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Synthesis and biological evaluations of chalcones, flavones and chromenes as farnesoid x receptor (FXR) antagonists

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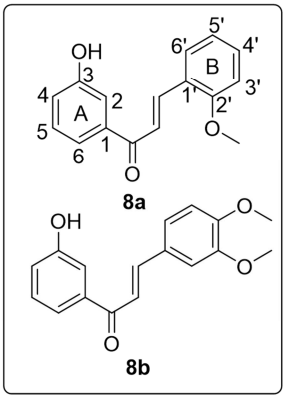
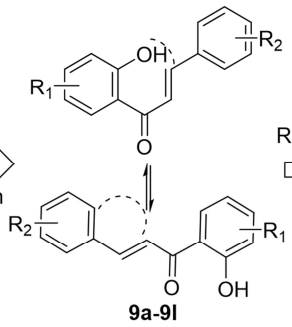


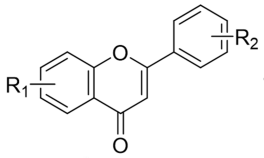

$IC_{50}$ for FXR antagonism: ~20μM

SAR exploration



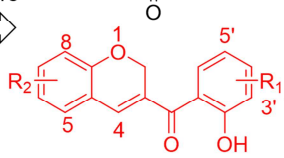

9a-9l

Ring closure




10a-10l

$R_1$= OH
$R_2$= OMe




11a-11l

Compound **11c** (R1= 3'-OH, R2=6-OMe)

$IC_{50}$ for FXR antagonism: 8.11μM

In KKay mice: plasma triglyceride level: ↓ 34.7%
hepatic triglyceride level: ↓ 42.5%
plasma ALT level: ↓ 22.1%

# Synthesis and biological evaluations of chalcones, flavones and chromenes as farnesoid x receptor (FXR) antagonists

Guoning Zhang[a], Shuainan Liu[a], Wenjuan Tan[b], Ruchi Verma[c], Yuan Chen[c], Deyang Sun[a], Yi Huan[a], Qian Jiang[a], Xing Wang[a], Na Wang[d,e], Yang Xu[f], Chiwai Wong[b], Zhufang Shen[a], Ruitang Deng[c], Jinsong Liu[d,e], Yanqiao Zhang[f], Weishuo Fang[a*1]

Farnesoid X receptor (FXR), a nuclear receptor mainly distributed in liver and intestine, has been regarded as a potential target for the treatment of various metabolic diseases, cancer and infectious diseases related to liver. Starting from two previously identified chalcone-based FXR antagonists, we tried to increase the activity through the design and synthesis of a library containing chalcones, flavones and chromenes, based on substitution manipulation and conformation (ring closure) restriction strategy. Many chalcones and four chromenes were identified as microM potent FXR antagonists, among which chromene 11c significantly decreased the plasma and hepatic triglyceride level in KKay mice.

**Keywords**: farnesoid X receptor, antagonist, chalcones, chromenes

## Introduction

The FXR (Farnesoid X receptor, NR1H4) belongs to the nuclear receptor superfamily which bind to cis-acting elements in the promoters of their target genes and modulate gene expression in response to metabolites.[1] It is highly expressed in tissues exposed to high concentration of bile acids (physiological ligands of FXR) such as the liver, kidney, intestine and other cholesterol-rich tissues such as adrenal glands.[2, 3]

FXR has an important role in maintaining bile acids and cholesterol homeostasis by regulating the expression of genes, such as cholesterol 7α-hydroxylase (Cyp7A1), $Na^+$-dependent taurocholate cotransporting polypeptide (NTCP) and bile salt export pump (BSEP) [4-6]. Apart from its crucial role in maintaining bile acids and cholesterol homeostasis, FXR also regulates the metabolism of lipid and glucose.[7-9] It has been shown that FXR is involved in various diseases such as metabolic disorders, hepatitis, arteriosclerosis and cancer.[10-14] Thus, FXR has been considered as an important drug target after the deorphanization of FXR in 1999.[15]

Since then, many efforts on discovering FXR ligands have been made and a handful of steroidal- and non-steroidal FXR ligands reported. Although increasing number of FXR agonists[16-18] have been discovered in the past decade, only three compounds (obeticholic acid, Px-102 and LJN-452) entered clinic trials. It is encouraging that obeticholic acid was approved by FDA in May 2016 for the treatment of primary biliary cholangitis (PBC) in combination with ursodeoxycholic acid

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*d. School of Life Sciences, University of Science and Technology of China, Hefei 230026, China*

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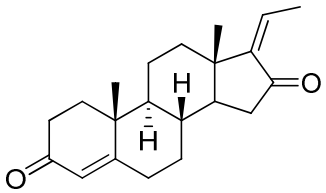
(UDCA) in adults with an inadequate response to UDCA, or as a single therapy in adults unable to tolerate UDCA, validating the utility of FXR interacting agents in human.[19]

It has been proved that FXR full activation by agonist may cause undesired side effects in animals and patients with diabetes and liver steatosis, such as the inhibition of bile acid synthesis and increased levels of low-density lipoprotein (LDL).[20-22] As an alternative, FXR antagonists have been actively pursued and reported in the past several years, although the amount and structure diversity of the antagonist is still very limited.

