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 antimycotic 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 (IC_{50}), assessed by parasite incorporation of [^{3}H]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 ($\approx$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 [^{3}H]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. [^{3}H]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, [^{3}H]hypoxanthine was added 15 min after parasite exposure to CLT and the incorporation 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 [^{3}H]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, [^{3}H]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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Table 1. *In vitro* inhibition of *P. falciparum* growth by CLT

Strain	$IC_{50} \pm SE$ (μM) (*b*)	
	Serum	Plasma
A4	1.09 ± 0.026 (8.2)	—
W2	0.553 ± 0.037 (3.6)	0.267 ± 0.004 (5.3)
FCR3	0.530 ± 0.022 (3.9)	0.210 ± 0.016 (2.8)
HB3	0.738 ± 0.045 (5.5)	0.431 ± 0.008 (6.8)
NF54	—	0.245 ± 0.014 (3.1)

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

Hall Institute, Melbourne, Australia, to H.G.). [8-^{3}H]Hypoxanthine was from Amersham Life Sciences. A stock solution of CLT (25 mM) was prepared in DMSO and further diluted in DMSO or culture medium as needed. These concentrations had no effect on parasite growth when tested in control cultures. Stock solutions of chloroquine were prepared in distilled water. Mefloquine was dissolved in ethanol and final dilutions were made in complete culture medium.

Results

CLT Inhibits *P. falciparum* Growth *in Vitro*. Parasite growth was assessed in all strains by measuring the incorporation of [^{3}H]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 IC_{50} 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 antimalarial action of the drug. [^{3}H]Hypoxanthine incorporation measurements in younger, ring-stage parasites of clone A4 showed a marginal increase in IC_{50} (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 IC_{50} 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-

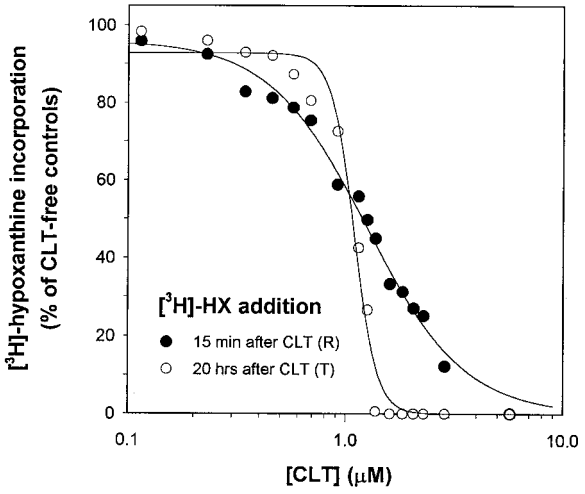

Fig. 1. Effect of CLT on *P. falciparum* growth at two different stages of parasite development (clone A4). CLT was added to ring-stage synchronized cultures. [^{3}H]Hypoxanthine was added either 15 min (ring trophozoites, R, ●) or 20 hr (mature-trophozoite stage, T, ○) after CLT. Parameter values for the concentration-response curves were determined as described in Table 1. The results were: young, ring-stage trophozoites, $IC_{50} = 1.24 \pm 0.05$ μM, $b = 1.9 \pm 0.1$; mature trophozoites, $IC_{50} = 1.09 \pm 0.03$ μM, $b = 8.2 \pm 1.3$.

