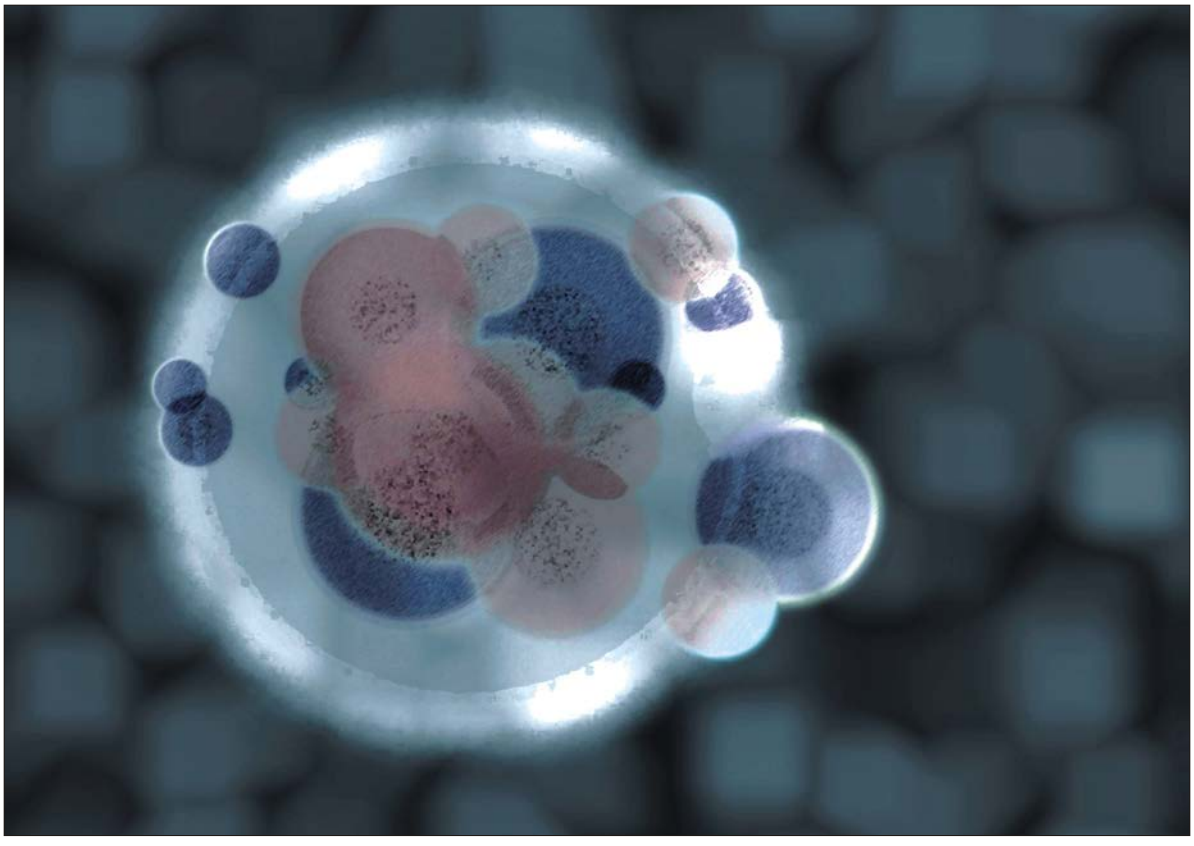

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Generation of Free Radicals and Enhancement of Hemin-induced Membrane Damage by a Catechol Iron Chelator in *Plasmodium falciparum*

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Abstract: We investigated whether FR160, a catechol iron chelator, affects the redox catalytic activity of heme towards membrane lipid, a more physiologically relevant oxidizable substrate. Formation of malondialdehyde (MDA), a major final product of lipid peroxidation initiated by reactive oxygen species, is used as indicator of membrane lipid oxidation. FR160 enhances the ability of hemin to generate MDA by 56%. Vitamin E, an oxygen-radical scavenger, decreases by 30% the formation of MDA by hemin in combination with FR160. When incubated in the presence of hemin, FR160 or artemisinin, alone, react with the amine groups of β-alanylhistidine (β-AH) and decreases the NBDF-reactive amines of β-AH. At 48 h of incubation in the presence of hemin, FR160 induces considerable decrease of NBDF-reactive amine of β-AH by 50%. The iron chelator FR160 acts by generation of radical species and enhance of hemin-catalysed oxidation of membrane lipids. FR160 neither affects the chemical heme crystallization activity nor the production of hemozoin in *P. falciparum* parasites.

Key words: Malaria, iron chelator, siderophore, free radical, lipid peroxidation, antimalarial drug

INTRODUCTION

The emergence and spreading of parasite resistance to currently used antimalarial drugs indicates that novel compounds need to be discovered and developed by identification of novel chemotherapeutic target. Iron chelation therapy has been considered a suitable treatment for various infectious diseases, including malaria[1]. Iron is needed for catalysis of DNA synthesis and for a variety of enzymes concerned in electron transport and energy metabolism. The withholding of iron from the parasite by iron chelators conceivably could disrupt the metabolism of the parasite by preventing DNA and pyrimidine synthesis, interfering with carbohydrate metabolism and inhibiting mitochondrial electron transport, antioxidant defenses, *de novo* synthesis of heme or disrupting proteolysis of host hemoglobin. Iron chelators have shown activity at preventing the growth of the malaria parasite in cell culture[2], in animal[3] and human studies[4]. Desferrioxamine, an iron chelator that has been extensively used for the treatment of iron overload disease[5], has detectable antimalarial activity in humans but has not found a role in the clinical treatment of malaria. This is at least partially related to the disadvantages involved in the administration of desferrioxamine[5]. Desferrioxamine is an hydrophilic agent that permeates rather slowly into parasitized erythrocytes and only at advanced stages of parasite growth[6]. The antimalarial action of iron chelators is dictated by three factors[6], i.e. iron (III)-binding capacity, chelator ingress into parasitized erythrocytes and chelator egress from parasites after treatment. However, the time window of action of desferrioxamine is relatively limited and the antimalarial activity is slow to develop, even after continuous *in vitro* or *in vivo* exposure to desferrioxamine[7]. Various iron chelators were assessed to

