Dramatic consequences of reducing erythrocyte membrane cholesterol on *Plasmodium falciparum*

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Abstract

During its blood stage development, the malaria parasite establishes an active cholesterol gradient across the various membrane systems within the infected erythrocyte. We have observed certain antimalarial compounds to disrupt cholesterol homeostasis in intraerythrocytic stages of *Plasmodium falciparum*. Previously, reduction of cholesterol from the erythrocyte membrane by treatment with methyl- β -cyclodextrin (M β CD) was shown to inhibit parasite invasion and growth. In addition, M β CD treatment of trophozoite stage *P. falciparum* was shown to result in parasite expulsion from the host cell. We have revisited these phenomena by using live video microscopy, ultrastructural analysis, and response to antimalarial compounds. By using time-lapse video microscopy of fluorescently tagged parasites, we show that M β CD treatment for just 30 min causes dramatic expulsion of the trophozoite stage parasites. This forceful expulsion occurs within 10 sec. Remarkably, the plasma membrane of the host cell from which the parasite has been expelled does not appear to be compromised. The parasitophorous vacuolar membrane (PVM) continued to surround the extruded parasite, but the PVM appeared damaged. Treatment with antimalarial compounds targeting PfATP4 or PfNCR1 prevented M β CD-mediated extrusion of the parasites, pointing to a potential role of cholesterol dynamics underlying the expulsion phenomena. We also confirmed the essential role of erythrocyte plasma membrane cholesterol for invasion and growth of *P. falciparum*. This defect can be partially complemented by cholesterol and desmosterol but not with epicholesterol, revealing stereospecificity underlying cholesterol function. Overall, our studies advance previous observations and reveal unusual cell biological features underlying cholesterol depletion of infected erythrocyte plasma membrane.

Keywords:

Malaria, *Plasmodium falciparum*, parasite extrusion, cholesterol dynamics, PfATP4 and PfNCR1 inhibitors, vomocytosis

Introduction:

A salient feature of malaria parasites is their dependence on the host to fulfil their nutrient requirements. In addition to various nutrients, *Plasmodium* salvages lipids and fatty acids from the host. While the parasite possesses pathways for synthesizing and modifying lipids (1, 2), it lacks the machinery for *de novo* cholesterol synthesis (3-6). Unlike the intrahepatic stages where the parasites have access to copious amounts of cholesterol (7), blood stage parasites can only access cholesterol present in the erythrocyte membrane (8). Recent studies from our laboratory have identified antimalarials that inhibit two parasite plasma membrane (PPM) transporters, PfATP4 and PfNCR1, which disrupt cholesterol and lipid homeostasis in the PPM (9, 10). Upon treatment with these compounds, there is a rapid accumulation of cholesterol in the PPM rendering the parasites sensitive to the cholesterol dependent detergent saponin. This was observed as the loss of cytosolic proteins in drug treated parasites exposed to saponin. This effect was reversible indicating an active mechanism of cholesterol dynamics within the parasite (9). These unexpected consequences of exposure to novel antimalarials suggest the presence of mechanisms that influence cholesterol transport within intraerythrocytic *P. falciparum*. Previously, it was shown that intra-erythrocytic *P. falciparum* growth requires a supply of fatty acids that cannot be substituted with lipids and cholesterol from serum derived lipoproteins (11). Furthermore, studies from the Halder laboratory have shown that cholesterol in the erythrocyte plasma membrane is essential for invasion and growth of *P. falciparum*. Interestingly, reduction of cholesterol from the erythrocyte plasma membrane by treatment with M β CD resulted in extrusion of the trophozoite stage parasites (12).

Given the observations regarding effects of new antimalarials on cholesterol dynamics and the consequences of cholesterol reduction from infected erythrocyte plasma membrane, we wished to examine a potential link between these phenomena. We have used live fluorescence video microscopy, transmission electron microscopy and treatment with antimalarials to revisit previous findings regarding the consequences of M β CD treatment of intra erythrocytic *P. falciparum*. Results from our experiments confirm and extend these observations and raise important mechanistic questions regarding the role of cholesterol in *P. falciparum* biology.

Results:

Dramatic expulsion of trophozoite stage parasites upon treatment with MβCD

A previous study showed that treatment of trophozoite stage parasites with MβCD releases the parasites from the erythrocyte. In this study, the authors used filipin and ethidium bromide staining of glutaraldehyde-fixed parasites and did not assess disposition of PVM and PPM in extruded parasites (13). We aimed to examine this effect in real-time using time lapse video microscopy as well as to assess the disposition of PVM and PPM. For this purpose, we used two different transgenic *P. falciparum* lines, one expressing PPM-localized PfVP1 tagged with mNeonGreen, and the other expressing PVM-localized EXP2 tagged with mRuby3 as well as the RhopH complex protein RhopH2 tagged with mNeonGreen. RhopH2 is initially stored in merozoite rhoptries and secreted into the parasitophorous vacuole (PV) during invasion, eventually trafficking to the host cell membrane (14). The use of these parasite lines permitted us to monitor the PPM, PVM and rhoptries of the parasite when exposed to MβCD. The trophozoites and early schizont stage-infected erythrocytes were attached to poly-L-lysine coated plates, stained with fluorescent probes for the RBC membrane and nuclei followed by treatment with MβCD for 30 mins. MβCD was removed by washing with the culture medium and the parasites were observed by live time-lapse fluorescent video microscopy. As shown in Fig. 1A and Supplementary Video 1, the parasite extruded out of the host erythrocyte in a dramatic fashion. This forceful transition from being intracellular to being extracellular occurred in less than 10 seconds. The extruded parasites were enclosed within the PPM (in green, Fig. 1B) as well as the PVM (in red, Fig. 1C). Depending upon the plane from which the parasite emerges from the erythrocyte, parts of the PVM were observed still tethered to the host cell. The extrusion phenomenon was limited to mature stage parasites. Cholesterol depletion of ring stage parasites did not result in extrusion; RhopH2 was partially localized to the erythrocyte plasma membrane in the ring stage parasites, but the parasite remained inside the host cell (Fig. 1D, Supplementary video 3). Quantification of this phenomenon showed that about 70% of the mature stage parasites extruded upon MβCD treatment (Fig. 1E).

