2-tert-Butyl-8-Quinolinammines Exhibit Potent Blood Schizontocidal Antimalarial Activity via Inhibition of Heme Crystallization

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We have recently reported that the attachment of a bulky metabolically stable tert-butyl group at the C-2 position of a quinoline ring in primaquine results in a tremendous improvement in the blood schizontocidal antimalarial activity of 8-quinolinamine. Because free heme released from hemoglobin catabolism in a malarial parasite is highly toxic, the parasite protects itself mainly by crystallization of heme into insoluble nontoxic hemozoin. We now demonstrate the ability of 2-tert-butylprimaquine to inhibit in vitro beta-hematin formation, to form a complex with heme with a stoichiometry of 1:1, and to enhance heme-induced hemolysis. The results described herein indicate that a major improvement in the blood-schizontocidal antimalarial activity of 2-tert-butylprimaquine might be due to a disturbance of heme catabolism pathway in the malarial parasite.

Malaria is one of the most common diseases in tropical countries. Over 300 million new malaria infections and millions of deaths due to malaria occur worldwide each year. The rapid spread of resistance to current quinoline antimalarials has made malaria a major global problem. Because a vaccine for malaria is not available, it is essential to find new antimalarial drugs and understand their antimalarial mechanism for treating patients.

8-Quinolinammines constitute a promising class of compounds because of their versatile biological and pharmacological activities, such as their antimalarial (38), antileishmanial (38), and antitubercular (3) activities. Primaquine (PQ) (Fig. 1) is the only 8-quinolinamine available to treat the malarial parasites in the infections caused by Plasmodium vivax and P. ovale. It has been reported that PQ has various degrees of activity against more life cycle stages of Plasmodium than any other currently employed antimalarials (30). However, drawbacks like toxicity, ineffectiveness as a blood schizontocide, and quick catabolism have limited the usefulness of PQ. Despite these drawbacks, in addition to excellent radical curative activity, PQ has broad range of antimalarial activities, including efficacy as a causal prophylactic, gametocytocide, and sporontocide. These encouraging pharmacological properties make PQ an ideal drug to emulate while designing new antimalarials with improved activities (27). Research efforts over the years have been directed toward finding analogues that retain the tissue schizontocidal activity of PQ with improved blood schizontocidal activity and reduced methemoglobin (MetHb) toxicity, and few derivatives with improved therapeutic index have been synthesized (8, 29). However, efforts to eliminate MetHb toxicity of PQ have proved to be unsuccessful after 40 years of research (30).

We recently reported that the placement of a metabolically stable tert-butyl group at the C-2 position of a quinoline ring in PQ results in a tremendous improvement in blood schizontocidal antimalarial activity (26, 27). 2-tert-Butylprimaquine (BPQ) (Fig. 1) exhibits potent in vivo blood schizontocidal antimalarial activities against both a drug-sensitive strain (P. berghei) and a multi-drug-resistant strain (P. yoelii nigeriensis) (27). Furthermore, BPQ also represents the first reported 8-quinolinamine completely devoid of MetHb toxicity associated with PQ. To continue our studies of PQ derivatives, here we investigate the mechanism of blood schizontocidal activity of BPQ.

During development and proliferation in host erythrocytes, the malarial parasite degrades hemoglobin in host erythrocytes to use the catabolic products as a source of amino acids. This degradation is accompanied by the release of free heme. The free heme is oxidatively active and toxic to both the host cell and the malarial parasite and causes parasite death (32). Because of the absence of heme oxygenase; the parasite is unable to cleave heme into an open-chain tetrapyrrole, which is required for cellular excretion (11). To protect itself, the malarial parasite detoxifies free heme via neutralization with histidine-rich protein 2 (22, 37), degradation with reduced glutathione (4, 19, 20), or crystallization into hemozoin, a water-insoluble malarial pigment produced in the food vacuole (17, 37). Egan et al. recently proposed that the primary pathway of heme detoxification is hemozoin formation (12). It has been shown that hemozoin is structurally and chemically identical to β-hematin (BH), a synthetic heme crystal (7, 33, 42). Several factors, such as heating (13), histidine-rich protein (22, 37), lipids (9, 34, 41), preformed BH (36), and alcohols (6, 21) have been reported to be responsible for promoting BH formation. Current reports indicate that blocking heme detoxification is one of the main mechanisms of current antimalarial quinolines and an ideal target for antimalarial screening (1, 14, 35, 36).

