Evaluation of P450 Inhibition and Induction by Artemisinin Antimalarials in Human Liver Microsomes and Primary Human Hepatocytes

Jie Xing, Brian J. Kirby, Dale Whittington, Yakun Wan, and David R. Goodlett

ABSTRACT:
Artemisinin drugs have become the first-line antimalarials in areas of multidrug resistance. However, monotherapy with artemisinin drugs results in comparatively high recrudescence rates. Autoinduction of cytochrome P450 (P450)-mediated metabolism, resulting in reduced exposure, has been supposed to be the underlying mechanism. To better understand the autoinduction and metabolic drug-drug interactions (DDIs), we evaluated the P450s (particularly CYP2B6 and CYP3A4) inhibited or induced by two artemisinin drugs, Qing-hao-su (QHS) and dihydroartemisinin (DHA) using human liver microsome, recombinant P450 enzymes, and primary human hepatocytes. The results suggested that QHS was a weak reversible inhibitor of CYP2B6 (K_i 4.6 μM), but not CYP3A4 (K_i 50 μM) and did not show measurable time-dependent inhibition of either CYP2B6 or CYP3A4. DHA inhibited neither CYP2B6 nor CYP3A4 (K_i > 125 μM). In addition, it was found that QHS induced the activity of CYP3A4 (E_max 3.5-fold and EC_50 5.9 μM) and CYP2B6 (E_max 1.9-fold and EC_50 0.6 μM). Of the other P450s, UDP glucuronosyltransferases, and transporters studied, QHS and DHA had no significant effect except for minor induction of mRNA expression of CYP1A2 (E_max 7.9-fold and EC_50 5.2 μM) and CYP2A6 (E_max 11.7-fold and EC_50 4.0 μM) by QHS. Quantitative prediction of P450-mediated DDIs indicates autoinduction of QHS clearance with the AUC/AUC ratio decreasing to 59%, as a result of a 1.9-fold increase in CYP3A4 and a 1.6-fold increase in CYP2B6 activity. These data suggest that QHS drugs are potential inducers of P450 enzymes, and the possible drug interactions (or lack thereof) with artemisinin drugs may be clinically relevant.

Introduction
Artemisinin, also known as Qing-hao-su (QHS), is a promising antimalarial agent originating from the Chinese medicinal herb *Artemisia annua* L. QHS and its derivatives, such as dihydroartemisinin (DHA), artemether, arteether, and artesunate, are effective against both chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum* (White, 2004; Dondorp et al., 2010). To avoid rapid drug resistance, QHS drugs are suggested to be used in the clinic as part of combination treatment [artemisinin combination therapies (ACTs)], i.e., the artemisinin compound acts early for a rapid parasite blood count reduction, whereas concomitant antimalarial drug with a prolonged half-life is included for eliminating residual parasitemia (Falade et al., 2008). The pharmacokinetics of QHS and DHA after ACT administration was comparable to values obtained in humans given QHS or DHA alone (Chinh et al., 2009; Hien et al., 2011).

However, limited data were available on the effect of QHS drugs on the disposition of their partner drugs.

Despite recommendations and warnings, QHS derivatives are available as monotherapy in many countries. Some evidence showed QHS and its derivatives (artemether and less convincingly artesunate) exhibited remarkable time-dependent pharmacokinetics. This has been confirmed in healthy volunteers and patients, as a severalfold decrease in plasma concentration of QHS with a corresponding increase in oral clearance. Autoinduction of drug metabolism has been suggested for this time dependence (Ashton et al., 1998a; Svensson and Ashton, 1999). The elimination of QHS in human liver microsomes (HLM) is mediated primarily by CYP2B6, with a probable secondary contribution of CYP3A4 and CYP2A6 (Svensson and Ashton, 1999). The relative contributions of CYP2B6, CYP3A4, and CYP2D6 to the metabolism of QHS in HLM were 10, 25, and 6.5%, respectively (Li et al., 2003). The autoinduction of QHS was probably attributed, at least in part, to induction of CYP2B6, but not CYP2C9 (Svensson and Ashton, 1999; Simonsson et al., 2003). Treatment of intestinal LS174T cells and primary human hepatocytes with increasing concentrations of QHS led to the specific induction of mRNA levels for...
CYP2B6, CYP3A4, CYP2C19, and multidrug resistance (MDR) 1 (Burk et al., 2005).

The CYP2B6-mediated N-demethylation of S-mephenytoin was found to increase 1.9-fold after multiple administrations of QHS to CYP2C19-poor metabolizers (Simonsson et al., 2003). QHS appears to induce other enzymes including CYP2C19 (Svensson et al., 1998; Mihara et al., 1999; omeprazole as a marker), CYP3A4 (midazolam hydroxylation; Asimus et al., 2007), CYP2A6 (coumarin hydroxylation; Asimus et al., 2008), and glucuronidation (7-hydroxycoumarin glucuronidation; Asimus et al., 2008).

