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FR258900, a Novel Glycogen Phosphorylase Inhibitor Isolated from Fungus No. 138354

I. Taxonomy, Fermentation, Isolation and Biological Activities

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Abstract FR258900 is a novel glycogen synthesis activator produced by Fungus No. 138354. This compound was isolated from the culture broth by solvent extraction and reverse-phase column chromatography. FR258900 stimulated glycogen synthesis and glycogen synthase activity in primary rat hepatocytes. FR258900 exhibited a potent inhibitory effect on the activity of liver glycogen phosphorylase, suggesting that this compound may activate hepatic glycogen synthesis *via* glycogen phosphorylase inhibition. Thus, this glycogen phosphorylase inhibitor may be useful in the treatment of postprandial hyperglycemia in type 2 diabetes.

Keywords fungal metabolite, glycogen phosphorylase inhibitor, diabetes

Introduction

Type 2 diabetes is a severe and prevalent disease. Tight control of plasma glucose reduced the incidence and progression of diabetic complications, however it is difficult to achieve complete glycemic control with current oral hypoglycemic agents. The liver maintains blood glucose homeostasis by uptake of glucose in the postprandial state, and by production of glucose from glycogenolysis and gluconeogenesis in the postabsorptive state. In type 2 diabetes, the rate of glycogen synthesis is impaired, and hence hepatic glucose uptake is reduced, which results in postprandial hyperglycemia [1, 2]. Increasing glycogen synthesis may therefore improve glucose disposal in patients with type 2 diabetes. To identify novel hypoglycemic agents, we used primary rat hepatocytes to screen various microbial products for their ability to stimulate glycogen synthesis *in vitro*. During the course of this screening, we discovered a novel glycogen synthesis activator FR258900, 2,3-bis(4-hydroxycinnamoyloxy)glutaric acid, from the cultured broth of fungal strain No. 138354 (Fig. 1). This compound potently stimulates glycogen synthesis in primary rat

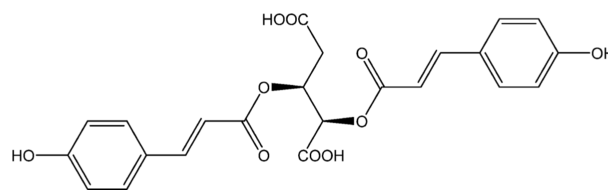

Fig. 1 Structure of FR258900

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hepatocytes, and exhibits a potent inhibitory effect on the activity of glycogen phosphorylase. In this paper, we describe the taxonomy of the producing microorganism, fermentation, isolation and biological activities of this compound.

Materials and Methods

Taxonomy

The producing fungus, strain No. 138354 was originally isolated from a decayed leaf, collected at Ayabe, Kyoto Prefecture, Japan. The observations were made after 14 days of cultivation at 25°C. The compositions of malt extract agar, Czapek's solution agar and MY20 agar were based on the JCM Catalogue of Strains [3]. The color names used in this study were taken from the Methuen Handbook of Colour [4]. The temperature range of growth was determined on potato dextrose agar.

HPLC Analysis

FR258900 was detected in the fermentation broth and the fractions obtained after purification by HPLC using a reverse-phase column YMC-Pack ODS-AM 302 (150× 4.6 mm i.d., YMC Co., Ltd., Kyoto, Japan). The mobile phase was 30% aqueous acetonitrile containing 0.05% trifluoroacetic acid. The flow rate was 1.0 ml/minute and the detection wavelength was set at 210 nm.

Measurement of Glycogen Synthesis

Rat primary cultured hepatocytes were isolated by the collagenase perfusion method [5] and cultured in collagen-coated 96-well plates in William's E medium supplemented with 5% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C for 5 hours in a humidified atmosphere of 5% CO_2 - 30% O_2. The cells were treated with FR258900 for 2 hours, and then washed twice with phosphate-buffered saline, and the intracellular glycogen content was measured. Glycogen levels were determined by digestion of glycogen using amyloglucosidase (Sigma, St. Louis, MO) and subsequent glucose measurement, as described previously [6].

