ASB14780, an orally active inhibitor of group IVA phospholipase A₂, is a pharmacotherapeutic candidate for non-alcoholic fatty liver disease

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List of Abbreviations:

ACC, acetyl-CoA carboxylase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCl₄, carbon tetrachloride; Col₁α₂, collagen 1α₂; CPT₁α, carnitine palmitoyl-transferase 1α; DAG, esterified diacylglycerol; DGAT₂, diacylglycerol acyltransferase 2; FAS, fatty acid synthase; FFA, free fatty acids; HE, hematoxylin and eosin; HFCD, high-fat cholesterol diet;
HPC, hydroxypropyl cellulose; HSC, hepatic stellate cell; HSL, hormone-sensitive lipase; IVA-PLA₂, group IVA phospholipase A₂; L-FABP, liver fatty acid binding protein; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; MTP, microsomal triglyceride protein; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; SCD-1, stearoyl-CoA desaturase 1; α-SMA, smooth muscle α-actin; SREBP1c, sterol regulatory element-binding protein 1c; TBS-T, Tris-buffered saline containing 0.05% Tween-20; VLDL, very-low-density lipoprotein.
Abstract

We have previously shown that high-fat cholesterol diet (HFCD)-induced fatty liver and carbon tetrachloride (CCl₄)-induced hepatic fibrosis are reduced in mice deficient in group IVA phospholipase A₂ (IVA-PLA₂), which plays a role in inflammation. We herein demonstrate the beneficial effects of ASB14780 (3-[1-(4-phenoxyphenyl)-3-(2-phenylethyl)-1H-indol-5-yl]propanoic acid 2-amino-2-(hydroxymethyl)propane-1,3-diol salt), an orally active IVA-PLA₂ inhibitor, on the development of fatty liver and hepatic fibrosis in mice. The daily co-administration of ASB14780 markedly ameliorated the liver injury and hepatic fibrosis following six weeks of treatment with CCl₄. ASB14780 markedly attenuated the CCl₄-induced expression of smooth muscle α-actin (α-SMA) protein and the mRNA expression of collagen 1a2, α-SMA and transforming growth factor-β1 in the liver, and inhibited the expression of monocyte/macrophage markers, CD11b and monocyte chemotactic protein-1, while preventing the recruitment of monocytes/macrophages to the liver. Importantly, ASB14780 also reduced the development of fibrosis even in matured hepatic fibrosis. Additionally, ASB14780 also reduced HFCD-induced lipid deposition not only in the liver, but also in already established fatty liver. Furthermore, treatment with ASB14780 suppressed the HFCD-induced expression of lipogenic mRNAs. The present findings suggest that an IVA-PLA₂ inhibitor, such as ASB14780, could be useful for the treatment of non-alcoholic fatty liver diseases, including fatty liver and hepatic fibrosis.
Introduction

Non-alcoholic fatty liver disease (NAFLD) is a complex liver disease, with a spectrum of hepatic histopathological changes ranging from mere intracellular fat deposition to non-alcoholic steatohepatitis (NASH), which may progress into hepatic fibrosis, cirrhosis, or hepatocellular carcinoma (Kleiner et al., 2005; Clark, 2006; Marchesini et. al., 2008). NAFLD and NASH are generally treated with weight reduction, exercise, and dietary changes. However, the curative effects of these non-pharmacotherapies are minimal, owing to high rates of non-compliance. In addition to guidance about changes in lifestyle, treatment with statins, antihypertensive agents, and β-blockers for concurrent metabolic disorders of obesity are also known to ameliorate NAFLD. However, these treatments have limited effects on the morbidity and mortality of patients with NAFLD and NASH.

Recently, pioglitazone, a synthetic peroxisome proliferator activated receptor γ (PPARγ) activator, has had considerable success in the treatment of NAFLD and NASH as a liver-directed pharmacotherapy. However, the positive effects of pioglitazone on NAFLD and NASH remain controversial. Although several clinical trials have demonstrated benefits for pioglitazone in patients with NAFLD and NASH (Belfort et al., 2006; Aithal et al., 2008; Sanyal et al., 2010), long-term treatment with pioglitazone is associated with weight gain (Sanyal et al., 2010), an increased risk of congestive cardiac failure (Lago et al., 2007) and bladder cancer (Piccinni et al., 2011), and reduced bone density (Lecka-Czernik, 2010). Thus, alternative pharmacotherapeutic options that improve the histological features of NAFLD and NASH are required.
Inflammation plays a central role in the development of NAFLD and NASH (Tilg and Moschen, 2010). The lipotoxicity of hepatic fats induces inflammatory and oxidative stresses (Takaki et al., 2014). Therefore, molecules that mediate inflammation may be promising candidate pharmacotherapeutic targets for these disorders. In the present study, we investigated the possibility of targeting group IVA phospholipase A2 (IVA-PLA2 or cytosolic PLA\(_2\alpha\)), which catalyzes the first step in the arachidonic acid cascade (Kita et al., 2006), for the treatment of these conditions. We previously demonstrated dramatic attenuation of high-fat cholesterol diet (HFCD)-induced fatty liver and carbon tetrachloride (CCl\(_4\))-induced hepatic fibrosis in IVA-PLA\(_2\)-deficient mice (Ii et al., 2009; Ishihara et al., 2012). These findings led us to hypothesize that IVA-PLA2 inhibitors may be promising candidates for the treatment of NAFLD and NASH. IVA-PLA2-specific inhibitors have already been developed by Wyeth Pharmaceuticals, Shionogi Pharmaceuticals, Astra Zeneca, and the Kokotos and Dennis groups, and include indole derivatives (McKew et al., 2003, 2006, and 2008; Lee et al., 2007), pyrrolidine-based compounds (Seno et al., 2000 and 2001; Ono et al., 2002; Flamand et al., 2006), propan-2-ones (Connolly et al., 2002; Ludwig et al., 2006; Hess et al., 2007; Fritsche et al., 2008), and 2-oxoamide compounds (Kokotos et al., 2002 and 2004; Stephens et al., 2006; Sic et al., 2007), respectively. Since none of these inhibitors is orally active, the prospect of using an IVA-PLA2 inhibitor has been limited. However, we recently developed an indole derivative, ASB14780 (3-[1-(4-phenoxyphenyl)-3-(2-phenylethyl)-1H-indol-5-yl]propanoic acid 2-amino-2-(hydroxymethyl)propane-1,3-diol salt), as a specific inhibitor of IVA-PLA2 (Tomoo et al., 2014). It has desirable bioavailability and oral efficacy, because it does not contain hydrophobic long
alkyl chains (Tomoo et al., 2014). The specificity of ASB14780 has been demonstrated, and ASB14780 inhibited human IVA-PLA$_2$ with an IC$_{50}$ value of 20 nM, which was excellent compared to other known sPLA$_2$s, such as sPLA$_2$-IA (cobra venom), sPLA$_2$-IIA (crotalus venom), sPLA$_2$-III (bee venom), and sPLA$_2$-IB (porcine pancreas), which have IC$_{50}$ values of ~10 µM in vitro (Tomoo et al., 2014). Based on these findings about ASB14780, we examined its effects on the progression of fatty liver and hepatic fibrosis in mice.
Materials and Methods

