Doctoral Dissertation

博士論文

AJS1669, a novel small-molecule muscle glycogen synthase activator, improves glucose metabolism and reduces body fat mass in *ob/ob* mice

(新規グリコーゲン合成酵素活性化剤 AJS1669 による

ob/ob マウスの耐糖能改善作用と体脂肪減少効果)

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Abstract

Glycogen is an important energy source for exercise. Aerobic exercise consumes glycogen in skeletal muscle, which is restored by food intake. Glycogen is also a component of whole body glucose metabolism that is synthesized primarily in liver and skeletal muscle. Impaired glycogen synthesis and turnover are a common defect in insulin resistance and type 2 diabetes. Thus, regulating glycogen metabolism might have crucial effects not only as an exercise-mimic, but also as an anti-diabetic. Glycogen metabolism is mainly regulated by two enzymes, glycogen synthase (GS) and glycogen phosphorylase (GP). When glucose is translocated to skeletal muscle, it is converted into glucose-6-phosphate (G6P), and this G6P is the substrate and allosteric activator of GS. On the other hand, G6P is a down-regulator of GP, and GP is activated when G6P is decreased. G6P cannot be precisely measured in the exercise condition because it is difficult to capture. However, G6P level presumably decreases during aerobic exercise because energy expenditure is more than energy intake.

I hypothesized that acceleration of glycogen metabolism could be an exercise-mimic and anti-diabetic. There are several reports of GS knockout mice and transgenic mice, but they did not provide convincing evidence for an exercise-mimic effect because effects observed are phenotypic in nature. Conditional knockout mice or a

pharmacological approach could be evaluated for the exercise-mimic effect. I used a pharmacological method. However, an activator of liver GS may induce liver gluconeogenesis, which would offset the effect of lowering blood glucose levels. Because GS is a key enzyme involved in the synthetic process, if a muscle-specific GS activator could be used, it would be a promising therapeutic target for treatment of type 2 diabetes. Moreover, there was no convincing evidence that muscle-specific glycogen activation could cause an exercise-mimic effect and accelerate glucose and glycogen metabolism in skeletal muscle. No muscle-specific GS activator was known. It took 2 years to prepare and identify the novel, potent, and orally available GS activator AJS1669. *In vitro*, AJS1669 activated GS subtype 1 (GYS1) in a concentration-dependent manner, with GYS1 stimulation further potentiated in the presence of glucose-6-phosphate (G6P), an allosteric activator of GS.

Because AJS1669 had potential to be a muscle-specific GS activator, I could test my hypothesis systematically as follows. The action of AJS1669 in skeletal muscle was elicited at lower concentrations than in liver. Additionally, AJS1669 dose-dependently increased glycogen levels and improved glycogen metabolism in human skeletal muscle cells. Repeated administration of AJS1669 over 4 weeks lowered blood glucose and HbA1c level in *ob/ob* mice. AJS1669 also improved glucose tolerance in a dose-

dependent manner and decreased body fat mass. These effects were not associated with excessive body weight gain or any adverse effects on liver function. The mRNA levels of genes involved in mitochondrial fatty acid oxidation and mitochondrial biogenesis were elevated in skeletal muscle tissue following AJS1669 treatment. Hepatic tissue of treated mice also exhibited elevated expression of genes associated with fatty acid oxidation. In contrast to *ob/ob* mice, AJS1669 administration in normal animals elicited no alterations in body weight or glucose-lowering effects. These results support my hypothesis that regulation of glycogen metabolism causes an exercise-mimic and anti-diabetic effects. The pharmacological agent AJS1669 can activate GYS1 and has potential for use as a new type of diabetes treatments aimed at improving glucose metabolism in skeletal muscle.

Abbreviations

DPP-IV: dipeptidyl peptidase-4

GLP-1: glucagon-like peptide-1

SGLT2: sodium/glucose co-transporter 2

AMPK: Adenosine monophosphate-activated protein kinase

WAT: White adipose tissue

BAT: Brown adipose tissue

13C-NMR: carbon-13 nuclear magnetic resonance spectroscopy

G6P: glucose 6 phosphate

GS: glycogen synthase

PPAR: peroxisome proliferator-activated receptor

ALT: alanine aminotransferase

AST: aspartate aminotransferase

AJS1669: {sodium2-[[5-[[4-(4,5-difluoro-2-methylsulfanyl-phenyl)phenoxy] methyl] furan-2-carbonyl]-(2-furylmethyl)amino] acetate}

GYS1: glycogen synthase 1

HbA1c: hemoglobin A1C

GPI: glycogen phosphorylase inhibitor

ATCC: American Type Culture Collection

FBS: Fetal bovine serum

BSA: Bovine serum albumin

GP: Glycogen phosphorylase

OGTT: oral glucose tolerance test

LPL: Lipoprotein lipase

HOMA-IR: homeostatic model for assessment of insulin resistance

Chapter 1: General introduction

The prevalence of diabetes (type 2 diabetes and type 1 diabetes) will increase by 54% to more than 54.9 million Americans between 2015 and 2030 and total annual medical and societal costs related to diabetes will increase by 53% to more than $622 billion by 2030 [1]. The pathogenesis of type 2 diabetes in particular involves increased gluconeogenesis in the liver or decreased glucose uptake in peripheral tissues, resulting from insufficient insulin secretion from the pancreas or attenuated insulin responsiveness [2]. Currently, the medications used to treat type 2 diabetes belong to two categories: insulin secretagogues and insulin sensitizers. Insulin secretagogues include sulfonylureas, glinides, dipeptidyl peptidase-IV (DPP–IV) inhibitors, or glucagon-like peptide-1 (GLP-1) analogues. Commonly used insulin sensitizers include pioglitazone and metformin. Sodium/glucose co-transporter 2 (SGLT2) inhibitors that act to reduce the re-absorption of glucose in the kidneys have also been marketed.

Currently available options for treatment, however, are adequate only for curbing the progression of diabetes but are ineffective in actually mitigating the condition. Moreover, these anti-diabetic drugs are associated with a number of undesirable side effects including hypoglycemia, weight gain, gastrointestinal effects, nausea, and edema [3]. There is, therefore, an acute need for the development of novel anti-diabetic drugs

that would avoid the side effects and actually improve the diabetic condition, while effectively controlling blood glucose level.

Exercise reduces body fat mass and attenuates the risk of type 2 diabetes. Given the numerous benefits of exercise on general health, identification of molecules involved in exercise and obesity or diabetes is crucial for understanding physiological and anti-diabetic effects. I was particularly interested in the mechanism of skeletal muscle in aerobic exercise.

When I explored the mechanism of exercise, the 5'-adenosine monophosphate-activated protein kinase (AMPK) signaling pathway was revealed. AMPK is a sensor of the ATP/AMP ratio in states of low cellular energy and acts to restore energy into the whole body by switching off anabolic pathways and switching on alternative catabolic pathways [4]. Therefore, AMPK is considered a metabolic master regulator and usually activated in response to hypoxia, caloric restriction, muscle contraction, and exhausting-exercise [5]. If AMPK could be activated forcedly, an exercise-mimic effect would be achieved. 5-amino-imidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) and biguanide can cause glucose uptake in skeletal muscle and inhibit glucose production in liver [6]. However, these AMPK activator, AICAR, can demonstrates significant toxicity as can cause bradycardia and hypoglycemia [7]. Since my interest was to

understand the physiological mechanism of skeletal muscle in exercise, I tried several approaches without using AMPK activators.

First, my hypothesis was contracted-muscle would secrete important products "myokines" that are beneficial for the whole body. I evaluated what kind of molecule was up or down regulated after soleus muscle contraction. Although several proteins were identified in this experiment, these proteins had been previously reported as novel myokines [8].

Second, I regarded exercise as a mechanism that decreases white adipose tissue (WAT) and increases brown adipose tissue (BAT). BAT is the fundamental tissue for maintaining glucose homeostasis. If BAT is provided to obese mice, these obese mice could get exercise effects. BAT transplantation methods in mice were established in the Goodyear laboratory. This model demonstrates BAT transplantation causes glucose utilization and has beneficial effects as an anti-diabetic [9]. Using this model, I analyzed muscle phenotype. However, an intriguing molecule in skeletal muscle was not identified.

Finally, I explored glycogen metabolism. In humans, skeletal muscle is the major site of glucose uptake and glycogen storage, with both processes regulated by insulin. Impaired glycogen synthesis is the major abnormality in type 2 diabetes [10, 11]. For

example, a method using carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy in subjects undergoing the hyperglycemic-hyperinsulinemic clamp has revealed that patients with diabetes have a 50% or greater decrease in glycogen synthesis compared to healthy individuals [12]. In analyses performed at the cellular level, patients suffering from type 2 diabetes involving insulin resistance have decreased GS activity in skeletal muscle [13, 14]. A potentially clinically relevant polymorphism of GYS1, the predominant GS subtype in skeletal muscle tissue, has been reported among patients with diabetes [15]. Insulin sensitivity is enhanced by exercise or weight loss associated with exercise [16-18]. Exercise activates GS and enhances insulin sensitivity [19-21]. Activating the GS that is reduced in type 2 diabetes and correcting glucose metabolism in skeletal muscle are regarded as effective approaches to treating diabetes [22].

In skeletal muscle, glucose transporter 4 (GLUT4) protein migrates to the membrane and functions to transport glucose from the blood into the cell. An increase in this uptake results in increased phosphorylation of glucose by hexokinase and its conversion to glucose-6-phosphate (G6P). One of the key enzymes in glycogen synthesis is GS. G6P is converted to UDP-glucose, and GS forms α-1,4-glycosidic linkages, while branching enzymes create α-1,6 glycosidic linkages [23]. There are two

GS isoforms in mammals with 69% homology. One is encoded by the GYS2 gene and is expressed only in liver, whereas the other is encoded by GYS1 and is expressed in skeletal muscle, brain, kidneys, pancreas, and adipose tissue [24, 25]. GYS2 protein is also involved in glucose homeostasis in liver. Liver-specific GS knockout animals are hypersensitive to fasting and have enhanced basal gluconeogenesis [26]. Liver glycogen shortage activates a liver–brain–adipose neural axis that plays an important role in switching the fuel source from glycogen to triglycerides under prolonged fasting conditions [27].

Therefore, I established my hypothesis as follows. In diabetic patients, skeletal muscle has lost the driver of glucose utilization. This driver can be recovered by exercise causing an up-regulation of glucose uptake and glycogen turnover in skeletal muscle. If skeletal muscle GS was activated specifically through a pharmacological chemical compound, glycogen accumulation would be increased, glycogen degradation would be induced through variations in G6P level and ATP/AMP ratio as in the exercise state, and up-regulated glycogen turnover would improve insulin resistance (Fig. 1). If I could identify a muscle-specific GS activator, I could not only reveal glycogen turnover as one of the most important processes in exercise, but also obtain candidates of novel anti-diabetic agents that have an exercise-mimic effect.

