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FR177391, A New Anti-hyperlipidemic Agent from *Serratia*

II. Pharmacological Activity of FR177391

Masamichi Inami†, Ikuo Kawamura†, Susumu Tsujimoto†, Tohru Yasuno†, Elizabeth Lacey†, Jiro Hirosumi†, Shoji Takakura†, Fusako Nishigaki†, Yoshinori Naoe†, Toshitaka Manda†, Seitaro Mutoh†

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Abstract The pharmacological effect of FR177391, isolated from *Serratia liquefaciens* No. 1821, was studied in normal animals and various types of animal models of hypertriglyceridemia. Treatment of normal mice with FR177391 resulted in an increase in heparin-releasable lipoprotein lipase (LPL) activity in the blood and epididymal fat tissue. FR177391 treatment decreased triglyceride (TG) and increased high-density lipoprotein cholesterol in the blood in normal rats following 7 days treatment, suggesting potent LPL activating properties of FR177391. Both Triton WR1339-induced severe and fructose-induced mild hypertriglyceridemia in rats were attenuated by FR177391 treatment. Severely elevated levels of TG in db/db mice, an insulin resistant diabetic animal model, also significantly decreased from 14 days of treatment with FR177391. FR177391 treatment for 9 days caused a decrease in the elevated levels of TG in mice induced by intraperitoneal inoculation of murine lymphoma EL-4. Overall, this study demonstrated that FR177391 can be possibly a LPL activating agent and that FR177391 treatment improved hypertriglyceridemia in various rat and mouse animal models. These results suggest that FR177391 is a promising candidate compound for the management of hypertriglyceridemia.

Keywords FR177391, lipoprotein lipase activator, hypertriglyceridemia

Introduction

In our screening program for anit-hyperlipidemic agents from microbial products, we found that *Serratia liquefaciens*. No. 1821 produced a compound, FR177391, that alleviated the decrease in lipid droplet formation in differentiated 3T3-L1 adipocyte cells, induced by the addition of tumor necrosis factor-α (TNF-α) [1]. FR177391 has a chemical structure as shown in Fig. 1. This result suggested that FR177391 possesses potent LPL activating activity, since the lipid droplet formation is thought to be associated with development of LPL activity in 3T3-L1 adipocyte cells [2]. Recently, a low molecular weight novel compound named after NO-1886 has been reported to be a potent LPL activator, and also effective in experimental atherosclerosis in rats and rabbits [3～7]. In this study, we investigated the LPL activating activity of FR177391 *in vivo*. We also evaluated the TG lowering activity of FR177391 in various animal models of hypertriglyceridemia, and compared them with those of NO-1886.

M. Inami (Correspondence author)**, I. Kawamura, S. Tsujimoto, T. Yasuno, E. Lacey, J. Hirosumi, S. Takakura, F. Nishigaki, Y. Naoe, T. Manda, S. Mutoh:** Medicinal Biology Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 2-1-6, Kashima, Yodogawa-ku, Osaka 532-8514, Japan.

E-mail: masamichi.inami@jp.astellas.com

† Present address: Astellas Pharma Inc., 2-1-6, Kashima, Yodogawa-ku, Osaka 532-8514, Japan

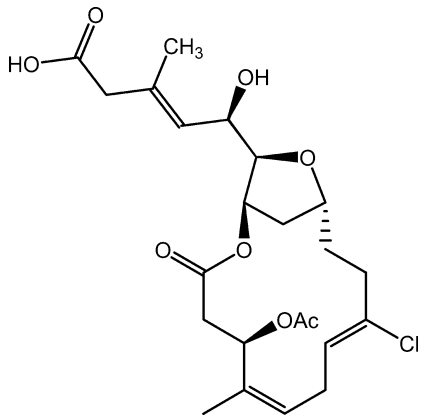

Fig. 1 Chemical structure of FR177391.

