Bicyclol: A Novel Drug for Treating Chronic Viral Hepatitis B and C

Geng Tao Liu*

Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing 100050, China

Abstract Chronic viral hepatitis B and C are diseases worldwide. At present, the number of effective and safe drugs for treatment of HBV and HCV is still limited. In order to develop novel anti-viral hepatitis drug, a number of analogues of the active component schizandrin C from *Fructus Schiznadrae*, a Chinese herb used in the therapy of viral hepatitis, were synthesized. Bicyclol, one of the analogues, was demonstrated to have actions of anti-hepatitis virus replication in duck hepatitis model and 2.2.15 cell line, anti-experimental liver injury induced by hepatotoxins such as CCl_4, acetaminophen and ConA, and anti-liver fibrosis in rats and mice. The active mechanism of bicyclol might be anti-apoptosis of hepatocytes through multiple signaling pathways mainly inducing the expressions of hepatic heat shock proteins (HSP27 and HSP70), molecular chaperons.

Clinical trial was performed by double blind, randomized and positive control or placebo method in multi-medical centers in China. Patients received bicyclol 25mg thrice daily for six months, then stopped treatment and followed up for 3 months. Oral administration of bicyclol normalized the elevated serum transaminases (ALT, AST) by approximately 50% in chronic viral hepatitis B and C, and also showed certain level of inhibiting HBV and HCV replication. No noticeable adverse reaction has been observed. In combination therapy of bicyclol with interferon alpha, lamivudine and adefovir dipivoxil in HBV or HCV, bicyclol may potentiate the anti-viral efficacy and reduce YMDD mutant and side effects. In 2004 China FDA issued license to manufacture bicyclol. Since then bicyclol has been widely used to treat chronic HBV and HCV in China.

Key Words: Chronic viral hepatitis, new drug, bicyclol, apoptosis, combination therapy, pegylated interferon, lamivudine, adefovir.

1. INTRODUCTION

Viral hepatitis is a disease worldwide. The number of people chronically infected by hepatitis B virus in China is at the top in the world. Approximately 20% of chronic hepatitis B (CHB) and hepatitis C patients (CHC) will develop to liver cirrhosis after a course of 5-20 years infection. Moreover, HBV infection is highly associated with the pathogenesis of primary hepatic cellular carcinoma (HCC). Despite the worldwide magnitude of CHB infection, effective and safe drug for the treatment of HBV and HCV is still limited. So far, interferon- α 2b and pegylated IFN-α 2α are generally accepted drugs for the specific treatment of CHC and CHB [1]. In addition, several anti-viral nucleoside/nucleotide analogues such as lamivudine, adefovir dipivoxil and entecavir have been developed to treat CHB [2]. However, the therapeutic efficacy of these drugs is still limited after withdrawal of drug medication, and the relapse rate and side effects are rather high. The therapy of nucleoside/nucleotide analogues has another problem of inducing HBV-DNA mutation (YMDD) which results in drug resistance particularly lamivudine [3, 4]. Therefore, it is very important to develop effective and safe new drugs for the treatment of CHB and CHC.

*Address correspondence to this author at the Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing 100050, China; Tel: +86-010-63165178; Fax: +86-010-63017757;
E-mail: liugt@imm.ac.cn / gtliu2002@yahoo.com

2. HISTORY OF BICYCLOL DISCOVERY

Traditional Chinese medicine has much experience in the treatment of CHB. In the early of 1970s clinical observation found that *Fructus Schizandrae,* one of the Chinese medicinal herbs, was effective in improvement of the abnormal liver function of CHB patients. Following this clinical lead, the study of pharmacology and chemistry of *F.Schizandrae* was systematically performed by researchers in our institute. Seven dibenzo (a, c) cycloocten lignans were isolated from the kernels of *F.Schizandrae* and their protective action against liver injury was screened in carbon tetrachloride-intoxicated mice. Of seven lignans, schizandrin C (Fig. **1**) was shown to be the most active compound in protection against liver injury in carbon tetrachloride-intoxicated mice. In order to develop novel drug for therapy of CHB, the chemists attempted to totally synthesized schizandrin C, but they were failure. Then, a number of analogues of natural schizandrin C were synthesized and screened in liver injury models. Fortunately, dimethyl-4, 4'-dimethoxy-5, 6, 5', 6'-dimethylene dioxybiphenyl-2, 2'-dicarboxylate (called DDB, Fig. **1**) was found to be effective in protection against liver injury in animals [5]. Through pre-clinical study, DDB was recommended for clinical trial. DDB markedly improved the impaired liver function and ameliorated the main symptoms of hepatitis patients. Side effects are rare and not serious. Since 1983 DDB has been widely used for the improvement of abnormal liver function of CHB hepatitis in China and also in several developing countries such as Korea and Egypt.

1573-4064/09 $55.00+.00

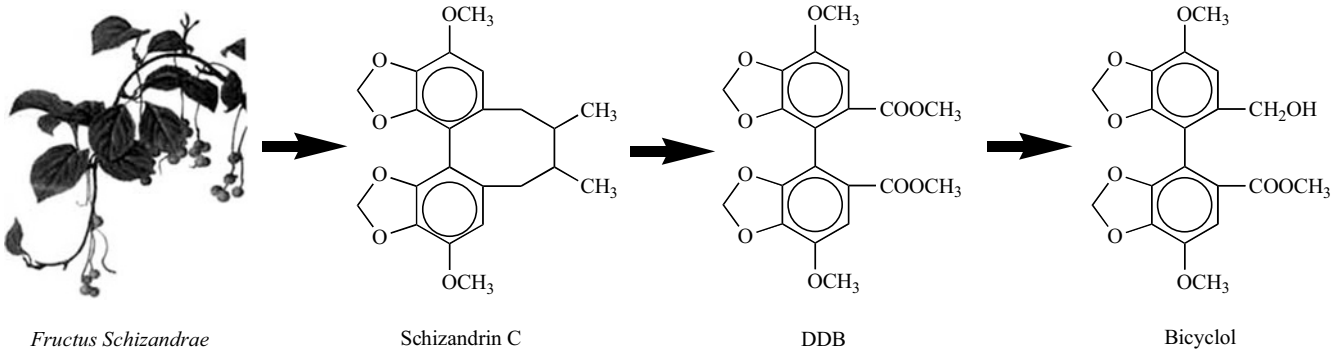

Fig. (1). The picture of *F. Schizandrae a*nd chemical structures of schizandrin C, DDB and bicyclol.

However, the anti-viral activity and bioavailability of DDB were poor. In order to develop a novel drug better than DDB in inhibition of HBV replication and bioavailability, a number of DDB derivatives were synthesized. The structure-activity relationship of DDB derivatives was studied in liver injury model. It was found that the hepatoprotective activity of DDB derivatives varied with position of methylene dioxide group, the length of the carboxylic acid on the biphenyl ring, displacement of the dicarboxylate group by hydroxyl or carboxyl group and heterocyclic ring between the two phenyl rings. Finally, a novel substitute of DDB (named bicyclol) by a 6-hydroxymethy (-CH_2OH) instead of 6-the carboxylate group (-$COOCH_3$) in the side chain (Fig. **1**) was found to be more effective than the parent DDB in protection against liver injury, and was also shown to inhibit hepatitis virus replication in 2.2.15 cell line and duck hepatitis [6a]. Bicyclol has two enantiomers. The enantioseparation of (±)-bicyclol was further performed by optically active alkaloid. Two enantiomers of (-) and (+) bicyclol were successfully obtained with crystallization. The biological profiles of both (-) and (+) bicyclol were compared in CCl_4-liver model in mice. The results showed that the protective effect of (-)-bicyclol at dosage 100 mg/kg was similar to that of the racemic bicyclol at 200 mg/kg. The potency of (+)-bicyclol at 200 mg/kg was inactive [6b]. From the industrial manufacture of bicyclol, the process of enantioseparation of (-)-bicyclol from (±)-bicyclol is rather complicated, (±)-bicyclol was finally selected and put into production. Bicyclol mentioned in the article was racemic (±)-bicyclol. Through study for 15 years, in 2004 China FDA issued license to manufacture bicyclol. Since then bicyclol has been widely used to treat chronic viral hepatitis in the mainland of China. The pharmacology and clinical application of bicyclol are briefly reviewed as follows.

3. PHARMACOLOGY OF BICYCLOL

3.1. Anti-Hepatitis Virus Activity of Bicyclol in 2.2.15 Cell Line and Duck Viral Hepatitis

(1). Assay of Anti-Viral Action of Bicyclol in 2.2.15 Cell Line

The 2.2.15 cell line is a human hepatic cellular carcinoma HepG2 cell which was established by stably transfected with human HBV genome, thus the cells can produce hepatitis antigens, HBV-DNA and virus-like particles. To testing the antiviral activity of bicyclol on HBsAg and HBeAg secretion by 2.2.15 cells, 1.2×10^5 2.2.15 cells per well were seeded in 24-well culture plates containing MEM medium and related substances, one day later, various concentrations of bicyclol ($5x10^{-4}$, $1x10^{-4}$ and $5x10^{-5}$mol/L) or equal volume of DMSO were added. At day 4 and day 8, the medium was removed and replaced with the same amount of bicyclol and medium. At day 12, the medium was removed and stored at –20°C for RIA assay of HBsAg and HBeAg with the same lot of test kits (Abbot). The results revealed that bicyclol at non-toxic concentrations of $5x10^{-4}$ and $1x10^{-4}$ mol/L inhibited the secretion of HBsAg by 59% and HBeAg by 35% in 2.2.15 cells. The total DNA of 2.2.15 cells was extracted for detection of HBV-DNA with ^{32}P-labeled HBV-DNA probe and dot blot hybridization method. The results showed that bicyclol at the concentrations of $5x10^{-4}$ and $1x10^{-4}$ mol/L reduced the amount of intracellular HBV-DNA, while $5x10^{-5}$ mol/L of bicyclol was ineffective.

(2). Assay of Anti-Viral Activity of Bicyclol in Duck Hepatitis

The research and development of anti-HBV drug was hampered by the lack of reliable HBV replication animal models in the past, although HBV transgenic mouse model is now available. Therefore, other hepadnaviruses such as woodchuck hepatitis virus (WHV) and duck hepatitis B virus (DHBV) models have been used for testing the anti-hepatitis activity of compounds. The WHV model is closely related to human viral hepatitis as the WHV may develop to liver cirrhosis and finally liver carcinoma. Since there is no woodchuck grown in China, accordingly, Beijing duckling infected with DHBV was proved to be a suitable model. After intravenous inoculation of DHBV-positive serum into 3 days-old Beijing ducklings, DHBV-DNA was found in the serum 4 days later, and persisted for 2 weeks. Furthermore, DHBV-DNA was found in liver extract 2 days post-injection, and proliferate intermediates were also found. The DHBV-DNA in serum and liver of ducks can be detected by a DNA-spot hybridization assay using DNA extracts. Therefore, duck viral hepatitis model is usually used for anti-hepatitis virus assay in China. So, the anti-hepatitis virus activity of bicyclol was first tested in the separated duck hepatocytes *in vitro* and then in Beijing ducklings infected with serum containing DHBV.

