Bempedoic acid unveils therapeutic potential in non-alcoholic fatty liver
disease: suppression of the hepatic PXR-SLC13A5/ACLY signaling axis

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**Running Title:** BA improves NAFLD via suppressing the PXR-SLC13A5/ACLY axis

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**Abbreviations:** ACC, acetyl-coenzyme A carboxylase; ACLY, ATP-dependent citrate lyase; ALT, alanine transaminase; AMPK, AMP-activated protein kinase; AST, aspartate transaminase; AUC, area under the curve; BA, bempedoic acid; BTA,
1,2,3-benzenetricarboxylic acid hydrate; DMEM, Dulbecco’s Modified Eagle Medium; DNL, de novo lipogenesis; FASN, fatty acid synthase; FBS, fetal bovine serum; HDL, high-density lipoprotein; H&E, hematoxylin-eosin; HFHFD, high-fat plus high-fructose diet; HMGCR, hydroxy-methyl-glutaryl coenzyme A reductase; ITT, insulin tolerance test; KEGG, Kyoto encyclopedia of genes and genomes; LDL, low-density lipoprotein; MEM, Minimum Essential Medium; MRM, multiple reaction monitoring; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity scores; NASH, non-alcoholic steatohepatitis; NCD, negative control diet; NEFA, non-esterified fatty acids; NRs, nuclear receptors; OGTT, oral glucose tolerance test; OPA, oleic acid and palmitic acid; PA, palmitate; PCN, pregnenolone 16α-carbonitrile; PXR, pregnane X receptor; PSR, picrosirius red; siRNA, small interfering RNA; SLC13A5 or PMCT, plasma membrane citrate transporter; SLC25A1 or CIC, mitochondrial citrate carrier; SREBP, sterol-regulatory element binding protein; TC, total cholesterol; TG, total triglyceride; UHPLC-MS/MS, ultra-high performance liquid chromatography-tandem mass spectrometry.
Abstract

The hepatic SLC13A5/SLC25A1-ACLY signaling pathway, responsible for maintaining the citrate homeostasis, plays a crucial role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). Bempedoic acid (BA), an ACLY inhibitor commonly used for managing hypercholesterolemia, has shown promising results in addressing hepatic steatosis. This study aimed to elucidate the intricate relationships in processes of hepatic lipogenesis among SLC13A5, SLC25A1 and ACLY, and to examine the therapeutic potential of BA in NAFLD, providing insights into its underlying mechanism. In murine primary hepatocytes and HepG2 cells, the silencing or pharmacological inhibition of SLC25A1/ACLY resulted in significant upregulation of SLC13A5 transcription and activity. This increase in SLC13A5 activity subsequently led to enhanced lipogenesis, indicating a compensatory role of SLC13A5 when the SLC25A1/ACLY pathway was inhibited. However, BA effectively counteracted this upregulation, reduced lipid accumulation, and ameliorated various biomarkers of NAFLD. The disease-modifying effects of BA were further confirmed in NAFLD mice. Mechanistic investigations revealed that BA could reverse the elevated transcription levels of SLC13A5 and ACLY, and the subsequent lipogenesis induced by PXR activation \textit{in vitro} and \textit{in vivo}. Importantly, this effect was diminished when PXR was knocked down, suggesting the involvement of the hepatic PXR-SLC13A5/ACLY signaling axis in the mechanism of BA action. In conclusions, SLC13A5-mediated extracellular citrate influx emerges as an alternative pathway to SLC25A1/ACLY in the regulation of lipogenesis in hepatocytes, BA exhibits therapeutic potential in NAFLD by suppressing the hepatic
PXR-SLC13A5/ACLY signaling axis, while PXR, a key regulator in drug metabolism may be involved in the pathogenesis of NAFLD.

**Significance statement:** This work describes that bempedoic acid, an ACLY inhibitor, ameliorates hepatic lipid accumulation and various hallmarks of NAFLD. Suppression of hepatic SLC25A1-ACLY pathway upregulates SLC13A5 transcription, which in turn activates extracellular citrate influx and the subsequent DNL. Whereas in hepatocytes or the liver tissue challenged with high energy intake, bempedoic acid reverses compensatory activation of SLC13A5 via modulating the hepatic PXR-SLC13A5/ACLY axis, thereby simultaneously downregulating SLC13A5 and ACLY.
1. Introduction

Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of histological conditions ranging from the benign hepatic steatosis to the more severe non-alcoholic steatohepatitis (NASH), the malign cirrhosis and even hepatoma (Younossi, 2019; Powell et al., 2021). Along with the gradually increased incidence, NAFLD has already become a global public health problem (Younossi et al., 2018; Younossi, 2019). However, there is still no approved pharmacotherapy for NAFLD/NASH due to its complicated pathogenesis.

The plasma membrane citrate transporter (PMCT, SLC13A5), mitochondrial citrate carrier (CIC, SLC25A1), and ATP-dependent citrate lyase (ACLY) play pivotal roles in maintaining citrate homeostasis (Kumar et al., 2021; Mosaoa et al., 2021). Increasing evidence supports their potential as targets for treating NAFLD. In recent years, a multitude of inhibitors designed specifically for these proteins have emerged. SLC13A5 has been found to be significantly upregulated in both animal models and patients with NAFLD (Schumann et al., 2020). Selective deletion or pharmacological inhibition of hepatic SLC13A5, using compounds like PF-06649298 and PF-06761281, effectively reduces cytosolic citrate and acetyl-CoA levels. This reduction mitigates lipid accumulation, as well as alleviates inflammation and fibrosis in the liver of NAFLD (Birkenfeld et al., 2011; Sauer et al., 2021). Similarly, CPTI-2, an inhibitor of SLC25A1, has shown potential in alleviating hepatic steatosis and hyperlipidemia (Tan et al., 2020).

Furthermore, ACLY has emerged as an attractive therapeutic target for hyperlipidemia and metabolic disorders (Ference et al., 2019; Morrow et al., 2022). Bempedoic acid
(BA), a novel non-statin drug prescribed for hypercholesterolemia, inhibits ACLY-mediated cleavage of citrate into acetyl-CoA with liver specificity. This inhibition effectively suppresses fatty acid and steroid biosynthesis (Nissen et al., 2023). In summary, targeting hepatic citrate disposition, encompassing its transport and metabolism regulated by the SLC13A5/SLC25A1-ACLY pathway, holds great promise as a therapeutic strategy for treating NAFLD.

Despite the potential benefits of targeting SLC13A5, SLC25A1, and ACLY individually to restore lipid homeostasis, the interconnections among these three targets remain unclear. It remains unknown whether ACLY inhibition affects the expression and activity of SLC25A1 or SLC13A5, and whether there is compensatory citrate supply in the absence of SLC25A1 or SLC13A5 alone. Additionally, the effectiveness of BA in treating hepatic steatosis suggests its potential for NAFLD treatment. However, as a liver-targeted ACLY inhibitor, it is important to investigate whether BA treatment alters the gene expression and/or function of SLC13A5 or SLC25A1, leading to disturbances in cytosolic citrate homeostasis in hepatocytes. The activation of pregnane X receptor (PXR), a master regulator of xenobiotic and endogenous metabolism, is consistently observed in high-fat diet mice during NAFLD progression (Li et al., 2018). Interestingly, SLC13A5 has been identified as a transcriptional target of PXR (Li et al., 2015). Moreover, several studies have suggested that PXR activation can upregulate lipogenic enzymes, including ACLY, independent of sterol regulatory element-binding protein (SREBP) activation (Gao and Xie, 2010; Bitter et al., 2015). Therefore, gaining a comprehensive understanding of the associations among PXR, SLC13A5, SLC25A1, and
ACLY in hepatocytes is crucial for advancing drug discovery and ensuring the rational utilization of available drugs for NAFLD treatment.

