Antifibrotic effects of tetrandrine on hepatic stellate cells and dimethylnitrosamine-intoxicated rats

Yi-Chao Hsu\textsuperscript{a}, Yung-Tsung Chiu\textsuperscript{b}, Ching-Chang Cheng\textsuperscript{b,c}, Yun-Lian Lin\textsuperscript{d}, Yi-Tsau Huang\textsuperscript{a}

\textsuperscript{a} Institute of Traditional Medicine, School of Medicine, National Yang-Ming University, Taipei,
\textsuperscript{b} Department of Medical Research and Education, Taichung Veterans General Hospital, Taichung,
\textsuperscript{c} Department of National Chung Hsing University College of Veterinary Medicine
\textsuperscript{d} National Research Institute of Chinese Medicine, Taipei,

\textit{Short title:} Tetrandrine reduced rat hepatic fibrosis \textit{in vitro} and \textit{in vivo}

\textit{Corresponding Author:} Yi-Tsau Huang, Institute of Traditional Medicine, School of Medicine, National Yang-Ming University, No. 155, Li-Nong Street, Sec. 2, Taipei 112, Taiwan. Tel. +886-2-28267179, Fax +886-2-28225044, E-Mail huangyt@ym.edu.tw
Abstract

Tetrandrine (C$_{38}$H$_{42}$O$_8$N$_2$, molecular weight: 622, Tet), an alkaloid isolated from the Chinese medicinal herb *Stephania tetrandra*, has been shown to elicit anti-inflammatory activity in pulmonary diseases and anti-fibrotic effects in various cell types. In this study, we investigated the *in vitro* and *in vivo* effects of Tet on hepatic fibrosis. A cell line of rat hepatic stellate cells (HSC-T6) was stimulated with transforming growth factor-$\beta$1 (TGF-$\beta$1) or tumor necrosis factor $\alpha$ (TNF-$\alpha$). The inhibitory effects of Tet (0.5 – 5.0 $\mu$M) on IKK$\alpha$ expression, I$\kappa$B$\alpha$ phosphorylation, NF$\kappa$B transcriptional activity, the mRNA expression of intercellular adhesion molecule (ICAM-1), and $\alpha$-smooth muscle actin ($\alpha$-SMA) secretion, were assessed. Fibrosis was induced by dimethylnitrosamine (DMN) administration in rats for 4 weeks. Fibrotic rats were randomly assigned to one of the four groups: vehicle (0.7% Carboxyl Methyl Cellulose, CMC), Tet (1 mg/kg), Tet (5 mg/kg), or silymarin (50 mg/kg), each given by gavage twice daily for 3 weeks starting after 1 week DMN pre-treatment. Tet (0.5 – 5.0 $\mu$M) concentration-dependently inhibited NF$\kappa$B transcriptional activity induced by TNF-$\alpha$. Tet reduced IKK$\alpha$ expression, TNF-$\alpha$-induced I$\kappa$B$\alpha$ phosphorylation, and attenuated TNF-$\alpha$-stimulated the mRNA expression level of ICAM-1 in HSC-T6 cells. In addition, Tet also inhibited TGF-$\beta$1-induced $\alpha$-SMA secretion and collagen deposition in HSC-T6 cells. Fibrosis scores of livers from DMN-treated rats with high dose of Tet (1.3 ± 0.3) were significantly reduced in comparison with DMN-treated rats receiving saline (2.0 ± 0.2). Hepatic collagen contents of DMN rats were significantly reduced by either Tet or silymarin treatment. Double staining results showed that $\alpha$-SMA positive cells with NF$\kappa$B activation were decreased in the fibrotic livers by Tet and silymarin treatments. The mRNA expression level of ICAM-1 was attenuated by Tet and silymarin treatment. In addition, the mRNA expression levels of $\alpha$-SMA, TGF-$\beta$1 were only attenuated by high dose of Tet, but not silymarin treatment. Moreover, levels of plasma AST and ALT activities were reduced by Tet and silymarin treatment. In conclusion, our results showed that Tet exerted anti-fibrotic effects in both HSC-T6 cells and in rats with DMN-induced fibrosis.

**Key Words:** Collagen, Hepatic fibrosis, NF$\kappa$B, $\alpha$-smooth muscle actin, tetrandrine, transforming growth factor-$\beta$1, tumor necrosis factor-$\alpha$. 

Abbreviations used: α-SMA, α-smooth muscle actin; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HSC, hepatic stellate cell; H$_2$O$_2$, hydrogen peroxide; ICAM-1, intercellular adhesion molecule 1; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; NAC, N-acetylcysteine; PDTC, pyrrolidinedithiocarbamate; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; TGF-β1, transforming growth factor-β1; TIMP-1, tissue inhibitor of metalloproteinase-1; TNF-α, tumor necrosis factor-α.
Introduction

Chronic injury leading to fibrosis in liver occurs in response to a variety of insults, including viral hepatitis (especially hepatitis B and C), alcohol abuse, drugs, metabolic diseases due to overload of iron or copper, autoimmune attack of hepatocytes, bile duct epitheliums or congenital abnormalities [Friedman, 1998]. Hepatic stellate cells (HSCs) are directly involved in mediating the pathological changes that lead to development of liver fibrosis [Friedman, 1993, 1998]. Following fibrogenic stimuli, HSCs change from quiescent to activated cells [McClain et al., 1993; Friedman 1998], which includes increased proliferation, a phenotypic transformation to myofibroblast-like cells, increased synthesis of extracellular matrix proteins, and contractility [Ogawa et al., 1986; Scheuer, 1991; Rockey, 1997; Friedman, 1996]. Activated HSCs are the predominant sources of the increased extracellular matrix proteins, which characterize hepatic fibrosis and cirrhosis [DeLeeuw et al., 1984; Ramadori et al., 1990; Pinzani, 1995]. Furthermore, activated HSCs have been implicated in hepatic inflammation through their ability to secrete cytokines [Friedman, 1993] and chemokines [Tiggelman et al., 1995; Rockey, 1997; Sprenger et al.] and express adhesion molecules [Marra et al., 1993].

