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Discovery of 9,11-Seco-Cholesterol Derivatives as Novel FXR Antagonists

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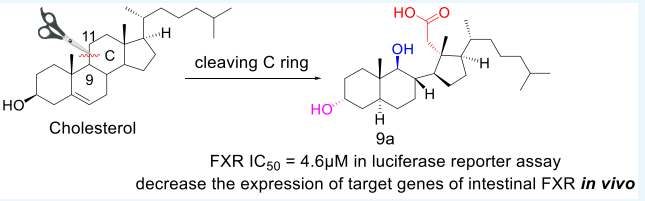
ABSTRACT: The farnesoid X receptor (FXR) plays an important role in the regulation of bile acid, lipid, and glucose homeostasis. Recent findings have shown that the inhibition of FXR is beneficial to improvement of related metabolic diseases and cholestasis. In the present work, 9,11-seco-cholesterol derivatives were designed and synthesized by cleaving the C ring of cholesterol and were identified as novel structures of FXR antagonists. Compound **9a** displayed the best FXR antagonistic activity at the cellular level (IC_{50} = 4.6 μM) and decreased the expression of the target genes of FXR *in vivo*.



INTRODUCTION

Bile acids (BAs) are atypical steroidal molecules generated by the liver from cholesterol and are important signaling molecules that activate bile acid activated receptors (BARs), including the farnesoid X receptor (FXR).[1,2] In hepatocytes and the distal ileum, FXR signaling regulates bile acid synthesis, metabolism, and transport.[3,4] In addition, FXR plays a key role in lipid, glucose, and energy metabolism.[4−9]

A growing number of studies have suggested that FXR antagonists exhibit beneficial effects in the treatment of metabolic diseases. The natural FXR antagonist tauro-β-muricholic acid (T-β-MCA, IC_{50} = 40 μM) inhibits intestinal FXR signaling and improves various metabolic end points in mice with established obesity.[10] Glycine-β-muricholic acid (Gly-MCA),[11] glycoursodeoxycholic acid (GUDCA, IC_{50} = 77.2 μM),[12,13] tauroursodeoxycholic acid (TUDCA, IC_{50} = 75.1 μM),[12,13] and hyocholic acid (HCA, IC_{50} = 70.1 μM)[14] have also been identified as FXR antagonists and reverse metabolic dysfunctions in HFD-fed obese mice by inhibiting intestinal FXR activity (Figure 1). In addition, some nonsteroidal FXR antagonists have been reported, including **3f** (IC_{50} = 0.58 μM),[15] **25** (IC_{50} = 9.2 nM),[16] **NDB** (IC_{50} = 3.4 μM),[17] and **FLG249** (IC_{50} = 32.0 nM)[18] (Figure 1). **FLG249** is the first reported nonsteroidal FXR antagonist exerted downstream of FXR in mouse ileum.[18] Therefore, novel potent FXR antagonists need to be developed to study FXR signaling as a potential therapeutic target for metabolic diseases.

Modifying the structure of natural products is a common strategy to discover novel drug candidates.[19,20] In our previous work, we reported a series of potent TGR5 agonists by modifying the natural product betulinic acid as a mimic of endogenous ligands of TGR5, including **13α**[21] and **11d-Na**[22] (Figure 2). In comparison to natural bile acids, such as CDCA, synthetic mimics share a hydrophobic ring system and a polar hydrophilic carboxylic acid group; the latter plays a key role in