In this study, we investigated the complex relationships among SLC13A5, SLC25A1, and ACLY in the process of lipogenesis using murine primary cultures of hepatocytes and HepG2 cells. We also assessed the therapeutic potential of BA in both *in vitro* and *in vivo* models of NAFLD. In addition to elucidating intricate interplays among SLC13A5, SLC25A1 and ACLY, this mechanistic study provided valuable insights into developing innovative strategies for effective managing NAFLD. Furthermore, our findings highlight the promising therapeutic benefits of BA in the treatment of NAFLD.
2. Materials and Methods

2.1. Chemicals and reagents

Bempedoic acid (>98%), BMS-303141 and PF-06649298 were purchased from MedChemExpress (Monmouth Junction, NJ, USA). [U-\textsuperscript{13}C\textsubscript{6}]-Citrate, [U-\textsuperscript{13}C\textsubscript{6}]-D-Glucose, Atorvastatin calcium, 1,2,3-benzenetricarboxylic acid hydrate (BTA) and Collagenase type IV were all obtained from Sigma-Aldrich (St Louis, MO, USA). Pregnenolone 16α-carbonitrile (PCN) was purchased from APExBIO (Shanghai, China). Palmitic acid, Oleic acid, and D-glucose were purchased from Aladdin Industrial Corporation (Shanghai, China). Lipofectamine 2000/3000 transfection reagents were obtained from Invitrogen (Carlsbad, CA, USA). Dulbecco’s Modified Eagle Medium (DMEM, high glucose or low glucose), Minimum Essential Medium (MEM), Opti-MEM and 0.25% trypsin-EDTA were purchased from GBICO (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from ExCell Bio (Shanghai, China). RNA isolater total RNA extraction reagent, HiScript III RT SuperMix (+gDNA wiper) and Taq Pro Universal SYBR qPCR Master Mix for qPCR were all purchased from Vazyme (Nanjing, China).

2.2. Animals

Male C57BL/6J mice aged 6-7 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mice were housed in specific pathogen-free environment (23-25 °C, 12 h light/dark cycle) and fed with food and water \textit{ad libitum}. All animal studies were carried out with strict compliance to the Guidelines for the Care and Use of Laboratory Animals issued by the NIH. The study design and
animal handling protocol were reviewed and approved by the Animal Ethics Committee of China Pharmaceutical University (Approval No. 2022-02-011, Nanjing, China).

2.2.1. High-fat plus high-fructose diet (HFHFD)-induced NAFLD mice

After one week of acclimatization, the mice were randomly divided into several groups, including negative control diet group (NCD, received a normal chow diet), HFHFD group (received 60% high-fat diet and water containing 20% fructose, ordered from Trophic Animal Feed High-tech Co., Ltd.) and BA groups (received HFHFD while treated with BA at 10 mg·kg⁻¹ or 30 mg·kg⁻¹ every day for the last 6 weeks). Body weights were recorded weekly. On the 17th week, the mice were fasted for 12 h or 4 h prior to the test, and fasting glucose levels were measured before glucose (2 g·kg⁻¹, i.g.) and insulin (0.75 U·kg⁻¹, i.p.) loading test. Then blood glucose levels were measured at 15, 30, 60, 90, 120 min and the AUC₀–120 min was calculated. After 18 weeks, mice were sacrificed after anesthetized by sodium pentobarbital (50 mg·kg⁻¹, i.p.) to collect the serum or plasma and the liver tissue, which were immediately cryopreserved at −80 °C.

2.2.2. Pregnenolone 16α-carbonitrile (PCN)-induced PXR-activation in mice

After one week of acclimatization, the mice were randomly divided into control group, PCN-treated group, and PCN plus BA-treated group. Specifically, PCN (100 mg·kg⁻¹) was injected intraperitoneally daily for five days, and BA was given by gavage at 10 mg·kg⁻¹ or 30 mg·kg⁻¹ 30 min after PCN administration, respectively. On the sixth day, mice were anesthetized and sacrificed to collect serum and liver tissue, which were frozen at -80 °C till analysis.

2.3. Cell cultures
Mouse primary hepatocytes were extracted from 6-week-old male C57BL/6J mice using a two-step perfusion method applied in our previous report (Sun et al., 2021). Briefly, the liver was perfused with collagenase to remove stray cells and disperse the hepatocytes, which were seeded in the six-well plates at the appropriate density. Mouse primary hepatocytes were cultured at 37 °C in an incubator with 5% CO₂ and stabilized for 12 h. Prior to experiments, the hepatocytes were stained with cytokeratin-18 immunofluorescence to identify the cell purity. The transcriptions of SLC13A5, SLC25A1, and ACLY as well as other enzymes were measured by qPCR. 300 μM citrate was added to mimic the physiological state.

HepG2 cells and HEK-293T cells were purchased from the Chinese Academy of Medical Sciences (Shanghai, China) and cultured in MEM or DMEM supplemented with 10% FBS and antibiotics (100 IU·mL⁻¹ penicillin and 0.1 mg·mL⁻¹ streptomycin) at 37 °C and 5% CO₂. When the cells density reached 70-80%, HepG2 cells were incubated in a mixture containing 300 μM oleic acid and palmitic acid (OPA, OA:PA=4:1) or 30 mM glucose for 24 h. In another set of experiment, HepG2 cells were stimulated with 10 μM atorvastatin for 24 h to detect the transcriptions of the target genes. 300 μM citrate was added to mimic the physiological state.

2.4. Plasmid construction and gene transfection

For SLC25A1, ACLY, SLC13A5 and PXR silencing assay, the mouse primary hepatocytes or HepG2 cells were incubated with 50 nmol·L⁻¹ siRNA and transfected using lipofectamine 2000/3000 transfection reagent according to the manuals. Scrambled siRNA was used as a negative control, and 300 μM citrate were added to the culture
medium to mimic physiological state. The siRNAs of human or mouse SLC25A1, ACLY, SLC13A5 and PXR (Table S1) were designed and synthesized by GenePharma (Shanghai, China). The silencing efficiency in transfected cells was verified and described in the Supplemental Material (Fig. S1).

For overexpression assay, cDNA sequences of mouse ACLY, SLC13A5, SLC25A1 were cloned into the pEX-3 plasmid while human SLC13A5 was cloned into the pcDNA3.1 plasmid (constructed by GenePharma, Shanghai, China). The control vectors were transfected as negative controls. Simply, mouse primary hepatocytes or HEK-293T cells were seeded and maintained in the appropriate medium containing 300 μM citrate for 12 h. Then, cells were transfected with the overexpressed plasmid using the lipofectamine 2000 reagent according to the product instructions. The overexpression efficiency of the target genes was verified and described in the Supplemental Material (Fig. S1 and S3).

