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Lipid accumulation inhibitory activities of novel isoxazole-based chenodeoxycholic acids: Design, synthesis and preliminary mechanism study

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ABSTRACT

In continuation of our drug discovery program on hyperlipidemia, a series of novel isoxazole-chenodeoxycholic acid hybrids were designed, synthesized and evaluated for their lipid-lowering effects. Preliminary screening of all the synthesized compounds was done by using a 3T3-L1 adipocyte model, in which the most active compound **16b** could significantly reduce the lipid accumulation up to 30.5% at a nontoxic concentration 10 μM. Further mechanism studies revealed that **16b** blocked lipid accumulation via activating FXR-SHP signaling pathway, efficiently down-regulated the expression of key lipogenesis regulator SREBP-1c.

Despite significant advances in the therapeutic management of cardiovascular disease (CVD), it still remains the number one killer in the developed countries. Hyperlipidemia, an abnormality in lipid homeostasis, is a major risk factor for progression of atherosclerotic disease which may account for up to 55% of CVD risk after correction for age and gender.[1] It is one of the most prevalent diseases, leading to considerable financial and social burden worldwide.[2] Present treatment of hyperlipidemia comprises of various classes of drugs which chiefly include statins, fibrates, niacin, cholesterol absorption inhibitors, bile acid sequestrants and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors.[3] Although there are many hypolipidemic drugs used to reduce cholesterol as well as triglycerides in the blood, many patients still exist residual cardiovascular risk or experience serious treatment side effects.[4] Therefore, it is still imperative to search for more active and less toxic agents.

Bile acids (BAs) are a class of cholesterol derived, amphipathic molecules that have early been recognized to aid in the transport, digestion and absorption of nutrients, fat and vitamins. Long before the era of modern medicine, traditional Chinese medicine recognized the therapeutic value of animal biles whose main component are BAs.[5] In 1999, the breakthrough discovery of BAs as the endogenous ligand of farnesoid X receptor (FXR) brought a new life to BAs.[6–8] FXR alters the transcription of several genes involved in fatty acid, triglyceride and lipoprotein metabolism, and administration of FXR agonists to rats reduces plasma triglyceride level.[9] The main mechanism of inhibition of triglyceride synthesis by FXR is inhibition of expression of the transcription factor sterol-regulatory-element-binding protein-1c (SREBP-1c) mediated by a signaling cascade that involves small heterodimer partner (SHP).[10] Chenodeoxycholic acid (CDCA), the most potent FXR endogenous ligand, used for the treatment of patients with cholesterol gallstone disease decreased plasma triglyceride level and was therefore suggested as a drug for the treatment of hypertriglyceridemia.[11–13]

Isoxazole, a five-membered ring containing oxygen and nitrogen atoms, have been reported to have many biological activities including antimicrobial,[14] antiviral,[15] anticancer,[16] anti-inflammatory,[17] immunomodulatory,[18] anticonvulsant,[19] antihyperlipidemic[20] and antidiabetic[21] properties. A series of non-steroidal FXR agonists like GW4064 and LY2562175 which contain isoxazole moiety possessed robust lipid modulating properties.[22,23] Furthermore, due to its easy synthesis and low cytotoxicity, isoxazole is a very popular fragment for the development of new agents with variable biological activities.[24]

In the design of new drugs, the development of hybrid molecules through the combination of different pharmacophores in one frame may possess interesting biological activities.[25,26] And combination endogenous metabolic products may provide a new molecule with less toxicity and better bioavailability. Inspired by this approach, we herein