Glycogen Synthase Activity

Glycogen synthase activity was measured as reported previously [7]. Primary rat hepatocytes were cultured in collagen-coated 90 mm diameter dishes, and treated with FR258900, glucagon or insulin for 2 hours. The cells were washed, and lysed in 0.5 ml of 10% glycerol containing 50 mM NaF and 10 mM EDTA by sonication on ice. Cell lysates were centrifuged at 15000×*g* for 20 minutes at 2°C. The supernatant were assayed for glycogen synthase activity in assay buffer (pH 7.5) containing 67 mM Tris-HCl, 5 mM DTT, 6.7 mM EDTA, 13 mg/ml glycogen and 8.9 mM [^{14}C]UDP-glucose (1 μCi/μmol) with or without 10 mM glucose-6-phosphate (G-6-P) for 20 minutes at 37°C. The reactions were spotted on 2-cm Whatman 3MM filters. Filters were washed several times in ice-cold 66% ethanol, and counted for incorporated ^{14}C radioactivity. Active glycogen synthase and total glycogen synthase were determined in the absence or presence of the allosteric activator G-6-P, respectively. Glycogen synthase activity is expressed as the activity ratio (−G-6-P/+G-6-P) [7]. Glycogen synthase is post-translationally modified by phosphorylation that directly inhibits the enzyme. An increase in this ratio reflects a decrease in the phosphorylation state of glycogen synthase.

Assays for Key Enzymes in Glycogen Metabolism

Glycogen phosphorylase (GP) was prepared as previously described [8], with modifications. A cDNA encoding human liver GP was subcloned into the GST expression vector pGEX-4T-2 (Amersham Biosciences Corp., Piscataway, NJ). The GST-GP fusion protein was expressed in *E. coli* DH5α cells, and was purified by using the Glutathione Sepharose 4B (Amersham Biosciences Corp.). The fusion protein was cleaved by thrombin protease (Amersham Biosciences Corp.). It was then reacted with phosphorylase kinase (Sigma) to obtain the phosphorylated active form of the enzyme (GPa), and subjected to anion exchange chromatography. Glycogen phosphorylase activity was measured in the direction of glycogen synthesis by the release of inorganic phosphate from glucose-1-phosphate in a buffer containing 50 mM Hepes (pH 7.2), 100 mM KCl, 2.5 mM EGTA, 2.5 mM $MgCl_2$, 0.5 mM glucose-1-phosphate, and 1 mg/ml glycogen at 25°C. Inorganic phosphate was measured by addition of 1M HCl containing 10 mg/ml ammonium molybdate and 0.38 mg/ml malachite green, and the absorbance was determined at 620 nm [9]. Glycogen synthase kinase-3 (GSK-3) activity was measured according to the method described by Coghlan *et al*. [7] using recombinant GSK-3β (Sigma) and phospho GS-2 peptide (Upstate Biotechnology, Lake Placid, NY) as a substrate. Glucose-6-phosphatase (G-6-Pase) activity was assayed according to the method described previously [10]. We used rat liver microsomes as the source of G-6-Pase. Protein tyrosine phosphatase-1B (PTP1B) activity was measured using a kit (Calbiochem, San Diego, CA).

Results

Characteristics of the Producing Strain

Cultural characteristics on various agar media are summarized in Table 1. This microorganism grew very restrictedly and formed dark grayish colonies on various agar media. Culture on potato dextrose agar grew very restrictedly, attaining 1.0~2.0 cm in diameter two weeks later at 25°C. The colony surface was raised, felty to cottony, wrinkly, and olive gray. The reverse was dark green. Colonies on corn meal agar grew at a similar rate as on potato dextrose agar, attaining 1.0~2.0 cm in diameter under the same conditions. The surface was plane, felty, olive and brownish gray at the center. The reverse was olive. This strain was able to grow at the temperature range from 5 to 28°C, with the growth optimum at 21 to 22°C.

