CTMI (2005) 295:293–324
© Springer-Verlag Berlin Heidelberg 2005

Bioavailable Iron and Heme Metabolism in *Plasmodium falciparum*

P. F. Scholl[1] · A. K. Tripathi[2] · D. J. Sullivan[2] (✉)

[1]Department of Environmental Health Sciences, Baltimore, MD 21205, USA

[2]W. Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, E5628, 615 North Wolfe Street, Baltimore, MD 21205, USA
dsulliva@jhsph.edu

1 Human Host Iron Metabolism . . . 294

2 Perturbations of Human Iron Metabolism by Malaria . . . 296

3 *Plasmodium* Iron Metabolism . . . 299
3.1 Hemozoin Structure and Function . . . 300
3.2 Biochemical Formation In Vivo and In Vitro . . . 301
3.3 Heme Crystal Inhibition . . . 307
3.4 *Plasmodium* Iron Sources and Pathways . . . 308
3.5 Heme Synthesis in Two Organelles . . . 310
3.6 Iron Chelation Therapy . . . 311

4 Hemozoin as a Biomarker of Malaria Infection . . . 312
4.1 Laser Desorption Mass Spectrometry . . . 312
4.2 Hemozoin Detection . . . 313

5 Perspectives . . . 316

References . . . 316

Abstract Iron metabolism is essential for cell function and potentially toxic because iron can catalyze oxygen radical production. Malaria-attributable anemia and iron deficiency anemia coincide as being treatable diseases in the developing world. In absolute amounts, more than 95% of *Plasmodium* metal biochemistry occurs in the acidic digestive vacuole where heme released from hemoglobin catabolism forms heme crystals. The antimalarial quinolines interfere with crystallization. Despite the completion of the *Plasmodium* genome, many 'gene gaps' exist in components of the metal pathways described in mammalian or yeast cells. Present evidence suggests that parasite bioavailable iron originates from a labile erythrocyte cytosolic pool rather than from abundant heme iron. Indeed the parasite has to make its own heme within two separate organelles, the mitochondrion and the apicomplast. Paradoxically, despite the abundance of iron within the erythrocyte, iron chelators are cytocidal to the *Plasmodium* parasite. Hemozoin has become a sensitive biomarker for laser desorption mass spectrometry detection of *Plasmodium* infection in both mice and humans.

Abbreviations

DMT	Divalent metal-ion transporters
ZnPPIX	Zinc protoporphyrin IX
HRP II	Histidine-rich protien II
VEPL	Vacuole enriched parasite lysates
DFO	Desferrioxamine
LDMS	Laser desorption mass spectrometry
MALDI	Matrix-assisted laser desorption ionization

1 Human Host Iron Metabolism

Iron is an essential participant in metabolic reactions in all living cells because as a transition metal it can undergo changes in electron oxidation states and therefore can both oxidize and reduce molecules. Inorganic iron is the second most abundant metal in the Earth's crust, yet from a biologic perspective is difficult to extract from its insoluble oxides. Likewise in humans 95% percent of the 3 g of iron is highly concentrated in erythrocytes or macrophages, with minute amounts bioavailable in tissues. Daily absorption and excretion is one-thousandth of the total pool (Fairbanks and Beutler 1995b; Hentze et al. 2004). Similarly, the intraerythrocytic *Plasmodium* parasite exists in the erythrocyte with 100 fg (20 mM) of iron per cell, but utilizes less than one-one-thousandth of this iron pool (Egan et al. 2002).

Iron exists only transiently as a free cation because it is carefully chaperoned to proteins that bind or incorporate it. Iron proteins comprise the heme proteins such as hemoglobin, myoglobin, catalase and cytochromes or iron flavoproteins such as xanthine oxidase, succinate dehydrogenase and NAD dehydrogenase (Hentze et al. 2004). Iron is essential for mitochondrial and chloroplast function as the electron transport chain, iron sulfur clusters and nearly half of the enzymes of the tricarboxylic acid cycle require iron. Because iron can also damage membranes and proteins with the production of oxygen radicals, its intracellular concentration is closely regulated with an array of storage and transport proteins that are transcriptionally regulated in eukaryotes. An iron responsive element and iron regulatory protein interact in the absence or presence of bioavailable iron to tightly regulate genes like ferritin, transferrin, aconitases, heme synthesis enzymes and the iron transporters such as divalent metal-ion transporters (DMT) or ferroportins (Hentze et al. 2004).

The human host of the malaria parasite has approximately 2,500 mg (female) to 3,500 mg (male) of total iron as depicted in Fig. 1. Hemoglobin contains approximately 1,700 mg (female)–2,400 mg (male) or about 65%

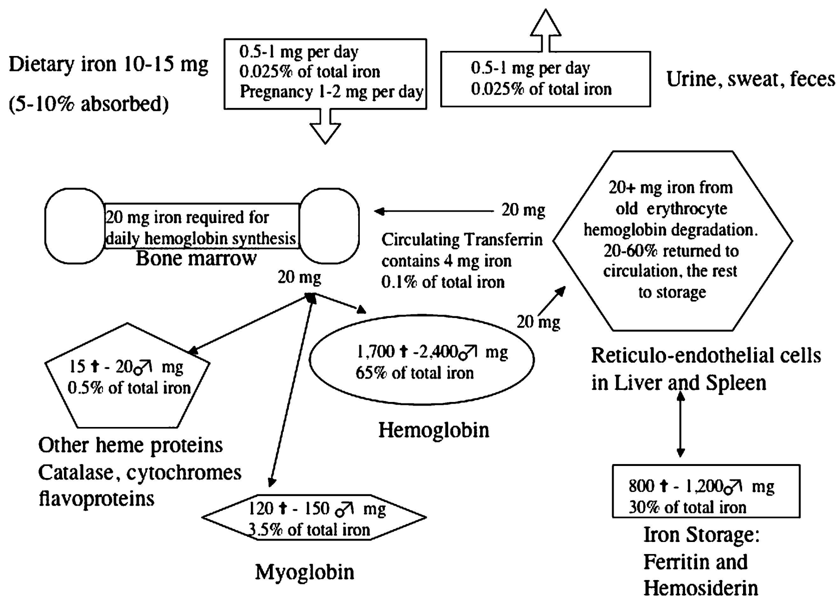

Fig. 1 Human iron compartments

of the iron total. The reticuloendothelial macrophages, predominately in the liver and spleen, contain approximately 600 mg in ferritin and hemosiderin; the bone marrow approximately 300 mg, the liver parenchyma approximately 1,000 mg and muscle and other cells approximately 400 mg. Only 3–4 mg circulate in a plasma labile pool bound to transferrin. This labile pool, also subject to diurnal variation, turns over seven to eight times a day resulting in approximately 20 mg that is loaded and delivered principally to the bone marrow for hemoglobin synthesis. While total dietary iron intake is about 10–15 mg, only 1 mg (10%) is absorbed through the intestine. One milligram is also excreted in sweat, urine or feces each day. Thus the majority of the daily hemopoietic iron requirements originates mainly from macrophage recycling of heme iron and also from ferritin store transfer. The spleen and liver reticuloendothelial cells normally digest phagocytosed erythrocytes at a rate sufficient to release 20% of the heme iron in a few hours with 20%–70% recycled back to hemoglobin in a few days (Fairbanks and Beutler 1995b; Hentze et al. 2004).

In the past 15 years, many aspects of the molecular iron metabolism in mammals have been elucidated. The majority of intestinal iron absorption is by heme endocytosis or transport into intestinal epithelial cells, called enterocytes where heme oxygenase degrades heme to release iron. The mechanism of heme transport across membranes is a large unanswered question. An al-

ternative enterocyte pathway, that is upregulated in iron deficiency, is direct iron cation transport by DMT-1 (Gushin et al. 1997). The enterocyte exports iron to the plasma via the transporter, ferroportin (McKie et al. 2000). Cellular iron uptake from plasma is by receptor-mediated uptake of transferrin. Acidification of the endocytotic vacuole releases iron which is transported into the cell cytosol again by DMT-1 (Hentze et al. 2004). Only specialized cells, including duodenal enterocytes, macrophages, hepatocytes, astrocytes and syncytiotrophoblasts, are capable of iron export via ferroportin. Iron export from nonintestinal cells requires ceruloplasmin or hephaestin (Garrick et al. 2003; Hentze et al. 2004; Kaplan and O'Halloran 1996; Miyajima 2002). Most of the cellular requirement for iron is for mitochondrial function and DNA synthesis.

Iron deficiency anemia affects over 500 million people including more than half of all children and pregnant women (Yip 1998). Low total iron body content results most commonly from insufficient dietary intake, but can also result, amongst diverse etiologies, from chronic blood loss, diversion of iron to the fetus in pregnancy or intravascular hemolysis with hemoglobinuria. Iron depletion is first evidenced with low to absent tissue ferritin storage, with intact plasma iron and blood hemoglobin levels. Clinical iron deficiency gradually progresses from low (20% of normal) plasma iron and transferrin saturation without anemia before progressing to low hemoglobin levels manifested by a hypochromic, microcytic anemia. The normal 120-day erythrocyte life span is decreased by 10%–20%. Cellular and erythrocyte ferritin decreases, while soluble and surface transferrin receptors increase along with erythrocyte zinc protoporphyrin IX (ZnPPIX). Serum erythropoietin is elevated in pure iron deficiency anemia (Fairbanks and Beutler 1995a).

Anemia of chronic disease caused by infection, inflammation or cancer is a systemic response that is hypothesized to limit bioavailable iron from pathogenic microbes or tumors in general (Gera and Sachdev 2002). Total body iron is normal, with high ferritin stores, but plasma iron is 20% of normal and dietary absorption decreases. Erythrocyte life span is also reduced by 20%. Most studies show ineffective erythropoiesis with a blunted production and response to erythropoietin. However, exogenous recombinant erythropoietin increases erythrocyte production in many chronic inflammatory diseases (Spivak 2002).

