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Hepatoprotection of auraptene from peels of citrus fruits against thioacetamide-induced hepatic fibrosis in mice by activating farnesoid X receptor

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Running title: Auraptene protects against hepatic fibrosis
Abstract

Hepatic fibrosis is a pathological process that eventually leads to development of cirrhosis and liver cancer by various types of chronic liver disease. Up to date, there is no standard treatment for the progression of liver fibrosis. This study aims to investigate the hepatoprotection of auraptene (AUR), a simple coumarin contained in the peels of citrus fruits such as grapefruit, against thioacetamide (TAA)-induced hepatic fibrosis in mice. The involvement of farnesoid X receptor (FXR) in the anti-fibrotic effect of AUR was further elucidated using in vivo and in vitro experiments. AUR was found to remarkably protect against liver injury induced by TAA in mice and maintain the homeostasis of bile acid via regulation of FXR-target genes including Bsep, Mrp2, Ntcp, Cyp7a1 and Cyp8b1. Masson and Sirius red staining indicated a reduction of collagen content in the liver of AUR treated mice. Furthermore, AUR inhibited the activation of hepatic stellate cells (HSCs) by down-regulating the expression of TGF-β1 and α-SMA and expressed anti-inflammatory effects via reducing the expression of NF-κB, TNF-α and IL-1β. However, the changes in these genes and protein, as well as ameliorative liver histology induced by AUR were abrogated by FXR antagonist guggulsterone in vivo and FXR siRNA in vitro. Overall, AUR protects against TAA-induced hepatic fibrosis due to reducing toxic bile acids, inhibiting hepatic stellate cells (HSCs) activation and inflammation, which were all in association with FXR activation. AUR might be efficacious for prevention and treatment of hepatic fibrosis in mice.

Key words Hepatic fibrosis; Farnesoid X receptor (FXR); Transporters; Thioacetamide; Auraptene
**Abbreviations**

ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUR, auraptene; Bsep, bile salt export pump; COL1-α1, collagen type I α1; Cyp7α1, cholesterol 7α-hydroxylase; Cyp8b1, oxysterol 12α-hydroxylase; ECM, extracellular matrix; FXR, farnesoid X receptor; H&E, hematoxylin and eosin; HSCs, hepatic stellate cells; IL-1β, interleukin-1β; Mrp2, multi-drug resistance-associated protein 2; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor-κB; Ntcp, Na+/taurocholate cotransporting polypeptide; TAA, thioacetamide; TB, total bile acids; TBIL, total bilirubin; TC, total cholesterol; TG, triglycerides; TGF-β1, transforming growth factor-β1; TIMP-1, tissue inhibitor of metalloproteinase-1; TNF-α, tumor necrosis factor-α; α-SMA, α-smooth muscle actin.
**Introduction**

Hepatic fibrosis represents a worldwide health threat affecting millions of people and resulting in significant morbidity and mortality.\(^1\) It is a part of the normal healing response to various kinds of chronic liver injury including alcoholic liver disease, nonalcoholic fatty liver disease, viral hepatitis, and cholestatic liver diseases.\(^2,3\) Liver fibrosis is usually characterized by the excessive accumulation of fibrous connective tissue in and around inflamed or damaged tissue mediated by activated myofibroblasts.\(^4\) Although clinical and translational implications have begun to impact significantly on the management and outlook of patients with liver fibrosis, there is still no FDA approved medication for liver fibrosis.\(^5\) Thus, intensifying to uncover the mechanisms underlying hepatic fibrosis and its attenuation is not only timely, but is more clinically relevant than ever.\(^6\)

Recently, the knowledge of the molecular mechanisms underlying hepatic fibrosis has been greatly enhanced. Hepatocytes, activated hepatic stellate cells (HSCs), endothelial and immune cells are all involved in the formation and relief of hepatic fibrosis.\(^7\) Stimulated Kupffer cells and liver sinusoidal endothelial cells activate HSCs via paracrine production of fibrosis-related factors including transforming growth factor-β1 (TGF-β1), α-smooth muscle actin (α-SMA), and tissue inhibitor of metalloproteinase-1 (TIMP-1).\(^8,9\) Among the many cytokines involved in hepatic fibrosis, TGF-β1 is the most crucial key factor for its indirectly stimulation of HSCs, resulting in the deposition of substantial amounts of extracellular matrix (ECM).\(^10\)

