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Evidences for the formation of bisbenzamidine–heme complexes in cell-free systems

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Abstract—Infrared and colorimetry data suggest that bisbenzamidines connected by various rigid or flexible linkers are able to interact with heme in cell-free systems. At pH 5.0 the inhibition of formation of β-hematin could be ascertained by infrared spectroscopy whereas at pH 7.0 the interaction yielded insoluble complexes for which a sandwich-type structure of stoichiometry 2:1, heme–drug, is tentatively proposed.

1. Introduction

Malaria[1] represents the most widespread and deadly parasitic disease in man, killing more than one million people each year essentially in the regions located between the tropics. Among the plethora of antimalarial drugs, chloroquine (**1**) is recognized as an exceptionally safe drug that has been successfully used for more than 50 years. However, its intensive use has led to the emergence of resistant strains of *Plasmodium falciparum*, the most predominant and dangerous of the four species of the parasite.

Plasmodium falciparum has a life cycle divided into three overall stages (mosquito, liver, and blood stages) that can be selectively targeted by chemotherapeutic agents. Chloroquine and most structurally related antimalarials (quinine, mefloquine) are generally thought to act during the blood stage, when the parasite digests hemoglobin to obtain the amino acids it requires. This reaction produces free heme (ferriprotoporphyrin IX, FPIX; hemin and hematin are the terms used to characterized FPIX chloride and FPIX hydroxide respectively), which is toxic to *Plasmodium falciparum*. Detoxication of heme can occur in the lysosomal vacuole of the parasite via formation of an insoluble polymer called hemozoin, or malaria pigment (β-hematin is the term used to characterize the synthetic form of hemozoin[2]). Chloroquine is believed to exert its antimalarial action primarily by inhibiting hemozoin formation either by binding to the free heme or by capping the end of the growing polymer.[1] Other modes of action that have been proposed for chloroquine and structurally related antimalarials include the inhibition of detoxication routes that are dependent on the peroxidative degradation of heme within the food vacuole[3] or on the glutathione-mediated reaction outside the vacuole.[4]

As part of our research program[5] aimed at the design of novel pentamidine (**2**) congeners as improved antimicrobial agents, we recently focused our attention on their antiplasmodial activity. Pentamidine is an important therapeutic agent that is clinically used in the treatment of *Pneumocystis carinii* pneumonia, African trypanosomiasis, and leishmaniasis. However, there are only limited reports on the antiplasmodial effects of pentamidine. Recently Stead et al.[6] suggested that the antiplasmodial action of pentamidine is based on its selective transport through a specific pore in the parasite and the subsequent binding to FPIX and inhibition of hemozoin formation. In order to assess whether other aromatic diamidines might act in a manner similar to pentamidine, we analyzed their behavior in the presence of FPIX in cell-free systems. These ex vivo experiments were conducted with the series of derivatives depicted in Figure 1.

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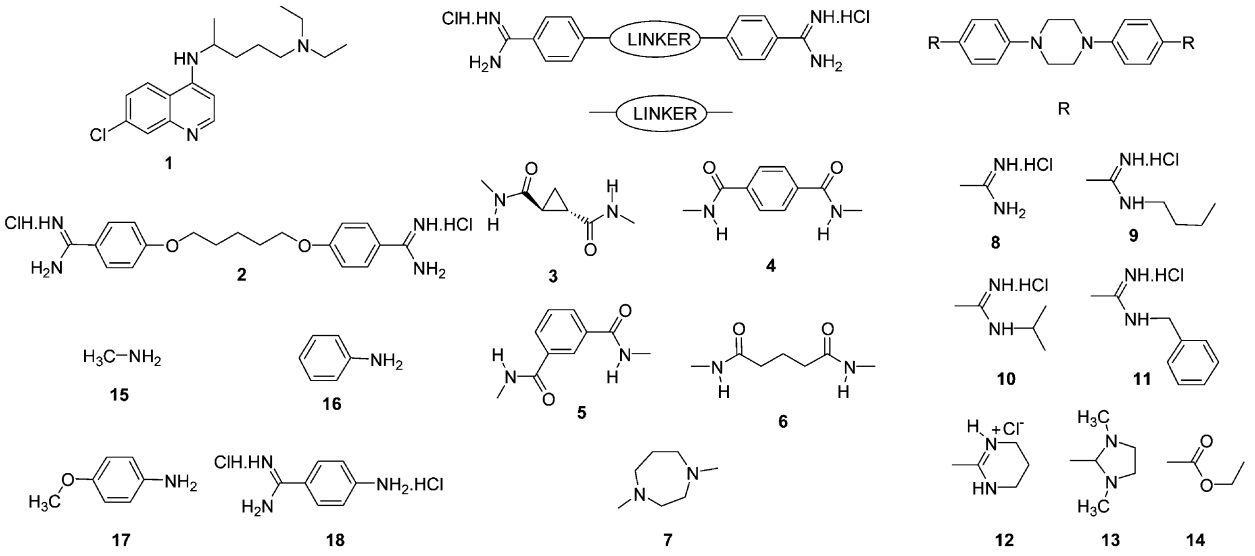

Figure 1. Structure of the compounds used in this study.

