Repurposing Approved Clinical Drugs for Protozoan Diseases

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Quinine Discovery
1638

1820 molecule identified by Pellitier and Caventou

QUINIDINE

QUININE
Perkins in 1826 while trying to make quinine from coal tar made aniline dye-mauve, which spawned dye industry and later stains for bacteria. Prontosil was made for its color fastness.
Paul Ehrlich—specific receptors in the parasite with higher avidity for drug than those present in host or ideally lacking in host. He developed arsenicals in a 606 compound screen on animals and methylene blue for the treatment of sleeping sickness and syphilis.

Analogs of methylene blue spawned the phenothiazines and other antipsychotic drugs.
World War II was the first major US “conflict in which fewer of our troops died of disease than of battle injuries and wounds. 1942-1945-494,299 cases of military malaria, 410,727 overseas (Mowrey “Statistics of Malaria” 1963 IM in WWII) with use of atabrine.

SN-13276 [pentaquine] and SN-7618 [chloroquine] identified in “Survey of Antimalarial Drugs” wartime effort. Screened 14,000 compounds for antimalarial activity-bird, primate and human studies (no mice yet) Spent 24 million in approximately 600 contracts with 133 universities, foundations, and commercial firms.

It examined pharmacology, metabolism, microbiology, and even funded Michael Heidelberger’s malaria vaccine work
Malarial Pigment

= Hemozoin

Formation of Hemozoin Detoxifies Reactive Heme By Coordinate Iron-Oxygen Bond.
Target of the Quinolines: Hemozoin Formation
Lipid Bodies

Isolated Hemozoin + Malachite Green

Delipidated Hemozoin
LC Mass Spectrometry Identifies Monostearate Glycerol ($m/z$ 365) and Monopalmitin Glycerol ($m/z$ 337) with no Monooleoyl Glycerol ($m/z$ 363) in Isolated Hemozoin along with DAG and Phospholipids
Diverse lipids promote Heme crystallization

Chloroquine (○) and quinidine (■) inhibition is reversible
**Hemozoin formation**
Requires pH less than 6.0
Can occur in water but occurs in lipid environment in parasite to exclude water.
Two step process to make heme dimer, then make large visible crystals

**Heme crystal inhibition**
Reversible
Requires heme binding
pH dependent
Interferes with heme dimer formation or large crystal formation

Three emission plumes of radiolabeled chloroquine localize directly to hemozoin
1. Not all drugs that bind heme inhibit heme crystal formation
2. Not all drugs that bind heme and inhibit heme crystals get to digestive vacuole
3. Need heme binding, hemozoin inhibition and target to DV
Heme Crystallization Assay for Rapid Drug Screening:

- After incubation of free heme with Neutral lipids and drug, 2% SDS, Sodium Bicarbonate solution is added to wells and mixed prior to measuring OD 405 of uncrystallized Heme.
- 1M NaOH next added to fully decrystallize all heme prior to second OD 405 measurement.
- **Difference between measurement indicates the amount of heme crystallized.**
- **Principle:** Low amounts of heme total to provide range for signal to background for 96-well plate.

<table>
<thead>
<tr>
<th>Inactive Drug</th>
<th>Significantly Active Drug</th>
<th>Ideal Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% of heme is crystallized</td>
<td>&lt;100% crystallization</td>
<td><strong>100% inhibition</strong></td>
</tr>
</tbody>
</table>

![Diagram with BC, SDS, and NaOH combinations for different drug activities]
Kinetics of Inhibition Crystal Extension Demonstrate Reversible, pH Dependent Inhibition

Reversible Drug Inhibition of Crystal Extension (ICE)

