Hormesis Effect of Methyl Triclosan on Cell Proliferation and Migration in Human Hepatocyte L02 Cells

Jing An,* Weiwei Yao, Waner Tang, Jingjing Jiang, and Yu Shang*

ABSTRACT: Methyl triclosan (mTCS) is a methylated derivative of triclosan (TCS), which is extensively used as an antimicrobial component of various nursing products and disinfectants. Current research studies of mTCS mainly focused on the environmental persistence and bioaccumulation potential. Knowledge regarding the toxicity and carcinogenicity of mTCS is limited until now. In this study, the human hepatocyte L02 cells were used to investigate the cellular effects of mTCS under different concentrations (0.1–60 μM). The hormesis effect was observed where a low dose of mTCS (≤5 μM) exposure stimulated the cell proliferation ability, while high-dose exposure (≥20 μM) inhibited cell proliferation. In the same time, low doses of mTCS (0.5 and 1 μM) induced enhanced anchorage-independent proliferation ability and cell migration ability, indicating a positive effect on malignant transformation in L02 cells. Moreover, reactive oxygen species productions were significantly increased after mTCS exposure (≥1 μM), as compared with the control group. Furthermore, expressions of tumor-related genes, mouse double minute 2 (MDM2), matrix metalloproteinase 9 (MMP9), and proliferating cell nuclear antigen (PCNA), and proto-oncogene MYC (c-Myc), Jun, and FosB were significantly upregulated, while no significant changes were observed on expressions of apoptosis-related and cell cycle-related genes in L02 cells after exposure of low-dose mTCS. In conclusion, these results indicated that a low dose of mTCS had a hormesis effect in L02 cells on cell proliferation and malignant transformation in vitro, which might be mediated through oxidative stress response.

INTRODUCTION

Methyl triclosan (mTCS) is a major biomethylation derivative of the environmental contaminant triclosan (TCS), which is a broad-spectrum antibacterial agent added to hand sanitizers, toothpastes, soaps, and other daily chemical products for more than 40 years. Generally, mTCS can be transformed by microorganisms under aerobic conditions. Replacing the hydrogen in TCS with a methyl group gives mTCS novel physicochemical properties including loss of antibacterial properties, increased resistance to photodegradation, environmental persistence, and longer half-life duration.2,3 Furthermore, mTCS is more hydrophobic and lipophilic than TCS, resulting in the widespread existence of mTCS in multiple environmental media including water, soil, and estuarine sediment,4 as well as in diverse organisms such as marine lives,5 freshwater algae,5 and earthworms.6 It has been reported that concentrations of mTCS in fish from lakes of Switzerland ranged from 4 to 365 ng/g,7 which were higher than that of TCS. In the biotransformation of the river biofilm, the concentration of mTCS and other biotransformation products reached 42% of the parental TCS level.8 Balmer et al. suggested that mTCS could be used as a suitable marker for wastewater treatment plant-derived prolipid contaminants in aquatic environments.8

Moreover, mTCS in various organisms could be enriched into the human body through foods such as eggs and milk.9 Goodbred et al. first reported the biomagnification capability of mTCS in the food network with a trophic amplification factor of 3.85.10 Until now, mTCS has been detected in human blood, urine,11 breast milk, liver tissue, brain tissue, and other tissues,12 which pose potential risks to human health. Moreover, increased production and application of disinfectant products against the novel coronavirus pneumonia had released more mTCS into the environmental media,13 making it urgent to promote the investigation on the toxicology effects and regulating mechanisms of mTCS.

Toxicology research studies have reported that mTCS induced embryonic development toxicity in zebrafish, manifested as delay in development of zebrafish (Danio rerio) embryo and sea urchin larvae,14 disruption of the metabolomes, and dysregulation on the biological pathways including nitrogen metabolism, energy metabolism, and fatty acid synthesis.15,16 In addition, mTCS could influence the thyroid hormone-responsive gene transcripts in rat pituitary GH3 cells, displaying the potential endocrine disruption property.17 Our preliminary experimental data proved that mTCS could cause...
Hormesis refers to a biphasic dose–response relationship of the chemical characterized by adaptive and stimulating effects at low doses. Long-term exposure to low-dose environmental stress triggers has been confirmed to be associated with many health risks in humans. Transcriptomic analysis of HepG2 cells revealed that mTCS might cause various biological responses including biosynthetic and metabolic processes and cellular organelle’s function. However, previous research studies were mainly conducted on the related high concentration of mTCS, while the toxicological effects and regulatory mechanism in organisms of environmental-related concentration are still limited.

