**In vitro** glucuronidation of the antibacterial triclocarban and its oxidative metabolites

N.H. Schebb, B. Franze, R. Maul, A. Ranganathan and B.D. Hammock

University of Veterinary Medicine Hannover, Institute for Food Toxicology and Chemical Analysis, Bischofsholer Damm 15, 30173 Hannover, Germany (N.H.S)

University of California, Davis, Department of Entomology and Cancer Research Center, One Shields Ave, Davis, CA 95616 (B.F., A.R., B.D.H)

³ BAM Federal Institute for Materials Research and Testing, Division Organic Chemical Analysis & Reference Materials; Richard-Willstätter-Straße 11, 12489 Berlin, Germany (R.M.)
Corresponding author: Bruce, D. Hammock, University of California, Davis, Department of Entomology, One Shields Ave, 95616 Davis, CA, USA, telephone (+1) 530 752 5109, fax (+1) 530 752 1537, bdhammock@ucdavis.edu

Number of text pages: 19
Number of Tables: 3 Number of Figures: 2
Number of references: 36 Words in the abstract: 239
Words in the introduction: 554 Words in the discussion: 1197

List of non-standard abbreviations:
ACN acetonitrile
CLM Cynomolgus monkey liver microsomes
DCC Dichlorcan, 4,4´-Dichlorcarbanilide
ESI-MS electrospray ionization mass spectrometry
GSH glutathione
m/z mass to charge ratio
LC liquid chromatography
HLM human liver microsomes
HKM human kidney microsomes
HIM human intestine microsomes
MLM mice liver microsomes
P450 cytochrome P450
RLM rat liver microsomes
TCC 3,4,4´-trichlorocarbanilide, triclocarbon
UDP uridine 5´ diphosphate
UDPGA uridine 5´-diphosphoglucuronic acid
UGT UDP glucuronosyltransferase
Abstract

Triclocarban (3,4,4'-trichlorocarbanilide, TCC) is widely used as an antibacterial in bar soaps. During usage 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. In order 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. Utilizing a new LC-UV-MS/MS 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-\textit{N}-glucuronide and TCC-\textit{N'}-glucuronide, were formed at very low conversion rates (<1%) by microsomal incubations. Low amounts of \textit{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 \textit{N}-glucuronidation of TCC. These results present an anomaly where in vivo the predominant urinary metabolites of TCC are \textit{N} and \textit{N'}-glucuronides, but these compounds are produced slowly 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% (ESA, 2005; Ahn et al., 2008). Due to its widespread usage and its 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 US 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 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., 2011c). 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 regarding 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; Schebb et al., 2011c) comparable to 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). 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 (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 the 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 in order to obtain quantitative data about the clearance of this metabolite.
Materials and methods

Chemicals

3,4,4′-Trichlorocarbanilide (Triclorcarban, TCC) was purchased from Aldrich (St Louis, MO) and further purified (≥ 99.9%) by repeated re-crystallization. The TCC metabolites, 2′-OH-TCC, 3′-OH-TCC, 6-OH-TCC, 2′-Gluc-TCC, the internal standards (I.S.) 4,4′-dichlorcarbanilide (DCC) and 2′-SO₃-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 displayed in Figure 1. Uridine 5′-diphosphoglucuronic acid (UDPGA) trisodium salt, alamethicin and D-saccharolactone were from Sigma (St Louis, MO). All other chemicals were from Fisher Scientific (Pittsburgh, PA) and were of the highest quality available.

Microsomes and human UGT isoforms

All microsomes and Supersomes®, i.e. microsomes from insect Sf-9 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 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 (TFP). The measured activities utilizing the respective substrates are shown in the supplementary material.