Most reported antagonists[23, 24] with steroidal scaffolds are originated from bile acids, the internal ligands, including the first known antagonist guggulsterone (**Figure 1**).[25-30] However, as the steroidal scaffold is a widely used preferential scaffold in drug design, it led to low selectivity of FXR antagonists against other nuclear receptors. There are also a few synthetic non-steroidal scaffolds reported to date (**Figure 2**), i.e., troglitazone (**1**),[31] substituted-isoxazole derivatives (**2a**-**2b**),[32] 1, 3, 4-trisubstituted-pyrazolone (**3**),[33] T3 (**4**)[34], NDB (**5**)[35], hydroxyacetophenone derivatives (**6a**-**6c**)[36] and 1,3-disubstituted-pyrazole-3-carboxamide (**7a**-**7c**)[37]. Natural products derived antagonists are rarely known, and only a family of sesterterpene suvanine except GS [38]. Besides, the marketed nonsteroidal anti-inflammatory drugs drug (NSAIDs) indomethacin was also identified as a potent FXR antagonist, and its FXR antagonism may account for the NSAIDs-induced liver injury.[39]




*Z*-guggulsterone

**Figure 1** Structure of the first steroidal FXR antagonist gugglesterone

Most of FXR antagonists known to date are only active at the micromolar level[40], except **4**，which inhibits the FXR activation induced by CDCA with an $IC_{50}$ value of 1 nM.[34] So it is still needed to find more potent, selective antagonists of new chemotypes. Here we report such an effort leading the discovery of chalcone and chromenes-based FXR antagonists and the metabolic regulation effect induced by a representative compound, chromene **11c**, in diabetic KKay mice.

