Toxicology and Applied Pharmacology 262 (2012) 117–123

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Cytotoxicity and inhibitory effects of low-concentration triclosan on adipogenic differentiation of human mesenchymal stem cells☆

Li-Wu Guo [a,1], Qiangen Wu [b,1], Bridgett Green [a], Greg Nolen [a], Leming Shi [c], Jessica LoSurdo [d], Helen Deng [e], Steven Bauer [d], Jia-Long Fang [b,*], Baitang Ning [a,**]

[a] Division of Personalized Nutrition and Medicine, National Center for Toxicological Research, Food and Drug Administration, Jefferson, AR 72079, USA
[b] Division of Biochemical Toxicology, National Center for Toxicological Research, Food and Drug Administration, Jefferson, AR 72079, USA
[c] Division of Systems Biology, National Center for Toxicological Research, Food and Drug Administration, Jefferson, AR 72079, USA
[d] Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892, USA
[e] Arkansas Department of Health, Little Rock, AR 72205, USA

ARTICLE INFO

Article history:
Received 14 February 2012
Revised 18 April 2012
Accepted 19 April 2012
Available online 1 May 2012

Keywords:
Adipocyte differentiation
Human mesenchymal stem cell
Triclosan
Cytotoxicity

ABSTRACT

Humans at all ages are continually exposed to triclosan (TCS), a widely used antimicrobial agent that can be found in many daily hygiene products, such as toothpastes and shampoos; however, the toxicological and biological effects of TCS in the human body after long-term and low-concentration exposure are far from being well understood. In the current study, we investigated the effects of TCS on the differentiation of human mesenchymal stem cells (hMSCs) by measuring the cytotoxicity, morphological changes, lipid accumulation, and the expression of adipocyte differentiation biomarkers during 21-day adipogenesis. Significant cytotoxicity was observed in un-induced hMSCs treated with high-concentration TCS (≥5.0 μM TCS), but not with low-concentration treatments (≤2.5 μM TCS). TCS inhibited adipocyte differentiation of hMSCs in a concentration-dependent manner in the 0.156 to 2.5 μM range as indicated by morphological changes with Oil Red O staining, which is an index of lipid accumulation. The inhibitory effect was confirmed by a decrease in gene expression of specific adipocyte differentiation biomarkers including *adipocyte protein 2*, *lipoprotein lipase*, and *adiponectin*. Our study demonstrates that TCS inhibits adipocyte differentiation of hMSCs under concentrations that are not cytotoxic and in the range observed in human blood.

Published by Elsevier Inc.

Introduction

Triclosan (TCS) has been used for more than 40 years in the United States as an antimicrobial agent in personal care products, household items, medical devices, and clinical settings (Jones et al., 2000). The general population is exposed to TCS through dermal contact with daily hygiene products and the consumption of food and drinking water containing TCS. In addition, workers may be exposed to TCS in the manufacturing environment by dermal contact and inhalation (Fang et al., 2010).

Allmyr et al. (2006) tested human plasma, breast milk, and urine collected from mothers using TCS-containing and TCS-free personal care products. This study showed that TCS-containing products were the dominant, but not exclusive, source of systemic exposure to TCS. It was reported that TCS was found in three out of five human breast milk samples randomly collected in the Stockholm area in Sweden, although this study only had a small sample size (n = 5) (Adolfsson-Erici et al., 2002). In the United States, TCS was detected in human milk (100 to 2100 μg/kg lipid) in 51 out of 62 samples from the Breast Milk Banks in California and Texas (Dayan, 2007). TCS was also detected in human blood plasma samples from Sweden (Sandborgh-Englund et al., 2006) and Australia (Allmyr et al., 2008, 2009). It has been reported that the maximum exposure of babies via breast milk is approximately 7.4 μg per kg body weight per day and that TCS in breast milk presents minimal risk to babies at this level of exposure (Dayan, 2007). Several studies have shown that the blood levels of total TCS in humans following use in either mouth rinses or dentifrices vary from 1.4 nM to 1.4 μM (Bagley and Lin, 2000; DeSalva et al., 1989; Sandborgh-Englund et al., 2006). After absorption, the major fraction of TCS is excreted in urine within 24 h. However, total urinary excretion of TCS varied among individuals, with 24% to 83% of the oral dose being excreted during the first 4 days after exposure (Sandborgh-Englund et al., 2006).

