Potent antimalarial activity of clotrimazole in in vitro cultures of Plasmodium falciparum

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Communicated by Helen M. Ranney, Alliance Pharmaceutical Corp., San Diego, CA, November 12, 1999 (received for review August 22, 1999)

The increasing resistance of the malaria parasite Plasmodium falciparum to currently available drugs demands a continuous effort to develop new antimalarial agents. In this quest, the identification of antimalarial effects of drugs already in use for other therapies represents an attractive approach with potentially rapid clinical application. We have found that the extensively used antifungal drug clotrimazole (CLT) effectively and rapidly inhibited parasite growth in five different strains of P. falciparum, in vitro, irrespective of their chloroquine sensitivity. The concentrations for 50% inhibition (IC50), assessed by parasite incorporation of [3H]hypoxanthine, were between 0.2 and 1.1 μM. CLT concentrations of 2 μM and above caused a sharp decline in parasitemia, complete inhibition of parasite replication, and destruction of parasites and host cells within a single intraerythrocytic asexual cycle (~48 hr). These concentrations are within the plasma levels known to be attained in humans after oral administration of the drug. The effects were associated with distinct morphological changes. Transient exposure of ring-stage parasites to 2.5 μM CLT for a period of 12 hr caused a delay in development in a fraction of parasites that reverted to normal after drug removal; 24-hr exposure to the same concentration caused total destruction of parasites and parasitized cells. Chloroquine antagonized the effects of CLT whereas mefloquine was synergistic. The present study suggests that CLT holds much promise as an antimalarial agent and that it is suitable for a clinical study in P. falciparum malaria.

In the course of an investigation on the homeostasis of human red cells infected in vitro with the human malaria parasite Plasmodium falciparum, we found that clotrimazole (CLT), an imidazole derivative used as an antifungal agent (1), had a powerful growth-inhibiting effect on the parasite. The vast clinical experience, proven tolerance, and unique lack of acquired fungal resistance to CLT prompted a detailed investigation of its antimalarial effects in cultures of P. falciparum to assess its clinical potential. In this study, we report the in vitro effects of CLT on P. falciparum cultures by assessing the time- and concentration-dependent changes in parasite growth, parasite morphology, stage-specific development, and parasite replication.

Materials and Methods

Cultures. Five different laboratory strains of P. falciparum [A4 (2), W2, NF54, HB3, and FCR] were cultured in human erythrocytes by standard methods (3) under a low oxygen atmosphere. The culture medium was RPMI 1640, supplemented with 40 mM Hepes, 25 mg/liter gentamicin sulfate, 10 mM d-glucose, 2 mM glutamine, and either 8.5% (vol/vol) pooled human serum (A4 clone), or 10% heat-inactivated plasma (strains W2, NF54, HB3, and FCR). Culture media, with or without CLT, were changed daily, unless otherwise indicated. Parasites were synchronized at the ring stage with D-sorbitol (4) for all experiments. Initial parasitemias varied between 2% and 7% for the different experiments.

Assessment of Parasite Morphology, Stage-Specific Development, and Replication. Parasite morphology, stage-specific development, and replication were evaluated in cultures by microscopic inspection of Giemsa-stained thin blood smears. Smears from drug-free cultures were always used as controls. Stage-specific development was assessed by examining a minimum of 600 parasitized cells on each smear, for differential counting of rings, trophozoites, schizonts, and pyknotic forms whose developmental stage could not be established. The fraction of each group was calculated as a percentage of the total parasitized cells. Parasitemia was measured by counting 2,000 red cells and is reported as the percent of parasitized erythrocytes. Morphologically normal and abnormal parasites were included in the measurements. Parasite replication was evaluated from successive parasitemia measurements at the beginning of each new asexual cycle.

