The role of neutral lipid nanospheres in *Plasmodium falciparum* haem crystallization

John M. PISCIOTTA*, Isabelle COPPENS*, Abhai K. TRIPATHI*, Peter F. SCHOLL†, Joel SHUMAN‡, Sunil BAJAD‡, Vladimir SHULAEV‡ and David J. SULLIVAN, JR*¹

*Malaria Research Institute, W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, 615 N. Wolfe Street, Baltimore, MD 21205, U.S.A., †Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, 615 N. Wolfe Street, Baltimore, MD 21205, U.S.A., and ‡Virginia Bioinformatics Institute, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, U.S.A.

The intraerythrocytic malaria parasite constructs an intracellular haem crystal, called haemozoin, within an acidic digestive vacuole where haemoglobin is degraded. Haem crystallization is the target of the widely used antimalarial quinoline drugs. The intracellular mechanism of molecular initiation of haem crystallization, whether by proteins, polar membrane lipids or by neutral lipids, has not been fully substantiated. In the present study, we show neutral lipid predominant nanospheres, which envelop haemozoin inside *Plasmodium falciparum* digestive vacuoles. Subcellular fractionation of parasite-derived haemozoin through a dense 1.7 M sucrose cushion identifies monoacylglycerol and diacylglycerol neutral lipids as well as some polar lipids in close association with the purified haemozoin. Global MS lipidomics detects monopalmitic glycerol and monostearic glycerol, but

not mono-oleic glycerol, closely associated with haemozoin. The complex neutral lipid mixture rapidly initiates haem crystallization, with reversible pH-dependent quinoline inhibition associated with quinoline entry into the neutral lipid microenvironment. Neutral lipid nanospheres both enable haem crystallization in the presence of high globin concentrations and protect haem from H₂O₂ degradation. Conceptually, the present study shifts the intracellular microenvironment of haem crystallization and quinoline inhibition from a polar aqueous location to a non-polar neutral lipid nanosphere able to exclude water for efficient haem crystallization.

Key words: haem crystallization, haemozoin, lipidomics, malaria, neutral lipid, *Plasmodium falciparum*, quinoline.

INTRODUCTION

The signs and symptoms of malaria coincide with rapid asexual multiplication in the bloodstream. In the case of lethal *Plasmodium falciparum*, the number of intraerythrocytic parasites can reach a density of hundreds of millions of organisms per ml of blood [1]. This rapid parasite multiplication depends on efficient proteolysis of approximately two-thirds of host erythrocyte haemoglobin over the 48 h of intraerythrocytic development. Catabolism of haemoglobin results in near molar amounts of toxic haem in an acidic, oxygen-rich lysosomal-like digestive vacuole [2,3]. The parasite quickly sequesters this released haem by the intracellular formation of inert haem crystals called haemozoin. A pair of haems where the central iron of one haem co-ordinates with the carboxylate oxygen of the propionic side chain of the adjacent haem composes the head-to-tail dimer [4]. These individual haem dimers hydrogen-bond into larger crystals containing millions of haem molecules. Slater and Cerami [5,6] first described *Plasmodium* trophozoite extracts which initiated haem crystallization.

The intracellular molecular mechanism of haem crystallization in an aqueous protein-rich environment, whether by HRP (histidine-rich proteins), polar membrane lipids or neutral lipids, has not been fully substantiated [7–9]. Bendrat et al. [9] first implicated polar lipids, later substantiated by Dorn et al. [10] with acetonitrile extracts of trophozoites promoting haem crystallization. Fitch et al. [11,12] later suggested a haem crystallization

role for neutral lipids based on *in vitro* initiation. On the basis of TEM [transmission EM (electron microscopy)] evidence, focus in the debate shifted back to polar membrane lipids, particularly the remnant parasitophorous vacuole membrane in the digestive vacuole as a membrane crystallization scaffold [13,14]. However, counter to the parasitophorous vacuole membrane as the lipid source for haem crystallization, Trager [15] demonstrated robust haem crystal formation in *P. falciparum* axenic cultures which lack a parasitophorous vacuole membrane. Tilley and co-workers [16] have recently demonstrated association of neutral lipids outside digestive vacuoles by fluorescence and TLC, postulating a haem crystallization role for di- and tri-acylglycerols produced from the parasitophorous vacuole membrane by phospholipases. While many classes of molecules in test tubes promote haem crystallization, the molecules within the *Plasmodium* digestive vacuole have not been defined.

