J. Pharm. Pharmacol. 2000, 52: 531–538
Received September 28, 1999
Accepted January 12, 2000

Effects of ZNC-2381, a New Oral Compound, on Several Hepatic Injury Models and on Hepatocellular Apoptosis in Mice and Rats

YOSHIHIKO ITOKAZU, YOSHIHIDE SEGAWA, TAKESHI OMATA, NAONORI INOUE, NAOKI TSUZUIKE, MASAAKI NAGASAWA, HIROYASU NISHIOKA, TADASHI KOBAYASHI*, YOSHIHISA NAKANO* AND TAKASHI KANDA*

*Central Research Laboratories, Zeria Pharmaceutical Company Ltd, 2512-1 Oshikiri, Kohnan-machi, Ohsato-gun, Saitama 360-0111 and *Research Laboratories, Nippon Chemiphar Company Ltd, 1-22 Hikokawato, Misato, Saitama 341-0005, Japan*

Abstract

The hepatoprotective effect of ZNC-2381 (1-(4-aminophenyl) methyl-3-(3-nitrophenyl)-1,3-dihydroimidazo[4,5-*b*]pyridine-2-one), a novel 2-one dihydroimidazopyridine derivative, has been evaluated in several experimental models of hepatic injury.

In mice, oral ZNC-2381, administered at doses of 3, 10 or 30 mg kg^{-1}, 1 h before induction of hepatic injury with concanavalin A, dose-dependently inhibited increases in serum alanine aminotransferase (ALT) activity. Apoptosis of liver cells, as indicated by DNA fragmentation (nucleosome assay) and DNA-ladder formation (electrophoresis), was also inhibited dose-dependently. ZNC-2381 dose-dependently inhibited concanavalin A-induced increases in serum tumour necrosis factor (TNF)-α levels, and TNF-α mRNA expression in the liver. Oral ZNC-2381 also dose-dependently inhibited increases in serum ALT activity in mice with hepatic injury induced by *Propionibacterium acnes* and a bacterial lipopolysaccharide (LPS) or D-galactosamine–LPS, and in rats with D-galactosamine-induced hepatic injury.

These results indicate that oral ZNC-2381 inhibits cytokine (TNF-α) production and cytokine-related hepatocellular apoptosis, and might thus prevent different types of hepatic injury.

Immune responses aimed at eliminating the hepatitis virus are involved in the development of hepatic injury during chronic viral hepatitis in man (Kita et al 1993; Rehermann et al 1995). Hepatocellular apoptosis, resulting from immune responses mediated by tumour necrosis factor (TNF)-α (produced by macrophages and Kupffer cells activated by the hepatitis virus), interferon (IFN)-γ (produced by lymphocytes), and Fas-Fas ligand and perforin interactions between cytotoxic T lymphocytes and hepatocytes, is thought to contribute to the development of hepatitis-associated liver injury (Ando et al 1993).

Concanavalin A-induced hepatic injury has been reported to result both from direct hepatocyte injury, mediated by activated $CD4^+$ lymphocytes, and from hepatocellular apoptosis, induced by TNF-α and IFN-γ (Tiegs et al 1992; Gantner et al 1995; Kusters et al 1996). Concanavalin A is frequently used to prepare animal models with high levels of cytotoxic T lymphocytes, because these cells are involved in the development of viral hepatitis in man (Kita et al 1993; Rehermann et al 1995). The development of concanavalin A-induced hepatic injury is inhibited by neutralizing anti-TNF-α antibodies, the phosphodiesterase (PDE) inhibitors motapizone (PDE III-selective) and rolipram (PDE IV-selective), the immunosuppressant agent FK-506, and dexamethasone (Tiegs et al 1992; Gantner et al 1997; Mizuhara et al 1998).

Although oral rolipram effectively inhibits TNF-α production in different models of hepatic injury (Gantner et al 1997), it has adverse effects on the central nervous and gastrointestinal systems (Eban & Ruther 1985; Lowe & Cheng 1992). In the search for a better-tolerated alternative, we discovered a novel 2-one dihydroimidazopyridine derivative, ZNC-2381 (1-(4-aminophenyl)methyl-3-(3-nitrophenyl)-1,3-dihydroimidazo[4,5-b]pyridine-2-one), which has almost no inhibitory effect on PDE III or IV activity, but inhibits TNF-α production and the proliferation of lymphocytes, thus

Correspondence: Y. Segawa, Central Research Laboratories, Zeria Pharmaceutical Company Ltd, 2512-1 Oshikiri, Kohnan-machi, Ohsato-gun, Saitama 360-0111, Japan.

preventing concanavalin A-induced hepatic injury. In this study we have investigated the hepatoprotective effect of ZNC-2381 in several models of experimental hepatic injury.

