METABOLISM OF β-ARTEETHER TO DIHYDROQINGHAOSU BY HUMAN LIVER MICROSONES AND RECOMBINANT CYTOCHROME P450

JAMES M. GRACE, ANTONIO J. AGUILAR, KIMBERLY M. TROTMAN, AND THOMAS G. BREWER

Walter Reed Army Institute of Research, Department of Pharmacology, and Armed Forces Research Institute of Medical Sciences

(Received June 5, 1997; accepted December 18, 1997)

This paper is available online at http://www.dmd.org

ABSTRACT:

β-Arteether (AE) is an endoperoxide sesquiterpene lactone derivative currently being developed for the treatment of severe, complicated malaria caused by multidrug-resistant Plasmodium falciparum. Studies were undertaken to determine which form(s) of human cytochrome P-450 catalyze the conversion of β-arteether to its deethylated metabolite, dihydroqinghaosu (DQHS), itself a potent antimalarial compound. In human liver microsomes, AE was metabolized to DQHS with a K_m of 53.7 ± 29.5 μM and a V_max of 1.64 ± 1.78 nmol DQHS/min/mg protein. AE biotransformation to DQHS was inhibited by ketoconazole and troleandomycin. Ketoconazole was a competitive inhibitor, with an apparent K_i of 0.33 ± 0.11 μM. Because AE is being developed for patients who fail primary treatment, it is possible that AE may be involved in life-threatening drug-drug interactions, such as the associated cardiotoxicity of mefloquine and quinidine. Coincubation of AE with other antimalarials showed mefloquine and quinidine to be competitive inhibitors with a mean K_i of 41 and 111 μM, respectively.

Metabolism of AE using human recombinant P450s provided evidence that cytochrome P450s 2B6, 3A4, and 3A5 were the primary isozymes responsible for its deethylation. CYP3A4 metabolized AE to dihydroqinghaosu at a rate approximately 10 times that of CYP2B6 and ~4.5-fold greater than that of CYP3A5. These results demonstrate that CYP3A4 is the primary isozyme involved in the metabolism of AE to its active metabolite, DQHS, with secondary contributions by CYP2B6 and -3A5.

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),1 a unique sesquiterpene lactone endoperoxide, is the active antimalarial moiety isolated from the Chinese medicinal herb, Artemisia annua (Klaymann, 1985). Arteether (see fig. 1), the ethyl ether derivative of the reduced lactol of QHS, dihydroqinghaosu (DQHS), 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 artemisinin analogs have not shown any neurotoxic side effects (Hien and White, 1993; Looareesuwan, 1994).

This work was supported by the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (TDR).

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

Send reprint requests to: Dr. James M. Grace, Walter Reed Army Institute of Research, Division of Experimental Therapeutics, Department of Pharmacology, Washington, DC 20307-5100.
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 diprophosphate (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, and BHT were purchased from Sigma. Ketoconazole, furafylline, 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), and 250 µM testosterone in 0.1 M potassium phosphate buffer (pH 7.4) was incubated at 37°C for 20 min. Quantitation of 6β-hydroxytestosterone formation was determined using a modified HPLC method previously described (Sonderfan et al., 1987). The product of the reaction, 6β-hydroxytestosterone, was detected at 242 nm, and quantitation was performed utilizing an external standard curve of the authentic metabolite.

HPLC Analysis. Quantitation of DQHS produced in microsomal incubations of AE was performed using HPLC with reductive electrochemical detection as described previously (Melendez et al., 1991).

Preparation of Human Liver Microsomes. Human liver from donors 5, 7, and 12 were obtained from the Washington Regional Transplant Consortium (Washington, DC). Human liver tissue was homogenized and fractionated by differential centrifugation as previously described (Wang et al., 1983), and the microsomal suspensions were stored in 0.10 M potassium phosphate buffer (pH 7.4) containing 20% glycerol at −80°C until used. Protein concentration was determined using the method of Lowry et al. (1951), and cytochrome P450 content was measured by the method of Omura and Sato (1964). Human liver microsomes from donors 12A, 13, 16, 17, and 18 were obtained commercially from Human Biologicals International (Scottsdale, AZ).

Microsomal Incubations. Human liver microsomes (0.20–1.0 mg/ml) were preincubated at 37°C in 0.10 M potassium phosphate buffer (pH 7.4) containing an NADPH regenerating system consisting of: 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 isozymes, 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 tolazoline 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 tolazoline. 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 isozymes 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 isoforms 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 $V_{max}$ for DQHS formation in human liver microsomes are presented in table 2.

