bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Antibiotic inhibition of the *Plasmodium* apicoplast decreases haemoglobin degradation and antagonises dihydroartemisinin action

Emily M. Crisafulli[1], Amanda De Paoli[2], Madel V. Tutor[1], Ghizal Siddiqui[2], Darren J. Creek[2], Leann Tilley[1], Stuart A. Ralph*[1]

1. Department of Biochemistry and Pharmacology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, 30 Flemington Road, Parkville, VIC 3052, Australia.
2. Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, VIC 3052, Australia.

* Email saralph@unimelb.edu.au

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Abstract

The World Health Organisation (WHO) recommends artemisinin (ART) combinations for treatment of uncomplicated *Plasmodium falciparum* malaria. Understanding the interaction between co-administered drugs within combination therapies is clinically important to prevent unintended consequences. The WHO guidelines recommend second line treatments that combine artesunate with tetracycline, doxycycline, or clindamycin—antibiotics that target the *Plasmodium* relict plastid, the apicoplast. In addition, antibiotics can be used simultaneously against other infectious diseases, leading to their inadvertent combination with ARTs. One consequence of apicoplast inhibition is a perturbation to haemoglobin uptake and trafficking—a pathway required for activation of ART derivatives. Here, we show that apicoplast-targeting antibiotics reduce the abundance of the catalyst of ART activation (free haem) in *P. falciparum*, likely through diminished haemoglobin digestion. We demonstrate antagonism between ART and these antibiotics, suggesting that apicoplast inhibitors reduce ART activation. These data have potential clinical implications due to the reliance on—and widespread use of—both ARTs and these antibiotics in malaria endemic regions.

Keywords. *Plasmodium*; malaria; apicoplast; delayed death; antibiotics; doxycycline; clindamycin; fosmidomycin; artemisinin.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Introduction

Malaria remains one of the deadliest diseases affecting humankind, responsible for an estimated 409,000 deaths in 2019 [1]. Incidence and mortality rates remain high despite substantial efforts in drug and vaccine development, and the widespread use of bed nets and vector control programs in malaria endemic regions. Of particular concern in recent years has been the development of increasing resistance to the current frontline antimalarial, artemisinin (ART). The lack of comparably safe, effective, fast-acting, and affordable antimalarials in the drug pipeline signifies that efforts toward monitoring and managing parasite sensitivity to ARTs is of the upmost importance to prevent worsening of a global health emergency.

ART derivatives are recommended for use with a second partner drug as ART combination therapies, or ACTs, for the treatment of uncomplicated *Plasmodium falciparum* malaria [2]. The rationale for this is multifaceted, combining mitigation of risks against treatment failure—due to the very short in vivo half-life (~1 h) of ART derivatives [3]—and against the development of drug resistance [4]—by hitting multiple drug targets within the parasite.

In some circumstances where preferred ART partner drugs are unavailable, the WHO recommends the use of artesunate (an ART derivative) plus either doxycycline or clindamycin [2]. There have also been calls to consider combining ART derivatives with antibiotics [5, 6], in part due to their dual anti-malarial and anti-bacterial activities. Currently, the WHO recommends treating patients presenting with suspected severe malaria with antibiotics in addition to malaria therapy—that is, until a bacterial infection can be excluded [2]. Use of these antibiotics in ACTs is appealing because many of them have anti-parasitic action through inhibition of the *Plasmodium* relict plastid, the apicoplast [7, 8]. Widespread use of these antibiotics in malaria endemic regions for malaria prophylaxis and the treatment of malaria or

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

bacterial infections means that they may already be circulating in patients who seek treatment with ACTs for malaria. The deliberate as well as unintended use of these drugs in combination in the field makes it important to understand their modes of action, and possible interaction, within *P. falciparum* parasites.

The ability of these antibiotics to kill *Plasmodium* spp. relies on the bacterial origins of the apicoplast [8]. While inhibitors that directly target apicoplast metabolism generally cause immediate parasite death [9], others that target the organelle's housekeeping functions—such as protein synthesis—cause a delayed death phenotype [10–12]. In delayed death, parasites survive the first cycle following treatment and it is not until the subsequent cycle that they cease proliferating and die. Isoprenoid synthesis is housed in the apicoplast, and antibiotic-treated parasites are depleted of the apicoplast-synthesised isoprenoid precursors required for the prenylation of trafficking machinery proteins, such as Rab proteins, resulting in a defect in the parasite's uptake and digestion of haemoglobin [13, 14]. Haem released during haemoglobin digestion is predominantly responsible for the activation of ARTs via the cleavage of the endoperoxide bond [15, 16], so inhibition of this process may have implications for efficacy of ART treatment when these drugs are combined.

In this study, we aimed to assess the nature of the interaction between apicoplast-targeting antibiotics and ART derivatives. We hypothesised that these antibiotics would behave antagonistically with ARTs due to their inhibition of haemoglobin uptake and subsequent reduced release of free haem. We showed that delayed death antibiotics do indeed reduce the abundance of free haem available in *P. falciparum* for ART activation in the cycle after treatment. Although combinations of ART and antibiotics have previously been tested for interactions, those assays were designed to identify effects during the first cycle of treatment, which would miss effects that manifest at the time when delayed death antibiotics exert their

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

biological impact. We instead tested for interactions in parasites that were treated with ART in the cycle after application of antibiotics. We found an antagonistic effect between these antibiotics and ART, presumably owing to the reduced activation of ART due to lower availability of free haem.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Methods

Plasmodium falciparum culture and synchronisation

Plasmodium falciparum 3D7 parasites were cultured as previously described [17]: at 2% (v/v) haematocrit in human O+ red blood cells (RBCs; Australian Red Cross Blood Service) and complete medium (RPMI-1640 with 25 mM sodium bicarbonate, 25 mM HEPES, 150 µM hypoxanthine, 20 µg/mL gentamicin (Sigma-Aldrich, G3632), and 0.5% (w/v) Albumax II (Thermo Fisher Scientific), pH 7.4); and maintained in malaria-mix gas (1% O_2, 5% CO_2, and 94% N_2) at 37°C. Ring stage synchronisation (~0–18 h p.i.) was achieved by a single treatment of infected RBCs with 5% (w/v) D-sorbitol (Sigma-Aldrich), unless otherwise indicated.

72 h in vitro single drug sensitivity assays

Synchronised ring stage 3D7 *P. falciparum* parasites (0.5% haematocrit; 1% parasitemia) were set up in V-bottom 96-well plates. Parasites were either: immediately treated with a dose gradient of atovaquone (Sigma-Aldrich, A7986), E-64 (Sigma-Aldrich, E3132), fosmidomycin (Sigma-Aldrich, F8682), proguanil (Sigma-Aldrich, G7048), or quinine (Sigma-Aldrich, 145904) in complete medium; or incubated for 24 h before treatment with various concentrations of dihydroartemisinin (DHA; Chem-Supply, D3793) or WR99210 (Jacobus Pharmaceutical) in complete medium. DHA-treated parasites were washed three times with complete medium 3 h post-drug treatment. Control wells containing parasites in the absence of drug or 100 µM chloroquine (Sigma-Aldrich, C6628) were prepared in parallel. All samples were prepared in triplicate.

72 h following set up, infected RBCs were incubated with lysis buffer (20 mM TRIS (pH 7.5), 5 mM EDTA, 0.008% (w/v) saponin, 0.08% (v/v) Triton X-100) and SYBR Green I (Thermo

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Fisher Scientific) for 1 h [18]. The fluorescent signal was detected using a FLUOstar Omega plate reader (BMG Labtech). Per cent survival was calculated by subtracting the background (chloroquine-treated) signal and normalising the data to the untreated control. GraphPad Prism (Version 9.1.2) was used to plot normalised data on XY scatter plots and calculate IC_{50}s (presented as mean ± SEM).

