In vitro metabolism of ferroquine (SSR97193) in animal and human hepatic models and antimalarial activity of major metabolites on Plasmodium falciparum.

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Running title page

Running title: Antimalarial properties of ferroquine metabolites

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List of abbreviations.
DMFQ: Mono-N-demethyl-ferroquine
FMO: Flavin-containing mono-oxygenase
HPLC-UV: High performance liquid chromatography coupled with UV detection
TOF MS/MS: quadrupole time-of-flight tandem mass spectrometry
HPLC-MS/MS: high-performance liquid chromatography tandem mass spectrometry
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 metabolised 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. CYP isoforms 2C9, 2C19, 3A4 and also 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 to 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 generalisation of *P. 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 absence of an efficient vaccine strategy.

Ferroquine (SSR97193), resulting from the incorporation of a metallocenic moiety to chloroquine (Fig. 1), was demonstrated to be a new drug with a powerful anti-malarial activity *in vitro* and *in vivo* (Biot et al., 1999a; Biot et al., 1999b; Biot et al., 1997; Delhaës et al., 2001; Delhaës et al., 2002). Tests on field isolates confirmed the susceptibility of all resistant parasites to ferroquine and the absence of a significant cross-resistance with major antimalarials currently used (Atteke et al., 2003; Domarle et al., 1998; Pradines et al., 2001; Pradines et al., 2002). Ferroquine is currently in clinical phase I of development and its toxicological and pharmacokinetic profiles must be assessed carefully. Expecting that ferroquine and chloroquine should be metabolised according to a similar pathway (Aderoumnu et al., 1983; Brown et al., 1985; Karbwang et al., 1993; Verdier et al., 1984), potential metabolites (mono-\(N\)-demethyl ferroquine and di-\(N\)-demethylferroquine) were previously synthesized and found active *in vitro* on *P. falciparum* (Biot et al., 1999b). However the putative pathways have not yet been elucidated experimentally and the metabolic enzymes involved in ferroquine metabolism still remained to be identified.

In the work described herein, ferroquine metabolism was investigated using animal and human hepatic models. We examined the CYP isoforms involved in the biotransformation of the compound and, based on our structural identification results, we proposed a metabolic scheme. Moreover, the main metabolites were synthesized and tested on chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. These results highlighted the importance of this molecule and justified the development of ferroquine as a new antimalarial drug.
METHODS

1. Experimental metabolism of ferroquine

1.1. 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 ultra-centrifugation as described in (Lake 1987). Incubation conditions were as follows: the test compound (5 μM) was incubated with 1 mg/mL of 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 addition of 1 volume of acetonitrile. The mixture was vortexed and centrifuged at 5,000 g for 10 min. The supernatant was retrieved and analysed by HPLC-UV. For the structural identification studies, supernatants were partly evaporated under a stream of N₂ and analysed by HPLC-UV and TOF-MS/MS detection.

1.2. In vitro metabolism by human recombinant enzymes preparation (Supersomes™)

Human recombinant CYP (isoforms ; 1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A4 + cytochrome b5) and flavin-containing mono-oxygenase (FMO) isoforms 1 and 3 were microsomal fractions prepared from Baculovirus infected insect cells, Supersomes™, obtained from Gentest (BD-Gentest, Wolburn, MA).

Incubation conditions were as follows: the test compound (5 μM) was incubated with 100 pmoles/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
addition of 1 volume of acetonitrile. The mixture was vortexed and centrifuged at 5,000 g for 10 min. The supernatant was retrieved and analysed by HPLC-UV.

