Malaria is endemic in most tropical and subtropical regions of the world. It is estimated that 300–500 million people are at risk of contracting malaria, with 900,000 new cases diagnosed each year (Murray and Lopez, 1994; Olliaro et al., 1996). There are 1–2 million deaths reported annually due to severe, cerebral malaria; with the majority of these deaths being children in Africa (Zuker and Campbell, 1993). Qinghaosu (QHS), a unique sesquiterpene lactone endoperoxide derived from the Chinese medicinal herb, Artemisia annua (Klaymann, 1985). Arteether (see fig. 1), the ethyl ether derivative of the reduced lactol of QHS, is currently being developed for use in severe and multidrug-resistant malaria, including cerebral malaria.

Recent studies have established a dose-dependent neurotoxicity in rats and dogs after repeated im administrations of high doses of AE (Brewer et al., 1994a, 1994b). DQHS, known to be more neurotoxic and efficacious than AE in vitro and in vivo (Brewer et al., 1993; Wesche et al., 1994), has been identified as a major metabolite in rat liver microsomes (Leskovac and Theoharides, 1991a). Large scale human studies with related artesinin analogs have not shown any neurotoxic side effects (Hien and White, 1993; Looreeeswan, 1994).

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1 Abbreviations used are: QHS, qinghaosu; DQHS, dihydroqinghaosu; AE, β-arteether; BHT, butylated hydroxytoluene; HPLC, high performance liquid chromatography.

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However, isolated case reports have implicated possible neurological dysfunction in humans after the administration of related artesinin compounds (Miller and Panosian, 1997; Senanayake and de Silva, 1994; van Hensbroek et al., 1996).

Arteether has been shown to be extensively metabolized using various in vitro and in vivo animal models (Chi et al., 1991; Leskovac and Theoharides, 1991a, 1991b). In the isolated perfused rat liver, AE biotransformation pathways include deethylation and hydroxylation followed by glucuronidation (Peggins et al., 1990). In rat liver microsomes, the NADPH-dependent, cytochrome P450-mediated O-deethylation of AE to DQHS has been identified as a major metabolic pathway (Leskovac and Theoharides, 1991a). The positive therapeutic effect of DQHS was determined in humans for a related artesinin analog, artemether. The plasma level of the active metabolite, DQHS, was measured by HPLC analysis, and the plasma antimalarial activity was assessed in vitro by bioassay for the same sample. The study demonstrated that the plasma concentration and the antimalarial activity profile for DQHS was similar, suggesting that other unidentified metabolites contributed little to the antimalarial activity of DQHS in vivo (Teja-Isavadharm, 1996). A related study in humans administered AE shows an identical trend in the DQHS plasma concentration and antimalarial effect profile (D. Kyle, personal communication). The current study was undertaken to determine which human cytochrome P450 isozyme(s) catalyze the conversion of AE to its active metabolite, DQHS.

Because AE is being developed for the treatment of multidrug-resistant malaria, it is not unusual for patients to receive multiple antimalarial drugs prior to the administration of AE. In general, antimalarial drugs have long elimination half-lives (t1/2) that can range from days to several weeks; consequently, clinically significant...
blood levels of other antimalarials will be present during AE administration. As a result, unexpected drug-drug interactions may occur when AE is given in combination with other antimalarials. Many antimalarials have associated cardiotoxicity (White, 1985), which may be magnified in the presence of AE. Therefore, an additional study was performed to elucidate potential drug-drug interactions that may occur when other antimalarials are administered prior to or in combination with AE.

Materials and Methods

Chemicals. Qinghaosu (WR249309), β-arteether (WR255131), dihydroqinghaosu (WR253997), mefloquine hydrochloride (WR142490), chloroquine diphosphate (W001544), and halofantrine hydrochloride (WR171669) were obtained from the Walter Reed Army Institute of Research repository (Washington, DC). Recombinant human CYP450 1A1, 1A2, 2B6, 2C9-Arg, 2C19, 2D6-Val, 2E1, 3A4, 3A5, and 4A11 microsomes were obtained from Gentest Corporation (Woburn, MA). Potassium phosphate monobasic, potassium phosphate dibasic, magnesium chloride hexahydrate, coumarin, diethyldithiocarbamate, which were dissolved in water, all inhibitors were dissolved in DMSO, and 100 mM furafylline, 50 mM SKF-525A, and sulfaphenazole were obtained from Research Biochemicals International (Natick, MA).

Determination of Testosterone 6β-Hydroxylation Activity in Human Liver Microsomes. A 250-μl reaction mixture containing 0.4 mg/ml microsomal protein, NADP" (0.5 mM), glucose-6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (1.0 IU/ml), and MgCl₂ (5 mM). The final incubation volume was 1 ml. The reaction was initiated by the addition of arteether at concentrations ranging from 0 to 320 μM and incubated for 30 min before being terminated by the addition of 5 ml of a 90:10 n-butyl chloride:ethyl acetate solution. The organic layer was transferred to a clean silanized test tube, and the sample was extracted with another 5 ml of 90:10 n-butyl chloride:ethyl acetate solution. The organic layers were combined and evaporated under nitrogen at ambient temperature. The samples were stored at −20°C and reconstituted in 50:50 ethanol:water 16 hr prior to HPLC analysis.

