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# 2′,4′,6′-Tris(methoxymethoxy) chalcone induces apoptosis by enhancing Fas-ligand in activated hepatic stellate cells

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ABSTRACT

Suppression of hepatic stellate cell (HSC) activation and proliferation, and induction of apoptosis in activated HSCs have been proposed as therapeutic strategies for the treatment and prevention of the hepatic fibrosis. We previously showed that 2′,4′,6′-tris(methoxymethoxy) chalcone (TMMC), a synthesized chalcone derivative, inhibits platelet-derived growth factor-induced HSC proliferation at 5–20 μM. Here, we showed that TMMC induces apoptosis in activated HSCs at higher concentrations (30–50 μM), but is not cytotoxic to primary hepatocytes. Moreover, TMMC induces hyperacetylation of histone by inhibiting histone deacetylase (HDAC) in activated HSCs. Interestingly, TMMC treatment remarkably increased Fas-ligand (FasL) mRNA expression in a dose-dependent manner. Cycloheximide treatment reversed the induction of TMMC on apoptosis, indicating that *de novo* protein synthesis was required for TMMC-induced apoptosis in activated HSCs. In addition, FasL synthesis by TMMC is closely associated with maximal procaspase-3 proteolytic processing. *In vivo*, TMMC reduced activated HSCs in $CCl_4$-intoxicated rats during liver injury recovery, as demonstrated by α-smooth muscle actin expression in rat liver. TMMC treatment also resulted in apoptosis, as demonstrated by cleavage of poly(ADP-ribose) polymerase in rat liver. In conclusion, TMMC may have therapeutic potential by inducing HSC apoptosis for the treatment of hepatic fibrosis.



## 1. Introduction

Hepatic stellate cells (HSCs) play a critical role in the development and maintenance of liver fibrosis (Pinzani and Marra, 2001; Lotersztajn et al., 2005). HSCs undergo phenotype transformation from vitamin A-storing quiescent cells to myofibroblast-like activated cells. Activated HSCs are proliferative and fibrogenic, with accumulation of extracellular matrix (Bataller and Brenner, 2005; Friedman, 2008). For many years liver fibrosis and cirrhosis were considered irreversible (Bonis et al., 2001). However, there has been a paradigm shift in the field with increasing clinical and experimental evidence for reversibility (Arthur, 2002). Therefore, suppression of HSC activation and proliferation as well as induction of apoptosis in activated HSCs has been proposed as therapeutic strategies for the treatment and prevention of hepatic fibrosis (Canbay et al., 2004; Elsharkawy et al., 2005; Henderson and Iredale, 2007). We have previously shown that 2′,4′,6′-tris(methoxymethoxy) chalcone (TMMC), a synthetic chalcone derivative, inhibits the proliferation of rat HSCs (Lee et al., 2006a). Therefore, here we examined the effect of TMMC on apoptosis in activated HSCs.

The balance between histone acetylation and deacetylation is regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC), and plays an important role in the transcriptional regulation of genes (Csordas, 1990; Grunstein, 1997; Hassig and Schreiber, 1997). Acetylation of lysine residues in histones results in an open chromatin structure and therefore activation of transcription, whereas hypoacetylation of histones causes chromatin condensation resulting in the suppression of transcription (Kouzarides, 1999; Wolffe, 1996; Hassig and Schreiber, 1997; Pazin and Kadonaga, 1997). Therefore, deregulation of HAT and HDAC has been suggested to play a causative role by changing gene expression patterns.

Recent accumulating evidences suggest that induction of histone hyperacetylation by HDAC inhibitors blocks proliferation and induces apoptosis (Han et al., 2000; Zhang et al., 2004; Boyle et al., 2005; Kumagai et al., 2007). Fas, an important apoptotic trigger, is present on the cell surface as a monomeric protein and can be bound by its natural ligand, Fas-ligand (FasL) (Nagata and Golstein, 1995; Nagata, 1999). Cross-linking of Fas by FasL leads to trimerization of Fas-associated death domain (FADD) in the inner membrane surface, a phenomenon that recruits different caspases and initiates a complex signaling pathway ultimately leading to apoptosis (Depraetere and Golstein, 1997).

In this study, we investigated whether TMMC could induce apoptosis in activated HSCs, and if so, we investigated whether TMMC is an HDAC inhibitor and the gene transcription involved in this activity. We also evaluated whether TMMC treatment after $CCl_4$ injection reduced activated HSCs in the liver.

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## 2. Materials and Methods

### 2.1. Reagents and Cell Isolation

TMMC was synthesized as described previously (Lee et al., 2006b). 3-(4,5-Dimethylthiazol-1-yl)2,5-diphenyl tetrazolium bromide (MTT) and cycloheximide (CHX) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Z-DEVD-FMK (a specific caspase-3 inhibitor) and Z-VAD-FMK (a general caspase inhibitor) were from R&D Systems, Inc (Minneapolis, MN, USA).

