Antimalarial combination therapy: lessons from mitochondrial physiology

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The Challenge of Feeding the Pipeline

- Resistance to currently used combination therapies will arise.
- Development of new drugs is a long process with many failures on the way (90%?; 99%?).
- Need to have a strategy that will feed the pipeline for a long period (50 years?; 100 years?).
- Basic understanding of parasite physiology and mechanisms of drug action is essential for an informed strategy for drug development pipeline.
- Drugs that work but for which MOAs are unknown should be subjected to in depth basic research to make informed decisions about their use in combination therapy.
The Story of Weird Mitochondria of Malaria Parasites
Plasmodium spp.
Abbreviated Electron Transport Chain in Plasmodium Mitochondrion

Five Mitochondrial Dehydrogenases Located in the Inner Membrane
The Q Cycle of Complex III
Ubiquinone

Atovaquone
Mitochondrial Functions in Malaria Parasites

- Pyrimidine biosynthesis
- [Fe-S] cluster assembly
- Ubiquinone synthesis
- Replication
- Recombination
- Repair
- Transcription
- Processing
- mtDNA replication
- mRNA
- rRNA
- Protein synthesis
- 3 proteins

Diagram showing metabolic pathways and protein synthesis in mitochondria of malaria parasites.
Pyrimidine Metabolism

Glutamine $\rightarrow$ Glutamate $\rightarrow$ Carbamoyl-P $\rightarrow$ N-carbamoyl-aspartate $\rightarrow$ Dihydroorotate

Carbamoyl-P synthase $\rightarrow$ Aspartyl carbamoyl transferase

L-Aspartate $\rightarrow$ Dihydroorotate dehydrogenase

Dihydroorotate $\rightarrow$ Orotate $\rightarrow$ Orotidine-5'-P $\rightarrow$ Cytidine-5'-P $\rightarrow$ Uridine-5'-P

Uridine-5'-P $\rightarrow$ Cytosine $\rightarrow$ Uracil $\rightarrow$ Deaminase; Phosphoribosyl transferase

Orotidine-5'-P $\rightarrow$ Orotate phosphoribosyl transferase

Orotidine-5'-P decarboxylase

UTP $\rightarrow$ CTP $\rightarrow$ TTP $\rightarrow$ dCTP $\rightarrow$ RNA $\rightarrow$ DNA
Two Types of Dihydroorotate Dehydrogenases (DHODH)

Cytochrome $bc_1$ Complex

CoQ $\rightarrow$ Mitochondrion $\rightarrow$ CoQH$_2$

Type 2 DHODH

Dihydroorotate $\rightarrow$ Orotate

Type 1A DHODH

Cytoplasm

Fumarate $\rightarrow$ Succinate

Cytochrome $bc_1$ Complex

CoQ $\rightarrow$ Mitochondrion $\rightarrow$ CoQH$_2$

Type 2 DHODH

Dihydroorotate $\rightarrow$ Orotate

Type 1A DHODH

Cytoplasm

Fumarate $\rightarrow$ Succinate

Most Eukaryotes

Pyrimidines

Many prokaryotes; Saccharomyces cerevisiae
LETTERS

Specific role of mitochondrial electron transport in blood-stage *Plasmodium falciparum*

Heather J. Painter¹, Joanne M. Morrisey¹, Michael W. Mather¹ & Akhil B. Vaidya¹
pHHyDHOD-GFP

**P. falciparum Expressing Type 1 DHODH Resistant to All Complex III Inhibitors**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$IC_{50}$ in D10 (wild type)</th>
<th>$IC_{50}$ in D10:yDHOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atovaquone</td>
<td>1 nM</td>
<td>&gt;2,250 nM</td>
</tr>
<tr>
<td>Myxothiazol</td>
<td>37 nM</td>
<td>&gt;3,300 nM</td>
</tr>
<tr>
<td>Antimycin</td>
<td>129 nM</td>
<td>&gt;3,300 nM</td>
</tr>
<tr>
<td>Pyridone</td>
<td>59 nM</td>
<td>&gt;16,600 nM</td>
</tr>
<tr>
<td>Acridone</td>
<td>&lt;50 pM</td>
<td>&gt;3,300 nM</td>
</tr>
<tr>
<td>Quinolone</td>
<td>2.8 nM</td>
<td>&gt;1000 nM</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>87 nM</td>
<td>62 nM</td>
</tr>
<tr>
<td>Artemisinin</td>
<td>40 nM</td>
<td>40 nM</td>
</tr>
</tbody>
</table>
So, what have we learned?

