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ORIGINAL ARTICLE

Treatment with Oxidized Phospholipids Directly Inhibits Nonalcoholic Steatohepatitis and Liver Fibrosis Without Affecting Steatosis

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Abstract

Background and Aims Previous studies demonstrated that toll-like receptors 4 and 2 (TLR-4 and TLR-2), which are expressed on liver-resident Kupffer, hepatic stellate cells, and circulating monocytes, play a role in nonalcoholic fatty liver disease. Lecinoxoids are oxidized phospholipids that antagonize TLR-2- and TLR-4-mediated activation of innate immune cells and inhibit monocyte migration. In this study, we tested the effect of two functionally different lecinoxoids on the development of nonalcoholic steatohepatitis and liver fibrosis in a mouse model.

Methods Two-day-old C57BL/6 mice were injected with streptozotocin and fed a high-fat diet from Week 4 after birth. At Week 6 post-birth, lecinoxoids VB-201 or VB-703 were given orally, once daily, for 3 weeks. Telmisartan was administered orally, once daily, for 3 weeks, as positive control. At experiment conclusion, biochemical indices were evaluated. HE stain and quantitative PCR were used to determine the extent of steatosis and steatohepatitis, and Sirius red stain was used to assess liver fibrosis.

Results Treatment with lecinoxoids did not alter the concentration of blood glucose, liver triglycerides, or steatosis compared with solvent-treated mice. However, whereas VB-201 inhibited the development of fibrosis and, to some extent, liver inflammation, VB-703 significantly lessened both liver inflammation and fibrosis.

Conclusions This study indicates that using lecinoxoids to antagonize TLR-2, and more prominently TLR-4, is sufficient to significantly inhibit nonalcoholic steatohepatitis and liver fibrosis. Inhibiting monocyte migration with lecinoxoids that are relatively weak TLR-4 antagonists may alter liver fibrosis and to some extent nonalcoholic steatohepatitis.

Keywords Nonalcoholic fatty liver disease · Toll-like receptor · Nonalcoholic steatohepatitis · Lecinoxoids · Fibrosis

Introduction

One of the major complications ensuing insulin resistance and metabolic syndrome is nonalcoholic fatty liver disease (NAFLD), which can progress from fatty liver (steatosis) to fatty liver with inflammation (steatohepatitis) and liver fibrosis. While the majority of subjects do not progress beyond steatosis, it is not clear why others continue to develop steatohepatitis. Supporting evidence suggests that modulations in gut microbiota can be central to the disease pathogenesis. In this context, it was shown that the integrity of the small intestine is impaired in patients with NAFLD and that the variety and proportion of gut flora in nonalcoholic steatohepatitis (NASH) patients are altered compared

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with healthy controls [1, 2]. This microbiota dysbiosis can promote NASH by increasing the levels of methylamines and alcohol [3, 4], compounds that induce liver inflammation and injury [5]. Alterations in gut microbiome were also shown to promote NASH by affecting bile acid metabolism, resulting in impaired FXR-driven signaling and consequently advancing steatosis [6]. Finally, wild-type mice demonstrated increased disease severity in a methionine–choline-deficient diet-induced NASH model when co-housed with mice with altered gut microbiota [7].

Bacterial components from residing gut microbiome such as lipopolysaccharides (LPS) and peptidoglycans (PGN) can travel through the portal vein into the liver and encounter toll-like receptors (TLRs) [8]. TLRs are a family of receptors imperative for the innate immune response against microbial invasion. Two of the TLRs, TLR-2 and TLR-4, recognize the bacterial products PGN and LPS, respectively [9, 10]. The interaction between these TLRs and their cognate agonists instigates a cascade of signals that include downstream phosphorylation events, which culminate in the secretion of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α. Several reports demonstrated the correlation between TLR-2 and TLR-4 and NAFLD pathogenesis. In TLR-2 KO mice, a lower rate of liver inflammation was observed when fed with a choline-deficient amino acid-deficient diet or high-fat diet [11, 12]. This decrease was manifested by a reduced number of macrophages and the expression of pro-inflammatory cytokines in the liver compared with wild-type mice. In addition, TLR-2 KO mice also exhibited lower rates of liver fibrosis [12]. Mice bearing nonfunctional mutated TLR-4 or TLR-4-targeted genes that were fed methionine–choline-deficient diets demonstrated reduced liver expression of TNF-α and TGF-β together with decreased liver content of collagen and α-SMA compared with control mice [13, 14].

Liver-resident Kupffer cells and hepatic stellate cells (HSC), which express TLR-2 and TLR-4, are central to the development of steatohepatitis and fibrosis [15–17]. The activation of TLR-2 and TLR-4 on Kupffer cells induces the production of TNF-α, TGF-β, and IL-1β, which in turn augment HSC activation. Combined with the direct TLR-4-mediated activation of HSC, ligation of these TLRs promotes fibrogenesis [8, 18]. The recruitment of circulating monocytes into injured liver was also shown to be of importance to the development of liver fibrosis. Indeed, in the absence of circulating monocytes, such as in CCR2-deficient mice, fibrosis following acute liver injury was reduced, suggesting that monocyte-derived macrophages promote liver fibrosis [19].

