Title

Atovaquone tolerance in *Plasmodium falciparum* parasites selected for high level resistance to a dihydroorotate dehydrogenase inhibitor

Running Title

Cross-tolerance between mitochondrial antimalarials

Authors

Jennifer L. Guler1*, John White III1, Margaret A. Phillips2, and Pradipsinh K. Rathod1#

Affiliations

1 Departments of Chemistry and Global Health, University of Washington, Seattle, Washington 98195-1700 USA.

2 Department of Pharmacology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390-9041 USA.

*Current address:

Department of Biology and Division of Infectious Disease, University of Virginia, Charlottesville, Virginia 22904 USA.

*Correspondence to: Pradipsinh K. Rathod (rathod@chem.washington.edu).
Abstract
Atovaquone is a component of Malarone, a widely prescribed antimalarial combination that targets malaria respiration. Here we show that parasites with high-level resistance to an inhibitor of dihydroorotate dehydrogenase demonstrate unexpected atovaquone tolerance. Fortunately, the tolerance is diminished with proguanil, the second partner in Malarone. It will be important to understand such “genetic cross talk” between respiration and pyrimidine biosynthesis since many antimalarial drug development programs target these two seemingly independent pathways.

Main body
Malarone is an antimalarial drug composition, often prescribed for travelers as a prophylactic but its high cost so far has limited its general use in endemic countries. The patent for this drug expired in 2013 and its global usage could soon surge. Atovaquone and proguanil are the synergistic pair of antimalarials that make up this effective, well-tolerated cocktail. While the mechanism of action for proguanil remains uncertain (1, 2), it is well know that atovaquone targets the cytochrome bc₁ complex of the *Plasmodium falciparum* respiratory chain (3, 4). One function of this mitochondrial pathway is to generate a proton gradient that is required to maintain membrane potential for important processes such as protein synthesis, heme biogenesis, and the citric acid cycle (reviewed in (2)). Importantly, the *P. falciparum* cytochrome bc₁ complex has been implicated in the direct regeneration of oxidized ubiquinone for dihydroorotate dehydrogenase (DHODH), a key enzyme in the biosynthesis of pyrimidine nucleotides (5-7). Thus, DHODH is physically and functionally tied to the respiratory chain.

Recently, *P. falciparum* parasites resistant to DSM1, a potent inhibitor of DHODH (8, 9), were generated at two levels (333nM for round 1, and 3 or 10 µM for round 2 (10)). Amplification of the genome segments that encompassed the DHODH gene were responsible for the observed phenotype. Whole genome sequence methods ruled out resistance conferring mutations elsewhere and no alterations in the sensitivities to several unrelated antimalarials were detected. However, here we report the unexpected development of tolerance to atovaquone in parasites resistant to higher levels of the DHODH inhibitor DSM1 (round 2).

Following DSM1 selection and subcloning (10), atovaquone sensitivity was tested. Round 1 parasites (~3-fold DSM1 resistance) exhibited wild type sensitivity to atovaquone in a 72 hour assay (Fig. 1A and Table 1). Long-term exposure to low levels of atovaquone confirmed the sensitivity of these parasites: 10 nM atovaquone completely prevented the proliferation of parental Dd2, as well as round 1 parasites within 2 days, and continued to do so over an extended period of 8 days (Fig. 1C and D, red
We further assessed the survival of Dd2 and round 1 clone C by exposing small clonal populations of parasites to atovaquone before extensive washing, re-planting in the absence of drug, and following the number of viable colonies over 60 days (11). In this clonal viability assay, with the exception of a slight increased survival of clone C at the lowest exposure level (24h, 10 nM), we observed the full cidal activity of atovaquone on these parasite clones (average survival of 0% when compared to untreated controls, Table 2).

