Proteomic analysis revealed alterations of the *Plasmodium falciparum* metabolism following salicylhydroxamic acid exposure

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Objectives: Although human respiratory metabolism is characterized by the mitochondrial electron transport chain, some organisms present a “branched respiratory chain.” This branched pathway includes both a classical and an alternative respiratory chain. The latter involves an alternative oxidase. Though the *Plasmodium falciparum* alternative oxidase is not yet identified, a specific inhibitor of this enzyme, salicylhydroxamic acid (SHAM), showed a drug effect on *P. falciparum* respiratory function using oxygen consumption measurements. The present study aimed to highlight the metabolic pathways that are affected in *P. falciparum* following SHAM exposure.

Design: A proteomic approach was used to analyze the *P. falciparum* proteome and determine the metabolic pathways altered following SHAM treatment. To evaluate the SHAM effect on parasite growth, the phenotypic alterations of *P. falciparum* after SHAM or/and hyperoxia exposure were observed.

Results: After SHAM exposure, 26 proteins were significantly deregulated using a fluorescent two dimensional-differential gel electrophoresis. Among these deregulated proteins, some were particularly involved in energetic metabolism. And the combinatory effect of SHAM/hyperoxia seems deleterious for the growth of *P. falciparum*.

Conclusion: Our results indicated that SHAM appears to activate glycolysis and decrease stress defense systems. These data provide a better understanding of parasite biology.

Keywords: *Plasmodium falciparum*, salicylhydroxamic acid, hyperoxia, glycolysis, proteomic
the metabolic changes of the mitochondrion contribute to environmental adaptations of parasites during its life cycle. Effective, in vivo, P. falciparum life cycle involves two hosts: the mosquito and humans during which the parasite is subjected to varying oxygen levels (from 5% oxygen in human venous blood to 13% oxygen in the human lungs and 21% oxygen in mosquito salivary glands).

Study of the rotenone drug effect underlined the divergence of the first component (complex I) of the P. falciparum mETC. The eukaryotic complex I is classically composed of a rotenone-sensitive nicotinamide adenine dinucleotide hydride (NADH) dehydrogenase. In contrast, P. falciparum encodes a rotenone-insensitive alternative complex I (type II NADH dehydrogenase, PfNDH2). This complex I is comparable to type II NADH dehydrogenase found in plants. Not surprisingly, parasite mitochondria have an endosymbiotic origin probably resulting from algal symbiont into a protoeukaryote. Furthermore, other mitochondrial drugs such as atovaquone inhibit electron transfer at the level of the bc1 complex (complex III) by interfering with the ubiquinol oxidation site of cytochrome b. The drug effect is lethal for the parasite, presumably by interruption of essential links to de novo pyrimidine biosynthesis and to the collapse of the mitochondrial transmembrane potential. This drug is already currently used in endemic regions in combination with proguanil (Malarone®). Also, the combination of atovaquone and salicylhydroxamic acid (SHAM), a specific inhibitor of alternative oxidase (AOX), inhibits parasite growth synergistically. The AOX, which is absent from mammals, has been most extensively studied in higher plants. The plant respiratory pathway includes an alternative respiratory chain, cyanide-resistant, and the classical mETC, cyanidesensitive. The AOX does not appear to contribute directly to mitochondrial transmembrane potential or the energy balance of the cell. However, it can contribute indirectly by accepting electrons from enzymes that provide electrons to coenzyme Q for the preservation of tricarboxylic acid cycle (TCA) turnover and the balance of carbon metabolism and electron transport. From the mitochondrial transmembrane potential and oxygen consumption measurements in presence of P. falciparum mETC inhibitors, the evidence of an alternative respiratory pathway in which electrons are transferred directly from coenzyme Q to oxygen was underlined. This cyanide-resistant alternative respiratory pathway was inhibited by two inhibitors, propyl gallate and SHAM, which are specific inhibitors of AOX activity in other organisms. The AOX gene has been characterized in several organisms such as plants, yeast, bacterium, and notably from other parasites, Trypanosoma brucei and Cryptosporidium parvum. Nevertheless, until now, no gene encoding AOX has been detected in the P. falciparum genome but 60% of the predicted genes could not be assigned to orthologous functions.

