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Research Paper

The antimalarial drug primaquine targets Fe–S cluster proteins and yeast respiratory growth

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ABSTRACT

Malaria is a major health burden in tropical and subtropical countries. The antimalarial drug primaquine is extremely useful for killing the transmissible gametocyte forms of *Plasmodium falciparum* and the hepatic quiescent forms of *P. vivax*. Yet its mechanism of action is still poorly understood. In this study, we used the yeast *Saccharomyces cerevisiae* model to help uncover the mode of action of primaquine. We found that the growth inhibitory effect of primaquine was restricted to cells that relied on respiratory function to proliferate and that deletion of *SOD2* encoding the mitochondrial superoxide dismutase severely increased its effect, which can be countered by the overexpression of *AIM32* and *MCR1* encoding mitochondrial enzymes involved in the response to oxidative stress. This indicated that ROS produced by respiratory activity had a key role in primaquine-induced growth defect. We observed that Δ*sod2* cells treated with primaquine displayed a severely decreased activity of aconitase that contains a Fe–S cluster notoriously sensitive to oxidative damage. We also showed that *in vitro* exposure to primaquine impaired the activity of purified aconitase and accelerated the turnover of the Fe–S cluster of the essential protein Rli1. It is suggested that ROS-labile Fe–S groups are the primary targets of primaquine. Aconitase activity is known to be essential at certain life-cycle stages of the malaria parasite. Thus primaquine-induced damage of its labile Fe–S cluster – and of other ROS-sensitive enzymes – could inhibit parasite development.

1. Introduction

Malaria is a mosquito borne infectious disease that causes flu-like symptoms and can lead to organ failure and death. In 2013, an estimated 198 million cases of malaria were registered by the World Health Organisation (WHO, 2014).

The disease is caused by a unicellular eukaryotic parasite of the genus *Plasmodium*. Among the five species known to infect humans, *P. falciparum* is the most dangerous and *P. falciparum* and *P. vivax* are the most frequently encountered worldwide. *Plasmodium* parasites have a complex life cycle during which they adopt different forms [1]. In the context of malaria control and elimination, an increasing effort is focused on the transmissible blood stage forms of the parasite known as gametocytes and on the quiescent liver stage forms of *P. vivax* and *P. ovale* that cause relapses, known as hypnozoites. Targeting these parasite reservoirs currently relies solely on drugs. Primaquine is the only approved drug effective against hypnozoites and is also an efficient transmission-blocking agent through its gametocytocide activity (for review [2]).

Primaquine (PQ)[1] is an 8-aminoquinoline developed in the 1940's that displays antimalarial activity at low micromolar concentrations against hypnozoites [3] and gametocytes [4–6]. Despite this remarkable activity, its precise mechanism of action is still poorly understood. Ultrastructural and functional studies suggest that PQ treatment affects the parasite mitochondrion [7–10]. In humans, PQ is transformed into several metabolites mainly by the cytochrome P450 (CYP450) 2D6 and by the monoamine oxidase A [11]. Some PQ phenolic metabolites have been described to be the active compounds against *Plasmodium* parasites and are

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most likely to act through an oxidative killing mechanism [12–14]. PQ was also shown to induce oxidative stress in mammalian cells [15,16]. In addition, PQ treatment provokes severe side effects in people deficient in erythrocytic enzymes controlling oxidative stress, such as the glucose-6-phosphate dehydrogenase (G6PD) and the NADH-methemoglobin reductase [17,18].

Here we explored the mechanism of action of PQ in the yeast *S. cerevisiae*. Yeast has well-known advantages for such studies, providing valuable genetic and biochemical tools. Deletion of non-essential genes is straightforward and collections of deletion mutants are available. In addition, yeast can survive in the absence of respiration by using the fermentation process as an energy source. Therefore the detrimental effect of a compound, such as PQ, can be tested in cells lacking enzymes involved in antioxidant defence with or without active respiratory function. Using this approach, we identified genes involved in PQ sensitivity. The data support key roles for oxidative damage and respiratory function in the action of PQ. We then tested the consequence of PQ exposure on Fe–S cluster containing enzymes known to be susceptible to oxidative damage.

[1] List of abbreviations: PQ, primaquine; SOD, superoxide dismutase; SO, superoxide; ROS, reactive oxygen species; OD, optical density; wt, wildtype strain; Fe–S, Iron–Sulphur.

2. Materials and methods

2.1. Chemicals

Primaquine, sodium L-ascorbate, DL-isocitric acid, L-malic acid, n-acetyl-L-cysteine, mitochondrial aconitase from porcine heart and *cis*-aconitate were purchased from Sigma Aldrich.

2.2. S. cerevisiae strains and culture media

S. cerevisiae strains from the series BY4742 (*MAT*α; *his3*Δ; *leu2*Δ; *lys2*Δ; *ura3*Δ), including this wt (wildtype) and derived isogenic deletion strains, were from Euroscarf (Frankfurt, Germany). The wt strain CWWT (*MAT*α *ade2-1 his3- leu2-3,112 trp1-1 ura3-1*) is derived from W303-1B.

The following growth media were used: YPD (1% yeast extract, 2% peptone, 2% glucose), YPEth (1% yeast extract, 2% peptone, 2% ethanol), YPG (1% yeast extract, 2% peptone, 3% glycerol), YPGal (1% yeast extract, 2% peptone, 0.2% glucose, 2% galactose), YP10 (1% yeast extract, 2% peptone, 10% glucose), and CSM (0.7% yeast nitrogen base, 2% glucose, 2% agar and 0.8 g/l of a complete supplement mixture minus uracil or minus leucine, supplied by Bio 101 (san Diego, CA, USA)).

2.3. Primaquine sensitivity test

PQ sensitivity was assessed by monitoring the inhibition of yeast cell proliferation. Yeasts were grown in 5 ml culture medium with increasing concentrations of primaquine. Cultures were inoculated from two-day-old cultures on YPG to an $OD_{600\ nm}$ of 0.1 and incubated at 28 °C. Cell density measured as $OD_{600\ nm}$ was estimated when the control cultures had reached stationary phase, one or three days after inoculation, depending on the culture medium. PQ sensitivity is presented for each strain and culture condition as the percentage of growth relative to control, *i.e.* untreated by PQ.

2.4. Generation of rho° mutants

Rho° mutants (devoid of mitochondrial DNA) were generated by growing cells on YPD medium containing 40 g/L of ethidium bromide for three rounds of culture.

