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

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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.

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

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

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

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

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

80 # Methods

81 ### *Plasmodium falciparum* culture and synchronisation

82 *Plasmodium falciparum* 3D7 parasites were cultured as previously described [17]: at 2% (v/v)
83 haematocrit in human O+ red blood cells (RBCs; Australian Red Cross Blood Service) and
84 complete medium (RPMI-1640 with 25 mM sodium bicarbonate, 25 mM HEPES, 150 µM
85 hypoxanthine, 20 µg/mL gentamicin (Sigma-Aldrich, G3632), and 0.5% (w/v) Albumax II
86 (Thermo Fisher Scientific), pH 7.4); and maintained in malaria-mix gas (1% O_2, 5% CO_2, and
87 94% N_2) at 37°C. Ring stage synchronisation (~0–18 h p.i.) was achieved by a single treatment
88 of infected RBCs with 5% (w/v) D-sorbitol (Sigma-Aldrich), unless otherwise indicated.

89 ### 72 h in vitro single drug sensitivity assays

90 Synchronised ring stage 3D7 *P. falciparum* parasites (0.5% haematocrit; 1% parasitemia) were
91 set up in V-bottom 96-well plates. Parasites were either: immediately treated with a dose
92 gradient of atovaquone (Sigma-Aldrich, A7986), E-64 (Sigma-Aldrich, E3132), fosmidomycin
93 (Sigma-Aldrich, F8682), proguanil (Sigma-Aldrich, G7048), or quinine (Sigma-Aldrich,
94 145904) in complete medium; or incubated for 24 h before treatment with various
95 concentrations of dihydroartemisinin (DHA; Chem-Supply, D3793) or WR99210 (Jacobus
96 Pharmaceutical) in complete medium. DHA-treated parasites were washed three times with
97 complete medium 3 h post-drug treatment. Control wells containing parasites in the absence of
98 drug or 100 µM chloroquine (Sigma-Aldrich, C6628) were prepared in parallel. All samples
99 were prepared in triplicate.

100 72 h following set up, infected RBCs were incubated with lysis buffer (20 mM TRIS (pH 7.5),
101 5 mM EDTA, 0.008% (w/v) saponin, 0.08% (v/v) Triton X-100) and SYBR Green I (Thermo

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

107 **120 h in vitro single drug sensitivity assays**

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

117 **Isobolograms**

118 Synchronised ring stage 3D7 *P. falciparum* parasites (0.5% haematocrit; 72 h: 1% parasitemia,
119 or 120 h: 0.1% parasitemia) were set up in V-bottom 96-well plates. Parasites were treated with
120 dose gradients of two drugs, producing a 96-well plate where each well consisted of a unique
121 combination (Fig. 1A). Various pairings of atovaquone, clindamycin, DHA, doxycycline, E-
122 64, fosmidomycin, proguanil, quinine, and WR99210 were tested. The combination of delayed
123 death drugs with DHA were done in both the presence and absence of 5 μM GGOH. As
124 described above, for combinations that included DHA, parasites were washed three times with

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

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

132 $$\mathbf{FIC_{50}\ (drug\ A)} = \frac{\mathbf{IC_{50}\ (drug\ A\ in\ presence\ of\ [\boldsymbol{x}]\ drug\ B)}}{\mathbf{IC_{50}\ (drug\ A)}}$$

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

135 $$\mathbf{\sum FIC_{50} = FIC_{50}\ (drug\ A) + FIC_{50}\ (drug\ B)}$$

136 Interpretation of these values utilised previously defined thresholds [19]: < 0.1, very strong
137 synergism; 0.1–0.3, strong synergism; 0.3–0.7, synergism; 0.7–0.85, moderate synergism;
138 0.85–0.9, slight synergism; 0.9–1.1, additive; 1.1–1.2, slight antagonism; 1.2–1.45, moderate
139 antagonism; 1.45–3.3, antagonism; 3.3–10, strong antagonism; and > 10, very strong
140 antagonism. GraphPad Prism (Version 9.1.2) was used to conduct unpaired *t*-tests with
141 Welch's corrections to test statistical significance of the ΣFIC_{50}s.

