In Vitro Activity of Artemisinin in Combination with Clotrimazole or Heat-treated Amphotericin B against Plasmodium falciparum

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Abstract. Currently available artemisinin-based combination therapies (ACTs) for malaria are inadequate. There remains an enormous unmet need for alternate artemisinin-based combination therapies. One of the fastest methods to identify promising artemisinin-based combination therapies is to look for synergistic or additive antimalarial interaction between artemisinin and an alternate drug against P. falciparum in vitro. Amphotericin B and clotrimazole are known drugs for treatment of human fungal infections. We repurposed clotrimazole or heat-treated amphotericin B in fixed ratio combination with artemisinin for antimalarial properties. Isobologram results show synergistic/additive interaction in both of the cases at therapeutically safe concentrations. Artemisinin, clotrimazole, and their synergistic combinations also decrease hemozoin production in parasitized erythrocytes. New permeation pathways induced in infected cells remain unaffected by drug combinations as indicated by sorbitol lysis. It would be interesting to extend the studies in vivo system.

INTRODUCTION

Malaria morbidity and mortality has been rising since the 1980s, mainly perhaps due to widespread emergence of drug-resistant strains of P. falciparum. Even in the twenty-first century malaria kills 1–2.7 million people annually—mostly young children in Africa.1 Artemisinin (ART) and its derivatives are the most potent and rapidly acting of all the available antimalarial drugs and the clinical resistance to these drugs has not yet been encountered. Most other antimalarials deployed have fallen to resistance. In response to this global threat of multidrug-resistant malaria and past experience, there are compelling reasons to believe that resistance to the artemisinin can be slowed by judicious use of appropriate drug combinations.

The use of artemisinin-based combination therapies (ACTs), rather than artemisinin alone (monotherapy), has thus been strongly supported by the WHO2 and is being effectively used for malaria treatment in many countries. Today ACTs have gained widespread recognition as the drugs of choice to cure malaria. There is an increasing demand for ACTs, especially from malaria-endemic countries, but they are in short supply due to the scarcity of artemisinin globally. Existing ACTs are expensive for the rural poor of endemic countries. There is an epidemic of fake artemisinin combination in Africa and Southeast Asia.3 The available ACTs are by no means ideal combinations for en masse deployments in malaria control, and sooner or later resistance will emerge to the currently used combinations. It is imperative, therefore, to search for alternate combinations that might decrease dependence on artemisinin without compromising the potency to cure malaria. It is an attractive proposition to identify cheaper, synthetic, prescribed drugs as a novel partner of artemisinin in malaria combination therapy. The concept of drug combination is based on the synergistic or additive potential of two (or more) structurally dissimilar drugs that have independent modes of action and different biochemical targets in the parasite, thus enhancing or maintaining the efficacy while ensuring mutual protection against resistance.

Amphotericin B (AMB) is an antimicrobial prescribed for treatment of serious fungal infections. This drug is also recommended as second-line treatment of visceral leishmaniasis.4 Heat treatment of AMB (70°C for 20 min) produces a novel formulation commonly referred to as heat-treated amphotericin B (HT-AMB).5 This superaggregated form possesses improved therapeutic index retaining its antifungal property.6 HT-AMB is less toxic than AMB, and has been reported to possess potent antiplasmodial activity in vitro.7 Clotrimazole (CLT) is also an antifungal agent with proven antiplasmodial activity in cultures.8 Both CLT and AMB are FDA-approved synthetic compounds and abundantly available. The vast experience and rarely acquired fungal resistance to these drugs prompted us to consider HT-AMB or CLT as the novel partner of artemisinin. We have thus investigated antiplasmodial interactions between ART and structurally unrelated (Figure 1) antifungal CLT or heat-treated amphotericin B by challenging P. falciparum erythrocytic stages in vitro. We have also determined the effect of drug combinations on hemozoin formation by the parasite and sorbitol-induced hemolysis of parasitized RBCs for mechanistic purposes.

MATERIALS AND METHODS

Parasite culture. Erythrocytic stages of P. falciparum, strain 3D7, were continuously cultured and maintained as stocks in 50-mm Petri dishes on human B+ red blood cells by candle-jar method7 at 37°C. The complete RPMI-1640 medium (Sigma) was prepared by the addition of sterile 5% sodium bicarbonate at the rate of 4 mL per 96 mL medium, and supplemented with 10% pooled B+ serum. The stock cultures were started with 5% hematocrit and parasitemia less than 1%. Subcultures were made at about 5% parasitemia. A chloroquine-resistant P. falciparum, RKL 303 strain, was similarly cultivated.

