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FR235222, a Fungal Metabolite, is a Novel Immunosuppressant that Inhibits Mammalian Histone Deacetylase (HDAC)

I. Taxonomy, Fermentation, Isolation and Biological Activities

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A cyclic tetrapeptide FR235222, a novel immunosuppressant, has been isolated from the fermentation broth of a fungus, *Acremonium* sp. No. 27082. FR235222 showed potent and selective inhibitory effects on both T cell proliferation and lymphokine production. Further study has revealed this compound exhibits potent inhibitory effects on the activity of mammalian histone deacetylases (HDACs).

Cyclosporin A (CsA) and tacrolimus (FK506), these two calcineurin inhibitors (CNIs) from microorganisms have changed the fields of solid organ and bone marrow transplantations dramatically. They have also proved to be effective in the treatment of various autoimmune diseases[1~6]. However, their clinical usage is limited by their side effects such as nephrotoxicity[7,8], neurotoxicity[9], and diabetogenicity[10], all of which are common to both agents suggesting the possibility of their relation to the mechanism of action. Furthermore, it has been reported that CNIs are rather less effective or sometimes have negative effects on the prevention of chronic allograft rejection[11~13] and the induction of tolerance[14,15], as compared with their powerful inhibitory effects in acute allograft rejection. Therefore, the seach for new immunosuppressants which are safer and meet the unmet needs with different mechanisms of action from CNIs is of great importance.

Taking the situation mentioned above into consideration, we have screened for new type inhibitors of T cell activation from microbial products. And we found a novel cyclic tetrapeptide FR235222 (originally designated WF27082B) that has potent and selective immunosuppressive activities. Further study has revealed this compound exhibits potent inhibitory effects on the activity of mammalian histone deacetylases (HDACs), as a different mechanism of action from that of CNIs. This paper describes the taxonomy of the producing microorganism, fermentation, isolation and *in vitro* biological activities of this compound.

Materials and Methods

Taxonomic Studies

The fungal strain No. 27082 was originally isolated from a soil sample, collected in Akita-city, Akita-prefecture, Japan. Cultural characteristics were determined using various agar media as follows; malt extract agar, potato dextrose agar (Difco 0013), Czapek's solution agar, Sabouraud dextrose agar (Difco 0190), Emerson Yp Ss agar (Difco 0739), corn meal agar (Difco 0386), MY20 agar and oatmeal agar (Difco 0552). The compositions of malt extract agar and Czapek's solution agar were based on the JCM Catalogue of Strains[16]. Morphological characteristics

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were determined principally from the cultures on a Miura's LCA plate[17]. These characteristics were observed after 28 days of incubation at 25°C. The color names used in this study were taken from the Methuen Handbook of Colour[18]. The temperature range of growth was determined on potato dextrose agar.

Fermentation

An aqueous seed medium (30 ml) containing 4.0% sucrose, 1.0% glucose, 2.0% soluble starch, 3.0% cotton seed flour, 1.5% soybean flour, 1.0% KH_2PO_4, 0.2% $CaCO_3$, 0.05% Adekanol LG-109 (defoaming agent, Asahi Denka Co., Ltd.) and 0.05% Silicone KM-70 (defoaming agent, Shin-Etsu Chemical Co., Ltd.) was poured into a 100-ml Erlenmeyer flask and sterilized at 121°C for 30 minutes. A loopful fungus strain No.27082 was inoculated from a slant culture into the flask and cultured at 25°C on a rotary shaker at 220 rpm (5.1 cm stroke) for 4 days. The seed culture (6 ml) was inoculated to 20 liters of sterile production medium consisting of 3.5% modified starch, 0.5% glycerol, 4.0% cotton seed flour, 2.0% corn gluten meal, 0.05% Adekanol LG-109 and 0.05% Silicone KM-70 (pH7.0) in a 30-liter jar fermentor. Fermentation was carried out at 25°C for 6 days under aeration of 20 liters/minute and agitation of 200~530 rpm.

Preparation of Splenocytes

Female Balb/c and C57BL/6 mice 6 to 8 weeks old (Charles River Japan Inc.) were killed and the spleens were removed aseptically. The spleens were teased into single-cell suspensions and filtered through nylon mesh. The cell suspension was freed of erythrocytes by treatment with ACK lysing buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$ and 0.1 mM Na_2EDTA, pH 7.4) and washed.