Hormesis refers to a biphasic dose–response relationship of the chemical characterized by adaptive and stimulating effects at low doses. Long-term exposure to low-dose environmental stress triggers has been confirmed to be associated with many chronic diseases. Given the fact that current toxicology experiments of mTCS were generally conducted on much higher concentrations than environmental dose, the toxicity evaluation induced by environmental relevant concentrations is of significance in formulating safety standards for environmental pollutants. The results of Wang et al. displayed that low concentration of BDE-47 ($10^{-10}$ to $10^{-9}$ M) induced the hormesis effect in HepG2 cells mediated with the DNA-dependent protein kinase catalytic subunit/phosphorylated protein kinase B pathway. The purpose of this study was to confirm the hormesis effect of environmental doses of mTCS in normal human liver cells and to explore the underlying molecular mechanism. This research will provide experimental support to formulate risk assessment and control policies for mTCS.

### RESULTS AND DISCUSSION

#### Effect of mTCS on Cell Proliferation

It was reported that both mTCS and TCS had toxic effects on embryo of sea urchin larvae, and mTCS could more efficiently influence the larval length than TCS. Gaume et al. investigated the cell viability of abalone blood cells exposed to mTCS for 24 h, finding that significant cytotoxicity was observed in mTCS-treated groups over concentrations of 4 μM. Our previous data showed that mTCS had a toxic effect on liver cancer cells HepG2, and the cell survival rate at 40 μM reduced to 91.5% compared to the control. Considering the environmental relevant mTCS concentration, as well as its low degradation rate and bioaccumulation tendency, the present study performed cell counting kit-8 (CCK-8) assay, plate cloning test, and soft agar cloning experiment to evaluate the effects of mTCS on cell proliferation of L02 under both low- and high-dose ranges.

As shown in Figure 1A, the cell viability after low-dose mTCS exposure (0.5, 1, and 5 μM) was slightly increased, and the stimulating effect was most pronounced in the 1 μM group (Figure 1A, p < 0.05). Cell viability of L02 exposed to a high dose of mTCS (20, 40, and 60 μM), however, was dose-dependently reduced compared to the control (Figure 1A). The results of plate cloning experiments displayed that low-dose mTCS (0.1, 0.5, 1, and 5 μM) stimulated the clone formation rates of L02 cells. Consistently, the highest clone formation rate was observed in the 1 μM mTCS group, with a 15.3% increase over the control (Figure 1B, p < 0.01).

Meanwhile, there was a significant decrease in the clone formation rate observed in the 40 and 60 μM mTCS groups compared to the control.26 Meanwhile, there was a significant decrease in the clone formation rate observed in the 40 and 60 μM mTCS groups compared to the control (Figure 1B, p < 0.01).

*Figure 1. Effect of mTCS exposure on cell proliferation of L02. (A): L02 cells were treated with different concentrations of mTCS (0, 0.1, 0.5, 1, 5, 10, 20, 40, and 60 μM) for 48 h, and then, the cell proliferation ability was determined by the CCK8 kit. (B): L02 cells were treated with different concentrations of mTCS (0, 0.5, 1, and 5 μM) for 14 d, and the plate clone formation ability was assayed. (C) L02 cells were treated with different concentrations of mTCS (0, 0.5, 1, and 5 μM) for 14 d, and the soft agar cloning formation ability was analyzed. (D): Representative images of soft agar cloning formation. A multiple comparison adjustment was conducted to compare the difference between groups, and the Bonferroni-corrected p-values less than 0.05 were considered statistically significant. *p < 0.05 and **p < 0.01, compared with the control group. C: control group: cells were treated with 0.1% dimethyl sulfoxide (DMSO)/Dulbecco’s modified eagle’s medium (DMEM) (v/v).*
formation rates observed in the groups of 40 and 60 μM mTCS (p < 0.05). Subsequently, 0.5, 1, and 5 μM were selected as the representative low-dose treatment groups for soft agar cloning experiment. The results consistently showed a stimulating effect on soft agar clone formation, which was also the most outstanding in the 1 μM mTCS group (Figure 1C,D, p < 0.05). In summary, the abovementioned experimental data indicated that mTCS exposure had obvious hormesis effects on cell proliferation of L02 cells.

