

Electronic Supporting Information

A potent free fatty acid receptor 1 agonist with a glucose-dependent antihyperglycemic effect

Xuekun Wang,^{‡a,b} Yurui Xu,^{‡a} Shujun Feng,^a Xinyu Huang,^a Xia Meng,^a Jiao Chen,^c Leilei Guo,^a Junliang Ge,^a Jikang Zhang,^a Jianmei Chen,^a Li Cheng,^a Kai Gu,^{a,d} Yu Zhang,^{*a} Qing Jiang^{*c} and Xinghai Ning^{*a}

a. Department of Biomedical Engineering, Nanjing National Laboratory of Microstructures, College of Engineering and Applied Sciences, Nanjing University, Nanjing 210093, P.R. China.

b. Department of Pharmaceutical Engineering, College of pharmacy; Liaocheng University, Liaocheng 252059, P.R. China.

c. Jiangsu Province Academy of Traditional Chinese Medicine, Nanjing 210028, P.R. China.

d. Colleges of Medicine, State Key Laboratory of Natural Medicines, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, P.R. China.

e. The Center of Diagnosis and Treatment for Joint Disease, Drum Tower Hospital Affiliated with the Medical School of Nanjing University, Nanjing 210008, P.R. China.

This PDF file includes:

1. Materials and Methods
2. Fig. S1 Development of agonists of free fatty acid receptor 1(FFA1).
3. Fig. S2 Synthesis of PAFA.
4. Fig. S3 ¹H NMR spectrum (500 MHz, DMSO-*d*₆) of PAFA.
5. Fig. S4 ¹³C NMR (125 MHz, DMSO-*d*₆) of PAFA.
6. Fig. S5 ClogP values of FFA1 agonists.
7. Fig. S6 Agonist activity of TAK-875 and PAFA.
8. Fig. S7 The specificity of PAFA to FFA1 receptor.
9. Fig. S8 The serum stability of TAK-875 and PAFA.
10. Fig. S9 The AUC_{0-120 min} of blood glucose levels.
11. Fig. S10 The AUC_{0-120 min} of blood glucose levels during OGTT in *db/db* mice.
12. Fig. S11 (A) Body weights of *db/db* mice during treatment. (B) The plasma levels of AST, ALT and TBIL of *db/db* mice during treatment.
13. Table. S1 The docking simulation studies of the PAFA-FAA1 complex.

1. Materials and Methods

Experimental Design

The primary objective of our study is to develop a novel FFA1 agonist for treating type 2 diabetes mellitus. A phenylthioacetic acid-based FFA1 agonist (PAFA) was designed using the pharmacophore based drug design approach. PAFA was synthesized and fully characterized by NMR and high resolution mass spectrometry. CHO cells stably expressing human FFA1 was used to test agonist activity of PAFA, and intracellular calcium flux signals were monitored by FLIPR Tetra system (Molecular Devices). GSIS was performed in insulin-secreting cell line (INS-1) to investigate the insulinotropic effects of PAFA. HPLC was used to test biostability of FFA1 agonists in serum. Dose-dependent and long-term antihyperglycemic effects of PAFA were tested in healthy ICR and *db/db* diabetic mice, received an oral administration of glucose (2 g/kg), and blood glucose levels and insulin secretion were monitored during OGTT studies. Adverse effects of PAFA were also systemically investigated, including risk of hypoglycaemia, cytotoxicity and hepatotoxicity. Power analyses were performed to determine minimum numbers of animals per group needed to attain significance of $P < 0.05$ with a 90 or 95% probability. At least 6 mice per group were used for animal studies. End points to assess FFA1 agonist activities on the basis of various established assays. All histological studies were performed by a blinded observer. Six cell cultures per group were used for in vitro experiments.

Mice

Male ICR mice (25 g, 6 weeks old) and *db/db* (30 g, 8 weeks old) were purchased from Nanjing Qinglongshan Experimental Animal Center, and acclimatized for 1 week before the initiation of experiments. The mice were randomly grouped ($n=6$) for the studies. All animal researches were performed in accordance with National Institute of Health Guidelines under the protocols, approved by the ethics committee at the Affiliated Drum Tower Hospital of Nanjing University Medical School.

Cells

CHO stably expressing FFA1 (M00273) was purchased from GenScript Inc. (China). CHO cell was cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS) (Gibco) and penicillin/streptomycin (1%, w/v). INS-1 and L02 cell lines were obtained from Jiangsu KeyGEN Bio TECH Corp., Ltd (China). INS-1 and L02 cell lines were cultured in RPMI1640 medium supplemented with 10% FBS and penicillin/streptomycin (1%, w/v). All cell lines were cultured at 37°C in 5% CO₂ atmosphere.

Materials and instrumentation

All starting materials, reagents and solvents were purchased and used without further purification unless otherwise indicated. Column chromatography over silica gel (200-300 mesh) was carried out for purifying the intermediates and final compound. NMR spectra were recorded on a Bruker DRX500 apparatus (500 MHz for ¹H NMR and 125 MHz for ¹³C NMR spectra in DMSO-*d*₆). Chemical shifts were expressed as values of ppm relative to TMS, and coupling constants (*J* values) were given in hertz (Hz). Mass spectrometry (MS) was recorded on Agilent 1290 Infinity LC/6460 QQQ MS, and elemental analyses were performed in the Heraeus CHNO-Rapid instrument.

