Cellular Signalling 53 (2019) 190–200

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

# Protective effect of lodoxamide on hepatic steatosis through GPR35

So-Yeon Nam, Soo-Jin Park, Dong-Soon Im*

*College of Pharmacy, Pusan National University, Busan 609-735, Republic of Korea*

ARTICLE INFO

*Keywords:*
GPR35
Steatosis
Lodoxamide
Hepatocyte
Fatty liver
GPCR

ABSTRACT

Although GPR35 is an orphan G protein-coupled receptor, synthetic agonists and antagonists have been developed. Recently, cromolyn, a mast cell stabilizer, was reported as an agonist of GPR35 and was shown to exhibit antifibrotic effects through its actions on hepatocytes and stellate cells. In this study, the role of GPR35 in hepatic steatosis was investigated using an *in vitro* model of liver X receptor (LXR)-mediated hepatocellular steatosis and an *in vivo* model of high fat diet-induced liver steatosis. GPR35 was expressed in Hep3B human hepatoma cells and mouse primary hepatocytes. A specific LXR activator, T0901317, induced lipid accumulation in Hep3B cells. Lodoxamide, the most potent agonist of GPR35, inhibited lipid accumulation in a concentration-dependent manner. The protective effect of lodoxamide was inhibited by a specific GPR35 antagonist, CID2745687, and by siRNA-mediated knockdown of GPR35. The expression of SREBP-1c, a key transcription factor for lipid synthesis, was induced by T0901317 and the induction was inhibited by lodoxamide. Through the use of specific inhibitors of cellular signaling components, the lodoxamide-induced inhibition of lipid accumulation was found to be mediated through p38 MAPKs and JNK, but not through $G_{i/o}$ proteins and ERKs. Furthermore, the protective effect of lodoxamide was confirmed in mouse primary hepatocytes. Lodoxamide suppressed high fat diet-induced fatty liver development, which suggested the protective function of GPR35 in liver steatosis. Therefore, the present data suggest that GPR35 may function to protect against fatty liver development.

## 1. Introduction

Non-alcoholic hepatic steatosis is characterized by the presence of steatosis in hepatocytes [1], which leads to the development of fatty liver [2]. It is strongly associated with obesity, insulin resistance, and type II diabetes [3]. They are major risk factors of non-alcoholic hepatic steatosis [4], which may progress to steatohepatitis, cirrhosis, and hepatocellular carcinoma [1].

*De novo* lipogenesis contributes to hepatic steatosis [5,6]. A nuclear receptor, liver X receptor α (LXRα), plays an important role in the regulation of fatty acid synthesis in hepatocytes through activating sterol regulatory element binding protein 1c (SREBP-1c), a key transcription factor regulating hepatic lipogenesis [7]. The hepatic expression of LXRα and SREBP-1c is increased in patients with steatosis [8].

Human GPR35, an orphan G protein-coupled receptor, is expressed in lung, stomach, small intestine, colon, spleen, and immune cells in humans [9–12]. Although several endogenous molecules, such as, cGMP, kynurenic acid, lysophosphatidic acid, and reverse T3, were reported as ligands of GPR35 [10,13–16], their potencies were subsequently found to be in the micromolar range, which was not supportive of their role as GPR35 ligands [17].

Recently, the chemokine CXCL17 was reported to be an endogenous ligand of GPR35 [18], although this was refuted by a later study [19]. However, many synthetic surrogate agonists and antagonists have been identified or developed [20–29]. Lodoxamide and CID-2745687 have been identified as a potent agonist and a selective antagonist for GPR35 [19,26]. Cromolyn, a mast cell stabilizer was identified as an agonist of GPR35 and was also shown to have antifibrotic effects through its actions on both hepatocytes and hepatic stellate cells [11,30]. Therefore, the function of GPR35 in hepatocytes especially in hepatic steatosis was investigated.

## 2. Materials and methods

### 2.1. Materials

Lodoxamide was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada), and CID-2745687 from Tocris (Ellisville,

* Corresponding author at: Laboratory of Signal Transduction, College of Pharmacy, Pusan National University, 63 Beon-gil 2, Busandaehag-ro, Geumjeong-gu, Busan 609-735, Republic of Korea.
*E-mail address:* imds@pusan.ac.kr (D.-S. Im).

https://doi.org/10.1016/j.cellsig.2018.10.001
Received 26 June 2018; Received in revised form 1 October 2018; Accepted 1 October 2018
Available online 07 October 2018
0898-6568/ 

Missouri, USA). The antibody used for the detection of GPR35 (NBP2-24640) was purchased from Novus Biologicals (Littleton, CO, USA). T0901317 was obtained from Cayman Chemical (Ann Arbor, MI, USA). Pertussis toxin (PTX) was purchased from Sigma-Aldrich (St. Louis, MO, USA). U0126, SB202190, and SP600125 were purchased from Tocris Bioscience (Bristol, UK).

### 2.2. Cell culture and treatment

Hep3B and HepG2 human hepatocellular carcinoma were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium with high glucose with 10% (*v*/v) fetal bovine serum, 100 units/mL penicillin, 50 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% $CO_2$. Cells were seeded into 6-well culture plates and allowed to adhere overnight (18 h). The medium was then changed to serum-free medium with 0.1% bovine serum albumin (BSA). Lodoxamide was pretreated at indicated concentrations for 1 h prior to incubation in the presence or absence of 1 μM T0901317. Each experiment was performed on three independent occasions.

### 2.3. Reverse transcription-PCR

Total RNA was isolated from the cells using Trizol reagent (Invitrogen, USA). RNA concentrations were determined by a Nanodrop ND-1000 spectrophotometer. One microgram of RNA was transcribed by using the Promega ImProm-II Reverse Transcription System (Madision, WI, USA) in accordance with the manufacturer's protocol. Synthesized cDNA products and primers for each gene were used for PCR with Promega Go-Taq DNA polymerase (Madision, WI, USA). Specific primers for mGAPDH (sense 5′-GCG CTA CCG GTC TTC TAT CA-3′, antisense 5′-TGC TGC CAA AAG ACA AGG G-3′), hGAPDH (sense 5′-GAG TCA ACG GAT TTG GTC GT-3′, antisense 5′-TTG ATT TTG GAG GGA TCT CG-3′), mGPR35 (sense 5′-CCA AGA TTC CCA GAT CCT GA-3′ antisense 5′-GGG GAG GGG TGT ATC CTA AA -3′), hGPR35a sense 5′-GTG TTC GTG GTC TGC TTC CT-3′, hGPR35b sense 5′-GTC CTT GCG TCT CTC TGA CC-3′, hGPR35 antisense 5′-GAG AGT CCT GGC TTT TGT GG-3′ were used to amplify gene fragments. For each sample, 7 μL of the PCR products were electrophoresed in 1.2% agarose gels and stained with nucleic acid gel stain (Real Biotech, Taiwan) [31].

### 2.4. Oil red O staining

Oil red O staining was performed in accordance with a previously described method [32]. Briefly, cells were fixed with 10% formalin for 15 min at room temperature and then rinsed with PBS. The slides were immersed in Oil red O working solution for 1 h, rinsed in tap water, counterstained with hematoxylin for 5 s, rinsed again with tap water, and mounted in an aqueous mounting medium.

### 2.5. Western blotting

Hep3B cells were harvested and resuspended in lysis buffer. Protein content was determined by using a BCA protein assay kit (Thermo scientific, Rockford, IL, USA) in accordance with the manufacturer's protocol. Cell lysates (30 μg protein) were separated by 8% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk and then incubated overnight with specific primary antibodies for GPR35 (Novus Biologicals, Littleton, CO, USA), SREBP-1c (Santa Cruz Biotechnology, CA, USA), or β-actin (Cell Signaling Technology, Danvers, MA, USA) at 4 °C. The blots were incubated with HRP-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA) and subsequently developed with ECL detection reagents [33]. All antibodies were used at a dilution of 1:1000. Luminescence was detected using a ChemiDoc Touch Imaging System (BioRad) and analyzed by using ImageLab software (BioRad).

### 2.6. Transfection for GPR35 silencing

The following oligonucleotides were purchased from Bioneer (Korea); GPR35 siRNA sense 5′-CCA CAA AAG CCA GGA CUC U(dTdT)-3′, antisense 5′-AGA GUC CUG GCU UUU GUG G(dTdT)-3′. Briefly, Hep3B cells were seeded at $1.0 \times 10^5$ cells/well in 12-well plates; after 24 h, GPR35 siRNA (200 nM) was introduced into cells by using Lipofectamine LTX reagent (Life Technologies, Carlsbad, CA) in accordance with the manufacturer's instructions. Non-silencing siRNA and siRNAs specific for GPR35 were transfected. The knockdown of the target gene and protein was confirmed by RT-PCR and Western blotting analyses at 24 h and 48 h after transfection, respectively.