NFκB is an essential regulator of the expression of a number of genes involved in immune, inflammatory, and growth responses [Roebuck et al., 1995; Baeuerle et al., 1996; Barnes et al. 2001]. DNA binding activity of NFκB is demonstrated in activated but not in quiescent HSCs, and activation of HSCs is associated with the nuclear translocation of NFκB [Hellerbrand et al., 1998]. Activated HSC predominantly expressed the classic NFκB p65:p50 complex. Several studies showed that HSC activation was associated with elevation of NFκB activity [Tsukamoto et al., 1990; Weinberg et al., 1994; Hellerbrand et al., 1998; Lang et al., 2000]. Once activated, NFκB dimers were translocated to the nucleus where they stimulated the transcription of genes that carried NFκB DNA binding motifs, including the genes encoding TGF-β and ICAM-1 [Hou et al., 1994; Liu et al., 1994; Lee et al., 1995; Ghost et al., 1998], this observation provides functional support for a critical role of NFκB in the activation of HSC.

Tetrandrine (Tet) is a bis-benzyl isoquinoline alkaloid derived from Stephania tetrandra (Moore). This compound has been characterized pharmacologically to exhibit hypotensive, immunosuppressive properties [Oh et al., 2003], inhibition of lipid
peroxidation [Hui et al., 1996], and downregulation of IκBα-kinases-IκBα-NFκB signaling pathway in human peripheral blood T cell [Ho et al., 2004]. It has also been shown to exhibit anti-fibrogenic activity against fibroblasts, particularly pulmonary fibroblasts [Reist et al., 1993]. It showed strong binding to alveolar macrophages [Ma et al., 1991], inhibition of proliferative activity of pulmonary fibroblast and had an inhibitory effect on types I and III collagen gene mRNA level in lung tissue of rats [Liu et al., 1994]. Tet also showed a blocking action of calcium channels [Weinberg et al., 1994; Liu et al., 1995]. It has been shown that calcium channels play an important role in the regulation of hepatic stellate cells contractility that is the marked phenotype of activated hepatic stellate cells [Bataller et al., 1998; Berlin, 1995]. Tet has an anti-fibrotic effect on liver fibrosis in rats induced by bile duct ligation and scission, implying that it might exert a direct effect on rat HSCs [Park et al., 2000], and induce HSCs apoptosis [Zhao et al., 2004]. We previously reported that Tet reduced the portal hypertensive state in bile duct ligated (BDL) rats [Huang et al., 1999]. Recently, we have reported that repeated administration of dimethylNitrosamine (DMN) induced fibrotic changes in the liver of rats, together with enhanced lipid peroxidation in hepatic mitochondria [Hsu et al., 2004]. In the present study, we used: (1) in vitro assays of HSCs, and (2) an in vivo rat model of fibrosis induced by DMN to investigate the anti-fibrotic effects of Tet. Silymarin, an extract from milk thistle (Silybum marianum) was included in the in vivo study for comparison with Tet, as it has a long history of usage by patients with liver diseases [Schuppan et al., 1999; Saller et al., 2001] and has been reported in the literature to exert anti-fibrotic effects in bile-duct-obstructed rats [Boigk et al., 1997; Jia et al., 2001] and DMN rats [Hsu et al., 2005].
Materials and Methods

**HSC-T6 Cell Line**

The HSC-T6 cell line, a generous gift of Prof. S.L. Friedman of the Mount Sinai School of Medicine (NY, USA), is an immortalized rat HSCs transfected with lipofectamine containing a cDNA in which the expression of the large T-antigen of SV40 is driven by the Rous sarcoma virus promoter [Vogel et al., 2000]. HSC-T6 cells were maintained in Waymouth medium (containing 10% FBS, pH 7.0) at 37 °C in 5% CO$_2$/95% air. The cultures were passaged by trypinization every 4$^{th}$ day and cells were plated in 150-mm culture dishes at a density of $1 \times 10^7$ cells per dish in Waymouth medium (containing 10% FBS, pH 7.0) and incubated under 5% CO$_2$ in air at 37 °C.

**Tetrandrine (Tet) solution**

Tet was purchased from Aldrich Chem. Co. (Milwaukee WII, USA.). For *in vitro* study, Tet was dissolved in dimethyl sulfoxide, Tet was diluted and added to the cultured medium to the final concentration of dimethyl sulfoxide (DMSO) was 0.1%. For in vivo study, Tet was mixed with 0.7% carboxyl methyl cellulose (CMC). Silymarin and other chemicals were from Sigma Chemical Co (St. Louis, MO, USA). Silymarin was also mixed with 0.7% CMC for *in vivo* administration.

**Measurement of cytotoxicity to HSCs**

The assays of reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were used to evaluate the potential of cytotoxicity of Tet. Cells were incubated in 24-well plates containing Waymouth medium (FBS-free) with or without Tet at different concentrations for 24 hrs at 37 °C. During the last hour, the cells were incubated with minimum essential medium containing 0.1 mg/ml MTT. The medium was aspirated, and the formazan particle was dissolved with DMSO. $A_{540}$ absorption intensity was measured by using enzyme-linked immunosorbent assay reader, according to the method of Hansen et al. [1989]. Relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The optical density of the formazan formed in the control cells was taken as 100% viability.
**Transiently transfected cells and luciferase assays**

$10^5$ cells/well were seeded on 24-well plates the day before transfection. Plasmid NFκB-Luc (1μg/well) (Strategene, La Jolla, CA) and pRL-SV40 (0.2 μg/well) (Promega, Madison. USA) were transfected into cells by lipofectamine (Invitrogen, California, USA). The pNFκB-Luc consists of NFκB-binding region, followed by the reporter gene firefly luciferase. Plasmid pRL-SV40 served as an internal control to normalize the transfection efficiency. After co-treatment with TNFα, LPS, PMA, H$_2$O$_2$ and Tet for 24 hours in 5% CO$_2$ incubator at 37°C, cells were harvested and lysed in 100μl of lysis reagent. Twenty μl of cell lysate was then mixed with 100μl of luciferin (the substrate of luciferase) right before luminescence detection. The luminescence, generated by luciferase activity, was measured with AutoLumat LB953 (Berthold technologies, Bad Wildbad, Germany). All reagents for luciferase assays were purchase from Promega (Madison. USA).

