TERUTROBAN, A TP-RECEPTOR ANTAGONIST, REDUCES PORTAL PRESSURE IN CIRRHOTIC RATS.

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Abbreviations

COX, cyclooxygenase; TXA_2, thromboxane; NO, nitric oxide; BDL, bile duct ligation; CCl_4, carbon tetrachloride; TP, Thromboxane-A_2/Prostaglandin endoperoxide; SEC, sinusoidal endothelial cells; HSC, hepatic stellate cells; TXAS, thromboxane synthase; eNOS, endothelial nitric oxide synthase.

Conflict of interest:

The authors confirm that there are no conflicts of interest.

ABSTRACT

An increased production of vasoconstrictive prostanoids, such as thromboxane A_2 (TXA_2), contributes to endothelial dysfunction and increased hepatic vascular tone in cirrhosis. TXA_2 induces vasoconstriction via activation of the Thromboxane-A_2/prostaglandin-endoperoxide (TP) receptor. This study investigates whether Terutroban, a specific TP receptor blocker, decreases hepatic vascular tone and portal pressure in rats with cirrhosis due to carbon tetrachloride (CCl_4) or bile duct ligation (BDL). Hepatic and systemic hemodynamics, endothelial dysfunction, liver fibrosis, hepatic Rho-kinase activity (a marker of hepatic stellate cell contraction), and the eNOS signaling pathway were measured in CCl_4 and BDL cirrhotic rats treated with Terutroban (30 mg/kg/day) or its vehicle for two weeks. Terutroban reduced portal pressure in both models without producing significant changes in portal blood flow, suggesting a reduction in hepatic vascular resistance. Terutroban did not significantly change arterial pressure in CCl_4-cirrhotic rats but decreased it significantly in BDL-cirrhotic rats. In livers from CCl_4 and BDL-cirrhotic Terutroban-treated rats, endothelial dysfunction was improved and Rho-kinase activity was significantly reduced. In CCl_4-cirrhotic rats, Terutroban reduced liver fibrosis and decreased α-SMA, collagen-I and TGF-β mRNA expression without significant changes in eNOS pathway. In contrast, no change in liver fibrosis was observed in BDL-cirrhotic rats but an increase in the eNOS pathway. **Conclusion:** Our data indicate that TP-receptor blockade with Terutroban decreases portal pressure in cirrhosis. This effect is due to decreased hepatic resistance, which in CCl_4-cirrhotic rats was linked to decreased hepatic fibrosis,

but not in BDL rats, in which the main mediator appeared to be an enhanced eNOS-dependent vasodilatation, which was not liver selective, as it was associated with decreased arterial pressure. The potential use of Terutroban for portal hypertension requires further investigation.

INTRODUCTION

In cirrhotic livers, increased resistance to portal blood flow resulting from architectural alterations of the liver parenchyma as well as from increased hepatic vascular tone is the primary factor in the pathophysiology of portal hypertension (1, 2). Increased hepatic vascular tone is partly due to an increased production of cyclooxygenase-1 (COX-1)-derived vasoconstrictive prostanoids, such as thromboxane (TXA_2) (3, 4) together with an insufficient intrahepatic availability of the vasodilator nitric oxide (NO) (5, 6).

We have previously demonstrated that, in isolated perfused cirrhotic livers, the blockade of the TXA_2/PGH_2 (TP) receptor with SQ29548 corrected the hyperresponse to methoxamine (3) and improved endothelial dysfunction (4) of the hepatic vascular bed. Moreover, sinusoidal endothelial cells (SEC) isolated from cirrhotic rats overexpress COX-1 (7) and thromboxane synthase (TXAS) (8), which represent an important source of vasoconstrictor prostanoids, such as TXA_2 (9). Importantly, COX inhibition not only reduces the exaggerated TXA_2 production of cirrhotic SEC but also restores, at least in part, its decreased NO bioavailability (8).

TP receptor ligands include TXA_2, PGH_2 and isoprostanes (10, 11). TXA_2 acts through its G-protein-coupled receptor leading to vasoconstriction by activating the RhoA/Rho-kinase pathway, and by increasing calcium levels in hepatic stellate cells (HSC) (12). Terutroban is an orally active, specific antagonist of the TP-receptor (13) that improves endothelial-dependent vasodilation (14), reduces inflammation (15), attenuates oxidative stress and exerts antifibrotic effects (16, 17) in different vascular disorders. In addition, Terutroban has been

shown to reduce RhoA/Rho-kinase-dependent signaling and restore NO

bioavailability in endotelial cells (18, 19).

The current study aimed at evaluating the long-term effects of the *in vivo*

blockade of TP receptor with Terutroban in two experimental rat models of

cirrhosis, carbon tetrachloride (CCl_4) and bile duct ligation (BDL).

MATERIALS AND METHODS

Induction of cirrhosis by carbon tetrachloride (CCl$_4$)

Male Wistar rats weighing 50 to 75g underwent inhalation exposure to CCl$_4$ three times a week as previously described (20). A high yield of micronodular cirrhosis was obtained after approximately 12 to 15 weeks of CCl$_4$ inhalation. When the cirrhotic rats developed ascites, administration of CCl$_4$ was stopped.

Induction of cirrhosis by bile duct ligation (BDL)

Secondary biliary cirrhosis was induced in male Sprague-Dawley rats (200 to 225 g) by BDL as previously described (21).

Terutroban treatment

Cirrhotic rats were randomized to receive Terutroban (30mg/Kg; n= 13 in CCl$_4$; n=14 in BDL; kindly supplied by Servier, Courbevoie Cedex, France) or its vehicle (1% hydroxyethylcellulose; n=8 in CCl$_4$; n=16 in BDL; Sigma-Aldrich, Tres Cantos, Madrid, Spain), administered orally by *gavage*, once a day, for 2 weeks. Treatment started one week after development of ascitis and stopping CCl$_4$ administration in a setting of advanced cirrhosis or after two weeks of BDL, in a precirrhotic stage. Experiments were performed 1 hour after the last dose of Terutroban or vehicle. Treatments were prepared by a third person and experimental studies were realized blindly. The code was kept sealed until the final analysis of the results. The dose of Terutroban used has been previously shown to have antivasoconstricting and antiatherosclerotic properties (16, 22, 23).

The animals were kept in environmentally controlled animal facilities at the Institut d'Investigacions Biomèdiques August Pi i Sunyer. All procedures were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with European Community guidelines for the protection of animals used for experimental and other scientific purposes *(EEC Directive 86/609).*

In vivo hemodynamic studies

Cirrhotic rats were anesthetised with intraperitoneal ketamine hydrochloride (100 mg/Kg; Merial Laboratories, Barcelona, Spain) plus midazolam (5 mg/kg intraperitoneally; Laboratorios Reig Jofré, Barcelona, Spain). The femoral artery and the ileocolic vein were cannulated with PE-50 catheters to measure mean arterial pressure (MAP; mmHg) and portal pressure (PP; mmHg) respectively. Perivascular ultrasonic transit-time flow probes connected to a flow meter (Transonic Systems Inc., Ithaca, NY, USA) were placed around the portal vein, as close as possible to the liver to measure portal blood flow perfusing the liver (PBF; mL/min/g liver) and around the superior mesenteric artery, in BDL cirrhotic rats, to measure superior mesenteric artery blood flow (SMABF, mL/minute/100g body weight). Hepatic vascular resistance (HVR, mmHg/mL/min/g liver) was calculated as: PP/PBF and superior mesenteric artery resistance (SMAR, mmHg/mL/min/100g body weight) was calculated as (MAP-PP)/SMABF. Blood pressures and flows were registered on a multichannel computer-based recorder (PowerLab; AD Instruments, Colorado Springs, CO). The temperature of the animals was maintained at 37± 0.5°C. Hemodynamic data were collected after a 20 min stabilization period.

