In Vitro Glucuronidation of the Antibacterial Triclocarban and Its Oxidative Metabolites


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Received August 10, 2011; accepted September 27, 2011

ABSTRACT:
Triclocarban (3,4,4′-trichlorocarbanilide; TCC) is widely used as an antibacterial in bar soaps. During use of these soaps, a significant portion of TCC is absorbed by humans. For the elimination from the body, glucuronidation plays a key role in both biliary and renal clearance. To investigate this metabolic pathway, we performed microsomal incubations of TCC and its hydroxylated metabolites 2′-OH-TCC, 3′-OH-TCC, and 6-OH-TCC. Using a new liquid chromatography-UV-mass spectrometry method, we could show a rapid glucuronidation for all OH-TCCs by the uridine-5′-diphosphate-glucuronosyltransferases (UGT) present in liver microsomes of humans (HLM), cynomolgus monkeys (CLM), rats (RLM), and mice (MLM). Among the tested human UGT isoforms, UGT1A7, UGT1A8, and UGT1A9 showed the highest activity for the conjugation of hydroxylated TCC metabolites followed by UGT1A1, UGT1A3, and UGT1A10. Due to this broad pattern of active UGTs, OH-TCCs can be efficiently glucuronidated in various tissues, as shown for microsomes from human kidney (HKM) and intestine (HIM). The major renal metabolites in humans, TCC-N-glucuronide and TCC-N′-glucuronide, were formed at very low conversion rates (<1%) by microsomal incubations. Low amounts of N-glucuronides were generated by HLM, HIM, and HKM, as well as by MLM and CLM, but not by RLM, according to the observed species specificity of this metabolic pathway. Among the human UGT isoforms, only UGT1A9 had activity for the N-glucuronidation of TCC. These results present an anomaly where in vivo the predominant urinary metabolites of TCC are N and N′-glucuronides, but these compounds are slowly produced in vitro.

INTRODUCTION

Triclocarban (3,4,4′-trichlorocarbanilide; TCC) (Fig. 1) is widely used as an antibacterial agent in bar soaps in the United States. It can generally be added to rinse-off personal care products in the United States and European Union in concentrations up to 1.5% [European Food Safety Authority, Opinion on Triclocarban (2005), Scientific Committee on Consumer Products, http://eca.europa.eu/health/ph_risk/committees/04_sccp/docs/scscp_o_016.pdf; Ahn et al., 2008]. Due to its widespread use and environmental persistence, TCC was detected in surface waters up to microgram per liter scale (Halden and Paull, 2005; Sapkota et al., 2007). Much higher levels of up to 0.44 g/kg were found in sludge, as shown in the Targeted National Sewage Sludge Survey, published by the U.S. Environmental Protection Agency in 2009. Moreover, TCC strongly accumulates in aquatic organisms such as algae (Cladophora spp.) and snails (log bioconcentration factor 3.2–3.4) (Coogan et al., 2007; Coogan and La Point, 2008).

Several studies have indicated that a significant portion of TCC in soaps is percutaneously absorbed by humans during and after showering (Scharpf et al., 1975; Schebb et al., 2011a). Approximately 0.4% of the applied TCC is found in the excreta and, thus, was absorbed and systemically available. Moreover, it has to be assumed that TCC from contaminated drinking water or food will be extensively absorbed, because TCC shows a high bioavailability after oral dosing (Hiles, 1977; Jeffcoat et al., 1977; Hiles and Birch, 1978a; Warren et al., 1978). These TCC exposures might be relevant to human health, because of unintended biological activities of TCC. By enhancing the action of steroids, TCC may have the potential to act as an endocrine disruptor (Ahn et al., 2008; Chen et al., 2008). Moreover, we recently showed that TCC inhibits the enzyme-soluble epoxide hydrolase, with an in vitro potency (IC_{50} 24 ± 5 nM) (Morisseau et al., 2009; Schebb et al., 2011b,c) comparable with synthetic inhibitors, which proved to alter the biological regulation of inflammation, pain, and blood pressure in vivo (Inceoglu et al., 2006; Imig and Hammock, 2009; Inceoglu et al., 2009).

