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Fumigaclavine C improves concanavalin A-induced liver injury in mice mainly via inhibiting TNF-α production and lymphocyte adhesion to extracellular matrices

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Abstract

Fumigaclavine C, an alkaloidal metabolite, was produced by *Aspergillus fumigatus* (strain No. CY018). This study examined the effect of this compound on concanavalin A (Con A)-induced liver injury in mice, a T cell-dependent model of liver damage. Con A administration resulted in severe liver injury, T lymphocyte activation and a strong increment in spleen cell adhesion, as well as in tumour necrosis factor-α (TNF-α) production. Against this liver injury, the intraperitoneal administration of fumigaclavine C dose-dependently inhibited the elevation in transaminase activity, TNF-α production in serum and the histological changes, including inflammatory infiltration, hepatocyte necrosis and degeneration and Kupffer cell hyperplasia. In addition, this compound in-vitro also inhibited the proliferation of spleen cells induced by Con A, and reduced their IL-2 and TNF-α production. Moreover, the intraperitoneal administration of fumigaclavine C inhibited the potential of spleen cells isolated from the liver-injured mice to adhere to fibronectin, laminin and type IV collagen. These results suggest that the improvement of this T cell-mediated liver injury by fumigaclavine C may be related to the inhibition of lymphocyte activation, proliferation and adhesion to extracellular matrices as well as the reduction in TNF-α production.

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Introduction

Concanavalin A (ConA)-induced liver injury, as a T cell-dependent model of liver damage (Tiegs et al 1992), has been regarded as an appropriate model of human liver diseases in many respects. Specifically, the cell types involved in the initiation and propagation of Con A-induced hepatitis (i.e., $CD4^+$ T cells, NKT cells, neutrophils, Kupffer cells) have been directly implicated in a number of liver diseases in man, including autoimmune, viral and alcohol hepatitis (Dienes et al 1987; Schumann et al 2000; Jaeschke 2002). This liver injury is thought to be mediated primarily by tumour necrosis factor-α (TNF-α) and could be prevented by the use of polyclonal TNF-α antiserum (Mizuhara et al 1994; Gantner et al 1995). In addition, clinical and experimental research has demonstrated that the adhesion of lymphocytes to extracellular matrix (ECM) is indispensable in lymphocytes migration and inhibition of the adhesion may lead to an alleviation of various inflammatory disorders (Shimizu & Shaw 1990; Shirin et al 1998). These findings suggest that inhibition of TNF-α production and lymphocyte adhesion may be a useful approach to the treatment of T cell-mediated liver diseases.

On the other hand, endophytes have been found to be a rich source of functional biomolecules with a diversity of biological actions as discerned with a limited amount of endophytic secondary metabolites (Tan & Zou 2001). In continuation of our characterization of biologically active and structurally novel metabolites from endophytic fungal cultures (Lu et al 2000; Zou et al 2000; Liu et al 2002, 2003), fumigaclavine C, an ergot alkaloid, was isolated from a column chromatography fraction derived from the ethyl acetate extract of a culture of *Aspergillus fumigatus* (strain no. CY018), an endophytic fungus isolated from the elder leaf of a salinity-tolerant medicinal plant

Cynodon dactylon (Gramineae) and identified by a combination of spectroscopic methods (Cole et al 1977). Despite the fact that this compound was reported as early as 1977 (Cole et al 1977), its biological activity is still rarely reported. Therefore, this study aimed to examine the effect of fumigaclavine C on the liver injury induced by Con A in mice and give a primary understanding of its characteristics in the aspect of T cell proliferation, adhesion and TNF-α production.

Materials and Methods

Drugs and reagents

The following drugs and reagents were used: fumigaclavine C (Figure 1, more than 90% of purity); ciclosporin (Sandoz Ltd, Basel, Switzerland); concanavalin A (Con A; Sigma, USA); kits for determining serum alanine transaminase (ALT), aspartate transaminase (AST) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); bovine serum albumin (BSA, Sigma); type IV collagen (Fluka, Chemie Gmbh); fibronectin (Sigma); laminin (CalBiochem, USA); 3-(4,5-dimethyl-2-thiazol)-2, 5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma); ELISA kits for TNF-α and IL-2 (Jingmei Biotech Co. Ltd, Shengzhen, China).

Animals

Male and female ICR mice, 18–20 g, 5–6 weeks old, were obtained from the Experimental Animal Center of Nanjing Medical University (Nanjing, China). They were maintained in plastic cages at 21 ± 2°C with free access to pellet food and water and kept on a 12-h light–dark cycle. This study complied with current ethical regulations on animal research in our university, and all mice used in the experiments received humane care.

