Synthesis of Ferroquine Enantiomers: First Investigation of Effects of Metallocenic Chirality upon Antimalarial Activity and Cytotoxicity

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Ferroquine (FQ) is a new antimalarial agent with a high blood schizotoncidal activity. Previous studies on this compound were done with racemate mixtures. As FQ possesses planar chirality, pure enantiomers were obtained by enzymatic resolution in order to compare their antimalarial activities and cytotoxicities. (+)-FQ and (−)-FQ were equally active in vitro, at nanomolar concentrations. Both enantiomers were slightly less active than the racemate in vivo; cytotoxicities were similar. Actually, the racemate represents the optimal formulation. To the best of our knowledge, this is the first investigation of biological activities of compounds with metallocenic chirality.

KEYWORDS:

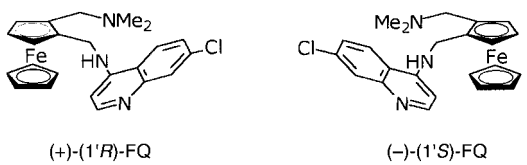
antimalarial activity · cytotoxicity · enzymatic resolution · ferroquine · metallocenes

Introduction

The most severe form of human malaria is caused by *Plasmodium falciparum*. An estimated 250 million people are infected every year, and the disease will prove fatal for 1.7 – 2.7 million of them.[1, 2] The emergence of multidrug-resistant *P. falciparum* has become a major problem in both prophylaxis and treatment of malaria. As chloroquine (CQ) has been the most extensively used drug, its efficacy is now compromised by the spread of resistance throughout endemic malarial areas.[1] In addition, the current lack of availability of an efficient malarial vaccine highlights the urgent need to develop alternative drugs that circumvent resistance to CQ.[3–5]

We have been focusing on chloroquine dicyclopentadienyl iron analogues, which have been reported to be effective against *P. falciparum* in vitro, and against *P. berghei*, *P. yoelii*, and *P. vinckei vinckei* in vivo.[6–10] The most active of these, 7-chloro-4-[2-(*N*′,*N*′-dimethylaminomethyl)-*N*-ferrocenylmethylamino]quinoline or ferroquine (FQ, Scheme 1), has previously been used as a racemic mixture and found to be active against all chloroquine-resistant *P. falciparum* field strains.[6–11]

Since many biological systems can discriminate between the members of a pair of enantiomers as different substances, different enantiomers may in many cases induce different responses in these systems. All the desired activity may reside in only one enantiomer, whilst the optical antipode may be totally inactive or even toxic. Moreover, enantiomers can exhibit large difference in their pharmacokinetic properties.[12, 13] Such properties may represent an impediment for drug development. Due to its 1,2-unsymmetrically substituted ferrocene structure, FQ possesses planar chirality. The aim of this investigation was to synthesize pure (+)-FQ and (−)-FQ enantiomers, in order to assess and to compare the antimalarial and cell antiproliferative



(+)-(1′*R*)-FQ (−)-(1′*S*)-FQ

Scheme 1. *Ferroquine (FQ) enantiomers.*

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activities of (±)-FQ, (+)-FQ, and (−)-FQ. This study constitutes, to our knowledge, the first contribution concerning the relationship between metallocene chirality and biological activity.

