



HAL
open science

Azithromycin disrupts apicoplast biogenesis in replicating and dormant liver stages of the relapsing malaria parasites *Plasmodium vivax* and *Plasmodium cynomolgi*

Nadia Amanzougaghene, Shahin Tajeri, Jean-François Franetich, Kutub Ashraf, Valérie Soulard, Pierre Bigeard, Cheick Oumar Guindo, Camille Bouillier, Julien Lemaitre, Francis Relouzat, et al.

► **To cite this version:**

Nadia Amanzougaghene, Shahin Tajeri, Jean-François Franetich, Kutub Ashraf, Valérie Soulard, et al.. Azithromycin disrupts apicoplast biogenesis in replicating and dormant liver stages of the relapsing malaria parasites *Plasmodium vivax* and *Plasmodium cynomolgi*. *International Journal of Antimicrobial Agents*, 2024, 63 (5), pp.107112. 10.1016/j.ijantimicag.2024.107112 . hal-04697389

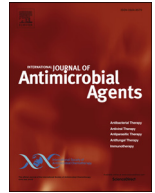
HAL Id: hal-04697389

<https://hal.science/hal-04697389v1>

Submitted on 24 Nov 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Azithromycin disrupts apicoplast biogenesis in replicating and dormant liver stages of the relapsing malaria parasites *Plasmodium vivax* and *Plasmodium cynomolgi*

Nadia Amanzougaghene^{a,b}, Shahin Tajeri^a, Jean-François Franetich^a, Kutub Ashraf^a, Valérie Soulard^a, Pierre Bigeard^a, Cheick Oumar Guindo^a, Camille Bouillier^b, Julien Lemaitre^b, Francis Relouzat^b, Roger Legrand^b, Clemens H.M. Kocken^c, Anne-Marie Zeeman^c, Wanlapa Roobsoong^d, Jetsumon Sattabongkot^d, Zhaoqing Yang^e, Georges Snounou^{b,**}, Dominique Mazier^{a,*}

^a Sorbonne Université, INSERM, CNRS, Centre d'Immunologie et des Maladies Infectieuses, CIMI-Paris, Paris, France

^b Université Paris-Saclay, Inserm, CEA, Immunologie des maladies virales, auto-immunes, hématologiques et bactériennes (IMVA-HB/IDMIT/UMR1184), Fontenay-aux-Roses and Kremlin-Bicêtre, France

^c Department of Parasitology, Biomedical Primate Research Centre, Rijswijk, The Netherlands

^d Mahidol Vivax Research Unit, Faculty of Tropical Medicine Mahidol University, Bangkok, Thailand

^e Department of Pathogen Biology and Immunology, Kunming Medical University, Chenggong New Town, Kunming, Yunnan Province, China

ARTICLE INFO

Article history:

Received 1 November 2023

Accepted 8 February 2024

Editor: D. Leitsch

Keywords:

Plasmodium vivax

Plasmodium cynomolgi

Hypnozoite

Apicoplast

Azithromycin

ABSTRACT

The control and elimination of malaria caused by *Plasmodium vivax* is hampered by the threat of relapsed infection resulting from the activation of dormant hepatic hypnozoites. Currently, only the 8-aminoquinolines, primaquine and tafenoquine, have been approved for the elimination of hypnozoites, although their use is hampered by potential toxicity. Therefore, an alternative radical curative drug that safely eliminates hypnozoites is a pressing need. This study assessed the potential hypnozoitocidal activity of the antibiotic azithromycin, which is thought to exert antimalarial activity by inhibiting prokaryote-like ribosomal translation within the apicoplast, an indispensable organelle. The results show that azithromycin inhibited apicoplast development during liver-stage schizogony in *P. vivax* and *Plasmodium cynomolgi*, leading to impaired parasite maturation. More importantly, this study found that azithromycin is likely to impair the hypnozoite's apicoplast, resulting in the loss of this organelle. Subsequently, using a recently developed long-term hepatocyte culture system, this study found that this loss likely induces a delay in the hypnozoite activation rate, and that those parasites that do proceed to schizogony display liver-stage arrest prior to differentiating into hepatic merozoites, thus potentially preventing relapse. Overall, this work provides evidence for the potential use of azithromycin for the radical cure of relapsing malaria, and identifies apicoplast functions as potential drug targets in quiescent hypnozoites.

© 2024 The Authors. Published by Elsevier Ltd.

This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

1. Introduction

Plasmodium vivax, an apicomplexan vector-borne parasite, is the most widely distributed human malaria parasite in tropical areas outside of Africa. It causes serious morbidity, with an estimated

2.8 billion people at risk of infection [1,2]. A major obstacle to *P. vivax* eradication is its ability to cause relapse episodes by the activation of dormant liver-stage hypnozoites weeks, months or even years after resolution of the primary blood-stage infection in individuals, who had no further infectious mosquito bites [3]. This

* Corresponding author. D. Mazier, Sorbonne Université, INSERM, CNRS, Centre d'Immunologie et des Maladies Infectieuses, CIMI-Paris, Paris, France.

** Corresponding author. G. Snounou, Université Paris-Saclay, Inserm, CEA, Immunologie des maladies virales, auto-immunes, hématologiques et bactériennes (IMVA-HB/IDMIT/UMR1184), 92265 Fontenay-aux-Roses and Kremlin-Bicêtre, France.

E-mail addresses: gsnounou@gmail.com (G. Snounou), dominique.mazier@sorbonne-universite.fr (D. Mazier).

salient biological feature also occurs in *Plasmodium ovalecurtisi* and *Plasmodium ovalewallikeri* infections, and in some non-human primate malaria parasites including *Plasmodium cynomolgi*, a species that is used as the model for *P. vivax* [3–6]. However, the biological mechanisms underlying hypnozoite formation, persistence and activation remain a mystery, and constitute a major obstacle to developing novel drugs against these non-replicating forms.

While several antimalarial drugs that can kill growing parasites in the liver are available, the 8-aminoquinoline primaquine remains the only therapeutic option to eliminate hypnozoites [2,4,7,8]. However, its long dosing schedule (for up to 14 days) and potential haemolytic toxicity in patients with glucose-6-phosphate dehydrogenase (G6PD)-deficient red cells, a genetic disorder that is fairly common in malaria-endemic areas [2,4,8], makes its deployment to achieve radical cure challenging. Tafenoquine, another 8-aminoquinoline, has been approved in some countries recently, but is yet to be deployed. Nonetheless, despite its longer half-life and shorter dosing schedule, tafenoquine can still cause severe haemolytic anaemia in G6PD-deficient individuals [2,9]. Thus, an efficacious alternative drug that eliminates hypnozoites safely is a pressing need, and will be crucial for vivax malaria elimination in the coming decades.

Antibiotics known to interfere with the apicoplast, a relict bacterium-like organelle found in most apicomplexan parasites [10], may offer an interesting alternative to eliminate hypnozoites. This organelle constitutes an attractive drug target as it harbours several metabolic pathways, such as the synthesis of fatty acids, isoprenoid precursors, haem and iron-sulphur complexes, that are essential for parasite survival [11–13]. The biogenesis, division and inheritance of the apicoplast during the repeated cycles of host cell invasion and parasite replication can be inhibited specifically by antibiotics by interfering with the housekeeping functions of this organelle, such as DNA replication, transcription or protein translation [10,13]. With few exceptions, in erythrocytic *P. falciparum* parasites, these antibiotics cause a distinctive 'delayed-death' phenotype, in which parasite growth is unaffected during the first asexual replication cycle after drug exposure, but is arrested during the subsequent division cycle, even after drug removal [14,15]. This effect is due to the fact that the progeny of antibiotic-treated parasites inherit non-functional apicoplasts that are unable to sustain their critical biological processes [10]. An effect comparable to the delayed-death phenotype is also observed for the intrahepatic stages of *Plasmodium berghei*, which show no apparent defect in maturation when treated early with apicoplast-targeting antibiotics, but subsequently fail to establish a blood-stage infection due to impaired apicoplast biogenesis [16,17].

Antibiotics that cause delayed death, such as doxycycline, clindamycin and the longer-acting azithromycin, are widely used for malaria treatment and prophylaxis [18–22]. The use of azithromycin is particularly attractive for antimalarial therapies because it displays a good safety profile in pregnant women and may also be used in young children, the most vulnerable populations at risk of malaria. Moreover, it is eliminated slowly from the body and accumulates in the liver [20,23], where the first obligatory replicative stage of the plasmodium infection occurs in mammalian hosts.

2. Materials and methods

2.1. Ethics statement

Adult cynomolgus macaques (*Macaca fascicularis*) that were captive-bred for research purposes and housed in the Infectious Disease Models for Innovative Therapies facilities at the Commissariat d'Énergie Atomique (CEA; Fontenay-aux-Roses, France)

were used for production of *P. cynomolgi* M strain sporozoites and liver cells. All animals were maintained in accordance with recommendations of the Weatherall report, and with French national regulations under the inspections of national veterinary inspectors. CEA complies with Standards for Human Care and Use of Laboratory Animals (Animal Welfare Assurance, OLAW number #A5826-01). The animals were used under the supervision of the veterinarians in charge of the animal facility, and the protocols employed were reviewed by the Ethical Animal Committee of CEA. Animal housing was in accordance with international guidelines for non-human primate care and use. Experimental procedures were conducted in strict accordance with the recommendations of the European guidelines for the care and use of laboratory animals (European directive 63/210). All protocols and the use of hepatocytes for the purpose of the work described here were approved by the Ethical Animal Committee of CEA (permit number A 92-032-02).

Adult captive-bred rhesus monkeys (*Macaca mulatta*, Indian origin) housed in the Biomedical Primate Research Centre (Rijswijk, The Netherlands) were used for production of *P. cynomolgi* M transgenic strain (PcyC-PAC-GFP_{hsp70}-mCherryLisp2) sporozoites. The research protocol was approved by the Central Committee for Animal Experiments (licence number AVD5020020172664), and the subprotocol was approved by the local independent ethical committee constituted conform Dutch law (BPRC Dier Experimenten Commissie, Instantie voor Dierenwelzijn, IvD; agreement number #007 C) prior to the start of the experiments. All experiments were performed according to Dutch and European laws. The Council of the Association for Assessment and Accreditation of Laboratory Animal Care has been awarded BPRC full accreditation. Thus, BPRC is fully compliant with the international demands on animal studies and welfare as set forth by European Council Directive 2010/63/EU, and Convention ETS 123, including the revised Appendix A as well as the 'Standard for Humane Care and Use of Laboratory Animals by Foreign Institutions' (identification number A5539-01), provided by the Department of Health and Human Services of the US National Institutes of Health and Dutch implementing legislation.

For production of *P. vivax* sporozoites, infected blood collection was approved by the Ethics Committee of Faculty of Tropical Medicine, Mahidol University (MUTM 201 L-040-05). Informed consent was obtained from each patient before sample collection.

All procedures involving murine models complied with European regulations. The protocol was ethically approved by the Ministère de l'Éducation Nationale, de l'Enseignement Supérieur et de la Recherche (authorization number 01737.03).

2.2. Primary cryopreserved hepatocyte culture conditions

Primary human and *M. fascicularis* hepatocytes were used in this study. *M. fascicularis* primary hepatocytes were isolated from liver segments taken from healthy *M. fascicularis* (kept at CEA), using a collagenase perfusion technique as described previously [24], and were cryopreserved immediately. Cryopreserved primary human hepatocyte vials were purchased from Biopredic (Lot no. LHuf17905A) (Biopredic International, Saint-Grégoire, France).

