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•Original article•

Dual-function natural products: Farnesoid X receptor agonist/inflammation inhibitor for metabolic dysfunction-associated steatotic liver disease therapy

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[ABSTRACT] Metabolic dysfunction-associated steatotic liver disease (MASLD) is the most prevalent chronic liver disease globally, with only one Food and Drug Administration (FDA)-approved drug for its treatment. Given MASLD's complex pathophysiology, therapies that simultaneously target multiple pathways are highly desirable. One promising approach is dual-modulation of the farnesoid X receptor (FXR), which regulates lipid and bile acid metabolism. However, FXR agonists alone are insufficient due to their limited anti-inflammatory effects. This study aimed to dto identify natural products capable of both FXR activation and inflammation inhibition to provide a comprehensive therapeutic approach for MASLD. Potential FXR ligands from the Natural Product Library were predicted *via* virtual screening using the Protein Preparation Wizard module in Schrodinger (2018) for molecular docking. Direct binding and regulation of candidate compounds on FXR were analyzed using surface plasmon resonance (SPR) binding assay, reporter gene analysis, and reverse transcription-polymerase chain reaction (RT-PCR). The anti-inflammatory properties of these compounds were evaluated in AML12 cells treated with tumor necrosis factor-alpha (TNF-α). Dual-function compounds with FXR agonism and inflammation inhibition were further identified in cells transfected with *Fxr* siRNA and treated with TNF-α. The effects of these dual-function compounds on lipid accumulation and inflammation were evaluated in cells treated with palmitic acid. Results revealed that 17 natural products were predicted *via* computational molecular docking as potential FXR agonists, with 15 exhibiting a strong affinity for FXR recombinant protein. Nine isoflavone compounds significantly enhanced FXR reporter luciferase activity and the mRNA expressions of *Shp* and *Ostb*. Structure-activity relationship analysis indicated that introducing isopropyl or methoxy groups at the C7 position or a methoxy group at the C6 position could enhance the agonistic efficacy of isoflavones. Three compounds (**2**, **6**, and **8**) were identified as dual-function natural products functioning as FXR agonists and inflammatory inhibitors, while one compound (**12**) acted as an FXR agonist to inhibit inflammation. These natural products protected hepatocytes against palmitic acid-induced lipid accumulation and inflammation. In conclusion, compounds **2**, **6**, and **8** (genistein, biochanin A, and 7-methoxyisoflavone, respectively) were identified as dual-function bioactive products that transactivate FXR and inhibit inflammation, serving as potential candidates or lead compounds for MASLD therapy.

[KEY WORDS] FXR agonist; Inflammation; Dual modulator; Natural products; MASLD

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Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD), formerly referred to as nonalcoholic fatty liver disease (NAFLD) [1], is the most prevalent chronic liver disease worldwide [2]. MASLD encompasses a progression of hepatic pathologies, from simple steatosis to metabolic dysfunction-associated steatohepatitis (MASH, previously termed nonalcoholic steatohepatitis), fibrosis, and potentially hepatocellular carcinoma (HCC). The etiology of MASLD is multifactorial, involving lipid accumulation, inflammation, hepatocellular injury, and progressive fibrosis [3, 4]. MASLD is now a leading cause of end-stage liver disease and a well-established risk factor for HCC. Despite the high global burden of MASLD, the FDA has approved only one therapeutic

agent, Resmetirom, emphasizing the urgent need for further pharmacological interventions to address this complex disease comprehensively.

Farnesoid X receptor (FXR), a member of the nuclear receptor superfamily of ligand-activated transcription factors, is highly expressed in the liver [5]. Research indicates that FXR and its target genes contribute to metabolic regulation and fibrogenesis suppression [5-7]. Furthermore, studies have shown that FXR suppresses inflammation by inhibiting NF-κB [8] or the NLRP3 inflammasome [9]. Consequently, FXR is widely recognized as a promising target for MASH treatment. Numerous FXR agonists with diverse chemical structures have been developed for MASH therapy [10]. Among these, obeticholic acid (OCA) emerged as the pioneering frontrunner and the first successful MASH therapy in clinical trials. However, OCA did not receive regulatory approval as a MASH pharmacotherapy due to its moderate benefits not outweighing its safety risks [11].

While OCA demonstrated notable improvements in liver histopathology for patients with MASH and achieved certain endpoints in clinical trials, its hepatoprotective efficacy remains suboptimal. In the phase 3 REGENERATE trial (NCT 02548351), the primary endpoint of MASH resolution did not reach statistical significance. This endpoint encompasses specific conditions: fatty liver disease without steatohepatitis or absence of fatty liver disease, a NAFLD activity score (NAS) of 0 for ballooning, and a NAS of 0−1 for inflammation, without fibrosis progression. Improvement in lobular inflammation by at least 1 point was observed in 35% of placebo patients, 39% in the OCA 10 mg group (P = 0.34), and 44% in the OCA 25 mg group (P = 0.032) [12]. The phase 2 FLINT trial (NCT01265498) showed improvement in lobular inflammation in 35% of placebo patients and 54% in the OCA group (P = 0.006), while portal inflammation improvement was observed in 13% of placebo patients and 12% in the OCA group (P = 0.90) [13]. These findings suggest that FXR agonists have a limited impact on inflammation.

Given its limited impact on inflammation, coadministration with anti-inflammatory agents may yield synergistic effects. C-C motif receptor 2/5 (CCR2/5) is a recognized target for inflammation and MASH [14]. Cenicriviroc, a CCR2/5 dual antagonist, is currently under evaluation for MASH treatment. A preclinical study demonstrated enhanced hepatoprotection when tropifexor, another FXR agonist, was combined with cenicriviroc [15]. However, a randomized, double-blind, multicenter, phase 2b clinical trial (NCT03517540) showed no significant improvement in ALT, body weight, or histological endpoints with combination therapy compared to monotherapy [16]. This outcome may be attributed to cenicriviroc's lack of efficacy against MASH (NCT03028740) [17]. Consequently, there is a pressing need to identify novel molecules with potent anti-inflammatory properties that can act synergistically with FXR agonists in combating MASH.

Numerous natural products demonstrate promising anti-inflammatory effects through diverse mechanisms [18-24]. This study aimed to investigate potential FXR agonists from natural sources that exhibit potent anti-inflammatory properties, potentially serving as candidates or lead compounds for MASLD therapy.

