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The Novel Gluconeogenesis Inhibitor FR225654 that Originates from *Phoma* sp. No. 00144

II. Biological Activities

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Abstract The novel gluconeogenesis inhibitor FR225654, isolated from the culture broth of *Phoma* sp. No. 00144, has an unique structure that consists of a highly oxygenated *trans*-decalin ring and a β-keto-enol, with a characteristic side chain. This compound selectively inhibited gluconeogenesis of rat primary hepatocytes and had hypoglycemic effects in several *in vivo* mouse models.

Keywords gluconeogenesis, hepatocyte, diabetes, FR225654

Introduction

In diabetes, hepatic glucose production is accelerated as a result of insulin resistance or insulin deficiency [1]. This phenomenon results in high blood glucose levels that cause many of the complications of this disease [2]. One way to control diabetes may be to inhibit this upregulated hepatic glucose production, specifically by inhibition of gluconeogenesis. Therefore, we screened microbial products for gluconeogenesis inhibitors using primary cultured rat hepatocytes. We isolated FR225654 from the culture broth of *Phoma* sp. fungal strain No. 00144 [3]. This compound has a highly oxygenated *trans*-decalin ring, a β-keto-enol in the core decalin moiety, and a characteristic side chain possessing a conjugated carboxylic acid and a tri-substituted olefin [4]. In this paper, we report its inhibitory activity both *in vitro* and *in vivo*.

Materials and Methods

Materials

Glucagon, insulin, and streptozotocin (STZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Glucose C-II test Wako was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A plasma insulin ELISA kit was purchased from Shibayagi Co., Ltd. (Gunma, Japan).

In Vitro Glucose Consumption Assay

To measure glycolysis, we used a glucose consumption assay using ddY mouse whole blood cells. Whole mouse blood was diluted 1 : 10 with FR225659-containing Dulbecco's Modified Eagle's Medium lacking glucose and incubated at 37°C in a humidified incubator (95% air, 5% CO_2) for 1 hour. After centrifugation, the glucose levels in the supernatant were determined enzymatically (Glucose C-II test Wako, Wako Pure Chemical Industries, Ltd).

In Vitro Glycogenolysis Assay

To measure glycogenolysis, we used two simple methods with cultured rat primary hepatocytes. First, hepatocytes were isolated from fed Wistar rats by the method described in the accompanying paper [3]. Isolated hepatocytes were

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twice washed with Dulbecco's Modified Eagle's Medium lacking glucose and sodium pyruvate. Washed cells were incubated in the same medium at 37°C in a humidified incubator (95% air, 5% CO_2) for 2 hours. After centrifugation, the glucose levels in the supernatant were determined enzymatically. We assumed that all glucose produced in the absence of gluconeogenic substrates was derived from glycogen. Second, hepatocytes were cultured for 4 hours at 37°C in a humidified incubator (95% air, 5% CO_2) in William's E medium containing fetal bovine serum 5% (v/v), glucose 30 mM and insulin 1×10^{-6} M. The cells were washed twice with William's E medium and incubated with William's E medium containing fetal bovine serum 5% (v/v), glucagon 1.5×10^{-6} M and FR225654 for 2 hours. After incubation, cells were washed twice with phosphate-buffered saline and subjected to two rounds of freeze-thaw. Amyloglucosidase (2.5 units/ml) was added and the solution incubated at 37°C for 2 hours. Glucose, originating from glycogen, was measured enzymatically.

Assay for Enzymatic Activity

The methods for measuring the activity of glucose-6-phosphatase (G6Pase) and fructose-1,6-bisphosphatase (FBPase) have been described previously [5, 6]. We used rat liver microsomes and cytosol as the source of G6Pase and FBPase, respectively.

The activity of glycogen synthase kinase-3 was measured using a commercially available enzyme (Glycogen synthase kinase-3 beta, Sigma-Aldrich) and substrate (GS-2 peptide, Upstate Biotechnology, Charlottesville, VA, USA).

