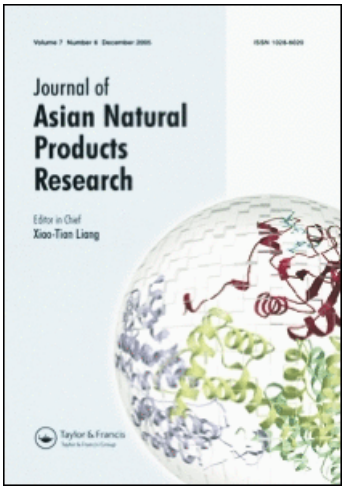

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Effects of bicyclol on immunological liver fibrosis in rats

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ORIGINAL ARTICLE

Effects of bicyclol on immunological liver fibrosis in rats

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Liver fibrosis results from chronic liver injury in conjunction with the accumulation of extracellular matrix proteins. The present study was performed to estimate the effect of bicyclol on bovine serum albumin (BSA)-induced immunological liver fibrosis in rats. Bicyclol (**1**) (100, 200, and 300 mg/kg) was given to rats by oral administration once a day for 5 weeks from the fourth week of intravenous injection of BSA. Blood and liver tissues were collected for the measurement of hydroxyproline (Hyp), procollagen type III (PIIIP), hyaluronic acid (HA), and transforming growth factor β-1 (TGF-β1) levels and liver pathological changes. The mRNA and protein expressions of hepatic TGF-β1, interleukin-1 (IL-1), IL-10, MMP-2, TIMP-1, phosphorylated p38 (Pp38), and Smad2/3 were detected by reverse transcription polymerase chain reaction and Western blot. As a result, bicyclol significantly protected against BSA-induced liver fibrosis as evidenced by the reduction of elevated serum HA, PIIIP, and hepatic Hyp in rats, while liver pathological changes were also alleviated. The overexpressions of hepatic TGF-β1, IL-1β, IL-10, MMP-2, and TIMP-1 were suppressed by bicyclol in BSA-treated rats. The phosphorylations of Pp38 and Smad2/3 were also inhibited after bicyclol treatment. The hepatoprotection of bicyclol was mainly due to the modulation on the expression of inflammatory/anti-inflammatory cytokines, downregulation of hepatic TGF-β1, and inhibition of hepatic collagen synthesis.

Keywords: bicyclol; bovine serum albumin; liver fibrosis

1. Introduction

Liver fibrosis is proved to be a treatable complication of advanced liver disease with increased deposition and altered composition of the extracellular matrix (ECM) in the liver [1]. As a pivotal and necessary stage to cirrhosis, liver fibrosis leads to the lethal complications and high mortality [2,3]. The main causes of liver fibrosis include chronic HBV/HCV infections, alcohol abuse, and non-alcoholic steatohepatitis [4–7]. Although much progress has been made in understanding the mechanisms of liver fibrosis and its therapeutic regimen, there is no ideal therapy. Therefore, it is of great significance to prevent liver fibrosis in theory and in practice.

Bicyclol, 4-dimethoxy-5,6,5′,6′-dimethylenedioxy-2-hydroxymethyl-2′-carbonyl biphenyl (**1**; Figure 1), is an antihepatitis drug for the treatment of chronic hepatitis B patients in clinical practice. Previous studies have shown that **1** markedly alleviated experimental liver

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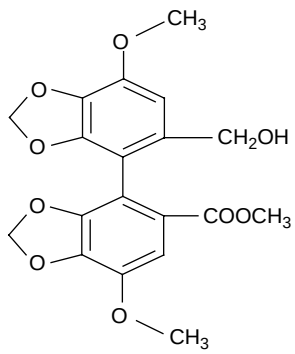

Figure 1. Chemical structure of bicyclol (**1**).

fibrosis induced by chemical toxins including carbon tetrachloride (CCl_4) and dimethylnitrosamine in murine [8,9]. The protective mechanism was related to the antioxidation, regulation of cytokine secretion, inhibition of apoptosis, etc. There has been no report for the effect of **1** on immunological liver fibrosis. Therefore, the present study was designed to investigate the effect of **1** on immunological liver fibrosis induced by bovine serum albumin (BSA) and possible mechanisms in rats.

