

The Ferroquine Antimalarial Conundrum: Redox Activation and Reinvasion Inhibition**

Faustine Dubar, Christian Slomianny, Jamal Khalife, Daniel Dive, Hadidjatou Kalamou, Yann Guérardel, Philippe Grellier, and Christophe Biot*

The idea of using organometallic complexes in drug discovery is becoming more and more popular among the medicinal chemistry community.^[1–8] Indeed, a number of organometallic compounds are presently undergoing evaluation in cancer^[9] and infectious disease^[10] clinical trials.^[11] Out of those, ferrocene–pharmacophore conjugates have paved the way for overcoming the problem of resistance. Ferrocenyl compounds are stable, lipophilic and redox active, and are usually devoid of serious toxicity.^[7]

One of the most advanced projects is ferroquine (FQ, SSR97193, Figure 1), a ferrocenyl analogue of the antimalarial chloroquine (CQ, Figure 1), in the Phase II Sanofi portfolio for uncomplicated *Plasmodium falciparum* (*P. falciparum*) malaria.^[12–14]

As no drug produces a single effect, there are several impediments to understanding the mechanisms of action (MOA) of the drug (or drug candidate) and the elucidation of the site of action or the nature of the drug–cell interaction. Previously, we established that FQ selectively targets the digestive vacuole (DV) of the *P. falciparum* parasite through other transport mechanism(s) than those involved for the organic parent drug CQ.^[15] Later, we found that CQ-resistant parasites treated by FQ accumulate a sulfur-containing compound, likely glutathione (GSH), in their DV suggesting the production of reactive oxygen species (ROS).^[16] The next

3D7	IC ₅₀ = 21.0 ± 4.0 nM	3D7	IC ₅₀ = 3.4 ± 0.3 nM
HB3	IC ₅₀ = 18.0 ± 8.0 nM	HB3	IC ₅₀ = 13.4 ± 2.6 nM
FcB1	IC ₅₀ = 105 ± 16 nM	FcB1	IC ₅₀ = 15.6 ± 2.1 nM

Figure 1. Structural formula and antimalarial activities of chloroquine (CQ) and Ferroquine (FQ) on CQ-susceptible (3D7 and HB3) and CQ-resistant (FcB1) strains. (See Table S1 for more details)

challenge is the identification and localization of reactive oxygen species production when treated by FQ in comparison with CQ, and the impact on the parasite reinvasion.

Herein, we investigate the oxidative conditions inside *P. falciparum* and the enhancement or inhibition of *P. falciparum* erythrocyte reinvasion, so as to understand the mechanism of action of FQ.

During its intra-erythrocytic phase, the parasite digests hemoglobin as its main source of amino acids^[17,18] and to preserve the osmotic integrity of the host.^[19] The process of degradation of hemoglobin generates not only a high amount of ferriprotoporphyrin IX (FP) but also a variety of reactive oxygen species, such as hydrogen peroxide, superoxide anion, singlet oxygen, and hydroxyl radical (HO•). Several analytical approaches have been used to estimate oxidative stress in different stages of *P. falciparum*.^[20,21] However, to our knowledge, ratiometric fluorescent probes which can be used for specific (and not general) intracellular reactive oxygen species detection have never been reported. The limitation was mainly due to the necessary requirements of such responsive probes: concentration, excitation intensity, absence of interaction with the cellular environment.

Recently, a ratiometric fluorescent probe **1** (Figure 2) has been successfully developed for detection of intracellular

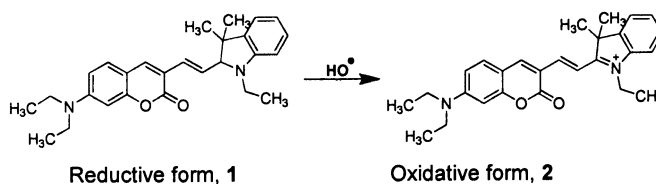


Figure 2. Ratiometric fluorescent probe with high selectivity for hydroxyl radicals.