Materials and Methods

Animals

Male and female BALB/c mice, 6 weeks, and male Sprague-Dawley rats, 5 weeks, were purchased from Charles River Japan (Atsugi, Japan) and acclimatized to a room temperature of $23 \pm 3^{\circ}C$, a humidity of $55 \pm 10\%$ and a 12 h : 12 h dark–light cycle for at least 4 days before use in the experiments. Commercial laboratory chow (solid, CRF-1; Oriental Yeast, Tokyo, Japan) and water were freely available. The experiments in this study were conducted in accordance with the guidelines of the Zeria Pharmaceutical Animal Care and Use Committee.

Drugs and chemicals

ZNC-2381 (MW 361·36; Zeria Pharmaceutical, Tokyo, Japan) was suspended in 0·5% methylcellulose solution for in-vivo experiments and dissolved in dimethylsulphoxide (DMSO; final concentration of DMSO 0·1%) for in-vitro experiments. Concanavalin A (Jack bean, type IV; Sigma, St Louis, MO) was dissolved in physiological saline (sterile, pyrogen-free; Ohtsuka Pharmaceutical, Osaka, Japan). *Propionibacterium acnes* (*P. acnes*; ATCC 6919) was heat-treated (100°C, 30 min) to inactivate and kill the bacteria, lyophilized, and suspended in physiological saline (sterile, pyrogen-free). Lipopolysaccharide (LPS; *Escherichia coli*, 055: B5, Sigma, for the D-galactosamine–LPS model, and *Salmonella enteritidis*, Sigma, for the *P. acnes*–LPS model) was dissolved in distilled water (sterile, pyrogen-free; Ohtsuka Pharmaceutical) then diluted with physiological saline (sterile, pyrogen-free). D-Galactosamine hydrochloride (Sigma) was dissolved in physiological saline (sterile, pyrogen-free) and adjusted to pH 7 with NaOH.

P. acnes–*LPS-induced hepatic injury*

Seven days after intravenous (i.v.) injection of $15\,mg\,kg^{-1}$ *P. acnes* into female BALB/c mice, LPS ($50\,\mu g\,kg^{-1}$) was injected intravenously to induce hepatic injury (Tiegs et al 1989). ZNC-2381 was administered orally 1 h before LPS injection. Blood was obtained from the orbital veins, under ether anaesthesia, 24 h after LPS injection, and the separated serum was used to measure alanine aminotransferase (ALT) activity.

D-Galactosamine–LPS-induced hepatic injury

ZNC-2381 was administered orally to male BALB/c mice 1 h before simultaneous injection of D-galactosamine (intraperitoneal (i.p.) $700\,mg\,kg^{-1}$) and LPS ($3\,\mu g\,kg^{-1}$, i.v.) (Leist et al 1995). Blood was obtained from the orbital veins, under ether anaesthesia, 8 h after D-galactosamine–LPS injection, and the separated serum was used to measure ALT activity.

D-Galactosamine-induced hepatic injury

ZNC-2381 was administered orally to male Sprague-Dawley rats 1 h before injection of D-galactosamine ($800\,mg\,kg^{-1}$, i.p.) (Nagakawa et al 1993). The rats were then fasted and 24 h later blood was collected from the abdominal aorta under ether anaesthesia. The separated serum was used to measure ALT activity.

Concanavalin A-induced hepatic injury

Concanavalin A ($13\,mg\,kg^{-1}$, i.v.) was injected into female BALB/c mice (Tiegs et al 1992) 1 h after oral administration of ZNC-2381. Blood was obtained from the orbital veins, under ether anaesthesia, 24 h after concanavalin A injection, and the separated serum was used to measure ALT activity. The liver was excised and used to detect DNA fragmentation and DNA-ladder formation, and for histopathological examination. Serum TNF-α, IFN-γ, interleukin (IL)-2 and IL-10 levels were measured 2 h after concanavalin A injection. TNF-α mRNA expression was also investigated in the excised livers.

Measurement of ALT activity and cytokine levels in serum

ALT activity was measured by colorimetry at 555 nm by means of transaminase CII-test (Wako, Osaka, Japan) or autosera S ALT (Daiichi Pure Chemicals, Tokyo, Japan) kits. TNF-α, IFN-γ, IL-2 and IL-10, respectively, were measured by use of Mouse TiterZyme TNFα EIA (PerSeptive Biosystems, USA), Mouse IFN-γ ELISA (enzyme-linked immunoassay; Endogen, USA), Mouse IL-2 ELISA (Endogen) and Mouse IL-10 ELISA (Endogen) kits.

Expression of TNF-α mRNA in the liver

The excised liver was homogenized in buffer containing 4 M guanidine thiocyanate, 25 mM sodium

citrate (pH 7·0), 0·5% sarkosyl, 0·1 M 2-mercaptoethanol and 2 M sodium acetate, and total RNA was extracted by the acid guanidium thiocyanate–phenol–chloroform method (Chomczynski & Sacchi 1987). DNA was amplified by the reverse transcription polymerase chain reaction (RT-PCR) method, electrophoresed on 2·0% agarose gel, and TNF-α mRNA expression in the liver was examined. In the RT-PCR method ATGAGCACAGAAAGCATGATC and TACAGGCTTGTCACTCGAATT were used as sense and antisense primers, respectively, for TNF-α. TGTGATGGTGGGAATGGGTCAG and TTTGATGTCACGGACGATTTCC (Stratagene, La Jolla, USA) were used as sense and antisense primers, respectively, for the control β-actin determination.