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improve the drug lipophilicity leading to increased access of drug to intracellular parasites and to faster speed of action[8]. We have shown previously the *in vitro* activity of FR160, a catecholate siderophore derived from spermidine, the N^1-nonyl,N^1,N^8-bis(2,3-dihydroxybenzoyl) spermidine hydrobromide[9]. FR160 acts on parasites at considerably faster rates and at all stages of parasite growth and is significantly more active than desferrioxamine[10]. FR160 shows minimal toxicities on mammalian cells or uninfected erythrocytes (ratio IC_{50} on Vero cells or uninfected red blood cells/IC_{50} on *P. falciparum* infected erythrocytes are >200). FR160 reaches and accumulates into infected *P. falciparum* erythrocytes and parasites[11]. 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[11]. Combinations of FR160 and tetracyclines, norfloxacin or atovaquone have synergistic or additive effects against *P. falciparum* parasites[10,12]. All these data 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[13].

The aim of the present study was to characterize the mechanisms of action of this iron chelator.

MATERIALS AND METHODS

Strain of *P. falciparum*: When required for drug assays, the chloroquine resistant clone W2 (Indochina) was synchronized by sorbitol lysis. Susceptibilities to FR160 and artemisinin derivatives were determined after suspension in RPMI 1640 medium (Invitrogen, United Kingdom), supplemented with 10% human serum and buffered with 25 mM HEPES and 25 mM $NaHCO_3$ (hematocrit of 1.5% and parasitemia of 0.5%).

Drugs and reagents: The synthesis of FR160 (Fig. 1) was previously described[9]. Artemisinin and chloroquine were obtained from Sigma Chemical (St. Louis, MO, USA). Stock solutions were prepared in methanol or ethanol.

Hemin chloride, thiobarbituric acid, vitamin C, vitamin E, β-alanylhistidine, 1,1,3,3 tetraethoxy-propane, NBDF (4-fluoro-7-nitrobenzo-2,1,3-oxadiazole) were obtained from Sigma Chemical.

***In vitro* assay:** For *in vitro* isotopic microtests, 200 µL of the suspension of parasitized erythrocytes 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 (Amersham, UK) to each well. Plates were incubated for 42 h at 37°C in an atmosphere of 10% O_2, 6% CO_2, 84% N_2 and 95% humidity. 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, Perkin Elmer, USA) and washed using a cell harvester (FilterMate™ Cell Harvester, Perkin Elmer). Filter microplates were dried and 25 µL of scintillation cocktail (Microscint™ O, Perkin Elmer) was placed in each well. Radioactivity incorporated by the parasites was measured using a scintillation counter (Top Count™, Perkin Elmer).

The IC_{50} (50% inhibitory concentration), 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 analysed after logarithmic transformation and expressed as the geometric mean IC_{50} and 95% confidence intervals (95% CI).

Effect of ascorbic acid (vitamin C), an antioxidant, on the activity of FR160: Activities of FR160, artemisinin and chloroquine were assessed in combination with different doses of ascorbic acid, ranging from 100 to 800 µM, in 5 independent experiments.

Oxidation of lipid membranes: measurement of malondialdehyde (MDA) generation: Red blood cells (A^+) was obtained from the bank of the Blood Transfusion Centre (Military Hospital, Toulon, France). Erythrocyte membranes were prepared according to the method described by Berman and Adams[14]. Hemin, FR160, artemisinin, vitamin E (radical scavenger) were added to aliquots of membrane suspension in a total volume of 1 mL for final concentrations of components in the assay mixture of : membranes 10% (v/v), hemin 0.5 mM, FR160 4 mM, artemisinin 2 mM, vitamin E 4 mM. After incubation of membranes preparations for 16 h at 37°C, malondialdehyde (MDA) was measured by method published previously[14]. Protein and lipid were removed by the sequential addition of H_2SO_4 (2/3 N) and Na_2WO_4 (10%) and centrifugation at 4°C for 15 min at 7,000 g. Supernatant was mixed with thiobarbituric acid (1%) in 50 mM NaOH and with concentrated H_3PO_4 and heated for 1 h at 95°C. After cooling, the pink chromogen was extracted into n-butanol and the fluorescence of the n-butanol layer measured on a fluorometer (Cytofluor 2300, Millipore, USA), using excitation and emission wavelengths of 530 and 590 nm, respectively. MDA was quantified by comparing the fluorescence to that of aqueous standards of 1,1,3,3 tetraethoxy-propane subjected to the identical thiobarbituric acid condensation reaction.

Results are expressed as a % of the MDA formed from membranes incubated alone and indicate the mean of 4 independent assays.

Generation of free radicals: FR160 and artemisinin were used as potential radical donors and amino acid-related compound (β-alanylhistidine) as radical recipient and the amounts of residual amine group of radical recipient were monitored by fluorometry. The reaction mixture was prepared according to the method described by Tanaka *et al.*[32]. Two hundred microliter of 10 mM of FR160 or 20 mM of artemisinin (in ethanol), 100 μL of 2.5 mM of β-alanylhistidine (β-AH) in sodium phosphate buffer, 20 μL of 10 mM hemin in 0.001 N NaOH and 680 μL of 20 mM sodium phosphate buffer (pH 7.2). During incubation at 37°C for 48 h, 100 μL aliquots were withdrawn at 0, 12, 24, 36 and 48 h, diluted to 1 mL with water and stored frozen until use for fluorometric assay. The amounts of residual amine were determined by fluorometry using amine-reactive NBDF (4-fluoro-7-nitrobenzo-2,1,3-oxadiazole). The 50 μL 10-fold diluted reaction mixture was added to 50 μL of 0.1 M borax (pH 9.3) and 100 μL of 0.4 mg mL^{-1} of NBDF in acetonitrile. The reaction mixtures were heated at 60°C for 2 min, then cooled in ice and diluted to 1 mL in 0.2 N HCl. The fluorescence of 200 μL of the acidified mixture was measured on a fluorometer (Cytofluor 2300, Millipore, USA), using excitation and emission wavelengths of 530 and 590 nm, respectively. Four independent assays were conducted.

