

Toxicity of novel anti-hepatitis drug bicyclol: A preclinical study

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Abstract

AIM: To study the toxicity of bicyclol to animals.

METHODS: Acute toxicity test was performed in Kunming strain mice that were orally given bicyclol at the doses of 3 and 5 g/kg body weight, respectively. Wistar rats were orally administered bicyclol at a dose of 5 g/kg body weight. Death and clinical symptoms of animals were recorded within 7 d. 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 d. Animal behaviors, blood biochemical markers, blood and urine pictures were examined. Chronic toxicity test was conducted in 80 Wistar rats of both sexes. The animals were orally administered with various doses of bicyclol [150, 300, 600 mg/kg, 100-400 folds corresponding to the proposed therapeutic dose (1.5 mg/(kg·d)) of bicyclol for patients] once daily for 6 mo except for Sunday. The control group was given the same volume of 0.2% sodium carboxyl methylcellulose (Na-CMC). Twenty-one beagle dogs received bicyclol (25, 75, 225 mg/kg, 16.6, 50, 150 folds corresponding to the proposed therapeutic dose of bicyclol for patients) once a day for 6 mo except for Sunday. 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 determined. 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, 1.0 g/kg bicyclol once daily from the 7th d of gestation for 10 d.

RESULTS: The oral LD₅₀ of bicyclol was over 5 g/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.

CONCLUSION: Bicyclol has no detectable chronic toxicity as well as mutagenicity and teratogenicity in animals.

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Key words: Bicyclol; Antiviral agents; Toxicity test

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INTRODUCTION

Chronic viral hepatitis is a worldwide disease. The incidence of chronic viral hepatitis B (CHB) in China is the highest in the world. Although many drugs have been used in the treatment of CHB and chronic viral hepatitis C (CHC) patients, no satisfactory drug is available. The long-term therapeutic efficacy of interferon-2 α and lamivudine on CHB patients is still limited^[1,2]. Therefore, it is necessary to develop effective and safe new drugs for the treatment of chronic viral hepatitis.

In order to develop effective and safe drugs against viral hepatitis, Professor Chun-Zhen Zhang in our institute has synthesized a series of derivatives of 4, 4'-dimethoxy-5, 6, 5', 6'-dimethylene-dioxy-2, 2'-dicarboxylate biphenyl (DDB), a widely used hepatoprotectant in China and foreign countries^[3-5]. 4, 4'-dimethoxy-5, 6, 5', 6'-dimethylene-dioxy-2-hydroxymethyl-2'-carbonyl biphenyl (bicyclol) has anti-liver injury, anti-liver fibrosis and anti-hepatitis B virus effects in animal models^[6-8]. In order to carry out the clinical trial of bicyclol in viral hepatitis patients, the toxicity of bicyclol was studied in animals. The results are described in this paper with its chemical structure shown in Figure 1.

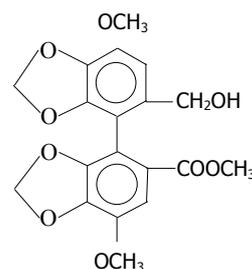


Figure 1 Chemical structure of bicyclol.

MATERIALS AND METHODS

Animals

Both sexes of Kunming strain mice weighing 18-22 g and Wistar rats weighing 50-70 g or 150-250 g were obtained from the Animal Center of Chinese Academy of Medical Sciences. The animals were fed laboratory chow and water *ad libitum* and maintained in an air-conditioned environment (23±1 °C, 50±5% humidity) in a 12 h light/dark cycle. Both sexes of beagle dogs weighing 8-12 kg (10 males, 11 females) were obtained from the Animal Center of Chinese Academy of Military Medical Sciences. All animals received care in compliance with the guidelines of Beijing Animal Control Center.

Drug

Bicyclol, a non-water soluble white powder with 99% purity, was kindly provided by Professor Chun-Zhen Zhang. Bicyclol was suspended in 0.2% sodium carboxyl methylcellulose (Na-CMC) for oral administration.

Acute toxicity test in mice and rats

Twenty mice were randomly divided into two groups, 10 (5 males and 5 females) each group. One group of mice received 3 g/(10 mL·kg) body weight of bicyclol by gavage, and the other group was given 5 g/kg of bicyclol. Ten rats weighing 150-200 g (5 males, 5 females) were orally administered 5 g/kg of bicyclol. Animal behavior and death were recorded within 7 d.

Subcutaneous injection toxicity test in mice

Ten mice (5 males, 5 females) were subcutaneously injected 2 g/kg of bicyclol. Animal behavior and death were observed within 7 d.

Subacute toxicity test in rats

Sixty-four rats were randomly divided into 4 groups, 16 each group. Bicyclol (150, 300, 600 mg/(10 mL·kg)) was administered by gavage to rats once a day for 14 d. Control group was given a corresponding volume of 0.2% Na-CMC vehicle. The intake of water and food, and body weight of each group rats were recorded every 2 d. All rats were sacrificed by decapitation 14 d after treatment with bicyclol. Animal general behavior and their serum ALP, GOT, BUN, LDH, TRIG, GLU, GPT, CHOL, TP, ALB, BIL, HB, WRC and RBC were determined. Pathological examination of main organs (heart, liver, kidney, lung, spleen, thymus, pituitary, adrenal, uterus, ovary, testis, prostate and seminal vesicle) was performed by microscopy.

