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A drug-selected *Plasmodium falciparum* lacking the need for conventional electron transport

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Abstract

Mitochondrial electron transport is essential for survival in *Plasmodium falciparum*, making the cytochrome (cyt) *bc*₁ complex an attractive target for antimalarial drug development. Here we report that *P. falciparum* cultivated in the presence of a novel cyt *bc*₁ inhibitor underwent a fundamental transformation in biochemistry to a phenotype lacking a requirement for electron transport through the cyt *bc*₁ complex. Growth of the drug-selected parasite clone (SB1-A6) is robust in the presence of diverse cyt *bc*₁ inhibitors, although electron transport is fully inhibited by these same agents. This transformation defies expected molecular-based concepts of drug resistance, has important implications for the study of cyt *bc*₁ as an antimalarial drug target, and may offer a glimpse into the evolutionary future of *Plasmodium*.

Unlike most other eukaryotic cells, Plasmodia do not rely on mitochondrial electron transport for energy production. Nonetheless, inhibition of cyt *bc*₁ complex electron transport is normally lethal to the parasite, presumably by interruption of essential links to *de novo* pyrimidine biosynthesis, via dihydroorotate dehydrogenase (DHODH), and to maintenance of the mitochondrial membrane potential. Although these interrelationships determine the viability of the parasite and our ability to reduce that viability, they are only generally understood. Review of Plasmodial electron transport and cyt *bc*₁ complex function are beyond the scope of this report, but in-depth discussions are available [1-3].

The anti-malarial drug atovaquone occupies the quinol oxidase (Q_o) site of mitochondrial cyt *b*, inhibiting electron flux through the cyt *bc*₁ complex (ubiquinol:cytochrome *c* oxidoreductase or complex III) and collapsing mitochondrial membrane potential with a potency 1000-fold greater in *Plasmodium* than mammalian cells [4,5]. Unfortunately, high rates of recrudescence infection and treatment failure were seen after anti-malarial use of atovaquone alone [6], and treatment failures after atovaquone-proguanil combination therapy (Malarone®) were soon evident [7,8] despite only limited worldwide anti-malarial use. The rapid development and the diversity of atovaquone-resistant phenotypes and genotypes (perhaps a consequence of its mechanism of action [9]), the parallel evolution of resistance-encoding mutations [10] and their appearance with or without atovaquone exposure [11] in dispersed geographic locations, all indicate the strong propensity for atovaquone resistance. The hope that this risk can be overcome by appropriate combination therapy or by novel inhibitors continues to drive efforts to discover and develop cyt *bc*₁ inhibitor anti-malarials [12].

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In such an effort, we had previously discovered 3-(6,6,6-trifluorohexyloxy)-6-amino-acridone (6-NH₂Ac) [13], a potent novel inhibitor of *in vitro* *P. falciparum* growth that was subsequently found to inhibit oxygen consumption in *P. yoelii* (unpublished data). We postulated *cyt b* as the target of 6-NH₂Ac and next sought to generate resistance to 6-NH₂Ac; at the same time, we expected to identify resistance-associated *cyt b* mutations that might aid in target validation. We cultured *P. falciparum* D6 under 6-NH₂Ac drug pressure (stepwise increase to 100nM over 1 week, followed by 3 weeks at 5μM), and were then able to isolate highly resistant clones by limiting dilution. The clone selected for study, SB1-A6, retains the sensitivity profile of the parent D6 to chloroquine, quinine, pyrimethamine, and 5-fluoroorotate, among others, but is highly resistant (>10,000-fold) to 6-NH₂Ac (Table 1).