Materials and Methods

Materials

FR177391 was prepared at our Research Laboratories. NO-1886 was synthesized at Fujisawa Pharmaceutical Co., Ltd. Bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM) and Triton WR1339 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fetal calf serum (FCS) was from GIBCO Laboratories (Grand Island, NY, U.S.A.). Heparin solution was from Shimizu Pharmaceutical Co., Ltd. (Shimizu, Japan). Tri-olein, lecithin from egg yolk, glycerol, LPL from *Pseudomonas* sp. and fructose were from Nacalai Tesque (Kyoto, Japan). FR177391 was dissolved in saline and administered orally to mice at a volume of 10 ml/kg of body weight, or to rats at a volume of 5 ml/kg of body weight. NO-1886 was suspended in 0.5% methylcellulose (MC) in water and administered orally to rats at a volume of 10 ml/kg of body weight, or to rats at a volume of 5 ml/kg of body weight. All other reagents were of the highest analytical grade commercially available.

Animals

All experimental procedures in this study were approved by our institutional Animal Care Committee. Specific pathogen-free male BALB/c and C57BL/6 mice were purchased from Charles River Japan Inc. (Kanagawa, Japan). Specific pathogen-free female C57BL/KsJ-+m/+m and C57BL/KsJ-db/db (db/db) mice were from CLEA Japan Inc. (Tokyo, Japan). Specific pathogen-free male Sprague-Dawley (SD) rats were from Japan SLC, Inc. (Shizuoka, Japan). The animals were kept in constant temperature and humidity conditions and fed a standard diet and water *ad libitum*.

Activation of Plasma LPL Activity in Mice

Compound was administered orally to BALB/c mice (8 weeks old) once daily for 3 days. Twenty four hours after the final compound treatment, mice were injected with heparin (300 units/ml, 0.2 ml/mice) *via* the tail vein. Blood was collected 15 minutes later under diethyl ether anesthesia. Plasma was then obtained by centrifugation and kept frozen at −80°C until analyzed.

The plasma heparin-releasable LPL activity was calculated by subtraction of NaCl-inactivated lipase activity from total lipase activity, as LPL is known to be sensitive and inactivated by NaCl [8]. The LPL activity in plasma was measured by a modification of the procedure of Nilsson-Ehle and Schotz [8]. Briefly, 1 g of tri-olein and 60 mg of lecithin (in chloroform) were mixed well. The solvent was evaporated off under nitrogen gas. The dried mixture was emulsified in 10 ml of glycerol by homogenization continuously for 5 minutes at 4°C. The emulsion was stored at 4°C as concentrated substrate. To prepare the substrate solution, 1 ml of concentrated substrate was diluted with 4 ml of 200 mM Tris-HCl buffer containing 3% BSA (w/v) (pH 8.0) and 1 ml of FCS, then shaken vigorously on a vortex mixer for 5 seconds. The substrate for assay was diluted immediately before use. Twenty μl of diluted substrate, 80 μl of sample or standard solution and 50 μl of 200 mM Tris-HCl buffer containing 3% FCS (pH 8.0) were incubated with or without 50 μl of 4 M NaCl solution at 37°C for 90 minutes to hydrolyze the substrate tri-olein. After incubation, the amount of released linoleic acid was measured using a non-esterified fatty acid (NEFA)-C TEST (Wako Pure Chemical Industries, Ltd., Osaka, Japan). LPL derived from *Pseudomonas* sp. was used as a standard.

Activation of LPL Activity in Epididymal Fat Tissue of Mice

Compound was administered orally to BALB/c mice (8 weeks old) once daily for 3 days. Twenty four hours after the final compound treatment, mice were anesthetized with diethyl ether, and the epididymal fat was removed, excised and incubated in DMEM medium containing 2% BSA and 10 units/ml heparin at 25°C for 2.5 hours (100 mg of fat tissue/0.1 ml of medium). Heparin-releasable LPL activity in supernatant was measured by the methods described above.

Effect on Blood Parameters in Normal Rats

Compound was given orally to SD rats (9 weeks old) once daily for 7 days. Four hours after the final compound treatment, rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneally), and blood was collected.

Serum was then harvested following the clotting of blood at room temperature for 1 hour. Serum was kept frozen at −80°C until analyzed. The levels of TG, total cholesterol and glucose were measured by Chemical Analyzer Model TBA-80FR (Toshiba). High-density lipoprotein (HDL) cholesterol levels in the serum were also measured using a HDL-cholesterol-test Wako kit (Wako Pure Chemical Industries, Ltd.).