142 **Haemoglobin fractionation**

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

145 24 h; or subjected to two D-sorbitol treatments, 8 h apart, and parasites (~8–18 h p.i.)
146 subsequently treated with 1 μM doxycycline or 25 nM clindamycin for 68 h. Negative controls
147 with the appropriate vehicle were set up in parallel. Following the incubation period, RBCs
148 were lysed with 0.15% (w/v) saponin and parasite pellets washed three times with PBS and
149 cOmplete™ mini EDTA-free protease inhibitor (Roche) at 4°C. Pellets were immediately
150 frozen at −80°C until use.

151 The haemoglobin fractionation assay was adapted from [20, 21] with minor adjustments.
152 Pellets were first resuspended in distilled water and sonicated for 5 min before addition of
153 HEPES (pH 7.5; final concentration 0.1 M). Samples were centrifuged (6,200 x *g*, 20 min) and
154 the pellets resuspended in distilled water before addition of 4% (w/v) sodium dodecyl sulfate
155 (SDS; final concentration 2% (w/v)). The samples were sonicated for 5 min, then incubated at
156 95°C for 5 min to solubilise the free haem. A solution of HEPES, NaCl, and pyridine was
157 added (final concentration 67 mM HEPES, 0.1 M NaCl, and 8.3% (v/v) pyridine) and samples
158 centrifuged (6,200 x *g*, 20 min). The supernatant—containing the free haem fraction—was
159 transferred to a clear, flat-bottom 96-well plate. The residual pellets were then resuspended in
160 distilled water before solubilisation with NaOH (final concentration 0.15 M). They were
161 sonicated for 15 min before a solution of HEPES, NaCl, and pyridine was added, as described
162 above. These samples—corresponding to the haemozoin fraction—were transferred to the 96-
163 well plate.

164 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)
165 pyridine) was included in triplicate for each study. The absorbance of each fraction was
166 measured at 405 nm using an Ensight plate reader (PerkinElmer). Samples were blank adjusted
167 and normalised to vehicle control. Data are presented as mean fold change compared to vehicle

168 control ± SEM, and GraphPad Prism (Version 9.1.2) was used to perform one sample *t*-tests.

169 All samples were prepared in triplicate.

170 # Results

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

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

187 To first validate this methodology, synchronised ring stage parasites were treated with the well-
188 established synergistic combination of atovaquone and proguanil (Fig. 2A). We analysed
189 growth at 72 h post-treatment (standard timing to assess first cycle death). The isobologram
190 indicated a moderately synergistic interaction and produced a mean ΣFIC_{50} of
191 0.72 ± 0.04—concordant with previous reports [27, 29, 30] (Fig. 2D and 3).

192 Testing the interaction of delayed death drugs (which kill in the second cycle) and DHA (which
193 kills in the first cycle) was a complex task, so we began by substituting the delayed death drug
194 for an apicoplast inhibitor, fosmidomycin, that causes first cycle killing. Fosmidomycin
195 directly blocks apicoplast metabolism by inhibiting 1-deoxy-D-xylulose-5-phosphate
196 reductoisomerase, an enzyme involved in the isoprenoid biosynthetic pathway [9]. Like other
197 apicoplast inhibitors, fosmidomycin perturbs haemoglobin uptake, albeit in the first cycle [13].
198 To specifically test the impact of the haemoglobin degradation defect, it was necessary to pre-
199 treat synchronised ring stage 3D7 parasites with a dose gradient of fosmidomycin prior to DHA
200 treatment. Further, to mimic the clinical scenario as closely as possible in an in vitro setting,
201 we pulsed parasites with DHA for 3 h at the trophozoite stage, 24 h after pre-treatment with
202 the apicoplast inhibitor (Fig. 2B). The experiment was designed to target the parasite life stage
203 for which ART derivatives are most active [24, 31] and to mimic the short in vivo half-life of
204 DHA [3]. Using this approach, fosmidomycin and DHA displayed moderate antagonism with
205 a mean ΣFIC_{50} of 1.29 ± 0.05 (Fig. 2D and 3). This phenotype had been observed previously
206 [32, 33], though using a different methodology that did not explore the clinically relevant half-
207 life of DHA [3], nor fosmidomycin-induced haemoglobin trafficking defects [13]. The
208 antagonism between fosmidomycin and DHA is concordant with the well-established
209 antagonistic interaction between DHA and E-64 [31]—a cysteine protease inhibitor that
210 prevents haemoglobin degradation, presumably reducing activation of DHA by free haem [34].
211 Consistent with this, we also saw a moderately antagonistic interaction between E-64 and
212 DHA—with a mean ΣFIC_{50} of 1.36 ± 0.04 (Fig. 2D and 3)—using the approach described
213 above (Fig. 2B).

