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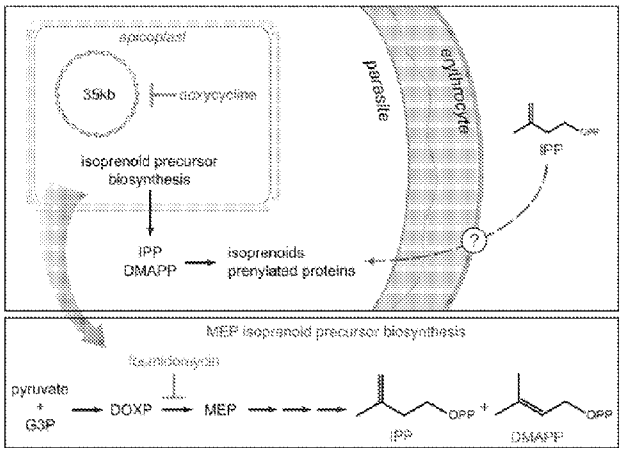
(54) Title: ATTENUATED STRAINS OF PLASMODIUM



(57) Abstract: The present disclosure relates to blood-stage malaria parasites. In particular, the present disclosure provides viable malarial parasites that lack an apicoplast as a consequence of conditions under which that are grown.

FIG. 7

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ATTENUATED STRAINS OF *PLASMODIUM*

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 61/467,834, filed March 25, 2011, which is hereby incorporated by reference in its entirety.

FIELD

[0002] The present disclosure relates to blood-stage malaria parasites. In particular, the present disclosure provides viable parasites that lack an apicoplast as a consequence of conditions under which they are grown.

BACKGROUND

[0003] Malaria is a life-threatening disease caused by *Plasmodium* parasites transmitted by the bite of infected *Anopholes* mosquitoes. Upon transmission in mosquito saliva, the parasites multiply in the liver before infecting red blood cells. Parasite replication in red blood cells causes lysis of the cells, and the characteristic symptoms of malaria (fever, headache, chills and vomiting).

[0004] Malaria is widespread in tropical and subtropical regions of sub-Saharan Africa, Asia and Latin America. According to the World Health Organization, there were nearly 250 million cases of malaria and nearly one million deaths in 2008. *Plasmodium* resistance to chloroquine and mosquito-resistance to dichlorodiphenyltrichloroethane (DDT) undermined early efforts to irradicate malaria. Growing parasite resistance to newer antimalarials, which is encouraged by the premature cessation of monotherapies, threatens to become a major public health problem.

[0005] Thus, the art needs vaccines and next generation antimalarials to reduce the prevalence of malaria parasites, and the morbidity and mortality caused by infection of humans.

SUMMARY

[0006] The present disclosure relates to blood-stage malaria parasites. In particular, the present disclosure provides viable parasites that lack an apicoplast as a consequence of conditions under which they are grown.

[0007] The present disclosure provides methods for producing a live, apicoplast-deficient *Plasmodium* parasite, comprising: i) treating erythrocytes infected with apicoplast-containing *Plasmodium* parasites with an apicoplast inhibitor to produce an apicoplast inhibitor-treated cell

culture; and ii) supplementing the apicoplast inhibitor-treated cell culture with isopentenyl pyrophosphate (IPP) to produce a live, apicoplast-deficient *Plasmodium* parasite. In some embodiments, the live, apicoplast-deficient *Plasmodium* parasite is of a species selected from the group consisting of *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale, Plasmodium knowlesi, Plasmodium chaubaudi, Plasmodium berghei*, and *Plasmodium yoelii*. In some preferred embodiments, the live, apicoplast-deficient *Plasmodium* parasite is a *Plasmodium falciparum* parasite. In some embodiments, the apicoplast inhibitor is selected from the group consisting of fosmidomycin, chloramphenicol, doxycycline, ciprofloxacin, trovofloxacin, rifampicin, rifabutin, clindamycin, azithromycin, spiramycin, thiostrepton, micrococcin, tetracycline, amythiamicin, thiolactomycin, cerulenin, clodinafop, quizlofop, haloxypop, fenoxaprop, tralkoxydim, diclofop, and FR-900098. In some embodiments, the apicoplast inhibitor is selected from the group consisting of fosmidomycin, chloramphenicol, and doxycycline. In some preferred embodiments, steps i) and ii) of the method are repeated at least once or twice, for at least three rounds of antibiotic treatment and IPP supplementation. In some embodiments, the live apicoplast-deficient *Plasmodium* parasite has an apicoplast genome to nuclear genome ration of less than 0.50, 0.25., 0.20, 0.15, 0.10, or 0.05. Also provided by the present disclosure are a live, apicoplast-deficient *Plasmodium* parasite obtained by these methods, as well as an isolated erythrocyte infected with the live, apicoplast-deficient *Plasmodium* parasite. In some embodiments, the erythrocyte is an O-negative human erythrocyte. In some embodiments, a composition comprising a live, apicoplast-deficient *Plasmodium* parasite or an isolated erythrocyte infected with the live, apicoplast-deficient *Plasmodium* parasite is provided. In some preferred embodiments, the composition is a vaccine further comprising an adjuvant. In some embodiments, the adjuvant is selected from the group consisting of mineral salts (e.g., aluminum hydroxide or "alum", aluminum phosphate, calcium phosphate, etc.), oil emulsions (e.g., MF59), particulate adjuvants (e.g., liposomes), microbial derivatives (e.g., monophosphoryl lipid A, CpG motifs, modified toxins, etc.), and plant derivatives (e.g., saponins such as QS-21).

[0008] In addition, the present disclosure provides methods for inducing an immune response in a mammalian subject to a *Plasmodium* parasite, comprising: i) providing a pharmaceutical formulation comprising: a) a live, apicoplast-deficient *Plasmodium* parasite; or b) an erythrocyte infected with the live, apicoplast-deficient *Plasmodium* parasite; and ii) administering the pharmaceutical formulation to a mammalian subject in an amount effective to induce an immune response in the mammalian subject to the *Plasmodium* parasite. In some embodiments, the live, apicoplast-deficient *Plasmodium* parasite is of a species selected from the group consisting of *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae,*

Plasmodium ovale, Plasmodium knowlesi, Plasmodium chaubaudi, Plasmodium berghei, and *Plasmodium yoelii*. In some preferred embodiments, the live, apicoplast-deficient *Plasmodium* parasite is a *Plasmodium falciparum* parasite. In some embodiments, the live, apicoplast-deficient *Plasmodium* parasite is a merozoite. In other embodiments, the live apicoplast-deficient *Plasmodium* parasite is a ring-stage parasite, a trophozoite or a schizont. In some embodiments, the mammalian subject is selected from the group consisting of a human, a monkey and a mouse. In some embodiments, the erythrocyte is an O-negative human erythrocyte. In some preferred embodiments, administration of the pharmaceutical formulation results in a transient infection of the subject's erythrocytes by the apicoplast-deficient *Plasmodium* parasite. In some embodiments, the immune response comprises one or more of a *Plasmodium* parasite-specific antibody response, cell proliferation, and cytokine secretion. In some embodiments, the immune response comprises a helper T cell response. The present disclosure further provides a pharmaceutical formulation comprising an effective amount of a live, apicoplast-deficient *Plasmodium* parasite or an erythrocyte infected with the live, apicoplast-deficient *Plasmodium* parasite, for inducing an immune response in a mammalian subject to the *Plasmodium* parasite. Additionally the present disclosure provides a live, apicoplast-deficient *Plasmodium* parasite or an erythrocyte infected with the live, apicoplast-deficient *Plasmodium* parasite, for preparation of a medicament for inducing an immune response in a mammalian subject to the *Plasmodium* parasite.

[0009] Moreover, the present disclosure provides methods for screening drug candidates, comprising treating erythrocytes infected with live, apicoplast-containing *Plasmodium* parasites with a drug candidate, in the presence and absence of isopentenyl pyrophosphate (IPP) supplementation, wherein replication of the parasites in the presence but not the absence of IPP supplementation indicates that the drug candidate is an apicoplast-inhibitor. In some embodiments, the *Plasmodium* parasites are of a species selected from the group consisting of *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale, Plasmodium knowlesi, Plasmodium chaubaudi, Plasmodium berghei*, and *Plasmodium yoelii*. In some preferred embodiments, the *Plasmodium* parasites are *Plasmodium falciparum* parasites. In some embodiments, the erythrocytes are O-negative human erythrocytes. In some embodiments, the replication of the parasites is determined by measuring parasitemia (e.g., staining and flow cytometry) or metabolism (e.g., update of radiolabeled compounds such as tritiated-hypothanine). In some embodiments, the *Plasmodium* parasites are ring-stage parasites at the onset of the treatment with the drug candidate. In some embodiments, the treatment with the drug candidate is done for at least 48 hours (e.g., one intraerythrocytic development cycle before the IPP supplementation). In some embodiments, the methods comprise at least two or

three rounds of the treatment and IPP supplementation. In some embodiments, the IPP supplementation comprises contacting the infected erythrocytes with IPP or analog or salt thereof IPP analogs include but are not limited to alcohols, esters, and monophosphates.

[0010] The present disclosure also provides further methods of screening drug candidates, comprising culturing erythrocytes infected with live, apicoplast-deficient *Plasmodium* parasites in the presence of isopentenyl pyrophosphate (IPP) supplementation, and in the presence and absence of a drug candidate, wherein replication of the parasites in both the presence and the absence of the drug candidate indicates that the drug candidate is not an apicoplast-inhibitor. . In some embodiments, the *Plasmodium* parasites are of a species selected from the group consisting of *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale, Plasmodium knowlesi, Plasmodium chaubaudi, Plasmodium berghei*, and *Plasmodium yoelii*. In some preferred embodiments, the *Plasmodium* parasites are *Plasmodium falciparum* parasites. In some embodiments, the erythrocytes are O-negative human erythrocytes. In some embodiments, the replication of the parasites is determined by measuring parasitemia (e.g., staining and flow cytometry) or metabolism (e.g., update of radiolabeled compounds such as tritiated-hypothanine). In some embodiments, the *Plasmodium* parasites are ring-stage parasites at the onset of the treatment with the drug candidate. In some embodiments, the treatment with the drug candidate is done for at least 48 hours (e.g., one intraerythrocytic development cycle before the IPP supplementation). In some embodiments, the methods comprise at least two or three rounds of the treatment and IPP supplementation. In some embodiments, the IPP supplementation comprises contacting the infected erythrocytes with IPP or analog or salt thereof IPP analogs include but are not limited to alcohols, esters, and monophosphates.