Beijing ducklings were infected intravenously with Shanghai duck hepatitis B virus (DHBV) serum. Seven days after infection of DHBV, ducklings were divided into five

groups and subject to different treatments. The drug groups were administered with doses of 0.2, 0.4, 0.6 g/kg of bicyclol suspended in 0.5% CMC-Na *p.o* or foscarnet (PFA) 0.5 g/kg *i.p.* (as positive anti-virus control drug) once daily for 10 days, respectively. Ducklings in the control group received the same volume of 5% CMC-Na *p.o.* After treatment for 10 days, bicyclol at 0.4, 0.6 g/kg significantly reduced the serum level of DHBV-DNA of ducklings (Table **1**). Bicyclol is ineffective at 0.2 g/kg. The potency of bicyclol (0.6 g/kg) corresponds to that of PFA (0.5 g/kg, *i.p*) [6]. When the effect of 0.4 and 0.6 g/kg of bicyclol on DHBV was repeated for two times, similar results were obtained. In another experiment, the ducklings infected with DHBV were treated with 0.6 g/kg of bicyclol or the vehicle once daily for 10 days, DHBV level in the livers was assayed by dot-blot hybridization method. It was found that bicyclol reduced liver DHBV DNA levels at day 5, day 10 of therapy and day 3 post therapy. The intracellular HBV-DNA in 2.2.15 cells was also measured. After cultivation of 2.2.15 cells with the above concentrations of bicyclol for 12 days, the total DNA in 2.2.15 cells was extracted for detection of HBV-DNA with ^{32}P-labeled HBV-DNA probe and dot-blot hybridization method. The results showed that bicyclol at concentrations of $5x10^{-4}$ and $1x10^{-4}$ mol/L markedly reduced the amount of intracellular HBV-DNA, while $5x10^{-5}$ mol/L of bicyclol was ineffective. No sign of intoxication was observed during the course of bicyclol treatment in the ducklings.

The anti-viral action of bicyclol in duckling hepatocytes was also assayed *in vitro*. Beijing ducklings within one day after hatching were infected intravenously with Shanghai duck hepatitis B virus (DHBV) serum. Seven days after infection, duckling hepatocytes were isolated and maintained in a culture medium containing bicyclol dissolved in DMSO for 7 days. DHBV-DNA was measured with dot-blot hybridization method. Bicyclol at non-toxic concentrations of $1x10^{-4}$ to $5x10^{-4}$ mol/L inhibited the replication of DHBV DNA *in vitro*.

(3).No inhibitory Effect on DHBV-DNA Polymerase (DHBV-DNA-P)

Various concentrations of bicyclol were incubated with DHBC-RCs and the substrate of enzymes at 37 °C for 2 h, the activity of DHBV-DNA-P was measured with radiolabeled method. Bicyclol at the concentrations of $1x10^{-3}$ to $1x10^{-5}$ mol/L showed no inhibitory effect on DHBV-DNA-P, indicating that bicyclol is not an inhibitor of DHBV-DNA-P.

All the above results suggest that bicyclol has anti-viral activity in duck hepatitis model and 2.2.15 cell line, and that bicyclol is not an inhibitor of DHBV-DNA-P. Further study found that the mechanism of anti-hepatitis virus action of bicyclol might be through modulation of cytotoxic T lymphocytes. As to by what mechanism bicyclol exerted its anti-viral action, it remains further study and elucidation.

3.2. Hepatoprotective Action of Bicyclol in Animal Models

CCl_4, acetaminophen and D-galactosamine are commonly used chemicals to induce liver injury through different mechanisms in animals CCl_4 is metabolized to $\cdot CCl_3$ radical by liver cytochrome p-450 activation, which induces injury of biomembrane. Overdose of acetaminophen is converted to reactive metabolite radical N-acetyl-p-benzoquinone imines (NAPQI) by liver cytochrome p-450, which induces exhaustion of glutathione in the hepatocytes and finally results in liver damage. D-galactosamine induces liver injury through its damage to DNA of hepatocyte nuclear and through release of cytokines. Besides the above liver injury models, liver injury can also be induced by immunological method such as Bacillus Chalmette Guerin (B.C.G.) or Corynebacterium parvum plus LPS or ConA alone in mice The aim of this study was to evaluate the protective action of bicyclol against liver injury mediated by different mechanisms in mice and rats. The results indicated that bicyclol has dose-effective action in protection against liver injury [6]. In general, 50, 100, 200 mg/kg of bicyclol significantly reduced the elevated serum ALT and AST levels in a dose-dependent manner, and also ameliorated the liver lesions. The results are briefly described as follows.

(1). Protection Against CCl_4-Induced Liver Injury in Mice

Male Kunming strain mice were i.p injected a dose of 0.5 % CCl_4 10ml/kg in peanut oil. Twenty four hours prior to injection of CCl_4, three doses of 200, 100 and 50mg/kg of bicyclol were orally administered to mice at an interval of 8 hours. It was found that the injection of CCl_4 induced significant elevation of serum ALT and AST levels and severe lesions of liver histology. The treatment of bicyclol 50, 100, 200 mg/kg significantly attenuated the elevated ALT and AST levels and liver lesions such as necrosis and inflammatory infiltration in a dose-dependent manner, suggesting that bicyclol has a hepatoprotective action in the mouse CCl_4 model [7].

Table 1. Effect of Bicyclol on Serum DHBV DNA Levels in DHBV-Infected Ducks

Group	Dosage (g/kg×10)	Inhibition Rate of DHBV DNA (%)		
		Treating 5 Days	Treating 10 Days	Post 3 Days
Control	–	15.3±32.6	14.3±42.8	61.9±27.8
PFA	0.25	64.8±34.3**	61.4±37.1**	21.9±27.7
Bicyclol	0.1	26.1±25.4	30.3±34.1	57.4±26.9
	0.2	22.3±30.2	70.1±16.3***	48.7±20.9
	0.3	68.0±20.8**	60.4±30.0**	23.3±49.8

**P<0.05, ***P<0.01 Vs control group.

(2). Protection Against D-Galactosamine Induced Liver Damage in Mice

Male Kunming strain mice were treated with 50, 100, 200 mg/kg of bicyclol as described in CCl_4 experiment above, one hour after dosing the drug,, all mice except the untreated control group were received an i.p. injection of a dose of D-galactosamine (1g/kg). As a result, all three dosages of bicyclol decreased the elevated ALT levels. The dosage of 200 mg/kg of bicyclol markedly reduced the serum AST level besides lowering serum ALT level. The mouse liver histology developed severe fat degeneration and necrosis after injection of D-galactosamine. The livers from mice treated with bicyclol (200 mg/kg) were subjected to histological examination. It *was* observed that bicyclol markedly reduced the lesions of the livers [8].

(3). Protection Against Acetaminophen Induced Liver Injury in Mice

Male mice were pre-treated with 50, 100, 200 mg/kg of bicyclol as mentioned in CCl_4 experiment.. The control mice received the same volume of vehicle. One hour later, a dose of 110 mg/kg acetaminophen was i.p. injected in to all the mice except untreated control to induce liver injury. After fasting for 16 hours, serum ALT and AST levels and liver pathological injury were examined. Bicyclol has a dose-dependent action of lowering serum ALT and AST. Bicyclol at dose of 200 mg/kg also markedly reduced the degree of liver lesions particularly piecemeal necrosis [9] (Fig. **2**). Moreover, the decrease of ATP/Pj and the elevation of Phospholipids/ATP in the hepatocyte mitochondria of acetaminophen-intoxicated mice were significantly inhibited by bicyclol (100,200mg/kg) pretreatment, indicating that bicyclol significantly improved the disturbance of energy metabolism (ATP) in mitochondria induced by acetaminophen [10].

(4). Protection Against ConA- or B.C.G. Plus LPS-Induced Immunological Liver Injury in Mice

Injection of B.C.G. vaccine plus LPS- or ConA alone-induced liver injury is through immune mechanism. Male Kunming strain mice were primed with an injection of B.C.G. vaccine into the tail vein, 10 days later, a dose of 7.5 μg/kg lipopolysaccharides (LPS) was intravenously injected to challenge the accumulated monocytes in the liver to release a number of cytokines, chemokines and free radicals to damage hepatocytes. Different dosages of bicyclol were administered p.o. to mice from the second day for 10 days after the injection of B.C.G. Like in CCl_4, D-galactosamine and acetaminophen liver injury models, bicyclol treatment also significantly protected against liver injury as indicated in lowering of the elevated serum ALT and AST levels and liver lesions. In addition, bicyclol reduced the elevation of TNF-α in serum of mice. Similarly, bicyclol markedly protected against ConA-induced liver injury in mice [11].

(5).Protection Against CCl_4 Induced Injury of Isolated Rat Hepatocytes

Wistar rat hepatocytes were separated by perfusion with 0.05% collagenase. The viability of isolated rat hepatocytes was over 85% in tryphen blue assay. Various concentrations of bicyclol dissolved in 95% ethanol were incubated with the isolated hepatocytes. The same volume of 95% ethanol was added onto the control cells. After culture for 3 hours, 10mM CCl_4 in 95% ethanol (10μl) was added to each well to induce hepatocyte intoxication. The release of ALT, AST and malondialdehyde (MDA) were measured as indication of hepatocyte injury. As a result, CCl_4 induced significant release of ALT and AST and production of MDA, an end product of lipid peroxidation of biomembrane. Prior addition of 10^{-3}, 10^{-4}mol/L bicyclol to the incubation system inhibited release of ALT and AST and production of MDA in the rat hepatocytes in a dose-dependent manner. And bicyclol also markedly increased the viability of rat hepatocytes and attenuated damages of cell surfaces such as aggregation and blabbing of microvillus examined by scanning electron microscopy, suggesting that bicyclol reduced these damages to the plasma membrane of rat hepatocytes (Fig. **3**).

