Clotrimazole Binds to Heme and Enhances Heme-dependent Hemolysis

PROPOSED ANTIMALARIAL MECHANISM OF CLOTRIMAZOLE*

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Nguyen Tien Huy, Kaeko Kamei‡, Takushi Yamamoto, Yoshiro Kondo, Kenji Kanaori, Ryo Takano, Kunihiko Tajima, and Saburo Hara

From the Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan

Two recent studies have demonstrated that clotrimazole, a potent antifungal agent, inhibits the growth of chloroquine-resistant strains of the malaria parasite, Plasmodium falciparum, in vitro. We explored the mechanism of antimalarial activity of clotrimazole in relation to hemoglobin catabolism in the malaria parasite. Because free heme produced from hemoglobin catabolism is highly toxic to the malaria parasite, the parasite protects itself by polymerizing heme into insoluble non-toxic hemozoin or by decomposing heme coupled to reduced glutathione. We have shown that clotrimazole has a high binding affinity for heme in aqueous 40% dimethyl sulfoxide solution (association equilibrium constant: $K_a = 6.54 \times 10^8 \text{M}^{-1}$). Even in water, clotrimazole formed a stable and soluble complex with heme and suppressed its aggregation. The results of optical absorption spectroscopy and electron spin resonance spectroscopy revealed that the heme-clotrimazole complex assumes a ferric low spin state ($S = \frac{1}{2}$), having two nitrogenous ligands derived from the imidazole moieties of two clotrimazole molecules. Furthermore, we found that the formation of heme-clotrimazole complexes protects heme from degradation by reduced glutathione, and the complex damages the cell membrane more than free heme. The results described herein indicate that the antimalarial activity of clotrimazole might be due to a disturbance of hemoglobin catabolism in the malaria parasite.

Malaria has become a key global threat due to quickly spreading resistance to quinoline-based antimalarial drugs such as quinine, chloroquine (CQ), and mefloquine (1). Furthermore, artemisinin-resistant strains of Plasmodium falciparum have been developed in the laboratory (2). Therefore, there has been extensive research into a new series of antimalarial drugs. The antifungal agent clotrimazole (CLT) (Fig. 1) has developed a means of detoxifying heme through polymerization to non-toxic, insoluble hemozoin (8) or by degradation with GSH (9–11), which is found at millimolar concentrations in red blood cells and parasite compartments (12, 13). About 30–50% of free heme is detoxified by polymerization at the trophozoite stage (10, 14, 15), and the remainder is detoxified by GSH-dependent degradation. The two detoxification processes of free heme are initiated by heme histidine-rich protein (HRP) 2 and heme-GSH complex formation, respectively. Antimalarials such as quinine and CQ also bind to free heme and inhibit its degradation. Furthermore, the imidazole moiety of CLT behaves as an axial ligand, binding free heme. We therefore considered that CLT exerts antimalarial activity by forming complexes with heme, similar to the heme-binding antimalarials, CQ and quinine.

In this study, the coordination reaction between CLT and heme was investigated by optical absorption spectroscopy and electron spin resonance (ESR) spectroscopy. The structure of the heme-CLT complex was characterized based on spectroscopic evidence. Furthermore, we compared the effects of CLT and CQ on GSH-dependent heme degradation and heme-induced hemolysis, and we propose a mechanism of antimalarial CLT action.

EXPERIMENTAL PROCEDURES

Materials—GSH, CLT, CQ, imidazole, and hemin (heme) were from Sigma. Mesoheme was from Porphyrin Products Inc. (Logan, UT). Human blood was drawn from healthy volunteers. Dimethyl sulfoxide (Me$_2$SO) was purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of the highest commercially available grade.

Heme Preparation—At the start of each experiment, a stock heme solution was prepared by dissolving hemin chloride in 20 mM NaOH and then removing the remaining hemin crystals by centrifugation for 10 min at 15,000 rpm. The heme concentration was estimated from absorbance at 385 nm ($e_{max} = 55,400$) in 100 mM NaOH (16) and adjusted to 1.0 mM. This stock reagent was stored in the dark on ice and used within 24 h.

