Metabolic interactions of selected antimalarial and non-antimalarial drugs with the major pathway (3-hydroxylation) of quinine in human liver microsomes

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Aims Nine antimalarial (plus two metabolites of proguanil) and twelve non-antimalarial drugs were tested for their possible interaction with CYP3A4-catalysed 3-hydroxylation of quinine by human liver microsomes *in vitro*.

Methods 3-Hydroxyquinine was assayed in the incubation mixture by an h.p.l.c. method using fluorometric detection. The respective IC_{50} values were estimated for the twenty-one drugs and two metabolites of proguanil tested herein.

Results Thirteen drugs exhibited an inhibitory effect on the 3-hydroxylation of quinine. According to the respective mean IC_{50} values, the inhibitory rank order of the drugs was: ketoconazole > troleandomycin (TAO, with preincubation) > doxycycline > omeprazole > primaquine > tetracycline = TAO (without preincubation) > nifedipine > erythromycin > verapamil > cimetidine > diltiazem > oleandomycin > hydralazine. Other drugs or metabolites showed little or no inhibition of quinine metabolism (mean $IC_{50} > 200$ or 500 μM). Among the antimalarial drugs, doxycycline showed relatively potent inhibition of quinine 3-hydroxylation with a mean IC_{50} value of 17 μM, followed by primaquine and tetracycline, with mean IC_{50} values of 20 and 29 μM, respectively.

Conclusions When the plasma/serum concentrations possibly attained after their usual therapeutic doses were taken into account, tetracycline, doxycycline, omeprazole, ketoconazole, nifedipine, TAO and erythromycin are likely to be inhibitors of quinine metabolism in patients when the drugs are co-administrated with quinine.

Keywords: quinine, 3-hydroxylation, CYP3A4, drug interactions, human liver microsomes, antimalarials

Introduction

Quinine is an important antimalarial drug with potent schizontocidal effects against all human *Plasmodium* species [1]. *Plasmodium falciparum* resistance to chloroquine, mefloquine and pyrimethamine/sulphadoxine has been rapidly increasing in endemic areas such as Southeast Asia, South America and East Africa [2–4] and this has resulted in an increased use of quinine, either singly or in combination with other antimalarials, for the treatment of malaria [1, 5, 6]. In addition, two recent clinical trials have shown that quinine is as effective as artemether, a promising antimalarial effective against *P. falciparum* malaria [7], in children with cerebral malaria [8] and in adults with severe falciparum malaria [9]. Thus, quinine is considered to be one of the most effective drugs for the treatment of malaria.

The primary route of elimination of quinine in humans is hepatic metabolism with less than 20% of the drug excreted unchanged in urine [1, 6, 10]. However, the detailed metabolism of quinine and cytochrome P450 (CYP) isoform(s) involved have been only recently elucidated; the formation of 3-hydroxyquinine from quinine is the major metabolic pathway [11, 12], and the 3-hydroxylation is catalysed mainly by CYP3A4 in human liver microsomes [13, 14]. In addition, a recent study [13] has shown that CYP2C19 (S-mephenytoin 4′-hydroxylase) is involved to a minor extent in this metabolic pathway of quinine.

Quinine has a low therapeutic index with adverse reactions such as cinchonism, hypoglycaemia and cardiac arrhythmias [1, 5, 6]. Plasma quinine concentrations between 8 and 15 $\mu g\ ml^{-1}$ are considered to be clinically effective and unlikely to be toxic in severe falciparum malaria [5]. Furthermore, many inhibitors and/or inducers of CYP3A4 [15–17] are widely used in clinical practice, and some substrates of CYP3A4 (e.g., diazepam, lignocaine) may be co-administered with quinine for the management of complications of severe falciparum malaria [18]. Quinine is also co-administered with other antimalarials for the treatment and/or chemoprophylaxis of malaria [1, 4–6]. The aim of the present study was to investigate the possible interactions of selected antimalarial and non-antimalarial drugs with quinine 3-hydroxylation.

