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Evaluation of CYPs inhibition and induction by artemisinin antimalarials in human liver microsomes and primary human hepatocytes

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

Department of Medicinal Chemistry, University of Washington, Seattle, WA 98195, USA (J.X., D.W. and D.R.G.)

Department of Pharmaceutics, University of Washington, Seattle, WA 98195, USA (B.J.K.)

Department of Pharmaceutical Sciences, Shandong University, Jinan, 250012, China (J.X.)

Institute for Systems Biology, Seattle, WA 98103, USA (Y.W.)

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Running title: CYPs inhibition and induction by artemisinin drugs

Corresponding author: Dr. David R. Goodlett

Postal address:

Department of Medicinal Chemistry

University of Washington, Box 357610

Seattle, WA 98195, USA

Telephone number: 206-616-4586

Fax number: (206) 685 3252

E-mail address: goodlett@u.washington.edu

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ABBREVIATIONS:

CYP, cytochrome P450; QHS, artemisinin; DHA, dihydroartemisinin; HLM, human liver microsome; UGT, UDP-glucuronosyltransferase; PXR, pregnane X receptor; CAR, constitutive androstane receptor; AHR, aryl hydrocarbon receptor; DDI, drug-drug interaction; TDI, time-dependent inhibition; MDR, multidrug resistance; MRP, multidrug-resistance related protein; PCR, polymerase chain reaction.

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ABSTRACT

Artemisinin drugs have become the first-line antimalarials in areas of multi-drug resistance. However, monotherapy with artemisinin drugs results in comparatively high recrudescence rates. Autoinduction of CYP-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 cytochrome P450s (especially CYP2B6 and 3A4) inhibited or induced by two artemisinin drugs, artemisinin (QHS) and dihydroartemisinin (DHA) using HLM, recombinant CYP 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 (IC_{50}, ~50 μM) and did not show measurable time dependent inhibition of either CYP2B6 or 3A4. DHA inhibited neither CYP2B6 nor 3A4 (IC_{50} > 125 μM). Additionally, 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 CYPs, UGTs 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 CYP-mediated DDIs indicate autoinduction of QHS clearance with the AUC_i/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.

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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 et al., 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, while 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 (Hien et al., 2011; Chinh et al., 2009). However, limited data was 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 several-fold decrease in plasma concentration of QHS with a corresponding increase in oral clearance. Autoinduction of drug metabolism has been suggested for this time-dependency (Ashton et al., 1998; Svensson and Ashton, 1999). The elimination of QHS in HLM is mediated primarily by CYP2B6 with a probable secondary contribution of CYP3A4 and 2A6 (Svensson and Ashton, 1999). The relative contributions of CYP2B6, 3A4 and 2D6 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 2C9 (Simonsson et al., 2003; Svensson and Ashton, 1999). Treatment of

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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 MDR1 (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 (PMs) (Simonsson et al., 2003). QHS appears to induce other enzymes including CYP2C19 (omeprazole as a marker; Svensson et al., 1998; Mihara et al., 1999), 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 PXR, CAR and AHR (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 EC_{50} of approximately 30 μM (10 to 15 times higher than C_{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 (HEK293), QHS activated CAR (maximally by 1.8-fold at 10 μM) but not PXR (Simonsson et al., 2006). 50 μM 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 (Asimus et al., 2007; Bapiro et al., 2005). Taken together, these results showed that QHS drugs may affect CYPs as both inducer and inhibitor, which may compromise the results of CYP activity in vivo.

The data listed above indicates that QHS and DHA have the potential to induce multiple CYP enzymes

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and possibly transporters. Because the net effect in vivo may be a combination of reversible inhibition, induction and irreversible inactivation of these CYP enzymes, we evaluated QHS and DHA for the potential to inhibit or inactivate CYP2B6 and 3A in recombinant systems as well as human liver microsomes. In addition, we evaluated QHS and DHA for their potential to induce multiple CYP 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 CYP enzymes in vivo, and evaluated the correlation between the predicted interaction potential from our in vitro studies and previously reported 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. (purity >99.0%, Yunnan, China). Bupropion, (±)-hydroxybupropion, midazolam, α-hydroxymidazolam, alprazolam (internal standard for LC-MS assay) were purchased from Cerilliant Corp. (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 Fisher Scientific, and were of highest purity available.