To determine the P450 isozyme(s) involved in the biotransformation of AE to DQHS, incubations were conducted using chemical inhibitors specific to various P450 isoforms (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 $0.3 \pm 0.1 \mu M$ and an IC$_{50}$ value of $0.79 \pm 0.045 \mu M$. The data in fig. 4 compare the rate of DQHS formation with testosterone $6\beta$-hydroxylase activity in human liver microsomes. Arteether $O$-deethylase activity showed a strong correlation ($r^2 = 0.70$) with CYP3A-mediated $6\beta$-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 $V_{max}$ parameters for CYP2B6, -3A4, and -3A5 are presented in table 2. The $V_{max}$ of the three isozymes varied by approximately 9-fold, with CYP3A4 having the highest $V_{max}$ 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 antimalarials. Table 3 contains the mean IC$_{50}$ values of several antimalarials co-incubated with AE ($100 \mu M$) 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 $\mu M$, respectively. Dixon analysis of mefloquine inhibition of DQHS formation in human liver sample 18 is shown in fig. 5.

**Results**

The metabolism of AE to DQHS by human liver microsomes and human recombinant P450 isoforms 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 $V_{max}$ for DQHS formation in human liver microsomes are presented in table 2.

To determine the P450 isozyme(s) involved in the biotransformation of AE to DQHS, incubations were conducted using chemical inhibitors specific to various P450 isoforms (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 $0.3 \pm 0.1 \mu M$ and an IC$_{50}$ value of $0.79 \pm 0.045 \mu M$. The data in fig. 4 compare the rate of DQHS formation with testosterone $6\beta$-hydroxylase activity in human liver microsomes. Arteether $O$-deethylase activity showed a strong correlation ($r^2 = 0.70$) with CYP3A-mediated $6\beta$-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 $V_{max}$ parameters for CYP2B6, -3A4, and -3A5 are presented in table 2. The $V_{max}$ of the three isozymes varied by approximately 9-fold, with CYP3A4 having the highest $V_{max}$ 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 antimalarials. Table 3 contains the mean IC$_{50}$ values of several antimalarials co-incubated with AE ($100 \mu M$) 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 $\mu M$, respectively. Dixon analysis of mefloquine inhibition of DQHS formation in human liver sample 18 is shown in fig. 5.

**Discussion**

Previous in vitro studies using rat hepatic microsomes have shown DQHS to be a major metabolite of AE (Baker et al., 1989; Leskovac and Theoharides, 1991). The present study demonstrates that AE is also metabolized to DQHS in human liver microsomes. Experiments performed using specific inhibitors to the major isozymes of cytochrome P450 (see fig. 3) resulted in significant inhibition of DQHS production by ketoconazole, SKF-5252, and TAO (70, 84, and 65% inhibition, respectively, compared with control). Ketoconazole and TAO are specific inhibitors of CYP3A4, whereas SKF-5252 is a general CYP450 inhibitor in vitro. These results indicate that CYP3A4 is the major human form of cytochrome P450 responsible for the metabolism of AE to DQHS.
for biotransformation of AE to DQHS. In human liver microsomes, the \( V_{\text{max}} \) varied 36-fold over eight livers, whereas the \( K_n \) varied by a factor of 4.5 (see table 2). Using microsomes prepared from a human lymphoblastoid cell line coexpressing human P450, CYPs 2B6, 3A4, and 3A5 were the only isozymes to catalyze the deethylation of AE to DQHS. Based on immunoblot analysis of 60 human liver donors, the relative content of CYPs 2B6 and 3A4 in human liver microsomes is approximately 0.2 and 29% of total P450 content, respectively (Shimada et al., 1994). The expression of CYP3A5, determined by immunoblot analysis, was found to be present in only 29% of all human livers analyzed and is approximately 10–30% the relative amount of CYP3A4 when expressed (Wrighton et al., 1990). As shown in table 2, the rate of DQHS formation in human recombinant P450s is approximately 4.5-fold less for CYP3A5 than CYP3A4, a trend previously described for testosterone 6β-hydroxylation (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-deethylase 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 antimarial drugs prior to AE administration. A list of antimarial 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 IC\(_{50}\) 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-450CYP3A4, now known as CYP3A4 (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 K_m ([S] < K_m). 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 antimalarials, 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 arteether, 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 antimalarials 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, DHQS, 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 antimalarials indicate that halofantrine and mefloquine inhibit DHQS formation in vitro. Because both antimalarials have IC_50 values greater than 10 μM, they probably do not represent a major risk in vivo if taken in combination.

Acknowledgments. The authors thank Dr. John Strong (Food and Drug Administration) for the gift of human livers 5, 7, and 12 used in this study and Dr. Kathleen Loe for her technical assistance in preparing this manuscript.

References