2.5. Biochemical analysis

Total cholesterols (TC), total triglycerides (TG), non-esterified fatty acids (NEFA), low density lipoprotein cholesterol (LDL), high density lipoprotein cholesterol (HDL), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured in serum of NCD- or HFHFD-fed mice using commercially available kits (Jiancheng Biotech, Nanjing, China). Liver tissue, mouse primary hepatocytes and HepG2 cells were lysed to determine the levels of the intracellular TG and TC. In addition, protein concentrations of cells or homogenate samples were determined with the BCA protein assay kit.
2.6. Histological analysis

For hematoxylin & eosin staining (H&E), paraffin sections of 4% paraformaldehyde (w/v)-fixed liver tissue (4 μm) were dewaxed and washed. Then the sections were immersed in hematoxylin stain and eosin stain, dehydrated with anhydrous ethanol and sealed with neutral gum. For Oil red O staining, liver sections were rapidly frozen by liquid nitrogen before placed at room temperature and dried. They were then immersed in oil red O stain and re-stained with hematoxylin, sealed with glycerol gelatin. The Oil red O staining sections were quantified by Image J. For picrosirius red staining (PSR), liver sections were stained in PSR staining solution and dehydrated in ethanol, then immersed in xylene and sealed with neutral gum. Finally, the sections were observed under light microscope and then photo-graphed. NAFLD activity score (NAS) were evaluated according to the NAFLD scoring system (Kleiner et al., 2005).

2.7. SLC13A5-mediated citrate uptake assay

The HepG2 cells or transfected HEK-293T cells were incubated with sodium buffer containing $^{13}$C-citrate (100 μM) and BA at 37 °C. Subsequently, the extracellular buffer was discarded and each well was washed using choline buffer. Cells were lysed by adding purified water and rapidly frozen at −80 °C. Finally, samples treated with acetonitrile were centrifuged and analyzed for intracellular $^{13}$C-citrate by UHPLC-MS/MS system.

2.8. Quantitative real-time PCR

Total RNA was extracted from liver tissues or cells using RNA isolater extraction reagent, the concentration of RNA was quantified by Micro-Spectrophotometer instrument (Allsheng, Nano-400A). Then, 1 μg total RNA in each sample was
reverse-transcribed into cDNA via HiScript III RT SuperMix, using Eppendorf Mastercycler nexus GSX1 gradient thermal cycler (Eppendorf, Shanghai, China). Subsequently, qRT-PCR technology with Taq Pro Universal SYBR qPCR Master Mix and a Real-Time PCR System (ABI Q3, Thermo Fisher Scientific, China) was used to quantify the relative transcription of the mRNAs calculated with the $2^{-\Delta\Delta CT}$ method. The primers used in this study were all shown in Supplemental Material (Table S2).

2.9. Western blotting

HFHFD-fed mice liver tissue and HEK-293T cells were lysed to extract total proteins. The loading buffer was also added to samples and heated at 100 °C. The individual proteins were analyzed by SDS-PAGE transferred to PVDF membranes (Millipore, Billerica, MA, USA) and blocked with BSA. Finally, the corresponding primary and secondary antibodies were incubated for image analyses. The immunoreactive bands were detected by ECL reagent (Tanon, Shanghai, China) using a gel imaging system (Tanon 5200 Multi, Tanon Science & Technology, Shanghai, China). The intensity of the bands was analyzed using the Image J. Anti-β-actin (Cell Signaling Technology Cat# 4970), Anti-SLC13A5 (Invitrogen, Cat# PA5-113058), Anti-SLC25A1 (Proteintech Group, Cat# 15235-1-AP), Anti-ACLY (Proteintech Group, Cat# 15421-1-AP) were used as primary antibodies. Goat anti-rabbit IgG (H+L) (Cell Signaling Technology, Cat# 7074S), Goat anti-mouse IgG (H+L) (Cell Signaling Technology, Cat# 7076S) were used as secondary antibodies.

2.10. RNA-sequencing
Total RNA was isolated using the RNAeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Purified total RNA was subjected to mRNA isolation, fragmentation, first-strand cDNA synthesis, second-strand cDNA synthesis, end repair, the addition of a single ‘A’ base, ligation of the adapter and enrichment according to the experimental instructions to complete the sequence library construction. Library fragments were quantified using an Agilent high sensitivity DNA assay. The libraries were constructed using a Qubit 2.0 Fluorometer to detect concentration and an Agilent 4200 to detect the size of the library. The Illumina NovaSeq6000 sequencer was used in PE150 sequencing mode.

2.11. UHPLC-MS/MS analysis

2.11.1. Determinations of citrate-derived $^{13}$C-labeled and unlabeled metabolites

Mouse primary hepatocytes or HepG2 cells were transfected with siRNA of SLC25A1, ACLY or treated with BA (100 μM), respectively. The culture medium was added with 5 mM isotope labeled or unlabeled glucose and 500 μM isotope labeled or unlabeled citrate for 48 h. Then cells were lysed and added with triple volume of acetonitrile to precipitate the intracellular proteins. After centrifuged at 4 °C for 10 min at 12,000 g, the supernatant was transferred and analyzed by UHPLC-MS/MS. Labeled and unlabeled citrate, citrate (m+2), citrate-derived acetyl-CoA and palmitate (PA) were quantitatively analyzed according to our published method with some modifications (Sun et al., 2021). The levels of citrate and citrate (m+2) were analyzed by AB Sciex 6500 Mass-spectrometry in negative multiple reaction monitoring (MRM) mode on ACE column (C18-AR, 4.6×100 mm). Acetonitrile and purified water (0.02% ammonia) were
designated as phase B (organic phase) and phase A (aqueous phase), respectively. Acetyl-CoA and malonyl-CoA were analyzed in positive MRM mode on SHIMADZU column (C18-AQ, 4.6×150 mm), acetonitrile and purified water (5 mM ammonium acetate) were designated as organic phase and aqueous phase, respectively. For the measurement of PA species, an Agilent column (Extend-C18, 4.6×30 mm) was used following acetonitrile and purified water (0.05% ammonia) as organic phase and aqueous phase, respectively. All the column temperatures were set at 40 °C and different target analytes were eluted by gradient elution. All data were acquired and analyzed by Analyst software (Version 1.6.3). The transitions of target analytes were summarized in Supplemental Material (Table S3).

2.11.2. Determinations of bempedoic acid and its metabolite ESP15228

The serum and liver homogenates of mice fed with HFHFD were harvested to determine the levels of BA and its metabolite ESP15228. The metabolites were extracted in the same way as mentioned above. An aliquot of 10 μL supernatant was injected into UHPLC-MS/MS (LC-40DXR-8045 mass spectrometer, Shimadzu, Kyoto, Japan). BA and ESP15228 were analyzed on a SHIMADZU column (C18-AQ, 4.6×150 mm) in negative MRM mode. Acetonitrile and purified water containing 0.1% formic acid were designated as organic phase and aqueous phase. The column temperature was at 40 °C and target analytes were eluted by gradient elution. Data were collected and analyzed by Lab Solutions LCMS (Version 5.80). The transitions of target analytes were summarized in Supplemental Material (Table S3).