The cryopreserved hepatocytes were thawed at 37°C and were seeded into 96- or 384-well plates (Greiner Bio-One, Frickenhausen, Germany) pre-coated with rat-tail collagen I (BD Biosciences, San Jose, CA, USA) at a density of approximately 80,000 or 25,000 cells per well, respectively. The hepatocytes were cultivated at 37°C in 5% CO₂ in William's E medium (Gibco) supplemented with 10% (v/v) fetal clone III serum (FCS; Hyclone, Cytiva, Chicago, IL, USA), 2 mM L-glutamine, 100 u/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies, Villebon sur Yvette, France), 5 × 10⁻³ g/L human insulin (Sigma Aldrich, St

Table 1
List of primary antibodies used in the study

Antibody	Source	Working dilution	References
Recombinant mouse, anti-PvUIS4	Noah Sather (Centre for Infectious Disease Research, Seattle, USA)	1:20,000	[55]
Mouse anti-PcUIS4, polyclonal	Laurent Rénia (Singapore Immunology Network, Agency for Science, Technology, and Research, Singapore)	1:500	[56]
Recombinant rabbit, monoclonal anti-PyACP	Scot E. Lindner (Huck Centre for Malaria Research, Penn State, USA)	1:250	[57]

Louis, MO, USA) and 5×10^{-5} M hydrocortisone (Upjohn Laboratories SERB, Paris, France). After 1 day, the cells were overlaid with matrigel (Corning Life Sciences, Amsterdam, The Netherlands), and were cultured for 1–4 days for *M. fascicularis* and human hepatocytes until infection with sporozoites.

2.3. *Plasmodium* sporozoite production and isolation

For *P. cynomolgi* (M strain), *M. fascicularis* was infected with the *P. cynomolgi* M strain by injection of frozen infected *M. fascicularis* blood. The resulting blood-stage parasitaemia was monitored by Giemsa-stained smears, and blood samples were collected at appropriate times and transferred safely to Centre d'Immunologie et des Maladies Infectieuses (CIMI; Paris, France) to infect *Anopheles stephensi* mosquitoes, bred in the insectary facility, using an Hemotek membrane feeding system (Hemotek Ltd, Blackburn, UK), as described previously [24].

For *P. vivax*, infected blood samples were collected from symptomatic patients attending malaria clinics in Sai Yok District, Kanchanaburi Province, and Tha Song Yang District, Tak Province, Thailand. *Anopheles dirus* mosquitoes were allowed to feed on the infected blood by membrane feeding, as described previously [25]. The infected mosquitoes were then shipped safely to CIMI where they were dissected to recover the sporozoites at the time of hepatocyte infection.

Two to three weeks after the blood meal, *P. vivax* and *P. cynomolgi* sporozoites were extracted from hand-dissected mosquito salivary glands crushed in a potter. The sporozoites were recovered after filtration through a 40- μ m filter (Cell Strainer, Becton Dickinson, Franklin Lakes, NJ, USA), as described previously [24,26]. The sporozoites were counted using a disposable Glasstic microscope slide (KOVA, Garden Grove, CA, USA) and kept on ice until hepatocyte infection.

2.4. Hepatocyte infection with *P. vivax* and *P. cynomolgi* sporozoites and drug assays

For infection, matrigel was removed from hepatocyte cultures and sporozoites were added. Briefly, *P. vivax* and *P. cynomolgi* sporozoites were resuspended in the complete medium used for hepatocyte culture. A total of 30,000 or 20,000 sporozoites per well were inoculated into hepatocyte cultures in 96- or 384-well plates, respectively. The infected cultures were then centrifuged at $560 \times g$ for 10 min at room temperature to allow fast sporozoite sedimentation on to the target cells, and thereafter were incubated at 37°C under 5% CO₂. Three hours later, cultures were washed and a layer of matrigel (Corning Life Sciences) was added, as per the manufacturer's recommendations. To allow for matrigel layer polymerization, the cultures were incubated for 45 min at 37°C prior to adding the medium, which was thereafter renewed every 24 h.

For the drug assays, complete medium supplemented with the appropriate azithromycin (Sigma-Aldrich) concentration was added and changed daily. In the first experiments, infected hepatocytes were exposed to a two-fold dilution azithromycin series, from 20 μ M to 1.25 μ M, from day 4 until fixation at day 8 post-sporozoite

infection for *P. vivax*, and from day 4 to day 10 post-infection for *P. cynomolgi*. In a second set of experiments, cultures were treated with 10 μ M azithromycin from day 4 to day 18 and fixed on days 10, 14, 16 and 18; or treated from day 4 to day 16 and fixed on days 10, 16, 20, 24 and 28. In any individual experiment, each concentration was tested in quadruplicate in 384-well plates or in triplicate in 96-well plates. For each defined time point, cultivation was ended by fixation with 4% (w/v) paraformaldehyde solution for 15 min at room temperature.

2.5. Parasite immuno-fluorescence assay, quantification and confocal imaging

The exoerythrocytic forms (EEFs) were stained using mouse polyclonal anti *Pc*-UIS4 (*P. cynomolgi*) and anti-PvUIS4 (*P. vivax*) (see Table 1), and revealed with an Alexa 488-conjugated goat anti-mouse immunoglobulin (Molecular Probes, Eugene, OR, USA). Host cell and parasite nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI). The EEFs were counted manually under fluorescence microscopy (Leica DMI4000B, Wetzlar, Germany), and their area was measured using a Cell Insight High Content Screening platform equipped with the Studio HCS software (Thermo Fisher Scientific, Waltham, MA, USA) in Celis Platform (ICM, La Pitié-Salpêtrière, Paris), as described previously [27].

To investigate the effect of azithromycin inhibition on apicoplast maturation, the EEFs were stained using acyl carrier protein (ACP) antibody, revealed with an Alexa Fluor 568-conjugated goat anti-rabbit immunoglobulin (Molecular Probes), and imaged using a confocal microscope (Leica SP8 white laser microscope) at 63X magnification in Quant Platform (ICM), as described previously [28]. For each condition, 100 parasites were observed. The images were analysed using ImageJ software (<https://fiji.sc/>).

2.6. Fluorescence-activated cell sorting of *P. cynomolgi*-GFP-infected hepatocytes and quantitative polymerase chain reaction quantification of apicoplasts

A. stephensi mosquitoes infected with *P. cynomolgi* M strain PcyC-PAC-GFP_{hsp70}-mCherryLisp2 parasites, provided by Clemens H M Kocken (Biomedical Primate Research Centre, Rijswijk, The Netherlands) [29] were used. Salivary gland sporozoites from these parasites were isolated from infected mosquitoes and added to hepatocytes as described above. Hepatocytes were seeded into collagen-coated six-well plates (Falcon by Becton Dickinson Labware Europe, France) at a density of approximately 2×10^6 cells per well, and 24 h later, 1×10^6 sporozoites were added per well. The infected hepatocytes were exposed to 10 μ M azithromycin from day 4 to day 10. At day 10 post-infection, the hepatocytes were harvested by trypsin treatment (0.25% trypsin-EDTA, 1 min at 37°C). Cells were pelleted by centrifugation, washed with phosphate buffered saline (PBS), resuspended in FACS solution, and transferred on ice to the cytometry platform (CYBIO, Cochin Institute, Paris, France). Hepatocytes containing GFP-expressing uninucleate forms (GFPlow) or liver schizonts (GFPhigh) were then FACS-sorted using a FACSAria flow cytometer equipped with a 488-nm coherent sapphire solid state 20 mW laser (BD Biosciences),

as described previously [30]. gDNA was isolated using a NucleoSpin tissue kit (Macherey-Nagel, Düren, Germany) as per the manufacturer's instructions. The quantity and quality of the extracted DNA were assessed using a NanoDrop ND-1000 (Thermo Fisher Scientific). Primers for quantitative polymerase chain reactions (qPCRs) were designed using the free Primer3 software, Version 4.0 (<https://primer3.org/>) to specifically amplify the apicoplast elongation factor Tu (EFTu) gene (PcyM_API01900, forward: 5'- TTCAAGCTCAATCCGGAGATA -3'; reverse: 5'- ATGACGAC-CTCCTTCTTCCTT -3'), the nuclear-encoded chitinase (CHT) genes (PcyM_0102900, forward: 5'- CCCCCAGGTAGAAGTACCAA -3'; reverse: 5'- GCACGGGTGAATATGACCTA -3') and the serine-tRNA ligase (stNRA) gene (PcyM_0317000-t36_1, forward: 5'- ACGTCA-GAAGGGAACAAAGGTA -3'; reverse: 5'- TCAGCAGGTCGAAGTG-GTAGTA -3'). All qPCR reactions were performed using an Applied Biosystems 7300 Real-Time PCR System with the Power SYBR Green PCR Master Mix kit (Applied Biosystems, Waltham, MA, USA), in accordance with the manufacturer's instructions. The cycling conditions were one cycle of 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s. Two biological replicates, with three technical replicates for each, were undertaken for each sample. The housekeeping tRNA gene was used for internal normalization [28]. Relative copy numbers of target genes relative to tRNA were calculated according to the $2^{-\Delta\Delta Ct}$ method [31].

2.7. *P. berghei* cultures and mouse infection

To determine the ability of azithromycin-treated late schizonts to establish blood-stage infections in naïve mice, HepG2 cell cultures were infected with sporozoites of *P. berghei* parasites and treated with 10 µM azithromycin. Briefly, 24 h prior to sporozoite infection, HepG2 cells (HB-8065; ATCC) were seeded in 24-well plates, pre-coated with collagen I, at a density of 15×10^3 cells/cm², in Dulbecco-modified Eagle medium supplemented with 10% fetal calf serum and antibiotics. On the day of infection, *P. berghei* (ANKA strain transgenic for GFP)-infected mosquitoes were dissected as described above, sporozoites were counted, and each well of a 24-well plate received approximately 3×10^4 sporozoites. The infected cultures were then incubated in the presence or absence of azithromycin at 37°C, 5% CO₂, with 90% humidity. At 66 h post-infection, parasites (all late schizonts and detached merozoites confused) were harvested from untreated and azithromycin-treated infected HepG2 cells, washed, resuspended in a drug-free medium, and then injected into susceptible mice. A total of six mice was used for each group, and for each mouse, an entire well of a 24-well plate, which contains an average of 1000 parasites, was injected intraperitoneally. Blood-stage parasitaemia was monitored by Giemsa-stained smears using light microscopy. All mice used in these experiments were female BALB/c mice (average weight was approximately 18 g) purchased from René Janvier (Le Genest-Saint-Isle, France).

To assess the effect of azithromycin treatment on the sporozoite stage, freshly dissected *P. berghei*-GFP sporozoites were incubated with varying concentrations of azithromycin for 30 min at room temperature. The sporozoites were recovered by centrifugation and resuspended in fresh culture medium. For quantification and confocal imaging, some sporozoites were placed on a coverslip then fixed for 10 min with 4% PFA followed by two washes with PBS, stained with DAPI and ACP antibody as described above, and imaged using a confocal microscope. To assess their hepatic development, these pre-incubated sporozoites were added to primary cultures of *M. fascicularis* hepatocytes. In total, 30,000 sporozoites per well were inoculated into hepatocyte cultures in 384-well plates

and cultivated until day 2 post-infection. The number of schizonts and area measurements were determined as described above.

2.8. Data analysis

GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) was used for data analysis and graphing. All values have been expressed as mean and standard deviation. A *P*-value ≤ 0.05 was considered to indicate significance.

3. Results

3.1. Liver-stage development in *P. vivax* and *P. cynomolgi* is blocked by azithromycin treatment

This study evaluated the effect of azithromycin on the in-vitro liver stages in primary hepatocytes of the relapsing parasites, *P. vivax* and *P. cynomolgi*. Using a radical cure mode assay, the parasites were exposed to escalating doses of azithromycin from day 4 to day 8 post-sporozoite infection for *P. vivax*, and from day 4 to day 10 post-infection for *P. cynomolgi* [32]. Parasite counts were carried out on days 8 and 10, respectively. Two types of EEF were observed for both species: large multi-nucleated schizonts and small uninucleated hypnozoite-like forms, as reported previously [26]. For both species, quantification of schizonts and uninucleate hepatic parasite numbers revealed no differences in azithromycin-treated cultures compared with untreated controls (Fig. 1A). However, the exposed schizonts showed a significant reduction in surface area in a dose-independent manner (Fig. 1B,C). More specifically, >67% of the untreated *P. vivax* schizonts had an area >1500 µm² (mean area 1419.10 µm²), whereas, at the highest dose, nearly all (97%) azithromycin-treated schizonts had an area <1500 µm² (mean area 449.29 µm²).

