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The role of haem in the activity of chloroquine and related antimalarial drugs

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Contents

Abstract

Advances made over the last decade indicate that the mechanism of action of important antimalarial agents, such as chloroquine, involves formation of π–π complexes between drugs and ferriprotoporphyrin IX. This process is believed to block the detoxification of host haemoglobin-derived haem in the food vacuole of the parasite. Detoxification of haem occurs via conversion to a coordination polymer involving the formation of an Fe(III)-carboxylate bond between the propionate group of one ferriprotoporphyrin IX molecule and

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the Fe(III) centre of the next. This compound is known as malaria pigment or haemozoin in vivo, but can also be prepared synthetically, in which case it is referred to as β-haematin. Literature relating to the structure and mechanism of formation of haemozoin/β-haematin, the mechanism of action of the drugs and thermodynamics and structures of ferriprotoporphyrin IX-drug complexes is reviewed.

Keywords: Malaria; Antimalarial drugs; Haemozoin; Ferriprotoporphyrin IX

1. Introduction

Malaria is the most serious parasitic disease in man, both from the point of view of mortality and morbidity and from its world-wide occurrence in tropical and subtropical regions. It is estimated that 300 million people are infected annually, with one to two million deaths [1,2]. The problem has been exacerbated by the appearance of drug resistant strains in the last three decades [3]. Since this resistance appears to be drug specific, rather than the result of a change in the biochemical target of these drugs, a thorough understanding of the mechanism of action of known drugs is an important priority [4] and may realistically afford the opportunity to develop new and effective substitutes.

Historically, chloroquine was the most effective drug for treatment and prophylaxis because of its strong therapeutic activity, low toxicity and low cost [5]. The mechanism of activity of chloroquine and a number of related antimalarials has been the subject of ongoing debate in the literature for many years [6], but advances since 1990 have contributed greatly to our understanding of the antimalarial activity of these drugs and may offer the opportunity for new breakthroughs in the development of novel and effective compounds. Current evidence suggests that the mechanism of action involves a unique bioinorganic process.

Fig. 1 illustrates the life cycle of *Plasmodium falciparum*, one of the four species of parasite which are pathogenic in man (and also the most dangerous). As can be seen, a portion of the life cycle occurs within the erythrocyte (red blood cell) of the human host. During this stage of the cycle, the parasite utilises host haemoglobin as a food source [7]. Haemoglobin is imported into a specialised acidic compartment in the parasite, known as a food vacuole and broken down by proteolytic enzymes called plasmepsins [8] to peptides which are subsequently degraded to amino acids. In the process four equivalents of haem (ferroprotoporphyrin IX, or Fe(II)PPIX) are released and oxidised (Fig. 2). Ferriprotoporphyrin IX (Fe(III)PPIX) is toxic to microorganisms in its free form (as discussed below) [9] and, in the parasite, is detoxified by conversion to an insoluble compound known as malaria pigment or haemozoin. As will be seen below, recent evidence suggests that it is this detoxification process which is the target of chloroquine and related drugs.

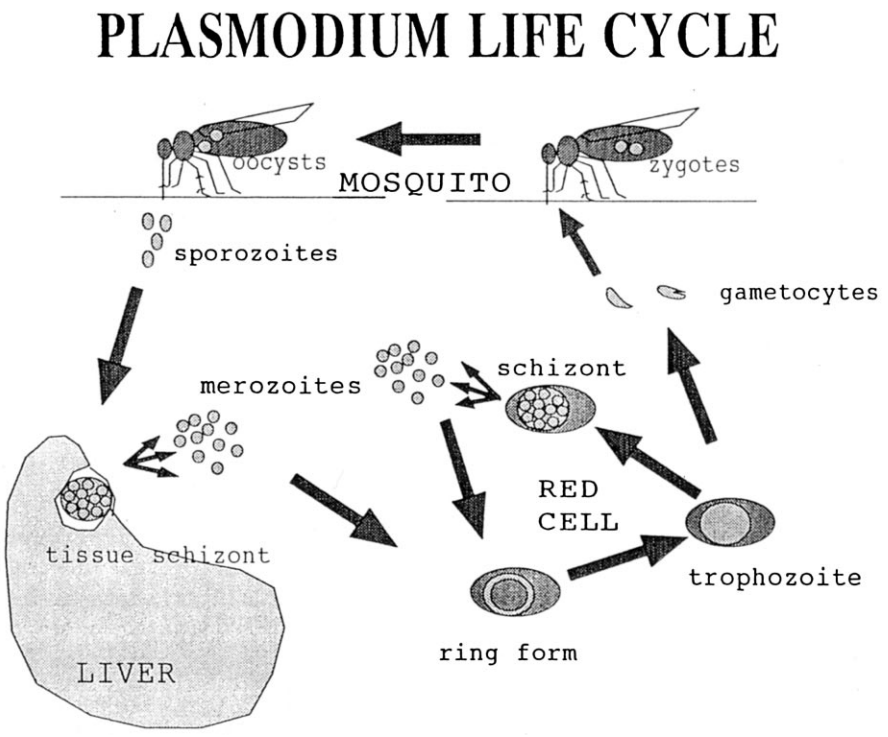

Fig. 1. A representation of the life cycle of *Plasmodium falciparum*. Ring forms, trophozoites and blood schizonts are collectively referred to as the blood stages of the cycle and are the specific targets of chloroquine and related antimalarial drugs. After invading red cells, most merozoites form ring stages and then trophozoites, but a small fraction instead develop into sexual forms called gametocytes which then reproduce in the gut of a mosquito when the insect feeds on the infected host.

2. Composition and structure of haemozoin

Haemozoin, or malaria pigment has been known since the eighteenth century, when discolouration of the internal organs of deceased chronic malaria suffers was first noticed [10]. This pigment was believed to be melanin until, in 1911, Brown first showed that it was constituted of haem [11]. Over the subsequent eight decades it was believed that haemozoin was either a specific haemoprotein (e.g. Ref. [12]) or else consisted of partially degraded haemoglobin [13]. In 1987, however, Fitch and Kanjananggulpan [14] were able to separate the haem component of haemozoin from proteins and noted its similarity to β-haematin, an insoluble and aggregated form of haematin (aqua- or hydroxyferriprotoporphyrin IX) [15] which at that time was of unknown structure.

A seminal paper in this field was published in 1991 [16], when Slater et al. showed that haemozoin is indeed β-haematin, and suggested that this compound is a polymer of Fe(III)PPIX in which the propionate side chain of one Fe(III)PPIX unit coordinates to the Fe(III) centre of the next. Evidence for this arrangement was