Inhibitory Effect on Cytochrome P450s. To measure the inhibition effects of QHS and DHA on the activities of CYP2B6 and 3A4, 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 3A4) activity in a direct manner using both pooled HLMs (n=5, mixed gender) and recombinant CYP enzymes (BD Gentest, Woburn, MA). In brief, duplicate incubations were conducted at 37°C in 200-µl incubation with the final

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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 rCYPs (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 prior to the initiation of metabolic reaction with NADPH and terminated after 3 min (CYP3A4) or 10 min (CYP2B6) by an equal volume of acetonitrile containing an appropriate internal standard. Precipitated protein was removed by centrifugation (1000 *g* 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 (IC_{50}) was determined at a substrate concentration equal to K_m. The apparent inhibition constant (K_i) 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 based on the IC_{50} value (approximately 1⁄20×IC_{50}, 1/5×IC_{50}, 1/2×IC_{50}, and 2×IC_{50}). The concentrations of marker substrates were chosen (approximately 1⁄4×K_m, K_m, and 3×K_m) with regard to their Michaelis-Menten kinetics (K_m). K_m values of CYP3A4 and CYP2B6 were 2 μM and 50 μM, respectively. The vehicle controls were analyzed in parallel. The K_i values were calculated by nonlinear regression analysis by fitting different models of enzyme inhibition to the kinetic data using WinNolin V5.0 (Pharsight, Mountainview, CA).

The time-dependent inhibitory (TDI) potency of CYP2B6 and CYP3A4 by QHS was investigated with pooled HLMs. QHS was preincubated at 37 ± 1°C, in duplicate, with HLMs (1 mg/ml) and NADPH (1 mM) in 0.1 M phosphate-buffered saline (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 K_m). The incubation was continued for 3 min (CYP3A4) or 10 min

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(CYP2B6) and quenched in ice-cold acetonitrile (containing IS) to measure residual P450 activity.

Inductive Effect on Cytochrome P450s, UGTs 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% CO_2 in 24 well plates in serum free Williams E medium supplemented with 100 nM dexamethasone and insulin-transferrin-selenium (Cellzdirect, Durham, NC). 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% DMSO in supplemented Williams E medium) 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 phosphate-buffered saline (PBS) twice before CYP activity was assessed. Induction of CYP3A4, 2B6 and CYP2C9 activities in primary human hepatocytes were determined based on two validated cocktail assays (Kenny et al., 2008; McGinnity et al., 2006). Human hepatocytes were incubated with bupropion (100 μM), midazolam (2 μM), diclofenac (9 μM) to measure the activity of CYP2B6, 3A4 and 2C9, respectively, after 72 hours 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

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indicated with the absorbance ratio of 260/280 nm between 1.8 and 2.0. 0.5 μg of total RNA was directly reverse-transcribed using the First Strand cDNA synthesis kit from Fermentas (Thermo Fisher Scientific, MD, USA). cDNAs were then diluted 100-fold for the following qPCR. The real-time PCR was done using a 7900HT fast real-time PCR system and a DyNAmo Flash SYBR green qPCR kit (F-415; NEB) with gene-specific oligonucleotides designed according to two previous reports (Westerink and Schoonen, 2007; Pérez et al., 2003). Genes evaluated in this study include CYPs (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5), UGTs (UGT1A9 and 2B7) and transporters (MDR1, MRP-1, MRP-2). β-actin was used as the endogenous control. Each sample was analyzed in duplicate. The mRNA levels of each test gene (CYPs, UGTs and transporters) were normalized to β-actin, according to the following formula: C_T (test gene)-C_T (β-actin) = ΔC_T. Thereafter, the relative mRNA levels of each gene were calculated using the $\Delta\Delta C_T$ method: ΔC_T (test gene) -ΔC_T (test gene in the DMSO control) = $\Delta\Delta C_T$ (test gene). The fold changes of mRNA levels were expressed as the relative expression $2^{-\Delta\Delta CT}$.

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). E_{max}, the maximum observed induction, and EC_{50}, the concentration that supports half-maximal induction were estimated by non linear regression of fold-induction versus concentration plots using Winnolin V5.0 (Pharsight, Mountainview, CA), and a simple E_{max} and EC_{50} 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 (HPLC)-tandem mass spectrometry methods. The mass

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spectrometric analyses were performed using a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters) with Shimadzu HPLC 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, Palo Alto, CA). The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile, which increased linearly from 50% of solvent B to 95% B during 6.0 min prior to 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 heated desolvation temperature at 350°C. Nitrogen was used as the desolvation gas and set to 800 L/hr. The cone voltage and collision energy was set at 30 V and 30 eV, respectively. The parameters of the multiple reaction monitoring transitions for the $[M+H]^+$ precursor ions to selected product ions were optimized with the following values for the analytes and internal standard: 1'-hydroxymidazolam, *m/z* 342.0 to 202.8; (±)-hydroxybupropion, *m/z* 256.0 to 138.6; 4'-hydroxydiclofenac, *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 internal standard alprazolam *m/z* 309.0 to 204.4. The lower limit of quantification (LLOQ) was 0.05 μM for three hydroxylated marker metabolites, and the LLOQ was 0.1 μM for QHS drugs, with acceptable precision and accuracy.

