# Non-iron porphyrins inhibit β-haematin (malaria pigment) polymerisation

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**Abstract Infrared spectroscopy was used to evaluate the effect of non-iron porphyrins (protoporphyrin IX and haematoporphyrin) on haematin polymerisation to β-haematin at acidic pH. Both molecules effectively inhibited the reaction, with haematoporphyrin 6 times as active as protoporphyrin IX. We postulated that the interaction between the π electron system of porphyrin rings leads to the formation of π–π adducts, which inhibit polymer elongation in the same way as antimalarial drugs (e.g., chloroquine); the presence of hydroxyl groups able to bind haem iron enhances activity.**

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*Key words:* Malaria; Haem; Haemozoin; β-Haematin; Protoporphyrin IX; Haematoporphyrin

## 1. Introduction

Haem (ferriprotoporphyrin IX) is a by-product of haemoglobin digestion, a process which takes place in an acidic compartment of intraerythrocyte-stage malaria parasites [1]. Haem must be detoxified by assembly into insoluble malaria pigment (haemozoin): one carboxylic oxygen of the propionate side chain of one haem molecule is coordinated to the central iron of an adjacent haem subunit to form oxygen–iron bonds [2–4]. Notwithstanding uncertainties about the details of the natural process in the parasite [5], polymerisation of soluble haematin has been shown to occur spontaneously in vitro at acidic pH [2,6,7]. The synthetic polymer, β-haematin, retains the solubility properties and the spectroscopic characteristics of native haemozoin [2,8].

Quinoline antimalarials (e.g., chloroquine, amodiaquine and quinine) act principally by forming adducts with ferriprotoporphyrin IX, thus blocking haemozoin formation [6,7,9]. Mössbauer spectroscopy data indicate that in the haem–quinoline complex the iron atom couples antiferromagnetically either directly or through the π electron system of chloroquine [10]. This observation let us hypothesize that any molecule able to intercalate or form π–π adducts with haem could inhibit haematin polymerisation. This may provide a rationale for screening new antimalarials.

To verify this hypothesis, we evaluated the inhibitory activity of non-iron porphyrins (protoporphyrin IX and haematoporphyrin), on the polymerisation of haematin to β-haematin at acidic pH by infrared (IR) spectroscopy. Protoporphyrin IX is a protoporphyrin isomer identical to haem (ferriprotoporphyrin IX) except that it lacks the central iron atom; haematoporphyrin is a non-iron porphyrin with two α-hydroxyethyl side chains (see Fig. 1). Both molecules share with haematin the possibility of giving π–π adducts [11].

## 2. Materials and methods

Haemin (ferriprotoporphyrin IX chloride), protoporphyrin IX, haematoporphyrin and chloroquine diphosphate were purchased from Sigma-Aldrich (Milan, Italy). β-Haematin was synthesized in accordance with the procedure reported by Egan et al. [6] with slight modifications. Briefly, haematin (ferriprotoporphyrin IX hydroxide) was prepared by dissolving 15 mg of haemin in 3 ml of 0.1 M NaOH. Then, a solution containing 0.3 ml of 1 M HCl plus 1.74 ml of 12.9 M sodium acetate (pH 5.00) pre-warmed at 60°C, was added. After 60′ of incubation at 60°C, the reaction mixture was filtered over Type HA Millipore filters (0.45 μm) and washed extensively with distilled water. Protoporhyrin IX and haematoporphyrin were dissolved in 3 ml NaOH 0.1 M in the presence of 15 mg haemin at different molar ratios (haemin/porphyrin ratios 1:0; 1:0.5; 1:1; 1:2; 1:3): the mixture was incubated as described above. Control inhibition of β-haematin formation was obtained by adding 3 equivalents of chloroquine to haemin, as described elsewhere [6,9]. The solid precipitate was then dried over phosphorus pentoxide under vacuum overnight and finally characterized FT-IR spectroscopy using a Jasco FT-IR spectrometer. To validate the sensitivity of FT-IR analyses 1 equivalent of solid β-haematin, previously synthesised, was ground with 3 equivalents of porphyrins in KBr matrix and characterized by FT-IR spectroscopy. Native haemozoin, isolated from human erythrocyte cultures infected with *Plasmodium falciparum*, was used as control (purified by Dr. S. Picot, University of Grenoble, France).