One-way Mixed Lymphocyte Reaction (MLR)

The MLR test was performed in flat-bottomed microtiter plates, with each well containing 5×10^5 Balb/c (H-2^d) responder cells and 5×10^5 X-irradiated C57BL/6 (H-2^b) stimulator cells in 100 μl RPMI1640 medium (Gibco) supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin (refered to as RPMI1640 complete medium). The cells were incubated at 37°C for 72 hours in humidified atmosphere of 5% CO_2-95% air. The test compound was dissolved in methanol, further diluted in RPMI1640 complete medium and added to the cultures in duplicate immediately after responder and stimulator cells were mixed. After the culture period, suppressive activities of the test samples were quantified by a MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (Sigma)] dye reduction assay. This MTT assay is used routinely by many investigators as an alternative to [^{3}H]-thymidine incorporation in determination of cell proliferation.

Anti-CD3 Antibody Induced T Cell Blastogenesis

Anti-CD3 antibody preparations were obtained from the ascites fluid of female nude mice 6 to 8 weeks old (BALB/c AnNCrj-nu/nu mice; Charles River Japan Inc.) inoculated intraperitoneally with anti-CD3 monoclonal antibody-producing 2C11 hybridoma cells (kindly gifted from Dr. BLUESTONE J.: Cell Biology and Metabolism Branch, NIH). The fluid was collected, heat-inactivated, titered and stored. Balb/c spleen cells (1×10^5 cells) suspended in 100 μl RPMI1640 complete medium containing 1 μg/ml of the anti-CD3 antibody were cultured in round-bottomed microtiter plates at 37°C for 72 hours in a CO_2 incubator with or without the test compound. After the culture period, suppressive activities of the test samples were quantified by the MTT assay.

TPA Induced Lymphocyte Blastogenesis

The assay was performed as the same manner as anti-CD3 antibody induced T cell blastogenesis described above except using 10 ng/ml 12-*O*-tetradecanoylphorbol 13-acetate (TPA; Sigma) as a stimulant instead of anti-CD3 antibody.

Hepatocyte Isolation and Culture

Hepatocytes were isolated from normal liver of 6~8-week old male Wister rats (Charles River Japan Inc.) by the two-step in situ collagenase perfusion technique created by Seglen to facilitate disaggregation of the adult liver as described previously[19]. Isolated hepatocytes (5×10^4 cells) in 100 μl Williams' medium E (Sigma) supplemented with 5% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin were cultured in collagen-coated flat-bottomed microtiter plates at 37°C for 24 hours in humidified atmosphere of 5% CO_2-30% O_2 with or without the test compound. After the culture period, the cell viability was quantified by the MTT assay.

Measurement of Lymphokine Production

Supernatants were collected after 24 and 72 hours in the culture of anti-CD3 antibody induced T cell blastogenesis described above. IL-2 and IL-4 levels in the 24- and 72-hour supernatants were assayed by ELISA kits (Quantikine M Mouse IL-2 ELISA kit and Quantikine M Mouse IL-4 ELISA kit; R&D systems), respectively. The assays were performed exactly as described by the manufacturer.

RAW 264.7 Cell Culture and Measurement of NO Production

0.5×10^5 RAW 264.7 cells (an Abelson leukemia virus-transformed murine macrophage cell line; RIKEN Gene Bank, Tsukuba, Japan) in 100 μl Eagle's minimal essential medium (MEM, phenol red-free; Nissui Phamaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin containing 1 μg/ml lipopolysaccharide (LPS; Sigma) were cultured in 96-well plates at 37°C for 20~24 hours in a CO_2 incubator with or without the test compound. After the culture period, nitrite concentration in the conditioned medium were determined by using Griess reagent according to the method described by GREEN *et al.*[20].

Assay of Histone Deacetylase (HDAC)

The partial purification of histone deacetylase, the preparation of [^{3}H] acetyl histones, and the assay for histone deacetylase activity were conducted according to the method as proposed by YOSHIDA *et al.*[21], except using human T cell leukemia Jurkat cells (ATCC) and mouse lymphoma EL-4 cells (ATCC) instead of mouse mammary gland FM3A cells.