Effect of mTCS Exposure on the Cell Migration.

Wound healing assay is a simple and economical test to investigate the cell migration capability in vitro, which could to some extent mimic the process of cell migration in vivo. A 24 h exposure of benzo[a]pyrene (1 μM) was reported to promote the migration capability of mouse hepatocarcinoma cell line Hepa1-6 cells. Furthermore, a low dose of tetrabromobisphenol A and its derivatives can also trigger the malignant migration in endometrial cancer cells. As shown in Figure 2A,B, compared with the control group (0.1% DMSO), the healing rate of scratch significantly increased in the low-dose mTCS treatment groups (0.5, 1, and 5 μM). The 1 μM mTCS group showed the highest healing rate, with a 113.7% increase over the control group after 24 h exposure (p < 0.05). Nevertheless, the healing rate of the 40 μM mTCS group was significantly lower than the control group. These results indicated that mTCS exerted a biphasic dose–response on wound healing, which is a stimulating effect at low doses (0.5, 1, and 5 μM mTCS) and inhibiting effect at high doses (40 μM mTCS). Thus, in the subsequent experiments, 1 μM mTCS was reserved as the representative adaptive dose and 40 μM mTCS was reserved as the inhibitory dose.

Oxidative Responses Induced by mTCS in L02 Cells.

L02 cells were treated with different concentrations of mTCS (0.1, 0.5, 1, 5, 10, 20, and 40 μM) for 24 h, and then, the production of reactive oxygen species (ROS) was detected with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assay. As shown in Figure 3, the ROS levels in L02 cells after a short-term mTCS exposure were significantly stimulated compared with the control group, with 16, 34.5, 53.7, 80.1, 74.9, 101.1, and 151.9% increase. ROS-mediated oxidative stress is associated with many processes, including cell proliferation, DNA damage, apoptosis, cell cycle, and so on. Generally, overproduction of ROS can inhibit cell

Figure 2. Effect of mTCS on cell migration. (A) L02 cell was treated with different concentrations of mTCS (0, 0.5, 1, 5, and 40 μM) for 24 h/48 h, and then, the cell migration ability was determined by wound healing assay. Images were captured with a microscope reader (Olympus, Tokyo, Japan) under 10× objective lens. (B) Quantitated data of the healing rate analyzed with ImageJ software. The Bonferroni-corrected p-values less than 0.05 among multiple comparisons were considered statistically significant. *p < 0.05, compared with the control group. (C) control group: cells were treated with 0.1% DMSO/DMEM (v/v).

Figure 3. Oxidative response induced by mTCS exposure in L02 cells. The L02 cell was treated with different concentrations of mTCS (0.1, 0.5, 1, 5, 10, 20, and 40 μM) for 24 h, and then, the ROS level was detected with the DCFH-DA probe. *p < 0.05 and **p < 0.01, compared with the control group. C: control group: cells were treated with 0.1% DMSO/DMEM (v/v). tBHP: tert-butyl hydroperoxide.
proliferation and induce DNA damage and cell death, which is proved to be an important toxicological mechanism for many toxicants, especially under high-dose exposure conditions.\textsuperscript{32,33} On the other hand, some chemicals at low levels induced only slightly increased ROS production, which can activate certain cytoprotective pathways, such as the extracellular regulated protein kinase signaling pathway, resulting in stimulatory effects on cell proliferation.\textsuperscript{34,35} The persistent stimulation of cell proliferation induced by long-term low-dose exposure was associated with the carcinogenic potential of many pharmaceutical and environmental compounds.\textsuperscript{36} In addition, Kasuba et al. found that low-dose glyphosate exposure slightly stimulated the proliferation of HepG2 cells, but there was no significant change in ROS production levels due to activation of the antioxidant system, which can effectively remove excess ROS produced during exposure.\textsuperscript{37} In our study, combined with the results of cell proliferation assays, the slightly increased ROS induced by low concentrations of mTCS triggered adaptive cellular processes to protect cells against oxidative damages, which may consequently result in enhanced cell proliferation.