2. Results and discussion

2.1 Effect of bicyclol on liver fibrosis induced by BSA in rats

Liver fibrosis induced by BSA was indicated by the elevated hepatic hydroxyproline (Hyp) content (3-fold, $P < 0.01$), serum levels of hyaluronic acid (HA, 2.3-fold, $P < 0.01$), procollagen type III (PIIIP, 5.2-fold, $P < 0.01$), and liver damages characterized by the hyperplasia of the lattice fibers and collagenous fibers in the portal area and extended outwards in rats. Bicyclol (**1**) treatment resulted in a significant decrease in liver Hyp and serum HA, PIIIP (Figures 2 and 3), while the liver histopathological changes were also improved in BSA-treated rats (Figures 4 and 5).

2.2 Effect of bicyclol on liver function in BSA-treated rats

In the BSA-treated group, the levels of alanin aminotransferase (ALT) and aspartate aminotransferase (AST) increased but had no significant difference compared with the control group, whereas the ratio of albumin and globulin (A/G) also decreased with no statistical significance (Table 1).

Transaminase activities and the ratio of A/G in the **1**-treated group tended to decrease but had no significance compared with the model group.

2.3 Effect of bicyclol on serum and hepatic transforming growth factor β-1, and signal pathways in BSA-treated rats

Hepatic transforming growth factor β-1 (TGF-β1) is the most potent profibrogenic mediator in liver fibrosis. p38 MAPK and Smad pathways are known to be involved

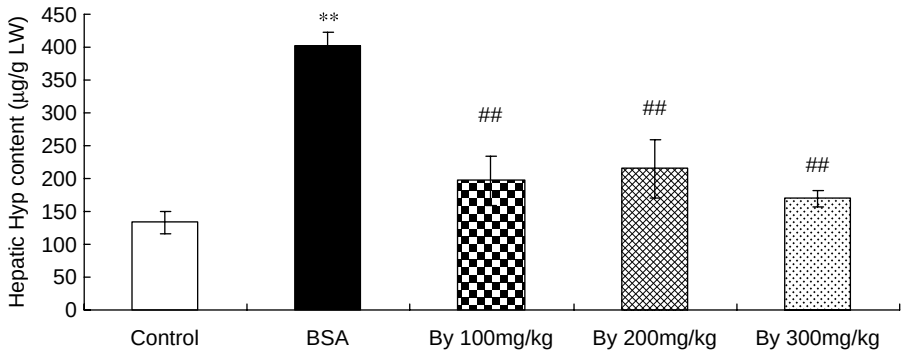

Figure 2. Effect of bicyclol on the elevation of liver Hyp content in BSA-treated rats ($n = 15$). Bicyclol (300 mg/kg) was administered orally after the fourth week of intravenous injection for 5 weeks. $^{**}P < 0.01$ vs. control group, $^{\#\#}P < 0.01$ vs. BSA group.

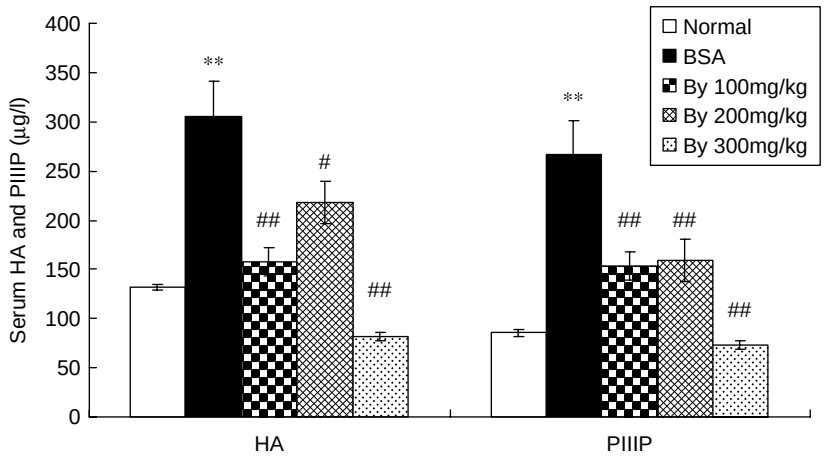

Figure 3. Effect of bicyclol on serum HA and liver PIIIP levels in BSA-treated rats ($n = 15$). Bicyclol (300 mg/kg) was administered orally after the fourth week of intravenous injection for 5 weeks. Rats were sacrificed after the last administration of bicyclol. $^{**}P < 0.01$ vs. control group, $^{\#\#}P < 0.01$ vs. BSA group.