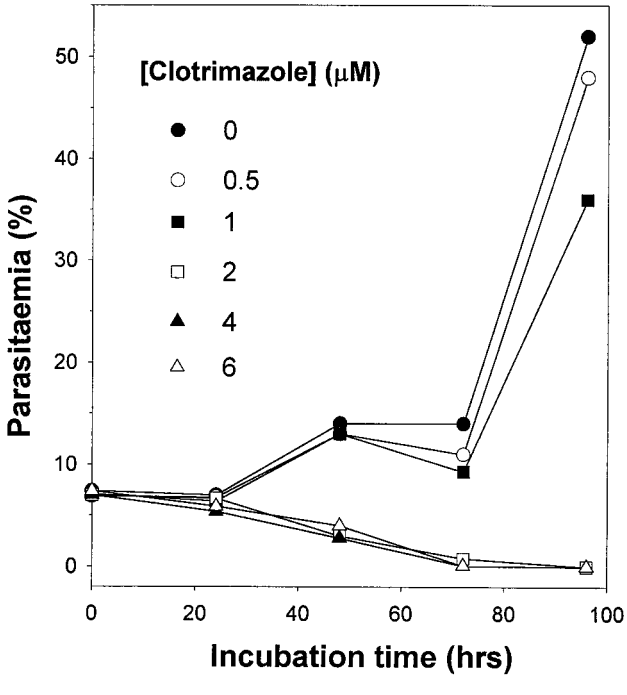

Fig. 2. Effect of CLT on *P. falciparum* parasitaemia *in vitro* (A4 clone). Shown are the time- and concentration-dependent effects of continuous incubation with CLT on parasitemia of *P. falciparum* cultures. All cultures were started with synchronized ring-stage parasites. Parasitemia was measured at the beginning of incubation (0 hr) and every 24 hr thereafter. Because parasite viability was not discernible from morphological criteria alone, morphologically normal and abnormal parasites were included in the measurements. Therefore, "viable parasitemia" may be lower than that shown. Parasitemias in drug-free cultures and cultures with CLT concentrations of 0.5 and 1 μM were diluted with fresh red cells at 72 hr; the values shown at 96 hr were corrected for this dilution.

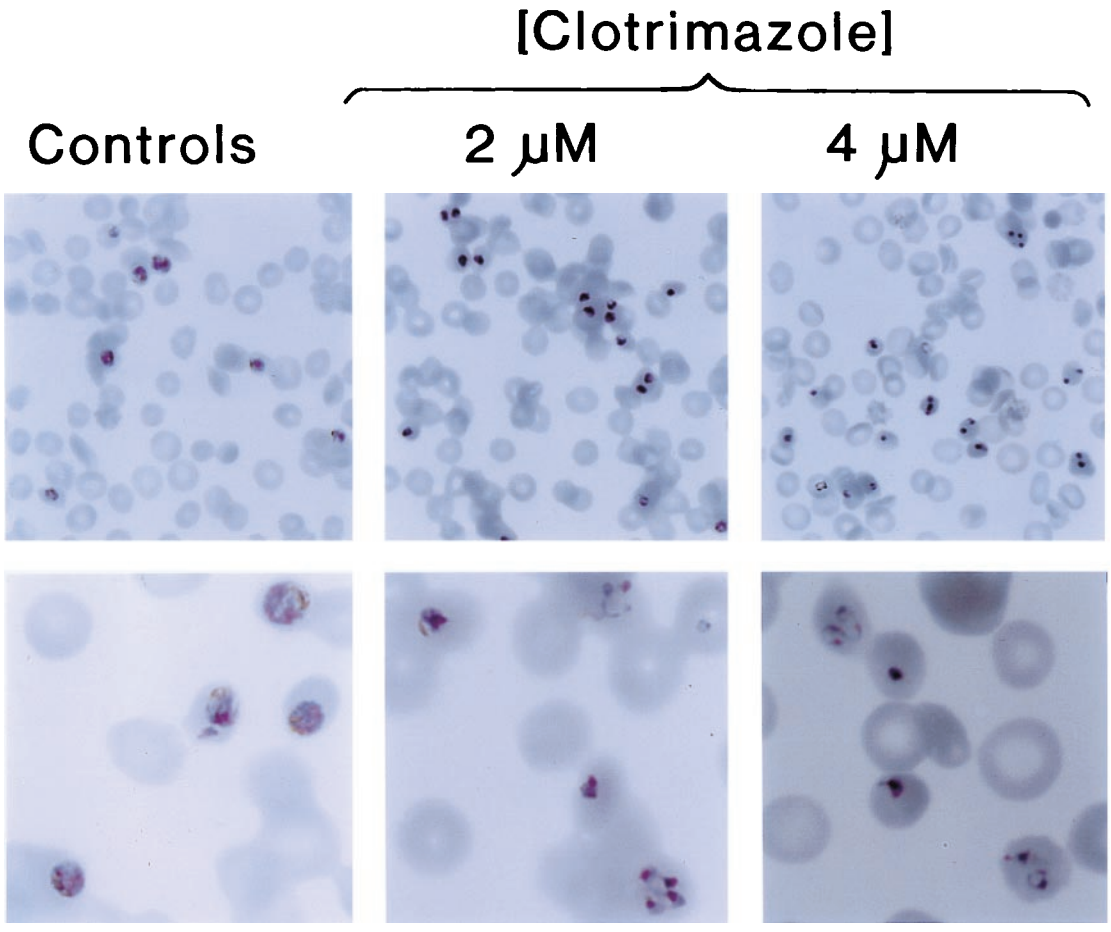

Fig. 3. Morphological appearance of *P. falciparum* cultures (A4 clone) after 24-hr incubation with CLT. Parasites were synchronized at the ring stage. Shown is the morphology of Giemsa-stained thin blood smears from drug-free control cultures (*Left*) and cultures incubated with either 2 μM CLT (*Middle*) or 4 μM CLT (*Right*) for 24 hr. The views are shown at two different magnifications: $\times$400 (*Upper*) and $\times$1,000 (*Lower*). Note parasite pyknotic changes and prevalence of ring forms in cultures treated with CLT.

