Identification of the human cytochrome P450 enzymes involved in the *in vitro* metabolism of artemisinin

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Aims The study aimed to identify the specific human cytochrome P450 (CYP450) enzymes involved in the metabolism of artemisinin.

Methods Microsomes from human B-lymphoblastoid cell lines transformed with individual CYP450 cDNAs were investigated for their capacity to metabolize artemisinin. The effect on artemisinin metabolism in human liver microsomes by chemical inhibitors selective for individual forms of CYP450 was investigated. The relative contribution of individual CYP450 isoenzymes to artemisinin metabolism in human liver microsomes was evaluated with a tree-based regression model of artemisinin disappearance rate and specific CYP450 activities.

Results The involvement of CYP2B6 in artemisinin metabolism was demonstrated by metabolism of artemisinin by recombinant CYP2B6, inhibition of artemisinin disappearance in human liver microsomes by orphenadrine (76%) and primary inclusion of CYP2B6 in the tree-based regression model. Recombinant CYP3A4 was catalytically competent in metabolizing artemisinin, although the rate was 10% of that for recombinant CYP2B6. The tree-based regression model suggested CYP3A4 to be of importance in individuals with low CYP2B6 expression. Even though ketoconazole inhibited artemisinin metabolism in human liver microsomes by 46%, incubation with ketoconazole together with orphenadrine did not increase the inhibition of artemisinin metabolism compared to orphenadrine alone. Troleandomycin failed to inhibit artemisinin metabolism. The rate of artemisinin metabolism in recombinant CYP2A6 was 15% of that for recombinant CYP2B6. The inhibition of artemisinin metabolism in human liver microsomes by 8-methoxypsoralen (a CYP2A6 inhibitor) was 82% but CYP2A6 activity was not included in the regression tree.

Conclusions Artemisinin metabolism in human liver microsomes is mediated primarily by CYP2B6 with probable secondary contribution of CYP3A4 in individuals with low CYP2B6 expression. The contribution of CYP2A6 to artemisinin metabolism is likely of minor importance.

Keywords: artemisinin, CYP2B6, CYP3A4, cytochrome P450, metabolism

Introduction

Artemisinin is the parent compound of an emerging class of antimalarial drugs of importance in the treatment of malaria in areas with multidrug resistant *Plasmodium falciparum*. Artemisinin is a sesquiterpene lactone with an internal peroxide bridge (Figure 1) necessary for its antiparasitic effect [1]. Limited data are available on artemisinin metabolism in both humans and animal

species. The fraction excreted unchanged in urine in humans is less than 1% of an oral administration [2]. In rats, the liver is the major organ of elimination [3]. Four metabolites, deoxy-artemisinin, deoxy-dihydroartemisinin, dihydroxyartemisinin and the so-called 'crystal-7' were identified in urine following oral administration of artemisinin to humans, but plasma metabolites remain unknown [4].

Artemisinin is commonly used together with other antimalarial drugs and information on its enzymatic pathways of elimination is important to avoid potential drug interactions. In addition, information on artemisinin metabolism is of particular interest with regard to the

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Figure 1 Chemical structure of artemisinin (MW 282).

remarkable time-dependent pharmacokinetics of artemisinin observed in patients and healthy volunteers after multiple oral administration [5–10]. A five-fold increase in oral clearance after 5 days of multiple administration of artemisinin has been found and auto-induction of hepatocellular activity has been proposed. In the rat, intestinal absorption is unaffected by multiple dosing and the time-dependent pharmacokinetics are not due to induction of P-glycoprotein intestinal efflux [11].

In this study, artemisinin metabolism in microsomes from human B-lymphoblastoid cell lines transformed with individual CYP450 complementary DNAs was investigated as well as the effect of selective chemical inhibitors on artemisinin metabolism in pooled human liver microsomes. The relative contribution of individual CYP450 isoenzymes to artemisinin metabolism in characterized human liver microsomes was evaluated with a tree-based regression model of artemisinin disappearance rates and specific CYP450 activities.