### 2.7. Isolation of mouse primary hepatocytes

Mouse primary hepatocytes were isolated from 8 weeks old Balb/c female mice by using a two-step collagenase perfusion method, as described previously [34]. The livers were perfused for 3 min with pre-warmed Hanks' Balanced Salt Solution (HBSS) without calcium and magnesium, and then with HBSS containing 0.1% collagenase at a flow rate of 8 mL/min for 5 min. The excised liver was gently minced in PBS, after which the suspension was filtered with a 70 μM filter and centrifuged at 50*g* for 1 min. After three washes with PBS and centrifuging steps to pellet the cells, the cell pellet was resuspended with DMEM containing 10% FBS, 100 units/mL penicillin, and 50 μg/mL streptomycin. Isolated mouse hepatocytes were seeded in collagen-coated 8-well chambers (SPL, Pocheon, Korea) at a density of $3.6 \times 10^5$ cells/mL.

### 2.8. Measurement of cellular triglyceride content in liver

Triglycerides in livers were extracted with methanol/chloroform (2:1; *v*/v). Solvent was evaporated in 60 °C and lipids were resuspended in deionized water. The levels of triglycerides were determined using a commercial kit from Asan Pharm (Chungcheong, South Korea).

### 2.9. Animals and diets

Male C57BL/6 mice were purchased from Daehan Biolink (DBL, Seoul, Korea) and housed under standard laboratory conditions (22 °C ± 2 °C under a 12-h light/dark cycle) with free access to food and water in the laboratory animal facility at Pusan National University (PNU). In this study, 8-week-old mice were randomly divided into 3 groups: control ($n = 5$), in wich mice were fed a normal chow for 7 weeks; high-fat diet (HFD), in which mice ($n = 5$) were fed a HFD (synthetic diet supplemented with 60% (*w*/w) fat) (Efeed, Korea) for 7 weeks; high-fat diet (HFD) plus lodoxamide, in which mice (n = 5) were fed a HFD for 7 weeks with lodoxamide (1 mg/kg) every day of the seventh week. The animal protocol used in this study was reviewed and approved by the PNU Institutional Animal Care Committee (PNU–IACUC) for compliance with the ethics of the procedures and animal care.

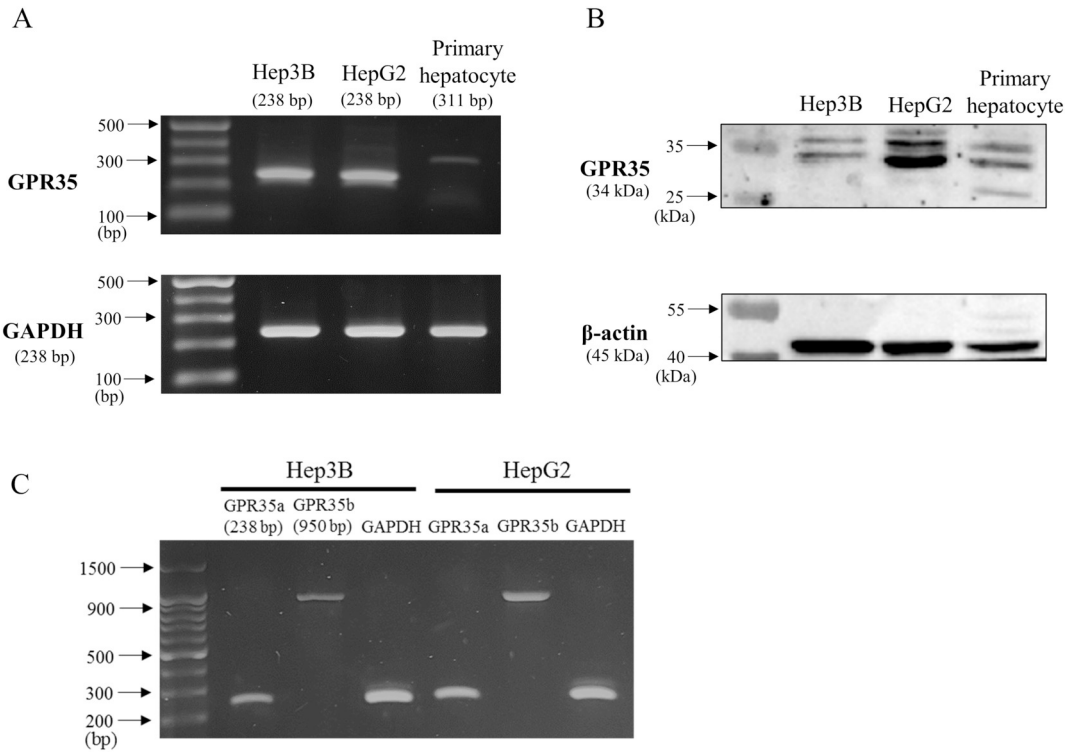


**Fig. 1.** Expression of GPR35 in Hep3B cells, HepG2 cells, and primary hepatocytes.
(A) RNA expression of GPR35 in Hep3B cells, HepG2 cells, and primary hepatocytes. (B) Western blot analysis of GPR35 in Hep3B cells, HepG2 cells, and primary hepatocytes. (C) RNA expression of GPR35a and GPR35b in Hep3B cells and HepG2 cells.

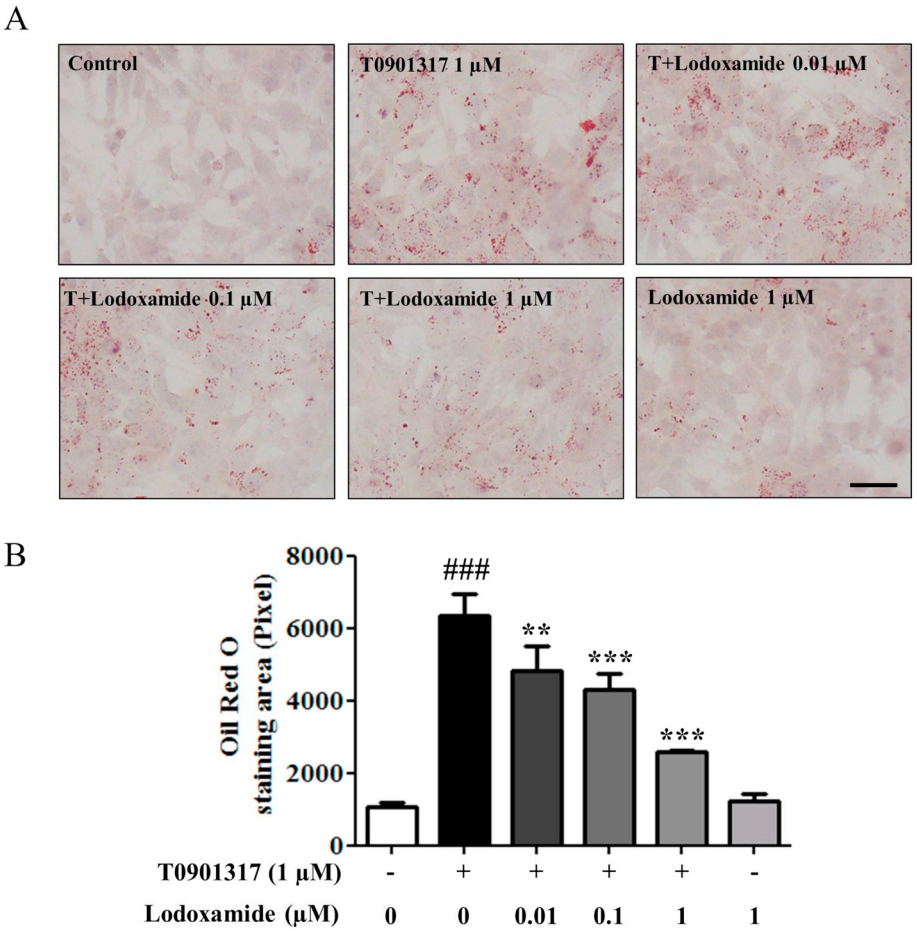


**Fig. 2.** Lodoxamide inhibits LXR-mediated lipid accumulation in Hep3B cells.
(A) Oil red O staining. Hep3B cells were pretreated with different concentrations of lodoxamide for 1 h and then treated with 1 μM T0901317 for 48 h. The red staining indicates the lipid droplets; scale bar = 50 μm. The data are representative of three independent experiments. (B) Histogram of lipid accumulation. Oil red O staining was analyzed using ImageJ software (NIH, Bethesda, MD, USA). The data are from three individual experiments and expressed as mean ± SD. ### $p < .001$ compared with the non-treated group, *** $p < .001$ and ** $p < .01$ compared with T0901317-treated group.

