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

II. Biological Activities in Animal Models

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(Received for publication October 18, 2002)

FR235222, a novel immunosuppressant which possesses potent inhibitory effects on the activity of mammalian histone deacetylases (HDACs), has been isolated from the fermentation broth of a fungus, Acremonium sp. No. 27082. FR235222 exhibited marked immunosuppressive effects on mouse ex vivo splenic T-lymphocyte proliferation, mouse delayed type hypersensitivity (DTH) response, rat adjuvant-induced arthritis (AA) and rat heterotopic cardiac transplantation. These results showed potential clinical use of this compound as a new type immunosuppressant in the fields of autoimmune diseases and organ transplantsations.

In the prior paper1), we showed a novel HDAC inhibitor FR235222 as a fungal metabolite that has potent and selective immunosuppressive activities. During the past ten years or more, several families of natural products have been discovered or rediscovered as HDAC inhibitors, such as short chain fatty acids (ex; n-butyrate2)), trichostatins (ex; trichostatin A3)), bicyclic depsipeptides (ex; FK2284)), depudecin5) and cyclic tetrapeptides (ex; trapoxin A6)). FR235222 was found to be a new constituent of the cyclic tetrapeptide family which include trapoxin A, trapoxin B7), HC-toxin8), Cyl-29), WF-316110), Chlamidocin11), apicidin12), TAN-174613), phoenistatin14) and so on.

Experiments by many investigators have revealed various beneficial activities of HDAC inhibitors so far. Above all, a number of reports have been submitted concerning their antitumour effect. HDAC inhibitors cause cell-cycle arrest15,16), differentiation17,18) and sometimes apoptosis19,20) in many tumourous cells, consequently exerting their potent efficacy in in vitro experiments. In reality, several HDAC inhibitors like FK22821) and MS-27522) are now under clinical investigation in this field. It also has been demonstrated that HDAC inhibitors stimulate the production of fetal hemoglobin23), and that butyrate showed some beneficial effects in the treatment of thalassemia and sickle cell disease in clinical trials24). Moreover, TOSHIRO NIKI et al. showed that TSA suppresses myofibroblastic differentiation of rat hepatic stellate cells in primary culture, indicating the possibility of HDAC inhibitors as therapeutic or prophylactic agents for fibroproliferative diseases like liver fibrosis25).

Immunosuppressive activity of HDAC inhibitors has been shown in recent several reports26). Most reports have been limited to the descriptions of in vitro activity. To the best of our knowledge, only one paper described by TAKAHASHI et al. showed the in vivo effectiveness of TSA in mouse DTH response27), although it seems less effective than that of CsA and not without toxicity. Therefore in this paper, we tested FR235222 in a mouse ex vivo splenic T-lymphocyte proliferation model, a mouse delayed type hypersensitivity (DTH) reaction model, a rat adjuvant-induced arthritis (AA) model and a rat heterotopic cardiac transplantation model, to ascertain not only the possibility of

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clinical usage of this compound but also the usefulness of HDAC(s) as (a) novel target(s) in developing immunosuppressants with good availability in vivo.

Materials and Methods

Ex Vivo Splenic T-Lymphocyte Proliferation and Bone Marrow Cell Proliferation Assay

7-Week old female Balb/c mice (Charles River Japan Inc., Atsugi, Japan) were randomly divided into five groups (each group consisted of three mice). FR235222 was dissolved in 10% aqueous HCO-60 (Nikko Chemicals Co., Ltd., Tokyo, Japan) and administered orally twice (28 hours and 4 hours before killing). After killing, spleen and right femur were removed from each mouse. Splenocytes were prepared, and anti-CD3 antibody induced T cell blastogenesis assay were performed in vitro according to the methods described in the prior paper without the addition of the test compound.

Femoral bone marrow cells from vehicle- or FR235222-treated mice were prepared and bone marrow cell proliferation assay were performed in vitro as follows. The marrow plugs were flushed out of the femurs and dissociated by repeated aspiration until a single-cell suspension was obtained. The cells were treated with ACK lysing buffer to lyse erythrocytes, washed, resuspended in RPMI1640 complete medium supplemented with 10% L-cell conditioned medium (LCM) prepared from monolayer cultures of mouse L-cells (ATCC) as previously described\(^28\) at 2.5×10^6 cells/ml, seeded and cultured in flat-bottomed 96-well plates in a volume of 100 μl/well at 37°C. After incubation for 72 hours in a CO₂ incubator, the proliferative response was quantified by the MTT assay.