Arteether O-Dealkylation by Isozymes of Human Recombinant Cytochromes P450. For experiments with recombinant human P450 microsomes, 0.5 mg of protein were incubated for 120 min using the same conditions described above for human microsomes. For CYP2A6, -2C9, and -4A11 isoforms, a 0.1 M Tris buffer (pH 7.5) was utilized. Control microsomes isolated from a cell line without cDNA inserts were included with each experiment to account for any metabolism of AE caused by enzymes native to the cell line.

Inhibition Studies of Arteether O-Dealkylation Activity by Selective P450 Inhibitors and Antimalarials. In experiments involving inhibition of DQHS formation by specific P450 inhibitors, incubations were carried out as described above. In these experiments, human liver microsomal protein (0.2 mg) isolated from liver samples HL 007 and 018 was preincubated for 5 min with various inhibitors prior to the addition of AE (100 μM). Furafylline and troleandomycin were preincubated for 15 min with microsomes containing an NADPH-regenerating system prior to initiating the reaction by addition of substrate. After the addition of substrate, all incubations were continued for an additional 30 min. Reactions were terminated by pipetting the sample into a tube containing 5 ml of a 90:10 n-butyl chloride:ethyl acetate solution (v/v).

Final inhibitor concentrations in these studies were 5 μM ketoconazole, 500 μM SKF-525A, 20 μM quinidine or sulfaphenazole, 25 μM furafylline, 50 μM diethyl dithiocarbamate, and 100 μM coumarin or troleandomycin. For inhibition studies involving other antimalarials, inhibitor concentrations ranged from 0 to 400 μM. For the water-soluble antimalarials chloroquine, quinine, and quinidine, concentrations in the range of 0–1500 μM were used. With the exception of quinidine, quinine, chloroquine, and diethyl dithiocarbamate, which were dissolved in water, all inhibitors were dissolved in methanol (final methanol concentration ≤0.8%).

Data Analysis. Data on DQHS formation in human liver microsomes and recombinant P450 isoforms were analyzed by nonlinear regression (Enzyme Kinetics, version 1.1; Trinity Software, Campton, NH) of the substrate concentration vs. velocity data using the Michaelis-Menten equation. The effect of specific P450 inhibitors and antimalarial compounds on the formation of DQHS was evaluated by estimating the IC₅₀ values using the logistical dose response equation in TableCurve 2.0 (Jandel Scientific). The inhibition constant (Kᵢ) for ketoconazole, quinidine, and mefloquine was determined by Dixon analysis.

To calculate the intrinsic clearance of AE in human liver microsomes, Vₘₐₓ scaling of each human liver sample was performed to account for total liver mass. Assuming a 70-kg body weight, the Vₘₐₓ for the liver was calculated using a typical yield of 20 mg of microsomal protein per gram of liver tissue and 21.43 grams of wet weight per kg of body weight. The calculation for Vₘₐₓ
The metabolism of AE to DQHS by human liver microsomes and human recombinant P450 isozymes displayed Michaelis-Menten kinetics. The formation of DQHS in human liver microsomes was linear up to 60 min and 1 mg/ml microsomal protein. Formation of DQHS for a representative human liver microsomal sample (HL 13) is depicted in fig. 2. The calculated values of $K_m$ and $V_{\text{max}}$ for DQHS formation in human liver microsomes are presented in table 1.

To determine the P450 isozyme(s) involved in the biotransformation of AE to DQHS, incubations were conducted using chemical inhibitors specific to various P450 isozymes (fig. 3). Ketoconazole and troleandomycin were found to inhibit DQHS formation to approximately 30 and 35% of control, respectively, implicating CYP3A4 involvement. Ketoconazole was a competitive inhibitor of DQHS formation with a mean $K_i$ of 0.3 ± 0.1 μM and an IC$_{50}$ value of 0.79 ± 0.045 μM. The data in fig. 4 compare the rate of DQHS formation with testosterone 6β-hydroxylase activity in human liver microsomes. Arteether 6-deethylase activity showed a strong correlation ($r^2 = 0.70$) with CYP3A-mediated 6β-hydroxylation of testosterone.

Using recombinant human CYP450 microsomes, AE was incubated with CYP1A1, -1A2, -2A6, -2B6, -2C9, -2C19-Arg, -2D6-Val, -2E1, -3A4, -3A5, and -4A11. Cytochromes 2B6, 3A4, and 3A5 were the only isozymes found to significantly de-ethylate AE to DQHS. The $K_m$ and $V_{\text{max}}$ parameters for CYP2B6, -3A4, and -3A5 are presented in table 2. The $V_{\text{max}}$ of the three isozymes varied by approximately 9-fold, with CYP3A4 having the highest $V_{\text{max}}$ and CYP2B6 the lowest.

