The In Vitro Hepatic Metabolism of Quinine in Mice, Rats and Dogs: Comparison with Human Liver Microsomes

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Accepted for publication August 14, 1997

ABSTRACT
The major metabolic pathway of quinine in the human has been shown to be 3-hydroxylation mediated by the human cytochrome P450 (CYP) 3A4. In this extended in vitro study, quinine 3-hydroxylation was further investigated using microsomes from mouse, rat, dog and human livers and was compared among them in terms of the in vitro enzyme-kinetic parameters and quinine-drug interaction screenings. In all species, 3-hydroxyquinine was the principal metabolite of quinine. There was intra- and interspecies variability among all the kinetic parameters, and dogs showed a closer resemblance to humans in terms of the mean kinetic data. Ketoconazole and troleandomycin inhibited the 3-hydroxylation of quinine in all species. Both α-naphthoflavone and diazepam showed an interspecies difference in quinine 3-hydroxylation: a trend toward an activation in dog and human, and a significant inhibition in mouse and rat, liver microsomes. Antisera did so with rat liver microsomes. Primaquine, doxycycline and tetracycline substantially inhibited the formation of 3-hydroxyquinine in rat, dog and human species, but proguanil had no such effect in any species. Chloroquine inhibited quinine 3-hydroxylation with rat and dog liver microsomes but not with human liver microsomes. There was a significant correlation (r = 0.986, P < .001) between the CYP3A contents and the formation rates of 3-hydroxyquinine in eight human liver microsomal samples. It is concluded that 3-hydroxyquinine is a main metabolite of quinine and that CYP3A/Cyp3a is a principal isoform involved in this metabolic pathway in the respective (rat, dog and human/mouse) species tested. The dog and possibly the rat may be qualitatively and quantitatively suitable animal models for exploring the quinine 3-hydroxylase activity and for screening quinine-drug interactions in vitro, at certain inconsistency with the human liver microsomal data.

Many consider malaria to be the most important infectious disease in the world. The antimalarial quinine still remains to be an important drug of choice for the treatment of severe and complicated malaria. Two recent clinical trials have shown that quinine is as effective as artemether (a qinghaosu derivative), a promising antimalarial that is effective against Plasmodium falciparum malaria (Hien and White, 1993), in children with cerebral malaria (Boele van Hensbroek et al., 1996) and in adults with severe falciparum malaria (Hien et al., 1996). In addition, the resistance of P. falciparum to chloroquine, mefloquine and pyrimethamine/sulfadoxine has been rapidly increasing in endemic regions such as Southeast Asia, South America and East Africa (Moran and Bernard, 1989; Wernsdorfer, 1991; Bradley, 1993), and this has resulted in an increased use of quinine as an alternative drug for treating multidrug-resistant P. falciparum malaria (White, 1996; Tracy and Webster, 1996). Furthermore, quinine is available not only as an oral form but also as an injectable formulation for malaria patients, and it has fewer life-threatening side effects if used correctly and at the normal therapeutic doses (White, 1992; Tracy and Webster, 1996). Thus quinine is considered one of the most effective and convenient drugs for the treatment of malaria.

The primary route of systemic elimination of quinine in the human is via an extensive hepatic biotransformation to hydroxylated metabolites; less than 20% of the drug is excreted unchanged in the urine (White et al., 1982; Tracy and Webster, 1996). However, despite its long history (at least 350 years) in the treatment of malaria, it was not until recently that the detailed metabolism of quinine and the cytochrome P450 (CYP) isoform(s) involved were clarified in humans. The formation of 3-hydroxyquinine is the major metabolic pathway (Wanwimolruk et al., 1995; Wanwimolruk et al., 1996; Zhang et al., 1997) in the human, and the 3-hydroxylation is catalyzed mainly by CYP3A4 (Zhao et al., 1996; Zhang et al., 1997) and to a minor extent by CYP2C19 (Zhao et al., 1996) in human liver microsomes.

So far as we know, the detailed quinine metabolism, the kinetics of quinine 3-hydroxylation and the CYP isoform(s)
involved in this metabolic pathway of quinine in animals have been neither elucidated nor compared among different animal species or between an animal species and the human. Thus the aims of this extended in vitro study were 1) to investigate and compare the formation and kinetics of quinine 3-hydroxylation by using different species (i.e., mouse, rat, dog and human) liver microsomes, and to identify the CYP isoform(s) involved in this metabolic pathway of quinine; and 2) to search for a suitable animal model for further study of quinine 3-hydroxylation and quinine-drug interactions in vitro.

