Rifaximin and lubiprostone mitigate liver fibrosis development by repairing gut barrier function in diet–induced rat steatohepatitis

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Abstract

Background: Although gut-derived lipopolysaccharide (LPS) affects the progression of non-alcoholic steatohepatitis (NASH) pathogenesis, few studies have focused on this relationship to develop treatments for NASH.

Aims: To explore the effects of combination with rifaximin and lubiprostone on NASH liver fibrosis through the modulation of gut barrier function.

Methods: To induce steatohepatitis, F344 rats were fed a choline-deficient L-amino acid-defined (CDAA) diet for 12 weeks and received oral administration of rifaximin and/or lubiprostone. Histological, molecular, and fecal microbial analyses were performed. Barrier function in Caco-2 cells were assessed by in vitro assays.

Results: Combination rifaximin/lubiprostone treatment significantly suppressed macrophage expansion, proinflammatory responses, and liver fibrosis in CDAA-fed rats by blocking hepatic translocation of LPS and activation of toll-like receptor 4 signaling. Rifaximin and lubiprostone improved intestinal permeability via restoring tight junction proteins (TJPs) with the intestinal activation of pregnane X receptor and chloride channel-2, respectively. Moreover, this combination increased the abundance of *Bacteroides*, *Lactobacillus,* and

Faecalibacterium as well as decreased that of *Veillonella* resulting in an increase of fecal short-chain fatty acids and a decrease of intestinal sialidase activity. Both agents also directly suppressed the LPS-induced barrier dysfunction and depletion of TJPs in Caco-2 cells.

Conclusion: The combination of rifaximin and lubiprostone may provide a novel strategy for treating NASH-related fibrosis.

Key words: non-alcoholic steatohepatitis, gut-liver axis, microbiome, lipopolysaccharide.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has emerged as the most prevalent chronic liver disease. NAFLD encompasses a continuum of liver abnormalities, from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [1, 2]. Importantly, significant fibrosis is the most powerful predictor of overall mortality, liver transplantation, and liver-related events in patients with NASH [3]. Although liver fibrosis regression is often a primary endpoint in clinical drug trials, no treatment for NASH-related fibrosis is available for clinical use.

The process of liver fibrosis in NASH is pathologically characterized by hepatic stellate cells (HSCs) activation and excessive accumulation of extracellular matrix (ECM) which are often caused by multiple factors, including the metabolic syndrome with visceral fat accumulation and insulin resistance, oxidative stress, genetic susceptibility, and gut dysbiosis [4, 5]. Dysbiosis is associated with gut barrier dysfunction involving intestinal hyperpermeability to microbial products such as lipopolysaccharide (LPS) [6, 7]. LPS enters the enterohepatic circulation, potentially exacerbating hepatic injury [6, 7]. In particular, LPS-mediated toll-like receptor 4 (TLR4)/nuclear factor-κB

(NF-κB) signaling is involved in the activation of Kupffer cells and HSCs resulting in the development of hepatic inflammation and fibrosis during NASH progression [8, 9]. Therefore, a potential therapeutic strategy against NASH fibrosis involves maintaining the gut barrier function and blunting the transfer of LPS into the liver [10].

Rifaximin is a poorly absorbed antibiotic widely applied for prevention of recurrent hepatic encephalopathy in the patients with cirrhosis [11, 12]. Remarkably, our clinical studies showed that rifaximin reduced plasma endotoxin activity and improved intestinal permeability in patients with cirrhosis [13, 14]. Furthermore, recent reports show that rifaximin inhibited liver fibrosis in rodent models of alcohol liver injury and NASH [15, 16]. However, the antifibrotic effect of rifaximin monotherapy seems to be partial and insufficient, such that augmentation of gut barrier integrity by combining rifaximin with another agent might be more effective against NASH fibrosis development.