In this study, the mechanism of blood schizontocidal activity
of BPQ was proposed, as demonstrated by its ability to inhibit in vitro BH formation, to form a complex with heme with a stoichiometry of 1:1, and to enhance heme-induced membrane destabilization.

**MATERIALS AND METHODS**

Materials. Hemin chloride (heme), chloroquine (CQ), and PQ were obtained from Sigma. Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemicals (Osaka, Japan). All chemicals were of the highest commercially available grade. BPQ was chemically synthesized from commercially available 6-methoxy-8-nitroquinoline as described earlier (27).

Heme preparation. A stock solution of heme was prepared by dissolving hemin chloride (16.3 mg) in 1 ml of DMSO and then removing the insoluble heme by centrifugation for 10 min at 7,000 × g. The concentration of heme in the solution was estimated from the absorbance at 400 nm after dissolution with 100 mM NaOH-2.5% sodium dodecyl sulfate solution. The molar extinction coefficient for heme is 105 at 400 nm as described previously (25). This stock reagent was stored at 4°C in the dark until used.

Antimalarial assay. Antimalarial activity against a chloroquine-sensitive \( P. falciparum \) clone (D6) was determined as previously described. Briefly, different drug dilutions were prepared in complete RPMI (RPMI 1640 medium plus 10% AB plus human serum). Fifty microliters of each dilution was transferred to the wells of a 96-well plate in triplicate. Parasited erythrocytes (mainly rings; 4% parasitemia; 5% hematoctrit) were added to each well. The volume in each well was filled up to 200 μl with complete RPMI. The plates were incubated at 37°C in a candle jar. After 48 h of incubation, thin smears from each well were made and stained with Giemsa. The number of parasited erythrocytes per 10,000 cells was counted. Percent inhibition by the drug over the negative control, which did not contain any drug, was plotted against the respective concentration of the drug. The 50% inhibitory concentration (IC\(_{50}\)) values (μM), concentrations that are required to inhibit 50% of parasite growth, were calculated graphically (28).

Inhibition assay of BH formation. An inhibition assay of BH formation was based on our previous method (40), with a slight modification. Briefly, Tween 20 (50 μg/ml), which was used as an initiator (24), was incubated with 100 μM heme in 1 ml of 0.5 M acetate buffer at pH 4.8 in the presence of various concentrations of drug (CQ, PQ, or BPQ). After incubation for 16 h at 37°C, BH was purified and then its concentration was determined as previously described (40). The values obtained from triplicate assays were plotted, and the IC\(_{50}\) values (μM), concentrations of drugs that are required to inhibit 50% of heme crystallization, were calculated graphically.

Absorption spectra. All absorption spectra were recorded on a Hitachi U-3300 double-beam spectrophotometer (Tokyo, Japan) using a 1.0-cm light path quartz cuvette at 23°C.

Drug-heme interaction assay. To examine the interaction of drugs and heme, a differential absorbance technique (Job's plot) was performed to determine the spectral changes as previously described (5). Briefly, series of solutions containing drug and heme were prepared in DMSO (40% vol/vol) buffered by 20 mM HEPES (pH 7.4). The total final combined concentration of heme and drug in the mixtures was constant at 10 μM. After incubation of the mixtures at room temperature for 30 min, the differential absorbance at 400 nm was recorded. The differential absorbance was obtained by subtracting the total absorbance at 400 nm of heme and drug from the absorbance of the heme-drug mixture.