Calibrators and quality control samples, containing metabolites of substrates in similar acetonitrile-0.1 M phosphate buffer and internal standard were assayed along with the samples. They were processed as described above and consisted of the above microsomal or media mix without NADPH.

Prediction of QHS as the Precipitant and Object of DDIs. The predicted net effect of QHS on CYP3A4 or 2B6 activity, included data from the three possible mechanisms: competitive inhibition, inactivation, and induction. The comprehensive mathematical model (Fahmi et al., 2008; Obach et al., 2007), which allows for predictions of net effect in the intestine and liver was used to predict the net effect

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in each organ as well as AUC of the object:

$$\frac{AUC_i}{AUC} = \left(\frac{1}{[A \times B \times C] \times f_m + (1 - f_m)}\right) \times \left(\frac{1}{[X \times Y \times Z] \times (1 - F_G) + F_G}\right) \quad (1)$$

The ratio of eq. 1 will yield the predicted AUC ratio of the object drug in the presence (AUC_i) relative to the absence (AUC) of a pharmacokinetic drug-drug interaction 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 condition: assuming a gut and liver effect and using unbound precipitant concentrations in the liver. Parameters f_m 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):

$$A = \frac{1}{1 + \frac{[I]_H}{K_I}} = \frac{1}{1 + \frac{C_{hep}, f_u}{K_I}} \qquad X = \frac{1}{1 + \frac{[I]_G}{K_I}} = \frac{1}{1 + \frac{C_G, f_u}{K_I}} \quad (2)$$

In the presence of reversible inhibition, $[I]_H$, K_I, C_{hep}, f_u represent the concentration of unbound QHS in the liver, apparent inhibition constant, total hepatic inlet C_{max} and unbound fraction in blood, respectively. In eq. 2, $[I]_G$ represents the concentration of unbound QHS in the intestinal enterocytes.

If one lets time-dependent inactivation term for hepatic (B) or intestinal portion (Y):

$$B = \frac{k_{deg}}{k_{deg,\,H} + \frac{[I]_H \times k_{inact}}{[I]_H + K_I}} \qquad Y = \frac{k_{deg}}{k_{deg,\,G} + \frac{[I]_G \times k_{inact}}{[I]_G + K_I}} \quad (3)$$

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 upon $[I]_H$, K_I, the first order inactivation rate constant (k_{inact})

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and the first-order rate of enzyme degradation (k_{deg}).

If one lets induction term for hepatic (C) or intestinal portion (Z):

$$C = 1 + \frac{d \cdot E_{max} \cdot [I]_H}{[I]_H + EC_{50}} \qquad Z = 1 + \frac{d \cdot E_{max} \cdot [I]_G}{[I]_G + EC_{50}} \qquad (4)$$

In the presence of induction, the rate of enzyme synthesis is determined by concentrations of QHS in the liver $[I]_H$, the maximum fold induction (E_{max}) and the concentration of QHS associated with half-maximum 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 enterocyte concentration was used (Obach et al., 2006):

$$C_{hep} = C_{max} + \frac{D \cdot k_a \cdot F_a}{Q_h} \qquad (5)$$

Prediction parameters, dose of QHS (D), plasma protein binding (f_u), fraction of precipitant drug absorbed (F_a), absorption rate (k_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 ml/min and 248 ml/min, respectively (Gautam et al., 2009; Gordi et al., 2005; Obach et al., 2007). The relative contributions of CYP3A4 (f_m, 0.25) and 2B6 (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. Two types of predictions were made: 1) predicting the net effect on each enzyme individually, and 2) the net effect on QHS and midazolam. No predictions were made for DHA

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mediated DDIs owing to lack of estimates for induction E_{max}, EC_{50} as well as high IC50s (>125 μM) for CYP3A and 2B6.

Results

In Vitro P450 Inhibition. The inhibitory effects of QHS and DHA on CYP2B6 and 3A4 activities were investigated in both HLMs and rCYPs. The inhibition constants derived from HLMs and rCYPs systems were consistent. QHS showed a stronger inhibitory effect on CYP2B6 (IC_{50}, 8.4 μM in HLM; 12.1 μM in rCYP) than DHA (IC_{50} >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 IC_{50}s of ~50 μM and > 125 μM, respectively.

QHS did not show time-dependent inhibition (TDI) (<10%) of CYP2B6 or 3A4. 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 3A4 in vivo.