In contrast, 3 or 10 µM level resistance to DSM1 (round 2, ~20- to 50-fold resistance, Table 1) conferred atovaquone “tolerance” (Fig. 1B and Table 1). This phenotype is not like full resistance seen in traditional studies (12-14) because proliferation stalls at ~50% and parasites persist at a low level even at atovaquone concentrations as high as 10 µM in a 72 hour study (as measured by SYBR green DNA labeling or by hypoxanthine uptake assays, Table 1, Fig. 1B). During longer exposures, unlike parental Dd2 clones and round 1 low level DSM- resistant clones, these round 2 parasites were not inhibited and continue to proliferate in the presence of 10 nM or 100 nM atovaquone. After 5 days, parasite growth slows but viable parasites continue to persist in culture even longer (Fig. 1E). When the relative growth is quantified from the later part of this experiment (from 3-8 days), round 2 DSM1 resistant clones continue to increase by ~40% in the presence of 10 nM atovaquone, while parental Dd2 and round 1 clones did not proliferate at all. Further supporting the atovaquone tolerance phenotype in round 2 clones, clonal viability assays on two round 2 clones displayed marked increase in survival under all conditions (average survival of up to ~90% when compared to untreated controls, Table 2).

Atovaquone-tolerance in round 2 DSM1 resistant parasites is diminished following the addition of the synergistic partner drug proguanil: the EC50 of round 2 clones shifted to Dd2 levels in a 72 hour assay (Table 1). In this way, the tolerance phenotype is similar to previous reports of atovaquone resistance (5, 13, 15). Occasionally, proliferation of round 2 clones did not disappear completely in these assays indicating that viable parasites may still be present (unpublished observation). Interestingly, tolerance was also observed when round 2 parasites were exposed to other cytochrome bc1 complex inhibitors such as myxothiazol and antimycin A (Fig. 2 and Table 1). These two inhibitors target different sites of the enzyme and therefore, cross-tolerance indicates that there may be a global perturbation of the complex that alters the binding of both inhibitors (3, 16).

At first, it is puzzling how high-level resistance to a DHODH inhibitor (DSM1) confers tolerance to inhibitors of the cytochrome bc1 complex. The present phenomenon is clearly different from traditional atovaquone resistance. While mutations in the mitochondrially-encoded cytochrome b gene are commonly observed in parasites that are resistant to antimalarials that target the cytochrome bc1
complex (3, 17-22), such parasites remain susceptible to DHODH inhibitors (23). Furthermore, whole 
genome sequencing of round 1 and 2 DSM1-resistant parasites failed to show causal mutations in the 
cytochrome bc₁ complex, nor anywhere in the nuclear or mitochondrial genome (10). This was also the 
case in a previous study where parasites developed resistance against a broad range of cytochrome bc₁ 
complex inhibitors; the genetic cause of this resistance was not identified (13). Since it had been 
proposed that the sole function of the respiratory chain was to regenerate ubiquinone for DHODH (5), 
the authors had speculated that DHODH had been uncoupled from the electron transport chain through 
an alternate source of ubiquinone oxidation. Despite the potential differences between atovaquone 
resistance and tolerance, a cytochrome bc₁ "bypass" could also explain the current observations made 
in DSM1 resistant clones. Fumarate reductase activity, for example, has previously been proposed as an 
alternate way to regenerate ubiquinone for the use of DHODH but this has not been biochemically 
investigated (24).

An alternative theory to explain atovaquone tolerance could involve some contribution of the 
neighboring up-regulated genes besides DHODH. In the DSM1-selected parasites, round 2 resistance 
always involved high-level amplification of at least 35kb of sequence surrounding the DHODH gene on 
chromosome 6. Intriguingly, the smallest conserved amplicon (8 genes) includes PlasmoDB ID 
PF3D7_0603200, which is annotated as a putative mitochondrial chaperone BCS-1. In both bacteria and 
mitochondria, this gene product is required for the translocation of an essential subunit of the 
cytochrome bc₁ complex from the mitochondrial matrix to the intermembrane space (25). This protein 
has not yet been investigated in P. falciparum but it is possible that amplification of the BSC-1 gene, 
along with DHODH, could confer enhanced functions to the complex and somehow, increase 
atovaquone tolerance.

