Corylin induces UGT1A1 via PPARs/AhR and exerts hepatoprotection in mice

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Abstract

Uridine-5'-diphosphate-glucuronosyltransferase 1A1 (UGT1A1) is a key enzyme in the regulation of bilirubin metabolism and detoxification of a variety of internal and external substances, and its insufficient function can lead to hyperbilirubinemia and affect the metabolic clearance of drugs, food additives, and environmental toxicants. However, there is a lack of safe and effective UGT1A1 inducers in the clinic. This study presents Corylin, an efficient UGT1A1 inducer identified from a series of natural flavonoids. Notably, Corylin demonstrated potent induction of intracellular UGT1A1, excellent cell membrane permeability, and a favorable safety profile. Furthermore, nuclear receptor reporter gene assays revealed that Corylin dose-dependently activated PPAR and AhR nuclear receptors, with the most robust activation observed in the PPARβ subtype. In vivo experiments indicated that Corylin exhibited favorable safety profiles and significant hepatoprotective effects in mice with APAP-induced liver injury, accompanied by marked UGT1A1 expression. Collectively, this study suggests that Corylin is a potential UGT1A1 inducer and a promising candidate for the intervention of liver-related diseases.

Keyword: Corylin; UGT1A1 inducer; PPAR; AhR; live protection

1. Introduction

Drug metabolizing enzymes play a crucial role in the metabolism and elimination of various clinical drugs and toxins, directly influencing their efficacy and safety. Additionally, these enzymes are involved in the biotransformation of numerous endogenous substances within the human body, which is essential for maintaining normal physiological functions. Consequently, drug metabolizing enzymes are not only pivotal proteins in determining the personalized application of clinical drugs but also serve as therapeutic targets for various metabolic diseases. UGT1A1 (Uridine 5'-diphospho- glucuronosyltransferases UGT1A1) is an important phase II drug metabolizing enzyme in the human body. Notably, UGT1A1 enzyme plays a key role in the metabolism and detoxification of bilirubin[1]; however, decreased activity of UGT1A1 enzyme results in the accumulation of unconjugated and bilirubin, which increases the risk of hyperbilirubinemia[2, 3], such as Gilbert syndrome and Crigler-Najjar syndrome[4] and other hyperbilirubinemia risks, and may even cross the blood-brain barrier and cause irreversible neurological damage[5-7]. Meanwhile, the accumulated high concentration of bilirubin increases the metabolic burden on the liver, leading to hepatotoxicity[8] and exacerbating liver injury caused by bilirubin metabolism disorders[9]. In addition, the reduction of UGT1A1 enzyme activity also affects the pharmacokinetic behavior of drugs, which triggers clinically relevant drug-drug interactions, leading to the occurrence of toxic side effects[10, 11] and predisposing patients to drug-induced liver injury[12]. For example, accumulation of irinotecan in the body may cause neutropenia and severe diarrhea[13]. Therefore, the study of new UGT1A1 inducers for enhancing the metabolic clearance of bilirubin and drugs has become an important strategy for combating hyperbilirubinemia and drug-associated liver injury.

In recent years, domestic and foreign researchers have carried out a series of work around the screening of bilirubin metabolizing enzyme inducers, but the representative bilirubin metabolizing enzyme inducers that have been reported so far suffer from the lack of discovery of inducer species, insignificant in vivo effects, and poor safety. The use of characteristic natural products as lead compounds or drug candidates is an

advantage and an important way for new drug research and development in China. The rich natural drug resources with structural diversity form the material basis for drug discovery and screening. Making full use of modern drug screening technology for efficient and rapid drug screening of natural products is not only an urgent need for excavation and development of traditional Chinese medicine resources, but also provides guarantee for innovative drug research.

Flavonoids are one of the most common and widely distributed groups of plant compounds and are present in almost all plant tissues[14, 15]. As dietary components, flavonoids are recognized to have significant health benefits[16, 17]. Their health-promoting effects are mainly attributed to their antioxidant, anti-inflammatory, and anticancer properties[18, 19], as well as their modulatory effects on the function of key cellular enzymes. To date, researchers have isolated and characterized more than 10,000 flavonoids, which exhibit a high safety profile[20]. Flavonoids such as Acacetin[21], Apigenin[22], and Neobavaisoflavone[23] have been shown to accelerate the metabolic clearance of bilirubin and drugs by activating specific nuclear receptor pathways and upregulating the expression and activity of UGT1A1. Therefore, studying the role of flavonoids in UGT1A1 induction and their mechanisms has become an important research direction at present. Meanwhile, further validation of the hepatoprotective effects of flavonoids[16, 24] through mouse experiments not only helps to clarify the safety of their clinical applications, but also provides theoretical support for the development of personalized therapeutic strategies.

In summary, bilirubin metabolizing enzymes can be used as therapeutic targets for the treatment of hyperbilirubinemia and as personalized drug targets for mitigating drug toxicity induced by their insufficient function. This study aimed to identify novel inducers of UGT1A1 from flavonoids and isoflavonoids. Through screening a variety of natural flavonoids, we discovered that Corylin exhibited significant induction of UGT1A1 in HepG2 cells, outperforming the known positive inducer, chrysin. Building on this finding, we further investigated the induction mechanism of Corylin and its hepatoprotective effects in the liver. Our goal is to develop safe and effective novel UGT1A1 inducers, thereby providing a theoretical foundation for the treatment of

hyperbilirubinemia and the advancement of hepatoprotective drugs.

2. Materials and methods

2.1 Chemicals and Reagents

All test flavonoids and isoflavonoids (purity >98%) were purchased from TargetMol, and a stock solution (10 mM) was prepared by dissolving each compound in DMSO and stored at -80°C until use. HepG2, Caco2, and HEK293 cells were purchased from ATCC (USA). Reverse transcription kit and TB Green® Premix Ex Taq™ (Tli RNaseH plus) kit were purchased from Takara. UGT1A1 and GAPDH primers were synthesized by Sangon Biotech（Table S1）. Primary antibody UGT1A1 (ab194697), primary antibody GAPDH (ab9485), and secondary antibody IgG H&L (HRP) (ab6721) were purchased from Abcam. chromatography grade DMSO, methanol, formic acid, and acetonitrile were purchased from Aladdin. Alanine aminotransferase (ALT) test kit, aspartate aminotransferase (AST) test kit, lactate dehydrogenase (LDH) test kit, and total bilirubin (TBIL) assay kit were purchased from Nanjing Jianjian Bio.

SPF grade wild-type C57BL/6 male mice were purchased from Beijing Viton Lihua Laboratory Animal Technology Co., Ltd, aged 6~8 weeks, weighing 21~26 g. The mice were acclimatized and fed for 1 week prior to the start of the experiment, and were allowed to ingest and drink freely at an ambient temperature of 22~26°C, an ambient humidity of 50%~60%, and alternated between light and dark for 12 hours each. The animal experiments of this thesis were reviewed by the Ethical Review Committee of the Chemistry Analysis Team of Linyi University Students, and the experiments followed the 3R principle of giving humane care for the use of experimental animals, and complied with the national regulations related to the welfare of animal experiments.

