An ACAT-1 Inhibitor, K-604, Ameliorates Hepatic Inflammation in NAFLD and NASH Models

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Abstract

A high level of cholesterol activates Kupffer cells, which subsequently triggers inflammation and ultimately leads to steatohepatitis. The number of Kupffer cells correlates with the inflammatory grade of Non-Alcoholic Fatty Liver Disease (NAFLD). In this study, we studied the role of 'foamy' Kupffer cells in the nexus between inflammation and steatosis and investigated whether K-604, a selective Acyl-Coenzyme A: Cholesterol Acyltransferase-1 (ACAT-1) inhibitor ameliorates hepatic steatosis and inflammation in rodent models of NAFLD and Non-Alcoholic Steatohepatitis (NASH). Methionine- and Choline-Deficient (MCD) diet-fed Zucker fatty rats were evaluated after 16, 16 and 12 weeks of K-604 treatment. The biochemical parameters, hepatic lipid levels, histopathological changes and gene expression levels were assessed. In MCD diet-fed KK-Ay mice, K-604 decreased the area and size of foamy Kupffer cells. In the Zucker fatty rats, K-604 could also inhibit hepatic steatosis. These results indicated that K-604 acts directly on Kupffer cells and inhibits hepatic inflammation, suggesting that ACAT-1 is involved in the progression of steatohepatitis. Therefore, ACAT-1 inhibition may be a new therapeutic target for NAFLD and NASH.

Keywords: ACAT-1; Kupffer cells; Inflammation; Nonalcoholic steatohepatitis

Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) is one of the most common chronic liver diseases and elevates the liver enzyme levels. NAFLD is frequently associated with obesity, hyperlipidemia, and type 2 (non-insulin-dependent) diabetes. Non-Alcoholic Steatohepatitis (NASH) defines a subgroup of NAFLD patients, where steatosis coexists with liver cell injury and inflammation. Hepatic inflammation is a key step in the pathogenesis of further liver damage and typically culminates in hepatic fibrosis, cirrhosis, and liver cancer. However, the mechanism driving the development of NASH from hepatic inflammation remains largely unknown. A high level of cholesterol activates Kupffer cells and subsequently triggers inflammation, which ultimately leads to steatohepatitis [1,2]. The number of CD68-positive Kupffer cells was correlated with the inflammatory grade in patients with NAFLD [3]. There were prominent enlargement and aggregation of Kupffer cells in the perivenular regions of patients with steatohepatitis, and scattered vacuoles of fat were located within the Kupffer cells [4]. Foamy or enlarged Kupffer cells have been observed in mice fed a Methionine- and Choline-Deficient (MCD) diet or a High-Fat and High-Cholesterol (HFC) diet, enabling the verification of the crucial role of Kupffer cells in the NASH model [2,5,6]. Recently, Tomita, et al. [7] reported that Acyl-Coenzyme A:Cholesterol Acyltransferase-1 (ACAT-1) is a key regulator of Free Cholesterol (FC) accumulation in hepatocellular liver cells, and it was concluded that ACAT-1 deficiency in mice increased cellular FC accumulation, leading to exaggerated liver fibrosis in response to bile duct ligation or carbon tetrachloride, even in the absence of Kupffer cells. This prompted us to publish results demonstrating that an ACAT-1 selective inhibitor, K-604, ameliorated hepatic steatosis and inflammation in rodent models of NAFLD and NASH.

K-604 inhibits ACAT-1, which mediates the conversion of FC into Cholesterol Ester (CE) (Figure S1). The IC_{50} values of K-604 for human ACAT-1 and ACAT-2 are 0.45 and 103 µmol/ L, respectively, indicating that K-604 is 229-fold more selective for ACAT-1 [8]. K-604 competitively inhibited the enzyme activity in the presence of oleoyl-CoA, with a K_{m} value of 0.38 µmol/ L. We previously demonstrated that K-604 directly prevented arterial macrophages from forming foam cells, without altering the plasma lipid levels in atherogenic F1B hamsters and apolipoprotein E-deficient mice [8,9]. We observed a remarkable reduction of Tumor Necrosis Factor-A (TNF-α) gene expression in the liver of Low-Density Lipoprotein Receptor-Deficient (Ldlr(−/−)) mice fed an HFC diet for 12 weeks during our evaluations of anti-atherosclerotic actions of K-604. Both liver macrophage foam cell formation and the progression of NASH and the arterial macrophages in advanced atherogenic lesions may use a similar mechanism that involves ACAT. In support of this suggestion, the ACAT-1 mRNA is up-regulated, resulting in cholesterol accumulation in hepatocytes in NAFLD or NASH [10,11]. Therefore, we verified the hypothesis that ACAT-1 up-regulation plays a pivotal role in regulating cholesterol metabolism in the...
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NASH liver by examining whether liver inflammation could be attenuated by ACAT-1 inhibition.

