Resistance to artemisinin in falciparum malaria parasites: A redox-mediated phenomenon

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A R T I C L E   I N F O

A B S T R A C T

Malaria remains a major public health disease due to its high yearly mortality and morbidity. Resistance to the gold standard drug, artemisinin, is worrisome and needs better understanding in order to be overcome. In this work, we sought to study whether redox processes are involved in artemisinin resistance. As artemisinin is known to act among others *via* production of reactive species, we first compared the production of reactive oxygen species and concomitant protein oxidation in artemisinin-sensitive and artemisinin-resistant parasites when treated with artemisinin. The results undoubtedly demonstrated, using different original methods, that the level of ROS, including superoxide production, and oxidized protein were lower in the resistant strain. Interestingly, the major in-between strain difference was reported at the earlier ring stages, which are the forms able to enter in a quiescence state according to the ART resistance phenomenon. Moreover, we demonstrated a better homeostasis regulation in relation with higher expression of antioxidants in the artemisinin-resistant parasites than their sensitive counterparts after artemisinin exposure, notably, superoxide dismutase and the glutathione (GSH) system. These findings enrich the body of knowledges about the multifaceted mechanism of artemisinin resistance and will help in the design and development of newer antimalarials strategies active against resistant parasites.

1. Introduction

Malaria has been an age-long epidemic that has been ravaging the world. Instead of a strong mortality decrease since 2000, recent reports show that malaria control has stalled in the last 5 years [1]. Among approaches deployed to curb this dramatic mortality, the antimalarials, artemisinin (ART) and its derivatives remain the most potent and fastest acting antimalarial characterized by rapid clearance of parasitemia and resolution of malaria symptoms. Regrettably, parasite resistance development to these endoperoxides emerged in Southeast Asia and threatens the rest of the world [1]. Many worrying signs of ART-resistance emergence have been reported in Africa, in relation or not with the *pfk13* gene polymorphism, the genetic marker of ART-resistance [2–5].

This raises concern over the future of global malaria control with artemisinins (ARTs), as Africa carries the greatest burden of 94% [1]. The artemisinin resistance is based on the ability of the parasite to enter into a quiescent state corresponding to a non-proliferative phase with a strongly reduced metabolism [6]. The parasite can resume its proliferation once the treatment is removed. This leads to a decrease of the parasite clearance and an overall treatment failure. Although the mechanism of ARTs-resistance through the *pfk13* gene is not fully understood, reports show that the *pfk13* polymorphism is associated with reduced hemoglobin endocytosis [7], acceleration of the unfolded protein response (UPR) pathways [8], improved ability to manage oxidative damages [9], changed DNA replication [10], increased basal level of phosphorylated elf2α [11] and/or increase in levels of

phosphatidylinositol-3-phosphoate [12].

The mechanism of action of ARTs is known to involve iron-catalyzed reductive homolytic cleavage of the endoperoxide bridge, generating an alkylating carbon-centered radicals that react with heme giving rise to the production of reactive oxygen species (ROS) [13] such as superoxide radicals [14], and with essential biomolecules, leading to the parasite death [15–17].

Therefore, this work seeks to understand whether redox processes could also be involved in *Plasmodium falciparum* resistance to artemisinins. We aim to compare redox markers, such as ROS production, protein oxidation, redox homeostasis and expression of antioxidants, in artemisinin-sensitive and artemisinin-resistant strains when treated with artemisinin. The response of both strains to different pro-oxidant molecules and antioxidant inhibitors are also compared.

2. Materials and methods

Unless mentioned, all reagents used were supplied by Sigma Aldrich. Artemisinin stock solution was made in DMSO (35.4 mM) and was diluted accordingly in the working solution of each experiment (PBS or RPMI). The doses used for artemisinin treatment in the different experiments were based on the clinically relevant values corresponding to the plasma peak in patients (1.5–3 µM). The percentage DMSO in the control ranged from 0.0085 to 1% just like in the ART treated groups.

2.1. Cell culture

Plasmodium falciparum cultures used in this study were an artemisinin-sensitive strain (F32-TEM) and its twin artemisinin-resistant strain (F32-ART5 called here, F32-ART), obtained by sequencing drug pressure cycles of increasing artemisinin concentrations from 2005 to 2014 and then regularly treated by 18 µM ART diluted with RPMI (for 48 h) to maintain ART resistance (phenotype and genotype of both strains being regularly checked) [2,18,19]. The cultures were maintained in RPMI (Fisher Scientific, France) with 5% type AB human serum and at 2% hematocrit [20,21]. The parasite growth was monitored daily by Giemsa staining and microscopy. For each experiment, a synchrony of each strain was obtained by D-sorbitol treatment [22] at the ring stage (0–24 h) and/or magnetic separation at the trophozoite stage [23]. To obtain 0–3 h old ring stages, the percoll-based approach was adopted [24].

2.2. Reactive oxygen species quantification

Firstly, overall ROS in both strains were measured by dichlorofluorescein (DCF) fluorescence [25] and secondly, the type of ROS (hydrogen peroxide (H_2O_2) and superoxide anion radical ($O_2^{\bullet-}$)), by liquid chromatography coupled to mass spectrometry (LC-MS), a new protocol that we developed [26]. Both the rings (0–24 h) and trophozoites (24–36 h) were subjected to ART treatment for 30–60 min under appropriate probes prior to quantification.

By DCF fluorescence, 100 µL of the parasite culture, at 10% parasitemia and 0.2% hematocrit, were prepared and introduced into a 96-well black plate. The *Plasmodium*-infected red blood cells (iRBCs) were probed with dichlorofluorescein diacetate (H_2DCFDA) (20 µM), then treated with various concentrations of ART or of DMSO (at the same concentration used for ART solution (0.5%)) as control. The fluorescence was read after 1 h of incubation to measure H_2DCFDA oxidation to the highly fluorescent DCF. The fluorescence of the plates (493 nm/522 nm) was taken on a Xenius® fluorimeter from SAFAS (Monaco, driven by SP2000 software version 7.4.13.0).

For the LC-MS approach, 15–20 million iRBCs were introduced into 1.5 mL tubes, probed with 10 µM each with either dihydroethidium (DHE) or coumarin boronic acid (CBA) to quantify superoxide radicals and hydrogen peroxide, respectively. The iRBCs were then treated with several concentrations of ART and incubated for 30 min. The drugs were

washed off and the cells lysed in order to get the intracellular products formed under reaction of the targeted reactive species with the probe, 2-hydroxyethidium (2-OHE$^+$) and 7-hydroxycoumarin (COH) for superoxide and hydrogen peroxide, respectively. The lysis was performed by treatment of the pellet with 100 µL of a lysing solution (5 mM Na_2HPO_4 and 1 mM EDTA (ethylenediaminetetraacetic acid), pH 8.0) which was immediately followed by two freezing/thawing cycles at −80 °C and 37 °C respectively. Methanol (100 µL) was added to the lysate and the entire set-up centrifuged for 30 min at 4 °C at 14,000 RPM to sediment the cell debris. The resulting supernatant which contains the adducts, 2-OHE$^+$ for superoxide and COH for hydrogen peroxide were taken for quantification by LC-MS. The resulting peaks were integrated using Xcalibur software and the corresponding concentrations of the reactive species calculated from the calibration curve of the standards (0–500 nM). LOD (limit of detection) values were 7 nM for superoxide and 11 nM for hydrogen peroxide.