ABBREVIATIONS: TCC, 3,4,4′-trichlorocarbanilide; ACN, acetonitrile; CLM, cynomolgus monkey liver microsomes; I.S., internal standards; DCC, 4,4′-dichlorocarbanilide; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; MS, mass spectrometry; LC, liquid chromatography; HLM, human liver microsomes; HKM, human kidney microsomes; HIM, human intestine microsomes; MLM, mouse liver microsomes; RLM, rat liver microsomes; SRM, selected reaction monitoring; UDPGA, UDP-diphosphoglucuronic acid; UGT, uridine-5′-diphosphate-glucuronosyltransferases.
glu et al., 2011). In mammals, TCC is rapidly metabolized. The main metabolite detected in human and monkey urine, accounting for 25% of TCC elimination products, results from direct N-glucuronidation at one of the nitrogen atoms of the urea moieties of TCC (Birch et al., 1978; Hiles and Birch, 1978a; Hiles et al., 1978; Schebb et al., 2011c). The majority of absorbed TCC is metabolized by cytochrome P450 enzymes to three hydroxylated TCC species, namely 2'-OH-TCC, 3'-OH-TCC, and 6-OH-TCC (Hiles, 1977; Jeffcoat et al., 1977; Birch et al., 1978; Hiles and Birch, 1978a; Hiles and Birch, 1978b; Hiles et al., 1978; Warren et al., 1978) (Fig. 1) with the ortho-hydroxylated species, 2'-OH-TCC, and 6-OH-TCC as main metabolites (Birch et al., 1978). All metabolites undergo extensive phase II metabolism, and the glucuronic acid conjugates of the hydroxylated TCC species account for the majority of TCC metabolites in mammalian bile (Birch et al., 1978). Thus, conjugation with glucuronic acid plays a key role in both renal and biliary elimination of TCC. In a recent study, it was also shown that glucuronides of phase I metabolites are the major metabolites in fish (Schebb et al., 2011a). However, no information is available about the UDP-glucuronosyltransferases (UGTs) involved in the conjugation of TCC and its metabolites, as well as the biochemistry and kinetics of the conversion. Therefore, in this study we investigated the activity of liver, kidney, and intestinal microsomes, and individual human UGT for the conjugation of TCC and its oxidative metabolites. Moreover, the enzyme kinetics for the glucuronidation of 2'-OH-TCC by liver microsomes was evaluated to obtain quantitative data about the clearance of this metabolite.

**Materials and Methods**

**Chemicals.** TCC was purchased from Sigma-Aldrich (St. Louis, MO) and further purified (≥99.9%) by repeated recrystallization. The TCC metabolites 2'-OH-TCC, 3'-OH-TCC, 6-OH-TCC, and 2'-Gluc-TCC, and the internal standards (I.S.) 4,4'-dichlorocarbanilide (DCC) and 2'-SO$_2$-O-TCC were synthesized by coupling the appropriate isocyanate and amine compounds, as described previously (Ahn et al., 2008; Baumann et al., 2010). The chemical structures of the analytes are shown in Fig. 1. Uridine 5'-diphosphoglucuronic acid (UDPGA) trisodium salt, alamethicin, and d-saccharolactone were obtained from Sigma-Aldrich. All other chemicals were from Thermo Fisher Scientific (Waltham, MA) and were of the highest quality available.

**Microsomes and Human UGT Isoforms.** All microsomes and Supersomes, i.e., microsomes from insect Sf9 cells infected with a baculovirus strain containing cDNA of human UGT isoforms, were obtained from BD Gentest (Woburn, MA). The following microsomal preparations were used (20 mg - protein $^{-1}$ · ml$^{-1}$): pooled human liver microsomes (HLM) from 25 mixed-gender donors, pooled rat liver microsomes (RLM) from 150 male Sprague-Dawley rats, pooled mouse liver microsomes (MLM) from 100 male B6C3F1 mice, pooled cynomolgus monkey liver microsomes (CLM) from 13 male animals, pooled human kidney microsomes (HKM) from mixed-gender donors, and pooled human intestine microsomes (HIM) from 10 mixed-gender donors. Supersomes of the following human UGT isoforms were used: UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17. The activity of the microsomal preparations was verified by monitoring their ability to conjugate the standard UGT substrates 4-(trifluoromethyl) umbelliferone (TFMU) and trifluoperazine. The measured activities using the respective substrates are shown in the supplemental data.

**Glucuronidation Assays.** The glucuronidation assay was performed as described by Maul et al. (2011) with slight modifications (Pfeiffer et al., 2006, 2009). Microsomes or individual UGTs were incubated with TCC and its hydroxylated metabolites in a total volume of 200 μl of 100 mM potassium phosphate buffer (pH 7.4). In a generic scheme, 20 μl of microsome solution containing 5 μg of protein were mixed with 76 μl of buffer and 40 μl of alamethicin solution (125 μg/ml) and placed on ice for 15 min. Alamethicin forms pores in the microsomal membrane and increases the substrate accessibility of the UGTs (Fisher et al., 2000). Subsequently, 4 μl of the substrate in dimethyl sulfoxide (DMSO; concentration in assay 10 μM, 2% DMSO), magnesium chloride, and the β-glucuronidase inhibitor saccharolactone (concentration in assay both 10 mM) were added, and the mixture was preincubated for 5 min at 37°C on a heated shaker. The reaction was initiated by the addition of 20 μl of UDPGA (20 mM) and incubated for an additional 30 min. After 5, 10, 15, and 30 min (for highly active preparations; 2, 10, and 15 min), 40-μl samples were transferred from each incubation mixture to a vial with 40 μl of acetonitrile (ACN) containing I.S. The final I.S. concentration was 1 μM. DCC was used as IS for the incubations of TCC, 2'-OH-TCC, and 6-OH-TCC. 2'-SO$_2$-O-TCC served as I.S. for incubations of 3'-OH-TCC. The resulting suspension was vigorously mixed and centrifuged at 16,000g for 10 min to remove precipitated protein and buffer salts. The supernatant was directly used for liquid chromatography-UV-electrospray ionization-tandem mass spectrometry (LC-UV-ESI-MS/MS) analysis.