BRIEF DESCRIPTION OF DRAWINGS

[0011] Figure 1 illustrates the chemical rescue of fosmidomycin-treated parasites with IPP precursors. Figure 1A provides EC_{50} curves for fosmidomycin inhibition in media supplemented with IPP, DMAPP, or both IPP and DMAPP. Figure 1B illustrates the dependence of chemical rescue on IPP concentration.

[0012] Figure 2 illustrates the chemical rescue of antibiotic-induced delayed death. Figure 2A provides EC_{50} curves with chloramphenicol +/- IPP rescue. Figure 2B provides EC_{50} curves with doxycycline +/- IPP rescue.

[0013] Figure 3 shows parasitemia and organelle genome-to-nuclear genome ratios under various growth conditions. Figure 3A shows the level of parasitemia as determined by flow cytometry for 1) no drug, 2) antibiotic only, 3) antibiotic plus IPP, 4) antibiotic plus IPP for three cycles followed by removal of IPP. Figure 3B shows the apicoplast genome-to-nuclear genome

ratio, while Figure 3C shows the mitochondrial genome-to-nuclear genome ration as determined by PCR comparing: 1) no drug, 2) antibiotic only, and 3) antibiotic plus IPP.

[0014] Figure 4 is a schematic of the intraerythrocytic development cycle of *Plasmodium spp.*

[0015] Figure 5 shows the rescue of antibiotic delayed death and apicoplast genome loss. Figure 5A shows survival of parasites over 4 life cycles treated with 1) doxycyline only, 2) doxycycline+IPP, or 3) doxycycline+IPP for 3 cycles followed by removal of both. Parasitemia is normalized to that of an untreated control. Figure 5B shows the apicoplast:nuclear genome ratio and Figure 5C shows the mitochondria:nuclear genome ratio of doxycycline only and doxycycline+IPP treated parasites over the same time course. Genome ratios are normalized to an untreated control. Data from three separate passages are shown.

[0016] Figure 6A-C shows the loss of protein processing of apicoplast-targeted proteins in antibiotic-treated, rescued parasites. Immunoblot using anti-GFP shows a time course of apicoplast-dependent protein processing of apicoplast-targeted GFP in untreated (Figure 5A), doxycycline-treated (Figure 5B), and doxycycline+IPP treated (Figure 5C) parasites.

[0017] Figure 7 provides a model of apicoplast function. The essential function of the apicoplast is the production of isoprenoid precursors, IPP and DMAPP, which are exported into the cytoplasm and used to synthesize small molecule isopremoids and prenylated proteins. Parasites that are unable to synthesize isoprenoid precursors either due to inhibition of the biosynthetic pathway by fosmidomycin or loss of the apicoplast following doxycycline inhibition can be chemically rescued by addition of exogenous IPP. The exogenous IPP enters the host cell through uknown membrane transporters and fulfills the missing biosynthetic function. Also shown is the reaction scheme for the MEP pathway biosynthesis of IPP and DMAPP.

BRIEF DESCRIPTION

[0018] *Plasmodium* parasites harbor an unusual plastid organelle called the apicoplast. Despite the annotation of several prokaryotically-derived biochemical pathways located in the apicoplast and the requirement for this organelle in all *Plasmodium* life stages (including the clinically-symptomatic intraerythrocytic stage), the function of the apicoplast remained a mystery prior to the development of the present disclosure. Previous work showed that treatment with fosmidomycin, an inhibitor of non-mevalonate isoprenoid precursor biosynthesis in the apicoplast, resulted in death of blood-stage *P. falciparum.* As described herein, fosmidomycin inhibition can be chemically rescued by supplementation with isopentenyl pyrophosphate (IPP), establishing drug specificity and definitively demonstrating that isoprenoid precursor biosynthesis is essential for blood-stage *P. falciparum* growth. Surprisingly, IPP supplementation also rescues

parasites from death following treatment with doxycycline and chloramphenicol, two antibiotics that cause loss of apicoplast function. An attenuated strain of *P. falciparum* was established by IPP supplementation through several cycles of antibiotic treatment. This strain has a specific and quantitative loss of the apicoplast genome. These findings indicate that isoprenoid precursor biosynthesis is the sole essential function of the apicoplast during blood-stage infection. The availability of rescued strains lacking an apicoplast provides an important tool with which apicoplast-specific antimalarials can be identified. In addition, the rescued *Plasmodium* strains provide live, chemically-attenuated, blood-stage immunogens for the prevention of malaria.

DETAILED DESCRIPTION

[0019] The present disclosure relates to blood-stage malaria parasites. In particular, the present disclosure provides viable parasites that lack an apicoplast as a consequence of conditions under which they are grown.

[0020] *Plasmodium* and other Apicomplexan parasites harbor an unusual plastid organelle called the apicoplast (Waller and McFadden, Curr Issues Mol Biol, 7:57-80, 2005; and Lim and McFadden, Phil Tran R Soc B, 365:749-763, 2010). The apicoplast is a relict chloroplast derived from secondary endosymbiosis of a plastid-bearing algae. It is therefore prokaryotic in origin containing pathways that have no counterpart in humans. During the course of evolution, the apicoplast has lost its photosynthetic function and exported most of its genome to the nucleus, requiring a dedicated protein targeting pathway to localize a majority of its required gene products to the apicoplast. Yet despite its minimalization, the apicoplast continues to serve essential though poorly-defined metabolic functions. In *Plasmodium*, apicoplast function is necessary for intraerythrocytic, intrahepatic, and mosquito stage development. Treatment of blood-stage *P. falciparum* with inhibitors of prokaryotic transcription and translation result in specific loss of the apicoplast and "delayed death" in the progeny of drug-treated parasites.

[0021] Despite its promise, very little is actually known about the function(s) the apicoplast serves, stymying efforts to develop apicoplast-directed therapies. A number of metabolic pathways have been described in the apicoplast, largely through functional assignment of annotated apicoplast-targeted nuclear genes with homology to known metabolic genes. Pathways identified include those for the biosynthesis of isoprenoid precursors, fatty acids, heme, Fe-S clusters, and ferredoxin. An astounding 5-10% of the nuclear genome is annotated with an apicoplast targeting signal, but 70% of these apicoplast-targeted gene products are of unknown function. Bioinformatic analysis also offers little insight into the timing of expression of putative apicoplast gene products during the complex life cycle of the parasite or their essentiality once expressed. For example, inhibitor studies initially suggested that fatty acid biosynthesis was

essential in blood-stage infection prompting their development as anti-malarials. Later genetic deletion of critical fatty acid biosynthetic genes definitively proved that the pathway is not required for blood stage growth and is instead critical in liver stage development. Experimental evidence is therefore required both to identify enzymes or pathways and to uncover their role during the parasite life cycle.

[0022] Among the annotated pathways, isoprenoid precursor biosynthesis, which generates the 5-carbon (C5) building blocks isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), is likely to be a main cytoplasmic output from the apicoplast. Other pathways appear to be involved in self-maintenance or support of the mitochondria. *Plasmodium* relies on a prokaryotic pathway variously known as the 1-deoxy-D-xylulose 5-phosphate (DOXP) or non-mevalonate pathway for synthesizing isoprenoid precursors, rather than the canonical mevalonate pathway used by most other eukaryotes and all mammals including humans. Both pathways produce IPP and DMAPP as the final products, but the enzymes and chemical intermediates leading to synthesis of these compounds are entirely different. Fosmidomycin, an inhibitor of the second enzyme in the non-mevalonate pathway, kills blood-stage parasites (Jomaa et al., Science, 285:1573-1576, 1999) and has been tested in clinical trials as an antimalarial. Inhibition by fosmidomycin suggests that isoprenoid precursor biosynthesis is essential in blood-stage infection, although the possibility of off-pathway targets as the cause of the drug effect (as was found to be the case for fatty acid biosynthesis inhibitors) has not been ruled out. Furthermore, IPP and DMAPP are not an end onto themselves but rather building blocks used to synthesize small molecule isoprenoids with a host of functions or C_{15}/C_{20} prenyl chains for the post-translational modification of proteins. Once IPP and DMAPP are exported into the parasite cytoplasm, the downstream isoprenoid products in *Plasmodium* and their function during infection are unknown.

[0023] As with other apicoplast pathways, the prokaryotic origin of isoprenoid precursor biosynthesis makes it an attractive therapeutic target. Furthermore, identification of the products derived from these building blocks could open the door to a host of new pathways and functions involved in pathogenesis. Investigation of apicoplast pathways is hampered by the inability to knockout essential genes, purify the organelle, and overexpress and purify *Plasmodial* proteins. Thus, an alternative strategy using drug inhibition/chemical rescue, in analogy to genetic deletion/complementation, was utilized herein to establish the essentiality of the isoprenoid precursor biosynthesis pathway. As determined during development of the present disclosure, production of isoprenoid precursors is the only essential function of the apicoplast during blood-stage infection.

[0024] The *P. falciparum* parasites generated during development of the present disclosure, which lack apicoplasts, as well as the methods for their production provide indispensable tools for the identification selective apicoplast-inhibitors. In particular, given the built-in specificity of the rescue method, growth inhibitory small molecules that target pathways involved in function, replication, or maintenance of the apicoplast can be identified. Additionally, the apicoplast-minus strains dependent on IPP for continued growth provide an attenuated strain for blood-stage vaccine development. These parasites can be injected as live parasites (e.g., infected erythrocytes or isolated merozoites) that can survive in blood for at most one cycle thereby stimulating an immune response, but would fail to reinvade host cells. A significant advantage of this approach is that attenuation is achieved chemically and does not require difficult genetic manipulation, thereby allowing for the incorporation of circulating field strains of *Plasmodium* in an immunogenic formulation.

