SUPPLEMENTS

sGC STIMULATION AND PDE-5 INHIBITION DECREASE SINUSOIDAL RESISTANCE AND REDUCE FIBROSIS IN BILE DUCT LIGATED RATS

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1. SUPPLEMENTARY METHODS

1.1. Shunting assessment

Porto-systemic shunting (PSS) was quantified after injection of 100µL of red microspheres (Triton Eosin Dye Trak, TRI-155-0531, 20ml, 15µm DM) into the portal vein, whereas 100µL of white microspheres (TRI-155-0370) were injected into the spleen to determine splenorenal shunting (SRS), as previously described.[1] Lung, liver, and spleen were explanted, digested in 4M KOH overnight, and filtered. Microparticle absorption was quantified photometrically (530nm for red and 370nm for white microspheres). The ratio of detected microspheres in lung/(liver+lung) and lung/(spleen+lung) was calculated to assess PSS and SRS, respectively. Total shunting represents the mean of both PSS and SRS.

1.2. Histological stainings

Liver tissue was formalin-fixed, paraffin-embedded, cut into 2µm thick slides, and mounted on super frost glass slides. CAB was stained as previously described,[2] using chromotrope-aniline blue (CAB) reagent solution (Sigma-Aldrich #C3143, #95290), and Picro-Sirius red (PSR) staining was conducted using a PSR staining kit (PSR, Morphisto #13425) according to the manufacturer's instructions.

For staining of smooth muscle actin alpha (αSMA) and cytokeratin 19 (CK19), 2µm thick liver sections were deparaffinized using xylol (CAS-No. 108-38-3, Sigma-Aldrich, Austria), then hydrated in decreasing concentrations of ethanol. Afterwards, epitope retrieval was performed by boiling in 10mM citrate buffer (pH 6). Subsequently, samples were subjected to blocking with appropriate serum and with avidin/biotin blocking kit (Vector Laboratories). Next, αSMA (DAKO #M0851) and CK19 (DAKO #M0888) antibodies were applied onto the liver sections overnight at +4°C. On the next day, antibodies were removed by washing with 50mM TRIS-buffered saline.

Incubation with secondary antibodies was followed by incubation with Vecta stain (Elite ABC HRP Kit, Vector Laboratories). Signals were then developed using 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma Aldrich).

For image analysis of CAB, six random pictures of each specimen were taken (Olympus BX51, 10x magnification), and equally color-filtered to highlight the blue-color tone. Quantification of CAB stained area was performed using ImageJ (V1.47, NIH, USA) in a semi-automated and blinded manner. Stained proportionate areas of PSR, αSMA, and CK19 were quantified on high-resolution 40x slide scans of whole liver lobe sections (Pannoramic MIDI Slidescanner; 3DHISTECH) and analyzed by automated quantification using the histomorphometry software Definiens TissueStudio ® (V4.3.1).

1.3. Hepatic hydroxyproline assessment

For measurement of hepatic hydroxyproline content, snap-frozen liver tissue pieces (50–100 mg) were homogenized in aqua dest., precipitated with 50% trichloroacetic acid (TCA,#T0699, Sigma-Aldrich, Germany), and washed with pure ethanol (#64-17-5, VWR, France). The protein pellet was hydrolyzed in 6M HCl (#13386, Merck, Germany) solution overnight at 95°C. The next day, the samples were oxidized with chloramine-T and then incubated with Ehrlich's reagent solution. Finally, hydroxyproline content was determined by measuring the absorbance at 562nm and normalized to liver sample weight.

