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Protective Effects of Chalcone Derivatives for Acute Liver Injury in Mice

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The hepatoprotective effects of chalcone derivatives were evaluated in D-galactosamine/lipopolysaccharide (D-GalN/LPS)-induced fulminant hepatic failure in mouse. Thirteen chalcone derivatives were synthesized for study and their hepatoprotective effects were evaluated by assessing aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in serum. Chalcone preparations were injected into mice at 12 h and 1 h before intraperitoneal injection of D-GalN/LPS. After abdominal administration, changes in AST and ALT between the control and treated groups were observed. Ten of the synthesized chalcone derivatives exhibited inhibitory effects on D-GalN/LPS-induced levels of AST and ALT in mice. Compounds **2**, **3**, **8**, **9**, and **12** markedly reduced serum AST and ALT at 8 h, inhibited hepatocyte necrosis and showed significant hepatoprotective activities. The activity of compound **3** was compared with the bifendate (DDB) through oral administration. Compound **3** showed much higher inhibitory effects than bifendate for decreasing AST and ALT activity. The results indicate that compound **3** has strong hepatoprotective activity through suppression of tumor necrosis factor-alpha (TNF-alpha) preduction, reduction of the histological change in the liver, and attenuated of hepatocyte apoptosis confirmed by DNA fragmentation assay.

Key words: Chalcone derivatives, Butein, Hepatoprotective activity, Fulminant hepatitis

INTRODUCTION

Fulminant hepatic failure is a clinical syndrome resulting from massive death (apoptosis or necrosis) of hepatocytes (Kosai *et al.*, 1999). Fulminant hepatic failure is induced in diverse pathological conditions such as hepatitis viral infection, drug and toxin exposure, alcohol, ischemia, metabolic disorder, massive malignant infiltration, septic shock, and chronic autoimmune hepatitis (Mas *et al.*, 1997). Different therapeutic options used to treat fulminant hepatic failure include antibiotics, diuretics, corticosteroids, blood transfusion, charcoal hemofusion, plasmaphoresis and liver transplantation (Kim *et al.*, 2000). However, none of these methods have been shown to effectively treat fulminant hepatic failure. A potent therapeutic agent that can prevent massive hepatocytic cell death is critical for the treatment of fulminant hepatic failure. Tumor necrosis factor-alpha (TNF-alpha)-induced massive hepatocyte apoptosis is a predominant mechanism functioning in this model (Kawaguchi, 1999; Sass *et al.*, 2002).

Chalcone derivatives, one of the large families of plant constituents, have various therapeutic benefits, including antioncogenic (Kumar *et al.*, 2003), anti-inflammatory (Hiseh *et al.*, 1998), analgeisc (Viana *et al.*, 2003), antiulcerative (Murakami *et al.*, 1991), antiviral (Wu *et al.*, 2003), antibacterial (Bekhit *et al.*, 2001), antifungal (Lopez *et al.*, 2001) and antimalarial (Liu *et al.*, 2001) properties. Butein (Fig. 1) is the nature chalcone derivative obtained from Dalbergia odorifera and has antioxidative activity (Cheng *et al.*, 1998). It had been reported by Woo et al that butein has weak hepatoprotective effects on rat liver injury (Woo *et al.*, 2003). To investigate whether other compounds had similar or more hepatoprotective acitivity than butein, the hepatoprotective effects of thirteen chalcone derivatives (Fig. 2) for D-galactosamine (D-GalN) and lipopolysaccharide (LPS)-induced fulminant hepatic failure in mice were determined. The GalN/LPS model provides a practical tool for the evaluation of drugs that interfere with hepatic apoptosis as well as inflam-

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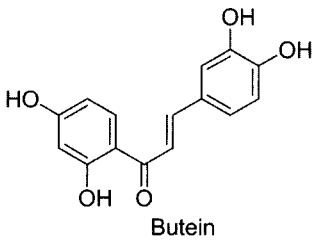
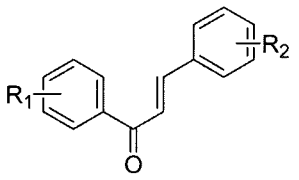