The exact mechanism of haem crystal inhibition is important to define, as this is also the target of the widely used quinoline antimalarials [6,17,18]. Most drug modelling has been based on drug activity in an aqueous polar environment postulated for haem crystallization in the digestive vacuole [19,20]. While haem binding is an important requirement for haem crystal inhibition, this alone does not correlate with parasite activity for quinolines [21].

In the present study, we utilized Malachite Green preservation of neutral lipids during fixation to identify haemozoin within neutral lipid nanospheres inside digestive vacuoles. Subcellular

Abbreviations used: DLG, dilinoleic glycerol; DOG, dioleic glycerol; DPG, dipalmitic glycerol; EM, electron microscopy; ESI-MS, electrospray ionization MS; FAME, fatty acid methyl ester; HRP, histidine-rich protein; MLG, monolinoleic glycerol; MOG, mono-oleic glycerol; MPG, monopalmitic glycerol; MSG, monostearic glycerol; Pfaldolase, *P. falciparum* aldolase; PfCRT, *P. falciparum* chloroquine-resistant transporter; PfHRP II, *P. falciparum* HRP II; SPHz, sucrose-purified haemozoin; TEM, transmission EM.

¹ To whom correspondence should be addressed (email dsullivan@jhspsh.edu).

fractionation of haemozoin through a dense 1.7 M sucrose cushion allowed identification of haemozoin-associated lipids. Characterization of haem crystallization kinetics and inhibition by the quinolines in a neutral lipid, non-polar environment shifts the focus of haemozoin formation and inhibition away from an aqueous polar environment.

EXPERIMENTAL

Parasite culture

P. falciparum clone 3D7 was grown at 2–4% haematocrit in RPMI 1640 medium supplemented with 10% (v/v) human serum [22]. Synchrony was maintained with sorbitol [23] and trophozoites were purified by saponin lysis [24]. Purified digestive vacuoles were isolated by the Goldberg et al. [25] method adapted by Tilley and co-workers [16]. Enriched digestive vacuoles refer to the washed hypo-osmotic lysis of parasites, rich in intact digestive vacuoles and membranes before sucrose/Percoll centrifugation yields pure vacuoles in pellet. Haemozoin isolation by ultracentrifugation through 1.7 M sucrose cushions was described previously [26]. Fresh saponin purified trophozoites were hypo-osmotically lysed, and the pellet was sonicated to disrupt digestive vacuoles before loading the sucrose cushions.

Microscopy

For TEM analysis of the intracellular disposition of haemozoin and neutral lipids, samples were fixed in the presence of 0.1% Malachite Green and processed by the method of Vielemeyer et al. [27], where 0.1% Malachite Green was again added during osmium post-fixation. Field emission in lens EM (LEO 1550) was performed on SPHz (sucrose-purified haemozoin), washed in 50 mM Tris/HCl (pH 8.0), then fixed in the presence of Malachite Green before electron micrograph processing [28]. The selectively fluorescent, hydrophobic phenoxazine dye, Nile Red (N 3013; Sigma), was used as a neutral lipid probe on SPHz and vacuole preparations before fixation on glass slides [16,27]. Images were obtained using a Nikon Eclipse E800 at $\times 1000$ magnification.

Western-blot analysis of sucrose cushion fractions

Sucrose cushion fractions were analysed by SDS/PAGE on 10% (w/v) gels and stained with Coomassie Brilliant Blue [29]. Protein content was determined with a Coomassie Brilliant Blue protein assay (Coomassie Plus; Pierce). Immunoblots were probed with antibodies directed to rabbit anti-PfCRT (*P. falciparum* chloroquine-resistant transporter; ATTCC-MR4), Pfaldolase (*P. falciparum* aldolase) and PfHRP II (*P. falciparum* HRP II), and immunoreactive signals were detected by chemiluminescence (Amersham Biosciences) [7,30].

TLC lipid analysis

Total lipids from extensively washed saponin-purified trophozoites, washed hypo-osmotic lysis trophozoite pellets representing enriched digestive vacuoles (fraction before Percoll/sucrose centrifugation) and SPHz with total haemozoin content, all equalized to 100 nmol, were extracted and separated by the method of Jackson et al. [16] with a polar lipid separation in chloroform/methanol/water (50:20:3, by vol.), followed by neutral lipid separation in hexane/di-isopropyl ether/acetic acid (16:10:1, by vol.). Standards were obtained from Sigma and were run in 10 μ g amounts.