Chemical inhibition of heme polymerization: A polymer identical to hemozoin, β-hematin, can be obtained *in vitro* from hematin at acidic pH. To identify molecules with inhibitory activity on heme polymerization, we used a quantitative *in vitro* spectrophotometric micro assay of heme polymerisation[15].

One hundred microliter of 4 mM solution of hematin, previously dissolved in 0.1 M of NaOH, were distributed in 96 well microplates (0.4 μmol/well). Fifty microliter of different doses of antimalarial drugs at a drug: heme ratio between 0:1 to 10:1 were added to triplicate test wells. Either 50 μL of water or 50 μL of the solvent used to solubilize the drugs were added to control wells. Hematin polymerization was initiated by adding 0.8 mmol of acetic acid (50 μL) at a final pH of 3 and the suspension was incubated at 37°C for 24 h to allow complete polymerization. Plates were then centrifuged at 3,300 g for 15 min and the soluble fraction of unprecipitated material collected. The remaining pellets were resuspended with 200 μL of DMSO to remove unreached hematin. Plates were then centrifuged again at 3,300 g for 15 min.

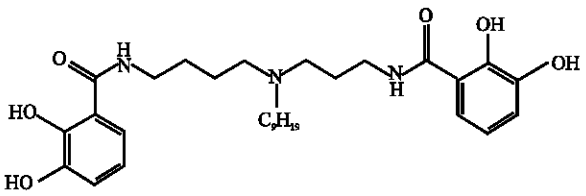

Fig. 1: Chemical structure of FR160 (N^4-nonyl,N^1,N^8-bis (2,3-dihydroxybenzoyl) spermidine hydrobromide)

The DMSO-soluble fraction was collected and the pellets, consisting of a pure precipitate of β-hematin, were dissolved in 0.1 M NaOH for spectroscopic quantification. A 150 μL aliquot of each fraction was transferred onto a new plate and serial four-fold dilutions in 0.1 NaOH were performed. The amount of hematin was determinated by measuring the absorbance at 405 nm using an automatic plate reader (Optimax, Molecular Devices, Sunnyvale, CA). The data are expressed as the molar equivalents of test compounds relative to hematin required to inhibit heme polymerization by 50%.

Measurement of hemozoin production in infected erythrocytes after exposure to FR160 and artemisinin: This method was performed as previously described by Orjih and Fitch[16] with some modifications. After synchronization of the parasites, erythrocytes were resuspended in RPMI 1640 medium supplemented with 10% human serum and buffered with 25 mM HEPES and 25 mM $NaHCO_3$ to a 5% hematocrit. The suspension was distributed under 2 mL/well into Falcon 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ). Antimalarial drugs (or ethanol) were added to final concentrations of 1 μM for FR160 and 100 nM for artemisinin at 0 or 20 h (0 to 20 h, 0 to 40 h and 20 to 40 h). The plate was gently shaken. Plates were then incubated for 20 or 40 h at 37°C in a atmosphere of 10% O_2, 6% CO_2 and 84% N_2 and a humidity of 95%. The medium was changed after the first 20 h of incubation. Hemozoin production was measured at 0, 20 and 40 h. At the end of the incubation, the parasites cultures were centrifuged at 4°C for 30 min at 27,000 g. The supernatant fluid was discarded and the pellet was washed 3 times in RPMI buffered with 25 mM HEPES and 25 mM $NaHCO_3$, then centrifuged at 4°C for 30 min at 27,000 g and the supernatant discarded. The cells were hemolyzed by adding 10 mL of cold 5 mM sodium phosphate buffer solution (pH 7.6) and shaken vigorously. The sample was frozen and thawed twice at -80°C before being centrifuged again for 30 min at 27,000 g. The supernatant was analysed spectrophotometrically for hemoglobin. The absorption spectrum at 500-700 wavelengths was drawn.

The pellet containing the hemozoin was washed once by resuspending it in 10 mL of 20 mM Tris-buffered solution pH 7.2 and centrifuging at 4°C for 30 min at 27,000 g. The supernatant was discarded and the pellet resuspended in 2 mL of 2.5% SDS buffered with 25 mM Tris to pH 7.8 and left for 2 h at room temperature. The pellet was then centrifuged for 30 min at 27,000 g and the supernatant was discarded. Hemozoin was converted in ferriprotoporphyrin IX by suspending the pellet in 1.8 mL of SDS/Tris-buffered solution and 0.2 mL of 1 N sodium hydroxide. The mixture was vigorously vortexed and allowed to stand for 1 h at room temperature for hydrolysis of β-hematin. The optical density of each well was measured using an automatic plate reader (Optimax, Molecular Devices, Sunnyvale, CA, USA) at 405 nm. Experiments were done 3 times.

RESULTS

Effect of ascorbic acid (vitamin C), an antioxidant, on the activity of FR160: As artemisinin (control +), the activity of FR160 is significantly ($p< 0.0005$) decreased by addition of ascorbic acid (Table 1).

