A novel mitoNEET ligand, TT01001, improves diabetes and ameliorates mitochondrial function in db/db mice

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TT01001, ethyl 4-(3-(3,5-dichlorophenyl)thioureido)piperidine-1-carboxylate;
Pioglitazone, 5-[[4-[2-(5-ethylpyridin-2-yl)ethoxy]phenyl]methyl]-1,3-thiazolidine-2,4-dione; hydrochloride; TNF-α, tumor necrosis factor-alpha; TZD, thiazolidinedione; IL-6, interleukin-6; PPARγ, peroxisome proliferator activated receptor gamma, PGC-1α; peroxisome proliferator-activated receptor coactivator-1α; MC, methyl cellulose; PMSF, phenylmethylsulfonyl fluoride; SPR, Surface Plasmon Resonance; TR-FRET, Time-resolved fluorescence resonance energy transfer; LBD, ligand binding domain; OGTT, oral glucose tolerance test; NEFA, nonesterified fatty acid; ELISA, enzyme-linked immunosorbent assay; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; TK1, thymidine kinase 1; CS, citrate synthase activity; DTNB, 5,5'-Dithiobis(2-nitrobenzoic acid); AUC, area under the curve; PK/PD, pharmacokinetics and pharmacodynamics.

**Recommended section**

Drug Discovery and Translational Medicine
Abstract

The mitochondrial outer membrane protein mitoNEET is a binding protein of the insulin sensitizer pioglitazone and is considered a novel target for the treatment of type II diabetes. Several small-molecule compounds have been identified as mitoNEET ligands using structure-based design or virtual docking studies. However, there are no reports about their therapeutic potential in animal model. Recently, we synthesized a novel small-molecule, TT01001 designed on the basis of pioglitazone structure. In this study, we assessed the pharmacological properties of TT01001 in both in vitro and in vivo studies. We found that TT01001 bound to mitoNEET without peroxisome proliferator activated receptor gamma activation effect. In type II diabetes model db/db mice, TT01001 improved hyperglycemia, hyperlipidemia, and glucose intolerance, and its efficacy was equivalent to that of pioglitazone, without the pioglitazone associated weight gain. Mitochondrial complex II+III activity of the skeletal muscle was significantly increased in db/db mice. We found that TT01001 significantly suppressed the elevated activity of the complex II+III. These results suggest that TT01001 improved type II diabetes without causing weight gain and ameliorated mitochondrial function of db/db mice. This is the first study that demonstrates the effects of a mitoNEET ligand on glucose metabolism and mitochondrial function in animal disease.
model. These findings support targeting mitoNEET as a potential therapeutic approach for the treatment of type II diabetes.
Introduction

Thiazolidinedione (TZD) derivatives such as pioglitazone and rosiglitazone are potent insulin sensitizers for the treatment of type II diabetes (Ahmadian et al., 2013). These ligands decrease serum triglycerides, free fatty acids, and inflammatory adipocytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) and increase the insulin-sensitizing hormone adiponectin by activating peroxisome proliferator activated receptor gamma (PPARγ) (Kadowaki et al., 2006; Quinn et al., 2008). These various effects of TZD derivatives via the activation of PPARγ lead to the improvement of insulin resistance and glycemic parameters. Meanwhile, clinical side effects such as weight gain or edema are frequently observed in patients with type II diabetes treated with TZD derivatives and are attributed to PPARγ activation (Tang and Maroo, 2007; Borsting et al., 2012). Therefore, the clinical use of TZD derivatives has been limited in patients with heart failure or a past history of heart failure or renal dysfunction (Nissen and Wolski, 2007).