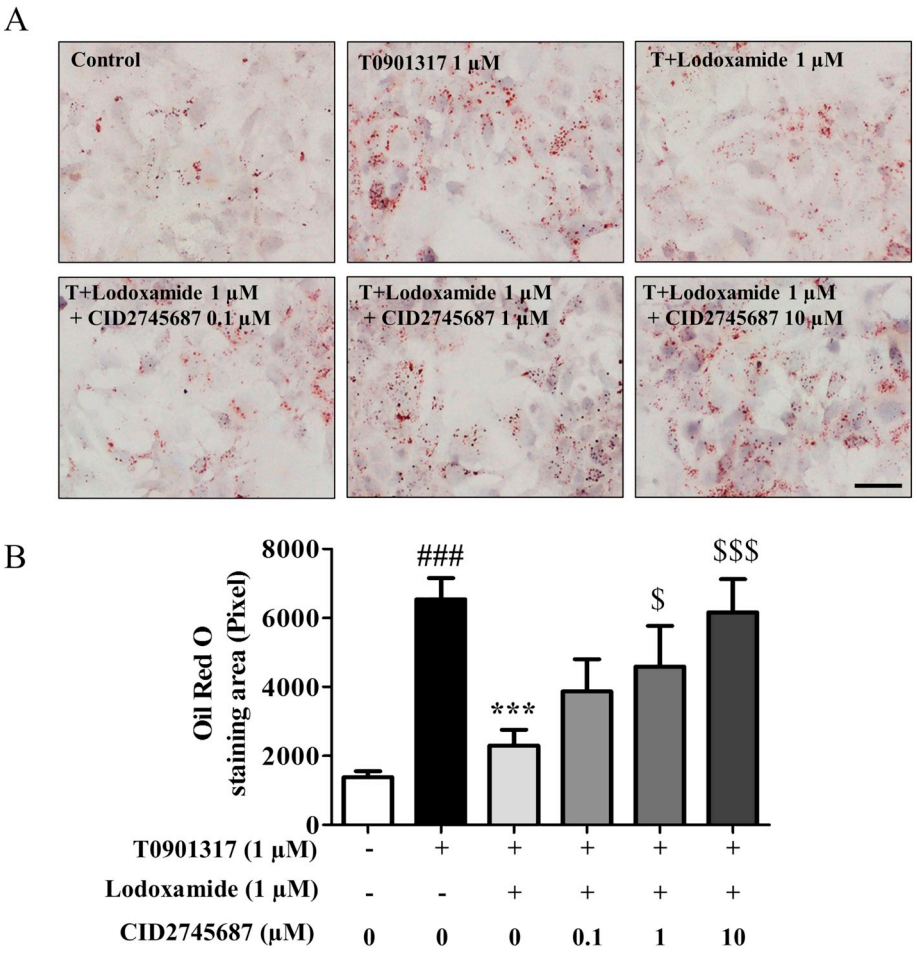


**Fig. 3.** CID2745687 antagonizes lodoxamide-induced inhibition of LXR-mediated lipid accumulation.
(A) Oil red O staining. Hep3B cells were pretreated with 1 μM lodoxamide for 1 h and then treated with 1 μM T0901317 for 48 h. The cells were pretreated with CID2745687 for 1 h prior to the treatment with lodoxamide and T0901317. The red staining shows lipid droplets; scale bar = 50 μm. The data are representatives of three independent experiments. (B) Histogram of lipid accumulation. Oil red O staining was analyzed using ImageJ software. The data from three individual experiments were expressed as mean ± SD. ### $p < .001$, compared with the non-treated group. *** $p < .001$ compared with T0901317-treated group, $ $p < .05$ and $$$ $p < .001$ compared with T0901317 plus lodoxamide-treated group.

### 2.10. Statistics

All results were expressed as mean ± SD. The data were analyzed by one-way ANOVA. A $p$ value < .05 was considered statistically significant.

## 3. Results

### 3.1. Lodoxamide inhibits LXR-induced lipid accumulation via GPR35 in Hep3B cells

Owing to the reported antifibrotic effects of cromolyn, an agonist of GPR35, in hepatocytes [11,30], the expression of GPR35 was first determined in Hep3B and HepG2 human hepatoma cell lines, and primary mouse hepatocytes. The mRNA and protein expression of GPR35 were detected in Hep3B cells, HepG2 cells, and mouse hepatocytes (Fig. 1-A and B). Because two isoforms of hGPR35 have been reported, specific primers were used to detect hGPR35a and hGPR35b [19]. Both isoforms of GPR35 mRNAs were detected in Hep3B cells and HepG2 cells (Fig. 1-C). Subsequently, lodoxamide, the most potent GPR35 agonist [19,26], was used to examine the role of GPR35 in lipid accumulation. Treatment of T0901317, a specific LXR activator, strongly increased the number of lipid droplets in Hep3B cells (Fig. 2). Lodoxamide treatment inhibited lipid accumulation in a concentration-dependent manner (Fig. 2). $EC_{50}$ value for lodoxamide was 85 nM.

CID2745687, a selective GPR35 antagonist, was used to determine whether the anti-lipogenic effect of lodoxamide was mediated by GPR35 [19,22]. As shown in Fig. 3, CID2745687 inhibited the lodoxamide-induced suppression of lipid accumulation in a concentration-dependent manner. $IC_{50}$ value for CID2745687 was 1.01 μM against 1 μM lodoxamide effect. In addition, the GPR35 expression was silenced in Hep3B cells by using RNA interference. The transfection of GPR35 siRNA led to silence the mRNA expression and, subsequently, protein expression (Fig. 4-A). The knockdown of GPR35 reversed the lodoxamide-induced suppression of lipid accumulation (Fig. 4-B). However, transfection with a scrambled siRNA sequence did not cause this effect (Fig. 4-B).

### 3.2. Involvement of GPR35 in lodoxamide inhibition of SREBP-1c in Hep3B cells

SREBP-1c, a key transcription factor for hepatic lipogenic genes, has an important role in hepatic steatosis [35,36] and SREBP-1c induction was shown to mediate LXR-induced lipid accumulation [37]. Thus, the

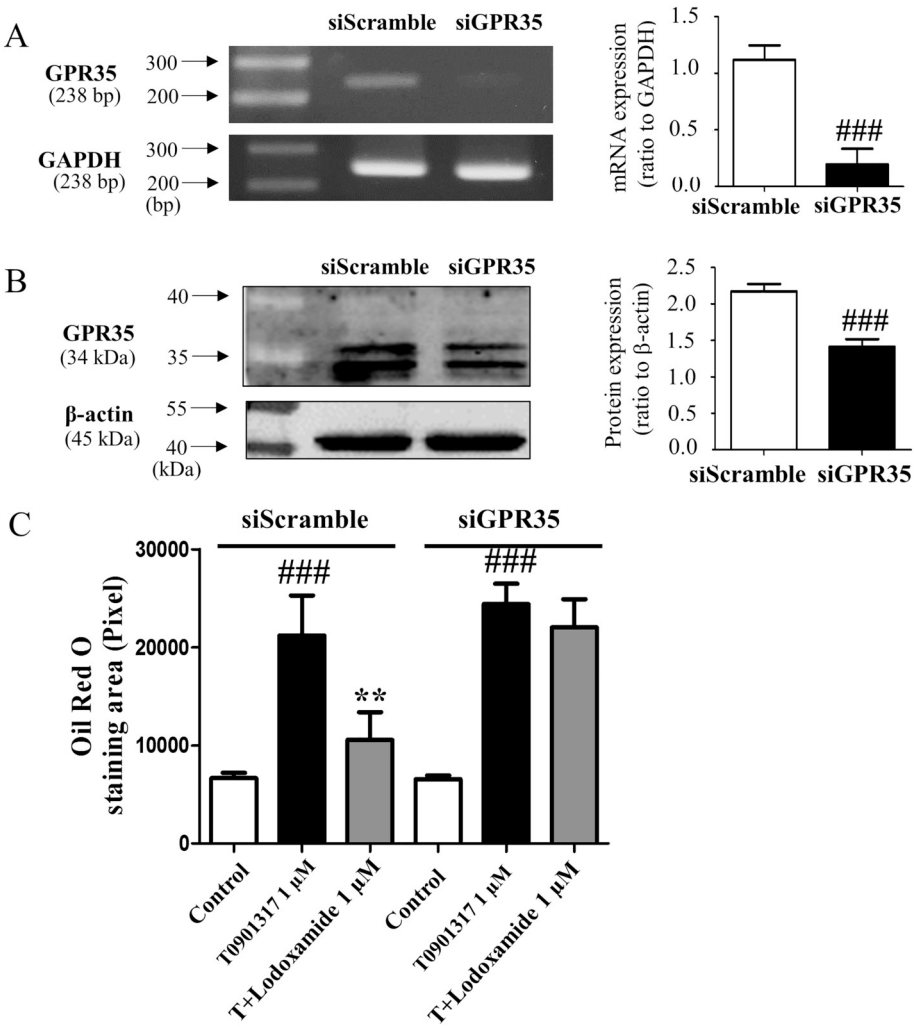


**Fig. 4.** Lodoxamide reduces LXR-mediated lipid accumulation through GPR35.
(A) Expression of GPR35 mRNA in siRNA-transfected Hep3B cells. (B) GPR35 protein expression in siRNA-transfected Hep3B cells. (C) Oil red O staining in GPR35-knockdowned Hep3B cells. Hep3B cells were transfected with GPR35 siRNA or scrambled siRNA and incubated for 48 h. Hep3B cells were then pretreated with 1 μM lodoxamide for 1 h, followed by 1 μM T0901317 for 48 h. Oil red O staining was analyzed using ImageJ software. The data from three individual experiments and expressed as mean ± SD. ### $p < .001$ compared with the non-treated group, ** $p < .01$ compared with T0901317-treated group.

effects of T0901317 and lodoxamide on SREBP-1c were determined. T0901317 treatment induced the expression of SREBP-1c protein (Fig. 5). Moreover, the LXR-mediated induction of SREBP-1c was markedly inhibited by lodoxamide treatment (Fig. 5). Treatment with CID2745687 significantly reversed the lodoxamide-induced suppression of SREBP-1c expression in Hep3B cells (Fig. 6). Therefore, these data show that the activation of GPR35 by lodoxamide suppressed the LXR-mediated expression of SREBP-1c in hepatocytes, which led to the inhibition of lipid accumulation.