Drug-metabolizing enzymes and transporters are regulated by nuclear receptors, such as pregnane X receptor (PXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (Köhle and Bock, 2009). CYP3A4 and CYP2B6 are most sensitive to the inductive effects of PXR and CAR activation, respectively. QHS, artemether, and arteether were found to activate hPXR as well as mouse and human CAR (Burk et al., 2005). QHS activated hPXR with an EC₅₀ of approximately 30 µM (10–15 times higher than the C₅₀ (max of QHS in vivo) in LS174T cells cotransfected with hPXR expression plasmid. Activation of hCAR required the same range of concentration as PXR. In another cell system (human embryonic kidney 293), QHS activated CAR (maximally by 1.8-fold at 10 µM) but not PXR (Simonsson et al., 2006). Fifty micromolar artemether and arteether activated hPXR and hCAR comparable to 100 µM QHS. In contrast, 25 µM DHA did not significantly activate hPXR (Burk et al., 2005).

In addition to the autoinduction phenomenon, both QHS and DHA were found to be potent inhibitors of CYP1A2 activity in vitro (Bapiro et al., 2001) and in vivo (Bapiro et al., 2005; Asimus et al., 2007). Taken together, these results showed that QHS drugs may affect cytochrome P450s (P450s) as both inducer and inhibitor, which may compromise the results of P450 activity in vivo.

The aforementioned data indicate that QHS and DHA have the potential to induce multiple P450 enzymes and possibly transporters. Because the net effect in vivo may be a combination of reversible inhibition, induction, and irreversible inactivation of these P450 enzymes, we evaluated QHS and DHA for the potential to inhibit or inactivate CYP2B6 and CYP3A in recombinant systems as well as HLM. In addition, we evaluated QHS and DHA for their potential to induce multiple P450 enzymes and transporters in freshly isolated human hepatocytes. Taking all of these data into account, we then predicted the net effect of QHS and DHA on P450 enzymes in vivo and evaluated the correlation between the predicted interaction potential from our in vitro studies and previously reported drug-drug interactions (DDIs) with QHS, as well as the potential for autoinduction of QHS and/or DHA.

Materials and Methods

Chemicals and Reagents. QHS and DHA were provided by Kunming Pharmaceutical Co. (Yunnan, China; purity > 99.0%). Bupropion, (±)-hydroxybupropion, midazolam, α-hydroxymidazolam, and alprazolam [internal standard (IS)] for liquid chromatography-mass spectrometry assay were purchased from Cerilliant Corporation (Round Rock, TX). Rifampin, diclofenac, 4′-hydroxydiclofenac, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used were purchased from Sigma-Aldrich or Thermo Fisher Scientific (Waltham, MA) and were of highest purity available.

Inhibitory Effect on P450s. To measure the inhibition effects of QHS and DHA on the activities of CYP2B6 and CYP3A4, the following marker substrates with high specificity for P450 enzymes were selected: midazolam 1′-hydroxylation for CYP3A4 and bupropion hydroxylation for CYP2B6. QHS and DHA were evaluated for their abilities to inhibit P450 (CYP2B6 and CYP3A4) activity in a direct manner using both pooled HLM (n = 5, mixed gender) and recombinant P450 (rP450) enzymes (BD Gentest, Woburn, MA). In brief, duplicate incubations were conducted at 37°C in 200 µl of incubation with the final content of organic solvent in the incubation mixture at 0.5%. The mixtures contained HLM (0.05 and 0.1 mg/ml microsomal protein for CYP3A4 and CYP2B6, respectively) or rP450s (2 pmol/ml for CYP3A4; 20 pmol/ml for CYP2B6), potassium phosphate buffer (0.1 M, pH 7.4), NADPH (1 mM), and P450 marker substrates. Reactions were preincubated for 3 min before the initiation of metabolic reaction with NADPH and were terminated after 3 min (CYP3A4) or 10 min (CYP2B6) by an equal volume of acetonitrile containing an appropriate IS. Precipitated protein was removed by centrifugation (1000g for 10 min at ambient temperature), and samples were injected onto the liquid chromatography-mass spectrometer to quantify the concentration of marker metabolites formed by individual P450 enzymes.

The concentration of QHS and DHA that caused 50% inhibition of the marker reaction (IC₅₀) was determined at a substrate concentration equal to Kₘ. The apparent inhibition constant (Kᵢ) of CYP2B6 for QHS was calculated using the inhibition model, which provides the inhibition mechanism. QHS was studied at four different concentrations that were chosen on the basis of the IC₅₀ value (approximately 1/20 × IC₅₀, 1/5 × IC₅₀, 1/2 × IC₅₀, and 2 × IC₅₀). The concentrations of marker substrate were chosen (approximately 1/4 × Kᵢ, 3 × Kᵢ, and 3 × Kᵢ) with regard to their Michaelis-Menten kinetics (Kₘ). Kᵢ values of CYP3A4 and CYP2B6 were 2 and 50 µM, respectively. The vehicle controls were analyzed in parallel. The Kᵢ values were calculated by nonlinear regression analysis by fitting different models of enzyme inhibition to the kinetic data using WinNonlin version 5.0 (Pharsight, Mountain View, CA).