1.3. *In vitro* metabolism by human hepatocytes

Freshly isolated human hepatocytes in primary culture were prepared from liver biopsies obtained from (n = 3) different cancer patients following partial hepatectomy. The two-step collagenase perfusion technique used for hepatocytes isolation has already been described (Fabre et al., 1988). Following isolation, cells were plated in collagen-coated 6-well plates (1.4 \(10^6\) cells/mL) in Isom's culture medium (Isom et al., 1984) supplemented with 10 % fetal calf serum and penicillin/streptomycin. 4 h later, the culture medium was renewed with identical culture medium but devoid of fetal calf serum. Several h (8-12 h) later, the culture medium was changed again and the incubations were started by addition of the test compound (5 \(\mu\)M). Hepatic biotransformation kinetics of the test compound (5 \(\mu\)M) as well as its major metabolite formation were measured over a 0-24 h period. These incubations were also performed in the absence or the presence of specific and potent CYP inhibitors, namely furafylline (CYP1A2), sulfaphenazole (CYP2C9), quinidine (CYP2D6) and ketoconazole (CYP3A4) (Bourrié 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 "intra-cellular + extra-cellular” mixture was then sonicated for 30 seconds, vortexed and centrifuged at 5,000 g for 10 min. Supernatants were retrieved and further analysed by HPLC-UV and TOF-MS/MS detection.

2. Separation and identification of ferroquine metabolites
Samples were analysed by HPLC-MS/MS using a Micromass Q-TOF (Waters-Micromass, Manchester, U.K.) 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: YMC-Pack Octyl 4 µm 250 x 2.1 mm column (Interchim, France), 5 to 95 % solvent B elution gradient using a mixture of solvent A (6.5 mM ammonium acetate , 0.1 % (v/v) formic acid pH 3.5 prepared in de-ionised water) and solvent B (acetonitrile). Flow rate was 250 µL.min⁻¹, injection volume was 100 µL, UV detection at λ = 254 nm, total run time was 50 min and column temperature was set at +38°C.

The analytical conditions for the human hepatocytes incubates were as follows: Aquasil C18 5 µm 250 x 3 mm column (Thermo Electron Corporation, France), 2 to 95 % solvent B elution gradient using a mixture of solvent A and solvent B as above. Flow rate was 500 µL.min⁻¹, injection volume was 100 µL, UV detection at λ = 254 nm, total run time was 75 min and column temperature was set at +38°C.

3. Synthesis of ferroquine and ferroquine metabolites

3.1. General

The ¹H 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 MALDI 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 (Biot et al., 1999a; Biot et al., 1999b; Biot et al., 1997). The 4-amino-7-chloroquinoline (named Met A) was obtained starting from 4, 7-dichloroquinoline as already described (Vippagunta et al., 1999).

3.2. Synthesis of metabolite 7-chloro-4-(2-(hydroxymethyl)ferrocenylmethylamino)quinoline (corresponding to Met G)

7-chloro-4-(2-(acetoxymethyl)ferrocenylmethylamino)quinoline

A mixture of ferroquine (530 mg, 1.22 mmol) and acetic anhydride (4mL, 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%). 1H 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, CHHOAc), 4.92 (1H, d, J = 12.2 Hz, CHHOAc), 4.03 (2H, m, Cp), 4.27 (2H, m, NHCH2), 4.18 (5H, s, Cp’), 4.09 (1H, m, Cp), 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 (50mL).
The mixture was stirred at 20°C for 4 h. The advancement of the reaction was followed by TLC. The mixture was extracted by CH$_2$Cl$_2$ (3 × 20mL). The combined organic layers were dried over Na$_2$SO$_4$ and evaporated. The resulting oil was purified by column chromatography (eluent CH$_2$Cl$_2$/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 + CH$_2$), 4.15 (5H, s, Cp$'$). MS 406 (M$^{35}$Cl), 407 (MH$^{35}$Cl), 408 (M$^{37}$Cl), 409 (MH$^{37}$Cl)

3.3. Synthesis of 7-chloro-4-(2-(N,N-dimethylaminomethyl)ferrocenylmethylamino)quinoline oxide (Met D)

3-Phenyl-2-(phenylsulfonyl)-1, 2-oxaziridine (Lee et al, 1996) (314 mg, 1.2 mmol) in dried CH$_2$Cl$_2$ (20 mL) was added to ferroquine (433 mg, 1 mmol) in dried CH$_2$Cl$_2$ (20 mL). After stirring at 20°C for 1 h, the solvent was evaporated and the resulting oil was purified by column chromatography (eluent methyl acetate, methyl acetate/triethylamine 95/5 then CH$_2$Cl$_2$/CH$_3$OH/triethylamine 50/45/5) giving Met D as brown crystals (397 mg, 88%). M.p.: decomposed before fusion. $^1$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 (MH$^{35}$Cl), 452 (MH$^{37}$Cl).

