



Effects of blocking chemokine receptor CCR1 with BX471 in two models of fibrosis prevention and rescue in mice

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ABSTRACT

Background: The induction, progression and resolution of liver fibrosis are influenced by multiple chemokines. The inhibition of CCR1 signalling by a specific non-peptide inhibitor (BX471) reduces kidney fibrosis after unilateral ureteral obstruction via suppression of leukocyte recruitment in mice. However, it remains unclear whether selective CCR1 inhibition also affects hepatic fibrogenesis. Therefore we aimed to study the effect of this intervention on liver fibrosis in prevention (CCL₄ administration) and rescue (ABCB4-deficient mice) mouse models.

Methods: In the prevention model, hepatic fibrosis was induced by repeated injections of CCL₄. Additionally, the verum group was treated with subcutaneous injections of BX471, while controls received vehicle only. ABCB4 deficient mice (on the BALB/c-background) with sclerosing cholangitis and biliary fibrosis received BX471 or vehicle, respectively (rescue model). Liver histopathology was assessed after Sirius red staining of collagen, and hepatic collagen contents were measured. In addition, we performed gene expression analyses of fibrosis-related genes.

Results: BX471 injections were tolerated moderately well by all mice, and all mice developed hepatic fibrosis. Significant differences were neither observed in serum aminotransferase activities after 6 weeks of treatment between the two groups in the prevention nor in the rescue model. Interestingly, hepatic collagen contents were significantly higher in mice treated with BX471 in the prevention model as compared to controls but histological stages of liver sections did not differ. Of note, we observed only moderate effects on liver fibrosis in the ABCB4 knock-out model.

Conclusions: Our data indicate that BX471 treatment did neither affect serum and tissue markers of liver injury and fibrosis in the CCL₄ model and only moderately in the *Abcb4*^{-/-} model of biliary fibrosis. The animal models indicate that treatment with BX471 alone is unlikely to exert major beneficial effects in chronic liver disease.

1. Introduction

Hepatic fibrosis is a response to chronic liver injury resulting in enhanced production of extracellular matrix (ECM) proteins. During this process, functional parenchyma is replaced by scar tissue leading to diminished liver function and distorted liver architecture [1]. The ECM proteins are produced by hepatic stellate cells (HSC), which become activated upon liver damage and express a variety of inflammatory mediators including chemokines [2]. Chemokines are small chemotactic molecules with cytokine-like functions that mediate inflammatory responses. Depending on their cysteine residues they are classified in C, CC, CXC and CX3C chemokines. They bind to distinct receptors on the

cell surface, induce signalling cascades inside the cell and are involved in a number of biological processes, including growth regulation, embryonic development, and angiogenesis [3]. It was shown that chemokines play an important role during hepatic fibrosis. CXCL9 attenuates liver fibrosis associated angiogenesis in mice [4], and CXCL10 exerts proapoptotic effects mediated by the non-cognate receptor TLR4 in hepatocytes [5]. The chemokine receptors CCR1 and CCR5 promote hepatic fibrosis in knockout mouse models [6], indicating that the blockade of CCR1 might be beneficial for fibrosis treatment. CCR1 is a universal chemokine receptor that binds various ligands with high affinity and is involved in many inflammatory diseases, including rheumatoid arthritis, atherosclerosis, psoriasis, and multiple myeloma [7].

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Also, it has recently been shown that CCR1 expression is increased in immune hyperactivation in patients with critical COVID-19 [8].

BX471 is a non-peptide antagonist that binds to CCR1 and thereby blocks its function. BX471 has been shown to reduce renal fibrosis when applied to unilateral ureteral obstructed mice [9]. Furthermore, BX471 protects mice against acute pancreatitis-associated lung injury [10], ventilator-induced lung injury [11], and it has been shown that this antagonist attenuates the systemic inflammatory response syndrome during sepsis [12]. Here, the authors could also show that BX471 led to reduced activity of myeloperoxidase, a marker of neutrophil infiltration in liver, indicating protection against liver injury. Therefore, BX471 provides a promising therapeutic approach for the treatment of hepatic inflammation and fibrosis.

ABCB4-deficient mice lack the hepatocanicular phospholipid translocase and develop sclerosing cholangitis and biliary fibrosis [13–15]. These mice represent a model for progressive familial intrahepatic cholestasis (PFIC type 3) in patients with severe mutations of the orthologous human gene, which may lead to biliary cirrhosis in children, and resemble primary sclerosing cholangitis in humans [16,17]. Thus, they are used as a model to study fibrogenesis, since no intoxication with chemicals like carbon tetrachloride (CCl_4) is needed. Based on these studies our aim was to block CCR1 with BX471 in prevention (wild-type mice treated with CCl_4) and rescue (ABCB4-deficient mice) fibrosis models to investigate the role of CCR1 in hepatic fibrogenesis and to assess its potential effects in these preclinical models.

