Isoprenoid Biosynthesis Inhibition Disrupts Rab5 Localization and Food Vacuolar Integrity in *Plasmodium falciparum*

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The antimalarial agent fosmidomycin is a validated inhibitor of the nonmevalonate isoprenoid biosynthesis (methylerythritol 4-phosphate [MEP]) pathway in the malaria parasite, *Plasmodium falciparum*. Since multiple classes of prenyltransferase inhibitors kill *P. falciparum*, we hypothesized that protein prenylation was one of the essential functions of this pathway. We found that MEP pathway inhibition with fosmidomycin reduces protein prenylation, confirming that *de novo* isoprenoid biosynthesis produces the isoprenyl substrates for protein prenylation. One important group of prenylated proteins is small GTPases, such as Rab family members, which mediate cellular vesicular trafficking. We have found that Rab5 proteins dramatically mislocalize upon fosmidomycin treatment, consistent with a loss of protein prenylation. Fosmidomycin treatment caused marked defects in food vacuolar morphology and integrity, consistent with a defect in Rab-mediated vesicular trafficking. These results provide insights to the biological functions of isoprenoids in malaria parasites and may assist the rational selection of secondary agents that will be useful in combination therapy with new isoprenoid biosynthesis inhibitors.

Severe malaria due to infection with the protozoan parasite *Plasmodium falciparum* has a significant impact on global health (1). Infections with *P. falciparum* contribute nearly 1 million deaths per year (2). Malaria control efforts are hampered by resistance to existing antimalarial agents, particularly chloroquine (3, 4). Clinical resistance to the more recently introduced artemisinin-based therapies has already been reported, highlighting the ongoing need to identify and exploit new targets for antimalarial drug development (5, 6).

Isoprenoid biosynthesis is a promising antimalarial drug target. Unlike mammalian cells, plasmodia do not use the classically described metabolic route via mevalonate. Instead, the malaria parasite produces isoprenoids through a mevalonate-independent pathway, which proceeds through a different key metabolite, methylerythritol 4-phosphate (MEP) (7, 8). *P. falciparum* requires isoprenoid biosynthesis through the MEP pathway during intraerythrocytic development, the stage of parasite growth responsible for the clinical symptoms of malaria. The genetic locus for the first dedicated enzyme of this pathway (deoxyxylulose-5-phosphate reductoisomerase [DXR]) is resistant to genetic disruption in *P. falciparum* and the related apicomplexan *Toxoplasma gondii*, and chemical inhibition of the MEP pathway by the small molecule fosmidomycin is lethal to malaria parasites (8–10). Fosmidomycin treatment of cells inhibits two enzymes of the MEP pathway (DXR and methylerythritol phosphate cytidyltransferase [IspD]), and growth inhibition by fosmidomycin is rescued by supplementation with downstream isoprenoids, such as isopentenyl pyrophosphate and geranylgeraniol (11, 12). Altogether, these studies validate the MEP pathway as an antimalarial drug target and establish the specificity of fosmidomycin as a chemical probe to address isoprenoid biology in *P. falciparum*.

Isoprenoids comprise a diverse class of cellular molecules, with over 20,000 natural isoprenoids described (13). The pathogenic stage of *P. falciparum* occupies a highly unusual ecological niche within human red blood cells and has several peculiar metabolic features that make it unclear which isoprenoids are essential in *P. falciparum.* For example, although isoprenoids contribute to membrane stability (as cholesterol), the malaria parasite acquires cholesterol from host cells and does not synthesize sterols *de novo* (14). In contrast, both ubiquinone biosynthesis and protein prenylation appear to be required for *P. falciparum* development. Ubiquinone, derived from isoprenoids, is an electron carrier and a necessary cofactor for the *P. falciparum* pyrimidine biosynthesis enzyme dihydroorotate dehydrogenase (DHODH), which is vital for malaria parasite growth (15). Protein prenylation is the posttranslational modification of proteins, such as small GTPases, with either farnesyl (15-carbon) or geranylgeranyl (20-carbon) isoprenyl groups. Isoprenyl moieties are covalently attached to C-terminal cysteines by one of three well-characterized prenyltransferases, which are expressed during the intraerythrocytic cycle (16, 17). Multiple classes of prenyltransferase inhibitors kill the malaria parasite, strongly suggesting that protein prenylation is an essential function of isoprenoid biosynthesis in malaria (18–21).

In our approach, we used the isoprenoid biosynthesis inhibitor fosmidomycin to address the role of protein prenylation as an essential function of isoprenoids in *P. falciparum*. In these studies, we confirm that ubiquinone is not the sole required isoprenoid in malaria parasites. We demonstrate that *de novo* isoprenoid biosynthesis via the MEP pathway generates the isoprenyl precursors for protein prenylation and that nonprenylated proteins are mislocalized upon fosmidomycin treatment. Finally, we demonstrate that inhibition of isoprenoid biosynthesis causes a late developmental arrest and vesicular trafficking defect in malaria parasites, consistent with a loss of protein prenylation.

MATERIALS AND METHODS

Materials. All buffer components, salts, and enzyme substrates were purchased from Sigma, unless otherwise indicated.