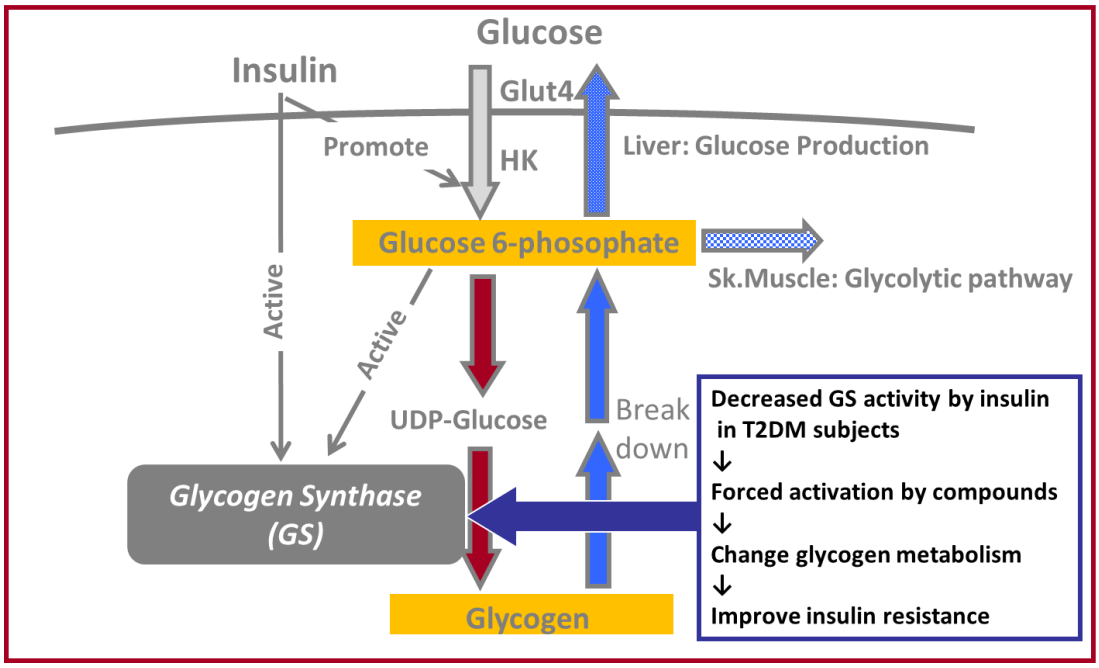


Fig 1. Schematic model outlining the hypothesis to acquire exercise mimic effect

Forcibly enhancing glycogen synthase (GS) activity, which is decreased in type 2 diabetic patients, promotes the flow of glucose into the skeletal muscle and the consumption of glucose 6-phosphate, which is an allosteric substance. That consumption will be a trigger for glycogen phosphorylase (GP) activation, which will up-regulate glycogen turnover. I hypothesized that muscle-specific activation is essential, because the action in liver causes gluconeogenesis, which returns glucose back into the blood.

Chapter 2: Execise mimic effects thtough glycogen synthase-activation on diabetic animal

2.1 Material and Methods

2.1.1 Chemicals and materials

Sodium 2-[[5-[[4-(4,5-difluoro-2-methylsulfanyl-phenyl) phenoxy] methyl]furan-2-carbonyl]-(2-furylmethyl) amino] acetate (AJS1669, Fig. 1A) was synthesized by AJINOMOTO PHARMACEUTICALS CO., LTD. using hGYS1 activation as an indicator.

Pioglitazone was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Glycogen phosphorylase inhibitor was purchased from Millipore (Bedford, MA).

2.1.2 In vitro hGYS1 assay

293T cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Human GYS1 gene (hGYS1) cDNA was derived from the muscle cDNA in the human MTC Panel I (Takara-bio, Shiga, Japan). pcDNA 3.1 (+) expression vector (Life Technologies Japan, Kanagawa, Japan) containing hGYS1 was transiently transfected into 293T cells.

GS (hGYS1) assay was performed as described previously [28]. In short, a solution containing AJS1669 at various concentrations with or without 10 mM G6P (Sigma-Aldrich Japan, Tokyo, Japan) was added to a polystyrene 96-well half area plate (12 μL/well). Next, 18 μL of a substrate solution containing 21.6 mM UDP-glucose (Sigma-Aldrich Japan, Tokyo, Japan), 21.6 mM phosphoenolpyruvic acid (Sigma-Aldrich Japan, Tokyo, Japan), and 4.05 mM NADH (Sigma-Aldrich Japan, Tokyo, Japan) were added to each well. Enzyme solution containing 0.17 mg/mL GS lysates and 1.5 μL pyruvate kinase/lactate dehydrogenase solution (Sigma-Aldrich Japan, Tokyo, Japan) were added (18 μL/well) to prepare a reaction solution. After the reaction solution was incubated at 37 °C for 25 min, the absorbance was measured at 340 nm using Benchmark Plus (Bio-Rad Laboratories, Tokyo, Japan).

The activities of the test compounds were calculated by the change in the absorbance (ΔA340). ΔA340 was calculated by subtracting the absorbance at 340 nm of the reaction solution containing AJS1669 and DMSO from the absorbance at 340 nm of a reaction solution containing only DMSO. The ΔA340 of the reaction solutions containing AJS1669 at final concentrations of 100 μM (without any G6P present) or 1 μM (with 2.5 mM G6P) were taken as normalization values (100%) to calculate the relative activity (%) at various concentrations. EC_{50}, representing the concentration of

the compound which elicits an increase in the relative activity by 50%, was calculated using XLfit (IDBS, Tokyo, Japan).

2.1.3 Animals

Male C57BL/6J mice (6 weeks old) and *ob/ob* mice (6 weeks old) were purchased from Charles River Laboratories (Kanagawa, Japan). The mice were fed standard laboratory chow (CRF-1, Charles River) and tap water *ad libitum* for the duration of the experiments. All animals were subjected to a reverse 12-hour light/dark cycle. The Institutional Animal Care and Use Committee of Pharmaceutical Research Laboratories of AJINOMOTO PHARMACEUTICALS CO., LTD., formerly EA Pharma Co. Ltd., approved all procedures involving the care and use of animals before they were performed.

2.1.4 Assay of GS activity in mouse tissue lysates

Lysates of muscle and liver tissues obtained from mice fasted for 16 h were homogenized using a Microson XL2000 (Qsonica, Newtown, CT, USA) in ice-cold buffer containing 50 mM KF (pH 7.0), 10 mM EDTA (pH 7.0), and 10% glycerol. After centrifugation (16,000 × g, 10 min), supernatant was collected and used as the enzyme

solution. The enzyme was incubated 3 times with each studied concentration of test compound for 30 min before the GS assay was performed. The GS assay was performed using previously described methods [29]. 30 μL mixture of enzyme and compounds was added to 60 μL of assay mixture containing 0.5 μCi/mL [^{14}C] UDP-glucose (PerkinElmer Japan, Kanagawa, Japan). After incubation for 20 min at 30 °C, 75 μL of aliquots were immediately spotted onto a filter paper. The filter paper, which completely absorbed the sample, was placed into a vial, washed twice with 10 mL of 66% (v/v) ethanol, and completely dried. Incorporation of [^{14}C] UDP-glucose into glycogen was measured using a scintillation counter (PerkinElmer Japan).

2.1.5 Glucose incorporation into glycogen assay in human muscle cells

Human muscle cells were purchased from Lonza (Basel, Switzerland). An assay evaluating glucose incorporation into glycogen was performed using previously published methods [30]. Cells were plated onto a collagen-coated 96-well plate with SkGM-2 (Lonza, Basel, Switzerland) containing 10% fetal bovine serum (FBS) for one day. Cells were subsequently differentiated using serum-reduced SkGM-2 containing 2% FBS for three days. After the medium was removed, the cells were incubated for 4 h with glucose-free DMEM (Life Technologies Japan, Kanagawa, Japan) containing 0.1%

bovine serum albumin (BSA). Cells were incubated for 3 h with DMEM containing 0.1% BSA, 1 g/L glucose, and 0.19 μCi/L [^{14}C] glucose (PerkinElmer Japan, Tokyo, Japan). The reaction media was removed and the cells were washed twice with ice-cold PBS. After disruption by incubation in 1 N NaOH for 10 min at 60 °C, cell homogenates were transferred to 96-well Multiscreen HTS plates (Millipore, Bedford, MA) containing 0.5 mg/mL glycogen in 100 μL of ice-cold ethanol and incubated for 20 minutes. The precipitate was filtered under vacuum and washed twice with ice-cold 66% ethanol. The filters were dried and Microscint 40 (PerkinElmer Japan) was added. The amount of [^{14}C] glucose incorporated into cellular glycogen was quantified in a TopCount 96-well liquid scintillation counter (PerkinElmer Japan). The decay rate (counts per minute, cpm) measured in the wells containing AJS1669 at the final concentration of 100 μM was taken as 100% in the calculations of the relative activity (%) after subtracting the cpm of the background wells which contained DMSO only.

2.1.6 Repeated administration study

Male *ob/ob* mice (8 weeks old) were divided into 4 groups (n = 6–8). Group allocation was performed to ensure that there were no significant differences in HbA1c level, body weight, and blood glucose level across groups. Vehicle (0.5%

methylcellulose) alone or with AJS1669 (3 or 10 mg/kg) or pioglitazone (10 mg/kg) was orally administrated to mice twice daily (at 9:00 AM and 4:00 PM) for 4 weeks. Body weight and food intake were monitored every 2 or 3 days. Blood samples for the measurement of basal blood glucose and HbA1c levels were taken from unrestrained mice by cutting off the tip of the tail. Blood glucose level was measured every 2 weeks using a Lifecheck sensor (Gunze, Osaka, Japan). HbA1c level was measured on day 28 (Tosoh, Tokyo, Japan). Blood lactate level was measured on day 28 (Arkray, Japan). Body composition was analyzed on day 26 using EchoMRI (EchoMRI LLC, Houston, TX). After 4 weeks of treatment, OGTT was performed following an overnight fast. After the OGTT, mice were fed for 3 days and sacrificed. The blood and tissue samples were collected for further analysis.

2.1.7 Oral glucose tolerance test

Single oral glucose test was performed using mice after fasting overnight. After measuring the body weight, 1 g/kg glucose (Otsuka, Tokushima, Japan) was orally administrated. Blood was collected from the tail vein 1 min before and 30, 60, and 120 min after the administration of glucose and blood glucose levels were determined using a Lifecheck sensor, as described above. The oral glucose tolerance of repeated

administration was performed as same as single oral glucose test. In brief, after 4 weeks of administration of compounds, mice were fasted overnight. After measuring the body weight, 1 g/kg glucose was orally administrated and evaluated blood glucose level as above.

2.1.8 Determination of glycogen content and pancreatic insulin

Frozen tissue samples were weighed and hydrolyzed in 30% (wt/vol) KOH solution in a boiling water bath for 30 min. At 15 min of hydrolysis, tubes were shaken vigorously to facilitate digestion. After cooling on ice, 25 μL of 6% Na_2SO_4 and 750 μL of 100% ethanol were added. After overnight storage at -80 °C, samples were centrifuged at 10,000 × g for 15 min. The glycogen pellet was dried and dissolved in 200 μL of water in a water bath at 55 °C. After verifying that the pellet was completely dissolved, 800 μL of water was added (total volume of 1 mL). The aliquots (25 μL) of resulting glycogen solution were used for subsequent measurements. Glycogen content was determined using a Glycogen assay kit (Biovision, Milpitas, CA, USA) according to the manufacturer's instructions. Pancreatic tissue was weighed, homogenized, and extracted with 2 mL 1.5% HCl–75% ethanol buffer overnight with shaking in the cold room. For measurements, samples were centrifuged at 3000 × g for 10 min. Insulin level

in the supernatant was measured using an insulin kit (Morinaga Institute of Biological Science, Tokyo, Japan) after 1:3000 dilution.