Butein

Fig. 1. The chemical structure of butein



	R_1	R_2
1	2, 4-$(OH)_2$	3, 4-$(OH)_2$
2	2, 4-$(OH)_2$	3-OCH_3, 4-OH
3	2, 4-$(OH)_2$	4-$N(CH_3)_2$
4	2, 4-$(OH)_2$	4-OCH_3
5	2, 4-$(OH)_2$	4-Cl
6	2, 4-$(OH)_2$	2-NO_2
7	2, 4-$(OH)_2$	3, 4-OCH_2O-
8	2, 4-$(OH)_2$	3-NO_2
9	2, 4-$(OH)_2$	2, 4-Cl_2
10	2, 4-$(OH)_2$	4-OH
11	4-OH	3-OCH_3, 4-OH
12	4-OH	4-$N(CH_3)_2$
13	4-OH	4-OH

Fig. 2. The chemical structures of compounds tested

matory liver injury (Xiong *et al.*, 1999; Nakama *et al.*, 2001; Arvelo *et al.*, 2002).

MATERIALS AND METHODS

Reagents

Chalcone derivatives were synthesized at the Department of Medicinal Chemistry, College of Pharmacy, Yanbian University. The compounds were dissolved in Tween-80. D-Galactosamine (GalN, *E.coli* 0111:B4) and lipopolysaccharide (LPS, *E.coli* 0111:B4) was purchased from Sigma-Aldrich (St. Louis, USA). Bifendate (DDB) was provided from a pharmaceutical factory in Guang Zhou, China (Batch number 980612).

Animal and experimental protocol

Male C57BL/6 mice (20~25 g) were purchased from the Laboratory of Animal Research, College of Medicine, Yanbian University, and fed with a normal standard chow Celsius diet and tap water, *ad libitum*. The mice were housed in plastic cages and maintained under conditions of 25 °C, 50-60% relative humidity, and 12 h lightdark cycles throughout the experiment. The mice were maintained in these facilities for at least one week prior to the experiment.

Chalcone derivatives preparations were administered intraperitoneally or orally to mice at 12 h and 1 h before GalN/LPS administration. The doses of the chalcone derivatives preparations administered are shown in Table I. An hour after the second chalcone derivative treatment, mice were given an intraperitoneal injection of GalN (700 mg/kg body weight), immediately followed by an intraperitoneal injection of LPS (10 μg/kg body weight) to induce fulminant hepatitis. Serum and liver tissue samples were obtained 1 h and 8 h after administration of GalN/LPS. There were at least 10 animals in each group.

Animal experiments were carried out under the "Guiding Principles in the Use of Animals in Toxicology" adopted by the Society of Toxicology (USA) in July 1989 and revised in March 1999. The animal care committee of our institution approved the present study.

Blood biochemistry and serum TNF-alpha assay

Blood was collected at 1 h and 8 h after GalN/LPS administration. Serum levels of AST and ALT at 8 h after GalN/LPS injection were quantified using an Autodry Chemistry Analyzer (SPOTCHEM™ SP4410, Arkray, Japan). Serum TNF-alpha level was determined using Quantikine®M Mouse TNF-alpha Immunoassay kit (R&D, Minneapolis, USA) at 1 h after GalN/LPS injection according to the manufacturer's protocol.

Determination of lethality

Chalcone derivative preparations were administered intraperitoneally at a dose of 10 mg/kg to mice at 12 h and 1 h before GalN/LPS injection. The numbers of dead mice were counted 24 h after GalN/LPS injection.

Histopathological analysis

The liver, fixed in formalin solution, was embedded in paraffin, 4-μm sectioned, stained with hematoxylin-eosin and photographed at 100 × magnification. Apoptotic bodies and nuclei displaying chromatin condensation were observed and compared between each treated group.

