Regular Article

Synthesis and Structure–Activity Relationships of 2-Aminoacetamide Derivatives as Peroxisome Proliferator-Activated Receptor $\alpha/\gamma$ Dual Agonists

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We describe the design, syntheses, and structure–activity relationships of novel zwitterionic compounds as nonthiazolidinedion-based peroxisome proliferator-activated receptor (PPAR) $\alpha/\gamma$ dual agonists. In our previous report, we obtained compound 1 showing potent PPAR $\alpha/\gamma$ dual agonistic activities, together with a sufficient glucose-lowering effect in $db/db$ mice. However, this compound possessed an issue, i.e., the 1,3,4-oxadiazole ring was not stable in acidic conditions. Thus, we carried out further optimization to improve the stability while maintaining the other favorable profile features including potent PPAR $\alpha/\gamma$ dual agonistic activity. We addressed the issue by changing the oxadiazole ring to a bioisostere amide group. These amide derivatives were stable in acidic conditions and decreased plasma glucose and plasma triglyceride levels significantly without marked weight gain.

Key words peroxisome proliferator-activated receptor; type 2 diabetes; bioisostere

In 2014, 4.9 million people died from diabetes.1) Diabetes is a serious health problem since the number of individuals with diabetes is 387 million at present and is estimated to be 592 million by 2035.2) Of these cases, the populations of type 2 diabetes currently account for >90% of all diabetes worldwide.2) The type 2 diabetic people who have insulin resistance are more susceptible to cardiovascular risk factors including dyslipidemia, coagulopathy, hypertension and obesity.3) Furthermore, it has been revealed that type 2 diabetes with insulin resistance and hyperinsulinemia is associated with the risk of cancer in recent years.4) In fact, the prevention and the treatment of type 2 diabetes becomes more and more important to maintaining the quality of life of people all over the world. Today, we have a wide variety of antidiabetic agents. Among these, the use of the thiazolidinedione (TZD) insulin-sensitizer makes sense for treating patients with type 2 diabetes since TZD activates peroxisome proliferator-activated receptor gamma (PPAR$\gamma$) and leads to abated insulin resistance by promoting hypertrophied adipocyte differentiation and improving the balance of physiological active substances.5,6) However, its activation also causes adverse effects including weight gain, fluid retention and edema.7) On the other hand, the activation of another subtype PPAR$\alpha$ has been identified to mediate the lipid-lowering activity in studies with the fibrate class of hypolipidemic drugs. Furthermore, some PPAR$\alpha$ agonists have been reported to reduce weight gain in rodents.8,9) Thus, PPAR$\alpha/\gamma$ dual agonists are expected to be a safer type 2 diabetic agent.

In our previous report,10) we obtained zwitterionic compounds showing the PPAR$\alpha/\gamma$ dual agonistic activities. In particular, compound 1 provided highly potent dual activities ($\alpha$: EC$_{50}$=0.55 nM, $\gamma$: EC$_{50}$=18 nM, shown in Fig. 1), accompanied by a pronounced glucose lowering effect and triglyceride remedying effect without body weight gain at 10 mg/kg in the $db/db$ mice. Moreover, the exchange of the ring system into this 1,3,4-oxadiazole ring reduced direct or mechanism-based inhibitions (MBI) of CYP3A4 inducing drug–drug interaction risks, which our early furan derivatives possessed.11) Although these oxadiazole derivatives showed excellent profiles as above, they were found to have a low stability in acidic media, which was not adequate for orally-administered agents. When...
the chemical stability of 1 was studied under Japanese Pharmacopoeia (JP) XIV 1st fluid (JP1, pH 1.2), JP XIV 2nd fluid (JP2, pH 6.8) and Britton–Robinson buffer (pH 4.0) at 37°C, it was revealed that the 1,3,4-oxadiazole ring was decomposed in JP1 acidic solution (Fig. 2). It was considered that this stability was not tolerable for the oral administration. Thus, our medicinal strategy to address the issue consisted of a conversion of the 1,3,4-oxadiazole to a bioisostere, i.e., amide group, which would ensure enough stability.

We report herein the optimization of the side chain on the nitrogen atom and the lipophilic tail of our lead with an aim to improve the chemical stability and to obtain antidiabetic drugs without adverse effects.

Results and Discussion

Chemistry Firstly, in order to confirm the potential of amide derivatives and to explore substituent effects on the nitrogen of the amide group, compounds 4a–i were designed and synthesized as depicted in Chart 1. The reductive alkylation of the secondary amine 2 with glyoxylic acid in the presence of NaBH(OAc)_3 in methanol gave a common key intermediate carboxylic acid, 3. The amidation reaction of 3 with the corresponding amine using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) and N,N-dimethylformamide (DMF), followed by hydrolysis provided 4a–i.

The syntheses of the (R)- or (S)-methyl group at the α-position introducing compounds (i.e., the alanyl amide compounds) were depicted in Chart 2. The treatment of the aldehyde 6 with alanine tert-butyl ester under reflux conditions to form the imine, followed by the reduction with NaBH_4 gave the secondary amines 7a and 7b. The reductive alkylation of 7a and 7b with commercially available aldehyde using NaBH(OAc)_3 in methanol afforded 8a and 8b, respectively. The acidic hydrolysis of 8a and 8b, followed by the amidation with methylamine under the conventional EDCI/HOBt condi-
tion gave 9a and 9b, respectively. Finally, the hydrolysis of 9a and 9b provided the carboxamides 10a and 10b, respectively.

Synthesis of a one-carbon homologation compound, 15, is shown in Chart 3. The treatment of the aldehyde 6 with commercially available 3-aminopropionic acid tert-butyl ester 11 under reflux conditions to form the imine, followed by...
the reduction with NaBH₄ gave the secondary amine 12. The alklylation of 12 with 4-(chloromethyl)-5-methyl-2-phenyl-1,3-oxazole in the presence of K₂CO₃ in acetonitrile gave 13. Acidic catalyzed hydrolysis of the tert-butyl ester group of 13, followed by amidation with methylamine provided 14. Finally, the hydrolysis of the ethyl ester group gave 15.

Synthesis of reverse amide compound 17 is depicted in Chart 4. The reductive alklylation of the secondary amine 2 with N-Boc-2-aminoacetaldehyde in the presence of NaBH(OAc)₃ in dichloromethane gave 16. The deprotection of the Boc group of 16, followed by acetylation and hydrolysis prepared 17.

Compound 22 was synthesized as shown in Chart 5. This compound was synthesized by using chloride 18 and secondary amine 19 in a similar manner to the synthetic approach of compound 15.

