BIOCHEMICAL PHARMACOLOGY 72 (2006) 1322–1333

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# 2′,4′,6′-Tris(methoxymethoxy) chalcone attenuates hepatic stellate cell proliferation by a heme oxygenase-dependent pathway

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ARTICLE INFO

*Article history:*
Received 1 June 2006
Accepted 8 August 2006

*Keywords:*
2′,4′,6′-Tris(methoxymethoxy) chalcone
GSH
ERK
AP-1
Hepatic stellate cell

*Abbreviations:*
AP-1, activator protein-1
DEM, diethyl maleate
ERK, extracellular signal-regulated kinase
HSC, hepatic stellate cell
HO-1, heme oxygenase-1
MAPK, mitogen-activated protein kinase
MEK, mitogen-activated protein kinase kinase
PI3K, phosphatidylinositol 3-kinase
SnPP, tin protoporphyrin
TMMC, 2′,4′,6′-tris(methoxymethoxy) chalcone

ABSTRACT

Proliferation of hepatic stellate cells (HSCs) is central for the development of fibrosis during liver injury. We have shown previously that butein (3,4,2′,4′-tetrahydroxychalcone) suppresses myofibroblastic differentiation of rat HSCs. Our aim in this study was to determine whether a new synthetic chalcone derivative, 2′,4′,6′-tris(methoxymethoxy) chalcone (TMMC) inhibits HSC proliferation induced by serum- or platelet-derived growth factor (PDGF). TMMC significantly inhibited growth factor-induced HSC proliferation. The inhibition of PDGF-induced proliferation by TMMC was associated with the phosphatidylinositol 3-kinase-Akt-$p70^{S6K}$ pathways. TMMC induced the expression of heme oxygenase 1 (HO-1) in HSCs. Using the chemical inhibitor tin protoporphyrin, we also found that the inhibitory action of TMMC on PDGF-induced proliferation is mediated by HO-1. Glutathione (GSH) depletion produced by TMMC activated extracellular signal-regulated kinase (ERK), which led to c-Fos expression and transactivation of activator protein 1 (AP-1) and HO-1 gene expression in the HSCs. These results demonstrate that TMMC preferentially activates ERK and that this activation leads to the transcriptional activation of AP-1 and consequently to HO-1 expression. HO-1 expression might be responsible for the antiproliferative effect of TMMC on HSCs.



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0006-2952/$ – see front matter 
doi:10.1016/j.bcp.2006.08.004

## 1. Introduction

Liver fibrosis is a common response to chronic liver injury and is characterized by increased deposition of extracellular matrix [1,2]. Hepatic stellate cells (HSCs) are the primary cells producing the extracellular matrix that contributes to hepatic fibrosis [3,4]. During liver fibrosis, HSCs become activated and transform into proliferating fibroblast-like cells, which produce large amounts of collagen. To prevent the progression of hepatic fibrosis, various types of compounds that interfere with HSC proliferation and activation have been developed as antifibrogenic agents [5,6].

We have shown previously that butein (3,4,2′,4′-tetrahydroxychalcone) suppresses myofibroblastic differentiation of rat HSCs [7]. However, pharmacokinetic studies have reported a low oral bioavailability of native butein [8,9]. That is, after administration of butein, large amounts of butein metabolites such as conjugated butein are found in the systemic circulation and are excreted into bile and urine. We have tried to chemically modify chalcone to improve the bioavailability of butein. In the process of synthesizing the chalcone derivatives, we found that 2′,4′,6′-tris(methoxymethoxy) chalcone (TMMC) [10] displays a potent antiproliferative effect, which is mediated by induction of heme oxygenase 1 (HO-1) in HSCs.

Glutathione (GSH) is a major cellular sulfhydryl source in nonprotein matter and plays a pivotal role in redox control in mammalian cells. It is also involved in the phase II drug metabolizing reaction as a cofactor of GSH S-transferase. Since GSH S-transferase is one of the abundantly expressed cytosolic proteins in most cells, the enzyme consumes GSH when a mass of the substrate fluxed resulting in its depletion and eventually cells to commit into the oxidative stress. It has been reported that GSH S-transferase substrates such as phorone and diethyl maleate (DEM) induce a decrease in GSH contents and a concomitant increase in HO activity in the liver of rats [11,12].

HO is the enzyme that catalyzes the degradation of the heme group to produce carbon monoxide, biliverdin, and free iron. Biliverdin is converted to bilirubin by biliverdin reductase, and free iron is used in metabolism or is sequestered by ferritin [13]. So far, three isoforms of HO have been fully characterized. HO-2 and HO-3 are constitutive isozymes [14,15], whereas HO-1 is induced by a variety of stimuli in various types of cells, including HSCs [16]. The exact functional role of HO-1 is not fully understood, although growing evidence indicates that HO-1 has antiproliferative effects [17–19]. Recent studies show that HO-1 is expressed in HSCs, is induced during chronic liver injury, and is part of an important pathway to limit progression of fibrosis during chronic liver diseases [16,20,21]. In light of the antifibrogenic role of HO-1, the specific activation of HO-1 gene expression by pharmacological modulation may represent a novel target for therapeutic intervention.

In this study, we demonstrated the effects of TMMC on growth factor-induced proliferation and the main intracellular signaling pathways elicited by platelet-derived factor (PDGF) in HSCs. We also show that the inhibitory effect of TMMC on cell proliferation is mediated by induction of HO-1 and that its molecular mechanisms involves the regulation of HO-1.

## 2. Materials and methods

### 2.1. Reagents and cell isolation

TMMC was synthesized as described previously [22]. All reagents were from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise indicated. Tin protoporphyrin (SnPP), an inhibitor of HO activity, was from Porphyrin Products, Inc. (Logan, UT, USA). The inhibitors of mitogen-activated protein kinase (MAPK), PD98059, U0126, SP600125, and SB203580 were from Calbiochem (San Diego, CA, USA). Rat HSCs were isolated from the livers of Sprague-Dawley rats as described previously [23–25]. Experimental manipulations were performed on cells between the third and fifth serial passages.

### 2.2. Cell proliferation analysis

Cell proliferation was evaluated by bromodeoxyuridine (BrdU) incorporation [26] using an ELISA kit (Roche Applied Science). HSCs were plated onto a 96-well microtiter plate at a density of $2 \times 10^4$ cells/well, washed three times in serum-free William's Medium E (WME, Gibco BRL, Gaithersburg, MD), and left in this medium for 1 h after the final wash. The cells were then incubated in fresh serum-free medium for 24 h. Serum-deprived HSCs were treated and incubated at 37 °C for another 24 h, including a 4 h pulse labeling with BrdU, as stated in the appropriate figures. After the culture medium was removed, the cells were fixed, incubated with anti-BrdU antibody conjugated with peroxidase, and the incorporated BrdU was detected with a subsequent substrate reaction according to the manufacturer's instructions.