Animals

Male six- to eight-week-old C57BL/6N mice were purchased from Japan SLC Inc. (Shizuoka, Japan). All mice were housed at 5/cage with a 12-h light/12-h dark cycle and *ad libitum* access to food and water. The study protocol was approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University (Kyoto, Japan).

Preparation of ASB14780 suspension

The IVA-PLA₂ inhibitor, ASB14780, was synthesized by Asubio Pharma Co., Ltd. (Hyogo, Japan) and was suspended in 0.5% hydroxypropyl cellulose (HPC) in sterile water. The inhibitor was administered daily by oral gavage in accordance with the body weight of each animal (0.1 or 0.3 g/kg body weight). Mice in the control group received 0.5% HPC via the same administration route.

Animal model of CCl₄-induced hepatic fibrosis, and treatment with ASB14780

Mice (eight weeks old) were randomly divided into four groups: a control group, a CCl₄ alone group, a CCl₄ plus low-dose ASB14780 group, and a CCl₄ plus high-dose ASB14780 group. Liver fibrosis was induced by twice weekly intraperitoneal administration of CCl₄ at 0.31 mL/kg body weight (diluted in corn oil; Sigma-Aldrich, St. Louis, MO, USA) for six weeks. ASB14780 was orally administered daily at 0.1 or 0.3 g/kg body weight, and was administered 1 h prior to CCl₄ injections. Control animals received 0.5% HPC via the same route of administration. In the
experiments performed to elucidate the effects of ASB14780 on the progression of established hepatic fibrosis, mice (eight weeks old) were randomly divided into the following four groups: Groups A and B, treated with CCl₄ alone for six (Group A) and nine weeks (Group B), respectively; Group C, treated with CCl₄ and ASB14780 (0.3 g/kg) for nine weeks and Group D, treated with CCl₄ for six weeks, followed by treatment with CCl₄ and ASB14780 for three weeks. The mice were sacrificed 48 h after the last injection.

Animal model of HFCD-induced fatty liver and treatment with ASB14780

The mice (six weeks old) were randomly divided into the following groups: a normal diet group (ND; 5.3% fat) treated with 0.5% HPC (Group 1), a ND group treated with ASB14780 (0.3 g/kg, once daily) (Group 2), an HFCD group (20% fat and 1.25% cholesterol; Research Diets Inc., Brunswick, NJ, USA) treated with 0.5% HPC (Group 3), and an HFCD group treated with ASB14780 (0.3 g/kg, daily) (Group 4) for 16 weeks. In another group, the mice were fed the HFCD for 16 weeks, and were administered ASB14780 (0.3 g/kg, daily) for the last six weeks (Group 5). After the end of a six-hour fasting period, the mice were sacrificed, and parameters related to fatty liver disease were assessed. The food consumption of each cage was measured daily at 5 pm during the last week of administration.

Serum biomarker measurements

Blood samples were collected from the inferior vena cava, and the serum was prepared by centrifugation at 10,000 × g for 10 min at room temperature. The activities of serum alanine
aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using Transaminase C II-Test kits; (Wako Pure Chemical Industries, Osaka, Japan), and the level of triglycerides was determined using Triglyceride E-test Wako kits (Wako Pure Chemical Industries).

**CYP2E1 activity assay**

The CYP2E1 activity was measured as described previously (Chang et al., 2006). Vehicle (HPC)-treated mice with or without ASB14780 treatment were then sacrificed 48 h after the last injection. After perfusion with ice-cold saline via the heart, the livers (30 mg) were homogenized in a buffer containing 50 mM Tris-HCl (pH7.4) and 0.15 M KCl using a Dounce Tissue Grinder. The liver homogenate was centrifuged at 9,000 × g for 20 min, then the supernatant was further ultracentrifuged at 105,000 × g for 60 min. The microsomal pellet obtained was suspended in 90 µL of Assay Buffer containing 50 mM KPO₄ (pH7.4). The concentration of microsomal protein was measured by a Bradford protein assay. The liver microsomal samples (45 µg protein) were incubated in Reaction Buffer containing 100 µM p-nitrophenol, NADPH regenerating system solution A (BD Biosciences, Boston, MA, USA) and NADPH regenerating system solution B (BD Biosciences, Boston, MA, USA) at 37°C for 90 min in a water bath. To assess the effect of ASB14780 on CYP2E1 activity in vitro, the liver microsomal samples (45 µg protein) were incubated in Reaction Buffer containing 100 µM p-nitrophenol, NADPH regenerating system solution A and NADPH regenerating system solution B at 37°C for 120 min in the presence of ASB14780. The enzymatic reaction was stopped by the addition of 3.3% (v/v) trichloroacetic acid.
acid, and samples were centrifuged at 10,000 × g for 5 min. The absorbance of the supernatant at 535 nm was measured after the addition of 0.67 M NaOH. The blank was prepared by adding the complete incubation mixture with heat-inactivated liver microsomal samples (95°C, 5 min). The CYP2E1 activity was calculated as the p-nitrophenol hydroxylation activity and was expressed as nanomoles of product formed/(minute × mg of microsomal protein).

**Histological analysis**

The mice were transcardially perfused with saline and excised livers were immersed in 10% buffered formaldehyde for one week. The fixed livers were embedded in paraffin for microtome slicing into 5 μm-thick sections. The tissue sections were mounted onto MS-coated glass slides, and were deparaffinized and stained with picric acid-Sirius red or hematoxylin and eosin (HE; Wako Pure Chemical Industries). Stained sections were photographed using a microscope (model IX71; Olympus, Tokyo, Japan) with a digital camera. Images were taken at full resolution with a single image dimension set at 1,360 × 1,024 pixels. Collagen fibers were stained with Sirius red, and were quantified by measuring the red areas using the U.S. National Institutes of Health ImageJ v1.47 software program (http://rsb.info.nih.gov/ij).