3.2. Azithromycin disrupts apicoplast integrity in *P. vivax* and *P. cynomolgi* multi-nucleate and uninucleate forms

Next, this study investigated whether the observed effect on schizont development was reflected by the inhibition of apicoplast maturation in the exposed parasites, and, more importantly, whether azithromycin was able to interfere with the apicoplast in the uninucleate forms. Apicoplast structure was visualized through staining of the parasites' ACP, a resident protein (Fig. 2A,C). In the liver stage, the apicoplast grows from a single dot/elongate structure that elongates progressively within the cytoplasm during the early schizogony to develop into a highly branched network structure in the late schizogony before dividing, such that each daughter merozoite inherits a single small apicoplast prior to release into the circulation [16,28,33]. In the untreated cultures, all the schizonts from both species showed the normal apicoplast biogenesis expected late in the schizogony; indeed, all the apicoplasts in the *P. vivax* and *P. cynomolgi* parasites were either as networks or segregated into formed merozoites (Fig. 2A,B). In contrast, exposure to azithromycin led to a disruption in the development of the apicoplasts or their complete loss in most of the parasites for both species (Fig. 2A,B). The proportion of schizonts with disrupted apicoplasts reached 71% for *P. vivax* and 80.5% for *P. cynomolgi*, with a concomitant decrease in the proportion of those with normal apicoplasts (Fig. 2B).

With respect to the uninucleate forms of both species, all showed the typical apicoplast development expected at this late period of development in the untreated cultures, with two distinguishable apicoplast morphologies: a dot or elongate-shaped apicoplast; or a branched morphology (Fig. 2C,D). In azithromycin-treated cultures of both species, more than half of the uninucleate forms had apicoplasts with diffuse punctate foci throughout

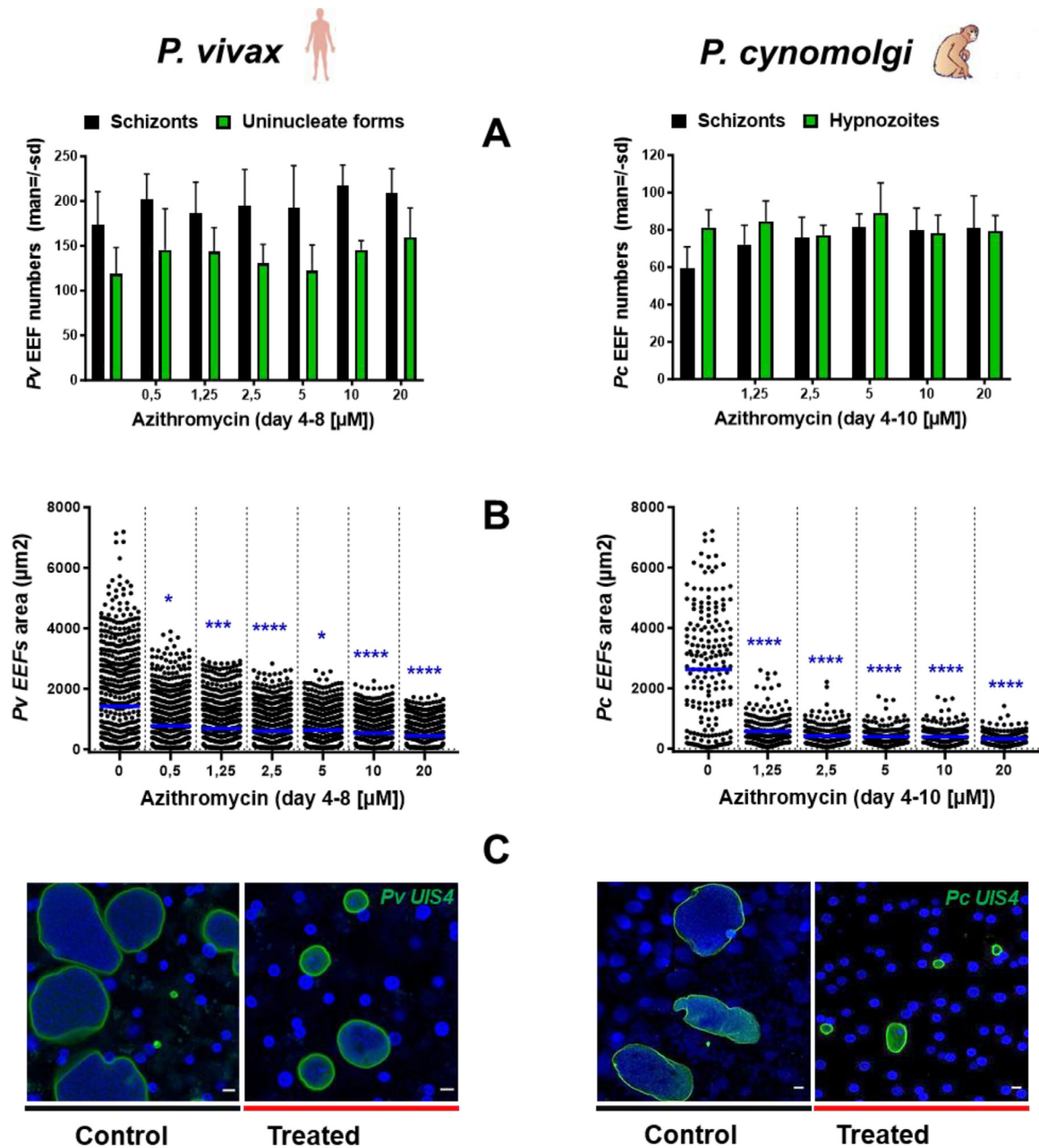


Fig. 1. Development of *Plasmodium vivax* and *Plasmodium cynomolgi* exoerythrocytic forms is blocked by azithromycin treatment of infected primary hepatocytes. Primary cultures of human and *Macaca fascicularis* hepatocytes were infected with *P. vivax* and *P. cynomolgi*, respectively, and exposed to different doses of azithromycin from day 4 post-infection until fixation on day 8 for *P. vivax*, or day 10 for *P. cynomolgi*. (A) The numbers of schizont (black) and uninucleate (green) forms were not different in azithromycin-treated cultures compared with untreated controls for *P. vivax* and *P. cynomolgi*. Data are from two independent experiments carried out in quadruplicate, and the results are expressed as mean \pm standard deviation. (B) Schizonts were significantly smaller in azithromycin-treated cultures compared with untreated controls for *P. vivax* and *P. cynomolgi*. Schizont size is expressed as surface area (μm^2). (C) Representative immunofluorescence images of *P. vivax* (day 8 post-infection) and *P. cynomolgi* (day 10 post-infection) schizonts in controls and azithromycin-treated cultures. Parasites were stained with an anti-UIS4 antibody (green), and DNA was labelled with DAPI (blue). Pictures are z-sections acquired by confocal microscopy with a $\times 63$ objective. Image z-stacks were deconvolved and then presented as a single combined image. Scale bar 10 μm . Statistical significance was determined using one-way analysis of variance Kruskal–Wallis test, followed by Dunn’s multiple comparisons with control groups. Significance was determined by $P < 0.0001$ (****), $P < 0.001$ (**), and $P < 0.05$ (*).

the cytoplasm, with some in which an apicoplast could not be detected. The proportion of uninucleate forms with disrupted apicoplasts increased in a dose-dependent manner to reach $>60\%$ at the highest azithromycin dose (Fig. 2D).

To confirm the azithromycin-induced defect in apicoplast segregation at the molecular level, hepatocytes harbouring GFP-expressing *P. cynomolgi* uninucleate forms (GFPlow) or liver schizonts (GFPhigh) were purified with FACS at day 10 post-infection, following exposure to 10 μM azithromycin from day 4 to day 10. Next, genomic DNA (gDNA) was isolated from azithromycin-treated

and untreated parasites, and the presence of apicoplast-encoded elongation factor Tu (EFTu) and the nuclear-encoded CHT genes was assessed quantitatively. As expected, azithromycin-treated and untreated schizonts or uninucleate forms had almost comparable quantities of the nuclear-encoded CHT genes (Fig. 2E). However, the gene copy numbers of the apicoplast-encoded EFTu were significantly reduced in the azithromycin-treated schizonts and the uninucleate parasites compared with those in the untreated parasites (Fig. 2E), which is consistent with an apicoplast multiplication defect. These results clearly demonstrate that the disruption of api-