The first model is based on MCD diet-fed KK-Ay mice, one of the most common NASH models that are used to cause severe steatohepatitis, similar to human NASH [5,12]. We also compared the efficacy of bezafibrate, well-known as a broad-spectrum Peroxisome Proliferator-Activated Receptor (PPAR) agonist, to modulate fatty acid metabolism and to eventually ameliorate steatohepatitis in animal models and tamoxifen-induced human NASH [5,12,13]. The second model is based on the HFC diet-fed Ldlr(-/-) mice that exhibit fatty liver and inflammatory cell infiltration. The Ldlr(-/-) mice are a promising model for investigating the onset of hepatic inflammation in NASH, and high levels of plasma cholesterol play a pivotal role in the formation of foamy Kupffer cells and hepatic inflammation [2,14–17]. We investigated whether the inhibition of ACAT-1 activity could affect the appearance of foamy Kupffer cells and liver inflammation when K-604 was administered to the HFC diet-fed Ldlr(-/-) mice. The third model uses Zucker fatty (ZF) rats, which exhibit severe obesity, hyperphagia, hyperlipidemia, severe fatty liver and hepatocyte hypertrophy, even when fed a normal diet [18]. We evaluated the efficacy of K-604 on steatosis as a model of NAFLD. Here, we revealed that the inhibition of ACAT-1 by K-604 suppressed the appearance of foamy Kupffer cells and ameliorated the inflammatory changes in the compromised liver. Our results suggest a crucial role for ACAT-1 in the progression of steatohepatitis, and ACAT-1 inhibition may be a novel therapeutic target for NAFLD and NASH treatments.

Materials and Methods

Animals

The twelve-week-old male KK-Ay mice were purchased from Clea Japan (Tokyo, Japan). The animals were individually housed in stainless steel cages, maintained at 23°C at a humidity of 50% under a 12 h light/dark cycle, and provided with tap water ad libitum throughout the experimental period. After a 1-week quarantine and acclimation period, the mice were divided into six groups (5–9 mice/group). Group 1 was fed a normal diet (CE-2; Clea Japan); group 2 was given an MCD diet (Oriental Yeast Co., Ltd., Tokyo, Japan); and groups 3–6 received the MCD diet supplemented with 0.03%, 0.1% and 0.2% K-604 (approximately 30, 100 and 200 mg/kg, respectively) or 0.06% bezafibrate (60 mg/kg). The study protocol was reviewed and approved by the Committee on Ethics of Animal Experiments, Kowa Company, Ltd. (Tokyo, Japan, Permit Number: W009054, W105025). After the 16-week treatment period, the animals were anesthetized by an intraperitoneal injection of pentobarbital, and blood samples and the liver were collected. K-604 was synthesized by Kowa Company, Ltd. Bezafibrate was purchased from Wako Pure Chemical Industries (Osaka, Japan).

The five-week-old male Ldlr(-/-) mice (Jackson Laboratory, Bar Harbor, ME) were divided into five groups (6–8 mice/group). Group 1 was fed a normal diet; group 2 was given a 1.25% cholesterol + 40 kcal% fat (HFC) diet (Research Diet, New Brunswick, NJ); and groups 3–5 received the HFC diet supplemented with 0.026% and 0.087% K-604 (30 and 100 mg/kg, respectively) or 0.052% bezafibrate (60 mg/kg) for 16 weeks. K-604 was also administered to the Ldlr(-/-) mice for 3 weeks to measure the K-604 concentrations in the plasma and liver (n = 4).

The eight-week-old male Zucker-fa/fa (ZF) rats and Zucker-?/+ (lean) rats (Charles River Laboratories Japan, Kanagawa, Japan) were divided into three groups (5–8 rats/group). Groups 1 and 2 (lean and ZF rats) were fed a normal diet; group 3 (ZF rats) was given a normal diet supplemented with 0.133% K-604 (100 mg/kg) for 12 weeks.

Plasma analysis

The plasma Alanine Aminotransferase (ALT), Total Cholesterol (TC), Triglyceride (TG) and glucose levels were measured using a LABOSPECT 003 (Hitachi High-Technologies Co., Tokyo, Japan). The plasma choline levels were measured using a commercial kit (Bioassay Systems, Hayward, CA).

Lipid content in the liver

The liver tissue was homogenized and subjected to lipid extraction according to the method described by Folch, et al. [19]. The hepatic FC, TC and TG contents were measured using commercially available kits (Wako Pure Chemical Industries). The hepatic Cholesteryl Ester (CE) levels were calculated by subtracting the hepatic FC levels from the hepatic TC levels.

Histopathology

The liver tissue was processed for histological analysis, and paraffin sections (6 μm) were stained with hematoxylin-eosin. Steatosis (lipid droplets) was scored using the method described by Kleiner, et al. [20] (0; < 5%, 1; 5%–33%, 2; > 33%–66%, 3; > 66%). The inflammatory foci were defined as accumulations of inflammatory cell nuclei, and the number of foci per field was quantified at 200 × magnification. Collagen deposition was detected by picrosirius red staining.