Lubiprostone, a chloride channel-2 (ClC-2) activator, has been used clinically to safely and effectively treat constipation [17, 18]. This bicyclic fatty acid derived from prostaglandin E1 is metabolized quickly and has limited systemic bioactivity, resulting in a localized lumen effect [17, 18]. Lubiprostone stimulates

Cl^- secretion, leading to water accumulation in intestinal segments, and was shown to suppress the intestinal hyperpermeability in mice with dextran sulfate sodium-induced colitis [17-19]. *Kato* et al. reported that lubiprostone improves intestinal barrier function in healthy volunteers after administration of diclofenac and was well tolerated and reduced the levels of liver enzymes in patients with NAFLD and constipation [20, 21]. However, the therapeutic potential of lubiprostone against NASH-based liver fibrosis is unclear.

In the present study, we investigate the effects of combination treatment with rifaximin and lubiprostone on liver fibrosis development and gut barrier function in diet-induced rat steatohepatitis model.

Materials and Methods

Animal treatment

Our previous reports have demonstrated that choline-deficient L-amino acid-defined (CDAA) diet induced a severe steatohepatitis as well as liver fibrosis and a remarkable gut hyperpermeability with increased LPS translocation into the liver [15, 22]. Thus, we employed this model to elucidate the effects of rifaximin and lubiprostone in the present study. A choline-sufficient amino acid-

defined (CSAA) diet was used as a negative control. CSAA and CDAA diets were purchased from Research Diets Inc. (New Brunswick, NJ, USA) and both ingredient compositions were shown in Supplementary Table 1. Six-week-old male Fischer 344 rats (CLEA Japan, Tokyo, Japan) were randomly divided into five groups and treated for 12 weeks as follows (Fig. 1A; n = 10 for each): i) CSAA with lactose hydrate (Wako Pure Chemical Corporation, Osaka, Japan) as vehicle [23]; ii) CDAA diet with lactose hydrate; iii) CDAA diet with rifaximin (ASKA Pharmaceutical Co. Ltd., Tokyo, Japan; 100 mg/kg); iv) CDAA diet with lubiprostone (Mylan N.V. , Tokyo, Japan; 10 μg/kg); and v) CDAA diet with rifaximin and lubiprostone [15, 16, 19, 24]. All drugs were administered daily by intragastric gavage. Another set of rats (n = 5) was applied to evaluate *in vivo* intestinal permeability. Rats were caged with free access to food and water under standard conditions (12:12 h day/night cycle, temperature of 23 ± 3 °C, humidity of 55 ± 10%, and continuous ventilation). Animal experiments were approved by the Animal Ethics Committee of Nara Medical University (No. 12639, No. 12768), and all protocols were performed according to the National Institutes of Health Guidelines for the Care and use of Laboratory Animals.

Gut permeability measurement

A 4-kDa fluorescein isothiocyanate (FITC)-dextran (Sigma–Aldrich, St. Louis, MO, USA) solution was used for intestinal permeability measurement [25]. Briefly, six hours after initiating fasting conditions, FITC-dextran was gently administered as oral gavage to rats (n = 5) in each group at 40 mg/kg, 200μl body weight. Blood was collected from the portal vein 1 hours after FITC-dextran administration. To evaluate the degree of gut permeability, blood was analyzed by fluorescence measurement of the concentration of FITC-labeled dextran at an excitation wavelength of 490 nm and an emission wavelength of 520 nm using NanoDrop 3300 fluorospectrometer (Thermo Scientific, Wenham, MA, USA).

Fecal microbiome analyses

Fecal samples from the colon of 5 rats in each group were stored at −80°C at the end of experiments, and then 16S rRNA next-generation sequencing was performed. Detailed methods are described in Supplementary information.

Fecal short-chain fatty acid (SCFA) measurement

SCFA concentrations in fecal samples were measured by the methods as previously described [26]. Fecal samples (300 mg) were mixed with 0.6 mL distilled water. Diluents were mixed with 90 μL 120 g/L perchloric acid. After centrifugation (15,000× *g*, 10 min, 4 °C), the supernatant fractions were filtered through a 0.45-μm cellulose acetate membrane filter (Cosmonice Filter W, Nakalai Tesque, Kyoto, Japan) and degassed under vacuum. The supernatant fractions (5 μL) were injected into an SIL-10 autoinjector (Shimadzu, Kyoto, Japan). SCFAs were separated by two serial organic acid columns (Shim-pack SCR-102H, Shimadzu) with a guard column (SCR-102HG; Shimadzu) at 45 °C with isocratic elution (0.8 mL/min) of 5 mM *p*-toluenesulfonic acid aqueous solution using a solvent delivery pump (LC-10ADvp; Shimadzu) with an online degasser (DGU-12A; Shimadzu, Kyoto, Japan). SCFAs were detected with an electronic conductivity detector (Waters 431; Waters Corporation, Milford, MA, USA) after post-column dissociation (0.8 mL/min) with 5 mM *p*-toluenesulfonic acid, 20 mM bis-Tris, and 100 μM ethylenediaminetetraacetic acid by using the solvent delivery pump. SCFAs were quantified with a system controller (CBM-20A; Shimadzu) and were represented as mM/g feces.