2.2 Cell and treatment

HepG2 and Caco2 cells were cultured in DMEM/high-glucose medium containing 1% penicillin-streptomycin solution and 10% or 20% fetal bovine serum under humidified conditions at 37°C, 5% CO_2 and 95% air. The medium was replaced with fresh medium daily. For the induction assay, DMSO was used as a negative control and Chrysin was used as a positive control, and all compounds to be tested used were stored in DMSO

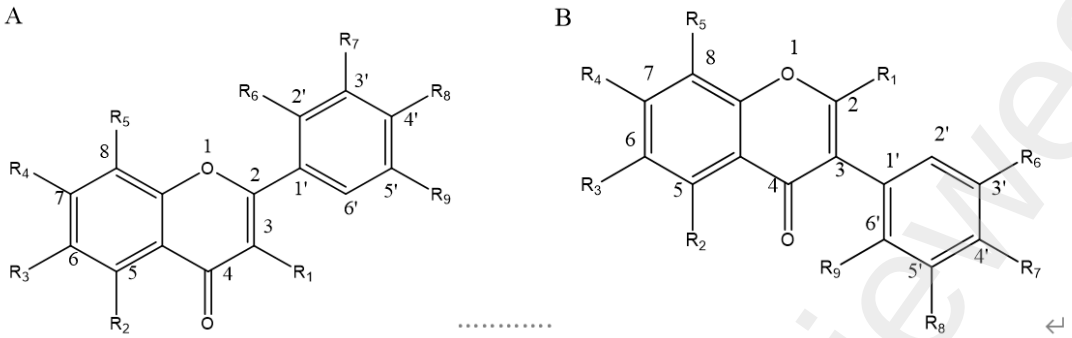
and diluted to the indicated concentrations with complete medium. The final concentration of DMSO in all experiments was less than 1% (V/V).

2.3 Real-time quantitative polymerase chain reaction analysis

For relevant gene expression analysis, HepG2 and Caco2 cells were inoculated in 12-well plates at a density of $8*10^4$ cells/well. When the cells reached approximately 60% fusion, albumin or the compound to be tested was diluted to a final concentration of 25 μM using complete medium and added to the well plates for 72 hours of incubation.

As shown in Figure S2, a Corylin concentration of 25 μM does not have a serious effect on the experiment. For time- and concentration-dependent induction of UGT1A1 mRNA assay, cells were divided into two groups, and one group of cells was used to study time-dependent induction by treating the cells with Corylin at final concentrations of 2, 10, and 25 μM in that order. One group of cells was incubated with Corylin (final concentration of 25 μM) for 0, 24, 48, and 72 hours for studying time-dependent induction.

Prior to the real-time quantitative PCR assay, total cellular RNA was extracted using the Trizol method and transcribed from RNA to cDNA using a reverse transcription kit according to the vendor's instructions. cDNA was then extracted from the cells using the TB Green® Premix Ex Taq™ (Tli RNaseH plus) kit according to the TB Green® Premix Ex Taq™ (Tli RNaseH plus) kit instructions in a QuantStudio™ 1 Plus Real - Time PCR Instrument for real-time fluorescence quantitative PCR experiments. Relative expression levels of target genes were normalized by the Ct value of human GAPDH ($2^{\Delta Ct}$ formula). The values displayed represent the normalized relative fold change in mRNA levels. Each sample was determined in triplicate.



Basic skeleton of (a)flavonoids and (b)isoflavonoids

Table 1. The inductive effects of tested compound on UGT1A1 gene expression.

No.	CAS	Compound	Skeleton	R1	R2	R3	R4	R5	R6	R7	R8	R9	n-folds of ctr
1	4143-74-2	4'-Methoxyflavone	A	H	H	H	H	H	H	H	OCH_3	H	6.55
2	577-85-5	3-Hydroxyflavone	A	OH	H	H	H	H	H	H	H	H	6.90
3	491-67-8	Baicalein	A	H	OH	OH	OH	H	H	H	H	H	3.71
4	22395-22-8	7-Methoxyflavone	A	H	H	H	OCH_3	H	H	H	H	H	2.36
5	480-40-0	Chrysin	A	H	OH	H	OH	H	H	H	H	H	8.66
6	21392-57-4	5,7-Dimethoxyflavone	A	H	OCH_3	H	OCH_3	H	H	H	H	H	1.09
7	973-67-1	5,6,7-Trimethoxyflavone	A	H	OCH_3	OCH_3	OCH_3	H	H	H	H	H	1.45
8	3570-62-5	5-hydroxy-7,8-dimethoxyflavone	A	H	OH	H	OCH_3	OCH_3	H	H	H	H	8.18
9	17290-70-9	Isosinensetin	A	H	OCH_3	H	OCH_3	OCH_3	H	H	OCH_3	OCH_3	2.77
10	520-28-5	Tectochrysin	A	H	OH	H	OCH_3	H	H	H	H	H	6.38
11	520-12-7	Pectolinarigenin	A	H	OH	OCH_3	OH	H	H	H	OCH_3	H	6.23
12	480-11-5	Oroxylin A	A	H	OH	OCH_3	OH	H	H	H	H	H	4.12
13	479-91-4	Casticin	A	OCH_3	OH	OCH_3	OCH_3	H	H	H	OCH_3	OH	5.07
14	152743-19-6	Lysionotin	A	H	OH	OCH_3	OH	OCH_3	H	H	OCH_3	H	1.95
15	529-53-3	Scutellarein	A	H	OH	OH	OH	H	H	H	OH	H	1.95
16	38183-03-8	7,8-Dihydroxyflavone	A	H	H	H	OH	OH	H	H	H	H	1.05
17	480-19-3	Isorhamnetin	A	OH	OH	H	OH	H	H	H	OH	OCH_3	1.82
18	117-39-5	Quercetin	A	OH	OH	H	OH	H	H	OH	OH	H	0.51
19	528-48-3	Fisetin	A	OCH_3	H	H	OH	H	H	H	OH	OH	0.64
20	481-53-8	Tangeretin	A	H	OCH_3	OCH_3	OCH_3	OCH_3	H	H	OCH_3	H	1.38

