

The Novel Gluconeogenesis Inhibitors FR225659 and Related Compounds that Originate from *Helicomyces* sp. No. 19353

II. Biological Profiles

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(Received for publication February 28, 2003)

The novel gluconeogenesis inhibitor FR225659 and four related compounds were isolated from the cultured broth of a fungal strain No. 19353. These compounds inhibited the glucagon-stimulated gluconeogenesis of rat primary hepatocytes and had hypoglycemic effects in two different *in vivo* models.

The liver is the main site of glucose production in mammals¹. Glucose homeostasis is normally very stringently controlled by hepatic glucose production and splanchnic glucose uptake in the healthy body². However, in diabetes, hepatic glucose production is accelerated³, which results in the high glucose levels that cause many of the complications of this disease⁴. One way to control diabetes may be to administer agents that retard this upregulated gluconeogenesis. In the search for such agents, we used primary cultured rat hepatocytes as a screening system and isolated FR225659 and four related compounds from the cultured broth of fungal strain No. 19353. These compounds have a novel structure that consists of three abnormal amino acids and an unusual acyl-group and reveals that they are potent inhibitors of hepatic gluconeogenesis *in vitro*. In this paper, we assess their biological profiles and their ability to suppress gluconeogenesis *in vivo*.

Materials and Methods

Animals

Male ddY mice were purchased from Charles River Japan Inc., Atsugi, Japan. Female C57BL/KsJ-db/db mice were purchased from Jackson Laboratories, Bar Harbor, ME, USA.

In Vitro Gluconeogenesis Assay

Rat primary cultured hepatocytes were isolated by the collagenase perfusion method⁵ and cultured overnight at 37°C in a humidified incubator (95% air, 5% CO₂) in 96-well plate in William's E medium containing fetal bovine serum 5% (v/v) and kanamycin 0.1 mg/ml. The cells were washed with phosphate-buffered saline (PBS) and incubated with FR225659 or its related compounds in the presence or absence of glucagon 1×10⁻⁷ M or dibutyryl-cAMP 0.2 mM in Dulbecco's Modified Eagle's Medium that lacks glucose but is supplemented with sodium pyruvate 20 mM, fetal bovine serum 1% (v/v), and kanamycin 0.1 mg/ml. After 13 hours, the glucose produced into the medium was determined enzymatically (Glucose C-II test Wako, Wako Pure Chemical Industries, Ltd.). The

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gluconeogenesis rate was determined to be the levels of glucose that were derived from the pyruvate in the medium.

Results

In Vivo Hypoglycemic Activity

(i) Glucagon-induced murine hyperglycemia model: Male ddY mice (4~5 weeks of age) that had fasted for 2 hours were given the compounds dissolved in olive oil by subcutaneous injection. Blood samples were collected just prior to the injections as baseline values. After 60 minutes, glucagon (100 $\mu\text{g}/\text{kg}$) dissolved in 0.01 M HCl/saline was injected subcutaneously and 30 minutes later, blood was obtained from the orbital vein. The blood samples were centrifuged and peripheral blood glucose levels were determined enzymatically. In other experiments, mice were injected orally with these compounds dissolved in methylcellulose 0.5% containing HCl 0.03 M and challenged with glucagon 90 minutes later.

(ii) Hypoglycemic activity with C57BL/KsJ-db/db (db/db) mice: Female C57BL/KsJ-db/db mice (10 weeks of age) that had fasted 2 hours received FR225659 dissolved in olive oil was administered subcutaneously. At 0, 3, 6, and 9 hours after administration, blood samples were collected from the orbital vein and peripheral blood glucose levels were determined.

Cytotoxicity Test

The cytotoxicity of the compounds was examined using EL-4 cells. After 3 days incubation with the compounds, cell viability was determined colorimetrically at 550 nm and 660 nm as a reference according to the MTT method. Cytotoxicity is expressed as the lowest concentration of the compound needed to reduce EL-4 cell viability by 50%.

In Vitro Glucose Consumption Assay

To measure glycolysis, we constructed the glucose consumption assay using ddY mouse whole blood cells. Whole mouse blood was diluted 1 in 10 with FR225659-containing Dulbecco's Modified Eagle's Medium lacking glucose and incubated at 37°C in a humidified incubator (95% air, 5% CO₂) for 1 hour. After centrifugation, the glucose levels in the supernatant were determined enzymatically.

Assay for Enzymatic Activity

The methods for measuring the activity of glucose-6-phosphatase (G6Pase) and fructose-1,6-bisphosphatase (FBPase) were described previously^{6,7}. We used rat liver microsomes and cytosol as the source of G6Pase and FBPase, respectively.