Materials and Methods

Agents

Natural Product Library (HY-L021) for screening, apigenin (HY-N1201, purity, 98.03%), genistein (HY-14596, purity, 99.18%), fisetin (HY-N0182, purity, ≥ 98%), formononetin (HY-N0183, purity, 97.67%), baicalein (HY-N0196, purity, 98.84%), biochanin A (HY-14595, purity, 99.01%), chrysin (HY-14589, purity, 99.55%), 7-methoxyisoflavone (HY-N6631, purity, 99.73%), calycosin (HY-N0519, purity, 99.8%), 5-methyl-7-methoxyisoflavone (HY-N1993, purity, 99.78%), ipriflavone (HY-N0094, purity, ≥ 98%), tectorigenin (HY-N0792, purity, 99.36%), glycitein (HY-N0016, purity, 98.03%), OCA (HY-12222, purity, ≥ 98%), and Z-guggulsterone (GS, HY-110066, purity, 98.88%) were acquired from MedChemExpress (Monmouth Junction, NJ, USA). Methylophiopogonone A (D266807, purity, 99.15%), methylophiopogonanone B (A1180, purity, 99.77%), 4′,7-dimethoxyisoflavone (D113596, purity, ≥ 98%) and glycyrrhisoflavone (D113348, purity, ≥ 98%) were obtained from the National Center for Standard Materials of China (Beijing, China). Palmitic acid (PA,76119) and dimethyl sulfoxide (67-68-5) were procured from Sigma–Aldrich (St. Louis, MO, USA). Recombinant murine TNF-α (315-01A) was obtained from PeproTech (Rocky Hill, USA). Recombinant FXR protein was sourced from Sangon Biotech (Shanghai, China). Modified Oil Red O Staining Kit (C0158S) was acquired from Beyotime Biotechnology (Shanghai, China).

Molecular docking

The ligand binding domain (LBD) of FXR (PDB code: 1OSH) was utilized for molecular docking [25]. The Protein Preparation Wizard module in Schrodinger (2018) was employed to perform various steps, including the removal of crystallized water molecules, the addition of missing loops, the repair of imperfect sidechains, and the determination of protonated states of the residues. The active pocket was defined based on the original ligand in the crystal complex. The FXR LBD was then designated as the receptor with the docking space set to 20 Å × 20 Å × 20 Å around the active pocket. A total of 3922 compounds from the Natural Product Library (HY-L021) were prepared using Discovery Studio 2019 to standardize their structures. Standard Glide docking (*Glide* SP) was employed to screen the library. Precision Glide docking (*Glide* XP) was utilized to analyze the top-scored 1,000 molecules. The top 500 molecules were subsequently clustered.

Surface plasmon resonance (SPR) binding assay

A surface plasmon resonance (SPR) binding assay was performed using a Biacore T200 (General Electric Company) to assess interactions between FXR protein and test compounds in real-time [26]. Recombinant FXR protein was immobilized on a CM5 sensor chip, while test compounds, di-

luted in phosphate-buffered saline (PBS) to concentrations ranging from 0.078 to 66.7 μmol·L^{-1} were flowed over the chip surface at a rate of 30 μL·min^{-1}. The contact and dissociation times were set to 60 s or 120 s, respectively. Blank flow cells without immobilized protein served as controls. The binding affinity of the test compounds to the FXR protein was quantified by calculating the equilibrium dissociation constant (K_D).

Cell culture and treatment

Murine AML12 cells were procured from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultivated in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum and antibiotics (100 U·mL^{-1} spenicillin and 100 μg·mL^{-1} streptomycin). Human HepG2 cells were acquired from ATCC and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin). Both cell lines were cultured at 37 ℃ in a humidified atmosphere with 5% CO_2.

To assess the impact of these compounds on FXR transactivation activity, AML12 cells were treated with the test compounds at 50 μmol·L^{-1}. To evaluate their effect on inflammation, AML12 cells were exposed to murine TNF-α (20 ng·mL^{-1}) for 2 h with or without the test compounds (10 μmol·L^{-1}). To examine the compounds' influence on MASH, hepatocytes were treated with PA-Na (0.4 mmol·L^{-1}) for 4 h in the presence or absence of the test compounds (25, 50, and 100 μmol·L^{-1} for compounds **2**, **8**, and **12**, or 6.25, 12.5, and 25 μmol·L^{-1} for compound **6**, respectively).

Cell transfection

Negative control siRNA and FXR siRNA were procured from Nanjing EKBIO Technology. Cell transfection with these siRNAs was performed using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions [27].

Reporter gene analysis

The cells underwent co-transfection with the FXR expression plasmid and the FXR target gene *OSTb* promoter reporter vector, utilizing Lipofectamine 3000 reagent according to previously described methods [6].

To assess the agonistic effect on FXR, cells were exposed to the test compounds or OCA, a known FXR agonist. For evaluating the antagonistic effect on FXR, cells were first treated with test compounds or GS (10 μmol·L^{-1}), a validated FXR antagonist, followed by OCA (10 μmol·L^{-1}). Subsequently, the cells were lysed, and luciferase activities were measured using a Bright-Lite Luciferase Assay System (Vazyme Biotech Co., Ltd., Nanjing, China).

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was conducted according to previously established protocols [28]. In summary, total RNA was extracted from cells using RNAiso Plus reagent (TaKaRa Biotechnology, Tokyo, Japan). The isolated RNA was then reverse transcribed utilizing the PrimeScript RT Reagent Kit with gDNA Eraser (Vazyme Biotech Co., Ltd, Nanjing, China). RT–PCR was subsequently performed using ChamQ SYBR quantitative PCR (qPCR) Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China). The sequences of primers employed are provided in Supporting Information Table S1.

Measurement of lipid content

HepG2 cells were stained with oil red O after treatment with compounds using the Modified Oil Red O Staining Kit, following the manufacturer's protocols. The positive area in the images was quantified using ImageJ software [29, 30].

Statistical analysis

analysis was conducted using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA), with results presented as mean ± standard error of the mean (SEM). Statistical comparisons between two groups were performed using a two-tailed Student's *t*-test, while comparisons among multiple groups employed one-way analysis of variance (ANOVA) with Tukey post hoc analysis. Statistical significance was established at *P* values less than 0.05.

Results

Prediction of FXR ligands from natural products via molecular docking

A structure-guided virtual screening and molecular docking approach was employed to identify potential FXR ligands from natural products. The FXR LBD (PDB code: 1OSH) was utilized for molecular docking (Fig. 1A). 3922 compounds from the Natural Product Library (HY-L021, MedChemExpress) were prepared using Discovery Studio 2019. OCA, a well-established FXR agonist, served as a positive control. The binding affinity, defined as the energy difference between the lowest binding energy state and the average binding energy, was recorded and used for ranking. OCA exhibited an affinity of −10.08 kcal·mol^{-1}. The docking results of OCA with FXR aligned well with previously published crystallographic data [25], validating the docking methodology. Using identical docking parameters, compounds from the Natural Product Library were docked into the LBD of FXR. Notably, 17 natural compounds were predicted to interact with the LBD of FXR with high affinity. Furthermore, compounds **1**, **2**, and **12** were predicted to have affinities comparable to OCA. Their chemical structures are illustrated in Fig. 1B, with their Glide scores presented in Table 1. In summary, the docking results suggest that 17 compounds from the Natural Product Library potentially bind to FXR, warranting further investigation as novel FXR ligands.