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

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

219 A longer pre-treatment was necessary to test the interaction between delayed death drugs and
220 DHA, as the haemoglobin trafficking defects observed when parasites are treated with these
221 apicoplast inhibitors are evident only in the cycle subsequent to treatment [14]. Ring stage
222 *P. falciparum* parasites were pre-treated with doxycycline or clindamycin for 72 h, to recreate
223 the delayed death effect, and subsequently pulsed with DHA for 3 h at the trophozoite stage in
224 the second cycle (Fig. 2C). Isobologram analysis of these data demonstrate an antagonistic and
225 moderately antagonistic interaction between DHA and doxycycline (mean ΣFIC_{50} of
226 1.49 ± 0.02), and DHA and clindamycin (mean ΣFIC_{50} of 1.44 ± 0.10), respectively (Fig. 2D
227 and 3). The ΣFIC_{50}s of these combinations significantly increased—approximately 1.5-
228 fold—from that of the additive interaction of atovaquone and DHA previously described
229 (Fig. 3). This interaction with delayed death antibiotics was specific to DHA. The combination
230 of delayed death drugs with WR99210—an inhibitor of dihydrofolate reductase—did not show
231 the same antagonism (Fig. 3, and S1C and D). Mean ΣFIC_{50} values for doxycycline and
232 clindamycin combined with WR99210 were 1.21 ± 0.05 and 1.16 ± 0.05, respectively (Fig. 3),
233 suggesting that this cohort of apicoplast inhibitors have a selective effect on DHA activity.
234 This interaction appeared to reverse when parasites were supplemented with exogenous
235 geranylgeraniol (GGOH), a polyprenol that restores haemoglobin trafficking by permitting
236 protein prenylation in the absence of isoprenoid biosynthesis [14] (Fig. S1B and D). However,
237 the timing of these experiments was complex as GGOH-restored parasites survive longer but
238 still die at a later stage that is yet to be thoroughly characterised [14], complicating attempts to
239 quantify this interaction.

240 **Apicoplast inhibitors reduce the abundance of haemoglobin degradation by-products**

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

259 **Discussion**

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

270 Previous studies have mapped the effects of apicoplast inhibition in asexual *P. falciparum*
271 parasites—from impeding IPP biosynthesis to downstream perturbation of haemoglobin uptake
272 [13, 14, 36]. Isoprenoids have multiple cellular fates, though the proximate cause of parasite
273 death through antibiotic treatment results from reduced prenylation of trafficking proteins,
274 potentially through Rab proteins involved in haemoglobin trafficking to the DV [13, 14]
275 (Fig. 5A). This prevents anchoring to vesicle membranes; and is associated with the aberrant
276 uptake of haemoglobin from the host RBC and fragmentation of the DV [13, 14] (Fig. 5B).
277 Further, delayed death drugs reduce the abundance of haemoglobin-like peptides in the cycle
278 following treatment [14], consistent with our finding that free haem and haemozoin levels are
279 lowered at this timepoint. Our data also demonstrate a reduction in abundance of these by-
280 products by fosmidomycin—a fast-acting apicoplast inhibitor—suggesting that reduced
281 haemoglobin degradation is an inevitable downstream effect of apicoplast inhibition in the
282 asexual blood stages.

283 We hypothesise that this reduced release of free haem is the root cause of the antagonism
284 between these antibiotics and DHA (Fig. 5). Consistent with this interpretation of the data is
285 the antagonistic interaction observed between the cysteine protease inhibitor, E-64, and
286 DHA—one that has been reported previously [31]. Indeed, a similar interaction has also been
287 reported for another haemoglobin degradation inhibitor (the cysteine protease inhibitor, N-
288 acetyl-L-leucyl-L-leucyl-L-norleucinal, or ALLN) [16]. In both studies, antagonism was
289 attributed to a haem-mediated reduction in ART activation. This antagonistic interaction with
290 apicoplast-inhibiting antibiotics may also extend to other ART-like compounds in the drug
291 pipeline that similarly rely on free haem for activation—a notable example being ozonides
292 [37, 38], the activation of which is directly inhibited by E-64 [39].