Drug solutions. ART (Sigma-Aldrich), CLT (Amoli Organics Pvt. Ltd., Mumbai, India) and AMB (Innovations Pvt. Ltd., India) were used. AMB 1 mg/mL aqueous stock solution was prepared and subjected to mild heating at 70°C for 20 min to prepare HT-AMB.5 ART and CLT were dissolved in DMSO to prepare 1 mg/mL stock each. The DMSO had no effect on the parasite growth at final concentrations used in the experiments. On the day of experiment, drug dilutions...
were prepared from stock solutions in gentamycin-free culture medium.

**Inhibitory concentration assay.** Concentration of each drug required to inhibit parasite growth in vitro was carried by exposing synchronous parasites, derived from stock culture, to graded concentration of the drug in 24-well plates in triplicate for 48 hours at 37°C in a candle-jar. The medium was changed in each well after 24 hours with or without drug. The percentage parasite inhibition in relation to control was calculated, and data plotted to determine 50% inhibitory concentration (IC50) at the end of an experiment by examining thin smear Giemsa-stained slides.

**Drug combinations.** For drug combination inhibitory assay six drug preparations, of which only four were combinations of the two drugs (e.g., A and B) in a fixed ratio of 4:1, 3:2, 2:3, and 1:4 of drug A and B, respectively were prepared. The first and the last of these six preparations had the drug A and drug B alone at a concentration approximately eight times higher than IC50 of the drug A and B (Table 1). For ART, 32 nM was taken as 8-fold IC50 and for CLT or HT-ART 8 μM. Thus, six combinations for ART (nM) and HT-AMB or CLT (μM) prepared were 32.0, 25.6:1.6, 19.2:3.2, 12.8:4.8, 6.4:6.4, and 0.8, respectively.

**Combination assay plate preparation.** The effect of each of the above drug or drug combination (along with their two-fold serial dilutions) on parasite growth and multiplication was assayed in triplicates, in flat-bottom, 96-well plates (BD Falcon™). Each experimental well contained a total volume of 200 μL medium with or without drug and 0.8% parasitized red blood cells with 2.5% hematocrit. Plates were prepared as described by Fivelman and others. Six preparations tested for each combination required two 96-well plates. After seeding of wells the plates were incubated in a candle-jar kept at 37°C for a 48-hour asexual cycle after which thin smear slides were prepared. In between after 24 hours the gas mixture in candle-jar was restored by burning the candle.

**Giemsa-stained slide preparation.** Thin blood film slides from each experimental well were methanol fixed and stained in freshly prepared Giemsa solution for counting the number of parasites in random adjacent microscopic fields equivalent to about 2500 erythrocytes under oil immersion. Percentage parasitemia was determined.

![Artemisinin](image1.png)  
**Artemisinin**  

![Amphotericin B](image2.png)  
**Amphotericin B**  

![Clotrimazole](image3.png)  
**Clotrimazole**  

**FIGURE 1.** Chemical structure of artemisinin, amphotericin B, and clotrimazole.

### Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mean IC50 ± SE *</th>
<th>3D7 strain</th>
<th>RKL 303 strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemisinin (ART)</td>
<td>3.97 nM ± 0.08</td>
<td>3.429 nM ± 0.3208</td>
<td></td>
</tr>
<tr>
<td>Heat-treated amphotericin B (HT-AMB)</td>
<td>0.822 μM ± 0.041</td>
<td>0.963 μM ± 0.0455</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B (AMB)</td>
<td>1.62 μM ± 0.011</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Clotrimazole (CLT)</td>
<td>0.946 μM ± 0.069</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* Standard error (N = 3); ND, not determined.
**Isobologram preparation.** The percentage parasitemia was used to calculate percentage inhibition of parasitemia in wells with drug in comparison to the control wells. Nonlinear concentration response curves of drug A, drug B, and each of the four combinations were made on Microsoft Excel. The fractional inhibitory concentration (FIC) for six triplicate preparations was interpreted by the following formula:

\[
FIC = \frac{\text{Concentration of drug in combination to produce IC}_{50}}{\text{Concentration of drug alone required to produce IC}_{50}}
\]

Isobologram was plotted with mean FIC to determine the interactions between drug A and B.

