A Novel Acetyl-CoA Carboxylase 2 Selective Inhibitor Improves Whole-Body Insulin Resistance and Hyperglycemia in Diabetic Mice through Target-Dependent Pathways

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ABSTRACT

Excess intramyocellular lipid (IMCL) deposition in skeletal muscle is closely associated with insulin resistance. Pharmacological inhibition of acetyl-CoA carboxylase (ACC) 2 offers a promising approach to treat insulin resistance through stimulation of mitochondrial fatty acid oxidation (FAO) and reduction of IMCL deposition. Previously reported experimental ACC2 inhibitors exhibited plasma glucose-lowering effects in diabetic rodents. However, their antidiabetic action may be potentially biased by off-target effects on triglyceride metabolism or by neurologic side effects. In this study, we investigated a safety profile, target dependency of its action, and antidiabetic efficacy of compound 2e, a novel olefin derivative potent ACC2 selective inhibitor. Four-day administration of suprapharmacological dose of compound 2e did not exhibit any obvious side effects in Sprague-Dawley rats. In db/db mice, single administration of compound 2e led to significantly elevated FAO and reduced IMCL deposition in skeletal muscle. In ACC2 knockout mice, treatment with pharmacological doses of compound 2e did not reduce plasma triglyceride levels, whereas A-908292, a previously reported ACC2 inhibitor, caused a significant triglyceride

reduction, showing that compound 2e was devoid of off-target triglyceride-lowering activity. Chronic treatment of db/db mice with compound 2e improved hyperglycemia but did not decrease plasma triglyceride levels. Additionally, compound 2e showed significant improvements of whole-body insulin resistance in the clamp study and insulin tolerance test. Collectively, compound 2e demonstrated a good safety profile and significant antidiabetic effects through inhibition of ACC2-dependent pathways. These findings provide further evidence that selective inhibition of ACC2 is an attractive strategy against insulin resistance and type 2 diabetes.

SIGNIFICANCE STATEMENT

This study shows that pharmacological inhibition of acetyl-CoA carboxylase (ACC) 2 leads to significant improvements in whole-body glucose homeostasis, independently of off-target metabolic pathways and toxicity, which were observed in previously reported ACC2 inhibitors. These findings support the concept that ACC2-selective inhibitors will be a novel remedy for treatment of type 2 diabetes.

Introduction

Insulin resistance, a major contributing factor to type 2 diabetes, is closely associated with dysregulation of lipid metabolism in skeletal muscle. Except for endurance-trained athletes (Goodpaster et al., 2001), it has been generally accepted that intramyocellular lipid (IMCL) accumulation in skeletal muscle has a causal relationship with whole-body insulin resistance (Krssak et al., 1999; Virkamäki et al., 2001). Considerable evidence suggests that chronic overnutrition causes reduced fatty acid oxidation (FAO) and increased IMCL deposition in skeletal muscle, driving the development of insulin resistance (Kelley et al., 1999; Kim et al., 2000;

Morino et al., 2006). Therefore, the strategy designed to accelerate FAO and diminish IMCL deposition is considered as an attractive approach for treating type 2 diabetes.

Acetyl-CoA carboxylase (ACC), the enzyme that catalyzes production of malonyl-CoA from acetyl-CoA, has a great impact on lipid storage by regulating both synthesis and oxidation of fatty acids. ACC has two distinct isoforms, ACC1 and ACC2. ACC1 produces malonyl-CoA in cytosol and promotes de novo fatty acid synthesis in lipogenic tissues, such as liver and adipose tissues (Brownsey et al., 2006; Wakil and Abu-Elheiga, 2009). On the other hand, ACC2 suppresses FAO in oxidative tissues, such as skeletal muscle, through malonyl-CoA-regulated inhibition of carnitine palmitoyltransferase 1, a key enzyme for long-chain acyl-CoA entry into mitochondrial FAO pathway (McGarry and Brown, 1997; Abu-Elheiga et al.,