**Western blot analysis for in vitro α-SMA, IKKα expression, IκBa phosphorylation and NFκB translocation**

Cells (5x10$^6$) were seeded in medium containing 10% FBS. After 24 hrs, cells were washed twice with PBS and the medium was replaced by serum-free medium. Cells (in serum-free medium) were pre-exposed to TGF-β1 (1 ng/ml) for 1 hour and washed twice with PBS, then treated with Tet for 12 hours in the study of α-smooth muscle actin (α-SMA) protein expression. On the other hand, cells were pretreated with Tet for 24 hours in 5% CO$_2$ incubator at 37°C, and then stimulated with TNFα for 20 minutes. After treatments, cytoplasmic containing α-SMA, IKKα, phospho-IκBα protein and nuclear extracts containing the NFκB active protein were prepared from cells using the nuclear extraction kit (Chemicon, Temecula, CA) according to the manufacturer’s instructions. In brief, cells were washed with cold PBS, scraped off the plates and transferred to a clean centrifuge tube. After centrifugation at 250 g for 5 min at 4°C, the supernatant was discarded. Add 5 cell pellet volumns of ice cold 1X cytoplasmic lysis buffer containing 0.5 mM DTT and 1/1000 dilution of proteinase inhibitor cocktail. Resuspend the cell pellet gently and centrifuge the cell suspension at 250 g for 5 min at 4°C. The supernatant was collected as cytoplasmic faction for the analysis of α-SMA, IKKα expression and IκBa phosphorylation. Pellets were resuspended in ice cold nuclear extraction buffer and
a fresh syringe was used with 27-gauge needle to disrupt the nuclei. Cell suspension were allowed to gently agitate for 30 min at 4°C and then centrifuged at 16,000 g for 10 min at 4°C. Transfer the supernatant to a fresh tube, this fraction is the nuclear extract for the analysis of NFκB translocation. Twenty μg of proteins in cytoplasmic fraction or nuclear fraction were separated on a 10% SDS-PAGE and transferred onto Immobilon-PVDF (Millipore, Bedford, MA, USA) in a transfer buffer (6.2 mM boric acid, pH 8.0). Blots were incubated initially with blocking buffer (5% BSA) for 1 hour at room temperature, and then with specific primary antibodies against mouse α-actin (Calbiochem-Novabiochem, San Diego, CA, USA), mouse IKKα, mouse phosphor-IκBα, mouse α-tubulin, mouse PCNA or mouse NFκB p65 (Santa Cruz Biotechnology, Santa Cruz, California, CA, USA). Primary antibodies had been diluted (1:100) with Tris-buffered saline-Tween 20 (TBS-T) containing 5% BSA and 0.01% sodium azide. After antibody incubation, the blots were washed with TBS-T for 1 hour and incubated with anti-mouse IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, California, CA, USA) for 1 hour at room temperature. After the washing of the secondary antibodies (1:2000) with TBS-T, immunodetection was performed, using an enhanced chemiluminescence kit for western blot detection (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). Film exposure ranged from a few seconds to 5 min.

**Quantification of Collagen Deposition in Cultured HSC-T6 Cells and in the Livers of DMN rats**

HSCs (in serum-free medium) were co-treated with TGF-β1 1 ng/ml and Tet for 24 hours. Cells were washed and collagen deposited in the wells was assayed using the Sircol collagen assay kit (Biocolor, Belfast, Northern Ireland) according to the manufacturer’s instructions and the method described by Williams et al. [2001]. Unbound dye was removed by washing and the bound complex dissolved in 0.5% sodium hydroxide. Collagen was quantitated by spectrophotometry at 540nm and results were expressed as percentage of the untreated controls. A portion of liver tissue was homogenized in acetic acid (0.5 M) at 4 °C using an ULTRA TURRAX® homogenizer (Ika Labotechnik, Staufen, Germany). The fraction of insoluble collagen after acid extraction, composed of cross-linked collagen, was then heated at 80 °C for 60 min for
conversion into soluble gelatin. The gelatin contents of the acid extracts were assayed using the Sircol collagen assay kit (Biocolor, Belfast, Northern Ireland) according to the manufacturer’s instructions and the method described by Shiba et al. [1998] and in our previous paper [Hsu et al., 2005].

**Hepato-fibrotic Animals**

Hepatic fibrosis was induced by DMN administration in male Sprague-Dawley rats and we have recently documented changes in molecular and cell biological parameters related to fibrosis in these rats [Hsu et al., 2004]. DMN (10 mg/kg) was injected intraperitoneally for 3 consecutive days per week for 4 weeks, according to the method of Jezequel et al. [1987]. DMN (1 g/ml) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and diluted 100 folds in saline with a final concentration of 10 mg/ml before injection. Control rats were injected with saline alone. Rats were maintained on a standard rat pellet diet and tap water ad libitum. Animal studies were approved by the Institutional Animal Care and Use Committee of the University and conducted humanely, in accordance with the *Guide for the Care and Use of Laboratory Animals* [National Academic Press, USA, 1996]. There were five groups of rats: (a) control rats receiving 0.7% CMC, (b) DMN rats receiving 0.7% CMC, (c) DMN rats receiving silymarin (50 mg/kg, mixed with 0.7% CMC), (d) DMN rats receiving Tet (1 mg/kg), and (e) DMN rats receiving Tet (5 mg/kg), each given by gavages twice daily for 3 weeks starting after 1 week pre-DMN administration. Four weeks after DMN or saline injection, the rats were examined for the parameters listed below. On the day of measurement, venous blood was withdrawn from each rat under anesthesia, and thereafter the rat was sacrificed by KCl injection to remove the liver for homogenization and biochemical analysis.

**Histopathological examination**

For morphometric studies, the liver fragments were taken from the left lobe of each rat. Liver specimens were preserved in 4% buffered paraformaldehyde and dehydrated in a graded alcohol series. Following xylene treatment, the specimens were embedded in paraffin blocks, cut into 5-μm thick sections and placed on glass slides. The
sections were then stained with picro-sirius red for collagen distribution [Lopez-De Leon and Rojkind, 1985]. A numerical scoring system for histologically assessing the extent of fibrosis was adapted from the formula of Scheuer [1991], with minor modification (Hsu et al., 2004). Briefly, fibrosis was graded as: 0: no fibrosis; grade 1: enlarged, fibrous portal tracts; grade 2: periportal or portal-portal septa, but intact architecture; grade 3: fibrosis with architectural distortion; grade 4: probable or definite cirrhosis. Additionally, hepatocyte necrosis or degeneration severity was also graded as: 0, no hepatocyte necrosis or degeneration; grade 1, focal necrosis or degeneration of hepatocyte (mild, lesion \( \leq 3 \)); grade 2, multifocal necrosis or degeneration of hepatocyte (moderate, lesion > 3); grade 3, locally extensive or diffuse necrosis or degeneration of hepatocyte (severe). The liver scoring examination was performed by the pathologist (Y.-T. C.) who was blinded to rats’ treatment assignment. Fibrosis and hepatocyte scores were given after the pathologist had examined throughout three different areas in the tissue slide for each rat.

