Quantitative characterization of hemozoin in *Plasmodium berghei* and *Plasmodium vivax*

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**A B S T R A C T**

The incidence and global distribution of chloroquine resistant (CR) *Plasmodium vivax* infection has increased since emerging in 1989. The mechanism of resistance in CR *P. vivax* has not been defined. The resistance likely relates to the formation and disposition of hemozoin as chloroquine's primary mechanism of action involves disruption of hemozoin formation. CR *P. berghei* strains, like CR *P. vivax* strains, are confined to reticulocyte host cells and reportedly they do not accumulate appreciable intra-erythrocytic hemozoin. Reports comparing hemozoin production between *P. berghei* and CR to chloroquine sensitive (CS) *P. berghei* are absent. Here we compare in *vivo* patterns of hemozoin formation and distribution in blood, spleen and liver tissue of male Swiss mice infected with CS or CR *P. berghei* not treated with chloroquine and CR *P. berghei* also treated with chloroquine. Light microscopy, laser desorption mass spectrometry and a colorimetric hemozoin assay detect trace hemozoin in the blood of CR *P. berghei* infected mice but significant hemozoin accumulation in liver and spleen tissue. Field emission in lens scanning electron microscopy reveals CR *P. berghei* hemozoin crystals are morphologically smaller but similar to those formed by CS parasites. CR *P. berghei* produces approximately five-fold less total hemozoin than CS strain. Lipid analysis of CS and CR *P. berghei* sucrose gradient purified bloodstage hemozoin indicates a similar lipid environment around the isolated hemozoin, predominately monopalmitic glycerol and monostearic glycerol. In contrast to CR and CS *P. berghei*, colorimetric hemozoin analysis of *P. vivax* strains indicates similar amounts of hemozoin are produced despite differing chloroquine sensitivities. These results suggest CR *P. berghei* forms significant hemozoin which accumulates in liver and spleen tissues and that the *P. vivax* chloroquine resistance mechanism differs from *P. berghei*. © 2017 The Authors. Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Intraerythrocytic *Plasmodium* parasites ingest and catabolize host erythrocyte hemoglobin in a digestive vacuole that arises from invagination of the parasitophorous vacuolar membrane and the underlying parasite membrane (Yayon et al., 1984). Hemoglobin comprises an estimated 95% of the total protein content of the mature erythrocyte. Hemoglobin catabolism releases copious monomeric iron protoporphyrin IX, or heme, which oxidatively damages membranes and inhibits parasite catabolic enzymes (Orjih et al., 1994). *Plasmodium* parasites avert heme-mediated damage by sequestering heme into a relatively inert, brown, birefringent biocystal called hemozoin. Chloroquine, a weak base, accumulates in the acidic digestive vacuole of drug sensitive parasites where it complexes free heme to cap and inhibit hemozoin elongation along the growing crystal face (Sullivan et al., 1996).

In chloroquine resistant (CR) *P. falciparum* a point mutation of the Pfcrt gene is associated with clinical treatment failure (Fidock et al., 2000). This mutation imparts a drug metabolite transporter mediated form of drug resistance that is verapamil-reversible...
The Pfmdr1 gene codes for another transporter also associated with chloroquine resistance in *P. falciparum* (Foote and Kemp, 1989; Das et al., 2013). The mechanism of *P. vivax* chloroquine resistance is not understood. Experimental testing is hampered because *P. vivax* is difficult to culture in vitro. *P. vivax* chloroquine resistance arose decades after it was noted in *P. falciparum*. The molecular basis of resistance clearly differs as *Pvmdr1* have not been found to impart chloroquine resistance in *P. vivax* amid conflicting clinical studies (Goncalves et al., 2014). One study found *P. vivax* chloroquine resistance was associated with enhanced expression of *pvct-o* and *pvmdr-1* (Melo et al., 2014). However, a different human study found increases in *pvmdr* copy number were associated with resistance to mefloquine yet actually enhanced susceptibility to chloroquine (Vargas-Rodriguez Rdel et al., 2012). Transcriptional upregulation of wild type pvcg10 (*pvct-o*) transfected into *CS P. falciparum* facilitates reduced chloroquine susceptibility, if not full resistance, but no direct role for transporters in *CR P. vivax* parasites has been established (Sa et al., 2005).