Growth Inhibition Assay. P. falciparum growth was assessed by measuring the incorporation of the radiolabeled nucleic acid precursor [3H]hypoxanthine (5) in media containing either human pooled serum or heat-inactivated plasma. All assays were started with ring stage-synchronized cultures at 2–4% parasitemia and processed as reported (6–8). Aliquots of stock solutions of CLT in DMSO were placed in the wells of flat-bottomed cell culture plates (Nunc), under sterile conditions, to render final concentrations of 0.1–5 μM CLT after the addition of either control or parasitized red cell suspensions in culture medium. DMSO concentrations did not exceed 0.2% (vol/vol) in cultures. The plates were placed in a gas-tight box that was flushed with a low oxygen gas mixture, sealed, and incubated at 37°C for about 16–20 hr. [3H]Hypoxanthine in culture medium (5–25 μCi/ml, final concentration) then was added to each well (10% vol/vol) and after a further 18- to 24-hr incubation, the cells were harvested and the cell-associated radioactivity was measured by scintillation counting. For the assessment of parasite growth in younger-ring-stage parasites of clone A4, [3H]hypoxanthine was added 15 min after parasite exposure to CLT and the incubation period was 20 hr. Suspensions of uninfected erythrocytes similarly treated were used for background subtraction. Parasite morphology was determined by Giemsa-stain smears immediately before the start of [3H]hypoxanthine uptake and at the end of it. For testing drug combinations, ring-stage parasites were exposed to increasing concentrations of either chloroquine or mefloquine, at a constant CLT concentration, and vice versa. After 18-hr incubation, [3H]hypoxanthine incorporation was measured as described. Isobole analysis was performed according to Berenbaum (9).

Chemicals and Solutions. Chemicals were from Sigma except for mefloquine (kind gift of Alan F. Cowman, The Walter and Eliza

Abbreviation. CLT, clotrimazole.

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§1734 solely to indicate this fact.
Table 1. In vitro inhibition of P. falciparum growth by CLT

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serum IC₅₀ ± SE (µM)</th>
<th>Plasma IC₅₀ ± SE (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>1.09 ± 0.026 (8.2)</td>
<td>—</td>
</tr>
<tr>
<td>W2</td>
<td>0.553 ± 0.037 (3.6)</td>
<td>0.267 ± 0.004 (5.3)</td>
</tr>
<tr>
<td>FCR3</td>
<td>0.520 ± 0.022 (3.9)</td>
<td>0.210 ± 0.016 (2.8)</td>
</tr>
<tr>
<td>HB3</td>
<td>0.738 ± 0.045 (5.5)</td>
<td>0.431 ± 0.008 (6.8)</td>
</tr>
<tr>
<td>NF54</td>
<td>—</td>
<td>0.245 ± 0.014 (3.1)</td>
</tr>
</tbody>
</table>

Concentrations of CLT for 50% inhibition of growth (IC₅₀), and slopes (b) of concentration-response curves for five different strains of P. falciparum. Kinetic (IC₅₀) and statistical (SE) parameters were obtained by least-mean squares fits to the equation \( y = aM/(bM + x^2) \), where \( a \) is the measured \([3H]\)hypoxanthine incorporation, expressed as a percent of that in CLT-free controls, at each CLT concentration (x); \( a \) is the IC₅₀, and b is the slope of the growth-inhibition curve.

Results

CLT Inhibits P. falciparum Growth In Vitro. Parasite growth was assessed in all strains by measuring the incorporation of \([3H]\)hypoxanthine in growing trophozoites starting at 20- to 24-hr postinvasion. The effects of CLT were tested on five P. falciparum strains: two chloroquine-resistant (W2 and A4), two chloroquine-sensitive (NF54 and HB3), and one mildly chloroquine-resistant (FCR3). CLT inhibited parasite growth with IC₅₀ values between 0.2 and 1.1 µM, irrespective of the chloroquine sensitivity of the strain (Table 1). The slopes (b) varied from 2.8 to 8.2, suggesting involvement of complex processes in the antimarial action of the drug. \([3H]\)Hypoxanthine incorporation measurements in younger, ring-stage parasites of clone A4 showed a marginal increase in IC₅₀ (1.24 ± 0.05 µM) with respect to older trophozoites of the same clone (1.09 ± 0.03 µM). The difference between the slopes of growth-inhibition curves for older trophozoites (b = 8.2) and ring-stage parasites (b = 1.9) (Table 1, Fig. 1) may be the result of drug cumulative damage in older trophozoites or reflect their higher vulnerability to CLT. Comparison between IC₅₀ values obtained in medium with plasma and medium with serum revealed systematically lower values in medium with plasma (Table 1).

CLT Alters P. falciparum Morphology and Interferes with Parasite Development and Replication. The effects of CLT on parasite morphology, development, and replication were evaluated in synchronized cultures of three strains of P. falciparum (A4, NF54, and W2). The effects were similar in all strains, at CLT concentrations characteristic for each strain (Table 1). Figs. 2–4 illustrate the effects observed in clone A4. CLT caused a marked decline in parasitemia at concentrations of 2 µM and above (Fig. 2). At these concentrations, there was complete inhibition of parasite replication as well as degeneration and destruction of parasites and their host cells, within a single asexual cycle. The effect on parasitemia was evident after 48-hr incubation when there was a sharp concentration-dependent transition between 1 and 2 µM CLT (Fig. 2). Lower concentrations of CLT (0.5 and 1 µM) caused a mild decrease in parasitemia relative to controls that was apparent only at 72 and 96 hr.