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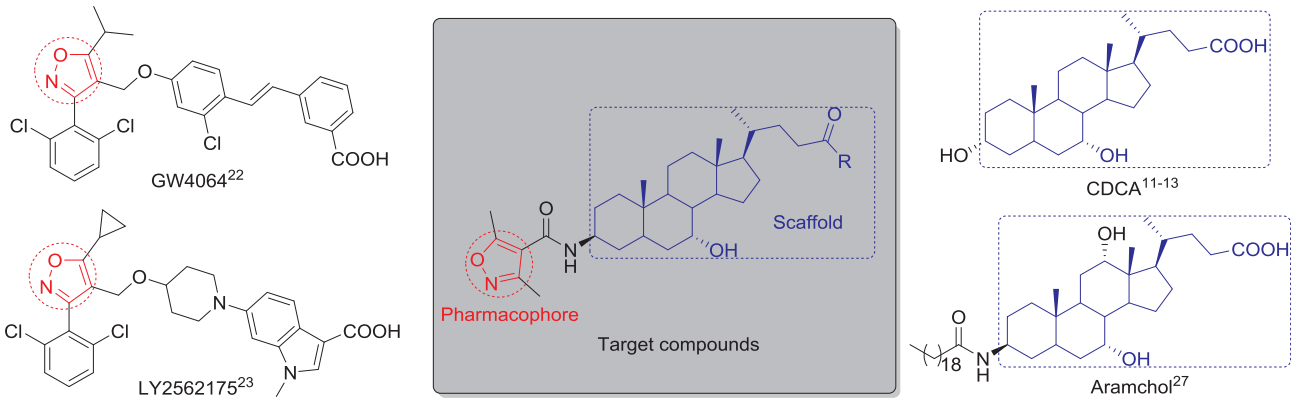
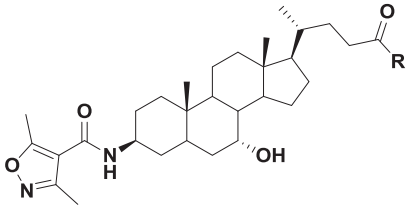
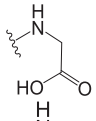
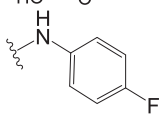
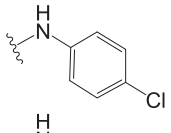
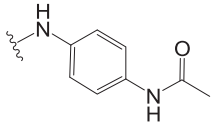
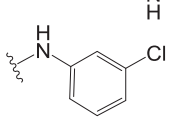


Fig. 1. Chemical structure of some potent lipid-lowering agents comprising isoxazole or BAs and general structure of our designed hybrids. (See above mentioned references for further information.)

Table 1
Structures of the designed compounds.



Compounds	R	Compounds	R
14		**16d**	
15		**16e**	
16a		**16f**	
16b		**16g**	
16c		**16h**	

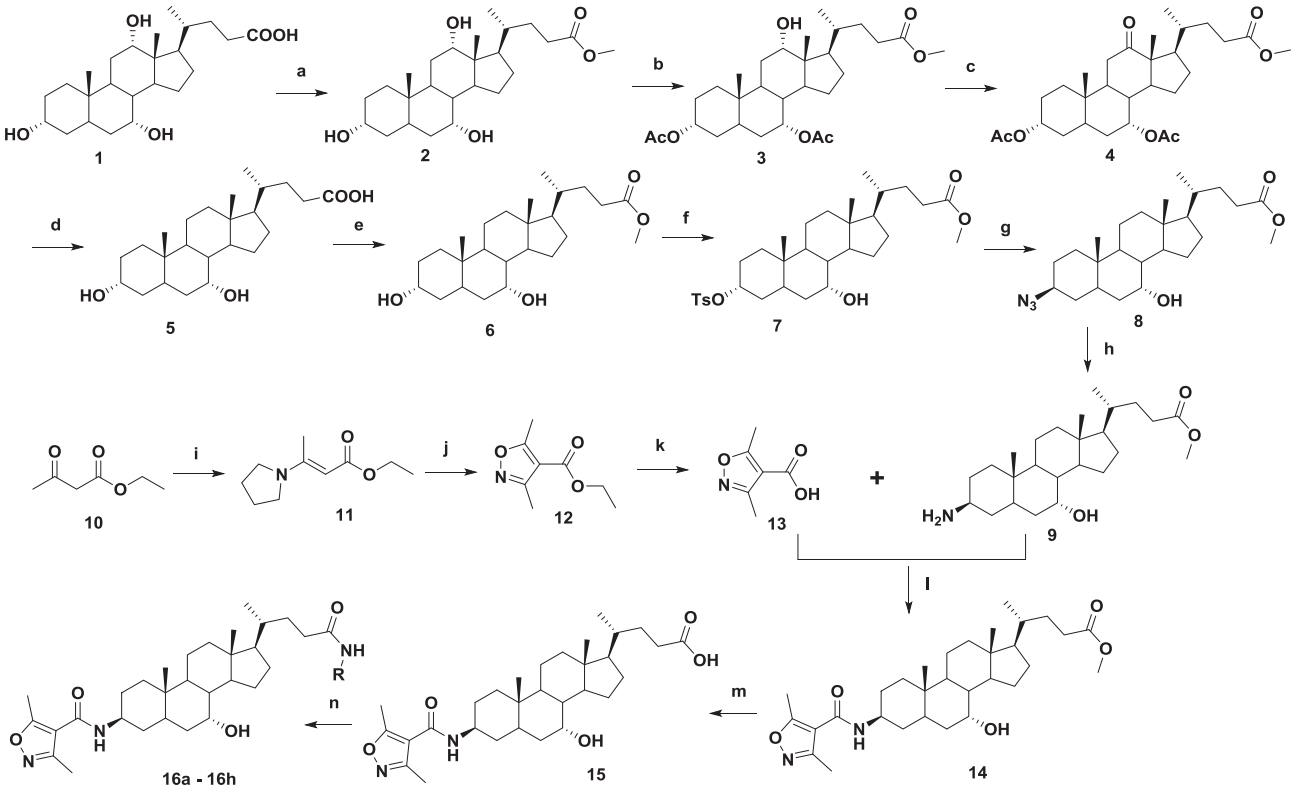
designed and synthesized a series of novel hybrids by a combination of isoxazole and CDCA, and evaluated them for their lipid-lowering activity (Fig. 1). The isoxazole pharmacophore was linked to the C-3 position of CDCA scaffold by amide bond which is widely existed in compounds used to treat the metabolic syndrome such as Aramchol.[27] In order to explore further the chemical space, we manipulated the side chain at C-24 carboxylic acid moiety, producing the small library reported in Table 1.