DNA fragmentation analysis

Liver tissue was obtained 8 h after GalN/LPS injection. Genomic DNA was extracted from liver tissues using a Wizard® genomic DNA purification kit (Promega, Madison, USA) according to the manufacturer's protocol. Extracted DNA was subjected to electrophoresis on a 2% agarose gel containing 0.1 μg/mL ethidium bromide.

Statistical analysis

All values are expressed as mean ± S.E.M. Treatment groups were compared using one-way ANOVA and Tukeys multiple comparison tests. Stastical significance was set a priori at a=0.05. Calculations were performed with the GraphPad Prism program (GraphPad Software, Inc., San Diego, USA).

RESULTS

The abdominal administeted GalN/LPS induced fulminant hepatic failure with marked increases in serum AST (7455 ± 2356 IU/L) and ALT levels (6236 ± 2015 IU/L). Thirteen chalcone derivatives reduced AST and ALT levels significantly at 8 h after GalN/LPS administration (Table I). Among them, compounds **2**, **3**, **8**, **9**, and **12** exhibited stronger activity (P<0.01), and compounds **5**, **6**, **10**, and **13** exhibited hepatoprotective activity (P<0.05), while compounds **1**, **7**, and **11** reduced AST and ALT, but did not have inhibitory effects on GalN/LPS.

We investigated whether oral administration of chalcone derivatives was effective for liver injury (Table II). Oral administration of compound **2** had no inhibitory activity against GalN/LPS, while compounds **3** and **9** exhibited stronger inhibitory activity (P<0.01). Furthermore, oral administration of compound **3** at 12 h and 1 h prior to GalN/LPS injection showed significantly higher inhibitory activity than at 8 h after GalN/LPS injection at 10 mg/kg. AST levels after GalN/LPS admninistration was 338 ± 161 IU/L and 239 ± 111 IU/L of ALT (P<0.01).

The orally administered compound **3** and DDB of GalN/LPS induced fulminant hepatic failure with marked increases of serum AST (5624 ± 712 IU/L) and ALT (4613 ± 634 IU/L) levels (Table III). Administration of DDB lowered, AST and ALT levels to 2657 ± 502 IU/L and 1275 ± 386 IU/L, respectively. However, administration of compound **3** lowered AST to 338 ± 161 IU/L and ALT to 239 ± 111 IU/L. Of the chalcone derivatives tested, compound **3** showed the strongest inhibitory activity (P<0.01).

Mice treated with saline instead of chalcone derivatives began to die within 8 h of GalN/LPS injection, and at 24 h the lethality rate reached 100%. However, pretreatment with compounds **3** and **9**, at doses of 10 mg/kg, markedly reduced lethality (Table IV). As the protective effects of compound **3** were suitable, we used compound **3** for the following study. We tested whether oral administration of compound **3** and DDB was effective in preventing liver injury. Oral administration with 10 mg/kg of compound **3** and 100 mg/kg of DDB for 12 h and 1 h before GalN/LPS injection significantly inhibited the elevation of serum AST and ALT levels seen 8 h after GalN/LPS injection. The

Table I. Reductions in serum AST and ALT from abdominal administered chalcone derivatives in GalN/LPS-induced fulminant hepatic failure

Preparations	Dose (mg/kg)	N	AST (IU/L)	ALT (IU/L)
Normal	-	5	45 ± 4**	16 ± 2**
Control(GalN/LPS)	-	10	7455 ± 2356	6236 ± 2015
1	10	10	4317 ± 140	3830 ± 1200
2	10	10	1344 ± 317**	1210 ± 322**
3	10	10	897 ± 528***	869 ± 629***
4	10	10	3915 ± 1267	3911 ± 1099
5	10	10	2301 ± 966*	1897 ± 780*
6	10	10	2394 ± 721*	2044 ± 737*
7	10	10	7042 ± 1840	7018 ± 1998
8	10	10	1953 ± 640**	1512 ± 480**
9	10	10	1637 ± 629**	1254 ± 533**
10	10	10	2803 ± 540	2630 ± 548*
11	10	10	8985 ± 2605	6528 ± 2572
12	10	10	1425 ± 432**	1226 ± 407**
13	10	10	2791 ± 1410*	3161 ± 1795