Docking simulation

The crystal structure of FFA1 (PDB ID: 4PHU) was obtained from the Protein Data Bank (PDB), and docking simulations were performed with MOE (version

2008.10, The Chemical Computing Group, Montreal, Canada). The structure of FFA1 was prepared with Protonate 3D, and a Gaussian Contact surface was drawn around the binding site. Subsequently, the active site was isolated and the backbone was removed. The ligand poses were filtered by Pharmacophore Query Editor. The molecular docking of compounds against the FFA1 receptor was performed. The structures of compounds were placed in the site with Pharmacophore method and then ranked with the London dG scoring function. For the energy minimization in the pocket, MOE Force Field Refinement was used and ranked based on the London dG scoring function.

Synthesis

Synthetic procedures and identification of 4-amino-3-fluorophenol (6): To a stirred solution of 3-fluoro-4-nitrophenol (5) (1.0 g, 6.53 mmol) in 80% ethanol aqueous solution (20 mL) was added Iron powder (2.19 g, 39.18 mmol) and ammonium chloride (1.40 g, 26.12 mmol), and the suspension was refluxed with stirring overnight. When the reaction was complete, the mixture was filtered and extracted with dichloromethane. The organic layers were washed with brine, dried over MgSO₄, and filtered. The filtrate was concentrated under vacuum and purified by column chromatography (EtOAc/petroleum ether, 1/4) to give (6) (0.49 g, 61%). ¹H NMR (500 MHz, DMSO-*d*₆) δ: 8.77 (s, 1H), 6.63-6.35 (m, 3H), 4.36 (s, 2H).

Synthetic procedures and identification of 4-mercapto-3-fluorophenol (7): To a stirred solution of 4-amino-3-fluorophenol (6) (0.45 g, 3.65 mmol) in concentrated hydrochloric acid (1.0 mL) was slowly added aqueous solution of sodium nitrite (0.28 g, 4.02 mmol). The mixture was stirred at 0 °C for 1 hour, and potassium ethylxanthate (1.17 g, 7.31 mmol) was then added. When the reaction was complete, the mixture was extracted with ethyl acetate. The organic layers were washed with brine, dried over MgSO₄, and filtered. The filtrate was concentrated under vacuum and re-dissolved in ethanol (20 mL), followed by adding KOH (0.41 g, 7.31 mmol) at room temperature. The reaction mixture was refluxed overnight, and 1 N HCl was added dropwise to neutralize the reaction. The mixture was then extracted with ethyl acetate and washed with brine, dried over MgSO₄ and filtered. The filtrate was concentrated under vacuum, and the residue was purified by column chromatography (EtOAc/petroleum ether, 1/2) to give (7) (0.16 g, 31%). ¹H NMR (500 MHz, DMSO-*d*₆) δ: 9.63 (s, 1H), 7.40-6.80 (m, 3H), 3.75 (s, 1H).

Synthetic procedures and identification of methyl 2-((4-hydroxy-2-fluorophenyl)thio)acetate (8): To a stirred solution of 4-mercapto-3-fluorophenol (7) (0.15 g, 1.07 mmol) and methyl chloroacetate (0.17 g, 1.60 mmol) in DMF was added K₂CO₃ (0.30 g, 2.14 mmol) at room temperature. The reaction mixture was stirred at room temperature overnight. The reaction mixture was filtered and extracted with ethyl acetate, and the organic layer was washed with brine, dried over MgSO₄ and filtered. The filtrate was concentrated under vacuum and purified by column chromatography (EtOAc/petroleum ether, 1/3) to give (8) (0.15 g, 66%). ¹H NMR (500 MHz, DMSO-*d*₆) δ: 9.55 (s, 1H), 7.22 (d, *J* = 8.4 Hz, 1H), 6.67 (d, *J* = 2.4 Hz, 1H), 6.58 (dd, *J* = 8.4, 2.6 Hz, 1H), 3.56 (s, 3H), 3.54 (s, 2H).

Synthetic procedures and identification of 2-((4-((2',6'-dimethyl-4'-(3-(methylsulfonyl)propoxy)-[1,1'-biphenyl]-3-yl)methoxy)-2-fluorophenyl)thio) acetic acid (PAFA): To a stirred solution of methyl 2-((4-hydroxy-2-fluorophenyl)thio)acetate (8) (52 mg, 0.24 mmol) in acetone was added K₂CO₃ (67 mg, 0.49 mmol) and 3'-(bromomethyl)-2,6-dimethyl-4-(3-(methylsulfonyl)propoxy)-1,1'-biphenyl (9) (100 mg, 0.24 mmol). The reaction mixture was refluxed overnight and filtered. The filtrate was concentrated under vacuum and the residue was re-dissolved in ethyl acetate. The organic layers was washed with brine, dried over MgSO₄ and filtered. The filtrate was concentrated under vacuum to give as a white solid (105 mg). The intermediate (100 mg, 0.18 mmol) was dissolved in the mixture of THF/CH₃OH/ H₂O (1:1:2), followed by adding aqueous NaOH (2 N, 0.36 mL, 0.72 mmol) at room temperature. The reaction mixture was stirred for 2 hours and acidified with HCl (1 N). The mixture was extracted with ethyl acetate, and the combined organic layer was washed with brine, dried over MgSO₄ and filtered. The filtrate was evaporated under vacuum, and the residue was purified by column chromatography (MeOH/CH₂Cl₂, 20/1) to give (PAFA) as a colorless solid (96 mg, 68.8%) as a white solid. ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.47 (t, *J* = 7.5 Hz, 1H), 7.41 (d, *J* = 11.9, Hz, 2H), 7.18 (s, 1H), 7.09 (d, *J* = 7.4 Hz, 1H), 7.00 (dd, *J* = 11.5, 2.5 Hz, 1H), 6.88 (dd, *J* = 8.7, 2.5 Hz, 1H), 6.72 (s, 1H), 5.18 (s, 2H), 4.10 (t, *J* = 6.2 Hz, 2H), 3.60 (s, 2H), 3.26 (s, 2H), 3.04 (s, 3H), 2.22 – 2.07 (m, 2H), 1.92 (s, 6H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 170.83, 160.06, 157.45, 140.82, 137.08, 134.66, 134.33, 129.51, 129.25, 129.14, 126.54, 113.80, 112.48, 103.71, 103.50, 70.18, 65.89, 51.06, 39.51, 36.63, 22.55, 21.18; MS (ESI, *m/z*): 531.1 [M-H]⁻. Anal. Calcd for C₂₇H₂₉FO₆S₂: C, 60.88; H, 5.49; Found: C, 60.91; H, 5.54.