Spectrometric titration. Differential absorption spectra were performed on a Hitachi U-3300 spectrophotometer as previously described (20). Briefly, the drug (CQ, BPQ, or PQ) was added sequentially to both a sample cuvette (containing 3.5 μM heme solution) and a reference cuvette. Both the sample cuvette and the reference cuvette contained 40% DMSO buffered by 20 mM phosphate buffer (pH 7.4). All differential spectra were recorded at wavelengths of between 350 and 650 nm, and the concentrations of heme-drug complexes were evaluated based on absorbance at 400 nm. The binding mode of drug to heme was analyzed by a Hill plot (20). The equilibrium association constants for the formation of heme-drug complexes as well as the number of ligand molecules (drug) that bind to heme were calculated from Hill plots using equation 1

\[
H + nL \rightleftharpoons H\text{L}_n
\]

and analyzed using the standard equation (equation 2)

\[
\log \frac{A - A_r}{A_r - A} = \log K_e + n \log [L]
\]

where \( A_o, A_r, \) and \( A \) are the absorbance of the initial, final, and mixed species, respectively; \( H \) represents heme; \( L \) is the ligand (drug); \( n \) is the number of ligand molecules that bind to heme; and \( K_e \) is the equilibrium association constant of the heme-ligand complex.

Hemolysis. Fresh blood from healthy donors was heparinized (1 mg of heparin/ml blood) to suppress clotting. The erythrocytes were separated from plasma by centrifugation at 1,500 × g for 3 min and washed six times with isotonic phosphate-buffered saline (PBS), pH 7.4. Thereafter, the erythrocyte was suspended in 10% cell suspensions in PBS. Sixty microliters of erythrocyte suspensions (final suspensions of cells ranged from 0 to 2% in serial 1:2 dilutions) was dispensed in a 96-well plate. Two hundred microliters of distilled water was added to the wells to lyse the erythrocyte or 200 μl of PBS was added. After shaking for 15 min, the plate was scanned at 630 nm using an MTP-120 microplate reader (Corona Electric Co., Ibaragi, Japan) to measure the turbidity of cells (Fig. 2A). Adding 200 μl of distilled water into wells caused 100% hemolysis of erythrocytes, as shown by turbidity at the baseline, while intact erythrocytes exhibited a linear turbidity in the range of 0 to 2% suspension. Thus, we used a 0.5% suspension of erythrocytes in all hemolytic assays.

To observe whether there is a good positive correlation between the percent of hemolysis and the reduction of turbidity over a wide range, we performed the following experiment. A series of solutions containing various concentrations of hemolyzed erythrocytes and intact erythrocytes were prepared in PBS. The total final combined suspension of cells in the mixtures was constant at 0.5%. The absorbance of mixtures recorded at 630 nm was found linearly over the whole range of hemolysis (Fig. 2B), indicating that this method can be used for the quantitation of hemolytic activity of drugs.

In the assay for investigation of the effect of drugs on heme-induced hemolysis, 15 μl of heme (final concentrations ranged from 0 to 20 μM) was incubated with or without drugs (10 μM) in 15 μl of PBS on a 96-well plate for 10 min. Thirty microliters of erythrocyte suspension (0.5%) in PBS was then added to the wells.
Antimalarial activity. In vitro antimalarial activities of CQ, PQ, and BPQ are shown as IC50 values for the inhibition of the chloroquine-sensitive human *P. falciparum* D6 strain (Table 1). PQ had no effect on the growth of *P. falciparum*, while BPQ displayed a slightly stronger activity than that of CQ against the *P. falciparum* D6 strain, in good agreement with our previous report (27). In addition, we previously indicated that BPQ has a potent blood schizontocidal antimalarial activity against *P. berghei* (a sensitive strain) and *P. yoelii nigeriensis* (a multidrug-resistant strain) in a rodent model, while PQ has no activity (27). These results suggest that the bulky tert-butyl group of BPQ plays an essential role in the improvement of antimalarial activity of PQ in vitro and in vivo.

