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**Modulation of human cytochrome P450 1B1 expression by 2,4,3',5'-
tetramethoxystilbene**

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Running title: Modulation of Human CYP1B1 by TMS

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator, CYP, cytochrome P450; DMBA, 7,12-dimethylbenz[*a*]anthracene; DRE, dioxin responsive element; EROD, 7-ethoxyresorufin O-deethylation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF, naphthoflavone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TMS, 2,4,3',5'-tetramethoxystilbene

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Abstract

We have previously shown that 2,4,3',5'-tetramethoxystilbene (TMS), a synthetic *trans*-stilbene analogue, is one of the most potently selective inhibitors of recombinant human cytochrome P450 (CYP) 1B1 *in vitro*. In the present studies, the effects of TMS on CYP1B1 expression were investigated in human cancer cells. TMS significantly inhibited CYP1-mediated 7-ethoxyresorufin O-deethylation (EROD) activity in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced MCF-7 cells or lung microsomes of Sprague Dawley rats treated with 7,12-dimethylbenz[*a*]anthracene (DMBA). TCDD-stimulated CYP1B1 protein and mRNA expression were significantly suppressed by TMS in a concentration-dependent manner in MCF-7, MCF-10A, and HL-60 cells. While TMS down-regulated TCDD-induced CYP1B1 gene expression, the levels of AhR and ARNT mRNA expression were not changed by TMS treatment. In human cancer cells, TMS induced apoptotic cell death and the cytotoxic effects of TMS were significant when the cells were incubated with TCDD. CYP1B1 was able to convert TMS to a metabolite(s) when incubated with NADPH. Metabolic activation of TMS by CYP1B1 induced by TCDD may mediate cellular toxicity of TMS in human cancer cells because the sensitivity to TMS in MCF-7 cells treated with TCDD were more significant than in HL-60 cells treated with TCDD. Taken together, our results indicate that TMS acts as a strong modulator of CYP1B1 gene expression as well as a potent selective inhibitor *in vitro*. The ability of TMS to induce apoptotic cell death in tumor cells, as well as CYP1B1 inhibition, may contribute to its usefulness for cancer chemoprevention.

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Introduction

Human cytochrome P450 1B1 (CYP1B1) is an important enzyme involved in the metabolic activation of diverse procarcinogens, such as arylamines, polycyclic and nitro aromatic hydrocarbons (Shimada et al., 1996). CYP1B1 is mainly found in extrahepatic steroidogenic tissues (e.g. ovary, testis, and adrenal gland), and in steroid-responsive tissues, e.g. breast, uterus, and prostate. The enzyme is also found in many other extrahepatic tissues, including kidney, thymus, lung, spleen, brain, heart, colon, and intestine (Shimada et al., 1996; Sutter et al., 1994).

CYP1B1 genes have been cloned and characterized from mice, rats, and humans (Savas et al., 1994; Shen et al., 1994; Walker et al., 1995; Tang et al., 1996). The human *CYP1B1* gene is located on chromosome 2 at 2p21-22 spanning approximately 12 kilobases of DNA and contains three exons and two introns (Tang et al., 1996). Expression of CYP1B1 is induced by polycyclic aromatic hydrocarbons, which act via the Ah receptor-mediated pathway. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a well-known agonist for activating transcription of the *CYP1B1* gene (Sutter et al., 1991). Several positive and negative regulatory DNA elements have been identified in the 5' region of the *CYP1B1* gene and may be involved in Ah receptor-mediated gene regulation (Wo et al., 1997).

A major interest in CYP1B1 arises from the fact that it is the most catalytically efficient 17 β -estradiol (E2) 4-hydroxylase characterized to date and 4-hydroxy E2 has been suggested to be carcinogenic (Hayes et al., 1996; Shimada et al., 1999). Moreover, CYP1B1 is expressed at a higher level in various human cancers, including brain, skin,

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testis, and breast (Murray et al., 1997; McKay et al., 1995). Because of the postulated significant role of CYP1B1 on carcinogenicity of E2, CYP1B1 is regarded as a target enzyme for blocking tumor initiation, and selective inhibition of CYP1B1 may prevent E2-related tumor formation (Liehr et al., 1997; Shimada et al., 1997; Li and Li, 1987; Liehr et al., 1986).

Many *trans*-stilbene analogues have been shown to inhibit the CYP1 family of proteins, which function in the metabolic activation of procarcinogens. Resveratrol inhibits CYP1A1 and CYP1B1 (Chun et al., 1999; Chang et al., 2000; Chen et al., 2004). Rhapontigenin and 3,5,3',4',5'-pentamethoxystilbene exhibit a potent and selective inhibition of CYP1A1, and 3,4'-dimethoxy-5-hydroxystilbene showed inhibition of EROD activity of both CYP1A1 and CYP1B1 with IC₅₀ values of 0.1 μ M, respectively (Chun et al., 2001; Lee et al., 2004). *Trans*-stilbene analogues also modulate expression of CYP1A1 and CYP1B1 in cultured human cells (Chen et al., 2004; Lee et al., 2004). Previously, we reported that 2,4,3',5'-tetramethoxystilbene (TMS), a methoxy derivative of oxyresveratrol, is a potentially selective inhibitor of CYP1B1 (Chun et al., 2001; Kim et al., 2002; Chun and Kim, 2003). TMS was a competitive inhibitor of recombinant human CYP1B1 with a K_i of 3 nM (Chun et al., 2001). It also strongly inhibited 4- and 2-hydroxylation of E2 by CYP1B1-expressing membranes or purified CYP1B1. The activation of 2-amino-3, 5-dimethylimidazo[4,5-*f*]quinoline (MeIQ) in an *Escherichia coli lac*-based mutagenicity tester system containing functional human CYP1B1 was strongly inhibited by TMS. TMS also inhibited human CYP1B1-mediated melatonin 6-hydroxylation with an IC₅₀ value of 30 nM (Ma et al., 2005).

The aim of the present study was to elucidate the effect of TMS on CYP1B1 activity and expression in human tumor cells and to determine the possibility of TMS as

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an adjuvant agent for cancer protection. The results show that TMS is able to inhibit activity and expression of CYP1B1 induced by TCDD in human tumor cells. Enhancement of sensitivity to TMS by TCDD in tumor cells emphasizes the possibility that CYP1B1-dependent metabolism may be involved in TMS-mediated cytotoxicity.

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Materials and Methods

Cell culture

MCF-7 cells were grown in MEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin. MCF-10A cells were incubated in DMEM and Ham's F-12 medium (1:1, v/v) containing 10 $\mu\text{g/ml}$ bovine insulin, 100 ng/ml cholera toxin, 0.5 $\mu\text{g/ml}$ hydrocortisone, 20 ng/ml recombinant human epidermal growth factor, 2 mM L-glutamine, 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 5% horse serum. HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. For treatment, 5 x 10⁵ cells were plated in 1 ml of culture medium and incubated for 1-3 days, as indicated. After incubation, the cells were harvested by scrapping in ice-cold 0.1 M potassium phosphate buffer (pH 7.4). Cells were centrifuged at 1,000x g for 5 min at 4°C and the pellets were resuspended in the same buffer. The cells were sonicated for 30 s at 4°C and stored at -70°C.