For the determination of the 2'-OH-TCC glucuronidation kinetics, various substrate concentrations (0.2, 0.5, 1, 2, 3, 5, 10, and 50 μM) were incubated with RLM, HLM, CLM, and MLM for 5, 10, 15, and 30 min, respectively. To keep the concentration of all samples within the linear range of the ESI-MS/MS detection, samples containing ±5 μM substrate were mixed 1:9 with ACN after incubation. Control incubations were conducted either in the absence of UDPGA or with Supersomes lacking an active UGT isoform in the
presence of UDPGA. Each incubation was carried out in three independent replicates.

**LC-UV-MS Analysis.** LC-UV-ESI-MS/MS analysis was performed using Waters Premier system (Waters, Milford, MA). Separation was performed on an RP-18 column with embedded polar groups of the dimensions $2 \times 100$ mm and a particle size of $2.2 \mu$m (ProntoSil C18 ace-EPS, BISCHOFF Chromatography, Leonberg, Germany). The analytes (injection volume $10 \mu$l) were separated by a binary gradient at a flow rate of $300 \mu$l/min of $25 \text{mM}$ ammonium acetate containing $0.1\%$ acetic acid as solvent A and pure ACN as solvent B. The following gradient was used: $0.0$ to $2.0$ min isocratic $30\%$ B, $2.0$ to $7.5$ min nonlinear with decreasing slope (convex gradient curve profile; Masslynx Waters) software setpoint $50$ to $85\%$ B, $7.5$ to $8.5$ min linear $80$ to $100\%$ B, $8.5$ to $9.5$ min isocratic $100\%$ B, $9.5$ to $9.7$ min return to initial conditions of $30\%$ B, and reconditioning between $9.7$ and $11.0$ min. Analytes were detected using a UV detector operating at $265$ nm and negative ESI MS/MS in sensitive and specific selected reaction monitoring (SRM) mode.

MS/MS conditions for TCC, $2'-\text{OH-TCC}$, $3'-\text{OH-TCC}$, $6-\text{OH-TCC}$, $2'-\text{Gluc-O-TCC}$, $2'-\text{SO}_3^-\text{-O-TCC}$, and DCC were optimized by infusing $100 \text{nM}$ of a solution of analytes in $80:20$ ACN/water at a flow rate of $10 \mu$l/min with a syringe pump. The optimization of separation and detection conditions for the further glucuronides was performed with biological samples because no standard substances were available. Human urine (diluted $1:1$ with ACN and centrifuged) of an exposed subject was used as source of the two $N$-glucuronides, as described previously (Schebb et al., 2011c). $3'-\text{Gluc-O-TCC}$ and $6\text{-Gluc-O-TCC}$ were generated by microsomal incubations with RLM, as described above. The optimized MS conditions and fragment spectra of the glucuronides are shown in the supplemental data (Table S1; Fig. S1).

The activity of the microsomes and individual UGTs was calculated based on the amount of formed product. For $2'-\text{Gluc-O-TCC}$, the quantification was performed by external calibration of the LC-UV-MS signal. The concentration of the other glucuronides was calculated based on their peak areas in the UV signal using the calibration function of their substrates. It is assumed that the glucuronides have the same molar absorbance as their aglycones.