Inhibition of Gluconeogenesis in Vitro

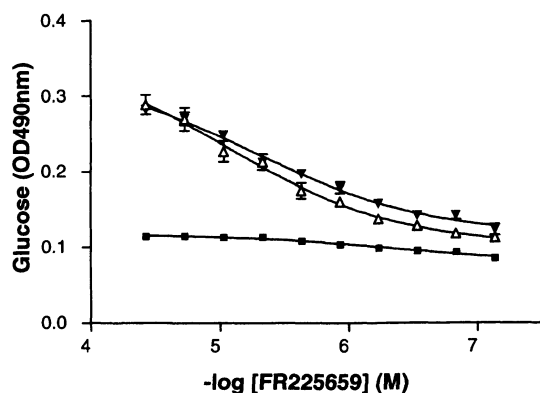
The abilities of FR225659 and its related compounds to inhibit glucagon-stimulated gluconeogenesis in primary rat hepatocytes *in vitro* are summarized in Table 1. FR225659 and its related compounds all strongly inhibited glucagon-stimulated glucose production from pyruvate present in the medium. However, the inhibitory activity of compound **3** was ten times weaker than that of FR225659. FR225659 differs from compound **3** in that it has a hydroxy group at position R₂. Thus, this group contributes to the high inhibitory capability of the molecule as well as the solubility. The substitution of hydroxy group to methoxy group at a position of R₁ leads to slightly increase the inhibitory activity. This may depend on its cell permeability. However, the inhibitory activity of the molecule was not affected when the methyl at position R₃ was altered to an ethyl group (see compounds **2** and **4**).

The basal gluconeogenesis rate of the cultured hepatocytes was not affected by FR225659 (Fig. 1). However, this compound does not seem to affect glycolysis since the consumption of glucose by blood cells was not inhibited by the presence of high concentrations of FR225659 (>30 μM , data not shown). To locate the target molecule of FR225659, we tested the ability of FR225659 to inhibit G6Pase and FBPase, two key enzymes of gluconeogenesis. However, the activities of these two enzymes were not affected by FR225659, even at high concentrations (>30 μM , data not shown). These observations suggest that this compound may selectively affect the glucagon signaling pathway. Consequently, we assessed whether this compound could inhibit the gluconeogenesis stimulated by dibutyryl-cAMP, the cell-permeable analogue of cAMP. cAMP is a second messenger

Table 1. Biological activities of FR225659 and its related compounds.

Compound	IC ₅₀ of hepatocyte gluconeogenesis (μM)	Cytotoxicity to EL-4 (μM)
FR225659 (1)	0.19	1.51
2	0.16	1.84
3	2.2	25
4	0.05	0.98
5	0.08	0.81

Fig. 1. Ability of FR225659 to downregulate *in vitro* gluconeogenesis stimulated by glucagon or dibutyryl-cAMP.



Hepatocytes were stimulated with 1×10^{-7} M glucagon (Δ), 0.2 mM dibutyryl-cAMP (\blacktriangledown), or neither agent (\blacksquare) in the presence of the indicated concentration of FR225659. The data are displayed as means \pm S.E.M. of medium glucose concentrations.

