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# Metabolism of the Antibacterial Triclocarban by Human Epidermal Keratinocytes to Yield Protein Adducts

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**ABSTRACT:** Previous studies of triclocarban suggest that its biotransformation could yield reactive metabolites that form protein adducts. Since the skin is the major route of triclocarban exposure, present work examined this possibility in cultured human keratinocytes. The results provide evidence for considerable biotransformation and protein adduct formation when cytochrome P450 activity is induced in the cells by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a model Ah receptor ligand. Since detecting low adduct levels in cells and tissues is difficult, we utilized the novel approach of accelerator mass spectrometry for this purpose. Exploiting the sensitivity of the method, we demonstrated that a substantial portion of triclocarban forms adducts with keratinocyte protein under the P450 inducing conditions employed. © 2012 Wiley Periodicals, Inc. *J Biochem Mol Toxicol* 26:230–234, 2012; View this article online at [wileyonlinelibrary.com](http://wileyonlinelibrary.com). DOI 10.1002/jbt.21411

**KEYWORDS:** Accelerator Mass Spectrometry; Biotransformation; Cytochrome P450; Keratinocytes; Protein Adducts; Reactive Metabolites; TCDD; Triclocarban

## INTRODUCTION

Triclocarban (TCC) is a common antimicrobial preservative in personal care products, particularly in bar soaps. Human exposure to TCC has come to public attention because it accumulates in the aquatic environment [1] and has biological effects on mammals. TCC is a potent inhibitor (IC<sub>50</sub> 24 ± 5 nM) of the human soluble epoxide hydrolase, an enzyme of the arachidonic cascade [2–4]. Similarly potent inhibitors have pronounced pharmacological effects on regulation of inflammation and pain [5]. Moreover, TCC may act as an endocrine disruptor at high concentration by enhancing the action of testosterone [6].

Bathing with TCC-containing soaps typically results in deposition of TCC on human skin of ≈0.3 μg/cm<sup>2</sup> [7]. A substantial portion traverses the epidermal barrier, is absorbed, and becomes systemically available [4,8]. During showering with antibacterial soap, ≈0.6% of the applied amount of TCC is dermally absorbed and is detected almost exclusively as metabolites [4]. In the plasma of mammals, TCC is found primarily as its sulfated oxidative metabolites, particularly 2'-SO<sub>3</sub>-O-TCC. In the bile, glucuronic acid conjugates of hydroxylated metabolites dominate, and in human, monkey and mouse urine primarily *N*-glucuronides are excreted [9,10]. Thus, both oxidative phase I metabolism, catalyzed by cytochrome-P-450 monooxygenases, and phase II metabolism, catalyzed by sulfotransferases and UDP-glucuronyltransferases, are important. Since keratinocytes in the epidermis and in culture express substantial phase I and phase II activities [11,12], this work explores the hypothesis that, upon dermal exposure, TCC undergoes biotransformation that produces potentially deleterious reactive metabolites. Use of accelerator mass spectrometry

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(AMS) offers the possibility to detect protein adducts of such metabolites with much greater sensitivity than with other methods [13–15].