21	529-44-2	Myricetin	A	OH	OH	H	OH	H	H	OH	OH	OH	1.42
22	42079-78-7	5-Methoxyflavone	A	H	OCH_3	H	H	H	H	H	H	H	6.69
23	548-83-4	Galangin	A	OH	OH	H	OH	H	H	H	H	H	5.23
24	632-85-9	Wogonin	A	H	OH	H	OH	OCH_3	H	H	H	H	3.63
25	491-78-1	5-Hydroxyflavone	A	H	OH	H	H	H	H	H	H	H	12.75
26	20316-62-5	Tiliroside	A	$C_{16}H_{19}O_7$	OH	H	OH	H	H	H	OH	H	2.48
27	1617-53-4	Amentoflavone	A	H	OH	H	OH	$C_{15}H_9O_5$	H	H	OH	H	3.38
28	478-01-3	Nobiletin	A	H	OCH_3	OCH_3	OCH_3	OCH_3	H	H	OCH_3	OCH_3	2.04
29	21967-41-9	Baicalin	A	H	OH	OH	$C_6H_9O_7$	H	H	H	H	H	1.57
30	6665-74-3	3-O-Methylgalangin	A	OCH_3	OH	H	OH	H	H	H	H	H	9.36
31	28978-02-1	Pectolinarin	A	H	OH	OCH_3	$C_{12}H_{21}O_{10}$	H	H	H	OCH_3	H	1.34
32	20126-59-4	Diosmetin-7-O-β-D-glucopyranoside	A	H	OH	H	$C_7H_{13}O_5$	H	H	H	OCH_3	OH	6.14
33	437-64-9	Genkwanin	A	H	OH	H	OCH_3	H	H	H	OH	H	11.9
34	520-34-3	Diosmetin	A	H	OH	H	OH	H	H	H	OCH_3	OH	4.7
35	480-44-4	Acacetin	A	H	OH	H	OH	H	H	H	OCH_3	H	11.47
36	520-36-5	Apigenin	A	H	OH	H	OH	H	H	H	OH	H	11.80
37	4443-09-8	Norwogonin	A	H	OH	H	OH	OH	H	H	H	H	1.48
38	76666-32-5	3'-Methoxyflavonol	A	OH	H	H	H	H	H	H	H	OCH_3	5.56
39	40957-83-3	Glycitein	B	H	H	OCH_3	OH	H	H	OH	H	H	1.32
40	1157-39-7	4',7-Dimethoxyisoflavone	B	H	H	H	OCH_3	H	H	OCH_3	H	H	1.63
41	17817-31-1	Desmethylglycitein	B	H	H	OH	OH	H	H	OH	H	H	1.96
42	82517-12-2	Methyl-7-methoxyisoflavone	B	H	CH_3	H	OCH_3	H	H	H	H	H	2.86
43	552-59-0	Prunetin	B	H	OH	H	OCH_3	H	H	OH	H	H	1.40

44	548-77-6	Tectorigenin	B	H	OH	OCH_3	OH	H	H	OH	H	H	1.61
45	548-76-5	Irigenin	B	H	OH	OCH_3	OH	H	OCH_3	OCH_3	OH	H	1.78
46	20633-67-4	Calycosin-7-O-β-D-glucoside	B	H	H	H	$C_6H_{11}O_6$	H	H	OCH_3	OH	H	2.99
47	446-72-0	Genistein	B	H	OH	H	OH	H	H	OH	H	H	9.45
48	53947-92-5	Corylin	B	H	H	H	OH	H	H	C_5H_8O	H	H	13.76

2.4 Western blot

To verify the induction of UGT1A1 protein expression levels by Corylin, HepG2 and Caco2 cells were inoculated in 6-well plates at a density of 4×10^5 cells/well. Cells were lysed using RIPA lysis buffer and centrifuged at 4°C for 15 min at 12,000 g to collect the supernatant. Subsequently, protein concentration was determined using the BCA protein assay kit. The cell homogenates were separated by 10% SDS-PAGE gel electrophoresis and the proteins were transferred to a PVDF membrane. After sealing the PVDF membrane using 5% skim milk powder, it was incubated overnight at 4°C with primary antibodies against UGT1A1 (ab194697, Abcam, dilution 1:500) or anti-GAPDH (ab9485, Abcam, dilution 1:2500). The PVDF membrane was washed with TBST buffer and incubated with secondary antibody (ab6721, Abcam, dilution 1:2000) for 1 hour at room temperature and again with TBST buffer. Finally, protein detection was performed using ultra-sensitive ECL Luminescent Reagent and Fluorescence Gel Imaging System and bands were analyzed for gray scale values using Image J software.

2.5 Luciferase reporter gene assays

To detect the activation of PPAR and AhR reporter genes, HEK293-PPAR-luc, HEK293-FXR-luc, HEK293-Nrf2-luc and HEK293-AhR-luc stably transfected cell lines constructed in the previous period were used in this study[23]. Cells were inoculated in 96-well plates, and when the cell fusion reached 50%, Corylin was added at final concentrations of 2, 10, and 25 μM, respectively. rosiglitazone and lignans were used as agonist positive controls for PPAR and AhR, respectively, and DMSO was used as a negative control. The final concentration of DMSO was less than 1% (V/V) in all experiments. After incubation for 48 h after dosing, the medium was discarded and the cells were washed with PBS, followed by measurement of luciferase activity in each well using the Firefly Luciferase Reporter Gene Assay Kit according to the supplier's instructions. In addition, to determine the PPAR isoforms involved in the induction of UGT1A1 by Corylin, HEK293-PPARα-luc, HEK293-PPARβ-luc, and HEK293-PPARγ-luc cells were inoculated and cultured in 96-well plates, and Corylin was added

at a final concentration of 2, 10, and 25 μM, respectively. after 48 h of incubation After that, the luciferase activity of each well was determined using the Firefly Luciferase Reporter Gene Assay Kit, also according to the supplier's instructions.

2.6 Glucuronidation activity assarys

To assay Corylin induced UGT1A1 to increase enzyme activity. In an incubation mixture (100 μL) containing Tris-HCL buffer solution (50 mM), HepG2 cell lysate (2.1 mg/mL), Alamethicin (20 μg/mL), $MgCl_2$ (4 mM), and β-Estradiol, the reaction was incubated for 5 min at 37°C, then UDPGA (3.5 mM) was added, and the reaction was performed at 37°C for 30 min, and finally the reaction was terminated by adding an equal volume of ice acetonitrile. The mixed samples were centrifuged at 4°C and 12,000 g for 10 min, and the supernatant was analyzed using high-performance liquid chromatography (UHPLC); all experiments were performed in triplicate. The mobile phase was a mixture of 0.1% formic acid (A) and methanol (B). The following gradient elution program was used: 0-30 min, 5%-95% B, 30-40 min, 95% B, 40-50 min, 95%-5% B. The system was operated at a flow rate of 1.0 mL/min with a 10 μL injection volume.

2.7 Animal experiments

Forty mice were randomly divided into normal group, model group, low-dose group (50 mg/kg), and high-dose group (100 mg/kg), 10 mice in each group. The low-dose group (50 mg/kg) and high-dose group (100 mg/kg) were injected intraperitoneally with Corylin diluted with 0.5% sodium hydroxymethyl cellulose every day, and the normal and model groups were injected with an equal volume of 0.5% sodium hydroxymethyl cellulose every day for 7 consecutive days. One hour after the last administration, APAP modeling agent was injected and the mice were starved (fasted without water), and 24 hours later the mice were executed and blood from the heart was collected and serum was extracted for further analysis. Liver and small intestine tissues were washed with ice-cold phosphate-buffered saline and then immediately snap frozen in liquid nitrogen. Tissue samples were stored at -80°C until analysis.

