Electrochemistry-Mass Spectrometry Unveils the Formation of Reactive Triclocarban Metabolites^[S]

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ABSTRACT:

Triclocarban (3,4,4'-trichlorocarbanilide, TCC) is a widely used antibacterial agent in personal care products and is frequently detected as an environmental pollutant in waste waters and surface waters. In this study, we report novel reactive metabolites potentially formed during biotransformation of TCC. The oxidative metabolism of TCC has been predicted using an electrochemical cell coupled online to liquid chromatography and electrospray ionization mass spectrometry. The electrochemical oxidation unveils the fact that hydroxylated metabolites of TCC may form reactive quinone imines. Moreover, a so-far unknown dechlorinated and hy-

droxylated TCC metabolite has been identified. The results were confirmed by *in vitro* studies with human and rat liver microsomes. The reactivity of the newly discovered quinone imines was demonstrated by their covalent binding to glutathione and macromolecules, using β -lactoglobulin A as a model protein. The results regarding the capability of the electrochemical cell to mimic the oxidative metabolism of TCC are discussed. Moreover, the occurrence of reactive metabolites is compared with findings from earlier *in vivo* studies and their relevance *in vivo* is argued.

Introduction

Triclocarban (3,4,4'-trichlorocarbanilide, TCC) (Fig. 1) has been widely used as an antibacterial agent in personal care products (Chen et al., 2008) for more than 45 years. TCC can be added to rinse-off products in the United States and European Union in concentrations up to 1.5% (European Commission, Scientific Committee on Consumer Products, 2005; Ahn et al., 2008). In the United States, 48% of all antimicrobial bar soaps contain TCC; it is a high-production chemical and approximately a million pounds are used per year (Halden and Paull, 2004). Because of its widespread usage and its environmental persistence, TCC was found in surface waters in concentrations up to micrograms per liter (Halden and Paull, 2005; Sapkota et al., 2007). Moreover, the Targeted National Sewage Sludge Survey, published in 2009 by the U.S. Environmental Protection Agency, cites the detection of TCC in all 84 sewage samples analyzed within the study. TCC was found at up to 0.44 g/kg, the highest concentration of all synthetic compounds screened for. These

data raised public concern regarding possible effects of TCC on human health (Ahn et al., 2008). In the 1970s, several studies indicated that a significant portion of TCC in soaps is percutaneously absorbed by humans during and after showering. Scharpf et al. (1975) demonstrated that approximately 0.4% of the applied TCC was found in the excreta and thus was absorbed and systemically available.

To evaluate the potential toxicological effects of TCC, knowledge of its metabolic fate is indispensable. Because TCC shows high bioavailability after oral dosing (Hiles, 1977; Jeffcoat et al., 1977; Hiles and Birch, 1978a; Warren et al., 1978), it can be assumed that TCC in contaminated drinking water or food will be extensively absorbed. *In vivo* studies in rodents, monkeys, and humans revealed that TCC is rapidly metabolized via two different pathways. The main metabolite found in human urine results from direct *N*-glucuronidation at one of the nitrogen atoms of the urea moiety of TCC (Birch et al., 1978; Hiles and Birch 1978, Hiles et al., 1978), accounting for 25% of TCC elimination from the body. The majority of absorbed TCC is metabolized by enzymes of the cytochrome P450 (P450) superfamily into different hydroxylated TCC species (phase I metabolites), which are subsequently conjugated to glucuronic acid or sulfated and predicted to be excreted via the feces (Jeffcoat et al., 1977; Birch et al., 1978; Hiles and Birch, 1978a,b; Hiles et al., 1978; Warren et al., 1978). The main phase I metabolites are 2'-OH TCC and 6-OH TCC, both bearing the hydroxyl group in the *ortho* position relative to the urea group (Fig. 1). In addition, 3'-OH TCC and dihydroxylated metabolites, including 2',6-diOH TCC and 3',6-diOH TCC, are formed (Fig. 1) (Birch et al., 1978; Warren et al., 1978).

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ABBREVIATIONS: TCC, 3,4,4'-trichlorocarbanilide, triclocarban; P450, cytochrome P450; OH, hydroxy; diOH, dihydroxy; LC, liquid chromatography; ESI, electrospray ionization; MS, mass spectrometry; EC, electrochemistry; HLM, human liver microsome(s); RLM, rat liver microsome(s); ACN, acetonitrile; β -LGA, β -lactoglobulin A.

Fig. 1. Structures of TCC (a) and its phase I metabolites: 6-OH TCC (b), 2'-OH TCC (c), 3'-OH TCC (d), 2',6-diOH TCC (e), and 3',6-diOH TCC (f). In addition, the suggested structures of quinone imines formed from 6-OH TCC (g) and 2'-OH TCC (h) are shown.

Based on these data and other earlier toxicological studies, the Scientific Committee on Consumer Products of the European Union regarded the usage of TCC in personal care products as safe (European Union, Scientific Committee on Consumer Products, 2005). However, recent findings from our group indicate a significant, unintended biological activity. By enhancing the action of steroids, TCC has the potential to act as an endocrine disruptor at high concentrations (Ahn et al., 2008; Chen et al., 2008). Moreover, TCC inhibits the enzyme-soluble epoxide hydrolase (Morisseau et al., 2009), with an in vitro potency (IC₅₀ 13 nM) comparable to that of synthetic inhibitors, which proved to alter the biological regulation of inflammation, pain, and blood pressure in vivo (Imig and Hammock, 2009).

