Is the Mitochondrion a Good Malaria Drug Target?

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Rapid emergence of resistance to atovaquone, which targets electron transport in the malaria parasite mitochondrion, relegated its use to prophylaxis and even cast a shadow over the development of drugs targeting other parasite mitochondrial pathways. Here we argue for a renewed focus on the mitochondrion as a drug target, focusing particularly on the issues of resistance. We posit a hypothesis for why atovaquone resistance emerges so quickly, and we explain how facile acquisition of resistance is apparently offset by an inability of parasites to spread this resistance. We also explore the utility and resistance issues for emerging new drugs targeting parasite mitochondria, concluding that the mitochondrion is indeed an excellent target.

Drug Resistance and Malaria Control

The goal of controlling, and ultimately eradicating, malaria is inexorably intertwined with the ongoing battles against resistance. Parasite resistance to antimalarials, and mosquito resistance to insecticides, are crippling our ability to control disease. We need to rethink our approaches to drug resistance (see Glossary) if we are ever to get off the antimalarial treadmill of a constant search for new targets and new drugs [1].

The spread of drug resistance is the product of two processes. First, parasites must develop a heritable characteristic (typically a gene mutation) that allows them to survive drug treatment. Second, that characteristic must spread through the parasite population. In Plasmodium, spread can only occur by transmission to new, naïve hosts via mosquitoes. The more difficult it is for the parasite to evolve resistance mechanisms, the less likely resistance is to emerge, and hence there are fewer opportunities for resistance to spread. Of concern is the realisation that the plethora of new antimalarial compounds are apparently focused on relatively few targets [2], which may allow parasites to recycle their mechanisms of resistance to overcome new drugs.

One such recurring target is the parasite mitochondrial electron transport chain (ETC), which responds to multiple chemotypes [3]. The rapid emergence of parasites resistant to the clinically approved ETC targeting atovaquone/proguanil drug combination (Malarone™) triggered concern about using such drugs as frontline treatments [4–6]. The basis for concerns about widespread use of resistance-amenable ETC targeting drugs is that a high rate of resistance emergence intuitively offers greater opportunity for that resistance to spread. However, the metabolic impact of resistance mutations to atovaquone combines with both the biology of the parasite in the insect stages and the unusual genetics of mitochondrial inheritance, to severely limit the ability of resistance mutations to transmit between vertebrate hosts [7]. This suggests that concerns about the spread of atovaquone resistance should be reassessed.

Unfortunately, concerns surrounding atovaquone resistance have coloured opinions on the validity of drugging the mitochondrion, even for drugs that target other mitochondrial pathways.
Recent investigations suggest that not all mitochondrial targeting drugs have the same resistance profiles as atovaquone, that ETC inhibitor combinations act synergistically and significantly reduce the ability of parasites to generate resistance [8], and that drugs with different mitochondrial targets offer single-dose, multistage antimalarial activity [9]. With the spectre of resistance somewhat allayed, the advantages of mitochondrial targeting compounds as tools to combat malaria are well worth reassessing.

The Plasmodium Mitochondrion–A Somewhat Surprising Drug Target

Given the limited number of metabolic processes retained by malaria parasite mitochondria [10,11], and the minimalists set of pathways that appear essential for parasite survival during the red blood cell stages of the disease [12,13], it is somewhat surprising that such a large and diverse set of compounds selectively target the Plasmodium mitochondrion. Although the canonical central pathways for carbon metabolism are maintained in the Plasmodium mitochondrion [10–12,14,15], we now understand that much of this machinery is quiescent and even dispensable during the blood stage of the life cycle, which generates the overwhelming proportion of its ATP requirements by cytosolic glycolysis [14,16–19]. Rather, the main role of the parasite mitochondrion during the blood stage appears to be the provision of precursors for pyrimidine synthesis, a process requiring a mitochondrion-located dihydroorotate dehydrogenase (DHODH) and a functioning ETC to maintain supporting ubiquinone turnover [13]. These pathways are the focus of the most comprehensive antimitochondrial drug development programs.