**Molecular Mechanism of the Hormesis Effect Induced by mTCS in L02 Cells.** Proto-oncogene MYC (c-Myc) and proliferating cell nuclear antigen (PCNA) are widely recognized as cellular proliferation markers.\textsuperscript{38,39} Changes in the expression levels of these genes are closely correlated with the cell proliferation ability. Increased c-Myc expression level, together with overexpression of PCNA, was associated with malignant transformation, tumor initiation, promoted tumor progression, and poor prognosis in a variety of malignancies.\textsuperscript{40−48} Mouse double minute 2 (MDM2) is an oncogenic protein and a negative regulator of the tumor suppressor p53 protein.\textsuperscript{44} Overexpression of MDM2 has been detected in many human malignancies, including lung cancer, colon cancer, and other malignancies.\textsuperscript{45} Guan et al. found that knocking down of MDM2 in osteosarcoma cells could significantly inhibit the cell proliferation, migration, and invasion capability.\textsuperscript{46} In this study, the mRNA expression levels of c-Myc, MDM2, and PCNA in L02 cells were significantly upregulated in the low-dose groups compared to the control, with a 36.7, 40.3, and 40.7% induction, respectively (p < 0.05, Figure 4A). Furthermore, the protein expression of MDM2 was also increased over 1.8-, 2.1-, and 1.8-fold after exposure of 0.5, 1, and 5 μM mTCS, respectively (Figure 4B,C). The abnormal expression of these genes may partially explain the enhanced proliferation ability and malignant transformation of L02 cells after low-dose mTCS exposure.

Tumor-related gene matrix metalloproteinase 9 (MMP9) is considered as a prognostic marker during cancer progression, mediating cancer cell invasion and metastasis.\textsuperscript{47} Overexpression of MMP9 was associated with enhanced migration of hepatocellular carcinoma cells,\textsuperscript{48} and downregulating the MMP2 and MMP9 activity could suppress the cell migration capability.\textsuperscript{49} Similarly, glioma-associated oncogene homolog 1 (GLI1) is highly expressed in human endometrial stromal cells. Reducing the expression of GLI1 led to inhibition of MMP2 and MMP9 expression and consequent attenuation of malignant migration, invasion, and proliferation.\textsuperscript{50} In the present study, MMP9 expression levels were over 1.5-, 2.3-, and 1.6-fold of that in the control group under 0.5, 1, and 5 μM mTCS exposure, respectively (Figure 5A,B), indicating that MMP9 participated in regulating the cell proliferation and migration induced by mTCS.

B-cell lymphoma 2 (BCL2)-associated X (BAX) and caspase 3 are downstream target genes of tumor protein p53, which plays an important role in mitochondrial pathway apoptosis.\textsuperscript{51} P53 inhibition in pancreatic cancer cells could enhance MDM2 expression and reduce BAX, caspase 3, caspase 9, and cyclin-dependent kinase inhibitor 1A (p21).\textsuperscript{52} Bai et al. found that cytosine-phosphorothioate-guanine oligodeoxynucleotides could induce apoptosis in human bladder cancer cells by enhanced expressions of pro-apoptotic-related factors caspase 3, BAX, and p53, as well as reduced BCL2 expression.\textsuperscript{53} Cytochrome c (Cyt-c) is one of the key mediators of the mitochondrial-mediated endogenous apoptotic pathway, which can be released from the mitochondria into the cytoplasm under oxidative stress.\textsuperscript{54,55} Li et al. found that the expression levels of Cyt-c and caspase 3 in human multiple myeloma cells exposed to zinc oxide nanoparticles were significantly higher than those in the control group.\textsuperscript{56} We previously found that a high dose of mTCS exposure resulted in significantly increased ROS production, enhanced p53 and caspase 3 expression, and