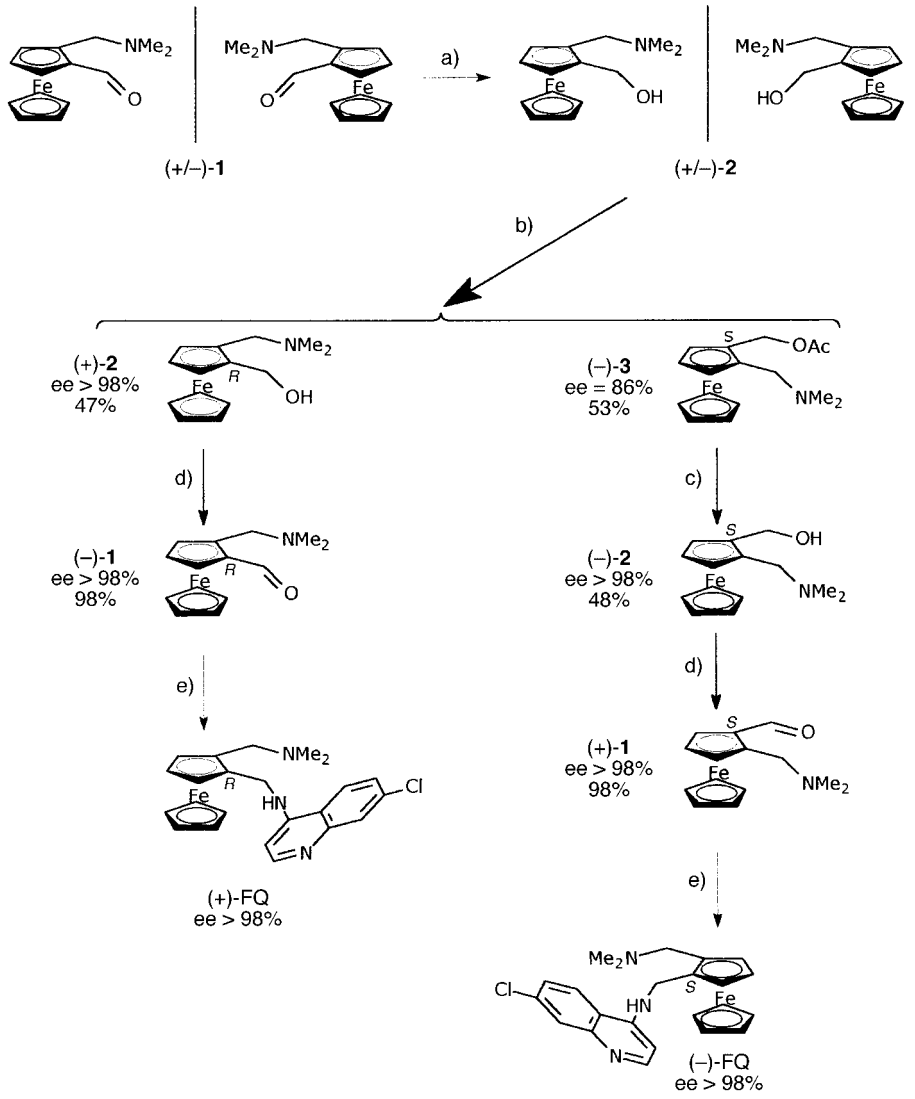
Results and Discussion

Chemistry

FQ possesses planar chirality due to its 1,2-unsymmetrically substituted ferrocene moiety. Despite their close structural relationship, the two enantiomers must be viewed as different pharmacological species in terms of their desired or undesired effects. A synthetic method to provide separate pure enantiomers of FQ in good yields was therefore developed (Scheme 2). The approach chosen included a lipase kinetic resolution according to Nicolosi's procedure, which explored the ability of enzymes to catalyze enantioselective reactions.[14] We used *Candida rugosa* lipase (*C.r.*l.), which is able to catalyze acetylation or transesterification more rapidly for (−) isomers than for (+) isomers.[14] All the relative configurations of the aromatic rings in the chiral ferrocenes were determined according to the nomenclature of Cahn, Ingold and Prelog, as extended to planar chiralities by Schlögl.[15]

Racemic 2-(*N*,*N*-dimethylaminomethyl)ferrocenecarboxaldehyde **1** was reduced in the presence of $NaBH_4$ in methanol to give the corresponding amino alcohol **2**. *C.r.*l. catalyzed the acetylation of **2** with vinyl acetate in *tert*-butyl methyl ether to give (−)-**3** in 53% yield and with 86% *ee*. The remaining untransformed (+)-**2** was isolated from the mixture in good chemical yield (47%) and extremely high enantiomeric excess (*ee* > 98%). The deacylation of (−)-**3** was undertaken by means of a transesterification process catalyzed by *C.r.*l. with *n*BuOH to provide (−)-**2** with high enantiomeric purity (*ee* > 98%) and in 48% yield.[16] Finally, the enantiopure amino alcohols were oxidized to the corresponding amino carboxaldehydes by use of a large excess of MnO_2. Starting from the pure (+)- and (−)-2-(*N*,*N*-dimethylaminomethyl)ferrocenecarboxaldehydes ((+)- and (−)-**1**), the subsequent syntheses of (+)-FQ and (−)-FQ were identical to that of (±)-FQ.[6, 7]