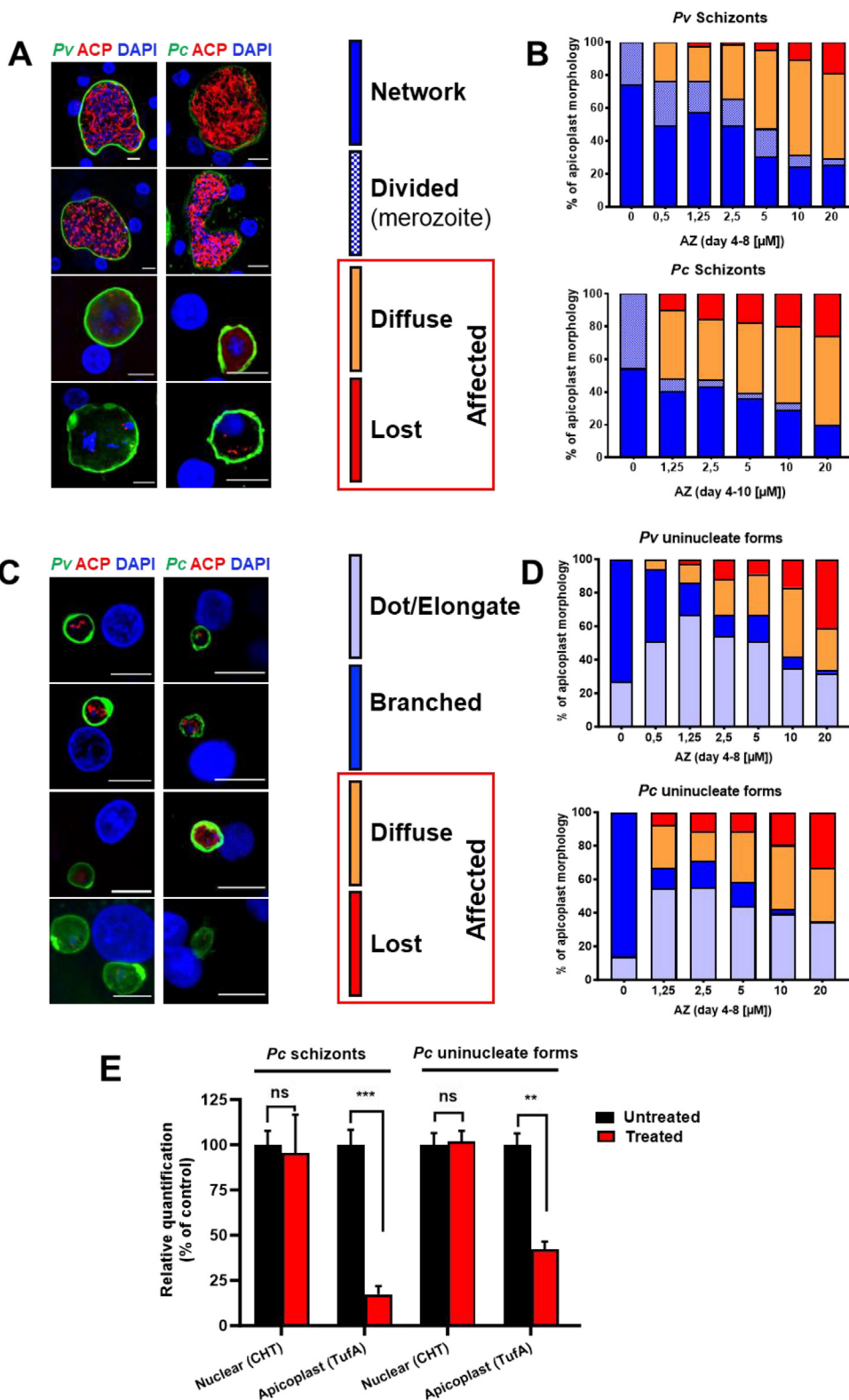


Fig. 2. Azithromycin disrupts apicoplast integrity in *Plasmodium vivax* and *Plasmodium cynomolgi* multi-nucleate and uninucleate forms. Representative immunofluorescence images of apicoplast morphology of (A) hepatic schizonts and (C) uninucleate forms in azithromycin-treated and untreated cultures. The parasites were visualized at day 10 post-infection with an anti-UIS4 antibody (green), nuclei were stained with DAPI (blue) and apicoplasts were stained with acyl carrier protein (ACP) antibody (red). Pictures are z-sections acquired by confocal microscopy with a $\times 63$ objective. Image z-stacks were deconvolved and then presented as a single combined image. Scale bar 10 μm . Proportion (as a mean of percentage) of the various apicoplast morphologies in (B) schizonts and (D) uninucleate forms in azithromycin-treated and untreated cultures. For each condition, 100 parasites were examined. Data are from two independent experiments carried out in quadruplicate. (E) Comparative analysis of *P. cynomolgi* genomic DNA (gDNA) of the nuclear gene (CHT) vs the apicoplast gene (EFTu) demonstrates a significant reduction in the EFTu copy number relative to the CHT in both azithromycin-treated schizont and uninucleate forms (Mann-Whitney *U*-test, $**P < 0.01$ and $***P < 0.001$; ns, not significant). gDNA was quantified by quantitative polymerase chain reaction from FACS-purified hepatocytes containing GFP-expressing uninucleate forms and schizonts at day 10 post-infection, from cultures exposed to 10 μM azithromycin from day 4 to day 10 post-infection or left untreated. gDNA copy numbers were normalized against tRNA genes. Error bars represent the standard error of the mean of two biological replicates.

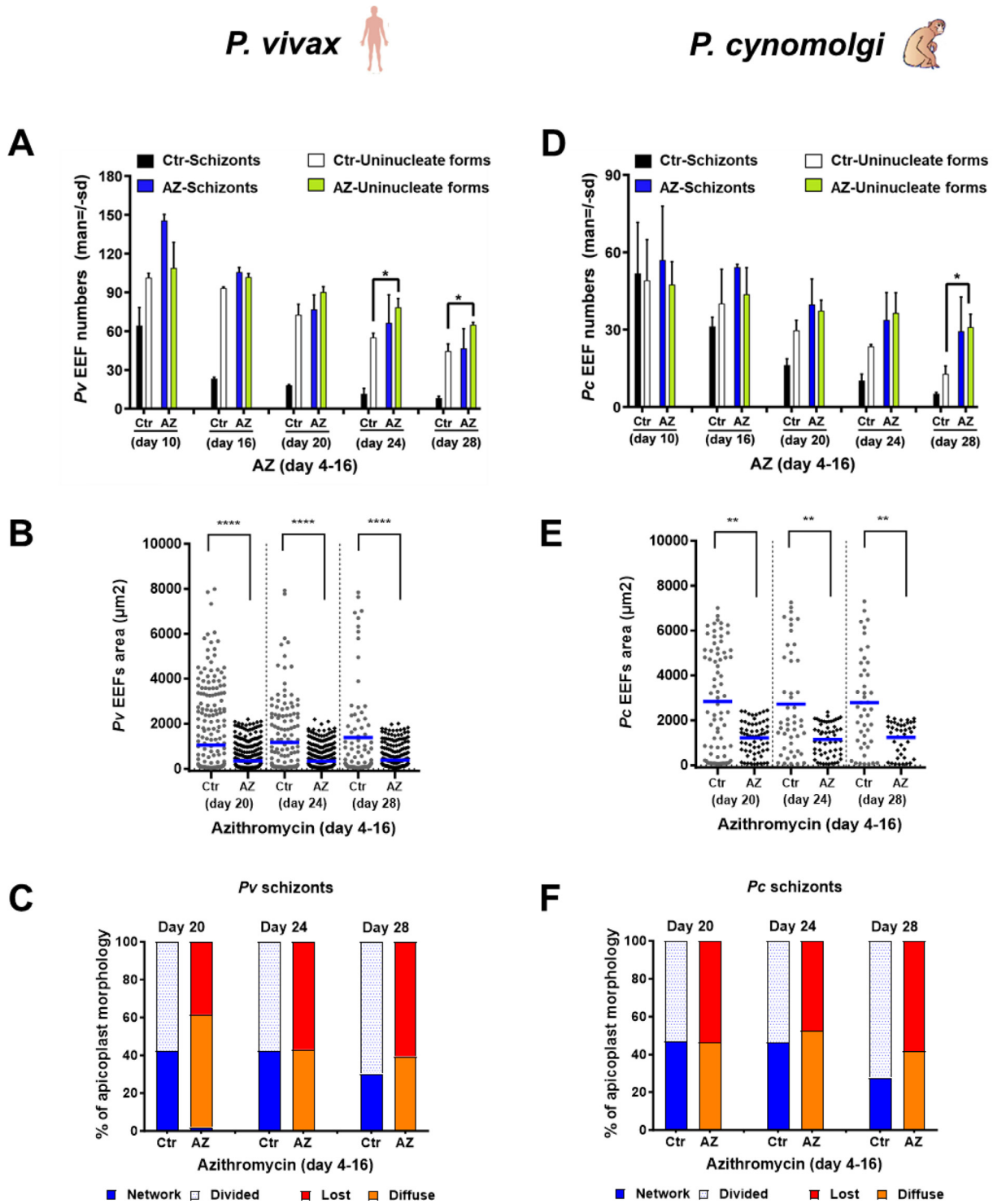


Fig. 3. Azithromycin dampens schizont maturation and uninucleate form reactivation rates. (A–F) *Plasmodium vivax* and *Plasmodium cynomolgi* hepatic cultures were exposed or not to 10 μM of azithromycin from day 4 to day 16 post-infection, and exoerythrocytic form (EEF) quantification was carried out at different time points. (A,D) Numbers of schizont (black) and uninucleate forms (green) were compared in azithromycin-treated and control *P. vivax* (A) and *P. cynomolgi* (D) cultures (unpaired Student's *t*-test: $P > 0.05$) for both species. Data were obtained from two independent experiments carried out in quadruplicate, and the results are expressed as mean \pm standard deviation. (B,E) The size of *P. vivax* (B) and *P. cynomolgi* (E) schizonts was significantly smaller in azithromycin-treated cultures compared with untreated controls. Schizont size is expressed as surface area (μm^2). (C,F) Quantification of the apicoplast morphologies in *P. vivax* (C) and *P. cynomolgi* (F) schizonts in azithromycin-treated and untreated cultures (means of percentages). One hundred parasites were analysed for each form. Data are from two independent experiments carried out in quadruplicate. Statistical significance was determined using a one-way analysis of variance Kruskal–Wallis test followed by Dunn's multiple comparisons with control groups. Significance was determined by $P < 0.0001$ (****), $P < 0.001$ (**) and $P < 0.05$ (*).

coplast division by azithromycin results in a loss of the organelle's DNA, corroborating results obtained elsewhere [34].

3.3. Azithromycin dampens schizont maturation and uninucleate form reactivation rate

Finally, in order to ascertain whether the azithromycin-exposed uninucleate parasites with altered apicoplast morphology were able to activate and resume normal hepatic development, a long-term hepatocyte culture system was used that allows maintenance of the primary hepatocytes in culture for 1 month post-seeding [26]. In preliminary experiments, the duration of azithromycin exposure required to damage the apicoplasts (diffuse ACP signal or loss of it) in all the uninucleate parasites was determined. Thus, *P. cynomolgi* hepatic cultures were exposed to 10 μ M azithromycin from day 4 post-infection until fixation at incremental time points (days 10, 14, 16 and 18), at which point they were immunostained for an apicoplast marker protein to assess the morphology of this organelle. This revealed that exposure to azithromycin from day 4 to day 16 led to the disruption of apicoplasts in all the uninucleate parasites (Fig. S1, see online supplementary material). Therefore, hepatic parasite development in cultures exposed to azithromycin from day 4 to day 16 was assessed until day 28 for *P. vivax* and *P. cynomolgi*, with control and treated cultures fixed at different time points (Fig. 3). It would be expected that between day 10 and day 16 post-infection, nearly all normally developing hepatic parasites would have fully matured to release merozoites into the medium (i.e. become lost from the culture), leaving some that might still be maturing, and others that derived from activated uninucleate forms, a process that will continue throughout the cultivation, that will also develop to yield merozoites. Therefore, a decrease in the number of uninucleate forms thereafter would reflect awakening from quiescence.

In both the control and the azithromycin-treated cultures, the activation of the uninucleate forms, as assessed by the decrease in their number over day 16 to day 28, proceeds at an even pace for both species, indicating a constant rate of activation to lead to mature parasites that then rupture, which is reflected by the rate of decrease in the number of schizonts over time (Fig. 3A,D and Fig. S2, see online supplementary material). However, the rate of activation appears slower for the azithromycin-treated cultures compared with the control cultures (Fig. S2, see online supplementary material). Schizont numbers are also observed to decrease in all cultures, possibly faster for *P. vivax* than *P. cynomolgi* (Fig. S2, see online supplementary material), either because the numbers in that species are lower, or because *P. vivax* schizonts are more susceptible to azithromycin. Nonetheless, the schizonts in the azithromycin-exposed cultures were significantly smaller in area than their control counterparts on day 16 and thereafter until day 28, during which time drug exposure had ceased (Fig. 3B,E). Furthermore, all the drug-exposed schizonts displayed apicoplasts with disrupted morphology, with an increasing proportion, as time went by, in which this organelle could not be detected (Fig. 3C,F) and where the nuclear material was abnormal (Fig. S3, see online supplementary material).

3.4. *P. berghei* parasites exposed to azithromycin during liver-stage growth fail to establish blood-stage infections

Using *P. berghei*-infected HepG2 cells exposed or not to 10 μ M azithromycin and then inoculated at 66 h post-infection into naïve mice (c. 1000 parasite forms per mouse), the present study confirmed a previous observation [17] that, although this treatment did not prevent hepatic-stage development, it ablated the ability of these parasites to lead to an erythrocytic infection (Fig. S4A, see online supplementary material). As expected, the drug-exposed

parasites were smaller in size and generally displayed affected apicoplast morphology (Fig. S4B, see online supplementary material). This study also demonstrated that exposure of *P. berghei* sporozoites to different doses of azithromycin for 30 min affected their apicoplast, in that most of them displayed the dot form and some lacked an apicoplast entirely ('lost' category) (Fig. S5A,B, see online supplementary material), in contrast to those in control sporozoites, where only 29% presented as a single dot with the others showing the mature elongated form. The exposure of sporozoites to azithromycin as above had no effect on the number of hepatic schizonts, although their size was reduced (Fig. S5C,D, see online supplementary material), a phenotype that is similar to that observed in parasites treated during hepatic development.

Finally, this 'prophylactic regimen' was repeated using in-vitro cultured *P. cynomolgi* hepatic stages, where the parasites were exposed to increasing azithromycin concentrations from the time of sporozoite inoculation until day 7 and then assessed. Although schizonts and uninucleate forms did not vary in number as drug concentration increased (Fig. S6A, see online supplementary material), the schizont area was reduced significantly at all doses (Fig. S6B, see online supplementary material), and the proportion of altered apicoplasts in both parasite forms increased with drug concentration, with nearly all affected in the 10 μ M azithromycin cultures (Fig. S6C,D).

