Novel Metabolites of Amodiaquine Formed by CYP1A1 and CYP1B1: Structure Elucidation Using Electrochemistry, Mass Spectrometry, and NMR

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ABSTRACT:

An aldehyde metabolite of amodiaquine and desethylamodiaquine has been identified. The aldehyde was the major metabolite formed in incubations with two recombinantly expressed human cytochromes P450 (rP450s), namely, CYP1A1 and CYP1B1. The aldehyde metabolite was also formed, to a lesser extent, in both human and rat liver microsomes. When comparing results from incubations with liver microsomes from 3-methylcholanthrene-treated rats (inducing CYP1A1 and CYP1B1) with those from non-induced rats, a 6-fold increase of the aldehyde metabolite was observed in the rat liver microsomes after 3-methylcholanthrene treatment. The metabolic oxidation was mimicked by the electrochemical system, and the electrochemical oxidation product was matched with the metabolite from the in vitro incubations. The electrochemical generation of the aldehyde metabolite was repeated on a preparative scale, and the proposed structure was confirmed by NMR. Trapping of the aldehyde metabolite was done with methoxyl amine. Trapping experiments with N-acetyl cysteine revealed that the aldehyde was further oxidized to an aldehyde quinoneimine species, both in the rP450 incubations and in the electrochemical system. Three additional new metabolites of amodiaquine and desethylamodiaquine were formed via rCYP1A1 and rCYP1B1. Trace amounts of these metabolites were also observed in incubations with liver microsomes from 3-methylcholanthrene-treated rats. Tentative structures of the metabolites and adducts were assigned based on liquid chromatography/tandem mass spectrometry in combination with accurate mass measurements.

Amodiaquine \(1\) was discovered in the 1950s as an effective antimalarial drug (Mein, 1951). It was withdrawn from clinical use in the 1970s because of its toxicity. However, the drug is gaining widespread use in Africa and Asia where chloroquine resistance is on the increase (Pussard et al., 1987). Because of severe side effects including hepatitis and agranulocytosis observed during prophylactic use of the drug, \(1\) is now only used for the treatment of the acute phase of malaria, during which no serious cases of toxicity have been reported (Laurent et al., 1993). It is administered in combination with other antimalarial agents such as artesunate in the artemisinin-based combination therapy currently recommended by the World Health Organization (Durrani et al., 2005).

Earlier studies have identified a quinoneimine metabolite as the likely cause of amodiaquine toxicity through various actions. The biochemical basis of the toxicity of \(1\) has been explored in depth by the group of Park as possibly being the result of the reactivity of the quinoneimine group (Maggs et al., 1988; Christie et al., 1989; Clarke et al., 1990, 1991; Harrison et al., 1992; Jewell et al., 1995; Naisbitt et al., 1998).

\(1\) is rapidly absorbed and extensively metabolized after oral administration to humans, resulting in low plasma exposure of the parent drug (Laurent et al., 1993). Desethylamodiaquine \(2\) is the main metabolite of \(1\), and other reported metabolites are bisdesethylamodiaquine and 2-hydroxydesethylamodiaquine (Churchill et al., 1985; Mount et al., 1986). \(1\) is rapidly converted to \(2\), whereas the elimination of \(2\) is very slow with a long terminal half-life (Pussard et al., 1987; Laurent et al., 1993). Both parent and metabolite have antimalarial activity, making the parent \(1\) act like a prodrug to the metabolite \(2\) (Churchill et al., 1985). In studies to identify the enzymes responsible for the metabolism of \(1\) to \(2\), it was found that CYP2C8 was the main enzyme responsible for this reaction (Li et al., 2002, 2003).