### 3.3. Lodoxamide inhibits LXR activation-induced lipid accumulation in primary mouse hepatocytes

To confirm the anti-lipogenic effects of lodoxamide, lipid accumulation was examined by Oil red O staining in primary mouse hepatocytes. Consistent with the results obtained in Hep3B cells, lipid accumulation induced by LXR activation was markedly decreased in a concentration-dependent manner by the treatment of primary hepatocytes with lodoxamide (Fig. 7). $EC_{50}$ value for lodoxamide was 6.1 nM in mouse hepatocytes. CID2745687 treatment also prevented this effect of lodoxamide in primary hepatocytes (Fig. 7). $IC_{50}$ value for CID2745687 was 98 nM against 1 μM lodoxamide effect. These results indicated that lodoxamide also exerted anti-steatosis effects in mouse hepatocytes.

### 3.4. Cellular signaling in the lodoxamide-induced inhibition of lipid accumulation in Hep3B cells

To investigate the signaling cascades involved in the lodoxamide-GPR35 response, specific inhibitors of $G_{i/o}$-type G proteins, ERKs, p38 MAPKs or JNK were used. GPR35 is reported to couple to $G\alpha_{i/o}$ and $G\alpha_{12/13}$ proteins [10,14,21,38,39]. As shown in Fig. 8, the inhibition of lipid accumulation by lodoxamide was not reversed by pretreatment with PTX (100 ng/mL, 24 h), a specific inhibitor for $G_{i/o}$ proteins; this suggested there was no involvement of $G_{i/o}$ proteins in the anti-steatosis response induced by lodoxamide (Fig. 8).

The lipid accumulation was measured in Hep3B cells in the presence of an ERK1/2 inhibitor, U0126 (10 μM); a p38 MAPK inhibitor,

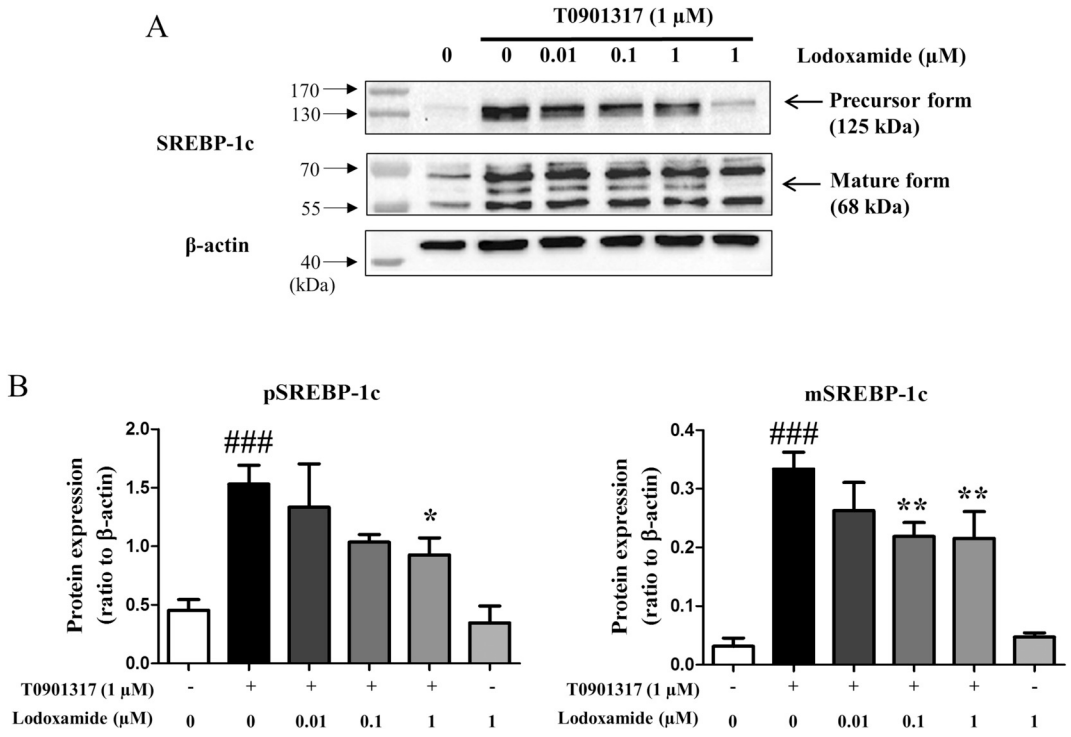


**Fig. 5.** Lodoxamide reduces SREBP-1c protein expression.
(A) Western blot analysis of SREBP-1c protein expression in Hep3B cells. Hep3B cells were pretreated with vehicle or 1 μM lodoxamide for 1 h, and then treated with 1 μM T0901317 for 48 h. (B) Quantitative Western blotting analysis of SREBP-1c in Hep3B cells. The data from three individual experiments were expressed as mean ± SD. ### $p < .001$ compared with the non-treated group, ** $p < .01$ compared with T0901317-treated group.

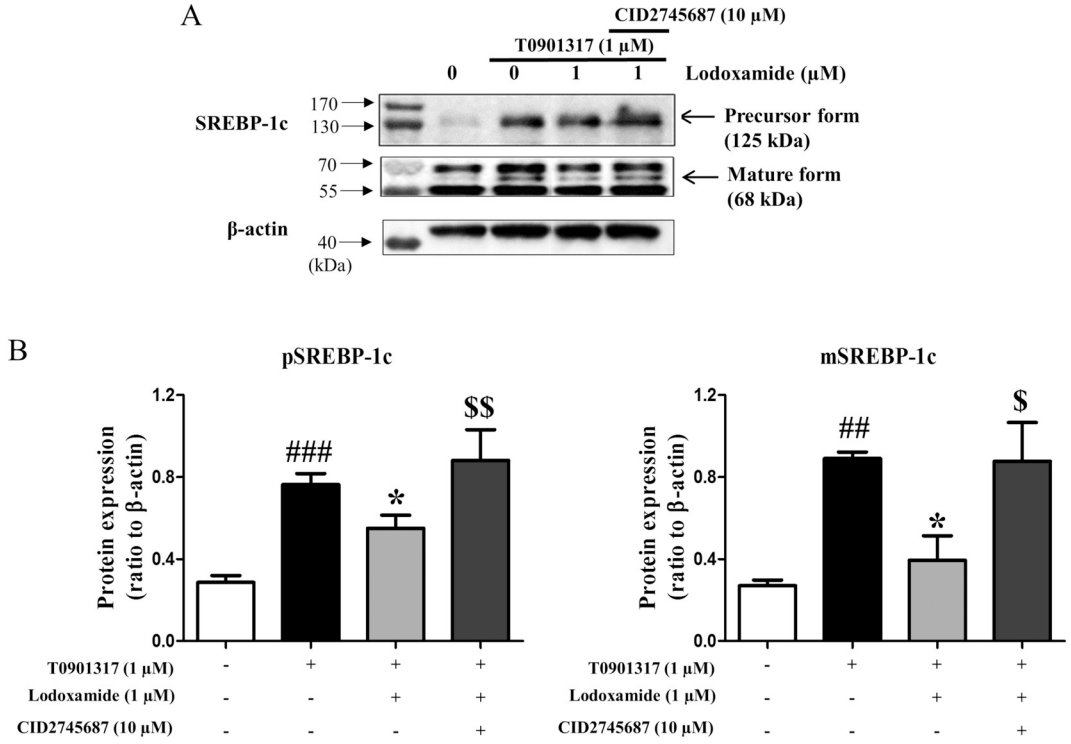


**Fig. 6.** CID2745687 inhibits lodoxamide-mediated reduction of SREBP-1c protein expression.
(A) Western blot analysis of SREBP-1c protein expression in Hep3B cells. Hep3B cells were pretreated with vehicle or 10 μM CID2745687 for 1 h, 1 μM lodoxamide for another 1 h, and then treated with 1 μM T0901317 for 48 h. (B) Quantitative analysis of Western blotting of SREBP-1c in Hep3B cells. The data from three individual experiments and expressed as mean ± SD. ## $p < .01$, and ### $p < .001$ compared with the non-treated group, * $p < .05$ compared with T0901317-treated group, $ $p < .05$ and $$ $p < .01$ compared with T0901317 plus lodoxamide-treated group.

