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FR171456, a Novel Cholesterol Synthesis Inhibitor Produced by *Sporormiella minima* No. 15604

II. Biological Activities

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Novel cholesterol synthesis inhibitors FR171456 and FR173945 were isolated from the culture broth of *Sporormiella minima* No. 15604. FR171456 strongly inhibited the cholesterol synthesis and up-regulated the LDL-receptor expression in human hepatoma cell line Hep G2. Single oral administration of FR171456 inhibited *in vivo* hepatic sterol synthesis in rats. And FR171456 shows a significant serum cholesterol-lowering effect in a cholesterol fed rabbit model.

In the previous paper[1], we reported novel and potent cholesterol synthesis inhibitors FR171456 (**1**) and FR173945 (**2**) (Fig. 1) produced by *Sporormiella minima* No. 15604. In Hep G2 cells, compounds **1** and **2** strongly inhibited cholesterol biosynthesis from mevalonic acid, which is the product of 3-hydroxy-3-methykglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34). They have IC_{50} values of 4.2 and 8.3 ng/ml, respectively. These compounds also inhibited cholesterol synthesis from acetic acid or farnesyl-diphosphate. Hence the inhibition point or target enzyme of **1** and **2** in the sterol synthesis pathway is thought to be later than the squalene synthetase catalized reaction. Squalene synthetase (farnesyl-diphosphate : farnesyl-diphosphate farnesyl transferase, EC 2.5.1.21) catalyses reductive dimerization of farnesyl-diphosphate to form squalene and is a first enzyme in the committed cholesterol synthesis pathway[2]. HMG-CoA reductase inhibitors could in principle suppress all post mevalonic acid biosynthetic steps and compromise supplies of biologically important non-steroidal isoprenoids[3,4]. Therefore, we have been focused on inhibitors of committed sterol biosynthesis as potential targets of lipid lowering agents. Here we show that FR171456 (**1**), a potent inhibitor of sterol synthesis, up-regulates LDL receptor *in vitro* and has a lipid lowering effect *in vivo* in a cholesterol fed rabbit model.

Materials and Methods

^{125}I-LDL Binding Assay

Hep G2 cells were cultured as described previously[1]. Human LDL (purchased from Sigma, USA) was used for ^{125}I iodination by the iodine monochloride method as described by Fielding *et al.*[5]. Hep G2 cells in 24-well culture plate were incubated with or without test samples for 48 hours at 37°C in Eagle's modified minimum essential medium (MEM) supplemented with 10% human lipoprotein deficient serum. After incubation, the cells were washed three times with MEM containing 10 mM HEPES

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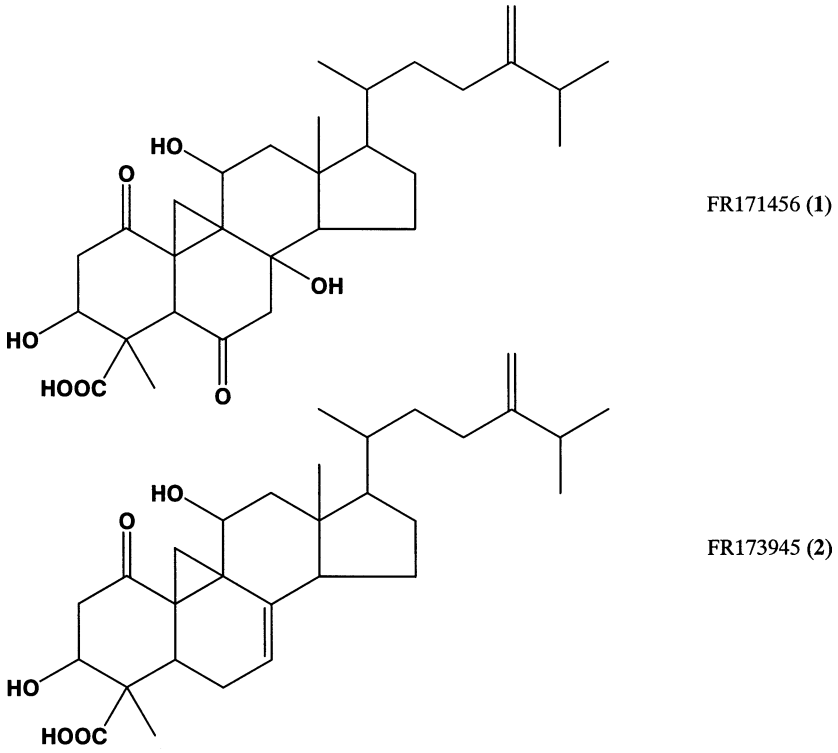
Fig. 1. Structures of FR171456 (**1**) and FR173945 (**2**).