Materials and Methods

Chemicals and reagents. Synthetic 3-hydroxyquinine was a generous gift from Dr. P. Winstanley (University of Liverpool, Liverpool, UK). Quinine, TAO, primaquine, doxycycline, chloroquine, diazepam and tetrabutylammonium bromide were purchased from Sigma Chemical Co. (St. Louis, MO). Quinidine, a-naphthoflavone, coumarin and p-nitrophenol were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and sulphonaphazole from Meiji Yakuhin Co. (Tokyo, Japan). Proguanil was kindly supplied by Zeneca Pharmaceuticals (Alderley Edge, UK). Acetonitrile, methanol and other reagents of analytical grade were purchased from Wako Pure Chemical Industries Ltd. NADP+, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan). Racemic mephenytoin was kindly donated by Dr. Käuper (University of Bern, Bern, Switzerland). S- and R-mephenytoin were separated from racemic mephenytoin on a Chiralcel OJ column (10 μm, 4.6 × 250 mm; Daicel Chemical Co. Ltd., Tokyo, Japan) according to the method of Yasumori et al. (1990). Rabbit polyclonal anti-rat CYP3A2, 2C11 and 2E1 antisera and preimmune serum were obtained from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan).

Preparation of microsomal fractions. Human liver samples were obtained from 12 patients who underwent a partial hepatectomy for metastatic liver tumor(s) in the Division of General Surgery, International Medical Center of Japan, Tokyo, as reported previously (Chiba et al., 1993; Zhao et al., 1996). The liver parenchyma of non-tumour-bearing part used for the study was shown later to be histopathologically normal in all cases. Use of human samples for the study had been approved by the Institutional Ethics Committee of the International Medical Center of Japan. Animal liver samples were obtained from 11 male C57/CD-1 mice, six male Wistar rats and five male beagle dogs (Japan River Co., Yokohama, Japan). Animals were fed a standard diet and had free access to drinking water. The animals were also allowed to acclimate in our animal facility for 1 week before the liver microsomal samples were prepared as described below.

Microsomal preparations from human and animal liver tissues tested herein were made according to standard procedures as previously described (Chiba et al., 1993;ECHizen et al., 1993). After the determination of protein concentration (Lowry et al., 1951), the individual microsomal samples were aliquoted, frozen and stored at −80°C until used.

Assay for quinine metabolism with liver microsomes. The assay of quinine metabolism was conducted in the same way as previously described (Zhao et al., 1996; Zhao and Ichikawa, 1997, in press). Microsomal fractions were incubated in the presence of an NADPH-generating system at 37°C for 10 to 15 min in test tubes. The incubation mixture consisted of 0.1 to 0.5 mg/ml microsomal protein, 4 mM MgCl2, 0.5 mM NADP+, 2.0 mM glucose-6-phosphate, 1 IU/ml glucose-6-phosphate dehydrogenase, 100 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA and 0.5 to 600 μM quinine, in a final volume of 250 μl. After incubation for 10 to 15 min, the reaction was stopped by addition of 500 μl of ice-cold methanol. The mixture was centrifuged at 1500 × g for 10 min, and the supernatant was injected onto a HPLC apparatus as described below.

3-Hydroxyquinine was determined in the incubation mixture by the HPLC method using fluorometric detection, according to a reported method (Wanwimolruk et al., 1996), with minor modifications as employed in our recent study (Zhao et al., 1996). Briefly, the HPLC 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 sulfate and 0.1 mM tetraphenylammonium bromide. The pH of the mobile phase was finally adjusted to 2.1, and it was delivered at a flow rate of 0.5 ml/min. Inter- and intraassay coefficients of variation for each procedure (n = 6) were less than 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.

Kinetics of the formation of 3-hydroxyquinine. Preliminary results indicated that the formation rates of 3-hydroxyquinine were linear at 37°C for incubation time up to 30 min (humans and dogs) or 10 min (rats and mice) and for microsomal protein concentration up to 0.25 mg/ml (humans and mice) or 1.0 mg/ml (dogs and rats) at the substrate quinine concentration of 50 μM. Accordingly, the kinetic studies were performed at 37°C with an incubation of 15 min (humans and dogs) or 10 min (rats and mice) at a microsomal protein concentration of 0.1 mg/ml (humans, rats and mice) or 0.5 mg/ml (dogs).

