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Type II NADH dehydrogenase of the respiratory chain of *Plasmodium falciparum* and its inhibitors

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ABSTRACT

Plasmodium falciparum NDH2 (pfNDH2) is a non-proton pumping, rotenone-insensitive alternative enzyme to the multi-subunit NADH:ubiquinone oxidoreductases (Complex I) of many other eukaryotes. Recombinantly expressed pfNDH2 prefers coenzyme CoQ_0 as an acceptor substrate, and can also use the artificial electron acceptors, menadione and dichlorophenol–indophenol (DCIP). Previously characterized NDH2 inhibitors, dibenziodolium chloride (DPI), diphenyliodonium chloride (IDP), and 1-hydroxy-2-dodecyl-4(1*H*)quinolone (HDQ) do not inhibit pfNDH2 activity. Here, we provide evidence that HDQ likely targets another *P. falciparum* mitochondrial enzyme, dihydroorotate dehydrogenase (pfDHOD), which is essential for de novo pyrimidine biosynthesis.

Malaria is a vector-born disease caused by a protozoan parasite of the genus *Plasmodium*. It accounts for 300–500 million cases each year and over a million deaths, mostly of children under the age of five in sub-Saharan Africa.[1] Given the emergence and spread of resistance to current antimalarials such as chloroquine, pyrimethamine, sulfadoxine, and atovaquone, the identification of novel drugs and drug targets in the *Plasmodium* parasite is critical for effective disease control.[2–12] The electron transport chain (ETC), a component of which is targeted by the commonly used drug, atovoquone, was examined to expand the repertoire of exploitable chemotherapeutic targets.[13] Although the ETC is generally well conserved across species, the first component (Complex I) of the *Plasmodium falciparum* ETC is DNA sequence divergent and relatively uncharacterized. The Complex I of eukaryotes is typically composed of a multi-subunit NADH:Ubiquinone oxidoreductase that oxidizes NADH in a rotenone-sensitive manner. In contrast, *P. falciparum* encodes an alternative single polypeptide non-proton pumping enzyme that is rotenone-insensitive. Alternative NADH dehydrogenase (NDH2) enzymes are flavoproteins that catalyze the transfer of electrons from NADH to ubiquinone (CoQ_n), using a ping-pong mechanism, in order to maintain a pool of oxidized NADH for reductive metabolic pathways, such as glycolysis or the TCA cycle.[14] NDH2 enzymes are found throughout the plant kingdom and in some bacteria, yeast, and protists.[15–23] These enzymes are characterized by their lack of a transmembrane domain, the presence of a conserved GxGxxG motif, and their insensitivity to Complex I inhibitors such as rotenone and piericidin A.[24] Like other NDH2 proteins, the respiratory chain NDH2 of *P. falciparum* (pfNDH2) is a single polypeptide, approximately 52 kDa in size.[14]

In an effort to explore targets along the ETC in *P. falciparum*, specific inhibitors for each component of the chain must be identified and characterized. While there are specific inhibitors to Complex III (cytochrome bc_1), such as antimycin A and

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Table 1
Kinetic properties of purified pfNDH2 in the presence of electron-accepting substrates

Electron acceptor	k_{cat} (1/min)[a]	K_m [a,b]
CoQ_0	20.0 ± 0.3	104.3 ± 6.6
CoQ_1	0.9 ± 0.1	2.2 ± 1.2
CoQ_4	n.a.	n.a.
CoQ_D	n.a.	n.a.
Menadione	4.6 ± 0.2	239.3 ± 22.4
DCIP	1.9 ± 0.0	4.6 ± 0.4

[a] K_m and k_{cat} values and standard deviations were calculated from three independent experiments. n.a., enzyme activity not observed.
[b] For each K_m determination, the concentration of either the electron acceptor or NADH was varied while the other substrate was fixed at 500 μM. K_m and k_{cat} values were determined using best-fit Michaelis–Menton curves.

atovaquone, the specificity of inhibitors to alternative Complex I, pfNDH2, has been debated.[25,26] Recombinant pfNDH2 has been difficult to express and purify, and previous biochemical studies have been confounded by the use of crude or partially purified lysate in enzymatic assays.[14,26,27] In this study, biochemically active pfNDH2 was recombinantly expressed in *Escherichia coli* in order to thoroughly evaluate substrate and inhibitor specificity. Full-length protein was expressed in-frame with a C-terminal 6xHis tag. The presence of detergent (0.5% Triton X-100) was critical for purification of active enzyme. The enzymatic activity of pfNDH2 was measured by chemical quantification of NAD^+ using an assay adapted from Putt et al. in which addition of acetophenone base, followed by incubation at 100 °C with formic acid, yields a product with strong fluorescence emission at 444 nm when excited at 372 nm (Supplemental Scheme 1).[28] The pH dependence of the pfNDH2 reaction was assessed using this fluorescence-based assay at fixed concentrations of NADH and CoQ_0 (0.1 mM for both substrates). pfNDH2 was maximally active at a pH range between 7.0 and 9.0 (Supplemental Fig. 1). Given these data, a HEPES buffer equilibrated to pH 8.0 was used in subsequent kinetics assays.