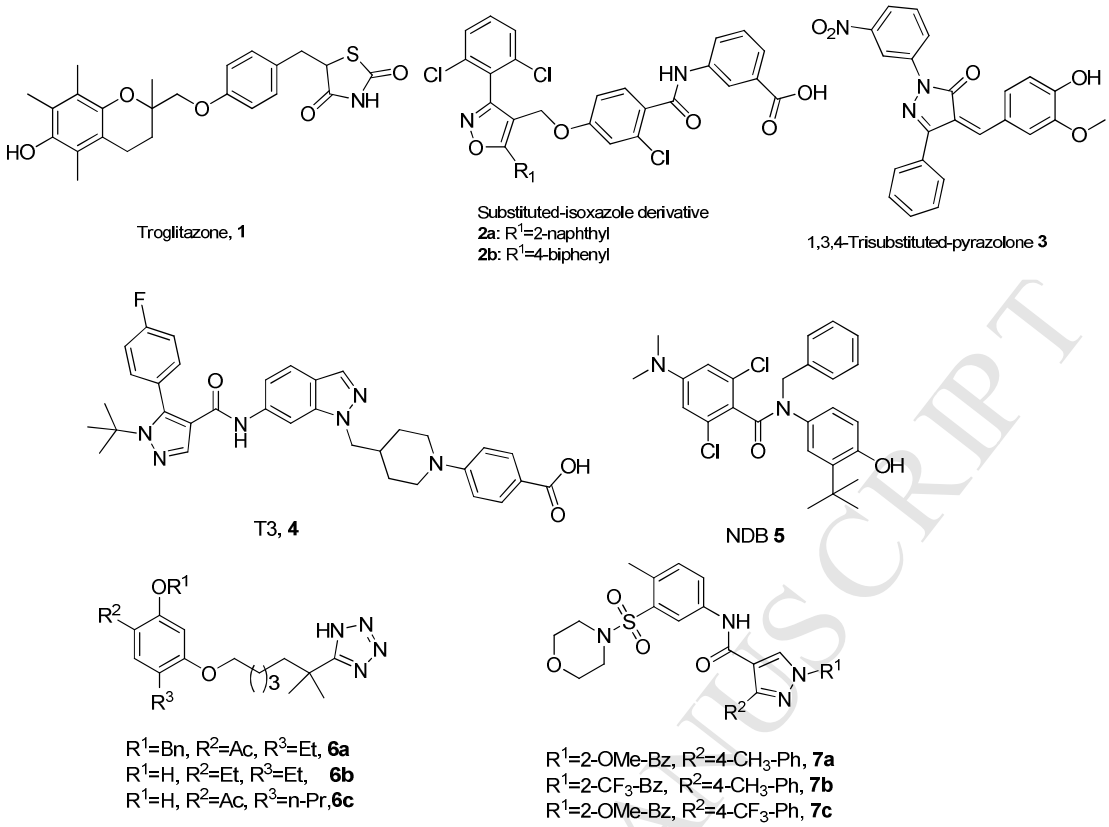



**Figure 2** Non-steroidal FXR antagonists reported in literatures.

## Results and discussion

Chalcones **8a** and **8b** were first identified in our lab as FXR antagonists from natural products, which abolished the FXR activation induced by 25 μM of chenodeoxycholic acid (CDCA) in 50% and 77% respectably at 10μM. Furthermore, their $IC_{50}$ values were found to be *ca.* 20 μM in the antagonistic assay.

Next, we intend to increase the binding affinity/activity of chalcones to FXR while maintaining its antagonistic effect. A small library consisting of twelve chalcones (**9a-9l**) was designed and synthesized. To our delight, ten of them retained the antagonistic effect and the most potent one exhibited an $IC_{50}$ of 8 μM.

To further increase the activity, we adopted a conformation restriction approach. Two libraries containing flavones **10a-10l** and chromenes **11a-11l** respectively were constructed. In these two newly designed libraries, most of the flavones lose activity completely, whereas four of the chromenes showed increased activity in FXR antagonistic assay. The chromene **11c** reduced the triglyceride level dramatically and shows a hepatic protection effect.

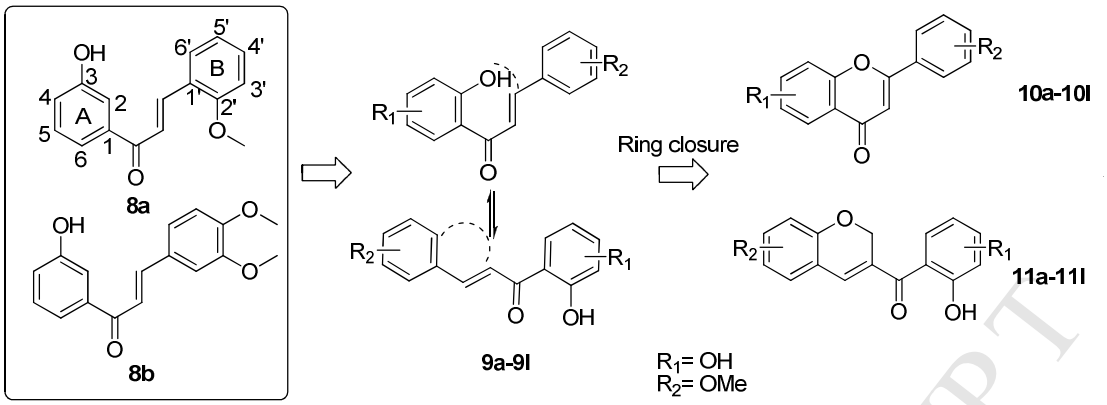
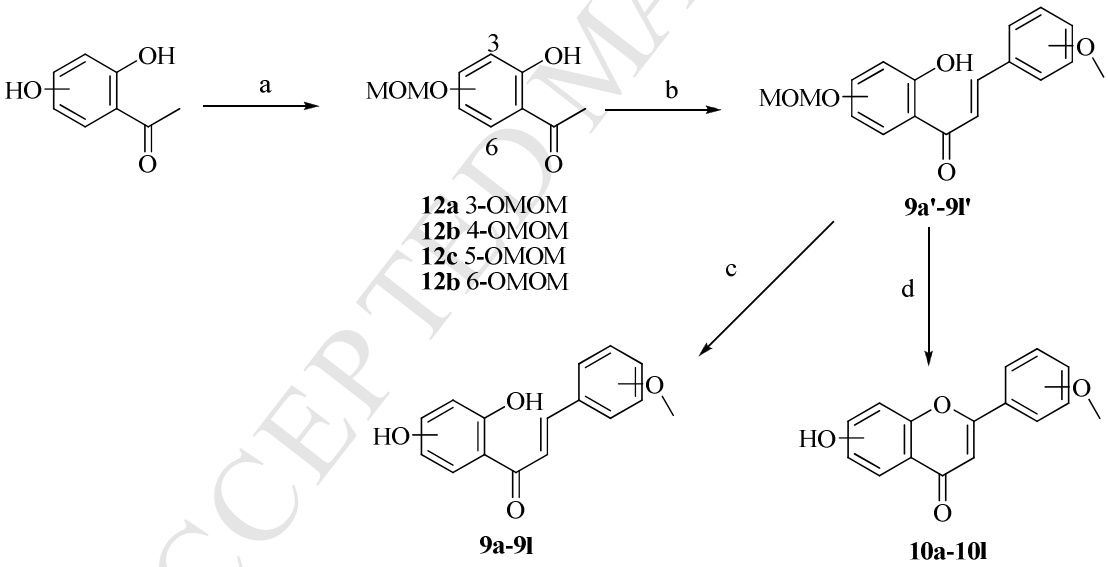