***Plasmodium falciparum* culture and strains.** *P. falciparum* strains were cultured *in vitro* in human erythrocytes, as described previously (12), with the following modifications: a 5% O_2–5% CO_2–90% N_2 atmosphere in RPMI 1640 medium supplemented with 27 mM sodium bicarbonate, 11 mM glucose, 5 mM HEPES, 1 mM sodium pyruvate, 0.37 mM hypoxanthine, 0.01 mM thymidine, 0.25 mg/ml gentamicin (Goldbio), and 0.5% Albumax (Invitrogen). The following strains were obtained from the Malaria Research and Reference Reagent Resource Center (MR4): wild-type strain 3D7 (MRA-102), D10 ACP-(leader)-GFP (MRA-568 [22]), and D10 ACP-(signal)-GFP (MRA-570 [22]). The following strains were kindly provided by Akhil Vaidya (Drexel University, Philadelphia, PA) (23): parental clone D10 and transgenic D10+pHHyDHOD-GFP (which heterologously expresses yeast DHODH [yDHODH]). The following strain was kindly provided by Daniel Goldberg (Washington University, St. Louis, MO): 3D7+pPlasmepsin-II-GFP (24).

Flow cytometric analysis. Cultures were treated twice with a 5% (wt/vol) D-sorbitol solution during ring-stage growth to produce a >90% synchronized culture. Each culture was resynchronized 24 h later by magnetic separation (MS) with MS columns and a MiniMACS separator (Miltenyi Biotech) to remove early-stage parasites, as previously described (25, 26). Giemsa-stained smears were used to monitor growth. Cell cycle analysis was performed using >95% synchronized *Plasmodium falciparum* 3D7 cultures. Four independent strains were split to 1% parasitemia (percentage of infected erythrocytes), and each was divided into four separate treatment groups (no drug, 5 μM fosmidomycin [Invitrogen], 5 μM fosmidomycin plus 5 μM geranylgeraniol, or 5 μM geranylgeraniol). At each time point, 100 μl of each culture was fixed with an equivalent volume of fixative solution (8% paraformaldehyde–0.015% glutaraldehyde in phosphate-buffered saline [PBS]). DNA content was determined by staining with the fluorophore acridine orange (1.5 μg/ml; Invitrogen), followed by resolution on a BD-FACS flow cytometer, as previously described (27). Data analysis was performed by using FlowJo (TreeStar Inc., Ashland, OR) and Prism 5 (GraphPad Software, La Jolla, CA) software.

Immunoblot analysis. *P. falciparum* 3D7 parasites were grown to approximately 10% parasitemia, synchronized by treatment with sorbitol as described above. Newly invaded ring-stage parasites were treated with or without 5 μM fosmidomycin for 24 h. Host erythrocytes were lysed with 0.1% saponin, and parasites were harvested by centrifugation, washed with PBS, and then stored at −80°C until use. Parasite pellets were lysed by sonication in lysis buffer (10% glycerol, 0.1% Triton X-100, 20 mM HEPES [pH 7.5], 150 mM sodium chloride, and protease inhibitors [Complete EDTA-free tablets; Roche]). Immunoblotting was performed as previously described, using rabbit antifarnesyl polyclonal antibody at a 1:500 dilution (Abcam, Cambridge, MA), rabbit anti-*Plasmodium falciparum* EIF1α antibody (kindly provided by Daniel Goldberg, Washington University [28]) at a 1:3,000 dilution, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin polyclonal antibody at a 1:10,000 dilution (Invitrogen).

Microscopic analysis. *P. falciparum* parasites were synchronized by treatment with sorbitol as described above. Newly invaded ring-stage parasites were treated with or without 5 μM fosmidomycin, 5 μM fosmidomycin plus 5 μM geranylgeraniol, or 5 μM wortmannin. Live cells were visualized as previously described (24), and images of live cells and Giemsa-stained slides were obtained with an Olympus BH8 microscope. Live cells were stained with Lysotracker Red DND-99 (LR) (Molecular Probes) at 75 nM for 15 min, washed in an equal volume of fresh culture medium, and visualized immediately for no longer than 15 min. For immunofluorescence microscopy, cells were fixed in 4% paraformaldehyde with or without 0.0075% glutaraldehyde and stored at 4°C. Fixed parasites were permeabilized, blocked, and incubated with antibodies, as previously described (29). Rabbit polyclonal antibodies against recombinant *P.*

falciparum Rab5a (Pf-Rab5a) and Pf-Rab5c were kindly provided by Gordon Langsley (Institut Cochin, France) (30) and were used at 1:500 and 1:1,000 dilutions, respectively. The secondary antibody used was Alexa Fluor 488 goat anti-rabbit IgG(H+L) (catalog number A11008; Invitrogen) at a 1:500 dilution, and Hoechst 33258 was used as a nuclear counterstain. Images were obtained with an Olympus Fluoview FV1000 confocal microscope. All images were analyzed by using ImageJ software (31). Minimal adjustments in brightness and contrast were employed and were applied equally to all samples.

For ultrastructural analysis, *Plasmodium*-infected red blood cells were fixed in 1% glutaraldehyde (Polysciences Inc., Warrington, PA)–1% osmium tetroxide (Polysciences Inc.) in 50 mM phosphate buffer (pH 7.2) for 1 h at 4°C. This low-osmolarity fixation was used to remove dense, soluble cytoplasmic components, allowing unobscured membrane analysis. Cells were washed in phosphate buffer and rinsed extensively in distilled water (dH_2O) prior to *en bloc* staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 h. Following several rinses in dH_2O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 90 nm were cut, stained with uranyl acetate and lead citrate, and viewed on a Jeol 1200 EX transmission electron microscope (Jeol USA, Peabody, MA) at an accelerating voltage of 80 kV.