The conversion rate was determined by linear regression of at least three data points of the product formation over the incubation time. Activity values were calculated based on the linear regression as mean and S.D. of three independent incubations. The $K_M$ and $V_{max}$ values for the glucuronidation of $2'-\text{OH-TCC}$ were obtained by fitting the conversion rates versus the concentration according to eq. 1 with the SigmaPlot (version 9.01; Systat Software, Inc., San Jose, CA).

$$v = \frac{V_{max} \times c}{K_M + c}$$  (1)

**In Vivo Experiments.** To verify the formation of the glucuronides in vivo, urine of rodents was analyzed in a preliminary metabolism study. Two 12-week-old male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) with a body weight of $458 \pm 25$ g and two 12-week-old male Swiss Webster mice (Charles River Laboratories, Inc.) of $34.4 \pm 0.7$ g were used. This study was approved by the University of California (UC) Davis Institutional Animal Care and Use Committee. Before the experiment, animals were housed in UC Davis facilities with access to food and water ad libitum. Animals were transferred into metabolic cages $24$ h before the experiment (Pakenham et al., 2002) and blank urine was collected. Two mice were placed in one metabolic cage and each rat was housed in a single cage. TCC was administered by oral gavage of a $1$ mg/ml solution of TCC in $20:80$ DMSO/PEG400 ($v/v$). Rats received a dose of $1$ mg/kg b.wt. and mice received a dose of $5$ mg/kg b.wt. After administration, urine was collected between $0$ to $24$ and $24$ to $48$ h. Urine was analyzed using online-LC-MS, as described previously (Schebb et al., 2011a).

**Results**

**LC-UV-MS Method.** The glucuronidation of TCC and its three monohydroxylated metabolites was investigated using different microsomes and individual UGTs. To quantitatively monitor the conjugate formation, analytical methods are needed to quantify the product formation. Therefore, a new LC-UV-MS method was developed. The application of an RP-column with embedded polar groups and $2.2-\mu$m particles yielded in a high-chromatographic resolution allowing a separation of TCC and all its metabolites in only $8$ min (Fig. 2A). For the metabolites with available reference standards, $2$-Gluc-TCC (peak

**FIG. 2.** Chromatographic separation with ESI-MS detection in SRM mode of TCC and its metabolites. Shown is an injection of a standard solution ($50$ nM) (A), $40$-min microsomal conversion of $6$-OH-TCC ($10$ nM) (B) with RLM and $3'$-OH-TCC ($10$ nM) (C) with RLM microsomes ($0.025$ mg protein/ml). D, analysis of a urine sample of a TCC exposed human is displayed. The peaks in the SRM chromatograms are $2'-\text{OH-gluc-TCC}$ (1), $2'-\text{SO}_3^-\text{-O-TCC}$ (2), DCC (I.S.) (3), $3'-\text{OH-TCC}$ (4), TCC (5), $2'-\text{OH-TCC}$ (6), $6$-OH-TCC (7), $6$-Gluc-O-TCC (8), $3'$-O-gluc-TCC (9), $N$-Gluc-TCC (10), and $N'$-Gluc-TCC (11).
1) eluted first, followed by the sulfate conjugate of 2'-OH-TCC, 2'-SO2-O-TCC (peak 2), used as IS. At a retention time of 4.61 min, 3'-OH-TCC eluted (peak 3), closely followed by the IS, DCC (peak 4) and TCC (peak 5). In contrast to earlier attempts for a reversed-phase separation (Birch et al., 1978; Baumann et al., 2010), the metabolites hydroxylated in ortho position to the urea moiety, 2'-OH-TCC (peak 6) and 6-OH-TCC (peak 7) were also baseline separated and eluted at 6.81 min and 7.45 min, respectively. The prolonged retention time of these hydroxylated metabolites compared with TCC can be explained by the formation of an intermolecular hydrogen bond between the hydroxyl and urea group (Birch et al., 1978; Warren et al., 1978; Baumann et al., 2010; Schebb et al., 2011c). Incubation of 3'-OH-TCC with RLM in the presence of UDPGA led to an abundant peak at 2.26 min (Fig. 2), which caused [M-H]− ions with m/z of 505, 507, and 509. The pattern of these ions indicated a compound containing three chlorine atoms. In addition, the fragment spectrum of this metabolite showed the same fragments as 2'-Gluc-O-TCC and was therefore tentatively identified as the 3'-Gluc-O-TCC. After incubation of 6-OH-TCC with RLM and UDPGA, peak 9 with a retention time of 2.51 min was detected. Showing the same isotopic pattern of the parent [M-H]− ions as 2'-Gluc-O-TCC and 3'-Gluc-O-TCC, the formed metabolite showed intense fragment ions at m/z 202, which is characteristic for the isocyanate fragment ions of TCC derivatives bearing a hydroxyl function in the dichloroaniline ring (Baumann et al., 2010; Schebb et al., 2011c) (Supplemental Fig. S1). To optimize the method with respect to the N-glucuronides of TCC, urine of a human subject that had been exposed to TCC was used as a source of N'-Gluc-TCC and N-Gluc-TCC for these conjugates (Fig. 1) (Schebb et al., 2011c). The N-Gluc-TCC eluted at 1.97 min and was almost baseline separated from N'-Gluc-TCC with a retention time of 2.07 min (Fig. 2).

