INTRODUCTION

Hundreds of millions of people are at risk of drug-resistant falciparum malaria infection. The worsening problems of drug resistance in many parts of the world and the limited number of antimalarial drugs available have led to increasing difficulties for adequate disease management. Artemisinin and its derivatives are among the most effective antimalarial drugs known today. They rapidly cure even drug-resistant falciparum infections. Artemisinin-based combination therapies (ACTs) are being used for treatment, instead of monotherapies, to delay the emergence of resistant strains of *Plasmodium falciparum* to this vital class of drugs. ACTs are increasingly being adopted as first-line treatments in malaria-endemic regions of the world that are afflicted with *P. falciparum* strains resistant to conventional antimalarial drugs. The history of drug resistance is replete with instances showing the uncanny ability of parasites to rapidly acquire resistance to any chemotherapeutic assault deployed en masse to treat uncomplicated malaria cases. Clinically artemisinin-resistant strains of *P. falciparum* have not yet been encountered. Thus, the demand for ACTs is increasing. Artemisinin is obtained from aerial parts of an herb, and this plant is globally scarce. Synthesis of artemisinin is not a commercially viable proposition at present. This makes ACT expensive for poor people in malaria-endemic regions. There is also reason to believe that, sooner or later, resistance to existing ACTs will emerge. Thus, there is need to search for synthetic synergistic drug partners of artemisinin and its derivatives to reduce dependence on this scarce natural product in ACTs. Use of a *P. falciparum* in vitro system is very potent in identifying novel lead compounds and combinations. We have evaluated the in vitro antimalarial activity of the synthetic compounds triclosan and ketoconazole as partner drugs in combination with artemisinin against erythrocytic stages of *P. falciparum* using a modified fixed-ratio isobologram method. Both of these combination partner drugs are FDA approved for human use and demonstrated antiplasmodial activity. In-depth studies of interactions between drugs may also provide clues to their mechanisms of action.

MATERIALS AND METHODS

**Parasite culture.** Stock culture of malaria parasite *P. falciparum* 3D7 strain was continuously maintained in vitro using the candle-jar method of Trager and Jensen. The parasites were maintained on B+ human red blood cells suspended in a complete culture medium. Each 960 mL of aqueous culture medium consisted of 10.4 g of powdered RPMI-1640 (with glutamine but without bicarbonate), 5.94 g of HEPES buffer, and 40 mg of gentamicin. Complete medium was constituted just before use by adding sterile 5% sodium bicarbonate at the rate of 4 mL per 96 mL and supplemented with 10% pooled B+ serum. The stock culture parasitemia was mostly kept between 1% and 5% with subculturing every fourth day. The starting hematocrit was 5%.

**Drug solutions.** Artemisinin, triclosan, and ketoconazole were obtained from Sigma-Aldrich (St. Louis, MO), Vivimed Laboratories Ltd. (Hyderabad, India), and Yanshu Chemicals Ltd. (Mumbai, India), respectively. Artemisinin and triclosan were dissolved separately in DMSO, and ketoconazole was dissolved in distilled water to obtain stock solutions of 0.2, 1, and 1 mg/mL concentrations, respectively. The stock solutions were diluted on the day of experiment to obtain the desired concentrations for each drug. The amount of DMSO in the diluted concentrations used had no effect on parasite growth.

**Inhibitory concentrations assay.** The inhibitory concentration of each individual drug needed to prevent the growth and multiplication of *P. falciparum* was determined in vitro using a dose–response assay in 24-well tissue culture plates in triplicates. Synchronous parasites were prepared and challenged with a graded concentration of a drug solution for 48 hours at 37°C by the candle-jar method. The medium was changed in each well after 24 hours with or without drug. The percentage inhibition of parasitemia in relation to control was calculated by examining thin-smear Giemsa-stained slides. Assay results were computed to determine the IC\textsubscript{50} value of each drug.
Drug combination preparations. For each combination assay, drug strength was made so that IC$_{50}$ of the individual drugs fell between third and fourth 2-fold serial dilutions, while the other four drug combinations were made in fixed ratios (Tables 2 and 3). Six combinations prepared in nM ratios of artemisinin/triclosan were 32:0, 25.6:1780, 19.2:3560, 12.8:5340, 6.4:7120, and 0:8900, respectively; and six concentrations of artemisinin (nM)/ketoconazole (µM) combinations were 32:0, 25.6:3.36, 19.2:6.72, 12.8:10.08, 6.4:13.44, and 0:16.8, respectively.