RESULTS

Electron transport bypass does not confer resistance to inhibition of isoprenoid biosynthesis. *De novo* isoprenoid biosynthesis through the MEP pathway is an essential metabolic pathway in malaria parasites. Because this pathway produces a large number of compounds, we are interested in which of these is required for development of the malaria parasite, *P. falciparum*. The isoprenoid ubiquinone is an electron acceptor for mitochondrial electron transport and is a necessary cofactor for the essential pyrimidine biosynthesis enzyme dihydroorotate dehydrogenase (DHODH). The cytochrome bc_1 complex of the electron transport chain is the target of the antimalarial agent atovaquone (32). Malaria parasites engineered to heterologously express a yeast dihydroorotate dehydrogenase (yDHODH) homolog, which does not require a ubiquinone cofactor, can survive without mitochondrial respiration and are resistant to atovaquone (23). Ubiquinone biosynthesis is therefore expected to be one of the essential functions of isoprenoids in malaria parasites.

In order to determine whether there are other essential functions of isoprenoids, we evaluated whether yDHODH expression also conferred resistance to inhibition of isoprenoid biosynthesis, using the validated MEP pathway inhibitor fosmidomycin. We obtained a strain of *P. falciparum* that heterologously expresses yDHODH. We independently confirmed that this strain is resistant to atovaquone, as was previously shown (data not shown) (23). We compared the fosmidomycin concentration that inhibited 50% of growth (IC_{50}) at 3 days between the yDHODH-expressing strain and its parental control line. The yDHODH-expressing strain was as sensitive to fosmidomycin as the control parasite line (mean IC_{50} of 1.2 μM [95% confidence interval {CI}, 0.66 to 2.3], compared to the mean IC_{50} of the wild-type strain, 0.88 μM [95% CI, 0.67 to 1.14]) (Fig. 1). Since expression of yDHODH does not confer fosmidomycin resistance, ubiquinone production is not likely to be the only essential function of isoprenoid biosynthesis in malaria parasites.

Inhibition of isoprenoid biosynthesis reduces protein prenylation. We evaluated whether inhibition of *de novo* isoprenoid biosynthesis by fosmidomycin, which should reduce the concentration of isoprenyl precursors, would reduce protein prenylation.
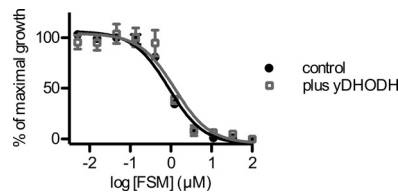
FIG 1 Bypass of electron transport does not confer fosmidomycin resistance. Shown are data for growth inhibition by the isoprenoid biosynthesis inhibitor fosmidomycin (FSM) in *P. falciparum* parasites (control) compared to parasites that heterologously express yeast dihydroorotate dehydrogenase, which does not require ubiquinone (control + yDHODH). Results are the means and standard deviations from three independent biological replicates.

We analyzed whole protein lysates from parasites grown with and without fosmidomycin (5 μM, approximately five times the IC_{50}) by immunoblotting with a rabbit polyclonal antifarnesyl antibody that recognizes both farnesyl and geranylgeranyl groups. Previous experiments in which *P. falciparum* was metabolically labeled with [³H]farnesol demonstrated a characteristic protein banding pattern of two dominant prenylated bands, one at 45 kDa and the other at 20 to 25 kDa (16, 33). We found that immunoblotting of parasite lysates with antifarnesyl antibody recapitulated this banding pattern, consistent with the identification of these bands as prenylated proteins (Fig. 2). We used a densitometric evaluation of band intensities to compare prenylation with and without fosmidomycin treatment. The intensity of antifarnesyl staining of the ~25-kDa lower band was reduced by 97% following fosmidomycin treatment (normalized to the density of control Pf-EF1α immunoblotting of the same blot). The intensity of the upper 45-kDa band, the identity of which is unknown, was reduced by 30% following fosmidomycin treatment. These results indicate that the isoprenyl moieties used for protein prenylation are derived from *de novo* isoprenoid biosynthesis in *P. falciparum* and that inhibition of isoprenoid biosynthesis reduces protein prenylation.

Rab GTPases are mislocalized when isoprenoid biosynthesis is inhibited. Fosmidomycin inhibited protein prenylation and particularly reduced prenylation of proteins of around 25 kDa. The malaria parasite expresses at least 11 small GTPases with predicted molecular masses of between 23 and 27 kDa, each of which is predicted to be geranylgeranylated (34, 35). These proteins likely comprise the dominant band that labels with [³H]farnesol and are recognized by antifarnesyl antibodies at 25 kDa (16). Since protein prenylation is typically required for the localization and function of small GTPases, such as Rab5 family members, we examined the localization of two candidate geranylgeranyltransferase substrates, Pf-Rab5a and Pf-Rab5c. Rab5 proteins are prototypical markers of early endosomal vesicles in most eukaryotic cells, and Pf-Rab5a was previously localized by immunoelectron microscopy to small hemoglobin-containing vesicles in *P. falciparum* (36). Pf-Rab5a and Pf-Rab5c were dispersed in punctae throughout malaria parasite cells (Fig. 3). Upon fosmidomycin treatment, a dramatic mislocalization of Rab5a occurred in the majority of treated cells, such that Rab5a was no longer present within the parasite cell but instead was found at the membrane of the host erythrocyte. Similarly, fosmidomycin treatment mislocalized Rab5c, which became diffusely localized throughout both the parasite cell and the host erythrocyte cytoplasm. Previous studies demonstrated that the antimalarial effects of fosmidomycin are rescued by medium supplementation with the downstream
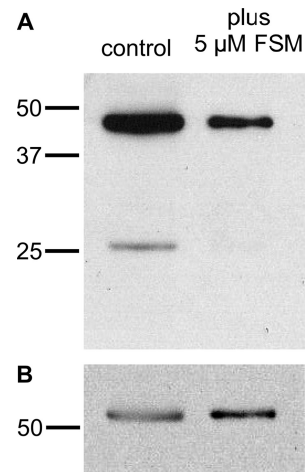

