



**HAL**  
open science

## **Superoxide: A major role in the mechanism of action of essential antimalarial drugs**

Chinedu Egwu, Ioannis Tsamesidis, Pierre Péro, Jean-Michel Augereau,  
Françoise Benoit-Vical, Karine Reybier

### ► To cite this version:

Chinedu Egwu, Ioannis Tsamesidis, Pierre Péro, Jean-Michel Augereau, Françoise Benoit-Vical, et al.. Superoxide: A major role in the mechanism of action of essential antimalarial drugs. *Free Radical Biology and Medicine*, 2021, 167, pp.271-275. 10.1016/j.freeradbiomed.2021.03.001 . hal-03203905

**HAL Id: hal-03203905**

**<https://hal.science/hal-03203905v1>**

Submitted on 24 Apr 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

# 1 **Superoxide: A major role in the mechanism of action of essential antimalarial** 2 **drugs**

3 **Chinedu O. Egwu<sup>1,2,3,4,5</sup>, Ioannis Tsamesidis<sup>1</sup>, Pierre Péro<sup>1</sup>, Jean-Michel Augereau<sup>3,4,5</sup>, Françoise**  
4 **Benoit-Vical<sup>3,4,5</sup><sup>†</sup> and Karine Reybier<sup>1</sup><sup>†\*</sup>**

5 <sup>1</sup> PharmaDev, UMR 152, Université de Toulouse, IRD, UPS, Toulouse, 31400, France.

6 <sup>2</sup> Medical Biochemistry, College of Medicine, Alex-Ekwueme Federal University, Ndufu-Alike Ikwo,  
7 P.M.B. 1010, Abakaliki, Ebonyi State, Nigeria

8 <sup>3</sup> CNRS, LCC, Laboratoire de Chimie de Coordination, Université de Toulouse, Toulouse, France

9 <sup>4</sup> Institut de Pharmacologie et de Biologie Structurale, IPBS, Université de Toulouse, CNRS, UPS,  
10 France

11 <sup>5</sup> New antimalarial molecules and pharmacological approaches, Inserm ERL 1289, Toulouse, France

12 <sup>†</sup> F.B.V. and K.R. share senior authorship.

13 \* Corresponding authors : FBV : [Francoise.Vical@inserm.fr](mailto:Francoise.Vical@inserm.fr) ; KR : [karine.reybier-vuattoux@univ-](mailto:karine.reybier-vuattoux@univ-tlse3.fr)  
14 [tlse3.fr](mailto:karine.reybier-vuattoux@univ-tlse3.fr), PharmaDEV, Université Toulouse 3 Paul Sabatier, Faculté de Pharmacie, 35 chemin des  
15 maraîchers - 31062 Toulouse Cedex 9

## 17 **Abstract**

18 Understanding the mode of action of antimalarials is central to optimizing their use and the discovery  
19 of new therapeutics. Currently used antimalarials belong to a limited series of chemical structures  
20 and their mechanisms of action are continually debated. Whereas the involvement of reactive  
21 species that in turn kill the parasites sensitive to oxidative stress, is accepted for artemisinins, little is  
22 known about the generation of such species in the case of quinolines or hydroxynaphtoquinone.  
23 Moreover, the nature of the reactive species involved has never been characterized in *Plasmodium*-  
24 infected erythrocytes. The aim of this work was to determine and elucidate the production of the  
25 primary radical, superoxide in *Plasmodium*-infected erythrocytes treated with artemisinin,  
26 dihydroartemisinin, chloroquine and atovaquone, as representatives of three major classes of  
27 antimalarial. The intracellular generation of superoxide was quantified by liquid chromatography  
28 coupled to mass spectrometry (LC-MS). We demonstrated that artemisinins, atovaquone and to a  
29 lesser extent chloroquine, generate significant levels of superoxide radicals in *Plasmodium*  
30 *falciparum* sensitive strains. More so, the production of superoxide was lowered in chloroquine-  
31 resistant strain of *Plasmodium* treated with chloroquine. These results consolidate the knowledge  
32 about the mechanism of action of these different antimalarials and should be taken into  
33 consideration in the design of future drugs to fight drug-resistant parasites.

34 **Keywords:** *Plasmodium falciparum*, antimalarials, superoxide radicals, mechanism of action, LC-MS.

## 38 Introduction

39 Malaria is one of the most killer-diseases in the world [1], for which several drugs are in use.  
40 Artemisinins (representative of endoperoxide drugs), quinolines, and hydroxynaphtoquinones are  
41 the three major classes of antimalarials, but their mechanisms of action are still debated. Indeed, the  
42 activation of artemisinins is thought to involve iron-catalyzed reductive homolytic cleavage of the  
43 endoperoxide bridge, generating carbon-centered radicals, an alkylating agent that reacts with  
44 essential biomolecules [2,3]. Hence, the carbon-centered radical formed can alkylate heme produced  
45 during the hemoglobin digestion [4,5], preventing its polymerization into hemozoin, leading to the  
46 accumulation of toxic redox-active iron complexes [6–9].

47 Several mechanisms of action have been proposed for chloroquine (representative of quinoline  
48 drugs): interaction with heme, blocking its detoxification into hemozoin [10], interaction with the  
49 DNA of *Plasmodium* [11], modulation of immune response [12] and/or inhibition of peroxidative  
50 degradation of heme [13].