Inhibition of heme crystallization by drugs. The antimalarial mechanism of CQ has been believed to act within the parasite food vacuole by blocking the formation of hemozoin (39). The blocking has been demonstrated in vitro by showing the ability of CQ to inhibit the formation of BH, a process that is closely similar to hemozoin synthesis within the parasite food vacuole (39). To explore the antimalarial mechanism of BPQ, we investigated its inhibitory effect on BH formation by using Tween 20 as an initiator. Under our experimental conditions, PQ had no effect on the formation of BH (Table 1). Under our encouragement, the inhibitory activity of BPQ against the formation of BH (IC50 = 2.9 ± 2.1 μM) was fivefold stronger than that of CQ (IC50 = 15.4 ± 5.6 μM), indicating that BPQ may possess the antimalarial mechanism in blocking heme detoxification of malarial parasite. To be worthy in vivo, BPQ is required to be concentrated to micromolar level in the parasite food vacuole, where hemozoin formation takes place. BPQ and PQ, bearing basic amino groups in their structures, are expected to accumulate in the food vacuole through pH trapping (14); however, its level in the food vacuole should be studied further.

Interaction of drugs and heme. The inhibition of heme crystallization by antimalarial drugs may be mediated by binding to heme (16). Here we investigated the interaction of drugs with heme by using a continuous variation technique (Job’s plot), as described in Materials and Methods. A solution of heme at pH 7.4 and 25°C. The total final combined concentration of heme and drug in the mixtures was constant at 20 μM in 40% aqueous DMSO. After incubation of the mixtures for 30 min, the differential absorbance at 400 nm was recorded. Values are the means ± standard errors of the means of three independent experiments. The results are reproducible.

### TABLE 1. IC50 values for inhibition of *P. falciparum* growth and heme crystallization

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 (μM) for inhibition of:</th>
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<tbody>
<tr>
<td></td>
<td><em>P. falciparum</em> D6 clone growth</td>
</tr>
<tr>
<td>CQ</td>
<td>0.3</td>
</tr>
<tr>
<td>PQ</td>
<td>ND*</td>
</tr>
<tr>
<td>BPQ</td>
<td>0.1</td>
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*ND, not detected.*

After shaking for 2 h at 37°C, the plate was scanned at 630 nm. The absorbance of the control well, which contained only erythrocytes, was considered to be 0% of hemolysis and was used to convert the remaining absorbance values to percentage of hemolysis. In another experiment, the effect of drugs in various concentrations on 10 μM of heme-induced hemolysis was also determined as above.

FIG. 3. Titration of the heme-CQ (A) and heme-BPQ (B) interaction. Differential spectral titration of drugs with heme proceeded as described in Materials and Methods. The concentration of CQ (A) was increased from 0 μM to 20 μM in 2-μM increments, and the concentration of BPQ (B) was increased from 0 μM to 60 μM in increments of 6 μM. Arrows indicate the effect of increasing the concentrations of drugs.
The interaction of heme and drugs in 40% DMSO was further explored by Job’s plot. The differential absorbance of heme-drug mixtures at 400 nm was recorded and plotted as shown in Fig. 4. Changes in differential absorption intensity were maximal when the molar fraction of heme to both CQ and BPQ was 1:1 (Fig. 4), supporting the notion that CQ and BPQ have affinities for the monomeric heme in our conditions. In the interaction of PQ with heme, Dorn et al. demonstrated that PQ could bind to μ-oxo dimer heme with an affinity between those of mefloquine and quinine (10). However, in our experiments, PQ exhibited no evidence of interaction with monomeric heme in 40% DMSO, in good agreement with a previous study (15).

To measure the affinity of heme for binding to drugs, spectrophotometric heme titration was performed by measuring the differential spectra between drug and heme-drug complex at various drug concentrations. The heme interactions with drugs were analyzed using Hill plots (20) to determine the number of molecules bound to heme in aqueous DMSO. Hill plots of our binding data in Fig. 5 show heme-drug complexes at 3.5 μM heme. The slopes of both linear graphs are approximately at one, within experimental error. The analysis using the Hill plot demonstrates that heme binds one CQ molecule with an association constant \( K_a \) of \( 3.24 \times 10^5 \text{M}^{-1} \), in good agreement with our previous data (20), whereas heme binds one molecule of BPQ with a \( K_a \) of \( 0.61 \times 10^5 \text{M}^{-1} \) in aqueous 40% DMSO at pH 7.4. In contrast, there was no reduction of Soret intensity of spectrophotometric heme titration when excess PQ was added to the heme solution (data not shown).