Induction of CYP2B6 mRNA Expression and Activity. Rifampin showed expected induction of CYP 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 EC_{50} of 0.6 μM. DHA showed minor induction of CYP2B6 activity and mRNA expression.

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Compared to rifampin at 10 μM, DHA at 10 μM (2-10 times of plasma C_{max}) induced CYP2B6 by 11.3% (activity) and 13.9% (mRNA expression), respectively. The fold induction of CYPs 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 hours, 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 to 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 to rifampin, DHA at 10 μM induced CYP3A4 by 5.2% (activity) and

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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 E_{max} of 3.5-fold and EC_{50} of 5.9 μM.

Induction of Other CYPs, 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 2A6 transcripts by 5.7- and 8.4-fold, respectively. QHS and DHA did not show induction of other CYPs (CYP2C8, 2C19, 2D6, 2E1, 3A5), UGTs (UGT1A9 and 2B7) or transporters (MDR1, MRP1, 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 2B6 were used simultaneously to predict the net effect on CYP3A and 2B6 in vivo. QHS exhibited in vitro reversible inhibition and induction of CYP2B6, and up to 38% of CYP2B6 activity remained based on the unbound precipitant hepatic inlet maximum concentration. The activity data of QHS was used for the prediction of induction. The fraction of CYP2B6 and 3A4 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_i/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 is shown in Table 2. Predicted fold changes of CYPs activity and AUC_i/AUC ratio of

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midazolam by QHS treatment are given in Fig.5.

The AUC_i/AUC ratio of midazolam (the substrate of CYP3A4) was estimated to be 40% using activity for induction after QHS treatment (Fig. 5).

Discussion

The inhibitory effects of QHS and DHA on two human P450 enzymes (CYP2B6 and 3A4) were investigated in this study. The P450 enzymes studied (mainly CYP2B6) were modestly inhibited by QHS, but not by DHA (IC_{50} value >125 μM). The inhibition mechanism of bupropion hydroxylation (CYP2B6) by QHS was competitive inhibition. Based on the K_i value (4.6 μM) and QHS C_{max} (total drug: 1.6 μM, and unbound drug: 0.58 μM) (Ashton et al., 1998; 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 IC_{50} value of QHS for CYP3A4 was approximately 50 μM, with K_i of 25 μM, assuming competitive inhibition. The value of $[I]/K_i$ 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 3A4. 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 (Mihara et al., 1999; Svensson et al., 1998), 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

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show potential TDI of CYP2B6 or 3A4, while 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 concentration of QHS increased, which suggested that at higher concentration of QHS, weak or slow inactivation may have dampened the observed increase in CYP3A4 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 2B6 transcripts and activity, and QHS was also a potent inducer of CYP1A2 and 2A6 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 FDA's cutoff value of 40% (“likely” to be an inducer) but higher than the cutoff value of 15% (“may”). At 10 μM, QHS showed minor induction of CYP3A4 in all three batches of hepatocytes. The average induction of CYP2B6 and 3A4 by DHA at 10 μM (2-10 times of C_{max}) was lower than the cutoff value of 15% (“may”).

Clearance of QHS drugs from the body is in part mediated by CYP2B6 and 3A4 (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 either CYP3A and/or CYP2B6. This has been shown for CYP2B6 in vivo (Simonsson et al., 2003; Elsherbiny et al., 2008). Induction of CYP3A4 and 2B6 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

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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, 2C9 and 2C19 by QHS drugs. For two QHS drugs, there was no change of CYP2C mRNA expression (CYP2C8, 2C9 and 2C19) 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 Burk's study, concentration-dependent induction of CYP2C19 mRNA expression by QHS was not clearly noticeable, and only half batches of human hepatocytes showed slight induction (Burk et al., 2005).

Another two inducible enzymes by QHS were CYP1A2 and CYP2A6. In two previous reports (Bapiro et al., 2001; Bapiro et al., 2005), QHS (K_i=0.43 μM) and DHA (K_i=3.67 μM) 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 2B7 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 MDR1 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 Pgp (Svensson et al., 1999). In

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another study (Burk et al., 2005), MDR1 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. Though, 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 2B6. The prediction of DDI showed QHS AUC_i/AUC ratio could decrease to 59% by its autoinduction. Additionally, the activity of CYP2B6 and 3A4 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 $f_{m,\,CYP}$ and F_G values of those object drugs. The AUC_i/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_{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 hepatocytes 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 EC_{50}. The variability of parameters, such as plasma protein binding ratio (f_u) and $f_{m,\,CYP}$ 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

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parent drug QHS. Unfortunately, an induction EC_{50} 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 inductive 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 CYP enzymes, and the most inducible are CYP2B6 and 3A4, 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 time-dependent inhibition (TDI) of CYP2B6 or 3A4. The induction of CYP2B6 and 3A was characterized for QHS, and this provided evidence for withdrawing monotherapy and recommending ACTs. Since the DDI prediction showed that QHS could result in a net induction rather than inhibition, the combined drugs (as well as QHS) which are cleared by CYP2B6 and CYP3A4 may require dose increase to maintain therapy.