Groups of 10 mice were injected i.p. with preparations at 12 h and 1 h before the injection of GalN (700 mg/kg)/LPS (10 μg/kg). Normal and GalN/LPS mice were treated with saline instead of chalcone derivatives preparations. Normal mice were also treated with saline instead of GalN/LPS. Serum was obtained 8 h after GalN/LPS injection. GalN/LPS-induced AST and ALT values were 7455 ± 2356 IU/L and 6236 ± 2015 IU/L, respectively. The results are shown as the mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001, significantly different from GalN/LPS.

Table II. Protective effects of orally administered chalcone derivatives on serum AST and ALT on GalN /LPS-induced fulminant hepatic failure

Preparations	Dose (mg/kg)	N	AST (IU/L)	ALT (IU/L)
Normal	-	5	52 ± 3*	18 ± 2*
Control(GalN/LPS)	-	10	2339 ± 773	2184 ± 889
2	10	10	1949 ± 431	1406 ± 359
2	20	10	2457 ± 512	1982 ± 483
3	10	10	338 ± 161*	239 ± 111*
3	20	10	521 ± 201*	521 ± 192*
9	10	10	496 ± 194*	470 ± 196*
9	20	10	985 ± 231*	964 ± 206*

Groups of 10 mice were injected with GalN (700 mg/kg) and LPS (10 μg/kg) and preparations were administered orally to mice with an intubation needle at 12 h and 1 h before the injection of GalN/LPS. Normal and GalN/LPS mice were treated with saline instead of chalcone derivatives preparations. Normal mice were treated with saline instead of GalN/LPS. Serum AST and ALT was obtained 8 h after GalN/LPS injection. GalN/LPS-induced AST and ALT levels were 2339 ± 773 IU/L and 2184 ± 889 IU/L, respectively. The results are shown as mean ± S.E.M. *P<0.01, significantly different from GalN/LPS.

TNF-alpha level in serum collected 1 h after GalN/LPS injection was also suppressed (Table V).

Table III. Protective effects of the orally administered compound 3 and bifendate on ALT and AST in GalN /LPS-induced fulminant hepatic failure

Preparations	Dose (mg/kg)	N	AST (IU/L)	ALT (IU/L)
Normal	-	5	41 ± 3*	20 ± 2*
Control(GalN/LPS)	-	10	5624 ± 712	4613 ± 634
DDB	100	10	2657 ± 502	1275 ± 386
Compound **3**	10	10	338 ± 161*	239 ± 171*

Groups of 10 mice were injected with GalN (700 mg/kg) and LPS (10 μg/kg) and preparations were injected administered orally to mice with an intubation needle. at 12 h and 1 h before the injection of GalN/LPS. Normal and GalN/LPS mice were treated with saline instead of compound **3** and bifendate. Normal mice were treated with saline instead of GalN/LPS. Serum was obtained 8 h after GalN/LPS injection. GalN/LPS-induced AST and ALT were 5624 ± 712 IU/L and 4613 ± 634 IU/L, respectively. The results are shown as mean ± S.E.M. *P<0.01 is significantly different from GalN/LPS

Table IV. Effects of chalcone derivatives on lethal toxicity induced by GalN/LPS in mice.

Preparations	Dead/Total		Lethality at 24 h (%)
	8 h	24 h	
GalN/LPS	6/10	10/10	100
3	1/12	2/12	16.7
2	6/12	9/12	75
9	2/12	3/12	25

Mice were injected with GalN (700 mg/kg) and LPS (10 μg/kg) and compounds **2**, **3**, and **9** at a dose of 10 mg/kg each were injected i.p. at 12 h and 1 h before the injection of GalN/LPS. GalN/LPS mice were treated with saline in place of compounds **2**, **3**, or **9**.