The time-dependent inhibitory (TDI) potency of CYP2B6 and CYP3A4 by QHS was investigated with pooled HLM. QHS was preincubated at 37 ± 1°C, in duplicate, with HLM (1 mg/ml) and NADPH (1 mM) in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) for up to 10 min. After the preincubation period, a 10-fold dilution was performed in the presence of NADPH (1 mM) and the marker substrate (at a concentration approximately equal to 15-fold of its Kᵢ). The incubation was continued for 3 min (CYP3A4) or 10 min (CYP2B6) and was quenched in ice-cold acetonitrile (containing IS) to measure residual P450 activity.

Inductive Effect on P450s, UDP Glucuronosyltransferases, and Transporters. Freshly isolated human hepatocytes with a Matrigel overlay from three individual donors were purchased from CellzDirect (Durham, NC). Hepatocytes were maintained at 37°C with 5% CO₂ in 24-well plates in serum-free Williams’ medium E supplemented with 100 nM dexamethasone and insulin-transferrin-selenium (CellzDirect). Forty-eight hours after plating, three preparations of cultured human hepatocytes from separate human livers were treated at 37°C once daily for 3 consecutive days with vehicle (0.2% dimethyl sulfoxide in supplemented Williams’ medium E) or vehicle containing QHS (1, 5, 10, 20, and 50 µM), DHA (1, 5, 10, 20, and 50 µM), or rifampin (positive control, 10 µM). During this period, drug-containing medium was replaced every 24 h, and the concentration of QHS or DHA in the media was determined to evaluate the depletion of drugs. Regular visual inspection of the hepatocyte cultures was performed to evaluate the cell morphology after vehicle or drug treatment. At the end of the treatment period, cells were washed with PBS twice before P450 activity was assessed. Induction of CYP3A4, CYP2B6, and FICP29 activities in primary human hepatocytes was determined on the basis of two validated cocktail assays (McGinnity et al., 2006; Kenny et al., 2008). Human hepatocytes were incubated with bupropion (100 µM), midazolam (2 µM), and diclofenac (9 µM) to measure the activity of CYP2B6, CYP3A4, and CYP2C9, respectively, after 72 h of treatment with QHS or DHA. To evaluate the potential of reversible inhibition, we determined the QHS drug concentrations in the media of hepatocytes during the activity assay.

Total RNA from the hepatocytes was isolated using the Qiagen RNeasy mini kit (Qiagen Sciences, Germantown, MD) according to the manufacturer’s instructions by using spin technology, including DNAse I digestion to minimize genomic DNA contamination from RNA samples. The concentration of purified RNA was determined by a NanoDrop spectrophotometer (Thermo Fisher Scientific), and the purity was indicated with the absorbance ratio of 260/280 nm between 1.8 and 2.0. Half a microgram of total RNA was directly reverse-transcribed using the First Strand cDNA synthesis kit from Fermentas (Thermo Fisher Scientific). cDNAs were then diluted 100-fold for the following quantitative polymerase chain reaction (PCR). The real-time PCR was done using a 7900HT fast real-time PCR system and a DyNAmo Flash SYBR green quantitative PCR kit (F-415; New England Biolabs, Ipswich, MA) with
gene-specific oligonucleotides designed according to two previous reports (Pérez et al., 2003; Westerink and Schoonen, 2007). Genes evaluated in this study include P450s (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5), UDP glucuronosyltransferases (UGTs; UGT1A9 and UGT2B7), and transporters (MDR1, multidrug resistance-related protein (MRP) 1, and MRP2). β-actin was used as the endogenous control. Each sample was analyzed in duplicate. The mRNA levels of each test gene (P450s, UGTs, and transporters) were normalized to endogenous control. Each sample was analyzed in duplicate. The mRNA levels of each gene were calculated using the ΔΔCt method: ΔCt (test gene) = Ct (β-actin) - ΔCt (endogenous control). The fold changes of mRNA levels were expressed as the relative expression 2^−ΔΔCt.

P450 activity and mRNA levels in the treated groups were expressed relative to that observed in the vehicle control. A greater than 2-fold increase in mRNA or activity of P450s, UGTs, and transporters, relative to the vehicle control, was defined to be induction. We also expressed the data relative to the induction produced by the positive control (rifampin at 10 μM). Emax, the maximal observed induction, and EC50, the concentration that supports half-maximal induction, were estimated by nonlinear regression of fold-induction versus concentration plots using WinNonlin version 5.0 (Pharsight), and a simple Emax and EC50 model. For the estimation of EC50, the depletion of QHS or DHA was taken into account.

