IN VITRO METABOLISM OF FERROQUEINE (SSR97193) IN ANIMAL AND HUMAN HEPATIC MODELS AND ANTIMALARIAL ACTIVITY OF MAJOR METABOLITES ON PLASMODIUM FALCIPARUM

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ABSTRACT:
Ferroquine (SSR97193) has been shown to be a promising antimalarial, both on laboratory clones and on field isolates. So far, no resistance was documented in Plasmodium falciparum. In the present work, the metabolic pathway of ferroquine, based on experiments using animal and human hepatic models, is proposed. Ferroquine is metabolized mainly via an oxidative pathway into the major metabolite mono-N-demethyl ferroquine and then into di-N,N-demethyl ferroquine. Some other minor metabolic pathways were also identified. Cytochrome P450 isoforms 2C9, 2C19, and 3A4 and, possibly in some patients, isoform 2D6, are mainly involved in ferroquine oxidation. The metabolites were synthesized and tested against the 3D7 (chloroquine-sensitive) and W2 (chloroquine-resistant) P. falciparum strains. According to the results, the activity of the two main metabolites decreased compared with that of ferroquine; however, the activity of the mono-N-demethyl derivative is significantly higher than that of chloroquine on both strains, and the di-N-demethyl derivative remains more active than chloroquine on the chloroquine-resistant strain. These results further support the potential use of ferroquine against human malaria.

The generalization of Plasmodium falciparum resistance to chloroquine and to some other antimalarials represents an urgent problem to cure malaria in endemic areas. Some drugs are currently available, alone or in association with other antimalarials (Wiesner et al., 2003; Baird, 2005), but the development of new molecules remains a priority in the absence of an efficient vaccine strategy.

Ferroquine (SSR97193), resulting from the incorporation of a metabolocenic moiety to chloroquine (Fig. 1), was demonstrated to be a new drug with a powerful antimalarial activity in vitro and in vivo (Biot et al., 1997, 1999a,b; Delhaës et al., 2001, 2002). Tests on field isolates confirmed the susceptibility of all resistant parasites to ferroquine and the absence of a significant cross-resistance with major antimalariaIs (Aderounmu, 1983; Verdier et al., 1984; Brown et al., 1985; Karbwang and Wernsdorfer, 1993), potential metabolites (mono-N-demethyl ferroquine and di-N-demethyl ferroquine) were previously synthesized and found active in vitro on P. falciparum strains. According to the results, the activity of the two main metabolites decreased compared with that of ferroquine; however, the activity of the mono-N-demethyl derivative is significantly higher than that of chloroquine on both strains, and the di-N-demethyl derivative remains more active than chloroquine on the chloroquine-resistant strain. These results further support the potential use of ferroquine against human malaria.

Materials and Methods

Experimental Metabolism of Ferroquine. In Vitro Metabolism by Animal and Human Liver Microsomal Fractions. Male CD1 mouse, male Sprague-Dawley rat, Beagle dog, macaque (Macaca fascicularis), and human liver microsomal fractions were prepared from either whole livers or from liver biopsies by differential ultracentrifugation as described in Lake (1987). Incu-

ABBREVIATIONS: P450, cytochrome P450; HPLC-UV, high-performance liquid chromatography coupled with UV detection; TOF-MS/MS, quadrupole-time-of-flight/tandem mass spectrometry; FMO, flavin-containing mono-oxygenase; Q-TOF, quadrupole-time of flight; DMFQ, mono-N-demethyl-ferroquine; HPLC-MS/MS, high-performance liquid chromatography/tandem mass spectrometry; DMSO, dimethyl sulfoxide; FQ, ferroquine; DMFQ, mono-N-demethyl ferroquine; HH, human hepatocyte metabolite.
bation conditions were as follows: the test compound (5 μM) was incubated with 1 mg/ml liver microsomal proteins for 20 min at 37°C in 100 mM KH₂PO₄, pH 7.4, buffer containing 5 mM MgCl₂ in the presence of 1 mM NADPH. At the end of incubation, the reaction was quenched by the addition of 1 volume of acetonitrile. The mixture was vortexed and centrifuged at 5000 g for 10 min. The supernatant was retrieved and analyzed by HPLC-UV. For the structural identification studies, supernatants were partly evaporated under a stream of N₂, and analyzed by HPLC-UV and TOF-MS/MS detection.