**Immuo-fluorescence staining (α-SMA and NFκB p65 double staining)**

Tissue sections were incubated with monoclonal mouse anti-human smooth muscle actin antibody (1:500 dilution, Dako) overnight at 37°C and then with rhodamine-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories Inc., USA) for 1 hour at 37°C. All the sections were subsequently incubated with fluorescein conjugated anti-rat NFκB p65 antibody (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, California, CA, USA) for 1 h at 37°C. All sections were observed under a laser confocal microscopy (TCS-SP2, Leica). The pictures of FITC images and those of rhodamine images were merged using Leica image analysis software.

**Western blot analysis for hepatic α-SMA and NFκB p65 expression**

Cytoplasmic extracts containing α-SMA protein and nuclear extracts containing the NFκB active protein were prepared from hepatic tissues using the nuclear extraction kit (Chemicon, Temecula, CA) according to the manufacturer’s instructions. The procedures of western blots were the same as we described above.
Biochemical Analysis of Plasma

Blood samples were collected (6 ml each from the femoral vein) and immediately centrifuged at 1300 g at 4 °C, and plasma were kept at -20 °C for liver and renal function tests. Alanine transaminase (ALT), aspartate transaminase (AST), and creatinine levels were measured using a colorimetric analyzer (Dri-Chem 3000, Fuji Photo Film Co, Tokyo, Japan), as we described previously [Hsu et al., 2004, 2005].

Quantitative realtime PCR for the Analysis of transcripts of α-SMA, TGF-β1 and ICAM-1

Cells (5x10^6) were seeded in medium containing 10% FBS. After 24 hrs, cells were washed twice with PBS and the medium was replaced by serum-free medium. Cells (in serum-free medium) were co-treated with TNF-α (20 ng/ml) and Tet for 2 hour for the mRNA expression of ICAM-1. Total RNA was isolated from cell lysates or hepatic tissues by the method of Chomczynski and Sacchi [1987]. For cDNA synthesis, 1 µg of total RNA was reverse-transcribed in a 30 µl of reaction mixture containing 10 µM dNTP mix, 500 µg/µl oligo(dT)_{12-18}, 0.2 µM DTT, 40 units of RNase inhibitor, 200 units of M-MLV reverse transcriptase, and 5×buffer (1.5 mM MgCl₂) (Invitrogen, California, USA). The reaction mix was incubated at 37 °C for 60 min and then denatured at 65°C for 10 min. Quantitative PCR analysis was performed using an ABI prism 7900 HT Sequence Detection System (Applied Biosystems). SyBR Green, a double-stranded DNA binding dye, was used for the fluorescent detection of DNA generated during the PCR. The PCR reaction was performed in a total volume of 20 µl with 0.4 pmol/µl of each primer, and 2x SyBR Green PCR master mix (Applied Biosystems); 1 µl cDNA corresponding to 100 ng of total RNA was used as template. The primer sequences for PCR amplification (α-SMA forward primer: 5’-TTC GTT ACT ACT GCT GAG CGT GAG A-3’, reverse primer: 5’-AAA GAT GGC TGG AAG AGG GTC-3’; TGF-β1 forward primer: 5’-TAT AGC AAC AAT TCC TGG CG-3’, reverse primer: 5’-TGC TGT CAC AGG AGC AGTG-3’; G3PDH forward primer: 5’-AGC CCA GAA CAT CAT CCC TG-3’, reverse primer: 5’-CAC CAC CTT CTT GAT GTC ATC-3’) were according to our previous report (Hsu et al., 2004). The primers of ICAM-1 (forward primer: 5’-CAC TAG AGG AGT GAG CAG GGT GAA AT-3’, reverse primer: 5’- TAT GAC TCG TGA
AAG AAA TCA GCT CTT-3’) were designed according to the published sequence of rat ICAM-1 (D00913) for PCR amplification.

Data analysis
Data are expressed as the mean ± SEM. One-way analysis of variance (ANOVA) was used for comparison of biochemical and molecular parameters. Statistical significance was accepted at $p < 0.05$. A non-parametric method (the Dunn procedure under the Kruskal–Wallis test) was used for multiple pairwise comparisons between groups for the histological grades of fibrosis. Statistical significance was accepted at $p < 0.05$. 
Results

In Vitro Effects of Tet on HSC-T6 Cells

(A) NFκB activation was determined by monitoring luciferase activity in cells

We first transfected the immortalized rat hepatic stellate cells (HSC-T6) with pNFκB-Luc plasmid, which contains the NFκB-responsive region followed by the firefly luciferase gene. Following TNFα treatments, NFκB proteins presumably will translocate into nucleus, bind to NFκB-binding sites on pNFκB-Luc DNA, and trigger expression of luciferase gene. Namely, luciferase activity corresponds to TNFα-induced NFκB activity. After exogenously adding luciferin to cell lysates, the luciferase-luciferin reactions generate luminescence with high sensitivity and can be measured. The NFκB responsive curve for different amount of TNFα is shown in [Figure 1A]. TNFα stimulated the luciferase activity in HSCs at 5, 10 and 20 ng/ml and reached plateau (223 ± 18% of controls) at 20 ng/ml, which was taken for further studies on Tet. We also investigated the effects of different known NFκB stimuli in HSC-T6 cells and observed that LPS stimulated the luciferase activity in HSCs at 0.5, 1.0 μg/ml and reached significant increase (313 ± 35% of controls) at 0.5 μg/ml. PMA (5 nM) and H2O2 (500 μM) also significantly stimulated the luciferase activity in HSCs and reached significant increase (247 ± 41% and 291 ± 22% of controls) [Figure 1A].

Secondly, we used N-acetylcysteine (NAC) and pyrrolidinedithiocarbamate (PDTC), well-known NFκB inhibitors in T-cells, as positive inhibitors in this assay. We observed that NAC (0.5-5.0 mM) and PDTC (1-25 μM) concentration-dependently reduced the NFκB activity induced by TNFα (20 ng/ml) in HSCs [Figure 1B]. Moreover, Tet (0.5- 5.0 μM) was shown to reduce the NFκB activity induced by TNFα [Figure 1B]. TNFα-stimulated luciferase activity was 273 ± 28% of controls, and this ratio were reduced to 99 ± 34% by co-administration of Tet (5.0 μM). The inhibitory effect of NAC, PDTC and Tet was not due to the cytotoxicity [Figure 1C]. We further investigated the inhibitory effect of Tet on NFκB activity induced by different stimuli and we demonstrated that LPS (0.5 μg/ml), PMA (5 nM) and H2O2 (500 μM)-stimulated luciferase activity was 168 ± 16%, 169 ± 6% or 177 ± 13% of control, and this ratio was reduced to 75± 12%, 66 ± 15% or 60 ± 8% by co-administration of Tet (5.0 μM) [Figure 1D].
(B) IKKα expression, IκBα phosphorylation and NFκB translocation in HSC-T6 Cells

Following TNFα stimulation for 20 mins, IKKα expression showed only mild increase (Figure 2A), but IκBα phosphorylation significant increased; this implied that IKKα activity was significantly induced by TNFα treatment. We also observed that the IKKα expression, IκBα phosphorylation was attenuated by pre-treatment of Tet [Figure 2A]. The amounts of the NFκB (p65) protein in nuclear extracts of cells were also concentration-dependently reduced by Tet treatment [Figure 2B].