Because the formation of 3-hydroxyquinine by liver microsomes obtained from all the humans, dogs and rats and a majority of the mice tested herein were consistent with a simple Michaelis-Menten kinetic behavior, the one-component enzyme kinetic parameters (Km, Vmax and Vmax/Km without the numerical subindices) for the formation of 3-hydroxyquinine from quinine (0.5–600 μM) were estimated by using linear regression analysis of unweighted raw data. On the other hand, because the formation of 3-hydroxyquinine gave a biphasic relationship in liver microsomes obtained from four mice studied, Michaelis-Menten kinetic parameters for the formation of 3-hydroxyquinine were estimated by fitting the data to the following equation:

\[
V = V_{\text{max1}} \cdot S/(K_{\text{m1}} + S) + V_{\text{max2}} \cdot S/(K_{\text{m2}} + S)
\]

where V is the velocity of the formation of 3-hydroxyquinine, S is the concentration of quinine in the incubation mixture, Km1 and Km2 are the affinity constants for the high- and low-affinity components and Vmax1 and Vmax2 are the maximum enzyme velocities for the high- and low-affinity components, respectively. The kinetic parameters were estimated initially by the graphical analysis of Eadie-Hofstee plots, and the values obtained were used as the first estimate for the nonlinear least-squares regression analysis MULTI (Yamaoka et al., 1981), in which unweighted raw data were fitted to the model equation.

Inhibition study. The effects of coinhibitor of inhibitor or substrate probes specific for different human CYP isoforms on the microsomal metabolism of quinine were studied separately. The specific inhibitors used were a-naphthoflavone for CYP1A (Kunze and Trager, 1993), sulphonaphazole for CYP2C9 (Goldstein and de Morais, 1994), quinidine for CYP2D6 (Kobayashi et al., 1989) and ketoconazole and TAO for CYP3A4 (Newton et al., 1995; Watkins et al., 1985). The substrate probes used were coumarin for CYP2A6 (Wrighton and Stevens, 1992), S-mephenytoin for CYP2C19 (Goldstein and de Morais, 1994) and p-nitrophenol for CYP2E1 (Tassaneeyakul et al., 1993). In addition, two so-called activators of CYP3A, a-naphthoflavone (Chang et al., 1994; Shou et al., 1994) and diazepam (Andersson et al., 1994; Pearce et al., 1996), were used for testing the possible activation of quinine 3-hydroxylation with the liver microsomes of all species. The concentrations of quinine were chosen...
Results

Metabolism of quinine. By using the optimized HPLC conditions described above, we observed similar metabolite formation (i.e., 3-hydroxylation) patterns of quinine within 60 min in all species tested. Quinine was extensively metabolized by the liver microsomes of all species, and the major peak had a retention time (16 min) identical to that of pure 3-hydroxyquinine (fig. 1). The formation of 3-hydroxyquinine was time-, NADPH- and microsomes-dependent (data not shown), which suggests the possible involvement of P450(s) in the metabolism.

In microsomes obtained from all the rat, dog and human livers and from 7 of the 11 mouse livers used, the formation of 3-hydroxyquinine exhibited a monophasic Michaelis-Menten kinetic profile (fig. 2A); this suggests that a single CYP isoform may be involved in the 3-hydroxylation of quinine in these microsomes. However, in the liver microsomes obtained from 4 of the 11 mice, the Eadie-Hofstee plots for the formation of 3-hydroxyquinine gave a biphasic behavior (fig. 2B), indicating that at least two CYP isoforms would be involved in this metabolic pathway of quinine. Accordingly, two different (i.e., one-enzyme and two-enzyme) kinetic analysis...
approaches were used for estimating the affinity constant ($K_m$), the maximum enzyme velocity ($V_{max}$) and the intrinsic clearance ($CL_{int}$), defined as $V_{max}/K_m$. The estimated kinetic parameters of quinine 3-hydroxylation in all species liver microsomes are listed in tables 1 and 2. All the kinetic parameters ($K_m$, $V_{max}$ and $V_{max}/K_m$) exhibited intra- and interspecies variability, dogs vs. humans and mice vs. rats having closer mean values between each other, respectively (table 1). The rank order of mean $CL_{int}$ for the formation of 3-hydroxyquinine with liver microsomes obtained from the four mammalian species was rats > mice > humans > dogs (table 1).