Mitochondria are known as the intracellular powerhouse of cells, and mitochondrial dysfunction is involved in a broad spectrum of diseases, both inherited and acquired (Andreux, et al., 2013). In type II diabetes, many studies have focused on the association between the pathology of diabetes and mitochondrial dysfunction. Lower
oxidative phosphorylation capacity was observed in muscle biopsy samples of patients with type II diabetes compared with those of healthy individuals (Kelley et al., 2008; Phielix et al., 2008; Ritov et al., 2010). The frequent reduction of mitochondrial contents was also shown in patients with type II diabetes (Hwang et al., 2010; Chomentowski et al., 2011). These reports indicate that mitochondrial function plays a key role in the pathology of type II diabetes. Recently, several reports have suggested that the TZD derivative pioglitazone directly influences mitochondrial function. For example, pioglitazone inhibits rat mitochondrial complex I activity in the liver and skeletal muscle tissue, indicating that alterations of cellular energy state by pioglitazone may contribute to the improvement in insulin sensitivity (Brunmair et al., 2004). Pioglitazone also increases the levels of the mitochondrial biogenesis regulator protein peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) in the skeletal muscle of db/db mice (Pagel-Langenickel et al., 2008). In patients with type II diabetes, pioglitazone treatment increases both mitochondrial DNA (mtDNA) copy numbers and the expression of PGC-1α in subcutaneous adipose tissue (Bogacka et al., 2005). These effects of pioglitazone are speculated to be independent of PPARγ activation (Feinstein et al., 2005). The mitochondrial outer membrane protein mitoNEET was identified as a novel binding protein of pioglitazone and has been considered a new target for type II
diabetes therapies (Colca et al., 2004). Although the physiological role of mitoNEET remains unclear, it is likely to modulate glucose metabolism or mitochondrial function. The mitochondria isolated from the heart of mitoNEET-deficient mice showed a decrease in state 3 respiration (Wiley et al., 2007). Overexpression of mitoNEET inside the adipose cell in genetic type II diabetic model ob/ob mice improved glycemic parameters and altered mitochondrial functions (Kusminski et al., 2012). Suppressed expression of mitoNEET in vitro decreased mitochondrial abilities (Sohn et al., 2013). Interestingly, the ligand for mitoNEET, NL-1 suppresses rotenone-induced toxicity in neuronal cells and mildly uncouples mitochondria (Geldenhuys et al., 2010). Other ligands for mitoNEET have also been reported by use of virtual docking studies (Bieganski and Yarmush, 2011). However, the in vivo effects of glucose metabolism or mitochondrial function have not been clarified for these mitoNEET ligands. Recently, we newly synthesized TT01001, an orally active, small molecule that is designed on the basis of the pioglitazone structure. In this study, we first assessed the in vitro pharmacological profile of TT01001 with PPARγ activity and mitoNEET binding. Next, we examined the in vivo effects of TT01001 on diabetes and mitochondrial function using the type II diabetes murine model, db/db mice.
Materials and Methods

Chemicals. The TT01001 (Fig. 1) was synthesized by the Pharmaceutical Laboratories, Toray Industries, Inc. (Kanagawa, Japan). Pioglitazone was purchased from Kemprotec Limited (Middlesbrough, Cambria, UK). All compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, MO, USA) for in vitro studies, or suspended in 0.5% (w/v) methyl cellulose (0.5% MC) (Nacarai Tesque, Kyoto, Japan) for in vivo studies.

Protein expression and purification. The protein expression and purification of the soluble human mitoNEET was performed as described previously with slight modification (Zuris et al., 2011). The gene of human mitoNEET (human kidney cDNA, Clontech, CA, USA) was amplified by PCR and cloned to the pCI-neo vector (Promega, Osaka, Japan). The fragment of mitoNEET encoding residues 33–108 was inserted into the pET-28b (+) vector (Novagen, WI, USA) via the Nde I and Xho I sites, and the expression vector was introduced into Escherichia coli BL21 (DE3) cells. The mitoNEET protein was induced by the histidine-tagged protein by adding isopropyl-1-thio-β-D-galactoside (final concentration, 0.5 mmol/L) and culturing for 4 h at 37°C. The Escherichia coli pellets were collected and lysed by sonication in buffer A [50 mM Tris-HCl, (pH 8.0), 250 mM NaCl, 5 mM imidazole] with 1 mM PMSF. The
clarified supernatant was loaded onto a HisTrap HP (GE Healthcare, Tokyo, Japan) column pre-equilibrated with buffer A. The column was washed with buffer A containing 100 mM imidazole, and the histidine-tagged mitoNEET protein was eluted with buffer A containing 500 mM imidazole. To remove the histidine-tag, the protein solution was incubated with restriction-grade thrombin (Novagen) for 6 h at room temperature. The solution was loaded onto a HiTrap-Benzamidine tandem column (HisTrap HP and HiTrap Benzamidine FF, GE Healthcare), and the column was washed with the buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 100 mM imidazole. The flowthrough and washing fractions were collected, and the protein was purified by size exclusion chromatography (HiLoad 16/60 Superdex 200 prep grade, GE Healthcare) in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl.