On screening a panel of 13 human recombinantly expressed cytochromes P450 (rP450s), in addition to the finding that CYP2C8 was mainly responsible for the elimination of \(1\) and the formation of \(2\), it was also found that CYP1A1 and CYP1B1 mediated the formation of an unidentified metabolite, M2. This metabolite was not observed in incubations with human liver microsomes (HLMs) (Li et al., 2002, 2003). This may be explained by the fact that CYP1A1 and CYP1B1 mainly are found in extrahepatic tissues. However, these two enzymes are expressed in low levels in human liver (Hakkola et al., 1997;
Stiborová et al., 2002, 2005), and at least CYP1A1 is expressed at low levels in rat liver (Harrigan et al., 2006; Stiborová et al., 2006). Previous studies have shown that 3-methylcholanthrene induces CYP1A1 and CYP1B1 in rat liver (Caron et al., 2005) and also CYP1A1 in human hepatocytes (Bowen et al., 2000). CYP1A1 and CYP1B1 are involved in the bioactivation of many procarcinogens (Shimada et al., 2001) and induced by a series of polyaromatic hydrocarbons (Pushparajah et al., 2008). Thus, it is of interest to establish the structure of M2 and assess its potential reactivity.

The electrochemistry (EC)/electrospray ionization/mass spectrometry (ESI/MS) technique combines EC online with ESI/MS (Zhou and Van Berkel, 1995). EC has been shown to be a useful means to mimic oxidative drug metabolism, including oxidation to reactive metabolites (Johansson et al., 2007; Lohmann and Karst, 2007; Madsen et al., 2007; Jurva et al., 2008). A previous publication presents a thorough study on which phase I drug metabolism reactions the EC system is able to mimic, including discussion on advantages and disadvantages of the technique (Johansson et al., 2007). In a study to show the utility of EC oxidation for generating electrophilic drug metabolites for characterization, 1 was investigated. In addition to the formation of the quinonemine species of 1, another electrochemical oxidation product of 1 was also generated for which an aldehyde structure was proposed (Jurva et al., 2008). The molecular mass of this oxidation product turned out to be the same as that of the unidentified metabolite M2, which was discovered in the previous metabolism study with CYP1A1 and CYP1B1 (Li et al., 2002; Jurva et al., 2008). Another research group has also proposed an aldehyde structure for an electrochemical oxidation product of 1 (Lohmann and Karst, 2007).

The aim of this present study was to identify and chemically characterize M2 (in this study called 3) using in vitro systems and electrochemical oxidation techniques. Structures 1 through 3 are presented in Fig. 1.

**Materials and Methods**

**Chemicals.** The following chemicals were obtained commercially: amodiaquine (I) dihydrochloride dihydrate (Sigma-Aldrich, St. Louis, MO); 7-ethoxyresorufin (Invitrogen, Carlsbad, CA); methoxylamine hydrochloride, 98% (Sigma-Aldrich); β-NADPH, reduced form tetrasodium salt, 98% (Sigma-Aldrich); leucine enkephalin acetate hydrate, 97% (Sigma-Aldrich); N-α-acetyl-l-lysine (Sigma-Aldrich); N-acetyl-l-cysteine, >99% (Sigma-Aldrich); deuterated acetaminol, 99.96% D (Armour Chemicals, Döttingen, Switzerland); desethylamodiaquine (2) dihydrochloride, 98.25% (LGC Standards, Teddington, Middlesex, UK); methanol (Rathburn Chemicals, Walkerburn, Scotland); formic acid, 98 to 100% (Merck, Darmstadt, Germany); and acetonitrile (Thermo Fisher Scientific, Waltham, MA). All the solvents were of analytical grade, and the water used in the experiments was obtained from a water purification system (Elgastat Maxima; ELGA, Lane End, UK).

**Incubations with rP450s.** The source of recombinant enzymes was bacterial membranes containing human cytochrome P450 (P450; CYP1A1, CYP1B1, and CYP2B1) coexpressed with NADPH-P450 reductase purchased from Cypex Ltd. (Dundee, Scotland, UK). The incubation mixture, containing 100 pmol/ml of enzyme and 5 μM amodiaquine 1 or desethylamodiaquine 2 in 0.1 M phosphate buffer, pH 7.4, was preincubated for 5 min at 37°C. Methoxylamine (dissolved in water) was added to a final concentration of 5 mM to trap the aldehyde metabolite 3, and N-acetyl cysteine (dissolved in water) was added to a final concentration of 5 mM to trapping the quinonemines. The reaction was initiated by adding NADPH to a final concentration of 1 mM, and the reaction mixtures (final volume 150 μl) were incubated for 30 min at 37°C.