We showed previously that a synthetic small molecule analog, VB-201, which belongs to the lecinoxoid family (lecin for lecithin—i.e., phospholipid, and oxoid for oxidized), directly binds to the CD14 and TLR-2 and consequently antagonizes TLR-2- and TLR-4-induced activation of monocytes, macrophages, and DCs [20]. Moreover, we demonstrated that VB-201 inhibits migration of monocytes toward various chemokines. In vivo treatment with VB-201 impaired migration of monocytes into the aorta and decreased the size of the aortic plaque in an atherosclerosis mouse model [21]. In the current report, we investigated the effect of VB-201 treatment on the development of NASH and liver fibrosis in a mouse model. In addition, we used VB-703, an unreported lecinoxoid designed in silico for improved efficacy, that does not affect monocytes migration, but exhibits increased inhibition of TLR-4 over VB-201. The results demonstrate that lecinoxoids restrict liver inflammation and profoundly ameliorate liver fibrosis.

Materials and Methods

Animals

C57BL/6 mice (15-day-pregnant females) were obtained from Japan SLC (Japan). All animals used in the study were housed and cared for in accordance with the Japanese Pharmacological Society Guidelines for Animal Use.

Tested Reagents

The bioactive compounds VB-201 [(R)-1-hexadecyl-2-(4-carboxy)butyl-*sn*-glycero-3-phosphocholine] and VB-703 [1-(2-octyl)dodecyl-2-(4′-carboxy)butyl-glycero-sn-3-phosphoric acid pyridiniumethyl ester] (Fig. 1) were synthesized in VBL's chemical laboratory (Or Yehuda, Israel). The

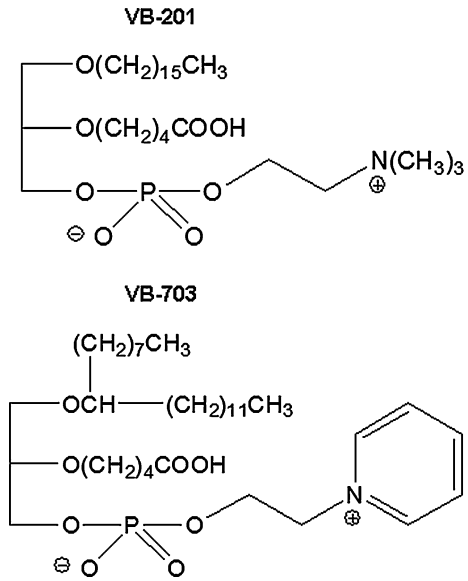

Fig. 1 Chemical structures of VB-201 and VB-703

generation of VB-201 was described previously [20]. VB-703 was synthesized from (S)-1-(2-octyl)dodecyl-2-(4′-carboxy)butyl-glycerol. The free acid was protected by esterification and reacted with phosphorus oxychloride followed by the addition of O-tosyl ethylene glycol. After aqueous hydrolysis, 1-(2-octyl)dodecyl-2-(4′-carboxy)butyl-glycero-sn-3-phosphoric acid tosylethyl ester was obtained. Heating of 1-(2-octyl)dodecyl-2-(4′-carboxy)butyl-glycero-sn-3-phosphoric acid tosylethyl ester in pyridine and purification by chromatography over silica gel yielded 1-(2-octyl)dodecyl-2-(4′-carboxy)butyl-glycero-sn-3-phosphoric acid pyridiniumethyl ester (VB-703). To prepare dosing solutions, VB-201 and VB-703 were dissolved in the solvent (0.5 % ethanol in PBS). Telmisartan (Micardis®) was purchased from Boehringer Ingelheim GmbH (Germany) and was dissolved in pure water.

Isolation of Human Monocytes and In Vitro Generation of Dendritic Cells

Venous blood samples were obtained from healthy male donors in compliance with the Institutional Review Board at the Sheba Medical Center, Ramat Gan, Israel. PBMCs were isolated on Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) using 50-ml Leucosep tubes (Greiner Bio-One, Frickenhausen, Germany). Cells were washed in PBS (Kibbutz Beit Haemek, Israel) and incubated at 4 °C for 15 min in a buffer containing PBS and 0.5 % bovine serum albumin (BSA) with human CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). To generate monocyte-derived DC (Mo-DC), CD14+ monocytes were counted, washed, and seeded (106/ml) in medium containing RPMI-1640, L-glutamine, β-mercaptoethanol, 10 % FCS, sodium pyruvate, nonessential amino acids, 0.01 M HEPES, antibiotics (Penicillin, Streptomycin), and 50 ng/ml human GM-CSF and 20 ng/ml human IL-4 (both from PeproTech Asia, Rehovot, Israel). Medium was replaced every 2–3 days.

Western Blotting

Monocytes (10^6/ml) were pretreated for 20 min with VB-201 or VB-703 followed by 15 min activation with the TLR-4 agonist LPS (100 ng/ml) (Sigma) or the TLR-2 agonist PGN (10 μg/ml) (InvivoGen). Cells were washed and resuspended in lysis buffer containing 1:100 dithiothreitol (DTT), phosphatase, and protease inhibitors (Thermo Scientific). Samples were loaded onto a precast Criterion TGX gel (Bio-Rad, Hemel Hempstead, UK) and transferred onto nitrocellulose membrane. Blots were blocked with 5 % milk or BSA in tris-buffered saline and Tween 20 (TBST) for 1 h, followed by incubation with primary and secondary antibodies. Membranes were developed using an ECL kit (Thermo Scientific). The following antibodies were used for immunoblotting.