Nuclear receptors regulate a variety of metabolic processes including bile acid homeostasis, fibrosis, even tumor formation. Farnesiod X receptor (FXR, NR1H4), a ligand-activated nuclear receptor highly expressed in liver, has been identified as a novel molecular target for the treatment of hepatic diseases.\(^11\) FXR regulates gene expression of bile salt export pump (Bsep), multidrug resistance-related protein 2 (Mrp2), Na\(^+\)/taurocholate cotransporting polypeptide (Ntcp), cholesterol 7α-hydroxylase (Cyp7a1), and oxysterol 12α-hydroxylase (Cyp8b1), which were
transporters and enzymes participating in the regulation of bile acid homeostasis.\textsuperscript{12} FXR also plays a crucial role in pathological process of hepatic fibrosis. FXR agonist was shown to relieve hepatic fibrosis by reducing hepatic gene expression of fibrotic markers.\textsuperscript{13} FXR affects α-SMA, TIMP-1, and TGF-β1 in animal models of chronic liver damage.\textsuperscript{14, 15} As an example hereof, FXR agonist has been shown to reduce fibrosis in cholestatic and non-alcoholic steatohepatitis (NASH) animal models.\textsuperscript{16, 17} Moreover, The activation of HSCs was inhibited significantly when FXR was activated.\textsuperscript{18} In addition, it has been shown that synthetic FXR agonist obeticholic acid, has good effect on hepatic inflammation by inhibiting hepatic pro-inflammatory gene TNF-α and IL-1β, and blocking nuclear translocation of nuclear factor-κB (NF-κB).\textsuperscript{19} FXR agonists might therefore represent useful agents to prevent or suppress liver cirrhosis and hepatocarcinogenesis.\textsuperscript{20}

Currently, natural products and herbal medicines have been widely used for management and treatment of liver diseases. Auraptene (7-geranyloxycoumarin, AUR) is a simple coumarin that among the most common component of \textit{Citrus} spp. Fruits.\textsuperscript{21} It has been revealed that AUR had various interesting pharmacological properties such as antioxidant, antimicrobial, anti-inflammatory, and cancer chemical preventive effects.\textsuperscript{22-25} It is also suggested that AUR could normalize lipid abnormalities and alleviate obesity and hepatic triglycerides (TG) accumulation.\textsuperscript{26} In our previous study, AUR was demonstrated as a FXR agonist protecting against lithocholic acid-induced cholestatic liver injury.\textsuperscript{27} However, a modulatory effect of AUR on liver fibrosis has not been investigated.

Herewith, it is hypothesized that AUR could exert favorable impacts on hepatic fibrosis induced by thioacetamide (TAA). \textit{In vivo} and \textit{in vitro} evidence were further provided to verify whether FXR and its target genes represent promising targets of AUR for the protection of hepatic fibrosis in mice.

\textbf{Materials and Methods}
Materials

AUR (purity > 98%) was purchased from Chengdu Must Bio-technology Co., Ltd. (Chengdu, China). Guggulsterone (GS, purity > 98%) was from Sigma-Aldrich (St. Louis, MO). Thioacetamide (TAA) and Masson’s Trichrome staining kit were purchased from Solarbio Company (Beijing, China). Primary antibodies against Bsep, Mrp2 and Ntcp were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and primary antibodies against TGF-β1, α-SMA, NF-κB, and β-actin were purchased from Proteintech Group (Chicago, IL, USA). Other biochemical indicators kits and chemicals were commercially available.

Animal treatments

All animals handling and experimental protocols were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Dalian Medical University, Dalian, China. Male C57BL/6 mice (25± 2 g, 8-10 weeks) were maintained in laboratory animal facilities on a standard 12 h light-dark cycle. Mice were randomly divided into seven groups (6 animals per group) and AUR was dissolved in vehicle (10% hydroxypropyl-beta-cyclodextrin in 500 mM phosphate, pH 7.0) for in vivo experiments: (1) normal saline (i.p.) + vehicle (gavage) as control group; (2) TAA (100 mg/kg, normal saline as solvent, i.p.) + vehicle (gavage); (3) TAA (100 mg/kg, i.p.) + AUR (7.5 mg/kg, gavage); (4) TAA (100 mg/kg, i.p.) + AUR (15 mg/kg, gavage); (5) TAA (100 mg/kg, i.p.) + AUR (30 mg/kg, gavage); (6) TAA (100 mg/kg, i.p.) + GS (10 mg/kg, i.p., 4 h before vehicle administration) + vehicle (gavage); (7) TAA (100 mg/kg, i.p.) + GS (10 mg/kg, i.p., 4 h before AUR administration) + AUR (30 mg/kg, gavage). TAA or normal saline were injected on Monday, Wednesday and Friday, AUR or vehicle were orally given on Tuesday, Thursday and Saturday. The experiment lasted for 8 weeks, and 12 h after AUR or vehicle administration for the last time, mice were sacrificed under anesthesia.
(65 mg/kg pentobarbital sodium, i.p.). Serum and livers were collected and snap frozen on liquid nitrogen, then stored at -80 °C until use.