**Glucuronidation assays**

The glucuronidation assay was carried out as described by Maul et al. (Maul et al., 2011) with slight modifications (Pfeiffer et al., 2006; Pfeiffer et al., 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 protein were mixed with 76 µL buffer and 40 µL of 125 µg/mL alamethicin solution 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 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 pre-incubated for 5 min at 37 °C on a heated shaker. The reaction was initiated by addition of 20 µL 20 mM UDPGA and incubated further for 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 ACN containing internal standard (I.S.). The final I.S. concentration was 1 µM. DCC was used as I.S. for the incubations of TCC, 2'-OH-TCC and 6-OH-TCC. 2'-SO₃-O-TCC served as I.S. for incubations of 3'-OH-TCC. The resulting suspension was mixed vigorously and centrifuged at 16,000 x g for 10 minutes to remove precipitated protein and buffer salts. The supernatant was directly used for 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. In order to keep the concentration of all samples within the linear range of the ESI-MS/MS detection the 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 on Waters Premier system (Waters Milford, MA). Separation was carried out on a RP-18 column with embedded polar groups of the dimensions 2 X 100 mm and particle size of 2.2 µm (Prontosil C18 ace EPS, Bischoff Chromatography, Leonberg, Germany). The analytes (injection volume 10 µL) were separated by a binary gradient at a flow rate of 300 µL/min of 25 mM ammonium acetate containing 0.1% acetic acid (HAc) as solvent A and pure acetonitrile (ACN) as solvent B. The following gradient was used: 0.0-2.0 min isocratic 30% B, 2.0-7.5 min nonlinear with decreasing slope (convex gradient curve profile nr. 5) 30-85% B, 7.5-8.5 min linear 80-100% B, 8.5-9.5 min isocratic 100% B, 9.5-9.7 min return to initial conditions of 30% B and reconditioning between 9.7-11.0 min. The analytes were detected by an UV detector operating at 265 nm and by negative electrospray ionization (ESI) tandem mass spectrometry (MS/MS) in sensitive and specific selected reaction monitoring (SRM) mode. MS/MS conditions for TCC, 2’-OH-TCC, 3’-OH-TCC, 6-OH-TCC, 2’-Gluc-O-TCC, 2’-SO3-O-TCC, and DCC were optimized by infusing a 100 nM of a solution of analytes in 80/20 ACN/water at a flow rate of 10 µl/ min with a syringe pump. The optimization of
separation and detection conditions for the further glucuronides was carried out 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'-Gluc-O-TCC and 6-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 supplementary material (Table S1 and Fig. S1).

The activity of the microsomes and individual UGTs was calculated based on the amount of formed product. For 2'-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 datum points of the product formation over the incubation time. Activity values were calculated based on the linear regression as mean and SD of three independent incubations. The $K_M$ and $v_{\text{max}}$ values for the glucuronidation of 2'-OH-TCC were obtained by fitting the conversion rates vs the concentration according to equation 1 with the software Sigmaplot Version 9.01 (Systat Software, Chicago, IL)

$$ v = \frac{v_{\text{max}} \cdot C}{K_M + C} $$

**In vivo experiments**

In order 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 Inc. Boston, MA) with a body weight of 458±25 g and two 12 week old male Swiss Webster mice (Charles River) of 34.4±0.7 g were used. This study was approved by the institutional UC Davis Animal Care and Use Committee. Prior to the experiment the animals were housed in UC Davis facilities with access to food and water ad libitum. 24 h hours before the experiment the animals were transferred into metabolic cages (Pakenham et al., 2002) and blank urine was collected. Two mice were placed in one metabolic cage and whereas 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 bodyweight and mice a dose of 5 mg/kg bodyweight. After administration urine was collected between 0-24h and 24-48 hours. The urine was analyzed by online-LC-MS as previously described (Schebb et al., 2011a).
Results