**Inhibition study.** Two previous in vitro studies (Zhao et al., 1996; Zhang et al., 1997) have demonstrated that CYP3A4 is a principal isoform involved in quinine 3-hydroxylation with human liver microsomes. In order to determine whether the CYP3A subfamily isoform also plays a predominant role in the formation of 3-hydroxyquinine from quinine in other species liver microsomes tested, we used two typical CYP3A inhibitors, ketoconazole and TAO, to perform an inhibition study with microsomes obtained from mouse, rat and dog livers. The effects of co-cultivation with the inhibitors on quinine 3-hydroxylation are shown in figure 3, together with our published data derived from human liver microsomes (Zhao et al., 1996), in order to facilitate comparison with those obtained from animal liver microsomal samples. All the plots showed that quinine 3-hydroxylation was inhibited in a concentration-related manner. The mean IC$_{50}$ (± S.D.) values with mouse, rat, dog and human liver microsomes were 0.021 (± 0.005), 0.087 (± 0.012), 0.056 (± 0.010) and 0.026 (± 0.013) μM for ketoconazole and 6.3 (± 1.4), 43.0 (± 4.2), 0.80 (± 0.13) and 29.0 (± 4.7) μM for TAO, respectively. The mean maximum inhibition on quinine 3-hydroxylation in mouse, rat, dog and human liver microsomes was about 94%, 91%, 88% and 90% for ketoconazole and about 85%, 66%, 93% and 70% for TAO, respectively. These observations suggest the possibility that the CYP3A (for rats, dogs and humans) or Cyp3a (for mice) subfamily (Nelson et al., 1996) may be involved in quinine 3-hydroxylation.

In four mouse liver microsomes used, the Eadie-Hofstee plots for the formation of 3-hydroxyquinine gave a biphasic profile (fig. 2B). Thus some relatively specific inhibitor or substrate probes (see “Materials and Methods”) were used to perform another inhibition study using three of the four mouse liver microsomes with two different substrate concentrations (10 and 120 μM) that were around the respective mean low $K_m$ ($K_{m1}$) and high $K_m$ ($K_{m2}$) values. The effects of these compounds on quinine 3-hydroxylation are shown in figure 4. In both concentrations of quinine (10 and 120 μM), p-nitrophenol, S-mephenytoin and sulfaphenazole inhibited the formation of 3-hydroxyquinine from quinine by more than 50% compared with the control, particularly in the case of low quinine concentration (10 μM). This indicates that mouse Cyp2e1 and Cyp2c (Nelson et al., 1996) may also be involved in this metabolic pathway of quinine in these mouse liver microsomes. To test whether the high concentrations of p-nitrophenol (4 mM and 10 mM) might also inhibit Cyp3a and/or other Cyp(s) in mouse liver microsomes, three different human liver microsomes were used to examine the possible effect of p-nitrophenol on quinine 3-hydroxylation. A considerable inhibition of quinine 3-hydroxylation was observed when p-

<table>
<thead>
<tr>
<th>Liver Microsomes</th>
<th>$n$</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (nmol/mg/min)</th>
<th>$V_{max}/K_m$ (μl/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>7</td>
<td>29.7 ± 5.6$^{b,c}$</td>
<td>2.39 ± 0.67$^{b,c}$</td>
<td>82.1 ± 22.9$^{b,c}$</td>
</tr>
<tr>
<td>Rat</td>
<td>6</td>
<td>22.7 ± 3.9$^{b,c}$</td>
<td>1.94 ± 0.25$^{b,c}$</td>
<td>86.3 ± 11.2$^{b,c}$</td>
</tr>
<tr>
<td>Dog</td>
<td>5</td>
<td>138 ± 22</td>
<td>0.77 ± 0.26</td>
<td>5.6 ± 1.8$^{a}$</td>
</tr>
<tr>
<td>Human</td>
<td>12</td>
<td>105 ± 18</td>
<td>1.31 ± 0.44</td>
<td>12.7 ± 4.7</td>
</tr>
</tbody>
</table>

The data are given as mean ± S.D.

$^a$ The number of liver microsomes where the Eadie-Hofstee plots for quinine 3-hydroxylation showed a monophasic profile as illustrated in figure 2A.

$^b$ Significantly ($P < .05$) different from the respective mean value in human.

$^c$ Significantly ($P < .05$) different from the respective mean value in dog.

$^d$ Significantly ($P < .05$) different from the respective mean value in human.
nitrophenol (4 mM and 10 mM) was coincubated with quinine (100 μM, data not shown). This observation appears to support the idea that p-nitrophenol, at a higher concentration, also inhibits CYP3A4 and/or other CYP(s), such as CYP2C19. Our previous study with human liver microsomes and recombinant human CYP isoforms (Zhao et al., 1996) showed that quinine 3-hydroxylation is catalyzed mainly by CYP3A4 and to a minor extent by CYP2C19.