There are several antimalarials in the drug development pipeline that target pyrimidine 
biosynthesis (23, 26) and ones that target the respiratory chain (17, 27-32). Some of the new 
cytochrome bc₁ inhibitors are effective against atovaquone-resistant parasites (33) but it would be of 
interest to determine if they also participate in the cross-talk described here. Since we routinely observe 
cross-resistance between DHODH inhibitors (unpublished observations), the present studies raise 
important questions about the mechanisms of action and of cross-resistance amongst antimalarials 
directed at mitochondrial functions. Alert must be tempered by the fact that atovaquone tolerance is 
only seen at very high-level amplifications of the DHODH locus for cell lines that had undergone two 
sequential rounds of selection, and that this tolerance is lessened with the addition of proguanil.
We thank Dr. Ali Guler for help with statistical analysis and Dr. Sreekanth Kokonda for DSM1 synthesis. PKR acknowledges support from the US NIH NIAID for the study of mutagenesis in malaria parasites (AI089688 and AI099280). Development of DHODH inhibitors is supported by NIH grants AI103947 (to MAP and PKR).

References


Table 1: EC₅₀ values (±CI) of DSM1 resistant clones for various antimalarials. The development of infected erythrocytes exposed to antimalarials was measured in triplicate by flow cytometry using SYBR green fluorescence (SYBR) or by hypoxanthine uptake assays (Hypo). EC₅₀ values were calculated as previously described (10). Values listed are from a single representative experiment. Tolerance (Tol) is assigned when EC₅₀ values could not be determined due to incomplete inhibition but parasites grew at attenuated rates (see Fig. 1B and E). (Nd), could not be determined. (-), experiment not performed.

<table>
<thead>
<tr>
<th>Antimalarials</th>
<th>Method</th>
<th>Dd2</th>
<th>Round 1</th>
<th>Round 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>D</td>
<td>C53-1</td>
</tr>
<tr>
<td>DSM1 selection (µM)</td>
<td>-</td>
<td>0</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Atovaquone (nM)</td>
<td>SYBR</td>
<td>1.2 (0.2)</td>
<td>1.0 (0.4)</td>
<td>0.5 (0.4)</td>
</tr>
<tr>
<td>Atovaquone (nM) + 1µM Proguanil *</td>
<td>SYBR</td>
<td>0.3 (0.1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Myxothiazol (µM)#</td>
<td>Hypo</td>
<td>0.3 (0.1)</td>
<td>0.2 (0.1)</td>
<td>0.5 (0.3)</td>
</tr>
<tr>
<td>Antimycin A (µM)#</td>
<td>Hypo</td>
<td>0.4 (0.07)</td>
<td>0.2 (0.1)</td>
<td>0.5 (0.4)</td>
</tr>
<tr>
<td>DSM1 (µM)$</td>
<td>SYBR</td>
<td>0.1 (0.02)</td>
<td>0.4 (0.04)</td>
<td>0.3 (0.04)</td>
</tr>
<tr>
<td>Atovaquone (nM)</td>
<td>Hypo</td>
<td>5.9 (0.3)</td>
<td>8.9 (1.1)</td>
<td>4.6 (0.3)</td>
</tr>
<tr>
<td>Proguanil (µM)</td>
<td>Hypo</td>
<td>14.1 (3.6)</td>
<td>14.8 (3.6)</td>
<td>14.8 (3.4)</td>
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<tr>
<td>Artemisinin (nM)</td>
<td>SYBR</td>
<td>12.5 (Nd)</td>
<td>8.3 (0.8)</td>
<td>7.4 (1.0)</td>
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<tr>
<td>Artemisinin (nM)</td>
<td>Hypo</td>
<td>5.2 (0.6)</td>
<td>5.3 (1.3)</td>
<td>5.2 (1.3)</td>
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</tbody>
</table>

*We have also performed this experiment with 100 and 500 nM proguanil. Similar to what we observed for 1µM proguanil (included in table), the lower nM proguanil concentrations shift the EC₅₀ values of atovaquone to Dd2 levels (unpublished observation).

$DSM1 EC₅₀ values were also measured by hypoxanthine uptake assay and published in (10).
Table 2: Survival of DSM1-resistant cell lines after exposure to lethal atovaquone concentrations (11).

