Bioorganic & Medicinal Chemistry 19 (2011) 3183–3191

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Structure-based design, synthesis, and nonalcoholic steatohepatitis (NASH)-preventive effect of phenylpropanoic acid peroxisome proliferator-activated receptor (PPAR) α-selective agonists

Shintaro Ban [a], Jun-ichi Kasuga [b], Izumi Nakagome [c], Hiromi Nobusada [a], Fusako Takayama [a], Shuichi Hirono [c], Hiromu Kawasaki [a], Yuichi Hashimoto [b], Hiroyuki Miyachi [a,*]

[a] Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 1-1-1, Tsushima-Naka, Kita-ku, Okayama 700-8530, Japan
[b] Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan
[c] School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

ARTICLE INFO

Article history:
Received 1 February 2011
Revised 26 March 2011
Accepted 30 March 2011
Available online 8 April 2011

ABSTRACT

A series of α-ethylphenylpropanoic acid derivatives was prepared as candidate peroxisome proliferator-activated receptor (PPAR) α-selective agonists, based on our PPARα/δ dual agonist **3** as a lead compound. Structure–activity relationship studies clearly indicated that the steric bulkiness and position of the distal hydrophobic tail part are critical for PPARα agonistic activity and PPARα selectivity, as had been predicted from a molecular-modeling study. A representative compound blocked the progression of nonalcoholic steatohepatitis (NASH) in an animal model.

1. Introduction

The peroxisome proliferator-activated receptors (PPARs) are members of the human nuclear receptor family, functioning as ligand-dependent transcription factors. These receptors are activated by the binding of various endogenous fatty acids and their metabolites, and by synthetic ligands.[1] Three subtypes, PPARα, PPARδ, and PPARγ have been isolated to date.[2] These PPAR subtypes share a high level of structural homology, but each has distinct physiological functions. Although PPARδ is ubiquitously expressed, the other two PPAR subtypes show unique tissue distribution patterns, that is, PPARα is expressed mostly in metabolically active tissues, such as liver, kidney, skeletal and cardiac muscle, and adrenal glands, while PPARγ is expressed mainly in adipose tissue, macrophages, and vascular smooth muscles.[3]

Among PPARs, PPARα regulates genes involved in uptake and oxidation of free fatty acids, triglyceride hydrolysis and up-regulation of reverse cholesterol transport, such as apolipoprotein A-I and apolipoprotein A-II.[4] Fibrate-class antihyperlipidemic agents, such as fenofibrate (**1**) and bezafibrate (**2**), which effectively lower elevated serum triglycerides and moderately increase high-density lipoproteins (HDL), can bind and activate PPARα. But, their affinity is weak in the high micromolar range), and the subtype selectivity is not high (Fig. 1).[5]

Nonalcoholic steatohepatitis (NASH), consisting of hepatic steatosis accompanied with inflammation and fibrosis, is a progressive liver disorder that occurs in patients without significant alcohol consumption.[6,7] NASH is the most prevalent cause of chronic liver disease in westernized countries, and patients with NASH and cirrhosis are at risk for hepatocellular carcinoma.[8]

Recent animal studies have demonstrated that acyl CoA oxidase (AOX)-null mice have defective peroxisomal β-oxidation and exhibit steatohepatitis.[9] When *PPARα*-null mice were fed with MCD diet, which is deficient in both methionine and choline and high in sucrose (40%) and fat (10%), they developed severe steatohepatitis compared with wild-type mice on the same diet. These data indicated the critical role of fatty acid disposal pathways in the progression of hepatic steatosis and steatohepatitis.[10] Therefore, PPARα agonists have therapeutic potential for the treatment of NASH.

In the continuous study directed toward the development of subtype-selective PPAR ligand,[11–15] we would like to report the design and synthesis of phenylpropanoic acid-type PPARα agonists. We also describe the preventive effect of a representative compound against progression of NASH in an animal model.

2. Inhibitor design

In order to create PPARα-selective agonists with phenylpropanoic acid structure as a basic framework, we focused on our PPARα/δ-dual agonist **3**.[11] Compound **3** is a potent PPARα/δ dual agonist with EC_{50} values of 10, 12, and 1900 nM for PPARα, PPARδ, and PPARγ, respectively.

Recently, we have succeeded in solving the X-ray crystallographic structure of **3** complexed with the hPPARδ ligand-binding

* Corresponding author. Tel.: +81 086 251 7930.
E-mail address: miyachi@pharm.okayama-u.ac.jp (H. Miyachi).

0968-0896/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2011.03.064

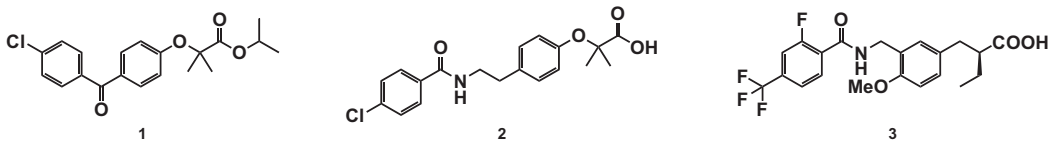


Figure 1. Representative fibrate class PPARα agonists (**1**, **2**) and our PPARα/δ dual agonist **3**.

domain (LBD).[16] The structure of this complex throws light on the *S*-enantiomer preference, the importance of the acidic functionality, and the effect of introduction of fluorine at the 2′-position for PPARδ activity. But, unfortunately, this complex lacks the six amino acid residues from the end of the H2′ helix to the beginning of the H3 helix (residues 265–270). Therefore, we computationally constructed a binding model of **3** complexed with full-length hPPARδ LBD structure,[17] based on the reported full structure deposited in PDB. We also constructed a binding model of **3** complexed with full-length hPPARα LBD, by superimposing **3** on the reported full-length hPPARα LBD. The results are depicted in Figure 2A and B. Comparing these two binding models, we noticed that the shapes of the hydrophobic pockets hosting the hydrophobic tail part of **3** (4-trifluoromethyl group) are somewhat different, that is, the hydrophobic pocket of hPPARα is wider than that of hPPARδ. Therefore, we anticipated that if we could introduce a sufficiently bulky substituent instead of the 4-trifluoromethyl group, we would be able to create a hPPARα-selective agonist, because the introduced substituent would enhance the interaction with the hydrophobic pocket in the case of the hPPARα LBD, whereas it would be too large to enter the pocket in the case of hPPARδ LBD. Based on this working hypothesis, we planned to synthesize derivatives of **3** bearing a bulky substituent at the hydrophobic tail part.

3. Chemistry

The synthetic routes to the racemic series of compounds are outlined in Scheme 1. Compound **9** was prepared from 4-methoxybenzaldehyde (**4**) in five steps. 4-Methoxybenzaldehyde was treated with triethyl 2-phosphonobutyrate in the presence of *t*BuOK as a base, followed by hydrogenolysis with palladium on carbon to afford the ethyl phenylpropionate derivative (**6**). The formylation of **6** with dichloromethylmethylether in the presence of $TiCl_4$ as a Lewis acid to afford **7**, which was treated with hydroxylamine hydrochloride and subsequent reduction afforded **9**. The benzoylation of **9** with substituted benzoyl chlorides in the presence of triethylamine as a base afforded benzamide derivatives (**10a**–**k**). Subsequent alkaline hydrolysis afforded the desired racemic products **11a**–**k**. Optically active phenylpropanoic acid derivatives, **14a–c**, were prepared as described previously (Scheme 1).