Control incubations were conducted in the absence of NADPH and in the absence of substrate. The reactions were quenched by adding 150 μl of ice-cold acetonitrile. The samples were vortexed for 10 s and then centrifuged for 10 min at +4°C, 4000 rpm (~2750g). All the incubations were performed in duplicate. The supernatants were stored at −18°C until analysis.

**Incubations with Liver Microsomes.** HLMs were purchased from BD Biosciences (San Jose, CA). Rat liver microsomes (noninduced and from 3-methylcholanthrene-treated rats) were from male Sprague-Dawley rats and were purchased from Celcius In Vitro Technologies (Baltimore, MD). 3-Methylcholanthrene induces CYP1A1 and CYP1B1 in rats (Caron et al., 2005). The incubation mixtures, containing 1.0 mg/ml liver microsomal protein and 10 μM 1 in 0.1 M phosphate buffer, pH 7.4, were preincubated for 5 min at 37°C. The reaction was initiated by adding NADPH to a final concentration of 1 mM, and the reaction mixtures (final volume 150 μl) were incubated for 60 min at 37°C. Control incubations were conducted in the absence of NADPH and in the absence of substrate. The reactions were quenched by adding 150 μl of ice-cold acetonitrile. The samples were vortexed for 10 s and then centrifuged for 10 min at +4°C, 4000 rpm (~2750g). The supernatants were stored at −18°C until analysis.

7-Ethoxyresorufin was included as a control substrate to confirm CYP1A1 activity and to get a measurement of ethoxyresorufin O-deethylation activity (Burke et al., 1985). The ethoxyresorufin O-deethylation activity was approximately 2 times higher in the rat liver microsomes (RLMs) from 3-methylcholanthrene-treated rats than in the noninduced RLMs.

**The EC/ESI/MS System.** The EC/ESI/MS system was set up as previously reported (Johansson et al., 2007). In short, samples were infused through an ESA Coulom 5011 analytical cell (ESA Inc., Bedford, MA) via a syringe pump at a flow rate of 5 μl/min. A make-up flow, consisting of 50% methanol and 50% 10 mM aqueous formic acid, was added before the electrochemical cell at a flow rate of 50 μl/min. The electrochemical cell was controlled by an ESA Coulom II potentiosat (ESA Inc.). The ESA working electrode was porous graphite, and all the reported cell potentials were measured versus a palladium reference electrode. The outlet from the ESA cell was connected to a Sciex API 4000 QTRap mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray interface. Full-scan spectra were acquired continuously.

**General Electrochemical Reaction Conditions.** The samples were infused through the electrochemical cell at a desired, fixed potential and were collected in glass vials. For electrochemical oxidation followed by LC/MS analysis, the substrate concentration in the syringe was 10 μM. For electrochemical synthesis for characterization by NMR, the concentration in the syringe was 1 mM. Amodiaquine 1 and desethylamodiaquine 2 were dissolved in methanol.

**Electrochemical Synthesis for Characterization by LC/MS.** The amodiaquine aldehyde 3 was generated by infusion of amodiaquine 1 or desethylamodiaquine 2 (10 μM) through the electrochemical cell at a total flow of 55 μl/min (5 μl/min from the syringe and 50 μl/min from the make-up flow). The potential of the first cell (E1) was maintained at +1000 mV, and the potential of the second cell (E2) was maintained at −100 mV. In the first cell, 1 or 2 was oxidized to the aldehyde but also to the corresponding quinonemine. To enhance the yields of the desired aldehyde product 3, the second cell was set
to reduce the quinoneimine byproducts back to aminophenols. The samples were collected for 10 min and were stored at −18°C until analysis.

Methoxyl amine adducts were generated by infusion of 1 or 2 (10 µM) through the electrochemical cell at a flow of 55 µl/min for 5 min with the potential of E1 maintained at +1000 mV and the potential of E2 at −100 mV. The oxidized sample was collected in a vial containing methoxyl amine (dissolved in water) to give a final concentration of 5 mM. To trap the quinoneimines, 1 or 2 (10 µM) was infused through the electrochemical cell at a flow of 55 µl/min for 5 min with the potential of E1 and E2 maintained at +1000 mV. The oxidized sample was collected in a vial containing N-acetyl cysteine (dissolved in water) to give a final concentration of 5 mM. After collection, the samples from the trapping experiments were kept at 25°C for 1 h before analysis by LC/MS.