All five glucuronic acid conjugates showed the same characteristic fragmentation of the urea backbone as previously described for TCC and its phase I metabolites (Baumann et al., 2010; Schebb et al., 2011c) (Supplemental Fig. S1; Table S1). Using these fragments for detection in SRM mode, TCC and all its metabolites could be specifically monitored. The method showed a high sensitivity with a detection limit of 3 fmol on column for most of the compounds (Table 1). Due to the narrow peak width, the detection limit in UV detection at 265 nm of 0.1 μM (300 fmol on column) was also relatively low. In UV detection, the slopes of 0.7 and 0.6 of the calibration curve for 2'-O-Gluc-TCC and its aglycone 2'-OH-TCC were very similar. Based on this difference of <15%, it was assumed that all TCC glucuronic acid conjugates show the same molar absorbance as their corresponding aglycones. Therefore, further quantification of the glucuronides was performed based on the LC-UV calibration of their parent compounds.

The sensitivity of the LC-UV-MS method allowed us to evaluate the product formation at four time points over the incubation time of a single incubation sample with a volume of 0.2 ml. The conversion rate for each incubation was calculated using linear regression of the product formation versus incubation time. This yields a more precise determination of the initial enzyme velocity than those values derived from common endpoint assays, which assume a linear product formation over the entire incubation time (Pfeiffer et al., 2006, 2009; Maul et al., 2011). The formation of all measured products was linear over an incubation time of 20 min or longer, and at least three data points of product formation were used for the linear regression. For highly active preparations and low product concentrations in the kinetic measurements, the incubation time was reduced and samples were taken at 2, 5, 10, and 15 min.

**Activity Screening for the Conjugation of TCC and Its Metabolites.** The activity of various enzyme preparations for the conjugation of TCC and its oxidative metabolites was investigated using microsomes from different species and different tissues to enable estimation of the tissues in which the glucuronidation may occur in vivo. To compare the activity of the microsomal preparations at their Vmax, this activity screening was performed at a high substrate concentration of 10 μM, which is above the expected KM. As shown in Table 2, the liver microsomes of the four mammalian species tested, namely HLM, CLM, RLM, and MLM, showed a high activity of at least 3 nmol · min−1 · mg protein−1 for the conjugation of the three hydroxylated TCC metabolites. The glucuronidation activity for 2'-OH-TCC of HLM, RLM, and MLM was very similar (mean 2.63 ± 0.06 nmol · min−1 · mg protein−1). Conjugation of 3'-OH-TCC and 6-OH-TCC occurred at a 2- to 3-fold higher rate, with remarkable species-dependent differences. Whereas 3'-OH-TCC was conjugated most extensively by RLM and MLM, HLM showed the highest activity for the conjugation of 6-OH-TCC. CLM showed higher activities for all hydroxylated TCC metabolites than the microsomes of the other species. Here, 2'-OH-TCC was conjugated the fastest, with an activity of 23 nmol · min−1 · mg protein−1, followed by 6-OH-TCC and 3'-OH-TCC. Microsomes from human, kidney and intestine overall showed a comparable activity to HLM for the conjugation of all hydroxylated TCC metabolites than the microsomes of the other species. Here, 2'-OH-TCC was conjugated the fastest, with an activity of 23 nmol · min−1 · mg protein−1, followed by 6-OH-TCC and 3'-OH-TCC. Microsomes from human, kidney and intestine overall showed a comparable activity to HLM for the conjugation of all hydroxylated TCC metabolites than the microsomes of the other species.

**TABLE 1**

*Performance of the analytical method*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time</th>
<th>LC-ESI(+)MS/MS LOD</th>
<th>UV LOD</th>
<th>Linear Range</th>
<th>UV LOQ Linear Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>fmol</td>
<td>nM</td>
<td></td>
<td>fmol</td>
</tr>
<tr>
<td>DCC</td>
<td>4.36 ± 0.03</td>
<td>3</td>
<td>1–1000</td>
<td></td>
<td>300</td>
</tr>
<tr>
<td>TCC</td>
<td>5.10 ± 0.04</td>
<td>3</td>
<td>1–1000</td>
<td></td>
<td>300</td>
</tr>
<tr>
<td>2'-OH-TCC</td>
<td>6.81 ± 0.07</td>
<td>3</td>
<td>1–1000</td>
<td></td>
<td>300</td>
</tr>
<tr>
<td>6'-OH-TCC</td>
<td>7.45 ± 0.05</td>
<td>3</td>
<td>1–1000</td>
<td></td>
<td>300</td>
</tr>
<tr>
<td>3'-OH-TCC</td>
<td>4.61 ± 0.02</td>
<td>3</td>
<td>1–1000</td>
<td></td>
<td>300</td>
</tr>
<tr>
<td>2'-SO2-O-TCC</td>
<td>3.46 ± 0.02</td>
<td>3</td>
<td>1–1000</td>
<td></td>
<td>300</td>
</tr>
<tr>
<td>N-Gluc-TCC</td>
<td>1.97 ± 0.01</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>N'-Gluc-TCC</td>
<td>2.07 ± 0.02</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>2'-O-gluc-TCC</td>
<td>2.50 ± 0.02</td>
<td>10</td>
<td>3–1000</td>
<td></td>
<td>300</td>
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<tr>
<td>6'-O-gluc-TCC</td>
<td>2.51 ± 0.02</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3'-O-gluc-TCC</td>
<td>2.26 ± 0.02</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

