Metabolic Activation of the Antibacterial Agent Triclocarban by Cytochrome P450 1A1 Yielding Glutathione Adducts

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ABSTRACT

Triclocarban (TCC) is an antibacterial agent used in widespread use in personal care products, particularly in bar soaps, with annual production totaling several million pounds (Halden and Paull, 2005). Due to its lipophilicity, TCC bioaccumulates in the aquatic environment and is one of the most abundant anthropogenic compounds found in sewage sludge (Langford et al., 2011). Moreover, significant TCC bioconcentration in aquatic organisms such as algae, snails (Coogan et al., 2007; Coogan and La Point, 2008), and fish (Schebb et al., 2011a) has been reported.

Despite initial reports that TCC is biologically inactive, more recent work suggests that this chemical may not be as innocuous as once thought. First, TCC may have the potential to act as an endocrine disruptor by enhancing the action of testosterone (Ahn et al., 2008; North-Root et al., 1984). A 2009 study characterized the reactive metabolites trapped as glutathione conjugates. Incubations were carried out with 14C-labeled TCC, recombinant CYP1A1 or CYP1B1, coexpressed with cytochrome P450 reductase, glutathione-transferases (GSH), and an NADPH-generating system. Incubations containing CYP1A1, but not CYP1B1, led to formation of a single TCC-GSH adduct with a conversion rate of 1% of parent compound in 2 hours. Using high-resolution mass spectrometry and diagnostic fragmentation, the adduct was tentatively identified as 3,4-dichloro-3'-glutathionyl-4'-hydroxyxycarbanilide. These findings support the hypothesis that TCC is activated by oxidative dehalogenation and oxidation to a quinone imine. Incubations of TCDD-induced keratinocytes with 14C-TCC yielded a minor radioactive peak coeluting with TCC-GSH. Thus, we conclude that covalent protein modification by TCC in TCDD-induced human keratinocyte incubations is mainly caused by activation of TCC by CYP1A1 via a dehalogenated TCC derivative as reactive species.

Introduction

Triclocarban (TCC) is an antimicrobial agent in widespread use in personal care products, particularly in bar soaps, with annual production totaling several million pounds (Halden and Paull, 2005). Due to its lipophilicity, TCC bioaccumulates in the aquatic environment and is one of the most abundant anthropogenic compounds found in sewage sludge (Langford et al., 2011). Moreover, significant TCC bioconcentration in aquatic organisms such as algae, snails (Coogan et al., 2007; Coogan and La Point, 2008), and fish (Schebb et al., 2011a) has been reported.

Despite initial reports that TCC is biologically inactive, more recent work suggests that this chemical may not be as innocuous as once thought. First, TCC may have the potential to act as an endocrine disruptor by enhancing the action of testosterone (Ahn et al., 2008; North-Root et al., 1984). A 2009 study characterized the reactive metabolites trapped as glutathione conjugates. Incubations were carried out with 14C-labeled TCC, recombinant CYP1A1 or CYP1B1, coexpressed with cytochrome P450 reductase, glutathione-transferases (GSH), and an NADPH-generating system. Incubations containing CYP1A1, but not CYP1B1, led to formation of a single TCC-GSH adduct with a conversion rate of 1% of parent compound in 2 hours. Using high-resolution mass spectrometry and diagnostic fragmentation, the adduct was tentatively identified as 3,4-dichloro-3'-glutathionyl-4'-hydroxyxycarbanilide. These findings support the hypothesis that TCC is activated by oxidative dehalogenation and oxidation to a quinone imine. Incubations of TCDD-induced keratinocytes with 14C-TCC yielded a minor radioactive peak coeluting with TCC-GSH. Thus, we conclude that covalent protein modification by TCC in TCDD-induced human keratinocyte incubations is mainly caused by activation of TCC by CYP1A1 via a dehalogenated TCC derivative as reactive species.

