ASB14780, an Orally Active Inhibitor of Group IVA Phospholipase A2, Is a Pharmacotherapeutic Candidate for Nonalcoholic Fatty Liver Disease

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Received October 7, 2015; accepted December 15, 2015

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 coadministration of ASB14780 markedly ameliorated liver injury and hepatic fibrosis following 6 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 (MCP-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 nonalcoholic fatty liver diseases, including fatty liver and hepatic fibrosis.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a complex liver disease with a spectrum of hepatic histopathological changes ranging from mere intracellular fat deposition to nonalcoholic 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 nonpharmacotherapies are minimal, owing to high rates of noncompliance. In addition to guidance about changes in lifestyle, treatment with statins, antihypertensive agents, and β-blockers for concurrent metabolic disorders of obesity is 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 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; Sanay et al., 2010), long-term treatment with pioglitazone is associated with weight gain (Sanay 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 histologic features of NAFLD and NASH are required.

Inflammation plays a central role in the development of NAFLD and NASH (Tigl 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 A₉ (IVA-PLA₂ or cytosolic PLA₂α), 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 (CCL₁)–induced hepatic fibrosis in IVA-PLA₂-deficient mice (Li 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, 2008; Lee et al., 2007), pyrrolidine-based compounds (Seno et al., 2000, 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, 2004; Stephens et al., 2006; Six 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-phenylethylo)-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-PLA₂ with an IC₅₀ value of 20 nM, which was excellent compared with other known secreted phospholipase A₂ (sPLA₂)α, such as sPLA₂–IA (cobra venom), sPLA₂–IIA (crotalus venom), sPLA₂–III (bee venom), and sPLA₂–IB (porcine pancreas), which have IC₅₀ 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 6- to 8-week-old C57BL/6N mice were purchased from Japan SLC Inc. (Shizuoka, Japan). All mice were housed at <5°cage with a 12-hour light/12-hour 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 according to 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 route of administration. In the experiments performed to elucidate the effects of ASB14780 on the progression of established hepatic fibrosis, mice (8 weeks old) were randomly divided into the following four groups: groups A and B, treated with CCL₁ alone for 6 (group A) and 9 weeks (group B), respectively; group C, treated with CCL₁ and ASB14780 (0.3 g/kg) for 9 weeks; and group D, treated with CCL₁ and ASB14780 for 6 weeks, followed by treatment with CCL₁ and ASB14780 for 3 weeks. The mice were sacrificed 48 hours after the last injection.

Animal Model of HFCD-Induced Fatty Liver and Treatment with ASB14780. The mice (6 weeks old) were randomly divided into the following groups: a normal diet group (ND; 5.3% fat) treated with 0.5% HPC (group 1), an 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) 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 6 weeks (group 5). At the end of a 6-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 minutes 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 hours after the last injection. After perfusion with ice-cold saline via the heart, the liver (30 mg) were homogenized in a buffer containing 50 mM Tris-HCl (pH 7.4) and 0.15 M KCl using a Dounce Tissue Grinder (Wheaton, Millville, NJ). The liver homogenate was centrifuged at 9000 × g for 20 minutes, then the supernatant was further ultracentrifuged at 105,000 × g for 60 minutes. The microsomal pellet obtained was suspended in 90 µl of assay buffer containing 50 mM potassium phosphate (pH 7.4). The concentration of microsomal protein was measured by a Bradford protein assay. The liver microsomal samples (45 µg of protein) were incubated in reaction buffer containing 100 µM p-nitrophenol, NADPH regenerating system solution A (BD Biosciences) at 37°C for 90 minutes in a water bath. To assess the effect of ASB14780 on CYP2E1 activity in vitro, the liver microsomal samples (45 µg of protein) were incubated in reaction buffer containing 100 µM p-nitrophenol, NADPH regenerating system solution A and NADPH regenerating system solution B (BD Biosciences) at 37°C for 60 minutes in the presence of ASB14780. The enzymatic reaction was stopped by the addition of 3.3% (v/v) trichloroacetic acid, and samples were centrifuged at 10,000 × g for 5 minutes. 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 minutes). CYP2E1 activity was calculated as the p-nitrophenol hydroxylating activity and was expressed as nanomoles of product formed/minute × mg of microsomal protein.