2. Infrared study

Conversion of hemin into β-hematin can be effected in non-biological media as reported by several authors.[6–10] In particular, Egan[9] demonstrated that the reaction readily proceeded under acidic conditions (acetate buffer, pH 5.0) yielding to the precipitation of β-hematin. However, this reaction can be inhibited by addition of antimalarials (quinine, chloroquine, amodiaquin) that are known to bind to heme and function as intra-erythrocytically active agents.

Inhibition of β-hematin formation can be readily monitored using IR spectroscopy by recording the intensities of several peaks typically attributed to β-hematin around 1710 (major peak corresponding to the carbonyl groups of porphyrin skeleton), 1660, and 1210 cm^{-1}.

In the present study, we first confirmed the formation of β-hematin from a basic solution of hemin (5 mM), which was subsequently neutralized and then acidified to pH 5.0 with 5 M acetate buffer. Such a final medium is supposed to mimic the conditions that exist in the digestive vacuole of the parasite, where the pH is about 5.0 and heme concentrations can reach values as high as 400 mM.[11–13] As described in the literature, inhibition of the reaction in the presence of chloroquine (15 mM, 3 equivalents) was observed. This experiment was also supposed to mimic the in vivo conditions because chloroquine is known[14–18] to accumulate in the digestive vacuole of the parasite to millimolar levels. Interestingly, addition of bisbenzamidines **3–12** (15 mM, 3 equivalents) also inhibited the polymerization as indicated by the weak intensity of the absorbance (if any) around 1710 cm^{-1} in the IR spectra of the precipitate. The intensity of the peaks at 1660 and 1210 cm^{-1} (also indicative of the formation of β-hematin) could hardly be monitored due to overlaps with other vibration bands arising from the bisbenzamidines. On the other hand, methylamine (**15**), aniline (**16**), *para*-anisidine (**17**), and even 4-aminobenzamidine (**18**, a monobenzamidine) did not prevent precipitation of β-hematin. Other 1,4-diarylpiperazines, which are structurally related to the bisbenzamidines **8–12** but which do not bear the amidine functions (namely compounds **13** and **14**), were also unable to inhibit formation of β-hematin.

3. Colorimetry study

Formation of a complex between pentamidine and ferriprotoporphyrin at micromolar concentrations in a neutral cell-free system could be observed by visible spectroscopy as reported by Stead et al.[6] The authors showed that the absorbance around 400 nm of a solution of FPIX (3 μM) decreased from 0.18 to 0.13 when one equivalent of pentamidine (3 μM) is present in a solution of HEPES buffer, pH 7.0.

In the first set of experiments, we reproduced that reaction and we noticed a comparable decrease of the absorbance either with pentamidine (**2**) or with the piperazine-linked bisbenzamidine **8**. However, this effect could not be duplicated with either chloroquine (**1**) or derivative **9**, a N-substituted bisbenzamidine. This prompted us to repeat the experiments using solutions 10 times more concentrated (because the values of the absorbance at 400 nm did not exceed 0.2 in the first set of experiments). Under the new experimental conditions, we could not record stable reproducible readings of the absorbance of a solution of heme (30 μM) with either **2** (30 μM) or **8** (30 μM), since it continuously decreased as a function of time. This phenomenon could readily be explained by visual inspection of the cuvettes in which a precipitate was formed leaving, after a few hours, a colorless supernatant as shown in Figure 2. Similar behaviors were also observed from **3–7**, **11**, and **12**. The fact that precipitation was not observed when using lower concentrations (3 μM), as we did in the

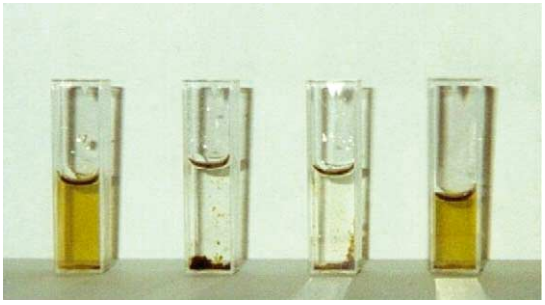

Figure 2. Reaction media obtained, after 4 h at rt and pH 7.0 in a HEPES buffer, from hemin and (from left to right) no reactant added, **8**, **2**, and **1**.

preliminary experiments, can reasonably be attributed to the solubility (K_s) of the complexes.

Unexpectedly, chloroquine (**1**, 30 μM), which is known to interfere in the process of malaria pigment formation, did not give rise to a decrease of the absorbance of the heme solution nor to the formation of a precipitate. Solutions of FPIX and compounds **9** or **10** were also stable. This demonstrates the importance of the solubility of the complex as a function of the pH. Indeed, acidification to pH 5 (with 5 M acetate buffer) of the solutions containing heme and **1**, **9**, or **10** afforded a precipitate accompanied by a discoloration of the mixture. On the other hand, solutions of FPIX and the amines **15**–**17**, 4-aminobenzamidine (**18**), or derivatives **13** and **14** were quite stable without any precipitate formation for several weeks at pH 7.0 as well as after acidification to pH 5.0.

