Effect of a New Immunosuppressant Histon Deacetylase (HDAC) Inhibitor FR276457 in a Rat Cardiac Transplant Model

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Histone deacetylase (HDAC) is a known modulator of gene transcription, and the immunosuppressive activity of HDAC inhibitors has been demonstrated in recent several reports. In this study, the HDAC inhibitor FR276457, a hydroxamic derivative, was found to have a similar inhibitory effect on all mammalian HDACs tested, but no isozyme selectivity. Both FR276457 and tacrolimus exerted an immunosuppressive effect on in vitro rat splenocyte proliferation stimulated with Concanavalin A. Next, the effect of FR276457 on allograft rejection when administered either as a monotherapy or in combination with tacrolimus was investigated in a rat heterotopic cardiac transplant model. Orally administered FR276457 prolonged the median survival times (MST) of the transplanted grafts in the vehicle group from 6 d to 17 or 21 d at doses of 20 or 40 mg/kg, respectively. Histopathological analysis showed the structures of the myocardium were not affected, but interstitial cellular infiltration could not be suppressed completely. Tacrolimus (0.032 mg/kg) prolonged allograft MST to 16 d. FR276457, when combined with tacrolimus, prevented allograft rejection at a dose lower than that of the monotherapy. The combination dose prolonged the MST in the groups treated with 10 and 20 mg/kg to >28 d, and cellular infiltration was suppressed completely. In conclusion, this study demonstrated that the oral administration of HDAC inhibitor FR276457 can prevent allograft rejection as a monotherapy, and has additive or synergistic effects when combined with tacrolimus.

Key words histone deacetylase inhibitor; tacrolimus; rat cardiac transplant model; combination therapy

It has been demonstrated that the administration of calcineurin inhibitors (CNIs), such as cyclosporin A (CsA) and tacrolimus (tacrolimus), reduces the rate of acute rejection associated with kidney transplantation and prolongs graft survival. However, clinical use of these inhibitors is limited by side effects such as nephrotoxicity, neurotoxicity, and diabetes.1,2) Furthermore, CNIs are less effective and sometimes might even negatively impact the prevention of chronic allograft rejection and induction of tolerance. For these reasons, new immuno-suppressive drugs are necessary to further improve the outcome of clinical organ transplantation.

Histone deacetylases (HDACs) are known as modulators of gene transcription. Gene transcription is important for proper cell function, proliferation and differentiation.2,3) There have been several reports on the relationship between HDAC inhibition and immunosuppression.4,5) For example, Edens et al. showed that HDAC inhibitors could induce antigen-specific anergy in lymphocytes.6) Furthermore, Tao and Hancock reported that HDAC inhibition promoted the generation and function of regulatory T cells.7) However, to date, only few studies have reported the use of HDAC inhibitors in a solid organ transplant model.

Two major classes of HDAC inhibitors are known, one class is the hydroxamic acid derivatives such as trichostatin A (TSA) or suberoylanilide hydroxamic acid (SAHA), and the other is the butyrates such as phenyl-butylate.8) In this report, the HDAC inhibitor FR276457, a hydroxamic derivative, was isolated and evaluated both as a monotherapy and in combination with tacrolimus in a rat heterotopic cardiac transplant model. FR276457 showed efficacy in preventing allograft rejection. Histological analyses and compound characterization were also performed.

MATERIALS AND METHODS

All animal experimental procedures were approved by the Committee for Animal Experiments of Astellas Pharma Inc.

Compound FR276457 (Fig. 1) was chemically synthesized at Astellas Pharma Inc. (Tokyou, Japan).

Purification of HDACs PEAK rapid cells (1–2×10^6) were transiently transfected with an individual expression plasmid for FLAG-tagged human HDACs (HDAC1, 2, 3, 4, 6), using the calcium phosphate method. The cells were harvested 72 h after transfection and lysed in protein lysis buffer (25 mm Tris–HCl, pH 7.4, 150 mm NaCl, 1% Triton X-100). The FLAG-tagged HDACs were purified with Anti-FLAG M2 agarose conjugated beads (Sigma-Aldrich) and kept at −80 °C before use.