The results are presented in relative units because the measured quantity of superoxide varies from an experiment to another depending on the parasitemia, the age of the parasites (and thus quantity of hemoglobin), the age of the red blood cells and the batch of blood, making not relevant the direct comparison of values. As an example, the measured values of superoxide, for F32-ART, ranged from 65 nM (for the DMSO control) to 119 nM (for ART treatment at 300 nM).

2.3. Antiplasmodial activity evaluation

2.3.1. Chemosensitivity assay

The SYBR Green method [27] was used for the determination of the IC_{50} values of different molecules and ART was used as reference molecule. The ring stages of F32-TEM and F32-ART strains, at 1% parasitemia, were treated with varying concentrations of the different molecules of interest and incubated for 48 h at 37 °C. The molecules were then washed off, the infected RBCs lysed and probed with SYBR Green. The fluorescence of the plates was taken on BioTek FLx800 Microplate Fluorescence Reader (485 nm/528 nm) and the IC_{50} values were determined using GraphPad Prism software.

2.3.2. Recrudescence assay

This method was used to differentiate the effect of the molecules tested on the artemisinin-sensitive and -resistant strains in order to evaluate their sensitivity to pro-oxidant molecules [19]. Each strain at 3% parasitemia at the ring stage (0–24 h) was subjected to high concentrations of each molecule for 48 h, using ART (18 µM) as the positive control. For each molecule, the concentrations were determined after a preliminary screening, which allowed for differentiation in phenotypic response from F32-TEM and F32-ART (usually a concentration between 1 and 1000 folds of the IC_{50} of each molecule) [28]. The drugs were then washed off and the pellet put back in drug-free medium. The parasite kinetics was followed for 30 days to monitor the parasite recrudescence. The ability of each strain to recrudesce was measured by the number of days (T_n) to reach initial parasitemia of 3%. The T_n was censored to 30 days if there was no recrudescence during the 30 days-monitoring.

2.4. Protein oxidation and cellular redox homeostasis

The impact of ART on artemisinin-sensitive and -resistant strains was investigated by measuring the levels of protein carbonyls (biomarker of protein oxidation) and redox homeostasis dysregulation after treatment.

The dinitrophenyhydrazine (DNPH) derivatization method was used to determine the protein oxidation [29]. Parasite cultures were prepared at 2% hematocrit and 5% parasitemia each for F32-TEM and F32-ART. They were treated with 3 µM ART and incubated for 6 h. The drugs were washed off and the cells were lysed by osmotic shock by adding 100 µL of a lysing solution (contains final concentration of 2.42 mg/mL Tris Base, 1.86 mg/mL EDTA, 0.08 mg/mL saponin, 0.8 µL/mL Triton X-100 in distilled water and pH 7.5). Equal volumes of the lysate and 10

mM DNPH prepared in 0.5 M H_3PO_4 were introduced into a new 96-well plate using lysate and 0.5 M H_3PO_4 mixture as the blank. Then 50 μL of 6 M NaOH were added to the sample to shift the absorbance of sample derivative (hydrazine) from 370 to 450 nm [29]. The absorbance was read at 450 nm against the blank.

The cellular homeostasis dysregulation leading to membrane alterations was measured using thiazole orange (TO), a DNA intercalant as described by Derick et al. with modifications [30]. The *Plasmodium* culture was prepared at 10% hematocrit (in RPMI) and 10% parasitemia for both strains. Then 100 μL of each strain was introduced into a 96-well black plate. The iRBCs were probed with 2 μM TO and incubated for 1 h at 37 °C. Each well was then treated with 1 μM ART or DMSO (0.5%) as control and the concentration of TO maintained at 2 μM by addition of TO accordingly. The fluorescence of TO intercalated with DNA was followed kinetically at 505/535 nm (excitation/emission) every 4–10 min for 2 h using a Xenius® fluorimeter from SAFAS (Monaco). Low intracellular fluorescence levels indicate a normal cell homeostasis while high levels of intracellular fluorescence show cell alteration, inducing an increase of the entry of TO. The kinetics of the fluorescence levels are thus representative of the ability of the cell to maintain or not redox homeostasis and fight oxidative dysregulation.

2.5. Antioxidant expression

The antioxidant expression was quantified by real-time quantitative polymerase chain reaction (RTqPCR) method, preceded by RNA extraction and cDNA synthesis. The antioxidant proteins investigated included iron superoxide dismutase (Fe-SOD or SOD1), an isoform of peroxiredoxin (nPrx), γ-glutamylcysteine synthetase (γ-GCS), glutathione synthetase (GS), glutathione-S-transferase (GST), glutathione reductase (GR) and thioredoxin reductase (TrxR). The values were expressed by comparison to the housekeeping gene 18S-rRNA.

2.5.1. RNA extraction

Prior to RNA extraction, the parasites were subjected to a 6 h ART (3 μM) treatment using DMSO (at the same percentage (0.0085%) used in ART solution) as the negative control. After the treatment, the drugs were washed off using PBS and the cell lysed with saponin (0.075%) to remove the red blood cells. The resulting pellet was introduced into a 2 mL cryotube and washed 3 times in ice-cold PBS to remove the RBC debris by spinning at 9000 RPM at 4 °C for 5 min each time. The parasites were further lysed and the RNA extracted using Qiagen kit according the manufacturer's instruction [31]. The resulting total RNA was quantified using nanodrop 2000 (Thermo Fisher) and their concentration adjusted to 30 ng/μL for cDNA synthesis.

2.5.2. Real-time quantitative PCR

Firstly, the cDNA was synthesized from the total RNA with random hexamers using the High-Capacity cDNA kit (Applied Biosystems). The resulting cDNA was used in combination with specific primers (Additional_file) for selected antioxidants with melting temperatures (T_m) between 55 and 65 °C. The real-time quantitative PCR was performed using Bio-Rad CFX96 Real-Time System in a 50 μL reaction volume with the PCR Master Mix Power SYBR™ Green Kit according to the manufacturer's instruction. Three independent biological samples were assayed in duplicates and analyzed with Bio-Rad CFX Maestro software. 18SrRNA sequence was used as the house-keeping gene for normalization. At the end of each reaction cycle, the melting curves were obtained and analyzed. Relative quantitative analysis was performed using the $2^{-\Delta\Delta Ct}$ method [32], where $\Delta\Delta Ct = (\mathbf{Ct}\text{target} - \mathbf{Ct}18S\ \text{rRNA})_{\text{treated}} - (\mathbf{Ct}\text{target} - \mathbf{Ct}18S\ \text{rRNA})_{\text{untreated}}$.

2.5.3. Glutathione quantification

The *Plasmodium* culture at 10% parasitemia and 2% hematocrit in PBS was treated with 3 μM ART or DMSO (0.4%) as negative control for 4 h. The N-ethylmaleimide (NEM) GSH derivatization was used for GSH quantification by LC-MS according to the method of Herzog et al. with slight modifications [33]. The concentration of GSH was determined from the integration of the chromatographic peak corresponding to the NEM derivatives and the standard curve.

3. Results and discussion

The aim of the present work was to decipher the redox mechanisms involved in the *Plasmodium falciparum* ART resistance. For this reason, we first compared the overall ROS generation and, more specifically, superoxide radical production under artemisinin treatment and its consequence towards protein oxidation. The resilience of parasites to oxidative stress with regards to their sensitivity to artemisinins was then measured by i) comparing the antiplasmodial effects of oxidant molecules (pro-oxidant or antioxidant inhibitors), ii) measuring the impact of artemisinin on parasite homeostasis dysregulation and iii) measuring their antioxidant capacity (antioxidant enzymes expression and GSH quantification).