Electrochemical Synthesis for Characterization by NMR. For electrochemical synthesis of amodiaquine aldelyde 3, amodiaquine 1 (1 mM) was infused through the electrochemical cell at a total flow of 11 µl/min (1 µl/min from the syringe and 10 µl/min from the make-up flow) for approximately 65 h with the potential of E1 maintained at +1500 mV and the potential of E2 at −200 mV. The oxidized sample was collected in an ice-cooled conical glass flask. The sample was kept on ice, and the methanol was removed under a stream of nitrogen gas. The remaining water was then removed by lyophilization, and the dry residue was kept at −18°C until analysis by NMR.

LC/MS/Solid-Phase Extraction/NMR. The sample derived from the electrochemical synthesis was dissolved in approximately 500 µl of acetonitrile/water (50:50). Separation was performed using an Agilent Technologies (Palo Alto, CA) 1100 series high-performance liquid chromatography system and an Agilent Zorbax (150 × 4.6 mm, 5 µm) column at a flow rate of 1 ml/min. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. At the start of the gradient, the acetonitrile content was 10% and was linearly increased to 55% over a period of 10 min. The acetonitrile content was then increased to 100% within 2 min. The initial mobile phase composition was then restored within 0.01 min. This condition was held for 0.69 min, and finally the initial mobile phase composition was restored within 0.01 min. The UPLC system was equipped with a Waters ACQUITY UPLC BEH (C18 2.1 × 50 mm, 1.7 µm) column, at a flow rate of 750 µl/min (the column temperature was set at 40°C). Mobile phase A consisted of 5% acetonitrile and 0.1% formic acid in water, and mobile phase B was 100% acetonitrile. At the start of the gradient, the acetonitrile content was 1% and was linearly increased to 50% over a period of 5 min. The acetonitrile content was then increased to 90% within 0.01 min. This condition was held for 0.69 min, and finally the initial mobile phase composition was restored within 0.01 min. The UPLC system was coupled to a Waters Q-TOF Premier instrument equipped with an ESI source. Leucine-enkephalin was used as the lock mass (m/z; 556.2771) for accurate mass calibration and introduced using the Lock Spray interface at 20 µl/min (500 pg/µl). All of the samples were diluted 1:2 in mobile phase A, and aliquots of 5 µl were injected onto the LC/MS system. Full-scan spectra were acquired in the positive ionization mode. For acquisition of tandem mass spectrometry spectra, the injection volume was 20 µl, and a collision energy ramp from 0 to 50 eV was used. The software used to process the data included Mass Lynx (version 4.1) and MetaboLynx (Waters). The mass-to-charge ratios of all the relevant ions in the MS mode were determined within 5 ppm from the exact mass of the proposed structure. Separate product ion spectrum was acquired for the 35Cl and the 37Cl isotopologs of the aldehyde metabolite 3 to establish whether fragment ions retained the chlorine atom.

Results

In the work by Li et al. (2002), an unidentified metabolite of amodiaquine (M2) with a pseudomolecular ion (MH+ at m/z 299) was described. This metabolite was formed via the recombinant enzymes CYP1A1 and CYP1B1 but was not observed in HLMs. In the present study, the electrochemical experiments with amodiaquine and desethylamodiaquine displayed an oxidation product (oxidation of 1 at +1000 mV followed by reduction at −100 mV) that gave a pseudomolecular ion at m/z 299 and eluted at 1.76 min. For comparison of the electrochemically generated oxidation product with the earlier described unidentified metabolite M2, incubations with rCYP1A1 and rCYP1B1 were performed. The other rP450s used in the study by Li et al. (2002) were not included in this present study because metabolite M2 was not formed via these rP450s. From the corresponding retention times, accurate mass, and product ion spectra, the oxidation product from the electrochemical system was shown to be identical with the major metabolite formed by the rP450s. This metabolite was the same as the unidentified metabolite M2 from the work by Li et al. (2002) because it had a pseudomolecular ion at m/z 299, a long retention time compared with 1, and was the major metabolite formed via rCYP1A1 and rCYP1B1. From now on this metabolite is referred to as metabolite 3. Incubations were also conducted with another rP450, rCYP2J2. In humans, CYP2J2 is mostly found in cardiovascular tissues but is also expressed in human monocytic leukocytes (Nakayama et al., 2008). Metabolite 3 was not formed in these incubations. Metabolite 3 was not seen in the control incubations without NADPH, which suggests an NADPH-dependent oxidation of amodiaquine to metabolite 3. A high-energy product ion spectrum of the 35Cl isotopolog of 3 is shown in Fig. 2. Proposed fragmentation pathways are available in the supplemental data.