### 2.3. Western blot analysis

Whole-cell lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrotransferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). Nitrocellulose membranes were incubated with specific antibodies against HO-1, total-ERK, and α-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibodies against phosphorylated ERK, Akt, and $p70^{S6K}$ were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Immunoreactive bands were detected by incubating with anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

### 2.4. Northern blot analysis

Total cellular RNA was extracted using TRIZOL® reagent (Invitrogen, Carlsbad, CA), separated by electrophoresis (20 μg/lane), and transferred to a nylon membrane as described previously [23]. The membranes were hybridized using [$^{32}$P]-labeled HO-1 cDNA. Specific cDNA probes were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using the selective primers and cloned in a TA vector (Promega, Madison, WI, USA). The primers used were: rat HO-1 sense, 5′-CAC GCA TAT ACC CGC TAC CT-3′, corresponding to nucleotides 510–529, and antisense, 5′-TCT GTC ACC CTG TGC TTG AC-3′ corresponding to nucleotides 699–718, according to

GenBank accession number NM_012580. Purified PCR products were sequenced to confirm gene identity. Prehybridization and hybridization were performed at 65 °C, and the membranes were then washed as described previously [23]. Reactive bands were detected by autoradiography using X-ray film (Agfa-Gevaert, Belgium).

### 2.5. Determination of GSH

Cells were washed twice with cold PBS and scraped into 100 μl of 4.3% sulfosalicylic acid and 392.5 μl of 0.1 M phosphate buffer. The mixture was centrifuged and the supernatants neutralized with 7.5 μl 5 M KOH per 500 μl total volume. The GSH recycling method was performed as described previously [10,27]. GSH standards were prepared in the background buffer and treated in the same way as the samples. Fifty-microliter samples of cell lysates or standards were transferred into the wells of microtiter plates. After addition of 100 μl of reaction mixture [0.1 M sodium phosphate buffer, pH 7.5, containing 2.5 mM EDTA, 0.15 mM 5,5′-dithio-bis (2-nitrobenzoic acid), 0.2 mM NADPH, and 1.0 U/ml GSH reductase], absorbance at 405 nm was read at 30 s intervals over 5 min. Cell protein was measured using the Bradford reagent (Bio-Rad, Hercules, CA, USA) with BSA as a standard.

### 2.6. Electrophoretic mobility shift assay

Nuclear extracts were prepared as previously described [10,28]. Electrophoretic mobility shift assay (EMSA) was performed according to the protocol accompanying Gel Shift Assay System (Promega). Briefly, double-stranded oligonucleotide probes containing activator protein 1 (AP-1) binding sequences were end labeled with [γ-$^{32}$P]-ATP using T4 polynucleotide kinase. The nuclear extract (5 μg) was incubated at room temperature for 20 min with a [$^{32}$P]-labeled probe in a binding buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 0.25 mg/ml of poly(dI–dC), and 20% glycerol. DNA-nuclear protein complexes were separated from the DNA probe by electrophoresis on a native 4% acrylamide gel, and the gel was vacuum dried and subjected to autoradiography.

### 2.7. RT-PCR

Total RNA (5 μg) was reverse transcribed using the Moloney Murine Leukemia Virus reverse transcriptase (Gibco BRL) and the resultant cDNA were diluted 10-fold for PCR. Oligonucleotide primer sequences were as follows: rat c-Fos sense, 5′-TAC TAC CAT TCC CCA GCC-3′, corresponding to nucleotides 219–236, and antisense, 5′-GCT CTA CTT TGC CCC TTC-3′, corresponding to nucleotides 508–525, according to GenBank accession number X06769. PCR reactions were carried out in the presence of 1.5 mM $MgCl_2$ for 30 cycles at the following temperatures and times: denaturation at 94 °C for 30 s; annealing at 57 °C for 30 s; extension at 72 °C for 10 s; followed by a final extension at 72 °C for 10 min. The amplified PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining. The integrity of cDNA samples was confirmed using primers specific for rat GAPDH [23]. Purified PCR products were sequenced using an autosequencer (ABI 377 DNA sequencer; Perkin-Elmer) and these sequences were compared with those in the BLAST nucleotide program (blastn) to confirm gene identity.

### 2.8. Statistical analysis

Data were analyzed using a one-way ANOVA and Tukey's multiple comparison tests when comparing means of more than three. Calculations were performed with the GraphPad Prism program (GraphPad Software, San Diego, CA, USA).

## 3. Results

### 3.1. Effect of TMMC on serum-stimulated and PDGF-induced cell proliferation

To evaluate the effect of TMMC on cell proliferation of cultured HSCs, we examined the incorporation of BrdU into chromosomal DNA. Serum-deprived HSC were either maintained in

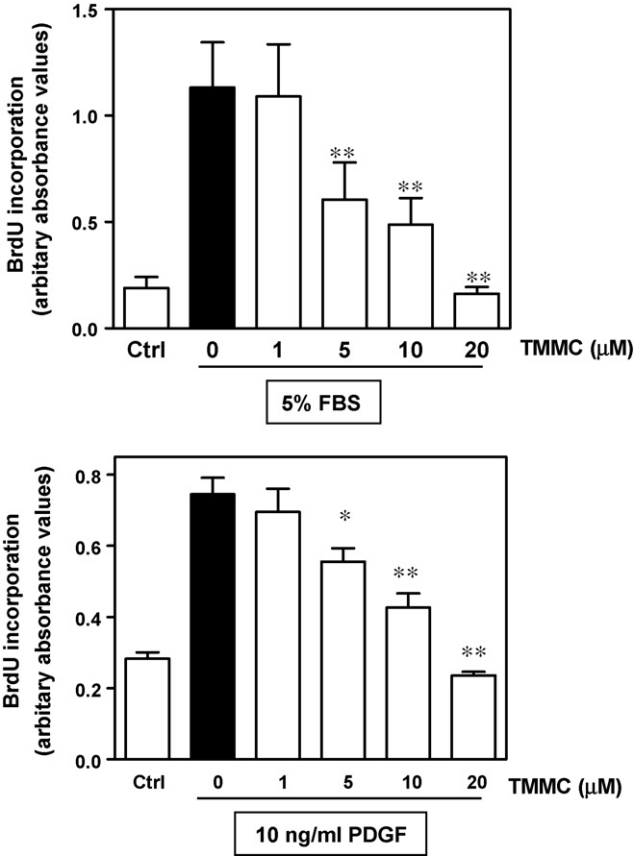


**Fig. 1 – Effect of TMMC on BrdU incorporation by HSCs. Seventy-five to 80% confluent cells were incubated for 24 h in serum-deprived medium and then pretreated for 1 h with increasing concentrations of TMMC before the start of the 24 h incubation with FBS (5%, upper panel) or PDGF (10 ng/ml, lower panel). Cells were pulsed with BrdU during the last 4 h of incubation. Results are means ± S.D. of three independent experiments, each performed using triplicate wells. $^{*}P < 0.05$ and $^{**}P < 0.001$ vs. FBS or PDGF alone.**

serum-rich media (5% FBS) or incubated in 10 ng/ml PDGF, with or without TMMC at the indicated concentrations for 24 h. As shown in Fig. 1, compared with control, 5% FBS increased the incorporation of BrdU into chromosomal DNA of cultured HSC by six times and 10 ng/ml PDGF increased BrdU incorporation by three times. TMMC significantly decreased the FBS-stimulated proliferation of HSC in a dose-dependent manner: to 53.4 ± 15.4% of control values with 5 μM TMMC, to 43.0 ± 11.0% with 10 μM TMMC, and to 14.4 ± 2.8% with 20 μM TMMC (Fig. 1A). In HSCs stimulated with 10 ng/ml PDGF, TMMC significantly decreased BrdU incorporation in a dose-dependent manner: by 25.4 ± 5.1% in cells incubated with 5 μM TMMC, by 42.7 ± 12.7% in cells incubated with 10 μM TMMC, and by 68.3 ± 9.0% in cells incubated with 20 μM TMMC (Fig. 1B).

