

# HDQ, a Potent Inhibitor of *Plasmodium falciparum* Proliferation, Binds to the Quinone Reduction Site of the Cytochrome $bc_1$ Complex

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The mitochondrial  $bc_1$  complex is a multisubunit enzyme that catalyzes the transfer of electrons from ubiquinol to cytochrome *c* coupled to the vectorial translocation of protons across the inner mitochondrial membrane. The complex contains two distinct quinone-binding sites, the quinol oxidation site of the  $bc_1$  complex ( $Q_o$ ) and the quinone reduction site ( $Q_i$ ), located on opposite sides of the membrane within cytochrome *b*. Inhibitors of the  $Q_o$  site such as atovaquone, active against the  $bc_1$  complex of *Plasmodium falciparum*, have been developed and formulated as antimalarial drugs. Unfortunately, single point mutations in the  $Q_o$  site can rapidly render atovaquone ineffective. The development of drugs that could circumvent cross-resistance with atovaquone is needed. Here, we report on the mode of action of a potent inhibitor of *P. falciparum* proliferation, 1-hydroxy-2-dodecyl-4(1*H*)quinolone (HDQ). We show that the parasite  $bc_1$  complex—from both control and atovaquone-resistant strains—is inhibited by submicromolar concentrations of HDQ, indicating that the two drugs have different targets within the complex. The binding site of HDQ was then determined by using a yeast model. Introduction of point mutations into the  $Q_i$  site, namely, G33A, H204Y, M221Q, and K228M, markedly decreased HDQ inhibition. In contrast, known inhibitor resistance mutations at the  $Q_o$  site did not cause HDQ resistance. This study, using HDQ as a proof-of-principle inhibitor, indicates that the  $Q_i$  site of the  $bc_1$  complex is a viable target for antimalarial drug development.

*Toxoplasma gondii* and *Plasmodium falciparum* are apicomplexan parasites causing toxoplasmosis and malaria, respectively. The latter disease is among the most serious health problems in the world, leading to more than 1 million deaths per year. Over recent years, an increase in malaria mortality has been attributed to the development of parasite resistance to first line therapies, which has raised calls for the urgent development of new drugs with novel modes of action (see, for example, reference 6). The mitochondrial respiratory chain is an effective target for antimicrobial agents directed against these pathogens. Differences between respiratory chain enzymes of mammals and pathogenic organisms have been exploited to develop compounds used for drug therapy such as atovaquone. Atovaquone is a hydroxynaphthoquinone active against different parasitic diseases (malaria, toxoplasmosis, and *Pneumocystis pneumonia* caused by the opportunistic pathogen fungus *Pneumocystis jirovecii*). Atovaquone inhibits the activity of the  $bc_1$  complex activity, a central enzyme of the respiratory chain (24). The mitochondrial  $bc_1$  complex is a multisubunit enzyme that catalyzes the transfer of electrons from ubiquinol to cytochrome *c* and couples this electron transfer to the vectorial translocation of protons across the inner mitochondrial membrane. The complex contains two distinct quinone-binding sites (the quinol oxidation site [ $Q_o$ ] and the quinone reduction site [ $Q_i$ ]), which are located on opposite sides of the membrane. Cytochrome *b*, the central membrane-embedded subunit, encoded by the mitochondrial genome, provides both  $Q_o$  and  $Q_i$  binding pockets. As a consequence of atovaquone-mediated  $bc_1$  complex inhibition, the electron transfer through the respiratory chain stops and the mitochondrial membrane potential of *P. falciparum* collapses. In addition, without a functioning  $bc_1$  complex to oxidize ubiquinol, the dihydro-orotate dehydrogenase oxidization (DHODH) comes to a halt as oxidized ubiquinone is required as an electron acceptor for the DHODH. Pyrim-

idine biosynthesis is thus inhibited which is lethal for the parasite (39, 44, 45). Unfortunately, resistance to atovaquone has been observed in both *P. falciparum* and *T. gondii*. This resistance is often associated with mutations in the target site, cytochrome *b*, the main subunit of the  $bc_1$  complex (reviewed in reference 21). Since atovaquone is effective against both circulating asexual stage parasites and liver stage parasites, it is a useful drug for both malaria treatment and prophylaxis. Therefore, new drugs that target the  $bc_1$  complex but that can circumvent atovaquone resistance and/or are more recalcitrant to resistance would be very welcomed. Currently, several different chemotypes targeting the  $bc_1$  complex have been developed, these include the hydroxynaphthoquinones (atovaquone analogues), pyridones (clopidol analogues), and acridine-related compounds (acridinediones, acridones, and quinolones [reviewed in reference 1]).

1-Hydroxy-2-dodecyl-4(1*H*)quinolone (HDQ) was recently shown to inhibit parasite replication of *T. gondii* and *P. falciparum* in nanomolar concentrations (40). HDQ treatment in *T. gondii* causes a loss of the mitochondrial inner-membrane potential and a severe ATP depletion due to the block of the electron flow (34). Because of the structural similarity between HDQ and ubiquinol,

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it seems likely that the drug could target ubiquinol binding sites of respiratory enzymes. Consistent with this hypothesis, HDQ has been shown to inhibit the mitochondrial alternative NADH dehydrogenase (NDH2) and complex I of the yeast *Yarrowia lipolytica*, albeit with different efficiencies (the 50% inhibitory concentrations [IC<sub>50</sub>s] were 0.2 and 2 μM, respectively) (16). The *T. gondii* type-II NADH dehydrogenase expressed in *Y. lipolytica* has also been shown to be inhibited by HDQ with an IC<sub>50</sub> of 0.3 μM (36). Furthermore, the HDQ-related compound HQNO (2-heptyl-4-hydroxyquinoline N-oxide) is a known inhibitor of mammalian and *S. cerevisiae* bc<sub>1</sub> complex. Mutations causing resistance to HQNO have been reported in yeast; they are located at the Q<sub>i</sub> site of the complex (reviewed in reference 10). This led us to question whether the antimalarial activity of HDQ may be mediated via bc<sub>1</sub> complex inhibition and, more specifically, by blocking the Q<sub>i</sub> site function.

We demonstrate here that in *P. falciparum*, HDQ, in addition to its inhibitory action toward the NADH:ubiquinone-oxidoreductase (PfNDH2), disrupts mitochondrial function through the potent inhibition of the bc<sub>1</sub> complex. By studying yeast with specific cytochrome *b* mutations in the Q<sub>o</sub> and Q<sub>i</sub> sites, and using molecular modeling, we show that HDQ inhibition of the bc<sub>1</sub> complex is mediated via Q<sub>i</sub> binding. HDQ therefore displays a novel inhibitory mode of action against an important antimalarial target.