Because the toxic effects of drugs and xenobiotics arise not only from the compound itself but also from its metabolites, the focus of the present study was to investigate the potential formation of reactive metabolites of TCC. As discussed above, TCC is metabolized to 2'-OH TCC and 6-OH TCC, bearing a hydroxyl group at the *ortho* position relative to the urea moiety. Upon oxidative dehydrogenation, catalyzed by P450 enzymes, *ortho*-quinone imines can be formed from these metabolites (Fig. 1) (Kalgutkar et al., 2002; Aynur et al., 2009). Quinones and quinone imines are well known reactive metabolites, which very often undergo adduct formation with cellular compounds such as GSH, proteins, or DNA (Bolton et al., 2000). As a consequence, these compounds can cause toxic side effects, especially in the case of depleted levels of cellular GSH (Evans et al., 2004; Zhou et al., 2005).

The detection of reactive metabolites by classic in vitro approaches such as incubation with microsomes is often hampered by covalent binding of the reactive metabolites formed to cellular compounds present in these fractions (Kremers, 1999). To detect these reactive intermediates, the metabolism of TCC was studied in an electrochemical cell. Several studies demonstrated that this electrochemical technique is capable of mimicking the majority of oxidative metabolism reactions, including aromatic hydroxylation, as well as the formation of quinones and quinone imines (Johansson et al., 2007; Lohmann and Karst, 2008; Baumann and Karst, 2010). Coupling an electrochemical cell online to a liquid chromatography (LC) and electrospray ionization (ESI) mass spectrometry (MS) system allows the direct detection of reactive metabolites in the absence of endogenous compounds (the EC/LC/ESI-MS setup is shown in Fig. 2a) (van Leeuwen et al., 2005; Lohmann and Karst, 2007). Moreover, nucleophiles such as GSH

or proteins can be added selectively to the online system to evaluate the reactivity of a specific metabolite (Lohmann and Karst, 2006; Lohmann et al., 2008). On the basis of the EC prediction of the TCC metabolism, we thoroughly investigated the P450-mediated metabolism of TCC with liver microsomes from humans (HLM) and rats (RLM) and compared the result with in vivo data known from the literature.

Materials and Methods

Chemicals. Acetonitrile (ACN) and methanol were purchased from Merck Biosciences (Darmstadt, Germany). Potassium dihydrogenphosphate, dipotassium hydrogenphosphate, ammonium acetate, and formic acid were obtained from Fluka (Buchs, Switzerland). TCC, magnesium chloride hexahydrate, β -lactoglobulin A (β -LGA) from bovine milk, L-glutathione (reduced) (GSH), and urea were purchased from Sigma-Aldrich (Steinheim, Germany). NADPH was purchased from AppliChem (Darmstadt, Germany). Pooled RLMs from male Sprague-Dawley rats (protein concentration, 20 mg/ml; P450 content, 520 pmol/mg protein) and pooled HLMs from 19 donors (protein concentration, 20 mg/ml; P450 content, 240 pmol/mg protein) were purchased from BD Biosciences (Woburn, MA). All chemicals used were the highest quality available.

Fig. 2. Experimental set-up used for (a) the electrochemical simulation of the oxidative metabolism of TCC and (b) the investigation of the reactivity of TCC oxidation products toward GSH.

Synthesis of Standards. The TCC metabolites, 2'-OH TCC and 3'-OH TCC (Fig. 1) were synthesized by coupling the appropriate isocyanate and amine compounds as described previously (Warren et al., 1978; Ahn et al., 2008). 6-OH TCC was synthesized as follows. 4,5-Dichloro-2-nitrophenol was prepared by ultrasound-assisted nitration of 3,4-dichlorophenol in a mixture of dichloromethane/H₂O containing tetra-*n*-butylammonium hydrogen sulfate (1 mol %) and nitric acid (2 Eq). The product ($R_f = 0.48$, 4:1 hexane-ethyl acetate) was isolated by flash chromatography on silica gel eluted with 15:1 hexane-ethyl acetate. Reduction with tin(II) chloride $\cdot$ 2H₂O in refluxing ethanol afforded 2-amino-4,5-dichlorophenol. Subsequent reaction with 4-chlorophenylisocyanate, followed by recrystallization from 1:2 hexane-ethyl acetate afforded 6-OH TCC (7% over three steps) as a white solid. All compounds were characterized by NMR and ESI-tandem mass spectrometry analysis, and purities were greater than 99% based on the peak areas in LC/UV analysis at $\lambda = 270$ and 254 nm.

Microsomal Incubations. A mixture of microsomal protein and the respective substrate (TCC, 2'-OH TCC, 3'-OH TCC, and 6-OH TCC) dissolved in 50 mM phosphate buffer solution (adjusted to pH 7.4) was preincubated for 5 min at 37°C. Magnesium chloride and NADPH were added to the incubation mixture, which was then further incubated at 37°C for 90 min. The total volume of incubation mixture was 500 μ l for RLM and 250 μ l for HLM, respectively. The final concentrations were as follows: 1.3 mg/ml microsomal protein, 50 μ M substrate added in dimethyl sulfoxide (1% dimethyl sulfoxide in the incubation mixture), 0.5 mM magnesium chloride, and 1.2 mM NADPH. Subsequent to the incubation, proteins were precipitated by adding an equal amount of ACN to the incubation mixture. After centrifugation (1700g), the supernatant was analyzed by LC/ESI-MS. To study adduct formation between metabolites and GSH, additional incubation mixtures with a concentration of 500 μ M GSH were prepared. Control incubations for all experiments were performed without addition of NADPH.

