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Design, synthesis and structure-activity relationship studies of novel partial FXR agonists for the treatment of fatty liver

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is now the most common chronic liver disease, while there is still no medicine available. Farnesoid X receptor (FXR) is considered as a potential target for the treatment of NAFLD, and there are several FXR agonists reached in clinical trials. Based on better safety, industry and academia are pursuing development of the partial FXR agonists. To extend the chemical space of existing partial FXR agonists, we performed a structure-activity relationship study based on previously reported partial agonist **1** by using bioisosteric strategy. All of these efforts resulted in the identification of novel partial FXR agonist **13**, which revealed the best agonistic activity in this series. Notably, compound **13** significantly alleviated the hepatic steatosis and hepatic function index in methionine-choline deficient (MCD) induced *db/db* mice, a classical nonalcoholic steatohepatitis (NASH) model widely used in preclinical evaluation. These results suggested that partial FXR agonist **13** might be a promising lead compound worthy further researches.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) includes a broad category ranging from simple liver steatosis to nonalcoholic steatohepatitis (NASH) and even cirrhosis [1,2]. NAFLD is closely related to other metabolic syndrome including obesity, diabetes and dyslipidemia [3–5]. NAFLD is now the most common chronic liver disease [6]. Although the morbidity of NAFLD is fast-growing, there is still no medicine available [7]. Therefore, it is urgent demand to develop effective drugs for the treatment of NAFLD. Currently, a lot of researches come from institutes and companies have focused on this field, including farnesoid X receptor (FXR) agonist and peroxisome proliferators-activated receptors agonist [8–12]. FXR, a sensor of chenodeoxycholic acid, regulates the synthesis and transport of bile acid [13,14]. FXR is highly expressed in the liver, intestine, and adipose tissue, and bile acids are the endogenous ligands of FXR [15]. The activation of FXR is related to glycometabolism, inflammation, and hepatoprotective effect [16,17]. Activation of FXR lowers the levels of triglycerides by modulating the expression of hepatic sterol regulatory element binding protein-1c and glucose-dependent lipogenic genes [18]. In addition, FXR improves glucolipid metabolism through regulation of peripheral insulin sensitivity and gluconeogenesis [19,20]. Moreover, FXR agonists reduced inflammation and fibrosis in the kidney, indicating the capacity of FXR agonists for the treatment of diabetic nephropathy and renal fibrosis [21]. Therefore, FXR is considered as a potential target for the treatment of metabolic syndrome [22,23].

There are several FXR agonists such as obeticholic acid, Cilofexor and LJN452 are studied in clinical trials for the treatment of NAFLD [23]. However, full FXR agonists may cause side effects by inhibiting cholesterol 7-hydroxylase, resulting in the interference of cholesterol metabolism [24]. The defect could be improved by partial activation of FXR to avoid total loss of cholesterol conversion [25,26]. There are a series of partial FXR agonist have been reported, exemplified by compound **1**, a candidate derived from pranlukast high potency on FXR (Fig. 1) [25]. Herein, we explore the further structure-activity relationship based on compound **1** by bioisosteric strategy to extend the chemical space of existing partial FXR agonists (Fig. 2).

2. Results and discussion

2.1. Chemistry

The compounds **2–13** were synthesized as shown in Scheme 1. Treatment of raw material **1a** with carboxylic acid **2a** under the

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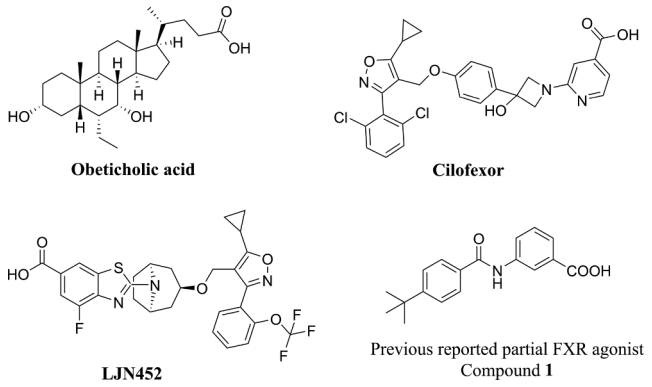


Fig. 1. Structure of representative FXR agonists.