2.12. Statistical analysis
The data were presented as mean ± SD. The scores were subjected to analysis using the Kruskal-Wallis test, followed by Dunn’s multiple comparison test as a post hoc analysis. For other experimental data, statistical significance was determined using an unpaired Student's t-test for comparisons between two groups, while comparisons among more than two groups were assessed using one-way ANOVA followed by Tukey’s test. All statistical analyses were conducted using GraphPad Prism 8.0 (San Diego, CA, USA) and IBM Statistical Package for the Social Sciences (SPSS) 25.0 software (Chicago, IL, USA). A significance level of $P < 0.05$ was considered statistically significant.
3. Results

3.1. Alteration of the SLC25A1-ACLY pathway leads to the upregulation of SLC13A5

To investigate the interconnections among SLC25A1, SLC13A5, and ACLY in hepatocytes, the transcription of one target was analyzed when the other was activated or suppressed. There were no marked changes in transcription of SLC25A1 or ACLY in mouse primary hepatocytes when one was knocked down or pharmacologically inhibited (Fig. 1A, C and S2A, C). However, overexpression of SLC25A1 or ACLY could upregulate each other (Fig. 1G, I). Interestingly, any change of ACLY or SLC25A1 substantially upregulated SLC13A5 (Fig. 1B, D, H, J and S2B, D) while alteration of SLC13A5 exhibited little impact on ACLY and SLC25A1 (Fig. 1E and F, K and L, and S2E and F). Similar interconnections among these three targets were also observed in HepG2 cells (Fig. S2G-L). Knockdown of SLC25A1 or ACLY significantly increased the transcription of SLC13A5 (Fig. S2H, J) while the transcript of SLC25A1 and ACLY remained stable upon the silencing of SLC13A5 (Fig. S2K and L). Moreover, there was no obvious interplay between SLC25A1 and ACLY (Fig. S2G, I). This information indicates that the SLC13A5-facilitated transport of extracellular citrate into the cytosol functions as an additional route when the typical SLC25A1-ACLY pathway is functioning normally or is activated. Moreover, this process also plays a crucial compensatory role when the primary pathway is inhibited.

3.2. Inhibition of the SLC25A1-ACLY pathway increases the extracellular citrate influx and the subsequent fatty acids biosynthesis
In order to investigate whether the compensatory upregulation of SLC13A5 might affect the subsequent lipogenesis in hepatocytes when the SLC25A1-ACLY pathway was suppressed, stable isotope labelled glucose or citrate was separately introduced to trace the carbon source utilization via determining the intracellular citrate species, citrate-derived acetyl-CoA and palmitate (PA). As the experimental design shown in Fig. 2A, when the mouse primary hepatocytes were cultured with U-13C-glucose (5 mM) and unlabeled citrate (500 μM), the intracellular amounts of 13C-labeled citrate (citrate+2), acetyl-CoA (acetyl-CoA+2) and PA (m+n, n=0, 2, 4, 6, 8, 10, 12, 14, 16) were significantly decreased with knockdown of SLC25A1 or ACLY (Fig. 2B-F). However, when the culture medium was replaced with unlabeled glucose (5 mM) and U-13C-citrate (500 μM), knockdown of SLC25A1 significantly drove the extracellular citrate-sourced lipid biosynthesis, proved by the increased amounts of U-13C-labeled citrate, acetyl-CoA and PA in hepatocytes (Fig. 2B-F). Likewise, ACLY knockdown also significantly elevated the extracellular citrate influx (Fig. 2B). Silencing ACLY appears to impede citrate cleavage into acetyl-CoA (Fig. 2C) and the subsequent PA synthesis (Fig. 2E and F) without discriminating the origin of citrate. In fact, less reduction of acetyl-CoA and PA generated from the SLC13A5-ACLY pathway was observed compared to that generated from the SLC25A1-ACLY pathway. These observations shed light on the fact that extracellular citrate uptake through SLC13A5 plays a role in subsequent lipid biosynthesis, particularly in situations where the SLC25A1-ACLY pathway is inhibited.

We extended our investigation to explore the potential compensatory activation of SLC13A5 when the SLC25A1-ACLY pathway is inhibited in HepG2 cells. The findings
aligned with those observed in mouse primary hepatocytes (Fig. 2G-K). Collectively, these results strongly suggest that the SLC13A5-mediated uptake of extracellular citrate could function as an alternative source to augment the cytosolic citrate pool in situations where the SLC25A1-ACLY pathway is suppressed. This mechanism might undermine the lipid-lowering effects of pharmacological inhibition targeting SLC25A1 or ACLY.

3.3. BA diminishes lipid accumulation by downregulating SLC13A5 and ACLY, while leaving canonical lipogenic enzymes unaffected

Expanding upon the preceding findings, we raised a question regarding whether BA (structure shown in Fig. 3A), a liver-specific ACLY inhibitor, also triggered a compensatory increase in SLC13A5 expression, thereby potentially counteracting its inherent liver-specific lipid-lowering advantage. Initially, we embarked on a stable isotope tracer study to investigate the immediate impact of BA on extracellular citrate influx and the consequent synthesis of fatty acids through the SLC13A5/SLC25A1-ACLY pathway. The results, as depicted in Figure 3B-D, diverged from the compensatory effect induced by ACLY inhibition or silencing as discussed earlier. Notably, BA distinctly diminished the cytosolic citrate derived from both extracellular influx and intracellular synthesis, leading to a subsequent reduction in acetyl-CoA generation and production of PA. Furthermore, we delved into citrate transport analysis in HepG2 cells and HEK-293T cells overexpressing SLC13A5. This was undertaken to evaluate BA’s potential to inhibit the transport activity of SLC13A5 directly. However, our findings (as illustrated in Figure S3) indicate that BA does not exert a direct inhibitory effect on extracellular citrate influx.
Subsequently, we proceeded to examine the transcriptional activity of SLC25A1/SLC13A5 and ACLY, along with downstream lipogenic enzymes, to validate the potential effects of BA on the SLC25A1/SLC13A5-ACLY pathway, the activated de novo lipogenesis (DNL) process, and lipid accumulation in hepatocytes induced by mixed OPA or high glucose. Consistent with our previous findings (Sun et al., 2021), the mRNA levels of both SLC13A5 and ACLY displayed significant upregulation in mouse primary hepatocytes subjected to high-energy challenge (Fig. 3E and F, K and L), mirroring the trends observed with crucial enzymes involved in lipid biosynthesis, such as acetyl-coenzyme A carboxylase 1 (ACC1), fatty acid synthase (FASN), and hydroxy-methyl-glutaryl coenzyme A reductase (HMGCR) (Fig. 3H-J, N-P), as well as subsequent lipid deposition including triglycerides (TG) and total cholesterol (TC) (Fig. S4A-H). However, SLC25A1 transcription remained relatively stable in OPA-challenged hepatocytes, with a slight increase observed in the high glucose group (Fig. 3G, M). These data suggest that the SLC13A5-ACLY pathway is upregulated in response to high-energy stimulation, positively correlating with activated DNL and ectopic lipid accumulation in hepatocytes. Notably, BA substantially mitigated lipid accumulation in hepatic cells (Fig. S4). Furthermore, BA markedly downregulated mRNA transcription of both SLC13A5 and ACLY, while exhibiting no discernible effects on the upregulated expression of FASN and HMGCR in hepatocytes. These results suggest that the inhibition of ACLY by BA does not induce compensatory activation of SLC13A5. Instead, BA suppresses the SLC13A5-ACLY pathway rather than the downstream lipogenic pathway, leading to the attenuation of DNL and alleviation of lipid accumulation in hepatocytes.
3.4. BA exhibits disease-modifying effects in in mice bearing NAFLD