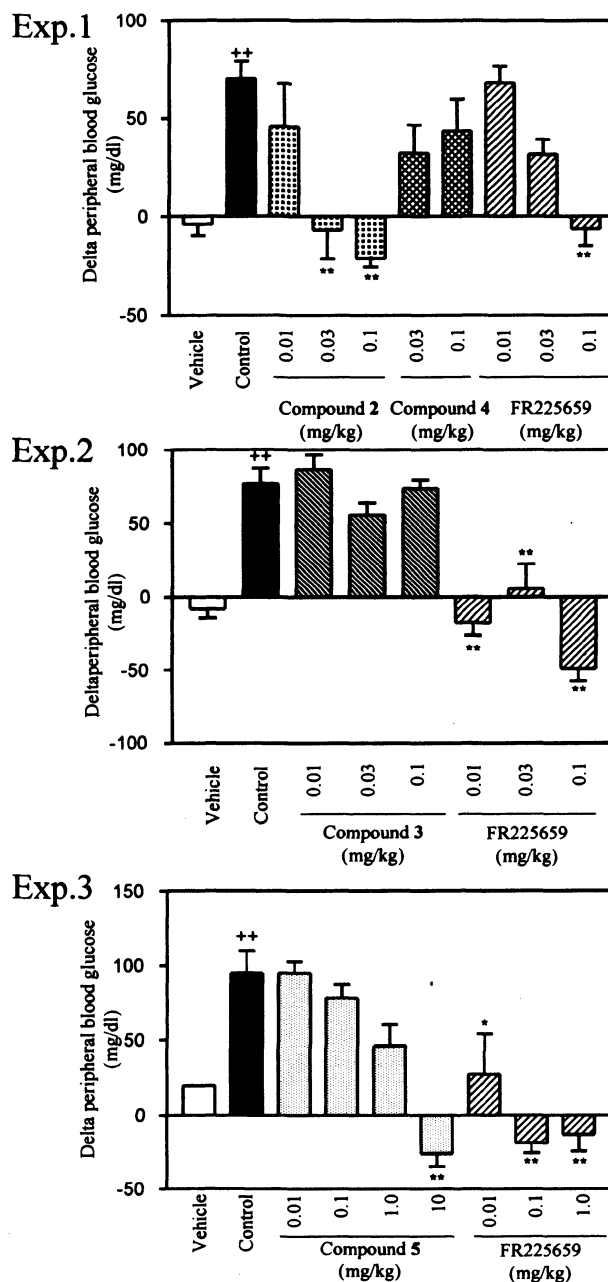
of glucagon signal transduction. We found that FR225659 inhibits cAMP-stimulated gluconeogenesis, which indicates that the target of this compound is downstream of cAMP in the glucagon signaling pathway (Fig. 1).

The amounts of the five compounds that inhibit EL-4 cell viability by 50% ranged from 0.81~1.84 μ M, except for compound 3 (25 μ M) (Table 1). Thus, the cytotoxicities of the compounds are ten times weaker than their gluconeogenesis inhibitory activities.

In Vivo Hypoglycemic Activities

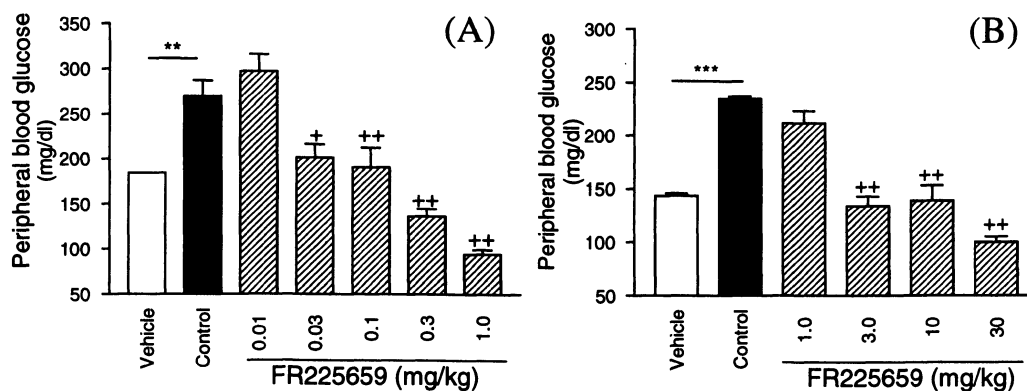
To examine the ability of FR225659 and its related compounds to inhibit hepatic glucose production *in vivo*, the glucagon-induced hyperglycemia murine model was first employed (Fig. 2). The glucagon-induced hyperglycemia in these mice was significantly suppressed by FR225659 and compound 2. Compound 3, which has weak activity *in vitro* (Table 1), also had little effect *in vivo*, even at a dose of 0.1 mg/kg. However, compounds 4 and 5, which both have potent *in vitro* activities (indeed they are more active than FR225659, see Table 1), had weak or no activity *in vivo*. These two compounds differ from FR225659 by the presence of a methoxy group rather than a hydroxy group at R_1 . The methoxy group of compounds 4 and 5 may diminish their absorption or increase their

Fig. 2. Ability of FR225659 and its related compounds to suppress glucagon-induced hyperglycemia in mice.



FR225659 or one of its related compounds was injected subcutaneously, followed by glucagon challenge as described in the Materials and Methods. The data are displayed as means \pm S.E.M. of individual delta peripheral blood glucose concentrations from basal (pre-glucagon) levels. $n=5$. $^{++}p<0.01$ vs. vehicle. $^{*}p<0.05$, $^{**}p<0.01$ vs. control.

Fig. 3. Ability of FR225659 to suppress glucagon-induced murine hyperglycemia when administered subcutaneously or orally.