Cultivation of the endophyte

The endophytic fungal strain CY018 was separated from the healthy leaves of *Cynodon dactylon* according to the procedure of Liu et al (2001). Furthermore, the endophytic nature of the isolated strains was robusted with the vitality test as described earlier (Lu et al 2000). The purified endophytic fungi were numbered and transferred to fresh potato carrot agar (PCA) medium separately and were kept at 4°C after being cultured at 28 ± 1°C for 7 days. The inoculum was prepared by introducing the periphery of 7-day-old Petri-dish cultures of the title endophyte into 1000-mL flasks containing 400 mL potato dextrose agar (PDA) broth, followed by shaking continuously (150 rev min^{-1}) for 4 days at 28 ± 1°C. The subsequent solid-matrix steady fermentation was accomplished by adding 15 mL inoculum into 250-mL flasks each containing 15 g millet, 0.5 g yeast extract, 0.1 g tartrate sodium, 0.1 g glutamine sodium, 0.01 g copperas and 0.1 mL pure corn oil at 28 ± 1°C for 35 days.

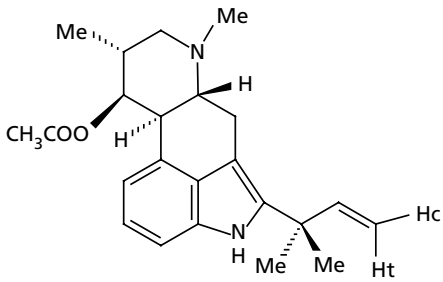

Figure 1 Chemical structure of fumigaclavine C.

Extraction, isolation and identification of fumigaclavine C

The afforded cultivation product (5.3 kg) was collected and extracted with 15 L methanol three times at room temperature. Evaporation of the solvent in-vacuo gave a brown oily residue (314 g), which was suspended in water (1000 mL). The suspension was extracted successively with ethyl acetate (EtOAc) (1000 mL × 3) and *n*-butanol (1000 mL × 3). Concentration of the EtOAc fraction in-vacuo gave a brown bioactive residue (153 g), which was subjected to chromatography on a silica gel column (1000 g, 200–300 mesh) eluting with a chloroform–methanol gradient (1:0, 6 L; 100:1, 7 L; 50:1, 8 L; 20:1, 6L; 10:1, 6L; 5:1, 6L; 0:1, 5L) to give five fractions (F-1, 103.5 g; F-2, 10.1 g; F-3, 12.8 g; F-4, 10.5 g; F-5, 15.0 g). F-2 was chromatographed further over silica gel (150 g, 200 ~ 300 mesh) using chloroform (CH_3Cl)–methanol (MeOH) mixtures of a growing polarity (200:1, 100:1, 50:1, 20:1, 10:1, each 1.2 L) to afford white needles (280 mg), which dissolved easily in $CHCl_3$ and were insoluble in H_2O. This metabolite was identified to be fumigaclavine C by comparison of the data of MS, ^{1}H NMR, ^{13}C NMR and optical rotation value with the literature (Cole et al 1977; Cole & Cox 1981).

Induction of Con A-induced liver injury

Considering the high mortality of Con A-induced liver injury and the good effect of fumigaclavine C and ciclosporin in our preliminary experiment, the size of each group was 8–10, except for 20 in the group only treated with Con A. Acute liver injury was induced by injecting mice with 18 mg kg^{-1} (body weight) of Con A in 200 μL phosphate-buffered saline (PBS) via the tail vein. Eight hours after Con A challenge (Schumann et al 2003), the mice were exsanguinated from the suborbital vein and the serum was collected for assaying ALT and AST activity by using the commercial kits as the protocols indicated. For histological examinations, mice were sacrificed and their abdomens were opened by a midline incision and portions from the right liver lobe were excised, fixed in 4% paraformaldehyde and embedded in paraffin. The tissue sections (5 μm) were stained with haematoxylin–eosin

and read on a 0 to 3 scale (0, no change; 1, mild; 2, moderate; and 3, severe) by a pathologist who had no knowledge of the induction of liver injury or other experimental data.

Preparation of splenocyte suspension

The spleen was aseptically taken from mice, crushed gently and separated into single cells by squeezing in 5 mL D-Hank's solution (GIBCO BRL). The cells obtained were passed through eight-layers of gauze and centrifuged at 1000 rev min^{-1} for 5 min at 4 °C. Pellets were added into 10 mL of sterile 0.17 M Tris (hydroxymethyl aminomethane)–0.75% NH_4Cl (pH 7.5) followed by centrifugation to remove erythrocytes. After washing twice with RPMI 1640 containing 100 U mL^{-1} of penicillin, 100 U mL^{-1} of streptomycin and 10% fetal calf serum (FCS) (RPMI 1640 medium), they were re-suspended in the RPMI 1640 medium and used for cell culture.