[*] Dr. Y. Guérardel, Prof. C. Biot
Université Lille1, Unité de Glycobiologie Structurale et Fonctionnelle, CNRS UMR 8576
IFR 147, 59650 Villeneuve d'Ascq Cedex (France)
E-mail: christophe.biot@univ-lille1.fr

Dr. F. Dubar
Université Lille1, Unité de Catalyse et Chimie du Solide—UMR CNRS 8181
B.P. 90108, 59652 Villeneuve d'Ascq Cedex (France)

Dr. C. Slomianny
Université Lille1, Inserm U1003—Laboratoire de Physiologie Cellulaire
Bâtiment SN3, 59655 Villeneuve d'Ascq Cedex (France)

Dr. J. Khalife, Dr. D. Dive, H. Kalamou
CIIL, Inserm U 1019, UMR CNRS 8024 Université Lille Nord de 15 France, Institut Pasteur de Lille
1 rue du Pr Calmette, 59019 Lille Cedex (France)

Prof. P. Grellier
National Museum of Natural History, UMR 7245 CNRS-MNHN, Team APE, CP 52
57 Rue Cuvier, Paris 75005 (France)

[**] This study was funded by a grant from the Ministère de l'Enseignement Supérieur to F.D.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/ange.201303690>.

HO[•].^[22] The hybrid coumarine–cyanine dye **1** has a maximum emission at 495 nm in its reductive form whereas it shifts to 651 nm in its oxidative form **2** (Figure 2). Furthermore, a 210-fold variation in the emission ratios (I_{651}/I_{495}) at two different wavelengths offers a very sensitive probe. Although this probe has already been used in cellular imaging in the relatively large HeLa cells (10–30 μm widths and 40–70 μm lengths), its use in malaria infected red blood cells (RBCs, approximately 6–8 μm) is unprecedented.

We have used probe **1** to assess HO[•] production at the trophozoite and schizont stages in CQ-susceptible (3D7) and CQ-resistant (FcB1) *P. falciparum* strains, and to examine the influence of FQ (in comparison to CQ) in generating HO[•]. DAPI (4',6-diamidino-2-phenylindole) was also used to stain the parasite nuclei.

As expected, without probe **1** uninfected red blood cells gave no fluorescence (see Figure S2 in the Supporting Information). A similar result was obtained when uninfected red blood cells were incubated with **1** indicating that uninfected red blood cells do not produce a detectable amount of HO[•]. We then proceeded to examine the potential usefulness of **1** for HO[•] imaging in *P. falciparum* infected red blood cells (iRBCs). In the absence of **1**, no fluorescence was detected in both strains (see Figure S3). After incubation with **1**, both strains exhibited fluorescence at the green and red wavelengths. Microscopic observations (see Figure S4) not only demonstrated the transport of the dye **1** across the red blood cell and parasite membranes, but also enabled for the first time the visualization of HO[•] in the cytosol and the digestive vacuole of trophozoite and schizont stages. As suspected, HO[•] is thus one of the reactive oxygen species generated by the mitochondrial respiratory chain and hemoglobin digestion. To avoid any misinterpretation owing to the absorption properties of hemozoin (Hz) crystals, calculations of the intensity ratios (I_{651}/I_{495}) has been performed close to Hz but in an area of the digestive vacuole devoid of Hz so as to minimize this phenomenon (see Figure S7). I_{651}/I_{495} ratios were approximately 10-fold higher in the cytosol than in the digestive vacuole at the trophozoite stage but this difference is much less at the schizont stage for which global OH[•] production decreases significantly (see Table S6). We obtained the first complete map of HO[•] compartmentalization in *P. falciparum*.

After drug treatment (FQ 40 nM, 30 min, Figure 3 or CQ, see Figure S5), we observed only small changes of the value of the I_{651}/I_{495} ratios (see Tables S6 and S11) at the trophozoite stage in the cytosol for both strains suggesting that redox properties of FQ has no influence over the HO[•] production within this cellular compartment (Figure 4). On the contrary, a significant difference between FQ and CQ was observed in the parasite digestive vacuole. Indeed, the addition of CQ decreased the I_{651}/I_{495} ratios (in comparison to the I_{651}/I_{495} ratios calculated in untreated red blood cells) whereas the addition of FQ resulted in a 6 to 10-fold increase in the I_{651}/I_{495}