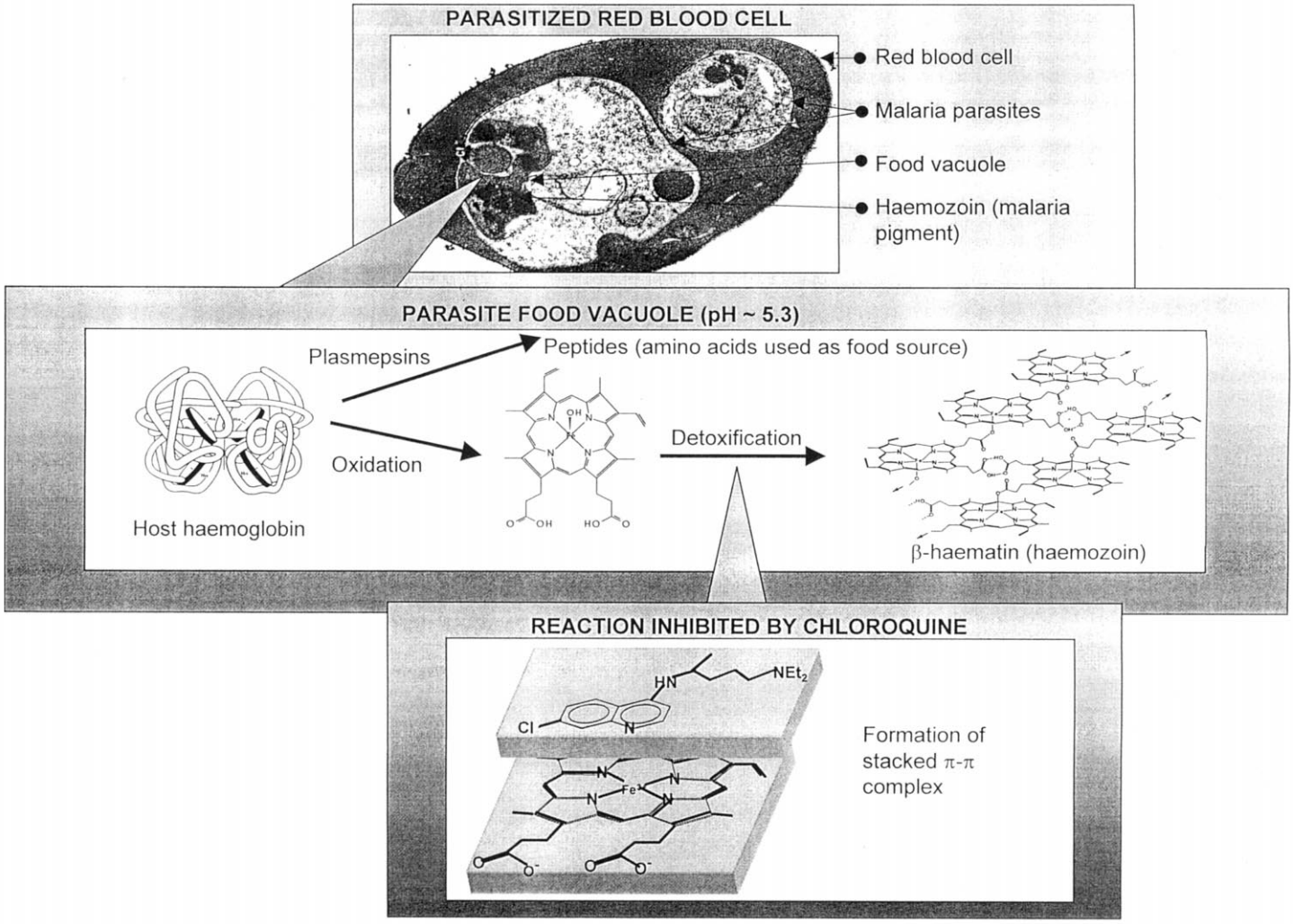

Fig. 2. (*Continued*)

obtained from infrared, ESR and EXAFS results. The infrared spectrum of haemozoin is quite distinct from that of haematin. A strong peak at about 1660 cm^{-1} which is present in β-haematin, but not in haematin, was interpreted as arising from the C=O stretch in an iron(III) coordinated, unidentate carboxylate since it occurs at essentially the same wavenumber as the corresponding stretch in acetato-[deuteroporphyrin IX dimethyl ester]iron(III) [17]. The strong β-haematin specific peak at about 1210 cm^{-1} is believed to be a C–O stretching frequency arising from the C–O–Fe(III) group. The solid state ESR obtained at 10 K was claimed to demonstrate that Fe(III) in β-haematin is low spin under these conditions, whereas in haematin or haemin (chloro-Fe(III)PPIX) it is high spin. This was suggested to be consistent with replacement of the weak field H_2O or OH^- axial ligand in haematin with the stronger field carboxylate ligand capable of π-overlap, although this particular interpretation has subsequently been disproved by magnetic susceptibility measurements [18] as well as low temperature ESR and magnetic Mössbauer spectroscopy which show that the iron is strictly high-spin ($S = 5/2$) Fe(III) in a slightly rhombic environment [19]. Finally, EXAFS [16] data for β-haematin is consistent with a model involving carboxylate coordination to Fe(III), viz. one oxygen atom at 1.92 Å and a carbon atom at 2.17 Å from the Fe(III) centre (unlike haematin which can be adequately modelled by considering the oxygen atom alone). Slater et al. also synthesised β-haematin by heating haematin in 4.5 M acetic acid (pH about 2.6) at 70°C overnight [16]. The synthetic material was shown to have an identical infrared spectrum and X-ray powder diffraction pattern to haemozoin. Furthermore, it was also reported to have very similar solubility properties (both being solubilised only at pHs above 10.5).

Subsequent to this initial work, questions were raised [20] as to whether formation of β-haematin could be an artifact of the haemozoin extraction and purification procedure. This question has been unequivocally answered by Bohle et al. [21] who have demonstrated that the X-ray powder diffraction pattern of haemozoin in situ in lyophilised, *P. falciparum* parasitised human erythrocytes is identical to that of β-haematin (Fig. 3). The high resolution X-ray diffraction technique, employing synchrotron radiation, also permitted determination of the unit cell dimensions of the crystal lattice and assignment of the structure to one of two space groups, $P\bar{1}$ or $P1$ with two haem molecules occupying the unit cell. Both are triclinic space groups, the former being more symmetrical in that the two haem units are related by a centre of inversion. Even with the excellent data obtained however, the structure could not be unequivocally assigned to one or other of these space groups. The structure shown in Fig. 4 is consistent with a $P\bar{1}$ unit cell and with the spectroscopic data discussed above. It is currently believed to be the structure of β-haematin/haemozoin.

Fig. 2. The proposed mechanism of action of chloroquine. The picture of the parasite is an electron micrograph of *P. lophurae*, a bird malaria parasite. It is magnified 36 000 × and is reproduced from Ref. [114] with copyright permission of the Rockefeller University Press. The structure of haemozoin is reproduced from Ref. [21] with copyright permission.

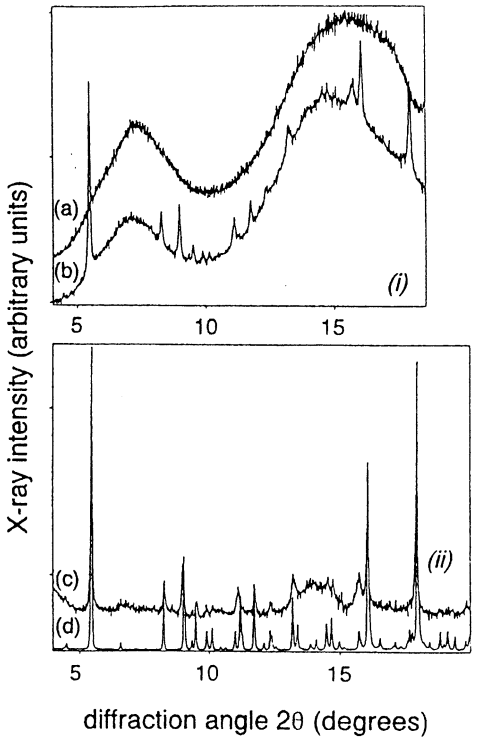

Fig. 3. High resolution X-ray powder diffraction patterns of: (a) lyophilised unparasitised red blood cells; (b) lyophilised red blood cells containing late *P. falciparum* trophozoites; (c) the difference (b)–(a); and (d) synthetic β-haematin prepared by the anhydrous route. The results show that haemozoin and β-haematin are identical. X-ray source: synchrotron radiation, $\lambda = 1.1495$ Å, $T = 293$ K. Reproduced from Bohle et al. [21] with copyright permission.