Methods

Chemicals

Artemisinin (batch 17/6/96) was obtained from the Institute of Malariology, Parasitology and Entomology, Hanoi, Vietnam. Ketoconazole was kindly provided by Janssen, Beerse, Belgium. Furaflavine was obtained from Salford Ultrafine Chemicals & Research Ltd, Manchester, England and S-mephenytoin was provided by Sandoz, Basle, Switzerland. Sulphaphenazole, quinidine, 8-methoxypsoralen, 3-amino-1,2,4-triazole and orphenadrine were all purchased from Sigma-Aldrich Sweden AB, Tyresö, Sweden. Dihydronicotinamide-adenine dinucleotide phosphate (NADPH) was obtained from Kebo Labs., Spånga, Sweden. All other reagents were purchased from commercial sources and were of the purest grade available.

Incubations with cDNA-expressed human CYP450 isoenzymes

Microsomes, derived from AHH-1 TK +/– human lymphoblastoid cell lines expressing individual CYP450 isoenzymes (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4), epoxide hydrolase and oxidoreductase were purchased from Gentest Corporation (Worburn, Massachusetts, USA). Artemisinin (final concentration 1000 ng ml^{−1}, MW 282, ≤1% acetone) was incubated with 1.0 and 2.5 mg ml^{−1} microsomal protein and NADPH (1.0 mg ml^{−1} mg^{−1} protein) in a 0.1 M Tris buffer, pH 7.4 consisting of 20% glycerol, 0.1 mM EDTA and 0.1 mM dithiothreitol. The final incubation volume was 1 ml. Incubations were initiated by the addition of substrate and were gently agitated at 37° C for 180 min in a water-bath (Haake SWB 20). Samples (150 µl) were removed at 0, 60, 120 and 180 min, mixed with 200 µl Tris buffer and the reaction terminated by heating the samples in boiling water for 3 min and then chilled on ice. After centrifugation (10 000 g, 10 min), 60 µl of the supernatant was injected directly onto the h.p.l.c. column. Experiments were performed in triplicate for each isoenzyme and protein concentration. Control incubations with the basal AHH-1 TK +/– cell line were incubated in parallel with the experiments. Artemisinin was stable in Tris buffer and unaffected by sample handling involving heating, chilling and addition of methanol.

Correlation experiments

Human liver microsomes from 10 donors, characterized for their ability to metabolize specific substrates were obtained from Human Biologics International (Scottsdale, Arizona, USA). All samples were from healthy donors and in all cases the cause of death was not due to any known biochemical deficiency in the liver. The microsomes were characterized for CYP450 activity with respect to caffeine N3-demethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), S-mephenytoin N-demethylation (CYP2B6), tolbutamide methyl-hydroxylation (CYP2C9), S-mephenytoin 4'-hydroxylation (CYP2C19), dextromethorphan O-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), dextromethorphan N-demethylation (CYP3A4), testosterone 6β-hydroxylation (CYP3A4/5) and lauric acid 12-hydroxylation (CYP4A11).

The disappearance of artemisinin was investigated by incubating (37° C, shaking water-bath) artemisinin (final concentration 2000 ng ml^{−1}, ≤1% methanol) with NADPH (1.0 mg ml^{−1} mg^{−1} protein) and 1.5 mg ml^{−1} microsomal protein^{−1} in Tris buffer (0.1 M, pH 7.4). The total incubation volume was 1 ml. After a

preincubation of 2 min, the reaction was initiated by the addition of ice-cold NADPH. Samples (100 µl) were removed at 0, 10, 20, 30 and 40 min, except for one donor where samples were taken at 0, 1, 2, 3, 4 and 5 min due to rapid elimination of artemisinin. The samples were mixed with 200 µl ice-cold methanol and chilled on ice. After centrifugation (10 000 g, 10 min), 60 µl of the supernatant was injected directly onto the h.p.l.c. column. The incubations were performed in triplicate with microsomes from each liver.