(6). Stimulation by Bicyclol of Protein Biosynthesis in Isolated Rat Hepatocytes

1). Effect on Normal Isolated Hepatocytes

The isolated Wistar rat hepatocytes (10^6cells per well) were obtained by collagenase perfusion as mentioned above. The isolated rat hepatocytes (10^6cells per well) were pre-incubated with various concentrations of bicyclol in DMF for 30 minutes, respectively. The control cells were incubated with the same volume of the vehicle. All the cells were

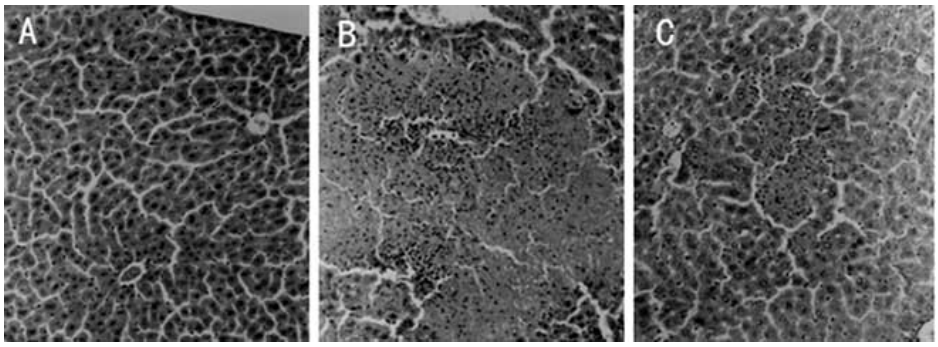

Fig. (2). Protective action of bicyclol against injury of liver tissues induced by acetaminophen in mice. Male Kunming strain mice were pretreated with three doses of bicyclol 200 mg/kg at an interval of 8 hours. The control mice received the same volume of vehicle. One hour later, except normal control group all mice were i.p. injected a dose of 110 mg/kg acetaminophen to induce liver injury. After fasting for 16 hours, liver histology was examined. H.E.staining. Magnitude x 100. A, normal mouse. B, acetaminophen-injected mouse showed severe piecemeal necrosis .C, mouse treated with bicyclol (200mg/kg), piecemeal necrosis was much less severe.

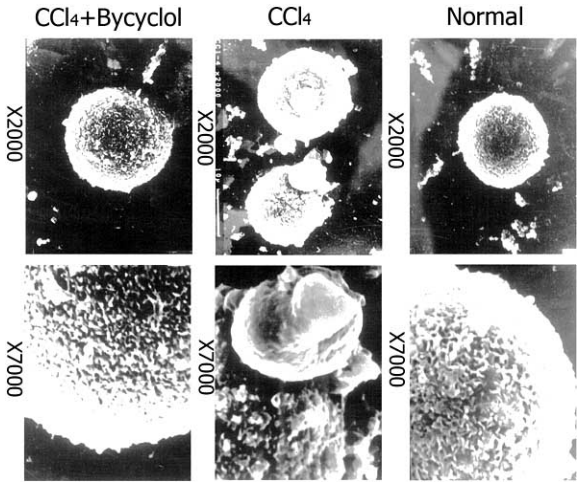

Fig. (3). Photographs of scanning electron microscope of isolated rat hepatocytes. Various concentrations of bicyclol dissolved in 95% ethanol were incubated with isolated Wistar rat hepatocytes. The same volume of 95% ethanol was added onto the control cells. After cultivation for 3 hours, 10mM CCl_4 in 95% ethanol (10μl) was added to each well to induce hepatocyte injury. The rat hepatocytes were examined by scanning electron microscopy, the surfaces of CCl_4 intoxicated hepatocytes showed severe injury expressed in aggregation and blabbing of microvillus. Bicyclol treatment markedly ameliorated injury of hepatocytes.

then cultured with ^{14}C-leucine (0.5μCi per well) for 60 minutes. The cells were lysesed with Triton x-100 and the protein was precipitated with trichloroacetic acid (TCA). The suspension was centrifuged at 4000 rpm for 10 minutes. After washing with TCA for three times and centrifugation, the precipitate was dissolved in 10N NaOH solution. The radioactivity of ^{14}C-leucine in the protein solution was counted by liquid scintillator. In comparison with untreated control hepatocytes, the addition of bicyclol (10^{-3} and 10^{-4} mol/L) into the cultured rat hepatocytes significantly increased the incorporation of ^{14}C-leucine into protein by 3 and 2 folds, respectively, indicating that bicyclol stimulated protein biosynthesis in rat hepatocytes.

2). Attenuation of Cycloheximide-Induced Inhibition of Protein Biosynthesis

Cycloheximide is an inhibitor of protein biosynthesis. The incubation condition of this experiment was the same as above. Rat hepatocytes ($5x10^5$ cells) were pre-incubated with various concentrations of bicyclol for 30 minutes and then with 10^{-4} mol/L cycloheximide for 30 minutes. The radioactivity of ^{14}C-leucine in the hepatocyte protein was counted as above. The addition of cycloheximide markedly inhibited the incorporation of ^{14}C- leucine into hepatocyte protein. Prior incubation of bicyclol 10^{-3} and 10^{-4}mol/L almost completely attenuated the inhibition of protein biosynthesis caused by cycloheximide.

3). Stimulation of Protein Biosynthesis of Rat Hepatocytes-Intoxicated with CCl_4

The results of above two experiments indicate that bicyclol can stimulate protein biosynthesis in isolated Wistar rat hepatocytes. This experiment was to further study whether bicyclol can also stimulate protein biosynthesis in CCl_4-intoxicated rat hepatocytes.The hepatocytes of normal rat were obtained as described above. Rat hepatocytes were precultured with various concentrations of bicyclol for one hour and then with 10μl CCl_4 (diluted 40 times with ethanol) for 2 hours. A dose of 5 μCi of ^{14}C-leucine was added into the cultured hepatocytes and continuously cultivated for I hour. The methods for extraction of protein and counting of ^{14}C-leucine radioactivity in the protein were the same as mentioned in the above experiments. Each group had four samples for counting. The incorporation of ^{14}C-leucine into hepatocyte protein (Dpm/$5x10^5$ cells) from 5205±166 of normal control group to 493±26 of CCl_4-intoxicated rat. Pretreatment of rat hepatocytes with 1mmol/L bicyclol significantly counteracted CCl_4-induced inhibition of protein biosynthesis. The radioactivity (Dpm/$5x10^5$ cells) in protein was 493 ±26 for CCl_4 group and 1504±157 for bicyclol group. The increase of protein biosynthesis by bicyclol was highly significant ($P<0.001$).

All the results of three *in vitro* experiments suggested that bicyclol has a stimulating effect on hepatocyte protein biosynthesis under injury. This action of bicyclol would be beneficial to repair of liver injury in chronic hepatitis patients.

3.3. Mechanisms of Bicyclol Against Liver Injury

The mechanism of liver injury is mainly related to mitochondria. Various stressors including virus infection induce damages of mitochondrial structure and function, which lead to disturbance of energy metabolism and protosome release such as cytochrome C and apoptosis inducing factor (AIF), thereby resulting in necrosis and apoptosis of hepatocytes [12]. As mentioned above, bicyclol has anti-viral and hepatoprotective actions. It is necessary to study by what mechanisms bicyclol exerts its action on virus and hepatocytes. For elucidating the active mechanism of bicyclol against liver injury, the following experiments were performed.

(1). Bicyclol is Not an Inhibitor of Liver and Serum ALT Activity

Since bicyclol has action to lower serum ALT and AST in different liver injury models; a question arises whether

bicyclol is an inhibitor of ALT and AST. To study this question, serum and liver homogenates were incubated with higher concentration (10^{-3} mol/L) of bicyclol in a water bath at 37 °C for 8h. No decline of both ALT and AST activity was demonstrated after incubation for 8h. To further study whether bicyclol reduces liver ALT protein content, liver ALT protein was separated and highly purified from normal mice by biochemical techniques. This purified ALT with adjuvant was injected into rabbit dermally to induce anti-ALT protein antibody production. Using this rabbit serum containing anti-ALT antibody, the ALT protein content in the livers from normal and bicyclol 150mg/kg-treated mice was assayed by immune-rocket electrophoresis. There was no decrease of liver ALT protein content in bicyclol treated mice in comparison with the mice of normal control. These results suggest that bicyclol neither directly inhibits ALT activity nor reduces ALT protein content in mouse liver [7].

(2). Bicyclol can Maintain Hepatocyte Membrane Stability Through Scavenging Free Radicals

It is known that CCl_4-induced liver injury is due to its metabolic activation to methyl trichloride radical ($\cdot CCl_3$) in liver. The formed $\cdot CCl_3$ radical is very toxic to proteins and lipids of biomembrane; thereby destroying the integrity of biomembrane. Free radicals can initiate lipid peroxidation of biomembrane, which results in damage of the structure and function of cells. If the hepatocyte membranes are injured by some factors, the ALT in cytosol and AST in mitochondria will be released from the cells. A question arises whether bicyclol can scavenge free radicals. To answer this question, lipid peroxidation of liver microsomes from normal mice was initiated with CCl_4 plus NADPH in the presence and absence of bicyclol *in vitro*. As a result, bicyclol inhibited lipid peroxidation in a dose-dependent manner. Bicyclol at the concentration of 10^{-4} mol/L almost completely inhibited MDA formation induced by CCl_4 in the microsomes. Further study demonstrated that bicyclol markedly reduced the covalent binding of $^{14}CCl_4$ to lipids and proteins of mouse liver microsomes. Using electron spin resonance (ESR) technique detected that bicyclol scavenged 63% of $\cdot CCl_3$ radicals generated from incubation of mouse liver microsomes with CCl_4 and NADPH [7]. Bicyclol could also inhibit oxygen free radicals generation in PMA-activated neutrophils. It is known that when human hepatocytes are infected with virus, the cells produce a number of inflammatory cytokines including oxygen free radicals to damage biomembrane, mitochondria and nuclear DNA. The free radical scavenging activity of bicyclol should be beneficial to maintain the membrane stability of hepatocytes.

(3).Protection Against Mitochondria Injury of Hepatocytes

It was reported that the mitochondria play a key role in necrosis and apoptosis of hepatocytes [12]. The AST exists in hepatocyte mitochondria. Both the results of pharmacological study and clinical trials of bicyclol indicate that bicyclol reduced not only serum ALT but also AST. There is a possibility that bicyclol has a protective action against mitochondrial damage in hepatocytes. To confirm this possibility, a higher dose of acetaminophen (AAP) was i.p. injected into mice to induce mitochondrial damage. Before AAP injection mice were treated with 150 mg/kg or 50 mg/kg of bicyclol once a day for 3 days. Bicyclol markedly protected against the damage of hepatocyte mitochondria as indicated in decrease of the injury of ultrastructure of mitochondria, the release of AST from the mitochondria, mitochondria fluidity and mitochondria swelling as well as the cytochrome C release from mitochondria [9]. The results indicate that the mitochondria are one of the target sites in bicyclol against liver injury.

(4). Protection Against Nuclear DNA Damage and Apoptosis of Hepatocytes

Human viral hepatitis is due to the virus integrated into nuclear DNA. It was reported that hepatocyte apoptosis is very important in the pathophysiology of viral hepatitis, and the inhibition of hepatocyte apoptosis would be a new strategy of treating viral hepatitis [13]. To investigate the effect of bicyclol on nuclear DNA damage and apoptosis, ConA-induced mouse liver injury model was used, because the active mechanism of ConA-induced liver damage is mediated by cytotoxic lymphocyte activation and apoptosis signaling. It was demonstrated that there were nuclear DNA fragmentation, DNA ladder and apoptosis as well as the cytochrome C release into the cytosol from mitochondria after injection of ConA (20mg/kg) into mice. When mice were pretreated with 150 mg/kg of bicyclol, once daily for 3 days before injection of 20 mg/kg ConA, all the above damages in liver were attenuated markedly. The increase of serum TNF-α level was also reduced by bicyclol. The injection of ConA induced up-regulation of TNF-α, IFN-α, Fas and FasLmRNA expression in liver tissues. Bicyclol significantly down-regulated the expression of IFN-α, Fas and FasLmRNA, but only slightly affected TNF-α mRNA expression in liver tissues. It is reasonable to speculate that the main active mechanism of bicyclol in protection against liver injury is anti-apoptosis of hepatocytes through inhibition of Fas/FasL mRNA expression in hepatocytes and TNF-α release in mice [14], although to assess the anti-apoptosis effect of bicyclol needs further study with more biomarkers of cell apoptosis. The *in vitro* study in macrophages demonstrated that bicyclol significantly inhibited iNOS expression and NF-κB activation induced by lipopolysaccharides [15]. It means that bicyclol has an anti-inflammatory action on liver.

