



HAL
open science

Identification of compounds active against quiescent artemisinin-resistant *Plasmodium falciparum* parasites via the quiescent-stage survival assay (QSA)

Thibaud Reyser, Lucie Paloque, Manel Ouji, Michel Nguyen, Sandie Ménard, Benoit Witkowski, Jean-Michel Augereau, Françoise Benoit-Vical

► To cite this version:

Thibaud Reyser, Lucie Paloque, Manel Ouji, Michel Nguyen, Sandie Ménard, et al.. Identification of compounds active against quiescent artemisinin-resistant *Plasmodium falciparum* parasites via the quiescent-stage survival assay (QSA). *Journal of Antimicrobial Chemotherapy*, 2020, 75 (10), pp.2826-2834. 10.1093/jac/dkaa250 . hal-02948398

HAL Id: hal-02948398

<https://hal.science/hal-02948398v1>

Submitted on 6 Oct 2021

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

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

1 **Identification of compounds active against quiescent artemisinin-resistant**
2 *Plasmodium falciparum* parasites via the Quiescent-stage Survival Assay

3
4 **Thibaud REYSER^{1,2,§}, Lucie PALOQUE^{1,2,§,*}, Manel OUJI^{1,2}, Michel NGUYEN^{1,2},**
5 **Sandie MENARD³, Benoit WITKOWSKI⁴, Jean-Michel AUGEREAU^{1,2},**
6 **Françoise BENOIT-VICAL^{1,2,5,*}**

7
8 ¹ LCC-CNRS, Laboratoire de Chimie de Coordination, Université de Toulouse, CNRS,
9 Toulouse, France.

10 ² Institut de Pharmacologie et de Biologie Structurale, IPBS, Université de Toulouse, CNRS,
11 UPS, France.

12 ³ Present adress: CPTP, Centre de Physiopathologie de Toulouse Purpan, INSERM U1043,
13 CNRS UMR5282, Université de Toulouse, Toulouse, France

14 ⁴ Malaria Molecular Epidemiology Unit, Pasteur Institute in Cambodia, Phnom Penh,
15 Cambodia.

16 ⁵ INSERM, Institut National de la Santé et de la Recherche Scientifique, France

17
18 **Running title:** Compounds active against quiescent artemisinin-resistant malaria parasites

19
20
* Author for correspondence: Francoise.Vical@inserm.fr

CNRS, LCC (Laboratoire de Chimie de Coordination), 205 route de Narbonne, BP 44099, F-
31077 Toulouse Cedex 4, France. Phone: +00 33 561 175 823

§ These authors contributed equally to this work. * LP and FBV share senior authorship.

21

22 **ABSTRACT**

23

24 **Background:** Quiescence is an unconventional mechanism of *Plasmodium* survival mediating
25 artemisinin resistance. This phenomenon increases the risks of clinical failures following
26 Artemisinin-based Combination Therapies (ACTs) by slowing parasite clearance and allowing
27 the selection of parasites resistant to partner drugs.

28 **Objectives:** To thwart this multi-resistance, the quiescent state of artemisinin-resistant parasites
29 must be taken into consideration from the very early stages of the drug discovery process.

30 **Methods:** We designed a novel phenotypic assay named Quiescent-stage Survival Assay
31 (QSA) to assess the antiplasmodial activity of drugs on quiescent parasites. This assay was first
32 validated on quiescent forms from different artemisinin-resistant parasite lines (laboratory
33 strain and field isolates), using two reference drugs with different mechanisms of action:
34 chloroquine and atovaquone. Furthermore, the efficacies of different partner drugs of
35 artemisinins used in ACTs were investigated against both laboratory strains and field isolates
36 from Cambodia.

37 **Results:** Our results highlight that because of quiescence-based mechanism and the respective
38 pharmacological targets of drugs, their efficacies on artemisinin-resistant parasites may be
39 different between quiescent parasites and their proliferating forms.

40 **Conclusions:** These data confirm the high relevance of adding the chemosensitivity evaluation
41 of quiescent parasites by the specific *in vitro* QSA to the antiplasmodial drug development
42 process in the current worrisome context of artemisinin resistance.