For immunohistochemistry, small pieces of the liver were embedded in OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan). Cryosections (8 μm) of the liver were stained with a rat anti-mouse CD68 monoclonal antibody (clone: FA-11, 1:50–1:100, Serotec, Oxford, UK) to detect the Kupffer cells/macrophages. Histoine Simple Stain Mouse MAX PO (#414351, Nichirei Biosciences Inc., Tokyo, Japan) was used as a horseradish peroxidase-conjugated secondary antibody. The immune complexes were visualized with diaminobenzidine and the slides were counterstained with hematoxylin. The area of the CD68-positive cells was quantified by image analysis software (Win ROOF; Mitani Co., Tokyo, Japan) and calculated as the ratio of the area of the CD68-positive cells relative to that of the sections. The size of the CD68-positive cells was calculated by dividing the area of the CD68-positive cells by the number of CD68-positive cells.
counted with Win ROOF. ACAT-1 was stained with a rabbit anti-human ACAT-1 polyclonal antibody (#100028, 1:100, Cayman Chemical, Ann Arbor, MI). Double immunofluorescence staining was performed using the anti-ACAT-1 and anti-CD68 antibodies as primary antibodies. Alexa Fluor® 488 donkey anti-rabbit IgG (1:2000, Molecular Probes, Eugene, OR) and Alexa Fluor® 594 donkey anti-rat IgG (1:2000, Molecular Probes) were used as the secondary antibodies.

**Quantitative RT-PCR**

Gene expression was analyzed as described below and normalized to the β-actin expression. The total RNA was extracted from the liver using ISOGEN (Nippon Gene, Tokyo, Japan) and reverse transcription was performed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). The mRNA levels of the various target genes were determined by TaqMan or SYBR Green-based assays using a 7900HT Fast sequence detection system (Applied Biosystems). Mouse *TNF-α* gene expression in the liver was measured using a Taqman® Gene Expression Assay (Applied Biosystems). The other primers and probes are listed in S2 Table.

**Measurement of K-604 concentrations in the plasma and liver**

The liver tissue was homogenized in saline. The plasma and homogenized liver samples were mixed with phosphate-buffered saline, tert-butyl methyl ether, and internal standard solution and centrifuged. The concentration of K-604 in the supernatant was measured by HPLC–MS/MS (Agilent 1100, Agilent Technologies, Carlsbad, CA). The cells were seeded onto 24-well plates at a density of 2×10^5 cells/well and cultured for 1 day.

**Cell culture**

The RAW 264.7 cells were cultured in Dulbecco’s Modified Eagle’s Medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% FBS (Tissue Culture Biologicals, Long Beach, CA) and penicillin (100 U/mL)/ streptomycin (100 μg/mL) (Life Technologies, Carlsbad, CA). The cells were seeded onto 24-well plates at a density of 2×10^5 cells/well and cultured for 1 day. K-604 was added into the culture medium for 18 h. After drug treatment, Lipopolysaccharide (LPS) was added to the culture medium and incubated for 1 or 3 h. The total RNA was extracted using ISOGEN, and quantitative RT-PCR was performed according to the method described above.

**Statistical analysis**

The results are expressed as the means ± SEM (Standard Error of the Mean). The SAS Preclinical Package (SAS Institute Japan, Tokyo, Japan) software was used for the statistical analysis. The Student’s t-test was used to examine the differences between the normal diet group/lean rats and the control groups (HFC diet-fed, MCD diet-fed, or ZF rats). Dunnett’s test for multiple comparisons or Student’s t-tests were used to determine the significance of the differences between the control and treatment groups. *P* < 0.05 indicated statistical significance.

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**Results**

**K-604 and bezafibrate suppressed hepatic lipid droplet deposition and inflammatory foci formation in the KK-Ay mice**

We first investigated whether ACAT-1 inhibition by K-604 could affect the histology of the liver sections in the MCD diet-fed KK-Ay mice. Histological analysis by hematoxylin-eosin staining revealed the presence of microvesicular lipid droplets in the hepatocytes of the normal diet group (Figure 1A). The MCD diet caused fatty droplet deposition and neutrophil infiltration in the liver (Figure 1B). The K-604 treatment ameliorated droplet deposition, and the low dose (30 mg/ kg) of K-604 also reduced the number of inflammatory foci (Figure 1C–E). As reported, bezafibrate decreased both droplet deposition and the number of inflammatory foci, although enlarged hepatocytes were present (Figure 1F). The steatosis score (droplet deposition) and a number of inflammatory foci (the number of inflammatory cell nuclei) demonstrated that 200 mg/ kg K-604 significantly suppressed lipid deposition and inflammatory foci formation to the same extent as bezafibrate (Figure 1G–H).