(5). Induction of Heat Shock Proteins is the Primary Target of Bicyclol Against Liver Injury

Heat shock proteins (HSPs) are molecular chaperons. They play important roles in cellular homeostasis during normal cell growth and in response to detrimental environmental stress [16,17]. Among several members of the HSP family, stress-inducible HSP27 and HSP70 are most intensively studied for their functions in protecting cells and tissues from injury caused by a variety of pathological agents [18, 19]. Studies on cell and animal models have shown that HSPs can be induced at the mRNA level by some agents to promote cell growth and protect tissues from injury. Thus, HSPs provide cells and tissues with an important defense mechanism to correct miss-folding of proteins. In order to further elucidate whether the molecular mechanism of bicyclol against liver injury is through HSP to correct miss-folding of proteins, we have carried out a set of *in vivo* experiments using ConA-and acetaminophen-induced liver

injury mouse models. Bicyclol through oral administration markedly alleviated ConA- and acetaminophen-induced liver injury in mice as indicated in reduction of serum aminotransferase, liver necrosis, the release of cytochrome C and apoptosis inducing factor (AIF) from mitochondria and hepatic DNA fragmentation. Correlated with these effects, bicyclol induced expressions of the mRNA and protein of hepatic HSP70/27, and activate heat shock factor-1(HSF1) in the mouse liver. Correspondingly, the elevated HSP27 and 70 proteins suppressed IκB degradation and NF-κB activation that were caused by ConA. These suppressive effects of bicyclol on ConA-and acetaminophen-induced mouse liver injury were markedly attenuated by quercetin, an inhibitor of HSPs biosynthesis [20]. Our results as described above suggest that bicyclol protects against liver injury by inducing the expression of HSP70/27-mRNA, and thus consequently inhibiting the NF-κB-mediated apoptosis (Fig. **4**).

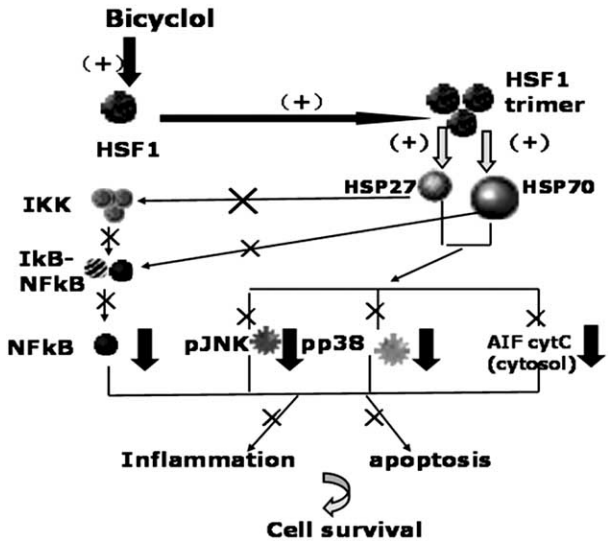

Fig. (4). Illustration of the mechanism of bicyclol against apoptosis of hepatocytes.

The "+" indicates the site of stimulation of bicyclol and the "X" indicates the site of inhibition of the elevated HSP70 and HSP27 by bicyclol. Mice were pretreated with 300mg/kg of bicyclol once daily for three days orally, and then were injected a dose of ConA 22 mg/kg-from the tail vein or 140mg/kg acetaminophen intraperitoneally, 2-3 hours later, the liver tissues were subjected to HSP70 and HSP27 related measurement by western blot and RT-PCR.

3.4. Anti-Liver Fibrosis of Bicyclol in Chronic CCl_4- and Dimethylnitosamine-Intoxicated Rats and Mice

Approximately 20% of chronic viral hepatitis patients will develop liver cirrhosis after a course of 5-20 years infection. One of the purposes of HBV and HCV therapy is to reduce liver fibrosis formation. It is interesting to study whether bicyclol has anti-liver fibrosis. Female Wistar rats were subcutaneously injected with 25% CCl_4 in olive oil (0.5 ml/kg) twice a week for 12 weeks. From 6 weeks till the end of 12 weeks, rats were treated with 75, 150 mg/kg of bicyclol once daily except Sundays. The control rats received the same volume of 0.5% CMC-Na. Serum ALT, AST, total protein, albumin and bilirubin were determined with different kits. Liver hydroxyproline and lesions were evaluated. After a course of treatment, bicyclol significantly reduced serum ALT, AST, globulin and liver hydroxyproline. The liver damage and fibrosis were ameliorated simultaneously (Fig. **5**). The hepatic transfer growth factor (TGF-α) which plays a key role in the production of fibrosis was inhibited significantly. It may be seen that bicyclol not only reduced liver damage, but also inhibited liver fibrosis [21]. Similar results were obtained in CCl_4- and dimethylnitosamine-induced liver fibrosis in mice [22].

4. PHARMACOKINETIC STUDY OF BICYCLOL IN RATS AND DOGS

The absorption, blood concentration, distribution and metabolism of bicyclol in rats and dogs were studied by HPLC. The results are briefly summarized as follows:

(1). Rat Blood Concentration of Bicyclol

Bicyclol in rat blood can be detected 15 minutes after oral administration of bicyclol, indicating that the absorption of bicyclol is rapid. The parameter of pharmacokinetics is in concord with the first model. The Cmax and AUC of bicyclol have a significant dose–concentration relationship. T1/2 (Ka), T1/2(Ke), and V/F are essentially the same for dosages of bicyclol 100, 200 and 400 mg/kg. T1/2(Ka) is 2.19, 2.16, 2.68 hours and for T1/2(Ke) is 3.33, 4.49 and 4.67 h for bicyclol 100, 200 and 400 mg/kg, respectively. CL/F is relatively large which is in concord with the wide distribution of bicyclol in various tissues of the body.

(2).Distribution of Bicyclol in Rats

Bicyclol was distributed in all the tissues including liver, lung, kidney, heat, brain, spleen, fat tissues and skeleton muscle, but mainly in the liver and reached maximum at 4 h, and secondly in fat tissues.

(3). Excretion from Feces, Urine and Bile in Rats

After oral administration of bicyclol, the total recovery rate of bicyclol from rat feces and G.I was 28.8% which included the unabsorbed and excreted from the bile duct into intestine. In other words, approximately 71.2 % of bicyclol administered was absorbed from gastro-intestinal tract. after oral administration. The amount of parent bicyclol excreted from the accumulated feces within 72 hours was 30.15 %, and from the urine was 1.3% for rats of 200mg/kg bicyclol-treated group. The amount of bicyclol excreted in the feces and urine for 100, 50 mg/kg of bicyclol group is lower than 200mg/kg bicyclol-treated group. The accumulated excretion of bicyclol from the bile within 24 hours was 0.03% of the administered bicyclol in rats given 200 mg/kg. Parent bicyclol was not detectable in the bile from rats treated with 100 and 50 mg/kg group.

(4). Plasma Protein Binding of Bicyclol

The average protein binding rate of bicyclol was 78.02±.69%.

(5). Identification of the Metabolites of Bicyclol in the Body

The metabolites of bicyclol were determined by HPLC and identified by MS and ^{1}H-NMR. 4- and 4'-hydroxyl bicyclol are the two main metabolites of bicyclol in urine, feces,

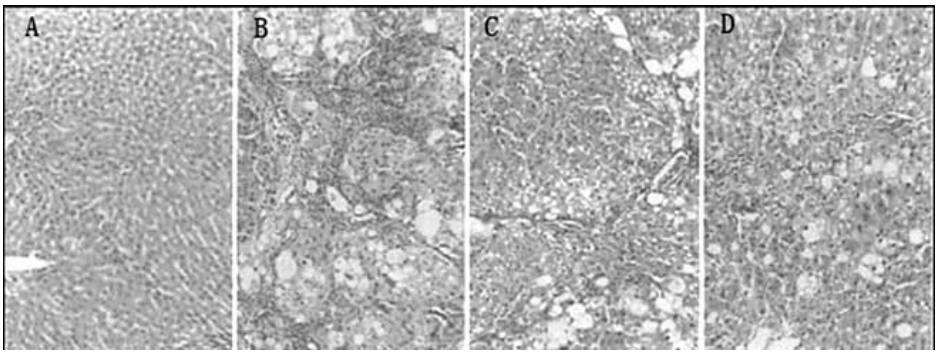

Fig. (5). Protective action of bicyclol against CCl_4 induced liver fibrosis in rats. Female Wistar rats were subcutaneously *injected* with 25% CCl_4 in olive oil 0.5 ml/kg twice a week for 12 weeks. From 6 weeks till the end of 12 weeks, rats were treated with 75, 150 mg/kg of bicyclol once daily except Sunday. Liver tissues were processed for examination of damage and fibrosis by microscopy. **A**, normal rat. **B**, CCl_4 model rat, inflammation, fatty degeneration and fibrosis formation were severe. **C**, Rat treated with bicyclol (100 mg/kg). D, Rat treated with bicyclol (200 mg/kg). Liver lesions of bicyclol 200mg/kg treated rat were significantly ameliorated. Bicyclol 100mg/kg was also effective but weaker than 200mg/kg of bicyclol.

plasma and liver. The hepatoprotective activity of both metabolites are weaker than the parent bicyclol [23].

(6). Pharmacokinetics of Bicyclol in Dogs

Five dogs received orally administration of 20 mg/kg bicyclol in 0.2% carboxyl methylcellulose (Na-CMC). The parent bicyclol in dog blood can be detected 15 minutes after dosing bicyclol, and reached a maximum 30 minutes after bicyclol medication. Three dogs still had bicyclol in their blood, and two dogs had no detectable bicyclol 24 hours after given bicyclol.

In summary, bicyclol is absorbed from G.I. rapidly and mainly distributed in the liver. Its main metabolites are 4- and 4'-hydroxyl bicyclol.