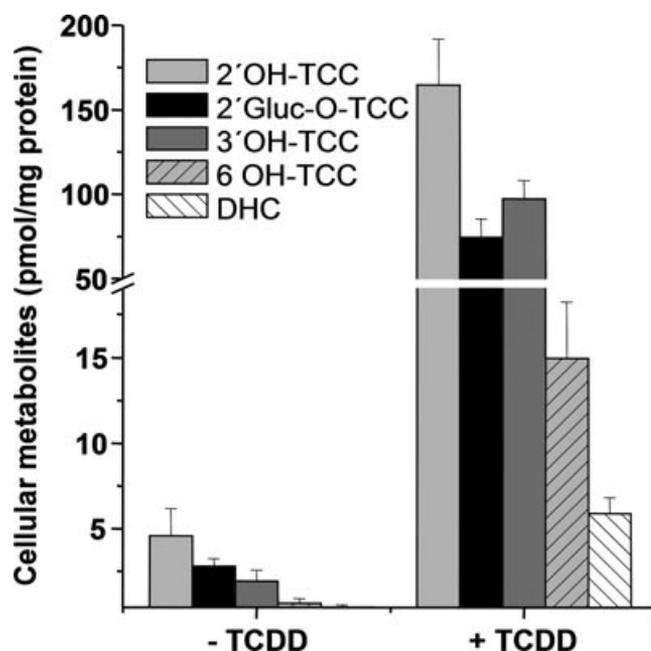
## MATERIALS AND METHODS

### Generation and Analysis of Metabolites in Cell Culture

Human epidermal spontaneously immortalized keratinocytes (SIK) were cultured with feeder layer support in a Dulbecco-Vogt modified Easgle's medium (DMEM)–Ham's F-12 mixture (2:1), as previously described [16]. For metabolite identification, near-confluent cultures were treated with or without 10 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for one day before the addition of 2  $\mu$ M TCC (1:1000 dilution of a 2 mM stock solution in DMSO). After 10 min or 20 h incubation, cultures were rinsed in 0.15 M sodium chloride–10 mM sodium phosphate (pH 7.2), drained, recovered by scraping from the dishes in 0.5 mL of 10 mM Tris (pH 8)–1 mM EDTA, sonicated, and stored at  $-80^{\circ}\text{C}$  in two equal aliquots. Fifty microliters of the homogenate were mixed with 200  $\mu$ L of acetonitrile/acetic acid [99/2 (v/v)] containing 12.5 nM  $^{13}\text{C}_6$ -TCC as internal standard and analyzed directly by online solid phase extraction-liquid chromatography-mass spectrometry (SPE-LC-MS) as previously described [17]. The second aliquot was dissolved in 2% sodium dodecyl sulfate (SDS) for protein assay using bicinchoninic acid (Pierce Chem Company, Rockford, IL). For the determination of protein binding, cells were incubated for 3, 8, and 24 h with 2  $\mu$ M [ $^{14}\text{C}$ ]TCC (specific activity 0.04 Ci/mol), rinsed twice with phosphate buffered saline, dissolved in 2% SDS containing 25 mM dithioerythritol (DTE), and sulfhydryls were alkylated with iodoacetamide. The protein was isolated by Sephadex G-25 gel filtration in 0.1% SDS and precipitated by the addition of 2.5 vol of ethanol. The pellet was rinsed 3 $\times$  in 67% ethanol and then analyzed for  $^{14}\text{C}$  content.

### AMS Analysis

Each protein pellet was placed in a quartz tube ( $\sim 6 \times 30$  mm, 4 mm i.d.) nested inside two borosilicate glass culture tubes (10  $\times$  75 mm in 12  $\times$  100 mm) and dried overnight in a vacuum centrifuge. An excess of CuO ( $\approx 40$  mg) was added and the inner quartz vials were transferred to quartz combustion tubes, evacuated, and sealed. The samples were combusted at  $900^{\circ}\text{C}$  for 3.5 h to oxidize completely all carbon to  $\text{CO}_2$  and then reduced to filamentous carbon, as previously described [18]. Carbon samples were packed into aluminum sample holders, and carbon isotope ra-



**FIGURE 1.** TCC biotransformation by human keratinocytes. Parallel SIK cultures pretreated one day  $\pm$  10 nM TCDD as indicated were then treated for 20 h with 2  $\mu$ M TCC. The metabolite levels detected were normalized to protein concentration ( $3.6 \pm 0.4$  mg/mL without TCDD;  $4.1 \pm 0.3$  mg/mL after TCDD treatment). DHC: 3,4-dichloro-4'-hydroxy-carbanilide. Each experiment was conducted in triplicate, and values are presented as means  $\pm$  standard deviation.

tios were measured on the compact 1-MV AMS spectrometer at the Lawrence Livermore National Laboratory centered around a National Electrostatics Corporation 3SDH-1 accelerator [19]. Typical AMS measurement times were 3–5 min/sample, with a counting precision of 0.8%–1.4% and a standard deviation among 3–7 measurements of 1%–3%. The  $^{14}\text{C}/^{13}\text{C}$  ratios of the unknowns were normalized to measurements of four identically prepared standards of known isotope concentration (Australian National University Sucrose) and converted to units of pmol TCC/mg protein [20].