In order to compare the biological activities of the FQ enantiomers with that of the racemic mixture, it was necessary to determine the purity of each enantiomer. As chiral HPLC was not efficient for this determination, the use of ^{1}H NMR spectroscopy with an optically active reagent was adopted. The ^{1}H NMR spectrum of racemic FQ with two equivalents of Pirkle's alcohol showed two singlets (dimethylamino signals) at $\delta = 2.22$ ppm, separated by 7.80 Hz and in a ratio of 50:50; this was useful for establishing the enantiomeric excess.[17] In contrast, the ^{1}H NMR spectra of (+)- and (−)-FQ measured under these conditions each showed only one dimethylamino resonance. Stereoselective analysis of the intermediate compounds in the final stages of the synthesis of (+)- and (−)-FQ (see Experimental Section for details) was also performed by ^{1}H NMR spectroscopy in the presence of two equivalents of Pirkle's alcohol.



Scheme 2. *Synthesis of (+)-FQ and (−)-FQ by enzymatic resolution. a) $NaBH_4$, MeOH, 0 °C, 20 min; b) vinyl acetate, C.r.l., tBuOMe, 45 °C, 16 h; c) n-BuOH, C.r.l., tBuOMe, 45 °C, 48 h; d) MnO_2, CH_2Cl_2, 0 °C; e) 1. $NH_2OH \cdot HCl$, EtOH, reflux, 3.5 h; 2. $LiAlH_4$, THF, reflux, 6 h; 3. 4,7-dichloroquinoline, K_2CO_3, TEA, NMP, 135 °C, 3.5 h. C.r.l. = Candida antartica lipase, THF = tetrahydrofuran, TEA = triethylamine, NMP = N-methylpyrrolidine.*

Biological activities

The screening procedure is briefly described in the experimental section. The antimalarial and cytotoxicity activities of the synthesized compounds are summarized in Table 1 and Table 2.

Table 1. *In vitro antimalarial activity and toxicity of the FQ enantiomers, FQ racemate and CQ.*

	(+)-FQ	(−)-FQ	(±)-FQ	CQ
antimalarial activity against culture-adapted strains of *Plasmodium falciparum*[a]				
HB3	12 ± 1	11 ± 2	11 ± 2	24 ± 4[b], [c]
Dd2	21 ± 6	21 ± 4	22 ± 4	130 ± 38[c]
cytotoxicity on mouse lymphoma cells[a]				
L5178Y	14 300 ± 2,800	16 600 ± 4,200	14 300 ± 4,400	> 50 000
security index	1 200 – 700[d]	1 500 – 800	1,300 – 600	> 2,000 – > 400

[a] Values are the mean IC_{50} values (± standard deviation) in nmol L^{-1} obtained from at least three independent experiments. [b] Chloroquine-susceptible threshold adopted is 100 nmol L^{-1}. [c] $p < 0.05$. Variance analysis (ANOVA system) was used to compare the IC_{50} values of CQ versus (+)-FQ, (−)-FQ, and (±)-FQ. [d] Values of the security index correspond to the ratio of the IC_{50} value in the L5178Y cell proliferative assay divided by the antimalarial activity IC_{50} value for chloroquine-susceptible and -resistant *P. falciparum* strains, respectively.

Table 2. *Comparative curative effect of FQ enantiomers on mice infected by* P. vinckei vinckei *strains and treated orally.*

Compound	Dose[a] [mg kg^{-1} day^{-1}]	*P. vinckei vinckei* strain[b] CQ-sensitive	CQ-resistant
(+)-FQ	5	0/10 (13.6; 9 – 17)[c]	2/10 (12; 7 – 16)
	10	10/10	8/10 (18; 18)
(−)-FQ	5	0/10 (10; 7 – 15)	0/10 (14.5; 10 – 16)
	10	7/10 (16.6; 17 – 16)	5/10 (18.8; 16 – 27)