Efficacy of TP receptor blockade

To determine if Terutroban correctly blocked the TP receptor, in a subgroup of CCl_4 and BDL cirrhotic rats (n=3) treated with Terutroban (30mg/kg) or vehicle for 2 weeks, measurements of MAP and PP were performed before and after the intravenous infusion of 10 µg/Kg U46619 (24). U46619 (9,11-dideoxy-$9\alpha,11\alpha$-methanoepoxy-prosta-5Z,13E-dien-1-oic acid; Cayman Chem. Co.) is a synthetic TXA_2 analogue that specifically activates the TP-receptor.

Evaluation of endothelial function

An additional group of cirrhotic rats were randomized to receive Terutroban (30 mg/Kg; n= 8 in CCl_4; n =8 in BDL) or vehicle (n= 9 in CCl_4; n =9 in BDL) for three days. Rats were anesthetized and livers were quickly isolated and perfused by a flow-controlled perfusion system as previously described (25). The perfused rat liver preparation was allowed to stabilize for 20 min before vasoactive substances were added. The intrahepatic microcirculation was preconstricted by adding the α1-adrenergic agonist methoxamine (Mtx; 10^{-4} mol/L; Sigma) to the reservoir. After 5 min, concentration–response curves to cumulative doses of acetylcholine (Ach; 10^{-7}, 10^{-6}, and 10^{-5} mol/L; Sigma) were evaluated. Responses to Ach were calculated as per cent change in portal perfusion pressure (26). The gross appearance of the liver, stable perfusion pressure, bile production over 0.4 ul/min/g of liver and a stable buffer pH (7.4 ± 0.1) were monitored during this period. If any viability criteria were not satisfied, the experiment was discarded.

Biochemical analysis

At the end of the *in vivo* hemodynamic study, serum samples from cirrhotic rats were collected by cardiac puncture to subsequently evaluate alanine aminotransferase (ALT), aspartate aminoransferase (AST), bilirubin and albumin, all by standard protocols.

Assesment of Rho-kinase activity

Hepatic samples were obtained as previously described (27). Rho-kinase activity was assessed by the phosphorylation of the endogenous Rho-kinase substrate, moesin at Thr[558] normalized to the level of total moesin expression. Moesin-phosphorylation and moesin total expression were assessed by Western Blot using a goat antiphosphorylated moesin at Thr[558] antibody (1:200; Santa Cruz Biotechnology, California, USA) and a mouse antibody recognizing moesin (1:200; Santa Cruz Biotechnology) overnight at 4°C followed by incubation with their associated horseradish peroxidase–conjugated secondary antibody (1:10000, 1 hour, room temperature, Stressgen Victoria, British Columbia, Canada). After stripping, blots were assayed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression as standardization of sample loading.

Evaluation of NO pathway

Western blot analysis of eNOS-phosphorylation and eNOS total protein expression

eNOS-phosphorylation (eNOS-P) and eNOS total expression were assessed in hepatic homogenates using a rabbit antiphosphorylated eNOS at Ser[1176]

(1:1000; Cell Signaling Technology Beverly, MA) and a mouse antibody recognizing eNOS (1:1000; BD Transduction Laboratories, Lexington, KY). Quantitative densitometric values of proteins were normalized to GAPDH.

cGMP levels

Measurements of cGMP, a secondary marker of NO bioavailability, were performed in rat liver homogenates treated with Terutroban or vehicle by enzyme immunoassay (Cayman Chem. Co. Tallin, Estonia) as previously described (28).

Evaluation of hepatic fibrosis

Quantification of hepatic fibrosis

Livers from cirrhotic rats were fixed in 10% formalin, embedded in paraffin, sectioned and stained with 0.1% Sirius red, photographed, and analyzed using a microscope equipped with a digital camera. Eight fields from each slide were randomly selected, and the red-stained area per total area was measured using AxioVision software {Rodriguez-Vilarrupla, 2012 4577 /id}. Values are expressed as the mean of 8 fields taken from Vehicle- (n=9) and Terutroban- (n=11) CCl_4-cirrhotic rats or n=10 animals *per* group in the BDL model.

Protein expression of α-smooth muscle actin (α-SMA)

Hepatic protein expression of α-SMA was determined by Western blot in hepatic samples using a mouse antibody against α-SMA (1:1000, Sigma-Aldrich).

Collagen I and TGF-β gene expression

Hepatic mRNA expression of Collagen I and TGF-*β* was analyzed by real-time PCR using predesigned gene expression assays obtained from Applied

Biosystems (AB, Foster City, CA) according to the manufacturer's protocol and reported relative to endogenous control GAPDH. All PCR reactions were performed in duplicate and using nuclease-free water as no template control.

Analysis of TP receptor expression in HSC

To directly study the expression of TP receptor, hepatic stellate cells (HSC) were isolated from control, CCl_4-, sham operated and BDL- rats as previously described (29). In the CCl_4 model, HSC were isolated one week after development of ascitis and in the BDL model two weeks after surgery (when Terutroban treatment was initiated in the *in vivo* studies). TP receptor protein expression was determined by Western Blot in hepatic samples using a mouse antibody against TP receptor (1:1000; Cayman Chem Co.).

Statistical analysis

Statistical analysis was performed with the SPSS 18.0 for Windows statistical package (IBM Corp., Armonk, NY). All results are expressed as mean ± SEM. Comparisons between groups were performed with the Student *t* test for unpaired data or with Mann-Whitney test when assumptions of normality could not be verified. Differences were considered significant at a *P* value < 0.05.

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RESULTS

Efficacy of TP receptor blockade in CCl$_4$ and BDL cirrhotic rats

As expected, infusion of U46619 produced a significant increase in MAP (23 ± 13%) and PP (11 ± 5%) in CCl$_4$-cirrhotic rats treated with vehicle (Fig 1A and 1B, black bars). By contrast, in CCl$_4$-cirrhotic rats treated with Terutroban, the increase of MAP (3 ± 5%) and PP (2 ± 3%) in response to TP agonist was markedly attenuated, indicating an effective blockade of the TP-receptor (Fig 1A and 1B, white bars). Terutroban produced a similar blockade of the TP-receptor in BDL-cirrhotic rats, as shown by the attenuation of the increase in MAP (5 ± 3% vs 18 ± 8% in vehicle) and in PP (3 ± 3% vs 12 ± 3% in vehicle) caused by U46619 (Fig 1D, 1E).