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Methods

Drugs and chemicals

Synthetic 3-hydroxyquinine was a generous gift from Dr P. Winstanley (University of Liverpool, Liverpool, UK). Quinine HCl, primaquine $2H_3PO_4$, doxycycline HCl, *rac*-verapamil, ketoconazole, troleandomycin (TAO), cimetidine, erythromycin, nifedipine, diltiazem HCl, oleandomycin H_3PO_4, hydralazine HCl, pyrimethamine, chloroquine $2H_3PO_4$, and norfloxacin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Omeprazole and lignocaine HCl were obtained from Fujisawa-Astra (Osaka, Japan) and Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan), respectively. Tetracycline HCl, quinidine, α-naphthoflavone, acetonitrile, methanol and other reagents of analytical grade were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Proguanil, cycloguanil and 4-chlorophenylbiguanide (CPB) were kindly supplied by Zeneca Pharmaceuticals (Alderley Edge, UK). $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan).

Human liver samples

Six histologically normal liver samples were obtained from Japanese patients with hepatic metastatic disease who underwent a partial hepatectomy at the Division of General Surgery, International Medical Center of Japan, Tokyo, Japan. Approval for the study was granted by the Institutional Ethics Committee of the International Medical Center of Japan. Livers were stored as 10–20 g portions at -80° C until required.

Preparation of liver microsomes

Washed microsomes were prepared by the classical differential centrifugation technique [19, 20]. After the determination of microsomal protein by the method of Lowry *et al.* [21], the individual microsomal samples were aliquoted and stored at -80° C until used.

Assay with human liver microsomes

The basic incubation medium contained 0.05 to 0.1 mg ml^{-1} human liver microsomes, 0.5 mM $NADP^+$, 2.0 mM glucose-6-phosphate, 1 iu ml^{-1} glucose-6-phosphate dehydrogenase, 4 mM $MgCl_2$, 0.1 mM EDTA, 100 mM potassium phosphate buffer (pH 7.4) and 100 μM quinine in a final volume of 250 μl. All the reactions, except for TAO, were initiated by addition of the NADPH-generating system without preincubation, and the mixture was incubated at 37° C in a shaking water bath for 15 min. The reactions were initiated for TAO in two different ways: incubation mixture containing TAO was once preincubated in the presence of the NADPH-generating system at 37° C for 15 min and then the reactions were initiated by addition of the substrate quinine. The reaction was also initiated without preincubation by addition of the NADPH-generating system with the incubation mixture containing quinine and TAO simultaneously, and the mixture was incubated at 37° C for 15 min. After the reaction was stopped by addition of 500 μl of cold methanol, the mixture was centrifuged at 1,500 *g* for 10 min, and 30 μl of supernatant was injected onto a high-performance liquid chromatography (h.p.l.c.) system as described below.

3-Hydroxyquinine was assayed in the incubation mixture by the h.p.l.c. method using fluorometric detection, according to a published method [12], with minor modifications as employed in our recent study [13]. Briefly, the h.p.l.c. system consisted of a model L-7100 pump (Hitachi Ltd, Tokyo, Japan), a model L-7200 autosampler (Hitachi), a model D-7500 integrator (Hitachi) and a 2×100 mm reversed-phase C18 column (Shandon, London, UK). The mobile phase consisted of a 40/60 (v/v) mixture of acetonitrile and 0.05 M sodium phosphate buffer, containing 10 mM sodium dodecyl sulphate and 0.1 mM tetrabutylammonium bromide. The pH of the mobile phase was finally adjusted to 2.1, and the mobile phase was delivered at a flow rate of 0.5 ml min^{-1}. Inter- and intra-assay coefficients of variation for each procedure ($n = 6$) were $< 10\%$, and the lowest limits of detection for both 3-hydroxyquinine and quinine, defined as the lowest concentration with a signal-to-noise ratio of 10, were 5 ng ml^{-1}.