Absorption Spectra—All absorption spectra were recorded on a Hitachi U-3300 double-beam spectrophotometer (Tokyo, Japan) using a methyl sulfoxide solution (association equilibrium constant: $K_a = 6.54 \times 10^8 \text{M}^{-1}$). Even in water, clotrimazole formed a stable and soluble complex with heme and suppressed its aggregation. The results of optical absorption spectroscopy and electron spin resonance spectroscopy revealed that the heme-clotrimazole complex assumes a ferric low spin state ($S = \frac{1}{2}$), having two nitrogenous ligands derived from the imidazole moieties of two clotrimazole molecules. Furthermore, we found that the formation of heme-clotrimazole complexes protects heme from degradation by reduced glutathione, and the complex damages the cell membrane more than free heme. The results described herein indicate that the antimalarial activity of clotrimazole might be due to a disturbance of hemoglobin catabolism in the malaria parasite.

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Absorption Spectra—All absorption spectra were recorded on a Hitachi U-3300 double-beam spectrophotometer (Tokyo, Japan) using a
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1.0-cm light-path quartz cuvette at 23 °C. A solution of 17 μM heme in 40% Me2SO and 20 mM HEPES buffer (pH 7.4) revealed Soret at 401 nm and Q band absorption maxima at 493 and 616 nm, and the ratio of absorption of the Soret (401 nm) and Q band (616 nm) was 28.72, indicating that the heme in the present system exists as a monomeric mode (17–19). The optical absorption spectra of the heme-CLT and heme-imidazole complexes were recorded 5 min after adding CLT (final concentration, 100 μM) and imidazole (final concentration, 500 μM) to heme (final concentration, 17 μM) in 40% Me2SO buffered with 20 mM HEPES (pH 7.4). The total volume of the reaction mixture was 1.0 ml.

Spectrometric Titration—Differential absorption spectra were measured on a Hitachi U-3300 spectrophotometer as follows. The drug, CLT or CQ, was added sequentially to a sample cuvette containing heme solution. The reference compartment held two cuvettes, one containing an identical heme solution aliquot to which a buffer other than the drug was added and the other containing a solution without heme and the same amount of the drug.

In the case of CLT titration, both the sample cuvette and the first reference cuvette contained 17 μM heme in 40% Me2SO buffered by 20 mM HEPES (pH 7.4), and the second reference cuvette contained the same solution without heme. Increasing amounts of CLT (0–105.6 μM in 6.6 μM increments) in Me2SO were titrated with the contents of the sample cuvette and the second reference cuvette, in which the total volume of the reaction mixture was maintained at 1.0 ml.

Before adding CQ, the sample cuvette and the first reference cuvette contained 5 μM heme in 40% Me2SO buffered by 20 mM HEPES (pH 7.4), and the second reference cuvette contained the same solution without heme. During the titration of heme-CQ complex formation, increasing amounts of CQ (0–36 μM in 4 μM increments) were added to both the sample and the second reference cuvettes, where the total volume of the reaction mixture was 1.0 ml during the titration. All differential spectra were recorded at wavelengths between 350 and 700 nm, and the concentrations of heme-CLT and heme-CQ complexes were evaluated based on absorbance at 416 and 401 nm, respectively.

The binding mode of CLT and CQ to heme was analyzed in terms of Hill (20, 21) and Scatchard (22, 23) plots. The equilibrium association constants for the formation of heme-CLT and heme-CQ complexes, as well as the number of ligands that bind to heme, were calculated from Hill plots using Eq. 1.

\[ H + nL \rightarrow H(L)_n \]  

(Eq. 1)

and analyzed using the standard equation (Eq. 2),

\[ \log \frac{A - A_0}{A_0 - A} = \log K_c + n \log[L] \]  

(Eq. 2)

where \( A \), \( A_0 \), and \( A \) are the absorbance of the initial, final, and mixed species, respectively; \( H \) represents heme; \( L \) is the ligand (CLT or CQ); \( n \) is the number of ligand molecules that bind to heme; and \( K_c \) is the equilibrium association constant of the heme-ligand complex.

ESR Measurements of Heme-CLT Complexes—ESR measurements were conducted for heme-CLT complexes that were formed in Me2SO at molar ratios of heme to CLT of 1:1, 1:1.5, 1:2, 1:4, and 1:8. After an overnight incubation at room temperature, the ESR spectrum of heme-CLT complex was recorded at 4.2 K by a JES-TE 300 spectrometer with 100-kHz field modulation. The integrated frequency counter monitored the microwave frequency of each measurement. The magnetic field strength was calibrated by hyperfine splitting of Mn(II) ion (8.69 militeslas/mT) in MnO powder. Powdered lithium-tetracyanoquinodimethane radical (g = 2.0025) was used as the standard g value. The ESR data were analyzed and calibrated using a Winrad system (Radical Research Inc., Tokyo). The typical conditions for ESR measurements were as follows: microwave power, 6.0 milliwatts; modulation magnitude, 0.68 mT; sweep range 30 mT to 500 mT; sweep time, 4 min; and time constant, 0.1 s.