![Figure 4. Effect of mTCS on the expressions of c-Myc, MDM2, and PCNA in L02 cells. (A) L02 cell was treated with 1 μM mTCS for 12 h, and then, the expressions of c-Myc, MDM2, and PCNA were determined by real time-quantitative polymerase chain reaction (RT-qPCR). (B) L02 cell was treated with 0.5, 1, and 5 μM mTCS for 24 h, and then, the expression of MDM2 protein was determined by western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as the internal reference. (C) Quantitation results of protein expression levels of MDM2. *p < 0.05 and **p < 0.01, compared with the control group. (C) Control group: cells were treated with 0.1% DMSO/DMEM (v/v).](https://doi.org/10.1021/acsomega.1c02127)
reduced MDM2 expression, indicating that high-dose mTCS could induce caspase-dependent mitochondrial apoptosis in HepG2 cells. However, in this study, the expressions of Cyt-c and caspase 3 in L02 cells exposed to a low dose of mTCS was only slightly increased to levels comparable to the control (Figure 6A, B). In addition, the mRNA levels of p53 and BAX were not significantly changed (Figure 6B), suggesting that the mitochondrial apoptotic process should not be the major regulating mechanism of mTCS toxicity under low-dose exposure.

The cell cycle process is also one of the main factors affecting cell growth. CyclinD1, a cell cycle regulator, regulates transition of the cell cycle from the G1/G0 phase to the S phase. P16 is a cyclin-dependent kinase inhibitor, playing an important role in negative regulation on G1/S transition. Inactivation of p16 was associated with uncontrolled cell hyperproliferation, which was involved in tumor initiation and progression. Data of this research showed that CyclinD1 expression was slightly upregulated, while p16 expression had a slight downregulated trend, which is consistent with the results of cell proliferation and migration (Figure 6C). However, neither of these changes is of statistical significance, and the effect of mTCS on the L02 cell cycle could not be determined.

Activating protein-1 (AP-1) is a dimer transcription factor, including proto-oncogene Jun and Fos, activating transcription factor (ATF), and other family members. The AP-1 family of dimeric transcriptional complexes is involved in almost all cellular and physiological processes and is implicated in many tumorigenic processes. Generally, Jun is a positive regulator

Figure 5. Effect of mTCS on the expressions of MMP9 in L02 cells. (A) L02 cell was treated with 0.5, 1, and 5 μM mTCS for 24 h, and then, the expression of MMP9 protein was determined by western blotting. GAPDH protein expression was used as the internal reference. (B) Quantization data of MMP9 protein expression levels. *p < 0.05 and **p < 0.01, compared with the control group. (C) Control group: cells were treated with 0.1% DMSO/DMEM (v/v).

Figure 6. Effect of mTCS on the gene expressions related with cell proliferation in L02 cells. (A) L02 cell was treated with 0.5, 1, and 5 μM mTCS for 24 h, and then, the expression of Cyt-c protein was determined by western blotting. GAPDH protein expression was used as the internal reference. (B–D) L02 cell was treated with 1 μM mTCS for 12 h, and then, the expression of related genes was detected with RT-qPCR. (B) Apoptosis related genes. (C) Cell cycle-related genes. (D) Genes related to AP-1 transcription. *p < 0.05, compared with the control group. (C) Control group: cells were treated with 0.1% DMSO/DMEM (v/v).
of cell proliferation, and aberrant Jun overexpression was associated with enhanced cell proliferation and malignant transformation.\textsuperscript{61,62} Proto-oncogene JunB has an opposite effect to Jun, and downregulated expression of JunB is found in many tumor cells, which is important in malignant development and progression.\textsuperscript{63} In this study, the expression of \textit{Jun} was increased and \textit{JunB} expression was decreased after 1 \textmu M mTCS exposure (Figure 6D). Furthermore, increased Fos expression leads to reversible malignant hepatocellular transformation, playing a promoting role in early HCC development.\textsuperscript{64} Our data showed that the expression of \textit{Fos} (one of the members of Fos) was significantly increased in both groups (Figure 6D). These results suggested that the AP-1 activity is involved in the cellular proliferation induced by mTCS.