The forceful expulsion of the parasite from the host cell remains mechanistically unexplained. MβCD treatment of mammalian cells has been shown to affect actin polymerization (15). Thus one possibility could be that rearrangement and polymerization of RBC cytoskeletal actin may underlie the parasite extrusion. To test this, we treated trophozoite-infected erythrocytes with the actin polymerization inhibitor, Cytochalasin D (CytD) (16). Treatment with CytD prior to MβCD treatment did not inhibit MβCD mediated extrusion of the parasites. Treatment with CytD alone did not cause the parasites to extrude out; however, we did observe defects in the PVM of the CytD treated parasites (Fig. 1F, Supplementary video 2). Although CytD did affect the morphology of the PVM, it failed to prevent extrusion of the parasites when exposed to MβCD.

To assess the disposition of membranes of extruded parasites in some detail, we performed transmission electron microscopy of mature stage parasites treated with MβCD. The extruded parasites were still surrounded by the PVM, but the PVM was compromised at multiple places (Fig. 1G). The PPM on the other hand remained intact with minimal changes in the intracellular structures. Images shown here are representative of multiple electron micrographs.

Prior Treatment with novel antimalarials abrogates MβCD mediated parasite extrusion

We have previously shown that inhibition of PPM transporters, PfATP4 or PfNCR1 alter cholesterol dynamics in the PPM of mature stage parasites (9, 10, 17). We therefore examined the effects of these cholesterol homeostasis disruptors on MβCD-mediated extrusion phenomenon. We treated mature stage parasites for 2 hours with KAE609 (a PfATP4 inhibitor), MMV009108 (a PfNCR1 inhibitor) or chloroquine (a heme detoxification inhibitor) followed by cholesterol depletion with MβCD. Interestingly, the parasite extrusion was greatly reduced by prior exposure to PfATP4 and PfNCR1 inhibitors but not by chloroquine (Fig. 2A). Quantitation of this phenomenon showed that 80% of parasites treated with DMSO or chloroquine extruded following MβCD treatment, whereas only about 25% did so following treatment with PfATP4 and PfNCR1 inhibitors (Fig. 2B). This quantitation was also supported by observation of intracellular and extracellular parasites in Giemsa-stained thin blood smear (Fig. 2C and D). These results suggest that disruption of normal

cholesterol dynamics in mature stage parasite results in inhibition of M β CD mediated extrusion of parasites.

Role of erythrocyte plasma membrane cholesterol in invasion and the intra-erythrocytic development of *P. falciparum*

Previous studies have shown that reduction of erythrocyte plasma membrane cholesterol by treatment with M β CD resulted in inhibition of parasite growth (12). Since we found that the ring stage parasites were not extruded from the erythrocyte by M β CD treatment, we went on to examine the effects of M β CD treatment of ring stage parasites for their intra-erythrocytic developmental cycle (IDC). In addition, we also examined the consequence of complementing cholesterol depleted ring stage parasites with different sterols such as cholesterol, desmosterol or epicholesterol (structures shown in Fig. 3A). We treated the ring stage infected erythrocytes with M β CD for 30 mins followed by washes with normal culture medium. Development of ring stage parasites was assessed by examination of Giemsa-stained thin smears. As shown in Fig. 3B, parasites in the control cells proceeded normally through the IDC and formed trophozoites and rings. In contrast, ring stage parasites growing in cholesterol depleted erythrocytes (M β CD treated) progressed to form what appeared to be trophozoites but failed to mature. Reconstitution with M β CD saturated with cholesterol (CD/Cho) or desmosterol (CD/Des) appeared to restore normal IDC progression. On the other hand, reconstitution with epicholesterol (CD/Epi) did not restore normal IDC, suggesting the importance of stereospecificity of cholesterol polar moiety in supporting parasite development. We also attempted to reconstitute with lanosterol or β -sitosterol but could not assess it since these resulted in lysis of erythrocytes. To assess the viability of the ring stage parasites growing in M β CD-treated or sterol-reconstituted erythrocytes, we split the culture 1:10 into untreated normal erythrocytes. This was followed by measuring parasitemia over a subsequent four-day period. As shown in Fig 3C, ring stage parasites growing in cholesterol-depleted or epicholesterol-reconstituted cells failed to propagate over the next two cycles. On the other hand, cholesterol- or desmosterol-reconstituted ring stage parasites were able to propagate at a level of about 50% compared to the control ring stage parasites. To assess progression of ring stage parasites, we measured hypoxanthine incorporation

over a 24-hour period. As shown in Fig 3D, there was significant reduction in hypoxanthine incorporation in M β CD treated and epicholesterol reconstituted parasites, whereas ring stage parasites in cholesterol or desmosterol reconstituted cells were able to incorporate hypoxanthine.

We examined the parasites by transmission electron microscopy at 24 and 48 hours in control and following M β CD treatment. As expected at 24 hours the trophozoites in the control group displayed normal morphology with distinctive internal structures and normal hemozoin formation and went on to form ring stages at 48 hours (Fig 3E, top panel; Supplementary Fig. 1). In contrast M β CD treated ring stages showed abnormal morphology with diminished food vacuoles at 24 hours. These parasites failed to develop into normal schizonts and did not egress at 48 hours after M β CD treatment. (Fig 3E, lower panel; Supplementary Fig 1). These results suggest a defect in normal trophozoite development in erythrocytes with depleted cholesterol content.