To exclude toxic effects of TMMC in these experimental conditions, we conducted a cell viability test using the MTT assay. Up to the concentration of 20 μM, TMMC had no significant cytotoxic effect (data not shown). Thus, we chose TMMC at concentrations of 1–20 μM for subsequent experiments.

### 3.2. Effect of TMMC on ERK, Akt, and p70$^{S6K}$ activation

Western blot analysis was performed to evaluate the effect of TMMC on the PDGF-signaling cascade. Activation of Ras by PDGF is followed by sequential activation of Raf, mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase (ERK) [29]. The phosphatidylinositol 3-kinase (PI3K)-Akt pathway is also activated after PDGF treatment of HSCs [30,31]. Because p70$^{S6K}$ is downstream of PI3K [32], its activation is an indirect measure of PI3K activation [33].

HSCs were pretreated for 1 h with the indicated concentrations of TMMC and then exposed to PDGF for 15 min. Contrary to our expectation, regardless of the concentration, TMMC did not inhibit PDGF-induced Akt and p70$^{S6K}$ activation. Moreover, TMMC-treated cells induced ERK phosphorylation beyond that induced by PDGF alone (Fig. 2).

### 3.3. TMMC induces HO-1 expression

We have shown previously that TMMC induces HO-1 in murine macrophage [10]. We investigated whether further increase in ERK phosphorylation by TMMC (Fig. 2) is associated with HO-1 expression. TMMC was added for various times (1, 2, 4, or 8 h) before the introduction of PDGF, and ERK phosphorylation and HO-1 expression were then determined by Western blot analysis. As shown in Fig. 3A, the marked increase in ERK phosphorylation observed at 1 h of pretreatment returned to the level of expression observed with PDGF alone at 8 h of pretreatment. In contrast, the increase in HO-1 expression was dependent on the pretreatment time.

To exclude the possibility that HO-1 induction was responsible for the effects of PDGF [34], we investigated the temporal response of HO-1 in HSCs treated with 20 μM TMMC at both the mRNA and protein levels in the absence of PDGF. The induction of HO-1 by TMMC was evident as early as 4 h, and this augmentation lasted for at least 24 h (Fig. 3B). This

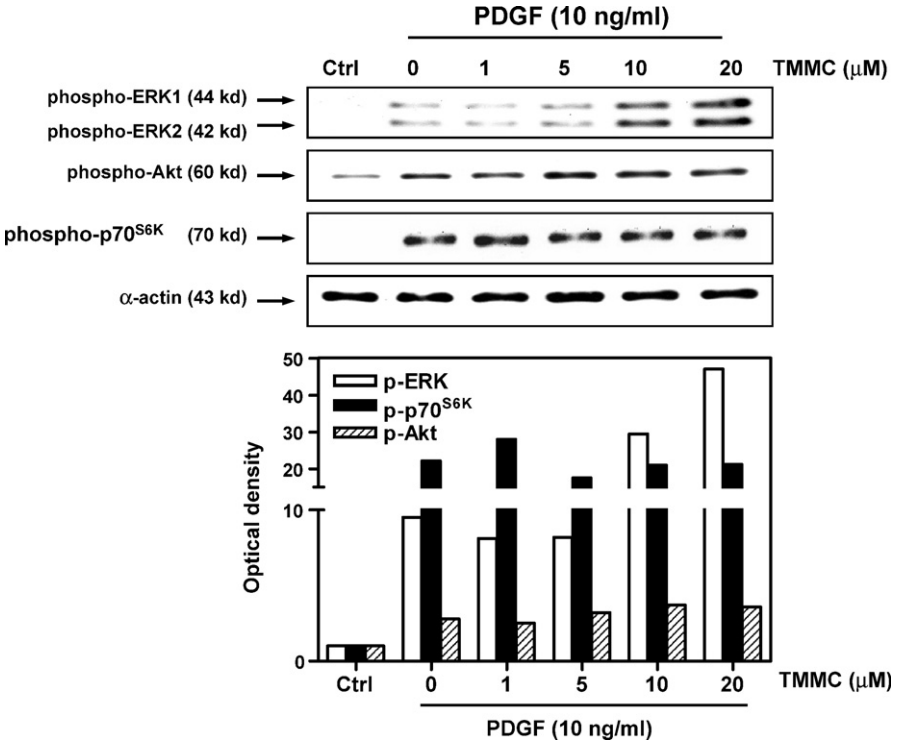


**Fig. 2 – Effect of TMMC on PDGF-induced ERK, Akt, and p70$^{S6K}$ activation of cultured HSCs. HSCs were pretreated with TMMC at the indicated doses for 1 h and then stimulated with 10 ng/ml PDGF for 15 min. Cell lysates were separated by electrophoresis, transferred to nitrocellulose, and then incubated with phosphospecific antibodies. α-Actin expression, as a marker of activated HSCs, was used to show equal loading. The densitometry values were normalized to their respective α-actin densitometry value.**

