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Cellular uptake of a catechol iron chelator and chloroquine into *Plasmodium falciparum* infected erythrocytes

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Abstract

Our study demonstrates the capacity of FR160, a catechol iron chelator, to reach and accumulate into infected *Plasmodium falciparum* erythrocytes and parasites (cellular accumulation ratio between 12 and 43). Steady-state FR160 accumulation is obtained after 2 hr of exposure. After 2 hr exposure, it reaches intracellular levels that are 4- to 10-fold higher in infected red blood cells than those attained in normal erythrocytes. There is quite a good correlation between the accumulation of chloroquine and FR160 in the different strains ($r = 0.939$) and in the IC_{50} values ($r = 0.719$). In contrast, the accumulation of FR160 and its activity is poorly correlated ($r = 0.500$), suggesting that activity of FR160 may be independent of its penetration into infected erythrocytes. The mechanism of accumulation is yet unknown but based on inhibitor studies, the uptake of FR160 seems to be not associated with the calcium pump or channel, the potassium channel or the Na^+/H^+ exchanger. Combinations of FR160 with verapamil, diltiazem, clotrimazole, amiloride, diazoxide, 4-aminopyridine, and picrotoxin should be avoided (antagonistic effects). The potent *in vitro* activity of FR160 on chloroquine-resistant strains or isolates, its lower toxicity against Vero cells, its mechanisms of action, its capacity to reach rapidly and accumulate into infected erythrocytes suggest that FR160 holds much promise as a new structural lead and effective antimalarial agent or at least a promising adjuvant in treatment of malaria.

Keywords: Malaria; Iron chelators; Siderophores; Chemotherapy; Accumulation

1. Introduction

The emergence and spreading of parasite resistance to currently used antimalarial drugs indicate that novel compounds need to be discovered and developed by identification of novel chemotherapeutic targets [1]. The development of novel chemotherapies depends largely on the identification of potential targets and elucidation of the mechanisms by which the drugs gain access to intracellular parasites. The strategy of targeting potential cytotoxic compounds to infected cells first requires basic knowledge about the permeability properties of the erythrocyte membrane and, in particular, how those properties differ in infected compared to uninfected red blood cells.

Iron chelation therapy was considered a suitable treatment for various infectious diseases, including malaria [2]. Iron is a prerequisite for all living organisms and is needed for catalysis of DNA synthesis and for a variety of enzymes concerned in electron transport and energy metabolism [3]. Recent experimental observations obtained *in vitro* [4,5] in rodent [6] and in primate models [7], and in clinical studies [8,9] showed antimalarial activity of desferrioxamine. The antimalarial action of iron chelators is dictated by three factors [10], i.e. iron(III)-binding capacity, chelator ingress into parasitized erythrocytes, and chelator egress from parasites after treatment. To reach the parasite cytosol, a

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Abbreviations: CQ, chloroquine; IC_{50}, 50% inhibitory concentration.

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drug would have to sequentially cross the concentric compartments enclosed within the various membranes. This process encompasses both membrane permeation steps and diffusion across aqueous compartments.

Various iron chelators were assessed to improve the drug lipophilicity leading to increased access of drug to intracellular parasites and to faster speed of action [11,12]. We have shown previously the *in vitro* activity on *Plasmodium falciparum* of FR160, a catecholate siderophore derived from spermidine, the N^4-nonyl,N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine hydrobromide [13]. FR160 was more effective at the late trophozoite and young schizont stages, although the drug affected rings and schizonts as well [14]. Combinations of FR160 and tetracyclines and norfloxacin have synergistic or additive effects against *P. falciparum* parasites [15]. We showed that the activity of FR160 differed significantly ($P < 0.0001$) between 75 isolates susceptible to chloroquine ($IC_{50} = 1.13$ μM) and 60 isolates resistant to chloroquine ($IC_{50} = 2.07$ μM) (Pradines, submitted).

The aim of this study was to assess the permeation of FR160 into infected and uninfected erythrocytes. To explore the cellular accumulation of FR160, we have prepared radiolabeled FR160 ([^{3}H]FR160). The accumulation of FR160 was compared with that of chloroquine. Chloroquine is the most used antimalarial drug. It exerted a huge drug pressure on *P. falciparum* populations and it is of major importance to delineate the potential for cross-resistance. Recent data suggest that iron chelation, by desferrioxamine, in *Plasmodium* spp. also influences the efficiency of the polymerization of hematin [16]. In addition, deprivation of the iron supply by interference with hemoglobin metabolism has also been proposed as a mode of action of chloroquine [17]. Chloroquine may play a role in iron metabolism, in particular, as an inhibitor of iron transport [17].