2.1.9 Determination of plasma ALT and AST levels

Plasma ALT and AST levels were measured using Colorimetric Slides (Fuji DRI-CHEM Slide; GPT/ALT-PIII and GOT/AST-PIII, Tokyo, Japan)

2.1.10 RNA extraction, real-time PCR, and measurement of the mitochondrial DNA content

Total RNA was extracted using RNeasy plus mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Concentration and purity of total RNA samples were measured using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Kanagawa, Japan). cDNA generated using a High capacity cDNA synthesis kit (Life Technologies Japan, Tokyo, Japan) was analyzed by quantitative PCR using Power SYBR premix (Life Technologies Japan). Expression data were normalized to the geometric mean of the levels of the housekeeping gene β-actin to control for the variability in expression levels and were analyzed using the $2^{-\Delta\Delta CT}$ method. Mitochondrial DNA content was quantified using primers for both NADH

dehydrogenase subunit 1 (ND1, an index of mitochondrial DNA) and lipoprotein lipase (LPL, index of genomic DNA). ND1 levels were normalized to LPL DNA content. The primer sequences are presented in Table 1

	F 5′-3′	R 5′-3′
Gys1	TCAGAGCAAAGCACGAATCCAG	CATAGCGGCCAGCGATAAAGA
Ucp3	CTGAAGATGGTGGCTCAGGA	CCGCAGTACCTGGACTTTCATTA
Tfam	CCTTCGATTTTCCACAGAACA	GCTCACAGCTTCTTTGTATGCTT
Ppargc1a	GCGCCGTGTGATTTACGTTG	CGGTAGGTGATGAAACCATAGCTG
Cpt1b	TATCCCAATCATCTGGGTGCTG	GCGGATGTGGTTCCCAAAG
Acadl	CCAAGAAGAAGTGATTCCTCACCA	ACCAATGCCGCCATGTTTCT
Acadm	CAACACTCGAAAGCGGCTCA	ACTTGCGGGCAGTTGCTTG
mt-Nd1	CCCATTCGCGTTATCTT	AAGTTGATCGTAACGGAAGC
Lpl	GGATGGACGGTAAGAGTGATTC	ATCCAAGGGTAGCAGACAGGT
Gys2	ACTGCTTGGGCGTTATCTCTGTG	ATGCCCGCTCCATGCAGTA
Acox1	CTGTGGCATTGGCATCGTG	GCAAATCTGATGGCTTTGACTTGA
Cd36	GATGGCCTTACTTGGGATTGGA	GGCTTTACCAAAGATGTAGCCAGTG
Fabp1	AAGTACCAATTGCAGAGCCAGGA	GGTGAACTCATTGCGGACCA
Cpt1a	TGTTCAGCTCAGACAGTGGTTTCA	AGGATCCACCAGGATGCCATA
Ucp2	CAGTACCACAGCGCAGGTCA	TCACTACGTTCCAGGATCCCAAG
Pck1	GGTGTCATCCGCAAGCTGAA	CTGCTCTTGGGTGATGATGACTG
G6pase	GTGCAGCTGAACGTCTGTCTGTC	TCCGGAGGCTGGCATTGTA
beta-actin	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA

Table 1. List of primers used for real-time PCR.

2.1.11 Effects of chronic administration of 30 mg/kg AJS1669 on normal mice

Normal mice were treated for 4 weeks with 30 mg/kg daily dose of AJS1669, administered as described above for *ob/ob* mice. Body weight and amount of food consumed were monitored. Blood glucose and HbA1c levels were monitored and the glucose tolerance test was performed as described above.

2.1.12 Statistical analysis

Data are expressed as mean ± SE. Differences between multiple groups (vehicle- vs. AJS1669-treated) and two groups (vehicle- vs. pioglitazone-treated) were evaluated using the Step-down Dunnett's multiple comparison test and the Student's *t*-test, respectively, with $p < 0.05$ considered to represent statistically significant differences. All statistical analyses were performed using EXSUS 8 software package (CAC EXICARE Corporation, Tokyo, Japan)

2.2 Results

2.2.1 Identification of AJS1669 for validation tool of the hypothesis verification, through GYS1 activity

Sequence Similarity of human and mouse GYS1 proteins is 97%. Therefore, I chose human GYS1 protein for screening, because the screened-tool compounds will be able to use for both mice human, and might have a character of novel anti-diabetic agents. Through the assay of hGYS1 activity, AJS1669 was identified as an appropriate tool for hypothesis testing (Fig. 2A). AJS1669 activated hGYS1 in a dose-dependent manner when incubated alone (Fig. 2B, EC_{50} = 5.2 μM) , and elicit an strong effect on hGYS1 activity with G6P (Fig. 1C, EC_{50} = 0.037 μM).

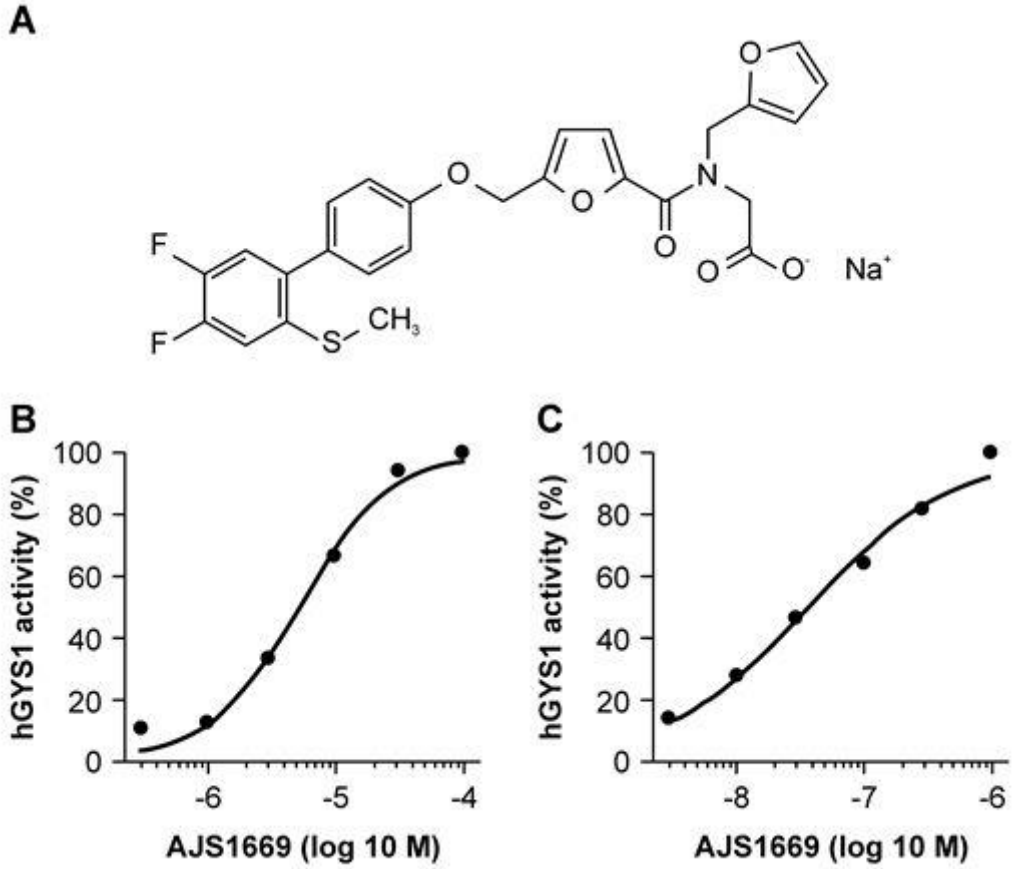


Fig. 2 Identification of the novel glycogen synthase activator AJS1669

(A) Chemical structure of AJS1669. (B) Effect of AJS1669 (0.3 – 100 μM) on human glycogen synthase 1 (hGYS1) activity. (C) Effect of AJS1669 (0.03 – 1 μM) on hGYS1 activity in the presence of 2.5 mM of glucose-6-phosphate (G6P)

2.2.2 GS activation in mouse tissue lysates and glycogen incorporation in human muscle cells by AJS1669

To assess the ability of AJS1669 to activate GS in each organ, mouse skeletal muscle and liver tissue were homogenized and [^{14}C] UDP-glucose was used to evaluate GS activity by measuring ^{14}C-labeled glycogen. Intriguingly, AJS1669 exhibited a concentration-dependent activation of GS in the skeletal muscle lysate (Fig. 3A), which was not observed in the liver lysate (Fig. 3B). Because activation of GS by G6P was detected, AJS1669 was determined to be a tool for evaluation of glycogen metabolism in skeletal muscle.

Next, to confirm the stimulation of glycogen production by AJS1669 in skeletal muscle tissue, human skeletal muscle cells were incubated with [^{14}C] glucose and AJS1669. The [^{14}C] glucose incorporated into cellular glycogen was measured by quantifying the produced [^{14}C] glycogen concentration. Incubation with AJS1669 alone increased [^{14}C] glycogen levels in a dose-dependent manner (Fig. 3C). AJS1669 showed a greater stimulation of glycogen production in the presence of glycogen phosphorylase inhibitor (GPI; Merck-Millipore cat no. 361515) than in the absence of GPI, whereas GPI alone did not increase the [^{14}C] glycogen level (Fig. 3C). These results indicate that the ability of AJS1669 to increase glycogen level is not a consequence of inhibition of

glycogenolysis, but rather reflects an activation of glycogen synthesis, with AJS1669 up-regulating the turnover of glycogen metabolism in the muscle cells by activating both glycogen synthesis and glycogenolysis (Fig. 3C).

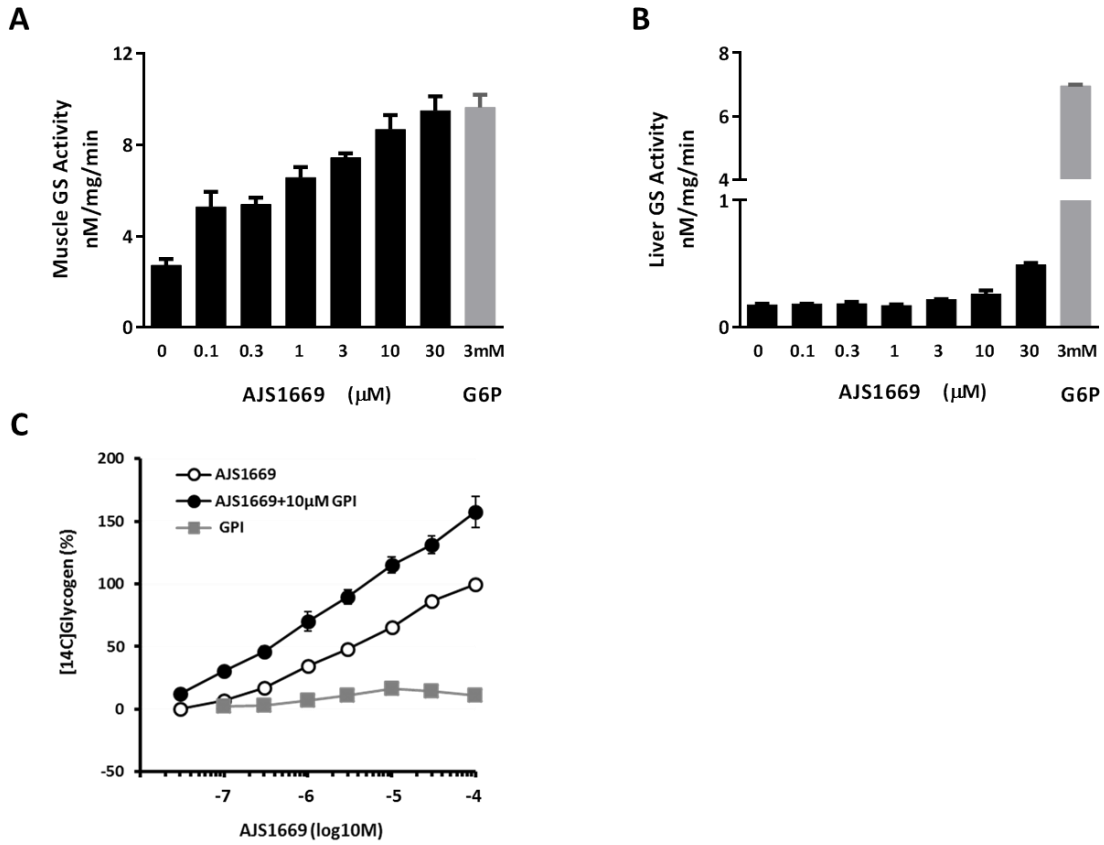


Fig. 3 Effect of AJS1669 on glycogen synthase activation in mouse tissue lysates in human muscle cells