The preparation of designed compounds (Table 1) was depicted in Scheme 1. As described in patent US9206220, CDCA (**5**) was obtained by methyl esterification, acetylation, oxidation and Wolff-Kishner-Huang reaction from CA (**1**).[28] Esterification of CDCA (**5**) with methanol, followed by sulfonylation with 4-tosyl chloride gave the sulfonate **7**. Treatment of **7** with NaN_3 gave **8** which was further reduced by PPh_3 to provide **9**. Isoxazole fragment **13** was prepared as follows: ethyl acetoacetate **10** was reacted with pyrrole to give **11**; cyclization reaction of **11** with nitroethane under the catalysis of phosphorus oxychloride, followed by ester hydrolysis afforded intermediate **13**. Amide **14** was obtained from **9** and **13** via condensation using DCC. After hydrolysis of **14** to the corresponding **15** with LiOH, target compounds **16a–16h** were obtained from **15** by reaction with EDCI and amines. The structures of all the target compounds were confirmed by ^{1}H NMR, ^{13}C NMR and HRMS. Details of the synthetic procedures and structural characterizations were described in the supplementary material.

It's well known that 3T3-L1 cell-based method has been shown to be an efficacious tool for screening beneficial compounds with lipid-lowering effects.[29] To avoid the impact of cytotoxicity on the lipid accumulation experiment using Oil Red O staining, our compounds were first tested for cell viability in 3T3-L1 cells. 3T3-L1 cells were treated with various concentrations (5, 10, 20, and 40 μM) of target compounds and CDCA for 48 h. Then, the cell viability was measured using MTT assays. As shown in Fig. 2, all of the tested compounds did not show significant cytotoxicity to the 3T3-L1 cells even at 40 μM, except compound **16g**. Due to all of the compounds did not obviously affect cell viability at 10 μM, the nontoxic concentrations of compounds selected for further biological investigation were at 10 μM.

3T3-L1 preadipocytes were differentiated for 6 days into mature adipocytes in accordance with the procedure which has been previously reported by our group.[30] To assess the effect of target compounds on lipid accumulation in 3T3-L1 adipocytes, Oil Red O staining and its subsequent quantification were performed to observe the intracellular lipid accumulation. As shown in Fig. 3A, 3T3-L1 preadipocyte have a fibroblast-like morphology. In the microscopic observation of Oil Red O staining in 3T3-L1 adipocytes, we found that a large number of lipid droplets were stained red (Fig. 3B). The biological evaluation results were depicted in Fig. 3C. All of the 10 hybrids decreased lipid accumulation and exhibited equal or greater activities than CDCA in 3T3-L1 adipocytes at 10 μM. Bulk amide group appeared to be unfavorable to inhibitory on lipid accumulation. Compound **16b** with N-methyl amide group showed the highest level of reduction in lipid accumulation, which reduced 30.5% lipid accumulation at 10 μM ($^*p < 0.05$).

