

## Effects of bicyclol on dimethylnitrosamine-induced liver fibrosis in mice and its mechanism of action

Qing-Wei Hu, Geng-Tao Liu \*

*Department of Pharmacology, Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, 1 Xian Nong Tan Street, Beijing, 100050, PR China*

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### Abstract

The aim was to investigate the suppressive effect of bicyclol on hepatic fibrosis induced by dimethylnitrosamine (DMN) in mice and the mechanism of its action. Hepatic fibrosis was established by intraperitoneal injection of  $8 \text{ mg kg}^{-1} \text{ day}^{-1}$  on three consecutive days of each week for 4 or 5 weeks. In the prophylactic experiment, bicyclol (100 and  $200 \text{ mg} \cdot \text{kg}^{-1}$ ) was administered by gavage in association with DMN injection. For the therapeutic experiment, mice were firstly injected with DMN for 5 weeks as in the prophylactic experiment, and then the mice in drug groups were orally administered bicyclol (100 and  $200 \text{ mg} \cdot \text{kg}^{-1}$ ) once daily for 5 weeks. As a result, the levels of alanine aminotransferase (ALT), total bilirubin, hydroxyproline (Hyp), prolidase, tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), transforming growth factor beta-1 (TGF $\beta_1$ ), type I collagen in serum and the score of liver fibrosis all significantly increased in the hepatic fibrosis model group in comparison with those in control group. The treatment with bicyclol markedly reduced all the above criteria. Bicyclol also attenuated the decrease of body weight of mice, serum total protein and albumin. In addition, bicyclol treatment inhibited liver TGF $\beta_1$  and tissue inhibitor of metalloproteinase 1 (TIMP-1) mRNA expression in the prophylactic experiment. Similarly, bicyclol reduced TIMP-1 levels in liver and serum and increased collagenase activity in the liver in the therapeutic experiment. The result suggest that bicyclol attenuates DMN-induced hepatic fibrosis in mice. Its mechanisms of action may be related to the hepatoprotective and anti-inflammation properties, the down-regulation of liver TGF $\beta_1$  and TIMP-1 expression and the increase of net collagenase activity in liver.

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### Introduction

Hepatic fibrosis (HF) represents the wound healing response of the liver to diverse repeated liver injuries, and is characterized by increased deposition and altered composition of extra-cellular matrix (ECM) in liver (Du et al., 1999). The hepatic stellate cell (HSC) is the main cell type responsible for the ECM production in liver. Collagen types I, III, IV, fibronectin, laminin and proteoglycans are the extracellular proteins produced during fibrogenesis, and collagen types I and III are the most abundant among these proteins (Bataller and Brenner, 2005). HF is a pivotal and necessary stage to cirrhosis,

which leads to lethal complications and high mortality. Patients with hepatitis B virus (HBV) and hepatitis C virus (HCV) may also develop liver cirrhosis in the ensuing years (Schwabe and Stremmel, 1998). The current investigation showed that HF, and even cirrhosis at early stage, may be reversible (Safadi and Friedman, 2002; Bonis et al., 2001). However, there is no acceptable therapy. Therefore, the prevention of HF has a very great significance both in theory and in practice.

Bicyclol (4,4'-dimethoxy-5,6,5',6'-dimethylene-dioxy-2-hydroxymethyl-2'-carbonyl biphenyl) is a novel anti-hepatitis drug (Liu, 2001). Oral administration of bicyclol (25–50 mg, thrice daily) markedly reduced the elevated levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in patients with chronic viral hepatitis B and C, and also partially inhibited HBV and HCV replication (Yao et al., 2002a,b,

\* Corresponding author. Tel.: +86 10 63165178; fax: +86 10 63017757.  
E-mail addresses: [liugt@imm.ac.cn](mailto:liugt@imm.ac.cn), [hqw123@imm.ac.cn](mailto:hqw123@imm.ac.cn) (G.-T. Liu).

2005). Pharmacologically, bicyclol had remarkable actions against liver injury and hepatitis virus (Zhao and Liu, 2001; Lu and Li, 2002; Li and Liu, 2004). Bicyclol was also shown to inhibit HF induced by carbon tetrachloride in rats (Li et al., 2004).

Dimethylnitrosamine (DMN)-induced liver fibrosis model can reproduce most of the features observed during human liver fibrosis (Wasser and Tan, 1999). This model has other advantages, such as progressive and remarkable pathological alterations, a high reproduction rate of fibrosis and a low mortality rate in experimental animals (Jezequel et al., 1989).

