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In Vitro Analysis of the Interaction between Atovaquone and Proguanil against Liver Stage Malaria Parasites

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The interaction between atovaquone and proguanil has never been studied against liver stage malaria, which is the main target of this drug combination when used for chemoprevention. Using human hepatocytes lacking cytochrome P450 activity, and thus avoiding proguanil metabolizing into potent cycloguanil, we show *in vitro* that the atovaquone-proguanil combination synergistically inhibits the growth of rodent *Plasmodium yoelii* parasites. These results provide a pharmacological basis for the high efficacy of atovaquone-proguanil used as malaria chemoprevention.

The drug combination atovaquone-proguanil (AP) is an efficient drug for malaria chemoprevention and treatment. The rationale for combining these two drugs originated from the observation that they produce a synergistic inhibitory effect against replicating blood stage parasites *in vitro* (1–3). In the context of malaria chemoprevention, however, AP exerts its protective effect primarily during the hepatic phase of the parasite infection (4, 5), and whether the AP synergy is operating during this preerythrocytic phase has not been explored.

To test this directly, we sought to evaluate *in vitro* the interaction between atovaquone and proguanil against liver stage *Plasmodium* infection through a fixed-ratio isobologram method. In hepatocytes, proguanil is partially metabolized by host cytochrome P450 enzymes into cycloguanil (6), which potently inhibits the development of liver stage *Plasmodium* infection (7). To address this confounding factor, we used HepG2-CD81 human hepatoma cells that retain many liver-specific properties and can be infected by some *Plasmodium* species (8) while displaying impaired cytochrome P450 activity (9).

To confirm the lack of or very minimal proguanil metabolism, HepG2-CD81 cells were incubated in the presence of 100 μM proguanil for 2 days, and cell culture supernatants were collected at 24 and 48 h after treatment to quantify drug levels. Proguanil and its metabolite cycloguanil were separated and quantified on a liquid chromatography mass detection mass spectrometer (TSQ Quantum Ultra; Thermo Fisher, France) using an Atlantis dC_{18} column (100 by 2.1 mm, 3 μm; Waters, France) and a calibration curve. Cycloguanil was not detected in any of the collected samples from the 24- and 48-h time points ($n = 8$ values) at the 0.04 μM limit of our assay (proguanil was detected as expected). In contrast, cycloguanil was detected in the corresponding samples of primary human hepatocytes used as positive controls (median, 0.32 μM; minimum, 0.21 μM; maximum, 0.97 μM; $n = 8$ values; $P < 0.001$, Mann-Whitney test). The same observations regarding cycloguanil production in the two cell types were made when using 20 μM instead of 100 μM proguanil ($P < 0.001$, Mann-Whitney test).

Subsequently, *Plasmodium*-infected hepatocytes were treated with single agents, and then combination treatments were performed to assess drug interaction. Primary human hepatocytes were isolated as previously described (8). HepG2 cells were derived from the liver tissue of a 15-year-old boy with differentiated hepatocellular carcinoma (10) and modified to induce CD81 expression (11). *Plasmodium yoelii* sporozoites (BY265 strain) were obtained from infected salivary glands of *Anopheles stephensi* 14 to 21 days after an infective blood meal on a parasite-infected Swiss-Webster mouse. Using a 96-well plate, 15,000 sporozoites were added per well, containing an average of 80,000 HepG2-CD81 or primary human hepatocyte cells in a monolayer at a density of 250,000 cells/cm^2. Drugs at various concentrations along with no-drug (solvent-only) controls were added together with sporozoites.