TP receptor protein expression in CCl$_4$ and BDL cirrhotic rats

TP receptor expression was determined in HSC from control, CCl$_4$- (Fig 1C) and BDL- cirrhotic rats (Fig.1F). Both cirrhotic models exhibited a significantly higher TP receptor expression compared to control rats.

Effects of chronic treatment with Terutroban in CCl$_4$-cirrhotic rats

TP receptor blockade lowers portal pressure in CCl$_4$-cirrhotic rats

PP was significantly lower in CCl$_4$-cirrhotic rats treated with Terutroban (11.9 ± 2.8 mmHg) as compared with vehicle-treated rats (14.5 ± 1.4 mmHg) (mean difference -17.9%; p=0.035). This reduction was not associated with a significant change in PBF reflecting a fall in HVR (7.9 ± 2.6 vs 10.3 ± 2.9 mmHg/ml/min/g in vehicle-treated rats) (mean decrease 25%; p=0.047). MAP was not significantly reduced by Terutroban (Fig 2).

TP receptor blockade attenuates Rho-kinase activity in CCl$_4$-cirrhotic rats

To further explore the intrahepatic molecular mechanisms behind TP receptor blockade, we evaluated moesin phosphorylation in hepatic samples, a marker of Rho kinase activity. TP receptor blockade with Terutroban reduced hepatic moesin phosphorylation indicating a reduction in Rho-kinase activity (Fig 3B). Since RhoA/Rho-kinase may modulate eNOS activity, we characterized eNOS expression and phosphorylation. However, Terutroban administration did not modify eNOS phosphorylation at Ser^{1176} (Fig 3C), total eNOS expression (Fig 3D) or hepatic cGMP levels (18.3 ± 2.9 pmol/ml vs 19.2 ± 3.4 pmol/ml in vehicle-treated rats) (Fig 3E).

TP receptor blockade attenuates hepatic fibrosis in CCl$_4$-cirrhotic rats

As expected, CCl$_4$-cirrhotic rats exhibited a marked distortion of the normal liver architecture, as identified by staining of liver sections with Sirius Red. Terutroban treatment produced a significant reduction in hepatic fibrosis, measured by the percentage of fibrosis area on Sirius Red stained liver sections (13.7% ± 4 vs 20.8% ± 3 in vehicle-treated rats) (Fig 4A). This was associated with a significant reduction in collagen I mRNA expression (Fig 4B), a marked decrease in α-SMA protein expression, a surrogate marker of HSC activation (Fig 4C), and decreased TGF-β mRNA levels (Fig 4D).

Effects of TP receptor blockade on liver function in CCl$_4$-cirrhotic rats

There were no significant differences in transaminases or bilirubin between CCl$_4$-cirrhotic rats treated with vehicle or Terutroban. However, albumin levels

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were significantly increased in Terutroban-treated rats. Liver, spleen and body weight were not different between groups (Table 1A).

TP receptor blockade improves endothelial dysfunction in CCl_4-cirrhotic rats

Improved vasorelaxation in response to Ach was observed in three days Terutroban-treated rats in comparison to cirrhotic rats treated with vehicle, that exhibited the expected impaired vasodilatory response to Ach (endothelial dysfunction) (Fig 3A). After NO synthase inhibition, Terutroban also improved the vasodilatory response to Ach (Ach 10^{-7} M: -4.3 ± 0.3%; 10^{-6} M: -8.0 ± 1.5%; 10^{-5}M: -14.3±2.4).

Effects of chronic treatment with Terutroban in BDL-cirrhotic rats

TP receptor blockade lowers portal pressure in BDL-cirrhotic rats

BDL cirrhotic rats treated with Terutroban also had a significantly lower PP than those treated with vehicle (15.2 ± 1.9 vs 17.3 ± 2 mmHg; p=0.007; mean difference -12.1%). Reduction in PP was observed without significant changes in PBF supporting a reduction in HVR (17.8 ± 5.2 vs 22.8 ± 3.8 mmHg/ml/min/g; p=0.038; mean decrease 22%). However, BDL rats treated with Terutroban exhibited a significantly lower MAP (70 ± 8 mmHg vs 91 ± 16 mmHg; p<0.05) than those receiving vehicle. As SMABF was similar in both groups, Terutroban produced a significant reduction in splanchnic arteriolar resistance (Fig 5).

TP receptor blockade attenuates Rho-kinase activity and improves NO bioavailability in BDL-cirrhotic rats

Moesin phosphorylation was significantly decreased in livers from Terutroban-treated BDL rats (Fig 6B). Contrary to CCl_4-cirrhotic rats, livers from BDL rats treated with Terutroban exhibited an enhanced eNOS phosphorylation at Ser^{1176} (Fig 6C) and increased total eNOS expression (Fig 6D), together with increased hepatic cGMP levels (7.2 ± 2.7 pmol/ml vs 4.1 ± 2.5 pmol/ml in vehicle-treated rats; $p < 0.05$) (Fig 6E).

TP receptor blockade does not attenuate hepatic fibrosis in BDL-cirrhotic rats

Contrary to CCl_4-cirrhotic rats, Terutroban administration to BDL rats did not reduce liver fibrosis as evaluated by the percentage of Sirius staining (36.9% ± 3.7 vs 34.7% ± 7.5 in vehicle) (Fig 7A), and did not significantly change α-SMA protein expression (Fig 7C), Type I collagen (Fig 7B) or TGF-β mRNA levels (Fig 7D).

Effects of TP receptor blockade on liver function in BDL-cirrhotic rats

There were no significant differences in transaminases, bilirubin or albumin between BDL cirrhotic rats treated with vehicle or Terutroban. Liver, spleen and body weight were not different between groups (Table 1B).

TP receptor blockade improves endothelial dysfunction in BDL-cirrhotic rats

Livers from cirrhotic rats treated with vehicle exhibited an impaired vasodilatory response to Ach. Terutroban treatment significantly improved vasorelaxation in response to Ach (Fig 6A).

DISCUSSION

An increase in the hepatic production of the vasoconstrictor prostanoid TXA_2 has been shown to increase hepatic resistance in cirrhotic livers, contributing to increased portal pressure (3, 20, 30-32). Up to now, *in vivo* efforts to reduce this increased hepatic resistance by reducing TXA_2 levels have been based on treatments with non-selective COX inhibitors (32, 33). However, the strategy to block TXA_2 production by non-selective COX inhibition is not acceptable in cirrhosis due to its demonstrated deleterious effects on sodium and water retention and renal function (34, 35). There are no previous reports of the effects of TP-receptor blockade *in vivo* in cirrhotic animals. We hereby report the effects of Terutroban, a specific TP-receptor blocker, in cirrhotic rats. Terutroban has been extensively used in clinical trials in vascular diseases and proved to be safe (14, 36, 37).