This model is also stable even after termination of DMN injection and is a reliable tool for screening antifibrotic agents (George et al., 2001).

To further evaluate the anti-hepatic fibrosis activity of bicyclol, the present study was designed to investigate whether bicyclol had anti-fibrotic effect on DMN-induced HF in mice and to study its mechanism of action.

## Materials and methods

### Materials

DMN ( $C_2H_6N_2O$ ) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Toshima, Japan). Bicyclol ( $C_{19}H_{18}O_9$ ) was kindly provided by Beijing Union Pharmaceutical Factory. It is a white crystal with purity over 99% and is not water soluble. The kits for ALT (No. 001020), albumin (No. 001024), total bilirubin (No. 006038) and total protein (No. 001023) determinations were purchased from Beihuakangtai Chemical Reagent Co., Ltd. (Beijing, China). Prolidase assay kit (No. 050320) was offered by Shanghai Navy Medical Research Center (Shanghai, China). Hydroxyproline (Hyp) assay kit (No. 050415) was a product of Nanjing Jiancheng Bioengineering Institute. Transforming growth factor beta-1 ( $TGF\beta_1$ ) enzyme-linked immunosorbent assay (ELISA) kit (No. 050410) and type I collagen ELISA kit (No. 050416) were products of TPI Incorporated (Washington, USA). Mouse tumor necrosis factor alpha ( $TNF\alpha$ , No. MTA00) was purchased from R&D Systems, Co. Ltd., USA. Mouse tissue inhibitor of metalloproteinase 1 (TIMP-1) ELISA assay kit (No. MTM100) was purchased from RapiBio Company (Calabasas, CA, USA). Trizol RNA extraction kit was purchased from Beijing Santaixinglong Technology Incorporated. The primers were synthesized by Sangon Biological Engineering and Technology Services Co., Ltd. (Shanghai, China). SYBR<sup>®</sup> Green Quantitative one-step RT-PCR kit (No. QR0100), thermo-fast 96-well PCR detection plates and adhesive PCR films were supplied by Agene (USA). Collagenase assay kit (No. 3001) was a product of Chondrex, Inc. (Redmond, USA). All other chemicals used were of analytical grade supplied by Beijing Chemical Agents Company (Beijing, China).

### Animals and treatment

Male ICR mice weighing 20–22 g were purchased from the Beijing Weitonglihua Experimental Animal Co., Ltd. All mice

were bred and maintained under constant conditions at temperature  $24\pm 1^\circ C$ , humidity  $55\pm 5\%$ , with 12h light and 12h dark cycles. Water and feed were accessible to mice ad libitum. Animal care and all experimental procedures were conducted in accordance with the health criteria for care of laboratory animals enacted by Beijing municipal government.

After a 2-day acclimation period, mice were randomly divided into 2 groups: group I was for prophylactic experiment, and group II was for therapeutic experiment. Mice of each group were further divided into four subgroups: control, model, bicyclol  $100\text{mg kg}^{-1}$ , bicyclol  $200\text{mg kg}^{-1}$  ( $n=15$  per each subgroup). In the prophylactic experiment, mice in control group received i.p. injection of normal solution (NS)  $5\text{mL kg}^{-1}$ , the mice in model group were i.p. injected with DMN  $8\text{mg kg}^{-1}\text{ day}^{-1}$  in NS on the first three consecutive days each week. The mice in bicyclol treatment groups received the same dose of DMN associated with oral administration of bicyclol ( $100, 200\text{mg kg}^{-1}$ , suspended in 0.5% sodium carboxymethylcellulose, Na-CMC) once daily for 4 weeks. The body weight of mice was monitored during the whole experiment period. At the end of the fourth week, all the surviving mice were sacrificed. Blood and the same lobe of the liver were collected for biochemical and histological examinations. The remainders of the livers were rapidly replaced in liquid nitrogen and stored for RT-PCR assay.

Mice in the group II for therapeutic experiment were differently treated as follows: Mice in the control group ( $n=15$ ) were administered NS  $5\text{mL kg}^{-1}$ , and mice in the DMN-treated group ( $n=55$ ) received i.p. injection of DMN  $8\text{mg kg}^{-1}\text{ day}^{-1}$  on the first three consecutive days of each week for 5 weeks. Then, all the mice injected with DMN were randomly divided into three subgroups ( $n=15$  in each group): model, bicyclol  $100\text{mg kg}^{-1}$  and bicyclol  $200\text{mg kg}^{-1}$  groups. Mice in the control group and model group were orally administered 0.5% Na-CMC  $5\text{mL kg}^{-1}$ , once daily for 5 weeks. Mice in the other two groups received bicyclol 100 and  $200\text{mg}\cdot\text{kg}^{-1}$  once daily for 5 weeks, respectively. The day after the last medication, all the surviving mice were sacrificed for examinations. The body weight of the mice was monitored during the whole experiment period.