EROD Enzyme Assay

EROD activity was determined for the measurement of P450 1 activities (Burke et al., 1985). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.4), 2 mg/ml bovine serum albumin (BSA), 2 μM ethoxyresorufin, and rat lung microsomes or cellular sonicates. The reaction mixtures were preincubated at 37°C for 3 min, and the reactions were initiated by addition of 120 μM NADPH. Incubations were performed in

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a shaking water bath at 37°C for 20 min and terminated by addition of 1 ml of methanol. The formation of resorufin was determined fluorometrically with a Perkin-Elmer LS 5 spectrofluorimeter with excitation and emission wavelengths of 550 nm and 585 nm, respectively. Protein concentrations were estimated using the bicinchoninic acid method according to the supplier's recommendations (Pierce Chemical Co., Rockford, IL) using BSA as a standard.

Western blot analysis

Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in 5% (w/v) nonfat dried milk in Tris-buffered saline with 0.05 % tween 20 (TBST) overnight at 4°C. Membranes were then incubated for 1 h with rabbit anti-CYP1B1 polyclonal antibodies at a 1:1000 dilution in TBST. After subsequent incubation with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody, proteins were visualized by an ECL method.

RT-PCR

Total RNA was extracted using Trizol reagent (Gibco BRL, Gaithersburg, MD). RT-PCR was performed using an Access RT-PCR system purchased from Promega (Madison, WI) according to the manufacturer's instructions. Human CYP1B1 cDNA was amplified by PCR using a sense primer (5'-AACGTCATGAGTGCCGTGTGT-3') and an antisense primer (5'-GGCCGGTACGTTCTCCAAATC-3') with 30 cycles by denaturation at 94°C for 20 s, annealing at 58°C for 20 s, and extension at 72°C for 40 s (Li et al., 1998). Human Ah receptor cDNA was amplified using a sense primer (5'-

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CATGCTTGGTCTTTTATGC-3') and an antisense primer (5'-TTCCCTTTCTTTTTCTGTCC-3') with 30 cycles by denaturation at 94°C for 20 s, annealing at 52°C for 20 s, and extension at 72°C for 40 s. Human ARNT cDNA was amplified using a sense primer (5'-GGAACAAGATGACAGCCTAC-3') and an antisense primer (5'-CAGAAAGCCATCTGCTGCC-3') with 30 cycles by denaturation at 94°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 40 s. Human β -actin cDNA was amplified using a sense primer (5'-CTACAATGAGCTGCGTGTGG-3') and an antisense primer (5'-TAGCTCTTCTCCAGGGAGGA-3') with 30 cycles by denaturation at 94°C for 20 s, annealing at 52°C for 20 s, and extension at 72°C for 40 s. The number of amplification cycles was optimized in preliminary experiments to ensure that the PCR has not reached its plateau. The amplified PCR products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining.

Determination of cell toxicity

Cells were plated onto 96-well plates and incubated at 37°C in a 5% CO₂ atmosphere. After incubation for the designated time, 10 μ g of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added per well. After incubating at 37°C for 4 h, cells were centrifuged at 1,000 x g for 5 min, the medium was removed by aspiration, and then MTT formazan crystal formed was dissolved by adding 0.15 ml of DMSO and shaking for 15 min. The absorbance at 540 nm was measured using a microplate reader. The percentages of cells surviving from each group relative to control, defined as 100% survival, were calculated.

Apoptosis assay

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Cells were harvested and washed twice with ice-cold PBS (pH 7.4). Cells were resuspended in 0.19 ml of binding buffer (pH 7.4) containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 140 mM NaCl, and 2.5 mM CaCl₂. Ten microliter of Annexin V-FITC (Zymed, South San Francisco, CA) was added per well and cells were incubated for 10 min at room temperature. Cells stained with Annexin V-FITC were washed with the binding buffer. Apoptotic cells were determined using fluorescence microscopy with excitation and emission wavelengths of 488 nm and 518 nm, respectively.

DNA fragmentation assay

Cells were harvested and lysed in 0.15 ml of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% (w/v) sodium dodecyl sulfate (SDS), and 0.5 mg/ml proteinase K. Proteolytic digestion was allowed to proceed at 50°C for 12~16 h. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with 1 volume of 7.5 M ammonium acetate and 2 volumes of absolute ethanol. Extracted chromosomal DNA was dissolved in 50 μ l of TE buffer (pH 8.0) containing 0.2% SDS, and 0.2 mg/ml DNase-free RNase. After incubation at 37°C for 1 h, DNA samples were analyzed in 1% agarose gel.

Expression of Recombinant Human CYP1B1

Coexpression (bicistronic) plasmids for human CYP1B1 and NADPH-P450 reductase were transformed into *Escherichia coli* DH5 α (Parikh et al., 1997). A single ampicillin-resistant colony of transformed cells was selected and grown in overnight culture to saturation at 37°C in LB medium containing 100 μ g/ml ampicillin. A 10-ml

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aliquot was used to inoculate each liter of Terrific Broth containing 0.2% bactopeptone (w/v), 100 $\mu\text{g/ml}$ ampicillin, 1 mM thiamine, trace elements, 0.5 mM δ -aminolevulinic acid, and 1 mM IPTG. The cultures were grown at 30°C with shaking at 200 rpm for 48 h. After incubation, cells were harvested by centrifugation at 6,500 x g for 20 min. Spheroplasts were prepared using lysozyme and disrupted by sonication. The cellular sonicates were centrifuged at 10,000 x g for 20 min and the membranes were collected by centrifugation at 110,000 x g for 90 min and were resuspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (v/v) (Guengerich et al., 1996). P450 content of whole *E. coli* cells and membranes was determined by the spectral method of Omura and Sato (1964) using an extinction coefficient of 91 $\text{mM}^{-1} \text{cm}^{-1}$ with a Shimadzu UV-160A spectrophotometer at ambient temperature. The isolated membrane fractions were stored at -70°C .

HPLC

TMS (50 μM) was incubated in 0.1 M potassium phosphate buffer (pH 7.4), containing bacterial membranes having 10 pmol of human CYP 1B1 and NADPH-P450 reductase in a final volume of 100 μl . After 5 min of preincubation at 37°C, the reaction was initiated by the addition of 1 mM NADPH and continued for 20 min with shaking. The same experiments were performed without NADPH or with heat-inactivated membranes. The reaction was terminated by the addition of 1 ml of dichloromethane. The reaction products were separated by HPLC on a 5 μm Nucleosil C18 reverse phase column (4.6 mm x 250 mm) with the mobile phase consisted of 40 % solvent A (glacial acetic acid in water, pH 2.0) and 60 % solvent B (20% solvent A and 80 % acetonitrile). The absorbance at 306 nm was measured using a Jasco UV-975 UV/VIS detector

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(Tokyo, Japan).

Data Analysis

Statistical analysis was performed by using one-way analysis of variance (ANOVA), followed by Dunnett's pairwise multiple comparison *t*-test with Graph-Pad Prism software (Graph-Pad, San Diego, CA) when appropriate. The difference was considered statistically significant at $P < 0.05$.