43

44

45

46

47 INTRODUCTION

48 Artemisinin-based combination therapies (ACTs), using artemisinin or its derivatives (ARTs)
49 combined with other antiparasmodial drugs, are recommended for the treatment of *Plasmodium*
50 *falciparum* malaria. ARTs produce a fast reduction in the parasite numbers, leaving the
51 remaining parasites exposed to the long-lasting partner drug. ¹ The widespread use of ACTs has
52 significantly contributed to the historical decrease of malaria mortality observed over the last
53 15 years in all endemic countries. ² However, *Plasmodium* resistance to ARTs emerged in 2006
54 and could now jeopardize malaria elimination programs. ³⁻⁷ ARTs-resistance is linked to a
55 mutation on the *kelch13* gene, ⁸ which encodes for a protein involved in different mechanisms,
56 among which hemoglobin uptake. ⁹ ARTs-resistance is mediated by a quiescent state upon
57 ARTs exposure, ¹⁰ in a mechanism that dramatically differs from other antimalarial drug
58 resistance mechanisms. This parasite cell cycle arrest is characterized by a drastic down-
59 regulation of the overall metabolism to allow parasites to face ARTs-induced cellular
60 damage, ^{11,12} and subsequent up-regulation to enable parasite recrudescence after drug
61 elimination. ^{10,13} In the field, ACT-resistance is always associated with ARTs-resistance. Either
62 ARTs-resistance facilitates emergence of ACT-resistance by allowing partner drug resistance
63 selection in artemisinin resistant parasites or ACT-resistance is acquired after recombination
64 between ARTs-resistant and partner drug-resistant parasite lineages. ^{7,14-19} Long-term
65 artemisinin drug pressure also triggers *in vitro* acquisition of a multidrug tolerance phenotype
66 based on the extended capacity of ARTs-resistant parasites to survive other antiparasmodial
67 drugs through a quiescence mechanism. ²⁰ Extending ACT treatment from 2 to 3 days in order
68 to improve clinical efficacy thus increases artemisinin drug pressure, leading to a higher
69 frequency of *P. falciparum* ARTs-resistance cases. ^{21,22} During ACT treatment, due to the
70 respective pharmacokinetics properties of both drugs, parasites are first exposed to ARTs. That

71 is why, in an ARTs-resistance scenario, ring-stage parasites first enter a quiescent state and are
72 then exposed to partner drugs. Therefore, a partner drug that is active against quiescent parasites
73 should be the gold standard for malaria eradication policies and programs. Because standard
74 chemo-sensitivity assays have shown their inability to study artemisinin resistance,^{8,10,20} we
75 propose a novel *in vitro* assay named Quiescent-stage Survival Assay (QSA) to assess the
76 efficacy of compounds against ARTs-induced quiescent parasites.

77

78 **MATERIALS AND METHODS**

79 **Parasite culture**

80 F32-ART5 (artemisinin-resistant), F32-TEM (artemisinin-sensitive) *Plasmodium falciparum*
81 laboratory strains were cultivated at 37°C in RPMI-1640 medium (Biowest) at 2 % hematocrit
82 in human red blood cells and 5 % AB human serum (EFS, French Blood Bank) under 5 % CO₂
83 in a humid atmosphere at 37°C.^{10,20,23,24} Artemisinin-resistant field isolates (from Pasteur
84 Institute of Cambodia) IPC8262, IPC8317, IPC6388 and IPC6938 were cultivated under 5 %
85 O₂, 5 % CO₂, and 90 % N₂ (FIVAL, Air Liquide) at 2 % hematocrit in RMPI-1640 medium
86 supplemented with 5 % human serum (EFS, French Blood Bank) and 0.55 % (w/w) albumax II
87 (Thermo Fisher).²⁵ The characteristics of the different strains used in this study are reported in
88 Table 1. Genotyping of field isolates were given by Pasteur Institute of Cambodia.

89

90 **Drugs**

91 Chloroquine diphosphate, lumefantrine and mefloquine hydrochloride were purchased from
92 Sigma-Aldrich (France), artemisinin from TCI Europe, atovaquone from GlaxoSmithKline,
93 dihydroartemisinin, *N*-desethyl amodiaquine and piperaquine tetraphosphate tetrahydrate were
94 provided by the WWARN. Drugs were used *in vitro* in combination assays at
95 pharmacologically relevant concentrations (700 nM dihydroartemisinin, 3 μM artemisinin,

96 200 nM chloroquine, 7 μ M atovaquone, 200 nM piperaquine, 964 nM mefloquine, 800 nM *N*-
97 desethyl amodiaquine, 5 μ M lumefantrine), equal or close to their plasma peak concentrations
98 in patients.²⁶ Stock solutions were obtained by dissolving piperaquine in 0.5 % lactic acid
99 (Sigma-Aldrich), *N*-desethyl amodiaquine and chloroquine in RPMI-1640, while the other
100 compounds were dissolved in DMSO (Sigma-Aldrich).