2.5. Assessment of rho$^-$ or rho° production

The mutant Δ*sod2* was grown in YPD at 1 mM PQ. Three rounds of 24 h culture were performed. Cells were then diluted and spread on YPD plates to obtain approx. 200 cells per plate. After three days growth, the colonies were replica-plated to YPG agar. The number of colonies able to grow on this respiratory medium was recorded. This was compared to data obtained with untreated cultures.

2.6. Isolation of multicopy suppressors of primaquine sensitivity

The PQ-sensitive Δ*sod2* mutant was transformed with a high-copy wild type genomic library made in the *URA3* 2μ vector pFL44L [19]. Approximatively 20,000 Ura$^+$ clones were selected and replica-plated onto YPG containing 500 μM PQ. Clones with increased resistance to PQ appearing after 4–5 days were analysed. The plasmid-borne resistance to PQ was rechecked. The chromosomal fragments present on the plasmids were then identified by sequencing.

2.7. Gene cloning on high copy number plasmid for gene overexpression

SOD2, MCR1, SOD1 and *LYS7* genes with their own promoter region were cloned in the multicopy vector pFL44-URA3 (*SOD2* and *MCR1*), YEp352-URA3 (*SOD1*) or YEp351-LEU2 (*LYS7*). The fragment encompassing the *SOD2* gene (open reading frame (ORF) and native promoter) was digested with *BamH*I and *Xma*I enzymes from a vector isolated from the high copy number library containing the *SOD2* gene and then ligated between the *BamH*I-*Xma*I sites of the pFL44 vector. Fragments encompassing the *SOD1* and *LYS7* genes were digested with *EcoR*I and *Xba*I enzymes (*SOD1*) or *Sal*I and *Sac*I enzymes (*LYS7*) from the pRS405-SOD1 vector and pRS403-LYS7 vectors respectively. Those two vectors were kindly supplied by P. Piper, University of Sheffield, UK. The fragments were then ligated between the corresponding sites in the relevant destination vectors (see above). The *MCR1* gene fragment was amplified from yeast genomic DNA with the *Sal*I_MCR1_F (CATAGTCGACAATGCAAACTCTCCCACCAG) and MCR1_R (CTGCCAAGAAGACGTTGGTT) primers, digested with *Sal*I and *Sph*I enzymes and then ligated between the *Sal*I-*Sph*I sites of the pFL44 vector.

2.8. Aconitase and fumarase measurement using cell extracts

The aconitase and fumarase activities were determined spectrophotometrically by monitoring the formation of cis-aconitate and fumarate at 240 nm and 25 °C [20–22]. Briefly, cell extracts were prepared from 2.0×10^8 cells (OD_{600} ~20) grown on YPGal. Lysis was performed at 4 °C in 10 mM MES buffer, pH6 containing 0.6 mM $MnCl_2$ and deprived of oxygen (by bubbling with nitrogen gas) with 0.5 mm glass beads (v/v), by vortexing for 30 s followed by incubation on ice for 30 s, repeating the process seven times. Cell debris were removed by centrifugation at 13,000 rpm for 5 min, and the resulting supernatant was aliquoted and frozen immediately in liquid nitrogen and kept at −80 °C (for a maximum of one month). Samples were thawed just before the assay [23]. Protein concentration was determined spectrophotometrically with the Bio-Rad protein assay kit, according to the manufacturer's instructions.

For the aconitase activity, the assay mixture contained 50 mM potassium phosphate buffer, pH 7.4, 30 mM sodium isocitrate, 0.6 mM $MnCl_2$, and 150–250 μg of protein for a final volume of 1 ml. For the fumarase activity, the assay mixture contained

50 mM potassium phosphate buffer, pH 7.4, 50 mM L-malic acid and 150–250 μg of protein for a final volume of 1 ml. The absorbance changes were measured for 5–30 min, and the activity was calculated from the slope of the linear portion; $\varepsilon_{240}=3.6\ mM^{-1}\ cm^{-1}$ for cis-aconitate and $\varepsilon_{240}=2.44\ mM^{-1}\ cm^{-1}$ for fumarate.

2.9. bc_1 complex and succinate dehydrogenase activity measurement using purified mitochondria

Yeast mitochondria were prepared as in [24]. Briefly, yeast cells grown in YPGal medium were harvested at mid-log phase. Protoplasts were obtained by enzymatic digestion of the cell wall using zymolyase in an osmotic protection buffer. Mitochondria were then prepared by differential centrifugation following osmotic shock of the protoplasts. Mitochondrial samples were aliquoted and stored at −80 °C.

The concentration of bc_1 complex in the mitochondrial samples was determined from dithionite-reduced optical spectra, using $\varepsilon=28.5\ mM^{-1}\ cm^{-1}$ at 562 nm–575 nm. Decylubiquinol-cytochrome *c* reductase activities were determined at room temperature by measuring the reduction of cytochrome *c* (final concentration of 20 μM) at 550 nm *versus* 540 nm over a one-minute time-course in 10 mM potassium phosphate pH 7, 0.01% (w/v) Lauryl-maltoside and 1 mM KCN. Mitochondria were added to obtain a final concentration of 4 to 10 nM bc_1 complex. Activity was initiated by the addition of 40 μM decylubiquinol. The measurements were repeated three to five times and averaged.

Succinate cytochrome *c* reductase activity was assessed by measuring the reduction of cytochrome *c* as described for bc_1 complex activity. With the exception that the activity was initiated by the addition of 10 mM succinate in a 50 mM phosphate buffer without Lauryl-maltoside. Mitochondria were added to obtain a final concentration of 20 to 30 nM bc_1 complex.

2.10. Oxygen uptake measurements with intact cells

Oxygen consumption rates were measured in a stirred reaction vessel of a Clark-type O_2 electrode at 25 °C. Whole cells oxygen uptake assays were carried out using around 10^8 cells mL^{-1} (grown in YPGal) in fresh YPGal medium with and without 3 μM CCCP (carbonyl cyanide m-chlorophenylhydrazone). After 2–3 min recording, PQ was added at various concentrations and the rate of oxygen consumption was recorded.

2.11. Aconitase measurement using porcine mitochondrial aconitase

100 mg of mitochondrial aconitase from porcine heart (Sigma) was dissolved in 25 mM Hepes pH 7.8 and loaded on a HiScreen Capto Q ImpRes column (GE Healthcare). Proteins were eluted with a linear NaCl gradient (0–1 M). Fractions containing aconitase were pooled and concentrated on a Vivaspin 30 K concentrator (Sartorius). Aconitase was reactivated by incubation for 1 h at room temperature under anaerobic conditions in a glove box (Jacomex, $O_2 < 9$ ppm) with 5X_Mohr's salt and Na_2S and loaded on a NAP-5 gel filtration column (GE Healthcare) equilibrated with 25 mM Hepes (pH 7.8) and 100 mM NaCl. Aconitase activity was measured in degazed 100 mM Tris–HCl pH 7.4 0.4 mM sodium citrate at 37 °C. The absorbance changes were measured for 15 min, and the activity was calculated from the slope of the linear portion using $\varepsilon_{240}=3.6\ mM^{-1}\ cm^{-1}$. The specific activity of purified mitochondrial aconitase was of 2500 u μg^{-1}.