Serum biochemical analysis
The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), total bile acids (TB), total cholesterol (TC) and TG values were measured with commercially available diagnostic kits (Nanjing Jiancheng Institute of Biotechnology, Nanjing, China).

Histopathological examinations
Before being snap frozen on liquid nitrogen, small pieces from different livers were collected and fixed in 10% neutral buffered formalin. After embedded in paraffin, 5 µm tissue sections were prepared and then stained with haematoxylin & eosin (H&E). Histopathological examinations of Masson’s Trichrome staining and Sirius red staining were further performed and graded as following: 0, normal liver and absence of fibrosis; 1+, fibrosis present; 2+, mild fibrosis; 3+, moderate fibrosis; 4+, severe fibrosis.

Primary mouse hepatocyte isolation and culture
Hepatocytes were isolated by the collagenase perfusion method as previously described from male C57BL/6 mice (8-10 weeks). The isolated mouse primary hepatocytes were cultured in the william’s E medium containing 100 nM dexamethasone, 10% fetal bovine serum, 100 U penicillin/streptomycin, 100 nM insulin and 1× glutamine on six-well plates coated with collagen for 5 h and then cultured in fresh william’s E medium overnight to a density of 6×10^5 cells per well. Cell viability after treated with TAA at different concentrations (0.5-4.0 mM) for 24 h was conducted using the MTT method. For AUR time- and dose-dependent study, incubations were carried out for various times (12, 24, and 48 h) with AUR (5, 10 and
20 µM) at 37 °C in an atmosphere of 5% CO₂ in air, then the culture medium was removed and 2.0 mM TAA was added for another 24 h. The cell viability was assessed using the MTT method.

**Transfection for RNA silencing**

After being cultured for 12 h, 200 nM siRNA for mouse FXR (siGENOME SMARTpool, Dharmacon) or negative control siRNA was transfected to mouse primary hepatocytes. Transfection was performed with lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer and cells were then incubated for 24 h. AUR (10 µM) was added to the culture medium for 24 h and then TAA (2.0 mM) was added for another 24 h. Cell viability was assessed using the MTT method and the mRNA level of Mrp2, Cyp7a1, TGF-β1, α-SMA, TNF-α, and IL-1β was further determined using quantitative real-time PCR.

**Quantitative real-time PCR**

After treatments, RNA from liver tissues or cells were extracted following the manufacturer’s instruction in Trizol reagent (Invitrogen, Grand Island, NY). RNA was analyzed for its quality and quantity, and complementary DNAs were synthesized using PrimeScript RT reagent kit (TaKaRa Biotech, Kyoto, Japan). Quantitative real-time PCR was operated on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Master Mix. The relative mRNA levels were normalized using β-actin as an internal standard. The primers for genes are shown in Table 1.

**Western blotting**

Liver tissues were first homogenized with protein lysis buffer containing 1 mM PMSF, and then incubated at 4 °C for 20 min followed by centrifugation. Protein concentration was determined by BCA assay kit (Solarbio Company, Beijing, China).
Protein aliquots (50 µg) were resolved by SDS polyacrylamide gel electrophoresis in a 8-12% gradient gel and transferred to PVDF membrane. The membranes were blocked with 5% non-fat milk, and probed overnight with primary antibodies including Bsep, Mrp2, Ntcp, TGF-β1, α-SMA, and NF-κB. Specific bands were incubated with specific horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 2 h and visualized by an enhanced chemiluminescence (ECL) method using Bio-Spectrum Gel Imaging System (UVP, USA). Protein expression level was normalized to the β-actin band intensity.