**Immunohistochemistry**

Frozen liver sections (10 μm thick) were stained with anti-α-smooth muscle actin (α-SMA; Sigma–Aldrich) or anti-F4/80 (CI: A3-1 clone; Abcam) antibodies. The sections were then incubated at 90°C in preheated Retrievagen A at pH 6.0 (BD Biosciences, Boston, MA, USA).
for 10 min. After incubation with Mouse-on-Mouse blocking solution (Vector Laboratories, Burlingame, CA, USA) for 1 h, the slides were incubated with blocking solution containing 10% horse (for α-SMA) or rabbit (for F4/80) serum in phosphate-buffered saline without calcium chloride and magnesium chloride (PBS(-)) containing 0.3% Triton X-100 and avidin-blocking solution (Vector Laboratories) for 1 h at room temperature. After being washed with PBS(-), the sections were incubated overnight with biotin-blocking solution (Vector Laboratories) and α-SMA (1:1,000 dilution) or F4/80 antibodies (1:100 dilution) in a humidified chamber at 4°C. The slides were washed with PBS(-) and endogenous peroxidases were quenched by incubation with 0.3% hydrogen peroxide in methanol for 30 min. The sections were then incubated with horse biotinylated mouse IgG (1:1,000 dilution; Vector Laboratories) or rabbit biotinylated rat IgG (1:800 dilution; Vector Laboratories) for 30 min. Detection of the antibody–antigen complexes was accomplished using a Vectastain Elite ABC kit (Vector Laboratories) and a metal-enhanced DAB substrate kit (Thermo Scientific, Rockland, IL, USA).

**Western blot analysis**

Frozen liver specimens were homogenized in ice-cold lysis buffer containing 20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, and 1% sodium deoxycholate supplemented with a complete protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN, USA) and phosphatase inhibitor cocktail solution (Wako Pure Chemical Industries). Protein extracts were collected after centrifugation at 10,000 x g for 10 min at 4°C. The protein concentrations were determined using the Bradford method. Protein
samples (90 μg) were denatured and electrophoresed on 7.5% (for IVA-PLA₂) and 10% (for α-SMA and α-tubulin) SDS–PAGE gels, and were then transferred onto nitrocellulose membranes. After being blocked with 3% skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T), the membranes were incubated with antibodies against IVA-PLA₂ (Cell Signaling Technology, Danvers, MA, USA; diluted 1:1,000 with Toyobo Can Get Signal solution 1), α-SMA (Sigma–Aldrich; diluted 1:1,000 with blocking solution), or α-tubulin (Cell Signaling Technology; diluted 1:2,000 with blocking solution) for 12 h at 4°C. After being washed with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG [for IVA-PLA₂; Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:5,000 with Can Get Signal solution 2] or goat anti-mouse IgG (for α-SMA or α-Tubulin; Santa Cruz Biotechnology; diluted 1:5,000 with 0.3% skim milk in TBS-T) for 1 h. After being washed again, immunoreactive bands were detected using Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan) with a LAS-3000 mini-image analysis system (Fujifilm, Tokyo, Japan). The band intensities were quantified using the Image J software program.

**Isolation of total RNA and quantitative real-time RT-PCR analysis**

Total RNA was extracted from liver specimens using an RNAiso Plus (Takara Bio, Shiga, Japan) according to the manufacturer’s instructions. Total RNA (2 μg) was reverse-transcribed using random hexamers and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). Quantitative real-time RT-PCR was then performed on cDNA samples using SYBR-Green I (Takara Bio) and an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA,
USA) or a LightCycler Nano Real-Time PCR system (Roche, Diagnostics, Mannheim, Germany). The specific primers used are shown in Supplementary Table S1. PCR was performed for 40 cycles with 5 sec of denaturation at 95°C and annealing and extension at 60 or 62°C for 34 sec (Supplementary Table S1). The gene expression was determined using the relative standard curve method and the expression levels were normalized to those of 18S ribosomal RNA or 36B4 after parallel amplification.

**Measurement of hepatic lipid contents**

In experiments with the fatty liver model, hepatic lipids were extracted from livers (50 mg) with chloroform-methanol (2:1, v/v) according to the procedures described by Folch et al. (1957). The hepatic triglyceride level was then determined using a commercial assay kit (Wako Pure Chemical Industries).

**Statistical analysis**

Data are indicated as mean ± standard error of means (SE). Since the data were unpaired, differences among means were analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer post-hoc test. P < 0.05 was considered as the lowest level of significance. Statistical calculations were performed using Mac statistics Ver. 2.0 (Excel addin) for Macintosh (ESUMI Co., Tokyo, Japan).
Results

*ASB14780 reduces CCl₄-induced hepatotoxicity in mice*

In a previous study, the hepatotoxicity induced by chronic administration of CCl₄ was less extensive in IVA-PLA₂-deficient mice than in WT mice (Ishihara et al., 2012). In order to evaluate the beneficial effects of the present orally active inhibitor of IVA-PLA₂ on CCl₄-induced liver damage (Figure 1), mice were administered ASB14780 (0.1 or 0.3 g/kg body weight) daily and CCl₄ twice weekly (0.31 mL/kg body weight) for six weeks. The body and liver weights are summarized in Table 1. No obvious adverse effects were observed in mice treated with ASB14780 at 0.3 g/kg body weight. In contrast, the liver weights and liver to body weight ratios were significantly increased in mice treated with CCl₄ alone (Table 1).

The serum levels of ALT and AST, markers of liver injury, were significantly increased by chronic CCl₄ administration (Figure 1A). The increases in the serum levels of ALT and AST induced by CCl₄ were significantly and dose-dependently reduced by oral administration of ASB14780 (Figure 1A). Histological HE staining showed that damaged areas were observed surrounding the central veins in CCl₄-treated mice, and this damage was dramatically and dose-dependently reduced by the oral administration of ASB14780 (Figure 1B).