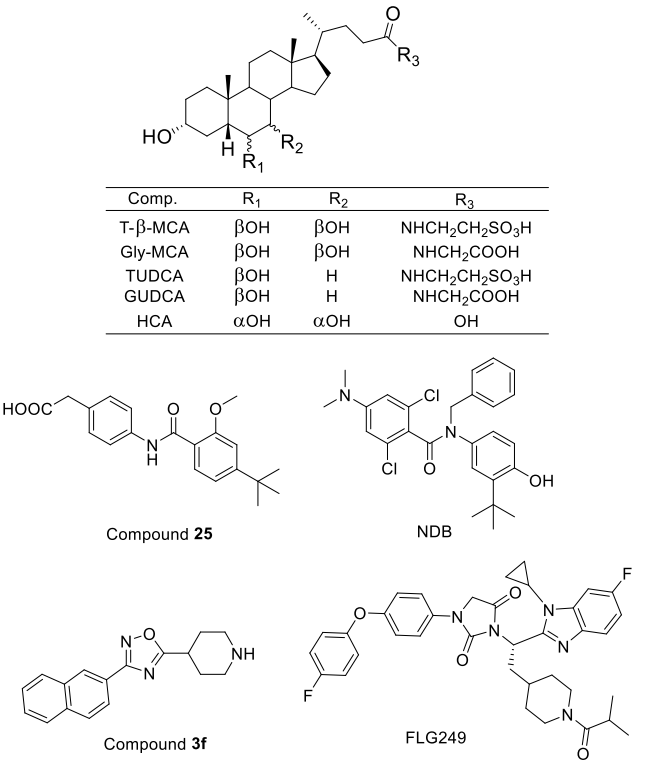


Figure 1. Structures of steroidal and nonsteroidal FXR antagonists.

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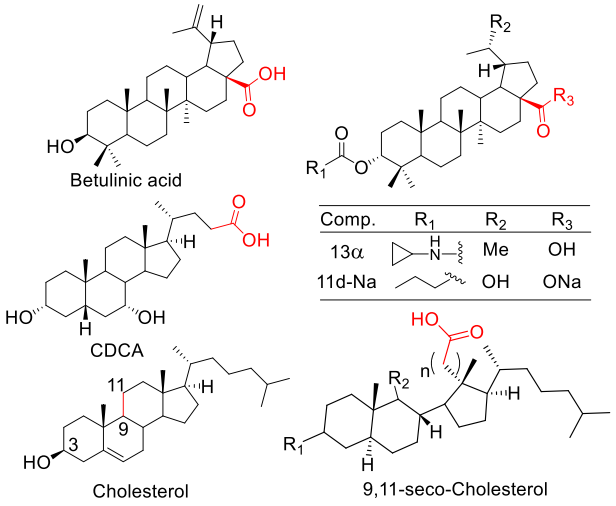


Figure 2. Structures of betulinic acid, 11d-Na, cholesterol, and 9,11-seco-cholesterol.

forming tight interactions with BARs. Cholesterol is the starting substance of bile acid biosynthesis and thus shares the same core structural skeleton as endogenous ligands of bile acid receptors, such as TGR5 and FXR. We propose an accessible strategy by modifying the structure of cholesterol through opening one of the rings of cholesterol to generate a carboxylic group and maintain the remaining hydrophobic ring system to create a novel seco-cholesterol as a mimic of endogenous ligands of FXR.