2.8 Statistical analysis

All data were expressed as mean ± standard deviation (SD) from triplicate assays. Statistical differences were determined by one-way ANOVA and differences were considered statistically significant at p value <0.05.

3. Results

3.1 Screening of UDP-Glucuronosyltransferase 1A1 Inducer at mRNA Levels

The induction of UGT1A1 on the mRNA levels by tested flavonoid and isoflavone compounds (list in Table 1) were analyzed by real-time PCR, with chrysin as positive control. As depicted in Table 1, 18 flavones exhibited excellent UGT1A1 inductive effects, with a 5-fold or higher inductive effects of than the negative control (DMSO only). These strong inducers include 4'-Methoxyflavone(No.1), 3-Hydroxyflavone(No.2), Chrysin(No.5), 5-hydroxy-7,8-dimethoxyflavone(No.8), Tectochrysin(No.10), Pectolinarigenin(No.11), 5-Methoxyflavone(No.22), Galangin(No.23), 5-Hydroxyflavone(No.25), 3-O-Methylgalangin(No.30), Diosmetin-7-O-β-D-glucopyranoside(No.32), Genkwanin(No.33), Acacetin (No.35), Apigenin(No.36), 3'-Methoxyflavonol(No.38), Genistein(No.47), Corylin(No.48). While Acacetin (No.35) [21] and Apigenin (No.36) [22] have been previously reported as UGT1A1 inducers. Among these tested compounds, Corylin (No.48) demonstrated the most potent UGT1A1-inducing effect, with an induction of UGT1A1 mRNA gene expression reaching up to 13.76-fold compared to the negative control, and a stronger induction effect than the positive control, chrysin. These findings encourage us to pursue further in-depth studies on Corylin.

3.2 Dose- and Time- Dependent UGT1A1 Induction Assays

The induction behavior of Corylin on UGT1A1 mRNA expression were evaluated using HepG2 and Caco2 cells. As shown in Fig. 1, In both HepG2 and Caco2 cells, Corylin showed a significant concentration-dependent induction effect, with a concentration range of 0-25 μM. In addition, a significant time-dependent induction

effect was also observed on both cells, with treatment times of 24 h, 48 h, and 72 h. These results suggest that the optimal induction concentration and induction time in the two cells are respectively 25 μM and 72 h. After a 3-day incubation period at a concentration of 25 μM, Corylin up-regulated UGT1A1 mRNA levels by 6.32-fold in HepG2 cells and 5.44-fold in Caco2 cells. These findings suggest that Corylin significantly enhances UGT1A1 mRNA expression in HepG2 and Caco2 cells, and Corylin had a better induction of UGT1A1 at the mRNA level in HepG2 cells.

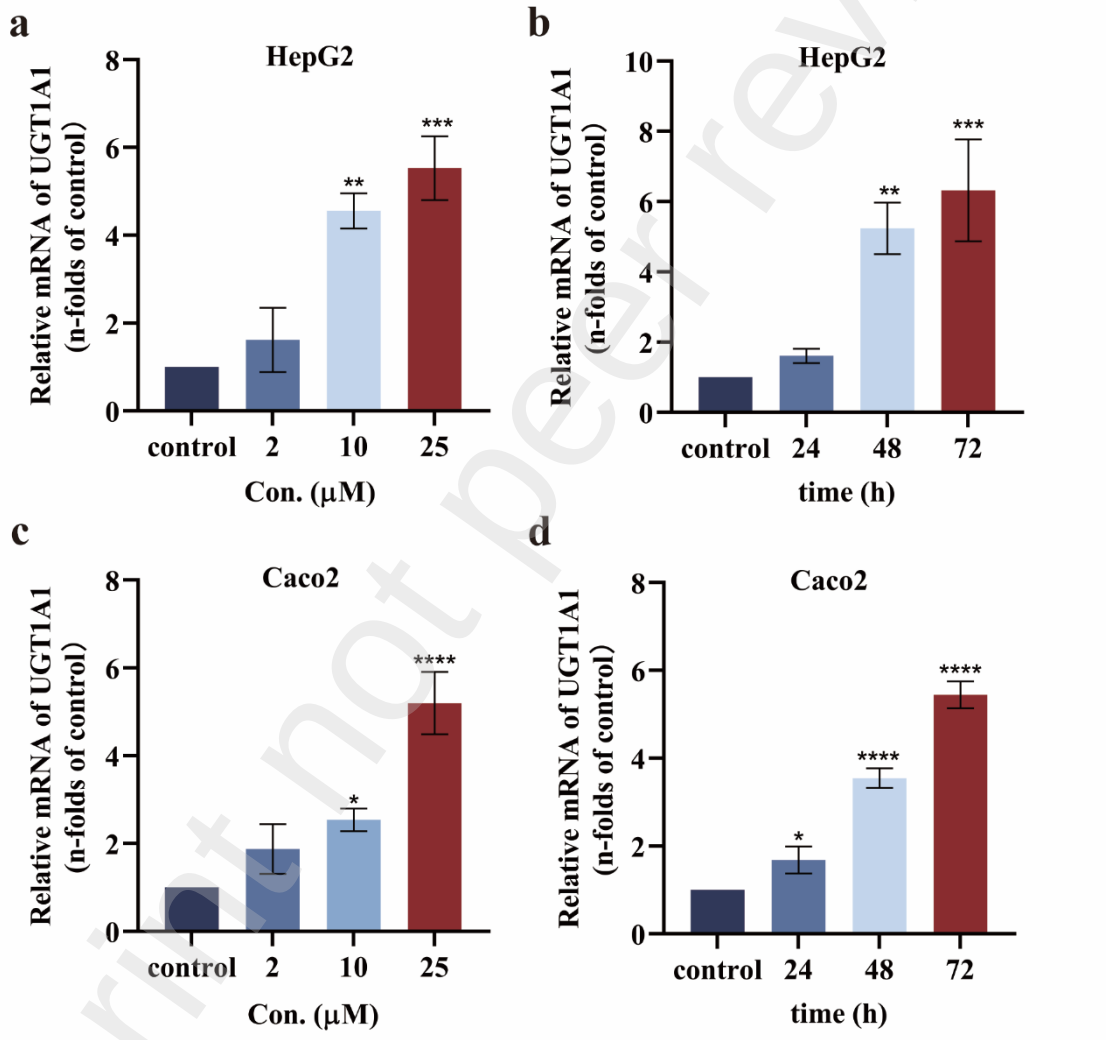


Fig. 1 The concentration-dependent (a) and time-dependent (b) induction of UGT1A1 mRNA expression by Corylin in HepG2 cells. The concentration-dependent (c) and time-dependent (d) induction of UGT1A1 mRNA expression by Corylin in Caco2 cells. The data were expressed as mean ± SD, *$p < 0.05$ and ****$p < 0.0001$ vs control group.