Rat HSCs and hepatocytes were isolated from the livers of Sprague Dawley rats as described previously (Park et al., 2005; Lee et al., 2006a). Apoptotic effects were studied in between passages 6 and 10 of activated HSCs. Isolated hepatocyte attachment to the culture dish was complete at 2 h after plating, and the medium was changed to remove unattached or dead cells. At 18 h after plating, hepatocytes were treated with TMMC.

### 2.2. Cell Viability Measurement

Cell viability was determined using the MTT assay. Cells were incubated in 96-well plates ($1\times10^4$ cells/well) and treated with TMMC at the indicated concentrations. Ten microliters of MTT was added to each well 1 h before the indicated periods of TMMC treatment and the cells were incubated for another 1 h. Medium containing MTT and TMMC was aspirated off, and 150 μl of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals formed in viral cells. The absorbance was read at 540 nm using a microplate reader (Molecular Devices Co., Sunnyvale, CA, USA). Each experiment was performed in quadruplicate and repeated at least twice.

### 2.3. Western Blot Analysis

Whole-cell lysates (for cleaved caspase-8, cleaved caspase-9, caspase-3, poly(ADP-ribose) polymerase [PARP], FasL and β-actin) and nuclear extracts (for Ac-Histone H3, Ac-Histone H4 and TATA binding protein [TBP]) were separated by 12 or 15% SDS-polyacrylamide gel electrophoresis. Nuclear extracts were prepared as previously described (Lee et al., 2004). Nitrocellulose membranes were incubated with specific antibodies against caspase-3, Ac-Histone H3, Ac-Histone H4, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibodies against cleaved caspase-8, cleaved caspase-9, and PARP were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-FasL and Anti-TBP antibodies were obtained from Abcam (Cambridge, UK). Immunoreactive bands were detected by incubating with anti-rabbit or anti-mouse IgG antibodies conjugated with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

### 2.4. HDAC Enzyme Activity Assay

HDAC activity was measured using the Colorimetric HDAC assay kit according to the instructions from the manufacturer (BioVisio Research Products, Mountain View, CA, USA). Briefly, the nuclear fraction was diluted to 10 μl with HDAC assay buffer. Following the addition of colorimetric HDAC substrate, the reaction mixture was incubated for 1 h at 37 °C, and stopped at 30 min with Lysine Developer. The colorimetric of the reaction mixture was monitored at 405 nm using a microplate reader (Molecular Devices Co., Sunnyvale, CA, USA). Each experiment was performed in triplicate and repeated at least twice.

### 2.5. Animals

Male Sprague–Dawley rats (200–220 g) were supplied by Dae Han Laboratory Animal Research and Co. (Chungbuk, Korea), and fed with a normal standard chow diet (Jae Il Chow, Korea) and tap water *ad libitum*. Animal experiments were performed under the "Guiding Principles in the Use of Animals in Toxicology" adopted by the Society of Toxicology (USA) in March, 1999. The Animal Studies Ethics Committee of Wonkwang University approved all of the present experiments.

### 2.6. Animal Experiments

*In vivo* study was carried out according to the previous reports with some modifications (Ikejima et al., 2001). Liver damage was generated in adult male rats with subcutaneous injection of $CCl_4$ (3 ml/kg, 50% in corn oil). Vehicle control animals were injected subcutaneously with corn oil (3 ml/kg). Either TMMC (50 mg/kg. p.o.) or vehicle alone was administrated to rats at 24 and 48 h after $CCl_4$ injection. Liver tissues were obtained under anesthesia 72 h after $CCl_4$ injection. For histological analysis, liver tissues were fixed with 10% buffered formalin, embedded with paraffin and hematoxylin-eosin (H&E) was performed. Serum samples and liver tissues for RNA preparation were frozen at −80 °C until assay.

### 2.7. Real-time Polymerase Chain Reaction (PCR)

Total RNA was obtained from HSCs and hepatic tissue using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse transcribed using the RETROscript kit (Ambion, TX, USA) with random decamers as primers. Real-time PCR amplification was performed using SYBR Green PCR Core Reagents (TAKARA, Warrington, Japan). The amount of target mRNA was determined using the comparative threshold (Ct) method by normalizing target mRNA Ct values to those for GAPDH (ΔCt). Statistical analysis of real-time PCR data was performed using ΔCt values. The primer sequences for target genes are described in Table 1.

**Table 1**
Sequences of the primers used for real-time PCR.

| Target genes (GenBank ID) | Primer sequences | |
|---|---|---|
| | Forward primer | Reverse primer |
| GAPDH (AF106860) | 5′-ACAAGATGGTGAAGGTCGGTGTGA-3′ | 5′-AGCTTCCCATTCTCAGCCTTGACT-3′ |
| Bax (NM017059) | 5′-TTGCTGATGGCAACTTCAACTGGG-3′ | 5′-TGTCCAGCCCATGATGGTTCTGAT-3′ |
| Bcl-2 (L14680) | 5′-TTGTGGCCTTCTTTGAGTTCGGTG-3′ | 5′-TCATCCACAGAGCGATGTTGTCCA-3′ |
| Fas (NM139194) | 5′-ACCACTGTTATCACTGCACCTCGT-3′ | 5′-AGGATCAGCAGCCAAAGGAGCTTA-3′ |
| FasL (NM012908) | 5′-AAGACCACAAGGTCCAACAGGTCA-3′ | 5′-TTCTCTTTGCCTCTGCATTGCCAC-3′ |
| FADD (NM152937) | 5′-GATCTGCGGGTGGCATTTGACATT-3′ | 5′-TGCCATCAATCTTGGCCTCAGACA-3′ |
| α-SMA (NM031004) | 5′-AATATTCTGTCTGGATCGGCGGCT-3′ | 5′-GAAGCATTTGCGGTGGACAATGGA-3′ |