[0025] General methods for working with *Plasmodium spp.* in vitro and in vivo are described in Moll et al., Methods of Malaria Research, fifth edition, MR4 / ATCC, Manassas, Virginia, and BioMalPar, Paris, France, 2008. Likewise, methods for testing malarial vaccines in rodents, monkeys and man are described in McCarthy and Good, Human Vaccines, 6:114-123, 2010, herein incorporated by reference. Specifically for testing vaccine candidates in mice, *P. berghei, P. chabaudi, P. vinkei,* or *P. yoelii* are employed. For testing vaccine candidates in monkeys (*Rhesus, Aotus, or Saimiri*), *P. falciparum or P. knowlesi* are employed. Vaccines developed for human use employ *P. falciparum, P. vivax, P. malariae, P. ovale,* or *P. knowlesi.*

[0026] In some embodiments of the present disclosure, a composition comprising one or both of a live, apicoplast-deficient *Plasmodium* parasite, and an erythrocyte infected with the live, apicoplast-deficient *Plasmodium* parasite are provided. In some embodiments, the composition is a vaccine. In some embodiments, the vaccine further comprises an adjuvant suitable for use in humans. Suitable adjuvants include but are not limited to mineral salts (e.g., aluminum hydroxide or "alum", aluminum phosphate, calcium phosphate, etc.), oil emulsions (e.g., MF59), particulate adjuvants (e.g., liposomes), microbial derivatives (e.g., monophosphoryl lipid A, CpG motifs, modified toxins, etc.), and plant derivatives (e.g., saponins such as QS-21).

EXAMPLES

[0027] Abbreviations: Cm (chloramphenicol); Dox (doxycycline); DMAPP (dimethylallyl pyrophosphate); EC_{50} (concentration required to achieve 50% of the maximum effect); GFP (green fluorescent protein); IPP (isopentenyl pyrophosphate); and RBC (red blood cell).

EXAMPLE 1

CHEMICAL RESCUE OF *PLASMODIUM FALCIPARUM* PARASITES LACKING AN APICOPLAST

[0028] This example describes the use of the combination of drug inhibition and chemical rescue, in analogy to genetic deletion and complementation, to establish essentiality of the isoprenoid precursor biosynthesis pathway for *P. falciparum* growth in erythrocytes. As demonstrated herein, the production of isoprenoid precursors is the only essential function of the apicoplast during blood-stage infection.

Materials and Methods

[0029] *P. falciparum cultures. Plasmodium falciparum* W2 (MRA-157), D10 (MRA-201), and D10 ACPL-GFP (MRA-568) were obtained from MR4 (Malaria Research and Reference Reagent Resource Center). Parasites were grown in human erythrocytes (2% hematocrit) in RPMI 1640 media supplemented with 0.25% Albumax II (GIBCO Life Technologies), 2 g/L sodium bicarbonate, 0.1 mM hypoxanthine, 25 mM HEPES (pH 7.4), and 50 μg/L gentamycin, at 37°C, 5% O_2, and 6% CO_2. Methods for culturing malarial parasites are known in the art (see, e.g., Trager and Jensen, Science, 193:673-675, 1976; and Schuster, Clinical Microbiological Reviews, 15:355-364, 2002, both herein incorporated by reference in their entirety). For D10 ACPL-GFP transgenic parasites, the media was also supplemented with 100 nM pyrimethamine (Sigma).

[0030] For passage of antibiotic-treated, IPP-rescued parasites, the media was supplemented with 50-100 μM chloramphenicol (Cm) or 1-2 μM doxycycline (Dox). 48 h after initiation of antibiotic treatment, rescued strains were supplemented with 100-200 μM IPP (Isoprenoids LC) for continuous passage. For comparison of growth between different treatment conditions, cultures were carried simultaneously and handled indentically with respect to media changes and addition of blood cells.

[0031] *Drug susceptibility assays.* Growth assays were performed in 96-well plates containing serial dilution of drugs in duplicate or triplicate. Media was supplemented with IPP or DMAPP (Isoprenoids LC) as indicated. To determine the EC_{50} of fosmidomycin (Invitrogen),

growth was initiated with ring-stage parasites (synchronized with 2.5% sorbitol treatment 48 hours prior) at 1% parasitemia (0.5-2% hematocrit). Plates were incubated for 72 h. To determine the EC_{50} of chloramphenicol and doxycycline at 48h, growth was initiated at 1% parasitemia and incubated for 48 h. To determine the EC_{50} of chloramphenicol and doxycycline at 96 h and observe the delayed death phenotype, cultures were initiated at 0.2% parasitemia, 75% of the media was exchanged at 48 h, and plates were incubated for an additional 48 h (total 96 h). For all assays, growth was terminated by fixation with 1% formaldehyde and parasitized cells were stained with 250 nM YOYO-1 (Invitrogen). Parasitemia was determined by flow cytometry. Data was analyzed by FlowJo, and EC_{50} curves plotted by GraphPad Prism.

[0032] *Quantitative real-time PCR.* Parasites from 200 μL of culture were isolated by saponin lysis followed by PBS wash to remove extracellular DNA. DNA was purified using QiaAMP blood kits (Qiagen). Primers were designed to target genes found on each organelle or nuclear genome: tufA (apicoplast) 5'-GATATTGATTCAGCTCCAGAAGAAA-3' (SEQ ID NO:1) / 5'-ATATCCATTTGTGTGGCTCCTATAA-3' (SEQ ID NO:2), cytb3 (mitochondria) 5'-AGATACATGCACGCAACAGG-3' (SEQ ID NO:3) / 5'-TCATTTGACCCCATGGTAAGA-3' (SEQ ID NO:4), and CHT1 (nuclear) 5'-TGTTTCCTTCAACCCCTTTT-3' (SEQ ID NO:5) / 5'-TGTTTCCTTCAACCCCTTTT-3' (SEQ ID NO:6). Reactions contained template DNA, 0.2 μM of each primer, and 1X LightCycler 480 SYBR Green I Master (Roche). PCR reactions were carried out on Lightcycler 6500 (Roche). Relative quantification of target genes was determined using the method of Pfaffl (Nucleic Acids Res, 29: e45, 2001). For each time point, the organelle:nuclear genome ratio of the antibiotic-treated control or antibiotic-treated IPP-rescued experiment was calculated relative to that of an untreated control collected at the same time. *Immunoblot.* Ring-stage D10 ACP_L-GFP parasites from 1 mL of culture were isolated by saponin lysis, washed with PBS, and resuspended in 1xNuPAGE LDS sample buffer (Invitrogen). Proteins were separated by electrophoresis on 12% Bis-Tris gel (Invitrogen) and transferred to nitrocellulose membranes. After blocking, membranes were probed with 1:1000 polyclonal rabbit anti-GFP (Clontech) antibody and 1:15,000 Alexa Fluor 810-conjugated anti-rabbit IgG secondary antibody (Invitrogen). Fluorescent antibody-bound proteins were detected with Odyssey Imager at 800 nm (LiCor Biosciences).

[0033] *Fluorescence microscopy.* Untreated and antibiotic treated/IPP rescued D10 ACP_L-GFP parasites were incubated in 2 μg/mL Hoescht 33342 stain for 30 min at 37 °C. Cells in culture media were settled onto 35 mm glass-bottom petri dishes (MakTek) coated with 1% polyethylenimine (Sigma). Wide-field epifluorescence live cell images were obtained on a Nikon Eclipse Ti-E equipped with a Coolsnap HQ2 camera (Photometrics) using a 100x/1.4 oil

immersion objective. Confocal live cell images were obtained on an A1 confocal mounted on a Nikon Eclipse Ti-E using a 60X/ 1.4 oil immersion objective. Images were analyzed by NIS-Elements software (Nikon).

Results

[0034] *Chemical rescue with isoprenoid precursors reverses fosmidomycin inhibition.* To demonstrate the specificity of fosmidomycin inhibition for the isoprenoid precursor biosynthesis pathway, the killing of blood-stage *P. falciparum* upon treatment with fosmidomycin was reversed by supplementation with isoprenoid precursors, IPP and DMAPP. Inhibition of blood-stage *P. falciparum* W2 growth by fosmidomycin was achieved with an EC_{50}=0.5-1.0 μM. When drug susceptibility was tested in media supplemented with IPP, DMAPP, or both IPP and DMAPP, IPP alone was sufficient to completely reverse the death phenotype (Figure 1A). Survival of parasites was dependent on the concentration of IPP in the media with 100-200 μM IPP sufficient for rescue (Figure 1B). Addition of DMAPP alone or in combination with IPP had no effect or was even slightly toxic (Figure 1A). Addition of up to 2.25 mM 3-methyl-3-butenol and 3-methyl-2-butenol, the alcohol analogs of IPP and DMAPP, which lack the pyrophosphate moiety, did not rescue fosmidomycin inhibition. Reversal of drug inhibition by addition of IPP was only seen with fosmidomycin and did not occur with chloroquine, a drug that does not target the isoprenoid precursor biosynthetic pathway. These findings establish that fosmidomycin inhibition is specific for the isoprenoid precursor biosynthetic pathway, isoprenoid precursor biosynthesis is essential for blood-stage *P. falciparum* growth, and exogenous IPP fulfills the endogenous biosynthetic function.

[0035] *IPP supplementation rescues antibiotic delayed death.* Treatment of blood-stage *P. falciparum* with antibiotics that block prokaryotic transcription or translation results in specific loss of apicoplast function. The deleterious effects were not immediately apparent in the first 48 hr life cycle of treated parasites during which time transcription (and translation) of apicoplast genes was inhibited, though organelle morphology, protein targeting, genome replication, and segregation remained intact. Instead, a "delayed death" phenotype was observed whereby the progeny of these treated parasites which have inherited non-functional organelles unable to import critical nuclear-encoded proteins fail to replicate the organelle genome or segregrate apicoplast into daughter cells. In this way progeny of treated parasites stall as schizonts unable to form merozoites for reinvasion in the second cycle following treatment.

