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REVIEW

Hemozoin Biocrystallization in *Plasmodium falciparum* and the antimalarial activity of crystallization inhibitors

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Quinine extracted from the bark of the South American *Cinchona* spp tree was the first of all antimalarials in western medicine and had been used since the 17th century. For more than 300 years, quinine was the only specific treatment for malaria. It was synthesized in 1944 (Woodward and Doering 1945) and has again become the antimalarial drug of choice in some parts of the world, both for uncomplicated and complicated forms of the disease and as monotherapy or as the backbone of combination therapy. The drug is a powerful schizonticide, active only against malaria pigment (hemozoin) producing stages (Warhurst et al. 2003). The drug has no activity against the sporozoites and exo-erythrocytic stages of the parasites (liver schizonts) which do not consume hemoglobin. Other blood schizontocidal antimalarial drugs (chloroquine, amodiaquine, mefloquine, halofantrine, and lumefantrine) are also likely to be inhibitors of heme detoxification (see Fig. 1) (Ezzet et al. 2000; Chong and Sullivan 2003; Warhurst et al. 2003; Tekwani and Walker 2005).

During the intra-erythrocytic phase of their life cycle, malaria parasites feeds on host cell hemoglobin to produce free amino acids and to regulate osmotic pressure (Lew et al. 2003). Toxic free hematin (Omodeo-Sale et al. 2005) is released along with oxygen and brownish-yellowish, inert malaria pigment (hemozoin) is produced as a result of heme detoxification (Peters 1964; Warhurst and Hockley 1967; Macomber et al. 1967; Homewood et al. 1975; Fitch and Kanjananggulpan 1987).

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The development of our knowledge of the function and formation of malaria pigment crystals and their place in chemotherapy has a fascinating history which merits a concise review. Recent reviews have highlighted the structure of hemozoin and its formation (Egan 2002, 2006; Sullivan 2002; Fitch 2004; Tekwani and Walker 2005; Bray et al. 2005). This microreview focuses primarily on historical perspectives and pigment biogenesis.

Historical perspectives

Meckel, a psychiatrist, in 1846, recorded innumerable black-brown pigment granules in the blood and spleen of a patient who had died in a hospital for the insane. Formation of the dark brown pigment is a most distinctive characteristic of the malaria parasite, and Meckel was looking at parasites without realizing it. He thought the pigment was melanin and described the condition as "melanemia" (Meckel 1847). A few years later, Virchow established that the brown pigment resulted from blood destruction due to malaria fever (Virchow 1849). The liver of persons affected by malarial fever has been described as having almost the colour of black lead and *febris intermittens* began to appear to be a blood disease (Frerichs 1858). The causal relationship of pigmentation to the parasite was established by Laveran, a French army surgeon living in the colonial world. Guided by the work of Meckel and Virchow, he examined microscopically, fresh unstained blood from 44 patients suffering from malaria and recognised pigmented bodies in red blood cells (Laveran 1880). In 1880, the technique of blood examination was imperfect, and his results were received with much scepticism (Smith and Sanford 1985). Spectroscopic examinations of malarial pigment by Carbone (1891) and

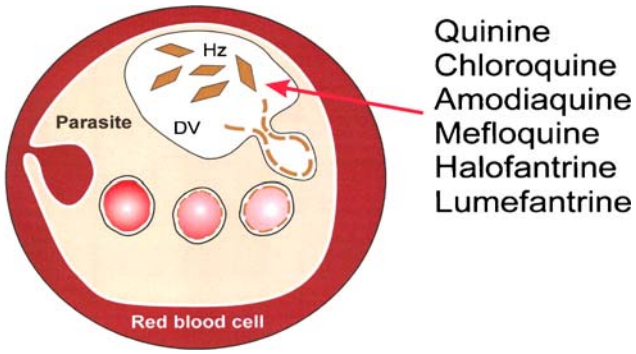

Fig. 1 Biocrystallization inhibitors currently in use. *Hz* Hemozoin, *DV* digestive vacuole