\section*{CONCLUSIONS}
In summary, this study showed that mTCS had hormesis effects on cell proliferation and migration in L02 cells. A low dose of mTCS promoted the malignant transformation of L02 cells \textit{in vitro}, as manifested by stimulation of cell proliferation, increased anchor-independent proliferation, and enhanced cell migration. C-Myc, PCNA, MDM2, and MMP9 participated in the stimulatory proliferation effect induced by a low dose of mTCS, while expressions of apoptosis-related and cell cycle-related genes were not significantly changed after low-dose mTCS exposure. The different cellular effects of L02 on cell proliferation and migration after low- and high-dose mTCS exposure may be explained by different ROS production levels and consequent genomic transcriptional responses related to cell proliferation. Considering the enhanced proliferation induced by low-dose mTCS and its environmental persistence, the carcinogenic potential of mTCS under long-term exposure and the regulating mechanisms are needed to be evaluated in future in-depth study.

\section*{MATERIALS AND METHODS}

\textbf{Chemicals and Reagents.} The standard mTCS powder (CAS: 4640-01-1, purity >97\%) was purchased from Dr. Ehrenstorfer (Germany). The storage solution of mTCS was dissolved in DMSO to a concentration of 200 mM, aliquoted, and stored at \textapprox{} 20 °C. The fetal bovine serum (FBS) and DMEM were obtained from Invitrogen (CA, USA). The reverse transcription kit and SYBR were purchased from TOYOBO (Osaka, Japan). DMSO, DCFH-DA fluorescent probe, tert-Butyl hydroperoxide (tBHP), RNase A, trypsin, and other analytical reagents used in this study were obtained from Sigma (MO, USA).

\textbf{Cell Lines and Culture Conditions.} The human L02 hepatocyte cell line was a gift of Professor Ping-Kun Zhou (Beijing Institute of Radiation Medicine, Beijing, China). Immortalized L02 cells were widely used in the toxicity examination of exogenous compounds, since they have similar proliferation characteristics with normal liver cells.\textsuperscript{65} L02 cells were incubated in DMEM complete medium containing 10% FBS and 1% penicillin–streptomycin, maintained in a constant temperature incubator at 37 °C with 5% CO\textsubscript{2}. When cells continuously grew to 80% confluency, various concentrations (0.1, 0.5, 1, 5, 10, 20, 40, and 60 \textmu M) of mTCS were added and coincubated for different times. After treatment, the cell viability, migration ability, redox status, gene expression at mRNA, and protein levels were measured. Cells of the control group were treated with 0.1% DMSO (\textit{v/v}) only. All experiments were carried out at least three times with more than three parallel samples.

\textbf{Analysis of Cell Viability.} CCK-8 (Dojindo, Kumamoto, Japan) was used to determine the cell viability according to the manufacturer’s instruction. L02 cells were digested with trypsin to prepare cell suspension (2 × 10\textsuperscript{5} cells/mL), which was then inoculated in 96-well plates (100 \mu L/well). After attachment for 24 h, cells were coincubated with different doses of mTCS for 48 h. The mTCS concentration gradient was set as low-dose groups (0.1, 0.5, 1, and 5 \textmu M) and high-dose groups (10, 20, 40, and 60 \textmu M). The control group was treated with DMEM complete medium containing 0.1% DMSO (\textit{v/v}). The cell viability after mTCS exposure was indirectly represented by absorbance at 450 nm measured with a multifunction microplate luminometer (Tecan, Switzerland).