1.4. Hepatic gene expression

RNA was isolated from frozen tissue samples using TRIzol® reagent (Thermo Fisher Scientific Waltham, MA, USA), according to the manufacturer´s instructions. Samples were homogenized, treated with chloroform and propanol-2, and then washed with ice-cold 75% ethanol in nuclease-free water. RNA concentration and purity were assessed using GeneQuant Spectrophotometer

(Biochrom, Cambridge, UK). The High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, US) was used to synthesize cDNA. After 10 minutes of RT incubation, samples were transcribed for 2 hours at 37°C. RT-PCR was performed using the TaqMan gene expression kit (TaqMan® Universal PCR Master Mix). For gene expression quantification by real-time PCR, a validated TaqMan Gene Expression kit (TaqMan® Universal PCR Master Mix) was used. Expression of the following fibrosis and inflammation markers was assessed: collagen 1 alpha 1 (*Col1a1*, Rn01463848_m1), transforming growth factor-beta 1 (*Tgfb1*, Rn00572010_m1), tissue inhibitor of metalloproteinases-1 (*Timp1*, Rn00587558_m1), smooth muscle actin alpha (*aSma*/*Acta2*, Rn01759928_g1), cGMP dependent protein kinase 1 (*Prkg1*, Rn01451055_m1), interleukin-6 (*Il6*, Rn01410330_m1), monocyte chemoattractant protein 1 (*Ccl2*/*Mcp1*, Rn00580555_m1), interleukin-1b (*Il1b*, Rn00580432_m1), chemokine (C-X-C motif) ligand 1 (*Cxcl1*, Rn00578225_m1), tumor necrosis factor-alpha (*Tnfa*, Rn99999017_m1) and vascular cell adhesion protein 1 (*Vcam*, Rn01521368_m1). Glyceraldehyde 3-phosphate dehydrogenase – (*Gapdh,* Rn01775763_g1) was used as a housekeeping gene. All used primers were ordered at Thermo Fisher Science. The RT-PCR was set to the following conditions: (1) polymerase activation at 95°C for 10 minutes, (2) 40 PCR cycles with a denaturing temperature of 95°C for 15 seconds and an extension temperature of 60°C for 1 minute and performed in the 7500 Fast Real-Time PCR System cycler (Applied Biosystems, Foster City, CA, US). The amount of target gene, normalized to *Gapdh* expression, was determined by the arithmetic equation $2^{-\Delta\Delta Ct}$ described in the comparative Ct method.

1.5. Western blot analysis

Liver tissue was homogenized with radioimmunoprecipitation assay (RIPA) buffer (with protease- and phosphatase inhibitors: phenylmethylsulfonyl fluoride (PMSF, #P7626, Sigma-Aldrich, USA), 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (Pefa block,#30827-99-7, Merck,

Germany), β-Glycerolphosphate (#50020, Fluka, Germany)) in Precellys Lysing Tubes (Bertin-instruments, France). Protein lysis was conducted on ice (40min) followed by a centrifugation step (12000rpm, 10min, 4°C). After quantification of total protein concentration (BCA Protein Assay Kit, Thermo Scientific) 30µg of the sample were separated by SDS polyacrylamide gel electrophoresis (4–20% gradient gels, Lonza, Basel, Switzerland) and transferred to PVDF membranes (GE Healthcare). Membranes were blocked with Western Blocking Reagent (Roche, Penzberg, Germany) and after the washing steps with TBS (10M Tris/HCl (pH 7.4), 5M NaCl) and TBST (10M Tris/HCl (pH 7.4), 5M NaCl, Tween-20) incubated with primary and secondary HRP-conjugated antibodies (Santa Cruz). Respective blots of liver proteins were incubated with the following primary antibodies: total endothelial nitric oxide synthetase (eNOS; N3893, Sigma Aldrich), phospho-eNOS (#9570, Cell Signaling), total-moesin (sc-6410, Santa Cruz), phospho-moesin (sc-12895, Santa Cruz), cGMP dependent protein kinase 1 (#3248, Cell Signaling) and αSMA (ab5694, Abcam). Bands were visualized using Prime Western Blotting Detection Reagent (GE Healthcare) in the Fusion Fx7 imaging system (Peqlab/VWR) and quantified by Fusion software. Protein expression was normalized to GAPDH (#5174, Cell Signaling) as a housekeeping gene. Membranes were stripped after each antibody, up to 3 times.