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Authorship Contributions:

Participated in research design: Xing, Kirby and Goodlett

Conducted experiments: Xing, Kirby, Wan and Whittington

Contributed new reagents or analytical tools: Not applicable

Performed data analysis: Xing and Kirby

Wrote or contributed to the writing of the manuscript: Xing, Kirby and Goodlett

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Footnotes:

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Figure legends:

Fig. 1 Dixon plot of QHS inhibition of CYP2B6-catalyzed bupropion hydroxylation in pooled HLMs.

Fig. 2 Induction of CYP2B6, 3A4 and 2C9 mRNA expression (A) and activity (B) by rifampin (Rif), artemisinin (QHS) and dihydroartemisinin (DHA) at the concentration of 10 μM in three primary human hepatocytes preparations (*: changes greater than 2-fold over negative control).

Fig. 3 Fold induction of CYP2B6 and 3A4 activity (−△−) and mRNA transcripts (−◆−) in three primary human hepatocytes preparations treated by artemisinin (QHS; A) or dihydroartemisinin (DHA, B) at different concentrations. In all instances, only changes greater than 2-fold over negative control were considered as induction.

Fig. 4 The plot of the ratio of induction of CYP3A4 (left) and 2B6 (right) activity to mRNA expression in three primary human hepatocytes preparations treated by artemisinin (QHS).

Fig. 5 Predicted fold changes of CYPs activity and AUC_i/AUC ratio of midazolam (MDZ) after QHS treatment.

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TABLE 1

Induction of CYPs, UGTs and transporters activity and mRNA expression by rifampin (Rif.), artemisinin (QHS) or dihydroartemisinin (DHA) at 10 μM in primary human hepatocytes derived from three donors. Data represent fold induction relative to negative vehicle control.

Genes	Rif.		QHS		DHA	
	ACT	mRNA	ACT	mRNA	ACT	mRNA
CYP2B6	13.9 ± 7.2	20.2 ± 7.4	3.1 ± 0.8	16.2 ± 0.9	2.9 ± 2.7	3.5 ± 0.8
CYP3A4	24.4 ± 9.7	74.8 ± 92.3	2.8 ± 0.7	10.2 ± 6.9	2.1 ± 0.7	3.4 ± 1.3
CYP2C9	3.6 ± 2.6	4.3 ± 3.1	1.2 ± 0.1	1.8 ± 0.4	1.2 ± 0	1.0 ± 0.2
CYP1A2	N.D.	1.1 ± 0.7	N.D.	5.7 ± 3.8	N.D.	1.1 ± 0.7
CYP2A6	N.D.	8.4 ± 4.3	N.D.	8.4 ± 4.0	N.D.	2.2 ± 1.4
CYP2C8	N.D.	1.3 ± 0.4	N.D.	1.1 ± 0.7	N.D.	0.8 ± 0.6
CYP2C19	N.D.	1.4 ± 0.3	N.D.	0.9 ± 0.3	N.D.	1.3 ± 0.4
CYP3A5	N.D.	2.6 ± 1.2	N.D.	1.2 ± 0.7	N.D.	1.0 ± 1.0
CYP2D6	N.D.	1.2 ± 0.8	N.D.	1.1 ± 0.3	N.D.	0.7 ± 0.1
CYP2E1	N.D.	0.7 ± 0.6	N.D.	1.1 ± 0.2	N.D.	0.6 ± 0.2
UGT1A9	N.D.	2.5 ± 2.3	N.D.	1.8 ± 1.2	N.D.	1.4 ± 1.1
UGT2B7	N.D.	1.2 ± 0.7	N.D.	1.1 ± 0.6	N.D.	0.7 ± 0.4
MDR1	N.D.	2.3 ± 1.1	N.D.	1.2 ± 0.3	N.D.	1.0 ± 0.5
MRP-1	N.D.	0.8 ± 0.4	N.D.	0.7 ± 0.3	N.D.	0.8 ± 0.4
MRP-2	N.D.	1.0 ± 0.4	N.D.	0.9 ± 0.5	N.D.	0.8 ± 0.5

N.D., not determined

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TABLE 2

Parameters used for the DDI prediction on QHS.