Considering the in vitro protective effects of BA against lipid deposition, we speculated whether BA can ameliorate NAFLD through its liver-specific regulatory mechanism. Mice were subjected to either NCD or HFHFD for a duration of 18 weeks. Over the final 6 weeks of this period, BA was administered via intragastric administration at doses of 10 or 30 mg·kg⁻¹ (Fig. 4A). Oral glucose tolerance tests (OGTT) and insulin tolerance tests (ITT) clearly indicated that BA substantially enhanced blood glucose tolerance and insulin sensitivity in mice fed with HFHFD (Fig. 4B and C). In comparison with the HFHFD group, BA treatment did not exert an impact on body weight or liver weight (Fig. 4D and E). The influence of BA on liver appearance is illustrated in Figure 4F. Moreover, histological assessments of liver tissues, including H&E, Oil red O, and PSR staining, revealed that BA treatment notably mitigated hepatocellular ballooning, lipid accumulation, inflammatory infiltration, and fibrosis (Fig. 4G). The NAFLD activity scores (NAS) of liver sections from mice, along with quantitative analysis of lipid droplet areas, provided further statistical support for the potential benefits of BA in addressing NAFLD (Fig. 4G).

In addition to the histopathological findings, we also assessed key indicators in serum and liver tissue related to NAFLD progression to underscore the favorable effects of BA. In comparison to the HFHFD group, BA exhibited distinct therapeutic effects on liver injury, as evidenced by the reduced serum ALT and AST levels (Fig. 5A and B). Furthermore, BA treatment significantly lowered lipid contents, including TG, TC, NEFA, and the LDL/HDL ratio, in serum (Fig. 5C-F), and TG levels in liver tissue (Fig. 5G),
although there was no significant impact on TC levels in the liver (Fig. 5H). Moreover, BA conspicuously alleviated the HFHFD-induced inflammatory response and fibrosis in the liver. This was supported by the diminished levels of pro-inflammatory cytokines such as Il-1β, Il-6, and Tnf-α (Fig. 5I), along with profibrotic factors including Col1α1, Acta2, and Timp1 (Fig. 5J). In accordance with reported findings, serum citrate levels were significantly elevated in HFHFD-treated mice, which BA was able to rectify (Fig. S5A). The influences of BA on other endogenous metabolites associated with liver lipogenesis are illustrated in Figure S5B-E. Concurrently, substantial quantities of BA and ESP15228 were detected in both plasma and liver tissue of BA-treated mice (Fig. S5F-I), ensuring an ample pharmacodynamic material basis for NAFLD treatment. Collectively, our experimental findings strongly suggest that BA exhibits disease-modifying effects in the context of NAFLD.

3.5. BA reduces lipid deposition through downregulating SLC13A5 and ACLY but not the canonical lipogenic enzymes in NAFLD mice

To delve into the specific molecular effects of BA on NAFLD, we carried out transcriptomic analysis on liver tissue obtained from both HFHFD and HFHFD-BA (30 mg·kg\(^{-1}\)) groups of mice. The results of volcano plot analysis highlighted significant changes in gene transcription profiles between these two groups (Fig. 6A). Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis of the RNA-seq dataset unveiled that BA’s positive impact on HFHFD-induced NAFLD primarily stems from its regulation of lipid metabolism. This includes processes like unsaturated fatty acid biosynthesis, degradation, and oxidation (Fig. 6B). The heatmaps generated based on the
KEGG analysis vividly depicted BA's robust effects on mitigating hepatic inflammation, apoptosis, and fibrosis (Fig. 6C). Notably, it's worth mentioning that transcription profiles of genes linked to lipogenesis, such as ACC, FASN, and HMGCR, were further upregulated by BA treatment (Fig. 6C). This data suggests that BA indeed has the ability to reverse NAFLD progression, likely by influencing hepatic lipid metabolism. Additionally, the upregulation of lipogenic enzymes indicates the involvement of other regulatory mechanisms in BA's ability to curtail lipid biosynthesis and deposition.

Given the pronounced efficacy of BA in curtailing fatty acid biosynthesis and lipid accumulation both in vitro and in vivo, we extended our inquiry to probe the regulatory impact of BA on gene transcription of SLC13A5/SLC25A1 and ACLY, as well as downstream enzymes implicated in DNL, within the liver tissues of mice subjected to a HFHFD. In alignment with the findings from in vitro experiments, the mRNA and protein levels of both SLC13A5 and ACLY experienced significant upregulation in the livers of mice fed the HFHFD. Intriguingly, BA treatment exhibited a dose-dependent reversal of this trend (Fig. 6D and E). In contrast, there were no notable changes in gene and protein expressions of SLC25A1 across all groups of mice (Fig. 6D and E).

Concordant with the outcomes derived from transcriptomic analysis, BA treatment, rather than causing downregulation, notably upregulated ACC1, FASN, and HMGCR—key enzymes pivotal for lipid biosynthesis (Fig. 6F-H). These results further underscore that BA attenuates hepatic lipogenesis potentially through the suppression of the SLC13A5-ACLY pathway, while not affecting the downregulation of downstream lipogenic enzymes.
3.6. BA mitigates lipid deposition by suppressing the hepatic PXR-SLC13A5/ACLY axis.

Given the significant roles played by the SLC13A5-ACLY pathway in the progression of NAFLD, further investigations were undertaken to delve into the underlying regulatory mechanism governing this metabolic pathway. Previous studies have proposed that PXR activation can induce the upregulation of SLC13A5, contributing to hepatic steatosis and metabolic disorders (Li et al., 2015). Given PXR's substantial impact on lipid homeostasis, we sought to determine whether the prominent upregulation of the SLC13A5-ACLY pathway was attributed to PXR activation in hepatocytes or within the liver of mice exposed to high-energy challenges.

As depicted in Figure 7, PCN, a rodent PXR activator, prominently elevated the gene transcriptions of both SLC13A5 and ACLY, concomitant with increased CYP3A11 transcription (a well-known PXR target gene), in primary mouse hepatocytes (Fig. 7A-C). Similar upregulations of SLC13A5 and ACLY were also observed in HepG2 cells stimulated with atorvastatin (Ator), a human PXR agonist (Fig. S6A-C). Importantly, BA effectively counteracted these upregulations triggered by PXR activation in primary mouse hepatocytes and HepG2 cells (Fig. 7A-C and Fig. S6A-C). In alignment with the in vitro findings, the transcriptional levels of SLC13A5, ACLY, and CYP3A11 were markedly elevated in the livers of mice treated with PCN, indicating that PXR activation could induce the transcription of hepatic SLC13A5 and ACLY (Fig. 7D-G). Furthermore, PCN treatment also led to the upregulation of ACC1 and HMGCR (Fig. 7H and I), accompanied by increased TG and TC levels (Fig. 7J and K), demonstrating that PXR
activation could stimulate DNL and foster lipid accumulation in the liver. In contrast, BA treatment significantly downregulated the relevant gene transcription and mitigated lipid accumulation. These findings collectively illustrate that PXR activation can induce the transcription of SLC13A5 and ACLY, thereby activating DNL and prompting lipid accumulation in the liver. However, BA effectively rectifies these disturbances related to lipid metabolism.