[0036] To determine whether rescue of the isoprenoid precursor biosynthesis pathway was sufficient to reverse the deleterious effects of apicoplast loss, blood-stage parasites were treated with chloramphenicol or doxycycline in the presence of IPP. Treatment with either

doxycycline or chloramphenicol alone resulted in "delayed death" as seen at a relatively high EC_{50} of 5-10 μM or >300 μM respectively at 48 h due to nonspecific inhibition, followed by apicoplast-specific inhibition apparent at 96 h with an EC_{50} of 0.4 μM for doxycycline or 10-20 μM for chloramphenicol (Figure 2 and Table 1-1). By supplementing with 200 μM IPP, reversal of apicoplast-specific inhibition at 96h by both drugs was observed with EC_{50} values reflective of only the nonspecific drug effects. In fact, when addition of IPP from 0-48 h, 48-96 h, or 0-96 h was compared, IPP was only required during the second cycle consistent with a deficiency in the progeny of treated parasites. Similar results were demonstrated with *P. falciparum* D10 strain, indicating that the rescue of antibiotic-induced delayed death is generally applicable and not specific to the original strain examined. Thus, IPP supplementation was found to be sufficient to rescue the delayed death phenotype induced by treatment with either doxycycline or chloramphenicol.

Table 1-1. Rescue of Apicoplast-Specific Growth Inhibition by Antibiotic

Strain	Drug	EC_{50} (μM)[a]				
		48 h		96 h		
		No Rescue	+IPP	No Rescue	+IPP 0-96 h	+IPP 48-96 h
W2	Chloramphenicol	>300[b]	>300[b]	13.2 (9.7-18.0)	>300[b]	>300[b]
W2	Clindamycin	>10[b]	>10[b]	0.004 (0.002-0.008)	>10[b]	ND
W2	Doxycycline	5.6 (3.9-8.0)	5.1 (3.5-7.5)	0.3 (0.2-0.4)	5.2 (3.6-7.5)	ND
D10	Chloramphenicol	>300[b]	>300[b]	19.8 (13.6-28.8)	>300[b]	>300[b]

ND, not determined.
[a]Represented as mean (95% confidence interval).
[b]50% inhibition above highest assayed concentration.
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[0037] *P. falciparum* W2 blood stage parasites were carried through several life cycles with simultaneous antibiotic treatment and IPP rescue in order to determine 1) how robust the rescued parasite was (e.g., assess presence of significant growth defects) and 2) the dependence of the surviving parasites on further supplementation with IPP (even after removal of the antibiotic). As shown in Figure 3A and Figure 5A, generation of the antibiotic-treated, IPP-rescued strain was compared with an untreated and antibiotic-treated only control that was carried simultaneously and handled identically with respect to media changes and addition of blood cells. The antibiotic-treated, IPP-rescued strain grew as well as the untreated strain throughout the treatment and rescue course. In contrast in the antibiotic treated only control, the parasites quickly died after the second cycle of treatment with undetectable parasitemia by the end of the third cycle. Of note, removal of IPP supplementation (in the absence of antibiotic treatment) from the rescued parasites at the start of the fourth cycle resulted in a rapid decline in parasitemia to undetectable levels by the following cycle. Again similar results were demonstrated with the *P. falciparum* D10 strain. These results show that antibiotic-treated parasites rescued by IPP

supplementation show no growth defect, but are entirely dependent on exogenous IPP for continued growth.

[0038] *Antibiotic-treated, IPP-rescued parasites lack an apicoplast.* In order to characterize the physical and functional status of the apicoplast in the strains resulting from continuous antibiotic treatment and IPP rescue, the presence of the organelle genome was assessed. To further characterize the apicoplast status, apicoplast protein markers expression and processing are also assessed. Loss of the apicoplast genome replication is one of the hallmarks of antibiotic treatment. During the generation of antibiotic-treated, IPP-rescued strain, quantitative PCR was employed to target genes on the apicoplast, mitochondrial, and nuclear genome to monitor the ratio of organelle: nuclear genome during the course of the antibiotic treatment and chemical rescue. Figure 3B and Figure 5B demonstrate a marked decline in the apicoplast:nuclear genome ratio after the second cycle in all antibiotic-treated parasites regardless of supplementation with IPP. At the end of the fourth cycle, the ratio is reduced by at least 100-fold. In contrast, no such decline is noted in the mitochondria: nuclear genome ratio as shown in Figure 3C and Figure 5C. Also of note, as parasitemia decreased in the antibiotic-treated (no rescue) culture, the apicoplast:nuclear ratio increases slightly reflecting the degradation of both apicoplast and nuclear genomes upon cell death. It is apparent therefore that IPP-rescued parasites undergo a specific loss of the apicoplast genome without loss of nuclear or mitochondrial genomes.

[0039] Array comparative genomic hybridization (aCGH) on the rescued parasites is also performed to determine whether all apicoplast genes are affected. In addition, immunofluorescence microscopy for apicoplast antigens is performed (e.g., apicoplast-targeted acyl-carrier protein, ACP, using anti-ACP antibody). Additionally or alternatively, fluorescence microscopy is performed with D10 ACPL-GFP transgenic strain in which GFP is targeted to the apicoplast. Immunoblots are done to examine protein processing by the apicoplast of ACP (in W2 strains) and GFP (in D10 ACPL-GFP transgenic strain). *Antibiotic-treated, IPP-rescued parasites lose protein import function.* A critical function of the apicoplast, required for the maintenance of its proteome, is the import of nuclear-encoded proteins into the organelle. A bipartite *N*-terminal sequence consisting of a signal sequence and a transit peptide is required to target proteins to the apicoplast [8]. Upon import into the apicoplast, the transit peptide is cleaved to produce a mature protein. Protein processing is therefore a marker of successful protein import into the apicoplast. A transgenic D10 strain expressing GFP fused to an *N*-terminal apicoplast targeting sequence (ACP_L-GFP) was used to assess apicoplast protein processing during the course of antibiotic treatment and IPP rescue [8]. The 33 kDa full-length GFP was cleaved to produce a predominant 30 kDa mature protein in untreated parasites as

shown in Figure 6A. Parasites treated with doxycycline only began to lose protein processing function during the second cycle as seen in the increased accumulation of full-length protein at 96h, but do not survive beyond this cycle as shown in Figure 6B. When doxycycline treatment was rescued with IPP, surviving parasites showed successive loss of protein processing with each treatment cycle such that only preprocessed GFP was detectable at 144 h, the start of the fourth cycle as shown in Figure 6C. A smaller, previously-described degradation band also became apparent in the rescued parasites [8]. The absence of protein processing activity indicates a loss of the critical protein import function of the apicoplast in these rescued parasites.

[0040] *Antibiotic-treated, IPP-rescued parasites lack an apicoplast.* The final outcome of antibiotic treatment is a failure of apicoplast replication and segregation during cell division, resulting in loss of the organelle and death [9]. The loss of the genome and protein import function indicates that parasites that survive antibiotic treatment are in fact apicoplast-minus. Localization of GFP in the D10 ACP_L-GFP strain was used to visualize the apicoplast. As expected, GFP localizes to a discrete structure in the parasite in untreated cells (data not shown). In contrast, in parasites that have been rescued from antibiotic death, GFP loses this discrete apicoplast localization and becomes diffuse. Confocal images show that numerous foci of GFP are scattered throughout the cytoplasm. The largest foci measure >200 nm, so these collections of GFP are less likely to be cytoplasmic protein aggregates but instead may represent vesicles containing protein. Combined with the absence of the apicoplast genome and protein import function, the loss of GFP localization indicates the absence of the apicoplast itself to which it is normally targeted.

Table 1-2. Components of RPMI Medium 1640 (Invitrogen)

	RPMI (mM)	Plasma[1] (mM)	Acquisition	Biosynthesis
Amino Acids*				
Glycine	0.133	0.2±0.06	Hb, plasma[2-4]	cytosol[5]
L-Arginine	1.15	0.1±0.02	Hb, plasma[2-4]	
L-Asparagine	0.379	0.13±0.008	Hb, plasma[2-4]	cytosol[5]
L-Aspartic acid	0.15	0.02±0.005	Hb, plasma[2-4]	cytosol[2,5]
L-Cystine	0.208	0.107±0.02	Hb, plasma[2-4]	
L-Glutamic Acid	0.136	0.06±0.02	Hb, plasma[2-4]	cytosol[2,5]
L-Glutamine	2.05	0.5±0.17	Hb, plasma[2-4]	cytosol[5]
L-Histidine	0.0968	0.09±0.03	Hb, plasma[2-4]	
L-Hydroxyproline	0.153	0.046±0.046	plasma[3]	
L-Isoleucine	0.382	0.059±0.03	plasma[4]	
L-Leucine	0.382	0.25±0.005	Hb, plasma[2-4]	
L-Lysine	0.274	0.43±0.02	Hb, plasma[2-4]	
L-Methionine	0.101	0.02±0.01	Hb, plasma[2-4]	
L-Phenylalanine	0.0909	0.17±0.003	Hb, plasma[2-4]	
L-Proline	0.174	0.263±0.15	Hb, plasma[2-4]	cytosol[5]
L-Serine	0.286	0.236±0.006	Hb, plasma[2-4]	
L-Threonine	0.168	0.26±0.01	Hb, plasma[2-4]	
L-Tryptophan	0.0245	0.044±0.007	Hb, plasma[2-4]	
L-Tyrosine	0.111	0.142±0.004	Hb, plasma[2-4]	
L-Valine	0.171	0.233±0.043	Hb, plasma[2-4]	
Vitamins				
Biotin	0.00082	0.00127±0.0007	plasma[6]	
Choline chloride	0.0214	0.011±0.002	plasma[3]	
D-pantothenate*	0.00052	0.005±0.0004	plasma[7,6]	
Folic acid*	0.00227	0.00008	plasma[8,9]	cytosol[9]
Nicotinamide*	0.0082	0.00044	RBC, plasma[10]	
PABA	0.0073	0.015	plasma[3]	
Pyridoxine*	0.00485	0.000025	RBC, plasma[6,10]	cytosol[6]
Riboflavin*	0.00053	0.000015	RBC, plasma[10]	
Thiamine*	0.00297	0.00012	RBC[6]	cytosol[6]
Vitamin B12	3.7E-06	0.0000003	RBC, plasma	
i-Inositol	0.194	0.03±0.019		cytosol[11]
Other				
D-Glucose*	11.11	4.44±0.37	plasma[12]	
Glutathione*	0.00326	0.037±0.0048		cytosol[13-15]
Hypoxanthine*	0.10	0.005±0.0004	RBC[16]	
HEPES	25.03			
Phenol Red	0.0133			

Concentrations of organic metabolites in RPMI medium were obtained from the manufacturer's website. The plasma concentration of each component indicates the availability of the nutrient in the extracellular milieu during human blood infection.[1] The mode of acquisition from plasma, host RBC, or hemoglobin (Hb) degradation and location of biosynthesis of each nutrient by blood-stage *Plasmodium* is indicated based on available evidence. * denotes nutrients which have been shown to be essential for blood-stage parasites.