Identification of potential FXR ligands that directly bind the FXR protein

To identify potential FXR ligands, we determined the binding potencies of the aforementioned 17 compounds to purified human FXR protein using SPR technology-based Biacore assay. OCA served as a positive control. Fig. 2 displays the sensor diagram and fitting curve. The results demonstrated that compounds **1**, **2**, **4** to **8**, and **10** to **17**, as well as OCA, directly bound to the FXR protein in a concen-

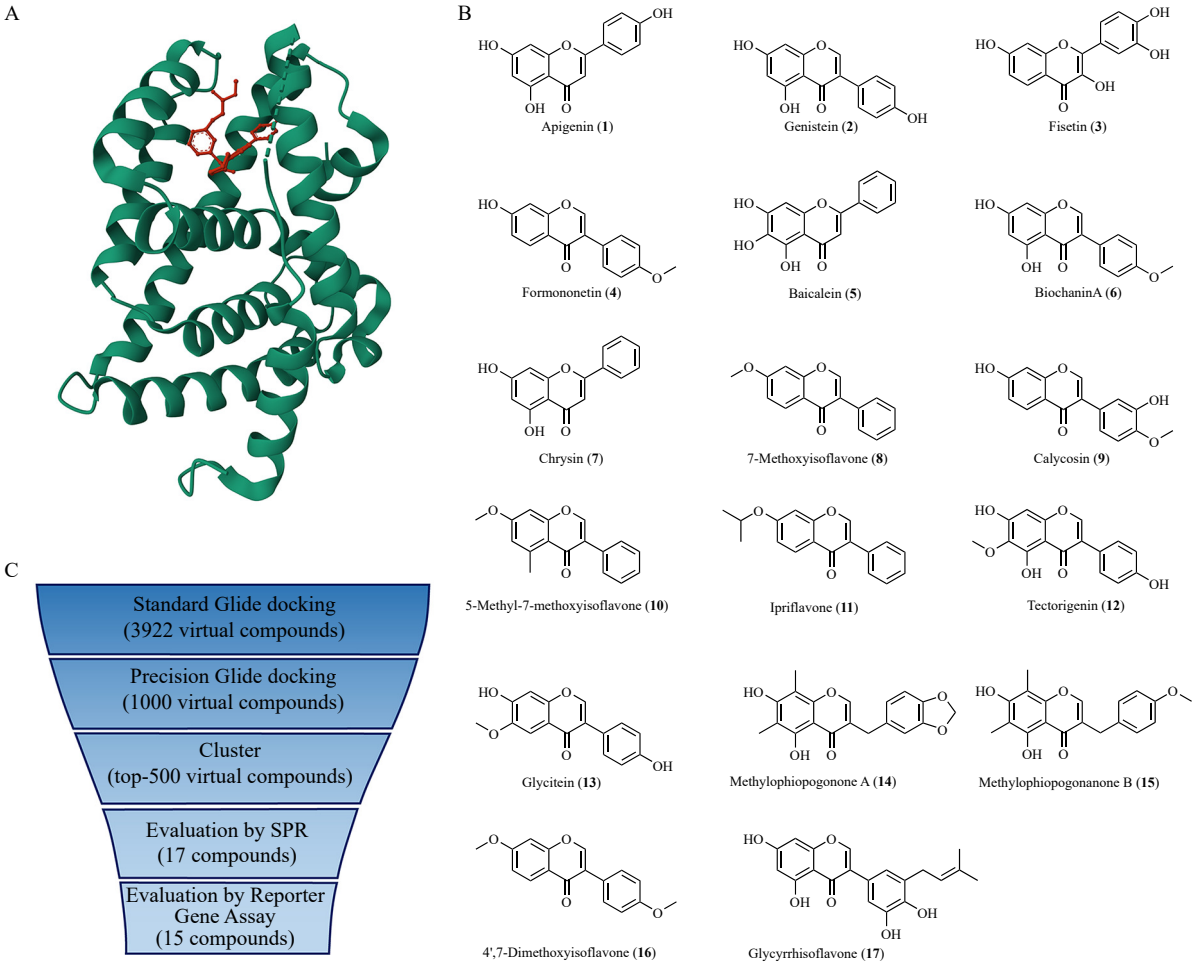


Fig. 1 ***In silico*** **prediction of FXR ligands by molecular docking. (A) Structure of FXR LBD. (B) Chemical structures of potential ligands. (C) Workflow for virtual screening of FXR ligands.**

tration-dependent manner. Compounds **3** and **9** exhibited minimal binding to the FXR protein fixed on the sensor chip surface. The binding affinity of OCA and the test compounds to the FXR protein was evaluated using K_D values (Table 1). We determined the K_D value of OCA binding to be 2.272×10^{-6} μmol·L^{-1}. Pleasantly, the K_D values for the interaction between 15 compounds and the FXR protein ranged from 8.156×10^{-5} to 3.469×10^{-7} μmol·L^{-1}. Furthermore, the K_D values for compounds **2**, **6**, **8**, and **15** were lower than that of OCA. Collectively, these results indicate that compounds **1**, **2**, **4**–**8**, and **10**–**17** directly bind to the FXR protein with high affinities.

Validation of FXR agonists and antagonists

We identified potential FXR ligands through SPR assays; however, distinguishing between agonists and antagonists remains challenging. To further investigate whether the aforementioned compounds (compounds **1**, **2**, **4**–**8**, and **10**–**17**) can transactivate FXR, their effects on FXR activation were examined using a luciferase reporter gene assay. FXR, a nuclear receptor, regulates the transcription of various target genes, including *Shp* and *Ostb*. A reporter gene system containing either the *Shp* promoter or the *Ostb* promoter was constructed. Results indicated that the *Ostb* promoter reporter is more sensitive than the *Shp* promoter reporter, consistent with their mRNA expression in the RT-PCR assay in response to OCA (Fig. S1). Consequently, the *Ostb* promoter reporter was employed in this study. AML12 cells were transiently cotransfected with an FXR expression plasmid and an FXR target gene *OSTb* promoter reporter vector. As anticipated, OCA treatment significantly increased the luciferase activity of the FXR reporter gene in a dose-dependent manner (Fig. 3A). GS significantly decreased the luciferase activity of the FXR reporter gene when coadministered with OCA but had a minimal effect on its luciferase activity when individually administered (Figs. 3 and 3C). These results suggest that this reporter gene system is suitable for identifying FXR agonists and antagonists. The efficacies of the aforementioned ligands on FXR transactivation activity were then assessed using this system. The potential FXR ligands exhibited varying activities toward FXR. Compounds **2**, **4**, **6**, **8**, **10**, **11**, **12**, **13**, and **16** significantly increased the luciferase activity of FXR. Comparing the activity of these natural compounds, compounds **2** and **6** demonstrated effects similar to OCA (Fig. 3D). To further validate the agonistic effect of these compounds on FXR,

Table 1 Glide scores and K_D values of 17 compounds that can fit into FXR LBD.