Our study demonstrates that *in vivo* chronic TP-receptor blockade with Terutroban produced a similar reduction in portal pressure in two different models, CCl_4 and BDL. The decrease in portal pressure was not associated with changes in portal blood flow, suggesting a reduction in hepatic vascular resistance. This beneficial effect of Terutroban on hepatic resistance should be attributed, in part, to the blockade of the vasoconstrictor effect of TXA_2 (3, 32). Indeed, the blunted increase in portal pressure after the infusion of the TXA_2 agonist U46619 in Terutroban-treated rats suggests an adequate TP-receptor blockade and inhibition of TXA_2-derived vasoconstriction of HSC and/or vascular smooth muscle cells in the hepatic vasculature. We also characterized the effects of Terutroban on Rho-kinase activity. Rho-kinase, which is activated among other factors by the TP-receptor, is a well known mechanism of HSC

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contraction (18, 38, 39) and it has been recently shown that its inhibition reduces hepatic vascular resistance (40, 41). Our results showing that Terutroban reduces hepatic Rho-kinase activity in both experimental models suggest that this may be an additional mechanism by which Terutroban decreases hepatic vascular resistance.

However, other effects of Terutroban were different according to the cirrhotic rat model. In CCl$_4$-cirrhotic rats, Terutroban ameliorated the architectural abnormalities of the liver, as shown by the reduction in liver fibrosis area on Sirius Red staining. This was associated with a decrease in collagen I mRNA expression, suggesting a reduced collagen synthesis as a consequence of TP-receptor blockade, as it was not observed in vehicle-treated rats. This effect was associated, and probably linked, to a reduction of HSC activation, as suggested by the decreased α–SMA protein expression. It has been suggested that isoprostanes, a natural ligand for TP receptor, have been identified in HSC and mediate HSC proliferation and collagen production (11). Furthermore, Terutroban significantly reduced TGF-β, which is one of the main fibrogenic cytokines that stimulates extracellular matrix deposition (42). These findings are in agreement with previous studies in an animal model of severe arterial hypertension showing that Terutroban was able to prevent fibrosis in the aorta by reducing TGF-β gene expression (16). Thus, in CCl$_4$-cirrhotic rats, both reduction in fibrosis and decreased hepatic vascular tone contribute to decrease the hepatic vascular resistance. Remarkably, the beneficial effects of Terutroban on fibrosis were not observed in the BDL model. Although we do not have an explanation for this, we may speculate that this differential effect on fibrosis may be due to the fact that the BDL model is characterized by a very

rapid and progressive fibrosis while CCl_4 represents a model with much slower fibrosis, susceptible of regression once CCl_4 inhalation is interrupted.

Another differential effect of Terutroban between models was that observed on the NO signaling pathway. In BDL rats, Terutroban promoted a significant increase of both eNOS protein expression, of its biologically active phosphorylated form, and the NO second messenger, cGMP, suggesting that in BDL rats, an increase in NO bioavailability may also play a role reducing hepatic vascular resistance. By contrast, TP-receptor blockade in CCl_4-cirrhotic rats did not produce significant changes in any of these parameters. At present, we have not a clear explanation for such differential effect of Terutroban. It is remarkable that although Terutroban did not change MAP in CCl_4-cirrhotic rats, this was not the case in BDL rats where a marked reduction was observed. It is possible that in the more severe ill rats with BDL cirrhosis, blocking the TXA_2 vasoconstrictive systemic pathway together with an increase in NO bioavailability probably also at the systemic level, may be responsible for such effect decreasing MAP.

It is important to emphasize that Terutroban reduces portal pressure in two different experimental settings of chronic liver disease. In the BDL model, Terutroban was administered after 2 weeks of bile duct ligation when cirrhosis and the portal hypertension syndrome is not fully established and there is still an ongoing active injury. In this situation, although we can not discard that longer periods of treatment may act on fibrosis, the main effect of Terutroban was over the dynamic component of resistance. By contrast, in the CCl_4 model, Terutroban was administered once the injury (CCl_4 inhalation) has been stopped in a setting of potential fibrosis reversal. Here, in addition to improving

the dynamic component of the hepatic resistance, Terutroban also facilitates fibrosis regression.

In conclusion, our data show that TP-receptor blockade with Terutroban significantly reduces portal pressure in cirrhotic rats by decreasing hepatic vascular resistance (with a similar and comparable order of magnitude in both cirrhotic models), suggesting that Terutroban may represent a useful agent in the treatment of portal hypertension in cirrhosis. However, in further translational steps of the investigation a special care must be taken in possible effects reducing MAP.

FIGURE LEGENDS:

Fig 1. Efficacy of Thromboxane/prostaglandin endoperoxide (TP) receptor blockade in CCl4- and BDL-cirrhotic rats.

Effects of the infusion of the thromboxane (TXA_2) agonist, U46619, in mean arterial pressure (MAP) and portal pressure (PP) in CCl_4- (Fig 1A, 1B) and BDL-cirrhotic rats (Fig 1D, 1E) treated with Vehicle or Terutroban. Measurements were obtained at baseline and after an intravenous infusion of the TXA_2 agonist. Data as presented as % of increment above baseline and are mean ± SEM (n=3 per group). *$p<0.05$ vs. Vehicle.

Representative Western Blot of TP expression in isolated hepatic stellate cells (HSC) from sham, CCl_4- and BDL-cirrhotic rats. Densitometry quantification in arbitrary units (AU) showed a significant increase in TP receptor expression in HSC from CCl_4 (Fig 1C) and BDL (Fig 1F) compared to HSC from sham rats. Data are presented as mean ± SEM (n=4 per group). * $p<0.05$ vs sham.

Fig 2. Effects of Terutroban on hepatic and systemic hemodynamics in CCl_4-cirrhotic rats.

Effects of Terutroban (30mg/Kg/day for 2 weeks) on portal pressure (PP), portal blood flow (PBF), hepatic vascular resistance (HVR) and mean arterial pressure (MAP) in CCl_4-cirrhotic rats. Data are presented as mean ± SEM (Vehicle, n=8; Terutroban, n=13). *$p<0.05$ vs. Vehicle.

Fig 3. Effects of Terutroban on endothelial function, Rho-kinase and eNOS pathways in CCl_4-cirrhotic rats.

(A) Endothelium-dependent vasorelaxation to Acetylcholine (Ach) in isolated and perfused livers of CCl$_4$-cirrhotic rats treated with Vehicle or Terutroban. Terutroban treatment significantly improved the impaired vasodilatory response to Ach in CCl$_4$-cirrhotic rat livers. Data are presented as mean ± SEM (Vehicle, n=9; Terutroban, n=8) *p<0.05 vs. Vehicle.

(B) Representative Western blot and analysis of moesin phosphorylation normalized to total moesin expression in CCl$_4$-cirrhotic livers treated with Vehicle or Terutroban. Densitometry quantification in arbitrary units (AU) showed a significant decrease in moesin phosphorylation at Thr^{558} in Terutroban-treated rats.

Representative Western blot and analysis of **(C)** P-eNOS and **(D)** eNOS in CCl$_4$-cirrhotic livers treated with Vehicle or Terutroban. Densitometry quantification in arbitrary units (AU), normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), showed no differences between both groups. Data are presented as mean ± SEM (n=7 *per* group). * p <0.05 vs Vehicle.