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Results

Suppression of CYP1B1 expression by TMS

In order to characterize whether TMS can suppress activity and expression of CYP1B1, the inhibition of EROD activity by TMS was determined in human MCF-7 tumor cells treated with TCDD (2 nM) and lung microsomes prepared from rats orally administered DMBA (200 mg/kg). TMS significantly suppressed TCDD-induced EROD activity in MCF-7 cells or DMBA-induced EROD activity in rat lung microsomes in a dose-dependent manner (Fig. 1). The suppression of TCDD-induced CYP1B1 gene expression by TMS in human tumor cell lines such as MCF-7, MCF-10A, or HL-60 was assessed by the RT-PCR and Western blot analyses (Fig. 2). TCDD (2 nM) significantly induced CYP1B1 expression in these human cell lines, especially in MCF-10A and HL-60 cells and TMS significantly suppressed the TCDD-induced CYP1B1 mRNA and protein expression (Fig. 2A and 2B). While TMS decreased TCDD-induced CYP1B1 gene expression, the levels of Ah receptor and ARNT mRNA expression were not changed by TMS treatment (Fig. 2C).

Cytotoxic effect of TMS

To determine the potential of TMS as an adjuvant agent for cancer chemoprotection, the cytotoxic effects of TMS in MCF-7 and HL-60 cells were determined. TMS exerted a concentration-dependent inhibition of cellular proliferation in the cultured cells for 72 h (Fig. 3A). As shown in Fig. 3B, treatment of MCF-7 or HL-60 cells with 1 μ M TMS significantly suppressed the cellular proliferation up to 96 h. To elucidate the ability of TMS to induce apoptotic cell death in MCF-7 and HL-60 cells, Annexin V-positive cells

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were determined using fluorescence microscopy. Fig. 3C showed that treatment with TMS (0-20 μM) for 24 h caused an appreciable increase in Annexin V-positive cells, which is indication of apoptotic cell death. TMS at 5 μM also generated the fragmentation of chromosomal DNA in HL-60 cells (Fig. 3D).

Enhancement of sensitivity to TMS by TCDD

Treatment with 2 nM TCDD for 72 h significantly decreased the cellular proliferation of MCF-7 and HL-60 cells (Table 1). TCDD (2 nM) treatment for 72 h caused 43% of suppression of in MCF-7 cell proliferation while the same concentration of TCDD suppressed 55% of HL-60 cell proliferation. The effect of TCDD on TMS-mediated cytotoxicity was determined in MCF-7 and HL-60 cells (Table 1). Interestingly, co-treatment with TCDD significantly enhanced the sensitivity to TMS in human tumor cells. Treatment with TMS (1 μM) for 72 h caused 95% of suppression of MCF-7 cell proliferation in the presence of 2 nM TCDD while the same concentration of TMS suppressed 92% of HL-60 cell proliferation. Treatment with 1 μM TMS alone for 72 h was able to block the cell growth for 66% and 73% in MCF-7 and HL-60 cells, respectively (Table 1).

Metabolism of TMS by CYP1B1

In vitro metabolism studies were performed with bacterial membranes of human CYP1B1 co-expressed with human NADPH-P450 oxidoreductase to examine whether CYP1B1 is able to metabolize TMS to its metabolite(s). As shown in Fig. 4A, TMS was metabolized by human CYP1B1 and one product (t_{R} , 7.2 min) was formed when the reaction was supported by NADPH (1 mM). The product peak was not formed when

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NADPH was not added (Fig. 4B) or membranes were heat-inactivated before added in reaction mixtures (Fig. 4C). Addition of α -naphthoflavone (NF) (10 μ M), a well-known CYP1 inhibitor, blocked the formation of a TMS metabolite (t_R , 7.2 min) by CYP1B1 (Fig. 5). We found that α -NF was also metabolized by CYP1B1 to produce a major metabolite (t_R , 8.4 min).

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DISCUSSION

We have previously demonstrated that TMS is a potent and selective competitive inhibitor of CYP1B1 (Chun et al., 2001). TMS significantly prevented CYP1B1-dependent P450 activities such as EROD and E2 hydroxylation in CYP1B1-expressing *E. coli* membranes and the purified CYP1B1 enzyme. TMS also blocked CYP1B1-dependent MeIQ genotoxicity. In this study, to evaluate the mechanisms of anticarcinogenesis by TMS in cells, we examined effect of TMS on the activity and expression of CYP1B1 in human cells. The results of these experiments indicate that TMS inhibits TCDD-induced EROD activity and also the expression of CYP1B1 in MCF-7 cells. In MCF-10A, an immortalized non-tumorigenic breast epithelium cell, and HL-60, an acute promyelocytic leukemic cell, TMS also down-regulates CYP1B1 mRNA and protein expression. Interestingly, suppression of TCDD-induced CYP1B1 expression by TMS was much more significant in MCF-10A and HL-60 cells than in MCF-7 cells. Western blot and RT-PCR analyses showed a similar concentration dependency (Fig. 2A and B). The induction of CYP1B1 expression by TCDD at 1 μ M concentration was almost completely blocked by TMS in HL-60 cells. As previously reported (Murray et al., 1997; McKay et al., 1995), we also found the basal expression of CYP1B1 in human cell lines such as MCF-7, MCF-10A, and HL-60. Although TMS significantly suppressed TCDD-induced CYP1B1 expression, Ah receptor and ARNT mRNA expression were not affected by TMS treatment. The recent demonstration that resveratrol acts as an Ah receptor antagonist and suppresses the Ah receptor-mediated DRE binding activity suggest that TMS may also suppress Ah receptor signal pathway

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(Chen et al., 2004; Ciolino et al., 1998; Casper et al., 1999). To determine the detailed mechanism of CYP1B1 modulation by TMS in cells, it will be necessary to investigate whether TMS may down-regulate *CYP1B1* mRNA expression by competing with TCDD at the Ah receptor binding site.

Because we have considered TMS as an adjuvant agent for cancer prevention, the cytotoxic effects of TMS were determined. TMS alone prevents cell proliferation in MCF-7 and HL-60 cells in a concentration- and time-dependent manner. We were also able to detect the indication of apoptotic cell deaths such as phosphatidylserine exposure measured by Annexin V-binding or chromosomal DNA fragmentation in cells treated with TMS at less than 10 μ M. Our prior work (Lee et al., 2002) and data from other laboratories (Huang et al., 1999; Clement et al., 1998; Wolter et al., 2002; Estrov et al., 2003) provide evidence that many *trans*-stilbene compounds also induce apoptotic cell death in various human tumor cells although cytotoxic effects were relatively lower than TMS. Geahlen and McLaughlin (1989) previously suggested that piceatannol (3,4,3',5'-tetrahydroxystilbene) acts as an inhibitor of protein tyrosine kinase p72^{Syk}. The ability of TMS to inhibit protein tyrosine kinases will need to be determined to understand the cytotoxic mechanism of TMS.