We also assessed the ability of merozoites to invade and establish infection in M β CD treated erythrocytes. M β CD treated erythrocytes were added to Percoll enriched schizonts. As shown in Fig 4A, reduced cholesterol content in uninfected erythrocytes prevented merozoite invasion. The merozoites appeared to remain attached to the surface of M β CD treated erythrocytes but failed to penetrate. Importantly, reconstitution of M β CD treated RBCs with cholesterol complemented this invasion defect (Fig. 4B). Furthermore, the parasites were able to grow in these reconstituted erythrocytes and undergo normal IDC progression. Splitting the cultures 1:10 with addition of normal erythrocytes resulted in continued normal IDC progression of parasites from control and cholesterol reconstituted cells but not from cholesterol deficient cells (Fig. 4C).

Different *P. falciparum* strains invade using different pathways: a sialic acid dependent or a sialic acid independent pathway (18, 19). To see if cholesterol in the erythrocyte membrane is selective for a given pathway, we added schizonts of either NF54 (sialic acid dependent) or Dd2 (sialic acid independent) strains to M β CD treated erythrocytes. Merozoites from neither strain were able to invade M β CD treated erythrocytes (Fig. 4D).

Discussion:

Using live video microscopy of fluorescently tagged parasites, we have extended previous observations showing expulsion of trophozoite stage *P. falciparum* upon treatment with M β CD. We also showed that this expulsion is inhibited by new antimalarial compounds that disrupt cholesterol homeostasis in the parasite. The dramatic expulsion of the trophozoite stage parasites following M β CD treatment remains unexplained. It is hard to perceive how an intracellular parasite can escape out of the host erythrocyte without causing the lysis of the host membrane in response to reduction in the cholesterol content in the erythrocyte plasma membrane. The force with which the expulsion appears to occur suggest an active process mediated either by the host or the parasite. Our observation that Cytochalasin D did not prevent expulsion of trophozoites indicates that actin polymerization is not required for this process. Osmotic pressure build-up through solute uptake in trophozoites resulting in their release from the host cells has been widely used for synchronization of blood stage *P. falciparum*. However, this requires incubation with high concentrations of solutes such as sorbitol. At 5 mM concentration, M β CD exposure does not pose an osmotic challenge, and at ~1300 Da molecular weight M β CD would not be taken up by the trophozoite-infected erythrocyte through its new permeability pathway. These observations suggest the possibility of a hitherto unrecognized process through which the parasite might sense cholesterol content of the erythrocyte membrane. M β CD-mediated expulsion of the parasite resembles expulsion of fungal pathogens from the mammalian cells through a process termed vomocytosis wherein the expulsion does not result in lysis of the host cell (20-22). However, vomocytosis occurs over a period of hours whereas the expulsion of the trophozoite happens in seconds, suggesting that these two phenomena occur through distinct mechanistic processes.

Inhibition of the parasite expulsion by compounds that inhibit PfATP4 or PfNCR1 was unexpected; this inhibition was not a result of parasite demise since chloroquine failed to do so. Since we have previously shown that these inhibitors induce cholesterol accumulation in the PPM in a reversible manner, we can envision dynamic movement of cholesterol between various membranes of infected erythrocytes. Recent investigations have also supported the notion of cholesterol gradients in

infected erythrocytes (23-27). Reduction of the plasma membrane cholesterol content, thus, appears to be sensed by the parasite in a manner that results in the expulsion of trophozoites.

The erythrocyte plasma membrane contains large amounts of cholesterol (50 mole% of total lipids). Upon infection by *Plasmodium*, the PVM acquires cholesterol but the PPM remains highly deficient in cholesterol. Indeed, this fact is the basis for widely used saponin mediated freeing of intra-erythrocytic parasites. Previous studies have suggested the importance of cholesterol for *P. falciparum* growth and invasion (12, 13, 24). A recent study using lattice light scattering microscopy demonstrated the importance erythrocyte membrane remodeling for the formation of the PVM, a process in which cholesterol is likely to play a significant role [11]. We observed that that ring stage parasites growing in cholesterol deficient erythrocytes fail to fully progress. Furthermore, the digestive vacuoles of trophozoite-like parasites at 24 hours after MβCD treatment of ring stages had unusual morphology (Fig.3E and Supplementary figure 1). At 48 hours after MβCD treatment of ring stages, parasites appeared to attempt segmentation without being able to form mature merozoites (Fig. 3E and Supplementary figure 1). One possible explanation could be that cholesterol dynamics assist lipid and/or fatty acid import to sustain parasite growth. The fact that cholesterol and desmosterol (but not epicholesterol) could substantially rescue these defects suggest stereospecificity in the role of cholesterol to sustain parasite growth. Further studies will be required to gain mechanistic insights into cholesterol dynamics and their contribution to parasite growth.

Methods:

Parasite lines and culture conditions

Experiments were carried out using different *P. falciparum* tagged lines. 1) the 3D7 strain in which endogenous PfVP1 gene was tagged with mNeon green (mNG) at the C-terminal end (PfVP1-mNG) (28). This line was used for monitoring the dynamics of the parasite plasma membrane; 2) the NF54 strain in which endogenous RhopH2 gene was tagged with mNG and

the endogenous Exp2 gene tagged with mRuby3 (NF54 Rhoph2/Exp2). Cloning was carried out with Infusion (Clontech) and NEBuilder HiFi (NEB). To generate an endogenous EXP2-mRuby3 fusion, 5' and 3' homology flanks targeting the 3' end of *exp2* were PCR amplified from plasmid pyPM2GT-EXP2-mNG (29) using primers

CACTATAGAACTCGAGGGAGAAACAATCTTTTATATAAAATGTACAGAGTTTGA
AAG and

TCCTCCACTTCCCCTAGGTTCTTTATTTTCATCTTTTTTTTCATTTTAAATAAATC
TCCAC and inserted between *XhoI* and *AvrII* sites in the plasmid pbPM2GT (30). The mRuby3 coding sequence was then amplified from plasmid pLN-HSP101-SP-mRuby3(31) using primers GATGAAAATAAAGAACCTAGGGGAAGTGGAGGAGTG and

TAACTCGACGCGGCCGTCCTTGTACAGCTCGTCCATGCC and inserted between *XhoI* and *AvrII*, resulting in the plasmid pbEXP2-mRuby3. This plasmid was linearized at the *AflIII* site between the 3' and 5' homology flanks and co-transfected with pUF-Cas9-EXP2-CT-gRNA into NF54^{attB}::HSP101^{DDD} (31). Parasites were maintained in 10 µM trimethoprim (TMP) to stabilize the HSP101^{DDD} fusion and selection with 2.5 µg/ml Blasticidin S was applied 24 hours post transfection. A clonal line bearing the EXP2-mRuby3 fusion was derived by limiting dilution after parasite returned from selection and designated NF54^{attB}::HSP101^{DDD}+EXP2-mRuby3.