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ABBREVIATIONS: (S)-9c, (S)-N-(4-(2-(4-isopropoxyphenoxy)thiazol-5-yl)but-3-yn-2-yl)acetamide; A-908292, (S)-methyl(4-(2-(4-isopropoxyphenoxy)thiazol-5-yl)but-3-yn-2-yl)carbamate; ACC, acetyl-CoA carboxylase; AUC_{0–24 h}, area under plasma concentration-time curve from 0 to 24 hours; BMIPP, β -methyl-p-iodophenyl-pentadecanoic acid; C_{max}, maximum plasma concentration; compound 2e, (S, E)-N-(4-(6-(7-chloro-2-ethoxybenzo[d]thiazol-6-yl)oxy)pyridin-3-yl)but-3-en-2-yl)acetamide; EMCL, extramyocellular lipid; FAO, fatty acid oxidation; HbA_{1c}, glycated hemoglobin; IMCL, intramyocellular lipid; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

2000; Brownsey et al., 2006; Wakil and Abu-Elheiga, 2009). Though whole-body deletion of ACC1 in mice is embryonic lethal (Abu-Elheiga et al., 2005), deletion of ACC2 in mice has been reported to decrease lipid accumulation by enhancing FAO in skeletal muscle and improve whole-body insulin resistance (Choi et al., 2007; Takagi et al., 2018). Pharmacological selective inhibition of ACC2 is thus currently regarded as a hopeful avenue for the therapy of insulin resistance.

To date, though many studies have reported the role of ACC2 in energy metabolism, only a few articles have described the antidiabetic effects of ACC2-selective inhibitors. A-908292, the first published ACC2-selective inhibitor, showed significant reductions in plasma triglyceride and glucose levels in ob/ob mice (Waring et al., 2008). However, its inactive enantiomer (R)-methyl(4-(2-(4-isopropoxyphenoxy)thiazol-5-yl)but-3-yn-2-yl)carbamate also showed similar effects (Waring et al., 2008). These results indicate that A-908292 could interfere with ACC2-independent metabolic pathways that have an impact on glucose homeostasis. In particular, its remarkable effect on triglyceride levels may be possibly attributed to the structure-based off-target action, considering the fact that genetic knockout of ACC2 did not show reduced triglyceride levels in high-fat diet-induced diabetic models (Choi et al., 2007; Takagi et al., 2018).

(S)-9c, another ACC2-selective inhibitor, was subsequently reported to show an acute reduction in skeletal muscle IMCL and then an improvement of hyperglycemia in db/db mice (Glund et al., 2012), suggesting the possible links between ACC2-regulated IMCL breakdown and antidiabetic effects. However, (S)-9c has highly similar structure to A-908292 and showed a marked reduction in plasma triglyceride (Gu et al., 2006; Glund et al., 2012), raising a considerable concern that (S)-9c may have the similar off-target metabolic effect to A-908292. Moreover, both A-908292 and (S)-9c have been reported to show structure-based neurologic toxicity (Gu et al., 2007), and there are not enough pharmacokinetic data to clarify relationships between their in vivo efficacy and toxicity. Therefore, despite much effort focusing on the therapeutic potential of ACC2 inhibition, it remains unclear whether pharmacological inhibition of ACC2 indeed has beneficial effects on glucose homeostasis, independently of off-target metabolic pathways and toxicity.

We have recently discovered compound 2e (Fig. 1), a novel olefin derivative, as a potent ACC2-selective inhibitor, with IC_{50} values of 1.9 and 1950 nM for ACC2 and ACC1, respectively (Nishiura et al., 2018). In the present study, we evaluated a safety profile of compound 2e in healthy rats and examined its ACC2-dependent or -independent metabolic effects with db/db mice and ACC2 knockout mice. Furthermore, we investigated the therapeutic impact of chronic treatment with compound 2e on whole-body insulin sensitivity and hyperglycemia in db/db mice under experimental conditions unaffected by off-target actions.

Materials and Methods

Test Compounds

Compound 2e and A-908292 were synthesized at the Shionogi Pharmaceutical Research Center (Osaka, Japan) according to the procedures described in previous reports (Gu et al., 2006; Nishiura et al., 2018). For toxicological study, compound 2e was dissolved in vehicle consisting of polyethylene glycol 400/Tween 80 (95:5 by

Fig. 1. Structure of compound 2e.

volume). For other experiments, compounds were suspended in 0.5% hydroxypropyl methylcellulose as vehicle. Compounds were administered to animals via the oral route.