**Time-resolved fluorescence resonance energy transfer (TR-FRET) assay.** The PPARγ activation effect was assessed by the TR-FRET method using the LanthaScreen™ TR-FRET PPARγ coactivator assay kit (Invitrogen, CA, USA). The test compounds or solvent vehicle (DMSO) were incubated together with the human PPARγ ligand-binding domain (LBD) tagged with GST, terbium-labeled anti-GST antibody, and fluorescein peptide with assay buffer. When binding of the test compound caused a conformational change in the PPARγ LBD, excitation of terbium at 340 nm resulted in
energy transfer and excitation of the fluorescein peptide, followed by emission at 520 nm. The signal at 520 nm was normalized by the signal obtained at 495 nm. Each assay was performed in quadruplicate and the data were expressed as mean ratios of 520 nm and 495 nm. The mean ratios were plotted against the concentration of the test compounds.

**Surface Plasmon Resonance (SPR) interaction analysis.** The SPR measurements were carried out by the Biacore™ S51 instrument (Biacore AB, Uppsala, Sweden). The mitoNEET protein was immobilized onto the sensor chip (CM-5, GE Healthcare) using the amine coupling method. Different concentrations of TT01001 (1, 2, 4, 8, 20 μmol/L) and pioglitazone (0.3, 0.6, 1.3, 2.5, 5.0 μmol/L) were injected for 60 s at a flow rate of 30 μL/min. The resonance unit (RU) curves were normalized by the reference surface and no-response concentration using S51 evaluation software (GE Healthcare).

**Animals and administration of compounds.** This study was reviewed by the Animal Care and Use Committee, approved by the head of the test facility, and performed in accordance with the Guidelines for Animal Experiments, Research and Development Division, Toray Industries, Inc. Male, 5-week-old mice of C57BL/6J and BKS.Cg-+Lepr<sup>db/db</sup>+Lepr<sup>db/db</sup> (db/db) were obtained from CLEA Japan, Inc. (Tokyo, Japan). All mice were group-housed in cages at 22–24°C with a 12-h light/dark cycle (lights on
at 7:00 am) for 1 week before the experiments began. Mice were given ad libitum access to food and water. In db/db mice, the vehicle (0.5% MC) or test compounds were orally administered once daily for 28 days. The vehicle was also given to C57BL/6J mice orally once daily for 28 days.

Analysis of blood glucose, glucose intolerance, and plasma parameters. We obtained whole blood samples (approximately 5 μL) from the tail vein under postprandial conditions and measured blood glucose with an automatic glucometer (Precision Exceed, Abbott Diabetes Care Ltd., CA, USA) on day 27 before final dosing. On day 28, we determined fasting blood glucose levels and performed oral glucose tolerance tests (OGTT) under 18 h fasting conditions. Blood glucose concentrations were quantified before and after glucose loading (1.5 g/kg, p.o.) at different time points (0, 30, 60, 90, 120, 150, and 180 min) by the above-mentioned method. On day 28, for plasma sampling, we also placed the mice under isoflurane anesthesia and collected whole blood from the inferior vena cava under postprandial conditions. Plasma samples were obtained by centrifugation at 3,000 rpm at 4°C for 10 min. Plasma insulin and nonesterified fatty acid (NEFA) levels were determined by enzyme-linked immunosorbent assay (ELISA) (Shibayagi, Gunma, Japan) and the colorimetric method (Wako Pure Chemical Industries, Osaka, Japan).
Mitochondrial DNA (mtDNA) determination by quantitative real time polymerase chain reaction (PCR). We sacrificed vehicle or test compound-treated mice on day 28 by bleeding under anesthesia, rapidly removed the skeletal muscle (soleus and gastrocnemius muscle), and immediately froze it with liquid nitrogen. We isolated total DNA from the skeletal muscle using the QIAamp DNA Mini Kit (QIAGEN, CA, USA). The mtDNA level was quantified using the TaqMan gene expression assay system (Applied Biosystems, CA, USA). The PCR reaction was carried out in a 20 μL volume containing 2 × TaqMan Universal Master Mix (Applied Biosystems, CA, USA), PCR grade water (Roche Diagnostic Japan, Tokyo, Japan), TaqMan probes for the D-loop and thymidine kinase 1 (TK1) regions, and the total DNA sample. The TaqMan probe sequence is shown in supplementary Table 1. We conducted PCR amplification with 40 cycles of the program at 95°C for 20 s, 95°C for 3 s, and 60°C for 30 s. Each sample was assayed in duplicate and the fluorescence spectra were continuously monitored by the 7500 Fast Real-Time PCR system (Applied Biosystems-Life Technologies, CA, USA) with sequence detection software version 1.4. Data analysis was based on measurement of the cycle threshold (Ct). The mtDNA copy number was determined from the standard curve and normalized by division of the D-loop value by the TK1 value. The data were expressed as relative values against the
mtDNA level in C57BL/6J mice and shown as mean ± S.E.M.