120 h in vitro single drug sensitivity assays

Synchronised ring stage 3D7 *P. falciparum* parasites (0.5% haematocrit; 0.1% parasitemia) were set up in V-bottom 96-well plates. In the presence or absence of 5 μM geranylgeraniol (GGOH; Sigma-Aldrich, G3278), parasites were incubated in varying concentrations of clindamycin (Sigma-Aldrich, C5269) or doxycycline (Sigma-Aldrich, D3447) for 72 h, before the drugs were washed off with complete medium. Control wells were prepared (as described above, 72 h in vitro drug sensitivity assays) with chloroquine added to the positive control wells only at 72 h post-treatment. All samples were prepared in triplicate. At the 120 h time-point, infected RBCs underwent lysis, data acquisition, and analysis (as described above, 72 h in vitro drug sensitivity assays).

Isobolograms

Synchronised ring stage 3D7 *P. falciparum* parasites (0.5% haematocrit; 72 h: 1% parasitemia, or 120 h: 0.1% parasitemia) were set up in V-bottom 96-well plates. Parasites were treated with dose gradients of two drugs, producing a 96-well plate where each well consisted of a unique combination (Fig. 1A). Various pairings of atovaquone, clindamycin, DHA, doxycycline, E-64, fosmidomycin, proguanil, quinine, and WR99210 were tested. The combination of delayed death drugs with DHA were done in both the presence and absence of 5 μM GGOH. As described above, for combinations that included DHA, parasites were washed three times with

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

complete medium 3 h post-drug treatment, to mimic the short in vivo half-life of ARTs. Controls and treatment regimens for each combination were as previously described (72 h in vitro drug sensitivity assays; 120 h in vitro drug sensitivity assays; Fig. 2A to C, and S1A to C). All samples were prepared in duplicate.

Parasites were lysed at the 72 or 120 h time-point (Fig. 2A to C, and S1A to C), data acquired, and per cent survival calculated for each data point (as described above, 72 h in vitro drug sensitivity assays). FIC_{50}s were calculated [19] (Fig. 1B):

$$\mathbf{FIC_{50}\ (drug\ A)} = \frac{\mathbf{IC_{50}\ (drug\ A\ in\ presence\ of\ [\boldsymbol{x}]\ drug\ B)}}{\mathbf{IC_{50}\ (drug\ A)}}$$

and GraphPad Prism (Version 9.1.2) used to plot data on XY scatter plots. ΣFIC_{50}s were calculated to quantify the drug interaction (presented as mean ± SEM) [19]:

$$\mathbf{\sum FIC_{50} = FIC_{50}\ (drug\ A) + FIC_{50}\ (drug\ B)}$$

Interpretation of these values utilised previously defined thresholds [19]: < 0.1, very strong synergism; 0.1–0.3, strong synergism; 0.3–0.7, synergism; 0.7–0.85, moderate synergism; 0.85–0.9, slight synergism; 0.9–1.1, additive; 1.1–1.2, slight antagonism; 1.2–1.45, moderate antagonism; 1.45–3.3, antagonism; 3.3–10, strong antagonism; and > 10, very strong antagonism. GraphPad Prism (Version 9.1.2) was used to conduct unpaired *t*-tests with Welch's corrections to test statistical significance of the ΣFIC_{50}s.

Haemoglobin fractionation

Ring stage 3D7 *P. falciparum* parasites were either: synchronised (as described above, *Plasmodium falciparum* culture and synchronisation) and treated with 3 μM fosmidomycin for

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

24 h; or subjected to two D-sorbitol treatments, 8 h apart, and parasites (~8–18 h p.i.) subsequently treated with 1 μM doxycycline or 25 nM clindamycin for 68 h. Negative controls with the appropriate vehicle were set up in parallel. Following the incubation period, RBCs were lysed with 0.15% (w/v) saponin and parasite pellets washed three times with PBS and cOmplete™ mini EDTA-free protease inhibitor (Roche) at 4°C. Pellets were immediately frozen at −80°C until use.

The haemoglobin fractionation assay was adapted from [20, 21] with minor adjustments. Pellets were first resuspended in distilled water and sonicated for 5 min before addition of HEPES (pH 7.5; final concentration 0.1 M). Samples were centrifuged (6,200 x *g*, 20 min) and the pellets resuspended in distilled water before addition of 4% (w/v) sodium dodecyl sulfate (SDS; final concentration 2% (w/v)). The samples were sonicated for 5 min, then incubated at 95°C for 5 min to solubilise the free haem. A solution of HEPES, NaCl, and pyridine was added (final concentration 67 mM HEPES, 0.1 M NaCl, and 8.3% (v/v) pyridine) and samples centrifuged (6,200 x *g*, 20 min). The supernatant—containing the free haem fraction—was transferred to a clear, flat-bottom 96-well plate. The residual pellets were then resuspended in distilled water before solubilisation with NaOH (final concentration 0.15 M). They were sonicated for 15 min before a solution of HEPES, NaCl, and pyridine was added, as described above. These samples—corresponding to the haemozoin fraction—were transferred to the 96-well plate.

A blank (0.2 M HEPES, 0.3 M NaOH, 0.3 M NaCl, 0.3 M HCl, 4% (w/v) SDS, and 25% (v/v) pyridine) was included in triplicate for each study. The absorbance of each fraction was measured at 405 nm using an Ensight plate reader (PerkinElmer). Samples were blank adjusted and normalised to vehicle control. Data are presented as mean fold change compared to vehicle

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

control ± SEM, and GraphPad Prism (Version 9.1.2) was used to perform one sample *t*-tests. All samples were prepared in triplicate.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Results

Apicoplast inhibitors behave antagonistically with the artemisinin derivative, dihydroartemisinin (DHA)

The Chou and Talalay isobologram method was used to test the efficacy of various drug combinations [22]. Specifically, we investigated whether apicoplast inhibitors and the ART derivative, dihydroartemisinin (DHA), behave antagonistically. Concentrations giving 50 per cent inhibition of growth of sorbitol-synchronised 3D7 ring stage *P. falciparum* (IC_{50} values) were first determined for individual drugs by means of a SYBR Green drug assay (Table S1). These were consistent with IC_{50} values from previous reports and allowed determination of dose gradients required to establish isobolograms [23–28]. For each isobologram, parasites were treated with varying combinations of two drugs across these gradients (Fig. 1A) at the relevant dosing regimens indicated (Fig. 2A to C, and S1A to C). The fraction of the IC_{50} concentration of each drug required to generate 50 per cent inhibition—the fractional IC_{50} (FIC_{50})—was then calculated for each dose held constant across the plate and plotted to form an isobologram (Fig. 1B). The shape of the isobologram and sum of the FIC_{50}s (ΣFIC_{50}s) were used to determine the type of drug interaction for each combination using previously defined thresholds [19].

To first validate this methodology, synchronised ring stage parasites were treated with the well-established synergistic combination of atovaquone and proguanil (Fig. 2A). We analysed growth at 72 h post-treatment (standard timing to assess first cycle death). The isobologram indicated a moderately synergistic interaction and produced a mean ΣFIC_{50} of 0.72 ± 0.04—concordant with previous reports [27, 29, 30] (Fig. 2D and 3).

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Testing the interaction of delayed death drugs (which kill in the second cycle) and DHA (which kills in the first cycle) was a complex task, so we began by substituting the delayed death drug for an apicoplast inhibitor, fosmidomycin, that causes first cycle killing. Fosmidomycin directly blocks apicoplast metabolism by inhibiting 1-deoxy-D-xylulose-5-phosphate reductoisomerase, an enzyme involved in the isoprenoid biosynthetic pathway [9]. Like other apicoplast inhibitors, fosmidomycin perturbs haemoglobin uptake, albeit in the first cycle [13]. To specifically test the impact of the haemoglobin degradation defect, it was necessary to pre-treat synchronised ring stage 3D7 parasites with a dose gradient of fosmidomycin prior to DHA treatment. Further, to mimic the clinical scenario as closely as possible in an in vitro setting, we pulsed parasites with DHA for 3 h at the trophozoite stage, 24 h after pre-treatment with the apicoplast inhibitor (Fig. 2B). The experiment was designed to target the parasite life stage for which ART derivatives are most active [24, 31] and to mimic the short in vivo half-life of DHA [3]. Using this approach, fosmidomycin and DHA displayed moderate antagonism with a mean ΣFIC_{50} of 1.29 ± 0.05 (Fig. 2D and 3). This phenotype had been observed previously [32, 33], though using a different methodology that did not explore the clinically relevant half-life of DHA [3], nor fosmidomycin-induced haemoglobin trafficking defects [13]. The antagonism between fosmidomycin and DHA is concordant with the well-established antagonistic interaction between DHA and E-64 [31]—a cysteine protease inhibitor that prevents haemoglobin degradation, presumably reducing activation of DHA by free haem [34]. Consistent with this, we also saw a moderately antagonistic interaction between E-64 and DHA—with a mean ΣFIC_{50} of 1.36 ± 0.04 (Fig. 2D and 3)—using the approach described above (Fig. 2B).