Liver DNA fragmentation assay

DNA fragmentation was measured by the method of Rodriguez et al (1996). In brief, the excised liver was homogenized in lysis buffer. After standing at room temperature for 30 min, the homogenate was centrifuged (700 *g*, 10 min), and the supernatant (20 μL, equivalent to 170 μg wet weight liver tissue) was examined by colorimetry at 450 nm using a cell death detection ELISA^{PLUS} (Boehringer Mannheim, Germany) kit. To detect DNA-ladder formation DNA was extracted from the liver by use of Isotissue (Nippon Gene, Toyama, Japan) and electrophoresed on 2·0% agarose gel.

Histopathological examination

The excised liver was fixed in 10% neutral buffered formaldehyde. Paraffin-embedded sections were prepared, stained with haematoxylin and eosin, then examined by light microscopy.

TNF-α production in activated macrophages

Peritoneal macrophages were prepared by the method of Nagakawa et al (1992). In brief, peritoneal exudate cells were collected from female BALB/c mice 7 days after administration of *P. acnes* (50 mg kg^{-1}, i.p.), and 1×10^6 cells mL^{-1} were cultured in RPMI 1640 medium (Gibco RBL, USA) containing 10% foetal bovine serum, 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, by use of 48-well culture plates (37°C, 5% CO_2–air). After 2 h culture the cells adhering to the plates were used as macrophages ($> 95\%$). LPS (100 ng mL^{-1}) and ZNC-2381 were added to these cells, culture (37°C, 5% CO_2–air) was continued for another 3 h, and the TNF-α content of the culture supernatant was measured.

PDE III and IV activity

Purified PDE III and IV enzymes were, respectively, from platelets and U937 cells from man. The effect of ZNC-2381 on PDE activity was measured by the method of Cortijo et al (1993). In brief, ZNC-2381 was added to Tris buffer (pH 7·5) containing the enzyme and [^{3}H]cAMP (0·01 μM), and incubated at 30°C for 20 min. To convert the reaction product, AMP, to adenosine, snake venom nucleotidase (10 mg mL^{-1}) was added, and incubation was continued at 30°C for a further 10 min. Unreacted cAMP was removed by absorption on AGI-X2 resin, then [^{3}H]adenosine in the aqueous phase was measured by scintillation counting.

Statistical analysis

Results are expressed as means ± s.e.m. The significance of differences was tested by use of Dunnett's one-tailed test for multiple group comparisons and Student's *t*-test for comparisons between two groups. The significance of changes in serum ALT activity was analysed after conversion of the measured values to natural logarithms. $P < 0.05$ was considered to be indicative of significance.

Results

Effects of ZNC-2381 on different animal models of hepatic injury

Oral administration of ZNC-2381 at doses of 1, 3, 10 or 30 mg kg^{-1} dose-dependently inhibited the increased serum ALT activity observed in control mice with *P. acnes*–LPS-induced and D-galactosamine–LPS-induced hepatic injury, and that seen in control rats with D-galactosamine-induced hepatic injury (Table 1).

Effect of ZNC-2381 on concanavalin A-induced hepatic injury in mice

Serum ALT activity and hepatocellular necrosis. Serum ALT activity was significantly higher in the control group with untreated concanavalin A-induced hepatic injury than in normal mice that had not received concanavalin A (Figure 1A), and hepatocellular necrosis was detected on histopathological examination 24 h after injection of concanavalin A (Figure 1B). Oral administration of ZNC-2381 at doses of 3, 10 or 30 mg kg^{-1} inhibited the concanavalin A-induced increase in serum ALT activity and dose-dependently reduced hepatocellular necrosis (Figure 1).

Table 1. Efficacy of ZNC-2381 in preventing increased serum alanine aminotransferase activity in different experimental models of hepatic injury in mice or rats.

Treatment	Dose ($mg\,kg^{-1}$)	Alanine aminotransferase activity ($IU\,L^{-1}$)		
		Propionibacterium acnes–lipopolysaccharide mice	D-Galactosamine–lipopolysaccharide mice	D-Galactosamine rats
Normal	–	26·2 ± 0·8	–	–
Control	–	788·0 ± 270·4	750·5 ± 228·8	439·0 ± 98·4
ZNC-2381	1	–	584·2 ± 172·0 (22)	371·9 ± 90·3 (15)
	3	507·8 ± 149·6 (36)	449·3 ± 100·0 (40)	245·6 ± 44·0 (44)
	10	290·3 ± 84·6 (63)	193·5 ± 63·9* (74)	174·9 ± 60·2* (60)
	30	170·6 ± 55·8 (78)	–	–

ZNC-2381 was administered orally 1 h before injection of each hepatic injury stimulant. Serum alanine aminotransferase activity was measured 8 h (D-galactosamine–lipopolysaccharide mice) or 24 h (*Propionibacterium acnes*–lipopolysaccharide mice, D-galactosamine rats) after induction of hepatic injury. Results are presented as means ± s.e.m., n = 6–14. Figures in parentheses indicate percentage inhibition. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ compared with control (Dunnett's one-tailed test). Normal group: no hepatic injury induced. Control group: hepatic injury induced without ZNC-2381 pretreatment.