Fig. 2. Our design strategy and structure-activity relationship study to extend the chemical space of existing partial FXR agonists.

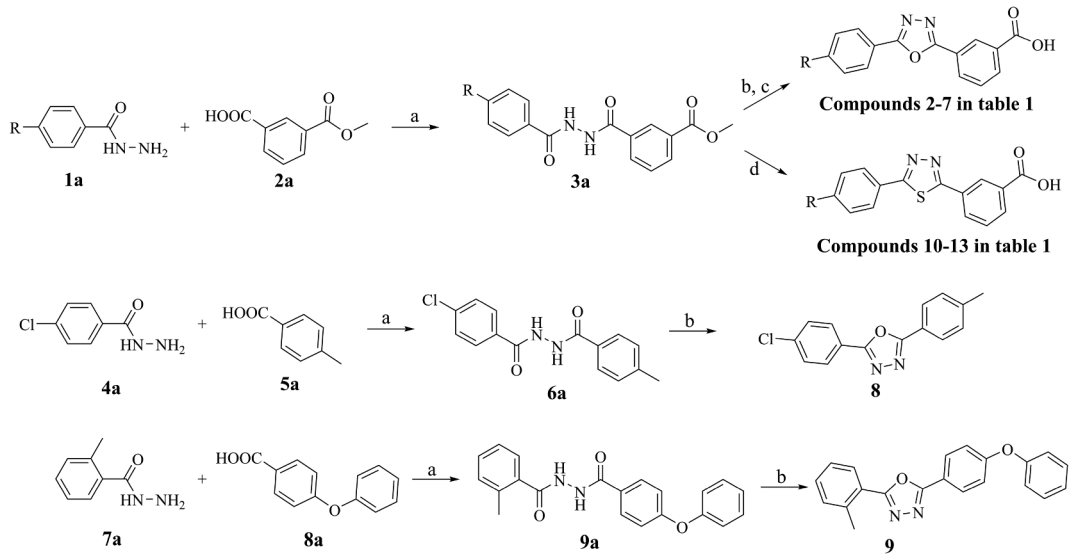
condition of condensating agent EDCI provided intermediate **3a**, which was converted into oxadiazoles ring under the dehydrating agent, followed by hydrolysis provided the target compounds **2–7**. A similar procedure provided compounds **8** and **9** with high yield. Cyclization of intermediate **3a** with Lawesson's reagent provided thiadiazoles ring, followed by hydrolysis afforded compounds **10–13**.

2.2. Structure-activity relationship study

The oxadiazole and thiadiazole rings are usually reported as the bioisosteres of amide scaffold [27]. As shown in Table 1, replacing amide of lead compound **1** with oxadiazole ring provided compound **2** with reduced potency on FXR, while maintaining the efficiency of maximal activation. To identify a better hydrophobic segment, we explored the ether substituents (compounds **3–7**) for the replacement of tertiary butyl based on previous report on amide series [25]. SAR studies have shown that large substituent (compounds **5–7**) is better than that of small substituent (compounds **3–4**), indicating that the hydrophobic interaction in this site is crucial to the agonistic activity on FXR. Among them, compound **5** revealed the best agonistic activity on FXR and keep partial agonistic efficiency. Inspired by previous research [28], we also explored the non-carboxylic acid derivatives **8** and **9**, but none of them exert potency on FXR. These results suggested that the previous SAR cannot be converted to our oxadiazole series. Notably, the thiadiazole series revealed better potencies on FXR than oxadiazole derivatives (**10** *vs* **2**, **11** *vs* **3**, **12** *vs* **4**, and **13** *vs* **5**). This difference might be attributed to the larger hydrophobic effect of thiadiazole than that of oxadiazole, which match better with hydrophobic pocket of FXR. Similar to oxadiazole series, the isobutoxy derivative **13** (EC_{50} = 142 nM, 28% max. efficiency) revealed the strongest activity on FXR.