51 The mechanism of action of atovaquone (representative of hydroxynaphtoquinone drugs) is quite  
52 different and related to the blocking of the mitochondrial electron transport chain at complex III of  
53 the respiratory chain of *Plasmodium*, which leads to parasite death [14]. Atovaquone is a competitive  
54 inhibitor of ubiquinol binding, which specifically inhibits the complex III (cytochrome bc1) of the  
55 electron transport chain of the parasite, leading to the loss of the membrane potential and  
56 consequently to the parasite death [15,16].

57 While the probable production of reactive species has been largely accepted and discussed in  
58 literature for artemisinins (ARTs) [8,17], the specific detection of such radical species has never been  
59 investigated up till date in *Plasmodium*-infected red blood cells (RBCs), even though the production  
60 of hydroxyl radicals by reaction of artemisinin with cancer cells (rich in iron) has been described [18].  
61 Most of the measurements have been carried out using nonspecific fluorescence probes such as  
62 CellRox or dichlorofluorescein (DCF) derivatives [6,8,9,19,20], but the nature of the primary radicals  
63 has never been determined in *Plasmodium*, for any of the antimalarial drugs mentioned above.  
64 Identifying the precise nature of the generated radicals will be useful to understand the mechanism  
65 of action of these drugs, and also the parasite resistance mechanisms, and could serve as a guide in  
66 the design and development of future antimalarials.

67 The aim of the present work is to investigate the involvement of reactive species in the mode of  
68 action of antiplasmodial drugs by killing parasites sensitive to oxidative stress, and to determine for  
69 the first time, the nature of such radicals using a very sensitive technique by LC-MS that we recently  
70 developed [21].

71

## 72 Materials and Methods

### 73 Materials

74 Unless otherwise stated, all reagents and the molecules tested (artemisinin (ART), atovaquone (ATQ)  
75 and chloroquine (CQ)) were supplied by Sigma Aldrich. Dihydroartemisinin (DHA) was synthesized by  
76 chemists from our laboratory. Stock solutions were prepared in DMSO except for CQ (in RPMI or  
77 PBS). The final concentration of each molecule was between 0.5-500 nM while the DMSO control was  
78 0.2 % which was similar to that contained in the solutions of the molecules of interest.

## 79 **Methods**

### 80 **Interaction of antimalarials with iron (II) by electron paramagnetic resonance**

81 Electron paramagnetic resonance (EPR) measurements were carried out using 5,5-dimethyl-1-  
82 pyrroline *N*-oxide (DMPO) as spin trap (stock solution 1 M in water). The analyses were performed on  
83 mixtures containing DMPO (100 mM), the molecule of interest (9 mM in DMSO or PBS) and a Fe(II)  
84 salt (Mohr's salt) (2 mM). The resulting solution was taken into an EPR flat quartz cell and the spectra  
85 immediately recorded. The amount of radicals was calculated from the double integration of the  
86 spectra.

87 EPR spectra were obtained at room temperature using the X-band on a Bruker EMX-8/2.7 (9.86 GHz)  
88 equipped with a gaussmeter (Bruker, Wissembourg, France) and a high-sensitivity cavity (4119/HS  
89 0205). Analyses were performed using a flat quartz cell (FZKI160-5 X 0.3 mm, Magnostech, Berlin,  
90 Germany). WINEPR and SIMFONIA software (Bruker, Wissembourg, France) were used for EPR data  
91 processing. Typical scanning parameters were: scan number, 6; scan rate, 1.2 G/s; modulation  
92 frequency, 100 kHz; modulation amplitude, 1 G; microwave power, 20 mW; sweep width, 100 G;  
93 sweep time, 83.88 s; time constant, 40.96 ms; magnetic field 3460-3560 G.

### 94 **Parasite culture and samples preparation**

95 The FcB1-Colombia (a chloroquine-resistant strain) and F32-Tanzania (a chloroquine-sensitive strain)  
96 were used at the trophozoite stage (24-36 h post-invasion). The parasites were maintained in RPMI  
97 at 5 % human serum and 2 % hematocrit. Parasites were firstly synchronized at the ring stage with 5  
98 % D-sorbitol solution [22]. Non-concentrated (5 % parasitemia) and concentrated cultures (>70 %  
99 parasitemia) were used respectively for the investigation. Indeed, to ensure that superoxide radicals  
100 detected are emanating exclusively from the parasites and not from oxidized red blood cells, the  
101 same analysis was conducted both on non-concentrated and concentrated *Plasmodium* cultures. To  
102 concentrate the parasite-infected erythrocytes, the parasite culture was passed through a magnetic  
103 column where the infected erythrocytes were trapped due to their high hemozoin content, allowing  
104 the uninfected ones to be eluted [23]. The use of the FcB1-Colombia *versus* F32-Tanzania was aimed  
105 at comparing the superoxide generating effect of chloroquine on both chloroquine-resistant and  
106 chloroquine-sensitive strains, respectively.