Serum from each mouse was obtained from blood by centrifugation at 3500rpm for 10min at  $4^\circ C$ . Liver specimens were removed immediately after the mice were sacrificed. The right lobe of each liver was fixed in  $40\text{g L}^{-1}$  phosphate-buffered saline (PBS)-buffered paraformaldehyde solution, and was processed for histopathological examination. The remaining liver tissue was immediately removed and saved for other assays.

### Histopathology

The fixed liver samples were processed and embedded in paraffin blocks. Tissue block sections were then mounted on slides, deparaffinized in xylene, dehydrated in alcohol, and sections of  $5\mu\text{m}$  in thickness were prepared. Then sections were stained with Van Gieson reticulum staining methods to analyze the extent of hepatic fibrosis. The stained sections were examined under Nikon microscope and analyzed by image Pro-Plus 7200 software (Nikon E600, Nikon Corporation,

Tokyo, Japan). A certified pathologist scored the degree of liver fibrosis in a blinded fashion. A score was given to each slide. The histological score criteria referred to semi-quantitative scoring system (Wang et al., 1998; Chevallier et al., 1994).

#### Biochemical determinations

The levels of ALT, albumin, total proteins, total bilirubin, and Hyp were measured using commercial kits. The mouse type I collagen, TNF $\alpha$  and TGF $\beta$ <sub>1</sub> ELISA assays were performed following the corresponding protocols of the kits. Liver tissue was homogenized in electron/glass homogenizer (DY89-I, Ningbo, China) using cold NS at 4°C. Then the homogenates were centrifuged, and aliquots of the supernatants were used for Hyp and TIMP-1 determinations. The total proteins in liver homogenates were determined by Coomassie Brilliant Blue G-250 methods using bovine serum albumin as the standard (Bradford, 1976). The assayed parameters were expressed in micrograms or nanograms per milligrams of liver protein. The optical density was measured using scanning full wavelength spectrophotometer (MQX200, BIO-TEK, USA).

#### Detection of mRNA expression

Total RNAs of the livers were extracted with Trizol and measured by ultraviolet visible spectrophotometer (UV-260, Shimadzu, Japan), and the OD260/OD280 value was between 1.8 and 2.0. The sequences of primers for RT-PCR were as follows:

Index	Up-stream primers	Down-stream primers
$\beta$ -actin	CCATC TACGAGG GCTAT	TGGCC AGTAA TGTCC AGG
TGF $\beta$ <sub>1</sub>	AGCACTACCA CCTGGACTG	TGGCCAGTAATGTCCAGG
TIMP-1	CCCAG AACCG CAGTG AAG	CGAAG AACCA AGGGA CCG

Total reaction volume was 20  $\mu$ L, and the final reaction concentrations or volumes were as follows: SYBR Green Taq Ready Mix for quantitative RT-PCR 10  $\mu$ L, MgCl<sub>2</sub> 3.0 mmol/L. Both up-stream primers and down-stream primers were 300 nmol/L, RNA template 25 ng/ $\mu$ L, TaqDNA polymerase 0.5 U/ $\mu$ L. Each sample had four replicates. Reverse transcription took place at 48°C for 30 min, AmpliTaq activation occurred at 94°C for 2 min. PCR parameters were as follows: denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 1 min (repeated 40 cycles, ABI-7000, Applied Biosystems, ABI, USA). Expression of mRNA was detected using the real-time fluorescence quantitative RT-PCR technique. The threshold cycle (Ct) value is in reverse proportion to the amount of starting target mRNA; the higher the Ct value, the less the amount of starting target mRNA (Lekanne Deprez et al., 2002). In this study,  $\beta$ -actin was employed as endogenous control gene (Thellin et al., 1999).