1.6. Platelet-rich plasma preparation

Blood was withdrawn from rats or cirrhotic patients and healthy controls with no history of liver disease under aseptic conditions in 3.2% sodium citrate VACUETTE blood tubes (#454327, Greiner Bio-one). Platelet-rich plasma pellets were obtained after whole blood centrifugation at low speed (160g/10min/24°C/no breaks), and subsequent centrifugation of the plasma (2000g/10min/4°C). Isolated pellets were washed with Dulbecco's phosphate-buffered saline (DPBS, #1420067, Gibco, UK) and incubated with RIPA buffer (incl. protease- and phosphatase inhibitors). Platelet lysis was conducted on ice (40min) followed by a centrifugation step

(12000rpm, 10min, 4°C). After quantification of total protein, 12µg of the sample were subjected to SDS gel electrophoresis and Western blotting, as described in Supplementary Methods 1.4. For protein analysis of platelet-rich plasma in rats and humans, total vasodilator-stimulated phosphoprotein (t-VASP, Cell Signaling, #3132), phosphorylated VASP (p-Ser239 VASP, Nano Tools, #0047-100/VASP-16C2) and cGMP dependent protein kinase 1 (#3248, Cell Signaling) were used. Protein expression was normalized to actin (#69100, ImmunO) as a housekeeping gene.

1.7. Assessment of the intrahepatic cGMP levels

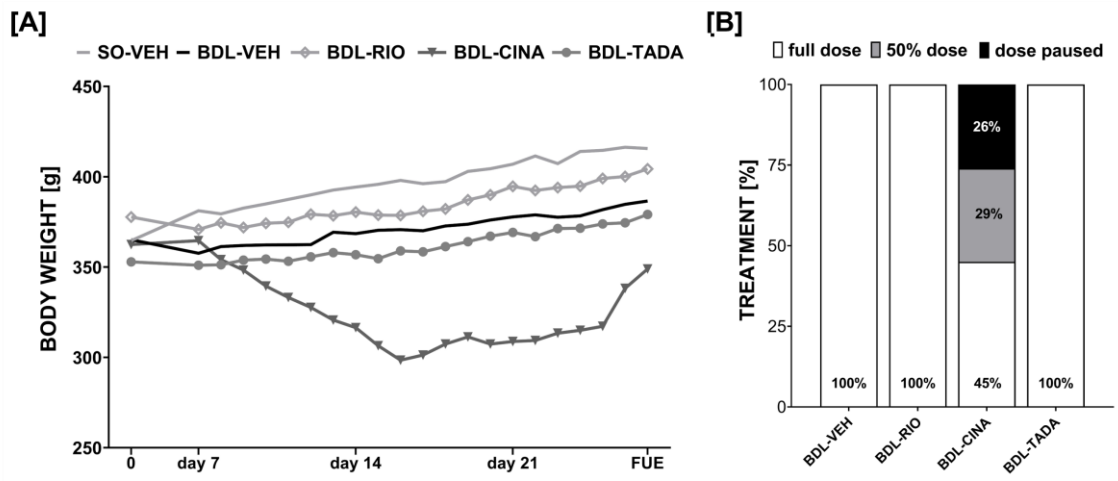
The determination of cGMP concentrations in the liver was performed using ultra-performance liquid chromatography with mass spectrometry (UPLC/MS). In brief, the cGMP level in each sample was analyzed using a relative quantitative assay comparing samples to the internal standard ($^{15}N_5$ cGMP). Snap-frozen tissue samples (approximately 100mg), collected during the follow-up measurements, were thawed and then homogenized with 6µL of 100mM IBMX (3-isobutyl-1-methylxanthine, phosphodiesterase inhibitor) in DMSO and with 400µL (per 100mg liver) 50nM $^{15}N_5$ cGMP in 2% H_3PO_4. Homogenate was centrifuged to purify the supernatant (20.817g/ 30min). To remove residual solid parts, the supernatant was centrifuged in an Amicon Ultra 30 K filter unit (14000g/ 60min). The clear filtrates were used for the solid-phase extraction process. Afterwards, samples were analyzed using UPLC with an AB Sciex Qtrap 5500 tandem mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with a Turbo Spray ion source in positive electrospray mode.