in the signal transmitting of TGF-β1. The expressions of TGF-β1 and phosphorylation of p38 MAPK and Smad2/3 were evaluated at both gene and protein levels after BSA treatment. As displayed in the results (Figures 6 and 7; Table 2), the elevation of hepatic TGF-β1 at gene (TGF-β1/β-actin is 1.09 and 1.14 in the control group and BSA-treated group, respectively) and protein (4-fold) levels was found, while the phosphorylated p38 (Pp38) MAPK (2-fold) and Smad2/3 (2.15-fold) were also increased in rats treated with BSA. Bicyclol (**1**) significantly inhibited the overexpression of TGF-β1 (4% at mRNA level and 68% at protein level), phosphorylations of p38 MAPK (31%) and Smad2/3 (37%), respectively.

2.4 Effect of bicyclol on the mRNA expression of inflammatory cytokines in BSA-treated rats

To determine the effect of **1** on the involvement of inflammatory and anti-inflammatory cytokines in liver fibrosis, the mRNA expressions of interleukin-1β (IL-1β) and IL-10 were examined by reverse transcription polymerase chain reaction (RT-PCR). As shown in Figure 6 and Table 2, the mRNA levels of hepatic IL-1β (17.4%) and IL-10 (12.6%) were all amplified by BSA. Bicyclol (**1**) significantly inhibited the elevation of IL-1β (32.5%, $P < 0.05$), while the increase in IL-10 expression was also reduced in BSA-treated rats (6.5%, $P < 0.01$).

2.5 Effect of bicyclol on the mRNA expression of hepatic MMP-2 and TIMP-1 in BSA-treated rats

Liver matrix metalloproteinases (MMPs) and their specific inhibitors – tissue inhibitor of metalloproteinases (TIMPs) – play an important role in both fibrogenesis and fibrolysis [10]. Our results showed that hepatic MMP-2 (14%) and TIMP-1 (7.9%) were significantly increased in rats after BSA treatment. Bicyclol (**1**) treatment resulted in a notable reduction in hepatic MMP (8.5%) and TIMP (9.6%) levels, respectively (Figure 6; Table 2).

2.6 Discussion

Previous studies showed that multiple injections of BSA can lead to liver fibrosis