LC-UV-MS method

The glucuronidation of TCC and its three mono-hydroxylated metabolites was investigated using different microsomes and individual UGTs. In order 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 a 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. 2 A). For the metabolites with available reference standards, 2'-Gluc-TCC (peak 1) eluted first, followed by the sulfate conjugate of 2'-OH-TCC, 2'-SO$_3$-O-TCC (peak 2), used as I.S. At a retention time of 4.61 min, 3'-OH-TCC eluted (peak 3), closely followed by the I.S. DCC (peak 4) and TCC (peak 5). In contrast to earlier attempts for an RP 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 to TCC can be explained by the formation of an intermolecular hydrogen bond between the hydroxyl and the 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 presence of UDPGA led to an abundant peak at 2.26 min (Fig. 2) which gave rise to [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, it gave rise to 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) (Supplementary Material Fig. S1). In order 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). Utilizing 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 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, the quantification of the further glucuronides was carried out 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 by linear regression of the product formation vs. incubation time. This yields in 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; Pfeiffer et al., 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 datum 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 from different tissues to enable an estimation of the tissues in which the glucuronidation may occur in vivo. In order to compare the activity of the microsomal preparations at their v_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 human (HLM), monkey (CLM), rat (RLM) and mouse (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 HLM 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 2-3 fold higher rate with remarkable species dependent differences. While 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 (HKM) and from human intestine (HIM) showed overall a comparable activity to HLM for the conjugation of the three monohydroxylated TCC metabolites. Interestingly, the activity of HIM and HKM to conjugate 2′-OH-TCC was higher (5.62±0.06 nmol min\(^{-1}\) mg protein\(^{-1}\)) compared to HLM, whereas the 3′-OH-TCC and 6-OH-TCC were glucuronidated at equal or 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\(^{-1}\) 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 (supplementary material Fig. S2), however no signals where observed in LC-UV chromatogram. Thus, quantification was not feasible and the activity of the microsomes to glucuronidate TCC was estimated semi-quantitatively 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. Similarly, none of the isolated UGTs (Supersomes\(^{®}\)) showed activity for TCC glucuronidation, except for UGT1A9, which gave rise to small \(N\)-glucuronide peaks in LC-ESI-MS (supplementary material Fig. S2). Even in incubations with elevated concentration of microsomal protein concentration (up to 2 mg mL\(^{-1}\)) 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\(^{-1}\) mg protein\(^{-1}\) and higher. The extrahepatic UGT1A8 showed the strongest
formation of glucuronides of all three phase I metabolites, being most active for transforming 3'-OH-TCC followed by 2'-OH-TCC (Table 2). The less active UGT1A9 showed a different substrate selectivity and conjugated the metabolites in the order 6-OH-TCC > 2'-OH-TCC > 3'-OH-TCC, whereas UGT1A7 showed very similar activities on all compounds between 1.0 and 1.5 nmol min⁻¹ mg protein⁻¹. UGT1A1, 1A3 and 1A10 had moderate activities for the conjugation of the hydroxylated TCC. UGT1A4, UGT1A6 and none of the tested enzymes of the UGT2 family showed significant activity for the glucuronidation of TCC and its metabolites.

In order to estimate the clearance of 2'-OH-TCC in the liver, its conjugation kinetics were investigated in different liver microsomes. Product formation was linear for at least three of the four incubation times investigated. For all four species, the conjugation followed Michaelis-Menten-type kinetics, as indicated by a linear correlation in the Lineweaver-Burk and Eadie-Hofstee plots (Shown for CLM in the supplementary material Fig. S3). A $K_M$ in the low µM range was calculated for the liver microsomes of the four mammalian species. RLM, HLM and MLM showed a similar $K_M$ between 2.5-3.3 µM (Table 3) whereas CLM showed a significantly lower $K_M$ of 1.4±0.9 µM. The $v_{max}$ of 26.7±4.8 nmol min⁻¹ mg protein⁻¹ was dramatically higher in CLM than in the other species, with 5.37±0.43 nmol mg⁻¹ min⁻¹ for HLM, 9.42±4.8 nmol min⁻¹ mg protein⁻¹ for RLM and 1.45±0.43 nmol min⁻¹ mg protein⁻¹ for MLM. It should be noted that the activity of CLM for the model substrate TFMU was also about two fold higher than of RLM, MLM and HLM. Thus, the calculated high $v_{max}$ might be caused by a higher UGT content in CLM compared to the other mammalian liver microsomes. Based on this kinetics a similar intrinsic clearance of about 1.5 mL min⁻¹ mg protein⁻¹ of 2'-OH-TCC was calculated for humans and mice. RLM and particularly CLM show a higher clearance of the oxidative TCC metabolites (Table 3).
The analysis of mouse and rat urine after oral administration confirmed earlier studies that glucuronides represent the major renal excretion form of TCC (Fig. 3). As described by Birch et al. (Birch et al., 1978) no N-glucuronides were detected in the rat urine and we found the glucuronide of 3’-OH-TCC as major urinary metabolite. By contrast this study showed that mice form N and N’-glucuronides similar to humans and monkeys as major urinary metabolites. Additionally, glucuronides of hydroxylated species were detected, indicating that at doses of 1-5 mg kg^-1 bodyweight (which is a high dose compared to the human exposure caused by soaps of about 1 µg kg^-1 body weight (Schebb et al., 2011c)) these metabolites contribute to the renal clearance of TCC (Fig.3).
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 (Birch et al., 1978). However, the present 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 1A1, 1A3 and 1A9 (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 favoured 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 well in line with in vivo observations, where glucuronides of hydroxylated TCC species are by far the dominating 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 to the HLM. The substrate selectivity pattern of HKM was 6-OH-TCC > 2'-OH-TCC > 3 OH-TCC, in line with the pattern of the highly active UGT1A9, which is expressed in the kidney (Tukey and Strassburg, 2000). Similarly, 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 UGT2 family showed significant activity for the conjugation of phase I TCC metabolites.