FIG 2 Fosmidomycin treatment inhibits protein prenylation. (A) Antifarnesyl immunoblot of extracts with or without treatment with 5 μM fosmidomycin (FSM) for 24 h. (B) Blot from panel A reprobed with antibodies to Pf-EIF1α to indicate equivalent protein loading. Results are representative of at least three independent biological replicates.

isoprenol geranylgeraniol, which we confirmed (see Fig. S1 in the supplemental material) (11, 12). Localizations of both Rab5a and Rab5c were restored by medium supplementation with geranylgeraniol, demonstrating that this effect of fosmidomycin was also due to inhibition of isoprenoid biosynthesis. To quantify these observations, a series of independent representative images (>50 cells under each condition) were scored on a scale of 1 to 3 (where 1 represents a typical intracellular localization and 3 represents primarily erythrocyte membrane staining) by a trained observer who was blinded to the treatment conditions (Fig. 3C). The cellular distributions of Pf-Rab5a and Pf-Rab5c were significantly altered upon fosmidomycin treatment ($P < 0.001$ for each [*t* test]).

Inhibition of isoprenoid biosynthesis causes developmental arrest during schizogony. Our data indicate that the MEP pathway generates the isoprenyl groups for protein prenylation in *P. falciparum* and that candidate prenylated proteins are mislocalized upon fosmidomycin treatment. We next evaluated the biological effects of isoprenoid depletion on *P. falciparum* to assess whether the phenotype of fosmidomycin-treated parasites was consistent with a loss of prenylated protein function. We examined gross effects on parasite development using Giemsa staining and light microscopy of parasites with or without fosmidomycin treatment. Within the erythrocyte host cell, untreated *P. falciparum* parasites mature from newly invaded parasites with a typical ring morphology into multinucleated schizonts. This is followed by egress as merozoites and reinvasion, in a cycle that typically takes approximately 48 h. We found that fosmidomycin-treated parasites have normal gross development over the first 24 h but arrest in the first cell cycle as schizonts, after the onset of nuclear division but prior to segmentation of daughter merozoites (Fig. 4). To quantify the stage of this arrest, we generated highly synchronized parasite cultures and monitored DNA content throughout the development of each culture using acridine orange staining and flow cytometry. At the initial time point (time zero), each culture was highly enriched for ring-stage parasites (>95% of total cells), which had not yet begun DNA replication
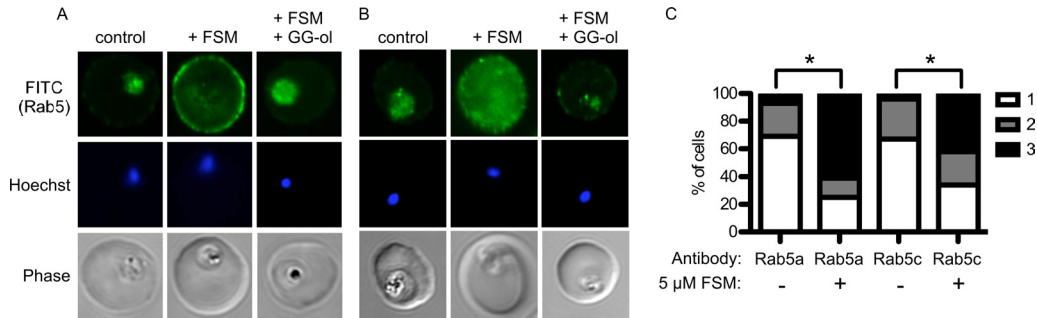
FIG 3 Mislocalization of Rab5 proteins by fosmidomycin treatment. (A and B) Confocal immunofluorescence with either anti-PfRab5a (A) or anti-PfRab5c (B) antibody in untreated parasites (control) compared to fosmidomycin-treated (+FSM) and fosmidomycin- and geranylgeraniol-treated (+FSM +GG-ol) parasites. FITC, fluorescein isothiocyanate. (C) Blinded scoring of >50 cells under each condition for severity of mislocalization of Rab5 (1, typical cellular punctae within parasite; 2, partial mislocalization to erythrocyte or erythrocyte membrane; 3, severe mislocalization to erythrocyte or erythrocyte membrane). *, $P < 0.001$ compared to untreated conditions.

(pre-S phase). Control and geranylgeraniol-treated parasites completed multiple rounds of DNA replication and produced daughter parasites (returning to pre-S phase). In contrast, the majority (>80%) of fosmidomycin-treated parasites entered S phase but failed to develop further (Fig. 5). These effects of fosmidomycin were reversed by medium supplemented with a downstream isoprenol, geranylgeraniol (Fig. 5), confirming that they are specific to isoprenoid blockade. Fosmidomycin-treated parasites arrest with an average DNA content of approximately 9.1 ± 1.0 n (where n refers to the haploid DNA content of ring-stage parasites), compared to control parasites, which contain an average maximum DNA content of 15.6 ± 1.3 n (see Fig. S2 in the supplemental material).