Primary Antibodies

p-p38 (cat. no. 4511; 1:1000) and p-IKK (cat. no. 2697; 1:1000) from Cell Signaling Technology (Danvers, MA, USA); tubulin (cat.no. T9026; 1:5000) and p-ERK (cat. no. M8159; 1:10 000) from Sigma (Israel); and heat shock protein (HSP) 90 (cat. no. 13119; 1:500) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) were used as primary antibodies.

Secondary Antibodies

HRP donkey anti-rabbit (1:5000) and HRP goat anti-mouse (1:3000) from Jackson ImmunoResearch (West Grove, PA, USA) were used as secondary antibodies.

ELISA

To measure the effect of VB-201 and VB-703 on IL-12/23p40 and IL-6 production, cells were collected 5–6 days post-culture, counted, and seeded (10^6/ml). Cells were pretreated for 1 h with VB-201 or VB-703 followed by 24-h activation with 100 ng/ml LPS to induce cytokine production. IL-12/23p40 and IL-6 concentration in supernatant was measured by ELISA. Cells activated with solvent (0.5 % ethanol in PBS) were used as control.

In Vitro Cell Migration Assay

Human monocytes were pre-incubated for 20 min with solvent (0.5 % ethanol/PBS), VB-201, or VB-703 as indicated. RANTES (100 ng/ml) and MCP-1 (100 ng/ml) were dissolved in 0.5 % FBS/RPMI-1640 and placed in the lower chamber of a QCM 24-well migration assay plate (Corning Costar; 5-mm pores). Migration assay was conducted by seeding 300,000 treated cells in the upper chamber, followed by incubation for 3 h. The number of cells that migrated into the medium in the lower compartment was determined by flow cytometry (BD FACSCalibur).

Induction of NASH and Treatment

In humans, diabetes mellitus is a risk factor for liver fibrosis, which in some cases may culminate in hepatocellular carcinoma; NASH is critical to the link between diabetes and liver fibrosis. The NASH model used in this study, which enables to assess the histopathological events that lead from diabetes to liver fibrosis, was induced in C57BL/6 male mice (Japan SLC Inc) by a single

subcutaneous injection of 200 μg streptozotocin solution (STZ, Sigma-Aldrich, USA) two days after birth and a high-fat diet (HFD, 57 kcal% fat, cat# HFD32, CLEA Japan, Japan) after 4 weeks of age. The injection of STZ induces inflammation in pancreatic islets that drives β-cell injury, leading to diabetic conditions. The subsequent, high-fat diet promotes liver steatosis and the recruitment and activation of macrophages in the liver, similar to what is seen in human NASH. At Week 6, after steatosis was established, solvent ($n = 8$), VB-201 ($n = 8$), VB-703 ($n = 8$), or telmisartan ($n = 8$) were administered by oral gavage from Week 6 for three more weeks. VB-201 and VB-703 were given at a dose of 4 mg/kg once daily. Telmisartan, which attenuates steatohepatitis progression, was administered at a dose of 10 mg/kg once daily and used as positive control. Normal mice ($n = 5$) were fed a normal diet without any treatment until 9 weeks of age.

Measurement of Whole Blood and Plasma Biochemistry

Nonfasting blood glucose was measured in whole blood using LIFE CHECK (EIDIA, Japan). For plasma biochemistry, blood was collected in polypropylene tubes containing anticoagulant (Novo-Heparin, Mochida Pharmaceutical, Japan) and centrifuged at $1000\times g$ for 15 min at 4 °C. The supernatant was collected and stored at −80 °C until use. Plasma alanine transaminase (ALT) levels were measured by FUJI DRI-CHEM 7000 (Fujifilm, Japan).

Measurement of Liver Triglyceride Content

Liver total lipid extracts were obtained by Folch's method [22]. Liver samples were homogenized in chloroform–methanol (2:1, v/v) and incubated overnight at room temperature. After washing with chloroform–methanol–water (8:4:3, v/v/v), the extracts were evaporated to dryness and dissolved in isopropanol. Liver triglyceride contents were measured by Triglyceride E-test (Wako Pure Chemical Industries, Japan).

Diagnosis and Scoring of Steatosis, NASH, and Liver Fibrosis

The expression level of inflammation mediators associated with steatohepatitis was used to determine NASH severity. To that end, RNA was prepared from livers using RNeasy mini kit (Qiagen). For cDNA preparation, 2 μg of RNA was mixed with qScript reaction mix and qScript reverse transcriptase (Quanta BioSciences) for 5 min at 22 °C and then for 30 min at 42 °C. Reaction was completed by incubating for an additional 5 min at 85 °C. All real-time PCR were performed using the 7300 Real-Time PCR System (Applied Biosystems). Q-PCR was performed with sets of probes with primers for mouse IL-1β, IL-6, IL-12/23p40, and MCP-1 (Applied Biosystems). GAPDH was used to normalize RNA levels. To assess steatosis and liver fibrosis, sections were cut from paraffin blocks of liver tissue prefixed in Bouin's solution (Wako Pure Chemical Industries). Steatosis score was calculated according to the criteria of Kleiner [23]. Coverage of collagen deposition in the liver was used as a marker to evaluate extent of fibrosis. To visualize collagen deposition, Bouin's fixed liver sections were stained using picro-Sirius red solution (Waldeck, Germany). For quantitative analysis, bright field images of Sirius red-stained sections were captured around the central vein using a digital camera (DFC280; Leica, Germany) at a 200-fold magnification, and the positive areas in five fields/section were measured using ImageJ software (National Institute of Health, USA).