2. Materials and methods

2.1. Mice

Mice were hosted under standard conditions (12L:12D photo period) and received water and a standard rodent diet *ad libitum*. All animal experiments were approved by the local government agency (Landesamt für Verbraucherschutz, Saarbrücken, Germany, TV42/08) and were carried out in accordance with Directive 2010/63/EU. All methods were carried out in accordance with the approved guidelines. BALB/c wild-type inbred mice were obtained from Charles River (Sulzfeld, Germany). *Abcb4* knock-out mice were generated by crossing mice with the FVB/NJ background (The Jackson Laboratory, Bar Harbor, USA) onto the BALB/c background for more than 10 generations [14].

2.2. Experimental design

For carbon tetrachloride (CCl_4) experiments, mice were injected

intraperitoneally with CCl_4 (0.7 ml/kg body weight) in mineral oil twice a week for six weeks (12 injections). BX471 and vehicle solution were prepared as described previously [9]. Mice were injected subcutaneously with a dose of 50 mg/kg body weight [18] twice a day during the week and once a day on the weekends or vehicle solution for six weeks (total of 68 injections per mouse), respectively.

For BALB/c wild-type mice ($n = 32$; 8 males and 8 females BX471; 8 males and 8 females vehicle), CCl_4 and BX471 treatment started at the age of seven weeks (Fig. 1A, prevention model). For ABCB4 deficient mice ($n = 24$; 6 male, 6 female BX471; 6 male 6 female vehicle), BX471 treatment started at the age of 10 weeks and was carried out for six weeks as described above (Fig. 1B, rescue model).

After six weeks of treatment mice were sacrificed and livers were harvested in 4% neutral buffered formalin for preparation of paraffin sections or snap-frozen in liquid nitrogen and stored at -80°C for molecular analyses.

For comparison without any treatment, untreated BALB/c wild-type mice ($n = 6$; 3 females and 3 males) and ABCB4 deficient mice ($n = 6$; 3 females and 3 males) were sacrificed at the age of 16 weeks and served as controls.

2.3. Chemical assays

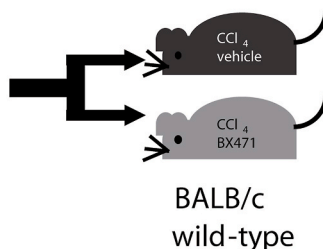
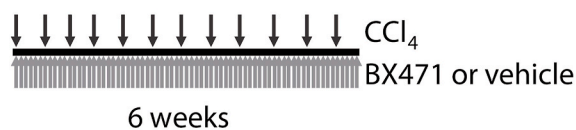
For determination of plasma alanine aminotransferase (ALT) activities, blood was taken prior to the first injection and after sacrifice. After centrifugation, plasma ALT activities were measured with an Olympus AU400 chemistry analyzer, using adapted reagents provided by Olympus [14].

The quantification of hepatic collagen contents was performed in liver hydrolysates via photometric measurement of the specific amino acid hydroxyproline as described [19,20].

2.4. Histopathology

Livers were fixed in 4% neutral buffered formalin at 4°C overnight, embedded in paraffin, and cut into 1–3 μm sections. Sections were stained with Sirius red. In brief, sections were rehydrated in a descending alcohol series, stained 3 min with Weigert, were differentiated with HCl-alcohol and stained 30 min with Sirius red solution (0.1%). After staining, sections were dehydrated in an ascending alcohol series and covered with mounting medium. The amount of Sirius red stained tissue was evaluated using LAS software (Leica, Wetzlar, Germany).

A Prevention model



B Rescue model

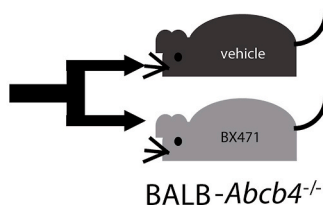


Fig. 1. Experimental design. (A) Prevention model: BALB/c wild-type mice ($n = 32$) were treated intraperitoneally with carbon tetrachloride (CCl_4) twice a week for six weeks (black arrows). Additionally, mice received BX471 ($n = 16$) or vehicle solution ($n = 16$) subcutaneously twice a day during the week and once at weekends (total of 68 injections; grey arrows). (B) Rescue model: ABCB4-deficient mice on the BALB/c background ($n = 24$) were treated with BX471 ($n = 12$) or vehicle solution ($n = 12$; grey arrows). Treatment was carried out for six weeks.

2.5. RNA and gene expression analyses

Total RNA from snap-frozen livers was extracted using Qiagen RNeasy Kit (Qiagen, Germany). One μg RNA was transcribed to cDNA with the High Capacity cDNA reverse transcription kit (Thermo Scientific, Germany) and quality was checked by common β -actin PCR. For quantitative gene expression analyses TaqMan® assays for *α -Sma* (Mm00725412_s1), *Ccr1* (Mm00438260_s1), *Col1a2* (Mm01165187_m1), *Mmp3* (Mm00440295_m1) and *Tgf β* (Mm03024053_m1; all Thermo Scientific, Germany) were used and results were normalized to 18S rRNA. All assays were run on a TaqMan® 7500 Fast real-time PCR system (Life Technologies, Germany). Relative gene expression results were normalized to untreated samples using the $\Delta\Delta\text{C}_t$ method.

