Supplementary Materials

Comparison of hepatotoxicity and mechanisms induced by triclosan (TCS) and methyl-triclosan (MTCS) in human liver hepatocellular HepG2 cells

Lu Wang [a#], Boyu Mao [c#], Huixin He [a], Yu Shang [a], Yufang Zhong [a], Zhiqiang Yu [d], Yiting Yang [b*], Hui Li [a], Jing An [a*]

[a] School of Environmental and Chemical Engineering, Shanghai University, Shanghai 200444, China

[b] Department of Neurology, Changhai Hospital, Second Military Medical University. Shanghai 200433, China

[c] Implant Dentistry Department, Jiangbei Dental Hospital, Ningbo 315000, China

[d] State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China

* Corresponding authors:

Jing An, School of Environmental and Chemical Engineering, Shanghai University, Shanghai 200444, China; Tel: +86 21 66137736; E-mail: peace74839@shu.edu.cn

Yiting Yang, Department of Neurology, Changhai Hospital, Second Military Medical University. Shanghai 200433, China; E-mail: yangytyd@163.com

[#] Co-first author

Materials and methods

Chemicals and reagents

TCS (CAS: 3380-34-5) and MTCS (CAS: 4640-01-1) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). The two compounds were dissolved in dimethyl sulfoxide (DMSO) to prepare the stock solutions (10 mM). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen (Paisley, U.K.). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was from BD Biosciences (Sparks, MD, USA). Mammalian protein extraction reagent (M-PER) and bicinchoninic acid (BCA) protein assay kits were from Thermo Fisher Scientific Inc (Waltham, MA, USA). Polyvinylidene difluoride (PVDF) membrane was from Millipore (Darmstadt, Germany). Propidium Iodide (PI), 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5- di-phenytetrazoliumromide (MTT), RNase A, Trypsin, and DMSO were purchased from Sigma (St. Louis, MO, USA). All other reagents were from Sigma (Saint Louis, MO, USA) and were analytical grade chemicals, if not stated otherwise.

All antibodies were purchased commercially as follows: anti-Bcl-2 associated X protein (Bax), anti-Caspase 3, anti-Caspase 9, anti-Cyclin E1, anti-CDK2, and anti-Cyclin D1 (Cell Signaling, Beverly, MA, USA), anti-heme oxygenase-1 (HO-1) (Abcam, Cambridgeshire, UK), anti-p53 (Proteintech, Chicago, IL, USA) anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Multisciences Biotechnology, Hangzhou, China), anti-Rabbit IgG (H + L)/HRP and anti-Mouse IgG (H + L)/HRP (Dingguo, China).

Cell culture and treatments

Human hepatocellular carcinoma cells (HepG2) were obtained from ATCC (American Type Culture Collection). HepG2 cells were conventionally cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, and incubated at 37˚C in a humidified atmosphere containing 5% CO_2. Based on preliminary experiments, four concentrations (5, 10, 20, and 40 μM) were selected in this study. HepG2 cells were treated with different concentrations of TCS or MTCS for 24 h, and then cell proliferation, apoptosis, cell cycle, DNA damage and related protein expressions were measured. The control group was incubated with 0.1% DMSO (v/v) only. All experiments were carried out in at least three times with more than three parallel samples.

Cell growth assay

In living cells, mitochondrial dehydrogenase could degrade MTT to insoluble blue violet crystal which is useful for indicating cytotoxicity. HepG2 cells were seeded in 96-well plates at a concentration of 10^4 cells per well. Cells were then exposed to 5, 10, 20, or 40 μM of TCS/MTCS in 100 μL of DMEM. After 24 h of treatment, 10 μL of 5 mg/mL MTT solution was added to culture medium, and incubated for 4 h at 37°C. The MTT reaction was terminated by addition of 150 μL DMSO and the absorbance at OD 490 nm was recorded using a Multiscan Mk3 plate reader (Thermo Electron Corporation, USA).