CYP2E1 in hepatocytes plays a role in CCl₄-induced liver injury through the generation of the CCl₃ radical (Noguchi et al., 1982; Avasarala et al., 2006). Similar CYP2E1 activity was detected in the livers of control and ASB14780-treated mice in the absence of CCl₄ (Figure 1C). To further evaluate the direct effects of ASB14780 on the CYP2E1 activity, the hepatic microsomal fraction was incubated in the presence of ASB14780. As shown in Figure 1D,
no inhibition of CYP2E1 activity was detected in the presence of ASB14780, suggesting that ASB14780 did not affect the generation of the CCl₃ radical in the treatment groups. Taken together with our previous observations showing a similar reduction of liver injury in IVA-PLA₂-deficient mice (Ishihara et al., 2012), these data suggested that IVA-PLA₂ deficiency or inhibition reduces CCl₄-induced hepatotoxicity.

**ASB14780 attenuates CCl₄-induced hepatic fibrosis in mice**

In further experiments, hepatic fibrosis was assessed in mice after chronic administration of CCl₄ by staining liver sections with picrosirius red and visualizing the hepatic collagen deposition. As shown in Figure 2A, the marked collagen deposition induced by CCl₄ was reduced in the livers of ASB14780-treated mice. Semi-quantification of the picrosirius red-stained areas revealed that the collagen accumulation in the livers of ASB14780-treated mice was significantly reduced in a dose-dependent manner (Figure 2C). In addition, quantitative RT-PCR for collagen 1a2 (Col1a2) mRNA also revealed that the CCl₄-induced increase in Col1a1 mRNA was significantly reduced in mice treated with ASB14780 (Figure 2E).

Chronic treatment with CCl₄ leads to the transformation of hepatic stellate cells to myofibroblast-like cells expressing smooth muscle α-actin (α-SMA) (Zerbe and Gressner, 1998; Tsukamoto et al., 1995). In subsequent immunohistochemical (Figure 2B), Western blot (Figure 2D), and quantitative RT-PCR (Figure 2E) analyses, α-SMA was not detectable in the liver sections from corn oil-treated control mice regardless of ASB14780 treatment, whereas CCl₄ treatment resulted in extensive α-SMA expression in portal areas. The treatment of mice with
ASB14780 reduced the expression of α-SMA induced by CCl₄ exposure (Figure 2B). These suppressive effects of ASB14780 were confirmed in Western blot and quantitative RT-PCR analyses of α-SMA (Figures 2D and E). In addition, the mRNA expression of a potent fibrogenic cytokine, transforming growth factor (TGF)-β₁, in the liver (Figure 2E) was increased by CCl₄ treatment, and was dose-dependently suppressed by ASB14780 treatment. These results are consistent with our previous study showing that CCl₄-induced hepatic fibrosis was reduced in IVA-PLA₂-deficient mice (Ishihara et al., 2012). Thus, the oral administration of ASB14780 inhibited hepatic fibrosis.

**CCl₄-induced recruitment of hepatic monocytes/macrophages is reduced in ASB14780-treated mice**

Hepatic monocyte/macrophage recruitment was increased in mice injected with CCl₄. Then effects of ASB14780 on CCl₄-induced infiltration of monocytes/macrophages were examined in the mouse livers (Figure 3). The immunohistochemical analyses of the macrophage surface marker F4/80 demonstrated that CCl₄-induced recruitment of hepatic macrophages around the central veins was reduced in mice administered ASB14780 (Figure 3A, lower panels), whereas F4/80-positive cells detected in mice in the absence of CCl₄ were still observed in mice treated with ASB14780 alone (Figure 3A). A quantitative RT-PCR analysis also showed significant dose-dependent reductions in the expression of CD11b, a monocyte/macrophage marker, in the livers of mice treated with ASB14780 (Figure 3B). These results suggest that CCl₄-induced recruitment of macrophages in the liver was diminished by the treatment of ASB14780. In
addition, the CCl₄-induced mRNA expression of monocyte chemotactic protein-1 (MCP-1), which stimulates the migration of monocytes, was significantly reduced by treatment with ASB14780 (Figure 3B).

Because T lymphocytes also participate in the development of hepatic fibrosis (Safadi et al., 2004), we assessed the liver expression of RANTES mRNA, which is a potent chemoattractant and activator of T lymphocytes. The RANTES mRNA levels were significantly increased in CCl₄-treated mice. However, concomitant treatment with ASB14780 had no significant effect on the expression of RANTES mRNA (Figure 3B). To assess the effects of ASB14780 on the expression level of IVA-PLA₂ in the liver, real-time RT-PCR and Western blot analyses were performed. As shown in Figure 4, the expression levels of the Pla2g4a mRNA and IVA-PLA₂ protein were significantly increased in mice treated with CCl₄, whereas these mRNA and protein levels were significantly attenuated by treatment with ASB14780. There was little effect of ASB14780 alone on the expression levels. These observations suggest that ASB14780 inhibited the CCl₄-induced inflammation in the liver. Thus, ASB14780 may reduce the progression of hepatic fibrosis by blocking the recruitment of hepatic monocytes/macrophages into the liver parenchyma.

**ASB14780 prevents further progression of established hepatic fibrosis**

Patients with hepatic fibrosis typically start pharmacotherapy at later stages of the disease. Therefore, the effects of ASB14780 on matured hepatic fibrosis were examined in the following four treatment groups: mice treated with CCl₄ for six weeks (Group A) or nine weeks (Group B),
mice treated with CCl4 and ASB14780 for nine weeks (Group C), and mice treated with CCl4 for six weeks followed by treatment with both CCl4 and ASB14780 for three weeks (Group D; Figure 5A). The serum AST and ALT levels (Figure 5B) and the areas stained with picrosirius red on the liver sections (Figures 5C and D) in the mice in groups A and B were equally high, suggesting that six weeks of exposure to CCl4 is sufficient to lead the matured hepatic fibrosis in mice. Notably, the levels of serum AST and ALT and the areas stained with picrosirius red in the mice in Groups C and D were significantly reduced in comparison with those in Group B (Figures 5B-D), suggesting that orally administered ASB14780 is effective even in the presence of established fibrosis.