Several methods are now available for synthesis of β-haematin. In 1993 Bohle and Helms [22] showed that it can be prepared by reacting haemin overnight at room temperature in dry methanol using an organic base, 2,6-lutidine, to abstract HCl (hereafter referred to as the anhydrous method). We have also shown [23] that β-haematin can be prepared in 4.5 M acetate at pH 4.5, by stirring for 30 min at 60°C (hereafter referred to as the mild thermal method). Infrared spectra of the products obtained by both these methods and the method developed by Slater et al. [16] (referred to as the vigorous thermal method) are identical and Mössbauer spectra obtained at 77 K [24] of parasite haemozoin and the product of the mild thermal method are also essentially identical. Notwithstanding these facts, there have been suggestions that the product of the mild thermal synthesis may in fact be a haem-acetate complex rather than β-haematin [25,26]. Recent studies in our laboratory employing X-ray powder diffraction and elemental analysis [27], however, have shown that the product is β-haematin. Indeed, the report of Ignatushchenko et al. [26] appears to be flawed, as scrutiny of the infrared spectra, even

of their proposed β-haematin products, are identical to those of haematin [16,23] (e.g. lacking peaks at 1210 and 1660 cm^{-1}). Furthermore, their elemental analyses do not agree with the known composition of β-haematin [21], but rather conform to that expected for haematin.

In addition to the above synthesis conditions, we have also shown that β-haematin can be formed under even milder conditions. For example, in 4.5 M acetate at pH 4.5 and 37°C in about 2 h. In 1 l of 0.5 M acetate at pH 4.5 and 37°C a sample consisting of 5 mg of haematin is about 50% converted to β-haematin in 7 days [23].

3. Mechanism of haemozoin and β-haematin formation

There is still substantial uncertainty over the mechanism of haemozoin formation in vivo. Originally Slater and Cerami [28] suggested that the reaction is enzyme-catalysed. This conclusion was based on the extreme conditions apparently required for synthetic β-haematin formation [16], and observations that an extract of plasmodial membranes apparently catalyses β-haematin formation. This so-called haem polymerase was reported to be heat-labile, supporting its identification as an enzyme. This enzymatic mechanism has been supported by Fitch et al. [29–33] who have reported both a heat-labile and a heat-stimulated haem polymerase activity in extracts of the mouse malarial parasite *P. berghei* [31]. The heat-labile polymerase

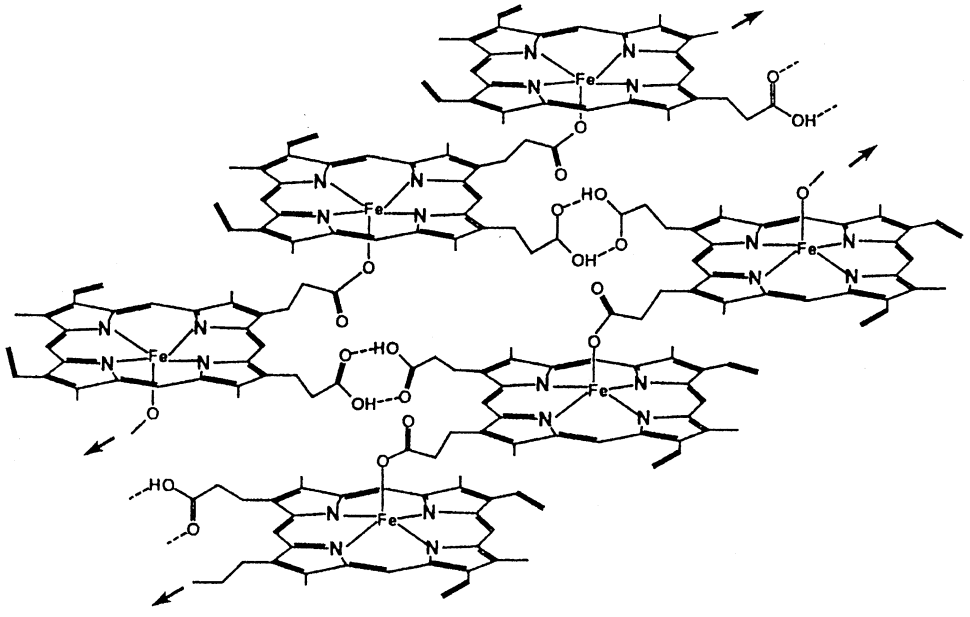

Fig. 4. The proposed structure of haemozoin and β-haematin from [21]. Reproduced from Ref. [21] with copyright permission.

was reported to be active at low acetate concentrations, while the heat-stimulated polymerase was reported active at high acetate concentrations. In contrast to the above observations, Dorn et al. [34] found that β-haematin formation is protein-independent, but suggested that it is autocatalytic and that the catalytic agent in trophozoite extract is in fact haemozoin itself. Subsequently, Bendrat et al. [35] reported that an acetonitrile extract of trophozoite lysate, probably lipid, catalyses β-haematin formation. Dorn et al. have supported and recently extended this observation, showing that various purified lipids can also catalyse β-haematin formation [36,37]. Since the products were directly observed by infrared spectroscopy [36] there can be little doubt that these lipids and extracts do indeed support β-haematin formation, although the question of whether they are actually involved in the reaction in vivo is still an open one. Sullivan et al. reported that histidine rich proteins (HRPs) secreted by the parasite catalyse or initiate haemozoin formation in vivo [38], but this too has recently been called into question when it was claimed that a synthetic peptide with a histidine repeat sequence the same as that found in HRP inhibits rather than promotes β-haematin formation [39]. This observation would seem to be chemically plausible given that histidine is likely to be a strong Fe(III)PPIX sequestering ligand and would be more likely to irreversibly bind Fe(III)PPIX. Clearly the mechanism of haemozoin formation in vivo is currently far from settled.

Some additional insight into haemozoin formation in vivo might be obtained by considering the mechanism of synthetic β-haematin formation. Such studies are hampered by the fact that under acid pH conditions required for the reaction, both the starting material and product are insoluble. This prevents the employment of conventional spectroscopic methods for observing the rate of reaction. We have therefore employed Mössbauer spectroscopy to monitor this reaction in 4.5 M acetate at pH 4.5 [40]. A remarkable feature of the reaction is that it appears to follow zero-order kinetics, i.e. the reaction proceeds at a constant rate until complete (Fig. 5). This observation is consistent with a mechanism in which the fixed concentration of dissolved reactant in a saturated haematin solution reacts at a steady velocity in the rate determining step to form β-haematin (Fig. 6). The role of acetate appears to be to catalyse the reaction through its ability [23] to increase the solubility of haematin under these conditions, thus essentially acting as a phase transfer catalyst. Under conditions in which there is sufficient solubilisation of haematin, the subsequent formation of β-haematin is then apparently spontaneous. This information suggests that any species which catalyses this reaction in vivo may play a similar role. This mechanism could explain why lipids catalyse β-haematin formation, as haematin partitions into detergent micelles [41] and is likely to be similarly solubilised by lipid micelles. If any proteins or peptides are also involved in the reaction in vivo, one might expect them to function by forming fairly weak complexes with Fe(III)PPIX thus providing a soluble source of reactant for β-haematin formation.