Animal Studies

The study of fatty acid uptake was conducted at Osaka University Graduate School of Medicine (Osaka, Japan), and all the other studies were conducted at Shionogi Pharmaceutical Research Center. All procedures for animal studies were approved by the Institutional Animal Care and Use Committee of Shionogi & Co., Ltd. (Osaka, Japan) or Osaka University Graduate School of Medicine. Sprague-Dawley rats were obtained from Charles River Japan (Kanagawa, Japan). BKS.Cg- $Lepr^{db}/+Lepr^{db}/Jcl$ (db/db) mice and their non-diabetic controls (db/m) were obtained from CLEA Japan (Tokyo, Japan). ACC2 knockout mice and wild-type mice were generated as previously described (Takagi et al., 2018). All studies were performed using male animals. All experimental animals were single-housed in cages at a temperature-controlled (20–23°C) environment under a 12-hour light/dark cycle and given access to a normal chow diet and water ad libitum. Rats and mice were fed CRF-1 (Charles River Japan) and CE-2 (CLEA Japan), respectively.

Toxicological Study of Compound 2e in Rats

Six-week-old Sprague-Dawley rats were randomized into two groups based on body weight and then orally given compound 2e (50 mg/kg per day) or vehicle once a day for 4 consecutive days. Clinical signs were observed three times a day (before dosing, after dosing, and afternoon). Body weight and food intake were measured daily. After 4 days administration of compound 2e, blood and tissue samples were collected from all rats. These collected samples were applied to hematology (complete blood count, differential white blood count, reticulocyte count, prothrombin time, and activated partial thromboplastin time), blood biochemistry (total protein, albumin, bilirubin, aspartate transaminase, alanine transaminase, alkaline phosphatase, lactate dehydrogenase, creatine kinase, amylase, urea nitrogen, creatinine, glucose, cholesterol, triglyceride, and electrolytes), and histopathology. For histopathological evaluation, H&E-stained sections of major organs (brain, heart, liver, spleen, lung, kidney, thigh muscle, adrenal gland, duodenum, ileum, jejunum, pancreas, pituitary gland, stomach, testis, thymus, and thyroid gland) were assessed. In a separate study, 6-week-old rats were orally given compound 2e (50 mg/kg), and blood samples were collected from the tail vein at 1, 3, 6, 8, and 24 hours after dosing for measurement of compound 2e concentrations.

Single Dosing Studies of Compound 2e in Mice

Pharmacokinetic Profile. The pharmacokinetic profile of compound 2e was evaluated in 7-week-old db/db mice in the nonfasted state, following a single oral administration (0.6 or 5 mg/kg). Blood samples were collected from the tail vein at 1, 3, 6, 8, 24, and 48 hours after dosing for measurement of compound 2e concentrations.

Evaluation of Malonyl-CoA Content in Skeletal Muscle. Ten-week-old db/db mice or 23-week-old ACC2 knockout mice were orally administered vehicle or compound 2e at the indicated doses in parallel with food removal. Mice were euthanized 6 hours after the administration,

and the quadriceps muscle was rapidly dissected. The muscle samples were stored at -80°C before analysis.

FAO. Indicators of FAO were examined in 8–12-week-old db/db mice following a single oral administration of compound 2e at a dose of 2.5 mg/kg. Fatty acid uptake in skeletal muscle was assessed using ^{123}I - β -methyl-p-iodophenyl-pentadecanoic acid (BMIPP; Nihon Mediphotics Co. Ltd., Tokyo, Japan). Mice were intravenously injected with approximately 100 kBq of ^{123}I -BMIPP 4 hours after the single dosing of compound 2e. Blood and quadriceps muscle samples were collected 2 hours after the ^{123}I -BMIPP injection, and radioactivity in the samples was measured with an automatic gamma-counter (Wizard 2480; PerkinElmer, Waltham, MA). The individual BMIPP uptake value was calculated as the ratio of quadriceps muscle to blood radioactivity. Acylcarnitine levels in quadriceps muscle (3 hours after dosing) as well as IMCL and extramyocellular lipid (EMCL) levels in tibialis anterior muscle (24 hours after dosing) were evaluated as previously described (Takagi et al., 2018).

Evaluation of Triglyceride-Lowering Effects in ACC2 Knockout Mice

ACC2 knockout mice at 21 weeks of age were randomized into three groups based on plasma triglyceride and glucose levels as well as body weight. Mice were orally given compound 2e (2.5 mg/kg), A-908292 (15 mg/kg), or vehicle twice a day for 4 consecutive days. After 4 days administration, blood samples were collected from the tail vein in the nonfasted state and plasma triglyceride levels were measured.