GSH-dependent Heme Degradation and Its Inhibition by CLT or CQ—Heme degradation by GSH was monitored by measuring spectral change as described by Atamna and Ginsburg (9). Fresh GSH stock solution (200 mM) was prepared in isotonic standard buffer (50 mM sodium phosphate containing 68 mM NaCl, 4.8 mM KCl, and 1.2 mM MnSO4, pH 7.4) (24, 25). Heme (final concentration, 3 μM) and GSH (final concentration, 2 mM) were mixed in isotonic standard buffer (pH 7.4) and incubated at 37 °C. Absorption spectra (300–600 nm) were recorded at 6-min intervals after mixing, using the same spectrophotometer. The rate constant and \( t_1/2 \) of GSH-dependent heme degradation in the absence of CLT and CQ were calculated from the decrease of absorbance at 365 nm in terms of first-order reaction kinetics.

In the presence of CLT (6 μM) or CQ (6 μM), the time-dependent spectral measurements were obtained by the same procedure. CLT in Me2SO and CQ in HEPES buffer (200 mM, pH 7.4) were all prepared as 3 mM stock solutions. Heme (3 μM), GSH (2 mM), and either CLT or CQ (6 μM) were mixed in 0.2 M HEPES buffer (pH 7.4) and incubated at 37 °C. In the control experiment, Me2SO (final concentration, 0.2%) and either CLT and CQ on heme induced by heme were examined in 0.5% cell suspensions in isotonic standard buffer. Erythrocyte suspensions (0.6 ml) were shaken with various concentrations of heme (0–20 μM) and CLT or CQ (0, 1, 5, and 10 μM) at 37 °C for 150 min at 140 cycles/min. Intact erythrocytes were then removed by centrifugation at 1,500 × g for 3 min, and the amount of hemoglobin released from the hemolysed erythrocytes into the supernatant was determined by measuring absorbance at 578 nm (26). After the pelleted intact erythrocytes were lysed with water and centrifuged to obtain the supernatant, the hemoglobin content in intact erythrocytes was measured as absorbance at 578 nm. The degree of hemolysis was calculated from the ratio of hemoglobin content released from erythrocytes hemolyzed by heme to the total hemolysis of the erythrocytes (26).

When using heme-bound erythrocytes, 0.5% of red blood cells in isotonic standard buffer, pH 7.4, were incubated with 10 μM heme at room temperature for 10 min. The erythrocyte suspension was separated by centrifugation at 1,500 × g for 3 min, and the pellet was washed three times with isotonic standard buffer to remove free heme, thus providing heme-bound erythrocytes. A sample of 0.6 ml of a 0.5% suspension of heme-bound erythrocytes was prepared in isotonic standard buffer, Me2SO (1%), CLT (10 μM), CQ (10 μM), or GSH (2.5 mM) was then added, and the mixture was incubated at 37 °C for 2 h. The hemolysis degree was calculated from three such experiments.

RESULTS

Absorption Spectrum of Heme Complexed with CLT—Fig. 2, curve 2, shows Soret and Q band absorption of 401, 493, and 616 nm by heme (17 μM) in 40% Me2SO, which is characteristic of high spin ferric complexes assuming a five-coordinate structure (27, 28) with weak axial ligand such as water or chloride anion. When excess CLT (final concentration, 100 μM) was added to the mixture, the Soret shifted toward red wavelengths at 412 nm, and Q band absorption was evident at 536 and 560 nm, as shown in Fig. 2, curve 2. The observed spectrum was classified into a six-coordinate ferric complex having nitrogenous ligands at both axial positions. In fact, the spectroscopic properties coincided with those of similar solutions of heme and imidazole (Soret, 410 nm; Q bands, 535 and 560 nm) shown in Fig. 2, curve 3. Furthermore, heme-bis-imidazole complexes have similar spectra (29, 30), as summarized in Table I. These results support the notion that CLT, like imidazole, has affinity for the heme chromophore. It is likely that the imidazole moiety of CLT is the nitrogenous donor.

