl sulfoxide and serially diluted with the culture medium. The mitomycin C-treated 1×10^5 stimulator PBL (50 μl), 1×10^5 responder PBL from another donor (50 μl), and the test sample solution (100 μl) were added into each well in a 96-well microtiter plate. The plate was incubated at 37°C for 7 days in a humidified 5% CO_2 atmosphere. Eighteen hours before cell harvesting using a cell harvester (PACKARD, MICROMATE196), 1 μCi of [^{3}H] thymidine was added to each well. The measurement of [^{3}H] thymidine incorporated into the cells and the computation of the inhibitory activity were performed as described for the mouse MLR.

Assays for Cytokine Secretion

IL-2, IL-6 and IFN-γ in the MLR supernatants were quantified with ELISA systems purchased from Genzyme (Cambridge).

Delayed-type Hypersensitivity (DTH) Response

The DTH assay was based on the method of UENO *et al.*[9]. Male BALB/c mice were immunized intravenously (i.v.) with sheep red blood cells (SRBC, 1×10^6 cells) on day 0. The mice were further injected with SRBC (1×10^8 cells) into subcutaneous tissue of the left hind footpads on day 3. On day 4, the increase of footpad thickness was measured with a dial-caliper. SNF4435C suspended in 0.9% NaCl solution containing 20% HCO-60 (Nikko chemicals) was administered into the mice through various routes once a day from day 0 to day 3. The compound treatment routes and doses are described in the Results. The maximum tolerated doses (MTDs) of the compound could not be determined in preliminary experiments because of the poor solubility of the compound. The dosages were significantly below the MTDs and arbitrarily chosen.

Skin Grafting

Skin allograft was performed using a technique described by YANAGAWA *et al.*[10] with minor modifications. Tail skin grafts (about 5×10 mm) from donor rats, WKAH (male, 7 weeks old) were transplanted to the dorsum of recipient rats, F344 (male, 7 weeks old) and covered with sterile bactericidal gauze. The affected part was then wrapped with an elastic bandage. After 3 days from the transplantation, the dressings were removed. Each graft was inspected daily until rejection, which was defined as more than 90% necrosis of the graft epithelium. The test compound suspended in 0.9% NaCl solution containing 20% HCO-60 (Nikko chemicals) was injected subcutaneously (s.c.) into the rats once a day for the 10 consecutive days, from the day of transplantation.

Statistical Analysis

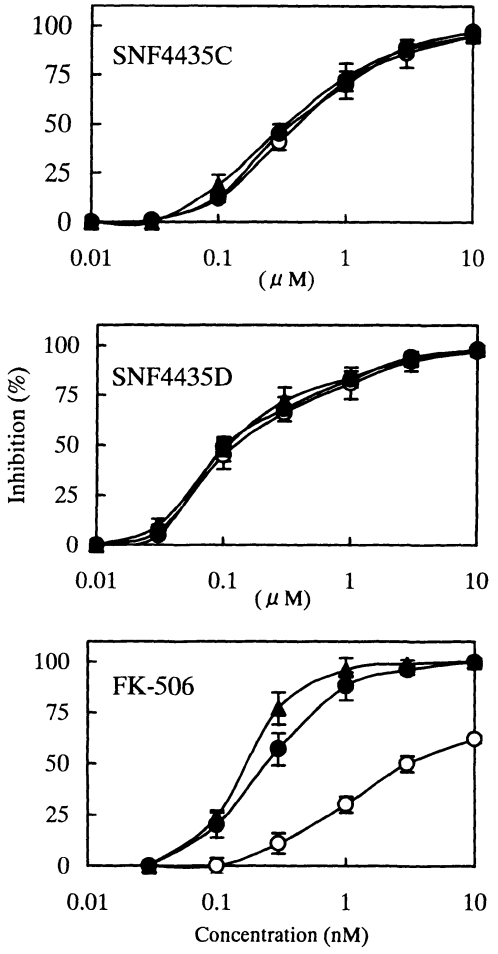
The results of the DTH assay were evaluated by the Dunnett two-tailed test, and skin graft survival times were compared by the Mann-Whitney *U* test. *P* values less than 0.05 were considered to be significant.