RESULTS

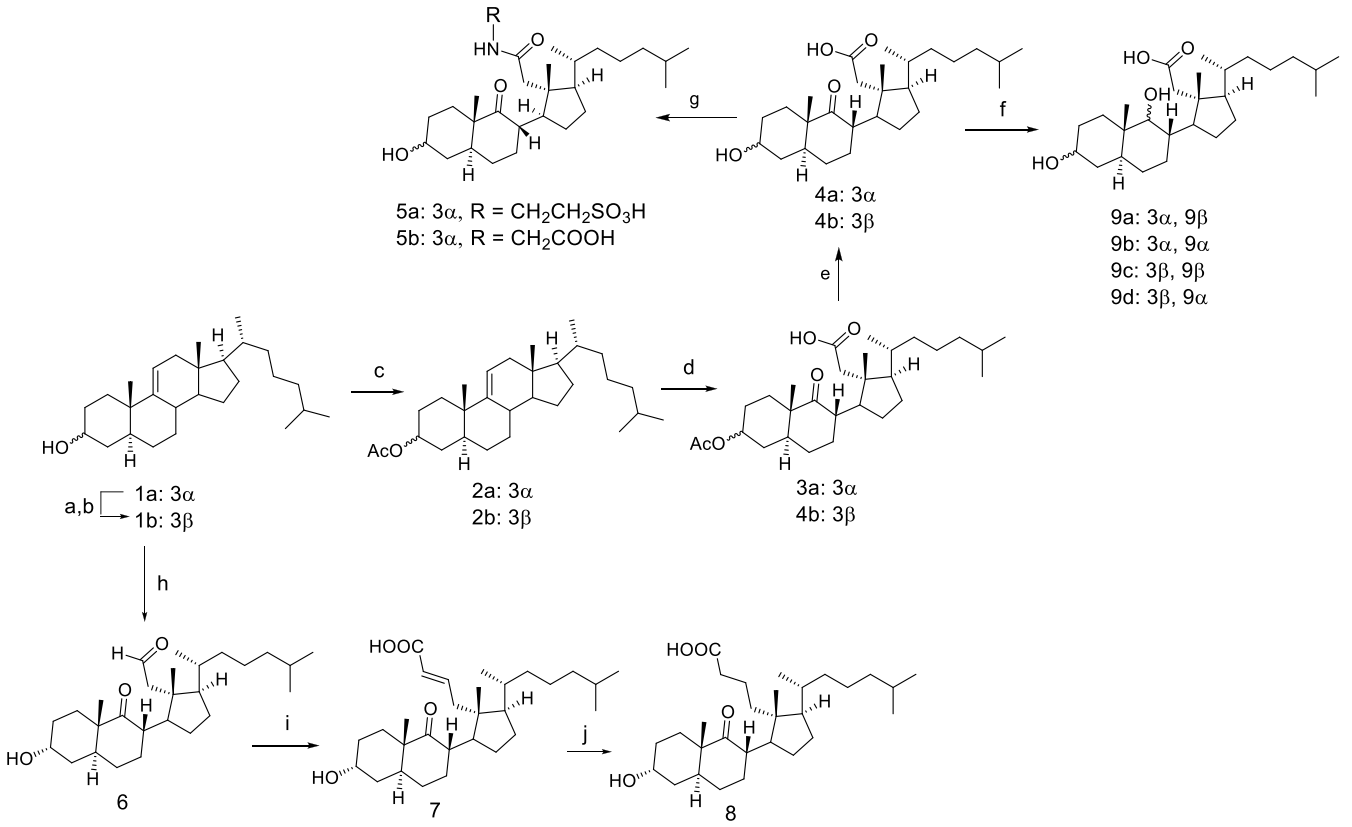
The synthesis of the desired seco-cholesterol compounds is outlined in Scheme 1. The C ring of cholesterol was cleaved in

Table 1. *In Vitro* FXR Inhibitory Rates of Seco-Cholesterol Derivatives

compd	inhibition (%)[a]	cell viability (%)
3a	54.7 ± 6.2	>100
3b	24.9 ± 2.0	>100
4a	53.1 ± 4.9	>100
4b	14.1 ± 4.1	>100
5a	0.7 ± 6.3	>100
5b	46.9 ± 4.5	>100
7	76.3 ± 1.9	>100
8	85.8 ± 2.4	>100
9a	90.1 ± 0.6	>100
9b	100 ± 0.2	>100
9c	100 ± 0.0	>100
9d	89.5 ± 2.4	>100

[a]The testing concentration was 10 μM.

