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A role for the succinate dehydrogenase in the mode of action of the redox-active antimalarial drug, plasmodione

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Summary

Malaria, caused by protozoan parasites, is a major public health issue in subtropical countries. An arsenal of antimalarial treatments is available, however, resistance is spreading, calling for the development of new antimalarial compounds. The new lead antimalarial drug plasmodione is a redox-active compound that impairs the redox balance of parasites leading to cell death. Based on extensive *in vitro* assays, a model of its mode of action was drawn, involving the generation of active plasmodione metabolites that act as subversive substrates of flavoproteins, initiating a redox cycling process producing reactive oxygen species. We showed that, in yeast, the mitochondrial respiratory chain NADH-dehydrogenases are the main redox-cycling target enzymes. Furthermore, our data supported the proposal that plasmodione is a pro-drug acting via its benzhydrol and benzoyl metabolites. Here, we selected plasmodione-resistant yeast mutants to further decipher plasmodione mode of action. Of the eleven mutants analysed, nine harboured a mutation in the FAD binding subunit of succinate dehydrogenase (SDH). The analysis of the SDH mutations points towards a specific role for SDH-bound FAD in plasmodione bioactivation, possibly in the first step of the process, highlighting a novel property of SDH.

Introduction

Malaria is one of the major public health issues in subtropical areas. This mosquito-borne infectious disease is caused by unicellular eukaryotic parasites of the genus *Plasmodium*, of which *P. falciparum* is responsible for the most severe forms of the disease. The parasites have a complex life cycle. In the human host, they mostly undergo asexual multiplication in red blood cells causing the malaria symptoms. A small population of the parasites differentiate in gametocytes that can infect mosquitoes upon a blood meal. Most drugs currently on the market target the symptomatic asexual forms. But increasing efforts are now made to develop drugs acting on the transmissible forms, with the aim of malaria control and elimination. A major concern, however, is the spread of drug resistances. Parasites have developed resistances to almost all drugs used so far to treat malaria, including artemisinin. There is thus an urgent need for new antimalarial compounds with different modes of action and low risk of resistance.

Plasmodione (3-[4-(trifluoromethyl)benzyl]-menadione) (PD) is a novel early lead compound displaying fast-acting antimalarial activity [1] [2]. It disrupts the redox balance of parasite-infected red blood cells [3] and is active against early asexual (rings) and sexual stages (young gametocytes). It also presents a safe toxicological profile for possible use in humans. The antimalarial selectivity of plasmodione largely comes from its specific bioactivation within *Plasmodium* parasitized-red blood cells. A mode of action was proposed based on extensive *in vitro* assays. The drug bioactivation starts with its benzylic oxidation to form the 3-benzoylmenadione (PDO), possibly through a benzhydrol (PD-bzol) intermediate [4], and likely catalysed by free or protein-bound hemes (including hemoglobin) *via* ferryl species (see [3] [5] and refs within). PDO then acts as a subversive substrate of flavoprotein(s). *In vitro*, PDO is able to cycle with the NADPH-dependent glutathione reductase and methemoglobin (metHb(Fe^{III})), the two major proteins of the parasitized red blood cells, especially in trophozoites that actively digest host hemoglobin. In treated parasitized cells, the redox-cycle would lead to redox unbalance and ultimately to parasite death (see Fig.5 and 6 in [3] for a detailed model of PD mode of action). However, PD is particularly active against early rings and gametocytes [2], at which stages, hemoglobin digestion is not the major metabolic process. Thus, in these stages, hemoglobin might not play a predominant role in PD activity and other actors need to be identified. We used yeast as a model to uncover enzymes and metabolic pathways required for PD activity.

PD is a potent inhibitor of yeast respiratory growth, acting most likely by triggering oxidative stress [5]. Genetic and biochemical analyses pointed towards a key role for the mitochondrial respiratory chain flavoprotein NADH-dehydrogenases (NDHs) in PD activity. We found that plasmodione metabolites PD-bzol and PDO act as subversive substrates of these enzymes. The reaction results in the production of superoxide anion radicals, which in turn leads to growth arrest (Fig.1). Yeast sensitivity towards PD correlates with NDH activity [5]. Also, increasing the dosage of genes encoding other flavoproteins such as *Glr1*, *Lpd1* and *Mcr* resulted in a moderate increased of PD sensitivity in cells with decreased NDH activity, suggesting those enzymes may as well contribute to PD activity [5].

Here, we selected and studied yeast mutants resistant to PD in order to further decipher the mode of action of PD and to uncover additional enzymes involved in PD activity. We found

that resistance is caused by defects in two components and one partner of components of the Krebs cycle: Lpd1, the mitochondrial lipoamide dehydrogenase, Lip2, the mitochondrial lipoyl transferase and SDH, the succinate dehydrogenase. Interestingly, of the eleven mutants obtained and analysed, nine harbour a mutation in SDH, and all SDH mutations are located in Sdh1, the FAD binding subunit, pointing towards a key role of that co-factor in PD activity. Further analyses suggest that SDH is involved in the bioactivation of PD into its active metabolites, but not in the redox cycling activity of these metabolites.

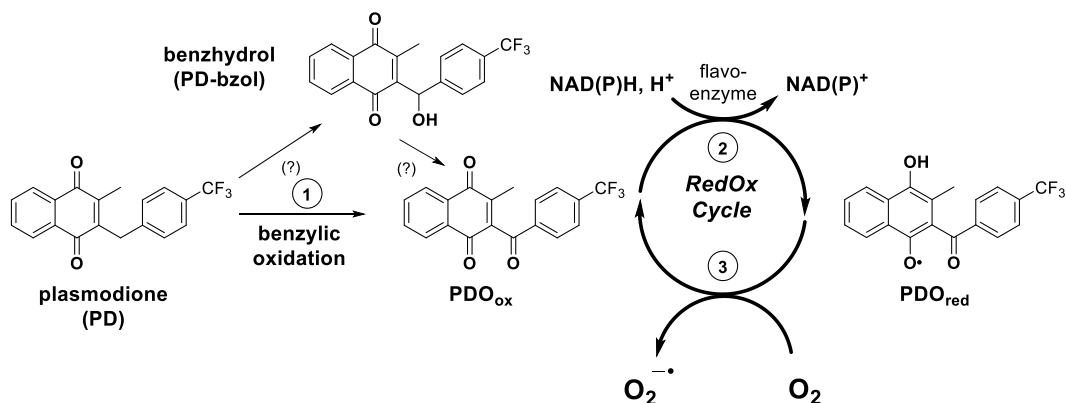


Figure 1. Simplified model of plasmidone activity pathway (adapted from [3]).