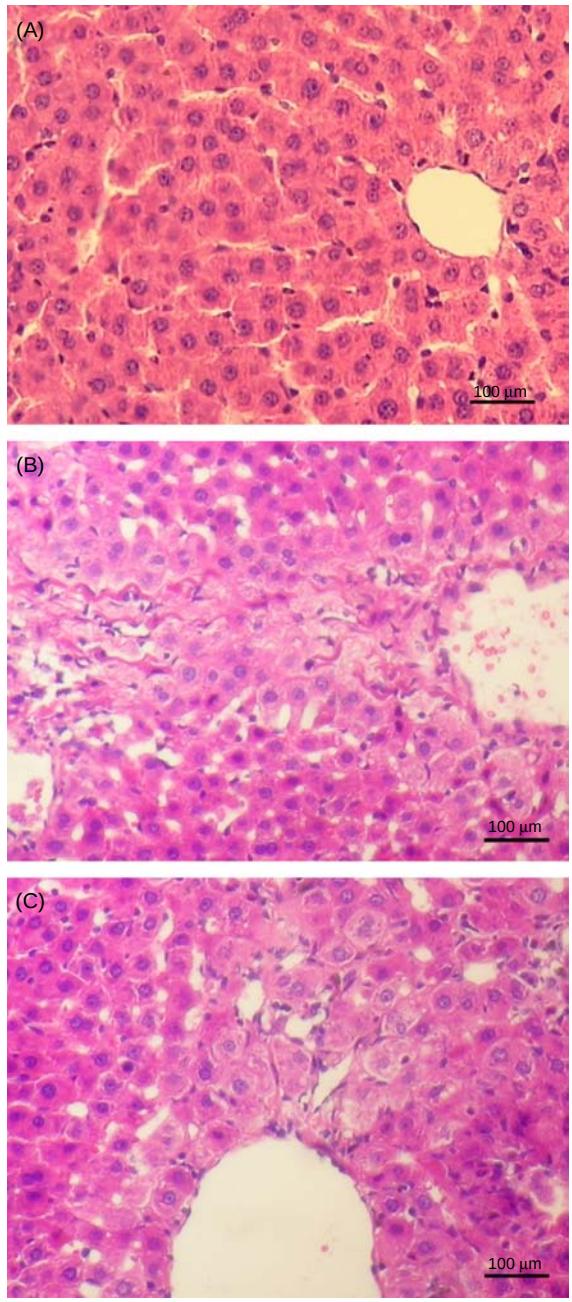

Figure 4. Effect of bicyclol on liver pathological injury induced by BSA in rats ($n = 15$). Bicyclol (300 mg/kg) was administered orally after the fourth week of intravenous injection for 5 weeks. Liver specimens were collected and liver sections were stained with H&E. (A) Control; (B) BSA-treated; and (C) bicyclol treatment (300 mg/kg). Original magnification ×100.

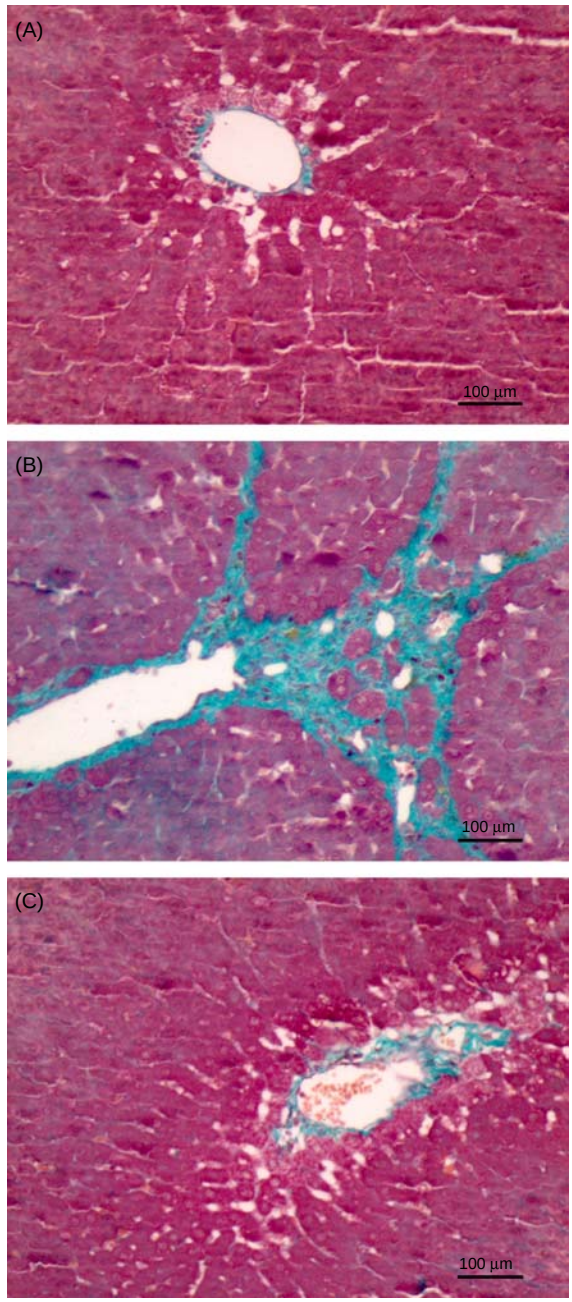

Figure 5. Effect of bicyclol on liver fibrosis induced by BSA in rats ($n = 15$). Bicyclol (300 mg/kg) was administered orally after the fourth week of intravenous injection for 5 weeks. Rats were sacrificed after the last administration of bicyclol. Liver specimens were collected and liver sections were stained with Masson. (A) Control; (B) BSA-treated; and (C) bicyclol treatment (300 mg/kg). Original magnification ×100.