2.12. ^{55}Fe-labelling and Fe-S cluster turnover in Rli1

For *in vitro* analysis of iron turnover, 200 ml culture (BY4741 transformed with high-copy *tet* bearing plasmid (pCM190) overexpressing HA-tagged Rli1) [25] that had been pre-incubated for 3 h with $^{55}FeCl_3$ (36 μCi) (Perkin-Elmer) was harvested by centrifugation (1500*g*, 5 min). Cells were washed and resuspended in lysis buffer (400 μl oxygen-free 50 mM phosphate buffer, pH 7.4, 3% (v/v) glycerol, 5 mM PMSF (Sigma-Aldrich, St. Louis, MO), EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN)) together with 500 μl of glass beads, diameter 425–600 μm. Samples were vortexed at maximum speed three times for 1 min, interspersed with three 1-min cooling periods on ice, before centrifugation at 16,000*g* for 5 min. Protein in the supernatant was determined with a Bradford assay kit (Bio-Rad, Hercules, CA). Protein (~500 μl) was mixed with 80 μl anti-HA beads (A2095; Sigma-Aldrich) for 1 h at 4 °C. Beads were washed four times with lysis buffer. Aliquots of the beads were incubated aerobically for 10 min at room temperature with 350 μM sodium L-ascorbate and 100 μM histidine [25,26] in the absence or presence of primaquine. Beads were collected by centrifugation, suspended in 5 ml scintillation fluid (Emulsifier Safe; Perkin Elmer-Cetus, Waltham, MA), and bead-associated ^{55}Fe measured with a Packard Tri-Carb 2100TR liquid scintillation analyzer (Meriden, CT).

3. Results

3.1. Primaquine inhibits yeast respiratory growth

Yeast can use both respiratory function and fermentation to produce energy. With ethanol or glycerol as carbon source (YPEth and YPG media) the cells exclusively rely on respiratory function to generate energy and thus to proliferate. With glucose or galactose as carbon source (YPGal and YPD media) and vigorous aeration, cells use both respiration and fermentation. Yeast use fermentation only in high glucose (YP10 medium) and low oxygen (without aeration) conditions. *Rho*° mutants that lack the mitochondrial genome and thus respiratory chain enzymes grow exclusively by fermentation in YPD or YPGal medium with vigorous aeration. By comparing drug effects on yeast growth in these culture conditions, the role of respiratory function in drug action could be assessed.

We first tested the effect of PQ on the respiratory growth of the wt strain CWWT. The strain was cultivated in YPEth medium with and without primaquine (PQ). Cultures were inoculated at an $OD_{600\ nm}$ of 0.1 and incubated at 28 °C with vigorous aeration. In parallel, the effect on fermentative growth was monitored. The CWWT strain was grown in YP10 medium without aeration with or without 2 mM PQ, and a *rho*° mutant derived from CWWT was cultured in YPGal medium with vigorous aeration with and without 2 mM PQ.

Cell densities were measured as $OD_{600\ nm}$ when the cultures reached the stationary phase. The data are presented as percentage of growth relative to control, *i.e.* untreated by PQ (Fig. 1A). At 2 mM PQ, respiratory growth was severely inhibited but not fermentative growth.

3.2. Sod2 deficiency increases the inhibitory effect of primaquine on yeast respiratory growth

As PQ was reported to induce oxidative stress in eukaryotic cells [15,16], and to interfere with mitochondrial ultrastructure and function in *Plasmodium* cells [7–10], we checked the inhibitory effect of PQ on the respiratory growth of a series of mutants with deletions of genes involved in oxidative stress defence (17 genes), in mitochondrial and/or nuclear DNA repair (three genes), in the mitochondrial respiratory chain (six genes), or in mitochondrial membrane organisation (one gene). The genes tested and the role and subcellular localisation of the gene products are listed in Table