In addition, we employed the two so-called activators of CYP3A, α-naphthoflavone (Chang et al., 1994; Shou et al., 1994) and diazepam (Andersson et al., 1994; Pearce et al., 1996), to determine the possible activation of the formation of 3-hydroxyquinine. The results, shown in figure 5, indicate that there was a marked interspecies difference in the activation or inhibition of quinine 3-hydroxylation when quinine was coincubated with α-naphthoflavone or diazepam: both α-naphthoflavone and diazepam tended to activate quinine 3-hydroxylation with dog and human liver microsomes, whereas they appreciably inhibited it with mouse and rat liver microsomes.

**Immunoinhibition.** The inhibition of quinine 3-hydroxylation by polyclonal antisera raised against purified rat CYP3A2 is shown in figure 6. The addition of anti-CYP3A antisera (25 μl—that is, 25 μg of IgG per microgram of microsomal protein) inhibited the 3-hydroxyquinine formation by about 96%, 84% and 92% in liver microsomes obtained from mouse, rat, dog and human livers, respectively, with no appreciable inhibition of quinine 3-hydroxylation by nonimmune IgG. These results were found to be in good agreement with our previous finding that polyclonal antibodies (10 mg of IgG per milligram of microsomal protein) raised against human CYP3A reduced the 3-hydroxylation activity of quinine by 72% in human liver microsomes (Zhao et al., 1996).

In contrast, both anti-rat 2C11 and 2E1 rabbit antisera (25 μl—that is, 25 μg of IgG per microgram of microsomal protein) did not appreciably inhibit the formation of 3-hydroxyquinine from quinine in rat liver microsomes or the liver microsomes obtained from mice in which the formation of 3-hydroxyquinine exhibited a biphasic Michaelis-Menten kinetics and was inhibited by p-nitrophenol, S-mephenytoin and sulfaphenazole (data not shown).

### Table 2

<table>
<thead>
<tr>
<th>Mouse Liver</th>
<th>$K_{cat}$ (μM)</th>
<th>$V_{max}$ (nmol/mg/min)</th>
<th>$V_{max}/K_{cat}$ (μmol/mg/min)</th>
<th>$K_{cat}$ (μM)</th>
<th>$V_{max}$ (nmol/mg/min)</th>
<th>$V_{max}/K_{cat}$ (μmol/mg/min)</th>
</tr>
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<tbody>
<tr>
<td>ML-2</td>
<td>0.7</td>
<td>0.04</td>
<td>50.0</td>
<td>53.4</td>
<td>1.38</td>
<td>25.9</td>
</tr>
<tr>
<td>ML-9</td>
<td>6.2</td>
<td>0.15</td>
<td>23.8</td>
<td>215.2</td>
<td>1.91</td>
<td>8.9</td>
</tr>
<tr>
<td>ML-10</td>
<td>3.7</td>
<td>0.21</td>
<td>57.0</td>
<td>118.2</td>
<td>1.41</td>
<td>11.9</td>
</tr>
<tr>
<td>ML-11</td>
<td>9.2</td>
<td>0.73</td>
<td>79.5</td>
<td>68.7</td>
<td>1.67</td>
<td>24.3</td>
</tr>
<tr>
<td>Mean</td>
<td>5.0</td>
<td>0.28</td>
<td>52.6</td>
<td>113.9</td>
<td>1.60</td>
<td>17.7</td>
</tr>
<tr>
<td>S.D.</td>
<td>3.6</td>
<td>0.31</td>
<td>23.0</td>
<td>73.0</td>
<td>0.25</td>
<td>8.6</td>
</tr>
</tbody>
</table>

**Fig. 3.** Effects of ketoconazole (panel A) and TAO (panel B) on quinine 3-hydroxylation in microsomes obtained from mouse (○), rat (●), dog (□) and human (■) livers. The plotted data are the mean values of experiments performed with microsomes obtained from three different livers of each species tested.
Metabolic drug interaction. Metabolic drug interactions between quinine and five other antimalarials that may be coadministered with quinine for the treatment and/or chemoprophylaxis of malaria (White, 1988, 1992 and 1996; Bradley, 1993; Tracy and Webster, 1996) were investigated by using rat, dog and human liver microsomes separately, each of which came from three different livers. The mean results are shown in figure 7, together with our recent data derived from human liver microsomes (Zhao and Ishizaki, 1997, in press) in order to facilitate comparison with those from animal liver microsomes. The results indicated that primaquine, doxycycline and tetracycline substantially inhibited the 3-hydroxyquinine formation (at least 55%) in both animal and human liver microsomes, although the respective IC50 values obtained from rat, dog and human liver microsomes differed somewhat among them (table 3). In addition, chloroquine appreciably reduced the formation of 3-hydroxyquinine from quinine (at least >40%) in rat and dog liver microsomes, but not in human liver microsomes, which suggests an interspecies difference in the interaction potential of these antimalarials with quinine. However, proguanil...
did not inhibit the 3-hydroxylation activity of quinine in any liver microsomes tested (fig. 7).