**Isolation of the mitochondrial fraction.** We obtained skeletal muscle from the sacrificed mice and homogenized it with homogenizing buffer (0.2 M sucrose, 0.13 M NaCl, 1.0 mM Tris-HCl, pH=7.4) on ice. The homogenized sample was centrifuged at 3,000 rpm for 10 min at 4°C and the supernatant was again centrifuged at 14,000 rpm for 10 min. The supernatant was removed and the pellet (i.e., the mitochondrial fraction) was dissolved in 0.25 M sucrose. The mitochondrial fraction was frozen and thawed twice to assess mitochondrial respiratory chain enzyme activity.

**Measurement of mitochondrial respiratory chain enzyme activity.** To evaluate the short-duration effect of the test compounds on mitochondrial function, we assessed respiratory chain enzyme activity of the skeletal muscle mitochondrial fraction in db/db mice. Moreover, the mitochondrial function of compound-treated db/db mice was used to evaluate the chronic effect of test compounds on mitochondrial function. The assay was conducted as described previously, with slight modifications (Spinazzi et al., 2012). The reaction was carried out in 200 μL volume and detected by a micro plate reader (SpectraMax 190, Molecular Devise, CA, USA). The test compounds or DMSO were directly added to the reaction buffer (final concentration of DMSO was 1%) to assess the short-duration effects of the test compounds. Mitochondrial complex specific
inhibitor, rotenone, malonate, and KCN were used as the reference compounds. Citrate synthase (CS) activity was measured as follows. The mitochondrial fraction was incubated in buffer containing 0.1 mM DTNB and 0.3 mM acetyl-CoA at 37°C for 5 min. The reaction was started by adding 0.5 mM oxaloacetic acid, and then we monitored the increase in absorbance at 412 nm for 3 min. Complex I+III and complex II+III activity were measured by reduction of oxidized cytochrome c at an absorbance of 550 nm. In complex I+III activity, 50 mM Tris-HCl, 1 mM KCN, 0.1 mM NADH, and 0.1 mM oxidized cytochrome c were incubated at 37°C for 5 min. The reaction was started by adding the sample, and the absorbance was observed for 3 min. In complex II+III activity, 5 mM potassium phosphate buffer, 2 mM KCN, 10 mM sodium succinate dibasic, and the sample were incubated at 37°C for 10 min. The reaction was started by adding 0.1 mM oxidized cytochrome c, and the absorbance change was observed for 3 min. Complex IV activity was measured by oxidation of reduced cytochrome c in absorbance at 550 nm. Reduced cytochrome c was prepared as described previously (Spinazzi et al., 2012). We incubated 5 mM potassium phosphate buffer and reduced cytochrome c at 37°C for 5 min. The reaction was started by adding the sample, and the absorbance change was observed for 3 min. The results of complex I+III, II+III, and IV activities were normalized by CS activity. Because of the variability
of CS activity in each sample, respiratory chain enzyme activities were also normalized to each protein concentration. Data were expressed as relative values against vehicle-treated C57BL/6J mice enzyme activities and shown as mean ± S.E.M.

