Soluble Guanylyl Cyclase Activator BI 685509 Reduces Portal Hypertension and Portosystemic Shunting in a Rat Thioacetamide-Induced Cirrhosis Model

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ABSTRACT

Portal hypertension (PT) commonly occurs in cirrhosis. Nitric oxide (NO) imbalance contributes to PT via reduced soluble guanylyl cyclase (sGC) activation and cGMP production, resulting in vasoconstriction, endothelial cell dysfunction, and fibrosis. We assessed the effects of BI 685509, an NO-independent sGC activator, on fibrosis and extrahepatic complications in a thioacetamide (TAA)-induced cirrhosis and PT model. Male Sprague–Dawley rats received TAA twice-weekly for 15 weeks (300–150 mg/kg i.p.). BI 685509 was administered daily for the last 12 weeks (0.3, 1, and 3 mg/kg p.o.; n = 8–11 per group) or the final week only (Acute, 3 mg/kg p.o.; n = 6). Rats were anesthetized to measure portal venous pressure. Pharmacokinetics and hepatic cGMP (target engagement) were measured by mass spectrometry. Hepatic Sirius Red morphology (SRM) and alpha-smooth muscle actin (αSMA) were measured by immunohistochemistry; portosystemic shunting was measured using colored microspheres. BI 685509 dose-dependently increased hepatic cGMP at 1 and 3 mg/kg (3.92 ± 0.34 and 5.14 ± 0.44 versus 2.50 ± 0.19 nM in TAA alone; P < 0.05). TAA increased hepatic SRM, αSMA, PT, and portosystemic shunting. Compared with TAA, 3 mg/kg BI 685509 reduced SRM by 38%, αSMA area by 55%, portal venous pressure by 26%, and portosystemic shunting by 10% (P < 0.05). Acute BI 685509 reduced SRM and PT by 45% and 21%, respectively (P < 0.05). BI 685509 improved hepatic and extrahepatic cirrhosis pathophysiology in TAA-induced cirrhosis. These data support the clinical investigation of BI 685509 for PT in patients with cirrhosis.

SIGNIFICANCE STATEMENT

BI 685509 is an NO-independent sGC activator that was tested in a preclinical rat model of TAA-induced nodular, liver fibrosis, portal hypertension, and portosystemic shunting. BI 685509 reduced liver fibrosis, portal hypertension, and portosystemic shunting in a dose-dependent manner, supporting its clinical assessment to treat portal hypertension in patients with cirrhosis.

Introduction

In 2017, there were 122.6 million prevalent cases of cirrhosis, causing more than 1.32 million deaths worldwide (2.4% of global deaths) (GBD 2017 Cirrhosis Collaborators, 2020). Portal hypertension is the primary consequence of cirrhosis, with increased portal vein pressure contributing to the pathogenesis and complications that arise during disease progression (Garcia-Tsao et al., 2017). Approximately 60% of patients with cirrhosis present with clinically significant portal hypertension (CSPH), defined as a hepatic venous pressure gradient of ≥10 mmHg (Groszmann et al., 2005; Garcia-Tsao et al., 2017). Portal hypertension also contributes to extrahepatic complications seen in patients with cirrhosis, including portosystemic shunting, splanchnic vasodilation, splenomegaly, and hypoalbuminemia (Bolognese et al., 2014). Currently, treatment guidelines for compensated cirrhosis focus on reducing the severity of portal hypertension and preventing progression to decompensation through etiologic treatments and nonselective beta-blockers (de Franchis, 2015; Garcia-Tsao et al., 2017), rather than treatment of the underlying pathomechanisms of cirrhosis and portal hypertension. Specifically, portal hypertension is driven by increased vascular resistance in the portal circulation caused by functional and structural changes in the liver and increased portal venous flow from splanchnic vessels (Sikuler and Groszmann, 1986). Intrahepatic changes include increased vasoconstriction and

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ABBRVIATIONS: αSMA, alpha-smooth muscle actin; BW, body weight; CSPH, clinically significant portal hypertension; HR, heart rate; HSC, hepatic stellate cell; LSEC, liver sinusoidal endothelial cell; MAP, mean arterial pressure; NO, nitric oxide; PT, portal hypertension; sGC, soluble guanylyl cyclase; SID, once daily; SRM, Sirius Red morphometry; TAA, thioacetamide.
endothelial dysfunction, contributing to collagen deposition in the liver (Garcia-Tsao et al., 2017; Simonetto et al., 2019).

One pathomechanism of cirrhosis and portal hypertension is the imbalance of nitric oxide (NO) (Wiest and Groszmann, 2002) and the reduction of NO's affinity to its receptor, soluble guanylyl cyclase (sGC) (Stasch et al., 2006; Oldenburger et al., 2021). This contributes to the structural and functional drivers of portal hypertension through reduced sGC activation and consequently reduced production of cyclic guanosine monophosphate (cGMP) (Schaffner et al., 2018; Hall et al., 2019). Under conditions of oxidative stress, such as those found in fibrosis (Roehlen et al., 2020), the heme prosthetic group of sGC is oxidized and therefore nonresponsive to NO. Reductions in cGMP levels lead to vasoconstriction and the activation of hepatic stellate cells (HSCs), which become contractile and produce excess extracellular matrix proteins, leading to collagen deposition in the liver (Thimangan and Yee, 1999; Elpek, 2014; Schaffner et al., 2018; Hall et al., 2019; Sandner et al., 2021). Furthermore, reductions in NO also cause capillarization of liver sinusoidal endothelial cells (LSECs), which may further promote activation of HSCs (Xie et al., 2012; DeLeve, 2015), enabling the constriction of portal blood flow and tissue remodeling that lead to portal hypertension and liver fibrosis (Thimangan and Yee, 1999; Elpek, 2014; Hall et al., 2019). Collectively, restoring cGMP levels and NO balance by augmenting sGC activity presents a potential new therapeutic approach for the treatment of portal hypertension in patients with cirrhosis.