2.3. Effects on NASH

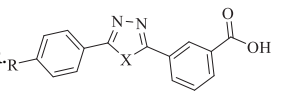
The effects of compound **13** on fatty liver were evaluated in *db/db* mice fed by MCD, a classical NASH model [29]. As shown in Fig. 3, the model group revealed significant liver steatosis, which was remarkably improved in compound **1** and **13** treated groups. The liver to brain weight ratio was significantly decreased in compound **13** treated group compared model control, which indicated that compound **13** alleviates fatty liver. Moreover, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were obviously decreased in treated groups (Fig. 3). These results suggested that partial FXR agonist **13** provides significant improvement on NASH.

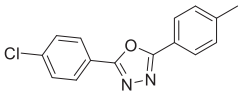


Scheme 1. Synthesis of target compounds **2–13**. Reagents and conditions: (a) EDCI, Et_3N, dichloromethane; (b) $POCl_3$, acetonitrile, reflux, 4 h; (c) $LiOH{\cdot}H_2O$, THF/MeOH/H_2O, r.t., 4 h; (d) Lawesson's reagent, THF, reflux, 4 h.

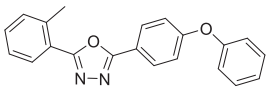
Table 1

In vitro activities of target compounds on FXR.

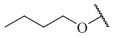
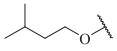




Compound 8



Compound 9

Compd.	R	X	EC_{50} (nM)[a]	max. activation
GW4064			246	100%
1			75	15%
2		O	362	23%
3	MeO	O	4560	31%
4	EtO	O	2175	19%
5		O	205	26%
6		O	328	17%
7		O	285	21%
8			> 10 μM	
9			> 10 μM	
10		S	227	14%
11	MeO	S	2058	25%
12	EtO	S	941	22%
13		S	142	28%

[a] EC_{50} value represents the mean of three determinations.

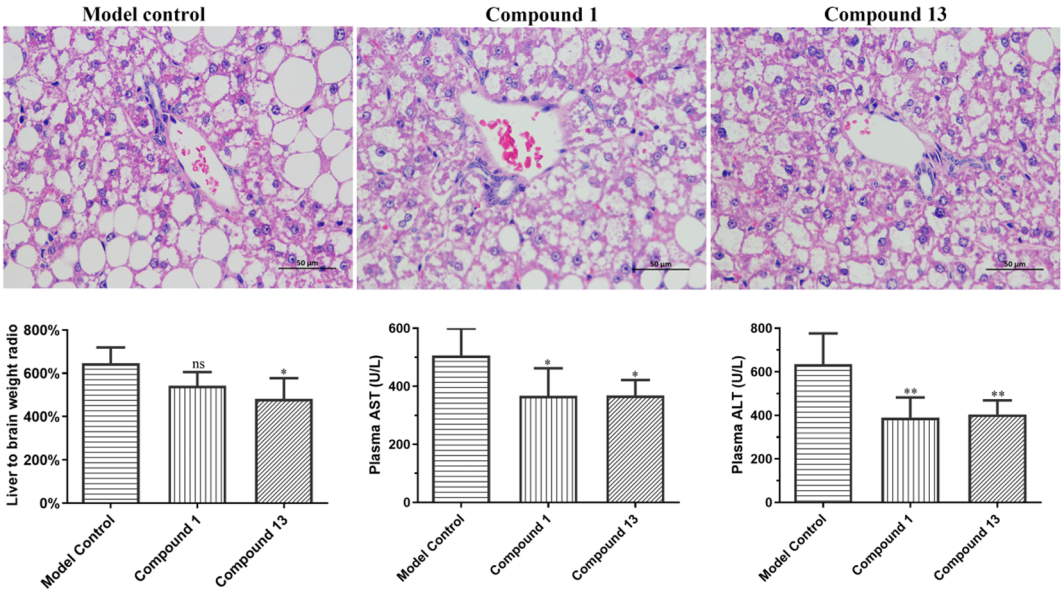


Fig. 3. Effect of compound **13** on NASH model. Representative photomicrographs of liver stained with Hematoxylin-Eosin at 400× magnification. Liver/brain-weight ratio, the plasma levels of AST and ALT. All the values are expressed as mean ± SD (n = 6). *$p \leq 0.05$ compared to model control were analyzed using a one-way ANOVA with Tukey's multiple-comparison post hoc test.