(pH 7.4), 10 mg/ml BSA (binding medium) at 4°C. Binding medium containing ^{125}I-LDL (2 μg) was added to the culture and incubated at 4°C for 2 hours. The cells were washed three times with 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 1 mg/ml BSA, and solubilized in 0.5 N NaOH. Total radioactivity was counted with gamma counter. Non-specific binding was measured by addition of 50-fold excess of non-radiolabeled LDL. Data are expressed as the average of duplicates.

Assay of *In Vivo* Sterol Synthesis in Rats

Compound **1** was administrated orally to male Sprague-Dawley rats. After 50 minutes, [1-^{14}C] sodium acetate was injected intraperitoneally. Fifty minutes after receiving the [^{14}C] acetate, blood (3 ml) was collected by heart puncture and plasma was separated. One ml of plasma was saponified in 15% KOH in 95% EtOH at 80°C for 2 hours. Non-saponifiable substances were extracted with *n*-hexane, and digitonin precipitable [^{14}C] sterols were measured through liquid scintillation counting. Data from this experiment were statistically analyzed by Student's *t*-test. (Values were expressed as mean±S.D.)

In Vivo Serum Cholesterol-lowering Effects in Cholesterol Fed Rabbits

Male Japanese white rabbits (9 weeks old) were fed with 0.5% cholesterol containing diet for one week. After grouping the rabbits by body weight and plasma cholesterol level, groups of 5 rabbits were fed with 0.5% cholesterol diet (control) or 0.5% cholesterol diet supplemented with **1** (0.0025%, 0.00025%, 0.000025% and 0.0000025% dosed 1, 0.1, 0.01, 0.001 mg/kg/day, respectively) for three weeks. Every one week, blood was collected and the plasma cholesterol level was measured with a commercial kit (Wako pure chemical, Co.). Data from this experiment were analyzed statistically by Dunnet's multiple comparisons test.

Results

Up-regulation of Cell Surface LDL Receptor

Since Hep G2 cells have been shown to possess specific LDL receptor similar to those demonstrated on extra hepatic tissue cells[6]. The binding activity of ^{125}I-LDL to Hep G2 cells was determined after incubation of the cells

Fig. 2. Effects of FR171456 (**1**) on the binding of ^{125}I-LDL to Hep G2 cells.

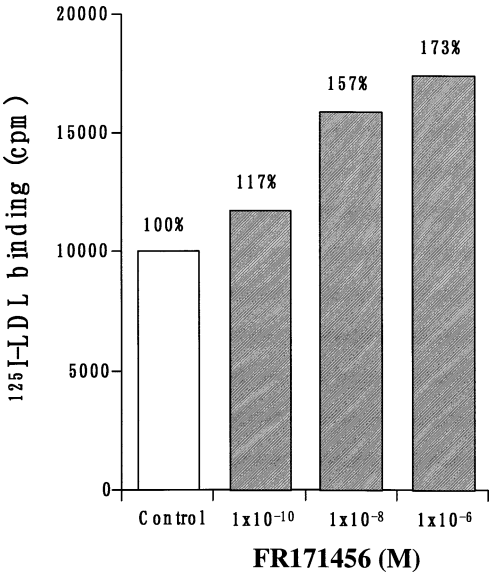
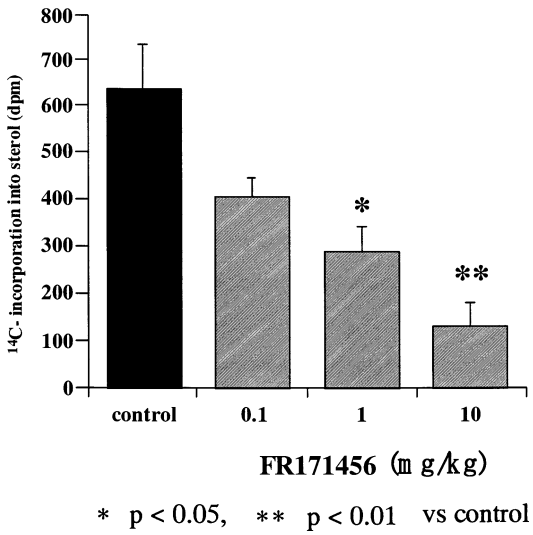
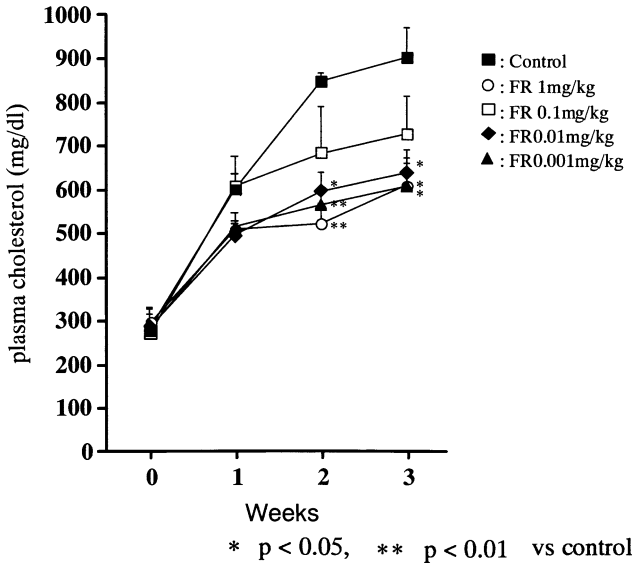