For generation of an endogenous RhopH2-mNG fusion, a gRNA target site was chosen upstream of the *rhoph2* stop codon (TCTTCACTGATTTCTTTGTA) and the gRNA seed sequence was synthesized as a sense and anti-sense primer pair (sense shown)

TAAGTATATAATATTTCTTCACTGATTTCTTTGTAGTTTGTAGAGCTAGAA, annealed and inserted into the *AflIII* site of the plasmid pAIO3 (32), resulting in the plasmid pAIO3-RhopH2-CT-gRNA. To integrate mNG at the 3' end of *rhoph2*, a 5' homology flank (up to but not including the stop codon) was amplified from NF54^{attB} genomic DNA using primers

256 AATTTTCATCATTATGAAAGTTCTCAGCTTAAGAAGCATATATTAAGAATATAGTT
257 TCAGA and
258 CCTCCACTTCCCCTAGGACTGCTCTTCAGAATATACAGGTTTTTTTATAAGATCCTC
259 CGATATCTCCTTATATGGATCAGATATATCTGAGAAA, incorporating several
260 synonymous mutations in the seed sequence of the gRNA target site within the *rhoph2*
261 coding sequence. A 3' homology flank (beginning at the endogenous stop codon) was
262 amplified using primers
263 GTGACACTATAGAACTCGAGTAAACGTTAAAAAAAAAAATATATATAAGGAGAAA
264 GCACTG and
265 TCTGAAACTATATTCTTAATATATGCTTCTTAAGCTGAGAACTTTCATAATGATGA
266 AATT, assembled in a second PCR reaction using primers
267 GTGACACTATAGAACTCGAGTAAACGTTAAAAAAAAAAATATATATAAGGAGAAA
268 GCACTG and
269 CCTCCACTTCCCCTAGGACTGCTCTTCAGAATATACAGGTTTTTTTATAAGATCCTC
270 CGATATCTCCTTATATGGATCAGATATATCTGAGAAA and inserted between *XhoI*
271 and *AvrII* sites in pyPM2GT-EXP2-mNG (29) resulting in the plasmid pyPM2GT-RhopH2-
272 mNG. This plasmid was linearized at the *AflIII* site between the 3' and 5' homology flanks
273 and co-transfected with pAIO3-RhopH2-CT-gRNA into NF54^{attB}::HSP101^{DDD}+EXP2-
274 mRuby3. Selection with 2 μ M DSM-1 was applied 24 hours post transfection (along with 10
275 μ M trimethoprim). After parasites returned from selection, a clonal line containing the EXP2-
276 mRuby3 and RhopH2-mNG fusions was obtained by limiting dilution and designated
277 NF54^{attB}::HSP101^{DDD}+EXP2-mRuby3+RhopH2-mNG. This line was used for monitoring the
278 parasitophorous vacuolar membrane and to mark the parasite. *P. falciparum* parasites were
279 cultured in O⁺ human blood from Interstate Blood Bank, TN in RPMI 1640 supplemented
280 with 2g/L sodium bicarbonate, 10mg/L hypoxanthine, 15mM HEPES, 50mg/L gentamycin

281 sulphate, 0.5% AlbuMax II. Parasite culture was maintained at 2.5% hematocrit at 37 °C in
282 90% N₂, 5% CO₂, 5% O₂.

284 **Preparation of MβCD and MβCD-sterol complexes**

285 Parasites were treated with 5mM MβCD diluted in appropriate medium from a stock solution
286 of 25mM MβCD (Catalog no. AC377110250) in PBS in appropriate medium. Sterols
287 [Cholesterol (Sigma, C3045-25G) , Desmosterol (Steraloids, catalog no. C3150-000),
288 Epicholesterol (Steraloids, catalog no. C6730-000)] were loaded onto MβCD according to
289 previously published protocol (33). Briefly, 10 μL of 15mg/ml sterols dissolved in ethanol
290 were added to 500 μL 5% w/v MβCD, heated for 10 mins at 80°C and mixed by inverting
291 several times until the solution was clear. The above step was repeated 4 times to add a total
292 of 50μL sterol stock solution. The solution was heated until the sterols were stably
293 incorporated in MβCD (clear solution). MβCD/sterol complexes were snap frozen in dry ice
294 for 2 mins. Subsequently, the MβCD/sterol complexes were lyophilized in a speed vac until
295 all liquid had evaporated and a fluffy powder remained at the bottom of the tube and stored
296 at -20°C. Immediately before use, 375μl of medium was added to the MβCD/sterol
297 complexes and vortexed until dissolved. The MβCD/sterol complexes were sterilized using
298 0.22μm syringe filter.