*Plasmodium berghei* infects rodents and is considered a useful model for studying *P. vivax* infection and alternative forms of chloroquine resistance (Espinosa et al., 2013). This is because CR *P. berghei*, like *P. vivax*, infects only reticulocyte host cells (Ocampo et al., 2002). CR *P. berghei* also does not secrete chloroquine more rapidly than the CS strain (Wang et al., 1998). Treatment with the P-glycoprotein transporter inhibitor rifampicin or verapamil does not restore susceptibility in CR strains (Peters et al., 1990; Platel et al., 1998). Consistent with the lack of chloroquine resistance transporter (CRT) based mutations, these facts indicate *P. berghei* and *P. vivax* chloroquine resistance differs from the transporter mediated resistance seen in *P. falciparum*.

CR *P. berghei* strains have for decades been reported to form little or no hemozoin pigment (Peters, 1964). Lin et al. recently created CR *P. berghei* strains that form little to no hemozoin by knocking out genes in the CS wild type that are associated with hemoglobin catabolism (Lin et al., 2015). The CS wild type could infect reticulocytes and mature RBCs alike and form abundant hemozoin, but the CR knockouts could develop only in reticulocytes. Chloroquine treatment of CS *P. berghei* reduces hemozoin formation by an order of magnitude and causes accumulation of deadly free heme; however, in CR *P. berghei* chloroquine treatment causes no detectable buildup of free heme (Fitch and Chou, 1997). Reduced glutathione (GSH), naturally elevated in reticulocytes, can degrade heme and might play a role in chloroquine resistance in *P. berghei* (Ginsburg et al., 1998). However, genetic upregulation of GSH does not impart chloroquine resistance to CS strain *P. berghei* (Vega-Rodriguez et al., 2015). Alternatively, membrane-bound heme and hemozoin may be ejected from reticulocytes using the native host reticulocyte process of exocytosis (Slomianny, 1990; Slomianny and Prensier, 1990). Hemozoin, if produced, would be expected to present in the liver and spleen tissue of mice infected with CR *P. berghei*. A shift to a circulating ring predominance in CR *P. berghei* with sequestration of trophozoites and schizonts in tissue (Jacobs and Warren, 1967; Schneider, 1968), also does not explain resistance to chloroquine as the sequestered parasites would have similar drug exposures. This study investigates *P. berghei* chloroquine resistance by investigating patterns of hemozoin formation and tissue distribution by multiple methods in CS and CR *P. berghei* parasites and their murine hosts. We also compared hemozoin formation in CR versus CS *P. vivax* isolates.

### 2. Materials and methods

#### 2.1. *P. berghei* parasite strains and infection of host mice

Male Swiss mice (CF-1) weighing approximately 25 g were purchased from Sasco, Inc. (Omaha, Neb.). They were given Purina Laboratory Chow and water ad libitum, and their care was provided in accordance with Saint Louis University guidelines. At St. Louis University blood from NYU-2 strain CR *P. berghei* infected mice isolated in 1974 and kept under chloroquine pressure as well as the chloroquine sensitive parent were utilized (Fitch et al., 1974). The CR line had been passaged every 2 weeks for two years under chloroquine pressure applied by intraperitoneal injection of 1 mg of chloroquine daily on Monday through Friday of each week. Frozen stocks of CR *P. berghei* were inoculated into 6 mice with chloroquine treatment at 1 mg per mouse intraperitoneally dose to revive the frozen isolate. After 14 days with parasitemia about 10% the infected blood was passed to 12 mice with continued chloroquine treatment 5 days of the week. After another 14 days the combined blood was inoculated into two groups with one on continued chloroquine and another off chloroquine. The frozen CS line of NYU-2 *P. berghei* was inoculated to a control set of donor mice not treated with chloroquine and on day 9 post infection of the CR and CRCT, a new CS NYU-2 *P. berghei* was begun such that on the same day the CS mice would be exsanguinated on day 5 of infection and day 15 for the CR and CRCT groups. Each passage used 100 μl of a 1:25 dilution of 25–50% parasitemia or 10 to 20 million infected parasites per passage given by the intraperitoneal route. 16 young male Swiss mice of 25 g each were divided into 4 groups of 4 mice per group. Mice of the chloroquine treated CRCT group were treated with 40 mg/kg of chloroquine base per day. Mice of the uninfected negative control group received no drug. Mice of the positive control group infected with CS *P. berghei* (NYU-2 strain) and the CR *P. berghei* also received no drug. Before death and following a 5 day time post infection period for CS infected mice and a 15 day time post infection period for the CR and CR/CT groups, due to their lower parasitemia, mice were anesthetized and sacrificed by exsanguination. Hematocrit levels were measured and two thin blood smears were prepared for each mouse. One smear was methanol fixed and Giemsa stained to permit quantitation of total parasitemia per 1000 RBCs. The other was fixed with methanol only. Livers and spleens were recovered, weighed before processing in formalin for histology or frozen at −80 °C.