**Biological Evaluation** Novel compounds were evaluated in a cell-based transcription assay using GAL4-PPAR chimeric receptors and plasmids for functional analysis-secreted alkaline phosphatase (pFA-SEAP) as a reporter vector, and meric receptors and plasmids for functional analysis-secreted.

The results of various amide derivatives are presented in Table 1. We have had a lot of information of the structure–activity relationship (SAR) of non-substituted phenyloxazole derivatives such as compound 23. And we aimed to avoid false effects on activities from high lipophilicity. Thus, we undertook SAR analysis of the amide group on the linker with low lipophilicity showing a non-substituted phenyloxazole group at the lipophilic tail. The derivatives 4a–c, which had unsubstituted or lower alkyl amide groups, showed acceptable agonistic activity. However, bulky amide compounds 4d–g showed weak activities. Among these, methoxyethyl amide 4f or thiazolyl amide 4g gave low selectivity for PPARα. The cyclic amino group introduced amide compounds 4h and i also showed weak activities. The introduction of the methyl group at the α-position of the amide (10a, b), a one-carbon homologation of the methylene chain to the ethylene one (15) or the reverse of the amide group (17) largely decreased the activities. Thus, we obtained encouraging results, showing that the amide variants possessed the potential as PPARα/γ dual agonists.

Then, we moved on to the balancing of the physicochemical property by further optimization of compound 4a, whose lipophilicity was slightly low to obtain a good oral exposure. We introduced the methyl group at 4-position on the lipophilic tail as a promising substituent in the previous study. As a result, our compound, 22 (Fig. 3), showed good activity and selectivity for PPARα (EC₅₀=2.8 nM, ten times stronger than EC₅₀=26 nM for PPARγ), which might be preferable to reduce the side effects of PPARγ activation, e.g., weight gain. Furthermore, 22 showed good physicochemical properties such as low lipophilicity, high metabolic stability and weak CYP inhibition.

With potent agonist 22 having a distinguished Absorption–Distribution–Metabolism–Excretion (ADME) profile, *in vivo* studies were carried out; oral dosing was once daily for 10 d in *db/db* mice, an obese animal model of type 2 diabetes characterized by severe insulin resistance and marked hypertriglyceridemia (six per group). Rosiglitazone, which is a selective PPARγ agonist, showed the effect of a significant decrease in plasma glucose and plasma triglyceride. However, this drug led to the adverse effects of an increase in the body weight on account of only PPARγ activation. Compound 22, on the other hand, showed a dose-dependent response and exhibited a good efficacy without marked weight gain at 1 mg/kg as depicted in Fig. 4 and Table 2. Although the inhibitory activities of 22 for PPARα and PPARγ in the *in vitro* assay were weaker than those of 1, the physicochemical properties improved 22 showed good *in vivo* efficacy in the lower dose as compared with 1, which showed the activity at 10 mg/kg.

Furthermore, a comparative study of 22 versus Muraglitazar (α: EC₅₀=0.3 μM, γ: EC₅₀=0.1 μM) was performed in Zucker Fatty rats, which develop type 2 diabetes with obesity, hyperglycemia and hyperinsulinemia. The results are displayed in Table 3. While both compounds showed a similar and significant blood glucose lowering effect and promoted triglyceride decreasing, compound 22, a PPARα dominant variant, produced less weight gain than Muraglitazar (dosed orally at 10 mg/kg in Zucker Fatty Rat [six per group] for 13 d).

**Conclusion** We described the design, syntheses and evaluation of novel zwitterionic compounds as PPARα/γ dual agonists. The issue faced by the 1,3,4-oxadiazole derivatives, *e.g.*, low stability under acidic conditions, was addressed by changing the oxa-
Table 1. *In Vitro* Activity and Physicochemical Properties of Compound 1 and Amide Compounds

<table>
<thead>
<tr>
<th>Compd</th>
<th>R</th>
<th>PPARα EC50 (nM)</th>
<th>PPARγ EC50 (nM)</th>
<th>Log Dα</th>
<th>CYP3A4 Direct inhibition (%)</th>
<th>MBI remaining (%)</th>
<th>Solubility JP1 (µg/mL)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Direct inhibition (%)</td>
<td>MBI remaining (%)</td>
<td>JP1 (µg/mL)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23^δ</td>
<td></td>
<td>2.9</td>
<td>33</td>
<td>1.1</td>
<td>28</td>
<td>110</td>
<td>Decomposed</td>
</tr>
<tr>
<td>4a</td>
<td></td>
<td>41</td>
<td>92</td>
<td>0.8</td>
<td>&lt;10</td>
<td>98</td>
<td>&gt;740</td>
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<tr>
<td>4b</td>
<td></td>
<td>25</td>
<td>53</td>
<td>0.9</td>
<td>26</td>
<td>89</td>
<td>&gt;760</td>
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<tr>
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<td>24</td>
<td>110</td>
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<td>16</td>
<td>122</td>
<td>&gt;720</td>
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<tr>
<td>4d</td>
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<td>27</td>
<td>230</td>
<td>1.8</td>
<td>21</td>
<td>NT^δ</td>
<td>&gt;640</td>
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<tr>
<td>4e</td>
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<td></td>
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<td>470</td>
<td>1.0</td>
<td>19</td>
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<td>&gt;920</td>
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<td>4g</td>
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<td>67</td>
<td>2.1</td>
<td>75</td>
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<td>810</td>
</tr>
<tr>
<td>4h</td>
<td></td>
<td>150</td>
<td>220</td>
<td>1.1</td>
<td>51</td>
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<td>&gt;690</td>
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<tr>
<td>4i</td>
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<td>60</td>
<td>340</td>
<td>2.2</td>
<td>37</td>
<td>NT</td>
<td>&gt;810</td>
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diazole ring to amide groups. Amide variant 22 exhibited the effects of a significant decrease in plasma glucose and plasma triglyceride levels without weight gain. The agonistic activities of amide derivatives are lower compared to the oxadiazole derivatives previously reported. However, we are convinced that this stable amide variant has superior quality for targeting orally-administered agents. Moreover, we are certain that PPARα dominant dual agonistic activity is necessary in order to reduce side effects of PPARγ activation by comparative study of the PPARγ dominant dual agonist. Finally, we are looking forward to contributing to improving the quality of life of patients by this PPARα/γ dual agonist.