We then assessed susceptibility to growth inhibition by a variety of *cyt bc*₁ inhibitors, comparing *in vitro* results between D6, SB1-A6 and TM90C2B (Table 1). TM90C2B is a clinical isolate, previously shown to be atovaquone resistant due to a mutation in the parasite *cyt b* gene resulting in a Y268S substitution within the Q_o site [14,15]. Parasite cultivation and drug-susceptibility assay methods were as previously described [13]. Compared to D6, SB1-A6 is highly resistant to all tested *cyt bc*₁ inhibitors, including those that target the proximal or distal niches of the Q_o site (atovaquone, stigmatellin, myxothiazol) or the quinone reductase (Q_i) site (antimycin A), and others for which the target site is less well-characterized (clopidol [16], WR243251 [17]). In contrast, TM90C2B is variably resistant to inhibitors that target the Q_o site (>25,000-fold to atovaquone, 20-fold to myxothiazole, and 2-fold to stigmatellin), but fully sensitive to antimycin A. Unlike the resistance phenotype of TM90C2B, which is consistent with a Q_o target site mutation, the resistance of SB1-A6 to drugs targeting widely separated sites within the *cyt bc*₁ complex excludes a classical molecular basis for *cyt b*-linked resistance.

To confirm the absence of an explanatory *cyt b* mutation, the circular mitochondrial DNA was isolated from intact *P. falciparum* D6 and SB1-A6 mitochondria using the alkali-lysis method, and the *cyt b*-encoding region of DNA fragments obtained by PCR. Previously described sense and antisense primers [18] were synthesized, together with an additional primer (5'-TTATATGTTTGCTTGGGAGCT-3'), constructed to obtain more accurate coverage of the mid region of *cyt b*. *Cyt b* sequencing, done at the Washington State University sequencing facility, revealed no differences among D6, SB1-A6, and the published sequence (GenBank accession number [NC002375](#)).

Mitochondria were then isolated in order to assess *cyt bc*₁ electron transport *in vitro* with and without inhibitors. Procedures followed the methods of Fry and Pudney [4], with some modifications. Parasites were concentrated by centrifugation after saponin lysis of host erythrocytes, the washed pellets passed through a Bio Neb Cell Disruptor (20 PSI, argon) (BioNeb®, Glas-Col, Terre Haute, IN), cell debris removed with centrifugation and mitochondria isolated by differential centrifugation. *Cyt bc*₁ complex activity was measured in buffer containing 50 mM tricine, 100 mM KCl, 50 μM *cyt c*⁺³, 50 μM decylubiquinol and 2 mM KCN. The reaction was initiated by the addition of solubilized mitochondrial fragments (6 mg/ml dodecylmaltoside) to the reaction about 15-20 seconds after the initiation of measurement to allow for the subtraction of background *cyt c*⁺³ reduction by decylubiquinol. Fresh decylubiquinol was generated just before experiments by adding a few crystals of NaBH₄ to 100 μl decylubiquinone suspended in 1:1 ethylene glycol:ethanol. Excess NaBH₄ was consumed by the addition of 3 μl of 0.1 N HCl. *Cyt c* reduction was measured spectrophotometrically by monitoring absorbance at 550nm.

Remarkably, despite marked SB1-A6 resistance (>10,000-fold) resistance to growth inhibition by atovaquone or 6-NH₂Ac, electron transport through cell-free *cyt bc*₁ complexes was fully susceptible to inhibition by either compound, as well as to stigmatellin or antimycin A. In both

SB1-A6 and D6, inhibition was complete at a single test concentration of 6-NH₂Ac (100nM), stigmatellin (2μM) or antimycin A (10μM) but dose-response curves were not determined. For atovaquone, the dose-response relationship was determined, revealing no difference in the atovaquone-sensitivity of the cyt *bc*₁ complex between SB1-A6 and D6 that could account for the lack of atovaquone effect on SB1-A6 proliferation (Fig. 1). Marked whole-parasite resistance to cyt *bc*₁ inhibitors (Fig. 1a) despite full sensitivity of the cell-free cyt *bc*₁ enzyme complex (Fig. 1b) indicates that SB1-A6 lacks a requirement for this component of active electron transport. A fitness cost of atovaquone resistance has been described [19] but, in contrast, we note that the growth of SB1-A6 is consistently the most robust of the many *P. falciparum* strains cultured in our laboratory.