4. Results and discussion

The SAR results are summarized in Figure 3. The introduction of a bulky substituent at the 4-position of the distal benzene ring was effective to improve PPARα agonistic activity, as expected, that is, the activity increased in the order of **11a** < **11b** < **11c** < **11d**. While the introduction of a substituent at the 4-position is important to elicit PPARδ agonistic activity (**11a** vs **11b**), the activity did not seem to correlate well with the bulkiness of the substituent. As regards activity towards PPARγ, the compounds were basically inactive (**11a**) or weak agonistic (**11b**–**11d**). The position of the bulky substituent is also important for PPARα agonistic activity. The PPARα agonistic activity increased in the order of 2-position (**11f**, **11i**) < 3-position (**11e**, **11h**) < 4-position (**11d**, **11g**). In the case of the ether linker connecting the distal benzene rings (**11d**, **11j**, and **11k**), one atom length (**11d**) might be optimal, because the

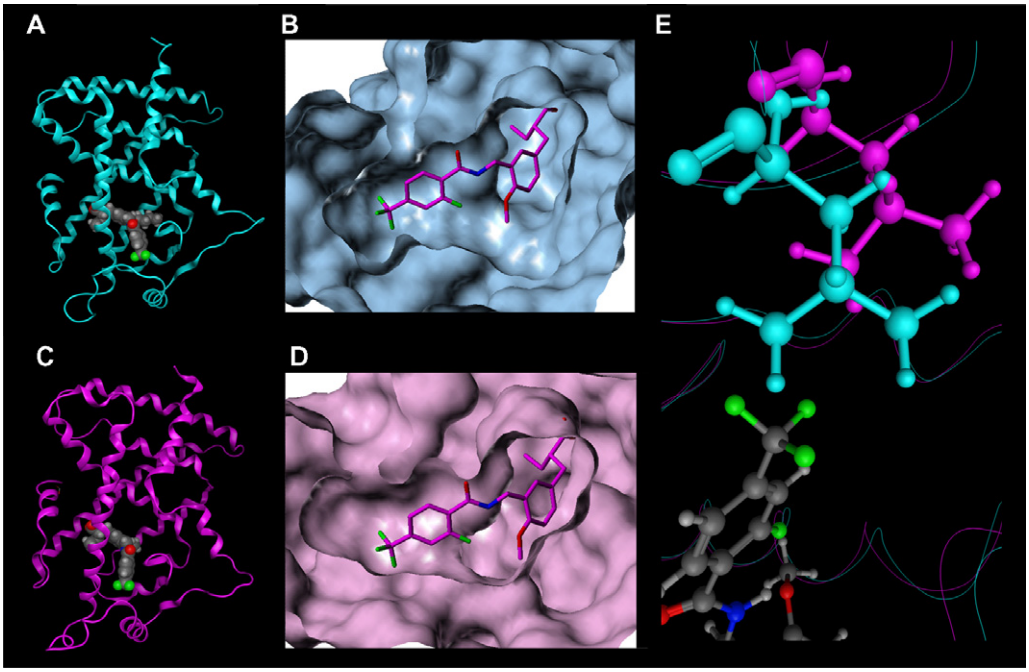
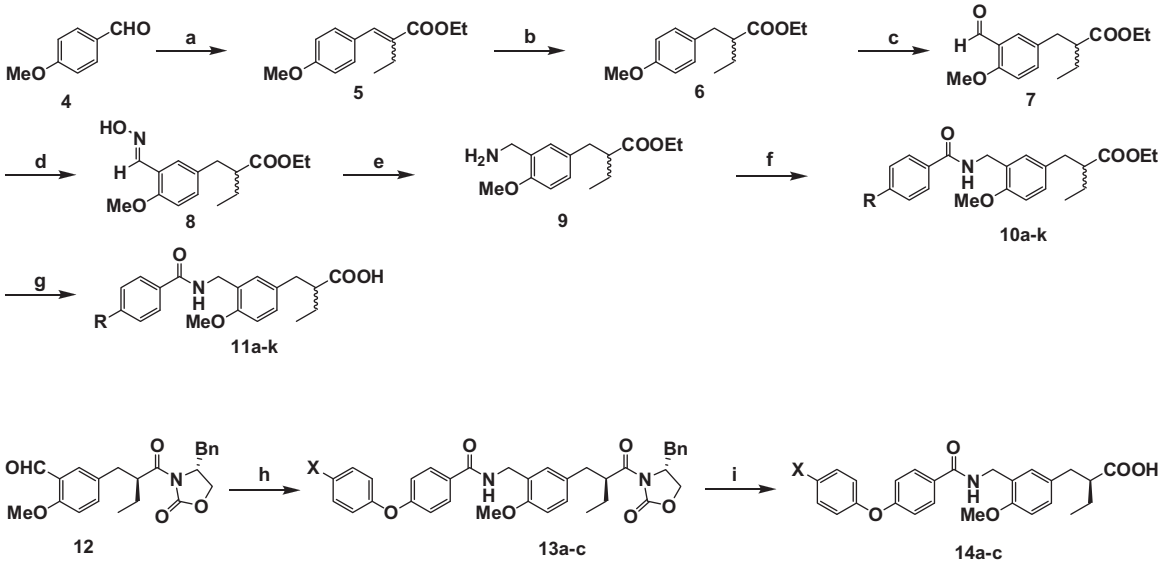
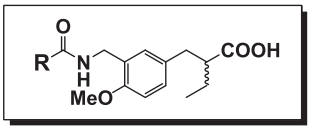


Figure 2. (A–E) Molecular modeling structures of PPAR LBD–**3** complexes. (A) PPARδ LBD–**3** complex model (amino acid residues other than 265–270 are taken from our previous X-ray data (PDB 2znp)); (B) binding mode of **3** to PPARδ LBD; (C) PPARα LBD–**3** complex model; (D) binding mode of **3** to PPARα LBD; (E) zoomed view of the ligand-binding domain near the trifluoromethyl group of 3. Cyan represents Ile of PPARδ, magenta represents Ile of PPARα.



Scheme 1. Synthetic route to the present series of compounds. Reagents and conditions: (a) $(EtO)_2POCH(Et)CO_2Et$, 60% NaH, THF, 0 °C to rt, 5 h, 85%; (b) H_2, 10% Pd–C, EtOH, rt, 2 h, quant.; (c) $MeOCHCl_2$, $TiCl_4$, CH_2Cl_2, −30 °C to 0 °C, 5 h, 70%; (d) NH_2OH HCl, pyridine, EtOH, reflux, overnight, 80%; (e) H_2, 10% Pd–C, EtOH, concd HCl, 50 °C, overnight, 90%; (f) substituted benzoyl chloride, triethylamine, CH_2Cl_2, rt, overnight, 60–80%; (g) aq NaOH, EtOH, rt to 60 °C, overnight, 55–90%; (h) substituted benzamide, triethylsilane, TFA, toluene, reflux, 48 h, 85–97%; (i) LiOH–H_2O, 30% H_2O_2, THF/H_2O = 4:1 (V/V), 0 °C, 2.5 h then rt, 3 h, 60–86%.



No.	R	stereo	EC_{50} (nM) PPARα	PPARδ	PPARγ
11a		rac	1700 ± 200	8100 ± 400	>10,000
11b	H_3C	rac	100 ± 12	360 ± 40	9600 ± 300
11c	F_3C–O	rac	12 ± 3	110 ± 12	4400 ± 400
11d	–O–	rac	8.8 ± 0.4	120 ± 10	820 ± 140
11e	O	rac	41 ± 4.	4800 ± 700	4600 ± 500
11f	O	rac	3600 ± 100	>10,000	>10,000
11g		rac	7.6 ± 1.9	210± 20	340 ± 40
11h		rac	74 ± 17	4100 ± 400	710 ± 600
11i		rac	3600 ± 300	>10,000	>10,000
11j	O	rac	52 ± 16	1400 ± 300	460 ± 40
11k	O	rac	60 ± 13	1400 ± 100	780 ± 100
14a	F, O	S	5.0 ± 1.3	300 ± 15	920 ± 40
14b	Cl, O	S	15 ± 3.0	580 ± 20	830 ± 30
14c	Br, O	S	29 ± 2.3	800 ± 30	990 ± 40

Figure 3. Potency of the compounds prepared in this study. Compounds were screened for agonist activity towards PPAR-GAL4 chimeric receptors in transiently transfected HEK-293 cells as described. The EC_{50} value is the molar concentration of the test compound that affords 50% of the maximal reporter activity. "rac" means racemic compound.

benzyl derivative (**11j**) and phenethyl derivative (**11k**) exhibited decreased PPARα agonistic activity.

Anticipating an improvement of PPARα agonistic activity when the 4-position trifluoromethyl group of **3** was changed to the phenoxy derivative, we next prepared the *S* enantiomer series of **11d**, for we have succeeded in solving the X-ray crystallographic structures of PPARα/δ-dual agonist **3**, complexed with both PPARα and PPARδ ligand binding domains as described above. The ethyl groups of **3** are placed at the deepest binding sites with the head carboxyl groups and contact the surrounding hydrophobic residues. When their *R* isomers approach the ligand-binding pocket, the ethyl groups may cause a steric clash with the surrounding

residues, particularly those on the central parts of the H3 helix, even though some structural rearrangements could occur in both the proteins and ligands.

Further addition of a halogen atom at the 4-position of the phenoxy moiety of **11d** was not effective in improving PPARα agonistic activity, especially in the case of the Cl (**14b**) and Br (**14c**) derivatives, but the 4-fluoro derivative (**14a**) exhibited comparable PPARα agonistic activity to **11d**.