Recent studies indicated that a further oxidation of 2'-OH-TCC leads to a reactive quinone imine metabolite, which 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 P-450 oxidation. All tested microsomes showed a low \( K_M \) and a high \( v_{\text{max}} \), 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-MU, 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 (E2) and its catecholic oxidative metabolites which are believed to play a role in the E2-mediated carcinogenesis (Pfeiffer et al., 2006). However, since both \( N \)- as well as \( O \)-glucuronides can be cleaved by glucuronidase from *E.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 (Figure 1) (Birch et al., 1978). Humans excrete about 25% of the total absorbed TCC via 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: i.) TCC-N-glucuronidation in vivo is catalyzed by other glucuronosyltransferases than the UGTs present in the microsomes. ii.) TCC-N-glucuronidation by UGT in vitro does not occur to the same extent than it occurs in vivo. There is some evidence favouring explanation ii.). First, there are several studies showing that N-glucuronidation reactions are underestimated by microsomal incubations (Anderson et al., 2009). Particularly TCC is not metabolized well 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.

In the activity screening using human UGTs only incubations with UGT1A9 gave rise to 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 \textit{in vivo}. For this purpose, metabolic studies in cell lines, precision tissue slices, optionally in combination with specific inhibitors, or transgenic animals transfected with human UGT isoforms or the UGT1\(-/-\) mouse, could be beneficial. Nevertheless, the tentative identification of UGT1A9 being the key enzyme in TCC-\(N\)-glucuronidation provides a convincing explanation for the observed species selectivity of this metabolic pathway. While 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 the 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 in line with the substrate specificity of UGT1A9. For example UGT1A9 is the key enzyme for the formation of the ternary \(N\)-hydroxy-PhIP-N3-glucuronide (Malfatti and Felton, 2001) and for the \(N\)-glucuronidation of the structurally similar \textit{para}-ethoxy phenyl urea (Uesawa et al., 2007).

In sum, 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.

**Author Contributions**

Participated in research design: Schebb and Hammock.
Conducted experiments: Franze and Schebb
Contributed new reagents or analytic tools: Ranganathan, Franze, Maul, and Schebb.
Performed data analysis: Franze, Schebb and Maul.
Wrote or contributed to the writing of the manuscript: Schebb, Franze, Maul, Ranganathan, and Hammock.
DMD #42283

References


ESA (2005) SCCP Triclorocarban.


Footnotes

This work was supported by the German Academic Exchange Service (DAAD, Bonn, Germany) in form of fellowships and grants from the US National Institute of Environmental Health Science [P42ES04699 and R01ES002710]. BDH is a senior fellow of the American Asthma Foundation.
Legends for Figures

**Fig. 1:** Overview of the UGT mediated TCC metabolism. Direct glucuronidation of TCC leads to N-glucuronides representing 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).

**Fig. 2:** Chromatographic separation with ESI-MS detection in SRM mode of TCC and its metabolites. Shown is an injection of a A) standard solution (50 nM), B) 40 min microsomal conversion of 6-OH-TCC (10 µM) with RLM and C) 3'-OH-TCC (10 µM) with RLM microsomes (0.025 mg protein/mL). In panel D) the analysis of a urine sample of a TCC exposed human is displayed. The peaks in the SRM chromatograms are 1) 2'-O-gluc-TCC, 2) 2'-SO3-O-TCC, 3) DCC (I.S.), 4) 3'-OH-TCC, 5) TCC, 6) 2'-OH-TCC, 7) 6-OH-TCC, 8) 6-O-gluc-TCC, 9) 3'-O-gluc-TCC, 10) N-Gluc-TCC and 11) N'-Gluc-TCC.