Proliferation assay of spleen cells

Spleen cells were cultured in 96-well flat-bottom microplates (Falcon) at a density of 5×10^5 cells/well in RPMI 1640 medium (0.2 mL) and stimulated with 5 μg mL^{-1} of Con A for 24 h and 72 h at 37 °C in 5% CO_2–air in the presence or absence of fumigaclavine C. Then the cell growth was evaluated with modified MTT assay (Sargent & Taylor 1989). Briefly, 20 μL of 5 mg mL^{-1} MTT in RPMI 1640 were added for a further 4-h incubation. After removing the supernatant, 200 μL of dimethyl sulfoxide (DMSO) were added to dissolve the formazan crystals. The plate was shaken for 10 min, and then read on an ELISA reader (Sunrise Remote/Touch Screen; TECAN, Austria) at 540 nm.

TNF-α and IL-2 assays

Spleen cells isolated from normal mice were suspended in RPMI 1640 medium at a density of 5×10^6/mL. Portions (0.1 mL) were seeded onto 96-well flat-bottom microplates and co-cultured with 5 μg mL^{-1} Con A in the presence or absence of fumigaclavine C at 37 °C in 5% CO_2–air. After 24 h, the supernatants were aspirated and stored at −20 °C until the ELISA assay. TNF-α in plasma 2 h after Con A inoculation together with IL-2 and TNF-α in the supernatant were assayed by ELISA kits as described by the manufacturer. In brief, standards and samples were added to the wells coated with immobilized monoclonal antibody to TNF-α or IL-2. After washing away any unbound substances, the anti-TNF-α or anti-IL-2 antibodies that were labelled with biotin and streptavidin conjugated to horseradish peroxidase were added in turn with a wash after each binding. Then a substrate solution consisting of stabilized hydrogen peroxide and tetramethylbenzidine was added and colour developed in proportion to the amount TNF-α or IL-2 bound in the initial step. The reaction was stopped by addition of 2 M sulfuric acid and optical density was read with an ELISA reader at 450 nm. The concentration of TNF-α or IL-2 in a sample was determined by interpolation from a standard curve, prepared with standard samples supplied by the manufacturer. The threshold of detection was 10 pg mL^{-1} for IL-2 and 26 pg mL^{-1} for TNF-α, while the standard curve's range was from 0 to 1000 pg mL^{-1} for IL-2 and from 0 to 2000 pg mL^{-1} for TNF-α, respectively.

Adhesion assay

Cell adhesion to ECM glycoproteins was performed essentially as previously described (Arai et al 1999) with some modifications. In brief, 96-well flat-bottom microplates were coated with 50 μL of fibronectin (10 μg mL^{-1}), laminin (10 μg mL^{-1}) or type IV collagen (50 μg mL^{-1}) in PBS overnight at 4 °C. Nonspecific binding sites were blocked with 0.2% BSA for 2 h at room temperature followed by washing three times with RPMI 1640. Spleen cells (5×10^5/well) suspended in RPMI 1640 were allowed to adhere in the microplate at 37 °C for 1 h. After adhesion, non-adherent cells were removed by washing three times with RPMI 1640. Then the cells remaining attached to the plates were quantified with MTT assay (Konrad et al 2000). All assays were run in triplicate and the results were expressed as percentage of bound cells. The absorbance of 5×10^5 cells added without previous washing was considered as 100% cell adhesion. The specificity of each cell adhesion assay was corroborated using BSA as substratum. In some cases, the effect of drug on the adhesion was expressed as a percent inhibition against the control without drug treatment, (bound $cells_{control}$ − bound $cells_{drug}$)/bound $cells_{control}$.

Statistical analysis

Data were expressed as mean ± s.e.m. Statistical analysis was evaluated by one-way analysis of variance, followed by Student's two-tailed t-test for the evaluation of the difference between two groups and Dunnett's t-test between control group and multiple dose groups. One-way analysis of variance revealed a significant effect at $P < 0.05$. Furthermore, the histopathological changes were expressed as median and range, and were analysed by the Kruskal–Wallis test, followed by post-hoc test (Rank Sums Test) for multiple comparisons, with the level of significance chosen as $P < 0.05$.

Results

Fumigaclavine C improves Con A-induced liver injury in mice

Fumigaclavine C (5, 10 and 20 mg kg^{-1} in PBS), ciclosporin (10 mg kg^{-1} in PBS) and PBS were administered intraperitoneally to fumigaclavine C groups (10 mice/group), the ciclosporin group (8 mice) and control group (20 mice) 3 times at an interval of 6 h. One hour after the final administration, 18 mg kg^{-1} of Con A was injected intravenously to induce the liver damage. Marked

elevation in serum ALT and AST activity associated with high mortality was observed in control mice 8 h after Con A injection (Table 1). Compared with the control, fumigaclavine C dose-dependently reduced ALT and AST levels and increased the survival rate of mice. Doses of 10 and 20 mg kg^{-1} of fumigaclavine C showed the same intensity of inhibition on the liver injury as did 10 mg kg^{-1} of ciclosporin. In addition, fumigaclavine C itself had no toxicity to the liver of mice, even at 40 mg kg^{-1} (Table 1).