MS

GLC fatty acid methyl ester analysis was done as described by Alderson et al. [31]. Synchronized trophozoites, isolated pure digestive vacuoles (Percoll/sucrose bottom) and SPHz were extracted by chloroform/methanol and processed as lithium adducts for ESI-MS (electrospray ionization MS) as described by Ham et al. [32]. At least four different biological isolations on different dates for each of the parasite fractionations were analysed by ESI-MS. Samples were infused at flow rate of 5 μ l per min into a Thermo-Finnigan TSQ quantum triple stage quadrupole ESI-MS.

Haem crystallization kinetic assays

Lipid extracts from uninfected erythrocytes, saponin-isolated trophozoites, pure digestive vacuoles and SPHz were quantified based on spot intensities as compared with known standards. Haemin, drugs, lipids and lipoproteins were obtained from Sigma. High-throughput assays of haem crystallization modified from Chong and Sullivan [33] were initiated in a 96-well format with 100 μ l incubations, 5 nmol of haemin and lipid. Reactions were stopped by addition of 100 μ l of 2% SDS/200 mM bicarbonate (pH 9.1) to quantify free haem at A_{405} , before subsequent addition of 20 μ l of 1 M NaOH that quantifies total haem present. The HTS 7000 Plus 96-well plate reader (PerkinElmer) has a molar absorption coefficient of $3.26 \times 10^8 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 405 nm for a 200 μ l volume.

H₂O₂ degradation assay

SPHz or purified digestive vacuole haemozoin was incubated with H₂O₂ as described by Noland et al. [28].

RESULTS

Neutral lipid nanospheres containing haemozoin are within digestive vacuoles

Ultrastructural localization of neutral lipids within early trophozoite stage digestive vacuoles after aldehyde fixation with Malachite Green prevented subsequent artefactual ethanol wash removal of neutral lipids. Electron-dense neutral lipid nanospheres devoid of an apparent polar lipid membrane bilayer were observed surrounding nascent haemozoin crystals (Figure 1A). A smaller remnant of the neutral lipid nanosphere was seen in late-stage trophozoites surrounding the larger haem crystal clusters (Figure 1B). Scanning EM of SPHz, fixed in the presence of Malachite Green, detected a film overlying haemozoin consistent with lipids (Figures 1C–1E), in contrast with haemozoin processed in the customary fashion with SDS detergent to remove lipids and processed for EM without and with Malachite Green (Figures 1F and 1G). Nile Red fluorescence, specific for a neutral lipid environment, also demonstrated neutral lipids within digestive vacuoles and also in close association with SPHz (Figure 2).

SPHz has minimal protein, but abundant neutral lipid content

Comparison of approx. 10 million saponin-purified trophozoites, which contain 10 nmol of haemozoin, with 10 nmol of SPHz by protein gel electrophoresis demonstrates a paucity of proteins in SPHz (Figure 3A). In a Coomassie Brilliant Blue protein assay comparing 10 nmol by haemozoin content of trophozoites with SPHz, no protein was detected in the SPHz preparation. A previous report [26] evaluated multiple erythrocyte and parasite membrane and cytosolic proteins in trophozoites and SPHz

Figure 1 Haemozoin is present within neutral lipid nanospheres

(A) TEM of early trophozoite stages highlights five to six small haem crystals surrounded by neutral lipid spheres inside digestive vacuoles. The inset shows lack of bilayer membrane at the periphery of lipid sphere. (B) A more mature trophozoite with larger haem crystals has a thinner rim of lipid around haemozoin. (C–E) Scanning EM of SPHs fixed in the presence of Malachite Green exhibits a film-like material around haemozoin, in contrast with (F) delipidated haemozoin or delipidated haemozoin processed with Malachite Green (G). Scale bar, 1 μ m (A, B) and 100 nm (C–G).