In the present work, a two dimensional-differential gel electrophoresis (2D-DIGE) approach, combined with mass spectrometry (MS) analysis, was used to define the consequences of SHAM on the P. falciparum proteome. Studies were previously reported that the hyperoxia induces a cycle delay on P. falciparum of 4 hours with a biological adaptation of the parasite to next cycle. The deleterious effect of this drug on the in vitro parasite growth in hyperoxia, the involved metabolic pathways, and its mode of action on glycolysis are discussed, supporting the hypothesis that the energetic metabolism may indeed provide an attractive chemotherapeutic target.

**Methods**

**SHAM or/and hyperoxia exposure of P. falciparum cultures**

The in vitro antimalarial activity of SHAM was determined using the isotopic semi microtest method as previously described by Desjardins et al. Drug concentrations were from 0.5 to 600 $\mu$M. The IC$_{50}$, ie, the drug concentration corresponding to 50% of the uptake of [H] hypoxanthine by the parasite in drug-free control wells, was determined by nonlinear regression analysis of log dose-response curves.

The 3D7 P. falciparum strain was cultured in human A+ erythrocytes, as previously described. Cultures were tightly synchronized (with 4 hour intervals) using combined D-sorbitol and CS columns on a VarioMACS (Miltenyi Biotec, Paris, France) according to standard procedures.

To evaluate the effect of SHAM, hyperoxia, and the combinatory effect, cultures were adapted to hyperoxic conditions (21% oxygen, 5% carbon dioxide, 74% nitrogen gas mixture) and regularly synchronized during six P. falciparum parasites’ cycles before the addition of SHAM. The effects of SHAM alone and combined SHAM/hyperoxia were observed at 0, 24, 32, 48, and 78 hours (0 hours corresponding to SHAM addition at the ring stage). Viability, parasitemia, and morphology were monitored daily by examining blood smears stained with RAL 555 (RAL Diagnostics, Martillac, France) from SHAM addition at the ring stage.

To test the SHAM effects on P. falciparum proteome (3% parasitemia and 6% hematocrit), the drug was added to 25 mL culture medium at IC$_{50}$ and the parasites were further
incubated for 12 hours and harvested. For each proteomic experiment, four biological replicates were performed.

Protein extraction

After SHAM pressure, parasitized erythrocytes (late ring stages aged 16–20 hours) were washed three times in phosphate buffered saline (PBS) medium (Invitrogen, Cergy Pontoise, France) and lysed in cold water-saponin (0.1%, Sigma, St Louis, MO) for 10 minutes. The lysate was then centrifuged at 1500 g for 5 minutes. The supernatant was discarded and the pellet containing free parasites was recovered by washing in cold PBS medium followed by a centrifugation step (1500 g for 5 minutes). The free parasites were washed until the supernatant became colorless. The pellet was then suspended in 4% (w/v) CHAPS (Sigma) and disrupted by ultrasonication (Vibracell 72412; Bioblock Scientific, Illkirch, France) 60 seconds on ice at maximum amplitude. The lysate was then centrifuged at 16,100 g for 15 minutes. The supernatant was further precipitated with 60% acetone (Sigma). The protein concentration for each sample was estimated using the BioRad Lowry-based DC assay (BioRad Laboratories, Hercules, CA), according to the manufacturer’s instructions. Total proteins were suspended in standard cell lysis buffer (7 M urea, 2 M thiourea, CHAPS, 30 mM Tris base, pH 8.5 [Sigma]) to obtain a protein concentration adjusted to 2.5 µg/µL. Protein samples were minimally labeled with CyDye according to the manufacturer’s protocols (GE Healthcare, Piscataway, NJ).

Briefly, protein extracts (50 µg) were labeled with 400 pmol of CyDye, freshly dissolved in anhydrous dimethyl formamide (Sigma) and incubated on ice for 30 minutes in the dark. The reaction was quenched with 1 µL of free lysine (10 nM, Sigma) by incubating for 10 minutes on ice. An equal volume of 2× sample buffer (8 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris base, pH 8.5 [Sigma]) obtained a protein concentration adjusted to 2.5 µg/µL. The samples were equilibrated in an equilibration buffer containing 50 mM Tris hydrochloride, pH 8.6, 6 M urea, 2% sodium dodecyl sulfate (SDS) and 30% glycerol supplemented with 1% (w/v) DTT for 15 minutes at room temperature. The reaction was quenched with 1 µL of free lysine (10 nM, Sigma) by incubating for 10 minutes on ice. An equal volume of 2× sample buffer (8 M urea, 2 M thiourea, 4% CHAPS, 10 mM Dithiothreitol (DTT) and 1% (v/v) IPG buffer 3–10 [GE Healthcare]) was added to the CyDye-labeled sample. The mixture of labeled proteins was then separated by 2D-DIGE.