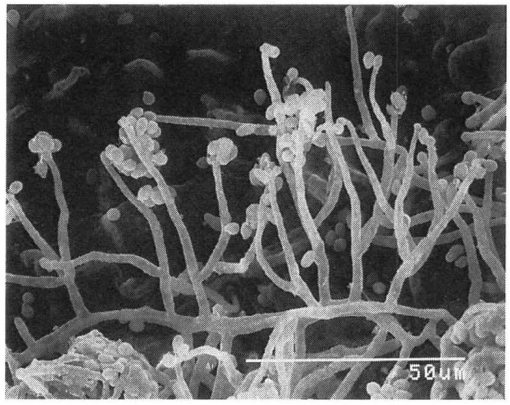
Results

Taxonomy of FR235222-Producing Strain No. 27082

The fungal strain No. 27082 grew very restrictedly on various culture media, and formed orange white to dark brown colonies. The strain produced conidial structures consisting of simple phialidic conidiogenous cells and conidia in slimy heads (Fig.1), while it did not form teleomorph on culture media. Its mycological characteristics were as follows.

Cultural characteristics are summarized in Table 1. Culture on potato dextrose agar grew very restrictedly, attaining 1.5~2.5 cm in diameter after four weeks at 25°C. This colony surface was centrally raised, cottony, radiately sulcate to wrinkly, exudate, brownish orange to grayish brown, but orange white at the margin. Conidial structures were not observed on the media. The reverse color was dark brown, and brown soluble pigments were produced. Colony on corn meal agar spread at a similar rate as on potato dextrose agar under the same conditions. The surface was plane, thin, powdery, olive at the center and orange white at the margin. Conidial structures were abundantly formed. The reverse was dark brown at the center and grayish orange to grayish brown at the margin, and pale orange soluble pigments were observed. This strain was able to grow at the temperature range from 2 to 26°C, with the growth optimum at 21 to 22°C.

Fig. 1. Scanning electron micrograph of conidial structures of strain No. 27082 (on the LCA plate, 25°C, 28 days).



Conidiophores were rarely present, micronematous, short, basitonous, and sometimes verticillate. Conidiogenous cells were discrete, acrogenous, phialidic, and nematogenous to plectonematogenous. They were hyaline, finely roughened, aciculate to subulate, with indistinct collarettes, 21~40 μm long, tapering from (1.5~)2~2.5 μm near the base to 1~2(~2.5) μm at the tip, and producing conidia in slimy drops. Conidia were hyaline at first, becoming dark olivaceous at maturity, smooth, one-celled, broadly ellipsoidal to ellipsoidal, sometimes pyriform, rounded at the tip, with a small projection at the base, and 3.5~5(~6)×2.5~3(~3.5) μm in size. Vegetative hyphae were hyaline, smooth, septate and branched. The hyphal cells were cylindrical, 2~3 μm in width. Chlamydospores were not observed.

On the basis of comparing the morphological characteristics with fungal taxonomic criteria by VON ARX[22] and DOMSCH *et al.*[23], strain No. 27082 was considered to belong to the hyphomycete genus *Acremonium* Link: Fr. 1821. Moreover, the above characteristics closely resembled to the species description of *Acremonium murorum* (Corda) W. GAMS 1971[23]. However, our strain grew slower than *A. murorum*, whose colony on malt extract agar reached 1.8~2.8 cm diameter in ten days at 20°C. Additionally, its conidia were clearly smooth, while ones of *A. murorum* were described as "coarsely warted". In conclusion, we classified this isolate as one strain of *Murorum* series in genus *Acremonium*, and named it *Acremonium* sp. No. 27082. The strain has been deposited

Table 1. Cultural characteristics of strain No. 27082.

