In Vitro Glucuronidation of the Antibacterial Triclocarban and Its Oxidative Metabolites


University of Veterinary Medicine Hannover, Institute for Food Toxicology and Chemical Analysis, Hannover, Germany (N.H.S.); University of California, Davis, Department of Entomology and Cancer Research Center, Davis, California (B.F., A.R., B.D.H.); and BAM Federal Institute for Materials Research and Testing, Division of Organic Chemical Analysis & Reference Materials, Berlin, Germany (R.M.)

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://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_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., 2011). 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 (IC50 24 ± 5 nM) (Morisseau et al., 2009; Schebb et al., 2011b) 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., 2011).

This work was supported by the National Institutes of Health National Institute of Environmental Health Sciences [Grants P42-ES04699, R01-ES002710] through the German Academic Exchange Service (DAAD, Bonn, Germany); and the American Asthma Foundation (B.D.H.).

Article, publication date, and citation information can be found at http://dx.doi.org/10.1124/dmd.111.042283.

The online version of this article (available at http://dmd.aspetjournals.org) contains supplemental material.

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, mice liver microsomes; RLM, rat liver microsomes; SRM, selected reaction monitoring; UDPGA, UDP-diphosphoglucuronic acid; UGT, uridine-5'-diphosphate-glucuronosyltransferases.
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 moiety 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 enzymes to three hydroxylated TCC species, namely 2'-OH-TCC, 3'-OH-TCC, and 6-OH-TCC. The resulting glucuronides account for the major metabolites in human urine (Schebb et al., 2011c). The glucuronides of the oxidative metabolites 2'-OH-TCC, 3'-OH-TCC, and 6'-OH-TCC are the major metabolites in mammalian bile (Birch et al., 1978).

**Glucuronidation Assays.** The glucuronidation assay was performed as described by Mau 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, 5, 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'-SO₃H-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.

**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'-dichlorocarbamidine (DCC) and 2'-SO₃H-2'-TCC were synthesized by coupling the appropriate isocyanate and amine compounds, as described previously (Ahn et al., 2011; 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 μg 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.
The glucuronidation of TCC and its 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-μm 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...
was therefore tentatively identified as the 3'-phase separation (Birch et al., 1978; Baumann et al., 2010), the 4) and TCC (peak 5). In contrast to earlier attempts for a reversed-

3-phase separation (Birch et al., 1978; Baumann et al., 2010; Schebb et al., 2011c). Incubation of 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 RLM in the presence of UDPGA led to an abundant

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 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 V_{	ext{max}}, this activity screening was performed at a high substrate concentration of 10 μM, which is above the expected K_{M}. 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 \cdot min^{-1} \cdot mg \cdot 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 \cdot min^{-1} \cdot mg \cdot 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 \cdot min^{-1} \cdot mg \cdot 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 the three monohydroxylated TCC metabolites. It is interesting to note that the activity of HIM and HKM to conjugate 2'-OH-TCC was higher (5.62 ± 0.06 nmol \cdot min^{-1} \cdot mg \cdot protein^{-1}) compared with HLM, whereas 3'-OH-TCC and 6-OH-TCC were glucuronidated at equal or lower rates than these tissues.