The first clinically approved drug targeting the Plasmodium mitochondrion was atovaquone, which specifically targets the cytochrome b component of the cytochrome bc1 complex, interrupting the ETC by acting as a ubiquinone analogue [20–22]. When paired with proguanil, atovaquone is an effective treatment for clinical malaria and has an excellent safety profile, making it useful for the treatment of pregnant women and children and also suitable for mass administration [23]. Atovaquone also has significant activity against the sexual stages of the parasite life cycle, both on gametocytes in the mammalian host and later in the mosquito midgut [24,25], making it one of the few clinically approved drugs with proven transmission blocking capability.

Compounds from several other chemotypes also target the cytochrome bc1 complex [3,26,27] and impact both the blood and vector stages of the disease. Several of these compounds have advanced to the preclinical stage of drug development [3,28,29]. Complex I of the parasite ETC has also been investigated as a drug target. In Plasmodium, complex I activity is performed by a bacterial-like type II NADH:ubiquinone oxidoreductase (NDH2) rather than the canonical NADH dehydrogenase [30]. In human malaria parasites, NDH2 is inhibited by compounds that also have antiparasitic activity [31]. However, the value of NDH2 as a drug target is somewhat ambiguous due to significant cross-reaction of inhibitory compounds with cytochrome bc1 and the finding that NDH2 is dispensable in blood stage P. berghei [16]. Recent work identified two tricarboxylic acid (TCA)-cycle components, fumarate hydratase and malate quinone oxidoreductase, as essential for P. falciparum survival in the blood stages, suggesting these enzymes may be future drug targets [18]. The downstream enzyme, DHODH, has also been successfully targeted [9,32]: the most promising compound (DSM 265) shows similar multistage effects, good safety profiles and, encouragingly, single-dose efficacy [9]. Unfortunately, the propensity of some parasite lines to develop resistance to DSM 265 is similar to that for atovaquone [9], suggesting the need for a cautious approach for this compound until the frequency of resistance can be assessed in a clinical setting.

Combine and Conquer: Synergy and Resistance in Drugs Targeting the Mitochondrial ETC

One advantage presented by a group of disparate compounds targeting a single metabolic pathway is the possibility of synergistic interactions. In the case of the Plasmodium
mitochondrial ETC synergy may also present an important counterbalance to the generation of resistance. The original trials with atovaquone monotherapy resulted in drug-resistant parasites almost immediately [23]. Administration in combination with the biguanide prodrug proguanil produced significant synergy and a great enough reduction in the incidence of resistance to permit clinical approval of the drug combination [23]. Proguanil has no antiparasitic activity as a monotherapy, although it is activated to the dihydrofolate reductase (DHFR) inhibitor cycloguanil in the liver. Cycloguanil is not synergistic with atovaquone, and the target of proguanil in combination therapy remains elusive. This is a major drawback for combating resistance, as any parasite developing resistance to atovaquone will not be impacted by proguanil alone, leaving mitochondrial function completely resistant to the combination treatment.

Recently, ELQ-300 has emerged as a potential new partner for atovaquone [33]. Like atovaquone, ELQ-300 targets cytochrome bc1 function but impacts a different functional domain of the cytochrome b protein [33]. ELQ-300 and atovaquone synergize in combination [8], although there appears to be less synergy than either drug has with proguanil [34,35]. Encouragingly, the combination of ELQ-300 and atovaquone significantly reduces the rates at which resistance to either compound appears compared to monotherapy [8], and parasites resistant to one drug remain sensitive to the other [8]. This suggests that, unlike atovaquone/proguanil, parasites becoming resistant to one combination partner are likely to succumb to the other—supporting the idea that resistance to the combination treatment will be much less likely to emerge during widespread treatment. If this is the case, the combination treatment of ELQ-300 and atovaquone could be an important addition to the antimalarial arsenal.