**Figure 3** Metabolic pattern of the TCC metabolites in rodent urine. In panel A the TCC metabolite concentration in mouse urine after oral gavage of 5 mg/kg bodyweight (t = 0 h) is shown. Results are shown as mean and deviation of two independent experiments. In panel B, TCC metabolites in rat urine after oral gavage of 1 mg/kg bodyweight (t = 0 h) are depicted. Results are shown as mean and deviation of threefold analysis of the combined urine of two animals.
### Tables

**Table 1** Performance of the analytical method. Retention time, limit of detection and linear range ($R^2 \geq 0.99$) for ESI-MS detection in SRM mode and UV detection at 265 nm are shown.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DCC</td>
<td>4.36 ± 0.03</td>
<td>3</td>
<td>1 – 1000</td>
<td>300</td>
<td>100 – 10 000</td>
</tr>
<tr>
<td>TCC</td>
<td>5.10 ± 0.04</td>
<td>3</td>
<td>1 – 1000</td>
<td>300</td>
<td>100 – 10 000</td>
</tr>
<tr>
<td>2’-OH-TCC</td>
<td>6.81 ± 0.07</td>
<td>3</td>
<td>1 – 1000</td>
<td>300</td>
<td>100 – 10 000</td>
</tr>
<tr>
<td>6’-OH-TCC</td>
<td>7.45 ± 0.05</td>
<td>3</td>
<td>1 – 1000</td>
<td>300</td>
<td>100 – 10 000</td>
</tr>
<tr>
<td>3’-OH-TCC</td>
<td>4.61 ± 0.02</td>
<td>3</td>
<td>1 – 1000</td>
<td>300</td>
<td>100 – 10 000</td>
</tr>
<tr>
<td>2’-SO3-O-TCC</td>
<td>3.46 ± 0.02</td>
<td>3</td>
<td>1 – 1000</td>
<td>300</td>
<td>100 – 10 000</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>300</td>
<td>100 – 10 000</td>
</tr>
<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>

n.d. = not determined because no reference compound was available
Table 2 Activities of various microsomes and human UGT-isoforms for the glucuronidation of 2'-OH-TCC, 3'-OH-TCC, 6-OH-TCC and TCC determined by LC-UV (265 nm). Activities are expressed as pmol min\(^{-1}\) mg protein\(^{-1}\) as the mean and SD of three independent measurements.

<table>
<thead>
<tr>
<th></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'-Gluc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RLM</td>
<td>2600 ± 130</td>
<td>5500 ± 1800</td>
<td>8700 ± 2800</td>
<td>nd</td>
</tr>
<tr>
<td>HLM</td>
<td>2700 ± 680</td>
<td>8500 ± 2200</td>
<td>5200 ±150</td>
<td>nd * +</td>
</tr>
<tr>
<td>CLM</td>
<td>22800 ± 100</td>
<td>16300 ± 700</td>
<td>9500 ± 1500</td>
<td>nd * ++</td>
</tr>
<tr>
<td>MLM</td>
<td>2600 ± 650</td>
<td>7900 ± 2400</td>
<td>14700 ± 690</td>
<td>nd * ++</td>
</tr>
<tr>
<td>HKM</td>
<td>5600 ± 1700</td>
<td>7700 ± 610</td>
<td>4200 ± 30</td>
<td>nd * +++</td>
</tr>
<tr>
<td>HIM</td>
<td>5700 ± 1200</td>
<td>3900 ± 1400</td>
<td>7100 ± 600</td>
<td>nd</td>
</tr>
<tr>
<td>1A1</td>
<td>850 ± 210</td>
<td>820 ± 260</td>
<td>520 ± 20</td>
<td>nd</td>
</tr>
<tr>
<td>1A3</td>
<td>390 ± 93</td>
<td>410 ± 50</td>
<td>540 ± 70</td>
<td>nd</td>
</tr>
<tr>
<td>1A4</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>1A6</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>1A7</td>
<td>870 ± 440</td>
<td>1100 ± 320</td>
<td>1700 ± 160</td>
<td>nd</td>
</tr>
<tr>
<td>1A8</td>
<td>5700 ± 2200</td>
<td>5000 ± 1100</td>
<td>9400 ± 250</td>
<td>nd</td>
</tr>
<tr>
<td>1A9</td>
<td>1200 ± 800</td>
<td>2400 ± 680</td>
<td>840 ± 190</td>
<td>nd * +</td>
</tr>
<tr>
<td>1A10</td>
<td>210 ± 160</td>
<td>280 ± 150</td>
<td>&lt;LOQ</td>
<td>nd</td>
</tr>
<tr>
<td>2B4</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>2B7</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>nd *</td>
<td>nd</td>
</tr>
<tr>
<td>2B15</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>2B17</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