Chronic toxicity test in rats

Eighty rats of both sexes weighing 50-70 g were equally divided into four groups (10 males and 10 females each group). Three groups of rats were administered 150, 300, 600 mg/(10 mL·kg) of bicyclol, respectively, once daily for 6 mo except for Sunday. The control group was given the same volume of 0.2% Na-CMC vehicle. On the next day after the last administration of bicyclol, all rats were decapitated. Blood and main organs of each rat were collected for pathological examination.

Chronic toxicity test in beagle dogs

Beagle dogs were placed in individual cages. The room temperature was kept at 16-22°C. Twenty-one beagle dogs were divided into four groups, 5 (2 females, 3 males) in control group, 5 (3 females, 2 males) in 25 mg/kg bicyclol group, 5 (2 females, 3 males) in 75 mg/kg bicyclol group, and 6 (4 females, 2 males) in 225 mg/kg bicyclol group. Bicyclol was mixed with a small piece of beef and given to each dog every day for 6 consecutive months before food was fed. One male dog from control group and 2 dogs (1 male and 1 female) from high dose (225 mg/kg) bicyclol group were sacrificed for biochemical and pathological examinations 3 mo after treatment. The remaining dogs in each group were continuously treated with bicyclol till to the end of 6 mo. Half of the dogs in each group were sacrificed 6 mo after bicyclol treatment, and the remaining dogs of each group were killed 1 mo after withdrawal of bicyclol medication.

General behavior, food intake and defecation of the animals were recorded every day. The dogs were weighed every 2 wk. Blood picture, biochemical parameters and urine were determined twice before bicyclol administration, and every 1.5 mo after bicyclol medication and 1 mo after withdrawal of bicyclol treatment. EKG (I, II, III, AVR, AVL, and ALF) was detected one time before and 1.5, 3, 6 mo after administration of bicyclol. The main organs including heart, liver, lung, kidney, spleen, brain, spinal cord, thymus, thyroid, pituitary, pancreas, stomach, jejunum, colon, mesentery, bladder, adrenal, uterus, ovary and testis were all removed for pathological examination.

Mutagenicity assay

The assay was carried out on TA97 and TA102 bacterial strains according to the classical method of Ames test. Both activating

and non-activating system were used. Briefly, 0.1 mL of TA 97 or TA 102 cultured at 37°C for 12 h was mixed with 0.1 mL of various concentrations of bicyclol (10, 100, 500, 1 000, 5 000 µg/plate) and 0.5 mL of phosphate buffer or S9 mixture. The above mixture was incubated at 37°C for 12 min, then added on the agar plate and incubated for another 48 h. The number of revertants on each plate was counted.

Micronucleus assay in mice

Male mice (3 each group) weighing 22-25 g were used in preliminary test. In brief, 3.0 g/kg bicyclol was orally administered to mice, and then the mice were sacrificed by decapitation at 18, 24, 30, 48 and 72 h after administration of bicyclol. The leg bone marrow of each mouse was removed for sliding smears. The smears were fixed with methanol and stained with 10% Giemsa. The number of micronucleus polychromatic erythrocytes (MNPCE) per 1 000 cells was counted under a light microscope. Male mice (6 each group) weighing 22-25g were used in regular test. Cyclophosphamide (50 mg/kg, i.p) was used as a positive control drug. Different doses of bicyclol (0.75, 1.5, 3.0 g/kg) were orally administered to mice. The mice were sacrificed by decapitation 24 h after drug treatment. The rest procedure was the same as described in preliminary test.

Chromosome aberration test of bicyclol

Chinese hamster lung (CHL) cells (10^6 cells/plate) were pre-incubated for 24 h. The test compounds were added to the plates and incubated for another 24 h. The cells were harvested and stained with 0.5% trepan blue. The survived cells of 100 cells per plate were counted and 50% inhibition rate of cell survival was calculated. The concentration of 50% inhibition rate of bicyclol on cell growth was 200 µg/mL.

The cultured medium contained RPMI 16 40, 10% fetal bovine serum, penicillin and streptomycin. Chinese hamster lung (CHL) cells were preincubated for 24 h. The non-metabolic activation group (-S9) was incubated with test compound for another 24 h. The metabolic activation group was incubated with test compound for 6 h in the absence of fetal bovine serum, then the incubation mixture was replaced by fresh medium and incubated for 48 h. Bicyclol was dissolved in PEG 400 and a same volume of PEG 400 was added onto the control plate. Colchicum (1 µg) was added to all groups and incubated for 3 h. The cells were harvested, centrifuged, fixed, sliced and stained with 10% Giemsa for 10 min. One hundred metaphase cells dispersed freely were examined for chromosome structure aberration and the incidence rate of mitiploids was determined under microscope.

Teratogenicity assay

Both sexes of Wistar rats (20 each group) weighing 200-250 g were used. Every early morning, the sperms were examined by vaginal smear for determination of pregnancy (d 0) after female and male rats were put into an animal cage at 2:1 ratio. Bicyclol (100, 1 000 mg/kg) was orally administered once a day from d 7 for 10 d. Control group was given a same volume of 0.2% Na-CMC.