3. Conclusion

In order to extend the chemical space of existing partial FXR agonists, we performed a SAR study based on previously reported partial agonist **1** by using bioisosteric strategy. The further exploration of amide bioisosteres, oxadiazole and thiadiazole, resulted in the identification of novel partial FXR agonist **13** (EC_{50} = 142 nM, 28% max. efficiency), which revealed the best agonistic activity in this series. To evaluate the potential of compound **13** on fatty liver, a NASH model was developed by fed *db/db* mice with MCD. Notably, the hepatic steatosis, hepatic function index, and liver to brain weight ratio were significantly alleviated in compound **13** treated group. In brief, we extend the new skeleton of existing partial FXR agonist, and the optimal compound **13** was considered as a potential lead structure worth in-depth study.

4. Experimental section

4.1. General chemistry

All starting materials were obtained from commercial sources. Purifications of chromatography were performed by silica gel and detected by thin layer chromatography using UV light at 254 and 365 nm. NMR spectra were recorded on a Bruker ACF-300Q instrument

(300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR spectra) and AVANCE NEO 400MHZ instrument (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR spectra). Chemical shifts (δ) are reported in ppm relative to tetramethylsilane (TMS) as reference; multiplicity: s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet; approximate coupling constants (J) are shown in hertz (Hz). High resolution mass spectrometry (HRMS) was performed on AB SCIEX X500R system.

*4.1.1. General synthetic procedure for intermediates **3a, 6a** and **9a***

The substituted benzoic acid (1 equiv) and EDCI (1.2 equiv) were dissolved in dichloromethane, and stirred for 30 min. To a stirred above solution added substituted benzoyl hydrazine (1 equiv) and Et_3N (2 equiv). The mixture was stirred at room temperature for 12 h. The organic layers were washed with brine (25 mL), dried over anhydrous sodium sulfate and filtered. The filtrate was concentrated to give intermediates **3a**, **6a** and **9a**, which was used for the next reaction without further purification.

*4.1.2. General synthetic procedure for target compounds **2–7***

To a solution of intermediates **3a** (1 equiv) in 15 mL acetonitrile was added $POCl_3$ (2 equiv) at ambient temperature. After addition was complete, the solution was heated to reflux for 4 h. The reaction mixture was concentrated in vacuo, then diluted with ethyl acetate (20 mL), and washed with water (2 × 20 mL), $NaHCO_3$ (2 × 25 mL) and brine (2 × 25 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The filtrate was evaporated and the residue was purified by silica gel column chromatography (petroleum ether/ethyl acetate, 10:1, v/v). To a solution of the obtained solid (1 equiv) in 2:3:1 THF/MeOH/H_2O (18 mL) was added LiOH·H_2O (3 equiv). After stirring at room temperature for 4 h, the volatiles were removed under reduced pressure. The residue was acidified with 1 N hydrochloric acid solution and then filtered and the filter cake was washed with 5 mL of cool water, dried in vacuum to afford a white powder. The white powder was purified by column chromatography using a mixture of petroleum ether/ethyl acetate (2:1–1:2, v/v) as eluent to afford the target compounds as white solid.

*4.1.2.1. 3-(5-(4-(tert-butyl)phenyl)-1,3,4-oxadiazol-2-yl)benzoic acid **(2)***. Yield 42%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.65 – 8.57 (m, 1H), 8.33 (d, J = 7.6 Hz, 1H), 8.17 (d, J = 7.4 Hz, 1H), 8.09–7.99 (m, 2H), 7.82–7.72 (m, 1H), 7.70–7.58 (m, 2H), 1.32 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 166.83, 164.73, 163.71, 155.56, 132.84, 132.64, 131.01, 130.40, 127.54, 127.09, 126.68, 124.28, 120.97, 35.29, 31.26. HRMS Anal. Calcd. for $C_{19}H_{18}N_2O_3$: m/z = 321.1317, $[M-H]^-$, found: m/z = 321.1315 $[M-H]^-$.