[a] Doses are expressed in terms of CQ base equivalent. CQ was applied as the diphosphate salt and FQ enantiomers as their ditartrates. [b] 10 mg kg^{-1} day^{-1} of (±)-FQ cured 100% of animals infected with either chloroquine-susceptible or chloroquine-resistant *P. vinckei vinckei*, even when CQ remained ineffective against the resistant strain at subtoxic doses (300 mg kg^{-1} day^{-1}).[10] [c] Compiled results from two independent experiments are presented as number of mice surviving up to 60 days after infection/number of treated mice. When malaria recrudescence and mice mortality were observed, mean and ranges of the delay in mice mortality expressed in days are given in parentheses.

In vitro assessment of the antimalarial activity: A comparison between the respective inhibitory properties of the FQ enantiomers, the racemic mixture, and CQ against the chloroquine-sensitive and chloroquine-resistant *P. falciparum* laboratory strains HB3 and Dd2 is shown in Table 1. Whatever its form ((+)-FQ, (−)-FQ, or the racemate), FQ was more active than CQ on the *P. falciparum* strains and particularly on the chloroquine-resistant one. The enantiomers were found to be equally efficient in vitro.

In vivo curative effect on mice: For this study we used a previously described model.[10, 18] *Plasmodium vinckei vinckei* preferentially invades mature erythrocytes and is fatal in untreated mice about 8 days after exposure, with parasitaemia of up to 80%.[10, 18] In comparison with the FQ racemate, which cured 100% of the animals infected with either chloroquine-susceptible or chloroquine-resistant *P. vinckei vinckei* strains at the oral dose of 10 mg kg^{-1} day^{-1} (in terms of CQ base equivalents) for 4 days,[10] (+)-FQ and (−)-FQ were not entirely curative at the same dose (Table 2). A minor difference in the potency of the enantiomers was observed in vivo. (+)-FQ displayed a better curative effect than (−)-FQ on both *P. vinckei vinckei* strains (Table 2). Like (+)-CQ in comparison to (−)-CQ,[19] (+)-FQ was more active than (−)-FQ against *P. vinckei vinckei*.

In vitro toxicity: The in vitro cytotoxicities of the drugs were compared by using mouse lymphoma cells (Table 1). Toxicities of all FQ forms, (+)-FQ, (−)-FQ or the racemate, appeared similar in the L5178Y cell proliferative assay ($p > 0.05$, Student's t test). FQ appeared to be more toxic than CQ, but its security index, ranging from 1500 to 600 for chloroquine-susceptible and chloroquine-resistant *P. falciparum* strains, respectively, confirmed that it should be a promising compound (Table 1).[20]

Chirality is an intrinsic property of macromolecular structures in the cell (enzymes, receptors, etc.), as becomes evident when they interact with chiral drugs. There is now a range of known examples of isomers with biological activities that may well reside predominantly in one enantiomer. The role of stereochemistry in antimalarial activity has been studied for most of the quinoline compounds,[19, 21–27] and the chirality of the organometallic compounds is also well known.[28, 29] To the best of our knowledge, however, there were no reported data concerning biologically active synthetic drugs with planar chirality. The aim of this study was therefore to evaluate the antimalarial activity and cytotoxicity of the FQ enantiomers versus the racemate.

We were able to prepare both FQ enantiomers in high enantiomeric excess, as assessed by NMR spectroscopy measurements, which allowed comparison of their biological properties. In vitro, the FQ enantiomers did not differ substantially in their ability to inhibit the growth of chloroquine-sensitive and chloroquine-resistant *P. falciparum* strains (Table 1). Meanwhile, in vivo, the racemic form had a higher curative effect than either enantiomer and (−)-FQ appeared to be less effective than the other enantiomer. These results are in accordance with previously published data on CQ, which contains an asymmetric carbon atom in its side chain.[19, 22–23] Indeed (+)-CQ was found to be significantly more active than (−)-CQ.[19, 23] Nevertheless, it should be keep in mind that FQ is a bulky organometallic compound exhibiting planar chirality rather than the central chirality of quinoline isomers. The sandwich structure of ferrocene renders it completely different from conventional aromatic molecules and modifies FQ in terms of redox properties and three-dimensional structure.[30, 31] Hence, these different kinds of chirality render any comparison between FQ and classical quinoline or amino alcohol enantiomers difficult, or perhaps impossible.[21, 24–27]