(C) Tet reduced the mRNA expression of ICAM-1 in TNFα-stimulated HSC-T6 cells

Following TNFα treatments, ICAM-1 expression showed significantly increase (fig 1E), TNFα-stimulated mRNA expression of ICAM-1 was 1093 ± 255% of controls, and this ratio was significantly reduced to 425 ± 90% by co-administration of Tet (5.0 μM) for 2 hours [Figure 2C].

(D) Effects of Tet on TGF-β1-treated HSC-T6 Cells

TGF-β1 (1 ng/ml) stimulated α-SMA secretion and collagen deposition in HSC-T6 cells [Figure 2A]. Tet (0.5-5.0 μM) concentration-dependently attenuated TGF-β1-stimulated α-SMA protein expression ratio, with higher concentrations of Tet (2.5 and 5.0 μM) achieving significant reduction [Figure 3A]. TGF-β1 (1 ng/ml)-stimulated collagen deposition was 143 ± 9% of controls, and this ratio was significantly reduced to 65 ± 23% by co-administration of Tet (5 μM) [Figure 3B].

In Vivo Effects of Tet on DMN Rats

(A) General Features

The body weight of DMN rats was significantly lower than that of control rats. DMN rats also displayed a sickened appearance, including less vigorous movements and less-smooth fir. The body weight of DMN rats was significantly improved by treatment with either the low or high dose of Tet (Table 1). DMN rats showed a decrease in liver weight compared with control rats. Both Tet and silymarin treatment increased the liver weight in DMN rats (Table 1).
(B) Plasma Biochemistry

DMN rats showed significantly higher plasma ALT and AST levels compared with control rats, indicating hepatic injury (Table 1). Levels of both ALT and AST in DMN rats were significantly decreased by low dose or high dose of Tet and silymarin, suggesting that Tet and silymarin ameliorated hepatic injury in DMN rats (Table 1). However, high dose of Tet reduced plasma creatinine levels in DMN rats, suggesting higher dose of Tet might cause renal impairment in DMN rats.

(C) Histological examination

Histological examination of livers from DMN rats revealed the following changes: progressive increase and expansion of fibrous septa and loss of hepatocytes (multifocal necrosis of hepatocyte and focal degeneration of hepatocyte), compared with control rats. Collagen fibers, as stained by Sirius red, were distinctly deposited in the liver of DMN rats (Figure 4A). Fibrosis scores of livers from DMN rats were significantly reduced in DMN rats treated with high doses of Tet (Table 2). The fibrosis scores were reduced by 35% and 25% with the high and low doses of Tet, and by 20% with silymarin. Hepatocyte necrosis scores in the livers from DMN rats were significantly reduced in DMN rats treated with the high and low doses of Tet, and silymarin (Table 2). The hepatocyte necrosis scores were reduced by 69% and 81% with the high and low doses of Tet, and by 81% with silymarin. Neither Tet nor silymarin could reduce the degeneration of hepatocyte in the livers of DMN rats.

(D) Hepatic Collagen Content

Hepatic collagen levels significantly increased in DMN rats compared with control rats, suggesting abundant accumulation of collagen in the liver of DMN rats. Hepatic collagen levels significantly decreased with treatment of low and high doses of Tet or silymarin, suggesting that Tet and silymarin ameliorate hepatic collagen deposition in DMN rats (Table 1). Hepatic collagen contents were reduced by 51% and 35% with high and low doses of Tet, respectively, and by 39% with silymarin.

(E) Immuno-fluorescence double staining for α-SMA and NFκB
In the immuno-staining for \(\alpha\)-SMA (red), \(\alpha\)-SMA-positive cells (activated HSCs) was observed in the fibrous septa, portal tracts and sinusoid of the livers from DMN rats (Fig. 4B). In double staining for \(\alpha\)-SMA (red) and NF\(\kappa\)B (green), we observed NF\(\kappa\)B translocated into the nucleus of \(\alpha\)-SMA-positive cells (activated HSCs), but not hepatocyte. Moreover, the \(\alpha\)-SMA-positive cells with NF\(\kappa\)B nuclear translocation were also accumulated in the fibrous septa, portal tracts of the fibrotic livers. The \(\alpha\)-SMA-positive cells with NF\(\kappa\)B nuclear translocation were reduced with the high and low doses of Tet, and with silymarin. Further analysis by western blot also demonstrated that \(\alpha\)-SMA protein expression in the cytoplasmic extracts of liver tissues from DMN rats were reduced with the high and low doses of Tet, and with silymarin (Fig. 4C). Western blot analysis for the nuclear extracts of liver tissues from DMN rats also demonstrated the amount of NF\(\kappa\)B in the nuclear extracts were reduced with the high and low doses of Tet, and with silymarin (Fig. 4D).

(F) Analysis of transcripts of \(\alpha\)-SMA, TGF-\(\beta\)1, and ICAM-1 genes

There were significant increases in hepatic mRNA expressions of \(\alpha\)-SMA, TGF-\(\beta\)1 and ICAM-1 genes relative to G3PDH in DMN rats compared with control rats (Figure 5). The mRNA expression level of ICAM-1 in DMN rats were attenuated in Tet- and silymarin-treated groups (Figures 5). In addition, the mRNA expression of \(\alpha\)-SMA, TGF-\(\beta\)1 in DMN rats was only reduced by Tet treatment. The mRNA expression levels of \(\alpha\)-SMA and TGF-\(\beta\)1 in DMN rats tended to decrease with silymarin treatment, but the \(p\) values were 0.305 and 0.067, and a significant difference may be reached if a larger number of DMN rats are studied or increase the dose of silymarin from 50 mg/kg to 100 mg/kg [Hsu et al., 2005].
Discussion