Measurement of sialidase activity

Sialidase, also known as neuraminidase, is one of the mucin-degrading enzymes produced by gut microbiota [27]. Thus, we assessed sialidase activity in the ileum of experimental rats. 10 mg of frozen ileum tissues were homogenized and centrifuged at 10,000 x g for 5 minutes at 4°C. Sialidase activity was measured using Neuraminidase Activity Assay Kit (Abcam) according to the manufacturer's instructions.

In vitro cell culture

Caco-2, a human intestinal epithelial cell line (Riken BRC, Ibaraki, Japan) was cultured in Dulbecco's modified Eagle's medium supplemented with 1% penicillin-streptomycin, 1% nonessential amino acids, 25 mmol/L glucose, and 10% fetal bovine serum at 37°C under 5% CO_2 [16, 28]. After 10–20 passages, cells were incubated as follows: LPS (*Escherichia coli* O55:B5) (Sigma–Aldrich) was added to the culture medium to evaluate cell viability and transepithelial electrical resistance. To the cells incubated with LPS (2 μg/mL) was added different concentrations of rifaximin (0, 0.1, 1, and 10 μM) or lubiprostone (0, 100, 250 and 500 nM), followed by incubation for 3, 6, 12, and 24 hours. The

human pregnane X receptor (PXR) antagonist, SPA70 (510 μM; Sigma–Aldrich) or ClC2 inhibitor GaTx2 (10 nM; Tocris Bioscience, Ellisville, MO, USA) was added to inhibit the pharmacological actions of rifaximin or lubiprostone, respectively [29, 30].

Statistical analyses

Mean ± standard deviation (SD) was used for data presentation. The difference between groups was determined for statistical significance by one-way analysis of variance (ANOVA) followed by Student's *t*-test for comparisons of 2 groups. Bartlett's test was used to determine homogeneity of variances. All statistical analyses were performed with GraphPad Prism version 9.0 software (La Jolla, CA, USA). A p-value of < 0.05 was considered statistically significant.

Additional methods can be found online in the Supplementary information.

Results

Effect of rifaximin and lubiprostone on hepatic steatosis in CDAA-fed rats

Twelve weeks on the CDAA diet markedly delayed the body weight gain and

increased the relative liver weight. These physical impairments were unchanged by treatment with rifaximin and lubiprostone (Figs. 1B and 1C). Although loose stool was observed in some of the rats treated with rifaximin and all rats treated with lubiprostone at the tested doses, these rats did not have renal dysfunction and electrolyte imbalance (Supplementary Fig. 1). The higher level of serum transaminases and the lower level of serum albumin were found in the CDAA-fed rats (Figs. 1D and 1E). Although the combination of rifaximin and lubiprostone inhibited the increase in serum transaminases levels, these agents did not affect hypoalbuminemia in the CDAA-fed rats (Figs. 1D and 1E). The CDAA-fed rats had the histological characteristics of hepatic steatosis and increased hepatic triglyceride levels; treatment with rifaximin and lubiprostone did not change this hepatic fat accumulation (Figs. 1F and 1G).

Rifaximin and lubiprostone attenuate hepatic macrophage expansion and LPS/TLR4 signaling in CDAA-fed rats

The CDAA-fed rats showed a marked expansion of $CD68^+$ macrophages in the liver and an upregulated hepatic *Cd68* mRNA level (Figs. 2A–2C). Treatment with rifaximin or lubiprostone ameliorated the $CD68^+$ macrophage

infiltration, which were enhanced by the concomitant treatment with both agents (Figs. 2A–2C). The concomitant treatment of both agents remarkably attenuated the increase in hepatic mRNA expressions of M1-macrophage markers, whereas it did not affect those of M2-macrophage markers (Figs. 2D and 2E).