Effect on Blood TG Levels in Triton WR1339-treated Rats

Triton WR1339 at a dose of 250 mg/kg was administered intravenously to SD rats (7 weeks of age). Compound was given orally to the rats twice, 30 minutes before and 20 hours after Triton WR1339 administration. Twenty four hours after the second compound treatment, rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneally) and blood was collected. Serum was then harvested following the clotting of blood at room temperature for 1 hour. Serum was kept frozen at −80°C until analyzed. The level of TG was measured using a commercial kit from Wako Pure Chemical Industries, Ltd.

Effect on Blood TG Levels in Fructose-fed Rats

At 7 weeks of age, SD rats were given a 20% fructose solution for 3 weeks. Control rats were given tap water to drink throughout the experimental period. Following the 2-week period of fructose treatment, compound was given orally to the rats once daily for 7 days. Two hours after the final compound treatment, rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneally) and blood was collected. Serum was then harvested following the clotting of blood at room temperature for 1 hour. Serum was kept frozen at −80°C until analyzed. The level of TG was measured using a commercial kit from Wako Pure Chemical Industries, Ltd.

Effect on Blood TG and Glucose Levels in db/db Mice

Compound was given orally to db/db mice, at 8 weeks of age, once daily for 14 days. One day after the final compound treatment, mice were anesthetized with diethyl ether and blood was obtained by capillary pipette from the retroorbital sinus. Heparin was used as an anti-coagulant. Plasma was then harvested and kept frozen at −80°C until analyzed. The levels of TG and glucose were determined using a commercial kit from Wako Pure Chemical Industries, Ltd.

Effect on Blood TG and NEFA Levels in EL-4 Lymphoma Bearing Mice

A cell suspension of EL-4 lymphoma (1.0×10^6 cells in 0.1 ml of buffered saline) was inoculated intraperitoneally into C57BL/6 mice, at 8 weeks of age. From the following day, compound was given orally once daily for 9 days. Four hours after the final compound treatment, mice were anesthetized with diethyl ether and blood was collected by cardiac puncture. Serum was harvested following the clotting of blood at room temperature for 1 hour. Serum was kept frozen at −80°C until analyzed. The levels of TG and NEFA were measured using a commercial kit from Wako Pure Chemical Industries, Ltd.

Statistical Analysis

All values are expressed as the mean±S.E. Analysis of variance was performed and the Student's *t*-test or Dunnett test was used to determine the significance of differences. A p value of 0.05 or less was considered significant.

Results

Effects of FR177391 and NO-1886 on Serum and Epididymal Fat Tissue LPL Activity in Normal Mice

FR177391 (0.032, 0.1 and 0.32 mg/kg) or NO-1886 (3.2 and 32 mg/kg) was orally administered to normal mice for 3 days, and serum heparin-releasable LPL activity was measured. FR177391 treatment resulted in a dose-dependent increase in serum LPL activity (Fig. 2A) and a significant increase in heparin-releasable serum LPL activity was observed in the 0.32 mg/kg FR177391-treated mice. NO-1886 at a dose of 32 mg/kg also caused a significant increase in serum LPL activity.

Figure 2B shows the LPL activity in epididymal fat tissue of mice after 3 days compound treatment. Both compounds showed a tendency to increase LPL activity, but neither effect was statistically significant. Thus, the potency of increase in serum and fat tissue LPL activity by FR177391 and NO-1886 was equally potent, but the effective dose of FR177391 was about 100-fold less than that of NO-1886.

Effects of FR177391 and NO-1886 on Blood Parameters in Normal Rats

FR177391 (0.1 and 0.32 mg/kg) or NO-1886 (10 and 32 mg/kg) was orally administered to normal rats for 7 days and blood parameters were measured. Both compounds showed a decrease in blood TG levels (Table 1) and the activity was almost the same. Significant and dose-dependent increase in blood levels of total cholesterol was observed in rats treated with FR177391 and NO-1886. Subsequently, in order to determine the mechanism of an increase in the circulating total cholesterol levels by both

compounds, HDL cholesterol levels were measured. As shown in Table 1, treatment with both compounds resulted in an increase in blood levels of HDL cholesterol. These results suggest that FR177391, like NO-1886, has the ability to increase HDL cholesterol, leading to an increase in total cholesterol in the blood. The blood glucose levels were not changed by either compound.