Strain No. 138354 formed neither teleomorph nor anamorph. Hyphae sometimes aggregated to a spherical mass like a pycnidium or a pericethium in old cultures, especially on corn meal agar. The aggregations were dark brown, globose to ellipsoidal, non-ostiolate, and did not produce spores. Their size was 40~90×35~70 μm with a length:breadth ratio of 1.0~1.4. Vegetative hyphae were brown, smooth, septate and branched. The hyphal cells were cylindrical and 2~5.5 μm thick. Chlamydospores were absent.

The above characteristics of the producing strain No. 138354 were insufficient for fungal identification, thus the strain was named simply as "Fungus No. 138354".

Fermentation

A loopful of fungus strain No. 138354 was inoculated from a slant culture into two 500 ml-Erlenmeyer flasks each containing 100 ml of the seed medium consisting of corn starch 2%, glycerin 1%, sucrose 1%, Pharmamedia 1%, gluten meal 1%, tween80 0.2%. The flasks were incubated at 25°C for 6 days on a rotary shaker (220 rpm). The resultant seed culture was inoculated into 20 liters of sterilized production medium in a 30-liter jar fermentor. The production medium was composed of soluble starch 6%, glucose 2%, KCl 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, KH_2PO_4 0.1%, β-cyclodextrin 1%, yeast extract 0.25%, Adekanol LG-109 (Asahi Denka Co., Ltd., Tokyo, Japan)

Table 1 Cultural characteristics of strain No.138354

Media	Cultural characteristics
Malt extract agar	G: Very restrictedly, 1.0~2.0 cm S: Circular, plane, felty to cottony, sectoring, greenish gray (30C2~30E2) R: Dark green (28F4)
Potato dextrose agar (Difco 0013)	G: Very restrictedly, 1.0~2.0 cm S: Circular, raised, felty to cottony, wrinkly, olive gray (1E2) R: Dark green (27F3~27F4)
Czapek's solution agar	G: Very restrictedly, 0.5~1.5 cm S: Circular, submerged, plane, felty, dark green (27F3) R: Dark green (27F3)
Sabouraud dextrose agar (Difco 0190)	G: Very restrictedly, 1.0~2.0 cm S: Circular, raised to capitate, felty, radiately sulcate, brownish gray (4D2) R: Dark green (27F3)
Emerson Yp Ss agar (Difco 0739)	G: Very restrictedly, 0.5~1.5 cm S: Circular, plane, felty, love brown (4E3~4F3) R: Dark green (28F4)
Corn meal agar (Difco 0386)	G: Very restrictedly, 1.0~2.0 cm S: Circular, plane, felty, olive (2F3), brownish gray (4C2) at center R: Olive (2F3)
MY20 agar	G: Very restrictedly, 1.0~2.0 cm S: Circular, plane to raised, felty to cottony, radiately sulcate, brownish gray (4E2~5E2) to gray (1E1) R: Dark green (28F3~28F4)
Oatmeal agar (Difco 0386)	G: Very restrictedly, 1.0~2.0 cm S: Circular, plane to raised, felty, gray (1D1~1E1), olive gray (2F2) to olive (2F3) at margin

Abbreviation: G, growth measured by colony size in diameter; S, colony surface; R, reverse.

0.05%, and Silicone KM-70 (ShinEtsu Chemical Co., Ltd, Tokyo, Japan) 0.05%. The pH of the medium was adjusted to 6.5 before sterilization. Fermentation was carried out at 25°C for 6 days at an aeration rate of 20 liters/minute and agitation speed of 200 rpm. The amount of FR258900 in the fermentation broth reached about 95 μg/ml at 6 days.