Autocatalysis was not evident in 4.5 M acetate (Fig. 5) as seeding the reaction mixture with preformed β-haematin had no significant influence on the rate of reaction. This finding is at odds with those of Ridley et al. [34,36,42] in lower

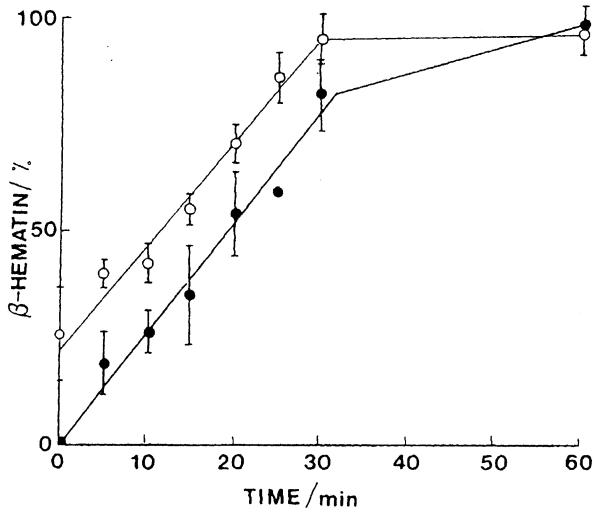

Fig. 5. Kinetics of β-haematin formation in 4.5 M acetate, pH 4.5 at 60°C determined by Mössbauer spectroscopy. Filled circles: unseeded; hollow circles: seeded with 10% preformed β-haematin at the beginning of the reaction. Note the zero order reaction (straight line dependence on time) and lack of any significant effect of seeding on the rate. Reproduced from Ref. [40] with copyright permission.

acetate concentrations. Several explanations are possible for this discrepancy. Firstly, it is possible that the sigmoidal shape of the curve is fairly subtle under high acetate conditions and is disguised by variability in the data. Secondly, it is possible that this could be a result of different reaction mechanisms under different conditions of acetate concentration. Finally, it could be a result of the different techniques used for monitoring the reaction. With the exception of our Mössbauer study [40], other attempts to quantify reaction products [16,28–39] have used solubilisation characteristics to distinguish between haematin and β-haematin. Mössbauer spectroscopy detects changes in the chemical environment of the Fe(III) ion and thus effectively directly monitors formation of the Fe(III)-propionate bond.

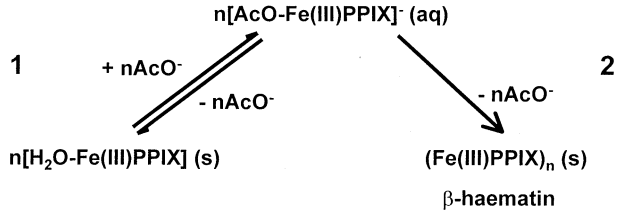

Fig. 6. Proposed mechanism of β-haematin formation in aqueous acetate. The proposed role of acetate is to solubilise haematin via a weak complex. Since there is a large excess of [H_2O-Fe(III)PPIX] (s) the concentration of [AcO–Fe(III)PPIX]$^-$ (aq) is constant virtually throughout the reaction and the formation of β-haematin will occur at a constant rate provided that reaction (2) is the rate determining step.

On the other hand, differential solubility monitors the tendency of haematin to be solubilised at lower pH than β-haematin. Since β-haematin is likely to be a polydisperse crystalline polymer, it is probably not actually a single compound but a mixture of compounds of different molecular weights, presumably exhibiting a range of solubilisation properties. The rate of solubilisation probably also depends on the size of the crystallites. Solubility studies would thus be expected to be sensitive, not only to formation of the Fe(III)-propionate bond, but also to subsequent chain extension reactions as well as crystal growth (which would not significantly affect the Mössbauer spectrum). Seeding with preformed β-haematin, on the other hand, could be expected to immediately result in chain extension of the preformed polymer, forming a less soluble product right away. Supporting this explanation, we have recently observed that synthetic β-haematin prepared by the mild thermal method is significantly more easily solubilised than haemozoin, despite having an identical structure and composition as determined by infrared spectroscopy, elemental analysis and X-ray diffraction [27]. The differences in ease of solubilisation probably reflect differences in crystal size and/or polymer chain length. In a possibly related observation, Blauer and Akkawi [43,44] have suggested existence of a β-haematin precursor which is very much more easily solubilised than β-haematin itself. They have suggested that this is a haematin oligomer involving Fe(III)-propionate bonds which they have called B-haematin. The supposed precursor products obtained by Blauer and Akkawi differ, however, in that their infrared spectra are not identical to that of β-haematin.

4. Mechanism of action of chloroquine and related antimalarials

Many hypotheses have been advanced over the last three to four decades to account for the mechanism of action of chloroquine. The literature dealing with this question up to 1993 has been critically discussed by Slater [6] and will not be detailed further here.

Hypotheses for the mode of action of chloroquine essentially fall into two broad categories: those in which the drug exerts its action outside the food vacuole of the parasite and those in which the activity is located inside the food vacuole. Intravacuolar mechanisms seem more plausible because of substantial accumulation of the drugs in the vacuole. A very brief summary of specific mechanisms which have been proposed is given in Table 1.

Most workers in the field currently favour an hypothesis in which quinoline antimalarial drugs inhibit formation of haemozoin. There is, however disagreement over how this occurs and there are essentially three variations of the hypothesis:

1. Slater and Cerami [28] originally suggested that these drugs inhibit the putative haem polymerase enzyme. This hypothesis was supported by the finding that the antimalarial amodiaquine does not interact directly with Fe(III)PPIX in benzene solution [69]. This indicated that the activity did not involve direct interaction with Fe(III)PPIX itself, but rather with the enzyme. Subsequent studies have shown, however, that amodiaquine forms complexes with Fe(III)PPIX in

Table 1
Some proposed mechanisms of action of chloroquine and related drugs

Proposed mechanism of action	Evidence presented in favour of the mechanism	Other comments
DNA binding (extravacuolar)	Chloroquine and quinine bind to DNA [45]. Chloroquine and related drugs exhibit antibacterial activity, blocking both DNA and RNA synthesis [46–48].	Required concentrations about 10^3 times too high [6]. No binding of mefloquine to DNA [49–51].
Inhibition of protein synthesis (extravacuolar)	Reported in *P. falciparum* at higher concs. (3 μM). Stimulated by haem [52].	Other studies found no evidence for this mechanism [53–55]. Not generally accepted.
Inhibition of polyamine metabolism (extravacuolar)	Chloroquine and mefloquine inhibit ornithine decarboxylase activity in trophozoite extracts [56].	Not consistent with blood-stage specificity of chloroquine [6].
Toxic haem-chloroquine complex (intravacuolar)	Blood-stage specific antimalarials form complexes with Fe(III)PPIX. First proposed by Chou et al. [57].	Free Fe(III)PPIX not likely to be present unless haemozoin formation is inhibited.
Increased vacuolar pH (intravacuolar)	Drugs are weak bases and accumulate in the food vauole [58]. Vacuolar protease activity reduced with increased pH [59].	Reports indicate that there is no change in vacuolar pH upon chloroquine treatment [60,61], or that the change ($\sim$0.25 pH units) [62] is too small [6]. Many weak bases are not antimalarial.
Inhibition of vacuolar lipase (intravacuolar)	Phospholipases are believed to break down membranes of endocytotic vesicles, when they release their contents into the food vacuole [63]. Phospholipase extracts from *P. falciparum* are inhibited by millimolar concs. of chloroquine and mefloquine [64].	Mechanism cannot explain specificity of the drugs and concentrations required are too high [6].
Inhibition of vacuolar protease (intravacuolar)	Aspartic protease enzymes from parasites which are responsible for haemoglobin degradation are partially inhibited by chloroquine [65–67]. Fe(III)PPIX itself is an even better inhibitor [68].	Process appears to require too high a concentration of drug.
Inhibition of haemozoin formation (intravacuolar)	See text.	See text.

aqueous media which are of comparable strength to those of chloroquine [70,71].

2. Fitch et al. [31] have extended this hypothesis by suggesting that these drugs potentiate regulators of the putative haem polymerase enzyme.
3. We have shown [23] that chloroquine, amodiaquine and quinine can directly inhibit synthetic β-haematin formation and suggested that activity of these drugs in vivo involves inhibition of haemozoin formation by direct interaction with Fe(III)PPIX. This hypothesis has also been proposed by Dorn et al. [34,36,72] and further support for this type of mechanism has been presented by several other laboratories [73–76], although there are some differences in detail [75].