Combination plate preparation. Drug dilutions of combinations were made in sterile, flat-bottom, 96-well tissue culture plates as described elsewhere. Each microtiter plate contains wells in eight rows (A–H) and 12 columns. Wells in row H, columns 3 to 11, contained a 2× stock concentration, and serial dilutions were made from solutions in these wells upward to wells in row B. Each well contained a total volume of 200 µL of drug, presynchronized infected RBCs (0.8% parasitemia at 2.5% hematocrit), and complete culture medium. Simultaneously, parasite control wells without drug were prepared in row A, columns 3 to 11, and rows A to H, columns 2 and 12, although only column 2 was taken for IC$_{50}$ calculations. The wells in column 1, rows A to H, were kept with a normal RBC suspension in complete medium, without drug. Six combinations (in triplicate) were tested in two plates. Except for row H, columns 3 to 11, in all wells 100 µL of complete medium was added. Serial dilution was done from row H to row B, with transfer of 100 µL each time after mixing, and 100 µL from the wells in row B was discarded finally. The plates were kept in a candle jar and candle-lighted to increase CO$_2$ concentration. The plates were incubated at 37°C for 48 hours with a brief interruption after 24 hours, and re-gassed by burning the candle.

Slide preparation and staining. The plates were removed from candle jar, and the material from each test well was transferred into the corresponding well numbered 1.5-mL microcentrifuge tube. After a fast spin, the supernatant was removed and the pellet was mixed thoroughly to prepare thin blood smear slide for each test well. The slides were air-dried, methanol-fixed, and stained in Giemsa stain for 40 minutes. After they were stained, slides were removed from the coupling jar, washed in running tap water, and air-dried. The Giemsa-stained slides were examined to count the number of parasites in random adjacent microscopic fields, equivalent to ~3000 erythrocytes at 1000× magnification. Percent parasitemia was calculated.

Construction of isobologram. For the combination assay, IC$_{50}$ was calculated from two sets of dose–response graphs (Figures 1 and 2), each containing a “drug-alone” curve and four drug-combination curves. Fractional inhibitory concentration (FIC) values were calculated separately for each drug concentration present in the combination by the following formula:

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

Drug interaction assessment. The sum of the fractional inhibitory concentrations (Σ FICs) of both the drugs for a particular combination shows the interaction pattern between the two drugs.

RESULTS

Sensitivity of P. falciparum. The sensitivity of malaria parasite, strain 3D7, to artemisinin, triclosan, and ketoconazole was assessed in vitro against erythrocytic stages. The computed IC$_{50}$ values given in Table 1 were used for combination assays.
TABLE 1
Baseline sensitivity of *P. falciparum* (strain 3D7) to artemisinin, triclosan, and ketoconazole in terms of IC₅₀

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mean IC₅₀ ± SE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemisinin</td>
<td>3.97 ± 0.08 nM</td>
</tr>
<tr>
<td>Triclosan</td>
<td>1.105 ± 0.059 μM</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>2.101 ± 0.0253 μM</td>
</tr>
</tbody>
</table>

* Standard error (n = 3).

Sensitivity to drug combinations. From combination experiments, the observed IC₅₀ values were analyzed in relation to data obtained with the single compounds by using the method of Berenbaum,¹ which yielded Σ FICs for the drug combination. Mean FIC values were calculated separately for both the drugs present in combinations 1 to 6. In these six preparations, combinations 1 and 6 contain drug A and drug B alone, respectively, so their IC₅₀ corresponded to the IC₅₀ obtained by their individual dose–response assay. From each triplicate, six sets of dose–response curves were obtained. The mean FICs of interaction between artemisinin/triclosan (Figure 3A) and artemisinin/ketoconazole (Figure 3B) were plotted in an isobologram for combination preparations 1 through 6.

Interaction between artemisinin and triclosan. The Σ FIC values of artemisinin and triclosan fixed-ratio combinations showed a synergistic interaction with combination 2 and trends toward mild synergism with combinations 3, 4, and 5 (Table 2).

Interaction between artemisinin and ketoconazole. Interaction of artemisinin with ketoconazole showed an additive pattern with combination 2, moderate antagonism with combination 3 and 4, and marked antagonism with combination 5 (Table 3).

