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

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Abstract

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

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

Introduction

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

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

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

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

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

Materials and Methods

Materials

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

Methods

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

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

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

Parasite culture and samples preparation

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

ROS quantification by LC-MS

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

O₂^{•-} produced was deduced from the integration of the peak of the chromatogram (Figure 2C) corresponding to the 2-hydroxyethidium (2-OH-E⁺, *m/z* 330), a specific adduct formed under the reaction of DHE with superoxide radicals, using the Xcalibur software and a calibration curve of the standard (Figure 2D).

Results and Discussion

As interference with heme metabolism is known to be involved in the mechanism of action of artemisinins [4,5] and quinolines [10], the ability of major antiparasitic drugs to interact with the iron (II) to generate radicals was firstly investigated chemically by EPR spectroscopy using DMPO as spin trap. The corresponding data (Figure 1A) clearly demonstrated that only artemisinin reacts with Fe(II) to produce radicals while no radicals were generated with CQ or ATQ. The six-lined spectrum recorded with artemisinin (Figure 1B) is characteristic of the DMPO-CH₃ adduct formed by the reaction of DMPO with [•]CH₃ produced when [•]OH reacts with DMSO.

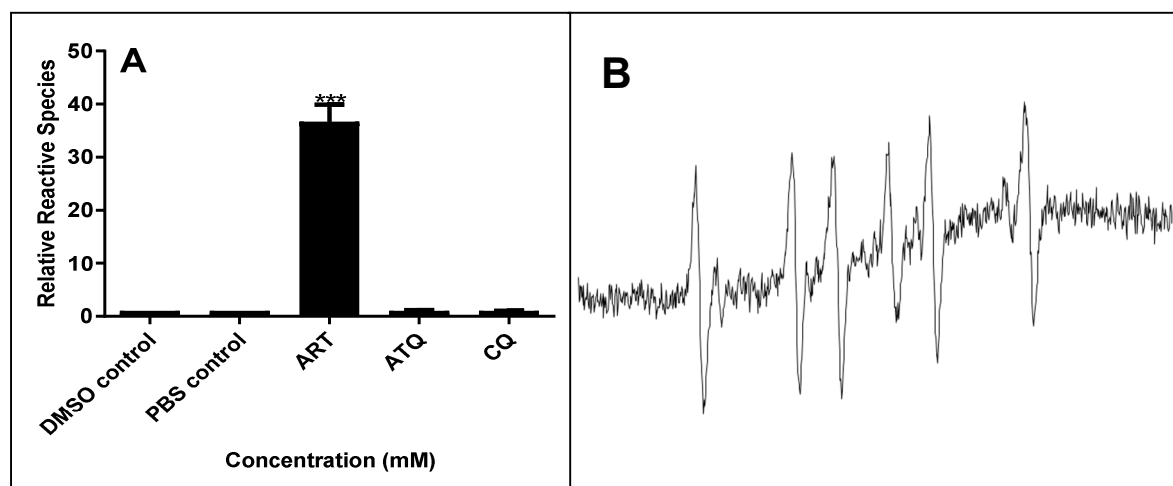


Figure 1: (A) Production of hydroxyl radical deduced from the double integration of the EPR spectra obtained in the presence of antimalarials (9 mM), Fe(II) salt (2 mM) and DMPO as spin trap (100 mM). Only ART interacts with Fe(II) to significantly generate hydroxyl radicals comparatively to the DMSO-control.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

The production of radicals, was then investigated on *Plasmodium falciparum* infected RBCs by targeting O₂^{•-}, the first radical formed by one electron reduction of oxygen. The total amounts of intracellular O₂^{•-} were determined by quantification of 2-hydroxyethidium (2-OH-E⁺) specifically formed under reaction of the probe DHE with superoxide (*m/z* 330) (Figures 2C and 2D). The analysis was performed after 30 min treatment of RBCs infected by the *P. falciparum* FcB1-Colombia strain (5% parasitemia) with ART, DHA, ATQ and CQ at 0.5-500 nM (Figure 2A). The tested concentrations was chosen according to their peak plasma concentration in patients. These results demonstrated that artemisinins (ART and DHA) and ATQ generate significant amounts of superoxide (from 1.66 ± 0.10 for ATQ to 1.92 ± 0.11 fold for ART with the highest concentration tested). By contrast, CQ