Figure 2 Neutral lipid-specific fluorescent dye indicates neutral lipids closely associated with haemozoin

(A, C) Bright-field and (B, D) fluorescent images of (A, B) an enriched digestive vacuole fraction (before sucrose/Percoll centrifugation) and (C, D) SPHs indicate the presence of neutral lipid molecules closely associated with purified haemozoin.

fractions observing more than 99% reduction of erythrocyte Band III, spectrin and catalase as well as parasite digestive vacuolar soluble plasmepsin 1, HRP II and also the integral

Figure 3 SPHs have minimal protein content and predominant neutral lipids

(A) Saponin-purified trophozoites (T) and SPHs equalized to 10 nmol of haemozoin, which approximates to 10 million parasites, were separated by gel electrophoresis and stained by Coomassie Brilliant Blue. Molecular-mass markers are in kDa. (B) Western-blot analysis of a digestive-vacuole integral membrane protein PfCRT (molecular mass = 48 kDa), soluble cytosolic Pfaldolase (ALD; molecular mass = 40 kDa) and soluble PfHRP II (molecular mass = 30 kDa, but runs as dimer at 60 kDa) located in digestive vacuoles and also infected erythrocyte cytosol shows more than 100-fold reduction of specific proteins. (C) TLC indicates significant amounts of monoacylglycerols. Isolated trophozoites (T), enriched digestive vacuoles (eDV) and SPHs were equalized to 100 nmol haemozoin content, and lipids were extracted and then separated by two mobile phases of TLC. Monoacylglycerols predominate in all the fractions. Diacylglycerols and triacylglycerols were diminished in SPHs compared with trophozoites. Lipid standards (STD) are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; MAG, monoacylglycerol; DAG, diacylglycerol; FA, non-esterified fatty acid; TAG, triacylglycerol.

membrane protein, Pfmdr-1 (*Plasmodium falciparum* multidrug resistance-1), which localizes to digestive vacuole. We also tested for the presence of the PfCRT, Pfaldolase and PfHRP II by Western blot, which all confirmed a paucity of proteins in this subcellular fraction of haemozoin (Figure 3B).

Significant amounts of monoacylglycerols were present in lipids extracted from trophozoites, enriched digestive vacuoles and SPHs, after separation by a dual-phase solvent TLC. A diminution of diacylglycerols and triacylglycerols was observed with

SPHz as compared with trophozoites and the enriched digestive vacuoles, all of which had been equalized to 100 nmol total haemozoin content (Figure 3C).

MS identifies neutral lipids associated with haemozoin

Fatty acid methyl ester analysis of trophozoites, pure digestive vacuoles and SPHz from freshly isolated trophozoites or residual bodies from culture supernatants identified predominantly saturated palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) fatty acids, with a smaller amount of unsaturated oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) fatty acids, and absent arachidonic acid ($C_{20:4}$) in SPHz fractions (Figure 4A). Trophozoites and pure digestive vacuoles have more oleic than stearic fatty acids.

We sought to identify through ESI-MS/MS (electrospray ionization tandem mass spectrometry) the precise parasite lipid species associated with haemozoin, focusing on SPHz. Significant amounts of the saturated lipids MSG (monostearic glycerol) and MPG (monopalmitic glycerol) were observed with a ratio of approx. 2:1 (Figure 4B, and see Supplementary Figures 1 and 2 at <http://www.BiochemJ.org/bj/402/bj4020197add.htm>). Surprisingly neither MOG (mono-oleic glycerol) nor MLG (monolinoleic glycerol) was detected in any of the parasite or SPHz preparations. The principal diacylglycerols associated with SPHz were distearic glycerol and 1-stearic-3-palmitic glycerol, with minimum amounts of DPG (dipalmitic glycerol), DOG (dioleic glycerol) and DLG (dilinoleic glycerol) (see Supplementary Table 1 at <http://www.BiochemJ.org/bj/402/bj4020197add.htm>). A mixture of 1,2-diacylglycerol and 1,3-diacylglycerol was also detected (see Supplementary Figure 3 at <http://www.BiochemJ.org/bj/402/bj4020197add.htm>). Only small amounts of the polar phosphatidylcholine and phosphatidylethanolamine and triacylglycerols were detected in the SPHz fractions relative to intact digestive vacuoles and trophozoites. Control incubations of synthetic β -haematin or delipidated haemozoin either with the lipid-rich fraction remaining at the top of sucrose cushion after sHZ isolation or with lipid standards for 30 min, followed by ultracentrifugation through sucrose showed by LC (liquid chromatography)-MS that lipids do not bind haem crystals non-specifically at time of experimental lysis (see Supplementary Figure 4 at <http://www.BiochemJ.org/bj/402/bj4020197add.htm>).