300 **Treatment of parasitized erythrocytes with MβCD and reconstitution with** 301 **MβCD/sterol complexes**

302 Parasite cultures were synchronized by treatment with 0.5M alanine for 10 minutes.
303 Erythrocyte infected with ring stage parasites were treated with 5mM MβCD for 30 mins at
304 37°C and washed 3 times with pre-warmed RPMI1640 media. Hematocrit was maintained at
305 2.5% throughout the culture and washing steps. The parasites were returned to normal culture

conditions. In experiments involving reconstitution with M β CD/sterol complexes, parasites were first treated with M β CD as described above. Following this, the cells were incubated with 1:7 dilution of M β CD/sterol complexes for 30 mins. Parasites were washed 3 times and returned to normal culture conditions. Giemsa-stained thin blood smears were prepared 24 and 48 hours after the treatment with M β CD or after reconstitution with M β CD/sterol complexes. The culture at 48 hours was split 1:10 in normal erythrocytes. Growth of the parasites in these cultures were followed for 4 days by examining Giemsa stained thin blood smears

Hypoxanthine incorporation of ring stage erythrocyte treated with M β CD or M β CD/sterol complexes

Ring stage erythrocyte (1.5% parasitemia) were treated with M β CD for 30 mins and washed 3 times with normal medium. The hematocrit was maintained at 2.5%. M β CD-treated ring stage erythrocytes were then treated with either M β CD/cholesterol, M β CD/desmosterol or M β CD/epicholesterol for 30 mins and washed 3 times with low hypoxanthine medium. The hematocrit was then adjusted to 1.5% by addition of low hypoxanthine medium to the cells. 200 μ l of cells were then plated in triplicates in a 96-well plate. Each well was pulsed with 22 μ l of 0.5 μ Ci/ml 3 H-hypoxanthine (PerkinElmer NET 177) and incubated for 24 hours at 37°C in 5% CO₂, 5% O₂, 90% N₂ chamber. Parasites were lysed by freeze/thaw and were collected on filters using a cell harvester (PerkinElmer LifeSciences). This was followed by addition of MicroScint O scintillation cocktail and incorporation of 3 H-hypoxanthine was measured using a TopCount scintillation counter (PerkinElmer Life-sciences).

Parasite invasion in M β CD or M β CD/Cholesterol treated erythrocytes

We treated 500 μ l of 50% hematocrit erythrocytes with 5mM M β CD for 30 mins followed by 3 washes. In addition, similarly treated erythrocytes were reconstituted with M β CD/Cholesterol for 30 mins followed by 3 washes. Synchronized late stages trophozoites were enriched by centrifugation over a 70% Percoll cushion (3000 RPM, 20 mins) and washed thrice with medium. Enriched late stage trophozoites were added to M β CD-treated, M β CD/Cholesterol-reconstituted and control erythrocytes. Giemsa-stained thin blood smears were prepared 24 and 48 hours following the addition. The culture at 48 hours was split 1:10 in normal erythrocytes. Growth of the parasites in these cultures were followed for 4 days by examining Giemsa-stained thin blood smears.

Live microscopy of parasites

Glass bottom culture dishes (35 mm) were coated with 0.1% poly-L-lysine overnight and washed 3 times with PBS. Parasite culture (250 μ l at 2.5% hematocrit) was added to the dishes and incubated for 30 mins to allow attachment. Culture dishes were washed 3 times with PBS followed by addition of medium. For M β CD extrusion experiments with NF54 Rhoph2/Exp2 line, all washes, incubation and imaging was done in medium supplemented with TMP. Parasites were attached to poly-L-lysine coated plates and incubated in RPMI containing wheat germ agglutinin-Alexa 350 (WGA-350) (Invitrogen, Catalog no. W7024) at 5 μ g/ml final concentration and incubated for 12 mins to stain the erythrocyte plasma membrane. In addition to WGA staining, PfVP1-mNG line was also stained with SYTO deep red nuclear stain for 30 min. Parasites were treated with 5mM M β CD followed by three washes with medium. The medium was replaced with phenol red free medium followed by live fluorescent microscopy. Imaging was done using the Nikon Ti microscope. WGA-Alexa-350 was visualized using DAPI filter set, mNeon green with FITC, SYTO deep red nuclear

stain with Cy5 and mRuby was visualized using TRITC filter set. Video microscopy parameters are given in the legends of Supplementary Videos.

Inhibition of MβCD mediated extrusion by novel antimalarials.

To look at the effects of antimalarials on MβCD mediated extrusion, parasites were stained with WGA-350 and SYTO deep red for PfVP-1mNG line or WGA-350 for NF54 RhopH2/Exp2 line as described above and treated with 10xEC₅₀ concentrations of Chloroquine (150nM), KAE609 (10nM) or MMV009108(1μM) for 2.5 hours prior to 30 mins treatment with MβCD and washed thrice with medium. Medium supplemented with drugs (at the concentration mentioned above) was added back to the dish and live fluorescent imaging was carried out. Quantification of parasites remaining inside/outside of erythrocytes was carried out by examining at least 100 different images of individual parasites for each experimental condition.

Cytochalasin D (Cyt-D) treatment prior to MβCD treatment of parasites

Attached NF54 RhopH2/Exp2 trophozoites were stained with WGA-350 for 12 mins, followed by three washes. The cells were then treated with 0.5μM Cyt-D for 45 mins. This was followed by the addition of 5mM MβCD for 30 mins. The culture dishes were washed 3 times and live fluorescent microscopy was performed to examine parasite extrusion. In the control condition, parasites were treated with Cyt-D without MβCD treatment.

Transmission electron microscopy of trophozoite infected MβCD treated erythrocytes

Erythrocytes infected with trophozoite stage parasites were treated with 5mM MβCD for 30 mins at 2.5% hematocrit followed by three washes with PBS and incubated in complete medium for 30 mins at 37 °C. Cells were washed once in PBS by gentle centrifugation and fixed with 2% paraformaldehyde, 2.5% glutaraldehyde in 100 mM cacodylate buffer and

were fixed for 1 hour at room temperature. Cells were gently pelleted by centrifugation, resuspended in cacodylate buffer and immediately stored on dry ice for shipment. Ring stage infected erythrocytes were treated with 5mM M β CD for 30 mins, washed thrice with PBS and returned to culture at 37 °C. At 24 and 48 hours after the treatment, infected erythrocytes were fixed and prepared for transmission electron microscopy as described above.