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

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

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

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

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

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

334 Arguably of greater global concern is that the combination of ART and apicoplast drugs could
335 expose parasites to suboptimal concentrations of activated ART, conceivably worsening the
336 already alarming spread of ART resistance and ART treatment failure that is currently
337 occurring in South-East Asia [1]. There are several reports of modulation of ART sensitivity
338 associated with mutations in or upstream of genes for apicoplast proteins, both in the field
339 [65, 66] and in cultured parasites [67]. The significance of this is so far unclear, but one
340 potential role could be through changes to apicoplast metabolism that impact isoprenoid
341 synthesis and thus haemoglobin uptake (Fig. 5C). Given the almost ubiquitous use of ACTs
342 throughout the malaria endemic world and the recent emergence of ART resistance in sub-
343 Saharan Africa [68, 69], protecting against the rise of resistance elsewhere is key to avoid the
344 worsening of malaria as a global health challenge.

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

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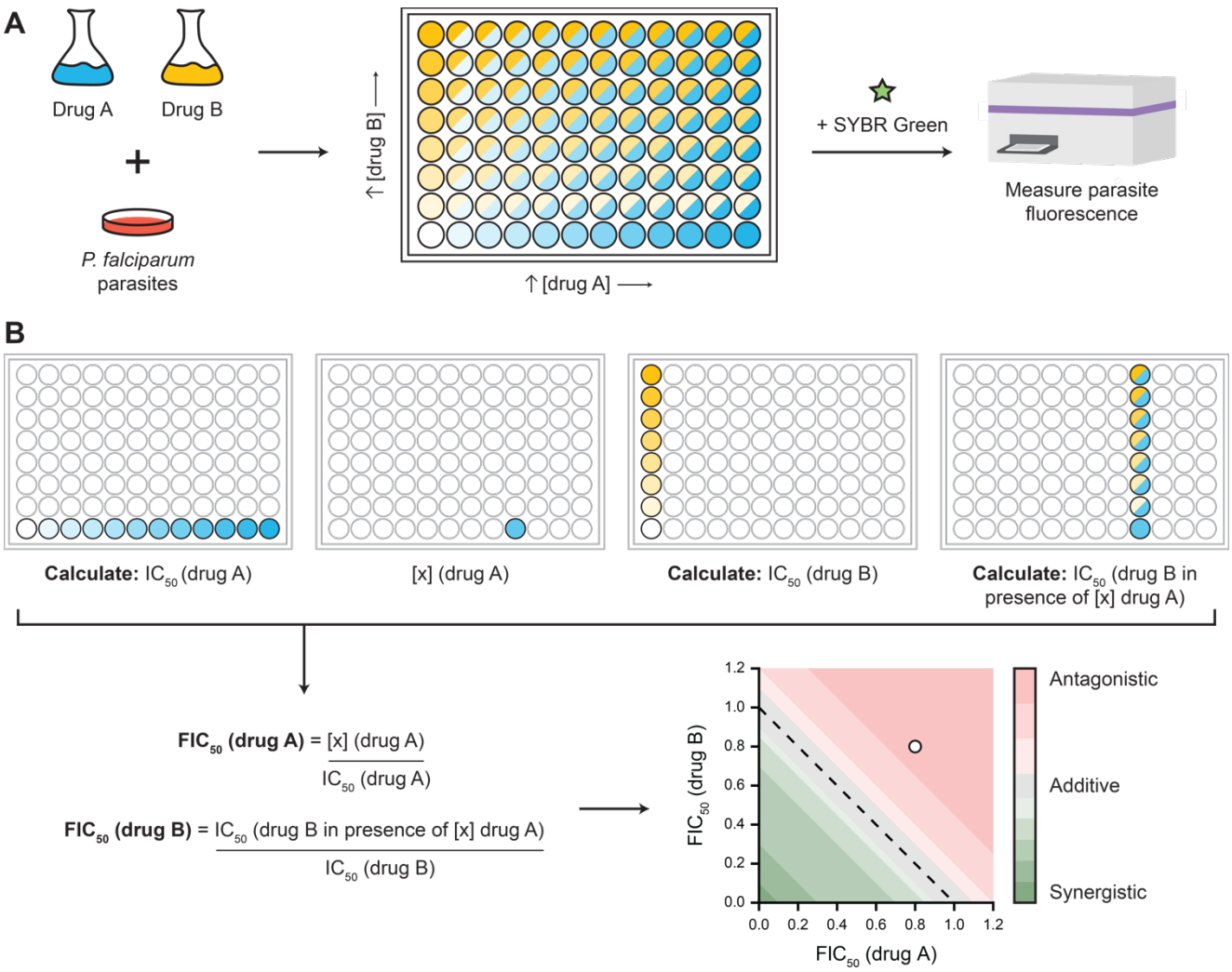
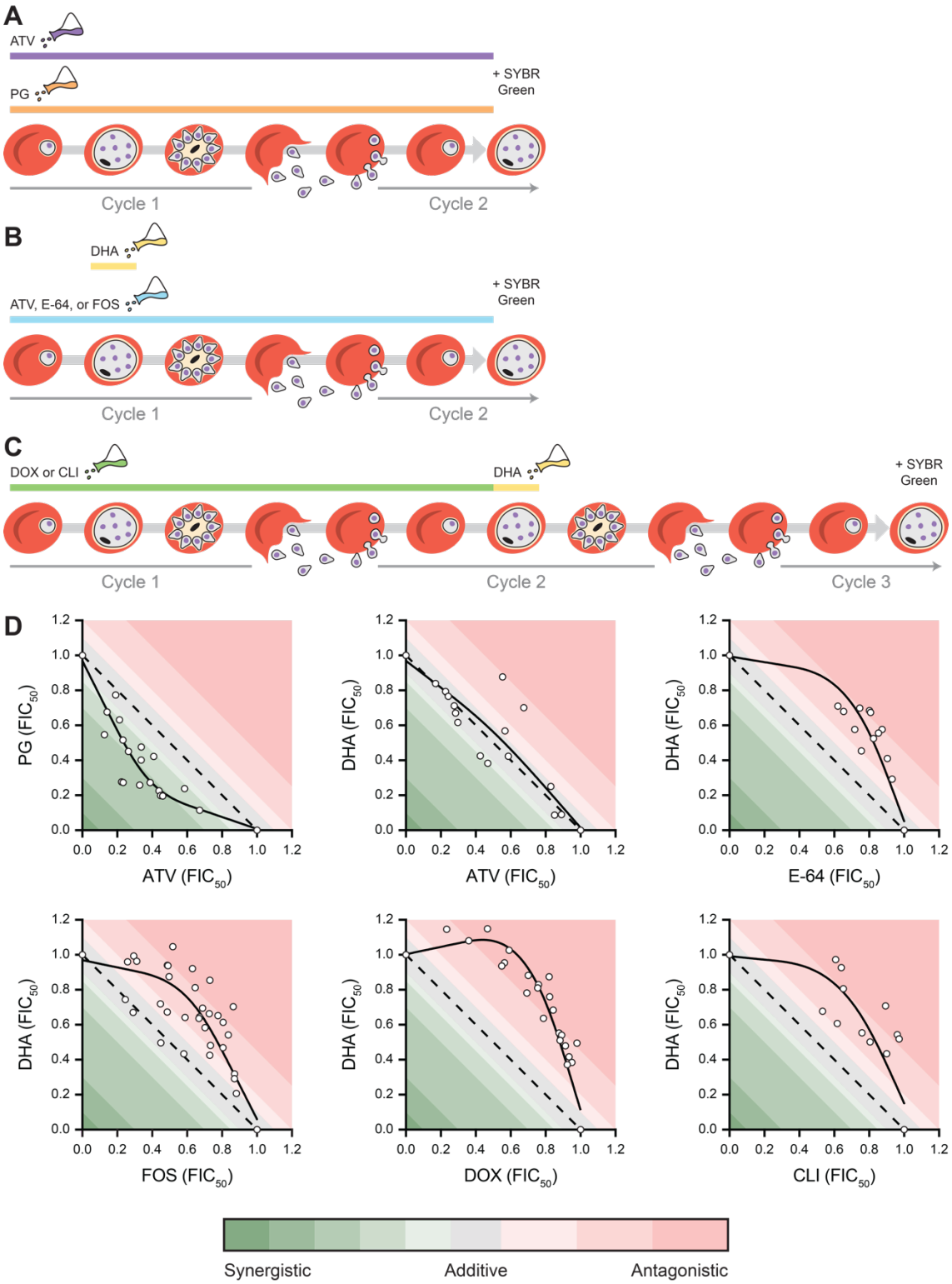