Highly expressed in hepatocyte and adipose tissue, SREBP-1c stimulates the expression of several lipogenic proteins, including acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD-1).[31] FXR activation suppresses the activity of SREBP-1c through FXR-SHP-SREBP-1c signaling pathway.[10] To uncover the molecular mechanisms by which our compounds inhibited the lipid accumulation, we examined whether the FXR signaling pathway was influenced by **16b** in HepG2 cells.[32] We chose GW4064 as positive control which has been successfully utilized as a research tool for FXR.[22] At first, GW4064 and **16b** were tested for cell viability in HepG2 cells. HepG2 cells were treated with various concentrations (5, 10, 20,



Scheme 1. Synthesis of **14**, **15** and **16a–16h**. Reagents and conditions: (a) Conc HCl, CH_3OH, reflux; (b) Ac_2O, DMAP, Et_3N, rt; (c) NaClO, AcOH, EA, 40 °C; (d) $N_2H_4{\cdot}H_2O$, KOH, triethyleneglycol, 120 °C, 2 h; then, 210 °C, 3 h; (e) Conc HCl, CH_3OH, reflux; (f) p-TsCl, Py, rt; (g) NaN_3, DMF, 60 °C; (h) PPh_3, THF, H_2O, reflux; (i) Pyrrole, $PhCH_3$, reflux; (j) $C_2H_5NO_2$, $POCl_3$, Et_3N, $CHCl_3$, rt; (k) NaOH, H_2O, CH_3OH, rt; (l) DCC, HOBT, THF, rt, N_2; (m) LiOH, H_2O, CH_3OH, rt; (n) RNH_2, EDCI, HOBT, DMF or $ClCO_2Et$, Et_3N, then RNH_2, rt.

40 and 80 μM) of corresponding compounds for 48 h. Then, the cell viability was measured using MTT assays. As shown in Fig. 4A, it was very obvious that GW4064 and **16b** did not show cytotoxicity to the HepG2 cells at 10 μM. Therefore, the concentration of the compounds selected for real-time PCR experiment was 10 μM. Similar to GW4064, **16b** treatment markedly increased the expression of FXR and SHP, while down-regulated the expression of SREBP-1c at 10 μM (Fig. 4B). These results indicated that **16b** inhibited lipid accumulation in 3T3-L1 adipocytes through FXR-SHP-SREBP-1c signaling pathway.

To help rationalize the effect of synthesized compounds on FXR-SHP-SREBP-1c signaling pathway, Discovery Studio 3.0/CDOCKER was used to determine the orientation of compounds bound in the active site of FXR (PDB ID: 3DCT) and the results of these studies were listed in Table 2. Results showed that the compounds **16b** bound effectively into the active site of FXR with minimal CDOCKER_ENERGY (ΔG) 2.62 kcal/mol as with reference to CDCA (ΔG) 9.54 kcal/mol (Table 2), but was much larger than GW4064 (ΔG) −61.08 kcal/mol. Compounds with bulk amide group like **16 g** (ΔG = 48.89 kcal/mol) appeared to be unfavorable to FXR binding compared to **16b** (ΔG = 2.62 kcal/mol). Molecular docking results concluded that interaction and environment at the binding site of FXR were more favorable for **16b** as compared to other synthesized compounds, which were consistent with the results of