2.6. Statistical analyses

Data are presented as means \pm SEM. Statistical analyses were carried out using GraphPad Prism 9.1.2 (GraphPad Software, La Jolla, CA, USA). Non-parametric Kruskal-Wallis tests were used and p values < 0.05 were considered as statistically significant.

3. Results

3.1. Tolerability of BX471 treatment

To assess the effect of BX471 for the treatment of hepatic fibrosis and to exclude a toxic effect in the combination with CCl_4 , two different mouse models were used. On the one hand, BALB/c wild-type mice were injected with CCl_4 and BX471 or vehicle, respectively (Fig. 1A, prevention model, no fibrosis at the start of treatment). ABCB4 deficient mice were injected with BX471 or vehicle (Fig. 1B, rescue model, established fibrosis at the start of treatment). The injections were tolerated moderately well by the mice. Overall, four BALB/c (3 BX471, 1 vehicle) and five *Abcb4*^{-/-} mice (all BX471) died, indicating toxic effects of BX471 treatment in long-term studies. The mice died for no obvious reason (no dramatic weight loss or abnormal behaviour), and

Supplementary Figure 1 provides detailed information on weight curves throughout the experiments.

The effect of BX471 was analysed by immunohistochemical staining and quantification of infiltrating macrophages (CD11b^+ ; Supplementary Figure 2). BX471 administration led to markedly reduced CD11b^+ cells as compared to vehicle-treatment except for male mice treated with CCl_4 , indicating a sex-dependent toxic effect of BX471 and CCl_4 .

3.2. Effects of BX471 treatment on hepatic fibrosis

As we observed sex differences when analysing the results, all data were stratified for male and female mice. As a marker of liver injury, serum alanine aminotransferase (ALT) activities were measured (Fig. 2A–D). As expected, ALT increased upon treatment in both models. Interestingly, male mice treated with CCl_4 and BX471 showed highest ALT levels (Fig. 2A) but there were neither significant differences between BX471- and vehicle-treated mice in female mice in the prevention model (Fig. 2B) nor in male or female *Abcb4*^{-/-} mice with persistent fibrosis (Fig. 2C and D).

To quantify the amount of fibrotic tissue in the livers accurately, the collagen-specific amino acid hydroxyproline was measured (Fig. 2E–H). Compared to non-treated mice hydroxyproline levels were significantly increased after BX471-treatment but showed only a slight increase compared to vehicle-treated mice in the prevention model in both sexes (Fig. 2E and F). Interestingly the opposite was shown for the rescue model as hydroxyproline levels were decreased in BX471-treated mice compared with non-treated animals. This decrease was significant in female mice but again, no difference was observed when comparing BX471- and vehicle-treated mice of both sexes (Fig. 2G and H).

Hepatic fibrosis in the different models was also assessed by quantifying Sirius red stained collagen in liver sections (Fig. 3). It appears that the treatment with BX471 and vehicle solution increases fibrosis in the prevention model and decreases fibrosis in the rescue model, as reflected by hepatic collagen contents. Significant differences were only observed for vehicle controls, but a trend was also visible for BX471 treatment.