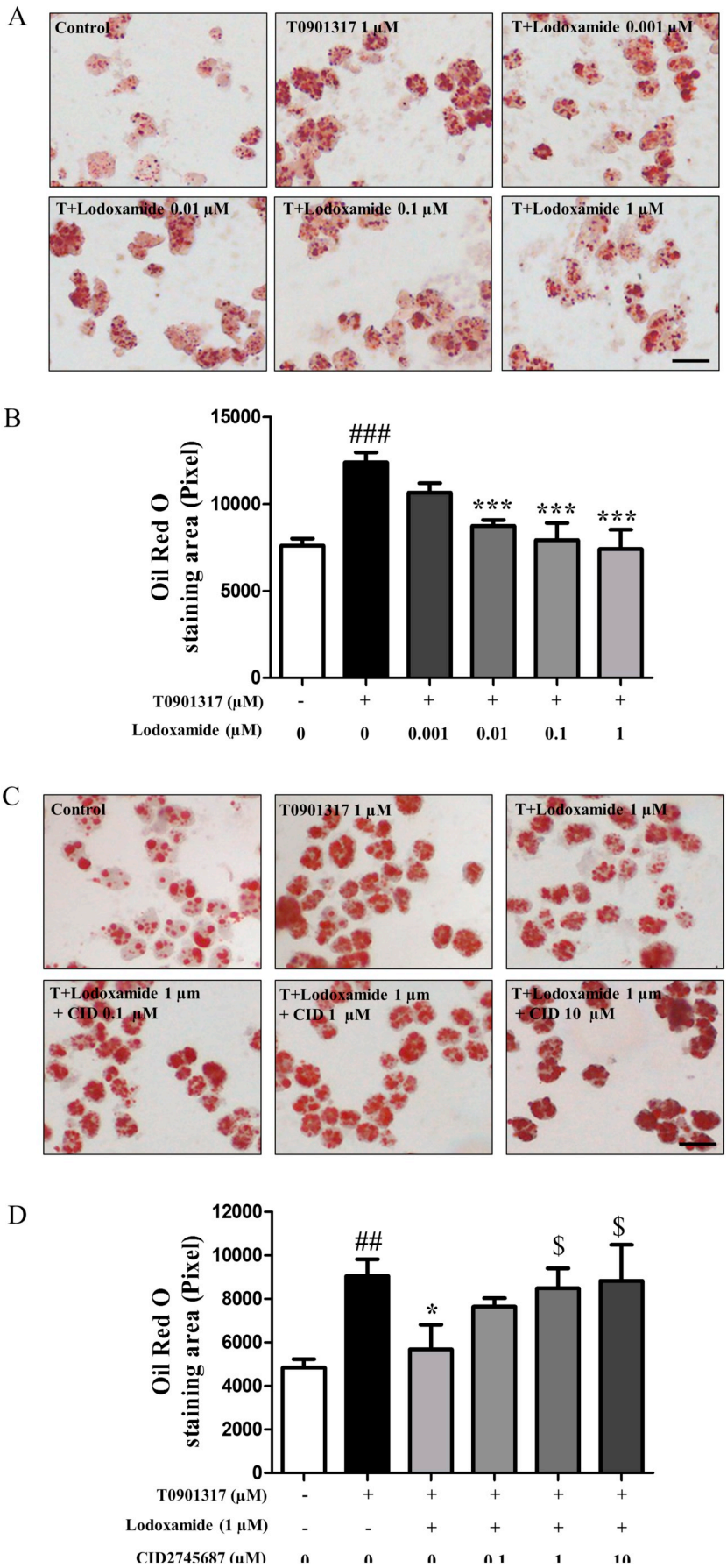


**Fig. 7.** Lodoxamide inhibits LXR-mediated lipid accumulation in mouse primary hepatocytes.
(A) Oil red O staining. Mouse primary hepatocytes were pretreated with different concentrations of lodoxamide for 1 h, and then treated with 1 μM T0901317 for 48 h. (C) Oil red O staining. Mouse primary hepatocytes were pretreated with vehicle or different concentrations of CID2745687 for 1 h and then treated with 1 μM of lodoxamide for 1 h, and then treated with 1 μM T0901317 for 48 h. (B, D) Oil red O staining was analyzed using ImageJ software. The data from three individual experiments were expressed as the mean ± SD. ## $p < .01$ and ### $p < .001$ compared with the non-treated group, * $p < .05$ and *** $p < .001$ compared with T0901317-treated group, $ $p < .05$ compared with T0901317 plus lodoxamide-treated group.

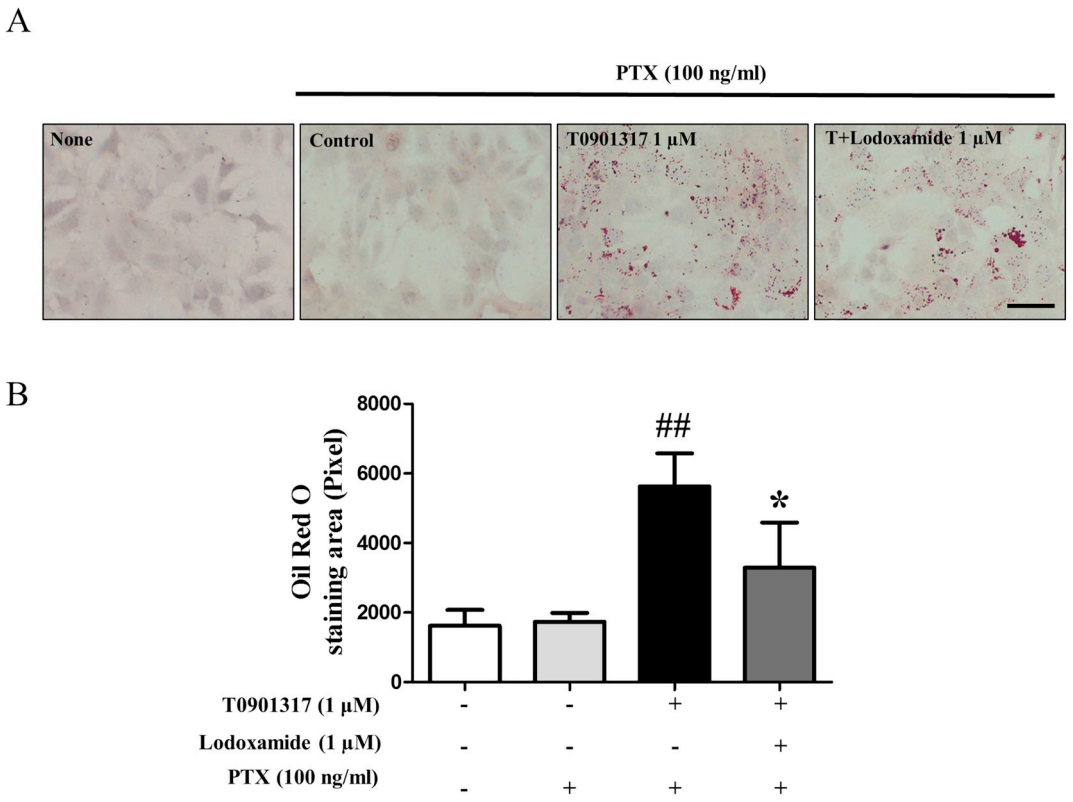


**Fig. 8.** Effect of pertussis toxin on the inhibitory effect of lodoxamide on lipid accumulation in Hep3B cells.
(A) Oil red O staining analysis of Hep3B cells pretreated with pertussis toxin (PTX). Hep3B cells were pretreated with PTX (100 ng/mL) for 24 h prior to treatment with lodoxamide and T0901317. (B) Histogram of lipid accumulation. The data from three individual experiments were expressed as the mean ± SD. ## $p < .01$ compared with the PTX treated group, * $p < .05$ compared with PTX plus T0901317 treated group.

SB202190 (10 μM); or a JNK inhibitor, SP600125 (10 μM). As shown in Fig. 9, SB202190 or SP600125 inhibited lodoxamide-induced inhibition of lipid accumulation in Hep3B cells, but U0126 did not. These results suggest the involvement of p38 MAPK and JNK in the lodoxamide-induced inhibition of lipid accumulation in Hep3B cells.

