The Propionate of Heme Binds N_4O_2 Schiff Base Antimalarial Drug Complexes

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Introduction

As recently as 40 years ago, only 10% of the world's population was at risk from malaria. Today, due to increased vector resistance to insecticides and parasite resistance to drugs, over 40% of the world's population is at risk.[1−3] One biochemical target for drug development that has received considerable attention is the catabolism of hemoglobin and subsequent detoxification of released heme by the malaria parasite *Plasmodium falciparum*.[4] During the intraerythrocytic phase of its life cycle, the parasite can degrade as much as 80% of an infected host's red blood cell hemoglobin for needed amino acids in a specialized digestive vacuole (pH 4.8−5.2).[5] Ironically, the parasite's source for amino acids also contains free heme (Fe^{III}PPIX), which at high concentrations is toxic to the parasite.[6] To balance the requisite need for amino acids against the toxic effects of heme, *P. falciparum* has evolved a detoxification pathway that aggregates free heme into an insoluble biomineral known as hemozoin.[7] The recently reported crystal structure of hemozoin[8] revealed a dimer of five-coordinate Fe^{III}PPIX's linked by reciprocating monodentate carboxylato linkages from one of the protoporphyrin IX's propionate moieties. The biomineral is composed of an extensive network of these dimeric units hydrogen-bonded together via the second propionic acid group of protoporphyrin IX.

The quinoline family of antimalarials, and chloroquine particularly, has been one of the most successful class of drugs ever developed.[9] Recent studies clearly showed that these drugs are capable of disrupting hemozoin formation.[10−12] Unfortunately, in almost every location where chloroquine prophylaxis has been used, resistant strains of *Plasmodia* are now present, making the need for new drugs critical. Particularly appealing are new drugs that act on previously characterized targets. Recently Sharma and Piwnica-Worms reported a new class of antimalarials that, like chloroquine, target hemozoin aggregation. Using the hexadentate ethylenediamine-*N*,*N*′-bis(2-hydroxy-R-benzylimino) ligand (R-ENBPI) and a reduced (R-benzylamino) analogue (R-ENBPA), Al(III), Fe(III), Ga(III), and In(III) complexes have demonstrated a wide range of activity against a variety of chloroquine-senstitive and -resistant strains of *P. falciparum*.[13−15] Herein, we report that the basis of the observed inhibition of hemozoin aggregation by N_4O_2 Schiff base complexes is likely the formation of a salt complex between the anionic propionate moiety of heme and the cationic drug complex.

Experimental Methods

General Information. Hemin chloride ((Fe^{III}PPIX)Cl) and protoporphyrin IX (PPIX) were purchased from Sigma. All other reagents were purchased from commercial sources and used as received. All aqueous solutions were prepared using doubly deionized water.

Syntheses of the N_4O_2 Complexes. Fe^{III}ENBPI (**1**) and Ga^{III}ENBPI (**2**) were prepared by a modification of the method of Sharma et al. in which the N_4O_2 ligand was reacted with the appropriate metal salt in ethanol.[13] Isolated complexes were characterized by ^{1}H NMR and IR spectroscopy, electrospray mass spectrometry, and elemental analysis, the results of which were found to be consistent with previously reported values. Mg^{II}ENBPI (**3**) was synthesized as previously described by Polyakov et al.[16] and similarly characterized (Supporting Information).

Physical Measurements. IR analyses of dried reaction mixtures in KBr matrixes were performed on a Perkin-Elmer 1760 X FT-IR spectrophotometer. Typically, 256 scans at 4 cm^{-1} resolution were collected. UV−vis measurements were performed on a Cary 3-E UV−vis spectrophotometer, fluorescence measurements were obtained on a Photon Technology International QM-1 fluorometer, and ^{1}H NMR spectra were recorded on a Bruker ACP-300 MHz spectrometer.

In Vitro Heme Aggregation Assays. The template (2−2.5 nmol) was incubated with 50−100 μM hemin in 2 mL of 25−500 mM acetate buffer (pH 4.8) for 48 h in the presence of varying concentrations of drug dissolved in DMSO. Control blanks of DMSO showed that the solvent had no effect on the assay. Assay development and analysis were performed according to literature procedures[17] with quantitation based on absorbance at 400 nm in 1 mL of 0.1 M NaOH solution.