Long-Term Efficacy Studies of Compound 2e in db/db Mice

Evaluation of Antidiabetic Effect. Six-week-old db/db mice were subjected to a habituation to oral administration for 1 week using 0.5% hydroxypropyl methylcellulose. At 7 weeks of age, mice were allocated to either the compound 2e- or vehicle-treated group based on body weight, food intake, plasma glucose levels, and glycated hemoglobin (HbA1c) values. Mice were orally given compound 2e (1.7 mg/kg) or vehicle twice a day for 8 weeks. Body weight and food intake were assessed daily. Blood metabolic parameters were measured between 5 and 7 weeks after the start of the treatment. Blood samples were collected from the tail vein under 5-hour fasted (at 5 weeks), ad libitum-fed (at 6 weeks), or overnight fasted (at 7 weeks) conditions. At the end of study, blood samples were collected from inferior vena cava under isoflurane anesthesia in the nonfasted state at 18 hours after the final administration, and tissue samples (quadriceps muscle and pancreas) were immediately dissected for measurement of malonyl-CoA and insulin content. The samples were stored at -80°C before analysis.

Evaluation of Whole-Body Insulin Sensitivity. db/db mice at 6 weeks of age (for hyperinsulinemic-euglycemic clamp study) or at 9 weeks of age (for insulin tolerance test) were subjected to the habituation and then to group allocation and treatment as described above. The clamp study was performed in mice treated with compound 2e or vehicle for 6 weeks as previously described (Takagi et al., 2018). Twenty percent glucose solution was used to maintain blood glucose levels in this study. Glucose infusion rate at the steady state was calculated from the average rate of 40 minutes of euglycemic conditions (approximately 90–130 mg/dl). For the insulin tolerance test, mice treated with compound 2e or vehicle for 7 weeks were fasted for 5 hours and injected intraperitoneally with Humulin R (0.8 U/kg; Eli Lilly, Kobe, Japan). Blood samples were collected from the tail vein at 0, 30, 60, 90, and 120 minutes after injection. A glucometer (Arkray, Tokyo, Japan) was used to measure blood glucose concentrations in the samples.

Measurements

Determination of Toxicokinetic and Pharmacokinetic Parameters of Compound 2e. Plasma samples were prepared from blood samples by centrifugation. Plasma concentrations of compound 2e were determined by liquid chromatography–tandem

TABLE 1

Toxicological study of compound 2e in rats

Male Sprague-Dawley rats at the age of 6 wk were orally given compound 2e at 50 mg/kg per day for 4 days, and toxicological parameters were compared with vehicle-treated group ($n = 3/\text{group}$). Toxicokinetic parameters were evaluated in 6-wk-old Sprague-Dawley rats after a single oral administration of compound 2e at 50 mg/kg ($n = 2$). Values are presented as means $\pm$ S.E.M.

Parameter	Results
Body weight	No significant difference from vehicle group
Food intake	No significant difference from vehicle group
Clinical signs	No abnormalities in general appearance and behavior. Loose stool was observed in both groups (one-third rat)
Histopathology	No histopathological abnormalities
Hematology	No significant difference from vehicle group
Blood biochemical parameters	Mild increase in plasma total cholesterol (vehicle vs. compound 2e: 76 ± 7 mg/dl vs. 101 ± 3 mg/dl; $P < 0.05$)
Toxicokinetic parameters	
-AUC _{0–24 hour} ($\mu\text{g}^*\text{h/ml}$)	620 ± 105
-C _{max} ($\mu\text{g/ml}$)	34.6 ± 5.0
-C _{24 hour} ($\mu\text{g/ml}$)	17.5 ± 4.4

C_{24 hour}, trough plasma concentration.

mass spectrometry (LC-MS/MS) analyses using API5000 with Analyst (AB Sciex, Framingham, MA). The area under plasma concentration-time curve from 0 to 24 hours (AUC_{0–24 hour}), maximum plasma concentration (C_{max}), and trough plasma concentration were calculated from the observed plasma concentration data at the sampling time points.

Blood Metabolic Parameters. Plasma and blood cell layer were obtained from blood samples by centrifugation at 22,400g for 5 minutes at 4°C . Plasma glucose, plasma triglyceride, and HbA1c levels were determined by enzymatic methods using a Hitachi 7180 automatic analyzer (Hitachi, Tokyo, Japan). Plasma insulin concentration was measured using a mouse insulin ELISA kit (Shionogi, Osaka, Japan) (Imai et al., 2015).