Oxidation of membrane lipids: Measurement of Malondialdehyde (MDA) generation: We investigated whether FR160 affects the redox catalytic activity of hemin towards membrane lipid, a more physiologically relevant oxidizable substrate. Formation of MDA, a major final product of lipid peroxidation initiated by reactive oxygen species, was used as indicator of membrane lipid oxidation. In Fig. 2, we show the effect of various compounds, alone or in combination on MDA generation. Hemin, FR160, artemisinin or vitamin E alone have little effect on basal MDA production. When hemin and FR160, hemin and artemisinin, hemin and vitamin E, hemin and artemisinin and vitamin E were added together, a synergistic effect was apparent. The ability of hemin to generate MDA is enhanced by FR160 by 56% ($p< 0.0005$), by artemisinin by 77% ($p< 0.0005$) and by vitamin E by 55% ($p< 0.0005$). The lipid-soluble radical scavenger vitamin E, which inhibits the MDA formation by 15%, doesn't induce a change in the MDA formation in combination with FR160 or artemisinin. Vitamin E decreases by 30% the formation of MDA by hemin in combination with FR160 ($p<0.0005$) and increases by 17% the MDA formation by hemin in combination with artemisinin ($p<0.025$).

Generation of free radicals: The Fig. 3 shows that when incubated in the presence of hemin, FR160 or artemisinin alone or the combination of FR160 and artemisinin react with the amine groups of β-alanylhistidine and decrease the NBDF-reactive amines of β-AH. Under the conditions where artemisinin causes 30% decrease of β-AH at 48 h of incubation in the presence of hemin, FR160 induces

Table 1: *In vitro* activities of FR160, artemisinin (control +) and chloroquine (control -) in combination with different concentrations of an oxygen-radical scavenger, ascorbic acid (vitamin C) against the *P. falciparum* W2 clone. The IC_{50} (50% inhibitory concentration), 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 analysed after logarithmic transformation and expressed as the geometric mean IC_{50} of 5 independent experiments and 95% confidence intervals (95% CI)

	IC_{50} in combination with increasing doses of ascorbic acid (95% confidence interval)		
Drugs	0 μM	400 μM	800 μM
FR 160	1.01 μM (0.97-1.05)	1.52 μM (1.18-1.86)	2.34 μM (1.95-2.73)
Artemisinin	8.59 nM (7.92-9.32)	14.01 nM (12.49-15.53)	18.70 nM (16.95-20.45)
Chloroquine	595 nM (545-645)	646 nM (565-726)	663 nM (563-763)

Values are means of 5 independent experiments

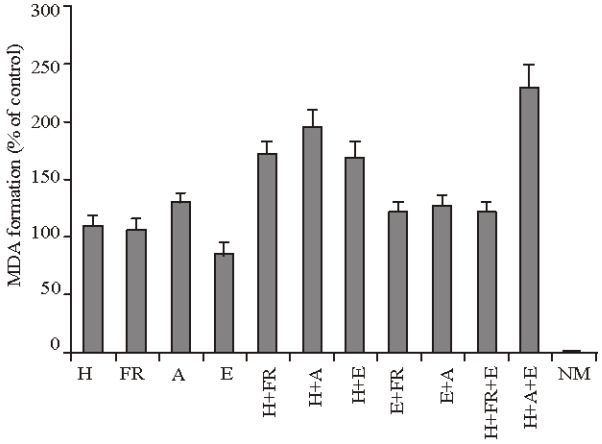

Fig. 2: Effect of hemin (H), iron chelator FR160 (FR), artemisinin (A) and vitamin E (E), alone or in combination, on malondialdehyde (MDA) generation in a model membrane lipid preparation. RBC membranes, washed free of hemoglobin, were incubated for 16 h at 37°C with 0.5 mM hemin (H), 4 mM iron chelator FR160 (FR), 2 mM artemisinin (A) and 4 mM vitamin E (E), either alone or in combination, as indicated. Membranes were removed by $H_2SO_4/NaWO_4$ precipitation and MDA in supernatant was measured fluorimetrically after reaction with TBA. Results are expressed as a % of the MDA formed from the membranes incubated alone and indicate the mean±SEM of 4 replicates. NM shows the result obtained when membranes were omitted