### 107 **ROS quantification by LC-MS**

108 The production of superoxide radicals ( $O_2^{\cdot-}$ ) after the treatment of *Plasmodium*-infected erythrocytes  
109 was monitored using the liquid chromatography coupled to mass spectrometry (LC-MS) [21] using  
110 dihydroethidium (DHE) as the probe. Precisely 681  $\mu$ L of the sample (parasitized RBCs culture) at a  
111 concentration of 15 million cells/mL in phosphate buffer saline (PBS) was introduced into a 1.5 mL  
112 tube and immediately probed with DHE at 10  $\mu$ M final concentration. Each tube was treated with  
113 either a molecule of interest or a control (i.e. the vehicle used for the corresponding molecule: DMSO  
114 for ART, DHA and ATQ or PBS for CQ) and the volume made up to 1 mL with PBS. The entire setup  
115 was incubated for 30 minutes at 37 °C. The tubes were then centrifuged and the supernatant  
116 removed. The pellet was lysed by treatment with 100  $\mu$ L of a lysing solution (5 mM sodium  
117 phosphate and 1 mM EDTA, pH 8.0) followed by two freezing/thawing cycles. Hundred microliters of  
118 methanol were then added to each tube and the entire setup was centrifuged for 30 minutes at 4 °C  
119 at 14,000 RPM to sediment the cell debris. The supernatant was taken for analysis. The amount of

120  $O_2^{\cdot-}$  produced was deduced from the integration of the peak of the chromatogram (Figure 2C)  
121 corresponding to the 2-hydroxyethidium (2-OH-E<sup>+</sup>,  $m/z$  330), a specific adduct formed under the  
122 reaction of DHE with superoxide radicals, using the Xcalibur software and a calibration curve of the  
123 standard (Figure 2D).

## 124 Results and Discussion

125 As interference with heme metabolism is known to be involved in the mechanism of action of  
126 artemisinins [4,5] and quinolines [10], the ability of major antiparasitic drugs to interact with the  
127 iron (II) to generate radicals was firstly investigated chemically by EPR spectroscopy using DMPO as  
128 spin trap. The corresponding data (Figure 1A) clearly demonstrated that only artemisinin reacts with  
129 Fe(II) to produce radicals while no radicals were generated with CQ or ATQ. The six-lined spectrum  
130 recorded with artemisinin (Figure 1B) is characteristic of the DMPO-CH<sub>3</sub> adduct formed by the  
131 reaction of DMPO with  $\cdot CH_3$  produced when  $\cdot OH$  reacts with DMSO.

132

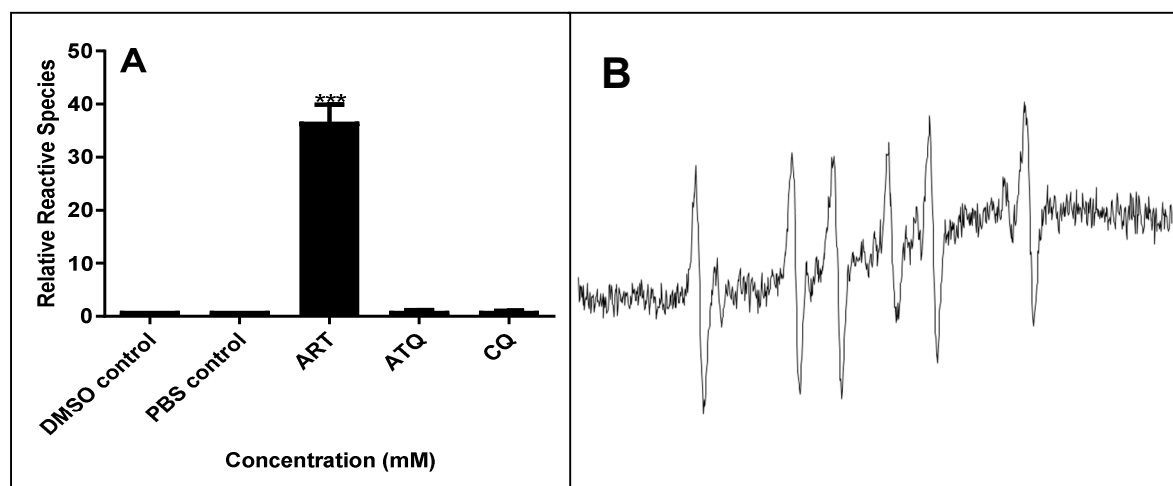
133

134

135

136

137



138 **Figure 1:** (A) Production of hydroxyl radical deduced from the double integration of the EPR spectra  
139 obtained in the presence of antimalarials (9 mM), Fe(II) salt (2 mM) and DMPO as spin trap (100  
140 mM). Only ART interacts with Fe(II) to significantly generate hydroxyl radicals comparatively to the  
141 DMSO-control. The results correspond to the mean of three independent experiments done in  
142 triplicates.\*\*\* = statistically significant ( $p < 0.001$ ). Artemisinin (ART); atovaquone (ATQ); chloroquine  
143 (CQ). (B) EPR spectrum recorded with ART.

144

145 The production of radicals, was then investigated on *Plasmodium falciparum* infected RBCs by  
146 targeting  $O_2^{\cdot-}$ , the first radical formed by one electron reduction of oxygen. The total amounts of  
147 intracellular  $O_2^{\cdot-}$  were determined by quantification of 2-hydroxyethidium (2-OH-E<sup>+</sup>) specifically  
148 formed under reaction of the probe DHE with superoxide ( $m/z$  330) (Figures 2C and 2D). The analysis  
149 was performed after 30 min treatment of RBCs infected by the *P. falciparum* FcB1-Colombia strain  
150 (5% parasitemia) with ART, DHA, ATQ and CQ at 0.5-500 nM (Figure 2A). The tested concentrations  
151 was chosen according to their peak plasma concentration in patients. These results demonstrated  
152 that artemisinins (ART and DHA) and ATQ generate significant amounts of superoxide (from  $1.66 \pm$   
153  $0.10$  for ATQ to  $1.92 \pm 0.11$  fold for ART with the highest concentration tested). By contrast, CQ

154 treatment did not lead to any increase of superoxide in *Plasmodium* cultures, regardless of the drug  
155 concentration used.