#### 2.2. Tissue processing and hemozoin analysis

The mice were infected on two separate occasions. After the first experiment, the liver and spleen were divided and half placed into neutral formalin and half frozen for hemozoin quantification and electron microscopy. In the second biologic experiment 80% of liver and spleen from each mouse went for quantification of hemozoin. Mouse livers and spleens were divided and preserved in 10% neutral formalin. Formalin preserved liver and spleen samples were paraffin embedded and thin sectioned. Histological slides were hematoxylin and eosin (H+E) stained and examined via brightfield and polarizing light microscopy. Individual microscopic field overlaps at 400x and 1,000x were photographed under brightfield and polarized light to document brown, birefringent hemozoin crystals in tissue and blood smears. The cryogenically stored blood, plasma, spleen and liver samples were thawed and processed to purify, detect and quantify hemozoin. Briefly, 400–500 μl of blood from each mouse were hypotonically lysed in 4.5 ml of DI H2O. Individual mouse livers or spleens were homogenized in 5 ml of DI H2O by pulsed sonication (Branson sonifier). Lysed blood and tissue were centrifuged at 14,000 times gravity for 15 min to pellet
hemozoin. The supernatant fraction was removed and the pellets resuspended in 1 ml 2% SDS, 100 mM sodium bicarbonate. This was spun down as above and the pellets washed in 2% SDS and centrifuged. Washed pellets were resuspended and incubated overnight at 60°C in 1 mg/ml protease K buffer. Following incubation, samples were washed in DI H2O. Purified hemozoin pellets were then decrystallized in 1 ml 2% SDS 20 mM NaOH for 1 h and spectrophotometrically quantitated by measuring absorbance either in a Beckman DU-640 cuvette with a 1 cm pathlength with a molar extinction coefficient at 400 nm of 1 x 10^5 or in a Perkin Elmer HTS 7000 96 well plate reader in 200 μl with molar extinction coefficient of 5.7 x 10^4 with a 405 nm filter for the 200 μl volume.

2.5. *P. vivax* monkey adapted strains and infection of primate hosts

*Saimiri* monkeys were infected with either the CS Salvador I strain *P. vivax* or the CR *P. vivax* Indonesian strain XIX at the Centers of Disease Control (Atlanta, GA). One additional *Aotus* monkey was infected with the CR *P. vivax* Indonesian strain XIX. Monkeys were housed with normal food and water and no drug treatment for a period of 5–7 weeks. Periodic blood samples were taken and parasitemia determined by optical microscopy of 5 μl thick smears. Hemoglobin levels at time of exsanguination were estimated by OD 405 nm measurements of 2 and 4 μl of packed RBCs suspended in 100 μl of DI H2O. Two blood samples from each monkey, approximately one ml each, were spun for 3 min at 3000xg and the plasma separated from pelletted packed RBCs.

2.6. Purification of *P. vivax* hemozoin

Packed RBC volumes from 460 to 650 μl from a ml of whole blood from each of the 3 monkeys infected with the three separate *P. vivax* isolates were split into 4 tubes of equal volume, for quadruplicate quantitation of hemozoin. Aliquotted packed RBCs were diluted 1:5 in DI H2O and sonicated. The lysate was spun down at 14,000xg for 20 min. Resuspended pellets were washed twice in 1 ml of 2% SDS to solubilize membranes. Hemozoin pellets were washed twice in 1 ml DI H2O before final pellet resuspension in 10 μl DI H2O. No protease K treatment was conducted due to the limited amount of *P. vivax* sample and the absence of liver or spleen tissue to digest. Hemozoin was purified and quantitated using the above SDS bicarbonate/NaOH decrystallization method and the total amount of hemozoin produced per 10^10 *P. vivax* parasites was calculated.