Inhibition of isoprenoid biosynthesis disrupts food vacuolar morphology. Because Rab GTPases are required for vesicular trafficking in most eukaryotic cells, we further examined the ultrastructural effects of isoprenoid biosynthesis inhibition on *P. falciparum* parasites. Highly synchronized cultures (enriched for ring-stage parasites) were cultured with or without fosmidomycin and then fixed at the indicated time points. Typical cellular features of *P. falciparum* include nuclei, mitochondria, apicoplast
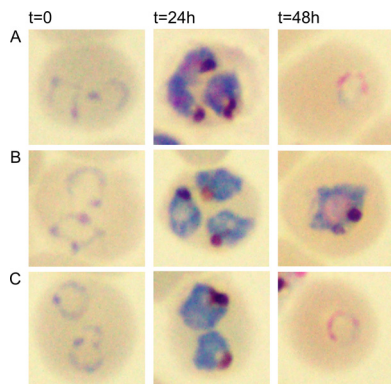
FIG 4 Fosmidomycin treatment causes growth arrest of malaria parasites during schizogony. Shown are Giemsa-stained light micrographs of synchronized ring-stage parasites at the indicated time points of culture for comparison of untreated parasites (A) to those treated with 5 μM fosmidomycin (B) or 5 μM fosmidomycin plus 5 μM the downstream isoprenol geranylgeraniol (C). Images of representative cells are indicative of results from at least five independent biological replicates.

organelles, as well as the food vacuole (FV), where hemoglobin digestion occurs. We found that fosmidomycin-treated parasites were ultrastructurally indistinguishable from control parasites at early stages of development through early trophozoite stages (through 16 h of drug treatment [data not shown]), including normal formation and development of the digestive FV. By 24 h of treatment, however, fosmidomycin-treated parasites began to demonstrate morphological abnormalities of the FV (Fig. 6). A typical malarial FV contains a single focus of hemozoin pigment crystals (the final product of heme detoxification from hemoglobin digestion) within a distinct food vacuolar membrane. In contrast, fosmidomycin-treated parasites demonstrated a variety of abnormal FV morphologies. In less severely affected parasites, these FVs appeared as multiple discontiguous vacuoles. In more severely affected parasites, the vacuolar membrane was absent, and hemozoin crystals were free within the cellular cytoplasm.

Inhibition of phosphatidylinositol 3-kinase function also disrupts food vacuolar morphology. The morphological effects of fosmidomycin-treated *P. falciparum* suggest a defect in hemoglobin trafficking in these cells. The phosphatidylinositol 3-kinase (PI3-K) inhibitor wortmannin was previously demonstrated to inhibit *P. falciparum* PI3-K *in vitro* and inhibit hemoglobin uptake in malaria parasites (37). Since Rab GTPases often function through the activation of PI3-Ks, we hypothesized that the ultrastructural abnormalities of fosmidomycin-treated parasites would be similar to those of wortmannin-treated parasites. We found that wortmannin caused a disruption in food vacuolar morphology similar to that caused by fosmidomycin. In wortmannin-treated parasites, the FV appeared to be fragmented into multiple discontiguous vacuoles (Fig. 6C). While a number of abnormal traits characterized the FV of both fosmidomycin- and wortmannin-treated cells, the most quantifiable change was the loss of FV membrane or apparent fragmentation of a single FV into multiple FVs. A series of representative electron micrographs (>25 cells under each condition) were scored for FV morphology. Abnormal FVs were defined by an absence of FV membrane or the presence of more than one focus of hemozoin. Using these criteria, FV morphology was significantly altered upon fosmidomycin and wortmannin treatments ($P < 0.001$ for each [t test]). While the ultrastructural effects that we observed differed slightly from what was previously reported for wortmannin-treated *P. falcipa-*
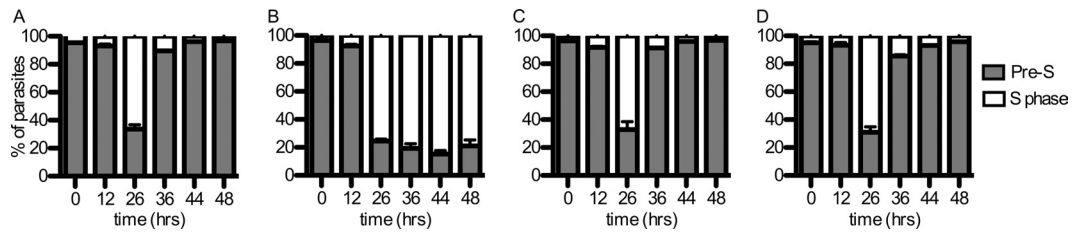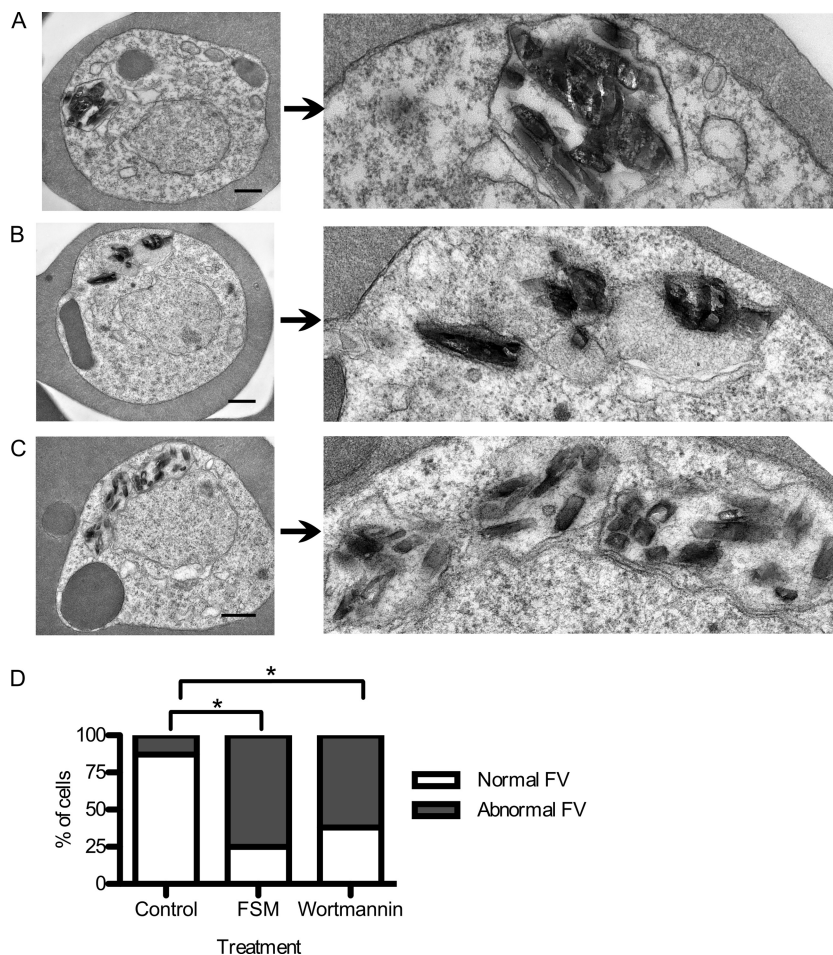
FIG 5 Fosmidomycin-treated parasites arrest during S phase. Shown are proportions of cells with unreplicated DNA (pre-S phase, morphologically early-ring-stage parasites, 12 h after invasion) compared to those in which DNA replication has begun (S phase), as determined by acridine orange staining and flow cytometric evaluation. Untreated parasites (A) are compared to fosmidomycin-treated parasites (B), fosmidomycin- and geranylgeraniol-treated parasites (C), and parasites with geranylgeraniol treatment alone (D). The cell cycle duration under these conditions is approximately 48 h; untreated parasites return to pre-S phase at 36 h.