#### Detection of collagenase activity

This assay consisted of four main steps: (1) Activation of latent collagenase in sample specimens. (2) Reaction with FITC-labeled soluble collagen for 60 min. (3) Denaturation and

further digestion of the cleaved collagen fragments into smaller fragments. (4) Extraction of the fragments by an organic solvent and determination of the fluorescent intensity (FI) of the extract. The FI was determined at  $\lambda_{em}$ =520 nm and  $\lambda_{ex}$ =490 nm (Fluorescence microplate reader, Spectra MAX-Geminix, Molecular Device, USA).

#### Statistical analysis

Arithmetic mean and standard deviation ( $\bar{x} \pm SD$ ) were calculated for the data. Significant differences between the groups were statistically analyzed using Student's *t*-test. All *P* values were two-tailed. The value of *P*<0.05 or less was considered statistically significant.

#### Results

During the period of prophylactic experiment, one mouse died in the model group. In the therapeutic experiment, one mouse died in the model group and one in the bicyclol 200 mg kg<sup>-1</sup> group. The data from the prophylactic experiment were listed in Table 1. DMN injection resulted in an increase of serum ALT and total bilirubin levels, while it induced a decrease of total protein and albumin levels as well as body weight in comparison with control group. Bicyclol (200 mg kg<sup>-1</sup>) treatment attenuated the retardation of increase of body weight in the mice that survived, reduced serum ALT and total bilirubin levels, and restored the serum total protein and albumin contents near to the levels in control group. Bicyclol 100 mg kg<sup>-1</sup> was also effective but the effect was weaker than in the 200 mg kg<sup>-1</sup> group. In the therapeutic experiment, bicyclol failed to improve the above levels of ALT, total bilirubin, albumin and total protein compared with model subgroup.

Hyp, a product of collagen metabolism, is an amino acid characteristic of collagen. The total collagen present in liver was, therefore, determined by estimating the Hyp content. Type

Table 1  
Protective effect of bicyclol on abnormal liver function in mice with dimethylnitrosamine-induced hepatic fibrosis (*n*=10)

Index	Control	Model	Bicyclol 100mg	Bicyclol 200mg
<i>Prophylactic experiment</i>				
Body weight (g)	36.5 $\pm$ 1.8**	30.9 $\pm$ 3.1	34.5 $\pm$ 2.5*	36.0 $\pm$ 2.2**
ALT (U/L)	24.6 $\pm$ 6.3*	37.2 $\pm$ 7.6	28.6 $\pm$ 7.2*	24.5 $\pm$ 4.5**
TP (g/L)	64.3 $\pm$ 4.3**	56.8 $\pm$ 3.9	61.6 $\pm$ 6.7	61.1 $\pm$ 3.4*
albumin (g/L)	37.9 $\pm$ 1.9**	33.5 $\pm$ 1.8	35.3 $\pm$ 1.9	36.3 $\pm$ 2.4*
TBIL ( $\mu$ mol/L)	1.61 $\pm$ 1.09**	7.21 $\pm$ 4.00	3.81 $\pm$ 1.93*	3.28 $\pm$ 1.90*
<i>Therapeutic experiment</i>				
Body weight (g)	42.2 $\pm$ 3.5**	30.6 $\pm$ 3.4	34.4 $\pm$ 3.0**	35.6 $\pm$ 3.7**
ALT (U/L)	17.2 $\pm$ 3.5*	22.2 $\pm$ 3.1	18.8 $\pm$ 4.4	18.6 $\pm$ 4.1
TP (g/L)	72.9 $\pm$ 4.3*	68.9 $\pm$ 2.9	69.4 $\pm$ 5.6	69.4 $\pm$ 5.5
Albumin (g/L)	37.6 $\pm$ 2.2*	34.9 $\pm$ 2.4	36.1 $\pm$ 2.6	35.3 $\pm$ 2.9
TIBL ( $\mu$ mol/L)	1.99 $\pm$ 1.15**	3.80 $\pm$ 1.06	3.22 $\pm$ 1.33	2.75 $\pm$ 1.10

ALT, alanine aminotransferase; TP, total protein; TBIL, total bilirubin. Values are means $\pm$ standard deviation. \**p*<0.05, \*\**p*<0.01 VS model.