2. Materials and methods

2.1. Synthesis of [^{3}H]FR160

2.1.1. Materials

^{1}H and ^{13}C NMR spectra were obtained on Bruker AC-200 or 250 spectrometers. Chemical shifts are reported in ppm relative to solvent $CDCl_3$ or CD_3OD (δ units). Mass spectra were recorded in the chemical ionization (CI) mode on a Riber-Mag R-10-10. Tritiated compounds were analyzed by chemical ionization-mass spectrometry (Nermag R-10-10). HPLC analyses were performed on a Waters 600 chromatographic system supplied with a Waters 996 photodiode array detector and a radioactive flow monitoring (LB507A analyzer; Berthold). Tritium determinations were made in an SL 3000 Intertechnique liquid scintillation counter. The automatic gas transfer unit used for tritiation has been previously described [18]. Carrier-free tritium gas was obtained from the Commissariat à l'Energie Atomique (CEA).

2.1.2. Synthesis of isotopically labeled dicatechols (Fig. 1)

*2.1.2.1. Toluene-4-sulfonic acid non-3-ynyl ester (**2**).* To a cold solution (5°) of toluene-4-sulfonyl chloride (6.0 g, 31 mmol) in 25 mL of dry CH_2Cl_2 and 5 mL of NEt_3 (36 mmol), 3.2 mL of non-3-yn-1-ol (**1**; 20 mmol) was added dropwise, and then allowed to warmup to room temperature. The mixture was stirred overnight. After washing with water and brine, the organic layer was dried (Na_2SO_4) and filtered. The solvent was removed under vacuum to give quantitatively 6.0 g of **1** as a viscous yellow oil.

^{1}H NMR ($CDCl_3$): 0.8 (t, 3 H) CH_3; 1.3 (m, 6 H) 3 × CH_2; 2.0 (t, 2 H) CH_2-5; 2.4 (s, 3 H) CH_3; 4.0 (t, 2 H) CH_2OTs; 7.3 (d, 2 H); 7.7 (d, 2 H).

^{13}C NMR ($CDCl_3$): 13.7 CH_3; 18.3 CH_2; 19.4 CH_2; 21.3 CH_2; 21.5 CH_2; 21.9 CH_2; 28.2 CH_3 (Ts); 30.7 CH_2; 68.1 CH_2OTs; 73.7 C-4; 82.6 C-3; 127.6 and 129.6 CH; 130.1 and 144.7 C.

Mass spectrum (CI), *m/z* 312 $[M + 18]^+$.

*2.1.2.2. 1-Bromo-non-3-yne (**3**).* To a solution of 5.9 g (20 mmol) of **1** in 50 mL of dry acetone was added 4.8 g (35 mmol) of NaBr. The mixture was stirred at reflux for 72 hr. After usual workup, 6.6 g of crude product was obtained and purified by distillation under vacuum (15 mmHg) providing 3.34 g of **3** as a colorless oil (53%), b.p.: 130–132°.

^{1}H NMR ($CDCl_3$): 0.9 (t, 3 H) CH_3; 1.2–1.6 (m, 6 H) 3 × CH_2; 2.1 (m, 2 H) CH_2; 2.7 (m, 2 H) CH_2; 3.4 (t, 2 H) CH_2Br.

^{13}C NMR ($CDCl_3$): 14.0 CH_3; 18.6 CH_2; 22.2 CH_2; 23.1 CH_2; 28.5 CH_2; 30.4 CH_2; 31.0 CH_2Br; 76.8 and 82.7 C≡C.

Mass spectrum (CI): *m/z* 202 (MH^+, ^{79}Br), 204 (MH^+, ^{81}Br).

*2.1.2.3. Preparation of N-alkylated spermidine (**4**).* 1-Bromo-non-3-yne (**3**; obtained in two steps from commercially available non-3-yn-1-ol (**1**) with N^1,N^8-bis-[2,3-(bis(benzyloxybenzoyl)spermidine)] (1.5 g, 1.48 mmol) was dissolved in dry acetone and to this Na_2CO_3 (1.5 g) and 1-bromo-non-3-yne (**3**; 0.3 g, 1.48 mmol) in 10 mL of dry acetone were added. The mixture was stirred at reflux overnight. The solvent was removed under vacuum and the residue was taken up in CH_2Cl_2. After usual workup and further purification by MPLC (CH_2Cl_2, MeOH 3–10%) product **4** was obtained in 40% yield.

^{1}H NMR ($CDCl_3$): 0.9 (t, 3 H) CH_3; 1.2–1.6 (m, 12 H) CH_2 × 6; 2.0–2.2 (m, 4 H) CH_2 × 2, 2.4 (m, 4 H) 2 × CH_2N, 2.6 (t, 2 H) CH_2N; 3.2 (m, 4 H) 2 × CH_2NH; 5.0–5.2 (2 s, 8 H) 4 × CH_2Br; 7.1–7.5 (m, 2 H) 2 × CH; 8.0 (2t, 2 H) 2 × NH.