362

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].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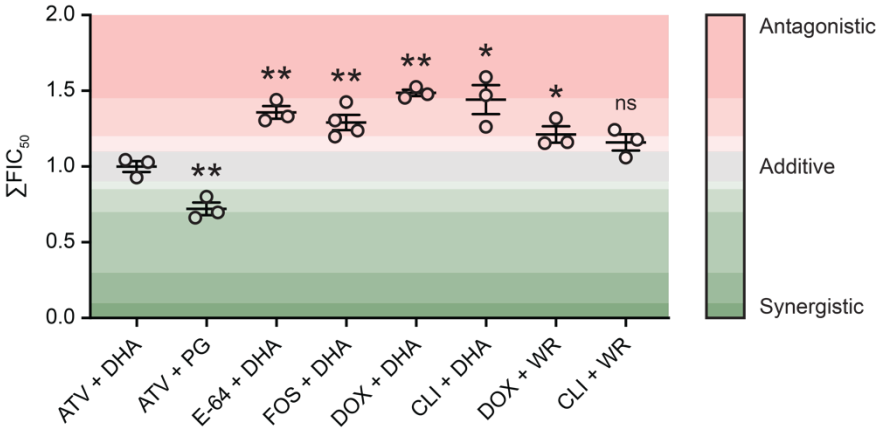
PG
+ SYBR Green
Cycle 1
Cycle 2
B
DHA
ATV, E-64, or FOS
+ SYBR Green
Cycle 1
Cycle 2
C
DOX or CLI
DHA
+ SYBR Green
Cycle 1
Cycle 2
Cycle 3
D
PG (FIC50)
ATV (FIC50)
DHA (FIC50)
ATV (FIC50)
DHA (FIC50)
E-64 (FIC50)
DHA (FIC50)
FOS (FIC50)
DHA (FIC50)
DOX (FIC50)
DHA (FIC50)
CLI (FIC50)
Synergistic
Additive
Antagonistic

371

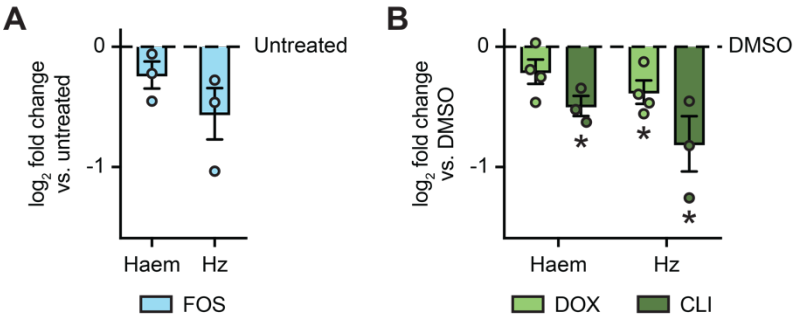
372 **Fig 2. Normalised isobolograms demonstrating antagonism between apicoplast inhibitors**
373 **and dihydroartemisinin (DHA).** A–C) Schematic of the treatment regimen of 3D7
374 *Plasmodium falciparum* ring stage parasites in the set-up of D) isobolograms. Parasites were
375 either: A) treated with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