Pregnant rats were weighed every 3 d. The rats were sacrificed by dislocation on d 20 (1 d before delivery). The living fetuses were taken from uterus and the number of corpora luteum, absorbed fetuses, dead and living fetuses was recorded, respectively. Meanwhile, the sex status and outside appearance of fetus were also observed. The fetuses were fixed with Bouin's solution and 95% ethanol for morphological examination of organs and bones.

Statistical analysis

All data were expressed as mean±SD and analyzed by Student's *t* test or χ^2 test between control and bicyclol-treated groups.

Table 1 Effect of bicyclol on blood picture and biochemical markers in rats (mean±SD)

Group (mg/kg)	Sex	Blood picture				Classification of leukocytes, RET and PT				
		RBC (10 ⁴ /mm ³)	WBC (/mm ³)	Hb (g/dL)	HCT (%)	Leukocytes (%)			RET (%)	Platelet (1×10 ⁹ /L)
						Neutrophil	Lymphocyte	Reticulocyte		
Control	M	713.8±95.8	14 400±3 969	17.3±1.4	37.5±5.2	39.3±7.05	58.6±4.32	4.5±2.06	1.55±0.99	900.75±136.07
Bicyclol 150		753.7±55.0	14 102±3 531	17.1±0.8	38.8±3.6	39.0±8.12	58.6±6.97	2.0±0.89	0.86±0.26	837.60±155.62
300		609.1±109	15 850±5 813	15.4±5.3	31.7±11	29.6±9.22	68.4±9.07	2.0±0.63	1.14±0.91	905.60±106.10
600		593.2±86.8 ^a	11 477±1 919	14.7±1.5 ^a	29.7±3.8	-	-	-	-	-
Control	F	681.9±104	13 230±3 671	16.8±1.99	35.1±5.0	29.4±13.47	67.8±13.9	2.4±2.5	1.04±0.62	763.20±144.50
Bicyclol 150		620.1±104	13 220±2 721	15.7±1.25	31.3±3.7	33.4±10.07	65.4±11.7	1.0±0.9	0.74±0.37	715.60±76.20
300		626.9±70	12 656±1 461	15.3±1.52	34.6±3.9	45.0±8.7	53.4±8.7	1.6±1.6	0.92±0.55	831.6±82.20
600		622.9±80	10 777±4 355	14.7±1.51	34.1±4.4	42.0±15.6	54.4±14.6	3.6±2.9	0.70±0.49	562.4±273.30

^aP<0.05 vs control group.

RESULTS

Acute toxicity test in mice and rats

No death and clinical symptoms of mice and rats treated with 3, 6, and 5 g/kg of bicyclol were observed within 7 d. Two mice and 2 rats from bicyclol treated groups were sacrificed and dissected on d 8. No abnormal changes of organs were observed by gross necropsy. The results suggested that the oral LD₅₀ of bicyclol was at least greater than 5 g/kg.

Subcutaneous injection toxicity test in mice

Subcutaneous injection of 2 g/kg bicyclol to mice did not induce death and abnormal changes in general behavior of mice within 7 d. Two mice were sacrificed and dissected for observation of lung, heart, liver, kidney and spleen by gross necropsy. No abnormal changes of the main organs were observed.

Subacute toxicity test in rats

After 14 d treatment of rats with 150, 300 and 600 mg/kg bicyclol, the general behavior and serum ALP, GOT, BUN, LDH, TRIG, GLU, GPT, CHOL, TP, ALB, BIL, HB, WRC, RBC values were all in normal range. The pathological observations of heart, liver, kidney, lung, spleen, thymus, pituitary, adrenal, uterus, ovary, testis, prostate and seminal vesicle were also normal under microscope (data not shown).

Chronic toxicity test in rats

For observation of chronic toxicity of bicyclol in rats, 4 groups of rats weighing 50–70 g were treated with 150, 300, 600 mg/kg bicyclol once a day for 6 mo. Four months after bicyclol treatment, 10 rats (5 males, 5 females) from bicyclol (600 mg/kg) group and 10 rats from control group were sacrificed by decapitation. The remaining rats in each group were given bicyclol for another 2 mo and then killed by decapitation. The following indexes were observed for toxicity assay: general behaviors including activity, fur color, feces, body weight and food intake; blood picture including RBC, WBC, Hb, HCT, classification of leukocytes (neutrophils, lymphocyte, reticulocyte), platelet, RET count; blood biochemical markers including GPT, GOT, ALP, LAP, total cholesterol triglyceride, D-bilirubin, total bilirubin, total protein, albumin, A/G ratio, glucose, BUN, creatine; urine test including glucose, protein, ketone body, and occult blood; pathological examination including heart, liver, lung, kidney, spleen, thymus, pituitary, adrenal, uterus, ovary, testis, seminal vesicle, prostate 6 mo after bicyclol treatment.

No toxic effect of bicyclol and no pathological changes in main organs were observed, although some variations in certain parameters were demonstrated (Tables 1–3), suggesting that bicyclol had no noticeable toxicity to rats at the test dosages.