In Vitro Metabolism by Human Recombinant Enzymes Preparation (Supersomes). Human recombinant P450 isoforms (1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A4 + cytochrome b₅) and flavin-containing monooxygenase (FMO) isoforms 1 and 3 were microsomal fractions prepared from Baculovirus-infected insect cells, Supersomes, obtained from Gentest (BD Gentest, Woburn, MA).

Incubation conditions were as follows: the test compound (5 μM) was incubated with 100 pmol/ml of each human recombinant enzyme for 20 min at 37°C in 100 mM KH₂PO₄, pH 7.4, in the presence of 1 mM NADPH. At the end of incubation, the reaction was quenched by the addition of 1 volume of acetonitrile. The mixture was vortexed and centrifuged at 5000g for 10 min. The supernatant was retrieved and analyzed by HPLC-UV.

In Vitro Metabolism by Human Hepatocytes. Freshly isolated human hepatocytes in primary culture were prepared from liver biopsies obtained from different cancer patients (n = 3) following partial hepatectomy. The two-step collagenase perfusion technique used for hepatocyte isolation has already been described (Fabre et al., 1988). Following isolation, cells were plated in collagen-coated six-well plates (1.4 × 10⁶ cells/ml) in Isom’s culture medium (Isom and Georgoff, 1984) supplemented with 10% fetal calf serum and penicillin/streptomycin. Four hours later, the culture medium was renewed with identical culture medium but devoid of fetal calf serum. Several hours (8–12 h) later, the culture medium was changed again, and the incubations were started by the addition of the test compound (5 μM). Hepatic biotransformation kinetics of the test compound (5 μM), as well as its major metabolite formation, were measured over a 0- to 24-h period. These incubations were also performed in the absence or the presence of specific and potent P450 inhibitors, namely furafylline (CYP1A2), sulfaphenazole (CYP2C9), quinidine (CYP3A4, and ketoconazole (CYP3A4) (Bourrie et al., 1996). At each sampling time, 1 volume of acetonitrile was added to the well, and the cells were scraped off the bottom of the well using a rubber cell scraper. The “intracellular + extracellular” mixture was then sonicated for 30 s, vortexed, and centrifuged at 5000g for 10 min. Supernatants were retrieved and further analyzed by HPLC-UV and TOF-MS/MS detection.

Separation and Identification of Ferroquine Metabolites. Samples were analyzed by HPLC-MS/MS using a Micromass quadruple-time of flight (Q-TOF) mass spectrometer (Waters-Micromass, Manchester, UK) fitted with a Micromass quadrupole-time of flight (Q-TOF) mass spectrometer (Waters-Micromass, Manchester, UK) fitted with an electrospray source in positive ion mode. HPLC column outlet was first connected to a UV detector for monitoring of the UV traces. The mobile phase flow was then split, and 100 μl/min were directed into the mass detector.

The analytical conditions for the microsomal fractions and the recombinant enzymes were as follows: a YMC-Pack Octyl 4-μm 250 × 2.1-mm column (Intechrom, Montluçon, France), 5 to 95% solvent B elution gradient using a mixture of solvent A [6.5 mM ammonium acetate and 0.1% (v/v) formic acid, pH 3.5, prepared in deionized water] and solvent B (acetonitrile) was used.

In Vitro Metabolism by Human Hepatocytes. The “intracellular” mixture was then sonicated for 30 s, vortexed, and centrifuged at 5000 g for 10 min. The supernatant was retrieved and analyzed by HPLC-UV. After cooling, water (50 ml) was added. The mixture was made alkaline by addition of K₂CO₃. The organic compounds were extracted by CH₂Cl₂ (3 × 20 ml). The organic layers were combined, dried over Na₂SO₄, and evaporated. The oil was purified by column chromatography (eluent, methyl acetate; then methyl acetate/triethylamine, 90:10) giving the ester as a red oil (507 mg, 92%). H NMR (CDCl₃) δ 8.53 (1H, d, J = 5.1 Hz, H2), 7.92 (1H, s, H8), 7.65 (1H, d, J = 9.3 Hz, H5), 7.29 (1H, d, J = 9.1 Hz, H6), 6.50 (1H, d, J = 5.4 Hz, H3), 5.54 (1H, s, NH), 5.09 (1H, d, J = 12.2 Hz, CHF=OAc), 4.92 (1H, d, J = 12.2 Hz, CH=OAc), 4.03 (2H, m, Cp), 4.27 (2H, m, NHCH₂), 4.18 (5H, s, Cp), 3.97 (1H, m), 3.96 (1H, m), 1.94 (3H, s, COCH₃).