To further elucidate whether BA's regulatory mechanism on the SLC13A5-ACLY pathway relies on PXR, we introduced PXR agonists and siRNA to activate or silence PXR in primary mouse hepatocytes and HepG2 cells. As illustrated in Figure 7L-M and Figure S6D-E, the PXR-mediated upregulation of SLC13A5 and ACLY induced by PCN or Ator was significantly attenuated by either BA treatment or siRNA intervention. Notably, when PXR was knocked down, BA exhibited no effect on the transcription of SLC13A5 and ACLY. These findings underscore that BA effectively counteracts the upregulated SLC13A5-ACLY pathway potentially by suppressing PXR activation, either in hepatocytes or within the liver, particularly under conditions of metabolic stress.
4. Discussions

Accumulating evidence has demonstrated that activated DNL is a central driving force in the development of NAFLD (Loomba et al., 2021; Powell et al., 2021). Many pharmacological inhibitors targeting key enzymes involved in DNL have reached late-stage development, with ACC inhibitors being considered promising candidates (Batchuluun et al., 2022). However, the unexpected occurrence of hypertriglyceridemia has limited the clinical applications as monotherapy (Goedeke et al., 2018). As an alternative approach, researchers have attempted to identify therapeutic targets upstream of DNL for NAFLD treatment. ACLY, responsible for cleaving citrate to provide lipogenic acetyl-CoA for lipid biosynthesis, has been suggested as a potential therapeutic target (Pinkosky et al., 2017; Morrow et al., 2022). Furthermore, both SLC13A5 and SLC25A1 play important roles in NAFLD progression (Tan et al., 2020). Since the fate of cytosolic citrate in hepatocytes is regulated by SLC13A5, SLC25A1, and ACLY, inhibition of one protein or pathway may be compensated for by the others, thereby complicating the pharmacological benefits of these inhibitors. Therefore, the present study aimed to investigate the interconnections among SLC13A5, SLC25A1, and ACLY, and to further explore the potential effect of BA, an approved liver-targeted ACLY inhibitor, on NAFLD and its underlying mechanism.

In the present study, we observed no change in the gene transcriptions of SLC25A1 and ACLY when SLC13A5 was silenced or pharmacologically suppressed. However, SLC13A5 transcript was upregulated when ACLY or SLC25A1 was knocked down, pharmacologically inhibited, or even overexpressed (Fig. 1). Stable isotope tracer studies
demonstrated that the increased expression of SLC13A5 resulted in the uptake of extracellular citrate, which sustained the supply of carbon sources for lipid biosynthesis when SLC25A1 was knocked down. These experimental results align with previous findings that SLC13A5 is upregulated as a compensatory supplier of citrate when SLC25A1 is deficient, as shown in HFD-fed mice treated with CTPI-2 (a specific SLC25A1 inhibitor) (Tan et al., 2020). Similarly, the knockdown of ACLY led to the transfer of more extracellular citrate, rather than mitochondrial citrate, into the cytosol via upregulated SLC13A5. This transfer enhanced fatty acid generation in hepatocytes and significantly compromised the lipid-lowering effect of solely targeting ACLY (Fig. 2). These results indicate that hepatocytes may attempt to compensate for the deficient activities of SLC25A1 or ACLY by promoting the uptake of extracellular citrate through upregulated SLC13A5. Another aspect of this compensation is likely to exacerbate the accumulation of cytosolic citrate when ACLY is inhibited, which could complicate the pharmacological benefits of ACLY inhibitors, considering the crucial role of citrate in linking carbohydrate and lipid metabolism.

BA is clinically employed for the management of dyslipidemia by suppressing lipid biosynthesis dependent on ACLY (Pinkosky et al., 2016; Banach et al., 2020). As a liver-targeting ACLY inhibitor, it is crucial to investigate whether BA treatment affects the transcription and functioning of SLC13A5 or SLC25A1, and how it further impacts lipid biosynthesis. In contrast to the findings obtained from ACLY suppression, BA was discovered to decrease the transport of cytosolic citrate species mediated by SLC25A1 and SLC13A5, respectively, thereby attenuating the subsequent generation of acetyl-CoA.
and palmitate derived from labeled citrate. Intriguingly, SLC13A5 and ACLY exhibited significant activation, while SLC25A1 did not, and this was accompanied by the upregulation of downstream lipogenic genes and ectopic lipid deposition in hepatocytes or the liver tissues of mice subjected to high-energy challenges (Fig. 3). These findings strongly indicate that the SLC13A5-ACLY pathway plays a substantial role in the activated DNL observed in NAFLD, by increasing the supply of citrate from the circulation and its subsequent cleavage into acetyl-CoA. Encouragingly, BA demonstrated the ability to downregulate the gene expressions of both SLC13A5 and ACLY, leading to a reduction in hepatic lipid deposition both in vitro and in vivo. Furthermore, additional analyses on citrate transport revealed that BA did not exert any inhibitory effect on the transport function of SLC13A5. Altogether, BA effectively reduces the cytosolic pools of citrate and acetyl-CoA, thereby diminishing the generation of fatty acids by downregulating both SLC13A5 and ACLY.

Our results demonstrated that BA exhibits significant benefits against the major characteristics of NAFLD both in vitro and in vivo. KEGG pathway enrichment analysis and heatmaps of the RNA sequencing data further highlighted notable differences in lipid metabolism and associated signaling pathways between overnutrition stimulation and BA treatment (Fig. 4-6). These findings provide further confirmation of the promising potential of BA as a treatment for NAFLD. However, the precise molecular mechanism underlying its effects remains unclear. In addition to its direct inhibition of ACLY, BA has been shown to activate AMPK, offering an alternative mechanism for modulating lipid metabolism (Pinkosky et al., 2013). Nevertheless, in our experiments, BA downregulated
the expressions of SLC13A5 and ACLY without exerting profound effects on the transcripts of ACC, FASN and HMGCR, and/or even showed up-regulatory effects on these genes in the liver tissue of mice exposed to high-energy challenges (Fig. 3 and Fig. 6). Moreover, a recent study reported that BA treatment significantly reduced hepatic lipid deposition in a modified NASH mouse model, albeit unexpectedly accompanied by upregulated expressions of ACC and FASN (Morrow et al., 2022). However, ACC, FASN and HMGCR, which are key enzymes involved in lipid biosynthesis, are known to be transcriptionally regulated by the AMPK-SREBP1/2 signaling pathway (Zhou et al., 2001; Lu et al., 2022). These observations suggest that the suppression of hepatic DNL by BA treatment may occur through an SREBP-s-independent mechanism.