5. TOXICITY STUDY OF BICYCLOL

The toxicity study of bicyclol included acute toxicity, chronic toxicity in rats and Beagle dogs, mutagenicity assay and reproductive toxicity as well as general pharmacology. Acute toxicity test was performed in Kunming mice and Wistar rats. Single dose of bicyclol (3 or 5 g/kg body weight) was administered to mice by gavage, respectively. A single dose of bicyclol (5g/kg body weight) was orally administered to Wistar rats. Mortality rate and clinical symptoms of animals were recorded for 7 days. Sub-acute toxicity test was carried out in rats that were treated with various doses of bicyclol (150, 300, 600 mg/kg) once daily for 14 days. Animal behaviors, blood biochemical markers, blood and urine pictures were examined. Chronic toxicity test was conducted in 80 Wistar rats of both sexes. Various doses of bicyclol 150, 300, 600mg/kg (100-400 folds corresponding to the proposed therapeutic dose 1.5 mg/ kg/day of bicyclol for patients) were orally administered into the rats once daily for 6 months except Sundays. The control rats were given the same volume of 0.2% Na-CMC. Twenty-one beagle dogs received 25, 75, 225mg/kg of bicyclol (16.6, 50, 150 folds corresponding to the proposed therapeutic dose of bicyclol for patients) once a day for 6 months except Sundays. The body weight, food intake, urine and feces, blood picture, blood biochemical markers, and pathological examination of main organs were determined. Mutagenicity and teratogenicity were assayed according to the regular methods. Mutagenicity assay included Ames's test, chromosome aberration test in CHL cells and micronucleus test in mice. For the teratogenicity assay, pregnant Wistar rats weighing 200-250 g were treated with 0.2, 1.0g/kg bicyclol once daily from the 7^{th} day of gestation for 10 days. The oral LD50 of bicyclol was over 5g/kg in mice and rats. No noticeable alterations in subacute and chronic toxicity of rats and dogs were demonstrated. No mutagenicity and teratogenicity of bicyclol were found [24]. It may be concluded that bicyclol is a drug with low toxicity in animals.

6. CLINICAL TRIAL OF BICYCLOL ON CHRONIC VIRAL HEPATITIS B AND C PATIENTS

6.1. Phase III Clinical Trial

Phase I and II trials of bicyclol have been completed. The results indicated that bicyclol is safe and bicyclol has a linear pharmacokinetic feature. The absorption of oral administration of bicyclol is higher after meal than before meal, but there was no accumulation in the body before and after meal [25]. Phase III trial of bicyclol was carried out by the method of double blind, randomized and control (positive drug or placebo) in eight hospitals in China.

(1). Efficacy in Chronic Hepatitis B Patients

The patients selected in the trial were all with elevated serum ALT and AST. Positive HBsAg, HBeAg and HBV-DNA lasted for at least 12 weeks. A total of 407 cases of CHB were randomly divided into bicyclol group (269 cases) and positive drug control group (138 cases) at the ratio of 2:1. DDB was used as positive control drug. The demographic (age, sex) and baseline features (ALT, AST level) were similar in both groups. The dosage, appearance and size of both bicyclol and DDB were all essentially the same. The patients were treated with bicyclol or DDB 25 mg, tid, p.o, for 24 weeks, drug treatment was then withdrawn and they were followed up for 12 weeks. Investigating items included clinical symptoms, liver function tests, serum hepatitis B (HBV) markers and safety profile. Serum ALT, AST and bilirubin were determined before treatment and every 4 weeks after starting therapy and cessation of medication. Serum

Table 2. Phase III Clinical Trial of Bicyclol on 269 Cases of CHB in 8 Hospitals

Criteria	6 Months Therapy	3 months after Stop Therapy
ALT normalized rate (%)	53.5(144/269)	40.2(108/269)
AST normalized rate (%)	48.7(131/269)	48.7(131/269)
HBeAg negative rate (%)	20.8(56/269)	29.0(78/269)
Anti-HBeAb positive rate (%)	15.6(42/269)	20.8(56/269)
HBV-DNA negative rate (%)	39.0(105 /269)	45.7(123/269)

Note: Bicyclol 25mg, t.i.d. p.o. x 6 months and followed up for 3 months after stopped medication.

Figures in parentheses are the numbers of part/total patients.

HBsAg, HBsAg and HBeAb were assayed with Abbott kits, and HBV-DNA was detected by dot-blot hybridization method. The measurement of HBV markers was monitored before treatment and at 12, 24 weeks after starting therapy and 12 weeks post cessation of medication, respectively. Efficacy evaluation was based on Intent-To-Treat (ITT) principle.

The results of bicyclol treatment on biochemical and virus hallmarkers are summarized in Table **2**. After bicyclol therapy, both clinical symptoms and serum ALT, AST levels were improved markedly. The normalization rates of ALT and AST were 53.5% and 48.7% at week 24. and kept sustained normal in 40.2% and 48.7% at 12 week after cessation of treatment. In DDB group, the ALT and AST normalization rates were 61.6% and 44.2% at week 24, and 45.7% and 50.0% at 12 week after stop of the treatment, respectively. The improvement of serum ALT and AST showed no statistically significant difference between bicyclol and DDB groups ($P > 0.05$). Regarding the HBV markers, in bicyclol group the negative rates of HBeAg, HBeAg/Anti-HBe seroconversion and negativity of HBV-DNA at the end of 24 week therapy were 20.8%, 15.6% and 39.0%, respectively, and then 29.0%, 20.8% and 45.7% at 12 week after cessation of treatment. The efficacy of bicyclol in inhibition of HBV markers is correlated with the level of ALT, the higher the ALT level, the higher the negative rate of HBeAg and seroconversion of HBeAb (Table **3**). Whereas in DDB group, those parameters (negative rates of HBeAg, HBeAg/Anti—HBe seroconversion and negativity of HBV-DNA) were 15.2%, 9.4% and 37.7% at week 24, and 21.0%, 14.5% and 38.4% at 12 week after cessation of DDB treatment. These results between bicyclol and DDB groups showed no significant difference statistically. However. in patients whose baseline ALT levels were higher than 5 times of upper limit of normal (>200 U /L), the HBeAg negative rate and seroconversion rate of anti-HBeAb at 12 weeks after cessation of treatment in bicyclol group were significant higher than those in DDB group, those were 48% versus 22% ($P = 0.015$) and 38% versus 12% ($P = 0.01$) (Table **4**). The difference between DDB and bicyclol group patients was significant statistically ($P<0.05$ or <0.01). The symptoms of patients such as fatigue, anorexia, anoxia and liver pain were markedly ameliorated. The adverse reactions of bicyclol group were mild and uncommon, only skin rash and dizziness occurred in one patient of each group, and skin rashes occurred in two and loss of appetite and nausea occurred in one patient who received DDB [26]. In short, bicyclol is an novel hepatoprotective drug with anti-hepatitis virus activity particularly for the improvement of the clinical symptoms and serum ALT and AST. Bicyclol is superior to DDB on the clearance of serum HBeAg and seroconversion (anti-HBeAb). It is well tolerated and safety throughout the course of tria1.

(2). Effect on HCV Patients

Thirty-nine CHC patients matched demographically and clinically were randomized into two groups: bicyclol group (group A, 20 cases) and placebo/bicyclol crossover group (group B, 19 cases). Group A patients received bicyclol (25mg, tid. p.o.) and group B patients received placebo (starch tablet 25mg, t.i.d. p.o.) for 3 months. Then the patients in group A received the same dosage of bicyclol for further 3 months and were observed for 3 months after the treatment was discontinued. The patients in group B received

Table 3. Correlation of Negative Rate of HBeAg /Seroconversion of Anti-HBeAb and Serum ALT Levels after 24 Weeks Treatment with Bicyclol and Withdrawal of Bicyclol for 12 Weeks in CHB Patients

Marker	ALT < 200IU/L		ALT >200IU/L	
	24 Weeks	12 Weeks*	24 Weeks	12 Weeks*
HBeAg negative rate (%)	18.5	23.3	28.6	47.6
HBeAb conversion rate (%)	12.6	15.5	25.4	38.1

Note: Bicyclol 25mg, t.i.d. p.o. x 6 months and followed up for 3 months after stopped medication.

* Cessation of bicyclol treatment for 12 weeks.

Table 4. Comparison of the Effect of Bicyclol and DDB on HBV Markers in Patients with Serum ALT>200U/L

Group	HBeAg (+) → (-) (%)		Anti-eAb Conversion (-) → (+) (%)	
	24 Week	36 Week	24 Week	36 Week
Bicyclol	28.6(18/63)	47.6(30/63)	25.4(16/63)	38.1(24/63)
DDB	18.8(6/32)	21.9(7/32)	15.6(5/32)	12.5(4/32)
P value	0.015		0.010	

Note: Bicyclol and DDB 25mg, t.i.d. p.o. x 6 months and followed up for 3 months after cessation of medication. Figures in parentheses are the numbers of part/total patients.

bicyclol 25mg, t.i.d. p.o for six months and were observed for 3 months after withdrawal of the drug treatment. Investigation items included clinical manifestations, 1iver function, serum HCV RNA and anti-HCV. Results in group A indicated that the serum ALT was 120±43 U/L before treatment and was 57±32 U/L after treatment (P<0.01). In group B the baseline serum ALT was 126±48 U/L. After the placebo administration for 3 months the serum ALT was 127±97 U/L (P>0. 05), and the clinical feature showed no improvement. After bicyclol treatment for 6 months, the serum ALT in group B was reduced to 68±45 U/L, significantly lower than that before bicyclol treatment (P<0.01), and clinical symptoms improved. The overall ALT normalization response rate for the total 39 patients was 64.1% at the end of bicyclol treatment, and 48.7% at 3 months after the treatment was discontinued. The serum HCV-RNA became negative in 5 patients at the end of treatment and was still negative in 2 patients at 3 months after the cessation of treatment. Adverse drug reactions were mild and uncommon. Mild dizziness occurred in 1 patient in each group. The results indicated that bicyclol is effective in improving ALT and AST as well as clinical manifestations of CHC patients [27]. It is safe and well tolerated and shows little adverse reaction.

6.2. Phase IV Clinical Trial

After China FDA issued a license to manufacture and sell bicyclol in China, an open trial of bicyclol in more than two thousand CHB patients and 100 cases of HCV were performed in 80 hospitals in China. Besides adult patients (18-65 y) the age of patients was extended to 12-17 years old. The dosage of bicyclol was 25-50 mg, tid, p.o. for six months as used in phase III clinical trial. The results were essentially similar to phase III trial.

(1).Effect on CHB

A total of 2064 cases of CHB patients (12-65 y) completed 6 months of treatment with bicyclol. Among them, 150 cases were young patients (12-17 y), 1914 cases were adult (18-65 y). In general, the results of phase IV trial were similar to those of phase III trial. After a course of 6 months treatment, the normalized rate of serum ALT was 59.6-70%, and AST was 48.3-59.4% [28]. The therapeutic efficacy of bicyclol in young patients (12-17 y) seemed to be higher than adult patients (Table **5**).