[0041] References for Table 1-2 only.

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Discussion

[0042] The function of the apicoplast in *Plasmodium spp* has been a critical missing link in our understanding of this organelle. The present disclosure demonstrates that isoprenoid precursor biosynthesis is the only essential function of the apicoplast during blood-stage growth. These results indicate that no other essential products are supplied by the apicoplast during the intraerythrocytic development cycle (IDC). This surprising revelation has several important implications. Because isoprenoid precursors are building blocks to synthesize cellular isoprenoid products with diverse functions, their key role now gives added urgency to the elucidation of these products and their downstream functions. At least one essential prenylated product is ubiquinone, a component of the mitochondrial electron transport chain. There are certainly other essential, as-yet-unidentified isoprenoid products, since transgenic parasites which express yeast dihydroorotic acid dehydrogenase and no longer require their electron transport chain are still susceptible to fosmidomycin and antibiotics and could be rescued with IPP supplementation (data not shown) [36]. Possible isoprenoid products include dolichols involved in protein *N*-glycosylation which have been detected in *Plasmodium* and prenylated proteins, such as Rab homologs required for vesicular trafficking and a recently identified tyrosine phosphatase [37-41].

[0043] The current findings also indicate that several annotated apicoplast pathways are in fact non-essential. Amongst both identified pathways and the 70% of apicoplast gene products with unknown function, only isoprenoid precursor biosynthesis and any pathways supporting this function in blood-stage parasites (including those required for organelle maintenance and replication) are essential and therefore viable apicoplast drug targets [20]. Assertions that type II fatty acid and, by implication, acetyl-CoA biosynthesis were essential apicoplast functions during blood-stage growth have already been disproven [23,24,42]. A parasite-derived pathway for heme biosynthesis contains steps that occur in the apicoplast, mitochondria, and cytosol. The present disclosure indicates that blood-stage parasites do not depend on *de novo* heme biosynthesis using this pathway, but instead rely on an extrinsic *de novo* pathway utilizing imported host enzymes or salvage of heme from the host by an unidentified mechanism [43,44]. Still other pathways such as Fe-S cluster biosynthesis supply cofactors for enzymes within the organelle but are not exported outside the organelle. These pathways become "non-essential" when the need for organelle maintenance is removed.

[0044] The complexity of the organelle and the simplicity of its blood-stage function pose an obvious contradiction. Approximately 5-10% of the *Plasmodium* genome is predicted to encode apicoplast-targeted gene products (although the localization and/or function of the majority of these gene products have not been validated) [20]. In order to import these proteins

into the apicoplast, parasites utilize a dedicated protein trafficking pathway [7,8]. In addition, the organelle undergoes complex morphological development during blood stage growth, requiring cellular machinery to faithfully replicate and segregate the organelle at every cell division [10]. Why are such huge resources consumed to maintain a single essential function? First, while the function of the apicoplast is limited during the blood stage, the need for more extensive organelle function during other developmental stages may dictate its maintenance in intraerythrocytic parasites as the organelle cannot be generated *de novo*. Fatty acid biosynthesis, for example, is an essential apicoplast function in liver stage parasites [23,24]. Second, *Plasmodium* may have been evolutionarily trapped in its bondage to the apicoplast. Having acquired the plastid early in its evolution, it may have been unable to dispense of it even after adopting an increasingly parasitic lifestyle due to the transfer of even a few essential functions to the organelle. In any case, this imbalance emphasizes the value of targeting housekeeping pathways involved in organelle maintenance and replication to interfere with its function.

[0045] An important consideration is whether these findings accurately reflect *in vivo* growth requirements of parasites during infection. Specifically, are there essential metabolites supplemented in culture that could not be acquired during *in vivo* growth and instead must be biosynthesized by the apicoplast? While parasitized RBCs during infection use human plasma as a source of extracellular nutrients, the cultures in this example were grown in RPMI medium 1640 supplemented with purified serum substitute, Albumax. Albumax could be replaced with 10% human serum with no effect on the survival of apicoplast-minus parasites in the presence of IPP (data not shown). RPMI medium contains salts, 20 amino acids, 11 vitamins, 4 other organic molecules, and glucose. The acquisition and biosynthesis of these nutrients by blood-stage *Plasmodium* and their essentiality for intraerythrocytic growth based on available evidence is shown in Table 1-2 above. In general, blood-stage *Plasmodium* biosynthesizes very limited amounts of just three amino acids. The blood-stage parasites are dependent on amino acids from either 1) hemoglobin degradation or 2) acquisition from patient plasma through newly established permeation pathways in the infected red cell [45-47]. Similarly, current knowledge of *Plasmodium* metabolism indicates that the remaining organic metabolites found in RPMI medium are biosynthesized by non-apicoplast pathways or can be acquired from the host red cell or patient plasma [46,48-50]. Consequently, the findings described herein can be extrapolated to *in vivo* requirements for the apicoplast to support parasite growth and development. These results also define a very minimal set of potential metabolites (IPP and components found in RPMI 1640 medium) that could be biosynthesized in the apicoplast.

[0046] Several aspects of the chemical rescue with isoprenoid precursors are notable. During chemical rescue, exogenous IPP could enter the parasite through permeation pathways

established in the parasitized erythrocyte or other uncharacterized membrane transporters [46,51]. The RBC is largely metabolically inactive and unlikely to have significant ongoing isoprenoid precursor biosynthesis via the host mevalonate pathway or stores of these metabolites [52]. It is also unlikely that these high-energy pyrophosphorylated molecules would accumulate to appreciable levels in plasma (200 μM was required for rescue in our experiments). Consistent with this notion, IPP was not present in the Serum Metabolome Database (SMDB), which contains 4229 detectable metabolites [53]. Therefore, acquisition of isoprenoid precursors *in vivo* by salvage of IPP from infected blood is improbable. Once in the parasite, exogenous IPP may fulfill its function in the cytoplasm with or without uptake into the apicoplast [54].

[0047] Although both IPP and DMAPP are required to synthesize isoprenoid products, supplementation with IPP alone is sufficient to fulfill endogenonous isoprenoid precursor biosynthesis, implying the presence of an IPP isomerase in the cytoplasm that converts IPP to DMAPP. This activity may be important in establishing the optimal cellular ratio of IPP to DMAPP, as toxicity was noted with increasing DMAPP concentrations. A putative IPP isomerase has been identified in the *Plasmodium* genome [54]. A recent report suggested that geranylgeraniol, the alcohol analog of a C_{20} prenyl chain, could rescue fosmidomycin inhibition [55]. Fosmidomycin inhibition could not be relieved with alcohol analogs of IPP and DMAPP, indicating either poor cellular penetration of the alcohols or the absence of a kinase to convert the alcohol analogs to the pyrophosphorylated and active metabolites (data not shown). Even with conversion of geranylgeraniol to geranylgeranyl pyrophosphate in the cell, it would seem that a C_5 building block, such as IPP, would almost certainly be required to extend the supplemented C_{20} unit for construction of polyprenyl chains, such as that found in ubiquinone, and to construct smaller prenyl chains, such as for protein farnesylation. The reported rescue with geranylgeraniol was performed at 1.5 μM fosmidomycin, which is above the concentration required for 50% growth inhibition but may be below that required for adequate inhibition of the biosynthetic pathway (since phenotypic growth inhibition can be apparent even at low levels of inhibition of the biosynthetic pathway) [55]. Therefore, the reported results may be complicated by ongoing biosynthesis of IPP and DMAPP contributing to the precursor pool. Consistent with this, neither farnesol nor geranylgeraniol was able to rescue fosmidomycin concentrations >10 μM, and both showed dose-related parasite toxicity (data not shown). In contrast, IPP rescue was demonstrated at fosmidomycin concentrations exceeding 100 μM, well above its EC_{90} for growth inhibition.

[0048] The consequences of apicoplast loss following antibiotic treatment and IPP rescue are no less intriguing. In the parasites that survive antibiotic treatment by chemical rescue, the organelle is irreversibly lost when it fails to segregate to daughter cells [9]. In these apicoplast-minus parasites, apicoplast gene products encoded in the nucleus may continue to be transcribed

and translated. These products may properly shuttle into the secretory pathway but cannot be diverted to the organelle [8]. Based on the microscopy results, apicoplast proteins are likely packaged into transport vesicles bound for the organelle but are unable to localize to the missing structure and therefore accumulate in the cytoplasm appearing as numerous foci. Although there may be structural remnants of the apicoplast still present, the observed foci are unlikely to support apicoplast functions. Apicoplast-targeted proteins likely require both cleavage of the long basic transit peptide and chaperones in the lumen of the apicoplast for proper folding. Consistent with this premise, cleavage of the transit peptide from targeted proteins, a critical apicoplast function, does not occur in rescued parasites.

[0049] The close physical and functional relationship between the apicoplast and the mitochondria raises the possibility that loss of the apicoplast might affect the ability of the mitochondria to replicate and divide. The mitochondrial genome could be detected by qPCR for the cytB3 gene and labeling of the mitochondria with Mitotracker by fluorescence microscopy in apicoplast-minus parasites was observed. Thus despite the loss of the apicoplast, these parasites apparently contain mitochondria.