(A) Activation of glycogen synthase (GS) in mouse muscle tissue lysates by AJS1669. (B) Activation of GS in mouse liver tissue lysates by AJS1669. (C) Effect of AJS1669 on glycogen accumulation in human muscle cells. Open circles: AJS1669, closed circles: AJS1669 + 10 μM glycogen phosphorylase inhibitor (GPI), closed squares: 10 μM GPI. G6P: glucose-6-phosphate

2.2.3 Oral glucose tolerance effect on diabetic *ob/ob* mice by single administration of AJS1669

After establishing this tool, AJS1669, I evaluated the oral glucose tolerance effect on diabetic *ob/ob* mice. However, the single administration of AJS1669 did not have any pharmacological effects on oral glucose test (Fig. 4)

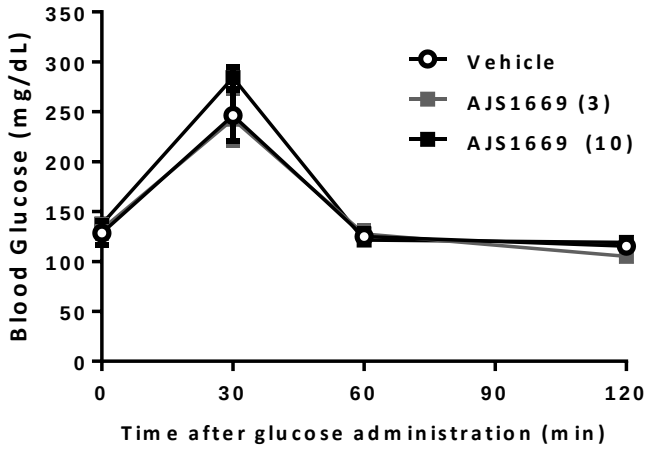


Fig. 4 Effects of single administration of AJS1669 and vehicle on glucose tolerance in *ob/ob* mice

Vehicle and AJS1669 were orally administrated with 1 g glucose.

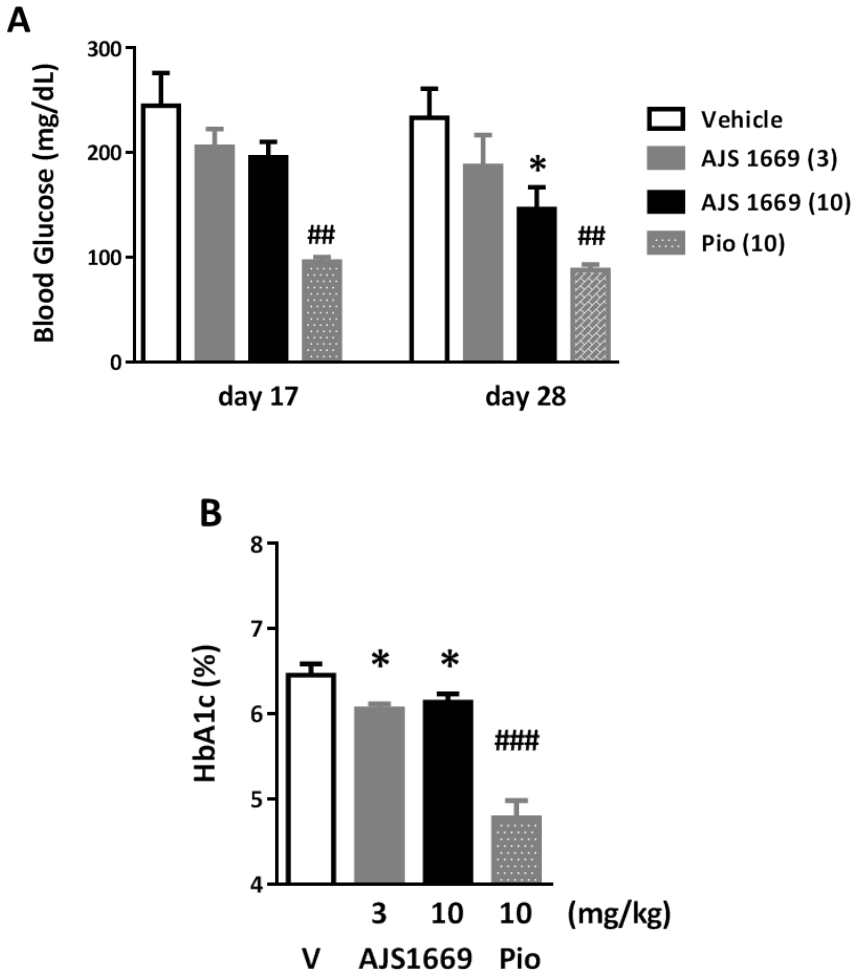
2.2.4 Evaluation of anti-diabetic effects of AJS1669 on diabetic *ob/ob* mice by chronic 4-week administration

Next, The ability of AJS1669 to act as a GYS1 activator with long-term administration was evaluated with pharmacological methods. *ob/ob* mice were orally administered 3 or 10 mg/kg AJS1669 twice daily. As a positive control, mice were administered pioglitazone, which is a PPARγ agonist known to regulate adipose tissue differentiation. Pioglitazone is an insulin sensitizer that causes significantly lower blood glucose levels and improves insulin resistance. On the other hand, it has an unattractive side effect profile that induces body weight gain as previously reported [31]. The group receiving 10 mg/kg AJS1669 exhibited a significant decrease in blood glucose level at day 28 (Fig. 5A). Additionally, a reduction in glycated hemoglobin (hemoglobin A1c, HbA1c) was observed at day 28 (Fig. 5B). HbA1c is an indicator of blood glucose levels over a period of red cell turnover. Lower HbA1c levels indicated that blood glucose was at a lower level during this period. These results showed AJS 1669 had hypoglycemic action.

Weight gain over the 4 weeks of administration was slightly lower in AJS1669-treated animals, as compared to the vehicle-treated group (Fig. 5C). No major differences in the amount of food consumed were observed between the treatment

groups (Fig. 5D). AJS1669 did not suppress food intake, unlike CNS-acting drugs.

Next, the plasma level of AJS1669 was measured using collected blood samples 2 h after the final administration. The plasma level rose linearly in a dose-dependent manner, with 0.92 ± 0.18 μM (mean ± S.E.M.) measured following administration of the 10 mg/kg dose (Fig. 5E). Because the EC50 was from 0.037 μM to 5.2 μM (G6P dependent), AJS1669 was sufficiently available at 2 h after administration.



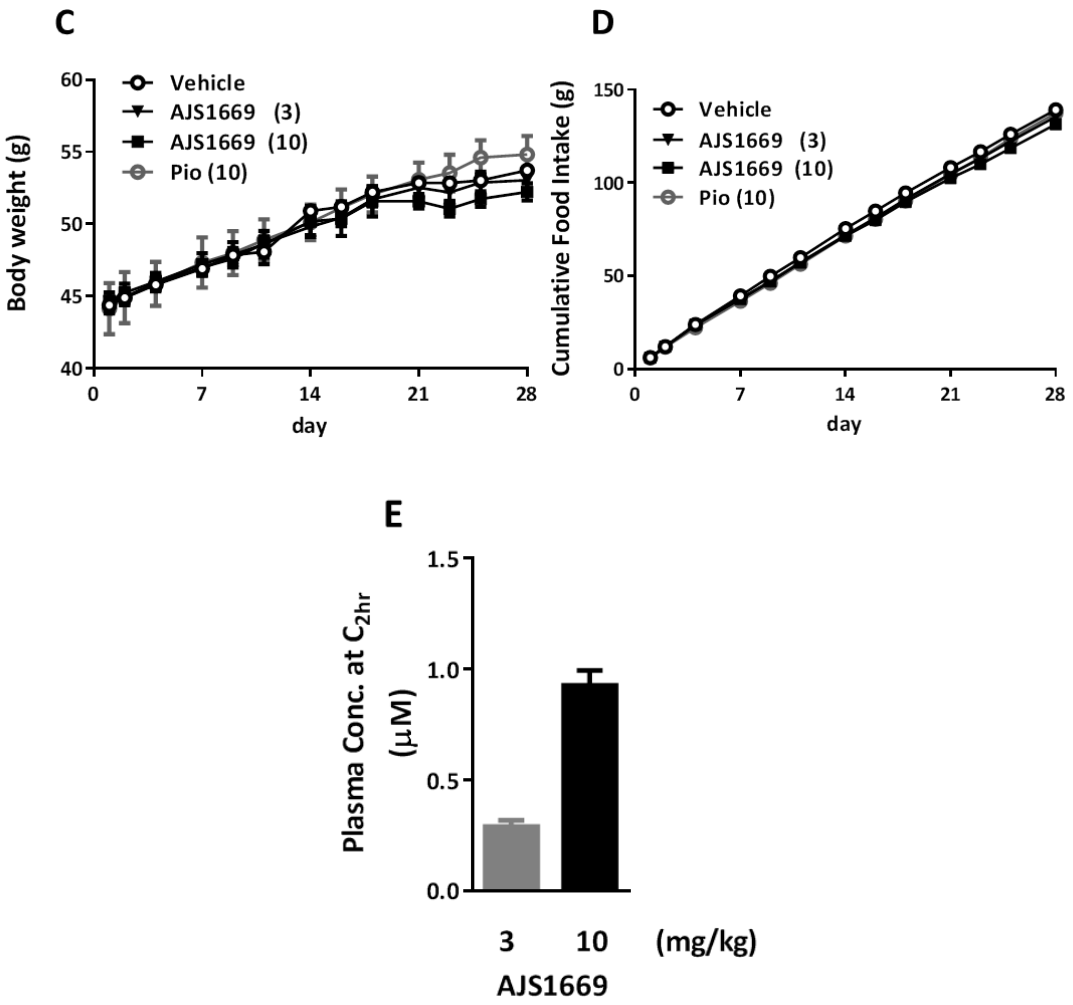


Fig. 5 Effect of chronic administration of AJS1669 on blood and pancreas parameters in *ob/ob* mice

Vehicle, AJS1669, and pioglitazone (Pio) were orally administrated twice daily for 4 weeks (n = 6-8). (A) Plasma glucose level, measured on days 17 and 28 of administration. (B) Blood level of glycated hemoglobin (HbA1c) on day 28. (C) Body weight and (D) cumulative food intake were monitored during the treatment. (E) Plasma concentration of AJS1669 at 2 hours following the final administration after 4 weeks. Data are expressed as mean ± S.E.M. *, $p < 0.05$ vs. vehicle-treated group, compared using Dunnett's multiple comparison test. ##, $p < 0.01$; ###, $p < 0.001$ vs. vehicle-treated group, compared using Student's *t*-test.

2.2.5 Oral glucose tolerance effect of AJS1669 on *ob/ob* mice as an insulin sensitizer

Because AJS1669 could improve the metabolic situation of diabetic mice, I evaluated whether AJS1669 could improve the diabetic condition with an oral glucose tolerance test as an insulin sensitizer. A glucose tolerance test in fasting condition measures how well the body is able to process glucose.