Results

In Vitro Immunosuppressive Activity

The effects of SNF4435C and D on mouse and human mixed lymphocyte reactions (MLR) were examined in comparison with that of FK-506. As demonstrated in Fig. 2, the compounds showed suppressive activity against the immune responses of both murine splenocytes and human peripheral blood lymphocytes to alloantigen stimulation, in a dose-dependent manner. The IC_{50} values of SNF4435C, D and FK-506 on mouse MLR were 0.5 μM, 0.2 μM and 0.3 nM, respectively. The IC_{50} values of the compounds on human MLR were 0.5 μM, 0.2 μM and 0.2 nM, respectively. The inhibitory effects were not affected by their cell toxicity (data not shown). The concentrations of SNF4435C and D required for the suppression of the MLR were much higher than that of FK-506. To determine whether exogeneous IL-2 could counteract the inhibitory actions of SNF4435C and D, 50 U/ml mouse recombinant IL-2 (MrIL-2) was added to the culture medium at the start of

Fig. 2. Effects of SNF4435C, D and FK506 on mixed lymphocyte reaction (MLR).



Symbols: ▲, human MLR; ●, mouse MLR; ○, mouse MLR in the presence of 50 U/ml MrIL-2. Values are means of triplicate assays ± SD (bars).

Table 1. Effects of SNF4435C, D and FK506 on cytokine production in the mouse MLR.

Compound		Concentration (pg/ml)					
		IL-2		IL-6		IFN-γ	
		48hr	72hr	48hr	72hr	48hr	72hr
Control		790±52	336±24	98±31	138±27	1350±159	6050±958
SNF4435C	0.1 μM	790±26	564±46	67±7	128±14	1530±218	6900±917
	0.3 μM	713±68	518±27	49±22	95±7	635±138	2290±731
	1 μM	601±47	702±58	12±6	18±12	585±79	610±89
SNF4435D	0.1 μM	689±94	469±49	91±22	140±38	1680±295	5710±785
	0.3 μM	661±41	420±19	21±3	62±23	550±44	2590±265
	1 μM	495±88	631±72	< 5	18±3	195±46	710±191
FK506	0.1 nM	403±15	258±58	86±24	135±28	2540±513	4720±458
	0.3 nM	310±36	49±19	53±15	112±2	795±79	2210±464
	1 nM	73±7	< 15	14±7	43±61	510±26	610±57

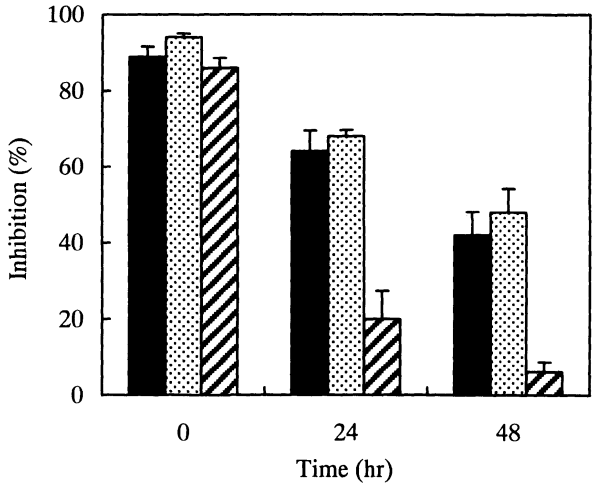
The values represent mean ± SD of triplicate assays.

the culture, in the mouse MLR assay system. Exogeneous MrIL-2 exhibited no restorative activity on the SNF4435C- and SNF4435D-induced immunosuppression. On the contrary, the FK-506-induced inhibition was significantly counteracted by the addition of MrIL-2.

The effects of SNF4435C, D and FK-506 on cytokine production in the mouse MLR were examined. After 48 hours and 72 hours culture, the concentrations of IL-2, IL-6 and IFN-γ in the supernatant were determined by ELISA. As shown in Table 1, the concentrations of IL-6 and IFN-γ in the culture broth of activated splenocytes decreased by the addition of SNF4435C or D in a dose-dependent manner. By contrast, SNF4435C and D did not suppress the production of IL-2. On the other hand, the addition of FK-506 to the culture media induced concentration-dependent suppressions of the IL-2, IL-6 and IFN-γ production. FK-506 at a concentration of 1 nM conspicuously suppressed the IL-2 production in the MLR.