FIG. 2. Ferroquine metabolism by different hepatic microsomal preparations. Liver microsomal fractions were prepared from either whole livers or from liver biopsies. The test compound was incubated with liver microsomal proteins. The supernatants of reaction mixtures were retrieved and analyzed by HPLC-UV. A, disappearance of ferroquine. B, formation of DMFQ (Met C).

Synthesis of Ferroquine and Ferroquine Metabolites. General. The H NMR spectra were recorded on a Brucker AC300 spectrometer using tetramethylsilane as the internal standard and deuterated chloroform (CDCl₃) and deuterated dimethylsulfoxide (DMSO-d₆) as the solvent. MS matrix-assisted laser desorption ionization TOF spectra were obtained using a Vision 2000 time-of-flight instrument (Finnigan MAT, Bremen, Germany) equipped with a nitrogen laser operating at wavelength of 337 nm. The matrix used was 2,4,6-trihydroxyacetophenone. SDS silica gel (35–70 mesh) was used for chromatography. Ferroquine and mono-N-demethyl-ferroquine (DMFQ or Met C) were synthesized as previously reported (Briot et al., 1997, 1999a,b). The 4-amino-7-chloroquinoline (named Met A) was obtained starting from 4,7-dichloroquinoline as already described (Yippagan et al., 1999).

Synthesis of Metabolite 7-Chloro-4-(2-(Hydroxymethyl)Ferrocenylmethylamino) Quinoline (Corresponding to Met G). 7-Chloro-4-(2-(acetoxyethyl)ferrocenylmethylamino)quinoline. A mixture of ferroquine (530 mg, 1.22 mmol) and acetic anhydride (4 ml, 42 mmol) were heated at 90°C for 5 min. After cooling, water (50 ml) was added. The mixture was made alkaline by addition of K₂CO₃. The organic compounds were extracted by CH₂Cl₂ (3 × 20 ml). The organic layers were combined, dried over Na₂SO₄, and evaporated. The oil was purified by column chromatography (eluent, methyl acetate; then methyl acetate/triethylamine, 90:10), giving the ester as a red oil (507 mg, 92%). H NMR (CDCl₃) δ 8.53 (1H, d, J = 5.1 Hz, H2), 7.92 (1H, s, H8), 7.65 (1H, d, J = 9.3 Hz, H5), 7.29 (1H, d, J = 9.1 Hz, H6), 6.50 (1H, d, J = 5.4 Hz, H3), 5.54 (1H, s, NH), 5.09 (1H, d, J = 12.2 Hz, CHF=OAc), 4.92 (1H, d, J = 12.2 Hz, CH=OAc), 4.03 (2H, m, Cp), 4.27 (2H, m, NHCH₂), 4.18 (5H, s, Cp), 3.97 (1H, m), 3.96 (1H, m), 1.94 (3H, s, COCH₃).

7-Chloro-4-(2-(hydroxymethyl)ferrocenylmethylamino)quinoline (Met G). An aqueous solution of K₂CO₃ (5 g, 25 ml) was added to a solution of 7-chloro-4-(2-(acetoxymethyl)ferrocenylmethylamino)quinoline (507 mg, 1.13 mmol) in MeOH (50 ml).

The mixture was stirred at 20°C for 4 h. The advancement of the reaction was followed by TLC. The mixture was extracted by CH₂Cl₂ (3 × 20 ml). The combined organic layers were dried over Na₂SO₄ and evaporated. The resulting oil was purified by column chromatography (eluent CH₂Cl₂/triethylamine, 90:10), giving 7-chloro-4-(2-(hydroxymethyl)ferrocenylmethylamino)quinoline as yellow
crystals (120 mg, 26%). $^1$H NMR (DMSO-$d_6$) $\delta$ 8.39 (1H, d, $J = 5.3$ Hz, H2), 8.18 (1H, d, $J = 9.0$ Hz, H5), 7.77 (1H, s, H8), 7.43 (1H, d, $J = 8.4$ Hz, H6), 7.31 (1H, s, NH), 6.63 (1H, d, $J = 5.1$ Hz, H3), 4.92 (1H, d, $J = 8.5$ Hz, CH$_2$N), 4.46 (1H, d, $J = 8.5$ Hz, CH$_2$N), 4.32–4.03 (5H, m, Cp), 4.15 (5H, s, CH$_3$). MS 406 (M $^{35}$Cl), 407 (M $^{37}$Cl), 408 (M $^{35}$Cl), 409 (M $^{37}$Cl).