**K-604 and bezafibrate reduced hepatic *TNF-α* and *COL1A1* gene expression in the KK-Ay mice**

The MCD diet induced hepatic *TNF-α* gene expression (Figure 2I), which reflected the accumulation of inflammatory foci in the liver sections. All doses of K-604 reduced *TNF-α* expression (~50% to ~66%), which correlated with the suppression of the inflammatory foci. Although the liver expression of type I procollagen (*COL1A1*, a fibrogenic gene) was decreased in the MCD diet-fed mice, bezafibrate significantly reduced both *TNF-α* and *COL1A1* expression by 86% and 84%, respectively. At doses of 100 and 200 mg/ kg, K-604 significantly reduced the *COL1A1* levels (~53% to ~57%, Figure 2I). The expression of acyl-CoA oxidase 1 (*ACOX1*, a PPARα target gene) was reduced in the MCD diet-fed group (Figure 2K). The K-604 treatment did not affect the expression of *ACOX1* while, bezafibrate increased its expression by 2-fold.

**K-604 and bezafibrate reduced the size of the Kupffer cells in the KK-Ay mouse livers**

We investigated the appearance of Kupffer cells in the KK-Ay mice by immunohistochemical staining with an anti-CD68 antibody. The Kupffer cells in the livers of the MCD diet-fed KK-Ay mice were larger than those in the normal diet-fed mice (Figure 2A–B). The K-604 (200 mg/ kg) and bezafibrate treatments inhibited the appearance of the enlarged Kupffer cells (Figure 2C–D). Quantification of the Kupffer cell size revealed that K-604 and bezafibrate tended to reduce the size of the Kupffer cells (Figure 2E). K-604 and bezafibrate did not affect the area occupied by Kupffer cells in the liver tissues (data not shown).
Body weights, plasma profiles, plasma concentrations of K-604, hepatic lipid levels and fibrosis assessments in the KK-Ay mice

The MCD diet-fed KK-Ay mice exhibited dramatic decreases in their body weights at the end of the experiment (Table 1). K-604 and bezafibrate did not affect the animals’ body weights. The plasma ALT levels in the MCD diet-fed group were not significantly elevated compared to the normal diet-fed group. While K-604 did not affect the plasma ALT levels, bezafibrate significantly decreased the plasma ALT levels. The plasma TC levels did not change in any of the treatment groups. The plasma TG and glucose levels were significantly decreased in the MCD diet-fed group. K-604 and bezafibrate did not affect the plasma TG and glucose levels. The plasma choline levels were significantly decreased in the MCD diet-fed group, likely due to the reduced supply of choline. K-604 and bezafibrate did not affect the plasma choline levels. The plasma concentrations of K-604 after a 16-week treatment with 30, 100 or 200 mg/kg were 43.3 ± 4.6, 214.3 ± 40.8 and 372.7 ± 39.2 nmol/L, respectively.
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The hepatic TC and FC levels were not affected in any of the treatment groups, although bezafibrate decreased the hepatic TC levels. The hepatic CE levels and CE/FC ratio remained the same in all groups because hepatic CE levels were very low in the KK-Ay mice. The hepatic TG levels in the MCD diet-fed group were comparable to those in the normal diet-fed group (Table 1). K-604 showed a tendency to decrease the hepatic TG levels. Bezafibrate significantly reduced the TG contents by 67%.

To analyze hepatic fibrosis, we performed picrosirius red staining on the liver sections. Although it was not obvious, collagen deposition appeared in the MCD diet-fed group (S2 Figure A-B). Neither K-604 nor bezafibrate exerted significant effects on liver fibrosis (S2 Figure C-D).

K-604 reduced the area and size of the Kupffer cells in Ldlr(−/−) mice

The first model of the MCD diet-fed KK-Ay mice was not suitable to observe the behavior of the Kupffer cells. We investigated whether the inhibition of ACAT-1 activity by K-604 could affect the appearance of foamy Kupffer cells in the Ldlr(−/−) mice. We also compared the effects of bezafibrate, as in the previous model. After 16 weeks of HFC diet feeding, the Kupffer cells in the liver were remarkably larger than those in the normal diet group (Figure 3A–B). K-604, but not bezafibrate treatment, decreased the size of the Kupffer cells (Figure 3C–D). K-604 (100 mg/kg) decreased the area and the size of the Kupffer cells by 54% and 36%, respectively (Figure 3 E–F).

K-604 ameliorated the hepatic inflammatory foci formation without altering lipid deposition in the Ldlr(−/−) mice

Liver histology revealed that the HFC diet promoted an accumulation of lipid droplets and inflammatory foci, which indicate neutrophil infiltration, in the liver (Figure 4A–B). K-604 suppressed the formation of inflammatory foci, without altering lipid deposition, compared to the control group (Figure 4C–D). Bezafibrate did not affect either the fatty droplet accumulation or inflammatory foci formation (Figure 4E).

K-604 reduced hepatic TNF-α and COL1A1 gene expression in the Ldlr(−/−) mice

The HFC diet significantly elevated hepatic TNF-α and COL1A1 mRNA levels compared to the normal diet group (Figure 4H–I). At doses of 30 and 100 mg/kg, K-604 reduced the TNF-α expression levels by 35% and 54%, respectively. Moreover, K-604 reduced the COL1A1 levels by 58–66%. On the other hand, bezafibrate did not suppress either TNF-α or COL1A1 expression. The K-604 treatment did not affect the expression of ACOX1 (Figure 4J). Conversely, bezafibrate increased the ACOX1 levels by 1.9-fold in the HFC diet-fed Ldlr(−/−) mice, similar to the MCD diet-fed KK-Ay mice.