Thus, we have successfully obtained a potent, selective, structurally novel PPARα agonist, **14a**. As compared to the PPARα/δ dual agonist lead **3**, **14a** showed twofold greater PPARα agonistic activity, while the PPARδ agonistic activity was considerably decreased to 1/25. Although the PPARγ agonistic activity was also increased by twofold as compared to **3**, this activity still weak compared to the PPARα agonistic activity. Therefore, we selected **14a** for further in vivo study. The docking modes are depicted in Figure 4A–D.

In order to investigate the therapeutic potential of **14a**, we evaluated the ability of **14a** to block progression of NASH in an animal model.[18,19] Although the pathogenesis of NASH has not yet been fully elucidated, the 'two-hit' theory is widely accepted.[20] The first hit is the accumulation of fatty acids in the liver to cause steatosis. Steatosis is relatively mild pathological process and is easily reversible. However, with secondary cellular stress, steatosis can progress to steatohepatitis.[21,22] The rat model used in this study mimics two hits, by continuous feeding of CDHF diets for 4 weeks as first hit. Then rats received CDHF diets for another 4 weeks and treated during this period with intraperitoneal injection of sodium nitrite every day to induce methemoglobinemia, as a second hit. The protocols are summarized in Figure 5, the results are summarized in Figures 6 and 7.

Representative histological sections of livers from the normal, choline deficient high-fat (CDHF) diet-fed, NASH (CDHF diet-fed with hypoxemia), and **14a**-treated NASH groups are shown in Figure 6. The livers from CDHF diet-fed rats were significantly greater in size than those of normal chow-fed rats (Fig. 6b). On the other hand, extensive liver atrophy was observed in NASH livers (Fig. 6c), and surface nodules were present. Administration of **14a** prevented atrophy and nodule formation, but had no apparent effect on the features of fatty liver (Fig. 6d). Histopathological examination of liver tissues stained with hematoxylin exhibited hepatic macrovesicular steatosis in all CDHF-fed rats (Fig. 6f and g). No fibrosis was seen in the livers of rats fed normal chow compared with those fed CDHF alone. In the case of the NASH rats, advanced liver fibrosis and necrosis were observed (Fig. 6k), but such liver fibrosis was clearly attenuated by **14a** (1 mg/kg/day) treatment (Fig. 6l).

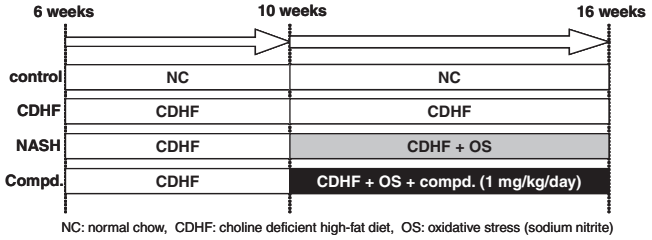


Figure 5. In vivo experimental design.

Figure 7 shows the changes of aspartate amino transferase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP), which are well-known markers of liver function.[23] In this experiment, levels were significantly higher in the NASH rats as compared to rats of the CDHF-fed group. In the 1 mg/kg/day **14a**-treated NASH group, liver function was improved, as compared

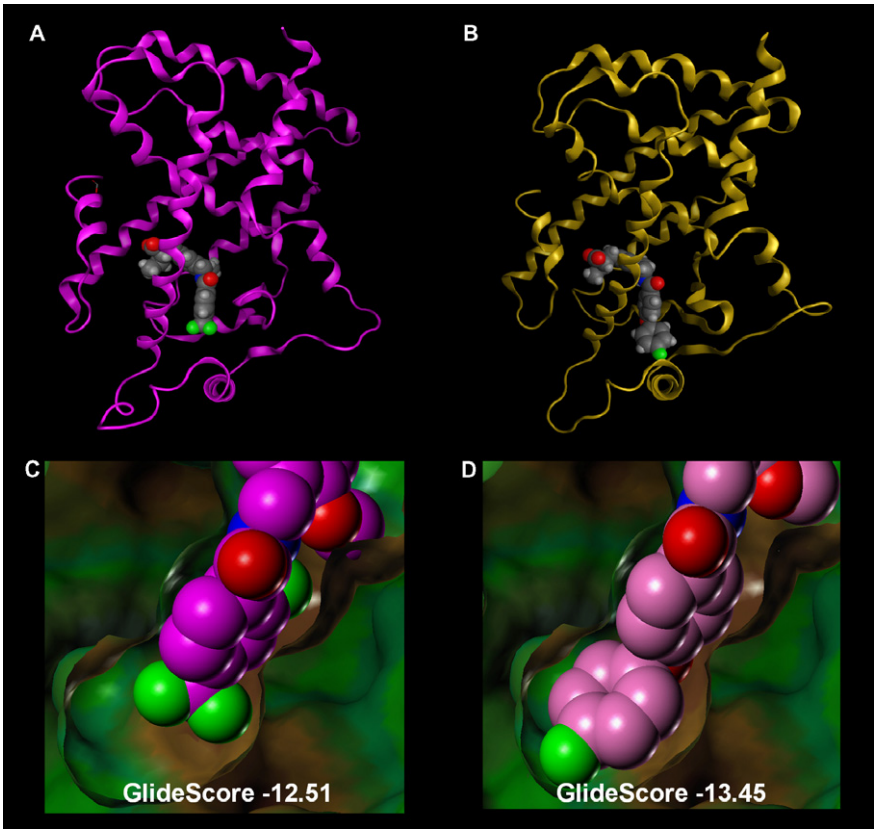


Figure 4. (A–E) Molecular modeling structures of PPARα LBD–**3**, and PPARα LBD–**14a** complexes. (A) PPARα LBD–**3** complex model (amino acid residues other than 265–270 are taken from our previous X-ray data (PDB 2znp)); (B) PPARα LBD–**14a** complex model; (C) zoomed view of the ligand-binding domain near the trifluoromethyl group of **3**; (D) zoomed view of the ligand-binding domain near the 4-(4-fluorophenoxy) group of **14a**.

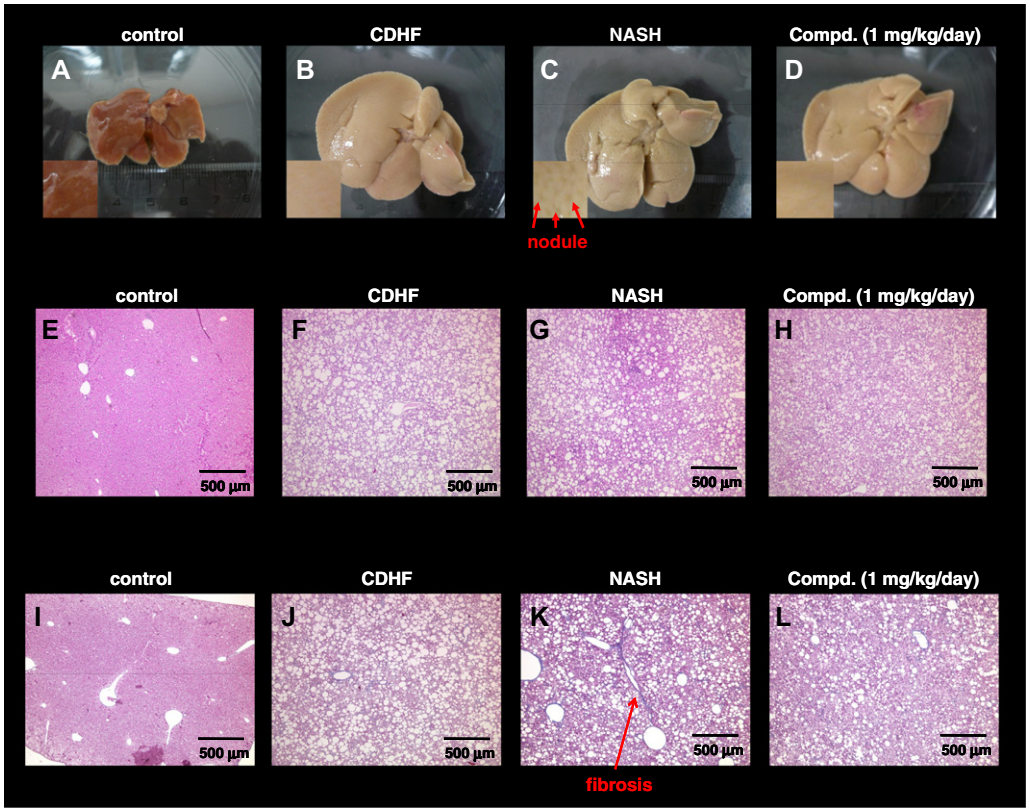


Figure 6. Histological evaluation of liver samples from rats of various groups. Histological evaluation of liver samples of rats from the normal (A), CDHF (B), NASH (C), and NASH + 1 mg/kg/day **14a** (D) groups. The effects of **14a** on liver structure were examined by hematoxylin and eosin staining in rats from the normal (e), CDHF (f), NASH (g), and NASH + 1 mg/kg/day **14a** (h) groups. The effects of **14a** on liver fibrosis were examined by Masson Trichrome staining in rats from the normal (I), CDHF (J) NASH (K), and NASH + 1 mg/kg **14a** (L) groups. Data show typical results. Scale bar = 500 μm.