Scheme 1. Synthesis of Seco-Cholesterol Derivatives[a]



[a]Reagents and conditions: (a) DMP, DCM, 0 °C, overnight; (b) $CeCl_3$, $NaBH_4$, MeOH:THF = 1:1, 30 min; (c) Ac_2O, DMAP, pyridine, 12 h; (d) O_3, DCM, −78 °C, 30 min, then H_2O; (e) KOH aqueous, MeOH, 2 h. (f) $NaBH_4$, *t*-BuOH, 70 °C, 30 min; (g) EEDQ, Et_3N, R-NH_2, DMF, 90 °C, overnight; (h) O_3, DCM, −78 °C, 30 min, then Me_2S, 0 °C, overnight; (i) propanedioic acid, piperidine, pyridine, 90 °C, 4 h; (j) Pd/C, H_2, MeOH, 3 days.

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Table 2. *In Vitro* FXR Inhibitory Activity of Compounds 8, 9a−d, and HCA

compd	IC_{50} (μM) FXR inhibition	cell viability (%)
8	7.7	41.1
9a	4.6	>100
9b	4.6	27.2
9c	3.4	85.4
9d	1.6	45
HCA	27.3	>100

the 9,11-position to generate a ketone acid, and the structure−activity relationship of the C3, C9, and C10 positions was investigated. The key intermediate olefin **1a** with C3−α-OH was synthesized according to Kido's method[23] from 7-dehydrocholesterol, and intermediate **1b** with C3−β-OH was synthesized from **1a** by oxidation with Dess−Martin periodinane in DCM, followed by reduction with sodium borohydride in MeOH. Then, the C3−OH was protected by acetic anhydride to afford **2a**,**b**, which were treated with ozone and water to cleave the double bond to give seco-cholesterols **3a**,**b** with a ketone and a carboxylic group. Hydroxylation of **3a**,**b** with potassium hydroxide gave **4a**,**b**, respectively. Then, **4a** was reacted with various amino acids under *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline in DMF to afford **5a**,**b**. A Knoevenagel condensation was used to extend the length of the carboxylic chain. First, olefin **1a** was treated with ozone in DCM and subsequently quenched with dimethyl sulfide to give **6** with a ketone and an aldehyde group. Then, **6** was reacted with propanedioic acid and piperidine in pyridine to obtain **7**. The double bone of **7** was reduced with Pd/C under H_2 to generate **8**, and the carbonyl group of **4a**,**b** at the C9 position was reduced by sodium borohydride to afford **9a**−**d** with different configurations of hydroxyl groups. **9a**−**c** could be distinguished by the chemical shifts and coupling constants of the H atom at the C9 position.

The cytotoxicity and efficacy on FXR antagonism of compounds were assessed by a Cell-Titer Blue assay and a cell-based luciferase reporter assay, respectively. FXR-driven SHP promoter transactivation was evaluated by transient cotransfection with a human FXR expression vector and luciferase reporter construct (SHP-luc) in the HEK293T cell line, and the transactivation activity was measured in the presence of GW4064 (10 μM) with or without test compounds.

As reported in Table 1, the inhibition rates of **3a**,**b** and **4a**,**b** were moderate at 10 μM; in general, the potency of **4a** with C3−α-OH was better than that of **4b** with C3−β-OH, and the subsequent replacement of the C3 position OH with an acetyl ester group had no significant improvement on FXR inhibition. These initial results encouraged us to further modify these seco-cholesterol skeletons. Although most natural steroidal FXR antagonists are conjugated bile acids, in contrast, there was no improvement in FXR inhibition when the carboxyl group was conjugated by taurine (**5a**) and glycine (**5b**). In comparison with **4a**, the FXR antagonistic activity was significantly increased when the length of the carboxylic chain was extended. Moreover, compound **8**, with a saturated chain, is better than compound **7**, with an unsaturated chain, for FXR inhibitory activity. The presence of the hydroxyl group at the C9 position substantially increased the FXR inhibitory rates, and the effect of the configuration of C3−OH on FXR inhibitory activity became weak.