^{13}C NMR ($CDCl_3$): 13.9 CH_3; 16.6; 18.6; 21.5; 22.1; 24.1; 26.6; 27.0; 28.4; 28.6 CH_2; 31.0 3 × CH_2N; 37.9 and 39.3 CH_2NH; 46.2; 51.3; 52.7 and 53.1 4 × OCH_3; 71.2 and 76.3

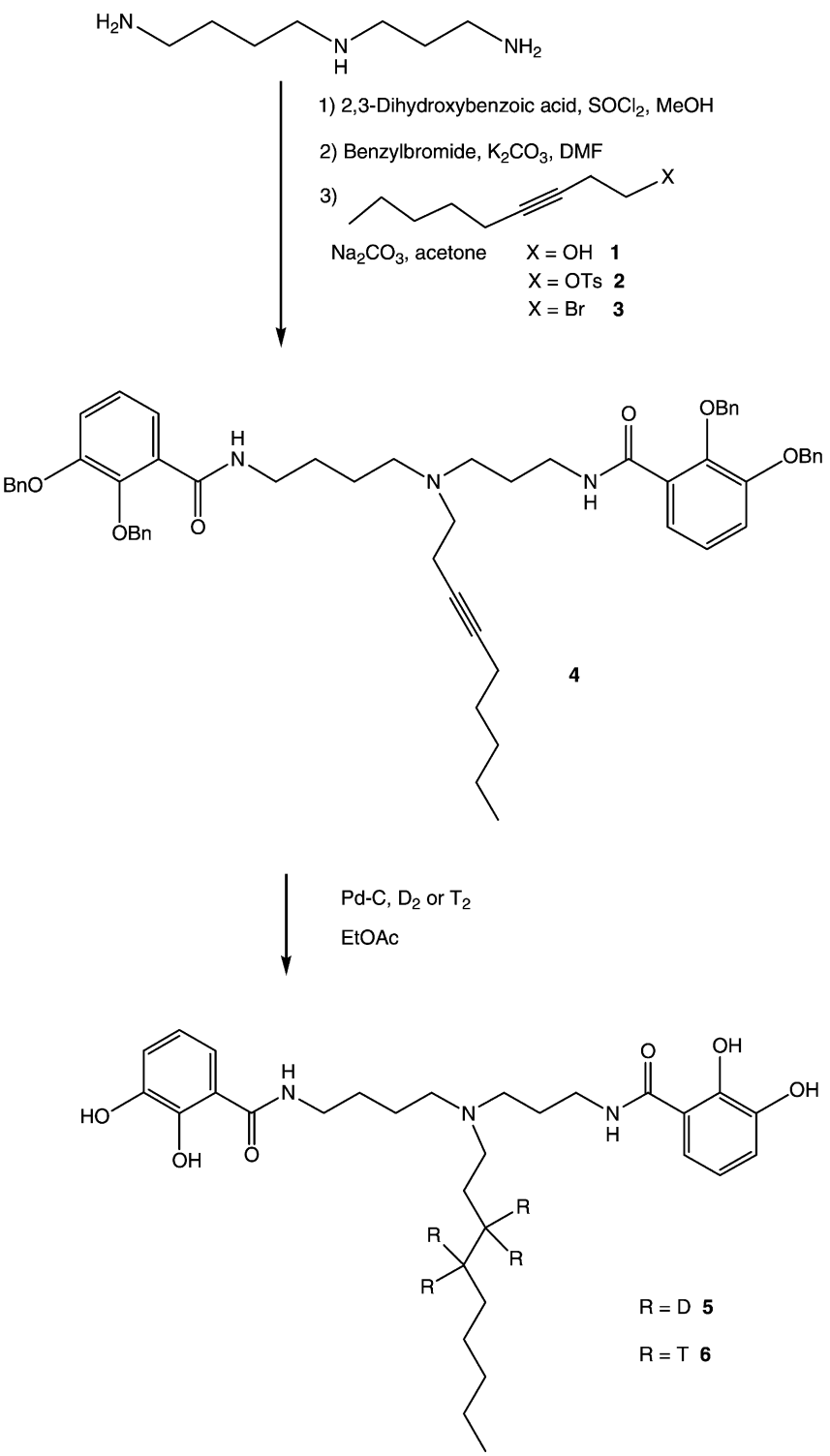

Fig. 1. Synthesis of isotopically labeled dicatechols.

C≡C; 116.8 CH; 123.1 CH; 124.3 CH; 136.3 C; 146.6 C; 151.6 C; 164.9 and 165.1 C=O.

Mass spectrum (CI): *m/z* 899 (M^+), 900 (MH^+).

*2.1.2.4. Preparation of deuterated product (**5**).* Compound **4** (10 mg, 0.011 mmol) and 10% Pd–C (3 mg) were suspended in EtOAc and deuterium gas was bubbled through the mixture for 5 hr. The Pd–C catalyst was filtered and the solvent was removed under vacuum to give quantitatively product **5**.

^{1}H NMR ($CDCl_3$): 0.8 (t, 3 H) CH_3; 1.25 (m, 12 H) 2 × CD_2 and 4 × CH_2; 1.5–1.8 (m, 6 H) 3 × CH_2; 1.9 (t, 2

H) CH_2; 2.6–2.9 (m, 6 H) 3 × CH_2N amine, 3.4 (t, 2 H) CN_2NH amide; 3.6 (t, 2 H) CH_2NH amide; 6.7 (dd, 2 H) 2 × CH; 6.9 (t, 2 H) 2 × CH; 7.1 (dd, 2 H) 2 × CH.