Synergy does not occur when the cytochrome b inhibitor atovaquone is combined with DSM 265, the current clinical candidate for a DHODH-inhibiting antimalarial [9]. In fact, there appears to be cross-resistance between DHODH inhibitors and cytochrome b inhibitors [6,36], although the biological mechanism remains unknown and some resistant parasites become hypersensitive to atovaquone/proguanil treatment [36]. Given the relative ease with which resistance to the monotherapy of either atovaquone or DHODH inhibitors can arise [9], it is unlikely that simple combinations of these two classes of drug will be a solution to the emergence of resistance in mitochondrial ETC-targeting drugs.

**Why Is Resistance Such a Problem for Some Drugs Targeting the Mitochondrial ETC?**

Since atovaquone-resistant parasites appeared during clinical trials, concerns about resistance have been a defining factor in the development and use of ETC-targeting drugs [23]. Drugs targeting a single enzyme are inherently susceptible to resistance so it is not surprising that resistance to atovaquone arises relatively easily in the laboratory and clinic. But there are two other factors that we think expedite resistance mutations to certain ETC targeting drugs: (i) the reduced requirements for mitochondrial energy metabolism during blood stages of the parasite life cycle, and (ii) the genome organization of the mitochondrion.

Malaria parasites rely almost solely on glycolysis to satisfy their energy requirements during the blood stages of the life cycle, largely eschewing chemiosmotic synthesis of ATP [12,14] (Figure 1). The primary purpose of the mitochondrial ETC in blood stage parasites is the provision of reducing power for DHODH function in the synthesis of pyrimidines [13]. Metabolomics shows that the tricarboxylic acid (TCA) cycle and ATP synthase activity are minimal during blood stages; findings supported by the dispensability of many proteins involved in the TCA cycle, ETC, and ATP generation during blood stage [16–19] (Figure 2). We propose that this facilitates the emergence of resistance mutations in ETC proteins by reducing the selective pressure on their enzymatic efficiency. Consistent with our proposition that selection is relaxed at blood stage, a great variety of mutations in cytochrome b can confer atovaquone resistance,
with four of these being observed in the clinic [37]. Although these cytochrome \( b \) mutations can have a quite severe impact on catalytic turnover, there is apparently minimal impact on parasite fitness, at least during blood stage [4,20,38]. The ability to tolerate a drastic reduction of function in cytochrome \( b \) during blood stage apparently means that many different individual point mutations can arise without the need for compensatory changes to retain high levels of enzymatic activity.

Mitochondrial genetics probably further facilitates certain resistance mutations arising and then persisting in a parasite population. Cytochrome \( b \) is one of only three proteins encoded on the \( P. falciparum \) mitochondrial genome [39], with the balance of mitochondrial proteins being encoded in the nucleus and post-translationally targeted from the cytoplasm into the mitochondrion [10,40]. Mitochondrial genomes typically have a much higher mutation rate because they lack many of the mechanisms to repair replication errors that limit mutation rate in the nucleus [41] so we predict that resistance mutations have a higher frequency of occurrence in
mitochondrion-encoded genes. To make matters worse, although each malaria parasite has only a single mitochondrion [42], there are multiple genomes in each mitochondrion [43]. This polyploidy may well allow a parasite to carry some suboptimal mutant gene copies—copies that could be preadapted to drug resistance. While conclusive evidence is lacking, it could be that the higher mutation rates may make atovaquone resistance mutations more common and that the multiple copies of the genome mean that these mutations can arise and be maintained in a population of parasites even in the absence of strong drug selective pressure. The presence of mutations conferring atovaquone resistance in clinical populations not widely exposed to atovaquone supports this possibility [44–46], but a survey of populations not subject to atovaquone pressure, either for malaria or other diseases such as Pneumocystis pneumonia, will be necessary to confirm this.