Histological examination of liver sections from fumigaclavine C-treated mice without Con A inoculation showed no difference to those from naive mice (Figure 2A–C and Table 2), while marked inflammatory infiltration, severe hepatocyte degeneration, hepatocyte necrosis and Kupffer cell hyperplasia were observed in the surviving mice, as detailed in Table 1, treated with Con A without medication in (Figure 2E, Table 2). Pre-treatment with fumigaclavine C dose-dependently reduced the extent of liver damage (Table 2) and livers showed near normal histology at high doses (Figure 2F–H, Table 2). The improvement in ciclosporin-treated mice was also significant (Figure 2D, Table 2).

Fumigaclavine C prevented the increase in spleen weights of mice induced by Con A (Table 3). Fumigaclavine C pre-treatment significantly inhibited this increase induced by Con A in a dose-dependent manner without influencing the body weights. Ciclosporin also significantly inhibited the increase in spleen weights. Fumigaclavine C, at a dose of 40 mg kg^{-1}, however, itself showed a slight tendency to decrease the spleen weights.

Fumigaclavine C inhibits the proliferation and IL-2 production of spleen cells induced by Con A in-vitro

Fumigaclavine C, when added simultaneously with Con A, dose-dependently inhibited the 24-h (Figure 3) and 72-h proliferation (data not shown) of spleen cells. At the same time, the increased production of IL-2 in the supernatant was also reduced by this compound. However, treatment for 24 h with this compound, at 10^{-7} to 10^{-5} g mL^{-1}, did not show any influence on the viability of the naive spleen cells (0.539 in OD_{540} at 10^{-5} g mL^{-1} of fumigaclavine C against 0.542 for medium), as well as of the spleen cells that had been activated for 48 h with Con A (0.456 in OD_{540} at 10^{-5} g mL^{-1} of fumigaclavine C against 0.450 for Con A).

The intraperitoneal administration of fumigaclavine C inhibits the ability of spleen cells from Con A-injected mice to adhere to fibronectin, laminin and type IV collagen

Spleen cells isolated from mice 2 h after Con A administration, which showed the highest adhesion activity (Yuan et al 2003), were allowed to adhere to fibronectin, laminin and type IV collagen for 1 h. As shown in Figure 4, the bound cells from the mice with Con A hepatitis remarkably increased as compared with those from naive mice. Compared with this increase, the intraperitoneal administration of fumigaclavine C significantly decreased the cells' ability to adhere to fibronectin, laminin and type IV collagen in a dose-dependent manner. The degree of inhibition of adherence at 20 mg kg^{-1} was 40.2% to fibronectin, 27.2% to laminin and 38.0% to type IV collagen.

Fumigaclavine C inhibits TNF-α production in-vivo and in-vitro

Con A injection significantly increased plasma TNF-α levels in mice 2 h later. In contrast to this marked elevation, fumigaclavine C pre-treatment dose-dependently caused a significant reduction in TNF-α production (Figure 5A).

Similarly, a significant increase in TNF-α level was seen in the supernatant of spleen cells activated by Con A for 24 h. Against this increase, fumigaclavine C showed a dose-dependent reduction (Figure 5B).

Table 1 Effect of fumigaclavine C on changes in serum transaminase activity and survival in mice with Con A-induced liver injury.

Group	Dose (mg kg^{-1})	No. of mice	No. of survivors	ALT (Karmen unit)	AST (Karmen unit)	Survival rate (%)
Naive	—	8	8	5.1 ± 2.8	88.8 ± 11.6	100
Fumigaclavine C	20	8	8	6.3 ± 2.9	118.5 ± 15.3	100
	40	8	8	10.4 ± 2.5	124.6 ± 8.4	100
Con A	18	20	9	6288 ± 545$^{\#\#}$	8632 ± 747$^{\#\#}$	45
Con A + Fumigaclavine C	5	10	9	3387 ± 1041*	4213 ± 879*	90
	10	10	10	1078 ± 519**	1423 ± 445**	100
	20	10	10	447 ± 271**	921 ± 328**	100
Con A + ciclosporin	10	8	8	650 ± 501**	1509 ± 841**	100

Mice were administered intraperitoneally with fumigaclavine C (5, 10, 20 or 40 mg kg^{-1}) or ciclosporin (10 mg kg^{-1}) in 200 μL PBS three times at an interval of 6 h. One hour after the final administration, they were injected intravenously with 18 mg kg^{-1} of Con A, followed 8 h later by bleeding. The serum was used for the measurement of ALT and AST. Values are means ± s.e.m. of mice surviving in each group. $^{\#\#}P < 0.01$ vs naive (one-way analysis of variance, Student's two-tailed t-test); $*P < 0.05$, $**P < 0.01$ vs Con A (one-way analysis of variance, Dunnett's t-test).