Statistical Analyses

Statistical analyses for in vivo studies were performed using Bonferroni's multiple comparison test on GraphPad Prism 4 (GraphPad Software, USA). p values <0.05 were considered statistically significant. A trend or tendency was assumed when a one-tailed t test returned p values <0.05. Results were expressed as mean ± SD. Student's t test was performed for the in vitro studies. p values ≤ 0.05 were considered statistically significant.

Results

Effect of Lecinoxoids on TLR-2 and TLR-4 Activation and on Chemokine-Induced Migration

We first compared the inhibitory effect of VB-201 on TLR-2- and TLR-4-mediated activation and chemokine-induced migration of human monocytes to its derivative VB-703. The results demonstrate that VB-703 inhibits TLR-4-mediated signaling events and cytokine production with a profoundly higher degree of activity than VB-201 (Fig. 2a, b) but similar to VB-201's inhibitory effect on TLR-2-mediated phosphorylation (Fig. 2c). Moreover, VB-703 showed annulled activity in the case of monocyte migration (Fig. 2d).

Effect of Lecinoxoids Treatment on Pathophysiological Characteristics of NASH-Induced Mice

Next, we assessed the effect of VB-201 and VB-703 on the development of steatosis, steatohepatitis and liver fibrosis

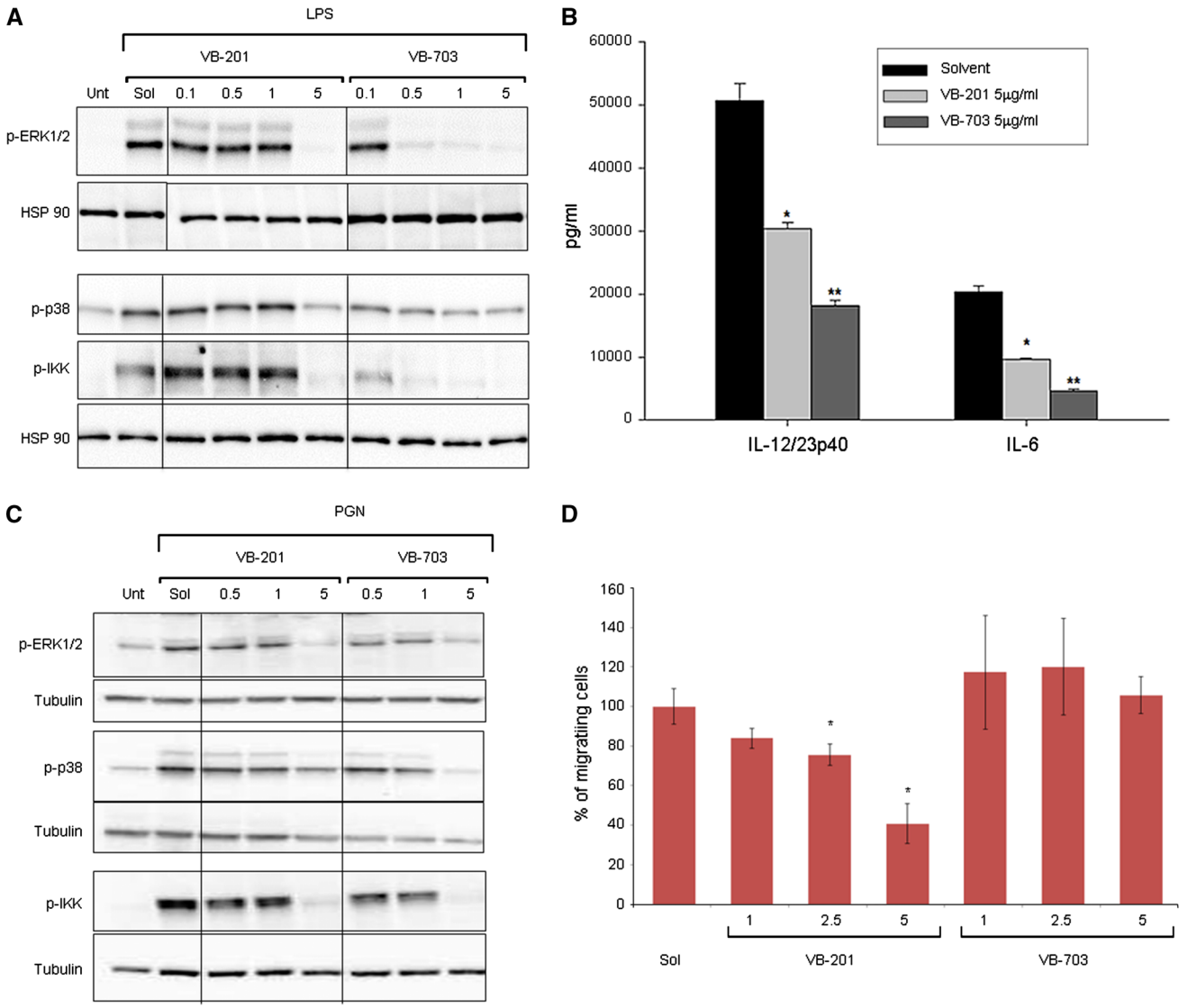

Fig. 2 VB-201 and VB-703 differentially affect TLR-4-mediated activation and migration of myeloid cells. Human monocytes (**a**, **c**) and human monocyte-derived dendritic cells (**b**) were pretreated at the indicated VB-201 or VB-703 concentrations (µg/ml) for 20 min and then activated with (**a**, **b**) TLR-4 or (**c**) TLR-2 agonists LPS and PGN, respectively. Samples were analyzed by Western blots and ELISA for inhibition of downstream phosphorylation event and cytokine production. Tubulin or HSP 90 was used in Western blotting for loading control. One out of at least three experiments is shown. For **b**, data are mean ± SD from triplicates. $*p < 0.001$ versus solvent; $**p < 0.001$ versus solvent and VB-201 (**d**) Human monocytes were pre-incubated for 20 min with solvent, VB-201, or VB-703 at the indicated concentrations (µg/ml) and then seeded in the upper part of a trans-well migration assay plate. RANTES (100 ng/ml) and MCP-1 (100 ng/ml) were placed in the lower chamber of the wells for 3 h. The number of cells that migrated into the medium in the lower compartment was determined by flow cytometry (BD FACSCalibur). $*p < 0.05$ versus solvent. Data are mean ± SD from triplicates, normalized to solvent-treated cells. One out three experiments is shown