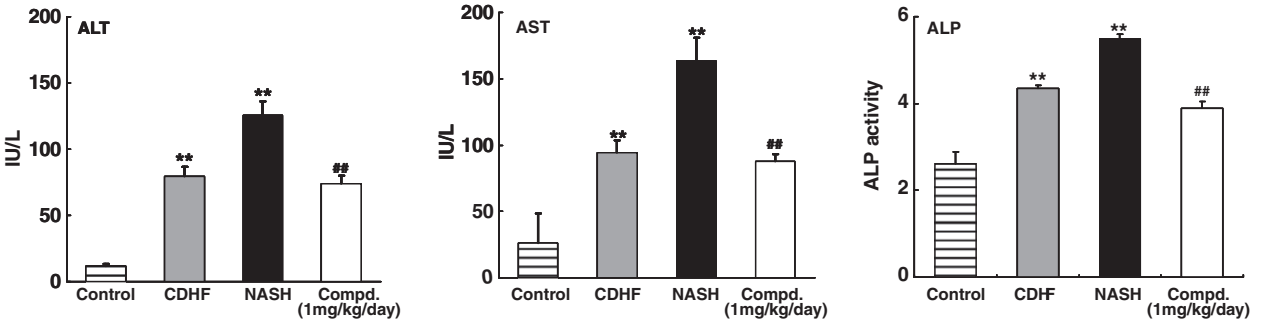


Figure 7. Biochemical markers of hepatobiliary damage (AST, ALT, and ALP levels in serum): AST, ALT, and ALP levels in the four experimental groups (see legend to Fig. 6) were measured at the end of the study period. Each value represents the mean ± SEM of five rats. $^{\#\#}p$ <0.01 versus normal control group, $^{**}p$ <0.01 versus CDHF group (by ANOVA followed by with Dunnett's multiple comparison test).

to the NASH group, although the enzyme levels were still increased compared to the normal chow-fed group. These in vivo findings indicate that our PPARα-selective agonist, **14a**, can block, or at least delay, the progression of NASH.

5. Conclusion

We have succeeded in creating a potent and selective PPARα agonist, **14a**. We also showed that **14a** can block progression of NASH in an animal model. Our results indicate that PPARα-selective activation can prevent or ameliorate liver dysfunction and delay the progression of fatty liver to fibrosis in this model of NASH.

6. Experimental

6.1. General methods

Melting points were determined with a Yanagimoto hot-stage melting point apparatus and are uncorrected. NMR spectra were recorded on a Varian VXR-300 (^{1}H 300 MHz) spectrometer. Proton chemical shifts were referenced to the TMS internal standard. Elemental analysis was carried out with a Yanagimoto MT-5 CHN recorder elemental analyzer and results were within 0.4% of the theoretical values. FAB-MS was carried out with a VG70-SE.

6.1.1. Ethyl 2-(4-methoxybenzylidene)butanoate (5)

Sodium hydride (214 mg, 5.35 mmol; 60% oil dispersion) was suspended in 10 mL of dehydrated tetrahydrofuran under argon and cooled with ice. Triethyl 2-phosphonobutyrate (1.34 g, 5.31 mmol) dissolved in 20 mL of dehydrated tetrahydrofuran was added dropwise. When the addition was completed, the mixture was stirred for 1 h, then 4-methoxybenzaldehyde (1.44 g, 5.33 mmol) dissolved in 25 mL of dehydrated tetrahydrofuran was added dropwise, and the mixture was stirred for a further 4 h at room temperature. The reaction mixture was poured into ice/water. The whole was extracted with ethyl acetate, and the organic phase was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated. The residue was purified by silica gel column chromatography (eluant, *n*-hexane/ethyl acetate = 5:1 v/v) to afford 1.45 g (74%) of the title compound as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.59 (s, 1H), 7.36 (d, J = 8.5 Hz, 2H), 6.92 (d, J = 8.5 Hz, 2H), 4.26 (q, J = 7.3 Hz, 2H), 3.83 (s, 3H), 2.57 (q, J = 7.3 Hz, 2H), 1.34 (t, J = 7.3 Hz, 3H), 1.18 (t, J = 7.3 Hz, 3H); MS (FAB) 235 $(M+H)^+$.

6.1.2. Ethyl 2-(4-methoxybenzyl)butanoate (6)

5 (9.31 g, 39.7 mmol) was dissolved in 200 mL of ethanol, 10% palladium on carbon (1.10 g) was added, and hydrogenation was carried out for 3 h at an initial pressure of 353 kPa. After completion of the reaction, the catalyst was removed by filtration and the filtrate was concentrated to afford 9.38 g (quant.) of the title compound as a yellow oil: ^{1}H NMR(500 MHz, $CDCl_3$) δ 7.07 (d, J = 8.5 Hz, 2H), 6.80 (d, J = 8.5 Hz, 2H), 4.07 (m, 2H), 3.78 (s, 3H), 2.86 (dd, J = 13.7, 8.5 Hz, 1H), 2.69 (dd, J = 13.7, 6.4 Hz, 1H), 2.53 (m, 1H), 1.63 (m, 1H), 1.55 (m, 1H), 1.16 (t, J = 7.3 Hz, 3H), 0.91 (t, J = 7.3 Hz, 3H); MS (FAB) 237 $(M+H)^+$.

6.1.3. Ethyl 2-(3-formyl-4-methoxybenzyl)butanoate (7)

To a solution of **6** (9.38 g, 39.7 mmol) and 500 mL of anhydrous dichloromethane was added titanium tetrachloride (35.0 mL, 318 mmol), followed by dichloromethyl methyl ether (13.4 mL, 148 mmol) at −20 °C, under an argon atmosphere. The mixture was stirred for 6 h at −20 °C, then poured into dilute HCl solution and the layers were separated. The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated. The residue was purified by silica gel column chromatography (eluant, *n*-hexane/ethyl acetate = 4:1 v/v) to afford 10.5 g (quant.) of the title compound as a pale-brown oil: ^{1}H NMR(500 MHz, $CDCl_3$) δ 10.43 (s, 1H), 7.62 (d, J = 2.6 Hz, 1H), 7.35 (dd, J = 8.5, 2.6 Hz, 1H), 6.90 (d, J = 8.5 Hz, 1H), 4.06 (m, 2H), 3.90 (s, 3H), 2.88 (dd, J = 13.7, 9.0 Hz, 1H), 2.72 (dd, J = 13.7, 6.4 Hz, 1H), 2.54 (m, 1H), 1.63 (m, 1H), 1.55 (m, 1H), 1.16 (t, J = 7.3 Hz, 3H), 0.91 (t, J = 7.3 Hz, 3H); MS (FAB) 265 $(M+H)^+$.

6.1.4. Ethyl 2-(3-((hydroxyimino)methyl)-4-methoxybenzyl) butanoate (8)

To a solution of **7** (6.8 g, 25.7 mmol), pyridine (3 mL) and ethanol (100 mL) was added hydroxylamine hydrochloride (2.1 g 30.2 mmol). The mixture was refluxed for 5 h, then concentrated and poured into water. The whole was extracted with ethyl acetate, and the organic phase was washed with water, brine, dil HCL, and saturated $NaHCO_3$ solution, then dried over anhydrous magnesium sulfate, and concentrated. The residue was purified by silica gel column chromatography (eluant, *n*-hexane/ethyl acetate = 5:1 v/v) to afford 6.80 g (95%) of the title compound as a yellow oil: ^{1}H NMR(500 MHz, $CDCl_3$) δ 8.46 (s, 1H), 7.51 (m, 2H), 7.14 (dd, J = 8.3, 2.6 Hz, 1H), 6.81 (d, J = 8.5 Hz, 1H), 4.07 (m, 2H), 3.83 (s, 3H), 2.87 (dd, J = 13.9, 8.5 Hz, 1H), 2.69 (dd, J = 13.9, 6.4 Hz, 1H), 2.54 (m, 1H), 1.63 (m, 1H), 1.55 (m, 1H), 1.17 (t, J = 7.3 Hz, 3H), 0.91 (t, J = 7.3 Hz, 3H); MS (FAB) 280 $(M+H)^+$.