**Data and statistical analysis.** All data were expressed as a mean ± S.E.M. We calculated areas under the curves (AUCs) using the trapezoidal rule from the blood glucose in the OGTT in C57BL/6J or db/db mice. We performed the statistical analysis using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison in three groups. A P-value of <0.05 was considered statistically significant.
**Results**

**TT01001 did not activate PPARγ but interacted with mitoNEET.** To evaluate the pharmacological properties of TT01001, we first examined the PPARγ activation effect by the TR-FRET method. No change in TR-FRET emission signal of TT01001 was seen in the concentration range of 0.001–100 μmol/L. On the other hand, pioglitazone increased the TR-FRET emission signals in a concentration-dependent manner (Fig. 2A).

Next, to evaluate the binding effect of TT01001 for mitoNEET, we assessed the effect of TT01001 on mitoNEET protein by the SPR method using the Biacore™ instrument. The injection of TT01001 onto immobilized mitoNEET increased the RU in a concentration-dependent manner (1, 2, 4, 8, 20 μmol/L) (Fig. 2B). Pioglitazone also increased the RU in a concentration-dependent manner (0.3, 0.6, 1.2, 2.5, 5.0 μmol/L) (Fig. 2C).

**TT01001 improved diabetes in db/db mice without causing weight gain.** We next evaluated whether TT01001 could exhibit an ameliorative effect on glycemic parameters in vivo. We orally administered either TT01001 (100 mg/kg) or pioglitazone (30 mg/kg) to a genetically obese rodent model, db/db mice, once daily for 28 days. There was no effect of TT01001 on body weight, but it was increased by pioglitazone in a duration-dependent manner (Fig. 3A). On day 24, there were no body weight changes
in TT01001-treated db/db mice; meanwhile, there was a significant increase in body weight in pioglitazone-treated db/db mice compared with vehicle treated-db/db mice (Fig. 3B). As for glycemic parameters, TT01001 treatment significantly decreased blood glucose levels (postprandial and fasting) (Fig. 3C, 3D). During the OGTT, blood glucose levels were lower in TT01001-treated db/db mice than in vehicle-treated db/db mice at all measurement points (Fig. 3E). Glucose AUCs (0–180 min in OGTT) were significantly decreased in TT01001-treated db/db mice (Fig. 3F). In plasma parameters of vehicle-treated db/db mice, a significant decrease was seen in the relative value of the endpoint of insulin concentration against the beginning of dosage compared with C57BL/6J mice. Neither TT01001 nor pioglitazone affected the plasma insulin levels (Fig. 3G). With regards to dyslipidemia, vehicle-treated db/db mice exhibited hyperlipidemia compared with C57BL/6J mice. There were significantly lower plasma NEFA levels in TT01001-treated db/db mice, similar to the changes seen with pioglitazone (Fig. 3H).

**Effect of TT01001 on mtDNA levels and mitochondrial respiratory chain enzyme activity.** To reveal the effect of TT01001 on mitochondrial function, we examined the effects on mtDNA levels and mitochondrial respiratory chain enzyme activity in the skeletal muscle. The mtDNA level was significantly decreased in vehicle-treated db/db
mice compared with that in C57BL/6J mice. Meanwhile, there were no differences in the mtDNA level of TT01001- or pioglitazone-treated db/db mice (Fig. 4). In addition to analyzing the mtDNA levels, we examined mitochondrial chain enzyme activity, complex I+III, II+III, and IV activity in db/db mice. First, we examined the short-duration effect of the test compounds on the mitochondrial respiratory chain enzyme activity of the skeletal muscle in db/db mice. There were no alterations in complex I+II, II+III, and IV activities with either TT01001 or pioglitazone (10 or 30 μmol/L, respectively) (Fig. 5A-C). We next examined the chronic effects of the test compounds on mitochondrial respiratory chain enzyme activity in the skeletal muscle of db/db mice. There was no change in complex I+III or IV activities of vehicle- or test compound-treated db/db mice compared with C57BL/6J mice (Fig. 6A, 6C). Meanwhile, complex II+III activity was approximately two-fold higher in vehicle-treated db/db mice than in C57BL/6J mice (Fig. 6B). We found that TT01001 significantly decreased elevated complex II+III activity compared with that in vehicle-treated mice.
**Discussion**

The mitochondrial outer membrane protein mitoNEET, a binding protein of the insulin sensitizer pioglitazone, is considered a novel drug target for the treatment of type II diabetes (Colca et al., 2004). Kushiminski and his colleagues demonstrated that the overexpression of mitoNEET in adipose tissues improved glycemic parameters, altered mitochondrial function, and decreased β-oxidation or membrane potentials (Kusminski et al., 2012). That report suggested an in vivo physiological role of mitoNEET in glucose metabolism or mitochondrial function. On the other hand, although targeting mitoNEET is considered a novel strategy for the treatment of type II diabetes, there were no reports on the mitoNEET ligand effect on diabetes or mitochondrial function using animal disease models. In this study, we demonstrated the first experimental observation of the effects of the mitoNEET ligand on diabetes and mitochondrial function in type II diabetic model db/db mice.