The heme-CLT complex in the absence of Me2SO gave a similar absorption spectrum, as indicated by a Soret band at 416 nm and Q bands at 533 and 564 nm (Table I). In contrast, the spectrum of the heme-imidazole complex in the absence of Me2SO included a broad peak at 433 nm (data not shown), which is derived mainly from the aggregated form of heme (31, 32).
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The heme-CLT complex, prepared in HEPES buffer in the absence of Me₂SO, was quite stable under ambient conditions. The values of the absorption maxima of the heme-CLT complex in Me₂SO and in HEPES buffer did not vary significantly, and heme precipitation was undetectable even in HEPES buffer. These results provide potent evidence of the ability of CLT to stabilize the monomeric form of heme and to inhibit the formation of polymeric heme in aqueous solution.

**Spectrophotometric Heme Titrations**—To characterize the binding of heme with CLT, spectrophotometric heme titration was performed by measuring the differential spectra between heme and heme-CLT complex at various CLT concentrations. As described under “Experimental Procedures,” aqueous Me₂SO (40% v/v) buffered by 20 mM HEPES buffer, pH 7.4, was used because heme in this solution should form a monomer at concentrations up to 26.6 μM heme; curve 3, heme-CLT complex (mixture of 100 μM CLT and 17 μM heme). Left y axis, Soret region; right y axis, Q region.

The absorption spectra were measured in 40% Me₂SO buffered by 20 mM HEPES, pH 7.4, and recorded after a 5-min mixing of heme and ligand when no further spectral change was observed. Curve 1, heme alone; curve 2, heme-imidazole complex (mixture of 0.5 μM imidazole and 17 μM heme); curve 3, heme-CLT complex (mixture of 100 μM CLT and 17 μM heme). Left y axis, Soret region; right y axis, Q region.

**Fig. 2.** Absorption spectra of heme, heme-imidazole complex, and heme-CLT complex. Absorption spectra were measured in 40% Me₂SO buffered by 20 mM HEPES, pH 7.4, and recorded after a 5-min mixing of heme and ligand when no further spectral change was observed. Curve 1, heme alone; curve 2, heme-imidazole complex (mixture of 0.5 μM imidazole and 17 μM heme); curve 3, heme-CLT complex (mixture of 100 μM CLT and 17 μM heme). Left y axis, Soret region; right y axis, Q region.

Inhibition of GSH-dependent Heme Degradation by CLT—The absorption spectrum of heme in HEPES buffer (200 mM, pH 7.4) exhibited two Soret bands at 385 and 342 nm, representing the monomer and dimer form, respectively (Fig. 7) (35), whereas the spectrum of heme in 40% Me₂SO revealed only one Soret band at 401 nm, indicating the monomer form of heme. Continuous addition of CLT into a heme solution achieves conversion of the heme spectrum to a form with lower Soret molar absorption and a Soret maximum shifted to a longer wavelength. The absorption spectra changed as the CLT concentration increased, through isosbestic points at 409, 467, 514, and 585 nm, indicating that only two absorbing species are present in the reaction mixture. During heme-CQ complex formation, the spectrum change was accompanied by a significant decrease in the intensity of monomeric heme at the Soret and Q bands (Fig. 3B), as described by Egan et al. (19), indicating interaction between CQ and heme.

The effects of heme interactions with CLT and CQ on titration behavior were analyzed using Hill plots (20, 21) to determine the number of molecules bound to heme in aqueous Me₂SO. Hill plots of our binding data in Fig. 4 show heme-CLT complexes at 17 μM heme and heme-CQ complexes at 5 μM heme. The slopes of these linear graphs are 2 and 1, respectively, within experimental error.

The same data are presented in Scatchard plots in Fig. 5 (22, 23). The straight line indicates the absence of cooperative interaction between heme and CQ, whereas a curved graph is observed for heme-CLT complex, indicating cooperation and the involvement of non-identical interacting binding sites. The analysis using the Hill plot demonstrates that heme binds two CLT molecules with an association constant (K_a) of 6.54 × 10⁸ M⁻², whereas heme binds one molecule of CQ with a K_a of 1.71 × 10⁶ M⁻¹ in aqueous 40% Me₂SO at pH 7.4.