Experimental
Chemistry Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. 1H-NMR spectra were determined on a JEOL JNM-EX400 spectrometer. Chemical shifts are reported in parts per million relative to tetramethylsilane (TMS) as an internal standard. Significant 1H-NMR data are tabulated in the following order: number of protons, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), and coupling constant(s) in hertz. Electron spray ionization condition (ESI) mass spectra were recorded on an Agilent 1100 and SCIEX API-150EX spectrometer. Fast atom bombardment ionization condition (FAB) mass spectra were recorded on a JEOL JMSHX110 spectrometer. Electron impact ionization condition (EI) mass spectra were recorded on a JEOL JMS-AX505W. Column chromatography was performed with Merck silica gel 60 (particle size 0.060–0.200 or 0.040–0.063 mm). Flash column chromatography was performed with Biotage FLASH Si packed columns. Thin layer chromatography (TLC) was performed on Merck pre-coated TLC glass sheets with silica gel 60 F254, and compound visualization was effected with a 5% solution of phosphomolybdic acid in ethanol, UV lamp, iodine, or Wako ninhydrin spray.

Table 1. Continued.

<table>
<thead>
<tr>
<th>Compd</th>
<th>R</th>
<th>PPARα EC50 (nM)</th>
<th>PPARγ EC50 (nM)</th>
<th>Log D</th>
<th>CYP3A4 Direct inhibition</th>
<th>MBI remaining</th>
<th>Solubility</th>
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<tr>
<td>10a</td>
<td></td>
<td>1500</td>
<td>2100</td>
<td>1.5</td>
<td>22</td>
<td>NT</td>
<td>&gt;700</td>
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<tr>
<td>10b</td>
<td></td>
<td>620</td>
<td>1400</td>
<td>1.6</td>
<td>22</td>
<td>86</td>
<td>&gt;780</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>180</td>
<td>1500</td>
<td>0.8</td>
<td>41</td>
<td>NT</td>
<td>&gt;860</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>290</td>
<td>1100</td>
<td>1.0</td>
<td>27</td>
<td>NT</td>
<td>&gt;570</td>
</tr>
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</table>

a) The logD values were determined from the partition coefficient for 1-octanol/phosphate buffer saline (PBS) at pH 7.4. b) The CYP3A4 direct inhibition values were shown in % inhibition at a 10 µM concentration of the compounds for 60 min incubation. c) The MBI values were shown in % remaining at a 100 µM concentration of the compounds reacted with CYP3A4 probe substrates after 30 min preincubation in human liver microsomes. d) Not tested. e) Water solubility was measured at JP1. f) Ref. 10.

Fig. 3. Profile of Compound 22
a) The value at pH 7.4. b) The % remaining value using human microsomes. c) The % inhibition value at a 10 µM concentration of compound 22. d) The % remaining value at a 100 µM concentration of compound 22 reacted with CYP3A4 probe substrates after 30 min preincubation in human liver microsomes.
Elemental analysis was performed using a PerkinElmer, Inc. CHNS/O 2400II or a Yokokawa Analysis IC7000RS, and analytical results were within ±0.4% of the theoretical values unless otherwise noted.

**2-N-{4-[(1-Ethoxy-2-methyl-1-oxopropan-2-yl)oxy]-3,5-dimethylbenzyl}-N-{[5-methyl-2-phenyl-1,3-oxazol-4-yl]methyl}glycine (3)** To a solution of ethyl 2-[2,6-dimethyl-4-[[[5-methyl-2-phenyl-1,3-oxazol-4-yl]methyl]amino]methyl]-phenoxyl]-2-methylpropanoate (2) (3.0 g, 6.9 mmol) in methanol (30 mL), glyoxylic acid monohydrate (0.84 g, 8.9 mmol) and sodium triacethoxyborohydride (2.3 g, 10 mmol) were added. The mixture was stirred at room temperature overnight. After the solvent was removed *in vacuo*, the residue was dissolved in ethyl acetate (EtOAc), washed with water and brine, dried over Na$_2$SO$_4$ and concentrated. The crude product was purified by column chromatography on silica gel (chloroform/methanol=19/1 as eluent, v/v) to provide 3 as a colorless solid (3.1 g, 91%): MS (ESI) $m/z$: 495 (M+H)$^+$. 

**General Procedure for 2-[4-([2-(Substituted)-amino]-2-oxoethyl)[[5-methyl-2-phenyl-1,3-oxazol-4-yl]methyl]-2,6-dimethylphenoxy]-2-methylpropanoic Acid (4a–i)** To a solution of 3 in DMF or acetonitrile (MeCN), commercially available amines, EDCI and HOBt were added. The mixture was stirred at room temperature overnight. After the solvent was removed *in vacuo*, the residue was dissolved in EtOAc, washed with water and brine, dried over Na$_2$SO$_4$ and concentrated. The crude product was purified by column chromatography on silica gel (hexane/EtOAc as eluent) to provide 4a–i ethyl ester form. To a mixture of 4a–i ethyl ester form and methanol was added NaOH (1 M aqueous solution) and stirred at 50°C overnight. After HCl (1 M aqueous solution) was added to the reaction mixture, the solvent was removed *in vacuo*. After EtOAc was added to the residue, the mixture was washed with water and brine, dried over Na$_2$SO$_4$ and concentrated. The crude product was purified by column chromatography on silica gel (chloroform/methanol). The residue was triturated with EtOAc–hexane or freeze-dried from 1,4-dioxane to provide 4a–i.

2-[2,6-Dimethyl-4-{{[2-(methylamino)-2-oxoethyl][(5-methyl-2-phenyl-1,3-oxazol-4-yl) methyl] amino}methyl}phenoxy]-2-methylpropanoic Acid (4b): This compound was obtained as a colorless solid in 66% yield by using 3 (5.9 mmol), methylamine (8.9 mmol), EDCI (12 mmol), HOBt (12 mmol), MeCN (2.0 mL) and NaOH (4.5 eq): $^1$H-NMR (400 MHz, DMSO-$d_6$) $\delta$: 1.31 (6H, s), 2.12 (6H, s), 2.22 (3H, s), 2.60 (3H, d, $J$=4.6 Hz), 3.09 (2H, s), 3.50 (2H, s), 3.56 (2H, s), 6.99 (2H, s), 7.48–7.53 (3H, m), 7.74–7.78 (1H, m), 7.91–7.93 (2H, m). MS (ESI) $m/z$: 480 (M+H)$^+$. Anal. Caled for C$_{27}$H$_{33}$N$_3$O$_5$: C, 67.62; H, 6.94; N, 8.76. Found: C, 67.57; H, 7.09; N, 8.56.