Neutral lipid nanosphere haem crystallization kinetics and inhibition by the quinolines

A high level of haem crystallization activity was found to be present in lipids extracted from SPHz (Figure 5A). Interestingly, although the saturated lipid MSG was largely inactive, MPG was found to be a potent promoter of haem crystallization and the combination of 1-stearic-3-palmitic glycerol was also able to promote crystallization (Figure 5A). Overall, monoacylglycerols are more potent catalysts of haem crystallization than are the diacylglycerols. Both MSG and distearic glycerol are inefficient catalysts. Erythrocyte membrane ghosts or the triacylglycerol triolein (either sonicated or not sonicated) in this direct comparison did not promote haem crystallization. Based on the results of TLC, FAME (fatty acid methyl ester) and ESI-MS, we postulated a mixture of predominantly neutral lipids to be present in the haemozoin-containing nanospheres. Even though stearic glycerol was not efficient at haem crystallization, this saturated lipid was still included in the reconstituted neutral lipid blend of MPG/MSG/DPG/DOG/DLG (2:4:1:1:1, by vol.). The neutral lipid blend produces haem crystals rapidly within 10 min, with near linear acceleration for 2 h to 80% substrate crystallized before a decreased acceleration (results not shown). The pH-dependence of neutral lipid nanosphere haem crystallization was above 70–

80% production up to pH 5.8 before rapidly decreasing to less than 10% production at pH 6.0 (results not shown). A 1:60 molar ratio of free haem to crystalline haem was quantified in SPHz, which was found to possess an approximate molar ratio of 15:100 lipid/haem. In the presence of neutral lipid nanospheres composed of MPG or the blend of neutral lipids, haem crystallization proceeds in the presence of 30–50 μ M globin, while 2 μ M globin inhibits the β -haematin seed haem crystal extension assay (Figure 5B). Neutral lipid nanospheres also protect haem crystals from peroxide degradation, as one-half of SDS-washed delipidated haemozoin was degraded in the presence of 50 mM H_2O_2 , while SPHz enveloped in neutral lipids required 90 mM H_2O_2 to degrade half of SPHz (results not shown) [28]. We postulated that inclusion of MSG by the parasite that does not crystallize haem efficiently might slow crystal growth kinetics at the growing face of crystal. We examined morphological appearance by scanning EM of crystals after production in MOG or the lipid blend. Crystals made in MOG have sharp tapered ends, in contrast with crystals made in the lipid blend, which were more blunt (Figures 6A and 6B).

To examine mechanisms by which quinoline anti-malarials inhibit neutral lipid nanosphere haem crystallization, we pre-incubated the neutral lipids with chloroquine, quinidine and the lipophilic mefloquine, all without and with haem, then washed away the water-soluble drug. Only pre-incubations of the chloroquine, quinidine and mefloquine with haem and mefloquine alone inhibited subsequent haem crystallization initiated by addition of fresh haem (Figure 7A). In concordance with Chong and Sullivan [33] and Egan and Ncokazi [34], we have also confirmed lipid-mediated haem crystal inhibition by chloroquine and quinidine to be a reversible time-dependent process, such that the IC_{50} for haem crystallization increases with longer periods of time (Figure 7B). Investigation into the effect of pH on chloroquine and quinidine inhibition revealed a sharp decrease in the amount of haem crystallized over a 0.4 pH unit increase from 5.2 to 5.6 at specific quinoline concentrations (Figure 7C).

DISCUSSION

For years the exact intracellular molecular mechanism(s) by which haem from haemoglobin is crystallized into biologically inert (to the parasite) haemozoin within the protein-rich aqueous acidic confines of the *Plasmodium* parasite's digestive vacuole has been vigorously disputed. In the present study, we provide ultrastructural evidence that neutral lipid nanospheres present within the digestive vacuole are the *in vivo* site of haemozoin formation. The multitude of previously published TEM images of *Plasmodium* parasites removed virtually all evidence of these neutral lipid nanospheres by the ethanol processing. Only when fixed in the presence of Malachite Green are neutral lipid nanospheres evident surrounding haemozoin by EM. Both scanning EM and Nile Red fluorescence of SPHz are evidence against a possible artefact, whereby Malachite Green might aggregate neutral lipids during fixation of trophozoites.