## RESULTS

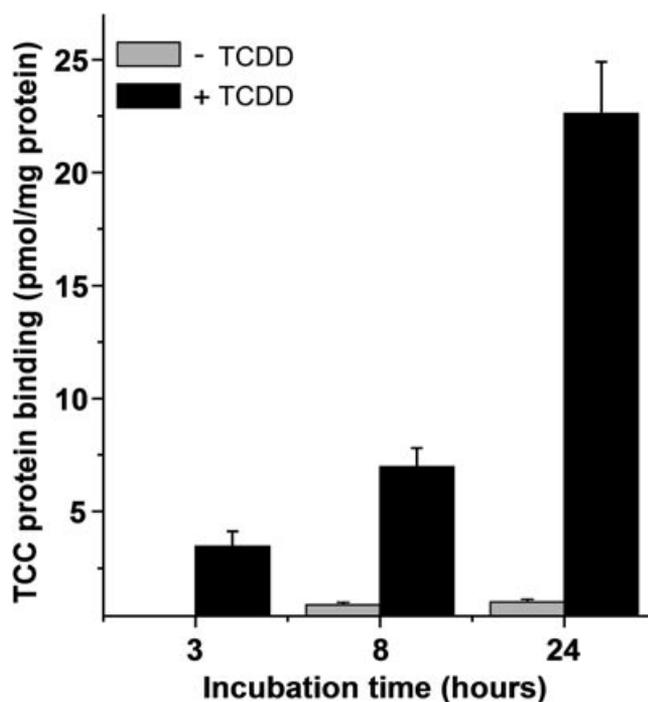
TCC was rapidly absorbed by the epidermal cells. After 10 min, the intracellular TCC concentration reached  $1.21 \pm 0.27$  nmol/mg protein and increased by 20 h incubation to a level of  $2.04 \pm 0.27$  nmol/mg protein. LC-MS/MS analysis revealed that a small portion of the absorbed TCC was metabolized by SIK. After 24 h,  $4.6 \pm 1.6$  pmol/mg protein 2'-OH-TCC was found, along with lower concentrations of the other known monohydroxylated TCC metabolites, 3'-OH-TCC ( $1.9 \pm 0.7$  pmol/mg protein) and 6-OH-TCC ( $0.4 \pm 0.7$  pmol/mg protein). The glucuronide of 2'-OH-TCC was the second most abundant detected

metabolite (Figure 1). Preincubation with 10 nM TCDD dramatically augmented metabolism of TCC in the keratinocytes, and the relative conversion (cellular metabolite concentration versus cellular TCC concentration) increased from  $\approx 0.5\%$  to 15% (Figure 1). In line with previous findings that TCC is not an aryl hydrocarbon receptor (AhR) agonist [21], this finding indicates that the CYP isoforms responsible for TCC metabolism (likely CYP1A1 and CYP1B1) are inducible by AhR agonists but are not well induced by TCC alone. After TCDD induction, unconjugated 2'-OH-TCC remained the major metabolite with a concentration of  $165 \pm 28$  pmol/mg protein, far exceeding the concentration of its conjugates. At  $5.91 \pm 0.93$  pmol/mg protein, the recently described oxidative metabolite 3,4-dichloro-4'-hydroxy-carbanilide (DHC) [22] was also formed in substantial amounts. 2'-SO<sub>3</sub>-O-TCC levels were  $<1$  pmol/mg protein, suggesting that 2'-OH-TCC is a poor substrate for the major sulfotransferase (SULT2B1b) reported in epidermis [11] and expressed in SIK [16]. Additionally, formation of 6-OH-Gluc-TCC was detected, whereas no evidence for direct glucuronidation of TCC to the major urine *N*-glucuronide metabolites of TCC [4] was found.