2
Perturbations of Human Iron Metabolism by Malaria

Malaria infection and disease impacts iron metabolism in many diverse ways (see the chapter by D. Roberts et al., this volume). Malaria infection geo-

graphically coincides with iron deficiency anemia. As a chronic infectious disease, malaria further decreases iron uptake from the intestine, contributes to ineffective erythropoeisis and sequesters bioavailable iron in ferritin stores (Spivak 2002). Lysis of infected erythrocytes may increase urinary losses of hemoglobin iron. The increased destruction of both infected and uninfected erythrocytes increases demand for bone marrow production. Asymptomatic malaria in semi-immune individuals is represented with parasitemias of approximately 1,000 parasites/µl or 0.025% infected erythrocytes. This fraction would represent 2.5% of daily erythrocytes destroyed. Symptomatic infections with 400,000 parasites/µl or 10% of erythrocytes infected represents a stress on the reticuloendothelial macrophages of 10 times the normal rate of destruction of erythrocytes. During maturation in the erythrocyte, the *P. falciparum* parasite sequesters 50%–65% of erythrocyte heme into an insoluble hemozoin crystal (Egan et al. 2002; Francis et al. 1997). Hemozoin is resistant to heme oxygenase degradation and accumulates in macrophages, monocytes and polymorphonuclear neutrophils (Schwarzer et al. 1999b). Essentially the *P. falciparum* parasite removes half of bioavailable heme presented to reticuloendothelial cells because macrophages are unable to degrade hemozoin heme to release iron. From a different perspective, a single erythrocyte has approximately 90–100 fg of iron with 50 fg present in hemozoin crystals at mature schizont stages, therefore 100 billion parasites contain 5 mg of iron in hemozoin (Egan et al. 2002). A single liter of whole blood contains 3–4 trillion erythrocytes. Therefore, a 2%–3% parasitemia represents 100 billion parasites in a liter (100,000/µl) or 5 mg of iron in hemozoin. Total blood volume for children is approximately 65–75 ml/kg lean body weight (Haddad et al. 2001). A 14-kg child then would have a liter of blood from which a 2%–3% parasitemia would remove 5 mg of iron in the form of hemozoin from the human iron metabolism cycle every 2 days. A 70-kg adult with 5 l of blood and a 2%–3% parasitemia, then has 30 mg of iron removed from bioavailable pools every 2 days. Hemozoin does not stimulate heme oxygenase production in macrophages/monocytes in vitro (Schwarzer et al. 1999a) and can persist for months (Levesque et al. 1999; Taliaferro and Mulligan 1937). Hemozoin has been shown to have specific inhibitory effects on macrophages and monocytes and also may directly inhibit hemoprogenitor cells in the marrow (Arese and Schwarzer 1997; Schwarzer et al. 1992). In summary, the malaria parasite causes anemia acutely by a destruction of both infected and uninfected erythrocytes, by a negative impact on erythropoiesis, by inducing an anemia of chronic diseases state and by sequestering away tens of milligrams of iron into nonbioavailable hemozoin which can persist for months before degradation to release iron.

The proportions of anemic individuals with malaria attributable anemia (Menendez et al. 1997) versus low dietary intake (Tatala et al. 1998) or losses

from intestinal helminths are often difficult to establish on a public health community level (Brooker et al. 1999; Leenstra et al. 2004; Nyakeriga et al. 2004; Stoltzfus et al. 2000). Both iron replenishment alone or malaria chemoprophylaxis or treatment increase hemoglobin levels by 1–2 g/dl (Massaga et al. 2003; Menendez et al. 2004). Often, however, with a high proportion of malaria attributable anemia in a population, iron repletion has little to no effect on hemoglobin levels (Desai et al. 2004; Stoltzfus et al. 2004). However, iron supplementation and malaria chemotherapy both increase hemoglobin values in a synergistic manner (Desai et al. 2003; Ekvall et al. 2000; Verhoef et al. 2002) although some studies still show minimal effect of combination therapy (Menendez et al. 2004).

A lingering debate in the malaria and nutrition community concerns community dietary iron supplementation, which may increase malaria disease in malaria endemic areas. Iron replenishment which improves anemia has both positive growth, development and cognitive benefits. However, early studies noted an increase in malaria disease especially with injection iron, but also with oral supplementation (Murray et al. 1975; Oppenheimer 2001). Other more recent studies mainly with oral replacement have not shown exacerbation of malaria disease (Berger et al. 2000; Mebrahtu et al. 2004; Menendez et al. 1997, 2004). A meta-analysis study did not show an increase in malaria disease from iron supplementation (Gera and Sachdev 2002). A hypothesis that seeks to balance the conflicting studies is that only the most severely anemic children with hemoglobin concentrations below 6–7 g/dl (less than 10%–20% of children in most malaria anemia study populations) are at risk for exacerbation of malaria disease with iron supplementation. The more rapid injection supplementation rather than the slower absorption by oral dosing increases the risk.

An hypothesized molecular mechanism that accounts for possible exacerbation of malaria disease with rapid iron supplementation involves high levels of ZnPPIX (Iyer et al. 2003). When bioavailable iron is low the human mitochondrial ferrochelatase inserts zinc instead of iron into protoporphyrin IX. Normal erythrocytes have concentration of ZnPPIX of 0.5 μM (25μmol ZnPPIX:1 mol heme). This can elevate to 5 μM (250 μmol ZnPPIX:1 mol heme) in severe iron deficiency anemia. Moderate iron deficiency anemia or anemia of chronic disease do not elevate ZnPPIX to these levels (Hastka et al. 1993; Lamola and Yamane 1974). ZnPPIX resides in the heme pocket of hemoglobin and can inhibit hemozoin extension in vitro with an IC_{50} of 5 μM (Iyer et al. 2003). Individuals with severe anemia have subpopulations of erythrocytes with greater elevations of ZnPPIX which are inhospitable to malaria infection alongside erythrocytes with slightly lower ZnPPIX levels that are able to support infection. Rapid iron repletion stimulates production of erythrocytes with lower levels of ZnPPIX and malaria disease is possibly exacerbated in

a few weeks with the replacement by a cohort of new erythrocytes capable of supporting high parasitemias.

3
Plasmodium Iron Metabolism

The intraerythrocytic *P. falciparum* parasite encounters two important issues that relate to iron. The first is how to handle the excess heme iron produced from hemoglobin catabolism and the second is how to acquire the iron necessary for cellular metabolism.

In absolute amounts more than 95% of *Plasmodium* metal biochemistry involves the sequestration of reactive, toxic heme in an acidic, oxygen-rich vacuole into an inert heme crystal called hemozoin that removes the heme iron moiety from solution chemistry (Egan et al. 2002; Francis et al. 1997). The appearance of microscopic birefringent hemozoin marks the *Plasmodium* transition from ring stage to trophozoite stage seen in Fig 2. Microscopically, schizont stage is the appearance of more than one nucleus. Gametocytes, ookinetes and even oocysts retain the hemozoin crystals, while sporozoites, liver stages and merozoites lack hemozoin. The intraerythrocytic parasite ingests 60%–80% of the host's 5 mM hemoglobin both as a source of amino acids and possibly to provide room for the parasite (Egan et al. 2002; Lew et al. 2003; Zhang et al. 1999). Interestingly, the *P. falciparum* parasite ingests 10 times the amount of hemoglobin protein than it retains for amino acid building blocks (Krugliak et al. 2002). *Plasmodium* aspartic and cysteine proteases efficiently degrade ingested hemoglobin releasing approximately 1 fmol of heme in a 2 fl volume of the acidic digestive vacuole. Conceptually, this approximates to almost 0.4 M heme in an oxygen-rich acidic environment capable of generating oxygen radicals by the Fenton reaction (Francis et al. 1997). The parasite lacks heme oxygenase activity or iron storage proteins

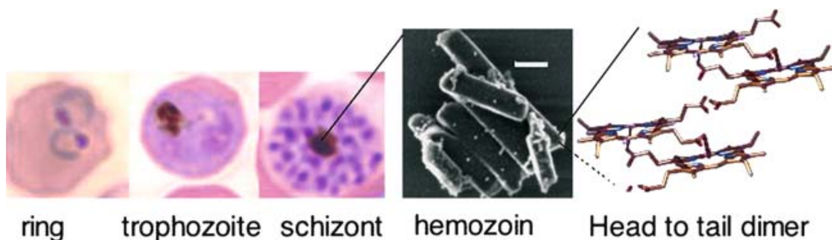

Fig. 2 Progressive magnification of hemozoin. Giemsa stain (×100) of ring, trophozoite and schizont stage intraerythrocytic *P. falciparum* with electron microscopy of crystals (*scale bar*, 200 nm) and model of hydrogen bonding of two heme crystal dimers

like ferritin. Indeed, the parasite has to synthesize its own heme rather than scavenge from the erythrocyte (Surolia and Padmanaban 1992).

Within the intraerythrocytic cycle, a single parasite undergoes rapid proliferation, multiplying 8–32-fold within 48 h. In a few weeks time, billions of infected erythrocytes result. This rapid replication of DNA requires iron for the ribonucleotide reductase for nucleotide synthesis (Mabeza et al. 1999). Despite thriving in a host cell with 20 mM concentrations of heme iron, the parasite is killed by low concentrations of iron chelators indicating that the amount of bioavailable iron is limited and crucial for parasite growth and survival (Mabeza et al. 1999; Raventos-Suarez et al. 1982).

3.1
Hemozoin Structure and Function

Even though the black malaria pigment or hemozoin was known to contain heme as described by Carbone in 1891 (Carbone 1891), 100 years passed before this molecule was demonstrated to be β-hematin, which spontaneously forms in vitro if heme is incubated at high (>50°C) temperatures in acidic, aqueous solutions (Fitch and Kanjananggulpan 1987; Slater et al. 1991). In 1991, Slater and Cerami reported, by different spectroscopic techniques, the unique iron–oxygen bond coordinating the propionate carboxylate oxygen of one heme to the central ferric molecule of another (Slater et al. 1991). They later demonstrated that parasite lysates were capable of catalyzing hemozoin formation in vitro (Slater and Cerami 1992). The initial model was that of heme polymers (Slater 1992). Later powder diffraction data by Bohle's group demonstrated instead that the biocrystal consists of two hemes linked by a reciprocal iron (Fe-1) head to carboxylate (O-31) tail (Pagola et al. 2000). The two coordinate hemes made a triclinic unit cell, a=12.196 (2) A°, b=14.684 (2) A°, c=8.040 (1) A°, a=90.22 (1)8, b=96.80 (1)8, ɣ=97.92 (1)8; V=1416.0 (3) A° 3; ϱ_{exp}=1.45 (1) g/cm^3; Z=2 (Pagola et al. 2000). As shown in Fig. 2 the head-to-tail dimers crystallize by hydrogen bonding of the remaining carboxylate groups into a lattice with a rectangular crystal morphology of size dimension 100 nm×100 nm×500 nm in the case of *P. falciparum* (Noland et al. 2003). β-hematin formation is a chemical process governed by time and temperature that absolutely requires an acidic pH in aqueous solutions (Egan 2002; Egan et al. 1994b). Blauer has described intermediate transition states by FTIR called B-hematin (Blauer and Akkawi 1997, 2000). Preformed heme crystals allow for rapid extension at 37°C (Chong and Sullivan 2003). Kinetics of formation is sigmoidal, characterized by a nucleation and then an extension process (Chong and Sullivan 2003; Egan 2002; Egan et al. 2001). Single head-to-tail dimers have not been isolated as they incorporate rapidly into the larger aggregate structure.