**Prevention of HFCD-induced fatty liver following treatment with ASB14780**

We have previously shown reduced development of fatty liver in HFCD-fed mice with IV A-PLA2 deficiency (Li et al., 2009). To elucidate the effects of ASB14780 on the development of fatty liver induced by an HFCD, mice were divided into the following four groups: ND (Group 1), ND with ASB14780 (Group 2), HFCD (Group 3), or HFCD with ASB1480 (Group 4) for 16 weeks. Significant increases in the body weight and liver weight were observed in the mice in Group 3, relative to those in Group 1 (Figure 6 and Table 2, p < 0.00001, two-way ANOVA), indicating that an HFCD for 16 weeks led to obesity and hepatic enlargement in mice.

ASB14780 did not affect the body and liver weights of ND-fed mice (Group 1 vs. Group 2, Figure 6 and Table 2, not significant, two-way ANOVA). However, ASB14780 almost entirely prevented the obesity and hepatic enlargement induced by HFCD (Group 3 vs Group 4, Figure 6...
and Table 2, p < 0.00001, two-way ANOVA). Furthermore, we examined the effects of ASB14780 on fatty liver, which has already started developing. Mice were fed an HFCD for 16 weeks and were administered ASB14780 during the last six weeks (Group 5). The body weights of the mice in Group 5 did not increase to the level of those in Group 3, but rather decreased to the level of those in Group 1 (Figure 6B) in spite of the fact that there was no change in the food consumption between the groups of mice fed an HFCD (Table 2).

Histological HE staining of the liver also showed dramatically reduced microvesicular steatosis in the mice in Groups 4 and 5 in comparison with that in Group 3 (Figure 7A). Moreover, the HFCD-associated increases in the liver triglyceride levels were reduced after treatment with ASB14780 (Group 3 vs. Groups 4 and 5, Figure 7B). In contrast, although higher levels of serum triglycerides were detected in HFCD-fed mice in Group 3 compared to the ND-fed mice in Group 1, these levels were not reduced after concomitant or delayed treatment with ASB14780 (Groups 4 and 5, Figure 7B), indicating that ASB14780 affects the triglyceride level increased by HFCD in the liver, but not in the serum. The food consumption was almost the same between the HFCD-fed mouse groups (Table 2). Taken together, these data indicate that ASB14780 reduces the HFCD-induced triglyceride levels in the liver without affecting the levels in the serum or the food consumption of the mice, and that ASB14780 can prevent the progression of fatty liver induced by HFCD, even after fatty liver started developing.

**ASB14780 inhibits the expression of lipogenesis-related mRNAs**

To identify the molecular mechanisms by which ASB14780 prevents the development of fatty
liver, the mRNA levels of genes related to lipid metabolism were quantified using real-time RT-PCR in HFCD-fed mice with and without ASB14780 treatment (Figure 8). Although the mRNA expression of CD36, which transports free fatty acids (FFA) within cells, was markedly increased in the HFCD-fed mice (Group 3) compared with ND-fed mice (Group 1) (Figure 8A), its expression in the livers of HFCD-fed mice treated with 0.3 g/kg ASB14780 (Group 4) was similar to that in the mice in Group 3. However, the mRNA expression of liver fatty acid binding protein (L-FABP), which transports FFA to mitochondria (Kim et al., 1992), and carnitine palmitoyl-transferase 1α (CPT1α), which performs the rate-limiting step in fatty acid oxidation (McGarry and Brown, 1997), did not differ between the mice in Groups 1 and 3 (Figure 8A).

The expression levels of genes encoding proteins involved in fatty acid synthesis and monounsaturated fatty acid synthesis, including acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD-1), were significantly increased in the livers of the mice in Group 3 compared with those in Group 1 (Figure 8B). Moreover, the mRNA expression of sterol regulatory element-binding protein 1c (SREBP1c), which is a transcription factor leading to the expression of ACC, FAS, and SCD-1 mRNA, was consistently increased in the livers of the mice in Group 3 compared with those in Group 1 (Figure 8B). However, the liver mRNA expression of several lipogenesis-related genes (SREBP1c, ACC, FAS, and SCD-1) was significantly decreased in the mice in Group 4 compared with the mice in Group 3, indicating that ASB14780 has significant anti-lipogenic activity. We further demonstrated that the mRNA expressions of SREBP1c, FAS, SCD1 and ACC increased in mice on HFCD. While the mice on HFCD with delayed treatment with ASB14780 after 10 weeks (Group 5) had
decreases in body weight and liver triglycerides, but the expression level of SREBP1c mRNA was comparable with that in the mice on an HFCD (Group 3). In contrast, mice in Group 5 appear to have decreased FAS and SCD-1 expression. The contradictory expressions of SREBP1c, FAS, SCD1 and ACC mRNAs in mice of Group 5 might be regulated by unknown factor(s) that suppress the transcriptions of lipogenic genes individually.

In further experiments, the mRNA expression of diacylglycerol acyltransferase 2 (DGAT2), which esterifies diacylglycerol (DAG) for de novo lipogenesis, was also reduced after treatment of HFCD-fed mice with ASB14780 (Group 4, Figure 8B). However, the levels of DGAT1 and microsomal triglyceride protein (MTP), which mediate very-low-density lipoprotein (VLDL) secretion, did not differ significantly among the three groups (Groups 1, 3 and 4; Figure 8C).

Finally, the mRNA expression of hormone-sensitive lipase (HSL), which catalyzes the hydrolysis of triglycerides, was significantly decreased in the liver from the mice in Group 4 compared with those from Group 3 (Figure 8C), indicating that there was decreased lipogenesis following treatment with ASB14780 (Figure 8B). In the mice fed the ND (Groups 1 and 2), there was little effect of ASB14780 alone on the mRNA expression levels of genes involved in lipid metabolism. Taken together, these findings suggest that ASB14780 suppresses HFCD-induced de novo lipogenesis in the liver.
Discussion

Following our observations of suppressed HFCD-induced fatty liver and CCl₄-induced hepatic fibrosis in IVA-PLA₂-deficient mice (Ii et al., 2009; Ishihara et al., 2012), we examined the effects of an orally active IVA-PLA₂ inhibitor, ASB14780, on the development of fatty liver and hepatic fibrosis in the present study. Daily administration of ASB14780 reduced CCl₄-induced hepatic fibrosis and HFCD-induced fatty liver, and subsequent quantitative RT-PCR analyses suggested that lipogenesis was significantly reduced in these mice. Notably, we also showed that ASB14780 was effective against established hepatic fibrosis and fatty liver. These findings suggest that ASB14780 may represent a candidate for pharmaceutical use in the treatment of NAFLD and NASH, and indicate that IVA-PLA₂ is a valid therapeutic target for the treatment of fatty liver and hepatic fibrosis.