3.4. Synthesis of 7-chloro-4-(2-(aminomethyl)ferrocenylmethylamino)quinoline (Met B)
A solution of 7-chloro-4-(2-(acetoxymethyl)ferrocenylmethylamino)quinoline (100 mg, 0.22 mmol) and NH₄OH (4 mL, 25%) in acetonitrile was stirring at 60°C. After 5 h, the solution was extracted by CH₂Cl₂ (3 x 10 mL). The organic layer was acidified by HCl (1N). The aqueous layer was neutralised by a potassium carbonate solution and extracted by CH₂Cl₂ (3 x 10 mL). The organic layers were combined, dried over Na₂SO₄ and evaporated to give Met B as yellow crystals (64 mg, 71%). ¹H NMR (CDCl₃ + D₂O) δ 8.58 (1H, d, J = 5.31 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, CHND₂). SM 428 (M⁺Cl + Na), 408 (MH⁺Cl), 406 (MH⁺Cl).

4. Antimalarial tests

4.1. Parasites.

Plasmodium falciparum clones 3D7 (provided by Paris Pasteur Institute Genopole) and W2 (provided by Dr B. Pradines, PHARO, Marseille, France) were routinely maintained in cultures (Trager et Jensen., 1976) in complete RPMI 1640 medium (RPMI medium; HEPES 25 mM; L-glutamine 300 mg/L - Invitrogen), enriched with 10% decomplemented human serum (AB⁺), and 6 % human red blood cells (O⁺) at 37°C under controlled atmosphere (O₂ 5 % - CO₂ 5 % - N₂ 90 %; Air Liquide, France). Serum and red blood cells were supplied by Centre Régional de Transfusion Sanguine, Lille, France. Cultures were controlled by thin smears stained with Giemsa (Merck). Parasitemia were monitored on 1000 red blood cells.

4.2. In vitro antimalarial assay on Plasmodium falciparum
The assays were conducted in vitro on asynchronous *P. falciparum* cultures using a modification of the semi-automated microdilution technique of Desjardin *et al.* (Desjardin *et al.*, 1979) based on radiolabelled hypoxanthine incorporation in nucleic acids of parasites. Chloroquine (provided by Pr. Jacques Brocard, USTL, Lille I) stock solutions were prepared in 70 % methanol and ferroquine and its synthetized metabolites (P.L, B.C, B.J authors) in DMSO. The final concentrations ranged from 4.5 to 581.5 nM. Tests starting conditions were 0.5 % initial parasitaemia and 0.5 % haematocrit. After addition of 0.5 µCi/well of [³H]-hypoxanthine (Amersham), microplates were incubated at 37°C under a controlled atmosphere (5 % O₂ - 5 % CO₂ - 90 % N₂, Air Liquide, France) for 48 h. Well contents were then harvested on Unifilter plate GF/C (Perkin Life Sciences) using a Skatron system and dried. Incorporation of radiolabelled [³H]-hypoxanthine into parasites nucleic acids was monitored in a fluorescence spectrometer (Perkin Elmer microplate scintillation and luminescence counter). Fifty percent and ninety percent inhibitory concentrations (IC₅₀ and IC₉₀) refer to molar concentrations of drug causing 50 % and 90 % reduction, respectively in [³H]-hypoxanthine incorporation compared to drug-free control wells. They were estimated by linear interpolation from dose-response curves.
RESULTS

1. Metabolism of ferroquine in hepatic microsomal systems

Ferroquine was incubated with liver microsomal preparations obtained from various species and both disappearance of the drug and formation of mono-N-demethyl ferroquine (DMFQ, Met C, HH-3) were monitored (Fig. 2). Strong inter-species 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.