Electrochemical Metabolism Simulation. The simulation of the oxidative metabolism was performed in an electrochemical thin-layer cell equipped with a boron-doped diamond working electrode and a Pd/H₂ reference electrode (reactor cell; Antec Leyden, Zoeterwoude, The Netherlands). Potentials were applied using either the electrochemical detector system ROXY (Antec Leyden) or a homemade potentiostat. The electrochemical cell was interfaced to the online EC/LC/ESI-MS system (Fig. 2a), in which the electrochemically generated oxidation products are directly injected onto LC column, separated, and detected by ESI-MS. For the electrochemical conversion, a solution of TCC [100 μ M in 50:50% (v/v) ACN 1 mM aqueous ammonium acetate] was passed through the electrochemical cell at a flow rate of 10 μ l/min (syringe pump model 74900; Cole-Parmer Instrument Co., Vernon Hills, IL) while applying a constant potential of 2500 mV versus the Pd/H₂ reference electrode. The oxidation products eluted from the cell and were collected in a 10-port valve, equipped with a 10- μ l injection loop. After filling of the loop, the valve was switched, and the loop contents were flushed onto the column.

For the investigation of GSH adducts of TCC and its metabolites, a slightly modified EC/ESI-MS set-up was used (Fig. 2b). The respective substrate [TCC, 2'-OH TCC, 3'-OH TCC, and 6-OH TCC] dissolved at a concentration of 10 μ M in 50:50% (v/v) ACN 1 mM aqueous ammonium acetate] was pumped through the electrochemical cell at a flow rate of 10 μ l/min. GSH at a concentration of 50 μ M in water was added to the effluent of the electrochemical cell via a T-piece and a syringe pump, operating at a flow rate of 10 μ l/min. Instead of using a constant potential, the potential in the cell was ramped between 0 and 2500 mV (10 mV/s), and the formation of adducts was directly monitored by coupling the electrochemical cell via a transfer capillary to the ESI interface. By converting the data into a three-dimensional plot, showing the formation of oxidation products in dependence on the potential and the m/z ratio, a so-called mass voltammogram can be generated (Lohmann and Karst, 2009).

Adduct formation between β -LGA and TCC, 2'-OH TCC, 3'-OH TCC, and 6-OH TCC was studied in an offline setup. The effluent (150 μ l) of the electrochemical cell was collected in a glass vial containing 150 μ l of protein solution (20 μ M β -LGA dissolved in 8 M urea). The flow rate in the electrochemical cell was set to 10 μ l/min, and the cell potential was kept constant at 1500 mV versus Pd/H₂. The concentration of the respective substrate was 100 μ M dissolved in 50:50% (v/v) ACN-1 mM aqueous ammonium acetate. After collection of the electrochemical cell effluent, the

sample was gently mixed, incubated at 37°C for 30 min, and analyzed by LC/ESI-MS.

LC/ESI-MS Conditions. All LC/ESI-MS measurements were performed on LC system from Antec Leyden coupled to a micrOTOF mass spectrometer, equipped with an ESI source (micrOTOF; Bruker Daltonics, Bremen, Germany). The LC system comprised two LC 100 pumps, an OR 110 organizer rack with a degasser and a pulse dampener, an AS 100 autosampler, and a ROXY column oven. ALEXYS software (Antec Leyden) and micrOTOFControl 1.1 with DataAnalysis 3.3 were used to control the LC and ESI-MS systems. LC separation of the oxidative metabolites was performed on a ProntoSIL 1203-C18-ace-EPS column (Bischoff Analysentechnik und -geräte GmbH, Leonberg, Germany) with the dimension 50 $\times$ 2 mm, particle size 3 μ m, and pore size 12 nm at a flow rate of 500 μ l/min. The injection volume was set to 10 μ l, and the oven temperature was 40°C. The mobile phase consisted of 90:10 (v/v) water (acidified with 0.1 vol % formic acid)-methanol (eluent A) and methanol (eluent B). The following gradient was applied: 0.0 to 2.0 min isocratic 50% B, 2.0 to 5.0 min linear from 50 to 80% B, 5.0 to 8.5 min linear from 80 to 90% B, 8.5 to 9.5 min isocratic 90% B and 9.5 to 12.5 min reequilibration at 50% B. ESI-MS detection of oxidative metabolites was performed in the negative ion mode using a capillary voltage of 4000 V.

For the LC/ESI-MS analysis of the protein adducts, a Discovery BIO Wide Pore C5 column (2.1 $\times$ 150 mm, 5 μ m, 30-nm pore size, Supelco; Sigma-Aldrich, St. Louis, MO) was used. At a flow rate of 300 μ l/min and an oven temperature of 40°C, 10 μ l of sample was injected. The following gradient of aqueous 0.1% formic acid (eluent A) and ACN (eluent B) was applied: 0.0 to 2.0 min isocratic 5% B, 2.0 to 12.0 min linear from 5 to 80% B, 12.0 to 14.0 min isocratic 80% B, and 14.0 to 20 min reequilibration at 5% B. ESI/MS was performed out in positive ion mode using a capillary voltage of -4000 V.