Table 2 Effect of bicyclol on organ weight in rats (*n* = 10, mean±SD)

Organ weight mg/100g BW	Sex	Control	Bicyclol (mg/kg)		
			150	300	600
Heart	M	308±38	300±42	310±74	360±70
Liver		2 960±310	3 120±610	3 200±810	3 300±700
Lung		570±120	620±280	580±160	700±180
Kidney		670±70	660±120	700±180	580±270
Spleen		160±30	170±40	170±43	140±45
Thymus		60±20	60±20	60±17	45±18
Pituitary		3±4	2±1	2±1	2±1
Adrenal		20±10	24±30	14±3	16±4
Prostate		110±30	120±30	109±29	110±40
Seminal		250±80	240±80	250±55	260±66
Testis		1150±160	1 120±180	1 040±301	1 130±296
Heart	F	350±40	360±38	380±40	370±41
Liver		3 200±330	3 300±220	3 300±210	3 300±290
Lung		970±320	890±280	700±80	680±291
Kidney		810±110	820±100	810±140	770±130
Spleen		220±44	210±36	240±34	320±54 ^b
Thymus		102±36	67±23 ^b	53±24 ^b	120±88
Pituitary		5±2	3±1	5±2	12±15
Adrenal		29±4	26±4	29±3	51±8
Uterus		173±50	147±48	163±31	158±57
Ovary		39±19	34±24	37±3	47±25

^bP<0.01 vs control group.

Chronic toxicity test in beagle dogs

The criteria of toxicology study were essentially the same as in chronic toxicity test in rats. Serum GPT, GOT, ALP, glucose, total cholesterol, total protein, total-bilirubin, albumin, creatine and BUN, RBC, WBC, Hb, RET, platelet (PLT) polymorphonuclear leukocyte, lymphocyte, monocyte and prothrombin (capillary method) and urine glucose, protein, ketone body, occult blood, pH were determined. Pathological examination of main organs included morphological change of organs by gross necropsy and light microscopy, weight of main organs and organ index. The results were as follows.

The body weight, stool, water and food intake of dogs treated with bicyclol were normal, except for 2 dogs in high dose (225 mg/kg) bicyclol group having less food intake in the first few days. No abnormal changes in heart rate and waveform from 6 leads were observed after bicyclol treatment. Most parameters of blood picture assay were in normal range. Although variations in certain parameters (Hb, lymphocytes, PLT, *etc.*) were demonstrated, there was no significant difference between control and bicyclol groups. Some changes in blood biochemical parameters such as total protein (TP) and albumin (ALB) increased, while T-BIL decreased in bicyclol-treated groups. However, there was no significant difference between control and bicyclol groups before and after bicyclol treatment (Tables 4–6).

Table 3 Effect of bicyclol on blood biochemical markers in rats ($n = 10$, mean \pm SD)

Parameter	Control	Bicyclol (mg/kg)		
		150	300	600
Male rats				
TP 10 g/L	6.68 \pm 0.31	6.30 \pm 0.63	7.30 \pm 0.61	6.47 \pm 0.41
ALB 10 g/L	3.82 \pm 0.22	3.55 \pm 0.29	3.79 \pm 0.42	3.68 \pm 0.21
GLB 10 g/L	2.86 \pm 0.22	2.75 \pm 0.43	3.51 \pm 0.38	2.80 \pm 0.32
A/G	1.34 \pm 0.13	1.31 \pm 0.17	1.09 \pm 0.15	1.33 \pm 0.17
SGOT IU/L	391 \pm 74	411 \pm 57	406 \pm 50	431 \pm 76
SGPT IU/L	63 \pm 10	50 \pm 8	46 \pm 8	62 \pm 10
ALP IU/L	293 \pm 42	255 \pm 53	303 \pm 67	285 \pm 68
LAP IU/L	63 \pm 5	62 \pm 7	69 \pm 8	65 \pm 5
T-CHO 10 mg/L	73.6 \pm 6.68	65.7 \pm 8.26	72.1 \pm 9.51	69.3 \pm 5.87
TG 10 mg/L	107.4 \pm 33.7	109.9 \pm 30.6	97.1 \pm 15.1	106.7 \pm 25.8
GLU 10 mg/L	4.2 \pm 5.33	2.2 \pm 1.87	8.5 \pm 12.7	8.2 \pm 8.8
D-BIL 10 mg/L	0.14 \pm 0.03	0.16 \pm 0.07	0.18 \pm 0.08	0.18 \pm 0.06
T-BIL 10 mg/L	1.33 \pm 0.22	1.32 \pm 0.71	1.58 \pm 0.67	1.68 \pm 0.53
BUN 10 mg/L	20.7 \pm 4.8	18.3 \pm 4.7	18.4 \pm 2.7	17.0 \pm 5.8
Creat 10 mg/L	1.14 \pm 0.19	1.13 \pm 0.16	1.13 \pm 0.23	0.97 \pm 2.2
Female rats				
TP 10 g/L	6.53 \pm 0.78	6.58 \pm 0.75	6.93 \pm 0.64	6.73 \pm 0.44
ALB 10 g/L	3.55 \pm 0.57	3.58 \pm 0.46	3.97 \pm 0.36	4.06 \pm 0.36
GLB 10 g/L	2.99 \pm 0.53	2.99 \pm 0.45	2.96 \pm 0.45	2.68 \pm 0.40
A/G	1.22 \pm 0.26	1.22 \pm 0.19	1.37 \pm 0.21	1.55 \pm 0.30
sGOT IU/L	439 \pm 95	403 \pm 70	520 \pm 119	433 \pm 100
sGPT IU/L	59 \pm 31	38 \pm 11	49 \pm 16	46 \pm 10
ALP IU/L	227 \pm 170	203 \pm 156	189 \pm 46	151 \pm 26
LAP IU/L	59 \pm 18	59 \pm 5	69 \pm 7	69 \pm 6
T-CHO 10 mg/L	66.4 \pm 14.5	63.2 \pm 10.5	63.5 \pm 4.20	60.4 \pm 9.2
TG 10 mg/L	108.6 \pm 49.5	117.5 \pm 58.7	121.9 \pm 23.1	120.8 \pm 4.3
GLU 10 mg/L	7.4 \pm 4.8	6.1 \pm 5.5	8.2 \pm 4.0	16.3 \pm 12.0
D-BIL 10 mg/L	0.36 \pm 0.45	0.27 \pm 0.09	0.19 \pm 0.05	0.16 \pm 0.08
T-BIL 10 mg/L	2.84 \pm 3.03	2.35 \pm 0.65	1.79 \pm 0.54	1.57 \pm 0.77
BUN 10 mg/L	23.0 \pm 7.2	32.7 \pm 16.7	24.6 \pm 9.6	23.5 \pm 4.5
Creat 10 mg/L	0.90 \pm 0.14	1.06 \pm 0.25	0.98 \pm 0.18	0.81 \pm 0.28