Recently, we synthesized a new small-molecule compound called TT01001 on the basis of the pioglitazone structure. Pioglitazone has been known as a PPARγ agonist (Lehmann et al., 1995), thus we first examined the PPARγ activation effect of TT01001. TT01001 did not change TR-FRET emission signal; meanwhile, pioglitazone showed the increase of TR-FRET emission signal with concentration dependent manner. These
Results suggest that TT01001 did not show the activation effect of PPARγ. We next assessed the binding effect of TT01001 to mitoNEET by using recombinant mitoNEET and the SPR method. TT01001 increased the RU with a concentration-dependent manner like pioglitazone on the immobilized recombinant mitoNEET. Previously, binding effect of pioglitazone to mitoNEET was observed using liver mitochondrial suspension (Geldenhuys et al., 2010). Therefore, our SPR data suggest that TT01001 has binding effect to mitoNEET. Collectively, TT01001 has the in vitro pharmacological characteristics as a mitoNEET binding effect, but not a PPARγ activation effect.

To clarify whether oral administration of TT01001 could exhibit an ameliorative effect on diabetes, we examined the effects of TT01001 on glycemic parameters in db/db mice. Oral administration of TT01001 for 28 days significantly reduced blood glucose levels (postprandial and fasting) and improved glucose intolerance and hyperlipidemia, but not plasma insulin levels. These effects of TT01001 were almost equivalent to those of pioglitazone, indicating that TT01001 has a potent antidiabetic effect. Pioglitazone improves hyperglycemia and hyperlipidemia of db/db mice via an insulin-sensitizing effect on peripheral tissues (Suzuki et al., 2000). Thus, our data suggest that TT01001 improved peripheral glucose and lipid utilization similar to pioglitazone. In contrast to the apparent effects on glucose metabolism, pioglitazone
significantly increased body weight in db/db mice. Weight gain is one of the major side effects of pioglitazone in clinical use (Gillies and Dunn, 2000), and it is attributed to pioglitazone’s enhancement of PPARγ activation (Borsting et al., 2012). Therefore, weight gain in pioglitazone-treated db/db mice is considered a hallmark side effect of pioglitazone in clinical use. Interestingly, TT01001 has equivalent efficacy with pioglitazone on glycemic parameters, whereas it has no effect on body weight in db/db mice. As the possible reason for this phenomenon, although TT01001 bound to mitoNEET similar to pioglitazone, it did not exhibit the PPARγ activation effect. Taken together, TT01001 has equivalent therapeutic efficacy to the insulin-sensitizer pioglitazone without causing weight gain in db/db mice.

Isolated heart mitochondria of mitoNEET-deficient mice show a decrease in state 3 respiration (Wiley et al., 2007). The overexpression of mitoNEET in genetic type II diabetic model ob/ob mice showed the alteration of mitochondrial functions (Kusminski et al., 2012). These reports may indicate that mitoNEET plays a physiological role in mitochondrial function. To examine the effects of TT01001 on mitochondrial function, we first examined the effect of TT01001 on mitochondrial biogenesis in the skeletal muscle, which is a major target organ of insulin and plays an essential role in glucose utilization (DeFronzo et al., 1979) in db/db mice. We observed significantly lower levels
of mtDNA in vehicle-treated db/db mice compared with C57BL/6J mice. Since mtDNA level is closely related to the pathology of type II diabetes (Lee et al., 1998, Song et al., 2001), decreased mtDNA levels in the skeletal muscle of vehicle-treated db/db mice were considered an alteration of mitochondrial function. However, we did not find that TT01001 and pioglitazone affected the mtDNA level in the skeletal muscle of db/db mice. Previous study showed that pioglitazone increased mtDNA copy numbers in the subcutaneous adipose tissue of patients with type II diabetes for 12 weeks after administration (Bogacka et al., 2005). Although the effects of TT01001 and pioglitazone on the mtDNA levels in adipose tissue are unknown, they may have no effect on the amount of mtDNA in skeletal muscle during an administration period of this length. Collectively, these data suggest that TT01001, at least in the skeletal muscle, did not effect on mitochondrial biogenesis.