Presently no consensus exists about how hemozoin is made within the parasite. Diverse hypotheses with some supporting in vitro data include spontaneous initiation (Egan et al. 1994b), seeded from preformed crystals (Ridley 1996), enzymatic by a heme 'polymerase or crystallase' (Slater and Cerami 1992), initiation by lipids (Bendrat et al. 1995; Fitch et al. 1999; Hempelmann and Egan 2002; Tripathi et al. 2002) or nucleation by parasite proteins (Choi et al. 1999; Sullivan et al. 1996a). The Pf histidine-rich protein II (HRP II)–that has 35% of its amino acids as histidine–is present in the digestive vacuole; it can bind heme at acidic pH and is able to initiate in vitro heme crystal formation inhibited by the quinolines (Sullivan et al. 1996a). However, the laboratory progeny, 3B-D7 from parents HB3 and Dd2, lacks both HRP II and HRP III and still makes hemozoin (Sullivan 2002b). While both *P. reichnowi* and *P. lophurae* have a protein with HRP II primary structure homology, the three mouse malaria's sequenced and *P. vivax* and *P. knowlesi*, all lack a close homolog of PfHRP II or III (Bahl et al. 2002). This indicates that PfHRP II is sufficient in vitro, but not absolutely necessary in vivo, to initiate hemozoin formation (Sullivan 2002a, 2002b). Tilley has also demonstrated that only a small fraction (but still several μM) of PfHRP II reaches the digestive vacuole (Papalexis et al. 2001).

Attention has recently focused on lipid initiation of heme crystal formation first described by Bendrat and Cerami, with further evidence of in vitro formation by Fitch et al. 1999, and also by Hempelmann et al. 2003, Pandey et al. 2003, and Tekwani (Tripathi et al. 2002). Unsaturated fatty acids and phospholipids accelerate hemozoin formation possibly by increasing solubility of heme in micelles. Likewise increasing dimethyl sulfoxide concentrations also accelerate hemozoin formation. However, some lipid formulations such as erythrocyte ghosts or the cholesterol-rich lipoproteins do not initiate hemozoin formation as efficiently (Fitch et al. 2003). While the lipid bilayers may contain a few unit head-to-tail crystal dimers, how the dimers are able to stack into the larger regular array of hemozoin crystals outside of a membrane remains unanswered.

3.2
Biochemical Formation In Vivo and In Vitro

Many different assays have been developed to compare the formation of hemozoin and inhibition by drugs. The assays can be divided by: (1) use of radioactivity (Dorn et al. 1995; Hawley et al. 1998; Slater and Cerami 1992) versus direct absorbance measurement of purified heme crystal (Basilico et al. 1998); (2) initiation by parasite lysates (Fitch and Chou 1996; Slater and Cerami 1992), preformed crystals (Dorn et al. 1998a, 1998b; Sullivan et al. 1996b), proteins (Sullivan et al. 1996a), or lipids (Bendrat et al. 1995;

Kurosawa et al. 2000; Tripathi et al. 2004) or nothing (Basilico et al. 1998); (3) purification by centrifugation or filter washes (Egan et al. 1994a) in mild basic solutions based on heme crystal being insoluble and unincorporated heme being soluble. The final product is quantified by dissolution of crystal in strong basic solutions of pH 14. Table 1 compares the elements of the published crystallization assays and the purification methods.

The assays range essentially from precipitation assays with millimolar heme at an acidic pH, to substrate binding assays with excess preformed heme crystal template. Only 3–5 μM heme is soluble at the pH less than 6 that is absolutely required for crystallization. Measurable product does not form at this low concentration, so 50 μM heme is the lowest concentration for routine assays even though most of the heme is precipitated. A nonradioactive, crystal extension assay with no centrifugation or filter washes was developed (Chong and Sullivan 2003). The basis of this assay lies in the difference in solubility and difference in visible absorption of free versus crystalline heme. Free heme is soluble in weak base, and has a very high molar extinction coefficient of 100,000 M/cm at 400 nm, whereas under these conditions heme crystal is insoluble and is spectroscopically invisible at the low concentrations used. In this assay, first, the absorption at 405 nm is measured of free heme mixed with β-hematin dissolved in a weak base and then the absorption is measured again after heme is decrystallized by concentrated NaOH. The difference in the two absorptions is then used to calculate the amount of heme crystal. The heme crystal extension reaction is kinetically and morphologically identical whether initiated with parasite-derived hemozoin or chemically synthesized β-hematin (Chong and Sullivan 2003). By using this more rapid assay the effects of pH, substrate and preformed crystal on the reaction kinetics were studied.

Presently all in vitro synthesis reactions have approximated but not duplicated the morphology of heme crystals isolated from the parasites in Fig. 3A. Duplication of the anhydrous anaerobic synthesis by Bohle to obtain larger crystals of dimension 200 nm×200 nm×500 nm (Bohle and Helms 1993) shows that these crystals in Fig. 3B most closely resemble *P. falciparum* hemozoin. An investigation into the effect of pH and the resultant morphology of initiation of heme crystallization by mono-oleoylglycerol, oleic acid, palmitoleic acid, low density liproprotein and high density lipoprotein, phosphatidylcholine and purified erythrocyte ghosts showed initiation of heme crystallization for all except the erythrocyte ghosts and high density lipoprotein. The lipids that initiated heme crystallization performed near equivalent to a hemozoin seeded reaction at pH 4 and 4.8. At pH 5.6, the lipids did not initiate heme crystallization, whereas the hemozoin seeded reaction was still at 100%. In Fig. 3C and D, field emission in-lens scanning electron microscopy examination of the in vitro incorporation on lipid formulations had long tapered edges consis-

Table 1 Published crystallization assays and purification methods.

Reference	Inititiator	Heme concentration (nmol)	Buffer; pH; temperature; time; volume	Washes	Comments and quinoline IC_{50}
Slater and Cerami 1992a	Trophozoite lysates 500 μg (approximately 125 nmol hemozoin)	400 μM and 140 μM ^{14}C hemin (400 and 140)	500 mM acetate; pH 5.0; 37°C; overnight; (?1 ml)	2× in 2% $SDS/NaHCO_3$ pH=9.1	Radioactive assay. First demonstration of hemozoin formation. IC_{50}: chloroquine, 120 μM; quinine, 300 μM; quinidine, 90 μM
Egan et al. 1994	Nothing	2.2 mM (11,000)	2.2 M acetate; pH 4.8; 60°C; 30 min; 5.04 ml	Water on filter	An acid precipitation with hemozoin formed. IC_{99}: 6 mM or three equivalents for chloroquine, quinine, quinidine
Dorn et al. 1995	Trophozoite lysates 25 μg, purified hemozoin or β-hematin –(?nmol)	140 μM ^{14}C hemin (140 ?)	500 mM acetate; pH 4.8; 37°C; overnight; (?1 ml)	2× in 2% $SDS/NaHCO_3$ pH=9.1	Lipid, preformed hemozoin promote formation. IC_{50}: chloroquine, 80–90 μM
Bendrat et al. 1995	Acetonitrile extract	50 μM (50)	165 mM acetate; pH 5.0; 37°C; overnight; 1 ml	2× in $NaHCO_3$ pH=9.1	Lipid component promotes formation
Sullivan et al. 1996a, 1996b	10 pmol HRP II or III; 5 nmol preformed hemozoin	50 μM (25)	500 mM acetate; pH 4.8; 37°C; overnight; 500μl	2% $SDS/NaHCO_3$ pH=9.1; 2%SDS	Nonradioactive initiation (HRP II) or extension of hemozoin formation. IC_{50}: chloroquine, 5 μM; quinine and quinidine, 1 μM

Table 1 (continued)

Reference	Inititiator	Heme concentration (nmol)	Buffer; pH; temperature; time; volume	Washes	Comments and quinoline IC_{50}
Basilico et al. 1998	Nothing	2 mM (400)	4 M; pH 3.0; 37°C; overnight; 200 µl	1× in DMSO	Acid precipitation with hemozoin formed. IC_{50}: chloroquine, 4 mM; quinidine, 8 mM (2–4×heme)
Hawley et al. 1998	50 µg β-hematin, 76 µmol	140 µM ^{14}C hemin (14)	500 mM acetate; pH 4.8; 37°C; overnight; 100µl	2% SDS/$NaHCO_3$, pH=9.1; $NaHCO_3$ pH=9.1; then 50 mM Tris pH 7.5	A binding assay as more than 1,000 times more preformed product than substrate added. IC_{50}: chloroquine, 24 µM; quinine, 64 µM; quinidine, 24 µM
Dorn et al. 1998a, 1998b	10–20 µmol β-hematin, Trophozoites lysates 25 µg=6 nmol hemozoin	140 µM ^{14}C hemin (14)	500 mM acetate; pH 4.8; 37°C; overnight; 100 µl	Filters. 2% SDS/$NaHCO_3$ pH 9.1; $NaHCO_3$ pH 9.1; 50 mM Tris pH 7.5	A binding assay as above. Trophozoite lysates. IC_{50}: chloroquine, 400 µM; quinine, 430 µM. Hemozoin template IC_{50}: chloroquine, 45 µM; quinine, 160 µM
Kurosawa et al. 2000	Acetonitrile extract of trophozoite lysates	100 µM cold and 0.56 nCi ^{14}C hemin (10)	500 mM acetate; pH 4.8; 37°C; overnight; 100 µl	Filters-2× 0.2% SDS/$NaHCO_3$, 2× 50 mM Tris pH7.5	No preformed polymer, just lipids to initiate. High throughput. IC_{50}: chloroquine, 80 µM

Table 1 (continued)

Reference	Inititiator	Heme concentration (nmol)	Buffer; pH; temperature; time; volume	Washes	Comments and quinoline IC_{50}
Chong and Sullivan 2003	1–5 nmol β-hematin	50 μM (10–50)	100 mM acetate; pH 5; 37°C; overnight; 1,000 or 200 μl	None	No centrifugation or filter washes based on low solubility and absorbance of β-hematin vs heme. IC_{50}: chloroquine, 4 μM
Tripathi et al. 2004	Lipid extracts	100 μM (100 or 20)	100 mM acetate; pH 5; 37°C overnight; 1,000 or 200μl	3× Tris/SDS; SDS-$NaHCO_3$;DW	Assay involves transfer to filters with three washes, then quantification

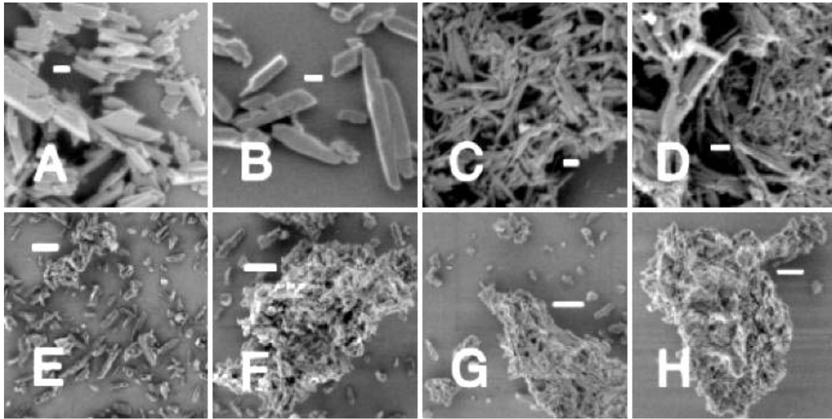

Fig. 3A–H Hemozoin from *P. falciparum* is similar to anhydrous base abstraction product, but contrasts with lipid initiated or HRP initiated heme crystals. **A** *P. falciparum* hemozoin. **B** β-Hematin produced from anhydrous base abstraction by the Bohle synthesis. Purified heme crystal product initiated by: **C** 50 μM mono-oleoylglycerol; **D** 50 μM palmitoleic acid; **E** VEPL + hemoglobin; **F** VEPL + 50 μM heme; **G** VEPL + HRP II + 50 μM heme; **H** HRP II and 50 μM heme. *Scale bar*, 200 nm for **A–D** and 1 μM for **E–H**

tent with rapid growth distinct from the apparently more controlled growth in parasites. Buller has modeled the rapid incorporation at 'growth faces' (Buller et al. 2002). Oxidized Fe^{3+} heme is a requirement for heme crystal formation. Strictly reduced conditions that have been demonstrated in aqueous conditions have not resulted in heme crystal formation (Monti et al. 1999). Protease treatment of acidified hemoglobin does not result in heme crystal formation.