Inhibition study on quinine 3-hydroxylation by selected drugs

The drugs tested for the possible inhibitory effect on quinine 3-hydroxylation were dissolved in methanol, except for chloroquine which was dissolved in 0.1 M phosphate buffer (pH 7.4). To identify the respective IC_{50} (a 50% inhibition of quinine 3-hydroxylation compared with the control) values, various inhibitor/substrate concentrations were chosen ranging from 0.001 to 500 μM and the quinine concentration was set at 100 μM, which is around the mean apparent K_m value for quinine 3-hydroxylation as determined previously by Zhao *et al.* [13]. Fifty μl of each drug dissolved in methanol was evaporated to dryness before addition of the other reaction constituents. Three to four different microsomal samples were used in the experiments, and the assays were carried out in duplicate. In all cases, the inhibited activities were compared with those from the respective control incubations.

The drugs tested for their possible interaction effects on quinine 3-hydroxylation were selected for the following reasons: primaquine, doxycycline, tetracycline, pyrimethamine, dapsone, chloroquine, norfloxacin, proguanil and quinidine have been used and may be co-administered with quinine for the treatment and/or chemoprophylaxis of malaria [1, 4–6, 22, 23]. Cycloguanil (active against *P. falciparum*) and CPB (inactive against *P. falciparum*) were selected because they are biotransformed from the prodrug proguanil in humans [24, 25]. Calcium-entry channel blockers (verapamil, nifedipine and diltiazem), omeprazole, ketoconazole, macrolide antibiotics (TAO, oleandomycin and erythromycin), cimetidine, lignocaine and dapsone were selected for the study, because they have been documented as the inhibitors/substrates of CYP3A4 [15–17] by which quinine is 3-hydroxylated [13, 14]. Quinidine is also a substrate toward CYP3A4 [16, 17]. Calcium-antagonists such as verapamil were selected for an additional reason: the reversal of chloroquine resistance against *P. falciparum* has

been observed by verapamil [26], and therefore they might be used for a combination treatment with antimalarial(s) in the future. Hydralazine was selected, because an animal *in vitro* study [27] has revealed that this antihypertensive drug is an inhibitor of aldehyde oxidase involved in the metabolism of cinchona antimalarials including quinine. We have been unable to evaluate the possible interaction between quinine and other antimalarials such as mefloquine, sulphadoxine, qinghaosu and its derivatives, halofantrine, amodiaquine, amopyraquine and atovaquone. This was because these antimalarials were not available for the study in Japan.

All the experimental data are expressed as mean ± s.d. throughout the text.

Results

Chromatograms and assessment of incubation conditions

Using the chromatographic conditions described above, except for quinidine, none of the drugs tested gave rise to any chromatographic peaks interfering with 3-hydroxyquinine and quinine. Quinidine, a diastereoisomer of quinine, was not studied further. Quinidine is metabolized to 3-hydroxyquinidine, a major metabolite of quinidine, by CYP3A4 [28], and 3-hydroxyquinidine has a retention time nearly identical to that of 3-hydroxyquinine [11, 12].

Preliminary studies revealed that the 3-hydroxylation of quinine with human liver microsomes was linear with regard to the incubation time from 5 to 60 min when 100 μM of quinine was incubated with microsomes equivalent to 0.1 mg of protein ml^{-1}. A linear relationship was also observed between the rate of the metabolite production in 15 min and protein concentration for up to 0.25 mg ml^{-1}. Accordingly, the subsequent kinetic and inhibition studies were performed with a 15-min incubation time and a microsomal protein content of 0.05 to 0.1 mg ml^{-1}.