(E) Intrahepatic cGMP levels in CCl$_4$-cirrhotic rats treated with Vehicle or Terutroban. cGMP levels were not significantly different between groups (n=9 per group). Data are presented as mean ± SEM.

Fig 4. Effects of Terutroban on hepatic fibrosis in CCl$_4$-cirrhotic rats.

(A) *Top*: Representative histological images of livers stained with Sirius red from Vehicle or Terutroban treated CCl$_4$-cirrhotic rats (original magnification 5X). *Bottom:* Quantification of liver fibrosis (% quantification of Sirius Red staining area = Sirius Red staining area/total area*100). Terutroban treated

CCl$_4$-cirrhotic rats showed a significant decrease in hepatic fibrosis. (Vehicle, n=9; Terutroban, n=11).

(B) Collagen I mRNA expression levels in livers from CCl$_4$-cirrhotic rats treated with Terutroban or Vehicle. Values were significantly decreased in Terutroban-treated rats. (Vehicle, n=9; Terutroban, n=12).

(C) Top: Representative Western blot analysis for α-SMA in livers from Vehicle or Terutroban-treated CCl$_4$ rats. *Bottom:* Densitometry quantification in arbitrary units (AU), normalized to GAPDH, showed a significant decrease in Terutroban-treated rats. (n=7 *per* group).

(D) TGF-β mRNA expression levels in livers from CCl$_4$-cirrhotic rats treated with Terutroban or Vehicle. Values showed a significant decrease in Terutroban-treated rats. (Vehicle, n=9; Terutroban, n=12). Data are presented as mean ± SEM. * p <0.05 vs Vehicle.

Fig 5. Effects of Terutroban on hepatic and systemic hemodynamics in BDL-cirrhotic rats.

Effects of Terutroban (30mg/Kg/day) on portal pressure (PP), portal blood flow (PBF), hepatic vascular resistance (HVR), mean arterial pressure (MAP), superior mesenteric artery blood flow (SMABF) and superior mesenteric arterial resistance (SMAR) in BDL-cirrhotic rats. Data are presented as mean ± SEM (Vehicle, n=16; Terutroban, n=14). *p<0.05 vs. Vehicle.

Fig 6. Effects of Terutroban on endothelial function, Rho-kinase and eNOS pathways in BDL-cirrhotic rats.

(A) Endothelium-dependent vasorelaxation to Acetylcholine (Ach) in isolated and perfused livers of BDL-cirrhotic rats treated with Vehicle or Terutroban. Terutroban treatment significantly improved the impaired vasodilatory response to Ach in BDL-cirrhotic rat livers. Data are presented as mean ± SEM (Vehicle, n=9; Terutroban, n=8) *p<0.05 vs. Vehicle.

(B) Representative Western blot and analysis of moesin phosphorylation normalized to total moesin expression, in BDL-cirrhotic livers treated with Vehicle or Terutroban. Densitometry quantification showed a significant decrease in moesin phosphorylation in Terutroban-treated rats. (n=13 per group).

Representative Western blot and analysis of **(C)** P-eNOS and **(D)** eNOS in BDL cirrhotic livers treated with Vehicle or Terutroban. Densitometry quantification in arbitrary units (AU), normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), showed a significant increase in eNOS phosphorylation and eNOS total protein expression in Terutroban-treated rats. (n=14 *per* group).

(E) Intrahepatic cGMP levels in BDL cirrhotic rats treated with Vehicle or Terutroban. cGMP levels were significantly increased in Terutroban treated rat livers. (n=13 per group). Data are presented as mean ± SEM. * p <0.05 vs Vehicle.

Fig 7. Effects of Terutroban on hepatic fibrosis in BDL-cirrhotic rats.

(A) *Top*: Representative histological images of livers stained with Sirius red from Vehicle or Terutroban treated BDL-cirrhotic rats (original magnification 5X). *Bottom:* Quantification of liver fibrosis (% quantification of Sirius Red staining area = Sirius Red staining area/total area*100). BDL cirrhotic rats treated with

Terutroban or vehicle showed no differences between both groups. (n=10 *per*

group).

 (B) Collagen I mRNA expression levels in livers from BDL-cirrhotic rats treated

with Terutroban or Vehicle, showed no differences between both groups. (n=14

per group).

(C) Top: Representative Western blot analysis for α-SMA in livers from Vehicle

or Terutroban-treated BDL rats. *Bottom:* Densitometry quantification in arbitrary

units (AU), normalized to GAPDH, showed no differences between both groups.

(n=14 *per* group).

(D) TGF-β mRNA expression levels in livers from BDL-cirrhotic rats treated with

Terutroban or Vehicle showed no differences between both groups. (n=14 *per*

group). Data are presented as mean ± SEM.

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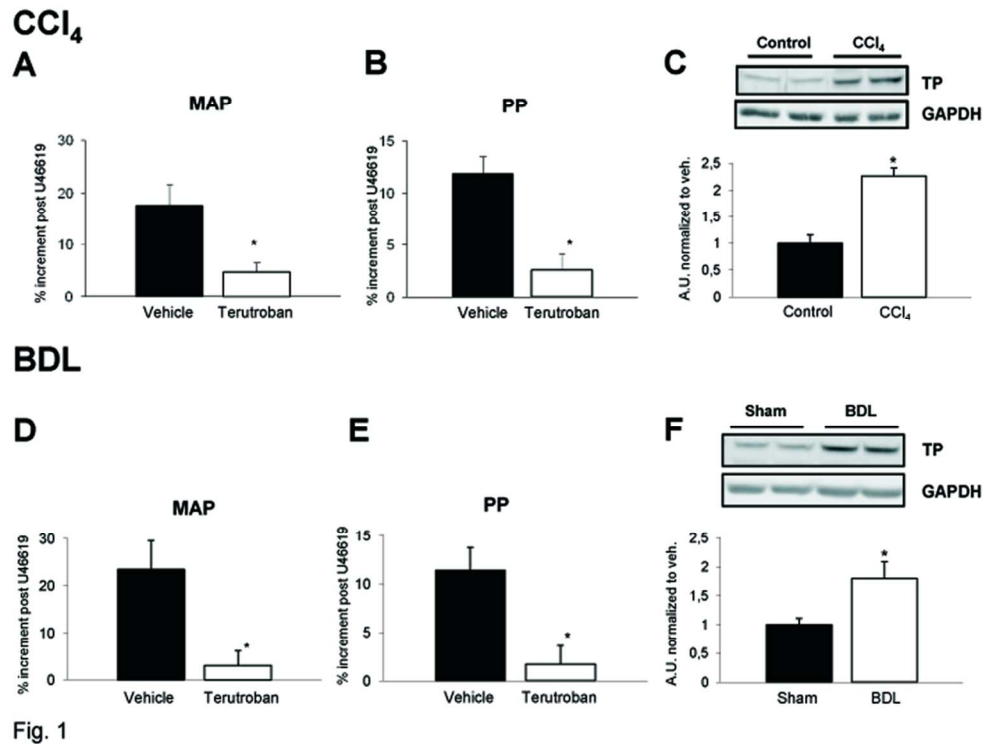42. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115:209-218.