### 3.5. *Lodoxamide suppressed high fat diet-induced fatty liver through GPR35 in vivo*

To confirm the protective actions of GPR35 against hepatic steatosis, an HFD-induced fatty liver model was used. In the HFD-feeding group, fatty liver was induced after 7 weeks, which was confirmed by H &E staining, Oil red O staining, measurement of triglycerides, and Western blotting of SREBP-1c. In the HFD plus lodoxamide group, mice were fed a HFD for 7 weeks with lodoxamide administration (1 mg/kg) daily orally for the seventh week. The administration of lodoxamide suppressed lipid accumulation and expression of SREBP-1c in the liver (Fig. 10).

### 3.6. *Discussion*

The present study reports the first demonstration of a GPR35–dependent effect on liver fat accumulation. Four key findings are reported. First, lodoxamide reduced LXR-mediated lipid accumulation in Hep3B cells and primary mouse hepatocytes *via* GPR35. Second, lodoxamide inhibited LXR-induced expression of lipogenic gene SREBP-1c *via* GPR35. Third, lodoxamide signaling was mediated *via* GPR35, p38 MAPK, and JNK. Forth, lodoxamide protects against HFD-induced fatty liver through GPR35 *in vivo* (Fig. 11).

CID2745687 was reported as a potent and effective antagonist on human GPR35 but not on rodent orthologs of GPR35 [27]. Therefore, the observed antagonism of CID2745687 on lodoxamide-induced action in mouse primary hepatocytes is surprising (Fig. 7D). This could be an off-target effect, because CID2745687 co-treatment did not reverse the lodoxamide effect at even 10 times higher dose of CID274687 (10 mg/kg) than lodoxamide (1 mg/kg) *in vivo* (data not shown). Lodoxamide was originally reported as a high potency agonist on human and rat GPR35 [26]. However, its potency at mouse GPR35 was reported > 4000 fold lower than human GPR35 [26]. In our study, lodoxamide $EC_{50}$ was 85 nM on Hep3B cells and 6.1 nM in mouse primary hepatocytes, which is contrasting to the previous study. Again, there might be an off-target effect of lodoxamide in mouse hepatocytes.

GPR35 has been reported to couple to both $G\alpha_{i/o}$ and $G\alpha_{13}$ subunits and to recruit β-arrestin-2 [10,14,21,38,39]. In the present study, $G\alpha_{i/o}$

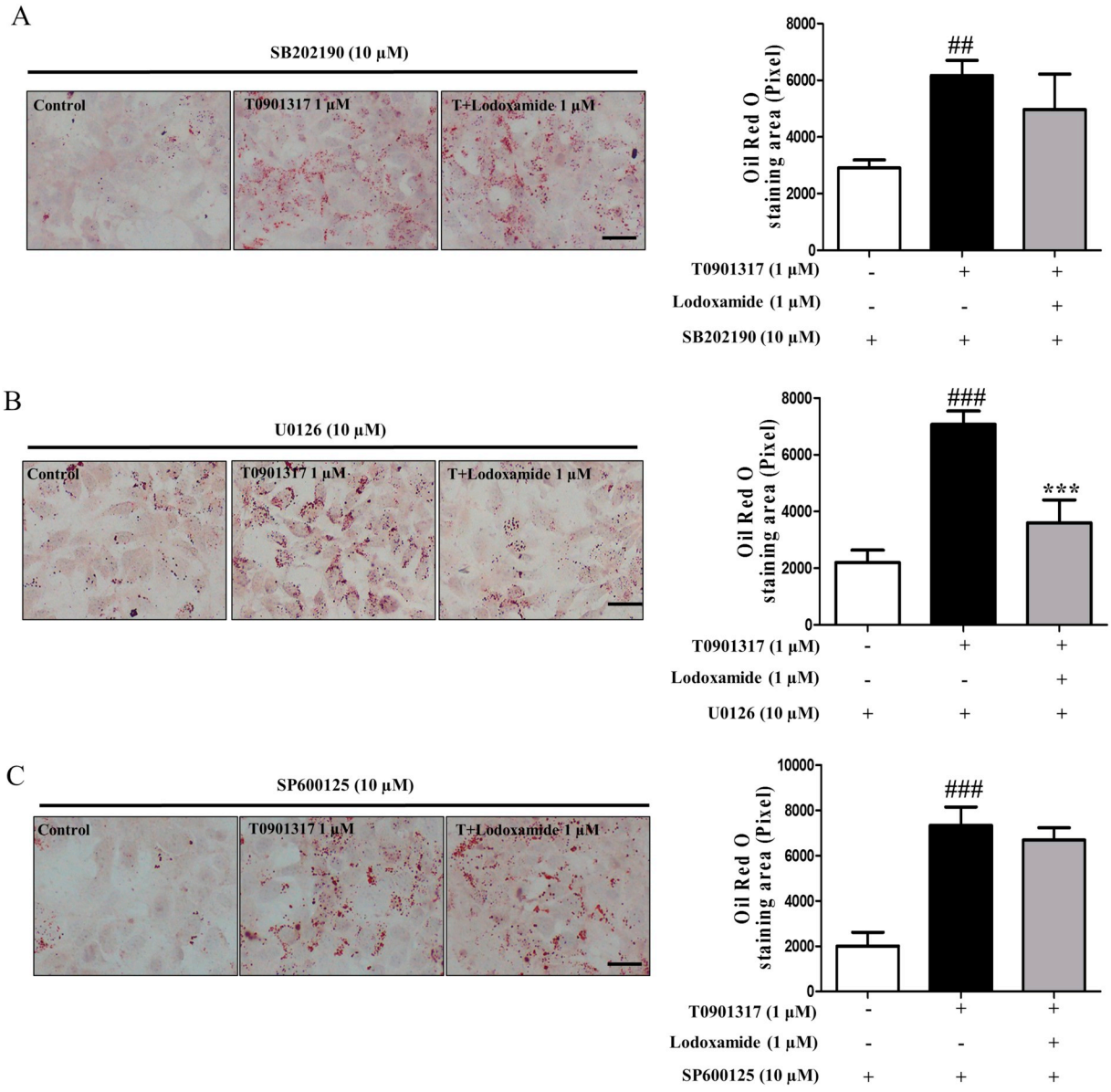


**Fig. 9.** Signaling pathways mediating the effect of lodoxamide on lipid accumulation in Hep3B cells.
Oil red O staining analysis of Hep3B cells pretreated with the p38 MAPK inhibitor, SB202190 (10 μM, A); ERK1/2 inhibitor U0126 (10 μM, B); or JNK inhibitor, SP600125 (10 μM, C) for 30 min prior to treatment with lodoxamide and T0901317. Hep3B cells were pretreated with 1 μM lodoxamide for 1 h and then treated with 1 μM T0901317 for 48 h. The data from three individual experiments were expressed as the mean ± SD. ## $p < .01$ and ### $p < .001$ compared with the control group, *** $p < .001$ compared with the T0901317 treated group.

was shown to be not a signaling component for the suppression of lipid accumulation. The p38 MAPK and JNK are known to regulate liver metabolism [40] and p38 MAPK pathway has been reported to contribute to the regulation of lipid metabolism in Hep3B cells [41]. Also, it has been reported that SREBP-1c is the most important transcriptional regulator of the lipogenesis gene in the liver [42]. In the present study, suppression of p38 MAPK or JNK resulted in the suppression of the lodoxamide effect, which suggested that the p38 MAPK and JNK pathways were involved in regulation of lipid metabolism in Hep3B cells. Therefore, p38 MAPK and JNK are thought to be the upstream kinases of SREBP-1c.

The present data indicated that the protective effect of lodoxamide occurred through GPR35 in liver steatosis and thus allowed the identification of potential therapeutic targets. These findings have identified the lodoxamide-GPR35 system as a promising therapeutic target for fatty liver diseases.