Inhibition experiments

Of the twenty-three tested compounds, thirteen exhibited an inhibitory effect on quinine 3-hydroxylation by human liver microsomes (Table 1 and Figure 1). The remaining eight drugs and metabolites of proguanil showed little or no inhibition of the 3-hydroxylation of quinine as judged by their mean IC_{50} values of >200 or 500 μM (Table 1). All of the thirteen drugs inhibited the microsomal metabolism of quinine in a concentration-dependent manner, but the magnitude of the inhibition differed among them (Figure 1a to c). The inhibitory rank order of these drugs was as follows: ketoconazole > TAO (with preincubation) > doxycycline > omeprazole > primaquine > tetracycline = TAO (without preincubation) > nifedipine > erythromycin > verapamil > cimetidine > diltiazem > oleandomycin > hydralazine, according to the respective mean IC_{50} values (Table 1).

Quinine 3-hydroxylation is catalysed mainly by CYP3A4 *in vitro* [13, 14]. TAO is a mechanism-based inhibitor of this isoform and requires an NADPH-dependent complexion for inactivation [29]. Thus, experiments were performed using two different protocols (i.e., with and without preincubation). First, the inhibitory effect of TAO was investigated after the co-incubation of quinine and TAO with human liver microsomes. With increasing TAO concentrations, a concentration-dependent inhibition of quinine 3-hydroxylation was observed (Figure 1c). The mean IC_{50} value for TAO was determined to be 29 μM (Table 1).

In the second protocol, human liver microsomes were preincubated for 15 min with TAO in the presence of the NADPH-generating system. The preincubated microsomes were then incubated with quinine concentration of 100 μM and the rate of quinine 3-hydroxylation was evaluated. After preincubation of microsomes with increasing TAO concentrations, a concentration-dependent inhibition of quinine 3-hydroxylation was observed (Figure 1c). The mean IC_{50} value for TAO obtained under this *in vitro* condition was calculated to be 8.3 μM (Table 1).

Otherwise, an activation (about 35%) of the formation of 3-hydroxyquinine from quinine was observed when quinine was incubated in the presence of diazepam (Figure 1b). However, no discernible activation was observed when

Table 1 Mean (± s.d.) IC_{50} values for inhibition of quinine 3-hydroxylation by various drugs in human liver microsomes *in vitro*.

Drugs	IC_{50} (μM)
Primaquine	20 ± 9
Doxycycline	17 ± 6
Tetracycline	29 ± 14
Verapamil	64 ± 32
Omeprazole	18 ± 14
Ketoconazole	0.026 ± 0.013
TAO⋆	29 ± 5
TAO⋆⋆	8.3 ± 2.3
Cimetidine	97 ± 16
Erythromycin	61 ± 15
Nifedipine	38 ± 6
Diltiazem	127 ± 62
Oleandomycin	143 ± 49
Hydralazine	197 ± 38
Lignocaine	>500
Pyrimethamine	>500
Dapsone	>200
Chloroquine	>200
Diazepam	>200
Norfloxacin	>200
Proguanil	>200
Cycloguanil	>200
CPB	>200
Quinidine	⋆⋆⋆

Abbreviations are: TAO, troleandomycin; CPB, 4-chlorophenylbiguanide; IC_{50}, a 50% inhibition of the 3-hydroxyquinine formation from quinine as compared with the control value.

⋆ = TAO was not preincubated with microsomes in the presence of the NADPH generating system for 15 min.

⋆⋆ = TAO was preincubated with microsomes in the presence of the NADPH generating system for 15 min.

⋆⋆⋆ = not detectable because of the interference with the assay of 3-hydroxyquinine.

The IC_{50} data are derived from three to four experiments with different human liver microsomes.