- Cytosolic bypass with yeast DHOD can render parasites independent of mtETC.
- Any additional evidence to support this conclusion?
- Parasites that lose the transgene become susceptible to mtETC inhibitors.
- Transgenic *P. falciparum* capable of salvaging pyrimidines are also resistant to mtETC inhibitors.
The Essential Function of Mitochondrial Electron Transport Chain in Blood Stages of *P. falciparum*

Dihydroorotate dehydrogenase
NADH dehydrogenase (rotenone insensitive)
Succinate dehydrogenase
Malate-quinone oxidoreductase
Glycerol 3-phosphate dehydrogenase

Electrons → Ubiquinone → Ubiquinol → Cytochrome $b_{c_1}$ complex

Atovaquone blocks

Cytochrome $c$ (ox.) → Cytochrome $c$ (red.) → Cytochrome $c$ oxidase

$H^+$ translocated

$O_2$ → $H_2O$ → $H^+$ pumped
Uses of the mtETC-independent Parasites

• Providing support for target validation for new antimalarials with potential anti-mtETC activity
• Assessing mitochondrial functions through gene knockout
• Assessing possible off-target activities of new parasite DHOD inhibitors
• Providing new selectable markers for gene transfer and knockout experiments
4(1H)-Pyridones

- Clopidol has been used as an anti-coccidial drug for over 30 years
- GSK, Spain, re-initiated the work on pyridones in 2002, supported by Medicines for Malaria Venture
- A lead compound identified and a large number of derivatives synthesized; cost likely to be low
- Plans are underway for initiating clinical trials
Ubiquinone

Clopidol

GW844520
Atovaquone-resistant *P. falciparum* are *hypersensitive* to GW844520.

**IC}_{50} for Compounds Targeting Cytochrome bc\textsubscript{1} Complex**

<table>
<thead>
<tr>
<th>Parasite Clone (cyt b)</th>
<th>Atovaquone</th>
<th>GW844520</th>
<th>Stigmatellin</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D7 (WT)</td>
<td>0.3</td>
<td>21</td>
<td>--</td>
</tr>
<tr>
<td>3D7 (M133I)</td>
<td>4</td>
<td>1.6</td>
<td>--</td>
</tr>
<tr>
<td>Dd2 (WT)</td>
<td>1.2</td>
<td>33</td>
<td>48</td>
</tr>
<tr>
<td>Dd2 (M133I)</td>
<td>49</td>
<td>0.7</td>
<td>84</td>
</tr>
<tr>
<td>Dd2 (Y268N)</td>
<td>&gt;1000</td>
<td>4.9</td>
<td>--</td>
</tr>
</tbody>
</table>
### Assessing Resistance Frequency in *P. falciparum* Clone Dd2

<table>
<thead>
<tr>
<th>Compound used</th>
<th>Seeded Parasites</th>
<th>Drug Dose, nM (xIC&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>Selection outcome*</th>
<th>Days to emergence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atovaquone</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>10 (10x)</td>
<td>0/2</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>10 (10x)</td>
<td>2/2</td>
<td>42, 42</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>10 (10x)</td>
<td>2/2</td>
<td>28, 42</td>
</tr>
<tr>
<td>GW844520</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>300 (10x)</td>
<td>0/2</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>300 (10x)</td>
<td>0/3</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>300 (10x)</td>
<td>0/3</td>
<td>--</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>--</td>
<td>1/1</td>
<td>13</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atovaquone</td>
<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>10 (10x)</td>
<td>2/2</td>
<td>50, 50</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>10 (10x)</td>
<td>2/2</td>
<td>24, 38</td>
</tr>
<tr>
<td>GW844520</td>
<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>300 (10x)</td>
<td>0/3</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>300 (10x)</td>
<td>0/3</td>
<td>--</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>--</td>
<td>1/1</td>
<td>16</td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GW844520</td>
<td>10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>150 (5x)</td>
<td>0/3</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>100 (3.3x)</td>
<td>0/3</td>
<td>--</td>
</tr>
</tbody>
</table>

* Ratios represent number of flasks yielding resistant parasites after 8 weeks of treatment.