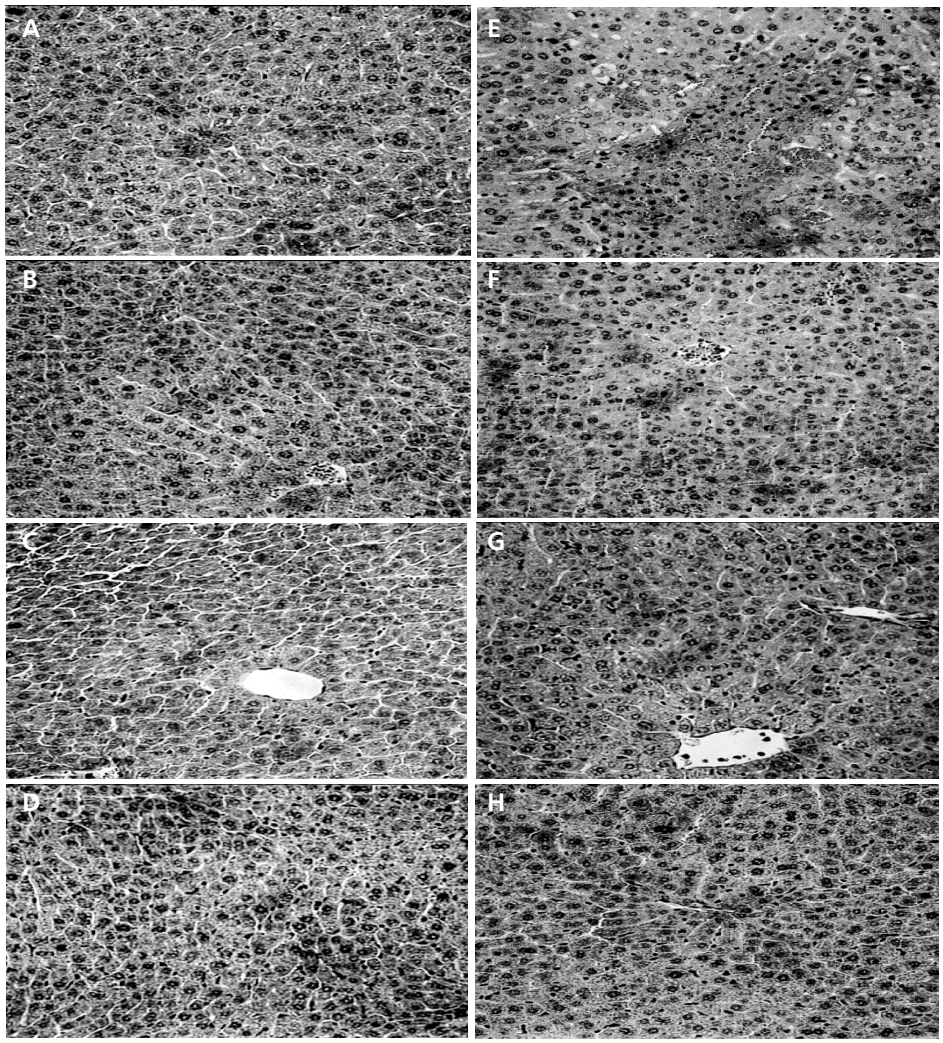

Figure 2 Effect of fumigaclavine C on the histopathological changes in the livers of mice with Con A-induced liver injury. Panels show liver from naive mouse (A); liver from mouse administered with 20 and 40 mg kg^{-1} of fumigaclavine C, respectively, three times at an interval of 6 h without Con A injection (B and C); liver from mouse treated with 10 mg kg^{-1} of ciclosporin 3 times at an interval of 6 h before Con A injection (D); liver from mouse treated with 18 mg kg^{-1} of Con A only (E); liver from mouse treated, respectively, with 5, 10, 20 mg kg^{-1} of fumigaclavine C 3 times at an interval of 6 h before Con A injection (F–H). (original magnification ×100).

Table 2 Effect of fumigaclavine C on the liver histopathological changes in mice with Con A-induced liver injury.