Step 1: The benzylic oxidation of plasmidone (PD) generates the 3-benzoylmenadione metabolite (PDO_{ox}), possibly through a benzhydryl (PD-bzol) intermediate. This reaction is likely catalysed by free or protein-bound hemes. Step 2: PDO_{ox} acts as a substrate of flavoenzymes and is reduced, generating PDO_{red}. Step 3: PDO_{red} transfers an electron to oxygen leading to superoxide anion radical production. Re-oxidized PDO binds again to and is reduced by flavoenzymes in a redox-cycle. The superoxide anion radicals generated by the process damage enzymes sensitive to oxidative stress. In addition, the continuous oxidation of NAD(P)H results in redox imbalance in the cells, increasing the protein oxidation and leading to growth arrest.

Materials and Methods

Growth media and yeast strains

The following growth media were used: YPD (1% yeast extract, 2% peptone, 3% glucose); YPEth (1% yeast extract, 2% peptone, 2% ethanol); YPG (1% yeast extract, 2% peptone, 2% glycerol).

Resistant mutants were selected from strain AD1-9 that lacks several membrane transporters (α , *ura3*, *his1*, Δ *yor1*, Δ *snq2*, Δ *pdr5*, Δ *pdr10*, Δ *pdr11*, Δ *ycf1*, Δ *pdr3*, Δ *pdr15*, Δ *pdr1*), kindly provided by M. Ghislain, UCL, Belgium.

Growth assays and drug sensibility tests

Drug sensitivity was assessed by monitoring the inhibition of yeast cell proliferation. Yeast were grown in 5 mL culture medium YPEth with increasing concentrations of drugs. Cultures were inoculated at an OD_{600nm} of 0.2 and incubated at 28°C with vigorous shaking for three days. OD_{600nm} were then measured. Data are plotted for each strain as the percentage of growth relative to untreated control. The experiments were repeated at least twice and the data

averaged. IC_{50s} (drug concentrations required to obtain 50% growth inhibition) were estimated from inhibitor titration plots.

Measurement of enzymatic activities

Mitochondria were prepared as in [6]. Protein concentration was determined by Bradford method.

Succinate- and NADH-cytochrome *c* reductase activities were measured by monitoring the rate of reduction of cytochrome *c* spectrophotometrically at 550-540 nm over 5-min time-course. Measurements were performed at room temperature in 1 mL of 10 mM potassium phosphate pH 7, 2 mM KCN and 20 μ M cytochrome *c*. Mitochondria were added at 70-120 μ g protein mL^{-1} for the succinate- cytochrome *c* reduction activities and around 25 μ g protein mL^{-1} for the NADH-cytochrome *c* reduction activities. The reaction was initiated by the addition 5 mM succinate or 0.8 mM NADH.

Lipoamide dehydrogenase activities were measured, as described in [7] by monitoring the rate of oxidation of NADH spectrophotometrically at 340 nm over 20 min time-course. Measurements were performed at room temperature in 1 mL of 50 mM potassium phosphate pH 6.5 and 0.9 mM of lipoamide. Mitochondria solubilized in triton X-100 for 10 min on ice were added at 60-100 μ g protein mL^{-1} . The reaction was initiated by addition of 0.2 mM of NADH.

The activities of the α -ketoglutarate dehydrogenase and pyruvate dehydrogenase complexes were measured by monitoring the rate of reduction of NAD spectrophotometrically at 340 nm over 20 min time-course. Measurements were performed at room temperature in 1 mL of 50 mM potassium phosphate pH 7.8, 1 mg mL^{-1} BSA (bovine serum albumin), 0.01 mM DTT (dithiothreitol), 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1 mM TPP (thiamine pyrophosphate), 1 mM NAD^+ and 2 mM α -ketoglutarate or 2 mM pyruvate. The mitochondria were added at 60-100 μ g protein mL^{-1} . The reaction was initiated with 0.25 mM coenzyme A trilithium salt.

The measurements were repeated at least thrice and averaged.

Sequencing analysis

Whole genome sequencing (WGS) and bioinformatics analysis were performed by the Next Generation Sequencing Core Facility of I2BC (www.i2bc.paris-saclay.fr). The sequences of the mutants were compared to the sequence of their parental strain, AD1-9. Up to 80 mutations per mutant were observed. Amongst them, the most likely mutations causing the phenotype were easily identified. The presence of mutations was then confirmed by PCR and sequencing of the mutated genes, using independent DNA preparations.

Results and discussion

Selection of the resistant mutants and identification of the mutations

The strain AD1-9, sensitive to plasmodione (PD), was used to select resistant mutants. Several subclones from AD1-9 were grown on YPD, and then incubated on respiratory medium (YPG) supplemented with 100 μ M PD that fully abolished AD1-9 growth. Resistant colonies appeared

after three to four week incubation. Eleven genetically independent resistant colonies, each from different subclones of the parental AD1-9 strain, were then analysed.

We first assessed their respiratory growth competence and level of resistance to PD. The mutants could be classed in three groups (Table 1). Most of the mutants presented a strong decrease in respiratory growth competence as the cell density (OD_{600nm}) after three-day culture reached only approximately 10% of the control density. The defect correlated with a strong resistance to PD as the IC_{50} increased by more than 6-fold compared to control. The other mutants showed a moderate or weak resistance, and similarly, a moderate or weak respiratory defect.

Table 1 Classification of the PD resistant mutants

phenotype group	OD_{600nm}	approx. IC_{50} (μM)	number of mutants
WT	45 ± 5	4	
strong	4 ± 1	>25	7
moderate	15 ± 2	15	3
weak	28 ± 3	8	1

The parental strain AD1-9 (WT) and mutants were grown in respiratory medium (YPEth) with increasing concentrations of PD. OD_{600nm} were measured after three-day culture. The experiments were repeated at least twice. The data were averaged and standard deviations estimated. IC_{50s} (drug concentrations required to obtain 50% growth inhibition) were estimated from the inhibitor titration plots of OD_{600nm} versus PD concentrations.

We then tested whether the resistance could be caused by a multidrug resistance mechanism, although the use of AD1-9 lacking several membrane transporters should make it unlikely. None of the mutants tested presented a cross-resistance to proguanil, azoxystrobin or atovaquone (not shown), suggesting a more specific mechanism of resistance to PD.

The mutants were then sequenced to identify possible resistance mutations. Nine mutants carry mutations in *SDH1*. The strong resistance mutants display the following amino-acid substitutions: G64S (in three mutants), W112L (in two mutants), D266G (in one mutant) and C431Y (in one mutant); G249C was found in two moderate resistance mutants. The two remaining mutants harbour a mutation in *LIP2* or in *LPD1*.