156 The uninfected RBCs control should be performed with RBCs from the same batch (same time and  
157 age) like those used for the parasite culture. Therefore the control has to be performed with few  
158 days old uninfected RBCs. However, uninfected RBCs can also produce  $O_2^{\cdot-}$  when treated with  
159 artemisinin [24]. For this reason the same experiment to quantify  $O_2^{\cdot-}$  was then carried out with  
160 concentrated cultures of *Plasmodium* in order to evaluate the amount of radicals specifically  
161 originating from the infected RBCs. The infected RBCs were sorted by magnetic separation and the  
162 analysis was performed after a 500 nM-treatment with ART, ATQ and CQ (Figure 2B). Increasing the  
163 parasitaemia from 5% to 70 % induced a 10-fold boost ( $17 \pm 5$  to  $157 \pm 33$  nM for the DMSO control  
164 and  $28 \pm 9$  to  $299 \pm 67$  nM for ART) in the level of  $O_2^{\cdot-}$  detected even in the absence of treatment  
165 from the drugs under study (supplementary data, [Figure S1](#)), confirming the strong redox activity in  
166 infected RBCs at the steady state. Moreover, by comparing the level of  $O_2^{\cdot-}$  formed with or without  
167 ART (supplementary data, [S1](#)), we estimated that the drug treatment led to a 60-fold higher  
168 production of  $O_2^{\cdot-}$  on infected RBCs than on uninfected erythrocytes. This result confirms that the  
169 increased amount of  $O_2^{\cdot-}$  recorded for ART in Figure 2A is mainly due to the effect of the drug on  
170 infected RBCs. The data obtained with *Plasmodium* cultures at 70 % parasitemia, presented in Figure  
171 2B, confirmed that ART and ATQ, but not CQ, induce a strong relative increase in superoxide in  
172 infected RBCs. Even though the relative increase was only  $2.0 \pm 0.1$  for ART and  $1.9 \pm 0.0$  for ATQ,  
173 that can be explained by the basal redox activity, obtained after infected RBCs sorting, which was 10  
174 fold higher even in the absence of drug treatment (supplementary data, [Figure S1](#)).

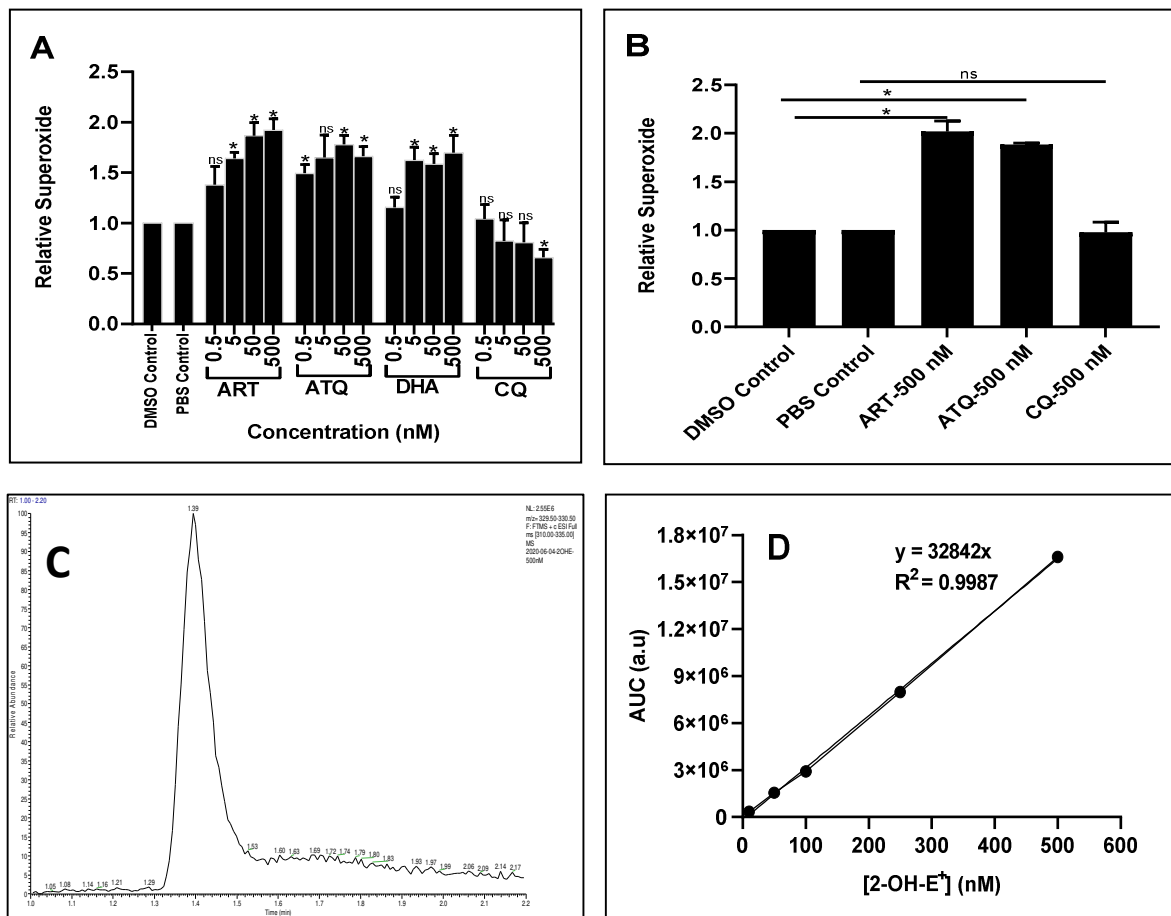
175 CQ is an age-long antimalarial drug whose activity is mediated by interference with heme  
176 metabolism [10,13]. It did not produce significant levels of  $O_2^{\cdot-}$  on the FcB1-Colombia strain in both  
177 conditions tested (Figure 2A and 2B). The lack of significant  $O_2^{\cdot-}$  production from CQ on FcB1 could be  
178 as a result of its lesser efficacy against this chloroquine-resistant strain as compared to the  
179 chloroquine-sensitive counterparts [25]. Indeed, in chloroquine-resistant strains, CQ is constantly  
180 effluxed from the food vacuole, the site of action of CQ [26], limiting its interaction with heme, which  
181 consequently leads to heme polymerization and parasite survival. This action could limit the  
182 availability of free heme, and consequently limiting the  $O_2^{\cdot-}$  generation. To confirm this hypothesis,  
183 the same analysis was carried out on a chloroquine-sensitive strain, F32-Tanzania (Figure 3). The  
184 results demonstrated that a significant amount of  $O_2^{\cdot-}$  ( $1.7 \pm 0.1$  fold of the PBS control) was  
185 produced after 30 min of CQ treatment on F32-Tanzania, confirming the reduced effect of CQ on the  
186 chloroquine-resistant strain reported in Figure 2. These data confirm that  $O_2^{\cdot-}$  detected herein are  
187 definitely linked with the antiplasmodial action of the tested molecules. However, the amount of  $O_2^{\cdot-}$   
188 was significantly lower than that observed with ART ( $1.7 \pm 0.1$  vs  $2.7 \pm 0.3$  folds) in the same  
189 conditions (Figure 3). Even though both drugs, CQ and ART, act, among other pathways, by inhibiting  
190 the heme polymerisation, their pharmacokinetics and pharmacodynamics are quite different [4–  
191 6,10,13,17] which consequently leads to varying production of superoxide.