Encouragingly, it appears that ELQ-300 resistance via cytochrome b mutations occurs at lower frequency than does resistance to atovaquone [9], and this suggests that not all targets encoded on the mitochondrial genome will necessarily share the high frequency of resistance seen for atovaquone. It will be interesting to explore the mechanisms that allow two drugs targeting different sites of cytochrome b to have differing amenity for resistance. Even if some drugs targeting the cytochrome bc1 complex have the biological deck stacked against them in terms of the ease with which resistance mutations can arise, this may not be all bad news.

Differential Metabolism and Mitochondrial Inheritance Create a Resistance Trap
The discovery that the Plasmodium mitochondrion could dispense with most of its canonical energy-related functions during the blood stage created controversy about whether Plasmodium was on the evolutionary path to losing its mitochondrion altogether [13]. However, it is now
clear that the mitochondrion remains essential to meet the metabolic demands on the parasite as it moves from the red blood cell to the other life stages in the mosquito and then the vertebrate liver (see Outstanding Questions). Metabolomic analysis demonstrates an increase in TCA cycle activity as the parasite transitions into the sexual gametocyte forms in readiness for uptake by a mosquito [14]. This activity apparently becomes increasingly important as the parasite progresses through the mosquito stages, with the loss of TCA cycle enzymes, ETC components, and ATP synthase activity causing parasite death at various stages in the mosquito midgut (Figures 1 and 2) [16–19]. It thus seems that upon entering the (presumably) less energy-rich environment of the mosquito midgut and then hemocoel, parasites require robust electron transport and active oxidative phosphorylation to survive and multiply.

What do the increased mitochondrial requirements in mosquito stages mean for atovaquone resistance mutations that significantly impair ETC function? We recently showed that strains of *P. berghei* and *P. falciparum* with various atovaquone resistance mutations in their cytochrome *b* genes die once they move from the vertebrate blood stage to the mosquito vector [7]. This strongly suggests that transmission of atovaquone-resistant parasites between mammalian hosts will be severely limited by the deleterious nature of these mutations and their severe impact on survival in the mosquito vector.

**Maternal Inheritance of Atovaquone Resistance — Genetics to the Rescue**

Malaria parasites undergo an unusual process of replication in the mosquito host, which impacts their ability to transmit deleterious mutations—especially mutations in the mitochondrial genome. Parasite sex ensues in the mosquito gut after uptake by blood feeding. Gametocytes produce gametes, male and female gametes fuse, their haploid nuclei fuse, and a short-lived diploid nucleus undergoes meiosis (Figure 3). However, at this point something unusual occurs: there is no cell division. Instead, the four haploid nuclei are replicated by mitosis numerous times without any cytokinesis. This results in a single, multinucleate (heterokaryotic) cell that develops into a cyst stage (the oocyst) lasting 12–14 days (Figure 3). During oocyst development, the parasite grows significantly and undergoes multiple nuclear divisions to produce a single cell (the sporoblast) containing hundreds of haploid, recombined nuclei. This cyst stage ends when the daughter cells (sporozoites), each with one haploid nucleus, bud from the sporoblast, egress, and begin their journey through the mosquito to her salivary glands, and ultimately into the next vertebrate host (Figure 3).

The presence of multiple recombinant nuclei in a single cytoplasm allows deleterious mutations in the nuclear genome to be carried through this replicative (oocyst) stage in the insect. This makes it possible for defects that would otherwise be lethal during the mosquito stage of the parasite life cycle to be passed on to the next generation of parasites as they enter a vertebrate host. This ability for heterozygous parasites to survive lethal mutations during the insect stage was demonstrated in the deletion of the NDH2 component of the ETC, which is lethal during the mosquito stages of the parasite life cycle but could be transmitted after crossing to wild-type parasites that effectively complemented the lesion [16]. Therefore, in the presence of a mixed parasite population, nuclear gene mutations that are lethal in the insect stage can be transmitted because these stages are not functionally haploid. But this does not hold true for mitochondrion-encoded genes.