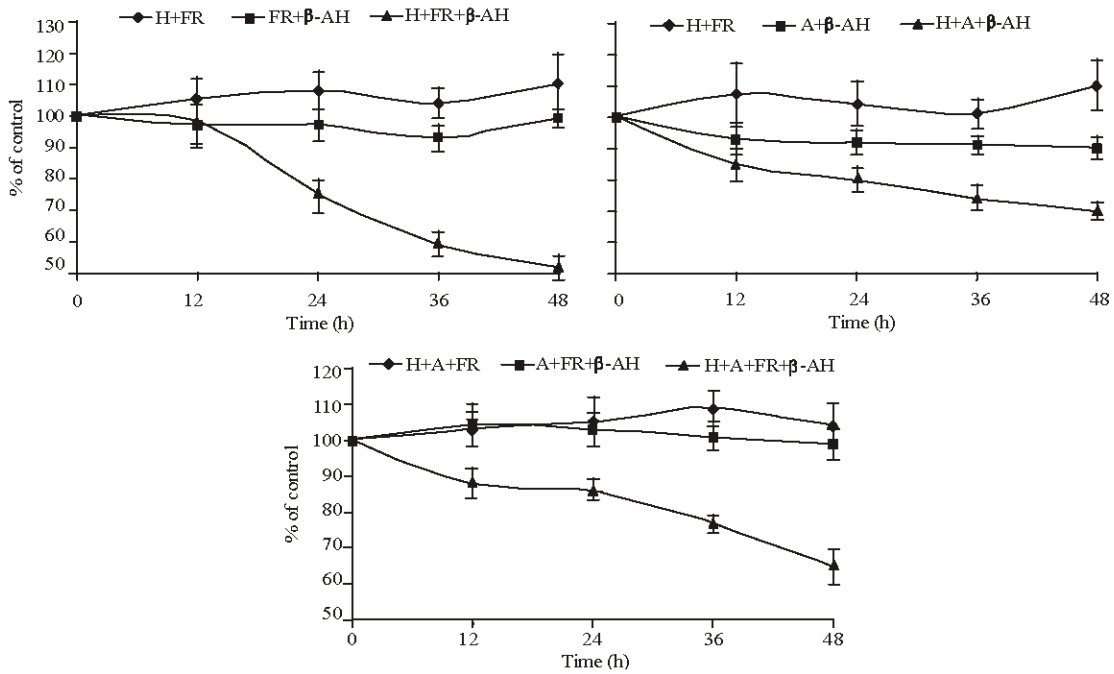

Fig. 3: Effect of hemin (H), iron chelator FR160 (FR) and artemisinin (A), alone or in combination, on β-alanylhistidine (β-AH). FR160 and artemisinin were used as potential radical donors and amino acid-related compound (β-AH) as radical recipient and the amounts of residual amine group of radical recipient were monitored by fluorometry. 10 mM of FR160 or 20 mM of artemisinin, 2.5 mM of β-AH in sodium phosphate buffer, 10 mM hemin in 0.001N NaOH and 20 mM sodium phosphate buffer (pH 7.2) were incubated at 37°C for 48h and aliquots were withdrawn at 0, 12, 24, 36 and 48 h, then diluted with water and stored frozen until use for fluorometric assay. The amounts of residual amine were determined by fluorometry using amine-reactive NBDF. The 10-fold diluted reaction mixture was added to 0.1 M borax (pH 9.3) and 0.4 mg mL^{-1} of NBDF in acetonitrile and the mixtures were heated at 60°C for 2 min, then cooled in ice and diluted in 0.2 N HCl. The fluorescence of the acidified mixture was measured on a fluorometer, using excitation and emission wavelengths of 530 and 590 nm, respectively. Four independent assays were conducted. Results are expressed as a % of the β-alanylhistidine formed from the membranes and indicate the mean±SEM of 4 replicates

considerable decrease of NBDF-reactive amine of β-AH by 50% ($p< 0.0005$). This decrease is also superior to that observed when FR160 is combined with artemisinin (35%, $p< 0.005$).

Chemical inhibition of heme polymerization: We don't obtain an inhibition of heme polymerization using FR160 (50% inhibition of heme polymerization IC_{50}>10 equivalents of FR160). In opposition, IC_{50} is observed using 1.60 equivalents of chloroquine (control +) and 1.45 equivalents of artemisinin (control +).

surement of hemozoin production in infected .. irocytes after exposure to FR160: The absorption ra of the supernatants of *P. falciparum* infected ..yunocytes hemolysates (after centrifugation at 27,000 g) drawn. FR160 and artemisinin don't change the absorption spectrum oh haemoglobin, which has characteristic peaks at 541 and 576 nm wavelengths. There is no peak at 630 nm wavelength, suggesting that detectable quantities of methaemoglobin are not formed in the cells.

FR160 doesn't inhibit the hemozoin production, while this one is dramatic when artemisinin is added to the culture at ring stages (Fig. 4). This decrease of production is also high when artemisinin is added at trophozoite stages (60% decrease in hemozoin production).

DISCUSSION

The antimalarial iron chelators can be placed into two major categories, depending on predominant mechanism of inhibition of parasite growth: withholding iron from plasmodial metabolic pathways or forming complexes with iron that are toxic to the parasite. For both of these