## MATERIALS AND METHODS

**Chemical synthesis of HDQ.** The synthesis of HDQ was based on the method of Woscheck, et al. (49) (see Fig. S1 in the supplemental material for route of synthesis). Briefly, ethyl 3-oxopentadecanoate (prepared according to the method of Tietze and Ma [46]) was condensed with aniline using a catalytic amount of *p*-toluenesulfonic acid and azeotropic removal of water. The crude enamine was cyclized in Dowtherm to give 2-dodecylquinolin-4(1*H*)-one. Treatment of this 4(1*H*)-quinolone with potassium *tert*-butoxide, followed by esterification with ethyl chloroformate, yielded the 4-(ethoxycarbonyloxy)quinoline. Oxidation of this with mCPBA yielded the N-oxide. Hydrolysis of the N-oxide with potassium hydroxide in aqueous ethanol, followed by acidic workup and recrystallization gave HDQ. All spectroscopic data and CHN analyses were in accord with the proposed structure.

**Parasites, culture, and drug sensitivity testing.** *P. falciparum* (3D7 strain) cultures consisted of a 2% suspension of O<sup>+</sup> erythrocytes in RPMI 1640 medium (R8758, glutamine, and NaHCO<sub>3</sub>) supplemented with 10% pooled human AB<sup>+</sup> serum, 25 mM HEPES (pH 7.4), and 20 μM gentamicin sulfate (47). Cultures were grown under a gaseous headspace of 4% O<sub>2</sub> and 3% CO<sub>2</sub> in N<sub>2</sub> at 37°C. Parasite growth was synchronized by treatment with sorbitol (32). Drug susceptibilities were assessed by the measurement of fluorescence after the addition of SYBR green I as described previously (42). Drug IC<sub>50</sub>s were calculated from the log of the dose-response relationship, as fitted with Grafit software (Erithacus Software, Kent, United Kingdom). The results are expressed as the means of at least three separate experiments. The atovaquone-resistant isolate TM90C2B (Thailand) was generously provided by Dennis Kyle (College of Public Health, University of South Florida, Tampa, FL).

**Transgenic parasites.** 3D7-γDHOD-GFP, a transgenic derivative of *P. falciparum* 3D7 containing yeast dihydroorotate dehydrogenase (DHODH), was generated via the electroporation of purified pHHyDHOD-GFP plasmid into ring stages of *P. falciparum* using a Bio-Rad GenePulser as described previously (39). Purified pHHyDHOD-GFP plasmid was generously provided by Akhil Vaidya (Drexel University College of Medicine, Philadelphia, PA). This plasmid contains a human dihydrofolate reductase gene as a WR99210-selectable marker (39).

**Preparation of *P. falciparum* cell extracts.** Free parasites were prepared from aliquots of infected erythrocytes (approximately 8 × 10<sup>9</sup> cells ml<sup>-1</sup>) by adding 5 volumes of 0.15% (wt/vol) saponin in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.76 mM K<sub>2</sub>HPO<sub>4</sub>, 8.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 5.5 mM D-glucose [pH 7.4]) for 5 min, followed by three washes using centrifugation and resuspension in HEPES (25 mM)-buffered RPMI containing a protease inhibitor cocktail (Complete Mini; Roche). Cell extract was prepared by repeated freeze-thawing in liquid N<sub>2</sub>, followed by disruption with a sonicating probe.

**Preparation of recombinant *P. falciparum* NADH dehydrogenase (PfNDH2).** Recombinant PfNDH2 was prepared from the *E. coli* heterologous expression strain F571 as described in reference 22 and used as a crude membrane preparation at a total protein concentration of 15 μg/ml for NADH:deacylubiquinone oxidoreductase activity measurement.

**Measurement of NADH:deacylubiquinone oxidoreductase activity.** Recombinant PfNDH2 and yeast NDH2 activities (using samples prepared as described in reference 19) were assayed in a reaction medium consisting of 50 mM potassium phosphate (pH 7.5), 2 mM EDTA, 200 μM NADH, and 10 mM KCN. NADH:deacylubiquinone oxidoreductase activity was initiated by the addition of 50 μM deacylubiquinone. Deacylubiquinone reduction was monitored at 283 nm (ε<sub>283</sub> = 8.1 mM<sup>-1</sup> cm<sup>-1</sup>) in a Cary 4000 spectrophotometer, with rate and IC<sub>50</sub> data determined as described for the cytochrome *c* reductase assay.

**Measurement of cytochrome *c* reductase activity in *P. falciparum* samples.** Cytochrome *c* reductase activity measurements were assayed in 50 mM potassium phosphate (pH 7.5), 2 mM EDTA, 10 mM KCN, and 30 μM equine cytochrome *c* (Sigma) at room temperature (4, 22). Cytochrome *c* reductase activity was initiated by the addition of deacylubiquinol (50 μM). Reduction of cytochrome *c* was monitored in a Cary 4000 spectrophotometer at 550 versus 542 nm. Initial rates (computer-fitted as zero-order kinetics) were measured as a function of deacylubiquinol concentration. Turnover rates of cytochrome *c* reduction were determined using ε<sub>550-542</sub> = 18.1 mM<sup>-1</sup> cm<sup>-1</sup>. Inhibitors of bc<sub>1</sub> activity were added without prior incubation. dimethyl sulfoxide (DMSO) in the assays did not exceed 0.3% (vol/vol). The IC<sub>50</sub>s were calculated by using the four-parameter logistic method.

As previously shown by Fisher et al. (22), the spectrophotometric challenge presented by the presence of hemozoin is countered by adjusting the protein concentration within the cuvette such that the total absorbance is under 2.0 U (i.e., 1% transmitted light), which, coupled with the use of exogenous cytochrome *c* for the enzymatic assay (ΔA = 0.02/min), is well within the operating parameters of the Cary 4000 spectrophotometer used in the assays. It should be noted that, in addition to the above, hemozoin is chemically inert under our assay conditions and so will not cause a drift in the measured absorbance.

**Real-time single-cell monitoring of membrane potential (Ψ<sub>m</sub>).** The rhodamine derivative, TMRE (tetramethylrhodamine ethyl ester), was used to monitor the membrane potential (Ψ<sub>m</sub>) of the plasma membrane and mitochondria of malaria-infected red blood cells, as described previously (4, 5).

**Yeast mutant strains.** The mutations of the cytochrome *b* gene are listed in Table 1. The mutated and wild-type (WT) mitochondrial genomes were transferred by cytoduction into AD1-9 (α *ura3 his1 yor1Δ::hisG snq2Δ::hisG pdr5Δ::hisG pdr10Δ::hisG pdr11Δ::hisG ycf1Δ::hisG pdr3Δ::hisG pdr15Δ::hisG pdr1Δ::hisG*; kindly donated by M. Ghislain, UCL, Belgium) and into BY4742 Δ*cox7* (α *ura3 his3 leu2 lys2 Δcox7::G418*; purchased from Euroscarf). All of the strains analyzed in the present study of the AD1-9 or the BY4742 series were isogenic. The BY4742 Δ*cox7* series were used for the cytochrome *c* reductase assays. Since the strain lacks a functional cytochrome oxidase due to the nuclear Δ*cox7* mutation, the quinol cytochrome *c* reductase activity could be measured without added KCN. The AD1-9 series were used for all of the growth experiments, since the multiple deletions in the ABC transporter genes render the strains more sensitive to drugs than standard yeast strains (26). The yeast culture media were as described earlier (19).