LOQ, limit of quantitation; n.d., not determined because no reference compound was available.
lower rates. Under similar conditions, none of the tested microsomes formed significant N-Glc-TCC or N'-Glc-TCC amounts, even at elevated protein concentrations up to 2 mg/ml in the microsomal incubations (Table 2). It is not likely that the absence of N-glucuronides can be explained by degradation during or after incubation, because similar investigations demonstrated that these metabolites are stable in urine and water ACN mixtures (Schebb et al., 2011c).

The SRM-ESI-MS signal showed a low formation of N-Glc-TCC and N'-Glc-TCC in HLM, CLM, MLM, and HKM incubations (Supplemental Fig. S2); however, no signals were observed in LC-UV chromatogram. Thus, quantification was not feasible, and the activity of the microsomes to glucuronidate TCC was estimated semiquantitatively based on the peak height in LC-MS (Schebb et al., 2011c). Activities are expressed as pmol \( \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1} \) as the mean and S.D. of three independent measurements.

<table>
<thead>
<tr>
<th>Microsomes/UGT-Isform</th>
<th>2'-OH-TCC</th>
<th>6-OH-TCC</th>
<th>3'-OH-TCC</th>
<th>TCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N-Gluc</td>
<td>N'-Glu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RLM</td>
<td>2600 ± 130</td>
<td>5500 ± 1800</td>
<td>8700 ± 2800</td>
<td>n.d.</td>
</tr>
<tr>
<td>HLM</td>
<td>2700 ± 680</td>
<td>8500 ± 2200</td>
<td>5200 ± 150</td>
<td>n.d.*</td>
</tr>
<tr>
<td>CLM</td>
<td>22800 ± 100</td>
<td>16300 ± 700</td>
<td>9500 ± 1500</td>
<td>n.d.*</td>
</tr>
<tr>
<td>MLM</td>
<td>2600 ± 650</td>
<td>7900 ± 2400</td>
<td>14700 ± 690</td>
<td>n.d.*</td>
</tr>
<tr>
<td>HKM</td>
<td>5600 ± 1700</td>
<td>7700 ± 610</td>
<td>4200 ± 30</td>
<td>n.d.*</td>
</tr>
<tr>
<td>HIM</td>
<td>5700 ± 1200</td>
<td>3900 ± 1400</td>
<td>7100 ± 600</td>
<td>n.d.*</td>
</tr>
<tr>
<td>A1</td>
<td>850 ± 210</td>
<td>820 ± 260</td>
<td>520 ± 20</td>
<td>n.d.</td>
</tr>
<tr>
<td>A3</td>
<td>390 ± 93</td>
<td>410 ± 50</td>
<td>540 ± 70</td>
<td>n.d.</td>
</tr>
<tr>
<td>A7</td>
<td>870 ± 440</td>
<td>1100 ± 320</td>
<td>1700 ± 160</td>
<td>n.d.</td>
</tr>
<tr>
<td>A8</td>
<td>5700 ± 2200</td>
<td>5000 ± 1100</td>
<td>9400 ± 250</td>
<td>n.d.</td>
</tr>
<tr>
<td>A9</td>
<td>1200 ± 800</td>
<td>2400 ± 680</td>
<td>840 ± 190</td>
<td>n.d.*</td>
</tr>
<tr>
<td>A10</td>
<td>210 ± 160</td>
<td>280 ± 150</td>
<td>&lt;LOQ</td>
<td>n.d.</td>
</tr>
<tr>
<td>B7</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>n.d.*</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

\(<\text{LOQ (limit of quantitation)}\), \text{glucuronide peak < LOQ of LC-UV detection (0.2 \mu M); n.d. (not determined because no reference compound was available), glucuronide peak < LOQ of LC-UV detection (60 \text{nm}).}

\(*\text{Low product amounts detected by ESI-MS.}\

\(+, +++, +++++\), The activity for the N-glucuronidation is rated semiquantitatively [(from low (+) to high (+++)) based on the peak height in the ESI-MS signal.

Discussion

The particular importance of a conjugation with glucuronic acid for the excretion of TCC in mammals has been known for more than 30 years. The excretion of TCC in mammals has been known for more than 30 years. The excretion of TCC in mammals has been known for more than 30 years.