<LOQ: Glucuronide peak < LOQ of LC-UV detection (0.2 µM)
nd: Glucuronide peak < LOQ of LC-UV detection (60 nM)
*: Low product amounts detected by ESI-MS.
**: The activity for the N-glucuronidation is rated semi-quantitatively (from low (+) to high (+++) based on the peak height in the ESI-MS signal.
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. Shown are the mean and SD of three independent measurements. The internal clearance for each tissue is calculated based on these results.

<table>
<thead>
<tr>
<th>microsomes</th>
<th>$K_M$ [µM]</th>
<th>$v_{max}$ [nmol min$^{-1}$ mg$^{-1}$]</th>
<th>clearance [ml min$^{-1}$ mg$^{-1}$]</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>1.45 ± 0.16</td>
</tr>
</tbody>
</table>
Figure 2

A

B

C

D

rel. intensity (%) of SRM signal

time (min)
Figure 3

A) mouse

B) rat

The graph shows the concentration of various compounds in urine samples over different periods. The x-axis represents the urine sampling period, while the y-axis shows the concentration in nM. Different compounds are indicated by various colors and patterns.

- TCC
- TCC-N-Glucs
- 2'-OH-TCC
- 2'-O-Gluc-TCC
- 2'-O-SO3-TCC
- 3'O-TCC
- 3'-O-Gluc-TCC
- 6-OH-TCC
- 6-O-Gluc-TCC

The data is presented for two species: A) mouse and B) rat. The sampling periods are indicated as -24-0 h, 0-20 h, and 20-48 h.
Supplementary Material

*In vitro* glucuronidation of the antibacterial triclocarban and its oxidative metabolites

Schebb N.H., Franze B., Maul, R., Ranganathan A. and Hammock B.D.

*Drug Metabolism and Disposition*

**Supplementary Table S1**
Optimized ESI-MS parameters for detection of TCC and its metabolites in SRM mode.

<table>
<thead>
<tr>
<th>compound</th>
<th>m/z [M-H]</th>
<th>cone voltage [V]</th>
<th>collision energy [V]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCC (I.S.)</td>
<td>279</td>
<td>126</td>
<td>28</td>
</tr>
<tr>
<td>TCC</td>
<td>313</td>
<td>160</td>
<td>26</td>
</tr>
<tr>
<td>2'-OH-TCC</td>
<td>329</td>
<td>168</td>
<td>24</td>
</tr>
<tr>
<td>6-OH-TCC</td>
<td>329</td>
<td>202</td>
<td>22</td>
</tr>
<tr>
<td>3'-OH-TCC</td>
<td>329</td>
<td>168</td>
<td>24</td>
</tr>
<tr>
<td>2'-SO3-O-TCC</td>
<td>409</td>
<td>168</td>
<td>28</td>
</tr>
<tr>
<td>N-gluc-TCC</td>
<td>489</td>
<td>336</td>
<td>30</td>
</tr>
<tr>
<td>N'-gluc-TCC</td>
<td>489</td>
<td>302</td>
<td>40</td>
</tr>
<tr>
<td>2'-O-gluc-TCC</td>
<td>505</td>
<td>168</td>
<td>40</td>
</tr>
<tr>
<td>6-O-gluc-TCC</td>
<td>505</td>
<td>343</td>
<td>40</td>
</tr>
<tr>
<td>3'-O-gluc-TCC</td>
<td>505</td>
<td>344</td>
<td>40</td>
</tr>
</tbody>
</table>
Supplementary Table S2

Activity of the microsomal preparations for the conjugation of 4-(trifluoromethyl)umbelliferone (TFMU) and trifluoperazine (TFP). Microsomal incubations with 100 µM TFMU and 200 µM were carried out as described for TCC and its metabolites. The glucuronide concentration was determined by HPLC as described previously (Maul et al. 2011; Uchaipichat et al. 2006). The activity and SD (nmol min⁻¹ mg protein⁻¹) are given as mean of three independent determinations.