We completed preliminary SAR screening for C3, C9, and C11, and further selected compounds with an FXR inhibitory rate of greater than 80% to test their IC_{50} and cell toxicity. As Table 2 shows, although **9a**−**d** had a great inhibitory rate, most of the compounds demonstrated apparent cytotoxicity, excluding compound **9a**. Compound **9a** was selected as the most potent FXR antagonist with an IC_{50} value of 4.6 μM. To further confirm FXR inhibition, we tested the effect of **9a** on the downstream target genes of FXR *in vivo*. C57BL/6J male mice (age 6−8 weeks) were given an antibiotic cocktail (bacitracin, neomycin, and streptomycin) in drinking water at 0.1% (w/v) for a consecutive 3 days to deplete gut microbiota and then gavaged with taurocholic acid (TCA, an FXR agonist, 400 mg/kg) and compound **9a** (10 mg/kg) 2, 24, and 48 h before they were sacrificed. In the ileum, **9a** substantially inhibited the mRNA expression of FXR target genes *Shp* and *Fgf15*, which was induced by TCA, but did not affect the *Tgr5* mRNA level (Figure 3A). Compound **9a** also inhibited the expression of *Fxr* and *Shp* in the liver (Figure 3B).

Recent reports indicated that intestine-selective FXR inhibition is beneficial to treat metabolic diseases.[10−13] Inhibition of intestinal FXR suppresses FGF15/19-FGF4R signaling, subsequently increasing BA synthesis and eliminating BAs via the feces, eventually reversing the accumulation of cholesterol in the liver.[13] In addition, the intestinal selective FXR antagonist Gly-MCA reduces the biosynthesis of intestine-derived ceramides, which reverses obesity, and metabolic syndrome by activating beige fat thermogenesis in obese mice.[9] However, in the liver, in the absence of FXR, the expression of CYP7A1, CYP8B1, and sodium-taurocholate cotransporting polypeptide (NTCP) increases, and the level of SHP expression decreases, which results in an increased level of hepatic BAs. The increased BA levels induce IL-1β, which

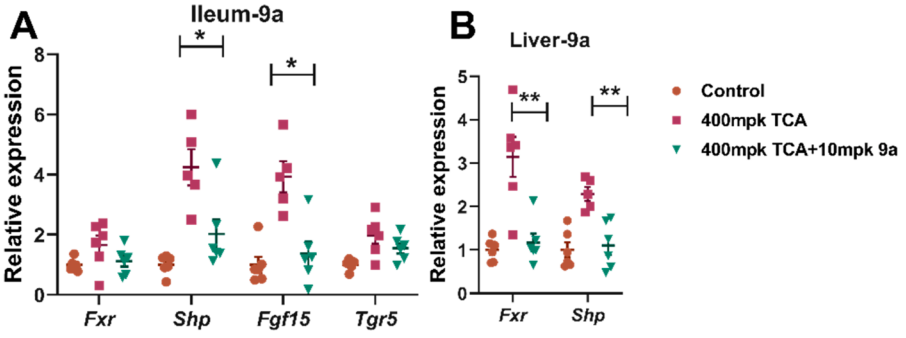

Figure 3. Compound **9a** inhibited the expression of genes downstream of FXR in the ileum (A) and liver (B) induced by TCA, an FXR agonist.

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eventually leads to tumorigenesis.[24,25] The present work identified some novel potent FXR antagonists with *in vitro* and *in vivo* FXR inhibition activity, while it also shows some antagonism of FXR signaling in the liver, which will be a problem[24−26] and requires further optimization in the future.

In conclusion, we discovered a series of novel FXR antagonists through cleavage of the cholesterol C-ring to mimic endogenous ligands. Compound **9a** exhibits potent inhibition of FXR with an IC_{50} value of 4.6 μM *in vitro* and effectively decreases expression on downstream genes of FXR *in vivo*. In comparison with other natural BA FXR antagonists, **9a** has a more potent inhibitory activity and lower cytotoxicity. Further optimization of these seco-cholesterol derivatives to improve their tissue specificity is in progress in our laboratory and will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c01567.

Experimental procedures and characterization data of new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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