192

193

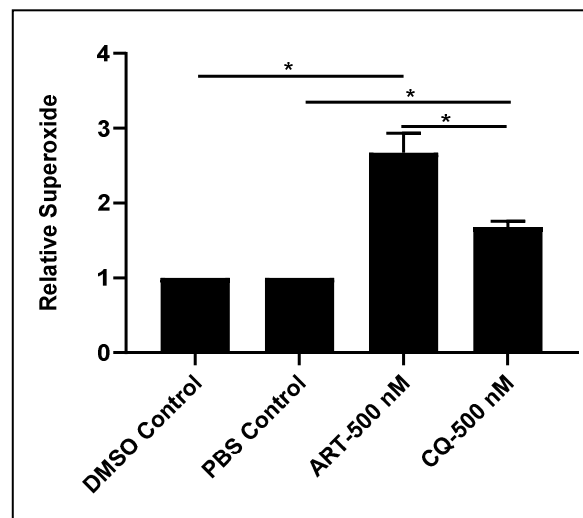
194

195  
 196  
 197  
 198  
 199  
 200  
 201  
 202  
 203  
 204  
 205  
 206  
 207  
 208  
 209  
 210  
 211  
 212  
 213  
 214  
 215  
 216  
 217  
 218  
 219  
 220  
 221  
 222  
 223  
 224  
 225



**Figure 2:** Quantification of superoxide radicals performed on the CQ-resistant strain, FcB1-Colombia in (A) non concentrated *Plasmodium* infected erythrocytes treated with increasing doses (0.5-500 nM) of artemisinin (ART), atovaquone (ATQ), dihydroartemisinin (DHA) and chloroquine (CQ), and (B) concentrated *Plasmodium* infected erythrocytes treated with ART, ATQ, and CQ at 500 nM. Artemisinins (ART and DHA) and ATQ generated significant amount of superoxide radicals ( $p < 0.05$ ) compared to the control (DMSO for ART, DHA and ATQ; PBS for CQ) unlike chloroquine. (C) Chromatogram of 2-OH-E<sup>+</sup> (D) Calibration curve of 2-OH-E<sup>+</sup>. The results correspond to the mean of six and three independent experiments done in triplicates for Figure 2A and Figure 2B, respectively. The level of significance was taken with reference to the control for each molecule. \*= statistically significant ( $p < 0.05$ ), ns=not significant.

226  
227  
228  
229  
230  
231  
232  
233  
234



235 **Figure 3:** Quantification of superoxide radicals on a non-concentrated culture of the CQ-sensitive *P.*  
236 *falciparum* strain, F32-Tanzania treated by ART and CQ at 500 nM. Both ART and CQ produced  
237 significant ( $p < 0.05$ ) levels of superoxide radicals when compared to their controls (DMSO for ART  
238 and PBS for CQ). Moreover, the level of superoxide was significantly ( $p < 0.05$ ) higher from ART than  
239 CQ. The results correspond to the mean of three independent experiments done in triplicates.\* =  
240 statistically significant ( $p < 0.05$ ).