The enhancement of sensitivity to TMS by TCDD in tumor cells is interesting. From in vitro metabolism studies with recombinant CYP1B1, we found a significant metabolism product of TMS using HPLC. Previously we suggested that the metabolic product might be an *O*-demethylated TMS (Chun et al., 2001). We considered the possibility that the cytotoxic effect of *O*-demethylated metabolite(s) may be more potent in tumor cells. Because induction of CYP1B1 expression by TCDD increases the production of TMS metabolite(s), simultaneous treatment with TCDD may significantly

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enhance TMS-mediated protection of tumor cell proliferation. The effect of TCDD on TMS cytotoxicity is stronger in MCF-7 cells than in HL-60 cells although TCDD induction of CYP1B1 expression is substantially higher in HL-60 than in MCF-7, likely due in part to the relatively stronger metabolic ability of MCF-7 cells than HL-60 cells.

Estrogen is a known risk factor in various human tumors, especially in breast tumor. Because CYP1B1 has been detected in over 70% of the human breast tumor samples analyzed and is known as a major enzyme responsible for carcinogenic metabolism of estrogen, this enzyme is regarded as a target enzyme for blocking tumor initiation and its inhibition remains a logical target for cancer protection. The data reported here suggests that TMS acts as a modulator of CYP1B1 gene expression as well as a potentially selective inhibitor in vitro and in cultured cells. The ability of TMS to induce apoptotic cell death in tumor cells as well as CYP1B1 inhibition may be beneficial in cancer prevention. A more detailed understanding of the precise mechanism(s) by which TMS inhibits CYP1B1 and induces apoptosis may facilitate the development of a new strategy for cancer chemoprevention.

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References

- Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T and Mayer RT (1985) Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochem Pharmacol* **34**: 3337-3345.
- Casper RF, Quesne M, Rogers IM, Shirota T, Jolivet A, Milgrom E and Savouret JF (1999) Resveratrol has antagonist activity on the aryl hydrocarbon receptor: implications for prevention of dioxin toxicity. *Mol Pharmacol* **56**: 784-790.
- Chang TK, Lee WB and Ko HH (2000) Trans-resveratrol modulates the catalytic activity and mRNA expression of the procarcinogen-activating human cytochrome P450 1B1. *Can J Physiol Pharmacol* **78**:874-881.
- Chen ZH, Hurh YJ, Na HK, Kim JH, Chun YJ, Kim DH, Kang KS, Cho MH and Surh YJ (2004) Resveratrol inhibits TCDD-induced expression of CYP1A1 and CYP1B1 and catechol estrogen-mediated oxidative DNA damage in cultured human mammary epithelial cells. *Carcinogenesis* **25**:2005-2013.
- Chun YJ, Kim MY and Guengerich FP (1999) Resveratrol is a selective human cytochrome P450 1A1 inhibitor. *Biochem Biophys Res Commun* **262**:20-24.
- Chun YJ and Kim S (2003) Discovery of cytochrome P450 1B1 inhibitors as new anti-cancer agents. *Med Res Rev* **23**: 657-668.
- Chun YJ, Kim S, Kim D, Lee SK and Guengerich FP (2001) A new selective and potent inhibitor of human cytochrome P450 1B1 and its application to antimutagenesis. *Cancer Res* **61**: 8164-8170.

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- Chun YJ, Ryu SY, Jeong TC and Kim MY (2001) Mechanism-based inhibition of human cytochrome P450 1A1 by rhapontigenin. *Drug Metab Dispos* **29**:389-393.
- Ciolino HP, Daschner PJ and Yeh GC (1998) Resveratrol inhibits transcription of CYP1A1 in vitro by preventing activation of the aryl hydrocarbon receptor. *Cancer Res* **58**: 5707-5712.
- Clement MV, Hirpara JL, Chawdhury SH and Pervaiz S (1998) Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. *Blood* **92**: 996-1002.
- Estrov Z, Shishodia S, Faderl S, Harris D, Van Q, Kantarjian HM, Talpaz M and Aggawal BB (2003) Resveratrol blocks interleukin-1beta-induced activation of the nuclear transcription factor NF-kB, inhibits proliferation, causes S-phase arrest, and induces apoptosis of acute myeloid leukemia cells. *Blood* **102**: 987-995.
- Geahlen RL and McLaughlin JL (1989) Piceatannol (3,4,3',5'-tetrahydroxy-trans-stilbene) is a naturally occurring protein-tyrosine kinase inhibitor. *Biochem Biophys Res Commun* **165**:241-245.
- Guengerich FP, Martin MV, Guo Z and Chun YJ (1996) Purification of functional recombinant P450s from bacteria. *Methods Enzymol* **272**: 35-44.
- Hayes CL, Spink DC, Spink BC, Cao JQ, Walker NJ and Sutter TR (1996) 17 β -estradiol hydroxylation catalyzed by human cytochrome P450 1B1. *Proc Natl Acad Sci USA* **93**:9776-9781.
- Huang C, Ma WY, Goranson A and Dong Z (1999) Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. *Carcinogenesis* **20**: 237-242.
- Kim S, Ko H, Park JE, Jung S, Lee SK and Chun YJ (2002) Design, synthesis, and

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- discovery of novel *trans*-stilbene analogues as potent and selective human cytochrome P450 1B1 inhibitors. *J Med Chem* **45**: 160-164.
- Lee SH, Ryu SY, Kim HB, Kim MY and Chun YJ (2002) Induction of apoptosis by 3,4'-dimethoxy-5-hydroxystilbene in human promyeloid leukemic HL-60 cells. *Planta Med* **68**: 123-127.
- Lee SK, Kim Y, Kim MY, Chun YJ and Kim S (2004) Potent inhibition of recombinant human cytochrome P-450 1A1 by pentamethoxystilbene. *J Toxicol Environ Health A* **67**:1987-2000.
- Li JJ and Li SA (1987) Estrogen carcinogenesis in Syrian hamster tissues: role of metabolism. *Fed Proc* **46**:1858-1863.
- Li W, Harper PA, Tang BK and Okey AB (1998) Regulation of cytochrome P450 enzymes by aryl hydrocarbon receptor in human cells: CYP1A2 expression in the LS180 colon carcinoma cell line after treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or 3-methylcholanthrene. *Biochem Pharmacol* **56**: 599-612.
- Liehr JG (1997) Dual role of oestrogens as hormones and pro-carcinogens: tumor initiation by metabolic activation of oestrogens. *Eur J Cancer Prev* **6**:3-10.
- Liehr JG, Fang WF, Sirbasku DA and Ari-Ulubelen A (1986) Carcinogenicity of catechol estrogens in Syrian hamsters. *J Steroid Biochem* **24**:353-356.
- Ma X, Idle JR, Krausz KW and Gonzalez FJ (2005) Metabolism of melatonin by human cytochromes P450. *Drug Metab Dispos* **33**:489-494.
- McKay JA, Melvin WT, Ah-See AK, Ewen SWB, Greenlee WF, Marcus CB, Burke MD and Murray GI (1995) Expression of cytochrome P450 CYP1B1 in breast cancer. *FEBS Lett* **374**:270-272.
- Murray GI, Taylor MC, McFadyen MC, McKay JA, Greenlee WF, Burke MD and