Body weights, plasma profiles, hepatic lipid levels and fibrosis assessments in the Ldlr(−/−) mice

The HFC diet-fed Ldlr(−/−) mice exhibited a significant increase in their body weights (Table 2). Neither K-604 nor
Table 1: Body weights, biochemical plasma parameters and hepatic lipid levels in the KK-Ay mice after 16 weeks of treatment.

<table>
<thead>
<tr>
<th></th>
<th>Normal diet</th>
<th>MCD diet</th>
<th>K-604 30 mg/kg</th>
<th>K-604 100 mg/kg</th>
<th>K-604 200 mg/kg</th>
<th>Bezafibrate 60 mg/kg</th>
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</thead>
<tbody>
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<td>Body weight (g)</td>
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<td></td>
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<td>17.9±0.4</td>
<td>17.5±0.4</td>
<td>16.4±0.4</td>
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<td>ALT (U/L)</td>
<td></td>
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<td>38.3±6.1</td>
<td>27.1±2.5</td>
<td>14.7±0.7</td>
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<td>TC (mg/dL)</td>
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<td>87.5±7.8</td>
<td>81.7±2.8</td>
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<td>TG (mg/dL)</td>
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<td></td>
<td>50.7±25.7</td>
<td>52.5±9.1</td>
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<td>42.4±5.5</td>
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<td>Glucose (mg/dL)</td>
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<td></td>
<td>129.7±5.6</td>
<td>114.5±5.7</td>
<td>108.4±6.7</td>
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<td>Choline (μM)</td>
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<td>6.4±0.1</td>
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<td>5.5±0.3</td>
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<td>Hepatic TC (mg/g)</td>
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<td>6.3±0.2</td>
<td>5.4±0.2</td>
<td>5.6±0.2</td>
<td>4.2±0.2**</td>
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<td>Hepatic TE/FC ratio</td>
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<td>0.37±0.06</td>
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<td>Hepatic TG (mg/g)</td>
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<td>7.0±0.9</td>
<td>47.6±0.7</td>
<td>28.4±5.6*</td>
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The data are expressed as the means ± SEM (n = 5–9). # P < 0.05, ### P < 0.001 compared to the normal diet group. * P < 0.05, ** P < 0.01 compared to the control group.

Figure 3: Immunohistochemistry of the Kupffer cells in the Ldlr(-/-) mice after 16 weeks of treatment.

The mice fed HFC diet exhibited elevated TC and TG contents in the liver (Table 2). K-604 decreased the hepatic TC levels (30 mg/kg: -29%, 100 mg/ kg: -49%). Moreover, at doses of 30 and 100 mg/ kg, K-604 markedly decreased the hepatic CE levels by 45% and 86%, respectively, and bezafibrate decreased the levels by 65%. Despite the reduction of the hepatic CE levels, the K-604-treated mice exhibited the same level of FC as the controls, but bezafibrate increased the FC levels in the liver. Both agents significantly decreased the CE/FC ratio.

The picrosirius red-stained liver sections exhibited weak
Figure 4: Steatosis, hepatic inflammation and gene expression in the Ldlr(−/−) mice after 16 weeks of treatment
(A-E): Hematoxylin-eosin-stained liver sections from the Ldlr(−/−) mice. The arrows indicate the inflammatory foci.
(F-G): Steatosis score and inflammatory foci in the liver sections. The data are expressed as the means ± SEM (4–10 fields/mouse, n = 6–8).
(H-I): Hepatic gene expression in the Ldlr(−/−) mice (n = 6–8). The data are expressed as the means ± SEM. # P < 0.05, ## P < 0.01, ### P < 0.001 compared to the normal diet-fed group. * P < 0.05, ** P < 0.01 compared to the control group.

Table 2: Body weights, biochemical parameters in the plasma, and hepatic lipid levels in the Ldlr(−/−) mice after 16 weeks of treatment.

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<tr>
<th>HFC diet</th>
<th>Normal</th>
<th>Control</th>
<th>K-604 30 mg/kg</th>
<th>K-604 100 mg/kg</th>
<th>Bezafibrate 60 mg/kg</th>
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<td>Liver/body weight</td>
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<td>0.049±0.001</td>
<td>0.047±0.001</td>
<td>0.047±0.001</td>
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<td>ALT (U/L)</td>
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<td>132.7±85.1</td>
<td>929.3±75.7*</td>
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<td>TG (mg/dL)</td>
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<td>Glucose (mg/dL)</td>
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<td>16.9±1.0###</td>
<td>12.0±0.8***</td>
<td>8.7±0.4***</td>
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<td>Hepatic CE (mg/g)</td>
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<td>5.4±0.3***</td>
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<td>Hepatic CE/FC ratio</td>
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<td>1.5±0.24###</td>
<td>0.85±0.06**</td>
<td>0.23±0.07***</td>
<td>0.36±0.08***</td>
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<td>Hepatic TG (mg/g)</td>
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<td>79.8±18.2</td>
<td>103.7±10.2</td>
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The data are expressed as the means ± SEM (n = 6–8). # P < 0.05, ## P < 0.01, ### P < 0.001 compared to the normal diet-fed group. * P < 0.05, ** P < 0.01, *** P < 0.001 compared to the control HFC diet-fed group.
Collagen deposition in the HFC diet group (S3 Figure A–B). Neither K-604 nor bezafibrate exerted significant effects on liver fibrosis (S3 Figure C–D).