Table 4 Blood test of female and male beagle dogs in control group before and after bicyclol treatment (mean \pm SD)

Parameter	Before treatment	After treatment (mo)				
		1.5	3	4.5	6	7 ($n = 1$)
Female ($n = 2$)						
RBC $\times 10^{12}$ /L	9.2 \pm 0.5	9.2 \pm 0.5	7.3 \pm 2.2	8.2 \pm 0.9	9.8 \pm 2.0	8.4
Hb g/L	140 \pm 14	140 \pm 7	145 \pm 7	143 \pm 11	148 \pm 11	140
RET (%)	0.9 \pm 0.3	0.8 \pm 0.3	0.9 \pm 0.3	0.7 \pm 0.3	0.8 \pm 0.1	0.6
PLT $\times 10^9$ /L	200 \pm 14	205 \pm 21	215 \pm 35	370 \pm 14 ^a	265 \pm 78	240
WBC $\times 10^9$ /L	14.6 \pm 2.2	13.7 \pm 2.0	15.2 \pm 3.7	13.6 \pm 2.6	16.2 \pm 7.2	14.5
Lymphocyte (%)	55 \pm 11	55 \pm 12	62 \pm 14	61 \pm 12	69 \pm 8	74
Polymorphonuclear leukocyte (%)	41 \pm 7	41 \pm 13	36 \pm 13	34 \pm 9	28 \pm 4	21
Monocyte (%)	5.5 \pm 2.1	5.0 \pm 1.4	2.5 \pm 0.7	4.0 \pm 1.4	3.5 \pm 0.7	5
Male ($n = 3$)						
RBC $\times 10^{12}$ /L	8.7 \pm 1.7	7.3 \pm 0.6	8.7 \pm 2.1	8.5 \pm 1.6	7.9 \pm 1.1	8.6
Hb g/L	135 \pm 10	140 \pm 5	135 \pm 9	145 \pm 7	150 \pm 7	140
RET (%)	0.9 \pm 0.4	0.6 \pm 0.1	0.6 \pm 0.2	0.9 \pm 0.4	0.8 \pm 0.4	0.8
PLT $\times 10^9$ /L	223 \pm 38	250 \pm 26	197 \pm 32	260 \pm 85	290 \pm 28	312
WBC $\times 10^9$ /L	12.1 \pm 3.1	12.7 \pm 0.8	10.6 \pm 2.3	11.9 \pm 2.1	12.2 \pm 1.6	20.1
Lymphocyte (%)	52 \pm 12	57 \pm 11	48 \pm 11	69 \pm 4	67 \pm 8	66
Polymorphonuclear leukocyte (%)	45 \pm 11	39 \pm 13	47 \pm 10	28 \pm 4	26 \pm 1	26
Monocyte (%)	3.7 \pm 0.6	3.3 \pm 1.5	2.7 \pm 0.6	3.5 \pm 0.7	7.0 \pm 1.4 ^a	8

^a $P < 0.05$ vs before dosing.

There were no abnormal changes in urine and prothrombin test 6 mo after bicyclol treatment. No abnormal changes of the main organs were observed by gross necropsy and light microscopy.

In a word, oral administration of bicyclol (25, 75, 225 mg/(kg·d)) for 6 mo, 16.6-150 folds corresponding to the proposed therapeutic dose of bicyclol for clinical use) to both sexes of beagle dogs had no detectable toxicity.

Mutagenicity test of bicyclol

The results of Ames test of bicyclol (reverse mutation test) in bacteria indicated that the positive mutagens induced a significant increase of revertants, whereas bicyclol showed no detectable mutagenicity in bacterial strains (Table 7).