Starting from freshly thawed parasites of low parasitemia, serial dilutions were performed to isolate clonal populations. Triplicate T25 flasks were seeded with 10 asynchronous infected erythrocytes and incubated with for 1 or 5 days with solvent-only (control), 10nM or 100nM atovaquone (tests). Each culture was treated at approximately the same time and at the end of incubation, the erythrocyte pellets were washed three times with complete media and transferred to 24 wells of a flat-bottom 96-well plate. Plated cultures were grown in inhibitor-free media under standard culture conditions with three media changes per week out to 60 days. Viable populations were identified using flow cytometry-detected SYBR green fluorescence after each media change. Average % Survival = 100 x (average positive wells (test) ÷ average positive wells (control)). NA, not applicable.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Dd2</th>
<th>C12</th>
<th>C53-1</th>
<th>C710-2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Day Exposure, 10nM ATO</td>
<td>0% (NA)</td>
<td>14% (16-18)</td>
<td>86% (9-13)</td>
<td>88% (11-13)</td>
</tr>
<tr>
<td>5 Day Exposure, 10nM ATO</td>
<td>0% (NA)</td>
<td>0%</td>
<td>29% (21)</td>
<td>63% (16-18)</td>
</tr>
<tr>
<td>1 Day Exposure, 100nM ATO</td>
<td>0% (NA)</td>
<td>0%</td>
<td>57% (25-28)</td>
<td>38% (23-25)</td>
</tr>
<tr>
<td>5 Day Exposure, 100nM ATO</td>
<td>0% (NA)</td>
<td>0%</td>
<td>14% (34)</td>
<td>25% (30-37)</td>
</tr>
</tbody>
</table>

* Recovered parasites were observable around the same time as the solvent-only controls (unpublished data).
Figure 1: Atovaquone tolerance in high-level DSM1-resistant parasites. Panels A and B. SYBR green-based dose-response of various *P. falciparum* clones to atovaquone are shown in the absence of DSM1 (combined from 3 independent experiments). A: DSM1-sensitive clonal Dd2 (black, filled circle) compared to partial DSM1-resistant round 1 clones (green, C (open square) and D (open triangle) from (10)). B: DSM1-sensitive clonal Dd2 (black, circle) compared to high-level DSM1-resistant round 2 clones (blue, C53-1 (square), C710-1b (circle), C710-2a (inverted triangle), and D73-1 (triangle) from (10)). Decrease in proliferation in the presence of atovaquone is calculated as a percentage of activity from DMSO controls. Lines on plots show the nonlinear curve fit of data points from individual clones. EC₅₀ values, where measurable, are listed in Table 1.

Panels C-E. Growth of different *P. falciparum* clones in the presence of continuous levels of 0 (black), 10 (red), or 100nM (purple) atovaquone over 8 days (see Table 1 for respective DSM1 concentrations). C: Clonal Dd2 (single values), D: Partially DSM1 resistant round 1 clones (mean of clone C and clone D with standard deviation), E: Highly DSM1-resistant round 2 clones (mean of C53-1, C710-b, C710-2a, and D73-1 clones with standard deviation). Cumulative % parasitemia was calculated by normalizing parasitemia values (measured using SYBR green-based flow cytometry method) to the starting parasitemia and calculating the percentage of the maximum value achieved over 8 days.

Figure 2: Tolerance to alternative cytochrome bc₁ inhibitors in high-level DSM1-resistant parasites. Hypoxanthine-uptake dose response of various *P. falciparum* clones to antimycin A (Panel A) or myxothiazol (Panel B) in the absence of DSM1. DSM1-sensitive clonal Dd2 (black, filled circle) compared to partial DSM1-resistant round 1 clones (green, C (open square) and D (open triangle) and high-level DSM-1 resistant round 2 clones (blue, C53-1 (square), C710-1b (circle), C710-2a (inverted triangle), and D73-1 (triangle)). Proliferation is calculated as a percentage of activity from DMSO controls. Lines on plots show the nonlinear curve fit of data points from individual clones. EC₅₀ values are listed in Table 1.