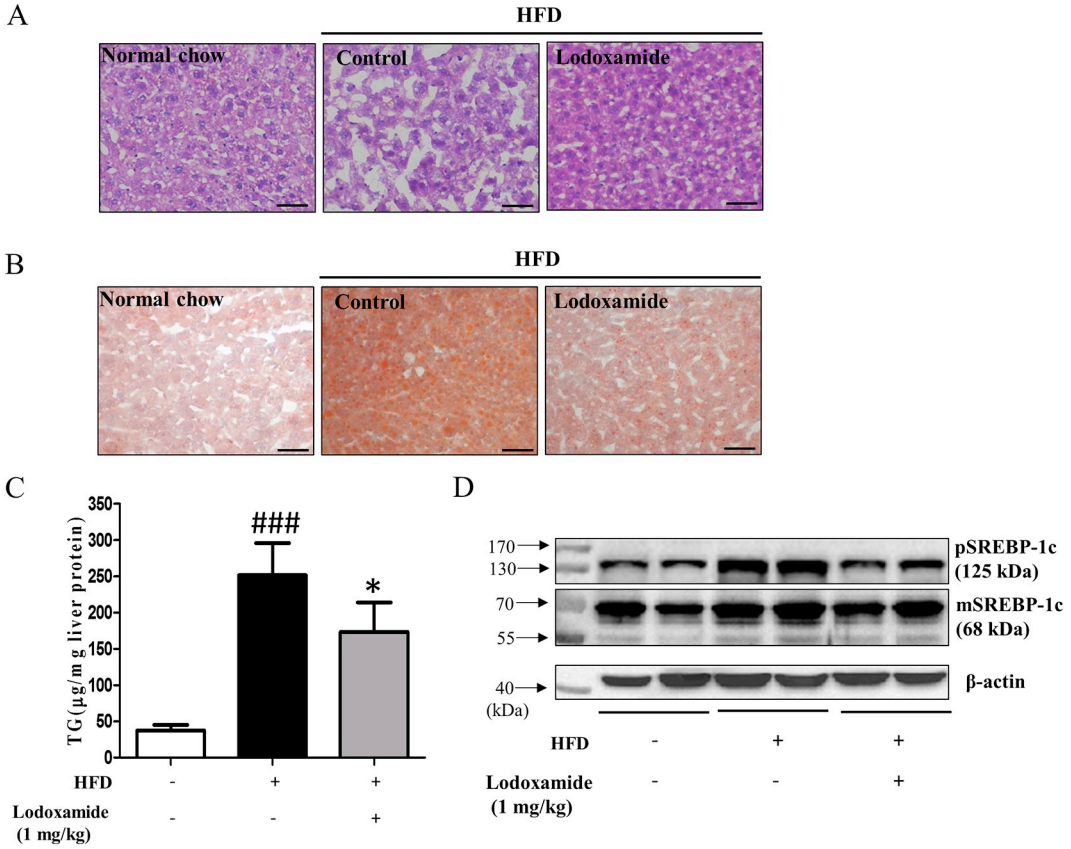


**Fig. 10.** Lodoxamide suppressed HFD-induced fatty liver in C57BL/6 mice.
C57BL/6 mice were fed a normal chow diet or an HFD for 7 weeks. Lodoxamide was administrated daily orally for the last week. Mice were sacrificed and livers were collected. (A) H&E staining of liver slices. (B) Oil red O staining of liver slices. (C) TG contents in liver. (D) Western blotting of SREBP-1c. The data were collected from five mice for each group and expressed as the mean ± SD. ### $p < .001$ compared with the normal chow group, * $p < .05$ compared with HFD treated group.

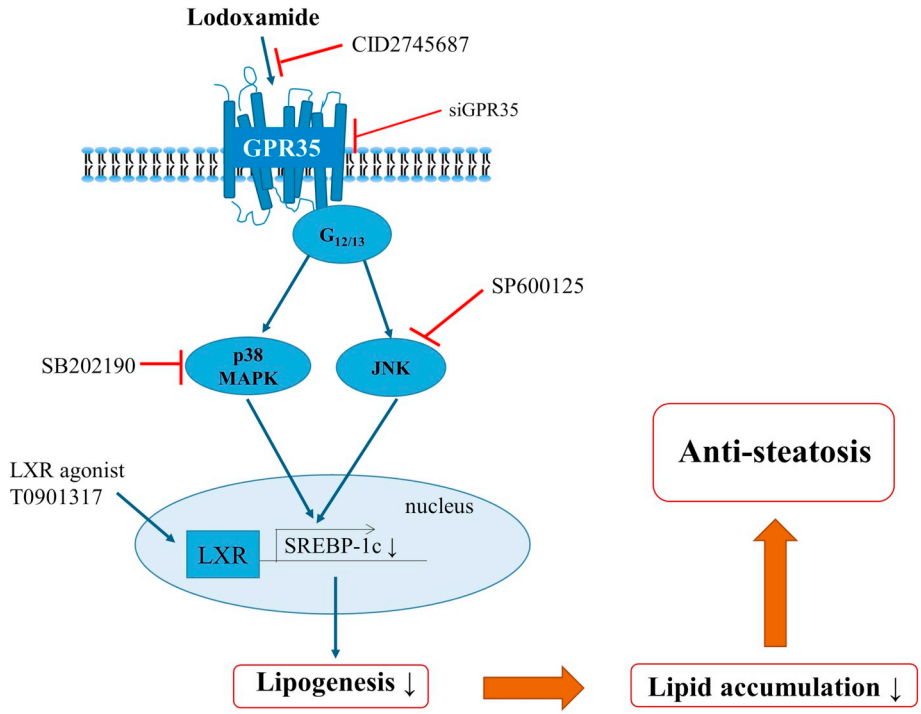


**Fig. 11.** Proposed signaling pathway and mechanism of lodoxamide on lipid accumulation in Hep3B cells.

## Acknowledgement

This research was supported by a grant from the Basic Science Research Program through the National Research Foundation (NRF) of Korea funded by the Ministry of Education, Science and Technology (#2016R1D1A1A009917086).