The exact chemical composition of the *P. falciparum* neutral lipid nanospheres by three complementary modalities indicates an absence of MOG and MLG, but the presence of significant amounts of MPG and MSG along with lesser amounts of various diacylglycerol species and polar lipids that are associated with SPHz. This represents the first demonstration of monoacylglycerols within the digestive vacuole. This does not rule out larger amounts of triacylglycerols or polar lipids within lipid nanospheres *in situ* in digestive vacuoles, but does indicate that triacylglycerols and polar lipids are not in closest approximation

Figure 4 MS identify neutral lipids associated with SPHz

(A) GLC-MS fatty acid methyl ester analysis was performed on Restek 37 component FAME mix standards (top), and enriched digestive vacuoles (1), SPHz (2), SPHz from residual bodies from culture supernatants (3), and asynchronous 3D7 parasites (4). Palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) fatty acids predominate over oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and arachidonic ($C_{20:4}$) fatty acids in SPHz. (B) ESI-MS total ionization chromatogram on trophozoites (1), pure digestive vacuoles (2) and SPHz (3). While m/z 300–400 is the monoacylglycerol region, diacylglycerols are clustered at m/z 550–650 and polar lipids at m/z 730–900. The asterisks (*) are peaks present in blank samples, and 'IS' represents internal standards. The inset shows lack of MOG (m/z 363) or MLG (m/z 361). Supplementary Figures 1–4 show MS/MS data of MPG, MSG and 1,3-distearic glycerol, and Supplementary Table 1 (at <http://www.BiochemJ.org/bj/402/bj4020197add.htm>) has a list of m/z values for parent ions and lithium adducts.

to haemozoin. The present study builds upon Bendrat et al.'s [9] previously described methyl esters of palmitic acid, stearic acid and oleic acid associated with haemozoin and upon

both Fitch et al.'s [11,12] and Tilley and co-workers's [16] identification of diacylglycerol and triacylglycerol in parasite fractions. Our observation is also in concordance with Oliveira

Figure 5 Neutral lipids rapidly promote haem crystallization

(A) Sonicated 50 μ M lipids were incubated in 50 μ M haemin in 100 μ l of sodium acetate (pH 4.8) for 20 h at 37°C. Organic lipid extracts of SPHz from parasites effectively catalyse crystallization of free haem compared with diverse individual neutral lipid molecules. The neutral lipid blend of MPG/MSG/DPG/DOG/DLG (2:4:1:1:1) was effective despite inclusion of MSG. Abbreviations: DOPC, dioleoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine. (B) The neutral lipid blend (filled bar) and MPG (striped bar) promote haem crystallization in the presence of greater than 50 μ M globin, while crystallization initiated by β -haematin seed (open bar) is effectively inhibited at 2 μ M globin. Error is S.D. of triplicate incubations.

et al.'s [35] description of haem crystal formation in lipid droplets.

Fitch et al. [11] have demonstrated that *in vitro*, unsaturated monoacylglycerols are effective promoters of haem crystallization, while unsaturated diacylglycerols and triacylglycerols are inefficient catalysts. On the basis of results with stearic acid, saturated lipids were described as incapable of haem crystallization. Counter to this unsaturated-lipid hypothesis, MPG demonstrated significant haem crystallization activity. While the mechanisms by which the *Plasmodium* parasite generate these catalytically active lipids in the digestive vacuole remain to be explored, the fact that a mixture of 1,2-diacylglycerol and 1,3-diacylglycerol was detected, and also that axenic *P. falciparum* cultures make haemozoin [15,36], indicates that possible phospholipase C

Figure 6 Haem crystals produced by blend are more blunt than crystals produced by MOG

Haem was incubated in 50 μ M MOG or lipid blend for 6 h, then washed with SDS/bicarbonate and then SDS alone and washed in water. Scanning EM of the crystals produced in (A) MOG have a sharp taper at ends, in contrast with haem crystals produced in (B) lipid blend, which have more squarish blunt ends.

activity on polar lipids is not the sole catabolic route to digestive vacuole diacylglycerols. The *P. falciparum* genome includes putative genes for phospholipase C (PF10_0132) and a lysosomal acid lipase (PF11_0276) which have not been characterized. The lysosomal acid lipase is similar to the mammalian gene implicated in Wolman disease, where triacylglycerols accumulate in lysosomes [37].