*4.1.2.2. 3-(5-(4-methoxyphenyl)-1,3,4-oxadiazol-2-yl)benzoic acid **(3)***. Yield 35%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.64–8.58 (m, 1H), 8.31 (d, J = 8.1 Hz, 1H), 8.15 (d, J = 8.2 Hz, 1H), 8.05 (d, J = 8.7 Hz, 2H), 7.82–7.74 (m, 1H), 7.14 (d, J = 8.7 Hz, 2H), 3.85 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 166.82, 164.62, 163.37, 162.59, 132.72, 132.44, 130.95, 130.38, 129.07, 127.42, 124.30, 115.92, 115.29, 55.98. HRMS Anal. Calcd. for $C_{16}H_{12}N_2O_4$: m/z = 295.0797, $[M-H]^-$, found: m/z = 295.0794 $[M-H]^-$.

*4.1.2.3. 3-(5-(4-ethoxyphenyl)-1,3,4-oxadiazol-2-yl)benzoic acid **(4)***. Yield 38%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.54–8.51 (m, 1H), 8.27 (d, J = 7.9 Hz, 1H), 8.13 (d, J = 7.8 Hz, 1H), 8.02–7.96 (m, 2H), 7.76–7.70 (m, 1H), 7.10–7.06 (m, 2H), 4.07 (d, J = 7.0 Hz, 2H), 1.33 (t, J = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.86, 164.61, 163.34, 161.88, 132.64, 131.09, 130.85, 130.36, 129.05, 127.40, 124.25, 115.64, 64.01, 14.94. HRMS Anal. Calcd. for $C_{17}H_{14}N_2O_4$: m/z = 309.0954, $[M-H]^-$, found: m/z = 309.0956 $[M-H]^-$.

*4.1.2.4. 3-(5-(4-ethoxyphenyl)-1, 3, 4-oxadiazol-2-yl)benzoic acid **(5)***. Yield 29%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.56–8.52 (m, 1H), 8.29 (d, J = 7.9 Hz, 1H), 8.14 (d, J = 7.9 Hz, 1H), 8.02–7.99 (m, 2H), 7.77–7.71 (m, 1H), 7.12–7.08 (m, 2H), 3.80 (d, J = 6.6 Hz, 2H), 2.05–1.97 (m, 1H), 0.97 (d, J = 6.8 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 166.76, 164.62, 163.31, 162.14, 132.68, 132.40, 130.94, 130.35, 129.03, 127.41, 124.33, 115.70, 74.45, 28.10, 19.39. HRMS Anal. Calcd. for $C_{19}H_{18}N_2O_4$: m/z = 337.1267, $[M-H]^-$, found: m/z = 337.1264 $[M-H]^-$.

*4.1.2.5. 3-(5-(4-butoxyphenyl)-1,3,4-oxadiazol-2-yl)benzoic acid **(6)***. Yield 25%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.57–8.51 (m, 1H), 8.32 (d, J = 7.8 Hz, 1H), 8.16 (d, J = 7.8 Hz, 1H), 8.03 (d, J = 8.6 Hz, 2H), 7.77–7.71 (m, 1H), 7.13 (d, J = 8.6 Hz, 2H), 4.05 (t, J = 6.4 Hz, 2H), 1.76–1.71 (m, 2H), 1.46–1.42 (m, 2H), 0.93 (t, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.85, 164.66, 162.09, 132.75, 132.41, 131.02, 130.42, 129.09, 127.45, 124.40, 115.71, 68.06, 31.06, 19.15, 14.16. HRMS Anal. Calcd. for $C_{19}H_{18}N_2O_4$: m/z = 337.1267, $[M-H]^-$, found: m/z = 337.1269 $[M-H]^-$.

*4.1.2.6. 3-(5-(4-(isopentyloxy)phenyl)-1,3,4-oxadiazol-2-yl)benzoic acid **(7)***. Yield 28%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.57–8.51 (m, 1H), 8.35–8.31 (m, 1H), 8.18–8.15 (m, 1H), 8.05 (d, J = 8.7 Hz, 2H), 7.78–7.72 (m, 1H), 7.15 (d, J = 8.7 Hz, 2H), 4.08 (t, J = 6.6 Hz, 2H), 1.82–1.74 (m, 1H), 1.64 (q, J = 6.7 Hz, 2H), 0.94 (d, J = 6.6 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 166.87, 164.70, 163.38, 132.37, 132.37, 130.46, 129.12, 127.46, 124.41, 115.77, 66.82, 37.72, 25.03, 22.89. HRMS Anal. Calcd. for $C_{20}H_{20}N_2O_4$: m/z = 351.1423, $[M-H]^-$, found: m/z = 351.1426 $[M-H]^-$.