3.3 Inductive Effects of Corylin on UDP-Glucuronosyltransferase 1A1 Protein Levels

Subsequently, the inducing effect of Corylin on UGT1A1 at the protein level was further examined in HepG2 cells and Caco2 cells. UGT1A1 protein expression levels were determined using western blotting after 72 h of incubation with Corylin-containing (final concentrations of 2 μM, 10 μM, and 25 μM) medium. As depicted in Fig. S3, treatment with Corylin at a final concentration of 25 μM resulted in a significant increase in UGT1A1 protein levels in HepG2 and Caco2 cells. After semi-quantitative analysis by Image J, Corylin increased an approximately 2.2-fold and 1.5-fold on the UGT1A1 protein expression (Fig. 2). The results showed that Corylin had a better induction of UGT1A1 at the protein level in HepG2 cells, which was the same as that of UGT1A1 mRNA, both suggesting that Corylin upregulates UGT1A1 protein expression more effectively in HepG2 cells.

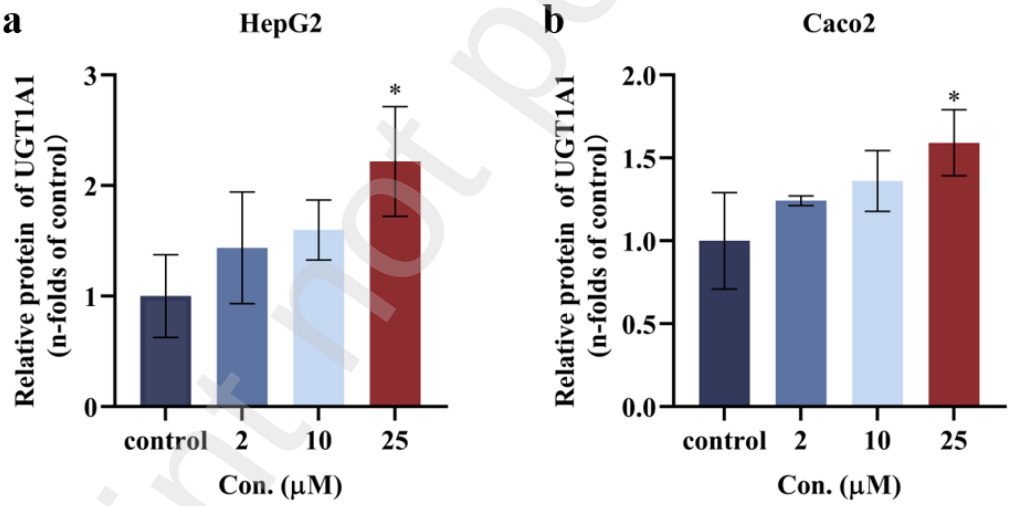


Fig. 2 Inductive effect of Corylin on UGT1A1 protein expression in HepG2 (a) and Caco2 (b). *$p < 0.05$ vs control group.

3.4 Identification of Nuclear Receptors Involved in UDP-Glucuronosyltransferase 1A1 Induction

Transcription of the UGT1A1 gene is regulated by a variety of nuclear receptors. These nuclear receptors regulate UGT1A1 expression by specifically recognizing and

binding to ligands, such as the aromatic hydrocarbon receptor (AhR), peroxisome proliferator-activated receptor (PPAR) and farnesoid X receptor (FXR). The results suggest that Corylin can regulate promoter activity by activating PPAR and AhR. To further identify the nuclear receptors responsible for Corylin regulation UGT1A1 expression, luciferase reporter gene assays were conducted. Firstly, the screening system of PPARα, PPARβ and PPARγ agonists based on luciferase reporter gene was successfully constructed (Fig. S4). As shown in the Fig. 3a, Corylin significantly increased the luciferase activity of the PPARs reporter gene by approximately 3.2-fold at a concentration of 2 μM, whereas the positive agonist rosiglitazone increased the activity of the PPARs reporter gene by only approximately 1.6-fold at a concentration of 25 μM. In addition, Corylin increased AhR reporter gene activity by approximately 3.63-fold at a concentration of 25 μM (Fig. 3b), which was significantly higher than the activation by the positive agonist lignocaine and was verified by using the AhR inhibitor CH223191 (Fig. S5). PPARs are the most well-studied fatty acid-activated nuclear receptors, and their activation is critical for the functioning of tissues and organs, such as the liver and muscles with a central role. Three subtypes of PPARα, PPARβ and PPARγ have been identified. As shown Fig. 4, Corylin had a significant activating effect on PPARs, especially significantly enhancing the luciferase activity of PPARβ and PPARα reporter genes by approximately 4.43-fold and 3.08-fold, respectively. Further molecular docking was performed using the ligand binding domain (LBD) of PPARα, PPARβ and PPARγ. The binding energies of Corylin to PPARα, PPARβ and PPARγ were -7.85 kcal/mol, -8.07 kcal/mol and -7.38 kcal/mol, respectively (Fig. S6). As shown in the Fig. S7, Corylin did not exhibit any significant activation of FXR or Nrf2 at the indicated concentrations, while the respective positive agonists obviously elevated the corresponding reporter activities. These results suggest that NBIF up-regulates the transcription of human UGT1A1 via activation of PPARs and AhR rather than FXR or Nrf2.

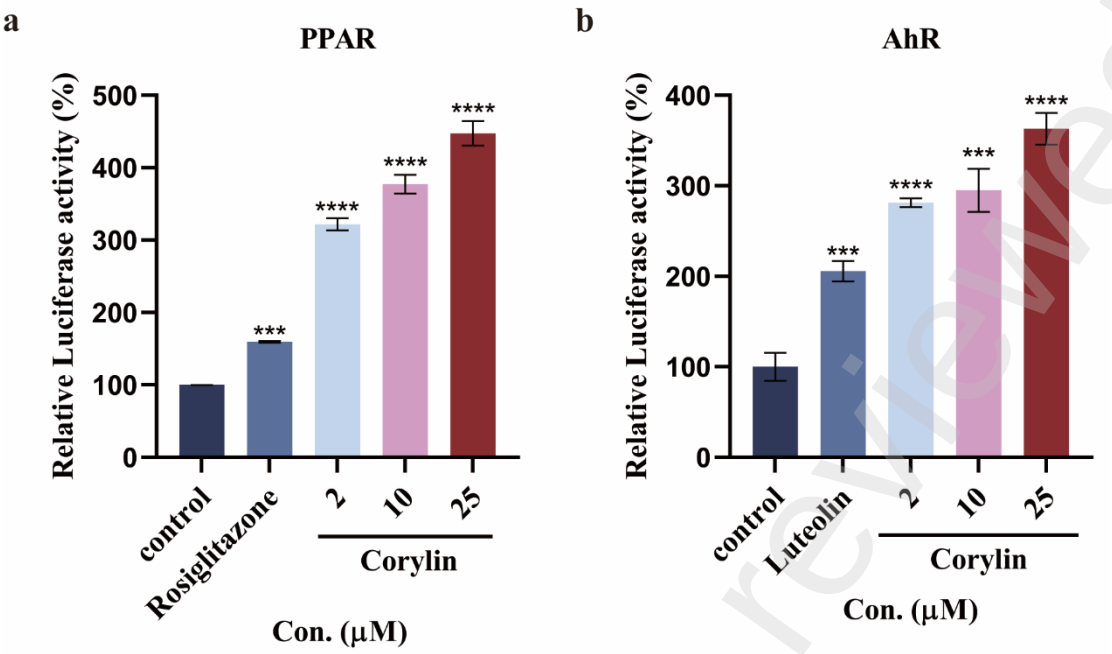


Fig. 3 Activation of nuclear receptors by Corylin (a)PPAR; (b)AhR.
The data were expressed as mean ± SD, ***p < 0.001 and ****p < 0.0001 vs control group.