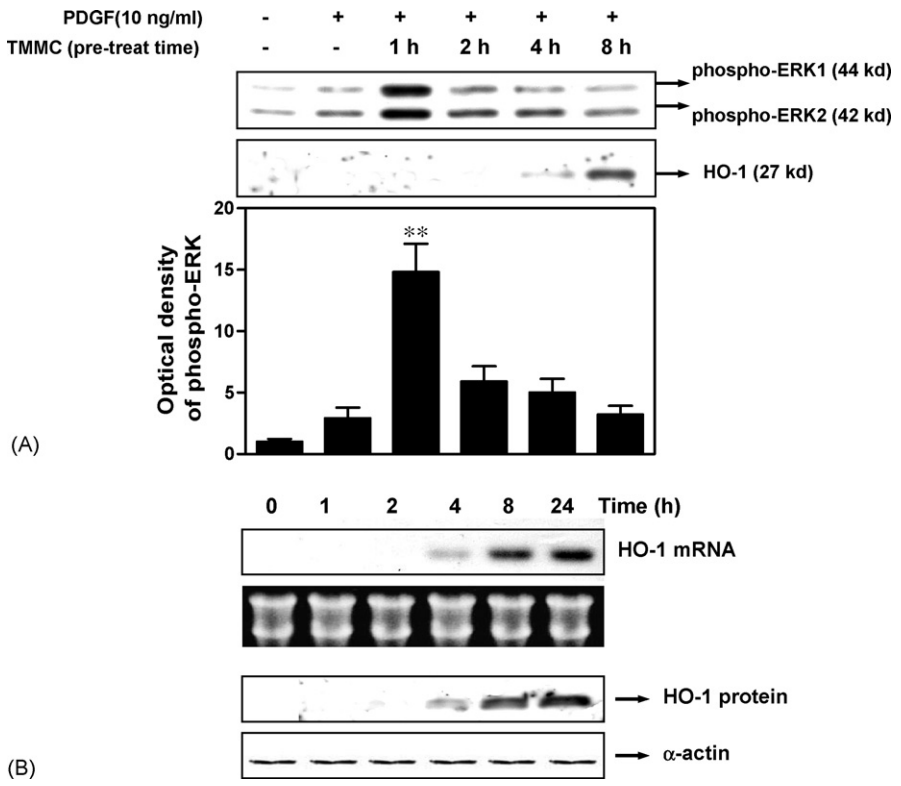


**Fig. 3 – (A) Preincubation with TMMC for different durations alters the expression of phosphorylated ERK and HO-1 in activated HSCs. Confluent cells were cultured for 24 h in serum-deprived medium and then incubated with 20 μM TMMC for various times (1, 2, 4, or 8 h) and then stimulated with 10 ng/ml PDGF. After 15 min, cell lysates were extracted from treated and untreated HSC and then analyzed by Western blot. The densitometric data of phosphorylated ERK expression are means ± S.D. from three independent experiments. $^{**}P < 0.001$ vs. PDGF alone. (B) Time-dependent induction of HO-1 mRNA and protein by TMMC. Cells were treated with 20 μM TMMC for various times (1, 2, 4, 8, or 24 h) and the expression of HO-1 mRNA and protein in cells was analyzed by Northern and Western blot, respectively. Ethidium bromide staining of ribosomal RNA (18S and 28S) and α-actin were used to show equal loading.**

result demonstrates that TMMC preferentially induce HO-1 in HSCs and that induction of HO-1 gene expression by TMMC occurs at both the transcriptional and translational levels.

### 3.4. *HO-1 mediates the inhibition of proliferation by TMMC*

Although results shown in Fig. 1 suggest that TMMC inhibits the proliferation of HSCs, pretreatment with TMMC for 1 h at any concentration did not inhibit the ERK, Akt and $p70^{S6K}$ phosphorylation in the PDGF-signaling event (Fig. 2). Because HO-1 expression was evident even 8 h after the incubation with TMMC (Fig. 3B), we pretreated HSCs for 12 h with increasing concentrations of TMMC and then exposed these HSCs to PDGF (10 ng/ml) for 15 min. As shown in Fig. 4A, in the absence of TMMC, PDGF markedly increased the activation of ERK, Akt, and $p70^{S6K}$. TMMC attenuated the level of phosphorylation of Akt and $p70^{S6K}$ under PDGF stimulation in a dose-dependent manner, whereas TMMC did not affect PDGF-induced ERK phosphorylation. This result suggests that TMMC does not modify the ERK pathway.

We found that TMMC inhibited PDGF-dependent HSC proliferation (Fig. 1) and TMMC caused a sustained upregulation of HO-1 in HSCs (Fig. 3). To determine whether the inhibitory actions of TMMC on PDGF-dependent proliferation are mediated by its induction of HO-1, we exposed cells to TMMC, PDGF, and SnPP, an inhibitor of HO activity (Fig. 4B). The addition of SnPP caused a loss of the suppressive effects of TMMC on PDGF-stimulated cell proliferation. Indeed, the decrease in DNA synthesis elicited by TMMC was significantly reduced in cells pretreated with SnPP. This result suggests that TMMC-mediated increases in HO activity contribute to the antiproliferative effects of TMMC.

### 3.5. *TMMC induction of HO-1 involves ERK phosphorylation*

Because TMMC did not affect PDGF-induced ERK phosphorylation, we hypothesized that TMMC modulates the phosphorylation of ERK. We determined whether HO-1 induction by TMMC is mediated through ERK activation. To confirm that the ERK pathway is not responsible for the inhibition of proliferation by TMMC in PDGF-stimulated HSCs, we examined HSC proliferation after preincubation with specific inhibitors of MAPKs. HSCs were preincubated with PD98059 (a specific inhibitor of ERK), SP600125 (a specific inhibitor of c-Jun N-terminal kinase or JNK), or SB203580 (a specific inhibitor of p38).

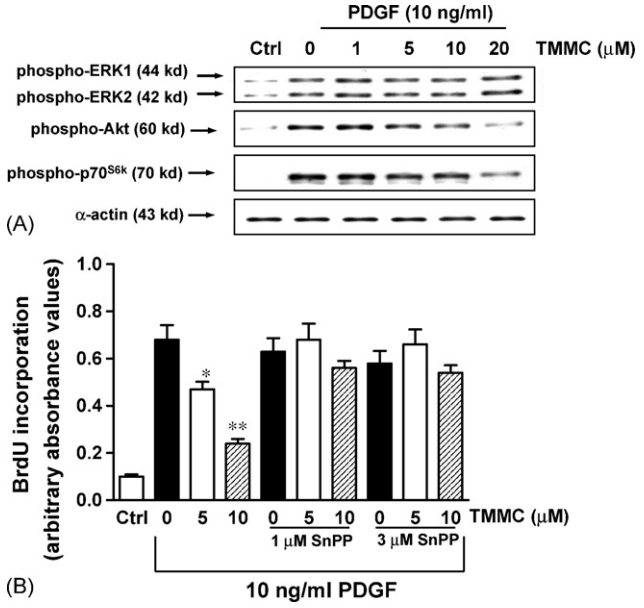


**Fig. 4 – (A) Effect of TMMC on PDGF-induced ERK, Akt, and $p70^{S6K}$ activation of cultured HSCs. HSCs were pretreated with TMMC at the indicated doses for 12 h and then stimulated with 10 ng/ml PDGF for 15 min. Cell lysates were separated by electrophoresis, transferred to nitrocellulose, and then incubated with phosphospecific antibodies. α-Actin expression, as a marker of activated HSCs, was used to show equal loading. (B) Antiproliferative effect of TMMC is mediated by HO-1 in rat HSCs. HSCs were pretreated for 1 h with or without SnPP and were stimulated further with 10 ng/ml PDGF together with 5 or 10 μM TMMC for 24 h. Cells were pulsed with BrdU during the last 4 h of incubation. Results are means ± S.D. of three independent experiments, each performed using triplicate wells. $^{*}P < 0.01$ and $^{**}P < 0.001$ vs. PDGF alone.**