Media	Cultural characteristics
Malt extract agar*	G: Very restrictedly, 1.5-2.5 cm S: Circular, plane, felty to cottony, formed someconidial structures, olive brown (4D3-4E3) at the center, orange white (6A2) or grayish orange(6B4-6B5) at the margin R: Brown (7E8) at the center, pale orange (5A3) at the margin
Potato dextrose agar (Difco 0013)	G: Very restrictedly, 1.5-2.5 cm S: Circular, centrally raised, cottony, radiately sulcate to wrinkly, exudate, formed no conidial structures, brownish orange (7C4) to grayish brown (7D3), and orange white (6A2) at the margin R: Dark brown (7F7-7F8), and producing brown soluble pigments
Czapek's solution agar*	G: Very restrictedly, 1.5-2.5 cm S: Circular to irregular, plane to centrally raised,submerged at the margin, formed no conidialstructures, dark brown (7F6) or orange white (6A2) at the center, yellowish white (4A2) at the margin, and brown (6E6-6E8) at the middle R: Dark brown (6F5-6F7), and yellowish white (4A2) at the margin
Sabouraud dextrose agar (Difco 0190)	G: Very restrictedly, 1.5-2.0 cm S: Circular, centrally raised, felty, radiately sulcate to wrinkly, formed no conidial structures, grayish orange (6B4-6B5) at the center, and light brown (6D6) to brown (6E6) at the margin R: Brown (7E7) to dark brown (7F7), and producing brown soluble pigments
Emerson Yp Ss agar (Difco 0739)	G:Very restrictedly, 1.5-2.5 cm S: Circular, plane, felty, exudate, formed no conidial structures, orange white (5A2), and light brown (6D6-6D7) at the margin R: Light brown (6D7) to dark brown (6F7)
Corn meal agar (Difco 0386)	G: Very restrictedly, 1.5-2.5 cm S: Circular, plane, thin, powdery, formed conidial structures abundantly, olive (3F5-3F6) at the center, and orange white (6A2) at the margin R: Dark brown (7F7) at the center, grayish orange (5B4) to grayish brown (5D3) at the margin, and producing of pale orange soluble pigments
MY20 agar*	G: Very restrictedly, 1.5-2.5 cm S: Circular, centrally raised, cottony to floccose, radiately sulcate, formed no conidial structures,and grayish orange (6B4-6B6) at the center, and orange white (5A2) at the margin R: Light brown (6D7-6D8), and light orange (5A5) at the margin
Oatmeal agar (Difco 0552)	G: Very restrictedly, 1.5-2.5 cm S: Circular, plane, felty to cottony, radiately sulcate, formed some conidial structures, dull green (30D4-30E4) at the center, orange white (6A2) at the margin, and producing grayish brown soluble pigments

Abbreviation G: growth, measuring colony size in diameter,
S: colony surface, R: reverse.

*: The compositions of malt extract agar, Czapek's solution agar and MY20 agar are based on JCM Catalogue of Strains (Nakase, T., 6th ed., pp.617, Japan Collection of Microorganisms, the Institute of Physical and Chemical Research, Saitama, 1995).

to the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-6539 (deposited date: Oct 2, 1998).

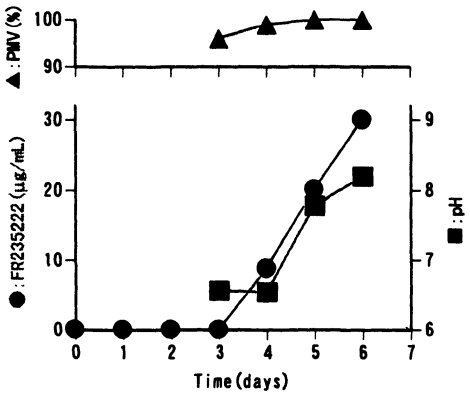
Fermentation

Fig. 2 shows the time course of FR235222 production by *Acremonium* sp. No. 27082 in a 30-liter fermentor, along with the pH and the packed mycelium volume (PMV; centrifuged at 3000 rpm for 10 minutes). A maximal yield of 30.0 μg/ml was observed after 6 days of cultivation.

Isolation

FR235222 was isolated according to the scheme as shown in Fig. 3. The cultured broth (20 liters containing

Fig. 2. Time course of FR235222 production in a 30-liter jar fermentor.