CYPs	f_m	K_i (μM)	E_{max} (fold)	EC_{50} (μM)
CYP2B6	0.1	4.6	3.5	5.9
CYP3A4	0.25	25.0	1.9	0.6

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Fig 1

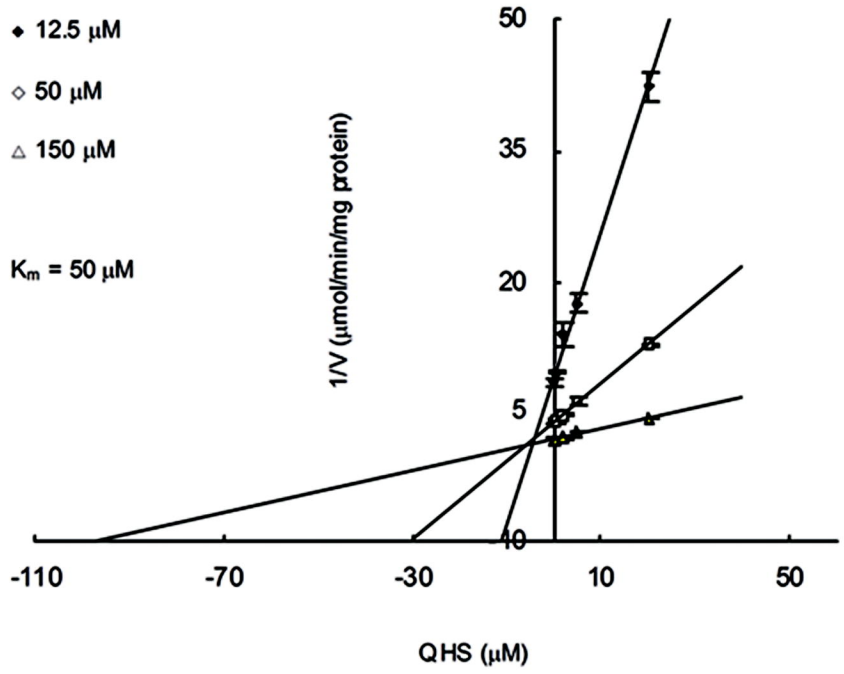