**ESR Spectrum of Heme-CLT Complex**—We further clarified the electronic and coordination structures of the heme-CLT complex by ESR spectroscopy. We recorded ESR spectra at 4.2 K for heme-CLT complexes prepared in Me₂SO, as described under “Experimental Procedures.” Before adding CLT, the observed ESR spectrum (Fig. 6, curve 1) of heme (0.5 mM) contained the line and g values (g = 6 and g = 2.0) typical of the ferric high spin (S = ½) species, taking five-coordinate geometry into account (33). A weak signal is always observed at about g = 4.3, which may be due to non-heme iron from decomposed heme (34) or impurities in the sample tube and Dewar assembly. On adding CLT (final concentration, 100 mM) to the reaction mixture, the ESR signal intensity of the high spin species decreased significantly with the concomitant formation of a new paramagnetic species with a distorted rhombic ESR line (Fig. 6, curves 2 and 3; g₁ = 1.46, g₂ = 2.66, and g₃ = 2.98), which was characteristic of ferric low spin complex (S = ½) having strong axial ligands at both axial positions. Upon the further addition of CLT, the molar ratio of CLT/heme reached 8, and the ESR signal of the new species was recorded exclusively, suggesting that CLT tended to shift the equilibrium to form the low spin ferric species (data not shown). The observed ESR shape of the low spin species line was quite similar to that recorded for a frozen mixture of heme-imidazole complex (data not shown). In addition, the observed g values of those complexes agreed well (e.g., protoheme-imidazole and protoheme-hexapeptide complex) (Table II). This provided experimental evidence that both complexes, heme-CLT and heme-imidazole complexes, have similar ligands at the axial positions.

Effect of CLT on Erythrocyte Hemolysis Induced by Heme—Hemolysis experiments were performed using fresh blood cells as described under “Experimental Procedures.” Up to only 2.5% hemolysis occurred in controls in which no heme was added. The hemolysis induced by the presence of heme was potentiated by CLT as well as by CQ (Fig. 9A), and the effects depended on the concentrations of both heme and the added agents. We also observed that CLT alone at up to 20 μM had no effect on hemolysis in the absence of heme (data not shown). Therefore, the enhancement of heme-dependent hemolysis of...
erythrocytes may be caused by the formation of heme-CLT complex. The amount of heme-dependent hemolysis enhanced by CQ was almost identical to that observed in a previous study (36).

The similar enhancement of heme-dependent hemolysis by CLT and CQ indicates that they have the same potential at high concentrations of heme (5–20 μM), as shown in Fig. 9A.

### Table I

| Sample          | Axial ligand | Additive | Solvent                  | Absorption | Reference |
|-----------------|--------------|----------|--------------------------|------------|-----------|
| Protoheme       | none         | none     | 40% Me₂SO³             | 401        | 493       | 616       | This work |
| Protoheme       | CLT          | none     | 40% Me₂SO³             | 412        | 536       | 560       | This work |
| Protoheme       | Imidazole    | none     | 40% Me₂SO³             | 410        | 535       | 560       | This work |
| Protoheme       | CLT          | none     | HEPES buffer               | 416        | 533       | 564       | This work |
| Protoheme       | CLT          | GSH      | HEPES buffer               | 416        | 533       | 564       | This work |
| Protoheme       | none         | CQ       | HEPES buffer               | 390        | 594       |           | This work |
| Protoheme       | none         | CQ + GSH | HEPES buffer               | 390        | 594       |           | This work |
| Protoheme       | N-methylimidazole | none | Phosphate buffer       | 413        | 535       | 564       | Ref. 29  |
| Protoheme       | Hexapeptide  | none     | Phosphate buffer       | 415        | 538       | 566       | Ref. 30  |

*⁴ 40% Me₂SO, 20 mM HEPES buffer at pH 7.4.
*⁵ 20 mM HEPES buffer, pH 7.4.
*⁶ pH 7.4.
*⁷ pH 7.6.

Fig. 3. Titration of the heme-CLT (A) and heme-CQ (B) interaction. Differential spectral titration of drugs with heme proceeded as described under “Experimental Procedures.” Concentration of CLT (A) was increased from 0 μM to 105.6 μM in 6.6 μM increments, and concentration of CQ (B) was increased from 0 μM to 36 μM in increments of 4 μM. Arrows indicate the effect of increasing the concentration of CLT and CQ.