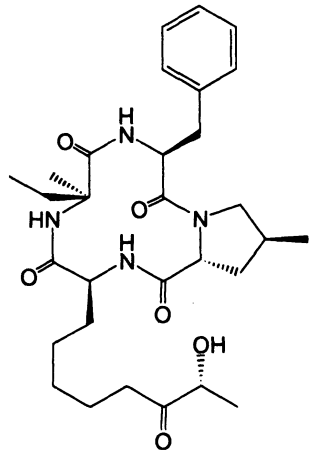


600 mg of FR235222) was extracted with 20 liters of acetone by intermittent mixing. The acetone extract was filtered with an aid of diatomaceous earth and diluted with the same volume of water. The diluted filtrate was passed through a column (1 liter) of Diaion HP-20 (Mistubishi Chemical Co., Ltd.). The column was washed with water and 70% aqueous methanol, and eluted with methanol. The eluate (1 liter) was diluted with 2 liters of water and applied on a column (180 ml) of YMC GEL ODS-AM 120-S50 (YMC Co., Ltd.) packed with 45% aqueous acetonitrile. The column was eluted with 45% aqueous acetonitrile and the elution of FR235222 was monitored by analytical high performance liquid chromatography (HPLC) using a YMC Pack ODS-AM AM303, S-5 120A (250 mm L.×4.6 mm i.d., YMC Co., Ltd.) at 210 nm with a mobile phase of 50% aqueous acetonitrile and a flow rate of 1.0 ml/minute. The retention time of FR235222 was 10.4 minutes. The portion corresponding to the FR235222 substance was concentrated *in vacuo* to give an aqueous residue. This residue was extracted with ethyl acetate and the extracts were concentrated *in vacuo* to give an oily residue (containing 554 mg of FR235222). The oily residue was dissolved in small volume of methanol, mixed with 20 ml of silica gel 60 (70~230mesh, MERCK) and concentrated to dryness. The dry powder was subjected to column chromatography of the same silica gel 60 (230 ml) which was packed with chloroform. The column was eluted with chloroform and elution was monitored by the analytical HPLC indicated above. The portion corresponding to the purified FR235222 substance was concentrated *in vacuo* to give 363 mg of a white powder. The structure of FR235222 is shown in Fig. 4. The details of the structural determination are reported in the "Notes" of this issue[24].

Fig. 3. Isolation procedure for FR235222.

Fermentation broth (20 L)
| added acetone (20 L)
Acetone extract
| diluted with water (same volume)
HP-20 column chromatography (1 L)
| washed with water and 70% aq MeOH
| eluted with MeOH (1 L)
| diluted with water (2 L)
ODS-AM column chromatography (180 mL)
| eluted with 45% aq acetonitrile
| concentrated *in vacuo* to an aqueous residue
| extracted with EtOAc
| concentrated *in vacuo* to an oily residue
Silicagel column chromatography (250 mL)
| eluted with chloroform
| concentrated *in vacuo*
White powder (363 mg)

Fig. 4. Structure of FR235222.



Biological Activities

The inhibitory effects of FR235222 on three types of lymphocyte blastogenesis, *i.e.* murine mixed lymphocyte reaction (MLR), anti-CD3 antibody induced murine T cell blastogenesis (termed anti-CD3-blast) and TPA induced

Table 2. Potent and selective inhibitory effect of FR235222 on both lymphocyte proliferation and lymphokine production.

	IC_{50} (ng/mL)
Lymphocyte proliferation	
Mixed lymphocyte reaction (mouse)	4.7
Anti-CD3 induced T cell blastogenesis (mouse)	3.0
TPA induced lymphocyte blastogenesis (mouse)	1.7
Normal cell viability	
Primary-cultured hepatocytes (rat)	>1000
Lymphokine and NO production	
IL-2 production from splenic T cells (mouse)	3.4
IL-4 production from splenic T cells (mouse)	0.35
NO production from RAW 264.7 cells (mouse)	>1000

Table 3. Inhibitory effect of FR235222 on the activity of mammalian histone deacetylases (HDACs).

Partially purified histone deacetylase from	IC_{50} (ng/mL)
Human T cell leukemia Jurkat cells	9.7
Mouse lymphoma EL-4 cells	27.9

murine lymphocyte blastogenesis (termed TPA-blast) were examined. As shown in Table 2, FR235222 exerted potent inhibitory activities against all the three proliferation types with the IC_{50} value of 4.7, 3.0 and 1.7 ng/ml, respectively. On the other hand, the cytotoxicity of FR235222 against primary cultured rat hepatocytes as a representative of normal cells was very weak (IC_{50}>1000 ng/ml). Next, the inhibitory effects of FR235222 on lymphokine production (IL-2 and IL-4) by anti-CD3 antibody stimulated T cells and NO production by LPS-stimulated murine macrophage cell line RAW 264.7 cells were examined. FR235222 exerted potent inhibitory activities against both IL-2 and IL-4 production with the IC_{50} value of 3.4 and 0.35 ng/ml, respectively. The former was virtually equivalent to, and the latter was almost ten times more potent than that of anti-proliferative activity against anti-CD3 blast. In contrast, it failed to inhibit NO production even at 1000 ng/ml. As a result, FR235222 showed potent and selective inhibitory effects on both lymphocyte proliferation and lymphokine production.

Furthermore, to determine the target molecule of FR235222, we examined whether this compound would inhibit histone deacetylases (HDACs), as its structure seemed to have some similarities to trapoxin, a known HDAC inhibitor. As shown in Table 3, we found an inhibitory effect on the activities of partially purified HDAC fractions from two mammalian lymphoidic cell lines, human T cell leukemia Jurkat cells and mouse lymphoma EL-4 cells with the IC_{50} value of 9.7 and 27.9 ng/ml, respectively.