Abbreviations: ACN, acetonitrile; DHC, 3,4-dichloro-4'-hydroxyxycarbanilide; ESI, electrospray ionization; GSH, glutathione; HPLC, high-performance liquid chromatography; LC, liquid chromatography; m/z, mass-to-charge ratio; MS, mass spectrometry; P450, cytochrome P450; TCC, 3,4,4'-trichlorocarbanilide, triclocarban.
TCDD or polycyclic aromatic hydrocarbons (Gotz et al., 2012). The present studies demonstrate the conversion of TCC by CYP1A1 to a reactive metabolite trapped as a glutathione conjugate. Conversion rates were monitored by LC/radioprofiling of extracts of incubations containing 14C-TCC, and the GSH conjugate was characterized by high-resolution mass spectrometry.

Materials and Methods

Radiochemical. 14C-(3,4,4′-Trichlorocarbanilide), uniformly labeled in the chlorophenyl ring, was obtained from Moravek Radiochemicals (Brea, CA) at a specific activity of 30 Ci/mmol. The material was tested for chemical and radioactive purity by reverse phase LC and found to be >99.5% pure.

Incubations with Recombinant CYP1A1 and CYP1B. Supersomes containing human CYP1A1 or CYP1B1 plus P450 reductase expressed in baculovirus-infected insect cells were obtained from BD Biosciences (San Jose, CA). Supersomes (500 pmole P450) were incubated with NADPH-generating solution (0.14 mM NADP, 3.8 mM glucose 6-phosphate, 0.1 IU glucose-6-phosphate dehydrogenase, 10 mM MgCl2). 14C-TCC (66 dpm/pmol, for metabolite profiling), or unlabeled TCC (for LC-ESI-MS/MS analysis) (10 μM final concentration, added in dimethyl sulfoxide to less than 0.1% of the total volume), glutathione (5 mM final concentration), and affinity purified mouse glutathione-S-transferases (conjugation activity: 5 μmol 1-chloro-2,4-dinitrobenzene per minute). Reactions were carried out in a shaking incubator at 37°C for 2 hours with a second addition of NADPH-generating system at 1 hour. Acetonitrile (3 vol, 4°C) was added to stop the reaction, precipitated protein was removed by centrifugation, and the supernatant was lyophilized to dryness.

Keratinocyte Culture. Human epidermal spontaneously immortalized keratinocytes (SIK) were cultured with 3T3 feeder layer support in a 4°C) was added to stop the reaction, precipitated protein was removed by centrifugation, and the supernatant was lyophilized to dryness.

Solid Phase Extraction. Waters Oasis HLB solid phase extraction columns (500 mg/12 ml extraction cartridge; Milford, MA) were prepared by washing with 5 ml of 100% ACN followed by 3 × 5 ml washes with 0.5% formic acid in water. The lyophilized sample was dissolved in 5 ml of 0.5% formic acid and applied to the column. The column was rinsed with 5 × 5 ml of 0.5% formic acid, and TCC metabolites were eluted with 15 ml of 75% ACN. After incubation for an additional 18 hours, the cells and medium were transferred to centrifuge tubes and mixed with 12 ml of acetonitrile (ACN) and frozen. After thawing the samples were centrifuged to remove precipitated protein and the supernatant was lyophilized.

HPLC Radioprofiling and LC-MS/MS Analysis. Samples were injected onto a Phenomenex (Torrance, CA) Kinetex XB-C18 column (2.6-μm particle size). For radioprofiling, the dimensions of the column were 150 × 4.6 mm, and a solvent flow rate of 1 ml/min was used. A 150–21.2-mm column of the same type was eluted at 210 μl/min for LC-ESI-MS/MS analysis. Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid–50% acetonitrile in water. The analytes were eluted with the following gradient: 0–100 minutes linear from 5% to 100% solvent B, 100–130 minute isocratic 100% B. For radioprofiling, fractions were collected at 30-second intervals and were counted for 2 minutes in a liquid scintillation counter.