rum, this may reflect the superior membrane preservation achieved by the low-osmolality fixation technique used here (37).

Inhibition of isoprenoid biosynthesis or PI3-K function disrupts food vacuolar integrity. Both fosmidomycin and wortmannin treatment of *P. falciparum* resulted in abnormal FV morphologies, as visualized by electron microscopy. To independently assess the integrity of the FV in fosmidomycin- and wortmannin-

treated parasites, we utilized a malaria parasite strain expressing the hemoglobin-digesting enzyme plasmepsin II tagged with green fluorescent protein (PMII-GFP). In malaria parasites, PMII-GFP is localized to the FV (24). Using confocal immunofluorescence, we confirmed that PMII-GFP was localized to the FV, which is distinguished microscopically in phase images by the presence of dark hemozoin pigment (Fig. 7, left). Following fos-

FIG 6 Food vacuolar defect in fosmidomycin- and wortmannin-treated parasites. (A to C) Transmission electron microscopic evaluation of control parasites (A) compared to parasites treated for 24 h with either the isoprenoid inhibitor fosmidomycin (B) or the PI3-K inhibitor wortmannin (C). On the right is a magnified view of a hemozoin-containing FV. (D) Scoring of electron micrographs of control versus fosmidomycin (FSM)- and wortmannin-treated cells. Abnormal FVs were defined as FVs that either lacked an FV membrane or contained more than one discontiguous membrane-bound collection of hemozoin ($n > 25$ under each condition). *, $P < 0.001$ compared to untreated conditions (Fisher's 2-tailed test).
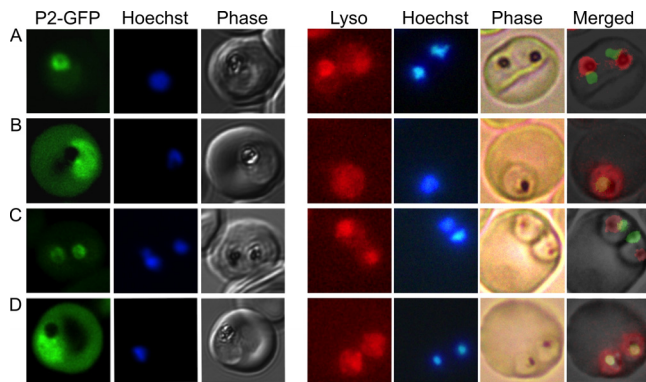
FIG 7 Loss of food vacuolar integrity upon fosmidomycin and wortmannin treatment. Shown is the confocal fluorescence microscopic localization of a plasmepsin II-GFP (P2-GFP) construct (left) or live fluorescence imaging of Lysotracker Red-stained malaria parasites (right). Control parasites (A) are compared to parasites treated for 24 h with either fosmidomycin (B), fosmidomycin plus geranylgeraniol (C), or wortmannin (D). Images are representative of at least three independent biological experiments. Visualization of PMII-GFP in FSM- and wortmannin-treated parasites required higher detector gain levels, resulting in increased observed erythrocyte autofluorescence.

midomycin treatment, PMII-GFP was no longer discretely localized within the FV. Instead, PMII-GFP was dispersed throughout the parasite cytoplasm, consistent with the physical disruption of the FV that was visualized by electron microscopy (Fig. 7). PMII-GFP localization was restored by medium supplementation with the isoprenoid geranylgeraniol, confirming that this effect of fosmidomycin is caused by an inhibition of isoprenoid biosynthesis. PMII-GFP was diffusely present throughout the cytoplasm of wortmannin-treated parasites, confirming that FVs are also disrupted upon PI3-K inhibition. Similar effects of fosmidomycin and wortmannin on FV integrity were confirmed in live parasite cells treated with Lysotracker Red (LR), a fluorophore that indicates acidic organelles. LR accumulated within the FV of control parasites but was dispersed throughout the cytoplasm of fosmidomycin- and wortmannin-treated parasites (Fig. 7, right). FV localization was restored in fosmidomycin-treated parasites that were rescued with medium supplementation with geranylgeraniol.