Moreover, the observed differences between in vivo antimalarial activities of FQ enantiomers might relate to pharmacokinetic properties. Studies of the stereoselective properties of CQ and its metabolites in humans have suggested preferential metabolism of (+)-CQ.[32–35] In the same way, highly stereospecific pharmacokinetic parameters were observed when mefloquine was administered orally in humans.[12, 36]

Hence, the origin of the differential in vivo FQ activity may involve one or more of the causes proposed above. Further experiments will be necessary to characterize the properties of FQ enantiomers. Notably, the metabolism and pharmacokinetic of these metallocenic derivatives need to be investigated, and the first steps of this research and development process are

underway. Nevertheless, our study clearly showed that the FQ racemic mixture represented the optimal formulation for the future development of this molecule as an antimalarial drug in terms of antimalarial properties and initial in vitro cytotoxicity investigation.

Experimental Section

Chemistry

General: Melting points were uncorrected. The ^{1}H and ^{13}C NMR spectra were recorded on a Brucker AC 300 spectrometer with tetramethylsilane (TMS) as the internal standard and $CDCl_3$ as the solvent. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra (MS) were obtained on a Vision 2000 time-of-flight instrument (Finnigan MAT, Bremen, Germany) equipped with a nitrogen laser operating at wavelength of 337 nm. Between 20 and 30 single-shot spectra in the reflector mode were accumulated to obtain a good signal-to-noise ratio. The matrix used was 2,5-dihydroxybenzoic acid (dhb). Merck Kieselgel 60 PF254 was used for chromatography. Enantiomeric excess (*ee*) values were determined by chiral high-performance liquid chromatography (HPLC) analysis with a Chiralcel OD column (250 × 4.6 mm) for **1** and **3**, and by ^{1}H NMR spectroscopic analysis with one (or two) equivalent(s) of Pirkle's alcohol for the other compounds. Optical rotations (α_D) were measured with a Perkin—Elmer 241 polarimeter.

(±)-2-(*N*,*N*-Dimethylaminomethyl)ferrocenylmethanol (2): $NaBH_4$ (593 mg, 15.6 mmol) was added with cooling (ice bath) to a solution of 2-(dimethylaminomethyl)ferrocenecarboxaldehyde (**1**, 715 mg, 2.6 mmol) in methanol (40 mL). The mixture was stirred at room temperature for 20 min, and then poured into water (40 mL) and extracted with CH_2Cl_2 (2 × 50 mL). The organic phase was dried over Na_2SO_4 and the solvents were removed to give (±)-**2** (696 mg, 2.55 mmol, 98%) as an oil. ^{1}H NMR ($CDCl_3$): $\delta = 4.75$ (d, J(H,H) = 12.20 Hz, 1H), 4.18 (m, 1H), 4.12 (d, J(H,H) = 12.20 Hz, 1H), 4.09 (m, 1H), 4.05 (s, 5H), 4.01 (m, 1H), 3.88 (d, J(H,H) = 12.50 Hz, 1H), 2.76 (d, J(H,H) = 12.50 Hz, 1H), 2.16 (s, 6H); MALDI-TOF MS: 274 $[M+H]^+$, 273 $[M]^{\bullet+}$, 242 $[M - CH_2OH]^+$, 228 $[M - NMe_2]^+$.

(+)-(1′*R*)-2-(*N*,*N*-Dimethylaminomethyl)ferrocenylmethanol ((+)-2) and (−)-(1′*S*)-2-(*N*,*N*-dimethylaminomethyl)ferrocenylmethyl acetate ((−)-3): Vinyl acetate (1.7 mL) and *Candida rugosa* lipase (2.9 g) were added to a solution of alcohol (±)-**2** (660 mg, 2.4 mmol) in *tert*-butyl methyl ether (70 mL). The mixture was continuously stirred (300 rpm) at 45 °C for 16 h. The enzyme was filtered off, and the filtrate was dried in vacuum. Column chromatography of the residue with diethyl ether/hexane/triethylamine (70:20:10) as the eluent afforded (−)-**3** (362 mg, 1.15 mmol, 48%, *ee* 86%) and (+)-**2** (300 mg, 1.10 mmol, 46%, *ee* > 98%).