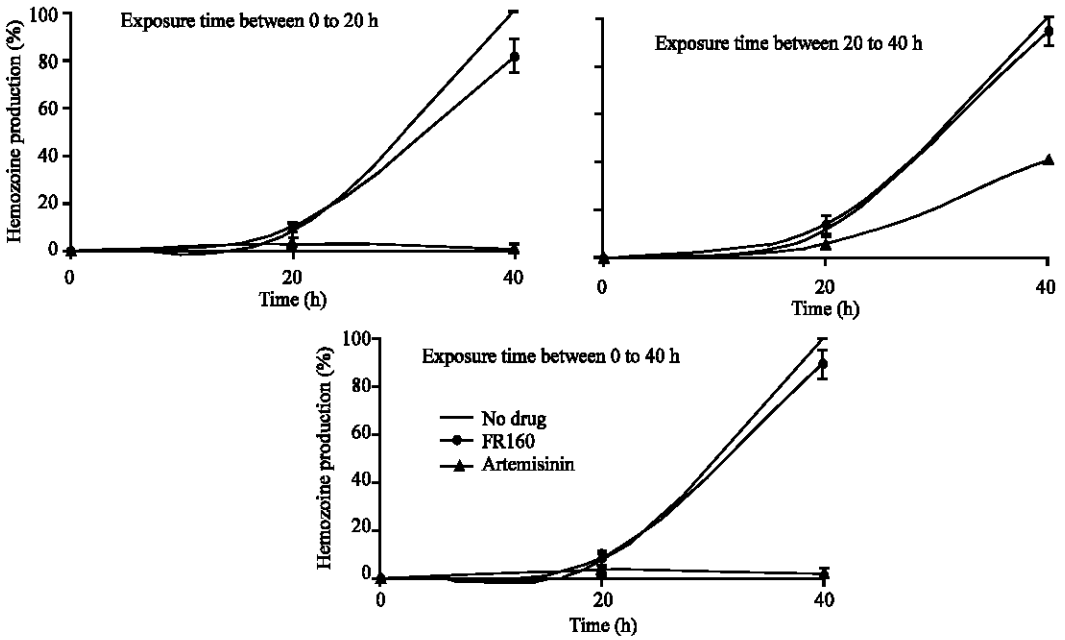

Fig. 4: Hemozoin production by *P. falciparum* without drug, with of FR160 or artemisinin. Concentrations of 1 μM for FR160 and 100 nM for artemisinin were added to *P. falciparum* parasitized erythrocytes at 0 or 20 h (0 to 20 h, 0 to 40 h and 20 to 40 h) and incubated for 20 or 40 h. Hemozoin production was measured at 0, 20 and 40 h. At the end of the incubation, the parasites cultures were centrifuged at 27,000 g. The pellet was washed 3 times then the cells were hemolyzed by adding cold 5 mM sodium phosphate buffer solution (pH 7.6). The sample was frozen and thawed twice at -80°C before being centrifuged at 27,000 g. The supernatant was analysed spectrophotometrically at 500-700 wavelengths. The pellet containing the hemozoin was resuspended in 2.5% SDS buffered with 25 mM Tris to pH 7.8 and left for 2 h at room temperature and then centrifuged at 27,000 g. Hemozoin was converted in ferriprotoporphyrin IX after addition of SDS/Tris-buffered solution and 1N sodium hydroxide. The optical density was measured at 405 nm. Three independent assays were conducted.

categories, an interaction with iron is the focus of the antimalarial activity.

The mechanism of antimalarial action of the first group of iron chelators appears to be the sequestration of iron necessary for plasmodial replication rather than a direct effect on the parasite or the withholding of other essential metals. The inhibitory action of these compounds on *P. falciparum* is fully abrogated when equimolar concentrations of iron are preincubated. This effect has been well documented for desferrioxamine[17], methyl-anthranilic desferrioxamine[17], desferrithiocin, desferricrocin[18], hydrazone derivatives[19], α-ketohydroxypyridones[20] and aminothiol derivatives [21]. Nevertheless, a number of other antimalarial drugs, which are not recognized as iron chelators, have ability to bind iron, such as bioflavonoid compounds or antibiotics[22,23].

Compounds of the second group, which includes aromatic metal chelators such as 8-hydroxyquinoline, seem to have an antiplasmodial effect other than the withholding of iron. It appears that a complex is formed extracellularly, which subsequently enters the parasitized red cell to produce a rapidly lethal free radical-mediated intracellular reaction[24].

In addition, some iron chelators, such as desferrioxamine, increase the concentration of soluble forms of hematin and enhance the rate of hematin crystallization[25].

In this study, we assess the ability of FR160 to generate radical species and enhance the hemin-catalysed oxidation of membrane lipids and to affect the chemical heme crystallization or the production of hemozoïn in *P. falciparum* parasites. We select artemisinin as a control + of generation of free radicals and inhibition of the heme crystallization. Artemisinin binds irreversibly to heme in the parasite food vacuole, preventing its polymerization to hemozoin and promoting heme-catalysed oxidation of the vacuolar membrane by molecular oxygen[26,27]. It has been shown that artemisinin inhibits heme polymerisation[15,28].

Nevertheless, the main antimalarial action of our iron chelator FR160 appears to be different that of desferrioxamine or iron chelators previously described.

FR160 acts as an oxidant drug. The use of antioxidant compounds, such as vitamin C or E, decreases the antimalarial activity and ability of FR160 to generate MDA in combination with hemin. The radical scavenger vitamin C, at the concentration of 800 μM, reduces the activities of FR160 and artemisinin by over 50%. However, vitamin C has been demonstrated to increase the release of iron in acidified lysates[29], which could decrease parasite susceptibility to FR160. We investigate whether FR160 affects the redox catalytic activity of hemin towards membrane lipid, a more physiologically relevant oxidizable substrate. Formation of MDA, a major final product of lipid peroxidation initiated by reactive oxygen species, is used as indicator of membrane lipid oxidation. We show the effect of various compounds, alone or in combination, on MDA generation. Two independent indices of redox activity were examined and both are significant in the presence of FR160: the hemin-catalysed oxidation of membrane lipid is increased by 56% in presence of FR160 (and by 77% in presence of artemisinin) and the generation of hemin-dependent radicals with a significant decrease in residual amine of β-alanylhistidine by 50% (30% for artemisinin). The observations on artemisinin, our control +, are consistent with previously published observation[14]. The effects on membrane lipid peroxidation by FR160 or artemisinin are unaffected by vitamin E, while this oxygen-radical scavenger significantly decreases the ability of the combination of hemin and FR160 to generate MDA by 30%. Vitamin E enhances the ability of hemin to generate MDA by 55% and that of the combination of hemin and artemisinin by 17%. This unexpected finding is in agreement with a previous study[14]. It has been demonstrated that under appropriate conditions, vitamin E may enhance peroxidation of lipid in low density lipoprotein[30]. Vitamin E contains two oxygen atoms that are the likely sites of interaction with hemin (the phenolic free hydroxyl group) and the oxygen atom in the heterocyclic ring. It was suggested that only the heterocyclic oxygen is reactive and may interact with the heme-iron in much the same way as does artemisinin[14]. The additive effect of artemisinin and vitamin E in potentiating the hemin-catalysed oxidation of lipid membranes suggests that either agent acts independently of the other[14].

The results on MDA production are consistent with those of generation of radical species. In the presence of hemin, FR160 induces considerable decrease of NBDF-reactive amine of β-alanylhistidine (β-AH) by 50%. Artemisinin causes, in the presence of heme, 30% decrease of β-AH, which is consistent with previously observations[31,32].

The presented evidence is sufficient to conclude that in these biochemical models, cell-free systems, both iron chelator FR160 and artemisinin act by generation of radical species and enhancement of hemin-catalysed oxidation of membrane lipids. Nevertheless, there are certainly other determinants involved in mechanism of action. However, the presented evidence is not sufficient to conclude on the antimalarial mechanisms of FR160 *in vivo*.

