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Simple Colorimetric Inhibition Assay of Heme Crystallization for High-Throughput Screening of Antimalarial Compounds▽

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Current assays for screening new antimalarials need initiators of β-hematin formation that require laborious preparation, special devices, and substrates. In this study, based on reduction of heme absorption in β-hematin formation, we developed a simple colorimetric assay using Tween 20 as an initiator and a microplate reader for high-throughput screening of inhibitors of β-hematin formation.

Plasmodium spp., which are malarial parasites, degrade hemoglobin in host erythrocytes to use the catabolic products as a source of amino acids during development and proliferation. This is accompanied by a release of free heme. The free heme is oxidatively active and toxic to both the host cell and the malarial parasite, and it causes parasite death. Due to the absence of heme oxygenase, the parasite is unable to cleave heme into an open-chain tetrapyrrole, which is necessary for cellular excretion (8). To protect itself, the parasite detoxifies free heme via neutralization with histidine-rich protein 2 (14, 23), degradation with reduced glutathione (2, 13), or crystallization into hemozoin (HZ), which is a water-insoluble malarial pigment produced in the food vacuole (12, 23). Chloroquine (CQ) and other antimalarial drugs have been demonstrated to inhibit the formation of a synthetic heme crystal, β-hematin (BH), which is structurally identical to hemozoin (21, 24), and are believed to inhibit hemozoin formation in the food vacuole of the malarial parasite. Current reports indicate that blocking the BH formation is an ideal target for antimalarial screening (1, 19, 22). Several factors such as temperature (9), histidine-rich protein (14, 23), lipids (6, 26), and preformed BH (22) have been reportedly responsible for promoting BH formation. However, the thermal BH crystallization is not natural and is a nonphysiological process. Some strains of *Plasmodium* spp. lack histidine-rich proteins but still form HZ (22), suggesting that histidine-rich proteins are sufficient but not completely necessary for HZ formation. Therefore, the two most important and natural initiators of BH formation are probably lipids and preformed BH. Several studies have used preformed BH (4, 6, 7) and lipids extracted from parasites (11), infected plasma (26), or commercial lecithin (25) as initiators of BH formation in assays. However, the preformed BH-based assay has a high background absorbance in the colorimetric assay (26) and requires fresh purification of BH, while using lipids requires laborious preparation and strict storage. In this study, we demonstrated that a surfactant (Tween 20), considered a good model for the biological membrane (3), can be used as an initiator for BH formation in assays in vitro.

Recently, high-throughput screening for BH inhibition of new antimalarial compounds has been suggested as a useful tool in the discovery of new antimalarial compounds (17, 18). Several methods have been described for the measurement of BH crystallization in high-throughput screening of antimalarials; however, they involve special filtration microplates (5, 27), expensive ^{3}H-labeled heme (17), a laborious transfer of samples to another 96-well plate (18), or the use of toxic substances such as pyridine and ^{3}H-labeled heme. Here, based on reduction of heme absorption in BH formation (16), we have developed a simple, reproducible, and fast colorimetric assay using Tween 20 as an initiator of BH for high-throughput screening of antimalarial candidates.

Hemin chloride (16.3 mg; Sigma) was dissolved in 1 ml of dimethyl sulfoxide. The solution was passed through a 0.2-μm-pore membrane filter to remove insoluble particles. The solution can be kept at 4°C up to 1 month as a stock solution (25) and diluted to 111.1 μM of heme with 1 M acetate buffer, pH 4.8, just before being used. In order to determine heme concentration in stock solution, the absorbance at 400 nm was measured after the solution was diluted with 2.5% sodium dodecyl sulfate in 0.1 M NaOH. The heme concentration was calculated with an extinction coefficient of 10^5 at 400 nm as described previously (13).

CQ (Sigma), amodiaquine (from MP Biomedical Inc.), and quinacrine (from Calbiochem, Canada) were dissolved in distilled water. Quinine sulfate (Q; Sigma), quinidine (from Wako, Osaka, Japan), and 8-hydroxyquinoline (Sigma) were dissolved in 20 mM sulfuric acid. Mefloquine (from Sigma) was prepared in methanol. pH was controlled at 4.8 in all assays. Tween 20 (1 g/100 ml) was dissolved in distilled water.