2. Metabolism of ferroquine in recombinant human cytochromes P-450 and FMO isoforms

In order to identify the main enzymes involved in ferroquine oxidative biotransformation in human liver, different recombinant human CYP and FMO isoforms were used (Fig. 3). CYP isoforms 2C9, 2C19, 2D6 and 3A4 were all able to oxidise ferroquine (Fig. 3A). The formation of DMFQ was mainly associated with CYP isoforms 2C9, 2C19 and 3A4 (Fig. 3B).

3. 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_int) of ferroquine ranged between 0.032 and 0.067 mL.hour\(^{-1}\).\(10^6\) hepatocytes\(^{-1}\) with a mean value of 0.053 mL.hour\(^{-1}\).\(10^6\) 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.hour\(^{-1}\).\(10^6\) hepatocytes\(^{-1}\) (n = 1 preparation). With two of the human hepatocytes preparations studied, ferroquine was also incubated in the presence of specific and potent CYP inhibitors. In the presence of 10 µM ketoconazole, a potent and
specific (under these experimental conditions) inhibitor 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 N-demethylation process suggested by the use of recombinant enzymes. A similar inhibitory effect was observed in both donor 1 and 2 in the presence of 10 µM 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 CYP 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 out of two, suggests a possible partial role of CYP2D6 in ferroquine biotransformation in certain individuals (Fig. 5).


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 Figure 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 quadrupole - Time of flight (Q-TOF) MS/MS detection (Fig. 7.a and 8.a). 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 deducted from these spectra and on the basis of co-elution with available reference compounds (i.e., FQ, Met B, DMFQ or Met C and Met D). Figure 7.b and Figure 8.b 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 the Figure) and human liver microsomal fractions (Fig. 7.b) or human hepatocytes (Fig. 8.b) as well as their fragmentation schemes and proposed structures. In order 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 (Figure 9). This spiking experiment confirmed that metabolites named B and C were the di- and mono-N-demethylated derivatives since they co-eluted with the corresponding synthetic references and showed identical MS and MS/MS spectra to those of these two references. Metabolite D co-eluted with the mono-N-oxide synthesised 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 co-elute with any chromatographic peak and exhibited a retention time slightly longer (T_R = 33.02 min) than that of the carboxylic acidic 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-hydroxymethylferrocenylmethylaminoquinoline (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 Figure 10). For metabolite D, the N-oxide on the tertiary amine structure was further confirmed by co-chromatography with the synthesised reference, while for metabolite E, the precise oxidation position could not be elucidated further in view of the mass spectrometry data available. Table 1 summarises the relative abundances of the metabolites identified in liver microsomal fractions from animal species and humans. This shows that metabolite C (DMFQ) is by far the main metabolite observed in all six species investigated.

Finally in human hepatocytes (Fig. 8.b), two glucuronic acid derivatives were also identified, namely HH-1 and HH-4, both formed from either the single oxidised derivative (Met E), or from the de-aminated derivative, i.e. 7-chloro-4-(2-hydroxymethylferrocenylmethyl) aminoquinoline (Met G), respectively.

The analysis of intra- and extra-cellular pooled mixtures shows the presence of a major mono-N-demethylated derivative (HH-3; DMFQ). The search for chlorinated compounds allowed to identify three additional minor metabolites either under their free form (HH-2, Met B, di-N-demethyl-ferroquine) or glucurono-conjugated (HH-1, glucuronide of a mono-hydroxylated derivative of ferroquine ; HH-4, glucuronide of a ferrocene-methanol derivative most likely resulting from the oxidative deamination of the primary amine HH-2).
5. Antimalarial activity of main ferroquine metabolites.

According to the metabolism pathways identified for ferroquine, the metabolites B, C, D, and G were synthesised and their antimalarial activities were tested on two strains of *P. falciparum* (strain 3D7 chloroquine-sensitive and W2 chloroquine-resistant). Results are shown in Table 2.