Determination of Malonyl-CoA Content. Frozen muscle samples were homogenized on ice using hand-held homogenizers in distilled water/60% perchloric acid/85% phosphoric acid (43:5:2 by volume) with the ratio of 1:10 (w/v). After centrifugation, the supernatant was transferred into LC-MS/MS system. LC-MS/MS system was performed on a Waters Micromass Quattro Ultima Pt (Micromass, Manchester, UK).

Measurement of Pancreatic Insulin Content. Frozen pancreas samples were homogenized on ice using hand-held homogenizers in 99.5% ethanol/distilled water/6 N hydrochloric acid (30:9:1 by volume) with the ratio of 1:6 (w/v). After standing at 4°C overnight, samples were centrifuged, and insulin concentrations in the supernatant were determined using a mouse insulin ELISA kit (Shionogi) (Imai et al., 2015).

Statistical Analysis

For long-term efficacy studies, the uniformity of the parameters for group assignment was confirmed by one-way analysis of variance. All results are presented as means $\pm$ S.E.M. Statistical comparisons between groups were analyzed by Student's *t* test. Statistical significance was set at $P < 0.05$.

Results

Toxicological Study of Compound 2e in Rats. Toxicological findings in the rats treated with compound 2e for 4 days at 50 mg/kg per day are shown in Table 1. In the clinical signs, there were no findings except for loose stool, which was observed in both groups and therefore considered to be due to the vehicle administration. Body weight and food intake were not affected by the treatment, and no abnormalities were

Fig. 2. Pharmacokinetic and pharmacological profiles of compound 2e in db/db mice. (A) Concentration-time profiles of compound 2e in plasma after oral administration of compound 2e at a dose of 0.6 or 5 mg/kg in db/db mice ($n = 3$). (B) Malonyl-CoA levels in skeletal muscle 6 hours after oral administration of vehicle or compound 2e (1.7 or 5 mg/kg) in db/db mice ($n = 4$). (C–F) The effects of compound 2e on skeletal muscle lipid metabolism were evaluated in db/db mice following a single oral administration of compound 2e at a dose of 2.5 mg/kg ($n = 4$ –7). The uptake values of ^{123}I - β -methyl-p-iodophenyl-pentadecanoic acid (BMIPP) in skeletal muscle were represented by the ratio of quadriceps muscle to blood radioactivity (C). Acylcarnitine levels in skeletal muscle of db/db mice (D). Intramyocellular lipid (IMCL) levels (E) and extramyocellular lipid (EMCL) levels (F) in skeletal muscle of db/db or db/m mice. IMCL and EMCL values were calculated as the ratio to total creatine (tCr). * $P < 0.05$; ** $P < 0.01$ vs. vehicle-treated mice; † $P < 0.01$ vs. db/m mice. All data are presented as means $\pm$ S.E.M.

found in hematology and histopathology. Only total plasma cholesterol value was mildly elevated, but there were no related findings in histopathological findings, and all the other blood chemistry parameters were unaffected. In addition, the systemic exposure of compound 2e at 50 mg/kg was the C_{max} value of 34.6 $\mu\text{g/mL}$ and the $\text{AUC}_{0-24 \text{ hour}}$ value of 620 $\mu\text{g}^*\text{h/mL}$, which were much higher than in pharmacology

studies. Taken together, the rats given compound 2e at 50 mg/kg per day were well tolerated.

Pharmacokinetic Profiles and Acute Pharmacological Actions of Compound 2e in db/db Mice. We evaluated pharmacokinetic parameters and ACC2 inhibitory action of compound 2e in db/db mice. Compound 2e showed good plasma exposure and dose-dependent pharmacokinetics at

Fig. 3. Evaluation of target specificity using ACC2 knockout mice. (A) Malonyl-CoA levels in skeletal muscle 6 hours after oral administration of vehicle or compound 2e (2.5 or 5 mg/kg) in ACC2 knockout mice ($n = 5$). (B) Off-target effects of ACC2 inhibitors on plasma triglyceride levels were evaluated in ACC2 knockout mice. Mice were orally given compound 2e (2.5 mg/kg), A-908292 (15 mg/kg), or vehicle twice a day for 4 consecutive days. After 4 days administration, plasma triglyceride levels were measured ($n = 7$). ** $P < 0.01$ vs. vehicle-treated mice. All data are presented as means $\pm$ S.E.M.