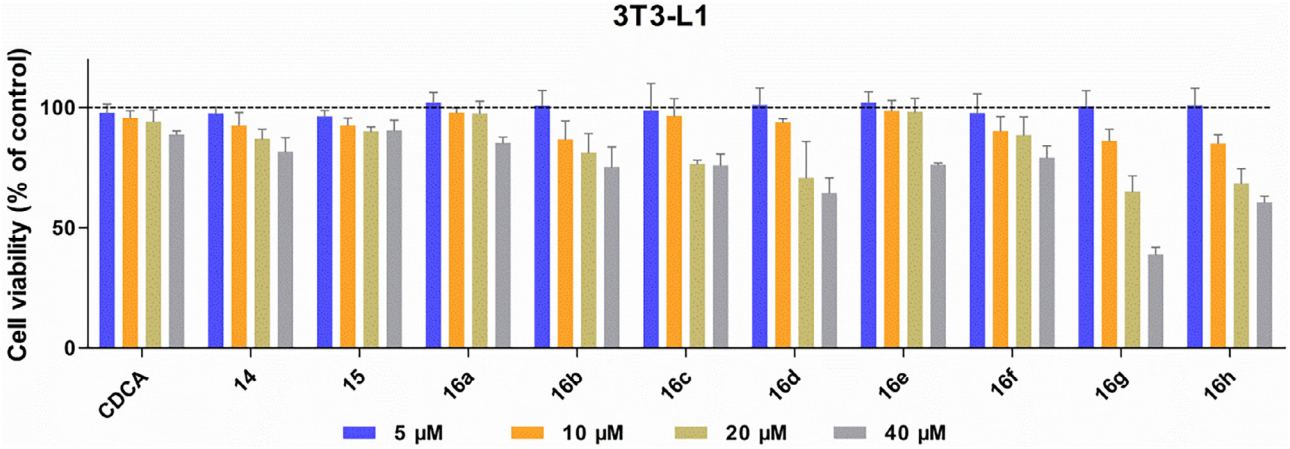


Fig. 2. The effect of synthesized compounds on cell viability in 3T3-L1 preadipocytes. The cells were treated with various concentrations (5, 10, 20, and 40 μM) of synthesized compounds and CDCA or control (1% DMSO) for 48 h, respectively. The results were expressed as the mean ± SD of two independent experiments.

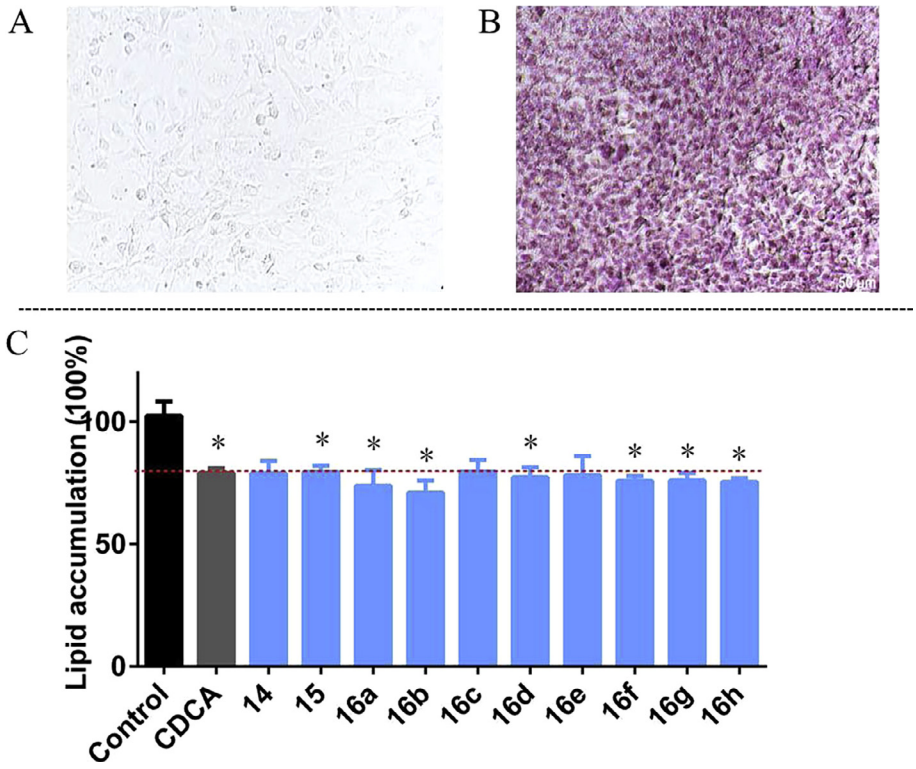


Fig. 3. (A) Microscopic images of 3T3-L1 preadipocyte. (B) Microscopic images of 3T3-L1 adipocytes stained with Oil Red O. (C) Screening of compounds inhibition on lipid accumulation in 3T3-L1 adipocytes at 10 μM. The results were expressed as the mean ± SD of two independent experiments ($^{*}p < 0.05$ vs vehicle).