Although the exact structure of the complexes, that is, the precipitates obtained in the colorimetry experiments, must still be elucidated, several arguments tend to indicate that all of them possess similar features. In particular, the IR spectra of the complexes exhibited many vibrational bands at comparable wavelengths (including an intense peak at 1700 cm^{-1}). Therefore, because the primary interaction between heme and quinolines might result from π–π interactions in a sandwich-type complex[19,20] of a 2:1 stoichiometry (heme–drug), our data suggest that bisbenzamidines **2**–**12** could behave in a similar way. An elemental analysis of a crude aggregate obtained from hemin and **9** also supported this assumption because the calculated and found values for C, H, and N differed by less than 0.4% for the monohydrate of a substance composed of 2 heme skeletons and one molecule of **9** ($C_{68}H_{64}Fe_2N_8O_8 + C_{26}H_{40}Cl_2N_6 + H_2O$). However, the proposed heme–drug (2:1) complex is tentative and further investigations are currently in progress to determine the association constants and to refine the structure of the precipitates that we isolated.

4. Conclusion

Numerous studies indicated that chloroquine and other antimalarials, for example, xanthones,[21] exert their antiplasmodial activities by complexation to heme and inhibition of hemozoin formation. Such a reaction can be mimicked in cell-free systems and our data[22–24] revealed that bisbenzamidines[25] structurally related to pentamidine also behaved in a similar manner. It is noteworthy that a good correlation between the binding of these compounds to heme in the cell-free experiments and their in vitro antiplasmodial activities could be established.[22] Indeed, bisbenzamidines **2**–**12** (except **3**) displayed potent inhibitory activity (IC_{50} values ranged from 3 to 200 nM) against two *Plasmodium falciparum* strains including a cloned chloroquine-susceptible strain from Haiti (Haiti 135) and a cloned chloroquine-resistant strain from Indochina (Indochina I), whereas, derivatives **13**–**18**, which did not form complexes with heme, were inactive against the two *Plasmodium falciparum* strains.

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23. *Infrared study*.[9,10] A solution of hemin (15.2 mg) in aqueous sodium hydroxide 0.1 M (3 mL) was thermostatted at 60 °C and diluted with warm solutions (60 °C) of hydrochloric acid 1.0 M (0.3 mL) and buffer acetate 5.0 M (pH 5.0; 1.8 mL). Three equivalents (70 mmol) of the compound to be tested were added and the mixture was stirred at 60 °C for 30 min. The reaction medium was cooled down in an ice bath for 5 min. The solid was filtered, washed with water and dried before analysis.
24. *Colorimetry study*.[6] A stock solution (10 mL) of the studied compound (solution A) was prepared by dissolving 2 μmol of that compound in a mixture of DMSO (1 mL) and HEPES buffer 0.2 M (pH 7.0). A stock solution (10 mL) of hemin (solution B) was prepared by dissolving 2 μmol (1.3 mg) of hemin in a mixture of aqueous sodium hydroxide 0.1 M (5 mL) and HEPES buffer 0.2 M. The solution (5 mL) of the studied compound that was scanned was obtained by mixing solution A (1 mL), solution B (1 mL), and HEPES buffer 0.2 M. The blank (5 mL) that was scanned was obtained in a similar way but in the absence of the compound and hemin. The solution (5 mL) of hemin that was scanned was also obtained in a similar way but in the absence of the compound. All solutions were prepared in volumetric flasks.
25. Compounds **1**, **2**, and **15**–**18** were commercially available. Compounds **3**–**5** and **7**–**14** were described in the literature.[5a,c,22,26–28] *N,N′-bis[4-(aminoiminomethyl)phenyl] pentanediamide, dihydrochloride salt,* (**6**) was obtained by heating under reflux for 30 min a mixture of 4-aminobenzamidine monohydrochloride (1.60 g; 10 mmol), glutaryl dichloride (0.85 g; 0.69 mL; 5 mmol), and pyridine (3.95 g; 4.07 mL; 50 mmol) in DMF (40 mL). After cooling, the solid was filtered and washed with acetone. Yield: 75%. M.p.: >300 °C. NMR (DMSO-d_6): 10.5 (s, 2H); 9.1 (br, 8H); 7.8 (m, 8H); 2.5 (t, 4H); 1.9 (quint, 2H) ppm. IR: 3400–2300; 3094; 1923; 1659; 1604; 1351 cm^{-1}. Anal. calcd for $C_{19}H_{22}N_6O_2{\cdot}2HCl$ (439.34): C, 51.94; H, 5.51; N, 19.13. Found: C, 51.79; H, 5.31; N, 18.89. (M-H-W Laboratories, Phoenix, AZ).
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