The small molecule BI 685509 is a potent, NO-independent sGC activator (Reinhart et al., 2023) shown to be well tolerated in phase I studies in patients with portal hypertension in compensated cirrhosis (Lawitz et al., 2021) and with chronic kidney disease (Cherney et al., 2021) and has progressed to phase II trials for CSPH (NCT05161481), chronic kidney disease (NCT04736628), and diabetic kidney disease (NCT04750577). In previous preclinical studies, BI 685509 was shown to be a potent activator of heme-free human sGC and was orally bioavailable with rapid absorption in mouse and rat models (Reinhart et al., 2023). Given that the pathophysiology of cirrhosis and portal hypertension involves endothelial cell dysfunction, our goal was to test the therapeutic benefit of the sGC activator BI 685509 on progression of liver fibrosis and extrahepatic complications in a preclinical rat model of thioacetamide (TAA)-induced endothelial damage. We used chronic application of TAA to induce clinically relevant features of cirrhosis in rats, including bridging, nodular liver fibrosis, portal hypertension, and portosystemic shunting. Our findings provided preclinical evidence supporting the therapeutic potential of BI 685509 and were instrumental in the decision to initiate clinical investigation in portal hypertension in patients with cirrhosis. Preliminary data from a phase Iib study in patients with mild hepatic impairment showed that BI 685509 was generally well tolerated and reduced portosystemic shunting (Lawitz et al., 2021), and phase II trials are now underway in patients with CSPH and cirrhosis (ClinicalTrials.gov, 2022).

Materials and Methods

Detailed methodology for this study is included in the Supplementary Material.

Ethics Statement. All animal procedures were approved by the Institutional Animal Care and Use Committee of Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT, USA), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal procedures are reported according to the Animal Research: Reporting of In Vivo Experiments guidelines (Percie du Sert et al., 2020).

Animals. Male Sprague–Dawley rats (10–11 weeks old, 300–350 g, n = 84) were supplied by Charles River Laboratories (Kingston, NY, USA) with surgically implanted telemetry transmitters (catalog no. HD-S10; Data Sciences International, St. Paul, MN, USA) in the abdominal artery to enable continuous monitoring of mean arterial pressure (MAP) and heart rate (HR). Upon arrival at Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT, USA), the rats were allowed to acclimate for 3 days prior to starting data collection. The rats were individually housed for the duration of the study.

Experimental Design. After 4 days of baseline telemetry recordings, the rats were administered TAA (first dose: 300 mg/kg; weeks 1–6; 200 mg/kg; weeks 7–15: 150 mg/kg, i.p. [catalog no. 63678-100G, lot no. BCCB7885; Sigma-Aldrich, St. Louis, MO, USA]) twice per week for 15 weeks to induce liver fibrosis (Guerra et al., 2010; Xie et al., 2012; Gracia-Sancho et al., 2019) and were compared with rats receiving no toxicant (naïve; n = 8; Fig. 1A). After 3 weeks of TAA, rats were randomly assigned (n = 9–11 per group) based on MAP, HR, and bodyweight (BW; Supplemental Fig. 1, A–C) to receive BI 685509 [0.3, 1, or 3 mg/kg p.o. once a day (SID)] therapeutically for the final 12 weeks of the study. To test the therapeutic concept in a model of advanced disease, a separate group received acute doses of BI 685509 only during the final week of the study (3 mg/kg p.o. SID, n = 6). Throughout the duration of the study, conscious MAP and HR were recorded over an interval of 10 seconds every 5 minutes using Ponemah Version 6.2 software (Data Sciences International) and reported as weekly 24-hour mean values. BW was recorded twice per week to monitor health status and to adjust TAA and compound dose volumes (weekly BW is shown in Supplemental Fig. 1D). Animals with weight loss exceeding 20% from baseline mass or advanced disease progression were euthanized according to animal welfare guidelines (Fig. 1B) (Wallace et al., 2015). The resulting group size for terminal analyses on Week 15 of the study is represented in Fig. 1A, and these animals are represented for randomization (Supplemental Fig. 1, A–C).

Statistical Analyses. Data were analyzed using GraphPad Prism version 8.3. Survival analysis is presented as a Kaplan–Meier curve. Simple linear regressions were used to present the relationship between pharmacokinetic and target-engagement outcomes. Continuous dyninata were analyzed using a two-way ANOVA with fixed effects of compound and week of study. Multiple comparisons were annotated for relevant comparisons by day within the study (naïve versus TAA; compound versus TAA). For terminal data, naïve and TAA groups were compared with a two-tailed t test, and all treatment groups were compared with the TAA group using a one-way ANOVA with Dunnett’s multiple comparisons. Data are presented as mean ± S.E., with individual data points represented and statistical significance defined as P < 0.05. When biologically meaningful, comparisons with P < 0.1 are discussed.