The significance of these findings, with regard to both our understanding of anti-malarial drug targets and the evolutionary biology of Plasmodia, hinges on unresolved questions about electron transport and atovaquone actions. One current model proposes that cyt *bc*₁ electron transport is essential only for its linkage to DHODH in support of *de novo* pyrimidine synthesis. In support of this model, a recent report described marked *P. falciparum* atovaquone resistance conferred by transgenic insertion of a single protein; a soluble, cytoplasmic DHODH (Type I) from *Saccharomyces cerevisiae* [20]. Those authors contend, therefore, that the link to the *Plasmodium* Type II DHODH function is the only essential role for cyt *bc*₁ electron transport, and that although atovaquone has other effects, its lethality (and that of other cyt *bc*₁ inhibitors) is primarily via prevention of pyrimidine synthesis. Noting the small size of the mitochondrial genome in *Plasmodium*, the absence of electron transport in some other Apicomplexans, and the ability of a single protein to render cyt *bc*₁ electron transport obsolete, they also suggest that *Plasmodium* may be evolving toward a future without mitochondrial electron transport. This raises the provocative question of whether SB1-A6 might represent one example of that future.

The mechanism by which the transgenic DHODH eliminates the need for cyt *bc*₁ electron transport remains controversial [21,22], but the resulting phenotype is strikingly similar to SB1-A6. SB1-A6, like the transgenic parasite, is pan-resistant to cyt *bc*₁ inhibitors and rendered hypersensitive to proguanil by the addition of 100nM atovaquone (Fig. 2). SB1-A6 is similarly sensitive to proguanil in the presence of 100nM 6-NH₂Ac (data not shown). No such effect is evident in TM90C2B (proguanil IC₅₀>5μM with or without atovaquone or 6-NH₂Ac), consistent with previous results in the TM90C2B [14] and other [23] atovaquone-resistant parasites with identified mutations at codon 268 of cyt *b*. A hypothesis to account for parasite-specific synergy between proguanil and atovaquone has been proposed, but remains speculative [20]. Presently, therefore, the similarities between SB1-A6 and the DHODH transgenic parasite are intriguing but of unclear meaning.

Others have suggested an alternative model in which electron flux upstream of cyt *bc*₁ is essential, in combination with cyt *bc*₁, for maintenance of the mitochondrial membrane potential and parasite survival [24,25]. In this model, a major role for the rotenone-insensitive, alternative (Type II, single-subunit) NADH dehydrogenase (NADH:quinone oxidoreductase or PfNDH2) has been proposed. These investigators and others [26] also contend that inhibition of pyrimidine biosynthesis does not adequately account for the actions of atovaquone, and they have argued that alternative explanations exist for the transgenic DHODH phenotype [21]. Interestingly, SB1-A6 shows modest (4-fold) resistance to the non-specific inhibitor of PfNDH2 diphenylene iodonium chloride (DPI) while TM90C2B does not (Table 1), and the addition of 100nM atovaquone does not enhance the susceptibility of SB1-A6 to DPI (IC₅₀=286.9nM alone; 302.7nM in the presence of atovaquone). If both cyt *bc*₁ and PfNDH2 do contribute to maintaining mitochondrial membrane potential, these results may suggest, at least in SB1-A6, the existence of an alternate source of membrane potential.

Resolving these questions and elucidating the mechanisms by which conventional electron transport may have become non-essential in SB1-A6 will require further study. Possible explanations for SB1-A6 include one or more of the following: a complete transformation in pyrimidine biosynthesis (i.e., not requiring DHODH); development of pyrimidine salvage capability; or uncoupling, biochemically if not positionally, of DHODH from cyt *bc*₁-mediated ubiquinol oxidation. The latter would require either an efficient alternate source of quinol oxidation in place of the cyt *bc*₁ complex, or that DHODH utilize an alternate readily-available electron acceptor other than ubiquinone. While succinate dehydrogenase [Complex II] can function in “reverse” to oxidize ubiquinol [27], its maximal capacity to do so is unknown; the capability of the Type II DHODH of *Plasmodium* to employ other electron acceptors has not yet been demonstrated in intact parasites.