We further investigated the impact of both treatments on the LPS-meaidtaed inflammatory signaling in the liver of CDAA-fed rats. The CDAA diet-feeding elevated LPS levels in the portal vein and increased hepatic mRNA level of LPS-binding protein (LBP), which recognizes and binds the lipid A moiety of LPS, enhancing host immune response to endotoxin (Fig. 2F and 2G). Increased hepatic *Lbp* expression accompanied with upregulation of *Tlr4* and its coreceptor *Cd14*, which play a central role in LPS recognition and signal initiation (Fig. 2G). Noticeably, treatment with rifaximin and lubiprostone alleviated the elevation of portal LPS level and upregulation of these genes, indicating that these drugs could abate hepatic translocation of LPS (Figs. 2F and 2G). Sequentially, the concomitant treatment with both agents effectively suppressed the phosphorylation of NF-κB p65 which plays an important role in the regulation of key inflammatory mediators in CDAA-fed rats (Fig. 2H).

Rifaximin and lubiprostone inhibit hepatic fibrogenesis in CDAA-fed rats

Given the anti-inflammatory properties, we assessed the effects on CDAA-induced hepatic fibrogenesis. CDAA-fed rats displayed hepatic fibrosis in centrilobular areas, which was stained by Sirius-Red, while treatment with rifaximin or lubiprostone significantly suppressed the CDAA diet-induced fibrosis (Figs. 3A and 3B). Along with the attenuation of fibrosis, the α-smooth muscle actin-positive areas, signifying the HSC activation, were markedly reduced, and consistently the hepatic accumulation of collagen 1 (COL-1), a representative ECM, was significantly ameliorated after the concomitant treatment (Figs. 3A, 3C and 3D). The observed ameliorations in the fibrotic phenotypes coincided with the reduction of profibrotic gene expressions, including *Acta2*, *Col1a1*, and *Tgfb1* (Fig. 3E).

Rifaximin and lubiprostone suppress the intestinal barrier dysfunction in the CDAA-fed rats

Both rifaximin and lubiprostone blunted the translocation of LPS into the liver, as indicated by the reduced expressions of TLR4-mediated inflammatory

mediators (Fig. 2). To investigate the mechanism underlying these properties, we next assessed tight junction proteins (TJPs) and intestinal permeability. Immunofluorescent analysis showed that the intestinal expression of zonula occludens-1 (ZO-1) and Claudin-1 were markedly lower in CDAA-fed rats but were restored after treatment with rifaximin and lubiprostone (Figs. 4A–4C). Western blotting confirmed the restoration of intestinal protein expression of both markers (Fig. 4D). As shown in Fig. 4E, concomitant treatment also upregulated the intestinal mRNA expressions of other TJP markers *Ocln* and *Cldn4*, encoding Occludin and Claudin-4, in addition to *Zo1* and *Cldn1*. Compared with the CSAA-fed control rats, the FITC-dextran content of CDAA-fed rats obviously increased showing the intestinal hyperpermeability (Fig. 4F). Meanwhile, either treatment with rifaximin or lubiprostone sharply lowered the FITC-dextran content in the CDAA-fed rats, and the combination of both agents augmented the effect of each single treatment (Fig. 4F).

Recent report has shown that rifaximin-mediated activation of intestinal pregnane X receptor (PXR) contributes to protect barrier function by dampening myosin light-chain kinase (MLCK) expression and c-Jun N-terminal kinases (JNK)1/2 activation in the intestinal mucosa [31]. Our experimental model

showed that consumption of CDAA diet increased intestinal MLCK expression and induced JNK1/2 phosphorylation which were suppressed by treatment with rifaximin (Fig. 4G). Moreover, lubiprostone-mediated ClC-2 activation has been reported to strengthen intestinal tight junction by downregulating caveolin-1, a key constituent of caveolae, cholesterol-enriched invaginations arising from lipid raft areas in the membrane [32]. Interestingly, we found that intestinal caveolin-1 protein was decreased in lubiprostone-treated groups as compared to vehicle-treated group in the CDAA-fed rats (Fig. 4H).