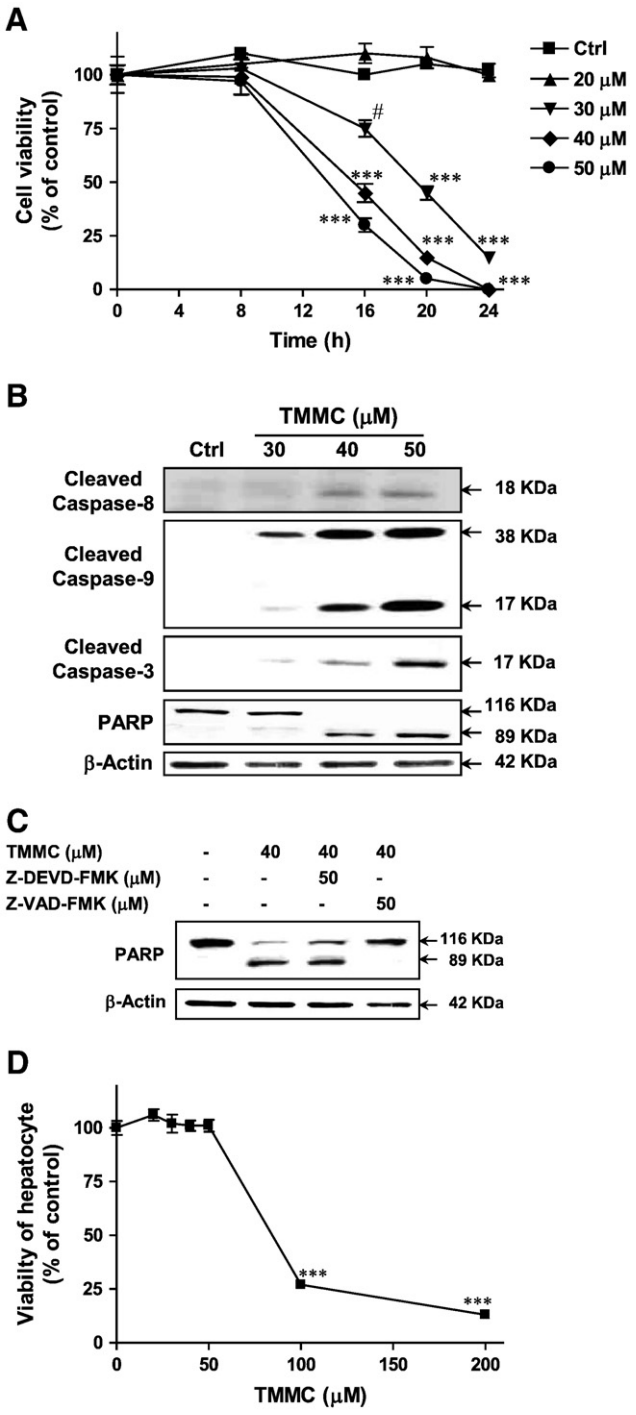


**Fig. 1.** TMMC induces apoptosis in activated rat HSCs. (A) Cytocidal effect of TMMC. Viability of cells treated with TMMC evaluated by the MTT assay. Results are expressed as % of control cell viability at the corresponding time point. Data are mean ± S.D. Each experiment was performed in quadruplicate and repeated at least twice. $^{\#}P<0.05$ and $^{***}P<0.001$ *vs.* control cells treated with DMSO instead of TMMC. (B) TMMC-induced cleavage of caspase-8, -9, -3 and PARP in activated rat HSCs. Lysates of cells treated with TMMC at the indicated concentrations for 20 h and subjected to SDS-PAGE followed by immunoblotting analysis. The membrane was incubated with anti-cleaved caspase-8, -9, -3 or anti-PARP antibodies. Actin was used as a protein-loading control. (C) Effects of caspase inhibitors on TMMC-induced apoptosis in activated rat HSCs. Cells were pretreated for 2 h with either Z-DEVE-FMK (caspase-3 inhibitor) or Z-VAD-FMK (pan-caspase inhibitor) before TMMC treatment (40 μM for 20 h). PARP cleavage was attenuated by pretreatment with caspase inhibitors. (D) Hepatotoxicity assay of TMMC in rat hepatocytes. Hepatocytes were incubated for 24 h in TMMC (20, 30, 40, 50, 100 or 200 μM) and viability was evaluated by the MTT assay. Results are expressed as % of control cell viability at the corresponding concentration. Data are mean ± S.D. Each experiment was performed in quadruplicate and repeated at least twice. $^{***}P<0.001$ *vs.* control cells treated with DMSO instead of TMMC.