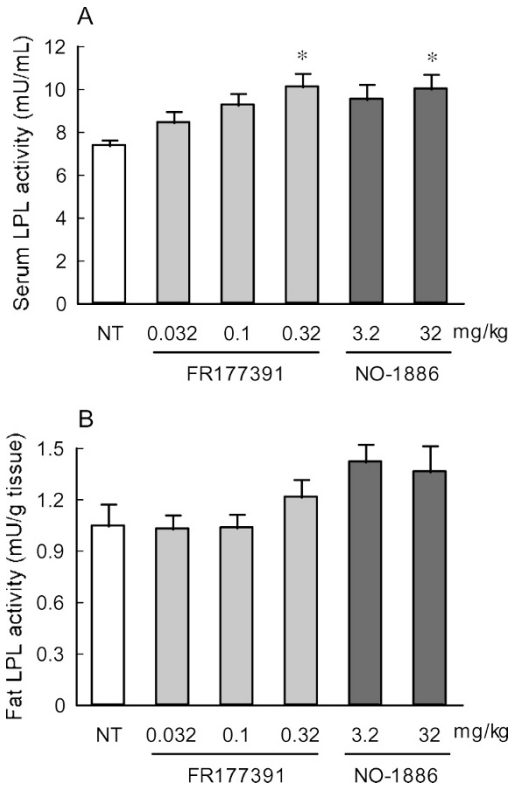

Fig. 2 Effects of FR177391 and NO-1886 on serum (A) and fat (B) LPL activity of normal mice.

Each column represents mean±S.E. of 5 mice. NT; non-treated. *: $p<0.05$, compared with NT mice (Dunnett test).

Effects of FR177391 and NO-1886 on Blood TG Levels in Triton WR1339-treated Rats

Intravenous administration of nonionic detergent, Triton WR1339 to experimental animals has been reported to cause a marked increase in the concentration of TG in the blood [9, 10]. It is generally assumed that this effect is the result of the inability of the LPL to hydrolyze the plasma triacylglycerols such as chylomicrons and VLDL in the presence of Triton WR1339 [9]. In addition, there is evidence that Triton WR1339 inhibits lipolysis due to coating of the surface of lipoproteins, leading to inhibition of interaction of the substrate and enzyme [9, 10]. Thus, in this study, we investigated whether or not FR177391 can attenuate the Triton WR1339-induced hypertriglyceridemia in rats due to its LPL activating activity. Triton WR1339 treatment caused a marked elevation in blood TG levels in rats (Fig. 3). However, when FR177391 at doses of 0.1 and 0.32 mg/kg was administered to rats twice, 30 minutes before and 20 hour after the Triton WR1339 treatment, this TG increase was attenuated, although the attenuation by FR177391 was not statistically significant. Treatment with NO-1886 at a dose of 32 mg/kg resulted in significant decrease in blood levels of TG in rats. Thus, these results suggest that FR177391 shows TG lowering activity in Triton WR1339-treated rats, although the potency is less than that of NO-1886.

Effects of FR177391 and NO-1886 on Blood TG Levels in Fructose-fed Rats

Fructose has been reported to induce hypertriglyceridemia associated with insulin resistance, hyperinsulinemia and hypertension [11～13]. Recent investigation showed that hypertriglyceridemia induced by fructose feeding is due to

Table 1 Effects of FR177391 and NO-1886 on blood parameters in normal rats

Treatment	Triglyceride (mg/dl)	Glucose (mg/dl)	Total cholesterol (mg/dl)	HDL cholesterol (mg/dl)
Saline	89.8±9.4	206.4±13.7	53.8±0.3	28.4±2.5
FR177391: 0.1 mg/kg	54.7±7.3*	193.3±4.6	64.9±4.6	38.0±2.4*
FR177391: 0.32 mg/kg	52.9±6.0*	186.9±7.4	68.5±4.6*	35.8±2.6
0.5% MC	87.3±15.6	205.5±15.2	51.9±3.0	38.2±2.3
NO-1886: 10 mg/kg	61.4±7.2	214.6±16.3	58.1±2.4	43.3±2.9
NO-1886: 32 mg/kg	53.8±6.1	182.2±11.6	68.5±3.1##	53.6±2.8##