Isolation and Purification

After the culture was completed, the culture broth was filtered with the aid of diatomaceous earth. The mycelial cake was extracted with 20 liters of acetone by stirring for an hour at room temperature. The extract was filtered with the aid of diatomaceous earth. The filtrate was evaporated to remove acetone and the resultant aqueous layer was extracted with ethyl acetate twice at pH 2.0. The organic layer was concentrated *in vacuo* to give a brown extract. The extract was dissolved in 50 ml of methanol and promptly passed through a column (2 liters) of Daisogel SP-120-ODS-B (15/30 μm, Daiso Co., Ltd., Osaka, Japan) packed with water containing 0.05% TFA. The column was eluted with 30% aqueous acetonitrile containing 0.05% TFA (6 liters). The elution was monitored by analytical HPLC as described in Material and Methods. The retention time of FR258900 was 8.5 minutes. The portion corresponding to FR258900 was evaporated to remove acetonitrile and the resultant aqueous layer was extracted with ethyl acetate twice. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to give an oily residue. This material was dissolved in a small amount of diethyl ether and concentrated to give a powder. The powder was dissolved in a small amount of hot water (70°C) and cooled to give crystals. The crystals were filtered and dried to give 1 g of FR258900.

Physico-chemical Properties

As shown in Table 2, FR258900 was soluble in methanol, acetonitrile, ethyl acetate and dimethyl sulfoxide, slightly soluble in water, and insoluble in *n*-hexane. The compound showed positive color reactions to iodine vapor, though negative against Morich, Ehrlich, $FeCl_3$, Dragendorf and ninhydrin, and showed UV absorption at 227 and 310 nm. The determination of the structure of FR258900 was accomplished primarily by a series of 2-D NMR techniques. Total synthesis of FR258900 was achieved, and the absolute configuration was determined as Fig 1. The details of the structure elucidation studies of FR258900 will be described elsewhere.

Biological Activities

FR258900 stimulated glycogen synthesis in primary rat hepatocytes in a dose dependent manner (Fig. 2a). We

Table 2 Physico-chemical properties of FR258900

Properties	FR258900
Appearance	White crystal
Molecular formula	$C_{23}H_{20}O_{10}$
Molecular weight	456
HRESI-MS (*m/z*)	
Found $(M+H)^+$	457.1148
Calcd. $(M+H)^+$	457.1134
$[\alpha]_D^{25}$	−216 (*c*=0.21, MeOH)
m.p.	212~214°C
UV λ_{max}^{MeOH} nm (ε)	227 (26100), 310 (51200)
Solubility	
Soluble	MeOH, acetonitrile, EtOAc DMSO
Slightly soluble	Water
Insoluble	*n*-Hexane
TLC Rf[a]	0.23

[a] Silicagel 60 F_{254} (E. Merck), *n*-butanol : ethanol : ammonia=1 : 1 : 1.

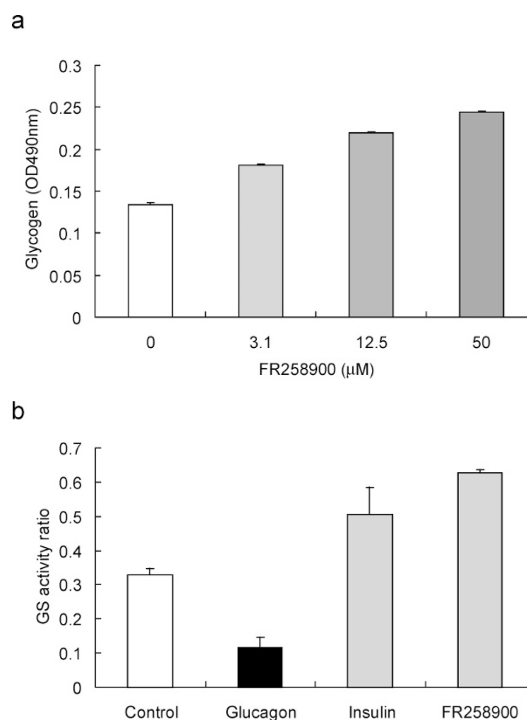

Fig. 2 Effects of FR258900 on glycogen synthesis in rat primary hepatocytes. (a) The intracellular glycogen content of primary cultured hepatocytes treated with the indicated concentrations of FR258900 for 2 hours. The data are displayed as means±S.E.M. of glucose concentration equivalent of glycogen (n=6). (b) Glycogen synthase activity in the hepatocytes treated with 30 nM glucagon, 1 μM insulin or 10 μM FR258900. The data are displayed as means±S.E.M. of GS activity ratio calculated as described in the Materials and Methods (n=3).