The accurate mass and product ion spectrum indicate that the structure of 3 corresponds to that of an aldehyde. To test this hypothesis, trapping experiments were performed with N-acetyl lysine and methoxyl amine. Trapping with N-acetyl lysine did not give any adducts, which may be explained by the protonation of N-acetyl lysine making it less suitable as a trapping agent at pH 7.4. When methoxyl amine was added to the electrochemical oxidation mixtures and the enzyme incubation mixtures, the pseudomolecular ion at m/z 299 completely disappeared, whereas a new pseudomolecular ion at m/z 328 appeared at a retention time shifted approximately 0.8 min in the chromatograms. This pseudomolecular ion corresponds to an addition of 29 Da to metabolite 3, implying that addition of methoxyl amine to the aldehyde had taken place. The product ion spectrum shows an abundant fragment at m/z 296, which corresponds to a loss of methanol (32 Da) from m/z 328. The product ion spectrum of this methoxyl amine adduct 5 is available in the supplemental data. The methoxyl amine adduct was formed in the incubations with rCYP1A1 and rCYP1B1 but not in the incubations with rCYP2J2. This is consistent with the fact that 3 was only formed in the incubations with rCYP1A1 and rCYP1B1. The methoxyl amine adduct was also formed when methoxyl amine was added to the electrochemically oxidized samples, whereas no adducts were formed when methoxyl amine was added to untreated samples. When the same trapping experiments were repeated with desethylamodiaquine, the same methoxyl amine adduct was formed as in the experiment with amodiaquine. Noteworthy is that desethylamodiaquine and the methoxyl amine adduct of 3 both gave pseudomolecular ions at m/z 328, but they were easily distinguished based on their accurate mass and retention times.
The electrochemical oxidation of 1 to 3 was performed on a larger scale to synthesize enough material for characterization by NMR. LC/MS/SPE/NMR was performed, and the isolated aldehyde 3 was dissolved in deuterated acetonitrile. The sample showed a pure compound with 11 signals in the aromatic region, including an OH and an NH signal. One-dimensional $^1$H NMR, one-dimensional nuclear Overhauser effect (NOE) experiments, as well as correlation spectroscopy and $^1$H-$^1$C correlated heteronuclear single quantum coherence experiments were performed, and chemical shifts were compared with a reference sample of 1 obtained by trapping on the LC/MS/SPE system using the same procedure as described above. From the NMR information, it was evident that 3 lacks signals from the ethyl group, and the CH$_2$ group connected to the phenol group in 1. The $^1$H and $^1$C chemical shifts and coupling pattern from the chloroquinolin group were almost identical in 1 and 3, indicating that this part of the molecule is unmodified in 3. A singlet in the proton spectrum at 9.97 ppm directly bonded to a carbon at 197.0 ppm was a clear indication of an aldehyde group. An NOE was observed between this singlet and a doublet at 7.71 ppm. This doublet showed a small coupling typical for aromatic meta coupling and was assigned to CH(10) according to the numbering in Fig. 3. Thus, comparison of chemical shifts between 3 and 1 and NOE information confirm that the structure of 3 is in accordance with the proposed aldehyde. The $^1$H NMR spectrum of 3 along with the structure is shown in Fig. 3. Additional NMR spectra and chemical shifts for 3 and 1 are available in the supplemental data.