**Data assessment.** The sum FIC value for each of the six preparations determined by the following formula was used to classify the drug–drug interaction.\(^{12-15}\)

\[
\Sigma \text{FIC} = \frac{\text{IC}_{50} \text{ of drug A in mixture}}{\text{IC}_{50} \text{ of drug A alone}} + \frac{\text{IC}_{50} \text{ of drug B in mixture}}{\text{IC}_{50} \text{ of drug B alone}}
\]

\(\Sigma \text{FIC} < 0.5\) represents substantial synergism, \(\Sigma \text{FIC} < 1\) represents synergism, \(\Sigma \text{FIC} \geq 1\) and < 2 represents additive interaction, \(\Sigma \text{FIC} \geq 2\) and < 4 represents slight antagonism whereas \(\Sigma \text{FIC} \geq 4\) represents marked antagonism.

**Hemozoin inhibition assay.** The hemozoin produced in *P. falciparum* cultures without drug or with IC\(_{50}\) drug concentrations was measured at the end of 48 hours.\(^{16}\) The cultures were centrifuged at 1300 rpm for 5 minutes to remove the medium. Pellet was subjected to 0.01% saponin lysis for 10 minutes at room temperature to free parasites from erythrocyte ghosts. The parasites were washed three times with PBS, re-suspended in 2.5% SDS in PBS, and subjected to spin at 20,000 g for 1 hour. The supernatant was discarded and the insoluble pellet was washed in 2.5% SDS in PBS and then dissolved in 20 mM NaOH. The hemozoin content was measured by determining the absorbance at 400 nm and using a standard curve prepared from beta hematin (Sigma). The amount of hemozoin formed in relation to control was calculated.

**Effect of drugs on sorbitol-induced hemolysis.** When 36- to 44-hour-old post invasion parasites are suspended in an isotonic sorbitol solution, there is net uptake of sorbitol and water into the infected erythrocytes, resulting in cell swelling and hemolysis. The hemolysis can be monitored spectrophotometrically by measuring absorbance at 540 nm in presence or absence of drugs at a concentration closer to the antiplasmodial IC\(_{50}\) concentration.\(^{17}\) Hemolysis is reduced in the presence of channel blockers.

To determine the effect of drugs on sorbitol-induced hemolysis, infected cell suspension was first prepared by harvesting 36- to 44-hour-old synchronized culture, initiated from ring stages with over 15% parasitemia, by 600 g spin for 5 minutes. Pellet was washed two times with a 7.4 pH solution of NaCl (150 mM)-HEPES (20 mM) and finally resuspended in this solution to give 50% hematocrit of infected cells. Stock solutions of drugs were diluted using sorbitol (300 mM)-HEPES (20 mM) buffer (pH 7.4) to make 0.1 mM buffered drug stock solutions; 100 \(\mu\)l of infected cell preparation (50% hematocrit) was mixed with the desired concentration of buffered drug solution in a total volume of 1 mL. This cell suspension was incubated at 37°C for 15 minutes, centrifuged, and aliquots of supernatant were used to estimate the amount of hemoglobin released by determining absorbance at 540 nm. The degree of hemolysis was compared with that of control; inhibition of hemolysis reflects the channel blocking effect of drugs, if any.

**RESULTS**

**Parasite drug sensitivity.** The susceptibility of erythrocytic stages of chloroquine-sensitive (3D7) and chloroquine-resistant (RKL 303) strains of *P. falciparum* to ART and HT-AMB *in vitro* is presented in Table 1. The two strains are equally susceptible to these drugs. ART is found to be the most active drug evaluated followed by HT-AMB, CLT, and AMB against 3D7 strain, as adjudged by the IC\(_{50}\) values. HT-AMB is about twice more active in its antiplasmodial activity than AMB.

**Sensitivity to drug combinations.** Two sets of five dose response curves are obtained from each replicate of combination assay with each set representing four combination solu-
tions and one curve for a drug alone. The IC\textsubscript{50} from each set was determined. Typical dose curve of ART and HT-AMB combination is depicted in Figure 2, whereas ART and CLT combination curve in Figure 3. Similar curves from other replicates are obtained to determine IC\textsubscript{50}. Mean FIC\textsubscript{50} values from triplicate is plotted for six drug preparations to obtain an isobologram of each combination experiment. The two isobolograms obtained from fixed ratio combinations evaluated on 3D7 strain of \textit{P. falciparum} are presented in Figure 4.

