

Zinc Protoporphyrin IX Binds Heme Crystals to Inhibit the Process of Crystallization in *Plasmodium falciparum*

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The intraerythrocytic *Plasmodium falciparum* parasite converts most of host hemoglobin heme into a nontoxic heme crystal. Erythrocyte zinc protoporphyrin IX, normally present at 0.5 μM , which is a ratio of 1:40000 hemes, can elevate 10-fold in some of the anemias associated with malaria disease protection. This work examines a binding mechanism for zinc protoporphyrin IX inhibition of heme crystallization similar to the antimalarial quinolines. Zinc protoporphyrin IX neither forms crystals alone nor extends on preformed heme crystals. Inhibition of both seed heme crystal formation and crystal extension occurs with an inhibitory concentration (IC_{50}) of 5 μM . Field emission in-lens scanning electron microscopy depicts the transition and inhibition of heme monomer aggregates to heme crystals with and without seeding of preformed hemozoin templates. In vitro zinc protoporphyrin IX, like the quinolines, binds to heme crystals in a saturable, specific, pH, and time-dependent manner. The ratio at saturation is approximately 1 zinc protoporphyrin IX per 250 hemes of the crystal. Unlike the quinolines, zinc protoporphyrin IX binds measurably in the absence of heme. Isolated ring and trophozoite stage parasites have an elevated zinc protoporphyrin IX to heme ratio 6 to 10 times that in the erythrocyte cytosol, which also corresponds to elevated ratios found in heme crystals purified from *Plasmodium* parasites. This work implicates protection from malaria by a mechanism where elevated zinc protoporphyrin IX in anemic erythrocytes binds to heme crystals to inhibit further crystallization. In endemic malaria areas, severe iron deficiency anemia should be treated with antimalarials along with iron replenishment.

INTRODUCTION

During the erythrocytic stages of its infection, the malaria parasite degrades host hemoglobin to acquire essential amino acids (1,2). Toxic free heme or iron protoporphyrin IX (FePPIX), a byproduct of this proteolysis, is not catabolized by heme oxygenase as it is in mammalian cells, but instead is converted into a unique FePPIX crystal called hemozoin (3,4). Inhibition of this FePPIX crystallization accounts for the mechanism by which the antimalarial quinolines act (5), although controversy still exists over the exact mechanism of action of crystal inhibition after the quinolines 1st bind to FePPIX (6–9). Numerous studies have shown that the quinolines bind FePPIX noncovalently (10–14). An additional theory suggests the FePPIX-quinoline complex incorporates into a growing crystal face that blocks further crystal extension (6,15–17). Indeed, this observation may explain why quinidine is a more potent in vitro crystallization inhibitor than chloroquine, despite having a lower FePPIX affinity (8).

Zinc protoporphyrin IX (ZnPPIX), present in normal erythrocytes at 0.5- μM concentrations, can elevate above 5- μM concentrations in erythrocyte anemias because of thalassemias, iron deficiency, hemolysis, or chronic inflammatory disease (18–20). When amounts of bioavailable iron in the reticulocyte are low, the ferrochelatase enzyme inserts zinc into protoporphyrin IX instead of iron, resulting in elevated ZnPPIX in the erythrocyte expressed as a ratio of μmol ZnPPIX to mol of Fe PPIX exceeding the normal value of approximately 25 μmol ZnPPIX to 1 mol FePPIX (19). The

ZnPPIX resides in the FePPIX pockets of hemoglobin (19). Stoltzfus and coworkers have recently assessed iron status indicators in 611 well, non-febrile preschool children from Zanzibar living in a highly endemic malarious area (21). Amongst children in the lowest hemoglobin quartile, a negative malaria blood film correlated with a markedly higher mean ZnPPIX to FePPIX ratio above 250 μmol ZnPPIX/mol FePPIX. This is interpreted as an important trend, but not conclusive in correlating an elevated ZnPPIX/FePPIX ratio with malaria protection.

Iron deficiency anemia affects over half of all young children and pregnant women in developing countries resulting in over 500 million cases of anemia worldwide (22). Endemic malaria and iron deficiency anemia coexist with mortality targeting young children and pregnant women (21,23). Many but not all clinical studies of iron deficiency have also reported protection from severe malaria disease and exacerbation of malaria disease in individuals undergoing iron repletion (24). Interestingly, for some of the hemoglobinopathies and also iron deficiency anemia, malaria disease protection occurs without changing incidence of parasitemia, although the degree of parasitemia was not determined (24,25).

Martiney and Cerami have already demonstrated ZnPPIX inhibition of FePPIX crystal formation in vitro and postulated a role for elevated ZnPPIX in β -thalassemic erythrocytes in protection from malaria (26). In vitro, nonmetal and noniron protoporphyrins have also been shown to inhibit the crystallization of FePPIX using, as a template for extension assays, FePPIX

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crystal derived from either from trophozoite lysates or from a chemical synthesis called β -hematin (27,28). The binding affinity, near $5.6 \times 10^5/M^{-1}$, of ZnPPIX to FePPIX by π - π interactions has been reported to be similar to chloroquine (28,29). The binding of a FePPIX-quinoline complex to preformed FePPIX crystals has been shown to be saturable and specific (15,16).

In this report, we investigated the mechanism of ZnPPIX inhibition of heme crystallization by binding to crystals. We also measured the amount of ZnPPIX bound to FePPIX crystals isolated from *P. falciparum*-infected erythrocytes. A new qualitative method, field emission in-lens scanning electron microscopy (FEISEM), compares the morphology of FePPIX crystal formation and inhibition growth extension. These biochemical data add further evidence for elevated ZnPPIX in the anemic erythrocyte providing protection from severe malaria disease by heme crystal inhibition.