The chromatography separation was performed at room temperature using a C18 (1.7 µM 2.1 x 150 mm, Waters) analytical column, and C18 (1.7 µm 2.1 x 5 mm, Waters) guarding column. The mobile phase was: A (0.1% HCOOH) and B (MeCN/MeOH/0.1% HCOOH, 1/2/4, v/v/v). The

gradient elution started with 100% eluent A for 0.5 min, followed by a 4 min gradient to 84% eluent A and 16% eluent B at a flow rate of 0.50 mL/min. The fractionated compounds were introduced directly in the electrospray source, ionized and monitored by multiple reaction monitoring. The source block temperature was set to 650°C and the electrospray capillary voltage to 5.0 kV. The dwell time for each fragmentation pathway was 50ms. Nitrogen was used as the collision gas. The results are present as a response ratio (ratio of areas of the MS-response from the cGMP and its heavy isotope-labelled internal standard).

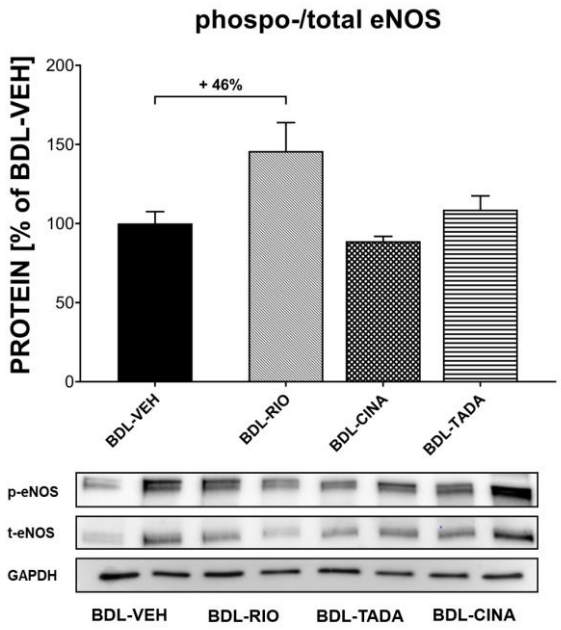
2. SUPPLEMENTARY FIGURES

Supplementary Figure-1. Body weight development and treatment interventions



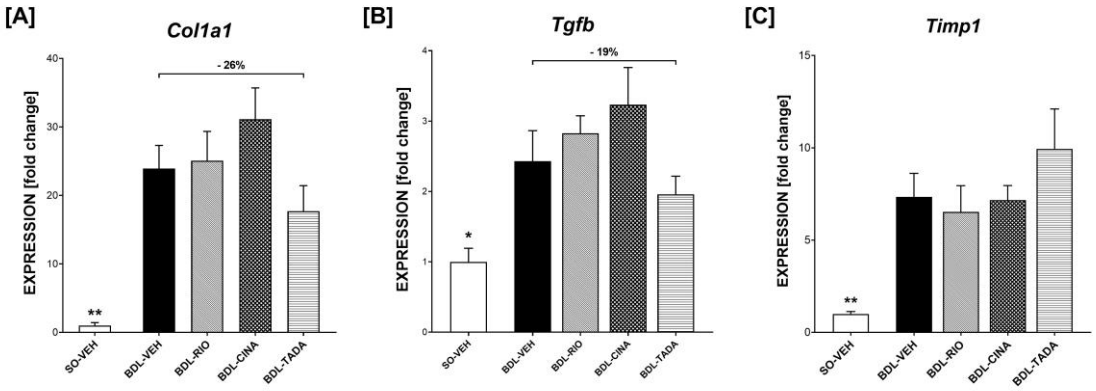
Supplementary Figure-1. [A] Body weight and health status were assessed daily. Cinaciguat treatment was associated with a significant decrease in body weight. **[B]** The treatment was halved (once body weight loss exceeded 10%) or paused (when the body weight loss was >15%) in the majority of BDL-CINA animals for at least one day. In total, 26% of CINA doses were abolished, 29% reduced by half, and only 45% performed as planned. All other treatment groups were treated as scheduled.