The location of the cytochrome *b* gene on the mitochondrial genome appears to prevent complementation by outcrossing from being a factor in the transmission of atovaquone resistance. The *Plasmodium* mitochondrion is only inherited from the female parent [47] (but see Outstanding Questions), and the mitochondrial genome does not undergo recombination [48]. In essence, the mitochondrion is a closed (uniparental haploid) system, and any deleterious mutations, such as atovaquone resistance in the cytochrome *b* gene, cannot be complemented.
Figure 3. Nuclear and Mitochondrial Inheritance During the Insect Stages of the *Plasmodium* Life Cycle. Schematic diagram of an outcross between parasites with different nuclear (+ or −) and mitochondrial genomes (red or green). The nuclear genomes of both male and female parents are retained and expressed in a single parasite following fertilization and meiosis. The developing oocyst is multinucleate and expresses genes from the recombinant nuclei. Segregation of the nuclear genome and a return to haploidy occurs when sporozoites form from the mature oocyst. The mitochondrion is inherited from the female parent alone and the genetic content of all mitochondria in the daughter sporozoites comes from the maternal mitochondrion. There is no recombination with, or complementation by, the paternal mitochondrial genome during the insect stages. Resistance genes encoded by the mitochondrial DNA (such as atovaquone resistance) must be inherited maternally and cannot be complemented by the male parent during the nonhaploid insect phases.
by functional (wild type) copies from a male parent. This means that the reduced catalytic activity of an atovaquone-resistant cytochrome b dooms the parasite in the insect stage, preventing transmission of the resistance mutation. This is confirmed in *P. berghei* where even high levels of outcrossing could not facilitate the transmission of atovaquone resistance [7].

Ironically, factors that apparently expedite acquisition of resistance to drugs targeting a component of ETC encoded by the mitochondrial DNA of malaria parasites make it very difficult for that resistance to spread through the parasite population. The lack of selective pressure to maintain optimal ETC function, and the location of the cytochrome b gene on the mitochondrial genome, apparently ease the acquisition of atovaquone resistance within patient. However, having developed resistance, the parasites are effectively trapped in that individual. When they attempt to extend their life cycle after being taken up by a feeding mosquito vector, the deleterious nature of the mutations, and the inability of the affected gene to be directly complemented, condemns the parasite and ultimately eliminates that mutation from the population.

**Concluding Remarks**

We are fortunate to have a number of effective, safe drugs and drug leads that target malaria parasite mitochondria. High cost has previously restricted the use of our current best anti-mitochondrial formulation (Malarone™), but the roll out of generics now the patent has expired will increase its accessibility. Historically, we have been worried about the apparent ease with which parasites become resistant to ETC-targeting compounds like atovaquone. Relaxed selection for mitochondrial ETC function at the blood stage, combined with mitochondrial genome polyploidy (where some of the targets are encoded), appears to expedite the development of atovaquone-resistance mutations (see Outstanding Questions) but similar factors do not necessarily apply to compounds that have other mitochondrial targets. Here we argue that new combinations of ETC-targeting drugs can dramatically reduce the rate at which resistance emerges. Also, the same factors facilitating resistance to atovaquone in the blood stages incur very serious, but delayed, fitness costs when the parasites eventually get an opportunity to try to complete their life cycle in mosquitoes. Reduced efficiency of the drug-resistant ETC enzyme blocks parasite development in the mosquito and severely constrains the ability of parasites to pass on these resistance mutations. Mitochondrial encoded ETC-targeting drugs can thus be considered a double-edged sword. Resistance will likely crop up at relatively high frequencies, but we might be able to contain its spread far better than we have historically done for other drugs. All these factors suggest that drugs targeting the parasite mitochondrion are well worth pursuing.

**References**

Trends in Parasitology