The effect of antimalarial drugs on synthetic β-haematin formation is readily monitored by infrared spectroscopy [23]. We found that the active drugs chloroquine, quinine and amodiaquine result in Fe(III)PPIX-drug complexes, with no evidence of infrared bands around 1660 and 1210 cm^{-1} which are characteristic of β-haematin. On the other hand, compounds with little or no antimalarial activity, 9-epiquinine and 8-hydroxyquinoline do not inhibit β-haematin formation. We collected data [23] on samples subjected to the conditions used in the mild thermal method of β-haematin synthesis, which are non-physiological. Basilico et al. [73] have found identical effects under the same conditions with chloroquine and metal-free porphyrins (protoporphyrin IX and haematoporphyrin) inhibiting β-haematin formation. Dorn et al. have reported that β-haematin formation is inhibited by eight known antimalarials under far milder conditions approximating more closely to those expected in vivo [36], while Hawley et al. [74] have also reported similar effects with a further twenty-three antimalarial compounds under the same conditions. An entirely novel gallium complex has also been reported recently which is able to inhibit β-haematin formation and which is antimalarially active [76]. Dorn et al. have noted [36] that the ability of chloroquine to inhibit β-haematin formation is maintained irrespective of the method used to promote the reaction. Furthermore, these compounds are capable of inhibiting the reaction at micromolar concentrations [36,72,74], well below the concentrations actually present in the food vacuole of the parasite. Table 2 lists all compounds which have to date been reported to inhibit β-haematin formation as well as a number of antimalarially inactive quinolines which do not inhibit the reaction (structures of the drugs are given in Fig. 7). A report that various quinolones can also inhibit β-haematin formation [26] is questionable, as the spectroscopic evidence and elemental analyses presented, strongly suggest that no β-haematin was formed, even in control experiments (see Section 2).

Goldberg et al. have argued [75] that in vivo chloroquine and quinine cap the growing haemozoin chain, thus preventing further sequestration of Fe(III)PPIX, rather than inhibiting the formation of β-haematin a priori. This interpretation is nevertheless in general accord with the hypothesis that inhibition of β-haematin formation occurs via direct interaction of the iron porphyrin functionality with the drug, rather than via mediation of a catalyst or enzyme.

While an indirect mechanism of β-haematin inhibition in vivo, such as those proposed by Slater and Cerami [28] or Fitch et al. [31] cannot be unequivocally

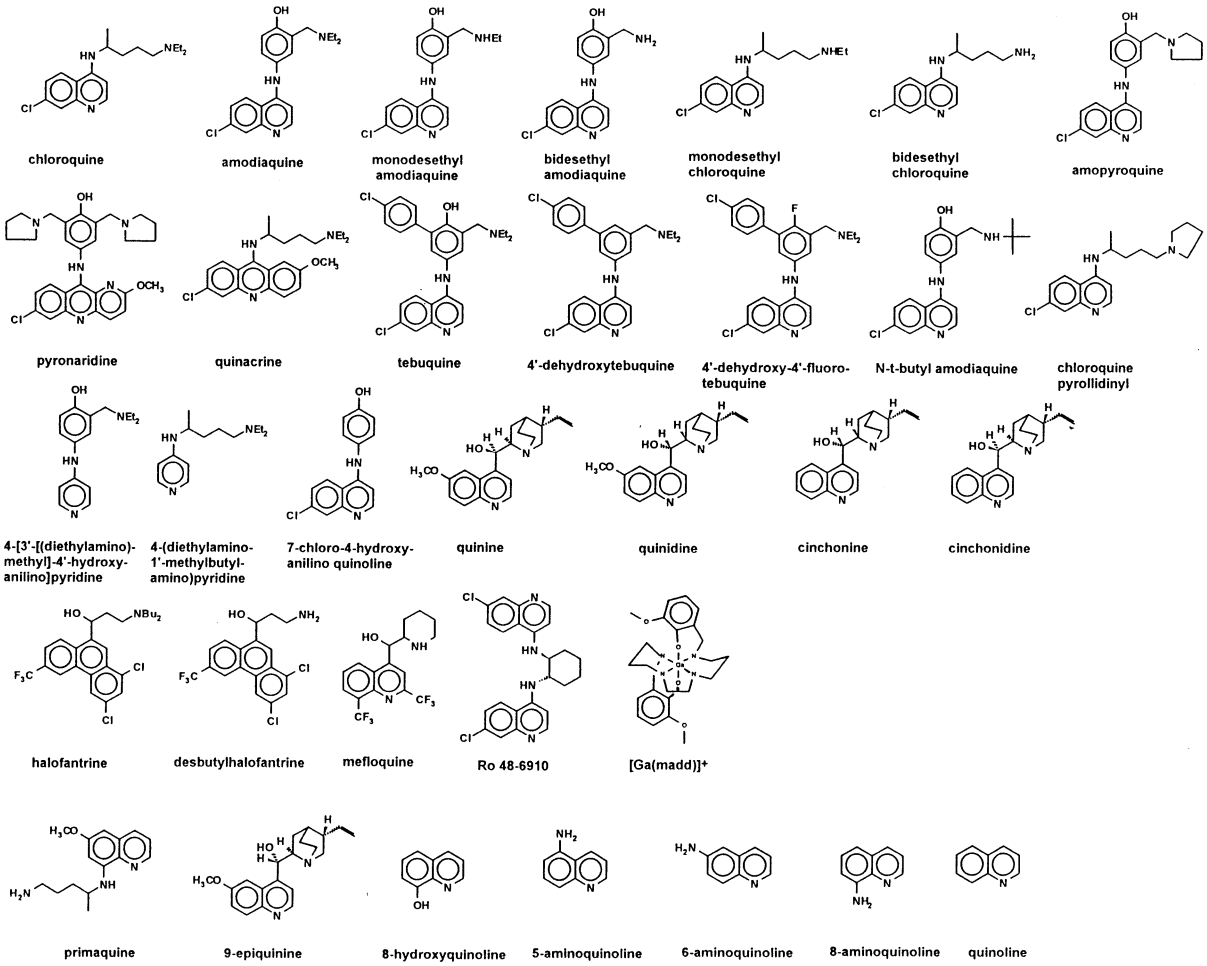

Fig. 7. Structures of the compounds referred to in Table 2.

Table 2
Correlation between inhibition of β-haematin formation and blood-stage antimalarial activity

Compound	Antimalarial activity	Inhibits β-haematin formation?
Chloroquine [23,36,73,74]	+	+
Amodiaquine [23,36,74]	+	+
Monodesethyl amodiaquine [74]	+	+
Bidesethyl amodiaquine [74]	+	+
Monodesethyl chloroquine [74]	+	+
Bidesethyl chloroquine [74]	+	+
Amopyroquine [74]	+	+
Pyronaridine [36,74]	+	+
Quinacrine [36,74]	+	+
Tebuquine [74]	+	+
4′-Dehydroxytebuquine [74]	+	+
4′-Dehydroxy-4′-fluorotebuquine [74]	+	+
N-*t*-Butyl amodiaquine [74]	+	+
Chloroquine-pyrollidinyl [74]	+	+
4-[3′-[(Diethylamino)methyl]-4′-hydroxyanilino]pyridine [74]	+	+
4-(Diethylamino-1′-methylbutylamino) pyridine [74]	+	+
7-Chloro-4-hydroxyanilino quinoline [74]	+	+
Quinine [23,36,74]	+	+
Quinidine [27,74]	+	+
Cinchonine [74]	+	+
Cinchonidine [74]	+	+
Halofantrine [27,36,74]	+	+
Desbutylhalofantrine [27]	+	+
Mefloquine [27,36,74]	+	+
Ro 48-6910 [36]	+	+
$[Ga(madd)]^+$ [76]	+	+
Primaquine [23,36,74]	+[a]	−
9-Epiquinine [23]	−	−
8-Hydroxyquinoline [23]	−	−
5-Aminoquinoline [27]	−	−
6-Aminoquinoline [27]	−	−
8-Aminoquinoline [27]	−	−
Quinoline [27]	−	−

[a] Strongly active against the liver stage where no haemozoin formation occurs, therefore believed to act by a different mechanism.

excluded at this stage, one nevertheless has to take account of the fact that chloroquine and other antimalarials of the same class have been shown to directly inhibit β-haematin formation under a wide range of conditions. These include acetate concentration (0.500–4.5 M), pH (4.5–6.5) and temperature (37–65°C) and also various methods of β-haematin synthesis (acetate, lipid or trophozoite extract promoted). There is thus no need to invoke an indirect mechanism and it is difficult to imagine how these drugs could fail to have similar direct effects on the reaction in the food vacuole.