Fig. 1

Fig 1. Efficacy of Thromboxane/prostaglandin endoperoxide (TP) receptor blockade in CCl4- and BDL-cirrhotic rats.
Effects of the infusion of the thromboxane (TXA2) agonist, U46619, in mean arterial pressure (MAP) and portal pressure (PP) in CCl4- (Fig 1A, 1B) and BDL-cirrhotic rats (Fig 1D, 1E) treated with Vehicle or Terutroban. Measurements were obtained at baseline and after an intravenous infusion of the TXA2 agonist. Data as presented as % of increment above baseline and are mean ± SEM (n=3 per group). *p<0.05 vs. Vehicle.
Representative Western Blot of TP expression in isolated hepatic stellate cells (HSC) from sham, CCl4- and BDL-cirrhotic rats. Densitometry quantification in arbitrary units (AU) showed a significant increase in TP receptor expression in HSC from CCl4 (Fig 1C) and BDL (Fig 1F) compared to HSC from sham rats. Data are presented as mean ± SEM (n=4 per group). * p<0.05 vs sham.
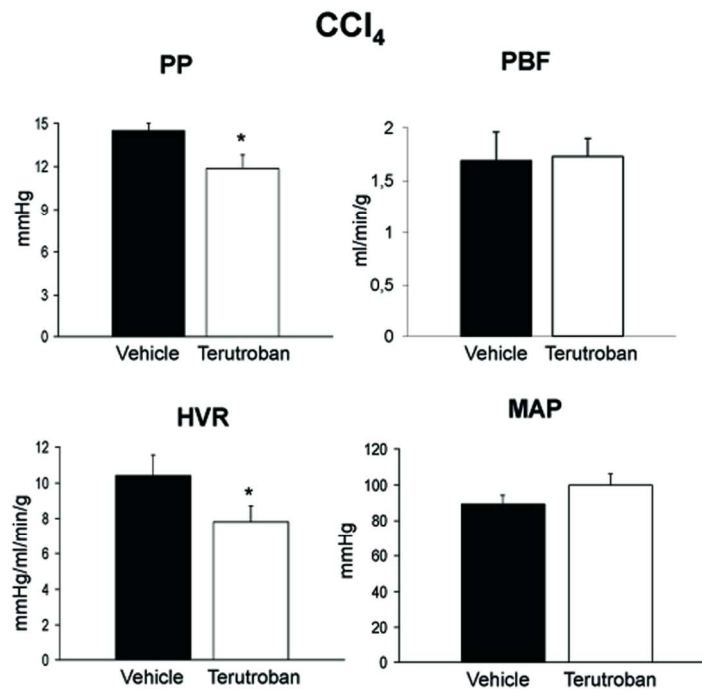
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Fig. 2

Fig 2. Effects of Terutroban on hepatic and systemic hemodynamics in CCl4-cirrhotic rats.
Effects of Terutroban (30mg/Kg/day for 2 weeks) on portal pressure (PP), portal blood flow (PBF), hepatic
vascular resistance (HVR) and mean arterial pressure (MAP) in CCl4-cirrhotic rats. Data are presented as
mean ± SEM (Vehicle, n=8; Terutroban, n=13). *p<0.05 vs. Vehicle.
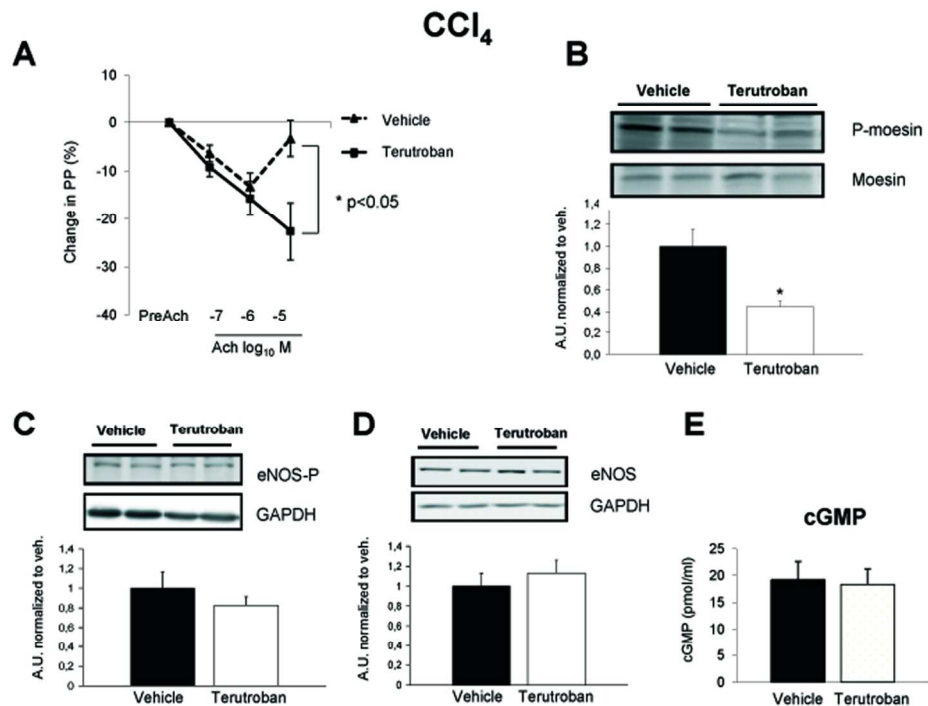
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Fig. 3

Fig 3. Effects of Terutroban on endothelial function, Rho-kinase and eNOS pathways in CCl4-cirrhotic rats. (A) Endothelium-dependent vasorelaxation to Acetylcholine (Ach) in isolated and perfused livers of CCl4-cirrhotic rats treated with Vehicle or Terutroban. Terutroban treatment significantly improved the impaired vasodilatory response to Ach in CCl4-cirrhotic rat livers. Data are presented as mean ± SEM (Vehicle, n=9; Terutroban, n=8) *p<0.05 vs. Vehicle.

(B) Representative Western blot and analysis of moesin phosphorylation normalized to total moesin expression in CCl4-cirrhotic livers treated with Vehicle or Terutroban. Densitometry quantification in arbitrary units (AU) showed a significant decrease in moesin phosphorylation at Thr558 in Terutroban-treated rats.

Representative Western blot and analysis of (C) P-eNOS and (D) eNOS in CCl4-cirrhotic livers treated with Vehicle or Terutroban. Densitometry quantification in arbitrary units (AU), normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), showed no differences between both groups. Data are presented as mean ± SEM (n=7 per group). * p <0.05 vs Vehicle.