oral doses of 0.6 and 5 mg/kg (Fig. 2A). A dose of 5 mg/kg showed the $C_{\max}$ value of 3.9 $\mu\text{g}/\text{ml}$ and the $\text{AUC}_{0-24 \text{ hour}}$ value of 74.2 $\mu\text{g}\cdot\text{h}/\text{ml}$, which were much (over eight times) lower than those in toxicological studies in rats. After single oral administration of compound 2e at doses of 1.7 and 5 mg/kg, skeletal muscle malonyl-CoA levels were significantly decreased (Fig. 2B). Single-dose treatment of compound 2e at 2.5 mg/kg elevated uptake of BMIPP, a fatty acid analog, and increased long-chain acylcarnitine levels in skeletal muscle, both as indicators of FAO (Fig. 2, C and D). Furthermore, compound 2e treatment acutely reduced skeletal muscle IMCL levels in db/db mice to the levels of nondiabetic control db/m mice (Fig. 2E). Interestingly, despite these significant elevations in fatty acid metabolism, compound 2e treatment did not reduce skeletal muscle EMCL levels in db/db mice (Fig. 2F), consistent with a previous observation in ACC2 knockout mice (Takagi et al., 2018). These results suggest that compound 2e enhanced FAO and lipid breakdown by ACC2 inhibitory action in skeletal muscle.

Evaluation of Target Specificity Using ACC2 Knockout Mice. We next assessed target specificity of compound 2e using ACC2 knockout mice. In contrast to the results in db/db mice, no significant reductions in malonyl-CoA levels were observed in ACC2 knockout mice with compound 2e at doses of 2.5 and 5 mg/kg (Fig. 3A). To determine whether compound 2e has similar off-target activity to A-908292, we compared its triglyceride-lowering effect with that of A-908292 in the short-treatment study. Four-day treatment with compound 2e (2.5 mg/kg twice daily) did not affect plasma triglyceride levels in ACC2 knockout mice, whereas A-908292 (15 mg/kg twice daily) markedly reduced plasma triglyceride levels (Fig. 3B), consistent with a previous report (Waring et al., 2008). These results indicate that compound 2e did not have such an off-target activity as to interfere with triglyceride metabolism independently of ACC2 inhibition, at least in pharmacological doses.

Antidiabetic Effect of Long-Term Treatment with Compound 2e in db/db Mice. The results of our short-term studies in mice indicate that a dose of 2.5 mg/kg compound 2e was adequate to induce ACC2 inhibitory action without the off-target metabolic effect. Based on the pharmacokinetic data of the relatively long half-life of compound 2e in mice (Fig. 2A), we adopted a dose of 1.7 mg/kg for the following long-term studies. To investigate antidiabetic effect, compound 2e was orally administrated to db/db mice at a dose of 1.7 mg/kg twice

daily (3.4 mg/kg per day) for 8 weeks. Body weight of mice treated with compound 2e was similar to that of vehicle-treated mice until 6 weeks and was slightly but significantly higher at 7–8 weeks (Fig. 4A). No significant difference in food intake was observed between both groups of mice throughout the study (Fig. 4B). Compared with vehicle-treated mice, compound 2e-treated mice showed significant decreases in blood glucose levels at 5 weeks (Fig. 4C) and HbA1c values at 6–7 weeks (0.64% points lower than vehicle-treated mice at 7 weeks; Fig. 4D). There were no reductions in plasma triglyceride levels of compound 2e-treated mice (Fig. 4E). Plasma insulin levels were slightly but not significantly higher in compound 2e-treated mice ($P = 0.09$ at 7 weeks; Fig. 4F), and no significant change in pancreatic insulin content was detected after compound 2e treatment (Fig. 4G). At the end of the study, pharmacokinetics and pharmacodynamics were assessed. Plasma concentration of compound 2e at 6 and 18 hours after the final administration were 1.7 and 0.8 $\mu\text{g}/\text{ml}$, respectively, which were more than 10-times lower than trough plasma concentration in the safety assessment study in rats (Table 1). Skeletal muscle malonyl-CoA levels were markedly reduced at 18 hours after the final administration compared with vehicle treatment (Fig. 4H). This reduction suggests that ACC2 inhibitory action was maintained throughout the study.