2D electrophoresis of parasite proteins, image analysis, and in-gel digestion

Isoelectric focusing was performed on 18 cm pH 3–10 linear IPG strips (GE Healthcare). Destreak buffer containing 1% (v/v) IPG buffer pH 3–10 was used for overnight rehydration of IPG strips. The samples were applied at the acidic end of the IPG strip using a cup-loading technique. Isoelectric focusing was carried out on an Ettan IPGphor II (GE Healthcare) electrophoresis unit at 20°C for a total of 45 kVh (ramp to 300 V in 3 hours, ramp to 1000 V in 6 hours, ramp to 8000 V in 3 hours, hold at 8000 V for 4 hours). IPG strips were equilibrated in a equilibration buffer containing 50 mM Tris hydrochloride, pH 8.6, 6 M urea, 2% sodium dodecyl sulfate (SDS) and 30% glycerol supplemented with 1% (w/v) DTT for 15 minutes at room temperature. The reaction was quenched with 1 µL of free lysine (10 nM, Sigma) by incubating for 10 minutes on ice. An equal volume of 2× sample buffer (8 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris base, pH 8.5 [Sigma]) was added to the CyDye-labeled sample. The mixture of labeled proteins was then separated by 2D-DIGE.

The samples were analyzed by nanoscale capillary liquid chromatography-tandem MS (nano LC-MS/MS). Purification and analysis were performed on a C18 capillary column using a CapLC system (Waters, Milford, MA) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF Ultima; Waters). Chromatographic separation was conducted on a reversed-phase capillary column (Atlantis™ dC18, 3 µm, 75 µm × 150 mm NanoEase™; Waters) with a 180–200 nL/minute flow. The gradient profile consisted of a linear
gradient from 95% A (water, 0.1% formic acid) to 60% B (80% acetonitrile, 0.1% formic acid) in 60 minutes followed by a linear gradient by 95% B in 10 minutes. Mass data acquisitions were piloted by MassLynx 4.0 software (Micromass Ltd, Manchester, UK) using automatic switching between MS and MS/MS modes. The internal parameters of Q-TOF were set as follows. The electrospray capillary voltage was set to 3.2 kV, the cone voltage was set to 30 V, and the source temperature was set to 80°C. The MS survey scan was m/z 400–1,300 with a scan time of 1 second and an interscan time of 0.1 second. When the intensity of a peak rose above a threshold of 15 counts, tandem mass spectra were acquired. Normalized collision energies for peptide fragmentation were set using the charge-state recognition files for +2 and +3 peptide ions. The scan range for MS/MS acquisition was from m/z 50 to 1500 with a scan time of 1 second and an interscan time of 0.1 second. Fragmentation was performed using argon as the collision gas and with the collision energy profile optimized for various mass ranges and charges of precursor ions. Mass data collected during a nano LC-MS/MS analysis were processed using ProteinLynx Global Server 2.2 software (Waters) with the following parameters: no background subtraction, smooth 3/2 Savitzky Golay, and no deisotoping to generate peak lists in the Micromass pkl format. Pkl files were then fed into a local search engine Mascot Daemon v2.2.2 (Matrix Science, London, UK). The data were searched against the *Homo sapiens* (218,356 sequences) and *P. falciparum* (13,110 sequences) National Center for Biotechnology Information nonredundant (NCBInr) protein databases (March 15, 2010). Search parameters allowed for one missed tryptic cleavage site, the carbamidomethylation of cysteine, and the possible oxidation of methionine; precursor and product ion mass error tolerance was <0.2 Da. All identified proteins had a Mascot score greater than 34 and 43 for *P. falciparum* and *H. sapiens*, respectively, corresponding to statistically significant identification (*P* < 0.05).