(−)-**3**: $[\alpha]_D^{20} = -8$ ($c = 1$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 4.92$ (s, 1H), 4.28 (m, 2H), 4.17 (m, 1H), 4.09 (s, 5H), 3.37 (d, J(H,H) = 12.50 Hz, 1H), 3.23 (d, J(H,H) = 12.48 Hz, 1H), 2.15 (s, 6H), 2.03 (s, 3H); MALDI MS: 315 $[M]^{\bullet+}$, 271 $[M - NMe_2]^+$, 256 $[M - OAc]^+$, 229 $[M - (NMe_2 + OAc)]^+$.

(+)-**2**: $[\alpha]_D^{20} = +33$ ($c = 1$ in $CHCl_3$); the ^{1}H NMR and MS MALDI TOF spectra were identical with those of (±)-**2**.

(−)-(1′*R*)-2-(*N*,*N*-Dimethylaminomethyl)ferrocenylmethanol ((−)-2): *Candida rugosa* lipase (1.6 g) and *n*-butanol (1 mL) were added to a solution of (−)-**3** (400 mg, 1.3 mmol) in *tert*-butyl methyl ether (70 mL), and the suspension was shaken (300 rpm) at 45 °C for 48 h. The reaction was stopped by filtering off the enzyme. The solvent was evaporated and the residue was puritfied by column chromatography on silica gel (AcOMe/MeOH/triethylamine (90:5:5) as the eluent) to give pure (−)-**2** (187 mg, 0.67 mmol, 53%, *ee* > 98%).

(−)-**2**: the ^{1}H NMR and MALDI-TOF mass spectra were identical with those of (±)-**2**. $[\alpha]_D^{20} = -34$ ($c = 1$ in $CHCl_3$).

(−)-(1′*R*)-2-(*N*,*N*-Dimethylaminomethyl)ferrocenecarboxaldehyde ((−)-1): Compound (+)-**2** (310 mg, 1.14 mmol) was dissolved in methanol (50 mL). The solution was cooled in an ice bath, and MnO_2 was slowly added with stirring. The reaction was monitored by thin layer chromatography until complete oxidation of starting material had occurred. The solid was filtered off, water (50 mL) was added, and the aqueous layer was extracted with diethyl ether (3 × 50 mL). After drying with Na_2SO_4 and removal of solvent, the product was purified by chromatography (Et_2O/hexane/triethylamine (70:20:10) as the eluent) to give (−)-**1** (279 mg, 1.03 mmol, 98%, *ee* > 98%). $[\alpha]_D^{20} = -23$ ($c = 1$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 10.10$ (s, 1H), 4.81 (m, 1H), 4.61 (m, 1H), 4.56 (m, 1H), 4.21 (s, 5H), 3.85 (d, J(H,H) = 13 Hz, 1H), 3.35 (d, J = 13 Hz, 1H), 2.23 (s, 6H); MALDI-TOF MS: 271 $[M]^{\bullet+}$, 256 $[M - CHO]^+$, 227 $[M - NMe_2]^+$.

(+)-(1′*S*)-2-(*N*,*N*-Dimethylaminomethyl)ferrocenecarboxaldehyde ((+)-1). The preparation of (+)-**1** was analogous to that of (−)-**1**: *ee* > 98%, $[\alpha]_D^{20} = +21$ ($c = 1$ in $CHCl_3$); the ^{1}H NMR and MALDI-TOF mass spectra were identical with those of the enantiomer (−)-**1**.

Pure enantiomers of 2-(*N*,*N*-dimethylaminomethyl)ferrocenecarboxaldehyde oxime and [2-(*N*,*N*-dimethylaminomethyl)ferrocenyl]-methylamine: The preparation of the pure enantiomers of these intermediate compounds (not shown) was analogous to that for the preparation of the racemates as described in the literature.[4] In each case the product obtained had an *ee* value of > 98%.