*4.1.2.7. 2-(4-chlorophenyl)-5-(o-tolyl)-1,3,4-oxadiazole **(8)***. Yield 57%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.14 (d, J = 7.8 Hz, 2H), 8.03 (d, J = 8.0 Hz, 2H), 7.71 (d, J = 8.0 Hz, 2H), 7.45 (d, J = 7.8 Hz, 2H), 2.42 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 165.00, 136.95, 133.26, 132.45, 131.46, 129.48, 129.31, 128.93, 120.95, 20.99. HRMS Anal. Calcd. for $C_{15}H_{11}ClN_2O$: m/z = 269.0560, $[M-H]^-$, found: m/z = 269.0563 $[M-H]^-$.

*4.1.2.8. 2-(4-phenoxyphenyl)-5-(o-tolyl)-1, 3, 4-oxadiazole **(9)***. Yield 41%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.13 (d, J = 8.7 Hz, 2H), 8.06 (d, J = 7.5 Hz, 1H), 7.54–7.49 (m, 5H), 7.27 (d, J = 7.3 Hz, 1H), 7.21 (d, J = 3.1 Hz, 1H), 7.20–7.17 (m, 2H), 7.15 (d, J = 1.8 Hz, 1H), 2.70 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.49, 163.70, 160.61, 155.61, 138.11, 132.25, 131.93, 130.84, 129.41, 129.32, 126.97, 125.17, 122.99, 120.31, 118.83, 118.44, 22.04. HRMS Anal. Calcd. for $C_{21}H_{16}N_2O_2$: m/z = 327.1212, $[M-H]^-$, found: m/z = 327.1215 $[M-H]^-$.

*4.1.3. General synthetic procedure for target compounds **10–13***

To a solution of **3a** (1 equiv) in 20 mL THF was added Lawesson's reagent (2 equiv), and the mixture was heated to reflux for 4 h. The reaction mixture was concentrated in vacuo, then diluted with ethyl acetate (25 mL), and washed with 1 N $NaHCO_3$ (3 × 15 mL) and brine (2 × 15 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (petroleum ether/ethyl acetate, 20:1, v/v). To a solution of the obtained solid (1 equiv) in 2:3:1 THF/MeOH/H_2O (18 mL) was added LiOH·H_2O (3 equiv). After stirring at room temperature for 4 h, the volatiles were removed under reduced pressure. The residue was acidified with 1 N hydrochloric acid solution and then filtered and the filter cake was washed with 5 mL of cool water, dried in vacuum to afford a white powder. The white powder was purified by column chromatography using a mixture of petroleum ether/ethyl acetate (2:1–1:2, v/v) as eluent to afford the target compounds as white solid.

4.1.3.1. 3-(5-(4-(tert-butyl)phenyl)-1,3,4-thiadiazol-2-yl)benzoic acid

(10). Yield 45%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.55–8.51 (m, 1H), 8.23 (d, J = 8.0 Hz, 1H), 8.13 (d, J = 7.8 Hz, 1H), 7.94 (d, J = 8.1 Hz, 2H), 7.76–7.71 (m, 1H), 7.59 (d, J = 8.1 Hz, 2H), 1.32 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.46, 167.12, 167.08, 154.97, 133.19, 132.29, 131.84, 130.38, 130.23, 128.46, 127.99, 127.14, 126.78, 35.21, 31.27. HRMS Anal. Calcd. for $C_{19}H_{18}N_2O_2S$: m/z = 337.1089, $[M-H]^-$, found: m/z = 337.1086 $[M-H]^-$.

4.1.3.2. 3-(5-(4-methoxyphenyl)-1,3,4-thiadiazol-2-yl)benzoic acid ***(11)***. Yield 45%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.53–8.47 (m, 1H), 8.20 (d, J = 7.8 Hz, 1H), 8.12 (d, J = 7.8 Hz, 1H), 7.99–7.90 (m, 2H), 7.76–7.71 (m, 1H), 7.17–7.05 (m, 2H), 3.84 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.31, 166.91, 162.22, 132.37, 132.05, 130.45, 130.36, 129.82, 128.29, 122.28, 115.31, 55.94. HRMS Anal. Calcd. for $C_{16}H_{12}N_2O_3S$: m/z = 311.0569, $[M-H]^-$, found: m/z = 311.0566 $[M-H]^-$.