Results

Pharmacokinetic and Target Engagement Profile of BI 685509. Oral administration of BI 685509 at 0.3, 1, and 3 mg/kg for 12 weeks produced dose-dependent increases in plasma and liver exposures, with similar exposures using acute treatment with 3 mg/kg for 1 week (Table 1). Hepatic cGMP levels, representative of target engagement, increased dose dependently with BI 685509 treatment at 1 mg/kg and 3 mg/kg BI 685509 for 12 weeks (3.92 ± 0.34 and 5.14 ± 0.44 nM, respectively) and also increased with acute treatment with 3 mg/kg BI 685509 for 1 week (4.36 ± 0.77 nM), compared with TAA-treated animals receiving vehicle (2.50 ± 0.19 nM;
**Effects of BI 685509 on Systemic Hemodynamics.** TAA produced acute hypertension and tachycardia during the first week of administration, followed by systemic vasodilation, represented by a reduction in MAP, and sustained HR compared with naïve rats, which is representative of hyperdynamic circulation observed in patients with cirrhosis (Supplemental Fig. 2, A and B). The reduction in MAP and normalization of HR was maintained for the duration of the study in rats treated with TAA compared with naïve rats (Fig. 3, A and D). Acute treatment with BI 685509 for 1 week did not produce any rapid response in MAP or HR (Fig. 3, B and E). Collectively, treatment with BI 685509 at any dose or duration produced no difference in the TAA-induced hypotension (MAP) and tachycardia (HR) during the final week of study compared with TAA-only treated animals (Fig. 3, C and F).

**Effects of BI 685509 on Components of Liver Fibrosis.** Treatment with TAA for 15 weeks induced severe nodularity and paleness in rat livers that could be observed at the macroscopic level (Fig. 4). Macroscopic improvements in liver morphology with BI 685509 treatment were observed, with lessening of the nodules and restoration of liver redness with increasing doses of BI 685509.

Hepatic fibrosis was measured by Sirius Red morphometry (SRM) at Week 15 and expressed as a percentage of liver area (Supplemental Fig. 5A, representative images in Fig. 5E). Hepatic SRM area was 12.3-fold greater in TAA-treated rats compared with naïve rats (16.08 ± 0.77 versus 8.9 ± 0.4 mmHg, respectively; P < 0.05) (Fig. 5, A and B). The reduction in MAP and normalization of HR was maintained for the duration of the study in rats treated with TAA compared with naïve rats (11.65% ± 1.80% and 11.70% ± 1.80%, respectively; P < 0.05). Acute treatment with 3 mg/kg BI 685509 for 1 week resulted in 36% less hepatic SRM area compared with TAA-treated rats (0.3 mg/kg: 2.73% ± 0.44%; 1 mg/kg: 2.68% ± 0.35%; 3 mg/kg: 2.30% ± 0.65%; P < 0.05). Furthermore, acute treatment with 3 mg/kg BI 685509 for 1 week resulted in 36% less hepatic zSMA area compared with TAA-treated rats (3.31% ± 0.03% versus 0.27% ± 0.02%, respectively; P < 0.05) (Fig. 5B, representative images in Fig. 5F). Hepatic zSMA area was lower in a dose-dependent manner in rats treated with BI 685509 for 12 weeks compared with TAA-treated rats (0.3 mg/kg: 2.73% ± 0.44%; 1 mg/kg: 2.68% ± 0.35%; 3 mg/kg: 2.30% ± 0.65%; P < 0.05). Furthermore, acute treatment with 3 mg/kg BI 685509 for 1 week resulted in 36% less hepatic zSMA area compared with TAA-treated rats (3.31% ± 0.03% versus 0.27% ± 0.02%, respectively; P < 0.05). Percentage hepatic ED-1 area, representative of macrophage infiltration, was 3.4-fold greater in rats treated with TAA compared with naïve rats, and no differences were observed with BI 685509 treatment (Supplemental Fig. 3).

To further describe changes in the hepatic fibrosis pattern in a clinically relevant manner, fibrosis and nodularity scoring was performed (Fig. 5, C and D). Thioacetamide for 15 weeks induced a fibrosis score of 3.91 ± 0.03 and nodularity score of 3.00 ± 0.00 compared with naïve rats (0.25 ± 0.06, 0 ± 0, respectively). Treatment with 1 mg/kg BI 685509 for 12 weeks produced lower fibrosis and nodularity scores compared with TAA treatment (3.11 ± 0.25 and 1.56 ± 0.44, respectively; P < 0.05 versus TAA).

**Effects of BI 685509 on Extrahepatic Complications of Cirrhosis.** Portal hypertension was evident, with 80.6% greater portal vein pressure measured in TAA-treated rats compared with naïve rats (16.08 ± 0.77 versus 8.9 ± 0.4 mmHg, respectively; P < 0.05) (Fig. 6A). Compared with TAA treatment, the portal vein pressure was 25.5%, 25.6%, and 21.6% less in rats treated with 1 mg/kg and 3 mg/kg BI 685509 for

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**TABLE 1**

<table>
<thead>
<tr>
<th>BI 685509 dosage (mg/kg/day)</th>
<th>Dosing duration (weeks)</th>
<th>Plasma C&lt;sub&gt;max&lt;/sub&gt; (nmol/L)</th>
<th>Plasma C&lt;sub&gt;min&lt;/sub&gt; (nmol/L)</th>
<th>Liver C&lt;sub&gt;min&lt;/sub&gt; (nmol/L)</th>
</tr>
</thead>
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<td>0.3</td>
<td>12</td>
<td>30.1 ± 7.0</td>
<td>34.0 ± 6.8</td>
<td>32.0 ± 4.6</td>
</tr>
<tr>
<td>1.0</td>
<td>12</td>
<td>70.1 ± 13.7</td>
<td>99.0 ± 29.2</td>
<td>116.6 ± 25.8</td>
</tr>
<tr>
<td>3.0</td>
<td>12</td>
<td>264.6 ± 73.6</td>
<td>490.3 ± 119.2</td>
<td>313.5 ± 63.2</td>
</tr>
<tr>
<td>3.0</td>
<td>1</td>
<td>170.6 ± 39.4</td>
<td>164.8 ± 89.5</td>
<td>163.1 ± 40.5</td>
</tr>
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Exposure concentrations are reported as mean ± S.E.
12 weeks and with acute treatment with 3 mg/kg for 1 week (11.98 ± 0.81, 11.97 ± 0.56, and 12.60 ± 0.85 mmHg, respectively; \( P < 0.05 \)).