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Fig. 2

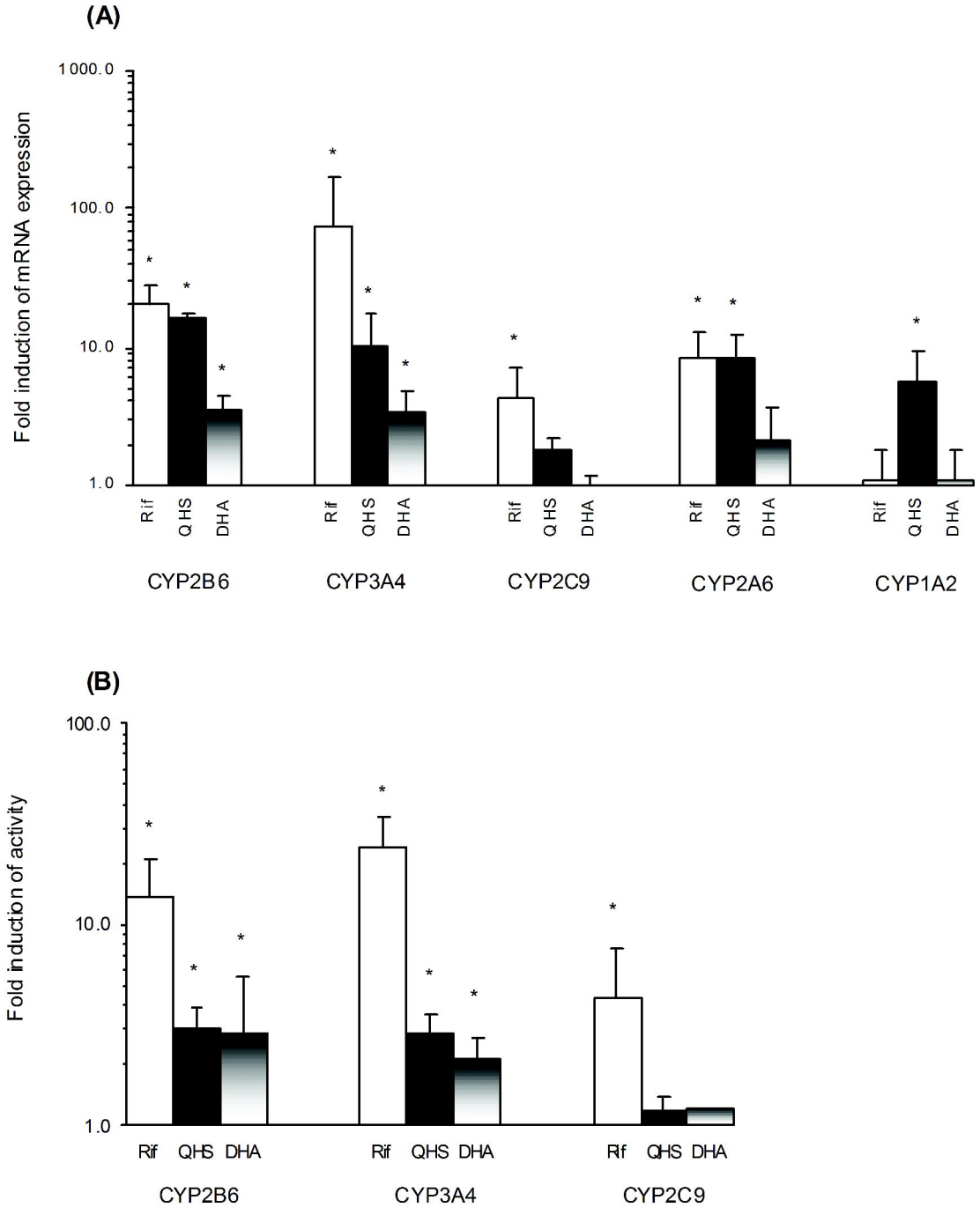

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Fig. 3

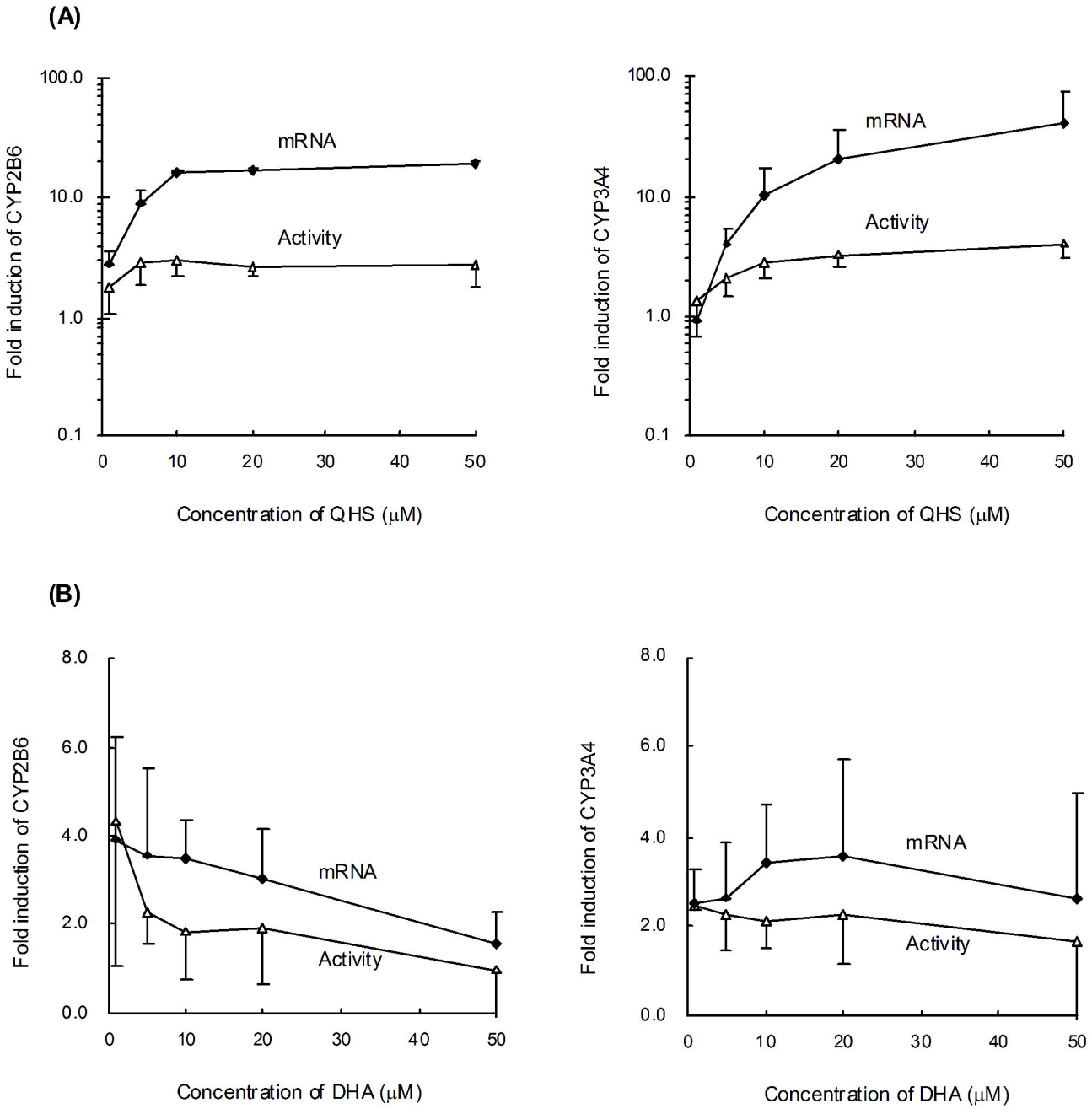

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Fig. 4