MATERIALS AND METHODS

Materials

Hemozoin from *P. falciparum* infected erythrocytes and synthetic β -hematin were isolated as described previously (16). ZnPPIX was obtained from Porphyrin Products Ltd (Logan, UT, USA). All other reagents were from Sigma (St. Louis, MO, USA) unless specified.

FePPIX Crystal Assay

Purified FePPIX crystals (from 5 to 100 nmol) were incubated with 50 nmol of FePPIX chloride monomer overnight in 1 mL of 100 mM sodium acetate, pH 4.8, in a 37 °C water bath with different concentrations of ZnPPIX or drug. The next day, the samples were centrifuged at $10000 \times g$ after the addition of 100 μ L of wash buffer—100 mM sodium bicarbonate, 0.5% Triton X-100. The supernate was removed and the pellet suspended in wash buffer by sonication at the lowest setting and recentrifuged. This step ensures that unbound FePPIX and ZnPPIX are washed off of insoluble FePPIX crystals. The FePPIX crystal pellet is washed additionally with 0.5% Triton X-100. The final pellet is dissolved in 2% SDS and 20 mM sodium hydroxide to measure protoporphyrin IX content by UV spectrophotometric analysis at 400 and 416 nm (Beckman DU 640 spectrophotometer). FePPIX and ZnPPIX were prepared fresh daily in dimethyl sulfoxide with an extinction coefficient $\epsilon_{400} = 100000 M^{-1} cm^{-1}$ and $260000 M^{-1} cm^{-1}$ respectively for FePPIX and ZnPPIX in SDS/ 20 mM sodium hydroxide.

ZnPPIX Determination

The amount of ZnPPIX bound to the crystals was directly assayed by an ethyl acetate:acetic acid HCl extraction method, where 20 μ L of the sample was dissolved in 1 mL of 3:1 of ethyl acetate (OmniSolv Grade, EM Science, Gibbstown, NJ, USA) and acetic acid, which was centrifuged in glass tubes, and decanted into another similar tube. One milliliter of ethyl acetate and 1 mL of 3 N hydrochloric acid (high-performance liquid chromatography grade) was then added, the sample was vortexed briefly and centrifuged again, and the extracted ZnPPIX fluorescence was read in an optical fluorometer (Model 4-7349 American Instrument Co, Silver Spring, MD, USA) (30). Control standards of known concentration of FePPIX and ZnPPIX were also set up and assayed to

correlate intensity of fluorescence to actual amounts of ZnPPIX bound. The lower limit of detection is approximately 20 pmol of ZnPPIX. Alternatively, 15 to 20 μ L of sample were placed onto a coverslip and the ZnPPIX to FePPIX ratio was read directly on a hematofluorometer (AVIV Biomedicals). The lower limit sensitivity is improved with this ratio determination. For all determinations, the purified crystals of FePPIX with ZnPPIX bound were treated with 20 mM sodium hydroxide to decrystallize hemozoin. FePPIX crystals or hemozoin absorb substantially less than monomeric FePPIX at 400 nm, which will artificially increase the ZnPPIX value in relation to FePPIX in intact crystals (31).

Binding Competition Assay

We incubated 10 nmol of ZnPPIX and 10 nmol of FePPIX crystals with and without 10 nmol of FePPIX monomer and 10 nmol of drugs in 1 mL of 100 mM sodium acetate overnight at 37 °C. The samples were purified by Triton X-100 and bicarbonate washes as above with quantification of FePPIX and ZnPPIX by visible spectroscopy and the ethyl acetate method. SEM was calculated for all assays on triplicate samples.

Parasite-associated ZnPPIX

Parasites were cultured in human erythrocytes and synchronized by sorbitol lysis (32,33). Ring, trophozoite, or schizont stage parasite cultures were harvested by saponin lysis (34). A ZnPPIX to FePPIX ratio was determined on the AVIV hematofluorometer. An additional trophozoite pellet was processed on a 60% sucrose cushion (15). A separate parasite harvest was processed for hemozoin isolation (3). The parasite pellet was washed several times in 2% SDS and treated with 2 mg/mL proteinase K, washed again with SDS, and treated with 6 M urea. Samples were taken at every wash step and ratio of ZnPPIX to FePPIX was determined on the AVIV hematofluorometer.

Field Emission In-lens Scanning Electron Microscopy (FEISEM)

Prior to sample application, 5 \times 5 mm silica chips (Ted Pella, cat#16008, Redding, CA, USA) were cleaned with ethanol, coated with poly-lysine, and then rinsed 3 times with distilled water. Incubations of 1 to 3 nmol FePPIX crystals with or without monomers were applied onto chips in distilled water or the sodium acetate buffer. After centrifugation at $3000 \times g$ for 10 min, FePPIX crystals formed a thin layer on the chip surface. The sodium acetate buffer was diluted 50 fold by addition of distilled water and recentrifuged. Chips were then stained with 2% uranyl acetate for 30 to 60 min. The chips and crystals or monomers coating them were continuously in aqueous solution until after uranyl acetate staining. The procedure was next modified from the previous report (35) to eliminate ethanol washes that dissolve protoporphyrin IX monomers before a critical point drying. Normally, after 1 quick rinse with 50% ethanol, chips are rinsed consecutively in 50%, 70%, 90% ethanol for 3 min each, followed by 3 rinses in 100% ethanol for 3 min each before critical point drying for 1 to 2 h. Chips processed either with ethanol washes and critical point drying or without ethanol were coated with chromium. Silica chips loaded with samples were ready for FEISEM on the LEO 1550 instrument.