3.1. ROS generation in Plasmodium falciparum and protein oxidation

The general and non-specific monitoring of the ROS generation followed by DCF fluorescence demonstrated that, under ART treatment, the artemisinin-resistant line (F32-ART), produced significantly less ROS than the artemisinin-sensitive strain (F32-TEM), at the ring stage whatever the concentration tested, while there was no difference at the trophozoite stage (Fig. 1). This data is particularly interesting since only ring stages are involved in artemisinin-resistance [19]. Comparing the treated to the untreated group, at the ring stage, significant levels of ROS were generated with lower ART concentration (200 nM) in the sensitive strain, F32-TEM, than in the resistant strain, F32-ART, whose significant ROS generation started at 50,000 nM (Fig. 1B) like for the trophozoite stages (Fig. 1A). Interestingly, at the ring stage, higher ART doses are thus required to generate significant levels of ROS in the ART-resistant parasites than in the sensitive ones.

For a more in-depth ROS quantification, we determined the levels of intracellular superoxide radical anion ($O_2^{\bullet-}$), the primary radical formed from the one electron reduction of oxygen, and hydrogen peroxide (H_2O_2) using LC-MS, a specific and sensitive method [26]. No H_2O_2 was detected on the two strains at both ring and trophozoite stages. *Plasmodium*, like most cells, contains superoxide dismutase enzymes (SODs) which dismutate the primary radical, $O_2^{\bullet-}$ to H_2O_2, which in turn can be reduced into water, not through catalase or glutathione peroxidase absent in the parasite [34], but through peroxiredoxins expressed in high levels.

As shown in Fig. 2, regardless of the stage of the parasite, when treated by 30 nM and 300 nM of artemisinin, both F32-TEM and F32-ART produced significant levels of $O_2^{\bullet-}$. However, while there was no in-between strain difference at the trophozoite stages, F32-ART produced significantly less $O_2^{\bullet-}$ than F32-TEM (Fig. 2B and C) at the ring stages (both at 0–3 h and 0–24 h post invasion). These data are in accordance with artemisinin resistance phenomenon which occurs at the ring stage and especially at earlier forms (0–3 h post invasion) [19,24].

Moreover, this finding is in tandem with the mechanism of artemisinin resistance due to mutations in the *pfk13* gene of the parasite. Indeed, the *pfk13* gene mutation affects the uptake and breakdown of hemoglobin in ART-resistant parasites [7,35], which consequently reduces the by-product, heme leading to a decreased heme-dependent activation of ART in these strains [35] and so, a decrease in the production of ROS. The reduced heme generation and attenuated metabolism characteristic of quiescence [19,28] definitely impact ROS production in the resistant strain of *P. falciparum*. The decrease of $O_2^{\bullet-}$ measured at very high concentrations of ART (3000 nM) could be explained by the generation of high concentration of $O_2^{\bullet-}$ transformed, by dismutation, into H_2O_2 [36].

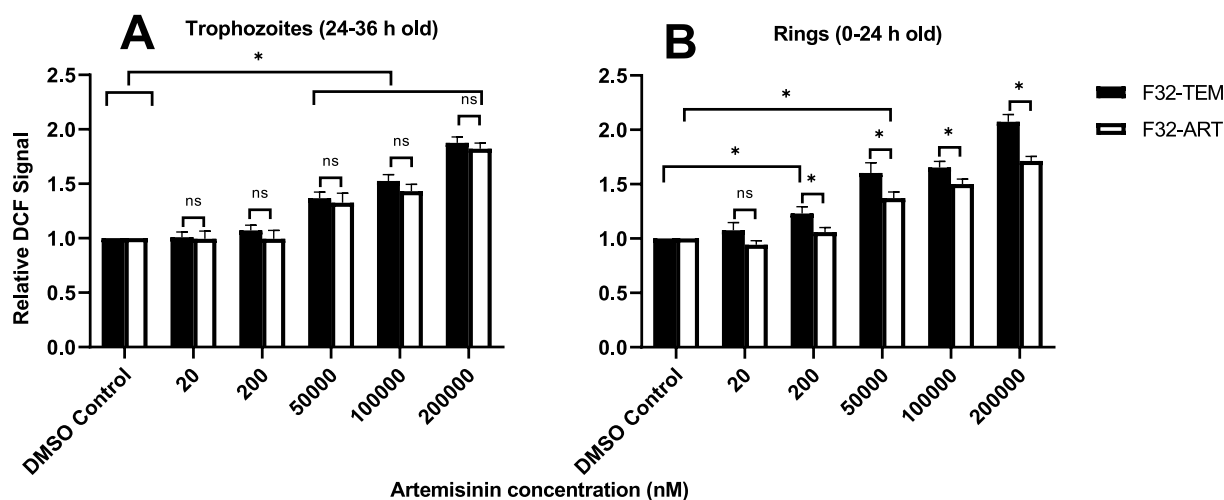ROS generated at low concentrations in physiological conditions are

Fig. 1. Overall ROS generation after artemisinin treatment determined by DCF-fluorescence from the 24–36 h trophozoites (A) and 0–24 h rings (B) post-invasion stages of artemisinin-resistant (F32-ART) and artemisinin-sensitive (F32-TEM) strains of *Plasmodium falciparum*. A significant difference in ROS production was measured between the two strains only at the ring stage. Moreover, at the trophozoite stage, both strains produced significant ($p < 0.05$) levels of ROS from $\geq$50,000 nM ART while at the ring stage, F32-TEM produced significant ($p < 0.05$) levels at a lower ART concentration (200 nM) than F32-ART. The results are triplicates from three independent experiments and values analyzed using *t*-test.
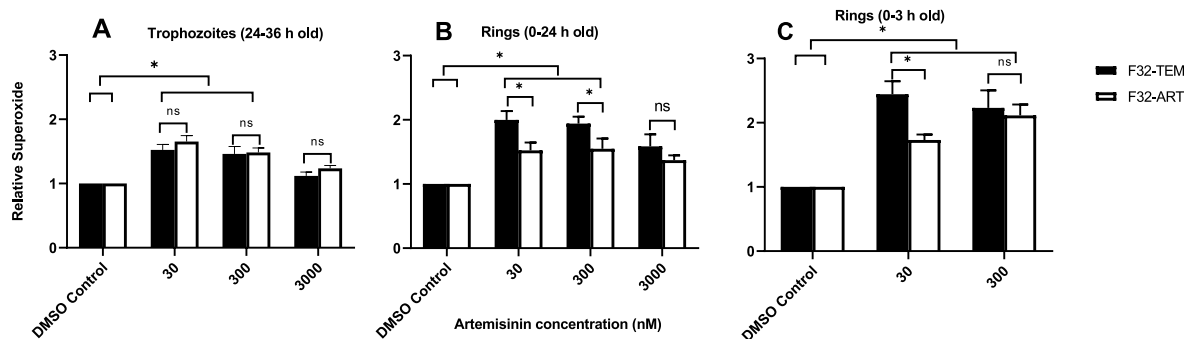

Fig. 2. Superoxide production after ART treatment from the 24–36 h trophozoites (A), 0–24 h rings (B) and 0–3 h rings (C) post-invasion stages of F32-TEM and F32-ART. At 30 and 300 nM, both strains produced significant ($p < 0.05$) levels of $O_2^{\bullet-}$ across all the stages with F32-TEM producing significantly ($p < 0.05$) higher $O_2^{\bullet-}$ than F32-ART at these concentrations at the ring stages. The results are triplicates from three, four and three independent experiments for A, B and C, respectively. The values were analyzed using *t*-test (n = 1 for 2B at 30 nM).

often beneficial for cells, playing important roles as regulatory mediators in signaling processes [37]. However, exacerbated levels of ROS as exemplified under ART treatment are deleterious, attacking and damaging bio-molecules ranging from lipids [15], proteins [16] to nucleic acids [17]. Therefore, protein oxidation leading to the generation of protein carbonyls can be considered as a biomarker of the organism's vulnerability to reactive species [38,39].