**Analytical Methods.** All analyses of P450 enzyme activities were performed with validated high-performance liquid chromatography/tandem mass spectrometry methods. The mass spectrometric analyses were performed using a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, MA) with Shimadzu high-performance liquid chromatography pumps and autosampler systems (Shimadzu, Kyoto, Japan). Chromatographic separation was performed on an Agilent SB-C18 column (5-μm particle size, 150 × 2.1 mm; Agilent Technologies, Santa Clara, CA). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile), which increased linearly from 50% of solvent B to 95% of solvent B during 6 min before column re-equilibration at a flow rate of 0.3 ml/min. The mass spectrometer was operated in the positive ionization electrospray mode. The spray voltage was set at 3.5 kV, and the heated desolvation temperature was set at 350°C. Nitrogen was used as the desolvation gas and set to 800 l/h. The cone voltage and collision energy were set at 30 V and 30 eV, respectively. The parameters of the multiple reaction monitoring transitions for [M + H]+ precursor ions to selected product ions were optimized with the following values for the analytes and IS: 1-hydroxymidazolam, m/z 342.0 to 202.8; (-)-hydroxybupropion, m/z 256.0 to 138.6; 4′-hydroxyclonofenan, m/z 312.3 to 231.2; QHS, m/z 283.1 to 151.0; DHA, m/z 267.1 to 163.1; and the IS alprazolam, m/z 309.0 to 204.4. The lower limit of quantification was 0.05 fmol for three hydroxylated marker metabolites and 0.1 μM for QHS drugs, with acceptable precision and accuracy.

Calibrators and quality-control samples, containing metabolites of substrates in similar acetonitrile-phosphate buffer (0.1 M) and IS were assayed along with the samples. They were processed as described above (under Inhibitory Effect on P450s) and consisted of the above microsomal (see Inhibitory Effect on P450s) or media mix (see Inductive Effect on P450s and UDP Glucuronosyltransferases, and Transporters) without NADPH.

**Prediction of QHS as the Precipitant and Object of DDIs.** The predicted net effect of QHS on CYP3A4 or CYP2B6 activity included data from the three possible mechanisms: competitive inhibition, inactivation, and induction. The comprehensive mathematical model (Obach et al., 2007; Fahmi et al., 2008), which allows for predictions of net effect in the intestine and liver, was used to predict the net effect in each organ as well as area under the concentration-time curve (AUC) of the object (eq. 1):

\[
\text{AUC} = \frac{1}{[A \times B \times C] \times f_u + (1 - f_u)} 	imes \frac{1}{[(X \times Y \times Z) \times (1 - F_G) + F_G]} \tag{1}
\]

The ratio of eq. 1 will yield the predicted AUC ratio of the object drug in the presence (AUCi) relative to the absence (AUC) of a pharmacokinetic DDI arising from simultaneous competitive inhibition (A and X), inactivation (B and Y), and induction (C and Z). AUC ratio predictions were made under the following conditions: assuming a gut and liver effect and using unbound precipitant concentrations in the liver. Parameters \(f_u\) and \(F_G\) represent the fraction of QHS metabolized by P450 enzymes in the liver and the fraction of QHS escaping intestinal metabolism, respectively.

If one lets reversible inhibition term for the hepatic (A) or intestinal portion (X) (eq. 2):

\[
A = \frac{1}{1 + \frac{[I]}{K_i}} = \frac{1}{1 + \frac{C_{hiu}}{K_i}} = \frac{1}{1 + \frac{C_{hiu}}{K_i}} = \Delta C T \text{ (test gene)} = \Delta C T \text{ (endogenous control)} \tag{2}
\]

In the presence of reversible inhibition, \([I]\), \(K_i\), and \(f_u\) represent the concentration of unbound QHS in the liver, apparent inhibition constant, total hepatic influx \(C_{max}\), and unbound fraction in blood, respectively. In eq. 2, \(C_T\) represents the concentration of unbound QHS in the intestinal enteroocytes. If one lets time-dependent inactivation term for hepatic or intestinal (Y) (eq. 3):

\[
B = \frac{k_{deg,G} \times [I]}{[I]_H + K_i} \quad Y = \frac{k_{deg,G} \times [I]}{[I]_H + K_i} \quad \tag{3}
\]

In the presence of inactivation, the overall rate of loss of the enzyme is determined by the pseudo-first-order apparent inactivation rate, which is dependent on \([I]_H\), \(K_i\), the first-order inactivation rate constant \((k_{deg,G})\), and the first-order rate of enzyme degradation \((k_{deg,G})\). If one lets induction term for hepatic (C) or intestinal portion (Z) (eq. 4):

\[
C = 1 + \frac{d \times E_{max} \times [I]}{[I]_H + EC_{50}} \quad Z = 1 + \frac{d \times E_{max} \times [I]}{[I]_H + EC_{50}} \tag{4}
\]

In the presence of induction, the rate of enzyme synthesis is determined by concentrations of QHS in the liver \([I]_H\), the maximal-fold induction \((E_{max})\), and the concentration of QHS associated with half-maximal induction \((EC_{50})\). The parameter \(d\) in eq. 4 represents an empirical calibration factor for the purposes of in vitro to in vivo induction scaling, and it is assumed to be 0.8 in this study. The driving force concentration used for the hepatic interaction was the estimated unbound steady-state \(C_{max}\) at the inlet to the liver, whereas for the intestinal interaction, estimated maximal enteroocyte concentration was used (Obach et al., 2006) (eq. 5):

\[
C_{Hep} = \frac{C_{max}}{Q_h} \tag{5}
\]