4. Discussion and conclusions

The lack of treatment options other than 8-aminoquinolines (primaquine and tafenoquine) to clear hypnozoites represents a major barrier to the control and elimination of *P. vivax*, *P. ovale-curtisi* and *P. ovale-wallikeri*, the three relapsing species of malaria parasites infecting humans [4]. Azithromycin, an antibiotic, is effective against erythrocytic parasites and has been favourably considered as an additional component in therapeutic or prophylactic regimes [22]. Azithromycin is thought to exert its antimalarial activity by inhibiting the prokaryote-like 50S ribosomal translation of the apicoplast [35]. The inhibitory effect of azithromycin on the liver stages was first demonstrated in *P. berghei*, where it impaired apicoplast biogenesis and prevented hepatic merozoite formation [16,17]. The dependence on this organelle to complete hepatic development is most likely due to specific metabolites or proteins whose synthesis is dependent on the apicoplast, such as some components of the type II fatty acid synthesis pathway [36–38], the plasmodium-specific apicoplast protein involved in liver merozoite formation [39], and enzymes of phosphatidic acid biosynthesis [40]. The requirement for metabolites during hepatic development is likely higher than that during the erythrocytic cycle, where the number of merozoites per infected cell is comparatively very small (4–32 compared with thousands for the hepatic schizont) [41].

This study exploited the long-term in-vitro cultivation of relapsing *P. vivax* and *P. cynomolgi* in primary hepatocytes to ascertain the potential of azithromycin exposure to impair hypnozoite formation, activation and subsequent maturation to form merozoites. In these cultures, the uninucleate forms observed on day 10, and especially after day 16, are considered as quiescent forms capable of activation, and thus, as hypnozoites [26].

The morphological and molecular observations confirmed that exposure to azithromycin treatment disrupts the normal development, division and functions of the apicoplast in hepatic schizonts, which consequently prevents their maturation to form merozoites. Furthermore, the data provide the first evidence that azithromycin disrupts the apicoplast in the quiescent uninucleate forms, and consequently impairs these forms that remain in the long-term in-vitro cultures. This is consistent with previous investigations where it was shown that the apicoplast is metabolically active in *P. vivax*

hypnozoites in a humanized mouse model [42], and in *P. cynomolgi* hypnozoites progressing to dormancy [43]. Although it is not yet known which and how the metabolic pathways present in the apicoplast are implicated in hypnozoite biology, upregulation of the apicoplast's FASII synthesis pathway genes may be essential [43]. In *Mycobacterium tuberculosis*, the acetyl-CoA is redirected in the TCA cycle for fatty acid synthesis in order to build up carbon storage in the latent stage [44], a requirement that may be similar for plasmodium hypnozoites [43]. Furthermore, a recent demonstration that the disruption of the metabolic pathways or the genesis of the toxoplasma apicoplast results in the loss of bradyzoite viability [45] lends support to these conclusions.

Two further observations in the long-term cultures are germane to the potential effect of azithromycin on hypnozoites. First, although azithromycin treatment did not prevent activation, the rate of activation of the uninucleate forms from day 16 onwards to resume development appears to be reduced in cultures exposed to azithromycin for 12 days, which may imply a lack of the metabolic resources needed for activation. Interestingly, this effect appears more pronounced in *P. cynomolgi* than in *P. vivax*. Second, the schizonts that result from those forms that have activated past day 16 appear not only to persist for longer periods, but also to have disrupted apicoplasts, implying that they would not be able to form merozoites. In addition, using *P. berghei*, a rodent malaria model, this study confirmed that hepatic parasites exposed to azithromycin *in vitro* could not establish blood-stage infection *in vivo*. Thus, these observations indicate that prolonged exposure to azithromycin would prevent hepatic parasite maturation, including that by activated hypnozoites, and thus prevent blood-stage infections, including relapses.

Early limited studies in macaques [46] and humans [47] excluded the potential for azithromycin to inhibit hypnozoites and prevent relapses. However, randomized, controlled, double-blind trials in Southeast Asia [48,49] revealed potent prophylactic activity against *P. vivax* infections, which must have included a significant antirelapse component because it persisted for many weeks following the last dose for *P. vivax* infections (9–10 weeks) but not *P. falciparum* infections (3–4 weeks). It is likely that the substantial accumulation of azithromycin in the liver, considered to be 10–100 times more concentrated in some tissues compared with serum [50], would have provided sufficient exposure of the hepatic stages to the drug. The fact that hepatic schizonts with disrupted apicoplasts may persist for long durations could also contribute to eliciting and maintaining protective immune responses against the liver-stage parasite, a vaccination strategy that has been proposed and validated [17,51–54]. Further investigations using other models of hypnozoite development, such as mice with livers engrafted with human or *M. fascicularis* hepatocytes [42], or preclinical trials in macaques would help to elucidate the mechanism of action of azithromycin. However, clinical trials designed to assess the efficacy of this drug, alone or in combination, against *P. vivax* relapses are warranted.

In conclusion, these findings provide the first indication that the apicoplast offers a valuable opportunity for hypnozoiticidal drug discovery, as this organelle may be susceptible to disruption by compounds other than 8-aminoquinolines to prevent relapses, and thus help to accelerate the elimination of *P. vivax*. Other cheap safe antimalarials, in addition to azithromycin, that target the apicoplast are therefore worthy of exploration as hypnocidal [34].

Author contributions

N.A., D.M. and G.S. designed the study. N.A., S.T., J.-F.F., K.A., A.-M.Z., W.R., F.R., J.L., C.B., C.-O.G., P.B. and V.S. performed the study. N.A., S.T., J.-F.F., D.M. and G.S. analysed the data. J.-F.F., G.S., J.S., C.H.M.K., V.S., Y.Z., R.L.G. and D.M. contributed essential materials.

N.A., S.T., D.M. and G.S. wrote the paper. All authors read and approved the manuscript.

Funding: Nadia Amanzougaghene and Shahin Tajeri acknowledge postdoctoral funding support from L'Agence Nationale de la recherche (Plasmodrug project N° 18-CE18-0009-01) and Fondation pour la Recherche Médicale (PALUKILL project N° DMC20181039565), respectively. The investigations on *P. cynomolgi* were funded through a grant from the Agence Nationale de la Recherche, France (ANR-17-CE13-0025-01). The IDMIT infrastructure is supported by the French Government 'Programme d'Investissements d'Avenir', under grant ANR-11-INBS-0008.

Competing interests: None declared.

Ethical approval: *P. cynomolgi* and liver cells: permit number A 92-032-02 and CCD license number AVD5020020172664. *P. vivax* production: permit number MUTM 201 L-040-05. Murine models: authorization number 01737.03.

Acknowledgements

The authors wish to thank Romain Duval for critical reading of the manuscript; Maurel Tefit and Thierry Houpert for their expertise and their valuable technical assistance in maintaining the *Anopheles stephensi* colony; Muriel Andrieu and Souganiya Many (CYBIO, Cochin Institute, Paris) for their technical help during the FACS experiments; the QUANT and CELIS microscopy platforms of the Institut du Cerveau et de la Moelle épinière, especially, David Akbar, Claire Lovo and Aymeric Millécamps, for their help in the analysis of microscopic images; and Noah Sather and Scott Lindner for the kind gift of anti-plasmodium antibodies.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ijantimicag.2024.107112](https://doi.org/10.1016/j.ijantimicag.2024.107112).

References

- Guerra CA, Howes RE, Patil AP, Gething PW, Van Boeckel TP, Temperley WH, et al. The international limits and population at risk of Plasmodium vivax transmission in 2009. *PLoS Negl Trop Dis* 2010;4:e774.
- Chu CS, White NJ. The prevention and treatment of Plasmodium vivax malaria. *PLoS Med* 2021;18:e1003561.
- White NJ, Imwong M. Relapse. *Adv Parasitol* 2012;80:113–50.
- Baird JK. Resistance to therapies for infection by Plasmodium vivax. *Clin Microbiol Rev* 2009;22:508–34.
- Sutherland CJ, Tanomsing N, Nolder D, Oguike M, Jennison C, Pukrit-tayakamee S, et al. Two nonrecombining sympatric forms of the human malaria parasite Plasmodium ovale occur globally. *J Infect Dis* 2010;201:1544–50.
- Snounou G, Sharp PM, Culleton R. The two parasite species formerly known as Plasmodium ovale. *Trends Parasitol* 2024;40:21–7.
- Edgcomb JH, Arnold J, Yount EH, Alving AS, Eichelberger L, Jeffery GM, et al. Primaquine, SN 13272, a new curative agent in vivax malaria; a preliminary report. *J Natl Malar Soc* 1950;9:285–92.
- Mazier D, Rénia L, Snounou G. A pre-emptive strike against malaria's stealthy hepatic forms. *Nat Rev Drug Discov* 2009;8:854–64.
- Lu K-Y, Derbyshire ER. Tafenoquine: a step toward malaria elimination. *Biochemistry* 2020;59:911–20.
- Low LM, Stanicic DI, Good MF. Exploiting the apicoplast: apicoplast-targeting drugs and malaria vaccine development. *Microbes Infect* 2018;20:477–83.
- Fichera ME, Roos DS. A plastid organelle as a drug target in apicomplexan parasites. *Nature* 1997;390:407–9.
- McFadden GI, Yeh E. The apicoplast: now you see it, now you don't. *Int J Parasitol* 2017;47:137–44.
- Biddau M, Sheiner L. Targeting the apicoplast in malaria. *Biochem Soc Trans* 2019;47:973–83.
- Dahl EL, Rosenthal PJ. Multiple antibiotics exert delayed effects against the Plasmodium falciparum apicoplast. *Antimicrob Agents Chemother* 2007;51:3485–90.