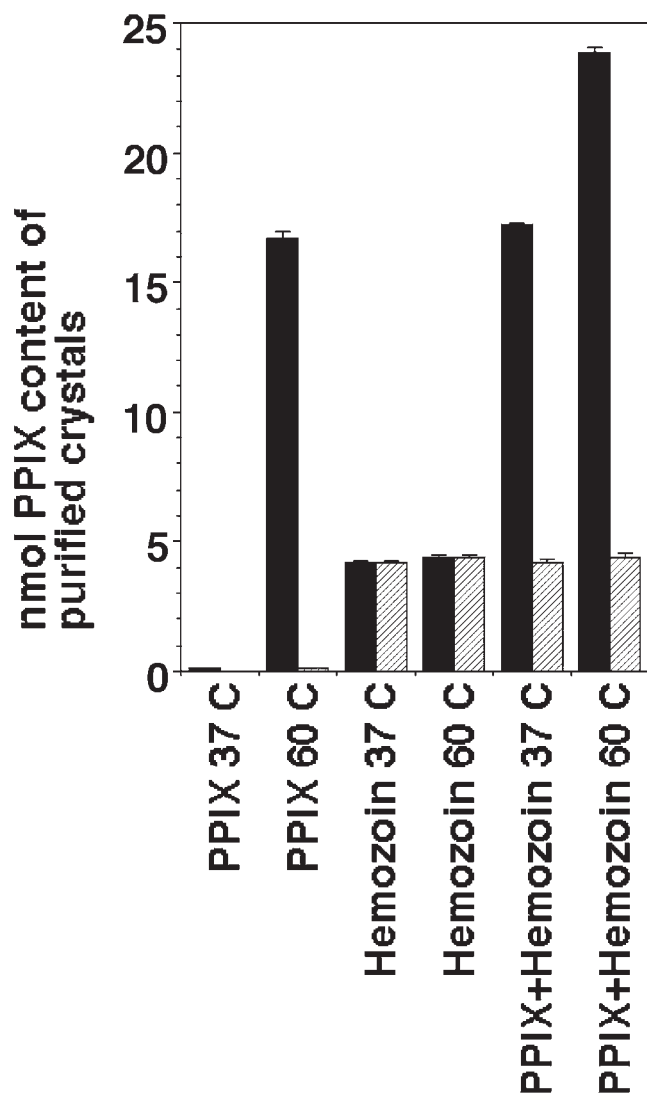


Figure 1. Crystal formation with FePPIX or ZnPPIX. Twenty-five nanomoles of FePPIX (solid bars) or ZnPPIX (hatched bars) were incubated overnight in 500 μ L of 100 mM sodium acetate, pH 4.8, at 37 $^{\circ}$ C and 60 $^{\circ}$ C with and without 5 nmol hemozoin. The incubation product was processed to purify away unincorporated protoporphyrin species, and the crystal content remaining was quantified. Only FePPIX alone at 60 $^{\circ}$ C or with hemozoin at 37 $^{\circ}$ C and 60 $^{\circ}$ C made measurable crystals. SEM is derived from triplicate samples.

RESULTS

ZnPPIX Does Not Measurably Form or Extend FePPIX Crystals

The yields of purified heme crystal conversion from monomer in acidic overnight incubations of 50 μ M FePPIX shows a temperature dependence (Figure 1). At 37 $^{\circ}$ C, less than 1% of substrate converts to crystals for FePPIX, whereas at 60 $^{\circ}$ C, approximately 70% of starting substrate converts to crystals for FePPIX. For similar 50 μ M ZnPPIX incubations, no measurable crystal conversion is detected at either temperature. The crystal conversion yield increases after addition of 5 nmol of purified

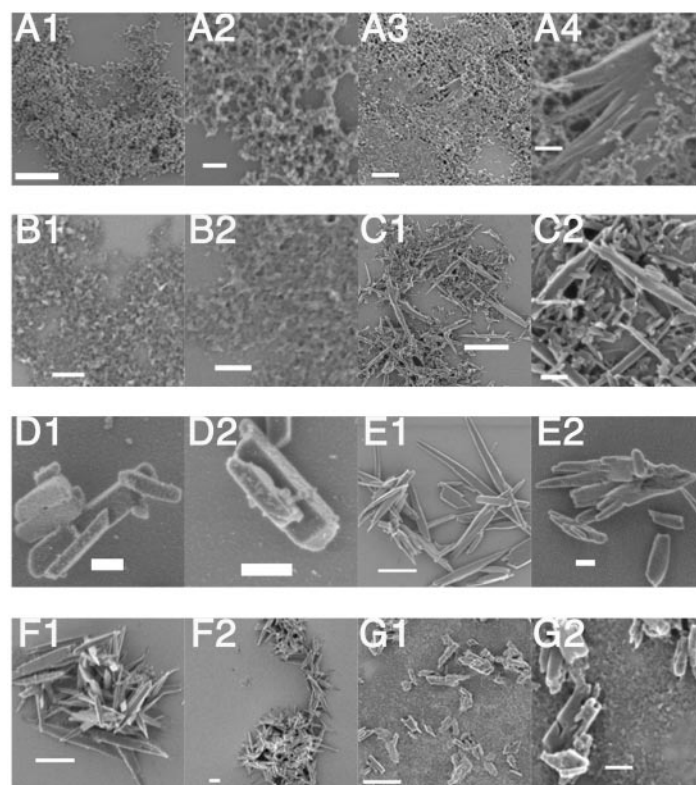


Figure 2. FESEM images of crystal formation and extension. The incubation products from Figure 1 were deposited onto a silica chip without ethanol washes or drying so that they would more closely represent solution morphology of the metal porphyrins. A: Incubations of 50 μ M FePPIX at 37 $^{\circ}$ C overnight remain 99% in moss-like aggregated state in A1 and A2 and are seen with a rare seed crystal in A3 and A4. B: ZnPPIX at 37 $^{\circ}$ C shows no seed crystals. C: When the temperature is raised to 60 $^{\circ}$ C, all of FePPIX monomer changes to long, thin, tapered crystals with no mossy aggregates seen. D: Extensively purified *P. falciparum* hemozoin has regular cuboidal smooth surfaces of about 100 nm \times 100 nm \times 300 nm. E: Extensively purified β -hematin is spiculated with a greater size range. F: Incubations of seeded hemozoin with 50 μ M FePPIX monomer show a spiculated appearance that resembles β -hematin more than the original *P. falciparum* hemozoin. G: ZnPPIX at both 37 $^{\circ}$ C and 60 $^{\circ}$ C shows no addition to preformed crystals. The size bar is 200 nm except for images A1, A3, C1, E1, F1, F2, and G1 where it is 1 micron.