A preparation of digestive vacuole enriched parasite lysates (VEPL) also known as 'crude vacuoles' (Goldberg et al. 1990) was used to initiate heme crystal incorporation in the standard acetate buffer pH 5.0. Field emission in-lens scanning electron microscopy showed closest resemblance to *P. falciparum* hemozoin with addition of only hemoglobin to the VEPL template in Fig. 3E, although new crystal incorporation was only 1.2 nmol. VEPL incubated with 50 μM heme (Fig. 3F), 1 μM recombinant HRP II and 50 μM heme (Fig. 3G), or just 1 μM recombinant HRP II and 50 μM heme and no VEPL (Fig. 3H) all showed a nonrectangular, almost amorphous addition of heme crystal dimers. The new synthesis of heme crystals was 7, 12 and 14 nmol, respectively, for the latter three conditions.

3.3 Heme Crystal Inhibition

Much of the interest in heme crystal formation derives from the extensive data showing many antimalarials like the quinolines, phenanthrenes and hydroxyxanthones inhibit β-hematin formation. Extensive work has been done on the heme binding interaction by the quinoline class of drugs (Egan et al. 1997; Fitch and Kanjananggulpan 1987; Leed et al. 2002; Moreau et al. 1985). Other drugs that bind heme have also been explored as antimalarials (Huy et al. 2002; Kalkanidis et al. 2002; Kristiansen and Jepsen 1985; Vennerstrom et al. 2000). Importantly, not all drugs that bind heme also inhibit hemozoin formation (Slater 1993). Heme binding affinity does not correlate with inhibition of heme crystallization in vitro (Egan et al. 2000). Not all drugs that bind heme and also inhibit hemozoin formation accumulate in the acidic digestive vacuole compartment to inhibit the parasite. Work has also been done that implicates the addition of basic side chains to heme binding drugs, such as the phenothiazines, to improve digestive vacuole localization and parasite inhibition (Kalkanidis et al. 2002).

Two mechanisms of β-hematin inhibition and an additional hybrid have been hypothesized. One is that the drug binds heme, sequestering away substrate from incorporation into the head-to-tail dimer or larger crystal (Fitch 1998; Leed et al. 2002; see also the chapter by Bray et al., this volume). Another is that drug binds directly to a growing face to prevent heme crystal dimer addition (Buller et al. 2002). The third, a hybrid, is that a drug–heme complex incorporates at the growing face to inhibit crystal growth (Chong and Sullivan 2003; Iyer et al. 2003; Sullivan et al. 1996b). Radiolabled chloroquine or quinidine copurify with hemozoin and in vitro bind measurably to growing heme crystals only with the addition of heme (Sullivan et al. 1996b, 1998). Examination of the kinetics of heme crystallization in the presence of quinoline antimalarials revealed that inhibition of heme crystallization by chloroquine and quinidine is reversible, and suggests previous studies that measured heme crystal formation at a fixed timepoint may underestimate the extent of inhibition. To distinguish between the inhibition mechanisms, the amount of heme substrate or heme crystal was increased to try to overcome drug inhibition. Addition of increasing amounts of heme substrate has no effect on the extent of chloroquine or quinidine inhibition, suggesting that these drugs do not act by sequestering free heme. Addition of increasing amount of heme crystal, however, reverses inhibition by these drugs, suggesting that the quinoline–heme complex somehow caps finite extension sites on the crystal (Chong and Sullivan 2003). This evidence suggests that a drug–heme complex binds at a growing face to inhibit crystal extension. Despite these and other findings, a consensus has not yet been reached on the mechanism of inhibition of heme crystallization.

A rational design strategy is hindered at present because of lack of knowledge concerning true inhibitory interaction, whether with heme, drug binding to the growing face of a crystal, drug–heme complex binding at the growing face, or competition with protein and/or lipid complex for initiation (Egan 2004). Molecular docking has been attempted to model interactions with hematin, and this had good agreement with NMR studies; this technique should be able to generate novel structures (Leed et al. 2002). Essential to this approach, but lacking at present, is the prediction of both heme binding and β-hematin inhibition. A manual docking strategy has been used to attempt to model drug alone to the well-defined structure of the β-hematin growing face (Buller et al. 2002). Egan has tried molecular mechanics and dynamics calculations to model quinoline interaction with a simplified porphyrin lacking substituents (Marques et al. 1996). The initial attempt did not correlate with structure–activity relationships or NMR evidence (Leed et al. 2002; O'Neill et al. 1997).

3.4 *Plasmodium* Iron Sources and Pathways

The *Plasmodium* parasite requires iron for DNA synthesis, glycolysis, pyrimidine synthesis, heme synthesis and electron transport. Debate continues on the critical source of iron for the intraerythrocytic parasite. An important paradox regarding iron is that millimolar desferrioxamine (DFO), an iron chelator, is cytostatic for mammalian cells and bacteria, while a 60-fold lower concentration of 15 μM DFO is cytocidal for *P. falciparum* despite the availability of 20 mM heme iron in hemoglobin (Mabeza et al. 1999). Postulated available sources susceptible to chelation include: (1) extracellular iron from transferrin or free iron in the media; (2) intracellular iron bound to low molecular weight proteins, heme iron or erythrocyte ferritin iron.

Despite early reports of transferrin mediated iron uptake (Haldar et al. 1986; Rodriguez and Jungery 1986), the bulk of experimental evidence excludes a requirement by parasites for extracellular iron. An important physiological study showed *Plasmodium* culture medium, which was depleted of transferrin by 500–1000-fold, supported *Plasmodium* growth well, while inhibiting fibroblasts dependent on transferrin uptake (Sanchez-Lopez and Haldar 1992). Free iron in *Plasmodium* culture medium has been determined to range from 1 to 10 μM. Dialysis removal of free iron to below 1 μM had no effect on parasite growth (Peto and Thompson 1986). Extracellular impermeable dextran-DFO also did not inhibit growth in culture (Scott et al. 1990). Intracellular low molecular weight proteins bind iron, but when DFO conjugates were loaded into resealed erythrocyte ghosts replication was not inhibited (Loyevsky et al. 1993; Scott et al. 1990). Egan has measured equal

amounts of total iron in uninfected and infected erythrocytes (Egan et al. 2002). In another study iron levels in the infected erythrocyte as measured by X-ray fluorimetry remained constant while zinc levels increased two- to threefold (Ginsburg et al. 1986). These constant iron levels suggest minimum import of extracellular iron. However with 20 mM iron levels the import of a few hundred micromolar equivalents or 1%–2% of total iron (more than enough to sustain the protozoan) may be within the standard error of measurement.

Lovesky has identified an intraerythrocytic labile pool of bioavailable iron with the fluorophore calcein (Loyevsky et al. 1999a). In the infected erythrocyte most of the labile pool of iron is in the parasite. In addition, uninfected erythrocytes had a greater total amount of labile iron than infected erythrocytes. Pollack has noted that a significant proportion of labile iron is bound to ATP in normal erythrocytes (Weaver et al. 1993; Zhan et al. 1990). This iron pool could be released with acidification, iron chelation and consumption of ATP by oxidative stress (Atamna and Ginsburg 1995; Gabay and Ginsburg 1993; Hershko and Peto 1988).

If even less than 1% of the 20 mM heme iron in hemoglobin were available, the parasite's iron requirements would be satisfied (Gabay and Ginsburg 1993; Gabay et al. 1994). The amount of heme that is uncoupled from crystallization, thus available for other metabolic needs is not known. Tilley has postulated hydrogen peroxide degradation of heme to release iron in the acidic digestive vacuole. Measurement of heme and iron in infected erythrocytes indicate that iron remains constant while total heme is decreased by 70% (Papalexis et al. 2001). At pH 7.0 reduced glutathione has also been demonstrated to catabolize heme, thereby releasing iron (Ginsburg et al. 1998). Ginsburg has proposed the transport of digestive vacuole heme to the parasite cytosol, where reduced glutathione releases iron by heme catabolism (Ginsburg and Krugliak 1999). Experiments demonstrated a loss of approximately half of the heme content. Chloroquine also interferes with heme degradation by glutathione (Famin and Ginsburg 2003; Famin et al. 1999). Elevated levels of reduced glutathione in mouse reticulocytes have also correlated with chloroquine resistance (Deharo et al. 2003). However, Egan in a separate set of experiments has demonstrated minimal loss of heme iron (Egan et al. 2002). Using subcellular fractionation and iron determinations, both infected and uninfected erythrocytes have the same amount of iron. Digestive vacuoles contained 90% of parasite iron in the form of heme with a minimal amount in parasite cytosol. This was confirmed by electron spectroscopic imaging of transmission electron microscopy.

Another potential source of iron is erythrocytic ferritin. Mature erythrocytes have 0.7 nM residual ferritin left over from heme synthesis by the reticulocyte (Gabay and Ginsburg 1993; Gabay et al. 1994). Ferritin accommodates up to 4,500 iron molecules (Mann et al. 1986). Erythrocytic ferritin could track to the digestive vacuole along with hemoglobin. Vacuolar proteases may de-

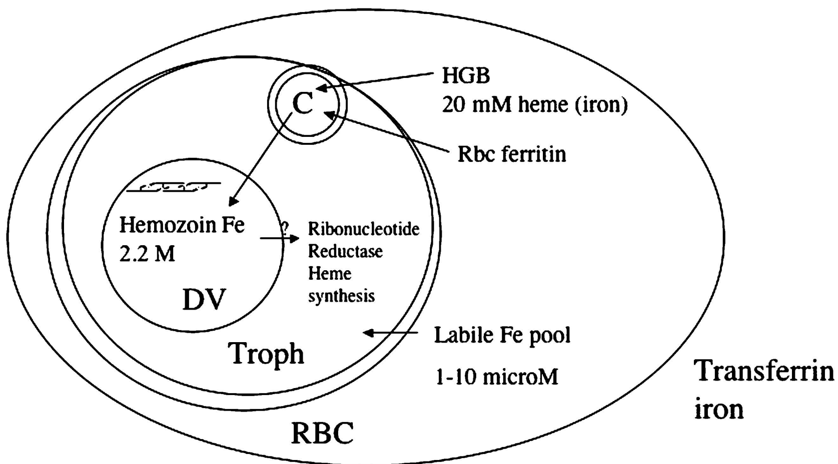

Fig. 4 *Plasmodium* heme and iron pathways. Hemoglobin (*HGB*), erythrocyte ferritin (rbc ferritin) and Pf are phagocytosed by the cytosome (*C*) and transported to the acidic digestive vacuole (*DV*) where proteases cleave hemoglobin, releasing toxic heme that is crystallized. Iron could enter the trophozoite (*TROPH*) from the erythrocyte (*RBC*) cytosol or originate from the DV from ferritin or heme. Quinolines (*Q*) enter the DV to cap crystallization

grade ferritin to release iron. Presently the data suggest that bioavailable iron is transported directly across the parasite plasma membrane rather than via the digestive vacuole or deriving from heme. Figure 4 depicts some of the postulated heme and iron pathways.