Results

The oxidation products of TCC were investigated by online EC/LC/ESI-MS (Fig. 2a) with special respect to the formation of reactive intermediates. The largest conversion of TCC in the electrochemical cell was achieved at a potential of 2500 mV versus Pd/H₂. The electrochemical oxidation of TCC gave rise to 10 products, as shown in the chromatogram in Fig. 3. All of these compounds showed a distinct isotopic pattern originating from the natural ³⁵Cl/³⁷Cl distribution, which indicates the presence of dichlorinated products in peaks 1 and 2 and trichlorinated products in peaks 3 to 10. On the basis of the measured exact masses for the monoisotopic ³⁵Cl species, molecular formulas of products 1 to 10 have been calculated and are listed in Table 1. For all compounds, the deviation between the theoretical and measured m/z is less than 5 ppm. Furthermore, fragment ions, generated by in-source fragmentation in the ESI interface, have been studied for locating the site of oxidative modification. As described by Warren et al. (1978), fragmentation of TCC takes place at both urea N-C bonds, giving rise to two aniline fragment ions (X1 and Y1) (Fig. 4) and two isocyanate fragment ions (X2 and Y2) (Fig. 4).

Fig. 3. EC/LC/ESI-MS-based prediction of the oxidative metabolites of TCC. At 2500 mV versus Pd/H₂, 10 different potential oxidative metabolites have been detected. Shown are the extracted ion traces of TCC and its oxidation products. Molecular formulas, based on exact masses and fragment ions occurring upon in-source fragmentation, have been used for identification (Table 1).

TABLE 1

Oxidative products of TCC detected in EC/LC/ESI-MS measurements (compare Fig. 3)

The deviation between the calculated and the measured m/z is less than 5 ppm for all compounds. Based on molecular formulas and the m/z of the characteristic fragment ions X1, Y1, X2, and Y2 (compare Fig. 4) of the carbanilides, the compounds have been tentatively identified. Structure of metabolites 3, 4, 5, 6, 8, and 10 are presented in Fig. 1.

Peak	Measured m/z	Molecular Formula $[M - H]^-$	Transformation of TCC	Fragmentations	Compound
1	292.9888	$C_{13}H_9Cl_2N_2O_2$	+O - H - Cl		
2	295.0052	$C_{13}H_9Cl_2N_2O_2$	+O + H - Cl	X1, Y2	4'-Hydroxy-3,4-dichlorocarbanilide
3	326.9492	$C_{13}H_6Cl_3N_2O_2$	+O - 2H		
4	326.9498	$C_{13}H_6Cl_3N_2O_2$	+O - 2H		
5	328.9650	$C_{13}H_8Cl_3N_2O_3$	+O	X1, X2	3'-OH TCC
6	344.9580	$C_{13}H_8Cl_3N_2O_3$	+2O	X1, Y1	3',6-DiOH TCC
7	342.9602	$C_{13}H_8Cl_3N_2O_3$	+2O - 2H		
8	344.9432	$C_{13}H_8Cl_3N_2O_3$	+2O	X1, Y1	2',6-DiOH TCC
TCC	312.9706	$C_{13}H_8Cl_3N_2O_1$		X2, Y2	TCC
9	328.9660	$C_{13}H_8Cl_3N_2O_2$	+O	X1, X2	2'-OH TCC
10	328.9657	$C_{13}H_8Cl_3N_2O_2$	+O	Y1, Y2	6-OH TCC

TCC is detected in negative ion mode as deprotonated $[M - H]^-$ ion, which is negatively charged at one of the urea nitrogens. Therefore, both aniline fragment ions (X1 and Y1) can be detected, whereas the isocyanate fragments (X2 and Y2) are not charged and thus cannot be detected in negative ion mode. When we compare the fragmentation of TCC and its metabolites, the change in the m/z values of the fragments provides distinct information about the side of modification. As an example, the metabolite 6-OH TCC bears an additional hydroxyl group at the dichloroaniline ring of TCC. Hence, one can observe a gain of 15.99 atomic mass units for the fragment ions Y1 and Y2. In this case, not only the aniline fragment ion Y1 but also the isocyanate fragment Y2 can be detected, because a deprotonation takes place at the hydroxyl group and gives rise to an intense isocyanate fragment ion (Table 1). On the basis of the isotopic patterns, the molecular formulas deduced from exact masses, and these distinct fragmentation patterns, the metabolites formed were tentatively identified as described in the following.

Three monohydroxylated metabolites (peaks 5, 9, and 10) were detected and identified as 3'-OH TCC, 2'-OH TCC, and 6-OH TCC by comparing retention time and mass spectra with a synthetic reference for each compound. Two dihydroxylated metabolites (peaks 6 and 8) were detected, aligned with X1 and Y2 ions, indicating a hydroxylation on both aromatic rings, which were identified as 2',6-diOH TCC (8) and 3',6-diOH TCC (6) (see below, results of HLM and RLM experiments). As can be seen from the chromatogram (Fig.

3), 2',6-diOH TCC coelutes with TCC. The possibility of 2',6-diOH TCC being an in-source-generated artifact of TCC can be excluded, because it is absent at a potential of 0 mV in the electrochemical cell.