• 4(1H)-pyridones are active against atovaquone-resistant \textit{P. falciparum}; these parasites are hypersensitive to some of the pyridones

• The frequency of pyridone resistance development in the Dd2 clone (a high frequency resistance developer) is lower than for atovaquone: $>10^{-8}$ vs. $10^{-7}$

• Low level pyridone-resistant parasites show mutations within cytochrome \textit{b} gene, but these do not affect atovaquone sensitivity

• The pyridone binding region appears to be distinct from atovaquone binding region in the parasite cytochrome \textit{bc}$_1$ complex

• \textit{Combination of two compounds with overlapping binding sites on the same target could be excellent for ACT}
What about Malarone® (Atovaquone/Proguanil Combination)?
Are the transgenic parasites susceptible to the atovaquone/proguanil combination?
Proguanil Sensitizes yDHOD-Transgenic Parasites to All mtETC Inhibitors

<table>
<thead>
<tr>
<th>Parasite Strain</th>
<th>Proguanil (1 μM)</th>
<th>Atovaquone</th>
<th>GW844520</th>
<th>Myxothiazol</th>
<th>Antimycin</th>
<th>Chloroquine</th>
</tr>
</thead>
<tbody>
<tr>
<td>D10</td>
<td>--</td>
<td>1.3</td>
<td>58.7</td>
<td>37.2</td>
<td>129.4</td>
<td>86.99</td>
</tr>
<tr>
<td>D10</td>
<td>+</td>
<td>0.23</td>
<td>37.8</td>
<td>14.9</td>
<td>71.45</td>
<td>62.6</td>
</tr>
<tr>
<td>D10::GFPyDHODH</td>
<td>--</td>
<td>&gt;2,250</td>
<td>&gt;16,600</td>
<td>&gt;3,300</td>
<td>&gt;33,300</td>
<td>62.1</td>
</tr>
<tr>
<td>D10::GFPyDHODH</td>
<td>+</td>
<td>0.68</td>
<td>45.01</td>
<td>21.9</td>
<td>67.44</td>
<td>62.7</td>
</tr>
</tbody>
</table>
Collapse of $\Delta \Psi_m$ in Parasites Exposed to Atovaquone/Proguanil Combination
Intermembrane Space (+)

(1.)

DHODH

Orotate

DHODH

Cyt $bc_1$

complex

Cyt c oxidase

$\frac{1}{2}O_2$

$H_2O$

$H^+$

$e^-$

Matrix (-)

Atovaquone
• \( \Delta p\text{H} \) portion of the protonmotive force across mitochondrial inner membrane force is dispensable for blood stage \( P. \text{falciparum} \)

• Electropotential \( (\Delta \Psi_m) \) portion of the protonmotive force across mitochondrial membrane is essential, and the parasite has two redundant paths to generate it

• Mitochondrial transport systems cannot operate without \( \Delta \Psi_m \)

• **Mitochondria in malaria parasites are essential**
Is proguanil the ideal partner for atovaquone?

(Is Malarone® formulation appropriate?)
Proguanil Metabolism and Activation

Proguanil → P450 enzyme → Dealkylation

4-Chlorophenyl biguanide

Cycloguanil = Parasite DHFR Inhibitor
• Proguanil seems to inhibit mitochondrial membrane potential generation, but its activity is masked by the dominant mitochondrial electron transport chain, the target for atovaquone

• Thus, atovaquone-resistant mutations in mtDNA will render proguanil ineffective as a prodrug partner

• Conversion to cycloguanil requires a CYP450 which is deficient in up to 25% of individuals

• Cycloguanil-resistant parasites are already widespread in S.E. Asia

• Malarone failure is beginning to be seen in various parts of the world

• It may be advisable to seek an alternative partner for atovaquone to save its efficacy; this may become important when Malarone goes off patent

• The decision about a partner for other mtETC inhibitors under development should be informed by these findings
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