[0050] While the survival of apicoplast-minus *P. falciparum* invokes a slew of intriguing questions, these same parasites promise to be a powerful and indispensable tool for further dissection of apicoplast biology. Apicoplast-minus *P. falciparum* strains generated herein can be used to assess organelle requirement during gametocytogenesis and mosquito stage development. These strains also provide novel avenues to identify isoprenoid products, generate conditional mutants of essential genes involved in apicoplast maintenance and replication, conduct metabolomic or proteomic profiling, and study protein trafficking to the organelle.

[0051] With regard to drug development, the chemical rescue strategy described herein also addresses the critical deficiency of current cell growth screening assays, namely lack of knowledge of the drug target. Candidate drug hits detected in phenotypic assays can be screened for chemical rescue of the growth inhibition. The reversal of growth inhibition by IPP supplementation specifically identifies inhibitors that target pathways involved in MEP pathway function, replication, or maintenance of the apicoplast, providing a pathway-specific drug screen to aid in discovery of new classes of anti-malarials. The ability to chemically complement the cell death phenotype will prevent false leads from off-target effects, like that seen with triclosan and its misconstrued effect on type II fatty acid biosynthesis [22].

[0052] Finally, the apicoplast-minus strains dependent on IPP for continued growth are a unique and ideal candidate for an attenuated blood-stage vaccine [56,57]. Unlike irradiated or drug-treated whole parasite vaccines, apicoplast-minus parasites continue to develop in blood at most one cycle, including a single erythrocyte rupture and reinvasion, thereby stimulating a

stronger immune response. However, judging by the effects of IPP withdrawal in culture, they would be unable to develop further in the absence of exogenous IPP. Lending support to this notion, a similar "limited survival" strategy targeting the apicoplast in liver-stage parasites has proven effective as a liver-stage vaccine candidate [58]. A significant advantage of the approach provided by the present disclosure is that attenuation is achieved chemically and does not require difficult or costly genetic manipulation (as is the case with genetically-modified vaccine strains), thereby allowing for the possibility of incorporating circulating field strains of *Plasmodium* in a vaccine formulation [59]. There would also be very little risk of reversion as it would be extremely difficult to reacquire apicoplast function by mutation.

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EXAMPLE 2

METHODS FOR IDENTIFICATION OF APICOPLAST INHIBITORS

[0054] Cell culture-based screening methods are currently available for identifying inhibitors that affect *P. falciparum* survival in red blood cells (Gamo et al., Nature 465: 305–310, 2010; and Guiquemde et al., Nature 465: 311–315, 2010). A major limitation to this phenotypic cell death screen is that the target of the inhibitor is not known and is frequently difficult to elucidate. Other approaches for screening potential anti-malarials are typically directed to known parasite targets, and therefore do not aid in identification of new classes of inhibitors. Identification of novel targets often requires more challenging methods such as laboratory generation of resistant organisms and genome sequencing to identify resistance-conferring mutations that may occur in the drug target.

[0055] The drug screening methods provided by the present disclosure are suitable for the identification of inhibitors that target pathways involved in apicoplast function, maintenance, and replication. Candidate compounds with promising in vitro and in vivo anti-malarial and pharmacokinetic characteristics are tested in the methods of the present disclosure to determine whether these candidates target the apicoplast. In particular, candidate compounds are tested in 48-hour "immediate death" and 96-hr "delayed death" growth assays with either standard media or media supplemented with 200 μM isopentenyl pyrophosphate. Small molecule compounds are tested over a nine-point series of concentrations (6-600 nM). To determine the EC_{50} for immediate death, growth is initiated with ring-stage parasites (synchronized with 2.5% sorbitol treatment 48 hours prior) at 1% parasitemia (0.5-2% hematocrit) in 96-well plates. Plates are incubated for 48 h at 37 °C. To determine the EC_{50} for delayed death, growth is initiated at 0.2% parasitemia, 75% of the media is exchanged at 48 h, and plates are incubated for an additional 48 h (total 96 h). For all assays, growth is terminated by fixation with 1% formaldehyde and parasitized cells are stained with 250 nM YOYO-1 (Invitrogen). Parasitemia is determined by flow cytometry on BD LSR II (BD Biosciences). Concentration response data is analyzed by a nonlinear regression logistic dose response model and the 50% and 90% inhibitory concentrations (IC_{50}s and IC_{90}s) for each compound are calculated.

[0056] Any compounds whose death phenotype is reversed by IPP supplementation either in 48 hour or 96 hour growth assays is identified as an inhibitor of a pathway involved in apicoplast function, maintenance, or replication. Conversely, anti-malarials that show no difference in drug effect in the presence or absence of IPP supplementation are determined to have a mechanism of action that does not involved the apicoplast.

CLAIMS

We claim:

1. A method for producing a live, apicoplast-deficient *Plasmodium* parasite, comprising: i) treating erythrocytes infected with apicoplast-containing *Plasmodium* parasites with an apicoplast inhibitor to produce an apicoplast inhibitor-treated cell culture; and ii) supplementing said apicoplast inhibitor-treated cell culture with isopentenyl pyrophosphate (IPP) to produce a live, apicoplast-deficient *Plasmodium* parasite.

2. The method of Claim 1, wherein the live, apicoplast-deficient *Plasmodium* parasite is of a species selected from the group consisting of *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale, Plasmodium knowlesi, Plasmodium chaubaudi, Plasmodium berghei*, and *Plasmodium yoelii*.

3. The method of Claim 1, wherein the live, apicoplast-deficient *Plasmodium* parasite is a *Plasmodium falciparum* parasite.

4. The method of any one of Claims 1-3, wherein the apicoplast inhibitor is selected from the group consisting of fosmidomycin, chloramphenicol, doxycycline, ciprofloxacin, trovofloxacin, rifampicin, rifabutin, clindamycin, azithromycin, spiramycin, thiostrepton, micrococcin, tetracycline, amythiamicin, thiolactomycin, cerulenin, clodinafop, quizlofop, haloxypop, fenoxaprop, tralkoxydim, diclofop, and FR-900098.

5. The method of any one of Claims 1-3, wherein the apicoplast inhibitor is selected from the group consisting of fosmidomycin, chloramphenicol, and doxycycline.

6. The method of any one of Claims 1-5, wherein steps i) and ii) are repeated at least once or twice.

7. The method of any one of Claims 1-6, wherein the live apicoplast-deficient *Plasmodium* parasite has an apicoplast genome to nuclear genome ration of less than 0.50, 0.25., 0.20, 0.15, 0.10, or 0.05.

8. A live, apicoplast-deficient *Plasmodium* parasite obtained by the method of any one of Claims 1-7.

9. An isolated erythrocyte infected with the live, apicoplast-deficient *Plasmodium* parasite of Claim 8.

10. A method for inducing an immune response in a mammalian subject to a *Plasmodium* parasite, comprising: i) providing a pharmaceutical formulation comprising: a) a live, apicoplast-deficient *Plasmodium* parasite; or b) an erythrocyte infected with the live, apicoplast-deficient *Plasmodium* parasite; and ii) administering said pharmaceutical formulation to a mammalian subject in an amount effective to induce an immune response in said mammalian subject to said *Plasmodium* parasite.

11. The method of Claim 10, wherein the live, apicoplast-deficient *Plasmodium* parasite is a merozoite.

12. The method of Claim 10 or 11, wherein the erythrocyte is an O-negative human erythrocyte.

13. The method of any one of Claims 10-12, wherein the mammalian subject is selected from the group consisting of a human, a monkey and a mouse.

14. The method of any one of Claims 10-13, wherein administration of said pharmaceutical formulation results in a transient infection of the subject's erythrocytes by said apicoplast-deficient *Plasmodium* parasite.

15. The method of any one of Claims 10-14, wherein the immune response comprises one or more of a *Plasmodium* parasite-reactive antibody response, cell proliferation and cytokine secretion.

16. A pharmaceutical formulation comprising an effective amount of a live, apicoplast-deficient *Plasmodium* parasite or an erythrocyte infected with the live, apicoplast-deficient *Plasmodium* parasite, for inducing an immune response in a mammalian subject to the *Plasmodium* parasite.

17. A method for screening drug candidates, comprising treating erythrocytes infected with live, apicoplast-containing *Plasmodium* parasites with a drug candidate, in the presence and absence of isopentenyl pyrophosphate (IPP) supplementation, wherein replication of said parasites in the presence but not the absence of IPP supplementation indicates that the drug candidate is an apicoplast-inhibitor.

18. The method of Claim 17, wherein the replication of said parasites is determined by measuring parasitemia or metabolism.

19. The method of Claim 17 or 18, wherein the *Plasmodium* parasites are ring-stage parasites at the onset of the treatment with the drug candidate.

20. The method of any one of Claims 17-19, wherein the treatment with the drug candidate is done for at least 48 hours before the IPP supplementation.

21. The method of Claim 20, comprising at least two or three rounds of the treatment and IPP supplementation.

22. The method of any one of Claims 17-21, wherein the IPP supplementation comprises contacting the infected erythrocytes with IPP or an analog or salt thereof.