^{13}C NMR ($CDCl_3$): 14.1 CH_3; 21.6; 22.6; 23.5; 26.1; 29.1; 29.7 and 31.7 CH_2; 37.9 CH_2NH; 52.6 CH_2N; 113.8 and 113.9 2 × CH; 116.9 and 117.2 2 × C; 118.1 and 118.7 2 × CH; 145.6 and 145.7 2 × C; 149.0 and 149.1 2 × C; 170.4 and 170.7 2 × C=O.

Mass spectrum (CI): *m/z* 908 (MH^+).

*2.1.2.5. Preparation of tritiated product (**6**).* The same batch of catalyst used for deuteration (10% Pd–C, 27 mg) was added to a solution of **4** (6 mg, 0.007 mmol) in 1 mL of EtOAc. The vial was connected to the tritiation apparatus [1]. The solution was frozen in liquid N_2. Carrier-free tritium gas was introduced and compressed to 1.3 bar. After thawing, the reaction mixture was kept at 20° and stirred for 4 hr. The 10% Pd–C catalyst was filtered and labile tritium atoms were exchanged by successive flash evaporation with 150 mL of MeOH. The crude tritiated product **6** was dissolved in 1 mL of MeOH (total radioactivity of 1.85 GBq) and purified by reverse-phase chromatography (Vydac C18 column; eluent A: 0.1% trifluoroacetic acid, eluent B: 90% acetonitrile in 0.1% trifluoroacetic acid; eluted at 1 mL/min with the following gradient: isocratic 40% B for 5 min, linear gradient from 40 to 60% B in 10 min and from 60 to 100% B in 15 min). Unambiguously, the tritiated product **6** was characterized by a retention time of 15 min and an absorption band (λ_{max}) at 215 nm. Compound **6** (111 MBq, 3.33 TBq/mmol) was obtained and stored at −80° in acetonitrile (33.3 MBq/mL) for several months without degradation.

Mass spectrum (CI): *m/z* 553 (MH^+).

2.2. Other drugs

Radiolabeled chloroquine diphosphate ([^{3}H]CQ) (26 Ci/mmol) was purchased from Amersham. Silicon oil 550 was purchased from Dow Corning (BDH Laboratory Supplies). Verapamil, clotrimazole, amiloride, diltiazem, diazoxide, 4-aminopyridine, and picrotoxin were obtained from Sigma Chemical.

2.3. Strains of Plasmodium falciparum

Two chloroquine-susceptible strains 3D7 (Africa) and D6 (Sierra Leone) and two chloroquine-resistant strains W2 (Indochina) and FCR3 (Gambia) were maintained in culture. When required for drug assays, cultures were synchronized by sorbitol lysis [19]. Susceptibility to the iron chelator FR160 was determined after suspension in RPMI 1640 medium (Life Technologies), supplemented with 10% human serum (pooled from different A^+ or AB sera from non-immune donors), and buffered with 25 mM HEPES and 25 mM $NaHCO_3$ (hematocrit of 1.5%, parasitemia of 0.5%).

2.4. In vitro assay

For *in vitro* isotopic microtests, 200 μL of the suspension of erythrocytes parasitized with ring stages was distributed in 96-well plates with antimalarial agents. Parasite growth was assessed by adding 1 μCi of [^{3}H]hypoxanthine with a specific activity of 14.1 Ci/mmol (NEN Products) to each well. Plates were incubated for 42 hr at 37° in an atmosphere of 10% O_2, 6% CO_2, 84% N_2 and a humidity of 95%. Immediately after incubation the plates were frozen and then thawed to lyse erythrocytes. The contents of each well were collected on standard filter microplates (Unifilter™ GF/B; Packard Instrument Company) and washed using a cell harvester (FilterMate™ Cell Harvester; Packard). Filter microplates were dried and 25 μL of scintillation cocktail (Microscint™ O; Packard) was placed in each well. Radioactivity incorporated by the parasites was measured using a scintillation counter (Top Count™; Packard).

The 50% inhibitory concentration (IC_{50}), i.e. the drug concentration corresponding to 50% of the uptake of [^{3}H]hypoxanthine by the parasites in drug-free control wells, was determined by non-linear regression analysis of log-dose/response curves. Data were analyzed after logarithmic transformation and expressed as the geometric mean of IC_{50} and 95% confidence intervals (95% CI) were calculated.