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 ($\approx$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.

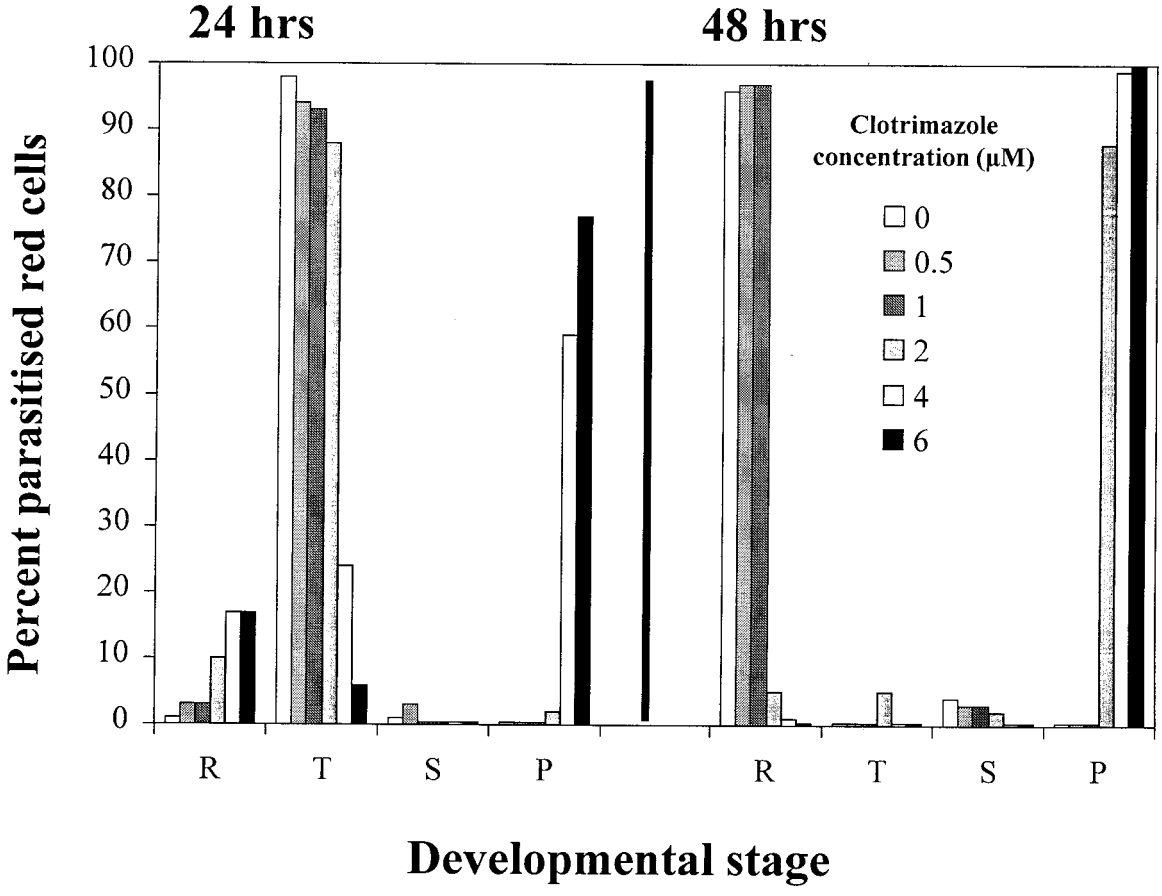

Fig. 4. Time- and concentration-dependent effects of CLT on *P. falciparum* stage-specific development *in vitro* (A4 clone). Ring-stage synchronized cultures were continuously exposed to CLT concentrations of 0.5, 1, 2, 4, and 6 μM. Parasite morphology and stage-specific development were assessed at the beginning of incubation (0 hr) and at 24 and 48 hr. The parasites were classified in four groups: rings (R), late trophozoites (T), schizonts (S), and pyknotic parasites whose stage of development could not be established (P). Parasite differential counts are reported as the percentage of total parasitized cells. The six bars within each group correspond to different CLT concentrations (left to right): 0, 0.5, 1, 2, 4, and 6 μM.