For purification of recombinant HDAC8, a FLAG-tagged human HDAC8 expression plasmid was transfected into the BL21 strain of Escherichia coli. A single colony was then cultured at 37 °C to an A600 of 0.3–0.5 and subsequently induced with 1 mm isopropyl β-D-galactopyranoside. After 6 h growth at 37 °C, the cells were collected and lysed by sonication in protein lysis buffer. The FLAG-tagged HDAC8 was purified with Anti-FLAG M2 agarose conjugated beads and kept at −80 °C before use.

![Fig. 1. Structure of FR276457](image)

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HDAC Activity Assay  HDAC activity was measured using a Cyclex HDAC Assay kit (Cyclex) according to the manufacturer’s instructions. Assays were performed at 30°C (HDAC1, 2, 3, 4, 6) or 15°C (HDAC8) for 1 h (HDAC1, 2, 3, 4, 6, 8). The fluorescence intensity was measured using a microplate (Black Clineplate, Labsystem) in a microplate fluorescence reader (SpectraFluor Plus, Teckan).

Inhibitory Effect on T Cell Proliferation  The spleens of 6- to 8-week-old female Lewis rats (Charles River Japan, Inc.) were removed aseptically and teased into single-cell suspensions. These splenocytes were suspended in RPMI1640 complete medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (referred to as RPMI1640 complete medium). This assay was performed in flat-bottomed microtiter plates, with each well containing 5×10^5 splenocytes. Splenocytes were incubated in medium containing 1 μg/ml Concanavalin A (ConA) and various concentrations of FR276457 and tacrolimus at 37°C for 72 h in a humidified atmosphere of 5% CO₂–95% air. The cells were pulsed with 1 μCi of H3-thymidine/well during the final 6 h, and harvested onto 5% CO₂–95% air. The cells were pulsed with 1 μCi of H3-thymidine/well during the final 6 h, and harvested onto 5% CO₂–95% air.

Calculation of IC₅₀ Values  IC₅₀ values were determined as the means of the concentrations calculated from independent dose–response curves by using logistic regression analysis. Each data point of the dose–response curves was the mean of duplicate assays. All analyses were performed using an SAS system (SAS Institute).

In Vitro Activity of FR276457  Heterotopic cardiac allografts were implanted using the cuff technique. Hearts from 8-week-old male ACI rats (Japan SLC, Inc.) were removed aseptically and teased into single-cell suspensions. These splenocytes were suspended in RPMI1640 complete medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (referred to as RPMI1640 complete medium). This assay was performed in flat-bottomed microtiter plates, with each well containing 5×10^5 splenocytes. Splenocytes were incubated in medium containing 1 μg/ml Concanavalin A (ConA) and various concentrations of FR276457 and tacrolimus at 37°C for 72 h in a humidified atmosphere of 5% CO₂–95% air. The cells were pulsed with 1 μCi of H3-thymidine/well during the final 6 h, and harvested onto 5% CO₂–95% air. The cells were pulsed with 1 μCi of H3-thymidine/well during the final 6 h, and harvested onto 5% CO₂–95% air. The uptake of H3-thymidine by proliferating cells was measured using a liquid scintillation counter.

RESULTS

Inhibitory Activities of FR276457 on HDAC Isozymes

### Table 1. Inhibitory Activities of FR276457 on Various Isozymes of HDAC (IC₅₀ nm)

<table>
<thead>
<tr>
<th>Subclass</th>
<th>Class I</th>
<th>Class II</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC</td>
<td>HDAC 1</td>
<td>HDAC 2</td>
</tr>
<tr>
<td>IC₅₀ (nm)</td>
<td>16.03</td>
<td>45.44</td>
</tr>
</tbody>
</table>

**Fig. 2.** Inhibitory Effect of FR276457 and Tacrolimus on T Cell Proliferation

FR276457 and tacrolimus inhibited splenocyte proliferation stimulated with ConA in a concentration-dependent manner.