Impact of Compound 2e on Whole-Body Insulin Sensitivity in db/db Mice. To further investigate the antidiabetic effect of compound 2e, we evaluated whole-body insulin sensitivity in db/db mice by hyperinsulinemic-euglycemic clamp and the insulin tolerance test. Steady-state glucose infusion rate in the clamp study was markedly increased in mice treated with compound 2e for 6 weeks compared with vehicle-treated mice (Fig. 5A). Likewise, insulin-stimulated glucose disposal in the insulin tolerance test was significantly improved in mice treated with compound 2e for 7 weeks (Fig. 5B). These results suggest that improved insulin sensitivity greatly contributed to glucose-lowering effect of compound 2e.

Discussion

Previous studies have revealed that ACC2 knockout mice showed favorable phenotype of improved glucose homeostasis, providing a notion of ACC2 as an antidiabetic target

Fig. 4. Antidiabetic effect of long-term treatment with compound 2e in db/db mice. Compound 2e was orally administered to db/db mice at a dose of 1.7 mg/kg twice daily (3.4 mg/kg per day) for 8 weeks. Body weight (A) and weekly food intake (B) in db/db mice ($n = 8$ to 9). (C–F) Blood metabolic parameters were evaluated under 5-hour fasted (at 5 weeks), ad libitum-fed (at 6 weeks), or overnight-fasted (at 7 weeks) conditions ($n = 8$ –9). Plasma glucose (C), glycated hemoglobin (D), plasma triglyceride (E), and plasma insulin (F) levels. At the end of the study, pancreatic insulin levels (G) and skeletal muscle malonyl-CoA content (H) were evaluated in nonfasted mice at 18 hours after the final administration ($n = 6$). * $P < 0.05$; ** $P < 0.01$ vs. vehicle-treated mice. All data are presented as means $\pm$ S.E.M.

(Choi et al., 2007; Takagi et al., 2018). Genetic manipulations, however, have potential limitations, such as complete absence of target molecule and nonuniformity of gene knockout strategy, which may partially account for phenotypic discrepancies between several lines of ACC2 knockout mice (Hoehn et al., 2010, 2012; Olson et al., 2010). Therefore, pharmacological studies are required to validate the therapeutic potential of ACC2 inhibition. Unfortunately,

previously reported ACC2 inhibitors were shown to potentially cause serious side effects or significantly affect cellular metabolic pathways, independently of ACC2 inhibition (Gu et al., 2007; Waring et al., 2008). The aim of this study was to determine the antidiabetic potential of ACC2 inhibition at pharmacological doses without obvious ACC2-independent action and toxicity, using a novel small-molecule ACC2 selective inhibitor, compound 2e.

Fig. 5. Impact of compound 2e on whole-body insulin sensitivity in db/db mice. Compound 2e was orally administered to db/db mice at a dose of 1.7 mg/kg twice daily (3.4 mg/kg per day) for 6 or 7 weeks. (A) After 6 weeks of treatment with compound 2e, the hyperinsulinemic-euglycemic clamp was conducted with continuous infusion of human insulin (10.5 mU/kg per minute) in overnight-fasted mice. Glucose infusion rate at the steady state was obtained from the average rate of 40 minutes of euglycemic conditions (approximately 90–130 mg/dl) ($n = 5-7$). (B) After 7 weeks of treatment with compound 2e, the insulin tolerance test was conducted with intraperitoneal injection of human insulin (0.8 U/kg) in 5-hour fasted mice ($n = 9-10$). * $P < 0.05$; ** $P < 0.01$ vs. vehicle-treated mice. All data are presented as means $\pm$ S.E.M.

One crucial issue related to ACC2 inhibitors is the structure-based off-target metabolic effect as exemplified in a study of short treatment with A-908292 (Waring et al., 2008). Based on the report by Abbott group, these off-target effects, characterized by a striking plasma triglyceride reduction, was probably through indirect activation of a peroxisome proliferator-activated receptor- α pathway. In addition, (S)-9c, another ACC2 inhibitor, also showed a marked reduction in plasma triglyceride (Glund et al., 2012). Notably, despite its great structural similarity with A-908292, no description and no data have been presented to identify the ACC2-dependent or -independent effects of (S)-9c. Because plasma triglyceride levels or peroxisome proliferator-activated receptor- α signaling pathways can greatly impact glucose homeostasis (Ye et al., 2001; Kim et al., 2003), previous studies using these inhibitors may not be sufficient to determine therapeutic efficacy of ACC2-specific inhibition. In the present study, administration of A-908292 caused acute reductions of plasma triglyceride levels in ACC2 knockout mice, further demonstrating its off-target effect observed in the previous study. Notably, in contrast to A-908292, short-term treatment with compound 2e did not evoke an ACC2-independent plasma triglyceride reduction, indicating that compound 2e was devoid of such off-target