ESI-MS/MS analysis was performed on an Orbitrap XL spectrometer (Thermo Scientific, Bremen, Germany). The analytes were ionized by electrospray ionization with mass resolution set to 15,000. Fragmentation was carried out on the two most abundant ions with collision energy of 35 V. To prevent contamination of the ion source, the first 5 minutes of the separation were directed to waste. The data were analyzed for GSH adducts utilizing product ion scans and neutral loss filtration was carried out with Metworks 1.3 (ThermoScientific). The data were analyzed for molecules showing the mass defect of TCC (0.0220 ± 0.05 Da). The range of ±0.05 Da covers most metabolic reactions, but the loss of chlorine or addition of GSH causes a greater increase in mass defect (Zhang et al., 2003; Zhu et al., 2006). Therefore, other filters were used for the mass defects of 2-OH-TCC GSH adduct –0.0411 Da (Baumann et al., 2010) and potential bis-GSH adduct –0.1093 Da, as well as DHC (–0.0119) and its GSH and bis-GSH adducts –0.0801 Da and –0.1482 Da each in a range of ≥0.05 Da.

Results and Discussion

The conversion of TCC to oxidative metabolites and GSH adducts was analyzed by LC-ESI-MS analysis. As shown in Fig. 1, A and B, the total ion current (TIC) using negative ESI of an extract from an incubation containing recombinant CYP1A1 showed two major peaks at 30 and 55 minutes followed by various signals eluting at the end of the chromatogram. Following positive ionization several additional peaks were detected, while corresponding peaks a and b were still clearly detectable (Supplemental Fig. 1). The ESI(−) MS signal obtained with extracts of incubations containing recombinant CYP1B1 displayed a single peak eluting at ~30 minutes, similar to a peak recovered from incubations containing recombinant CYP1A1. Since the GSH adducts appear to be generated slowly, thereby yielding very small TIC signals, the MS scan data were analyzed for diagnostic fragmentation of GSH adducts, i.e., formation of a fragment at m/z 272 in negative ESI and neutral loss of 129 Da in positive ionization mode (Dieckhaus et al., 2005; Ma and Subramanian, 2006). As shown in Fig. 2 both analyses showed a single signal at the retention time of peak b, indicating that a GSH adduct elutes at ~55 minutes. No peaks were found in the analysis of extracts of incubations containing CYP1B1.

In a complementary strategy, the high-resolution MS chromatograms of extracts of CYP1A1 incubations were analyzed for TCC metabolites. With three chlorine atoms, TCC and its metabolites show both a distinct isotopic pattern as well as a high mass defect, allowing a specific analysis for unknown metabolites. However, no signals for metabolites bearing three chlorine atoms were found. Since TCC can be oxidatively dehalogenated to DHC (Baumann et al., 2010), the same analysis was carried out for the isotopic pattern of molecules containing two chlorine atoms. Here a peak eluting at 55 minutes resulted from the incubation with CYP1A1 (Fig. 2C), suggesting that this metabolite has two chlorines. Similarly, mass defect filtering for the GSH adduct of DHC shows a clear signal at 55 minutes (Fig. 2D), while no further signals could be found for the mass defect of the TCC-GSH adduct. Analyses of extracts prepared from incubations containing CYP1B1 failed to reveal signals consistent with the formation of TCC-GSH adducts.

The MS spectra of peak b showed dominant ions at m/z 600.0723 in negative ESI mode, with the characteristic pattern of a single charged ion of a compound bearing two chlorine atoms (Fig. 1, Supplemental Fig. 1). These are likely the [M-H]1 ions of a TCC metabolite since, in positive ESI, corresponding ions at 2.000 Da higher mass were found (Supplemental Fig. 1). The exact (monoisotopic) mass of this metabolite is 601.0801 amu, which corresponds exactly to the calculated mass of a DHC-GSH adduct. Moreover, the observed fragmentation pattern is consistent with the suggested structure of a DHC-GSH adduct. As shown in Fig. 3, fragmentation in negative ion mode leads to four major fragments, while positive ESI gave rise to two major fragments. The main fragment at m/z 413.1130 can be attributed to scission of the carbon nitrogen bond (a in Fig. 3A) of the urea, leading to an aniline fragment. This is a major route of fragmentation of TCC and its derivatives following negative ESI
The other fragment ions can be assigned to the fragmentation of GSH. Accordingly, the ions at $m/z$ 326.9796 and $m/z$ 272.0887 are both sides of the product resulting from fragmentation of the carbon thiol bond of GSH (Fig. 3B, Supplemental Fig. 2). The signal at 254.0784 results from further fragmentation at site c. Fragmentation of the amide bonds of GSH yielded fragments at $m/z$ 473.0442 and 527.0544. All major fragments support the suggested structure of the DHC-GSH adduct. Moreover the exact mass of the