**Co-localization of ACAT-1 and CD68 in the liver and the plasma and liver concentrations of K-604 in the Ldlr(−/−) mice**

Immunostaining for ACAT-1 in the liver indicated that ACAT-1 was expressed at low levels in the sinusoidal endothelia in the livers of the normal diet-fed Ldlr(−/−) mice, but was enriched in those of the HFC diet-fed mice (S3 Figure E–F). The sections were double stained with anti-ACAT-1 and anti-CD68 antibodies, and the CD68-positive Kupffer cells overlapped with the ACAT-1 signal (S3 Figure G).

The plasma and liver concentrations of K-604 in the Ldlr(−/−) mice were measured after a 3-week treatment with 30 and 100 mg/kg K-604. While the concentrations of K-604 in the plasma from each treatment group were 0.12 ± 0.05 and 0.54 ± 0.21 µmol/L, respectively, those in the liver were 0.58 ± 0.16 and 3.82 ± 1.17 µmol/kg liver, respectively. Compared to the IC_{50} values for human ACAT-1 (0.45 µmol/L) and the K_{i} value (0.38 µmol/L), the concentration of K-604 (100 mg/kg) in the liver would be sufficient to suppress ACAT-1.

**K-604 suppressed hepatic steatosis and hepatocyte hypertrophy in ZF rats**

K-604 prevented lipid droplet formation in the MCD diet-fed KK-Ay mice, but not in the HFC diet-fed Ldlr(−/−) mice. Therefore, we examined the effects of K-604 on fatty liver using ZF rats as simple steatosis model. The livers of the ZF rats exhibited severe fatty liver and hypertrophy of the hepatocytes compared to the lean rats (Figure 5A–B). Few inflammatory foci were observed in the control groups. K-604 normalized the fatty liver and enlarged hepatocytes (Figure 5C). We did not evaluate the Kupffer cells in the ZF rats because the ZF rats did not develop steatohepatitis without inflammatory stimuli or a high-fat diet [18].

**K-604 tended to decrease the hepatic expression of the TNF-α and COL1A1 genes in the ZF rats**

There were no differences in the hepatic expression of the TNF-α and COL1A1 mRNAs between the lean rats and control rats (Figure 5D–E), which may reflect the histopathological analysis that showed little inflammation in the liver (Figure 5B). Similar to the Ldlr(−/−) and KK-Ay mice, K-604 also exhibited a tendency to reduce the expression levels of these genes. K-604 did not affect ACOX1 expression (Figure 5F).

**Body weights, plasma profiles, plasma concentrations of K-604 and hepatic lipid levels in the ZF rats**

The ZF rats exhibited a significant increase in their body weights (Table 3). K-604 did not affect the animals’ body weights while it significantly decreased the liver/body weight ratio. The plasma ALT, TC, TG and glucose levels in the ZF rats were significantly elevated compared to the lean group. K-604 significantly decreased the ALT and glucose levels, although the

### Table 3: Body weights, biochemical parameters in the plasma, and the hepatic lipid levels in the lean and ZF rats after 12 weeks of treatment.

<table>
<thead>
<tr>
<th>ZF rats</th>
<th>Lean</th>
<th>Control</th>
<th>K-604 100 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>414.7±11.2</td>
<td>670.4±20.1###</td>
<td>641.3±19.3</td>
</tr>
<tr>
<td>Liver/body weight ratio</td>
<td>0.03±0.000</td>
<td>0.057±0.004#</td>
<td>0.045±0.002*</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>37.9±3.7</td>
<td>176.8±30.6##</td>
<td>71.2±10.4*</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>51.9±2.5</td>
<td>148.6±17.9##</td>
<td>230.6±32.2*</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>147.1±11.9</td>
<td>1073.3±195.1##</td>
<td>1117.4±376.4</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>191.8±10.9</td>
<td>343.9±30.1##</td>
<td>228.6±15.4**</td>
</tr>
<tr>
<td>Hepatic TC (mg/g)</td>
<td>2.4±0.1</td>
<td>5.4±0.7##</td>
<td>3.3±0.1*</td>
</tr>
<tr>
<td>Hepatic CE (mg/g)</td>
<td>0.4±0.1</td>
<td>2.4±0.6#</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>Hepatic FC (mg/g)</td>
<td>2.0±0.1</td>
<td>3.0±0.2##</td>
<td>2.1±0.1**</td>
</tr>
<tr>
<td>Hepatic CE/FC ratio</td>
<td>0.21±0.07</td>
<td>0.81±0.21</td>
<td>0.57±0.08</td>
</tr>
<tr>
<td>Hepatic TG (mg/g)</td>
<td>13.5±1.9</td>
<td>119.3±12.4###</td>
<td>43.6±4.6***</td>
</tr>
</tbody>
</table>