The drug-containing cell culture media were replaced 2 and 24 h after infection, and the development of liver stage parasites was stopped 48 h after infection by fixation with methanol. Liver stage parasites were stained with a polyclonal antibody specific for *Plasmodium* HSP70 (12), and host and parasite nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole). Fluorescent labeling was visualized using a fluorescence microscope with ×400 magnification (Leica DMI4000 B), and parasites were counted visually. We measured the drug effects on parasite maturation into liver stage schizonts as a proxy for parasite growth. Only small parasites with a single nucleus were considered to display arrested maturation and thus were not counted as schizonts (Fig. 1A). We

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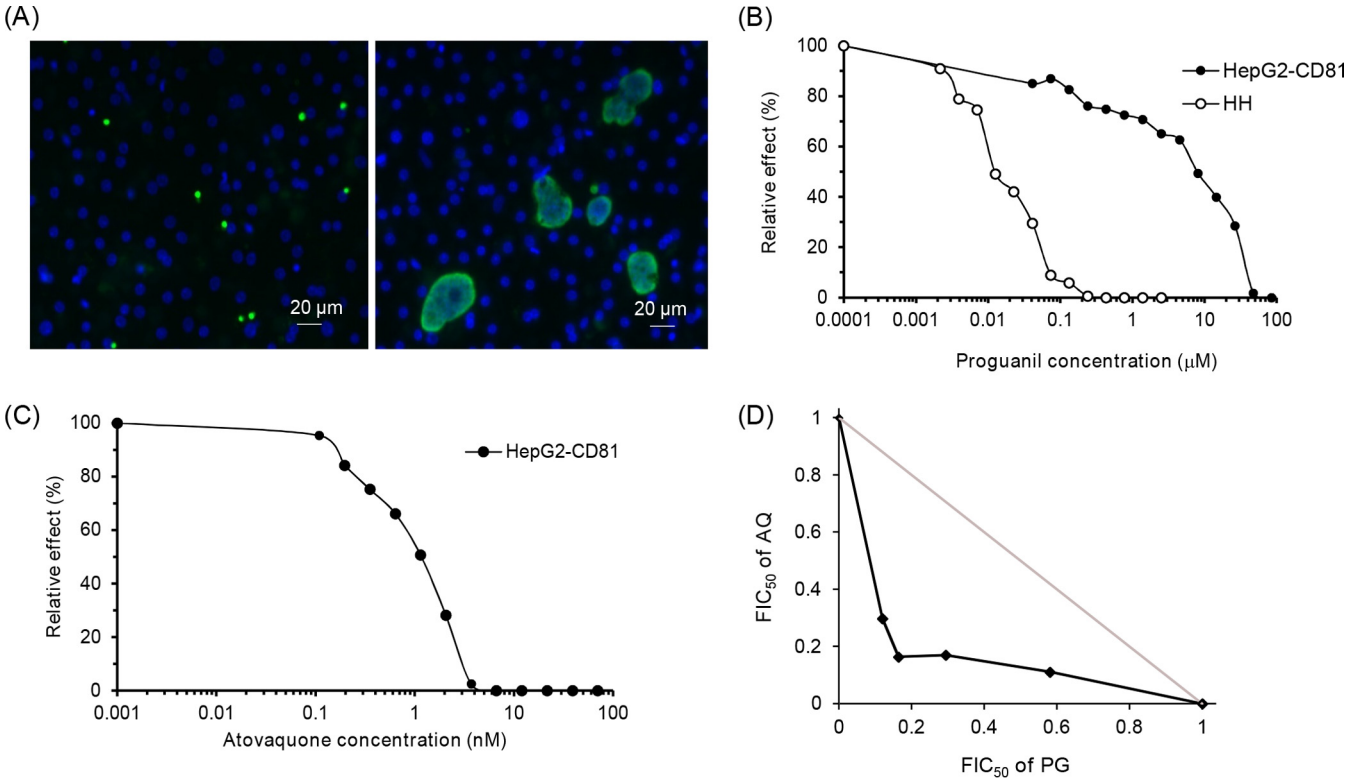