Cell apoptosis assay

The Annexin V- FITC/PI detection kit was used to identify and quantify the apoptotic cells according to the instructions of supplier. Annexin V can bind with the extracellular phosphatidylserine to detect early apoptotic cells. PI is a nucleic acid dye, which can selectively penetrate the incomplete cell membrane of late apoptotic and necrotic cells. After treatment with TCS or MTCS (5, 10, 20, and 40 μM) for 24 h, cells were collected with trypsin digestion. Approximately 4×10^5 cells from each sample were suspended in 400 μL binding buffer. The cell suspensions were incubated with Annexin V-FITC at 37°C for 15 min, and then with 10 μL of PI for 5 min. The stained cells were immediately analyzed by flow cytometry using the FL2 detector (Becton Dickinson FACScan) with an excitation wavelength at 488 nm. Notably, the staining procedure was conducted in the dark. The data were analyzed with Summit 5.2 Software. The apoptosis rates referred the percentage of apoptotic cells in total cells (10,000 cells per sample).

Cell cycle assay

Cell cycle in HepG2 was assessed using flow cytometry (Becton Dickinson FACScan) based on cellular distribution of DNA content. PI can combine with the intracellular DNA and RNA, and the fluorescence intensity of PI directly reflects the amount of intracellular DNA content. Briefly, cells were plated in six-well plates at a density of 10^5 cells per well for 24 h, and then treated with TCS or MTCS (5, 10, 20, and 40 μM) for 24 h. Cells were harvested with trypsin and fixed with 70% ethanol at -20°C for 24 h. Then cell pellets were washed with D-Hank's and resuspended in 1 mL of D-Hank's containing 0.1 mg/mL RNase A at 37°C for 30 min, and then stained

with PI (50 μg/mL) for another 30 min. At last, cell samples were filtered with 300 mesh nylon filter and then evaluated by flow cytometry. All data were analyzed by Multicycle software. For each sample, a total of 10,000 cells were recorded.

Comet assay

DNA single-strand beaks were evaluated by alkaline single-cell gel electrophoresis (comet assay) according to our previous study. Alkaline comet assay is a sensitive method to detect DNA damage induced by xenobiotics. Briefly, cells treated with TCS or MTCS (5, 10, 20, and 40 μM) for 24 h were wrapped in "sandwich" gel, and then were lysed for 1.5 h in a cooled cell lysis solution. After lysis, the slides were subjected to a horizontal gel electrophoresis in alkaline electrophoresis buffer at 25 V and 300 mA at 4°C for 20 min. Then the slides were immersed in the neutralization buffer at 4°C for 2 × 8 min and air dried. Finally, the slides were stained with PI (20 μg/mL), and the fluorescence intensity was evaluated using a fluorescence microscope (Olympus BX-51, Japan). For each sample, 300 randomly captured cells by an investigator were collected and measured. The DNA damage was evaluated through the olive tail moment (Olive TM) using the CASP software version (University of Wroclaw, Poland). Tert-Butyl hydroperoxide (tBHP, 10 μM) was used as positive control.

Western blotting

Western blotting was performed according to our previous study to measure the related protein expressions in HepG2 cells. After treatment of TCS or MTCS (5, 10, 20, and 40 μM) for 24 h, HepG2 cells were lysed using M-PER to collect the total

protein. Equal amount of protein samples (60-80 μg) were subjected to 8-12% sodiumdodecylsulfate- polyacrylamide gel electrophoresis (SDS-PAGE) at 70 V for 30 min and then at 120 V for 2 h, and then transferred from gel to a PVDF membrane. After blocking with 5% skim milk, these PVDF membranes were incubated with the primary antibodies overnight at 4°C, and followed with secondary antibodies for 1 h at room temperature. The blots were visualized using chemiluminescence, and the optical densities of individual bands were quantitated using the Chemi-Imager digital imaging system (Alpha Innotech, San Leandro, CA, USA).