**Interaction between ART and HT-AMB.** Mean FIC of each combination is calculated at different fixed ratios and the isobologram plot prepared from FICs shows clear synergism, whereas substantial synergistic values are seen in combination preparations 2 and 3 (Table 2) on 3D7 strain of \textit{P. falciparum}.

**Interaction between ART and CLT.** The isobologram prepared from the fixed ratio values show synergism between the two drugs with highest synergism seen in preparation 2 (Table 3) whereas combination 5 shows additive interaction (\textit{FIC} ≥ 1 and < 2 represents additive interaction).

**Effect of drugs on hemozoin formation.** The amount of hemozoin formed within intraerythrocytic parasite food vacuole is related to the ingested and degraded hemoglobin of the host cell. The heme released during hemoglobin digestion is converted into hemozoin, a nontoxic, insoluble polymer of hematin in the oxidized ferric state. The effect of IC\textsubscript{50} concentrations of ART, HT-AMB, and CLT on hemoglobin digestion as reflected by the hemozoin formation is shown in Table 4. The table also shows the effect of pyrimethamine, an antimalarial with known mode of action, where hemoglobin digestion pathway is not affected—acting as an additional negative control. The percent hemozoin formation in parasites treated with HT-AMB is similar to the parasites treated with pyrimethamine, indicating that HT-AMB does not affect hemoglobin digestion. However, ART and CLT show less than expected 50% hemozoin formation, and substantially less hemozoin formation as compared with pyrimethamine-treated parasites indicates that these drugs interfere with hemozoin formation. Similar results are obtained with the best synergistic combinations evaluated (Table 5) for hemozoin formation. ART and CLT in 4:1 ratio the hemozoin formation was only 47%, indicating an additive effect.

**Effect of drugs on sorbitol-induced hemolysis.** Growing intraerythrocytic parasite induces new permeation pathways in the membrane of the host erythrocyte that are absent from the membrane of uninfected cells. The new channels induced by the parasite allow the passage of otherwise impermeable sorbitol. Desai and others\textsuperscript{18} also identified a 140-pS channel

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**Figure 3.** Drug concentration-response curves of artemisinin and clotrimazole are presented for six different preparations/combinations.

**Figure 4.** (A) Isobologram showing interaction between artemisinin and heat-treated amphotericin B. (B) Isobologram showing interaction between artemisinin and clotrimazole. Mean FICs of drug A and B were taken on y and x axis, respectively, along with error bars. The straight line joins both axes at combination 1 and 6 mean FIC values.
that readily transports amino acids and monosaccharides across PVM in the malaria parasite. Compounds are said to be good inhibitors of sorbitol-induced lysis if percent hemolysis is \( \leq 40\% \) relative to the control.\(^1\) Our results showed that the percent sorbitol-induced hemolysis for drugs alone or in combinations were not significant (Tables 4 and 5). Frusemide is an efficient inhibitor of parasite-induced channels\(^1\) and has been used as a positive control; at 10 \( \mu M \) it inhibits about 45% sorbitol-induced hemolysis.

### DISCUSSION

History of drug resistance in malaria shows that any synthetic antimalarial deployed en masse as monotherapy has soon fallen to resistance. A few natural products have escaped this uncanny ability of malaria parasites. These natural products include artemisinin and quinine. Age-old artemisinin has been in use for fever resolving in China for over 2,000 years\(^1\) and quinine has a recorded history of over 350 years for treatment of malaria. These products could retain their prolonged effectiveness because for the majority of their life span they have been used as a mixture, contained in a decoction of the foliage or bark, and not as a purified compound. Mixtures contained more than one ingredient that were active against malaria parasites; thus combination therapies were inadvertently being used for malaria treatments. Some of the alkaloids contained in *Cinchona* bark, for instance, are more potent antiplasmodial than quinine.\(^2\)\(^,\)\(^3\)\(^,\)\(^4\) Combination therapies are, therefore, desirable to delay the selection and spread of resistant mutants to antimalarials. Artemisinin and its derivatives are now the only compounds effective against drug-resistant malaria parasites.\(^2\)^\(^,\)\(^5\) Combination therapies involving artemisinin and its derivatives hopefully will delay the emergence of resistance to this vital class of antimalarials.