2-[2,6-Dimethyl-4-{{[2-(dimethylamino)-2-oxoethyl][(5-methyl-2-phenyl-1,3-oxazol-4-yl) methyl]amino}methyl]phenoxyl]-2-methylpropanoic Acid (4c): This compound was obtained as HCl salts of a colorless solid in 52% yield by using 3 (4.5 mmol), dimethylamine (6.7 mmol), EDCI (8.9 mmol), HOBt (8.9 mmol), DMF (10 mL) and NaOH (2.9 eq), and being hydrochlorinated with HCl (4 M dioxane).
solution): $^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$: 1.36 (6H, s), 2.18 (6H, s), 2.42 (3H, s), 2.84 (3H, s), 2.89 (3H, s), 4.11–4.40 (6H, m), 7.31 (2H, s), 7.54–7.59 (3H, m), 7.97–7.99 (2H, m). MS (ESI) $m/z$: 494 (M+H)$^+$. Anal. Calcd for C$_{29}$H$_{35}$N$_3$O$_5$·HCl·2H$_2$O·0.25EtOAc: C, 60.19; H, 6.74; N, 7.36.  

2-[4-{[[2-(Ethylamino)-2-oxoethyl][[5-methyl-2-phenyl-1,3-oxazol-4-yl]methyl]amino)methyl]-2,6-dimethylphenoxy]-2-methylpropanoic Acid (4d): This compound was obtained as a colorless solid in 36% yield by using 3 (0.40 mmol), ethylamine (0.81 mmol), EDCI (0.81 mmol), HOBt (0.81 mmol), DMF (2.0 mL) and NaOH (5.6 eq), and being hydrochlorinated with HCl (4 M dioxane solution): $^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$: 0.99 (3H, t, $J=7.1$ Hz), 1.36 (6H, s), 2.18 (6H, s), 2.41 (3H, s), 3.08 (3H, s), 3.79–3.90 (2H, m), 4.01–4.06 (2H, m), 4.24–4.39 (4H, m), 7.27 (2H, s), 7.54–7.57 (3H, m), 8.00–7.96 (2H, m). MS (ESI) $m/z$: 494 (M+H)$^+$. Anal. Calcd for C$_{29}$H$_{35}$N$_3$O$_5$·HCl·2H$_2$O·0.2EtOAc·0.1hexane: C, 61.49 H, 7.20; N, 7.32. Found: C, 61.75; H, 7.12; N, 6.97.  

2-[4-{[[2-(Cyclopropylamino)-2-oxoethyl][[[5-methyl-2-phenyl-1,3-oxazol-4-yl]methyl]amino)methyl]-2,6-dimethylphenoxy]-2-methylpropanoic Acid (4e): This compound was obtained as a colorless solid in 52% yield by using 3 (0.28 mmol), cyclopropylamine (0.42 mmol), EDCI (0.42 mmol), HOBt (0.42 mmol), DMF (2.0 mL) and NaOH (9.5 eq): $^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$: 0.36–0.40 (2H, m), 0.59–0.63 (2H, m), 1.32 (6H, s), 2.11 (6H, s), 2.25 (3H, s), 2.66–2.68 (1H, m), 3.06 (2H, s), 3.53 (2H, s), 3.57 (2H, s), 6.94 (2H, s), 7.50–7.55 (3H, m), 7.83 (1H, d, $J=4.4$ Hz), 7.92–7.95 (2H, m). MS (ESI) $m/z$: 506 (M+H)$^+$. Anal. Calcd for C$_{29}$H$_{35}$N$_3$O$_5$·0.75H$_2$O·0.1hexane: C, 61.69; H, 6.05; N, 8.98; S, 5.62. Found: C, 61.72; H, 6.69; N, 7.93.  

2-[4-{[[2-(2-Methoxyethyl)amino]-2-oxoethyl][5-methyl-2-phenyl-1,3-oxazol-4-yl]methyl]amino)methyl]-2,6-dimethylphenoxy]-2-methylpropanoic Acid (4f): This compound was obtained as a colorless solid in 43% yield by using 3 (0.28 mmol), 2-methoxyethylamine (0.42 mmol), EDCI (0.42 mmol), HOBt (0.42 mmol), DMF (2.0 mL) and NaOH (9.5 eq): $^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$: 1.33 (6H, s), 2.13 (6H, s), 2.66–2.68 (1H, m), 3.06 (2H, s), 3.53 (2H, s), 3.57 (2H, s), 6.94 (2H, s), 7.50–7.55 (3H, m), 7.83 (1H, d, $J=4.4$ Hz), 7.92–7.95 (2H, m). MS (ESI) $m/z$: 494 (M+H)$^+$. Anal. Calcd for C$_{29}$H$_{35}$N$_3$O$_5$·HCl·2H$_2$O·0.25EtOAc: C, 60.04; H, 7.05; N, 7.00. Found: C, 60.08; H, 6.72; N, 6.60.  

### Table 2. Parameters of in Vivo Study with 22 on db/db Mice

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Compd</th>
<th>Dose (mg/kg)</th>
<th>Plasma glucose (mg/dL)</th>
<th>Change (%)</th>
<th>Plasma triglyceride (mg/dL)</th>
<th>Change (%)</th>
<th>BW change (%)</th>
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<tr>
<td>1</td>
<td>Vehicle</td>
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<td>563 ± 7.6</td>
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<td>251 ± 36</td>
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<td></td>
<td>22</td>
<td>1</td>
<td>227 ± 15</td>
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<tr>
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<td>Rosiglitazone</td>
<td>10</td>
<td>223 ± 17</td>
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<td>77 ± 6.2</td>
<td>−69$^{a}$</td>
<td>+9.9$^{a}$</td>
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</table>

$^a$ Mean±standard deviation (n=6). $b$ p<0.001 versus vehicle control (t-test).