Delayed-type Hypersensitivity (DTH) Reaction

7-Week old female Balb/c mice were immunized with sheep red blood cells (1×10^8 cells/head) by subcutaneous injection. Each group consisted of five mice. FR235222 was dissolved in 10% HCO-60-saline and 10% aqueous HCO-60, and administered once a day (u.i.d.) for 8 consecutive days beginning at one day before the immunization, subcutaneously and orally, respectively. Six days after the immunization, sheep red blood cells (1.25×10^8 cells/head) were injected into the right rear footpad, and 24 hours later, footpad swelling was measured with a dial guage (Ozaki MFG Co., Ltd.). The magnitude of the DTH was expressed as the thickness of the challenged right footpad as compared with the untreated left footpad.

Adjuvant-induced Arthritis (AA)

Adjuvant-induced arthritis was produced by subcutaneous injection of 0.5 mg of heat-killed *Mycobacterium tuberculosis* (Difco) suspended in 0.05 ml liquid paraffin into the right hind paw of 8-week old female Lewis rats (Charles River Japan Inc., Atsugi, Japan) on day 0. Each group consisted of ten rats. FR235222 was dissolved in 10% HCO-60-saline and 10% aqueous HCO-60, and administered twice a day (b.i.d.) for 17 consecutive days beginning at the day of inoculation, subcutaneously and orally, respectively.

On day 0 and day 17, the right (injected) and left (uninjected) hind paw volumes were measured with an electronic water plethysmograph.

Heterotopic Cardiac Transplantation

Heterotopic cardiac allografts were implanted using the cuff technique. Hearts from 8-week old male Lewis rats weighing 200~230 g (Charles River Japan Inc., Atsugi, Japan) were removed and implanted into the neck of 8-week old male ACI rats weighing 150~200 g (CLEA Japan Inc., Tokyo, Japan). Anesthesia was conducted with 40 mg/kg phenobarbital intraperitoneally. The recipients whose heartbeats stopped within 3 days after grafting or which died with living grafts were excluded from stastical analysis. FR235222 was dissolved in 10% aqueous HCO-60, and administered orally twice a day (b.i.d.) for 14 consecutive days beginning at the next day of transplantation.

Results

We examined the immunotherapeutic effectiveness of a novel HDAC inhibitor FR235222 in the following animal models, i.e. a mouse ex vivo splenic T-lymphocyte proliferation model, a mouse delayed type hypersensitivity (DTH) reaction model, a rat adjuvant-induced arthritis (AA) model and a rat heterotopic cardiac transplantation model.

First, immunosuppressive activity of FR235222 in mice was assessed by ex vivo splenic T-lymphocyte proliferation assay, and bone marrow toxicity of FR235222 was assessed by ex vivo bone marrow cell proliferation assay. As shown in Fig. 1A, the ex vivo splenic T-lymphocyte proliferation of the groups treated with 3.2, 10, 32 and 100 mg/kg (p.o., twice) of FR235222 was significantly suppressed as compared with that of vehicle-treated group (all P<0.01 by Student's t-test), producing 30, 25, 36 and 43% inhibition, respectively. On the other hand, there were no significant
differences in the number of splenocytes per body (Fig. 1B), the bone marrow cell proliferation (Fig. 1C) and the number of bone marrow cells per right femur (Fig. 1D) between the vehicle-treated control and the FR235222-treated groups.

Second, we evaluated the effect of FR235222 on the DTH reactions in mice where sheep red blood cell (SRBC) was used as an antigen. As shown in Fig. 2, the footpad swelling was markedly suppressed by the administration of FR235222 both subcutaneously and orally in a dose-dependent fashion without any significant changes of body weight gain as compared with vehicle-treated groups. Subcutaneous dosings at 0.1, 0.3, 1, 3.2 and 10 mg/kg produced 8, 20, 31, 42 and 61% inhibition, respectively. Moreover, oral administrations at 10, 32 and 100 mg/kg, u.i.d. showed 20, 34 and 47% inhibition respectively, with a good correlation to the % inhibition in the ex vivo T-lymphocyte response described above.