Two products were formed with a m/z of 326.95 (peaks 3 and 4), indicating that they are dehydrogenation products of monohydroxylated metabolites. Peak 4 was identified as quinone imine of 2'-OH TCC. The identification is based on the comparison with the chromatogram of the HLM incubation mixture of 2'-OH TCC (see below). Furthermore, when 2'-OH TCC in the electrochemical cell is oxidized at a potential of 1500 mV, an intense formation of peak 4 can be observed. This result is in agreement with the known electrochemical formation of quinone imines at moderate electrochemical potentials (300–1500 mV) (Johansson et al., 2007; Lohmann and Karst, 2007). When the electrochemical cell potential is increased up to 2500 mV, the signal of the quinone imine of 2'-OH TCC (peak 4) decreases, whereas a new signal of peak 3 appears. Hence, peak 3 is assumed to be an oxidation product of the quinone imine of 2'-OH TCC. A structure for peak 3 could not be assigned, but a ring formation via a nucleophilic attack of a urea nitrogen on the quinone imine might have taken place. That peak 3 is the quinone imine of 6'-OH TCC can be excluded, because the electrochemical oxidation of 6'-OH TCC at a potential of 1500 mV results in the formation of the quinone imine eluting at 4 min, whereas peak 3 has a retention time of 5 min. In addition to the dehydrogenation products 3 and 4, the molecular formula of peak 7 indicates the formation of a quinone imine, via dehydrogenation of 2',6-diOH TCC or 3',6-diOH TCC (Table 1). As expected for quinone imine structures, none of these dehydrogenated metabolites (peaks 3, 4, and 7) show the characteristic fragmentation of carbanilides, in contrast to all other metabolites (Table 1).

Besides the different hydroxylated TCC derivatives, dechlorinated species bearing two chlorine atoms (peaks 1 and 2) have been detected in the chromatogram. The molecular formula of peak 2 and the m/z of the in-source fragments indicate a substitution of the chlorine with a hydroxyl group in the monochloroaniline ring of TCC. Presumably peak 1 has been formed from 2 via a dehydrogenation reaction, exhibiting ions with an m/z decreased by 2.01 Da. It should be noted that two further dechlorinated products, correlating to dechlorinated dihydroxylated TCC species, have been observed (ion traces not shown) but are not discussed in more detail, because they only occur in low intensities and are detected neither as in vitro nor in vivo metabolites.

The EC-based oxidation studies provide information about sites in the molecule that are susceptible to oxidation, and they point out potential metabolites of TCC, allowing the identification of retention times and mass spectrometric properties of these products. Based on these results, RLM and HLM incubations of TCC and its known major

Fig. 4. Fragmentation pattern of TCC according to Warren et al. (1978). The fragments X1 and Y1 were only detected for hydroxylated derivatives by means of (-)-ESI-MS.

FIG. 5. Extracted ion traces of m/z 328.96, correlating to the hydroxylated TCC metabolites 3'-OH TCC (peak 5), 2'-OH TCC (peak 9), and 6-OH TCC (peak 10). All three metabolites were found in the TCC incubation mixture with RLM and HLM. Identification was performed on the basis of in-source fragmentation (m/z 175.96 and 201.95) and comparison with authentic reference compounds.

metabolites 2'-OH TCC, 3'-OH TCC, and 6-OH TCC were performed and analyzed by means of LC/ESI-MS. The extracted ion traces of the metabolites found in these *in vitro* studies are presented in Figs. 5 to 7. Figure 5 depicts the ion traces of the hydroxylated metabolites detected in the RLM and HLM incubation mixtures of TCC. As predicted by EC, 2'-OH TCC (peak 9) and 6-OH TCC (peak 10) are predominantly formed by the liver microsomes of both species, and 3'-OH TCC (peak 5) is produced to a lesser extent. An unexpected finding was that not only hydroxylated metabolites but also a dechlorinated metabolite (peak 2) are formed in RLM and HLM incubation mixtures of TCC (Fig. 6). The exact masses, the distinctive isotope pattern (Fig. 6), and the fragment ions (Table 1) indicate a dechlorination at the 4' position and hydroxylation in the same or another position of the monochloroaniline ring of TCC.

Dihydroxylated TCC metabolites have been found in *in vivo* studies (Birch et al., 1978) and were predicted by EC oxidation. However, these metabolites have not been found in the microsomal incubation of TCC with RLM and HLM (data not shown), which is probably caused by the low conversion rate (<2%) of TCC to oxidative metabolites in the microsomal incubations. Nonetheless, they can be formed by P450 enzymes from monohydroxylated compounds as shown by incubating 2'-OH TCC, 3'-OH TCC, and 6-OH TCC with RLMs (Fig. 7). The fragmentation of peak 6 reveals hydroxylation of both aromatic rings (Table 1). This metabolite was assigned as 3',6-diOH TCC, because it is formed from both 3'-OH TCC and 6-OH TCC. In a similar manner, metabolite 8 was assigned as 2',6-OH TCC. In comparison with the EC studies (Fig. 3), one additional dihydroxylated compound (peak X, Fig. 7) was detected in the RLM incubation mixture of 2'-OH TCC. The X1 fragment ions of this metabolite (Table 1) indicate dihydroxylation of the monochloroaniline ring. However, on the basis of the fragmentation data, the exact position of the hydroxylation cannot be determined. Only minor conversion of 6-OH TCC to 2',6-diOH TCC and no further hydroxylation of 2'-OH TCC and 3'-OH TCC by HLM were detected. This result is in line with *in vivo* studies showing the formation of dihydroxylated compounds in the rodent animal model but not in humans (Birch et al., 1978).