**Figure 3** Two FXR antagonists identified by our lab and new compounds generated in this study.

The synthesis of chalcones was based on a known methodology[41], and the route outlined in **Scheme 1** (for details, please refer to Supporting Information). Briefly, the different substituted dihydroxy-acetophenones were selectively protected for one hydroxyl by methyloxymethyl (MOM) group. Then the mono-protected compounds were condensed with the corresponding benzaldehydes in a solution of ethanol and aqueous KOH. The MOM-protective group was then removed under acidic condition to afford corresponding chalcones.

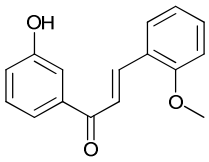
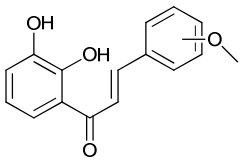
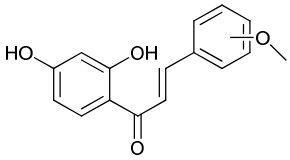
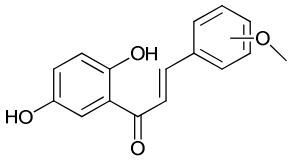
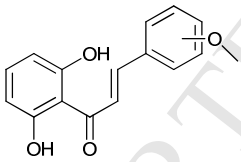



**Scheme 1** Synthesis of compounds **9a-9l** and **10a-10l**. *Reagents and Conditions*: a. MOMCl, DIEA, DCM, RT. b. EtOH / KOH (40%) = 1:1 (v/v). 0°C-RT. c. HCl (3M) / MeOH = 5:2 (v/v). d. $I_2$, DMSO, 160°C.

For the primary assay, the agonistic and antagonistic activities of these compounds were screened using the mammalian one-hybrid FXR coactivator association assay. Encouragingly, ten compounds (**9a-9i** and **9l**) showed moderate to high antagonistic activities against the FXR activated by 25 μM of CDCA (**Table 1**). It was found that the chalcones bearing 2,3-, 2,4- and 2,5-dihydroxyl groups on ring A exhibited antagonistic activity, whereas 2,6-dihydroxyl substituted chalcones are less or not active (**9a-9i** vs. **9j-9l**). In addition, all above chalcones with

2,3-, 2,4- and 2,5-dihydroxyls showed the highest potency in combinations with a 2′-OMe (*ortho*) substitution (**9a**, **9b**, **9d** or **9g** vs. **9c**, **9e**, **9f**, **9h** or **9i**).

**Table 1.** Chemical structures of compounds **8a**, **9a-9l**, and their activities based on the mammalian one-hybrid assay.

| Cmpd | Structure | R | Antagonist rate at 10 μM (%)[a] | $IC_{50}$ (μM) | Agonist rate at 10 μM (%)[a] |
|---|---|---|---|---|---|
| **8a** |  | - | 20.39 | 21.2 | 3.43 |
| **9a** |  | *o*-OMe | 75.19 | 16.2 | 4.16 |
| **9b** | | *m*-OMe | 81.53 | 17.3 | 2.43 |
| **9c** | | *p*-OMe | 27.60 | -[b] | 5.19 |
| **9d** |  | *o*-OMe | 81.51/97.00[c] | 8.2±1.1[d] | 7.37 |
| **9e** | | *m*-OMe | 33.96 | 15.1 | 4.91 |
| **9f** | | *p*-OMe | 34.39 | -[b] | 3.97 |
| **9g** |  | *o*-OMe | 56.22 | 15.3 | 2.18 |
| **9h** | | *m*-OMe | 30.42 | 18.2 | 2.44 |
| **9i** | | *p*-OMe | 42.69 | -[b] | 4.38 |
| **9j** |  | *o*-OMe | < 5 | -[b] | 5.09 |
| **9k** | | *m*-OMe | < 5 | -[b] | 4.50 |
| **9l** | | *p*-OMe | 33.82 | -[b] | 3.99 |

a. The luciferase activity of HEK293T cells treated by 25μM of CDCA was set as 100%. b. Not determined. c. Antagonist effect on activation induced by 1μM of GW4064. d. Mean value of three independent experiments.