Micronucleus test of bicyclol in mice

The results of preliminary test showed that there was no

Table 5 Blood test of female and male beagle dogs before and after treatment with 225 mg/kg bicyclol (mean±SD)

Parameter	Before treatment	After treatment (mo)				
		1.5	3	4.5	6	7 (n = 1)
Female (n = 4)						
RBC ×10 ¹² /L	8.9±2.1	9.0±2.5	8.1±1.6	10.0±0.8	9.0±2.3	8.8
Hb g/L	141±9	151±9	141±6	153±12	165±13 ^a	155
RET (%)	1.0±0.2	0.6±0.1	0.8±0.2	0.7±0.1	0.8±0.2	0.8
PLT ×10 ⁹ /L	195±55	230±37	217±29	237±15	313±25 ^a	368
WBC ×10 ⁹ /L	10.6±2.2	10.2±0.7	12.2±3.2	10.5±2.4	12.2±3.0	10.9
Lymphocyte (%)	51±6	52±8	60±11	59±11	59±10	63
Polymorphonuclear						
Leukocyte (%)	44±6	43±8	37±6	39±9	36±8	31
Monocyte (%)	3.8±1.3	4.5±0.6	3.0±1.2	2.0±2.0	5.3±3.1	6
Male (n = 2)						
RBC ×10 ¹² /L	8.9±0.3	10.4±0.0 ^a	9.9±1.6	7.4	7.9	7.5
Hb g/L	140±14	142±9	140±7	155	160	145
RET (%)	1.1±0.1	0.9±0.2	1.0±0.4	0.9	0.8	0.6
PLT ×10 ⁹ /L	215±7	290±0 ^a	138±23 ^a	210	260	248
WBC ×10 ⁹ /L	9.3±0.6	9.5±0.7	11.2±1.9	6.9	12.8	14.4
Lymphocyte (%)	55±4	51±11	52±6	48	55	67
Polymorphonuclear						
Leukocyte (%)	41±3	46±11	46±6	48	40	26
Monocyte (%)	3.5±0.7	3.0±0.0	4.0±1.4	4	5	7

^aP<0.05 vs before dosing.**Table 6** Blood biochemical parameters of female and male beagle dogs 6 mo after bicyclol treatment (mean±SD)

Parameter	Control	Bicyclol (mg/kg)		
		25	75	225
Female				
Glu mmol/L	4.94±0.83	6.06±0.38	5.60±0.08	6.36±0.62
T-CHO mmol/L	2.56±1.08	2.82±0.60	2.97±1.02	3.43±1.39
TP g/L	71.3±0.2	74.3±4.2 ^a	73.5±3.3	76.1±6.2
ALB g/L	37.0±0.9	39.9±1.7	41.0±3.7	44.0±4.1
Crea μmol/L	119±28	138±10	145±42	143±27
BUN mmol/L	4.25±0.05	3.51±0.24	4.22±0.29	3.60±0.78
T-BIL μmol/L	2.80±1.70	4.00±2.26	3.60±0.57	1.20±0.40
SGPT 10 U/L	26.2±1.3	28.3±15.0	12.8±8.9	25.2±8.1
SGOT 10 U/L	61.8±14.9	41.3±14.6	45.1±8.0	45.8±23.8
ALP 10 U/L	2.65±1.51	1.95±0.39	2.55±0.38	3.14±1.12
Male				
Glu mmol/L	5.79±0.29	5.19±0.44	5.58±0.88	4.65
T-CHO mmol/L	2.37±0.35	2.82±0.60	2.39±0.06	2.65
TP g/L	74.2±5.2	71.6±9.8	79.1±12.2	70.6
ALB g/L	34.9±1.6	38.7±1.5	42.4±7.5	33.4
Crea μmol/L	136±10	136±9.8	165±39	105
BUN mmol/L	3.43±0.63	3.94±0.58	2.95±0.34	3.20
T-BIL μmol/L	4.40±0.57	4.00±0.00	3.20±0.80	2.80
SGPT 10 U/L	34.6±9.9	23.1±6.9	25.7±9.9	36.0
SGOT 10 U/L	57.9±11.6	37.9±5.8	54.5±7.3	53.3
ALP 10 U/L	2.51±0.46	5.41±4.59	2.23±1.16	1.94

^aP<0.05 vs control group.

significant alteration in the number of micronucleated polychromatic erythrocytes (MNPCE) per 1 000 cells in mice after treatment with 3 g/kg bicyclol. Then, the regular test was performed in mice treated with different doses of bicyclol (0.75, 1.5, 3.0 g/kg, P.O.) or cyclophosphamide (50 mg/kg, i.p). The results showed that bicyclol exhibited no mutagenicity in micronucleus test (Table 8) while cyclophosphamide induced remarkable mutagenicity of MNPCE.

Chromosome aberration test of bicyclol

The results indicated that there was no significant difference in chromosome aberrations between control and bicyclol-treated cells (χ^2 test). The positive control compounds, carboplatin and cyclophosphamide, induced significant chromosome aberrations in CHL (Table 9).

Teratogenicity test of bicyclol

No abortion, premature labor and other toxic effects of bicyclol

were found in pregnant rats except for a pregnant rat in high dose group died of pneumonia on d 10 (Table 10).

There were no significant differences in body height and weight, ratio of sex, outward appearance, living and dead fetus between control and bicyclol treatment groups, suggesting that bicyclol had no toxic effect on embryonic development and fetus (Tables 11, 12).

Bicyclol had no effect on the development of occipital, cervical, sacral bones and coccyx. Defect of sternum was observed in all groups.

No abnormal changes in development of bones were observed in all groups, except for multi-rib in high dose bicyclol group rats (Tables 13, 14). Bicyclol had no effect on development of main organs of fetus.