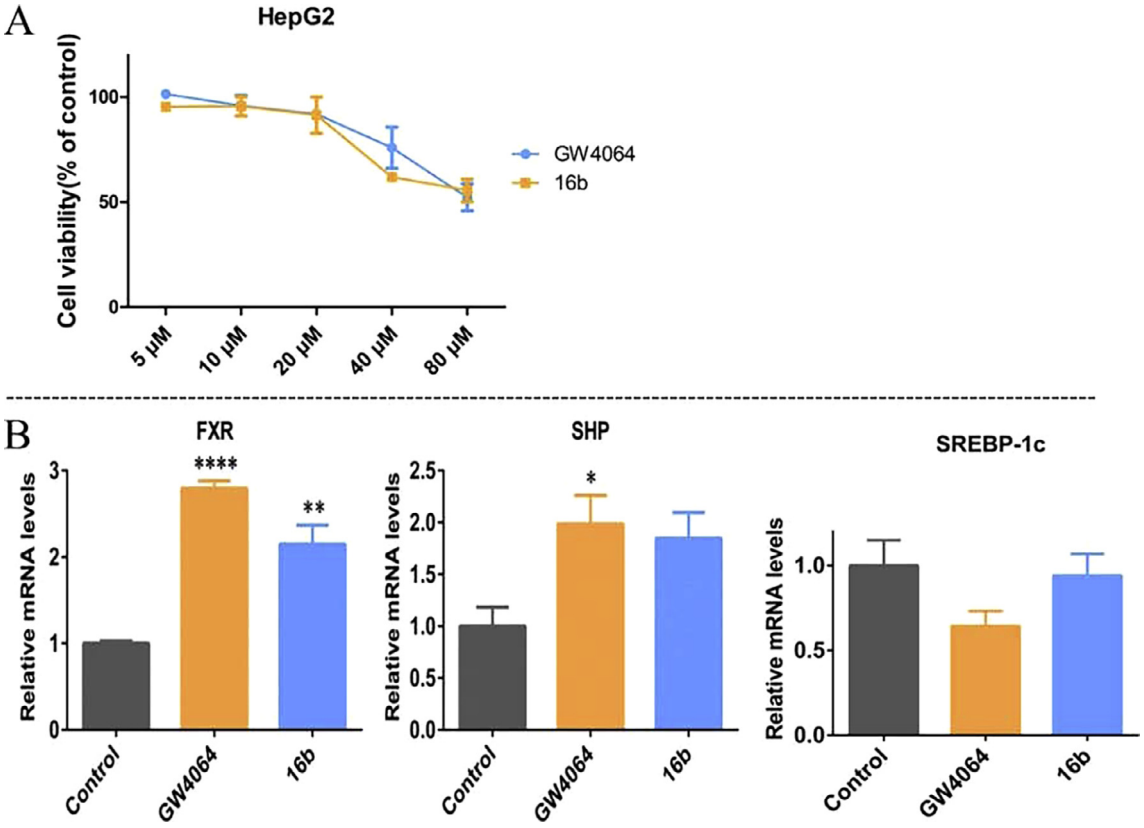
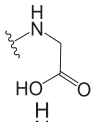
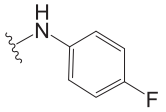
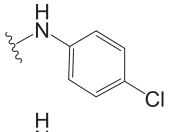
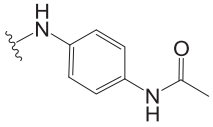
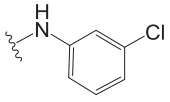


Fig. 4. (A) The effect of GW4064 and **16b** on cell viability in HepG2 cells. (B) Real-time PCR analysis of mRNA expression of FXR-SHP-SREBP-1c in HepG2 cells treated with compound **16b** and GW4064 at 10 μM. The results were expressed as the mean ± SEM (n = 3). ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs vehicle (0.1%DMSO)).

Table 2
Docking energy of FXR with compounds.

Compounds	R	CDOCKER_ENERGY
14		3.94
15		5.19
16a		16.05
16b		2.62
16c		2.78
16d		4.67
16e		38.17
16f		27.65
16g		48.89
16h		35.82
CDCA	–	9.54
GW4064	–	−61.10

lipid accumulation experiment.