Brown (1911) proved that malaria pigment was equivalent to hematin and not melanin. The degradation of heme to nontoxic metabolites was considered most unlikely because the hematin content of infected cells remains constant, and degradation products of hematin have never been found (Ball et al. 1948; Scholl et al. 2005). However, hemozoin is significantly different from pure hematin: it was noted by Deegan and Maegraith 1956 that hematin dissolves rapidly in solvents in which hemozoin is insoluble. An aggregation of hematin can be separated from hemozoin by its differential solubility in mildly alkaline bicarbonate buffer: hematin dissolves in this solution, whereas hemozoin (pigment) does not. Pigment dissolves in trisodium/disodium phosphate buffer, at approximately pH 11 (Egan et al. 1999). The composition of hemozoin remained unknown until the explanation for the observed low solubility was given. Malaria pigment is chemically and structurally similar to a distinctive hematin pigment, called β-hematin (Fitch and Kanjananggulpan 1987). The term β-hematin was coined by Hamsik (1925) for sodium bicarbonate insoluble hematin. The facts that β-hematins are insoluble in sodium bicarbonate solution, that hematin esters are not transformed into β-hematin compounds and that β-hematins are reconverted into hemin esters by esterification indicate that the carboxylic acid groups of hematin are involved in β-hematin formation. Lemberg and Legge (1949) used the term β-hematin to allude to the presence of a specific linkage in which iron of one hematin unit is bound to the propionic acid group of another; β-hematin can either be a cyclic dimer or a linear polymer. Today, all synthetic hematin—crystals with iron of one hematin bound to the propionic acid group of another hematin are called β-hematin. It should be noted that this terminology is silent about possible variations in three-dimensional structure.

For many years, malaria pigment was thought to be formed of high molecular weight chains of hemes linked in a coordination complex with the ferric-iron of each heme moiety bound to a carboxyl side chain of the adjacent heme molecule (Slater et al. 1991). Chloroquine was thought to inhibit a unique plasmodial "heme polymerase" that is responsible for linking hematins together to form a polymer (Slater and Cerami 1992). From 1992, the search for the postulated "heme polymerase" was an important focus of malaria research for more than 10 years, with many researchers supporting the polymerization concept. In 1994 Hempelmann and Marques (1994) drew attention to the fact that malaria pigment was readily split into a soluble form at