Normal rats were orally treated with compound once daily for 7 days. Four hours after the final compound treatment, blood was collected and the blood parameters were measured. Rats were used in groups of 10. Values are shown as mean±S.E. *: $p<0.05$, compared with saline-treated rats (Dunnett test). ##: $p<0.01$, compared with 0.5% MC-treated rats (Dunnett test).

direct stimulation of hepatic VLDL-TG secretion [14]. In this experiment, the effect of FR177391 on fructose-induced hypertriglyceridemia was studied in rats. The rats given fructose solution for 3 weeks showed a mild elevation in blood TG levels, as shown in Fig. 4. When FR177391 (0.1 and 0.32 mg/kg) was orally administered to fructose-fed rats for 7 days, the blood levels of TG significantly decreased to the levels of normal rats untreated with fructose. NO-1886 treatment also caused a decrease in TG levels in the blood. These results show that FR177391, like NO-1886, has the ability to ameliorate the fructose-induced mild hypertriglyceridemia in rats.

Effect of FR177391 on Blood TG and Glucose Levels in db/db Mice

In order to determine the ameliorative effect of FR177391 on hypertriglyceridemia associated with insulin resistance, FR177391 at doses of 0.1 and 0.32 mg/kg was given orally to db/db mice, an insulin resistant diabetic animal model [15], once daily for 14 days and blood parameters were measured. As shown in Fig. 5, db/db mice had elevated levels of TG and glucose in the blood. FR177391 (0.1 and 0.32 mg/kg) treatment resulted in a dose-dependent and significant decrease in TG levels of db/db mice. Blood glucose levels in db/db mice treated with FR177391 were not different from vehicle-treated db/db mice.

Effect of FR177391 on Blood TG and NEFA Levels in Mice Bearing EL-4 Lymphoma

Recently, we found that the inoculation of EL-4 into mice caused severely elevated blood levels of TG and NEFA, associated with impaired LPL activity in the blood and cachectic symptoms [16]. Therefore, we investigated whether FR177391 could attenuate the increased levels of

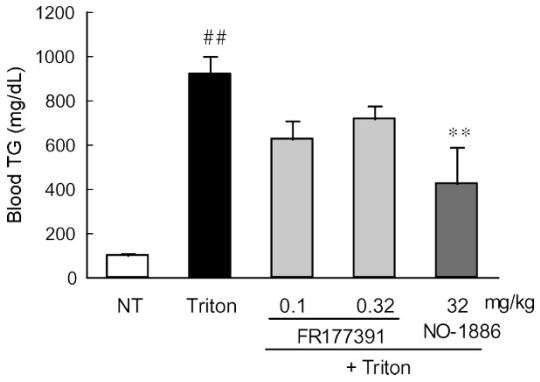

Fig. 3 Effects of FR177391 and NO-1886 on blood TG levels in Triton WR1339-treated rats.

Each column represents mean±S.E. of 10 rats. NT; non-treated, Triton; Triton WR1339-treated. ##: $p<0.01$, compared with NT rats (Student's *t*-test). **: $p<0.01$, compared with Triton WR1339-treated rats (Dunnett test).

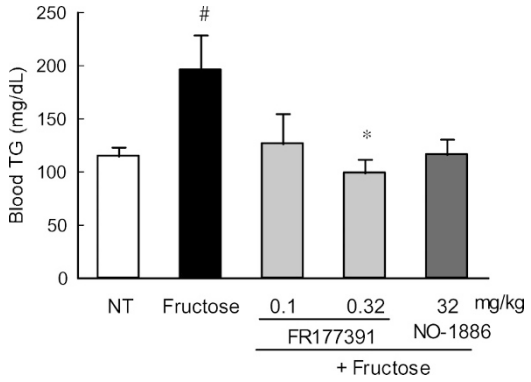

Fig. 4 Effects of FR177391 and NO-1886 on blood TG levels in fructose-fed rats.