Table 2

Inhibitory effect of bicyclol on levels of hydroxyproline, type I collagen and score of liver fibrosis in mice with dimethylnitrosamine-induced hepatic fibrosis ( $n=10$ )

Index	Control	Model	Bicyclol 100mg	Bicyclol 200mg
<i>Prophylactic experiment</i>				
Serum Hyp ( $\mu\text{g/ml}$ )	3.88 $\pm$ 0.47**	5.79 $\pm$ 0.87	4.59 $\pm$ 1.05*	4.39 $\pm$ 0.66**
Liver Hyp ( $\mu\text{g/mg}$ )	0.62 $\pm$ 0.09**	1.17 $\pm$ 0.24	0.86 $\pm$ 0.09**	0.85 $\pm$ 0.09**
Serum Collagen I ( $\mu\text{g/L}$ )	387 $\pm$ 129**	634 $\pm$ 188	500 $\pm$ 122	452 $\pm$ 129*
Fibrosis score	0.0 $\pm$ 0.0**	12.6 $\pm$ 1.9	8.7 $\pm$ 1.5**	6.9 $\pm$ 1.2**
<i>Therapeutic experiment</i>				
Serum Hyp ( $\mu\text{g/ml}$ )	3.73 $\pm$ 0.25**	4.76 $\pm$ 0.39	4.21 $\pm$ 0.29**	4.14 $\pm$ 0.43*
Liver Hyp ( $\mu\text{g/mg}$ )	0.52 $\pm$ 0.10**	1.22 $\pm$ 0.25	0.83 $\pm$ 0.14**	0.74 $\pm$ 0.10**
Serum Collagen I ( $\mu\text{g/L}$ )	322 $\pm$ 78**	665 $\pm$ 144	475 $\pm$ 81**	439 $\pm$ 108**
Fibrosis score	0.0 $\pm$ 0.0**	12.8 $\pm$ 2.1	8.9 $\pm$ 2.0**	6.4 $\pm$ 0.9**

Hyp, hydroxyproline. Values are means $\pm$ standard deviation. \* $p<0.05$ , \*\* $p<0.01$  VS model.

I collagen, one of the most abundant collagens in fibrotic liver, was also measured using ELISA methods. As shown in Table 2, Hyp and type I collagen contents in the model group were all significantly higher than those of the control group in both prophylactic and therapeutic experiments, indicating that the liver fibrosis model was successfully established. The treatment with bicyclol significantly decreased the levels of Hyp and type I collagen, and the score of liver fibrosis as compared with corresponding model group.

In histological examination, the injection of DMN caused hepatic fibrosis, as reflected in disruption of tissue architecture and the marked increase in fibrosis score in all DMN-injected

mice. The architecture of the liver lobule became unclear. Centrilobular necrosis, focal and piecemeal necrosis occurred, and neutrophils and mononuclear cells infiltrated the liver of mice injected with DMN. Formed collagen fibers extended toward the hepatic lobule. Hepatic cords became disarranged and hepatic sinusoid was distorted. The connective tissue showed hyperplasia and was widely spread in the fibrotic liver. The degree of fibrosis and the intensity of fiber accumulation were dramatically reduced by bicyclol treatment in prophylactic and therapeutic experiments (Figs. 1 and 2).

In both prophylactic and therapeutic experiments, the serum levels of prolidase,  $\text{TNF}\alpha$  and  $\text{TGF}\beta_1$  were markedly elevated in DMN-injected model group as compared with control group. Treatment with bicyclol significantly lowered prolidase,  $\text{TNF}\alpha$  and  $\text{TGF}\beta_1$  levels in comparison with model group.

In the prophylactic experiment, bicyclol significantly down-regulated the elevated TIMP-1 mRNA expression in fibrotic liver made by DMN (Table 3). Collagenase activity such as TIMP-1 plays a very important role in ECM degradation. In the therapeutic experiment, the activity of liver collagenase in the model group was clearly elevated, whereas the collagenase activity in mice treated with bicyclol was much higher than that of the model group. Bicyclol also decreased TIMP-1 content both in serum and in liver (Table 3).

## Discussion

HF is the result of imbalance between ECM production and degradation. The components of hepatic ECM include several families of structural and supporting molecules, and HSCs are the major connective tissue-producing cell type in liver (Schuppan et al., 2001). Because collagen is the main element of ECM when liver fibrosis occurs, the collagen content was