TABLE 1 Mutations in cytochrome *b*

Mutation(s)	Source or reference <sup>a</sup>
G33A	This study
G37S	This study
T127I	26
G143A	20
I147V	26
S152A	26
H204Y	11
S206T/V	11
N208V	This study
R218K	This study
M221Q	11
F225L	This study
K228M	This study
K228I	This study
L275F	26
Y279C	19
Y279S	20
G143A+K228M	This study

<sup>a</sup> For mutations derived in this study, site-directed mutations were introduced in the mitochondrial cytochrome *b* gene by microprojectile bombardment-mediated mitochondrial transformation as described previously (26).

**Measurement of quinol cytochrome *c* reductase activity in yeast mitochondria.** Mitochondria were prepared as described by Lemaire and Dujardin (33). Quinol cytochrome *c* reductase activity measurements were performed in 10 mM potassium phosphate (pH 7) and 20  $\mu$ M equine cytochrome *c* at room temperature. Mitochondria were diluted to 5 to 30 nM *bc*<sub>1</sub> complex. Concentrations were determined from reduced optical spectra, using  $\epsilon = 28.5 \text{ mM}^{-1} \text{ cm}^{-1}$  at 562 to 575 nm. Activity was initiated by the addition of 40  $\mu$ M decylubiquinol. Cytochrome *c* reduction was recorded at 550 nm versus 540 nm over a 3-min time course in a Beckmann DU 640 spectrophotometer. Initial rates were measured. The IC<sub>50</sub>s were determined by inhibitor titration over a 10 to 100% inhibition range. The measurements were repeated at least twice and averaged.

**Molecular docking.** Equilibrium geometry was performed on HDQ using Spartan 08 (<http://www.computational-chemistry.co.uk/spartan08.html>) with molecular mechanics. Molecular docking of HDQ into the Q<sub>i</sub> site of yeast *bc*<sub>1</sub> complex (Protein Data Bank [PDB] 3CX5) was performed using GOLD 5.0.1 (48). Hydrogen atoms were added to the protein, and all crystallographic water molecules were removed. The yeast protein was aligned with that of bovine *bc*<sub>1</sub> complex (PDB 1SQX), which is highly conserved at the Q<sub>i</sub> site, and cocrystallized with ubiquinone. The location of ubiquinone was used in the modeling to define the search space for the docking algorithm. The site for docking was defined to be all residues within 5 Å of ubiquinone in the yeast protein. Docking was also performed including waters within 3 Å of the binding site but did not yield different results compared to no waters present. No water molecules were close enough to the various docking poses to form hydrogen bonds. Analysis is based on results with no water molecules present. GoldScore fitness function was used to perform the docking. GoldScore is a molecular mechanics-based scoring function that uses a protein-ligand hydrogen bond and Van der Waals terms to optimize for the prediction of ligand-binding poses. The docking was repeated 10 times, with early termination criteria disabled and default GA settings applied.

## RESULTS

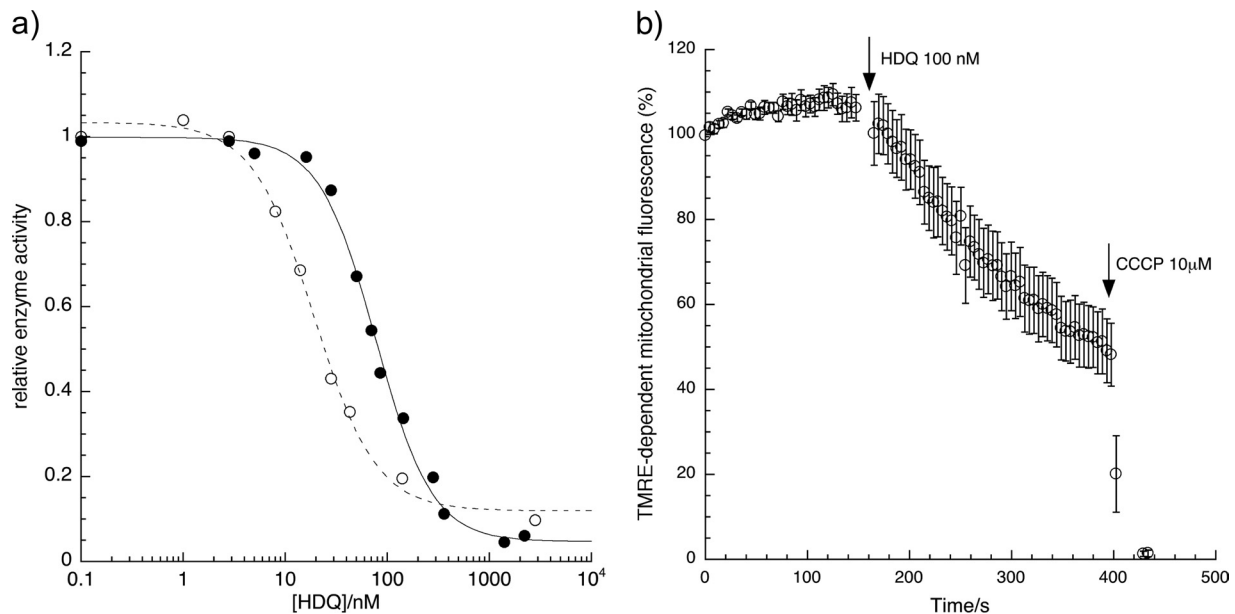
**HDQ disrupts *P. falciparum* mitochondrial function and inhibits PfNDH2 and the *bc*<sub>1</sub> complex.** HDQ displayed potent inhibitory activity of *P. falciparum* growth proliferation with an IC<sub>50</sub> of  $86.5 \pm 2.6$  nM against the control strain 3D7 (all values are derived from three or more independent experiments performed as