Synthesis of 7-Chloro-4-(2-(Aminomethyl)Ferrocenylmethylamino) Quinoline (Met B).

A solution of 7-chloro-4-(2-(acetoxymethyl)ferroceny1- amidino) Quinoline Oxide (Met D).

After addition of 0.5

$^1$H NMR (DMSO-$d_6$) $\delta$ 10.32 (1H, s, NH), 8.35 (1H, d, $J = 5.1$ Hz, H2), 8.12 (1H, d, $J = 8.8$ Hz, H5), 7.23 (1H, d, $J = 8.8$ Hz, H6), 6.61 (1H, d, $J = 5.1$ Hz, H3), 4.50–4.15 (7H, m, Cp + 2 CH$_2$), 4.22 (5H, s, Cp'), 3.23 (3H, s, CH$_3$), 2.87 (3H, s, CH$_3$). MS 450 (M $^{35}$Cl), 452 (M $^{37}$Cl).

Synthesis of 7-Chloro-4-(2-(Aminomethyl)Ferrocenylmethylamino) Quinoline (Met B).

3-Phenyl-2-(phenylsulfonyl)-1,2-oxaziridine

$^1$H NMR (CDCl$_3$) $\delta$ 8.58 (1H, d, $J = 5.3$ Hz, H2), 7.91 (1H, d, $J = 1.53$ Hz, H8), 7.81 (1H, d, $J = 8.96$ Hz, H5), 7.25 (1H, m, H6), 6.47 (1H, d, $J = 5.34$ Hz, H3), 4.35 (1H, d, $J = 13.13$ Hz, CHNDQ), 4.28 (1H, m, Cp), 4.21 (1H, m, Cp), 4.13 (1H, m, CHNDQ), 4.12 (5H, s, Cp'), 4.10 (1H, m, Cp), 3.74 (2H, 2s, CHNDQ). SM 428 (M $^{35}$Cl + sodium), 408 (M $^{37}$Cl), 406 (M $^{35}$Cl).

Antimalarial Assays. Parasites. Plasmodium falciparum clonal 3D7 (provided by Dr. B. Pradines, Institut de Médecine Tropicale du Service de Santé des Armées, Marseille, France) were routinely maintained in cultures (Trager and Jensen, 1976) in complete RPMI 1640 medium (25 mM HEPES and 300 mg/l l-glutamine; Invitrogen), enriched with 10% decomplemented human serum (Quality Biological, Gaithersburg, USA) and 0.5% human red blood cells (O$^+$, PerkinElmer microplate scintillation and luminescence counter; PerkinElmer Life and Analytical Sciences). Fifty and 90% inhibitory concentrations (IC50 and IC90) were determined using a corrected $\log_{10}$ IC50.

trations (IC\textsubscript{50} and IC\textsubscript{90}) refer to molar concentrations of drug causing 50 and 90% reduction, respectively, in \[^{3}H\]hypoxanthine incorporation compared with drug-free control wells. They were estimated by linear interpolation from dose-response curves.

**Results**

**Metabolism of Ferroquine in Hepatic Microsomal Systems.** Ferroquine was incubated with liver microsomal preparations obtained from various species, and both the disappearance of the drug and formation of mono-\(\text{N}\)-demethyl ferroquine (DMFQ, Met C, HH-3) were monitored (Fig. 2). Strong interspecies differences were observed, and consumption appeared to be much more rapid in rodent and monkey hepatic microsomes than in dog or human hepatic microsomes (Fig. 2A). The extent of formation of DMFQ (Fig. 2B) was well correlated with ferroquine consumption, and it certainly represents the major pathway involved in ferroquine metabolism in all species studied.

**Metabolism of Ferroquine in Recombinant Human Cytochromes P450 and FMO Isoforms.** To identify the main enzymes involved in ferroquine oxidative biotransformation in human liver, different recombinant human P450 and FMO isoforms were used (Fig. 3). P450 isoforms 2C9, 2C19, 2D6, and 3A4 were all able to oxidize ferroquine (Fig. 3A). The formation of DMFQ was mainly associated with P450 isoforms 2C9, 2C19, and 3A4 (Fig. 3B).