The effects of delayed addition of the compounds to the culture on the inhibition of mouse MLR were examined. SNF4435C (3 μM), D (3 μM) or FK-506 (3 nM) was added to the culture after 0, 24 or 48 hours from the start of the culture, and the percent inhibition was compared in the MLR (Fig. 3). The potent inhibitory activities of SNF4435C and D were still observed even when these compounds were added after 48 hours. By contrast, FK-506 exhibited little inhibitory activity when added after 48 hours (percent inhibition by the addition of the compounds at 0, 24 and 48 hours later: SNF4435C, 90%, 65%, 42%; SNF4435D, 95%, 68%, 48%; FK-506, 87%, 20%, 7%).

Fig. 3. Effects of delayed addition of SNF4435C, SNF4435D and FK-506 on the inhibition of mouse MLR.

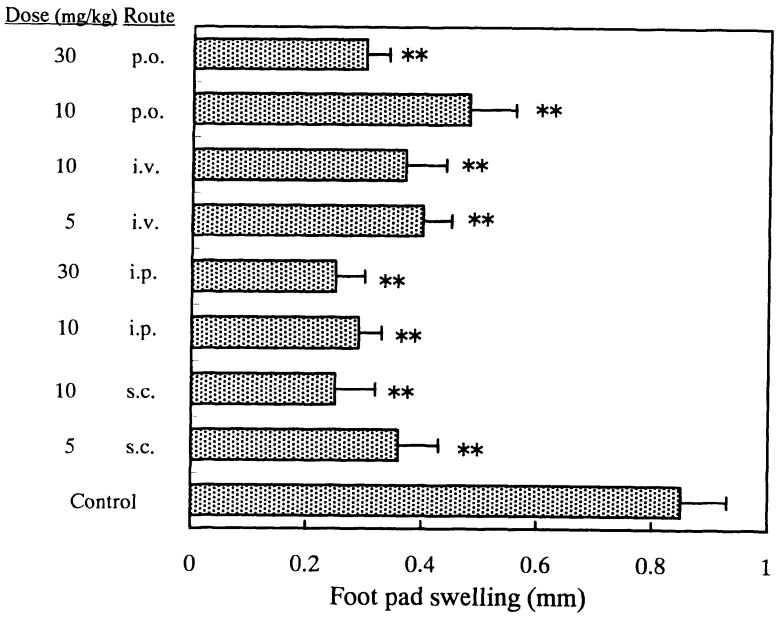


SNF4435C (■; 3 μM), SNF4435D(⊡; 3 μM) or FK506 (▨; 3 nM) was added at 0, 24 or 48 hours after the start of the culture. Values are means of triplicate assays ± SD (bars).

In Vivo Immunosuppressive Activity

To elucidate the potency and efficacy of SNF4435C, the

Fig. 4. Effect of SNF4435C on delayed type hypersensitivity (DTH) response to SRBC in mice.



Each group consisted of eight mice. Dunnett two-tailed test: ** $p<0.01$ v.s. control.

Table 2. Effect of SNF4435C on skin allograft survival in rats.

Compound	Dose (mg/kg)	No. of rats	Skin allograft survival day	MST (range)
Vehicle		7	7, 7, 7, 8, 8, 8, 8	8.0 (7~8)
SNF4435C	3	6	8, 8, 8, 8, 9, 9	8.0 (8~9)
SNF4435C	10	8	9, 9, 9, 9, 9, 10, 10, 11	9.0 (9~11)**
SNF4435C	30	7	14, 16, 16, 16, 16, 16, 17	16.0 (14~17)**
FK506	0.3	8	13, 13, 14, 14, 15, 17, 17, 18	14.5 (13~18)**
FK506	1	8	18, 18, 18, 18, 19, 19, 19, 19	18.5 (18~19)**
FK506	3	7	17, 18, 18, 18, 18, 18, 18	18.0 (17~18)**

** $P<0.01$ calculated by Mann-Whitney U test.
MST: Median survival time (days).

major component, for *in vivo* immunosuppressive activity, subsequent experiments using two animal models were performed.

The effect of SNF4435C on the mouse delayed type hypersensitivity (DTH) reaction to sheep red blood cells (SRBC) was appraised. Fig. 4 shows that SNF4435C significantly restrained the DTH reaction in all tested groups. The oral, intravenous, intraperitoneal and subcutaneous administration of 10 mg/kg SNF4435C suppressed the footpad swelling by 47%, 59%, 68% and 73%, respectively, as compared with the control group. The subcutaneous or intraperitoneal administration of SNF4435C was more effective than the oral application.