BNT Binding Assays. Binding studies were based on the UV−vis difference titration methods of Morgan.[18] Aliquots of a stock solution of the N_4O_2 complex substrate (≈1 mM) dissolved in DMSO were added to acetate buffer solutions (25−500 mM, pH 4.8) containing appropriate amounts of the nucleating template (1−10 nmol) and to the buffer solution blank. Samples were equilibrated for 15 min prior to measurements.

Porphyrin Aggregation Fluorescence Studies. Stock solutions of the fluorophore (N_4O_2 complex **1**, **2**, or **3**, dissolved in DMSO) and heme quencher (1.0 mM in 100 mM NaOH) were used. Fluorescence titrations were performed in a 4.0 mL quartz cell by adding varying aliquots of Fe^{III}PPIX quencher (final concentrations: 4−320 μM) to 20−40 μM fluorophore (**1**, **2**, or **3**) solutions in 100 mM, pH 4.8, acetate buffer. All samples were excited at their respective excitation maxima,

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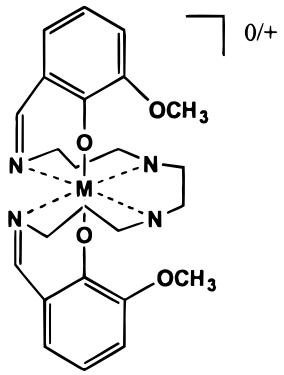

Figure 1. Representation of the N_4O_2 Schiff base antimalarial complexes. M = Fe(III), Ga(III), or Mg(II).

Fe^{III}ENBPI (**1**) at 310 nm, Ga^{III}ENBPI (**2**) at 380 nm, and Mg^{II}ENBPI (**3**) at 350 nm, and monitored at the emission maxima of the corresponding fluorophores.

Results and Discussion

The hexadentate ENBPI complexes (Figure 1) represent an adaptable class of antimalarial compounds due to the modular nature of the coordination complex reflected by the widely available choice of ligand components and potential metals. Piwnica-Worms and co-workers have taken advantage of this flexibility to explore a wide range of ligand substituents and central metal ions to map interesting metal and ligand dependencies for these drugs against both chloroquine-sensitive and -resistant strains of *P. falciparum*.[13−15] Goldberg et al. demonstrated that in vivo hemozoin formation was greatly diminished in parasites cultured in the presence of Fe^{III}ENBPI (**1**) and Ga^{III}-ENBPI (**2**), while control parasites matured normally. Further examination showed that **1** was effective at inhibiting the in vitro histidine-rich protein II (HRP II) mediated aggregation of heme ($IC_{50} \sim 4\ \mu M$).[13] While a detailed analysis of several crystal structures and NMR solution data suggested that the spatial orientation of the ligand's aromatic periphery was critical to imparting the favorable biotransport properties of these drugs,[14] the specific role of the coordination complex in the prevention of hemozoin aggregation has remained unclear.

Recently, we showed that dendrimeric peptide bionucleating templates (BNT II) based on the putative heme-binding domain of HRP II function as excellent HRP analogues in in vitro hemozoin aggregation assays.[19] Using the in vitro BNT II system, the dose responses of hemozoin formation to the complexes Fe^{III}ENBPI, Ga^{III}ENBPI, and Mg^{II}ENBPI (**1**−**3**) were evaluated. At 25 mM acetate buffer, pH 4.8, the half-maximal inhibitory effects (IC_{50}) for **1** and **2** were 9.5 and 58 μM, respectively. These values were comparable to previously reported IC_{50} values.[13] The neutral Mg^{II}ENBPI complex (**3**) did not inhibit hemozoin aggregation. Although the antimalarial activity of **3** had not previously been examined, Piwnica-Worms and co-workers compared the activities of cationic and neutral Schiff base complexes to probe the important role that charge may play in the efflux transport of these agents by MDR1 P-glycoprotein in cancerous tumors.[16] By contrasting the cationic complexes **1** and **2** with neutral **3**, they demonstrated the importance of the charge of the drug in its biolocalization properties. In our analysis of these complexes as hemozoin aggregation inhibitors, the difference in activity between the cationic complexes **1** and **2** and the neutral complex **3** revealed that the charge of the complex was critical for inhibition of heme aggregation.