![Fig. 1. Dixon plot of QHS inhibition of CYP2B6-catalyzed bupropion hydroxylation in pooled HLM.](image-url)
Prediction parameters, dose of QHS (D), plasma protein binding (f_p), fraction of precipitant drug absorbed (f_a), hepatic blood flow (Q_H), and enterocytic blood flow (Q_g) were assumed to be 500 mg, 0.36, 1.0, 0.3 h⁻¹, 1500, and 248 ml/min, respectively (Gordi et al., 2005; Obach et al., 2007; Gautam et al., 2009). The relative contributions of CYP3A4 (f_m, 0.25) and CYP2B6 (f_m, 0.1) to the metabolism of QHS in HLM were obtained from a previous report (Li et al., 2003). The f_m value at 0.93 was used for the metabolism of midazolam. The following two types of predictions were made: 1) the net effect on each enzyme individually and 2) the net effect on QHS and midazolam. No predictions were made for DHA-mediated DDIs because of lack of estimates for induction E_max, EC50, and high IC50s (>125 μM) for CYP3A and CYP2B6.

Results

In Vitro P450 Inhibition. The inhibitory effects of QHS and DHA on CYP2B6 and CYP3A4 activities were investigated in both HLM and rP450s. The inhibition constants derived from HLM and rP450 systems were consistent. QHS showed a stronger inhibitory effect on CYP2B6 (IC50, 8.4 and 12.1 μM in HLM and rP450, respectively) than DHA (IC50, >125 μM in both systems). The mechanism of inhibition of CYP2B6 by QHS was competitive, and the K_i was estimated to be 4.6 μM. The representative Dixon plot for the inhibitory effect of QHS on bupropion hydroxylation in HLM is shown in Fig. 1. QHS and DHA were both found to be weak inhibitors of CYP3A4 with IC50s of ~50 and >125 μM, respectively.

QHS did not show TDI (<10%) of CYP2B6 or CYP3A4. However, rapid depletion of QHS was observed during the incubation, and less than 15% of 50 μM QHS remained after a 10-min incubation. Therefore, it is difficult to accurately assess from these data whether QHS may act as a mechanism-based inhibitor of CYP2B6 or CYP3A4 in vivo.

Induction of CYP2B6 mRNA Expression and Activity. Rifampin showed expected induction of P450 enzymes (Fig. 2). The induction potency of CYP2B6 decreased in order as follows: rifampin > QHS > DHA, and the magnitude of induction of CYP2B6 by QHS was concentration-dependent (Fig. 3). At 10 μM, QHS induced CYP2B6 activity by 3.1-fold relative to the vehicle control. QHS at 10 μM (2–6 times of plasma C_max) induced CYP2B6 18.0 (activity) and 89.3% (mRNA expression) as effectively as the induction positive control rifampin at 10 μM. QHS showed the induction of CYP2B6 activity with E_max of 1.9-fold and EC50 of 0.6 μM. DHA showed...
minor induction of CYP2B6 activity and mRNA expression. Compared with rifampin at 10 μM, DHA at 10 μM (2–10 times of plasma Cmax) induced CYP2B6 by 11.3 (activity) and 13.9% (mRNA expression), respectively. The fold induction of P450 activity and mRNA expression by rifampin, QHS, and DHA at 10 μM is shown in Table 1. CYP2B6 activity was not induced to the same degree as mRNA expression with increasing concentrations of QHS drugs. The plot of the ratio of induction of CYP2B6 activity to mRNA expression across QHS concentrations is shown in Fig. 4. The ratio decreased as the concentration of QHS increased, which suggested that inactivation or reversible inhibition might exist.

In the media of hepatocytes after the treatment with QHS drugs for 24 h, approximately 38 and 8% of QHS and DHA, respectively, remained. The depletion of QHS and DHA was concentration-dependent, i.e., the greater depletion (P < 0.05) was observed in the lower concentrations (5 μM) compared with the higher concentrations (50 μM). For the estimation of EC50, the depletion of QHS and DHA was taken into account.

In addition, no significant difference was observed, upon visual inspection, in the viability of cells treated with the vehicle and cells treated with rifampin, QHS, or DHA (<50 μM). Cytotoxic effects were observed by 50 μM DHA.

**Induction of CYP3A4 mRNA Expression and Activity.** The magnitude of induction of CYP3A4 activity and transcripts by QHS was concentration-dependent (Fig. 3). At 10 μM, rifampin (positive control of CYP3A4), QHS, and DHA induced CYP3A4 activity by 24.4-, 2.8-, and 2.1-fold, respectively, and mRNA expression by 74.8-, 10.2-, and 3.4-fold, respectively (Table 1). QHS at 10 μM was 9.1 (activity) and 26.0% (mRNA expression) as effective as rifampin. DHA showed minor induction of CYP3A4 activity and mRNA expression. Compared with rifampin, DHA at 10 μM induced CYP3A4 by 5.2% (activity) and 7.7% (mRNA expression). As expected, CYP3A4 activity was not induced to the same degree as mRNA expression with increasing concentrations of QHS drugs. The plot of the ratio of induction of CYP3A4 activity to mRNA expression across QHS concentrations is shown in Fig. 4. The ratio decreased as the concentration of QHS increased, which suggested that inactivation or reversible inhibition might exist. QHS showed induction of CYP3A4 activity with Emax of 3.5-fold and EC50 of 5.9 μM.