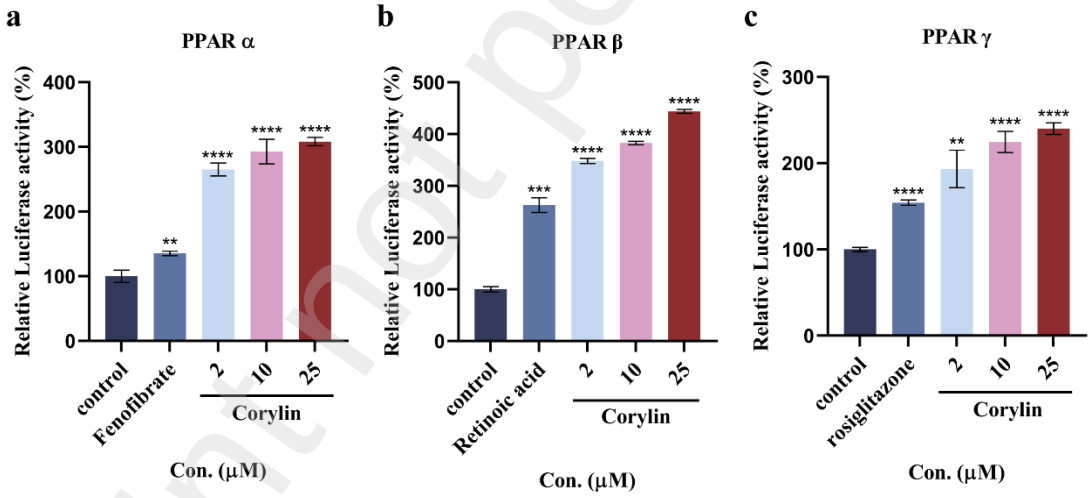


Fig. 4 Activation of PPAR isoforms by Corylin (a)PPARα; (b)PPARβ; (c)PPARγ.
The data were expressed as mean ± SD, **p < 0.01， ***p<0.001 and ****p < 0.0001 vs control group.

3.5 UGT1A1 activity assay in HepG2 cells

UGT1A1 activity assay in HepG2 cells was further examined using a substrate-based biochemical assay, due to β-Estradiol can be metabolized to Estradiol 17- (β-D-Glucuronide) by UGT1A1. As depicted in Fig. S8, the peaks of the substrate estradiol

and the product Estradiol-3-glucuronide were well shaped, completely separated without interference from impurity peaks, and in good linear relationship. As shown in the Fig. 5, the cellular exocytosis of Estradiol 17- (β-D-Glucuronide) was significantly increased in HepG2 cell homogenates prepared after Corylin induction, indicating that Corylin can significantly enhance the activity of UGT1A1 enzyme, which in turn promotes the metabolism of estradiol.

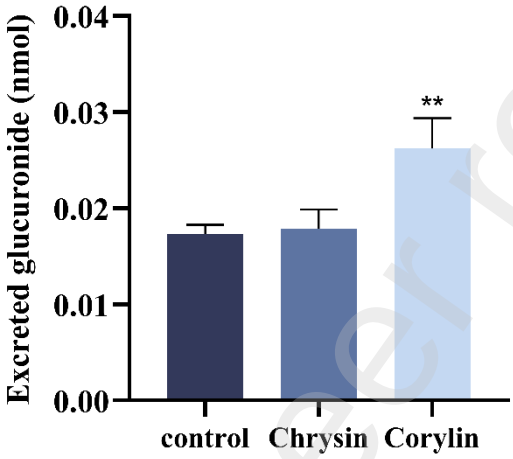


Fig. 5 *In vitro* metabolism of cell homogenates β-Estradiol

3.6 Corylin significantly alleviates liver injury mice

Encouraged by the above findings, the *in vivo* hepatoprotective effect of Corylin on liver injury mice were further investigated. After As depicted in Fig. 6a, b and c, APAP significantly increased the activities of LDH, ALT and AST compared with the control group, indicating that the model of APAP-induced acute liver injury was successfully established. The Corylin-treated APAP mice significantly reduced serum LDH, AST and ALT activities in a dose-dependent manner compared with the model group. Bilirubin concentration was used to assess chemically induced liver injury[25, 26]. APAP caused significant damage to the liver parenchyma, resulting in a substantial increase in bilirubin levels (Fig. 6d). TBIL levels were reduced in both the low and high dose groups of Corylin, and the effect was more pronounced in the high dose group. Meanwhile, the treatment group could reduce liver enlargement (Fig. S9) and reduced liver coefficient (Fig. S10) caused by APAP liver injury. To further assess the protective effect of Corylin against APAP-induced acute liver injury, liver histopathology was

performed. In the control group (Fig. 6e), hepatocytes were arranged in a radial pattern, and the lobular structure was complete and clear; in the APAP model group (Fig. 6f), the cells were swollen and the cytoplasm was loose and pale, accompanied by inflammatory cell infiltration and a small amount of necrotic cell debris; in the low-dose group, the liver morphology improved, but a small amount of granulocyte infiltration (Fig. 6g) could be seen; in the high-dose group(Fig. 6h), the liver morphology was closer to that of the normal group, with occasional hepatocellular edema, and no obvious inflammatory cell infiltration. There was no obvious inflammatory cell infiltration. These observations clearly showed that Corylin significantly alleviates APAP-induced liver injury mice e *in vivo*.

Furthermore, Corylin dose-dependently increased the level of UGT1A1 in colonic and hepatic tissues and of APAP-treated mice (Fig. 7 and Fig.S11). Corylin These results suggested that APAP-induced liver injury could reduce the expression of UGT1A1, which may attenuate the metabolic clearance of bilirubin and drugs, whereas Corylin can restore the metabolic function of the liver by up-regulating the expression of UGT1A1, thereby alleviating liver injury.