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References:

1. Cassera MB, Gozzo FC, D'Alexandri FL, Merino EF, del Portillo HA, Peres VJ, Almeida IC, Eberlin MN, Wunderlich G, Wiesner J, Jomaa H, Kimura EA, Katzin AM. 2004. The methylerythritol phosphate pathway is functionally active in all intraerythrocytic stages of *Plasmodium falciparum*. *J Biol Chem* 279:51749-59.
2. Gerold P, Schwarz RT. 2001. Biosynthesis of glycosphingolipids de-novo by the human malaria parasite *Plasmodium falciparum*. *Mol Biochem Parasitol* 112:29-37.
3. Holz GG, Jr. 1977. Lipids and the malarial parasite. *Bull World Health Organ* 55:237-48.
4. Maguire PA, Sherman IW. 1990. Phospholipid composition, cholesterol content and cholesterol exchange in *Plasmodium falciparum*-infected red cells. *Mol Biochem Parasitol* 38:105-12.
5. Vial HJ, Ancelin ML, Philippot JR, Thuet MJ. 1990. Biosynthesis and dynamics of lipids in *Plasmodium*-infected mature mammalian erythrocytes. *Blood Cells* 16:531-55; discussion 556-61.
6. Vial HJ, Philippot JR, Wallach DF. 1984. A reevaluation of the status of cholesterol in erythrocytes infected by *Plasmodium knowlesi* and *P. falciparum*. *Mol Biochem Parasitol* 13:53-65.
7. Labaied M, Jayabalasingham B, Bano N, Cha SJ, Sandoval J, Guan G, Coppens I. 2011. *Plasmodium* salvages cholesterol internalized by LDL and synthesized de novo in the liver. *Cell Microbiol* 13:569-86.

8. Grellier P, Rigomier D, Clavey V, Fruchart JC, Schrevel J. 1991. Lipid traffic between high density lipoproteins and *Plasmodium falciparum*-infected red blood cells. *J Cell Biol* 112:267-77.
9. Das S, Bhatnagar S, Morrissey JM, Daly TM, Burns JM, Jr., Coppens I, Vaidya AB. 2016. Na⁺ Influx Induced by New Antimalarials Causes Rapid Alterations in the Cholesterol Content and Morphology of *Plasmodium falciparum*. *PLoS Pathog* 12:e1005647.
10. Istvan ES, Das S, Bhatnagar S, Beck JR, Owen E, Llinas M, Ganesan SM, Niles JC, Winzeler E, Vaidya AB, Goldberg DE. 2019. *Plasmodium* Niemann-Pick type C1-related protein is a druggable target required for parasite membrane homeostasis. *Elife* 8.
11. Coppens I, Vielemeyer O. 2005. Insights into unique physiological features of neutral lipids in Apicomplexa: from storage to potential mediation in parasite metabolic activities. *Int J Parasitol* 35:597-615.
12. Samuel BU, Mohandas N, Harrison T, McManus H, Rosse W, Reid M, Haldar K. 2001. The role of cholesterol and glycosylphosphatidylinositol-anchored proteins of erythrocyte rafts in regulating raft protein content and malarial infection. *J Biol Chem* 276:29319-29.
13. Lauer S, VanWye J, Harrison T, McManus H, Samuel BU, Hiller NL, Mohandas N, Haldar K. 2000. Vacuolar uptake of host components, and a role for cholesterol and sphingomyelin in malarial infection. *The EMBO journal* 19:3556-3564.
14. Ahmad M, Manzella-Lapeira J, Saggu G, Ito D, Brzostowski JA, Desai SA. 2020. Live-Cell FRET Reveals that Malaria Nutrient Channel Proteins CLAG3 and RhopH2 Remain Associated throughout Their Tortuous Trafficking. *mBio* 11.
15. Guerra FS, Sampaio LdS, König S, Bonamino M, Rossi MID, Costa ML, Fernandes P, Mermelstein C. 2016. Membrane cholesterol depletion reduces breast tumor cell migration by a mechanism that involves non-canonical Wnt signaling and IL-10 secretion. *Translational Medicine Communications* 1:3.
16. Casella JF, Flanagan MD, Lin S. 1981. Cytochalasin D inhibits actin polymerization and induces depolymerization of actin filaments formed during platelet shape change. *Nature* 293:302-5.
17. Bhatnagar S, Nicklas S, Morrissey JM, Goldberg DE, Vaidya AB. 2019. Diverse Chemical Compounds Target *Plasmodium falciparum* Plasma Membrane Lipid Homeostasis. *ACS infectious diseases* 5:550-558.
18. Camus D, Hadley TJ. 1985. A *Plasmodium falciparum* antigen that binds to host erythrocytes and merozoites. *Science* 230:553-6.
19. Dolan SA, Miller LH, Wellems TE. 1990. Evidence for a switching mechanism in the invasion of erythrocytes by *Plasmodium falciparum*. *J Clin Invest* 86:618-24.
20. Cruz-Acuña M, Pacifici N, Lewis JS. 2019. Vomocytosis: Too Much Booze, Base, or Calcium? *mBio* 10.
21. Nicola AM, Robertson EJ, Albuquerque P, Derengowski Lda S, Casadevall A. 2011. Nonlytic exocytosis of *Cryptococcus neoformans* from macrophages occurs in vivo and is influenced by phagosomal pH. *mBio* 2.
22. Seoane PI, May RC. 2020. Vomocytosis: What we know so far. *Cell Microbiol* 22:e13145.
23. Fraser M, Jing W, Bröer S, Kurth F, Sander LE, Matuschewski K, Maier AG. 2021. Breakdown in membrane asymmetry regulation leads to monocyte recognition of *P. falciparum*-infected red blood cells. *PLoS Pathog* 17:e1009259.
24. Geoghegan ND, Evelyn C, Whitehead LW, Pasternak M, McDonald P, Triglia T, Marapana DS, Kempe D, Thompson JK, Mlodzianoski MJ, Healer J, Biro M,