Compounds **9a, 9b, 9d, 9e, 9g and 9h** were further selected to determine their FXR antagonist activity at different concentrations. All of these six compounds dose-dependently inhibited CDCA induced FXR activation with $IC_{50}$ values of 8.2~18.2 μM, whereas the reference compound **8a** exhibited an $IC_{50}$ at 21.2 μM. These compounds were also determined for their FXR agonist activity and none of them acted as FXR agonists (**Table 1**).

To distinguish the antagonistic activity from the inhibition of normal cell function, the cytotoxic effect for these compounds was determined in MTT assay at 10 μM. All six compounds showed no or very weak cytotoxicity (**Figure 4**), indicating the antagonistic activity was not due to non-specific cytotoxicity.

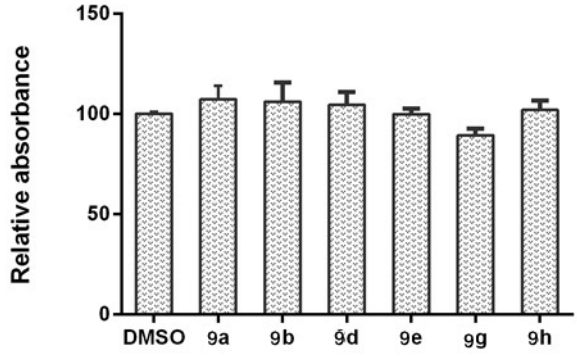



**Figure 4**. Cell toxicity of selected compounds determined by MTT. 293T Cells in 0.1% fetal bovine serum were treated with 10 μM of compounds for 24 hr before assessing toxicity by MTT assay; DMSO treatment was set at 100%.

Among these six compounds, **9d** which exhibiting the highest antagonistic effect for CDCA, also inhibits the activation induced by 1 μM of a more potent synthetic agonist GW4064 nearly completely in the mammalian one-hybrid *h*-FXR assay (**Table 1**). Then compound **9d** was assigned to a transactivation experiment to confirm its FXR antagonistic activity in Huh 7 cells. As shown in **Figure 5A**, the compound dramatically decreased BSEP transactivation dramatically at high concentrations. Compared with the data in 293T cells, a higher concentration of **9d** is required to exhibit the same antagonistic rate. This may be arisen from the easier metabolism of **9d** in Hub7, a cell line derived from hepatocyte containing a lot of enzymes. A fast metabolic rate in mouse liver microsomes ($t_{1/2}$=3.0min) was detected, verifying the correctness of this hypothesis. Furthermore, **9d** was found to be a selective FXR antagonist as it has minimal effects on estrogen signaling pathway even at a concentration of 100 μM (**Figure 5B**)

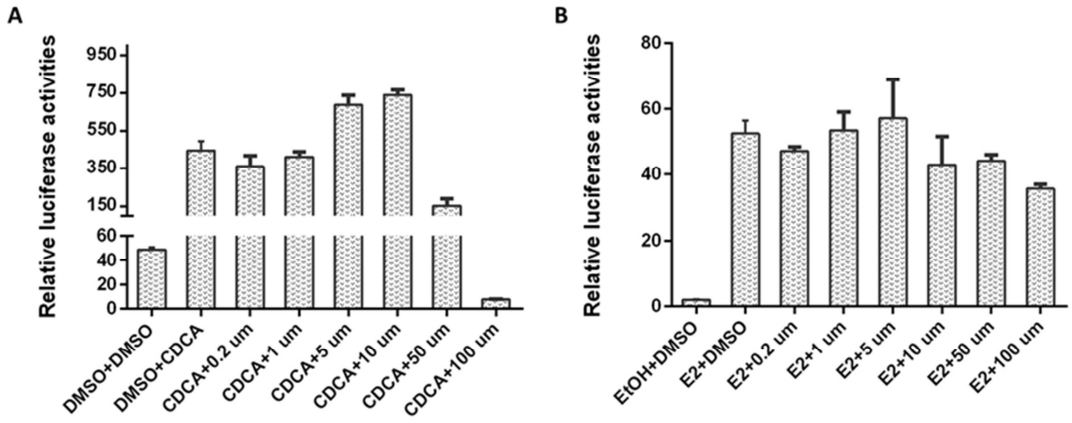



**Figure 5**. The effects of the compound **9d** on FXR and estrogen signaling pathway. A. Huh 7 cells were co-transfected with human BSEP promoter luciferase reporter, phBSEP (-2.6kb and FXR, followed by treatment with CDCA (10μM) and various concentrations of the compound **9d**. B. Huh 7 cells were transfected with estrogen response element (ERE) reporter and estrogen receptor α (ERα), followed by treatment with 17β-estrodial (E2) (100 nM) and various concentrations of the compound.