\textbf{Colony Formation Experiment.} Colony formation experiment is an important method to detect cell proliferation and invasiveness, and the colony formation rate indicates the independent viability of cells. \textit{In vitro}, when a single cell proliferates for more than six generations (about 10–14 d), the cell population becomes a clone containing more than 50 cells with a size between 0.3 and 1.0 mm.\textsuperscript{66} The long-term survival of L02 cells exposed to mTCS was monitored by plate cloning assay. Cell suspensions (300 cells/well) were prepared and seeded in 60 mm Petri dish, which were then treated with different concentrations of mTCS (0.1, 0.5, 1, 5, 10, 20, 40, and 60 \textmu M) for 14 d. The visible cell colonies were fixed with methanol and stained with Giemsa (Sigma, MO, USA) solution for 15 min. The colony-forming ability of L02 cells was further evaluated with soft agar colony formation assay, which was slightly adjusted based on previous experimental procedures.\textsuperscript{66} In brief, the 6 g/L lower agarose and 3 g/L upper agarose containing 150 cells were prepared in a six-well plate. Cells in soft agar were incubated in a constant temperature incubator at 37 °C for 14 d. Cell clones were stained with crystal violet (Sigma, MO, USA). Typical colony images were recorded with a microscope reader (Olympus, Tokyo, Japan), and the number of cell colonies was counted.

\textbf{Wound Healing Assay.} Wound healing assay is widely used to evaluate the migration ability of cells. Briefly, approximately 5 × 10\textsuperscript{5} cells were inoculated in a 24-well plate, using the overnight monolayer as the standard. Cell scratches in the plate well were prepared with a 10 \mu L pipette tip perpendicular. After washing with phosphate-buffered saline, cells were incubated with different concentrations of mTCS solution (0.5, 1, 5, and 40 \textmu M) diluted with serum-free medium for 24 or 48 h. The cell scratches at the same location in each well were photographed using a microscope reader (Olympus, Tokyo, Japan). The photographs were analyzed with ImageJ software to quantify the area of the scratches and relative healing rate.

\textbf{Oxidative Stress Detection.} The DCFH-DA fluorescence probe was used to detect the ROS levels in L02 cells. Once penetrated into cells, DCFH-DA could be hydrolyzed by esterase to form DCFH, and then, DCFH could be further oxidized by ROS to form the strong fluorescent product DCF. The fluorescence intensity of DCF could thus indirectly reflect the level of ROS in the cell. After exposure to various concentrations of mTCS (0.1, 0.5, 1, 5, 10, 20, and 40 \textmu M), cells were incubated with 10 \mu M DCFH-DA in the dark for 20 min. In the same time, tBHP was applied as the positive control. The ZOE fluorescent cell imager (Bio-Rad, CA, USA) was used to take photographs under the green channel. The
Table 1. Primer Sequence for RT-qPCR