As a control, we substituted the apicoplast inhibitor for atovaquone, an inhibitor of cytochrome *b* that was not predicted to interact with DHA. The mean ΣFIC_{50} of atovaquone

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

and DHA was 1.00 ± 0.04, indicating a simple additive interaction and demonstrating that the unusual pre-treatment approach employed in our methodology did not contribute to the observed phenotype (Fig. 2D and 3).

A longer pre-treatment was necessary to test the interaction between delayed death drugs and DHA, as the haemoglobin trafficking defects observed when parasites are treated with these apicoplast inhibitors are evident only in the cycle subsequent to treatment [14]. Ring stage *P. falciparum* parasites were pre-treated with doxycycline or clindamycin for 72 h, to recreate the delayed death effect, and subsequently pulsed with DHA for 3 h at the trophozoite stage in the second cycle (Fig. 2C). Isobologram analysis of these data demonstrate an antagonistic and moderately antagonistic interaction between DHA and doxycycline (mean ΣFIC_{50} of 1.49 ± 0.02), and DHA and clindamycin (mean ΣFIC_{50} of 1.44 ± 0.10), respectively (Fig. 2D and 3). The ΣFIC_{50}s of these combinations significantly increased—approximately 1.5-fold—from that of the additive interaction of atovaquone and DHA previously described (Fig. 3). This interaction with delayed death antibiotics was specific to DHA. The combination of delayed death drugs with WR99210—an inhibitor of dihydrofolate reductase—did not show the same antagonism (Fig. 3, and S1C and D). Mean ΣFIC_{50} values for doxycycline and clindamycin combined with WR99210 were 1.21 ± 0.05 and 1.16 ± 0.05, respectively (Fig. 3), suggesting that this cohort of apicoplast inhibitors have a selective effect on DHA activity. This interaction appeared to reverse when parasites were supplemented with exogenous geranylgeraniol (GGOH), a polyprenol that restores haemoglobin trafficking by permitting protein prenylation in the absence of isoprenoid biosynthesis [14] (Fig. S1B and D). However, the timing of these experiments was complex as GGOH-restored parasites survive longer but still die at a later stage that is yet to be thoroughly characterised [14], complicating attempts to quantify this interaction.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Apicoplast inhibitors reduce the abundance of haemoglobin degradation by-products

To determine if the observed antagonistic interactions between apicoplast inhibitors and DHA were indeed due to reduced DHA activation, we sought to examine the effects of these antibiotics on the downstream products of haemoglobin digestion. Trafficking of haemoglobin is perturbed in parasites treated with fosmidomycin [13] or delayed death drugs [14], though the effect on by-products of haemoglobin digestion—free haem and haemozoin—has yet to be explored. We quantified the effects of fosmidomycin, doxycycline, and clindamycin on the abundance of these by-products in *P. falciparum* using a haem fractionation method, whereby pyridine is used to indirectly quantify haem products [20, 21]. The delayed death drugs, doxycycline and clindamycin, both significantly reduced haemozoin abundance in trophozoite parasites 72 h following treatment—a 1.3- ($p = 0.0215$) and 1.7-fold ($p = 0.0416$) reduction compared to the vehicle control, respectively (Fig. 4B). Fosmidomycin treatment caused a 1.5-fold reduction in haemozoin abundance in the same cycle as treatment. Though these latter changes were not statistically significant ($p = 0.0854$) (Fig. 4A), the magnitude of reduction is similar to that seen for other inhibitors of haemozoin formation assayed using the same methodology [20, 21]. While haemozoin appears reduced in abundance, the effect on free haem appears less stark for both fast-acting and delayed death apicoplast inhibitors (Fig. 4). However, this may be explained by it being a labile and toxic by-product of haemoglobin digestion that is quickly converted into chemically inert haemozoin by the parasite.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Discussion

Combining drugs to enhance potency and reduce the risk of antimalarial resistance is a central plank in the strategies for malaria treatment. A notable example is the pairing of atovaquone with proguanil, a highly synergistic combination used for prophylaxis and treatment, frequently sold under the brand name Malarone [2, 29]. However, there are many documented examples of suboptimal antimalarial combinations [35], making choice of drug combinations key for effective treatment and reduced risk of drug resistance. Here, we demonstrate that antibiotics targeting the *P. falciparum* apicoplast behave antagonistically with the ART derivative, DHA, when the antibiotics are administered in the cycle before DHA. We propose that this interaction is underpinned by an antibiotic-mediated reduction in free haem that reduces the activation of DHA necessary for cytotoxicity (Fig. 5).

Previous studies have mapped the effects of apicoplast inhibition in asexual *P. falciparum* parasites—from impeding IPP biosynthesis to downstream perturbation of haemoglobin uptake [13, 14, 36]. Isoprenoids have multiple cellular fates, though the proximate cause of parasite death through antibiotic treatment results from reduced prenylation of trafficking proteins, potentially through Rab proteins involved in haemoglobin trafficking to the DV [13, 14] (Fig. 5A). This prevents anchoring to vesicle membranes; and is associated with the aberrant uptake of haemoglobin from the host RBC and fragmentation of the DV [13, 14] (Fig. 5B). Further, delayed death drugs reduce the abundance of haemoglobin-like peptides in the cycle following treatment [14], consistent with our finding that free haem and haemozoin levels are lowered at this timepoint. Our data also demonstrate a reduction in abundance of these by-products by fosmidomycin—a fast-acting apicoplast inhibitor—suggesting that reduced haemoglobin degradation is an inevitable downstream effect of apicoplast inhibition in the asexual blood stages.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

We hypothesise that this reduced release of free haem is the root cause of the antagonism between these antibiotics and DHA (Fig. 5). Consistent with this interpretation of the data is the antagonistic interaction observed between the cysteine protease inhibitor, E-64, and DHA—one that has been reported previously [31]. Indeed, a similar interaction has also been reported for another haemoglobin degradation inhibitor (the cysteine protease inhibitor, N-acetyl-L-leucyl-L-leucyl-L-norleucinal, or ALLN) [16]. In both studies, antagonism was attributed to a haem-mediated reduction in ART activation. This antagonistic interaction with apicoplast-inhibiting antibiotics may also extend to other ART-like compounds in the drug pipeline that similarly rely on free haem for activation—a notable example being ozonides [37, 38], the activation of which is directly inhibited by E-64 [39].

The antagonistic interaction between the fast-acting apicoplast inhibitor, fosmidomycin, and DHA described here is consistent with prior reports [32, 33]. By contrast, findings vary when testing the interaction between delayed death drugs and ART derivatives, although to our knowledge, such studies are restricted to analyses of interactions in the same cycle, which would ignore the impact of delayed death. For example, in vitro studies have reported interactions ranging from additivity [40–43] to synergy [42]; while there is a single in vivo report of synergy [44]. However, these studies were conducted using growth assays where the ART and antibiotic were administered at the same time and/or were terminated before the delayed death effect would have manifested. It is probable then that any effect captured results from the influence of secondary targets within the parasite, or only capture killing based on the very start of inhibition due to the antibiotic. Our experimental design specifically incorporates the delay in onset of the haemoglobin trafficking defect [14].