### Table 3. Parameters of in Vivo Study with 22 on Zucker Fatty Rat

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Compd</th>
<th>Dose (mg/kg)</th>
<th>Plasma glucose (mg/dL)</th>
<th>Change (%)</th>
<th>Plasma triglyceride (mg/dL)</th>
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<td>Rosiglitazone</td>
<td>10</td>
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<td>−57$^{b}$</td>
<td>175 ± 18</td>
<td>−70</td>
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<td>274 ± 39</td>
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<td>Muraglitazar</td>
<td>10</td>
<td>89 ± 5.8</td>
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<td>132 ± 6.8</td>
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<td>Rosiglitazone</td>
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<td>83 ± 11</td>
<td>−70$^{b}$</td>
<td>147 ± 11</td>
<td>−58$^{b}$</td>
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$^a$ Mean±standard deviation (n=6). $b$ p<0.01 versus vehicle control (t-test).
3,5-dimethylbenzyl]-alaninate (7a, b). The mixture of tert-butyl alaninate 5 (a or b), ethyl 2-(4-formyl-2,6-dimethylphenoxy)-2-methylpropanoate (6) and triethylamine (TEA) in tetrahydrofuran (THF) was stirred under reflux conditions. After the insoluble matter was removed by filtration through celite, the filtrate was concentrated. Sodium borohydride was added to the solution and the mixture was stirred at room temperature. After the solvent was removed in vacuo, the residue was dissolved in EtOAc, washed with saturated sodium bicarbonate aqueous solution and brine, dried over Na2SO4 and concentrated to provide 7 (a or b).

tert-Butyl N-[4-[1-(Ethoxy-2-methyl-1-oxopropan-2-yl)oxy]-3,5-dimethylbenzyl]-alaninate (7a): This compound was obtained as a pale yellow oil in 93% yield by using tert-butyl ester hydrochloride (5a, 1.7 mmol), TEA (1.7 mmol), THF (10 mL), sodium borohydride (1.1 mmol) and methanol (5.0 mL); MS (ESI) m/z: 394 (M+H)+.

tert-Butyl N-[4-(1-Ethoxy-2-methyl-1-oxopropan-2-yl)oxy]-3,5-dimethylbenzyl]-alaninate (7b): This compound was obtained as a pale yellow oil in 80% yield by using d-alanine tert-butyl ester hydrochloride (5b, 1.7 mmol), TEA (1.7 mmol), THF (10 mL), sodium borohydride (1.1 mmol) and methanol (5.0 mL). 1H-NMR (400 MHz, CDCl3) δ: 1.28 (3H, d, J = 6.9 Hz), 1.35 (3H, t, 1.7 Hz), 1.45 (6H, s), 1.49 (9H, s), 2.18 (6H, s), 3.28–3.32 (1H, m), 3.52 (1H, d, J = 12.3 Hz), 3.66 (1H, d, J = 12.3 Hz), 4.29 (2H, q, J = 7.1 Hz), 6.93 (2H, s). MS (ESI) m/z: 394 (M+H)+.

General Procedure for tert-Butyl N-[4-[1-(Ethoxy-2-methyl-1-oxopropan-2-yl)oxy]-3,5-dimethylbenzyl]-alaninate (8a, b): To a solution of tert-butyl N-[4-[1-(Ethoxy-2-methyl-1-oxopropan-2-yl)oxy]-3,5-dimethylbenzyl]-alaninate 7 (a or b) and 5-methyl-2-phenyl-1,3-oxazole-4-carbaldehyde in dichloromethane, sodium triacethoxyborohydride was added and stirred at room temperature overnight. After the solvent was removed in vacuo, the residue was dissolved in DMF, methylamine hydrochloric acid, NMM, EDCI and HOBt was added to the reaction solution and stirred at room temperature overnight. After EtOAc was added to the residue, the mixture was washed with water, 10% citric acid aqueous solution, satd. sodium bicarbonate aqueous solution and brine, dried over Na2SO4 and concentrated. The crude product was purified by column chromatography on silica gel (hexane/EtOAc as eluent) to provide 8 (a or b).

tert-Butyl N-[4-[1-(Ethoxy-2-methyl-1-oxopropan-2-yl)oxy]-3,5-dimethylbenzyl]-N-[5-[methyl-2-phenyl-1,3-oxazol-4-yl]-methyl]o-alaninate (8a): This compound was obtained as a colorless oil in 58% yield by using 7a (0.51 mmol), 5-methyl-2-phenyl-1,3-oxazole-4-carbaldehyde (0.51 mmol), dichloromethane (3.0 mL) and sodium triacethoxyborohydride (0.76 mmol); 1H-NMR (400 MHz, CDCl3) δ: 1.31–1.35 (6H, m), 1.42 (6H, s), 1.51 (9H, s), 2.14 (6H, s), 2.26 (3H, s), 3.65 (5H, dq, J = 5.62, 14.0 Hz), 4.27 (2H, q, J = 7.1 Hz), 6.94 (2H, s), 7.38–7.43 (3H, m), 7.96–7.99 (2H, m). MS (ESI) m/z: 565 (M+H)+.

tert-Butyl N-[4-[1-(Ethoxy-2-methyl-1-oxopropan-2-yl)oxy]-3,5-dimethylbenzyl]-N-[5-[methyl-2-phenyl-1,3-oxazol-4-yl]-methyl]o-alaninate (8b): This compound was obtained as a colorless oil in 73% yield by using 7a (0.51 mmol), 5-methyl-2-phenyl-1,3-oxazole-4-carbaldehyde (0.51 mmol), dichloromethane (3.0 mL) and sodium triacethoxyborohydride (0.76 mmol); 1H-NMR (400 MHz, CDCl3) δ: 1.31–1.36 (6H, m), 1.42 (6H, s), 1.51 (9H, s), 2.14 (6H, s), 2.26 (3H, s), 3.51–3.77 (5H, m), 4.27 (2H, q, J = 7.1 Hz), 6.94 (2H, s), 7.38–7.43 (3H, m), 7.96–7.99 (2H, m). MS (ESI) m/z: 565 (M+H)+.

General Procedure for Ethyl 2-[2,6-Dimethyl-4-([1-(methylamino)-1-oxopropan-2-yl][5-methyl-2-phenyl-1,3-oxazol-4-yl)methyl]methyl]phenoxy]-2-methylpropanoate (9a, b): To a solution of 8 (a or b) in dichloromethane, HCl (4 M dioxane solution) was added and stirred at room temperature overnight. After the solvent was removed in vacuo, the residue was dissolved in DMF. Methylamine hydrochloric acid, NMM, EDCI and HOBt was added to the reaction solution and stirred at room temperature overnight. After EtOAc was added to the residue, the mixture was washed with water, 10% citric acid aqueous solution, satd. sodium bicarbonate aqueous solution and brine, dried over Na2SO4 and concentrated. The crude product was purified by column chromatography on silica gel (hexane/EtOAc as eluent) to provide 9 (a or b).