Next, we performed the evaluation of FR235222 in the rat developing adjuvant arthritis (AA) model. Fig. 3 shows the results demonstrating that the paw edema was markedly suppressed and body weight was significantly recovered by the administration of FR235222 both subcutaneously and orally. Subcutaneous dosing at 0.05, 0.16, 0.5 and 1.6 mg/kg, b.i.d. produced 25, 34, 50 and 69% inhibition in injected paw (right hind paw) edema, and 37, 64, 97 and 105% inhibition in uninjected paw (left hind paw) edema, respectively, in a dose dependent manner. Especially, marked recovery of body weight was observed at 0.5 and 1.6 mg/kg (56 and 51%, compared with normal control as 100%). Oral administrations at 0.16, 0.5, 1.6 and 5 mg/kg b.i.d. produced 21 (20), 34 (32), 39 (58) and 51 (76) % inhibition in injected (uninjected) paw edema in a dose-dependent fashion, and 73% of body weight recovery was produced at the highest dosage (5 mg/kg).

Moreover, we examined the ability of FR235222 to prevent allograft rejection in a rat heterotopic cardiac transplantation model. As shown in Table 1, in allografting between Lewis donors and ACI recipients, the median graft survival time in the group of rats treated orally with 5 mg/kg, b.i.d. of FR235222 was dramatically longer than that in the vehicle-treated rats (MST; >100 days vs. 9 days). One of the FR235222-treated rats died with its active graft at day 13. But there was no significant difference between the mean value of body weight gain in FR235222-treated rats (n=12) and that of vehicle-treated rats (25.5±2.2 g vs. 26.4±3.0 g).
Fig. 2. Effect of FR235222 on mouse delayed type hypersensitivity (DTH) reaction.

Footpad swelling of vehicle- or FR235222-treated mice subcutaneously (A) and orally (C), and body weight gain of them subcutaneously (B) and orally (D) are shown, respectively. Values are expressed as the mean ± S.E. (n=5). *, **, ***; P<0.05, 0.01, 0.001 as compared with vehicle-treated group (Student’s t-test).

Fig. 3. Effect of FR235222 on adjuvant-induced arthritis (AA) in rats.

Edema of vehicle- or FR235222-treated rats subcutaneously (A) and orally (C), and body weight of them subcutaneously (B) and orally (D) are shown, respectively. Values are expressed as the mean ± S.E. (n=10). **; P<0.01 as compared with vehicle-treated group (Dunnett’s test).
Discussion

In this paper, virtually for the first time, we demonstrated the powerful and safe effectiveness of an HDAC inhibitor as a new immunosuppressant in several animal models of immune response using our novel compound FR235222. Furthermore, most importantly, this compound is orally bioactive suggesting its great possibility of clinical use in the fields of autoimmune diseases and organ transplantations.

In the mouse ex vivo T cell/bone marrow differential proliferation assay, oral administration of FR235222 exerted an inhibitory effect on splenic T cell proliferation without affecting the number of splenocytes per body, the bone marrow cell proliferation and the number of bone marrow cells per femur. These results, we think, show the possibility of selective immunotherapeutic effect of this compound without myelotoxicity. In the mouse DTH reaction and the rat AA model, subcutaneous and oral dosing of FR235222 showed powerful and dose-dependent suppression of edema, without any significant changes of body weight gain compared with vehicle-treated control in the former model, and with marked recovery of body weight in the latter model. These results strongly suggest that it might serve as a well-tolerated drug with a wide therapeutic window for the treatment of autoimmune diseases like rheumatoid arthritis (RA). Moreover, oral treatment of FR235222 markedly prolonged the survival of heterotopic heart allografts in our Lewis to ACI rat transplantation model. Long term graft acceptance after drug cessation (MST>100 days) suggested the possibility of tolerance. One rat died with active graft as described above. We think the death wasn’t likely due to its toxicity but rather due to a technical error or something, because there was no influence of FR235222 treatment on body weight as a sign of adverse effects. This marked efficacy with good tolerability indicates the potential clinical use of this compound in the transplantation field.