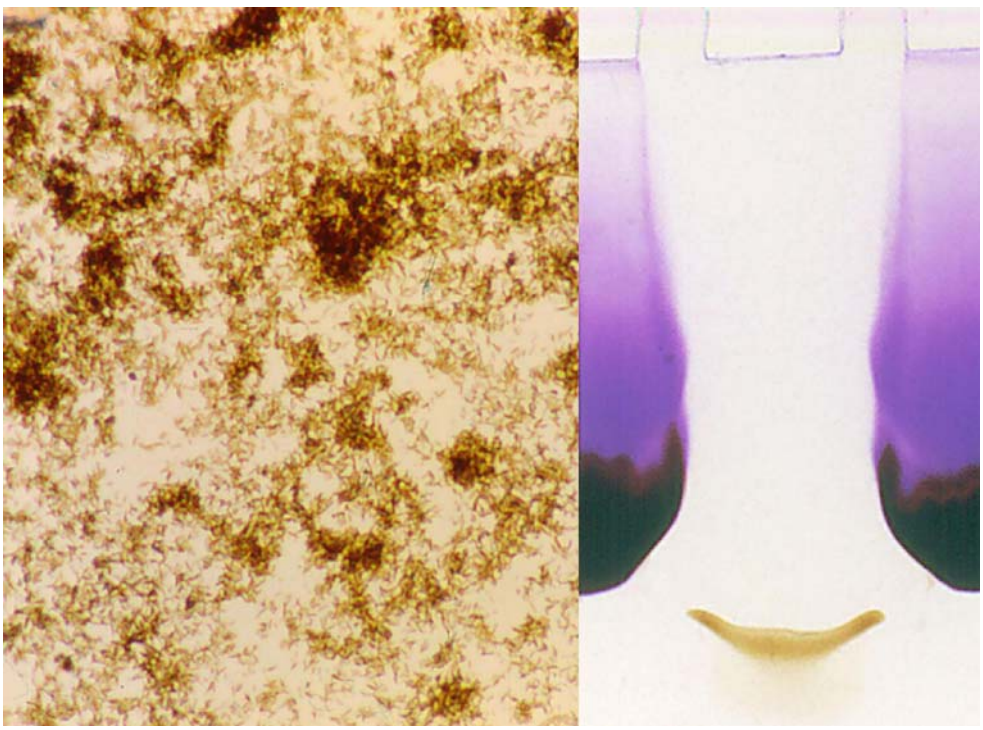

Fig. 2 Isolated hemozoin from *P. falciparum*. Stained with Giemsa's stain (magnification× 3,000) (*left*). Hemozoin electrophoresis at pH 11.2 (*right*): isolated pigment was applied to the *middle lane* and red cell lysate was applied to the *two outer lanes*; staining was done with Coomassie Blue

pH 11.2. Hydrogen, hydrophobic and ionic bonds are broken easily by reagents such as urea, SDS and mild alkaline buffer, but coordination polymers are stable. A polymer would, therefore, produce a "ladder" of different "mers" after electrophoretic separation in SDS gels at pH 11. Five different parasite strains and six different pH conditions were tested, but neither a "ladder" nor degradation products were ever found (see Fig. 2). The inescapable conclusion was that hemozoin could not consist of polymerized hemes and that an enzyme "heme polymerase" was not present. Pigment is not formed by polymerisation but by biocrystallization. The determination of the structure later confirmed the non-polymeric nature of synthetic β-hematin and hemozoin. Reciprocal iron carboxylate bonds form heme dimers which are linked through hydrogen bonding between the remaining propionate groups (Pagola et al. 2000).

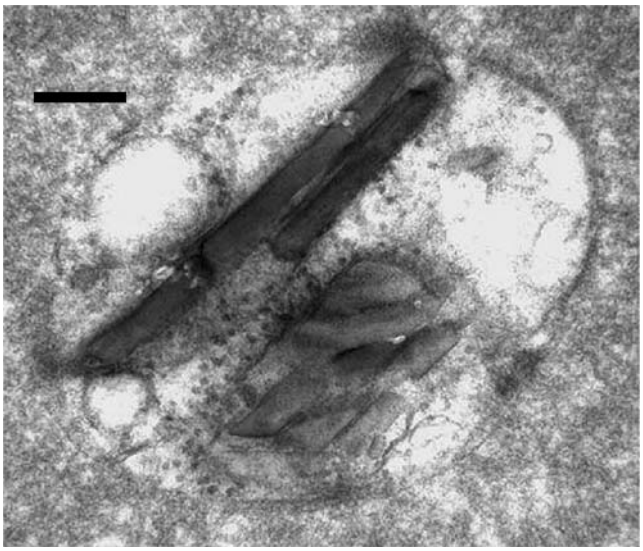

Fig. 4 A transmission electron micrograph of a section through a food vacuole (*Plasmodium falciparum*) containing groups of hemozoin crystals. *Scale bar*=200 nm

Hemozoin biogenesis

Hemozoin is formed in a process known as biomineralization or biocrystallization (Egan et al. 2001; Ziegler et al. 2001). Biomineralization is common in nature and describes the deposition of low molecular weight inorganic materials (e.g., calcium carbonate or calcium phosphate, which form the hard mineralized body parts like bones, teeth and shells) within or outside the cells of living organisms. This process is not enzymatic and proceeds on organic matrices produced by the organism (Lowenstam and Weiner 1989; Mann 2002; Bäuerlein 2004); biocrystallization refers to the process by which living organisms form crystals from high molecular weight, organic materials (Hempelmann and Egan 2002). The sequestration of toxic macromolecules in intracellular crystalline assemblies is a very efficient means for detoxification. Hemoglobin in the red cell cytosol has an extraordinarily high concentration with more than 340 mg/ml (5 mM), and no crystal formation would be possible in a solution of such high viscosity. Therefore, pigment crystals must be enveloped in specific membranes (Hempelmann et al. 2003). The first step of pigment formation is the removal of water from aquaferriprotoporphyrin IX, which most probably, is carried out by lipids. The lipidic environment would provide conditions that favour the formation of the iron–carboxylate bond of the β-hematin dimer. Formation of this bond in aqueous solutions has a high activation energy that requires non-physiological conditions of temperature or ionic strength to grow hemozoin crystals in vitro. In aqueous solution, the high concentration of water (55.5 M) may