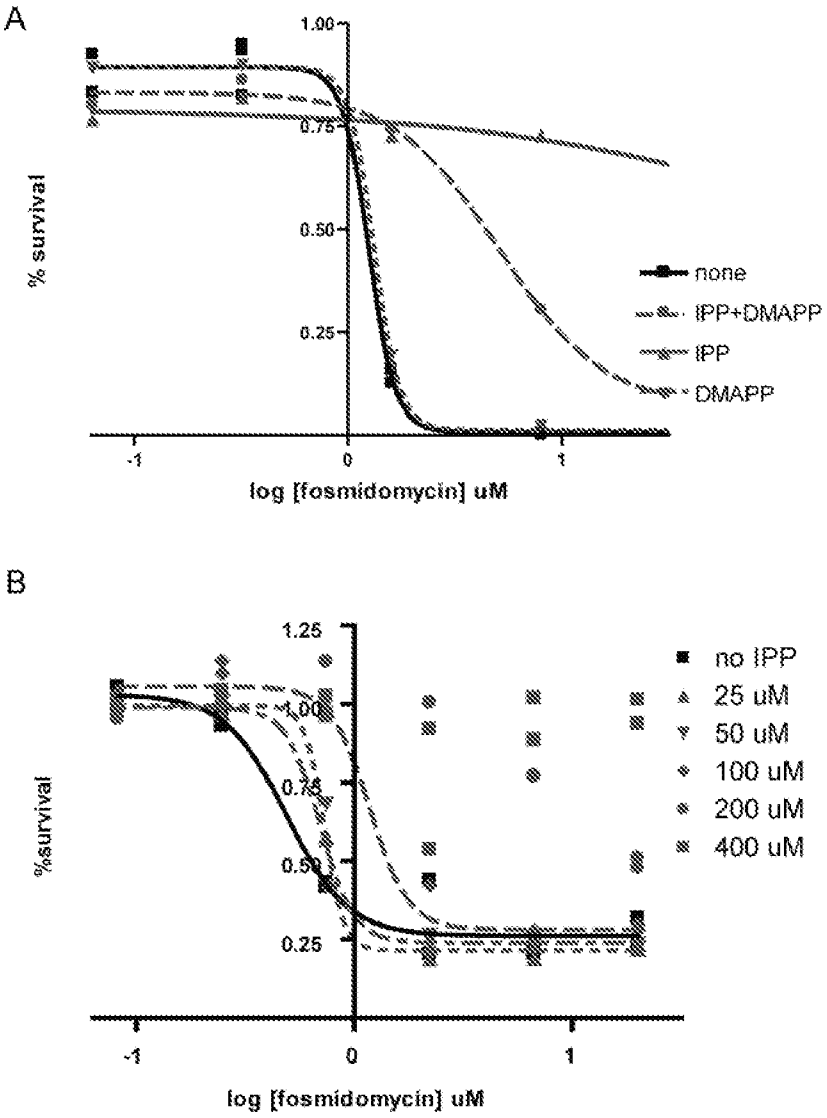
A
1.00
0.75
0.50
0.25
% survival
-1
0
1
log [fosmidomycin] uM
none
IPP+DMAPP
IPP
DMAPP
B
1.25
1.00
0.75
0.50
0.25
%survival
-1
0
1
log [fosmidomycin] uM
no IPP
25 uM
50 uM
100 uM
200 uM
400 uM

FIG. 1

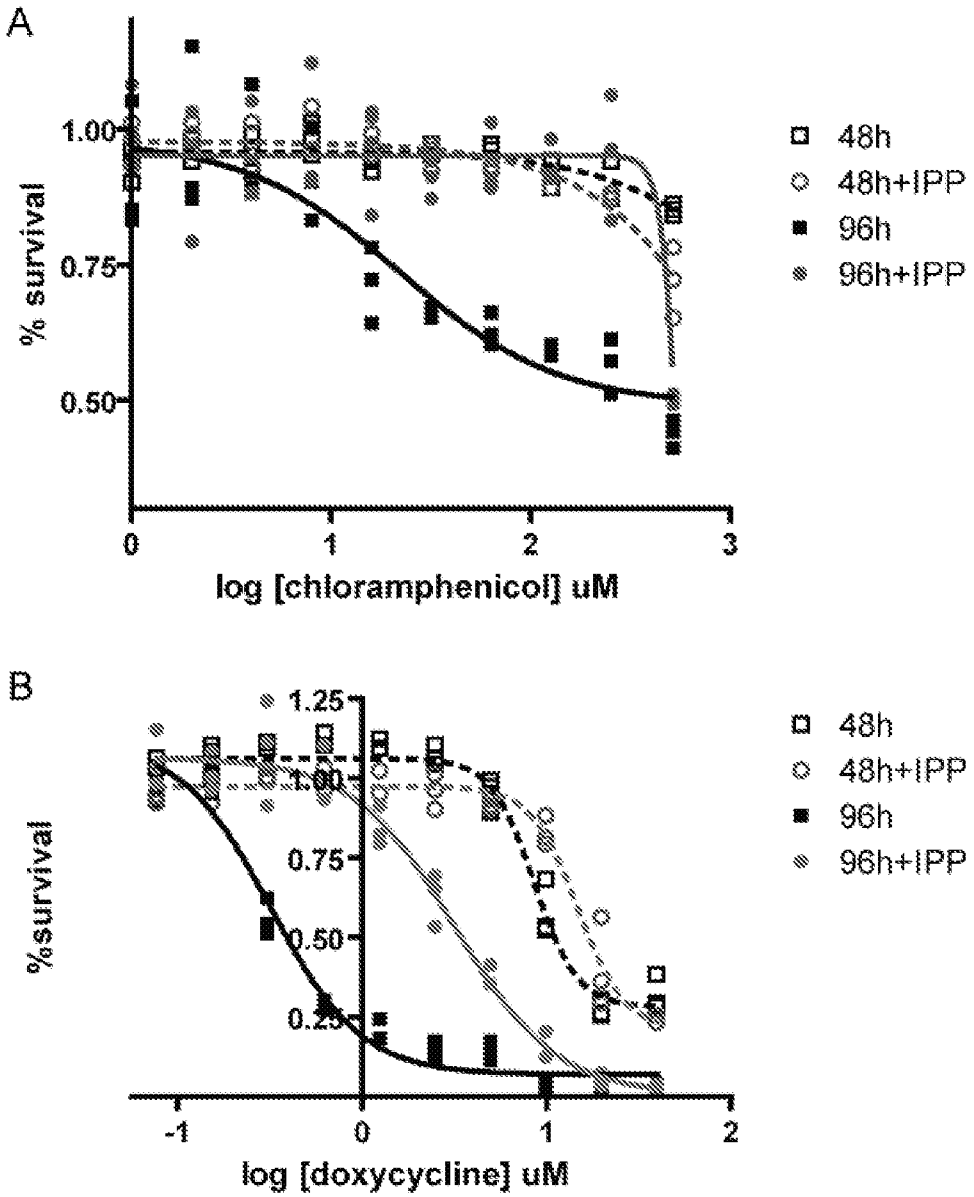
A
1.00
0.75
0.50
% survival
0
1
2
3
log [chloramphenicol] uM
48h
48h+IPP
96h
96h+IPP
B
1.25
1.00
0.75
0.50
0.25
%survival
-1
0
1
2
log [doxycycline] uM
48h
48h+IPP
96h
96h+IPP

FIG. 2

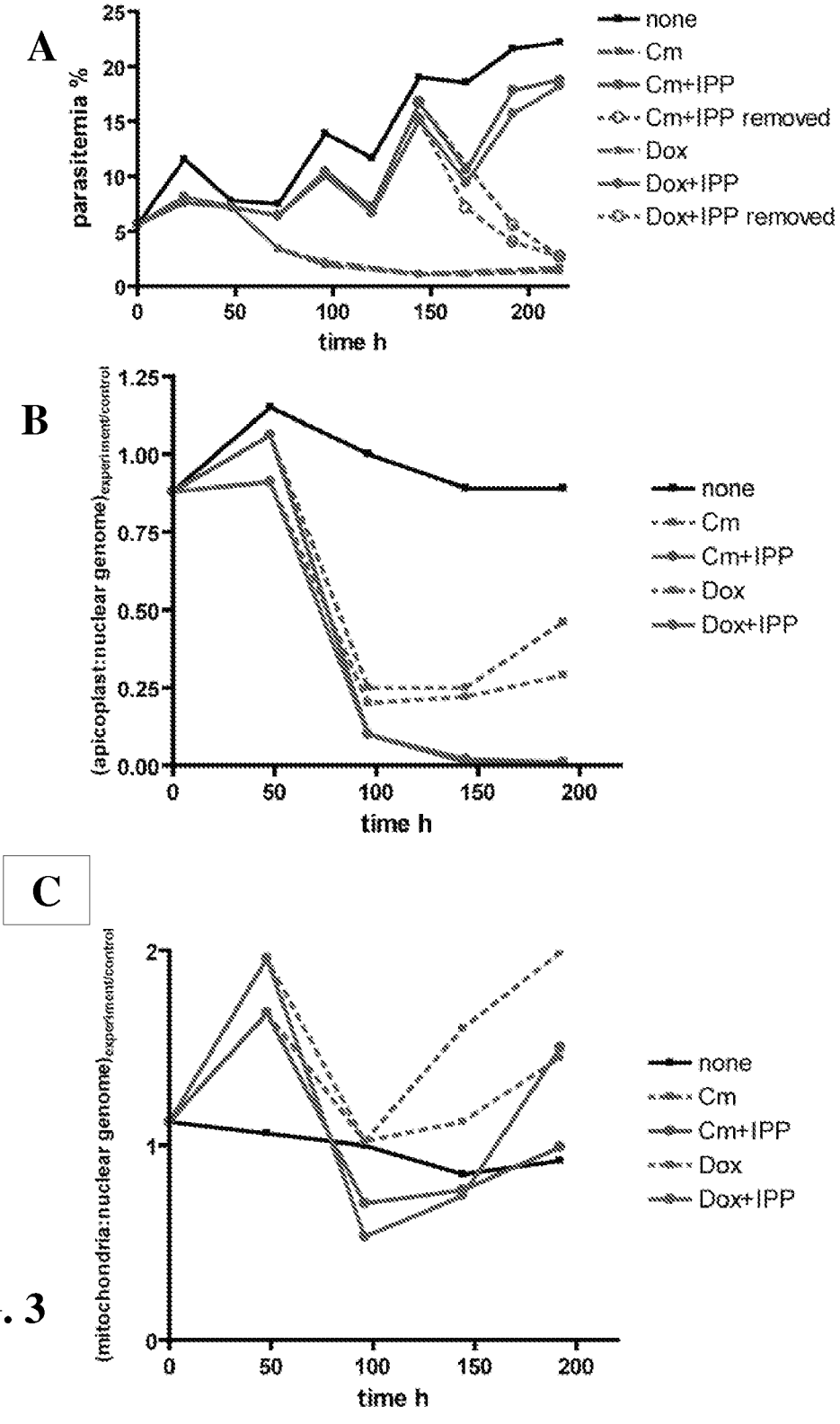
A
parasitemia %
time h
none
Cm
Cm+IPP
Cm+IPP removed
Dox
Dox+IPP
Dox+IPP removed
B
(apicoplast:nuclear genome)experiment/control
time h
none
Cm
Cm+IPP
Dox
Dox+IPP
C
(mitochondria:nuclear genome)experiment/control
time h
none
Cm
Cm+IPP
Dox
Dox+IPP