(−)-7-Chloro-4-[(1′*S*)-2-(*N*′,*N*′-dimethylaminomethyl)-*N*-ferrocenylmethylamino]-quinoline ((−)-FQ): The preparation of compound (−)-FQ was analogous to that of the racemic FQ as described in the literature.[4] *ee* > 98%; $[\alpha]_D^{20} = -43$ ($c = 0.39$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 8.53$ (d, J(H,H) = 5.39 Hz, 1H), 7.91 (d, J(H,H) = 2.14 Hz, 1H), 7.61 (d, J(H,H) = 8.96 Hz, 1H), 7.26 (dd, J(H,H) = 2.14, 8.96 Hz, 1H), 6.46 (d, J(H,H) = 5.39 Hz, 1H), 4.35 (d, J(H,H) = 13.12 Hz, 1H), 4.28 (m, 1H), 4.17 – 4.15 (m, 2H), 4.15 (s, 5H), 4.09 (m, 1H), 3.80 (d, J(H,H) = 12.57 Hz, 1H), 2.88 (d, J(H,H) = 12.57 Hz, 1H), 2.22 (s, 6H); ^{13}C NMR ($CDCl_3$): $\delta = 152.2$ (CH), 150.1 (C^{IV}), 149.3 (C^{IV}), 134.6 (C^{IV}), 128.3 (CH), 124.7 (CH), 122.2 (CH), 117.9 (C^{IV}), 98.9 (CH), 83.9 (2 × C^{IV}), 71.4 (CH), 70.5 (CH), 69.2 (5 × CH), 65.9 (CH), 58.1 (CH_2), 44.9 (2 × CH_3), 42.5 (CH_2); MALDI-TOF MS: 436 $[MH^{37}Cl]^+$, 434 $[MH^{35}Cl]^+$, 397, 391 $[M - HNMe_2{}^{37}Cl]^+$, 389 $[M - NMe_2{}^{35}Cl]^+$; elemental analysis calcd for $C_{23}H_{24}N_3FeCl$ (433.5): C 63.67, H 5.54, N 9.69; found C 63.69, H 5.86, N 9.48.

(+)-7-Chloro-4-[(1′*R*)-2-(*N*′,*N*′-dimethylaminomethyl)-*N*-ferrocenylmethylamino]-quinoline ((+)-FQ): The preparation of compound (+)-FQ was analogous to that of the racemic FQ as described in the literature.[4] *ee* > 98%; $[\alpha]_D^{20} = +44$ ($c = 0.38$ in $CHCl_3$); elemental analysis calcd for $C_{23}H_{24}N_3FeCl$ (433.5): C 63.67, H 5.54, N 9.69; found C 63.66, H 5.67, N 9.45; the ^{1}H and ^{13}C NMR and MALDI-TOF mass spectra were identical with those of the enantiomer (−)-FQ.

7-Chloro-4-[(1′S)-2-(*N*′,*N*′-dimethylammoniomethyl)-*N*-ferrocenylmethylamino]-quinolinium ditartrate ((−)-FQ · 2L-(+)-tartaric acid): The preparation of this compound was analogous to that of the racemate as described in the literature.[4] ^{1}H NMR (D_2O): $\delta = 8.40$ (d, J(H,H) = 7.08 Hz, 1H), 8.18 (d, J(H,H) = 9.01 Hz, 1H), 7.92 (s, 1H), 7.67 (d, J(H,H) = 9.01 Hz, 1H), 6.98 (d, J(H,H) = 7.08 Hz, 1H), 4.75 – 4.33 (m, 12H), 4.31 (m, 4H), 2.85 (s, 3H), 2.75 (s, 3H); UV (H_2O) $\lambda_{max} = 204$, 218, 252, 311 nm.

7-Chloro-4-[(1′S)-2-(*N*′,*N*′-dimethylammoniomethyl)-*N*-ferrocenylmethylamino]-quinolinium ditartrate ((+)-FQ · 2L-(+)-tartaric

acid): The preparation of this compound was analogous to that of the racemate as described in the literature.[4] The ^1H and ^{13}C NMR and MALDI-TOF mass spectra were identical to those of the salt (−)-FQ·2 L-(+)-tartaric acid.