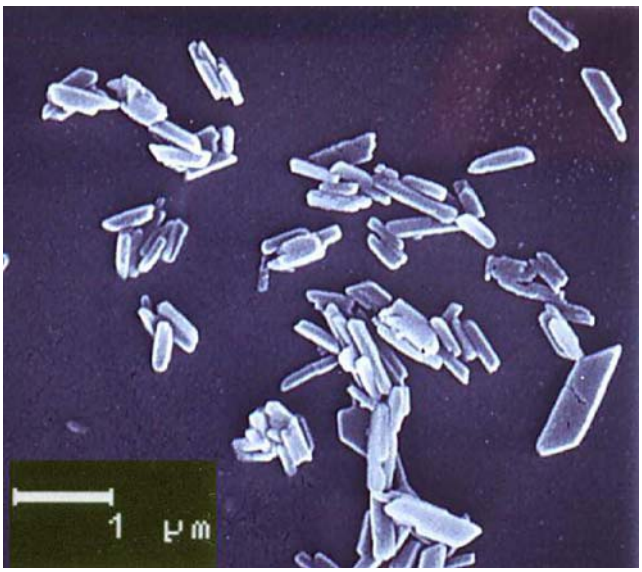

Fig. 3 Scanning electron micrograph of isolated *Plasmodium falciparum* hemozoin. Microscocopy was carried out with a DSM 950 microscope (Zeiss, Oberkochen, Germany)

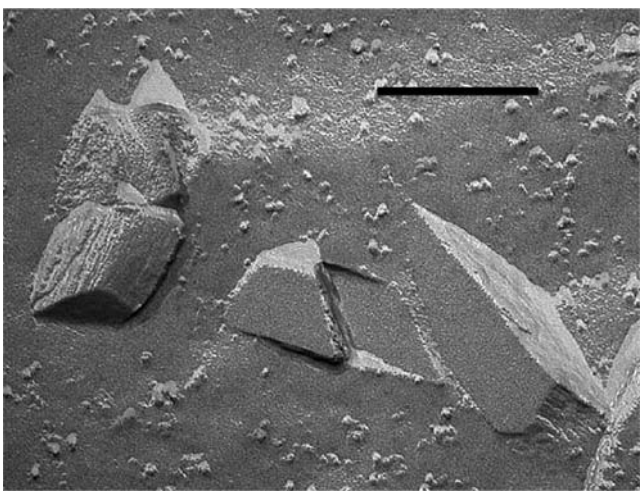

Fig. 5 A freeze-fracture preparation showing a group of hemozoin crystals orientated at various angles. *Scale bar*=200 nm