After the 4 weeks of AJS1669 administration, AJS1669 and food was not administrated to mice overnight. These mice were given 1 g/kg of D-glucose orally following a 16 h fast. Blood was collected at 0, 30, 60, and 120 min to measure glucose levels. Repeated administration of 3 or 10 mg/kg AJS1669 elicited significant dose-dependent decreases in blood glucose level for 30 min after administration (Fig. 6A). As a result, AJS1669 significantly improved glucose tolerance in a dose-dependent manner (Fig. 6B). Fasting blood glucose and insulin levels were measured at the same time, with the calculated homeostatic model assessment-insulin resistance (HOMA-IR). Low HOMA-IR numbers indicate high insulin sensitivity. HOMA-IR was decreased at higher doses in comparison with the vehicle-treated group, although there was no significant difference (Fig. 6C).

AJS1669 could improve glucose tolerance to a similar extent as pioglitazone. As previously described, pioglitazone causes excessive body weight gain through up-regulation of glucose uptake in WAT. If AJS1669 has an exercise-mimic effect, body fat mass should be reduced. Therefore, the body composition of these mice was evaluated.

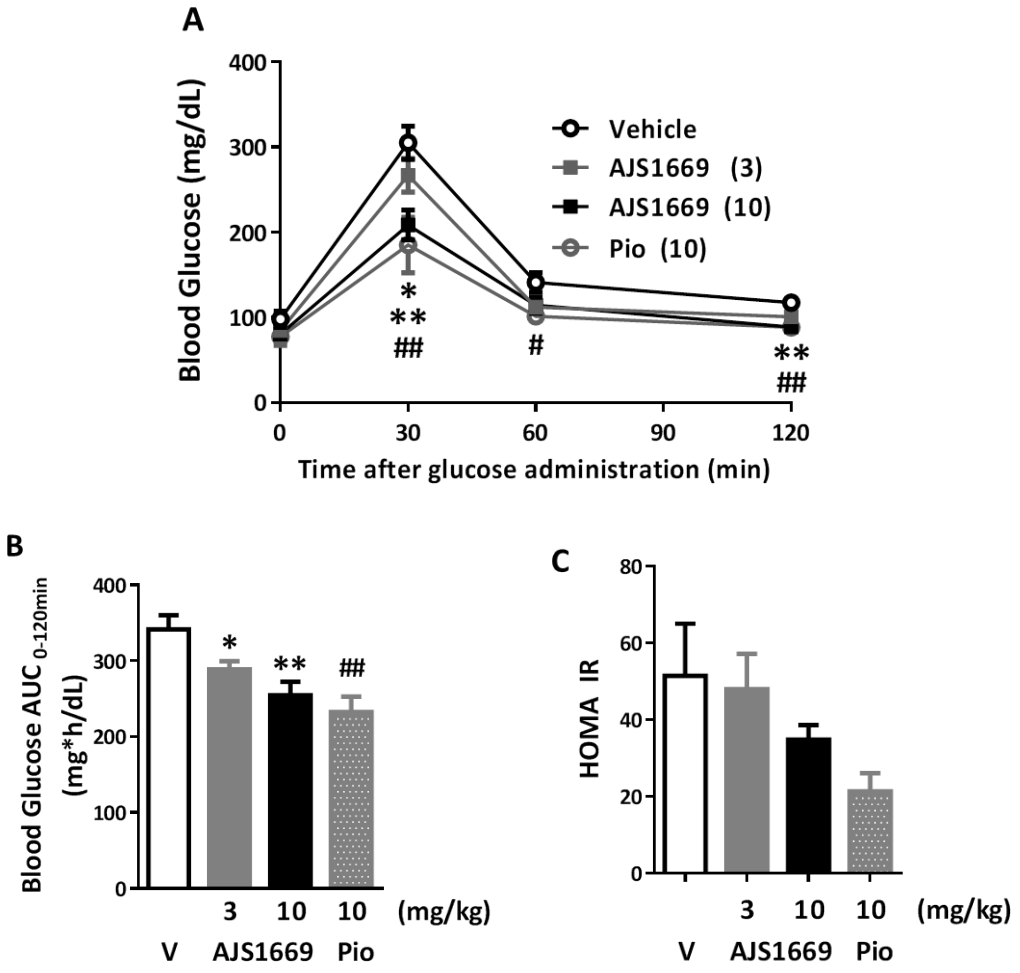


Fig. 6 Effects of chronic administration of AJS1669, pioglitazone, and vehicle on glucose tolerance in *ob/ob* mice

(A) Changes in blood glucose level in the oral glucose tolerance test (OGTT) performed in mice fasted overnight after the final administration. (B) Area under the curve from 0 to 120 min (AUC_{0-}

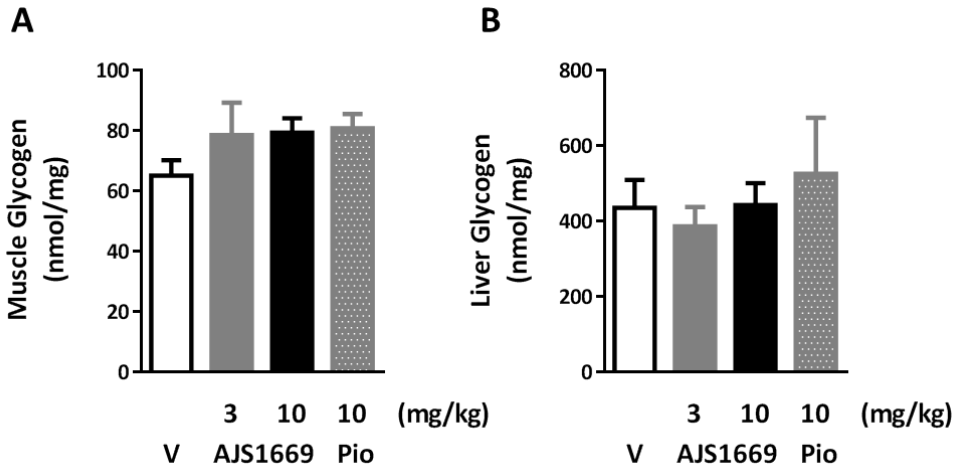
$_{120min}$) of blood glucose level (C) Homeostatic model assessment-insulin resistance (HOMA-IR) calculated using blood samples collected before the OGTT. Data are expressed as mean ± S.E.M. (n = 6-8). *, $p < 0.05$, **, $p < 0.01$ vs. vehicle-treated group compared using the Dunnett's multiple comparison test. #, $p < 0.05$; ##, $p < 0.01$ vs. vehicle-treated group, compared using Student's *t*-test. V: vehicle-treated; Pio: pioglitazone-treated

2.2.6 Chronic administration-effects of AJS1669 on body composition and blood parameters on diabetic *ob/ob* mice

As described in Fig. 3C, the GS activator AJS1669 induced glycogen accumulation with GPI in muscle cells. On the other hand, diabetic mice have lower glycogen levels than non-diabetic mice. I assumed the glycogen level in skeletal muscle would be increased in the AJS1669 treatment group, but this accumulation level was not excessive, because AJS1669 could regulate turnover of glycogen storage in muscle. Moreover, I predicted that liver glycogen would not change in the AJS1669 treatment group, because AJS1669 did not have strong activity in liver.

As expected, the glycogen level in skeletal muscle was increased dose-dependently and the glycogen level in both skeletal muscle and liver were not significantly increased after 4 weeks of repeated AJS1669 administration compared to vehicle-treated animals (Fig. 7A, B). Insulin content in the pancreas showed a tendency to increase at higher doses (Fig. 7C). This data indicated that the pancreas prevented glucose toxicity and maintained its function.

Treatment-induced changes in body composition were assessed using the EchoMRI™, with measurements performed on the day before the start of administration and following 4 weeks of administration. EchoMRI™ measures body composition without sacrificing mice. Body composition was measured by EchoMRI™ before fasting step of OGTT. A significant decrease in body fat mass was observed following administration of 10 mg/kg AJS1669. Conversely, animals treated with pioglitazone exhibited a significant elevation in fat mass (Fig. 7D). No difference in lean mass change was observed between the groups (Fig. 7E). These results support my hypothesis. I demonstrated that AJS1669 have an anti-diabetic effect through an exercise-mimic action.



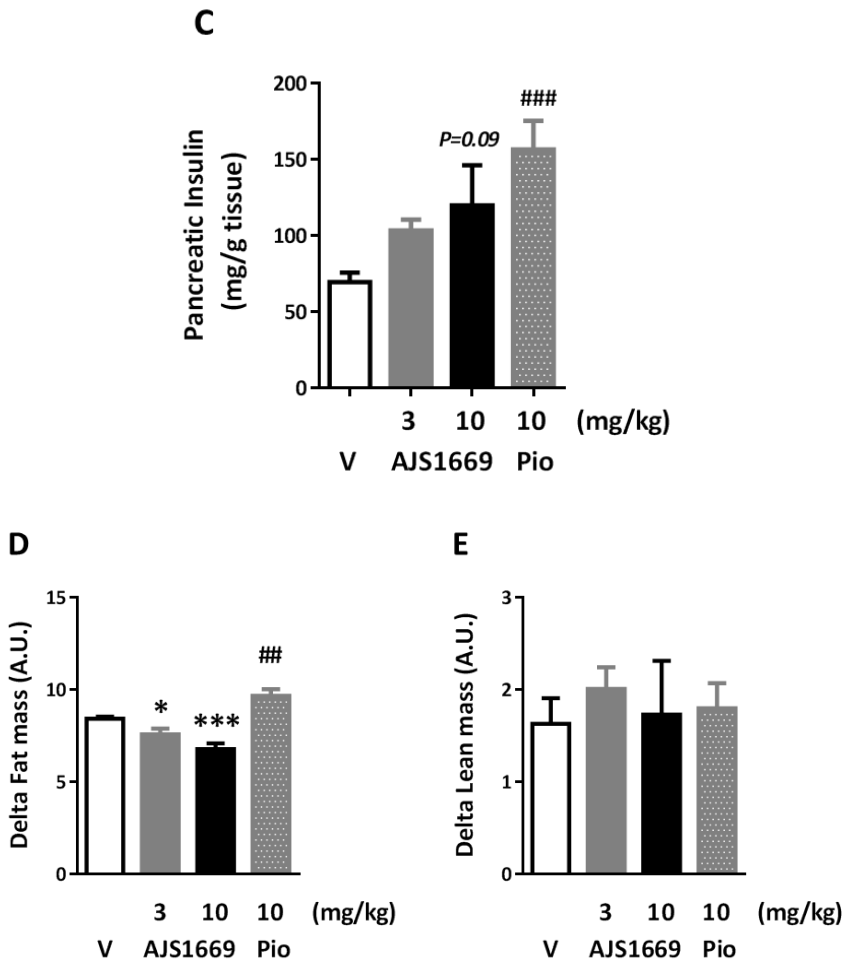


Fig. 7　Chronic effects of AJS1669 on body composition

(A) Muscle glycogen concentration. (B) Liver glycogen level. (C) Pancreatic insulin level. (D) Delta fat mass calculated using EchoMRI. (E) Delta lean mass calculated using EchoMRI. Data are expressed as mean ± S.E.M. (n = 6-8). *, $p < 0.05$, **, $p < 0.01$ vs. vehicle-treated group, compared using Dunnett's multiple comparison test. #, $p < 0.05$; ##, $p < 0.01$ vs. vehicle-treated group, compared using Student's *t*-test. V: vehicle-treated; Pio: pioglitazone-treated

2.2.7 Effect of repeated administration of AJS1669 on mitochondrial biogenesis in skeletal muscle and hepatic fatty acid oxidation

AJS1669 could activate GS and accelerate turnover of glycogen accumulation in skeletal muscle. Chronic administration of AJS1669 exerted anti-diabetic effects and improved insulin sensitivity. An acute bout of exercise increases skeletal muscle glucose uptake. However, chronic exercise training improves mitochondrial function, increases mitochondrial biogenesis, and increases the expression of numerous metabolic genes [32]. I assessed whether AJS1669 caused an aerobic exercise-mimic effect or anaerobic exercise-mimic effect.