Statistical analysis
Statistical analysis was performed with Prism version 5 (GraphPad Software Inc., San Diego, CA). All data were expressed as the means ± SD. When comparing two groups, statistical significance was determined using Student’s t test. When more than two groups and two factors were investigated, a one-way ANOVA was applied for comparisons. Differences with $P < 0.05$ were considered statistically significant.

Results
AUR protects against TAA-induced liver injury
The chemical structure of AUR was shown in Fig. 1A. Eight weeks of treatment with TAA resulted in significantly lower body weight and increased liver to body weight ratio compared with control, which was attenuated by 30 mg/kg AUR (Fig. 1B-D). H&E staining revealed that TAA treatment caused acute focal necrosis and vacuolisation in some liver cells, with inflammatory cell infiltration. However, clear recovery effects in response to the damage caused by TAA were exhibited after 30 mg/kg AUR was treated (Fig. 2A). Serum ALT and AST are indicators typically used to test liver function. TBIL and TB are the biomarkers of cholestasis which are used to test hepatobiliary function. The ALT, AST (Fig. 2B), TBIL, and TB (Fig. 2C-D) levels were considerably higher in the TAA group than they were in the AUR 30 mg/kg...
treated group. In addition, TC and TG levels increased in the TAA group, and these effects were also attenuated by 30 mg/kg AUR (Fig. 2E). These results indicated that the livers of the mice in the TAA group were severely impaired and AUR exhibited protective effect in TAA-induced fibrosis mice.

**AUR regulates FXR target genes involved in bile acid homeostasis**

Significant increases in serum bile acid level after TAA administration was observed (Fig. 2C-D), suggesting that hepatotoxicity induced by TAA may impair bile acid homeostasis. To understand the mechanism underlying decreased bile acids in AUR-treated mice, the expression of hepatic transporters and enzymes involved in bile acid homeostasis was examined. As shown in Fig. 3A-B, TAA increased the expression of efflux transporter Mrp2 and decreased expression of uptake transporter Ntcp. This may be an adaptive response to TAA-induced hepatotoxicity. The hepatic expression of Bsep and Mrp2 were significantly increased by AUR. Besides, a significant decreased protein level of Ntcp was observed in AUR-treated mice. Besides transporters, bile acid synthetic enzymes were also changed in TAA model mice. Cyp7a1 is the rate-limiting enzyme in bile acid synthesis. Cyp8b1 is also important for the synthesis of bile acid from cholesterol. As shown in Fig. 3C, the mRNA levels of Cyp7a1 was increased significantly by TAA. However, AUR decreased the gene expression of Cyp7a1 and Cyp8b1.Taken together, these findings suggested that the protective effect of AUR on TAA induced liver injury may be due to its regulation of hepatic FXR-regulated genes including Bsep, Mrp2, Ntcp, Cyp7a1, and Cyp8b1, which contribute to the increased transport and decreased synthesis of toxic bile acids.

**AUR prevents the activation of HSCs and relieves TAA-induced liver fibrosis**

Masson’s Trichrome staining and Sirius red staining (Fig. 4A-B) were used to detect collagen distribution in liver and estimate the level of liver fibrosis. Livers from
TAA-treated mice showed apparent collagen deposition between liver lobules and this pattern was significantly attenuated by AUR. Fig. 4C showed that treatment with AUR (30mg/kg) significantly lower the grade of fibrosis as compared to TAA group. To elucidate the anti-fibrosis mechanism of AUR, gene expression involved in HSCs activation and fibrosis were evaluated. As shown in Fig. 4D, 8 weeks of treatment with TAA increased the gene expression of TIMP-1 and COL1-α1. Besides, the protein level of TGF-β1 and α-SMA, which can be considered as indicators of the existence of activated HSCs, were also up-regulated by TAA. Upon treatment with AUR, the TGF-β1 and α-SMA protein levels were reduced (Fig. 4E). These results showed that AUR was effective at inhibiting the activation of HSCs and relieving TAA-induced liver fibrosis.

**AUR suppresses inflammation induced by TAA in mice**

To elucidate the underlying protection mechanism of AUR against inflammation induced by TAA, hepatic inflammatory markers were determined. As shown in Fig. 5A, the mRNA levels of TNF-α, IL-1β, and IL-6 were significantly increased in TAA-treated mice, which were restored by AUR. To further clarify the mechanism of anti-inflammatory effect of AUR, protein expression level of FXR target gene NF-κB was also determined. AUR inhibited hepatic NF-κB during TAA-induced liver fibrosis (Fig. 5B). These data suggested that the protective effect of AUR against inflammation during TAA-induced liver fibrosis may be due to its inhibition of hepatic FXR-regulated gene NF-κB.