Agonist activity of PAFA towards FFA1 (FLIPR assay)

CHO cells stably expressing human FFA1 were seeded into 96-well plates (6×10⁴ cells/well) for 24 h. CHO cells were then incubated in culture buffer (containing 2.5 mg/mL fluorescent calcium indicator Fluo 4-AM, 2.5 mM probenecid and 0.1% fatty acid-free BSA) for 1 h at 37 °C, followed by adding various concentrations of PAFA, TAK-875 or γ-linolenic acid. The intracellular calcium flux signals were monitored by FLIPR Tetra system (Molecular Devices). Agonist activity of PAFA on human FFA1 was expressed as [(A-B)/(C-B)] X 100 (intracellular concentrations of calcium in CHO cells treated with PAFA(A), vehicle (B), and 10 mM γ-linolenic acid(C)). The EC₅₀ value was calculated using Graphpad Prism 5.

Insulinotropic effects of PAFA in INS-1 cells

Rat insulinoma INS-1 cells were seeded into 96-well plates (6×10⁴ cells/well), and were subsequently cultured for 2 days. INS-1 cells were then incubated in Krebs–Ringer bicarbonate Hepes (KRBH) buffer (composed of HEPES, sodium chloride, sodium bicarbonate, potassium dihydrogen phosphate, calcium chloride, magnesium sulfate and glucose) in presence of 11 mM glucose for 2 h. The buffer solution was discarded, and INS-1 cells were incubated with different agents. After different incubation times, the supernatants were collected, and insulin levels were measured with rat Insulin radioimmunoassay Kit (Beijing North Institute of Biological Technology, China).

The specificity of PAFA to FFA1 receptor.

Rat insulinoma INS-1 cells were seeded into 96-well plates (6×10^4 cells/well), and were subsequently cultured for 2 days. INS-1 cells were then incubated in Krebs–Ringer bicarbonate Hepes (KRBH) buffer in presence of 11 mM glucose for 2 h. The buffer solution was discarded, and INS-1 cells were incubated with PAFA (3 μ M) and DC260126 (MCE, a FFA1 antagonist, 6 μ M). After different incubation times, the supernatants were collected, and insulin levels were measured with rat Insulin radioimmunoassay Kit (Beijing North Institute of Biological Technology, China).

The serum stability of TAK-875 and PAFA

The serum stability of TAK-875 and PAFA was investigated by incubating in PBS containing 0.5% methylcellulose and 50% FBS at 37 °C for 24 h. At predetermined time intervals, aliquots were withdrawn and then determined by the HPLC method. The mobile phase for TAK-875 and PAFA analysis was consisted of gradient of phase A (10–95%, 0.1% H₃PO₄ in acetonitrile) and phase B (0.1% H₃PO₄ in water). The detection was conducted at wavelength of 210 nm and the flow rate was 1 mL/min.

Dose-dependent antihyperglycemic effects of PAFA during OGTT in male ICR

Eight-week-old male ICR mice were fasted overnight before the initiation of animal experiments. Mice were orally administered with PBS, PAFA (5 mg/kg, 10 mg/kg, 20 mg/kg, 40 mg/kg) or TAK-875 (20mg/kg) solution containing 0.5% methylcellulose, and 30 min later, mice were fed with glucose (2 g/kg). Plasma glucose levels were measured by blood glucose test strips (Sannuo GA-3 type, Changsha, China) at the different time points (0, 15, 30, 60 and 120 min). In addition, plasma insulin levels were measured by mouse insulin RIA kit (Beijing North Institute of Biological Technology, China).

Antihyperglycemic effects of PAFA during OGTT in *db/db* diabetic mice

Db/db mice (10-week old) were fasted overnight before the initiation of animal experiments. Mice were orally administrated with PBS, PAFA (20 mg/kg) or TAK-875 (20 mg/kg) solution containing 0.5% methylcellulose, and 30 min later, mice were fed with glucose (2 g/kg). Plasma glucose levels were measured by blood glucose test strips (Sannuo GA-3 type, Changsha, China) at the different time points (0, 15, 30, 60 and 120 min). In addition, plasma insulin levels were measured by mouse insulin RIA kit (Beijing North Institute of Biological Technology, China).

Long-term antihyperglycemic effects of PAFA *in vivo*

Db/db mice (10-week old) were fasted overnight before the initiation of animal experiments. Mice were orally administrated with PBS, PAFA (20 mg/kg) or TAK-875 (20 mg/kg) solution containing 0.5% methylcellulose every day, and mice were fed with normal diet. Plasma glucose levels were measured every week by blood glucose test strips (Sannuo GA-3 type, Changsha, China) after fasted overnight.