First, I checked that AJS1669 was not a GS protein inducer. In skeletal muscle, treatment with AJS1669 elicited no change in *Gys1* mRNA levels. A trend towards a decrease was observed in mRNA encoding Uncoupling Protein 3 (*Ucp3*), a mitochondrial uncoupling molecule. A significant increase was observed, however, in mRNA encoding mitochondrial transcription factor A (*Tfam*), which is involved in the replication and translation of mitochondrial DNA (Fig. 8A). Subsequent evaluation of mRNA level of genes involved in fatty acid oxidation detected a trend towards elevated levels of carnitine palmitoyltransferase 1B (*Cpt1b*), which plays an important role in skeletal muscle mitochondrial fatty acid β-oxidation, long chain acyl-CoA

dehydrogenase (*Acadl*), and medium chain acyl-CoA dehydrogenase (*Acadm*), which are essential for converting these particular fatty acids to energy and causes fatty acid oxidation in mitochondria, following AJS1669 treatment. Additionally, AJS1669 administration increased mitochondrial DNA content in skeletal muscle tissue (Fig. 8B). These results demonstrate the possibility that AJS1669 improved mitochondrial biogenesis. Serum lactate level was not increased 2 h after the final administration of AJS1669 in this chronic test at 4 weeks. Lactate level was not changed in the AJS1669 treatment group compared to the vehicle treatment group (Fig. 8D).

The gene expression in liver was also evaluated. Significant increases in *Cd36*, fatty acid binding protein 1 (*Fabp1*), *Cpt1a*, and *Ucp2* genes involved in fatty acid oxidation were observed (Fig. 8C). No change, however, was observed in the transcription of phosphoenolpyruvate carboxykinase 1 (*Pck1*) and Glucose 6-phosphatase (*G6pase*) genes, which are involved in hepatic gluconeogenesis. Fatty acid oxidation was enhanced in liver as well as in skeletal muscle. AJS1669 did not have the potential to suppress hepatic glucose production as well as pioglitazone [33].

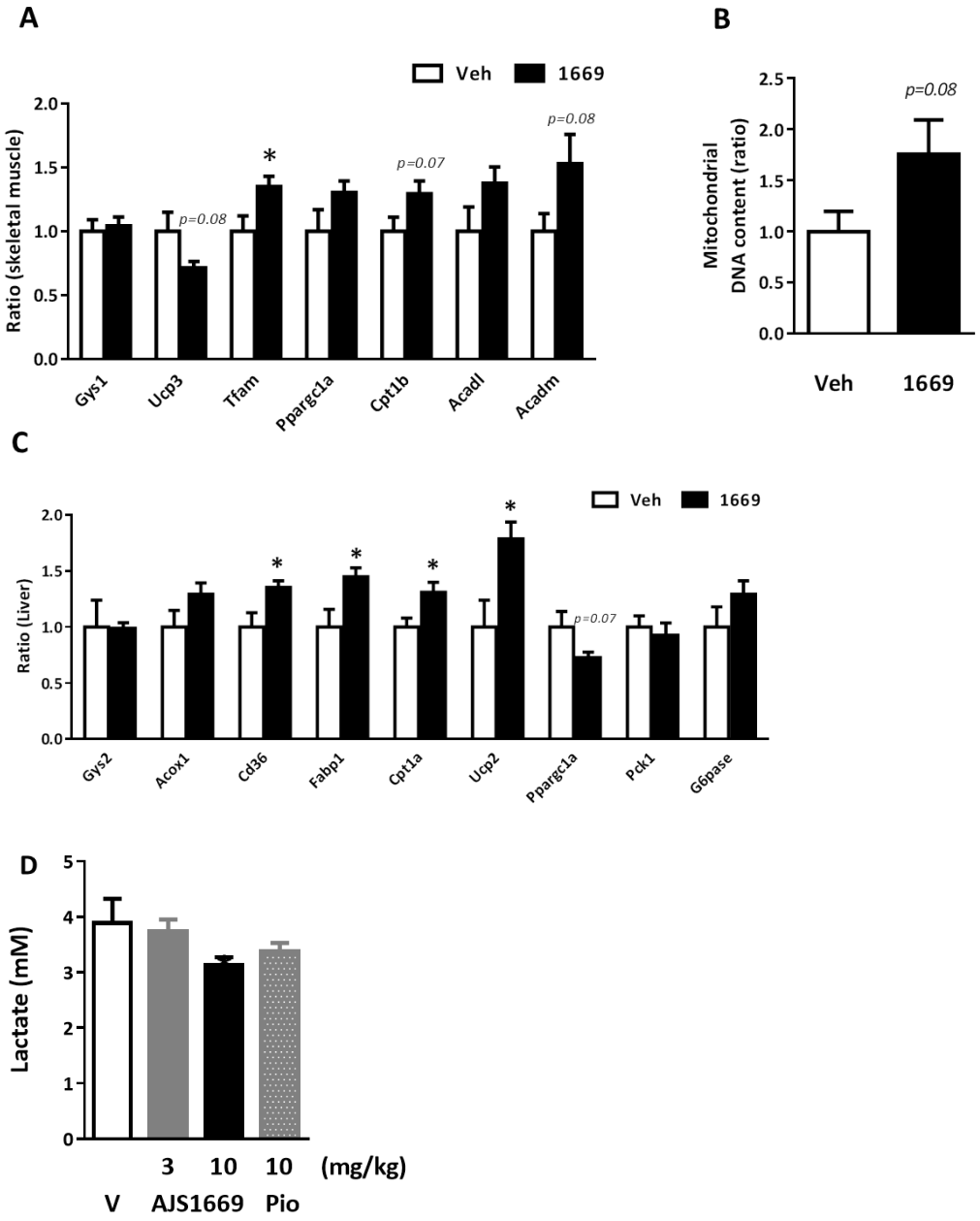


Fig. 8 Effects of AJS1669 or vehicle chronic administration on gene expression in muscle and liver tissue

(A) Gene expression level in mixed gastrocnemius muscle. (B) Mitochondrial DNA content, as assessed by mitochondrial DNA copy number. (C) Gene expression level in hepatic tissue. (D) Serum lactate level at the end of chronic administration. Data are expressed as mean ± S.E.M. (n = 7-8). *, $p < 0.05$ vs. vehicle-treated group by Student's *t*-test. V: vehicle-treated; Pio: pioglitazone-treated; 1699: AJS1669-treated

2.2.8 Effects of chronic administration of 30 mg/kg AJS1669 on normal mice

Although AJS1669 did not directly activate liver GS, the gene expression levels of hepatic fatty acid oxidation were elevated. Frozen blood samples and liver samples were used for confirming beneficial effects in liver by AJS1669. The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) increase with increase in age. In *ob/ob* mice, an agent regulating fatty acid metabolism in liver can decrease AST and ALT level [32, 33]. Thus, serum levels of ALT and AST are not only a liver injury index, but also related to hepatic steatosis in *ob/ob* mice. In addition to ALT and AST levels, triglyceride level (TG) in liver was measured as an index of liver lipid accumulation.

As expected, ALT and AST had a decreasing trend in AJS1669-treated animals compared to vehicle-treated animals (Fig. 9A, B). Moreover, AJS1669 did not cause liver injury based on its chemical structure. On the other hand, an insignificant change was observed in the AJS1669 treatment group (Fig. 9D).

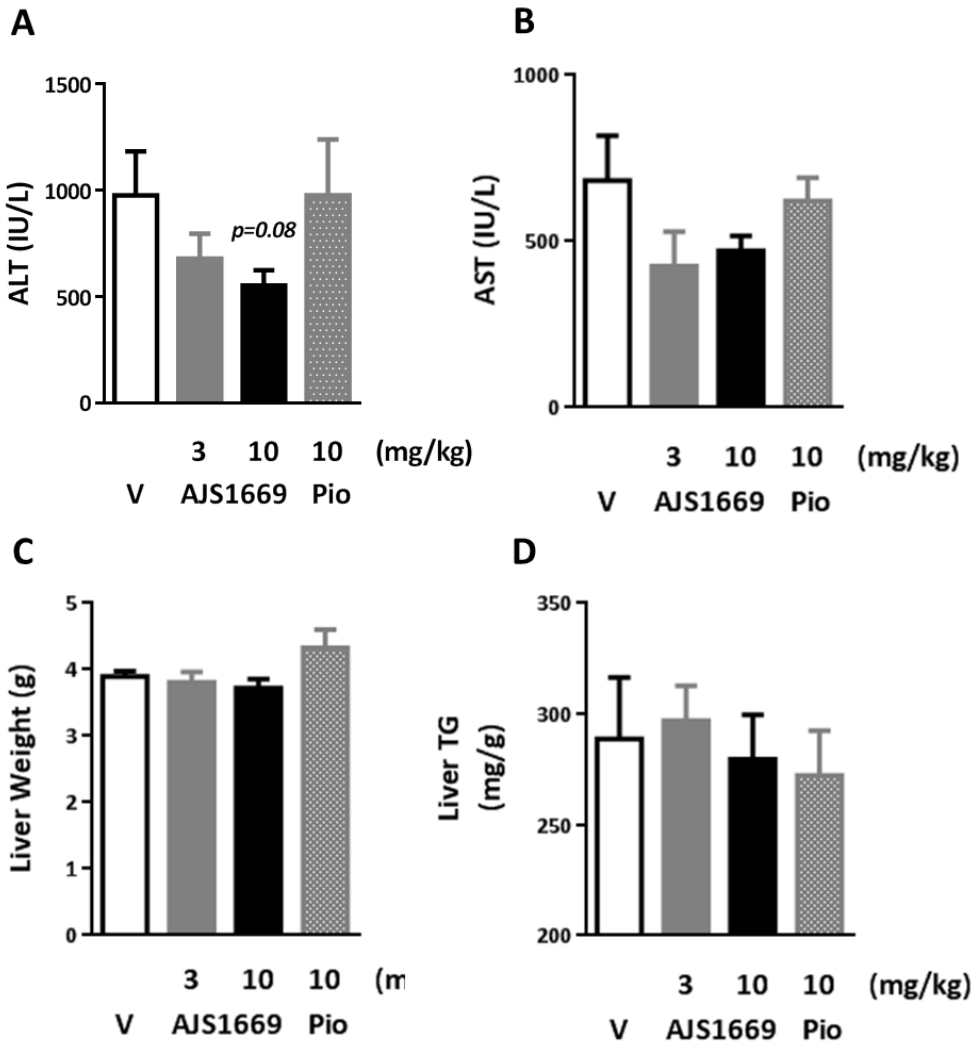


Fig. 9 Liver safety index

(A) Alanine aminotransferase (ALT) and (B) aspartate aminotransferase (AST) levels. (C) Liver weight and (D) Liver Triglyceride (TG) level. V: vehicle-treated; Pio: pioglitazone-treated; 1699: AJS1669-treated

2.2.9 Evaluation of safety index in chronic administration of 30 mg/kg AJS1669 on normal mice

AJS1669 is the activator of muscle GS. If AJS1669 improves impaired glycogen turnover, this compound should not affect normal mice which have unbroken glycogen metabolism. To confirm this logic, normal mice were treated for 4 weeks with 30 mg/kg AJS1669 twice daily, a chronic dose three times higher than the effective dose in *ob/ob* mice. No significant differences were observed between AJS1669- and vehicle-treated animals in body weight, amount of food consumed, blood glucose level, HbA1c level, and glucose tolerance (Fig. 10A-E). Additionally, no major changes in plasma ALT and AST level were observed (Fig. 10F, G).