CLT inhibited parasite development and altered normal morphology in all strains (Fig. 3). Pyknosis and vacuolation were the most prominent morphological changes. Fig. 4 shows the effects of CLT on parasite development (A4 clone). After 24-hr incu-
bation, most parasites exposed to CLT concentrations above 2 μM were morphologically abnormal, more than half were shrunk, reduced to a pyknotic mass, and a significant fraction (17%) failed to develop beyond the ring-trophozoite stage (Figs. 3 and 4). After 48 hr, only pyknotic forms were present (Fig. 4). Incubation with 2 μM CLT showed that by 24 hr most parasites had developed from ring forms to more mature trophozoites except for a fraction of rings (10%) that showed delayed or arrested development. However, the trophozoites were smaller than controls and morphologically abnormal. Most of them failed to develop to schizonts, indicating that development was arrested mainly at mid-trophozoite stage. The effects with 2 μM CLT were more dramatic by 48 hr postincubation, by which time most parasites were markedly pyknotic; there was no progression to a second asexual cycle. Lower CLT concentrations (0.5 and 1 μM) caused only a slight but consistent retardation of parasite development.

Effects of Transient in Vitro Exposure of P. falciparum to CLT. The effects of transient in vitro exposure to CLT were investigated in synchronized cultures (A4 clone) to assess the potential effectiveness of short-term treatments. Ring-stage and late trophozoite-stage cultures were exposed for 12 hr to both 1.5 and 2.5 μM CLT after which the cells were washed and cultured in drug-free media. Parasitemia and stage-specific development were assessed before and after 12-hr incubation, thereafter, every 24 hr, for a total of 72 hr (trophozoites) or 96 hr (rings), and compared with drug-free controls. Ring trophozoites were not susceptible to a 12-hr exposure to CLT concentrations of 1.5 and 2.5 μM. The only documented abnormality was a transient delay in development in a fraction of parasites which remained in the ring stage (R) (controls: 28% R; 1.5 μM CLT: 50% R; 2.5 μM: 73% R). Normal development resumed after the cells were washed and incubated in a drug-free medium. By contrast, late trophozoite-stage parasites were susceptible to 12-hr treatment with 2.5 μM CLT (there was no effect with 1.5 μM). After 12-hr incubation, coincident with the beginning of the second parasite asexual cycle, there was a large proportion (~30%) of pyknotic forms that did not appear to recover after removal of the drug; a significant fraction of schizonts were slightly shrunken. In the second asexual cycle, parasitemia was 70% lower than in controls, indicating a marked inhibition of parasite replication. Nevertheless, normal development resumed after removal of CLT, as shown by the increase in parasitemia in the subsequent cycle. These results suggest that the effect of CLT on ring trophozoites is cytostatic rather than cytotoxic, and that the cytotoxic effect is cumulative with time.

A longer incubation period of 24 hr with 2.5 μM CLT was tested in synchronized ring-stage parasites (A4 clone). After 24-hr incubation, CLT was removed and the incubation continued for a further 96 hr. No parasites could be found in culture at 72 hr or later, indicating parasite death and disintegration of the infected cells.
Combined Effects of CLT with Other Antimalarials. Combinations of CLT with mefloquine or chloroquine were tested against a \textit{P. falciparum} chloroquine-sensitive (HB3) strain and a chloroquine-resistant (W2) strain. Isobole analysis (9) of the results with strain HB3 (Fig. 5) demonstrated a substantial antagonism between CLT and chloroquine and a significant synergy between CLT and mefloquine. CLT antagonism with chloroquine also was obtained with strain W2; however, in this strain, the data on the interaction of CLT with mefloquine were inconclusive.

Discussion
This study demonstrates a potent antimalarial effect of CLT on five different strains of \textit{P. falciparum in vitro}. CLT had time- and concentration-dependent effects with a broad stage specificity of action. However, the drug was more effective at the late-trophozoite and schizont stages than at the ring-trophozoite stage. Parasite growth was inhibited with an IC\textsubscript{50} \(\approx 1\) \(\mu\)M, as assessed both morphologically and by the incorporation of \(^{[3]}\text{H}\)hypoxanthine. The transient effects observed after a 12-hr exposure of ring forms to 2.5 \(\mu\)M CLT suggest that at this concentration CLT has a cytostatic effect on younger asexual stages. For cytotoxic effects to develop, a longer parasite exposure to the drug is necessary. The total disappearance of parasites and disintegration of parasitized cells observed after a 24-hr exposure of a ring-stage culture to 2.5 \(\mu\)M CLT suggests that CLT may be potentially effective in relatively short-term treatments.