As described above, FR235222 is a member of cyclic tetrapeptide structural group. However, FR235222 possesses unique structural features which are distinct from the other cyclic peptides in the following points. FR235222 has three unique nonproteinogenic amino acid residues, namely a methylproline residue, an isovaline residue and an intriguing amino acid (2-amino-9-hydroxy-8-oxodecanoic acid) residue which contains a hydroxyketone element. Its methylproline residue is a quite novel structure, because all the other known cyclic peptides have proline or pipecolinic acid residues. And its isovaline residue and hydroxyketone element are both rare, because in all the known cyclic peptides only phoenistatin and TAN-1746 have the former and the latter structures, respectively. We think these structural features might contribute to its exertion of powerful and safe effectiveness in animal models. At least, according to our experiments, its hydroxyketone element was much more stable in the incubation with rat liver S-9 fraction and mouse whole blood at 37°C, compared with the epoxyketone element which the majority of the known compounds have. Indeed, we have evaluated several epoxyketone-containing compounds in in vivo models and certified their limited effects (data not shown). Therefore, we think this hydroxyketone element can give FR235222

<table>
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<th>Treatment</th>
<th>Dose (mg/kg, p.o., b.i.d.)</th>
<th>n</th>
<th>Graft survival time (days)</th>
<th>Median survival time (days)</th>
<th>Body weight gain (g)</th>
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<td>Vehicle</td>
<td>-</td>
<td>11</td>
<td>6, 7, 8, 9, 9, 9, 9, 10, 11, 12, 16</td>
<td>9</td>
<td>26.4 ± 3.0</td>
</tr>
<tr>
<td>FR235222</td>
<td>5</td>
<td>13</td>
<td>7, 7, 9, (13), 22, &gt;100, &gt;100, &gt;100, &gt;100, &gt;100</td>
<td>&gt;100</td>
<td>25.5 ± 2.2</td>
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Table 1. Effect of FR235222 on heterotopic heart transplantation in rats.

*Vehicle or FR235222 was administered orally twice a day for 14 consecutive days, beginning at the next day of transplantation.

b Number of rats per group.

c Body weight gain was expressed as the increment of body weight from day 0 to day 14 (mean±S.E.).

d Died with active graft.

e P<0.05 as compared with vehicle-treated group (Mann-Whitney's U-test).
the powerful efficacy in vivo, especially its oral bioavailability.

At least 17 HDAC isozymes have been identified in humans\(^\text{39}\) so far, and it is strongly suggested that different isozymes may have different functions in different cells, tissues and organs. Although we haven’t ascertained the isozyme selectivity of FR235222 yet, we think its potent and selective immunosuppressive activities in vitro and in vivo might be due to its selectivity to (a) immuno-related isozyme(s), and that its selectivity might be distinct from those of other known HDAC inhibitors. Indeed, Yoshida M. et al. reported that HDAC6, but not HDAC1 or HDAC4, was resistant to trapoxin and cyclic hydroxamic-acid-containing peptide 1 (CHAP1), both of which belong to the cyclic tetrapeptide family, while TSA inhibited these HDAC6 to a similar degree\(^{30}\). This suggests the possibility that structural difference may lead to difference in isozyme selectivity.

As mentioned in the prior paper, some investigators have reported that calcineurin inhibitors (CNIs) are rather less effective or sometimes have negative effects on the prevention of chronic allograft rejection and the induction of tolerance. We haven’t evaluated the effectiveness of FR235222 in chronic rejection models yet. Recently, it has been reported that HDAC inhibitors have inhibitory effects on the expressions of MCP-1\(^{31}\), VEGF\(^{32}\), VEGF receptor\(^{33}\), collagen\(^{35}\) etc., all of which are considered as accelerating factors of chronic rejection\(^{34-36}\). So we think FR235222 may have a beneficial effect on chronic rejection. Moreover, regarding the possibility of tolerance induction by HDAC inhibitors, Kathleen M. Gilbert et al.\(^{37}\) showed potential clinical use of butyrate derivatives to induce antigen-specific T cell inactivation associated with increased levels of p21\(^{\text{CIP1}}\) and p27\(^{\text{KIP1}}\), though they didn’t mention the relationship between tolerance induction and HDAC inhibition. Therefore we have the expectation that FR235222 might have a beneficial effect on tolerance induction. In other words, FR235222 might meet the unmet needs which CNIs cannot meet sufficiently in the transplantation fields.

In conclusion, we have verified the worth of further evaluation of FR235222 as a candidate of new immunotherapeutic agents. We have also ascertained the usefulness of HDAC(s) as (a) novel target(s) in generating new immunosuppressants. Further studies on its isozyme selectivity and its mechanism of immunosuppression at the molecular level are being pursued. The results will be published elsewhere.

### References