P. falciparum lacks a ferroportin, ferritin, metallothione, ferroxamine-based transport systems, or ferredoxin or bacterial iron siderophore orthologs in the sequence database (Rasoloson et al. 2004). Loyevsky has charatereized a *Plasmodium* iron regulatory protein (gb AJ012289; PF13_0229) demonstrating that it can bind mammalian IREs and also *P. falciparum* IREs which are different from the mammalian consensus sequence (Loyevsky et al. 2001, 2003). A DMT-1 ortholog (PFE1185w) localized to the plasma membrane and has not yet been expressed for functional characterization (D.J. Sullivan, unpublished results).

3.5
Heme Synthesis in Two Organelles

Plasmodium requires heme for heme dependent protein synthesis (Surolia and Padmanaban 1992) and as prosthetic group in parasitic cytochromes (Fry and Beesley 1991). Succinylacetone, a specific inhibitor of heme biosyn-

thesis, inhibited the in vitro growth of parasite with an IC_{50} of 2 μM (Surolia and Padmanaban 1992; Wilson et al. 1996). These observations suggest that heme biosynthesis is vital for the parasite and represents an attractive target for therapeutic intervention. Whole genome sequence shows the presence of the complete heme biosynthesis pathway in *P. falciparum* (except uroporphyrinogen III synthase whose orthologs are most disparate at primary sequence level than are other heme biosynthetic enzymes (Panek and O'Brian 2002)). Microarray analysis has shown expression of all the genes of the heme biosynthesis pathway throughout the erythrocytic stage, with the maximum level of expression during the metabolically most active trophozoite stage (Bozdech et al. 2003). Ferrochelatase has been recently cloned and reported to rescue the ferrochelatase null mutant of *Escherichia coli*, suggesting that the parasite encodes active ferrochelatase (Sato and Wilson 2003).

Protein sequence analysis of different members of the pathway divides them into two major classes: one that contains apicoplast targeting signals—ALAD, PBG deaminase and uroporphyrinogen decarboxylase;and a second group that lacks the apicoplast targeting signals—ALAS, coproporphyrinogen oxidase, protoporphyringen oxidase and ferrochelatase—which presumably are located in the mitochondria. Detailed localization studies of different enzymes of heme biosynthesis pathway is ongoing. These observations suggest that heme biosynthesis involves at least two compartments—plastids and mitochondria. The close proximity of both organelles within *Plasmodium* suggests cooperation of heme biosynthesis. The transport of intermediates to and from both the organelles would be of interest and may provide another area of therapeutic intervention.

3.6 Iron Chelation Therapy

Iron chelation therapy inhibits erythrocytic *Plasmodium* parasites in vitro and in vivo (Mabeza et al. 1999). Ultrastructural studies show a stage specific effect on a trophozoite-to-schizont stage transition with an enlarged nucleus rather than an enlarged digestive vacuole as seen with the quinolines (Atkinson et al. 1991). Iron chelation also inhibits mouse malaria development in hepatocytes at appropriate concentrations (Loyevsky et al. 1999b). The mechanism of action with diverse groups of iron chelators can be grouped to either withholding of iron or by formation of a toxic complex with iron (Mabeza et al. 1999). Iron withholding has a direct effect on the ribonucleotide reductase necessary for DNA synthesis and/or δ-aminolevulinate synthase for heme synthesis. Because of minimal effects on host cells and concurrent development of iron chelators as adjunctive cancer chemotherapy, several trials of iron chelators have been performed for both severe malaria and uncompli-

cated malaria. Intravenous deferioxamine B when added to quinoline therapy shortened recovery from coma and increased the rate of parasite clearance (Gordeuk et al. 1992). A later study showed an increase in mortality in cerebral malaria patients treated with deferioxamine B (Thuma et al. 1998). Other oral agents have also been tried with no therapeutic success in uncomplicated malaria. Iron chelation may potentially influence immune modulation of severe disease by interference with nitric oxide or cytokines (Mabeza et al. 1999). Iron chelation is antagonistic in vitro to artemisinin action (Eckstein-Ludwig et al. 2003). The increasing use of artemisinin combination therapy, then theoretically, precludes a role for adjunctive iron chelation therapy.

4 Hemozoin as a Biomarker of Malaria Infection

Clinical experience and light microscopic examination of blood films have endured for the past century as the primary tools for diagnosing malaria infection. However, a single low parasitemia or noninfected film can require examination for more than 30 min. Examination under polarized light detects birefringent hemozoin present in trophozoites of *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*, but will miss the rings of *P. falciparum* (Lawrence and Olson 1986). Theoretically, *P. falciparum* gametocytes should be detectable by hemozoin under polarized light. Immunochromatographic platforms are able to detect and speciate *Plasmodium* infections from a drop of blood in 10 min (Moody 2002). PCR based methods provide the greatest sensitivity and specificity, however they are the more labor, reagent and instrument intensive of the diagnostic techniques and are not adaptable to field work (Moody 2002). Laser desorption mass spectrometry (LDMS) was recently demonstrated to be an extremely rapid and sensitive tool for detecting hemozoin as a pan-species biomarker of infection (Demirev et al. 2002; Scholl et al. 2004).

4.1 Laser Desorption Mass Spectrometry

MS is the measurement of the relative abundance and mass-to-charge ratio (*m/z*) of ions generated from a sample in a vacuum. Mass spectrometric detection can be profoundly sensitive and enable the detection of femtomole amounts of heme under ideal conditions. LDMS samples (0.3–1 μL) are deposited on a metal plate and dried before introduction into the instrument. Twenty to several hundred samples can be deposited on a single plate depending on the instrument. The sample plate is held at a high (±3–20 kV) potential relative to the exit of the ion source region. Laser desorption lacks

addition of denaturing matrix solutions routinely used to achieve ionization with the more common matrix assisted laser desorption ionization (MALDI) (DeHoffman et al. 1996). In LDMS, a pulse (~3 nsec) of ultraviolet or infra red wavelength laser light (~1–10 MW/cm^2) illuminates a sample area on the order of 100×50 μm resulting in its conversion to gas phase ions. These ions are accelerated by the electric field in the source region until they exit into the mass analyzer. In time of flight (TOF)-MS, the *m/z* of these ions can be calculated by measuring the time required for ions to traverse a fixed distance before striking the ion detector (Cotter 1997). A useful mass spectrum can be obtained from a single laser shot; however, 20–100 single-shot spectra are usually acquired, filtered and averaged for presentation. The sample can be interrogated by rastering the laser over its surface and thereby enable the acquisition of many spectra from fresh areas within a ~ 2-mm^2 area. Spectra can be acquired from a fixed sample position until it is depleted (Scholl et al. 2004).

By adjusting the laser intensity, ions produced by LD ionization can fragment to yield daughter ions that are structurally characteristic of the parent ion. The degree of fragmentation can be controlled by adjusting the laser intensity. Fragment ions are useful in the interpretation of mass spectra to determine chemical structure and increase specificity of analyte detection. Following the interpretation of a mass spectrum, the molecular identities of sample components can be determined, and in some circumstances, quantitatively measured. Bioinformatic approaches are being developed to automate the interpretation of mass spectra and diminish the need for technical expertise to exploit mass spectrometric detection strategies. The combined sensitivity and specificity achievable using mass spectrometric detection makes it an attractive choice for many biomedical applications including the detection of malaria parasites in blood samples.

4.2
Hemozoin Detection

The LDMS analysis of purified hemozoin crystals using a N_2 laser (337 nm) yields the identical mass spectrum of heme alone demonstrating that heme is desorbed from hemozoin crystal surfaces by laser illumination. Hemozoin is thus able to act as its own matrix, absorbing the laser energy to yield the intact parent ion of 616 *m/z* and characteristic structurally rich fingerprint fragment ions from consecutive cleavages of the two propionic acid side chains to yield structurally characteristic ions at *m/z* 571 (M-COOH), 557 (M-CH_2COOH), 512 (M-CH_2COOH-COOH) and 498 [M-$(CH_2COOH)_2$] seen in Fig. 5A (Demirev et al. 2002; Scholl et al. 2004). The head-to-tail dimer *m/z* ratio of 1,232 is not seen even if pure hemozoin is subjected to LDMS.

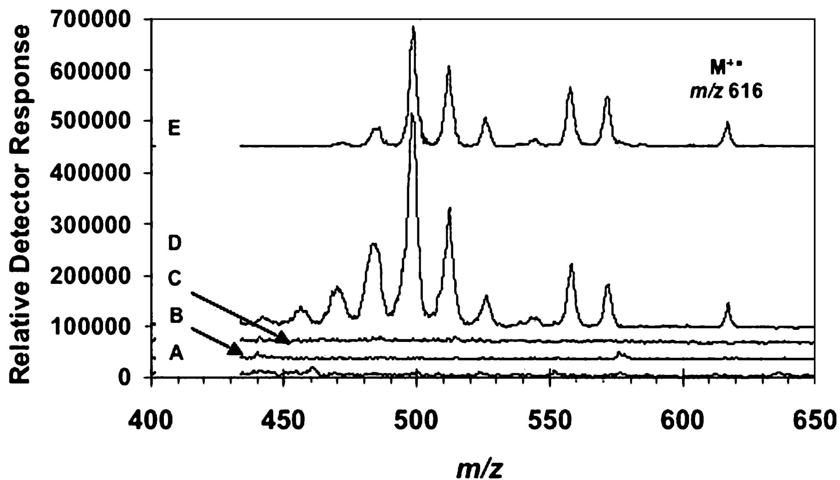

Fig. 5 LDMS spectra of tenfold diluted whole blood from the mouse presented in Fig. 1 and heme. (*A*) Day 1; (*B*) day 0, pre-infection; (*C*) day 1; (*D*) day 2 after infection; (*E*) heme standard. The parent molecular heme cation radical (*m/z* 616) fragments via consecutive cleavages of the two propionic acid side chains to yield structurally characteristic ions at *m/z* 571 (M-COOH), 557 (M-CH_2COOH), 512 (M-CH_2COOH-COOH) and 498 (M-$(CH_2COOH)_2$). Each spectrum represent the average of 100 individual laser shot spectra acquired from the equivalent of <0.3 μl of whole blood

Infected erythrocytes contain the bioanalyte hemozoin which has extremely high local heme concentrations of 2.2 M within the crystal (Pagola et al. 2000). A single laser energy shot is able to detect heme signal spectra consistent with hemozoin.