Supplementary Figure-2. Hepatic expression of total and phospho-eNOS



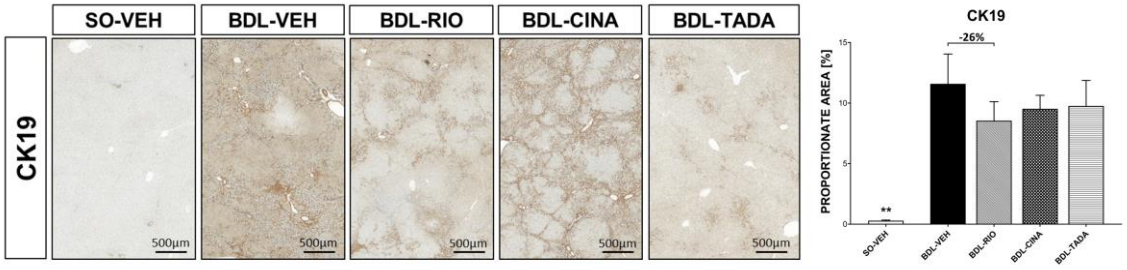
Supplementary Figure-2. Hepatic content of phosphorylated eNOS (relative to total eNOS) tended to be increased in the BDL-RIO group (+46%). However, there were no changes in phosphorylated to total eNOS levels observed in CINA or TADA treated rats.

Supplementary Figure-3. Hepatic expression of markers of liver fibrosis



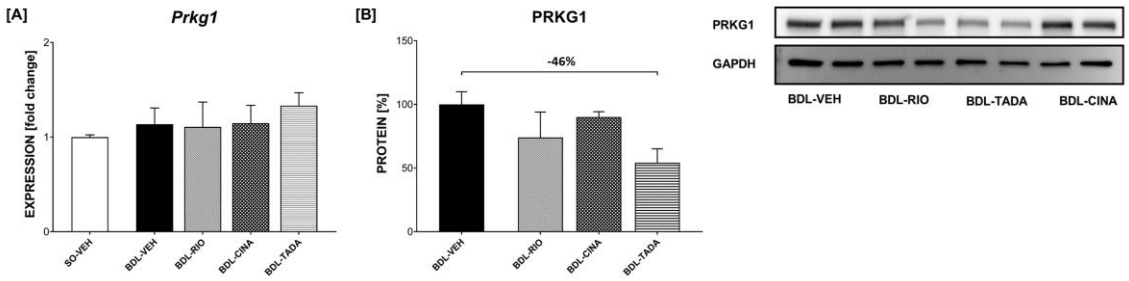
Supplementary Figure-3. [A] Hepatic *Col1a1*, **[B]** *Tgfb,* and **[C]** *Timp1* expression was significantly upregulated in the BDL model. However, only a non-significant decrease of *Col1a1* (-26%) and *Tgfb* (-19%) was noted in the BDL-TADA group, while the other treatments showed no effect on the hepatic gene expression of these markers. *p<0.05, **p<0.01 vs. BDL-VEH.