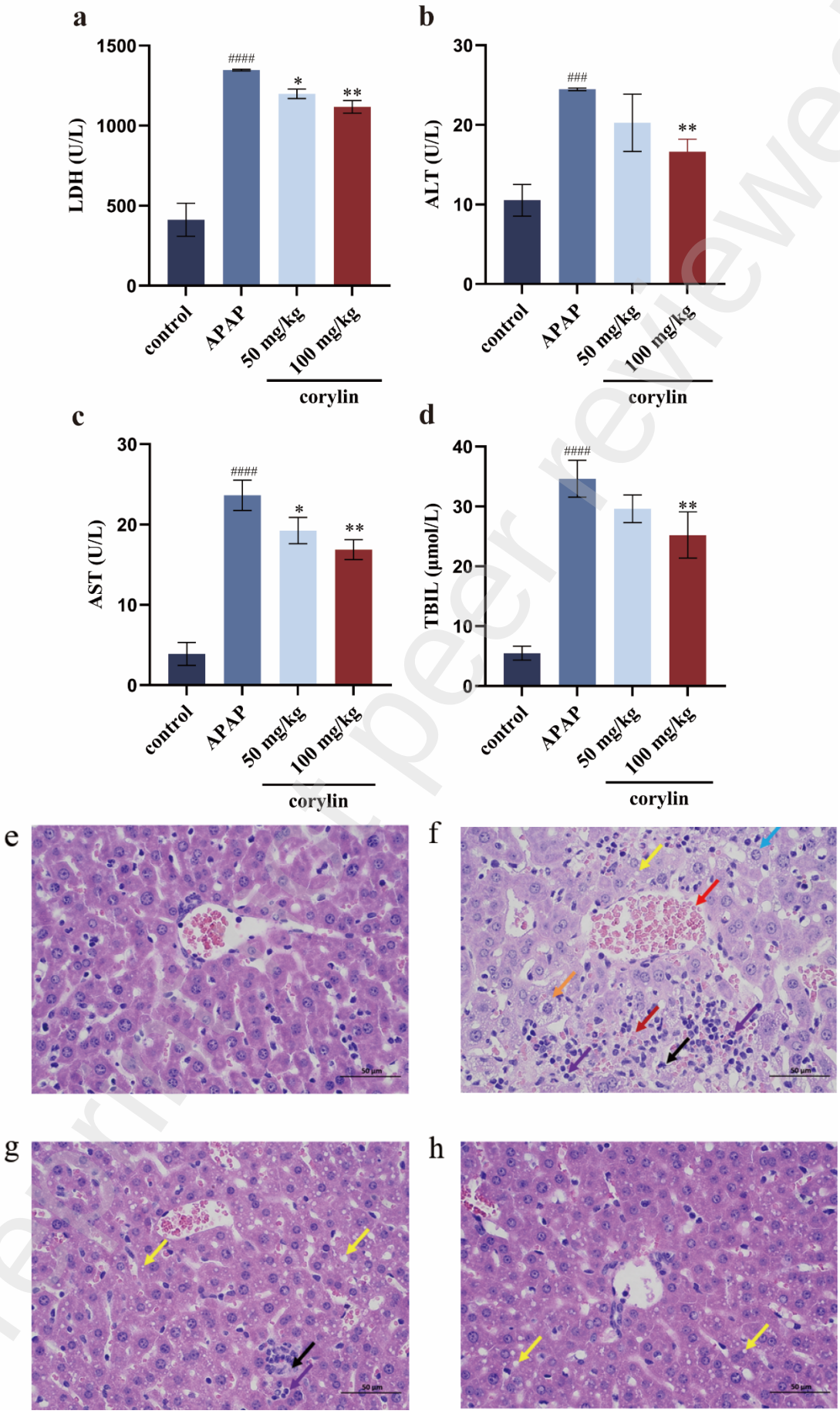


Fig. 6 Effects of tonic isoflavones on serum biochemical indices in a APAP-induced liver injury model in mice (a)LDH; (b)ALT; (c)AST; (d)TBIL; Influence of Corylin on the histopathology of the liver in mice with APAP-induced liver injury (e)control; (f)APAP; (g)Corylin 50 mg/kg; (h)Corylin 100 mg/kg. The data were expressed as mean ± SD, *p < 0.05 and **p < 0.01 vs APAP group, ###p < 0.001 and ####p < 0.0001 vs APAP group.

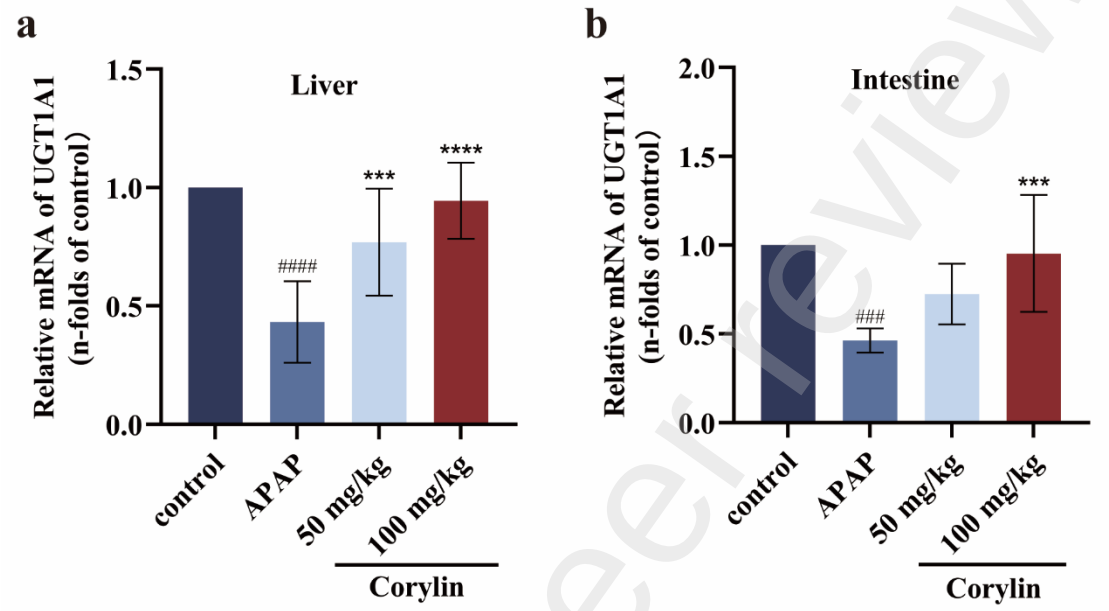


Fig. 7 Effects of Corylin on UGT1A1 mRNA expression in tissues from mice with APAP-induced liver injury (a)liver; (b)intestine. The data were expressed as mean ± SD, ***p < 0.001 and ****p < 0.0001 vs APAP group and ####p < 0.0001 vs APAP group.