Hypoglycemic risk of PAFA

Eight-week-old male ICR mice were fasted overnight before the initiation of animal experiments. Mice were orally administered with PBS, PAFA (60 mg/kg), glibenclamide (5 mg/kg) or TAK-875 (60 mg/kg) solution containing 0.5% methylcellulose. Plasma glucose levels were measured by blood glucose test strips (Sannuo GA-3 type, Changsha, China) at the different time points (0, 15, 30, 60, 120 and 180 min).

Cytotoxicity of PAFA against L02 cell lines

MTT assay was performed to determine cytotoxicity of PAFA. L02 cells were incubated with different concentrations of PAFA for 48 h, followed by adding MTT (Sigma, 5 mg/mL). The plates were incubated for an additional 4 h. The culture medium was removed by aspiration, and the blue MTT-formazan product was extracted with DMSO. After 10 min extraction at rt, and the absorbance at 570 nm was measured on a microplate reader (Infinite M200 Pro, Tecan).

The hepatotoxicity assessment of PAFA in *db/db* mice

Db/db mice (8-week old) were orally treated with PBS, PAFA (20 mg/kg) or TAK-875 (20 mg/kg) solution containing 0.5% methylcellulose for 4 weeks. The body weight and food consumption of mice were monitored every week for 4 weeks. The fasting blood glucose levels were measured every week. After 4-week treatment, mice were fasted overnight, and blood samples were collected for measuring plasma levels of ALT, AST and TBIL by automatic biochemical analyzer (Beckman Coulter, AU5811, Tokyo, Japan). The major organs, including heart, liver, lung and kidney, were collected, and a small piece of tissues were stained with hematoxylin and eosin (H&E) for histopathological examination.

Statistical analyses

All experiments were repeated at least 3 times with 6 replicates. Error bars represent standard error of the mean from independent samples assayed within the experiments. Statistical analysis was done with GraphPad Prism 6 software. Statistical significance was calculated using unpaired Student's t-test or one-way ANOVA. * represents $p < 0.05$, ** represents $p < 0.01$ and *** represents $p < 0.001$.

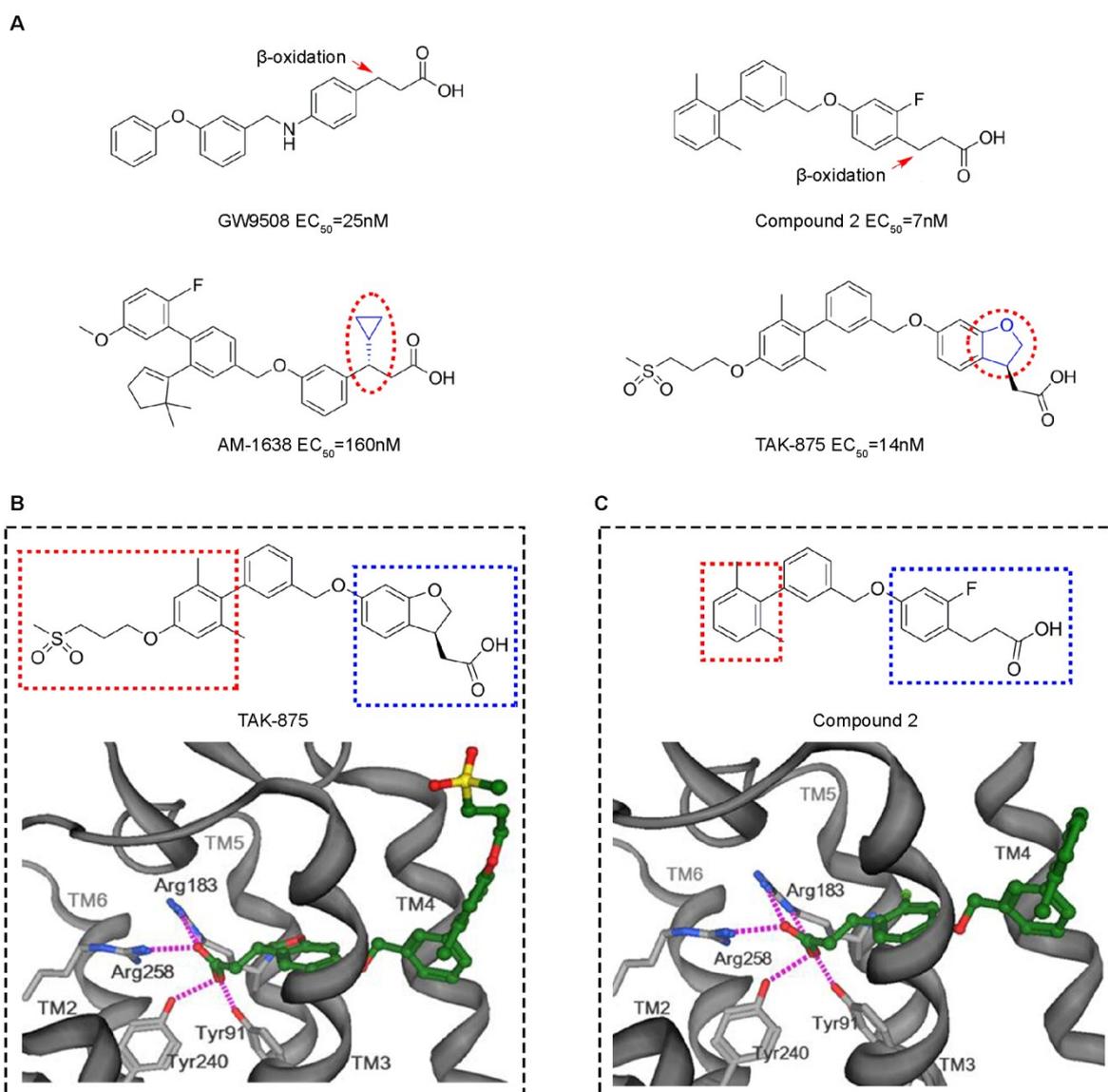


Fig. S1 Development of agonists of free fatty acid receptor 1(FFA1). (A) Representative agonists of FFA1. (B) Interaction of TAK-875 with the charge cluster. The carboxylate moiety is coordinated with several key residues on FFA1. (C) Interaction of compound 2 with the charge cluster. The carboxylate moiety is highly coordinated with several key residues on FFA1.