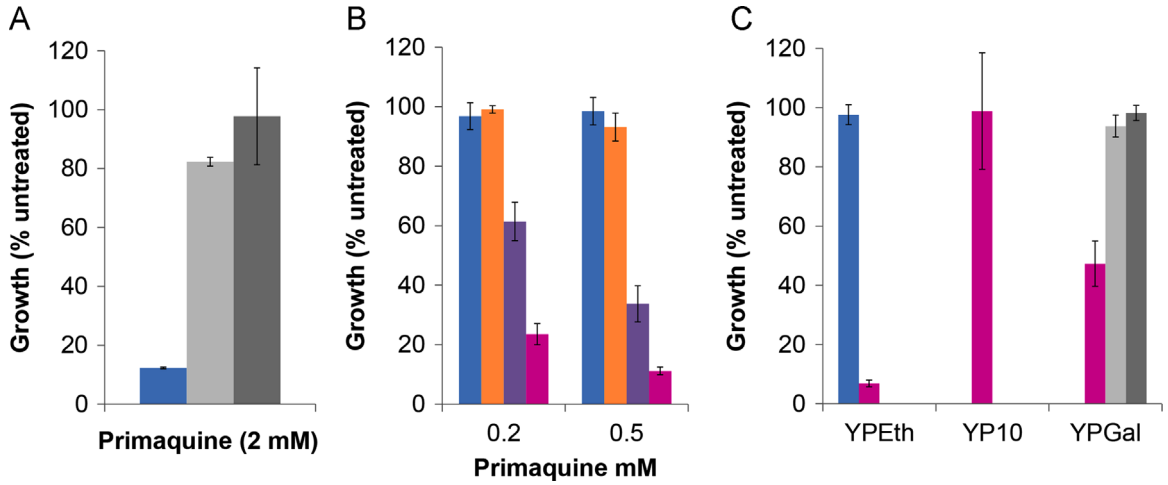

Fig. 1. Inhibitory effect of primaquine on yeast growth. Yeast wt and mutant strains were grown in different culture media in the absence or presence of PQ. Cells from a two-day-old culture on YPG agar were inoculated to an OD600 nm of 0.1 in 5 ml. Cell densities (OD600 nm) were estimated after one to three days growth. The data are presented as percentages of the culture OD600 nm without PQ. The measurements were repeated two to eight times and the data averaged. Error bars represent the standard deviation. A. Effect of primaquine on respiratory and fermentative growth. The CWWT strain was grown in YPEth with vigorous aeration (blue) and in YP10 without aeration (light grey). A rho° mutant derived from CWWT was cultured in YPGal with vigorous aeration (dark grey). B. Effect of gene deletion on primaquine sensitivity. Wt BY4742 (blue), Δccp1 (orange), Δsod1 (purple) and Δsod2 (magenta) strains were grown in YPEth in the absence or presence of 0.2 and 0.5 mM PQ with vigorous shaking for aeration. C. Effect of primaquine on respiratory and fermentation growth of Δsod2 cells. The cultures were supplemented with 1 mM PQ. Δsod2 was grown in YPEth (magenta), in YP10 without aeration (magenta), in YPGal (magenta) and in YPGal plus 0.4 μM azoxystrobin (light grey), with vigorous aeration. A rho° mutant derived from Δsod2 was cultured in YPGal with vigorous aeration (dark grey). Its control wt strain BY4742 was grown in YPEth (blue).

S1 (supplemental data). The wt strain B4742 and its derived deletion mutants were cultured in the presence or absence of PQ and the cell density (estimated by the OD at 600 nm) was measured after three days of incubation at 28 °C with vigorous shaking for good aeration. A first screen was performed in YPG medium (supplemental Fig. S1) and the data confirmed by monitoring the growth in YPEth medium, as illustrated in Fig. 1B. Of the 27 mutants tested, only two mutants clearly displayed an increased susceptibility to PQ: Δ*sod2*, lacking the mitochondrial superoxide dismutase and Δ*sod1*, lacking the mainly cytosolic superoxide dismutase. The Δ*sod2* mutant showed a more severe sensitivity than Δ*sod1*.

We then compared the effect of PQ on the respiratory and fermentative growth of Δ*sod2*. To achieve exclusive respiratory growth, the strain was cultivated in YPEth with vigorous aeration. To obtain exclusive fermentation, three culture conditions were used: Δ*sod2* grown in YP10 without agitation; Δ*sod2* cultured in YPGal with vigorous aeration in the presence of 0.4 μM azoxystrobin, which fully and specifically inhibits respiratory chain complex III activity; Δ*sod2 rho°*, generated by ethidium bromide treatment, cultured in YPGal with vigorous aeration.

As shown in Fig. 1C, the Δ*sod2* mutant growing by fermentation (YP10, YPGal plus azoxystrobin and *rho°* mutant in YPGal) was found to be insensitive to 1 mM PQ, while its respiratory growth (YPEth) was severely decreased. The respiratory growth of the parental wt strain BY4742 was not decreased by 1 mM PQ. That strain was found to be less sensitive to PQ than the CWWT strain. At 1 and 2 mM PQ, CWWT growth yield reached respectively 50% and 12% of control yield while, at the same concentrations, BY4742 growth yield reached 100% and 67% of control yield. The Δ*sod1* mutant displayed a behaviour similar to Δ*sod2*; its PQ sensitivity was observed only when cells grew by respiration (not shown).

We also monitored the effect of PQ on Δ*sod2* with an active respiratory function grown in YPGal with vigorous aeration. Addition of 1 mM PQ in YPGal (supporting both respiration and fermentation) decreased the growth yield by approximately 50% while in YPEth (exclusively respiration), PQ had a more severe effect (Fig. 1C). The increase in PQ sensitivity correlated with increasing reliance on respiration.

The results indicated that an active respiratory function is required for the inhibitory action of PQ on Δ*sod2* and Δ*sod1* cells, as observed with the control strain CWWT.

3.3. Chemical and genetic rescue of primaquine sensitivity of Δsod2

In order to get more insight to the inhibitory action of PQ, we tested possible means to rescue PQ sensitivity during respiratory growth of Δ*sod2* cells.

First we checked whether the decreased respiratory growth of the Δ*sod2* mutant in the presence of PQ could be rescued by antioxidant agents. As shown in Fig. 2, the addition of 5 mM ascorbate or n-acetyl cysteine (NAC) to the culture medium largely restored the growth of Δ*sod2*.

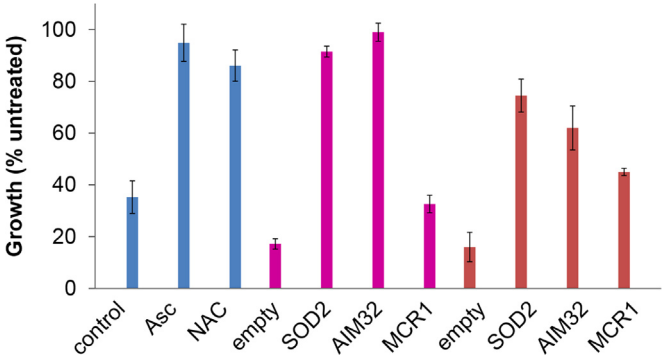

Fig. 2. Effect of ascorbate and N-acetyl cysteine, and of the over-expression of *SOD2*, *AIM32* and *MCR1* on primaquine susceptibility of the Δ*sod2* mutant. Cells from a two-day-old culture on YPG agar were inoculated to an $OD_{600\ nm}$ of 0.1 in 5 ml of YPEth or YPG with or without PQ. Ascorbate (Asc) or N-acetyl cysteine (NAC) were added at a concentration of 5 mM. The cultures were incubated at 28 °C with vigorous shaking for aeration. Cell densities were estimated after three days. The sensitivity to PQ is presented for each condition or strain as the percentage of growth relative to the control, *i.e.* $OD_{600\ nm}$ without PQ. Values represent the averages of two or three independent cultures, with error bars representing standard deviation. Δ*sod2* grown in YPEth with 0.2 mM primaquine with or without Asc or NAC (blue); Δ*sod2* and Δ*sod2* overexpressing *SOD2*, *AIM32* or *MCR1* grown in YPEth with 0.3 mM primaquine (magenta); Δ*sod2* and Δ*sod2* overexpressing *SOD2*, *AIM32* or *MCR1* grown in YPG with 0.1 mM primaquine (brown).