in a NASH mouse model, using telmisartan as standard of care. Mean body weight of the solvent group at experiment conclusion was lower than that of the normal group. Mean body weight of the telmisartan group was significantly lower than that of the solvent group. No significant differences were observed in mean body weight between the solvent or the telmisartan group and the groups treated with VB-201 or VB-703. Nevertheless, the solvent group exhibited a significant increase in mean liver weight compared with the normal group. The telmisartan group showed a significant decrease in mean liver weight compared with the solvent group. There were no significant differences in mean liver weight between the solvent group and the groups treated with VB-201 or VB-703.

Table 1 Effect of treatment regiments on pathophysiological characteristics of NASH-induced mice

	Normal	Solvent	VB-201	VB-703	Telmisartan
Body weight (g)	21.32 ± 0.10	18.77 ± 0.70	18.15 ± 0.70	18.74 ± 0.96	15.2 ± 0.63*
Liver weight (mg)	844.20 ± 45.77	1267.37 ± 37.33[a]	1366.75 ± 79.98	1341.00 ± 66.05	986.38 ± 46.48*
Whole blood glucose (mg/dL)	144.20 ± 9.35	709.00 ± 58.34[a]	628.62 ± 42.25	648.70 ± 40.06	751.00 ± 55.28
Plasma ALT (U/L)	21.20 ± 5.31	62.00 ± 20.95	41.00 ± 5.40	32.60 ± 3.36	34.25 ± 2.90
Liver Tg (mg/g liver)	1.98 ± 0.20	38.27 ± 5.15[a]	40.02 ± 4.27	31.20 ± 3.07	12.48 ± 1.54**
Steatosis score	0	1.25 ± 0.20	1.00 ± 0.19	1.00 ± 0.31	0.25 ± 0.16**

All data presented as mean ± SD. Normal $n = 5$, solvent $n = 8$, VB-201 $n = 8$, VB-703 $n = 8$, telmisartan $n = 8$

* $p < 0.01$ telmisartan versus solvent

** $p < 0.001$ telmisartan versus solvent

[a] $p < 0.001$ solvent versus normal

Furthermore, we found that solvent-treated mice had elevated blood glucose and plasma ALT levels, and that treatment with lecinoxoids or telmisartan did not affect these parameters. Next, we sought to determine whether lecinoxoids can decrease liver fat content in NAFLD. The results show that solvent-treated mice had significantly increased liver triglyceride content compared with the normal group, and that while telmisartan treatment