FIG 1 Inhibitory effect of atovaquone and proguanil on the development of liver stage rodent *Plasmodium* infection. *P. yoelii* sporozoites were added to HepG2-CD81 cell monolayers together with different drug concentrations. The drug-containing cell culture media were replaced 2 and 24 h after infection, and the development of liver stage parasites was stopped 48 h after infection by fixation with methanol. Liver stage parasites were stained with a polyclonal antibody specific for *Plasmodium* HSP70 (green), while host and parasite nuclei were stained with DAPI (blue). The numbers of arrested and nonarrested parasites were evaluated by microscopic examination. (A) Representative image of *P. yoelii* parasites 48 h after infection. Small arrested parasitic forms (<7 μM) with a single nucleus are shown in the left panel, and fully developed schizonts are displayed in the right panel. (B) Dose-response curves for proguanil against *P. yoelii* in HepG2-CD81 and primary human hepatocytes (HH). Data are representative of those from three independent assays. (C) Dose-response curve for atovaquone against *P. yoelii* in HepG2-CD81 cells. Data are representative of those from three independent assays. (D) Isobologram showing the interaction between atovaquone and proguanil against *P. yoelii* infecting HepG2-CD81 cells. Drug interactions are represented by the normalized FIC_{50}s for atovaquone and proguanil plotted against each other. Data are representative of those from three independent assays. The gray line is the line of additivity.

estimated the 50% inhibitory concentration (IC_{50}) by plotting the relative reduction in the number of schizonts per well on the *y* axis against drug concentration on the *x* axis (13). Experiments were repeated at least three times with different sporozoite and hepatocyte batches. No hepatocyte toxicity was observed at any of the highest atovaquone and proguanil concentrations used in the study (0.039 and 154 μM, respectively). Two or three wells per drug concentration were used to analyze parasite counts in the IC_{50} and synergy experiments, respectively. The median numbers of schizonts per well in drug-free controls were 100 (minimum, 53; maximum, 319) and 505 (minimum, 311; maximum, 527) for the IC_{50} and synergy experiments, respectively.

We first compared the IC_{50}s for proguanil against *P. yoelii* parasites obtained from HepG2-CD81 and human primary hepatocytes. The IC_{50}s were about 100-fold higher in HepG2-CD81 (IC_{50}s, 2.2, 3.2, and 18.4 μM; median, 3.2 μM) than in primary human hepatocytes (IC_{50}s, 0.02, 0.03, and 0.04 μM; median, 0.03 μM) (Fig. 1B). Altogether, the drug dosage and IC_{50} measurements indicated a deficiency in proguanil metabolism in HepG2-CD81 cells. Regarding atovaquone, the median IC_{50} in HepG2-CD81 cells was 0.92 nM (IC_{50}s, 0.76, 0.92, and 1.1 nM) (Fig. 1C). The proguanil and atovaquone IC_{50} values in HepG2-CD81 cells were consistent with those in a previous report (7).

Because we had evidence that cycloguanil production is dramatically reduced or prevented in HepG2-CD81 cells, we explored the effect of the AP combination against *P. yoelii* infection through a fixed-ratio isobologram method, as previously described (14). Briefly, six starting solutions containing six fixed atovaquone/proguanil molar ratios were prepared and then serially diluted 2-fold seven times. The starting solutions 1 to 6 were prepared at atovaquone/proguanil nanomolar concentrations of 6:0, 4.8:4,000, 3.6:8,000, 2.4:12,000, 1.2:16,000, and 0:20,000, respectively. The fractional inhibitory concentration (FIC) was calculated for each drug in each combination according to the following equation:

$$FIC_{50} = \frac{IC_{50} \text{ of drug in combination}}{IC_{50} \text{ of drug alone}}$$

The isobologram curve was generated by plotting the FIC_{50} of atovaquone against the FIC_{50} of proguanil. The mean $FIC_{50\ index}$ was calculated according to the following equation:

$$FIC_{50\ index} = \text{average } FIC_{50}\,(\text{atovaquone}) + \text{average } FIC_{50}\,(\text{proguanil})$$

The median isobologram (of 3 independent experiments) for the interaction between atovaquone and proguanil is shown in

Fig. 1D. The $FIC_{50\ index}$ had a median value of 0.64 (0.62, 0.64, and 0.69), which together with the isobolograms indicated that the interaction between the two drugs is synergistic.

In conclusion, we report here the first *in vitro* study, to our knowledge, that formally investigated the effect of a drug combination on liver stages of malaria. We showed that the synergism between atovaquone and proguanil against rodent malaria parasites *in vitro* is conserved across liver and blood stages, which provides a pharmacological basis for the high efficacy of AP when used as malaria chemoprevention.

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