FR225659 was injected subcutaneously (A) or orally (B), followed by glucagon challenge as described in the Materials and Methods. The data are displayed as means \pm S.E.M. of individual peripheral blood glucose concentrations. $n=5$. ** $p<0.01$, *** $p<0.001$ vs. vehicle. + $p<0.05$, ++ $p<0.01$ vs. control.

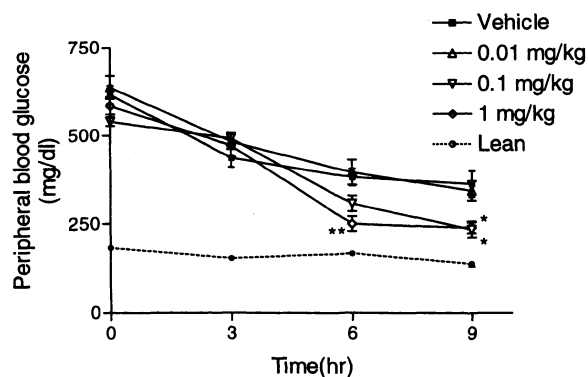
metabolism *in vivo*. We also tested the ability of orally administered FR225659 to suppress glucagon-induced hyperglycemia and found that it was effective at >3 mg/kg (Fig. 3).

FR225659 was also tested with db/db mice, an animal model of spontaneous type 2 diabetes. These mice have severe insulin resistance and show hyperglycemia at 8~10 weeks of age, when they were used in the experiment described here. The peripheral blood glucose levels of the db/db mice were significantly decreased by the administration of FR225659 in a dose-dependent manner (Fig. 4).

Discussion

It is now well established that in diabetes, hepatic glucose production, especially by the gluconeogenesis pathway, is continuously upregulated³. Inhibitors of gluconeogenesis may thus be useful in the treatment of diabetes since they could normalize the inappropriately high rates of hepatic gluconeogenesis in the pathological state. This hypothesis is supported by the fact that inhibitors of two key enzymes in gluconeogenesis, namely, FBPase^{8,9} and G6Pase¹⁰, indeed have the inhibitory activities of hepatocyte gluconeogenesis *in vitro* and the hypoglycemic activities *in vivo*. Consequently, we screened microbial products using primary cultured rat hepatocyte to identify novel gluconeogenesis inhibitors and identified

Fig. 4. Hypoglycemic effect of FR225659 in db/db mice that spontaneously develop diabetes.



Female db/db mice (10 weeks of age, $n=5$ per group) were received FR225659 at the dose of 0.01 mg/kg (open triangle), 0.1 mg/kg (reversed triangle), 1 mg/kg (open diamond) and vehicle (closed square) subcutaneously. Corresponded lean mice, injected vehicle, indicate closed circle. At 0, 3, 6, and 9 hours after administration, blood samples were collected and peripheral blood glucose levels were determined. The data are displayed as means \pm S.E.M. of individual peripheral blood glucose concentrations. * $p<0.05$, ** $p<0.01$ vs. vehicle.

FR225659 and four related compounds in the cultured broth of *Helicomyces* sp. No.19353. We found that four of the five compounds strongly inhibit gluconeogenesis *in*

vitro and that two, FR225659 and compound 2, have potent hypoglycemic effects in the glucagon-induced murine hyperglycemic model. Furthermore, FR225659 had a potent hypoglycemic effect in db/db mice.

The gluconeogenesis inhibitory activity of FR225659 does not involve the inhibition of G6Pase and FBPase. Rather, it seems to inhibit gluconeogenesis by blocking glucagon stimulation. In particular, it appears that this compound acts on downstream of cAMP in the glucagon signaling pathway. It is possible that this compound affects the factors in signal transduction, *e.g.* cAMP-dependent kinase (PKA), or suppresses the induction of enzymes by glucagon. It is suggested that FR225659 specifically inhibits glucagon-mediated gluconeogenesis, since it did not affect the basal gluconeogenesis rate of hepatocytes and the glycolysis rate of blood cells *in vitro*. Supporting our observations is that it is known that glucagon plays an important role in not only the two animal models utilized in this report but also in type 2 diabetes in humans¹¹). Thus, it appears that FR225659 inhibits glucagon responses and that this mediates the hypoglycemic activity of this compound in animal models.

In summary, we screened novel gluconeogenesis inhibitors using primary cultured rat hepatocytes and identified five structurally related compounds, two of which show hypoglycemic activity *in vivo*. This validates the use of the cell-based assay we used for screening gluconeogenesis inhibitors. We are currently further characterizing the inhibitory mechanism employed by these compounds.

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