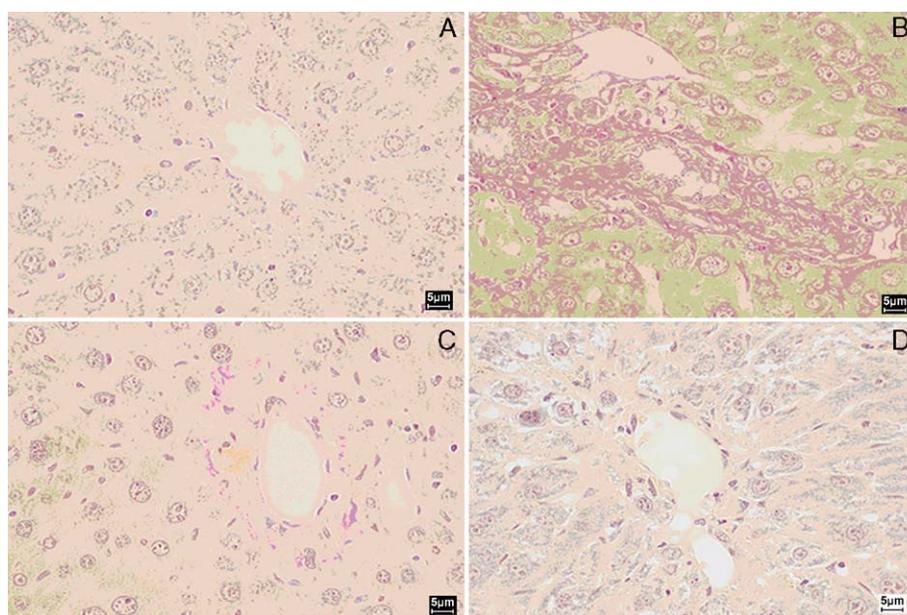


Fig. 1. Prophylactic effect of bicyclol on hepatic fibrogenesis induced by injection of dimethylnitrosamine (DMN) for 4 weeks in mice. Van Gieson (VG) stained sections of mouse liver for collagen fibrils, in which fibrous materials were stained red. 40 $\times$ C0.45 mount. (A) Control mouse. (B) Model mouse, mouse with DMN-injection for 4 weeks. (C) Mouse treated with bicyclol 100mg  $\text{kg}^{-1}$ . (D) Bicyclol 200mg  $\text{kg}^{-1}$  treated mouse.

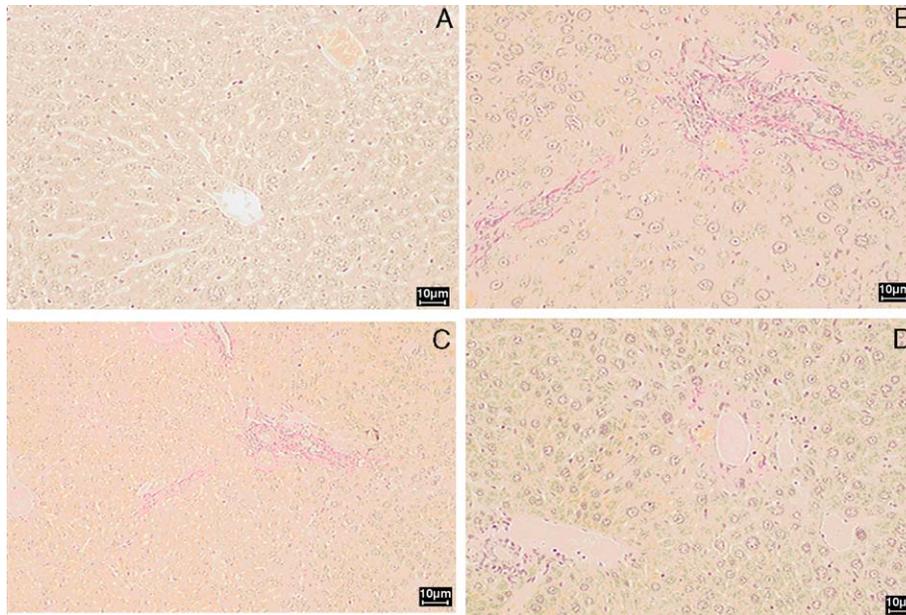


Fig. 2. Therapeutic effect of bicyclol on established hepatic fibrosis induced by DMN in mice. VG stained sections of mouse liver for collagen fibrils.  $20\times C0.45$  mount. (A) Control mouse. (B) Model mouse. (C) Mouse treated with bicyclol  $100\text{mg kg}^{-1}$ . (D) Mouse treated with bicyclol  $200\text{mg kg}^{-1}$ .

determined quantitatively to evaluate the efficacy of the anti-fibrosis effect of bicyclol in the present study. The results showed that bicyclol had anti-fibrogenesis activity expressed in decrease of Hyp, type I collagen levels and fibrosis score.