Animals

Male C57BL/6N mice were purchased from Charles River Japan Inc. (Atsugi, Japan). Male C57BL/KsJ-db/db mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA).

Male 6-week old ddY mice, purchased from Japan SLC Inc. (Shizuoka, Japan), were made diabetic by intraperitoneal injection of a bolus of streptozotocin (STZ; 225 mg/kg) dissolved in 2 mM citrate buffer (pH 4.5). After 10 days, plasma glucose levels were determined using the Glucose C-II test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Animals with levels >400 mg/dl were selected for the study.

The glucagon-induced mouse hyperglycemia model has been described previously [7].

In all experiments, FR225654 was dissolved in PEG-400 and given orally at a volume of 10 ml/kg.

Statistical Analysis of *in Vivo* Experiments

Data are expressed as means±SEM. Statistical analysis was conducted using an unpaired Student's *t*-test or a one-way ANOVA.

Results

Inhibition of Gluconeogenesis *in Vitro*

FR225654 strongly inhibited gluconeogenesis from pyruvate in cultured primary rat hepatocytes *in vitro* (Table 1). This compound also inhibited gluconeogenesis when glycerol was added to the culture medium as a gluconeogenesis substrate (data not shown). These results indicate that FR225654 inhibited gluconeogenesis downstream from aldolase. However, FR225654 did not affect glycogenolysis in hepatocytes. Furthermore, this compound did not affect glycolysis since the consumption of glucose by blood cells was not inhibited in the presence of high concentrations of this compound (Table 1).

Consequentially, we tested the ability of FR225654 to inhibit glucose-6-phosphatase and fructose-1,6-bisphosphatase which are key enzymes in the gluconeogenesis pathway, and glycogen synthase kinase-3 which is a key enzyme in glycogen synthesis. However, the activities of these enzymes were not affected by FR225654, even in the presence of high concentrations of this compound (Table 1).

In Vivo Hypoglycemic Effects of FR225654

We examined the acute hypoglycemic activity of FR225654 on C56BL/6N mice. Peripheral blood glucose was significantly decreased by 3 hours after oral administration at a dose of 10 mg/kg FR225654. In contrast, plasma insulin levels were unchanged (Fig. 1).

To examine whether this hypoglycemic activity was due to the inhibition of hepatic glucose production, we tested the effects of FR225654 in a glucagon-induced

Table 1 IC_{50} values of FR225654 against several metabolic pathways and related enzymes

Pathway or enzyme	IC_{50}
Gluconeogenesis of hepatocytes*	1.1×10^{-7} M
Glycogenolysis of hepatocytes	$>1\times10^{-4}$ M
Glycolysis of blood cells	$>5\times10^{-5}$ M
Glucose-6-phosphatase	$>1\times10^{-4}$ M
Fructose-1,6-bisphosphatase	$>1\times10^{-4}$ M
Glycogen synthase kinase-3	$>1\times10^{-5}$ M

* The method of this pathway is described in Ref. 3. The other methods are described in Materials and Methods.

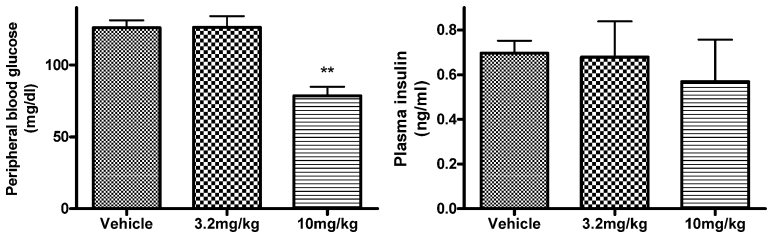

Fig. 1 Hypoglycemic activity of FR225654 in normal mice. FR225654 was orally administered to male C57BL/6 N mice (n=5). After 3 hours, blood samples were collected from the orbital vein and plasma glucose (panel A) and insulin (panel B) were measured. The data are displayed as means±S.E.M. ** $P<0.01$ *vs.* vehicle control.