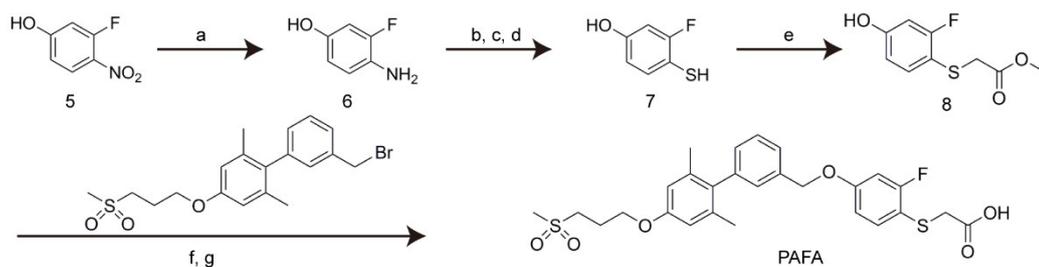


Fig. S2 Synthesis of PAFA. Reagents and conditions: (a) Fe, NH₄Cl, EtOH, H₂O, 70°C, 12 h; (b) NaNO₂, HCl, 0°C, 1 h; (c) EtOCS₂K, H₂O, 0°C, 12 h; (d) KOH, EtOH, 80°C, 12 h; (e) BrCH₂COOCH₃, K₂CO₃, DMF, KI, rt, 12 h; (f) K₂CO₃, acetone, KI, 45°C, 12 h; (g) NaOH, THF/MeOH/H₂O, rt, 4 h.

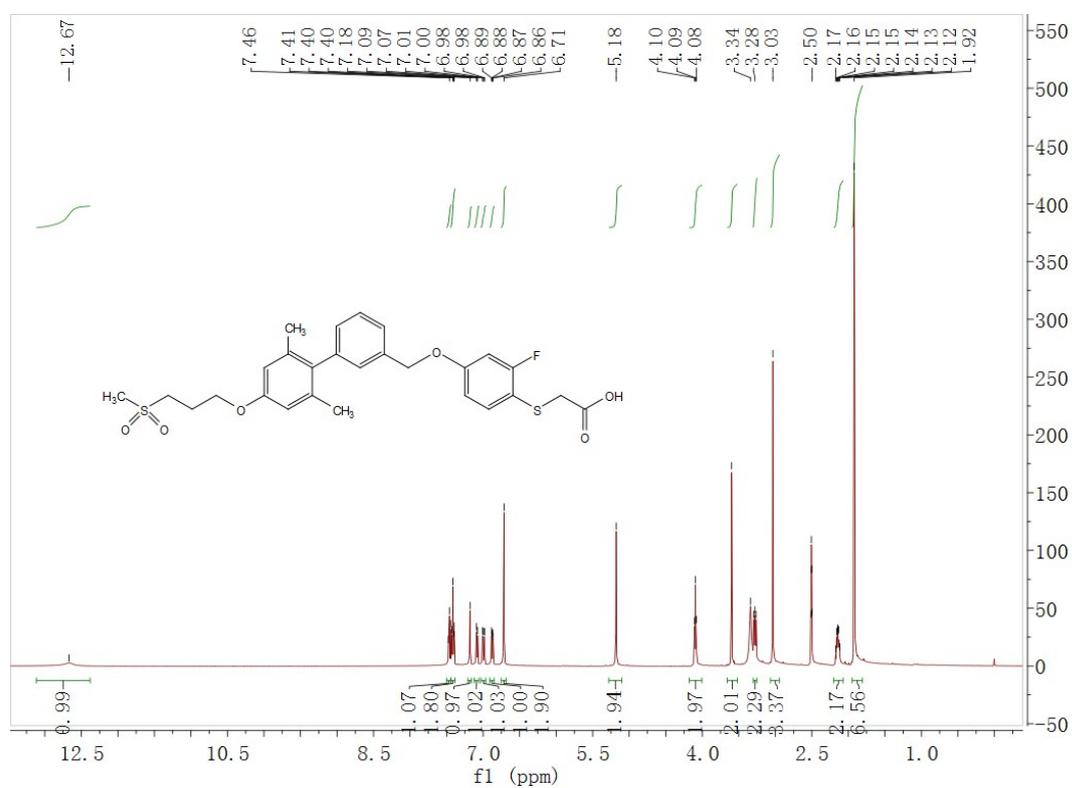


Fig. S3 ¹H NMR spectrum (500 MHz, DMSO-*d*₆) of PAFA.