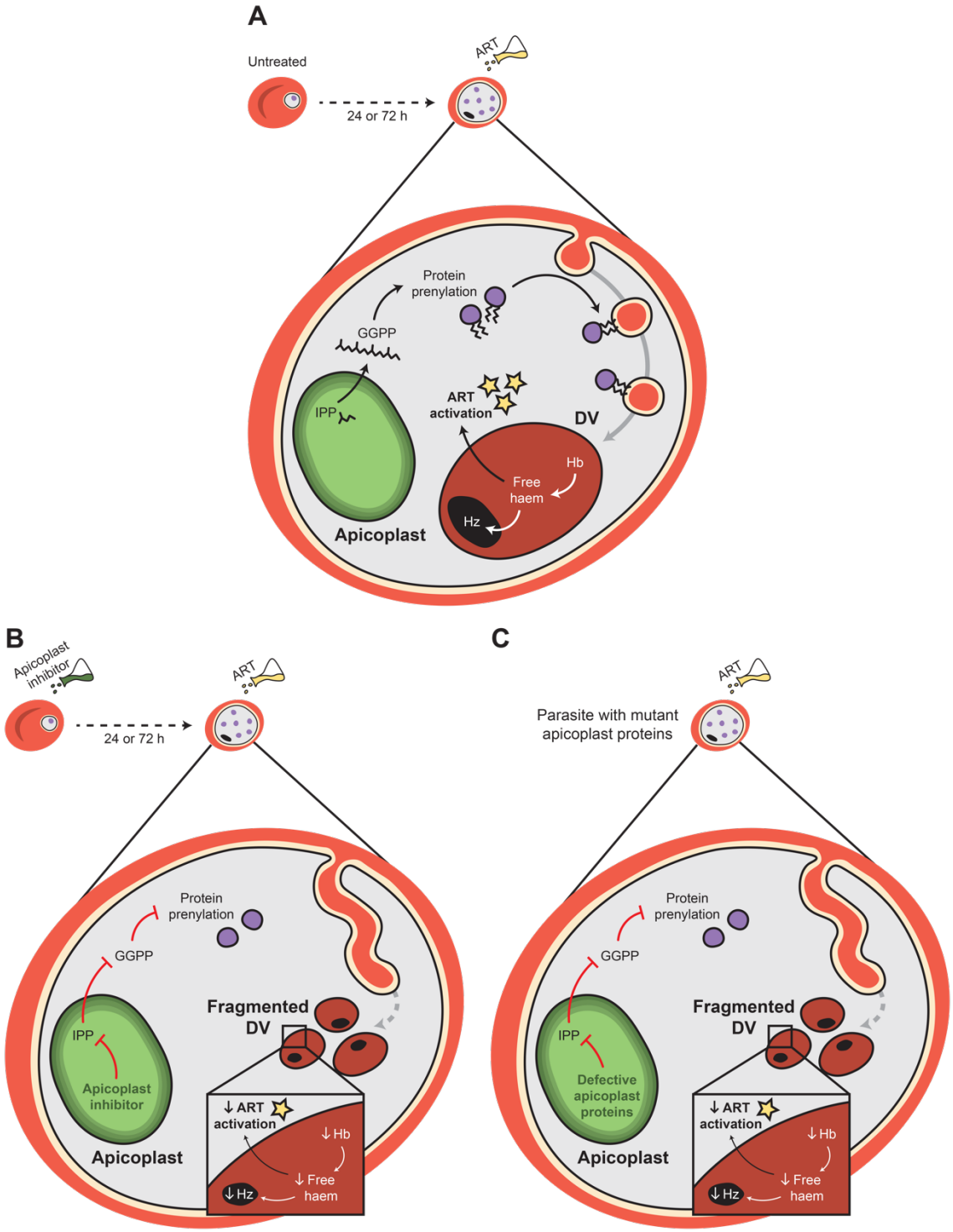
Drug combination	$\sum FIC_{50}$ (mean ± SEM)	Interaction
ATV + DHA	1.00 ± 0.04	Additive
ATV + PG	0.72 ± 0.04**	Moderate synergism
E-64 + DHA	1.36 ± 0.04**	Moderate antagonism
FOS + DHA	1.29 ± 0.05**	Moderate antagonism
DOX + DHA	1.49 ± 0.02**	Antagonism
CLI + DHA	1.44 ± 0.10*	Moderate antagonism
DOX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

390



391 **Fig 4. Apicoplast inhibitors reduce the abundance of haemoglobin degradation by-**
392 **products.** 3D7 *Plasmodium falciparum* ring stage parasites were treated with vehicle or:
393 A) 3 μM fosmidomycin (FOS) for 24 h (~24–42 h p.i.); or B) 1 μM doxycycline (DOX) or
394 25 nM clindamycin (CLI) for 68 h (~76–86 h p.i.). Parasites were harvested and sequentially
395 fractionated to determine the relative levels of free haem and haemozoin (Hz). Data were
396 normalised to the vehicle control and are presented as mean $\log_2$ fold change ± SEM ($n \geq 3$).
397 One sample *t*-tests were performed: $*p \leq 0.05$.


A
Untreated
ART
24 or 72 h
Protein
prenylation
GGPP
IPP
ART
activation
DV
Hb
Free
haem
Hz
Apicoplast
B
Apicoplast
inhibitor
ART
24 or 72 h
Protein
prenylation
GGPP
IPP
Apicoplast
inhibitor
Fragmented
DV
↓ ART
activation
↓ Hb
↓ Free
haem
↓ Hz
Apicoplast
C
ART
Parasite with mutant
apicoplast proteins
Protein
prenylation
GGPP
IPP
Defective
apicoplast
proteins
Fragmented
DV
↓ ART
activation
↓ Hb
↓ Free
haem
↓ Hz
Apicoplast

398

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

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

[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

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

418 [b] Attributed to possible inadequate azithromycin dosage.

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