preformed *P. falciparum* hemozoin seed crystals to the 50 μ M FePPIX substrate to about 50% and 80% of substrate at 37 $^{\circ}$ C and 60 $^{\circ}$ C, respectively. In contrast, addition of hemozoin seed crystals to 50 μ M ZnPPIX does not measurably extend the preformed FePPIX crystals.

FESEM qualitatively evaluates the morphology of the above experiments shown in Figure 1 in greater detail. The FESEM imaging protocol was adapted to a fully aqueous deposition and metal coating, which to a degree partially preserves noncrystal heme aggregate morphology by eliminating ethanol washes and critical point drying. The amount of FESEM distortion of the monomer or mu-oxy dimer that appears as moss-like aggregates is not known, yet crystals can easily be distinguished from noncrystal aggregates. Almost all FESEM images of incubations with 50 μ M FePPIX at 37 $^{\circ}$ C demonstrate no visible crystal formation (Figure 2A1 and 2A2). However in rare images, formation of a

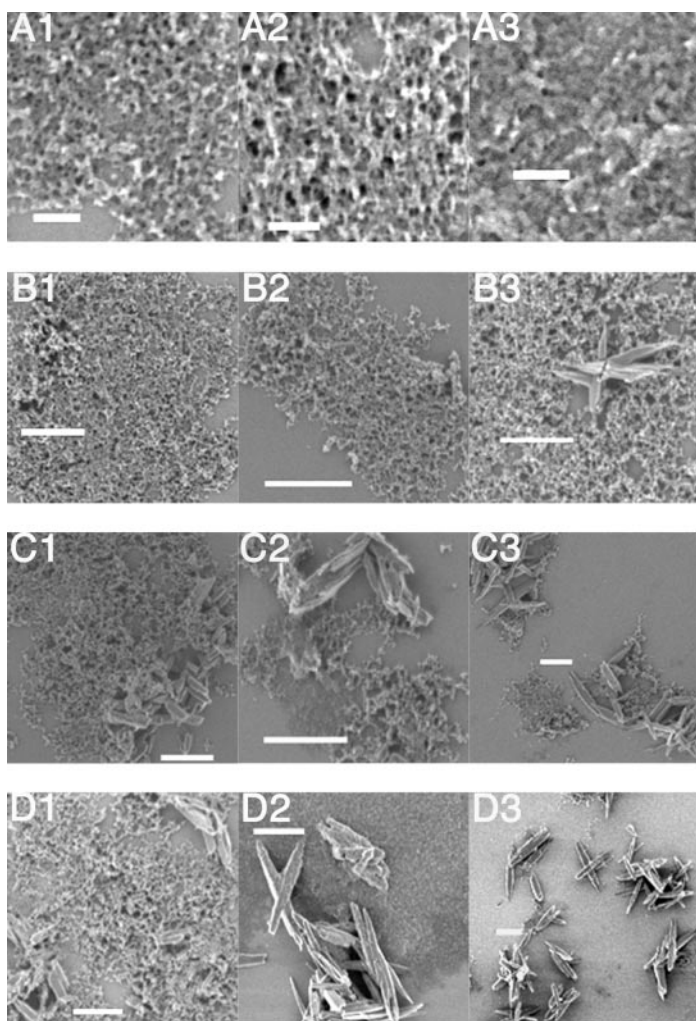


Figure 3. FESEM images of 10 μM PPIX or drugs with FePPIX with and without hemozoin. A: 10 μM ZnPPiX, FePPIX, and chloroquine were mixed in solution with 100 mM sodium acetate, pH 4.8, vortexed, and spun onto the silica chips for FESEM. A1: 10 μM FePPIX and chloroquine. A2: 10 μM ZnPPiX and FePPIX. A3: 10 μM ZnPPiX and chloroquine. B: Overnight incubations of 10 μM chloroquine, ZnPPiX, or artemisinin (B1, B2, B3, respectively) with 50 μM FePPIX showing only seed crystals with artemisinin. C and D: Incubations of 50 μM FePPIX with 10 μM chloroquine, ZnPPiX, artemisinin or no drug and the addition of 5 nmol seed crystals of hemozoin at 0 h, 12 h, and 24 h. C1: Time 0 h for chloroquine, hemozoin, and 50 μM FePPIX. Time 0 h is identical for FePPIX and hemozoin (not shown). C2: 12 h incubation of ZnPPiX, hemozoin, and 50 μM FePPIX. C3: 12 h incubation of hemozoin and 50 μM FePPIX. Incubation of artemisinin, hemozoin, and FePPIX is identical to C3 (not shown). D1: 24 h incubation of chloroquine, hemozoin, and 50 μM FePPIX showing no extension of crystal. The 12-h time point is identical (not shown). D2: 24-h incubations of ZnPPiX, hemozoin, and 50 μM FePPIX showing some spicule crystal extension, but largely monomer aggregates. D3: 24-h incubations of artemisinin, hemozoin, and 50 μM FePPIX showing almost total conversion of FePPIX substrate into FePPIX crystals. The size bar is 200 nm in A and 1 micron in B, C, and D.