6.1.5. Ethyl 2-(3-(aminomethyl)-4-methoxybenzyl)butanoate HCl (9)

Compound **5** (6.80 g, 24.3 mmol) was dissolved in 150 mL of ethanol, 2.5 mL of concd HCl, 10% palladium on carbon (0.70 g) was added, and hydrogenation was carried out overnight at an initial pressure of 0.3 MPa. After completion of the reaction, 100 mL of water was added, the catalyst was removed by filtration, and the filtrate was concentrated. Ether (300 mL) was added to the residue and triturated to afford 6.10 g (83%) of the title compound as a colorless amorphous solid: ^{1}H NMR(500 MHz, $CDCl_3$) δ 8.03 (s, 3H), 7.17 (m, 2H), 6.97 (d, J = 8.2 Hz, 1H), 4.02 (m, 2H), 3.92 (s, 2H), 3.79 (s, 3H), 2.76 (dd, J = 13.6, 8.2 Hz, 1H), 2.64 (dd, J = 13.6, 6.7 Hz, 1H), 2.52 (m, 1H), 1.49 (m, 2H), 1.11 (t, J = 7.0 Hz, 3H), 0.84 (t, J = 7.6 Hz, 3H); MS (FAB) 266 $(M+H)^+$.

6.1.6. 2-(3-(Benzamidomethyl)-4-methoxybenzyl)butanoic acid (11a)

To a solution of **9** (302 mg, 1.0 mmol), triethylamine (0.3 mL) and dichloromethane (10 mL) was added benzoyl chloride (0.12 mL, 1.0 mmol) at 0 °C, and the mixture was stirred for 30 min at 0 °C, then for 5 h at room temperature. The reaction mixture was washed with dil. HCL and saturated $NaHCO_3$ solution, dried over anhydrous magnesium sulfate, and concentrated. The residue was purified by silica gel column chromatography (eluant, *n*-hexane/ethyl acetate = 2:1 v/v) to afford 297 mg (90%) of the ester intermediate **10a** as a yellow oil: ^{1}H NMR(500 MHz, $CDCl_3$) δ 7.75 (d, J = 5.3 Hz, 2H), 7.4 (m, 3H), 7.14 (s, 1H), 7.06 (d, J = 8.1 Hz, 1H), 6.80 (d, J = 8.1 Hz, 1H), 6.65 (s, 1H), 4.60 (d, J = 6.0 Hz, 2H), 4.05 (m, 2H), 3.85 (s, 3H), 2.86 (dd, J = 13.7, 8.5 Hz, 1H), 2.68 (dd, J = 13.7, 6.8 Hz, 1H), 2.53 (m, 1H), 1.62 (m, 1H), 1.54 (m, 1H), 1.16 (t, J = 7.3 Hz, 3H), 0.90 (t, J = 7.7 Hz, 3H); MS (FAB) 370 $(M+H)^+$.

A mixture of **10a** (290 mg, 0.79 mmol), 1 mol/L LiOH (2 mL) and ethanol (5 mL) was stirred at 80 °C overnight. The reaction mixture was neutralized with HCl and extracted with $CHCl_3$. The extract was washed with water, brine, dil HCL, and saturated $NaHCO_3$ solution, then dried over anhydrous magnesium sulfate, and concentrated. The residue was purified by silica gel column chromatography (eluant, *n*-hexane/ethyl acetate = 2:1 v/v) to afford 235 mg (85%) of the title compound as a colorless crystalline powder: ^{1}H NMR(500 MHz, $CDCl_3$) δ 7.75 (d, J = 7.7 Hz, 2H), 7.47 (m, 1H), 7.40 (t, J = 7.7 Hz, 2H), 7.16 (d, J = 1.7 Hz, 1H), 7.08 (d, J = 8.1 Hz, 1H), 6.79 (d, J = 8.1 Hz, 1H), 6.72 (s, 1H), 5 (br, 1H), 4.58 (d, J = 6.0 Hz, 2H), 3.85 (s, 3H), 2.88 (dd, J = 13.7, 8.5 Hz, 1H), 2.70 (dd, J = 13.7, 6.4 Hz, 1H), 2.55 (m, 1H), 1.64 (m, 1H), 1.56 (m, 1H), 0.94 (t, J = 7.7 Hz, 3H); MS (FAB) 342 $(M+H)^+$; HRMS (M+H) calcd for 342.1705, found 342.1707 $(M+H)^+$.

6.1.7. 2-(3-(4-Methylbenzamidomethyl)-4-methoxybenzyl) butanoic acid (11b)

This compound was prepared from **9** and 4-methylbenzoyl chloride, using the same procedures as described for the preparation of **11a**: ^{1}H NMR(500 MHz, $CDCl_3$) δ 7.65 (d, J = 8.1 Hz, 2H), 7.2 (br, 1H), 7.19 (d, J = 7.7 Hz, 2H), 7.15 (d, J = 2.1 Hz, 1H), 7.07 (dd, J = 8.1, 2.1 Hz, 1H), 6.78 (d, J = 8.1 Hz, 1H), 6.71 (s, 1H), 4.57 (d, J = 5.6 Hz, 2H), 3.83 (s, 3H), 2.88 (dd, J = 13.7, 8.1 Hz, 1H), 2.68 (dd, J = 13.7, 6.4 Hz, 1H), 2.55 (m, 1H), 2.37 (s, 3H), 1.63 (m, 1H), 1.56 (m, 1H), 0.94 (t, J = 7.3 Hz, 3H); MS (FAB) 356 $(M+H)^+$; HRMS (M+H) calcd for 356.1862, found 356.1849 $(M+H)^+$.

6.1.8. 2-(4-Methoxy-3-((4-(trifluoromethoxy)benzamido) methyl)benzyl)butanoic acid (11c)

This compound was prepared from **9** and 4-methylbenzoyl chloride, using the same procedures as described for the preparation of **11a**: ^{1}H NMR(500 MHz, $CDCl_3$) δ 7.78 (d, J = 8.6 Hz, 2H), 7.21 (d, J = 8.5 Hz, 2H), 7.14 (d, J = 2.1 Hz, 1H), 7.08 (dd, J = 8.1,

2.1 Hz, 1H), 6.79 (m, 2H), 4.56 (d, *J* = 6.0 Hz, 2H), 3.83 (s, 3H), 2.88 (dd, *J* = 13.7, 8.5 Hz, 1H), 2.69 (dd, *J* = 13.7, 6.4 Hz, 1H), 2.54 (m, 1H), 1.65 (m, 1H), 1.57 (m, 1H), 0.94 (t, *J* = 7.3 Hz, 3H); MS (FAB) 426 $(M+H)^+$; HRMS (M+H) calcd for 426.1528, found 426.1539 $(M+H)^+$. Anal. Calcd for $C_{21}H_{21}NO_4$: C, 59.29; H, 5.21; N, 3.29. Found: C, 59.30; H, 5.45; N, 3.10 $(M+H)^+$.

6.1.9. 2-(4-Methoxy-3-((4-phenoxybenzamido) methyl)benzyl)butanoic acid (11d)

This compound was prepared from **9** and 4-methylbenzoyl chloride, using the same procedures as described for the preparation of **11a**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.72 (d, *J* = 9.0 Hz, 2H), 7.36 (t, *J* = 8.1 Hz, 2H), 7.16 (m, 2H), 7.07 (dd, *J* = 8.6, 2.1 Hz, 1H), 7.02 (d, *J* = 8.1 Hz, 2H), 6.97 (d, *J* = 8.5 Hz, 2H), 6.79 (d, *J* = 8.1 Hz, 1H), 6.66 (t, *J* = 4.6 Hz, 1H), 4.57 (d, *J* = 6.0 Hz, 2H), 3.84 (s, 3H), 3.5 (br, 1H), 2.87 (dd, *J* = 13.7, 8.6 Hz, 1H), 2.71 (dd, *J* = 13.7, 6.4 Hz, 1H), 2.55 (m, 1H), 1.65 (m, 1H), 1.58 (m, 1H), 0.95 (t, *J* = 7.3 Hz, 3H); MS (FAB) 434 $(M+H)^+$; HRMS (M+H) calcd for 434.19675, found 434.1960 $(M+H)^+$. Anal. Calcd for $C_{26}H_{27}NO_5$: C, 72.04; H, 6.28; N, 3.23. Found: C, 71.79; H, 6.38; N, 3.00.