Hepatic nuclear receptors (NRs) are upstream transcriptional regulators that play important roles in the pathophysiology of NAFLD by controlling various metabolic processes (Moreau et al., 2008; Cariello et al., 2021; Puengel et al., 2022). Among these NRs, PXR has been extensively studied for its role in regulating the transcription of numerous hepatic genes involved in xenobiotic/endogenous metabolic enzymes and transporters (Sayaf et al., 2021). It has been reported that PXR activation promotes DNL independently of SREBP signaling, while PXR knockout significantly alleviates lipid disorders (Gao and Xie, 2010; Karpale et al., 2021). Besides, PPARα and PPARγ can induce the expression of key genes involved in lipogenesis (ACC and FASN), fatty acid uptake (CD36 and FABPs), and fatty acid oxidation (CPT and HMGCS2) (Zhou et al., 2006; Sayaf et al., 2021). Consistent with these findings, our study revealed that BA treatment exerted significant regulatory effects on hepatic NRs, including PXR.
suppression and PPARs activation (Fig. 6 and Fig. 7). This modulation might give some useful explanations for the further upregulation of lipogenic enzymes in hepatocytes or liver tissues from in vitro and in vivo NAFLD models upon BA treatment. Some studies have shown that SLC13A5 can be directly induced by PXR activation (Li et al., 2015; Li et al., 2021). Considering the important roles of SLC13A5 and ACLY in citrate transport and metabolism, it is reasonable to postulate that both SLC13A5 and ACLY may be dually induced by PXR activation (Li et al., 2021). In our experiments, besides the regulatory effect on the SLC13A5-ACLY pathway, BA treatment reversed the upregulation of CYP3A11, a known target gene of PXR activation in the livers of HFHFD-fed mice, suggesting mechanistic relationships among PXR activity, the SLC13A5-ACLY pathway, and BA intervention. Furthermore, the transcripts of SLC13A5 and ACLY were profoundly induced in mouse primary hepatocytes and HepG2 cells stimulated with PXR agonist (Fig. 7 and S6). PXR activation also led to ectopic lipid accumulation in the livers of PCN-stimulated mice, accompanied by upregulation of ACC and HMGCR. In contrast, BA significantly attenuated hepatic lipid accumulation by concurrently downregulating SLC13A5 and ACLY through PXR suppression. These observations suggest that BA can downregulate both SLC13A5 and ACLY by modulating PXR activity, thereby reversing the compensatory activation of SLC13A5 resulting from ACLY inhibition. Moreover, growing evidence has shown that well-known PXR-activating drugs such as rifampicin, phenytoin, ritonavir, and statins can induce hypercholesterolemia or hepatic steatosis (Zhou et al., 2009; Shehu et al., 2019). In contrast, BA exhibits potency in modulating PXR to alleviate lipid disorders, indicating
its potential application in the management of metabolic disorders.

The insights gained from this study also highlight safety considerations regarding drugs or xenobiotics that modulate PXR. Given that PXR activation enhances DNL, it could potentially expedite the progression of NAFLD and contribute to drug-induced liver injury. Furthermore, it appears that BA exerts its disease-modifying effects in NAFLD by downregulating the PXR signaling pathway. Considering that PXR governs various mechanisms of drug metabolism and disposition, the possibility of drug-drug interactions between BA and medications that are substrates of PXR-induced enzymes or transporters cannot be overlooked. However, the available information in these domains is limited, underscoring the significant scientific interest in delving further into these issues.

Finally, based on the findings of our study, we would like to bring attention to several unresolved issues and potential avenues for future research. While SLC25A1 has been suggested as a potential therapeutic target for NAFLD/NASH (Tan et al., 2020), we did not observe any changes in the gene expression of SLC25A1 in our prior and current NAFLD models (Sun et al., 2021). Recent emerging evidence indicates that the activation of the SLC25A1-mediated citrate export pathway serves as a central signaling mechanism in inflammation and cancer. Moreover, it has been demonstrated that IL-1β induces mitochondrial citrate export to the cytosol for DNL by regulating the phosphorylation of SLC25A1 in adipocytes, rather than its protein expression (Liu et al., 2022). These findings imply a potential correlation between the gene or protein expressions of SLC25A1 and disease progression, suggesting that posttranslational modifications might
play a more substantial role in the pathophysiology of NAFLD/NASH. Another limitation of this research lies in the regulatory mechanism study of BA for NAFLD treatment. Our study proposes that BA mitigates lipid biosynthesis by suppressing the PXR-SLC13A5/ACLY axis in hepatocytes. However, comprehending the integrated function of coactivators, repressors of PXR, and even other Nuclear Receptors (NRs) in the pharmacological mechanisms of BA represents a challenging yet informative area for investigation. Overall, these unresolved issues and future prospects offer vital directions for further exploring the molecular mechanisms underlying NAFLD/NASH and the therapeutic potential of BA.

In summary, our findings underscore the significance of the SLC25A1-ACLY pathway and its compensatory activation of SLC13A5 within the context of lipid metabolism and NAFLD. This compensatory mechanism weakens the inherent lipid-lowering effect, particularly in instances of ACLY inhibition. However, our research demonstrates that BA effectively addresses this compensation by modulating the PXR-SLC13A5/ACLY axis. Through this modulation, BA attenuates DNL, reduces lipid deposition, and ameliorates other key features of NAFLD both \textit{in vitro} and \textit{in vivo}. Our study illuminates a novel mechanism of action for BA and underscores its potential as a therapeutic avenue for NAFLD.
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Data Availability Statement

The authors declare that all data supporting the findings of this study are available within the paper and its Supplemental Data.

Authorship contribution

Participated in research design: Z.Q., F.H., H.L.

Conducted experiments: Q.S., Y.G., M.Z., S.W.

Contributed new reagents or analytical tools: H.M., N.L., P.X.

Performed data analysis: W.H., Y.L., Z.L., H.L.

Wrote or contributed to the writing of the manuscript: Z.Q., Q.S., H.L.
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Footnote

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Figure legends

Fig. 1. Knockdown or overexpression of SLC25A1 or ACLY upregulates the transcription of SLC13A5 in mouse primary hepatocytes. (A and B) Transcriptions of Slc25a1 and Slc13a5 upon Acly knockdown. (C and D) Transcriptions of Acly and Slc13a5 upon Slc25a1 knockdown. (E and F) Transcriptions of Acly and Slc25a1 upon Slc13a5 knockdown. (G and H) Transcriptions of Slc25a1 and Slc13a5 upon Acly overexpression. (I and J) Transcriptions of Acly and Slc13a5 upon Slc25a1 overexpression. (K and L) Transcriptions of Acly and Slc25a1 upon Slc13a5 overexpression. All data are presented as mean ± SD (n = 6). * P<0.05, ** P<0.01, *** P<0.001 vs control group. Acly, ATP-dependent citrate lyase; OE, overexpression; Slc13a5, plasma membrane citrate transporter; Slc25a1, mitochondrial citrate carrier.