| gene name | primer | primer sequence (5′–3′) | MW | Tm/0.05 M (°C) | GC % |
|-----------|--------|--------------------------|----|----------------|------|
| e-Myc     | forward| GGCTCCTGGCAGAAAAGGCTCA  | 5837.84 | 60 | 58 |
|           | reverse| CTCGGTACCTGTCTGCTATGT | 6170.04  | 55.3 | 50 |
| MDM2      | forward| GAATCATCGGACTCGAGATTCATC | 7032.64 | 58 | 48 |
|           | reverse| TCTGTCCTCAATTAATGCCTCTCT | 6891.64 | 56.21 | 43 |
| PCNA      | forward| CCTGCTGGGATAATTAGCTCCA  | 6397.24 | 57.59 | 52 |
|           | reverse| CAGCGCTGATGGTGCAGAAGGC | 5893.84  | 62 | 63 |
| Jun       | forward| TCCAGTGGCCGAAAAGGGGAAG | 6497.24 | 55.63 | 48 |
|           | reverse| CGATTTCTGACTGGCTTCAGGT | 6452.24 | 55.63 | 48 |
| JunB      | forward| ACGACTCATACACAGCTACGG  | 6384.24 | 57.59 | 52 |
|           | reverse| GCTCGGTTTCAGAGGATTGAGT | 7116.64 | 58 | 48 |
| FosB      | forward| GCTGCAAGATCCCTACGAGAAG | 6400.24 | 59.54 | 57 |
|           | reverse| ACAGAGAAGTGGTACGAGAAGGT | 6872.44 | 59.54 | 45 |
| Caspase3  | forward| CATGGAAGCGAATCAATGGACT | 6792.44 | 55.94 | 45 |
|           | reverse| CTTGACCAAGACGGGAGATGCA | 6415.24 | 57.59 | 52 |
| p53       | forward| CAGCAGATGGGAGGATTGTG | 6182.04 | 57.59 | 55 |
|           | reverse| TCATCCTAAATACCTCAGACGC | 6279.24 | 55.63 | 48 |
| BAX       | forward| CCCAGGAGGTCTTCTTCCGAG  | 6413.24 | 59.54 | 57 |
|           | reverse| CAGCCCAATGGTGGTTTGTGAT | 6397.24 | 57.59 | 52 |
| CyclinD1  | forward| GCTTGCGATGGGGAAGGCTC  | 6151.04 | 57.59 | 55 |
|           | reverse| CTCCTCTTCTGCACAGTCTGAA | 6605.44 | 59.54 | 45 |
| p16       | forward| GATCCGAGTGTTGAGGAAGTGC | 6551.24 | 59.54 | 57 |
|           | reverse| CCCCTGCAAATCTGCTGCC | 6596.84 | 60 | 58 |
| GAPDH     | forward| GGACGAGATCCCTCCTCATAAT | 6424.24 | 57.59 | 52 |
|           | reverse| GGCTGTGGTTCATACCTCTCATGG | 7036.64 | 58 | 48 |

Fluorescent photographs were analyzed with Ipwin32 software, and the average fluorescence intensity of different concentration groups was quantitated.

**Western Blotting Analysis.** Cells were treated with mTCS (0.5, 1, and 5 μM) for 24 h, and then, the total protein was collected and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein samples were transferred to the polyvinylidene fluoride membrane (Sigma, MO, USA). The primary and corresponding secondary antibodies were then incubated with protein samples according to the manufacturer’s instructions. Finally, blots were visualized using chemiluminescence, and the optical density of each band was quantified using the ChemiDoc imaging system (Bio-Rad, CA, USA).

Primary antibodies used in this experiment were as follows: anti-MMP9 and anti-Cyt-c antibodies were purchased from Epitomics (CA, USA), the anti-MDM2 antibody was purchased from Milipore (MA, USA), and the anti-GAPDH antibody was purchased from Abcam (Cambridgeshire, UK). The secondary antibodies and anti-mouse immunoglobulin G (IgG) were purchased from Bio-Tech (Beijing, China), and anti-rabbit IgG was purchased from Zhong Shan Bio Tech (Beijing, China).

**RT-qPCR Assay.** After treatment of 0.1% DMSO or 1 μM mTCS for 12 h, the total RNA of L02 cells was extracted using TRIzol reagents. The agarose gel electrophoresis image of total RNA is shown in Figure S1. The qualified RNA samples were reversely transcribed into cDNA using a reverse transcription kit (TOYOBO, Osaka, Japan). RT-qPCR reaction was then performed to detect the expression level of related target genes. The amplification procedure was started at 95 °C for 60 s and then followed by 50 cycles of denaturation at 95 °C for 15 s, annealing at 63 °C for 15 s, and extension at 72 °C for 45 s. The primer pairs were purchased from Sunny Biotechnology (Shanghai, China), as shown in Table 1. The ΔΔCt method was used to quantify the gene expression levels after normalization to the internal reference gene (GAPDH).

**Statistical Analysis.** The experimental data were expressed as the mean ± standard error and analyzed using Excel 2010 software. The imaging data were processed using ImageJ software. Multiple comparisons between groups were analyzed using one-way ANOVA with the Bonferroni post hoc test. The significance of the difference was determined by the Bonferroni-corrected p-values less than 0.05.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at [https://pubs.acs.org/doi/10.1021/acsomega.1c02127](https://pubs.acs.org/doi/10.1021/acsomega.1c02127). Agarose gel electrophoresis image of total RNA (PDF)

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18910
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