3. Results

3.1. Microscopy shows CR and CS *P. berghei* hemozoin production and host tissue accumulation

No brown pigment particles or birefringent crystals are seen in the blood of uninfected animals (Fig. 1A and B). Brightfield examination of Geimsa blood films shows CS *P. berghei* parasites infect normocytes and reticuloocytes, often have multiple parasites per infected erythrocyte and produce dark intracellular pigment particles consistent with hemozoin (Fig. 1C). Overlapping polarized light micrographs demonstrate that the position of the brown pigment corresponds directly to areas of birefringence (Fig. 1D). Blood films from CR and CRCT *P. berghei* have much less birefringent crystals (Fig. 1E–H).

Histological examination of livers and spleens under brightfield and polarizing light microscopy demonstrates that CS as well as CR parasites produced hemozoin that heterogeneously accumulates in liver and spleen (Figs. 2 and 3, respectively). Relatively less birefringent, brown crystal accumulates in the liver and spleen of CR infected as compared to CS infected animals with the least accumulation being in CRCT animals (Figs. 2 and 3). No brown pigment or birefringent crystals are visualized in liver (Fig. 2A and B) or spleen (Fig. 3A and B) of uninfected animals.

Examination of hemozoin purified from blood samples by FEI-SEM demonstrated the occurrence of crystals consistent with hemozoin in the blood of CS infected mice (Fig. 4A). Hemozoin was not purified from the blood of CR or CRCT *P. berghei* infected mice in sufficient quantities to allow for characterization of blood hemozoin by FEI-SEM. However, crystals morphologically identical to wild type CS hemozoin were identified by FEI-SEM in combined liver and spleen tissue isolated from either CR or CRCT infected animals (Fig. 4B–D). CR *P. berghei* forms roughly 70 nm x 70 nm x 200 nm
smooth faced crystals in the presence or absence of chloroquine. In contrast the CS *P. berghei* crystals were 135% larger along a single width or height face at 95 nm × 95 nm × 200 nm. No crystals were observed in the uninfected group. CR strain *P. berghei* forms hemozoin crystals morphologically identical to hemozoin produced by wild type CS *P. berghei*.

Fig. 1. Photomicrographs of Geimsa stained blood smears from: An uninfected mouse (1A, B), a CS infected mouse (1C, D), a CR infected (1E, F), and a CR infected chloroquine treated mouse (1G, H). Fig. 1B, D, F and H are photomicrographs of the same fields taken under polarizing light. Birefringent crystals appear as intracellular points of light. The arrows in F and H indicate a single visible hemozoin crystal. Total magnification = 1000x.

Fig. 2. Histological photomicrographs of H + E stained liver sections from: An uninfected mouse (2A, B), a CS infected mouse (2C, D), a CR infected mouse (2E, F), and a CR infected chloroquine treated mouse (2G, H). Fig. 2B, D, F and H are photomicrographs of these identical fields taken under polarizing light. Areas of extracellular accumulation of birefringent crystal in liver appear as white areas. Inset in sections C and D show an infected erythrocyte on the left which polarizes light on darkfield. Total magnification = 1000x.

3.2. Hemozoin production differs between strains

On the day of exsanguination, mean hematocrit values were roughly two-fold higher in the blood of uninfected animals; 43% (±2.4) as compared to CRCT mice at 23.5% (±6.8%), CS mice at 20% (±2.2%), and CR mice at 15.8% (±1.7%) (Table 1). Mean percent of infected erythrocytes at time of exsanguination ranged from a CS group high of 69% (total intraerythrocytic parasites/total RBCs) to 29% for the CR group and 15% for the CRCT group (Table 1). Mouse 1 in the CR/CT group was of interest in that its 4% parasitemia was substantially lower than the other 3 members of this group.
Because of multiple parasites invading a single erythrocyte we also counted the total number of parasites per 1000 erythrocytes to normalize total parasites. The means were 1303, 897 and 464 per 1000 erythrocytes respectively for CS, CR and CRCT P. berghei.