Inhibition experiments

The effect of selective inhibitors on the rate of artemisinin disappearance was investigated in microsomes pooled from 10 livers (Human Biologics International, Scottsdale, Arizona, USA). The selective inhibitors 8-methoxypsoralen (500 µM), orphenadrine (500 µM), ketoconazole (4 µM) and troleandomycin (50 µM) were used to investigate the involvement of CYP2A6 [12], CYP2B6 [13, 14], CYP3A4 [15, 16] and CYP3A4 [17], respectively. The effect of orphenadrine in combination with ketoconazole or 8-methoxypsoralen was investigated in these microsomes as well.

Inhibition studies were also performed with characterized microsomes. Microsomes (Human Biologics International, Scottsdale, Arizona, USA) from one human liver (HBI 102) with high S-mephenytoin *N*-demethylation (CYP2B6) and dextromethorphan *N*-demethylation (CYP3A4) activity and microsomes from one liver (HBI 103) with low CYP2B6 and CYP3A4 activity were incubated with artemisinin in the presence of the CYP2B6 inhibitor orphenadrine (500 µM). The effect of the CYP3A4 inhibitor ketoconazole (4 µM) was also investigated in these two livers. In addition, artemisinin was coincubated with the antimalarial R,S-mefloquine (200 µM).

The inhibitors were dissolved in dimethylsulphoxide (DMSO) (final concentration <0.5%). The total incubation volume was 1 ml and the incubation was maintained at 37°C in a shaking water-bath. The reaction was initiated by addition of ice-cold NADPH after 2 min preincubation (10 min preincubation with troleandomycin) and the metabolic activity was terminated by addition of ice-cold MeOH (200 µl) to 100 µl sample at 0, 10, 20, 30 and 40 min. After centrifugation at 10 000 g for 10 min, 60 µl of the supernatant was injected directly onto the h.p.l.c. column. Control experiments were performed in parallel with artemisinin incubated without inhibitor but with 0.5% DMSO. All inhibition incubations were performed in triplicate.

In addition, the specificity of orphenadrine, ketoconazole and 8-methoxypsoralen in inhibiting artemisinin metabolism was investigated in triplicate in microsomes

from human B-lymphoblastoid cell lines expressing CYP2B6, CYP3A4 and CYP2A4, respectively.

Artemisinin analysis

Artemisinin was quantified by h.p.l.c. with on-line postcolumn alkali derivatization as described by Edlund *et al.* [18]. The detection was linear within the concentration span used for the standard curve (20–700 ng ml⁻¹). Two quality control samples at each of three concentrations (40, 240 and 600 ng ml⁻¹) were run at the same time. The inhibitors did not interfere chromatographically with artemisinin.

Data analysis

In incubations with microsomes from human lymphoblastoid cell lines expressing different isoenzymes, artemisinin disappearance rates (nmol min⁻¹ mg⁻¹ protein), adjusted for native activity were calculated. In incubations with characterized microsomes and pooled microsomes, artemisinin first-order elimination rate constants were estimated by log-linear regression of drug concentration–time data. The inhibition of artemisinin metabolism by the specific inhibitors was evaluated by comparing the first-order elimination rate constants (*k*) in the presence and absence of inhibitors [% inhibition = 100 × (*k*_{absence} – *k*_{presence}) / *k*_{absence}].

To compare artemisinin disappearance rates and specific CYP450 activities in characterized human liver microsomes, regression analysis was performed on log-transformed data for both dependent and independent variables due to skew distribution and in particular microsomes from one donor exhibiting generally very high enzyme activities and artemisinin disappearance rates. Since simple linear regressions of artemisinin metabolism rates against individual, specific enzyme activities in microsomes from different livers do not apply when enzyme activities correlate (coregulation), analysis by multiple regressions was initially considered for investigating the relative contribution of individual isoenzymes to the disappearance of artemisinin in characterized liver microsomes. After both forward and backward stepwise multiple regression, CYP2B6 activity was selected as explaining most of the variability in artemisinin disappearance rates, whereas the inclusion of other enzymes into the regression model was inconsistent (results not shown). The relationship between log artemisinin disappearance rate constants and log S-mephenytoin *N*-demethylation activities (CYP2B6 activity) (slope = 0.97 ± 0.04 (s.e.mean), *r*² = 0.94) obtained from a simple regression analysis (Statistica, StatSoft, Tulsa, Okla) was by visual inspection of the plot not completely linear. Due to possible nonlinearities and subgroups with different contributions of individual

isoenzymes, a tree-based model [19] was therefore chosen for the final regression analysis using Xpose 2.0 software [20]. This is further considered in the discussion section. Results are presented as median (range).