LDMS, surprisingly, is able to distinguish hemozoin from hemoglobin. Heme complexed with hemoglobin is also abundantly present at 20 mM concentrations in infected and noninfected blood samples. Therefore, hemoglobin was expected to preclude the specific LDMS detection of heme in hemozoin crystals. However, heme bound to hemoglobin or other serum proteins such as albumin or hemopexin is LDMS ‘silent’. Denaturation of these proteins produces a heme positive signal from noninfected blood samples. Thus standard sample preparation methods that denature molecules using trifluoroacetic acid and organic solvents like acetonitrile cannot be used to specifically detect hemozoin. However, MALDI could be used to detect parasite species-specific proteins to complement LDMS hemozoin detection methods.

LDMS was initially used to detect hemozoin in 0.5-μl samples containing as few as 10 parasite equivalents/μl following centrifugal concentration and

washing of hemozoin crystals from *P. falciparum* cultured in human blood. The heme spectral intensity linearly correlated with the number of parasites deposited (Demirev et al. 2002). Follow-up studies in a mouse malaria model and infected human clinical samples demonstrated that the extensive sample cleanup of blood was not necessary (Scholl et al. 2004). In a mouse time course study employing inoculating doses ranging from 10^2 to 10^6 parasites, LDMS detected *P. yoelli* infections 3–5 days earlier than light microscopy or a colorimetric hemozoin assay. Minimal sample preparation required only 10-fold dilution of whole blood samples followed by the analysis of 0.3 μl deposited on metal slides. LDMS analysis required less than approximately 1 min per sample. Although the equivalent of <0.03 μl of blood was analyzed and a four-log range of inoculating parasites used, the LDMS heme signal rapidly saturated, precluding the more interesting correlation of the mass spectrometric signal intensity with parasitemia. However, data are now emerging that demonstrate LDMS can be used to rapidly detect *P. falciparum* and *P. vivax* in human clinical blood samples and that, at low parasitemia levels (<5%), LDMS signal intensity correlates linearly with parasitemia (Nyunt et al. 2005 AJTMH in press). This has generated interest in developing LDMS for the high throughput, semi-quantitative analysis of malaria infections. The detection of *P. falciparum* infections by the LDMS detection of hemozoin crystals in blood is remarkable because the majority of erythrocytes infected with hemozoin laden trophozoites are sequestered from general circulation by attachment to tissue capillaries. Both nonmicroscopically visible hemozoin in ring stages and circulating leukocytes containing hemozoin crystals may contribute to hemozoin signal by LDMS (Scholl et al. 2004).

The unique combination of hemoglobin physical chemistry, malaria biochemistry and the operating principles of LDMS have unexpectedly enabled its use, without the need for significant sample preparation, for the potentially high throughput detection of malaria parasites. In addition to the high throughput screening of blood samples for malaria, LDMS may find additional applications in the screening of candidate anti-malaria drugs. A great deal of additional work is required to validate the use of LDMS for the detection of heme as a biomarker of malaria infection. In addition to the analytical sensitivity and specificity limits of LDMS, the persistence of hemozoin in chronically infected populations, the effect of thalassemias and sickle-cell anemia, and other viral and parasitic infections need to be evaluated. In any event, the greatest advantage in the potential use of LDMS for detecting malaria infections may ultimately be in its use to triage samples for further light microscopic examination.

5 Perspectives

Aspects of human and *Plasmodium* iron metabolism interrelate with malaria anemia and disease pathogenesis, mechanisms of malaria chemotherapy, diagnostic tests, human iron deficiency and eukaryotic iron biology. Unresolved research questions include etiology of malaria anemia both in the different physiologic setting of an iron-replete or an iron-depleted host. The exact molecular intracellular assembly of heme crystals and the inhibition mechanism of the quinoline class remains. Acquisition, intracellular shuttle and utilization of iron by *Plasmodium* in heme synthesis and other iron pathways will diversify knowledge of eukaryotic iron metabolism. A new development for *Plasmodium* hemozoin is a mass spectrometric diagnostic test.

References

Arese P, Schwarzer E (1997) Malarial pigment (haemozoin): A very active 'inert' substance. Ann Trop Med Parasitol 91:501–516

Atamna H,Ginsburg H (1995) Heme degradation in the presence of glutathione: A proposed mechanism to account for the high levels of non-heme iron found in the membranes of hemoglobinopathic red blood cells. J Biol Chem 42:24876–24883

Atkinson CT, Bayne MT, Gordeuk VR, Brittenham GM, Aikawa M (1991) Stage-specific ultrastructural effects of desferrioxamine on *Plasmodium falciparum* in vitro. Am J Trop Med Hyg 45:593–601

Bahl A, Brunk B, Coppel RL, Crabtree J, Diskin SJ, Fraunholz MJ, Grant GR, Gupta D, Huestis RL, Kissinger JC, Labo P, Li L, McWeeney SK, Milgram AJ, Roos DS, Schug J, Stoeckert CJ, Jr. (2002) Plasmodb: The *Plasmodium* genome resource. An integrated database providing tools for accessing, analyzing and mapping expression and sequence data (both finished and unfinished). Nucl Acids Res 30:87–90

Basilico N, Pagani E, Monti D, Olliaro P, Taramelli D (1998) A microtitre-based method for measuring the haem polymerization inhibitory activity (hpia) of antimalarial drugs. J Antimicrob Chemother 42:55–60

Bendrat K, Berger BJ, Cerami A (1995) Haem polymerization in malaria. Nature 378:138

Berger J, Dyck JL, Galan P, Aplogan A, Schneider D, Traissac P, Hercberg S (2000) Effect of daily iron supplementation on iron status, cell-mediated immunity, and incidence of infections in 6–36 month old Togolese children Eur J Clin Nutr 54:29–35

Blauer G, Akkawi M (1997) Investigations of b- and β-hematin. J Inorg Chem 66:145–152

Blauer G, Akkawi M (2000) On the preparation of beta-haematin. Biochem J 346:249–250

Bohle DS, Helms JB (1993) Synthesis of β-hematin by dehydrohalogenation of hemin. Biochem Biophys Res Comm 193:504–508

Bozdech Z, Llinas M, Pulliam BL, Wong ED, Zhu J, DeRisi JL (2003) The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum.* PLoS Biol 1:86–100

Brooker S, Peshu N, Warn PA, Mosobo M, Guyatt HL, Marsh K, Snow RW (1999) The epidemiology of hookworm infection and its contribution to anaemia among pre-school children on the Kenyan coast. Trans R Soc Trop Med Hyg 93:240–246

Buller R, Peterson ML, Almarsson O, Leiserowitz L (2002) Quinoline binding site on malaria pigment crystal:A rational pathway for antimalaria drug design. Cryst Growth Des 2:553–562

Carbone T (1891) Sulla natura chimica del pigmento malarico G. Accad Med Torino 39:901

Choi YHC, Cerda FJ, Chu H-a, Babcock TG, Marletta AM (1999) Spectroscopic characterization of the heme-binding sites in *Plasmodium falciparum* histidine-rich protein 2. Biochemistry 38:16916–16924

Chong CR, Sullivan DJ (2003) Inhibition of heme crystal growth by antimalarials and other compounds:Implications for drug discovery. Biochem Pharm 66:2201–2212

Cotter RJ (1997) Time-of-flight mass spectrometry: Instrumentation and applications in biological research. American Chemical Society, Washington, D.C

Deharo E, Barkan D, Krugliak M, Golenser J, Ginsburg H (2003) Potentiation of the antimalarial action of chloroquine in rodent malaria by drugs known to reduce cellular glutathione levels. Biochem Pharm 66:809–817

DeHoffman E, Charette J, Stroobant V (1996) Mass spectrometry: Principles and applications. John Wiley and Sons, Chichester, UK

Demirev PA, Feldman AB, Kongkasuriyachai D, Scholl P, Sullivan D, Jr., Kumar N (2002) Detection of malaria parasites in blood by laser desorption mass spectrometry. Anal Chem 74:3262–3266

Desai MR, Dhar R, Rosen DH, Kariuki SK, Shi YP, Kager PA, Ter Kuile FO (2004) Daily iron supplementation is more efficacious than twice weekly iron supplementation for the treatment of childhood anemia in western Kenya. J Nutr 134:1167–1174

Desai MR, Mei JV, Kariuki SK, Wannemuehler KA, Phillips-Howard PA, Nahlen BL, Kager PA, Vulule JM, ter Kuile FO (2003) Randomized, controlled trial of daily iron supplementation and intermittent sulfadoxine-pyrimethamine for the treatment of mild childhood anemia in western Kenya. J Infect Dis 187:658–666

Dorn A, Stoffel R, Matile H, Bubendorf A, Ridley RG (1995) Malarial haemozoin/beta-haematin supports haem polymerization in the absence of protein. Nature 374:269–271

Dorn A, Vippagunta SR, Matile H, Bubendorf A, Vennerstrom JL, Ridley RG (1998a) A comparison and analysis of several ways to promote haematin (haem) polymerisation and an assessment of its initiation *in vitro.* Biochem Pharm 55:737–747

Dorn A, Vippagunta SR, Matile H, Jaquet C, Vennerstrom JL, Ridley RG (1998b) An assessment of drug-haematin binding as a mechanism for inhibition of haematin polymerisation by quinoline antimalarials Biochem Pharm 55:727–736

Eckstein-Ludwig U, Webb RJ, Van Goethem ID, East JM, Lee AG, Kimura M, O'Neill PM, Bray PG, Ward SA, Krishna S (2003) Artemisinins target the SERCA of *Plasmodium falciparum.* Nature 424:957–961

Egan T, Ross D, Adams P (1994a) Quinoline anti-malarial drugs inhibit spontaneous formation of beta-haematin (malaria pigment). FEBS Letters 352:54–57

Egan TJ (2002) Physico-chemical aspects of hemozoin (malaria pigment) structure and formation. J Inorg Biochem 91:19–26

Egan TJ (2004) Haemozoin formation as a target for the rational design of new antimalarials. Drug Design Reviews Online 1:93–110

Egan TJ, Combrinck JM, Egan J, Hearne GR, Marques HM, Ntenteni S, Sewell BT, Smith PJ, Taylor D, Van Schalkwyk DA, Walden JC (2002) Fate of haem iron in the malaria parasite *Plasmodium falciparum.* Biochem J 365:343–347

Egan TJ, Hunter R, Kaschula CH, Marques HM, Misplon A, Walden J (2000) Structure-function relationships in aminoquinolines: Effect of amino and chloro groups on quinoline-hematin complex formation, inhibition of beta-hematin formation, and antiplasmodial activity. J Med Chem 43:283–291

Egan TJ, Mavuso WW, Ncokazi KK (2001) The mechanism of beta-hematin formation in acetate solution. Parallels between hemozoin formation and biomineralization processes. Biochemistry 40:204–213

Egan TJ, Mavuso WW, Ross DC, Marques HM (1997) Thermodynamic factors controlling the interaction of quinoline antimalarial drugs with ferriprotoporphyrin IX. J Inorg Biochem 68:137–145

Egan TJ, Ross DC, Adams PA (1994b) Quinoline anti-malarial drugs inhibit spontaneous formation of beta-haematin (malaria pigment). FEBS Lett 352:54–57