241 The generation of reactive species under artemisinin treatment has been largely reported as their  
242 main mechanism of action, *via* an interruption of heme metabolism [4,8,30–33]. The definitive  
243 identification of these reactive species remained a subject of debate, since most of the quantification  
244 of these reactive species in *Plasmodium* had been carried out by non-specific approaches using DCF  
245 or CellRox fluorescence [6,8,19,20]. Therefore the present work demonstrates, for the first time, that  
246 treatment of *Plasmodium* by artemisinins leads to superoxide generation. Superoxide radicals from  
247 ART and DHA (the active metabolites of all artemisinin derivatives currently used) could be as a result  
248 of: i) re-oxidation of the unpolymerized heme and consequent electron transfer to molecular oxygen  
249 generating the superoxide radical-anion [34] and/or ii) alkylation of essential biomolecules which can  
250 lead to cellular dysfunction such as mitochondrial dysfunction through the depolarization of the  
251 mitochondria membrane [6,8,17], mitochondrial dysfunction being often associated with an  
252 increased reactive oxygen species production [29].

253 Concerning atovaquone, the production of ROS under ATQ treatment has already been described in  
254 cancer cells [35] but not in *Plasmodium*. The mode of action of ATQ is based on the inhibition of  
255 mitochondrial cytochrome *bc1* complex *via* competitive inhibition of ubiquinol binding [15,36]. As a  
256 consequence, ATQ induces the collapse of the mitochondrial membrane potential and blocks the  
257 energy supply of the parasites [16,37]. Moreover, as the main metabolic function of cytochrome *bc1*  
258 activity in *P. falciparum* appears to involve the regeneration of ubiquinone from ubiquinol, the  
259 inhibition of this site might lead to the ubiquinol accumulation, which can undergo oxidation to  
260 generate significant amount of superoxide radicals.

261



## 262 **Conclusion**

263 Superoxide radical was analytically and specifically detected here for the first time, as a major  
264 reactive species produced by artemisinins, atovaquone, and at a lower level by chloroquine, in their  
265 antiparasitic action. This radical is rapidly transformed chemically or enzymatically (by superoxide  
266 dismutase) into hydrogen peroxide that reacts with iron(II) to form hydroxyl radical  $\cdot\text{OH}$  (Fenton  
267 reaction). This very reactive species having high oxidation potential reacts rapidly (lifespan  
268 nanoseconds) with essential biomolecules (DNA, protein, lipids...) leading to the death of the  
269 parasites which makes its intracellular detection extremely difficult.

270 Our findings clarify the specifics and identities of the reactive species, i.e. superoxide radical  
271 produced by artemisinins and also categorize atovaquone as one of the antimalarials that acts *via*  
272 intermediary reactive species generation. This study will help further in the understanding of the  
273 mechanism of action of these molecules and help in the development of antimalarials, to fight drug-  
274 resistance.

275

## 276 **Declaration of competing Interest**

277 None. The study design, data collection, analysis and interpretation, report writing, and the choice of  
278 publishing this work are solely the responsibility of the authors.

279

## 280 **Acknowledgements**

281 This work was supported through a PhD fellowship program to ECO from Alex-Ekwueme Federal  
282 University, Ndufu-Alike, Ikwo (AE-FUNAI) and Campus France. This study was also supported in part  
283 by the French "Agence Nationale de la Recherche" (ANR grant INMAR ANR16 CE35 0003), the CNRS  
284 (Centre National de la Recherche Scientifique, France), the IRD (Institut de Recherche pour le  
285 Développement, France) and the University Toulouse III.

286

## 287 **References**

- 288 [1] WHO, World Malaria Report 2020, (2020). [https://www.who.int/teams/global-malaria-](https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2020)  
289 [programme/reports/world-malaria-report-2020](https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2020) (accessed November 30, 2020).
- 290 [2] S.R. Meshnick, A. Thomas, A. Ranz, C.M. Xu, H.Z. Pan, Artemisinin (qinghaosu): the role of  
291 intracellular heme in its mechanism of antimalarial action, *Mol. Biochem. Parasitol.* 49 (1991)  
292 181–189. [https://doi.org/10.1016/0166-6851\(91\)90062-B](https://doi.org/10.1016/0166-6851(91)90062-B).
- 293 [3] N. Klonis, D.J. Creek, L. Tilley, Iron and heme metabolism in *Plasmodium falciparum* and the  
294 mechanism of action of artemisinins, *Curr. Opin. Microbiol.* 16 (2013) 722–727.  
295 <https://doi.org/10.1016/j.mib.2013.07.005>.
- 296 [4] A. Robert, F. Benoit-Vical, C. Claparols, B. Meunier, The antimalarial drug artemisinin alkylates  
297 heme in infected mice, *Proc. Natl. Acad. Sci. U. S. A.* 102 (2005) 13676–13680.  
298 <https://doi.org/10.1073/pnas.0500972102>.
- 299 [5] A. Robert, Y. Coppel, B. Meunier, Alkylation of heme by the antimalarial drug artemisinin,  
300 *Chem. Commun.* (2002) 414–415. <https://doi.org/10.1039/b110817b>.
- 301 [6] J. Wang, L. Huang, J. Li, Q. Fan, Y. Long, Y. Li, B. Zhou, Artemisinin directly targets malarial