The activity of DMFQ on 3D7 strain is very close to that of ferroquine itself and similar to that of chloroquine. The IC$_{90}$/IC$_{50}$ index of DMFQ is similar to that of ferroquine and significantly lower than that of chloroquine. Concerning the chloroquine-resistant strain W2, DMFQ is less active than ferroquine (3.4-fold), but remains much more active than chloroquine with a lower IC$_{90}$/IC$_{50}$ index. Activity of Met D appeared much lower than those of ferroquine and chloroquine on a chloroquine-sensitive strain, but the product remains as active as chloroquine on the W2 strain. Met B and Met G were less active than chloroquine on both *P. falciparum* strains tested.
DISCUSSION

In humans, the antimalarial drug chloroquine is metabolized into one major metabolite, \( N \)-desethylchloroquine and then into \( N \)-didesethylchloroquine. The formation of \( N \)-desethylchloroquine was reported to be catalysed mainly by CYP3A4, CYP2C8 and CYP2D6 (Projean et al., 2003). In this study, we examined the metabolism of ferroquine in different \textit{in vitro} hepatic models. Our results clearly showed that the drug is metabolised 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.

\textit{In vitro} studies using human liver microsomes as well as human recombinant enzymes demonstrated that several CYP isoforms were mainly involved in ferroquine metabolism (\textit{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, \textit{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 (Aderoumnu et al., 1983; Brown et al., 1985; Karbwang et al., 1993; Verdier et al., 1984).

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, Met G) were less effective than ferroquine (8 to 10-fold according to their IC₅₀ values) and their IC₉₀ (>> 100 nM) 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-folds 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., 1999a; Biot et al., 1997; Delhaës et al., 2001; Delhaës et al., 2002). Concerning 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 allows to determine the probable metabolic pathways of ferroquine and the antimalarial properties of the main identified metabolites.

Based on i) the relatively low metabolism rate of ferroquine in various human hepatic in vitro systems compared with rodent systems, ii) the genetic stability and the plurality of the main CYP isoforms involved in ferroquine biotransformation in humans, and iii) the significant antimalarial activity of DMFQ, we expect strong efficacy of ferroquine (SSR97193) in human malaria therapy.
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References.


Karbwang J and Wernsdorfer W (1993) Clinical Pharmacology of Antimalarials. Faculty of Tropical Medicine, Madihol University \textbf{p107}. 


Footnotes

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Reprint request:

Daniel Dive; INSERM U547, Institut Pasteur, 1 rue du Professeur P. Calmette, B.P. 245, 59019 Lille Cedex, France
Legends of figures

Figure 1: Chemical structures of chloroquine and ferroquine.

Figure 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 analysed by HPLC-UV. A: disappearance of ferroquine. B: formation of DMFQ (Met C).

Figure 3: *In vitro* metabolism of ferroquine by human recombinant CYP isoforms and FMO isoforms (microsomal fractions prepared from *Baculovirus* infected insect cells: Supersomes™). The supernatants of reaction mixtures were retrieved and analysed by HPLC-UV. A: disappearance of ferroquine. B: formation of DMFQ (Met C).

Figure 4: Metabolism of ferroquine by freshly isolated human hepatocytes in primary cultures. Hepatocytes preparation protocol is detailed in Material and Methods. The supernatants from reaction mixtures were retrieved and further analysed by HPLC-UV and TOF-MS/MS detection. A: ferroquine disappearance. B: formation of DMFQ (Met C).

Figure 5: Effect of specific and potent CYP inhibitors on the metabolism of ferroquine in 2 primary culture human hepatocytes preparations (A, B: donor 1; C, D: donor 2). A, C: ferroquine disappearance. B, D: formation of DMFQ (Met C).

Figure 6: HPLC-UV chromatogram and LC-ESI+--MS and MS/MS spectra of ferroquine (SSR97193, FQ), Met B, DMFQ (Met C), Met D and Met G. A: HPLC-UV (254 nm) chromatogram of a mixture of ferroquine (SSR97193, FQ), synthetic Met B, synthetic DMFQ (Met C), synthetic Met D and synthetic Met G. B: *Left panel* - LC-ESI+--MS spectra of ferroquine (SSR97193, FQ), synthetic Met B, synthetic DMFQ (Met C), synthetic Met D and synthetic Met G showing the parent ion fragmented during the subsequent MS/MS analysis. *Right panel* - LC-ESI+--MS/MS spectra,
structures and fragmentation schemes of ferroquine (SSR97193, FQ), synthetic Met B, synthetic DMFQ (Met C), synthetic Met D and synthetic Met G. These MS/MS spectra correspond to the fragmentation of the precursor ion detected after single MS analysis.