Besides being further hydroxylated, the *ortho*-hydroxy TCC metabolites 9 (2'-OH TCC) and 10 (6-OH TCC) (Table 1) are likely to undergo a P450-catalyzed dehydrogenation, which results in the for-

FIG. 6. Extracted ion traces of m/z 295.01, found in the RLM and HLM incubation mixture of TCC. For HLM-based experiments with TCC, the isotope pattern is shown, indicating the loss of a chlorine atom. A possible structure, deduced from exact masses, the isotope pattern, and fragment ions (Table 1), is presented below the chromatogram. *, peak resulting from the RLM matrix.

mation of quinone imines (Guengerich, 2007). This is shown in Fig. 8 for 2'-OH TCC. The same quinone imine as predicted by EC was identified in the HLM incubation mixture of 2'-OH TCC. However, no quinone imines were detected in the HLM and RLM incubation mixture of TCC, 3'-OH TCC, and 6-OH TCC.

Both the EC-based and *in vitro* investigations point out that presumably reactive quinone imine species (compounds 1, 3, 4, and 7) are formed. With the aim of evaluating the reactivity of the respective quinone imines, their binding to GSH was investigated using the EC/ESI-MS system depicted in Fig. 2b (Lohmann et al., 2008). Earlier EC-based oxidation studies have shown that dehydrogenated quinones and quinone imines can be very well formed by EC (Lohmann and Karst, 2007; Madsen et al., 2007; Lohmann et al., 2008). However, the oxidation potential has to be carefully optimized, because at too high a potential, quinones and quinone imines can undergo subsequent reactions. Using mass voltammograms, one can easily screen through the entire potential range, whereas the emerging oxidation products and GSH adducts are monitored online by ESI-MS. A representative mass voltammogram of 2'-OH TCC and GSH is shown in Fig. 9. 2'-OH TCC is oxidized in the electrochemical cell at increasing potential, and GSH is added to the effluent of the cell. At a potential greater than 1000 mV versus Pd/H₂, the signal of 2'-OH TCC decreases, whereas an increasing signal of a GSH adduct with the m/z 634.02 is observed. The molecular formula determined and the isotope pattern correlate with a GSH adduct formed between 2'-OH TCC and GSH. A similar adduct (same m/z and isotope pattern) was identified for 6-OH TCC and GSH. For TCC and 3'-OH TCC, no GSH adducts have been identified (data not shown). Different isomeric GSH adducts cannot be distinguished solely on the basis of their mass voltammograms. Therefore, the experiments were repeated for

FIG. 7. Extracted ion traces of m/z 344.94, correlating with the dihydroxylated TCC metabolites 3',6-diOH TCC (peak 6) and 2',6-diOH TCC (peak 8), found in the RLM incubation mixture of 2', 3'- and 6-OH TCC and HLM incubation mixture of 6-OH-TCC. For RLM, 2'-OH TCC and 3'-OH TCC, fragment ions (m/z 183.98 and 201.95) are shown. Fragment ions of peak X correlate with the incorporation of two hydroxyl groups in the monochloroaniline ring of TCC. *, peaks resulting from the RLM matrix.

2'-OH TCC and 6-OH TCC using the EC/LC/ESI-MS online system (EC potential 1500 mV versus Pd/H₂, GSH to the effluent of EC) (Fig. 2a), and the LC/ESI-MS chromatograms were compared with the RLM and HLM incubation mixtures of 2'-OH TCC and 6-OH TCC with GSH (supplemental data). In the EC/LC/ESI-MS chromatograms of 2'-OH TCC and 6-OH TCC with GSH, a peak with m/z of 634.02 appears, which elutes at 5.2 min in both chromatograms. In the case of the 2'-OH TCC GSH adduct, the fragments m/z 473.05 (X1) and 447.07 (X2) were detected, indicating a hydroxyl group and a GSH moiety in the monochloroaniline ring of TCC. In the case of 6-OH TCC, no fragments in significant intensities could be used for identification. Even though both adducts (2'-OH TCC GSH and 6-OH TCC GSH) show similar retention times, it is very likely that they are isomeric and coeluting but not identical compounds. The same GSH adduct eluting at 5.2 min was detected in the RLM incubation mixture of 6-OH TCC and GSH (data not shown). Besides this GSH adduct, no further adducts in the HLM incubation mixture of 6-OH TCC and

FIG. 8. Investigation of an incubation of 2'-OH TCC with HLM for the formation of quinone imine species. In the extracted ion trace of m/z 326.95, the same dehydrogenation product (peak 4) as found after EC oxidation of TCC was detected (Fig. 3). *, peak resulting from a dehydrogenation reaction of m/z 328.96 (Fig. 5) in the ESI interface.

the RLM/HLM incubation mixtures of 2'-OH TCC have been found, even though the reactive quinone imine itself has been detected for 2'-OH TCC (Fig. 4). These results can be interpreted in different ways: either reactive metabolites are not formed in significant amounts or they are not detectable because of their direct binding to cellular macromolecules. As expected, 3'-OH TCC adduct formation with GSH was not observed in the electrochemical studies or in the microsomal incubations, because the required *meta*-quinone imine intermediate structure does not exist.