FIG. 3

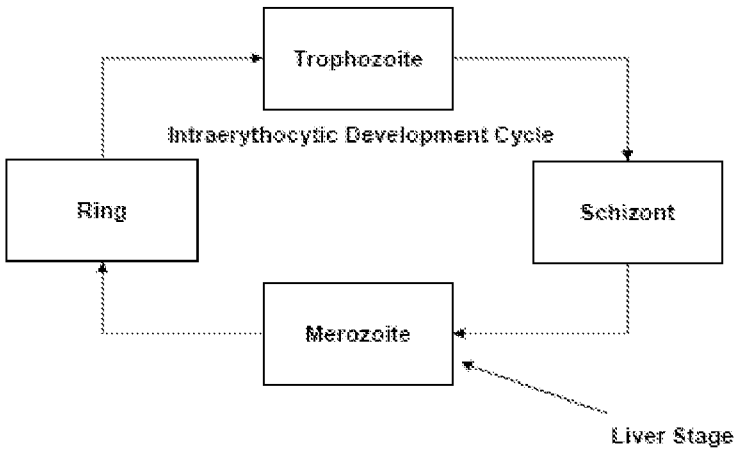
Trophozoite
Intraerythocytic Development Cycle
Ring
Schizont
Merozoite
Liver Stage

FIG. 4

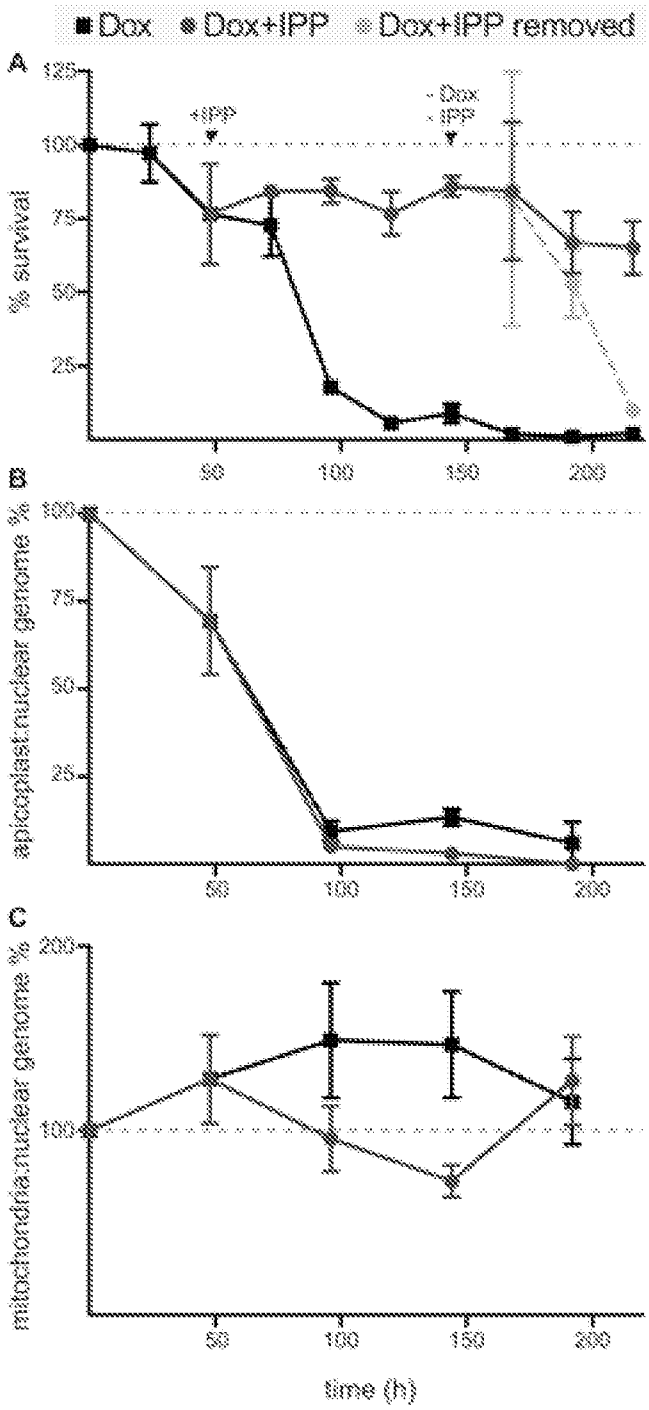
Dox
Dox+IPP
Dox+IPP removed
A
+IPP
-Dox
-IPP
% survival
125
100
75
50
25
50
100
150
200
B
apicoplast:nuclear genome %
100
75
50
25
50
100
150
200
C
mitochondria:nuclear genome %
200
100
50
100
150
200
time (h)

FIG. 5

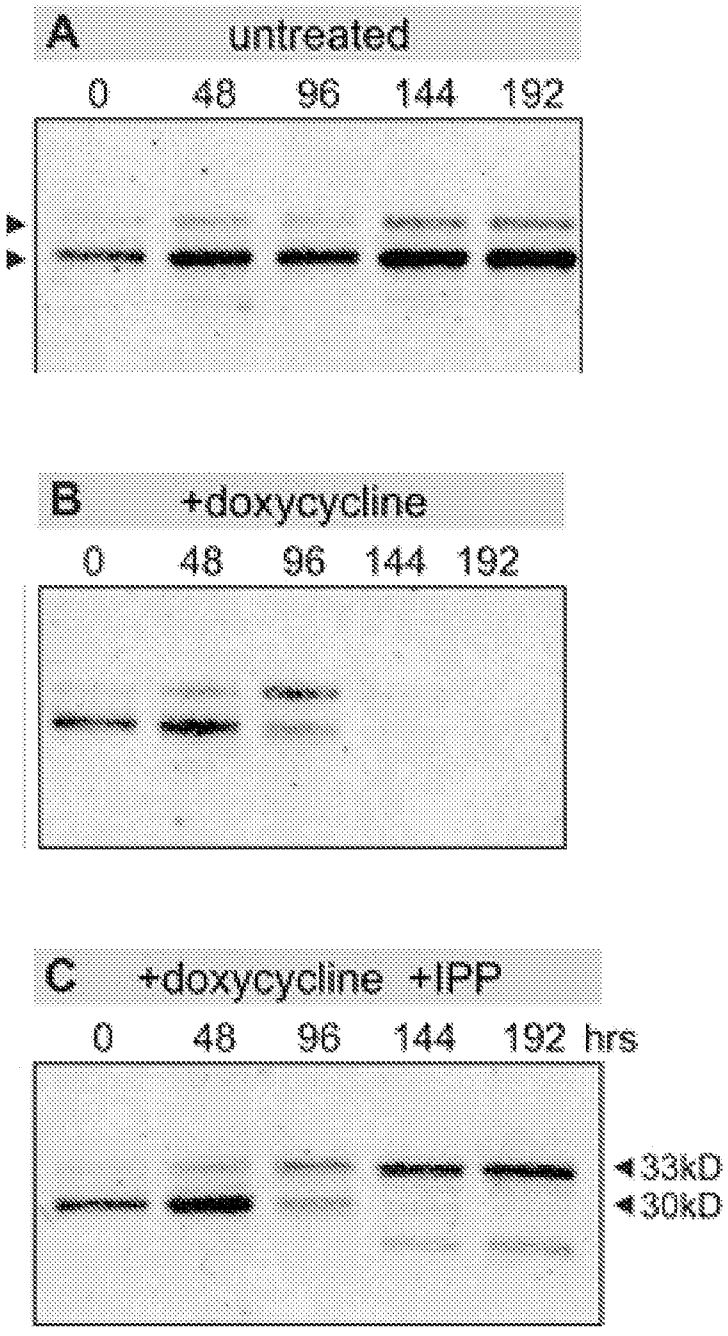
A untreated
0 48 96 144 192
B +doxycycline
0 48 96 144 192
C +doxycycline +IPP
0 48 96 144 192 hrs
33kD
30kD

FIG. 6

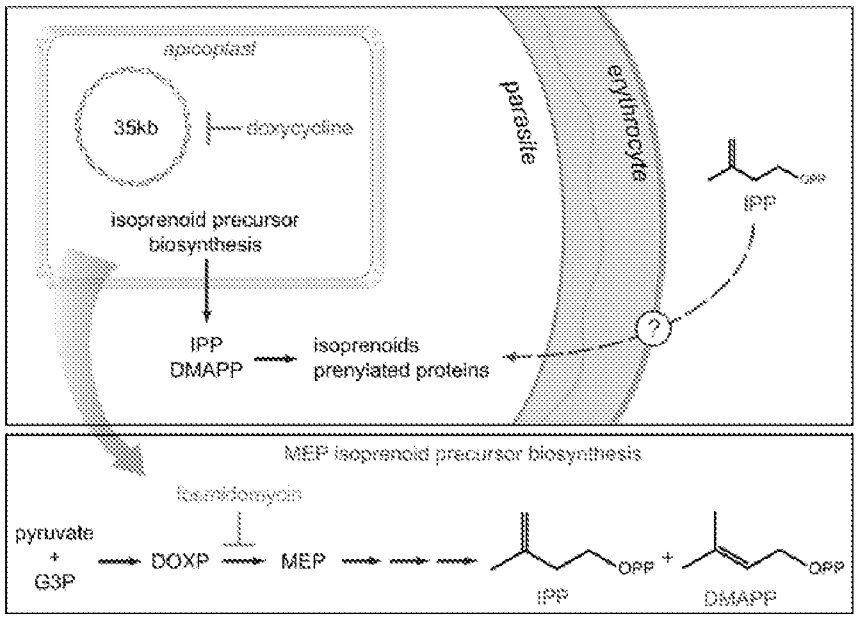
apicoplast
35kb
doxycycline
isoprenoid precursor biosynthesis
IPP
DMAPP
isoprenoids
prenylated proteins
parasite
erythrocyte
?
IPP
MEP isoprenoid precursor biosynthesis
fosmidomycin
pyruvate
+
G3P
DOXP
MEP
OPP
IPP
+
OPP
DMAPP

FIG. 7