## References

[1] N. Chalasani, Z. Younossi, J.E. Lavine, A.M. Diehl, E.M. Brunt, K. Cusi, M. Charlton, A.J. Sanyal, A. American Gastroenterological, D. American Association for the Study of Liver, G. American College of, The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology, Gastroenterology 142 (7) (2012) 1592–1609.
[2] C.S. Lieber, D.P. Jones, L.M. Decarli, Effects of prolonged ethanol intake: production of fatty liver despite adequate diets, J. Clin. Invest. 44 (1965) 1009–1021.
[3] C. Postic, J. Girard, The role of the lipogenic pathway in the development of hepatic steatosis, Diabet. Metab. 34 (6 Pt 2) (2008) 643–648.
[4] H.M. Parker, N.A. Johnson, C.A. Burdon, J.S. Cohn, H.T. O'Connor, J. George, Omega-3 supplementation and non-alcoholic fatty liver disease: a systematic review and meta-analysis, J. Hepatol. 56 (4) (2012) 944–951.
[5] K.L. Donnelly, C.I. Smith, S.J. Schwarzenberg, J. Jessurun, M.D. Boldt, E.J. Parks, Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease, J. Clin. Invest. 115 (5) (2005) 1343–1351.
[6] C. Postic, J. Girard, The role of the lipogenic pathway in the development of hepatic steatosis, Diabet. Metab. 34 (6) (2008) 643–648.
[7] D.J. Peet, S.D. Turley, W. Ma, B.A. Janowski, J.-M.A. Lobaccaro, R.E. Hammer, D.J. Mangelsdorf, Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXRα, Cell 93 (5) (1998) 693–704.
[8] N. Higuchi, M. Kato, Y. Shundo, H. Tajiri, M. Tanaka, N. Yamashita, M. Kohjima, K. Kotoh, M. Nakamuta, R. Takayanagi, Liver X receptor in cooperation with SREBP-1c is a major lipid synthesis regulator in nonalcoholic fatty liver disease, Hepatol. Res. 38 (11) (2008) 1122–1129.
[9] B.F. O'Dowd, T. Nguyen, A. Marchese, R. Cheng, K.R. Lynch, H.H. Heng, L.F. Kolakowski Jr., S.R. George, Discovery of three novel G-protein-coupled receptor genes, Genomics 47 (2) (1998) 310–313.
[10] J. Wang, N. Simonavicius, X. Wu, G. Swaminath, J. Reagan, H. Tian, L. Ling, Kynurenic acid as a ligand for orphan G protein-coupled receptor GPR35, J. Biol. Chem. 281 (31) (2006) 22021–22028.
[11] Y. Yang, J.Y. Lu, X. Wu, S. Summer, J. Whoriskey, C. Saris, J.D. Reagan, G-protein-coupled receptor 35 is a target of the asthma drugs cromolyn disodium and nedocromil sodium, Pharmacology 86 (1) (2010) 1–5.
[12] S. Fallarini, L. Magliulo, T. Paoletti, C. de Lalla, G. Lombardi, Expression of functional GPR35 in human iNKT cells, Biochem. Biophys. Res. Commun. 398 (3) (2010) 420–425.
[13] S. Oka, R. Ota, M. Shima, A. Yamashita, T. Sugiura, GPR35 is a novel lysophosphatidic acid receptor, Biochem. Biophys. Res. Commun. 395 (2) (2010) 232–237.
[14] L. Jenkins, E. Alvarez-Curto, K. Campbell, S. de Munnik, M. Canals, S. Schlyer, G. Milligan, Agonist activation of the G protein-coupled receptor GPR35 involves transmembrane domain III and is transduced via Galpha(13) and beta-arrestin-2, Br. J. Pharmacol. 162 (3) (2011) 733–748.
[15] C. Southern, J.M. Cook, Z. Neetoo-Isseljee, D.L. Taylor, C.A. Kettleborough, A. Merritt, D.L. Bassoni, W.J. Raab, E. Quinn, T.S. Wehrman, A.P. Davenport, A.J. Brown, A. Green, M.J. Wigglesworth, S. Rees, Screening beta-arrestin recruitment for the identification of natural ligands for orphan G-protein-coupled receptors, J. Biomol. Screen. 18 (5) (2013) 599–609.
[16] H. Deng, H. Hu, Y. Fang, Multiple tyrosine metabolites are GPR35 agonists, Sci. Rep. 2 (2012) 373.
[17] N. Divorty, A.E. Mackenzie, S.A. Nicklin, G. Milligan, G protein-coupled receptor 35: an emerging target in inflammatory and cardiovascular disease, Front. Pharmacol. 6 (2015) 41.
[18] J.L. Maravillas-Montero, A.M. Burkhardt, P.A. Hevezi, C.D. Carnevale, M.J. Smit, A. Zlotnik, Cutting edge: GPR35/CXCR8 is the receptor of the mucosal chemokine CXCL17, J. Immunol. 194 (1) (2015) 29–33.
[19] S.J. Park, S.J. Lee, S.Y. Nam, D.S. Im, GPR35 mediates lodoxamide-induced migration inhibitory response but not CXCL17-induced migration stimulatory response in THP-1 cells; is GPR35 a receptor for CXCL17? Br. J. Pharmacol. 175 (1) (2018) 154–161.
[20] Y. Taniguchi, H. Tonai-Kachi, K. Shinjo, Zaprinast, a well-known cyclic guanosine monophosphate-specific phosphodiesterase inhibitor, is an agonist for GPR35, FEBS Lett. 580 (21) (2006) 5003–5008.
[21] L. Jenkins, J. Brea, N.J. Smith, B.D. Hudson, G. Reilly, N.J. Bryant, M. Castro, M.I. Loza, G. Milligan, Identification of novel species-selective agonists of the G-protein-coupled receptor GPR35 that promote recruitment of beta-arrestin-2 and activate Galpha13, Biochem. J. 432 (3) (2010) 451–459.
[22] P. Zhao, H. Sharir, A. Kapur, A. Cowan, E.B. Geller, M.W. Adler, H.H. Seltzman, P.H. Reggio, S. Heynen-Genel, M. Sauer, T.D. Chung, Y. Bai, W. Chen, M.G. Caron, L.S. Barak, M.E. Abood, Targeting of the orphan receptor GPR35 by pamoic acid: a potent activator of extracellular signal-regulated kinase and beta-arrestin2 with antinociceptive activity, Mol. Pharmacol. 78 (4) (2010) 560–568.
[23] Z. Neetoo-Isseljee, A.E. MacKenzie, C. Southern, J. Jerman, E.G. McIver, N. Harries, D.L. Taylor, G. Milligan, High-throughput identification and characterization of novel, species-selective GPR35 agonists, J. Pharmacol. Exp. Ther. 344 (3) (2013) 568–578.
[24] D. Thimm, M. Funke, A. Meyer, C.E. Muller, 6-Bromo-8-(4-[(3)H]methoxybenzamido)-4-oxo-4H-chromene-2-carboxylic acid: a powerful tool for studying orphan g protein-coupled receptor GPR35, J. Med. Chem. 56 (17) (2013) 7084–7099.
[25] M. Funke, D. Thimm, A.C. Schiedel, C.E. Muller, 8-Benzamidochromen-4-one-2-carboxylic acids: potent and selective agonists for the orphan G protein-coupled receptor GPR35, J. Med. Chem. 56 (12) (2013) 5182–5197.
[26] A.E. MacKenzie, G. Caltabiano, T.C. Kent, L. Jenkins, J.E. McCallum, B.D. Hudson, S.A. Nicklin, L. Fawcett, R. Markwick, S.J. Charlton, G. Milligan, The antiallergic mast cell stabilizers lodoxamide and bufrolin as the first high and equipotent agonists of human and rat GPR35, Mol. Pharmacol. 85 (1) (2014) 91–104.
[27] L. Jenkins, N. Harries, J.E. Lappin, A.E. MacKenzie, Z. Neetoo-Isseljee, C. Southern, E.G. McIver, S.A. Nicklin, D.L. Taylor, G. Milligan, Antagonists of GPR35 display high species ortholog selectivity and varying modes of action, J. Pharmacol. Exp. Ther. 343 (3) (2012) 683–695.
[28] S. Heynen-Genel, R. Dahl, S. Shi, M. Sauer, S. Hariharan, E. Sergienko, S. Dad, T.D.Y. Chung, D. Stonich, Y. Su, M. Caron, P. Zhao, M.E. Abood, L.S. Barak, Selective GPR35 antagonists - Probes 1 & 2, probe reports from the NIH Molecular Libraries Program, Bethesda (MD), (2010).
[29] S. Heynen-Genel, R. Dahl, S. Shi, M. Sauer, S. Hariharan, E. Sergienko, S. Dad, T.D.Y. Chung, D. Stonich, Y. Su, P. Zhao, M.G. Caron, M.E. Abood, L.S. Barak, Selective GPR35 Antagonists - Probe 3, Probe Reports from the NIH Molecular Libraries Program, Bethesda (MD), (2010).
[30] J.S. Choi, J.K. Kim, Y.J. Yang, Y. Kim, P. Kim, S.G. Park, E.Y. Cho, D.H. Lee, J.W. Choi, Identification of cromolyn sodium as an anti-fibrotic agent targeting both hepatocytes and hepatic stellate cells, Pharmacol. Res. 102 (2015) 176–183.
[31] J.M. Lee, S.J. Park, D.S. Im, Calcium signaling of lysophosphatidylethanolamine through lpa1 in human SH-SY5Y neuroblastoma cells, Biomol. Ther. 25 (2017) 194–201.
[32] S.-J. Park, K.-P. Lee, S. Kang, J. Lee, K. Sato, H.Y. Chung, F. Okajima, D.-S. Im, Sphingosine 1-phosphate induced anti-atherogenic and atheroprotective M2 macrophage polarization through IL-4, Cell. Signal. 26 (10) (2014) 2249–2258.
[33] A.Y. Lee, S. Kang, S.J. Park, J. Huang, D.S. Im, Anti-Allergic effect of oroxylin a from *Oroxylum indicum* using in vivo and in vitro experiments, Biomol. Ther. 24 (3) (2016) 283–290.
[34] J. Chu, H. Zhang, X. Huang, Y. Lin, T. Shen, B. Chen, Y. Man, S. Wang, J. Li, Apelin ameliorates TNF-alpha-induced reduction of glycogen synthesis in the hepatocytes through G protein-coupled receptor APJ, PLoS One 8 (2) (2013) e57231.
[35] H. Shimano, N. Yahagi, M. Amemiya-Kudo, A.H. Hasty, J.-i. Osuga, Y. Tamura, F. Shionoiri, Y. Iizuka, K. Ohashi, K. Harada, Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes, J. Biol. Chem. 274 (50) (1999) 35832–35839.
[36] N. Yahagi, H. Shimano, A.H. Hasty, M. Amemiya-Kudo, H. Okazaki, Y. Tamura, Y. Iizuka, F. Shionoiri, K. Ohashi, J.-i. Osuga, A crucial role of sterol regulatory element-binding protein-1 in the regulation of lipogenic gene expression by polyunsaturated fatty acids, J. Biol. Chem. 274 (50) (1999) 35840–35844.
[37] J.J. Repa, G. Liang, J. Ou, Y. Bashmakov, J.-M.A. Lobaccaro, I. Shimomura, B. Shan, M.S. Brown, J.L. Goldstein, D.J. Mangelsdorf, Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRα and LXRβ, Genes Dev. 14 (22) (2000) 2819–2830.
[38] H. Ohshiro, H. Tonai-Kachi, K. Ichikawa, GPR35 is a functional receptor in rat dorsal root ganglion neurons, Biochem. Biophys. Res. Commun. 365 (2) (2008) 344–348.
[39] J. Guo, D.J. Williams, H.L. Puhl, S.R. Ikeda 3rd, Inhibition of N-type calcium channels by activation of GPR35, an orphan receptor, heterologously expressed in rat sympathetic neurons, J. Pharmacol. Exp. Ther. 324 (1) (2008) 342–351.
[40] A. Lawan, L. Zhang, F. Gatzke, K. Min, M.J. Jurczak, M. Al-Mutairi, P. Richter, J.P. Camporez, A. Couvillon, D. Pesta, R.J. Roth Flach, G.I. Shulman, A.M. Bennett, Hepatic mitogen-activated protein kinase phosphatase 1 selectively regulates glucose metabolism and energy homeostasis, Mol. Cell. Biol. 35 (1) (2015) 26–40.
[41] J. Huang, S. Kang, S.J. Park, D.S. Im, Apelin protects against liver X receptor-mediated steatosis through AMPK and PPARalpha in human and mouse hepatocytes, Cell. Signal. 39 (2017) 84–94.
[42] X.M. Feng, J. Xiong, H. Qin, W. Liu, R.N. Chen, W. Shang, R. Ning, G. Hu, J. Yang, Fluoxetine induces hepatic lipid accumulation via both promotion of the SREBP1c-related lipogenesis and reduction of lipolysis in primary mouse hepatocytes, CNS Neurosci. Ther. 18 (12) (2012) 974–980.