described in Materials and Methods). The drug was markedly less effective against the transgenic strain containing yeast dihydroorotate dehydrogenase (3D7-yDHODH-GFP) with an IC<sub>50</sub> of  $6.1 \pm 0.9$   $\mu$ M, as previously reported (15). The decreased sensitivity clearly indicates that HDQ inhibits mitochondrial function, since the expression of the yeast dihydroorotate dehydrogenase has been shown to bypass the need for an efficient quinol oxidation by the *bc*<sub>1</sub> complex (39). The addition of proguanil (1  $\mu$ M final concentration) was observed to reverse the resistance of the transgenic 3D7-yDHODH-GFP strain to HDQ, as has been shown for atovaquone (39). Interestingly, the isolate TM90C2B that carries the cytochrome *b* mutation Y268S (yeast Y279S) and shows a high level of atovaquone resistance (IC<sub>50</sub> mutant/control > 1,000), is highly sensitive to HDQ (IC<sub>50</sub> =  $64 \pm 7.2$  nM), which suggests that these inhibitors have a different mode/site of action. As described earlier, HDQ has previously been reported to act as an inhibitor of the type II NADH dehydrogenase (NDH2) in *Y. lipolytica* (16) and of NDH2 from *T. gondii* expressed in *Y. lipolytica* (36). In agreement with these published data, we found that the activity of the recombinant malaria parasite PfNDH2 was sensitive to submicromolar concentrations of HDQ with a measured IC<sub>50</sub> of  $77 \pm 4.2$  nM (Fig. 1a). A previous study reported that PfNDH2 was not sensitive to HDQ (15). The discrepancy is likely to originate from the significant differences in the heterologous expression strategy used for this enzyme, as well as differences in the assay conditions, these issues have been discussed previously (22).

Interestingly, the parasite *bc*<sub>1</sub> complex activity was also found to be inhibited by nanomolar concentration of HDQ with a IC<sub>50</sub> =  $19 \pm 1.3$  nM (Fig. 1a), which is in the same range as the IC<sub>50</sub> for the Q<sub>o</sub> site inhibitors atovaquone and the acridinedione WR249685 (IC<sub>50</sub> = 3 to 5 nM) (4). Thus, the quinol analog appears to have a dual action, targeting two respiratory enzymes, the PfNDH2 and the *bc*<sub>1</sub> complex. Consistent with HDQ inhibiting these respiratory enzymes, perfusion of HDQ to trophozoites-stage parasites resulted in a rapid depolarization of mitochondrial membrane potential (Fig. 1b).

Furthermore, we observed that the *bc*<sub>1</sub> complex activity of the atovaquone-resistant isolate TM90C2B was as sensitive to HDQ as the wild-type (atovaquone sensitive) strain 3D7, with 200 nM HDQ reducing QH2-cytochrome *c* reductase activity by 74 and 77%, respectively. The TM902CB and 3D7 strains were similarly sensitive to the Q<sub>i</sub> site inhibitor antimycin (5  $\mu$ M), displaying 80 and 73% inhibition, respectively, whereas 50 nM atovaquone reduced 3D7 *bc*<sub>1</sub> activity by 75%, while only affecting TM902CB *bc*<sub>1</sub> activity by 10% (all experiments performed in triplicate as described in Materials and Methods). These data confirm that the atovaquone resistance mutation Y268S found in the TM902CB strain does not confer cross-resistance to HDQ, indicating that the two drugs have distinct binding sites in the *bc*<sub>1</sub> complex. In order to further probe the specific binding site of HDQ to the *bc*<sub>1</sub> complex, we used yeast as a model system, owing to its tractability in being genetically/biochemically manipulated. We then generated a number of yeast mutants with specific point mutations in the Q<sub>o</sub> and Q<sub>i</sub> sites and monitored their impact on HDQ sensitivity.

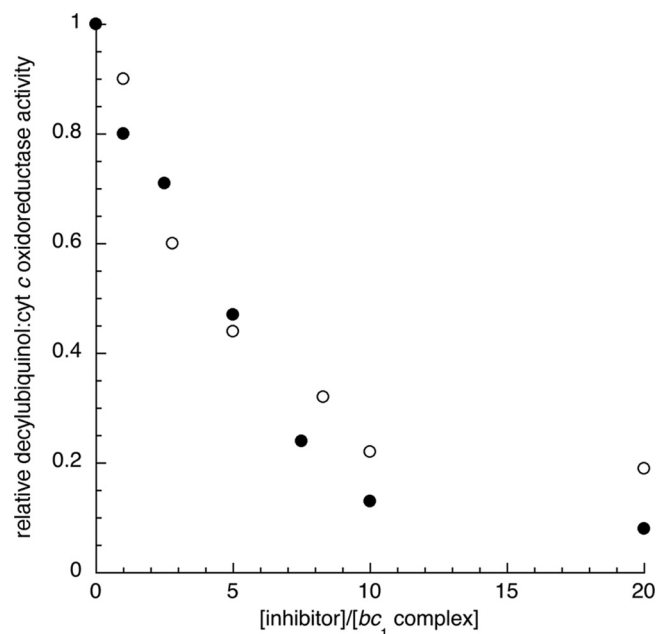
**HDQ blocks the respiratory growth of *S. cerevisiae* through inhibition of the *bc*<sub>1</sub> complex.** We first monitored the effect of HDQ on the growth of yeast cells cultured in respiratory medium, using ethanol as a substrate, and in fermentable medium, using glucose as substrate (10% glucose without aeration) (data not



**FIG 1** Effect of HDQ on *P. falciparum* PfNDH2,  $bc_1$  complex and mitochondrial membrane potential. (a) The PfNDH2 activity (●) was determined by monitoring NADH oxidation and concomitant decylquinone reduction; the  $bc_1$  activity (○) was determined by monitoring cytochrome *c* reduction using decylubiquinol as an electron donor (see Materials and Methods). All data have been acquired from multiple observations from at least three separate preparations. DMSO in the assays did not exceed 0.3%. The  $IC_{50}$ s were calculated by using the four-parameter logistic method. (b) Effect of HDQ on the mitochondrial membrane potential ( $\Psi_m$ ) of *P. falciparum*. The time course of TMRE-dependent fluorescence after the addition of HDQ (100 nM) to *P. falciparum*-infected erythrocytes is shown. The data were normalized to 100% in untreated and to 0% in CCCP (10  $\mu$ M)-treated cells. Graph shows the mean data derived from experiments performed independently  $\pm$  the standard errors ( $n \geq 3$ ).

shown). HDQ inhibited the respiratory growth with an  $IC_{50}$  of  $\sim 1$   $\mu$ M. Yeast growth in fermentable medium was not affected by 20  $\mu$ M HDQ, indicating that HDQ has no effect on cell growth supported by fermentation. For comparison, the  $IC_{50}$ s for atovaquone and HQNO were approximately 20 and 5  $\mu$ M, respectively. The sensitivity of the yeast NDH2 activity to HDQ was then assayed. We found that HDQ was a weak inhibitor of the *S. cerevisiae* NDH2 (<10% inhibition of NADH:decylubiquinol oxidoreductase activity with 1  $\mu$ M HDQ, measured as described in Materials and Methods). Yeast  $bc_1$  complex activity, on the contrary, was sensitive to HDQ (Fig. 2), with a ratio of  $IC_{50}$  per  $bc_1$  complex of 4 to 5, which is similar to the inhibitory effect of azoxystrobin, a  $bc_1$  complex inhibitor acting at the  $Q_o$  site and used to control plant pathogenic fungi.