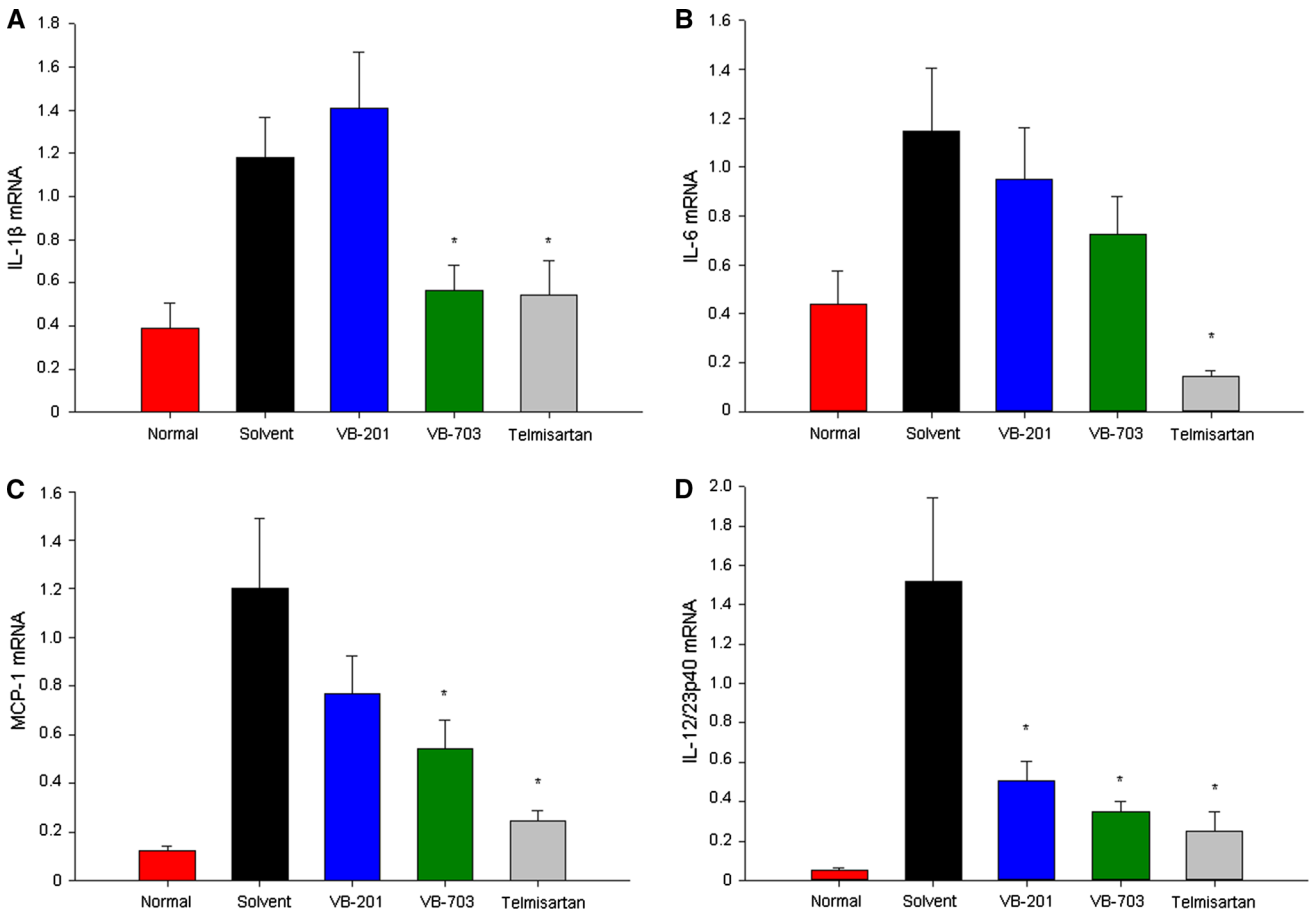

Fig. 3 VB-703 inhibits steatohepatitis. Mice were induced for NASH, and the indicated compounds were administered orally at a dose of 4 mg/kg once daily from Week 6 to Week 9. Telmisartan was administered at a dose of 10 mg/kg once daily. Q-PCR was performed on RNA extracted from livers of mice on Week 9 to detect IL-1β (**a**), IL-6 (**b**), MCP-1 (**c**), and IL-12/23p40 (**d**). GAPDH was used to normalize RNA levels. Normal $n = 4$, solvent $n = 7–8$, VB-201 $n = 7–8$, VB-703 $n = 8$, telmisartan $n = 5–6$. Data are mean ± SE from triplicates. *$p \leq 0.05$