<table>
<thead>
<tr>
<th>microsomes / UGT</th>
<th>activity TFMU nmol min⁻¹ mg⁻¹</th>
<th>activity TFP pmol min⁻¹ mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM</td>
<td>55.7±3.93</td>
<td>12.7±3.6</td>
</tr>
<tr>
<td>MLM</td>
<td>46.4±5.32</td>
<td>&lt;LOD (5)</td>
</tr>
<tr>
<td>CLM</td>
<td>107±1.48</td>
<td>127±3.7</td>
</tr>
<tr>
<td>RLM</td>
<td>70.8±3.76</td>
<td>&lt;LOD (5)</td>
</tr>
<tr>
<td>HIM</td>
<td>10.4±0.82</td>
<td>&lt;LOD (5)</td>
</tr>
<tr>
<td>HKM</td>
<td>30.0±1.28</td>
<td>&lt;LOD (5)</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>2.30±0.17</td>
<td>&lt;LOQ (0.2)</td>
</tr>
<tr>
<td>UGT1A3</td>
<td>1.93±0.32</td>
<td>488±20.6</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>&lt;LOD (0.04)</td>
<td></td>
</tr>
<tr>
<td>UGT1A6</td>
<td>20.7±0.45</td>
<td></td>
</tr>
<tr>
<td>UGT1A7</td>
<td>6.69±0.75</td>
<td></td>
</tr>
<tr>
<td>UGT1A8</td>
<td>2.44±0.03</td>
<td></td>
</tr>
<tr>
<td>UGT1A9</td>
<td>10.8±0.96</td>
<td></td>
</tr>
<tr>
<td>UGT1A10</td>
<td>2.44±0.03</td>
<td></td>
</tr>
<tr>
<td>UGT2B4</td>
<td>0.67±0.13</td>
<td></td>
</tr>
<tr>
<td>UGT2B7</td>
<td>2.73±0.10</td>
<td></td>
</tr>
<tr>
<td>UGT2B15</td>
<td>3.19±0.05</td>
<td></td>
</tr>
<tr>
<td>UGT2B17¹</td>
<td>&lt;LOQ (0.2)</td>
<td></td>
</tr>
</tbody>
</table>

¹ Activity of 0.37 nmol min⁻¹ mg protein⁻¹ for the conjugation of the substrate eugenol (According to manufacturer)
Figure S1:
Left: Negative electrospray ionization product ion spectra of the TCC-glucuronide conjugates. A: 2'-O-gluc TCC; B: 3'-O-gluc-TCC; C: 6-O-gluc-TCC; D: N'-gluc-TCC, E: N-gluc-TCC. Right: Structures of the glucuronides. The lines depict the fragmentation sites leading to the major fragments. The adjacent numbers show the m/z values of the resulting fragments.
Figure S2:
LC-ESI-MS/MS chromatograms of microsomal incubations of TCC. Shown are the SRM signals of \( N \)-Gluc-TCC- (black) and \( N' \)-Gluc-TCC (grey) of 30 minute incubations (25 µg/ml protein) of TCC with (A) HKM and (B) UGT1A9. Analysis after incubation with (C) UGT1A7 and (D) UGT1A4 are shown exemplary for microsomal preparations not converting TCC.
**Figure S3**

Determination of $K_M$ and $v_{max}$ values by fitting to the Michaelis-Menten function and utilization of various linearization methods. The data derived from a glucuronidation experiment of 2'-OH-TCC by CLM is shown exemplarily. **A:** Mathematical fitting  **B:** Lineweaver-Burk plot;  **C:** Hanes plot;  **D:** Eadie-Hofstee plot.