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

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

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

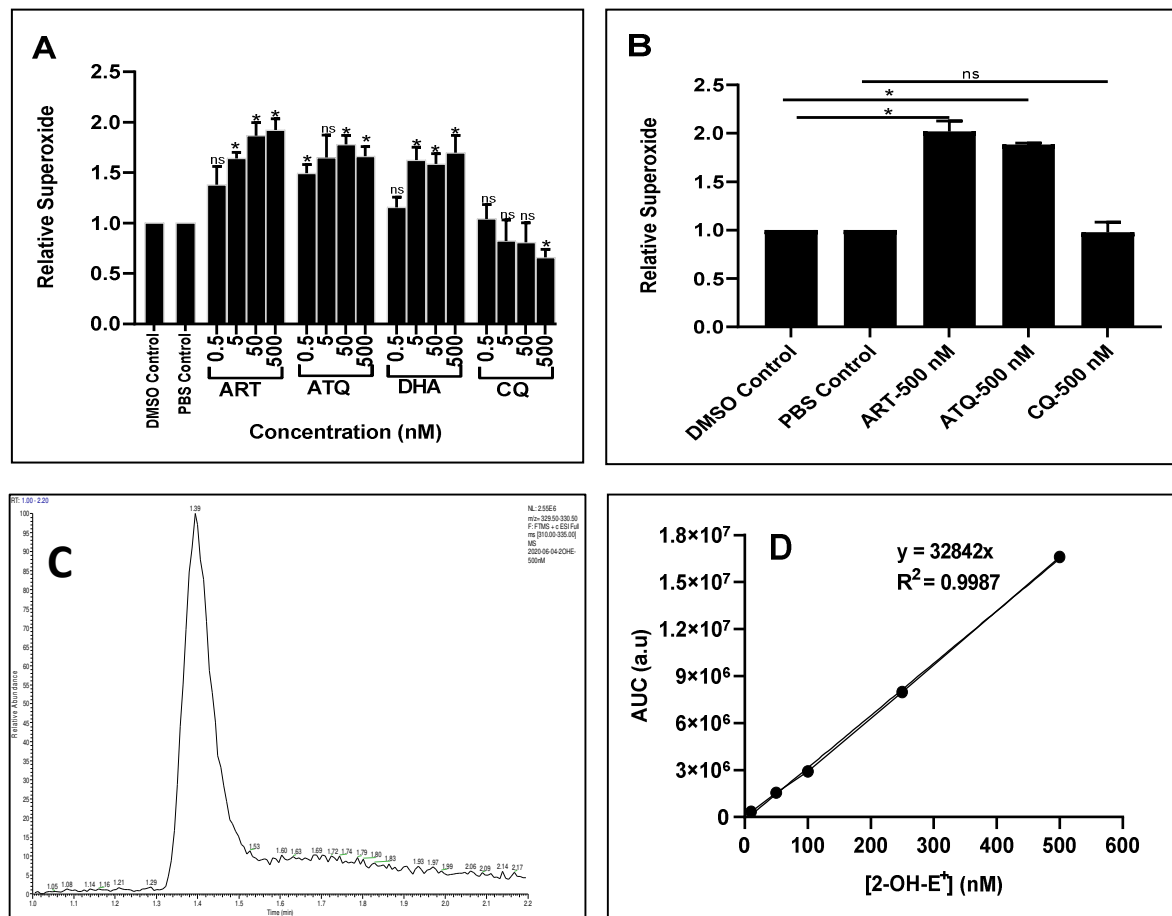


Figure 2: Quantification of superoxide radicals performed on the CQ-resistant strain, FcB1-Colombia in (A) non concentrated *Plasmodium* infected erythrocytes treated with increasing doses (0.5-500 nM) of artemisinin (ART), atovaquone (ATQ), dihydroartemisinin (DHA) and chloroquine (CQ), and (B) concentrated *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⁺ (D) Calibration curve of 2-OH-E⁺. 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.

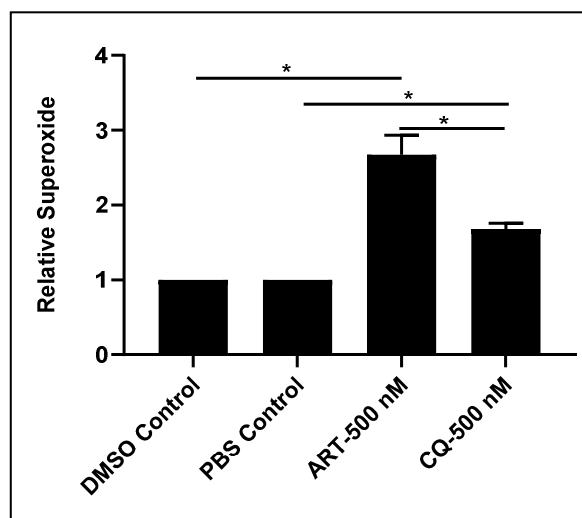


Figure 3: Quantification of superoxide radicals on a non-concentrated culture of the CQ-sensitive *P. falciparum* strain, F32-Tanzania treated by ART and CQ at 500 nM. Both ART and CQ produced significant ($p < 0.05$) levels of superoxide radicals when compared to their controls (DMSO for ART and PBS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

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

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

Conclusion

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

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

Declaration of competing Interest

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

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