FR160 and artemisinin show different ability to inhibit chemically the heme crystallization. FR160 unaffects the chemical heme crystallization while chloroquine or artemisinin inhibit the polymerization of heme. In previous study, we demonstrated that desferrioxamine unaffects the crystallization of heme[10]. Such results have also been shown[33]. Nevertheless, it has been also demonstrated that desferrioxamine increases the concentration of soluble forms of hematin, enhances the rate of hematin polymerization and also initiates hematin crystallization[25]. The heme polymerization inhibitory activity of artemisinin has been still debated. Some works demonstrated the absence of heme crystallization inhibitory activity of artemisinin derivatives[34,35], while Pandey *et al.*[28] showed that artemisinin is a potent inhibitor of heme crystallization activity. The decrease of the production of hemozoin by *P. falciparum* after artemisinin exposure has been shown[36]. In this report, we demonstrate that the production of hemozoin decreases by over 100% when artemisinin is added at ring stages and by over 60% when added at trophozoite stages. This results are comparable to those previously described[36]. Parasites don't product more hemozoin when artemisinin is discarded at before trophozoite stages, indicating that hemoglobin catabolism by *P. falciparum* is irreversibly inhibited. At 20 h, we measure negligible amounts of hemozoin in ring stages, while in some works, amounts in ring stages represent 50% of amounts in trophozoite stages[36]. The present study suggest that artemisinin derivatives have a comparable mechanism of action as chloroquine and quinolines[37,38] and FR160 a different mode of action than quinoleines or artemisinin derivatives (inhibition of heme crystallization) and desferrioxamine (initiation of hematin crystallization).

In conclusion, the presented evidence is sufficient to conclude that in the biochemical model, cell-free systems, both iron chelator FR160 and artemisinin act by generation of radical species and enhancement of hemin-catalysed oxidation of membrane lipids. FR160 neither affects the chemical heme polymerization activity nor the production of hemozoin in *P. falciparum* parasites. However, the presented evidence is not sufficient to conclude on the antimalarial mechanisms of FR160 *in vivo*. The potent *in vitro* activity of FR160 on chloroquine resistant strains or isolates, its lower toxicity, 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 and that it is suitable for *in vivo* studies.

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REFERENCES

1. Pradines, B., J. Millet and M. Henry, 2003. Chelation du fer dans la thérapie antipaludique. Med. Trop., 63: 119-130.
2. Hershko, C. and T.E.A. Peto, 1988. Desferoxamine inhibition of malaria is independant of host iron status. J. Exp. Med., 168: 375-387.
3. Pollack, S., R.N., Rosan, D.E. Davidson and A. Escajadillo, 1987. Desferrioxamine suppresses *Plasmodium falciparum* in *Aotus* monkeys. P. Soc. Exp. Biol. Med., 184: 162-164.
4. Mabeza, G.F., G. Biemba and V.R. Gordeuk, 1996. Clinical studies of iron chelators in malaria. Acta. Haematol., 95: 78-86.
5. Richardson, D.R., 1999. Therapeutic potential of iron chelators. Expert Opin. Invest. Drug, 8: 2141-2158.
6. Cabantchik, Z.I., 1995. Iron chelators as antimalarials: The biochemical basis of selective cytotoxicity. Parasito, Today, 11: 74-78.
7. Cabantchik, Z.I., H. Glickstein, J. Golenser, M. Loyevsky and A. Tsafack, 1996. Iron chelators: Mode of action as antimalarials. Acta. Haematol., 95: 70-77.
8. Lytton, S.D., B. Mester, I. Dayan, H. Glickstein, J. Libman, A. Shanzer and I. Catbanchik, 1993. Mode of action of iron (III) chelators as antimalarials: I. Membranes permeation properties and cytotoxic activity. Blood, 81: 214-221.
9. Pradines, B., F. Ramiandrasoa, L.K. Basco, L. Bricard, G. Kunesch and J. Le Bras, 1996. *in vitro* activities of novel catecholate siderophore against *Plasmodium falciparum*. Antimicrob. Agent Chemother., 40: 2094-2098.
10. Pradines, B., J.M., Rolain, F. Ramiandrasoa, T. Fusai, J. Mosnier, C. Rogier, W. Daries, E. Baret, G. Kunesch, J. Le Bras and D. Parzy, 2002. Iron chelators as antimalarial agents: *In vitro* activity of dicatecholate against *Plasmodium falciparum*. J. Antimicrob. Chemother., 50: 177-187.
11. Hammadi, A., F. Ramiandrasoa, V. Sinou, C. Rogier, T. Fusai, J. Le Bras, D. Parzy, G. Kunesch and B. Pradines, 2003. Cellular uptake of a catechol iron chelator and chloroquine into *Plasmodium falciparum* infected erythrocytes. Biochem. Pharmacol., 65: 1351-1360.
12. Pradines, B., F. Ramiandrasoa, J.M. Rolain, C. Rogier, J. Mosnier, W. Daries, T. Fusai, G. Kunesch, J. Le Bras and D. Parzy, 2002. *In vitro* potentiation of antibiotics activities by a catecholate siderophore against *Plasmodium falciparum* chloroquine resistant parasites. Antimicrob. Agent Chemother., 46: 225-228.
13. Pradines, B., T. Fusai, C. Rogier, V. Sinou, J. Millet, M. Henry and D. Parzy, 2002. Progress in the development of antimalarial drugs: Iron chelation and reversal of chloroquine resistance as therapy for malaria. Recent Res. Devel. Antimicrob. Agent Chemother., 5: 25-43.
14. Berman, P.A. and P.A. Adams, 1997. Artemisinin enhances heme-catalysed oxidation of lipid membranes. Free Radical Bio. Med., 22: 1283-1288.
15. Basilico, N., E. Pagani, D. Monti, P. Olliaro and D. Taramelli, 1998. A micro-based method for measuring the Haem Polymerization Inhibitory Activity (HPIA) of antimalarial drugs. J. Antimicrob. Chemother., 42: 55-60.
16. Orjih, A.U. and C.D. Fitch, 1993. Hemozoin production by *Plasmodium falciparum*: Variation with strain and exposure to chloroquine. Biochim. Biophys. Acta., 1157: 270-274.
17. Loyevsky, M., S.D. Lytton, B. Mester, J. Libman, A. Shanzer and Z.I. Cabantchik, 1993. The antimalarial action of desferal involves a direct access route to erythrocytic (*Plasmodium falciparum*) parasites. J. Clin. Invest., 91: 218-224.
18. Fritsch, G., G. Sawatzki, J. Treumer, A. Jung and D.T. Spira, 1987. *Plasmodium falciparum*: Inhibition *in vitro* with lactoferrin, desferrithiocin and desferricrocin. Exp. Parasitol., 63: 1-9.
19. Tsafack, A., M. Loyevsky, P. Ponka and Z.I. Cabantchik, 1996. Mode of action of iron (III) chelators as antimalarials: IV. Potentiation of desferal action by benzoyl and isonicotinoyl hydrazone derivatives. J. Lab. Clin. Med., 127: 574-582.
20. Heppner, D.G., P.E. Hallaway, G.J. Kontoghiorghes and J.W. Eaton, 1988. Antimalarial properties of orally active iron chelators. Blood, 72: 358-361.
21. Loyevsky, M., C. John, I. Zaloujnyi and V. Gordeuk, 1997. Aminothiol-multidentate chelators as antimalarials. Biochem. Pharmacol., 54: 451-458.