Chronic hepatitis virus infection, alcohol or drug intoxication or any other factors that cause damage to hepatocytes elicit an inflammatory reaction in the liver. Membrane components of the damaged hepatocytes, metabolites of toxic agents and infiltrating inflammatory cells are the activators of Kupffer cells (KCs). The activated KCs release a number of soluble agents, including cytokines, reactive oxygen species and other factors (Jaeschke et al., 2002). Under stimulation of soluble factors from damaged hepatocytes or activated KCs, HSCs undergo morphological transition to myofibroblast-like cells, and

proliferate. This transition of HSCs is characterized by an accelerated production of large amounts of ECM (Hautekeete and Geerts, 1997; Kmiec, 2001). During the complicated cytokine-mediated cross-talking of various cell types, hepatocellular damage is an initiating event where the activated KCs serve as the mediators, while HSCs act as the effectors (Alcolado et al., 1997). Damaged hepatocytes can also generate reactive oxygen intermediates which exert paracrine stimulation of HSCs (Maher and Bissell, 1993). In the present study, bicyclol was shown to protect the hepatocytes from fibrosis and significantly improve the abnormalities in liver function induced by DMN-injection in mice.

Liver fibrosis is associated with an increase of circulating  $\text{TNF}\alpha$  which is secreted by KCs once stimulated by bacteria or viral infection, immune complex or chemical drugs.  $\text{TNF}\alpha$

Table 3  
Effect of bicyclol on levels of  $\text{TNF}\alpha$ , prolidase,  $\text{TGF}\beta_1$ , TIMP-1 and collagenase activity in fibrosis liver induced by dimethylnitrosamine in mice ( $n=10$ )

Index	Control	Model	Bicyclol 100mg	Bicyclol 200mg
<i>Prophylactic experiment</i>				
Serum Prolidase (U/L)	$1284\pm 252^{**}$	$1879\pm 256$	$1559\pm 213^*$	$1455\pm 194^{**}$
Serum $\text{TNF}\alpha$ (ng/L)	$8.2\pm 1.6^{**}$	$16.7\pm 4.9$	$10.9\pm 2.4^*$	$11.3\pm 1.9^*$
Serum $\text{TGF}\beta_1$ ( $\mu\text{g/L}$ )	$12.81\pm 0.75^{**}$	$17.34\pm 1.75$	$14.32\pm 1.05^{**}$	$15.37\pm 1.74^*$
$\text{TGF}\beta_1/\beta\text{-actin}$	$0.941\pm 0.020^{**}$	$0.901\pm 0.018$	$0.928\pm 0.012^{**}$	$0.932\pm 0.017^{**}$
$\text{TIMP-1}/\beta\text{-actin}$	$0.855\pm 0.016^{**}$	$0.799\pm 0.012$	$0.834\pm 0.016^{**}$	$0.832\pm 0.023^{**}$
<i>Therapeutic experiment</i>				
Serum Prolidase (U/L)	$1614\pm 309^{**}$	$2431\pm 307$	$1804\pm 385^{**}$	$2104\pm 434$
Serum $\text{TNF}\alpha$ (ng/L)	$11.2\pm 1.3^{**}$	$14.7\pm 0.9$	$12.8\pm 2.3^*$	$12.0\pm 1.4^{**}$
Serum $\text{TGF}\beta_1$ ( $\mu\text{g/L}$ )	$13.40\pm 0.84^{**}$	$15.60\pm 0.84$	$14.3\pm 1.18^*$	$14.8\pm 0.94$
Serum TIMP-1 ( $\mu\text{g/L}$ )	$3.4\pm 0.3^{**}$	$4.1\pm 0.3$	$3.9\pm 0.3^*$	$3.7\pm 0.3^{**}$
TIMP-1 (ng/mg liver)	$117.8\pm 7.0^{**}$	$132.4\pm 9.0$	$123.4\pm 6.9^*$	$122.7\pm 7.7^*$
Collagenase activity (U/g liver)	$1.28\pm 0.39^{**}$	$2.50\pm 0.81$	$5.81\pm 1.93^{**}$	$4.33\pm 1.08^{**}$

$\text{TNF}\alpha$ , tumor necrosis factor- $\alpha$ ;  $\text{TGF}\beta_1$ , transforming growth factor  $\beta_1$ ; TIMP-1, tissue inhibitor of metalloproteinase 1. Values are means $\pm$ standard deviation. \* $p<0.05$ , \*\* $p<.001$  VS model.

not only can propel HSCs to transform into myofibroblast cells to synthesize more collagen and proteoglycans, but also indirectly induces the production of other fibrogenic factors, such as IL-1, IL-6, etc. (Liu et al., 2001; Luckey and Petersen, 2001). The results of the present study indicated that bicyclol treatment decreased DMN-induced elevation of serum TNF $\alpha$  levels.