Table V. Protective effects of orally administered compound **3** and DDB on serum transaminases and tumor necrosis factor-alpha after GalN/LPS-treatment

Preparation	Dose (mg/kg)	AST(IU/L)	ALTIU/L)	TNF-α (ng/mL)
Normal	-	25 ± 12*	17 ± 10*	22± 3*
GalN/LPS	-	5422 ± 823	5002 ± 564	652 ±102
3	10	346 ± 152*	254 ± 165*	482 ± 92*
DDB	100	2712 ± 456*	1356 ± 371*	496 ± 87*

Groups of no fewer than 10 mice were injected with GalN (700 mg/kg) and LPS (10 μg/kg). Preparations were administered orally to mice with an intubation needle 12 h and 1 h before the injection of GalN/LPS. Normal and GalN/LPS mice were treated with saline instead of compound **3** and DDB preparations. Normal mice were treated with saline instead of GalN/LPS. Serum was obtained 8 h after GalN/LPS injection. AST and ALT values of GalN/LPS-treated mice were 5422 ± 823 IU/L and 5002 ± 564 IU/L, respectively. Serum TNF-alpha level of GalN/LPS-treated mice was 652 ± 102 ng/mL. Results are shown as mean ± S.E.M. *P<0.001, significantly different from GalN/LPS.

Eight hours after GalN/LPS administration, severe liver damage including numerous apoptotic hepatocytes and massive necrosis with intralobular hemorrhage were seen in the livers of GalN/LPS-treated mice (Fig. 3). GalN/LPS injected livers showed only spotty necrosis of hepatocytes, and GalN/LPS injected livers pretreated with compound **3** and DDB resembled those of normal mice (Fig. 3).

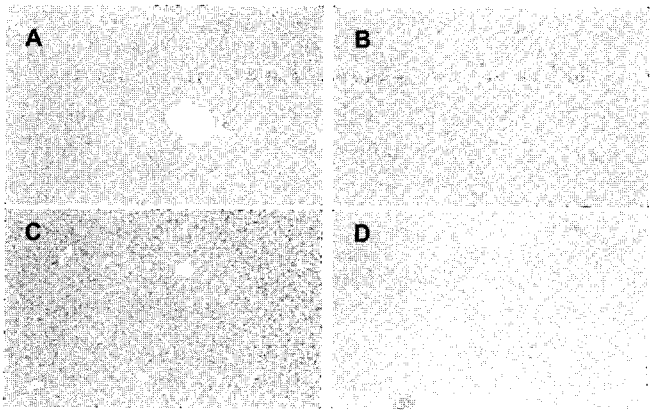


Fig. 3. Histological changes in the liver of mice 8 h after GalN/LPS injection with and without compound **3** and bifendate (hematoxylin and eosin staining, original magnification ×100). Mice were injected with GalN (700 mg/kg) and LPS (10 μg/kg). Compound **3** and DDB were injected i.p. at a dose of 10 mg/kg and 100 mg/kg, respectively at 12 h and 1 h before injection of GalN/LPS. GalN/LPS mice were pretreated with saline instead of compound **3** and DDB at 12 h and 1 h before injection of GalN/LPS. Normal mice were injected with saline instead of compound **3**, DDB, or GalN/LPS. A: Normal, B: GalN/LPS, C: compound **3**, D: DDB.