In the present study, we observed that Tet exerted inhibitory effects on HSC-T6 cells and DMN-intoxicated rats, including (a) Tet significantly reduced IKKα expression and TNFα-induced NFκB activity, IκBα phosphorylation and NFκB nuclear translocation in HSC-T6 cells. (b) Regardless of the NFκB stimulus was TNFα, LPS, PMA or H2O2, Tet could reduce the NFκB activity in HSC-T6 cells. (c) Tet also reduced the mRNA expression of NFκB responsive gene, ICAM-1, induced by TNFα. (d) Tet concentration-dependently attenuated TGF-β1-stimulated α-SMA secretion and collagen deposition by HSC-T6 cells. (e) Our in vivo study showed that fibrosis scores of livers from DMN-treated rats with high dose of Tet was significantly reduced in comparison with DMN-treated rats receiving saline. Levels of plasma AST and ALT activities in DMN rats were reduced by Tet treatment. Hepatic collagen contents in DMN rats were significantly reduced by Tet treatment. Hepatic mRNA expression levels of α-SMA, TGF-β1, and ICAM-1 genes were significantly reduced by Tet treatment. (f) Activated HSCs (α-SMA-positive cells) with NFκB nuclear translocation in the fibrotic livers were decreased by Tet treatment. Overall, treatments with Tet and silymarin at 50 mg/kg in DMN rats yielded comparable benefits in terms of reductions in hepatic fibrosis scores, plasma AST activities indicative of hepatic injury, and fibrosis-related mRNA expressions of α-SMA, TGF-β1, and ICAM-1 genes. But high dose of Tet exerted better benefits than silymarin in terms of reductions in fibrosis scores (35% vs. 20%) hepatic collagen contents (51% vs. 39%), and mRNA expressions of α-SMA and TGF-β1 gene.

Tumor necrosis factor-α (TNFα) is elevated during hepatic inflammation, such as alcoholic liver disease [McClain et al., 1993], and contribute to the activation of HSCs. TNFα is a potent inducer of nuclear factor κB (NFκB), a key transcription factor that induces genes involved in inflammation, responses to infection, and stress [Thanos and Maniatis, 1995; Barnes and Karin, 1997]. Activated HSC predominantly expressed the classic NFκB p65:p50 complex. Once activated, NFκB dimers were translocated to the nucleus where they stimulated the transcription of genes that carried NFκB DNA binding motifs, including the genes encoding TGF-β and ICAM-1 [Hou et al., 1994; Ghost et al., 1998; Ledebur et al., 1995]. Several studies showed that HSC activation was associated with elevation of NFκB activity [Hellerbrand et al., 1998; Lang et al., 2000; Wright et al.,
To our knowledge, the present study is the first to use the double staining technique to co-localize the NFκB with activated HSCs (α-SMA-positive cells) in the DMN-induced hepatic fibrosis. The important observation suggested that in vivo NFκB activation and translocation into the nucleus of activated HSCs during hepatic fibrogenesis. In our laboratory, we have also observed that TNFα could induce NFκB translocation into the nucleus of HSC-T6 cells (data not shown).

Recently, Tet is shown to downregulate the IκBα-kinases-IκBα-NFκB signaling pathway in human peripheral blood T cell [Ho et al., 2004], the authors found that Tet itself did not affect NFκB binding to its corresponding DNA sequence but could prevent the degradation of IκB-α and inhibit nuclear translocation of p65 by blocking IκB-α kinases α and β activities. In our study, we observed that Tet could attenuate the ICAM-1 gene expression induced by TNFα and the NFκB activity induced by different stimuli in HSC-T6 cells. We also demonstrated that Tet significantly inhibited IKKα expression, TNFα-induced IκBα phosphotylation and NFκB translocation in HSCs. There is another report showing that green tea polyphenol epigallocatechin-3-gallate (EGCG) also inhibit acetaldehyde-induced NFκB activity in HSCs [Chen et al., 2002]. One interesting difference between our study on Tet and the study on EGCG is that Tet affects the upstream signaling molecules of NFκB pathway, whereas the latter one only demonstrated that EGCG reduced DNA binding ability of NFκB in HSCs [Chen et al., 2002].

In the literature, Tet also mediate anti-proliferative effects and are demonstrated typically by inhibition of cancer proliferation, which includes suppression of cell proliferation [Chen, 2002; Jin et al., 2002; Lai, 2002; Kwan and Achike, 2002; Kuo and Lin, 2003; Oh and Lee, 2003; Weinberg et al., 1994], angiogenesis [Chen, 2002] and multidrug resistance (MDR) to other anticancer drugs [Fu et al., 2002; Jin et al., 2002], and promotion of apoptosis [Jin et al., 2002; Kuo and Lin, 2003] and radio sensitivity [Chen, 2002]. In leukemia, breast carcinoma, lung cancer, heptoblastoma and neuroblastoma cells, Tet-induced anticancer actions display the following common features. (a) Tet arrests cell cycle progression, mostly in the G1 phase [Jin et al., 2002; Lee et al., 2002; Yoo et al., 2002; Kuo and Lin, 2003]. (b) The antiproliferative action of tetrandrine is associated with increased p53 and/or p21 expression [Jin et al., 2002; Lee et
al., 2002; Kuo and Lin, 2003; Oh and Lee, 2003], and decreased cyclin D1 expression [Lee et al., 2002] (c) Tet also increases several apoptotic signals including APO-1 (CD95, Fas) [Kuo and Lin, 2003] and caspase-3 activity [Yoo et al., 2002], and causes the release of cytochrome c together with downregulation of Bcl-x [Oh and Lee, 2003]. Thus, the anti-proliferative actions of Tet on tumors are due to: (a) induce cell cycle arrest in the G1 phase; and (b) simultaneously activate apoptotic pathways. Recently, Tet was shown to induce apoptosis in HSCs via increased sub-G1 DNA content and the activation of caspase-3 protease at doses of 40 and 80 μM treatments for 12 hours [Zhao et al., 2004]. In the present study, the inhibitory effect of Tet (0.5-5.0 μM) was not due to its cytotoxicity, as the cell viability was above 80% when HSCs treated with Tet 5.0 μM for 24 hours.