- 302 mitochondria through its specific mitochondrial activation, *PLoS One*. 5 (2010) 1–12.  
 303 <https://doi.org/10.1371/journal.pone.0009582>.
- 304 [7] J. Wang, C. Xu, Z.R. Lun, S.R. Meshnick, Unpacking ‘Artemisinin Resistance,’ *Trends Pharmacol.*  
 305 *Sci.* 38 (2017) 506–511. <https://doi.org/10.1016/j.tips.2017.03.007>.
- 306 [8] A.M. Gopalakrishnan, N. Kumar, Antimalarial action of artesunate involves DNA damage  
 307 mediated by reactive oxygen species, *Antimicrob. Agents Chemother.* 59 (2015) 317–325.  
 308 <https://doi.org/10.1128/AAC.03663-14>.
- 309 [9] S. Gunjan, T. Sharma, K. Yadav, B.S. Chauhan, S.K. Singh, M.I. Siddiqi, R. Tripathi, Artemisinin  
 310 Derivatives and Synthetic Trioxane Trigger Apoptotic Cell Death in Asexual Stages of  
 311 *Plasmodium*, *Front. Cell. Infect. Microbiol.* 8 (2018) 256.  
 312 <https://doi.org/10.3389/fcimb.2018.00256>.
- 313 [10] D.J. Sullivan, H. Matile, R.G. Ridley, D.E. Goldberg, A common mechanism for blockade of  
 314 heme polymerization by antimalarial quinolines, *J. Biol. Chem.* 273 (1998) 31103–31107.  
 315 <https://doi.org/10.1074/jbc.273.47.31103>.
- 316 [11] F. Kwakye-Berko, S.R. Meshnick, Binding of chloroquine to DNA, *Mol. Biochem. Parasitol.* 35  
 317 (1989) 51–55. [https://doi.org/10.1016/0166-6851\(89\)90141-2](https://doi.org/10.1016/0166-6851(89)90141-2).
- 318 [12] A. Ramos-Avila, J.L. Ventura-Gallegos, A. Zentella-Dehesa, C. Machuca-Rodríguez, M.M.  
 319 Moreno-Altamirano, V. Narváez, M. Legorreta-Herrera, Immunomodulatory role of  
 320 chloroquine and pyrimethamine in *Plasmodium yoelii* 17XL infected mice, *Scand. J. Immunol.*  
 321 65 (2007) 54–62. <https://doi.org/10.1111/j.1365-3083.2006.01869.x>.
- 322 [13] P. Loria, S. Miller, M. Foley, L. Tilley, Inhibition of the peroxidative degradation of haem as the  
 323 basis of action of chloroquine and other quinoline antimalarials, *Biochem. J.* 339 (1999) 363–  
 324 370. <https://doi.org/10.1042/0264-6021:3390363>.
- 325 [14] A.C. Chou, C.D. Fitch, Control of heme polymerase by chloroquine and other quinoline  
 326 derivatives, *Biochem. Biophys. Res. Commun.* 195 (1993) 422–427.  
 327 <https://doi.org/10.1006/bbrc.1993.2060>.
- 328 [15] M. Fry, M. Pudney, Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4'-  
 329 chlorophenyl) cyclohexyl]-3- hydroxy-1,4-naphthoquinone (566C80), *Biochem. Pharmacol.* 43  
 330 (1992) 1545–1553. [https://doi.org/10.1016/0006-2952\(92\)90213-3](https://doi.org/10.1016/0006-2952(92)90213-3).
- 331 [16] I.K. Srivastava, H. Rottenberg, A.B. Vaidya, Atovaquone, a broad spectrum antiparasitic drug,  
 332 collapses mitochondrial membrane potential in a malarial parasite, *J. Biol. Chem.* 272 (1997)  
 333 3961–3966. <https://doi.org/10.1074/jbc.272.7.3961>.
- 334 [17] T. Antoine, N. Fisher, R. Amewu, P.M. O’Neill, S.A. Ward, G.A. Biagini, Rapid kill of malaria  
 335 parasites by artemisinin and semi-synthetic endoperoxides involves ROS-dependent  
 336 depolarization of the membrane potential, *J. Antimicrob. Chemother.* 69 (2014) 1005–1016.  
 337 <https://doi.org/10.1093/jac/dkt486>.
- 338 [18] K. Tsuda, L. Miyamoto, S. Hamano, Y. Morimoto, Y. Kangawa, C. Fukue, Y. Kagawa, Y.  
 339 Horinouchi, W. Xu, Y. Ikeda, T. Tamaki, K. Tsuchiya, Mechanisms of the pH- And oxygen-  
 340 dependent oxidation activities of artesunate, *Biol. Pharm. Bull.* 41 (2018) 555–563.  
 341 <https://doi.org/10.1248/bpb.b17-00855>.
- 342 [19] N. Klonis, M.P. Crespo-Ortiz, I. Bottova, N. Abu-Bakar, S. Kenny, P.J. Rosenthal, L. Tilley,  
 343 Artemisinin activity against *Plasmodium falciparum* requires hemoglobin uptake and  
 344 digestion, *Proc. Natl. Acad. Sci. U. S. A.* 108 (2011) 11405–11410.  
 345 <https://doi.org/10.1073/pnas.1104063108>.
- 346 [20] R.A.C. Sussmann, W.L. Fotoran, E.A. Kimura, A.M. Katzin, *Plasmodium falciparum* uses vitamin