- [15] Kennedy K, Cobbold SA, Hanssen E, Birnbaum J, Spillman NJ, McHugh E, et al. Delayed death in the malaria parasite *Plasmodium falciparum* is caused by disruption of prenylation-dependent intracellular trafficking. *PLoS Biol* 2019;17:e3000376.
- [16] Stanway RR, Witt T, Zobiak B, Aepfelbacher M, Heussler VT. GFP-targeting allows visualization of the apicoplast throughout the life cycle of live malaria parasites. *Biol Cell* 2009;101:415–30.
- [17] Friesen J, Silvie O, Putrianti ED, Hafalla JCR, Matuschewski K, Borrmann S. Natural immunization against malaria: causal prophylaxis with antibiotics. *Sci Transl Med* 2010;2 40ra49.
- [18] Taylor WR, Widjaja H, Richie TL, Basri H, Ohrt C, Tjitra E, et al. Chloroquine/doxycycline combination versus chloroquine alone, and doxycycline alone for the treatment of *Plasmodium falciparum* and *Plasmodium vivax* malaria in northeastern Irian Jaya, Indonesia. *Am J Trop Med Hyg* 2001;64:223–8.
- [19] Borrmann S, Issifou S, Esser G, Adegnikaa AA, Ramharter M, Matsiegui P-B, et al. Fosmidomycin-clindamycin for the treatment of *Plasmodium falciparum* malaria. *J Infect Dis* 2004;190:1534–40.
- [20] Dunne MW, Singh N, Shukla M, Valecha N, Bhattacharyya PC, Dev V, et al. A multicenter study of azithromycin, alone and in combination with chloroquine, for the treatment of acute uncomplicated *Plasmodium falciparum* malaria in India. *J Infect Dis* 2005;191:1582–8.
- [21] Noedl H, Krudsood S, Chalermratana K, Silachamroon U, Leowattana W, Tangpakdee N, et al. Azithromycin combination therapy with artesunate or quinine for the treatment of uncomplicated *Plasmodium falciparum* malaria in adults: a randomized, phase 2 clinical trial in Thailand. *Clin Infect Dis* 2006;43:1264–71.
- [22] Rosenthal PJ. Azithromycin for malaria? *Am J Trop Med Hyg* 2016;95:2–4.
- [23] Andersen SL, Ager AL, McGreevy P, Schuster BG, Ellis W, Berman J. Efficacy of azithromycin as a causal prophylactic agent against murine malaria. *Antimicrob Agents Chemother* 1994;38:1862–3.
- [24] Dembele L, Gego A, Zeeman A-M, Franetich J-F, Silvie O, Rametti A, et al. Towards an in vitro model of *Plasmodium* hypnozoites suitable for drug discovery. *PLOS One* 2011;6:e18162.
- [25] Sattabongkot J, Kumpitak C, Kiattitubtr K. Membrane feeding assay to determine the infectiousness of *Plasmodium vivax* gametocytes. *Methods Mol Biol* 2015;1325:93–9.
- [26] Dembélé L, Franetich J-F, Lorthiois A, Gego A, Zeeman A-M, Kocken CHM, et al. Persistence and activation of malaria hypnozoites in long-term primary hepatocyte cultures. *Nat Med* 2014;20:307–12.
- [27] Bosson-Vanga H, Primas N, Franetich J-F, Lavazec C, Gomez L, Ashraf K, et al. A new thienopyrimidinone chemotype shows multistage activity against *Plasmodium falciparum*, including artemisinin-resistant parasites. *Microbiol Spectr* 2021;9 e0027421.
- [28] Ashraf K, Tajeri S, Arnold C-S, Amanzougaghene N, Franetich J-F, Vantaux A, et al. Artemisinin-independent inhibitory activity of Artemisia sp. infusions against different *Plasmodium* stages including relapse-causing hypnozoites. *Life Sci Alliance* 2022;5:e202101237.
- [29] Voorberg-van der Wel AM, Zeeman A-M, Nieuwenhuis IG, van der Werff NM, Klooster EJ, Klop O, et al. A dual fluorescent *Plasmodium cynomolgi* reporter line reveals in vitro malaria hypnozoite reactivation. *Commun Biol* 2020;3:7.
- [30] Voorberg-van der Wel A, Roma G, Gupta DK, Schuierer S, Nigsch F, Carbone W, et al. A comparative transcriptomic analysis of replicating and dormant liver stages of the relapsing malaria parasite *Plasmodium cynomolgi*. *Elife* 2017;6:e29605.
- [31] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C T method. *Nat Protoc* 2008;3:1101–8.
- [32] Gupta DK, Dembele L, Voorberg-van der Wel A, Roma G, Yip A, Chuenchob V, et al. The *Plasmodium* liver-specific protein 2 (LISP2) is an early marker of liver stage development. *Elife* 2019;8:e43362.
- [33] Stanway RR, Mueller N, Zobiak B, Graewe S, Froehle U, Zessin PJM, et al. Organelle segregation into *Plasmodium* liver stage merozoites. *Cell Microbiol* 2011;13:1768–82.
- [34] Uddin T, McFadden GI, Goodman CD. Validation of putative apicoplast-targeting drugs using a chemical supplementation assay in cultured human malaria parasites. *Antimicrob Agents Chemother* 2018;62 e01161–17.
- [35] Sidhu ABS, Sun Q, Nkrumah LJ, Dunne MW, Sacchetti JC, Fidock DA. In vitro efficacy, resistance selection, and structural modeling studies implicate the malarial parasite apicoplast as the target of azithromycin. *J Biol Chem* 2007;282:2494–504.
- [36] Yu M, Kumar TRS, Nkrumah LJ, Coppi A, Retzlaff S, Li CD, et al. The fatty acid biosynthesis enzyme *FabI* plays a key role in the development of liver-stage malarial parasites. *Cell Host Microbe* 2008;4:567–78.
- [37] Vaughan AM, O'Neill MT, Tarun AS, Camargo N, Phuong TM, Aly ASI, et al. Type II fatty acid synthesis is essential only for malaria parasite late liver stage development. *Cell Microbiol* 2009;11:506–20.
- [38] Pei Y, Tarun AS, Vaughan AM, Herman RW, Soliman JMB, Erickson-Wayman A, et al. *Plasmodium* pyruvate dehydrogenase activity is only essential for the parasite's progression from liver infection to blood infection. *Mol Microbiol* 2010;75:957–71.
- [39] Haussig JM, Matuschewski K, Kooij TWA. Inactivation of a *Plasmodium* apicoplast protein attenuates formation of liver merozoites. *Mol Microbiol* 2011;81:1511–25.
- [40] Lindner SE, Sartain MJ, Hayes K, Harupa A, Moritz RL, Kappe SHI, et al. Enzymes involved in plastid-targeted phosphatidic acid synthesis are essential for *Plasmodium yoelii* liver stage development. *Mol Microbiol* 2014;91:679–93.
- [41] Roques M, Bindschedler A, Beyeler R, Heussler VT. Same, same but different: exploring *Plasmodium* cell division during liver stage development. *PLoS Pathog* 2023;19:e1011210.
- [42] Mikolajczak SA, Vaughan AM, Kangwanransan N, Roobsoong W, Fishbaugh M, Yimamnuaychok N, et al. *Plasmodium vivax* liver stage development and hypnozoite persistence in human liver-chimeric mice. *Cell Host Microbe* 2015;17:526–35.
- [43] Bertschi NL, Voorberg-van der Wel A, Zeeman A-M, Schuierer S, Nigsch F, Carbone W, et al. Transcriptomic analysis reveals reduced transcriptional activity in the malaria parasite *Plasmodium cynomolgi* during progression into dormancy. *Elife* 2018;7:e41081.
- [44] Baek S-H, Li AH, Sasseti CM. Metabolic regulation of mycobacterial growth and antibiotic sensitivity. *PLoS Biol* 2011;9:e1001065.
- [45] Sanchez SG, Bassot E, Cerutti A, Mai Nguyen H, Aïda A, Blanchard N, et al. The apicoplast is important for the viability and persistence of *Toxoplasma gondii* bradyzoites. *Proc Natl Acad Sci USA* 2023;120:e2309043120.
- [46] Puri SK, Singh N. Azithromycin: antimalarial profile against blood- and sporozoite-induced infections in mice and monkeys. *Exp Parasitol* 2000;94:8–14.
- [47] Ranque S, Badiaga S, Delmont J, Brouqui P. Triangular test applied to the clinical trial of azithromycin against relapses in *Plasmodium vivax* infections. *Malar J* 2002;1:13.
- [48] Taylor WR, Richie TL, Fryauff DJ, Picarima H, Ohrt C, Tang D, et al. Malaria prophylaxis using azithromycin: a double-blind, placebo-controlled trial in Irian Jaya, Indonesia. *Clin Infect Dis* 1999;28:74–81.
- [49] Heppner DG, Walsh DS, Uthaimongkol N, Tang DB, Tulyayon S, Permpanch B, et al. Randomized, controlled, double-blind trial of daily oral azithromycin in adults for the prophylaxis of *Plasmodium vivax* malaria in Western Thailand. *Am J Trop Med Hyg* 2005;73:842–9.
- [50] Lode H. The pharmacokinetics of azithromycin and their clinical significance. *Eur J Clin Microbiol Infect Dis* 1991;10:807–12.
- [51] Butler NS, Schmidt NW, Vaughan AM, Aly AS, Kappe SHI, Harty JT. Superior antimalarial immunity after vaccination with late liver stage-arresting genetically attenuated parasites. *Cell Host Microbe* 2011;9:451–62.
- [52] Matuschewski K, Hafalla JC, Borrmann S, Friesen J. Arrested *Plasmodium* liver stages as experimental anti-malaria vaccines. *Hum Vaccin* 2011;7(Suppl):16–21.
- [53] Belnoue E, Costa FTM, Frankenberg T, Vigário AM, Voza T, Leroy N, et al. Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment. *J Immunol* 2004;172:2487–95.
- [54] Roestenberg M, McCall M, Hopman J, Wiersma J, Luty AJF, van Gemert GJ, et al. Protection against a malaria challenge by sporozoite inoculation. *N Engl J Med* 2009;361:468–77.
- [55] Schafer C, Dambrauskas N, Steel RW, Carbonetti S, Chuenchob V, Flannery EL, et al. A recombinant antibody against *Plasmodium vivax* UIS4 for distinguishing replicating from dormant liver stages. *Malar J* 2018;17:370.
- [56] Chua ACY, Ananthanarayanan A, Ong JY, Wong JY, Yip A, Singh NH, et al. Hepatic spheroids used as an in vitro model to study malaria relapse. *Biomaterials* 2019;216:119221.
- [57] Lindner SE, Sartain MJ, Hayes K, Harupa A, Moritz RL, Kappe SHI, et al. Enzymes involved in plastid-targeted phosphatidic acid synthesis are essential for *Plasmodium yoelii* liver-stage development. *Mol Microbiol* 2014;91:679–93.

1 **Supporting Information for**

2 **Azithromycin disrupts apicoplast biogenesis in replicating and dormant liver stages of the**
3 **relapsing malaria parasites *Plasmodium vivax* and *P. cynomolgi***

4 Nadia Amanzougaghene^{1,2}, Shahin Tajeri^{1,†}, Jean-François Franetich¹, Kutub Ashraf¹, Valérie
5 Soulard¹, Pierre Bigeard¹, Cheick Oumar Guindo¹, Camille Bouillier², Julien Lemaitre², Francis
6 Relouzat², Roger Legrand², Clemens H M Kocken³, Anne-Marie Zeeman³, Wanlapa
7 Roobsoong⁴, Jetsumon Sattabongkot⁴, Yang Zhaoqing⁵, Georges Snounou^{2*} and Dominique
8 Mazier ^{1*}

9

10 ***Corresponding authors:**

11 Dominique Mazier (e-mail: dominique.mazier@sorbonne-universite.fr)

12 Georges Snounou (e-mail : gsnounou@gmail.com)

13

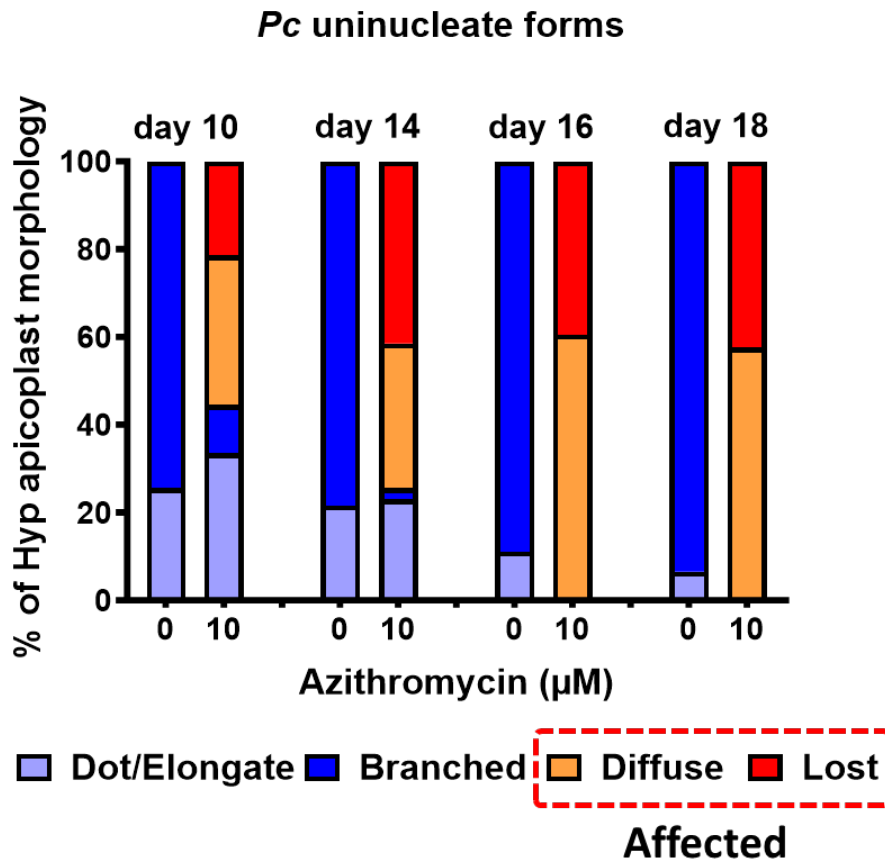
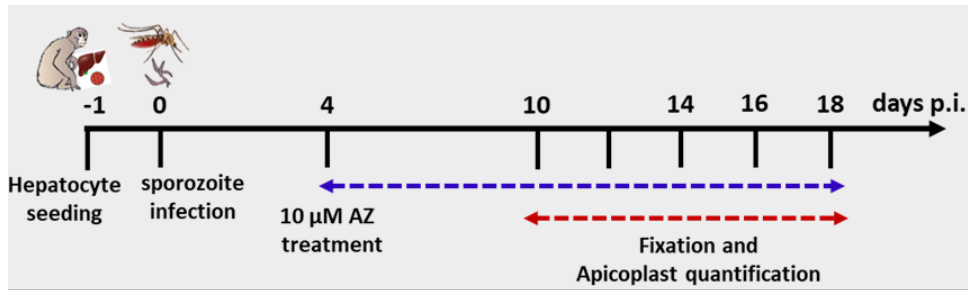
14