As ACTs become more popular, a variety of artemisinin-based combinations will be needed to meet the global demand—and to combat local parasite resistance with specific susceptibility profile. Erythrocytic cultures of *P. falciparum* have been useful to determine the susceptibility of clinical isolates to different antimalarial drugs *in vitro*. Monitor spread of drug resistance; derive clones from an isolate; and to obtain sufficient number of synchronized stages of the parasite for biochemical and molecular studies. Absence of immunity interference in culture provides an added advantage to screen schizonticidal properties of a novel compound *in vitro*. It is a potent system to accurately assay antiplasmoidal drug–drug interactions by isobologram analysis, which is expected to show synergistic, additive, or antagonistic outcome in inhibiting the parasite growth and multiplication. We obtained isobolograms using a fixed ratio method,\(^1\) wherein the concentration ratio of both the drugs varies by a fixed ratio. Using known synergistic antimalarial drugs, sulfadoxine and pyrimethamine, in combination assay (data not presented) we found this method to be simple and reproducible in our laboratory.

In combination treatment the partner medicines should have different modes of action resulting in the improved efficacy of treatment and slowing down the emergence of resistance by mutual protection. Artemisinin is a potent, rapidly acting blood schizontocide and active against all *Plasmodium* species.\(^2\) There are varied views regarding its mechanism of action on *P. falciparum*. Its active component is an endoperoxide bridge, in sesquiterpene lactone structure, which is believed to interact with the iron in the parasitized red blood cells to form toxic free radicals, atoms with unpaired electrons that then destroy proteins in nanodomain vicinity, crucial to the parasite’s survival, leading to demise of the parasite.\(^2\)^\(^,\)\(^19\)^\(^,\)\(^25\)^\(^,\)\(^24\) It has also been demonstrated that ART and its derivatives inhibit an essential calcium adenosine triphosphatase (PfATPase) enzyme,\(^2\) disrupting calcium homeostasis. Inhibition of this vital enzyme causes global release of calcium stored in endoplasmic reticulum into the cytoplasm, thereby interfering with parasite ability to maintain calcium stores, disrupting cellular physiology and function.**

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### Table 2

<table>
<thead>
<tr>
<th>Combination preparation</th>
<th>Ratio of drugs (in 100 ( \mu L ))</th>
<th>Drug A (Artemisinin) Mean FIC(_{50})*</th>
<th>Drug B (HT-AMB) Mean FIC(_{50})*</th>
<th>( \Sigma ) FICs, interaction †</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 5 0</td>
<td>1.0236 ± 0.0083</td>
<td>0.0</td>
<td>1.0256, ADD</td>
</tr>
<tr>
<td>2</td>
<td>4 4 1</td>
<td>0.3733 ± 0.0182</td>
<td>0.1123 ± 0.0052</td>
<td>0.4856, SUB-SYN</td>
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<tr>
<td>3</td>
<td>3 3 2</td>
<td>0.2656 ± 0.0077</td>
<td>0.2093 ± 0.0089</td>
<td>0.4749, SUB-SYN</td>
</tr>
<tr>
<td>4</td>
<td>2 2 3</td>
<td>0.3143 ± 0.0072</td>
<td>0.5873 ± 0.0108</td>
<td>0.9016, SYN</td>
</tr>
<tr>
<td>5</td>
<td>1 1 4</td>
<td>0.1053 ± 0.0039</td>
<td>0.5083 ± 0.0200</td>
<td>0.6136, SYN</td>
</tr>
<tr>
<td>6</td>
<td>0 0 5</td>
<td>0.0</td>
<td>1.0276 ± 0.0286</td>
<td>1.0276, ADD</td>
</tr>
</tbody>
</table>

* Standard error (N = 3) † SUB-SYN, substantial synergism; SYN, synergism; ADD, additive.