We have previously shown that CCl₄-induced events that collagen deposit, recruitment of monocyte/macrophage into the liver, and induction of MCP-1 mRNA were reduced in IVA-PLA₂-deficient mice (Ishihara et al., 2012). In the present study, ASB14780 also reduced the development of hepatic fibrosis with suppression of recruitment of monocyte/macrophage into the liver and induction of MCP-1 mRNA. In a similar fashion, reduced development of fatty liver was also observed in mice with ASB14780 likely to in IVA-PLA₂-deficient mice. Thus, ASB14780 is thought to exert inhibitory effects on the development of hepatic fibrosis and fatty liver through its inhibitory activity for IVA-PLA₂. In contrast, ASB14780 almost completely prevented body weight gain in mice fed the HFCD. However, the body weights of IVA-PLA₂-deficient HFCD-fed mice were equivalent to those of WT mice on HFCD (Ii et al., 2009),
suggesting that the pharmacological effects of ASB14780 on HFCD-induced obesity may be independent of IVA-PLA₂ activity.

We have shown that CCl₄-induced liver injury was reduced by deficiency or inhibition of IVA-PLA₂ in mice (Ishihara et al., 2012 and the current study). In contrast, Fas-induced liver injury was reportedly reduced in transgenic mice overexpressing IVA-PLA₂ in hepatocytes, suggesting that IVA-PLA₂ protects against Fas-induced hepatocyte apoptosis (Li et al., 2011). In contrast, IVA-PLA₂ overexpression sensitized hepatocytes to lipopolysaccharide (LPS)/D-galactosamine-induced hepatic toxicity, resulting in marked increases in the serum ALT and AST levels in hepatic IVA-PLA₂-overexpressing mice (Li et al., 2011). Thus, the IVA-PLA₂-mediated hepatic responses may vary depending on the context of liver injury. These findings in models with IVA-PLA₂ overexpression are compatible with the present data demonstrating that inhibition of IVA-PLA₂ protects against the hepatic injury induced by chronic-CCl₄ exposure, because LPS/D-galactosamine- and CCl₄-induced hepatotoxicity are reportedly mediated by oxidative stress (Osakabe et al., 2002).

In further experiments, ASB14780 prevented the progression of lipid accumulation and fibrosis in the liver, even after 10 weeks of exposure to the HFCD to induce fatty liver development or six weeks of exposure to CCl₄ to induce hepatic fibrosis. This is very important for the development of pharmacotherapy for NASH and NAFLD, because most patients with these conditions begin to receive treatment at the advanced stage of the diseases. A previous meta-analysis indicated that thiazolidinediones, especially pioglitazone, improved steatosis and inflammation, but were associated with weight increases in approximately 70% of patients, and
with edema in less than 10% of patients (Musso et al., 2010). In contrast, Cochrane reviews suggested that there is insufficient evidence to support the use of bile acids, antioxidant supplements, metformin, or thiazolidinediones in the treatment of NAFDL patients without diabetes (Lirussi et al., 2007; Angelico et al., 2007; Orlando et al., 2007). Thus, pharmacotherapy for NAFLD has not yet been established. The present inhibitor suppressed the accumulation of lipids and collagen in the liver, even after fatty liver and hepatic fibrosis had already developed, indicating that ASB14780 may be a promising agent for the treatment of NAFLD.

In the present study, the levels of lipogenic genes, such as FAS, SCD-1, and ACC were not always closely correlated to the level of SREBP1c mRNA. In the group of mice administered with ASB14780 during last 6 weeks, expression level of SREBP1c was high likely to mice on HFCD, although expressions of FAS and SCD-1 mRNAs were basal level. While it has been suggested that the transcriptions of FAS, SCD1 and ACC mRNAs are regulated by the activity of SREBP1C, contradictory results on expressions of SREBP1c and its downstream genes such as FAS, ACC and SCD-1 have been also shown in the model of hepatic steatosis by tamoxifen (Lee et al., 2010). Lelliott et al. (2005) reported that the level of SREBP1 mRNA in the rat liver shows no change in response to tamoxifen treatment, whereas the levels of FAS, SCD1 and ACC mRNAs in tamoxifen-treated rat livers are lower than those in the control group. Cole et al. (2010) have shown that there are no differences in the levels of mice liver SREBP1c and ACC mRNAs in the livers between control mice and tamoxifen-treated mice, but the levels of FAS and SCD1 mRNAs in tamoxifen-treated mice are significantly increased and decreased, respectively. It has been also suggested that tamoxifen does not affect the activities and levels of SCD1
mRNA in the rat liver, but tamoxifen can decrease the activities of rat liver ACC and FAS (Gudbrandsen et al., 2006).

The removal of causative factors is the most effective treatment for hepatic fibrosis, because liver fibrosis is reversible (Bataller and Brenner, 2005). Accordingly, hepatic fibrosis was reversed in a patient with chronic hepatitis B virus infection after successful treatment with the anti-viral agent, lamivudine (Kweon et al., 2001). Since it is accepted that oxidative stress is central to the pathogenesis of NASH (Marchesini and Forlani, 2002; Angulo, 2002), the antioxidants vitamin E, silymarin, and phosphatidylcholine all inhibit the activation of hepatic stellate cells (HSCs), protect hepatocytes from apoptosis, and attenuate experimental hepatic fibrosis (Tome and Lucey, 2004). Because inflammation precedes and promotes the progression of hepatic fibrosis, anti-inflammatory drug therapies have been considered, and corticosteroids are widely used in the treatment of hepatic fibrosis in patients with autoimmune hepatitis and acute alcoholic hepatitis (Czaja and Carpenter, 2004). In addition, pentoxifylline, which acts as an anti-inflammatory agent by inhibiting TNFα production (Tilg, 2010), has been considered for the treatment of NASH (Zein et al., 2011). Similar to corticosteroids and pentoxifylline, ASB14780 prevented the development of NAFLD symptoms, such as fatty liver and hepatic fibrosis, probably through its activity as an inhibitor of IVA-PLA₂ (Tomoo et al., 2014).