6.1.10. 2-(4-Methoxy-3-((3-phenoxybenzamido)methyl) benzyl)butanoic acid (11e)

This compound was prepared from **9** and 4-methylbenzoyl chloride, using the same procedures as described for the preparation of **11a**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.46 (d, *J* = 7.7 Hz, 1H), 3.39 (s, 1H), 7.3 (m, 3H), 7.1 (m, 4H), 7.00 (d, *J* = 8.5 Hz, 2H), 6.78 (d, *J* = 8.5 Hz, 1H), 6.72 (t, *J* = 5.1 Hz, 1H), 4.55 (d, *J* = 5.6 Hz, 2H), 3.81 (s, 3H), 2.89 (dd, *J* = 13.7, 8.1 Hz, 1H), 2.68 (dd, *J* = 13.7, 6.8 Hz, 1H), 2.55 (m, 1H), 1.63 (m, 1H), 1.57 (m, 1H), 0.94 (t, *J* = 7.3 Hz, 3H). HRMS (M+H) calcd for 434.19675, found 434.1960 $(M+H)^+$.

6.1.11. 2-(4-Methoxy-3-((2-phenoxybenzamido)methyl) benzyl)butanoic acid (11f)

This compound was prepared from **9** and 4-methylbenzoyl chloride, using the same procedures as described for the preparation of **11a**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.36(t, *J* = 5.6 Hz, 1H), 8.25 (dd, *J* = 8.1, 1.3 Hz, 1H), 7.9 (br, 1H), 7.3 (m, 3H), 7.17 (m, 2H), 7.13 (d, *J* = 1.7 Hz, 1H), 7.02 (d, *J* = 8.1 Hz, 3H), 6.79 (d, *J* = 8.1 Hz, 1H), 6.67 (d, *J* = 8.1 Hz, 1H), 4.60 (d, *J* = 6.0 Hz, 2H), 3.54 (s, 3H), 2.86 (dd, *J* = 13.7, 7.7 Hz, 1H), 2.63 (dd, *J* = 13.9, 6.8 Hz, 1H), 2.52 (m, 1H), 1.60 (m, 1H), 1.53 (m, 1H), 0.92 (t, *J* = 7.3 Hz, 3H). HRMS (M+H) calcd for 434.1967, found 434.1968 $(M+H)^+$.

6.1.12. 2-(4-Methoxy-3-((4-phenylbenzamido)methyl)benzyl) butanoic acid (11g)

This compound was prepared from **9** and 4-methylbenzoyl chloride, using the same procedures as described for the preparation of **11a**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.82 (d, *J* = 8.1 Hz, 2H), 7.62 (d, *J* = 8.1 Hz, 2H), 7.59 (d, *J* = 7.7 Hz, 2H), 7.45 (t, *J* = 7.3 Hz, 2H), 7.37 (t, *J* = 7.2 Hz, 1H), 7.18 (d, *J* = 2.1 Hz, 1H), 7.09 (dd, *J* = 8.6, 2.1 Hz, 1H), 6.80 (d, *J* = 8.1 Hz, 1H), 6.77 (m, 1H), 4.60 (d, *J* = 5.6 Hz, 2H), 3.85 (s, 3H), 2.89 (dd, *J* = 13.7, 8.5 Hz, 1H), 2.71 (dd, *J* = 13.6, 6.4 Hz, 1H), 2.56 (m, 1H), 1.65 (m, 1H), 1.58 (m, 1H), 0.95 (t, *J* = 7.3 Hz, 3H); MS (FAB) 418 $(M+H)^+$; HRMS (M+H) calcd for 418.2018, found 418.1998. Anal. Calcd for $C_{26}H_{27}NO_4$: C, 74.80; H, 6.52; N, 3.35. Found: C, 74.62; H, 6.57; N, 3.21 $(M+H)^+$.

6.1.13. 2-(4-Methoxy-3-((3-phenylbenzamido)methyl)benzyl)butanoic acid (11h)

This compound was prepared from **9** and 4-methylbenzoyl chloride, using the same procedures as described for the preparation of **11a**: ^{1}H NMR(500 MHz, $CDCl_3$) δ 7.99 (s, 1H), 7.69 (m, 2H), 7.59 (d, *J* = 7.7 Hz, 2H), 7.44 (m, 3H), 7.36 (t, *J* = 7.7 Hz, 1H), 7.17 (d, *J* = 2.1 Hz, 1H), 7.08 (dd, *J* = 8.5, 2.1 Hz, 1H), 6.79 (br, 1H), 6.78 (d, *J* = 8.6 Hz, 1H), 4.60 (d, *J* = 6.0 Hz, 2H), 3.83 (s, 3H), 2.89 (dd, *J* = 13.7, 7.9 Hz, 1H), 2.69 (dd, *J* = 13.7, 6.9 Hz, 1H), 2.55 (m, 1H), 1.63 (m, 1H), 1.56 (m, 1H), 0.93 (t, *J* = 7.3 Hz, 3H); MS (FAB) 418 $(M+H)^+$; HRMS (M+H) calcd for 418.2018, found 418.2007. Anal. Calcd for $C_{26}H_{27}NO_4$: C, 74.80; H, 6.52; N, 3.35. Found: C, 74.52; H, 6.38; N, 3.15 $(M+H)^+$.

6.1.14. 2-(4-Methoxy-3-((2-phenylbenzamido)methyl)benzyl) butanoic acid (11i)

This compound was prepared from **9** and 4-methylbenzoyl chloride, using the same procedures as described for the preparation of **11a**: ^{1}H NMR(500 MHz, $CDCl_3$) δ 7.71 (d, *J* = 6.8 Hz, 1H), 7.45 (t, *J* = 7.3 Hz, 1H), 7.38 (t, *J* = 7.7 Hz, 1H), 7.33 (d, *J* = 7.3 Hz, 1H), 7.3–7.2 (m, 5H), 7.01 (dd, *J* = 8.5, 2.1 Hz, 1H), 6.93 (d, *J* = 2.1 Hz, 1H), 6.57 (d, *J* = 8.1 Hz, 1H), 5.82 (br, 1H), 4.30 (m, 2H), 3.56 (s, 3H), 2.86 (dd, *J* = 13.7, 8.1 Hz, 1H), 2.65 (dd, *J* = 13.7, 6.8 Hz, 1H), 2.54 (m, 1H), 1.64 (m, 1H), 1.57 (m, 1H), 0.95 (t, *J* = 7.7 Hz, 3H); MS (FAB) 418 (M+H); HRMS (M+H) calcd for 418.2018, found 418.1998 $(M+H)^+$.

6.1.15. 2-(3-((4-(Benzyloxy)benzamido)methyl)-4-methoxybenzyl)butanoic acid (11j)

This compound was prepared from **9** and 4-methylbenzoyl chloride, using the same procedures as described for the preparation of **11a**: ^{1}H NMR(500 MHz, $CDCl_3$) δ 7.71 (d, *J* = 9.0 Hz, 2H), 7.4 (m, 5H), 7.16 (d, *J* = 2.1 Hz, 2H), 7.07 (dd, *J* = 8.5, 2.1 Hz, 1H), 6.96 (d, *J* = 8.5 Hz, 2H), 6.78 (d, *J* = 8.1 Hz, 1H), 6.64 (s, 1H), 5.09 (s, 2H), 4.55 (m, 2H), 3.84 (s, 3H), 2.87 (dd, *J* = 13.7, 8.5 Hz, 1H), 2.70 (dd, *J* = 13.7, 6.0 Hz, 1H), 2.54 (m, 1H), 1.64 (m, 1H), 1.56 (m, 1H), 0.94 (t, *J* = 7.7 Hz, 3H); MS (FAB) 448 $(M+H)^+$; HRMS (M+H) calcd for 448.2124, found 448.2111 $(M+H)^+$. Anal. Calcd for $C_{27}H_{29}NO_5$: C, 72.46; H, 6.53; N, 3.13. Found: C, 72.29; H, 6.76; N, 2.99.