Finally, the implications of the SB1-A6 phenotype to the consideration of the cyt *bc*₁ complex as an anti-malarial drug target are unclear, but important questions are raised. Was the adaptation leading to SB1-A6 a unique event, a reproducible event uniquely related to 6-NH₂Ac, or a possible consequence of exposure to other cyt *bc*₁ inhibitors, as well? The possibility that adaptations similar to those observed in SB1-A6 might arise in clinical isolates deserves consideration, particularly in light of the failure of identified mutations to consistently account for either the presence or the degree of atovaquone resistance. Since adaptations analogous to those in SB1-A6 can be ruled out by sensitivity to antimycin A, such testing might be considered in the analysis of atovaquone-resistant isolates lacking resistance-associated mutations. We would also suggest that SB1-A6 will prove a useful screening tool for cyt *bc*₁ inhibitor activity, without the need for cell-free mitochondrial extracts, and that efforts determine the validity and value of this approach are warranted. Investigations are underway to determine the adaptive mechanisms resulting in the SB1-A6 phenotype, the elucidation of which may clarify our understanding of, and approach to, mitochondrial drug targets in *Plasmodium*.

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Fig. 1.

SB1-A6 is highly resistant to atovaquone, but there is no evidence of cytochrome bc_1 atovaquone resistance. **(A)** *In vitro* growth of *P. falciparum* D6 and SB1-A6 assessed after 72 hours in varying concentrations of atovaquone. SB1-A6 is >100,000-fold less susceptible to growth inhibition. Growth was measured by SYBR Green I fluorescence. (n=4). Error bars represent SEM. **(B)** Comparison of *in vitro* cytochrome bc_1 activity between D6 and SB1-A6 in the presence of varying concentrations of atovaquone. There is no difference in susceptibility to inhibition by atovaquone, indicating that this is not the basis for atovaquone resistance in SB1-A6. Using mitochondria isolated from cultured parasites, activity was determined spectrophotometrically by measuring the reduction of cytochrome c in the presence of decylubiquinol and cyanide (n=3). Error bars represent SEM.

Fig. 2.

SB1-A6 is rendered hypersensitive to proguanil by the addition of atovaquone. *In vitro* growth of *P. falciparum* SB1-A6 (diamonds) and TM90C2B (circles) assessed after 72 hours in varying concentrations of proguanil, in the absence (open shapes) and presence (closed shapes) of 100nM atovaquone. In SB1-A6, the addition of atovaquone decreased the proguanil IC₅₀ from >5000nM to 149nM, while having no such effect in TM90C2B. Growth was measured by SYBR Green I fluorescence. Error bars represent SEM.

Table 1Comparison of parasite susceptibility to *in vitro* growth inhibition by several compounds.

Tested compounds ²	IC ₅₀ , nM ¹		
	D6	SB1-A6	TM90C2B
Chloroquine	7.8	8.8	145.3
Quinine	7.4	10.9	-
Pyrimethamine	0.34	0.36	-
5-fluoroorotate	1.8	1.2	-
6-NH ₂ Ac	0.48	>2,500 (8,657) ³	54
Atovaquone	0.093	>2,500 (19,510) ³	>2,500 (4,049) ²
Stigmatellin	79.2	609	147.5
Myxothiazol	33	1310	695
Antimycin A	13	>2,500 (11,560) ³	10.7
Clopidol	1395	>2,500 (>100,000) ³	892
WR243251 ⁴	<5	>250	<5
DPI	73.4	286.9	69.6

¹ The concentration resulting in 50% growth inhibition, averaged from 2-10 experiments, each run in quadruplicate² Compounds in the shaded area are cyt *bc*1 inhibitors.³ The IC₅₀ exceeded 2.5μM, the highest tested concentration in most experiments. The value in parentheses represents the average from the few experiments in which higher concentrations were tested and an actual IC₅₀ could be determined⁴ Single experiment only