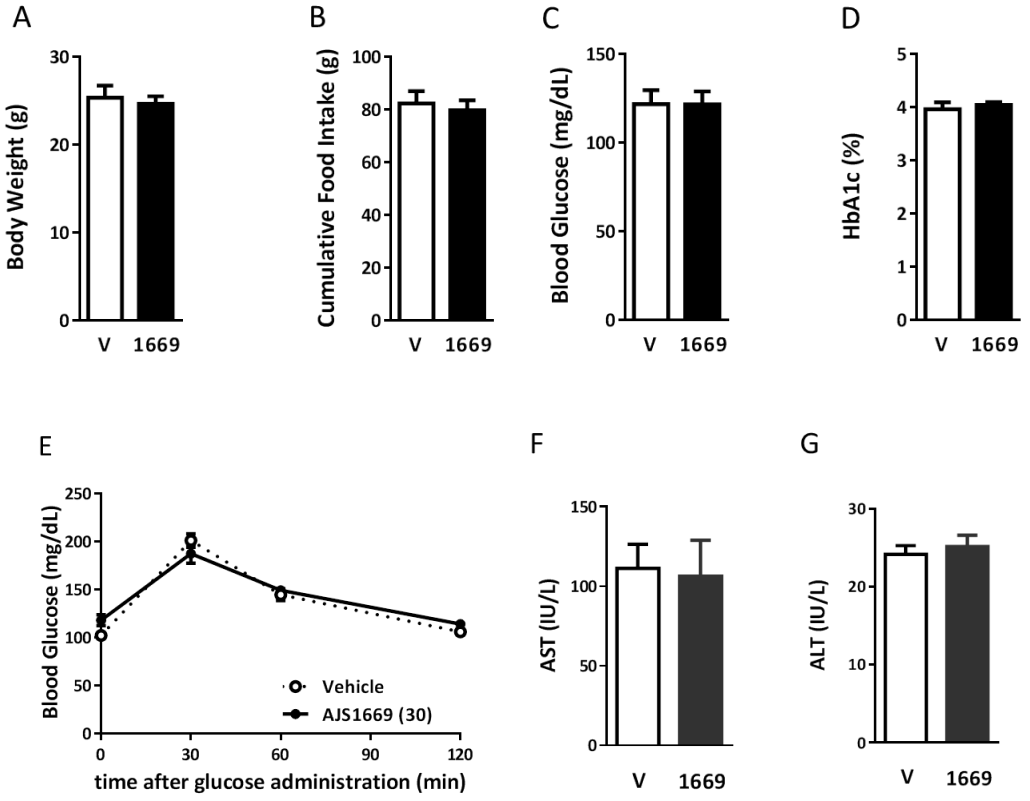


Fig. 10 Effects of chronic administration of 30 mg/kg AJS1669 on normal mice

(A) Changes in body weight. (B) Cumulative food intake. (C) Basal blood glucose level on day 28. (D) HbA1c level, assessed on day 28. (E) Oral glucose tolerance test (OGTT) performed on day 29. (F) Plasma aspartate aminotransferase (AST) level on day 32. (G) Plasma alanine aminotransferase (ALT) level on day 32. (n = 7-8) V: vehicle-treated; 1699: AJS1669-treated

2.3 Discussion

I assessed the hypothesis that up-regulation of muscle-specific GS activation would be an exercise-mimic through regulation of glycogen turnover (Fig. 1). To confirm this hypothesis, several approaches are considered. I decided to use the pharmacological approach. However, the concept of identifying the pharmacological tool of muscle GS activator was an unconventional idea in pharmacological theory, because enzyme activators are more difficult to identify than inhibitors.

AJS1669 was synthesized as a novel allosteric activator of GYS1 (Fig. 2A). *In vitro* testing showed this compound activated hGYS1 in a dose-dependent manner when administered alone, and its action was further potentiated in the presence of 2.5 mM G6P (Fig. 2B). A lower dose of AJS1669 with G6P elicits an effect on hGYS1 activity, compared to the dose-response relationship without G6P (Fig. 2C). These facts indicated that AJS1669 had a potential of strong efficacy under high G6P concentration. High concentration of G6P is converted by high glucose. Therefore, AJS1669 might be more effective in diabetic subjects.

Prior to evaluating its action *in vivo*, I confirmed that the muscle specific activity of AJS1669 using skeletal muscle lysate and liver lysate from C57BL/6 mice. AJS1669 exhibited a concentration-dependent activation of GS in the skeletal muscle lysate,

which was not observed in liver lysate (Fig. 3A,B). GS activity is regulated by G6P, which is converted from glucose by hexokinase and glucokinase. Glucokinase is mainly in liver and has low affinity to bind to glucose. On the other hand, hexokinase in skeletal muscle has high affinity for glucose. Even though AJS1669 has a same level of GS activity in between these 2 organs, AJS1669 exerted GS activity in skeletal muscle from low-dose glucose due to the activation of hexokinase. Thus, AJS1669 acted potently in skeletal muscle, and exhibited greater activation with a lower dose in the presence of high G6P concentration

AJS1669 increased the glycogen level in a concentration-dependent manner in human skeletal muscle cells. These data demonstrate that AJS1669 was a low-dose skeletal muscle-specific GS activator in mice, which would act on both mouse and human. To assess the effects on the phenotypic level, skeletal muscle cells were incubated with AJS1669. AJS1669 increased the glycogen level in a concentration-dependent manner. Intriguingly, incubation with AJS1669 and GPI increased glycogen levels higher than AJS1669 alone. On the other hand, no increase was observed following incubation with GPI alone. These observations suggest that AJS1669, in addition to promoting glycogen synthesis, caused a degradation of some glycogen, apparently enhancing the glycogen metabolism turnover. G6P is an important allosteric

inhibitor of glycogen phosphorylase (GP) and this enzyme is in an inactive state in resting muscle [34]. On the other hand, activation of GS causes G6P consumption. I hypothesize that AJS1669 forcedly decreased G6P levels and this decreased-G6P switched on GP activity. Because up-regulated glycogen turnover is one aspect of aerobic exercise, AJS1669 was a prospective compound that might cause an anti-diabetic effect through exercise-mimic activity. Thus, AJS1669 acted potently in skeletal muscle, and exhibited greater activation with a lower dose in the presence of high G6P concentration, I decided to use AJS1669 for verifying my hypothesis.

At first, I evaluated oral glucose tolerance effect by OGTT of AJS1669 single administration. Unfortunately, AJS1669 did not exhibit a blood glucose-lowering effect after a single administration (Fig. 4).

Pioglitazone, which is well known as an insulin sensitizer, also does not affect diabetic animals after a single administration, but lowers blood glucose by repeated administration. The underlying mechanism of pioglitazone is activation of peroxisome proliferator-activated receptor gamma (PPARγ) . PPARγ, which is a member of the nuclear receptor superfamily. PPARγ is highly expressed in adipose tissue and regulates adipogenesis and insulin sensitivity [35,36]. Reportedly, PPARγ and PPARδ agonists do not directly activate glycogen synthesis, but rather act to enhance glycogen formation

by stimulation of insulin receptors [37,38]. Therefore, I decided to use pioglitazone as the positive control for evaluation of the anti-diabetic effect of AJS1669.

Although the structures of AJS1669 and pioglitazone are different, I confirmed whether AJS1669 potentiates PPAR activity using a reporter assay system. Each PPAR ligand-binding domain was overexpressed in CV-1 kidney fibroblast cells derived from African green monkey as a protein fused to the yeast GAL4 DNA-binding domain and the luciferase activity was measured. AJS1669 did not elicit any increase in PPARα, γ, or δ activity at a concentration of 100 μM (Table 2), which is higher than the physiological level resulting from administration of 10 mg/kg AJS1669 (Fig. 3E).

	PPAR α		**PPAR γ**		**PPAR δ**	
	EC_{50} (μM)	E_{max} (%)	EC_{50} (μM)	E_{max} (%)	EC_{50} (μM)	E_{max} (%)
AJS1669	>100	9	>100	29	>100	0
WY-14643	11	100				
Pioglitazone			0.28	100		
GW501516					0.0027	100

Table 2. Effect of AJS1669, WY-14643, pioglitazone and GW501516 on PPAR subtypes.

WY-14643 = PPARα agonist, Pioglitazone = PPARγ agonist, GW501516 = PPARδ agonist

Based on the potential of AJS1669 for improving glycogen metabolism determined through *in vitro* tests, we selected *ob/ob* mice, which are hyperinsulinemic, bulimic, and

do not actively exercise. Diabetic-induced mice (DIO) were not used because they are easily affected by food consumption and exercise capacity. Additionally, *db/db* mice were not used because they may experience weight loss as a result of severe hyperglycemia. To determine the quantity for oral administration to mice, we sought a value in between the *in vitro* activity and blood concentration level. Because the 3 mg/kg AJS1669 concentration *in vivo* (Fig. 3E) was sufficient to activate GYS1 *in vitro*, and following the 3Rs policy, we decided to use 10 mg/kg as the maximum dose for the *in vivo* experiments. The concentration of AJS1669 was much higher in liver than in muscle. As previously reported, the furanyl group of AJS1669 has high stability with respect to liver metabolism, as it is a 5-membered ring [39].

Repeated administration demonstrated the ability of AJS1669 to lower the blood glucose level and decrease the HbA1c level (Fig. 5). Pioglitazone also does not affect diabetic animals after a single administration, but exerts anti-diabetic effects in chronic treatment. An insulin sensitizer could improve diabetic body conditions, and that improvement includes enhanced glucose uptake and consumption throughout the body. The function of pioglitazone is to differentiate adipose tissue into small adipose tissue and provide beneficial adipokines to the body. Although AJS1669 did not reduce blood glucose levels as much as pioglitazone (Fig. 5), if AJS1669 could improve insulin

sensitivity in skeletal muscle, glucose tolerance would be improved. An oral glucose tolerance was performed after 4 weeks of repeated administration. In the analysis with area under curve, AJS1669 ameliorated glucose tolerance to the same level as pioglitazone (Fig. 6). These results indicate that AJS1669 is an insulin sensitizer with a different mechanism than pioglitazone.

Pioglitazone has not only a great potential for reducing blood glucose level, but also causes differentiation of adipose tissue and blockage of fatty acid release from adipose tissue [40]. Hence, it changes fat distribution and causes body weight gain. Thus, it is not an appropriate drug for Type 2 diabetes patients. AJS1669 could improve not only blood glucose level but also glucose tolerance. AJS1669 did not cause excessive body weight gain (Fig. 5C). Given that AJS1669 was an exercise-mimic through glycogen turnover, I expected body fat mass would be decreased.

Body composition assessment using EchoMRI™ showed that pioglitazone treatment significantly increased whole body fat mass whereas AJS1669 markedly decreased the fat mass (Fig. 7C). Interestingly, repeated administration of AJS1669 did not elicit a major change in the amount of food consumed compared to the amount consumed by the vehicle-treated group. These results demonstrate that AJS1669 could exert anti-diabetic effects through a different mechanism than pioglitazone, and the underlying

mechanism of AJS1669 might be similar to the exercise effect.