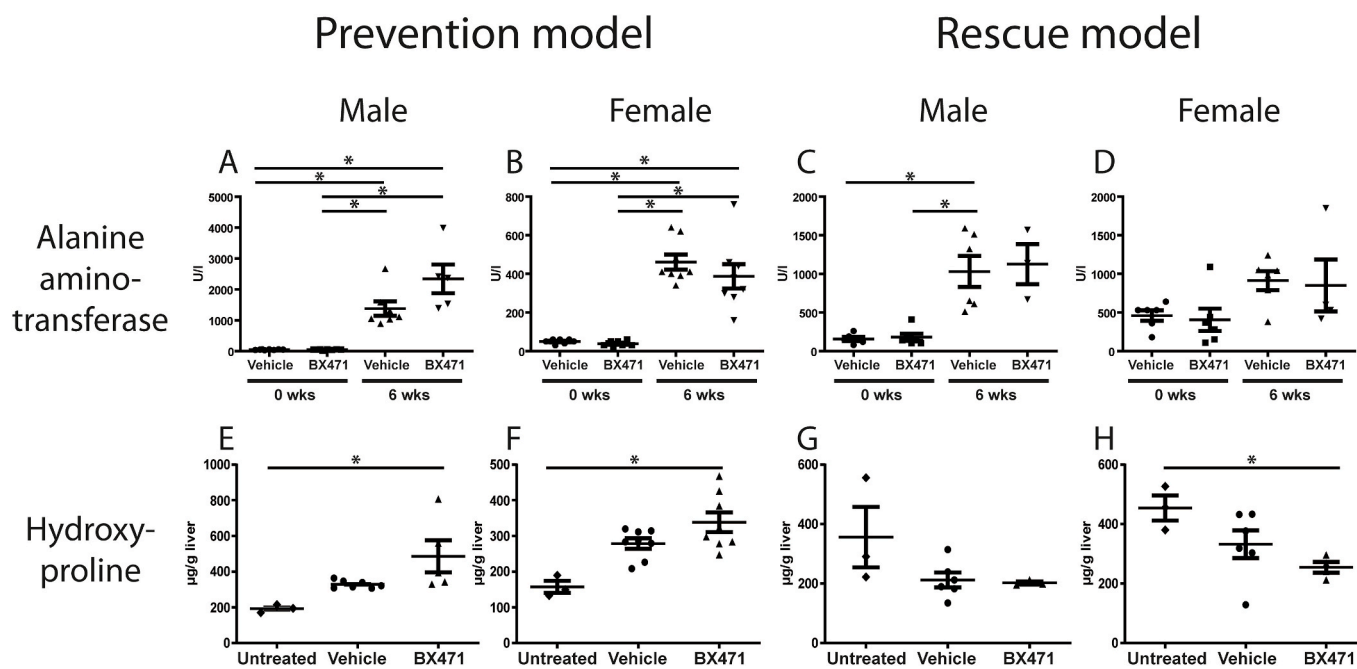


Fig. 2. Phenotypic characterization of liver injury and fibrosis. To analyse the amount of hepatic damage and fibrosis, different parameters were assessed. Plasma alanine aminotransferase activities were measured in the prevention (A, B) and the rescue model (C, D) before and after treatment. Hepatic hydroxyproline contents in the prevention (E, F) and the rescue model (G, H) were quantified. *, p < 0.05.