Biology

In vitro activity studies:

Preparation of tested compounds: Chloroquine diphosphate was supplied by Sigma. Ditartrate salts of (±)-FQ, (+)-FQ, and (−)-FQ were prepared according to the reported procedure.[6, 7] Stock solutions were prepared at 5 mg mL^{-1} in 70% ethanol and stored at −20 °C until the assays were performed.

Parasite strains: Two culture-adapted strains of *P. falciparum* were used: the chloroquine-resistant strain Dd2 (Indochina) and the chloroquine-sensitive strain HB3 (Honduras). All stock parasite cultures were maintained by Trager and Jensen's method.[37, 38]

In vitro measurement of parasite growth inhibition by drugs: The assays were conducted in vitro by a modification of the semi-automated microdilution technique of Desjardins et al., based on radiolabeled [^{3}H]hypoxanthine incorporation.[39] Drug testing was carried out in 96-well microtiter plates. Stock solutions of each compound (5 mg mL^{-1}) were prediluted in complete culture medium (RPMI 1640 supplemented with 10% pooled human AB+ serum), and titrated in duplicate in serial twofold dilutions. The final concentrations ranged from 4.5 – 581.5 nmol L^{-1} for CQ, the FQ enantiomers, and the racemate. After addition of a suspension of parasitized erythrocytes in complete culture medium (200 μL per well, 0.5% initial parasitaemia with a majority of ring stages, 2% haematocrit) and [^{3}H]hypoxanthine (Amersham, Little Chalfont, UK, 0.5 μCu per well), the test plates were incubated at 37 °C for 48 h in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2. Growth of the parasites was estimated from the incorporation of radiolabeled [^{3}H]hypoxanthine into the parasites' nucleic acids, measured in a liquid scintillation spectrometer (Beckman LS 1701). Fifty percent inhibitory concentration (IC_{50}) values refer to molar concentrations of drug causing 50% reduction in [^{3}H]hypoxanthine incorporation, compared to drug-free control wells. IC_{50} values were estimated by linear regression analysis of log-dose-response curves.

In vivo antimalarial studies:

Parasites and animals: Two strains of *P. vinckei vinckei* were used in this study: a chloroquine-susceptible strain (*P. vinckei vinckei* Katanga (provided by Dr. Wery, Antwerpen, Belgium) and a chloroquine-resistant strain. *P. vinckei vinckei* strains were maintained by serial blood-passage in mice. The chloroquine-resistant *P. vinckei vinckei* strain was developed by the single fixed dose relapse technique.[10, 40] All handling of animals conformed with national guidelines.

Studies on mice infected with *P. vinckei vinckei*: Groups of five female Swiss mice (Janvier) weighing 30 g were intraperitoneally inoculated with 10^7 parasitized erythrocytes in phosphate-buffered saline (PBS, 0.5 mL). The first treatment dose was given orally 1 h after infection on day 0 and was repeated once daily for 3 days ("4-day-blood schizontocidal test"[41, 42]). From day 7 of infection, thin blood smears from mouse tail blood were made every day for the following two weeks, and then every third day. They were fixed in methyl alcohol and stained with Giemsa. At least 1000 cells were counted to determine parasitaemia and 100 fields were examined before a preparation was considered negative. Negative mice were held for 2 months. For treatment, each mouse received (+)-FQ, (−)-FQ, (±)-FQ, or CQ dissolved in PBS (0.2 mL), and control animals received only PBS. All dosages given in Table 2 are expressed in terms of CQ base equivalents.

In vitro toxicity:

In vitro toxicity was estimated on mouse lymphoma (L5178Y) cells. The cell line was routinely cultured in RPMI 1640 supplemented with 10% horse serum (Gibco, France). For determination of in vitro toxicity of each compound, 2×10^5 cells in 100 μL were distributed in each well of 96-well plates, and 100 μL of culture medium containing the drug was then added to each well.[20] Drugs were titrated in duplicate in serial threefold dilutions, with final concentrations ranging from 500 000 – 686 nmol L^{-1}. Cell growth was estimated from [^{3}H]hypoxanthine incorporation after 48 h incubation. The results were analysed in the same manner as for *P. falciparum* cultures and are expressed as the mean of three independent experiments.

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