Each column represents mean±S.E. of 10 rats. NT; non-treated, Fructose; 20% fructose solution-fed. #: $p<0.05$, compared with NT rats (Student's *t*-test). *: $p<0.05$, compared with fructose-fed rats (Dunnett test).

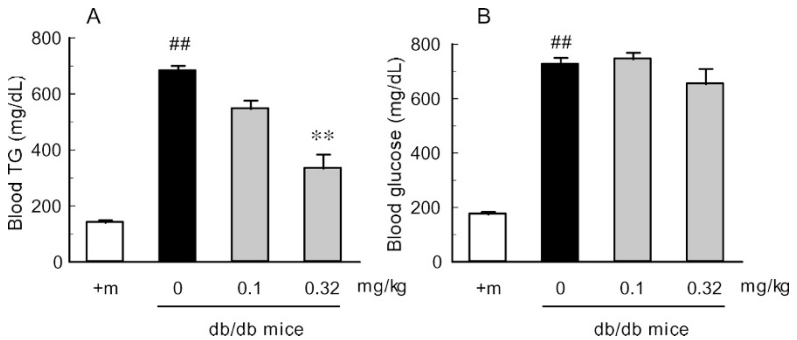

Fig. 5 Effect of FR177391 on blood TG (A) and glucose (B) levels in db/db mice.

Each column represents mean±S.E. of 10 mice. +m; +m/+m mice. ##: $p<0.01$, compared with +m/+m mice (Student's *t*-test). **: $p<0.01$, compared with vehicle-treated db/db mice (Dunnett test).

TG and NEFA in EL-4 lymphoma bearing mice, following 9 days of oral treatment. As shown in Fig. 6, the EL-4 bearing mice had elevated levels of TG and NEFA in the blood. FR177391 (0.1, 0.32 and 0.56 mg/kg) treatment resulted in a dose-dependent decrease in the serum TG and NEFA levels.

Discussion

FR177391 is a compound isolated from microbial products in our screening program for LPL activating agents [1]. As the addition of FR177391 to differentiated 3T3-L1 adipocyte cells resulted in alleviating the decrease in lipid droplet formation induced by TNF-α [1], FR177391 appeared to have the ability to enhance LPL activity. Therefore, the principal objectives of this investigation were to study the LPL activating activity of FR177391 *in vivo*, and to further examine the pharmacological activity of FR177391 in various animal models of hypertriglyceridemia.

Our data demonstrate that FR177391, like NO-1886, increases the LPL activities of blood heparin-releasable and epididymal fat tissue in normal mice following 3 days treatment, and decreases the blood levels of TG in normal rats, following 7 days of treatment (Fig. 2 and Table 1). The potency of LPL activating and TG lowering effects by FR177391 was almost equal to NO-1886, but its effective dose was less than 100-fold that of NO-1886. Recent studies by Tsutsumi *et al.* have revealed that NO-1886 increases LPL expression in adipose tissue and increases LPL activity in post-heparin plasma in rats at doses of 25 and 50 mg/kg [3, 4]. In the present study, NO-1886 significantly enhanced LPL activities of both blood and fat tissue in mice and decreased the circulating TG levels in normal rats at a dose of 32 mg/kg. Thus, the present results concerning NO-1886 are consistent with previous studies [3, 4]. As shown in Table 1, FR177391-treated normal rats had elevated levels of HDL and total cholesterols, associated with reduced TG levels in the blood. It is recognized that following the hydrolysis of the core TG by LPL in VLDL, the VLDL shrink and surface components, mainly phospholipids, are transferred to HDL [17, 18]. Recent investigation showed that in LPL knockout mice surviving up to 18 hours after birth, severe hypertriglyceridemia was accompanied by low levels of plasma HDL [19], and conversely, overexpression of human LPL in mice drastically lowered VLDL and increased HDL [20]. Therefore, the increased HDL cholesterol observed in FR177391-treated rats was thought to be due to LPL activation by FR177391. These results suggest the possibility that FR177391, like NO-1886, is a LPL activating agent *in vivo*, and also that FR177391 has the ability to lower circulating TG in normal animals.