### 2.8. Immunohistochemical Staining

To detect α-smooth muscle actin (α-SMA) in the liver, tissue sections were deparaffinized and immunohistochemical staining was performed by the strepavidin-biotin-peroxidase complex method using LSAB® 2 kit (DAKO Co., Carpentaria, CA, USA) and monoclonal anti-α-SMA (Clone 1A4, Sigma Chemical Co.) as primary antibody.

### 2.9. Statistical Analysis

Data were analyzed using Student's *t* test when appropriate or by a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests when comparing more than three means. Calculations were performed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

## 3. Results

### 3.1. TMMC-induced Apoptosis in Activated Rat HSCs

The cytotoxic effects of TMMC (20, 30, 40, or 50 μM) on HSCs were assessed using the MTT assay. TMMC at 20 μM did not affect cell viability (Lee et al., 2006a), but higher concentrations (30 to 50 μM) reduced cell viability (Fig. 1A). Cell death was >50% after 12 h of exposure to the TMMC. We used the immunoblot analysis for biochemical hall markers of apoptosis. TMMC dose-dependently increased the activation of caspase-8, -9, -3 and the cleavage of PARP (Fig. 1B), indicating TMMC-induced cell death of activated HSCs is due to apoptosis. To confirm the role of caspases in TMMC-mediated apoptosis, HSCs were pretreated with specific cell-permeable caspase inhibitors and then treated with TMMC. z-DEVD-FMK (caspase-3 inhibitor) and z-VAD-FMK (pan-caspase inhibitor) inhibited TMMC-induced PARP cleavage (Fig. 1C). In contrast, TMMC did not affect the viability of primary rat hepatocytes, even at 50 μM (Fig. 1D).

### 3.2. Epigenetic Regulation of Histones by TMMC in Activated Rat HSCs

Recently, various HDAC inhibitors have been shown to induce apoptosis (Han et al., 2000; Zhang et al., 2004; Boyle et al., 2005;

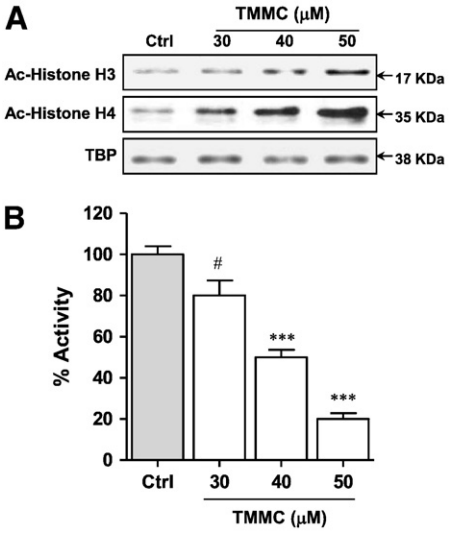


**Fig. 2.** TMMC induces accumulation of acetylated histone H3 and H4 and inhibits HDAC activity. (A) Activated HSCs were treated with TMMC for 20 h and then nuclear extracts were subjected to immunoblotting with anti-acetylated Histone H3 or H4 antibodies. The anti-TBP antibody was used as a nuclear protein loading control. (B) Activated HSCs were treated with TMMC for 20 h and then nuclear extract were subjected to HDAC enzyme activity assay. Data are mean ± S.D. Each experiment was performed in triplicate and repeated at least twice. $^{\#}P<0.05$ and $^{***}P<0.001$ *vs.* control cells treated with DMSO instead of TMMC.

Kumagai et al., 2007). We therefore tested whether TMMC-induced apoptosis in activated HSCs resulted from HDAC inhibition. We first examined the ability of TMMC to affect histone acetylation in our cellular system. Indeed, TMMC dose-dependently induced histone H3 and histone H4 acetylations in HSCs (Fig. 2A). To examine the sensitivity of HSC HDAC to TMMC, we next analyzed the activity of HDAC enzyme by treatment with TMMC. TMMC directly inhibited HDAC activity dose-dependently (Fig. 2B) confirming that TMMC inhibits HDAC in HSCs.

### 3.3. TMMC enhances Fas-ligand mRNA Expression in Activated Rat HSCs

Although a number of HDAC inhibitors have been shown to induce apoptosis in different cells, the mechanism underlying this appears to vary (Medina et al., 1997; Cao et al., 2001; Bernhard et al., 2001). We therefore examined the expression of apoptosis-related genes such as Fas, FasL, FADD, Bax, and Bcl-2 by real-time PCR. TMMC treatment increased Fas, FADD, and Bax mRNA expression and decreased Bcl-2 mRNA expression (Fig. 3A). But compared with control group, the differences of Fas, FADD and Bax mRNA expression were not significant. Interestingly, however, TMMC treatment remarkably enhanced FasL mRNA expression by about 35-fold compared with control; Bcl-2 mRNA expression was also significant decrease but less marked than FasL mRNA expression. In addition, TMMC treatment significantly increased the FasL mRNA expression in a dose-dependent manner (Fig. 3B).