Table 1. Effect of bicyclol on serum ALT, AST activities, and A/G value in BSA-treated rats ($n = 15$, mean ± SD).

Groups	ALT (U/l)	AST (U/l)	A/G
Normal	57.22 ± 13.14	99.41 ± 24.86	2.03 ± 0.17
Model	67.91 ± 24.84	105.74 ± 11.33	2.39 ± 0.27
By 100 mg/kg	67.65 ± 7.70	102.41 ± 19.11	2.17 ± 0.25
By 200 mg/kg	67.21 ± 16.36	101.32 ± 21.32	2.36 ± 0.30
By 300 mg/kg	52.63 ± 19.08	98.23 ± 13.66	2.09 ± 0.19

mediated by an immune complex, which is considered to be similar to the process of fibrogenesis in clinics [11]. The result of the present study demonstrated that BSA caused liver fibrosis as evidenced by the elevation of hepatic Hyp, serum HA and PIIIP, and pathological changes including the hyperplasia of lattice fibers and collagenous fibers, which is consistent with the previous studies [11,12]. Bicyclol (**1**) had an overall protective effect against BSA-induced liver fibrosis by the inhibition of liver fibrogenesis biochemically and pathologically, although no significant changes in serum ALT, AST levels, and the ratio of A/G were observed as that seen in severe hepatic injury and liver fibrosis induced by CCl_4 intoxication [9].

Liver fibrosis is a scarring process associated with an increased and altered deposition of liver extracellular matrix. It is mainly characterized by the cellular activation of hepatic stellate cells (HSCs), aberrant activity of TGF-β1, and its downstream cellular mediators [13]. TGF-β was known to regulate the expression of both components of the ECM network, such as fibrillar collagens, fibronectin, and protease inhibitors including TIMPs [14]. Based on the previous

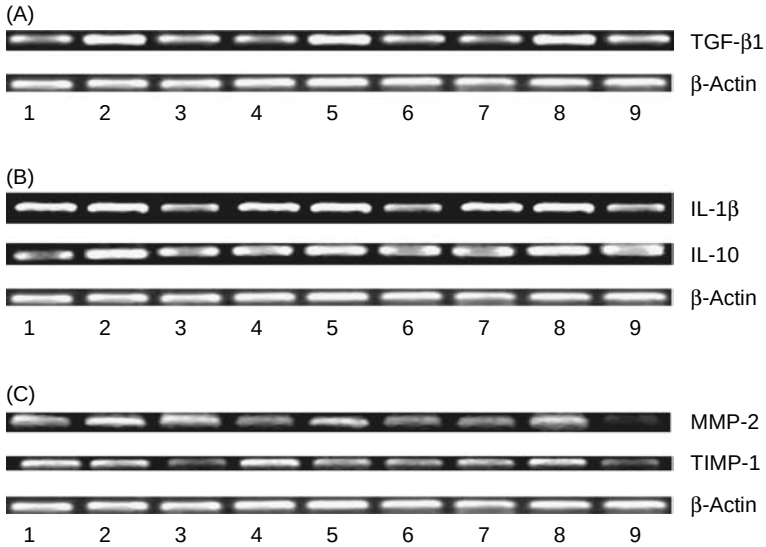

Figure 6. Effect of bicyclol on the mRNA expression of hepatic (A) TGF-β1, (B) IL-1β, IL-10, (C) MMP-2, and TIMP-1 in BSA-treated rats. Bicyclol (300 mg/kg) was administered orally after the fourth week of intravenous injection for 5 weeks. Rats were sacrificed after the last administration of bicyclol. Lanes 1, 4, 7, control; lanes 2, 5, 8, BSA; lanes 3, 6, 9, bicyclol (300 mg/kg).