It has been previously shown that the quinoneimine metabolite of 1 can be trapped by glutathione or cysteine (Jewell et al., 1995; Lohmann and Karst, 2007; Jurva et al., 2008). Incubations and electrochemical experiments were performed to elucidate whether quinoneimine species were formed in the same systems as the aldehyde metabolite. For these experiments, N-acetyl cysteine was used as the trapping agent. In the incubations of 1, together with N-acetyl cysteine in rCYP1A1 and rCYP1B1, two new compounds with pseudomolecular ions at m/z 460 appeared in the chromatograms. The fact that these two compounds did not form in the incubations with rCYP2J2 indicates that the new compounds originate from the aldehyde species 3. Successful trapping with N-acetyl cysteine implies that the intermediate may be of quinoneimine nature. The proposed structures of the adducts (6 and 7), with numbering in the aromatic ring of interest, are shown in Fig. 4. The two chromatographically well separated compounds with the same accurate mass suggest conjugation with N-acetyl cysteine in two different positions of the aromatic ring of the aminophenol moiety. Because of a higher steric hindrance in position 2 of the aromatic ring, N-acetyl cysteine conjugation at positions 5 and 6 seems to be more probable. The product ion spectra were dominated by a loss of 129 Da, resulting from a cleavage of the thioether on the N-acetyl cysteine. The product ion spectra of 6 and 7, together with proposed mechanism for the loss of 129 Da, are shown in the supplemental data. The experiments were repeated with 2, and the same adducts were formed as in the experiments with 1. The same adducts were also formed with the electrochemical system. The observed metabolism mediated by CYP1A1 and CYP1B1, involving the aldehyde moiety, is shown in Fig. 4. The adducts formed in the trapping experiments are also included in the same figure.

In addition to the N-acetyl cysteine adducts of the quinoneimine species of the aldehyde 3, the quinoneimines of 1 and 2 were also trapped with N-acetyl cysteine, giving the expected adducts with pseudomolecular ions at m/z 517 and 489, respectively. These two adducts were observed in rCYP1A1, rCYP1B1, and rCYP2J2 and have not been previously shown in rP450s. Both the product ion spectra revealed a loss of 129 Da, the same as observed for the N-acetyl cysteine adducts above. In addition, a loss of diethylamine (73 Da) from 517 and a loss of ethylamine (45 Da) from 489 were observed. The product ion spectra of the N-acetyl cysteine adducts of 1 and 2 formed in the rP450s are shown in the supplemental data.

In addition to the aldehyde metabolite 3, three other, to our knowledge, not previously reported metabolites (8-10) of amodiaquine 1 and desethylamodiaquine 2 were discovered in incubations with the rP450s. These metabolites appeared in incubations with rCYP1A1 and rCYP1B1 but were not formed by rCYP2J2. Metabolite 8 gave a
pseudomolecular ion at m/z 301 and eluted at 1.38 min. The product ion spectrum is dominated by a fragment at m/z 283, which corresponds to a loss of water. The proposed structure is a benzylic alcohol as shown in Fig. 5. Metabolite 9 gave a pseudomolecular ion at m/z 315 and eluted at 1.66 min. The product ion spectrum shows a fragment at m/z 285, which corresponds to a loss of formaldehyde (30 Da). This is in agreement with the fragmentation pattern of the aldehyde metabolite 3. Because m/z 315 corresponds to an addition of 16 Da to the aldehyde (m/z 299) and the product ion spectrum indicates a loss of formaldehyde, metabolite 9 was tentatively assigned as the aldehyde with an additional aromatic hydroxylation as shown in Fig. 5. Metabolite 10 gave a pseudomolecular ion at m/z 317 and eluted at 1.21 min. The product ion spectrum of 10 is dominated by a fragment at m/z 299, which corresponds to a loss of water. Thus, metabolite 10 was tentatively assigned as the benzylic alcohol (8) with an additional aromatic hydroxylation. Interpretation of the product ion spectra of metabolites 9 and 10 indicated that the hydroxylation occurred in the quinoline moiety. Proposed structures of metabolites 8 through 10 are available in the supplemental data. Table 1 shows relative quantities of parent (1 or 2, respectively) and metabolites formed by rCYP1A1 and rCYP1B1 after incubation for 30 min as evaluated from the peak areas of the extracted ion chromatograms.