In bacteria, sub-lethal concentrations of TCS inhibit enoyl-reductase, a type II fatty acid synthase (FAS) enoyl-reductase (FabI) that is

☆ The views presented in this article do not necessarily reflect those of the U.S. Food and Drug Administration.

* Correspondence to: J.-L. Fang, National Center for Toxicological Research, 3900 NCTR Road, HFT110, Jefferson, AR 72079, USA. Fax: +1 870 543 7136.
** Correspondence to: B. Ning, National Center for Toxicological Research, 3900 NCTR Road, HFT100, Jefferson, AR 72079, USA. Fax: +1 870 543 7773.
E-mail addresses: jia-long.fang@fda.hhs.gov (J.-L. Fang), baitang.ning@fda.hhs.gov (B. Ning).

[1] These authors contributed equally to the work.

0041-008X/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.taap.2012.04.024

involved in bacterial lipid biosynthesis. Since FabI-synthesized fatty acids are essential for reproducing and building up cell membranes, bacteria are sensitive to TCS. Although there is no direct evidence suggesting that TCS is hazardous to humans, several recent studies have found that it may facilitate antibacterial resistance (Aiello et al., 2007) or interfere with endocrine functions in rats (Paul et al., 2010; Stoker et al., 2010). In mammals, TCS was demonstrated to be cytotoxic to MCF-7 and SK-BR-3 breast cancer cells at such concentrations (Liu et al., 2002a). Rodricks et al. reported an increased incidence of liver tumors induced by TCS in an oral 18-month carcinogenicity bioassay in mice (Rodricks et al., 2010). These authors proposed that the carcinogenicity of TCS might be attributed to its activation of the peroxisome proliferator-activated receptor α (PPARα).

Stem cells have become attractive tools for toxicity testing because of their sensitivity to external stimuli during differentiation (Stewart and Bolt, 2011). Testing the toxicity of human drugs in adult animal models is not likely to detect adverse effects on development and cell differentiation that occur prenatally. Stem cells express a unique set of proteins that direct cellular metabolism and differentiation; exposure to a drug may change the expression of these important proteins, disrupt communication between cells, and cause pathological changes. It was reported that exposure to 50 μM TCS prevented preadipocyte differentiation in mouse 3T3-L1 cells (Schmid et al., 2005). Wiaderkiewicz et al. reported that exposure of Sprague–Dawley rats to electromagnetic fields combined with TCS blocked the differentiation of liver mesenchymal stem cells, thus affecting regeneration of injured liver (Wiaderkiewicz et al., 2007). Moreover, the high potency of TCS as an inhibitor of estrogen sulfotransferase activity raises concerns about its possible effects on fetal growth and development (James et al., 2009). It is of interest to investigate the effects of TCS at varying concentrations on human stem cells, since stem cells may exhibit differential sensitivities to low dose and non-cytotoxic chemicals (Trosko and Chang, 2010).

Adverse effects on human stem cell differentiation should be considered when performing a human risk assessment for exposure to low concentrations of TCS. Mesenchymal stem cells (MSCs) are multipotent cells that can be differentiated into a variety of cell types (adipocytes, osteoblasts and chondrocytes) with specific inducers (Jiang et al., 2002; Pittenger et al., 1999). In the current study, we investigated the biological effects of exposure to low concentrations of TCS in hMSCs by measuring toxicological and phenotypic characteristics, such as cell viability, lipid accumulation during adipogenesis, and the expression of specific adipocyte differentiation biomarkers *adipocyte protein 2* (*aP2*), *lipoprotein lipase* (*LPL*), and *adiponectin*.

Materials and methods

Chemicals and primers. Triclosan [5-chloro-2-(2, 4-dichlorophenoxy) phenol, TCS] was obtained from Alfa Aesar (a Johnson Matthey Company, Ward Hill, MA) and dissolved in 99.5% dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO). Dexamethasone, isobutylmethylaxanthine, and indomethacin were obtained from Sigma-Aldrich. RNeasy Mini Kit was purchased from QIAGEN (Valencia, CA). RNA Nano Chips and reagents Part I and II were obtained from Agilent Technologies (Santa Clara, CA). All other chemicals and biochemicals were of analytical grade and used without further purification. TaqMan® assays for genes of interest (*aP2*: probe Hs01086177_m1; *LPL*: probe Hs00173425_m1; and *adiponectin*: probe Hs00605917_m1) were obtained from Life Technologies (Carlsbad, CA).