Because the potential for combination drug therapy with AE is significant, studies were undertaken to determine possible drug-drug interactions between AE and other commonly used antimalariais. Table 3 contains the mean IC$_{50}$ values of several antimalariais co-incubated with AE (100 μM) in microsomes from human livers 007 and 018. Halofantrine, mefloquine, and quinidine were found to be the most potent inhibitors.

Because mefloquine and quinidine are often used in combination therapy with other artemisinin analogs, the inhibition constants ($K_i$) were determined for both compounds in human livers 007 and 018. Mefloquine and quinidine were competitive inhibitors of AE metabolism to DQHS with a mean $K_i$ of 41 and 111 μM, respectively. Dixon analysis of mefloquine inhibition of DQHS formation in human liver samples 7 and 18 is shown in fig. 5.
Arteether was incubated with microsomes from a human liver bank, and the rate of DQHS formation was correlated to its CYP3A activity evaluated as 6β-hydroxysteroid dehydrogenase activity (Wrighton et al., 1989). Due to the polymorphic expression of CYP3A5, it seems that this isozyme does not substantially contribute to the large interindividual variability seen for arteether O-deethylation in the representative sample of human liver donors used in this study. Although AE was shown to be a substrate for CYP2B6 in vitro using recombinant enzyme, the overall contribution of this isozyme in human liver microsomes based on relative P450 content suggests that this isozyme does not contribute significantly to DQHS formation in vivo.

AE is indicated as second line therapy for severe, complicated malaria in patients that have failed traditional drug treatments. For this reason, a patient receiving AE will most likely have been treated with several antimalarial drugs prior to AE administration. A list of antimalarial drugs that might be utilized in combination therapy is found in table 3. Many of these other antimalarials, such as mefloquine and halofantrine, have very long elimination half-lives, making it likely that significant blood levels will be present when AE is administered (White, 1985). Therefore, we examined the potential for drug-drug interactions between AE and several of these compounds. Halofantrine, the most potent inhibitor based on IC50 values (see table 3). Mefloquine and quinidine were found to be modest, competitive inhibitors of AE O-deethylation in vitro.

Halofantrine, a 9-phenanthrenemethanol antimalarial drug, is largely metabolized by CYP3A4 with significant correlations toward CYP3A4 protein levels and the rate of felodipine metabolism. Inhibition studies demonstrated that ketoconazole is a potent inhibitor of halofantrine N-debutylation, further implicating the role of CYP3A4 in the metabolism of the drug (Halliday et al., 1995).

Mefloquine is a 4-quinolinemethanol analog of quinine that is efficacious against chloroquine-resistant and chloroquine-sensitive strains of Plasmodium falciparum (Zannoni, 1985). Ketoconazole inhibits the formation of 4-carboxymefloquine, a major urinary metabolite of mefloquine, suggesting the involvement of CYP3A4 in vitro (Bangchang, 1992). The anti-arrhythmic drug, quinidine, has been shown to be an effective antimalarial drug, and iv quinidine is the current standard of care for severe, multidrug-resistant malaria. Although quinidine is a potent inhibitor of CYP2D6 (Mikus, 1986), oxidation of the drug is induced by barbiturates and rifampicin (Data et al., 1976; Twum-Barima and Carruthers, 1981), implicating other CYP isozymes. In this regard, Guengerich and co-workers (1986)
demonstrated that quinidine is metabolized to 3-hydroxy and N-oxide products in human liver microsomes by P-450 CYP3A4, now known as CYP3A4A (Nelson et al., 1993).

In humans, the steady state plasma concentrations of AE is approximately 100–200 ng/ml or 0.3–0.6 μM. In the case of AE, the in vivo concentrations are significantly less than the in vitro Kₘ (IS) (< Kₘ). Based on reported literature values for the plasma concentrations of mefloquine (Hellgren et al., 1991) and quinidine (Verme et al., 1992), the anticipated percentage of inhibition for AE O-deethylation in vivo is less than 10%; halofantrine would inhibit approximately 20%. Therefore, it is anticipated that these antimarial drugs, which have been characterized as being substrates of human liver CYP3A4, would not cause significant drug-drug interactions at the metabolic level. This is supported by the observation that clinical trials using artemether, the methyl ether derivative of artemisinin, in combination with mefloquine showed no apparent drug-drug interaction (Shwe et al., 1988). However, these criteria do not exclude more potent CYP3A4 substrates, such as ketoconazole or erythromycin, from inhibiting AE metabolism. Conversely, this study does not address the issue of AE inhibiting the metabolism of other antimarial drugs that may result in cardiovascular toxicity often associated with these types of drugs (White, 1985, 1996).

In this report, we studied the deethylation of AE to its active metabolite, DQHS, and found that it is mediated primarily by the CYP3A4 enzyme in human liver microsomes with minor contributions by CYPs 2B6 and 3A5. In addition, drug-drug interaction studies using other antimarial indicators that halofantrine and mefloquine inhibit DQHS formation in vitro. Because both antimarial drugs have IC₅₀ values greater than 10 μM, they probably do not represent a major risk in vivo if taken in combination.

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References