Rifaximin and lubiprostone directly improve LPS-induced barrier impairment in enterocytes

We further investigated the pharmacological action of rifaximin and lubiprostone on *in vitro* barrier function in Caco-2 enterocytes. The LPS stimulus dose-dependently (0.1-2 μg/ml) decreased the values of transepithelial electrical resistance (TEER), the indicator of the integrity of the cellular barriers, without affecting cell viability (Figs. 5A and 5B); this finding suggests that LPS-stimulated barrier impairment does not lead to cell death within above concentration. As shown in Fig. 5C, treatment with rifaximin suppressed the

LPS-stimulated reduction in TEER values in a dose-dependent manner, which was abolished by addition of SPA70 as a human PXR antagonist. These findings suggest that rifaximin might prevent the intestinal barrier impairment via PXR activation. Likewise, lubiprostone also suppressed the LPS-induced reduction in TEER values of Caco-2 cells (Fig. 5D). Interestingly, this suppressive effect of lubiprostone was negated by treatment with GaTx2, a selective inhibitor of ClC-2, indicating that the lubiprostone-mediated activation of ClC-2 protects intestinal barrier function (Fig. 5D). Consistent with the recovery of electrical resistance, both rifaximin and lubiprostone restituted the LPS-induced deprivation of intestinal protein expressions of ZO-1 and Claudin-1 in Caco-2 cells (Fig. 5E). Notably, we confirmed that the restoration of TJPs by rifaximin and lubiprostone was reversed by treatment with SPA70 and GaTx2, respectively (Fig. 5E).

Modification of fecal microbiota and SCFA metabolism by rifaximin and lubiprostone in CDAA-fed rats

We next examined whether rifaximin and lubiprostone affected the gut microbiota in the present rodent models. A total of 782,766 paired-end reads

were obtained from the 25 samples after sequencing, with 543,930 clean tags after alignment and filtering. Samples contained 21,757 ± 2,674 clean tags on average. The α-diversity (Observed features and Shannon) were profoundly lower in the CDAA-fed/vehicle-treated rats than CSAA-fed rats (Supplementary Fig. 2A and Fig. 6A). Rifaximin treatment did not cause a change in this parameter, but interestingly, lubiprostone treatment led to a recovery in this impaired α-diversity (Supplementary Fig. 2A and Fig. 6A). In addition, we used weighted UniFrac analyses to compare the similarity of microbial communities as β-diversity. The PCoA plots showed a clear shift of CDAA-fed/vehicle-treated group from CSAA-fed group, and this shift was attenuated by treatment with lubiprostone (Fig. 6B). The PERMANOVA with FDR correction proved that the observed differences were statistically significant ($p < 0.05$ in all comparisons) supporting that lubiprostone could improve CDAA-induced alteration of microbial diversity in rats. Meanwhile, the composition of fecal microbiota was markedly modified by both rifaximin and lubiprostone in the CDAA-fed rats (Figs. 6C-6H). We found that the CDAA-fed rats showed a decrease in the relative abundance (RA) of *Bacteroidetes* and *Desulfobacterota* but an increase in the RA of *Firmicutes*, *Actinobacteriota*, and *Proteobacteria* as compared to

CSAA-fed rats at the phylum level (Fig. 6C). These alteration at phylum level were inhibited by treatment with rifaximin and lubiprostone (Fig. 6C). For the genera in abundance, CDAA diet consumption significantly altered 43 genera in the feces, and 18 genera were influenced by combination treatment of rifaximin and lubiprostone in CDAA-fed rats (PERMANOVA with FDR correction; $p < 0.05$ in all comparisons) (Fig. 6D and Supplementary Fig. 2B). Among them, we focused on the increased RA of the genera, *Bacteroides*, *Lactobacillus*, and *Faecalibacterium* which are known for their production of SCFAs which increase TJPs expression in the gut to decrease potential gut hyperpermeability (Fig. 6E-6G) [33-35]. Fecal SCFAs analysis showed that the fecal level of n-butyric acid was decreased in the CDAA-fed rats, and this decrease was suppressed by combination treatment (Fig. 6I). On the other hands, there were no significant differences in the fecal levels of acetic acid, propionic acid, and i-butyric acid among experimental groups (Fig. 6I). Moreover, we found a decrease in the RA of *Veillonella*, one of the sialidase-rich species to degrade intestinal mucin leading to impairment of barrier integrity (Fig. 6H) [36]. In fact, the intestinal activity of sialidase was changed in parallel with the RA of *Veillonella* (Fig. 6J).