### Table 3

<table>
<thead>
<tr>
<th>Combination preparation</th>
<th>Ratio of drugs (in 100 ( \mu L ))</th>
<th>Drug A (Artemisinin) Mean FIC(_{50})*</th>
<th>Drug B (Clotrimazole) Mean FIC(_{50})*</th>
<th>( \Sigma ) FICs, interaction †</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 5 0</td>
<td>1.0111 ± 0.0043</td>
<td>0.0</td>
<td>1.011, ADD</td>
</tr>
<tr>
<td>2</td>
<td>4 4 1</td>
<td>0.423 ± 0.0092</td>
<td>0.108 ± 0.0036</td>
<td>0.531, SYN</td>
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<tr>
<td>3</td>
<td>3 3 2</td>
<td>0.527 ± 0.0006</td>
<td>0.397 ± 0.0033</td>
<td>0.924, SLT-SYN</td>
</tr>
<tr>
<td>4</td>
<td>2 2 3</td>
<td>0.389 ± 0.0066</td>
<td>0.609 ± 0.0094</td>
<td>0.998, SLT-SYN</td>
</tr>
<tr>
<td>5</td>
<td>1 1 4</td>
<td>0.369 ± 0.0026</td>
<td>1.560 ± 0.0154</td>
<td>1.929, ADD</td>
</tr>
<tr>
<td>6</td>
<td>0 0 5</td>
<td>0.0</td>
<td>1.098 ± 0.0316</td>
<td>1.098, ADD</td>
</tr>
</tbody>
</table>

* Standard error (N = 3) † SYN, synergistic; SLT-SYN, slightly synergistic; ADD, additive.

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** Footnotes:
1. Standard error (N = 3)
2. SUB-SYN, substantial synergism; SYN, synergism; ADD, additive.
Table 4

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration used (Mean IC_{50} ± SE)*</th>
<th>% Hemozoin formation*</th>
<th>Concentration</th>
<th>% Sorbitol-induced hemolysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ART</td>
<td>3.9 nM (3.97 nM ± 0.08)</td>
<td>48.16 ± 0.173</td>
<td>5 nM</td>
<td>90.93 ± 0.1934</td>
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<tr>
<td>HT-AMB</td>
<td>0.8 μM (0.82 μM ± 0.041)</td>
<td>56.46 ± 0.228</td>
<td>1 μM</td>
<td>60.36 ± 0.303</td>
</tr>
<tr>
<td>CLT</td>
<td>0.9 μM (0.94 μM ± 0.069)</td>
<td>46.006 ± 0.174</td>
<td>1 μM</td>
<td>83.04 ± 0.2292</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>3.5 nM</td>
<td>56.27 ± 0.98</td>
<td>10 nM</td>
<td>94.12 ± 0.6955</td>
</tr>
<tr>
<td>Frusamide</td>
<td>ND</td>
<td>ND</td>
<td>10 μM</td>
<td>54.63 ± 0.198</td>
</tr>
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</table>

* Standard error (N = 3); ND, not determined.

causing parasite death. This multi-targeted attack on the parasite by ART may be responsible for the non-emergence of resistance to this drug, despite its use in China for fever treatment over thousands of years. The partner drug of ART or its derivatives in combination should preferably have comparable half-lives, so as not to leave the other drug unprotected from monotherapy selection pressure. Peak ART plasma concentrations occur around 3 hrs after oral administration.25,26 The elimination half-life is approximately 1 hr.26

Clotrimazole is a synthetic azole derivative, [1-(α-2-chlorotrityl) imidazole], effective against a wide range of fungal pathogens. The fungistatic mechanism of CLT is believed to be associated with inhibition of sterol 14α-demethylase and microsomal P-450 dependent enzymes.27,28 CLT shows diverse microbial actions including effective inhibition of *P. falciparum* growth in vitro.29,30 The total disappearance of parasite and disintegration of parasitized cells was observed on 48-hr exposure of ring-stage culture to 2.5 μM of CLT.30 This suggests that CLT may be potentially effective in relatively short-term treatments. The anti-malarial mechanism of CLT is not well understood. It has been suggested that the antiplasmodial effect may be exerted due to its ability to inhibit heme catabolism in the malaria parasite and by enhancing heme-induced membrane damage.29 Inhibition of oxidized glutathione export from infected red cells or from the parasite is also considered to be one of the possible mechanisms of antiplasmodial effect of CLT.31 CLT inhibits heme-peroxidase that increases the accumulation of hydrogen peroxide in *P. falciparum* and causes oxidative stress.31 A major change in calcium ion fluxes32–34 has also been attributed as the cause of CLT toxicity to parasite. High doses of CLT for treatment of mycotic infections have been well tolerated without significant clinical, hematological, or biochemical side effects.35 In healthy subjects after a single oral dose of 1g CLT, plasma levels reach mean peak concentration of about 2 μM within 2–4 hrs of administration.36 CLT has a short elimination half-life of about 3hrs37 and steep slope of the concentration-response curve38 are additional features associated with a low probability of resistance development along with the multiple target attack in the parasite. CLT has lower toxicity relative to other imidazoles. These characteristics make CLT an attractive candidate medicine to be evaluated as an antiplasmodial in combination with ART in vivo system. ART-CLT an antiplasmodial combination has been found to be synergistic, but not all ART plus azoles38 behave in a synergistic manner. Drug interaction may also alter the mechanism of combination action. Not much is known about ART-CLT mode of action on malaria parasites. ART and CLT did not inhibit sorbitol-induced hemolysis to a significant extent alone or in combination. We have shown here that ART-CLT interaction is additive in the hemozoin inhibition indicating another target site of action in the parasite by this synergistic combination.