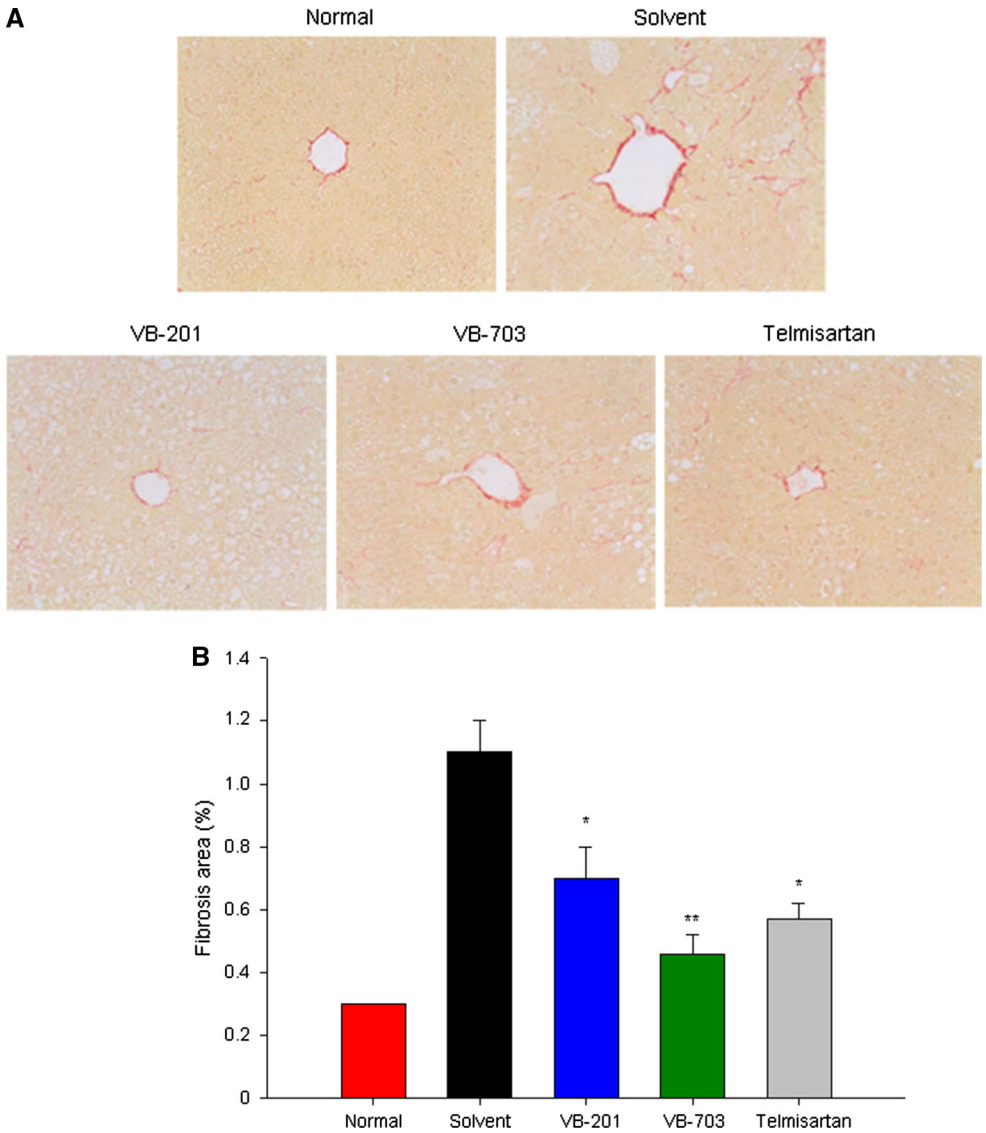

Fig. 4 VB-201, and more profoundly VB-703, restricts liver fibrosis. Mice were induced for NASH, and the indicated compounds were administered orally at a dose of 4 mg/kg once daily from Week 6 to Week 9. Telmisartan was administered at a dose of 10 mg/kg once daily. Liver sections prepared at the end of the experiment were stained with picro-Sirius red to visualize collagen deposition. **a** Representative stain of each group is shown. **b** Percent of fibrosis for each tested group. Normal $n = 5$, solvent $n = 8$, VB-201 $n = 8$, VB-703 $n = 8$, telmisartan $n = 6$. Data are mean ± SE. $^{*}p \leq 0.05$, $^{**}p < 0.001$. One out of two experiments is shown

significantly decreases liver triglycerides, the administration of lecinoxoids had no apparent effect (Table 1). In accord with the latter result, telmisartan significantly reduced steatosis, whereas treatment with lecinoxoids had no effect on steatosis (Table 1).

VB-201 and VB-703 Effect on Steatohepatitis

We now examined the effect of each of the lecinoxoids on NASH, where NASH is defined as increased expression levels of inflammation mediators in the liver compared with normal mice. For that, upon killing, RNA was prepared from livers and the differences between tested groups in the expression of inflammation mediators associated with steatohepatitis were determined by quantitative PCR. Analysis of inflammation mediators IL-1β, IL-6, MCP-1, and IL-12/23p40 in the liver of NASH-induced mice showed that VB-703 for the most part significantly inhibited expression of pro-inflammatory mediators, whereas VB-201 significantly attenuated only the expression of IL-12/23p40 (Figs. 3a–d).

Lecinoxoids Restrict Liver Fibrosis

In order to evaluate whether treatment with lecinoxoids restricts liver fibrosis, liver samples were stained with

Sirius red to assess collagen content, as a marker for the presence of fibrogenesis. The extent of fibrosis was determined by calculating the percent of positively stained area in five fields within a section. The results, presented in Fig. 4a, b, demonstrate that VB-703, more than VB-201 and even telmisartan, significantly restricted the development of liver fibrosis.

Discussion

NAFLD is a progressive disease that begins with a fatty liver and that, if left untreated, may advance to liver inflammation and in few cases might culminate in liver fibrosis. The majority of drugs currently tested in clinical trials focus on targeting molecules that are involved in the regulation of lipid and glucose metabolism. These drugs include agonists of nuclear receptors, such as FXR and PPAR-α that induce anti-steatotic responses in NASH patients [24], a niacin analog that reduces triglycerides levels, and a glucagon-like peptide-1 receptor agonist that induces insulin secretion. In this study, we evaluated an alternative approach according to which oxidized phospholipid small molecules are used to target pathways directly associated with liver inflammation and fibrosis for treating NASH and ensuing liver fibrosis. Two major stages were suggested to promote NASH and liver scarring: the recruitment of circulating monocytes into the fatty liver and activation of these monocytes, together with resident Kupffer and stellate cells, through TLR-2 and TLR-4. In virtue of the biological function of the lecinoxoids, treatment of mice with VB-201 or VB-703 did not alter steatosis. However, when indices directly associated with inflammation and fibrosis were analyzed, differences in the efficacies of VB-201 and VB-703 were revealed. Functionally, VB-201 differs from VB-703 in its ability to also inhibit monocytes migration, but it is a weaker antagonist of TLR-4 than is VB-703. Nevertheless, our results demonstrate that significant effects of lecinoxoids on liver inflammation could be attained by targeting TLR-2 and predominantly TLR-4, since VB-703 was superior to VB-201 in inhibiting all of the inflammation mediators tested. Although VB-201 showed a weaker influence than VB-703 on steatohepatitis, these results do not exclude the therapeutic role VB-201 may have on fibrosis. Indeed, limiting monocytes migration to ameliorate liver fibrosis has already been shown with cenicriviroc mesylate, a CCR2 and CCR5 antagonist, which reduced fibrosis by 50–65 % both in a rat thioacetamide model and in a mouse NASH model and is currently undergoing evaluation in a phase II clinical study in patients with liver fibrosis. Future efficacy studies either in animal models or in clinical practice should therefore include test compounds that are preferentially strong antagonists to TLR-4 or combination therapies to decrease monocytes migration and TLR-4 activation to treat both steatohepatitis and liver fibrosis. In this study, we did not test the effect of VB-201 and VB-703 at the cellular level, in the sense that hepatic cells involved in NASH and liver fibrosis such as HSC and Kupffer cells were not isolated and analyzed for their response in the presence of VB-201 and VB-703. Moreover, in this model, the extent of fibrosis reached in the control group was relatively low. Accordingly, in vitro studies are currently underway to test the effect of these and other lecinoxoids on pure resident liver macrophages and HSC, alongside in vivo experiments in other liver fibrosis models. To conclude, small molecules oxidized phospholipids that strongly antagonize TLR-4 and inhibit monocytes migration should be further explored for their potential to treat subjects with NASH and liver fibrosis.