Table 2 Identified mutations

phenotypic group	gene	amino-acid substitution	number of mutants
strong	<i>SDH1</i>	G64S	3
	<i>SDH1</i>	W112L	2
	<i>SDH1</i>	D266G	1
	<i>SDH1</i>	C431Y	1
moderate	<i>SDH1</i>	G249C	2
	<i>LIP2</i>	G172V	1
weak	<i>LPD1</i>	G94D	1

We previously observed that the deletion of *NDE1*, encoding the main NDH, caused PD resistance. It thus could be intriguing that no mutation in that gene was found. However Δ nde1 mutant presented a moderate PD resistance with a IC_{50} value around 15 μ M [5] and the PD dose used in the screening presumably favoured the selection mutants with high level of resistance. It is also likely that by selecting more mutants, Nde1 mutations would eventually be found.

Resistance mutations in *LPD1* and *LIP2*

Both *LPD1* and *LIP2* encode mitochondrial enzymes involved in the pyruvate dehydrogenase (PDH) and α -ketoglutarate dehydrogenase (KGDH) complexes that are central to energy metabolism (Fig.2A). Lpd1 is a dihydrolipoamide dehydrogenase (or lipoamide dehydrogenase, LPD) located in the mitochondrial matrix. It functions as a homodimer, forming the E3 component of the PDH and KGDH complexes. Lpd1 catalyses the reoxidation of the lipoic acid co-factor bound to E2 with the reduction of NAD^+ into NADH. Lpd1 also possesses a diaphorase activity and can catalyse the oxidation of NADH using different electron acceptors, in accordance to the former nomenclature of LPD denominated menadione reductase. The mitochondrial lipoyl transferase Lip2 participates in the attachment of the co-factor lipoic acid to the E2 component of the two complexes.

To confirm that the observed mutation in *LIP2* is the cause of the moderate respiratory defect and the decreased sensitivity to PD, we transformed the WT strain and the lip2 mutant with a multi-copy plasmid bearing a WT copy of *LIP2* under the control of its own promoter, and tested the growth and PD sensitivity of the resulting strains (Fig.2 C and D). The increased gene copy resulted in a slight growth defect in the WT strain while the WT *LIP2* copy restored the respiratory growth and PD sensitivity of lip2-G172V to WT level, as expected.

A PD resistance mutation in *LPD1* was not unexpected. Indeed, we previously showed that the overexpression of *LPD1* in a Δ *NDE1* background, increased the sensitivity to PD, indicating that this flavoprotein could be involved in PD activity, in concert with other flavoproteins such as the NADH-dehydrogenases (NDHs) [5].

The PD resistance mutation identified in *LPD1* results in the replacement of a glycine by an aspartate at position 94. Residue G94 is located at the interface between the two monomers in the yeast Lpd1 structure. The nearest residue is N104 located in the other monomer (Fig.2B). We propose that G94D might induce a steric (and/or electrostatic) clash and destabilize the structure, causing a defect in the enzyme activity. To test this, we measured the NADH oxidation activity of LPD using lipoamide as electron acceptor (diaphorase activity) in mitochondrial samples of WT and mutants. As expected, mutation *sdh1-D266G* had no effect on LPD activity. By contrast, we observed a severely decreased activity in mutant *lpd1-G94D* and an increased activity in *lip2-G172V* (Fig.2E). The reduced lipoylation of E2 in mutant lip2 mutant might favour the diaphorase activity of LPD, at least in our experimental conditions, hence the increase in NADH oxidation observed in this mutant.

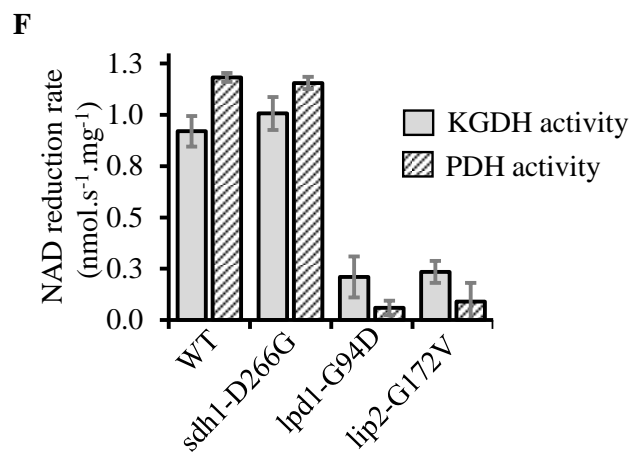
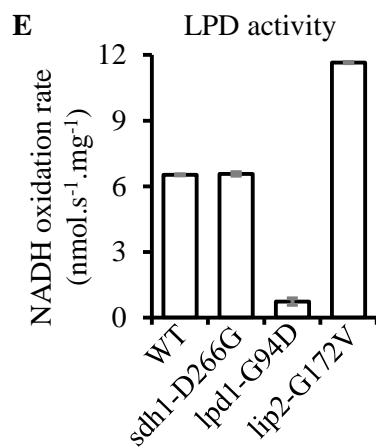
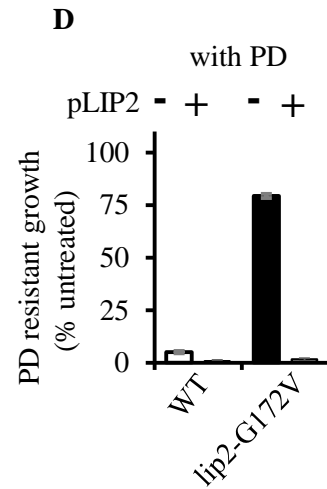
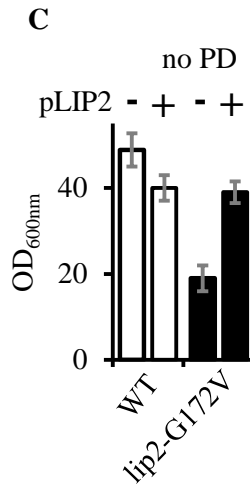
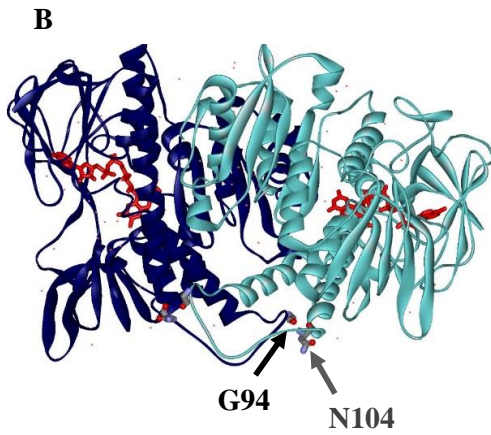
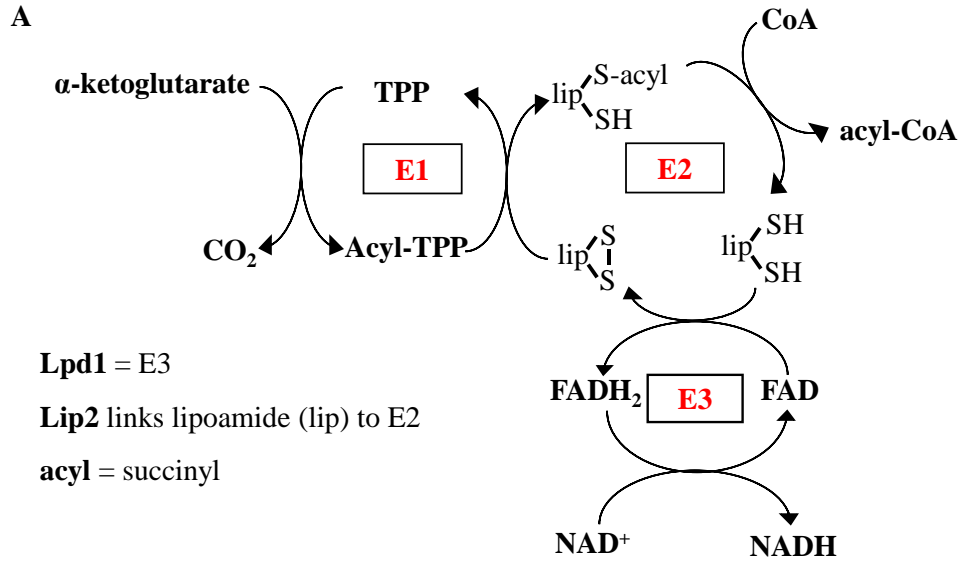