Subsequently, we evaluated the TG lowering activity of FR177391 in 4 different types of animal models, in order to determine whether FR177391 is effective in the management of hypertriglyceridemia. FR177391 treatment caused a tendency to reduce Triton WR1339-induced severely elevated TG levels in the blood (Fig. 3). Fructose-induced mild hypertriglyceridemia was almost completely inhibited by FR177391, following 7 days of treatment (Fig. 4). This result is consistent with the recent observation that NO-1886, a potent LPL activator, reduces circulating TG levels without causing increased muscle TG accumulation or deterioration in glucose tolerance in a fructose-fed rat model of hypertriglyceridemia and insulin resistance [21]. In addition, as shown in Fig. 5, severe hypertriglyceridemia in db/db mouse, an insulin resistant diabetic animal model, was dose-dependently attenuated by FR177391, following 14 days of treatment. However, the circulating levels of glucose in this mouse model were not affected, suggesting

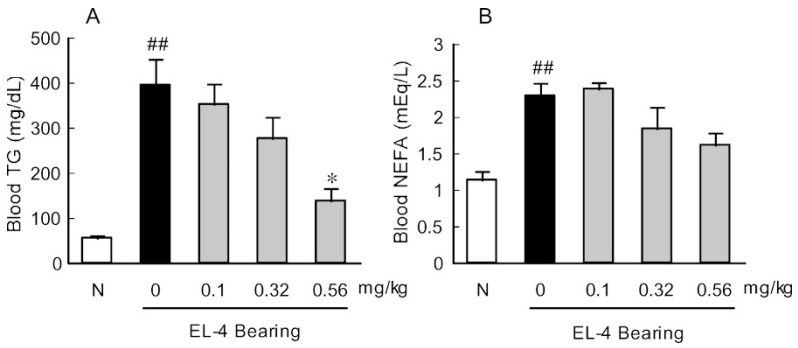

Fig. 6 Effect of FR177391 on blood TG (A) and NEFA (B) levels in EL-4 bearing mice.

Each column represents mean±S.E. of 9 mice. N; normal mice. ##: $p<0.01$, compared with normal mice (Student's *t*-test). *: $p<0.05$, compared with vehicle-treated EL-4 bearing mice (Dunnett test).

that the TG lowering effect of FR177391 may not be derived from improvement of insulin resistance but due to LPL activation.

In the last experiment of this study, we evaluated the effect of FR177391 on murine lymphoma EL-4 induced severe hypertriglyceridemia in mice, as we recently found that the inoculation of EL-4 into mice caused severely elevated blood levels of TG and NEFA associated with cachectic symptoms [16]. The severely elevated levels of TG and NEFA in the blood were significantly attenuated by FR177391, following 9 days of treatment (Fig. 6). Thus, these results indicate that FR177391 treatment resulted in an amelioration against hypertriglyceridemia in different types of animal models, suggesting that FR177391 is a potential candidate compound for the management of hypertriglyceridemia.

Elevated plasma TG levels are increasingly recognized as a risk factor for cardiovascular disease, and also commonly embedded in the context of metabolic syndromes, including obesity, diabetes and hypertension [22, 23]. Several classes of pharmacological agents aiming at improving hypertriglyceridemia have been developed. Recent clinical evidence demonstrates that nicotinic acid, fibrates and statins are effective in lowering TG levels by approximately 20% to 40% [24～26]. However, the benefit of TG lowering by these drugs on the incidence of cardiovascular events has not been fully demonstrated. Therefore, the development of novel agents that have different mechanisms of action from these drugs and show more powerful TG lowering activity alone or combined with these drugs is strongly sought. It is possible that FR177391, a potent LPL activating agent, may be an interesting candidate compound that has the ability to produce clinical benefits for patients with hypertriglyceridemia, either alone or combined with statins or fibrates. Further pre-clinical and clinical investigations are required to verify the benefit of this compound.

In conclusion, the present study demonstrated that FR177391 can be possibly a LPL activating agent and also that FR177391 treatment improved hypertriglyceridemia in various rat and mouse animal models. These results suggest that FR177391 is a promising candidate compound for the management of hypertriglyceridemia.

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