FEBS Letters 352 (1994) 54–57

FEBS 14534

Quinoline anti-malarial drugs inhibit spontaneous formation of β-haematin (malaria pigment)

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Received 2 August 1994

Abstract Polymerisation of haematin to β-haematin (haemozoin or malaria pigment) in acidic acetate solutions was studied using infrared spectroscopy. The reaction was found to occur spontaneously between 6 and 65°C, in 0.1–4.5 M acetate and pH 4.2–5.0. The anti-malarial drugs quinine, chloroquine and amodiaquin were found to block spontaneous β-haematin formation, while the anti-malarially inactive 9-epiquinine and 8-hydroxyquinoline had no effect on the reaction, as did primaquine, a drug which is active only against exo-erythrocytic stages of infection. It is argued that the intra-erythrocytically active anti-malarial agents act by binding to haematin, blocking β-haematin formation and leaving toxic haematin in the parasite food vacuoles.

Key words: Quinoline anti-malarial drug; Haem; β-Haematin; Haemozoin

1. Introduction

The high incidence of malaria, together with the appearance of drug-resistant strains of the organism, has rendered this disease a major human health problem [1]. The parasite infects host red blood cells, ingesting and degrading haemoglobin in acidic food vacuoles [2]. Released haem, which is toxic to the parasite [3], is converted into insoluble and inert malaria pigment (haemozoin) [4], a haem polymer referred to as β-haematin [5]. The mode of action of quinoline anti-malarial drugs remains uncertain. It has been proposed that the drugs either raise the pH of the food vacuole [6], inhibiting haemoglobin proteolysis, alternatively that they bind to haem [7], killing the organism by toxic effects [8], or that they inhibit a putative haem polymerase enzyme, preventing haemozoin formation [9]. In the current study it is shown that spontaneous formation of β-haematin is inhibited by the anti-malarial agents chloroquine, quinine and amodiaquin, but not by the therapeutically inactive substances 9-epiquinine and 8-hydroxyquinoline, nor by primaquine, a quinoline anti-malarial drug not active against the intra-erythrocytic stages of the parasite. This suggests that the mode of action of the intra-erythrocytically active anti-malarials is to simply complex haem and prevent polymerization to β-haematin. Based on these results, criteria for the rational design of these anti-malarial drugs can be envisaged.

2. Materials and methods

The formation of β-haematin can be brought about non-biologically, i.e. in the absence of proteins, peptides etc., and it has been reported that product formation can be monitored by the differential solubilities of haematin and β-haematin in carbonate buffer (pH 10.2), the former being soluble and the latter insoluble [5,9]. In the current study, unreliable and non-reproducible results were obtained by this method due to difficulties encountered in centrifuging β-haematin and to the propensity of haem to adsorb onto submicron filters. Infrared spectroscopy was therefore used to characterize the reaction products [5]. This technique unequivocally distinguishes between haematin and β-haematin (see Fig. 1). The latter has sharp bands at 1660 and 1207 cm^{-1} which are absent in the former.

Haematin was obtained by dissolving 15 mg haemin (Sigma; used as supplied or recrystallized from chloroform/acetic acid) in 3.0 ml of 0.1 M NaOH. This solution was stirred in a glass titration cell connected to a thermostatted bath. To the solution was added 0.30 ml of 1.0 M HCl and 1.74 ml of 12.9 M acetate (pH 5) pre-incubated at 60°C. After 0, 10 or 30 min the reaction mixture was removed from the cell, cooled on ice for 5 min and then filtered on an 8 μm cellulose acetate/nitrate Millipore filter type SC and extensively washed with water. The solid was dried over silica gel and phosphorus pentoxide at 37°C for 48 h. Infrared spectra were obtained from discs of each of the solids (2–2.5 mg) in a CsI matrix (250 mg) on a Perkin-Elmer 983 infrared spectrometer.