### 3.4. TMMC-induced Apoptosis requires Fas-ligand Synthesis in Activated Rat HSCs

We next checked whether FasL protein synthesis was required for TMMC-induced apoptosis using CHX, a protein synthesis inhibitor. HSCs were pretreated for 1 h with CHX followed by addition of TMMC for 20 h. The pretreatment with CHX reduced the level of FasL protein induced by TMMC and almost completely inhibited TMMC-induced cleavages of procaspase-3 and PARP (Fig. 4A), indicating that *de novo* protein synthesis is required for TMMC-induced apoptosis. To further confirm FasL protein synthesis required for TMMC-induced apoptosis,

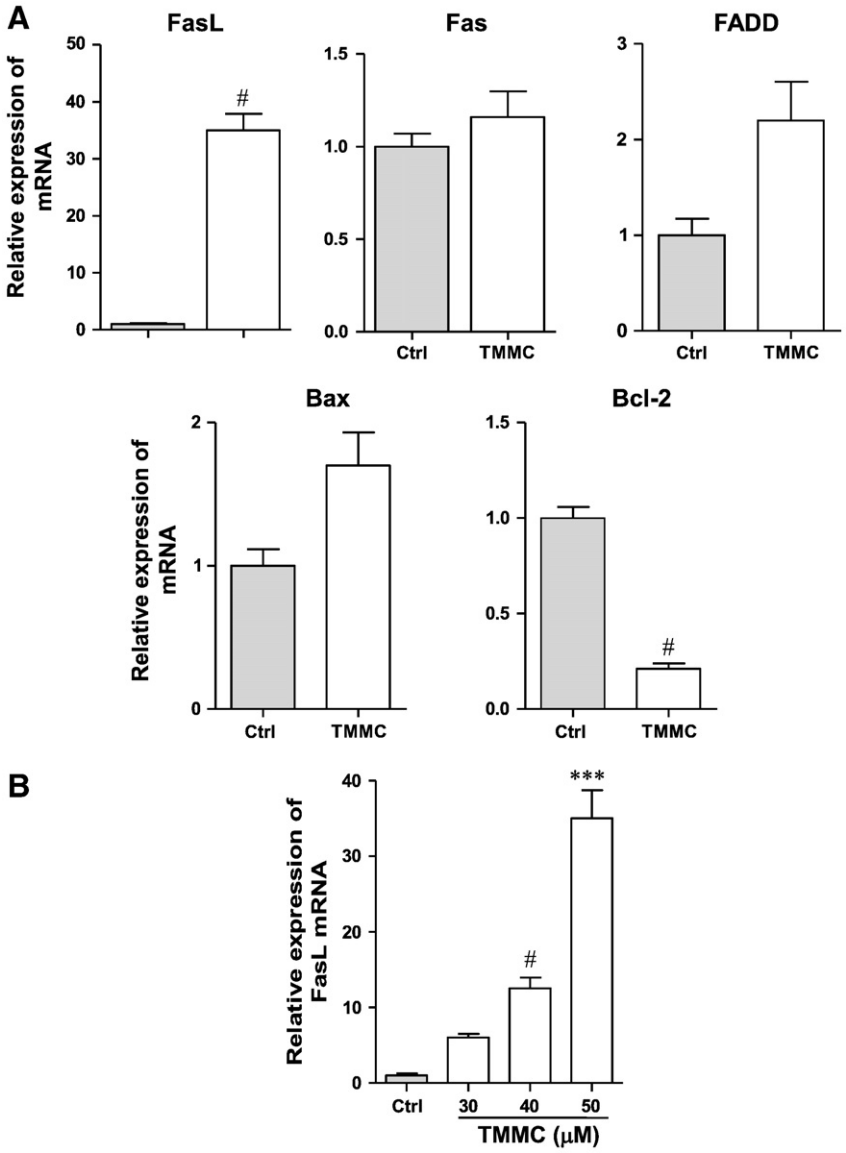


**Fig. 3.** TMMC strongly up-regulates Fas-ligand mRNA. (A) Activated rat HSCs were treated with TMMC (50 μM) for 16 h before total RNA was isolated and then subjected to Real-time PCR analyses for Fas-ligand (FasL), Fas, FADD, Bax, and Bcl-2 mRNA. Data are mean ± S.D. of triplicate from three independent experiments. $^{\#}P<0.05$ *vs.* control cells treated with DMSO instead of TMMC. (B) Activated HSCs were treated with various concentrations of TMMC for 16 h and then subjected to Real-time PCR analyses for FasL mRNA. Data are mean ± S.D. of triplicate from three independent experiments. $^{\#}P<0.05$ and $^{***}P<0.001$ *vs.* control cells treated with DMSO instead of TMMC.