To determine the cellular activity of **9d** is FXR specific, we measured the direct binding of compound **9d** to the ligand binding domain of FXR (FXR-LBD). Compound **9d** is able to thermally stabilize the conformation of FXR-LBD by increasing its melting temperature (Tm) by 3.1 °C in a circular dichroism (CD) assay (Supporting Information, Table S1), while CDCA can increase the Tm by 6.0 °C. Using Alphascreen technology, we demonstrated that compound **9d** significantly inhibited CDCA induced co-activator recruitment (**Figure. 6**) at the concentration of 50 μM,

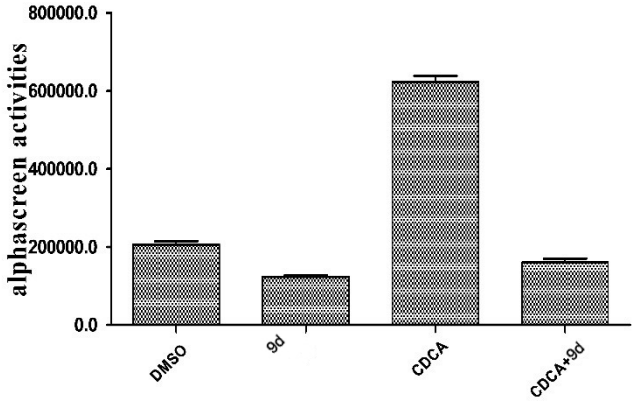
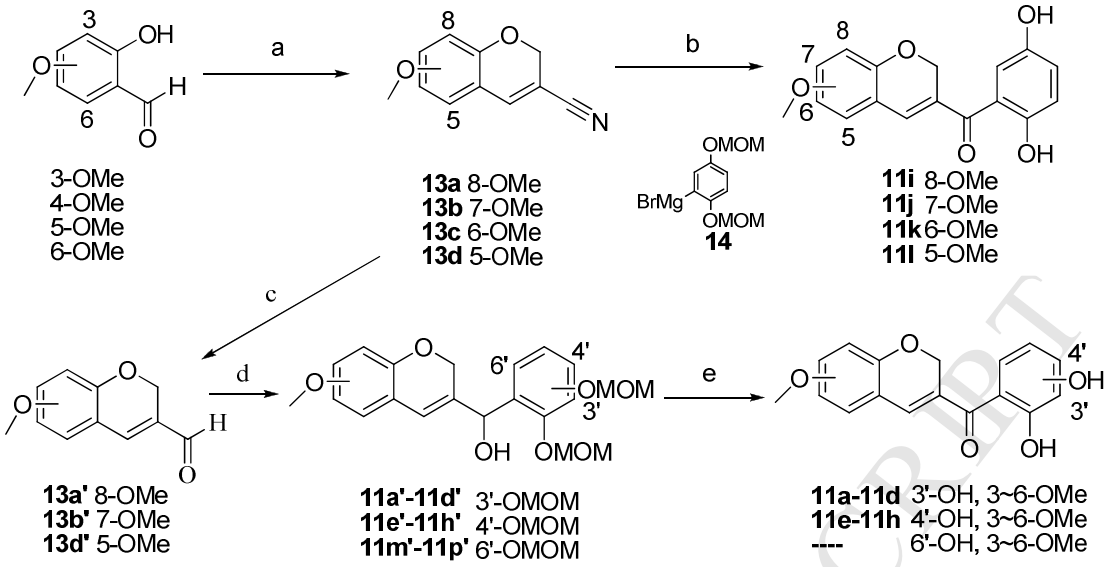



**Figure 6** The activity of **9d** was tested by Alphascreen assay at a concentration of 50 μM. Values are the means ± S.D. of three independent experiments.

After analyzing all of the available data, we decided to manipulate the activity and stability of these compounds next. As compound **9d** bearing a flexible chalcone skeleton, we hypothesized that its relatively low affinity is related to the conformation change upon binding to FXR and also the entropy penalty during this process. Hence, restricting the conformational flexibility of chalcones properly may reduce the entropy penalty and thus increase free binding energy. Besides, this structure manipulation may also increase the metabolic stability.