Similar to other CoQ_n-utilizing enzymes, NDH2 homologs show differential substrate specificities for various CoQ_n. To gauge the substrate specificity of recombinant pfNDH2, the concentration of NADH was held constant, and in excess at 200 μM, while the substrates, CoQ_0, CoQ_1, CoQ_4, CoQ_D, menadione, and DCIP, were varied from 0.5 μM to 2 mM. The $K_{m,app}$ and k_{cat} for the various substrates were calculated based on best fit Michaelis–Menten kinetic curves, and are reported in Table 1 (Supplemental Fig. 2).

Among the CoQ_n substrates, only CoQ_0 and CoQ_1 showed detectable catalysis with pfNDH2. CoQ_0 afforded the maximal catalytic rate; however, the $K_{m,app}$ was significantly higher for CoQ_0 than CoQ_1, reducing the catalytic efficiency ($k_{cat}/K_{m,app}$) of the substrate. Electron acceptors with longer carbon chains such as CoQ_4, and CoQ_D were not efficiently utilized by pfNDH2. It should be noted that in vivo, pfNDH2 is predicted to be membrane-associated, however, the in vitro enzyme assay is carried out in the absence of membrane components. CoQ_0 does not have the carbon tail of the endogenous substrate, but the catalytic rings of the molecule are intact.[29] The observed differences in k_{cat} may be due to differential binding affinities of CoQ_n in the absence of mitochondrial membrane association. The inorganic electron acceptor, DCIP, functioned with similar efficiency to CoQ_0, albeit with increased substrate specificity and lower catalytic rate. In comparison, the efficiency of menadione was significantly reduced due to an increase in the $K_{m,app}$.

In the absence of a crystal structure for NDH2 enzymes, predictions for the flavin-binding region and binding sites for NADH and CoQ_n have been based on sequence and structural similarities to other redox enzymes.[30] The biochemical relevance of these predictions, however, has not yet been demonstrated. An N-terminal truncation product of pfNDH2 (NΔ214) was expressed and purified in order to determine if the conserved GxGxxG domains are necessary for catalytic activity. Truncated pfNDH2 showed significantly less activity compared to full-length product arguing that the N-terminal region of the enzyme is critical for full catalytic activity (data not shown).

NDH2 has been implicated as an activator of the plant-derived antimalarial, artemisinin.[31] A knockout screen using *Saccharamyces cerevisiae* homozygous deletion strains showed that deletion of two *S. cerevisiae* NDH2 genes resulted in artemisinin resistance. Although expression of pfNDH2 in *S. cerevisiae* NDH2 knockout strains partially restored artemisinin sensitivity, and over-expression of the *S. cerevisiae* NDH2 genes was shown to increase artemisinin sensitivity, no biochemical link between pfNDH2 and artemisinin has yet been established.[31] Therefore artemisinin was tested for either activation or inhibition of pfNDH2 activity. We found that arteminsin, even at high concentrations, did not perturb catalysis by pfNDH2 (Table 2). These results suggest that artemisinin may act via an indirect mechanism with NDH2 rather than by direct enzyme binding.

Previously described inhibitors of Complex I and NDH2 were evaluated for inhibition of recombinant pfNDH2 activity. Inhibition of enzymatic activity was assessed in the presence of excess NADH (200 μM) and CoQ_0 approximately equivalent to the $K_{m,app}$ (100 μM). As expected, Complex I inhibitors had no effect on pfNDH2 enzyme activity (Table 2). Eschemann et al. identified 1-hydroxy-2-dodecyl-4(1*H*)quinolone (HDQ) as an inhibitor of yeast (*Yarrowia lipolytica*) NDH2 by isolating mitochondrial membrane fractions in lieu of purified protein.[14] In addition, HDQ was shown to be a potent inhibitor of *P. falciparum* parasite proliferation.[14,32] Dibenziodolium chloride (DPI) and diphenyliodonium chloride (IDP) have been reported to inhibit pfNDH2 activity in

Table 2
Inhibition of in vitro enzyme activity and in vivo parasite proliferation

Compound	IC_{50} (μM) of enzyme activity[a]			IC_{50} (μM) of *P. falciparum* proliferation[a,b]	
	pfNDH2	pfDHOD	scDHOD	DD2	DD2-scDHOD
Atovaquone	>10	>10	>10	0.002 ± 0.0002	>10
Antimycin A	>10	>10	>10	3.4 ± 1.8 mg/mL[c]	>10
HDQ	>10	4.0 ± 0.1	>10	0.24 ± 0.09	>10
DPI	>10	>10	>10	2.7 ± 0.5	3.0 ± 0.6
IDP	>10	>10	>10	12.4 ± 2.0	10.6 ± 3.8
Rotenone	>10	>10	>10	>10	>10
Flavone	>10	>10	>10	>10	>10
Artemisinin	>10	>10	>10	0.01 ± 0.0006	0.006 ± 0.0001

[a] IC_{50} values and standard deviations were calculated from three independent experiments.
[b] IC_{50} values for DD2 and DD2-scDHOD parasite strains are based upon [^{3}H]-hypoxanthine dose–effect curves.
[c] IC_{50} values are given in mg/mL as antimycin A was a mixture of the components A_1, A_2, A_3, and A_4.