The data are expressed as the means ± SEM (n = 5–8). # P < 0.05, ## P < 0.01, ### P < 0.001 compared to the lean rats. * P < 0.05, ** P < 0.01, *** P < 0.001 compared to the control group.
plasma TC levels were increased. The plasma concentrations of K-604 after 12-weeks of treatment with a dose of 100 mg/kg were 3.3 ± 0.3 μmol/ L. This concentration was sufficient to inhibit not only ACAT-1 but also ACAT-2 in the liver.

An analysis of the hepatic lipid levels revealed that the ZF rats exhibited increased hepatic TC and TG levels compared to the lean rats (Table 3). The K-604 treatment significantly decreased both the hepatic TC and TG levels. In addition, K-604 decreased both the hepatic CE and FC levels, resulting in a reduction of CE/FC ratio, but the difference was not significant.

**K-604 did not affect the LPS-induced TNF-α expression in RAW 264.7 cells**

To assess whether K-604 directly inhibits LPS-induced macrophage inflammation in vitro, RAW 264.7 cells were treated with LPS (10 ng/mL) for 1 h or 3 h. LPS-induced TNF-α expression was not affected by pretreatment with K-604 (S4 Figure A–B).

**Discussion**

In the KK-Ay mice, the MCD diet did not elevate the plasma ALT and hepatic TG levels, and severe fibrosis was not observed in the liver (Table 1 and S2 Figure). However, the normal diet group exhibited obesity and high levels of hepatic TGs. After being fed the MCD diet for 16 weeks, the mice exhibited a dramatic weight loss and a decreased supply of fatty acids, resulting in reduced lipid accumulation and COL1A1 mRNA expression in the liver (Table 1 and Figure 1J). As several groups reported, marked fibrosis was not observed in the MCD diet-fed mice [5,12,21], which is different from the observation that the MCD diet-fed Wistar rats exhibited severe fibrosis [22,23]. The MCD diet-induced the progression of steatohepatitis in the KK-Ay mice, as demonstrated by lipid droplet accumulation, inflammatory foci formation, and TNF-α gene expression. K-604 significantly ameliorated steatosis, hepatic inflammation and inflammatory gene expression, similar to bezafibrate. Bezafibrate effectively up-regulates the catabolism of fatty acids by inducing ACOX1 expression, thus leading to reduced lipid accumulation in the liver [5,12]. While K-604 did not affect ACOX1 gene expression, K-604 reduced the number of inflammatory foci and the gene expression in the liver, suggesting a crucial role for ACAT-1 in inflammation. It was noteworthy that a high dose (200 mg/kg) of K-604 reduced both inflammation and steatosis. The size of the Kupffer cells in the MCD diet-fed KK-Ay mice was not larger than that in the HFC diet-fed LDLr(-/-) mice (Figure 2E and 3F). We concluded that the size could be affected by the hepatic cholesterol levels (control group: 5.6 mg/g, KK-Ay mice vs 16.9 mg/g, LDLr(-/-) mice, Table 1 and 2).

The HFC diet-fed LDLr(-/-) mice represent a promising model for investigating the onset of hepatic inflammation and steatosis in two components of NASH pathophysiology [2,14-17]. By comparing K-604 with the controls and bezafibrate, we demonstrated that the selective ACAT-1 inhibitor K-604 effectively suppressed inflammatory foci formation. The lipid analysis indicated that both K-604 and bezafibrate decreased the plasma TC and liver TC and CE levels while tending to increase the liver TG levels. While K-604 did not affect the plasma TG levels, bezafibrate decreased the plasma TG levels and significantly increased the liver TG levels compared to HFC diet-fed controls. Notably, bezafibrate was reported to significantly reduce the liver microsomal ACAT activities in rats [24]; thus, the reduction of the CE levels and an increase in the FC levels can be explained by ACAT inhibition. A question was raised of where to draw the line between the efficacies of K-604 and bezafibrate if both agents inhibit ACAT activities. To date, two ACAT isoforms have been identified. ACAT-1 is ubiquitously expressed in various human tissues and cells, such as various types of macrophages (including Kupffer cells), the adrenal glands and the liver while ACAT-2 is primarily expressed in the rodent intestine and liver and is involved in the absorption of cholesterol from the diet [25,26]. We presumed that bezafibrate might effectively inhibit ACAT in hepatocytes (ACAT-2), but not in Kupffer cells (ACAT-1).