## 3. Results and discussion

Haematin can polymerise chemically in 4.5 M acetate buffer at 60°C [6]. The reaction product was characterized by FT-IR; this technique unequivocally distinguishes haematin from β-haematin. Both the synthetic polymer and native haemozoin show bands at 1662 and 1209 $cm^{-1}$ characteristic of the iron–carboxylate bonds [2], which are not present in the spectrum

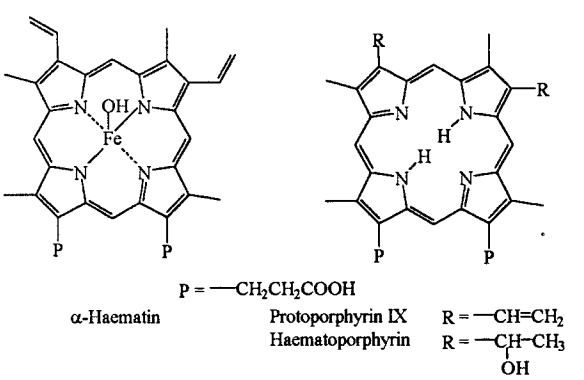


Fig. 1. Chemical structure of porphyrins used in this study.

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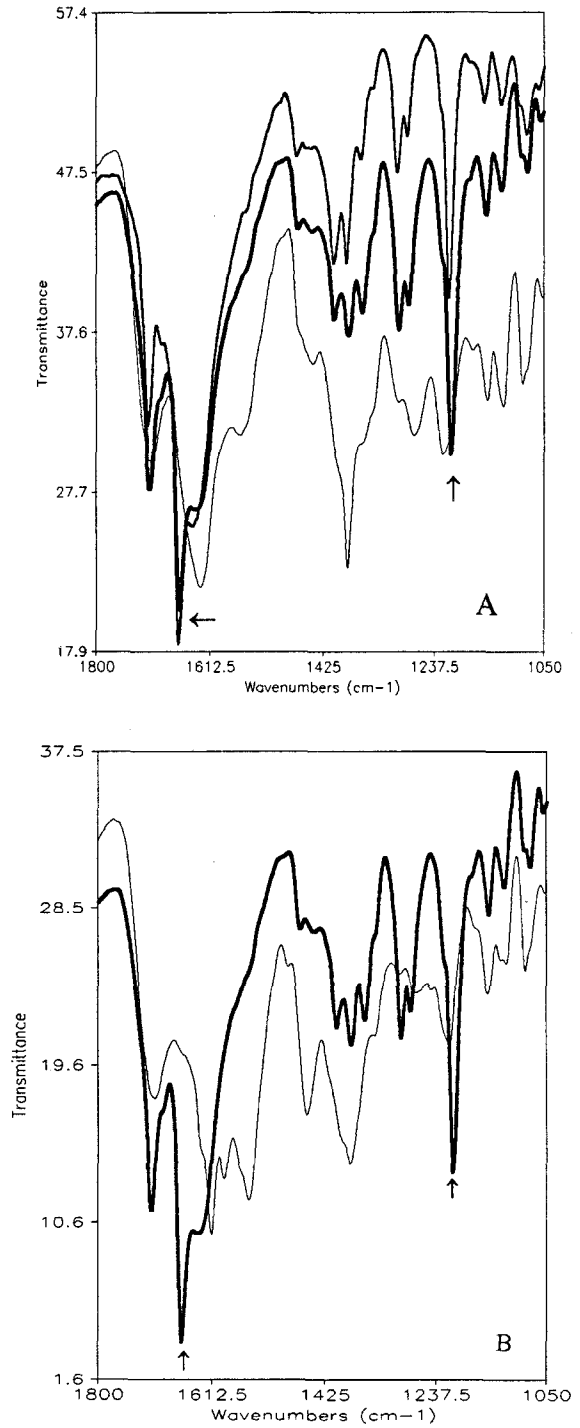