- 464 Cowman AF, Rogers KL. 2021. 4D analysis of malaria parasite invasion offers
465 insights into erythrocyte membrane remodeling and parasitophorous vacuole
466 formation. *Nat Commun* 12:3620.
- 467 25. Hayakawa EH, Yamaguchi K, Mori M, Nardone G. 2020. Real-time cholesterol
468 sorting in *Plasmodium falciparum*-erythrocytes as revealed by 3D label-free imaging.
469 *Sci Rep* 10:2794.
- 470 26. Tokumasu F, Crivat G, Ackerman H, Hwang J, Wellems TE. 2014. Inward
471 cholesterol gradient of the membrane system in *P. falciparum*-infected erythrocytes
472 involves a dilution effect from parasite-produced lipids. *Biol Open* 3:529-41.
- 473 27. Koch M, Cegla J, Jones B, Lu Y, Mallat Z, Blagborough AM, Angrisano F, Baum J.
474 2019. The effects of dyslipidaemia and cholesterol modulation on erythrocyte
475 susceptibility to malaria parasite infection. *Malar J* 18:381.
- 476 28. Solebo O, Ling L, Zhou J, Fu T-M, Ke H. 2021. *Plasmodium*
477 *falciparum* vacuolar pyrophosphatase 1 for ring stage development and its
478 transition to trophozoite. *bioRxiv*
479 doi:10.1101/2021.10.25.465524:2021.10.25.465524.
- 480 29. Glushakova S, Busse BL, Garten M, Beck JR, Fairhurst RM, Goldberg DE,
481 Zimmerberg J. 2017. Exploitation of a newly-identified entry pathway into the
482 malaria parasite-infected erythrocyte to inhibit parasite egress. *Sci Rep* 7:12250.
- 483 30. Beck JR, Muralidharan V, Oksman A, Goldberg DE. 2014. PTEX component
484 HSP101 mediates export of diverse malaria effectors into host erythrocytes. *Nature*
485 511:592-5.
- 486 31. Glushakova S, Beck JR, Garten M, Busse BL, Nasamu AS, Tenkova-Heuser T,
487 Heuser J, Goldberg DE, Zimmerberg J. 2018. Rounding precedes rupture and
488 breakdown of vacuolar membranes minutes before malaria parasite egress from
489 erythrocytes. *Cell Microbiol* 20:e12868.
- 490 32. Nessel T, Beck JM, Rayatpisheh S, Jami-Alahmadi Y, Wohlschlegel JA, Goldberg
491 DE, Beck JR. 2020. EXP1 is required for organisation of EXP2 in the
492 intraerythrocytic malaria parasite vacuole. *Cell Microbiol* 22:e13168.
- 493 33. Luu W, Gelissen IC, Brown AJ. 2017. Manipulating Cholesterol Status Within Cells.
494 *Methods Mol Biol* 1583:41-52.

Figures

Figure 1:

Figure 1: Trophozoites extrude out from erythrocyte upon treatment with MβCD.

(A) Still frames from Supplementary video 1 from time-lapse video microscopy performed at. Parasites extrude out from the erythrocyte with the PVM still tethered to the erythrocyte membrane without causing the lysis of erythrocyte (indicated by arrows). (B) Representative images (>50) of PfV/P1- mNG line after MβCD treatment. Nucleus is stained with SYTO-deep red (Pink). (C) Representative images (>50) of NF54 RhopH2/Exp2 line after MβCD

525 treatment. Erythrocyte membrane is stained with WGA-Alexa-350 (Blue). Infected
526 erythrocytes were subjected to live fluorescent microscopy after treatment with MβCD.
527 Parasites extrude out from erythrocyte without causing lysis of the erythrocyte with their PPM
528 (B) and PVM (C) still attached to the erythrocyte as indicated by the arrows. (D) Representative
529 images of erythrocytes infected with ring stages of the NF54 RhopH2/Exp2 line treated with
530 MβCD. Ring stage parasites do not extrude out from erythrocytes. (E) Quantification of
531 parasites remaining inside/outside of erythrocytes after MβCD treatment was carried out by
532 examining at least 100 different images of individual parasites for each experimental condition
533 (100 for ring stages and 330 for trophozoites). (F) Upper panel: Treatment of NF54
534 RhopH2/Exp2 trophozoites with 0.5 μM Cyt-D for 45 mins prior to MβCD treatment does not
535 inhibit parasite extrusion. Lower panel: Treatment with 0.5 μM Cyt-D alone does not cause
536 extrusion of trophozoites but did alter PVM morphology (as indicated by arrows). (G)
537 (Left) Treatment with MβCD causes extrusion of *P. falciparum* stage trophozoites without
538 causing erythrocyte rupture unlike the control group where the trophozoite is still inside the
539 erythrocyte (Scale bar- 2μM, Direct magnification-5,000x). (Right) Zoomed in image of
540 parasite extruded out of erythrocyte after MβCD treatment shows the PVM ruptured at multiple
541 places indicated by black arrows. Electron microscopy of extruded *P. falciparum*. Left panels
542 show lower magnification views of control and MβCD-treated trophozoites. A higher
543 magnification view of an extruded parasite (right panel) shows that the PVM is compromised
544 at multiple places (arrows) (Scale bar-500nm, Direct magnification-12,000x).
545

Figure 2:

Figure 2 : Treatment with PfATP4 or PfNCR1 inhibitors prior to MβCD treatment inhibited MβCD mediated extrusion of parasites: (A) Trophozoite stage parasites from PfVP1-mNG line were treated with KAE609 (10nM), MMV009108 (1μM) and chloroquine (150nM) prior to treatment with MβCD. Live fluorescent imaging showed that parasite extrusion was inhibited by prior treatment with KAE609 and MMV009108 (indicated by yellow arrows) while treatment with chloroquine like MβCD did not inhibit this extrusion (indicated by white arrows). Nucleus is stained with SYTO-deep red (pink). Erythrocyte membrane is stained with WGA-Alexa-350 (Blue). Representative images from 3 independent experiments for each condition (B) Quantification from live fluorescent microscopy experiments of parasite extrusion upon drug treatment followed by MβCD treatment. Approximately 250 cells for each experimental condition over 4 independent experiment were assessed. (C) Giemsa stained thin

blood smears prepared from cultures after treatment with KAE609 (10nM), MMV009108 (1μM) and chloroquine (150nM) prior to treatment with MβCD. (D) Quantitation of parasite extrusion upon drug treatment followed by MβCD treatment from Giemsa-stained thin blood smears (~1000 cells were counted).