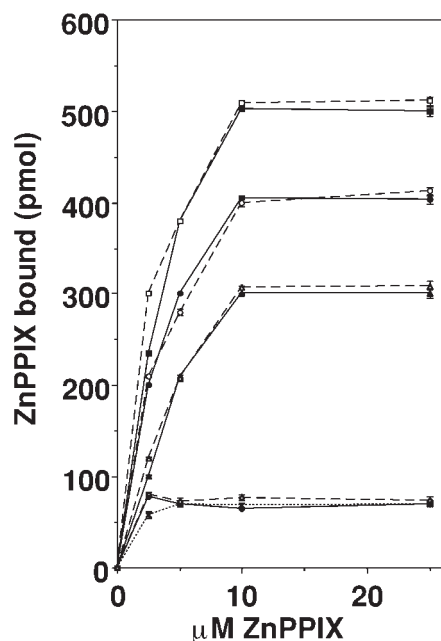
seed crystal is seen amidst aggregated uncrystallized FePPIX (see Figure 2A3 and 2A4). The purified crystal yield remains less than 1% starting heme substrate under these conditions, even with the rare small FePPIX crystals seen. ZnPPiX at all time points and temperatures remains aggregated with no nidus of crystal formation detected despite scanning many fields (see Figure 2B).

Heme crystals from *Plasmodium* parasites, historically termed “hemozoin”, are structurally identical to synthetic β -hematin made in vitro (3,4). Both are β -hematins containing a reciprocal coordinate bond of the central iron to adjacent oxygen of the heme side chain propionate carboxylate group. The head-to-tail Fe1-O41 dimer has been defined by powder diffraction data (4). Synthetic heme crystals of β -hematin can vary in microscopic morphology (36,37), but biochemically are similar to parasite hemozoin. Here we show that FESEM analysis of unpurified β -hematin product at 60 $^{\circ}\text{C}$ shows little moss-like aggregates, but large quantities of long thin tapered heme crystals of varying size (see Figure 2C). In contrast, purified *P. falciparum* hemozoin crystals have rectangular dimensions and smooth surfaces (see Figure 2D). Purified β -hematin prepared by the Egan method of heating mM amounts of heme in concentrated Molar solutions of acetic acid also has long thin tapering morphology, but less of the smaller crystals are recovered (see Figure 2E). Seeding the crystallization process with preformed hemozoin crystals accelerates the monomer to crystal conversion yield. The incubation mixture of preformed hemozoin FePPIX crystals from parasites with 50 μM FePPIX at both temperatures depicts a long, thin, tapered extension with the resultant product morphology more typical of β -hematin rather than the more cuboidal *P. falciparum* hemozoin (see Figure 2F). In contrast to FePPIX, 50 μM ZnPPiX alone as substrate incubated with hemozoin seed crystals shows no discernible addition of ZnPPiX to preformed crystals (see Figure 2G). A moss-like aggregate of unincorporated ZnPPiX is seen covering some of the preformed hemozoin crystals. Buller and colleagues have modeled the theoretical growth of heme crystals with discussion of a fast-growing dimension that may produce the long, thin, tapered crystals indicative of rapid in vitro growth. Conditions for growth in a test tube have not yet perfectly replicated the crystal morphology of the parasite, which may be more controlled. However, the biochemical characteristics of in vitro formation and inhibition approximate parasite biology.

ZnPPiX Inhibits Heme Crystal Extension

Spectroscopy has previously suggested π - π porphyrin-porphyrin ring interaction of ZnPPiX with FePPIX is similar to binding of many quinolines. FESEM imaging of 10 μM solutions of ZnPPiX plus FePPIX were compared with chloroquine plus FePPIX and chloroquine plus ZnPPiX in 100 mM sodium acetate, pH 4.8 (Figure 3A). The aggregated porphyrins were similar in appearance. At these concentrations, chloroquine does not deflect downward the optical density of the ZnPPiX indicating minimal to no interactions with this quinoline (data not shown). In contrast, the optical density of FePPIX is deflected downward by chloroquine. The artemisinin class of antimalarials, sesquiterpene lactones, also bind heme, but do not inhibit heme crystallization. Instead, the artemisinin endoperoxide bridge after interacting with free iron produces an oxygen radical essential for antimalarial action. This drug has been shown to inhibit the PfATPase calcium pump and not accumulate in the digestive vacuole that chloroquine targets (38). Incubations of the heme binding drugs chloroquine (10 μM), ZnPPiX and artemisinin with 50 μM FePPIX for 24 h show no nidus of FePPIX crystal formation with chloroquine or ZnPPiX

A



B

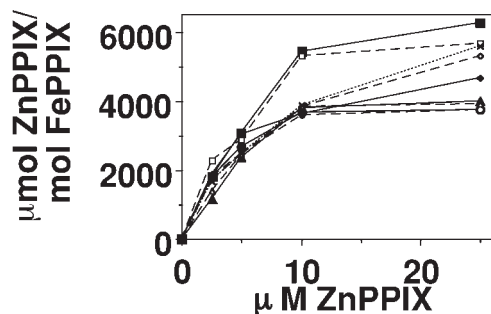


Figure 4. Saturable ZnPPiX binding to FePPiX crystals. A: ZnPPiX binding during a FePPiX crystal extension assay or directly to crystal rises with an increase in the amount of preformed FePPiX crystals used to seed the reaction. 100 nmol (■), 75 nmol (●), 50 nmol (▲), and 10 nmol (◆) of β -hematin or 10 nmol of parasite hemozoin (x) by FePPiX content was incubated with (filled symbols, solid lines) or without (empty symbols, dashed lines) 50 nmol FePPiX substrate and increasing concentrations of ZnPPiX in 1 mL of 100 mM sodium acetate, pH 4.8, at 37 °C overnight. The absolute amount determined by the ethyl acetate method of ZnPPiX bound after crystal purification was similar with or without the addition of FePPiX substrate. B: Molar ratios of ZnPPiX:FePPiX in purified FePPiX crystals saturated at 3000 to 6000 μ mol ZnPPiX/mol FePPiX. Picomoles of bound ZnPPiX in A are divided by nmol FePPiX content of recovered crystals. The symbols are the same as in A. SEM is calculated on triplicate samples.