Therefore, two libraries containing flavones **10a-l** and chromenes **11a-l** corresponding to the structures of above chalcones were constructed. As shown in **Scheme 1**, Mono-protected chalcones **9a′-l′** were treated with catalytic amount of iodine in a solution of DMSO at 160°C to afford the corresponding flavones, and the MOM group was also removed under this condition. Chromenes **11a-l** was synthesized as shown in **Scheme 2**. Treatment of 3-cyano chromenes **13a-d** with Grignard reagent **14** and followed by deprotection with aq. HCl solution in one-pot afforded the target products **11i-l** in 27%-36% yields. Because of the low yield of directed addition reaction of 3-cyano chromenes, 3-cyano chromenes **13a-d** were first reduced to 3-formyl chromenes **13a′-d′** and then treated with corresponding aryl lithium at -50°C to form the compounds **11a′-h′** and **11m′-p′**. After the $MnO_2$ oxidation and deprotection under acidic condition, the targeted chromenes **11a-h** were furnished. However, for the specific substitution, **11m-11p** were not afforded after the same process from **11m′-p′,** which were converted to by-products not further characterized.



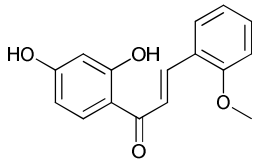

**Scheme 2** Synthesis of compounds **11a-11l**. *Reagents and Conditions*: a. acrylonitrile, DABCO, reflux. b. i. Grignard reagent **14**, THF, 60°C. ii. HCl (3M) / MeOH = 5:2 (v/v). c. DIBAl-H, toluene, -20°C-RT. d. corresponding phenyl lithium -50°C. e. i. $MnO_2$, DCM. ii. MeOH / HCl (3M) = 5:2 (v/v).

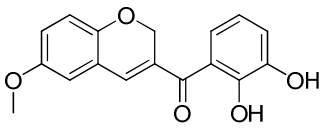
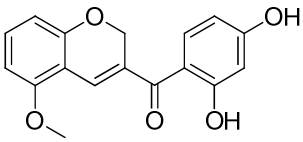
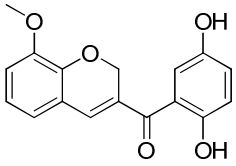
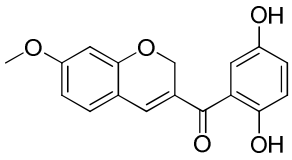
The flavone and chromene libraries were tested, and most of the flavones lose FXR antagonistic potency, among which only **10a**, **10d** and **10g** showed weak antagonist activity (antagonism 14.41-34.49%) at 10 μM, although the corresponding chalcones **9a**, **9d** and **9g** showed much more potent antagonistic activity (antagonism 56.22-81.51%) at the same concentration. We reasoned that the conformational restriction of chalcones to flavones may not reflect the correct bioactive conformation of the chalcone, thus resulted in the significant loss of activity.

In contrast, the chromenes **11c** and **11h-j** were determined as potent FXR antagonists which inhibited the FXR activation induced by 10 μM of CDCA. All of these four compounds dose-dependently inhibited CDCA induced FXR activation and their $IC_{50}$ values were measured as 3.58-8.11μM, whereas the $IC_{50}$ of **9d** was measured as 4.29μM (**Table 2**).

The chromenes **11c** and **11h-j** were more potent in inhibiting the FXR activation than the corresponding chalcones (**11c** *vs.* **9b**, **11h** *vs.* **9d**, **11i** *vs.* **9h** and **11j** *vs.* **9i**), indicating the ring closure of chalcone to chromene locked the right conformation of chalcone binding to FXR.

**Table 2.** Chemical structures of compounds **9a**, **11c, 11h-11j**, and their $IC_{50}$s based on the mammalian one-hybrid assay

| Cmpd | structure | $IC_{50}$ (μM)[a,b] |
|---|---|---|
| **9d** |  | 4.29 |

| | | |
|---|---|---|
| **11c** |  | 8.11 |
| **11h** |  | 3.58 |
| **11i** |  | 3.87 |
| **11j** |  | 3.98 |

a. The luciferase activity of HEK293T cells treated by 25μM of CDCA was set as 100%. b. Mean value of three independent experiments.

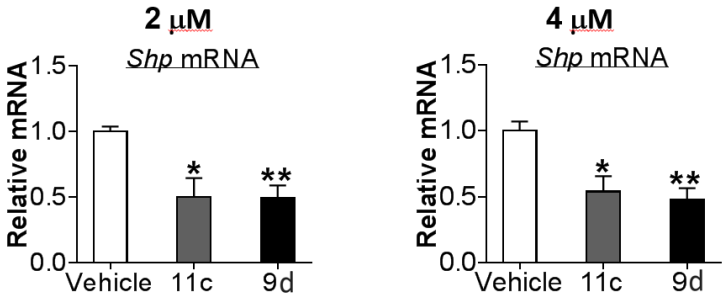



**Figure 7** Relative Shp mRNA leves quantified by qRT-PCR in primary hepatocytes

In order to determine whether **9d** and **11c** could antagonize FXR activity in hepatocytes, we isolated mouse primary hepatocytes and treated them with 2 μM **9d** and **11c** for 24 h. Compared to the control group, treatment with **9d** or **11c** reduced small heterodimer partner (Shp) mRNA levels by 50% (Figure 7). SHP is a well-characterized FXR target gene (PMID: 11030331 and 11030332). These data demonstrate that both **9d** and **11c** can antagonize FXR activity in hepatocytes.