Fig. 2. Knockdown of SLC25A1 or ACLY increases the influx of the extracellular citrate and the subsequent fatty acid biosynthesis. (A) Schematic diagram of the labeled carbon utilization for DNL sourced from the extracellular [U-13C6] glucose or [U-13C6] citrate. Labeled carbons are shown in red and unlabeled in black. The determined contents of 13C-citrate or 13C-citrate (m+2), 13C-acetyl-CoA and 13C-palmitate (m+n, n=2, 4, 6, 8, 10, 12, 14, 16) as well as the palmitate enrichment in mouse primary hepatocytes (B-F) or in HepG2 cells (G-K) cultured with mixed labeled and unlabeled carbon source upon knockdown of Slc25a1 or Acly. All data are presented as mean ± SD (n = 5-6). * P<0.05, ** P<0.01, *** P<0.001 vs control group. Acly, ATP-dependent citrate lyase; Slc13a5, plasma membrane citrate transporter; Slc25a1, mitochondrial citrate carrier.
**Fig. 3.** Bempedoic acid reduces both endogenous synthesis and exogenous uptake of citrate, lipogenic pools of acetyl-CoA, and palmitate via downregulating both SLC13A5 and ACLY. (A) Chemical structure of bempedoic acid. (B-D) The determined contents sourced from labeled [U-13C₆] glucose or [U-13C₆] citrate in mouse primary hepatocytes. (B) 13C-citate or 13C-citrate (m+2). (C) 13C-acetyl-CoA. (D) 13C-palmitate (m+n, n=2, 4, 6, 8, 10, 12, 14, 16) and the palmitate enrichment. (E-P) The transcriptions of lipogenic enzymes in mouse primary hepatocytes treated with OPA or glucose. (E, K) Acly. (F, L) Scl13a5. (G, M) Scl25a1. (H, N) Acc1. (I, O) Fasn. (J, P) Hmgcr. All data are presented as mean ± SD (n = 5-6). P<0.05, ## P<0.01, ### P<0.001 vs control group; * P<0.05, ** P<0.01, *** P<0.001 vs OPA/Glucose-challenged group. Acc1, acetyl-coenzyme A carboxylase; Acly, ATP-dependent citrate lyase; BA, bempedoic acid; BSA, bovine serum albumin; Fasn, fatty acid synthase; Hmgcr, hydroxy-methyl-glutaryl coenzyme A reductase; NC, negative control; OPA, oleic acid and palmitic acid; Scl13a5, plasma membrane citrate transporter; Scl25a1, mitochondrial citrate carrier.

**Fig. 4.** Bempedoic acid improves glucose tolerance and histopathological changes in the hepatic tissues of HFHFD-fed mice. (A) Experimental flow chart. (B and C) Blood glucose curve and AUC of OGTT and ITT. (D) Body weight. (E) Liver weight. (F) Representative photos of the liver. (G) H&E, Oil red O and picrosirius red staining, NAFLD activity score and Oil red O positive in the indicated groups. All data are presented as mean ± SD (n = 8). P<0.05, ## P<0.01, ### P<0.001 vs NCD group; * P<0.05, ** P<0.01, *** P<0.001 vs HFHFD group. Scale bars: 50 μm. AUC, area under the
curve; BA, bempedoic acid; H&E, hematoxylin-eosin; HFHFD, high-fat plus high-fructose diet; ITT, insulin tolerance test; NAS, NAFLD activity scores; NCD, negative control diet; OGTT, oral glucose tolerance test.

**Fig. 5.** Bempedoic acid alleviates hepatic injury, lipid accumulation, inflammatory response and fibrosis in HFHFD-fed mice. (A) Serum ALT. (B) Serum AST. (C) Serum TG. (D) Serum TC. (E) Serum NEFA. (F) Serum LDL/HDL. (G) Liver TG. (H) Liver TC. (I) Hepatic mRNA levels of Il1β, Il6, Tnf-α. (J) Hepatic mRNA levels of Col1α1, Acta2, Timp1. All data are presented as mean ± SD (n = 6-8). *P <0.05, **P <0.01, ###P <0.001 vs NCD group; *P <0.05, **P <0.01, ###P <0.001 vs HFHFD group. Acta2, actin alpha 2; ALT, alanine transaminase; AST, aspartate transaminase; BA, bempedoic acid; Col1α1, Collagen type I alpha 1; HDL, high-density lipoprotein; HFHFD, high-fat plus high-fructose diet; Il1β, interleukin 1 beta; Il6, interleukin 6; LDL, low-density lipoprotein; NCD, negative control diet; NEFA, non-esterified fatty acids; TC, total cholesterol; TG, total triglycerides; Timp1, tissue inhibitor of metalloproteinase 1; Tnf-α, tumor necrosis factor alpha.

**Fig. 6.** Bempedoic acid protects against NAFLD via regulating hepatic SLC13A5 and ACLY but not downstream lipogenic enzymes in HFHFD-fed mice. (A) Volcano plot analysis of the RNA-seq data. (B) KEGG enrichment analysis of pathways (lipid metabolic pathways were marked in red). (C) Heatmaps of lipid metabolism, inflammation, fibrosis, apoptosis related mRNA expression profiles based on the
RNA-seq data set. (D-H) The hepatic levels of mRNA or protein of Acly, Slc13a5, Slc25a1, Acc1, Fasn, Hmgcr. (D and E) Acly, Slc13a5, Slc25a1. (F) Acc1. (G) Fasn. (H) Hmgcr. All data are presented as mean ± SD (n = 3-8). # P<0.05, ## P<0.01, ### P<0.001 vs NCD group; * P<0.05, ** P<0.01, *** P<0.001 vs HFHFD group. Acc1, acetyl-coenzyme A carboxylase 1; Acly, ATP-dependent citrate lyase; BA, bempedoic acid; CIC, Slc25a1, mitochondrial citrate carrier; Fasn, fatty acid synthase; HFHFD, high-fat plus high-fructose diet; Hmgcr, hydroxy-methyl-glutaryl coenzyme A reductase; NCD, negative control diet; PMCT, Slc13a5, plasma membrane citrate transporter.

**Fig. 7.** Bempedoic acid downregulates both SLC13A5 and ACLY via suppressing PXR activation. (A-C) The mRNA levels of Acly, Slc13a5, Cyp3a11 in PCN-stimulated mouse primary hepatocytes. (A) Acly. (B) Slc13a5. (C) Cyp3a11. (D) Experimental flow chart. (E-I) The mRNA levels of Acly, Slc13a5, Cyp3a11, Acc1, Hmgcr in the liver of PCN-treated mice. (E) Acly. (F) Slc13a5. (G) Cyp3a11. (H) Acc1. (I) Hmgcr. (J) Liver TG. (K) Liver TC. (L and M) The mRNA levels of Acly and Slc13a5 in mouse primary hepatocytes treated with si Pxr. (L) Acly. (M) Slc13a5. All data are presented as mean ± SD (n = 6). # P<0.05, ## P<0.01, ### P<0.001 vs control group; * P<0.05, ** P<0.01, *** P<0.001 vs PCN group. Acc1, acetyl-coenzyme A carboxylase 1; Acly, ATP-dependent citrate lyase; BA, bempedoic acid; Cyp3a11, cytochrome P450 3A11; Hmgcr, hydroxy-methyl-glutaryl coenzyme A reductase; NC, negative control; PCN, pregnenolone 16α-carbonitrile; Slc13a5, plasma membrane citrate transporter; Slc25a1, mitochondrial citrate carrier; TC, total cholesterol; TG, total triglycerides.
Fig. 5

A. Serum ALT

B. Serum AST

C. Serum TG

D. Serum TC

E. Serum NEFA

F. Serum LDL/HDL

G. Liver TG

H. Liver TC

I. Inflammation

J. Fibrosis

- NCD
- HFHD
- HFHD+BA (10 mg·kg⁻¹)
- HFHD+BA (30 mg·kg⁻¹)
Fig. 6

A

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C

Lipid metabolism

HFF HD

BA

Inflammation

HFF HD

BA

Fibrosis

HFF HD

BA

Apoptosis

HFF HD

BA

D

mRNA

(relative transcription)

NCD

HFF HD

HFF HD+BA (10 mg·kg⁻¹)

HFF HD+BA (30 mg·kg⁻¹)

E

ACLY

PMCT

CIC

β-actin

F

Acc1

G

Fasn

H

Hmger

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