Splenic congestion, consistent with the observed portal hypertension, was evident with double the spleen size detected in TAA-treated rats compared with naïve rats (275.79 ± 22.72 versus 141.69 ± 6.46 mg spleen/100 g BW, respectively; \( P < 0.05 \)) (Fig. 6B). Spleen weight was not different in rats treated with BI 685509 compared with TAA, although the response pattern was consistent with that observed with portal vein pressure.

Secondary to portal hypertension, extrahepatic angiogenesis and a reduction in oncotic pressure is observed during cirrhosis through portosystemic shunting, hypoalbuminemia, and mesenteric neovascularization. Minimal portosystemic shunting was observed in naïve rats (7.62% ± 1.23%), whereas this was 2.9-fold greater in rats treated with TAA for 15 weeks (21.89% ± 4.03%; \( P < 0.05 \)) (Fig. 6C). Portosystemic shunting was reduced in rats treated with 1 and 3 mg/kg BI 685509 for 12 weeks (9.78% ± 1.39% and 10.44% ± 1.59%, respectively; \( P > 0.06 \)) compared with TAA. No difference in portosystemic shunting was observed with acute 3 mg/kg BI 685509 for 1 week compared with TAA.

Hypoalbuminemia was observed in TAA-treated rats compared with naïve rats (3.02 ± 0.07 versus 3.34 ± 0.06 g/dL, respectively; \( P < 0.05 \)) (Fig. 6D). Albumin concentrations tended to be preserved with treatment of BI 685509 at 0.3 mg/kg and 1 mg/kg for 12 weeks (\( P = 0.08 \)). Albumin concentrations were greater after acute treatment with 3 mg/kg BI 685509 for 1 week (3.40 ± 0.09; \( P < 0.05 \)) compared with TAA treatment. Serum concentrations of the liver function enzymes alanine transaminase, aspartate transaminase, alkaline phosphatase, and direct bilirubin were also measured and were variably greater in TAA-treated rats compared with naïve rats, with minimal differences observed with BI 685509 treatment (Supplemental Fig. 4).

Mesenteric neovascularization was detected by an 80% to 90% increase in CD31 staining in the jejunal (Fig. 7, A and C) and colonic (Fig. 7B) region of TAA-treated rats compared with naïve rats (jejunal: 10.43% ± 1.32% versus 5.57% ± 0.88%, respectively; \( P > 0.05 \); colon: 13.07% ± 0.98% versus 7.39% ± 0.53%, respectively; \( P > 0.05 \)). The jejunal and colonic mesenteric CD31 area did not differ in rats treated with BI 685509 compared with TAA-treated rats.

**Discussion**

We tested the therapeutic benefit of sGC activation on the progression of liver fibrosis and extrahepatic complications using a preclinical rat model of TAA-induced endothelial damage to support the advancement of BI 685509 into clinical trials in patients with CSPH. We demonstrated that the sGC activator BI 685509 effectively prevented the progression of cirrhosis in a preclinical model that induced clinically relevant nodular, ...
bridging liver fibrosis, portal hypertension, portosystemic shunting, splenomegaly, mesenteric neovascularization, and hypoalbuminemia. These effects were consistent with target engagement in the target organ, as reflected by an increase in cGMP concentrations in the liver and which positively correlated with plasma exposures of BI 685509. Collectively, these data supported the decision to initiate clinical development of BI 685509 in patients with CSPH in compensated cirrhosis (Lawitz et al., 2021; ClinicalTrials.gov, 2022).

In liver cirrhosis and portal hypertension, restoration of cGMP through sGC modulation is of therapeutic interest as it addresses both vasoactive and antifibrotic pathomechanisms (Xiao et al., 2015; Schwabl et al., 2018), offering potential benefits over the current standard of care using beta-blockers.
In other models of cirrhosis and nonalcoholic steatohepatitis, sGC modulation was shown to reduce the severity of fibrosis, decrease portal pressure, and prevent all three aspects of nonalcoholic steatohepatitis pathophysiology (steatosis, inflammation, and fibrosis), supporting development of sGC-targeting therapies (Knorr et al., 2008; Sandner and Stasch, 2017; Flores-Costa et al., 2018; Schwabl et al., 2018; Hall et al., 2019; Brusilovskaya et al., 2020). These investigational agents can act as either sGC stimulators (Stasch et al., 2001, 2002), which bind the reduced, heme-containing form, or sGC activators (Stasch et al., 2006), which bind the heme-free, oxidized form, to increase the production of cGMP independently from NO (Sandner et al., 2021). Under conditions of oxidative stress, the sGC heme becomes oxidized, rendering the enzyme nonresponsive to stimulators (Sandner et al., 2021). Therefore, activators of sGC are known to have greater pharmacological activity under pathological conditions and oxidative stress, as shown with the sGC activator BAY 41-2272 (Thoonen et al., 2015). We have previously shown that sGC activator monotherapy inhibits the