**Results**

**SHAM and hyperoxia combinatorial effect on the asexual blood cycle of *P. falciparum***

To study the deleterious effect of SHAM on asexual blood stages of *P. falciparum* in hyperoxia, 21% oxygen-adapted cultures of 3D7 were exposed to SHAM IC$_{50}$ of 246 ± 4 μM. Consistent with previously published results, SHAM inhibited growth of the 3D7 strain with an average IC$_{50}$ of 246 ± 4 μM. To determine the SHAM effect on asexual blood stages of *P. falciparum*, the drug was added at the IC$_{50}$ concentration on synchronous cultures of 3D7 at the ring stage. The parasitemia and parasitic stage percentages were monitored during two life cycles by blood smears at 0, 24, 32, 48, and 78 hours in three independent experiments (0 hours corresponding to SHAM addition at the ring stage). The parasitemia of SHAM-treated cultures was low at 32 hours compared to untreated parasites (Figures 1A and 1D, 0.6% parasitemia vs 0.25%, respectively). At the end of 48 hours under SHAM exposure, 54% of the parasites were degenerated but the parasites not affected by SHAM continued their cycle without phenotypic changes and lengthening of the parasitic cycle. Thus, the SHAM perturbed the parasite growth between the 24th and 32nd hours of the cycle at IC$_{50}$ concentration.

**SHAM-alteration to the *P. falciparum* proteome***

To study the SHAM effect on *P. falciparum*, a proteomic approach was performed in synchronized parasites exposed to SHAM IC$_{50}$. To identify altered *P. falciparum* proteins, 2D-DIGE experiments coupled to MS were performed. Four independent cultures of untreated and SHAM-treated *P. falciparum* were included in this analysis. After protein separation by 2D-DIGE using pH 3–10 IPG strips and
homogeneous 10% SDS-polyacrylamide gel electrophoresis, each gel was individually imaged, and all gel images were analyzed using DeCyder 6.5 software. Among 1998 matched protein spots, 18 spots were differentially modulated (|fold change| ≥ 1.5, P ≤ 0.05 t-test) between the control and SHAM treatment (seven and eleven spots were up- and down-modulated, respectively; Figure 2). The corresponding protein spots were excised from gels, analyzed by LC-MS/MS, and searched against *P. falciparum* and *H. sapiens* databases (NCBI). Eight protein spots not fulfilling the selection criteria were still selected and so submitted to MS analysis. Their P values were significant or very close to significance with
Salicylhydroxamic acid (SHAM) effect on *Plasmodium falciparum* 3D7 strain proteome. Representative data from a two-dimensional-differential gel electrophoresis experiment using a 10% homogenous sodium dodecyl sulfate polyacrylamide gel with pH range 3–10 are shown. The proteins from untreated *P. falciparum* parasites or treated parasites with 250 µM SHAM were labeled with Cy3 and Cy5, respectively. As determined by Decyder 6.5 software, protein spots that were up- and down-expressed on *P. falciparum* under SHAM treatment (*|FC|* ≥ 1.5, *P* ≤ 0.05 t-test) were marked with master numbers (Table 1). Bold and italicized numbers correspond, respectively, to identified proteins from *P. falciparum* and *Homo sapiens*.

|FC| closed from 1.5 (Table 1). With this last selection, a total of 26 protein spots were identified by MS that correspond to 10 *P. falciparum* and 16 *H. sapiens* specific proteins (Table 1). Among these eight last selected proteins, six correspond to isoforms supporting the abundance variation of significant deregulated proteins. Thus, some proteins were detected in more than one spot (eg, four for glyceraldehyde-3-phosphate dehydrogenase, three for catalase), suggesting the presence of different deregulated isoforms. So, the number of distinct proteins identified was five for *P. falciparum* and nine for *H. sapiens* (Table 1). The importation of human proteins into the parasite during erythrocytic cycle is a well-known phenomenon, as previously described in other studies.39

To determine the metabolic pathways perturbed under SHAM pressure, the identified proteins were classified using the NCBI Clusters of Orthologous Groups of proteins database (Table 1). Among the functional categories, some proteins were predicted to play a role in glycolysis, chaperone-assisted protein folding, and redox metabolism. Among the antioxidant proteins, human catalase, carbonic anhydrase I, and carbonic anhydrase II were altered in SHAM-treated *P. falciparum*.