4.1.3.3. 3-(5-(4-ethoxyphenyl)-1,3,4-thiadiazol-2-yl)benzoic acid ***(12)***. Yield 40%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.53–8.47 (m, 1H), 8.19 (d, J = 7.7 Hz, 1H), 8.11 (d, J = 7.6 Hz, 1H), 7.91 (d, J = 8.4 Hz, 2H), 7.76–7.70 (m, 1H), 7.06 (d, J = 8.4 Hz, 2H), 4.08 (q, J = 6.9 Hz, 2H), 1.35 (t, J = 7.0 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.30, 166.93, 161.51, 132.39, 132.13, 132.03, 130.40, 129.79, 128.31, 122.13, 115.66, 63.95, 14.95. HRMS Anal. Calcd. for $C_{17}H_{14}N_2O_3S$: m/z = 325.0725, $[M-H]^-$, found: m/z = 325.0722 $[M-H]^-$.

4.1.3.4. 3-(5-(4-isobutoxyphenyl)-1,3,4-thiadiazol-2-yl)benzoic acid ***(13)***. Yield 36%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.54–8.46 (m, 1H), 8.20 (d, J = 7.7 Hz, 1H), 8.12 (d, J = 7.7 Hz, 1H), 7.93 (d, J = 8.3 Hz, 2H), 7.76–7.70 (m, 1H), 7.08 (d, J = 8.3 Hz, 2H), 3.81 (d, J = 6.5 Hz, 2H), 2.11–1.96 (m, 1H), 0.99 (d, J = 6.6 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.32, 166.91, 166.38, 161.78, 132.40, 132.13, 132.06, 130.43, 129.81, 128.32, 122.17, 115.77, 74.44, 28.10, 19.42. HRMS Anal. Calcd. for $C_{19}H_{18}N_2O_3S$: m/z = 353.1038, $[M-H]^-$, found: m/z = 353.1035 $[M-H]^-$.

4.2. Evaluation for FXR

The human BSEP proximal promoter was amplified from a BAC clone containing the upstream genomic region of human BSEP and cloned into the pGL3 Basic vector create a BSEP promoter-reporter plasmid (named pGL3-hBSEP-luc). HEK293T cells were transfected with pGL3-hBSEP-luc and expression vectors encoding human FXR (NR1H4) using Fugene 6 (Promega) according to manufacturer instructions. Following transfection, test compounds were added in a 10 point dose response and cells were incubated at 37 °C with 5% CO_2 incubator for 24 h. Promoter activity was determined by using Steady-Glo reagent (Promega). The maximum efficacy of the positive control agonist GW4064 is arbitrarily set at 100%.

4.3. Animals

Eight weeks old *db/db* mice were purchased from Model Animal Research Center of Nanjing University (Jiangsu, China). All animals were acclimatized for 1 week before the experiments. The animal room was maintained under a constant 12 h light/black cycle with the temperature at 23 ± 2 °C and relative humidity 50 ± 10% throughout the experimental period. Mice were allowed ad libitum access to standard pellets and water unless otherwise stated, and the vehicle used for drug administration was 0.5% sodium salt of Carboxy Methyl Cellulose aqueous solution for all animal studies. The methionine-choline deficient (MCD) diet was purchased from Nantong Trofi Feed Technology Co., Ltd. All animal experimental protocols were approved by the ethical committee at Yancheng Teachers' University and adhered to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication NO. 85-23, revised 2011).

4.4. Effects of compound ***13*** *on NASH*

db/db mice were randomly divided into groups (n = 6 per group), and fed the MCD in parallel with daily oral gavage with vehicle, compound **1** (30 mg/kg) or compound **13** (30 mg/kg). After treatment for 40 days, mice were fasted overnight and sacrificed under deep anesthesia. Liver and serum samples were collected and processed for histological and serological analysis. Serum levels of ALT and AST were measured by automatic biochemical analyzer (Beckman Coulter, AU5811, Tokyo, Japan). Liver tissue was fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) staining according to a standard procedure.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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