The levels of protein carbonyls detected in both strains reflect the direct and indirect attack of ART on plasmodial proteins. We demonstrated that both the F32-TEM and F32-ART strains produce protein carbonyls after ART exposure with a significant increase (2.07 ± 0.39 folds) in treated F32-TEM with reference to its DMSO control (Fig. 3). Even though there was no significant difference between the two strains, proteins from F32-ART appeared less impacted by ART damages.

The higher relative protein carbonyl level from the F32-TEM consolidates the fact that a lower amount of ROS is produced in the ART-resistant strain than in the sensitive one.

However, a lower amount of ROS generated associated with a lower protein oxidation could also be due to a better antioxidant machinery, able to reduce oxidative stress. To verify this hypothesis, we measured (i) the response of ART-sensitive and ART-resistant parasites to pro-oxidants (ii) their respective antioxidant expression.
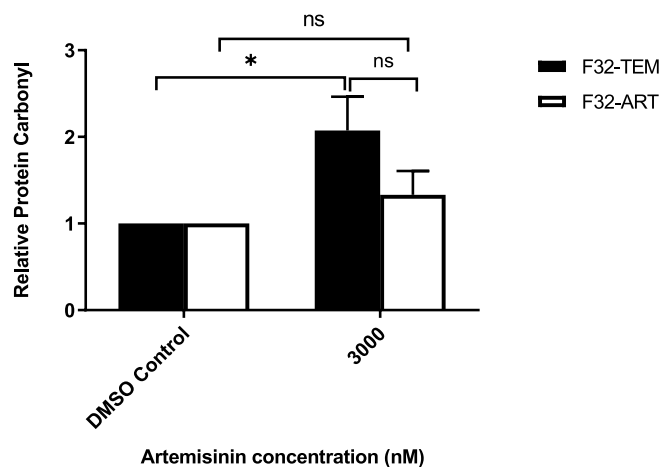

Fig. 3. Effect of artemisinin treatment (3 μM for 6 h) on protein carbonylation in *Plasmodium falciparum* at the ring stage (0–24 h post-invasion). There was no significant difference between the F32-ART and F32-TEM ($p = 0.07$) treated groups; however, comparing each strain to its respective control, there was a significant ($p < 0.05$) increase in the F32-TEM but no significant difference for F32-ART. The values are triplicates of four independent experiments and values analyzed using *t*-test.

3.2. Antioxidant capacity of ART-sensitive and ART-resistant parasites

3.2.1. Response to pro-oxidants

The sensitivity of the two strains to pro-oxidant/antioxidant inhibitors molecules such as H_2O_2, menadione, methylene blue (MB, inhibitor of glutathione reductase) DL-buthionine-(SR)-sulfoximine (BSO, inhibitor of γ-glutamylcysteine synthetase), 4-nitro-2,1,3- benzothiadiazole (4NBTD, inhibitor of thioredoxin reductase), conoidin A (inhibitor of peroxiredoxins), usnic acid (inhibitor of *Plasmodium* vitamin E biosynthesis) [40] and 6-amino-5-nitroso-3-methyluracil (a SOD inhibitor) were measured. While usnic acid and 6-amino-5-nitroso-3-methyluracil did not show any antiplasmodial activities even at 100 μM, the IC_{50} values of the other molecules obtained on both strains were quite similar (Table 1). The absence of differences in response of F32-TEM and F32-ART to oxidative stress generated by redox molecules, like for artemisinin, is in accordance with the fact that a chemosensitivity assay based on the parasite proliferation is not adapted to evaluate the *Plasmodium* artemisinin resistance based on a quiescence state [19,28]. However, H_2O_2 IC_{50} values showed that the resistant strain seems less vulnerable to H_2O_2 than its sensitive counterpart (11 ± 2 *vs* 23 ± 4 mM, respectively).

Because artemisinin resistance is managed by a quiescence stage phenomenon, IC_{50} values based on a proliferative assay are not relevant to highlight the resistance to artemisinin, that is why a recrudescence assay was performed. The recrudescence assay is based on the time to reach the initial parasitemia and was used to compare the vulnerability of the ART-resistant strain, in comparison to the sensitive one, towards oxidants and antioxidant inhibitors. The absence of effect of menadione, H_2O_2, BSO and conoidin A on *P. falciparum* recrudescence after treatment did not permit to differentiate F32-ART from F32-TEM as both strains reached initial parasitemia from the day 2 of the kinetics (data not shown). However, like for ART treatment, F32-ART recrudesced significantly faster (more than 7 days earlier) than F32-TEM after treatment with the pro-oxidant compounds MB and 4NBTD (Fig. 4A, B and C). These results, which indicate that the ART-resistant strain could manage the oxidative stress better than the sensitive one, led us to quantify the antioxidant expression in order to confirm this hypothesis.

Indeed, central to the plasmodial antioxidant machinery are the reduced glutathione (GSH) and thioredoxin (Trx) systems which donate electrons from their sulphohydryl groups to reduce oxidized molecules in order to maintain their active conformations, hence acting as redox buffers to the cells [41,42]. While GSH is regenerated in part from glutathione disulfide (GSSG) through a salvage pathway catalyzed by glutathione reductase (GR), thioredoxin (Trx) is regenerated by thioredoxin reductase (TrxR) [43]. GR and TrxR are inhibited specifically by
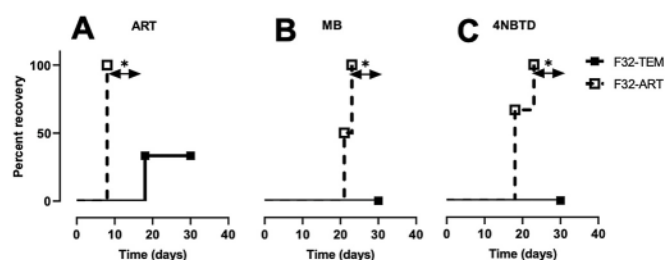

Fig. 4. Kaplan Meier survival curves showing the recovery time (days) of artemisinin-sensitive and -resistant strains after drug pressure from pro-oxidants: artemisinin (ART) at 18 μM (A), methylene blue (MB) at 0.5 μM (B) and 4-nitro-2,1,3-benzothiadiazole (4NBTD) at 20 μM (C). The recovery of F32-ART treated with MB and 4NBTD was ≥7 days earlier than that of F32-TEM which was a similar pattern to the ART treatment control ($p < 0.001$), suggesting a more vulnerability of F32-TEM to stressors. Ring stages (0–24 h old) were used for the experiment. The level of significance of the data was analyzed by log ranking between the two strains. Results represent three independent experiments. The recrudescences up to 30 days were censured.