While combination treatment options would normally involve the simultaneous administration of ART with an antibiotic, several treatment regimens could mirror a scenario in which ART

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

is present in the cell at a time where delayed death is relevant. One scenario that presents is the purposeful use of apicoplast inhibitors and ARTs in combination. The WHO currently recommends treating patients presenting with an unknown illness with antibiotics in addition to antimalarials—that is, until a bacterial infection can be excluded [2]. Antibiotics can also be used as partner drugs in ACTs: calls for these to be considered as frontline therapies have been made due to the added benefit that comes by concurrently treating any present bacterial infections prevalent in malaria endemic regions [5, 6]. While pre-treatment with antibiotics is not done purposefully in these instances, the need for multiple doses and the longevity of the antibiotic mode of action means that ultimately a similar "pre-treatment" scenario would eventuate, for example in the second or later dose of a combination therapy.

The widespread use of antibiotics in regions with high malaria transmission presents another—more complex—scenario whereby these drugs are combined inadvertently. Use of apicoplast-targeting antibiotics in mass drug administration efforts to treat bacterial infections in malaria endemic regions is one such situation—a notable example being administration of azithromycin for trachoma [45]. Azithromycin would consequently be circulating in patients seeking treatment for malaria infections with ACTs in these regions. There are also documented, and presumably many more undocumented, cases where non-compliance with a prescribed course of prophylactic doxycycline (which is rather commonplace [46–48]) results in a malaria infection [49]—an infection that will presumably be treated with an ACT. Both scenarios create conditions for possible suboptimal activation of the ART component of the ACT.

The many possible contributors (e.g. ART resistance) make it very difficult to deconvolve the root cause of any treatment failure and attribute it to this apicoplast drug interaction. However, clinical trial data—while presenting extremely varied reports of efficacy—suggest treatment

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

failure is a very real possibility. A number of clinical trials report combinations of apicoplast inhibitors and ART derivatives that produced inferior cure rates and increased rates of recrudescence and treatment failure compared to other therapies [50–64] (Table 1).

Arguably of greater global concern is that the combination of ART and apicoplast drugs could expose parasites to suboptimal concentrations of activated ART, conceivably worsening the already alarming spread of ART resistance and ART treatment failure that is currently occurring in South-East Asia [1]. There are several reports of modulation of ART sensitivity associated with mutations in or upstream of genes for apicoplast proteins, both in the field [65, 66] and in cultured parasites [67]. The significance of this is so far unclear, but one potential role could be through changes to apicoplast metabolism that impact isoprenoid synthesis and thus haemoglobin uptake (Fig. 5C). Given the almost ubiquitous use of ACTs throughout the malaria endemic world and the recent emergence of ART resistance in sub-Saharan Africa [68, 69], protecting against the rise of resistance elsewhere is key to avoid the worsening of malaria as a global health challenge.

The clinical uses of apicoplast-targeting antibiotics mean that they are often either purposefully or inadvertently used in combination with ART derivatives in the field. Although extrapolation of clinical relevance from in vitro data should be done carefully, these data flag potential concerns against combining ARTs with apicoplast-inhibiting antibiotics and reinforce the need to consider the molecular modes of action of any drugs used in combination in the field.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Acknowledgments

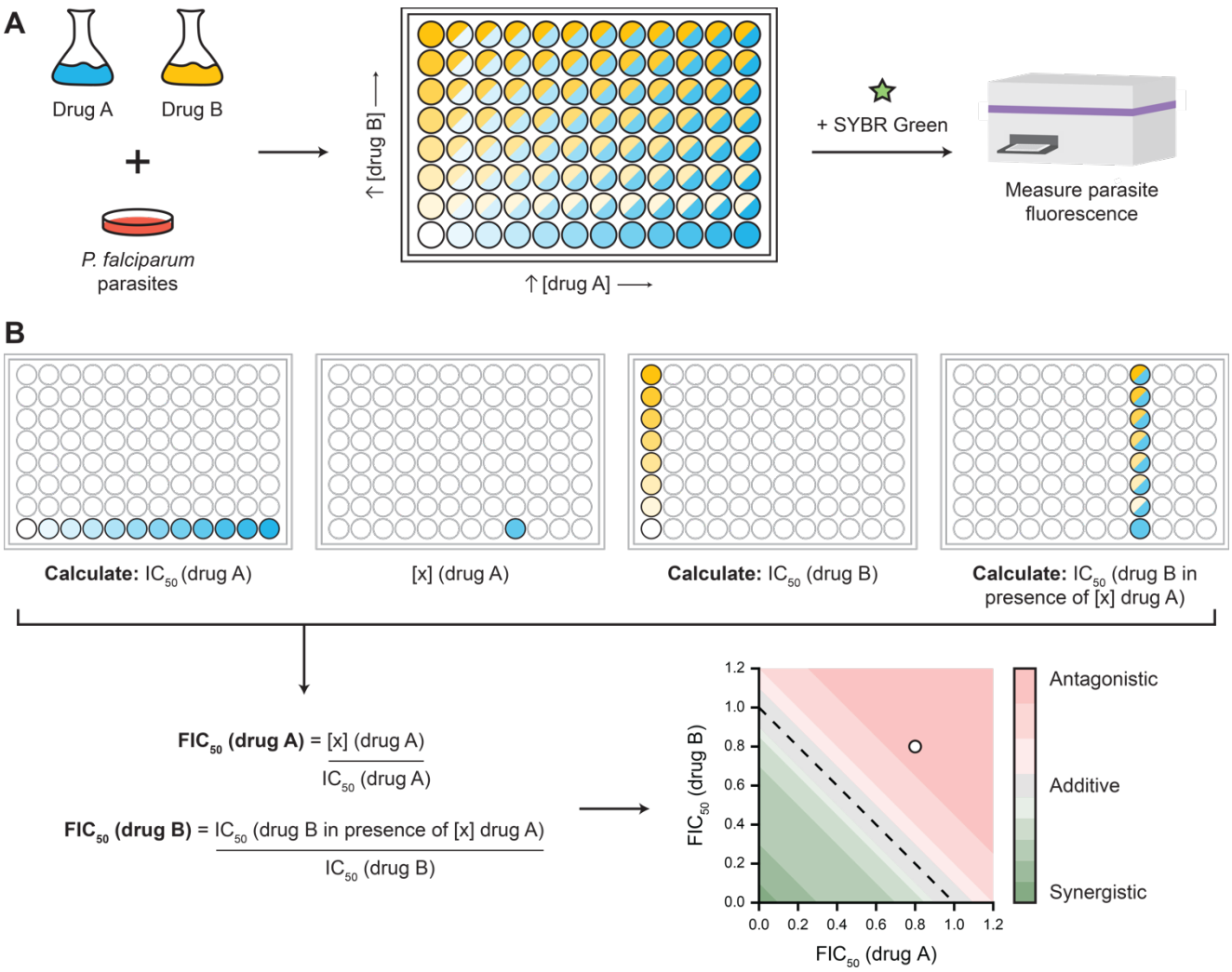
We are grateful to Geoffrey I. McFadden, Christopher D. Goodman, Stanley C. Xie (University of Melbourne), Kit Kennedy (Weill Cornell Medicine), Matthew P. Challis (Monash Institute of Pharmaceutical Sciences), and Christina Spry (The Australian National University) for helpful discussions. We thank Jacobus Pharmaceutical for the kind gift of WR99210 and the Australian Red Cross Blood Service for donation of red blood cells for in vitro culturing of parasites.

This study was funded through grants from the Australian National Health and Medical Research Council (Grant #1181336, 1139884 and #1163235).

We also wish to acknowledge the Traditional Custodians of the lands on which this project was conducted, the Wurundjeri People of the Kulin nation.

Conflict of interest statement. Nil.