Nonetheless, the present data suggest that ASB14780, as a selective inhibitor of IVA-PLA₂, may ameliorate NAFLD and NASH as well as HFCD-associated obesity. Moreover, ASB14780 prevented hepatic lipid accumulation when administered before and after the development of fatty liver and hepatic fibrosis. Hence, although further studies are necessary to
determine the clinical efficacy of ASB14780, this orally active agent has potential as a pharmaceutical treatment for NAFLD and NASH.
Acknowledgements

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Authorship Contributions

Participated in research design: Ishihara, Nagahira, Hayashi, and Akiba.

Conducted experiments: Kanai, Tomoo, and Nagahira.

Performed data analysis: Ishihara and Akiba.

Wrote or contributed to the writing of the manuscript: Kanai, Ishihara, Kawashita, and Akiba.
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Hess M, Schulze Elfringhoff A, and Lehr M (2007) 1-(5-Carboxy- and 5-carbamoylindol-1-yl)propan-2-ones as inhibitors of human cytosolic phospholipase A2α: bioisosteric


crystallized 4-triphenylmethylthio derivative 'pyrrophene'. *Bioorg Med Chem Lett* **11:**587-590.


Footnotes

# S.K. and K.I. contributed equally to this work.

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Figure legends

Figure 1. Inhibitory effects of the IVA-PLA$_2$ inhibitor, ASB14780, on CCl$_4$-induced liver injury

Six-week-old male C57BL/6N mice were intraperitoneally administered CCl$_4$ in corn oil at 0.31 mL/kg (2 times/week) for 6 weeks. Some mice were concomitantly administered 0.1 or 0.3 g/kg of ASB14780 by oral gavage (once a day 1 h before CCl$_4$ injection). All of the mice were sacrificed 48 h after the last injection. (A) The serum levels of AST and ALT were determined using enzymatic assays. (B) Paraffin-embedded liver sections were stained with hematoxylin and eosin; scale bar, 40 $\mu$m. (C) CYP2E1 catalyzes the oxidation of $p$-nitrophenol in mitochondrial fractions and is involved in the metabolism and toxicity of CCl$_4$. The CYP2E1 activity was determined spectrophotometrically. The data are presented as the means ± SE (n = 6–7/group) and differences were identified using an ANOVA with a Tukey–Kramer post-hoc analysis; **$P$ < 0.01. (D) CYP2E1 activity was assessed in vitro in the absence or presence of ASB14780. The data are presented as mean activity (n = 4) relative to control (0 µM) ± SE and differences were identified using an ANOVA with a Tukey–Kramer post-hoc analysis.

Figure 2. Inhibitory effects of ASB14780 on CCl$_4$-induced collagen deposition and activation of hepatic stellate cells

(A) Liver sections were stained with picrosirius red; the scale bar corresponds to 200 $\mu$m. (B) $\alpha$-SMA expressing cells were detected using immunohistochemistry; the scale bar corresponds to 200 $\mu$m. (C) The collagen fibers stained with picrosirius red in A were quantified using the NIH
ImageJ software program, and the expression of the (D) \( \alpha \)-SMA (top blot) and \( \alpha \)-tubulin (bottom blot) proteins in liver extracts was assessed using Western blot analyses. The band intensities were quantified using the NIH ImageJ software program as arbitrary units (A.U.). (E) The expression levels of Col1a2, \( \alpha \)-SMA, and TGF-\( \beta \)1 mRNA were assessed using real-time RT-PCR, and were normalized to that of 18S rRNA. The data are presented as the means ± SE (A.U., n = 6–7/group), and differences were identified using an ANOVA with a Tukey–Kramer post-hoc analysis; *\( P < 0.05 \), **\( P < 0.01 \).

**Figure 3. Reduced CCl\(_4\)-induced infiltration of macrophages and Kupffer cells into the liver after administration of ASB14780**

(A) The CCl\(_4\)-induced infiltration of macrophages and Kupffer cells was assessed by immunohistochemical staining with an anti-F4/80 antibody; the scale bar corresponds to 40 \( \mu \)m. (B) The expression levels of MCP-1, CD11b, and RANTES mRNA were determined using real-time RT-PCR and were normalized to that of 18S rRNA. The data are expressed as the means ± SE (arbitrary units (A.U.), n = 6–7/group) and differences were identified using an ANOVA with a Tukey–Kramer post-hoc analysis; *\( P < 0.05 \), **\( P < 0.01 \).

**Figure 4. Reduction of CCl\(_4\)-induced IVA-PLA\(_2\) levels after the administration of ASB14780**

(A) The expression levels of Pla2g4a mRNA were assessed using real-time RT-PCR, and were normalized to that of 18S rRNA. (B) The levels of IVA-PLA\(_2\) (top blot) and \( \alpha \)-tubulin (bottom blot) proteins in the liver extracts were assessed using a Western blot analysis. The band
intensities were quantified using the NIH ImageJ software program. The data are expressed as the means ± SE (arbitrary units (A.U.), n = 6–7/group) and differences were identified using an ANOVA with a Tukey–Kramer post-hoc analysis; **P < 0.01.

Figure 5. Reduction of CCl₄-induced hepatic fibrosis by the administration of ASB14780 after hepatic fibrosis had already developed

Hepatic fibrosis was induced in six-week-old mice following twice-weekly intraperitoneal injections of CCl₄ in corn oil (0.31 mL/kg). The mice were sacrificed 48 h after the last injection. (A) Group A was injected with CCl₄ for six weeks and groups B, C, and D were injected with CCl₄ for nine weeks. In addition, group C received 0.3 g/kg ASB14780 by oral gavage every day, with it being administered 1 h before CCl₄ injection. After six weeks of CCl₄ treatment, group D received 0.3 g/kg ASB14780 for another three weeks. (B) The serum AST and ALT levels were determined using enzymatic assays. (C) Liver sections were stained with picrosirius red; the scale bar corresponds to 200 μm. (D) The number of collagen fibers was quantified by measuring the red areas using the ImageJ software program. The data are presented as the means ± SE (n = 6–7/group), and differences were identified using an ANOVA with a Tukey–Kramer post-hoc analysis; **P < 0.01, ##P < 0.01.

Figure 6. ASB14780 suppressed HFCD-induced weight gain in mice

Mice were fed an HFCD for 16 weeks to induce fatty liver. The mice in the treatment groups were administrated the IVA-PLA₂ inhibitor, ASB14780, at 0.1 or 0.3 g/kg by oral gavage every
day. After a 6-h fast, the mice were sacrificed and parameters related to fatty liver were assessed.