2.5. Measurement of the uptake of [^{3}H]FR160 by erythrocytes parasitized with ring stages

Accumulation of [^{3}H]FR160 and [^{3}H]CQ was carried out essentially according to the protocol of Bray *et al.* [20] with some modifications [21]. Infected erythrocytes were suspended in RPMI 1640 medium buffered with 25 mM HEPES and 25 mM $NaHCO_3$, at a parasitemia of 2% and hematocrit of 2%. Eppendorf microfuge tubes were loaded with 400 μL of silicon oil 550, 1 mL of reaction buffer containing 10 nM [^{3}H]FR160 or 3 nM [^{3}H]CQ on top of the oil, and then with 25 μL of appropriately concentrated cell suspension. The cell suspension was mixed with the reaction buffer and incubated for 2 hr at 37° in an atmosphere of 10% O_2, 6% CO_2, 84% N_2 and 95% relative humidity. After 1 min of centrifugation at 13,000 *g*, 100 μL of the buffer was processed for scintillation counting. Infected erythrocytes pellets were lysed by distilled water and by 5:5:2 quaternary hydroxide:glacial acetic acid:hydrogen peroxide and left in an oven at 50° for 2 hr. They were then processed for scintillation counting. FR160 and chloroquine accumulation is expressed as the cellular accumulation ratio, which is the ratio of the amount of radiolabeled drug in parasitized erythrocytes (amount of [^{3}H]FR160 or [^{3}H]CQ in parasitized erythrocytes − amount of [^{3}H]FR160 or [^{3}H]CQ in uninfected red cells) to the amount of [^{3}H]FR160 or [^{3}H]CQ in a similar volume of buffer after incubation [22].

2.6. Measurement of the time course of the uptake of [^{3}H]FR160 by parasitized erythrocytes at ring stages

The cellular accumulation ratio for FR160 was assessed after several different times of exposure.

2.7. Measurement of the uptake of [^{3}H]FR160 by parasitized erythrocytes at ring stages in presence of pharmacological blockers

The cellular accumulation ratio of FR160 was assessed in presence of several pharmacological blockers, such as verapamil (L-type Ca^{2+}-channel blocker) [23], clotrimazole (inhibitor of the Ca^{2+}-activated K^+ channel) [24], amiloride (inhibitor of transmembrane Na^+ entry and Na^+-K^+-ATPase) [25], diltiazem (L-type Ca^{2+}-channel antagonist) [23], diazoxide (ATP-sensitive K^+-channel opener) [26], 4-aminopyridine (non-specific K^+-channel blocker) [27], and picrotoxin (antagonist of GABA–Cl^-–receptor–ionophore complex) [28], at the concentration of 5 μM.

2.8. Measurement of the uptake of [^{3}H]FR160 by parasites at ring stages

Infected erythrocytes were incubated in 10 nM [^{3}H]FR160 or 3 nM [^{3}H]CQ as described in Section 2.5. After 1 min of centrifugation at 13,000 *g*, the supernatant was discarded. Infected erythrocytes pellets were lysed by 1 mL 0.02% saponine to obtain intact parasites. The parasites seem to be not altered in microscopy. However, we are not sure that some drug had escaped out of the parasite. The estimate of radioactivity associated with the parasite may be underestimated. Eppendorf microfuge tubes were loaded with 400 μL of silicon oil 550. One hundred microliters of the supernatant with hemoglobin was processed for scintillation counting. The pellet of hemolysate and intact parasites was lysed by distilled water and by 5:5:2 quaternary hydroxide:glacial acetic acid:hydrogen peroxide and left in an oven at 50° for 2 hr. They were then processed for scintillation counting. Comparison was assessed between the amount ([^{3}H]FR160 in pellets of infected erythrocytes − amount of [^{3}H]FR160 in pellets of uninfected red cells) and the amount ([^{3}H]FR160 in hemoglobin supernatant of infected red cells − amount of [^{3}H]FR160 in hemoglobin supernatant of normal red cells).

2.9. Drug combinations

Combinations of FR160 with the several blockers were tested three independent times against the *P. falciparum* W2 clone. To evaluate drug interactions, isobolograms were constructed by plotting a pair of fractional IC_{50} for each combination of FR160 and blockers. The different blocker fractional IC_{50} was calculated by dividing their fixed concentrations (12 concentrations) by the IC_{50} of tested drugs alone and plotted on the horizontal axis. The corresponding FR160 fractional IC_{50} was calculated by dividing the IC_{50} of FR160 combined with fixed concentrations of blockers by the IC_{50} of FR160 alone and plotted on the vertical axis. A curve was then drawn through the resulting pairs of fractions from the ends of both axes on the graph. Points lying above the straight diagonal line (corresponding to the points where there is no interaction between the drugs) are antagonistic, points below the straight diagonal line are considered to be synergistic [29].

3. Results and discussion

The elucidation of the mode of action of antimalarial agents involves the study of the routes and modes of entry of drugs into infected red blood cells and drug interference with parasite functions. It is of interest to assess whether the permeation properties of normal vs. infected erythrocytes are sufficiently different to ensure preferential access of drugs to infected cells. We show that the rapid inhibitory potency of FR160 (less than 6 hr on ring stages and 3 hr on trophozoite stages to reduce parasites growth by 50–70% [14]) is dictated by its capacity for rapid penetration into parasitized erythrocytes. Previous studies showed that desferrioxamine enters the parasite through an aqueous duct leading to the parasite outer membrane [30] and permeates into parasite [31]. One of the salient features of desferrioxamine and methylanthranilic-desferrioxamine uptake into infected cells is that even after hours of incubation with drug only 5–15% of the external concentration is attained within the infected cell [31]. Results demonstrated a 3-fold higher permeation into infected compared with non-infected cells. The apparently poor inhibitory effect of desferrioxamine on rings might merely reflect its poor accessibility to the parasite at early stages of growth.