**Metabolism of Ferroquine in Freshly Isolated Human Hepatocytes in Primary Culture.** The fate of ferroquine was also studied in vitro in freshly isolated human hepatocytes (Fig. 4). The in vitro intrinsic metabolic clearance (Cl\textscripts{int}) of ferroquine ranged between 0.032 and 0.067 ml h\(^{-1}\) (10\(^6\) hepatocytes\(^{-1}\)) with a mean value of 0.053 ml h\(^{-1}\) (10\(^6\) hepatocytes\(^{-1}\)) (\(n = 4\) preparations), which, in our experimental conditions, can be considered as a low to intermediary value. When tested simultaneously, chloroquine exhibited a low metabolic clearance value of 0.029 ml h\(^{-1}\) (10\(^6\) hepatocytes\(^{-1}\)) (\(n = 1\) preparation). With two of the human hepatocytes preparations studied, ferroquine was also incubated in the presence of specific and potent P450 inhibitors. In the presence of 10 \(\mu\)M ketoconazole, a potent and specific inhibitor (under these experimental conditions) of CYP3A4, the amount of DMFQ (HH-3) formed appeared to decrease in both hepatocyte preparations studied (Fig. 5), thus confirming the implication of CYP3A4 in the \(\text{N}\)-demethylation process suggested by the use of recombinant enzymes. A similar inhibitory effect was observed in both donors 1 and 2 in the presence of 10 \(\mu\)M sulfaphenazole, a potent and specific inhibitor of CYP2C9, also suggesting a role of CYP2C9 in contributing to this pathway. In addition, and in donor 2 only, quinidine also exhibited a certain inhibitory effect on ferroquine disappearance kinetics. However, in agreement with the results obtained with recombinant human P450 isoforms, this inhibition by quinidine did not produce any significant decrease in DMFQ (HH-3) formation kinetics, thus confirming that CYP2D6 does not support this particular metabolic route. This result, observed in only one human hepatocyte preparation of two, suggests a possible partial role.

**FIG. 5.** Effect of specific and potent P450 inhibitors on the metabolism of ferroquine in two primary culture human hepatocytes preparations (A and B, donor 1; C and D, donor 2). A and C, ferroquine disappearance. B and D, formation of DMFQ (Met C).
of CYP2D6 in ferroquine biotransformation in certain individuals (Fig. 5).

Metabolic Pathways of Ferroquine in Human Hepatic Models.
The use of human hepatic in vitro models enabled us to establish the major metabolic pathways of ferroquine. The HPLC-UV chromatograms and MS and MS/MS spectra of available synthetic standards (i.e., FQ, Met B, DMFQ or Met C, Met D, and Met G) are shown in Fig. 6. Although it was only present as its glucuronide derivative in human hepatocyte incubates, metabolite G [i.e., 7-chloro-4-(2-hydroxymethyl)ferrocenylmethylamino) quinoline] was included in these analyses.