**Induction of Other P450s, UGTs, and Transporters.** Rifampin showed expected induction of CYP2C9 activity and transcripts by 3.6- and 4.3-fold, respectively. Neither QHS nor DHA showed significant induction of CYP2C9 activity or mRNA expression (Table 1).

At 10 μM, QHS showed induction of CYP1A2 and CYP2A6 transcripts by 5.7- and 8.4-fold, respectively. QHS and DHA did not show induction of other P450s (CYP2C8, CYP2C19, CYP2D6, CYP2E1, and CYP3A5), UGTs (UGT1A9 and UGT2B7), or transporters (MDR1, MRP1, and MRP2) mRNA expression (Table 1).

**Prediction of Autoinduction and DDIs on QHS.** In vitro data of QHS from the three possible mechanisms (competitive inhibition, inactivation, and induction of CYP3A4 and CYP2B6) were used simultaneously to predict the net effect on CYP3A and CYP2B6 in
vivo. QHS exhibited in vitro reversible inhibition and induction of CYP2B6, and up to 38% of CYP2B6 activity remained according to the unbound precipitant hepatic inlet maximal concentration. The activity data of QHS was used for the prediction of induction. The fraction of CYP2B6 and CYP3A4 activity remaining by induction was estimated to be 2.6 - and 2.1-fold, respectively. The net fold change in CYP2B6 activity was calculated to be 1.6-fold, and the value was 1.9-fold for CYP3A4 activity. DDI was observed with QHS, and the AUC/AUC ratio of QHS in response to itself was estimated to be 59% using activity for induction. The main parameters used for DDI prediction on QHS are shown in Table 2. Predicted fold changes of P450 activity and AUC/AUC ratio of midazolam by QHS treatment are given in Fig. 5. The AUC/AUC ratio of midazolam (the substrate of CYP3A4) was estimated to be 40% using activity for induction. The inhibitory effects of QHS and DHA on two human P450 enzymes (mainly CYP2B6) were modestly inhibited by both QHS and DHA (IC50 values for CYP2B6 were 74.8 and 12.5 μM, respectively). In contrast, CYP3A4 was more significantly inhibited by QHS (IC50 values for CYP3A4 were 7.4 and 1.4 μM, respectively). The IC50 value of QHS for CYP3A4 was approximately 50 μM, with Kι of 25 μM, assuming competitive inhibition. The value of [I]/Kι for CYP3A4 is below the cutoff value at 0.1 (a "remote" inhibitor). These results point to the potential of weak inhibition type DDIs for QHS during multidrug treatment, especially with those drugs metabolized by CYP2B6 or CYP3A4. However, few reports on the clinically relevant inhibition of CYP2B6 exist for this class of compounds. The potential inhibition of CYP2B6 may be compromised by its induction, which could lead to the discrepancy of activity. Previous in vivo studies did not show inhibition of CYP3A4 by QHS (Svensson et al., 1998; Mihara et al., 1999), and the activation of CYP3A4 (1-hydroxymidazolam/midazolam 4-h plasma concentration ratio was increased 2.66-fold) by QHS drugs in healthy subjects was found (Asimus et al., 2007).

Human hepatocytes are commonly used to evaluate P450 induction via an enzyme activity endpoint; however, TDI of P450 can confound data interpretation (McGinnity et al., 2006). In this work, QHS did not show potential TDI of CYP2B6 or CYP3A4, whereas QHS underwent rapid depletion. The clinical consequence of weak or slow TDI may be significant, and this property may compromise the activity readout in cultured cells. The present induction study showed that there was more induction of CYP3A4 transcripts than activity as the concentra-

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<tr>
<th>Genes</th>
<th>Rifampin (ACT)</th>
<th>Rifampin (mRNA)</th>
<th>QHS (ACT)</th>
<th>QHS (mRNA)</th>
<th>DHA (ACT)</th>
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<td>8.4 ± 4.0</td>
<td>N.D.</td>
<td>2.2 ± 1.4</td>
</tr>
<tr>
<td>CYP2C8</td>
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<td>1.3 ± 0.4</td>
<td>N.D.</td>
<td>1.1 ± 0.7</td>
<td>N.D.</td>
<td>0.8 ± 0.6</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>N.D.</td>
<td>1.4 ± 0.3</td>
<td>N.D.</td>
<td>0.9 ± 0.3</td>
<td>N.D.</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>N.D.</td>
<td>2.6 ± 1.2</td>
<td>N.D.</td>
<td>1.2 ± 0.7</td>
<td>N.D.</td>
<td>1.0 ± 1</td>
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<td>CYP2D6</td>
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<td>1.2 ± 0.8</td>
<td>N.D.</td>
<td>1.1 ± 0.3</td>
<td>N.D.</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>N.D.</td>
<td>0.7 ± 0.6</td>
<td>N.D.</td>
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<td>N.D.</td>
<td>0.6 ± 0.2</td>
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<td>UGT1A9</td>
<td>N.D.</td>
<td>2.5 ± 2.3</td>
<td>N.D.</td>
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<td>N.D.</td>
<td>1.4 ± 1.1</td>
</tr>
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<td>UGT2B7</td>
<td>N.D.</td>
<td>1.2 ± 0.7</td>
<td>N.D.</td>
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<td>N.D.</td>
<td>0.7 ± 0.4</td>
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<tr>
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<td>2.3 ± 1.1</td>
<td>N.D.</td>
<td>1.2 ± 0.3</td>
<td>N.D.</td>
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<tr>
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<td>N.D.</td>
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<td>0.8 ± 0.5</td>
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N.D., not determined.