Figure 7.a: HPLC-UV chromatograms (254 nm) of incubation of ferroquine (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 co-factor is shown for mouse only; it did not produce any metabolites in any of the species tested.

Figure 7.b: LC-ESI+-MS and MS/MS spectral data of metabolites observed in animal and human liver microsomal incubates - data shown obtained with mouse liver microsomes. *Left panel* - 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 panel* - 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.

Figure 8.a: HPLC-UV chromatograms of incubation of ferroquine (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).

Figure 8.b: LC-MS/MS spectral data of metabolites observed in primary human hepatocytes incubated with ferroquine - structural identification studies.

Figure 9. Mouse microsomal incubate spiked with a mixture of synthetic references B, C, D and G.

Figure 10: Metabolic pathways of ferroquine in human hepatic models. The analytical HPLC conditions were detailed in Material and Methods. Samples were analysed by HPLC-MS/MS using a Micromass Q-TOF fitted with an electrospray source in positive ion mode.
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>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;A&quot;</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>&quot;B&quot;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>&quot;C&quot;</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>+</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>&quot;D&quot;</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>&quot;E&quot;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>&quot;F&quot;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2: Antimalarial activity of ferroquine and its main metabolites on two *P. falciparum* strains.

IC<sub>50</sub> and IC<sub>90</sub> are given as values ± standard deviations. Number of experiences in parentheses.

<table>
<thead>
<tr>
<th>Product</th>
<th>strain 3D7</th>
<th></th>
<th></th>
<th>strain W2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; nM</td>
<td>IC&lt;sub&gt;90&lt;/sub&gt; nM</td>
<td>IC&lt;sub&gt;90/IC&lt;sub&gt;50&lt;/sub&gt;&lt;/sub&gt;</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; nM</td>
<td>IC&lt;sub&gt;90&lt;/sub&gt; nM</td>
<td>IC&lt;sub&gt;90/IC&lt;sub&gt;50&lt;/sub&gt;&lt;/sub&gt;</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>11.6±5.2 (29)</td>
<td>37.5±16.3 (25)</td>
<td>3.1</td>
<td>146.9±61.1 (33)</td>
<td>&gt;1000 (25)</td>
<td>&gt;6.9</td>
</tr>
<tr>
<td>Ferroquine</td>
<td>8.0±2.5 (29)</td>
<td>15.1±7 (25)</td>
<td>1.8</td>
<td>13.6±4.2 (33)</td>
<td>15.1±7.0 (25)</td>
<td>1.1</td>
</tr>
<tr>
<td>Met C</td>
<td>12.0±7.7 (3)</td>
<td>23.0±13.3 (3)</td>
<td>1.9</td>
<td>46.4±35.5 (4)</td>
<td>58.6±24.7 (4)</td>
<td>1.3</td>
</tr>
<tr>
<td>Met D</td>
<td>77.2±25.3 (6)</td>
<td>301.8±121.5 (6)</td>
<td>3.8</td>
<td>94.7±25.0 (4)</td>
<td>&gt;1000 (4)</td>
<td>&gt;10</td>
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<tr>
<td>Met B</td>
<td>73.8±8.5 (3)</td>
<td>259.1±80.2 (3)</td>
<td>3.5</td>
<td>108.3±28.2 (3)</td>
<td>743.4±59.7 (3)</td>
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<tr>
<td>Met G</td>
<td>226.1±31.1 (6)</td>
<td>&gt;100 (6)</td>
<td>&gt;4.4</td>
<td>359±99.3 (4)</td>
<td>&gt;100 (4)</td>
<td>&gt;9</td>
</tr>
</tbody>
</table>
Figure 1

CQ

FQ
Figure 2

(A) Ferrocyan oxidative metabolism (%) of Swiss CD1-Mouse, SD Rat, Beagle Dog, Macaca Monkey, and Human.