No.	Name	Glide scores (kcal·mol^{-1})	K_D values
1	Apigenin	−10.0934	4.412×10^{-6}
2	Genistein	−9.9588	5.941×10^{-7}
3	Fisetin	−9.8189	Non-convergence
4	Formononetin	−9.3634	1.323×10^{-6}
5	Baicalein	−9.3222	2.761×10^{-6}
6	Biochanin A	−9.2301	3.469×10^{-7}
7	Chrysin	−9.2117	2.850×10^{-6}
8	7-Methoxyisoflavone	−9.4862	8.929×10^{-7}
9	Calycosin	−9.3139	Non-convergence
10	5-Methyl-7-methoxyisoflavone	−9.3758	4.232×10^{-6}
11	Ipriflavone	−8.9740	4.969×10^{-6}
12	Tectorigenin	−10.0182	2.612×10^{-6}
13	Glycitein	−9.5116	8.156×10^{-5}
14	Methylophiopogonone A	−8.4636	2.156×10^{-5}
15	Methylophiopogonanone B	−8.2072	6.172×10^{-7}
16	4′,7-Dimethoxyisoflavone	−8.7459	1.849×10^{-6}
17	Glycyrrhisoflavone	−8.8813	3.075×10^{-6}
/	OCA	−10.08	2.272×10^{-6}

RT–PCR assays were conducted. Corroborating with the results from reporter gene analysis, compounds **2**, **4**, **6**, **8**, **10**, **11**, **12**, **13**, and **16** significantly upregulated the mRNA levels of *Shp* and *Ostb* (Fig. 3E).

In addition to the aforementioned validated agonists, compounds **1**, **5**, **7**, **14**, **15**, and **17,** which directly bind to the FXR protein in SPR assays, were identified as potential FXR ligands. Some of these compounds may function as FXR antagonists, inhibiting FXR transactivation, while others may bind without significantly affecting its trans-activities. To identify potential FXR antagonists among these ligands, we employed a reporter gene system. Compounds **14**, **15**, and **17** significantly inhibited FXR luciferase activity in cells co-treated with OCA (Fig. 3F). This observation was further corroborated by RT-PCR assay (Fig. 3G). However, compounds **1**, **5**, and **7** neither trans-activated FXR nor antagonized OCA-induced trans-activation (Fig. S2).

Collectively, these findings demonstrate that compounds **2**, **4**, **6**, **8**, **10**, **11**, **12**, **13**, and **16** function as FXR agonists, activating FXR transactivation. Conversely, compounds **14**, **15**, and **17** act as FXR antagonists, inhibiting FXR transactivation. Compounds **1**, **5**, and **7** exhibit minimal impact on FXR trans-activity.

Structure-activity relationship analysis

Given that these newly identified FXR agonists and antagonists share a common chemical skeleton, we conducted a structure-activity relationship analysis. The antagonist belongs to the flavone family, while the agonists are part of the isoflavone family, indicating that the positioning of the B ring at the C3 position induced an agonistic effect on FXR. Subsequently, we performed a structure-activity relationship (SAR) analysis of the aforementioned FXR agonists. Fig. 4 displays the concentration-activating activity plots for all agonists. Their EC_{50} values, simulated using Prism software, are presented in Table 2. Among the nine isoflavone analogs, compounds **8**, **11**, and **16** demonstrated potent agonistic activity with EC_{50} values below 0.1 μmol·L^{-1}, followed by compounds **4**, **6**, **10**, and **12** with EC_{50} values under 0.5 μmol·L^{-1}, while compound **13** exhibited the weakest activity. Based on the EC_{50} values of agonists listed in Table 2, the introduction of a substituent at the C7 position (R1) is crucial for its activity. Specifically, the addition of isopropoxyl or methoxyl