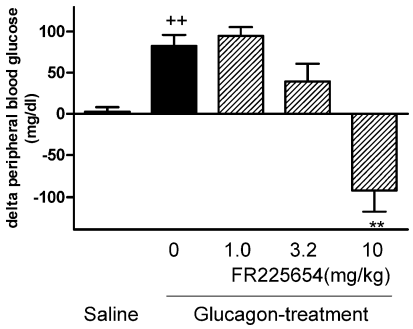

Fig. 2 Ability of FR225654 to suppress glucagon-induced hyperglycemia in normal mice. FR225654 was orally administered and followed by glucagon challenge, as described in Ref. 7. The data are displayed as the means±S.E.M. of changes in peripheral blood glucose relative to basal (pre-glucagon) levels. n=5. $^{++}P<0.01$ *vs.* saline challenge control. ** $P<0.01$ *vs.* vehicle control (with glucagon challenge).

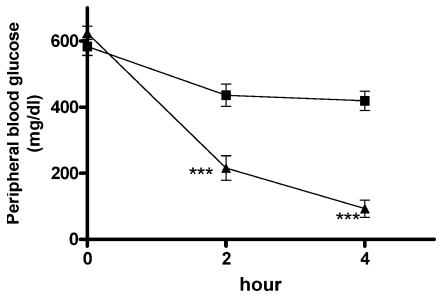

Fig. 3 Hypoglycemic effect of FR225654 on STZ-mice. FR225654 (10 mg/kg, triangles) and vehicle (PEG-400, squares) were orally administered to randomized STZ-treated mice (n=10). After 2 or 4 hours, blood samples were obtained from the orbital vein and plasma glucose levels were measured. The data are displayed as means±S.E.M. *** $P<0.001$ *vs.* vehicle control at each time point.

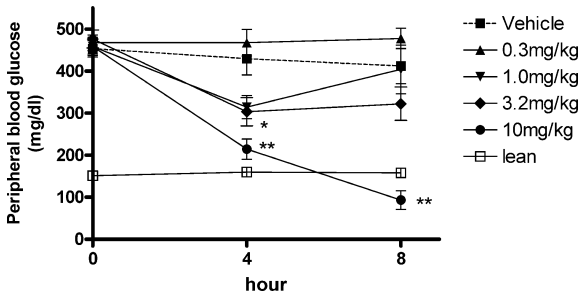

Fig. 4 Hypoglycemic effect of FR225654 on db/db mice. Male db/db mice (n=5) received FR225654 orally at doses of 0.3 mg/kg (triangle), 1 mg/kg (reverse triangle), 3.2 mg/kg (diamond), 10 mg/kg (circle), or 0 mg/kg (vehicle control; square). After 4 and 8 hours, blood samples were obtained from the orbital vein and plasma glucose levels were measured. The data are displayed as means±S.E.M. * $P<0.05$, ** $P<0.01$ *vs.* vehicle control at each time point.

hyperglycemia mouse model. In this model, glucagon leads to uncontrolled increase of hepatic glucose production. FR225654 significantly suppressed glucagon-induced hyperglycemia in mice (Fig. 2).

Next, we examined the effects of FR225654 in an experimental type 1 diabetic mouse model, STZ-treated mice. FR225654 significantly decreased the peripheral blood glucose levels of STZ-treated mice at a dose of 10 mg/kg (Fig. 3). Furthermore, we examined the effects of FR225654 in a spontaneous type 2 diabetic mouse model, the db/db mice, which are characterized by severe insulin resistance and increased hepatic glucose production. The peripheral blood glucose levels of these mice showed a significant decrease in the dose dependent manner following oral administration of FR225654 (Fig. 4).

In all experiments, this compound brought on severe hypoglycemia at a dose of 32 mg/kg. Also, the hypoglycemic effects of this compound (10 mg/kg) were turned back to normal glucose levels after refeeding and overnight period (data not shown).