**Correlation between human CYP3A and quinine 3-hydroxylation.** To confirm further the major role of human CYP3A4 isoform in quinine 3-hydroxylation, the CYP3A contents of each of eight human liver microsomes used for determining the kinetic parameters in the present study were measured by Western blot analysis (Berthou et al., 1994). A significant correlation \((r = 0.968, P < .001)\) was found between the CYP3A contents and the 3-hydroxyquinine formation rates in the eight human liver microsomal samples (fig. 8), a result that strongly supports the previous finding that CYP3A is a principal isoform involved in the formation of 3-hydroxyquinine from quinine (Zhao et al., 1996; Zhang et al., 1997).

**Discussion**

Recently, two *in vitro* studies (Zhao et al., 1996; Zhang et al., 1997) with human liver microsomes obtained from two distinct ethnic groups (Japanese and Caucasians) have revealed that quinine 3-hydroxylation, in both groups, is mediated mainly by human CYP3A4 with the approximate mean \(K_m\) and \(V_{max}/K_m\) (i.e., \(CL_{int}\)) values between each other. The current *in vitro* study is, so far as we know, the first to compare quinine 3-hydroxylation with liver microsomes obtained from different species (i.e., mouse, rat, dog and human) or to search for a suitable animal model resembling human in terms of the *in vitro* enzyme-kinetic parameters and quinine-drug interaction screenings. The results provided further *in vitro* evidence that the formation of 3-hydroxyquinine is predominantly mediated via the CYP3A/CYP3A subfamily in all the species tested and that the dog and/or rat may be more suitable as an animal model for exploring the quinine 3-hydroxylation activity and for screening quinine-drug interactions *in vitro*, though the results will be at variance with the human liver microsomal data. Nevertheless, whether dogs would be better than rats for further *in vitro* metabolism research for quinine remains unanswered from the present study, because the quinine-drug interaction data obtained from dog and rat liver microsomes were similarly discrepant with those from the human liver microsomes, as discussed later.

With all species tested, the formation of 3-hydroxyquinine in liver microsomes showed a quite similar chromatographic pattern with 3-hydroxyquinine as the major metabolite of quinine (fig. 1). The rate of quinine 3-hydroxylation was also similar between dog and human and between mouse and rat liver microsomes (table 1). On the basis of these results, one may assume that, from a qualitative point of view, the dog, rat and even mouse could be an appropriate species for conducting an *in vitro* study on the metabolism of quinine. However, the dog may be quantitatively more suitable for exploring the quinine 3-hydroxylation activity *in vitro*, because the mean kinetic parameters for quinine 3-hydroxylation \((K_m, V_{max}\) and \(V_{max}/K_m\) derived from dog liver microsomes, compared with the other two rodent species, were closer to those obtained from human liver microsomes (table 1).

We observed a potent and concentration-dependent inhibition of quinine 3-hydroxylation in all species when quinine was coincubated with two CYP3A inhibitors, ketoconazole and TAO (fig. 4), which indicates that CYP3A/CYP3a is likely to be the major hepatic isoenzyme responsible for the formation of 3-hydroxyquinine. However, a recent study (Eagling et al., 1996) has revealed that ketoconazole, at a relatively low concentration (mean IC\(_{50} = 1–6 \mu M\)), also inhibits other CYP isoforms in rat liver microsomes. This suggests that a differential selectivity of CYP isoform inhibitors such as ketoconazole may exist in the liver microsomes of different species. Nevertheless, in the present study, the mean IC\(_{50}\) values for ketoconazole were less than 0.1 \(\mu M\) among the four species tested (fig. 2), and the mean \(K_i\) value for ketoconazole in human liver microsomes was 0.015 \(\mu M\) (unpublished data), a result that supports the previous observations of the selective inhibition of the CYP3A isoform by ketoconazole at a low concentration (<1 \(\mu M\)) (Newton et al., 1995; Bourrie et al., 1996). The results derived from the two chemical inhibitors of CYP3A were also in good agreement with our immunoinhibition results; that is, anti-CYP3A antisera (25 \(\mu g\) of IgG per microgram of microsomal protein) inhibited 3-hydroxyquinine formation by about 96%, 84% and 92% in liver microsomes obtained from mouse, rat and dog, respectively (fig. 6).