Liver DNA fragmentation. Increased liver DNA fragmentation and DNA-ladder formation were detected 24 h after injection of concanavalin A. Oral administration of ZNC-2381 dose-dependently inhibited the increase in liver DNA fragmentation and ladder formation induced by concanavalin A (Figure 2).

Serum cytokine levels and TNF-α mRNA expression in the liver. Serum TNF-α levels were increased, and TNF-α mRNA expression was detected in the liver, 2 h after injection of concanavalin A. Pretreatment with oral ZNC-2381 at doses of 1, 3, 10 or $30\,mg\,kg^{-1}$ dose-dependently suppressed the increase in serum TNF-α and inhibited TNF-α mRNA expression in the liver (Figure 3).

ZNC-2381 also dose-dependently inhibited concanavalin A-induced increases in serum IFN-γ and IL-2 levels. In contrast, dose-dependent increases in serum IL-10 levels were observed (Table 2).

Effect of ZNC-2381 on TNF-α production and PDE activity in-vitro

ZNC-2381 at 0·1 μM inhibited LPS-stimulated TNF-α production by 41% in *P. acnes*-primed macrophages; 1·0 μM inhibition was 68% (Table 3). At 0·1 and 10 μM ZNC-2381 had almost no effect on PDE III or IV activity (Table 3).

Discussion

This study has demonstrated that oral ZNC-2381 inhibits the increase in serum ALT activity in concanavalin A-induced hepatic injury and in other models of hepatic injury.

The concanavalin A-induced hepatic injury model involves the triggering of hepatocellular apoptosis (Leist et al 1995). This is followed by increased serum ALT activity and hepatocellular necrosis. Pretreatment with anti-TNF-α antibodies has been shown to prevent both apoptosis and necrosis, suggesting that TNF-α-induced hepatocellular apoptosis is important in the development of concanavalin A-induced hepatic injury (Trautwein et al 1998). Similarly, TNF-α is involved in the induction of hepatocellular apoptosis in *P. acnes*–LPS- and D-galactosamine–LPS-induced hepatic injury in mice (Nagakawa et al 1990; Leist et al 1995) and in D-galactosamine-induced hepatic injury in mice and rats (Itokazu et al 1999; Stachlewitz et al 1999).

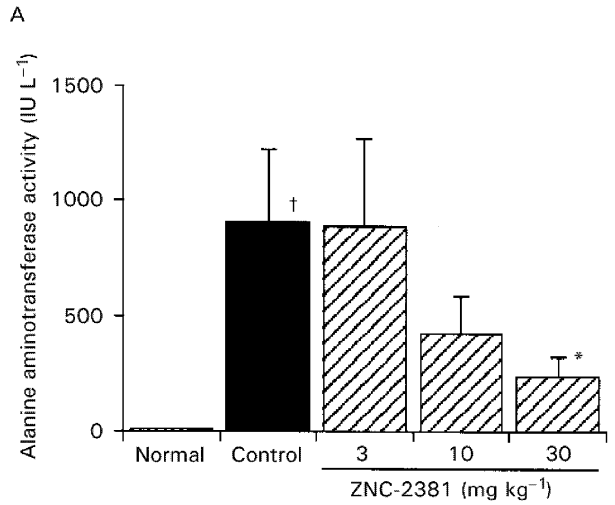
Increases in DNA fragmentation and DNA-ladder formation in hepatocytes are markers of apoptosis in the liver. ZNC-2381 suppressed these increases and prevented histopathological progression to hepatocellular necrosis in the concanavalin A-induced hepatic injury model. ZNC-2381 also inhibited concanavalin A-induced increases in serum TNF-α levels and TNF-α mRNA expression in the liver, and suppressed LPS-induced TNF-α production in *P. acnes*-primed macrophages in-vitro. These findings suggest that suppression of TNF-α production is involved in the inhibition of hepatocellular apoptosis by ZNC-2381. ZNC-2381 also increased serum IL-10 levels in mice with concanavalin A-induced hepatic injury. It has been reported that IL-10 inhibits TNF-α production, thereby preventing hepatic injury in concanavalin A-induced or D-galactosamine–LPS-induced hepatic injury in mice (Louis et al 1997a, b); it is, therefore, possible that ZNC-2381 inhibits TNF-α production by promoting IL-10 production. The

PDE inhibitors motapizone and rolipram also prevent hepatic injury by inhibiting TNF-α production and enhancing IL-10 production (Gantner et al 1997). However, ZNC-2381 inhibited concanavalin A-induced TNF-α production and also TNF-α mRNA expression in the liver without affecting PDE III or IV activity, further studies will be needed to elucidate the mechanism by which ZNC-2381 inhibits TNF-α production.