(B) DMFQ formed (µM, Eq) of Swiss CD1-Mouse, SD Rat, Beagle Dog, Macaca Monkey, and Human.
Figure 5

Donor 1

A

FQ Concentration (µM)

FQ disappearance

Incubation Time (Hours)

B

N-Demethyl Metabolite (µM-Eq.)

DMFQ formation

Incubation Time (Hours)

Donor 2

C

FQ Concentration (µM)

FQ disappearance

Incubation Time (Hours)

D

N-Demethyl Metabolite (µM-Eq.)

DMFQ formation

Incubation Time (Hours)
Figure 6a
Figure 6b-1

Ferroquine

ESI⁺-MS

ESI⁺-MS/MS of ion m/z = 434

Metabolite B

ESI⁺-MS

ESI⁺-MS/MS of ion m/z = 406

Metabolite C

ESI⁺-MS

ESI⁺-MS/MS of ion m/z = 420
Figure 6b-2

Metabolite D

ESI⁺-MS

ESI⁺-MS/MS of ion m/z = 450

Metabolite G

ESI⁺-MS

ESI⁺-MS/MS of ion m/z = 407
Figure 7a

Mouse
  No
  NADPH

Mouse
  +
  NADPH

Rat
  +
  NADPH

Rabbit
  +
  NADPH

Dog
  +
  NADPH

Macaque
  +
  NADPH

Human
  +
  NADPH
Figure 7b-1

Metabolite A

ESI⁻-MS

ESI⁺-MS/MS of ion m/z = 434

Metabolite B

ESI⁻-MS

ESI⁺-MS/MS of ion m/z = 406
Figure 7b-2

**Metabolite C**

**ESI⁺-MS**

- 242
- 243
- 389
- 420
- 422
- 391
- 423

**ESI⁺-MS/MS of ion m/z = 420**

![Chemical structure of Metabolite C](image)

**Metabolite D**

**ESI⁺-MS**

- 79
- 120
- 189
- 389
- 391
- 450
- 392
- 452

**ESI⁺-MS/MS of ion m/z = 450**

![Chemical structure of Metabolite D](image)

**Metabolite E**

**ESI⁺-MS**

- 203
- 213
- 256
- 257
- 285
- 287
- 388
- 407
- 452

**ESI⁺-MS/MS of ion m/z = 450**

![Chemical structure of Metabolite E](image)
Metabolite F

**ESI\(^{+}\)-MS**

**ESI\(^{+}\)-MS/MS of ion m/z = 421**
Figure 8b-1

Ferroquine

**ESI**⁺-MS

<table>
<thead>
<tr>
<th>100</th>
<th>256</th>
<th>213</th>
<th>389</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>434</td>
<td>391</td>
<td>436</td>
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</table>

**ESI**⁺-MS/MS of ion m/z = 434

<table>
<thead>
<tr>
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<th>256</th>
<th>389</th>
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<tbody>
<tr>
<td>50</td>
<td>214</td>
<td>257</td>
<td>434</td>
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Metabolite HH1

**ESI**⁺-MS

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**ESI**⁺-MS/MS of ion m/z = 626

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<tbody>
<tr>
<td>50</td>
<td>582</td>
<td>406</td>
<td>626</td>
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Metabolite HH2

**ESI**⁺-MS

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<tbody>
<tr>
<td>20</td>
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</table>

**ESI**⁺-MS/MS of ion m/z = 406

<table>
<thead>
<tr>
<th>100</th>
<th>199</th>
<th>229</th>
<th>389</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>259</td>
<td>408</td>
<td>450</td>
</tr>
</tbody>
</table>
Figure 8b-2

Metabolite HH3

ESI'-MS

ESI'-MS/MS of ion m/z = 420

Metabolite HH4

ESI'-MS/MS of ion m/z = 583