**Discussion**

**SHAM and hyperoxia exposure affects *P. falciparum* growth**

Previous studies provided evidence for the existence of a *P. falciparum* AOX40 and supported the ability of SHAM
Plasmodium falciparum metabolism following salicylhydroxamic acid exposure

Other studies have examined the effects of cyanide, a complex IV inhibitor, on P. falciparum respiratory metabolism using a polarographic assay. In the presence of high concentrations of cyanide, 25% of the parasite oxygen consumption was residual, suggesting that the parasite exploits a cyanide-resistant alternative respiratory pathway. This residual oxygen consumption was totally inhibited by SHAM (coenzyme Q analog), a specific inhibitor of AOX enzymatic activity involved in this alternative pathway.

### Table 1

Proteins identified from the two dimensional-differential gel electrophoresis analysis in salicylhydroxamic acid-treated *Plasmodium falciparum*

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<tr>
<th>gi number</th>
<th>Gene identification</th>
<th>Protein name</th>
<th>MW (kDa)</th>
<th>pl</th>
<th>Master spot number</th>
<th>Significance (Mascot score)</th>
<th>Average ratio</th>
<th>t-test</th>
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<td></td>
<td>Glycolysis</td>
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<td>gi</td>
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<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>L-lactate dehydrogenase</td>
<td>34.33</td>
<td>7.12</td>
<td>2478*</td>
<td>146</td>
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<td>enolase</td>
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<td>Chaperone assisted protein folding</td>
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<td>60.93</td>
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**Notes:** The spot number corresponds to the same numbers as indicated in Figure 2. The Mascot gi number of the spots, their gene identification (gene corresponding as found in PlasmoDB), their name, the theoretical MW, and pl values, as well as the corresponding Mascot score are listed for MS/MS analysis (scores greater than 34 for *Plasmodium falciparum* and 43 for *Homo sapiens* are considered as significant [P < 0.05]). Paired average volume ratio (experiment salicylhydroxamic acid versus control) and P values (t-test) were obtained using DeCyder 6.5 software. *Protein spots do not fulfill both criteria of selection, but which were nevertheless submitted to MS analysis thanks to their P values or FC significant.

**Abbreviations:** MW, molecular weight; pl, isoelectric point, MS, mass spectrometry.

To potentiate the activity of atovaquone, a complex III inhibitor, in vitro chemosusceptibility assays. Other studies have examined the effects of cyanide, a complex IV inhibitor, on *P. falciparum* respiratory metabolism using a polarographic assay. In the presence of high concentrations of cyanide, 25% of the parasite oxygen consumption was residual, suggesting that the parasite exploits a cyanide-resistant alternative respiratory pathway. This residual oxygen consumption was totally inhibited by SHAM (coenzyme Q analog), a specific inhibitor of AOX enzymatic activity involved in this alternative pathway.
Our results indicate that SHAM (IC$_{50}$ 250 µM) induced the death of effectively 54% of the parasites but in hyperoxic conditions and to the same IC$_{50}$, parasite death is 100%. Therefore, in hyperoxic conditions, SHAM exposure has a deleterious effect on parasite survival. SHAM seems to inhibit the metabolic adaptation involved in hyperoxia, which is essential to parasite survival.

Consequently, as described in plants, SHAM action seems to prevent the decrease in the levels of reactive oxygen species (ROS), which are exceedingly produced in hyperoxia by the mETC. Because the AOX activity reduces oxygen, its activity should be dependent on the oxygen concentration and also on the coenzyme Q concentration and its redox state. This alternative pathway could provide a regulatory function for excess electrons when the mETC is saturated (as during hyperoxia) and thus, decrease ATP production (unpublished data). Additionally, this “electron leak” mechanism may improve potential repression of the TCA cycle by elevated levels of NADH and ATP.

**SHAM alters the P. falciparum proteome**

To investigate the metabolic pathways altered by the activity of SHAM, a comparative study on the P. falciparum proteome was performed using 2D-DIGE coupled to MS for identification. The experimental design (parasite stage, drug dose, incubation time) was chosen based on our preliminary results. In our study, SHAM was exposed at the IC$_{50}$ concentration to ring stage parasites for 12 hours.