Combined Effects of CLT with Other Antimalarials. Combinations of CLT with mefloquine or chloroquine were tested against a *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 *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_{50} $\approx$1 μM, as assessed both morphologically and by the incorporation of [^{3}H]hypoxanthine. The transient effects observed after a 12-hr exposure of ring forms to 2.5 μM 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 μM 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 μM 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 *in vivo* is sufficient to inhibit up to 92% of the intermediate conductance K^+ (Gardos) channel (16), another CLT target (18), with an IC_{50} of 1 μM 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

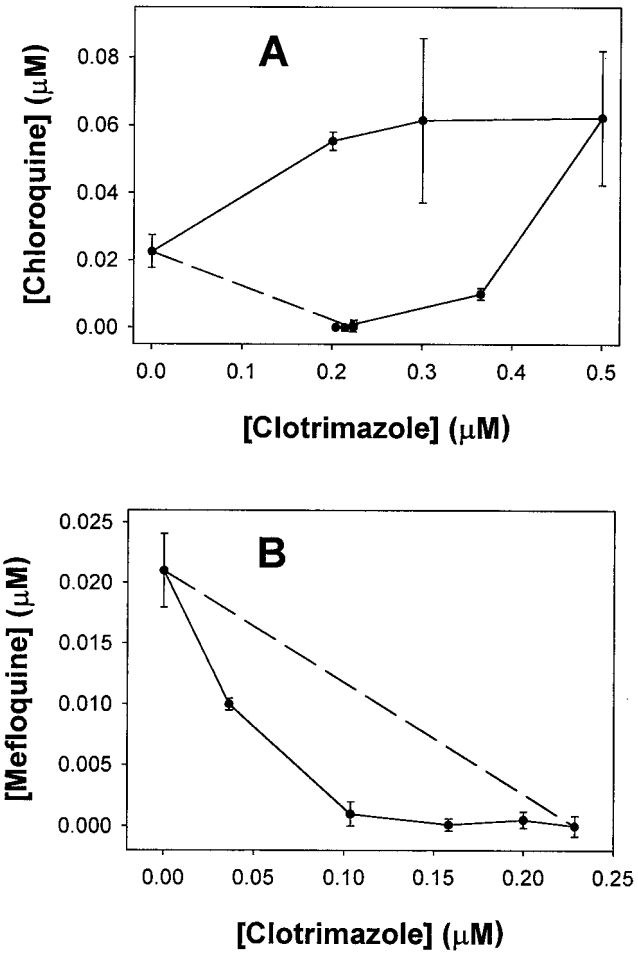

Fig. 5. Effects of combinations of CLT with chloroquine or mefloquine on *P. falciparum* growth *in vitro* (HB3 strain). Isobolograms show the effect of combinations of both CLT with chloroquine (*A*) and CLT with mefloquine (*B*). Values on both axis are IC_{50}. The dashed line represents a simple additive effect. Error bars represent the SE of three replicates. Where error bars are not seen, they are smaller than the symbol size.

underestimation of the potential effectiveness of CLT *in vivo*. The short elimination half-life of CLT (≈3 hr) (15) suggests that multiple dosing over a period of four parasite cycles may be necessary for optimal cytotoxic antimalarial effects *in vivo* (19).

In contrast with other antifungal agents, development of fungal resistance to CLT has not been observed *in vivo* (14, 20) and has been extremely difficult to elicit *in vitro.* Attempts to induce the emergence of fungal mutants with enhanced resistance to CLT, after multiple subcultures, have failed to demonstrate significant changes in the drug's IC_{50} in a variety of strains (14). Whether or not this behavior would apply to malaria parasites remains to be determined. CLT's short elimination time and the steep slope of the concentration-response curve shown here for trophozoites (Fig. 1) are additional features associated with a low probability of development of resistance (21).

The main aims of combined therapy are to delay development of drug resistance and to find combination partners that potentiate the individual antimalarial effects (21–23). The antagonism documented here between CLT and chloroquine indicates that this combination should be avoided. However, further research is required to confirm unequivocally our preliminary results on the synergistic effect between CLT and mefloquine. These results are reminiscent of the interactions between artemisin, or arteether, with chloroquine and mefloquine (24–27).

The antimalarial effects of imidazole agents have been known since the early eighties (28–31). However, the mechanism of the antimalarial activity of CLT is unknown. CLT has been shown to have a multiplicity of effects on cell proliferation (32), steroid metabolism (33–35), and sickle cell dehydration (36, 37). The mechanism of these actions has been variously attributed to its inhibitory effect on cytochrome P450-dependent enzymes (38), sarcoplasmic reticulum Ca^{2+} pump (39), capacitative Ca^{2+} 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 other 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 Ca^{2+} pump and capacitative Ca^{2+} channels (32, 39, 49), which induces depletion of intracellular Ca^{2+} 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 IC_{50} for the effect of CLT on viability was found to be similar to the IC_{50} 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.

We thank Chris Newbold and the members of the Molecular Parasitology Group, Institute of Molecular Medicine, especially Bob Pinches, for their invaluable help and support and Britta Urban for providing us with a *P. falciparum* clone (A4) free of mycoplasma contamination. This work was supported by funds from The Wellcome Trust, the Department of Physiology, University of Cambridge, and the Sir Halley Stewart Trust and the Leverhulme Trust for B.C.E.

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