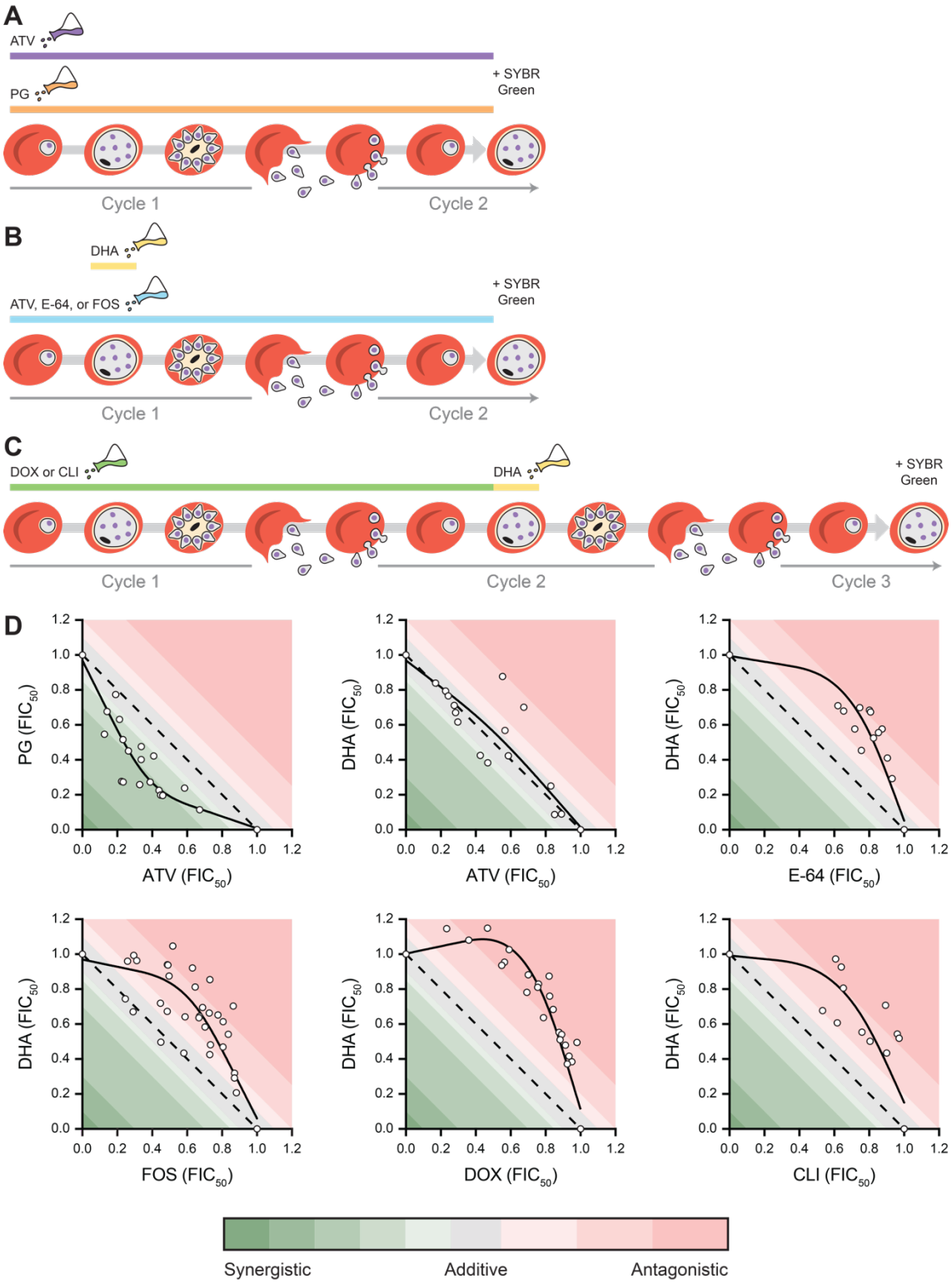
bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



362

363 **Fig 1. Drug interactions were determined by isobologram analysis.** A) Schematic of the
364 isobologram set-up pipeline, whereby 3D7 *Plasmodium falciparum* ring stage parasites were
365 added to a V-bottom 96-well plate containing dose gradients of two drugs (blue, yellow).
366 Following drug incubation, parasites were lysed and stained with SYBR Green for 1 h before
367 fluorescent signal was detected using a microplate reader. B) Isobologram data analysis
368 pipeline required calculation of fractional IC_{50}s (FIC_{50}s) to be plotted on an XY scatter plot.
369 Drug combinations were evaluated to be synergistic (green), additive (grey), or antagonistic
370 (red) based on previously defined thresholds [19].

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

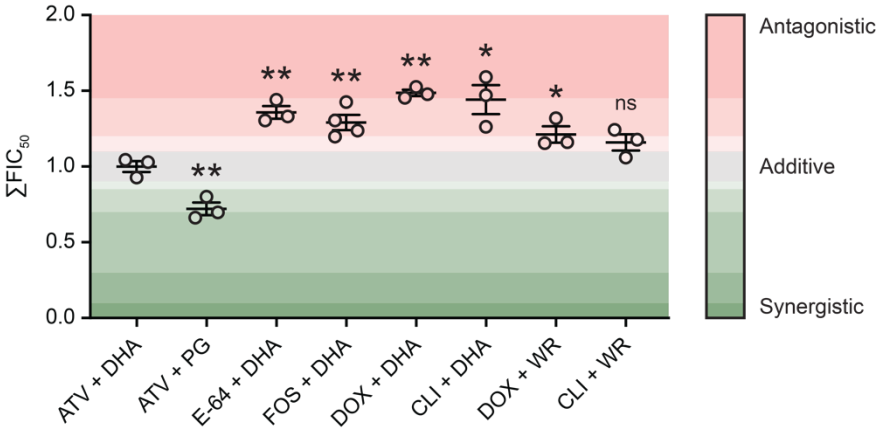


371

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Fig 2. Normalised isobolograms demonstrating antagonism between apicoplast inhibitors and dihydroartemisinin (DHA). A–C) Schematic of the treatment regimen of 3D7 *Plasmodium falciparum* ring stage parasites in the set-up of D) isobolograms. Parasites were either: A) treated with dose gradients of atovaquone (ATV) and proguanil (PG) for 72 h; B) pre-treated for 24 h with ATV, E-64, or fosmidomycin (FOS); or C) pre-treated for 72 h with doxycycline (DOX) or clindamycin (CLI). Pre-treated parasites were pulsed with a dose gradient of DHA for 3 h. Parasites were lysed and stained with SYBR Green at A and B) 72 h or C) 120 h post-initial drug treatment. Fractional IC_{50}s (FIC_{50}s) are presented ($n \geq 3$). Interaction thresholds as previously defined [19].

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



Drug combination	$\sum FIC_{50}$ (mean ± SEM)	Interaction
ATV + DHA	1.00 ± 0.04	Additive
ATV + PG	0.72 ± 0.04**	Moderate synergism
E-64 + DHA	1.36 ± 0.04**	Moderate antagonism
FOS + DHA	1.29 ± 0.05**	Moderate antagonism
DOX + DHA	1.49 ± 0.02**	Antagonism
CLI + DHA	1.44 ± 0.10*	Moderate antagonism
DOX + WR	1.21 ± 0.05*	Moderate antagonism
CLI + WR	1.16 ± 0.05	Slight antagonism

Fig 3. Mean ΣFIC_{50}s demonstrating antagonism between apicoplast inhibitors and dihydroartemisinin (DHA). 3D7 *Plasmodium falciparum* ring stage parasites were either: treated with atovaquone (ATV) and proguanil (PG); pre-treated for 24 h with ATV, E-64, or fosmidomycin (FOS); or pre-treated for 72 h with doxycycline (DOX) or clindamycin (CLI). Pre-treated parasites were pulsed with a dose gradient of DHA for 3 h or WR99210 (WR) for 48 h. Parasites were lysed and stained with SYBR Green at 72 or 120 h. Data are presented as mean ± SEM ($n \geq 3$). Unpaired *t*-tests with Welch's corrections were performed: *$p \leq 0.05$, **$p \leq 0.01$. Interaction thresholds as previously defined [19].