The prolonging effects of SNF4435C and FK-506 on rat skin allograft survival were examined in MHC-incompatible rat strains of WKAH donors and F344 recipients. The test compounds were administered subcutaneously for 10 consecutive days from the day of

transplantation. In this model, the rejection process became visible from day 6 to 7 after transplantation and immediately culminated into complete graft necrosis. As shown in Table 2, the median survival time of skin grafts was 8.0 days in the vehicle-treated group. SNF4435C at a dose of 3 mg/kg did not prolong the graft rejection. A significant prolongation of skin graft survival was observed at doses of more than 10 mg/kg in a dose-dependent manner. SNF4435C at a dose of 30 mg/kg prolonged median allograft survival time to 16 days. At this dose, no severe loss of body weight was observed. On the other hand, a significant prolongation of skin graft survival was obtained at a dose of 0.3 or 1 mg/kg in the FK-506-treated groups.

Discussion

In this report, we demonstrate the pharmacodynamaical profiles of SNF4435C and D, immunosuppressants produced by a strain of *Streptomyces spectabilis*.

The kinetics of immunosuppression by SNF4435C and D was examined using the *in vitro* mixed lymphocyte reaction (MLR). SNF4435C and D suppressed the alloantigen-driven proliferative responses of both murine splenocytes and human peripheral blood lymphocytes in a concentration-related manner with an IC_{50} of 0.5 μM and 0.2 μM, respectively. To determine whether these suppressive activities were IL-2-dependent, the effects of SNF4435C and D were further studied in mouse MLR. Our present experiments showed that SNF4435C and D did not suppress IL-2 production and the compounds-induced suppression was not restored by the addition of exogeneous IL-2. These results were obviously distinct from that with FK-506. SNF4435C and D exerted potent suppressive activity even when added 24~48 hours after the start of MLR cultures, whereas FK-506 exerted little activity under the same conditions. The IL-2 production by T-cells occurs early following activation[11,12]. Our results suggest that SNF4435C and D suppress T-cell immune reaction in an IL-2-independent manner. Moreover, the results also suggest that the compounds might affect a late stage rather than an early stage in T-cell activation. Thus, the immunosuppressive mechanisms of SNF4435C and D are evidently different from those of FK-506. It is known that FK-506 or CsA binds to intracellular binding protein immunophilin (FKBP or cyclophilin, respectively), and then the drug-immunophilin complex suppresses the nuclear transport of a cytoplasmic subunit of NFAT in T cells, through inhibition of the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin[13,14]. The existence of binding proteins for SNF4435C and D such as FKBP and cyclophilin has not been demonstrated at this time yet. The mechanisms of immunosuppressive action of the compounds remain to be elucidated. The clinical use of FK-506 or CsA has been hampered by the appearance of renal dysfunction and other side effects[15]. Accordingly, safer immunosuppressive agents with different mechanisms have been searched for. In accordance with the requirements, SNF4435C and D might be uniquely earnest candidates for immunosuppressants that can be used in combination with FK-506 or CsA.

SNF4435C, the major component, was effective in suppressing the immune responses in both mouse delayed type hypersensitivity (DTH) reaction and rat allogeneic skin graft rejection. SNF4435C suppressed the DTH responses in all tested administration routes. But the efficacy of SNF4435C by oral administration was inferior to that by intraperitoneal or subcutaneous administration at the same dose (10 mg/kg), suggesting poor bioavailability of the orally administered compound. In the skin graft model, subcutaneous administration of 10~30 mg/kg SNF4435C was effective for prolongation of the survival. FK-506 was 50 to 100 times more potent than SNF4435C in prolongation of rat skin allograft survival. An acute toxicity test revealed that the LD_{50} of subcutaneously administered SNF4435C in mice and rats was more than 100 mg/kg, which is over 10-fold higher than the effective doses in *in vivo* immunosuppression. It is likely that SNF4435C is less toxic than FK-506, although detailed comparative studies with FK-506 are necessary.

Immunosuppressants are used not only to prevent rejection against the transplanted organs or tissues, but also to remedy autoimmune or allergic diseases. However, strategies for the chemotherapy of the diseases have not been established yet. The absolute potency of SNF4435C may be inferior to that of FK-506, when assessed by the skin graft model. However, SNF4435C and D have different immunosuppressive mechanisms from FK-506 and CsA, and preferentially suppress the mitogen-induced B-cell proliferation compared with T-cell proliferation[1]. From the point of view of these possible mechanisms, SNF4435C and D might become more effective means for immunosuppressive therapy. The results in this paper will countenance further preclinical evaluation of the compounds as candidates for immunosuppressive agents.

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