6.1.16. 2-(4-Methoxy-3-((4-phenethoxybenzamido)methyl) benzyl)butanoic acid (11k)

This compound was prepared from **9** and 4-methylbenzoyl chloride, using the same procedures as described for the preparation of **11a**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.67 (d, *J* = 8.5 Hz, 2H), 7.25 (m, 5H), 7.13 (s, 2H), 7.05 (d, *J* = 8.3 Hz, 1H), 6.85 (d, *J* = 8.5 Hz, 2H), 6.76 (d, *J* = 8.3 Hz, 1H), 6.62 (s, 1H), 4.53 (d, *J* = 6.0 Hz, 2H), 4.17 (t, *J* = 6.6 Hz, 2H), 3.81 (s, 3H), 3.08 (t, *J* = 6.0 Hz, 2H), 2.85 (dd, *J* = 13.7, 8.5 Hz, 1H), 2.67 (dd, *J* = 13.7, 6.4 Hz, 1H), 2.52 (m, 1H), 1.61 (m, 1H), 1.54 (m, 1H), 0.91 (t, *J* = 7.3 Hz, 3H); MS (FAB) 462 $(M+H)^+$; HRMS (M+H) calcd for 462.2280, found 462.2280 $(M+H)^+$.

6.1.17. *N*-(5-((*S*)-2-((*R*)-4-Benzyl-2-oxo-oxazolidine-3-carbonyl)butyl)-2-methoxybenzyl)-4-(4-fluorophenoxy) benzamide (13a)

A mixture of **12** (235 mg, 0.59 mmol), 4-(4-fluorophenoxy)benzamide (560 mg, 2.42 mmol), triethylsilane (0.29 mL, 1.8 mmol), trifluoroacetic acid (0.14 mL, 1.9 mmol), and 15 ml of dehydrated toluene was refluxed for 2 days. The mixture was evaporated, and the residue was purified by silica gel column chromatography (eluant; *n*-hexane/ethyl acetate = 3:1 v/v) to afford 274 mg (76%) of the title compound as a yellow oil. ^{1}H NMR (500 MHz $CDCl_3$) δ 7.71 (d, *J* = 8.8 Hz, 2H), 7.33–7.18 (m, 6H), 6.98 (d, *J* = 8.8 Hz, 2H), 6.97–6.94 (m, 2H), 6.87–6.79 (m, 3H), 6.73 (ddd, *J* = 10.0 Hz, 2.0 Hz, 2.0 Hz, 1H), 6.52 (t, *J* = 5.6 Hz, 1H), 4.68–4.55 (m, 3H), 4.15–4.08 (m, 2H), 4.05 (dd, *J* = 9.2 Hz, 2.4 Hz, 1H), 3.84 (s, 3H), 3.02–2.96 (m, 2H), 2.77 (dd, *J* = 13.6 Hz, 6.4 Hz, 1H), 2.48 (dd, *J* = 13.6 Hz, 9.2 Hz, 1H), 1.82–1.67 (m, 1H), 1.66–1.53 (m, 1H), 0.93 (t, *J* = 7.6 Hz, 3H); MS (FAB) 611.

6.1.18. *N*-(5-((*S*)-2-((*R*)-4-Benzyl-2-oxo-oxazolidine-3-carbonyl) butyl)-2-methoxybenzyl)-4-(4-chlorophenoxy)benzamide (13b)

This compound was prepared from **12** and 4-(4-chlorophenoxy)benzamide, using the same procedures as described for the preparation of **13a**. ^{1}H NMR (400 MHz $CDCl_3$) δ 7.69 (d, J = 8.8 Hz, 2H), 7.32 (d, J = 8.8 Hz, 2H), 7.27–7.18 (m, 5H), 6.99–6.92 (m, 6H), 6.82 (d, J = 8.4 Hz, 1H), 6.50 (t, J = 5.6 Hz, 1H), 4.68–4.55 (m, 3H), 4.15–4.08 (m, 2H), 4.05 (dd, J = 8.8 Hz, 2.4 Hz, 1H), 3.84 (s, 3H), 3.02–2.96 (m, 2H), 2.76 (dd, J = 13.6 Hz, 6.4 Hz, 1H), 2.48 (dd, J = 13.6 Hz, 9.2 Hz, 1H), 1.82–1.68 (m, 1H), 1.66-1.53 (m, 1H), 0.93 (t, J = 7.6 Hz, 3H); MS (FAB) 627, 629 $(M+H)^+$.

6.1.19. *N*-(5-((*S*)-2-((*R*)-4-Benzyl-2-oxo-oxazolidine-3-carbonyl) butyl)-2-methoxybenzyl)-4-(4-bromophenoxy)benzamide (13c)

This compound was prepared from **12** and 4-(4-bromophenoxy)benzamide, using the same procedures as described for the preparation of **13a**. ^{1}H NMR (500 MHz $CDCl_3$) δ 7.70 (d, J = 8.5 Hz, 2H), 7.46 (d, J = 9.0 Hz, 2H), 7.29-7.17 (m, 5H), 7.00-6.89 (m, 6H), 6.82 (d, J = 8.0 Hz, 1H), 6.51 (t, J = 4.0 Hz, 1H), 4.67-4.61 (m, 2H), 4.58 (dd, J = 14.5 Hz, 7.0 Hz, 1H), 4.14–4.09 (m, 2H), 4.05 (dd, J = 9.0 Hz, 3.5 Hz, 1H), 3.84 (s, 3H), 3.02–2.96 (m, 2H), 2.77 (dd, J = 13.5 Hz, 6.5 Hz, 1H), 2.48 (dd, J = 13.5 Hz, 9.0 Hz, 1H), 1.81–1.70 (m, 1H), 1.64-1.54 (m, 1H), 0.93 (t, J = 7.5 Hz, 3H); MS (FAB) 671, 673 $(M+H)^+$.

6.1.20. (*S*)-2-(3-((4-(4-Fluorophenoxy)benzamido)methyl)-4-methoxybenzyl)butanoic acid (14a)

Compound **13a** (267 mg, 0.437 mmol) was dissolved in 24 mL of tetrahydrofuran and 6 mL of water under an argon atmosphere with ice-cooling. To this solution was added 30% aqueous hydrogen peroxide (0.50 mL, 4.4 mmol). Then a solution of lithium hydroxide monohydrate (80 mg, 1.8 mmol) in water (1 mL) was added, and the mixture was stirred further for 2.5 h at 0 °C, and for 3 h at room temperature. An aqueous solution of sodium hydrogen sulfite (1.00 g/6 mL) was added to the mixture and the whole was stirred for 30 min, then acidified with dil HCl, and extracted with ethyl acetate. The extract was washed with brine, dried over anhydrous magnesium sulfate and concentrated. The residue was purified by silica gel column chromatography (eluant; *n*-hexane/ethyl acetate = 2:1 v/v) to afford 97 mg (36%) of the title compound as a colorless amorphous solid. ^{1}H NMR (500 MHz $CDCl_3$) δ 7.75 (d, J = 8.8 Hz, 2H), 7.30 (ddd, J = 8.4 Hz, 8.4 Hz, 6.8 Hz, 1H), 7.16 (d, J = 2.4 Hz, 1H), 7.08 (dd, J = 8.4 Hz, 2.4 Hz, 1H), 7.01 (d, J = 8.8 Hz, 2H), 6.85 (ddd, J = 8.4 Hz, 2.4 Hz, 0.8 Hz, 1H), 6.81–6.77 (m, 2H), 6.73 (ddd, J = 10.0 Hz, 2.4 Hz, 2.4 Hz, 1H), 6.70 (t, J = 5.6 Hz, 1H), 4.62–4.53 (m, 2H), 3.85 (s, 3H), 2.88 (dd, J = 14.0 Hz, 8.8 Hz, 1H), 2.70 (dd, J = 13.6 Hz, 6.4 Hz, 1H), 2.59–2.51 (m, 1H), 1.71–1.51 (m, 2H), 0.94 (t, J = 7.6 Hz, 3H); $[\alpha]_D^{20} = +19$; HRMS (FAB) calcd for 512.1073; found: 512.1055 $(M+H)^+$.