Results

Incubations with cDNA-expressed human CYP450 isoenzymes

In incubations with microsomes from the AHH-1 TK + / - human lymphoblastoid cell line, the most rapid disappearance of artemisinin, about $6 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein, was observed in microsomes expressing CYP2B6 (Figure 2). The rate of artemisinin metabolism was 7-fold and 10-fold higher in microsomes with cDNA-expressed CYP2B6 compared to cDNA-expressed CYP2A6 and CYP3A4, respectively. Low disappearance rates were seen in incubations with recombinant CYP1A1 and oxidoreductase. No or negligible disappearance of artemisinin was found with microsomes expressing CYP1A2, 2C19, 2C8, 2C9, 2D6, 2E1 and epoxide hydrolase. There was no difference in the relative order of the velocities between the different cDNA expressed enzymes in incubations with 1.0 mg ml^{-1} microsomal protein compared with 2.5 mg ml^{-1} protein, with the exception of CYP2E1 where the rate of artemisinin disappearance ($\text{mg}^{-1} \text{ protein}$) was more rapid when incubated with 2.5 mg ml^{-1} compared with 1.0 mg ml^{-1} protein.

Correlation experiments

When artemisinin was incubated with commercially obtained microsomes from 10 human livers, a 119 fold

variation in artemisinin first-order disappearance rate constants was observed (median = 0.028 min^{-1} , range $0.011\text{--}0.998 \text{ min}^{-1}$). Of all covariates (CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, 3 A4/5 and 4A11) CYP2B6 and CYP3A4 activities were selected in the regression tree as important for artemisinin disappearance rate (Figure 3). With the length of the vertical lines connecting nodes being proportional to the increase in the goodness of fit of the model, most of the variability in artemisinin disappearance rates was explained by CYP2B6 activity. Three subgroups were identified, liver microsomes with S-mephenytoin *N*-demethylation activity (CYP2B6 activity) greater than $189 \text{ pmol mg}^{-1} \text{ protein min}^{-1}$ (subgroup III); liver microsomes with S-mephenytoin *N*-demethylation activity (CYP2B6 activity) less than $189 \text{ pmol mg}^{-1} \text{ protein min}^{-1}$ and dextromethorphan *N*-demethylation activity (CYP3A4 activity) greater than $52 \text{ pmol mg}^{-1} \text{ protein min}^{-1}$ (subgroup II); and finally liver microsomes with S-mephenytoin *N*-demethylation activity (CYP2B6 activity) less than $189 \text{ pmol mg}^{-1} \text{ protein min}^{-1}$ and dextromethorphan *N*-demethylation activity (CYP3A4 activity) less than $52 \text{ pmol mg}^{-1} \text{ protein min}^{-1}$ (subgroup I). Of the 10 livers, two were classified as belonging to subgroup III with artemisinin being metabolized predominantly by CYP2B6. For the other eight livers, both CYP2B6 and CYP3A4 were important for artemisinin metabolism. Four livers belonged to the group with low CYP2B6 activity and high CYP3A4 activity (subgroup II) and four livers belonged to subgroup I with low CYP2B6 and CYP3A4 activity. The predicted artemisinin first-order disappearance rate constant for subgroups I, II and III were 0.014, 0.031 and 0.255 min^{-1} corresponding to artemisinin half-lives ($t_{1/2}$) in

Figure 2 Artemisinin disappearance rate ($\text{nmol min}^{-1} \text{ mg protein}^{-1}$) in microsomes prepared from human B-lymphoblastoid cell lines transformed with individual cytochrome P450 complementary DNAs (triplicate).