22. Silfen, J., P. Yanai and Z.I. Cabantchik, 1988. Bioflavonoid effects on *in vitro* cultures of *Plasmodium falciparum*. Inhibition of permeation pathways induced in the host cell membrane by the intraerythrocytic parasite. Biochem. Pharmacol., 37: 4269-4276.
23. Pradines, B., C. Rogier, T. Fusai, J. Mosnier, W. Daries, E. Baret and D. Parzy, 2001. *In vitro* activities of antibiotics against *Plasmodium falciparum* are inhibited by iron. Antimicrob. Agent Chemother., 45: 1746-1750.
24. Scheibel, L.W. and G.G. Stanton, 1986. Antimalarial activity of selected aromatics chelators IV. Cation uptake of *Plasmodium falciparum* in the presence of oxines and siderochromes. Mol. Pharmacol., 30: 364-369.
25. Vippagunta, S.R., A. Dorn, A. Bubendorf, R.G. Ridley and J.L. Vennerstrom, 1999. Deferoxamine: stimulation of hematin polymerization and antagonism of its inhibition by chloroquine. Biochem. Pharmacol., 58: 817-824.
26. Meshnick, S.R., A. Thomas, A. Ranz, C.M. Xu and H.Z. Pan, 1991. Artemisinin (qinghaosu): The role of intracellular hemin in its mechanism of antimalarial action. Mol. Biochem. Parasitol., 49: 181-189.
27. Wei, N. and S.M. Sadrzadeh, 1994. Enhancement of hemin-induced membrane damage by artemisinin. Biochem. Pharmacol., 48: 737-741.
28. Pandey, A.V., B.L. Tekwani, R.L. Singh and V.S. Chauhan, 1999. Artemisinin, an endoperoxide antimalarial, disrupts the hemoglobin catabolism and heme detoxification system in malarial parasites. J. Biol. Chem., 274: 19383-19388.
29. Gabay, T., M. Krugliak, G. Shalmiev and H. Ginsburg, 1994. Inhibition by antimalarial drugs of hemoglobin denaturation and iron release in acidified red blood cell lysates-a possible mechanism of their antimalarial effect? Parasitology, 108: 371-381.
30. Bowry, V.W., K.U. Ingold and R. Stocker, 1992. Vitamin E in human low-density lipoprotein-When and how this antioxidant becomes a prooxidant. Biochem. J., 288: 341-344.
31. Tanaka, Y., F. Fang, C.G. Zhang, X.W. Zhang and S. Omura, 1998. Heme-dependent radical generation from antimalarial fungal metabolites, radicicol and heptelidic acid. J. Antibiot., 51: 451-453.
32. Tanaka, Y., K. Kamei, K. Otoguro and S. Omura, 1999. Heme-dependent radical generation: Possible involvement in antimalarial action of non-peroxide microbial metabolites nanaomycin A and radicicol. J. Antibiot., 52: 880-888.
33. Pandey, A.V. and B.L. Tekwani, 1997. Depolymerization of malarial hemozoin: A novel reaction initiated by blood schizontocidal antimalarials. FEBS Lett., 402: 236-240.
34. Asawamlahasakda, W., I. Ittarat, C.C. Chang, P. Mcelroy and S.R. Meshnick, 1994. Effects of antimalarials and protease inhibitors on plasmodial hemozoin production. Mol. Biochem. Parasitol., 67: 183-191.
35. Hawley, S.R., P.G. Bray, M. Mungthin, J.D. Atkinson, P.M. O'neill and S.A. Ward, 1998. Relationship between antimalarial drug activity, accumulation and inhibition of heme polymerisation in *Plasmodium falciparum in vitro*. Antimicrob. Agent Chemother., 42: 682-686.
36. Orjih, A.U., 1996. Haemolysis of *Plasmodium falciparum* trophozoite-infected erythrocytes after artemisinin exposure. Brit. J. Haematol., 92: 324-328.
37. Vennerstrom, J.L., E.O. Nuzum, R.E. Miller, A. Dorn, L. Gerena, P.A. Dande, W.Y. Ellis, R.G. Ridley and W.K. Milhous, 1999. 8-aminoquinolines active against blood stage *Plasmodium falciparum in vitro* inhibit hematin polymerization. Antimicrob. Agent Chemother., 43: 598-602.
38. Sullivan, D.J., H. Matile, R.G. Ridley and D.E. Goldberg, 1998. A common mechanism for blockade heme polymerisation by antimalarial quinolines. J. Biol. Chem., 273: 31103-31107.