Figure 2. Characterisation of *lpd1* and *lip2* mutants

A) Schematic representation of the α -ketoglutarate dehydrogenase complex activity. The enzymatic complex is formed by three components: TPP dependant α -ketoglutarate dehydrogenase (E1); dihydrolipoyl transacetylase (E2) and lipoamide dehydrogenase (E3, Lpd1 in yeast). Lip2 is responsible for the lipoylation of E2. TPP, thiamine pyrophosphate; lip, lipoamide; CoA, coenzyme A.

B) Location of the PD resistant mutation in yeast Lpd1 homodimer structure (PDB: 1JEH). The monomers are shown in dark and in light blue. The FAD co-factor is in red. G94 is the residue mutated in the PD resistant mutant. N104, located on the other monomer, is the nearest residue to G94.

C, D) Restoration of respiratory growth competence and PD sensitivity in the *lip2* mutant. WT (white) and *lip2* mutant (black), without (-) and with (+) plasmid-borne wt copies of *LIP2* (pLip2) were grown in respiratory medium (YPEth) for three days. OD_{600nm} were then monitored. C) OD_{600nm} reached in absence of PD; D) Growth yield in medium supplemented with 6 μ M PD. Data are presented, for each strain, as % of OD_{600nm} reached in absence of PD.

E) LPD activity in WT and mutants *sdh1-D266G*, *lpd1-G94D* and *lip2-G172V*. Lipoamide-driven NADH oxidation rates were monitored at 340 nm using 0.9 mM lipoamide as electron acceptor. Detergent-solubilized mitochondria were added at 60-100 μ g protein mL⁻¹. The reaction was initiated by addition of 0.2 mM of NADH. The measurements were repeated at least thrice and the data averaged. Error bars represent standard deviation.

F) Activity of the complexes α -ketoglutarate dehydrogenase (grey) and pyruvate dehydrogenase (hatched) in WT and mutants *sdh1-D266G*, *lpd1-G94D* and *lip2-G172V*. NAD⁺ reduction rates were monitored at 340 nm using either 2 mM α -ketoglutarate or 2 mM pyruvate as electron donor. Mitochondria were added at 60-100 μ g protein mL⁻¹. The measurements were repeated at least thrice and the data averaged. Error bars represent standard deviation.

Based on the structure of LipB (PDB: 1W66), the *Mycobacterium tuberculosis* homolog of Lip2, substitution G172V found in the PD resistant Lip2 mutant is located in the catalytic domain [8], [9], [10], [11]. An impaired Lip2 activity should result in a defect in the lipoylation of the E2 component of KGDH and PDH and thus affect the activities of both complexes. To test this, we measured the KGDH and PDH activities in WT and mutants (Fig.2F). As expected, both activities reached WT levels in mutant *sdh1-D266G* but were severely decreased in both *lpd1-G94D* and *lip2-G172V* mutants.

How to explain the resistance to PD of *lpd1-G94D* and *lip2-G172V* mutants? We hypothesize that the decreased activities of the pyruvate dehydrogenase complex and the α -ketoglutarate dehydrogenase complex, one of the limiting steps of the Krebs cycle [12], would result in a decreased activity of the whole Krebs cycle and a lower production of NADH. In consequence, the activities of the NADH-dependent flavoenzymes, such as NDHs that catalyse the damaging redox-cycling with PD metabolites, would be decreased, leading to a reduced sensitivity to PD. Indeed a lower NADH/NAD⁺ ratio was observed in correlation with decreased KGDH activity [13] [14] and refs within. In *lpd1-G94D*, the impaired activity of the flavoenzyme Lpd1 itself could contribute to the observed PD resistance. However, Lpd1 seems a minor actor in PD activity [5].

Resistance mutations in *SDHI* and importance of the FAD binding domain

The succinate dehydrogenase SDH is composed of four subunits named Sdh1-4 in yeast (Fig.3A). Sdh1 forms the site of succinate oxidation and contains a covalently bound FAD. Sdh2 binds three iron-sulphur clusters, while the membrane anchored subunits Sdh3 and Sdh4 form the ubiquinone reduction site and bind a heme *b*. Electrons are transferred from the succinate oxidation site to FAD, then to the three iron-sulphur groups and finally to the ubiquinone reduction site. The role of the heme *b* remains poorly understood (for a review, see for instance [15]).

Of the eleven PD resistant mutants (Table 2), nine harboured mutations in *SDHI* corresponding to five different amino-acid substitutions, namely G64S, W112L, G249C, D266G and C431Y. Residues G64, G249, D266 and C431 are located in the FAD binding domain (Fig.3B) and their substitution is likely to affect FAD binding or properties, notably the replacement of D266 by a glycine as the side-chain of D266 is H-bonded to FAD in the structure of the porcine SDH [16]. W112 is outside the catalytic domain. The effect of its replacement by a leucine is more difficult to predict, but it might affect the proper folding of Sdh1 and thus the correct binding of FAD.