MB and 4NBTD respectively [44,45]. This ability of the parasites to manage ROS as we saw by measuring ROS produced (Figs. 1 and 2) or indirectly in the recrudescence assay with MB and 4NBTD (Fig. 4) has to be put in connection with the mechanism of resistance (which involves quiescence and recrudescence) and reveals its importance in the survival of the parasites exposed to artemisinins.

3.2.2. Cellular redox homeostasis

The homeostasis dysregulation was measured *via* the thiazole orange (TO) fluorescence. This technique is of particular interest since TO targets the DNA, which is only found in the parasites, thereby preventing ambiguous analysis due to interferences from uninfected erythrocytes, which do not have DNA. TO intercalates the DNA [46], but its access into the cells are regulated by the cell membrane, which is suggestive of a cell with intact cellular homeostasis. A membrane alteration as a consequence of ART exposure [15] leads to increased entry of TO into the cells to reach the DNA. An increase in the fluorescence level characterizes an increase in the intracellular oxidative stress through membranes alterations. A weak fluorescence level after ART exposure is indicative of lower impact of ART and so, a higher parasite antioxidant capacity.

At the trophozoite stage, from 0 to 30 min, the linear regression of the slope showed no difference between F32-TEM and F32-ART (5.4×10^{-3} *vs* 4.8×10^{-3}). From 30 to 60 min, the ART-sensitive strain showed a higher slope than the resistant one (2.8×10^{-3} *vs* 1.3×10^{-3}), even though the difference was not statistical (Fig. 5A).

At the ring stage, between 0 and 4 min, the slope was significantly higher in the sensitive strain than in the resistant one (3.6×10^{-2} *vs* 1.0×10^{-2}). This gap suggests a higher level of oxidative stress in the F32-TEM parasites; however, after 4 min, there was no significant difference between the two strains (slope: 3.2×10^{-3} *vs* 3.2×10^{-3}) (Fig. 5B) even though the level of fluorescence was very different between the two strains. This result shows that F32-TEM is more vulnerable to homeostasis dysregulation due to ART exposure, which is totally in accordance with the previous results demonstrating that a lower amount of ROS was produced in the ART-resistant strain comparatively to the sensitive one. Moreover, this could mean that at the ring stage, the deleterious effect of ART is experienced later in the ART-resistant parasites than the sensitive ones. These data also confirm that ART resistance is associated with the ring stage. Our results showing that vulnerability to homeostasis dysregulation from ART treatment is less pronounced at the ring stage for F32-ART than F32-TEM (Fig. 5B) can be explained by the lower activation of ART by heme (due to reduced hemoglobin endocytosis) at the ring stage in the ART-resistant than in the ART-sensitive parasites [7,35, 47] and could also be as a result of a higher expression of antioxidant machinery.

Table 1
Pro-oxidants IC_{50} values on artemisinin-sensitive (F32-TEM) and artemisinin-resistant (F32-ART) strains.

Molecules	IC_{50} (μM)		p value
	F32-TEM	F32-ART	
Menadione	6.7 ± 1	8.5 ± 1	0.193
H_2O_2	11 ± 1 (x 10^3)	23 ± 2 (x 10^3)	0.005
Artemisinin	20 ± 1 (x 10^{-3})	23 ± 1 (x 10^{-3})	0.070
Methylene blue	2.8 ± 0.7 (x 10^{-3})	1.4 ± 0.1 (x 10^{-3})	0.051
DL-buthionine-(SR)-sulfoximine	59 ± 13	55 ± 2	0.340
4-nitro-2,1,3-benzothiadiazole	57 ± 5	57 ± 0.8	0.481
Conoidin A	5.0 ± 1	4.6 ± 2	0.500
Usnic acid	>100	>100	
6-amino-5-nitroso-3-methyluracil	>100	>100	

The IC_{50} of H_2O_2 was statistically ($p < 0.05$) higher in F32-ART than that of F32-TEM while there were no in-between strain differences statistical ($p > 0.05$) for other pro-oxidants/antioxidant inhibitors tested. The results are triplicates from three independent experiments (geometric means ± SEM) and values analyzed using *t*-test.
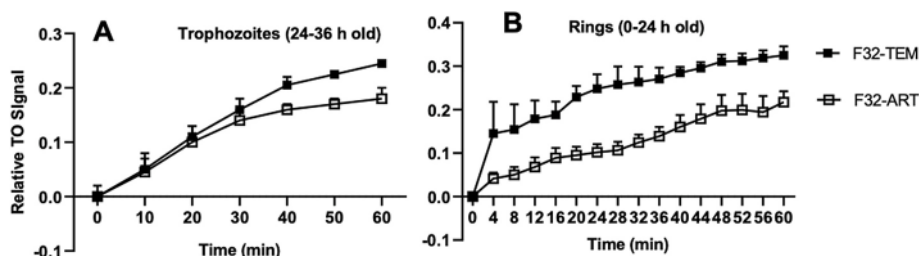
Fig. 5. Impact of ART (1 μM) on *Plasmodium* cellular homeostasis on trophozoite (24–36 h old) (A) and ring (0–24 h post invasion) (B) stages. ART treatment had significantly ($p < 0.05$) more impact on F32-TEM than F32-ART in the first 4 min at the ring stages while there was no statistical difference at the trophozoite stage. The results are triplicates from 3 independent experiments and *p*-values obtained after a regression analysis of each graph at 95% confidence interval.

3.2.3. Parasite antioxidant expression

To verify the hypothesis that the lower production of ROS highlighted for the ART-resistant strain could be due to a higher efficacy of the antioxidant machinery, the quantification of the expression (transcriptional profiles) of several antioxidants (Fig. 6) was performed by RTqPCR for both strains at basal conditions and under ART exposure.

At the ring stage, the ART-resistant strain had, at the basal level, significantly higher expression of Fe-SOD and TrxR than its sensitive counterpart (Fig. 7). There was no statistical in-between strain difference in the expression of γ-GCS, GST, GR, GS and nPrx.

The higher basal expression of Fe-SOD in F32-ART than F32-TEM, shows the better preparedness of the ART-resistant parasites to handle $O_2^{\bullet-}$, like already reported on a dihydroartemisinin-resistant and chloroquine-resistant clones [48,49]. These results could explain why there was lesser ROS production and weaker homeostasis dysregulation impact in F32-ART than in F32-TEM, at the ring stage, as early as the first minutes after ART exposure (Fig. 5B).

However, ART exposure for 6 h led to an increased overall expression (except for GR) of antioxidants in both strains as shown by the transcriptional profiles in Fig. 8. ART treatment generates ROS, among which superoxide [14] that mounts oxidative pressure on the parasite. Although enzyme synthesis is gene regulated, it can also be induced or repressed by the action of specific substrates or products like ROS [50]. These primary radicals, which emanate from the cytosol or mitochondria, are handled by Fe-SOD and Mn-SOD. However, Fe-SOD is more

relevant in the fight against malaria parasites due to its strategic location into the cytosol and its sensitivity to inhibition by drug candidates [34, 51]. Moreover, the expressions of Fe-SOD which converts $O_2^{\bullet-}$ to H_2O_2, and peroxiredoxins which finally converts H_2O_2 to water are elevated in the parasite under ART treatment [52]. In the same way, we showed that there was a more significant expression of Fe-SOD in the F32-ART than F32-TEM (1.9 ± 0.01 *vs* 1.3 ± 0.15-fold compared to untreated) (Fig. 8A). This overexpression could be partly responsible for the lower amount of superoxide radical anions (Fig. 2) and the lower protein oxidation (Fig. 3) measured in the ART-treated ART-resistant parasites.