**Discussion**

The inhibitory effects of QHS and DHA on two human P450 enzymes (CYP2B6 and CYP3A4) were investigated in this study. The P450 enzymes studied (mainly CYP2B6) were modestly inhibited by QHS, but not by DHA (IC50 > 125 μM). The inhibition mechanism of bupropion hydroxylation (CYP2B6) by QHS was competitive inhibition. On the basis of the \( K_ι \) value (4.6 μM) and QHS \( C_{\text{max}} \) (total drug, 1.6 μM; unbound drug, 0.58 μM) (Ashton et al., 1998b; Li et al., 2003), CYP2B6 activity is predicted to be inhibited 12.5 (unbound) and 34.8% (total), which suggested that QHS is a “possible” CYP2B6 inhibitor. The IC50 value of QHS for CYP3A4 was approximately 50 μM, with \( K_ι \) of 25 μM, assuming competitive inhibition. The value of \([I]/K_ι \) for CYP3A4 is below the cutoff value at 0.1 (a “remote” inhibitor). These results point to the potential of weak inhibition type DDIs for QHS during multidrug treatment, especially with those drugs metabolized by CYP2B6 or CYP3A4. However, few reports on the clinically relevant inhibition of CYP2B6 exist for this class of compounds. The potential inhibition of CYP2B6 may be compromised by its induction, which could lead to the discrepancy of activity. Previous in vivo studies did not show inhibition of CYP3A4 by QHS (Svensson et al., 1998; Mihara et al., 1999), and the activation of CYP3A4 (1-hydroxymidazolam/midazolam 4-h plasma concentration ratio was increased 2.66-fold) by QHS drugs in healthy subjects was found (Asimus et al., 2007).

Human hepatocytes are commonly used to evaluate P450 induction via an enzyme activity endpoint; however, TDI of P450 can confound data interpretation (McGinnity et al., 2006). In this work, QHS did not show potential TDI of CYP2B6 or CYP3A4, whereas QHS underwent rapid depletion. The clinical consequence of weak or slow TDI may be significant, and this property may compromise the activity readout in cultured cells. The present induction study showed that there was more induction of CYP3A4 transcripts than activity as the concentra-
tion of QHS increased, which suggested that at higher concentration of QHS, weak or slow inactivation may have dampened the observed increase in CYP3A4A activity relative to mRNA transcripts. No QHS or DHA was found in the media of hepatocytes washed by PBS twice, which implies there is no significant reversible inhibition caused by the remaining drug in the media.

We found that QHS was a potent inducer of CYP3A4 and CYP2B6 transcripts and activity, and QHS was also a potent inducer of CYP1A2 and CYP2A6 transcripts. QHS appeared to be a more potent inducer of P450 enzymes than DHA. In these three preparations of hepatocytes treated with QHS at 10 μM, the relative effectiveness for CYP2B6 induction was lower than the U.S. Food and Drug Administration’s cutoff value of 40% ("likely" to be an inducer) but higher than the cutoff value of 15% ("may" to be an inducer). At 10 μM, QHS showed minor induction of CYP3A4 in all three batches of hepatocytes. The average induction of CYP2B6 and CYP3A4 by DHA at 10 μM (2–10 times of \(C_{\text{max}}\)) was lower than the cutoff value of 15% ("may").

Clearance of QHS drugs from the body is in part mediated by CYP2B6 and CYP3A4 (Svensson and Ashton, 1999). The expression levels of these two enzymes are very variable, and these enzymes are also highly inducible by xenobiotics. Therefore, the autoinduction observed with QHS drugs may be a result of autoinduction of CYP3A and/or CYP2B6. This has been shown for CYP2B6 in vivo (Simonsson et al., 2003; Elsherbiny et al., 2008). Induction of CYP3A4 and CYP2B6 mRNA expression by QHS has been observed in primary human hepatocytes and in the human intestinal cell line LS174T (Burk et al., 2005), and activation of CYP3A4 by QHS drugs was found in healthy subjects (Asimus et al., 2007). However, another study showed CYP3A4 enzymatic activity was not induced in humans (Svensson et al., 1998). As discussed above, the discrepancy of CYP3A4 activity may be caused by the net effect of induction and inhibition. In addition, the choice of in vivo probe substrates may lead to variable results.