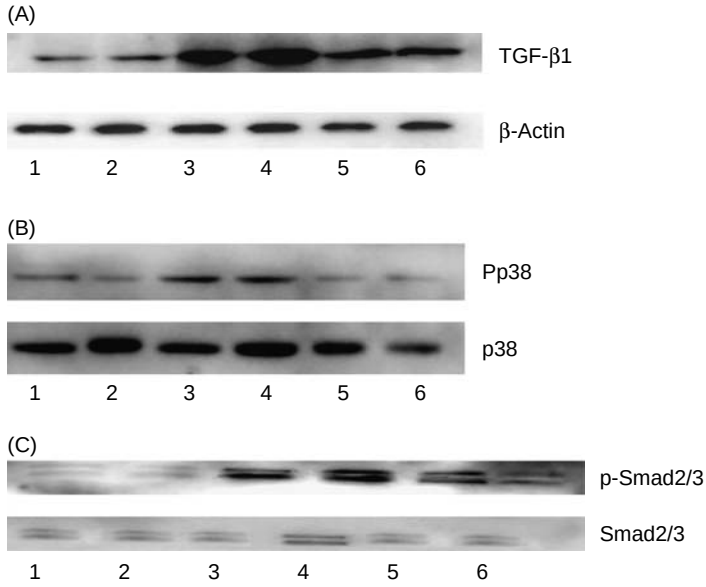

Figure 7. Effects of bicyclol on the hepatic TGF-β1 protein expression and signaling pathway in BSA-treated rats. Bicyclol (300 mg/kg) was administered orally after the fourth week of intravenous injection for 5 weeks. Effects of bicyclol on (A) hepatic TGF-β1 protein expression, (B) the phosphorylation of hepatic p38 MAPK, and (C) the phosphorylation of hepatic Smad2/3 in BSA-treated rats. Lanes 1, 2, control; lanes 3, 4, BSA; lanes 5, 6, bicyclol (300 mg/kg).

Table 2. Effect of bicyclol on serum and hepatic TGF-β1, p38 MAPK, Smad2/3, IL-1β, IL-10, MMP-2, and TIMP-1 in BSA-treated rats ($n = 6$, mean ± SD).

Index	Normal	Model	By 300 mg/kg
TGF-β1/β-actin mRNA	1.09 ± 0.01	1.14 ± 0.02*	1.09 ± 0.01$^{\#}$
TGF-β1/β-actin Protein	1.23 ± 0.17	4.93 ± 0.1**	1.56 ± 0.08$^{\#\#\#}$
Pp38/p38 Protein	0.61 ± 0.08	1.20 ± 0.01**	0.83 ± 0.03$^{\#\#}$
p-Smad2,3/Smad2,3 Protein	0.58 ± 0.005	1.25 ± 0.29*	0.79 ± 0.006$^{\#}$
IL-1β/β-actin mRNA	2.12 ± 0.16	2.48 ± 0.04*	1.68 ± 0.46$^{\#}$
IL-10/β-actin mRNA	1.05 ± 0.008	1.18 ± 0.01**	1.10 ± 0.02$^{\#\#}$
MMP-2/β-actin mRNA	1.00 ± 0.03	1.15 ± 0.03*	1.05 ± 0.04$^{\#}$
TIMP-1/β-actin mRNA	0.88 ± 0.005	0.95 ± 0.01**	0.89 ± 0.03$^{\#\#}$

Notes: $*P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. BSA group. $**P < 0.01$ vs. control group; $^{\#\#}P < 0.01$ vs. BSA group. $^{\#\#\#}P < 0.001$ vs. BSA group.

studies, the TGF-β1 signal transduction pathway has become an effective target for the prevention or treatment of liver fibrosis [15]. As shown in the present study, **1** was found to reduce the expression of liver TGF-β1 with a significant difference both on mRNA and protein levels. It suggested that **1** protected liver from fibrogenesis through the downregulation of liver TGF-β1.

The previous studies revealed that TGF-β can induce the activation of p38 MAPK signaling pathway, leading to the phosphorylation of Smad3 at the linker region [16], which promoted extracellular matrix production both *in vitro* and *in vivo* [17]. Our results demonstrated that **1** inhibited the phosphorylation of both Smad2/3 and p38. Thus, it is hypothesized that the inhibitory effect of **1** on TGF-β1 expression was at least in part via the Smad2/3 and p38 signaling pathways.