Intriguingly, although muscle glycogen levels in all animals in the group treated with 10 mg/kg AJS1669 increased in comparison with the vehicle-treated group levels, the change was not significant. The same results were observed for pioglitazone-treated animals. Both AJS1669 and pioglitazone improved glucose tolerance (Fig. 6A) suggesting that glycogen levels could be increased by administration of these compounds. In this study, utilizing *ad libitum* feeding in *ob/ob* mice, a significant increase in muscle glycogen may not have been detected (Fig. 7A). However, AJS1669 increased the glycogen level in muscle cells, and this level was additively increased under treatment with both AJS1669 and GPI (Fig. 3C). Therefore, AJS1669 increased not only glycogen accumulation but also glycogen degradation, and this machinery could improve glycogen metabolism. In exercise, both glycogen accumulation and glycogen degradation are involved in energy homeostasis and regulation of insulin sensitivity [41]. Since AJS1669 improves the energy capacity of skeletal muscles, it was necessary for us to choose diabetic animals for evaluating this compound. Evaluation of these mice showed that pioglitazone treatment significantly increased the whole body fat mass (a harmful side effect), whereas AJS1669 markedly decreased the fat mass as we hypothesized (Fig. 7A). AJS1669 thus appears to induce both glycogen synthesis

and glycogen turnover directly, and therefore may be the first in a new class of insulin sensitizers with aerobic exercise-mimic effect through the regulation of glycogen turnover.

To examine the possibility that the effect of AJS1669 on muscle glycogen causes a metabolic response in muscle, β-oxidation-related genes were also evaluated. Mitochondria are critical organelles responsible for regulating the metabolism of skeletal muscle. Mitochondria exhibit plasticity by volume, structure and function in response to chronic exercise, aging and disease [42]. Dysregulation of mitochondrial function has implications for diabetes [43]. Therefore, I evaluated increases in expression level of β-oxidation-related genes involved in lipid metabolism in skeletal muscle and liver, as well as genes involved in mitochondrial biogenesis. As I expected, the up-regulation of these genes were observed following 4 weeks of repeated administration of AJS1669 (Fig. 8A, C). While AJS1669 treatment did not modify the expression of *Gys1* in skeletal muscle tissue, it significantly increased the expression of *Tfam*, a gene involved in the stability and transport of mitochondrial gene transcripts. TFAM is ap promoter specific enhancer of mitochondrial DNA [44]. Additionally, a trend towards an increase in expression of genes involved in β-oxidation, such as *Cpt1b* and *Acadm*, was also observed. CPT1B is the muscle isoform of CPT, which is the rate-

limiting enzyme governing long-chain fatty acid entry into mitochondria. Deletion of one allele of CPT1B in mice caused impairment of muscle insulin signaling that led to severe insulin resistance upon high fat feeding [45]. The *Acadm* gene encodes an enzyme called medium-chain acyl-CoA dehydrogenase (MCAD). MCAD is essential for fatty acid oxidation, which is the multistep process that breaks down (metabolizes) fats and converts them to energy [46]. Thus AJS1669 changed mitochondrial biogenesis and medium and long chain fatty acid oxidation related genes in skeletal muscle. These regulation of genes involved in mitochondrial biogenesis a by AJS1669 is likely to be associated with an increase in mitochondrial DNA content (Fig. 8B) and that of oxidation genes is related with decrease in fat mass (Fig. 7D). It is well known that aerobic exercise training changes in insulin sensitivity and muscle oxidative capacity [47]. Recently, CPT1B and MCAD are recognized as aerobic exercise related genes [48]. Therefore, I have demonstrated that AJS1669 has an exercise mimic effect through gene regulation.

In liver, no significant modulation of genes controlling gluconeogenesis genes, such as *pck1* and *G6Pase* was observed. As evidenced by the lack of effect on the liver observed through *in vitro* experiments, AJS1669 may not alter glucose metabolism by directly affecting the liver, because the excessive liver glycogen accumulation would be

inhibited by the fat mass decrease [26]. Thus, in muscle, the glycogen level was changed, mitochondrial biogenesis gene expression increased, and β-oxidation-related gene expression increased. On the other hand, in liver, glycogen was not changed, but surprisingly, lipid metabolism gene expression was significantly increased. However, TG levels in liver of AJS1669-treated animals were the same as vehicle-treated. There are some possibilities for this discrepancy. Following up-regulation of the β-oxidation-related genes that are involved in lipid metabolism in skeletal muscle, lipid flow might be changed across the body to affect the level of oxidation in liver. Even though lipid gene levels were changed, it might take more time to influence phenotypes. In addition, *ob/ob* mice are a spontaneous diabetic model and show a severe diabetic phenotype. If AJS1669 is provided earlier, the results might be more significant. Additionally, I assessed whether AJS1669 is a safe drug in comparison with pioglitazone. 4 weeks of repeated administration of AJS1669 did not elevate plasma ALT and AST level, and no abnormalities were detected in the liver safety profile (Fig. 9A, B). Similarly, C57BL/6 mice treated for 4 weeks with 30 mg/kg exhibited no change in body weight, food intake, blood glucose level, or ALT and AST levels (Fig. 10). AJS1669, therefore, does not appear to reduce fat mass by any toxic mechanism.

Another factor in the effect on body composition may be related to the difference in

glycogen metabolism between muscle and liver tissue. In mice, the basal amount of glycogen in skeletal muscle at satiation is approximately one-tenth of that in liver, and glycogen metabolism in liver is thought to affect glucose metabolism more significantly than in skeletal muscle [49]. In humans, however, skeletal muscle accounts for nearly 40% of body composition [50] and plays a central role in glucose metabolism (Fig. 11) [51]. Results of studies using mouse tissue lysates showed AJS1669 elicited a stronger effect in skeletal muscle than liver (Fig. 3A, B). However, intense effects were not achieved in mice (Fig. 5). I therefore postulate that the pharmacological effect of AJS1669 observed in mice should be far more pronounced in humans (Fig. 11).

In skeletal muscle tissue of patients suffering from type 2 diabetes or obese patients, a decrease in mitochondrial oxidative capacity has been reported [52,53], along with lower expression levels of genes related to mitochondrial biogenesis [54,55]. Increases in physical activity and weight loss interventions in these patients are the main inducer of improvements in mitochondrial functional capacity and these mitochondrial changes are associated with improved insulin resistance [56,57]. Considering the association between GS activity and mitochondrial function, a number of unanswered questions remain regarding the molecules that are directly involved. It is, however, conceivable that repeated administration of AJS1669 alters glycogen metabolism and may therefore

mimic the effects of exercise and weight loss.

Past reports have suggested that effects of physical exercise are beneficial for type 2 diabetes patients through altering glycogen metabolism. However, whether enhancing muscle-specific GS activity attenuated diabetes was unknown. Through these experiments, one aspect of an exercise-mimic effect has occurred through a muscle-specific GS activator. AJS1669 enhanced glycogen metabolism, improved mitochondrial biogenesis, and consequently elicited a reduction in body fat mass and exhibited anti-diabetic activity. Fortunately, AJS1669 does not have side effects in the pharmacological dose range. Therefore, AJS1669 exhibits potential for use as a new insulin resistance-improving drug with a mechanism of action distinct from that of pioglitazone. AJS1669 did not elevate plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). AJS1669 was administered at 30 mg/kg, which is 3 times dose of pharmacological effects, for 4 weeks to C57BL/6 mice. There were no observed increase in weight gain or decrease in blood glucose levels. These evaluations suggest that AJS1669 might be a novel insulins sensitizer that safely improves glycogen metabolism in skeletal muscle and lowers blood glucose levels.

Finally, I estimated translation efficiency in human body, beucause AJS1669 has a potential for human drug. Comparison with pioglitazone, AJS1669 did not lower blood

glucose level. Pioglitazone induces adipocyte differentiation *in vivo*. On the other hand, AJS1669 showed their efficacy through muscle glycogen turnover. In postabsorptive humans, there are 100 g of glycogen in the liver and 400 g of glycoge in muscle [58]. As in mice, 200mg in the liver and 50mg in muscle. These facts demonstrates the hypoglyemic action is mainly regulated by skeletal muscle in human. Even if AJS1669 could provide to human, the pharmacological effect might be exerted than Actos® (pioglitazone) (Fig. 11).

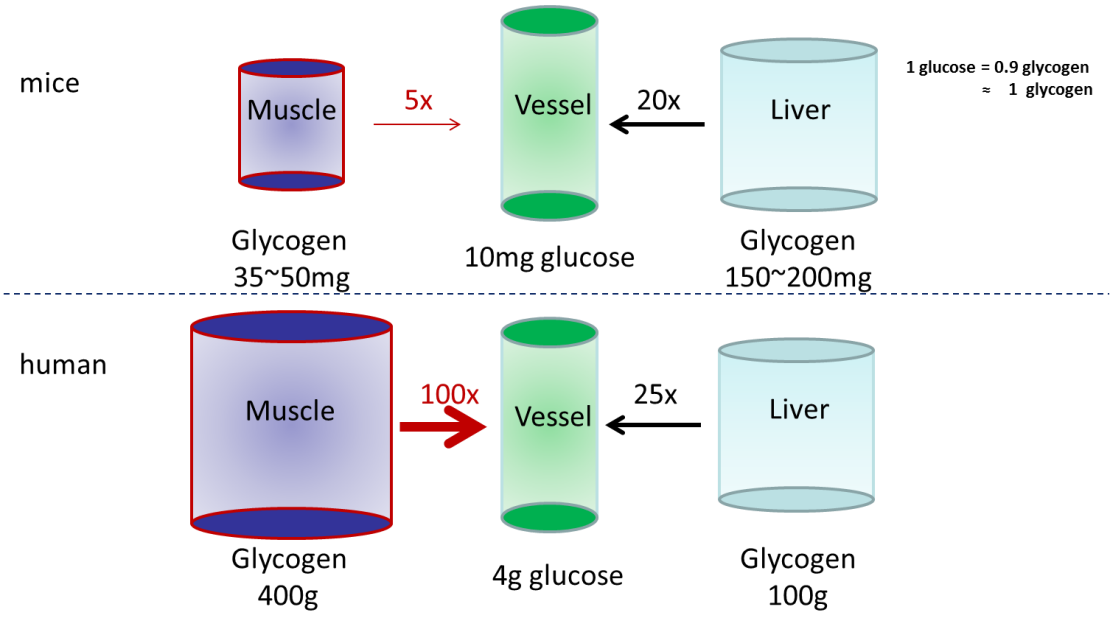


Fig. 11 Schematic model of differences in glycogen metabolism between muscle and liver tissue in mice and humans.

The hypoglycemic action exerted by glycogen metabolism from the conversion of its glycogen accumulation ability and organ weight and the influence on blood glucose level exerted by liver glycogen accumulation is about four times stronger than skeletal muscle in mice. On the other hand,

the influence of blood glucose level by glycogen metabolism in human is about 4 times stronger in skeletal muscle than in liver. Therefore, the medicinal effect in mice is about 10 ~ 16, which is considered approximately twice as strong.

Glycogen turnover is accelerated in aerobic exercise and this turnover is lower in diabetic patients. Notably, this is the first demonstration that acceleration of glycogen turnover in skeletal muscle causes exercise mimic effect, lowering blood glucose, decreasing body fat mass and intensifying mitochondrial biogenesis. Moreover, novel GS activator, AJS1669 has a possibility to be novel insulin sensitizer. Here I elucidate GS activation of muscle specific in diabetic animals leads to exercise mimic through the perspective of physiology and molecular biology [59],.

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