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- Melvin WT (1997) Tumor-specific expression of cytochrome P450 CYP1B1. *Cancer Res* **57**:3026-3031.
- Omura T and Sato R (1964) The carbon-monoxide binding pigment of liver microsomes. *J Biol Chem* **239**: 2370-2378.
- Parikh A, Gillam EMJ and Guengerich FP (1997) Drug metabolism by *Escherichia coli* expressing human cytochromes P450. *Nature Biotechnol* **15**: 784-788.
- Savas Ü, Bhattacharyya KK, Christou M, Alexander DL and Jefcoate CR (1994) Mouse cytochrome P-450EF, representative of a new 1B subfamily of cytochrome P-450s. Cloning, sequence determination, and tissue expression. *J Biol Chem* **269**:14905-14911.
- Shen Z, Liu J, Wells RL and Elkind MM (1994) cDNA cloning, sequence analysis, and induction by aryl hydrocarbons of a murine cytochrome P450 gene, *CYP1B1*. *DNA Cell Biol* **13**:763-769.
- Shimada T, Gillam EMJ, Sutter TR, Strickland PT, Guengerich FP and Yamazaki H (1997) Oxidation of xenobiotics by recombinant human cytochrome P450 1B1. *Drug Metab Dispos* **29**:617-622.
- Shimada T, Hayes CL, Yamazaki H, Amin S, Hecht SS, Guengerich FP and Sutter TR (1996) Activation of chemically diversified procarcinogens by human cytochrome P-450 1B1. *Cancer Res* **56**:2979-2984.
- Shimada T, Watanabe J, Kawajiri K, Sutter TR, Guengerich FP, Gillam EMJ and Inoue K (1999) Catalytic properties of polymorphic human cytochrome P450 1B1 variants. *Carcinogenesis* **20**:1607-1613.
- Sutter TR, Guzman K, Dold KM and Greenlee WF (1991) Targets for dioxin: genes for plasminogen activator inhibitor-2 and interleukin-1. *Science* **254**:415-418.

DMD#6502

Sutter TR, Tang YM, Hayes CL, Wo YY, Jabs EW, Li X, Yin H, Cody CW and Greenlee WF (1994) Complete cDNA sequence of a human dioxin-inducible mRNA identifies a new gene subfamily of cytochrome P450 that maps to chromosome 2. *J Biol Chem* **269**:13092-13099.

Tang YM, Wo YY, Stewart J, Hawkins AL, Griffin CA, Sutter TR and Greenlee WF (1996) Isolation and characterization of the human CYP1B1 gene. *J Biol Chem* **271**:28324-28330.

Walker NJ, Gastel JA, Costa LT, Clark GC, Lucier GW and Sutter TR (1995) Rat CYP1B1: an adrenal cytochrome P450 that exhibits sex-dependent expression in livers and kidneys of TCDD-treated animals. *Carcinogenesis* **16**:1319-1327.

Wo YY, Stewart J and Greenlee WF (1997) Functional analysis of the promoter for the human CYP1B1 gene. *J Biol Chem* **272**:26702-26707.

Wolter F, Clausnitzer A, Akoglu B and Stein J (2002) Piceatannol, a natural analog of resveratrol, inhibits progression through the S phase of the cell cycle in colorectal cancer cell lines. *J Nutr* **132**: 298-302.

DMD#6502

Footnote

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Legends for figures

Fig. 1. Inhibition of EROD activity by TMS. *A.* MCF-7 cells (5×10^5 /ml) were incubated with TMS (0, 0.01, 0.1, 0.5, 1 or 5 μ M) for 48 h in the absence or presence of TCDD (2 nM). Cells were harvested and EROD activities were determined. Results are the mean \pm S.D. of three separate experiments. *Significantly different from TCDD-treated group ($p < 0.05$). *B.* Rats were orally administered with DMBA (200 mg/kg) for 5 days. Lung microsomes were isolated and EROD activity was determined in the presence of TMS (0, 0.01, 0.1, 0.5, 1 or 5 μ M). Results are the mean \pm S.D. of three separate experiments. *Significantly different from DMBA-treated group ($p < 0.05$).

Fig. 2. Effects of TMS on CYP1B1 protein and mRNA expression in cultured human cells. *A.* Western blot analyses for TCDD-induced CYP1B1 expression. MCF-7, MCF-10A, or HL-60 cells were incubated with TMS (0, 1, 5, 10 or 20 μ M) for 48 h in the absence or presence of TCDD (2 nM). Total cellular sonicates were prepared and equal amount of protein (25 μ g) in individual cellular lysates were used for Western blot analyses with antibody directed against CYP1B1. β -Actin was used as a loading control. *B.* RT-PCR analysis of TCDD-induced CYP1B1 mRNA expression. Total RNA was isolated from MCF-7 (5×10^6), MCF-10A (5×10^6), or HL-60 (1×10^7) cells. The partial coding sequence of human CYP1B1 was amplified with specific primers. The PCR products were separated on a 2% agarose gel containing ethidium bromide and

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visualized under UV light. *C.* RT-PCR analysis of AhR and ARNT mRNA expression. Total RNA was isolated from isolated from MCF-7 (5×10^6), MCF-10A (5×10^6), or HL-60 (1×10^7) cells. The partial coding sequences of human AhR, ARNT, and β -actin gene were amplified with specific primers.

Fig. 3. Induction of apoptosis by TMS. *A.* Cells (5×10^5 /ml) were incubated with the indicated concentration of TMS for 72 h. The percentage of cells surviving from each groups relative to controls were determined by MTT reduction assay. Results are the mean \pm SD of three separate experiments. *B.* Cells (5×10^5 /ml) were incubated with 1 μ M TMS for 24, 48, 72, or 96 h. MTT assay was performed and the percentage of cells surviving from each groups relative to controls were determined. Results are the mean \pm S.D. of three separate experiments. *Significantly different from DMSO-treated control group ($p < 0.05$). *C.* Cells were incubated with TMS (0, 5, 10, or 20 μ M) for 24 h. After staining cells with Annexin V-FITC, positive cells were analyzed by fluorescence microscopy. *D.* HL-60 cells were incubated with TMS (1-5 μ M) for 24 h. Chromosomal DNA was isolated and the formation of DNA fragmentation was analyzed by 1% agarose gel electrophoresis.

Fig. 4. HPLC analysis of TMS metabolism by recombinant CYP1B1. TMS (50 μ M) was incubated with bacterial membranes expressing CYP1B1 (0.1 μ M) in 0.1 M potassium phosphate buffer (pH 7.4) in the presence of 1 mM NADPH for 20 min at 37 °C. The metabolite generated was analyzed using HPLC (C_{18} reverse phase column, 4.6 mm x 250 mm). The retention times of TMS and its metabolite (M1) were 18.8 min and

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7.2 min, respectively. *A.* CYP1B1 membranes were incubated with TMS in the absence of NADPH. *B.* CYP1B1 membranes were incubated with TMS in the presence of 1 mM NADPH. *C.* CYP1B1 membranes were incubated at 90°C for 10 min for heat inactivation. Heat-inactivated CYP1B1 membranes were incubated with TMS in the presence of 1 mM NADPH.