Fig. 4. Hill plots of heme-CLT (○) and heme-CQ (●) association. The pH and the temperature were constant at pH 7.4 and 22 °C. The n values correspond to individual slopes. ○, heme-CLT association at 17 μM heme in which absorbance was monitored at 416 nm. The n and Kₐ values were 2.03 and 6.54 × 10⁵ M⁻¹, respectively. ●, heme-CQ interaction at 5 μM heme in which absorbance was monitored at 401 nm. The n and Kₐ values were 1.06 and 1.71 × 10⁵ M⁻¹, respectively.

Fig. 5. Scatchard plots of heme-CLT association (○) at 17 μM heme and of heme-CQ association (●) at 5 μM heme. Conditions were similar to those described in the legend to Fig. 4. α indicates [A₁ - A/A₁ - A₀]. The unit of α/[Ligand] is 1 × 10⁴ M⁻¹ for heme-CLT complex and 1 × 10⁶ M⁻¹ for heme-CQ complex.

However, at lower heme concentration (1.5 μM), CLT was more effective than CQ (Fig. 9B).

To understand this potency of CLT in greater detail, we treated erythrocytes with 10 μM heme followed by sedimentation and then washing the sample three times. The erythro...
The malaria parasite has developed processes to detoxify free heme that were not hemolyzed by heme but which had membranes bound to heme (9). These erythrocytes were incubated with CLT or CQ to determine the effect of these drugs on the susceptibility of erythrocytes to hemolysis. Fig. 10 shows that CLT but not CQ increased the hemolysis of heme-bound erythrocytes, whereas Me₆SO and GSH had no effect.

**DISCUSSION**

We propose a mechanism for the antimalarial activity of CLT based on the results of spectroscopic and physiological measurements. We investigated interactions occurring between heme and CLT using optical absorption and electron spin resonance. The observed optical and ESR parameters of the heme-CLT complex (Tables I and II) were compared with those of related complexes to assign the axial ligand of the complex. The ESR and optical absorption spectra indicated six-coordinate complexes having nitrogenous ligands at both axial positions of heme, as has frequently been observed for naturally occurring heme enzymes and synthetic iron porphyrin complexes (29, 30). For example, spectroscopic parameters of cytochrome b-559 indicate that the heme chromophore has two histidine moieties at both axial positions (29). In addition, the spectroscopic parameters of heme-CLT and heme-imidazole complexes agree closely. This suggests that the imidazole moiety of CLT is likely to be the axial ligand of the complex.

The crystal field parameters, rhombicity ($|R|/\mu$) and tetragonality ($\mu/\lambda$), of the heme-CLT complex and related complexes were calculated in terms of Bohan’s proposal (37). The calculated crystal field parameters of heme-CLT complexes agree well with those of heme-imidazole complexes, as summarized in Table II. On the basis of the results obtained from ESR and optical measurements, as well as from the crystal field calculation, we concluded that the axial ligand of the heme-CLT complex is therefore the nitrogenous donor derived from the imidazole moiety of CLT (Fig. 1).

The proposed coordination structure of heme-CLT complex is consistent with the results of the Hill plot in that the heme-CLT complex bears two molecules of CLT at both axial positions. The equilibrium association constant ($K_e$) for the formation of the heme-CLT complex was $6.54 \times 10^8$ M⁻². In comparison with the $K_e$ values of heme-imidazole derivatives (38), CLT is one of the strongest axial ligands for heme. We presently cannot explain why CLT has pronounced affinity for heme under our reaction conditions. However, the hydrophobic group of CLT, three aromatic rings with a chlorine atom, should stabilize axial ligation to heme. Taking into account the results of the Hill and Scatchard plots, we found that CLT forms a stable six-coordinate iron porphyrin complex with a high equilibrium association constant.

The malaria parasite has developed processes to detoxify free heme released as a result of hemoglobin catabolism. Some of the free heme (30–50%) is subsequently polymerized to non-toxic hemozoin (3) through the formation of complex with malarial HRP. The remaining non-polymerized free heme passes through the membrane of food vacuoles and also reaches the cytosol of the parasite (39–42), where free heme may be efficiently decomposed by reaction with GSH (10, 11). It is reported that oral CLT (single dose of 1 g) was absorbed rather efficiently, reaching a concentration in plasma of 2 μM within 2–4 h of administration (43) without serious side effects, and easily diffused into erythrocytes (43, 44). Therefore, CLT should be able to reach the parasite compartment, enabling production of stable heme-CLT complexes in both erythrocytes and parasites.