These findings further confirm the dominant role of the CYP3A/CYP3a subfamily in the formation of 3-hydroxyquinine from quinine in the animal species tested.

Although ketoconazole and TAO strongly inhibited quinine 3-hydroxylation in all species, we observed a marked inter-

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**Table 3**

The mean IC\(_{50}\) values of antimalarials for quinine 3-hydroxylation with microsomes obtained from rat, dog and human livers \((n = 3)\)

<table>
<thead>
<tr>
<th>Antimalarial</th>
<th>IC(_{50}) ((\mu M))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat</td>
</tr>
<tr>
<td>Primaquine</td>
<td>37.5</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>62.4</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>117.7</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>47.2</td>
</tr>
<tr>
<td>Proguanil</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

\(^a\) \(P < .01\) compared with the rat or dog group.
species difference in the inhibition potency (e.g., dog vs. human in the case of TAO; fig. 3). This may occur because TAO can function as an “inducer” by stabilizing CYP3A4, and it can act as an inhibitor of CYP3A4 substrates with a low $K_{m}$. Thus TAO appears not to be an ideal choice to study as an inhibitor of CYP3A4, compared with another well-known inhibitor of CYP3A4, ketoconazole. This interspecies difference also existed when $\alpha$-naphthoflavone and diazezapam, two so-called activators of CYP3A in human liver microsomes (Chang et al., 1994; Andersson et al., 1994; Pearce et al., 1996), were coincubated with quinine to conduct an activation study. As expected, both $\alpha$-naphthoflavone and diazezapam tended to activate quinine 3-hydroxylation with human and dog liver microsomes, whereas they substantially inhibited it with mouse and rat liver microsomes (fig. 5). The reason for these discrepant findings is entirely obscure, but they may be explained in part by interspecies differences in CYP3A/Cyp3a isoform(s) that have been observed and characterized among humans (Nelson et al., 1996; Wrighton and Stevens, 1992), dogs (Nelson et al., 1996; Eguchi et al., 1996), rats (Nelson et al., 1996; Soucek and Gut 1992; Nedelcheva and Gut 1994; Funae and Imaoka, 1993) and mice (Nelson et al., 1996; Funae and Imaoka, 1993) with respect to structures, functions and properties. In addition, activation of CYP3A by $\alpha$-naphthoflavone is not always observed; some reactions mediated via CYP3A can be inhibited by this compound (Yun et al., 1992; Berthou et al., 1994). This has been interpreted to indicate an allosteric mechanism (Raney et al., 1992) as well as an interspecies difference in CYP3A isoform(s) (Nelson et al., 1996). In addition, diazezapam is also a substrate not only of CYP3A4 but also of 2C19 with human liver microsomes (Andersson et al., 1994), which may inhibit the metabolism of CYP3A4 or 2C19-mediated substrates like quinine. Thus we must exercise caution when we interpret the effects of selective modifiers of P450-catalyzed reactions, particularly their effects on the interaction results obtained from different species, such as those observed in this study.

With mouse liver microsomes in which the formation of 3-hydroxyquinine from quinine showed a biphasic Michaelis-Menten kinetics (fig. 2B), a marked inhibition (>50%) of quinine 3-hydroxylation was observed when quinine was coincubated with $p$-nitrophenol [a substrate of Cyp2e1 (Forkert et al., 1994)], $S$-mephénytoin and sulfaphenazole [a substrate and inhibitor of the CYP2C subfamily, respectively (Goldstein and de Morais, 1994)] (fig. 4); this suggests the possible involvement of Cyp2e1 and Cyp2c in quinine 3-hydroxylation in these mouse liver microsomes. However, these findings seem to be inconsistent with our data obtained from the immunoinhibition study: anti-rat CYP2E1 and 2C11 antisera (25 μl) did not appreciably inhibit the formation of 3-hydroxyquinine with the same mouse liver microsomes (data not shown). The reason for this discrepancy remains unknown, but it is possible that the anti-rat CYP 2E1 and 2C11 antisera have a weak ability to cross-react with mouse Cyp2e1 and Cyp2c and/or that $p$-nitrophenol, $S$-mephénytoin and sulfaphenazole also inhibit mouse Cyp(s) other than Cyp2e1 and Cyp2c. The latter possibility may be particularly likely with the high concentrations of $p$-nitrophenol (4 mM and 10 mM) we used. We assume that at such high concentrations, $p$-nitrophenol might also inhibit Cyp3a and/or other Cyp(s) in mouse liver microsomes, because $p$-nitrophenol is not solely, though it is largely, metabolized by Cyp2e1 in some animal and human liver microsomes (Monostory and Vereczkey, 1994; Tassaneeyakul et al., 1993). This assumption appears to be supported by the fact that some compounds that are metabolized by one enzyme can also bind to another enzyme and inhibit it. For example, quinidine is a substrate of CYP3A4 (Guengerich et al., 1986) but inhibits CYP2D6 (Kobayashi et al., 1989).