Group	Dose (mg kg^{-1})	No. of mice	Hepatocyte necrosis	Hepatocyte degeneration	Inflammatory infiltration	Kupffer cell hyperplasia
Naive	—	8	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Fumigaclavine C	20	8	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
	40	8	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Con A	18	9	2 (1–2)$^{\#\#}$	3 (1–3)$^{\#\#}$	2 (1–3)$^{\#\#}$	2 (1–2)$^{\#\#}$
Con A + Fumigaclavine C	5	9	1 (0–2)*	2 (1–3)	1 (1–2)	1 (1–2)*
	10	10	0 (0–1)**†	1 (0–2)**†	1 (1–2)*	1 (1–2)**
	20	10	0 (0–1)**†	1 (0–1)**†	1 (0–2)**	1 (0–1)**
Con A + Ciclosporin	10	8	0 (0–2)**	1 (0–3)*	1 (1–2)*	1 (0–2)**

Mice were treated as described in Table 1. The liver tissue sections were stained with haematoxylin–eosin. The histological changes were read on a scale of 0–3 (0, no change; 1, mild; 2, moderate; 3, severe). Each value indicates median (range) of 8–10 mice surviving as shown in Table 1. $^{\#\#}P < 0.01$ vs naive (Kruskal–Wallis test); $^{*}P < 0.05$, $^{**}P < 0.01$ vs Con A; $^{\dagger}P < 0.05$ vs Con A + fumigaclavine C (5 mg kg^{-1}) (post-hoc test).

Table 3 Effect of fumigaclavine C on changes in spleen weights of mice with Con A-induced liver injury.

Group	Dose (mg kg^{-1})	No. of mice	Body weights (g)	Spleen weights (mg)	Spleen weights/body weights (mg/g)
Naive	—	8	20.9 ± 0.4	80.4 ± 3.0	3.85 ± 0.06
Fumigaclavine C	20	8	20.6 ± 0.5	80.2 ± 5.4	3.89 ± 0.48
	40	8	20.3 ± 0.4	66.1 ± 7.6	3.24 ± 0.26
Con A	18	9	20.0 ± 0.4	210.9 ± 20.9$^{\#\#}$	10.51 ± 0.65$^{\#\#}$
Con A + fumigaclavine C	5	9	20.9 ± 0.6	162.7 ± 19.7*	7.82 ± 0.77*
	10	10	20.6 ± 0.4	121.4 ± 11.6**	5.98 ± 0.31**
	20	10	20.8 ± 0.5	111.6 ± 6.7**	5.35 ± 0.24**
Con A + ciclosporin	10	8	20.5 ± 0.5	124.4 ± 9.3**	6.10 ± 0.45**

Mice were treated as described in Table 1. Eight hours after the injection of Con A, the surviving mice were weighed. After bleeding, their spleens were then taken out and weighed. Each value indicates the mean ± s.e.m. of 8–10 mice surviving as shown in Table 1. $^{\#\#}P < 0.01$ vs naive (one-way analysis of variance, Student's two-tailed *t*-test); $^{*}P < 0.05$, $^{**}P < 0.01$ vs Con A (one-way analysis of variance, Dunnett's *t*-test).

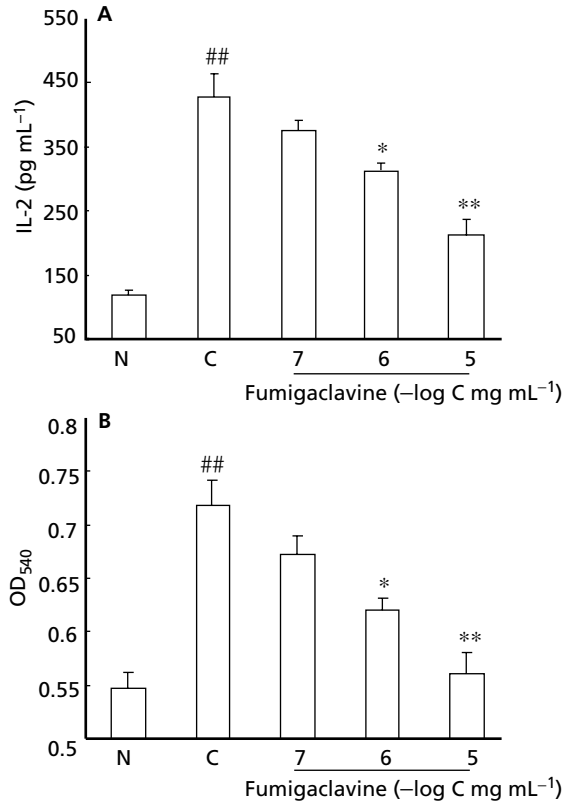

Figure 3 Effect of fumigaclavine C on the proliferation and IL-2 production of mouse spleen cells induced by Con A in-vitro. Spleen cells isolated from naive mice were cultured with 5 μg mL^{-1} of Con A for 24 h in the presence or absence of fumigaclavine C. IL-2 levels in the culture supernatant were measured by ELISA (A). At the same time, MTT assay was used for measuring the cell viability (B). Each column represents the mean ± s.e.m. of three experiments using 3 mice with triplicate sets in each assay. N, cells from naive mice; C, Con A-activated spleen cells. $^{\#\#}P < 0.01$ vs N (one-way analysis of variance, Student's two-tailed *t*-test); $^{*}P < 0.05$, $^{**}P < 0.01$ vs C (one-way analysis of variance, Dunnett's *t*-test).