The effect of drugs was studied by performing the reaction as described above, except that three equivalents of quinine hemi-sulphate (Sigma), chloroquine phosphate (Sigma), amodiaquin dihydrochloride (Sigma), 9-epiquinine hydrochloride (Buchler GmbH), primaquine diphosphate (Sigma) or six equivalents of 8-hydroxyquinoline (Merck)

Table 1
The relationship between intra-erythrocytic anti-malarial activity, ability to bind to haem, and inhibition of β-haematin formation for various substances

Substance	Intra-erythrocytic anti-malarial activity	Ability to bind to haem	Inhibition of β-haematin formation
Chloroquine	+[a]	+[d]	+
Quinine	+[a]	+[e]	+
Amodiaquin	+[a]	+[f]	+
9-Epiquinine	−[b]	−[b]	−
8-Hydroxyquinoline	−[a,c]	ND	−
Primaquine	−[g]	−[h]	−

ND, not determined, but no thick black drug-haematin clumping observed under acidic conditions and no evidence of drug in the infrared spectrum (Fig. 3F).
[a] [16]; [b] [18]; [c] not reported to be anti-malarial; [d] [7,13]; [e] [7,12]; [f] [7,14,15]; [g] [17]; [h] [19].

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In this work haem refers to iron protoporphyrin IX (often abbreviated as FPIX in the literature), haemin is ferriprotoporphyrin IX chloride, haematin is ferriprotoporphyrin IX hydroxide/aquo and β-haematin is a polymer of haem in which the propionate side chain of the porphyrin of one haem molecule is complexed to the Fe(III) of the next via a carbon-oxygen-iron bond.

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SSDI 0014-5793(94)00921-X

was added to the reaction mixture prior to acidification, and the reaction was terminated after 30 min in each case.

The solubility of haematin in acetate solution at pH 5.0 was studied by adding 9.0 μl of 5×10^{-3} M haemin dissolved in DMSO to 3.0 ml of each of the various acetate solutions ranging between 0.0 and 6.0 M. Precipitate was removed by centrifugation in an Eppendorf microcentrifuge, and spectra of the solutions were obtained on a Hewlett-Packard 8450A diode array spectrophotometer.

3. Results

Formation of β-haematin from a basic (0.1 M NaOH) haematin solution acidified with glacial acetic acid to 4.5 M acetate concentration (final pH of about 2) and heated to 70°C has been reported to proceed overnight [5]. In the current study it was found that the reaction was essentially complete within 30 min at 60°C, 2 hours at 37°C, and 8 days at 6°C. The reaction rate thus appears to follow the usual dependence on temperature. Slater and Cerami [9] postulate that a haem polymerase enzyme is required for the reaction. They report no evidence of reaction in 0.5 M acetate, pH 4.8, at 37°C in the absence of trophozoite extract (see Fig. 1b in [9]). In the current study, however, when 5 mg of haematin was incubated at the same temperature in 1 l of the identical solution, the reaction was at least 50% complete as judged from the infrared spectrum after 7 days. This corresponds to a rate of approximately 380 pmol/min. Even in the presence of trophozoite extracts those authors obtained rates of the order of 1 pmol/min (see Fig. 3 in [9]). The reason for this discrepancy is not known, but may result from their method of assay which is based on differential solubility. The current study strongly suggests that the reaction is spontaneous under conditions prevailing in the food vacuole. The reaction even proceeds in a mixture of 0.1 M glutamate and 0.4 M glycine, pH 4.5 (complete within 91 h at 65°C, approximate rate of 1400 pmol/min), and in 0.1 M acetate, pH 4.2 (complete within 25 h at 65°C, about 5000 pmol/min). No difference in the reaction rates was observed whether or not the haemin was recrystallized.

Under acidic conditions haematin is essentially insoluble, but it was found that increasing concentrations of acetate solubilize an increasing proportion of the haematin (Fig. 2). This phenomenon most likely accounts in part for facilitation of the polymerization by acetate, as the reaction probably proceeds in solution. This was corroborated by the invariable observation of a marked increase in reaction rate when the reaction volume was increased with the amount of haematin being kept constant. This is because a greater proportion of the haematin is in solution in the larger volume, with both solutions being saturated. In the food vacuole of the parasite carboxylate groups on free amino acids and/or peptides (derived possibly from haemoglobin degradation [11]) or even a protein [9–11] probably play a role analogous to the acetate, although our data suggest that a specific haem polymerase enzyme is probably not required as the reaction is chemically facile.