15 This PDF file includes:

16 Figures S1 to S6

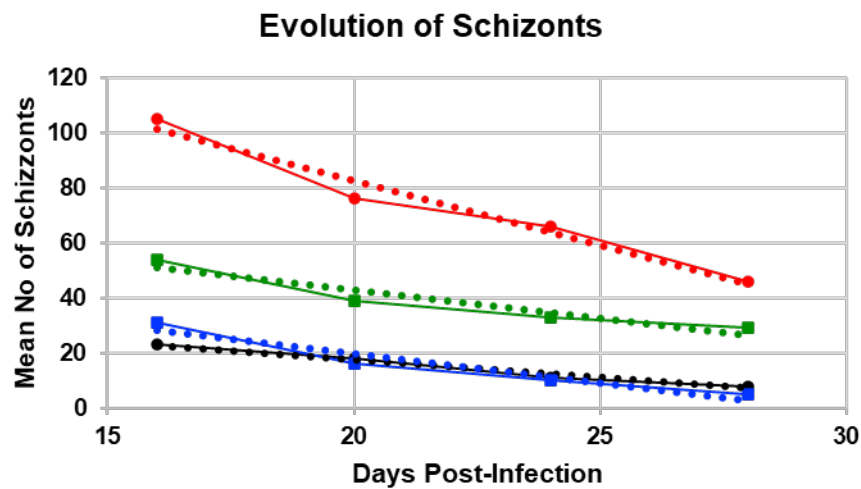
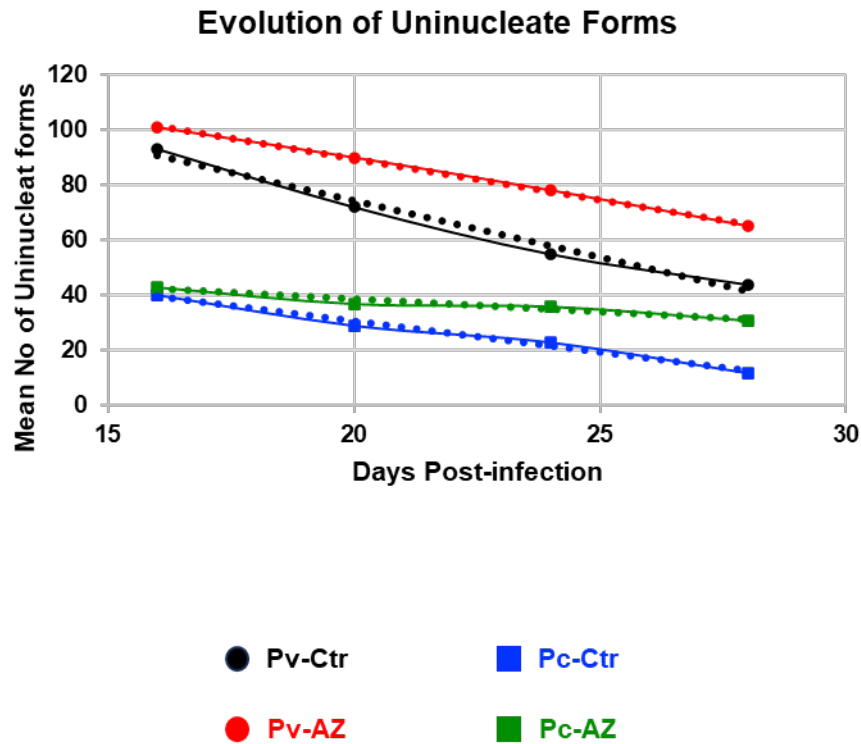
17

18



19

20 **Figure S1. Duration of azithromycin exposure required to damage 100% of the**
 21 **uninucleate form's apicoplasts.** *P. cynomolgi* cultures were exposed to 10 μM of azithromycin
 22 from day 4 to day 10, 14, 16, or 18 post-infections and fixed at days 10, 14, 16 and 18 post-
 23 infections, respectively. The apicoplast morphologies were quantified in uninucleate forms in
 24 azithromycin-treated and untreated cultures. 100 parasites were analyzed for each form. Data
 25 are from two independent experiments performed in quadruplicates. Uninucleate forms are
 26 shown as means of percentages.

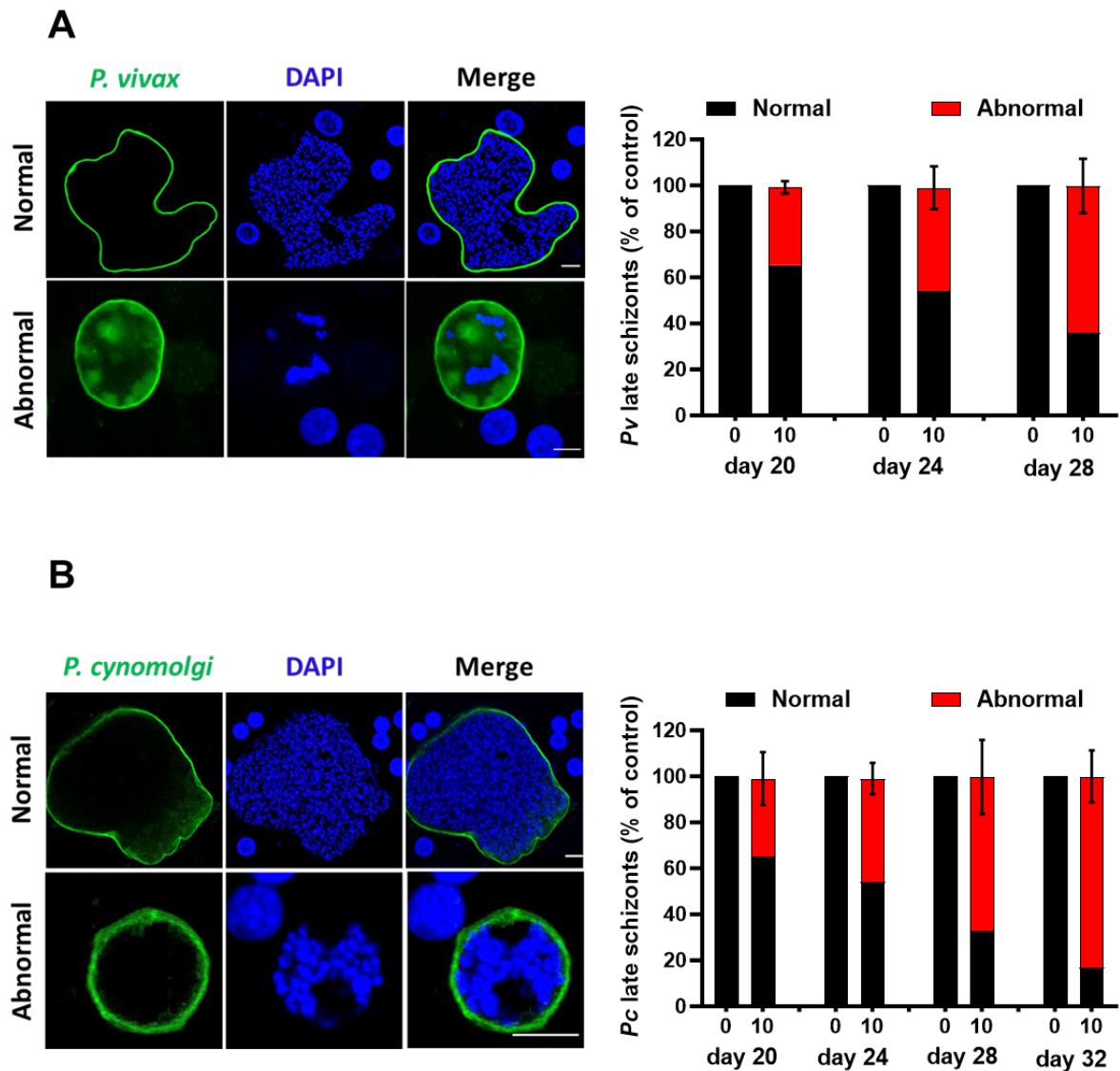


27

28

29 **Figure S2. Decrease in the numbers of uninucleate forms and schizonts over time in**
 30 **azithromycin-treated and control cultures.** The data for the mean number of forms are
 31 derived from Fig. 3 A and C, *P. vivax* and *P. cynomolgi*, respectively. The fitted lines are
 32 represented as dotted lines to allow visual comparison.

33



35

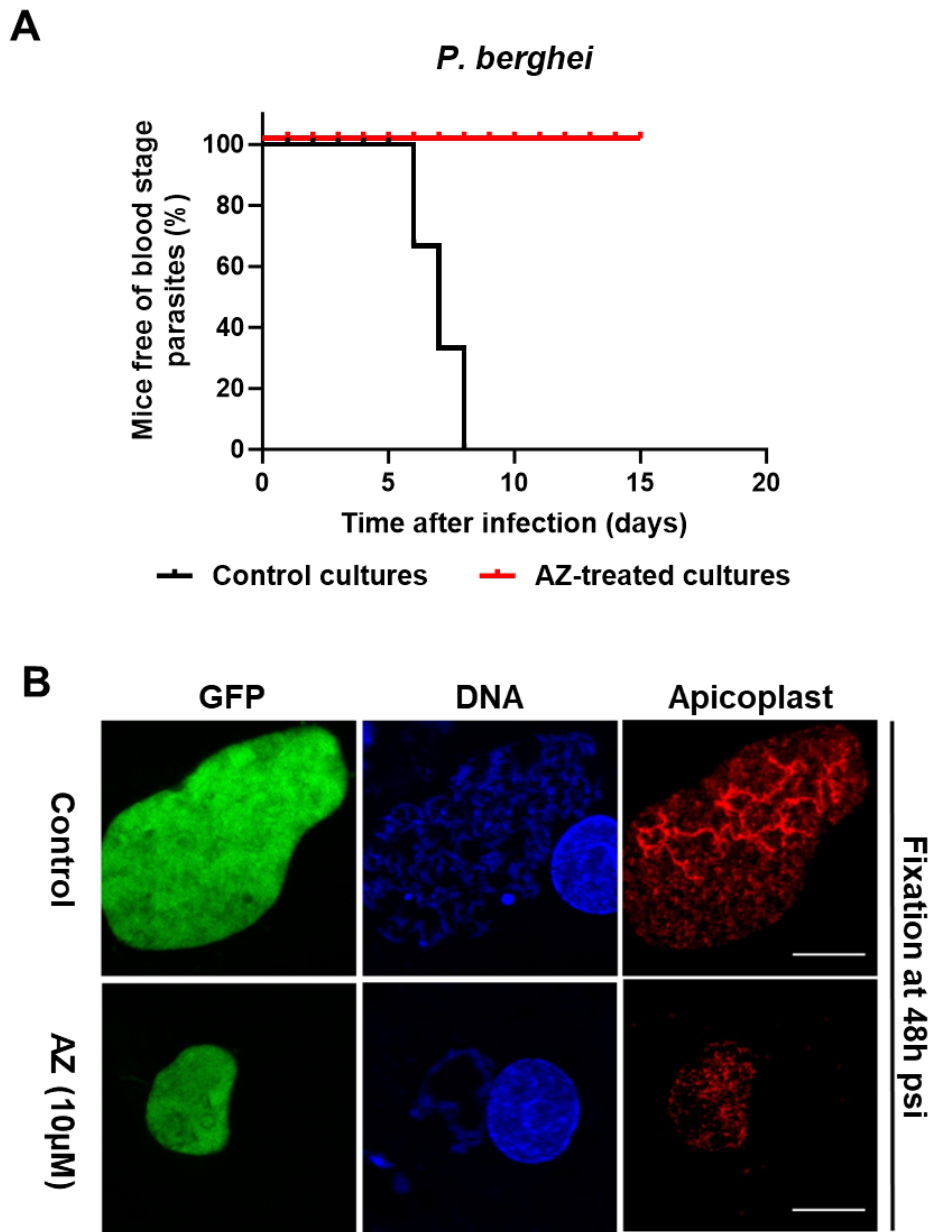
36

37 **Figure S3. Altered morphology of late schizonts in treated cultures.** Quantification of late
 38 schizonts morphology in *P. vivax* (A) and *P. cynomolgi* (B) schizonts in azithromycin-treated
 39 and untreated cultures (means of percentages). 100 parasites were analyzed for each form. Data
 40 are from two independent experiments carried out in quadruplicates.