We also investigated the induction of CYP2C8, CYP2C9, and CYP2C19 by QHS drugs. For two QHS drugs, there was no change of CYP2C mRNA expression (CYP2C8, CYP2C9, and CYP2C19) and activity (CYP2C9). It is probably due to the fact that the CYP2C isoenzymes are induced less than the CYP2B isoenzymes by even potent CAR or PXR activators, even though they are regulated by the same receptor. In the study by Burk et al. (2005), concentration-dependent induction of CYP2C19 mRNA expression by QHS was not clearly noticeable, and only half batches of human hepatocytes showed slight induction.

Another two inducible enzymes by QHS were CYP1A2 and CYP2A6. In two previous reports (Bapiro et al., 2001, 2005), QHS (\(K_i = 0.43 \mu M\)) and DHA (\(K_i = 3.67 \mu M\)) were found to be potent inhibitors of CYP1A2. The inhibitory effect on CYP1A2 by QHS and DHA was indicated by a decrease in the paraxanthine/caffeine ratio between day 6 and day 1 after administration of QHS drugs to healthy subjects. A significant increase was also observed in the CYP1A2 index from day 1 to day 5 in the QHS group (Bapiro et al., 2005), suggesting an induced activity of CYP1A2, which is consistent with our study. It was speculated that QHS plays a dual role in the regulation of CYP1A2, i.e., its expression was induced and activity was inhibited.

The results of induction of phase II enzymes showed that there was no induction of their transcripts by QHS or DHA, even though UGT1A9 and UGT2B7 were the main enzymes involved in the metabolism of DHA (Ilett et al., 2002). We also demonstrated that the mRNA expression of three main transporters including MDRI was not induced by QHS or DHA. Results obtained with an in situ rat perfusion model have shown that pretreatment with QHS did not change transport activity of intestinal P-glycoprotein (Svensson et al., 1999). In another study (Burk et al., 2005), MDRI mRNA expression was partially induced up to 5-fold by QHS at a higher concentration (300 μM).

Given the concurrent of inhibition and induction of CYP2B6 by Artemisinin, the prediction of DDI from in vitro data was complicated. Although the effectiveness of inhibition or induction of CYP3A4 is lower than the cutoff in some HLM or hepatocytes preparations, QHS drugs may still produce DDI with coadministered drugs, especially when the drugs are metabolized by both CYP3A4 and CYP2B6. The prediction of DDI showed that QHS AUCi/AUC ratio could decrease to 59% by its autoinduction. In addition, the activity of CYP2B6 and CYP3A4 will increase to 1.6- and 1.9-fold, respectively, after QHS treatment. The DDI between QHS and other coadministered drug will depend on the \(f_{\text{in}}\) and \(E_{\text{max}}\) values of those object drugs. The AUCi/AUC ratio of midazolam (the substrate of CYP3A4) was estimated to be 40% using activity for induction. The DDI results presented here are supported by the finding that increased CYP3A4 activity (1.6-fold) by QHS administration was observed even after a single oral dose (Asimus et al., 2007).

Several factors may impose a degree of uncertainty in the DDI prediction on QHS. \(E_{\text{max}}\) values obtained from mRNA induction and enzyme activity data can be altered by factors such as variations in rates of transcription, translation, and protein synthesis. The metabolic instability of inducers in hepatocyte cultures during the incubation time may lead to additional uncertainty. Consumptions of inducers could decrease the actual concentrations in the medium and thus differ from the nominal concentrations used for the calculation of kinetic parameters. In this study, the depletion of QHS was taken into account in the estimation of \(E_{\text{SP}}\). The variability of parameters, such as plasma protein binding ratio \(f_{\text{P}}\) and \(f_{\text{u}}\), will also play a role in the DDI prediction.

From the point of inhibition and induction, DHA seems to be a more promising antimalarial than its parent drug QHS. Unfortunately,
an induction EC50 estimate was not obtainable in this study because of several irregular values in the induction-response dataset. The DDI prediction on DHA was therefore not performed. However, the present results showed the inducing capacity is different among QHS drugs, which is important when selecting drugs to be used in antimalarial combination therapy such that the potential for DDIs is minimized.

In conclusion, our data showed QHS drugs are potential inducers of P450 enzymes, and the most inducible are CYP2B6 and CYP3A4, which are believed to be the main enzymes involved in the autoinduction of QHS drugs. Several main UGT enzymes and transporters were not induced by QHS or DHA. QHS also showed weak inhibition of CYP2B6, and it did not show TDI of CYP2B6 or CYP3A4. The induction of CYP2B6 and CYP3A was characterized for QHS, and this provided evidence for withdrawing monotherapy and recommending ACTs. Because the DDI prediction showed that QHS could result in a net induction rather than inhibition, the combined drugs (as well as QHS) that are cleared by CYP2B6 and CYP3A4 may require a dose increase to maintain therapy.

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Authorship Contributions

Participated in research design: Xing, Kirby, and Goodlett.
Conducted experiments: Xing, Kirby, Whittington, and Wan.
Performed data analysis: Xing and Kirby.
Wrote or contributed to the writing of the manuscript: Xing, Kirby, and Goodlett.

References


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