Fig. 5. Assessment of liver fibrosis. Liver fibrosis was assessed by SRM (A: quantification; E: representative images) and αSMA (B: quantification; F: representative images). Histopathology scoring was performed to assess (C) fibrosis and (D) nodularity according to Kleiner et al. (Kleiner et al., 2005). Data were analyzed by a t-test to detect differences between naive and TAA alone, and then a one-way ANOVA with Dunnett’s post hoc multiple comparisons test versus TAA alone was performed. Data are expressed as mean ± S.E., with individual values represented.
progression of diabetic nephropathy in ZSF1 rats, a disease also characterized by oxidative stress (Boustany-Kari et al., 2016). Additionally, BI 685509 has been shown to be a potent activator of heme-free sGC and to demonstrate a low nM potency for the activation of the human and mammalian sGC α1/1 heterodimer (Reinhart et al., 2023). This was contrasted against the sGC stimulator praliciguat (Tobin et al., 2018), which had no effect on heme-free sGC activity (Reinhart et al., 2023). Here Fig. 6. Extrahepatic hemodynamics. (A) Anesthetized portal vein pressure, (B) splenic congestion, (C) portal–systemic collateralization, and (D) serum albumin concentrations were measured at the study’s conclusion. Data were analyzed by a t test to detect differences between naïve and TAA alone, and then a one-way ANOVA with Dunnett’s post hoc multiple comparisons of treatment groups compared with TAA alone was performed. Data are expressed as mean ± S.E, with individual values represented.

Fig. 7. Mesenteric neovascularization. Mesenteric neovascularization was quantified from (A) the jejunal region and (B) the colonic region using CD31 staining. (C) Representative images (10×) from the jejunal region of cirrhotic rats. Data were analyzed by a t test to detect differences between naïve and TAA alone, and then a one-way ANOVA with Dunnett’s post hoc multiple comparisons test versus TAA alone was performed. Data are expressed as mean ± S.E., with individual values represented.
we show that specific activation of sGC via BI 685509 is an effective therapeutic approach in cirrhosis and portal hypertension.

Portal hypertension and cirrhosis drive development of extrahepatic complications. The increased intrahepatic resistance during portal hypertension leads to increased splenic and mesenteric inflow through splanchnic vasodilation and new vessel formation, with splenic congestion actively contributing to maintenance of portal hypertension (Bolognesi et al., 2014). This congestion contributes to splenomegaly and portosystemic shunts and accompanies development of hyperdynamic circulatory syndrome, characterized by increased HR and cardiac output and low arterial blood pressure in patients (Bolognesi et al., 2014). Here we used chronic TAA treatment to induce clinically relevant pathophysiology to test the therapeutic benefit of BI 685509. Using continuous telemetry, we characterized systemic hemodynamics under conscious conditions to capture, in part, hyperdynamic circulatory dysfunction in the presence of TAA, which, to our knowledge, has not been previously described. Taken together, our results demonstrate that TAA treatment resulted in acute tachycardia that preceded renal compensation. As the pathology worsened, systemic vasodilation was apparent, suggestive of increased circulatory volume, which is characteristic of hyperdynamic circulation in patients with portal hypertension (Iwakiri and Groszmann, 2006; Garcia-Pagan et al., 2012). The ensuing hyperdynamic circulation (especially evident after 10 weeks of TAA), and the development of extrahepatic complications, strengthens the pathologic relevance of the rat TAA model in assessing the impact of therapies on portal hypertension and associated complications.

BI 685509 treatment did not affect the reduction in MAP seen with TAA-induced hyperdynamic circulation; however, treatment with 1 or 3 mg/kg BI 685509 changed the trajectory of the impact of TAA on MAP after week 10, with MAP reductions appearing to plateau after this point. This observation is notable as increased cGMP levels, via activation of sGC, act as a vasodilator, and therefore treatment with BI 685509 in otherwise healthy animals would be expected to acutely reduce systemic blood pressure (Reinhart et al., 2023). In previous preclinical studies of BI 685509, single high doses were associated with decreased MAP and increased HR; however, these effects could be attenuated with repeat dosing (Reinhart et al., 2023). In the present study, we specifically evaluated the acute and chronic effects of BI 685509 on MAP and HR, with no worsening in diseased rats, despite the intrinsic vasodilatory effect of this compound. These results, together with previous data, show that effects on blood pressure are unlikely to increase over time and that the risks may be reduced with dose titration.

Treatment with BI 685509 reduced hepatic fibrosis and other extrahepatic complications of cirrhosis. Notably, chronic TAA induced bridging fibrosis with a histopathology score of 4, demonstrating that the disease severity tested is consistent with cirrhosis in patients. Treatment with BI 685509 not only reduced the hepatic fibrosis area but also produced a lower fibrosis stage preclinically than treatment with TAA, which has been proposed as an endpoint in registration trials of cirrhosis (Rivera-Esteban et al., 2021).

Beyond the liver, we demonstrate the therapeutic potential of BI 685509 to alleviate complications of cirrhosis, including portal hypertension, portosystemic shunting, and hypoalbuminemia. Modulation of sGC can help to ameliorate these extrahepatic complications by reducing the intrahepatic resistance observed in portal hypertension and increasing hepatic vasodilation, through both vasoactive and antifibrotic mechanisms (Schwabl et al., 2018; Brusilovsky et al., 2020). Portosystemic shunting in portal hypertension occurs via vasodilation and angiogenesis as a result of the increased intrahepatic resistance. Consequences of portosystemic shunting include gastrointestinal hemorrhage, ascites, and hyperdynamic circulation in patients with cirrhosis (Bolognesi et al., 2014). Treatment with BI 685509 reduced portal vein pressure and portosystemic shunting in TAA-induced portal hypertension, showing the potential vasoactive mechanisms of this compound. Also encouraging was the observed trend for BI 685509 to restore hypoalbuminemia produced with TAA treatment. Albumin concentrations have been shown to be reduced in cirrhosis, with human albumin being used as a treatment of patients with cirrhosis and hypoalbuminemia to improve hemodynamic imbalances (Arroyo, 2002). Collectively, although preclinical rodent models do not reliably recapitulate gastrointestinal hemorrhaging and ascites, we demonstrate a clear potential for BI 685509 treatment to benefit the angiogenic pathophysiology of cirrhosis within the preclinical limitations.