To examine the possibility that the mislocalization of PMII-GFP was due to general defects in protein traffic or membrane permeability, we evaluated the ability of drug-treated cells to appropriately localize two additional GFP fusion proteins with well-characterized localizations: the apicoplast localization sequence from acyl carrier protein (ACP), ACP$_L$-GFP (which traffics to the apicoplast), and the signal sequence from ACP, ACP$_s$-GFP (which is exported to the parasitophorous vacuole space) (22). Both ACP$_s$- and ACP$_L$-GFP were appropriately localized in both fosmidomycin- and wortmannin-treated cells (Fig. 8). ACP$_L$-GFP fluorescence in parasites with and without drug treatments demonstrated a typical apicoplast appearance, with a long, thin, single structure early in intraerythrocytic development (38). With and without drug treatment, ACP$_s$-GFP fluorescence was present within the parasitophorous vacuole and was frequently seen in the tubovesicular network. These results indicate that general protein trafficking mechanisms are still in place in both fosmidomycin- and wortmannin-treated cells and support that the FV phenotype and PMII-GFP localization observed in these cells are not an indirect effect of acute cellular injury.

DISCUSSION

Isoprenoid biosynthesis via the nonmevalonate (MEP) pathway is required for intraerythrocytic development of *P. falciparum* malaria parasites. Fosmidomycin-treated parasites arrest in the first cell cycle following drug treatment, in contrast to the "delayed death" that occurs in the second cell cycle of parasites treated with other apicoplast-targeting antimalarial agents (such as clindamycin and doxycycline) (34). This is not unexpected, given that isoprenoids are expected to have multiple cellular functions outside apicoplast maintenance. We find that fosmidomycin-treated parasites successfully generate digestive food vacuoles (FVs), initiate hemoglobin digestion, and begin DNA replication prior to developmental arrest. *De novo* isoprenoid biosynthesis therefore does not appear to be required for these complex and energy-intensive cellular tasks, and this finding indicates a narrower role for isoprenoids in malaria cellular functions than had been suspected.

Our results suggest that the biological effects of isoprenoid inhibition by fosmidomycin in *P. falciparum* are in part due to an
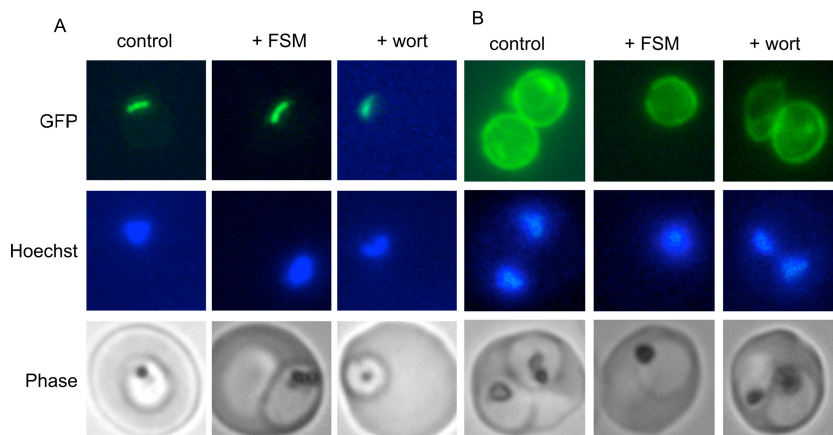
FIG 8 Apicoplast and parasitophorous vacuolar targeting in fosmidomycin- and wortmannin-treated parasites. Shown is live-cell fluorescence of malaria parasites that express either the leader sequence (ACP$_L$-GFP) (A) or signal sequence (ACP$_s$-GFP) (B) from *P. falciparum* acyl carrier protein, fused to GFP, which traffic to the apicoplast or parasitophorous vacuole, respectively (27). Untreated parasites (control) are compared to fosmidomycin (+FSM)- and wortmannin (+wort)-treated parasites. Images are representative of at least three independent biological experiments.

inhibition of protein prenylation. While the mitochondrial electron carrier ubiquinone is also likely essential, we find that malaria parasites that have been engineered to bypass the need for electron transport remain highly sensitive to fosmidomycin. Our results implicate at least one further essential function of isoprenoid biosynthesis in malaria parasites and are consistent with findings reported previously by Yeh and DeRisi (unpublished data described in reference11). We present the first evidence that the isoprenyl metabolites used in protein prenylation are derived from *de novo* isoprenoid biosynthesis, since protein prenylation is reduced by isoprenoid biosynthesis inhibition. Protein prenylation is necessary for proper protein localization and function of prenylated proteins, and indeed, we find that inhibition of isoprenoid biosynthesis causes a dramatic mislocalization of two candidate prenyltransferase substrates (Rab5a and Rab5c). Finally, inhibition of isoprenoid biosynthesis causes food vacuolar morphological defects that are consistent with a disruption of protein prenylation. Inhibition of protein prenylation directly with prenyltransferase inhibitors causes a more rapid cell cycle arrest than we observed (16); however, this is consistent with our previously reported findings that the levels of isoprenoid precursors do not change for 6 h following fosmidomycin treatment (12).