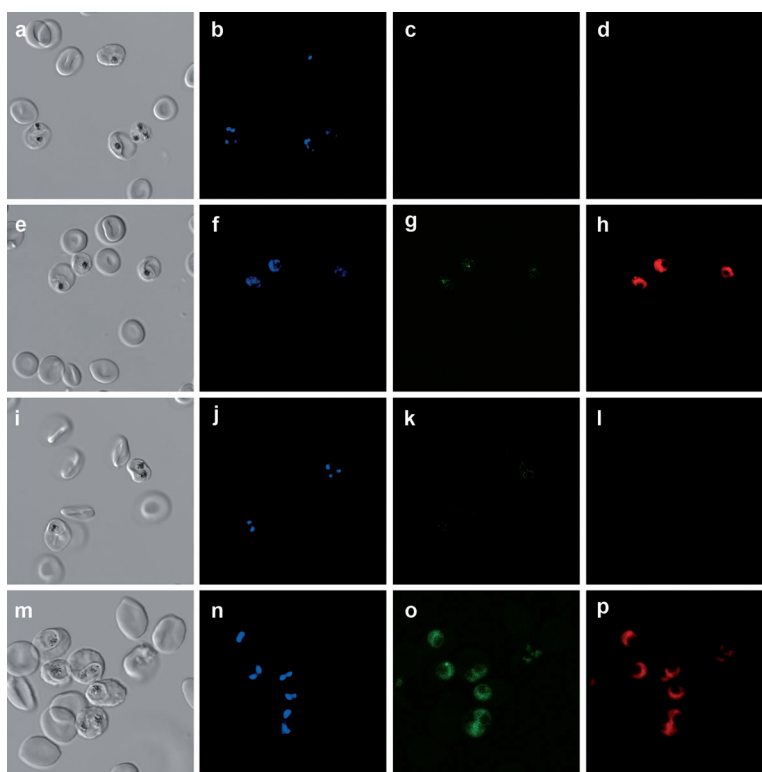


Figure 3. a)–d) Red blood cells infected by the CQ-susceptible strain (3D7) pre-treated by FQ (40 nM, 30 min), fixed and stained with DAPI; e)–h) red blood cells infected by the CQ-susceptible strain (3D7) pre-treated by FQ (40 nM, 30 min), incubated with **1** (40 μM for 5 min), fixed and stained with DAPI; i)–l) red blood cells infected by the CQ-resistant strain (FcB1) pre-treated by FQ (40 nM, 30 min), fixed and stained with DAPI; m)–p) red blood cells infected by the CQ-resistant strains (FcB1) pre-treated by FQ (40 nM, 30 min), incubated with **1** (40 μM for 5 min), fixed and stained with DAPI. Bright-field images (a, e, i, m) and corresponding DAPI fluorescence emission (b, f, j, n), green fluorescence emission (c, g, k, o), and red fluorescence emission (d, h, l, p).

ratios according to the clone studied. In other words, the purely organic drug CQ exerts an antioxidant effect by quenching HO[•]^[23] whereas the organometallic FQ increases the generation of HO[•].

At the schizont stage (Figure 4), the addition of CQ led to a 5 to 16-fold increase of the I_{651}/I_{495} ratios (in comparison to the I_{651}/I_{495} ratios in untreated parasites) in the cytosol (see Tables S6 and S11). Nevertheless, no significant difference was observed in the digestive vacuole (see Tables S6 and S11). So, CQ exerts no influence or, as in cytoplasm, a quenching effect on HO[•] production in its site of action. Moreover, recently, Egan et al. have shown that after CQ treatment, a high level of iron was detected in the cytosol compared to in the digestive vacuole, suggesting a free heme diffusion across the digestive vacuole membrane into the cytosol.^[24] These data suggest that heme released, through the action of CQ, is able to accumulate in the cytosol where it can react with H₂O₂ and form HO[•], thus explaining the increase in the level of these radicals in the cytosol compared to the digestive vacuole. After FQ treatment, although the level of I_{651}/I_{495} ratios are similar in the cytosol as those with CQ, the FQ gave a 17-fold increase of I_{651}/I_{495} ratios in the digestive vacuole (compared to the I_{651}/I_{495} ratios in controls), contrary