(E) Intrahepatic cGMP levels in CCl4-cirrhotic rats treated with Vehicle or Terutroban. cGMP levels were not significantly different between groups (n=9 per group). Data are presented as mean ± SEM.
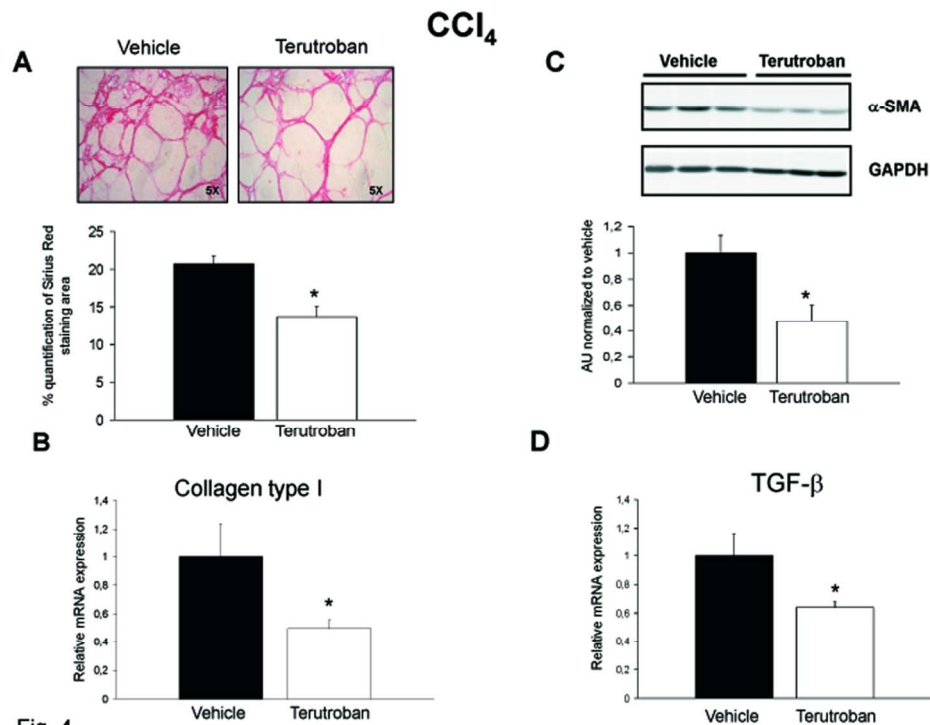
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Fig 4. Effects of Terutroban on hepatic fibrosis in CCl4-cirrhotic rats.
(A) Top: Representative histological images of livers stained with Sirius red from Vehicle or Terutroban treated CCl4-cirrhotic rats (original magnification 5X). Bottom: Quantification of liver fibrosis (% quantification of Sirius Red staining area = Sirius Red staining area/total area*100). Terutroban treated CCl4-cirrhotic rats showed a significant decrease in hepatic fibrosis. (Vehicle, n=9; Terutroban, n=11).
(B) Collagen I mRNA expression levels in livers from CCl4-cirrhotic rats treated with Terutroban or Vehicle. Values were significantly decreased in Terutroban-treated rats. (Vehicle, n=9; Terutroban, n=12).
(C) Top: Representative Western blot analysis for α-SMA in livers from Vehicle or Terutroban-treated CCl4 rats. Bottom: Densitometry quantification in arbitrary units (AU), normalized to GAPDH, showed a significant decrease in Terutroban-treated rats. (n=7 per group).
(D) TGF-β mRNA expression levels in livers from CCl4-cirrhotic rats treated with Terutroban or Vehicle. Values showed a significant decrease in Terutroban-treated rats. (Vehicle, n=9; Terutroban, n=12). Data are presented as mean ± SEM. * $p < 0.05$ vs Vehicle.
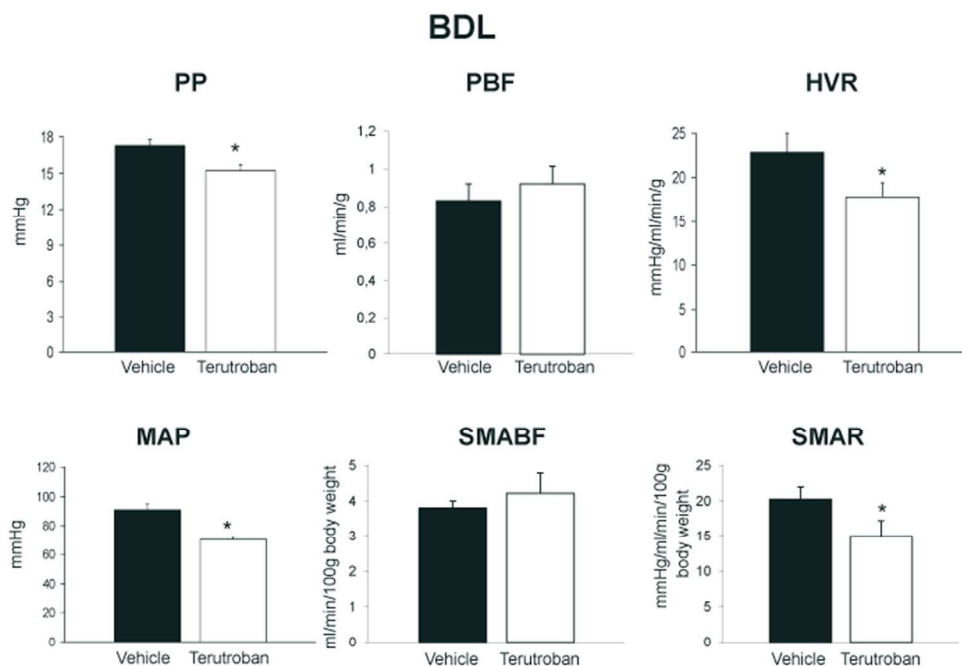
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Fig. 5

Fig 5. Effects of Terutroban on hepatic and systemic hemodynamics in BDL-cirrhotic rats.
Effects of Terutroban (30mg/Kg/day) on portal pressure (PP), portal blood flow (PBF), hepatic vascular resistance (HVR), mean arterial pressure (MAP), superior mesenteric artery blood flow (SMABF) and superior mesenteric arterial resistance (SMAR) in BDL-cirrhotic rats. Data are presented as mean ± SEM (Vehicle, n=16; Terutroban, n=14). *p<0.05 vs. Vehicle.
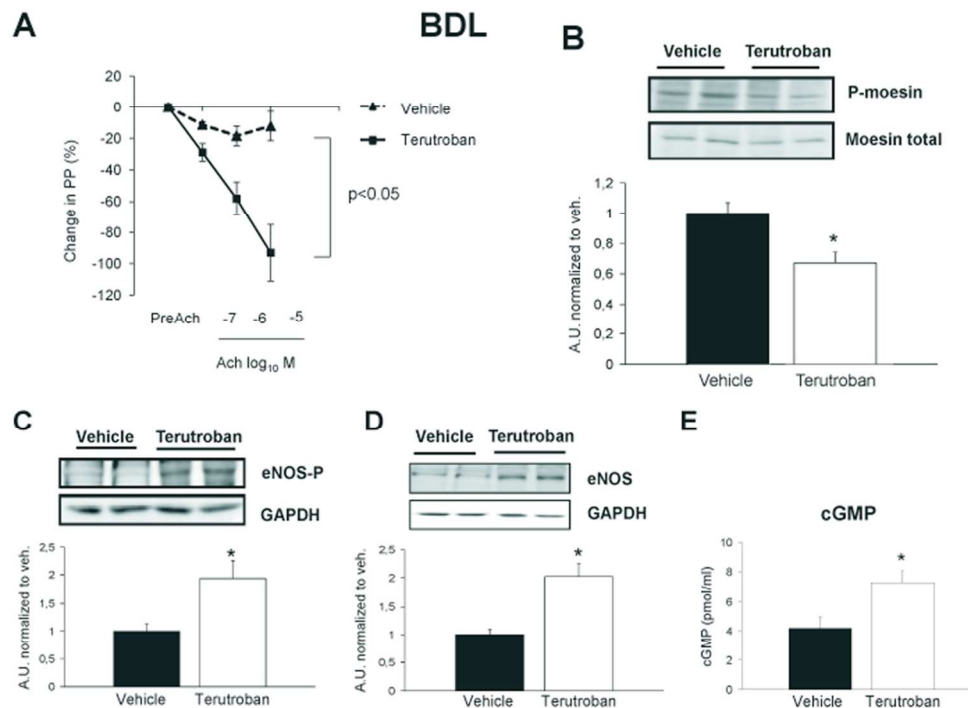
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Fig. 6