DISCUSSION

Artemisinin and its four derivatives (dihydroartemisinin, artesunate, artemether, and arteether) are rapidly acting antimalarial drugs effective against even chloroquine-resistant malaria infections. To improve their efficacy and delay the onset of resistance to this vital class of antimalarial drugs, they are used in combination with other effective antimalarial drugs. At present, they play an essential role in malaria management and control. This, however, depends on ensuring that they are affordable, readily available, and of acceptable quality. Unfortunately, the quality and effectiveness of these antimalariales have been declining, and the supply is often inefficient and unreliable. Fake preparations of the frequently used ACT—artemether and lumefantrine combination—have hit some of the Southeast Asian markets.¹² The global scarcity of artemisinin, leading to its high cost, and the lack of quality control in resource-poor countries where malaria is rampant are some of the factors for these maladies. The widespread misuse of ACTs suggests that they will succumb to resistance sooner rather than later. The current grim situation demands reduced dependence on artemisinins and a search for synthetic, inexpensive partner drugs to augment the antimalarial activity of new combinations.

In vitro cultures of *P. falciparum* blood stages form a good first screen for identifying potential partner drugs for ACTs to be developed as anti-malaria therapies. Combining artemisinin with another in vitro–effective schizonticidal drug may lead to altered pharmacokinetics, decreases in efficacy, and modified toxicity levels of combined drugs. Many of these aspects cannot be predicted using this system. However, in vitro drug–drug interactions interpretation for antimalarial activity by an isobologram is obtainable and can provide information regarding synergistic, additive (indifferent), or antagonistic outcomes in inhibiting parasite growth and multiplication. Isobolograms are prepared either by the fixed-ratio method,¹ where concentration ratios of both drugs differ by a fixed ratio, or by the checkerboard method,¹³ where one drug concentration is kept constant while the other is varied. Both of these methods have been used in drug-combination studies for antitumor, antibacterial, and antifungal agents.¹⁴–¹⁶ The fixed-ratio method using known synergistic antimalarial drugs—sulfadoxine and pyrimethamine—in combination assay was validated (data not presented) and found to be accurate, simple, and straightforward. In-depth study of antimalarial drug interactions is of great significance to both development of combination therapies and understanding the mode of action of these drugs.⁷

The candidate partner drug in ACT should have an independent mode of action to delay the selection of resistance. Artemisinins are sesquiterpene lactones containing an endoperoxide bridge. There are several views regarding the mode of action of artemisinin on *P. falciparum*. The mechanism of action is considered to be at least two-step processes, involving an activation step and alkylation. In the activation step, intraparasitic iron catalyzes cleavage of the endoperoxide bridge and generates a highly reactive free radical, which in the second step forms a covalent bond with parasite proteins and incapacitates them.¹⁷,¹⁸ Eventually killing the parasite. It seems to act on many targets within a nanodomain vicinity of the parasite. Perhaps this is one reason why resistance to this vital class of drug has not emerged despite its long historic use for fever resolution in China. A yeast model showed artemisinin to interact with the electron transport chain, generating a local reactive oxygen species and causing depolarization of the mitochondrial membrane.¹⁹ It has also been shown that
artemisinins exert their antimalarial action by disrupting calcium homeostasis; they promote discharge of calcium ions from intracellular stores of malaria parasite by specifically inhibiting a metabolic enzyme: the malarial, calcium-dependent, endoplasmic reticulum ATPase. Inhibition of this enzyme causes uncontrolled release of calcium stored in the endoplasmic reticulum into cytoplasm, causing parasite death.

Triclosan is synthetic 2-hydroxydiphenyl ether that has been evaluated as a drug partner with artemisinin. Triclosan has a favorable safety profile and is well tolerated by humans. It is neither an acute oral toxicant nor does it act as a carcinogen, mutagen, or teratogen. It is found in many commercial products, such as mouthwashes and deodorants. Its use as a systemic antimicrobial has not been established, but antimalarial activity was demonstrated when it was administered subcutaneously in an in vivo system. Triclosan also inhibits the growth of the blood stages of *P. falciparum* in vitro and is effective against chloroquine-resistant and chloroquine-sensitive strains.

Triclosan inhibits the fatty acid biosynthesis II (FAS II) pathway in *P. falciparum* through inhibition of *FabI* enzyme, which is necessary for the conversion of crotonyl-ACP (acyl carrier protein) in butyryl-ACP. The FAS II pathway is completely lacking in humans and is, therefore, potentially an attractive target for antimalarial therapeutics. On the basis of these facts, triclosan was considered to be a suitable partner drug for evaluation with artemisinin. A modified fixed-ratio isobologram method was used to determine the effect of triclosan in combination with artemisinin in strain 3D7 of *P. falciparum*. The experiments conducted in combination showed a mild synergistic interaction in each combination ratio (Table 2), with synergism at different combination solutions ± SE.* It also inhibits growth of falcipain (FP) crystals and will be of importance for its mechanistic implications in the design of novel antimalarial drugs.