Discussion

Hepatic glucose production, especially through the gluconeogenesis pathway, is reported to be continuously

upregulated in diabetes [8]. Therefore a gluconeogenesis inhibitor which normalizes the inappropriate upregulation of hepatic glucose production is a very useful agent in the treatment of diabetes. This hypothesis is supported by the fact that an inhibitor of fructose-1,6-bisphosphatase has hypoglycemic effects in animal models and type 2 diabetic subjects [9].

We have screened gluconeogenesis inhibitors from microbial products using primary cultured hepatocytes, and identified a novel gluconeogenesis inhibitor FR225654 from the culture broth of *Phoma* sp. No. 00144 [3]. FR225654 showed a potent inhibition of gluconeogenesis in primary cultures of rat hepatocytes. FR225654 specifically inhibited gluconeogenesis, especially in the downstream of the aldolase reaction, and did not affect glycolysis or glycogenolysis. We have not yet identified the molecular target of this compound.

FR225654 had potent plasma glucose-lowering effects in several mouse models. These effects were independent of insulin action, because FR225654 did not alter plasma insulin levels in normal mice and had potent hypoglycemic effects in STZ-mice, which are a toxin induced insulin-deficient mouse model. These data raise the possibility that this insulin-independent hypoglycemic compound could be used in treatment of obesity induced diabetes, or for control of glucose levels in type 1 diabetes.

We examined short-term *in vivo* experiments because we have screened rapidly acted inhibitor of gluconeogenesis using the assay system of primary cultured hepatocytes. However, it is continuously exploring the long-term effects and toxicity of this compound, now.

In summary, we used primary cultures of rat hepatocytes to screen extracts from fungal cultures and found a novel gluconeogenesis inhibitor, FR225654. This compound displays a potent inhibition of gluconeogenesis and had potent plasma glucose-lowering effects in several animal models. We are continuing to characterize the inhibitory mechanism of this compound.

References

1. Consoli A. Role of liver in pathophysiology of NIDDM. Diabetes Care 15: 430–441 (1992)
2. Rossetti RA, Giaccari A, Defronzo RA. Glucose toxicity. Diabetes Care 13: 610–630 (1990)
3. Ohtsu Y, Sasamura H, Tanaka M, Tsurumi Y, Yoshimura S, Takase S, Shibata T, Hino M, Nakajima H. The novel gluconeogenesis inhibitor FR225654 that originates from *Phoma* sp. No. 00144 I. Taxonomy, fermentation, isolation and physico-chemical properties. J Antibiot 58: 447–451 (2005)
4. Ohtsu Y, Yoshimura S, Kinoshita T, Takase S, Nakajima H. The novel gluconeogenesis inhibitor FR225654 that originates from *Phoma* sp. No. 00144 III. Structure determination. J Antibiot 58: 479–482 (2005)
5. Swanson MA. Methods Ezmymol 2: 541–543 (1955)
6. McGilvery RW. Methods Enzymol 2: 543–546 (1955)
7. Ohtsu Y, Sasamura H, Shibata T, Nakajima H, Hino M, Fujii T. The novel gluconeogenesis inhibitors FR225659 and related compounds that originate from *Helicomyces* sp. No.19353 II. Biological profiles. J Antibiot 56: 689–693 (2003)
8. Consoli A, Nurjhan N, Capani F, Gerich J. Predominant role of gluconeogenesis in increased hepatic glucose production in NIDDM. Diabetes 38: 550–557 (1989)
9. Potter SC, Chandramouli V, Landou BR, Linemeyer DL, Erion MD, van Poelje PD. Effect of MB06322, a potent and selective inhibitor of fructose-1,6-bisphosphatase, on gluconeogenesis in the ZDF rat as assessed by the deuterated water technique. Diabetes 53 (Suppl. 2): A364 (2004)