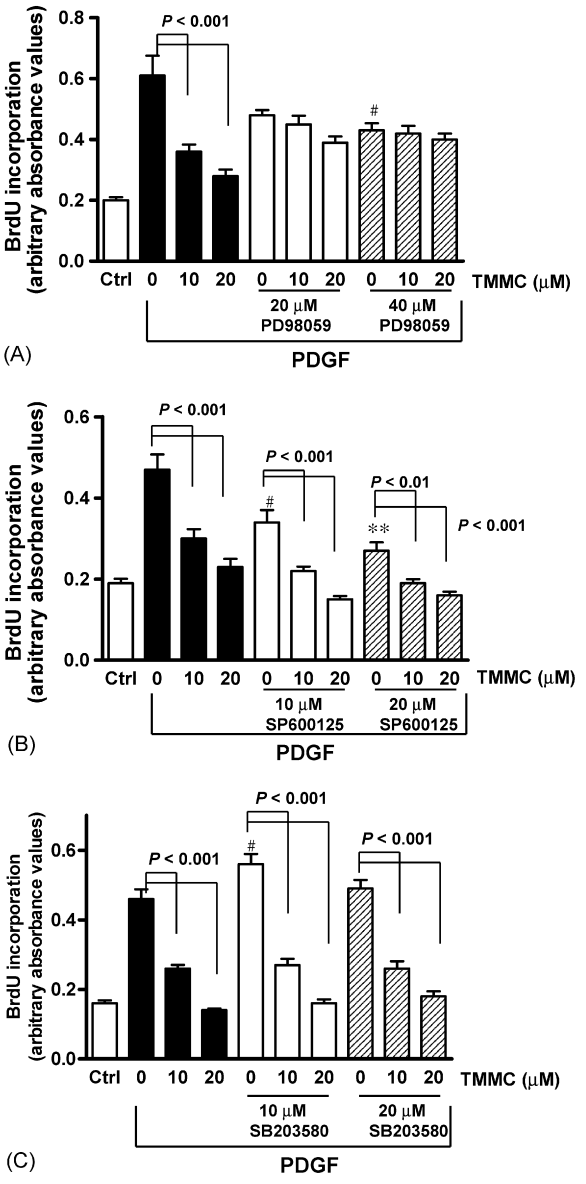


**Fig. 5 – Effect of different kinase inhibitors on inhibition of proliferation by TMMC in PDGF-induced HSCs. HSCs were preincubated with PD98059 (A), SP600125 (B), or SB203580 (C) for 1 h and then stimulated further with 10 ng/ml PDGF together with 10 or 20 μM TMMC for 24 h. Cells were pulsed with BrdU during the last 4 h of incubation. Results are means ± S.D. of three independent experiments, each performed using triplicate wells. $^{\#}P < 0.05$ and $^{**}P < 0.001$ vs. PDGF alone-treated cells in the absence of inhibitors.**

As shown in Fig. 5, blocking ERK activity before PDGF stimulation inhibited HSC proliferation. Inhibition of JNK also regulates HSC proliferation, although inhibition of p38 increases HSC proliferation. These results confirm that the ERK and JNK pathway are involved in PDGF-stimulated HSC proliferation [35,36]. Suppression of HSC proliferation by TMMC was abrogated by PD98059 (Fig. 5A), whereas pre-incubation with SP600125 (Fig. 5B) or SB203580 (Fig. 5C) had no effect on the inhibition of proliferation by TMMC in PDGF-stimulated HSCs. Together with the observation that the TMMC-mediated increase in HO activity contributes to the antiproliferative effects of TMMC (Fig. 4B), these results indicate that the TMMC-mediated increase in HO-1 expression can be blocked by PD98059. These data suggest that the ERK pathway is involved in the regulation of HO-1 expression by TMMC.

We next used time-course experiments to confirm the activation of ERK in HSCs incubated with 20 μM TMMC. As shown in Fig. 6A, HSCs exhibited a basal level of phosphorylated ERK. The TMMC-induced increase in ERK phosphorylation peaked after 60 min of exposure. The level of phosphorylated ERK increased significantly by 5.2 times after 60 min of exposure to TMMC.

To finally confirm the role of ERK signaling in the HO-1 expression events, we assessed the effects of another MEK inhibitor, U0126, on the antiproliferative effect by TMMC in PDGF-stimulated HSCs. As shown in Fig. 6B, the effect of U0126 on proliferation in PDGF-stimulated HSCs was virtually identical to that of PD98059 (i.e., compared with condition in Fig. 5A). These data confirm and further strengthen the involvement of ERK signaling in HO-1 expression by TMMC.

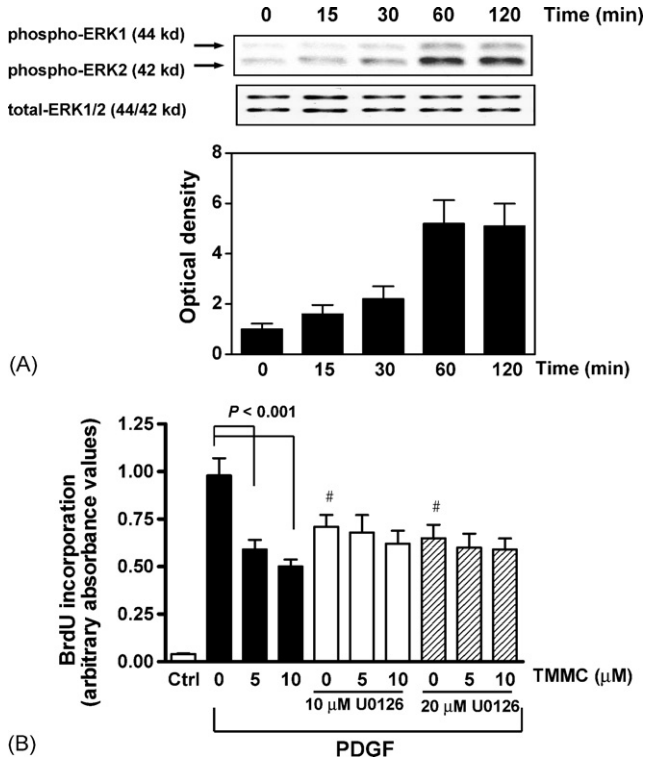


**Fig. 6 – (A) Time-course experiment on the effect of TMMC on ERK phosphorylation. Confluent cells were incubated for 24 h in serum-deprived medium and then treated with TMMC (20 μM) for the indicated periods (15, 30, 60 or 120 min). Cell lysates were obtained and separated by electrophoresis, transferred to nitrocellulose, and then incubated with anti-phospho-ERK antibody. Representative data from three independent experiments are shown. Equal loading of proteins in each lane was confirmed by probing the membrane with anti-total ERK antibody. Densitometric data are means ± S.D. from all experiments. (B) Effect of MEK inhibitor on inhibition of proliferation by TMMC in PDGF-stimulated HSCs. HSCs were preincubated with U0126 for 1 h and then stimulated further with PDGF together with 5 or 10 μM TMMC for 24 h. Cells were pulsed with BrdU during the last 4 h of incubation. Results are means ± S.D. of three independent experiments, each performed using triplicate wells. #P < 0.05 vs. PDGF alone-treated cells in the absence of inhibitors.**

### 3.6. *Effects of GSH content modification by TMMC on HO-1 induction*

We next investigated the molecular mechanisms of HO-1 expression induced by TMMC in HSCs. We have previously described the decrease in intracellular GSH levels by TMMC exposure, which plays a role in triggering HO-1 expression [10]. As shown in Fig. 7A, TMMC-induced GSH depletion also occurred rapidly and was already detectable after 1 h in HSCs. GSH levels decreased further during the next 2 h, although, after 4 h, the cells started to replenish their intracellular pool. This result suggests that the intracellular GSH level may play a role in the effect of TMMC. We then examined the effects of intracellular GSH on HO-1 expression in TMMC-treated HSCs. The cells were treated with glutathione ethyl ester, an established method to increase cellular levels of GSH [37] or DEM, an electrophilic agent that rapidly depletes intracellular GSH by conjugation through the transferase reaction [38]. As shown in Fig. 7B, pretreatment with glutathione ethyl ester abolished HO-1 induction by TMMC. In contrast, TMMC was a more potent inducer of HO-1 when GSH was depleted by DEM. These results indicate that intracellular GSH levels modulate TMMC-induced HO-1 expression. In addition, pretreatment of cells with the ERK inhibitor PD98059 blunted HO-1 protein induction elicited by TMMC in a dose-dependent manner. This result is consistent with the observation that TMMC stimulates ERK phosphorylation.