Spectrophotometric hemozoin quantitation (OD 400 nm) showed highest mean hemozoin content to be in the blood of CS strain infected mice followed by the CR group and CRCT groups respectively (Table 1). Thus in blood, CR and CRCT hemozoin was strain infected mice followed by the CR group and CRCT groups showed highest mean hemozoin content to be in the blood of CS.

Neutral lipids associated with P. berghei hemozoin

Hemozoin from both CS and CR P. berghei may exist in a similar lipid environment for formation. Sucrose cushion purification of hemozoin from both CS and CR P. berghei in blood copurifies detectable amounts of the monoacylglycerides monopalmitic glycerol (MPG) (337 m/z) and monostearic glycerol (MSG) (365 m/z) (Fig. 6A). These lipids are identified in wild type CS P. berghei intact parasites isolated by saponin (Fig. 6B) and to a lesser extent in sucrose gradient purified hemozoin from bloodstage CR P. berghei (Fig. 6C). The identity of MPG and MSG in sucrose purified hemozoin and CS P. berghei parasites was confirmed by MS/MS analysis as shown in Supplementary Fig. 2, 3 and 4.

In summary, MSG and MPG appear to be the dominant MAG...
neutral lipids associated with sucrose purified hemozoin in *P. berghei* while little to no saturated MOG or MLG is seen. As with CS *P. berghei*, sucrose purified hemozoin from CR *P. berghei* also contains above background MPG and MSG as verified by MS2 analysis (Supplementary Fig. 4A and D) and absent MLG and MOG (Supplementary Fig. 3, 4B and 4C).

### Table 1
Spectrophotometric quantification of hemozoin in blood, spleens and livers.

|                | CS          | CR          | CRCT        | Uninfected |
|----------------|-------------|-------------|-------------|------------|
| **Blood**      |             |             |             |            |
| % Hematocrit   | 20 ± 2.2    | 15.8 ± 1.7  | 23.5 ± 6.8  | 43 ± 2.4   |
| Percent infected erythrocytes | 69%         | 29%         | 15%         | 0%         |
| Parasites per 1000 erythrocytes | 1303 ± 214 | 897 ± 379   | 464 ± 298   | 0          |
| Volume blood processed µl | 400         | 400         | 400         | 400        |
| nmols hz./ml.  | 864.1 ± 191.5 | 12.7 ± 1.05 | 0.1 ± 0.03  | 0.03 ± 0.002 |
| nmols hz. blood total | 1728.2 ± 383 | 25.3 ± 2.1  | 0.2 ± 0.06  | 0.06 ± 0.004 |
| **Spleen**     |             |             |             |            |
| Weight (mg.)   | 233 ± 22    | 867 ± 84    | 928 ± 143   | 76 ± 9     |
| mg processed   | 196 ± 22    | 693 ± 84    | 742 ± 143   | 61 ± 9     |
| nmols hz./gm.  | 682 ± 116   | 34 ± 20     | 10 ± 7      | 3.9 ± 1.1  |
| nmols hz spleen total weight | 160 ± 26    | 29.7 ± 13.8 | 6.6 ± 0.9   | 0.3 ± 0.1  |
| **Liver**      |             |             |             |            |
| Weight (mg.)   | 1482 ± 97   | 2104 ± 34   | 2276 ± 501  | 1559 ± 96  |
| mg processed   | 1142 ± 97   | 1683 ± 34   | 1821 ± 501  | 1247 ± 96  |
| nmols hz./gm.  | 1530 ± 177  | 350 ± 100   | 21 ± 10     | 0.3 ± 0.02 |
| nmols hz liver total weight | 2266 ± 262  | 736 ± 217   | 47 ± 18     | 0.67 ± 0.028 |
| **Total**      |             |             |             |            |
| nmols hz. average mouse | 4155 ± 333  | 790 ± 231   | 54 ± 26     | NA         |

Blood, spleen and liver hemozoin was isolated, decrystallized by NaOH treatment and quantified via OD 400 nm. CS *P. berghei* infected mice had highest hemozoin (hz.) accumulations followed by CR and CR/CT infected mice. CR parasites produce 0.49 μmoles of hemozoin per 10^10, or one fourth as much as an equivalent number of CS parasites. Chloroquine treatment attenuates production of hemozoin by CR parasites roughly 16-fold as compared to chloroquine untreated CR *P. berghei*.