The TAA model of cirrhosis also reflects the hepatic cellular mechanisms of human cirrhosis, inducing LSEC capillarization, HSC activation, and persistent collagen deposition (via Sirius Red staining) (Xie et al., 2012). In conjunction with severe portal hypertension, the TAA model produces a lower incidence of ascites than other toxic models of cirrhosis, providing a model of cirrhosis that remains stable for several weeks, even after TAA is withdrawn (Liedtke et al., 2013). Although LSEC capillarization was not investigated in the present study, Xie et al. have shown that TAA-induced capillarization could be reversed with an sGC activator, which, in turn, promoted HSC quiescence (Xie et al., 2012). Indeed, in the present study, HSC activation, represented by greater hepatic sSMA area, was dose dependently decreased with BI 685509 treatment, and this raises the potential for a benefit in LSEC capillarization.

The results of the present study are consistent with studies of other sGC modulators in various preclinical models of liver fibrosis and therefore strengthen the therapeutic concept of modulating sGC in cirrhosis. The sGC stimulator riociguat decreased portal pressure, liver fibrosis, and inflammatory markers in bile-duct-ligated rats (Brusilovsky et al., 2020). In addition, a study of the sGC stimulator BAY 41-2272 used the bile-duct-ligation model to show that BAY 41-2272 treatment significantly decreased collagen and expression of fibrotic markers (Sandner and Stasch, 2017). The preclinical studies of the sGC activator BAY 60-2270 used two different models of fibrosis, including carbon tetrachloride and a pig serum model (Knorr et al., 2008). The scar collagen formation in the carbon tetrachloride model is due to hepatic inflammation and necrosis, whereas the pig serum model induces septal fibrosis with minimal necrosis. Once-daily treatment with BAY 60-2270 prevented 60% to 75% of fibrosis across both models (Knorr et al., 2008). Although the preclinical evidence for these compounds is promising, they have yet to enter human trials, unlike BI 685509, which has already been shown to be well tolerated in phase Ib trials in patients with chronic kidney disease and mild/moderate hepatic impairment (Cherney et al., 2021; Lawitz et al., 2021), and is currently under investigation in patients with cirrhosis (ClinicalTrials.gov, 2022).

Similar to cirrhosis, impaired NO signaling is implicated in diabetic nephropathy, with conditions of oxidative stress...
Conclusions

Compounds that restore cGMP through sGC activation are suitable for patients with pathophysiologies involving oxidative stress. Here we demonstrate the therapeutic potential of the sGC activator BI 685509 in cirrhosis and the associated outcomes. By modeling cirrhosis and portal hypertension preclinically with TAA, we observed the benefit of BI 685509 for key parameters, including liver fibrosis, portal vein pressure, portosystemic shunting, and hypoalbinemia. These therapeutic benefits were observed with both chronic application and acute application in established disease, without worsening of systemic hemodynamic parameters. Collectively, these data support the possibility that BI 685509 could address a critical clinical need in treating patients with cirrhosis and portal hypertension.

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Authorship Contributions

Participated in research design: Jones, Fryer.
Conducted experiments: Jones, Chen, Ng, Villalona, McHugh, Zeveleva, Wilks, Brilisauer, Bretschneider.
Performed data analysis: Jones, Chen, Ng, Villalona, McHugh, Zeveleva, Wilks, Brilisauer, Bretschneider, Qian, Fryer.
Wrote or contributed to the writing of the manuscript: Jones, Chen, Qian, Fryer.

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SUPPLEMENTARY MATERIAL

Materials and Methods

Animals

Rats were pair-housed with the same companion for the duration of study in a hut with a chew stick for enrichment. Rats were maintained in a 12-hour light–dark cycle with standard rodent chow (Teklad Global 16% protein rodent diet #2016C; Envigo, Indianapolis, IN, USA) and water provided ad libitum.

Terminal Procedures

Blood Sampling. Blood samples were collected by tail-vein venipuncture on Week 15 of treatment for pharmacokinetic analyses at 0.5 hours and 24 hours post dosing of BI 685509 to obtain systemic maximum concentration (C_{max}) and minimum concentration (C_{min}), respectively. Whole blood was transferred into EDTA tubes and plasma separated by centrifugation (10,000 rpm × 10 min at 4°C).
**Portal Vein Pressure.** After 15 weeks of thioacetamide (TAA), rats were anesthetized with 2.5% isoflurane in dorsal recumbence and a mid-line incision was performed to expose the abdominal cavity. The portal vein was separated from the surrounding tissue and cannulated with polyethylene (PE)-50 tubing filled with saline. Portal vein pressure was measured via a fluid-filled physiologic pressure transducer (Statham P23XL; Spectramed, Oxnard, CA, USA) and recorded using Ponemah Version 5.1 Software (DSI, St. Paul, MN, USA) in three 10-second intervals after the signals were stabilized, and the values were averaged per animal.