No drug
Chloroquine
Quinidine
Ketoconazole

pH dependent ICE
<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (μM) [\text{FP}]/ICG \times 30]</th>
<th>IC_{50} CQ-susceptible parasites (strain, reference)</th>
<th>IC_{50} CQ-resistant parasites (strain, reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Classic antimalarials</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Amodiaquine</td>
<td>1.2 ± 0.2 [42]</td>
<td>7.8 nM (3D7, [43])</td>
<td>18.5 nM (K1, [43])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.5 nM (HB3, [43])</td>
<td>13.8 nM (PH3, [43])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0 nM (NF54, [47])</td>
<td></td>
</tr>
<tr>
<td>Chloroquine</td>
<td>4.3 ± 0.2 [12]</td>
<td>14.0 nM (3D7, [43])</td>
<td>192.0 nM (K1, [43])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.5 nM (HB3, [43])</td>
<td>158.8 nM (PH3, [5])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15.0 nM (NF54, [48])</td>
<td>122.0 nM (W2, [49])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.0 nM (D6, [49])</td>
<td>140.0 nM (K1, [50])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0 nM (NF54, [47])</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.5 nM (CDC1, [50])</td>
<td></td>
</tr>
<tr>
<td>Quinacrine</td>
<td>4.9 ± 0.7 [10]</td>
<td>8.0 nM (NF54, [47])</td>
<td>3.1 nM (K1, [50])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.0 nM (CDC1, [50])</td>
<td></td>
</tr>
<tr>
<td>Quinidine</td>
<td>1.5 ± 0.2 [33]</td>
<td>21.5 nM (3D7, [43])</td>
<td>50.6 nM (K1, [43])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.9 nM (HB3, [43])</td>
<td>43.6 nM (PH3, [43])</td>
</tr>
<tr>
<td>Quinine</td>
<td>17.1 ± 0.7 [3]</td>
<td>34.2 nM (3D7, [43])</td>
<td>81.2 nM (K1, [43])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36.8 nM (HB3, [43])</td>
<td>74.3 nM (PH3, [43])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.0 nM (NF54, [22])</td>
<td></td>
</tr>
<tr>
<td><strong>Antifungal cytochrome P450 inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>12.9 ± 0.6 [4]</td>
<td>431.0 nM (HB3, [51])</td>
<td>1.1 μM (A4, [51])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>245.0 nM (NF54, [51])</td>
<td>553.0 nM (W2, [51])</td>
</tr>
<tr>
<td>Ecosazolole</td>
<td>19.7 ± 1.9 [3]</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>6.5 ± 0.6 [8]</td>
<td>~9.4 μM (Honduras I/CDC, [53,54])</td>
<td>~1.0 μM (Indochina I/CDC, [54])</td>
</tr>
<tr>
<td>Miconazole</td>
<td>21.4 ± 2.2 [2]</td>
<td>–</td>
<td>~400.0 nM (I/CDC, [54])</td>
</tr>
<tr>
<td><strong>Antimalarial dyes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azure A</td>
<td>33.7 ± 1.7 [1]</td>
<td>10.2 nM (D6, [49])</td>
<td>10.5 nM (W2, [49])</td>
</tr>
<tr>
<td>Brilliant cresyl blue</td>
<td>29.0 ± 0.9 [2]</td>
<td>9.7 nM (D6, [49])</td>
<td>5.5 nM (W2, [49])</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>28.5 ± 2.3 [2]</td>
<td>3.6 nM (D6, [49])</td>
<td>4.0 nM (W2, [49])</td>
</tr>
<tr>
<td>Nile blue</td>
<td>1.7 ± 0.2 [50]</td>
<td>51.0 nM (D6, [49])</td>
<td>42.0 nM (W2, [49])</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin</td>
<td>1.7 ± 0.4 [29]</td>
<td>–</td>
<td>~400.0 nM (I/IO, [54,55])</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>6.6 ± 1.3 [8]</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Biliverdin</td>
<td>0.6 ± 0.1 [91]</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Biochomin A</td>
<td>20.8 ± 1.1 [2]</td>
<td>164.0 μM (pW, [56])</td>
<td>–</td>
</tr>
<tr>
<td>Distamycin</td>
<td>16.1 ± 0.5 [3]</td>
<td>0.7 μM (ITO4, [57])</td>
<td>618 nM (FCR3, [58])</td>
</tr>
<tr>
<td>Elliotiine</td>
<td>2.0 ± 0.2 [61]</td>
<td>–</td>
<td>330 nM (FCR3, [59])</td>
</tr>
</tbody>
</table>
Drug discovery

1. Approximate the target

2. Screen the parasite
Figure 1 | The existing universe of 9,990 drugs and their availability.
Library Construction

Merck Index Therapeutic Index (16,000 entries)