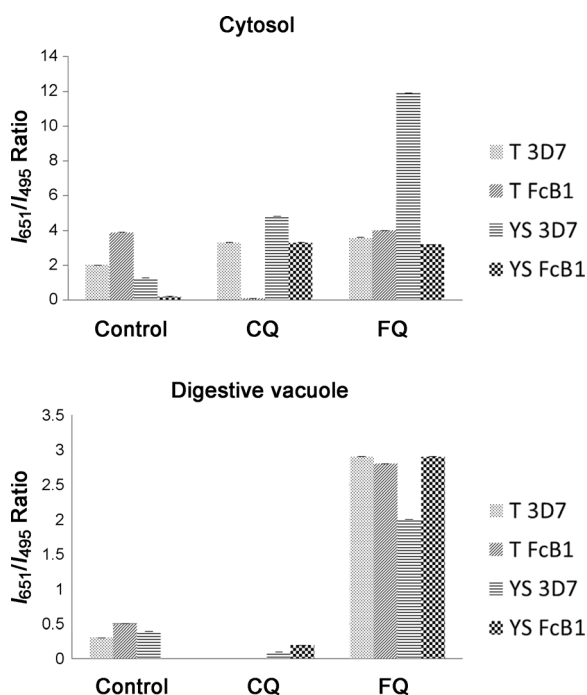


Figure 4. Quantification of the I_{651}/I_{495} ratio in the cytosol (top) and the digestive vacuole (bottom) of *P. falciparum* parasites (CQ-susceptible strain 3D7 and CQ-resistant strain FcB1) without drug (control) or after drug treatment (CQ or FQ) at the trophozoite (T) and young schizont (YS) stages.

to CQ. These data suggest that the level of free heme release during the hemozoin inhibition is not sufficient to explain the high levels of $\text{HO}^\bullet$ in the digestive vacuole. Once again, only the organometallic FQ is clearly able to induce an additional oxidative stress in the parasite which in turn disrupts the intracellular redox balance. The parasitic antioxidant defense system is then overflowed which results in membrane alterations leading to the death of the parasite.

Enhancement by FQ of the $\text{HO}^\bullet$ formation in the digestive vacuole is not yet documented, but it probably involves a mechanism similar to that of the Fenton reaction. Note that other ferrocene-based compounds have been shown to generate H_2O_2 by autooxidation, forming the ferricinium cation.^[25,26] FQ thus combines Hz inhibition activity, redox activity, and antimalarial activity against both CQ-susceptible and CQ-resistant strains. These results clearly demonstrate that the $\text{HO}^\bullet$ increase has a direct role in its antimalarial action.

Along the *Plasmodium* erythrocytic life-cycle, the trophozoite undergoes schizogony and develops into a schizont, later releasing merozoites. Upon rupture of the schizont, merozoites are released to rapidly invade new red blood cells and perpetuate the cycle. So, we monitored the influence of both drugs (FQ and CQ) upon the reinvasion step of the CQ-resistant strain (FcB1). Highly synchronized parasite cultures at the old-trophozoite-schizont stages were exposed for 5 h to 25 nM or 100 nM of FQ (or CQ). Exposed red blood cells were then washed three times and put back in culture with new red blood cells at a parasitemia of 0.8%. The ability of parasites to develop and to reinvade was followed up at different times

for more than 120 h. Indeed, the effect of FQ on parasite growth during the subsequent cycles when the parasite is no longer exposed to FQ was not known.