We demonstrate here that [^{3}H]FR160 is taken up by erythrocytes infected with *P. falciparum* and that infected red blood cells quickly exhibit accumulation of FR160 from medium (10- to 40-fold) (Table 1). The time course of FR160 uptake by a chloroquine-susceptible and a chloroquine-resistant *P. falciparum* at a fixed concentration of 10 nM is shown in Fig. 2. Steady-state FR160 accumulation of the two strains is obtained after 2 hr. Steady state of the resistant strain is one-fourth that of the susceptible strain. Maximum uptake was attained after more than 8 hr for desferrioxamine and after 10–15 min to 8 hr for reversed siderophores [12]. The estimated amount of [^{3}H]FR160 in pellet which contained parasites (and erythrocytes membranes, hemozoin ...) is 5-fold higher for W2 to 18-fold higher for 3D7 than those measured in pellets of uninfected erythrocytes (data not shown). These results suggest that FR160 is taken up by parasites and not only by infected erythrocytes.

Table 1
Measurement of cellular accumulation ratio of FR160 at a fixed extracellular concentration of 10 nM and chloroquine at 3 nM in *Plasmodium falciparum* infected erythrocytes

Strain	FR160		Chloroquine	
	Mean IC_{50} (nM) (95% confidence interval)	Cellular accumulation ratio (95% confidence interval)	Mean IC_{50} (nM) (95% confidence interval)	Cellular accumulation ratio (95% confidence interval)
3D7	790 (760–820)	43 (27–60)	19 (9–29)	251 (232–271)
D6	170 (130–210)	27 (19–37)	22 (18–27)	287 (247–333)
W2	1040 (970–1110)	13 (7–18)	832 (665–999)	17 (13–22)
FCR3	1450 (1230–1670)	12 (9–16)	890 (762–1019)	11 (7–16)

Values are means of three to seven independent experiments.

After 2 hr of exposure, FR160 reaches intracellular levels that are 4- to 10-fold higher in infected red blood cells than those attained in normal erythrocytes. In a previous study, Fritsch and Jung [32] showed that [^{13}C]desferrioxamine was taken up by erythrocytes and after 5 hr exposure it reached intracellular levels that were 3- to 4-fold higher than those attained in normal red blood cells. The cellular FR160 accumulation ratio is 3- to 4-fold higher in parasites, which are 2- to 5-fold more susceptible to FR160. Nevertheless, the values of accumulation may be underestimated because during the saponin lysis some drug could have escaped out the parasite. The parasitized erythrocytes seemed to be intact and not altered in microscopy. The parasites, which present the highest accumulation ratio for FR160 and are the more potent susceptible to FR160, are also the strains the more susceptible to chloroquine (14-fold) and present a high cellular accumulation ratio for chloroquine (20-fold higher than accumulation in chloroquine-resistant parasites). There is quite a good correlation between the accumulation of both drugs in the different strains ($r = 0.939$) and in the IC_{50} values of both drugs ($r = 0.719$). We have shown in previous study that the activity of FR160 differed significantly ($P < 0.0001$) between 75 isolates susceptible to chloroquine ($IC_{50} = 1.13$ μM) and 60 isolates resistant to chloroquine ($IC_{50} = 2.07$ μM) (Pradines, submitted). A positive correlation between the IC_{50} of two antimalarial drugs and between the accumulation values may suggest common features in drug uptake and/or mode of action or resistance.

The mode of action of chloroquine and the mechanism of resistance to it are not well understood. It has been hypothesized that, as a weak base, chloroquine and its close analogues follow the pH gradient and accumulate in the food vacuole of the susceptible parasites [33]. The most convincing explanation of its activity lies in its capacity to interfere with hemoglobin degradation in food vacuole by raising the vacuolar pH [34] and/or by inhibition of the crystallization of the free heme by the formation of toxic heme–chloroquine complex [35,36]. In previous study, Vippagunta *et al.* [16] showed that desferrioxamine increased the concentration of soluble forms of hematin, enhanced the rate of hematin crystallization, and also could initiate hematin crystallization. In contrast, chloroquine decreased the concentration of soluble forms of hematin and inhibited hematin crystallization. We have shown that FR160 could act by generation of radical species and enhancement of heme-catalyzed oxidation of lipid membranes, but it neither affects the chemical heme polymerization activity nor the production of hemozoin in *P. falciparum* parasites (Pradines, submitted). Deprivation of the iron supply by interference with hemoglobin metabolism has also been proposed as a mode of action of chloroquine [17]. Chloroquine may play a role in iron