Ekvall H, Premji Z,Bjorkman A (2000) Micronutrient and iron supplementation and effective antimalarial treatment synergistically improve childhood anaemia. Trop Med Int Health 5:696–705

Fairbanks Vf, Beutler E. (1995a) Iron deficiency. In: Beutler E, Lichtman M, Coller B, Kipps T (eds) Williams hematology, McGraw-Hill, New York, pp490–511

Fairbanks Vf, Beutler E. (1995b). Iron metabolism. In: Beutler E, Lichtman M, Coller B, Kipps T (eds) Williams hematology, McGraw-Hill, New York, pp369–379

Famin O, Ginsburg H (2003) The treatment of plasmodium falciparum-infected erythrocytes with chloroquine leads to accumulation of ferriprotoporphyrin IX bound to particular parasite proteins and to the inhibition of the parasite's 6-phosphogluconate dehydrogenase. Parasite 10:39–50

Famin O, Krugliak M, Ginsburg H (1999) Kinetics of inhibition of glutathione-mediated degradation of ferriprotoporphyrin IX by antimalarial drugs. Biochem Pharm 58:59–68

Fitch C, Cai G, Chen Y, Shoemaker J (1999) Involvement of lipids in ferriprotoporphyrin IX polymerization in malaria. Biochim Biophys Acta 1454:31–37

Fitch CD (1998) Involvement of heme in the antimalarial action of chloroquine. Trans Am Clin Climatol Assoc 109:97–105; discussion 105–106

Fitch CD, Chen YF, Cai GZ (2003) Chloroquine-induced masking of a lipid that promotes ferriprotoporphyrin IX dimerization in malaria. J Biol Chem 278:22596–22599

Fitch CD, Chou AC (1996) Heat-labile and heat-stimulable heme polymerase activities in *Plasmodium berghei.* Mol Biochem Parasitol 82:261–264

Fitch CD, Kanjananggulpan P (1987) The state of ferriprotoporphyrin IX in malaria pigment. J Biol Chem 262:15552–15555

Francis SE, Sullivan DJ Jr, Goldberg DE (1997) Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum* Annu Rev Microbiol 51:97–123

Fry M, Beesley JE (1991) Mitochondria of mammalian *Plasmodium* spp. Parasitology 102:17–26

Gabay T, Ginsburg H (1993) Hemoglobin denaturation and iron release in acidified red blood cell lysate-a possible source of iron for intraerthrocytic malaria parasites. Exp Parisitol 77:261–272

Gabay T, Krugliak M, Shalmiev G, Ginsburg H (1994) Inhibition by anti-malarial drugs of haemoglobin denaturation and iron release in acidified red blood cell lysates–a possible mechanism of their anti-malarial effect? Parasitology 108:371–381

Garrick MD, Nunez MT, Olivares M, Harris ED (2003) Parallels and contrasts between iron and copper metabolism. Biometals 16:1–8

Gera T, Sachdev HP (2002) Effect of iron supplementation on incidence of infectious illness in children: Systematic review. BMJ 325:1142

Ginsburg H, Famin O, Zhang J, Krugliak M (1998) Inhibition of glutathione-dependant degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarials mode of action. Biochem Pharmacol 56:1305–1313

Ginsburg H, Gorodetsky R, Krugliak M (1986) The status of zinc in malaria (*Plasmodium falciparum*) infected human red blood cells: Stage dependent accumulation, compartmentation and effect of dipicolinate. Biochimica et Biophysica Acta 886:337–344

Ginsburg H, Krugliak M (1999) Chloroquine—some open questions on its antimalarial mode of action and resistance. Drug Resist Update 2:180–187

Goldberg DE, Slater AFG, Cerami A, Henderson GB (1990) Hemoglobin degradation in the malaria parasite *Plasmodium falciparum*: An ordered process in a unique organelle. Proc Natl Acad Sci USA 87:2931–2935

Gordeuk V, Thuma P, Brittenham G, McLaren C, Parry D, Backenstose A, Biemba G, Msiska R, Holmes L, McKinley E, et al. (1992) Effect of iron chelation therapy on recovery from deep coma in children with cerebral malaria. N Engl J Med 327:1473–1477

Gushin H, Mackenzie B, Berger UV, Gushin Y, Romero MR, Boron WF, Nussberger S, Gollan JL, Hediger MA (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388:482–487

Haddad S, Restieri C, Krishnan K (2001) Characterization of age-related changes in body weight and organ weights from birth to adolescence in humans. J Toxicol Environ Health A 64:453–464

Haldar K, Henderson CL, Cross GAM (1986) Identification of the parasite transferrin receptor of *Plasmodium falciparim*- infected erythrocytes and its acylation via 1,2-diacyl-sn-glycerol. Proc Natl Acad Sci USA 83:8565–8569

Hastka J, Lasserre J, Schwarzbeck A, Strauch M, Hehlmann R (1993) Zinc protoporphyrin in anemia of chronic disorders. Blood 81:1200–1204

Hawley SR, Bray PG, Mungthin M, Atkinson JD, O'Neill PM, Ward SA (1998) Relationship between antimalarial drug activity, accumulation, and inhibition of heme polymerization in *Plasmodium falciparum in vitro* Antimicrob Agents Chemother 42:682–686

Hempelmann E, Egan TJ (2002) Pigment biocrystallization in *Plasmodium falciparum.* Trends Parasitol 18:11

Hempelmann E, Motta C, Hughes R, Ward SA, Bray PG (2003) *Plasmodium falciparum*: Sacrificing membrane to grow crystals? Trends Parasitol 19:23–26

Hentze MW, Muckenthaler MU, Andrews NC (2004) Balancing acts: Molecular control of mammalian iron metabolism. Cell 117:285–297

Hershko C, Peto TE (1988) Deferoxamine inhibition of malaria is independent of host iron status. J Exp Med 168:375–387

Huy NT, Kamei K, Yamamoto T, Kondo Y, Kanaori K, Takano R, Tajima K, Hara S (2002) Clotrimazole binds to heme and enhances heme-dependent hemolysis: Proposed antimalarial mechanism of clotrimazole. J Biol Chem 277:4152–4158

Iyer JK, Shi L, Shankar AH, Sullivan DJ, Jr. (2003) Zinc protoporphyrin IX binds heme crystals to inhibit the process of crystallization in *Plasmodium falciparum*. Mol Med 9:175–182

Kalkanidis M, Klonis N, Tilley L, Deady LW (2002) Novel phenothiazine antimalarials: Synthesis, antimalarial activity, and inhibition of the formation of beta-haematin. Biochem Pharmacol 63:833–842

Kaplan J, O'Halloran TV (1996) Iron metabolism in eukaryotes: Mars and venus at it again. Science 271:1510–1512

Kristiansen JE, Jepsen S (1985) The susceptibility of *Plasmodium falciparum in vitro* to chlorpromazine and the stereo-isomeric compounds cis (z)- and trans (e)-clopenthixol. Acta Pathol Microbiol Immunol Scand [B] 93:249–251

Krugliak M, Zhang J, Ginsburg H (2002) Intraerythrocytic *Plasmodium falciparum* utilizes only a fraction of the amino acids derived from the digestion of host cell cytosol for the biosynthesis of its proteins. Mol Biochem Parasitol 119:249–256

Kurosawa Y, Dorn A, Kitsuji-Shirane M, Shimada H, Satoh T, Matile H, Hofheinz W, Masciadri R, Kansy M, Ridley RG (2000) Hematin polymerization assay as a high-throughput screen for identification of new antimalarial pharmacophores. Antimicrob Agents Chemother 44:2638–2644

Lamola AA, Yamane T (1974) Zinc protoporphyrin in the erythrocytes of patients with lead intoxication and iron deficiency anemia. Science 186:936–938

Lawrence C, Olson JA (1986) Birefringent hemozoin identifies malaria. Am J Clin Pathol 86:360–363

Leed A, DuBay K, Ursos LM, Sears D, De Dios AC, Roepe PD (2002) Solution structures of antimalarial drug-heme complexes Biochemistry 41:10245–10255

Leenstra T, Kariuki SK, Kurtis JD, Oloo AJ, Kager PA, ter Kuile FO (2004) Prevalence and severity of anemia and iron deficiency: Cross-sectional studies in adolescent schoolgirls in western Kenya. Eur J Clin Nutr 58:681–691

Levesque MA, Sullivan AD, Meshnick SR (1999) Splenic and hepatic hemozoin in mice after malaria parasite clearance. J Parasitol 85:570–573

Lew VL, Tiffert T, Ginsburg H (2003) Excess hemoglobin digestion and the osmotic stability of *Plasmodium falciparum*-infected red blood cells. Blood 101:4189–4194

Loyevsky M, John C, Dickens B, Hu V, Miller JH, Gordeuk VR (1999a) Chelation of iron within the erythrocytic *Plasmodium falciparum* parasite by iron chelators. Mol Biochem Parasitol 101:43–59

Loyevsky M, LaVaute T, Allerson CR, Stearman R, Kassim OO, Cooperman S, Gordeuk VR, Rouault TA (2001) An IRP-like protein from *Plasmodium falciparum* binds to a mammalian iron-responsive element. Blood 98:2555–2562

Loyevsky M, Lytton SD, Mester B, Libman J, Shanzer A, Cabantchik ZI (1993) The antimalarial action of desferal involves a direct access route to erythrocytic (*Plasmodium falciparum*) parasites. J Clin Invest 91:218–224

Loyevsky M, Mompoint F, Yikilmaz E, Altschul SF, Madden T, Wootton JC, Kurantsin-Mills J, Kassim OO, Gordeuk VR, Rouault TA (2003) Expression of a recombinant IRP-like *Plasmodium falciparum* protein that specifically binds putative plasmodial ires. Mol Biochem Parasitol 126:231–238

Loyevsky M, Sacci JB, Jr., Boehme P, Weglicki W, John C, Gordeuk VR (1999b) *Plasmodium falciparum* and *Plasmodium yoelii*: Effect of the iron chelation prodrug dexrazoxane on in vitro cultures. Exp Parasitol 91:105–114

Mabeza GF, Loyevsky M, Gordeuk VR, Weiss G (1999) Iron chelation therapy for malaria: A review. Pharmacol Ther 81:53–75

Mann S, Bannister JV, Williams RJP (1986) Structure and composition of ferritin cores isolated from human spleen, limpet (*Patella vulgata*) hemolymph and bacterial (*Pseudomonas aeruginosa*) cells. J Mol Biol 188:225–232

Marques HM, Voster K, Egan TJ (1996) The interaction of the heme-octapeptide, n-acetylmicroperoxidase-8 with antimalarial drugs: Solution studies and modeling by molecular mechanics methods. J Inorgan Biochem 64:7–23

Massaga JJ, Kitua AY, Lemnge MM, Akida JA, Malle LN, Ronn AM, Theander TG, Bygbjerg IC (2003) Effect of intermittent treatment with amodiaquine on anaemia and malarial fevers in infants in Tanzania: A randomised placebo-controlled trial Lancet 361:1853–1860