There was no statistical in-between strain difference for the expression of nPrx; nevertheless, the trend revealed that there was more overexpression (9.4 ± 2.13 *vs* 5.1 ± 0.95-folds) in the F32-ART *vs* F32-TEM exposed to ART (Fig. 8B). This result is consistent with the fact that *Plasmodium* lacks catalase and glutathione peroxidase but is highly dependent on peroxiredoxins (Fig. 9) to detoxify H_2O_2 [53]. Peroxiredoxins are kept in reduced forms by Trx and GSH dependent systems (Fig. 9), whose expressions were also elevated under the ART exposure (except GR) (Fig. 8) in order to continually keep the Prxs in functional states in the presence of oxidative stress.

The increased expression of peroxiredoxins in both strains in response to ART treatment corroborates the previous findings showing increased expression and high affinity of peroxiredoxin that lead to H_2O_2 reduction [52,54]. This could explain the non-detection of H_2O_2 by LC-MS, as we reported in this study. Furthermore, the high expression
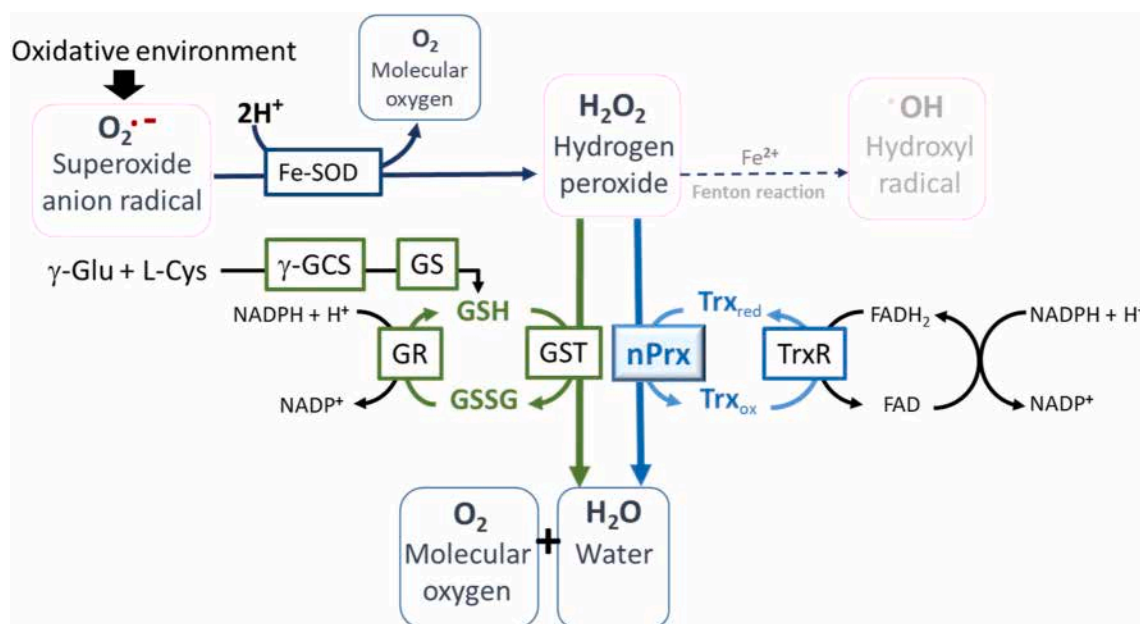

Fig. 6. *Plasmodium* antioxidant system allowing redox homeostasis. Fe-SOD (iron superoxide dismutase), nPrx (an isoform of peroxiredoxin), GR (glutathione reductase), γ-GCS (γ-glutamylcysteine synthetase), GS (glutathione synthetase), GST (glutathion-S-transferase) and TrxR (thioredoxin reductase). The peroxiredoxins prevent production of hydroxyl radical (Fenton reaction), the most reactive and deleterious ROS[34].
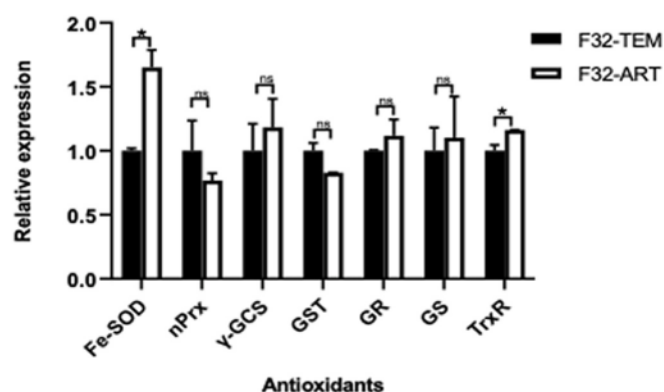
Fig. 7. Relative basal transcriptional profiles of antioxidant expression in *Plasmodium falciparum* at the ring stage (0–24 h post-invasion) of F32-ART compared to F32-TEM. The ART-resistant strain (F32-ART) expressed higher basal levels of Fe-SOD and TrxR than the sensitive one (F32-TEM) while the reverse was the case for nPrx, which was expressed more in the ART-sensitive strain. The basal level of other antioxidants was not statistically different between both strains. This result represents duplicates from three independent biological samples (0–24 h rings). Fe-SOD = iron superoxide dismutase, nPrx = an isoform of peroxiredoxin, GR = glutathione reductase, γ-GCS = γ-glutamylcysteine synthetase, GS = glutathione synthetase, GST = glutathione-S-transferase and TrxR = thioredoxin reductase. The values were analyzed using *t*-test.

of peroxiredoxins could also explain the very low activity of H_2O_2 against F32-TEM and F32-ART strains with huge IC_{50} values in millimolar range (Table 1) in comparison to micromolar range in other cell lines and the other tested compounds [55–57]. Moreover, the higher peroxiredoxin level in F32-ART than F32-TEM (Fig. 8B) can explain the significantly higher IC_{50} of H_2O_2 against the ART-resistant strain.

Beyond SODs and peroxiredoxins, the two other most important antioxidant systems are GSH and Trx dependent systems, which play interconnecting roles in mopping up ROS and keeping biomolecules in reduced and functional states. The expression of γ-GCS, involved in *de novo* GSH synthesis is significantly increased (4.5 ± 1.3 *vs* 3.2 ± 0.23-folds) under ART treatment in F32-ART and F32-TEM (Fig. 8C). Although the difference was not significant, there was also a tendency to overexpress GS in the resistant strain. These two enzymes are the keys of the *de novo* pathway and main source of GSH [58]. The expression of GR decreased (0.3 ± 0.12 *vs* 0.4 ± 0.14-fold) significantly in both strains under ART exposure (Fig. 8E) but there was no in-between strain statistical difference. This can be explained by the fact that GR is a repressible enzyme whose level is decreased due to elevated expression of γ-GCS and/or under oxidative stress [59]. Indeed, GST, that plays an interconnecting role in the reductive functions of GSH, and helps in the reduction of H_2O_2 using GSH as a cofactor [34], increased significantly in both strains but more in F32-ART than F32-TEM (Fig. 8D).