Cell culture and adipocyte differentiation. Human adult mesenchymal stem cells (hMSCs) were originally provided by AllCells (Emeryville, CA). hMSCs were grown in alpha Minimum Essential Medium (αMEM), supplemented with 16.5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine, and penicillin/streptomycin at a concentration of 100 μg/ml each (Sigma-Aldrich), in a humidified incubator at 37 °C, with 5% CO_2. Aliquots of cells (2×10^4) were seeded in each well of 24-well plates. When cells reached 70–80% confluence, a fat differentiation medium (FDM, αMEM supplemented with 0.5 μM dexamethasone, 0.5 μM isobutylmethylxanthine, 50 μM indomethacin, 16.5% fetal bovine serum, 2 mM L-glutamine, and antibiotics) was used to initiate adipocyte differentiation. The media were changed every 3–4 days for 21 days. During the adipocyte differentiation process, lipid droplets became observable at the light microscopic level around day 10 and usually reached maximal around day 21. To determine the effect of TCS exposure on this differentiation, varying concentrations of TCS were added to FDM at the beginning of the 21-day incubation for adipocyte differentiation.

Cytotoxicity of TCS on hMSCs. The cytotoxicity of TCS was assessed using an MTS CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI). Briefly, un-induced stem cells (i.e., cultured with αMEM) were plated into 24-well plates and treated with TCS at 0.625 to 40 μM for 2 days. For the induced stem cells (i.e., cultured with FDM), hMSCs were treated with TCS at 0.156 to 2.5 μM for 21 days. Control cells were fed with complete cell culture medium free of TCS and containing 0.1% dimethylsulfoxide. At the end of the treatment, the 24-well cultures were incubated with MTS solution for 2 h at 37 °C. The absorbance at 570 nm, with 690 nm as the reference wavelength, was measured with a BioTek Synergy 2 Reader (BioTek Instruments, Inc., Winooski, VT). The viability of the cells was calculated by comparing the absorbance of the treated cells to the controls.

Lactate dehydrogenase (LDH) released into cell culture media was used as a marker of cell membrane damage, which indicates cell necrosis. The amount of LDH released into cell culture media was measured with an LDH kit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, 150 μl of cell culture media from each well of all groups were collected at days 2, 6, 9, 12, 15, 18, and 21, and then centrifuged at 1000 *g* for 10 min. After centrifugation, 100 μl of supernatant from each well was used for the LDH release assay and its amounts determined by subtracting the background absorbance at 690 nm from the primary wavelength measurement (490 nm), using a BioTek Synergy 2 Reader. The amount of LDH from each well was presented as the percentage of LDH released in cell culture media without TCS treatment (control).

Oil Red O staining and quantification. At day 21, cultured cells were washed 3 times with PBS and then fixed in 10% buffered formalin solution (Sigma-Aldrich) at room temperature for 1 h. They were then stained with freshly filtered Oil Red O (Sigma-Aldrich) solution at room temperature for 20 min followed by rinsing 3 times with PBS. Oil Red O-stained cells were morphologically characterized using a microscope. To quantitate the uptake of this dye, the cells were washed three times using a total volume of 300 μl 100% isopropanol. The absorbance (490 nm) of the combined isopropanol extracts was measured using a UV spectrophotometer.

Real time-PCR. At the end of the TCS treatments, total RNA from each sample was isolated from the treated and control cells (~5×10^5 cells) using an RNeasy Mini Kit (QIAGEN) and their qualities and quantities were measured using a 2100 Bioanalyzer (Agilent Technologies). RNA Integrity Numbers for all samples were above 9.0. Total RNA (0.5 μg) was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit with random hexamers (Life Technologies). The resulting cDNAs were quantified with TaqMan® real-time PCR on a 7900 HT Fast Real Time PCR System (Life Technologies). Data were normalized for the internal control, *GAPDH*, and analyzed using the $_{\Delta\Delta}$Ct method.

Statistical analyses. Data are presented as mean ± standard deviation of three independent experiments. Statistical significance was determined by one-way analysis of variance followed by the Dunnett's