Table 1. Acetate Buffer Dependence of the IC_{50} of N_4O_2 Schiff Base Antimalarial Complexes for the Inhibition of BNT II Mediated Hemozoin Aggregation

	IC_{50}, μM		
complex	25 mM acetate buffer	100 mM acetate buffer	500 mM acetate buffer
1	9.5	15	NI
2	58	NI	NI
3	NI	NI	NI
CQ[19]	40	40	40

[a] NI = no inhibition; CQ = chloroquine.

The proposed mechanisms for hemozoin aggregation inhibitors fall into three classes: (1) chemisorption of the drug onto crystallized hemozoin, leading to inhibition of further aggregation,[20] (2) inhibition of proteins that are responsible for the nucleation of hemozoin,[11] and (3) direct binding of the drug to heme, which consequently disrupts the crystallization of hemozoin.[21] Pagola et al. have argued that the structure of hemozoin provides support for a chemisorption mode of action by planar aromatic-rich compounds such as the quinolines.[8] The topology of the N_4O_2 Schiff base complexes does not, however, immediately suggest the planar π-rich environments of traditional heme aggregation inhibitors. Additionally, the control experiments below show that complexes **1**−**3** do not appreciably bind to the bionucleating template. Considering the minimal hemozoin aggregation assay system employed in this study, a readily envisioned mode of action for these cationic complexes is that they prevent the formation of the critical axial linkage necessary for the dimeric unit of the hemozoin aggregate by interacting with the anionic propionate moiety of heme. The formation of such a drug−heme salt complex would explain the observed charge dependence of the inhibition of hemozoin aggregation by ENBPI complexes.

If the mode of action of these antimalarial coordination complexes originates from specific interactions with the heme's propionate moiety, then their efficacy should be sensitive not only to the complex's charge but also to the concentration of a competing receptor, such as acetate. Table 1 shows the effect on IC_{50} values for complexes **1** and **2** as the concentration of the acetate buffer increases. At 500 mM acetate buffer, all of the complexes are ineffective hemozoin inhibitors. In contrast, assays performed with 25 mM acetate buffer and increasing concentrations of NaCl reveal no effect on either hemozoin aggregation or the IC_{50} values of complexes **1** and **2** (Figure 2). This suggests that the interactions between the drug complexes and the heme propionate are not simply charge dependent but specific for the carboxylate moiety. The observed sensitivity of the cationic ENBPI complexes to the concentration of acetate in the buffer is in sharp contrast to the behavior of other classes of antimalarial heme aggregation inhibitors, such as the quinolines, hydroxyxanthones, and porphyrins. The latter compounds, believed to act via a different mechanism, demonstrate no sensitivity to acetate buffer concentration in either the template-based heme aggregation assay[19] or the heme polymerization inhibition activity assay of Egan et al.[22]

The fluorescence quenching studies of the Schiff base chromophores **1**−**3** by Fe^{III}PPIX provide additional evidence

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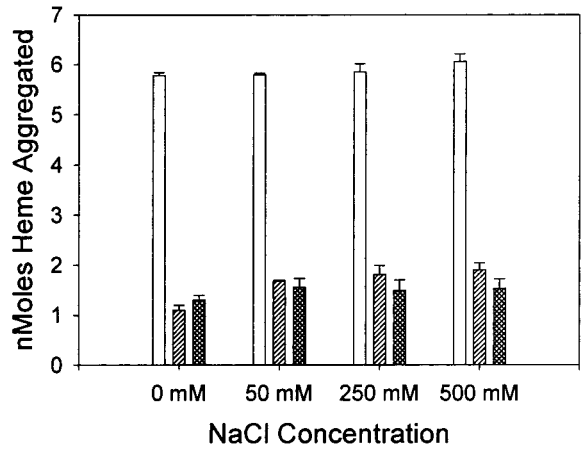

Figure 2. Effect of increasing salt concentration on hemozoin aggregation and drug inhibition. Assays were performed in 25 mM acetate buffer (pH 4.8) with increasing concentration of NaCl as described in the Experimental Section. Drug concentrations for **1** and **2** (50 and 200 μM, respectively) were selected near maximal inhibitory concentrations. Vertical bar identifications in each set: left, no drug; middle, Fe^{III}ENBPI; right, Ga^{III}ENBPI.