The analysis of mouse and rat urine after oral administration confirmed earlier studies that glucuronides are the major renal excretion form of TCC (Fig. 3). As described by Birch et al. (1978), no N-glucuronides were detected in the rat urine, and we found that the glucuronide of 3'-OH-TCC was the major urinary metabolite. By contrast, this study showed that mice form N and N'-glucuronides as major urinary metabolites similar to humans and monkeys. In addition, glucuronides of hydroylated species were detected, indicating that at doses of 1 to 5 mg/kg b.w. [a high dose compared with the human exposure caused by soaps of approximately 1 \mu kg/b.w. (Schebb et al., 2011c), these metabolites contribute to the renal clearance of TCC (Fig. 3).

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years (Birch et al., 1978). However, this study is the first investigation of the biochemical formation of these glucuronides. By performing microsomal incubations in combination with a new LC-MS method, we showed that various human UGTs rapidly glucuronidate the TCC phase I metabolites 2'-OH-TCC, 3'-OH-TCC, and 6-OH-TCC.

The hepatic UGTs (UGT1A1, UGT1A3, and UGT1A9) (King et al., 2000; Tukey and Strassburg, 2000) showed high activities for the conjugation of all hydroxylated metabolites. The highest activity was found for UGT1A9, which favored 6-OH-TCC as substrate, followed by 2'-OH-TCC and 3'-OH-TCC. As expected from these results, HLM showed high activity for the conjugation of the hydroxylated TCC metabolites, with a descending conversion rate order of 6-OH-TCC > 3'-OH-TCC > 2'-OH-TCC. Rapid conjugation of the oxidative TCC metabolites was also found in the liver microsomes of mouse, monkey, and rat, indicating that mammalian liver UGTs generally show a high affinity for hydroxylated TCC metabolites. This is consistent with in vivo observations, where glucuronides of hydroxylated TCC species are by far the dominant metabolites found in mammalian bile (Birch et al., 1978).

The UGTs 1A1, 1A3, and 1A9 possessing high activity for the conversion of hydroxylated TCC are also expressed in other organs, such as the kidney and gastrointestinal tract (King et al., 2000; Tukey and Strassburg, 2000). Our activity screening also unveiled very high conjugation activity for the extrahepatic UGTs 1A7 and 1A8 (Table 2). Thus, it is concluded that oxidative TCC metabolites will also be rapidly conjugated in other tissues. This assumption is supported by the investigation of microsomes of kidney and intestine, which conjugated oxidative TCC metabolites at rates comparable with HLM. The substrate selectivity pattern of HKM was 6-OH-TCC > 2'-OH-TCC > 3'-OH-TCC, consistent with the pattern of the highly active UGT1A9, which is expressed in the kidney (Tukey and Strassburg, 2000). Likewise, HM conjugated 3'-OH-TCC the fastest, followed by 2'-OH-TCC and 6-OH-TCC, which is identical to the pattern observed for the intestinal UGT1A8 (Tukey and Strassburg, 2000). None of the enzymes of the UGT2 family showed significant activity for the conjugation of phase I TCC metabolites.

Recent studies indicate that further oxidation of 2'-OH-TCC leads to a reactive quinone imine metabolite that covalently binds to proteins in vitro (Baumann et al., 2010). The formation of significant amounts of free 2'-OH-TCC may be of concern for human health. Therefore, we investigated the kinetics of the glucuronidation of 2'-OH-TCC as a possible detoxifying reaction in mammalian liver microsomes competing with a further cytochrome P450 oxidation. All tested microsomes showed a low Km and a high Vmax resulting in a high apparent intrinsic clearance (Table 3). The clearance of 2'-OH-TCC by MLM and RLM was comparable or higher than the reported values for reference UGT substrates including 4-methylumbellifereone, para-nitrophenol, propofol, and mycophenolic acid (Shiratani et al., 2008). With a conversion rate of 1.74 ml·mg⁻¹·min⁻¹, the clearance of HLM was also significantly higher than for estradiol and its catecholic oxidative metabolites, which are believed to play a role in estradiol-mediated carcinogenesis (Pfeiffer et al., 2006). However, because both N- and O-glucuronides can be cleaved by glucuronidase from Escherichia coli (Schebb et al., 2011c), enterohepatic circulation will probably occur for biliary-excreted TCC metabolites.