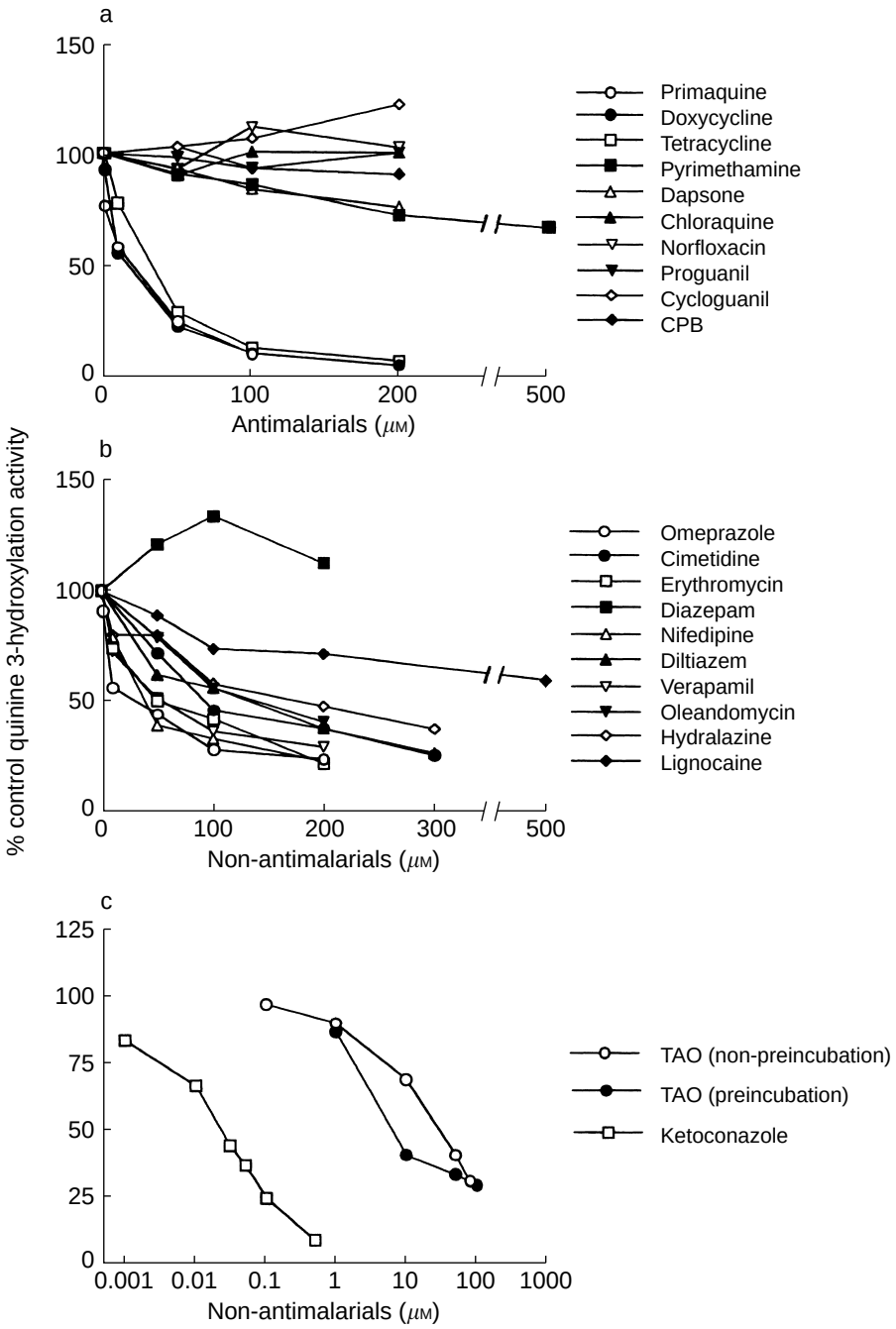

Figure 1 Effects of antimalarial (a) and non-antimalarial drugs (b) and (c) on the formation of 3-hydroxyquinine from quinine by human liver microsomes. Data plotted are the mean values of experiments performed with microsomes from three to four different livers. The horizontal (x) axes in (a) and (b) are shown as an arithmetic scale, whereas the horizontal axis in (c) as a logarithmic scale for the clarification of data plots. CPB = 4-chlorophenylbiguanide; TAO = troleandomycin.

quinine was incubated with α-naphthoflavone (25 μM), a well-known activator of CYP3A4/5 [30].