Competitive interactions between antimalarial drugs in the presence of trophozoite extracts have been proposed to indicate an indirect mechanism of inhibition of haemozoin formation [33]. Dorn et al. have shown [36], however, that trophozoite membrane extracts increase the concentration of chloroquine required to inhibit β-haematin formation, suggesting that components in the extract compete with Fe(III)PPIX for chloroquine binding. These could provide sites for competitive drug binding which are unrelated to their mechanism of action. Significantly, no such effect was observed with food vacuole extracts [36], which are more appropriate as a model of the process in vivo. Additional support for the hypothesis that antimalarial activity stems from direct interaction between the drugs and Fe(III)PPIX comes from the apparent correlation between the so-called IC_{50} for β-haematin inhibition (concentrations of drug at which conversion of Fe(III)PPIX to β-haematin is 50% inhibited under the standard conditions used in the particular synthesis) and the IC_{50} for antimalarial activity against cultured parasites [72] (the concentration at which 50% inhibition of parasite growth occurs). Not surprisingly, the correlation is imperfect as it takes no cognisance of the necessity of the drug to reach and accumulate in the food vacuole. Indeed, Hawley et al. [74] found a similar correlation with a variety of amodiaquine analogs only when they were accumulation normalised.

The precise basis of the toxic effects of Fe(III)PPIX on the parasite is also still unresolved. Sugioka and Suzuki [77,78] have suggested that this activity results from peroxidative activity of Fe(III)PPIX and Fe(III)PPIX-drug complexes with pre-formed lipid peroxides (a process which occurs in other systems such as isolated mitochondria [79] and which is believed to be the cause of the bactericidal activity of Fe(III)PPIX [80]), while Fitch et al. [81–83] have suggested that Fe(III)PPIX lyses malaria parasites via a colloid-osmotic mechanism, possibly by inhibiting the maintenance of cation gradients. Since these studies have not directly investigated the effect on food vacuole membranes, their relationship to any processes which may occur in the parasite in vivo are uncertain. Indeed, available evidence suggests that the vacuoles swell, but do not lyse upon chloroquine treatment [6,84]. Whatever the mechanism, it should be noted that relatively little unpolymerised Fe(III)PPIX may be required to exert a toxic effect, and it may not be necessary for complete inhibition of β-haematin synthesis to occur. Indeed, it is possible that only traces of free Fe(III)PPIX are required, so that gross changes in haemozoin may be virtually undetectable upon treatment of parasites with drugs.

5. The interaction of antimalarial drugs with Fe(III)PPIX; thermodynamics and structure

There is a large body of literature reporting various types of spectroscopic evidence for complex formation between antimalarial drugs and Fe(III)PPIX [24,57,69–71,77,85–93], including among others chloroquine, amodiaquine, quinine, quinidine, halofantrine and mefloquine, but quantitative data is much sparser. Chou et al. [57] reported association constants for several antimalarial drug

complexes of Fe(III)PPIX in aqueous solution at pH 7.4. Under these conditions Fe(III)PPIX exists in an aggregated state [94], probably consisting at least partially of μ-oxo dimers [95], but possibly further aggregated by non-covalent interactions [94,96] the structure and stoichiometry of which is not clearly defined. The study was performed using equilibrium dialysis to distinguish between bound and free drug concentrations. Recently Dorn et al. [72] have reported association constants for chloroquine and eight other antimalarial drug–Fe(III)PPIX complexes, also in aqueous solution at pH 6.5 using titration calorimetry. Enthalpy and entropy data were also reported for these complexes. They interpreted their results as indicating that Fe(III)PPIX itself exists almost exclusively as a μ-oxo dimer under these conditions. Although such data provide information on conditions prevailing under physiological conditions, interpretation of the data is likely to be complicated by possible changes in the extent of non-covalent aggregation upon drug binding. In addition, there are several reports suggesting that studies performed on Fe(III)PPIX in aqueous solution can be unreliable and non-reproducible [97–99]. For this reason we investigated the association of antimalarials with monomeric Fe(III)PPIX in 40% aqueous-DMSO, pH 7.5 [93]. This investigation was carried out using visible spectrophotometric titration, as the Soret band of Fe(III)PPIX at 402 nm is strongly quenched upon association with the drugs. Entropies and enthalpies of association were also reported, based on van't Hoff plots. Thermodynamic constants reported in the above study and in the study of Dorn et al. [72] are shown in Table 3. Addition of acetonitrile to the 40% aqueous-DMSO system was found to weaken the association constants, at least in the cases of chloroquine and quinine indicating a significant hydrophobic component to these associations [93]. This probably explains discrepancies which have been reported for amodiaquine [69] and 9-epiquinine [85] which were reported not to form complexes with Fe(III)PPIX in studies conducted in benzene solution, while complexes were observed in aqueous medium [57,70,71,77,93]. It is likely that complex formation would either not occur, or be very weak in organic solvents such as benzene.

Several interesting observations emerge from these studies. Firstly, a comparison of the association constants obtained by Dorn et al. [72] in aqueous solution at pH 6.5 using titration calorimetry and those which we obtained in aqueous-DMSO at pH 7.5 with monomeric Fe(III)PPIX [93] show an excellent correlation (Table 3). More unexpectedly, the actual values of the constants are remarkably similar. This is probably fortuitous, since evidence from our study [93] suggests that the constants in aqueous solution would be much higher (if the Fe(III)PPIX were monomeric). This increase may be coincidentally compensated by the unfavourable free energy required to disaggregate Fe(III)PPIX. This seems to be supported by the widely different ΔS and ΔH values obtained in the two studies (in addition, the values are not directly comparable due to differences in stoichiometry). Secondly, the association constants do not appear to be particularly sensitive to pH (there is a small decrease in log K in the case of chloroquine and a small increase in the case of quinine when pH is decreased from 7.5 to 5.4 [93]). Thirdly, 9-epiquinine forms a complex almost as strong as quinine [93,100], in spite of its apparent inability to inhibit β-haematin formation. This shows that detailed structural factors must be