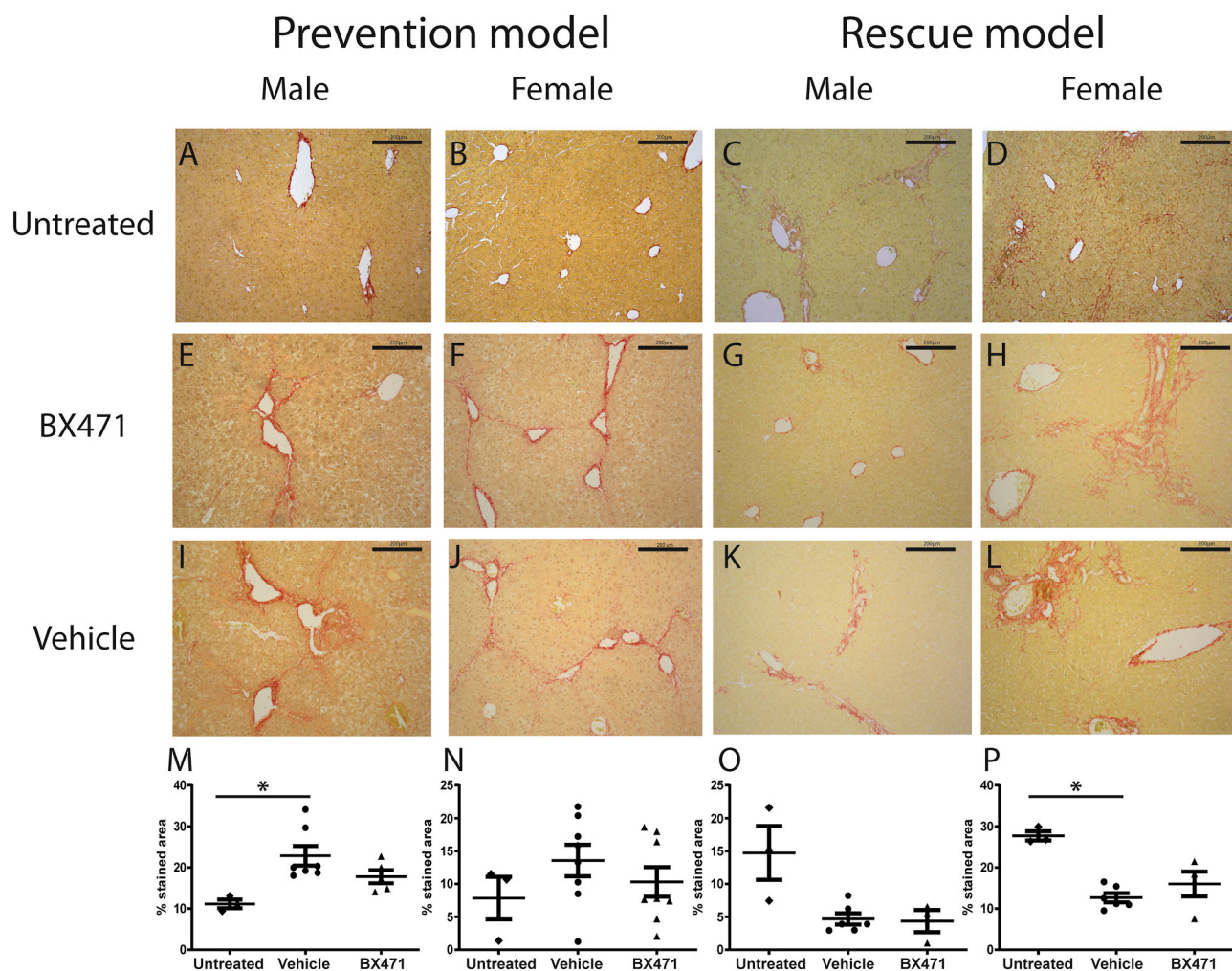


Fig. 3. Histopathology of hepatic fibrosis. Representative images of the different groups stained with Sirius Red to visualize collagen fibers (A–L). Scale bars indicate 200 μm . The areas of Sirius red stained tissue in the livers of the prevention and the rescue model were quantified (M–P). *, $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Gene expression of fibrosis-related markers

The molecular consequences of BX471 treatment in both models were analysed at the mRNA level. Five specific transcripts were selected: *Ccr1* as indicator of BX471 antagonism, *Tgfb* as critical profibrogenic mediator, *Col1a2* and α -SMA as markers for fibrosis, and *Mmp3* as marker of extracellular matrix turnover (Fig. 4).

BX471 administration led to a decrease of *Ccr1* expression as compared to vehicle-treated mice, except for female mice in the prevention model where *Ccr1* expression was induced. Interestingly, in the prevention model, *Tgfb* mRNA steady-state levels were markedly increased in the BX471-treated mice of both sexes as compared to vehicle (Fig. 4E and F), while *Col1a2* and α -Sma levels were not affected (Fig. 4I, J, M and N). *Mmp3* was reduced in female BX471-treated mice (Figure 4Q and R) as compared to vehicle.

In the rescue model, BX471 had no influence on gene expression of any of the markers except for *Col1a2*, which was markedly decreased in female BX471-treated ABCB4-deficient mice (Fig. 4H) as compared to vehicle.

4. Discussion

This study investigates the potential effects of BX471, a non-peptide CCR1 antagonist, in the treatment of hepatic fibrosis. To analyse its efficacy during liver fibrogenesis, two different mouse models were used,

a prevention (CCl₄ challenge) and a rescue model (ABCB4-deficient mice). Mice underwent BX471 and vehicle treatment, respectively. The fibrotic process could only partly be prevented by the therapy with BX471 in the rescue model as shown by hepatic collagen contents in female ABCB4 deficient animals, and BX471 rather increased fibrosis in the prevention model.

Previously BX471 has been reported to have effects on different fibrotic diseases in murine models [9,10,12,18,21]. For example, renal fibrosis could be ameliorated as shown by decreased collagen expression in ureteral obstructed mice after treatment with this CCR1 antagonist [9, 18]. The fact that this does not prove true for the liver is consistent with the notion that core but also organ-specific pathways contribute to fibrosis in different organs [22]. The determination of Sirius red stained area and collagen amount via hydroxyproline lead to mixed results, which might be explained by the fact that by Sirius red, fibrillary collagen deposits in the tissue are detected (type I and III), whereas the hydroxyproline measurement includes fragmented collagen [23].

In contrast to our findings, it has been shown that the lack of CCR1 in CCR1-deficient mice leads to a significant decrease of liver fibrosis [6]. This was shown using toxic (CCl₄) and surgical (bile duct ligation, BDL) models for the induction of liver fibrosis. There might be several explanations for the contrary findings. On the one hand, the effect of a constitutive knockout is much stronger than the transient blockade of small signalling proteins, and on the other hand, there might be toxic interactions of CCl₄ and BX471, which increase liver injury, as indicated