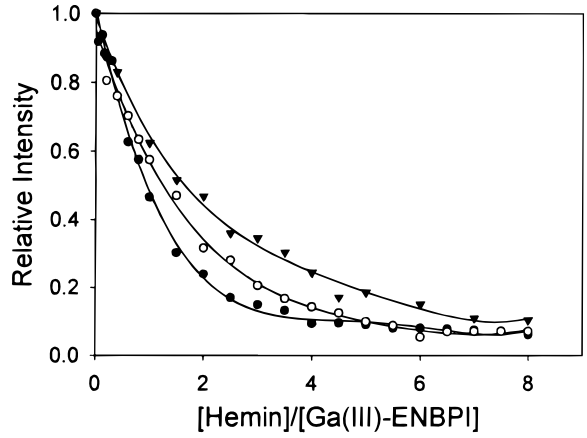

Figure 3. Representative study of fluorescence quenching of Ga^{III}-ENBPI by Fe^{III}PPIX at varying concentrations of acetate buffer (pH 4.8): ●, 25 mM; ○, 100 mM; ▼, 500 mM. Each point represents the average of three individual experiments with a standard deviation of no more than 5% for any given point.

for interactions between the drugs and heme. A variety of porphyrins were used previously in fluorescence quenching experiments as indirect probes for solution interactions among π-stacked complexes, salt complexes, and pH-induced aggregates.[23,24] Complexes **1** and **2** are moderately fluorescent and **3** is weakly fluorescent in aqueous acetate buffer, pH 4.8 (Supporting Information). The titration of dilute solutions of complexes **1**−**3** with Fe^{III}PPIX resulted in the partial quenching of the fluorophore Schiff base complexes (Figure 3 and Supporting Information). At ratios of 1:1 Fe^{III}PPIX to complex, the fluorescence values for **1**−**3** were 70%, 50%, and 60%, respectively, of the original intensities. Complete quenching of **1** and **2** was only achieved with 4−6-fold excesses of Fe^{III}-PPIX, whereas complete quenching of **3** was achieved with 7−8.5-fold excesses of heme. Quenching studies at increasing acetate buffer concentrations showed a systematic decrease in the quenching of fluorescence by Fe^{III}PPIX as the buffer concentrations increased for complexes **1** and **2**. Mg^{II}ENBPI showed little sensitivity to acetate buffer concentration after an initial decrease in quenching from 25 to 50 mM.

Controls for the effect of possible demetalation reactions were negative.[13] NMR stability studies (48 h, 37 °C, pH 4.8, 25 or 500 mM acetate buffer) of complexes **1**−**3** showed no signs of hydrolysis. Unlike Polyakov et al.,[16] we do not, under these reaction conditions, observe the appearance of a proton resonance associated with an aldimino proton, an expected signature of demetalation (Supporting Information). Additional controls that monitored the fluorescence emissions of the individual complexes at either 25 or 500 mM acetate buffer concentration (pH 4.8), 37 °C showed no decrease in the intensities of the emissions over a 48 h period. Possible binding of **1**−**3** to the biomineralization template as a mode of drug action is ruled out by binding studies based on the UV−vis difference titration methods of Morgan[18] showing that none of the Schiff base complexes appreciably bind BNT II. This contrasts to a number of metal-substituted heme analogues which are effective inhibitors of hemozoin formation and do bind to BNT II.[19]

Summary

The Schiff base N_4O_2 complexes offer a flexible template on which to develop novel antimalarial drug complexes which inhibit the aggregation of hemozoin. The high degree of synthetic control over the ligand scaffolding and the wide choice of coordinating metals offer considerable potential for long-range viability against developing resistance. Detailed structural studies, both solid state and solution, have highlighted the importance of the spatial arrangement of the ligand periphery in obtaining selective biotransport and localization properties that would be effective against chloroquine-sensitive and -resistant strains of *P. falciparum*.[13−15] The studies presented here demonstrate that the overall charge of the complex is critical to the drug's ability to inhibit hemozoin formation. Furthermore, evidence supports the hypothesis that these cationic complexes inhibit hemozoin formation via the formation of a specific drug/heme propionate salt. The formation of such a salt complex limits the propionate's ability to form the requisite axial linkage of the repeating dimeric unit in the hemozoin aggregate.

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Supporting Information Available: Text presenting descriptions of the ligand and complex syntheses (**1**−**3**) and complex characterizations, plots of hemozoin inhibition for complexes **1**−**3** at varying acetate buffer concentrations, fluorescence spectra of **1**−**3**, fluorescence quenching plots for studies between Fe^{III}PPIX and complexes **1**−**3** at varying concentrations of acetate buffer, and NMR and fluorescence stability plots for **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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