In a word, the results indicated that when bicyclol (200, 1000 mg/kg) was given orally once daily from the 7th d for 10 d, no significant effects on pregnant rats, development of embryo, skeleton and organs of fetuses were observed.

Table 7 Mutagenicity of bicyclol in bacteria (revertants/plate) (mean±SD)

(µg/plate)	TA97		TA102	
	-S9	+S9	-S9	+S9
Bicyclol 0	86±15	130±15	227±15	272±12
10	68±8	111±1	237±13	230±10
100	80±1	116±20	259±33	304±40
500	57±14	120±6	236±16	279±5
1 000	88±8	140±4	237±35	344±18
5 000	80±4	88±1	259±23	153±2
9-aminoacridine 0.2	938±10			
2AF 10		1 736±509		
DMC 20				
MMS 2 µL			1 606±48	
Vp16 1 mg				1 270±37

Table 8 Mutagenicity in mice after bicyclol treatment

Group	Dosage (mg/kg)	n	MNPCE (‰)	P/N value	P
Control		6	2.2±0.04	100/56	1.8
Bicyclol	750	6	1.8±0.09	135/65	2.1
	1 500	6	2.5±0.14	126/74	1.7
	3 000	6	1.7±0.08	130/70	1.9
Cyclophosphamide	50	6	38.2±3.0	100/250	0.4

Table 9 Chromosome aberrations in mice after bicyclol treatment

Dosage (µg/kg)	-S9		+S9	
	Aberration (%)	P	Aberration (%)	P
Control (DMSO) 100	100	2	100	3
Bicyclol 50	100	2	100	2
				>0.05
	100	3	100	3
	200	2	100	3
Carboplatin 100	50	84		
CY 100			50	66

Table 10 Effect of bicyclol on pregnant rats (mean±SD)

Group	n	Number of pregnancies	Death	Body weight (g)		
				D 0	D 9	D 18
Control	20	15	0	251.9±6.8	271.0±6.8	337.7±8.2
Bicyclol 200 mg/kg	20	15	1	253.5±5.4	295.4±5.5	373.3±6.7
Bicyclol 1 000 mg/kg	20	17	0	258.2±5.7	277.4±5.64	352.9±7.5

Table 11 Effect of bicyclol on embryonic development

Dosage (mg/kg)	n	Corpus luteum	Living fetus(%)	Dead fetus (%)	Resorption (%)
Control	15	177	171 (96.6)	0	6 (3.4)
Bicyclol 200	15	181	155 (93.3)	6 (3.6)	5 (3.0)
Bicyclol 1 000	17	225	203 (97.1)	1 (0.5)	5 (2.4)

Even though defect of sternum was observed in high dose bicyclol group, no significant difference in the development of skeleton between control and bicyclol groups was found by χ^2 test. The multi-rib in individual fetuses was considered to be normal variation.

DISCUSSION

The acute, chronic and special toxicity of any new drug should be studied before recommended for clinical trial. Acute toxicity is mainly to determine the lethal dose (LD₅₀) of a drug to induce 50% death of experimental animals. Results of the present study indicated that 5 g/kg bicyclol given orally to mice and rats did not induce clinical intoxication and death, suggesting that the LD₅₀ of bicyclol is at least greater than 5 g/kg body weight. In general, if the LD₅₀ of a single drug is over 5 g/kg, it may be regarded as a low toxicity drug. Bicyclol belongs to this kind of

drugs. Chronic toxicity test should be performed in 2 species of animals and 3 doses should be tested. One of the doses should be the effective dosage in pharmacology, and the biggest dose should be large enough to produce toxicity in animals. In the study of chronic toxicity, 3 doses of bicyclol (150, 300 and 600 mg/kg) were used. Among the 3 doses of bicyclol tested, 150 mg/kg was the pharmacologically effective dose, and 600 mg/kg was 400 folds higher than the proposed therapeutic dose of bicyclol (1.5 mg/(kg·d)) for patients. As a result, the 3 doses of bicyclol given for 6 mo have no detectable toxic effects on rats. All parameters obtained from blood picture, urine, blood biochemistry and pathological examinations of main organs were in normal range. In chronic toxicity test of beagle dogs for 6 mo, 25, 75 and 225 mg/kg of bicyclol were used. The highest dose of 225 mg/kg was 150 folds corresponding to the proposed therapeutic dose of bicyclol for patients. Like the results of chronic toxicity in rats, bicyclol

Table 12 Effect of bicyclol on fetus (mean±SD)

Dosage (mg/kg)	Brood number (X)	BW (g)	Height (cm)	Tail length (cm)	Sex ratio (F/M)
Control	39.9±4.1	3.5±0.01	3.4±0.02	1.28±0.02	89/82
Bicyclol 200	43.3±3.1	4.3±0.08	3.4±0.02	1.26±0.01	81/74
Bicyclol 1 000	47.7±2.2	4.0±0.09	3.3±0.02	1.17±0.01	97/106

Table 13 Effect of bicyclol on ossification of fetus, *n* (%)

Dosage (mg/kg)	<i>n</i>	Ossification of sternum		
		Defect of the 5th and 6th sternums	Defect of the 6th sternum	Number of sternum defects
Control	87	0	8 (9.2)	79 (91.0)
Bicyclol 200	77	1 (1.3)	6 (7.8)	70 (90.9)
Bicyclol 1 000	108	9 (8.3)	1 (0.9)	94 (87.0)

Table 14 Effect of skeleton development of fetus

Dosage (mg/kg)	<i>n</i>	Cervical -rib	Defect of the 13th rib	Defect of the 14th rib
Control	87	0	0	0
Bicyclol 200	77	0	0	0
Bicyclol 1 000	108	0	0	4 (3.7%)

also had no noticeable toxic effects on all biochemical indices and pathological examinations of the main organs, although there were variations of certain parameters before and after bicyclol treatment, there were no statistically significant difference between control and bicyclol groups, suggesting that bicyclol is a low toxicity drug.