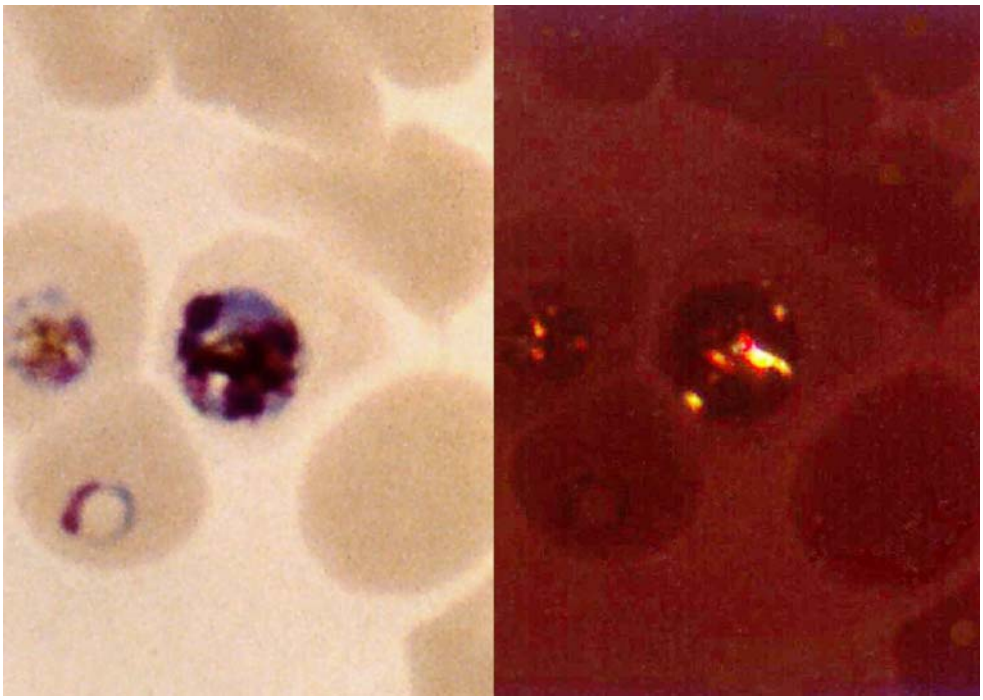

Fig. 6 Birefringence of hemozoin present in mature stage parasites. *Left*: Giemsa-stained thin blood smear viewed with ordinary light and showing three *P. falciparum* parasitised red blood cells. *Right*: the same field examined using polarized light and showing the birefringence of the crystalline hemozoin (magnification×3,000)

compete with the carboxylate groups for the Fe (III) centres. Thus initial formation of the iron carboxylate bond is probably thermodynamically unfavourable. In a hydrophobic environment (e.g., a lipid bilayer), the iron carboxylate bond is likely to be more stable due to the low water concentration. This may well contribute to a significant reduction in activation energy as any intermediate stages would be thermodynamically much more stable than in water. Moreover, in a growing hemozoin crystal, lipids would promote hydrogen bonding between propio-