Cytokines of the transforming growth factor β (TGFβ) family influence a wide spectrum of cellular processes including differentiation, proliferation, apoptosis and migration. The observed TGF-β1 expression of activated HSCs, the potency of TGF-β1 to up-regulate ECM expression, and the expression of TGF-β receptors on HSCs has led to a widely accepted model in which persistent auto-/paracrine stimulation of activated HSCs by TGF-β1 is the key pro-fibrogenic response in liver fibrosis [Bauer and Schuppan, 2001]. Therefore we used it in the present study to activate HSC-T6 cells and then evaluate the inhibitory effects of Tet, Tet (0.5-5.0 μM) concentration-dependently attenuated TGF-β1-stimulated α-SMA protein expression ratio, with higher concentrations of Tet (2.5 and 5.0 μM) achieving significant reduction [figure 2A]. Tet (5.0 μM) also reduced collagen deposition by HSC-T6 cells [figure 2B].

In a previous study, Park et al. [2000] observed that administration of Tet in BDL rats can reduce the hepatic hydroxyproline content, AST, ALT and alkaline phosphatase level. In the present study using a different animal model, we additionally included hepatic collagen contents and protein as well as mRNA expressions of fibrosis-related genes as biomarkers of hepatic fibrosis. Either DMN or BDL is commonly used to induce hepatic fibrosis; there are important differences between these two models. Firstly, DMN does not cause fatty change in hepatocyte but generate significant hepatocyte necrosis, associated with marked inflammation [Tsukamoto et al., 1990]. The model of BDL is with low degrees of cell damage and inflammation. Secondly, bile duct ligation in rats
generally causes an increase in liver weights, whereas DMN intoxication leads to a reduction in liver weights [Shimizu, 2000; Hsu et al., 2004]. It is highly recommended that to validate the efficacy of any anti-fibrotic molecules should use these two different kinds of model in parallel [Lotersztajn et al., 2005]. In our laboratory, we have also investigated the effects of Tet in BDL rats, and this part of study is currently under processing in our laboratory.

Although there are as yet no clinically efficacious anti-fibrotic agents, experimental studies have been unremittingly conducted to assess the potentials of agents targeting the reduction of inflammation, inhibition of HSC activation or proliferation, induction of HSC apoptosis, or promotion of scar matrix degradation [Poli, 2000; Friedman, 2003]. Recently, there are some interesting reports of plant-derived anti-fibrotic agents in experimental animals [Schuppan et al., 1999; Shimizu et al., 1999; Gebhardt, 2002]. Herbs such as Sho-saiko-to [Sakaida et al., 1998; Shimizu et al., 1999], silymarin [Boigk et al., 1997; Jia et al., 2001], Inchin-ko-to [Sakaida et al., 2003], and *Salvia miltiorrhiza* [Nan et al., 2001; Oh et al., 2002; Hsu et al., 2005] have been shown respectively to reduce the severity of hepatic fibrosis in treated rats. In the present study, we incorporated both “fibrogenic- or fibrosis-related markers” markers for the evaluation of Tet.

In conclusion, our results showed that Tet treatment exerted anti-fibrotic effects in DMN-induced fibrotic rats. Tet also inhibited fibrogenic responses of HSC-T6 cells to TGF-β1 and TNFα.
Acknowledgments

We gratefully acknowledge the kind provision of HSC-T6 cells by Dr Scott L. Friedman, Division of Liver Diseases, The Mount Sinai School of Medicine, New York, NY, and USA. This work was supported in part by grants of National Science Council, Taiwan (NSC 92-2320-077-006, NSC 93-2320-010-072 and NSC93-2320-010-001).
References


Friedman, SL. 1998. in Diseases of the Liver (Schiff, E., Sorrell, M., and Maddrey, W.,


Hsu YC, Lin YL, Chiu YT, Shiao MS, Lee CY, Huang YT. 2005. Anti-fibrotic effects of


Reist RH, Dey RD, Durham JP, Rojanasakul Y and Castranova V. 1993. Inhibition of


Table 1. General profiles in control rats and dimethylnitrosamine (DMN)-induced fibrotic rats receiving tetrandrine (Tet), silymarin or vehicle treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Control + vehicle</th>
<th>DMN + vehicle</th>
<th>DMN + sil (50 mg/kg)</th>
<th>DMN + Tet (1mg/kg)</th>
<th>DMN + Tet (5 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>419 ± 9</td>
<td>286 ± 9 *</td>
<td>314 ± 8 *#</td>
<td>300 ± 9 *#</td>
<td>309 ± 8 *#</td>
</tr>
<tr>
<td>LW (g)</td>
<td>13.5 ± 0.4</td>
<td>7.7 ± 0.5 *</td>
<td>13.0 ± 0.7 #</td>
<td>9.5 ± 1.5 *#</td>
<td>10.1 ± 0.8 *#</td>
</tr>
<tr>
<td>SW (g)</td>
<td>1.01 ± 0.07</td>
<td>1.46 ± 0.13 *</td>
<td>1.44 ± 0.10 *</td>
<td>1.57 ± 0.16 *</td>
<td>1.58 ± 0.19 *</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>28.6 ± 1.7</td>
<td>115.1 ± 1.9 *</td>
<td>104.4 ± 5.1 *</td>
<td>86.1 ± 3.4 *#</td>
<td>64.8 ± 7.9 *#</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>70.4 ± 3.5</td>
<td>223.1 ± 44.1 *</td>
<td>150.4 ± 10.7 *#</td>
<td>117.4 ± 7.1 *#</td>
<td>101.9 ± 8.6 *#</td>
</tr>
<tr>
<td>Cr (mg/dl)</td>
<td>0.58 ± 0.02</td>
<td>0.60 ± 0.29</td>
<td>0.63 ± 0.03</td>
<td>0.54 ± 0.02</td>
<td>0.45 ± 0.27 *#</td>
</tr>
<tr>
<td>Collagen (mg/g liver)</td>
<td>12.0 ± 2.1</td>
<td>26.1 ± 1.9 *</td>
<td>15.8 ± 1.5 #</td>
<td>17.0 ± 1.5 *#</td>
<td>12.9 ± 1.3 #</td>
</tr>
</tbody>
</table>