We also demonstrated that TrxR increased significantly in both strains after ART exposure, with no in-between strain difference (Fig. 8G). TrxR with its reductive function, is required in the generation of several isoforms of Trx, which are supportive of the GSH system and directly involved in reducing oxidized bio-molecules and ROS [41]. We could consider that the higher expression of TrxR in F32-ART both at
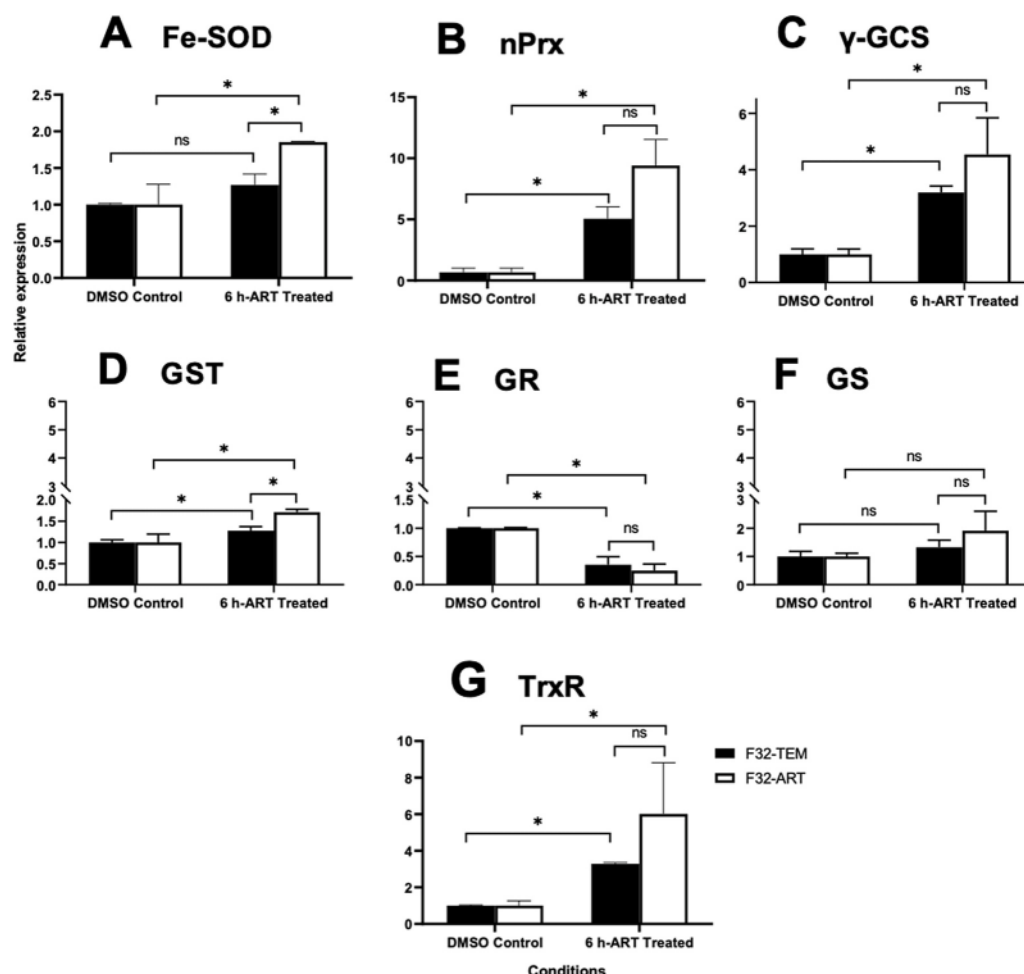

Fig. 8. Impact of ART treatment on the transcriptional profiles of antioxidants. Relative expression of antioxidant enzymes (A:Fe-SOD = Iron-superoxide dismutase, B: nPrx = Peroxiredoxin, C: γ-GCS = γ-glutamylcysteine synthetase, D: GST = Glutathione-S-transferase, E: GR = Glutathione reductase, F: GS = Glutathione synthetase and G: TrxR = Thioredoxin reductase) in F32-ART (ART-resistant) and F32-TEM (ART-sensitive) strains treated with artemisinin (at 3 µM during 6 h) comparatively to the DMSO control (corresponding to the value 1). The results are duplicates from three independent biological samples (performed with 0–24 h old rings). The values were analyzed using *t*-test. *$p < 0.005$.
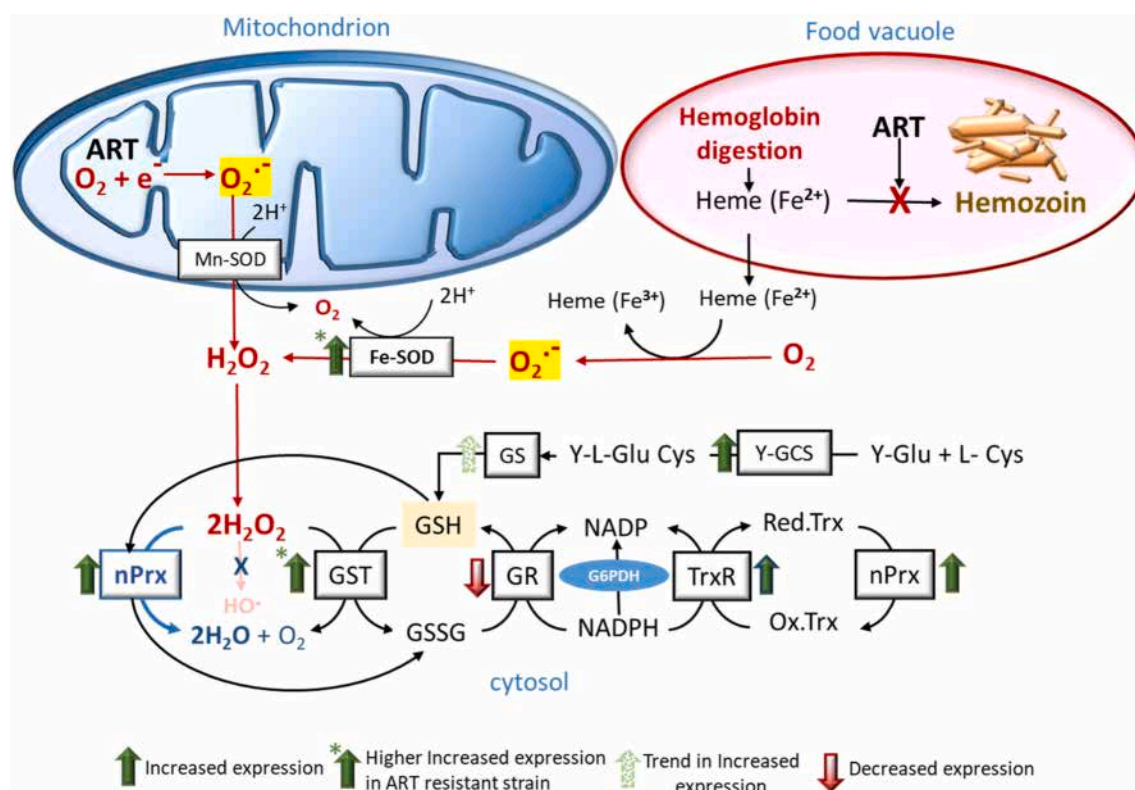
Fig. 9. Global overview of antioxidant expression in artemisinin-resistant and artemisinin-sensitive *Plasmodium falciparum* under ART treatment. The key enzymes involved in mopping up reactive species (except glutathione reductase) increased under ART treatment in *P. falciparum*. nPrx by converting H_2O_2 to H_2O prevents the formation of the hydroxyl radical HO$^•$. This scheme was inspired by Nogueira et al. [49]. Fe-SOD = iron superoxide dismutase, nPrx = an isoform of peroxiredoxin, GR = glutathione reductase, γ-GCS = γ-glutamylcysteine synthetase, GS = glutathione synthetase, GST = glutathione-S-transferase and TrxR = thioredoxin reductase. G6PDH = glucose-6-phosphate dehydrogenase; NADPH = reduced nicotinamide adenine dinucleotide phosphate; NADP = oxidized nicotinamide adenine dinucleotide phosphate; Red.Trx = reduced thioredoxin; Oxi.Trx = oxidized thioredoxin; GSH = reduced glutathione, GSSG = oxidized glutathione; H_2O_2 = hydrogen peroxide and $O_2^{•-}$ = superoxide radical. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