Incubations with 1 in HLMs and RLMs were performed. Low levels of the aldehyde 3 were formed in both HLMs and RLMs. The identification was made by comparing the retention times and the product ion spectra with those from the electrochemical standard and the rP450s. Incubations of 1 with liver microsomes from 3-methylcholanthrene-induced rats and noninduced rats were performed. This was done to elucidate whether larger amounts of 3 were formed in the liver microsomes from 3-methylcholanthrene-induced rats compared with noninduced RLMs because 3-methylcholanthrene is a known inducer of CYP1A1 and CYP1B1. Extracted ion chromatograms of metabolite 3 formed from amodiaquine via rCYP1A1, rCYP1B1, induced RLM, noninduced RLM and HLM compared with the standard generated electrochemically are presented in Fig. 6. A rough estimation (based on duplicate incubations) of the contribution of the aldehyde to the total metabolism gave a 6-fold increase of formed aldehyde in the liver microsomes from 3-methylcholanthrene-induced rats compared with the noninduced RLMs. Trace amounts of the metabolites 8 through 10 were formed in the liver microsomes from 3-methylcholanthrene-induced rats. Trace amounts of metabolite 8 were formed in the noninduced RLMs, but metabolites 9 and 10 were not observed. None of these three metabolites (8-10) was observed in HLMs.

An unknown metabolite with a pseudomolecular ion at m/z 372 appeared in the incubations with RLMs but was not observed in the incubations with HLMs. This metabolite may have been formed via hydroxylation or N-oxidation because the mass corresponds to an addition of 16 Da to amodiaquine. The product ion spectrum, shown in the supplemental data, displays one single fragment at m/z 283. Because m/z 283 results from a loss of the diethylamine from amo-
diaquine, this suggests that the oxidation has taken place on the diethylamine part of the molecule. Based on this and the fact that the retention time of the metabolite was 0.23 min longer than that of amodiaquine, the structure of the metabolite formed in RLMs was tentatively assigned as an N-oxide on the tertiary amine. The different metabolites’ contribution to the total metabolism and percentage
Discussion

This study has shown that the aldehyde metabolite 3 was formed in rCYP1A1, rCYP1B1, HLMs, noninduced RLMs, and RLMs from 3-methylcholanthrene-induced rats, and generated electrochemically. The aldehyde was the major metabolite in rCYP1A1 and rCYP1B1 but was formed only in small amounts in HLMs and RLMs. This may be explained by the fact that CYP1A1 and CYP1B1 are mainly extrahepatic enzymes that are expressed in the liver only to a small extent. Thus, these P450s might have a larger impact on extrahepatic metabolism (e.g., in the leukocytes) than on the metabolism in the liver. CYP1A1 might also play a part in the hepatic metabolism in case of induction because CYP1A1 is a known subject for induction in humans and rats (Bowen et al., 2000; Caron et al., 2005). Human monocytic leukocytes express CYP2J2 (Nakayama et al., 2008), and one severe toxicity of amodiaquine is agranulocytosis. Thus, it was of interest to perform incubations to find out whether the aldehyde is formed by rCYP2J2. Because the aldehyde was not observed in the incubations with rCYP2J2, no further experiments were performed.

In the study by Li et al. (2002) the aldehyde metabolite 3 (in that study referred to as the unidentified metabolite M2) was not observed in incubations with HLMs. In the present study, small amounts of 3 were observed in HLMs, which may be explained by our slightly different experimental conditions, e.g., higher protein concentration, longer incubation time, and a much more sensitive method for analysis compared with the former study.

Previously the group of Park studied the metabolism of 1 in the rat. Three metabolites were described and designated M4, M5, and M6. M6 was assigned to be a carboxylic acid, whereas M4 and M5 were not identified (Jewell et al., 1995). The new metabolites observed in this present study may correspond to one or more of metabolites M4 through M6 proposed in the former study. In addition, the formation of a carboxylic acid metabolite in vivo may further support the in vitro findings of the aldehyde in the present study. Aldehydes are rarely seen in vivo because oxidation to corresponding carboxylic acids is frequent.
Common observation under the acidic chromatographic conditions is the retention times as shown in Table 1. This is a hydroxylation of 5, higher than that of the corresponding parent metabolite. Furthermore, in a previous study of a liver microsome incubations and separate the metabolite of interest from other formed metabolites and the biological matrix. The data estimated from integration of extracted ion chromatograms. Values shown are averaged numbers from duplicate incubations.