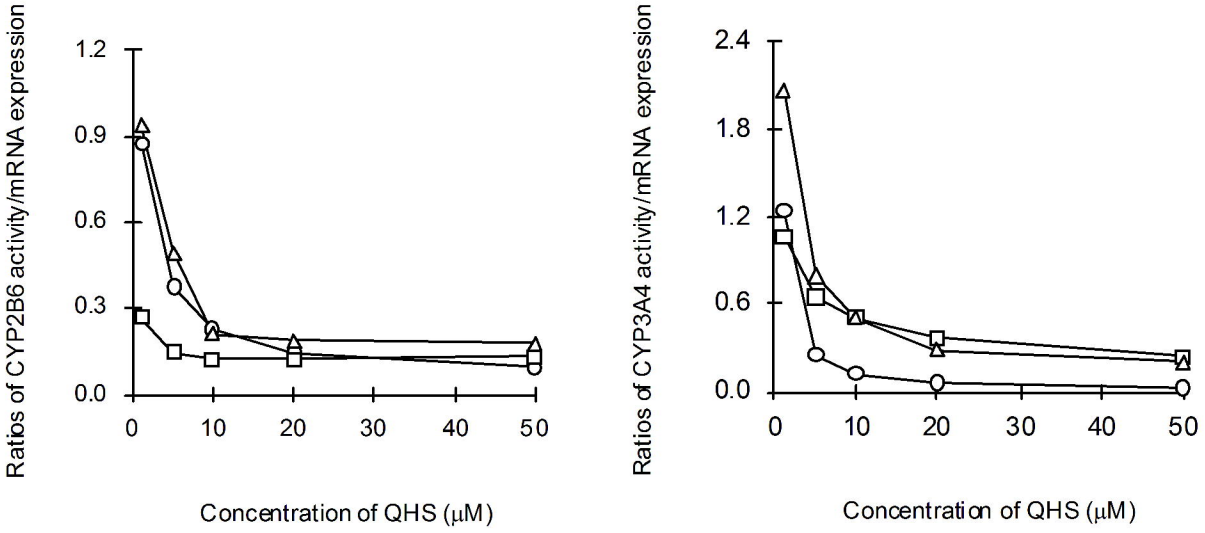

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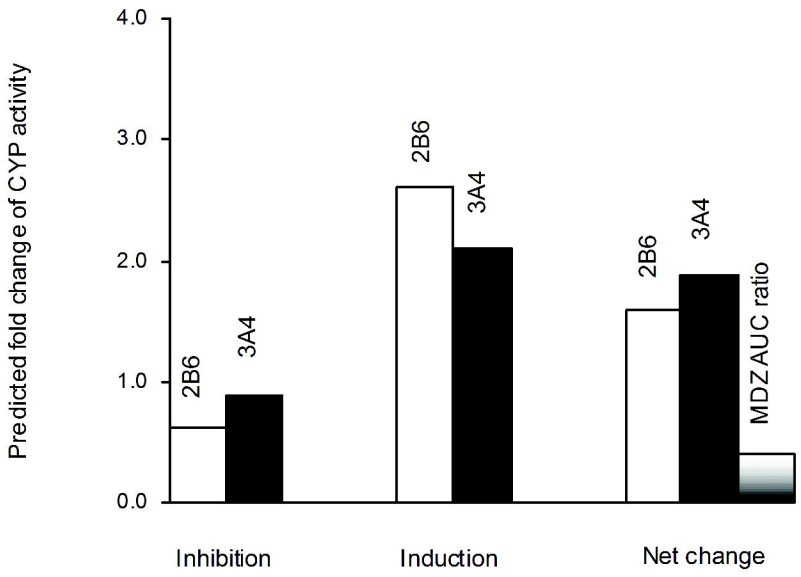
Fig. 5



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