To further investigate whether the decreased intracellular GSH level directly accounts for TMMC-induced ERK phosphorylation, we investigated the effects of glutathione ethyl ester or DEM on TMMC-induced ERK phosphorylation. Treatment with glutathione ethyl ester markedly inhibited TMMC-induced ERK phosphorylation, whereas treatment with DEM increased TMMC-induced ERK phosphorylation. Incubation of cells with DEM alone also slightly induced ERK phosphorylation (Fig. 7C). These results suggest that the decrease in the GSH level by TMMC lies upstream of ERK activation.

### 3.7. *TMMC induction of HO-1 mediated through AP-1 activation*

Because AP-1 is the major transcription factor involved in HO-1 gene transcription [39,40], we explored the effect of TMMC on the transactivity of AP-1. To determine whether TMMC can activate AP-1 in HSCs, cells were exposed to 20 μM TMMC for 1, 2, 4, or 8 h and AP-1 binding activity was analyzed by EMSA. As shown in Fig. 8A (left panel), DNA binding activity to the AP-1 consensus sequence increased in HSCs exposed to TMMC at 4 h and 8 h after exposure. In addition, analysis of the AP-1 binding site showed that TMMC stimulated the binding activity in a dose-dependent manner at 8 h after TMMC treatment (Fig. 8A, right panel).

The transcription complex AP-1 is a dimer composed of c-Jun and c-Fos family members, whose expression and activity are regulated tightly by the MAPK family of serine-threonine kinase [41,42]. In the case of c-Fos, the activation of ERK leads to increased expression of c-Fos transcriptional activity [43,44]. Because our findings suggested that the ERK pathway is involved in the regulation of HO-1 expression by TMMC, we investigated the effects of TMMC on induction of c-Fos mRNA expression. Treatment with increasing concentrations of TMMC provoked dose-dependent increases in the levels of c-Fos mRNA (Fig. 8B). Notably, the profile of the response to different concentrations of TMMC of c-Fos mRNA expression was consistent with that of TMMC-induced activation of AP-1 (Fig. 8A, right panel). Because our findings suggested that the TMMC-induced decreased GSH level lies upstream of ERK activation, we evaluated whether intracellular GSH levels and ERK phosphorylation are involved in the TMMC-induced c-Fos mRNA expression. HSCs were pretreated with GSH-modulating agents and ERK inhibitors in the presence of 20 μM TMMC (Fig. 8C). Glutathione ethyl ester suppressed the TMMC-mediated increase of c-Fos mRNA expression in a

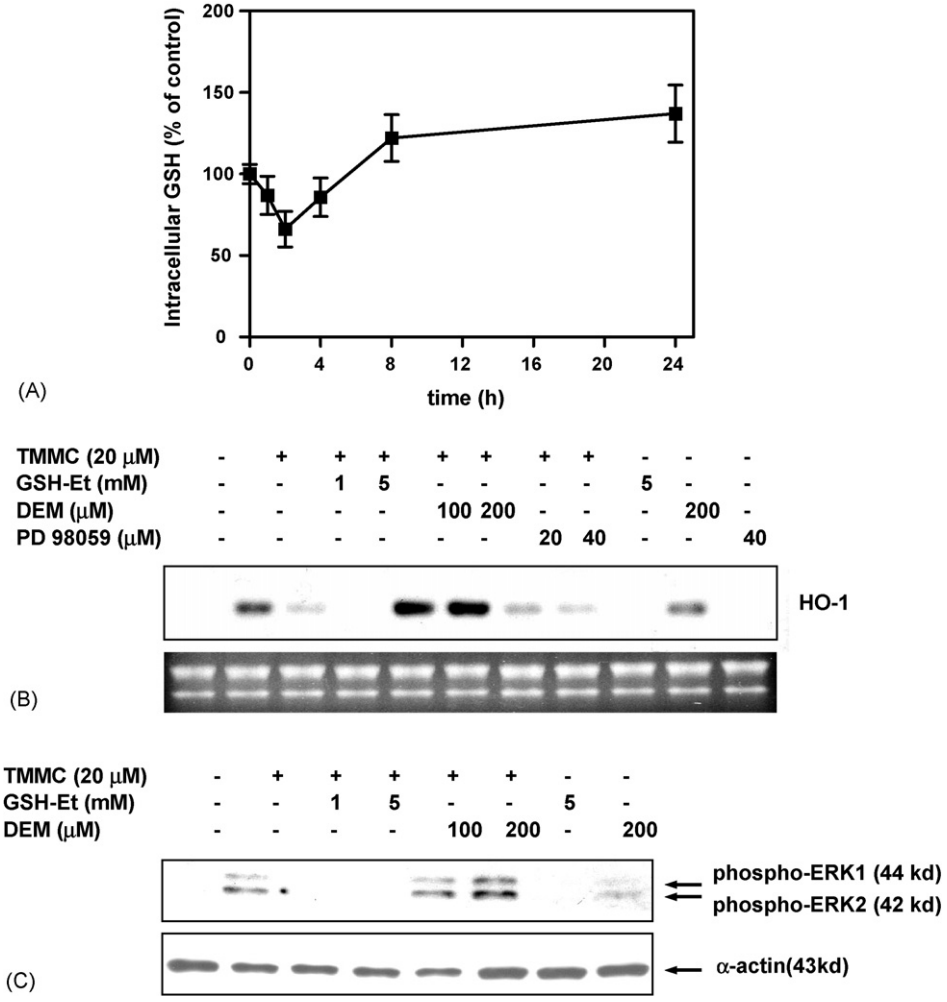


**Fig. 7 – (A) Intracellular GSH level of HSCs incubated with 20 μM TMMC for 1, 2, 4, 8, or 24 h. Data are expressed as the percentage of the untreated control value, which was set at 100%. Results are presented as means ± S.D. of three experiments. (B) Effects of GSH-modulating agents or ERK kinase inhibitor on HO-1 mRNA expression in the presence of TMMC. Cells were pretreated with glutathione ethyl ester (GSH-Et, 1 or 5 mM), DEM (100 or 200 μM), or PD98059 (20 or 40 μM) for 1 h and then incubated with 20 μM TMMC for 5 h. Total RNA was extracted and the level of HO-1 mRNA was determined by Northern blot. Ethidium bromide staining of ribosomal RNA (18S and 28S) is shown as the control. (C) Effects of GSH-modulating agents on ERK phosphorylation in the presence of TMMC. Cells were pretreated with glutathione ethyl ester (GSH-Et, 1 or 5 mM) or DEM (100 or 200 μM) for 1 h and then incubated with 20 μM TMMC for 2 h. Cell lysates were obtained and separated by electrophoresis, transferred to nitrocellulose, and then incubated with anti-phospho-ERK antibody. Representative data from three independent experiments are shown. α-Actin expression, as a marker of activated HSCs, was used to show equal loading.**

dose-dependent manner. Blocking MEK phosphorylation with U0126 also inhibited TMMC-stimulated c-Fos mRNA expression. In contrast, treatment with DEM did not significantly alter TMMC-stimulated c-Fos mRNA expression. These results suggest that GSH depletion and activation of the ERK play an obligatory role in induction of c-Fos mRNA expression by TMMC in HSCs.