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

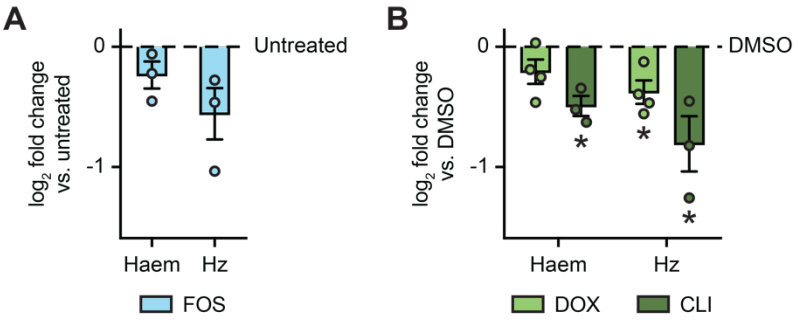
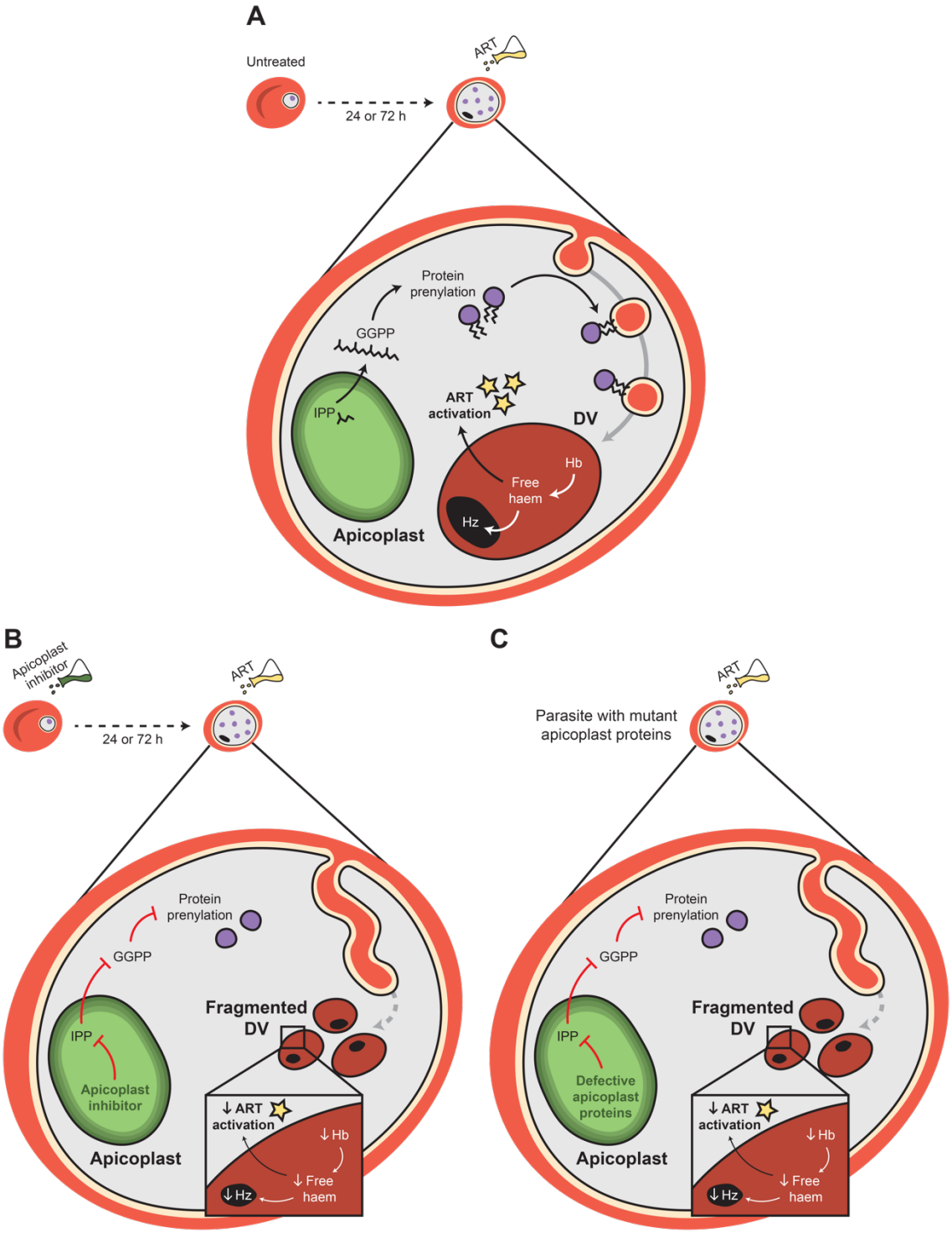


Fig 4. Apicoplast inhibitors reduce the abundance of haemoglobin degradation by-products. 3D7 *Plasmodium falciparum* ring stage parasites were treated with vehicle or: A) 3 µM fosmidomycin (FOS) for 24 h (~24–42 h p.i.); or B) 1 µM doxycycline (DOX) or 25 nM clindamycin (CLI) for 68 h (~76–86 h p.i.). Parasites were harvested and sequentially fractionated to determine the relative levels of free haem and haemozoin (Hz). Data were normalised to the vehicle control and are presented as mean $\log_2$ fold change ± SEM ($n \geq 3$). One sample *t*-tests were performed: *$p \leq 0.05$.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



398

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Fig 5. Model of apicoplast inhibition of isoprenoid biosynthesis decreasing haemoglobin (Hb) degradation and antagonising artemisinin (ART). A) In the absence of apicoplast inhibition, ART is activated by a product of Hb degradation—free haem—generated in the digestive vacuole (DV). Hb trafficking and degradation is dependent on the prenylation of trafficking proteins, a process that itself relies on production of the isoprenoid precursor, isopentenyl pyrophosphate (IPP), in the apicoplast. B) Apicoplast inhibition—both by fast-acting direct inhibitors of isoprenoid biosynthesis (24 h) and delayed death antibiotics (72 h)—reduces isoprenoid biosynthesis, preventing formation of geranylgeranyl pyrophosphate (GGPP), which forms the prenyl group on trafficking proteins required for Hb uptake and trafficking to the DV. In the absence of prenylated trafficking proteins, the cytostome becomes elongated and the DV fragmented. Hb degradation is reduced, reducing the abundance of free haem and haemozoin (Hz), and, subsequently, the activation of ART. C) Mutations in genes for apicoplast proteins reduce apicoplast metabolic processes, such as isoprenoid biosynthesis. In a similar fashion to drug inhibition, these mutations reduce prenylation and perturb haemoglobin uptake, such that there is less free haem available to activate ART.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

414 **Table 1. Summary of findings from clinical studies that combined apicoplast inhibitors**

415 **with artemisinin derivatives[a].**

Study	Apicoplast inhibitor + artemisinin derivative		Control	
	Drugs	**Finding**	**Drug(s)**	**Finding**
[54]	Azithromycin + artemether	14.8% cure rate[b]	N/A	N/A
[55]	Azithromycin + artesunate	30% recrudescence rate	Artesunate monotherapy	30% recrudescence rate
[56]	Azithromycin + artesunate	56% cure rate	Artesunate monotherapy	44% cure rate
			Mefloquine + artesunate	98% cure rate
[60]	Azithromycin + artesunate	88.9–92% cure rate	Azithromycin + quinine	73.3–92% cure rate
[61]	Azithromycin + artesunate	58% recrudescence rate (of those, 32% failure rate)	Lumefantrine + artemether	20% recrudescence rate (of those, 9% failure rate)
[63]	Azithromycin + artesunate	94.6% cure rate	Lumefantrine + artemether	97% cure rate
[64]	Azithromycin + artesunate	96.7% cure rate	Artesunate monotherapy	90% cure rate
[57]	Azithromycin + dihydroartemisinin	69.7% cure rate	Mefloquine + dihydroartemisinin	100% cure rate
[59]	Clindamycin + artesunate	87% cure rate	Clindamycin + quinine	94% cure rate
[54]	Doxycycline + artemether	53.3% cure rate	N/A	N/A

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

[52]	Doxycycline + artemisinin	67% recrudescence rate 3 cases of treatment failure	Quinine monotherapy	16% recrudescence rate 1 case of treatment failure
			Quinine + artemisinin	28% recrudescence rate 0 cases of treatment failure
[51]	Doxycycline + artesunate	80% cure rate	Doxycycline + mefloquine	96% cure rate
[58]	Fosmidomycin + artesunate	100% cure rate	N/A	N/A
[50]	Tetracycline + artemisinin	9.5% recrudescence rate	Artemisinin monotherapy	10–50% recrudescence rate
[53]	Tetracycline + artesunate	80% cure rate	Tetracycline + quinine	77% cure rate
[62]	Tetracycline + artesunate	95.5% cure rate	Tetracycline + quinine	91.1% cure rate

[a] Metrics defined by the original authors are presented here. These are internally consistent but vary between studies.