McKie AT, Marciani P, Rolfs A, Brennan K, Wehr K, Barrow D, Miret S, Bomford A, Peters TJ, Farzaneh F, Hediger MA, Hentze MW, Simpson RJ (2000) A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol Cell 5:299–309

Mebrahtu T, Stoltzfus RJ, Chwaya HM, Jape JK, Savioli L, Montresor A, Albonico M, Tielsch JM (2004) Low-dose daily iron supplementation for 12 months does not increase the prevalence of malarial infection or density of parasites in young Zanzibari children J Nutr 134:3037–3041

Menendez C, Kahigwa E, Hirt R, Vounatsou P, Aponte JJ, Font F, Acosta CJ, Schellenberg DM, Galindo CM, Kimario J, Urassa H, Brabin B, Smith TA, Kitua AY, Tanner M, Alonso PL (1997) Randomised placebo-controlled trial of iron supplementation and malaria chemoprophylaxis for prevention of severe anaemia and malaria in Tanzanian infants. Lancet 350:844–850

Menendez C, Schellenberg D, Quinto L, Kahigwa E, Alvarez L, Aponte JJ, Alonso PL (2004) The effects of short-term iron supplementation on iron status in infants in malaria-endemic areas. Am J Trop Med Hyg 71:434–440

Miyajima H (2002) Genetic disorders affecting proteins of iron and copper metabolism: Clinical implications. Intern Med 41:762–769

Monti D, Vodopivec B, Basilico N, Olliaro P, Taramelli D (1999) A novel endogenous antimalarial: Fe (ii)-protoporphyrin IX alpha (heme) inhibits hematin polymerization to beta-hematin (malaria pigment) and kills malaria parasites. Biochemistry 38:8858–8863

Moody A (2002) Rapid diagnostic tests for malaria parasites. Clin Microbiol Rev 15:66–78

Moreau S, Perly B, Chachaty C, and Deleuze C (1985) A nuclear magnetic resonance study of the interactions of antimalarial drugs with porphyrins. Biochem Biophys Acta 840:107–116

Murray MJ, Murray NJ, Murray AB, Murray MB (1975) Refeeding-malaria and hyperferraemia. Lancet 1:653–654

Noland GS, Briones N, Sullivan DJ (2003) The shape and size of hemozoin crystals distinguishes diverse *Plasmodium* species. Mol Biochem Parasitol 130:91–99
Nyakeriga AM, Troye-Blomberg M, Dorfman JR, Alexander ND, Back R, Kortok M, Chemtai AK, Marsh K, Williams TN (2004) Iron deficiency and malaria among children living on the coast of Kenya. J Infect Dis 190:439–447
O'Neill PM, Willock DJ, Hawley SR, Bray PG, Storr RC, Ward SA, Park BK (1997) Synthesis, antimalarial activity, and molecular modeling of tebuquine analogues. J Med Chem 40:437–448
Oppenheimer SJ (2001) Iron and its relation to immunity and infectious disease. J Nutr 131:616S-633S; discussion 633S–635S
Pagola S, Stephens PW, Bohle DS, Kosar AD, Madsen SK (2000) The structure of malaria pigment beta-hematin. Nature 404:307–310
Pandey AV, Babbarwal VK, Okoyeh JN, Joshi RM, Puri SK, Singh RL, Chauhan VS (2003) Hemozoin formation in malaria: A two-step process involving histidine-rich proteins and lipids. Biochem Biophys Res Commun 308:736–743
Panek H, O'Brian MR (2002) A whole genome view of prokaryotic haem biosynthesis. Microbiology 148:2273–2282
Papalexis V, Siomos MA, Campanale N, Guo X, Kocak G, Foley M, Tilley L (2001) Histidine-rich protein 2 of the malaria parasite, *Plasmodium falciparum*, is involved in detoxification of the by-products of haemoglobin degradation. Mol Biochem Parasitol 115:77–86
Peto TE, Thompson JL (1986) A reappraisal of the effects of iron and desferrioxamine on the growth of *Plasmodium falciparum 'in vitro'*: The unimportance of serum iron Br J Haematol 63:273–280
Rasoloson D, Shi L, Chong CR, Kafsack BF, Sullivan DJ (2004) Copper pathways in *Plasmodium falciparum* infected erythrocytes indicate an efflux role for the copper P-ATPase. Biochem J
Raventos-Suarez C, Pollack S, Nagel RL (1982) *Plasmodium falciparum*: Inhibition of *in vitro* growth by desferrioxamine. Am J Trop Med Hyg 31:919–922
Ridley RG (1996) Hemozoin formation in malaria parasites: Is there a haem polymerase? Trends Microbiol 4:253–254
Rodriguez MH, Jungery M (1986) A protein on *Plasmodium falciparum*-infected erythrocytes functions as a transferrin receptor. Nature 324:388–391
Sanchez-Lopez R, Haldar K (1992) A transferrin-independent iron uptake activity in *Plasmodium falciparum*-infected and uninfected erythrocytes. Mol Biochem Parasitol 55:9–20
Sato S, Wilson RJ (2003) Proteobacteria-like ferrochelatase in the malaria parasite Curr Genet 42:292–300
Scholl PF, Kongkasuriyachai D, Demirev PA, Feldman AB, Lin JS, Sullivan DJ, Kumar N (2004) Rapid detection of malaria infection *in vivo* by laser desorption mass spectrometry. Am J Trop Med Hyg 71:546–551
Schwarzer E, Datteis DF, Giribaldi G, Ulliers D, Valente E, Arese P (1999a) Hemozoin stability and dormant induction of heme oxygenase in hemozoin-fed human monocytes. Mol Biochem Parasitol 100:61–72
Schwarzer E, Turrini F, Ulliers D, Giribaldi G, Ginsburg H, Arese P (1992) Impairment of macrophage functions after ingestion of *Plasmodium falciparum*-infected erythrocytes or isolated malarial pigment. J Exp Med 176:1033–1041

Scott MD, Ranz A, Kuypers FA, Lubin BH, Meshnick SR (1990) Parasite uptake of desferroxamine: A prerequisite for antimalarial activit.y Br J Haematol 75:598–602

Slater AF (1993) Chloroquine: Mechanism of drug action and resistance in *Plasmodium falciparum*. Pharmacol Ther 57:203–235

Slater AF, Cerami A (1992) Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites. Nature 355:167–169

Slater AF, Swiggard WJ, Orton BR, Flitter WD, Goldberg DE, Cerami A, Henderson GB (1991) An iron-carboxylate bond links the heme units of malaria pigment. Proc Natl Acad Sci USA 88:325–329

Slater AFG (1992) Malarial pigment. Experim Parasitol 74:362–365

Spivak JL (2002) Iron and the anemia of chronic disease. Oncology (Huntingt) 16:25–33

Stoltzfus RJ, Chway HM, Montresor A, Tielsch JM, Jape JK, Albonico M, Savioli L (2004) Low dose daily iron supplementation improves iron status and appetite but not anemia, whereas quarterly anthelminthic treatment improves growth, appetite and anemia in Zanzibari preschool children. J Nutr 134:348–356

Stoltzfus RJ, Chwaya HM, Montresor A, Albonico M, Savioli L, Tielsch JM (2000) Malaria, hookworms and recent fever are related to anemia and iron status indicators in 0- to 5-y old Zanzibari choldren and these relationships change with age. J Nutr 130:1724–1733

Sullivan D (2002a) Hemozoin: A biocrystal synthesized during the degradation of hemoglobin. In Matsumura S, Steinbüchel A (eds) Miscellaneous biopolymers, biodegradation of synthetic polymers, Wiley-VCH Verlag GmbH & Co, Weinheim, Germany, pp129–163

Sullivan DJ (2002b) Theories on malarial pigment formation and quinoline action. Int J Parasitol 32:1645–1653

Sullivan DJ Jr, Gluzman IY, Goldberg DE (1996a) *Plasmodium* hemozoin formation mediated by histidine-rich proteins. Science 271:219–222

Sullivan DJ Jr, Gluzman IY, Russell DG, Goldberg DE (1996b) On the molecular mechanism of chloroquine's antimalarial action. Proc Natl Acad Sci USA 93:11865–11870

Sullivan DJ Jr, Matile H, Ridley RG, Goldberg DE (1998) A common mechanism for blockade of heme polymerization by antimalarial quinolines. J Biol Chem 273:31103–31107

Surolia N, Padmanaban G (1992) De novo biosynthesis of heme offers a new chemotherapeutic target in the human malarial parasite. Biochem Biophys Res Commun 187:744–750

Taliaferro WH, Mulligan HW (1937) The histopathology of malaria with special reference to the function and origin of macrophages in defense. Indian Med Mem 29:1–12

Tatala S, Svanberg U, Mduma B (1998) Low dietary iron availability is a major cause of anemia: A nutrition survey in the Lindi district of Tanzania. Am J Clin Nutr 68:171–178

Thuma PE, Mabeza GF, Biemba G, Bhat GJ, McLaren CE, Moyo VM, Zulu S, Khumalo H, Mabeza P, M'Hango A, Parry D, Poltera AA, Brittenham GM, Gordeuk VR (1998) Effect of iron chelation therapy on mortality in zambian children with cerebral malaria. Trans R Soc Trop Med Hyg 92:214–218

Tripathi AK, Garg SK, Tekwani BL (2002) A physiochemical mechanism of hemozoin (beta-hematin) synthesis by malaria parasite Biochem Biophys Res Commun 290:595–601

Tripathi AK, Khan SI, Walker LA, Tekwani BL (2004) Spectrophotometric determination of de novo hemozoin/beta-hematin formation in an *in vitro* assay. Anal Biochem 325:85–91

Vennerstrom JL, Ager AL Jr, Andersen SL, Grace JM, Wongpanich V, Angerhofer CK, Hu JK, Wesche DL (2000) Assessment of the antimalarial potential of tetraoxane WR 148999. Am J Trop Med Hyg 62:573–578

Verhoef H, West CE, Nzyuko SM, de Vogel S, van der Valk R, Wanga MA, Kuijsten A, Veenemans J, Kok FJ (2002) Intermittent administration of iron and sulfadoxine-pyrimethamine to control anaemia in Kenyan children: A randomised controlled trial. Lancet 360:908–914

Weaver J, Zhan H, Pollack S (1993) Erythrocyte haemolysate interacts with atp-fe to form a complex containing iron, ATP and 13 800 MW polypeptide. Br J Haematol 83:138–144

Wilson CM, Smith AB, Baylon RV (1996) Characterization of the delta-aminolevulinate synthase gene homologue in *P. falciparum.* Mol Biochem Parasitol 79:135–140

Yip R (1998) Iron deficiency. Bull WHO 76:121–123

Zhan H, Gupta RK, Weaver J, Pollack S (1990) Iron bound to low MW ligands: Interactions with mitochondria and cytosolic proteins. Eur J Haematol 44:125–131

Zhang J, Krugliak M, Ginsburg H (1999) The fate of ferriprotoporphyrin IX in malaria infected erythrocytes in conjunction with the mode of action of antimalarial drugs. Mol Biochem Parasitol 99:129–141