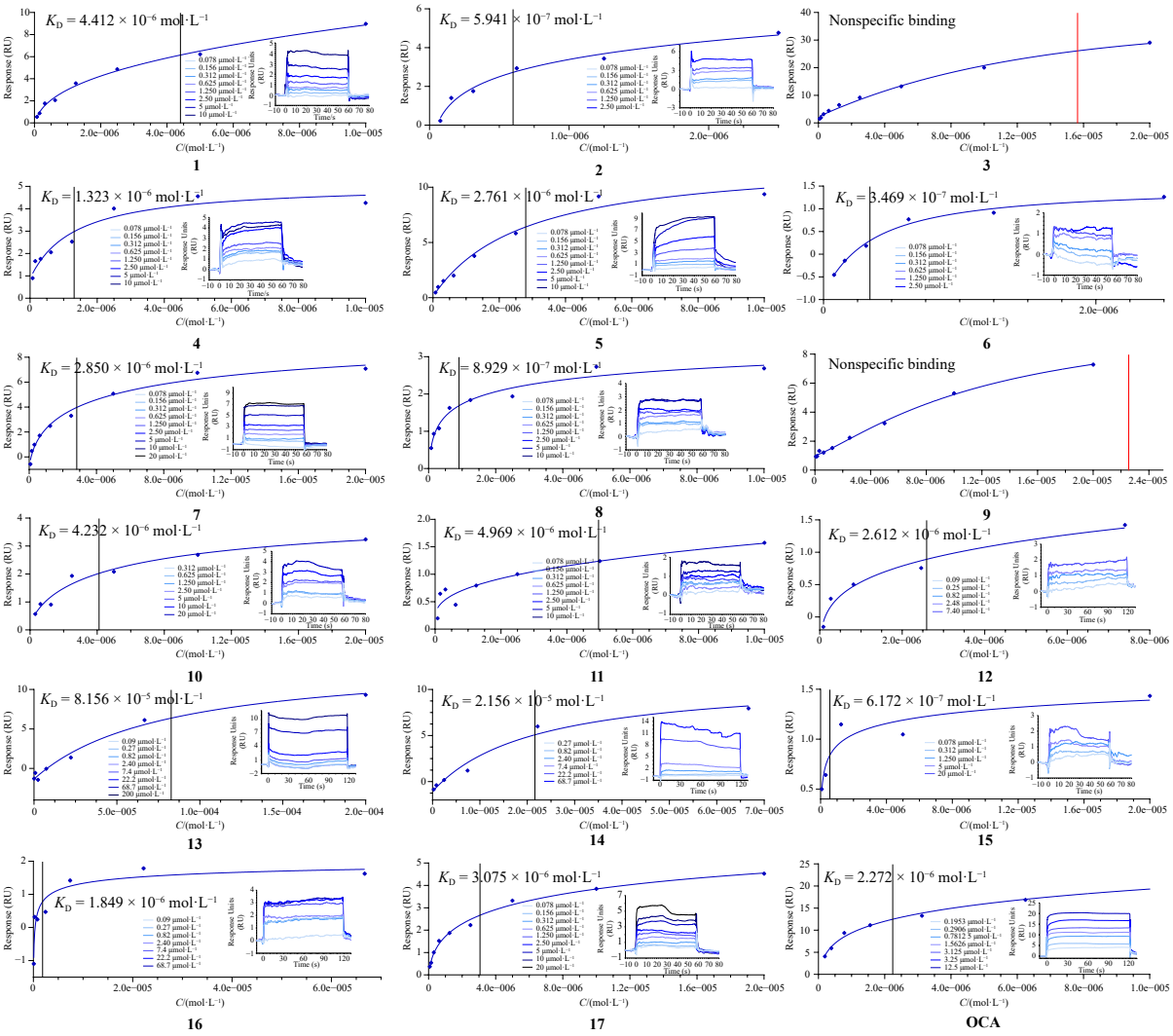


Fig. 2 Affinity sensing diagram and fitting curve of potential ligands with FXR.

groups significantly enhances the agonistic efficacy of isoflavones. Furthermore, introducing a methoxyl group at the C6 position (R2) also augments the agonistic efficacy, as evidenced by comparing compounds **2** and **12**. However, a comparison of compounds **8** and **10** reveals that the methyl group at the C5 position (R3) diminishes the agonistic efficacy. The hydroxyl group at the C5 position may also influence its agonistic activity, with this effect largely dependent on the substituent at the C6 position. The introduction of a hydroxyl group at the C5 position decreases its agonistic activity when the C6 position lacks a substituent (compounds **4** *vs*. **6**), while it increases agonistic activity when the C6 position contains a methoxy group (compounds **12** *vs* **13**).

Furthermore, the methoxy group at the C4' position (R4) enhances agonistic efficacy in comparison to the hydroxyl group.

FXR-dependent and independent anti-inflammatory effects of the above agonists

The aforementioned studies successfully identified several FXR agonists. Subsequently, we evaluated their anti-inflammatory effects. An inflammatory response in hepatocytes was induced by TNF-α treatment. Notably, treatment with compounds **2**, **6**, **8**, and **12** significantly suppressed the mRNA levels of *Tnfa* (Fig. 5A). Previous research has shown that FXR activation can inhibit inflammation by suppressing NF-*κ*B. We sought to determine whether the anti-inflammatory effects of these natural compounds were dependent on their FXR agonistic activity. To this end, *Fxr*-specific siRNA was used to knock down its expression in hepatocytes. In cells transfected with scramble siRNA, treatment with compounds **2**, **6**, **8**, and **12** significantly reduced *Tnfa* mRNA expression (Fig. 5B). In cells transfected with *Fxr* siRNA, compounds **2**, **6**, and **8** significantly decreased *Tnfa* mRNA levels, while compound **12** failed to reduce its expression (Fig. 5B). These results suggest that compounds **2**, **6**, **8**, and **12**, exhibit potent anti-inflammatory effects. The effect of compound **12** is largely dependent on its FXR agonistic efficacy, while compounds **2**, **6**, and **8** are FXR agonists with additional anti-inflammatory properties.