Fig. 5. Inhibition of CYP1B1-mediated TMS metabolism by α -NF. TMS (50 μ M) was incubated with bacterial membranes expressing CYP1B1 (0.1 μ M) in 0.1 M potassium phosphate buffer (pH 7.4) in the presence of 1 mM NADPH and α -NF (10 μ M) for 20 min at 37°C. The metabolite generated was analyzed using HPLC (C₁₈ reverse phase column, 4.6 mm x 250 mm). The retention times of TMS, M1 (a TMS metabolite), α -NF, and M2 (a α -NF metabolite) were 18.8, 7.2, 21.2 and 8.4 min, respectively. *A.* CYP1B1 membranes were incubated with TMS in the presence of 1 mM NADPH. *B.* CYP1B1 membranes were incubated with α -NF in the presence of 1 mM NADPH. *C.* CYP1B1 membranes were incubated with TMS and α -NF in the presence of 1 mM NADPH.

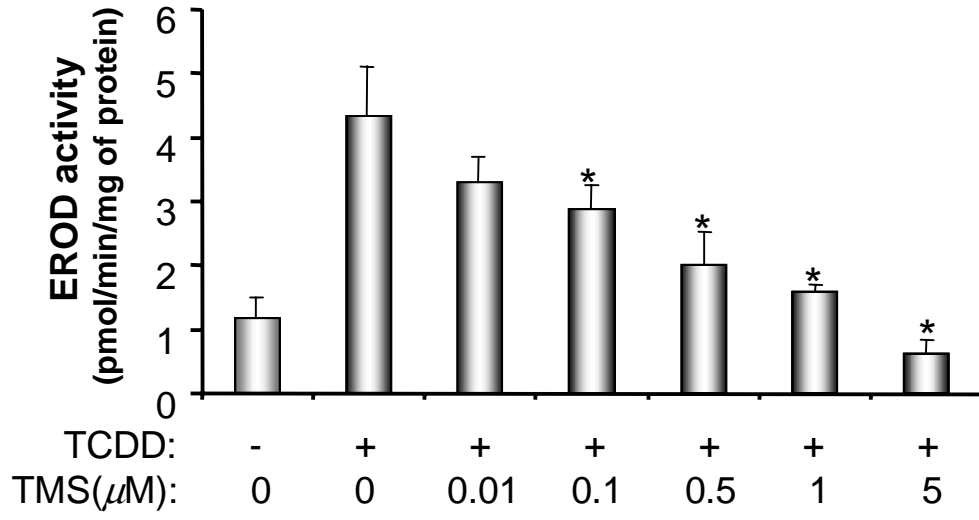
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Table 1. Effect of TCDD on cytotoxicity of TMS in human tumor cells. MCF-7 and HL-60 cells were treated with 2 nM TCDD in the presence or absence of TMS (1 μ M) for 72 h. MTT assays were performed to measure cell proliferation. The level in control cells at 0 h was set at 100 %. Results represent the means \pm S.D. of five different experiments. *Significantly different from DMSO-treated groups ($p < 0.05$). # Significantly different from TMS-treated groups ($p < 0.05$).

Treatment	MCF-7 proliferation (% of control)		HL-60 proliferation (% of control)	
	-TCDD	+TCDD	-TCDD	+TCDD
DMSO	914 \pm 164	526 \pm 79*	655 \pm 54	296 \pm 26*
TMS	313 \pm 66*	49 \pm 13*.#	178 \pm 21*	52 \pm 10*.#