Figure 5 clearly shows the absence of parasites after one cycle when the schizonts were pretreated by FQ whereas parasitaemia reached more than 2% when the cultures were

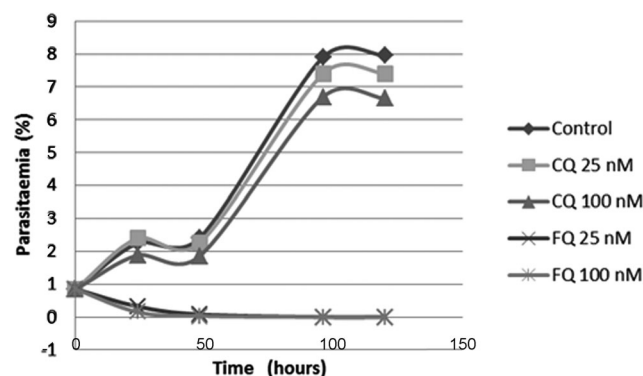


Figure 5. Evolution of the parasitaemia as a function of time of cultures of synchronized parasites at the trophozoite-schizont stages of the CQ-resistant strain FcB1 pretreated during 5 h with 25 and 100 nM FQ and CQ, or in the absence of drug (control).

pretreated by CQ under the same conditions. We hypothesized that the oxidative stress due to FQ occurring in the trophozoite and schizont stages also alters the merozoite formation, thus inhibiting reinvasion. Similar results were obtained with pretreated schizonts of the CQ-susceptible strain HB3 (see Figure S8 and S9).

In addition to a drastic reduction of parasitemia, a significant difference of parasite stage distribution is observed between FQ and CQ or controls 24 h after treatment (Figure S9 and S10). CQ treatment does not affect parasite reinvasion and new ring and young trophozoites stages are observed as for the controls. In contrast, after FQ treatment, the parasites able to reinvade are unable to develop as demonstrated by the small number of parasites with a typical young trophozoite morphology and the increasing number of parasites with pycnotic appearance. All parasites were dead 48 h after the pretreatment.

While further work will be required to better understand the effects of FQ upon the reinvasion steps and the development of new ring stages, these results provide the first conclusive evidence for the specificity of an organometallic drug candidate. Indeed, FQ and CQ are involved in the detoxification process of the parasite.^[27] Therefore, their action can lead to a free heme accumulation in the parasite, and thus release of $\text{HO}^\bullet$. However, the level of these radicals is much higher in the presence of FQ than CQ, demonstrating that the ferrocene is involved in the production of $\text{HO}^\bullet$. We have previously shown that the efflux phenomenon observed with CQ in CQ-resistant strains, is not observed with FQ. The concentration of FQ coupled to its redox properties therefore explain the greater effectiveness of the FQ, including the CQ-resistant strains of *P. falciparum*.

In summary, we report the first successful subcellular mapping of HO[•] generation in *P. falciparum* red blood cells treated with the drug candidate FQ using a ratiometric fluorescent probe. The resulting oxidative damage is consistent with the breakdown of the digestive vacuole membrane, leading then to the death of the parasites. In addition to the primary mechanism of quinoline action (inhibition of the heme detoxification process), an important implication is that the metallocenic moiety is part of the mechanisms of action. Based on the evidence that different stages of the *Plasmodium* erythrocytic life cycle (including rings) are targeted, we conjecture that FQ could play a key role in the inhibition of merozoites reinvasion. This finding clearly shows the advantage of an organometallic-based drug versus the purely organic parent drug, and can provide the next generation of drugs.

Received: April 30, 2013

Published online: June 13, 2013

Keywords: bioorganometallic chemistry · ferroquine · malaria · reactive species · subcellular imaging