Discussion

This study shows that the combination of rifaximin and lubiprostone markedly suppressed liver inflammation and fibrogenesis in CDAA-diet–induced rat steatohepatitis. Our investigation of the molecular mechanism underlying these effects revealed an improvement in gut permeability induced by both agents, resulting in a potent blockade of LPS translocation into the liver that led to suppression of the hepatic LPS/TLR4 pathway. Disruption of the intestinal barrier arising from TJP deprivation has been shown to play a key role in the progression of NASH pathogenesis [37-39]. *Rahman* et al. described that the defects in intestinal permeability induced by genetic deletion of junctional adhesion molecule A exacerbated high-fat-diet–induced murine steatohepatitis [37]. Recent meta-analyses also reported that NASH patients exhibit intestinal hyperpermeability compared with healthy controls [38, 39].

The present study revealed the involvement of multiple mechanisms underlying the rifaximin and lubiprostone-mediated improvement in intestinal permeability (Fig. 6K). First, we hypothesized that both rifaximin and lubiprostone could directly affect enterocytes. As mentioned above, rifaximin and lubiprostone have been reported to protect intestinal barrier integrity by

enhancing tight junction via PXR/MLCK/JNK1/2 and ClC-2/caveolin-1 pathway, respectively [31, 32]. According to these pharmacological evidences, our findings demonstrated that rifaximin reduced MLCK expression and inhibited JNK1/2 phosphorylation while lubiprostone reduced caveolin-1 expression in the ileum of CDAA-fed rats. To reinforce this notion, our *in vitro* studies showed that rifaximin or lubiprostone directly suppress LPS-induced epithelial paracellular permeability and TJP downregulation in enterocytes, and both effects were negated by treatment with the antagonists of PXR or ClC-2, respectively.

Second, both agents modified the profiles of gut microbiota in CDAA-fed rats. Fecal microbial analysis revealed that combination treatment with both agents enriched *Bacteroides*, a finding that coincides with recent studies in a murine ankylosing spondylitis model [40]. Notably, the abundance of *Lactobacillus* and *Faecalibacterium* increased in CDAA-fed rats treated with both agents. These microbial species are known to be involved in the pathogenesis of NAFLD and NASH as producers of SCFAs, which increase TJPs expression in the gut to decrease potential gut hyperpermeability [33, 34, 41, 42]. In fact, we found that the combination treatment suppressed a decrease in fecal n-butyric acid level.

Moreover, our previous human studies have shown that rifaximin depleted *Veillonella*, a part of the normal microbiota of the mouth and gastrointestinal tract [13, 14]. *Patel* et al. also recently suggested that rifaximin improves hepatic encephalopathy in cirrhosis patients by repairing gut barrier function via suppression of mucin-degrading sialidase-rich species, including *Veillonella* [36]. In consistent with these reports, our results elucidated that combination treatment reduced the RA of *Veillonella* as well as decreased the intestinal sialidase activity. These findings support that the microbial changes by both agents were at least partially associated with improvement of intestinal permeability in the CDAA-fed rats.

The present study has several limitations. First, lubiprostone recovered the decreased diversity and impaired composition of the fecal microbiota in CDAA-fed rats, although it exerted neither antibacterial nor probiotic effects. In this context, we hypothesize that the property changes in the inner mucus layer could influence on the gut microbial status. *Musch* et al. previously showed that lubiprostone changed the composition of the inner layer mucus in the colon of murine cystic fibrosis model [43]. They also documented that lubiprostone increased *Lactobacillus,* which were attributed to compositional changes of the

mucus. Second, clinical evidence suggests that small intestine bacterial overgrowth is associated with TLR signaling genes in NASH pathogenesis [44, 45]. Also, both rifaximin and lubiprostone are reported to modify the status of small intestine bacterial overgrowth [46, 47]. Therefore, our future studies will pursue these lines of research in the present model.