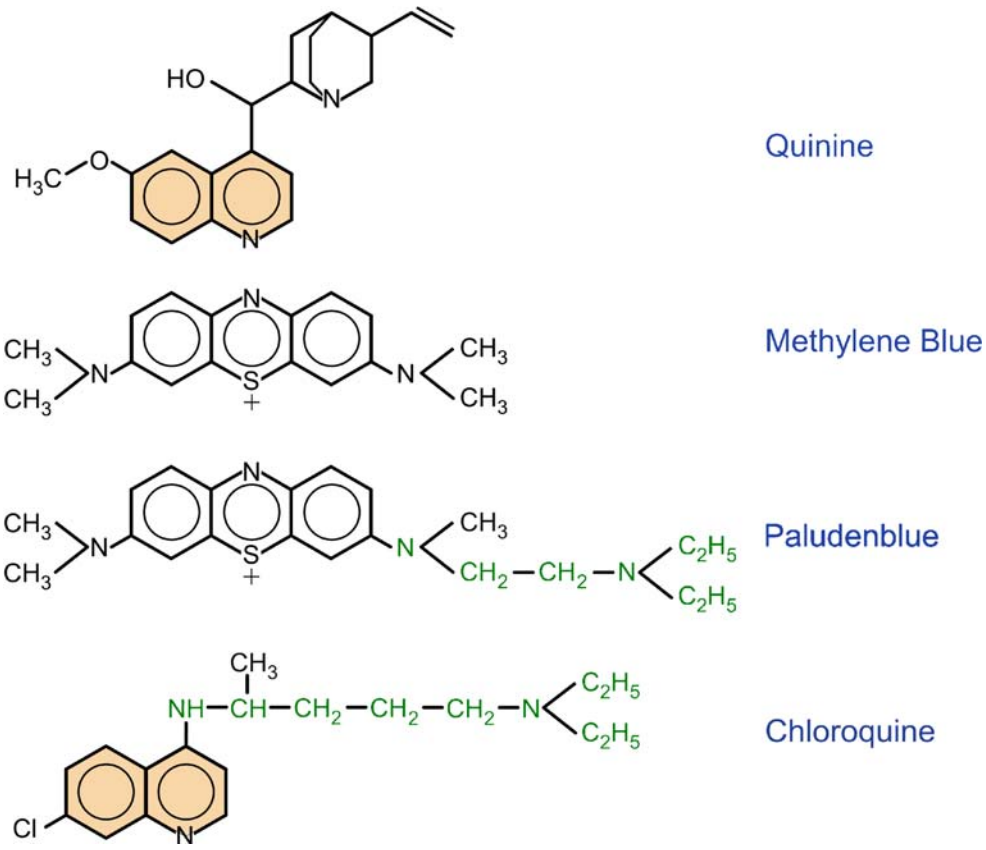

Fig. 7 The derivation of chloroquine

nate side chains providing rigidity and complexity to the crystal (Egan 2002; Sullivan 2002; Fitch 2004).

Biocrystallization as an antimalarial drug target

The first synthetic drug with a slight antimalaria activity was a phenothiazine, methylene blue (Guttmann and Ehrlich 1891; Vennerstrom et al. 1995; Atamna et al. 1996). Methylene blue is a strong inhibitor of hemozoin crystal growth (Deharo et al. 2002; Chong and Sullivan 2003). One methyl side chain of methylene blue was further modified to yield a better drug (Paludenblue) (Schulemann 1932; Dünschede 1971). This drug never gained a place in therapy but the new type of chemical side chain—the dialkylaminoalkylamino group—was of practical use (Wainwright and Amaral 2005) and is now part of the chloroquine (Resochin) molecule (7-chloro-4-[[4-(diethylamino)-1-methylbutyl] amino] quinoline) (see Fig. 7). Resochin was initially synthesized in 1934 by H. Andersag, who worked in Bayer's Elberfeld laboratories of the "Interessen-Gemeinschaft Farbenindustrie AG" (IG Farben) consortium. Resochin has a narrow safety margin, is very dangerous in overdosage and was considered to be too toxic for practical use in humans. This "Resochin Error" was corrected in the 1940s by the American partners of the chemical giant IG Farben and Resochin was renamed chloroquine (Coatney 1963). Chloroquine is safe when used at the correct dose, it is cheap, and it has been one of the most successful antimalarials ever developed. It was a reliable first-line prophylaxis that was used extensively for 40 years. The first cases of chloroquine resistance were reported from South America (Moore and Lanier 1961) and independently from Southeast Asia (Harinasuta et al. 1962). Chloroquine resistance spread progressively from the initial foci, and this cheap and formerly most widely used antimalarial drug is now useless in most endemic areas. Chloroquine resistance is not due to inactivation, breakdown or neutralisation of chloroquine but is linked to multiple mutations in a transporter protein in the parasite's digestive vacuole membrane (PfCRT, *Plasmodium falciparum* chloroquine resistance transporter) (Wellems and Plowe 2001).

Macomber et al. (1967) suggested that quinoline containing antimalarial drugs are inhibitors of heme detoxification. Today, the blood schizontocidal antimalarial action of quinolines is attributed primarily to their inhibition of hemozoin biocrystallization (Bray et al. 1998; Tekwani and Walker 2005; Figs. 3, 4, 5 and 6), and pigment biogenesis has attracted significant interest for new antimalarial drug discovery research. High field NMR experiments have been carried out by Leed et al. (2002), indicating a previously unrecognized key role for the chloroquine aliphatic chain in stabilizing aquaferriprotoporphyrin IX–chloroquine complexes (Fig. 7). There is a recent revival of interest for the syntheses of quinine, the first biocrystallization inhibitor (Kaufman and Ruveda 2005) because no widespread quinine resistance has yet been reported. Heme is not a parasite-encoded molecule and biocrystallization is, therefore, a non-mutatable target. A better understanding of the process of biocrystallization and its uniqueness is needed to develop new drugs (Dascombe et al. 2005; Sharma 2005).

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