[b] Attributed to possible inadequate azithromycin dosage.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

References

1. World Health Organization. World malaria report 2020: 20 years of global progress and challenges. Geneva: World Health Organization, **2020**.

2. World Health Organization. WHO Guidelines for malaria. Geneva: World Health Organization, **2021**.

3. White NJ. Qinghaosu (artemisinin): the price of success. Science **2008**; 320(5874):330–4.

4. White NJ. Preventing antimalarial drug resistance through combinations. Drug Resist Updat **1998**; 1(1):3–9.

5. Noedl H. ABC – antibiotics-based combinations for the treatment of severe malaria? Trends Parasitol **2009**; 25(12):540–4.

6. Gaillard T, Boxberger M, Madamet M, Pradines B. Has doxycycline, in combination with anti-malarial drugs, a role to play in intermittent preventive treatment of *Plasmodium falciparum* malaria infection in pregnant women in Africa? Malar J **2018**; 17(1):469.

7. Geary TG, Jensen JB. Effects of antibiotics on *Plasmodium falciparum* in vitro. Am J Trop Med Hyg **1983**; 32(2):221–5.

8. Goodman CD, Su V, McFadden GI. The effects of anti-bacterials on the malaria parasite *Plasmodium falciparum*. Mol Biochem Parasitol **2007**; 152(2):181–91.

9. Jomaa H, Wiesner J, Sanderbrand S, et al. Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. Science **1999**; 285(5433):1573–6.

10. Dahl EL, Rosenthal PJ. Multiple antibiotics exert delayed effects against the *Plasmodium falciparum* apicoplast. Antimicrob Agents Chemother **2007**; 51(10):3485–90.

11. Goodman CD, Pasaje CFA, Kennedy K, McFadden GI, Ralph SA. Targeting protein translation in organelles of the Apicomplexa. Trends Parasitol **2016**; 32(12):953–65.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

12. Kennedy K, Crisafulli EM, Ralph SA. Delayed death by plastid inhibition in apicomplexan parasites. Trends Parasitol **2019**; 35(10):747–59.

13. Howe R, Kelly M, Jimah J, Hodge D, Odom AR. Isoprenoid biosynthesis inhibition disrupts Rab5 localization and food vacuolar integrity in *Plasmodium falciparum*. Eukaryotic Cell **2013**; 12(2):215–23.

14. Kennedy K, Cobbold SA, Hanssen E, et al. Delayed death in the malaria parasite *Plasmodium falciparum* is caused by disruption of prenylation-dependent intracellular trafficking. PLoS Biol **2019**; 17(7):e3000376.

15. Meshnick SR, Taylor TE, Kamchonwongpaisan S. Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. Microbiological Reviews **1996**; 60(2):301–15.

16. Klonis N, Crespo-Ortiz MP, Bottova I, et al. Artemisinin activity against *Plasmodium falciparum* requires hemoglobin uptake and digestion. Proc Natl Acad Sci U S A **2011**; 108(28):11405–10.

17. Trager W, Jensen JB. Human malaria parasites in continuous culture. Science **1976**; 193(4254):673–5.

18. Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob Agents Chemother **2004**; 48(5):1803–6.

19. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev **2006**; 58(3):621–81.

20. Combrinck JM, Fong KY, Gibhard L, Smith PJ, Wright DW, Egan TJ. Optimization of a multi-well colorimetric assay to determine haem species in *Plasmodium falciparum* in the presence of anti-malarials. Malar J **2015**; 14:253.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

21. Birrell GW, Challis MP, De Paoli A, et al. Multi-omic characterization of the mode of action of a potent new antimalarial compound, JPC-3210, against *Plasmodium falciparum*. Mol Cell Proteomics **2020**; 19(2):308–25.

22. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Advances in Enzyme Regulation **1984**; 22:27–55.

23. Liu J, Istvan ES, Gluzman IY, Gross J, Goldberg DE. *Plasmodium falciparum* ensures its amino acid supply with multiple acquisition pathways and redundant proteolytic enzyme systems. Proc Natl Acad Sci U S A **2006**; 103(23):8840–5.

24. Klonis N, Xie SC, McCaw JM, et al. Altered temporal response of malaria parasites determines differential sensitivity to artemisinin. Proc Natl Acad Sci U S A **2013**; 110(13):5157–62.

25. Sanders NG, Meyers DJ, Sullivan DJ. Antimalarial efficacy of hydroxyethylapoquinine (SN-119) and its derivatives. Antimicrob Agents Chemother **2014**; 58(2):820–7.

26. 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 **2017**; 62(1):e01161–17.

27. Skinner-Adams TS, Fisher GM, Riches AG, et al. Cyclization-blocked proguanil as a strategy to improve the antimalarial activity of atovaquone. Commun Biol **2019**; 2:166.

28. Remcho TP, Guggilapu SD, Cruz P, et al. Regioisomerization of antimalarial drug WR99210 explains the inactivity of a commercial stock. Antimicrob Agents Chemother **2021**; 65(1):e01385–20.

29. Canfield CJ, Pudney M, Gutteridge WE. Interactions of atovaquone with other antimalarial drugs against *Plasmodium falciparum* in vitro. Exp Parasitol **1995**; 80(3):373–81.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

30. Fivelman QL, Adagu IS, Warhurst DC. Modified fixed-ratio isobologram method for studying in vitro interactions between atovaquone and proguanil or dihydroartemisinin against drug-resistant strains of *Plasmodium falciparum*. Antimicrob Agents Chemother **2004**; 48(11):4097–102.

31. Xie SC, Dogovski C, Hanssen E, et al. Haemoglobin degradation underpins the sensitivity of early ring stage *Plasmodium falciparum* to artemisinins. J Cell Sci **2016**; 129(2):406–16.

32. Wiesner J, Henschker D, Hutchinson DB, Beck E, Jomaa H. In vitro and in vivo synergy of fosmidomycin, a novel antimalarial drug, with clindamycin. Antimicrob Agents Chemother **2002**; 46(9):2889–94.

33. Chaijaroenkul W, Pruktal P, Muhamad P, Na-Bangchang K. Assessment of in vitro antimalarial interactions between dihydroartemisinin and fosmidomycin. The Southeast Asian Journal of Tropical Medicine and Public Health **2007**; 38(5):791–5.

34. Giannangelo C, Siddiqui G, De Paoli A, et al. System-wide biochemical analysis reveals ozonide antimalarials initially act by disrupting *Plasmodium falciparum* haemoglobin digestion. PLoS Pathog **2020**; 16(6):e1008485.

35. Bell A. Antimalarial drug synergism and antagonism: mechanistic and clinical significance. FEMS Microbiol Lett **2005**; 253(2):171–84.

36. Yeh E, DeRisi JL. Chemical rescue of malaria parasites lacking an apicoplast defines organelle function in blood-stage *Plasmodium falciparum*. PLoS Biol **2011**; 9(8):e1001138.

37. Giannangelo C, Anderson D, Wang X, Vennerstrom JL, Charman SA, Creek DJ. Ozonide antimalarials alkylate heme in the malaria parasite *Plasmodium falciparum*. ACS Infect Dis **2019**; 5(12):2076–86.