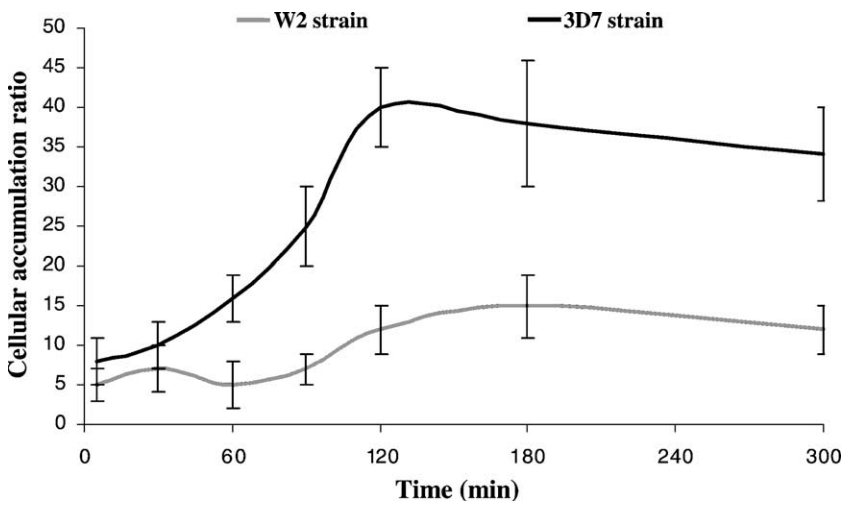

Fig. 2. Time course of FR160 accumulation by chloroquine-susceptible strain (3D7) and chloroquine-resistant strain (W2).

Table 2
Measurement of cellular accumulation ratio of FR160 in combination with pharmacological channel blockers in *Plasmodium falciparum* parasites with different susceptibilities to FR160 and chloroquine

Drug	3D7 strain		W2 strain	
	Cellular accumulation ratio	95% Confidence interval	Cellular accumulation ratio	95% Confidence interval
Without drug	43	27–60	13	7–18
Picrotoxin	50	35–65	16	8–24
Clotrimazole	40	24–55	8	5–11
Diazoxide	30	24–36	10	6–14
Amiloride	58	46–70	11	6–16
Diltiazem	42	30–54	13	10–17
Verapamil	38	27–51	9	5–14
4-Aminopyridine	41	29–53	14	10–18

Values are means of three independent experiments.

metabolism, in particular, as an inhibitor of iron transport in *Saccharomyces cerevisiae* [17]. However, such mechanism has not been shown in *Plasmodium*. The correlation between IC_{50} of chloroquine and FR160 could be ascribed to common features on iron homeostasis.

The hypothesis that correlation between the accumulation values of chloroquine and FR160 could be ascribed to common mechanisms of uptake is significantly weakened by the finding that the pharmacological blockers, which modulate response to chloroquine, have no effect on the accumulation of FR160 (Table 2) and antagonize its activity (Fig. 3). Chloroquine reaches the parasite food vacuole, where it accumulates due to the local acid pH and the weak base properties of the drug [33]. The chloroquine activity depends on a high level accumulation within the parasite and drug resistance stems from reduced drug accumulation. Several compounds, like verapamil [37,38], desipramine [39], and dihydroanthracene derivatives [40,41], demonstrated in the past decade promising capability to reverse the chloroquine resistance in parasite isolates *in vitro* in animal models [42] and human malaria [43]. The reversal of chloroquine resistance by verapamil was proposed to occur by modulating the activity of the parasitic Na^+/H^+ exchanger *via* the calcium/calmodulin-dependent pathway [44]. Nevertheless, Bray *et al*. [20] provided definitive evidence that chloroquine uptake is determined by the binding of chloroquine to ferriprotoporphyrin IX. Recently, it has been shown that the digestive vacuolar pH of a chloroquine-resistant strain is more acidic relative to chloroquine-susceptible parasites [45]. Verapamil normalizes the vacuolar pH of the chloroquine-resistant parasites to a value near that measured for chloroquine-susceptible parasite (increase of the pH) and has no effect on chloroquine-susceptible parasites [46]. In fact, it seems that acidification of digestive vacuole contributes to drug resistance *via* the effects that pH has on the solubility of unpolymerized heme found in the vacuole [47]. Reversers of chloroquine resistance increase the pH of acid vesicles where ferriprotoporphyrin IX is generated and, therefore, this increases the affinity of chloroquine-ferriprotoporphyrin IX binding. To evaluate if similar mechanisms are responsible for the access of FR160 and chloroquine into infected erythrocytes, we assessed the interaction of FR160 with several channel blockers, which have been known to reverse chloroquine resistance, such as verapamil or diltiazem, or which inhibit chloroquine uptake as amiloride and clotrimazole, or never used with chloroquine as diazoxide, 4-aminopyridine, and picrotoxin. These results show that accumulation of FR160 into infected erythrocytes seems to be not associated with the calcium pump or channel, the potassium channel or the Na^+/H^+ exchanger. The hypothesis that correlation between the accumulation values of chloroquine and FR160 could be ascribed to common mechanisms of uptake is significantly weakened by the finding that the pharmacological blockers, which modulate response to chloroquine, have no effect on the accumulation of FR160 and antagonize its activity. Nevertheless, a larger number of strains should be tested to provide a meaningful analysis. The weak difference of the accumulation values for FR160 between chloroquine-susceptible and chloroquine-resistant strains (3- to 4-fold) associated to a weak difference of FR160 IC_{50} (2- to 5-fold) in contrast with the differences of the accumulation values for chloroquine (20-fold) and IC_{50} (40-fold) also suggest that cellular uptake of both drugs may involve different transport systems. The identification of these transport systems is now required.