To pursue the analysis, we chose mutants D266G and G249C, presenting a strong and a moderate phenotype, respectively. To confirm the effect of the mutations in *SDHI* on both respiratory growth and PD sensitivity, these two mutants were transformed with a plasmid bearing the WT copy of *SDHI* under the control of its own promoter. As expected, this led to the restoration of a respiratory growth and sensitivity to PD similar to controls (Fig.3C and D).

To test the effect of the PD resistance mutations on SDH activity, we measured the succinate-driven cytochrome *c* reductase activity in mitochondrial samples of WT and mutants (Fig.3E). In that assay, we monitored the combined activity of SDH (succinate-ubiquinone oxidoreductase) and complex III (ubiquinol-cytochrome *c* oxidoreductase), as we followed the rate of cytochrome *c* reduction. The activity of SDH being the limiting factor of the reaction ([17] and references within), a decrease in SDH activity would result in a lower cytochrome *c* reduction rate. In the *sdh1* mutants, cytochrome *c* reduction was indeed decreased by 70-85% compared to control activity. In *lpd1-G94D*, the rate was slightly reduced, while in *lip2-G172V*, the rate was decreased by around 45 % compared to WT rate. The cause of this decrease was not investigated further.

In the previous experiment, cytochrome *c* could be reduced either by complex III in a fully functioning SHD/complex III chain, or by reactive oxygen species produced by a malfunctioning SDH, as cytochrome *c* can be directly reduced by superoxide anion radicals [18]. To distinguish between these two possibilities, we measured the rates of cytochrome *c* reduction in the samples with fully inhibited complex III (i.e. after addition of 10 μ M of the complex III specific inhibitor atovaquone) over a 20 min time course (Fig.3F). As expected, in WT extracts, the rate of cytochrome *c* reduction fell to 2% of the rate of untreated sample with active complex III. The same behaviour was observed in the *lip2* and *lpd1* mutants. By contrast, the rates were significantly higher in both *sdh1* mutants and increased with time, reaching 39% (*sdh1-D266G*) and 14% (*sdh1-G249C*) of the untreated rate after 10 min reaction. These results thus suggest that the mutated SDH produces superoxide anion radicals. As residues D266 and

G249 are located near FAD, their substitutions might alter the properties of the flavin, resulting in an increased level of flavosemiquinone intermediates that could react with oxygen producing superoxide anion radicals. This hypothesis is coherent with several previous reports demonstrating that (i) superoxide anion radicals are the major free radical species originating from the FAD site of SDH [19] [20] (ii) flavosemiquinone can be detected in mammalian SDH [21], and (iii) amino-acid substitutions in the vicinity of the FAD of the ferredoxin-NADP⁺ reductase modify the reduction potential of the flavin [22].

If alterations of SDH activity could cause PD resistance, it was intriguing that no mutation in other subunits of the complex was found in our screen. To test whether mutations in other SDH locations could also result in PD resistance, we constructed mutants harbouring mutations in the ubiquinone-binding site and substitutions of the heme ligands.

Three previously studied mutations located in the ubiquinone binding site were chosen, namely *sdh4*-Y120I, *sdh4*-D119N and *sdh3*-R97K. Residue *sdh4*-Y120 is required for ubiquinone reduction and might have a role in the protonation of ubiquinone [23]. Mutations *sdh4*-D119N and *sdh3*-R97K result in a decreased ubiquinone reduction activity and increased superoxide anion radical production [24]. *Sdh4*-Y120I presented a severe respiratory growth defect, as observed with *sdh1*-D266G. *Sdh3*-R97K and *sdh4*-D119N presented a moderate growth defect resembling that of *sdh1*-G249C. However, in contrast to the two *sdh1* mutants, the *sdh3* and *sdh4* mutants remained sensitive to PD (Fig.3G and H). Thus, mutations in the SDH ubiquinone binding site, causing growth defect and (as reported elsewhere) enzymatic activity defect, do not result in PD resistance.

We then tested the effect of mutating the heme *b* ligands *sdh3*-H156 and *sdh4*-C109. Replacements of these residues by an alanine caused a 80% decrease in heme content with little or no effect on respiratory growth and SDH activity [25]. Even the double mutation H156A-C109A resulting in the complete heme loss had only very weak effect [26], showing that the heme is not required for yeast SDH. Of note, a later report even questioned the presence of a heme in yeast SDH [27]. In agreement with published data, both *sdh3*-H156A and *sdh4*-C109A mutants showed a moderately decreased respiratory growth, and again, they remained fully sensitive to PD (Fig.3G and H).

In conclusion, we identified, through a selection screen, several mutations in *Sdh1* conferring PD resistance, mostly in the FAD binding domain, and we observed that PD sensitivity was not decreased in other mutants with defect in another catalytic domain. This fact points toward a key role of *Sdh1* and its FAD co-factor in PD sensitivity.

We then addressed the role of SDH in PD activity and first checked whether SDH could play the same role as NDHs and be involved in the redox-cycling of PD metabolites.

Plasmidione metabolites, as subversive substrates of SD

As previously reported, PD metabolites PDO and PD-bzol can act as subversive substrates of flavoproteins and initiate a redox-cycling process producing superoxide anion radicals [1]. In yeast, NDHs appear to be the major redox-cycling enzymes responsible for PD activity [5]. We previously hypothesized that PDO and PD-bzol interact with NDHs in a manner similar to