Discussion

In this paper, we have presented a novel fungal product HDAC inhibitor FR235222 as a potent and selective immunosuppressant. As mentioned above, FR235222 exerted potent inhibitory activities against all the three types of lymphocyte proliferation we tested, *i.e.* MLR, anti-CD3-blast and TPA-blast. We also evaluated the inhibitory effects of FK506 in the same three assay systems. FK506 showed potent inhibitory activities against MLR and anti-CD3-blast with the IC_{50} value of 0.10 and 0.20 ng/ml respectively, but it failed to inhibit TPA-blast even at 1000 ng/ml. So there was a difference between the effect of FR235222 and that of FK506 against TPA-blast, indicating

their distinct mechanisms of action. Moreover, we demonstrated FR235222 exerts potent inhibitory effect on lymphokine production (IL-2 and IL-4). It has been reported that one of the most conventional HDAC inhibitors trichostatin A (TSA) selectively inhibits IL-2 production at the transcriptional level[25]. Our preliminary experimental data also demonstrated FR235222 showed a similar result to that of TSA (unpublished data). Therefore, we think the effect of FR235222 on lymphokine production was likely not due to decreased cell viability, but rather due to direct transcriptional inhibition. Moreover, we certified that FR235222 showed much less effects on the function of nonlymphoidic cells, *i.e.* the viability of primary cultured rat hepatocytes and the NO production of LPS-induced murine macrophage cell line RAW 264.7 cells. We think these potent and selective immunosuppressive activities of FR235222 may be a result of the inhibition of HDAC(s) in T lymphocytes, because results on several chemical derivatives of FR235222 showed a correlation between immunosuppressive activities and HDAC inhibitory activities (data not shown).

In general, HDAC inhibitors are known as modulators of gene transcription. Gene transcription is one of the most important processes in proper cell function, proliferation and differentiation. It is strictly regulated by chromatin structure and the binding of various regulatory proteins to DNA, both of which can be modulated by the acetylation levels of histones, as well as non-histone proteins. These acetylation levels are controlled by the equilibrium of histone acetyltransferases (HATs) and HDACs. HDAC inhibitors bring the equilibrium of the acetylation levels to the state of hyperacetylation through the blockade of deacetylation, consequently affecting gene transcriptions.

The connection between HDAC inhibition and immunosuppression has been suggested in several reports, although the precise mechanism underlying involvement of HDAC(s) in immune systems has not yet been defined. For instance, TAKAHASHI *et al.* found that TSA has selective inhibitory activity against IL-2 gene expression in Jurkat T cells[25], as described above. NILAMADHAB MISHRA *et al.* revealed that TSA reverses skewed expression of CD154, interleukin-10, and interferon-γ gene and protein expression in lupus T cells[26]. It has been revealed that HDAC inhibitors specifically upregulate or downregulate the transcription of several inducible genes, without altering the transcription of housekeeping or constitutive genes. As demonstrated by differential display, the expression of only 2% of cellular genes is changed by TSA treatment[27]. The hyperacetylation of histone is generally considered to provoke relaxation of the chromatin structure to make several regulatory proteins (including transcriptional factors) accessible to DNA. Therefore, it is easy to understand that HDAC inhibitors induce transcriptional activations. Indeed, it has been reported that HDAC inhibitors enhance the gene expression of $p21^{WAF1/CIP1}$[28], gelsolin[29], c-fos[25] and so on. But, in fact, it has been well known that transcriptional inhibitions such as IL-2, IL-8[30] and c-myc[31] gene suppressions are also induced by HDAC inhibitors. It seems rather difficult to reveal how HDAC inhibitors cause the transcriptional down regulations. It might be assosiated with the hyperacetylation of non-histone substrates, because some transcriptional factors such as p53[32], EKLF[33], HMG-Y[34] and SP3[35] are regulated their DNA-binding and transcriptional activities through their acetylation and deacetylation by HAT(s) and HDAC(s). We think, at the moment, HDAC inhibitors may exert immunosuppressive activities *via* both upregulation of immunosuppressive genes and downregulation of immunopromotive genes.

In this report, we showed a novel HDAC inhibitor FR235222 as a fungal product that has potent and selective immunosuppressive activities. Its interesting activities strongly motivated us to evaluate this compound in animal models. The results are reported in the accompanying paper[36].

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