This study also showed that ZNC-2381 inhibits the increases in serum IFN-γ and IL-2 levels induced by concanavalin A injection, and prevents concanavalin A-induced lymphocyte proliferation in-vitro (unpublished observation). These findings suggest that inhibition of lymphocyte activation is also involved in the prevention of hepatic injury by ZNC-2381.

It has been reported that IFN-γ is a critical mediator similar to TNF-α in concanavalin A-induced hepatic injury (Tiegs 1997). Systemic concanavalin A injection also induced the release of IFN-γ and anti-mouse IFN-γ antiserum and protected against concanavalin A-induced hepatic injury (Kusters et al 1996). IFN-γ also induces apoptotic cell death in mouse hepatocytes, the cell death was accelerated by TNF-α (Morita et al 1995) and increases the number of TNF-α receptors on hepatocytes in-vitro (Aggarwal et al 1985). At least two inflammatory cytokines, TNF-α and IFN-γ, mediate hepatic injury in a concanavalin A-induced mouse model.

Figure 2 shows that ZNC-2381 inhibited DNA fragmentation in concanavalin A-induced mouse liver damage and that this involved inhibition of TNF-α production by ZNC-2381. It is already known that IFN-γ-potentiated TNF-α-induced DNA fragmentation and hepatotoxicity (Kusters et al



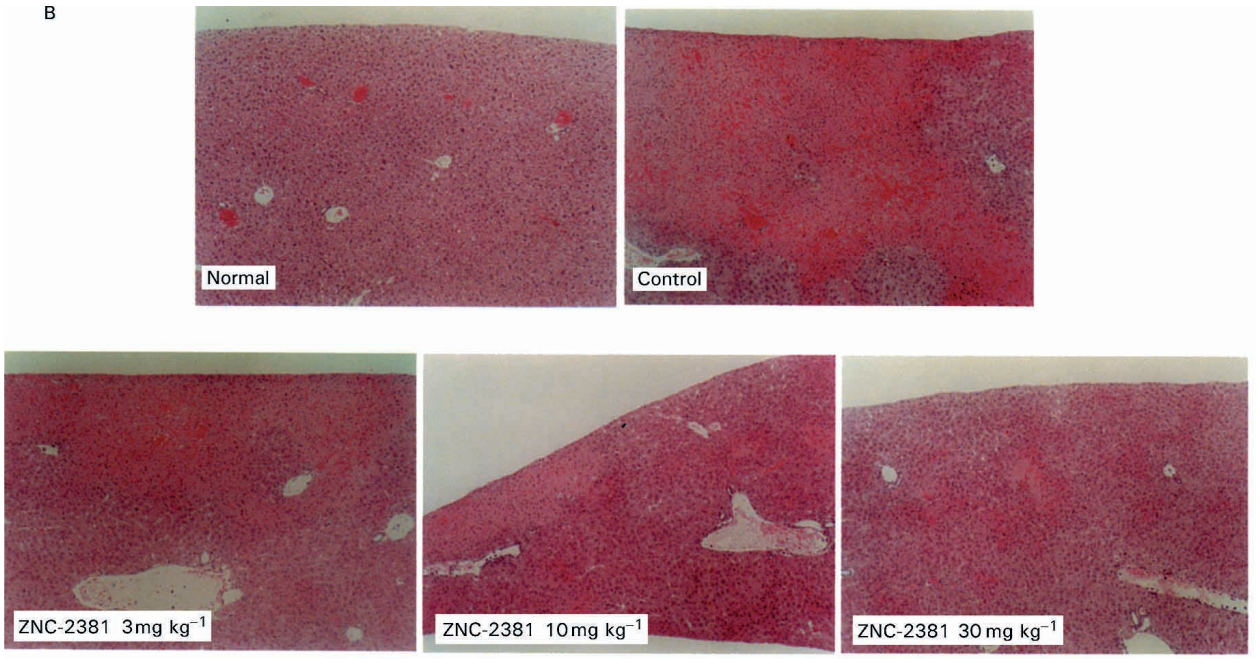


Figure 1. Efficacy of ZNC-2381 in preventing A. increased serum alanine aminotransferase activity and B. histopathological changes in the liver after concanavalin A-induced hepatic injury in mice. ZNC-2381 was administered orally 1 h before intravenous injection of concanavalin A (13 mg kg^{-1}) into female BALB/c mice. Serum aminotransferase activity was measured 24 h after concanavalin A injection. The results are presented as means ± s.e.m. (n = 6). Liver sections were harvested 24 h after concanavalin A injection and stained with haematoxylin and eosin (× 25). † $P < 0{\cdot}01$ compared with the normal group (Student's t-test); *$P < 0{\cdot}05$ compared with control (Dunnett's one-tailed test). Normal group: no hepatic injury induced. Control group: hepatic injury induced without ZNC-2381 pretreatment.