Table 3 Inhibitory effects of FR258900 on various enzyme activities

Enzyme	IC_{50} (μM)
Glycogen phosphorylase	2.5
Glucose-6-phosphatase	>200
Glycogen synthase kinase-3	>200
Protein tyrosine phosphatase 1B	>200

examined whether the ability of FR258900 to stimulate glycogen synthesis resulted from activation of glycogen synthase, the rate-determining enzyme in glycogen synthesis. As shown in Fig. 2b, glucagon suppressed glycogen synthase activity. In contrast, FR258900 stimulated glycogen synthase activity as well as insulin. To determine the target molecule of FR258900, we examined the ability of FR258900 to inhibit key enzymes in glycogen metabolism [11]. Glycogen phosphorylase catalyzes the first step in glycogen degradation. G-6-Pase catalyzes the terminal reaction of glycogenolysis. GSK-3 is reported to play an important role in the regulation of glycogen synthesis *via* inhibitory phosphorylation of glycogen synthase [12], and PTP1B is implicated as a negative regulator of insulin signaling [13]. As shown in Table 3, FR258900 inhibited glycogen phosphorylase with IC_{50} of 2.5 μM. In contrast, FR258900 exhibited no inhibition of other enzymes, such as GSK-3, G-6-Pase, or PTP1B.

Discussion

Defects in insulin-stimulated glycogen synthesis are a major contributor to postprandial hyperglycemia in patients with type 2 diabetes. In the current study, we presented a novel fungal product FR258900 as an activator of hepatic glycogen synthesis. We also demonstrated the ability of FR258900 to stimulate glycogen synthase activity. We speculate that the activity of FR258900 to stimulate glycogen synthesis may be due to glycogen phosphorylase inhibition, because results on several chemical derivatives of FR258900 showed a correlation between upregulatory activities of glycogen synthesis and inhibitory activities of glycogen phosphorylase (data not shown). These results suggest that glycogen phosphorylase is a potential candidate target for controlling postprandial hyperglycemia in type 2 diabetes.

Glycogen phosphorylase activity is regulated by allosteric mechanisms and by phosphorylation [14]. The dephosphorylated form (GPb) is less active than the phosphorylated form (GPa). GPa is a potent inhibitor of PP1G (glycogen targeted protein phosphatase-1) which dephosphorylates and activates glycogen synthase [15]. Therefore, FR258900 may restore PP1G activity from GPa-mediated inhibition, resulting in the indirect activation of glycogen synthase.

Glycogen phosphorylase is the rate-controlling enzyme of the glycogenolytic pathway [14], hence the inhibition of glycogen phosphorylase could suppress glycogenolysis and hepatic glucose output. Thus, inhibition of glycogenolysis, as well as activation of glycogen synthesis, may be also involved in increased glycogen content in hepatocytes treated with FR258900. Several inhibitors of glycogen phosphorylase described recently have demonstrated glucose-lowering effects by inhibiting hepatic glucose produciton in type 2 diabetes [9, 16]. In the present study, we demonstrated that glycogen phosphorylase inhibitor could activate glycogen synthesis, suggesting that phosphorylase inhibitors should also be effective in promoting hepatic glycogen synthesis in the absorptive state. We describe in our accompanying paper1 [7] whether FR258900 can improve oral glucose disposal by increasing liver glycogen synthesis, and also lower blood glucose by reducing hepatic glucose output in diabetic animal models.

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