Ethyl 2-[2,6-Dimethyl-4-([[(2R)-1-(methylamino)-1-oxopropan-2-yl][5-methyl-2-phenyl-1,3-oxazol-4-yl)methyl]methyl]phenoxy]-2-methylpropanoate (9a): This compound was obtained as a colorless oil in 78% yield by using 8a (0.28 mmol), HCl (4 M dioxane solution, 2.0 mL), dichloromethane (5.0 mL), DMF (5.0 mL), methyl amine hydrochloric acid (0.43 mmol), NMM (0.43 mmol), EDCI (0.43 mmol) and HOBt (0.43 mmol); MS (ESI) m/z: 522 (M+H)+.

Ethyl 2-[2,6-Dimethyl-4-([(2S)-1-(methylamino)-1-oxopropan-2-yl][5-methyl-2-phenyl-1,3-oxazol-4-yl)methyl]methyl]phenoxy]-2-methylpropanoate (9b): This compound was obtained as a colorless oil in 27% yield by using 8b (0.35 mmol), HCl (4 M dioxane solution, 5.0 mL), dichloromethane (5.0 mL), DMF (5.0 mL), methyl amine hydrochloric acid (0.43 mmol), NMM (0.43 mmol), EDCI (0.43 mmol) and HOBt (0.43 mmol); 1H-NMR (400 MHz, CDCl3) δ: 1.31–1.35 (6H, m), 1.42 (6H, s), 2.11 (6H, s), 2.22 (3H, s), 2.91 (3H, d, J = 4.9 Hz), 3.31–3.61 (5H, m), 4.26 (2H, q, J = 7.1 Hz), 6.84 (2H, s), 7.43–7.47 (3H, m), 7.99–8.01 (2H, m), 8.50 (1H, s). MS (ESI) m/z: 522 (M+H)+.

General Procedure for N-[4-[1-(Ethoxy-2-methyl-1-oxopropan-2-yl)oxy]-3,5-dimethylbenzyl]-N-[5-[methyl-2-phenyl-1,3-oxazol-4-yl]methyl]alanine (10a, b): To a mixture of 9 (a or b) in methanol, NaOH (1 M aqueous solution) was added and stirred under reflux conditions. After HCl (1 M aqueous solution) was added to the reaction mixture, the solvent was removed in vacuo. After EtOAc was added to the residue, the mixture was washed with water and brine, dried over Na2SO4 and concentrated. The crude product was purified by column chromatography on silica gel (chloroform/methanol as eluent). The residue was hydrochlorinated with HCl (4 M dioxane solution), and then concentrated. Recrystallization from EtOAc–hexane produced 10 (a or b) HCl salts.

2-[2,6-Dimethyl-4-([(2R)-1-(methylamino)-1-oxopropan-2-yl][5-methyl-2-phenyl-1,3-oxazol-4-yl)methyl]methyl]phenoxy]-2-methylpropanoic Acid (10a): This compound was obtained as a colorless solid in 67% yield by using 9a (0.21 mmol), methanol (5.0 mL) and NaOH (1 M aqueous solution, 2.0 mL); 1H-NMR (400 MHz, DMSO-d6) δ: 1.34 (6H, s), 2.14 (6H, s), 2.69 (3H, d, J = 4.2 Hz), 3.10–3.61 (4H, m), 3.97–4.41 (4H, m), 7.06–7.60 (5H, m), 7.97–7.99 (2H, m).
8.23–8.75 (1H, m). MS (ESI) m/z: 494 (M+H)^+. Anal. Calcd for C_{28}H_{35}N_{3}O_{5}·HCl·0.75H_{2}O·0.2EtOAc: C, 62.14; H, 6.99; N, 7.21. Found: C, 62.26; H, 7.09; N, 7.22.

tert-Butyl N-[[1-Ethoxy-2-methyl-1-oxopropan-2-yl]oxy]-3,5-dimethylbenzyl]-β-alaninate (12) To a solution of 6 (0.19 g, 0.72 mmol) in THF (5.0 mL), 11 (0.16 g, 0.86 mmol) and TEA (0.12 µL, 0.86 mmol) were added and stirred under reflux condition for 3.5 h. After the solvent was removed in vacuo, the residue was dissolved in methanol (5.0 mL). Sodium borohydride (40 mg, 1.1 mmol) was added to the reaction solution at 0°C and stirred at the same temperature for 40 min. After water and satd. sodium bicarbonate aqueous solution were added and the organics was extracted with dichloromethane. The organic layer was dried over Na_{2}SO_{4} and concentrated. The residue was purified by column chromatography on silica gel (hexane/EtOAc) to provide 12 (0.30 g, crude product). This compound was used at the next step without further purification.

tert-Butyl N-[[1-Ethoxy-2-methyl-1-oxopropan-2-yl]oxy]-3,5-dimethylbenzyl]-N-[[5-methyl-2-phenyl-1,3-oxazol-4-yl]methyl]alaninate (13) To a solution of 12 (0.30 g, ca. 0.72 mmol) and 4-((chloromethyl)-5-methyl-2-phenoxyborohydride (0.49 g, 0.84 mmol) was added and stirred at room temperature for 16 h. After the reaction mixture was cooled to room temperature, HCl (1 M aqueous solution, 2.5 mL) and water were added and the organic was extracted with dichloromethane. The organic layer was dried over Na_{2}SO_{4} and concentrated. The residue was purified by column chromatography on silica gel (hexane/EtOAc=9:1 then 2:1 as eluent, v/v) to provide 13 (300 mg, crude product).

Ethyl 2-[2,6-Dimethyl-4-[[3-(methylamino)-3-oxopropyl][5-methyl-2-phenyl-1,3-oxazol-4-yl]methyl]amino]methyl]phenoxy]-2-methylpropanoate (15) To a solution of 14 (0.30 g, 0.58 mmol) in THF (5.0 mL), NaOH (0.5 M aqueous solution, 5.0 mL) was added and stirred under reflux condition overnight. After the reaction mixture was cooled to room temperature, HCl (1 M aqueous solution, 2.5 mL) and water were added and the organic was extracted with dichloromethane. The organic layer was dried over Na_{2}SO_{4} and concentrated. The residue was purified by column chromatography on silica gel (dichloromethane/methanol=95:5 then 91:9 as eluent, v/v) to provide 15 as a colorless solid (0.24 g, 80%). 1H-NMR (400 MHz, CDCl_{3}) δ: 1.35 (3H, t, J=7.1 Hz), 1.42 (9H, s), 1.45 (6H, s), 2.17 (6H, s), 2.23 (3H, s), 2.47 (2H, t, J=7.2 Hz), 2.91 (2H, t, J=7.2 Hz), 3.47–3.56 (4H, m), 4.28 (2H, q, J=7.1 Hz), 6.95 (2H, s), 7.36–7.45 (3H, m), 7.96–8.03 (2H, m). MS (ESI) m/z: 494 (M+H)^+. Anal. Calcd for C_{28}H_{35}N_{3}O_{5}·0.5H_{2}O·C: 66.91; H, 7.22; N, 8.36. Found: C, 67.12; H, 7.35; N, 8.13.