On the addition of GSH to heme, the Soret absorption blue-shifted to 365 nm due to the formation of heme-GSH intermediate complexes, and the GSH-dependent heme degradation proceeded quickly (Fig. 7). On the contrary, GSH caused no spectral variation in the heme-CLT complex (Table I), and the Soret absorption of the complex remained unchanged (Fig. 8). Thus, the axial-ligated CLT completely inhibited GSH-dependent heme degradation. The concentration of GSH in red blood cells and in the parasite compartment reaches millimolar levels (12, 13). Nevertheless, the high equilibrium association constant of CLT ($6.54 \times 10^8$ M⁻²) should allow the heme-CLT complex to exist in the erythrocyte and malaria parasite.

Furthermore, free heme, which would otherwise bind to malarial HRP initiating heme polymerization into non-toxic hemozoin, is also changed to heme-CLT complex through the axial ligand exchange reaction caused by minimal amounts of CLT in vitro. The ESR spectrum due to heme-CLT complex was recorded after a minimal addition of CLT to a solution of the heme-HRP model peptide (27 amino acids) complex in which two histidine residues are bound at the axial position (data not shown). This means that the heme, including heme bound to HRP, can be easily converted to heme-CLT complex in the presence of CLT. These findings indicate that the formation of heme-CLT complex considerably affects malarial defense systems against toxic free heme, heme polymerization by HRP, and heme decomposition by GSH.

Taking into account the molecular structure of CLT, the heme-CLT complex in which two CLT molecules bind to both axial positions is considered to be hydrophobic and bulky. The hydrophobic and bulky species tend to localize in the hydrophobic layer of the membrane, destabilizing its bilayer structure. Comparing the hemolysis that proceeds in the absence and presence of CLT, we found that the free heme-dependent hemolysis was effectively facilitated by the ligation of CLT to free heme (Figs. 8 and 9). The hydrophobicity and the molecular size of the heme-CLT complex may therefore be important factors for hemolysis. When the population of heme-CLT complexes becomes critical, malaria-infected erythrocytes are expected to be hemolyzed, causing the malarial parasites to die. In addition, heme-CLT complexes accumulating in the malarial membrane may destroy the membrane, fatally damaging the malaria parasite. Furthermore, CLT was found to be more effective than CQ in promoting hemolysis (Fig. 9). The Hill plots shown in Fig. 4 indicate that CQ has higher affinity to heme than does CLT with an ~10-fold lower concentration of CQ required for half saturation in 40% Me₆SO solution. Therefore, we are unable to explain why CLT demonstrated a higher ability to promote hemolysis. However, in other work, we found that the concentrations of CLT and CQ required for half saturation for binding with 10 μM heme were around 10 μM and nearly identical in 0.2 M HepES, pH 7.4, containing 1 mg/ml bovine serum albumin. This result indicates that CLT has 2 Unpublished data.
high affinity to heme similar to CQ under physiological conditions. Thus, the difference in the effects of hemolysis between CLT and CQ might have arisen due to the difference of hydrophobicity and structure of these complexes with heme but not due to a difference in the affinity to heme. In addition, we have also found that CLT promotes intracellular heme-induced hemolysis.\(^3\) We have found that CLT (5 \(\mu\)M) enhances hemolysis from 8 to 60% in cases in which free heme had been released from hemoglobin inside erythrocytes by pretreatment with menadione.\(^2\) Based on these results and the fact that CLT has

\[\text{TABLE II}\]

| Sample                                           | \(g_1\) | \(g_2\) | \(g_3\) | \(B/\mu\) | \(\mu/\lambda\) | Reference       |
|--------------------------------------------------|---------|---------|---------|-----------|----------------|----------------|
| \(\text{Fe}^{3+}\) mesoporphyrin IX (CLT)\(^a\)   | 1.46    | 2.26    | 2.98    | 0.566     | 3.121          | This work       |
| \(\text{Fe}^{3+}\) mesoporphyrin IX (imidazole)\(^b\) | 1.48    | 2.24    | 2.98    | 0.536     | 3.316          | This work       |
| Cytochrome 6-559 (from maize)\(^b\)              | 1.54    | 2.27    | 2.94    | 0.570     | 3.364          | Ref. 29         |
| \(\text{Fe}^{3+}\) protoporphyrin IX (N-methylimidazole)\(^b\) | 1.52    | 2.26    | 2.95    | 0.560     | 3.342          | Ref. 29         |
| Chlorotheophyrin dimethyl ester (imidazole)\(^c\) | 1.53    | 2.25    | 2.92    | 0.552     | 3.592          | Ref. 33         |

\(^a\) 100% Me\(_2\)SO.