MASH is characterized by lipid accumulation and in-

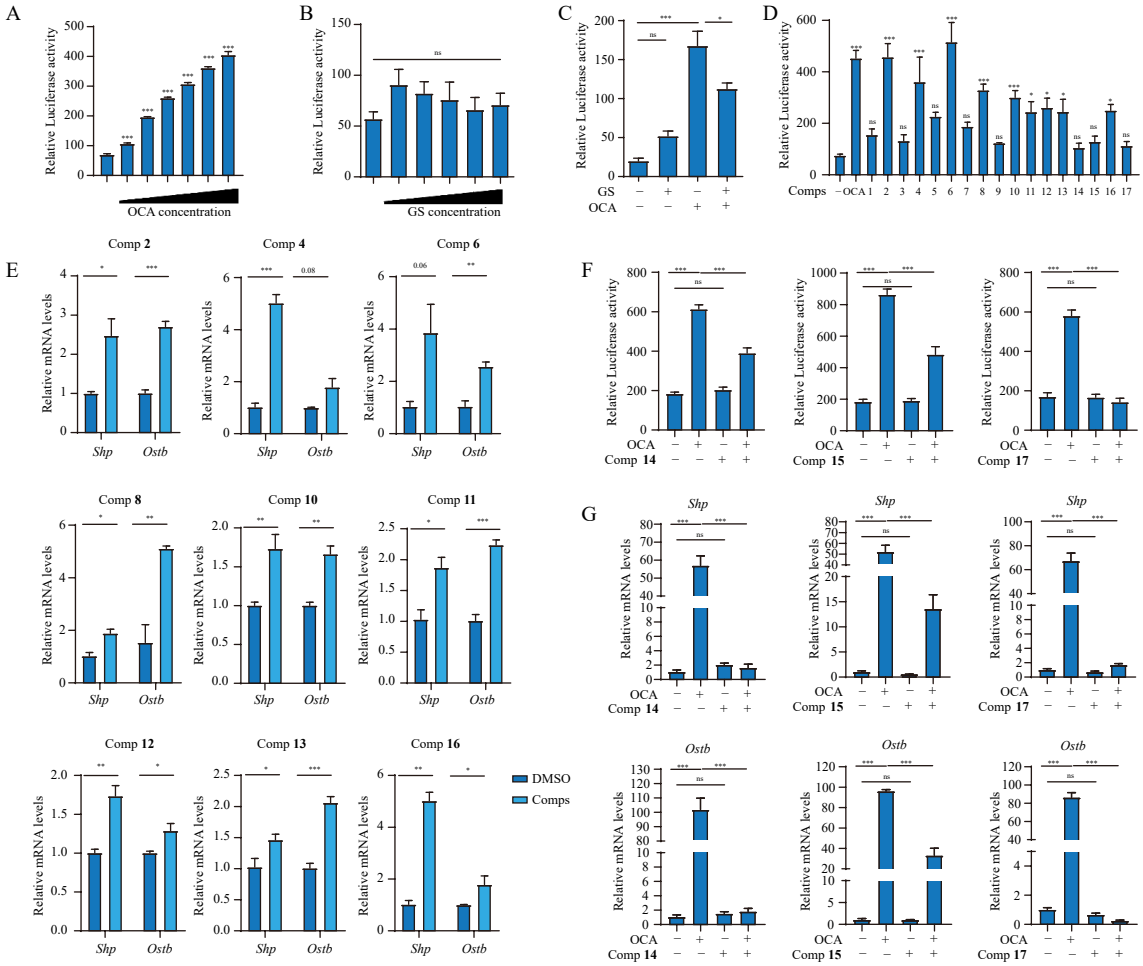


Fig. 3 Identification of FXR agonists and antagonists. (A) Effect of OCA (0.25−10 μmol·L^{-1}) on luciferase activity of FXR (*n* = 5). (B) Effects of GS (3−100 μmol·L^{-1}) on luciferase activity of FXR (*n* = 8). (C) Effect of GS (10 μmol·L^{-1}) in the presence or absence of OCA (10 μmol·L^{-1}) on luciferase activity of FXR (*n* = 5). (D) Effects of potential FXR ligands from natural products on luciferase activity (*n* = 5). (E) Effects of potential FXR agonists from natural products on mRNA expression of *Shp* and *Ostb* (*n* = 3) . (F−G) Effects of potential FXR antagonists on FXR luciferase activity (*n* = 8) (F) and mRNA expression of *Shp* and *Ostb* (*n* = 3) (G). Results are presented as mean ± SEM. Statistical significance was determined by a two-tailed paired *t*-test (E) or ANOVA (F−G). * *P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns, not statistically significant.

flammation. To assess the potential therapeutic effects of compounds **2**, **6**, **8**, and **12** on MASH, an *in vitro* evaluation was conducted. HepG2 cells were treated with PA-Na to induce fat accumulation and inflammation. Treatment with compounds **2**, **6**, and **8** resulted in a significant reduction of fat accumulation (Fig. 5C). Furthermore, these compounds significantly decreased the mRNA expression levels of inflammation-related genes (Fig. 5D). Similarly, compound **12** demonstrated efficacy in reducing cellular lipid accumulation and mRNA levels of inflammation-related genes (Fig. 5C, 5D). These findings suggest that compounds **2**, **6**, and **8** may be promising candidates or lead compounds for MASH therapy.

Discussion

MASLD represents a complex spectrum of liver diseases driven by multiple factors, including metabolic imbalance and inflammatory processes [3]. The scientific community has recognized that addressing this multifaceted disorder requires a multifaceted therapeutic approach to target its diverse pathological components [31, 32]. In this investigation, we identified four naturally occurring compounds that concurrently activate FXR and suppress inflammation.

MASLD represents a leading cause of chronic liver disease globally [3]. Despite its high prevalence and substantial burden, no effective medication has been approved for MASLD treatment. A primary reason for this lack of medication is the multifaceted nature of the disease, involving various pathologic factors such as metabolic dyshomeostasis, inflammation activation, and oxidative stress. These factors simultaneously or sequentially drive the development and progression of MASLD. The coadministration of two or more medicines targeting different pathogenic factors is anticipated to achieve complementary or synergistic effects [11]. Consequently, combination therapies have gained widespread acceptance as a rational alternative approach to address the

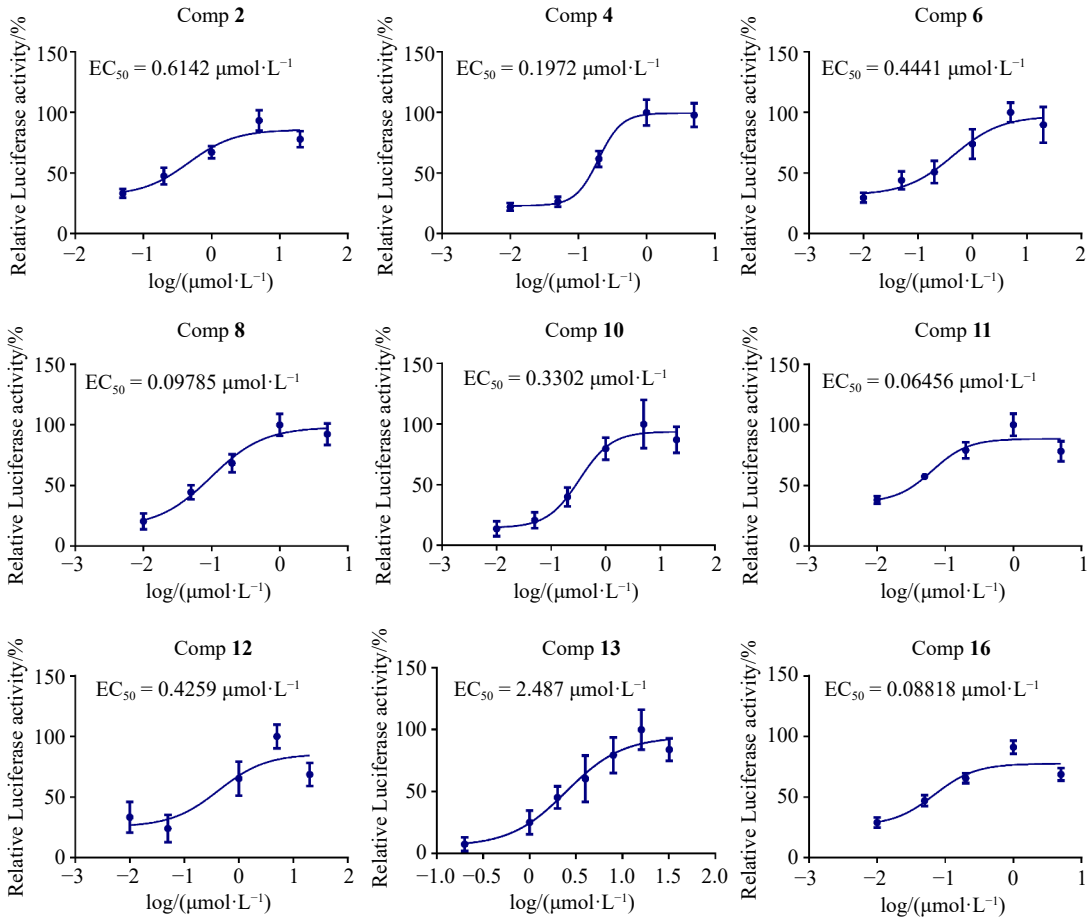


Fig. 4 Dose-dependent effects of potential agonists on FXR luciferase activity.