Discussion

Two recent *in vitro* studies with human liver microsomes [13, 14] have indicated that the major pathway (i.e., 3-hydroxylation) of quinine is mediated mainly by CYP3A4. In this extended *in vitro* microsomal study, we observed that antimalarial drugs, doxycycline, primaquine and tetracycline, inhibited quinine 3-hydroxylation in human liver microsomes with mean IC_{50} values of 17, 20 and 29 μM, respectively (Table 1). Thus, on a theoretical basis, a drug interaction may occur between quinine and these drugs *in vivo*. Nevertheless, such a prediction must be confirmed in the clinical situation by a kinetic study on quinine in patients with malaria given quinine concurrently with other antimalarials. This is because an *in vivo* drug interaction is dependent on blood or more importantly hepatic tissue drug concentrations.

Primaquine and verapamil may not have any clinical significance since their maximum serum concentrations (0.4 and 0.8 μM, respectively) attained after their usual doses [31] are much (50 and 80 times) lower than their mean IC_{50} values (20 and 64 μM, respectively) (Table 1). On the other hand, doxycycline and tetracycline reach serum concen-

trations of about 4 and 8 μM after normal doses [32]. Although these concentrations of doxycycline and tetracycline are not close to their mean IC_{50} values (17 and 29 μM, respectively) (Table 1), there is the possibility that these antibiotics may cause an interaction when co-administered with quinine *in vivo*. Indeed, Karbwang *et al.* [33] have observed a pharmacokinetic interaction between doxycycline and quinine (i.e., plasma levels of quinine were elevated by doxycycline), although another clinical study [34] failed to detect an interaction in patients with acute falciparum malaria.

Among the drugs listed in Table 1, calcium-antagonists (verapamil, nifedipine and diltiazem), macrolide antibiotics (TAO, erythromycin and oleandomycin), ketoconazole, omeprazole and cimetidine are inhibitors/substrates of CYP3A4 [15–17, 20, 35, 36]. Although to date no information is available on the involvement of CYP isoform(s) in the metabolism of primaquine, doxycycline and tetracycline in humans, our data suggest that they might be substrates or inhibitors of CYP3A4. Supporting this, Na Bangchang *et al.* [37] have indicated that ketoconazole, a CYP3A4 inhibitor [15–17, 38], was a potent inhibitor of primaquine metabolism in human liver microsomes. However, the authors employed a >25 μM concentration of ketoconazole, and the IC_{50} and apparent K_i values yielded by this inhibitor for the primaquine metabolism were 15 and 6.7 μM, respectively [37]. At such concentrations, ketoconazole inhibits several CYP isoforms including CYP3A4 [38], 2C [39, 40] and 1A2 [41]. Studies [42, 43] have shown the specificity of ketoconazole toward CYP3A4 when used at a low concentration (<1 μM). Therefore, from the data of Na Bangchang *et al.* [37], no definite conclusion can be drawn that primaquine is a substrate or inhibitor of CYP3A4, although our data suggest this possibility.

Different inhibitory effects of TAO were observed with and without preincubation of the microsomal samples (Figure 1c). Preincubation of microsomes with TAO for 15 min gave a mean IC_{50} value of 8.3 μM. Without preincubation, the mean IC_{50} value was 29 μM (Table 1). A considerable loss of quinine 3-hydroxylase activity occurs during the 15 min preincubation with TAO, suggesting that the mechanism-based inactivation is important for the inhibitory effects. Thus, the observation that TAO is a mechanism-based inhibitor of quinine 3-hydroxylation, mediated dominantly by CYP3A4 [13, 14], is in agreement with the results reported by Watkins *et al.* [29].