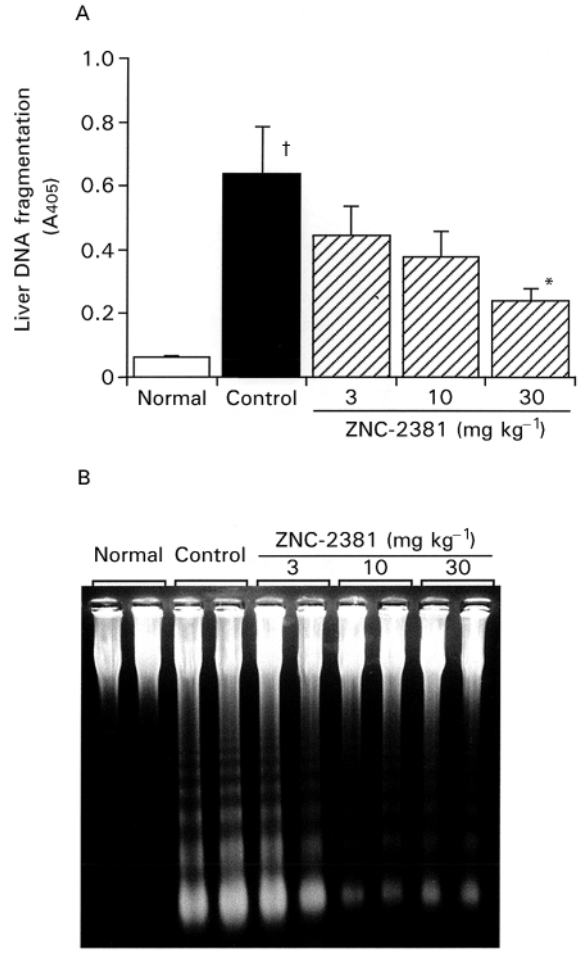


Figure 2. Efficacy of ZNC-2381 in preventing DNA fragmentation and DNA-ladder formation in the liver after concanavalin A-induced hepatic injury in mice. ZNC-2381 was administered orally 1 h before intravenous injection of concanavalin A (13 mg kg^{-1}) into female BALB/c mice. DNA fragmentation (A) was measured 24 h after concanavalin A injection; the results are presented as means ± s.e.m., n = 6. DNA-ladder formation (B) was detected by agarose gel electrophoresis. †$P < 0{\cdot}01$ compared with the normal group (Student's *t*-test); *$P < 0{\cdot}05$ compared with control (Dunnett's one-tailed test). Normal group: no hepatic injury induced. Control group: hepatic injury induced without ZNC-2381 pretreatment.

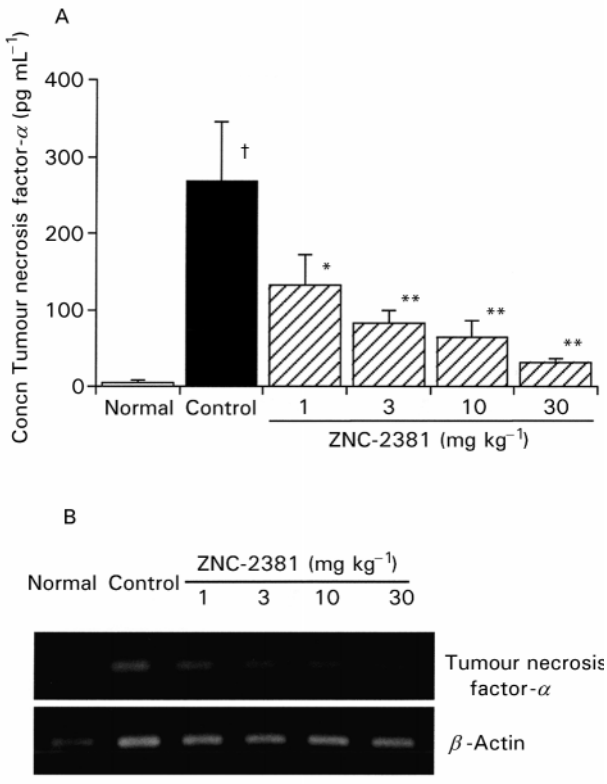


Figure 3. Efficacy of ZNC-2381 in preventing increases in serum tumour necrosis factor-α levels and inhibiting tumour necrosis factor-α mRNA expression in the liver after concanavalin A-induced hepatic injury in mice. ZNC-2381 was administered orally 1 h before intravenous injection of concanavalin A (13 mg kg^{-1}) into female BALB/c mice. Serum tumour necrosis factor-α levels (A) were measured 2 h after concanavalin A injection; the results are presented as means ± s.e.m., n = 6. Liver tumour necrosis factor-α mRNA expression (B) was determined by reverse transcription polymerase chain reaction analysis. †$P < 0{\cdot}05$ compared with the normal group (Student's *t*-test); *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ compared with control (Dunnett's one-tailed test). Normal group: no hepatic injury induced. Control group: hepatic injury induced without ZNC-2381 pretreatment.