4. Discussion

Bilirubin is the major end product of heme catabolism and heme oxygenase[27]. Recent studies have shown that bilirubin has significant anti-inflammatory and antioxidant effects[28, 29], and elevated levels within the normal range may play a protective role against cardiovascular and inflammatory diseases such as coronary microvascular dysfunction and coronary atherosclerosis[30, 31]. However, elevated levels of unconjugated bilirubin can cause jaundice-related disorders. As a fat-soluble substance, bilirubin can cross the blood-brain barrier, leading to severe hyperbilirubin encephalopathy and permanent neurological damage[7, 32]. UGT1A1 is a key metabolizing enzyme of bilirubin, and also plays an important role in the metabolism of many endogenous substances or exogenous drugs. Therefore, a reduction in UGT1A1 enzyme activity can trigger a variety of physiologic and pathologic

disorders[3]. UGT1A1 enzyme activity is affected by a variety of factors, and genetic mutations can lead to defects in the enzyme's structure or function, causing a reduction or absence of glucuronidation, which in turn can trigger hyperbilirubinemia[33, 34]. Exogenous drugs can also induce or inhibit UGT1A1 enzyme activity[35]. And there is still a large gap in research on UGT1A1 inducers compared to other drug metabolizing enzymes.

Currently, UGT1A1 inducers are mainly studied for their ability to detoxify bilirubin and drug metabolism by increasing enzyme activity. For example, phenobarbital activates constitutive androstane receptor (CAR) and pregnane X receptor (PXR) to up-regulate UGT1A1 expression[36, 37], which is used to treat Crigler-Najjar syndrome[4], but long-term use has side effects such as nerve damage and liver complications[38, 39]. Oleanolic acid (OA) and its isomer Ursolic acid (UA) activate PXR-induced UGT1A1[40], and rifampicin is also a classical UGT1A1 inducer[41], but is limited by potential drug-drug interactions in clinical applications[42, 43]. Therefore, the search for potentially safe and effective UGT1A1 inducers from flavonoid natural products may provide new solutions to the problems of improving abnormal drug metabolism and reducing adverse drug reactions. In the present study, Corylin, a UGT1A1 inducer with significant induction effect, was identified from flavonoids or isoflavonoids, and its induction mechanism was investigated, and its hepatoprotective effect was also confirmed in further studies.

Our results indicate that Corylin has a time- and concentration-dependent induction of UGT1A1 mRNA expression in HepG2 and Caco2 cells (Fig. 1) and also exhibits a concentration-dependent effect at the protein level (Fig. 2). To further explore the molecular mechanism of Corylin effect on UGT1A1 expression, we found that Corylin is a pan agonist capable of activating all PPAR isoforms. This multi-modulatory property may give it a unique advantage in the future treatment of metabolic disorders while reducing the potential risks associated with multiple drug interactions[44]. Specifically, Corylin mainly activates PPARβ and PPARα (Fig. 4a and 4b) in a concentration-dependent manner, whereas it has a weak activating effect on PPARγ, which may be related to the low expression level of PPARγ in liver tissue[45]. In

addition, Corylin can also activate AhR(Fig. 3b), and the activation of AhR affects glucose and lipid metabolism in the liver, but the specific mechanism of action is usually dependent on the cellular environment and regulatory pathways, so further in-depth studies are needed[46-48]. These findings suggest that Corylin is not only a UGT1A1 inducer, but also a potential drug for the treatment of diseases such as diabetes, lipid metabolism disorders and inflammation. Meanwhile, in vitro metabolic experiments confirmed that Corylin could effectively increase glucuronidation levels.

Corylin effectively attenuates acetaminophen (APAP)-induced acute liver injury and has a significant protective effect on the liver. DILI is one of the major causes of liver injury and various substances are known to cause liver injury[49]. Multiple chemical-induced liver injury in animal models was used to test novel substances for their protective and therapeutic effects. Among these models, APAP overdose is an important paradigm for liver[50] research as it is the only model of acute DILI in mice that is consistent with the cause of the main entity of ALF within individuals[51-53]. The stability and reproducibility of this model is unavoidable. APAP is a widely used analgesic within the therapeutic context[54], however overdose of APAP is metabolized in the liver by cytochrome P450 enzymes (CYP2E1, CYP3A4) to generate the toxic intermediate N-acetyl-p-benzoquinone imine (NAPQI), which leads to hepatic glutathione (GSH) depletion[55, 56], triggering Oxidative stress and mitochondrial dysfunction ultimately lead to hepatocellular necrosis and inflammation[57], and APAP-induced liver injury was found to have a similar mechanism of toxicity in humans and mice. serum liver biomarkers of AST, ALT, LDH, and TBIL are typical sensitive indicators of early acute liver injury[58, 59]. Corylin pretreatment significantly reduced APAP-induced elevation of serum AST, ALT, LDH, and TBIL (Fig. 6a、6b、6c and 6d). In addition, histopathological examination was performed to confirm the biochemical findings. Necrosis, vacuolar degeneration and inflammatory cell infiltration were observed after APAP administration[60]. Corylin reduced histological liver injury in a dose-dependent manner, with higher doses of Corylin showing greater protection against hepatic injury (Fig. 6g and 6h). The results of the histopathological investigations were consistent with those of the biochemical analysis.

The histopathological findings were consistent with the biochemical results, thus confirming the hepatoprotective effect of Corylin. APAP damage to hepatocytes may alter the integrity of the cell membrane, resulting in the inability of the liver to regulate metabolism. It was observed that the gene and protein expression levels of UGT1A1 in the livers and intestines of APAP-induced liver-injured mice were significantly lower than that of the blank control group, and Corylin could slightly enhance its expression (Fig. 7 and Fig. S11). The above experiments proved that Corylin has a protective effect on liver injury in mice.

Corylin is an isoflavonoid derived from boneset and has a wide range of applications in the field of herbal medicine[61]. It can be used to treat osteoporosis, diabetic complications, and has antioxidant properties[62, 63]. In addition, some studies suggest that Corylin may have anti-cancer potential[64]. Safety is always the most critical consideration in the clinical application of drugs. As a natural compound, Corylin generally has a favorable safety profile. Studies have shown that Corylin has potential as a potential UGT1A1 inducer in UGT1A1 enzyme induction and hepatoprotection. With further research, combined with personalized therapy and drug safety considerations, Corylin is expected to be an effective drug for the treatment of hyperbilirubinemia and liver disease. However, personalized therapeutic strategies still need to be rigorously monitored in the clinic to ensure that their safety and efficacy are maximized[65].

Conclusion

In conclusion, this study reports a safe and effective natural flavonoid UGT1A1 inducer, Corylin, which significantly induced UGT1A1 expression at both gene and protein levels. Further experiments demonstrated that Corylin is a PPAR pan agonist with the strongest effect on PPARβ activation, and also activates AhR nuclear receptors. APAP-induced liver injury experiments in mice demonstrated that Corylin has a significant hepatoprotective effect. Corylin shows promising applications in basic research, and its properties as a UGT1A1 inducer may provide a new solution for future Corylin as a UGT1A1 inducer may provide a new solution for future clinical treatment.

Supporting information

Additional supporting information can be found online in the Supporting Information section. (Supporting Information)

Declaration of competing interest

These authors have no conflict of interest to declare.

Acknowledgments

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