Ketoconazole was also evaluated for drug interactions with artemisinin. This synthetic compound is an imidazole and has wide-spectrum antimicrobial activity, including *P. falciparum*. Ketoconazole inhibits the enzyme 14α-demethylase, thereby interrupting the synthesis of ergosterol, an important component of plasma membrane of apicomplexan parasites like *P. falciparum*. This enzyme is also necessary for conversion of lanosterol into zymosterol, which is converted to ergosterol. It also inhibits growth of falcipain (FP) crystals by a surface-binding mechanism and interferes with other heme crystal-sensitive pathways in *P. falciparum*, leading to death of the parasite. Falcipain-2 (FP-2) plays an important role in degradation of erythrocyte proteins like hemoglobin, thus rendering an important target in the design of novel antimalarial drugs.

### Table 2

<table>
<thead>
<tr>
<th>Combination solution</th>
<th>Ratio of drugs (in 100 µL)</th>
<th>Mean FIC&lt;sub&gt;50&lt;/sub&gt; ± SE*</th>
<th>Σ FICs, interaction†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Drug A (artemisinin)</td>
<td>Drug B (triclosan)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.0 ± 0.029</td>
<td>0</td>
<td>1.0, ADD</td>
</tr>
<tr>
<td>2</td>
<td>0.416 ± 0.0078</td>
<td>0.1093 ± 0.0027</td>
<td>0.525, SYN</td>
</tr>
<tr>
<td>3</td>
<td>0.568 ± 0.0087</td>
<td>0.386 ± 0.0079</td>
<td>0.954, SLT-SYN</td>
</tr>
<tr>
<td>4</td>
<td>0.3233 ± 0.0047</td>
<td>0.494 ± 0.006</td>
<td>0.817, SLT-SYN</td>
</tr>
<tr>
<td>5</td>
<td>0.1483 ± 0.00145</td>
<td>0.666 ± 0.0245</td>
<td>0.824, SLT-SYN</td>
</tr>
<tr>
<td>6</td>
<td>0.0</td>
<td>1.0103 ± 0.0399</td>
<td>1.0103, ADD</td>
</tr>
</tbody>
</table>

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

### Table 3

<table>
<thead>
<tr>
<th>Combination solution</th>
<th>Ratio of drugs (in 100 µL)</th>
<th>Mean FIC&lt;sub&gt;50&lt;/sub&gt; ± SE*</th>
<th>Σ FICs, interaction†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Drug A (artemisinin)</td>
<td>Drug B (ketoconazole)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.019 ± 0.01266</td>
<td>0.0</td>
<td>1.019, ADD</td>
</tr>
<tr>
<td>2</td>
<td>1.217 ± 0.03037</td>
<td>0.301 ± 0.005686</td>
<td>1.518, ADD</td>
</tr>
<tr>
<td>3</td>
<td>1.233 ± 0.03851</td>
<td>0.809 ± 0.02771</td>
<td>2.042, SLT-A NT</td>
</tr>
<tr>
<td>4</td>
<td>1.208 ± 0.05028</td>
<td>1.793 ± 0.06917</td>
<td>3.001, SLT-A NT</td>
</tr>
<tr>
<td>5</td>
<td>0.780 ± 0.01472</td>
<td>3.110 ± 0.03166</td>
<td>3.890, MKD-A NT</td>
</tr>
<tr>
<td>6</td>
<td>0.0</td>
<td>1.001 ± 0.084096</td>
<td>1.001, ADD</td>
</tr>
</tbody>
</table>

* Standard error (n = 3).
† ADD, additive; SLT-A NT, slightly antagonistic; MKD-A NT, markedly antagonistic.
showed a combination of artemisinin and ketoconazole to be largely antagonistic in antiplasmodial activity in vitro (Table 3). The antimalarial activity of ketoconazole is enhanced with an increase in oxygen concentration.27 Older P. falciparum-infected erythrocytes are more susceptible to ketoconazole than younger infected RBCs, as older RBCs are more susceptible to oxidative stress.28 Because we used the candle-jar method, where the concentration of oxygen in the cultures could not be controlled, it would be premature to conclude that interactions between artemisinin and ketoconazole were antagonistic without supporting evidence from in vivo or in vitro model system wherein oxygen levels could be controlled. Although the concentrations of gentamicin used in the experiments do not affect the parasite growth in vitro,29 even small amounts may affect drug interactions.

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REFERENCES