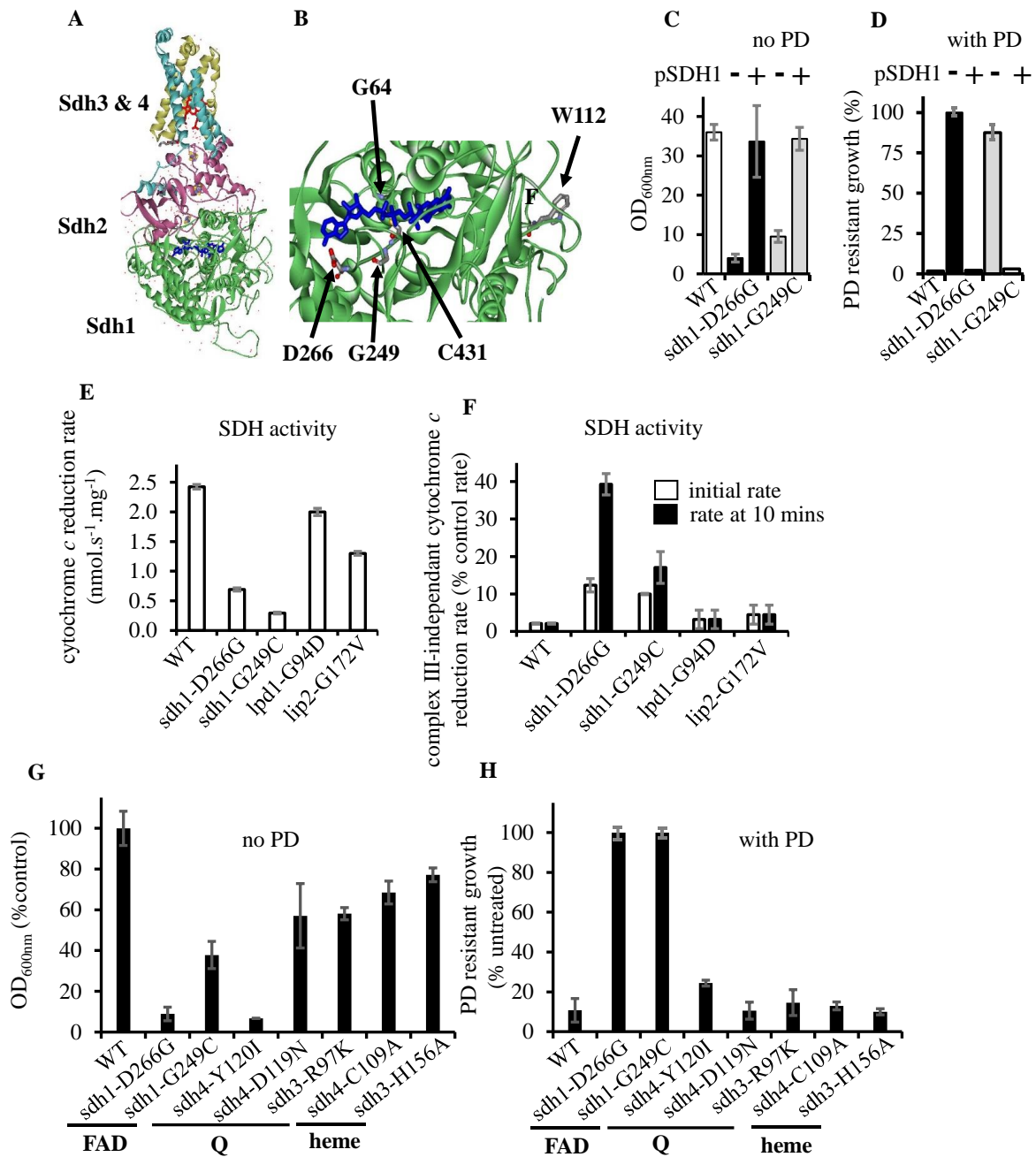


Figure 3. Characterisation of Sdh1 mutations

A) Structure of SDH, formed by four subunits. Sdh1 (green) covalently binds the FAD co-factor (blue) and forms the succinate oxidation site; Sdh2 (purple) binds the iron-sulphur clusters; The membrane-anchored subunits Sdh3 and Sdh4 (blue and yellow) bind a heme *b* (in red) and form the ubiquinone-binding site. B) Location of the PD resistance mutations in the FAD binding pocket of Sdh1. The figures were drawn using PDB: 1ZPO and 2H88.

C, D) Restoration of respiratory growth competence and PD sensitivity in *sdh1* mutants. WT (white) and *sdh1* mutants (black and grey), without (-) and with (+) plasmid-borne wt copies of *SDH1* (pSDH1) were grown in respiratory medium (YPEth) for three days. OD_{600nm} were then monitored. C) OD_{600nm} reached in absence of PD; D) Growth yield in medium supplemented with 8 μM PD. Data are presented, for each strain, as % of OD_{600nm} reached in absence of PD.

E, F) SDH activity in control and PD resistant mutants. Succinate driven-cytochrome *c* reductase activities were measured by monitoring the reduction of cytochrome *c* at 550-540 nm. Mitochondria were added at around 120 μg protein mL⁻¹. The reaction was initiated by the addition of 5 mM succinate. E) Cytochrome *c* reduction rates

resulting from SDH and complex III combined activities. F) Cytochrome *c* reduction rates independent of complex III activity (in presence of 10 μ M atovaquone that fully inhibits complex III), presented as % of control rates (with active complex III, as in E). White, initial rates; black, rates after 10 min reaction. The measurements were repeated at least twice and the data averaged. Error bars represent standard deviation.

G,H) Effect of Sdh3 and Sdh4 mutations on respiratory growth and PD sensitivity. WT and mutants were grown in YPEth medium (G) without and (H) with 6 μ M PD. Cell density (OD_{600nm}) were measured after three days culture. H) The data are presented as % of OD_{600nm} of WT. F) For each strain, the data are presented as percentage of the OD_{600nm} of the untreated culture. The experiments were repeated at least twice and the data averaged. Error bars represent standard deviation. FAD, Q and heme indicate mutations of the FAD-site, the ubiquinone binding-site and of the heme ligands.

menadione whose binding in the ubiquinone site was modelled *in silico* [28]. Once in that site, the compounds could be reduced via one or two electron transfer from the NADH-reduced FAD, and then react with and transfer electrons to O_2 , producing superoxide anion radicals.

To investigate whether PDO could also enter in a redox cycle with SDH, we monitored the rates of succinate-driven cytochrome *c* reduction using mitochondrial sample of the WT strain, as done in Fig.3E and 3F, and compared them to the rates NADH-driven activity.

First, we observed that the control rate of NADH-driven activity (i.e. the rate of cytochrome *c* reduction with a fully active complex III) was seven-fold higher than that of succinate-driven activity (Fig.4A). As expected, addition of atovaquone, an antimalarial compound blocking complex III, fully inhibited both NADH- and succinate-driven activities (Fig.4B and C). Addition of PDO to the atovaquone treated samples initiated cytochrome *c* reduction by both succinate and NADH, however with dramatically different rates. In the NADH-driven reaction, after addition of 5 and 50 μ M PDO, the rates reached 100% and 250% of the control rate, respectively (Fig.4B). By contrast, after addition of 5 and 50 μ M PDO, the succinate-driven rates reached 10% and 20% of the control rate (Fig.4C). Thus, SDH appears ten-fold less active than NDH in catalysing the PDO-driven cytochrome *c* reduction reaction. Furthermore, addition of SOD and catalase in both NADH- and succinate-driven reactions resulted in a more than two-fold decrease of the PDO-caused cytochrome *c* reduction. We propose that PDO can be reduced by SDH, as by NDH, although in a less efficient way. The PDO-radicals thus generated can then react with O_2 producing superoxide anion radicals, which in turn can reduce cytochrome *c*. Addition of SOD and catalase, lowering superoxide level, decrease cytochrome *c* reduction rate.