(see Figure 3B1 and 3B2). However, incubations of artemisinin and FePPiX result in rare, small seed crystals (see Figure 3B3) similar to that of FePPiX alone, seen in Figure 2A3 and 2A4.

Chloroquine and ZnPPiX have previously been shown to have similar inhibitory concentrations to inhibit 50% of radioac-

tive heme incorporation into preformed hemozoin (26). The heme crystal assay here uses significantly lower FePPiX concentrations and seed crystals of purified hemozoin. Both ZnPPiX and chloroquine inhibit FePPiX crystal extension with an inhibitory concentration of approximately 3 to 5 μ M for 50% of the monomer to crystal conversion yield. Again FEISEM is employed to visualize at 12 h and 24 h the amount of crystal formation (see Figure 3C). At time 0, no extension products are visualized for all incubations with FePPiX, chloroquine and hemozoin as a representative sample (see Figure 3C1). The lower right corner illustrates preformed hemozoin mixed in with noncrystalline aggregated heme. The incubations of 10 μ M ZnPPiX with 50 μ M FePPiX plus hemozoin template show most of the substrate to be aggregated at 12 h (see Figure 3C2). After 12 h, substantially more of the FePPiX aggregates have converted to heme crystals in the reaction with no drug inhibition (see Figure 3C3). At 24 h, chloroquine still prevented any crystal growth (see Figure 3D1). The crystals evident at the corners are the preformed crystals that show minimal to no extension. Similar to chloroquine, ZnPPiX has still prevented most of the crystal extension (see Figure 3D2) with little change from the 12-h time point. In contrast, artemisinin, which also binds FePPiX, shows no inhibition of crystal growth (see Figure 3D3).

ZnPPiX Binds to FePPiX Crystals During Extension

Because ZnPPiX is naturally fluorescent, we assay the amount of ZnPPiX association to the growing FePPiX crystals in a similar crystal extension assay to that used for the quinoline antimalarials (15). Using 10 nmol of FePPiX crystals as a seed template either purified from parasites, or from synthetic β -hematin, approximately 70 pmol of ZnPPiX binds at saturation (Figure 4). Increasing the quantity of β -hematin FePPiX crystals to 50, 75, and 100 nmol, increases the amount of ZnPPiX bound at saturation. The more available synthetic β -hematin was used for the larger amounts of heme crystal, because biochemically it has almost identical characteristics to hemozoin measured by ZnPPiX binding with 10 nmol FePPiX crystals, as well as drug inhibition and susceptibility to hydrogen peroxide degradation (35,37). Control incubations with ZnPPiX alone or ZnPPiX plus 100 nmol of FePPiX monomer did not pellet detectable ZnPPiX in this assay (data not shown). Unlike the antimalarial quinolines that require the addition of FePPiX monomers for measurable binding to FePPiX crystals, the addition or deletion of 50 μ M FePPiX did not affect ZnPPiX saturation levels. A calculated μ mol ZnPPiX to mol FePPiX ratio levels off at 3000 to 6000, for saturating amounts of ZnPPiX despite increasing amounts of FePPiX crystal template or deleting FePPiX monomer (see Figure 4B). This indicates that ZnPPiX is binding to limited sites of the FePPiX crystals at a ratio of 1 ZnPPiX per 167 to 333 FePPiX molecules for preformed crystal templates. Similar to FePPiX extension, ZnPPiX binding to crystal template is at its highest at low pH of 4.0 decreasing to 10% binding by pH 6.0 in both MES and sodium acetate buffers (data not shown). A time course assay shows no measurable ZnPPiX bound to crystals at time 0 with increasing amounts measured up to a maximum at 16 h (data not shown).

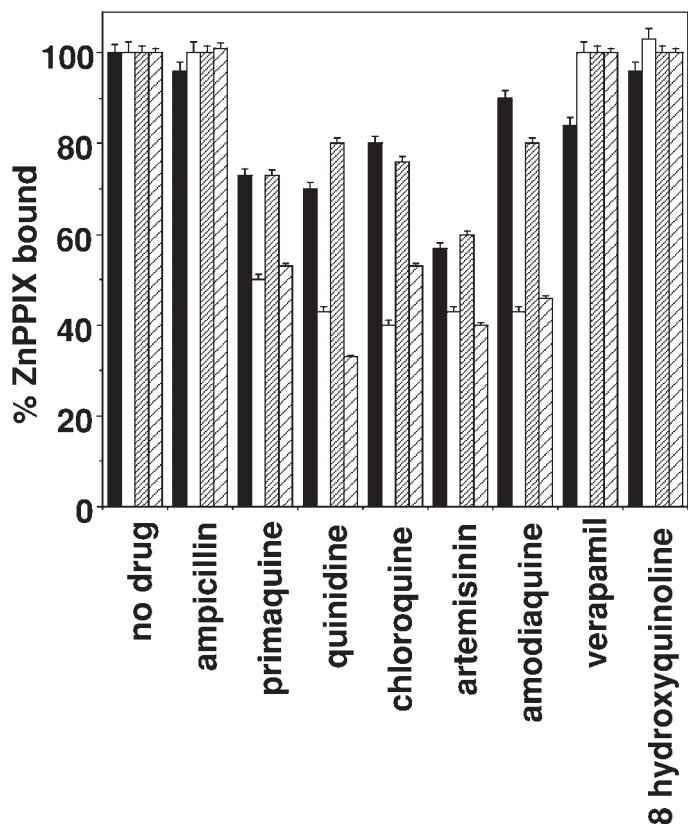


Figure 5. Antimalarial quinoline competition of ZnPPiX binding to crystals. ZnPPiX (10 nmol) and drug (10 nmol) were incubated with 10 nmol hemozoin (□, ■) or β-hematin (▨, ▩) without (■, ▩) or with (□, ▨) 10 nmol FePPiX, overnight in 1 mL of 100 mM sodium acetate, pH 4.8. The percent of ZnPPiX binding by the ethyl acetate method to recovered FePPiX crystal was measured. SEM is calculated on triplicate samples.