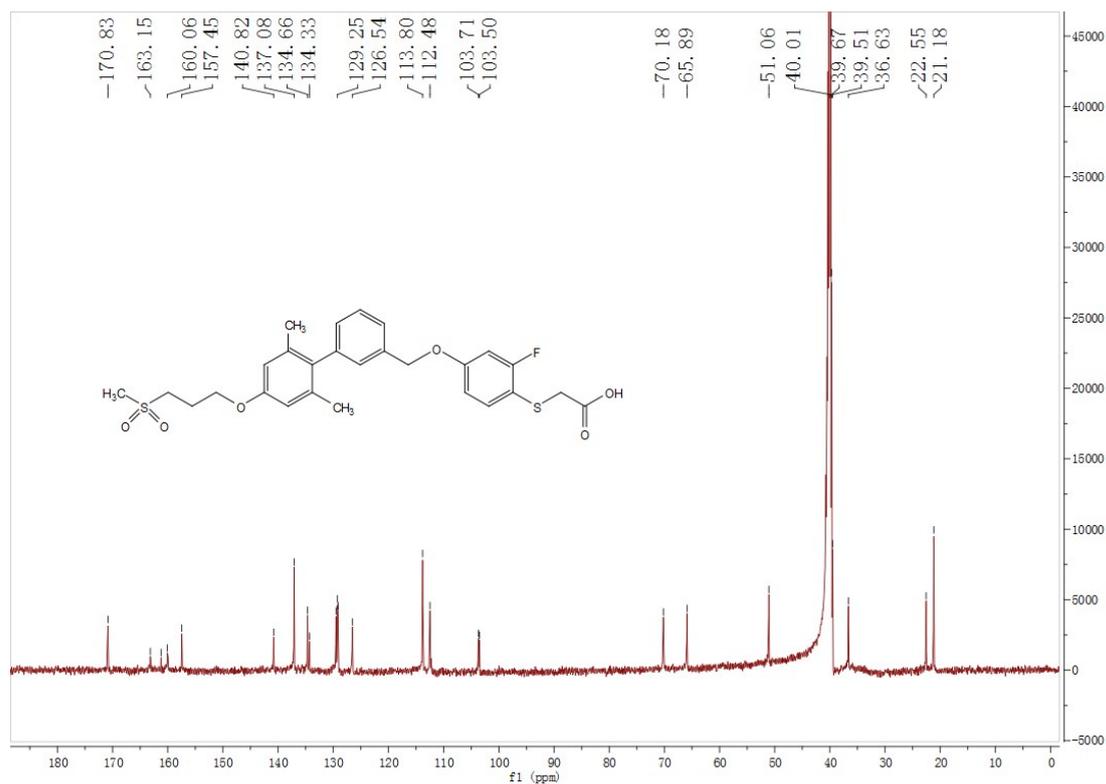


Fig. S4 ¹³C NMR (125 MHz, DMSO-*d*₆) of PAFA.

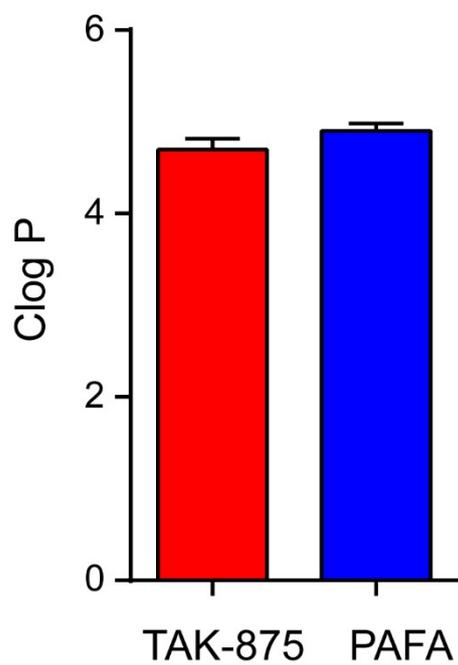


Fig. S5 ClogP values of FFA1 agonists, which were calculated by the BioByte's algorithm implemented in ChemBioDraw Ultra 12.0. Values are mean ± SD (n = 6).

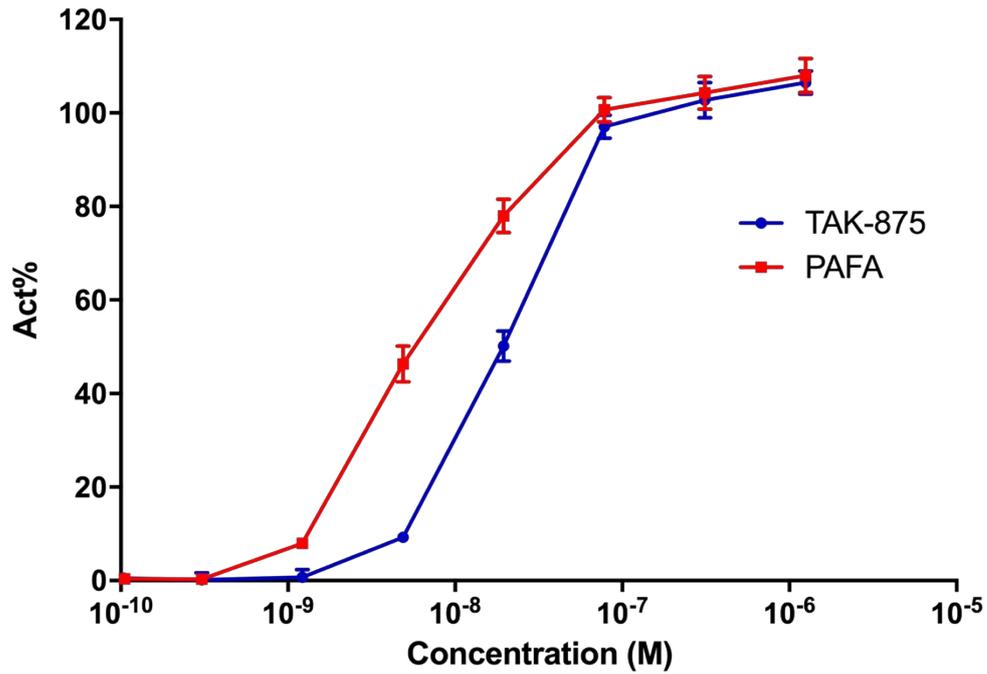


Fig. S6 Agonist activity of TAK-875 and PAFA. Values are mean \pm SD (n = 6).