### Table 2. Effect of FR276457 on Graft Survival Time in a Rat Heart Transplant Model

<table>
<thead>
<tr>
<th>FR276457 (mg/kg, p.o.)</th>
<th>n</th>
<th>Graft survival time (d)</th>
<th>MST (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>7</td>
<td>5, 5, 5, 6, 6, 7</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>4, 5, 6, 6, 7, 9</td>
<td>6</td>
</tr>
<tr>
<td>20</td>
<td>7</td>
<td>5, 6, 16, 17, 23, 27, &gt;28</td>
<td>17**</td>
</tr>
<tr>
<td>40</td>
<td>6</td>
<td>18, 20, 21, 26, 28, &gt;28</td>
<td>21**</td>
</tr>
</tbody>
</table>

**++p<0.01 vs. vehicle group.**

The HDAC-inhibitory activity of FR276457 was evaluated by using recombinant human HDACs prepared from PEAK rapid cells (HDAC1, 2, 3, 4, 6) or _E. coli_ (HDAC8). The IC₅₀ values were shown as the means (n=3). FR276457 showed no isozyme selectivity, and had almost the same inhibitory effect on the activity of mammalian HDACs (Table 1).

In Vitro Activity of FR276457  FR276457 inhibited the proliferation of splenocytes stimulated with ConA in a concentration-dependent manner with an IC₅₀ value of 31.6 nm (Fig. 2). That of tacrolimus was 1.58 nM (Fig. 2).

Monotherapy  The ability of orally administered FR276457 to prevent allograft rejection was assessed in a rat heterotopic cardiac transplant model. In this model, vehicle-treated allografts were rejected within 7 d after transplantation (Table 2). Although 10 mg/kg of FR276457 had no effect on the prolongation of MST (6 d), a daily dose of 20 or 40 mg/kg produced MST of 17 or 21 d, respectively (Table 2).

Combination Therapy with Tacrolimus  To determine the efficacy of orally administered FR276457 in combination with tacrolimus, its effect was evaluated when administered intramuscularly in combination with tacrolimus 0.032 mg/kg. This dose of tacrolimus was considered to be subtherapeutic,
**Table 3. Effect of Combination Therapy with ER276457 and Tacrolimus on Graft Survival Time in Rat Heart Transplant Model**

<table>
<thead>
<tr>
<th>FR276457 (mg/kg, p.o.)</th>
<th>n</th>
<th>Graft survival time (d)</th>
<th>MST (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle 7</td>
<td>7</td>
<td>10, 12, 13, 16, 17, 17</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>11, 13, 16, 20, &gt;28</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>26, &gt;28, &gt;28, &gt;28, &gt;28</td>
<td>&gt;28**</td>
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<tr>
<td>20</td>
<td>7</td>
<td>24, 26, &gt;28, &gt;28, &gt;28</td>
<td>&gt;28**</td>
</tr>
</tbody>
</table>

**+** p < 0.01 vs. vehicle group.

In the allograft treated with 40 mg/kg of FR276457 (the effective dose), the degree of interstitial cell infiltration was not acceptable (Fig. 3C). In the isograft, the myocardium was intact, and no cell infiltration was observed (Fig. 3A). In the allograft treated with 40 mg/kg of FR276457 (the effective dose), the degree of interstitial cell infiltration was not acceptable (Fig. 3C). In the allograft treated with 40 mg/kg of FR276457 (the effective dose), the degree of interstitial cell infiltration was not acceptable (Fig. 3C). In the allograft treated with 40 mg/kg of FR276457 (the effective dose), the degree of interstitial cell infiltration was not acceptable (Fig. 3C).

**DISCUSSION**

In this report, the novel HDAC inhibitor FR276457 effectively prevented allograft rejection in a rat cardiac transplant model. This compound had strong efficacy as a monotherapy, and also exerted additive or synergistic effects in combination therapy with tacrolimus.