In addition to a significant lowering effect of bicyclol on serum ALT and AST, bicyclol also inhibited HBV replication to a certain extent. After 6 months of treatment with bicyclol the negative conversion rate of HBeAg was approximately 20%, and the seroconversion rate of HBeAb was about 15%. The negative rate of HBeAg and seroconversion rate of HBeAb were correlated with the age and ALT levels of patient. The response of those patients (12-65y) with serum ALT>5ULN and young patient group (12-17y) seemed to be more sensitive to bicyclol therapy (Table **6**). The efficacy of bicyclol in inhibition HBV replication still remained

Table 5. The Normalization Rates of Serum ALT and AST in Patients with Chronic Viral Hepatitis B after Treatment with Bicyclol

Patients	Age	Marker	Normalization rate %		
			12 Weeks Therapy	24 Weeks Therapy	12 Weeks Post Therapy
All patients	12-65	ALT	44.9(926/2064)	60.4 (1246/2064)	71.9(846/1177)
		AST	32.3(628/1946)	49.1(956/1946)	75.8(692/913)
Young patients	12-17	ALT	52.7(79/150)	70.0 (105/150)	76.7 (79/103)
		AST	37.1(53/143)	59.4(85/143)	80.0 (68/85)
Adult patients	18-65	ALT	44.3 (847/1914)	59.6(1141/1914)	71.4 (767/1704)
		AST	31.9(575/1803)	48.3(871/1803)	75.4(624/828)

Note: Bicyclol 25mg, t.i.d. p.o. x 6 months. Figures in parentheses are the numbers of part/total patients.

Table 6. The Changes of Serum HBV DNA Markers in Chronic Viral Hepatitis B Patients after Bicyclol Treatment for 24 Weeks and then Cessation of Medication for 12 Weeks

Marker	Treatment	ALT Level before Therapy		Total Rate
		ALT ≤ 5 x ULN	> 5 x ULN	
HBeAg (-)	Treat 24 wks	21.19(263/1241)	29.6(117/395)*	23.23(380/1636)
%	Stop 12 wks	22.35(262/1171	31.5(117/371)*	24.56(379/1546)
HBeAb (+)	Treat 24 wks	11.37(140/1231)	16.5(65/393)*	12.62(205/1624)
%	Stop 12 wks	12.88(149/1157)	20.5(76/370)*	14.73(225/1527)
HBVDNA (-)	Treat 24 wks	13.10(171/1305)	20.4(66/323)*	14.56(237/1628)
%	Stop 12 wks	7.20(211/1227)	22.0(70/309) **	18.29(281/1536)

Note: Bicyclol 25mg, t.i.d. p.o. x 6 months and followed up for 3 months after stopped medication. Figures in parentheses are the numbers of part/total patients. X^2 analysis, * P<0.05, ** P<0.01, compared with ALT ≤ 5 x ULN before treatment.

and even tended to increase after withdrawn of drug therapy for three months.

(2). Effect on 100 Cases of CHC

One hundred HCV patients with ALT and AST levels >5 ULN and anti-HCV and HCV-RNA positive were selected for phase IV clinical trial [28]. Some of the patients were failures in previous interferon-2α therapy. The dosage of bicyclol was 25-50mg t.i.d. p.o. and the course of treatment was six months as described in phase IV clinical trial. After 6 months of treatment with bicyclol, the elevated serum ALT returned to normal was by 54.0% (54/100 cases) and AST by 38.0%(35/92 cases). Both ALT and AST simultaneously became normal by 31.5% (29/92 cases). The HCV-RNA negative rate (detected by RT-PCR) was 18.8%(18/96 cases) after a course of 6 month therapy, and 25.3 %(22/87 cases) after cessation of bicyclol treatment for 3 months [28]. In phase III and IV clinical trials, the main symptoms of CHC patients such as fatigue, nausea, anorexia, and vomiting, were markedly ameliorated. No noticeable side effect was observed.

6.3. Clinical Reports Post Phase IV Trial Till 2008

In addition to papers from the above phase III and IV clinical trials, a number of clinical papers reported the efficacy of bicyclol in the treatment of CHB and HCV in China. It is hard work to describe the results of individual papers. Only the main results of all these papers are summarized as follows: A total of more than 2000 CHB patients were treated with bicyclol at dosage of 25-50mg, tid, p.o. for 3-6 months. The elevated serum ALT became normal by 63.4-87.0%, AST by 45.5-79.5%, HBeAg negative rate was 10.9-43.6% and HBV-DNA negative rate 10-53.0%. In terms of normalization of ALT and AST levels and negative conversion of HBeAg, bicyclol 150 mg daily was more effective than 75 mg daily [29]. No noticeable side effects were observed. All the papers concluded that bicyclol is an effective and safe hepatoprotectant with ant-HBV activity. In addition, two papers reported the efficacy of bicyclol used alone or bicyclol plus ribavilin in the treatment of CHC [30, 31]. Seventy patients with chronic HCV were randomly divided into bicyclol (34 cases) group and control group (36 cases). The patients of treatment group were received bicyclol 50mg, tid. p.o. Control group patients were given vitamins and liver protectants. The course of therapy was 6 months. At the end of the 6 month trial, serum HCV-RNA in 18 out of 34 cases of bicyclol group turned to negative(52.9%), while only 3 cases of 36 control patients became negative (8.3%). The difference in the ratio of HCV-RNA turning to negative between two groups was significant (P<0.01). The rate of serum ALT level returning to the normal limit was 83.9 % for bicyclol group and 62.8% for control group [30]. Another paper reported the efficacy of bicyclol in the treatment of 21 cases of CHC (12 failure to previous interferon-2α therapy, 9 cases intradiction to interferon therapy). The patients were administered bicyclol 50mg and ribavilin 300mg, thrice daily for 24 weeks. Fifteen control patients received vitamins, amino acids and glycyrrhizin. At the end of the trial, the negative rate of HCVRNA was 42.9% (9/21 cases) in bicyclol group and 6.7% (1/15cases). in control group. There was significant difference between bicyclol and control groups (P<0.01). The elevated serum ALT became normal by 76.2 % (16/21 cases) in bicyclol treatment group and 33.3 (5/ cases) in control group [31].

6.4. Combination Therapy of Bicyclol with Lamivudine, Adefovir or Interferon-2α in CHB

Eight papers reported the efficacy of the combination therapy of lamivudine plus bicyclol and lamivudine or bicyclol used alone [32-39]. The results are briefly summarized as follows: A total of more than 200 CHB patients enrolled in the trials were previously treated with lamivudine, some of them had already developed drug resistance to lamivudine because of YMDD development. The schedule of treatment was lamivudine 100mg, qd. plus bicyclol 25 or 50mg, t.i.d. p.o. for 24 or 48 weeks. Generally, at the end of combination therapy of lamivudine plus bicyclol, the effectiveness of anti-HBV DNA and negative of HBeAg were higher than lamivudine or bicyclol used alone, particularly theYMDD mutant and relapse rate of virus markers was reduced. Example 1[39], to investigate and compare clinical curative effect of single drug therapy or combination of lamivudine and bicy-

lol in treatment of chronic hepatitis,130 cases of chronic hepatitis B with positive HBsAg, HBeAg and HBV-DNA were randomly divided into three groups : group A (41 patients, lamivudine 100mg/d, p.o, qd, for 12 months). Group B (45 patients, bicyclol 75mg/d, p.o, t.i.d, for 12 months) and group C (44 cases, lamivudine 100mg/d, p.o. qd, and bicyclol 75mg/d, p.o, t.i.d, for 12 months). At the end of treatment, the normalization rates of ALT and AST in group A were 43.95% and 48.8%, respectively; And in Group B was 71.1% and 71.1%. There was a significant difference between group A and group B (P<0.05). The normalization rates of ALT and AST in group C were 90.9% and 93.2%, respectively. There were significant differences between group C and group A (P<0.01) or group B(P<0.05). There were no obvious differences among three groups regarding the rates of seroconversion of HBeAg/HBeAb. The rates of turning to negative of HBV- DNA in group A, B and C were 60.98%, l5.6% and 79.5%, respectively. There were significant differences among three groups (P<0.05). The results suggested that bicyclol was shown to have obvious hepatoprotective effect in normalization of the elevated ALT and AST levels, and potentiated antiviral effect of lamivudine. Another paper reported that 60 HBV patients with at least six months history of lamivudine administration were randomly assigned into 2 groups. Group A (treatment group) was treated with a sequential administration of lamivudine and followed by bicyclol, group B(control group)was treated only with lamivudine for six months, and the two groups were observed for another 6 months. The serum ALT was assayed monthly and the level of HBV-DNA(quanti-tative PCR) was detected every three months. The results indicated that from 4, 8 to 12 weeks, the normalization rate of ALT in bicyclol group was higher than that in lamivudine group(P<0.05). But from 24 to 48 wk, there was no difference in the ALT recovery rate between bicyclol and lamivudine groups (P>0.05). At the end of 24 wk, HBV-DNA negative conversion rate in the lamivudine group was higher than that in the bicyclol group. But at 48 wk, the difference of HBeAg negative rate or seroconversion of anti-HBeAg rate between both groups was not significant, although HBV-DNA negative rate of lamivudine treated group was higher than bicyclol group. It appears that in the early stage of treatment the ALT lowering effect of bicyclol is better than that of lamivudine, while the anti-HBV-DNA effect of lamivudine was higher than bicyclol. At the end of 48 weeks treatment, the liver function recovery and anti-HBV efficacy of bicyclol and lamivudine were similar [34]. Example 3, to survey the therapeutic effect of lamivudine alone or in combination with bicyclol on chronic viral hepatitis B, 90 patients with chronic viral hepatitis B were divided into A, B and C groups : Patients of group A (30 patients) were given 100mg lamivudine p.o qd, plus 25 mg bicyclol p.o, tid, and the course of treatment lasted for 72 weeks. Patients of group B (30 patients) were subjected to lamivudine treatment alone at the same dosage as that of patients in group A. Patients of group C (30 cases) were treated with conventional hepatinica and symptomatic therapeutic measures, serving as control. The course of treatment lasted for 72 weeks. Bicyclol combined with lamivudine reduced YMDD mutant rate. At the end of 72 weeks, the rate of YMDD mutant was 16.7% in group A and 36.7% in group B, the reduction of YMDD mutant rate by bicyclol treatment was significant (P< 0.05) [36]. The above results of three papers suggested that combination therapy of bicyclol with lamivudine might potentiate the improvement of abnormal liver function and negative of HBeAg and reduced the rate of YM DD mutant.

Similar results were obtained from the combination therapy of bicyclol with adefovir dipivoxil [40]. Sixty three CHB patients received adefovir dipivoxil 10 mg daily (p.o.) and bicyclol 25mg, tid daily for 48 weeks. Another 62 cases of CHB received adefovir dipivoxil 10 mg daily for 48 weeks alone. Compared with pretreatment, the ALT/AST lowering effect of adefovir plus bicyclol was more pronounced than adefovir alone group (P<0.05-0.01).The negative rate of HBV DNA was 58.7% *VS* 40.3% (P<0.05), and HBeAg negative rate was 31.8% *VS*. 16.1% (P<0.05), for adefovir plus bicyclol and adefovir alone group, respectively. Adefovir dipivoxil combined with bicyclol therapy seemed to be more effective and safe in the treatment of CHB. Also, combination therapy of bicyclol with FCV increased the antiviral efficacy [41].