Of note, addition of 50 μ M of the parental drug PD did not initiate cytochrome *c* reduction (data not shown), suggesting that PD itself cannot enter in a redox cycle with SDH, which is in agreement with our previous data showing that PD is a poor substrate of NDH and does not initiate superoxide anion radicals production [5].

As a control, we also tested the effect of PD and PDO on the succinate-driven cytochrome *c* reductase activity in the absence of atovaquone, i.e. when complex III is functional. A 25-30 % decrease in the reaction rate was observed at 50 μ M PD or PDO (data not shown), indicating that the compounds could interfere with the enzyme activity. However a maximum of 50%

decrease was reached at 90 μM PD and higher doses of PD did not inhibit further the reaction. It is thus clear that PD-induced respiratory growth inhibition is not caused by the inhibition of SDH by PD.

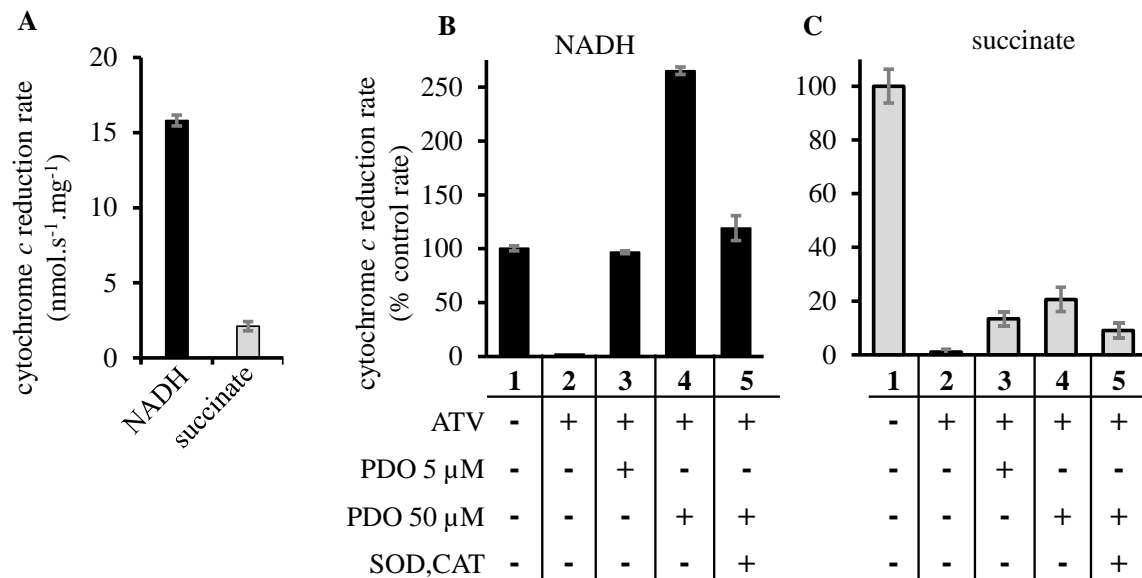


Figure 4. Effect of PD metabolites PDO on SDH and NDH activities.

NADH- and succinate-cytochrome *c* reductase activities were measured by monitoring the rates of cytochrome *c* reduction at 550-540 nm. Mitochondria were added at around 75 μg protein mL^{-1} for the succinate- cytochrome *c* reduction assays and around 25 μg protein mL^{-1} for the NADH-cytochrome *c* reduction assays. The reactions were initiated by the addition 0.8 mM NADH or 5 mM succinate. The measurements were repeated at least twice and the data averaged. Error bars represent standard deviation. A) Control rates recorded after addition of NADH (black) or succinate (grey). B) NADH- C) succinate-cytochrome *c* reduction rates expressed as % of control rates (shown in A) and recorded addition of (2) 10 μM atovaquone (ATV); (3) 5 μM PDO and ATV; (4) 50 μM PDO and ATV; (5) 50 μM PDO and ATV with superoxide dismutase and catalase both at 300 U mL^{-1} (SOD, CAT).

Taken together, our data indicate that SDH could catalyse PDO reduction and redox cycling, leading to superoxide anion radical production, however to a much lower level than NDHs.

If we assumed that SDH plays the same role as NDH in PD mode of action, it would be difficult to conciliate SDH comparatively low redox cycling activity with PD metabolites and the observed PD resistance of the *sdh1* mutants.

Role of SDH-bound FAD in PD bioactivation

Should NDH and SDH play the same role, i.e. enter in a redox cycle with PD metabolites, leading to superoxide anion radical production and growth arrest, we reasoned that increasing NDH activity by overexpressing *NDE2* in *sdh1* mutants should restore PD sensitivity.

While the overexpression of *NDE2* in WT increased the sensitivity to PD (as previously shown [5]), it completely failed to do so in *sdh1-D266G* (Fig.5A), further indicating a different role for SDH and NDHs.

In addition, while the resistance of *sdh1-D266G* to PD was more pronounced than that of $\Delta nde1$ (Fig.5B), this resistance profile was fully inverted upon exposure to PD-bzol (Fig.5C). (In these growth assays, we used PD-bzol instead of PDO, as the WT AD1-9 yeast strain was insensitive to the compound in accordance with its low activity on parasitized red blood cells, presumably because it cannot easily penetrate into cells [1]). $\Delta nde1$ was more resistant to PD-bzol, whereas *sdh1* mutant was more sensitive than WT.

The resistance of $\Delta nde1$ to both PD and PD-bzol is easily explained by the decreased activity of PDO/PD-bzol redox-cycling NDH. The same profile of resistance to both PD and PD-bzol was also observed with the *lip2* mutant (data not shown), supporting our suggestion that its resistance would result from a decreased activity of the NADH-dependent flavoenzymes, such as NDHs.

By contrast, in *sdh1-D266G*, the PD-bzol redox-cycling activity was not affected as the mutant was sensitive to PD-bzol. But the process of PDO/PD-bzol generation from PD is likely to be impaired, which causes the resistance to PD. The increased PD-bzol sensitivity of the *sdh1* mutant, compared to WT could be due to the overproduction of superoxide anion radicals by the malfunctioning SDH (see Fig.3F), which would add to the superoxide anion radicals produced by PD-bzol reacting with flavoenzymes and exacerbate the redox stress. Also, if the *Sdh1* substitution increases the level of FAD semiquinone and superoxide anion radicals, benzylic oxidation of PD-bzol to PDO could be favoured, which might render cells more sensitive to the added PD-bzol.