6.1.21. (*S*)-2-(3-((4-(4-Chlorophenoxy)benzamido)methyl)-4-methoxybenzyl)butanoic acid (14b)

This compound was prepared from **13b**, using the same procedures as described for the preparation of **14a**. Mp 108–109 °C; ^{1}H NMR (400 MHz $CDCl_3$) δ 7.73 (d, J = 9.2 Hz, 2H), 7.32 (d, J = 9.2 Hz, 2H), 7.16 (d, J = 2.4 Hz, 1H), 7.08 (dd, J = 8.0 Hz, 2.4 Hz, 1H), 6.97 (d, J = 8.8 Hz, 2H), 6.96 (d, J = 8.8 Hz, 2H), 6.79 (d, J = 8.4 Hz, 1H), 6.68 (t, J = 5.6 Hz, 1H), 4.62–4.52 (m, 2H), 3.85 (s, 3H), 2.88 (dd, J = 14.0 Hz, 8.4 Hz, 1H), 2.71 (dd, J = 13.6 Hz, 6.4 Hz, 1H), 2.59–2.52 (m, 1H), 1.71–1.51 (m, 2H), 0.95 (t, J = 7.2 Hz, 3H); $[\alpha]_D^{20} = +20$; MS (FAB) 468, 470 $(M+H)^+$; HRMS (FAB) calcd for 468.1578; found: 468.1555 $(M+H)^+$.

6.1.22. (*S*)-2-(3-((4-(4-Brophenoxy)benzamido)methyl)-4-methoxybenzyl)butanoic acid (14c)

This compound was prepared from **13c**, using the same procedures as described for the preparation of **14a**. Mp 106–107 °C; ^{1}H NMR (500 MHz $CDCl_3$) δ 7.73 (d, J = 9.0 Hz, 2H), 7.46 (d, J = 9.0 Hz, 2H), 7.16 (d, J = 2.4 Hz, 1H), 7.08 (dd, J = 8.5 Hz, 2.4 Hz, 1H), 6.97 (d, J = 9.0 Hz, 2H), 6.90 (d, J = 9.0 Hz, 2H), 6.79 (d, J = 8.5 Hz, 1H), 6.66 (t, J = 5.6 Hz, 1H), 4.61–4.53 (m, 2H), 3.85 (s, 3H), 2.88 (dd, J = 13.5 Hz, 9.0 Hz, 1H), 2.71 (dd, J = 14.0 Hz, 6.0 Hz, 1H), 2.58–2.54 (m, 1H), 1.70–1.53 (m, 2H), 0.95 (t, J = 7.5 Hz, 3H); $[\alpha]_D^{20} = +19$; MS (FAB) 512, 514 $(M+H)^+$; HRMS (FAB) calcd for 512.1073; found: 512.1055 $(M+H)^+$.

6.2. Animals

Six-week-old male Wistar rats were purchased from Shimizu Animal Co. (Kyoto, Japan) and maintained at the Okayama University animal facility in accordance with the Okayama University Guidelines on the Care and the Use of Laboratory Animals. The rats were housed in individual cages in air-conditioned rooms with a controlled 12-h light-dark cycle. The rats were allowed to acclimate to the animal facility and allowed free access to diet and water ad libitum.

6.3. Experimental design

At 6 weeks of age, the rats were divided into groups and fed either control normal diet or choline deficient high-fat (CDHF) diet (Oriental Yeast Co., Tokyo, Japan) for 4 weeks. At the end of this period, the 6-week experimental period began. The rats of the normal control group were continued on normal chow (MF laboratory chow, Oriental Yeast Co., Tokyo, Japan). The rats of the CDHF group were continued on CDHF diet. On the other hand, the rats of the CDHF + hypoxemia (NASH) group continued on CDHF diet and were also treated with intraperitoneal injection of 40 mg/kg of sodium nitrite ($NaNO_2$) every day to induce methemoglobinemia. The rats of the **14a**-treated NASH group were treated in the same way as the NASH group, but also received **14a** 1 mg/kg/day. At the end of the 6-week experimental period, the rats were sacrificed under light ether anesthesia, blood was collected from the inferior vena cava for serum analysis, and livers were resected for histopathological and biochemical analyses. The experimental design is summarized in Figure 5.

6.4. Biochemical analysis

Serum biochemical markers, AST, ALT, and ALP were determined spectrophotometrically by using standard kits (Wako Pure Chemical Industries, Osaka, Japan).

6.5. Histological examination

Liver tissues were collected, fixed overnight in 10% formaldehyde in phosphate-buffered saline, then dehydrated and embedded in paraffin. Liver tissue sections (4 μm thick) were stained with hematoxylin and with Masson trichrome (MT) staining for histological examination.

6.6. Statistical analysis

Data are expressed as mean ± SEM of five rats. Differences among groups were examined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A p value less than 0.05 was considered statistically significant.

Acknowledgments

This work was supported in part by the Targeted Proteins Research Program of the Japan Science and Technology Corporation (JST) and by the Uehara Memorial Foundation, and the Terumo Life Science Foundation.

References and notes

1. Banner, C. D.; Göttlicher, M.; Widmark, E.; Sjövall, J.; Rafter, J. J.; Gustafsson, J. A. *J. Lipid Res.* **1993**, *34*, 1583.
2. Nuclear Receptor Nomenclature Committee *Cell* **1999**, *97*, 161.
3. Willson, T. M.; Brown, P. J.; Sternbach, D. D.; Henke, B. R. *J. Med. Chem.* **2000**, *43*, 527.
4. Staels, B.; Auwerx, J. *Curr. Pharm. Des.* **1997**, *3*, 1.
5. Issemann, I.; Prince, R. A.; Tugwood, J. D.; Green, S. *J. Mol. Endocrinol.* **1993**, *11*, 37.
6. Portincasa, P.; Grattagliano, I.; Palmieri, V. O.; Palasciano, G. *Curr. Med. Chem.* **2006**, *13*, 2889.
7. London, R. M.; George *J. Clin. Liver Dis.* **2007**, *11*, 5.
8. Cuadrado, A.; Orive, A.; García-Suárez, C.; Domínguez, A.; Fernández-Escalante, J. C.; Crespo, J.; Pons-Romero, F. *Obes. Surg.* **2005**, *15*, 442.
9. Meyer, K.; Jia, Y.; Cao, W. Q.; Kashireddy, P.; Rao, M. S. *Int. J. Oncol.* **2002**, *21*, 175.
10. Roden, M. *Nat. Clin. Pract. Endocrinol. Metab.* **2006**, *2*, 335.
11. Kasuga, J.; Yamasaki, D.; Araya, Y.; Nakagawa, A.; Makishima, M.; Doi, T.; Hashimoto, Y.; Miyachi, H. *Bioorg. Med. Chem.* **2006**, *14*, 8405.
12. Kasuga, J.; Nakagome, I.; Aoyama, A.; Sako, K.; Ishizawa, M.; Ogura, M.; Makishima, M.; Hirono, S.; Hashimoto, Y.; Miyachi, H. *Bioorg. Med. Chem.* **2007**, *15*, 5177.
13. Kasuga, J.; Yamasaki, D.; Ogura, K.; Shimizu, M.; Sato, M.; Makishima, M.; Doi, T.; Hashimoto, Y.; Miyachi, H. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1110.
14. Kasuga, J.; Ishida, S.; Yamasaki, D.; Makishima, M.; Doi, T.; Hashimoto, Y.; Miyachi, H. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 65959.
15. Kasuga, J.; Ishikawa, M.; Yonehara, M.; Makishima, M.; Hashimoto, Y.; Miyachi, H. *Bioorg. Med. Chem.* **2010**, *18*, 7164.
16. Oyama, T.; Toyota, K.; Waku, T.; Hirakawa, Y.; Nagasawa, N.; Kasuga, J.; Hashimoto, Y.; Miyachi, H.; Morikawa, K. *Acta Crystallogr., Sect. D* **2009**, *65*, 786.
17. 17. The calculations were performed using the Tripos force field in SYBYL7.3.
18. Takayama, F.; Egashira, T.; Kawasaki, H.; Mankura, M.; Nakamoto, K.; Okada, S.; Mori, A. *J. Clin. Biochem. Nutr.* **2009**, *45*, 335.
19. Nakamoto, K.; Takayama, F.; Mankura, M.; Hidaka, Y.; Egashira, T.; Ogino, T.; Kawasaki, H.; Mori, A. *J. Clin. Biochem. Nutr.* **2009**, *44*, 239.
20. Day, C. D.; James, O. F. W. *Gastroenterology* **1998**, *114*, 842.
21. Tokushige, K.; Takakura, M.; Tsuchiya-Matsushita, N.; Taniai, M.; Hashimoto, E.; Shiratori, K. *J. Hepatol.* **2007**, *46*, 1104.
22. Burt, A. D.; Mutton, A.; Day, C. P. *Semin. Diagn. Pathol.* **1998**, *15*, 246.
23. Holoman, J.; Glasa, J.; Galbavy, S.; Danis, D.; Molnarova, A.; Kazar, J.; Bednarova, A.; Misianik, J. *Bratisl. Lek. Listy.* **2002**, *103*, 70.