## 4. Discussion

HSCs play a central role in the development of liver fibrosis. During this process, HSCs proliferate, accumulate in the diseased liver, and secrete fibrosis components [3,4]. Blocking HSC proliferation is considered an important target for the development of antifibrotic drugs. We have shown previously that butein, a type of chalcone derivative, has beneficial effects, including antifibrogenic effects, in the liver [7,45] and anti-inflammatory activity [28]. However, pharmacokinetic studies have reported a low oral bioavailability of native butein [8,9]. Thus, we tried to chemically modify chalcone to improve the bioavailability of butein. In the process of synthesizing the chalcone derivatives, we found that TMMC inhibits HSC proliferation. We have also reported previously that TMMC inhibits the lipopolysaccharide-induced increase in nitric oxide production and activation of expression of the inducible

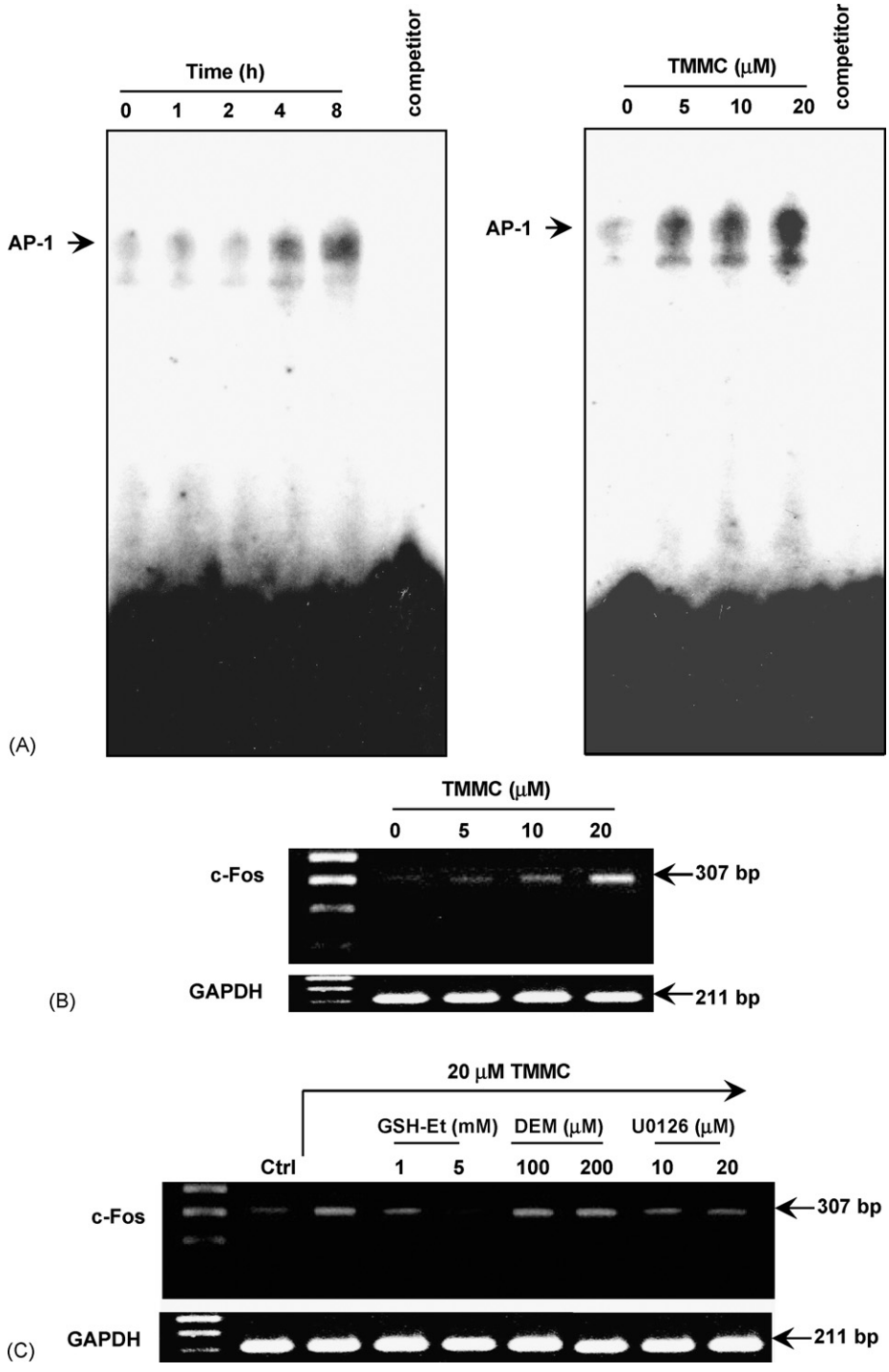


**Fig. 8 – (A) Induction of AP-1 binding activity by TMMC: time-course study of AP-1 binding activity after TMMC treatment. Nuclear extracts from HSCs treated with 20 μM TMMC at the indicated times (1, 2, 4 or 8 h) were incubated with radiolabeled AP-1 oligonucleotide (left panel). Dose-dependent induction of AP-1 binding activity by TMMC. Cells were treated with different concentrations of TMMC for 8 h. AP-1-binding activity was assessed by EMSA (right panel). (B) Dose-dependent c-Fos mRNA expression in response to TMMC in HSCs. Cells was treated with different concentration of TMMC for 4 h and total RNA was extracted. c-Fos mRNA expression was analyzed by RT-PCR. The amount of RNA loaded in each lane was confirmed by GAPDH. (C) Effects of GSH-modulating agents or MEK kinase inhibitor on c-Fos mRNA expression in the presence of TMMC. Cells were pretreated with glutathione ethyl ester (GSH-Et, 1 or 5 mM), DEM (100 or 200 μM), or U0126 (10 or 20 μM) for 1 h and then incubated with 20 μM TMMC for 4 h. Total RNA was extracted and the level of c-Fos mRNA was determined by RT-PCR. The amount of RNA loaded in each lane was confirmed by GAPDH.**

nitric oxide synthase gene [10]. Our current data indicate that TMMC, a synthetic chalcone derivative, inhibits growth factor-induced HSC proliferation. We note that the effect of TMMC is dependent on its action as an HO-1 inducer.

In the dose range 5–20 μM, TMMC inhibited cell proliferation induced by serum or PDGF without affecting cell viability. To elucidate which steps of PDGF signaling are affected by TMMC, we investigated the effects of this drug on the downstream pathways induced by PDGF. Preincubation with TMMC for 1 h did not affect signaling events, including the phosphorylation of Akt and $p70^{S6K}$. Moreover, TMMC-treated cells induced ERK phosphorylation beyond that induced by PDGF alone. At this point, we investigated whether the further increase in ERK phosphorylation by TMMC is associated with HO-1 expression. We have shown previously that TMMC induces HO-1 in murine macrophage [10]. As expected, TMMC induced HO-1 in HSCs and induction of HO-1 gene expression at both the mRNA and protein levels. Based on our observation that HO-1 expression was evident even 8 h after incubation with TMMC, HSCs were pretreated for 12 h with TMMC and then exposed to PDGF. Although the effects of PDGF require activation of ERK [30,35], the inhibitory effect of TMMC on PDGF-induced proliferation is not exerted through interference with this kinase. However, our data indicate that TMMC attenuates the PDGF-induced activation of Akt and $p70^{S6K}$ in a dose-dependent manner; this activation is necessary for PDGF-induced proliferation in HSCs [30,31]. Because Akt is positioned downstream of PI3K and $p70^{S6K}$ lies downstream of Akt, these findings indicate that inhibition of the PI3K-Akt-$p70^{S6K}$ pathway, a crucial step in proliferative signaling in HSCs [46], is responsible for the inhibitory effect of TMMC on PDGF-induced proliferation.