Since TCC can be metabolized in epidermal keratinocytes, it is anticipated to be biotransformed during dermal absorption from personal care products. The observed metabolites are consistent with those detected in mammals with 2'-OH-TCC as the major oxidative metabolite [9]. By contrast to the metabolite patterns in blood, bile, and urine, the amount of unconjugated oxidative metabolites of TCC exceeded by far the level of conjugated (phase II) species. Nearly two fold more 2'-OH-TCC was formed than 2'-Gluc-O-TCC with or without TCDD pretreatment (Figure 1). Because further oxidation of 2'-OH-TCC can yield a reactive quinone imine [22], we hypothesized that TCC metabolism in keratinocytes can lead to protein adduct formation. As shown in Figure 2, up to  $23 \pm 2$  pmol TCC adducts/mg protein were detected after 24 h incubation in the presence of TCDD. As illustrated, protein adducts increased in a time-dependent manner, but only very low adduct formation of about 1 pmol/mg protein, slightly above the background signal, was detected in the absence of TCDD, in line with the low metabolic conversion of TCC (Figure 1).

## DISCUSSION

This study demonstrates that TCC oxidative conversion leads to reactive intermediates that can bind covalently to protein, a phenomenon clearly manifest upon induction of metabolizing enzymes. It is noteworthy that the low level of TCC adducts produced



**FIGURE 2.** Covalent protein adduct formation by TCC (2  $\mu$ M) in spontaneously immortalized keratinocytes (SIK) determined by accelerator mass spectrometry. A background value of 0.5 pmol/mg (0.5 h incubation without TCDD induction) has been subtracted. Each experiment was conducted in triplicate and values are presented as means  $\pm$  standard deviation.

a substantial <sup>14</sup>C signal, which was only  $\approx 20\times$  fold higher than the natural <sup>14</sup>C/C concentration of  $1.2 \times 10^{-12}$ . Because of the high sensitivity of AMS, quantitative information about very low protein adducts of TCC can be obtained, enabling investigation of their formation mechanistically. The relatively low protein adduct formation of TCC and high sensitivity of AMS are well suited for obtaining quantitative information on the distribution of protein adducts in vivo, especially if individual proteins are isolated to identify specific targets.

Human epidermal cells cultured in the Rheinwald-Green system provide a close approximation to natural epidermis [23]. The SIK line, a minimally deviated epidermal model, has been utilized previously to demonstrate the importance of CYP induction to produce toxic effects of agents that are themselves poor inducers [24]. The reactivity of a chemical to produce a complete antigen by covalently adducting carrier protein is the major factor in producing allergic skin sensitization [25]. Among the numerous compounds for which the importance of epidermal cytochrome P450 activity is known to activate nonreactive chemicals is the common rubber constituent and known sensitizer diphenylthiourea [26]. In the absence of P450 induction, the low level of

TCC-protein adducts suggests only a low probability of adverse effects in keratinocytes. However, the substantial level of protein adducts with TCDD treatment raises the possibility of skin sensitization in special circumstances. P450 inducers such as AhR agonists [27], for which TCDD in this work serves as a proxy, are widely encountered in tobacco smoke, pharmaceuticals, food constituents, and other consumer products, as well as in environmental pollutants, and even from the skin microflora [28]. The unrecognized possibility that TCC, with wide human exposure to the integument, contributes to the burden on society of allergic contact dermatitis or other toxic effects, particularly in individuals exposed to ubiquitous AhR agonists, merits further investigation. Moreover, in view of the ability of Langerhans cells to participate in activation of chemicals in the epidermis [29], present work could underestimate the risk posed by TCC.

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