Fig. 1. Radioactivity profile of LC fractions and LC-ESI(−)-MS chromatograms of incubation of TCC with CYP1A1 (A) and CYP1B1 (B). Inset: MS spectrum of the peak eluting at 55.3 minutes.

Fig. 2. Extracted ion signals for diagnostic fragments of GSH adducts of incubation of TCC with CYP1A1. (A) ESI(−)-MS/MS of the GSH fragment at $m/z$ 227 and (B) ESI(+)-MS/MS neutral loss scan of 129 Da. (C) Isotopic pattern analysis for two chlorine atoms. (D) Mass defect analysis for the DHC-GSH adduct.
parent molecule as well as product ions are fully consistent with the calculated masses of the fragments (Fig. 3).

The DHC-GSH conjugate can be formed from TCC by dehalogenation and hydroxylation followed by nucleophilic addition to the mono-chloraniline ring. To support the assumption that the GSH adduct arises via the intermediate formation of DHC, incubation of CYP1A1 with DHC as precursor was carried out. This incubation yielded a peak at the same retention time showing the same ESI-MS, ESI-MS/MS ions as the incubation of TCC (Supplemental Fig. 3). Thus, TCC is metabolically activated by oxidative dehalogenation at the mono-chloraniline ring and hydroxylation to a p-quinone imine that generates the glutathione adduct. Incubation of TCC with CYP1A1 but not 1B1 gave rise to GSH adducts (Fig. 1). No radioactive peak was observed in the retention-time window for CYP1B1 incubations. In both CYP1A1 and CYP1B1 incubations, a smaller peak eluted from the column at 31 minutes with a maximum radioactivity of ~1500 dpm (0.3% of the added radioactivity). No structural information could be obtained on this peak due to the small amounts present (Fig. 1). Dominant ions using negative ESI observed in extracts from both CYP1A1 and CYP1B1 incubations included those at m/z 413, 497, 565, and 597, which revealed neither a chlorine isotope pattern nor typical GSH fragments (data not shown).

A 2-hour incubation with 0.5 nmol of CYP1A1 led to the formation of 0.5 nmol of GSH adduct, or product formation as low as 1 nmol/nmol P450 per 2 hours. It is difficult to extrapolate from the observed formation rate in vitro to the generation of metabolites in tissues in vivo. TCC conversion to both oxidative metabolites and N-glucuronides is substantially underpredicted by in vitro microsomal incubations (Baumann et al., 2010; Schebb et al., 2012b). This low predictability is likely related to the poor water solubility of the compound, 142 nM (Snyder et al., 2010), causing a low effective concentration in the assays. Thus, TCC activation might occur to a much greater extent in vivo. In fact, the suggested precursor of the reactive metabolite has been found in a number of different organisms ranging from medaka fish to human keratinocytes (Schebb et al., 2011a, 2012a).