In the mitochondrial respiratory chain enzyme activity assay, TT01001 did not affect complex I+III, II+III, or IV of the isolated mitochondrial fraction of the skeletal muscle from db/db mice during a short time period. On the other hand, in a chronic examination, TT01001 led to a significant reduction in the increase of complex II+III activity without effect on complex I+III or IV activity in the skeletal muscle of db/db mice. In db/db mice, mitochondrial respiration of the glycolytic skeletal muscle is enhanced under
Excess lipid increased the oxidative capacity and expression level of the mitochondrial respiratory chain subunit in the skeletal muscle of db/db mice (Turner et al., 2007). Possibly as a result of enhanced complex II+III activity in db/db mice might indicate that it is a compensatory response to diabetic conditions, hyperglycemia or hyperlipidemia. The skeletal muscle mitochondrial complex II has been reported to produce superoxide and/or hydrogen peroxide at relatively high rate (Quinlan et al., 2012). Accordingly, we speculate that compensatory enhanced complex II+III activity leads to high production of superoxide and/or hydrogen peroxide, and they, at least in part, contribute to type II diabetes condition in db/db mice. mitoNEET possibly contributes to modulate oxidative stress via transfer iron into mitochondrial matrix (Zuris et al., 2011). TT01001 might ameliorate mitochondrial function towards normalization through the modulation of oxidative stress in the skeletal muscle of db/db mice. Therefore, mitoNEET may be a key regulatory protein of mitochondrial function, especially respiratory chain enzyme complex II or oxidative stress modulation. On the other hand, it was not clear how TT01001 suppressed the increase in complex II+III activity with no effect to mitochondrial biogenesis or complex I+III and complex IV activity. The practical concentration of TT01001 in target tissues and the binding affinity of it to mitoNEET
have been unknown. Thus, a detailed analysis of mitoNEET on type II diabetes or mitochondrial function and PK/PD profiles of TT01001 is needed to elucidate the mechanism of action of TT01001. Further examination might identify the physiological role of mitoNEET in type II diabetes or mitochondrial function.

In conclusion, our results show that the orally active, small molecule TT01001 showed binding affinity for mitoNEET without PPARγ activation effect. It improved diabetes and ameliorated mitochondrial function in the skeletal muscle of db/db mice. This is the first study that demonstrates the effects of a mitoNEET ligand on glucose metabolism and mitochondrial function in a type II diabetic animal model. Although further study is needed to clarify the physiological role of mitoNEET in diabetes or mitochondrial function, our data suggest that the alteration of mitochondrial function via mitoNEET may be valuable for the treatment of type II diabetes. Finally, this study would support targeting mitoNEET as a useful therapeutic approach for the treatment of type II diabetes.
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Authorship Contributions

Participated in research design: Takahashi, Yamamoto, Ogasawara, Hayashi, Nakada, Kainoh.


Contributed new reagents or analytical tool: Yamamoto, Aoki, Kawai.

Performed data analysis: Takahashi

Wrote or contributed to the writing of the manuscript: Takahashi, Ogasawara, Nakada, Kainoh
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Footnotes

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d)
Figure Legends

Fig. 1. The chemical structure of TT01001

Fig. 2. TT01001 did not activate PPARγ but interacted with mitoNEET. The activity of PPARγ was assessed by the TR-FRET method using the Lanthascreen™ TR-FRET PPARγ coactivator assay kit. TT01001 or pioglitazone (PIO) were incubated with the human PPARγ ligand-binding domain tagged with GST, terbium-labeled anti-GST antibody, and fluorescein peptide. Each line is expressed as a mean emission ratio of 520 nm and 495 nm (N=4) (A). The interaction of TT01001 and PIO was determined by the SPR method using Biacore™ S51. Different concentrations of TT01001 or PIO were injected onto the immobilized mitoNEET on a sensor chip (CM-5, GE Healthcare) for 60 s at a flow rate of 30 μL/min. Each line indicates the resonance unit curves of 1, 2, 4, 8, 20 μmol/L TT01001 (B) and 0.3, 0.6, 1.2, 2.5, 5.0 μmol/L PIO (C), respectively.