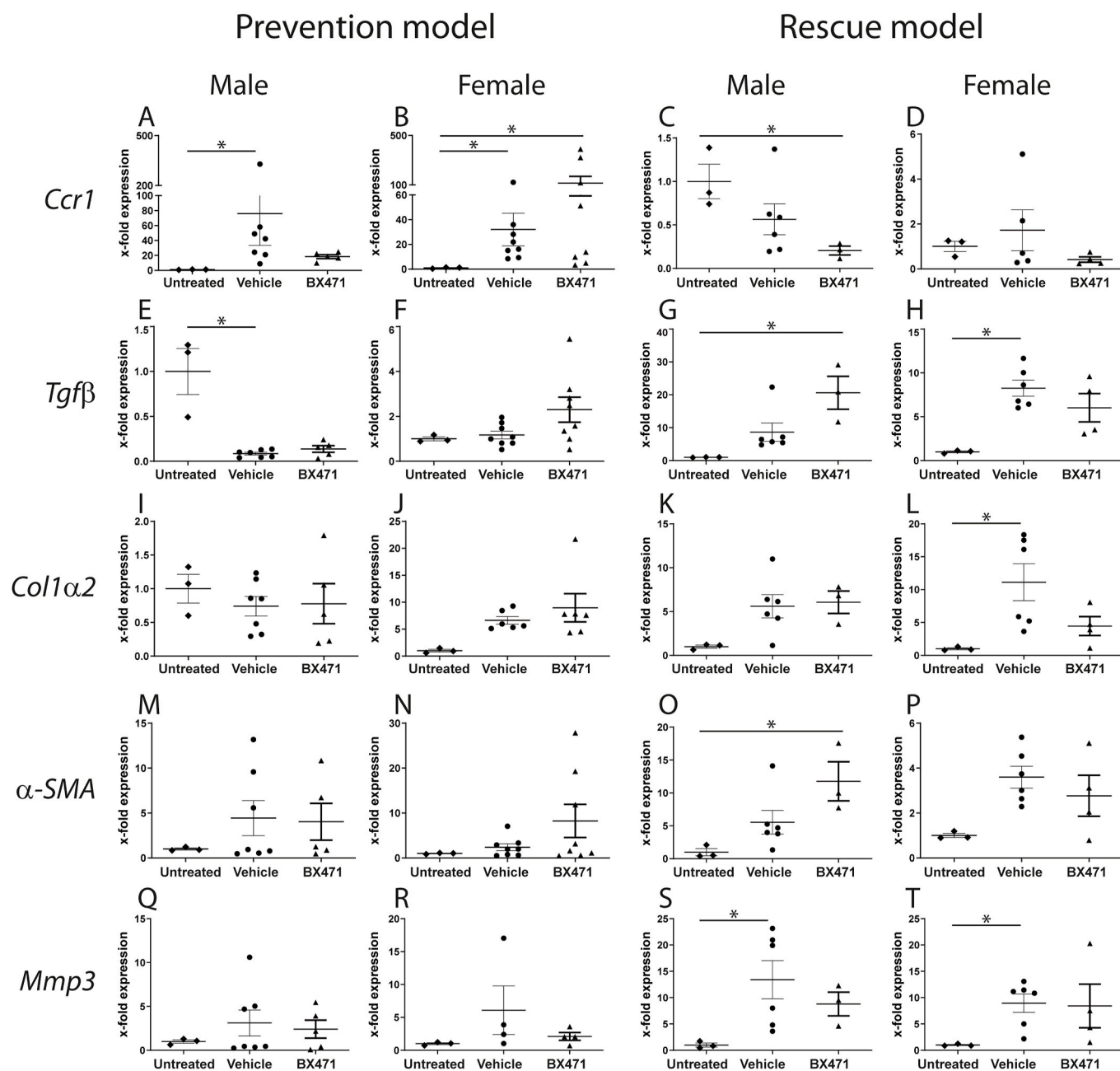


Fig. 4. Gene expression analyses of fibrosis markers. The hepatic mRNA steady-state levels of *Ccr1* and different fibrosis-associated genes were determined in the prevention (A, B, E, F, I, J, M, N, Q, R; left panel) and the rescue model (C, D, G, H, K, L, O, P, S, T; right panel). Abbreviations: *Ccr*, C-C chemokine receptor; *Col*, Collagen; *Mmp*, Matrix metalloproteinase; *Sma*, Smooth muscle actin, *Tgf*, Transforming growth factor.

by elevated ALT levels in male mice in particular in the CCl₄ model. Also, different genetic backgrounds (CCR1-deficient mice were bred to C57BL/6J and BALB/c mice were used in this study) could explain, at least in part, the results. BX471 has been evaluated in patients with multiple sclerosis in a randomized controlled trial, but no differences in the development of new lesions could be observed, assuming that there are other regulatory mechanisms bypassing CCR1 blockade [7]. In addition, Gilchrist et al. [24] tested several CCR1 antagonists with regards to their binding efficiency and reported BX471 to be a member of the “lower binding group”.

Our data suggest that the blockade of CCR1 by BX471 is not sufficient to decrease liver fibrosis in a prevention preclinical model and only had moderate effects in a rescue model of fibrosis. Although the chemokine receptor CCR1 binds multiple chemokines and is therefore involved in numerous chemokine-mediated signalling pathways, the fibrotic process

in liver appears to depend predominantly on alternative factors.

Author contributions

SNW: designed and performed the experiments, wrote the manuscript.

IN: performed experiments.

FG: supervised experiments, reviewed the manuscript.

FL: wrote and reviewed the manuscript, discussed results.

Declaration of competing interest

The authors declare that they have no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2021.101077>.