(A) The schedule of the treatment with ASB14780 and the high-fat cholesterol diet (HFCD). The mice were divided into five groups according to the treatment. Broken and solid lines with arrowheads indicate the presence and absence of ASB14780, respectively. Blue and red arrows indicate that the mice were fed a normal diet (ND) and the HFCD, respectively. (B) The body weights were measured every week for 16 weeks for mice fed the ND or HFCD, and (C) the body weights were measured at week 10 and 16. The data are presented as the means ± SE (n = 4–8/group), and differences were identified using a two-way ANOVA; *P < 0.05, **P < 0.01.

Figure 7. Suppression of HFCD-induced fatty liver by ASB14780

(A) Paraffin-embedded liver sections were stained with hematoxylin and eosin; the scale bar corresponds to 20 μm. (B) The liver and serum triglyceride levels were determined using enzymatic assays. The data are presented as the means ± SE (n = 4–8/group), and differences were identified using an ANOVA with a Tukey–Kramer post-hoc analysis; *P < 0.05, **P < 0.01.

Figure 8. Suppression of hepatic lipogenesis-related mRNA expression by ASB14780

The expression levels of CD36, L-FABP, CPT1α (A), SREBP1c, ACC, FAS, SCD-1, DGAT2 (B), and DGAT1, MTP, and HSL mRNA (C) were assessed using real-time RT-PCR and were normalized to that of 36B4. The data are presented as the means ± SE (arbitrary units (A.U.), n = 4–8/group), and differences were identified using an ANOVA with a Tukey–Kramer post-hoc analysis; *P < 0.05, **P < 0.01, #P < 0.05.
Table 1. The effects of ASB14780 and CCl4 on the body weight, liver weight, and liver to body weight ratios of control and treated animals

<table>
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<tr>
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<th>ASB (0)/Veh</th>
<th>ASB (0.1)/Veh</th>
<th>ASB (0.3)/Veh</th>
<th>ASB (0)/CCl4</th>
<th>ASB (0.1)/CCl4</th>
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<tr>
<td>Body weight (g)</td>
<td>27.6 ± 0.9</td>
<td>26.0 ± 0.5</td>
<td>26.2 ± 0.5</td>
<td>26.2 ± 0.7</td>
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<td>Liver weight (g)</td>
<td>1.20 ± 0.06</td>
<td>1.15 ± 0.08</td>
<td>1.27 ± 0.05</td>
<td>1.63 ± 0.09*</td>
<td>1.42 ± 0.12</td>
<td>1.50 ± 0.08</td>
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<td>Liver weight/Body weight (%)</td>
<td>4.35 ± 0.21</td>
<td>4.41 ± 0.29</td>
<td>4.86 ± 0.17</td>
<td>6.2 ± 0.2*</td>
<td>5.76 ± 0.53</td>
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The data are presented as the means ± SE for 6–7 individuals. Tukey–Kramer *post-hoc* analysis

*P < 0.05 vs. ASB(0)/Veh
Table 2. The effects of ASB14780 and HFCD on the tissue weights, serum biomarker levels, and food intake of control and treated animals

<table>
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<tr>
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<th>ASB(0)/ND (Group 1)</th>
<th>ASB(0.3)/ND (Group 2)</th>
<th>ASB(0)/HFCD (Group 3)</th>
<th>ASB(0.3)/HFCD (Group 4)</th>
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<td>Body weight (g)#</td>
<td>27.5 ± 1.1</td>
<td>28.6 ± 1.3</td>
<td>37.3 ± 0.8**</td>
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<td>Liver weight (g)</td>
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<td>1.44 ± 0.06*</td>
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<td>Fat pad weight (g)</td>
<td>0.72 ± 0.11</td>
<td>0.80 ± 0.10</td>
<td>2.25 ± 0.15**</td>
<td>1.07 ± 0.06##</td>
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<tr>
<td>Serum AST (IU/L)</td>
<td>21.54 ± 0.91</td>
<td>22.67 ± 0.91</td>
<td>30.42 ± 2.57</td>
<td>31.46 ± 2.09</td>
<td>26.71 ± 1.84</td>
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<td>Serum ALT (IU/L)</td>
<td>6.29 ± 0.52</td>
<td>5.57 ± 0.15</td>
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<td>Food intake (g)</td>
<td>2.79 ± 0.07</td>
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<td>2.68 ± 0.08</td>
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<td>2.40 ± 0.06</td>
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The data are presented as the means ± SE for 4–7 individuals. Tukey–Kramer post-hoc analysis

#Body weights were measured after a 6-hour fast

*P < 0.05 vs. ASB(0)/ND, **P < 0.01 vs. ASB(0)/ND

#P < 0.05 vs. ASB(0)/HFCD, ##P < 0.01 vs. ASB(0)/HFCD
Figure 3

(A) IHC: F4/80

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(B) mRNA level (A.U.)

![Graph showing mRNA levels of CD11b, MCP-1, and RANTES with statistical significance markers.](image-url)
Figure 4

(A) Pla2g4a mRNA level (A.U.)

(B) IVA-PLA2 protein level (A.U.)

ASB (g/kg) 0 0.3 0 0.3
Veh CCl4

ASB (g/kg) 0 0.3
Veh CCl4

IVA-PLA2 α-Tubulin

**

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Figure 5

(A) Time course of CCl₄ treatment in the four groups.

(B) Time course of AST (IU/L) and ALT (IU/L) levels in each group.

(D) Time course of picrosirius red-stained area (mm²) in each group.

(C) Picrosirius red staining of liver tissues from each group.

Group A: 200 µm
Group B: 200 µm
Group C: 200 µm
Group D: 200 µm
Figure 6

(A) Graph showing the progression over time for different groups marked as 1 to 5. The x-axis is labeled as 0 to 16 weeks with two sections: ~10 weeks and 10~16 weeks. The y-axis indicates the sample groups and treatments such as ASB(0)/ND and ASB(0.3)/HFC.

(B) Line graph displaying body weight (g) against week, with markers for Group 1 to 5. The x-axis ranges from 0 to 16 weeks.

(C) Bar charts illustrating body weight (g) at Week 10 and Week 16 for Groups 1 to 5. Significant differences are indicated with **.
Figure 7

(A) Hematoxylin and Eosin staining

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(B) Liver triglyceride (mg/g protein) and Serum triglyceride (mg/dL)

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<td>0.7</td>
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<td>Serum</td>
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