Histologic Analysis. The mice were transcardially perfused with saline, and excised livers were immersed in 10% buffered formaldehyde for 1 week. The fixed livers were embedded in paraffin for microtome slicing into 5-µm-thick sections. The tissue sections were mounted onto MAS-coated Superfrost glass slides (Matsunami Glass, Osaka, Japan), and were deparaffinized and stained with picric acid–sirius red or H&E (Wako Pure Chemical Industries). Stained sections were photographed using a microscope (model IX71; Olympus, Osaka, Japan) and were deparaffinized and stained with picric acid–sirius red or H&E (Wako Pure Chemical Industries).
Tokyo, Japan) with a digital camera. Images were taken at full resolution with a single image dimension set at 1360 × 1024 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–smooth muscle α-actin (α-SMA; Sigma-Aldrich) or anti-F-4/80 (Cl:43-1 clone; Abcam, Cambridge, UK) antibodies. The sections were then incubated at 90°C in preheated Retrievagen A at pH 6.0 (BD Biosciences) for 10 minutes. After incubation with Mouse-on-Mouse blocking solution (Vector Laboratories, Burlingame, CA) for 1 hour, the slides were incubated with blocking solution containing 10% horse (for α-SMA) or rabbit (for F-4/80) 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 hour at room temperature. After being washed with PBS(−), the sections were incubated overnight with biotin-blocking solution (Vector Laboratories) and α-SMA (1:1000 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 minutes. The sections were then incubated with horse biotinylated mouse IgG (1:1000 dilution; Vector Laboratories) or rabbit biotinylated rat IgG (1:800 dilution; Vector Laboratories) for 30 minutes. 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).

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) and phosphatase inhibitor cocktail solution (Wako Pure Chemical Industries). Protein extracts were collected after centrifugation at 10,000 × g for 10 minutes 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; diluted 1:1000 with Toyobo Can Get Signal solution 1 (Toyaob, Osaka, Japan)), α-SMA (Sigma-Aldrich; diluted 1:1000 with blocking solution), or α-tubulin (Cell Signaling Technology; diluted 1:2000 with blocking solution) for 12 hours 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; diluted 1:5000 with Can Get Signal solution 2) or goat anti-mouse IgG (for α-SMA or α-tubulin; Santa Cruz Biotechnology; diluted 1:5000 with 0.3% skim milk in TBS-T) for 1 hour. 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 ImageJ software program.

Isolation of Total RNA and Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction Analysis. Total RNA was extracted from liver specimens using 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. Quantitative real-time reverse transcription (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) or a LightCycler Nano Real-Time PCR system (Roche Diagnostics, Mannheim, Germany). The specific primers used are shown in (Supplemental Table S1). PCR was performed for 40 cycles with 5 seconds of denaturation at 95°C and annealing and extension at 60 or 62°C for 34 seconds (Supplemental 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 by 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 the mean ± S.E. Since the data were unpaired, differences among means were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post-hoc test. P < 0.05 was considered as the lowest level of significance. Statistical calculations were performed using Mac statistics version 2.0 (Excel add-in) 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−/− mice than in wild-type mice (Ishihara et al., 2012). To evaluate the beneficial effects of the present orally active inhibitor of IVA-PLA2 on CCl4-induced liver damage (Fig. 1), mice were administered ASB14780 daily (0.1 or 0.3 g/kg body weight) and CCl4 twice weekly (0.31 ml/kg body weight) for 6 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 (Fig. 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 (Fig. 1A). Histologic H&E 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 (Fig. 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 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 suggest that IVA-PLA2 deficiency or inhibition reduces CCl3-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 Fig. 2A, the marked collagen deposition induced by CCl₄ was reduced in the livers of ASB14780-treated mice. Semiquantification 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 (Fig. 2C). In addition, quantitative RT-PCR for Col1a2 mRNA also revealed that the CCl₄-induced increase in Col1a1 mRNA was significantly reduced in mice treated with ASB14780 (Fig. 2E).

Chronic treatment with CCl₄ leads to the transformation of hepatic stellate cells to myofibroblast-like cells expressing...
α-SMA (Zerbe and Gressner, 1988; Tsukamoto et al., 1995). In subsequent immunohistochemical (Fig. 2B), Western blot (Fig. 2D), and quantitative RT-PCR (Fig. 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 (Fig. 2B). These suppressive effects of ASB14780 were confirmed in Western blot and quantitative RT-PCR analyses of α-SMA (Fig. 2, D and E). In addition, the mRNA expression of a potent fibrogenic cytokine, transforming growth factor-β1, in the liver (Fig. 2E) was increased by CCl4 exposure, 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 3B).

Because T lymphocytes also participate in the development of hepatic fibrosis (Safadi et al., 2004), we assessed the liver expression of regulated on activation normal T cell expressed and secreted (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 (Fig. 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 Fig. 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 response 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 6 weeks (group A) or 9 weeks (group B), mice treated with CCl4 and ASB14780 for 9 weeks (group C), and mice treated with CCl4 for 6 weeks followed by treatment with both CCl4 and ASB14780 for 3 weeks (group D; Fig. 5A). The serum AST and ALT levels (Fig. 5B) and the areas stained with picrosirius red on the liver sections (Fig. 5, C and D) in the mice in groups A and B were equally high, suggesting that 6 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 (Fig. 5, B–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 IVA-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 body weight and liver weight were observed in the mice in group 3 relative to those in group 1 (Fig. 6; 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; Fig. 6; 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; Fig. 6; Table 2; P < 0.00001, two-way ANOVA). Furthermore, we examined the effects of ASB14780 on fatty liver, which had already started developing. Mice were fed an HFCD for 16 weeks and were administered ASB14780 during the last 6 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 (Fig. 6B) despite the fact that there was no change in the food consumption in the liver. Thus, ASB14780 inhibited the CCl4-induced inflammation in the liver. This effect of ASB14780 may reduce the progression of hepatic fibrosis by blocking the recruitment of hepatic monocytes/macrophages into the liver parenchyma.