In the quinine-antimalarial interaction study, chloroquine showed a moderate inhibition (about 45%) of quinine 3-hydroxylation in rat and dog liver microsomes, whereas no inhibition was seen with human liver microsomes (fig. 7 and table 3). In addition, the inhibition potency of doxycycline and tetracycline on quinine 3-hydroxylation differed somewhat between animals (i.e., rats and dogs) and humans (fig. 7). The reason for this observation is unclear, but it may be associated with an interspecies difference in substrate affinity (e.g., binding constants to the protein), enzyme activity and/or susceptibility to inhibition. In addition, the possibility cannot be excluded that quinine is more specifically metabolized by different isoforms belonging to the same CYP3A subfamily (e.g., CYP3A3, 3A4, 3A5 and 3A7 in humans, CYP3A12 in dogs, CYP3A1, 3A2 and 3A9 in rats and Cyp3a11, 3a13 and 3a16 in mice (Nelson et al., 1996)) among the species tested, although quinine is 3-hydroxylated mainly via CYP3A4 by human liver microsomes (Zhao et al., 1996; Zhang et al., 1997), and the CYP3A contents correlated significantly with the quinine 3-hydroxylation activities in the eight human liver microsomes assessed in the present study (fig. 8). Furthermore, the possibility of CYP(s)/Cyp(s) other than CYP3A also being involved in quinine 3-hydroxylation with animal liver microsomes cannot be totally ruled out. In this respect, further studies are definitely required.

In conclusion, our data have shown that 3-hydroxyquinine is a main metabolite of quinine and that CYP3A/Cyp3a is a principal isoform involved in this metabolic pathway in all species (rat, dog and human/mouse) tested. Overall, the dog and possibly the rat appear to be qualitatively and quantitatively suitable animal models for exploring the quinine 3-hydroxylase activity and for screening quinine-drug interactions in vitro, though some variance from the human liver microsomal data exists (e.g., an inhibition potency by TAO and discrepant interaction result with chloroquine). Finally, we are tempted to assert that an interspecies in vitro comparison study with animal and human liver microsomes will provide useful information on species differences and similarities in the metabolism of antimalarials such as quinine, thereby helping to identify an appropriate animal species for evaluating and/or forecasting the safety and toxicity of those antimalarial drugs. Liver microsomal samples obtained from such an animal species can be used to assess the possible involvement of candidate CYP(s) in the metabolism and then can be used to evaluate the drug-drug interactions that should be specifically investigated during the early clinical development program of antimalarial drugs. This seems to be particularly important during the early development of a new antimalarial drug in light of the fact that the detailed metabolic profile and involved CYP isoforms of quinine have only recently been clarified (Zhao et al., 1996; Zhang et al., 1997; Zhao and Ishizaki, 1997, in press) despite its 350-year history of clinical use (Tracy and Webster, 1996). Given that CYP isoform(s) involved in the metabolic pathways of many antimalarial drugs has (have) not been known (White, 1992),
the search for an animal model, as undertaken by this study, is required for bridging the gap between studies of human and of animal liver microsomal P450.

Acknowledgments

The authors thank Dr. P. Winstanley, University of Liverpool, for the generous donation of 3-hydroxyquinine, Zeneca Pharmaceuticals, Alderley Edge, UK, for the donation of propranolol and Dr. A. Küper for the donation of racemic mephenytoin as the respective in vitro assay standards in the present study. They also thank Dr. Wanwimolruk, University of Otago, New Zealand, for supplying the column as a tool for analyzing 3-hydroxyquinine.

References


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