It would be interesting to know the reason for the differential effects of PQ and BPQ on the interaction with heme. The effects could be due to their different structures, in which BPQ has a bulky hydrophobic tert-butyl group at position 2 of the quinoline ring, which would help to increase the \( \pi-\pi \) interaction of quinoline and porphyrin ring of heme, but the reason requires further study.

**Effect of drugs on erythrocyte hemolysis induced by heme.**

We recently demonstrated that CQ and antifungal azoles, which have antimalarial activities, possess abilities for binding heme and enhancing the toxicity of heme (19, 20, 23). Here, to explore the effect of BPQ on heme-induced membrane destabilization, hemolytic experiments were performed using fresh erythrocytes as described in Materials and Methods. The hemolysis induced by heme was potentiated by CQ as well as by BPQ (Fig. 6). The activity of CQ in the enhancement of heme-induced hemolysis was significantly stronger than the activity of BPQ. The hemolytic effects of CQ and BPQ depended on the concentrations of both heme and drugs. On the other hand, PQ had no effect on the heme-induced hemolysis, supporting our previous results that PQ cannot bind to monomeric heme, while BPQ can bind to heme. We also observed that drugs alone at concentrations of up to 30 μM had no effect on hemolysis in the absence of heme. Based on these results, the enhancement of heme-dependent hemolysis by BPQ may be caused by the formation of a heme-drug complex. This heme-drug complex may be a more hydrophobic and bulky species than free heme is, probably resulting in a high destabilization of membrane bilayer structure. The hydrophobicity and the molecular size of the heme-BPQ complex, therefore, may be important factors for the destabilization of membrane, which causes the malarial parasites to die.

In addition, BPQ was found to be less effective than CQ for the promotion of heme-induced hemolysis (Fig. 6) as well as for affinity for binding monomeric heme (Fig. 3 and 5), but BPQ is stronger than CQ in antimalarial activity and the inhibition of heme crystallization. Therefore, the difference in the effects of antimalarial activities between BPQ and CQ might have arisen due to the difference in their abilities to inhibit hemozoin formation but not due to a difference in their enhancement of the toxicity of free heme on cell membrane or their affinities for binding monomeric heme.

It has been proposed that the toxicity of heme on the biological cell membrane results from direct binding or incorporation, which may affect the reciprocal interactions between membrane and cytoskeleton proteins (31). It has also been suggested that CQ can complex with heme and enhance the
toxicity of heme, probably by increasing the transfer of heme in solution to the phospholipid bilayer membrane (18). Thus, BPQ may possess a similar mechanism for increasing the transfer of heme in solution to the membrane, but the mechanism requires further study. It is generally believed that the pK_a contributes substantially to the antimalarial activity of CQ (pK_a = 8.41), and activity is reduced due to any deviation in the pK_a. The role of pK_a in antimalarial activity is not very clear in the case of BPQ. The pK_a levels of 4.92 and 10.01 for ring nitrogen and a side-chain primary amino group, respectively, possibly help in the accumulation of the drug in acidic food vacuoles for strong hematin binding.

In summary, introduction of the 2-tert-butyl group in the quinoline ring of PQ produced a very pronounced increase in the toxicity of free heme against malaria parasites. However, further studies of the concentration of this new compound in the parasitized erythrocyte and the food vacuole are needed to confirm this observation. In addition, study of the radical curative antimalarial activity of BPQ is also needed.

It has been established that PQ and related compounds enhance the conversion of oxyhemoglobin to MetHb, and the severity of malaria correlates with MetHb levels (2); thus, the use of 8-quinolinamines as blood schizontocidal antimalarials requires further study. It is generally believed that the pK_a levels of 4.92 and 10.01 for ring nitrogen and a side-chain primary amino group, respectively, possibly help in the accumulation of the drug in acidic food vacuoles for strong hematin binding.

REFERENCES