### Table 1

**Performance of the analytical method**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time</th>
<th>LC-ESI(−)-MS/MS</th>
<th>UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCC</td>
<td>4.36 ± 0.03</td>
<td>LOD: 3 fmol</td>
<td>LOD: 300 nM</td>
</tr>
<tr>
<td>TCC</td>
<td>5.10 ± 0.04</td>
<td>LOD: 3 fmol</td>
<td>LOD: 300 nM</td>
</tr>
<tr>
<td>2'-OH-TCC</td>
<td>6.81 ± 0.07</td>
<td>LOD: 3 fmol</td>
<td>LOD: 300 nM</td>
</tr>
<tr>
<td>6'-OH-TCC</td>
<td>7.45 ± 0.05</td>
<td>LOD: 3 fmol</td>
<td>LOD: 300 nM</td>
</tr>
<tr>
<td>3'-OH-TCC</td>
<td>4.61 ± 0.02</td>
<td>LOD: 3 fmol</td>
<td>LOD: 300 nM</td>
</tr>
<tr>
<td>2'SO₂-O-TCC</td>
<td>3.46 ± 0.02</td>
<td>LOD: 3 fmol</td>
<td>LOD: 300 nM</td>
</tr>
<tr>
<td>N'-Gluc-TCC</td>
<td>1.97 ± 0.01</td>
<td>LOD: n.d.</td>
<td>LOD: n.d.</td>
</tr>
<tr>
<td>N'-Gluc-TCC</td>
<td>2.07 ± 0.02</td>
<td>LOD: n.d.</td>
<td>LOD: n.d.</td>
</tr>
<tr>
<td>2'-O-gluc-TCC</td>
<td>2.50 ± 0.02</td>
<td>LOD: 10 fmol</td>
<td>LOD: 300 nM</td>
</tr>
<tr>
<td>6'-O-gluc-TCC</td>
<td>2.51 ± 0.02</td>
<td>LOD: n.d.</td>
<td>LOD: n.d.</td>
</tr>
<tr>
<td>3'-O-gluc-TCC</td>
<td>2.26 ± 0.02</td>
<td>LOD: n.d.</td>
<td>LOD: 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-Gluc-TCC or N'-Gluc-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-Gluc-TCC and N'-Gluc-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 (Table 2). HKM showed the highest activity, followed by MLM, CLM, and HLM. RLM incubations did not lead to any formation of TCC-N-glucuronides. Likewise, none of the isolated UGTs (Supersomes) showed activity for TCC glucuronidation, except for UGT1A9, which caused small N-glucuronide peaks in LC-ESI-MS (Supplemental Fig. S2). Even in incubations with elevated microsomal protein concentration (up to 2 mg/ml), none of the other UGTs formed detectable levels of N-glucuronides in a 30-min incubation. By contrast, a broad variety of human UGTs showed high activities for the conjugation of the three hydroxylated TCC metabolites. Here, UGT1A7, UGT1A8, and UGT1A9 were most active with activities of 1 nmol/min·mg protein"
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, HIM 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 \( K_M \) and a high \( V_{max} \), 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-methylumbellif erone, para-nitropheno1, propofol, and myco- phenolic acid (Shiratani et al., 2008). With a conversion rate of 1.74 ml \( \cdot \) mg\(^{-1}\) \( \cdot \) 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 as 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 carbanilide 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.

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 \( K_M \) and a high \( V_{max} \), 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-methylumbellif erone, para-nitropheno1, propofol, and myco-phenolic acid (Shiratani et al., 2008). With a conversion rate of 1.74 ml \( \cdot \) mg\(^{-1}\) \( \cdot \) 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 as 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 carbanilide 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.

### Table 3

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>( K_M )</th>
<th>( V_{max} )</th>
<th>Clearances</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLM</td>
<td>3.29 ± 0.89</td>
<td>9.42 ± 4.80</td>
<td>2.86 ± 1.46</td>
</tr>
<tr>
<td>HLM</td>
<td>3.08 ± 1.37</td>
<td>5.37 ± 0.43</td>
<td>1.74 ± 0.31</td>
</tr>
<tr>
<td>CLM</td>
<td>1.40 ± 0.90</td>
<td>26.7 ± 4.80</td>
<td>19.1 ± 5.33</td>
</tr>
<tr>
<td>MLM</td>
<td>2.46 ± 0.19</td>
<td>1.45 ± 0.16</td>
<td>0.59 ± 0.16</td>
</tr>
</tbody>
</table>

---

**Table 3**

\( K_M \) and \( V_{max} \) values for the glucuronidation of 2'-OH-TCC by the UGTs of different microsomes determined by LC-ESI-MS

Mean and S.D. of three independent measurements are shown. Internal clearance for each tissue was calculated based on these results.
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 isoforms. 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 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-N-glucuronide (Malfatti and Felton, 2001) and for the N-glucuronidation of the structurally similar para-ethoxy phenyl urea (Uesawa et al., 2007).

In our study, we 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 activity 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, Franze, 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.

References


Ohno S 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-N-glucuronide (Malfatti and Felton, 2001) and for the N-glucuronidation of the structurally similar para-ethoxy phenyl urea (Uesawa et al., 2007).

In our study, we 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 activity 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, Franze, Maul, and Ranganathan.

Wrote or contributed to the writing of the manuscript: Schebb, Franze, Maul, Ranganathan, and Hammock.