The finding that the TCC-GSH adduct—and thus reactive metabolite—formation is catalyzed by CYP1A1 is consistent with data showing that TCC-protein–adduct formation in keratinocytes is increased substantially following induction with TCDD. As shown in Fig. 4, incubation of 14C-TCC with TCDD-induced keratinocytes resulted in the formation of multiple polar radiolabeled metabolites resolved by HPLC (a–f). The peaks b, c, d, e, and f coeluted with known TCC metabolites including DHC (b), 2-O-Gluc TCC (c), 3’OH-TCC (d), 2’OH-TCC and 6 OH-TCC (e), and TCC (f). In addition the small peak (a) was tentatively identified as DHC-GSH, based on its retention time between 55–58 minutes. Although this peak represents only a small portion of the metabolites generated (about 1% of the amount of TCC added to the cells), it is qualitatively and quantitatively consistent with the formation of small amounts of protein-bound metabolite and demonstrates the ability of keratinocytes to inactivate the electrophile through conjugation with GSH.
In conclusion, our study supports the hypothesis that TCC can be oxidatively converted to reactive metabolites (Baumann et al., 2010; Schebb et al., 2012a). The GSH adduct formed by CYP1A1 was tentatively identified as 3,4-dichloro-3'-glutathionyl-4'-hydroxyxanilide, a metabolite that is likely formed via an initial substitution of an aromatic chlorine atom by a hydroxyl group. This is not a common P450-catalyzed reaction but has been reported for certain halogenated aromatics [for review see Guengerich (2001)]. The resulting metabolite, DHC, can be further oxidized to a p-quinone imine, which is a well-established electrophilic intermediate generated during the activation of several drugs, including acetaminophen. Activation of TCC by CYP1A1 to glutathione-reactive intermediates is consistent with the large increases in protein adduct observed in TCDD-induced compared with control keratinocyte incubations (Schebb et al., 2012a).

The biologic consequences of reactive metabolite formation from TCC cannot be assessed with certainty, but the slow rates of metabolism to both protein- and glutathione-reactive intermediates would suggest minor if any untoward effects. One concern is whether these reactive metabolites bind to nucleophilic sites on DNA. The quinone imine intermediates generated from TCC are soft Lewis acids and would be less likely to react with the hard nucleophilic sites (strong Lewis bases) on DNA bases. A preliminary mutagenicity study of TCC in TCDD-induced keratinocytes showed no detectable mutagenic activity (Supplemental Table 1), a finding consistent with the characteristics of the electrophiles trapped as glutathione conjugates.

Overall, the formation of reactive metabolites, particularly at the levels described here, does not translate to a high level of concern for adverse health effects for use of TCC in personal care products. Given the high production volume of TCC and the widespread human exposure, it is surprising that these reactive metabolites and GSH adducts of this chemical have not been detected in the last 50 years of its use.

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Address correspondence to: Dr. Nils Helge Schebb, Institute for Food Toxicology and Chemical Analysis, University of Veterinary Medicine, Hanover Germany (N.H.S.); Department of Molecular Biosciences, School of Veterinary Medicine (J.B.M., D.M., A.R.B.), and Department of Entomology and Comprehensive Cancer Center (B.D.H.), and Environmental Toxicology, College of Agricultural and Environmental Sciences (R.H.R.), University of California, Davis, California

Authorship Contributions

Performed in research design: Schebb, Muvalva, Morin, Buckpitt, Hammock, Rice.

Contributed reagents or analytic tools: Schebb, Hammock.

Performed data analysis: Schebb, Muvalva, Morin, Rice.

Wrote or contributed to the writing of the manuscript: Schebb, Muvalva, Morin, Buckpitt, Rice.

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Schebb NH, Franssen F, Muvvala J, Morin M, Buckpitt A, Rice R, and Chemical Analysis, University of Veterinary Medicine, Hanover, Germany (N.H.S.); Department of Molecular Biosciences, School of Veterinary Medicine (J.B.M., D.M., A.R.B.), and Department of Entomology and Comprehensive Cancer Center (B.D.H.), and Environmental Toxicology, College of Agricultural and Environmental Sciences (R.H.R.), University of California, Davis, California

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Address correspondence to: Dr. Nils Helge Schebb, Institute for Food Toxicology and Chemical Analysis, University of Veterinary Medicine, Bischofsholer Damm 15, Hanover, Germany. E-mail: schebb@wwu.de