Figure 3 Results from a tree-based model with artemisinin first-order disappearance rate constants in human liver microsomes (10 livers, triplicates) regressed against specific cytochrome P450 activities. CYP2B6 activity was selected as the covariate explaining most of the variability in artemisinin disappearance rate. The length of the vertical lines connecting nodes are proportional to the increase in the measure of goodness of fit of the model, wherefore the first split with CYP2B6 activity clearly explains the data more than the lower split with CYP3A4 activity. Three terminal nodes were identified; Subgroup I (low activity of both CYP2B6 and CYP3A4), Subgroup II (low CYP2B6 and higher CYP3A4 activity) and Subgroup III (high CYP2B6 activity).

k_{disapp} = predicted artemisinin first-order disappearance rate constant in human liver microsomes estimated by log-linear regression

$t_{1/2}$ = predicted artemisinin disappearance half-life in human liver microsomes

microsomal incubations of 50.6, 22.5 and 2.7 min, respectively (Figure 3).

Inhibition experiments

Co-incubation of artemisinin with the CYP2B6 inhibitor orphenadrine in characterized human liver microsomes expressing high and low activity of S-mephenytoin-*N*-demethylation, decreased artemisinin disappearance rates by 75 (69, 78)% and 45 (39, 52)%, respectively. Ketoconazole, a CYP3A4 inhibitor, decreased the disappearance of artemisinin in incubations with microsomes with high and low dextromethorphan *N*-demethylation by 45 (34, 57)% and 33 (23, 42)%, respectively. The percentage inhibition of artemisinin disappearance rate in pooled human liver microsomes in the presence of inhibitors are presented in Figure 4. Ketoconazole inhibited artemisinin metabolism by 46%. However, incubations with ketoconazole together with orphenadrine did not increase the inhibition of artemisinin metabolism (74%) compared with orphenadrine alone (76%). Troleandomycin, known to inhibit CYP3A4 [17] metabolism did not inhibit artemisinin metabolism in microsomes from the same pool of 10 human livers. The CYP2A6 inhibitor 8-methoxypsoralen decreased artemisinin metabolism by 82%. 8-methoxypsoralen together with orphenadrine inhibited artemisinin metabolism in pooled human liver microsomes by 90%.

The efficiency and selectivity of orphenadrine, ketoconazole and 8-methoxypsoralen in inhibiting artemisinin

metabolism were tested in microsomes derived from human B-lymphoblastoid cell lines expressing CYP2B6, CYP3A4 and CYP2A6, respectively. Orphenadrine inhibited artemisinin metabolism in recombinant CYP2B6 by 67 (64, 71)%, while only modest inhibition was seen by ketoconazole 5 (3, 33)% and 8-methoxypsoralen 26 (13, 48)% in recombinant CYP3A4 and CYP2A6, respectively.

When artemisinin was incubated in the presence of the antimalarial mefloquine, artemisinin disappearance in pooled microsomes was reduced by 20 (18, 39)%.

The percentage of artemisinin remaining at the end of the incubations with microsomes used in the inhibition experiments was 0% (pool of microsomes; Human Biologics International), 0% (ID102; Human Biologics International) and 63 (58, 64)% (ID103; Human Biologics International) in the absence of inhibitors.