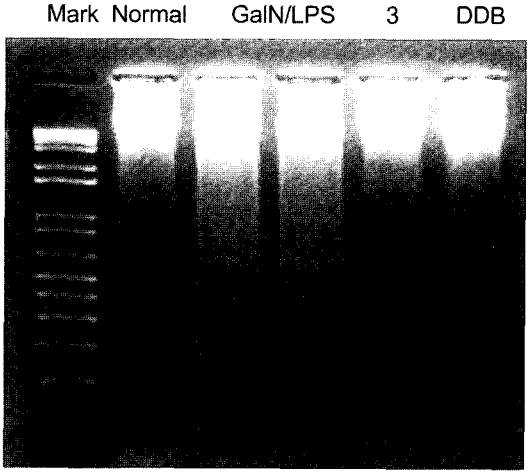


Fig. 4. DNA fragmentation in the liver of mice 8 h after GalN/LPS injection. Mice were injected with GalN (700 mg/kg) and LPS (10 μg/kg). Preparations were injected i.p. at a dose of 10 mg/kg of compound **3** and 100 mg/kg dose of bifendate at 12 h and 1 h before the injection of GalN/LPS. GalN/LPS mice were pretreated with saline instead of compound **3** and bifendate at 12 h and 1 h before the injection of GalN/LPS. Normal mice were injected with saline instead of compound **3**, DDB and GalN/LPS.

To confirm the suppressive effects of compound **3** and DDB on hepatocyte apoptosis, genomic DNA fragmentation was assayed. Genomic DNA fragmentation was observed in the livers of mice treated with GalN/LPS alone 8 h after GalN/LPS injection, while very little DNA fragmentation was observed in the livers of mice pretreated with 10 mg/kg of compound **3** and with 100 mg/kg of DDB (Fig. 4).

DISCUSSION

At present many experimental animal models of liver injury have been established with CCl_4, D-GalN, or D-GalN/LPS (Kiichiro *et al.*, 1999; Toshio *et al.*, 2002; Takahashi *et al.*, 2001; Tran *et al.*, 2002). Administration of a subtoxic dose of GalN together with or followed by LPS is a well established model for fulminant hepatic failure (Galanos *et al.*, 1979).

The present study is the first report that chalcone derivatives have protective effects on acute liver injury in mice. To assess compounds with potential hepatoprotective effects, thirteen chalcone derivatives were synthesized in the lab and administered abdominally at varying doses. Except for compounds **1**, **7**, and **11**, it was found that ten chalcone derivatives exhibited inhibitory effects on GalN-/LPS-induced increases in AST and ALT levels in mice. Compounds **2**, **3**, **8**, **9**, and **12** markedly reduced serum AST and ALT at 8 h, which inhibited hepatocyte necrosis and showed significant hepatoprotective activities.

We tested whether the oral administration of compounds **2**, **3**, and **9** at different doses was effective against liver injury. The results of this study (shown in Table II) indicated that compound **2** had no protective effect compared with compounds **3** and **9**, perhaps because compound **2** is a weak lipid having low solubility and bioactivity. However, both compounds **3** and **9** showed strong hepatoprotective activities, with compound **3** showing the strongest activity.

Finally, in comparison with the clinical hepatoprotective drug bifendate assessed in our previous study (unpublished data) intraperitoneal injection of compound **3** showed a stronger hepatoprotective effect as indicated by reduced serum AST and ALT levels. Compound 3 had nearly a 10-fold higher hepatoprotective activity than bifendate. Our results indicated that intraperitoneal injection of compound **3** markedly reduced hepatocyte necrosis, reduced histological changes and DNA fragmentation, and suppressed lethality induced by GalN/LPS. Remarkably, serum TNF-alpha levels had decreased with pretreatment of compound **3**. It was also shown that compound **3** suppressed LPS-induced increases in TNF-alpha release in a dose-dependent manner, which could explain the inhibitory effects of compound **3** against LPS-induced hepatotoxicity observed in the present study.

The structure of compound **3** contains a dimethylin group at the phenyl ring position, which may increase electron density of carboxide. Consequently, the nitrogen atom of dimethylin could form hydrogen bonds with the electron-deficient hydrogen atom of –OH, –COOH, -SH, and thus it has greater reactivity.

Based on our findings, further investigation of the hepatoprotective effects, liver fibrosis, and harmful effects of compound **3** is warranted.

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