Serum prolidase activity reflects not only HF, but also the degree of liver injury (Myara et al., 1987). In the present study, bicyclol decreased the elevated prolidase activity. Taken together, it may be speculated that the antifibrotic effect of bicyclol may be attributed, at least in part, to suppressing HSC activation via hepatoprotective mechanism. This protection may integrate the elimination of free radicals, the protection against nuclear DNA damage and apoptosis of hepatocytes (Liu, 2001; Zhao and Liu, 2001; Liu et al., 2005).

Cytokines of the transforming growth factor (TGF) family influence a wide spectrum of cellular processes including differentiation, proliferation, apoptosis and migration. TGF $\beta_1$  participates in initiation and maintenance of fibrogenesis in many organs including the liver (Border and Noble, 1994). There is much evidence that TGF $\beta_1$  is the most potent profibrogenic mediator that directly and indirectly promotes the proliferation of HSCs (Sato et al., 2003; Bauer and Schuppan, 2001). Therefore, the effect of bicyclol on TGF $\beta_1$  mRNA expression was assessed using the real-time fluorescence quantitative RT-PCR technique. The results showed that bicyclol reduced TGF $\beta_1$  mRNA expression in liver and TGF $\beta_1$  levels in serum. The suppression of TGF $\beta_1$  mRNA expression by bicyclol should be a very important mechanism of its anti-fibrotic action.

The mechanism of resolution and reversion of HF involves the balance between matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs), the apoptosis of activated HSCs, and augment of collagenase activity. (Iredale et al., 1998). Collagenase, including MMP1, MMP8 and MMP13, which degrade collagen types I, II, III, plays a pivotal role in the degradation of ECM. In addition, collagen types I and III are most abundantly found in liver with fibrosis. Recent studies revealed that ECM degradation was mostly influenced by MMPs and TIMPs. MMPs are usually useful for ECM degradation. TIMPs include four members: TIMP-1, TIMP-2, TIMP-3 and TIMP-4, and TIMP-1 and TIMP-2 are expressed in liver. TIMPs not only inhibit pro-enzyme activation, but also can fully activate enzymes. TIMP-1 can inhibit collagenase activity, and also inhibits MMP9 and stromelysin activity (Murphy et al., 2002). The decrease of TIMPs and the increase of collagenase activity often coincide when hepatic fibrosis begin to reverse. In this paper, the treatment with bicyclol resulted in the increase of collagenase activity and the reduction of TIMP1 content in fibrotic liver. It is still uncertain whether the increase of collagenase activity was a direct effect of bicyclol or an indirect effect of bicyclol resulting from inhibition of TIMP-1 expression.

## Conclusion

Combining the present and previous results, it may be concluded that bicyclol counteracts liver fibrosis induced by

CCl<sub>4</sub> or DMN. The mechanism of its antifibrotic effect might be mediated by multiple pathways, including suppression of hepatic inflammation, particularly inhibition of TGF $\beta_1$  and TIMP-1 mRNA expression, and increase of net collagenase activity in liver.

HF is a prepathologic state of cirrhosis, which plays a pivotal role in the carcinogenesis of hepatocellular carcinoma (HCC). It is known that HCC is substantially increased in patients with severe liver fibrosis (Sorensen et al., 1998). So, the prevention of HF may serve as a potential pathway for chemoprevention of HCC (Ginseng-HCC chemopreventive study Osaka group, 2001). Another investigation in our laboratory demonstrated that bicyclol markedly prevented malignant transformation of WB-F344 rat liver epithelial cells induced by 3-methylcholanthrene with 12-*O*-tetradecanoyl phorbol 13-acetate (Sun and Liu, in press), and also markedly reduced the occurrence of hepatic carcinoma induced by diethylnitrosamine plus phenobarbital in mice (personal communication). In China, thousands of patients with chronic viral hepatitis B have received bicyclol treatment; the abnormalities of liver function were markedly improved in most patients and hepatitis B virus replication was also inhibited in some patients. No noticeable adverse effect was found. So, it is worthwhile to extend clinical trials to evaluate the antifibrotic effect of bicyclol in patients with liver fibrosis.

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