IL-1, as an inflammatory cytokine, was involved in the liver fibrosis mainly through the activation of p38 [18] and upregulation of TIMP-1 mRNA expression in HSCs [19]. IL-10 is considered as an anti-inflammatory cytokine which inhibits the production of several inflammatory cytokines, including IL-1β [20]. Several reports also demonstrated that the exogenous IL-10 can inhibit the liver fibrosis *in vivo* and *in vitro*. In the present study, it was found that the mRNA level of both hepatic IL-1β and IL-10 was enhanced after BSA treatment. Bicyclol (**1**) was shown to reduce the expressions of IL-1β and IL-10 in BSA-induced rats. The above results suggested that the hepatoprotective effect of **1** on liver fibrogenesis was via inhibiting IL-1. It was presumed that **1** significantly downregulated the gene expression of liver pro-inflammatory cytokines, and thereby attenuated feedback increase of IL-10.

MMPs and TIMPs were believed to be involved in the deposition and degradation of the ECM in liver fibrosis [21,22]. The expression of TIMP-1, especially in the activated HSCs, is upregulated, resulting in the inhibition of MMP activity and subsequent accumulation of matrix proteins in extracellular space [23]. In our study, **1** reduced the mRNA expression of MMP-2 and TIMP-1 significantly. Also, the ratio of MMP-2/TIMP-1 was downregulated by **1**. Namely, **1** alleviates ECM accumulation by suppressing MMP-2, and then ameliorates the expression of TIMP-1.

In conclusion, **1** showed significant hepatoprotective effect on BSA-induced liver fibrosis in rats. The hepatoprotection of **1** is mostly due to its ability to diminish the liver fibrogenesis by inhibiting the hepatic TGF-β1 and related signaling pathways, to attenuate the overexpression of inflammatory cytokines, and to maintain the balance of the deposition and degradation of the ECM by regulating MMP-2/TIMP-1.

3. Materials and methods

3.1 Materials

Bicyclol (**1**) (purity $>$ 99%) was kindly provided by Beijing Union Pharmaceutical Factory (BUPF, Beijing, China). BSA fraction V was purchased from Beijing Tianxiangbangding Biotech Co. Ltd (Beijing, China). Incomplete Freund's adjuvant was obtained from Beijing Biosynthesis Biotechnology Co. Ltd (Beijing, China). The kits for ALT, AST, albumin, and globulin determinations were purchased from Beihuakangtai Chemical Reagent Co. Ltd (Beijing, China). Hyp assay kit was a product of Nanjing Jiancheng Bioengineering Institute (Nanjing, China). TGF-β1, PIIIP, and HA ELISA kits were the products of Senxiong Biotech (Shanghai, China). Trizol was obtained from BioDev Tech Co. Ltd (Beijing, China). RT-PCR kits were provided by Takara Biotechnology Co. Ltd (Osaka, Japan). TGF-β1, p38, and Pp38 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were of analytical grade and were obtained from the local market.

3.2 Animals

Male adult Sprague–Dawley rats weighing 100–120 g were obtained from Beijing Vital River Experiment Animal Co. Ltd (Beijing, China). The animals were maintained at 24 ± 1°C, humidity 55 ± 5%, with a 12 h light/dark cycle and had free access to rodent chow and tap water. All experimental procedures were performed according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Bioethics Committee of the Institute of Materia Medica, Chinese Academy of Medical Sciences.

3.3 Experimental protocol

After a 2-day acclimation period, BSA (9 mg/ml)-incomplete Freund's adjuvant was given by a multi-point hypodermic injection in a volume of 0.5 ml/each to all rats except for those in the control group. Serum BSA antibody was detected by a double diffusion test after five BSA injections (the intervals of the first two injections and the last three injections were 2 weeks and 1 week, respectively). Then, BSA (2–4 mg/rat) was intravenously injected to antibody positive rats twice a week for 8 weeks.