Fig 6. Effects of Terutroban on endothelial function, Rho-kinase and eNOS pathways in BDL-cirrhotic rats.
(A) Endothelium-dependent vasorelaxation to Acetylcholine (Ach) in isolated and perfused livers of BDL-cirrhotic rats treated with Vehicle or Terutroban. Terutroban treatment significantly improved the impaired vasodilatory response to Ach in BDL-cirrhotic rat livers. Data are presented as mean ± SEM (Vehicle, n=9; Terutroban, n=8) *p<0.05 vs. Vehicle.
(B) Representative Western blot and analysis of moesin phosphorylation normalized to total moesin expression, in BDL-cirrhotic livers treated with Vehicle or Terutroban. Densitometry quantification showed a significant decrease in moesin phosphorylation in Terutroban-treated rats. (n=13 per group).
Representative Western blot and analysis of (C) P-eNOS and (D) eNOS in BDL cirrhotic livers treated with Vehicle or Terutroban. Densitometry quantification in arbitrary units (AU), normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), showed a significant increase in eNOS phosphorylation and eNOS total protein expression in Terutroban-treated rats. (n=14 per group).
(E) Intrahepatic cGMP levels in BDL cirrhotic rats treated with Vehicle or Terutroban. cGMP levels were significantly increased in Terutroban treated rat livers. (n=13 per group). Data are presented as mean ± SEM. * p <0.05 vs Vehicle.
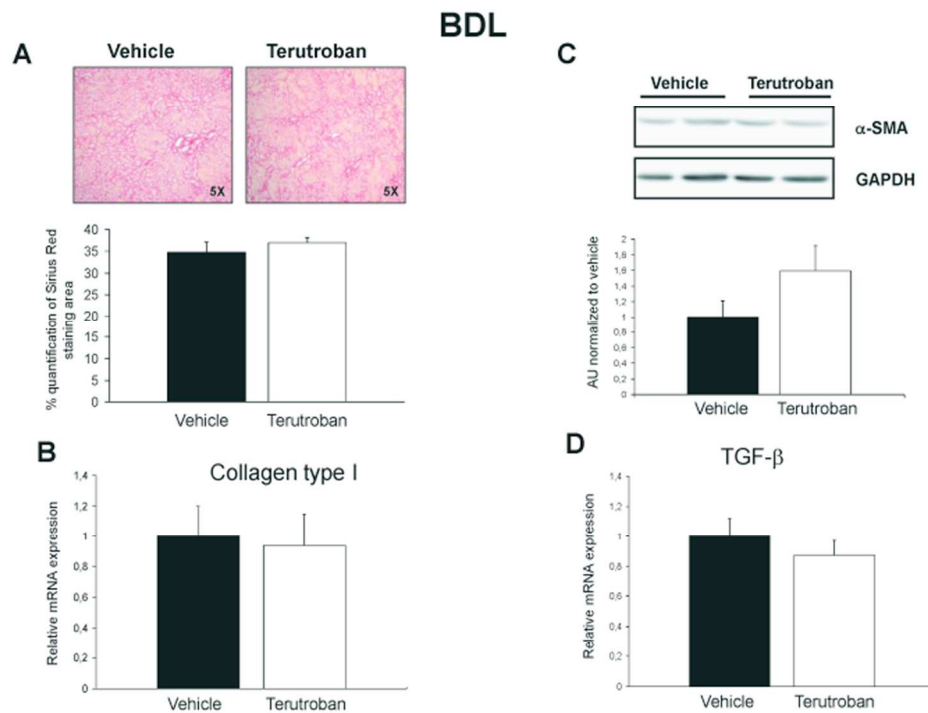
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Fig. 7

Fig 7. Effects of Terutroban on hepatic fibrosis in BDL-cirrhotic rats.
(A) Top: Representative histological images of livers stained with Sirius red from Vehicle or Terutroban treated BDL-cirrhotic rats (original magnification 5X). Bottom: Quantification of liver fibrosis (% quantification of Sirius Red staining area = Sirius Red staining area/total area*100). BDL cirrhotic rats treated with Terutroban or vehicle showed no differences between both groups. (n=10 per group).
(B) Collagen I mRNA expression levels in livers from BDL-cirrhotic rats treated with Terutroban or Vehicle, showed no differences between both groups. (n=14 per group).
(C) Top: Representative Western blot analysis for α-SMA in livers from Vehicle or Terutroban-treated BDL rats. Bottom: Densitometry quantification in arbitrary units (AU), normalized to GAPDH, showed no differences between both groups. (n=14 per group).
(D) TGF-β mRNA expression levels in livers from BDL-cirrhotic rats treated with Terutroban or Vehicle showed no differences between both groups. (n=14 per group). Data are presented as mean ± SEM.

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CCl$_4$

	Vehicle (n=8)	Terutroban (n=10)	pvalue
AST (U/L)	276 ± 170	206 ± 117	ns
ALT (U/L)	106 ± 57	105 ± 43	ns
Bilirubin (mg/dL)	0.3 ± 0.3	0.2 ± 0.4	ns
Albumin (g/L)	22.8 ± 4	27.6 ± 2	< 0,05
Body weight (g)	361 ± 29	356 ± 17	ns
Liver weight (g)	8.6 ± 2,4	9.4 ± 1	ns
Spleen weight (g)	0.93 ± 0.2	0,92 ± 0.1	ns

BDL

	Vehicle (n=16)	Terutroban (n=14)	pvalue
AST (U/L)	480 ± 181	667 ± 382	ns
ALT (U/L)	95 ± 37	112 ± 46	ns
Bilirubin (mg/dL)	7.4 ± 1.2	6.4 ± 1.8	ns
Albumin (g/L)	23 ± 4	20 ± 4	ns
Body weight (g)	324 ± 26	315 ± 33	ns
Liver weight (g)	18.1 ± 3.9	17.1 ± 4.8	ns
Spleen weight (g)	1.7 ± 0.3	1.6 ± 0.4	ns

Data are presented as mean ± SD.

AST, aspartate aminotransferase; ALT, alanine aminotransferase.