**HDQ acts at the  $Q_i$  site of the  $bc_1$  complex.** In order to determine the site of binding of HDQ, we monitored the impact of mutations introduced in the  $Q_o$  and  $Q_i$  sites on the sensitivity to HDQ. First, we measured the quinol-cytochrome *c* reductase activity as described in Fig. 2 and determined the  $IC_{50}$  for HDQ. For comparison, we estimated the  $IC_{50}$  for two other  $bc_1$  complex inhibitors: the  $Q_i$  site binding compound, antimycin and the  $Q_o$  site binding compound, azoxystrobin (Table 2). Two  $Q_i$  site mutations G33A and K228M, previously reported as causing resistance to HQNO and antimycin, respectively (8, 14), decreased the sensitivity of the  $bc_1$  complex toward HDQ (Table 2): the  $IC_{50}$ s increased approximately 7- and 10-fold for G33A and K228M, respectively. K228M had a moderate effect on antimycin sensitivity while the sensitivity toward the  $Q_o$  site inhibitor (azoxystrobin) was unchanged. The  $Q_o$  site mutation G143A that causes a high level of resistance toward azoxystrobin, myxothiazol, and atovaquone (20) had no effect on HDQ sensitivity. When G143A and



**FIG 2** Sensitivity of the yeast  $bc_1$  complex activity to HDQ and to the  $Q_o$  site inhibitor azoxystrobin. The quinol cytochrome *c* reductase activity was measured using mitochondria prepared from WT yeast cells (see Materials and Methods). The measures were repeated at least three times and averaged. The errors did not exceed 10% of the presented values. The data are presented as the ratio of the inhibitor concentration (○, azoxystrobin; ●, HDQ) to the  $bc_1$  complex concentration. An  $IC_{50}$  ratio of 4 to 5 (inhibitor molecules per  $bc_1$  complex) was estimated from the plots.

**TABLE 2** Inhibitory effect of HDQ on the quinol cytochrome *c* reductase of WT and mutant mitochondria

Cytochrome <i>b</i> WT and mutation(s)	Turnover no. <sup>a</sup> (s <sup>-1</sup> )	IC <sub>50</sub> /bc <sub>1</sub> complex <sup>b</sup>		
		HDQ	Antimycin	Azoxystrobin
WT	33	4	0.5	5
<b>Q<sub>i</sub> site</b>				
G33A	20	30	0.4	ND
G37S	20	4	0.4	ND
K228M	16	30–50	0.8	4
<b>Q<sub>o</sub> site</b>				
G143A	15	4	0.5	>20
<b>Q<sub>i</sub> and Q<sub>o</sub></b>				
G143A+K228M	18	30–40	0.9	>20

<sup>a</sup> The turnover number is the amount of cytochrome *c* reduced per bc<sub>1</sub> complex per s using 40 μM decylubiquinol (see Materials and Methods). The measurements were repeated at least twice. The errors did not exceed 10% of the presented values.

<sup>b</sup> Values are presented as the ratio of the IC<sub>50</sub> to the concentration of the monomeric bc<sub>1</sub> complex (estimated using the cytochrome optical signal as described in Materials and Methods). For example, 0.5 molecules of the tight binding inhibitor antimycin were added per monomeric bc<sub>1</sub> complex to inhibit the quinol cytochrome *c* reductase activity by 50%. ND, not determined.

K228M mutations were combined, the resulting mutant showed resistance toward both Q<sub>o</sub> and Q<sub>i</sub> site inhibitors. We tested a third Q<sub>i</sub> site change, G37S, reported as causing weak resistance to ilicicolin H (13). This mutation had no effect on HDQ sensitivity. For all of the mutations located in the active sites (Q<sub>o</sub> or Q<sub>i</sub>), we observed a 1.5- to 2-fold decrease in bc<sub>1</sub> complex activity.

We then monitored the inhibitory effect of HDQ on the respiratory growth of a collection of Q<sub>i</sub> mutants chosen on the basis of the structure (28, 29) and/or on previous report of their effect on HQNO sensitivity (10) (Table 3). In line with the enzymatic data presented in Table 2, G33A and K228M (and the double-change G143A+K228M) conferred a high level of resistance (IC<sub>50</sub> of >10 μM compared to 1 μM for the WT). These mutants also showed a marked (>10-fold) resistance to HQNO (data not shown). Two other Q<sub>i</sub> mutations (causing HQNO resistance [10]) resulted in an increased resistance toward HDQ, H204Y and M221Q, whereas mutations in positions 208, 218, and 225 had little effect. Interestingly, the HDQ sensitivity of mutant K228I was similar to the WT, in direct contrast to K228M. S206T/V and G37S appeared to be more sensitive to HDQ. The increased sensitivity of the respiratory growth of G37S might seem surprising, since the sensitivity of the mutated bc<sub>1</sub> complex was unchanged compared to the WT. However, this behavior can be explained by the decreased activity of the bc<sub>1</sub> complex in the mutant (Table 2). Since the level of bc<sub>1</sub> complex activity is lower in the mutant cells, its titration by HDQ in the culture gave a lower value. A similar behavior explains the increased sensitivity of S206V/T. Note that the respiratory growth of the mutants with lower bc<sub>1</sub> activity was not decreased in the absence of inhibitor. The bc<sub>1</sub> complex activity is in excess in WT yeast cell, and only a severe decrease in the activity or level of the complex results in a defective respiratory growth.

For comparison, we monitored the inhibitory effect of HDQ on respiratory growth of Q<sub>o</sub> site mutants (Table 3). T127I, I147V, S152A, and L275F are mutations found in the human pathogenic fungus *P. jirovecii*, after atovaquone treatment (26); Y279C and Y279S are atovaquone resistance mutations reported in *P. falciparum*.