Fig. 2. A: Infrared spectra of haematin, synthetic β-haematin, obtained after 60′ incubation in 4.5 M acetate, pH 5, 60°C, and native haemozoin, purified from cultures of *P. falciparum*. The characteristic peaks of haemozoin and β-haematin at 1662 and 1209 $cm^{-1}$ are marked with arrows. β-haematin (bold line); haemozoin (line, medium thickness); haematin (thin line). B: Infrared spectra of β-haematin or haematin polymerised in the presence of 3 equivalents of chloroquine. β-haematin (bold line); haematin+chloroquine (thin line).

of haematin (Fig. 2A). FT-IR is also a valid and reproducible method for studying the inhibition of haemozoin formation by antimalarial drugs, such as quinoline antimalarials [6]. As shown in Fig. 2B, the FT-IR spectra of the precipitate in the presence of 3 equivalents of chloroquine differ significantly from untreated controls: the characteristic peaks at 1662 and 1209 $cm^{-1}$ are completely lost.

FT-IR proved to be suited to visualizing the distinctive bands of β-haematin even in the presence of large quantities of unpolymerised porphyrins: the infrared spectra of β-haematin mixed with 3 equivalents of protoporphyrin IX or haematoporphyrin at the time of sample preparation can be seen in Fig. 3. Both spectra are superimposable on that of β-haematin.

Different equivalents of protoporphyrin IX or haematoporphyrin added to haemin at the beginning of the reaction showed the ability to inhibit β-haematin polymerisation. The characteristic peaks of β-haematin disappeared progressively with increasing molar doses of protoporphyrin IX. Complete inhibition of β-haematin formation was observed at a haemin/protoporphyrin IX ratio of 1:3 (Fig. 4A). Haematoporphyrin was more effective in inhibiting polymerisation since it was active also at a molar ratio of 1:0.5 (Fig. 4B).

Both iron- and non-iron-porphyrins undergo spontaneous dimerisation primarily due to π–π interactions between the electron rings of adjacent molecules [11]. The interactions between quinoline antimalarials and the haematin ring are also mediated by co-facial links, as demonstrated by Mössbauer spectroscopy studies [8,12]. Quinoline-type antimalarials from haem–drug complexes, whereby the quinoline ring lies parallel to the porphyrin ring [8,10]. Such adducts prevent haemozoin polymer extension [9]. The quinoline ring was reported to interact in a similar way with uroporphyrin I [13,14]. Our

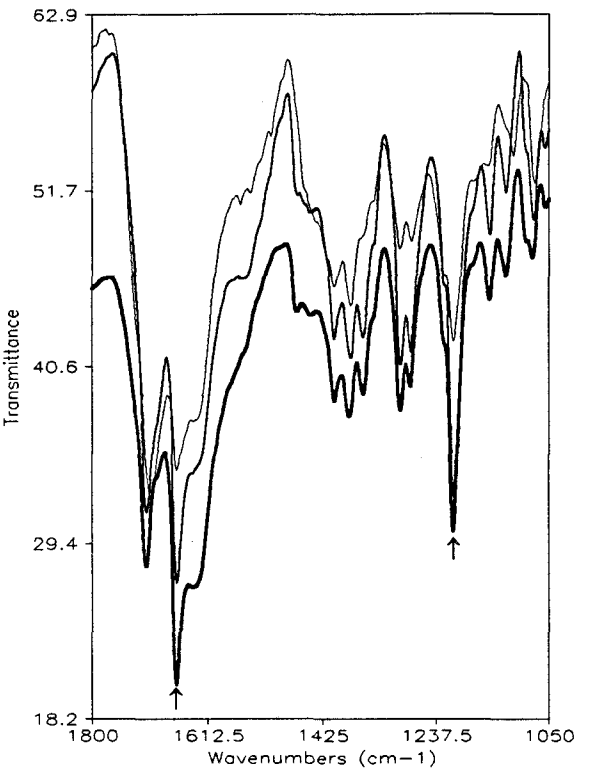


Fig. 3. Comparison between the infrared spectra of β-haematin and of β-haematin ground together with 3 equivalents of haematoporphyrin or protoporphyrin IX in KBr matrix. The characteristic peaks of β-haematin at 1662 and 1209 $cm^{-1}$ are marked with arrows. β-haematin (bold line); + protoporphyrin IX (line, medium thickness); + haematoporphyrin (thin line).