Two hundred microliters of heme buffered by 1 M acetate buffer, pH 4.8 (final concentrations ranged from 2.4 to 76 μM in serial 1:2 dilutions), was dispensed in a 96-well plate. The plate was scanned at 415/630 nm (absorbance at 630 nm was subtracted from absorbance at 415 nm) using an MTP-120 microplate reader (Corona Electric Co., Ibaragi, Japan). The

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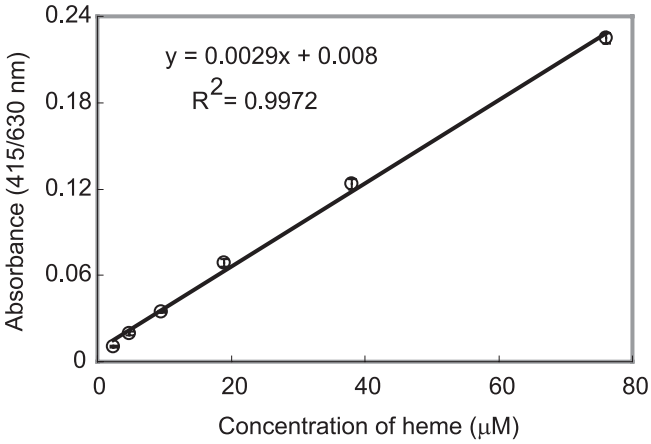

FIG. 1. Linear relationship between absorbance at 415/630 nm and heme concentration.

heme absorbance at 415 nm must be corrected by subtraction of turbidity of BH. The recorded absorbance at 415/630 nm was found linearly over the heme concentration ranges of 2.4 to 76 μM (Fig. 1). Thus, we used 50 μM of heme in all assays to evaluate the effect of drugs on BH formation.

To establish effective conditions for BH formation, we studied the efficiency of BH formation induced by various concentrations of Tween 20 in vitro at 37°C. One hundred microliters of Tween 20 (various concentrations in serial 1:2 dilutions by distilled water) was added in each well of a 96-well plate. Next, 100 μl of heme (50 μM, buffered by 1 M acetate buffer, pH 4.8) was added to each well and incubated at 37°C for 250 min. The plate was read at 415/630 nm as described above. Based on our previous study (16), the yield of heme converted to BH was calculated by the following equation:

$$(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \quad (1)$$

where A_{control} is the absorbance of the heme without Tween 20 at 415/630 nm, while A_{sample} represents the absorbance of the heme in the presence of Tween 20.

The results are shown in Fig. 2. The yield of BH reached maximum, approximately 80%, at 0.012 g/liter of Tween 20 (Fig. 2, top) and at 250 min (Fig. 2, bottom), indicating a relatively high efficiency in converting heme into BH in the presence of Tween 20. The crystal growth curves showed a sigmoidal pattern (Fig. 2, bottom), in good agreement with previous reports, describing the transition from a metastable state to a stable equilibrium state, suggesting that Tween 20 plays a role in stabilizing nuclei of BH (25). To our surprise, the rate of Tween 20-induced BH formation is higher than that of other assays using initiators such as lipids and preformed BH.

The characteristics of purified BH were further confirmed by infrared spectroscopy as described previously (15), demonstrating the expected peaks at 1,210 and 1,664 cm^{-1} (data not shown). Based on the above results, heme in all experiments for high-throughput screening antimalarial drugs was incubated in the presence of 0.012 g/liter of Tween 20 for 250 min.

To evaluate the availability of the BH formation induced by Tween 20 for high-throughput screening of new antimalarial

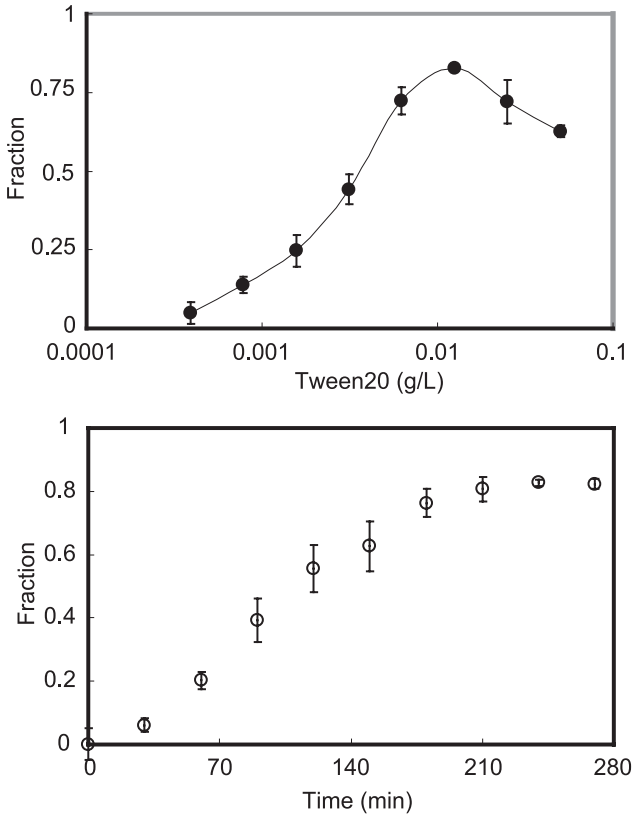

FIG. 2. Catalysis of BH formation induced by Tween 20. (Top) Dose response of Tween 20-induced BH formation. (Bottom) Time course of BH crystallization induced by Tween 20 (0.012 g/liter). BH crystallization was expressed as percent heme converted into BH.