\(^b\) Phosphate buffer, pH 7.4.

\(^c\) Chloroform.

\[\text{FIG. 7. Heme degradation by GSH.}\] Heme (3 \(\mu\)M) in isotonic buffer was mixed with 2 mM GSH and incubated at 37 \(^\circ\)C. Absorption spectra (300–600 nm) were measured at 6-min intervals immediately (six upper lines) and at 1 and 2 h (two lower lines) after mixing. Broken line, absorption spectrum of heme (3 \(\mu\)M). Inset, absorbance at 365 nm was regressed against time to fit first-order reaction.

\[\text{FIG. 8. GSH-dependent heme degradation in presence of CLT and CQ.}\] Heme (3 \(\mu\)M) and GSH (2 mM) were mixed in 0.2 M HEPES buffer (pH 7.4) in the absence (■) and presence of CLT (○), 6 \(\mu\)M, CQ (●), 6 \(\mu\)M, or Me\(_2\)SO (□), 0.2% v/v. GSH-dependent heme degradation was monitored by changes in absorbance at 396 nm.

\[\text{FIG. 9. Potentiation of heme-dependent hemolysis by CLT and CQ.}\] Hemolysis of erythrocytes was monitored by measuring hemoglobin absorbance at 578 nm. Percent of hemolysis was calculated from the ratio of hemoglobin content released from erythrocytes hemolysed by heme to the total heme content of erythrocytes as described under “Experimental Procedures.” A, suspensions of erythrocytes were incubated for 2.5 h without (+) or with 1 \(\mu\)M (○), 5 \(\mu\)M (△), and 10 \(\mu\)M (□) agents; then, the degree of hemolysis was measured. Closed and open symbols indicate CLT and CQ, respectively. B, suspensions of erythrocytes were incubated with 1.5 \(\mu\)M heme and 10 \(\mu\)M CLT or 10 \(\mu\)M CQ at 37 \(^\circ\)C for 150 min; then, the degree of hemolysis was measured.

\[\text{FIG. 10. Effects of CLT and CQ on hemolysis of heme-bound erythrocytes.}\] Me\(_2\)SO (DMSO) (1%), CLT (10 \(\mu\)M), CQ (10 \(\mu\)M), or GSH (2.5 mM) was added to heme-bound erythrocyte suspensions and incubated at 37 \(^\circ\)C for 2 h. Hemolysis rate was calculated from three experiments as described under “Experimental Procedures.”

\(^3\) N. T. Huy, K. Kamei, R. Takano, and S. Hara, submitted for publication.
the ability to compete with HRP and GSH, we expect that CLT at clinically achievable concentrations in plasma would invade erythrocytes and parasites, form a complex with heme released from hemoglobin, and damage cell membranes. The decomposition of the membrane triggered by the heme-CLT complex is thought to play an important role in the antimalarial action of CLT.

One proposal states that 2-chlorophenyl-bis-phenyl methanol, one of several in vivo metabolites of CLT lacking the imidazole, has weaker antimalarial activity (50% growth inhibitory concentration (IC50) of ~1 μM) than CLT (IC50 of ~1 μM) (3). This indicates that the imidazole group is not essential but is involved in the antimalarial activity of CLT. The antimalarial mechanism of 2-chlorophenyl-bis-phenyl methanol might be due to inhibition of the Ca2+ pump and Ca2+ channels (5, 6, 43). However, the much higher antimalarial activity of CLT than of 2-chlorophenyl-bis-phenyl methanol might be caused by the formation of complexes with heme through an imidazole group, supporting our theory of the antimalarial mechanism of CLT involving malarial heme catabolism.

Previous reports (3, 4) indicate that CLT inhibits the growth of CQ-resistant strains of malaria. Furthermore, the low IC50 value of CLT against malaria makes it a practical antimalarial drug because a plasma CLT concentration of 2 μM is achievable with an oral dose of 1 g. Therefore, CLT is promising as a new antimalarial drug.

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Clotrimazole Binds to Heme and Enhances Heme-dependent Hemolysis: PROPOSED ANTIMALARIAL MECHANISM OF CLOTRIMAZOLE
Nguyen Tien Huy, Kaeko Kamei, Takushi Yamamoto, Yoshiro Kondo, Kenji Kanaori, Ryo Takano, Kunihiro Tajima and Saburo Hara

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