<table>
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<tr>
<th>Structure</th>
<th>m/z</th>
<th>Induced RLMS</th>
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<th>HLMs</th>
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<td>Percentage of metabolites after incubation of 1 for 60 min</td>
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</table>

a Trace, trace amounts observed.

Metabolites 9 and 10 are proposed to be formed by aromatic hydroxylation of 3 and 8, respectively, rather than via N-oxygenation. This hypothesis is based on the observation that 9 behaves as being more polar than 3 and 10 as being more polar than 8 based on their chromatographic retention times as shown in Table 1. This is a common observation under the acidic chromatographic conditions used in this study as the pKₐ of the protonated amine is significantly higher than that of the corresponding N-oxide. Thus, the N-oxide would behave chromatographically as being more lipophilic, whereas the hydroxylated metabolite would be expected to behave as more polar than the respective parent metabolite. Furthermore, in a previous study of 1 in humans, the major metabolite besides 2 was tentatively identified as 2-hydroxylaminoleumidine by ¹H NMR (Churchill et al., 1985). This further strengthens the proposal that metabolite 9 and metabolite 10 are formed via aromatic hydroxylation (in the chloroquinoline moiety) of 3 and 8, respectively. The metabolite with a pseudomolecular ion at m/z 372, observed only in the incubations with RLMS, was tentatively assigned as an N-oxide of the tertiary amine moiety of amodiaquine. This is in accordance with the discussion above because the metabolite had a longer retention time, thus behaving as being more lipophilic, than the parent.

As described, all of the experiments (the incubations and the electrochemical experiments) were repeated with 2, and the same metabolites and adducts were formed as when the experiments were performed with 1. This may be important from a therapeutic point of view as desethylamodiaquine is the major metabolite of amodiaquine in vivo and because the half-life of desethylamodiaquine is significantly longer than that of amodiaquine.

Both desethylamodiaquine and the aldehyde are formed via metabolic N-dealkylation. Mechanistically, N-dealkylations may proceed via hydroxylation at the α-carbon to form a carbinolamine that will spontaneously degrade to the aldehyde and amine, or undergo dehydrogenation to an iminium ion. An equilibrium exists between the carbinolamine and the iminium ion, and a general discussion on the N-dealkylation of tertiary amines was published previously (Rose and Castagnoli, 1983).

This study presents the utility of electrochemistry in generating the same oxidation products as those formed by different in vitro systems and, after repeating the oxidation on a large scale, synthesizing enough material for characterization by NMR. Electrochemical generation of metabolites is a facile method, easy to set up, and it usually results in relatively clean product mixtures without many byproducts. An alternative for metabolite generation would be to perform very large liver microsome incubations and separate the metabolite of interest from other formed metabolites and the biological matrix. The disadvantages of this method compared with the electrochemical system are the greater expense, the complex matrix, and that optimizing for a certain metabolite would be difficult. In the present study, the flow through the electrochemical cell was kept low to get a high yield of the aldehyde product. The use of a preparative electrochemical cell would further speed up the product formation because such a preparative cell would be able to handle higher concentrations and thus produce a higher yield of product per time unit.

In conclusion, this study has shown novel metabolic pathways of amodiaquine. Other metabolites were formed via CYP1A1 and CYP1B1 in comparison with those previously reported to be formed in liver microsome incubations. These enzymes, CYP1A1 and CYP1B1, are mainly expressed in extrahepatic tissues, e.g., in human leukocytes rather than in the liver. This may be of interest as one of the severe toxicities of amodiaquine treatment is agranulocytosis. Metabolites of particular interest might be the aldehyde metabolite 3 and the aldehyde quinoneimine 4. Further studies are needed to elucidate whether these metabolites contribute to the amodiaquine toxicity in vivo.

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