Table 2. Effects of ZNC-2381 on serum interferon-γ, interleukin-2 and interleukin-10 levels after concanavalin A-induced hepatic injury in mice.

Treatment	Dose (mg kg^{-1})	Interferon-γ (ng mL^{-1})	Interleukin-2 (ng mL^{-1})	Interleukin-10 (ng mL^{-1})
Normal	–	3·5 ± 2·1	0·0 ± 0·0	0·04 ± 0·03
Control	–	10·4 ± 0·3†	26·2 ± 2·1†	0·45 ± 0·04†
ZNC-2381	1	9·8 ± 1·2 (9)	–	–
	3	9·1 ± 0·9 (20)	25·4 ± 4·2 (3)	0·47 ± 0·09
	10	7·8 ± 0·6* (38)	22·3 ± 1·0 (15)	1·39 ± 0·45
	30	4·7 ± 0·5** (82)	15·5 ± 1·8** (41)	3·83 ± 1·41**

ZNC-2381 was administered orally 1 h before intravenous injection of concanavalin A (13 mg kg^{-1}) into female BALB/c mice. Serum interferon-γ, interleukin-2 and interleukin-10 levels were measured 2 h after concanavalin A injection. Results are presented as means ± s.e.m., n = 4–6. Figures in parentheses indicate percentage inhibition. †$P < 0{\cdot}01$ compared with normal group (Student's *t*-test). *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ compared with control (Dunnett's one-tailed test). Normal group: no hepatic injury induced. Control group: hepatic injury induced without ZNC-2381 pretreatment.

Table 3. Effects of ZNC-2381 on phosphodiesterase activity and lipopolysaccharide-induced tumour necrosis factor-α production in cultured *Propionibacterium acnes*-primed macrophages.

Treatment	Dose (μM)	Tumour necrosis factor-α (pg mL^{-1})	Phosphodiesterase (% inhibition)	
			III	IV
Spontaneous[a]	–	2·0 ± 2·0	–	–
Control	–	970·7 ± 49·3	–	–
ZNC-2381	0·1	576·5 ± 63·7* (41)	−8	11
	1	307·1 ± 65·4* (68)	–	–
	10	–	−1	30

The tumour necrosis factor-α content of the culture medium was measured 3 h after addition of lipopolysaccharide (100 ng mL^{-1}) and ZNC-2381 to macrophages harvested 7 days after injection of *Propionibacterium acnes* into mice. The results are presented as means ± s.e.m., n = 4. The PDE activity assay used platelets and U937 cells from man as sources of phosphodiesterases III and IV, respectively. Results are presented as the means from duplicate experiments. Figures in parentheses indicate percentage inhibition. *$P < 0.01$ compared with control (Dunnett's one-tailed test).[a]Spontaneous: spontaneous tumour necrosis factor-α production in unstimulated macrophages. Control: no ZNC-2381 added to the culture.

1996). These findings suggest that inhibition of IFN-γ levels by ZNC-2381 might also be related to the prevention of concanavalin A-induced DNA fragmentation in the liver.

In conclusion, we have shown that oral ZNC-2381 inhibits cytokine (TNF-α, IFN-γ) production and prevents hepatocellular apoptosis and hepatic injury after the administration of concanavalin A. The mechanism underlying these effects seems to involve both inhibition of cytokine production and a direct effect on the hepatocellular apoptosis signal pathway.

References

Aggarwal, B. B., Eessalu, T. E., Hass, P. E. (1985) Characterization of receptors for tumour necrosis factor and their regulation by γ-interferon. Nature 318: 665–667

Ando, K., Moriyama, T., Guidotti, L. G., Wirth, S., Schreiber, R. D., Schlicht, H. J., Huang, S., Chisari, F. V. (1993) Mechanisms of class I restricted immunopathology. A transgenic mouse model of fulminant hepatitis. J. Exp. Med. 178: 1541–1554

Chomczynski, P., Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162: 156–159

Cortijo, J., Bou, J., Beleta, J., Cardelus, I., Llenas, J., Morcillo, E., Gristwood, R. W. (1993) Investigation into the role of phosphodiesterase IV in bronchorelaxation, including studies with human bronchus. Br. J. Pharmacol. 108: 562–568

Eban, E., Ruther, E. (1985) Animal trial prediction and clinical effect of new chemical substances to be used as antipsychotic agents. Pharmacopsychiatry 18: 69–70

Gantner, F., Leist, M., Lohse, A. W., Germann, P. G., Tiegs, G. (1995) Concanavalin A-induced T-cell-mediated hepatic injury in mice: the role of tumour necrosis factor. Hepatology 21: 190–198

Gantner, F., Kusters, S., Wendel, A., Hatzelmann, A., Schudt, C., Tiegs, G. (1997) Protection from T cell-mediated murine liver failure by phosphodiesterase inhibitors. J. Pharmacol. Exp. Ther. 280: 53–60