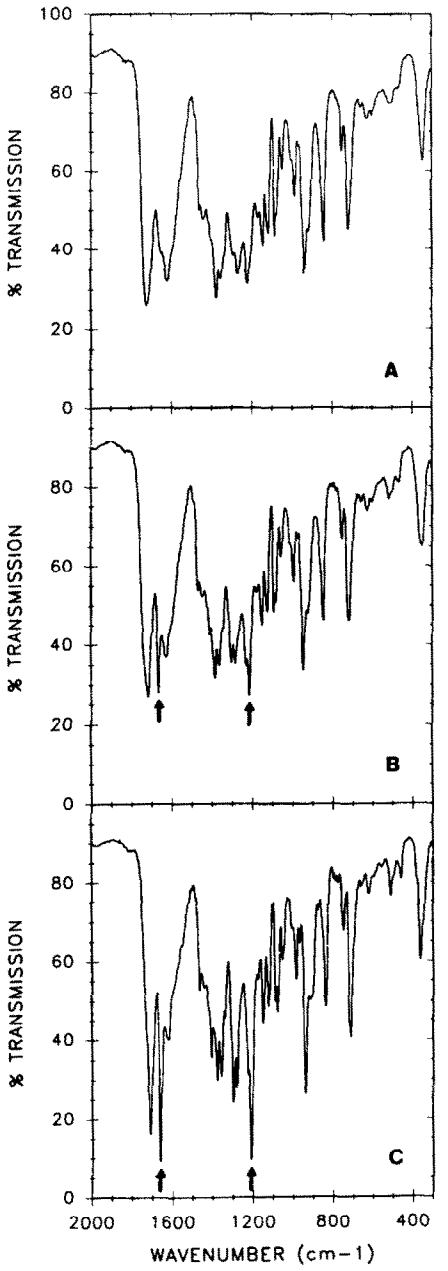

Fig. 1. Infrared spectra of haematin after 0 (A), 10 (B) and 30 min (C) incubation in 4.5 M acetate, pH 4.5, 60°C. The definitive peaks for β-haematin at 1660 cm^{-1} and 1207 cm^{-1} are marked with arrows.

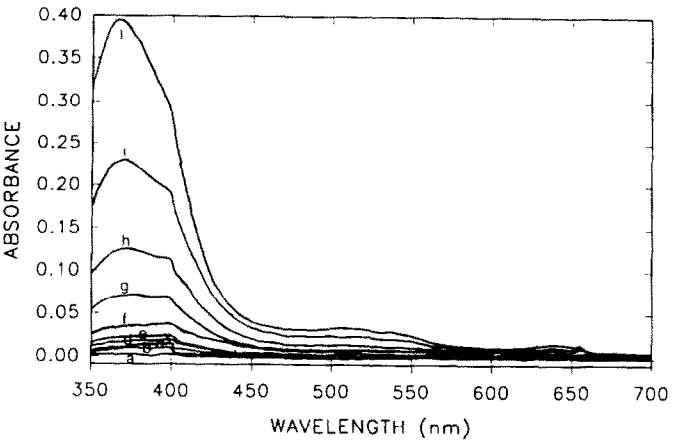

Fig. 2. Visible spectra of saturated solutions of haematin in (a) 0, (b) 2.0, (c) 2.5, (d) 3.0, (e) 3.5, (f) 4,0, (g) 4.5, (h) 5.0, (i) 5.5 and (j) 6.0 M acetate, pH 5.0. Spectra were obtained at 23°C.