Quinolines Compete for ZnPPiX Binding to FePPiX Crystals

To investigate if specific antimalarial quinolines would compete for ZnPPiX binding, 10 μM drug concentrations are coincubated with 10 μM ZnPPiX in a FePPiX crystal binding assay without and with 10 μM FePPiX substrate. This binding assay differs from the extension assay because the lower concentration of FePPiX substrate results in no measurable increase in heme crystal (16). Active quinoline antimalarials incubated without FePPiX substrate compete with the ZnPPiX binding to both parasite hemozoin and β-hematin in the range of 20% to 40% (Figure 5). Verapamil, 8-hydroxyquinoline, and ampicillin do not compete. The metal chelators such as ferrozine, desferoxime, neocuprione, EDTA, and EGTA also do not compete for ZnPPiX binding (data not shown). Interestingly adding 10 μM FePPiX increases the amount of competition by the quinolines to 50% to 60%. Five μM of ZnPPiX also competes 82% of ³H-chloroquine and 57% of ³H-quinidine binding to FePPiX crystal in a similar radioactive binding assay reported previously to assess quinoline binding to hemozoin (data not shown) (16). This suggests that ZnPPiX binds to the same sites on

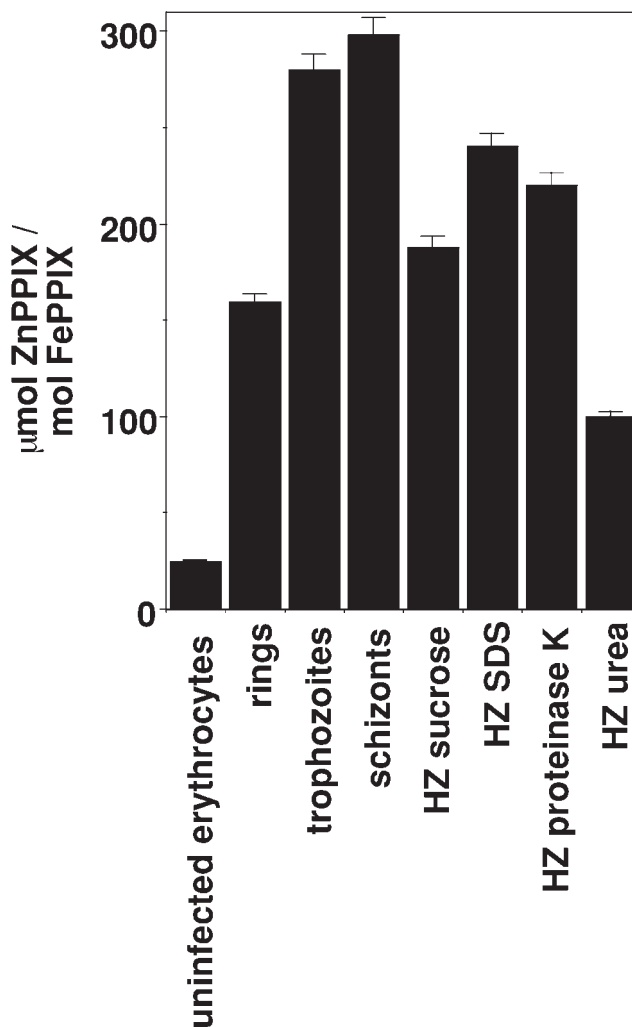


Figure 6. Elevated ratio of ZnPPiX to FePPiX in isolated parasite hemozoin. Parasites (3D7 strain of *P. falciparum*) were cultured in human erythrocytes and a saponin lysis purification was done at the stages of rings, trophozoites, and schizonts with greater than 90% synchrony. Trophozoite stages were also sonicated and placed on 60% sucrose cushions for ultracentrifugations to purify hemozoin (HZ sucrose). This hemozoin was washed with SDS (HZ SDS), treated with Proteinase K (HZ proteinase K), and urea (HZ urea). ZnPPiX:FePPiX ratios by the AVIV hematofluorometer were measured after treatment with 20 mM sodium hydroxide to decrystallize hemozoin.

the FePPiX crystals as the active quinoline antimalarials and that ZnPPiX binds in the absence of free FePPiX.

ZnPPiX Copurifies with FePPiX Crystals Within the Parasite

An important validation of ZnPPiX inhibition of FePPiX crystallization is demonstration of increased ZnPPiX to FePPiX ratios in isolated parasites and more specifically in purified FePPiX crystals (Figure 6). A synchronized population of parasites shows increasing ratios of ZnPPiX to FePPiX in saponin-lysed purified parasite pellets as more FePPiX crystal is made. A sucrose cushion purification that separates hemozoin from membranes and solu-

ble proteins shows a 10-fold elevation of ZnPPIX to FePPIX in hemozoin compared with ratios in normal erythrocytes. A more rigorous standard laboratory purification of hemozoin from parasite material involves washes with the detergent, SDS, followed by proteinase treatment and urea denaturation and further SDS and water washes (3,37). Radioactive chloroquine and quinidine are stripped from parasite hemozoin with this purification (data not shown). However the ZnPPIX to FePPIX ratio remains elevated after SDS and proteinase K treatment, but decreases to half the ratio value with urea treatment.