Table 3
Association constants of some Fe(III)PPIX-drug complexes

Compound	In 40% aqueous-DMSO, pH 7.5, 25°C. 1:1 Stoichiometry. [93][a]			In aqueous solution, pH 6.5, 37°C. Stoichiometry (drug:Fe(III)PPIX) in {}[b]		
	log K ($\pm$ SEM, $n=3$)[d]	ΔH[c] ($\pm$ SEM, $n=3$)[d] (kJ mol^{-1})	ΔS[c] ($\pm$ SEM, $n=3$)[d] (J K^{-1} mol^{-1})	log K	ΔH (kJ mol^{-1})	ΔS (J K^{-1} mol^{-1})
Chloroquine	5.52 ± 0.03	-4.7 ± 0.9	90.3 ± 3	5.6 ± 0.2 {1:4}	-42 ± 4	-30 ± 16
Amodiaquine	5.39 ± 0.04	-11.8 ± 4.0	63 ± 13	5.0 ± 0.1 {1:4}	-39 ± 3	-32 ± 12
Quinine	4.10 ± 0.02	-28.9 ± 3.0	-18 ± 10	4.32 ± 0.04 {1:5}	-56 ± 6	-98 ± 22
9-Epiquinine	4.04 ± 0.03	-34.6 ± 2.9	-39 ± 10	–	–	–
Mefloquine	3.90 ± 0.08	-35.9 ± 5.1	-46 ± 16	4.1 ± 0.1[e] {1:3}	-19 ± 4[e]	17 ± 16[e]
Quinacrine	–	–	–	5.69 ± 0.04 {1:4}	-39.1 ± 0.8	-17 ± 3
Pyronaridine	–	–	–	5.47 ± 0.03 {1:7}	-46 ± 2	-42 ± 5
Halofantrine[f]	–	–	–	4.66 ± 0.07 {1:1}	-24.0 ± 0.8	12 ± 2
Primaquine	Not detected	Not detected	Not detected	4.2 ± 0.2 {1:7}	-38 ± 6	-42 ± 22
Ro 48-6910	–	–	–	5.8 ± 0.1 {1:19}	-24 ± 1	38 ± 7

[a] By spectrophotometric titration.
[b] By titration calorimetry; values calculated from Ref. [72], stoichiometries are rounded off.
[c] From van't Hoff plots.
[d] Standard error of the mean on three determinations.
[e] Unbuffered.
[f] In 80% aqueous ethanol.

involved in the inhibitory process. Fourthly, no primaquine association with Fe(III)PPIX was observed in aqueous DMSO at pH 7.5 [93], but it was observed in aqueous solution at pH 6.5 [72], although it is not able to inhibit β-haematin formation [23,72,74]. The stoichiometry of the complex in aqueous solution in fact suggests that it may induce Fe(III)PPIX aggregation. Finally, there appears to be some correlation between the strength of binding and antimalarial activity of many of the drugs (Fig. 8). Not surprisingly, this correlation is imperfect, as the activity of the drug must also depend strongly on its ability to accumulate in the food vacuole of the parasite as was noted earlier [74]. Nevertheless, this correlation seems to further support the hypothesis that drug activity results from Fe(III)PPIX-drug association.

Evidence for the structures of Fe(III)PPIX-drug complexes comes from NMR studies. Moreau et al. [89,90] investigated the proton NMR spectra of Fe(III)PPIX- and uroporphyrin-chloroquine and quinine complexes by monitoring peak shifts in the drugs when they were titrated with small mole percentages (0–1.6%) of the porphyrin compounds (higher percentages of Fe(III)porphyrins destroy the signal through paramagnetic line broadening). These were strongly indicative of cofacial interactions, commonly referred to as π–π interactions. There was no evidence to suggest coordination of either the quinoline ring nitrogen, or of the alkyl nitrogen atoms to the Fe(III) centre as the signals of H atoms on or adjacent to these nitrogens did not experience especially large shifts. This is not surprising, at least as far as the quinoline ring nitrogen is concerned, since H8 (on the benzene aromatic ring) would be expected to sterically inhibit coordination of the N atom to

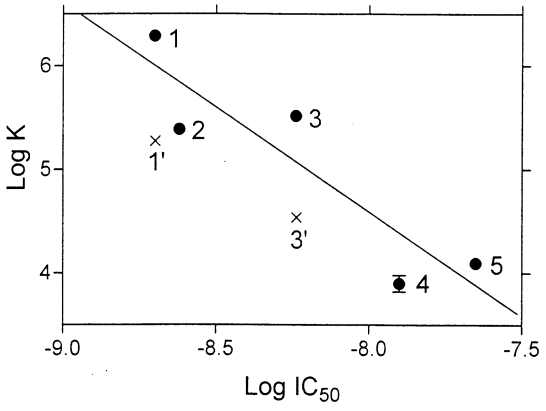

Fig. 8. An apparent correlation between log K values of Fe(III)PPIX-drug complexes in 40% aqueous DMSO, pH 7.5 (from [93] except for halofantrine which is from [27]) and log IC_{50} values of the drugs against the NF54 strain of *P. falciparum* from [72]. Drugs are: 1-halofantrine (log K estimated), 2-amodiaquine, 3-chloroquine, 4-mefloquine and 5-quinine. The log K for 1′ is that of halofantrine in 40% DMSO, 30% acetonitrile and 30% H_2O (pH 7.5) and for 3′ is that of chloroquine in the same solvent system. In 1, the log K for halofantrine has been estimated assuming an identical effect of acetonitrile as that found for chloroquine (which is assumed based on the fact that the effect is identical with quinine [93]).

Fe(III)PPIX. Further evidence that the complex does not involve coordination to the Fe(III) centre comes from the fact that metal free uroporphyrin I itself forms complexes with chloroquine and quinine of comparable strength [90]. A ring current model was used to predict the structure of the complex and indicated that the quinoline nucleus is situated above the porphyrin ring, rather than at the periphery, as is more usually the case with such π–π interactions. The quinoline substituents appeared to be oriented away from the porphyrin ring. Similar studies on the interaction of chloroquine and quinine with both Fe(III)uroporphyrin I and uroporphyrin I using ^{13}C-NMR were undertaken by Constantinidis and Satterlee [91,92]. These also indicated co-facial complexation of the quinoline rings and the porphyrin rings, although in this case there was evidence for coordination of the 9-hydroxy group on quinine to the Fe(III) centre of Fe(III)uroporphyrin [91]. Chloroquine, however was also proposed to lie directly above the porphyrin ring with no coordinative interactions. A blue shift observed in the charge transfer band of the haem-peptide *N*-acetylmicroperoxidase-8 (*N*-AcMP-8) when it is titrated with quinine or 9-epiquinine [101] seems to provide support for the suggestion that these drugs coordinate to the Fe(III) centre of the metalloporphyrin through a deprotonated 9-hydroxy group, although our subsequent study in 40% aqueous-DMSO [93] shows that similar changes occur in the spectrum of Fe(III)PPIX titrated with chloroquine which has no hydroxyl groups. Furthermore, the association constant of the Fe(III)PPIX-quinine complex increases slightly with decreasing pH [93], which is inconsistent with coordination of a deprotonated donor atom.

The stoichiometry of Fe(III)PPIX-drug interactions appear to vary between different studies. Moreau et al. [89,90] and Dorn et al. [72] have proposed a 1:4 (drug:Fe(III)PPIX) ratio for most of the complexes in aqueous solution, corresponding to one chloroquine molecule intercalated between the porphyrin rings of two stacked μ-oxo dimers. Some drugs gave even higher stoichiometries, with Ro 48-6910 exhibiting a stoichiometry of 1:19. This indicates the presence of higher Fe(III)PPIX aggregates in aqueous solution. In the case of Fe(III)uroporphyrin I, which is monomeric in aqueous solution, Constantinidis and Satterlee [91,92] found ratios of 1:2 for both quinine and chloroquine. Mössbauer spectra [24] of Fe(III)PPIX complexes of quinine and chloroquine precipitated from aqueous acetate solution (pH 4.5) seem to be consistent with the model of Constantinidis and Satterlee, and are inconsistent with the presence of μ-oxo dimers (except possibly for chloroquine) due to the absence of evidence of strong antiferromagnetic coupling. In 40% aqueous-DMSO we found 1:1 complex formation between Fe(III)PPIX and chloroquine, amodiaquine, quinine, 9-epiquinine and mefloquine [93], although chloroquine exhibited evidence of a 1:2 complex when concentrations of chloroquine were low compared to Fe(III)PPIX. With *N*-AcMP-8, 1:1 complexes were observed in the cases of quinine and 9-epiquinine and a 2:1 (drug:N-AcMP-8) complex was seen with chloroquine [101]. These results suggest a strong influence of solvent and pH on the stoichiometry of the interaction which is directly related to the propensity of the Fe(III)porphyrin to aggregate under the prevailing conditions. This seems to follow the order Fe(III)PPIX (aq., pH > 6.5) > Fe(III)PPIX (aq., pH 4.5) ≈ Fe(III)uroporphyrin I > Fe(III)PPIX (40% aq-DMSO) and the correspond-

ing drug:Fe(III)PPIX ratios follow the order 1:4 (or higher), 1:2, 1:2 and 1:1, respectively.