Fig. 3. TT01001 improved diabetes in db/db mice without causing weight gain. The vehicle (0.5% MC), TT01001 (100 mg/kg), or pioglitazone (PIO, 30 mg/kg) were orally administered to db/db mice once daily for 28 days. The vehicle was also orally
administered to C57BL/6J mice. The courses of body weight (N=14) (A), body weight on day 24 (N=14) (B), postprandial blood glucose levels (N=14) (C), fasting blood glucose levels (N=8) (D), blood glucose levels during the OGTT (N=8) (E), blood glucose AUCs (0–180 min) (N=8) (F), ratio of plasma insulin concentration between the beginning of dosage and end of dosage (N=6) (G), plasma concentration of NEFA (N=6) (H) are shown as means ± S.E.M. *P < 0.05, **P < 0.01 compared with the vehicle-treated db/db mice by Dunnett’s multiple test.

**Fig. 4.** TT01001 and pioglitazone did not affect the mtDNA level in the skeletal muscle of db/db mice. The vehicle (0.5% MC), TT01001 (100 mg/kg), or pioglitazone (PIO, 30 mg/kg) was orally administered to db/db mice once daily for 28 days. The vehicle was also orally administered to C57BL/6J mice. The mtDNA levels were determined by quantitative real time PCR. Data are shown as means of the mtDNA ratio against C57BL/6J mice ± S.E.M. **P < 0.01 compared with the vehicle-treated db/db mice by Dunnett’s multiple test (N = 6 animals per group).

**Fig. 5.** TT01001 and pioglitazone did not affect mitochondrial respiratory chain enzyme activity during a short time period. DMSO, TT01001, or pioglitazone (PIO) was applied
to the skeletal muscle mitochondrial fraction of db/db mice, and mitochondrial respiratory chain enzyme activity was measured by complex I+III (A), complex II+III (B), and complex IV (C) activities. Complex activity was normalized by citrate synthase activity in each sample. Mitochondrial complex specific inhibitor, rotenone, malonate, and KCN were used as the reference compounds. Each assay was performed in triplicate and the data are shown as means of ratios against the control (DMSO treatment) ± S.E.M.. Incubation time of TT01001 and pioglitazone is 5 min for complex I+III and complex IV activity assay, 10 min for complex II+III activity assay, respectively.

**Fig. 6.** TT01001 decreased elevated complex II+III activity in db/db mice. The vehicle (0.5% MC), TT01001 (100 mg/kg), or pioglitazone (PIO, 30 mg/kg) was orally administered to db/db mice once daily for 28 days. The vehicle was also orally administered to C57BL/6J mice. The mitochondrial fraction was isolated from vehicle- or compound-treated mice, and mitochondrial respiratory chain enzyme activity was measured by complex I+III (A), complex II+III (B), and complex IV (C) activities. The data are shown as means of ratios against vehicle-treated C57BL/6J mice ± S.E.M. *P < 0.05, **P < 0.01 compared with the vehicle-treated db/db mice by Dunnett’s multiple test (N = 6 animals per group).
Figure 1
Figure 2

A

![Graph showing emission ratio (520/490) vs. concentration (μmol/L) for TT01001 and PIO.]

B

![Graph showing resonance units over time for TT01001 at concentrations of 20, 8, 4, 2, and 1 μmol/L.]

C

![Graph showing resonance units over time for PIO at concentrations of 5.0, 2.5, 1.2, 0.6, and 0.3 μmol/L.]

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Figure 4
Figure 5

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Figure 6

Complex I+III activity (% of C57BL6)

Vehicle  Vehicle  TT01001  PIO

Complex II+III activity (% of C57BL6)

Vehicle  Vehicle  TT01001  PIO

Complex IV activity (% of C57BL6)

Vehicle  Vehicle  TT01001  PIO

Supplemental Table 1 TaqMan probes sequence for quantitative real time PCR

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<tr>
<th>Genes</th>
<th>Forward primer sequence (5→3')</th>
<th>Probe sequence (5→3')</th>
<th>Revers primer sequence (5→3')</th>
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<td>GTCATATTTGGGAAACTAGAAATTGATC</td>
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