Compliance with ethical standards

Conflict of interest The authors are employees and stock option holders of VBL Therapeutics.

References

1. Miele L, Valenza V, La Torre G, et al. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. *Hepatology*. 2009;49:1877–1887.
2. Compare D, Coccoli P, Rocco A, et al. Gut–liver axis: the impact of gut microbiota on non alcoholic fatty liver disease. *NMCD*. 2012;22:471–476.
3. Spencer MD, Hamp TJ, Reid RW, Fischer LM, Zeisel SH, Fodor AA. Association between composition of the human gastrointestinal microbiome and development of fatty liver with choline deficiency. *Gastroenterology*. 2011;140:976–986.
4. Zhu L, Baker SS, Gill C, et al. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. *Hepatology*. 2013;57:601–609.
5. Zeisel SH, Wishnok JS, Blusztajn JK. Formation of methylamines from ingested choline and lecithin. *J Pharmacol Exp Ther*. 1983;225:320–324.
6. Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. *Nature*. 2012;489:242–249.
7. Henao-Mejia J, Elinav E, Jin C, et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature*. 2012;482:179–185.
8. Roh YS, Seki E. Toll-like receptors in alcoholic liver disease, non-alcoholic steatohepatitis and carcinogenesis. *J Gastroenterol Hepatol*. 2013;28:38–42.

9. Blasius AL, Beutler B. Intracellular toll-like receptors. *Immunity*. 2010;32:305–315.
10. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol*. 2010;11:373–384.
11. Ehses JA, Meier DT, Wueest S, et al. Toll-like receptor 2-deficient mice are protected from insulin resistance and beta cell dysfunction induced by a high-fat diet. *Diabetologia*. 2010;53: 1795–1806.
12. Miura K, Yang L, van Rooijen N, Brenner DA, Ohnishi H, Seki E. Toll-like receptor 2 and palmitic acid cooperatively contribute to the development of nonalcoholic steatohepatitis through inflammasome activation in mice. *Hepatology*. 2013;57:577–589.
13. Csak T, Velayudham A, Hritz I, et al. Deficiency in myeloid differentiation factor-2 and toll-like receptor 4 expression attenuates nonalcoholic steatohepatitis and fibrosis in mice. *Am J Physiol Gastrointest Liver Physiol*. 2011;300:G433–G441.
14. Rivera CA, Adegboyega P, van Rooijen N, Tagalicud A, Allman M, Wallace M. Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. *J Hepatol*. 2007;47:571–579.
15. Paik YH, Schwabe RF, Bataller R, Russo MP, Jobin C, Brenner DA. Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. *Hepatology*. 2003;37:1043–1055.
16. Schwabe RF, Seki E, Brenner DA. Toll-like receptor signaling in the liver. *Gastroenterology*. 2006;130:1886–1900.
17. Thobe BM, Frink M, Hildebrand F, et al. The role of MAPK in Kupffer cell toll-like receptor (TLR) 2-, TLR4-, and TLR9-mediated signaling following trauma-hemorrhage. *J Cell Physiol*. 2007;210:667–675.
18. Seki E, Tsutsui H, Nakano H, et al. Lipopolysaccharide-induced IL-18 secretion from murine Kupffer cells independently of myeloid differentiation factor 88 that is critically involved in induction of production of IL-12 and IL-1beta. *J Immunol*. 2001;166:2651–2657.
19. Karlmark KR, Weiskirchen R, Zimmermann HW, et al. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology*. 2009;50: 261–274.
20. Mendel I, Feige E, Yacov N, et al. VB-201, an oxidized phospholipid small molecule, inhibits CD14- and Toll-like receptor-2-dependent innate cell activation and constrains atherosclerosis. *Clin Exp Immunol*. 2014;175:126–137.
21. Feige E, Yacov N, Salem Y, et al. Inhibition of monocyte chemotaxis by VB-201, a small molecule lecinoxoid, hinders atherosclerosis development in ApoE(-)/(-) mice. *Atherosclerosis*. 2013;229:430–439.
22. Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*. 1957;226:497–509.
23. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005;41:1313–1321.
24. Ratziu V, Pienar L. Pharmacological therapy for non-alcoholic steatohepatitis: how efficient are thiazolidinediones? *Hepatol Res*. 2011;41:687–695.