Aside from oxidative metabolism with subsequent conjugation, direct N-glucuronidation is a major pathway in the metabolism and excretion of TCC (Fig. 1) (Birch et al., 1978). Humans excrete approximately 25% of the total absorbed TCC in the urine as N- and N'-glucuronide (Scharpf et al., 1975; Schebb et al., 2011c). These metabolites are also the major urinary TCC metabolites in monkeys and in mice, whereas no N-glucuronidation was observed in rats (Fig. 3) (Birch et al., 1978). According to this species specificity, TCC-N-glucuronides were detected after incubation with HLM, CLM, and MLM, but not after incubation with RLM. However, none of the microsomal incubations showed a formation of TCC-N-glucuronides at a rate higher than 1%. We therefore have to conclude that microsomal incubations poorly predict the in vivo importance of this metabolic pathway. There are two possible explanations for this observation: TCC-N-glucuronidation in vivo is catalyzed by other glucuronosyltransferases than the UGTs present in the microsomes; or TCC-N-glucuronidation by UGT does not occur to the same extent in vitro than it occurs in vivo. There is some evidence favoring the latter explanation. First, there are several studies that showed N-glucuronidation reactions are underestimated by microsomal incubations (Anderson et al., 2009). In particular, TCC is not well metabolized in microsomal incubations, as shown for oxidative metabolic conversions (Baumann et al., 2010). However, earlier findings show that the structurally similar carbonanilide sorafenib, bearing more polar groups at both aniline rings than TCC, is converted by HLM to N-glucuronides at significant conversion rates (Sparidans et al., 2009). Therefore, urea derivatives, bearing a phenyl moiety on both nitrogen atoms, have to be regarded as possible substrates for UGT.

![Graph](https://via.placeholder.com/150)

**Fig. 3.** Metabolic pattern of the TCC metabolites in rodent urine. A, the TCC metabolite concentration in mouse urine after oral gavage of 5 mg/kg b.w. (t = 0 h) is shown. Results are shown as mean and S.D. of two independent experiments. B, TCC metabolites in rat urine after oral gavage of 1 mg/kg b.w. (t = 0 h) are depicted. Results are shown as mean and S.D. of 3-fold analysis of the combined urine of two animals.
In the activity screening that used human UGTs, only incubations with UGT1A9 caused significant, although low, levels of N-glucuronides. The very low conversion rates did not allow a quantitative activity measurement. This observation indicates that it would be easy to overlook N-glucuronide formation by other isoenzymes. Therefore, the identification of UGT1A9 as the exclusive UGT catalyzing the N-glucuronidation of TCC should be confirmed by more predictive models consistent with the high conjugation rates found in vivo. For this purpose, metabolic studies using cell lines, precision tissue slices, transgenic animals transfected with human UGT isoforms, or the UGT1A9 mouse model could be beneficial. Optionally, enzyme-specific inhibitors could be used in the cell culture experiments. Nevertheless, the tentative identification of UGT1A9 as being the key enzyme in TCC-N-glucuronidation provides a convincing explanation for the observed species selectivity of this metabolic pathway. Whereas human, monkey, and mouse express an active enzyme, UGT1A9 is a pseudogene in rat (Mackenzie et al., 2005). Consequently, only incubation with RLM did not lead to any N-glucuronides in our experiments. With respect to tissue specificity, the observed activity of HLM, HIM, and HKM is also in accordance with the Nal., 2005). Consequently, only incubation with RLM did not lead to any N-glucuronides in our experiments. With respect to tissue specificity, the observed activity of HLM, HIM, and HKM is also in accordance with the expression pattern of UGT1A9 being expressed in all these tissues (Ohno and Nakajin, 2009). Finally, the conjugation of the urea-nitrogen in TCC is consistent with the substrate specificity of UGT1A9. For example, UGT1A9 is the key enzyme for the formation of the ternary N-hydroxy-PhIP-αN-glucuronide (Malfatti and Felton, 2001) and for the N-glucuronidation of the structurally similar para-ethoxy phenyl urea (Uesawa et al., 2007).

In summary, our study showed that all major oxidative metabolites of TCC are rapidly conjugated with glucuronic acid by microsomes from liver, kidney, and intestine. A broad variety of UGTs have high affinity for the hydroxylated TCC metabolites, with high activities particularly for UGT1A7, UGT1A8, and UGT1A9. By contrast, hardly any N-glucuronides of TCC are formed in microsomal incubations. Nevertheless, based on sensitive LC-ESI-MS detection of low amounts of the formed product, UGT1A9 could be tentatively identified as a major UGT in this metabolic pathway of TCC.

Acknowledgments

We thank Robert Tukey and Mei-Fei Yueh (University of California, San Diego) for helpful discussions.

Authorship Contributions

Participated in research design: Schebb and Hammock.
Conducted experiments: Schebb and Franze.
Contributed new reagents or analytic tools: Schebb, Fanze, Maul, and Ranganathan.
Performed data analysis: Schebb, Franze, and Maul.
Wrote or contributed to the writing of the manuscript: Schebb, Franze, Maul, Ranganathan, and Hammock.

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