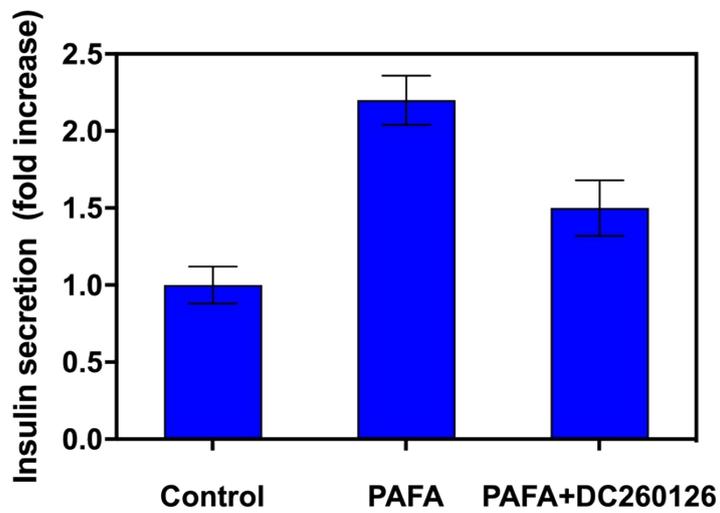


Fig. S7 The specificity of PAFA to FFA1 receptor. INS-1 cells were incubated with 11 mM glucose in the presence of 3 μ M PAFA and DC260126 (6 μ M) for 2 h, and the levels of insulin were measured. Values are mean \pm SD (n = 6).

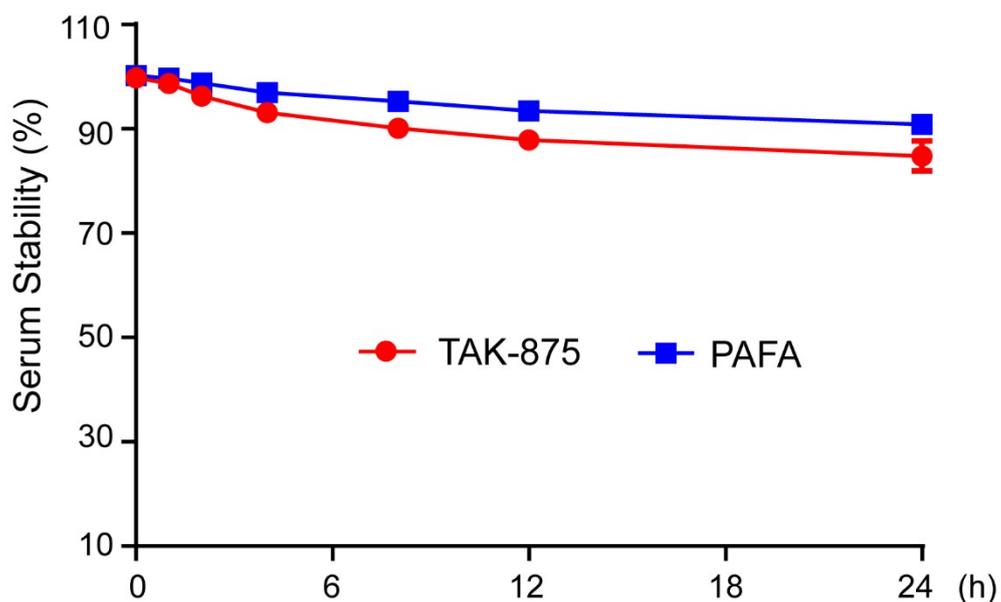


Fig. S8 The serum stability of TAK-875 and PAFA. Values are mean \pm SD (n = 6).