Discussion

The catalytic capacity of CYP2B6 for artemisinin was demonstrated by rapid artemisinin disappearance in microsomes from human B-lymphoblastoid cell line expressing CYP2B6 (Figure 2), inhibition by orphenadrine (76%) in pooled human liver microsomes from different donors (Figure 4) and S-mephenytoin *N*-demethylation activity explaining most of the variability in artemisinin disappearance rate (Figure 3). Orphenadrine inhibited artemisinin metabolism to a lower degree (46%) in microsomes from a liver sample with low CYP2B6

Figure 4 Percent inhibition of artemisinin disappearance rate in pooled human liver microsomes by selective inhibitors (triplicate).

activity, compared with a liver with high CYP2B6 activity (75%). This degree of inhibition was also related to the rate of artemisinin disappearance, being much higher in the microsomal sample with high expression of CYP2B6. In the microsomes from the B-lymphoblastoid cell line used in the present study, the CYP2B6 activity, measured as 7-ethoxy-4-trifluoromethylcoumarin deethylase activity is reported to be similar to a human liver microsome pool (order catalogue, Gentest Corp., Wornburn, USA). The high affinity of artemisinin for the recombinant CYP2B6 should therefore be comparable with the *in vivo* situation. A large variation in CYP2B6 mRNA levels has been observed in human liver specimens [21] which is compatible with the highly variable pharmacokinetics of artemisinin. CYP2B6 is not one of the major drug metabolizing CYP450 enzymes, but has been shown to catalyse the 4-hydroxylation of cyclophosphamide [22] and partly mediate the metabolism of an artemisinin derivative, β -arteether in human liver microsomes [23].

Recombinant CYP3A4 was catalytically competent in metabolizing artemisinin, although the rate was 10-fold lower compared with recombinant CYP2B6 (Figure 2). The CYP3A4 activity (measured as testosterone 6 β -hydroxylase activity) in microsomes from the B-lymphoblastoid cell lines expressing CYP3A4, was half of that in a human liver microsome pool (order catalogue, Gentest Corp., Wornburn, USA). The affinity between artemisinin and CYP3A4 might therefore be underestimated. Even though ketoconazole inhibited artemisinin disappearance in pooled human liver microsomes by 46%, ketoconazole in combination with orphenadrine did not increase the inhibition of artemisinin disappearance (74%) compared to incubation with orphenadrine alone (76%) (Figure 4). The ketoconazole concentration used in the inhibition studies (4 μ M), only inhibited artemisinin disappearance with 5% in microsomes from cDNA-expressed CYP3A4, indicating maybe a suboptimal ketoconazole concentration. Troleandomycin failed to

inhibit artemisinin disappearance rate in the pooled human liver microsomes. However, the tree-based regression model identified CYP3A4 to be of importance for artemisinin metabolism in individuals with low expression of CYP2B6 (Figure 3). Substantial interindividual variability in the CYP3A4 levels exists [24, 25] and some variability in artemisinin pharmacokinetics might therefore also be attributed to differences in expression of CYP3A4.

Artemisinin is often combined with mefloquine in the treatment of malaria. The formation of 4-carboxymefloquine from mefloquine has been suggested to be mediated by CYP3A4 *in vitro* [26]. Co-incubation of artemisinin with mefloquine decreased the disappearance of artemisinin in pooled human liver microsomes (20%) which possibly could be attributed to inhibited artemisinin CYP3A4 metabolism by mefloquine.

The possible involvement of CYP2A6 in artemisinin metabolism was indicated by artemisinin being metabolized by recombinant CYP2A6 and inhibition by 8-methoxypsoralen. The rate of artemisinin metabolism was 7-fold lower in recombinant CYP2A6 compared with in recombinant CYP2B6 (Figure 2). 8-methoxypsoralen inhibited artemisinin disappearance in pooled liver microsomes by 82% and the inhibition increased by 90% when 8-methoxypsoralen and orphenadrine were combined (Figure 4). However, CYP2A6 activity was not included in the tree-based regression model. In tree-based regression, splitting of data in different subgroups continues until either the node is deemed homogenous or contains too few observations (≤ 5). Artemisinin metabolism might be mediated by CYP2A6 but to a lesser extent than by CYP2B6 and CYP3A4.

cDNA-expressed CYP1A1 and oxidoreductase were competent in metabolizing artemisinin (Figure 2). However, the contribution of these enzymes to the overall metabolism *in vivo* is probably negligible. CYP1A1 is absent or present at very low levels in human liver [27, 28] and the activity of oxidoreductase in microsomes

from cDNA-transformed human B-lymphoblastoid cell line is around 20% higher compared with human liver microsomes (order catalogue, Gentest Corp., Wornburn, USA).