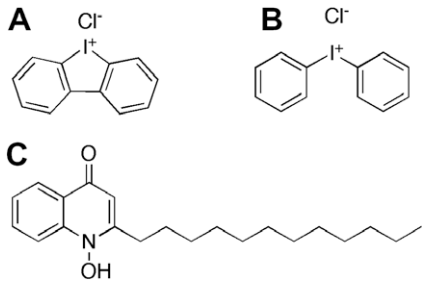

Figure 1. Chemical structures of (A) DPI, (B) IDP, and (C) HDQ.

crude lysate fractions (IC_{50} = 0.24 ± 0.03 and 5.99 ± 0.36, respectively), and both show efficacy against whole parasite proliferation.[26] It has been suggested that the antimalarial mechanisms of DPI, IDP, and HDQ may be attributed to the inhibition of pfNDH2 activity, however, dose–effect profiles using purified recombinant pfNDH2 did not corroborate these findings. In fact, these compounds did not inhibit pfNDH2 activity at concentrations of up to 10 μM (Table 2). Both DPI and IDP are well-known flavoprotein oxidoreductase inhibitors, suggesting that previous observations of reduction in NADH consumption using crude parasite lysate may have been due to inhibition of a different flavoenzyme-dependent reaction.[33–38]

The chemical structure of HDQ is similar to that of the CoQ_n substrate (Fig. 1) and thus, we speculated that its mode of action might be related to another CoQ_n-dependent enzyme, *P. falciparum* Type II dihydroorotate dehydrogenase (pfDHOD). pfDHOD is a mitochondrial flavoenzyme that catalyzes the oxidation of dihydroorotate (L-DHO) using a FMN cofactor that is re-oxidized by CoQ_n.[39] The malaria parasite relies upon pfDHOD as it catalyzes the rate-limiting step for de novo pyrimidine biosynthesis.[40] Inhibition of pfDHOD activity by HDQ was assessed in the presence of excess L-DHO (500 μM) and CoQ_0 approximately equivalent to the $K_{m,app}$ (115 μM), by coupling the assay to the chromogen, DCIP (Supplemental Scheme 2). HDQ inhibited pfDHOD activity with an IC_{50} of 4.0 ± 0.1 μM (Table 2).

A DD2 transgenic *P. falciparum* strain expressing a Type I cytoplasmic DHOD from *S. cerevisiae* (scDHOD) was used to pinpoint the antimalarial mechanism of HDQ (Supplemental methods). Painter et al. had previously shown that the role of the mitochondrial electron potential in the asexual stage of *P. falciparum* growth was to maintain a pool of CoQ_n in order to sustain pfDHOD activity and subsequent de novo pyrimidine biosynthesis.[40] It was demonstrated that addition of exogenous scDHOD results in a bypass of the endogenous electron transport chain through Complex III.[40] ScDHOD utilizes fumarate or NAD^+ rather than CoQ_n to reoxidize the flavin (FMN) prosthetic group in the second half-reaction of the redox process.[41–45] Therefore, inhibition of scDHOD activity was assessed in the presence of excess L-DHO (500 μM) and fumarate approximately equivalent to the $K_{m,app}$ (115 μM), by coupling the assay to the chromogen, DCIP (Supplemental Scheme 2). None of the compounds tested inhibited scDHOD enzyme activity. Proliferation of the scDHOD-expressing transgenic strain was unaffected by HDQ at a drug concentration of up to 10 μM, while the parental strain showed half-maximal growth inhibition at 0.24 ± 0.09 μM (Table 2) (Supplemental Fig. 3). This rescued growth phenotype associated with the addition of exogenous yeast DHOD argues that pfDHOD is the likely in vivo target of HDQ rather than pfNDH2 as formerly described.

The results of this study have shown that previously characterized NDH2 inhibitors are not effective inhibitors of pfNDH2. Such an observation is not unexpected since inhibitors for alternative rotenone-insensitive NADH dehydrogenases are rare and non-specific.[46] The essentiality of pfNDH2 remains undetermined, therefore additional work is required to identify specific inhibitors of pfNDH2 for further biochemical characterization.[47]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.11.071.

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