All metabolites formed in vitro were separated by HPLC-UV and identified using on-line triple Q-TOF MS/MS detection (Figs. 7a and 8a). They were named according to their HPLC retention time (i.e., A to F in microsomal incubates and HH-1 to HH-4 in hepatocyte incubates). The structures of the metabolites were proposed based on the MS/MS spectra, on fragmentation patterns deduced from these spectra, and on the basis of coelution with available reference com-
FIG. 6. Continued.
Fig. 6. Continued.
FIG. 7. a, HPLC-UV chromatograms (254 nm) of incubation of FQ with animal and human liver microsomal fractions. Metabolites of ferroquine are named A, B, C, D, E, F, and G according to their chromatographic retention time. The control incubation in the absence of NADPH cofactor is shown for mouse only; it did not produce any metabolites in any of the species tested. b, LC-ESI-MS and MS/MS spectral data of metabolites observed in animal and human liver microsomal incubates; data shown were obtained with mouse liver microsomes. Left, LC-ESI-MS spectra of ferroquine (SSR97193, FQ), Met A, Met B, DMFQ (Met C), Met D, Met E, and Met F showing the parent ion fragmented during the subsequent MS/MS analysis. Right, LC-ESI-MS/MS spectra and proposed fragmentation schemes and structures of ferroquine (SSR97193, FQ), Met A, Met B, DMFQ (Met C), Met D, Met E, and Met F. These MS/MS spectra correspond to the fragmentation of the precursor ion detected after single MS analysis.
FIG. 7. Continued.
FIG. 7. Continued.
FIG. 8. A, HPLC-UV chromatograms of incubation of FQ with fresh human primary culture human hepatocytes. Metabolites of ferroquine are named HH-1, HH-2, HH-3, and HH-4 according to their chromatographic retention time (only the peaks related to ferroquine or its metabolites are named). B, LC-MS/MS spectral data of metabolites observed in primary human hepatocytes incubated with ferroquine—structural identification studies.
FIG. 8. Continued.
Fig. 8. Continued.
pounds (i.e., FQ, Met B, DMFQ or Met C, and Met D). Figure 7b and Fig. 8b represent the chemical structure and the ESI/MS and ESI/MS/MS spectra of the different metabolites of ferroquine produced in animal (mouse data shown in Fig. 7) and human liver microsomal fractions (Fig. 7b) or human hepatocytes (Fig. 8b) as well as their fragmentation schemes and proposed structures. To further confirm the structures of the main metabolites, a sample of mouse liver microsomal incubate was spiked with the mixture of available metabolites B, C, D, and G (Fig. 9). This spiking experiment confirmed that metabolites named B and C were the di- and mono-N-demethylated derivatives because they coeluted with the corresponding synthetic references and showed identical MS and MS/MS spectra to those of these two references. Metabolite D coeluted with the mono-N-oxide synthetic reference and it showed identical MS and MS/MS spectra.

On the other hand, as expected, the synthetic reference corresponding to metabolite “G” (only observed as its glucuronide derivative in human hepatocyte incubates) did not coelute with any chromatographic peak and exhibited a retention time slightly longer ($T_R = 33.02$ min) than that of the carboxylic acid derivative named metabolite “F” ($T_R = 32.22$ min).

The analysis of mouse liver microsomal incubates allowed to identify several metabolites related to an oxidative process (oxidation and/or N-dealkylation). In humans, the metabolism was moderate. The main metabolism route (accounting for at least 50% of total ferroquine disappearance in all species studied) consisted in an oxidative N-demethylation of ferroquine on the terminal tertiary amine group, yielding mono-N-demethyl ferroquine (DMFQ, Met C), and, subsequently, di-N-demethyl ferroquine (Met B). A second pathway, N-dealkylation on the amino-quinoline secondary amine, was quantitatively less important and led to the loss of the lateral chain carrying the ferrocene ring and the formation of 7-chloro-4-aminoquinoline (Met A). A third pathway resulted in the elimination of the terminal nitrogen from the lateral chain, thus leading in turn to the formation of 7-chloro-4-(2-hydroxymethyl)ferrocenymethylaminquinoline (Met G) and the corresponding carboxylic acid (Met F). This pathway represented a minor part of the original amount of ferroquine added.

Two other minor pathways resulted in the formation of two ferroquine N-oxides or hydroxylated derivatives (Met D and E, also named “FQ + O”; see Fig. 10). For metabolite D, the N-oxide on the tertiary amine structure was further confirmed by cochromatography with the synthesized reference, whereas for metabolite E, the precise oxidation position could not be elucidated further in view of the mass spectrometry data available. Table 1 summarizes the relative abundance of the metabolites identified in liver microsomal fractions from animal spe-
The formation of N-desethylchloroquine was reported to be catalyzed mainly by CYP3A4, CYP2C8, and CYP2D6 (Projean et al., 2003). In this study, we examined the metabolism of ferroquine in different in vitro hepatic models. Our results clearly show that the drug is metabolized more slowly by human liver microsomes than by rodent liver microsomes. This strongly suggests a lower clearance of ferroquine in humans compared with rodents and a longer persistence of efficient concentrations in patients. However, this hypothesis has to be confirmed in clinical pharmacokinetic studies.