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consumption between the groups of mice fed an HFCD (Table 2).

Histologic H&E staining of the liver also showed dramatically reduced microvesicular steatosis in the mice in groups 4 and 5 in comparison with those in group 3 (Fig. 7A). Moreover, the HFCD-associated increases in the liver triglyceride levels were reduced after treatment with ASB14780 (group 3 vs. groups 4 and 5; Fig. 7B). In contrast, although higher levels of serum triglycerides were detected in HFCD-fed mice in group 3 compared with the ND-fed mice in group 1, these levels were not reduced after concomitant or delayed treatment with ASB14780 (groups 4 and 5; Fig. 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 starts 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 (Fig. 8). Although the mRNA expression of CD36, which transports free fatty acids within cells, was markedly increased in the HFCD-fed mice (group 3) compared with ND-fed mice (group 1) (Fig. 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, which transports free fatty acids to mitochondria (Kim and Storch, 1992), and carnitine palmitoyl-transferase 1a, 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 (Fig. 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 (Fig. 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 (Fig. 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 antilipogenic activity. We further demonstrated that the mRNA expressions of SREBP1c, FAS, SCD-1, and ACC increased in mice on an HFCD. The mice on an 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, SCD-1, and ACC mRNAs in the mice of group 5 might be regulated by unknown factor(s) that suppresses the transcriptions of lipogenic genes individually.

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

Finally, the mRNA expression of hormone-sensitive lipase, which catalyzes the hydrolysis of triglycerides, was significantly decreased in the livers from the mice in group 4 compared with those from group 3 (Fig. 8C), indicating that lipogenesis decreased following treatment with ASB14780.
by deficiency or inhibition of IVA-PLA2 in mice (Ishihara et al., 2012). In contrast, ASB14780 almost completely prevented body weight gain in mice fed the HFCD. However, the body weights of IVA-PLA2–deficient mice (Ishihara et al., 2012) were equivalent to those of –deficient HFCD-fed mice were equivalent to those of wild-type mice on an HFCD (Iii et al., 2009), suggesting 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.

For CCl4-induced events, we have previously shown that hepatic 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.

For CCl4-induced events, we have previously shown that hepatic 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.

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), in the present study, we examined the effects of an orally active IVA-PLA2 inhibitor, ASB14780, on the development of fatty liver and hepatic fibrosis. 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.

For CCl4-induced events, we have previously shown 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 similar to 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 wild-type mice on an 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/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

**Fig. 8.** Suppression of hepatic lipogenesis-related mRNA expression by ASB14780. The expression levels of CD36, liver fatty acid binding protein (L-FABP), and carnitine palmitoyl-transferase 1 (CPT1a) (A); SREBP1c, ACC, FAS, SCD-1, and DGAT2 (B); and DGAT1, microsomal triglyceride protein (MTP), and hormone-sensitive lipase (HSL) mRNA (C) were assessed using real-time RT-PCR and were normalized to that of 36B4. The data are presented as the means ± S.E. (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).
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 lipopolysaccharide/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 6 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 disease. 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 NAFLD patients without diabetes (Angeli, 2007; Lirussi et al., 2007; Orlando et al., 2007). Thus, pharmacotherapy for NASH 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 ASB14780 during the last 6 weeks, the expression level of SREBP1c was high similar to mice on HFCD, although expressions of FAS and SCD-1 mRNAs were basal level. Although it has been suggested that the transcriptions of FAS, SCD-1, 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 also been 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, SCD-1, 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 mouse liver SREBP1c and ACC mRNAs between control mice and tamoxifen-treated mice, but the levels of FAS and SCD-1 mRNAs in tamoxifen-treated mice are significantly increased and decreased, respectively. It has also been suggested that tamoxifen does not affect the activities and levels of SREBP1c 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 of 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 antiviral agent lamivudine (Kweon et al., 2001). Since it is accepted that oxidative stress is central to the pathogenesis of NASH (Angulo, 2002; Marchesini and Forlani, 2002), the antioxidants vitamin E, silymarin, and phosphatidylcholine all inhibit the activation of hepatic stellate cells, 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 tumor necrosis factor α 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 of NAFLD and NASH.

Acknowledgments
The authors thank Ami Takeuchi and Yuki Ito, Kyoto Pharmaceutical University, for their technical help with this study.

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Participated in research design: Ishihara, Nagahira, Hayashi, Akiba.
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Performed data analysis: Ishihara, Akiba.
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