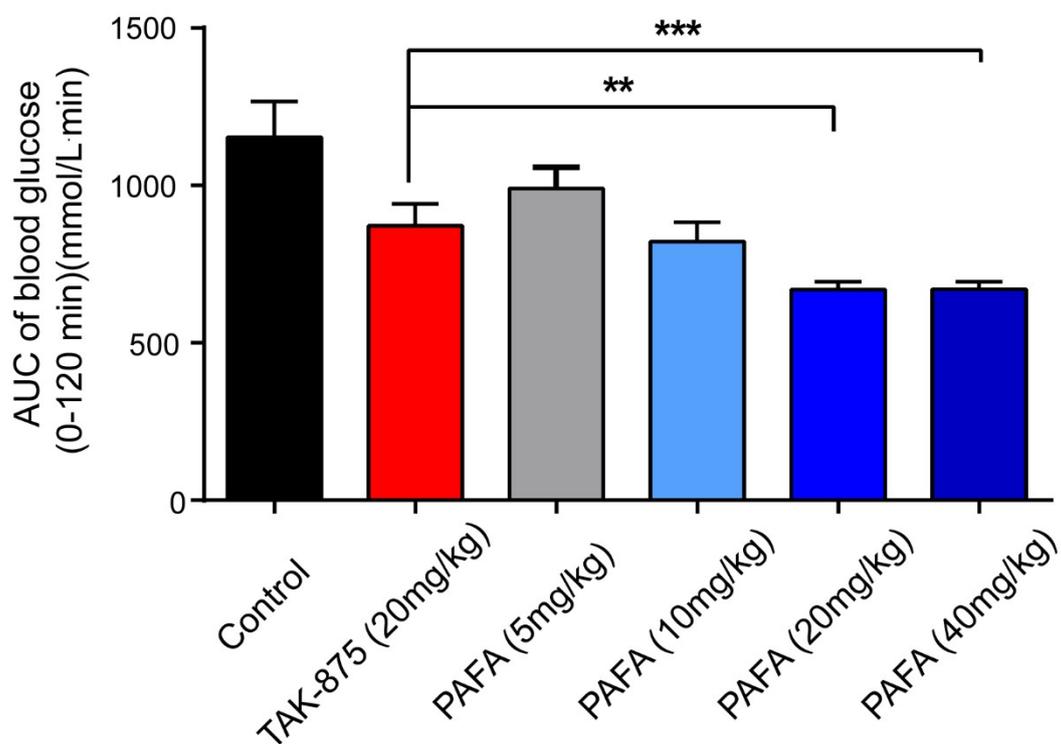


Fig. S9 The AUC_{0-120 min} of blood glucose levels. ICR mice were treated with PAFA or TAK-875. Values are mean \pm SD (n = 6). P values were determined by Student's t-test (**p < 0.01, *** p < 0.001).

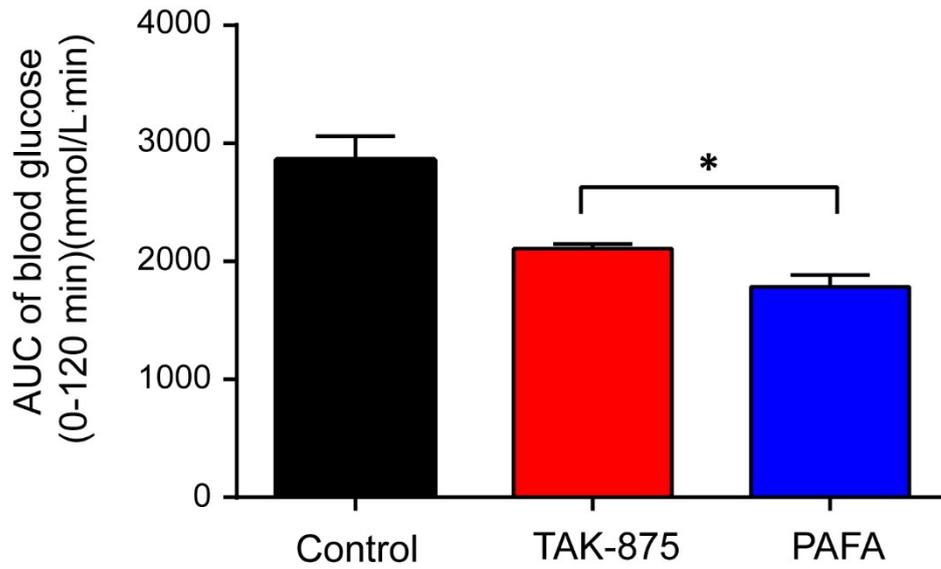


Fig. S10 The $AUC_{0-120 \text{ min}}$ of blood glucose levels during OGTT in *db/db* mice. Values are mean \pm SD (n = 6). P values were determined by Student's t-test (*p < 0.05).

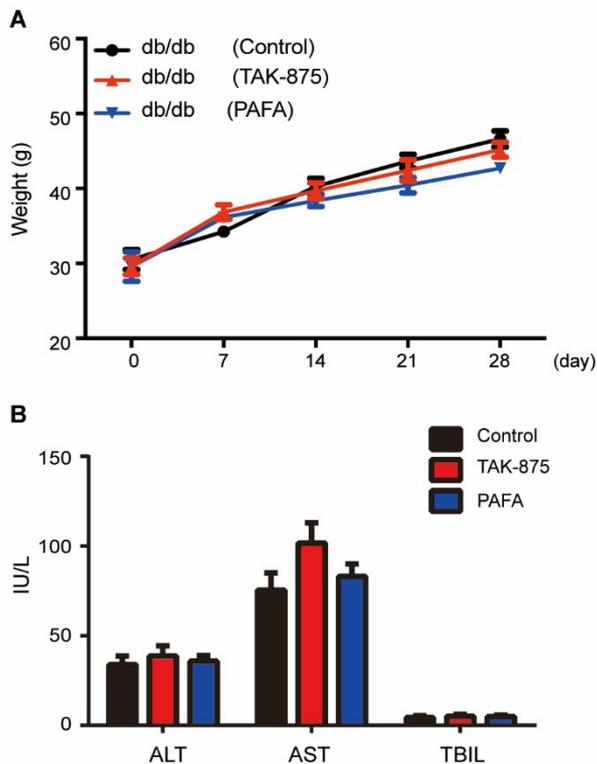


Fig. S11 (A) Body weight changes of *db/db* mice during treatment. (B) The plasma levels of AST, ALT and TBIL of *db/db* mice during treatment. Values are mean \pm SD (n = 6).

Type	Docking score
Compound 2	-14.29
TAK-875	-12.32
PAFA	-14.24

Table. S1 The docking simulation studies of the PAFA-FAA1 complex.