effects as to interfere with triglyceride metabolism and that ACC2 inhibition per se does not directly reduce plasma triglyceride levels, at least in the short period. In db/db mice, compound 2e treatment acutely decreased malonyl-CoA content, increased FAO, and diminished IMCL levels in skeletal muscle, as reasonably expected actions from ACC2 inhibition. Furthermore, the present observation that chronic treatment with compound 2e led to improved glucose homeostasis without affecting plasma triglyceride levels agreed well with the previous reports describing the phenotypes of ACC2 knockout mice (Choi et al., 2007; Takagi et al., 2018). Collectively, these results indicate that compound 2e treatment improves glucose homeostasis through the target-dependent pathway.

Neurologic toxicity is another concern about previously reported ACC2 inhibitors. Both A-908292 and (S)-9c were reported to show neurologic side effects such as seizures in the anesthetized rats (Gu et al., 2007). However, despite the potential toxicological concern, there is a lack of their pharmacokinetic data about nontoxic doses as well as therapeutic doses, making it difficult to know whether their neurologic toxicity may have any effects on the therapeutic effects. In the present study, compound 2e raised no serious safety concerns with no abnormal behavior in rats at repeated high dosage (50 mg/kg per day). More importantly, our pharmacokinetic data revealed that the enhanced IMCL breakdown and improved glucose homeostasis by compound 2e treatment were observed at much lower plasma concentrations than those of nontoxic doses. These results confirm the antidiabetic effects of compound 2e without toxicological concerns and encourage further toxicological studies to fully characterize safety profiles of compound 2e.

In the long-term efficacy studies, compound 2e treatment improved insulin-stimulated glucose disposal but did not significantly affect plasma insulin levels in db/db mice, indicating that the improvement of whole-body insulin resistance plays an important role in the glucose-lowering effects of compound 2e. Although the detailed mechanism underlying antidiabetic effects of compound 2e has not been identified, the acute and robust IMCL reductions by compound 2e treatment are likely to primarily contribute to the enhancement of insulin sensitivity. Recently, among IMCL species, long-chain acyl-CoA, ceramide, and diacylglycerol have been reported to directly impair insulin action and be especially important for the development of type 2 diabetes (Yu et al., 2002; Adams et al., 2004; Stratford et al., 2004; Szendroedi et al., 2014). Genetic deletion of ACC2 has been shown to reduce long-chain acyl-CoA and diacylglycerol levels and enhance insulin signaling in skeletal muscle (Choi et al., 2007). Thus, further investigations of compound 2e-induced changes in IMCL composition may provide mechanistic explanations for the therapeutic effects of ACC2 inhibition. Additionally, because ACC2 can play key roles in regulating hepatic and adipose tissue metabolism (Abu-Elheiga et al., 2003, 2012; Oh et al., 2005), future studies focusing on these tissues are warranted to expand understanding of the overall benefit of pharmacological ACC2 inhibition.

In the present study, we observed that chronic treatment of compound 2e resulted in greater body weight than that of vehicle treatment in db/db mice, inconsistent with previous reports of high-fat diet studies with ACC2 knockout mice (Choi et al., 2007; Takagi et al., 2018). Several works have

shown that body weight gain of db/db mice disappears along with the progression of diabetic phenotype, and antidiabetic treatments contribute to their continuous body weight gain (Gibbs et al., 1995; Arakawa et al., 2001). Thus, the greater body weight, which was observed only in the later period of the experiment, may be accounted for by the amelioration in hyperglycemia and insulin sensitivity by compound 2e treatment.

In summary, we demonstrated that compound 2e, a recently discovered ACC2 inhibitor, improved insulin resistance and hyperglycemia in db/db mice. Importantly, these beneficial effects of compound 2e were linked with activation of on-target metabolic pathways and were not accompanied with ACC2-independent actions that were observed in previously reported ACC2 inhibitors. Our findings provide strong evidence that pharmacological inhibition of ACC2 is a promising approach for treating type 2 diabetes.

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Authorship Contributions

Participated in research design: Takagi, Tanimoto, Shimazaki.

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