Two papers reported the therapeutic effect of INFα-2b alone and in combination with bicyclol in CHB [42,43]. In general, as comparison with INFa-2b treatment alone, the combination of bicyclol with INFa-2b increased the improvement of the abnormal liver function and HBV-DNA negative, and reduced adverse reaction of INFa-2b. A paper reported that 30 patients of group A were received injection of 5 MU INFa-2b twice a week and simultaneously bicyclol 25mg, t.i.d. p.o. The course of treatment lasted for 72 week. At the end of 24, 48 and 72 weeks of treatment, the rates of serum turning negative of HBVDNA were 41.0%, 52.7%, 63.3%, respectively, in patients of group A, and 26.7%, 37.3%, 42.7%, respectively, in group B patients. The HBVDNA negative rate of group A patients was significantly higher than that of group B (p<0.05). The efficacy of INFa-2b plus bicyclol is superior to INFa-2b treatment alone [42].

Most of the clinical reports focused on the evaluation of the therapeutic efficacy and side effects of bicyclol in the treatment of CHB and CHC, Only two papers reported the effect of bicyclol treatment on the cellular immune function of chronic viral hepatitis B patients [44, 45]. The levels of interleukin (IL)-4, IL-10 and interferon (IFN)- in culture supernatant of peripheral blood mononuclear cells (PBMCs) from 30 CHB patients and 7 healthy volunteers were measured after bicyclol treatment. At 3 months during bicyclol (25mg,t.i.d) therapy, the level of IFN- significantly increased in CHB patients from 36.25±19.92 pg/ml before therapy to 53.19±7.28 pg/ml (P<0.05), meanwhile the level of IL-4 significantly decreased from 17.18±7. 43 pg/ml to 9.74±7.75 pg/ml (P<0.0 1). The decrease of IL-4 level and increase of IFN- after 3 months of therapy of bicyclol were more obvious in positive HBeAg than in HBeAg-negative patients (P<0.05). It appears that bicyclol might promote Th l type cytokine-mediated immune response and also down-regulates Th2, which may be involved in against liver inflammation and anti-HBV activity of bicyclol [44].

6.5. Extending Clinical Application of Bicyclol in Liver Fibrosis

One of the purposes of treatment of chronic viral hepatitis is to reduce development of liver fibrosis. There were four papers dealing with the therapeutic effect of bicyclol on liver fibrosis of CHB patients [46-49]. Twenty CHB patients were diagnosed by liver biopsy and serum fibrosis biochemical markers. The patients received bicyclol 50mg, tid, p.o.for 6 months. After six months treatment with bicyclol, the elevated serum fibrosis markers such as hyaluronic acid (HA), PIIIP, IV type collagen and laminin (LN) in serum were all reduced significantly, and 8 out of 12 patients with elevated serum alpha fetal protein returned to the normal limit (66.7%). Histology and Immunohistochemical observation of the specimens from liver biopsy showed that the hepatic inflammation and fibrosis were ameliorated, and the content of hepatic TGF-β, type I and type llI collagen were significantly reduced as compared with pre-treatment [46,47]. Another paper reported the therapeutic effect of bicyclol on 40 cases of liver fibrosis of CHB. After six months treatment of bicyclol, the serum biochemical markers of liver fibrosis such as HA, PIIIP, IV type collagen and LN were all decreased significantly, and the ultrasound examination of liver also showed improvement. This paper did not perform liver biopsy to observe the changes of liver histology before and after bicyclol treatment [48]. Bicyclol combined with ribavirin in the treatment of hepatocirrhosis of hepatitis C patients also showed better efficacy [49]. The above reports showed that bicyclol may be useful in the therapy of liver fibrosis induced by chronic viral hepatitis B. This possibility is supported by the animal study that bicyclol markedly ameliorated liver fibrosis induced by CCl_4 and dimethylnitosamine in rats and mice, respectively [21, 22].

7. PERSPECTIVE

Chronic viral hepatitis B and C are complicated diseases. At present, drugs used in the treatment of viral hepatitis including interferons and the new generation of anti-viral nucleosides/neucleotides such as adefovir dipivoxil merely inhibited hepatitis B or C virus replication but not eradicated virus. The efficacy of these drugs in the treatment of CHB and CHC is still limited. Therefore, to develop novel drug for therapy of chronic viral hepatitis is of necessity particularly in China since the incidence of HBV is at the top in the world. This paper provides information of the novel drug bicyclol for the treatment of viral hepatitis B and C. In animals, bicyclol has multiple pharmacological effects such as anti-liver injury, anti-liver fibrosis, anti-hepatitis virus replication and stimulating protein synthesis of hepatocytes *in vivo* and *in vitro*. Clinically,bicyclol not only significantly normalized the elevated serum ALT and AST levels, but also inhibited HBV and HCV replication at a certain levels particularly in those patients with high ALT level (> 200U/L). Adverse reactions to bicyclol were rare and not serious. Combination therapy of bicyclol with INFa-2b or anti-viral nucleosides/neucleotides such as lamivudine or adefovir dipivoxil might potentiate the anti-hepatitis virus effect and reduce the adverse reactions. In addition, bicyclol is also beneficial for treatment of liver fibrosis. As to by what mechanism bicyclol exerted its action against liver injury and hepatitis virus replication, the accumulated evidence from our study indicated that bicyclol seems to be a multi-targeting drug as it can maintain biomembrane stability of hepatocyte *via* eliminating free radicals, protects against injury of mitochondria particularly inhibiting the release of cytochrome C to initiate apoptosis, inhibits hepatocyte nuclear DNA damage and related cellular signaling. All these effects of bicyclol should be the pharmacological bases of bicyclol in the treatment of chronic viral hepatitis patients. Rust and Gores pointed out that apoptosis plays very important role in the pathogenesis of viral hepatitis, and the inhibition of hepatocyte apoptosis would be a new strategy of treating viral hepatitis [13]. From our results it may be seen that the active mechanism of bicyclol against liver injury might be anti-apoptosis of hepatocytes through multiple signal transduction pathways particularly induction of hepatic heat shock protein 70 and 17. These effects of bicyclol seemed to be different from that of interferons and anti-viral nucleosides/nucleotides such as lamivudine. The main mechanism of anti-virus action of interferes is through .immune-modulation. The anti-viral action of nucleoside/nucleotide such as lamivudine is the result of inhibition of HBVDNA polymerase activity. As mentioned above, bicyclol was shown to inhibit hepadnavirus replication in duck model of DHBV infection and in 2.2.15 cells as well as in viral hepatitis patients, while it has no direct inhibitory effect on DHBV-DNA polymerase activity, indicating that bicyclol is not an inhibitor of DHBV-DNA polymerase. So, the mechanisms underlying the anti-viral activities of bicyclol are still mysterious. To open this mystery needs more investigations.

Furthermore, it is quite possible to get more clinical benefits if increasing the dosage of bicyclol from 75-150mg to 150-300mg daily and prolongation of the course of treatment for 2 or 3 years. In addition, bicyclol was shown to have anti-liver fibrosis [21, 22] and chemopreventive effects on hepatic carcinoma development induced by chemical carcinogens [50-52]. Since the pathogenesis of hepatocellular carcinoma is closely associated with hepatitis virus infection, and the most important purpose of pharmacotherapy of viral hepatitis is to arrest development of liver fibrosis and carcinoma, the efficacy of long term medication of bicyclol in liver fibrosis and chemoprevention of hepatic carcinoma is of very interesting to study further.

In a word, bicyclol is a promising novel drug for treating chronic viral hepatitis particularly in improvement of the abnormal liver function. However, the active mechanism of bicyclol and clinical assessment such as liver histology and the comparison of the clinical efficacy of bicyclol with interferons and anti-viral nucleosides/nucleotides all need more study. From our research and development of bicyclol, it may be seen that there is a great potential for discovery new drugs from traditional Chinese medicine. The key problem is to integrate traditional Chinese medicine with knowledge and techniques of modern medicine and pharmacy.

ACKNOWLEDGEMENTS

This work was supported by grant (No.94-ZD-02) from the Ministry of Sciences and Technology of China, and a grant (1995) from China Medical Board Foundation in New York. U.S.A.

ABBREVIATIONS

HBV	=	Hepatitis B virus
HCV	=	Hepatitis C virus
CHB	=	Chronic hepatitis B
CHC	=	Chronic hepatitis C
ALT	=	Anlanine transferase
AST	=	Asparate transferase
DHBV	=	Duck hepatitis B virus
DDB	=	Dimethyl-4, 4'-dimethoxy-5, 6, 5', 6'-dimethylene dioxybiphenyl-2, 2'-dicarboxylate
WHV	=	Woodchuck hepatitis virus
YMDD mutant	=	Hepatitis B virus(HBV) DNA polymerase mutations in the tyrosine-methionine-aspartate-aspartate (YMDD) motif
PFA	=	Foscarnet
HCC	=	Hepatic cellular carcinoma
DMF	=	Dimethylformamide
DMSO	=	Dimethyl sulphoxide
Na-CMC	=	Sodium carboxymethylcellulose
MDA	=	Malondialdehyde
PIIIP	=	Procollagen III peptide
TNF-α	=	Tumor necrosis factor-α.
NADPH	=	Reduced form of nicotinamide-adenine dinucleotide phosphate
IFN-α	=	Interferon-α
HSPs	=	Heat shock proteins
TGF	=	Transfer growth factor
HPLC	=	High performance liquid chromograph
P.O.	=	Oral administration
T.i.d.	=	Three times daily
B.i.d.	=	Twice daily
Qd	=	Once daily

REFERENCES

[1] Yeh, S.H.; Chen, D.S.; Chen, P.J. A prospect for pharmacogenomics in the interferon therapy of chronic viral hepatitis. *J. Antimicrob. Chemother.*, **2003**, *52*, 149-151.

[2] Lia, Y.F. Therapy of chronic hepatitis B: current challenges and opportunities. *J. Viral Hepatol.*, **2002**, *99*(6), 393-399.

[3] Borgia, G.; Gentile, I. Treating chronic hepatitis B: today and tomorrow. *Curr. Med. Chem.*, **2006**, *13*, 2839-55.

[4] Wong, S.N.; Lok, A.S. Update on viral hepatitis: 2005. *Curr. Opin. Gastroenterol.*, **2006**, *22*(3), 241-247.

[5] Liu, G.T. Therapeutic effects of biphenyl dimethyl dicarboxylate (DDB) on chronic viral hepatitis B. *Proc. CAMS. PUMC.*, **1987**, *2*(4), 228-232.

[6] Liu, G.T. The anti-virus and hepatoprotective effect of bicyclol and its mechanism of action. *Chin. J. New Drugs*, **2001**, *10*, 325-327.

[6b] Hu, Wei.; Li,Y.; Zhang, C.Z. Enantioseparation of racemic anti-hepatitis new drug. bicyclol with crystallization. *Chin. Chem. Lett.*, **2005**, *16*(11), 1471-1473.

[7] Liu, G.T.; Li, Y.; Wei, H.L.; Zhang, H.; Xu, J.Y.; Yu, L.H. Mechanism of protective action of bicyclol against CCl_4-induced liver injury in mice. *Liver Int.*, **2005**, *25*, 872-879.