We then sought to identify gene functions that could suppress the PQ sensitivity. To that end, the Δ*sod2* mutant was transformed with a multicopy genomic library and PQ resistant transformants were selected (Materials and Methods). Six transformants showed a clear co-segregation between plasmid and PQ resistance. The inserts of the six isolated plasmids were sequenced. Four plasmids contained a segment of chromosome VIII that included *SOD2*. One plasmid had a fragment of chromosome XIII that included two partial genes and the complete *AIM32* gene. One plasmid had a fragment of chromosome XI including two partial genes, the arginine t-RNA and seven other genes (*RPS27A, RSM22, SRP102, GPM1, YKL151C, MCR1*, and *DBR1*). Amongst these genes, *MCR1* seemed the most likely candidate as it encodes the mitochondrial NADH-cytochrome b_5 reductase. In order to verify that the suppression of PQ sensitivity resulted from overexpression of *MCR1*, the gene with its own promoter was cloned into a multicopy plasmid (Materials and Methods) and used to transform Δ*sod2*. As a control, the *SOD2* gene with its own promoter was also cloned in a multicopy plasmid and used to transform Δ*sod2*.

As shown in Fig. 2, the overexpression of *SOD2* and *AIM32* suppressed the inhibitory effect of PQ on Δ*sod2* cells, both in YPEth and YPG. The overexpression of *MCR1* resulted in a partial suppression of PQ sensitivity, more clearly observed in YPG.

We also tested if *SOD1* overexpression could suppress the PQ susceptibility of *Δsod2* cells. As previously reported [27], increased Sod1 activity can only be obtained if Ccs1, the chaperone that recruits copper to Sod1, is also expressed at higher level. Therefore, the *Δsod2* mutant was transformed with two multicopy vectors, one containing *SOD1* and the second containing *CCS1* (also named *LYS7*). The overexpression of *SOD1* and its cofactor did not suppress PQ susceptibility of the *Δsod2* mutant (data not shown).

We sought to check whether the PQ sensitivity of the wt strain CWWT (see Fig. 1A) could be rescued by the overexpression of *SOD2* or *AIM32*. However, overexpression of *SOD2* had a deleterious effect on the respiratory growth of CWWT (in the absence of PQ) as the growth yield of the transformed strain reached only 25% of the growth yield of the control strain. Analysis of the *SOD2* overexpressing strain was not pursued further. The overexpression of *AIM32* had no effect on the respiratory growth and PQ sensitivity of CWWT. At 2 mM PQ, the growth yield, monitored as described in Fig. 1A, reached 10–15% of the control yield. It seems therefore that Aim32 confers protection only in Sod2-deficient cells. By comparison, we tested the effect of NAC on the PQ sensitivity of CWWT. The strain was grown in YPEth with 5 mM NAC (as in Fig. 2) with or without 2 mM PQ. Addition of NAC partly rescued the PQ sensitivity as the growth yield reached 45% of the untreated growth yield, while it reached 12% in absence of NAC.

It would have been interesting to check whether overexpression of *SOD2*, *AIM32* or *MCR1* could rescue the PQ sensitivity of Δ*sod1* cells in respiratory medium. However overexpression of these genes had a deleterious impact on Δ*sod1* respiratory growth (in the absence of PQ), precluding the test.

The impact of deleting *AIM32* and *MCR1* on PQ sensitivity was tested. The respiratory growth of Δ*aim32* and Δ*mcr1* mutants in YPEth and YPG was not more sensitive to PQ than that of the wild type (*i.e. SOD2*), so deletion of these genes did not affect wild-type PQ sensitivity.

Thus, by searching for multicopy suppressors of Δ*sod2* sensitivity to PQ, two genes were identified; *AIM32* and *MCR1*. Mcr1 is a NADH-cytochrome b_5 reductase located in the inter-membrane space of mitochondria, involved in oxidative stress defence [28–30]. The function and location of Aim32 are not well defined. Sequence analysis showed that the protein is located in the mitochondria and contains a thioredoxin-like domain [31,32].

3.4. Primaquine impairs activity of the Fe–S cluster enzyme aconitase in Δsod2 cells

The absence of the mitochondrial Sod2 markedly enhanced PQ inhibitory action on yeast respiratory growth, which could be countered by anti-oxidant molecules and by the overexpression of genes encoding mitochondrial proteins with known or possible anti-oxidant action.

We thus asked whether PQ treatment could have a detrimental effect on mitochondrial function.

We first checked whether PQ could act as a direct inhibitor of respiratory function. To that end, the effect of PQ on the oxygen consumption of intact cells was tested using a Clark-type oxygen electrode (Materials and Method). Addition of 1 mM PQ in the assays had no effect on the oxygen uptake activities of Δ*sod2* and wt cells. It thus appears that PQ did not act as an inhibitor of the respiratory function.

We also checked whether more prolonged treatment to PQ could impair the overall respiratory function of cells. Δ*sod2* and wt cells were grown in YPGal with or without 1 mM PQ for 24 h. The cells were then harvested and their oxygen consumption activities were directly monitored (Materials and Methods). The activities were unaltered by PQ exposure (Table S2). Thus prolonged treatment with 1 mM PQ had no significant deleterious effect on the overall oxygen consumption of Δ*sod2* whereas its growth yield was decreased by 50% in YPGal and by 90% in YPEth (Fig. 1C).

In parallel, we tested whether PQ treatment might result in the loss of mtDNA, and thus increase the production of *rho*° cells. Δ*sod2* cells were cultured in YPGal with 1 mM PQ and the production of *rho*° cells was assessed as described in the Materials and Methods. The treatment had no effect. Thus at the doses tested, PQ did not appear to alter mtDNA stability.

We then tested the effect of PQ treatment on aconitase activity as the TCA cycle enzyme, located in the mitochondrial matrix is known to be highly sensitive to oxidative damage. As a control, the activity of the mitochondrial enzyme fumarase, that is not sensitive to oxidative damage, was also monitored. Wt and Δ*sod2* cells were cultured for 24 h in YPGal in the absence or in presence of 0.3 or 1 mM PQ. Aconitase and fumarase activities were monitored as described in Materials and Methods.

Under basal conditions (no PQ treatment), aconitase activity was decreased about 1.5 fold in Δ*sod2* compared to wt cell extracts, whereas the fumarase activity was similar in wt and Δ*sod2*. After PQ treatment, the aconitase and fumarase activities of wt were not altered compared to untreated wt controls. By contrast, the aconitase activity of Δ*sod2* was further decreased (2.5 fold for 0.3 mM PQ and 4.5 fold for 1 mM PQ) compared to wt while the fumarase activity remained unchanged. Thus as presented in Fig. 3, the aconitase/fumarase ratio of 0.65 remained unchanged in the wt upon PQ treatment whereas in Δ*sod2* the ratio decreased from 0.35 (untreated) to 0.2 (0.3 mM PQ) and 0.1 (1 mM PQ).