2004 Physician’s Desk Reference (4,000 entries)

FDA-approved drug list & USP dictionary (~12,000 entries)

Specialty vendors (Tocris, BioMol, Calbiochem, LOPAC, Schreiber)

NINDS (1,038 compounds ~70% FDA approved)

ChemACX.Com
Searches 400 vendor catalogs

Johns Hopkins Hospital Pharmacy Storeroom (832 drugs)

USP Drug standards (~1,400 drugs and metabolites)
### Clinical compound libraries

<table>
<thead>
<tr>
<th></th>
<th>NINDS</th>
<th>Prestwick</th>
<th>LOPAC</th>
<th>JHCCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>1,040</td>
<td>880</td>
<td>1,280</td>
<td>2,895</td>
</tr>
<tr>
<td>FDA or phase II</td>
<td>700</td>
<td>600</td>
<td>500</td>
<td>2,687 (1780)</td>
</tr>
</tbody>
</table>
Existing drug space

- Existing drug space (~1,500 drugs)
- The Johns Hopkins Clinical Compound Library (2,687 drugs)
- MicroSource Discovery Spectrum Collection (1,161 drugs)
- Prestwick Collection (836 drugs)
- Sigma-Aldrich Library of Pharmacologically Active Compounds (617 drugs)

Existing FDA drug space

- Existing FDA drug space (~3,400 drugs)
- The Johns Hopkins Clinical Compound Library (1,937 FDA-approved drugs)
- MicroSource Discovery Spectrum Collection (821 FDA-approved drugs)
- Prestwick Collection (590 FDA-approved drugs)
- Sigma-Aldrich Library of Pharmacologically Active Compounds (418 FDA-approved drugs)
A clinical drug library screen identifies the antihistamine astemizole as an antimalarial agent

Curtis Chong, Lirong Shi, Jun Liu

Heme Crystallization and Drugs
Astemizole inhibition of heme crystallization

- Potent (IC5010 microM)
- Reversible and pH dependent

○ Chloroquine ■ astemizole ● desmethylandastemizole

Hydrophilic metabolite of astemizole (norastemizole) not active against *P. falciparum* or *P. vinckei* parasites

<table>
<thead>
<tr>
<th>Plasmodium falciparum strain</th>
<th>Astemizole IC₅₀ (nM)</th>
<th>Desmethylandastemizole IC₅₀ (nM)</th>
<th>Norastemizole IC₅₀ (nM)</th>
<th>Chloroquine IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D7</td>
<td>227 ± 6.4</td>
<td>117 ± 1.4</td>
<td>4,477 ± 15</td>
<td>31.8 ± 3.5</td>
</tr>
<tr>
<td>Dd2</td>
<td>457 ± 12.3</td>
<td>106.2 ± 10.3</td>
<td>3,590 ± 16</td>
<td>79.3 ± 6.8</td>
</tr>
<tr>
<td>ITG</td>
<td>734 ± 2.2</td>
<td>56.8 ± 27</td>
<td>2,230 ± 934</td>
<td>107.3 ± 13.8</td>
</tr>
</tbody>
</table>
How safe is astemizole?

- Acrivastine: 3 reports/million DDDs sold
- Astemizole: 233 reports/million DDDs sold
- Cetirizine: 286 reports/million DDDs sold
- Loratadine: 86 reports/million DDDs sold

- Total rate & rhythm disorders
- Selected reactions *
- Cardiac deaths + sudden death
A Progression of Quinoline Antimalariais

<table>
<thead>
<tr>
<th>DRUG</th>
<th>ICG IC50, uM (SE)</th>
<th>3D7 IC50 48 H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>1.49 (0.05)</td>
<td>0.02 uM</td>
</tr>
<tr>
<td>Atemizole</td>
<td>0.724 (0.096)</td>
<td>0.479 uM (0.015)</td>
</tr>
<tr>
<td>Rifampin</td>
<td>2.05 (0.18)</td>
<td>21.2 uM (0.45)</td>
</tr>
<tr>
<td>Terfenadine</td>
<td>2.19 (0.74)</td>
<td>17 uM (2.0)</td>
</tr>
<tr>
<td>Loratadine</td>
<td>2.46 (0.42)</td>
<td>&gt; 100 uM</td>
</tr>
<tr>
<td>Flutrimazole</td>
<td>0.71 (0.28)</td>
<td>2.64 uM (0.78)</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>12.9 (0.6)</td>
<td>200</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>6.5 (0.6)</td>
<td>5</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>&gt;50</td>
<td>&gt; 100 uM</td>
</tr>
<tr>
<td>Methylene Blue</td>
<td>29 (2.3)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