To investigate potential adduct formation between quinone imine metabolites of TCC and proteins, offline EC experiments have been performed. TCC, 2'-OH TCC, 3'-OH TCC, and 6-OH TCC were oxidized in the electrochemical cell at 1500 mV (optimum potential for GSH adduct formation) (Fig. 9). The effluent of the cell was directly mixed with a solution of β -LGA. β -LGA was selected as a model protein because of its structural homogeneity, its relatively low molecular mass (162 amino acids, 18.4 kDa), and a single free cysteine thiol group, thus allowing an easier elucidation of its modification after reaction with reactive metabolites. The protein mixtures have been analyzed by LC/ESI-MS. Figure 10 shows the charge distribution and the deconvoluted mass spectra of the peak of unmodified β -LGA (Fig. 10a), β -LGA after reaction with oxidized TCC (Fig. 10b), and oxidized 2'-OH TCC (Fig. 10c). In the case of TCC, the deconvoluted spectrum reveals a mass gain of 292.9 Da, which correlates to adduct formation between β -LGA and metabolite 1 (dechlorinated hydroxylated dehydrogenated TCC species) (Fig. 6). The reaction between oxidized 2'-OH TCC and β -LGA yields the formation of a protein adduct with a mass gain of 328.9 Da relative to unmodified β -LGA. The deconvoluted mass (18,693.4 Da) corresponds well to the theoretical value of a covalent 2'-OH TCC- β -LGA adduct of 18,692.7 Da. The same adducts were detected for 6-OH TCC and, as expected from the GSH studies, no adduct was detected for 3'-OH TCC and β -LGA.

Discussion

The first step in this novel TCC metabolism study comprises the electrochemical simulation of the oxidative transformation reactions. Regarding these preliminary data, it is important to note that EC-based studies simply reveal the most oxidation-prone sites in a molecule. No steric influences by protein-substrate interactions are accounted for. Hence, the oxidation products formed do not perfectly predict actual phase I metabolites from a biological system. However, the electrochemical metabolism simulation of TCC demonstrates, as has by now been observed in a number of studies (Johansson et al., 2007; Lohmann and Karst, 2008; Baumann and Karst, 2010), that the EC-based

FIG. 9. Mass voltammogram of 2'-OH TCC and GSH generated in the EC/ESI-MS set-up (Fig. 2). At an oxidation potential of 1500 mV, a maximum of GSH adduct formation is observed.

approach has the potential to simulate the majority of oxidative metabolism reactions. When EC-based studies are compared with in vivo and in vitro results, very good agreement can be found in many cases (Johansson et al., 2007; Lohmann and Karst, 2008; Baumann and Karst, 2010). For TCC, the three major metabolites, 2'-OH TCC, 3'-OH TCC, and 6-OH TCC, which are known biotransformation products in the human organism (Birch et al., 1978; Hiles and Birch,

1978a), are successfully predicted by EC (Fig. 3). Moreover, the rate of their formation is in line with in vivo studies, because 2'-OH TCC is predominantly formed, followed by 6-OH TCC and 3'-OH TCC, which only forms in low quantities. The dihydroxylated metabolites 2',6-diOH TCC and 3',6-diOH TCC, which have been identified in in vivo studies with rats (Jeffcoat et al., 1977; Hiles and Birch, 1978b; Hiles et al., 1978; Warren et al., 1978) are also found in the LC/

FIG. 10. Mass spectra and deconvoluted mass spectra of unmodified β -LGA (a), β -LGA after reaction with oxidized TCC (b), and β -LGA after reaction with oxidized 2'-OH TCC (c). The shifted deconvoluted masses of b and c in comparison with a indicate protein adduct formation between β -LGA and TCC metabolites.

ESI-MS chromatogram of EC-oxidized TCC (Fig. 3). Besides these well known metabolites of TCC, dechlorinated (peaks 1 and 2) and dehydrogenated species (peaks 3, 4, and 7) (Fig. 3; Table 1) have been identified for the first time upon electrochemical oxidation. Besides the successful simulation of all known oxidative metabolites of TCC, it is important to note that no false biotransformation reactions were predicted by EC. Even while using a high potential of 2500 mV, no *N*-oxidation or cleavage of the urea moiety was observed, which is consistent with results obtained in vivo (Hiles and Birch, 1978; Warren et al., 1978).

With use of knowledge about the chemical identity of the potential metabolites gained from the EC investigation, conventional in vitro studies using RLMs and HLMs have been conducted. The observed conversion rates of TCC in these microsomal incubations were less than 2%, which confirm previously described conversion rates by Warren (1976). In contrast, in in vivo studies in rats, monkeys, and humans, the hydroxylated metabolites are present at higher concentrations than TCC itself in blood and bile (Jeffcoat et al., 1977; Birch et al., 1978; Hiles and Birch 1978a). A possible reason for the low conversion rate in vitro is assumed to be the low water solubility of TCC. Another reason is a possible P450 inhibition by TCC or its metabolites, as discussed by Condie and Buhler (1979). However, recent findings from our group, which show no significant CYP1A2, CYP2C6, and CYP2C9 inhibition by TCC, are contradictory to this assumption (Morisseau et al., 2009). Regardless of the underlying molecular mechanism, the low conversion rate renders the detection and characterization of unknown metabolites formed in vitro a difficult task. Because the EC investigations provided crucial information about potential metabolites, including LC retention time, molecular formulas, and fragment ions, the microsomal samples could be sensitively and specifically screened by LC/ESI-MS for metabolites.

As expected, RLM and HLM incubations led to the major hydroxylated metabolites 2'-OH TCC, 3'-OH TCC, and 6-OH TCC (Fig. 5), but dihydroxylated TCC derivatives could only be observed after further incubation of the hydroxylated metabolites of TCC (Fig. 7). In these experiments, the metabolites 2',6-diOH TCC and 3',6-diOH TCC were found in the RLM incubation mixture, plus one previously unknown dihydroxylated species, bearing two hydroxyl groups in the monochloroaniline ring of TCC. Even though the dihydroxylated metabolites have not been reported as human biotransformation products (Birch et al., 1978), they occur in the HLM incubation mixture of 6-OH TCC.