- 347 e to avoid oxidative stress, *Parasites and Vectors*. 10 (2017) 461.  
 348 <https://doi.org/10.1186/s13071-017-2402-3>.
- 349 [21] I. Tsamesidis, C.O. Egwu, P. Pério, J.M. Augereau, F. Benoit-Vical, K. Reybier, An LC–MS assay  
 350 to measure superoxide radicals and hydrogen peroxide in the blood system, *Metabolites*. 10  
 351 (2020) 175. <https://doi.org/10.3390/metabo10050175>.
- 352 [22] C. Lambros, J.P. Vanderberg, Synchronization of *Plasmodium falciparum* Erythrocytic Stages in  
 353 Culture, *J. Parasitol.* 65 (1979) 418–420. <https://doi.org/10.2307/3280287>.
- 354 [23] C. Ribaut, A. Berry, S. Chevalley, K. Reybier, I. Morlais, D. Parzy, F. Nepveu, F. Benoit-Vical, A.  
 355 Valentin, Concentration and purification by magnetic separation of the erythrocytic stages of  
 356 all human *Plasmodium* species, *Malar. J.* 7 (2008) 45. <https://doi.org/10.1186/1475-2875-7-45>.
- 357
- 358 [24] I. Tsamesidis, P. Pério, A. Pantaleo, K. Reybier, Oxidation of erythrocytes enhance the  
 359 production of reactive species in the presence of artemisinins, *Int. J. Mol. Sci.* (2020).  
 360 <https://doi.org/10.3390/ijms21134799>.
- 361 [25] F. Benoit-Vical, J. Lelièvre, A. Berry, C. Deymier, O. Dechy-Cabaret, J. Cazelles, C. Loup, A.  
 362 Robert, J.F. Magnaval, B. Meunier, Trioxaquines are new antimalarial agents active on all  
 363 erythrocytic forms, including gametocytes, *Antimicrob. Agents Chemother.* 51 (2007) 1463–  
 364 1472. <https://doi.org/10.1128/AAC.00967-06>.
- 365 [26] R.E. Martin, R. V. Marchetti, A.I. Cowan, S.M. Howitt, S. Bröer, K. Kirk, Chloroquine transport  
 366 via the malaria parasite’s chloroquine resistance transporter, *Science* (80-. ). 325 (2009) 1680–  
 367 1682. <https://doi.org/10.1126/science.1175667>.
- 368 [27] A. Santo, H. Zhu, Y.R. Li, Free Radicals: From Health to Disease, *React. Oxyg. Species*. 2 (2016)  
 369 245–263. <https://doi.org/10.20455/ros.2016.847>.
- 370 [28] S. Müller, Redox and antioxidant systems of the malaria parasite *Plasmodium falciparum*, *Mol.*  
 371 *Microbiol.* 53 (2004) 1291–1305. <https://doi.org/10.1111/j.1365-2958.2004.04257.x>.
- 372 [29] M.P. Murphy, Mitochondrial dysfunction indirectly elevates ROS production by the  
 373 endoplasmic reticulum, *Cell Metab.* 18 (2013) 145–146.  
 374 <https://doi.org/10.1016/j.cmet.2013.07.006>.
- 375 [30] S.R. Meshnick, Y.Z. Yang, V. Lima, F. Kuypers, S. Kamchonwongpaisan, Y. Yuthavong, Iron-  
 376 dependent free radical generation from the antimalarial agent artemisinin (qinghaosu),  
 377 *Antimicrob. Agents Chemother.* 37 (1993) 1108–1114.  
 378 <https://doi.org/10.1128/AAC.37.5.1108>.
- 379 [31] S. Kamchonwongpaisan, S.R. Meshnick, The mode of action of the antimalarial artemisinin  
 380 and its derivatives, *Gen. Pharmacol.* 27 (1996) 587–592. [https://doi.org/10.1016/0306-3623\(95\)02047-0](https://doi.org/10.1016/0306-3623(95)02047-0).
- 381
- 382 [32] R.K. Haynes, S.C. Vonwiller, The behaviour of qinghaosu (artemisinin) in the presence of non-  
 383 heme iron(II) and (III), *Tetrahedron Lett.* 37 (1996) 257–260. [https://doi.org/10.1016/0040-4039\(95\)02142-6](https://doi.org/10.1016/0040-4039(95)02142-6).
- 384
- 385 [33] R.K. Haynes, S.C. Vonwiller, The behaviour of qinghaosu (artemisinin) in the presence of heme  
 386 iron(II) and (III), *Tetrahedron Lett.* 37 (1996) 253–256. [https://doi.org/10.1016/0040-4039\(95\)02141-8](https://doi.org/10.1016/0040-4039(95)02141-8).
- 387
- 388 [34] C. Bonaventura, R. Hensens, A.I. Alayash, S. Banerjee, A.L. Crumbliss, Molecular controls of  
 389 the oxygenation and redox reactions of hemoglobin, *Antioxidants Redox Signal.* 18 (2013)  
 390 2298–2313. <https://doi.org/10.1089/ars.2012.4947>.
- 391 [35] M. Fiorillo, R. Lamb, H.B. Tanowitz, L. Mutti, M. Krstic-Demonacos, A.R. Cappello, U.E.

392 Martinez-Outschoorn, F. Sotgia, M.P. Lisanti, Repurposing atovaquone: Targeting  
393 mitochondrial complex III and OXPHOS to eradicate cancer stem cells, *Oncotarget*. 7 (2016)  
394 34084–34099. <https://doi.org/10.18632/oncotarget.9122>.  
395 [36] D. Birth, W.C. Kao, C. Hunte, Structural analysis of atovaquone-inhibited cytochrome bc 1  
396 complex reveals the molecular basis of antimalarial drug action, *Nat. Commun.* 5 (2014).  
397 <https://doi.org/10.1038/ncomms5029>.  
398 [37] V. Barton, N. Fisher, G.A. Biagini, S.A. Ward, P.M. O’Neill, Inhibiting Plasmodium cytochrome  
399 bc1: A complex issue, *Curr. Opin. Chem. Biol.* 14 (2010) 440–446.  
400 <https://doi.org/10.1016/j.cbpa.2010.05.005>.  
401  
402  
403