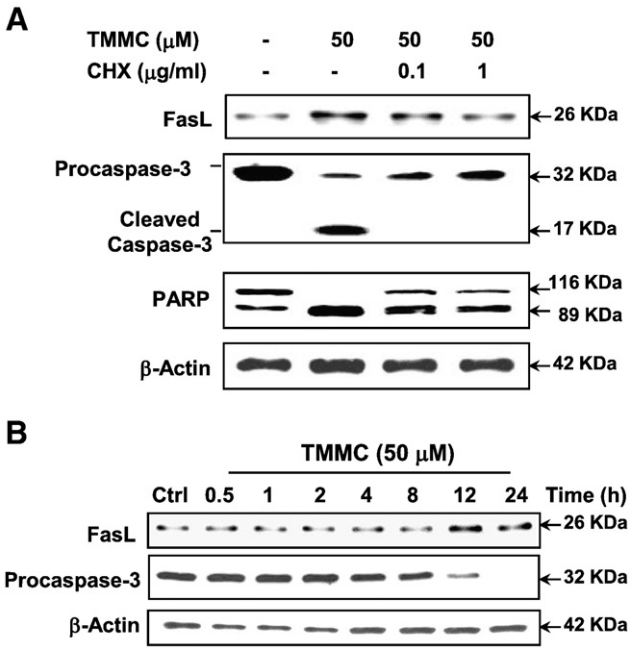


**Fig. 4.** FasL synthesis is required for TMMC-induced apoptosis in activated rat HSCs. (A) Activated rat HSCs were pretreated with cycloheximide (0.1 or 1 μg/ml) for 1 h followed by treatment with 50 μM TMMC for 20 h. Cell lysate was subjected to SDS-PAGE followed by immunoblotting analysis. The membrane was incubated with anti-FasL, anti-caspase-3 or anti-PARP antibodies. Actin was used as a protein-loading control. (B) Activated rat HSCs were incubated with TMMC (50 μM) for 24 h. At indicated time point after treatment with TMMC, isolated cellular extract were subject to immunoblotting with anti-FasL or anti-caspase-3 antibodies. Actin was used as a protein-loading control.

HSCs were incubated with TMMC for various times and cell lysates were subjected to immunoblot analysis using anti-FasL and anti-caspase-3 antibodies. As shown in Fig. 4B, TMMC induced expression of FasL starting from 12 h after treatment. While treatment with TMMC for up to 8 h caused a slight reduction of procaspase-3, incubation with TMMC for longer time (12 to 24 h) led to an almost complete disappearance of procaspase-3, suggesting that FasL expression by TMMC treatment is associated with induction of apoptosis via caspase-3. This result is consistent with the observation that >50% HSC death was observed after 12 h of TMMC exposure (Fig. 1A).

### 3.5. TMMC reduces activated HSCs in acute $CCl_4$ in vivo

To evaluate whether TMMC reversed liver fibrosis in a rat model of acute $CCl_4$ intoxication, TMMC was treated at 24 h and 48 h after $CCl_4$ injection. Liver sections stained with hematoxylin and eosin showed $CCl_4$ administration produced inflammatory cell infiltration and hepatocyte degeneration, but these changes were negligible observed in TMMC-treated liver intoxicated with $CCl_4$ (Fig. 5A). There were no significant differences in the histological appearance in rats treated with TMMC alone when compared with untreated control rats (data not shown).

Acute $CCl_4$ administration activates quiescent HSCs into α-SMA-positive, proliferating myofibroblast-like cells (Ikejima et al., 2001). To investigate the effect of TMMC on activated HSCs *in vivo*, α-SMA protein and mRNA expression in the liver were assessed by immunohistochemistry and real-time PCR, respectively. $CCl_4$ increased α-SMA immunoreactivity compared with control liver, but TMMC attenuated this induction (Fig. 5B). There were no significant differences in α-SMA immunoreactivity in rat treated with TMMC alone when compared with untreated control rats (data not shown). Similarly, TMMC inhibited the increase in α-SMA mRNA expression induced by $CCl_4$ (Fig. 5C).

As apoptosis is suggested as an elimination process of activated HSCs in liver injury (Canbay et al., 2004; Elsharkawy et al., 2005; Henderson and Iredale, 2007), we examined whether the reduction in α-SMA-positive cells (Fig. 5 B and C) was due to apoptosis. We therefore measured cleaved PARP, a substrate of active caspase-3, in whole liver tissues using immunoblot analysis. Fig. 5D shows cleaved PARP was readily detected in TMMC-treated liver intoxicated with $CCl_4$.

## 4. Discussion

Since HSCs are the key cell type involved in hepatic fibrosis (Pinzani and Marra, 2001; Lotersztajn et al., 2005), induction of apoptosis in activated HSCs is an appealing therapeutic approach to halting or reversing hepatic fibrosis (Canbay et al., 2004; Elsharkawy et al., 2005; Henderson and Iredale, 2007). As proof of concept, it has recently been observed that resolution of hepatic fibrosis in rats was associated with apoptosis of HSCs, and some antifibrogenic chemicals are proposed to act by the mechanism of inducing HSC apoptosis (Li et al., 2001; Wright et al., 2001; Kweon et al., 2003).