The dechlorinated, hydroxylated TCC oxidation product (peak 2) (Fig. 3; Table 1), predicted by EC, was also unambiguously identified in both the HLM and RLM incubation mixtures of TCC. Regarding the electrochemical formation of dechlorinated species, a radical driven mechanism can be assumed. On the surface of boron-doped diamond electrodes, OH radicals are formed at a potential of 2500 mV (Kraft, 2007). These electrophilic species are known to interact with π electrons of chlorinated aromatic compounds (Vrtacnik and Voda, 2003), yielding different hydroxylated and dechlorinated oxidation products (Liu et al., 2009). In vivo, dechlorination reactions have been described for several polychlorinated hydrocarbons, such as the polychlorinated biphenyls (Schnellmann et al., 1984; Ariyoshi et al., 1997; Haraguchi et al., 2005). The mechanism presumably proceeds via an epoxidation of TCC by P450 TCC or 3',4'-epoxy TCC, followed by a dechlorination to 3,4-dichloro 4'-hydroxycarbanilide, as discussed by Ariyoshi et al. (1997) for the metabolism of polychlorinated biphenyl 153. By a rearrangement (the so-called NIH shift) of the epoxide intermediate and subsequent dechlorination, 3,4-dichloro-3'-hydroxycarbanilide could also be formed as described for the oxidative metabolism of similar chlorinated compounds (Ariyoshi et al.,

1997; Koerts et al., 1998). However, based on the fragment ions provided in Table 1, the exact position of the hydroxyl group remains unknown. It can only be confirmed that the dechlorination and hydroxylation take place in the monochlorinated and not the dichlorinated aromatic ring of TCC.

Further metabolites, predicted by EC, are the dehydrogenation products (peaks 1, 3, 4, and 7). Only one of these metabolites, the quinone imine of 2'-OH TCC (peak 4) has been detected as a minor product in the HLM incubation mixture of 2'-OH TCC (Fig. 8). This finding can be interpreted in different ways: either the dehydrogenated metabolites are not formed in significant amounts in vitro by hepatic P450 or they are not detectable because they are reactive metabolites and undergo subsequent reactions with biological macromolecules. The latter assumption is supported by the occurrence of dihydroxylated TCC metabolites, which proves that TCC can undergo two subsequent P450-catalyzed oxidation reactions.

To obtain a deeper insight into the potentially reactive metabolites of TCC (peaks 1, 3, 4, and 7), their reactivity toward GSH and the model protein β -LGA was studied. Again, EC was used as a valuable tool to selectively generate these dehydrogenated metabolites. The results shown in Figs. 9 and 10 explain why these metabolites have rarely been found in microsomal studies. The dehydrogenated 2'-OH TCC and 6-OH TCC metabolites rapidly bind to GSH and β -LGA. For β -LGA, no unmodified β -LGA is present after the reaction with the oxidation products. Moreover, the reaction between oxidized TCC and β -LGA reveals that the quinone imine of the dechlorinated hydroxylated TCC metabolite (1) forms an adduct with β -LGA. The deconvoluted mass spectra in Fig. 10a (adduct formation between oxidized TCC and β -LGA) indicates that further protein adducts of TCC might have been formed. However, those could not be identified within this study.

These findings clearly prove for the first time that the oxidative metabolism of TCC may lead to quinone imine species, which are reactive toward cellular nucleophiles. Even though these metabolites have not been reported within the extensive investigation of the fate of this compound in vivo, these findings are not contradictory to previous results. Several studies suggest the formation of adducts, which could result from these reactive metabolites. Warren and colleagues (Warren, 1976; Warren et al., 1978) reported that during the analysis of feces and urine from rats as well as during analysis of microsomal incubation mixtures, considerable radioactivity was found at the origin of a thin-layer chromatography plate. They assumed that these are highly polar materials, which are not cleaved by β -glucuronidase or arylsulfatase, and, therefore, might be glutathione or mercapturic acid conjugates of reactive metabolites. Similar observations were reported by Jeffcoat et al. (1977), who detected polar unidentified metabolites in rat bile and Birch and colleagues (Birch et al., 1978; Hiles and Birch 1978a), who detected unknown polar metabolites in monkey bile. Furthermore, Hiles and Birch (1978b) published data indicating strong protein binding of a TCC metabolite.

In conclusion, on the basis of the combination of the purely instrumental EC technique and in vitro studies with liver microsomes, novel reactive metabolites of TCC have been identified. A dechlorinated hydroxylated TCC metabolite has been discovered, as well as reactive quinone imine species, which tend to bind to proteins and glutathione. These findings may explain the previously reported observation of unidentified polar TCC metabolites and protein binding in vivo. Thus, it is likely that these metabolites are relevant biotransformation intermediates in vivo. However, neither microsomal studies nor the data obtained by the EC-based metabolism simulation can completely depict processes taking place in vivo. The fact that reactive metabolites of TCC have been identified and have also been shown to undergo adduct formation does not consequently imply that they

cause toxicity (Evans et al., 2004) although it has to be considered that with depletion of GSH, intermediately formed reactive quinone imines are no longer effectively trapped and excreted from the organism and can potentially cause toxic effects, e.g., by adduct formation with proteins (Evans et al., 2004; Zhou et al., 2005).

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