Fig.1

A



B

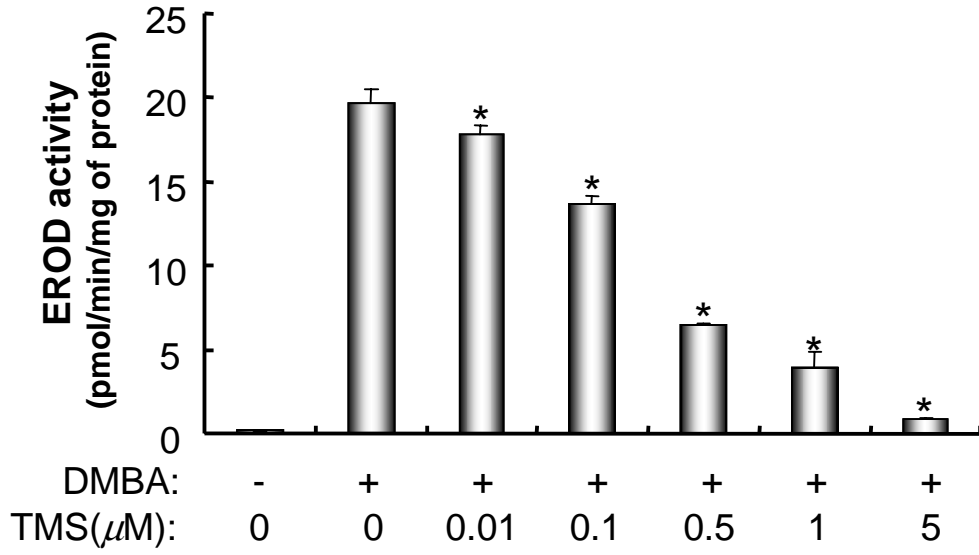


Fig.2

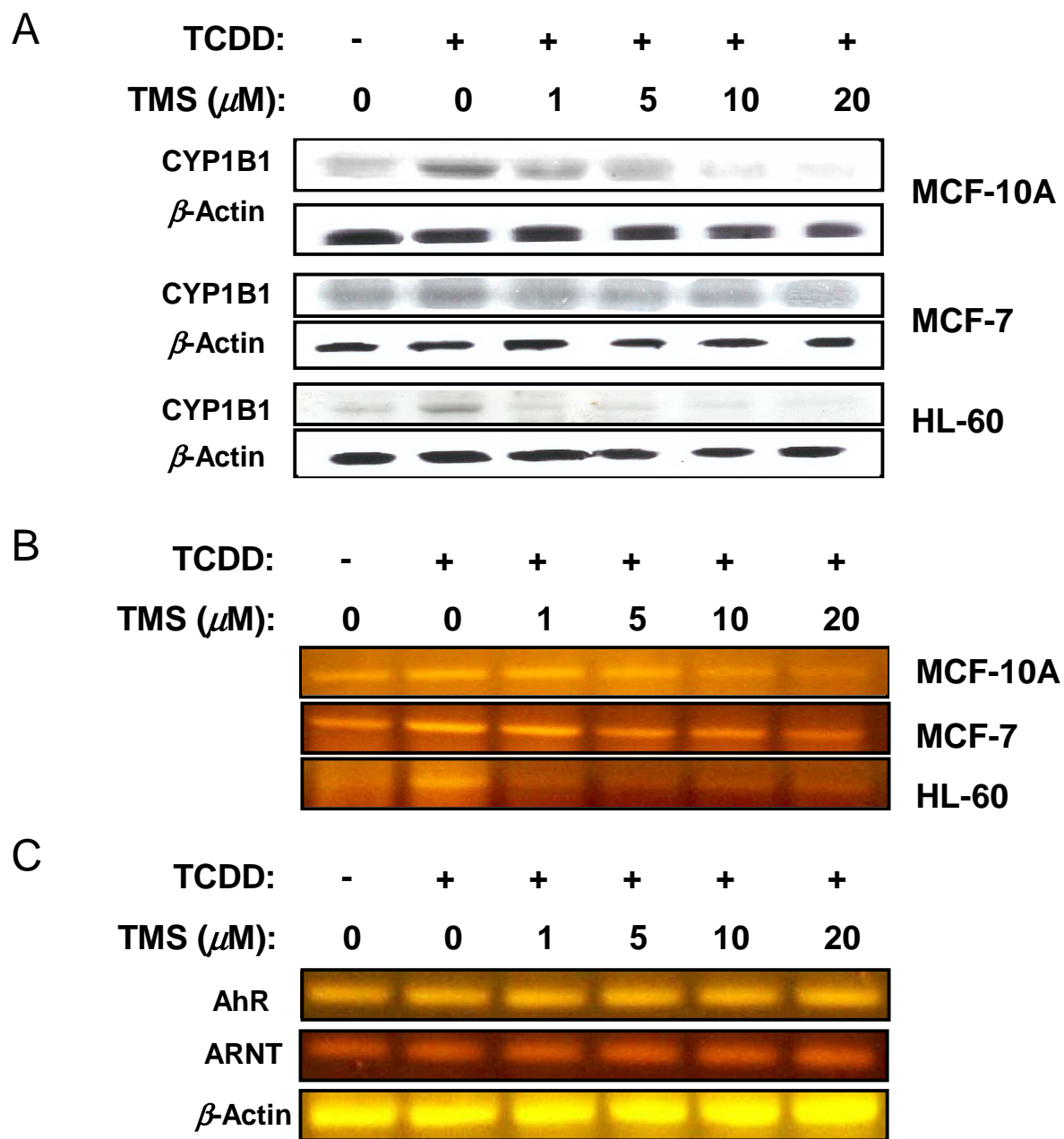


Fig.3

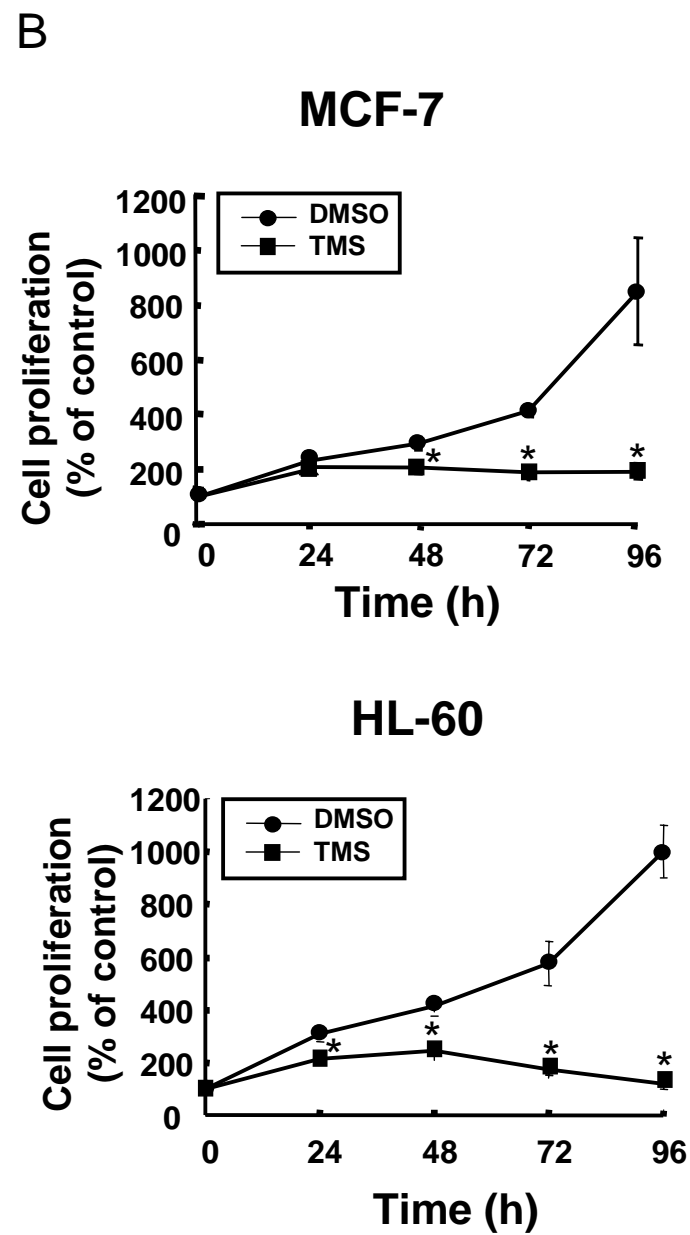
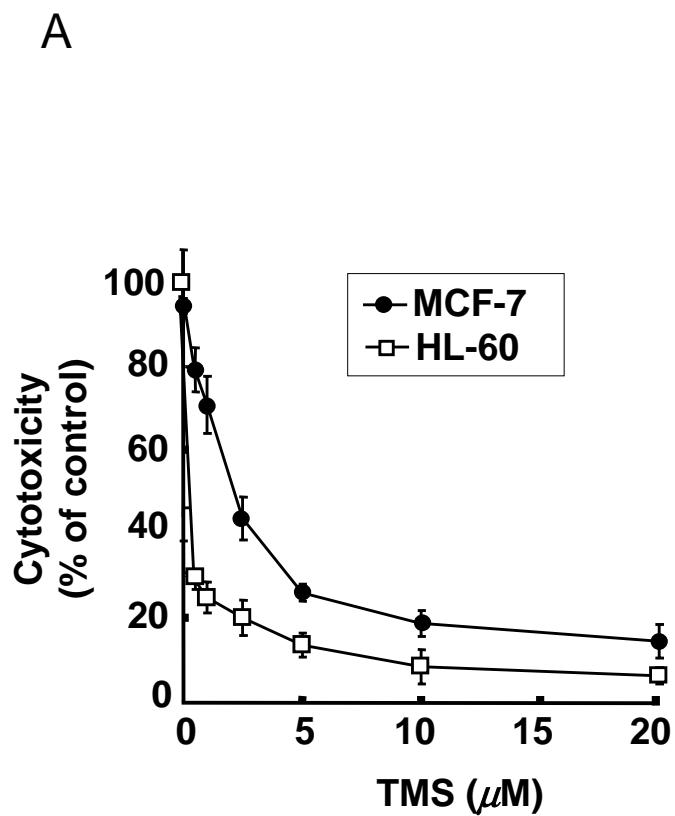