The histopathological analysis indicated that K-604 also decreased the size of the CD68-positive Kupffer cells in a dose-dependent manner, accompanied by suppressed inflammatory foci formation, but not the accumulation of lipid droplets. This observation prompted us to focus on the relationship between ACAT and Kupffer cells. Macrophages take up modified low-density lipoprotein, and the esterification of cholesterol by ACAT-1 leads to foam cell formation. Foamy macrophages are thought to contribute to the development of atherosclerotic lesions, and the inhibition of ACAT is the basis for potential therapies targeting atherosclerosis [27,28]. To determine the correlation between ACAT-1 and Kupffer cells, we conducted double immunofluorescence analysis, which indicated that the ACAT-1 and CD68 proteins were co-localized in the liver (S3 Figure G). These observations suggest that K-604 inhibited ACAT-1 and thus modulated the cholesterol levels in the Kupffer cells. As previously reported, impaired Kupffer cell function is important in the pathogenesis of NASH [29-31]. Thus, within the paradigm of the NASH etiology, it appears plausible that the inhibition of ACAT-1 by K-604 may suppress foam cell formation and lead to the normalized phagocytic function and morphology of the Kupffer cells. Leroux, et al. [32] reported that fat-laden Kupffer cells are ‘primed’ to exhibit a proinflammatory phenotype and are sensitive to inflammatory stimuli, resulting in enhanced production of inflammatory cytokines, regardless of the presence or absence of LPS.

In contrast, bezafibrate showed no effects on liver inflammation or steatosis, despite the reductions in the plasma and liver cholesterol levels, which were similar to those observed with K-604. One of the differences between the K-604 and bezafibrate treatments was the hepatic FC levels (K-604 30 mg/kg: 6.6, K-604 100 mg/kg: 7.4 vs. bezafibrate: 10.3 mg/g, Table 2), and the increase in the hepatic FC levels (46% increase) in response to bezafibrate may lead to inflammatory reactions. Of note, Tomita, et al. [7] indicated that the FC accumulation in hepatic stellate cells results in enhanced Toll-like receptor 4 signaling and an exacerbation of liver fibrosis [7]. This theory
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might be plausible to support the behavior of bezafibrate. The dysregulation of cholesterol homeostasis also alters membrane fluidity and phagocytosis, which is disturbed in an animal model of NASH [33]. Kupffer cell dysfunction may promote steatohepatitis by sensitizing the hepatocytes to LPS [33]. These observations were in contrast with the reports on the suppression of fatty droplet formation and inflammatory reactions following activation of PPARα by bezafibrate or Wy-14,643 in the MCD diet-fed mice [34,35].

Considering the differences between the KK-Ay mice and Ldlr(−/−) mice in this study, bezafibrate significantly decreased the liver TG levels in the MCD diet-fed KK-Ay mice, but not in the HFC diet-fed Ldlr(−/−) mice, although the expression of the ACOX1 gene was induced in both mouse models. Based on the remarkable reduction in the hepatic TG levels (67% decrease, Table 1), bezafibrate suppressed fatty liver and resulted in a reduction in the steatosis score and inflammatory foci formation in the KK-Ay mice. In contrast, K-604 did not decrease the liver TG levels and fatty droplet accumulation as well as bezafibrate, but reduced the TNF-α and COL1A1 gene expression in the livers of the Ldlr(−/−) mice. We presumed that K-604 acts by directly preventing the formation of foamy Kupffer cells and exhibits anti-inflammatory activity.

The result showing that the ZF rats exhibit severe steatosis by overfeeding as a model of NAFLD appear to elucidate the efficacy of ACAT inhibition [18]. K-604 ameliorated fatty liver and normalized the enlarged hepatocytes in the ZF rats. This indicates that a potent inhibition of ACAT-1 (100 mg/kg) may promote favorable effects on lipid metabolism in the ZF rats. We noted that genetic deletion of ACAT-2 or treatment with antisense oligonucleotides of ACAT-2 could protect the mouse liver from dietary cholesterol-induced steatosis by facilitating TG secretion to the plasma [36,37]. In the mouse liver, ACAT-2 is primarily expressed in hepatocytes [7]. Although K-604 exhibits 229-fold selectivity in human CHO cell lines [8], it cannot be always reflected in rodent models. Furthermore, K-604 could inhibit ACAT activity in hepatocytes and in Kupffer cells because of the high concentrations of K-604 (100 mg/kg) that were administered to the ZF rats.

Recently, Tomita, et al. [7] proposed that ACAT-1 deficiency leading to FC accumulation could promote liver fibrosis induced by bile duct ligation or carbon tetrachloride. This discrepancy between the previous report and our study may be attributed to the different experimental conditions, such as a complete genetic deficiency of ACAT-1 and its enzyme function. Additional extensive investigations are required to clarify the potential role of ACAT-1 in NAFLD/NASH pathophysiology. Here, ACAT-1 inhibition resulted in the suppression of foamy Kupffer cell formation, with a subsequent reduction in inflammatory gene expression and an improvement of inflammatory cell infiltration, even when fatty liver had progressed. ACAT-1 plays a pivotal role in the formation of foamy Kupffer cells, followed by liver inflammation. Liver-specific inhibition of ACAT-1 may provide a useful basis for novel therapeutic strategies targeting steatosis and liver inflammation in NAFLD and NASH.

References

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