It is poorly correlated between accumulation and IC_{50} for FR160 ($r = 0.5$), while it is highly correlated for chloroquine ($r = 0.994$). These data suggest that the activity of FR160 seems to be independent of its accumulation in infected erythrocytes. In contrast, desferrioxamine does indeed penetrate the infected red cell and its antimalarial activity is dependent on this [48].

In conclusion, our study demonstrates the capacity of FR160 to reach and quickly accumulate into infected erythrocytes. The mechanism of accumulation is yet unknown but it seems to be not associated with calcium pump or channel, potassium channel or Na^+/H^+ exchanger. The potent *in vitro* activity of FR160 on chloroquine-resistant strains or isolates, its lower toxicity, its mechanisms of action, its capacity to reach rapidly and accumulate

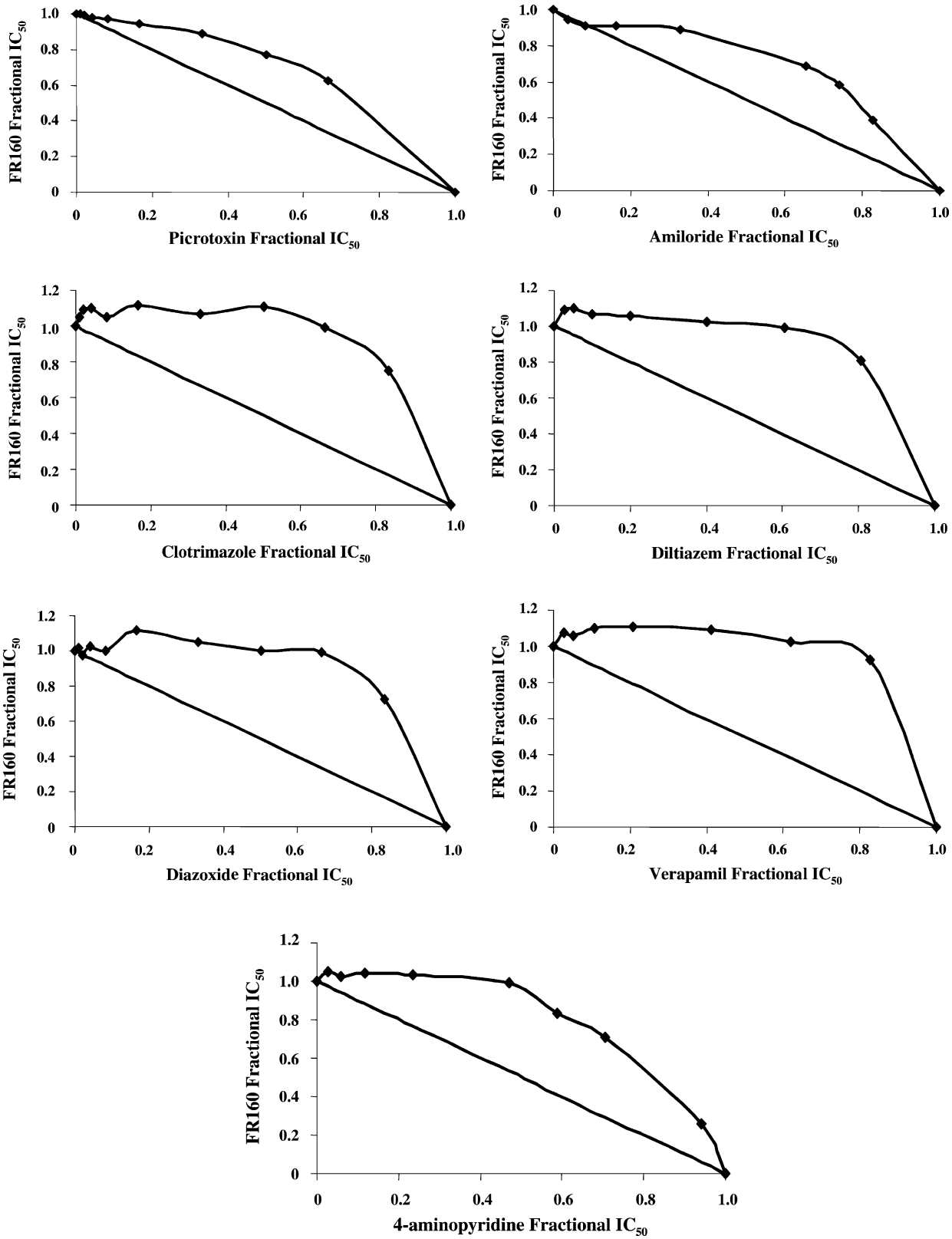

Fig. 3. *In vitro* combination of FR160 with several pharmacological channels blockers against the *Plasmodium falciparum* W2 strain.

into infected erythrocytes suggest that FR160 holds much promise as a new structural lead and effective antimalarial agent or at least a promising adjuvant in treatment of malaria and that it is suitable for *in vivo* studies.

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