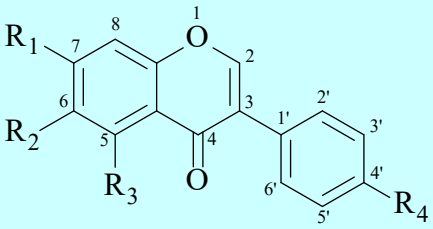
complex pathophysiology of the disease [32, 33]. However, the resulting polypharmacy, involving multiple medications, presents disadvantages, such as intricate and problematic drug-drug interactions and additional adverse effects. Many limitations of polypharmacology can be mitigated by employing multitarget agents that simultaneously address multiple desired therapeutic mechanisms.

FXR, a member of the nuclear receptor superfamily of ligand-activated transcription factors, is highly expressed in the liver. FXR and its target genes have been reported to contribute to metabolic regulation [34] and fibrogenesis suppression [6]. Significant efforts have been dedicated to screening and developing FXR agonists for the treatment of liver diseases [10]. In this study, we identified several FXR agonists from natural products. Compounds **2**, **4**, **6**, **8**, **10**, **11**, **12**, **13**, and **16** function as FXR agonists that directly bind to and trans-activate FXR. Consistent with our findings, the binding of compound **2** (genistein) to FXR was recently predicted and reported through molecular docking [35]. Our study provides substantial evidence for the direct binding and regulation of compound **2** (genistein) on FXR. Compound **4** (formononetin) and compound **12** (tectorigenin) were recently reported to alleviate alpha-naphthylisothiocyanate-induced cholestatic liver injury and upregulate the expression of multidrug resistance protein 2 and bile salt export pump, classical target genes of FXR [36, 37]. These results suggest that the compounds regulate FXR signaling, and our present study further provides compelling evidence that the compounds directly bind to FXR and exert hepatoprotective effects.

While FXR demonstrates various beneficial properties, therapy based solely on pure FXR agonists appears insufficient for patients with MASH. Consequently, drug combinations incorporating FXR agonists have been proposed and validated for enhanced hepatoprotection. Several strategies combining FXR agonists with apoptosis inhibitors, glucagon-like peptide-1 receptor agonists, dipeptidyl peptidase-4 inhibitors, SIRT1 activators, or SUMO inhibitors have been suggested [11]. Furthermore, researchers have developed dual modulators that simultaneously regulate FXR and other targets, including FXR/PPARγ dual agonists, FXR/fatty acid binding protein 1 dual modulators, and FXR/soluble epoxide hydrolase (sEH) dual modulators [11]. These combinations and dual modulators demonstrate excellent hepatoprotective effects.

To address the limitations in anti-inflammatory efficacy observed in clinical trials, crucial strategies for drug combinations have been proposed. Preclinical studies have demonstrated superior hepatoprotection with the combination of

Table 2 EC_{50} value of FXR agonists identified by report gene system.

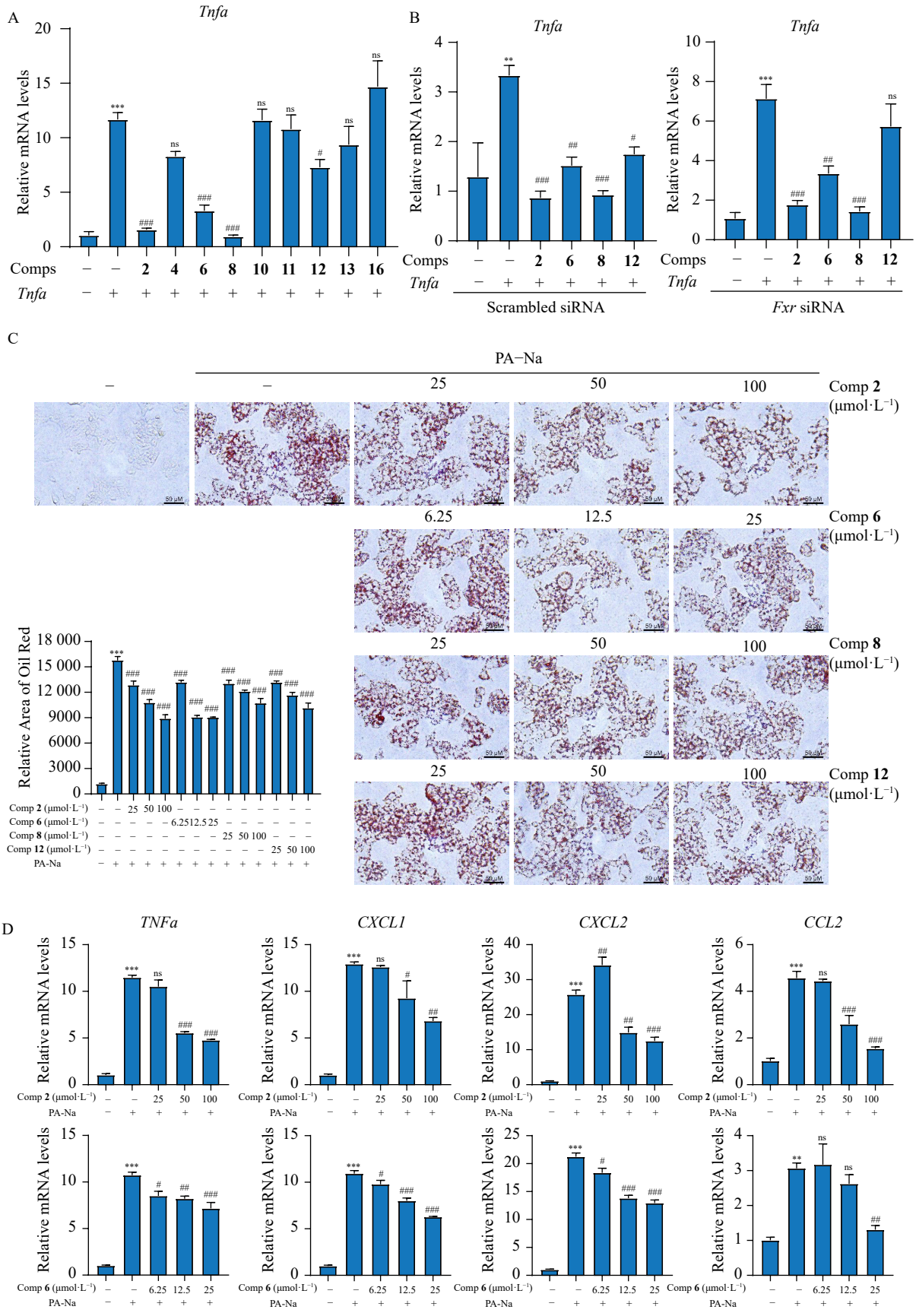


No.	Name	R1	R2	R3	R4	EC_{50} (μmol·L^{-1})
2	Genistein	HO	H	HO	HO	0.6142
4	Formononetin	HO	H	H		0.1972
6	Biochanin A	HO	H	HO		0.4441
8	7-Methoxyisoflavone		H	H	H	0.0979
10	5-Methyl-7-methoxyisoflavone		H	CH_3	H	0.2685
11	Ipriflavone		H	H	H	0.0646
12	Tectorigenin	HO		HO	HO	0.4259
13	Glycitein	HO		H	HO	2.4870
16	4′,7-Dimethoxyisoflavone		H	H		0.0882