In vitro studies using human liver microsomes as well as human recombinant enzymes demonstrated that several P450 isoforms were mainly involved in ferroquine metabolism (i.e., isoforms 2C9, 2C19, 2D6, and 3A4). More extensive inhibition studies using freshly isolated human hepatocytes in primary culture have also demonstrated that CYP3A4 and, to a lesser extent, CYP2C9 both play a major role in the overall hepatic metabolic clearance of ferroquine and more particularly in the major metabolic pathway identified, i.e., the N-demethylation of the terminal tertiary amine. In one preparation of hepatocytes, a noticeable inhibitory effect was exerted by quinidine thus suggesting some partial contribution by CYP2D6 also in some subjects. Thus, the effect of genetic polymorphisms would not be a limiting factor on ferroquine metabolism and on its effectiveness.

The metabolic pathways determined during our study (Fig. 10) confirm, at least in part, the hypothesis previously proposed concerning ferroquine metabolism (Biot et al., 1999b). The main degradation pathway is an oxidative route resulting in the formation of mono-N-demethyl ferroquine (DMFQ, Met C, or HH-3) and di-N-demethyl ferroquine (Met B or HH-2). Another pathway results in the formation of 4-amino-7 chloroquinoline (Met A). These two main metabolic pathways are very similar to those reported previously for chloroquine (Aderounmu, 1983; Verdier et al., 1984; Brown et al., 1985; Karbwang and Wermesdorfer, 1993).

Examination of antimalarial activities of major ferroquine metabolites showed that DMFQ remains as active as ferroquine on the chloroquine-sensitive strain 3D7 and less active than ferroquine but much more active than chloroquine on chloroquine-resistant strain W2. The other metabolites (Met B, Met D, and Met G) were less effective than ferroquine (8- to 10-fold according to their IC_{50} values), and their IC_{50} (\geq 100 nM) values show that they would not be efficient in parasite elimination. In a previous study (Biot et al., 1999b), DMFQ was found as active as ferroquine on chloroquine-sensitive strain (HB3) and on a moderate chloroquine-resistant strain (Dd2). Met C was found only 2-fold less active than ferroquine on Dd2.

Thus, DMFQ is significantly involved in the global antimalarial activity of ferroquine previously observed on rodent Plasmodium species (Biot et al., 1997, 1999a; Delhaës et al., 2001, 2002). Con-

### Table 1

**Relative abundances of ferroquine metabolites observed in animal and human microsomal fractions**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mouse</th>
<th>Rat</th>
<th>Rabbit</th>
<th>Dog</th>
<th>Macaque</th>
<th>Humans</th>
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-- metabolite not detected; + to ++++, metabolite detected and classified according to its relative abundance in extracts.

### Table 2

**Antimalarial activity of ferroquine and its main metabolites on two P. falciparum strains**

<table>
<thead>
<tr>
<th>Product</th>
<th>Strain 3D7</th>
<th>Strain W2</th>
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<tr>
<td></td>
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<td>IC_{90}</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>11.6 ± 5.2 (29)</td>
<td>37.5 ± 16.3 (25)</td>
</tr>
<tr>
<td>Ferroquine</td>
<td>8.0 ± 2.5 (29)</td>
<td>15.1 ± 7.25 (25)</td>
</tr>
<tr>
<td>Met C</td>
<td>12.0 ± 7.7 (3)</td>
<td>23.0 ± 13.3 (3)</td>
</tr>
<tr>
<td>Met D</td>
<td>77.2 ± 25.3 (6)</td>
<td>301.8 ± 121.5 (6)</td>
</tr>
<tr>
<td>Met B</td>
<td>73.8 ± 8.5 (3)</td>
<td>259.1 ± 80.2 (3)</td>
</tr>
<tr>
<td>Met G</td>
<td>226.1 ± 31.1 (6)</td>
<td>&gt;100 (6)</td>
</tr>
</tbody>
</table>

IC_{50} and IC_{90} are given as values ± standard deviations. Number of experiences in parentheses.
cerning potential ferroquine therapy in human malaria, the present results obtained with human hepatic in vitro systems indicate that DMFQ may also be involved. This point remains to be further documented by clinical pharmacokinetic studies.

Conclusion

Our study allowed us to determine the probable metabolic pathways of ferroquine and the antimalarial properties of the main identified metabolites. Based on 1) the relatively low metabolism rate of ferroquine in various human hepatic in vitro systems compared with rodent systems, 2) the genetic stability and the plurality of the main P450 isoforms involved in ferroquine biotransformation in humans, and 3) the significant antimalarial activity of DMFQ, we expect strong efficacy of ferroquine (SSR97193) in human malaria therapy.

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References


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