Tree-based models provide an alternative to linear and additive models for regression problems [29] and automatically take into account possible nonlinearities and interactions between covariates. Although the correlation between artemisinin disappearance rates in human liver microsomes and S-mephenytoin *N*-demethylation rates (CYP2B6 activity) was high, the relationship was not linear. A tree-based regression model was therefore favoured over a linear regression model in this case. Further, since the expression of CYP2B6 has been reported to be low in human liver [30], the contribution of other isoenzymes could be more important in individuals with low CYP2B6 expression. The use of tree-based models may therefore offer an advantage over multiple linear regression models since the model can be different for different subgroups when the interaction and contribution of isoenzymes are not the same in the entire population. In the case of artemisinin disappearance rates, two covariates were selected (CYP2B6 and CYP3A4 activity) and the data split into three terminal nodes representing Subgroups I, II and III. The results from tree-based models are also easy to interpret since it gives predictions of parameters corresponding to each terminal subgroup of the tree. In the case of artemisinin disappearance rate in human liver microsomes, cut-off values for CYP2B6 and CYP3A4 activities as well as predicted artemisinin first-order disappearance rate constants were provided for each of the three different subgroups.

Even if CYP3A4 may contribute to artemisinin metabolism *in vivo*, the pronounced decrease in bioavailability during multiple dosing can not be explained by induction of CYP3A4. Artemisinin caused a three-fold increase in oral clearance of omeprazole in healthy subjects by inducing the formation of hydroxyomeprazole, associated with CYP2C19 activity whereas the CYP3A4 mediated formation of omeprazole sulphone was unaffected. The lack of induction of hydroxyomeprazole by artemisinin, but increase in clearance of artemisinin and omeprazole in a subject classified as poor metabolizer for CYP2C19 was indicative of artemisinin inducing also an isoenzyme other than CYP2C19 [10]. CYP2B6 is an inducible enzyme [31] but whether the time-dependent pharmacokinetics of artemisinin is an effect of autoinduction of this enzyme remains to be shown.

In the present study the rate of artemisinin metabolism was measured as a disappearance rate instead of metabolite formation rate. This places a limitation of the interpretability of the inhibition experiments especially if the inhibitors are only partially selective. Ketoconazole has

previously been shown to be a selective inhibitor of CYP3A4 at the concentration used in the present study (4 μ M) [16, 17] and orphenadrine has been shown to inhibit CYP2B6 activity [13, 14]. Orphenadrine (500 μ M) caused 84% decrease in S-mephenytoin *N*-demethylase activity in human liver microsomes [13] and cyclophosphamide hydroxylation was inhibited by 47% by orphenadrine (300 μ M) [27]. However, in a recent report orphenadrine (500 μ M) decreased not only the activity of the CYP2B6 marker, 7-ethoxytrifluoromethylcoumarin *O*-deethylase by 45% but also partly decreased CYP1A2, CYP2A6, CYP3A4 and CYP2C19 activity by 20–40% and strongly inhibited CYP2D6 activity by 80% [32]. This suggests orphenadrine not to be a selective inhibitor of CYP2B6. Although it cannot be ruled out that the decrease in artemisinin metabolism by orphenadrine is partly due to inhibition of other CYP450 enzymes, the inhibition by orphenadrine was $67 \pm 5\%$ in recombinant CYP2B6. It is therefore likely that the major part of the inhibition by orphenadrine can be explained by inhibited CYP2B6 activity.

This study suggests artemisinin metabolism in human liver microsomes to be primarily mediated by CYP2B6 and with secondary contribution of CYP3A4 in individuals with low CYP2B6 expression. The contribution of CYP2A6 to artemisinin metabolism is probably of minor importance. Drug-drug interactions may occur with substrates for these isoenzymes and has to be taken into consideration when combining artemisinin with other antimalarial drugs with the aim to reduce recrudescence rates and resistance development.

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