In conclusion, our data demonstrate that SDH and NDH do not play the same role in PD mode of action, that PD resistance in *sdh1-D266G* mutant cannot be explained by the decrease of SDH-driven PDO/PD-bzol-reducing activity, and that SDH likely acts upstream of PD-bzol/PDO. We propose that SDH may be essential to produce PD redox-active metabolites, such as PD-bzol/PDO, that could then enter into a damaging redox-cycle with other flavoenzymes such as NDHs (and possibly *Lpd1* and *Glr1*), producing superoxide anion radicals and leading to growth arrest. SDH contribution to redox-cycling, albeit possible, would be limited.

Furthermore, the key role of SDH in PD bioactivation is likely to be mediated by its bound FAD, as indicated by the analysis of the SDH mutants. Interestingly, in SDH, the flavin co-factor is covalently bound to the protein, contrarily to NDH. The covalent linkage increases the redox potential of the bound co-factor to permit succinate oxidation (see [29] [15] and refs within). In yeast, the substitution of the FAD ligand H90 results in an assembled enzyme lacking succinate oxidation activity but retaining the fumarate reductase activity, i.e. the reverse reaction [30]. The FAD reduction potential in yeast NDH is around -370 mV [31] while it is -79 mV in the mammalian SDH [16]. Furthermore, in SDH, FAD transfers the electrons from a two-electron donor, succinate, to a one-electron acceptor, the first iron-sulphur group. By contrast, NDH mediates electron transfer from a two-electron donor (NADH) to a two-electron

acceptor (quinone), without involving semi-reduced flavin [31](see [32] for a review on flavin electron transfer). These features of SDH flavin co-factor may be important for its role in PD bioactivation.

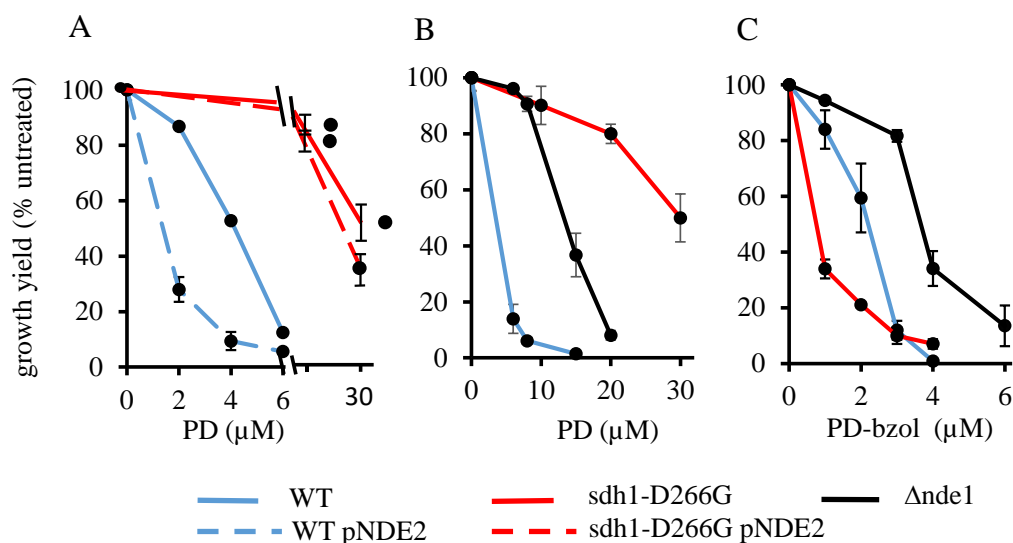


Figure 5. Effect of *SDH1* mutation and *NDH* gene dosage on growth sensitivity to PD and PD-bzol. WT and mutants were grown in YPEth medium with increasing concentrations of drugs. Cell density ($\text{OD}_{600\text{nm}}$) were measured after three days culture. For each strain, the data are presented as percentage of the $\text{OD}_{600\text{nm}}$ of the untreated culture. The experiments were repeated at least twice and the data averaged. Error bars represent standard deviation. A) Effect of increased NDH level on PD sensitivity of WT and *sdh1*-D266G (*sdh1*). WT (blue) and *sdh1* (red) (straight lines) compared to WT and *sdh1* transformed with a multicopy plasmid bearing *NDE2* (pNDE2) (dotted lines); B and C) Effect of SDH and NDH decreased level on PD and PD-bzol sensitivity. WT (blue); mutant Δnde1 (black); mutant *sdh1*-D266G (red).

The transformation of the pro-drug PD in the active metabolite PDO, possibly via the PD-bzol intermediate requires benzylic oxidation that could be catalysed by free hemes and/or protein-bound hemes. It was hypothesized that the benzylic oxidation would be preceded by a reduction of PD catalysed by a NAD(P)H-dependent flavoenzyme (see Fig. 2 in [1]). A possible role for SDH-bound FAD in that process could thus be envisioned. However, a direct reduction of PD by FAD seems unlikely.

More work is needed to uncover PD bioactivation mechanism and the possible site of reaction of PD in SDH. To this end, the design and synthesis of several fluorine-based chemical tools as mechanism-based inhibitors are in progress, which would be used on PD-sensitive and -resistant yeasts. In addition, attempts are being made to develop biomimetic conditions for the transformation of PD into PDO and/or PD-bzol, under a cascade of redox reactions producing superoxide anion radicals or under enzyme catalysis

Plasmodium SDH

The transformation of PD into its active metabolites is crucial for its activity. Uncovering the actors involved in that transformation is key to deciphering PD mode of action. Our data obtained with the yeast model point towards an important role for SDH in PD bioactivation. Is this finding relevant to PD mode of action in malaria parasites? *Plasmodium* possesses a SDH that is dispensable in asexual blood stages but required for sexual stages [33] [34], as also observed for the parasite NADH dehydrogenase (NDH2) [35] [36]. *Plasmodium* SDHa is the homolog of yeast flavoprotein Sdh1. As *NDH2*, it is expressed at very low levels at the ring and early trophozoite stages, and at higher levels in late trophozoites and schizonts as well as during gametocytogenesis [37] [38]. At the protein level, it is more readily identified in gametocytes, and especially in late gametocyte stages, than in asexual stages [39] [40]. This reflects the importance of the Krebs cycle during parasite development, where its activity is dispensable in asexual stages but its inhibition during gametocytogenesis results in growth arrest and cell death [41].

As for *NDH2*, *SDHa* expression pattern is not fully coherent with the stage specificity of plasmodione that is more effective on early rings and gametocytes. Still, low level of the enzyme might be sufficient to catalyse PD bioactivation and generate the active metabolites. Alternatively, other *Plasmodium* enzymes might play the role of SDH in PD biotransformation. The identification of the mechanism and actors of PD bioactivation and mode of action in the parasite will be aided by the results obtained in the yeast model.

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