Neutral lipid nanospheres also may serve as transient repositories for lipophilic haem initially released into the aqueous digestive vacuolar milieu during the process of haemoglobin degradation. While neutral lipid nanospheres absorb and concentrate free haem, charged cytoplasmic proteins such as globin may be effectively screened out of the nanosphere's hydrophobic interior. Thus neutral lipid nanospheres appear to accumulate and concentrate lipophilic free haem while promoting haemozoin formation at the surface interface where acidic conditions favour lipid-mediated crystallization. Our results suggest that the irreversible head-to-tail β -haematin dimer formation is accelerated under conditions of a pH less than 6 and the neutral lipid environment which favours formation of haem crystal because the competing solvent water to axial iron is absent, thus permitting release of axial water molecules. This would thermodynamically favour haem crystal product formation. The newly formed β -haematin dimer, almost a cubic nanometre [4], then stacks into larger crystals of 100 nm $\times$ 100 nm $\times$ 500 nm in the case of *P. falciparum*. Although the lipid blend made crystals that were less tapered and more blunt than crystals made by MOG, the lipid blend crystals did not exactly replicate haemozoin made by *P. falciparum*. This may require the presence of non-specific proteins or other molecular species. The divergent morphology of non-mammalian and non-*Plasmodium* haem crystals may result from different amounts, compositions, sizes or timing of neutral lipid nanospheres merging intracellularly or extracellularly [28]. Histidine-rich protein II or other *Plasmodium* orthologues may function as chaperones of haem from haemoglobin to the neutral lipid nanospheres.

A controversial observation has been that that chloroquine-resistant *P. falciparum* isolates can be segregated from chloroquine-sensitive isolates on the basis of a 0.2–0.4 pH unit decrease in vacuolar pH [38]. Our observed sharp decrease in the amount of haem crystallization which takes place over a small 0.4 pH unit increase from 5.2 to 5.6 occurred only at specific quinoline concentrations. Increasing or decreasing drug concentrations by

Figure 7 Quinolines inhibit haem crystallization

(A) Chloroquine (50 μM; horizontal striped bar), 50 μM quinidine (diagonally striped bar), 50 μM mefloquine (dotted bar) or no drug (filled bar) was pre-incubated (PI) with 50 μM haem (PI + haem) or without 50 μM haem (PI minus haem) in 500 μl of sodium acetate (pH 4.8) for 2 h at 37 °C. All samples were washed twice by centrifugation (14 000 *g* and 15 min) with 1 ml of 50 mM ammonium bicarbonate (pH 8.3). Fresh 50 μM haem was added after washing the pellets to commence a 6 h haem crystallization assay. A control group included no pre-incubations (No PI) but same drug concentrations. (B) The IC₅₀ for inhibition of haem crystallization was determined from at least six chloroquine (▲) and quinidine (■) concentrations at indicated times. Error is S.D. of three line plot determinations from triplicate incubations for each point on the lines. (C) pH-dependent profile of drug inhibition of blend-initiated haem crystallization reactions was measured as a percentage of no-drug control with 25 μM quinidine (□, solid line) and 15 μM chloroquine (△, solid line) for 24 h, or 10 μM quinidine (■, dashed line) and 5 μM chloroquine (▲, dashed line) for 4 h. No-drug control yielded 4 ± 0.7 nmol of haem crystals until pH 5.8, with a steep 80% decrease in yield at pH 5.9. Error is S.D. of triplicate incubations.

as little at 10 μM or changing time of incubation removed the sharp decline. Accurate determinations of aqueous quinoline concentrations separate from concentrations in lipid or bound to haem or haemozoin are still necessary to model further the complex associations of digestive vacuolar pH, haem crystallization inhibition, lipophilicity and specific drug export.

Chloroquine and other quinolines may inhibit crystallization either by prevention of head-to-tail β-haematin dimer formation

or by inhibition of β-haematin dimer integration into the growing face of the larger haem crystal leading to accumulation of haem dimers which may be just as toxic as haem monomers in binding to enzymes or increasing permeability of membranes. Important for quinoline action and also drug resistance, we show that drug–haem complexes enter a non-polar lipid environment to inhibit crystallization. This is a shift in the conception of quinoline action taking place in an aqueous environment to a lipid non-polar environment.

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