The synthetic chalcone, TMMC, has potent anti-inflammatory effects in murine macrophages and human intestinal epithelium (Lee et al., 2006b, 2007). We previously showed that TMMC at lower concentrations (5–20 μM) inhibits HSC proliferation induced by platelet derived growth factor (Lee et al., 2006a). Here, TMMC at higher concentrations (30–50 μM) induces apoptosis in activated HSCs, which was prevented by Z-DEVD-FMK, a specific caspase-3 inhibitor, and Z-VDA-FMK, a pan-caspase inhibitor. These data suggested that TMMC-induced apoptosis in activated HSCs involves the caspase-dependent pathway. Because hepatocyte damage might result in HSC activation, HSC selectivity is a crucial for apoptosis-inducing agent (Elsharkawy et al., 2005; Hagens et al., 2006). TMMC at these concentrations ranges did not affect the viability of rat primary hepatocytes *in vitro* (Fig. 1D). Therefore, our results suggested that TMMC exerted antifibrotic effects, possibly through two different mechanisms, anti-proliferation and apoptosis induction, depending on its concentrations.

Epigenetic mechanisms have emerged as major determinants of gene expression, both under physiological and pathological conditions. In addition to promoter methylation, histone modification, particularly histone acetylation, is considered as a key principle of epigenetic regulation (Khan and Krishnamurthy, 2005; Fuks, 2005). Histone deacetylation, which is associated with a repressed chromatin state, is tightly controlled by two enzymes, histone acetyltransferase (HAT) and histone deacetylases (HDAC) (Csordas, 1990; Grunstein, 1997; Hassig and Schreiber, 1997). Recent study reports that HDAC inhibitor, trichostatin A, exerts antifibrotic effect by suppressing myofibroblast differentiation of HSCs (Niki et al., 1999; Rombouts et al., 2002). However, the effect of HDAC inhibitor on apoptosis of activated HSC has not been studied so far. Therefore, we tested whether the effect of TMMC on apoptosis in activated HSCs was attributable to the inhibition of HDAC. TMMC increased intracellular levels of acetylated histone H3 and H4 (Fig. 2A) and dose-dependently inhibited HDAC activity (Fig. 2B), indicating that the induction of histone hyperacetylation by TMMC most likely results from inhibition of HDAC. Because HDAC inhibitors can modulate the expression of apoptosis-related genes (Medina et al., 1997; Cao et al., 2001; Bernhard et al., 2001), we examined the expression of apoptosis-related genes to identify the gene regulated by HDAC inhibitor, TMMC. TMMC strongly up-regulates the expression of FasL mRNA in a dose-dependent manner expression (Fig. 3).

Fas is a member of the tumor necrosis factor receptor superfamily. This cell surface receptor together with its cognate ligand, FasL, induces apoptosis (Nagata and Golstein, 1995; Depraetere and Golstein, 1997;