tropifexor (an FXR agonist) and cenicriviroc (a C-C motif receptor 2/5 (CCR2/5) antagonist). A phase 2 clinical trial is currently evaluating the effect of this combination in patients with MASH (NCT03517540) [38]. Considering the drawbacks of drug combinations, we aimed to identify dual modulators that simultaneously function as FXR agonists and inflammation inhibitors. We successfully identified three dual modulators from natural compounds, exhibiting excellent agonistic efficacy against FXR and robust anti-inflammatory activities. Previous studies have shown that FXR agonists can inhibit inflammation [39]. Among these identified dual modulators, compound **12** (tectorigenin) exhibits anti-inflammatory activity by regulating FXR, while compounds **2** (genistein), **6** (biochanin A), and **8** (7-methoxyisoflavone) demonstrate anti-inflammatory activities independent of their FXR regulation. We have previously demonstrated that FXR protein degrades in inflammatory hepatocytes. Moreover, the loss of FXR protein is one of the main factors limiting the hepatoprotective efficacy of FXR agonists [33]. Since compound **12** exhibited potent anti-inflammatory effects primarily through its FXR agonistic efficacy, we posit that the loss of FXR protein may limit both its agonistic and anti-inflammatory effects. Conversely, compounds **2**, **6**, and **8** displayed anti-inflammatory properties irrespective of their FXR agonist activity. Inhibition of inflammation would preclude FXR protein degradation, facilitating the metabolic regulation and anti-inflammatory effects of these compounds *via* FXR. Hence, we hypothesize that the effectiveness of compounds with FXR-independent anti-inflammatory activity would be greater than that of compounds with FXR-dependent anti-inflammatory activity. Indeed, these dual modulators show powerful hepatoprotective effects in PA-treated hepatocytes. Further investigation is needed to evaluate their hepatoprotective effects against MASH in animal models. Compounds **4** (formononetin) and **12** (tectorigenin) have been reported to exhibit excellent effects against cholestasis in animals [36, 37]. More recently, compound **4** (formononetin) has demonstrated excellent hepatoprotective effects against MASLD [40]. Based on this evidence, we believe these compounds possess drug-like properties. Natural products are valuable resources for developing novel drugs and can serve as promising candidates, or at least lead compounds, for MASH therapy.

Conclusion

In summary, this study identified 15 ligands of the FXR protein derived from natural products, with 9 demonstrating FXR transactivation. Notably, compounds **2**, **6**, and **8** (genistein, biochanin A, and 7-methoxyisoflavone, respectively) exhibited significant anti-inflammatory activity independent of FXR, while compound **12** (tectorigenin) displayed FXR-dependent activity. These compounds effectively reduced lipid accumulation and inflammatory response in PA-loaded hepatocytes, positioning them as potential candidates for MASH therapy.


A
Tnfa
Relative mRNA levels
Comps
Tnfa
B
Tnfa
Scrambled siRNA
Fxr siRNA
C
PA−Na
Comp 2 (μmol·L⁻¹)
Comp 6 (μmol·L⁻¹)
Comp 8 (μmol·L⁻¹)
Comp 12 (μmol·L⁻¹)
Relative Area of Oil Red
D
TNFa
CXCL1
CXCL2
CCL2

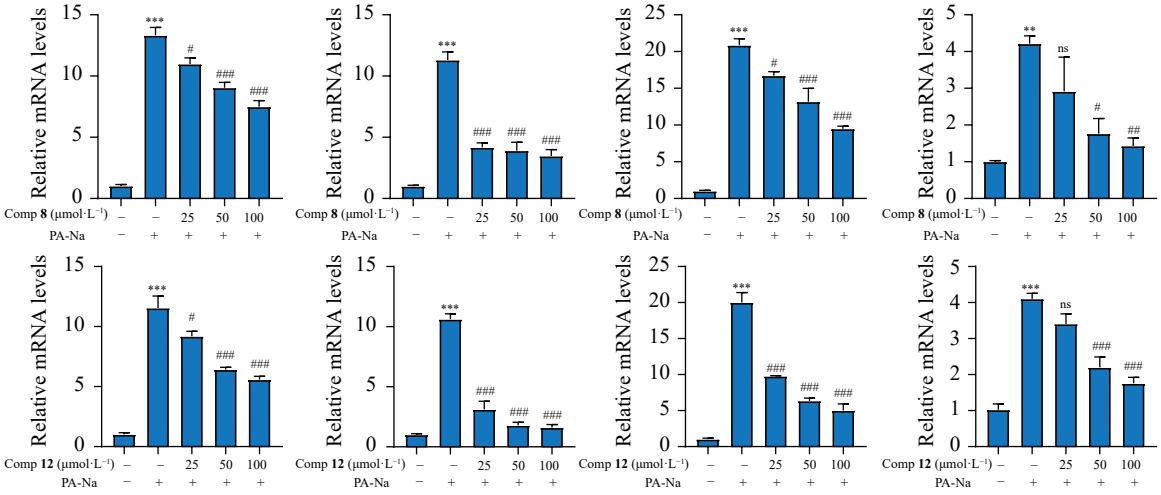


Fig. 5 Hepatoprotective effects of FXR agonists. (A) Impact of FXR agonists on *Tnfa* expression in cells under inflammatory conditions (n = 3). (B) Influence of compounds 2, 6, 8, and 12 on *Tnfa* mRNA in inflammatory cells transfected with control siRNA (left) or *FXR*-specific siRNA (right) (n = 3). (C) Effects of compounds 2, 6, 8, and 12 on lipid accumulation induced by Palmitic acid (PA)-Na treatment (n = 3). (D) Impact of compounds 2, 6, 8, and 12 on inflammatory-related genes in cells treated with PA-Na (n = 3). Statistical significance was determined by ANOVA. Results are presented as mean ± SEM. Statistical significance was determined by ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* control; $^{\#}P < 0.05$, $^{\#\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *vs* model; and ns, statistically not significant.

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