Fig. 3. Effects of FR171456 (**1**) on hepatic sterol synthesis in rat.



for 48 hour with compound **1** at the indicated concentrations (Fig. 2). Compound **1** up-regulated the receptor mediated binding of ^{125}I-LDL in Hep G2 cells in a dose-dependent manner.

Fig. 4. Serum cholesterol lowering effect of **1** on cholesterol fed rabbits.



Inhibition of Sterol Synthesis in Rats by **1**

Single oral administration of compound **1** inhibited rat hepatic sterol synthesis *in vivo* with an ED_{50} value at 4.6 mg/kg (Fig. 3). This demonstrates that compound **1** can serve as an effective inhibitor of cholesterol synthesis *in vivo*.

In Vivo Serum Cholesterol Lowering Effect in Cholesterol Fed Rabbits

As shown in Fig. 4, daily dosing of compound **1** reduced serum cholesterol levels in cholesterol fed rabbits at doses of 1 to 0.001 mg/kg. In these dosages, **1** did not show any significant toxic effects. In 0.1 mg/kg dosed group, one rabbit did not respond to treatment.

Discussion

In this paper, we show the *in vitro* and *in vivo* effects of FR171456 (**1**). LDL receptor mediates the uptake of plasma LDL to supply cholesterol to the cell[7]. The synthesis of LDL receptor is regulated by a sterol-mediated negative feedback mechanism[8]. When cells are cholesterol depleted, the LDL receptor gene transcription is activated and the cell surface number of LDL receptors is up-regulated to satisfy cellular demands *via* the uptake of plasma LDL. In

contrast, when sterols accumulate within cells, the synthesis of LDL receptor is suppressed. The inhibition of cholesterol biosynthesis depletes the cellular cholesterol level and activates the transcription and synthesis of LDL receptor, thereby lowering in the plasma cholesterol level. This is a plausible mechanism of HMG-CoA reductase inhibitors used clinically for amerolating an elevated level of plasma cholesterol[9].

To investigate the effect of FR171456 on the cell surface LDL receptor, we carried out the receptor binding assay by using Hep G2 cells and ^{125}I-LDL as a ligand. The Hep G2 cell line is thought be a suitable model for investigating lipid metabolism in human[10,11]. And HepG2 cells have been shown to possess specific LDL receptor similar to those demonstrated on extra hepatic tissue cells[6]. When the cells were cultured 48 hours in the presence of 1 μM of compound **1**, the specific binding of ^{125}I-LDL was increased by 173%. These result indicated that **1** activated the synthesis of LDL receptor *in vitro*.

Next we tested the effect of 1 on hepatic sterol synthesis *in vivo*. As shown in Fig. 3, single oral administration of compound **1** was shown to inhibit hepatic sterol synthesis in rat dose dependently with an ED_{50} value of 4.6 mg/kg.

Finally daily oral administration of compound **1** reduced serum cholesterol levels in cholesterol fed rabbits without any significant toxicity *in vivo* (Fig 4). Especially very low dose, such as 0.01 to 0.001 mg/kg/day, multiply administration of FR171456 significantly reduce the serum cholesterol level in cholesterol fed rabbits. Therefore FR171456 and/or FR173945 are expected to have hypolipidemic effect in humans.

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