Ethyl 2-[2-[[2-(tert-Butyloxycarbonyl)amino]ethyl][5-methyl-2-phenyl-1,3-oxazol-4-yl]methyl]amino]methyl]-2,6-dimethylphenoxy]-2-methylpropanoate (16) To a solution of 2 (0.43 g, 0.98 mmol) and tert-butyl (2-oxoethyl)carbamate (0.28 g, 1.7 mmol) in dichloromethane (10 mL), sodium triacetoxyborohydride (0.49 g, 0.84 mmol) was added and stirred at room temperature for 16 h. After the reaction mixture was diluted with EtOAc, the mixture was washed with satd. sodium bicarbonate aqueous solution and brine, dried over Na_{2}SO_{4} and concentrated. The residue was purified by column chromatography on silica gel (hexane/EtOAc=9:1 then 2:1 as eluent, v/v) to provide 16 as a colorless oil (0.40 g, 71%). MS (ESI) m/z: 580 (M+H)^+.
stirred at room temperature for 2 d. HCl (1 M aqueous solution, 2.5 mL) and water were added and the organics was extracted with EtOAc. The organic layer was washed with water and brine, dried over Na₂SO₄ and concentrated. The residue was purified by column chromatography on silica gel (chloroform/methanol=9/1) as eluent, v/v and triturated with EtOAc–hexane to provide 17 as a colorless solid (51 mg, 31%): ¹H-NMR (400 MHz, CDCl₃) δ: 1.33 (6H, s), 1.76 (3H, s), 2.13 (6H, s), 2.27 (3H, s), 2.52–2.55 (2H, m), 3.17–3.22 (2H, m), 3.49 (2H, s), 3.52 (2H, s), 6.97 (2H, s), 7.48–7.53 (3H, m), 7.68–7.71 (1H, m), 7.93–7.97 (2H, m). MS m/z: 494 (M+H)⁺. Anal. Calcd for C₃₆H₄₂N₄O₁₂: C, 65.12; H, 7.35; N, 8.44. Found: C, 65.43; H, 7.35; N, 7.74.

**Ethyl 2-[(2-tert-Butoxy-2-oxoethyl)[5-methyl-2-(4-methylphenyl)-1,3-oxazol-4-yl]methyl]amino[methyl]-2,6-dimethylenephenoxy]-2-methylpropanoate (20)** To a solution of 18 (1.0 g, 4.5 mmol) and 19 (2.0 g, 5.2 mmol) in MeCN (20 mL), K₂CO₃ (0.94 g, 6.8 mmol) was added and stirred under reflux conditions for 2 h. After the solvent was removed in vacuo, the product was purified by column chromatography on silica gel (hexane/EtOAc=9/1 then 3/1 as eluent, v/v) to provide 20 as a pale yellow oil (2.5 g, 98%): ¹H-NMR (400 MHz, CDCl₃) δ: 1.35 (3H, t, J=7.2 Hz), 1.45 (6H, s), 1.47 (9H, s), 2.17 (6H, s), 2.29 (3H, s), 2.39 (3H, s), 3.30 (2H, s), 3.71 (2H, s), 3.74 (2H, s), 4.28 (2H, q, J=7.2 Hz), 6.99 (2H, s), 7.23 (2H, d, J=8.3 Hz), 7.89 (2H, d, J=8.11 Hz). MS (ESI) m/z: 565 (M+H)⁺.

**Ethyl 2-[(2-Amino-2-oxoethyl)[5-methyl-2-(4-methylphenyl)-1,3-oxazol-4-yl]methyl]amino[methyl]-2,6-dimethylenephenoxy]-2-methylpropanoate (21)** To a solution of 20 (1.5 g, 2.7 mmol) in dichloromethane, trifluoroacetic acid (10 mL, 0.13 mol) was added at 0°C and stirred at room temperature for 5 d. After the solvent was removed in vacuo, the residue (0.15 g, 0.29 mmol) was dissolved in DMF (5.0 mL), and then NH₃Cl (20 mg, 0.44 mmol), TEA (0.16 mL, 1.2 mmol), EDCI (80 mg, 0.44 mmol) and HOBr (40 mg, 0.29 mmol) were added to the reaction solution and stirred at room temperature for 20 h. After the reaction mixture was diluted with chloroform, the mixture was washed with water, dried over Na₂SO₄ and concentrated. The residue was purified by column chromatography on silica gel (chloroform/methanol=20/1 as eluent, v/v) to provide 21 as a colorless oil (0.13 g, 89%): ¹H-NMR (400 MHz, CDCl₃) δ: 1.35 (3H, t, J=7.1 Hz), 1.45 (6H, s), 2.16 (6H, s), 2.24 (3H, s), 2.40 (3H, s), 3.23 (2H, s), 3.53 (2H, s), 3.60 (2H, s), 4.27 (2H, q, J=7.1 Hz), 5.44 (1H, br), 6.91 (2H, s), 7.25 (2H, m), 7.69 (1H, br), 7.87 (2H, d, J=8.2 Hz). MS (ESI) m/z: 508 (M+H)⁺.

**2-[(2-Amino-2-oxoethyl)[5-methyl-2-(4-methylphenyl)-1,3-oxazol-4-yl]methyl]amino[methyl]-2,6-dimethylenephenoxy]-2-methylpropanoic Acid (22)** To a solution of 21 (0.13 g, 0.26 mmol) in methanol (2.0 mL) and THF (5.0 mL) NaOH (1 M aqueous solution, 1.3 mL) was added and stirred under reflux conditions for 15.5 h. After the reaction mixture was cooled to room temperature, water was added and the aqueous layer was washed with diethyl ether. HCl (1 M aqueous solution) was added to the aqueous layer and the organics were extracted with chloroform. The organic layer was dried over Na₂SO₄ and concentrated. The residue was purified by crystallization from Hexane-Diethyl ether to provide 22 as a colorless solid (56 mg, 46%): ¹H-NMR (400 MHz, CDCl₃) δ: 1.50 (6H, s), 2.20 (6H, s), 2.28 (3H, s), 2.40 (3H, s), 3.22 (2H, s), 3.57 (2H, s), 3.62 (2H, s), 5.75 (1H, br), 6.95 (2H, s), 7.26 (2H, m), 7.75 (1H, br), 7.87 (2H, d, J=8.2 Hz). IR (ATR) cm⁻¹: 3334, 2920, 1730, 1678, 1120, 733, 700. FAB-MS m/z: 480 (M+H)⁺. HR-FAB-MS m/z: 480.2500 (Calcd for: C₂₈H₃₅N₃O₅·1.25H₂O). Anal. Calcd for C₂₈H₃₅N₃O₅·1.25H₂O: C, 65.43; H, 7.35; N, 8.44. Found: C, 64.92; H, 6.73; N, 8.20.