Itokazu, Y., Segawa, Y., Inoue, N., Omata, T. (1999) D-galactosamine-induced mouse hepatic apoptosis: possible involvement with tumour necrosis factor, but not with caspase-3 activity. Biol. Pharm. Bull. 22: 1127–1130

Kita, H., Moriyama, T., Kaneko, T., Harase, I., Nomura, M., Miura, H., Nakamura, I., Yazaki, Y., Imawari, M. (1993) HLA B44-restricted cytotoxic T lymphocytes recognizing an epitope on hepatitis C virus nucleocapsid protein. Hepatology 18: 1039–1044

Kusters, S., Gantner, F., Kunstle, G., Tiegs, G. (1996) Interferon gamma plays a critical role in T cell-dependent liver injury in mice initiated by concanavalin A. Gastroenterology 111: 462–471

Leist, M., Gantner, F., Bohlinger, I., Tiegs, G., Germann, P. G., Wendel, A. (1995) Tumour necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. Am. J. Pathol. 146: 1220–1234

Louis, H., Moine, O. L., Peny, M. O., Quertinmont, E., Fokan, D., Goldman, M., Deviere, J. (1997a) Production and role of interleukin-10 in concanavalin A-induced hepatitis in mice. Hepatology 25: 1382–1389

Louis, H., Moine, O. L., Peny, M. O., Gulbis, B., Nisol, F., Goldman, M., Deviere, J. (1997b) Hepatoprotective role of interleukin 10 in galactosamine/lipopolysaccharide mouse liver injury. Gastroenterology 112: 935–942

Lowe, J. A., Cheng, J. B. (1992) The PDE IV family of calcium-independent phosphodiesterase enzymes. Drugs Fut. 17: 799–807

Mizuhara, H., Kuno, M., Seki, N., Yu, W. G., Yamaoka, M., Yamashita, M., Ogawa, T., Kaneda, K., Fujii, T., Senoh, H., Fujiwara, H. (1998) Strain difference in the induction of T-cell activation-associated, interferon gamma-dependent hepatic injury in mice. Hepatology 27: 513–519

Morita, M., Watanabe, Y., Akaike, T. (1995) Protective effect of hepatocyte growth factor on interferon-gamma-induced cytotoxicity in mouse hepatocytes. Hepatology 21: 1585–1593

Nagakawa, J., Hishinuma, I., Hirota, K., Miyamoto, K., Yamanaka, T., Tsukidate, K., Katayama, K., Yamatsu, I. (1990) Involvement of tumour necrosis factor-α in the pathogenesis of activated macrophage-mediated hepatitis in mice. Gastroenterology 99: 758–765

Nagakawa, J., Hishinuma, I., Miyamoto, K., Hirota, K., Abe, S., Yamanaka, T., Katayama, K., Yamatsu, I. (1992) Protective effects of (2*E*)-3-[5-(2,3-dimethoxy-6-methyl-1,4-benzoquinoyl)]-2-nonyl-2-propenoic acid on endotoxin-mediated hepatitis in mice. J. Pharmacol. Exp. Ther. 262: 145–150

Nagakawa, J., Hirota, K., Hishinuma, I., Miyamoto, K., Sonoda, J., Yamanaka, T., Katayama, K., Yamatsu, I. (1993) Protective effect of E3330, a novel quinone derivative, in galactosamine-induced hepatitis in rats. J. Pharmacol. Exp. Ther. 264: 496–500

Rehermann, B., Fowler, P., Sidney, J., Person, J., Redeker, A., Brown, M., Moss, B., Sette, A., Chisari, F. V. (1995) The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J. Exp. Med. 181: 1047–1058

Rodriguez, I., Matsuura, K., Ody, C., Nagata, S., Vassalli, P. (1996) Systemic injection of a tripeptide inhibits the intracellular activation of CPP32-like proteases in vivo and fully protects mice against Fas-mediated fulminant liver destruction and death. J. Exp. Med. 184: 2067–2072

Stachlewitz, R. F., Seabra, V., Bradford, B., Bradham, C. A., Rusyn, I., Germolec, D., Thurman, R. G. (1999) Glycine and uridine prevent D-galactosamine hepatotoxicity in the rat: role of Kupffer cells. Hepatology 29: 737–745

Tiegs, G. (1997) Experimental hepatitis and role of cytokines. Acta. Gastroenterol. Belg. 60: 176–179

Tiegs, G., Wolter, M., Wendel, A. (1989) Tumour necrosis factor is a terminal mediator in galactosamine/endotoxin-induced hepatitis in mice. Biochem. Pharmacol. 38: 627–631

Tiegs, G., Hentschel, J., Wendel, A. (1992) A T cell-dependent experimental liver injury in mice inducible by concanavalin A. J. Clin. Invest. 90: 196–203

Trautwein, C., Rakemann, T., Brenner, D. A., Streetz, K., Licato, L., Manns, M. P., Tiegs, G. (1998) Concanavalin A-induced liver cell damage: activation of intracellular pathways triggered by tumour necrosis factor in mice. Gastroenterology 114: 1035–1045