The molecular origin of the drug–Fe(III)PPIX interactions is not currently understood. It is not clear why, for example, some aminoquinolines such as 5-, 6- and 8-aminoquinoline and the 8-aminoquinoline derivative primaquine do not appear form complexes with monomeric Fe(III)PPIX, while others such as chloroquine do. In the case of 1,10-phenanthrolines, which are neutral molecules, Shelnutt [102–105] demonstrated that the strength of association of π–π complexes formed with metallouroporphyrins is directly proportional to the Hammett constants of substituents on the phenanthroline rings and that this could in turn be related to shifts in various Raman bands in the porphyrin, which indicate transfer of electron density from the porphyrin ring to the phenanthroline. These interactions are thus dominated by porphyrin-to-phenanthroline charge transfer. Shelnutt noted, however, that cationic species, such as methyl viologen and (protonated) chloroquine, do not exhibit the same trends [104,105]. The interactions in these cases are probably essentially electrostatic cation–π interactions. This appears to be borne out by the fact that there does not seem to be any simple correlation between the association constants of antimalarial drug–Fe(III)PPIX complexes and the identity of the quinoline ring substituents [93].

Only two attempts appear to have been made to computationally model the interactions between antimalarials and iron porphyrins [101,106]. In both cases molecular mechanics and molecular dynamics methods were used. In the earlier study [101] the interactions between quinine, 9-epiquinine and chloroquine on the one hand and a simplified model of *N*-AcMP-8 ([Fe(III)(porphine)(H_2O)-(imidazole)]$^+$) on the other, were modelled. The structures of the drugs and the iron–porphyrin were optimised by molecular mechanics and these structures were then used as a starting point for modelling the interactions in their complexes. Quenched dynamics calculations were then used to identify flexible regions of the complexes. The study showed that coordination of the 9-hydroxyl group of quinine (and 9-epiquinine) to the Fe(III) centre is feasible, with considerable flexibility in the complex. The benzene ring of quinoline was found to overlie a meso carbon atom in the porphyrin ring, while the pyridine ring overlies a pyrrole β carbon. The quinuclidine ring was found to be orientated up and away from the porphyrin ring. No significant difference between the complexes of quinine and 9-epiquinine could be detected which could easily account for the inability of 9-epiquinine to inhibit β-haematin formation. A cofacial interaction of the quinoline ring of chloroquine with the porphyrin ring was shown to result in a minimum energy structure, but this structure proved to be unstable when molecular dynamics simulations were attempted, as the two molecules simply moved apart. A weakness of this study was that no cognisance was taken of solvent, which experimental studies indicate may play a key role in the interactions. The second study [106] used molecular mechanics methods to optimise the structures of the drugs and Fe(III)PPIX. Drug structures were then further optimised using semi-empirical molecular orbital calculations. The interactions between the drugs and Fe(III)PPIX were simulated by molecular mechanics calculations in vacuo. This somewhat more

sophisticated study converged on energy minimised structures for amodiaquine– and tebuquine–Fe(III)PPIX complexes which involved cofacial interactions between the quinoline ring and Fe(III)PPIX and also hydrogen bonding between the protonated terminal amino groups of the drugs and the propionate carboxylate groups of Fe(III)PPIX. These structures proved stable when immersed in a cube of water and subjected to molecular dynamics simulation.

In spite of the findings of the modelling study described above, there is reason to doubt that the interaction of the terminal amino group in the side chain of the drug with the propionate group of Fe(III)PPIX is essential for strong complex formation. De et al. [107] have shown that chloroquine analogs with side chains ranging from ethyl to dodecyl and all containing a terminal diethylamino group are equally active against chloroquine sensitive parasites, while the ethyl, propyl, isopropyl, decyl and dodecyl derivatives are also fully active against chloroquine resistant strains. It is difficult to believe that all of these compounds could form equally strong H-bonds between the amino group and the Fe(III)PPIX propionate group. Furthermore, preliminary results in our laboratories indicate that a side chain amino group is not essential for formation of a strong complex with Fe(III)PPIX, since 4-aminoquinoline itself forms such a complex.

A full understanding of the interactions of antimalarial drugs with Fe(III)PPIX will probably require an integrated investigation involving synthesis, spectroscopy and molecular modelling of compounds specifically designed to isolate the effects of the various functional groups on these molecules.

6. Additional requirements for antimalarial activity and prospects for rational design

Based on current evidence presented above a hierarchical set of requirements for rational design of a novel antimalarial compound can be envisaged [108]. It should:

1. form a relatively strong complex with Fe(III)PPIX which should persist around pH 5;
2. inhibit β-haematin formation;
3. accumulate in the food vacuole of the parasite;
4. be relatively non-toxic;
5. be bio-available and be sufficiently water soluble.

The current status of (1) and (2) have been discussed above. The question of drug accumulation appears to involve essentially two aspects:

Firstly, the drug must be able to cross cell membranes in its deprotonated form. There is evidence that the 7-chloro group of chloroquine is important for uptake [109], since so called 7-hydrochloroquine which lacks this Cl atom accumulates to only about 1/20th of the extent to which chloroquine accumulates and has comparably reduced activity. It has been suggested that this is due to interactions with a specific uptake protein [109], but it could possibly also be due to the considerable lipophilicity of the Cl atom [110] which may facilitate transport of the drug across the membrane. In support of the latter suggestion, the Cl atom in

chloroquine or amodiaquine can be replaced by other hydrophobic groups such as CF_3 and CH_3 [111] and still retain reasonable activity, although it would appear that steric factors also come into play. Alternatives in which these substituents are at the 6-position are active, while those at the 5- and 8-positions are inactive, or exhibit very much less activity [111]. It is not clear whether these effects are due to some influence on the structures or stabilities of their Fe(III)PPIX complexes or due to interactions with an as yet unidentified uptake protein. It can be envisaged that the 6-methoxy group in quinine may play a similar role.

Secondly, it seems to be quite well established that the terminal amino groups found in these drugs are essential for their accumulation in the food vacuole of the parasite. This hypothesis is based on the premise that only the unprotonated form of chloroquine can traverse membranes. Since chloroquine has two sites of protonation, both with pK_as well above 7.4 (8.3 and 10.2 [112]) at the pH of the serum there would be 10^4-fold more deprotonated chloroquine than at pH 5.4, the approximate pH of the vacuole. As a result one would expect about a 10^4-fold accumulation of the drug in the vacuole. Less accumulation would be expected with quinoline alcohols such as quinine, in which there is one high pK_a (9.7) and one very much lower pK_a (quinoline ring N, $pK_a = 5.07$ [112]). Although this appears to be the major mechanism of uptake, it has been argued that weak base properties alone cannot fully account for accumulation of the drugs [109,113] and some additional, as yet unknown uptake sites may also be involved.

7. Conclusions

Considerable progress towards understanding the mechanism of action of chloroquine and related antimalarials has been made over the last seven years. A combination of bioinorganic chemistry, organic synthesis, pharmacology and basic biology have provided a great deal of new information on the unique processes occurring in the food vacuole of the parasite and it may be hoped that their continued application will lead to the rational design of new drugs in the foreseeable future. In this respect, the recent report of a relatively cheap and simple microtitre assay for haem polymerisation inhibitory activity may make a valuable contribution towards the identification of molecules which have potential antimalarial activity [115].

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