References

- [1] R. Bataller, D. Brenner, Liver fibrosis, *J. Clin. Invest.* 115 (2005) 209–218, <https://doi.org/10.1172/JCI200524282>. The.
- [2] H.E. Wasmuth, F. Tacke, C. Trautwein, Chemokines in liver inflammation and fibrosis, *Semin. Liver Dis.* 30 (2010) 215–225, <https://doi.org/10.1055/s-0030-1255351>.
- [3] C.R. Mackay, Chemokines: immunology's high impact factors, *Nat. Immunol.* 2 (2001) 95–101, <https://doi.org/10.1038/84298>.
- [4] H. Sahin, E. Borkham-Kamphorst, C. Kuppe, M.M. Zaldivar, C. Grouls, M. Al-samman, A. Nellen, P. Schmitz, D. Heinrichs, M.-L. Berres, D. Doleschel, D. Scholten, R. Weiskirchen, M.J. Moeller, F. Kiessling, C. Trautwein, H. E. Wasmuth, Chemokine Cxcl9 attenuates liver fibrosis-associated angiogenesis in mice, *Hepatology* 55 (2012) 1610–1619, <https://doi.org/10.1002/hep.25545>.
- [5] H. Sahin, E. Borkham-Kamphorst, N.T. do O, M.-L. Berres, M. Kaldenbach, P. Schmitz, R. Weiskirchen, C. Liedtke, K.L. Streetz, K. Maedler, C. Trautwein, H. E. Wasmuth, Proapoptotic effects of the chemokine, CXCL 10 are mediated by the noncognate receptor TLR4 in hepatocytes, *Hepatology* 57 (2013) 797–805, <https://doi.org/10.1002/hep.26069>.
- [6] E. Seki, S. De Minicis, G.-Y. Gwak, J. Kluwe, S. Inokuchi, C.A. Bursill, J.M. Llovet, D.A. Brenner, R.F. Schwabe, CCR1 and CCR5 promote hepatic fibrosis in mice, *J. Clin. Invest.* 119 (2009) 1858–1870, <https://doi.org/10.1172/JCI37444.1858>.
- [7] R. Horuk, BX471: a CCR1 antagonist with anti-inflammatory activity in man, *Mini Rev. Med. Chem.* 5 (2005) 791–804, <http://www.ncbi.nlm.nih.gov/pubmed/16178722>.
- [8] R.L. Chua, S. Lukassen, S. Trump, B.P. Hennig, D. Wendisch, F. Pott, O. Debnath, L. Thürmann, F. Kurth, M.T. Völker, J. Kazmierski, B. Timmermann, S. Twardziok, S. Schneider, F. Machleidt, H. Müller-Redetzky, M. Maier, A. Krannich, S. Schmidt, F. Balzer, J. Liebig, J. Loske, N. Suttrop, J. Eils, N. Ishaque, U.G. Liebert, C. von Kalle, A. Hocke, M. Witzentrath, C. Goffinet, C. Drost, S. Laudi, I. Lehmann, C. Conrad, L.E. Sander, R. Eils, COVID-19 severity correlates with airway epithelium-immune cell interactions identified by single-cell analysis, *Nat. Biotechnol.* 38 (2020) 970–979, <https://doi.org/10.1038/s41587-020-0602-4>.
- [9] H.-J. Anders, V. Vielhauer, M. Frink, Y. Linde, C.D. Cohen, S.M. Blattner, M. Kretzler, F. Strutz, M. Mack, H.-J. Gröne, J. Onuffer, R. Horuk, P.J. Nelson, D. Schlöndorff, A chemokine receptor CCR-1 antagonist reduces renal fibrosis after unilateral ureter ligation, *J. Clin. Invest.* 109 (2002) 251–259, <https://doi.org/10.1172/JCI14040>.
- [10] M. He, R. Horuk, M. Bhatia, Treatment with BX471, a nonpeptide CCR1 antagonist, protects mice against acute pancreatitis-associated lung injury by modulating neutrophil recruitment, *Pancreas* 34 (2007) 233–241, <https://doi.org/10.1097/mpa.0b013e31802e7598>.
- [11] J. Blázquez-Prieto, I. López-Alonso, L. Amado-Rodríguez, E. Batalla-Solís, A. González-López, G.M. Albaiceta, Exposure to mechanical ventilation promotes tolerance to ventilator-induced lung injury by Ccl3 downregulation, *Am. J. Physiol. Lung Cell Mol. Physiol.* 309 (2015) L847–L856, <https://doi.org/10.1152/ajplung.00193.2015>.
- [12] M. He, R. Horuk, S.M. Mochhala, M. Bhatia, Treatment with BX471, a CC chemokine receptor 1 antagonist, attenuates systemic inflammatory response during sepsis, *Am. J. Physiol. Gastrointest. Liver Physiol.* 292 (2007) G1173–G1180, <https://doi.org/10.1152/ajpgi.00420.2006>.
- [13] K. Hochrath, C.S. Stokes, J. Geisel, M.J. Pollheimer, P. Fickert, S. Dooley, F. Lammert, Vitamin D modulates biliary fibrosis in ABCB4-deficient mice, *Hepatology* 59 (2014) 443–452, <https://doi.org/10.1007/s12072-014-9548-2>.