The antiproliferative effect of the HO-1 pathway in HSCs was described *in vitro* in rat HSCs and then confirmed *in vivo* in animal models of $CCl_4$-induced fibrotic liver [16]. Our current data provide pharmacological evidence that HO-1 induction is responsible for the observed TMMC-mediated inhibition of HSC proliferation. The antiproliferative effect of TMMC on PDGF-stimulated cell growth was blocked by chemical inhibition of HO activity using SnPP. This is consistent with the finding by Li et al. [20] that HO-1 is a major antifibrogenic pathway in the liver.

In general, HO-1 gene and protein expression is modulated by MAPK activation. Three major subgroups of the MAPK family identified to date include ERK, JNK, and p38 MAPK. Depending on the stimuli specificity, contradictory results on the regulatory role of different MAPK pathways for HO-1 gene expression have been observed [47,48]. In this study, we found the effect of TMMC on HO-1 induction is dependent on ERK, but not on JNK or p38. We showed that suppression of HSC proliferation by TMMC was abrogated by PD98059, whereas preincubation with SP600125 or SB203580 had no effect on the inhibition of proliferation by TMMC in PDGF-stimulated HSCs. Similarly, another MEK inhibitor, U0126, also abrogated the antiproliferative effects of TMMC in PDGF-stimulated HSCs. Considering that TMMC-mediated increases in HO activity contribute to the antiproliferative effects of TMMC (Fig. 4B), this raises the possibility that TMMC-mediated increase in HO-1 expression might be blocked by ERK MAPK inhibitor. That is, the ERK pathway might be involved in the regulation of HO-1 expression by TMMC. This observation is consistent with our previous study showing ERK MAPK activation by TMMC in RAW 264.7 macrophages [10].

In addition, our data demonstrate that transient depletion of cellular GSH by TMMC induces expression of HO-1 mRNA in HSCs. Addition of glutathione ethyl ester decreased the induction of HO-1 mRNA expression by TMMC, whereas DEM upregulated TMMC-induced HO-1 expression. Our results indicate that the induction level of HO-1 mRNA coincides with the decreased cellular GSH level. Recently, it is reported that DEM elicit reduction of DNA synthesis and inhibition of α1(I) collagen mRNA expression in human myofibroblasts [21]. GSH synthesis occurs in the cytosol of all mammalian cells and its rate of synthesis is determined by two key factors: the availability of cysteine and the activity of γ-glutamylcysteine synthetase [49,50]. In contrast, Choi et al. [51] recently reported that decreased hepatic GSH levels correlate with a decrease in GSH synthetase activity. At this point, more work is needed to clarify the exact molecular mechanism governing GSH depletion by TMMC (e.g., alterations in the enzymes governing GSH synthesis or metabolism). In addition, our results show that GSH depletion by TMMC preferentially induces ERK activation in HSCs. Treatment with glutathione ethyl ester markedly reduced TMMC-induced ERK phosphorylation, whereas treatment with DEM increased TMMC-induced ERK phosphorylation. These results suggest that the TMMC-induced decrease of the intracellular GSH level may trigger a primary signal that induces later activation of the ERK cascade.

The molecular mechanisms underlying HO-1 induction are complex and tightly regulated at the level of transcription. Deletion and mutational analyses of the regulatory element of the HO-1 gene indicate that the AP-1-binding site plays an important role in mediating HO-1 gene regulation and is a commonality in response to multiple agents in the activation mechanism of HO-1 [39,40]. Therefore, we examined the role of AP-1 in the induction of the HO-1 by TMMC in HSCs. TMMC induced AP-1 binding activity in a time and dose-dependent manner. Our data suggest that TMMC is capable of inducing AP-1 activation in HSCs, which, as previously indicated with strong evidence [39,40], leads to the induction of HO-1 gene transcription. In addition, we also found that TMMC treatment induced c-Fos transcription in dose-dependent manner that coincided with the dose-dependent increase of AP-1 binding activity in the nuclear extract of the HSCs. These results indicate that TMMC activates c-Fos transcription, thus leading to AP-1 activation and consequently to HO-1 gene expression in HSCs. Our data suggest that GSH depletion and activation of ERK play an obligatory role in the induction of c-Fos mRNA expression by TMMC in HSCs. These findings are consistent with the observation that activation of ERKs leads to the coordinated stimulation of c-Fos expression by acting on transcription factors bound at the c-Fos promoter [52]. Thus, the data presented here are compatible with the schematic representation shown in Fig. 9. As suggested in this diagram, TMMC decreases the intracellular GSH level and the decreased GSH level may play a critical role in HO-1 expression by activation of ERK and AP-1.

In conclusion, we have shown that TMMC inhibits the proliferation of rat HSCs induced by PDGF through an inhibiting the PI3K-Akt-$p70^{S6K}$ pathway. We found that TMMC

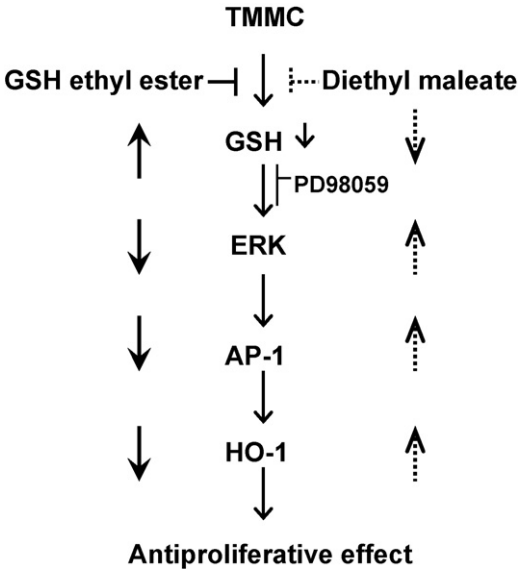


**Fig. 9 – Schematic diagram of TMMC-induced HO-1 expression in HSCs. TMMC decreases the intracellular GSH level and activates ERK. Such ERK activation leads to activation of AP-1, resulting in expression of HO-1, an antifibrogenic protein. Solid and dotted arrows indicate series of reaction by addition of diethyl maleate and GSH ethyl ester, respectively.**

increases the expression of HO-1 in HSCs and that the antiproliferative effects may be mediated by this induction. Our results also show that GSH depletion by TMMC promotes HO-1 expression in HSCs by sequential activation of the ERK MAPK pathway and AP-1. HO-1 can be regard as a potential therapeutic target in liver fibrosis. In this respect, further study of TMMC, a novel and more potent inducer of HO-1, will facilitate the development of new applications for the prevention and treatment of hepatic fibrosis.

## Acknowledgment

This work was supported by the Korea Research Foundation Grant (KRF-2004-005-E00093).

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