Omeprazole, a substrate of CYP2C19 [17, 19, 35], inhibited the 3-hydroxylation of quinine with a mean IC_{50} value of 18 μM (Table 1). This is in agreement with our recent study [13] that the 3-hydroxylation of quinine is partly catalysed by CYP2C19. However, omeprazole is also a substrate for CYP3A4 [17, 35]. In addition, the content of CYP3A is the highest in human liver microsomes (about 30% of the total CYPs) [44], whereas the content of CYP2C19 is only about 1% of the total CYPs [44]. Therefore, omeprazole might inhibit the activity of CYP3A4 more than that of CYP2C19, since quinine is principally 3-hydroxylated by CYP3A4 [13, 14]. On the other hand, the apparent K_m values for omeprazole metabolism were about 8 μM via CYP2C19 and about 49 μM via CYP3A4 [35], whereas the mean IC_{50} value of omeprazole for quinine 3-hydroxylation was 18 μM (Table 1). Therefore, omeprazole appears to have the greater inhibition potential for CYP3A4 compared with CYP2C19. Christians *et al.* [45] have also reported that omeprazole inhibits the metabolism of an immunosuppressive macrolide, tacrolimus (a substrate of CYP3A4 [16, 17]), competitively with a mean apparent K_i value of 34 μM.

Diazepam stimulated the 3-hydroxylation of quinine by about 35% at a 100 μM concentration of diazepam (Figure 1b), which is consistent with previous reports [36, 46] that diazepam can stimulate its own metabolism by CYP3A4/5. In contrast, α-naphthoflavone (25 μM) failed to enhance the 3-hydroxylation of quinine in the present study. Interaction data with α-naphthoflavone appear to conflict among different *in vitro* studies with human liver microsomes [46–49]: Depending on concentration CYP3A4-catalysed reactions are either stimulated [46, 47], or inhibited by α-naphthoflavone [48, 49].

Of the non-antimalarial drugs tested, some were known substrates/inhibitors of CYP3A [15–17, 35, 36]. Therefore, it is reasonable to observe inhibition of quinine 3-hydroxylation by these drugs. Indeed, ketoconazole was a potent inhibitor of quinine metabolism (Figure 1c), with a mean IC_{50} value of 0.026 μM, followed by TAO (with and without preincubation), nifedipine and erythromycin. Because the plasma/serum concentrations of these drugs attained after their usual therapeutic doses [31, 32] are near their respective IC_{50} values (Table 1), a significant interaction may occur when these drugs are administered concomitantly with quinine *in vivo*. Other substrates/inhibitors of CYP3A4, which were weaker inhibitors (e.g., diltiazem with a mean $IC_{50}>100$ μM) of quinine metabolism (Table 1), may not have any significant inhibitory effects on quinine 3-hydroxylation *in vivo*. Also lignocaine, a substrate of CYP3A4 [15–17], produced no inhibition ($IC_{50}>500$ μM). Lignocaine may have a low affinity for CYP3A4 and/or be metabolized very readily and thus eliminated from the incubation mixture rapidly. Therefore, lignocaine would not be able to exert its inhibitory effect over the duration of the incubation. This observation is also consistent with a previous study [50], indicating that various CYP3A4 substrates interact differently with the enzyme or perhaps with different isoforms. This may partly explain the observed lack of the *in vivo* correlation between some substrates.

In conclusion, although we recognise that a drug identified as an inhibitor in an *in vitro* study is not necessarily an inhibitor in the *in vivo* situation, drugs that fail to inhibit the metabolism of a substrate *in vitro* will not be inhibitors *in vivo* [45]. Thus, antimalarial drugs such as chloroquine, pyrimethamine, proguanil and its metabolites (cycloguanil and CPB), norfloxacin as well as dapsone screened in the present study (Table 1) are unlikely to cause a clinically significant drug interaction with quinine. On the other hand, for the antimalarial or non-antimalarial drugs, which showed inhibition of quinine metabolism *in vitro* (Table 1), further assessment of the possible interactions is required in clinical studies, particularly for doxycycline, tetracycline, omeprazole, ketoconazole, TAO, nifedipine and erythromycin. When these drugs and quinine are prescribed concurrently in patients with malaria, a close monitoring of the

blood (plasma or serum) concentrations of quinine may be required to avoid supratherapeutic quinine concentrations (e.g., $>20\ \mu g\ ml^{-1}$ [5, 6]) and thereby related toxicity.

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