Discussion

Con A-induced hepatitis in the mouse is a well-characterized model of T cell-mediated liver disease. In this study, we examined the effect of fumigaclavine C, an ergot alkaloid, on Con A-induced liver injury. As a result, the intraperitoneal administration of fumigaclavine C, at doses of 5–20 mg kg^{-1}, significantly reduced the serum ALT and AST levels and raised the survival rate against Con A-induced liver damage (Table 1). The pathological observation also showed that the liver damage was improved by fumigaclavine C, especially at doses of 10 and 20 mg kg^{-1} (Figure 2, Table 2).

As to the pathogenesis of Con A-induced liver damage, there is increasing evidence that many cell types (T lymphocytes, macrophages, neutrophils, eosinophils and NKT cells) are involved in this liver damage (Mizuhara et al 1994; Schumann et al 2000; Ajuebor et al 2003; Bonder et al 2004). Among them, the activation and infiltration of T lymphocytes into the liver are critical in this model as shown by the resistance of severe combined immunodeficiency disorder mice or mice pre-treated with a T cell-specific immunosuppressive drug, FK 506 or ciclosporin (Tiegs et al 1992; Mizuhara et al 1994; Santucci et al 2000). In an attempt to elucidate the protective mechanism of fumigaclavine C, we first found that it could dose-dependently reduce the increase in spleen weights in Con A-injected mice in-vivo (Table 3), and then examined its effect on T lymphocyte activation and proliferation in-vitro. Fumigaclavine C, when co-cultured with spleen cells for 24 h from the beginning of the cultivation, not only inhibited Con A-induced lymphocyte proliferation, but also reduced IL-2 production (Figure 3). However, when added 48 h after the incubation of spleen cells with Con A, it exerted no inhibitory or cytotoxic effect on the activated spleen cells. These results suggested that fumigaclavine C may mainly act at the early stage of T lymphocyte activation. It should be

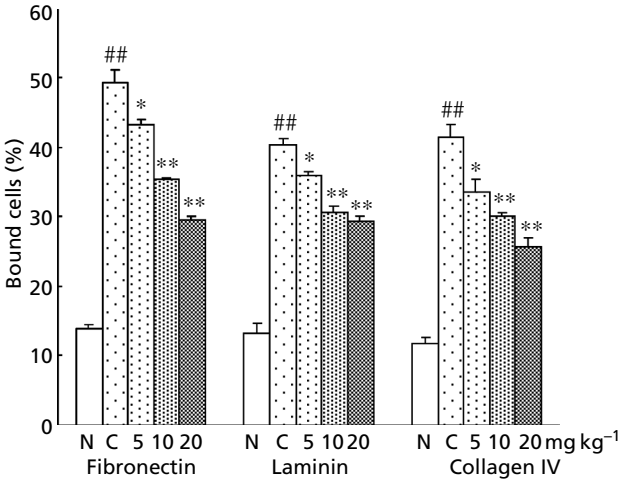

Figure 4 In-vivo effect of fumigaclavine C on the adhesion of mouse spleen cells to fibronectin, laminin and collagen IV. Mice were treated as described in Table 1. Spleen cells were isolated from naive mice or from mice 2 h after Con A injection, and 5×10^5 of the cells were applied to the adhesion assay for 1 h. Data are expressed as mean ± s.e.m. of three experiments using 3 mice and each assay was performed in triplicate. N, cells from naive mice; C, cells from Con A-treated mice without medication. $^{\#\#}P < 0.01$ vs N (one-way analysis of variance, Student's two-tailed *t*-test); $^{*}P < 0.05$, $^{**}P < 0.01$ vs C (one-way analysis of variance, Dunnett's *t*-test).