**The protection of AUR against TAA-induced liver fibrosis is FXR-dependent**

To verify that AUR activate FXR to regulate expression level of genes in bile acid homeostasis and to exert protection against TAA-induced liver fibrosis *in vivo*, we blocked FXR using FXR antagonist GS in mice. H&E staining indicated that the hepatoprotective effect of AUR was reduced by GS administration (Fig. 6A).
Masson’s Trichrome staining and Sirius red staining suggested that the increased collagen distribution in liver induced by TAA was further aggravated by GS, and the reduced collagen distribution induced by AUR was increased by GS (Fig. 6B-C). GS also abrogated the decrease in mRNA expression of Ntcp, Cyp7a1 (Fig. 6D), TNF-α, IL-1β and IL-6 (Fig. 6E), as well as the fibrosis grade induced by AUR (Fig. 6F). At the same time, GS abrogated the decrease in mRNA expression of COL1α-1, TGF-β1, α-SMA, TIMP-1 (Fig. 6G) and the regulation of Bsep, Mrp2 and NF-κB expression by AUR (Fig. 6H). These results clearly demonstrated that AUR protected against TAA-induced liver fibrosis through activating FXR in vivo.

To further prove the above results of in vivo experiment, in vitro test of inhibiting FXR using siRNA was carried out in mice primary cultured hepatocytes. As shown in Fig. 7A, exposure to TAA for 24 h induced a dose-dependent decrease in viability of mice primary cultured hepatocytes. Compared with control group, the viability of mice primary cultured hepatocytes was decreased to 96.2%, 68.2%, 52.3% and 32.1% by TAA (0.5-4.0 mM). Therefore, the concentration of TAA 2.0 mM was chosen for the following study. Fig. 7B illustrated that AUR could dose-dependently increased cell viability for 12, 24 and 48 h, with a maximal increase at the dose of 10 µM AUR for 24 h. Therefore, AUR at the dose of 10 µM for 24 h was selected for the following study. Western blot analysis ensured that FXR expression was decreased by approximately 80% after transfection of specific siRNA sequence targeting FXR mRNA (data not shown). As shown in Fig. 7C, AUR markedly restored the viability of mice primary cultured hepatocytes reduced by TAA, whereas the changes were abrogated by FXR siRNA. The significant decreases in ALT and AST by AUR were also abrogated by FXR silencing (Fig. 7D). Notably, FXR siRNA abrogated the increase in Mrp2 and the decrease in Cyp7a1, TGF-β1, α-SMA, TNF-α and IL-1β by AUR (Fig. 7E). These results further demonstrated the involvement of FXR activation in the hepatoprotective effect of AUR. Taken together, both in vivo and in vitro results suggested that the protection of AUR against TAA-induced liver fibrosis was
FXR-dependent.

Discussion

Liver fibrosis is a major health distress that causes significant morbidity and mortality with no satisfactory treatment and cure. AUR is one of the main nutraceutical isolated from the Citrus spp. Fruits with antioxidant and anti-inflammatory activities. The current study demonstrated that the mechanism underlying AUR protection is at least three roles. One role is to reduce bile acids in liver by increasing their efflux and reducing their uptake and synthesis. The second role is to inhibit activation of HSCs through down-regulating the expression of level of TGF-β1, α-SMA, TIMP-1, and COL1-α1, which were selective markers of HSCs activation. The third role is to inhibit inflammation via an reduction in NF-κB protein expression and consequently inhibit the downstream inflammatory cascade which was evidenced by decreasing the expression of TNF-α and IL-1β. Further in vivo and in vitro studies both indicated that the hepatoprotective effect of AUR against TAA-induced liver fibrosis was FXR-dependent.

TAA has been shown to induce liver fibrosis development in several different mouse strains. The major changes in TAA-induced liver damage is the altered biomarkers such as AST, ALT, TC, and TG. In the present study, the increased levels of AST, ALT, TC and TG in TAA treated mice were observed. These effects as well as the histopathological changes were all restored with AUR treatment.