![Methylene blue](image.png)

![Quinacrine](image.png)

![Rifampin](image.png)

![Loratadine](image.png)

![Clotrimazole](image.png)

![Ketoconazole](image.png)

![Fluconazole](image.png)
Parasite inhibition does not correlate with heme crystal inhibition unless you are selective
LHTS shows gefitinib inhibits lipid mediated heme crystallization in vitro.

Gefitinib inhibits \textit{P. falciparum} parasites as shown by 72 hour Syber green assay.
Gefitinib also inhibits hemozoin formation in *P. falciparum*

Sub-micromolar gefitinib abolishes formation of dark, birefringent hemozoin crystals in parasites (top) when dosed at the early ring stage and grown to shizont stage.
Conclusions:

New lipid based drug screening method (LHTS) identified Gefitinib as a potent inhibitor of lipid mediated heme crystal formation.

Optical microscopy and colorometric analysis show gefitinib inhibits *P. falciparum* hemozoin formation in a reversible, dose dependent manner.

The drug also inhibits chloroquine resistant parasites (IC50 of 420 nM).

This lipid based drug screening assay (LHTS) enables discovery of novel antimalarial compounds, like gefitinib.

By screening libraries of approved drugs for non-obvious new uses we may accelerate deployment of new malaria treatments.
Enteric protozoa - the continued need for new therapeutics

- **Cryptosporidium**
  - Consistently effective therapeutic needed for the treatment of cryptosporidiosis in immune compromised patients
  - Nitazoxanide – gold standard for clinical care of the immunocompetent patients but efficacy in immune compromised equivocal

- **Entamoeba histolytica**
  - Few effective drugs available (metronidazole and nitazoxanide preferred)
  - Potential for development of drug resistance demonstrated in the laboratory

- **Giardia intestinalis**
  - Several drugs available but drug resistant strains against almost all
Substituted quinoline

Anti-parasitic properties

- FDA approved drug for treatment of *Enterobius* (pinworms) but no longer used in U.S.
- Effective against malaria at low dose (3 nM) in vitro
- Not appreciably absorbed from the gut

*Pyrvininium may inhibit growth of other enteric protozoan parasites of public health importance*
Growth inhibition by pyrvinium is 2000 fold greater than by paromomycin

Pyrvinium: $IC_{50} = \sim 350 \text{ nM}$

Chloroquine: $IC_{50} = \sim 30 \text{ \mu M}$

Paromomycin: $IC_{50} = \sim 700 \text{ \mu M}$
Significant reductions in oocyst shedding by pyrvinium treated mice

No Drug 5 mg/kg Pyrvinium 100 mg/kg Paromomycin
Significant reduction in trophozoite stages in ileum of pyrvinium treated mice

Pyrvinium at 5 mg/kg for 4 days

No drug control
85% reduction in trophozoite stages in ileum of pyrvinium treated mice
Conclusions on pyrvinium activity against *Cryptosporidium*

- Pyrvinium pamoate is a potent inhibitor of parasite growth in vitro
  - IC\textsubscript{50} of 350nM is ~2000x lower than the IC50 for paramomycin

- Pyrvinium pamoate has significant anti-cryptosporidial activity in vivo
  - 90-96% reduction in oocyst shedding (IFA) compared to untreated control mice
  - Reduction was equivalent to that seen in paramomycin treated mice, though the pyrvinium dose was 20x lower indicating stronger potency
  - Significant reduction in intestinal trophozoite stages compared to untreated mice, with most parasite infection limited to small focal areas instead of the diffuse pattern of infection seen in controls.
  - No statistical difference in trophozoite reduction compared to paromomycin

- Pyrvinium pamoate is a potential drug candidate for treatment of cryptosporidiosis
  - Already an FDA-approved drug so less time and cost to move to market
Drug discovery

1. Approximate the target

2. Screen the parasite
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