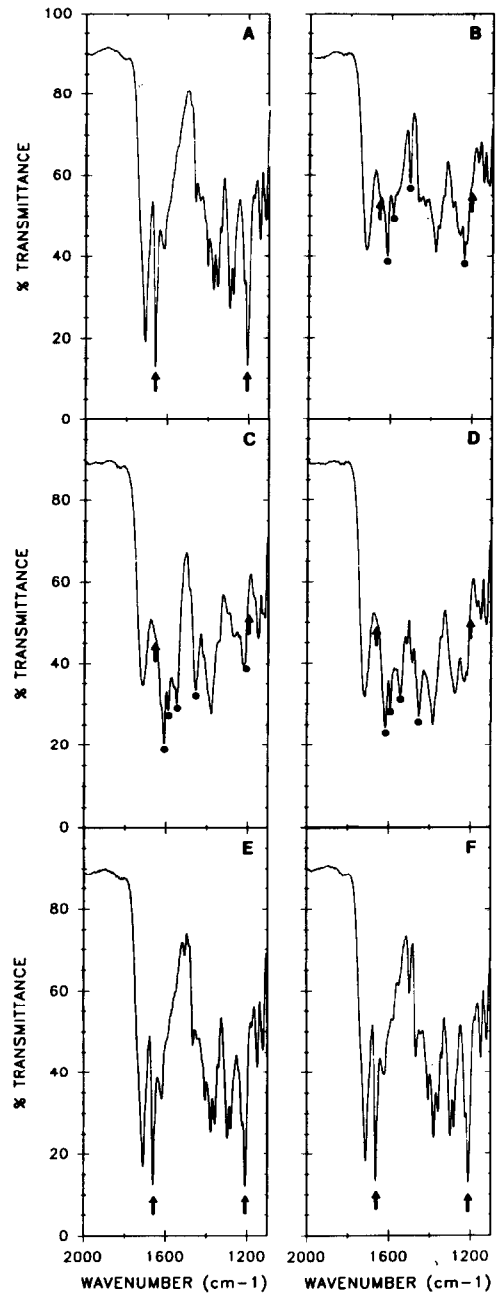

Fig. 3. As for Fig. 1 (after 30 min reaction time), except that drug was added to each of the haematin solutions prior to acidification. Spectra are for solutions containing (A) no drug, (B) three eqv of quinine, (C) three eqv of chloroquine, (D) three eqv of amodiaquin, (E) three eqv of 9-epiquinine and (F) six eqv of 8-hydroxyquinoline. The positions at which β-haematin peaks occur are indicated with arrows. Peaks due to drug associated with the haematin are marked with filled circles.

In order to ensure conveniently short reaction times, the effects of quinoline drugs on spontaneous β-haematin formation were studied under the reaction conditions pH 4.5, 4.5 M acetate and 60°C. Under these conditions a reaction time of 30 min is sufficient for complete reaction in the absence of drug (Fig. 3A). Inclusion of three equivalents of quinine (Fig. 3B), chloroquine (Fig. 3C) or amodiaquin (Fig. 3D) resulted in complete inhibition of β-haematin formation. In contrast, three equivalents of 9-epiquinine (Fig. 3E) or primaquine (result not shown), or six equivalents of 8-hydroxyquinoline (Fig. 3F) did not inhibit the reaction. The first three substances are known to bind to ferriporphyrins [7,12–15] and are intra-erythrocytically active anti-malarial drugs [16], while primaquine is active only against exo-erythrocytic stages of infection [17], and 9-epiquinine and 8-hydroxyquinoline are anti-malarially inactive [16,18]. 9-Epiquinine [18] and primaquine [19] have been shown not to bind to haem. Evidence of association of quinine, chloroquine or amodiaquin with haem under acidic reaction conditions can be seen in the infrared spectra (Fig. 3B–D, filled circles), while there is no evidence of similar association in the case of 9-epiquinine, 8-hydroxyquinoline (Fig. 3E,F) or primaquine (not shown). The characteristics of these compounds are summarized in Table 1. The haematin complexes of the intra-erythrocytically active drugs form thick black precipitates under the prevailing acidic conditions in the reaction vessel which are reminiscent of the 'clumping' observed in parasites treated with chloroquine [6]. This thick precipitate was also observed at room temperature.

4. Discussion

It has been demonstrated in the present work that spontaneous formation of β-haematin can be prevented by complexation of a suitable ligand to the haem molecule at low pH. Furthermore, there is a direct relationship between intra-erythrocytic anti-malarial activity and inhibition of spontaneous β-haematin formation among the six substances tested. As the formation of β-haematin does not require a catalyst, even if a polymerase is involved in vivo it would not seem to be the primary target for intra-erythrocytic anti-malarial drugs.

These findings provide a rational explanation for the hypothesis that haem is the 'drug receptor' [7,8]. The main criterion for selection or design of intra-erythrocytic anti-malarial drugs is thus that they bind to haem at low pH and so block conversion of toxic haem to polymeric non-toxic β-haematin. It is not yet clear whether the drugs actually enhance the toxicity of the haem. Clearly, the drug would also have to be able to enter the parasite and accumulate in the food vacuole, at least in part due to weak basic diprotic properties [20], and be relatively non-toxic to the host.

Finally, the method presented in this work provides a powerful and very simple chemical technique for screening substances for potential intra-erythrocytic anti-malarial activity.

Acknowledgements: We thank Dr.L.R. Purves, Department of Chemical Pathology, Institute of Child Health, Red Cross War Memorial Children's Hospital, for laboratory facilities. We would also like to thank Prof. David Thornton, Department of Chemistry, University of Cape Town, for making available materials and equipment for infrared spectroscopy. We would also like to thank Buchler GmbH, Braunschweig, Germany, for very generously donating 9-epiquinine to us. D.C.R. acknowledges the Medical Research Council of South Africa for financial support.

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