The binding mode of **16b** in the active site of FXR was shown in Fig. 5. An excellent superimposition of compound **16b** over the structure of GW4064 was observed in the binding pocket of FXR, suggested that the designed compounds exhibited the similar binding patterns as that of GW4064 (Fig. 5A). The docking model of **16b** in Fig. 5B showed that the isoxazole moiety appeared to make a hydrogen bond with Thr288 and Pi-Pi interaction with Phe284. Moreover, the terminal amide formed the essential hydrogen bonds interaction with Arg331

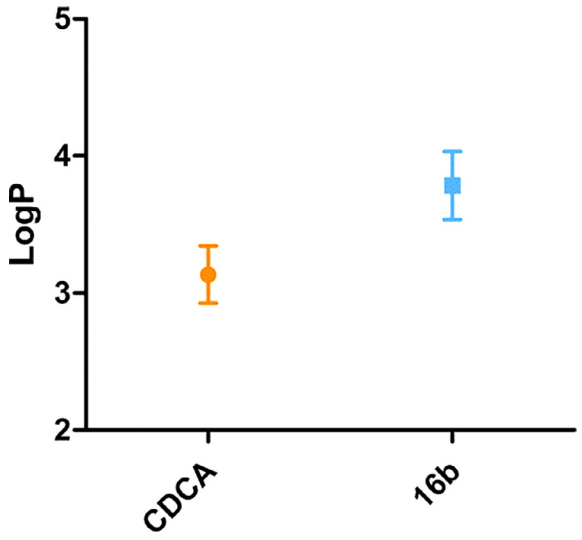


Fig. 6. Partition coefficients of compound **16b** and CDCA. The partition coefficient represents $P = C_o/C_w$ of the compound between octanol (o) and water (w). The results were expressed as the mean ± SEM (n = 3).

which play important roles in the binding to FXR.[34] These interactions were postulated to be the primary reason for the **16b** activity.

Oil-water partition coefficient (P) is an important standard to estimate the druglike physical properties of compounds, which was measured by the distribution between octanol and water. The concentrations of compounds in each phase were measured by HPLC and partition coefficient was characterized by log P = log (C_o/C_w).[33] As shown in Fig. 6, compound **16b** displayed satisfied physical property (log P = 3.78), similar to the endogenous product CDCA (log P = 3.13).

In this study, we successfully designed and synthesized a series of isoxazole-CDCA hybrids. Screening of all the synthesized compounds by using a 3T3-L1 adipocyte model led to identify compound **16b** as a potent blocker of lipid accumulation in 3T3-L1 adipocytes at a nontoxic concentration 10 μM. Further mechanism studies revealed that **16b** reduced lipid accumulation via activating FXR-SHP signaling pathway, efficiently down-regulated the expression of key lipogenesis gene SREBP-1c. Moreover, **16b** displayed satisfied lipophilicity in a preliminary study. In summary, our work revealed a new hybrid molecule with therapeutic effects and pharmacological activities on hyperlipidemia.

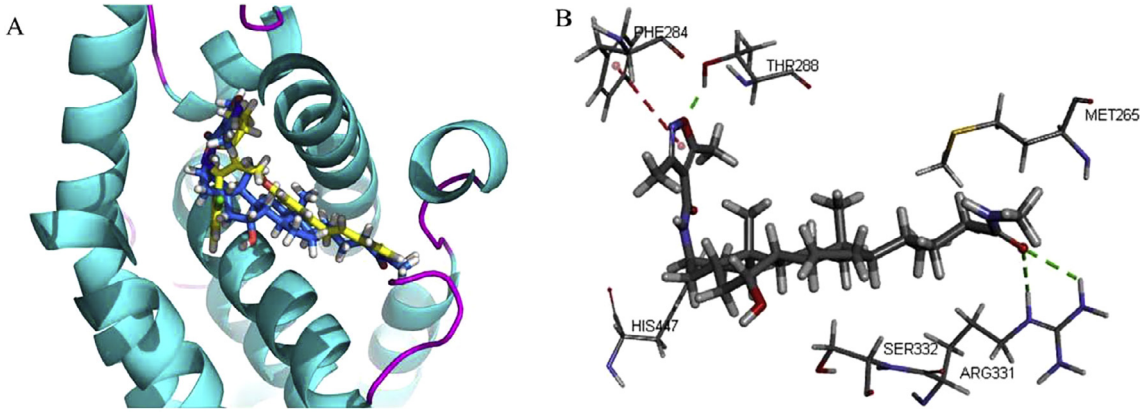


Fig. 5. (A) Superimposed poses of **16b** (blue) with representative derivatives GW4064 (yellow) binding to FXR. (B) Detailed interaction of **16b** with FXR. Green dashed lines indicate potential hydrogen bonds to key residues and red dashed lines indicate Pi-Pi interaction.

Acknowledgments

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A. Supplementary data

Supplementary data (the ^{1}H NMR, ^{13}C NMR and HRMS spectra of target compounds) associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.07.026.

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