Bicyclol (**1**) (100, 200, and 300 mg/kg) was administered (suspended in 5% carboxymethyl cellulose) to rats by gavage once a day for 5 weeks since the fourth week of intravenous injection of BSA. Other groups received an equal volume of vehicle as a control. The animals were sacrificed 16 h after the last administration of **1**. Blood samples were collected for the measurement of serum ALT, AST, PIIIP, HA, and TGF-β1. One piece of tissue from the same lobe of liver in each rat was fixed properly for histopathological examination. The remaining liver tissues were stored at −80°C for biochemical assays and RNA isolation.

3.4 Histopathology

Liver tissues were fixed with 10% neutral formalin and embedded in paraplast. Tissue sections (5 μm) were cut and stained by hematoxylin and eosin (H&E), and Masson.

3.5 Biochemical determinations

Serum levels of ALT, AST, the ratio of A/G, and hepatic Hyp content were determined colorimetrically by the diagnostic kits according to the instructions provided. Serum PIIIP, HA, and TGF-β1 were measured by ELISA assay kits based on the corresponding protocols.

3.6 Detection of cytokine expressions by RT-PCR

Total liver RNA was extracted using TRIzol reagent, and was reverse-transcribed to cDNA using a PR-PCR kit. Corresponding primer sets for PCR are shown in Table 3, and the final volume of reaction was 40 μl. The samples were loaded into a thermal cycler after determining the optimal number of cycles. For each gene, the final cycle was followed by extension for 7 min at 72°C. PR-PCR products were subjected to electrophoresis using 2% agarose gel, and then the bans were visualized with ethidium bromide and analyzed by BandScan software (Glyko Inc., Novato, CA, USA). Results are expressed as ratios relative to β-actin (density of PCR product/β-actin).

3.7 Detection of protein expressions by Western blot

The extracts of liver tissue were prepared by homogenization in lysis buffer (50 mM Tris, pH 7.5, 150 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1% Nonidet P-40, 50 mM sodium fluoride, 10 μg/ml proteinase inhibitor mixture, 10% glycerol) at 4°C, followed by centrifugation at 20,000*g*, 4°C for 10 min. After quantification of protein concentrations, the supernatants were mixed with Laemmli loading buffer, boiled for

Table 3. Primers and reaction conditions used for RT-PCR.

Index	Upstream primers	Downstream primers	Annealing temperature (°C)	PCR cycle no.	Product size (bp)
β-Actin	ACCACAGCTGAGAGGGAAATCG	AGAGGTCTTTACGGATGTCAACG	55	35	277
TGF-β1	GGACTCTCCACCTGCAAGAC	CTCTGCAGGCGCAGCTCTG	55	35	392
IL-1β	CCCATACACACGGACAACTAGA	ATCCCAAACAATACCCAAAGAA	51	35	508
IL-10	GACTTTAAGGGTTATTGGGTTGC	CACTGCCTTGCTCTTATTTTCACA	57	40	201
MMP-2	TATGGCTTCTGCCCTGA	TCCAAACTTCACGCTCTTCA	58	35	969
TIMP-1	TCCCCAGAAATCATCGAGAC	ACGGCTGTCTAGCCCTTCT	52	30	408

4 min, and then subjected to Western blot analysis. Membranes were blotted against primary antibodies at 4°C for 16 h, washed with 0.1% (v/v) Tween-20 in Tris-buffered saline (pH 7.4), and incubated with horseradish peroxidase-conjugated secondary antibodies for 45 min. A Western blot was performed using isolated total protein (50 μg), and specific primary antibodies (Santa Cruz Biotechnology) to TGF-β1 (sc-146), Pp38 (sc-7973), p38 (sc-7149), p-Smad2/3 (sc-11769), Smad2/3 (sc-8332), and β-actin (sc-1616), followed by the incubation with horseradish peroxidase-conjugated anti-rabbit IgG antibody. The immunoreactive bands were visualized by an ECL Western blot detection system (Kodak Inc., San Leandro, CA, USA).

3.8 Statistical analysis

All results expressed as mean ± SD were analyzed by one-way analysis of variance with the SPSS 11.0 statistical software package. The differences between means were analyzed by the Student–Newman–Keuls test for multiple comparisons. A *P*-value of less than 0.05 was considered to be statistically significant.

Acknowledgements

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