Considering there is no significant difference for the FXR antagonistic activity of the four chromenes and the preparation of compound **11c** is easier, we chose this compound to evaluate its metabolic regulation activity *in vivo*. We administered **11c** orally (200 mg/kg, qd) in diabetic KKay mice for 28 days. The plasma triglyceride level in **11c** treated group decreased by 21.4% and 34.7% ($P<0.01$, **Figure 8A**) after 16 and 28 days of treatment, respectively. Hepatic triglyceride level was also decreased by 42.5% ($P<0.01$, **Figure 8B**) after 28 days' treatment. The increased level of ALT (also named as GPT, glutamic pyruvic transaminase) and AST (also named as GOT, glutamic oxaloacetic transaminase) in plasma is usually associated with damaged liver. Compound **11c** was found to reduce the plasma ALT level by 22.1% ($P<0.05$, **Figure 8A**) and had no effect on the plasma AST

level after 28 days of treatment, indicating this antagonist does not have hepatitis damage and even could play a hepatic protection role. Besides, it also did not affect the food and water intake and the body weight of mice.

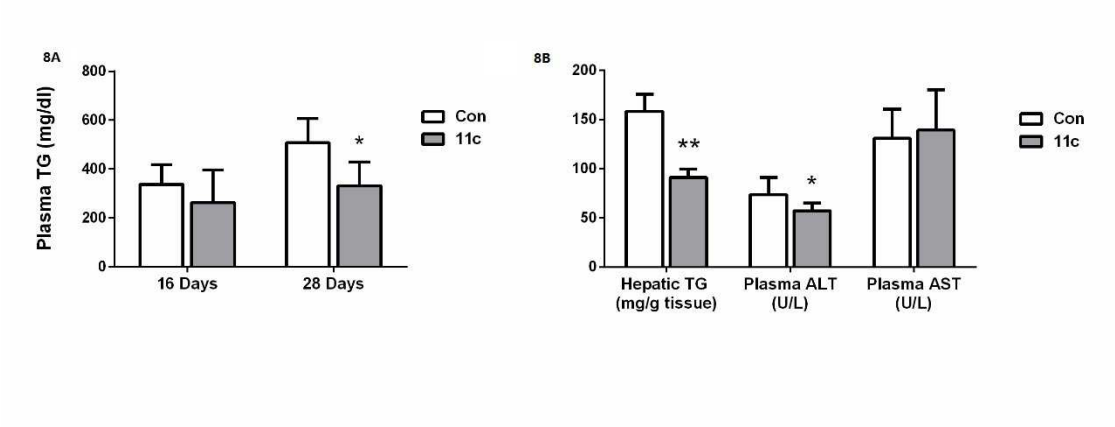



**Figure 8** Effects of **11c** (200 mg/kg) on triglyceride metabolism and plasma ALT and AST level in diabetic KKay mice after 28 days treatment. (A)The plasma triglyceride levels at 16 and 28 days treatment. (B) The hepatic triglyceride level and plasma ALT and AST level after 28 day of treatment. n=10. **P*<0.05, ***P*<0.01, compared with control.

## Conclusions

In summary, we reported a new series of potent chalcone and chromenes-based FXR antagonists with $IC_{50}$ values of 3.6~19.2μM. Among these chalcones, the most potent compound **9d** is a confirmed FXR binder exhibiting antagonism at micromolar level. Chromenes were further found to be active as FXR antagonist after the conformational restriction of chalcones, and the chromene **11c** significantly reduce the triglyceride in plasma and hepatic and plasma ALT level upon its treatment after 28 days in KKay mice.

As there is less research on FXR antagonist compared with that on agonist, the pharmacological role of the antagonist and its potential in the disease treatment remains in debate, even some FXR antagonists have been found to be beneficial in the treatment of cholestasis and hypercholesterolemia in animal models.[33] This research provided another example for the pharmacological activity of the antagoinst and shed the light on its therapeutic potential in the treatment of hypertriglyceridemia and in hepatic protection.

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New series of chalcone and chromene are first reported as potent FXR antagonists.

A chromene compound (11c) significantly reduce the plasma and hepatic triglyceride level and plasma ALT level in KKay diabetic mice.

Pharmacological role of FXR antagonist and its potential in the disease treatment is revealed.