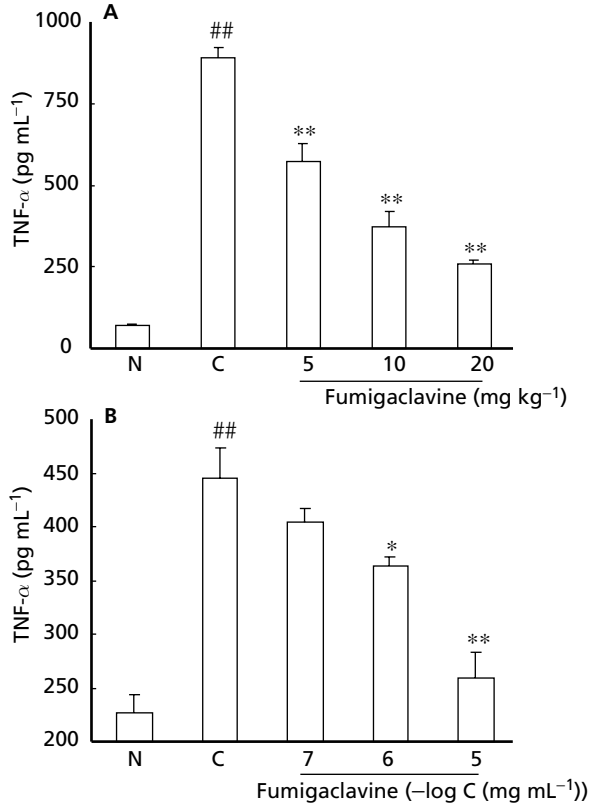

Figure 5 Effect of fumigaclavine C on the TNF-α level in serum and in culture supernatant of mouse spleen cells activated by Con A for 24 h. Mice were treated as described in Table 1. Two hours after Con A injection, mice were bled. Spleen cells isolated from naive mice were cultured with 5 μg mL^{-1} of Con A for 24 h in the presence or absence of fumigaclavine C. TNF-α levels in serum (A) and in culture supernatant were measured by ELISA (B). Each column represents the mean ± s.e.m. of eight mice (A) or three experiments using 3 mice with triplicate sets in each assay (B), respectively. N, Normal mice (A) or cells from naive mice (B); C, control mice without medication (A) or Con A-activated spleen cells (B). $^{\#\#}P < 0.01$ vs N (one-way analysis of variance, Student's two-tailed *t*-test); $^{*}P < 0.05$, $^{**}P < 0.01$ vs C (one-way analysis of variance, Dunnett's *t*-test).

noted that fumigaclavine C influenced neither the body weights of mice in-vivo (Table 3) nor the naive spleen cells without activation in-vitro, suggesting that it may have low side effects when used for the treatment of liver diseases.

The localization of lymphocytes to the inflammatory site is a complicated coordinated multi-step process. Cell adhesion to ECM glycoproteins is a prerequisite for their subsequent homing to the inflammatory sites (Chalupny et al 1992; Shirin et al 1998). Immune cell–ECM interactions dictate the subsequent recognition and activation, proliferation, differentiation and secretion of cytokines by the immune cells, as well as affecting the recruitment of additional immune cells to inflammatory loci (Nathan et al 1989; Shimizu & Shaw 1990). To find out how fumigaclavine C affects the inflammatory infiltration, as observed in histological examinations (Figure 2, Table 2), we next examined the effect of fumigaclavine C on the immune cell–ECM interaction. We found that the intraperitoneal administration of fumigaclavine C dose-dependently inhibited the adhesion of spleen cells to laminin, fibronectin and type IV collagen (Figure 4). Such reduction in adhesion ability by fumigaclavine C might be related to its inhibition on Con A-induced T lymphocytes activation and proliferation, and may contribute to the protection against the liver injury induced by Con A.

On the other hand, TNF-α also plays a critical role in Con A-induced liver damage, as TNF-α immunoneutralization or gene ablation (Mizuhara et al 1994; Gantner et al 1995; Nakamura et al 2001) conferred protection against the liver damage. As shown in Figure 5, pre-treating mice with fumigaclavine C significantly decreased the TNF-α levels in plasma, which reached a peak in serum 2 h after Con A injection and decreased rapidly thereafter as reported by Santucci et al (2000), and this compound in-vitro also inhibited TNF-α production in the supernatant of spleen cells incubated with Con A for 24 h. The reduction in TNF-α may lead to the inhibition of inflammatory infiltration, as TNF-α can up-regulate cell surface adhesion molecules to promote lymphocyte adhesion and transmigration (Vassalli 1992; Hershkoviz et al 1994; Springer 1994), and induce macrophage inflammatory protein-2 (MIP-2), a chemokine, to facilitate the recruitment of neutrophils (Nakamura et al 2001). Furthermore, neutrophils, the major cell type recruited to the liver within 4 h after Con A administration, are also critical in the initiation of T lymphocyte recruitment in this liver

damage (Bonder et al 2004). Therefore, the inhibition of TNF-α by fumigaclavine C at 2 h may confer the improvement of histological changes, which was confirmed in the surviving mice (Table 2, Figure 2). In addition, since macrophage/monocyte is the major producer of TNF-α and neutrophils can also produce TNF-α in acute inflammation (Bonder et al 2004), we cannot exclude some possible effects of fumigaclavine C on these cell types. The detailed mechanism is now under study.

Conclusions

Our results indicate that fumigaclavine C is useful for improving Con A-induced liver injury. Its mechanisms may include inhibition of lymphocyte activation, proliferation and adhesion to extracellular matrices, as well as reduction in TNF-α production.

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