Bile acids play a mechanistic role in the pathogenesis in a number of hepatic diseases. Accumulation of toxic bile acids results in hepatocellular death and ultimately leading to liver fibrosis. In liver cells, FXR is bound to and activated by bile acid leading to the regulation of a cohort of genes that function to decrease the concentration of bile acids. Specifically, FXR induces the expression of the genes encoding Bsep and Mrp2 while repressing the expression of Ntcp. In this study, the expression of Mrp2 was increased and Ntcp was decreased by TAA administration,
that may be due to the intrinsic repair mechanism in liver. AUR through FXR activation further enhanced the changes of the above genes, resulting in decreased accumulation of toxic bile acid in hepatocytes.

Liver fibrosis is characterized by quantitative and qualitative amendments of hepatic ECM. It is well known that HSCs activation and over expression is the key initial event in the pathogenesis of hepatofibrosis. Activated HSCs were also responsible for secreting collagen scar tissue, which can lead to liver cirrhosis. The up-regulation of α-SMA and COL1-α1 genes is a fundamental step that convoys HSCs activation and liver fibrosis. The decreasing of TIMP-1, a known inhibitor of metalloproteinases, suggests an additional increase in extracellular matrix degradation, further ameliorating the existing misbalance between accumulation and degradation of collagens of fibrosis. TGF-β1 plays a crucial role in promoting the activation of HSCs and increasing the expression of COL1-α1. In the current study, AUR regulated several profibrogenic genes expressed in activated HSCs, such as TIMP, TGF-β1 and COL1-α1.

Inflammation is also a key factor in HSCs activation. Pathological inflammation has been suggested to activate transcription of pro-apoptotic genes and damage hepatocytes. Thus attenuation of the inflammatory response in hepatocytes has been given much attention. Several targets, including NF-κB, TNF-α and IL-1β were proposed as mainly part of the inflammatory signaling. A recent study found that TAA induced a significant increase in hepatic NF-κB expression together with significant elevation of the TNF-α and IL-1β level, which indicated amplified inflammatory responses induced by TAA challenge. By down-regulating NF-κB and IL-1β, the liver fibrosis has improved. FXR is an immune regulator with anti-inflammatory activity. FXR agonist was shown to have potential beneficial effect on hepatic fibrosis associated with a marked overall decrease in NF-κB activation. Subsequently, the anti-inflammatory mechanism of AUR was evaluated by studying different markers of inflammation. Results revealed that AUR induced a significant
decrease in TNF-α and IL-1β expression. Furthermore, NF-κB, a downstream gene of FXR, was also decreased by AUR treatment.

Previous study reported that auraptene is an inhibitor of cholesterol esterification and a modulator of estrogen receptors, which are two targets involved in the control of sterol and lipid metabolism. The inhibition of cholesterol esterification in hepatocytes will modify the pool of free sterols and oxysterols which could impact on other nuclear receptors and sterol metabolic enzymes. Possibly, AUR through inhibition of cholesterol esterification and regulation of estrogen receptors, exerts hepatoprotection against TAA-induced liver injury. This issue requires to be further studied in future. Maybe AUR has other pharmacological roles in hepatoprotection such as the effect of anti-oxidative stress, and this issue requires to be further studied.

Conclusions

We present evidences to characterize the reduction of hepatic bile acids, the anti-fibrotic and anti-inflammatory effects of AUR in this study. All these effects are in association with FXR activation. The present results indicate that AUR may possess important properties to treat liver diseases. Besides, the development of potent FXR agonist might be beneficial in the treatment of liver fibrosis in clinical practice.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Table 1: The primer sequences used for the real-time PCR assay

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<th>Gene</th>
<th>GenBank accession</th>
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Figure legends

**Fig. 1.** The effects of AUR on body weight and the liver to body weight ratios in TAA-induced liver fibrosis mice. (A) The chemical structure of AUR. (B) Effect of AUR on body weight gain. (C) Effect of AUR on liver weight. (D) Effect of AUR on the liver to body weight ratios. Data are presented as mean ± S.D. (n=6). * P < 0.05 versus Control; # P < 0.05 versus TAA alone.

**Fig. 2.** Hepatoprotection of AUR on eight weeks of TAA treatment in mice. (A) Representative photomicrographs of liver sections stained by H&E (200 × magnification, scale bar, 100 µm). (B) Effect of AUR on serum ALT and AST activities. (C-D) Effect of AUR on serum TBIL and TB activities. (E) Effect of AUR on serum TC and TG levels. Data are presented as mean ± S.D. (n=6). * P < 0.05 versus Control; # P < 0.05 versus TAA alone.