To date, multiple forms of HDACs have been identified in mammalian cells. In humans, at least 11 HDACs have been reported, which are divided into class I (HDAC1, 2, 3, 8, 11) and class II (HDAC 4, 5, 6, 7, 9, 10).9—11) The fact that several HDACs have been identified in humans suggest that they might play distinct roles in cellular functioning; however, their specific roles and the functional differences between them still remain to be elucidated. FR276457 was examined to determine whether it possesses isozyme-selective inhibitory activity (HDAC 1, 2, 3, 4, 6, 8). No isozyme selectivity was observed, and almost the same inhibitory effect was exerted on the activity of all mammalian HDACs tested.

Histone deacetylases (HDACs) regulate the addition and removal of acetyl groups on specific lysine residues in proteins and maintain a dynamic balance of steady-state protein acetylation. In previous studies, it was shown that HDAC inhibitors induced protein hyperacetylation and caused chromatin remodeling, transcriptional activation and repression, cell-cycle arrest, cell differentiation, and cell death.12—17) In recent years, the relationship between HDAC inhibition and immunosuppression has been reported.4,5) These data suggest that HDAC inhibitors may be a potential therapeutic agent in the fields of autoimmune disease and organ transplantation. First, the immunosuppressive effect of FR276457 was examined in a ConA-stimulated assay. FR276457 as well as tacrolimus inhibited T cell proliferation dose-dependently and showed immunosuppressive effects in vitro.

There have been few reports on the efficiency of HDAC inhibitors during organ transplantation. However, Böhning et al. did report that n-butyric acid prolonged the MST a little for Brown Norway-to-Lewis rat cardiac transplantations.18) Mori et al. also have reported that the HDAC inhibitor FR235222 exerted marked immunosuppressive effects on Lewis-to-ACI rat heterotopic cardiac transplantation.19) In this study, the ability of FR276457 to prevent allograft rejection was examined in an ACI-to-Lewis rat heterotopic cardiac transplant model which is a severe transplant model. In an orally administered monotherapy, FR276457 prolonged graft survival time at doses of at least 20 mg/kg. This shows that FR276457 has a strong immune-suppressive effect as FR23522 does. Furthermore, histological analyses of samples 5 d after transplantation showed that FR276457 had not disrupted the myocardium. However, contrary to expectations, cell infiltration was not suppressed. It has been reported that HDAC inhibitors induce antigen-specific anergy in lymphocytes.6,20,21) Taking this into consideration, it is possible that the infl-
treated cells in these allografts might be anergic or lacking in cytotoxic activity. In addition, it has also been reported that HDAC inhibitors can induce regulatory T cell. Further examination of these cells is necessary to determine the exact mechanisms behind these phenomena.

FR276457 is effective when combined with tacrolimus because calcineurin inhibitors (CNIs), tacrolimus and cyclosporin A are strong preventers of allograft rejection, but their clinical usage is limited by their side effects. However, combining CNIs with FR276457 seems to significantly reduce the side effects in the clinical setting, which suggests additive or synergistic effects. Combination therapy with doses of at least 10 mg/kg FR276457 prolonged graft survival time; however, no efficacy was apparent when given as a monotherapy. Allograft histopathology also improved with combination therapy; interstitial cell infiltration was suppressed almost completely.

It has been shown that tacrolimus inhibits Ca2+/calmodulin-regulated phosphatase calcineurin by forming a complex with the immunophilin FK506-binding protein 12 (FKBP12). In addition, inhibition of calcineurin blocks dephosphorylation of the transcription factor NF-AT in the cytoplasm, which is required for nuclear translocation and the NF-AT-regulated expression of cytokine genes such as IL-2. The differences between the mechanisms of FR276457 and tacrolimus might be the reason that additive or synergistic effects are seen when the two are coadministered. This combination is expected to be applicable in the clinical setting.

In conclusion, FR276457 was found to be a suitable candidate for use as a new immunosuppressant. Its efficacy was determined not only as a monotherapy, but also in combination with tacrolimus in a rat heterotopic cardiac transplant model. The results suggest that HDAC inhibitors have strong efficacy in the field of organ transplantation.

REFERENCES