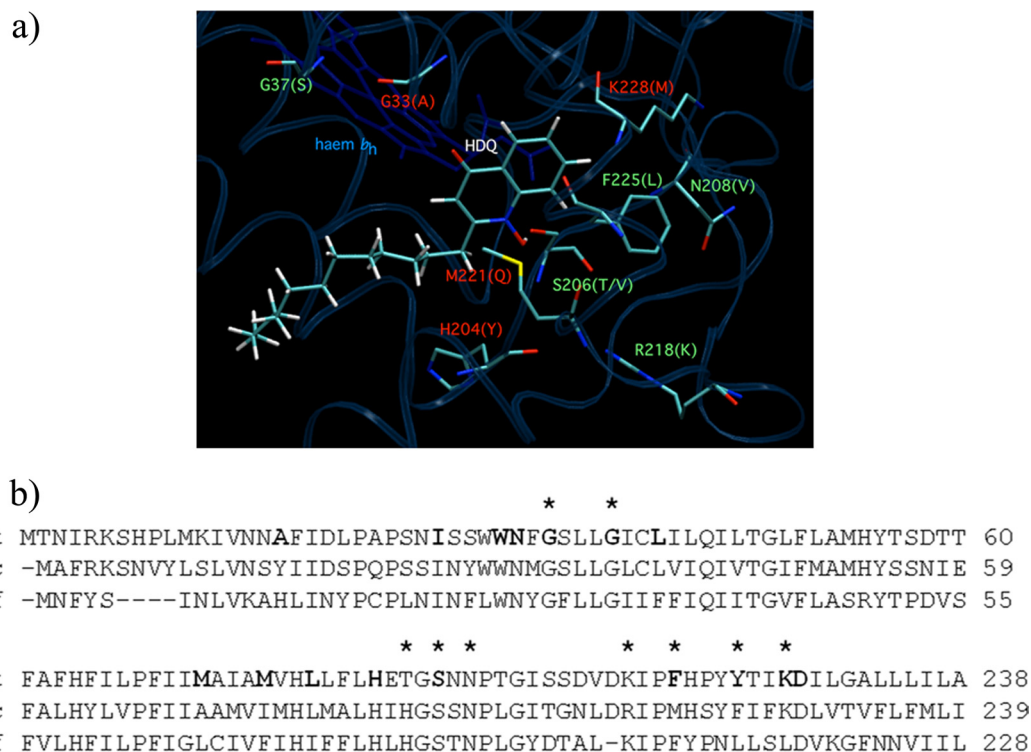
**TABLE 3** Differential effects of cytochrome *b* mutations on the sensitivity of the respiratory growth toward HDQ

WT and site mutation(s)	IC <sub>50</sub> (μM) <sup>a</sup>
WT	1
<b>Q<sub>i</sub> site</b>	
G33A	>10
G37S	<0.05
H204Y	>10
S206T/V	<1
N208V	1–2
R218K	1–2
M221Q	>10
F225L	1–2
K228M	>10
K228I	1
G143A+K228M	>10
<b>Q<sub>o</sub> site</b>	
T127I	1
G143A	0.05–0.1
I147V	0.5
S152A	0.05
L275F	0.5–1
Y279C	<0.05
Y279S	<0.05

<sup>a</sup> The IC<sub>50</sub> values were estimated as described in Materials and Methods. Cells were cultured in respiratory medium (ethanol) in the presence of increasing concentrations of HDQ. The cell density was measured at the early stationary phase. The cell density of the mutants in the absence of inhibitors was similar to that of the WT. The measurements were repeated at least twice.

*arum* (Y268C/S in *P. falciparum*). G143A causes a high level of resistance toward Q<sub>o</sub> site fungicides in plant pathogenic fungi and was shown to confer atovaquone resistance when introduced in yeast (20; for a review of cytochrome *b* mutations, see reference 21). None of the atovaquone resistance mutations conferred cross-resistance to HDQ. An increased sensitivity toward HDQ was observed for several mutants. The increased sensitivity correlates with a decreased activity of the mutated bc<sub>1</sub> complex caused by the Q<sub>o</sub> site mutation. T127I and I147V had no or mild effect on the bc<sub>1</sub> complex activity (26) and on the sensitivity toward HDQ, whereas S152A caused a 5-fold decrease in activity (26) and a marked sensitivity toward HDQ. The G143A mutation resulted in a 2-fold decrease in quinol cytochrome *c* reductase activity and a >10-fold decrease in the sensitivity of the respiratory growth to HDQ (Tables 2 and 3). L275F had no effect on the respiratory growth sensitivity and the bc<sub>1</sub> complex activity (not shown). Y279C/S has previously been shown to severely impair bc<sub>1</sub> complex activity (31) and, accordingly, the IC<sub>50</sub> on respiratory growth was markedly more sensitive to HDQ (<0.05 μM). Consistent with these data, Q<sub>i</sub> site mutants with reduced bc<sub>1</sub> activity showed an increased sensitivity toward the Q<sub>o</sub> site inhibitor atovaquone (data not shown). The double mutant that combines Q<sub>o</sub> and Q<sub>i</sub> site resistance mutations G143A+K228M expressed the combined resistance toward Q<sub>o</sub> and Q<sub>i</sub> inhibitors.

In summary, four Q<sub>i</sub> site HQNO-resistance mutations caused a marked cross-resistance to HDQ, whereas none of the atovaquone-resistant Q<sub>o</sub> site mutations tested conferred cross-resistance to HDQ. It is interesting that, as described above for *P.*



**FIG 3** Model of HDQ binding in the  $Q_1$  site and localization of  $Q_1$  mutations. (a) Structural model (obtained as described in Materials and Methods) showing mutations causing resistance to HDQ (red) and mutations without effect on HDQ resistance (green). (b) Comparison of the sequences of  $Q_1$  site region between bovine (Bt), yeast *S. cerevisiae* (Sc), and *P. falciparum* (Pf) cytochromes *b*. Residues in close contact ( $>4 \text{ \AA}$ ) with the antimycin bound at the  $Q_1$  site in the bovine enzyme are indicated in boldface (28). The mutated residues analyzed in the present study are marked with an asterisk (\*).

*falciparum* TM902CB, the mutation Y279S (Y268S in *P. falciparum*), when introduced in yeast, caused a marked decreased in  $bc_1$  complex activity, a high level of resistance to atovaquone (20, 31) but no cross-resistance toward HDQ. These data are consistent with the hypothesis that HDQ targets the  $Q_1$  site of the  $bc_1$  complex. Molecular modeling was then performed to further explore the molecular interactions of HDQ binding at the  $Q_1$  site of the  $bc_1$  complex.

**Molecular modeling of HDQ binding at the  $Q_1$  site of the *S. cerevisiae*  $bc_1$  complex.** The analysis was performed using GOLD 5.0.1 with PDB 3CX5 as a template (see Materials and Methods). The best scoring pose had a GoldScore of 59.4, indicative of a good binding orientation within the  $Q_1$  site. Subsequent poses supported the predicted orientation. A detailed description of the model of docking and associated data are presented in the supplementary data (see Fig. S2 and Table S1 in the supplemental material). For comparison purposes, *in silico* docking of ubiquinone-6 within the  $Q_1$  site of yeast  $bc_1$  resulted in a “best solution” binding pose with a GoldScore of 50.4. Docking of stigmatellin at the  $Q_0$  and  $Q_1$  sites of yeast  $bc_1$  resulted in GoldScores of 111.6 and 56.3, respectively, in agreement with the expected binding site for this inhibitor. Interestingly, the favored docking pose for HDQ within the  $Q_0$  site had a GoldScore value of 65.4, which is suggestive of an apparently more favorable interaction than that observed at  $Q_1$ . However, it should be noted that hydrogen bonds are more highly weighted than hydrophobic and van der Waals interactions as determined by the GOLD docking algorithm, and the more hydrophilic nature of the  $Q_0$  site compared to  $Q_1$  may artificially

favor HDQ docking *in silico* at  $Q_0$ . The mobility of the ectodomain of the Rieske protein and the presence of protein-bound water within  $Q_0$  also presents challenges for *in silico* modeling methods.