[8] Wang, H.P.; Li, Yan. Protective effect of bicyclol on acute hepatic failure induced by lipopolysaccharides and D-galactosamine in mice. *Eur. J. Pharmacol.*, **2006**, *534*, 194-201.

[9] Zhao, D.M.; Liu, G.T. Protective effect of bicyclol on acetaminophen induced liver mitochondria injury in mice. *Chin. J. New Drugs*, **2002**, *11*, 536-540.

[10] Li, Y.; Dai, G.W.; Li. Y.; Liu, G.T. Effect of bicyclol on acetaminophen-induced hepatotoxicity: Energy metabolism and mitochondrial injury in acetaminophen intoxicated mice. *Acta Pharmacol. Sin.*, **2001**, *36*, 723-726.

[11] Zhao, D.M.; Liu, G.T. Protective effect of bicyclol on concanavalinA-induced liver nuclear DNA injury in mice. *Natl. Med. J. China*, **2001**, *81*(14), 844-848.

[12] Kaplowitz, N. Mechanism of liver injury. *J. Hepatol.*, **2000**, *32*, 39-43.

[13] Rust, C.; Gores, G.J. Physiology in Medicine. Apoptosis and liver disease. *Am. J. Med.*, **2000**, *108*, 567-571.

[14] Li, M.; Liu, G.T. Inhibition of Fas/FasL mRNA expression and TNF-a release in concanavalin A-induced liver injury in mice by bicyclol. *World J. Gastroenterol.*, **2004**, *10*(12), 1775-1779.

[15] Li, M. Liu, G.T. Inhibitory effect of bicyclol on iNOS expression and NF-KB activation degradation in macrophages induced by lipopolysaccharides. *Chin. Pharmacol. Bull.*, **2006**, *22*(12), 1438-1443.

[16] Hartl, F.U. Molecular chaperones in cellular protein folding. *Nature*, **1996**, *381*, 571-579.

[17] Fink, A.L. Chaperone-mediated protein folding. *Physiol. Rev.*, **1999**, *79*, 425-449.

[18] Jaattela, M. Heat shock proteins as cellular lifeguards. *Ann. Med.*, **1999**, *31*, 261-271.

[19] Kultz, D. Molecular and evolutionary basis of the cellular stress response. *Ann. Rev. Physiol.*, **2005**, *67*, 225-257.

[20] Bao, X.Q.; Liu, G.T. Bicyclol: A novel anti-hepatitis drug with hepatic heat shock protein27/70 inducing activity and cytoprotective effects in mice. *Cell Stress Chaperones*, **2008** Apr 8. [Epub ahead of print].

[21] Li, Y.; Li, Y.; Liu, G.T. Protective effect of bicyclol on experimental liver fibrosis induced by carbon tetrachloride. *Natl. Med. J. China*, **2004**, *84*(24), 2096-2101.

[22] Hu, Q.W.; Liu, G.T. Effects of bicyclol on dimethylnitosamine-induced liver fibrosis in mice and its mechanism of action. *Life Sci.*, **2006**, *79*(6), 606-612.

[23] Ju, M.H.; Li, Y. Metabolism of bicyclol in rat and human liver microsomes in vitro. *Acta Pharm. Sin.*, **2005**, *40*(2), 111-116.

[24] Liu, G.T.; Li, Y.; Wei, H.L.; Lu, H.; Zhang, H.; Gao, Y.G.; Wang, L.Z. Toxicity of novel anti-hepatitis drug Bicyclol: A preclinical study. *World J. Gastroenterol.*, **2005**, *11*(5), 665-671.

[25] Ji, Y.Y.; Cheng, N.N.; Yao, G.B. Pharmacokinetic study of bicyclol in thirty health volunteers. *Chin. J. Clin. Pharmacol. Ther.*, **2001**, *6*(3), 218-221.

[26] Yao, G.B.; Ji, Y.Y.; Wang, Q.H.; Zhou, X.Q.; Xu, D.Z.; Chen, X.Y.; Zhang, Q.B. A randomized double-blind controlled trial of bicyclol in treatment of chronic hepatitis B. *Chin. J. New. Drugs Clin. Rem.*, **2002**, *21*, 457-461.

[27] Yao, GB.; Ji, Y.Y.; Wang, Q.H.; Wu, W.F. Treatment of chronic hepatitis C by bicyclol, a randomized double-blind placebo controlled trial. *Natl. Med. J. China*, **2002**, *82*, 958-960.

[28] Yao, G.B.; Xu, D.Z.; Lan, P.; Xu, C.B. Wang, C.; Luo, J.; Sheng, Y.M.; Li, Q. Efficacy and safety of bicyclol in treatment of 2200 chronic viral hepatitis. *Chin. J. New Drugs Clin. Rem.,* **2005**, *24*(6), 421-425.

[29] Jiang, J.J.; Kan, Z.C.; Jia, J. Analysis of chronic hepatitis B treated by bicyclol at different doses. *Infect. Dis. Inform.*, **2005**, *18*, 80-81.

[30] Guo, Q.; Zhang, Y.J.; Wang, J.; Su, C.L.; Chang, X. Evaluation of clinical usage of bicyclol in the treatment of chronic HCV. *Evaluat. Anal. Drug Use Hospitals China*, **2003**, *3*(6), 337-339.

[31] Sun, J.M. Efficacy of bicyclol combined with rivabilin in the treatment of chronic hepatitis C patients failure or contradiction to interferon therapy. *China Clin. Pract. Med.*, **2007**, *1*(8), 45-46.

[32] Ye, X.G.; Wang, R.L. Comparison of anti-HBV and liver function recovery between bicyclol and lamivudine in treating chronic hepatitis B. *Chin. J. Clin. Pharm.*, **2005**, *14*(2), 74-76.

[33] Tao, X.H. Therapy of bicyclol combined with lamivudine in 28 patients resistance to lamivudine. *ZheJiang Tradit. Chin. West. Med.*, **2005**, *15*, 428-429.

[34] Huang, Y.C.; Xu, D.; Zhang, Z.G.; Tian, D.Y.; Qi, J.Y. Clinical study of sequential treatment of bicyclol in patients after treatment of lamivudine. *Chin. J. Pharmacoepidemiol.*, **2005**, *14*(6), 321-323.

[35] Zhu, C.L.; Chen, J.M.; Huangpu, Y.S.; Zhang, L.X. Clinical observation of the efficacy of sequential therapy of bicyclol and lamivudine in rebound after stopped lamivudine in chronic hepatitis B. *Infect. Dis. Inform.*, **2006**, *19*(2), 79.

[36] Wan, X.F.; Yang, Z.J.; Zhang, L.M.; Zhu, Y.F.; Zhuge, C.D. Lamivudine alone or in combination with bicyclol to treat chronic viral hepatitis B : A clinical study. *Chin. J. Nosocomiol.*, **2006**, *16*(7), 811-813.

[37] Shen, W.; Wang, X.Y.; Dong, X.; Xie, C.J.; Chen, B.L. Dynamic observation of combination therapy on serum HBV markers of chronic hepatitis B patients. *Clin. Focus*, **2004**, *19*(23), 1359-1360.

[38] Zeng, J.H.; Yang, T.X. Effect of lamivudine combined bicyclol therapy on mutant hepatitis B virus. *Pract. Med. J.*, **2007**, *239*(17), 2741.

[39] Wu, X.L.; Wang, H.X.; Zhong, G.Q. Study of clinical curative effect of bicyclol combined with lamivudine in treatment of chronic hepatitis B and the influence on T cell subset. *Eval. Anal. Drug Use Hospitals China*, **2005**, *5*(4), 235-237.

[40] Yu, J.G.; Shang, Q.H.; An, Y. Effectiveness and safety of adefovir dipivoxil combined with bicyclol in treatment of chronic hepatitis B. *Infect. Dis. Inform.*, **2007**, *20*(5), 299-301.

[41] Yu, B.L.; Liu, T.Y.; Wei, S.Y. Efficacy of bicyclol combined with FCV in the treatment of chronic hepatitis B. *Infect. Dis. Inform.*, **2004**, *17*(2), 57.

Received: 06 September, 2008 Revised: 27 October, 2008 Accepted: 13 November, 2008

[42] Wan, X.F.; Wu, L.C.; Yun, B. Interferon$_{\alpha}$-2b alone and combined with bicyclol in the treatment of chronic viral hepatitis B. *ZheJiang Clin. Med.*, **2006**, *8*(9), 904-905.

[43] Liang, X.G.; Liu, W.D.; Wang, J.F.; Chen, B.X. Clinical observation of combination therapy of bicyclol with interferon-α in the therapy of positive HBeAg patients. *Infect. Dis. Inform.*, **2007**, *20*(2), 115-116.

[44] ShI, B.B.; Peng, J.; Wen, F.Y.; Hou, J.L. Effect of bicyclol tablets on cellular immune responses in patients with chronic hepatitis B. *J. First Mil. Med. Univ.*, **2005**, *25*(6), 705-706.

[45] Xia, Q.Y.; Zheng, J.L.; Zhou, Y.J.; Wang, C.X. Effect of bicyclol on cellular immune function of chronic viral hepatitis B patient. *Infect. Dis. Inform*, **2005**, *18*(3), 137-138.

[46] Yi, J.H.; Li, W.; Xiong, Y. Clinical study on bicyclol against liver fibrosis induced by chronic hepatitis B. *J. Clin. Intern. Med.*, **2006**, *23*(1), 57-59.

[47] Yi, J.H.; Li, W.; Zeng, L.L.; Luo, D.D. Effect and mechanism of bicyclol on the hepatic fibrotic patients with chronic hepatitis B. *J. Dalian Univ.*, **2006**, *27*(4), 27-30.

[48] Wang, X.H. Clinical observation of bicyclolin the treatment of early liver fibrosis of chronic hepatitis B. *Proc. Clin. Med. J.*, **2005**, *14*,143-44.

[49] Sun, J.M.; Zhu, Z.; Hu, Q.J. Clinical observation on bicyclol combined with ribavirin in the treatment of hepatitis C hepatocirrhosis. *Chin. J. Dial. Artif. Organs*, **2007**, *18*(3),16-17.

[50] Zhu, B.; Liu, G.T.; Wu, R.S.; Strada, S.J. Chemoprevention of bicyclol against hepatic preneoplastic lesions. *Cancer Biol. Ther.*, **2006**, *5*(12), 1665-1673.

[51] Sun, H.; Liu, G.T. Chemopreventive effect of bicyclol on malignant transformation of WB-F344 rat liver epithelial cells and its effect on related signal transduction in vitro. *Cancer Lett.*, **2006**, *236*, 239-249.

[52] Zhu, B.; Liu, G.T.; Zhao, Y.M.; Wu, R.S.; Strada, S.J. Chemosensitizing multiple drug resistance of human carcinoma by bicyclol involves attenuated p-glycoprotein, GST-P and Bcl-2. *Cancer Biol. Ther.*, **2006**, *5*, 536-543.