Malaria parasites belong to the phylum Apicomplexa and harbor a variety of unusual intracellular organelles like the inner membrane complex (a flattened patchwork of vesicles) and the apicoplast (a chloroplast-like organelle acquired by secondary endosymbiosis of an algal ancestor). Amphotericin B interacts with the outer membrane sterols in yeasts, algae, and protozoa resulting in pore formation thus altering permeability and leading to death of the organism.39 AMB binding to ergosterol and disrupting membrane integrity in fungal infections has been demonstrated.39,40 It also causes peroxidation of unsaturated fatty acids on the parasitized red cell membrane.41 AMB perhaps also affects multiple targets within the intracellular parasite, acting primarily on parasite plasma membrane. Heat-treated AMB has an enhanced *in vitro* antiplasmodial effect than AMB against both chloroquine-resistant and chloroquine-sensitive *P. falciparum* strains.7 The IC_{50} of AMB or HT-AMB on 3D7 strain was found to be similar to that reported for other strains. Heat treatment of AMB forms a ‘superaggregated form’ having significantly increased stability, marked decrease in hemolytic efficiency, improved therapeutic index for mycoses treatment with reduced toxicity to mammalian cells, and decreased renal cytotoxicity.41–42 HT-AMB was thus considered a good candidate for *in vivo* evaluation of interactions with artemisinin. Resistance to polyene antibiotics like amphotericin B is rare, with resistance mostly restricted to the less common species of *Candida*.43 ART plus HT-AMT do not affect

Table 5

<table>
<thead>
<tr>
<th>Combination ratio</th>
<th>Drug concentrations in 5 mL</th>
<th>% Hemozoin formation*</th>
<th>% Sorbitol-induced hemolysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ART: CLT (4:1)</td>
<td>3.17 nM: 0.1892 μM</td>
<td>47.01 ± 0.2703</td>
<td>4 nM: 0.2 μM 86.68 ± 0.4882</td>
</tr>
<tr>
<td>ART:HT-AMB (4:1)</td>
<td>3.17 nM: 0.1644 μM</td>
<td>50.74 ± 0.2699</td>
<td>4 nM: 0.2 μM 79.97 ± 0.4030</td>
</tr>
<tr>
<td>ART:HT-AMB (3:2)</td>
<td>2.38 nM: 0.3288 μM</td>
<td>52.48 ± 0.2847</td>
<td>3 nM: 0.4 μM 77.40 ± 0.2967</td>
</tr>
</tbody>
</table>

* Standard error (N = 3).
hemozoin formation and this combination is not a channel blocker for sorbitol lysis.

The principle of one drug, one target in drug discovery has yielded notable successes. Most synthetic drugs working on this principle against infectious diseases, like antimalarial pyrimethamine, soon succumbed to resistance. Drugs affecting more than one target in parasites are likely to retain their effectiveness for longer periods. Artemisinin, CLT, and HT-ATB all seem to qualify this attribute. The stage-specific inhibition assay (data not presented) using synchronized erythrocytic stages of *P. falciparum* showed that artemisinin is predominantly toxic to schizont stages whereas HT-AMB to ring stages, indicating yet again a differential mode of drug action on the parasite. The interactions of ART and HT-AMB against 3D7 parasite strain yielded synergistic results, showing cooperative interaction that creates an enhanced combined effect. It will be interesting to evaluate these interactions using these combinations in an *in vivo* model system.

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