**Portosystemic Collateralization.** Immediately after portal pressure values were recorded, portosystemic shunting was measured by injecting 150,000 yellow microspheres (15 µm diameter, Dye-Trak VII+ Microspheres; Triton Technology, San Diego, CA, USA) into the spleen of each rat. Microspheres were prepared in neutral saline containing 0.05% Tween80 and diluted for injection of 100 µl per rat. Five minutes after injection, rats were euthanized by cardiac puncture and exsanguination. The entire lung and ~2 g of liver were collected. The microspheres were recovered by sedimentation according to the manufacturer’s protocol, using 10,000 Blue Dye-Trak microspheres as a processing control for each sample. Absorbance was recorded for each organ recovery at 390 nM (yellow) and 670 nM (blue) in a 96-well format. Liver absorbance was corrected for total liver weight. The proportion of microspheres recovered in the lung represents the divergence of blood flow away from the liver and toward the periphery. (Chojkier and Groszmann, 1981; Gallego et al., 2017) Thus, portosystemic shunting equals the proportion of lung absorbance divided by lung and liver absorbance combined.

**Sample Collection and Processing,** After collecting the portal pressure and portosystemic collateralization data, animals were euthanized by exsanguination and thoracotomy. Whole blood was transferred into EDTA tubes on ice or allowed to clot at room temperature for 30 minutes. Plasma and serum were separated by centrifugation (10,000 rpm × 10 min at 4°C).
Subsequently, the liver (right lobe), spleen, and mesenteric tissue were dissected and weighed. All hepatic analyses were performed on liver tissue obtained from the right lobe.

Pharmacokinetic and Biochemical Measurements

**Pharmacokinetics.** Quantitative bioanalysis was performed by Covance (Princeton, NJ, USA) with a fit-for-purpose assay using protein precipitation followed by liquid chromatography-tandem mass spectrometry. An API 4000 triple quadrupole mass spectrometer (AB Sciex, Toronto, ON, Canada) was operated in multiple reaction monitoring (MRM) mode for simultaneous multi-analyte quantification using the following MRM transitions (Q1/Q3): BI685509 (583.4/244.2) and internal standard 13C2,2H5 and BI685509 (590.5/244.2), and internal standards verapamil (455.4/165.2) and propranolol (260.2/116.2) in positive-ion mode. Protein precipitation of plasma samples was performed with 10 µl plasma and 70 µl acetonitrile. Hepatic exposures were determined from liver tissue (~200 mg) transferred into 2 ml Precellys® tubes (1.4/2.8 mm beads; 91-PCS-CKM; Bertin Instruments, Montigny-le-Bretonneux, France). Acetonitrile–methanol (1:1)/H2O (75/25) was added in an amount four-fold the tissue weight. Tissues were homogenized using a Precellys® Evolution tissue homogenizer (Bertin Instruments) with settings at 5500 rpm/three cycles of 40 seconds on/30-second pause. Liver supernatants were collected after centrifugation for 4 min at 4000 rpm and 4°C, then further processed as described above for plasma.

**Cyclic Guanosine Monophosphate (cGMP).** Liver samples were immediately frozen in liquid nitrogen and stored at −80°C. Liver tissue samples were diluted 1:5 (w/v) in an extraction buffer (2% formic acid + 2 mM 3-isobutyl-1-methylxanthine) and homogenized using a Precellys® Evolution tissue homogenizer (Bertin Instruments). After centrifugation of the homogenate, 250 µl of the supernatant fluid was mixed with 5 µl of the internal standard (13C15N2-labeled cGMP, 1 µM in 2% formic acid) and transferred to solid-phase extraction (Oasis WAX 96-well plate, 30 µm, 10 mg; Waters, Eschborn, Germany). Standard curves and cGMP-
spiked quality controls were prepared in the extraction buffer and mixed with the internal standard before solid-phase extraction. For solid-phase extraction, the 96-well plate was preconditioned with 200 µl methanol, followed by 200 µl 2% formic acid. After sample loading, the plate was washed initially with 200 µl 2% formic acid, followed by 200 µl methanol + 2% formic acid. The analytes were eluted twice each with 75 µl methanol + 5% NH₃. The eluate was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 µl 0.1% formic acid and analyzed by mass spectrometry.

An Agilent 1290 Infinity II liquid chromatography (LC) system (Agilent, Santa Clara, CA, USA) was coupled to an API 6500+ (AB Sciex, Darmstadt, Germany) mass spectrometer, and 10 µl of sample were injected. The LC was equipped with an Acquity Premier UPLC HSS T3 1.8 µm 2.1 × 100 mm (Waters) at 40°C. Solvent A consisted of 0.1% formic acid and solvent B of acetonitrile + 0.1% formic acid. The LC gradient (total 5 min) started at 0% solvent B for 0.5 min and increased to 5% solvent B within 0.3 min. From 0.8 to 2.5 min, the gradient was kept at 5% solvent B and increased to 100% solvent B within 0.7 min. From 3.2 to 4.0 min, the gradient was kept at 100% solvent B and decreased to 0% solvent B within 0.2 min. The column was conditioned for 0.8 min at 0% solvent B. The flow rate of the LC was 400 µl/min.

The mass spectrometer was operated in positive-ion mode with a source temperature of 400°C, CAD gas = 8, gas 1 = 50 and gas 2 = 50, curtain gas = 40. The analytes were monitored by MRM, and the following transitions were recorded: cGMP Q1 = 346.2, Q3 = 152.1, DP = 100, CE = 35; 13C15N₂-cGMP Q1 = 349.1, Q3 = 155.1, DP = 100, CE = 35. The peak area of cGMP was normalized to the internal standard signal of 13C15N₂-cGMP and quantified using Analyst 1.6 (AB Sciex).