Thus PQ treatment resulted in marked decrease in the activity of the mitochondrial aconitase that contains ROS-sensitive FeS cluster. It is most likely that the PQ-induced loss of aconitase activity in Δ*sod2* cells results from the damage of the FeS cluster and not from the loss of apoprotein because it has been shown that the apoprotein is required for the maintenance of mtDNA in yeast [33] and we found that PQ treatment did not increase the production of *rho*° cells (cells lacking mtDNA).

By comparison, we tested whether PQ could also impair the

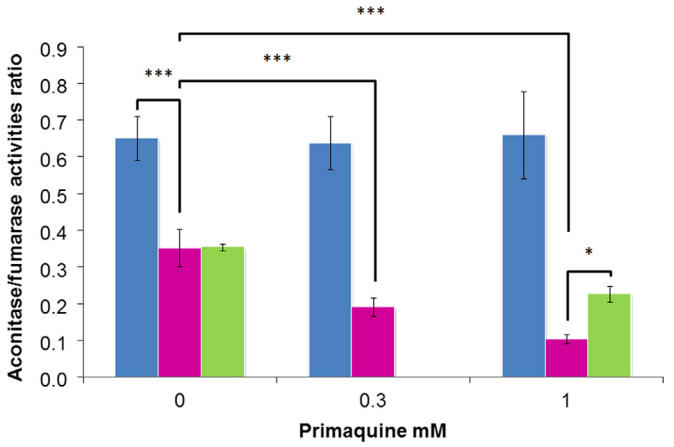

Fig. 3. Effect of primaquine treatment on aconitase activity in cells. Aconitase and fumarase activities were assessed spectrophotometrically at 240 nm. Total cell extracts were obtained from a 24 h culture in YPGal with 0, 0.3 or 1 mM PQ (see Materials and Methods). The reaction was started by addition of the cell extract to phosphate buffer containing isocitrate as substrate for the aconitase or malate as substrate for the fumarase. The data are presented as the ratio of the aconitase activity and the fumarase activity (the individual activities are given in Table S2). Values represent the averages of at least three measurements, with error bars representing standard deviation. *, $p < 0.05$; and ***, $p < 0.001$ according to Mann–Whitney *U* test. Wt (blue); Δ*sod2* (magenta) and Δ*sod2* overexpression *AIM32* (green).

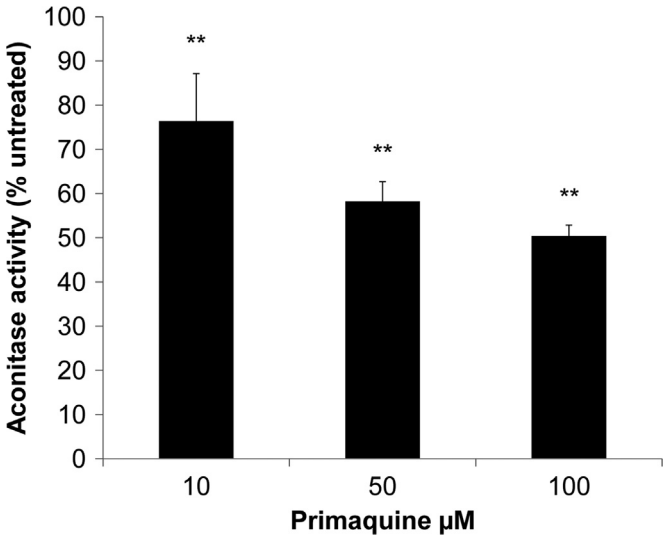

Fig. 4. Effect of primaquine on mitochondrial aconitase activity *in vitro*. Purified mitochondrial aconitase (33 ng μL^{-1}) was incubated for 20 min at room temperature with buffered PQ at different concentrations. The reaction was started by addition of the cis-aconitate and activity was monitored spectrophotometrically at 240 nm at 37 °C. The activity is expressed as a percentage of the activity obtained after incubation of the enzyme in the absence of PQ and is the average of four measurements, with error bars representing standard deviation. **, $p < 0.01$ according to Mann–Whitney *U* test.

activity of two other respiratory enzymes containing Fe-S clusters that are not readily damaged by oxidative stress: complex III (or bc_1 complex), containing a [2Fe-2S] cluster and complex II (or succinate dehydrogenase), containing three Fe-S clusters ([2Fe-2S], [4Fe-4S], and [3Fe-4S]). The Δ*sod2* cells were grown in the conditions used for the aconitase assay, and measurements of the enzymatic activities were performed on purified mitochondria. Complexes II and III activities were unaffected by 0.3 mM PQ treatment when compared to untreated controls (Table S2).

We then asked whether the overexpression of *AIM32* found to rescue PQ sensitivity of Δ*sod2* respiratory growth would protect the aconitase activity against PQ-induced damage. Δ*sod2* transformed with the multicopy plasmid carrying *AIM32* was cultured for 24 h in YPGal with or without 1 mM PQ. Aconitase and fumarase activities were monitored. As shown in Fig. 3, *AIM32* overexpression partly protected aconitase activity against PQ-induced damage. The aconitase/fumarase ratio of PQ treated cells increased from 0.1 in Δ*sod2* to 0.2 in Δ*sod2* overexpressing *AIM32*. However, overexpression of *AIM32* had no effect on the basal rate (without PQ treatment), suggesting different roles for Aim32 and Sod2.

3.5. Primaquine attack on Fe–S containing enzymes in vitro

It has been shown PQ is transformed into several metabolites mainly by the cytochrome P450 (CYP450) 2D6 and by the monoamine oxidase A in mammalian cells [11]. Yeast cells do not have a monoamine oxidase A. They have two CYP450 enzymes but none is equivalent to the enzymes involved in PQ metabolism in humans. Thus PQ is likely to be the active molecule causing growth defects and decreased aconitase activity in treated yeast cells.

We thus checked whether PQ could damage purified aconitase *in vitro* and observed that PQ exposure decreased aconitase activity in a dose dependent manner (Fig. 4).

As shown in Figs. 3 and 4, PQ impaired aconitase activity both *in vivo* (in treated cells) and *in vitro* (using purified mitochondrial aconitase).