#### 3.5. Quantitation of *P. vivax* hemozoin production by strain

In *P. vivax* both CS and CR isolates inhabit reticulocytes similar to CR *P. berghei*. The CS Sal 1 parasitemia was 8200/µl while CR Indo Strain was 6900/µl. Hemoglobin content was slightly lower in the Indo strain. After normalization for parasitemia and host cells as determined by hemoglobin content, colorimetric measurements of decrystallized hemozoin isolated from a ml of whole blood indicated that 3.29 ± 1.1 and 4.55 ± 0.8 μmol of hemozoin are produced...
per $10^{10}$ Indo and Sal-I, respectively. That is to say only 1.4 fold more hemozoin was produced by the CS 
Plasmodium vivax
strain than the CR 
P. vivax
strain, which is within error in measurement. Giemsa and polarized microscopy show similar hemozoin in CS and CR 
P. vivax
(Fig. 7).

4. Discussion

The worldwide distribution and incidence of CR 
P. vivax
has expanded significantly since emerging in Papua New Guinea in 1989 (Rieckmann et al., 1989) (Goncalves et al., 2014). Understanding into the molecular basis of alternative forms of chloroquine resistance is needed to retain quinolines as effective frontline drugs (Baird et al., 2012). In this study, hemozoin production and distribution was analyzed in blood, spleen and liver tissue from CR or CS 
P. berghei
infected mice. Results found that 1830 ($\pm$210) nmoles hemozoin are produced per $10^{10}$ asynchronous NYU-2 CS strain 
P. berghei
parasites in blood circulation. This is consistent with the CS strain’s hemozoin levels previously measured by Fitch of 2100 ($\pm$360) nmoles per ml of packed male Swiss mouse iRBCs. For CR 
P. berghei
infected mice, the hematocrit adjusted quantitation of 80.14 ($\pm$13.48) nmoles per $10^{10}$ is consistent with the previously reported 71 $\pm$16 for the CR strain (Fitch and Chou, 1997). Therefore, circulating CR 
P. berghei
parasites contain only 3–4% of the hemozoin content of CS strain infected blood cells. Blood film microscopic analysis is generally supportive of the long held designation of the CR strain as lacking substantial hemozoin and thus being unpigmented. However, evaluation of tissue hemozoin in liver and spleen of CR infected mice clearly shows that measurement of blood hemozoin alone is an inaccurate indication of actual hemozoin production by CR strain 
P. berghei
. This agrees with older data of a ring predominance in the blood with CR 
P. berghei
with substantial measurable schizonts in the spleen (Schneider, 1968) and liver (Jacobs and Warren, 1967). In previous studies 95% of residual hemozoin persistent for months is present in the liver and spleen of murine malaria (Levesque, 1989). On a normalized per parasite basis at the time of exsanguination CR 
P. berghei
achieves close to a 28% total hemozoin production capacity compared with the CS strain, and the CRCT 
P. berghei
is 4% of
Sanchez et al., 2009). In *P. falciparum* from 4 to 10 μmoles of hemozoin is reportedly formed per 10^10 late trophozoite stage parasites (Orjih and Fitch, 1993; Zhang et al., 1999). Gligorijevic reports up to 15 μmoles produced by the late trophozoite to early schizont transition in *P. falciparum* with little productivity difference seen between CR and CS strains (Gligorijevic et al., 2006). This is supported by Zhang and Ginsburg while a somewhat older measurement of 9.60 μmoles per 10^10 CS versus 5.15 for CR has also been reported (Orjih and Fitch, 1993).

Roughly 50% higher chloroquine dosage is required to impart an equivalent level of inhibition than when CS *P. berghei* grows in the reticulocyte (Dei-Cas et al., 1984). Clearly, CR strain specific factors in conjunction with reticulocyte specific host cell conditions contribute to resistance. An important morphological difference between sensitive and resistant *P. berghei* is the digestive vacuole. In CS parasites abundant intracellular hemozoin crystals are seen in a single digestive vacuole while 2 or 3 smaller vacuoles generally devoid of pigment are usually present in CR strains (Saxena et al., 1989). Ultrastructural studies indicate disruption of the normal cytosomal tubal system in drug resistant parasites with only small, double membrane cytoplasmic food vesicles derived by micropinocytosis; only in infected mature erythrocytes does both micropinocytosis and cytosomal ingestion occur (Slomianny et al., 1985; Slomianny, 1990; Slomianny and Prensier, 1990). Paradoxically, the rate of hemoglobin catabolism appears higher in *P. berghei* infected reticulocytes than mature erythrocytes despite the lack of a cytosomal system. This counters the notion that the reported lack of free heme and pigment in CR *P. berghei* can be explained by a failure of the CR strain to digest hemoglobin resulting from access to some alternative reticulocyte-specific nutrient source. While hemoglobin catabolism rates are comparable in rat reticulocytes infected with either CS or CR *P. berghei*, CR infected reticulocytes contained less total heme which is apparently coupled to a net loss of heme from the CR infected cell (Wood and Eaton, 1993). This is in agreement with the observation that free heme is absent from CR and CRCT parasites (Fitch and Chou, 1997).