- [14] K. Hochrath, M. Krawczyk, R. Goebel, M. Langhirt, B. Rathkolb, K. Micklich, J. Rozman, M. Horsch, J. Beckers, M. Klingenspor, H. Fuchs, V. Gailus-Durner, E. Wolf, M. Acalovschi, D. a Volmer, M. Hrabě de Angelis, F. Lammert, The hepatic phosphatidylcholine transporter ABCB4 as modulator of glucose homeostasis, *Faseb. J.* 26 (2012) 5081–5091, <https://doi.org/10.1096/fj.12-209379>.
- [15] J.J. Smit, a H. Schinkel, R.P. Oude Elferink, a K. Groen, E. Wagenaar, L. van Deemter, C. a Mol, R. Ottenhoff, N.M. van der Lugt, M. a van Roon, Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease, *Cell* 75 (1993) 451–462, [https://doi.org/10.1016/0092-8674\(93\)90380-9](https://doi.org/10.1016/0092-8674(93)90380-9).
- [16] R. Barikbin, D. Neureiter, J. Wirth, A. Erhardt, D. Schwinge, J. Kluwe, C. Schramm, G. Tiegs, G. Sass, Induction of heme oxygenase 1 prevents progression of liver fibrosis in Mdr2 knockout mice, *Hepatology* 55 (2012) 553–562, <https://doi.org/10.1002/hep.24711>.
- [17] B. Delire, P. Stärkel, I. Leclercq, Animal models for fibrotic liver diseases: what we have, what we need, and what is under development, *J. Clin. Transl. Hepatol.* 3 (2015) 53–66, <https://doi.org/10.14218/JCTH.2014.00035>.
- [18] V. Vielhauer, E. Berning, V. Eis, M. Kretzler, S. Segerer, F. Strutz, R. Horuk, H.-J. Gröne, D. Schlöndorff, H.-J. Anders, CCR1 blockade reduces interstitial inflammation and fibrosis in mice with glomerulosclerosis and nephrotic syndrome, *Kidney Int.* 66 (2004) 2264–2278, <https://doi.org/10.1111/j.1523-1755.2004.66038.x>.
- [19] S. Hillebrandt, C. Goos, S. Matern, F. Lammert, Genome-wide analysis of hepatic fibrosis in inbred mice identifies the susceptibility locus Hfib1 on chromosome 15, *Gastroenterology* 123 (2002) 2041–2051, <https://doi.org/10.1053/gast.2002.37069>.
- [20] I.S. Jamall, V.N. Finelli, S.S. Que Hee, A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues, *Anal. Biochem.* 112 (1981) 70–75, [https://doi.org/10.1016/0003-2697\(81\)90261-X](https://doi.org/10.1016/0003-2697(81)90261-X).
- [21] M. Liang, C. Mallari, M. Rosser, H.P. Ng, K. May, S. Monahan, J.G. Bauman, I. Islam, A. Ghannam, B. Buckman, K. Shaw, G.P. Wei, W. Xu, Z. Zhao, E. Ho, J. Shen, H. Oanh, B. Subramanyam, R. Vergona, D. Taub, L. Dunning, S. Harvey, R. M. Snider, J. Hesselgesser, M.M. Morrissey, H.D. Perez, Identification and characterization of a potent, selective, and orally active antagonist of the CC chemokine receptor-1, *J. Biol. Chem.* 275 (2000), <https://doi.org/10.1074/jbc.M001222200>, 19000–8.
- [22] W.Z. Mehal, J. Iredale, S.L. Friedman, Scraping fibrosis: expressway to the core of fibrosis, *Nat. Med.* (2011), <https://doi.org/10.1038/nm0511-552>.
- [23] A. Baghdasaryan, C.D. Fuchs, C.H. Österreicher, U.J. Lemberger, E. Halilbasic, I. Pählman, H. Graffner, E. Krones, P. Fickert, A. Wahlström, M. Ståhlman, G. Paumgartner, H.-U. Marschall, M. Trauner, Inhibition of intestinal bile acid absorption improves cholestatic liver and bile duct injury in a mouse model of sclerosing cholangitis, *J. Hepatol.* (2015), <https://doi.org/10.1016/j.jhep.2015.10.024>.
- [24] A. Gilchrist, T.D. Gauntner, A. Fazzini, K.M. Alley, D.S. Pyen, J. Ahn, S.J. Ha, A. Willett, S.E. Sansom, J.L. Yarfı, K.A. Bachovchin, M.R. Mazzoni, J.R. Merritt, Identifying bias in CCR1 antagonists using radiolabeled binding, receptor internalization, β -arrestin translocation, and chemotaxis assays, *Br. J. Pharmacol.* 171 (2014), <https://doi.org/10.1111/bph.12835> n/a-n/a.
- [25] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis. <https://doi.org/10.1038/nmeth.2089>, 2012.