The intraerythrocytic stage of *P. falciparum* depends on digestion of host cell hemoglobin as its source for most amino acids (27). Hemoglobin is transported from the host cell to a specialized acidic digestive structure, the FV, where proteolysis and heme detoxification occur. Hemoglobin trafficking to the FV is a complicated, actin-dependent process, the molecular details of which have not been fully elucidated (36, 39, 40). The Rab family of small GTPases is likely to be important for this process. There are at least 11 Rab family homologs in the *P. falciparum* genome (41). Of these, Rab5 homologs in particular are important for early endocytosis in other eukaryotes, including other protozoans, such as the related apicomplexan *Toxoplasma gondii* as well as *Leishmania donovani* and *Entamoeba histolytica* (42–44). In *P. falciparum*, Rab5a, a prototypical early endosomal marker, has been localized to small hemoglobin-containing vesicles by immunoelectron microscopy, suggesting that these are endocytic vesicles en route to the FV (36).

Activated GTP-bound Rab5s typically function through activation of phosphatidylinositol-3 kinases (PI3-Ks) to increase local levels of phosphatidylinositol 3-phosphate [PI(3)P]. Although casein kinase physically associates with Rab5b, the downstream effectors of Rab5s in malaria are not known (30). A single essential, wortmannin-sensitive PI3-K has been described for *P. falciparum*, which can synthesize PI(3)P, PI(3,4)P_2, and PI(3,4,5)P_3 (35, 37). PI(3)P is the dominant phosphorylated phosphatidylinositol in malaria-infected erythrocytes and appears to have a large number of functional roles in malaria parasites (35). Using specific PI(3)P-binding proteins, PI(3)P has been localized to the food vacuole, the apicoplast, and the endoplasmic reticulum in malaria parasites (35, 45, 46). Surprisingly, PI(3)P binding by export signals appears to be required for malaria protein export to the host cell, and PI3-K itself is exported to the host erythrocyte (37, 45).

The growth arrest and morphological changes of fosmidomycin-treated malaria parasites may reflect multiple cellular insults. Decreased protein prenylation upon fosmidomycin treatment is likely to result in mislocalization of multiple prenyltransferase substrates, including all Rab family members. For example, a loss of Rab5 prenylation and defective trafficking to the FV could ex-
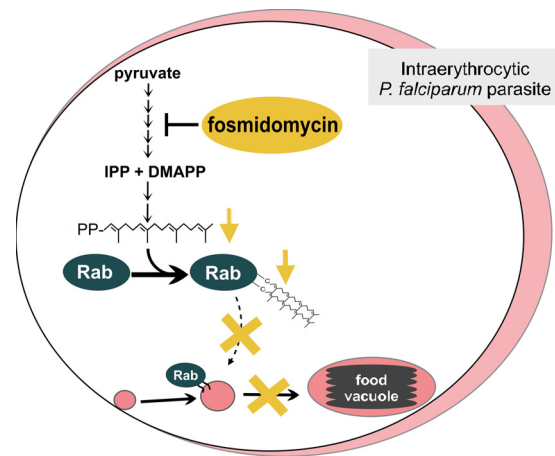
FIG 9 Model of fosmidomycin effects on malaria parasites. Fosmidomycin blocks isoprenoid biosynthesis and causes a defect in growth and vesicular trafficking to the food vacuole (FV). These effects are rescued by geranylgeraniol (GG-ol), indicating that the essential isoprenoids in malaria are metabolically derived from geranylgeranyl pyrophosphate (GG-PP). GG-PP is the substrate for geranylgeranyltransferase (GGTase), which modifies the endocytosis regulator Rab5 (a small GTPase) in most eukaryotes. Blocking of isoprenoid biosynthesis (with fosmidomycin) decreases protein prenylation, causes Rab5 mislocalization, and alters FV morphology. IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate.

plain the ultrastructural defects observed for fosmidomycin-treated parasites (graphically represented in Fig. 9). Expression of a constitutively active Rab5a (Q102L) allele produces a bloated, enlarged FV, the opposite effect of what we observed upon fosmidomycin treatment and Rab5 mislocalization (36). In addition to the effects on food vacuolar morphology that we observed, fosmidomycin was also previously reported to interrupt apicoplast development in *P. falciparum* (9). The lipid PI(3)P has been localized to both the apicoplast and food vacuole, and PI3-K inhibition with wortmannin disrupts apicoplast development (35, 47) and alters food vacuolar morphology (this study). While PI(3)P signaling and isoprenoid biology are both complex, these phenotypic similarities suggest that the two pathways could converge and are consistent with the hypothesis that PI3-K may be a Rab5 effector in *P. falciparum*.

Upon fosmidomycin treatment, both Rab5a and Rab5c are found within the host erythrocyte. This mislocalization does not appear to be due to general membrane permeability, since other protein constructs are retained within the parasite plasma membrane or parasitophorous vacuole (PMII-GFP [Fig. 7] and ACP$_L$-GFP and ACP$_s$-GFP [Fig. 8]). Since neither Rab5a nor Rab5c is predicted to contain a host-targeting (HT) or *Plasmodium* export element (PEXEL) motif, the localization of these proteins to the host cell suggests an alternate mechanism of export that is unmasked when the proteins are not tethered by prenylation. Further experimentation is required to determine whether there is a population of Rabs in *P. falciparum* that have biological functions within the host erythrocyte under normal growth conditions.

As novel inhibitors are developed to target nonmevalonate isoprenoid biosynthesis, an understanding of the biological consequences of isoprenoid biosynthesis inhibition in *P. falciparum* may inform the rational selection of secondary agents that will be useful in combination therapy. For example, since fosmidomycin-treated parasites arrest prior to completion of DNA synthesis,

small molecules that act on parasite egress, such as PfSUB1 protease inhibitors (48), may be less useful alongside inhibition of isoprenoid biosynthesis. Our results suggest that parasite-specific prenyltransferase inhibitors may be particularly useful in combination antimalarial therapy with isoprenoid biosynthesis inhibitors.

ACKNOWLEDGMENTS

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