Slomianny originally proposed that hemozoin is generally not seen accumulated intracellularly in CR *P. berghei* because it is expelled from the developing reticulocyte via exosomal release (Slomianny et al., 1985). As demonstrated by LDMS, optical spectrometry, tissue histology and FEISEM, significant accumulation of hemozoin in liver and spleens of CR *P. berghei* infected mice but not the uninfected mice is consistent with CR *P. berghei* producing significant hemozoin. We noted the hemozoin was 70% smaller in width of a single face in the CR *P. berghei*. A major limitation of our study is not being able to measure hemozoin production with equal numbers of CR and CS *P. berghei* grown *ex vivo* in culture, which would provide a side by side cellular comparison in addition to the organ quantification reported here.

Bannister and coworkers also published microscopic evidence of exosomal export vesicles in *P. falciparum* (Bannister et al., 2004). Exosomes from *Plasmodium yoelii* infected reticulocytes contain parasite derived components suggesting *Plasmodium* species can access the host cell’s export process (Martin-Jaular et al., 2011). Subversion of the native exocytosis pathway might provide parasites with a resistance mechanism whereby chloroquine, known to bind heme and hemozoin, can be expelled from the cell along with pigment. Exocytosis is a known drug resistance mechanism in other types of eukaryotic cells (Azmi et al., 2013). Alternatively, there could be sequestration of late trophozoites and schizonts in the tissues, which mask blood stage hemozoin. We do see trophozoites from bloodstage CR and CRCT parasites and isolated hemozoin from these mice is detectable by mass spectrometry, and biochemical means with a small amount seen in blood films.

Hemozoin-associated monooacylglycerides such as MPG have been implicated as catalyzing heme crystal formation (Egan et al., 2006) (Ambele et al., 2013). Here, saturated monooacylglycerides observed in close association with sucrose purified hemozoin isolated from blood stage parasites, namely MPG and MSG, were conserved in CS and CR *P. berghei* (Fig. 6). That these neutral lipids

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Fig. 7. Photomicrographs of Geimsa stained blood smears from: CS *P. vivax* Sal 1 (7A), CR *P. vivax* Ind (7C), and photomicrographs of the same fields taken under polarizing light: CS *P. vivax* Sal 1 (7B), CR *P. vivax* Ind (7D). Total magnification — 1000x.
are consistently associated with hemozoin and occur at relatively stable ratios in both \textit{P. falciparum} and \textit{P. berghei} suggests a conserved mechanism for hemozoin formation may exist (Pisciotto et al., 2007). Neutral lipid compositional differences probably do not primarily mediate chloroquine resistance (Supplementary Fig \textit{2}–4). This is consistent with this the morphological uniformity in the crystal structure seen between hemozoin purified from the CS, CR and CRCT infected mice (Fig. \textit{4}). Insufficient material was available to determine if neutral lipids are associated with \textit{P. vivax} hemozoin.

Literature comparing hemozoin production in CR and CS strains of \textit{P. vivax} of is lacking. Here, we observed CR \textit{P. vivax} produced 72\% of the hemozoin compared to CS \textit{P. vivax}, although the monkey sample size was low. This suggests the mechanism of chloroquine resistance in \textit{P. vivax} may differ from \textit{P. berghei}. Considering the asynchronous nature of \textit{P. vivax}, the hemozoin yields of 3–4 \textmu moles per 10^{10} parasites suggest \textit{P. vivax} produces hemozoin at close to the same rate as \textit{P. falciparum}.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jpddr.2017.02.001.

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