Figure 3:

Figure 3: Cholesterol in the erythrocyte membrane is essential for growth and proliferation of *P. falciparum*.

(A) Structure of M β CD, Cholesterol, Epicholesterol and Desmosterol. The differences between the structure of sterols are highlighted in red. (B) Giemsa-stained thin blood smears prepared from control, M β CD and erythrocytes reconstituted with cholesterol, desmosterol or epicholesterol at 24 and 48 hours after treatment. C) Parasitemia in 1:10 split of ring stage parasites from control, M β CD (CD) or erythrocytes reconstituted with cholesterol (CD/Cho), desmosterol(CD/Des) or epicholesterol (CD/Epi) over 4 days (~3000 cells were counted). Based on one tailed unpaired *t*-test (* $p < 0.05$). (D) Relative (^3H)-hypoxanthine incorporation by ring stage parasites over a 24 hour period following treatment with M β CD or reconstituted with cholesterol, desmosterol and epicholesterol. Based on unpaired t-test with Welch's correction (* $p < 0.05$, *** $p < 0.001$). Error bars=mean $\pm$ SD N=8. (E) TEM of the ring stage erythrocyte treated with 5mM M β CD and imaged after 24 or 48 hours. (Left)After 24 hours, in erythrocyte treated with M β CD, parasites are unable to form normal trophozoites. The nuclear membrane is indistinct, hemozoin is dispersed (indicated by arrows) and food vacuole is small compared to control erythrocytes. (Right) After 48 hours, parasites in the control culture were able to invade the erythrocyte and form rings while the parasites in the M β CD treated

Figure 4:

Figure 4: Reducing the cholesterol content in the erythrocyte membrane inhibits *P. falciparum* invasion

(A) Giemsa stained thin blood smears from control and MβCD treated erythrocytes after 24 hours. While ring stages were seen in the control erythrocytes, no invasion was seen in the MβCD treated erythrocytes but merozoites appeared to remain attached to the surface as indicated by arrows. (B) Percoll enriched trophozoites were added to erythrocytes treated with MβCD or reconstituted with cholesterol and giemsa stained thin blood smears were prepared at 24 and 48 hours. Reconstituted erythrocytes were able to support parasite growth. (C) Parasitemia from 1:10 spilt of culture from MβCD treated and reconstituted with cholesterol in normal erythrocytes over a period of 4 days (~3000 cells were counted). Based on Mann-Whitney test (* $p < 0.05$) (D) Percoll synchronized trophozoites from NF54 and Dd2 strains

were added onto MβCD treated erythrocytes. Parasitemia from 1:10 split of these cultures by examining Giemsa-stained thin blood smears (~1000 cells were counted).

Supplementary Videos

Supplementary video 1: Treatment with MβCD causes extrusion of trophozoites from erythrocytes

Representative time lapse video microscopy of the NF54 RhopH2/Exp2 line after MβCD treatment. Erythrocyte membrane is stained with WGA-Alexa-350 (Blue). Infected erythrocytes were subjected to live fluorescent microscopy after treatment with MβCD. Parasites extrude out from erythrocyte without causing lysis of the erythrocyte. Video was acquired using the Nikon Ti microscope. The interval between each frame was 4 sec and the video was captured for a total 20 min (93 frames, FPS:0.09, frames 59-64 shown in the video). Images were deconvoluted by Richardson Lucy algorithm using the Nikon NIS elements software package and converted to .avi files.

Supplementary video 2: Cyt-D treatment does not inhibit MβCD mediated parasite extrusion

Representative time lapse video microscopy of the NF54 RhopH2/Exp2 line after MβCD treatment. Erythrocyte membrane is stained with WGA-Alexa-350 (Blue). Infected erythrocytes were subjected to live fluorescent microscopy after treatment with Cyt-D followed by treatment with MβCD. Treatment with Cyt-D does not inhibit MβCD mediated extrusion of the parasite. Video microscopy was performed as mentioned above for video 1. The interval between each frame was 31 sec and the video was captured for a total 5 min (11 frames, FPS:0.04, all 11 frames shown in the video)

Supplementary movie 3: Treatment with M β CD does not cause extrusion of ring stage parasites

Erythrocytes infected with ring stages of the NF54 RhopH2/Exp2 were treated with M β CD .

Erythrocyte membrane is stained with WGA-Alexa-350 (Blue). Ring stage parasites do not extrude out after M β CD treatment. Video microscopy was performed as mentioned for video

1. The interval between each frame was 31 secs and the video was captured for a total 5 mins (11 frames, FPS:0.04, all 11 frames shown in the video)

Supplementary Figure 1:

653 **Supplementary figure 1:** TEM of the ring stage erythrocyte treated with 5mM MβCD and
 654 imaged after 24 (Day 1) or 48 hours (Day2)
 655 (A) After 24 hours, the trophozoite like parasites in the MβCD treated cultures (lower panel)
 656 have indistinct membranes, compromised food vacuole and dispersed hemozoin compared to
 657 the controls (Upper panel) (B) After 48 hours, in the control culture (Upper panel) the
 658 parasites invaded and formed rings while the parasites in the MβCD treated cultures (Lower
 659 panel) failed to form mature merozoites (Scale bar-500nm, Direct magnification-15,000x).