**Fig. 3.** AUR alters gene and protein expression of hepatic transporters and enzymes involved in bile acid homeostasis in TAA-induced liver fibrosis mice. (A-B) Western blot analysis was used to measure the expression of FXR downstream proteins Bsep, Mrp2 and Ntcp. Specific band intensity was quantified, normalized to β-actin. (C) Quantitative real-time PCR analysis was performed to measure the expression of FXR target gene Cyp7a1 and Cyp8b1. Data are the mean ± S.D. (n=6). * P < 0.05 versus Control; # P < 0.05 versus TAA alone.

**Fig. 4.** The effect of AUR on hepatic fibrosis in TAA-treated mice. (A) Effect of AUR on liver histopathological examination by Masson staining (200 × magnification). (B) Sirius red staining indicated a reduction in liver collagen content in mice with AUR treatment (400 × magnification). (C) The grade of fibrosis with Masson staining: 0 = no fibrosis; 1+, fibrosis present; 2+, mild fibrosis; 3+, moderate fibrosis; 4+, severe fibrosis.
fibrosis. (D) Quantitative real-time PCR analysis was performed to measure the gene expression of TIMP-1 and COL1-α1. (E) Western blot analysis was used to measure the expression of TGF-β1 and α-SMA protein expression. Specific band intensity was quantified, normalized to β-actin. Data are the mean ± S.D. (* P< 0.05 versus Control; # P< 0.05 versus TAA alone).

**Fig. 5.** AUR suppresses inflammation in TAA-induced liver fibrosis mice. (A) Quantitative real-time PCR analysis was performed to measure the gene expression of inflammatory gene TNF-α, IL-1β and IL-6. (B) Western blot analysis was used to measure NF-κB protein expression. Specific band intensity was quantified, normalized to β-actin. Data are the mean ± S.D. (* P< 0.05 versus Control; # P< 0.05 versus TAA alone).

**Fig. 6.** AUR protects against TAA-induced liver fibrosis via FXR in mice. The images of (A) H&E stained liver sections (200 × magnification, scale bar, 100 µm), (B) Masson staining (200 × magnification) and (C) Sirius red staining (400 × magnification) after GS administration were shown. The protective effect of AUR on the TAA-treated mice was abrogated by GS. Quantitative real-time PCR analysis was performed to measure the gene expression of (D) Ntcp, Cyp7a1, (E) TNF-α, IL-1β and IL-6 after GS administration. (F) The grade of fibrosis after GS administration. 0, no fibrosis; 1+, fibrosis present; 2+, mild fibrosis; 3+, moderate fibrosis; 4+, severe fibrosis. (G) The gene expression of COL1-α1, TGF-β1, α-SMA and TIMP-1 after GS administration. (H) Western blot analysis was used to measure the protein expression of Bsep, Mrp2 and NF-κB. Specific band intensity was quantified, normalized to β-actin. Data are the mean ± S.D. (* P< 0.05 versus corresponding TAA alone; # P< 0.05 versus corresponding TAA+AUR).

**Fig. 7.** *In vitro* evidences on FXR activation by AUR. (A) The viability of mice
primary cultured hepatocytes after exposed to TAA (0, 0.5, 1.0, 2.0, and 4.0 mM) for 24 h. Data are the mean ± S.D. (n=5). *P< 0.05 versus 0 mM TAA. (B) Effects of AUR (5, 10 and 20 µM) on the viability of mice primary cultured hepatocytes administered with TAA (2.0 mM) for 12, 24 and 48 h. Data are the mean ± S.D. (n=5). *P< 0.05 versus corresponding Control respectively; # P< 0.05 versus corresponding TAA respectively. (C) Effects of AUR (10 µM) on the viability of mice primary cultured hepatocytes administered with TAA (2.0 mM) with or without FXR siRNA. (D) Effects of AUR on ALT and AST in mice primary cultured hepatocytes administered with TAA (2.0 mM) with or without FXR siRNA. (E) Effects of AUR on the mRNA levels of Mrp2, Cyp7a1, TGF-β1, α-SMA, TNF-α, IL-1β with or without FXR siRNA. Data are the mean ± S.D. (n=5). *P< 0.05 versus corresponding TAA alone; # P< 0.05 versus corresponding TAA+AUR.
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A table of contents

Graphical abstract:

Textual abstract:

Auraptene protects against hepatic fibrosis