- [1] G. Jaouen, N. Metzler-Nolte, R. Alberto, *Medicinal Organometallic Chemistry*, Springer, Heidelberg, **2010**.
- [2] E. Alessio, *Bioinorganic Medicinal Chemistry*, Wiley-VCH, Weinheim, **2011**.
- [3] M. Navarro, W. Castro, C. Biot, *Organometallics* **2012**, *31*, 5715–5727.
- [4] M. Adams, Y. Li, H. Khot, C. De Kock, P. J. Smith, K. Land, K. Chibale, G. S. Smith, *Dalton Trans.* **2013**, *42*, 4677–4685.
- [5] L. Glans, W. Hu, C. Jöst, C. de Kock, P. J. Smith, M. Haukka, H. Bruhn, U. Schatzschneider, E. Nordlander, *Dalton Trans.* **2012**, *41*, 6443–6450.
- [6] L. Glans, A. Ehnbon, C. de Kock, A. Martínez, J. Estrada, P. J. Smith, M. Haukka, R. A. Sánchez-Delgado, E. Nordlander, *Dalton Trans.* **2012**, *41*, 2764–2733.
- [7] D. R. van Staveren, N. Metzler-Nolte, *Chem. Rev.* **2004**, *104*, 5931–5985.
- [8] C. G. Hartinger, P. J. Dyson, *Chem. Soc. Rev.* **2009**, *38*, 391–401.
- [9] G. Gasser, I. Ott, N. Metzler-Nolte, *J. Med. Chem.* **2011**, *54*, 3–25.
- [10] M. Navarro, C. Gabbiani, L. Messori, D. Gambino, *Drug Discovery Today* **2010**, *15*, 1070–1078.
- [11] E. A. Hillard, G. Jaouen, *Organometallics* **2011**, *30*, 20–27.
- [12] G. Mombo-Ngoma, C. Supan, M. P. Dal-Bianco, M. A. Missinou, P.-B. Matsiegui, C. L. Ospina Salazar, S. Issifou, D. Ter-Minassian, M. Ramharther, M. Kombila, et al., *Malaria J.* **2011**, *10*:53.
- [13] C. Supan, G. Mombo-Ngoma, M. P. Dal-Bianco, C. L. Ospina Salazar, S. Issifou, F. Mazuir, A. Filali-Ansary, C. Biot, D. Ter-Minassian, M. Ramharther, et al., *Antimicrob. Agents Chemother.* **2012**, *56*, 3165–3173.
- [14] D. Dive, C. Biot, *ChemMedChem* **2008**, *3*, 383–391.
- [15] F. Dubar, S. Bohic, C. Slomianny, J.-C. Morin, P. Thomas, H. Kalamou, Y. Guérardel, P. Cloetens, J. Khalife, C. Biot, *Chem. Commun.* **2012**, *48*, 910–912.
- [16] F. Dubar, S. Bohic, D. Dive, Y. Guérardel, P. Cloetens, J. Khalife, C. Biot, *ACS Med. Chem. Lett.* **2012**, *3*, 480–483.
- [17] M. Aikawa, P. K. Hepler, C. G. Huff, H. Sprinz, *J. Cell Biol.* **1966**, *28*, 355–373.
- [18] J. Liu, E. S. Istvan, I. Y. Gluzman, J. Gross, D. E. Goldberg, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 8840–8845.
- [19] V. L. Lew, T. Tiffert, H. Ginsburg, *Blood* **2003**, *101*, 4189–4194.
- [20] Y. Fu, L. Tilley, S. Kenny, N. Klonis, *Cytometry Part A* **2010**, *77*, 253–263.
- [21] Y. Fu, N. Klonis, C. Suarna, G. J. Maghzal, R. Stocker, L. Tilley, *Cytometry Part A* **2009**, *75*, 390–404.
- [22] L. Yuan, W. Lin, J. Song, *Chem. Commun.* **2010**, *46*, 7930.
- [23] Y. Miyachi, A. Yoshioka, S. Imamura, Y. Niwa, *Ann. Rheum. Dis.* **1986**, *45*, 244–248.
- [24] J. M. Combrinck, T. E. Mabotha, K. K. Ncokazi, M. A. Ambele, D. Taylor, P. J. Smith, H. C. Hoppe, T. J. Egan, *ACS Chem. Biol.* **2013**, *8*, 133–137.
- [25] R. Kovjazin, T. Eldar, M. Patya, A. Vanichkin, H. M. Lander, A. Novogrodsky, *FASEB J.* **2003**, *17*, 467–469.
- [26] E. Hillard, A. Vessièrès, L. Thouin, G. Jaouen, C. Amatore, *Angew. Chem.* **2006**, *118*, 291–296; *Angew. Chem. Int. Ed.* **2006**, *45*, 285–290.
- [27] F. Dubar, T. J. Egan, B. Pradines, D. Kuter, K. K. Ncokazi, D. Forge, J.-F. Paul, C. Pierrot, H. Kalamou, J. Khalife, et al., *ACS Chem. Biol.* **2011**, *6*, 275–287.