Fig.3

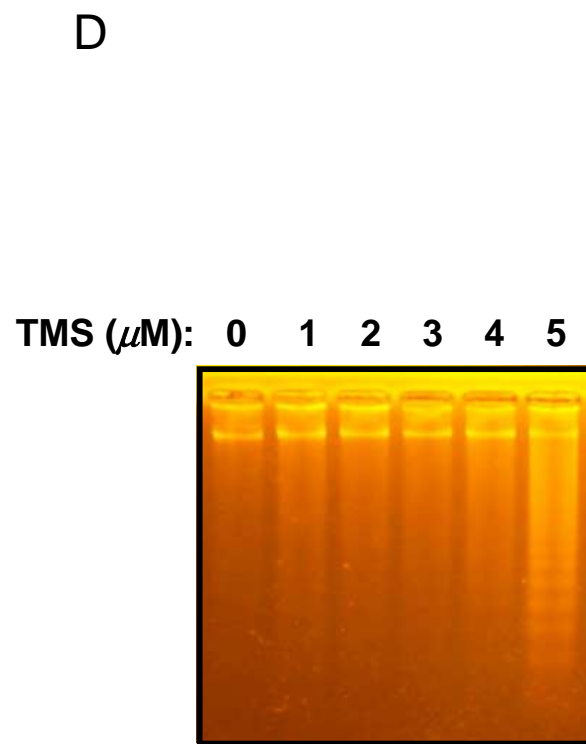
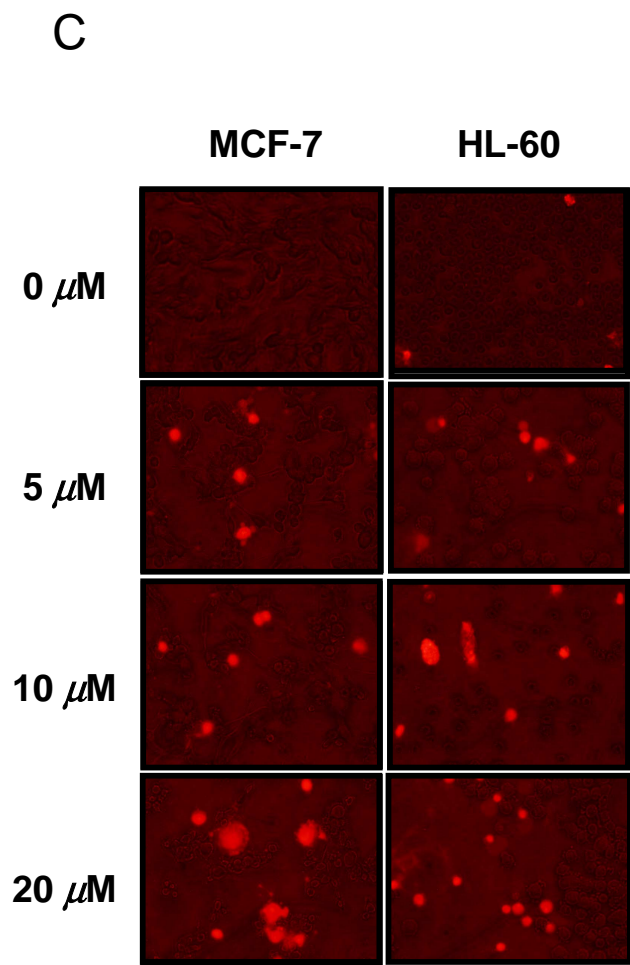


Fig.4

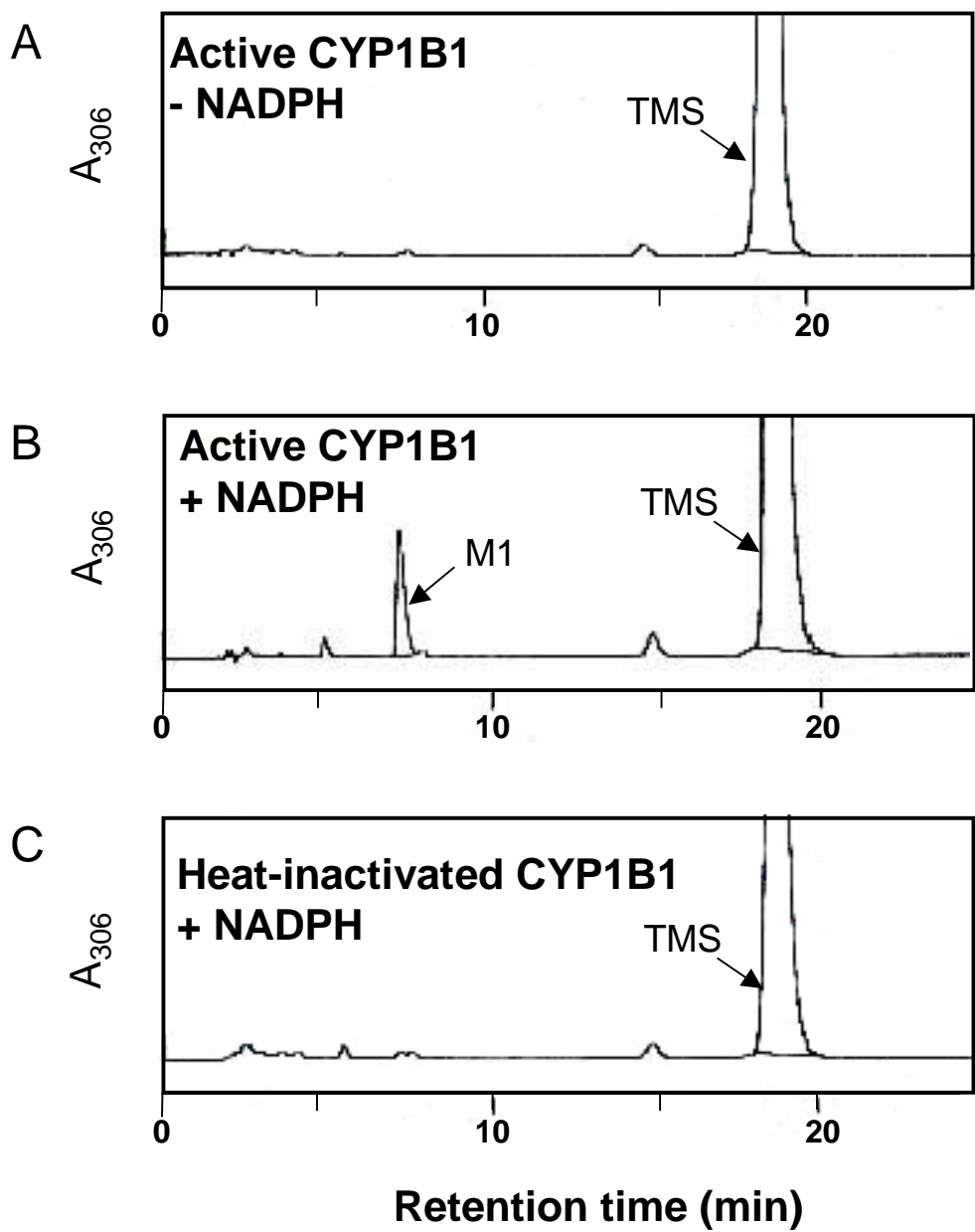


Fig.5