Supplementary Figure-4. Immunohistochemistry of CK19 as a marker of ductular proliferation



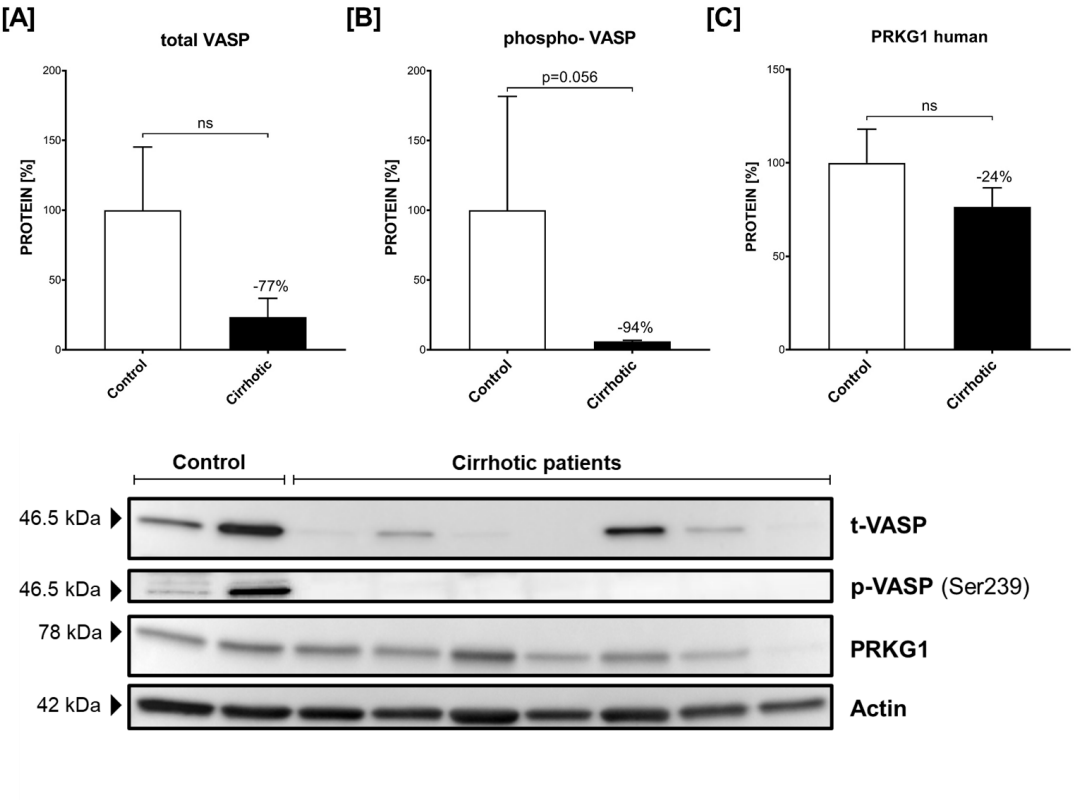
Supplementary Figure-4. Representative liver slides stained for CK19. RIO treated rats showed less CK19 density as compared to cirrhotic controls (-26%); however, this effect was statistically not significant.

Supplementary Figure-5. Gene expression and protein synthesis of *Prkg1*/PRKG1 in the liver



Supplementary Figure-5. [A] The hepatic *Prkg1* expression remained unchanged between all BDL groups. **[B]** However, the protein expression of PRKG1 was slightly lower in BDL-TADA animals, compared to BDL-VEH controls.

Supplementary Figure-6. Protein expression of total-VASP, phospho-VASP, and PRKG1 in platelet-rich plasma from patients with liver cirrhosis and healthy controls



Supplementary Figure-6. [A, B] Patients with liver cirrhosis presented with lower total VASP protein expression in platelet-rich plasma, and even lower levels of phosphorylated VASP (-94%, p=0.056). **[C]** The PRKG1 protein expression decreased by 24% in cirrhotic patients.

3. SUPPLEMENTARY REFERENCES

1. Schwabl P, Payer BA, Grahovac J, et al. Pioglitazone decreases portosystemic shunting by modulating inflammation and angiogenesis in cirrhotic and non-cirrhotic portal hypertensive rats. *Journal of hepatology*. 2014; 60: 1135-42.
2. Roque AL. Chromotrope aniline blue method of staining Mallory bodies of Laennec's cirrhosis. *Laboratory investigation; a journal of technical methods and pathology*. 1953; 2: 15-21.