The purposes of specific toxicity tests including mutagenicity, teratogenicity and carcinogenesis are to study whether the tested drug would induce fetal deformation and mutation or carcinogenesis. Mutagenicity test includes the classical Ames test, chromosome aberrations in Chinese hamster lung (CHL) cells and micronucleus assay. In Ames test, the biggest dose of any test drug should reach 5 000 µg/plate. For chromosome aberration assay, the concentration of 50% inhibition rate of test compound on growth of CHL cells should be tested. In both Ames and chromosome aberration assays, positive mutagenic compounds should also be used as control in presence and absence of liver microsomes (S9 mixtures). In micronucleus assay, 1/2 LD₅₀ of test drug should be used. The data of our current study show that bicyclol at doses of 5 000 µg/plate in Ames test, 200 µg/mL (IC₅₀) in chromosome aberration assay and 3 g/kg in mice all have no mutagenic effect, while the positive control compounds all induce mutation. In other word, bicyclol has no mutagenicity.

The purpose of teratogenicity assay is to evaluate whether the test drug induces fetal deformation of pregnant patients who take the tried drug. As shown in the present study, bicyclol at a rather high dose of 1 g/kg, which was 667 folds of the proposed therapeutic dose (1.5 mg/(kg·d)) for patients, did not induce fetal deformation. All the above results indicate that bicyclol is a low toxicity drug. This conclusion is supported by the results of clinical trial of bicyclol on chronic viral hepatitis. More than 2 000 chronic hepatitis B patients were treated with bicyclol for 6 mo. As a result, bicyclol significantly normalized the elevated serum transaminases (ALT and AST) by about 50%, and also turned positive virus markers such as HBeAg to negative by about 20%. Side effects such as headache and skin eruption occurred in only about 1% of these patients^[9,10]. No other noticeable side effects were observed. It was reported that adverse effects (influenza-like syndrome) occur frequently during treatment with interferons^[11], and that lamivudine can

induce mitochondrial toxicity^[12]. The side effect and toxicity of bicyclol are lower than those of interferon-2α and lamivudine.

REFERENCES

- 1 **Lok AS.** Hepatitis B infection: pathogenesis and management. *J Hepatol* 2000; **32**: 89-97
- 2 **Liaw YF, Chien RN, Yeh CT, Tsai SL, Chu CM.** Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. *Hepatology* 1999; **30**: 567-572
- 3 **Liu GT.** Therapeutic effects of biphenyl dimethyl dicarboxylate (DDB) on chronic viral hepatitis B. *Proc Chin Acad Med Sci Peking Union Med Coll* 1987; **2**: 228-233
- 4 **Liu GT.** From *Fructus Schizandrae* to biphenyl dimethyl dicarboxylate (DDB), a new hepatoprotector for hepatitis. In: Zhou JH, Liu GZ, eds. *Recent Advances in Chinese Herbal Drugs-Actions and Uses*. Beijing: Science Press, Beijing China & S.A.T.A.S. Belgium 1991: 112-125
- 5 **Fu TB, Liu GT.** Protective effects of dimethyle-4,4'-dimethoxy-5,6,5',6'-dimethylene dioxybiphenyl-2,2'-dicarboxylate on damages of isolated rat hepatocytes induced by carbon tetrachloride and D-galactosamine. *Biome Environ Sci* 1992; 5185-5194
- 6 **Liu GT.** The anti-virus and hepatoprotective effect of bicyclol and its mechanism of action. *Zhongguo Xinyao Zazhi* 2001; **10**: 325-327
- 7 **Li Y, Dai GW, Li Y, Liu GT.** Effect of bicyclol on acetaminophen-induced hepatotoxicity: energetic metabolism and mitochondrial injury in acetaminophen-intoxicated mice. *Yaoxue Xuebao* 2001; **36**: 723-726
- 8 **Gu XJ.** The efficacy of bicyclol in treatment of chronic hepatitis B. *Zhongguo Xinyao Zazhi* 2004; **13**: 940-942
- 9 **Yao GB, Ji YY, Wang QH, Zhou XQ, Xu DZ, Chen XY.** A randomized double-blind controlled trial of bicyclol in treatment of chronic viral hepatitis. *Zhongguo Xinyao Linchuang Zazhi* 2002; **21**: 457-461
- 10 **Yin H.** Results of phase IV clinical trial of bicyclol on chronic viral hepatitis. *China Prescription Drug* 2004; **9**: 25-27
- 11 **Haria M, Benfield P.** Interferon-alpha-2a. A review of its pharmacological properties and therapeutic use in the management of viral hepatitis. *Drugs* 1995; **50**: 873-896
- 12 **Kakuda TN.** Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. *Clin Ther* 2000; **22**: 685-708