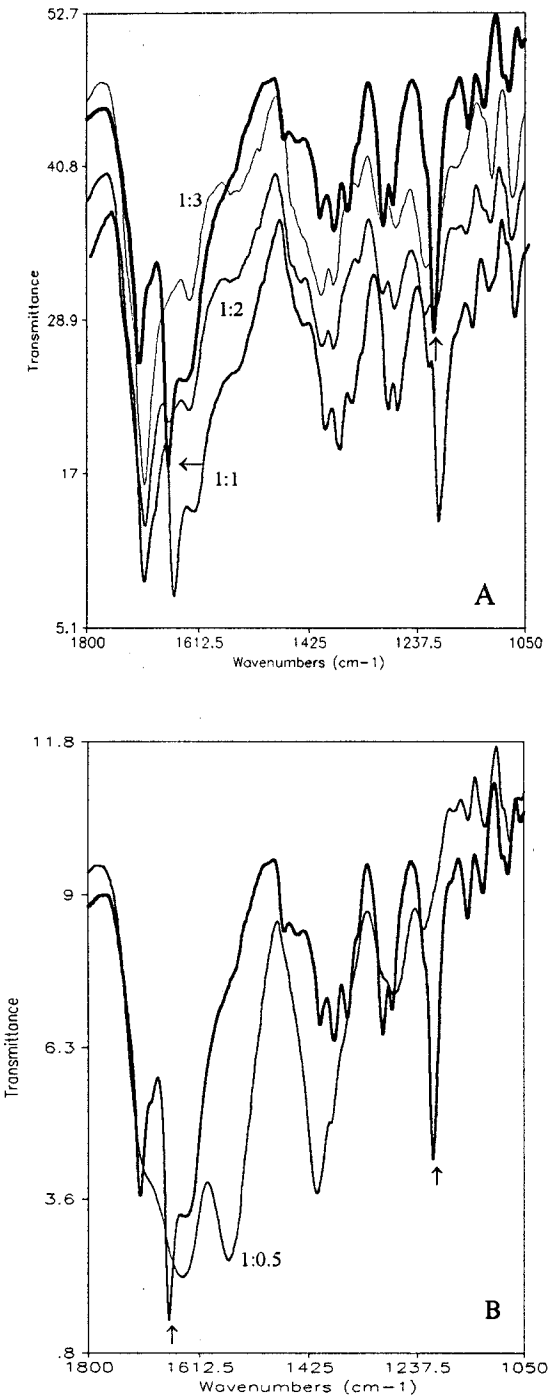


Fig. 4. A: Infrared spectra of β-haematin or haematin polymerised in the presence of different equivalents of protoporphyrin IX, indicated by lines in order of decreasing thickness: β-haematin; + 1:1 protoporphyrin IX; + 1:2 protoporphyrin IX; + 1:3 protoporphyrin IX. B: Infrared spectra of β-haematin or haematin polymerised in the presence of 0.5 equivalents of haematoporphyrin. β-haematin (bold line); + 1:0.5 haematoporphyrin (thin line).

data substantiate the hypothesis that molecules able to give co-facial π–π interactions with haematin can interfere with haem polymerisation.

The greater inhibitory activity of haematoporphyrin than of protoporphyrin IX could be attributed to two secondary hydroxy side groups in the benzylic position; the presence of hydroxy side groups in the quinoline antimalarial drugs, quinine and quinidine, is also noteworthy. The crucial role of these groups for the antimalarial activity of 4-quinolinecarbinolamine compounds was recently emphasised [15].

In conclusion, in this paper we show that non-iron porphyrins inhibit β-haematin polymerisation by coordinating with haematin via co-facial π–π links. Potency increases in the presence of hydroxyl groups. Therefore, we postulate that compounds able to give both π–π link and binding between hydroxyl groups and haematin iron are best suited to form drug–haem complexes and thus to inhibit haem polymerisation and detoxification.

These results are of potential relevance not only to the design of new antimalarials, but also to the treatment of disorders of porphyrin metabolism, such as porphyrias.

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