As PQ was found to attack Fe–S containing aconitase, we reasoned that the drug might also impair other Fe–S cluster containing enzymes sensitive to oxidative damage, for instance, Rli1, a [4Fe-4S] protein previously shown to be susceptible to oxidant-induced Fe–S turnover *in vitro* [25]. Thus we checked whether PQ could disrupt Rli1 Fe–S clusters *in vitro* by monitoring the release of Fe. Rli1-HA

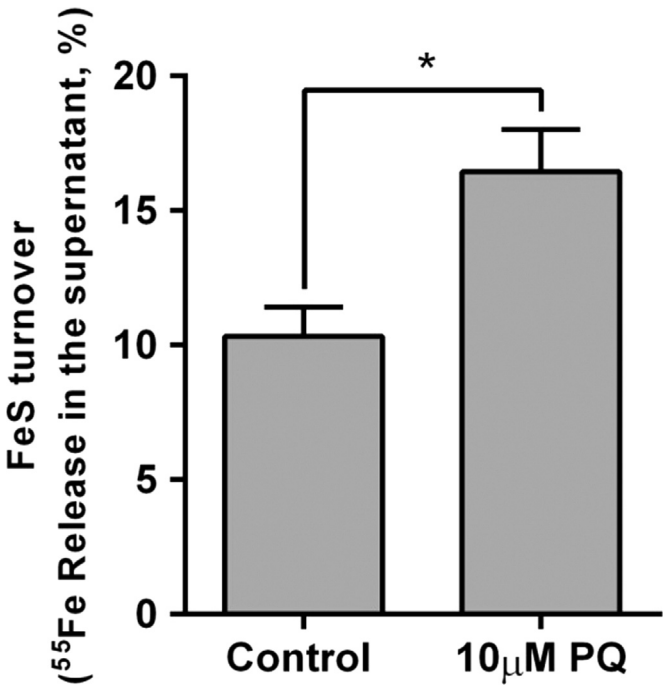

Fig. 5. Release of Fe from Rli1 during primaquine exposure. Yeast expressing an HA-tagged construct of Rli1 under *tet* control were cultured in the absence of doxycycline to maximise expression. Rli1-HA was immunoprecipitated from protein extracts of cells preincubated with $^{55}FeCl_3$. ^{55}Fe release from Rli1 was calculated from ^{55}Fe determinations after 10 min incubation *in vitro* with 350 µM ascorbate/100 µM histidine [25,26] supplemented or not with 10 µM PQ. *, $p < 0.05$ according to Student's *t*-test, two tailed. Data are means from three independent experiments ± SEM.

was immunoprecipitated from cells preincubated with $^{55}FeCl_3$ and ^{55}Fe release assayed during subsequent incubation with 10 μM PQ. PQ treatment increased the rate of Fe–S turnover from Rli1 by more than 50% (Fig. 5).

4. Discussion

4.1. Defence mechanism against primaquine attack

In this study we investigated the mode of action of the antimalarial drug primaquine (PQ) using yeast as a model. We found that the deletion of genes encoding the superoxide dismutase, *SOD2* and to a lesser extent *SOD1* increased the sensitivity to PQ, while the deletion of other genes involved in oxidative stress defence were without effect.

Sod2, located in the mitochondrial matrix [34,35], is the main protection against superoxide produced by the respiratory chain. Our data indicate that Sod2 plays also a key role in the protection against PQ. We found no redundancy in the protective effects of Sod activity, as overexpression of *SOD1* did not protect Δ*sod2* cells from PQ toxicity. This might be because the two Sods differ in their cellular location, Sod1 being predominantly cytosolic [36].

In the absence of Sod2, addition of the anti-oxidant compounds ascorbate or of N-acetyl cysteine (NAC) to the culture or the overexpression of *AIM32* and *MCR1* decreased the sensitivity to PQ.

The cell defence mechanisms against ROS include Sods, catalase, peroxidases and antioxidants such as ascorbate. In yeast, erythroascorbate is found instead of ascorbate but may play the same antioxidant role [28]. Interestingly, Mcr1 is involved in the regeneration of erythroascorbate when the molecule becomes oxidised through scavenging free radicals [28]. Mcr1 is also involved in ubiquinone-dependent antioxidant protection [29]. The enzyme is located in the mitochondrial intermembrane space [30]. Overexpression of *MCR1* could speed up the regeneration of erythroascorbate and ubiquinone, which would provide higher antioxidant protection and thus protect from PQ toxicity. In human erythrocytes, the Mcr1 equivalent (NADH-cytochrome b_5 reductase or NADH methemoglobin reductase) is essential to reduce methemoglobin back to haemoglobin and limit its concentration below toxic levels [37]. PQ produces increased levels of methemoglobinemia in patients with deficiency in NADH methemoglobin reductase [17]. Although the yeast and erythrocyte cells differ in many aspects, NADH-cytochrome b_5 reductase might provide protection from PQ toxicity in both cellular systems. In *P. falciparum* parasites, a putative NADH-cytochrome b_5 reductase is reported (PF3D7_1367500) with a likely mitochondrial location. It will be interesting to establish whether this enzyme could have a role in the PQ sensitivity of malaria parasites.

Aim32, predicted to be located in the mitochondria, contains a thioredoxin-like ferredoxin family domain [32]. Thioredoxin-like ferredoxins are a large family of Fe–S proteins with unknown function [38]. Their thioredoxin fold and ferredoxin domain are suggestive of a role in redox sensing and electron transfer. They may function as thiol-based molecular switches by modulating disulphide bond formation in their target proteins [39,40]. In absence of Sod2, Aim32 might protect PQ-sensitive Fe–S clusters by buffering PQ-induced oxidative damage. Proteins with similar function, albeit poorly conserved sequence, are likely to be present in humans and in the parasite.

4.2. Primaquine attack on Fe–S clusters

Fe-S clusters are notoriously vulnerable to oxidative damage, which makes several proteins whose function depends on these co-factors highly sensitive to ROS and possibly to PQ.

The mitochondrial matrix aconitase is a dehydratase catalysing the reversible interconversion of citrate to isocitrate. The protein's [4Fe-4S] cluster is ligated by three cysteine residues and water molecules. This cluster is exposed to solvent, which is key for the catalytic activity, but makes the cluster easily accessible to oxidants. Superoxide and hydrogen peroxide can readily oxidise this Fe–S cluster [41]. Upon oxidation, the active [4Fe–4S4] cluster converts to an inactive [3Fe–4S] form (for review [42]). The absence of Sod2 (and Sod1) which shields the enzyme from oxidative damage results in a two-fold decrease in aconitase activity [43]. We found that PQ treatment caused a further and severe loss of aconitase activity in Δ*sod2* cells. In addition, PQ exposure of purified aconitase resulted in decreased activity.

I*n vitro* assays showed also that PQ disrupted the Fe–S cluster of the [4Fe–4S] containing protein Rli1, known to be ROS-sensitive *in vitro* [25].