If hemozoin with ZnPPIX bound that is recovered from parasites cultured in normal erythrocytes or hemozoin with ZnPPIX bound in vitro (incubations with 10 or 50 μM ZnPPIX washed or diluted tenfold) is used to seed the in vitro FePPIX extension assay, less than 15% inhibition is seen. Chloroquine and quinidine also give the same minimal inhibition result.

DISCUSSION

This work demonstrates a role for the naturally occurring ZnPPIX in erythrocytes to inhibit FePPIX crystallization in *Plasmodium* by binding to the surface of FePPIX crystals. Here we demonstrate that ZnPPIX binding to FePPIX crystals is saturable and specific similar to the quinoline binding and inhibition. Unlike the quinolines, free heme is not a requirement for measurable ZnPPIX binding to crystals. Ring stage parasites also begin to make hemozoin and also were shown to have an elevated ZnPPIX/FePPIX ratio like trophozoites. The demarcation to trophozoite stage is the point that hemozoin is visible under oil immersion microscopy, but 3 to 12 h before that point, heme crystals are still made (2,39).

When ZnPPIX or the quinoline/FePPIX complex binds on a point of FePPIX crystal, extension could proceed adjacent to the site of inhibitor binding (17). ZnPPIX may need to reach more than 10 times the in vitro IC_{50} to have an inhibitory effect by surface binding. Because ZnPPIX, like FePPIX, readily absorbs proteins nonspecifically, a higher concentration would be necessary to inhibit large crystal formation. If the π - π interactions of ZnPPIX with FePPIX strictly acted by prevention of substrate from incorporation into formation of crystal, one should observe no increase in the ratios of ZnPPIX to FePPIX from purified FePPIX crystals. Bohle and colleagues have powder diffraction data for a head to tail heme crystal dimer rather than a heme polymer (4). Even though the reactive iron is no longer able to participate in Fenton chemistry to make oxygen radicals once the coordinate bond is made, the head-to-tail dimer may still be able to intercalate in membranes like heme monomer. Formation of the large hemozoin crystals, targeted by the quinolines and ZnPPIX, is then postulated to be a means of preventing membrane toxicity of the crystal dimers.

The IC_{50} of 5 μM is substantially less than the 500 μM previously reported by Martiney and Cerami or the 1.1 mM reported by Cole and Wright. However the assay used by Martiney uses approximately 125 nmol of hemozoin crystals and 15 μmol of FePPIX substrate (26). The assay may be described in a sense as a radioactive FePPIX binding assay with minimal extension. Chloroquine in this assay has an IC_{50} of 120 μM . The Cole/Wright

assay mirrored that of Egan who reported a 6 mM IC_{50} for chloroquine. This assay uses a very high insoluble concentration of 2.2 mM FePPIX at 60 °C and in a sense is an acid precipitation assay performed at the high temperature of 60 °C. The IC_{50} of 5 μM reported here in this assay with 50 μM FePPIX is close to that noted for chloroquine and quinidine (15). In all the different assays, the IC of ZnPPIX is close to chloroquine.

The results that show minimal inhibition of crystal extension in vitro with prebound ZnPPIX, chloroquine, or quinidine are explained by a mixture of covalent and noncovalent interactions for ZnPPIX and the noncovalent interactions for chloroquine and quinidine. The washes or dilution allows for a dissociation of noncovalent inhibitor, which then permits FePPIX extension to occur at a few sites. At equilibrium without washes or dilution of inhibitor association and disassociation in the test tube or parasite digestive vacuole inhibition of heme crystallization is achieved.

Normal erythrocytes have a 1:40000 ratio of ZnPPIX to FePPIX from hemoglobin equal to 25 μmol ZnPPIX:1 mol FePPIX. In *Plasmodium*, ZnPPIX accumulates in the parasite 10-fold more than in normal erythrocytes to 1:4000 or 250 μmol ZnPPIX to 1 mol FePPIX. In iron deficiency, the starting concentration of ZnPPIX in the erythrocyte is up 10-fold to 5 μM , which also equals to 1:4000 or 250 μmol ZnPPIX to 1 mol of FePPIX. Theoretically, in the erythrocyte of iron deficiency, the *Plasmodium* parasite may then encounter 100-fold concentration increases in ZnPPIX to 50 μM or 1:400, which would also equal 2500 μmol ZnPPIX to 1 mol FePPIX. This concentration is sufficient for saturable binding and inhibition of FePPIX crystal formation in vitro. Unique to *Plasmodium* infection, the normal erythrocyte has a potential drug, ZnPPIX, already present in the host cell cytosol at 0.5 μM . Alteration in the erythrocyte environment increases this potential drug 10- to 100-fold in the case of iron deficiency. ZnPPIX is delivered along with hemoglobin to the site of action of the quinolines in the digestive vacuole of the *Plasmodium* parasite. The ZnPPIX concentration increase should be regarded as an average for millions of heterogeneous erythrocytes counted. A small portion of the erythrocytes will have a lower concentration of ZnPPIX that is capable of supporting *Plasmodium* multiplication in the face of a high average concentration of ZnPPIX. The moderate elevation of ZnPPIX associated with anemia of chronic disease or acute infection is most likely not high enough to exert a significant effect on the inhibition of the parasite.

The implications for public health are that only the severely anemic individuals may have an intrinsic mechanism of protection from very high parasitemias. The ZnPPIX:FePPIX ratio is the average of a population of erythrocytes and still may support parasitemia. Most moderate and some severely anemic individuals should not have a ZnPPIX to FePPIX ratio above 250 and would not have exacerbation of malaria with iron replenishment therapy. The authors strongly support iron supplementation for the positive growth and cognitive benefits (24). Iron supplementation should not be withheld for fear of exacerbating malaria. Rather, severely anemic individuals with elevated ZnPPIX:FePPIX (above 250) should be targeted for antimalarial chemoprophylaxis. Further epidemiological studies designed specifically to this question of protection from severe malaria disease are a next step.

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