**Serum Biomarkers.** Concentrations of albumin, aspartate transaminase, alanine transaminase, alkaline phosphatase, and direct bilirubin were analyzed by Crown Bioscience,
Inc. (New Iberia, LA, USA) using a Beckman Coulter AU480 Chemistry Analyzer (2018072087; Beckman Coulter, Brea, CA, USA).

Tissue Histology

Liver fibrosis. The right liver lobe was sliced (3 mm), placed into a histo-cassette, fixed by immersion in 10% phosphate-buffered formalin for 48 hours, then transferred into 70% ethanol for storage. Samples were later embedded and sectioned at 4 μm thickness for Sirius Red morphometry (SRM) and immunohistochemical alpha-smooth muscle actin (αSMA) and ED-1 staining by Wax-it Histology Services (Vancouver, BC, Canada). For quantification of SRM, all 70 slides were scanned using a Vectra® 3 multispectral imager (Akoya, Marlborough, MA). An average of 72 multispectral images per liver were captured at 10× magnification and analyzed using the InForm® 2.4.8 image analysis platform (Akoya, Marlborough, MA). The lumens of large vessels were excluded from the analysis, and portal tracts were included. Data were expressed as a percentage of SRM staining area.

Separately, liver sections were submitted to Probetex, Inc. (San Antonio, TX, USA) for histopathologic assessment. Liver sections were stained with hematoxylin and eosin and subsequently scored by a trained histopathologist who was blinded to the treatment groups. First, a general assessment of liver fibrosis was conducted on 10 low-power fields at 10× magnification using the Kleiner grading system.(Kleiner et al., 2005) Secondly, nodularity, fragmentation, and carcinomas were evaluated based on the number of lesions in the liver section.

Liver αSMA, ED-1. Slides of 4 μm liver sections were air dried overnight at 37°C, dewaxed, and rehydrated in graded ethanol to phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked by immersion in 3.0% hydrogen peroxide in methanol for 15 min. Sections were then washed and incubated with the primary antibody for αSMA (ab124964; Abcam,
Cambridge, MA, USA) at a dilution of 1:1000 and the primary antibody for ED-1 (MCA341R; Bio-Rad, Hercules, CA, USA) at a dilution of 1:500 and were subsequently incubated with the respective secondary antibodies (ImmPress Reagent Kit; Vector Laboratories, Burlingame, CA, USA) for 30 minutes in a hydration chamber. Immunoperoxidase detection was performed using the avidin–biotin complex method (Vector Laboratories) using 3,3’-diaminobenzidine tetrahydrochloride as substrate. The primary antibody was replaced with PBS as a negative control. For quantification of immunohistochemistry (IHC) of αSMA and ED-1, all slides were scanned using Vectra® 3 multispectral imager (Akoya, Marlborough, MA). An average of 83 αSMA-labeled and 63 ED-1-labelled multispectral images per liver were captured at 10× magnification and analyzed using the InForm® 2.4.8 image analysis platform. Most of the lumen and the laminar collagen from large vessels were excluded from the analysis, and portal tracts were included. Data are expressed as a percentage of positive αSMA- and ED-1-stained area.

Mesenteric Neovascularization. The entire mesentery adjacent to the jejunum or colon was separately dissected at the end of the study, fixed by immersion in 10% phosphate-buffered formalin for 48 hours, then transferred to 70% ethanol for storage. A section from each of the four middle branches proximal to the jejunum or colon was dissected for tissue processing, microtomy, chromogenic IHC, and quantification of the CD31-positive area. The IHC stain was performed on the Leica Bond Rx (Leica Biosystems, Wetzaler, Germany), using recombinant monoclonal anti-CD-31 antibody (1:2000 [0.279 µg/ml] [ab182981; Abcam]) and a pH 6 antigen-retrieval solution (ER1). The CD31-IHC-stained slides were scanned using the Leica APERITO AT2 Scanner (Leica Biosystems). Scanned images were analyzed for the percentage CD31-IHC-positive area using HALO V3.0 software (Indica Labs, Albuquerque, NM, USA). The percentage of positive stain for each animal was obtained from the combined results from the four analyzed images in each slide.
Supplementary figures

Fig. S1.

Randomization and BW response. After 3 weeks of TAA treatment, rats were randomized to begin treatment with BI 685509 based on MAP (A), HR (B), and BW (C and D). Weekly BW was monitored throughout study duration for animal health and to adjust dosages. Data are expressed as mean ± S.E., with individual means represented for discrete data.

BW, body weight; HR, heart rate; MAP, mean arterial pressure; TAA, thioacetamide.
Fig. S2.

Systemic hemodynamics response to TAA. Representative data of the effect of TAA on MAP (A) and HR (B) in rats prior to the start of treatment with BI 685509. Continuous data were analyzed by two-way ANOVA with repeated measures. Data are expressed as mean ± S.E.

HR, heart rate; MAP, mean arterial pressure; TAA, thioacetamide.
Assessment hepatic ED-1. Macrophage infiltration was assessed by ED-1 area (A: Quantification; B: Representative images) Data were analysed by a t-test to detect differences between naïve and TAA alone and then a one-way ANOVA with Dunnett’s post hoc multiple comparisons test versus TAA alone was performed. Data are expressed as mean ± S.E., with individual values represented.

TAA, thioacetamide.
Serum biomarkers. Serum (A) ALT, (B) AST, (C) ALP, and (D) D. bilirubin concentrations were measured at the study conclusion. Data were analysed by a t-test to detect differences between naïve and TAA alone and then a one-way ANOVA with Dunnett’s post hoc multiple comparisons test versus TAA alone was performed. Data are expressed as mean ± S.E., with individual values represented.

ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; TAA, thioacetamide.
Supplementary References