The data suggested that ROS-labile Fe–S groups may be primary targets of PQ. In addition to aconitase and Rli1, other ROS-sensitive Fe–S containing proteins might be affected by PQ treatment, such as Nar1. Rli1 and Nar1 are essential proteins. Rli1 is required for nuclear export of ribosomal subunits, translation termination and ribosome recycling [25,44]. Nar1 is involved in the biosynthesis of cytosolic and nuclear Fe–S proteins, so is required for the essential activity of Rli1 [45]. PQ-induced damage of their Fe–S clusters would compromise cell growth.

4.3. Primaquine damage, respiratory function and growth defects

PQ inhibited proliferation when cells relied on respiratory function while the drug had no or little effect on cells relying on fermentation.

Respiratory growth requires TCA cycle function, thus aconitase activity. In Δ*sod2* cells grown in YPGal with 1 mM PQ, the aconitase activity was 4.5-fold lower than in wt cells. However the oxygen consumption of cells was unaffected, indicating that the remaining aconitase activity was sufficient for the respiratory function. When cells are grown in YPEth, the decrease in aconitase might reach the threshold and become a limiting factor of cell respiration and growth.

The PQ-induced decrease in aconitase activity would not –or not fully -account for the observed growth defect, especially in YPGal. PQ-induced damage to essential proteins such as Rli1 and Nar1 would cause a growth defect.

Rli1 and Nar1, however, are required during both fermentative and respiratory growth. The PQ-induced growth defect was observed only when cells relied on respiration. For instance, *rho*° mutants that lack the mtDNA and therefore the respiratory chain, derived from the control strain CWWT and from mutants Δ*sod2* and Δ*sod1*, were unaffected by PQ treatment. This indicates that respiratory activity enhances the deleterious effect of PQ. The respiratory chain is generally the major source of endogenous ROS. It can be hypothesised that the damage to Fe–S clusters results from the combined action of PQ and ROS produced by the respiratory chain-and overproduced in absence of Sod2.

The oxidative damage would result from the additive detrimental effects of respiratory ROS and PQ. It could also be suggested that PQ would react with the endogenous ROS to produce a more active compound.

4.4. Primaquine attack in the malaria parasite

We found that PQ treatment impaired aconitase activity in yeast and hypothesized that the drug might alter other FeS containing enzymes. Recent studies in *P. falciparum* reported that the TCA cycle while not needed for parasite survival in asexual blood stages was required for gametocyte and mosquito stages [46,47].

In addition, during the sexual (gametocyte) stage, the organization of mitochondria is more complex and enzymes of the TCA cycle and of the oxidative phosphorylation pathway as well as many other mitochondrial proteins are up-regulated [48], compared to the asexual blood stage. Remarkably, using *P. falciparum* knockout lines, aconitase (PF3D7_1342100) was found to be essential for gametocytogenesis [46,47]. The parasite aconitase is likely to be ROS-sensitive as are the yeast and mammalian enzymes. Interestingly, the parasite fumarase resembles the Fe–S containing 'class I'-type enzymes found in some Bacteria and Archaea instead of the superoxide insensitive 'class II' type enzyme found in yeast and mammalian cells [49–51]. In addition, fumarase might be required at all the stages of the parasites life cycle [46]. Both enzymes might be sensitive to PQ-induced damage. A severe decrease of their activities upon PQ treatment would affect the respiratory activity and thus the ATP supply, but also biosynthetic pathways such as haem biosynthesis and pyrimidine synthesis, and halt parasite development.

It is interesting to note that PQ exerts its toxic effect across diverse eukaryotic cells when compromised for antioxidant defence, such as erythrocytes with deficiencies in G6PD or methemoglobin reductase and in Δ*sod2* yeast. It is tempting to speculate that *Plasmodium* parasite stages that are exquisitely sensitive to PQ might have constitutively weak antioxidant defences and/or oxidative pathways that are highly activated. *Plasmodium* sp. possesses several oxidative stress defence enzymes [52], which include a cytosolic SOD1 (PF3D7_0814900) and a mitochondrial SOD2 (PF3D7_0623500). However, whereas eukaryotes usually possess cytosolic Cu/Zn-SOD and mitochondrial Mn-SODs, most of the protozoan parasites contain only Fe-SODs [53], and the *P. falciparum* Fe-SOD enzyme was found to be sensitive to oxidative inactivation [54]. Therefore, a plausible hypothesis is that exposure to PQ and its metabolites would cause oxidative damage to the Fe-SODs of the parasite and overwhelm its antioxidant defence system, which then would not be able to shield the aconitase and maybe other redox-sensitive enzymes. That could result in oxidative killing of the parasites, especially at stages requiring fully active mitochondria such as gametocytes. Regarding the metabolism of hepatic quiescent forms of *P. vivax* and *P. ovale*, little is known except that these forms are cleared *in vitro* and *in vivo* by PQ. It has been reported that addition of interleukin inhibited the development of the rodent malaria *P. yoelii* hepatic cultures and that the inhibition was the result of an oxidative burst [55], indicating that the parasites at the hepatic stage might be susceptible to oxidative damage.

5. Conclusions

We have used the yeast *S. cerevisiae* model to uncover the mode of action of primaquine (PQ). We observed that yeast sensitivity to PQ: 1) was markedly increased in a genetic knockout strain that lacks the gene *SOD2* encoding the mitochondrial superoxide dismutase, *2)* was restricted to cells relying on the respiratory function to proliferate, and 3) in absence of Sod2, could be suppressed by the overexpression of two mitochondrial enzymes involved in the response to oxidative stress, Aim32 and Mcr1. This indicated a role for respiratory function – more specifically for ROS produced by the respiratory chain-in PQ sensitivity.

We found that PQ treatment impaired the activity of aconitase and damaged its Fe–S cluster. The Fe–S cluster of the essential protein Rli1 was also found to be susceptible to PQ attack *in vitro*, which leads to the hypothesis that ROS-labile Fe–S clusters would be primary targets of PQ.

In the malaria parasite, PQ treatment might also damage the Fe–S cluster of aconitase that is essential for parasite development at certain stages of its life cycle. Interestingly, in the parasite, the mitochondrial fumarase, likely to be an essential enzyme, is also a Fe–S containing enzyme and is possibly ROS-sensitive. In addition, the SODs –especially the mitochondrial SOD2- that could shield the Fe–S groups of these proteins against PQ-induced damage might be prone to oxidative inactivation. The link between PQ susceptibility and these mitochondrial enzymes should now also be investigated in the malaria parasites.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2015.10.008.

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