basal condition and under ART treatment highlights the better preparedness of ART-resistant parasites in handling ROS than ART-sensitive ones. However, like after artemisinin treatment, F32-ART showed faster recrudescence than F32-TEM after exposure of MB and 4-NBTB, both inhibitors of TrxR (Fig. 4). Therefore, TrxR does not appear to be the most important factor involved in the resistance of the F32-ART strain to ROS. This led us to examine other components of the *Plasmodium* redox system to differentiate the two strains in their ability to manage oxidative stress.

Although both the ART-sensitive and ART-resistant parasites had significant increases in TrxR, γ-GCS, GST and nPrx, the significant higher in-between strain expression of Fe-SOD and GST give the ART-resistant parasites an advantage over sensitive ones, enabling them to withstand oxidative stress better under ART treatment as shown in Fig. 9.

To confirm these results, proteomic experiments and enzymatic activity assays will be carried out.

3.2.4. GSH quantification

Glutathione system plays a huge role in the plasmodial antioxidant system, acting as buffers. Among several other roles, GSH is directly and indirectly involved in ROS detoxification [42,60] which can lead to GSH depletion [61]. The GSH level quantification carried out using LC-MS revealed that the GSH level in F32-ART was less depleted than that in F32-TEM after a 4 h treatment with ART as presented in Fig. 10.

A higher basal GSH level in ART-resistant strains has been already reported [62,48]. The level of GSH depends on its rate of oxidation (GSH is oxidized by ROS or bio-molecules) and re-generation. The lower ROS generation (Figs. 1 and 2) and overall higher expression of GSH generating enzymes (γ-GCS and GS) in F32-ART under ART exposure (Fig. 8C

and F) could explain the lower decrease of GSH in these ART-resistant parasites.

It has been shown that *Plasmodium* does not take up GSH from the host and that the genes coding the enzymes in this *de novo* pathway are essential for its survival [63]. Our results (Fig. 8C, F and 10), which
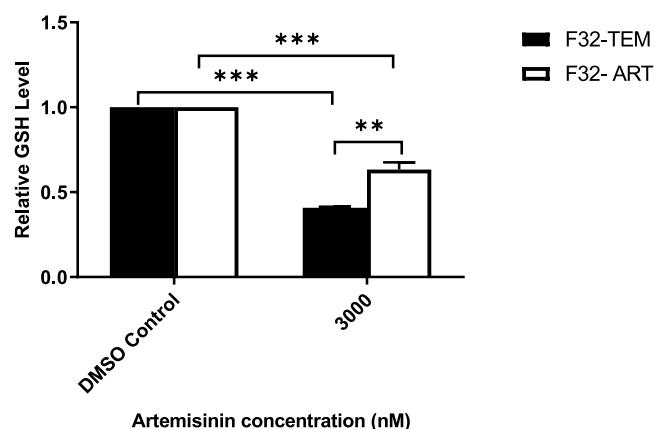

Fig. 10. Impact of artemisinin exposure (3 μM, after a 4 h treatment) on *Plasmodium falciparum* reduced glutathione (GSH) at the ring stage (0–24 h post invasion). F32-TEM showed significantly ($p < 0.05$) higher susceptibility to GSH depletion than F32-ART under ART treatment. These results are triplicates from one experiment. The fold depletion in GSH was 0.41 ± 0.00 for F32-TEM and 0.63 ± 0.04 for F32-ART, compared to untreated control. ** = $p < 0.01$ and *** = $p < 0.001$.

show a trend to overexpress the *de novo* pathway enzymes and a lower impact of ART on GSH depletion in the ART-resistant strain, underline the importance of this pathway in this resistance context.

4. Conclusion

The currently reported redox-mediated mechanism is a new piece in the complex ART resistance puzzle. Our results can be put in relation with previous findings such as the reduced hemoglobin endocytosis [7], acceleration of the unfolded protein response (UPR) pathways [8], improved ability to manage oxidative damages [9] and increased DNA replication at the ring stage [10].

We demonstrated for the first time, that the parasite antioxidant system is enhanced in the ART-resistant strain both at the steady state and under artemisinin exposure, especially Fe-SOD and TrxR, thus showing that artemisinin resistance is connected to the ability of *Plasmodium* to manage oxidative stress. The lower ROS generation, lower response to pro-oxidants, lower impact of ART on biomolecules and the higher expression of some antioxidants in the artemisinin-resistant parasites than their sensitive counterparts after ART exposure, show clearly that *Plasmodium falciparum* resistance to artemisinin is redox-mediated. The lower depletion of GSH in the ART-resistant strain under artemisinin exposure highlights the importance of the GSH system in this resistance. The use of molecules that specifically inhibit GSH synthesis in combination with artemisinin could be a viable approach in winning the war against malaria due to artemisinin resistance. These findings enrich the body of knowledge about the mechanism of artemisinin resistance and might help in the design and development of newer antimalarials to evade the abilities of the parasite to efficiently manage ROS.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgement

Additional file

Primers used for RTqPCR.

Gene ID	Direction	RNA primer (5′-3′)	Length	Tm
18S r RNA	F	TGACTACGTCCCTGCCCTT	19	64.7
	R	ACAATTCATCATATCTTTCATCGG	24	63.7
GR PF14_0192	F	GGTGGAAGTGGAGGAATGGC	20	60.68
	R	CGTTGACACACGTTCCACCT	20	60.81
γ-GCS PFI0925W	F	TGGTTCACATTGGACTCCAGAA	22	59.56
	R	TCTAGCTCCTTGAACGGCAC	20	59.75
GS PFE0605C	F	TGCCTTACAAGTAGACCCATCT	22	58.55
	R	TCCCTCCTTCTCTTTGAGGTT	21	58.01
Fe-SOD PF08_0071	F	CTGATTGTGGTGGTGAGCCT	20	59.96
	R	TCCCCAACCGGAACCAAAAT	20	59.81
TrxR PFI1170C	F	GGCTTCAGCCAAAGAAGCTG	20	59.76
	R	CGTTCACACACGTTCCACCT	20	60.81
nPrx PF10_0268	F	ACTGCTGAGGACCAACTCAA	20	58.87
	R	GTTGCTGACTTTCCTGGGGA	20	59.89
GST PF14_0187	F	AACGGTGATGCTTTTGTTGAATT	23	65.3
	R	GCTTTGAGCTAATATCAAATCTCCAA	26	64.2

The primers were designed from the portal of National Centre for Biotechnology information.

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