The protein-HDQ interactions were similar to that of  $Q_1$ -bound substrate ubiquinone-6 in PDB entry 1EZV and to the binding interactions of the quinolone headgroup of NQNO (2-nonyl-4-hydroxyquinoline N-oxide), the C9-alkyl variant of HDQ, in the bovine structure (25). The  $Q_1$  site mutations analyzed above were reexamined in light of this model (Fig. 3). G33A (a conserved residue in mammalian, yeast, and *P. falciparum* cytochrome *b* and located in transmembrane helix A) has been previously shown to cause resistance to HQNO but not to antimycin (8). In our binding model, the  $\alpha$ C atom of G33 has a closest approach of 4.4  $\text{\AA}$  to the quinolone headgroup of HDQ, so it is reasonable to suggest that introduction of a methyl group at this position may result in steric hindrance with  $Q_1$ -bound molecules. H204, conserved in yeast and *Plasmodium*  $bc_1$  complex, is replaced by a threonine in mammals and has a closest approach of 10  $\text{\AA}$  to the quinolone headgroup of NQNO in the bovine atomic structure (25). A similar separation is observed in our HDQ-docked yeast model. It is thus not immediately apparent why this mutation should affect quinolone binding and yet not be deleterious for  $bc_1$  complex activity (9), although it may be postulated that introduction of the bulkier tyrosyl side chain results in local reorganization in the packing of side chains or conformation in the DE loop. M221Q (transmembrane helix E) causes resistance to HQNO but not antimycin and has a nearly WT level of  $bc_1$  com-

plex activity (8). The εC methyl group of M221 is predicted to be in a stabilizing hydrophobic interaction with the quinolone ring of HDQ in our yeast structural model (3.6 Å closest approach). This residue is replaced by phenylalanine in mammalian and *Plasmodium* cytochrome *b*. In the NQNO-inhibited bovine enzyme, this phenylalanine side chain participates in a stabilizing aromatic-aromatic interaction with the bound quinolone. Mutation to the more polar glutamine side chain removes this interaction. K228M (transmembrane helix E) confers weak resistance to antimycin but marked resistance to HDQ (Table 2). The terminal amino group of this residue participates in a water-mediated H-bond to the formyl amino oxygen atom of bound antimycin in bovine bc<sub>1</sub> complex (28). A similar bridged hydrogen-bonding association has been suggested for this residue and substrate ubiquinone in the yeast bc<sub>1</sub> structure, where it was proposed to form part of a proton-uptake pathway for quinone redox chemistry at Q<sub>i</sub> (30). Interestingly, the side chain appears to demonstrate considerably mobility and can be modeled in two different conformations (the terminal NE atoms of K228 point in different directions in the yeast and bovine crystal structures [25]). This residue has a closest approach of 5.5 Å to NQNO in the bovine structure, although it is unclear whether it is participating in a water-bridged H-bond with the quinolone N-oxide moiety. In our structural model, which was based on the yeast coordinates (3CX5 [43]), the side chain of K228 is oriented “distal” to the Q<sub>i</sub> pocket, and so we observe a separation of 13 Å to the quinone headgroup of bound HDQ. Rotation of this residue into the Q<sub>i</sub> “proximal” position would facilitate a bridged hydrogen bond between the terminal amino group and the HDQ quinolone headgroup. It should be noted, however, that the mutation of this residue to isoleucine did not confer resistance to HDQ (and K228 is replaced by leucine in *P. falciparum* cytochrome *b*), and so it is unlikely that hydrogen bonds to this position are a significant factor in quinolone binding. It is therefore unclear why the K228M mutation should confer HDQ resistance, it may arise from distortion of the nearby aA loop, a region of the Q<sub>i</sub> site predicted to contain several residues in close association with bound HDQ.

G37S (transmembrane helix A) has been reported previously as causing resistance to ilicicolin H (13); no resistance to HDQ was observed here. This residue has a closest approach of 8.3 Å to HDQ in our docking model. S206 (DE loop) is conserved in eukaryotic cytochrome *b* and is predicted to be a H-bond donor via its hydroxyl moiety to the methoxy oxygen atom of Q<sub>i</sub>-bound ubiquinone in the bovine and yeast bc<sub>1</sub> complex crystal structures (28, 29). Mutation to threonine or valine, however, has no effect on bc<sub>1</sub> complex catalytic activity (9) and confers no resistance to HDQ. F225L (transmembrane helix E) has previously reported as causing resistance to diuron (14). The phenyl ring of this residue has a closest contact of 3.1 Å with the quinolone group of HDQ in our docking model, with the rings oriented at 120 degrees with respect to each other and a van der Waals contact surface area of 19 Å<sup>2</sup>. The isobutyl group of leucine is likely to be able to form a similar stabilizing hydrophobic interaction with HDQ in the F225L mutant and, indeed, leucine is found in this position in the sequence of *P. falciparum* cytochrome *b*. N208V and R218K, both located in the DE loop (proposed as a proton input pathway for quinone redox chemistry at Q<sub>i</sub> [30]), are ~10 Å from HDQ in our binding model.

## DISCUSSION

HDQ has been shown previously to display potent antimalarial activity (40). Here, we confirm this observation and additionally show that this compound is active against the atovaquone resistance parasite TM90C2B (carrying the Y268S mutation), suggesting a different target site of action to atovaquone. The lack of activity of HDQ against the transgenic 3D7-γDHODH-GFP strain (39), indicated that this compound targets mitochondrial function. Consistent with this, assessment of mitochondrial function using single-cell imaging of parasite mitochondria revealed that addition of HDQ rapidly depolarized the mitochondrial membrane potential. Assessment of the electron transport chain enzymes NADH:ubiquinone oxidoreductase (PfNDH2 [18]) and bc<sub>1</sub> complex revealed that HDQ is a potent inhibitor of both enzymes. HDQ therefore displays characteristics of a privileged pharmacophore able to inhibit more than one enzyme. It is not clear, however, from our data alone whether parasite kill is afforded via the inhibition of bc<sub>1</sub> alone or via a combination of the inhibition of bc<sub>1</sub> and PfNDH2. A recent study, performed in the rodent malaria *P. berghei*, indicates that deletion of the NDH2 gene is not lethal to erythrocytic stages of the parasite (7). Our observation that proguanil reverses the resistance of the transgenic 3D7-γDHODH-GFP to HDQ, is consistent with similar observations using bc<sub>1</sub>-acting inhibitors (39). However, this same experiment has not been performed with a known PfNDH2-selective drug and is therefore difficult to interpret. Clearly further, definitive investigations are required to determine the essentiality of PfNDH2 in *P. falciparum*. It is worth noting, however, that, historically, anti-infectives displaying polypharmacology show greater efficacy over single-targeting inhibitors (27). In *Toxoplasma gondii*, the deletion of type II NADH:dehydrogenase genes is not lethal but is required for optimal tachyzoite growth (35). Interestingly, HDQ has been shown to be synergistic with atovaquone for growth inhibition (36), which may be attributed to the polypharmacological effect of HDQ against the type II NADH:dehydrogenases as well as the Q<sub>o</sub> and Q<sub>i</sub> sites of the bc<sub>1</sub> complex.