Tet, tetrandrine; Sil, silymarin; BW, Body weight; LW, liver weight; SW, spleen weight; ALT, alanine transaminase; AST, aspartate transaminase; Cr, creatinine; collagen, Collagen content (mg/g liver dry weight). Data are expressed as the mean ± SEM. The number of rats in each column is 8. *p< 0.05 vs. control group; #p< 0.05 vs. DMN group.
Table 2. Fibrosis scores in control rats and dimethylnitrosamine (DMN)-induced fibrotic rats receiving tetrandrine (Tet), silymarin or vehicle treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Control +vehicle</th>
<th>DMN +vehicle</th>
<th>DMN + sil (50 mg/kg)</th>
<th>DMN + Tet (1mg/kg)</th>
<th>DMN + Tet (5 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fibrosis score</strong></td>
<td>0 ± 0</td>
<td>2.0 ± 0.2 **</td>
<td>1.6 ± 0.2 *</td>
<td>1.5 ± 0.2 *</td>
<td>1.3 ± 0.3 **#</td>
</tr>
<tr>
<td><strong>Hepatocyte necrosis</strong></td>
<td>0 ± 0</td>
<td>1.6 ± 0.3 **</td>
<td>0.3 ± 0.2 # #</td>
<td>0.5 ± 0.2 * #</td>
<td>0.3 ± 0.2 # #</td>
</tr>
<tr>
<td><strong>Hepatocyte degeneration</strong></td>
<td>0 ± 0</td>
<td>0.5 ± 0.2 *</td>
<td>0.4 ± 0.2 *</td>
<td>0.5 ± 0.3</td>
<td>0.4 ± 0.2</td>
</tr>
</tbody>
</table>

Tet, tetrandrine; Sil, silymarin; Data are expressed as the mean ± SEM. The number of rats in each column is 8. *p< 0.05, **p< 0.01 vs. control group; #p< 0.05, # #p< 0.01 vs. DMN group.
Figure legends

Figure 1. (A) Effects of TNFα, LPS, PMA and H₂O₂ on transcriptional activity of NFκB in HSC-T6 cells. * p < 0.05 vs. Control, n=3. (B) Effects of NAC, PDTC and tetrandrine (Tet) on TNFα-induced NFκB transcriptional activity in HSC-T6 cells at 24 hours after treatment. * p < 0.05 vs. Control, # p < 0.05 vs. TNFα alone, n=3. (C) Effects of NAC, PDTC and Tet on cell viability of HSC-T6 cells at 24 hours after treatments. (n=3) (D) Effects of Tet on NFκB transcriptional activity induced by LPS, PMA and H₂O₂ in HSC-T6 cells at 24 hours after treatment. * p < 0.05 vs. Control, # p < 0.05 vs. LPS, PMA or H₂O₂. (n=3)

Figure 2. (A) Effects of Tet on IKKα expression and TNFα-induced IκBα phosphorylation in cytoplasmic extract of HSC-T6 cells. (B) Effects of tetrandrine on TNFα-induced nuclear translocation of NFκB (p65) in nuclear extract of HSC-T6 cells. Representative results from three independent experiments are shown here. (C) Quantitative real-time PCR analysis for mRNA expression of ICAM-1 after TNFα (20 ng/ml) and Tet treatment in HSC-T6 cells.*p<0.05 vs. control cells; #p<0.05 vs. TNFα-treated cells, (n=3).

Figure 3. (A) Tet reduced the protein expression of α-SMA induced by TGF-β1 (1 ng/ml) in HSC-T6 cells. Representative results from three independent experiments are shown here. (B) Effects of Tet on collagen deposition by HSC-T6 cells after TGF-β1 stimulation for 24 hours. Collagen deposition by HSC-T6 cells was quantified by sircol collagen assay. * p < 0.05 vs. control group; # p < 0.05 vs. TGF-β1 alone, (n=3).

Figure 4. (A) Histological examination of liver sections in control and dimethylnitrosamine (DMN)-treated rats. Representative liver sections were obtained from control rats (a), DMN rats receiving saline (b), DMN rats receiving 50 mg/kg silymarin (c), DMN rats receiving 1 mg/kg Tet (d), and DMN rats receiving 5 mg/kg Tet (e). Sections were stained with Sirius red. Scale bar=200 μm. (B) Double immunofluorescence staining was performed with anti-α-SMA IgG (red) and an anti-NFκB (p65) IgG (green) as described before. Scale bar=50 μm. The pictures of FITC images and those of rhodamine images were merged by using Leica image analysis.
software. (C) Tet reduced the protein expression of α-SMA in the cytoplasmic extracts of liver tissues from rats. (D) Tet and silymarin reduced the nuclear translocation of NFκB (p65) in nuclear extract of liver tissues from rats.

**Figure 5.** Quantitative realtime PCR analysis for the expressions of transforming growth factor-β1 (TGF-β1), α-smooth muscle actin (α-SMA) and intercellular adhesion molecule-1 (ICAM-1) transcripts in control rats, DMN rats receiving vehicle, Tet (1 and 5 mg/kg) or silymarin (50 mg/kg). The number of rats in each column is 8. *p* < 0.05 vs. the control group; #*p* < 0.05 vs. the DMN group receiving vehicle.
Figure 1.

(A)

(B)
Figure 2.

(A)

<table>
<thead>
<tr>
<th>TNFα (20 ng/ml)</th>
<th>Tet (µM)</th>
<th>DMSO (0.1%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

IKK α

phospho-IκB α

α -tubulin

84 KD

37 KD

52 KD

(B)

<table>
<thead>
<tr>
<th>TNFα (20 ng/ml)</th>
<th>Tet (µM)</th>
<th>DMSO (0.1%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

NFκB (p65)

PCNA

65 KD

35 KD
(C)

<table>
<thead>
<tr>
<th></th>
<th>TNFα (20 ng/ml)</th>
<th>Tet (μM)</th>
<th>DMSO (0.1%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

M

ICAM-1

409 bp

G3PDH

209 bp

Expression of ICAM-1 (% of Control)

*
Figure 3.

(A)

<table>
<thead>
<tr>
<th>Condition</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β (1 ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tet (μM)</td>
<td></td>
<td>-</td>
<td>0.5</td>
<td>1.0</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>DMSO (0.1%)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

α-SMA 42KD

α-tubulin 52KD

(B)

Collagen deposition by HSCs (% of control)

<table>
<thead>
<tr>
<th>Condition</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β (1 ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tet (μM)</td>
<td></td>
<td>-</td>
<td>0.5</td>
<td>1.0</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>DMSO (0.1%)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* #
Figure 4.

(A)
<table>
<thead>
<tr>
<th></th>
<th>Phase</th>
<th>α-SMA</th>
<th>NFκB</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
</tr>
<tr>
<td>b</td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
</tr>
<tr>
<td>c</td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
</tr>
<tr>
<td>d</td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
</tr>
<tr>
<td>e</td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
</tr>
</tbody>
</table>
(C)  
\[ \alpha \text{-SMA} \quad 42\text{KD} \]
\[ \alpha \text{-tubulin} \quad 52\text{KD} \]

(D)  
\[ \text{NFk\(\kappa\)B (p65)} \quad 65\text{KD} \]
\[ \text{PCNA} \quad 35\text{KD} \]
Figure 5.