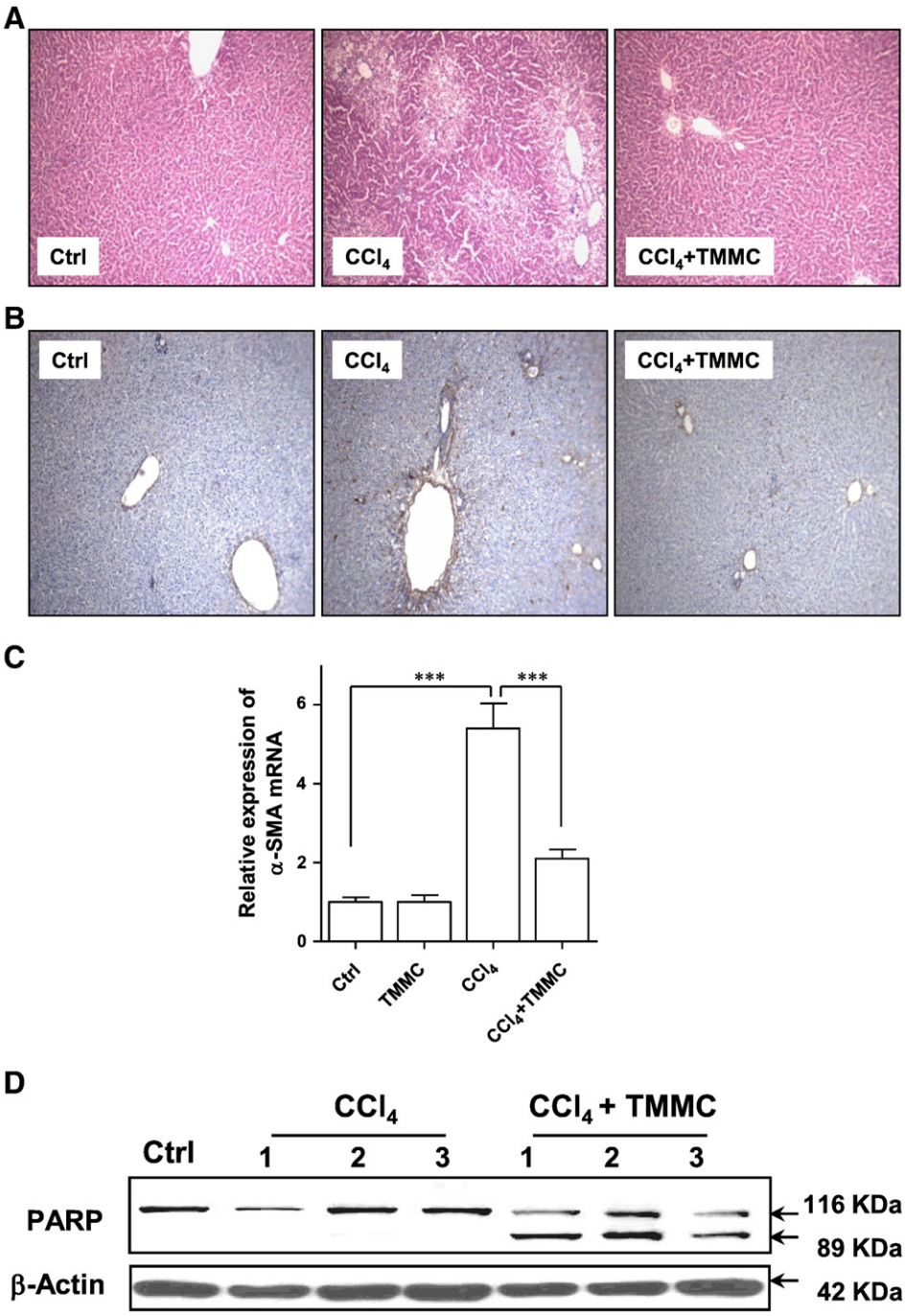


**Fig. 5.** Effects of TMMC on liver histology and expression of α-SMA in $CCl_4$-intoxicated rat liver. TMMC (50 mg/kg, p.o.) or vehicle alone was administrated to rats at 24 and 48 h after $CCl_4$ injection (3 ml/kg, s.c.). Liver tissues were obtained 72 h after $CCl_4$ injection. (A) Representative photographs of liver histology from control (*left panel*), $CCl_4$ treatment alone (*middle panel*), and $CCl_4$ + TMMC (*right panel*) are shown (H&E staining, original magnification ×100). (B) α-SMA expression in liver was detected by immunohistochemical staining. Representative photographs from control (*left panel*), $CCl_4$ treatment alone (*middle panel*), and $CCl_4$ + TMMC (*right panel*) are shown (original magnification ×100). (C) α-SMA mRNA expression was determined by real-time PCR analysis of whole liver. Data are mean ± S.D., n = 3 rats. $^{***}P<0.001$. (D) Cleavage of PARP. Whole liver lysate was subjected to SDS-PAGE and immunoblotting analysis with anti-PARP antibody and actin as a protein-loading control.

Nagata, 1999). To test whether TMMC-induced apoptosis required FasL synthesis, we incubated HSCs with CHX, which inhibits protein synthesis. Preincubation with CHX inhibited TMMC-induced cleavage of caspase-3 and PARP, indicating that *de novo* protein synthesis is required for TMMC-induced apoptosis. In addition, TMMC induced FasL synthesis paralleled maximal procaspase-3 proteolysis (Fig. 4B). These results suggest that TMMC enhances FasL expression resulting in caspase-3 activation and subsequent induction of apoptosis.

Moreover, we determined whether TMMC reversed liver fibrosis in a rat model of acute $CCl_4$ intoxication. Treatment with TMMC at 24 and 48 h after injection of $CCl_4$ significantly reduced the levels of aspartate transaminase and alanine transaminase in serum (data not shown). TMMC also improved hepatocyte necrosis and inflammatory cell infiltration in $CCl_4$-injuried liver (Fig. 5A). These results suggest that TMMC protects against $CCl_4$-induced acute liver injury. TMMC treatment also decreased α-SMA expression and mRNA levels, indicating elimination of activated HSCs. TMMC treatment after injection of $CCl_4$ increased hepatic accumulation of the PARP cleavage product (Fig. 5D). This result suggests that TMMC reduced activated HSCs *in vivo*, at least in part, through the induction of apoptosis. Taken

together, TMMC may be a successful application of HSC apoptosis-inducing strategy for the treatment of liver fibrosis in the future. However, the effect of TMMC on other cell types in the liver must be investigated.

In conclusion, we found that TMMC enhances FasL expression resulting in caspase-3 activation and subsequent induction of apoptosis in activated HSCs *in vitro*. This is the first study elucidating the possible link between HDAC inhibition and apoptosis in activated HSCs. However, the underlying mechanism remains to be defined. Our results also show that TMMC reduces hepatic fibrosis in the $CCl_4$ acute rat *in vivo*. Thus, TMMC may have therapeutic potential by inducing HSC apoptosis and inhibiting liver fibrogenesis.

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