In summary, combination treatment with rifaximin and lubiprostone showed a potent antifibrotic effect on CDAA-fed rat steatohepatitis. Notably, the effects of both agents were involved in the reduction of endogenous LPS overload based on the repair of gut barrier integrity. We underscore that these drugs are clinically available and that the above-mentioned effects were exerted without hepatic and renal toxicity in rats. Overall, this combination regimen may constitute a novel therapy for the prevention of NASH progression for future clinical application.

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Figure legends

Figure 1. Rifaximin and lubiprostone on hepatic steatosis in CDAA-fed rats. (A) Experimental protocols. (B) Changes in body weights during experimental period. (C) Ratio of liver weight to body weight at the end of experiment. (D) Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). (E) Serum levels of albumin. (F) Representative macrophotographs of liver, and microphotographs stained with hematoxylin-eosin (H&E). Scale Bar; 50 μm. (G) Hepatic triglyceride (TG) concentration. Data are mean ± SD (n = 10), ** $p < 0.01$. N.S; not significant. Rif; rifaximin, Lub; lubiprostone.

Figure 2. Rifaximin and lubiprostone on hepatic macrophages and inflammation in CDAA-fed rats. (A) Representative microphotographs of CD68-stained liver sections. Scale Bar; 50 μm. (B) Semi-quantitative analysis of CD68-positive macrophages in high-power field by NIH imageJ software. Histochemical quantitative analyses included five fields per section. (C-E) Relative mRNA levels of *Cd68* (C), *Tnfa*, *Il1b*, *Il6*, and *Nos2* (D), *Il10*, *Arg1* and

Cd163 (E) in the liver of experimental rats. (F) Plasma lipopolysaccharide levels in the portal vein. (G) Relative mRNA levels of *Lbp*, *Tlr4* and *Cd14* in the liver of experimental rats. (H) Western blots for NF-κB p65 phosphorylation in liver tissues (left panel) and quantitative phosphorylation rate of phosphorylated p65/ total p65 (right panel). Actin was used as the loading control. The mRNA expression levels were measured by qRT-PCR, and *Gapdh* was used as internal control (C-E and G). Quantitative values are indicated as fold changes to the values of CSAA group (C-E, G, and H). Data are mean ± SD (B-G; n = 10, H; n = 6), * $p < 0.05$ and ** $p < 0.01$. Rif; rifaximin, Lub; lubiprostone.

Figure 3. Rifaximin and lubiprostone on hepatic fibrosis development in CDAA-fed rats. (A) Representative microphotographs of liver sections stained with Sirius-red and α-SMA. Scale Bar; 50 μm. (B, C) Semi-quantitative analysis of Sirius-red (B), and α-SMA (C) immune-positive areas in high-power field by NIH imageJ software. Histochemical quantitative analyses included five fields per section. (D) Western blots for COL-1 in liver tissues (left panel) and semi-quantitation (right panel). Actin was used as the loading control. (E) Relative mRNA expression levels of *Acta2*, *Col1a1* and *Tgfb1* in the liver of experimental

rats. The mRNA expression levels were measured by qRT-PCR, and *Gapdh* was used as internal control. Quantitative values are indicated as fold changes to the values of CDAA + Vehicle group (B and C) or CSAA group (D and E). Data are mean ± SD (B, C and E; $n = 10$, D; $n = 6$), * $p < 0.05$ and ** $p < 0.01$. N.D, not detected. Rif; rifaximin, Lub; lubiprostone.

Figure 4. Rifaximin and lubiprostone on intestinal barrier function in CDAA-fed rats. (A) Representative microphotographs of ileum sections stained with zonula occludens-1 (ZO-1) and Claudin-1 in the experimental groups. Nuclei counterstained with 4',6-diamidino-2-phenylindole (DAPI). Scale Bar: 50 μm. (B, C) Semi-quantitation of ZO-1 and Claudin-1 immuno-positive areas in high-power field by NIH imageJ software. Quantitative analyses included five fields per section. (D) Western blots for ZO-1 and Claudin-1 in the intestinal tissues. Actin was used as the loading control. (E) Relative mRNA expression levels of *Zo1*, Occuldin (*Ocln*), Claudin-1 (*Cldn1*) and Claudin-4 (*Cldn4*) in the intestine of experimental rats. The mRNA expression levels were measured by qRT-PCR, and *Gapdh* was used as internal control. (F) Blood levels of fluorescein isothiocyanate (FITC)-dextran (4kDa) 4 h after oral administration.