**PPAR Transactivation Assay** The fusion protein (PPARα ligand binding domain–GAL4 DNA binding domain) expression plasmid (pFA-PPARα/GAL4 or pFA-PPARα/GAL4) and the reporter plasmid (pFA-SEAP, Stratagene) were used. HEK 293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. After 24 h of culture, the cells were co-transfected with pFA-PPARα/GAL and pFA-SEAP using Lipofectamine (Invitrogen) and Plus Reagents (Invitrogen) according to the manufacturer’s protocol. After 5 h of transfection, the cells were treated with DMEM-FBS containing a test compound at 37°C in 5% CO₂. After 48 h of incubation, the conditioned medium was collected and the SEAP activity in it was measured using the Reporter assay kit—SEAP (TOYOBO) according to the manufacturer’s protocol. Chemiluminescence was read by ARVOxx, PerkinElmer, Inc. The fold increase in chemiluminescence in the presence of a test compound compared to that in the absence of it was calculated, and then the EC₅₀ value was obtained.

**Animal** Animal facilities, animal care, and study programs were in accordance with the in-house guideline of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd. Female db/db (C57BLKS/J-m+/+Leprdb) mice were purchased at 10 weeks old from CREA Japan, Inc. (Tokyo, Japan) and used as a type 2 diabetic animal model. Male Zucker Fatty (Crlj:ZUC-Leprfa) rats were purchased at 6 weeks old from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and used as a type 2 diabetic animal model. All of the animals were housed six per cage and maintained on an 8 a.m. light/8 p.m. dark schedule. Rodent chow and water were given *ad libitum*.

**In Vivo db/db Mouse Studies** Mice (six per group) received a once daily oral dosing of a test compound or vehicle (0.5% methylcellulose) by oral gavage for 10 d. Blood was collected from the tail vein immediately prior to the next dosing on days 0, 5 and 10 for measurement of the plasma glucose and triglyceride levels.

**In Vivo Zucker Fatty Rat Studies** Mice (six per group) received a once daily oral dosing of a test compound or vehicle (0.5% methylcellulose) by oral gavage for 13 d. Blood was collected from the tail vein immediately prior to the next dosing on days 1, 7 and 13 for measurement of the plasma glucose and triglyceride levels.

**Distribution Coefficient** The distribution coefficients (logD) between 1-octanol and phosphate buffered saline (PBS) were assayed by a shaking flask method.¹⁶ Equal amounts of PBS and 1-octanol were shaken and left for over 12 h. The upper layer (1-octanol) and lower layer PBS were collected individually. Each compound was dissolved in 1-octanol or PBS (200 µL). The same amount of either PBS or 1-octanol was added and the mixture was shaken vigorously for 30 min at room temperature. Then, both phases were separated and assayed using LC-MS methodologies (LC-Mass spectrometer: 1100 Series LC/MSD, Agilent; Analytical Column: X Terra®
The measurement sample solution, 5% solution (1:1 (v/v)) were prepared to create a calibration curve. Liver microsomes.14) 30-min preincubation of the test compounds in pooled human activity (1 CYP3A4 is estimated as the percentage of the enzymatic compounds.

Column: X Terra® MSC18 3.5 µM, 3.0×30 mm, Waters; Mobile Phase: 10 mM ammonium acetate buffer (pH 4.5)/0.05% acetic acid in acetonitrile=95/5 to 10/90 v/v). The values of logD were analyzed using Analyst software program (version 1.4, Applied Bio. Systems).

CYP3A4 Direct Inhibition Assay P450 3A4 inhibition activities were measured with a high throughput inhibitor screening kit (Baculovirus-insect cell-expression system, SupersomesTM, and a fluorescent substrate (7-benzoyloxy-tri-fluoromethylcoumarin)) available from CORNING.15) Percent inhibition was estimated by fluorescence at 10 µM of the test compounds.

MBI Assay Mechanism based inactivation against CYP3A4 is estimated as the percentage of the enzymatic activity (1'-hydroxylation of midazolam) remaining, after the 30-min preincubation of the test compounds in pooled human liver microsomes.16)

Solubilities The solubilities were determined by HPLC analysis. Ten millimolars of compound solution in DMSO (50 µL) was freeze-dried. To the residue JP XIV 1st fluid (250 µL, pH 1.2) was added, and the mixture was stirred by pipette operation. The mixture was saved under shading over 12 h. After filtration of the mixture, the resulting filtrate was diluted 20 times by adding aqueous DMSO solution (1:1 (v/v)) and 100 µM of compound solution in DMSO solution (1:1 (v/v)) to obtain the measurement sample solution. Five micromolars of compound solution in aqueous DMSO solution (1:1 (v/v)) and 100 µM of compound solution in aqueous DMSO solution (1:1 (v/v)) were prepared to create a calibration curve. The measurement sample solution, 5 µM solution and 100 µM solution were assayed using HPLC methodologies (Analytical Column: X Terra® MSC18 3.5 µM, 3.0×30 mm, Waters; Mobile Phase: 10 mM ammonium acetate buffer (pH 4.5)/0.05% acetic acid in acetonitrile=95:5 to 10:90 v/v; Wave length: PDA 220–420 nm). The solubilities were analyzed using Millenium software (Waters).

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Conflict of Interest Yoshihiro Shibata, Katsuji Kagechika, Masahiro Ota, Mitsuhiko Yamaguchi, Masaki Setoguchi, Kiyoshi Chiba, Hiromichi Takano, Chiyuki Akiyama, Mayumi Ono, Mina Nishi and Hiroyuki Usui are employees of Daiichi Sankyo Co., Ltd. Hideo Kubo is an employee of Daiichi Sankyo RD Novare Co., Ltd.

References and Notes
13) We judged that MBI was considered sufficient below 80% remaining from an examination of previously reported drugs.