41

42

43



45

46 **Figure S4. Parasites emerging from azithromycin-treated cultures are non-infectious. (A)**

47 Kaplan-Meier curves of the appearance of parasitaemia in inoculated mice. Groups of six

48 animals were injected intraperitoneally with *P. berghei*-infected hepatoma cells at 66 hours post

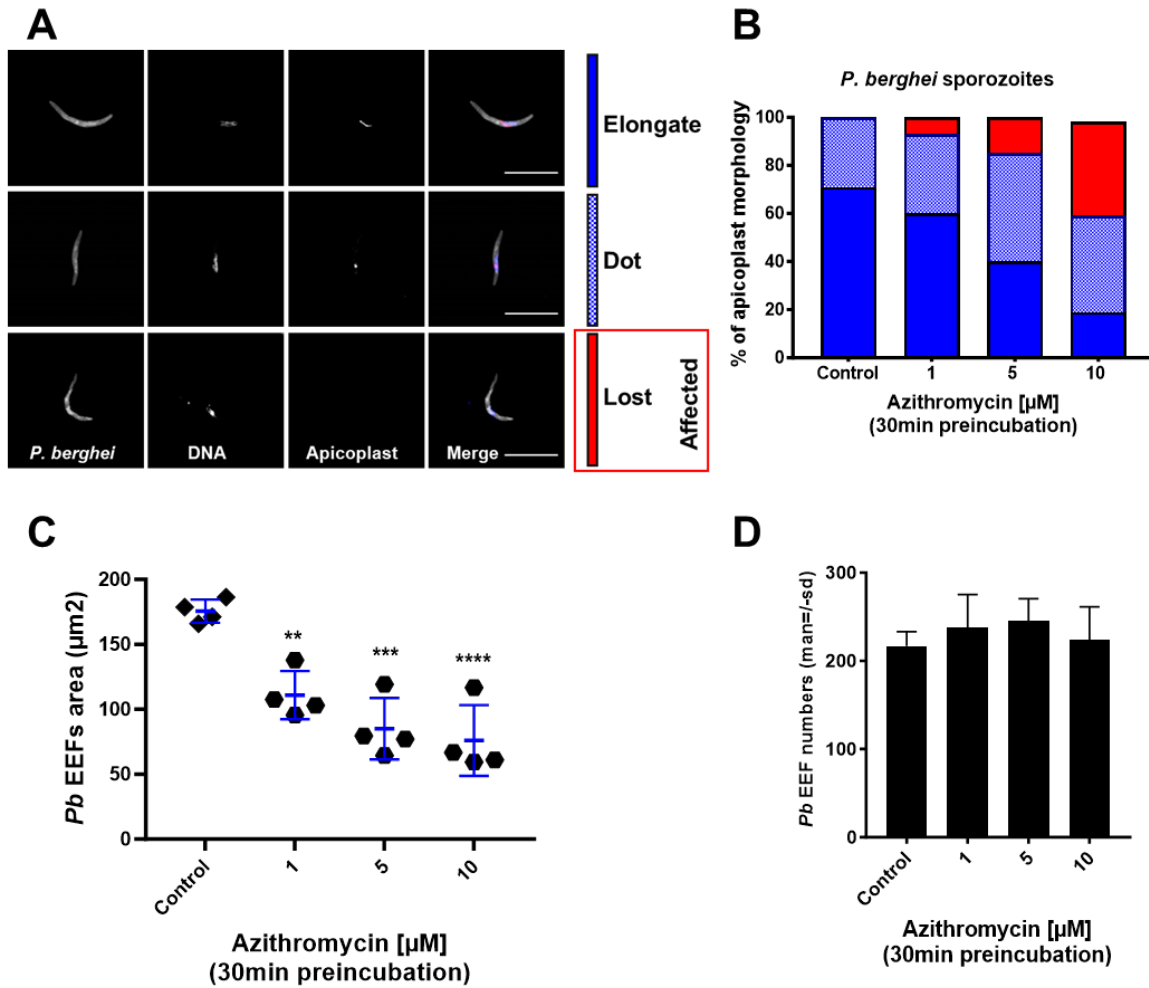
49 sporozoites infection from azithromycin-treated or untreated cultures. **(B)** Inhibition of

50 apicoplast maturation during liver-stage development of *P. berghei*. Shown are

51 immunofluorescence images of 48-hour liver stages *P. berghei* GFP (green) parasites stained

52 with antibody to PbACP (red) and nuclei stained with DAPI (blue). Note the highly branched
 53 structure of mature apicoplasts in control parasites stained with an antibody to PbACP in
 54 contrast to azithromycin-treated parasites. Scale bars 10 μm .

55



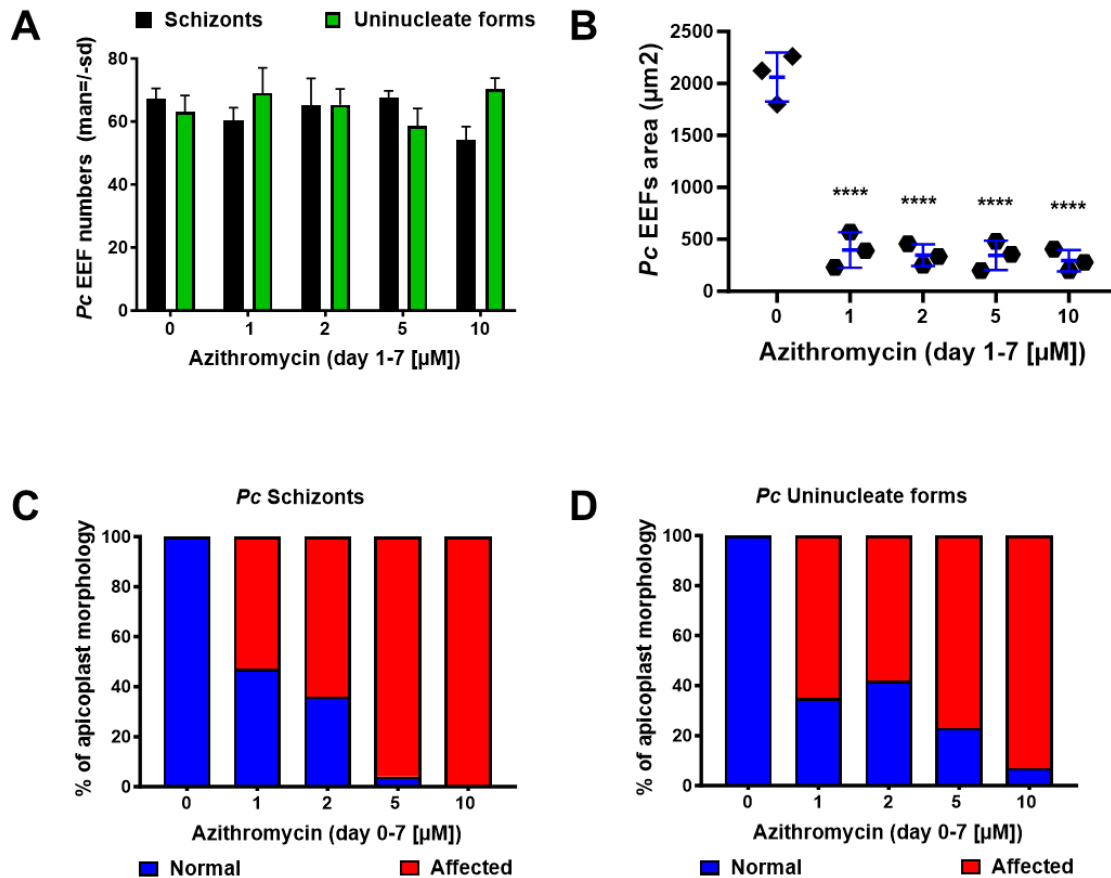
56

57 **Figure S5. Disruption of apicoplast integrity in *P. berghei* sporozoites stage by**
 58 **Azithromycin.** (A) Representative immunofluorescence images of apicoplast morphology of
 59 *P. berghei* sporozoites preincubated or not with azithromycin for 30 min. The sporozoites were
 60 visualized with GFP (gray), nuclei were stained with DAPI (blue) and apicoplast with ACP-
 61 antibody (red). Pictures are z-sections acquired by confocal microscopy with a $\times 63$ objective.
 62 Image z-stacks were deconvolved and then presented as a single combined image. Scale bar,

63 10µm. (B) Proportion (as mean percentage) of the various apicoplast morphologies in
64 sporozoites exposed or not to azithromycin. For each condition, 100 parasites were examined.
65 Data are from two independent experiments each carried out in quadruplicates. (C and D)
66 Primary cultures of *M. fascicularis* hepatocytes were infected with *P. berghei* sporozoites
67 preincubated or not with different doses of azithromycin and fixed at day 2 post-infection. (C)
68 Sizes of schizonts were significantly smaller in cultures infected with sporozoites preincubated
69 with azithromycin as compared to control. Schizonts size is expressed as surface area (µm²).
70 (D) No difference in the numbers of schizonts in cultures infected with sporozoites preincubated
71 with azithromycin as compared to control. Data are from two independent experiments carried
72 out in quadruplicate and the results are expressed as mean ± standard deviation (SD). Statistical
73 significance was determined using a one-way ANOVA Kruskal-Wallis followed by Dunn's
74 multiple comparisons to control groups, where significance is determined by P < 0.0001 (****),
75 P < 0.001 (**), and P < 0.05 (*).

76

77



78

79 **Figure S6. *P. cynomolgi* EEFs development is blocked by azithromycin treatment of**
80 **infected primary simian hepatocytes. *P. cynomolgi* M-infected primary hepatocyte cultures**
81 **were exposed to the different doses of azithromycin from day 0 to day 7 pi and fixed on day 7.**
82 (A) The numbers of schizont (dark) and uninucleate forms (green) were not significantly
83 different in azithromycin-treated cultures as compared to untreated controls. The experiments
84 were performed in triplicate and the results were expressed as mean \pm standard deviation (SD).
85 (B) The size of schizonts was significantly smaller in azithromycin-treated cultures compared
86 to untreated control. Each dot represents mean EEFs area per well in a 96-well plate ($n = 3$
87 wells). (C and D) Quantification of the various apicoplast morphologies in schizonts (C) and
88 uninucleate forms (D) in AZ-treated and untreated cultures. For each condition, 100 parasites
89 were observed. Statistical significance was determined using a one-way ANOVA Kruskal-

90 Wallis test followed by Dunn's multiple comparisons to control groups, where significance is
91 determined by $P < 0.0001$ (****), $P < 0.001$ (**) and $P < 0.05$ (*).

92

93