Most SHAM-altered proteins are involved in glycolysis and redox metabolism. The major implication of glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase, L-lactate dehydrogenase, enolase, and phosphoglycerate mutase) suggests the need to sustain the major energy dependence of the parasite on glycolysis. Although the function of the P. falciparum mitochondrion remains unclear, its contribution to ATP production is considered to be minimal in asexual stages. P. falciparum relies principally on anaerobic glycolysis for energy production. Therefore, the parasites are dependent on glycolysis in a microaerophilic environment while in hyperoxia conditions; our previous results showed that the respiratory metabolism is favored at the expense of glycolysis. This observation could explain the lethal effect of SHAM in hyperoxic conditions. The NADH generated during glycolysis are reoxidized through glycerol-3-phosphate in the cytosol and by PfNDH2 in the mitochondria. In plants, the alternative respiratory pathway involves an alternative NADH dehydrogenase, coenzyme Q, and AOX. Mitochondrial AOX exists under two forms, covalent and noncovalent homodimers. The oxidized covalent form of the enzyme is inactive, whereas reducing the disulfide bond generated by NADH activates the enzyme, possibly mediated by a glutathione/thioredoxin coupling system. Interestingly, glycolysis activation under SHAM pressure could be the result of a metabolic adaptation in a microaerophilic environment. The end products of P. falciparum glycolysis are pyruvate, lactate, and glycerol. Firstly, pyruvate is a substrate for the mETC under stress conditions. Finally, glycerol production by the glycerol-3-phosphate permitted the reoxidation of NADH and the activity of the mETC.

Figure 3 A schematic representation of the Plasmodium falciparum metabolic pathways perturbed under salicylhydroxamic acid treatment. Glycolysis and mitochondrial respiratory chain pathways based on the Ginsburg website are represented.
of AOX activity involved several compounds, including those in redox states (NAD/NADH) and allosteric effectors (pyruvate). Thus, the existence of an AOX function in *P. falciparum* could be beneficial for the survival of the parasite in hyperoxic environments as in the salivary glands of mosquitoes or perhaps in the human lungs.

SHAM treatment leads to an inhibition of antioxidant proteins. Among the proteins identified, some originated from the human erythrocyte host and correspond to host proteins imported by the parasite or proteins arising from host–parasite interaction. Consequently, cytosolic catalase and carbonic anhydrase I were down-represented under SHAM pressure. While *P. falciparum* lacks the antioxidant enzyme catalase, host catalase was imported into the food vacuole and accomplished hydrogen peroxide detoxification. Thus, the down-importation of catalase could be attributed to a SHAM effect against oxidative damage by diminishing hydrogen peroxide production. Carbonic anhydrase has been identified as a major intracellular peroxidation target in erythrocytes and could be attributed to oxidative stress induced by SHAM in the parasitized erythrocytes.

In our study, a number of identified proteins (enolase, catalase, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase) have been previously found as potential glutaredoxin target proteins. In higher plants, the reduction of AOX in its active form involved a glutathione/thioredoxin coupling system, suggesting a SHAM effect on feedback regulation of AOX activity as described in higher plants. The other deregulated proteins were particularly involved in protein processing (chaperonin-containing T-complex 1), as well as translation (elongation factor 2), consequences of a general stress state of the parasite. Chaperone-assisted protein folding was described to contribute to protein folding, assembly, and translocation, inducing expression of proteins in these pathways under several types of cellular stress.

**Conclusion**

Although the AOX gene was not characterized yet in the *P. falciparum*, SHAM, an AOX specific inhibitor, disturbed the in vitro growth of *P. falciparum* in hyperoxic conditions. Additionally, recent studies sustained the existence of the alternative respiratory pathway in *P. falciparum*, illustrated by the identification of alternative NADH dehydrogenase, mostly described in some detail for plants. Despite the fact that the *P. falciparum* AOX gene was not yet identified, the metabolic pathways involved in SHAM response illustrates the existence of a similar AOX function. The failure of a comparative sequence analysis is mainly due to the extreme adenine-thymine bias (80%) of the parasite genome or the high gene variability between other species and *P. falciparum*. Nevertheless, an AOX-like function could play a role in oxidative stress defense and could be a major benefit to *P. falciparum*, which appears to be sensitive to hyperoxic conditions. Indeed, the respiratory metabolism was the predominant source of ROS on *P. falciparum*, and partial inhibition of this metabolism led to a decrease of ROS. This hypothesis is in agreement with a publication reporting the AOX protective role in preventing ROS production.

Several studies on the use of mETC inhibitors underlined the crucial role of respiratory metabolism to parasite survival. However, much work remains to scrutinize the specific biochemical pathways of the *P. falciparum* mitochondria. This unique particularity of the alternative respiratory pathway could be a promising target for the development of a new group of antimalarial drugs.

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**Disclosure**

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