(G and H) Western blots for MLCK, p-JNK1/2 and t-JNK (G) and Caveolin-1 (H) in the intestinal tissues. Actin was used as the loading control. Quantitative values are indicated as fold changes to the values of CSAA group (B, C and E). Data are mean ± SD (B, C and E; $n = 10$, F; $n = 5$), * $p < 0.05$ and ** $p < 0.01$. Rif; rifaximin, Lub; lubiprostone.

Figure 5. Effect of rifaximin and lubiprostone on LPS-stimulated Caco-2 cells.

(A) Cell viability of lipopolysaccharide (LPS) (0.1-2 μg/ml)-stimulated Caco-2 cells. Cell viability was estimated as the ratio to the value of non-treatment group (NT) at 0 hr. (B) *In vitro* paracellular permeability in LPS (0.1-2 μg/ml)-stimulated Caco-2 cells determined as transepithelial electrical resistance (TEER). (C and D) The effects of rifaximin (0-10 μM) or lubiprostone (0-500 nM) on TEER in LPS (2 μg/ml)-stimulated Caco-2 cells. (E) Western blots for the effects of rifaximin (10 μM) or lubiprostone (500 nM) on the protein expression of ZO-1 and Claudin-1 in LPS (2 μg/ml)-stimulated Caco-2 cells. Actin was used as the loading control. SPA70 (510 μM) and GaTx2 (10 nM) were used as a human pregnane X receptor antagonist and a chloride channel 2 inhibitor,

respectively (C-E). Data are mean ± SD (n=3 independent experiments with n=6 (A-D) or n=3 (E) samples per condition), ^{a} $p < 0.05$ and ^{aa} $p < 0.01$ vs non-treatment (NT) group, ^{b} $p < 0.05$ and ^{bb} $p < 0.01$ vs LPS-treated group, ^{c} $p < 0.05$ and ^{cc} $p < 0.01$ vs LPS + Rif (10 μM)-treated group, ^{d} $p < 0.05$ and ^{dd} $p < 0.01$ vs LPS + Lub (500 nM)-treated group, Rif; rifaximin, Lub; lubiprostone.

Figure 6. Rifaximin and lubiprostone on fecal microbiota, SCFA and intestinal sialidase in CDAA-fed rats.

(A) Comparative analysis of α-diversity (Shannon index) in the feces of experimental groups. (B) PCoA plot based on unweighted UniFrac distance matrices for the experimental groups. The percentages on each axis indicate the proportion of each dimension. (C) Bacterial taxonomic analysis of fecal microorganisms at the phylum level. (D) Heatmap for altered 43 genera in abundance in fecal microbiota by the consumption of CDAA diet. (E-H) Relative abundance of the genus *Bacteroides* (E), *Lactobacillus* (F), *Faecalibacterium* (G), and *Veillinella* (H) in fecal microbiota of experimental groups. (I) Fecal levels of SCFAs (Acetic acid, Propionic acid, n-Butyric acid and i-Butyric acid) of

experimental groups. (J) Intestinal activity of sialidase of experimental groups. Quantitative values are indicated as fold changes to the values of CSAA group. (K) Graphic scheme of the effect of rifaximin and lubiprostone on the CDAA-induced liver fibrosis. Data are mean ± SD (n = 5), * $p < 0.05$ and ** $p < 0.01$. Rif; rifaximin, Lub; lubiprostone, ClC-2: Chloride channel-2; iNOS: Inducible nitric oxide synthase; LPS: Lipopolysaccharide; PXR: Pregnane X receptor; TGF-β1: Transofroming growth factor β1; TLR4: Toll-like receptor 4; TNFα: Tumor necrosis factor α.