Given orally, a substantial fraction of CLT is absorbed; it diffuses readily into all tissues and is rapidly metabolized (10, 11). Absorption is more efficient when given in oil solution than in tablets (12). Infants and children have shown good absorption and delayed excretion, compared with adults (13). Pediatric and adult administrations of high doses of CLT (100 mg/kg per day in four six-hourly oral doses) for treatment of mycotic infections have been well tolerated without significant clinical, hematological, or biochemical side effects (14). Pharmacokinetic information on CLT in humans indicates that, in healthy subjects after a single oral dose of 1 g CLT (\(\approx 15\) mg/kg body weight), plasma levels reach mean peak concentrations of about 2 \(\mu\)M within 2–4 hr of administration (15, 16). Similar and even higher CLT levels have been found by others after oral administration of comparable doses (12, 17). Most of CLT in plasma is bound to lipoproteins, 4\% is bound to albumin, and less than 1\% appears to be free (15). However, CLT partition into red cells \textit{in vivo} is sufficient to inhibit up to 92\% of the intermediate conductance K\textsuperscript{+} (Gardos) channel (16), another CLT target (18), with an IC\textsubscript{50} of 1 \(\mu\)M in whole blood. This finding suggests that binding to plasma proteins is loose and of low affinity relative to targets with micromolar dissociation constants. Intracellular targets with high CLT affinity may thus efficiently extract the drug from plasma components. Moreover, persistence of Gardos channel inhibition after discontinuation of CLT administration, sometimes even when plasma levels had become undetectable (16), suggests that attention to plasma levels alone may lead to
Values on both axis are IC₅₀. The dashed line represents a simple additive combinations of both CLT with chloroquine (documented here between CLT and chloroquine indicates that antagonism of drug resistance and to find combination partners that potentiate the individual antimalarial effects (21–23). The mechanism of these actions has been variously attributed to its inhibitory effect on cytochrome P450-dependent enzymes (38), sarcoplasmic reticulum Ca²⁺ pump (39), capacitative Ca²⁺ channels (32, 40, 41), and the intermediate-conductance Gardos channel (18). The diversity of reported actions suggests that CLT may act independently on a surprising variety of targets. From the effects of CLT in other biological systems a number of possible modes of action may be considered. The fungicidal action, shared with other imidazoles, results mainly from inhibition of cytochrome P450-dependent enzyme systems (38, 42, 43). Alternative mechanisms shown for some imidazoles have been related to inhibition of catalase and peroxidase activities (44), of potential significance because the malaria parasite is inherently under considerable oxidant stress (45, 46), and also because various pro-oxidants have been shown to have antimalarial activity (45, 47, 48). Oxygen enhances the antimalarial activity of some imidazoles; the imidazole drug ketoconazole has a greater antimalarial effect in older red cells, which are more susceptible to oxidative stress, than in younger red cells (31), effects that may be shared by CLT. The antiproliferative action of CLT results mainly from inhibition of the sarcoplasmic reticulum Ca²⁺ pump and capacitative Ca²⁺ channels (32, 39, 49), which induces depletion of intracellular Ca²⁺ stores. Store depletion activates protein kinase R, causing phosphorylation of the eukaryotic translation initiation factor 2α and inhibition of protein synthesis (49).

While this work was in progress, Saliba and Kirk (50) reported the effect of CLT on the viability of a P. falciparum strain (FAF6), monitored by measuring the parasite-derived lactate dehydrogenase produced over a 48-hr period. They also showed that the imidazole group plays a significant role in the antimalarial activity of CLT. The IC₅₀ for the effect of CLT on viability was found to be similar to the IC₅₀ values reported here for hypoxanthine incorporation. Thus, CLT has been shown to be effective in six different P. falciparum strains, irrespective of their chloroquine sensitivity.

The potent in vitro antimalarial activity of CLT on chloroquine-sensitive and chloroquine-resistant strains of P. falciparum demonstrated here, the lower toxicity of CLT relative to other imidazoles, the failure to develop fungal drug resistance to CLT, the adequate plasma levels obtained after oral administration of the drug, the short elimination time, the high partition in erythrocyte targets, and the proven clinical safety and tolerance in infants and adults suggest that CLT holds much promise as an effective antimalarial agent and new structural lead, and that it is suitable for a pilot clinical study in uncomplicated P. falciparum malaria.

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