In order to determine the mode of action of HDQ against the bc<sub>1</sub> complex, yeast mutants were generated carrying specific amino acid substitutions in the two catalytic sites, Q<sub>o</sub> and Q<sub>i</sub> (Table 1). The specific Q<sub>i</sub> point mutations tested were chosen because of their involvement in the binding of antimycin and ubiquinol in the Q<sub>i</sub> site of the bovine enzyme, as revealed by crystal structures (28, 29) and/or by a previous report of the effect on HQNO sensitivity (10): S206T/V, H204Y, K228M/I, G33A, G37S, M221Q, and F225L. Two residues located in a possible proton pathway toward the Q<sub>i</sub> site (30), R218K and N208V, were also tested. The Q<sub>o</sub> site mutations were atovaquone-resistant mutations reported in human and plant pathogens. None of the Q<sub>o</sub> site mutations conferred cross-resistance to HDQ, while four Q<sub>i</sub> site mutations caused a cross-resistance toward HDQ and HQNO, namely, G33A, H204Y, M221Q, and K228M (Tables 2 and 3). Thus, it could be suggested that these residues are involved in the stabilization of HDQ in the Q<sub>i</sub> site. As described above, molecular modeling of HDQ bound to the Q<sub>i</sub> site supports the importance of residues G33, H204, and M221 for the binding interaction; however, it is not clear at this stage why the K228M mutation affects this process.

The mutational study cannot exclude that HDQ could also bind at the Q<sub>o</sub> site with a lower affinity. Inhibitors have been

described that bind at both Q<sub>o</sub> and Q<sub>i</sub> sites. For instance, spectroscopic studies have shown that Ascochlorin acted at both sites of the bacterial and vertebrate bc<sub>1</sub> complex and crystallographic analyses has revealed its precise binding sites in the two quinone pockets of the chicken enzyme (3). Crystallographic analysis of the bovine bc<sub>1</sub> complex showed that 2-nonyl-4-hydroxyquinoline N-oxide (NQNO) binds at both sites in the crystal structure (25). It is likely that HQNO could also bind at the Q<sub>o</sub> site. However, amino acid substitutions causing resistance to HQNO have only been found in the Q<sub>i</sub> site (10). This suggests that the affinity of NQNO/HQNO for the Q<sub>o</sub> site would be lower; thus, resistance could not develop by mutation of the Q<sub>o</sub> site.

Comparison of sequences (Fig. 3B) shows that Q<sub>i</sub> site residues involved in HDQ stabilization (as judged by mutational analysis) are well conserved between yeast and *P. falciparum* enzyme. It is likely that HDQ would also target the Q<sub>i</sub> site of *P. falciparum*, which is in agreement with the observation that the atovaquone resistance *P. falciparum* isolate TM90C2B (with the Q<sub>o</sub> site mutation Y279S [Y268S in *P. falciparum*]) is sensitive to HDQ.

The bc<sub>1</sub> complex of the malaria parasite is a proven drug target and is an essential component for various stages of the parasite life cycle, including the liver stages and the circulating asexual stages. This enzyme is therefore the only validated malarial drug target that has utility for both curative and prophylaxis treatment. Unfortunately, atovaquone-resistant parasites have been observed in the field following atovaquone or Malarone treatment failures (see, for example, references 2, 23, 38, and 41). Atovaquone, as do the fungicides and pesticides targeting the bc<sub>1</sub> complex, binds at the Q<sub>o</sub> site (the only exception is the fungicide cyazofamid that targets the Q<sub>i</sub> site and is only active against oomycetes [37]). Mutations in the Q<sub>o</sub> site have been reported that compromise the pathogen control (reviewed in reference 21). In addition, the Q<sub>o</sub> inhibitors have similar modes of binding, and thus cross-resistance toward the drugs is observed. Thus, inhibitors acting at the Q<sub>i</sub> site would be invaluable tools against pathogens.

With the exception of cyazofamid, most bc<sub>1</sub> inhibitors that have been developed into drugs or pesticides do not target the Q<sub>i</sub>. The Q<sub>i</sub> site is less conserved among species than the Q<sub>o</sub> site. From an alignment of 16 diverse cytochrome *b* sequences, including vertebrates, invertebrates, fungi, plants, and protozoa, the estimated sequence similarity at the Q<sub>o</sub> site is 48% for the ef loop and 60% for the C terminus of the helix C and the cd1 loop; the sequence similarity at the Q<sub>i</sub> site is 30% for the aA loop and N terminus of helix A, 36% for the C terminus of helix D and the DE loop and 44% for the N terminus of helix E (data not shown). Thus, from the drug development point of view, the relative sequence diversity of the Q<sub>i</sub> pocket should have favored selectivity. On the other hand, Q<sub>i</sub> appears to be more structurally rigid (25, 28), with no evidence of movement on antimycin binding, whereas the Q<sub>o</sub> site shows more mechanical flexibility on inhibitor binding (17). It was previously reported that in the Q<sub>o</sub> pocket, H-bonds between side chains of a few conserved residues hold together the components of the site that are regions of the cytochrome *b* distant in the sequence. Crofts et al. described the feature as a “loose stitching” that would allow the expansion of the site upon binding of inhibitors (12). That property presumably allows the site to accommodate more diverse molecules and could explain the development of specific Q<sub>o</sub> inhibitors active against pathogens.

We showed here that HDQ, active against *P. falciparum*, is a

proof-of-concept molecule that targets the Q<sub>i</sub> site and that can therefore circumvent atovaquone resistance. HDQ is not active *in vivo* using the *P. berghei* rodent model (7), but given its potent activity against bc<sub>1</sub>, this is most probably due to poor drug exposure. Drugs developed on the HDQ scaffold targeting the Q<sub>i</sub> site, but with improved pharmacokinetic features, may therefore be a valuable tool in combating parasite drug resistance as we would predict that if used in combination, Q<sub>i</sub> and Q<sub>o</sub> site inhibitors would be more recalcitrant to the emergence of resistance. Double mutations at both active sites, if they arose, would result in a severe loss of function and thus of cell fitness and are unlikely to be tolerated. Further synthetic studies are now under way to develop more drug-like inhibitors of the Q<sub>i</sub> site of the bc<sub>1</sub> complex.

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