38. Adoke Y, Zoleko-Manego R, Ouoba S, et al. A randomized, double-blind, phase 2b study to investigate the efficacy, safety, tolerability and pharmacokinetics of a single-dose regimen

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

514 of ferroquine with artefenomel in adults and children with uncomplicated *Plasmodium*
515 *falciparum* malaria. Malar J **2021**; 20(1):222.
516 39. Giannangelo C, Stingelin L, Yang T, Tilley L, Charman SA, Creek DJ. Parasite-mediated
517 degradation of synthetic ozonide antimalarials impacts in vitro antimalarial activity.
518 Antimicrob Agents Chemother **2018**; 62(3):e01566–17.
519 40. Chawira AN, Warhurst DC. The effect of artemisinin combined with standard antimalarials
520 against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum* in
521 vitro. The Journal of Tropical Medicine and Hygiene **1987**; 90(1):1–8.
522 41. Fivelman QL, Walden JC, Smith PJ, Folb PI, Barnes KI. The effect of artesunate combined
523 with standard antimalarials against chloroquine-sensitive and chloroquine-resistant strains of
524 *Plasmodium falciparum* in vitro. Trans R Soc Trop Med Hyg **1999**; 93(4):429–32.
525 42. Ramharter M, Noedl H, Winkler H, et al. In vitro activity and interaction of clindamycin
526 combined with dihydroartemisinin against *Plasmodium falciparum*. Antimicrob Agents
527 Chemother **2003**; 47(11):3494–9.
528 43. Noedl H, Krudsood S, Leowattana W, et al. In vitro antimalarial activity of azithromycin,
529 artesunate, and quinine in combination and correlation with clinical outcome. Antimicrob
530 Agents Chemother **2007**; 51(2):651–6.
531 44. Chawira AN, Warhurst DC, Robinson BL, Peters W. The effect of combinations of
532 qinghaosu (artemisinin) with standard antimalarial drugs in the suppressive treatment of
533 malaria in mice. Trans R Soc Trop Med Hyg **1987**; 81(4):554–8.
534 45. World Health Organization. WHO Alliance for the global elimination of trachoma by 2020:
535 progress report, 2019. Weekly Epidemiological Record **2020**; 95:349–60.
536 46. Watanasook C, Singharaj P, Suriyamongkol V, et al. Malaria prophylaxis with doxycycline
537 in soldiers deployed to the Thai-Kampuchean border. The Southeast Asian Journal of Tropical
538 Medicine and Public Health **1989**; 20(1):61–4.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

47. Sánchez JL, DeFraites RF, Sharp TW, Hanson RK. Mefloquine or doxycycline prophylaxis in US troops in Somalia. Lancet **1993**; 341(8851):1021–2.

48. Shanks GD, Roessler P, Edstein MD, Rieckmann KH. Doxycycline for malaria prophylaxis in Australian soldiers deployed to United Nations missions in Somalia and Cambodia. Mil Med **1995**; 160(9):443–5.

49. Tan KR, Magill AJ, Parise ME, Arguin PM. Doxycycline for malaria chemoprophylaxis and treatment: report from the CDC expert meeting on malaria chemoprophylaxis. Am J Trop Med Hyg **2011**; 84(4):517–31.

50. Nguyen DS, Dao BH, Nguyen PD, et al. Treatment of malaria in Vietnam with oral artemisinin. Am J Trop Med Hyg **1993**; 48(3):398–402.

51. Looareesuwan S, Viravan C, Vanijanonta S, et al. Randomized trial of mefloquine-doxycycline, and artesunate-doxycycline for treatment of acute uncomplicated falciparum malaria. Am J Trop Med Hyg **1994**; 50(6):784–9.

52. Bich NN, de Vries PJ, Van Thien H, et al. Efficacy and tolerance of artemisinin in short combination regimens for the treatment of uncomplicated falciparum malaria. Am J Trop Med Hyg **1996**; 55(4):438–43.

53. Duarte EC, Fontes CJ, Gyorkos TW, Abrahamowicz M. Randomized controlled trial of artesunate plus tetracycline versus standard treatment (quinine plus tetracycline) for uncomplicated *Plasmodium falciparum* malaria in Brazil. Am J Trop Med Hyg **1996**; 54(2):197–202.

54. Na-Bangchang K, Kanda T, Tipawangso P, et al. Activity of artemether-azithromycin versus artemether-doxycycline in the treatment of multiple drug resistant falciparum malaria. The Southeast Asian Journal of Tropical Medicine and Public Health **1996**; 27(3):522–5.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

55. de Vries PJ, Le NH, Le TD, et al. Short course of azithromycin/artesunate against falciparum malaria: no full protection against recrudescence. Trop Med Int Health **1999**; 4(5):407–8.

56. Krudsood S, Silachamroon U, Wilairatana P, et al. A randomized clinical trial of combinations of artesunate and azithromycin for treatment of uncomplicated *Plasmodium falciparum* malaria in Thailand. The Southeast Asian Journal of Tropical Medicine and Public Health **2000**; 31(4):801–7.

57. Krudsood S, Buchachart K, Chalermrut K, et al. A comparative clinical trial of combinations of dihydroartemisinin plus azithromycin and dihydroartemisinin plus mefloquine for treatment of multidrug resistant falciparum malaria. The Southeast Asian Journal of Tropical Medicine and Public Health **2002**; 33(3):525–31.

58. Borrmann S, Adegnika AA, Moussavou F, et al. Short-course regimens of artesunate-fosmidomycin in treatment of uncomplicated *Plasmodium falciparum* malaria. Antimicrob Agents Chemother **2005**; 49(9):3749–54.

59. Ramharter M, Oyakhirome S, Klouwenberg PK, et al. Artesunate-clindamycin versus quinine-clindamycin in the treatment of *Plasmodium falciparum* malaria: a randomized controlled trial. Clin Infect Dis **2005**; 40(12):1777–84.

60. Noedl H, Krudsood S, Chalermratana K, 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(10):1264–71.

61. Sykes A, Hendriksen I, Mtove G, et al. Azithromycin plus artesunate versus artemether-lumefantrine for treatment of uncomplicated malaria in Tanzanian children: a randomized, controlled trial. Clin Infect Dis **2009**; 49(8):1195–201.

bioRxiv preprint doi: https://doi.org/10.1101/2021.10.31.466372; this version posted November 2, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

62. Taj A, Sharif MA, Mahmood A, Luqman M. To compare the relapse rate of artesunate with tetracycline and quinine with tetracycline in uncomplicated falciparum malaria. Pakistan Journal of Medical Sciences **2009**; 25(2):274–8.

63. Thriemer K, Starzengruber P, Khan WA, et al. Azithromycin combination therapy for the treatment of uncomplicated falciparum malaria in Bangladesh: an open-label randomized, controlled clinical trial. J Infect Dis **2010**; 202(3):392–8.

64. Phong NC, Quang HH, Thanh NX, et al. In vivo efficacy and tolerability of artesunate-azithromycin for the treatment of falciparum malaria in Vietnam. Am J Trop Med Hyg **2016**; 95(1):164–7.

65. Miotto O, Amato R, Ashley EA, et al. Genetic architecture of artemisinin-resistant *Plasmodium falciparum*. Nat Genet **2015**; 47(3):226–34.

66. Miotto O, Sekihara M, Tachibana SI, et al. Emergence of artemisinin-resistant *Plasmodium falciparum* with kelch13 C580Y mutations on the island of New Guinea. PLoS Pathog **2020**; 16(12):e1009133.

67. Zhang M, Wang C, Otto TD, et al. Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis. Science **2018**; 360(6388):eaap7847.

68. Balikagala B, Fukuda N, Ikeda M, et al. Evidence of artemisinin-resistant malaria in Africa. N Engl J Med **2021**; 385(13):1163–71.

69. Uwimana A, Umulisa N, Venkatesan M, et al. Association of *Plasmodium falciparum kelch13* R561H genotypes with delayed parasite clearance in Rwanda: an open-label, single-arm, multicentre, therapeutic efficacy study. Lancet Infect Dis **2021**; 21(8): 1120–8.