drugs, the inhibitory effects of quinoline antimalarial drugs on BH formation were investigated using Tween 20 (Fig. 3) as an inducer. The procedure for high-throughput screening of new antimalarial drugs was performed in the following order of

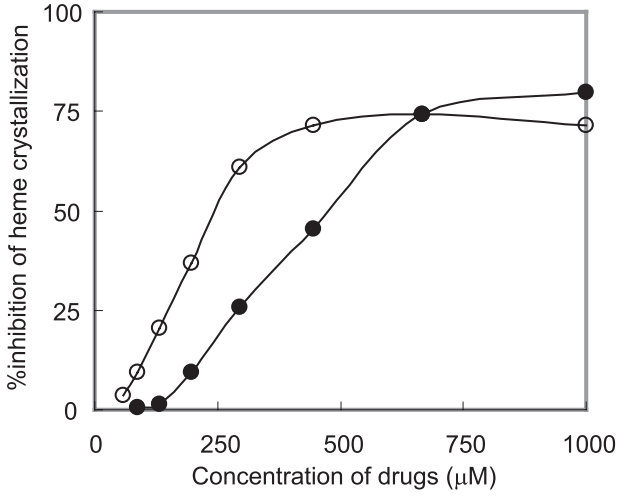

FIG. 3. Inhibition of heme crystallization by antimalarials. After incubation of heme with various concentrations of CQ (open circles) and Q (closed circles) in duplicate, the absorbance at 415/630 nm was recorded and the fraction (*f*) of heme converted to BH was calculated as described in the text. The results are representative data from three independent experiments.

steps. Firstly, drugs were prepared at various concentrations using distilled water and sulfuric acid (20 mM). Secondly, 90 μl of heme solution (50 μM), freshly buffered by 1 M acetate buffer (pH 4.8) from dimethyl sulfoxide stock, was added into the plate. Finally, Tween 20 was added into each well at 0.012 g/liter. After incubation at 37°C for 250 min, samples were mixed by being pipetted three times, and then the plate was read at 415/630 nm. The fraction (*f*) of heme converted to BH was calculated as in a previous study (16):

$$f = (A_{\text{control}} - A_{\text{sample}})/(A_{\text{control}} - A_{\text{min}}) \quad (2)$$

where A_{control} is the absorbance of the heme without Tween 20 or an antimalarial at 415/630 nm, while A_{sample} represents the absorbance of the heme in the presence of both Tween 20 and drugs and A_{min} is the absorbance of the heme with Tween 20 in the absence of an antimalarial at 415/630 nm.

Percentage of inhibition of BH by drugs was then described by the following equation:

$$\% \text{ inhibition} = (1 - f) \times 100 = 100 \times (A_{\text{sample}} - A_{\text{min}})/(A_{\text{control}} - A_{\text{min}}) \quad (3)$$

The values obtained from duplicate assays were plotted, and the IC_{50} values, the concentrations inhibiting 50% of heme crystallization, were calculated graphically. The results, obtained from four independent duplicate experiments, showed that all quinoline antimalarials used in this study can inhibit Tween 20-induced BH formation (IC_{50} values of 252 ± 49 μM, 365 ± 103 μM, 315 ± 123 μM, 167 ± 59 μM, 325 ± 87 μM, and 292 ± 99 μM for CQ, Q, mefloquine, amodiaquine, quinidine, and quinacrine, respectively). The mechanisms of the drugs are believed to relate to heme crystallization; they bind heme and inhibit hemozoin formation in the lysosomal digestive vacuole of the parasite (20). The IC_{50} values for these drugs were higher than those of assays based on preformed BH-based and lecithin (25). The reason is obscure, but the slight difference in the abilities of antimalarial drugs to bind to heme in the presence of inducers and the difference in the mechanism of initiation of heme crystallization between Tween 20 and other inducers might affect the values. In addition, the IC_{50} value is widely varied depending on the incubation time of the assay (10). However, these values were within a threefold range, in good agreement with previous reports (4, 7, 26), suggesting that the IC_{50} values in our assay of inhibition of Tween 20-induced BH formation are acceptable for high-throughput screening of new antimalarial drugs.

Our results showed that using Tween 20 has several advantages over using current initiators: for instance, (i) the assay is fast and based on a simple technique using a microplate reader, which is available in most labs; (ii) the Tween 20-based assay is not expensive for large-scale screening; (iii) the method is a convenient method that requires no fresh preparation of inducers; (iv) Tween 20 can be prepared in stock solution and requires no strict conditions for storage; (v) the assay does not use pyridine or mutagenic and carcinogenic agents; (vi) the assay uses a simple 96-well plate, which requires no purification or transfer steps, promising an ideal high-throughput screening of antimalarial candidates.

In conclusion, our assay of inhibition of BH formation using Tween 20 is cheap, reproducible, nontoxic, and more convenient than other assays for large-scale screening of novel antimalarials.

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