101

102 **Standard chemosensitivity assay**

103 The chemosensitivity assay was carried out in 96-well culture plates on D-sorbitol (Sigma-
104 Aldrich) ring-stage synchronized parasites.²⁷ The antiplasmodial activities were evaluated
105 using the SYBR Green I method.²⁸ Each drug concentration was tested in triplicates. The
106 parasites were incubated with the drugs for 48 h. Parasite pellets were then washed in 1X PBS
107 prior to lysing red blood cells at -20 °C overnight. Then, the plates were thawed and 100 μ L of
108 each well were transferred into a black 96-well plate. 100 μ L of SYBR Green I (Thermo Fisher),
109 diluted at a final concentration of 2X in lysis buffer, were added to each well and incubated for
110 1 h at room temperature prior to reading the plates on BioTek FLx800 Microplate Fluorescence
111 Reader ($\lambda_{\text{excitation}} = 485 \text{ nm}$, $\lambda_{\text{emission}} = 528 \text{ nm}$). IC₅₀ values were determined using GraphPad
112 Prism software.

113

114 **Quiescent-stage Survival Assay (QSA)**

115 To evaluate drug activity in quiescent artemisinin-resistant parasites, the Quiescent-stage
116 Survival Assay (QSA) was first developed using the F32-ART5 strain. D-sorbitol synchronized
117 ring-form cultures at 3 % parasitemia and 2 % hematocrit (5 mL of culture per well in 6-well
118 plate) were first exposed to dihydroartemisinin at 700 nM for 6 h to induce quiescence of
119 artemisinin-resistant parasites. Then parasite cultures were exposed to the combination,
120 dihydroartemisinin + compound to be tested, for 48 h (Fig. 1A, condition a). Keeping parasites

121 under dihydroartemisinin treatment during exposure to the compound to be tested ensured
122 quiescence maintenance. In parallel, other combinations were tested as control conditions (Fig.
123 1A, conditions b-d). The compounds were also evaluated on proliferating artemisinin-resistant
124 parasites i.e. without any pre-treatment (Fig. 1A, condition e). Between each drug treatment,
125 parasite cultures were washed with 40 mL of RPMI-1640 medium and put back in culture in
126 new wells for the next treatment. After drug exposure, parasite cultures were washed with
127 40 mL of RPMI-1640 medium and cultured in drug-free conditions supplemented with 10 %
128 human serum in new wells. The parasitemia for each condition was then microscopically
129 monitored every 2 days to determine the time required to reach the initial parasitemia (3 %),
130 this time point being referred to as recrudescence day. Culture media were renewed twice a
131 week and 25 μ L of fresh red blood cells were added once a week. If no recrudescence occurred
132 after 30 days, the experiment was stopped.^{10,20} Delay in recrudescence time is the difference
133 between the median recrudescence time from different independent experiments obtained with
134 the drug combination (dihydroartemisinin 6 h / (dihydroartemisinin + drug) 48 h) (Fig. 1A,
135 condition a) and the median recrudescence time obtained with dihydroartemisinin alone
136 (dihydroartemisinin 6 h / dihydroartemisinin 48 h) (Fig. 1A, condition b). This delay is
137 representative of the capacity of the tested compound to be active against quiescent parasites.
138 Concentrations and treatment durations were chosen for their pharmacological relevance.
139 During the QSA development, several conditions were tested to optimize the assay: i) 24-h or
140 48-h incubation of the compounds, ii) use of a physiological dose of 3 μ M of ART or 700 nM
141 of DHA to induce quiescence, iii) repetition of the drug combination treatments to mimic ACT
142 pharmacology, and iv) 2 sorbitol treatments, at 30 h and 54 h during drug exposure to be sure
143 that parasites were in a quiescent state.

144

145 **Recrudescence assay**

146 Recrudescence assay was carried out as previously reported to evaluate the ability of the F32-
147 ART5 parasite line to survive drug exposure comparatively to the artemisinin-sensitive F32-
148 TEM parasite line.^{10,20} After 48-h drug exposure, synchronized ring-stage parasites were
149 washed with RPMI-1640 medium and placed in normal drug-free culture conditions. The time
150 to parasite recrudescence was determined as the time required to reach initial parasitemia. The
151 experiment was stopped if no recrudescence was microscopically observed within 30 days.^{10,20}

152

153 **Ring-stage Survival Assay (RSA)**

154 The Ring-stage Survival Assay (RSA) was carried out as previously reported.²⁹ Briefly, 0-
155 3 hours old ring-stage parasites (obtained after a Percoll® (Sigma-Aldrich)-sorbitol treatment)
156 were exposed to 700 nM DHA or 0.1 % DMSO (control) for 6 hours in duplicate, then washed
157 in RPMI-1640 and replaced in normal culture conditions for the next 72 h. Survival rates were
158 measured microscopically on Diff-Quick® (RAL Diagnostics)-stained thin blood smears. The
159 parasitemia was calculated by counting 10,000 red blood cells per assay by two independent
160 microscopists. A parasite growth rate ≥ 2 in non-exposed culture was deemed necessary to
161 validate the assay. The strains with a survival rate of dihydroartemisinin-treated parasites
162 relative to control above 1 % in RSA were referred to as artemisinin-resistant.²⁹

163

164 **Piperaquine survival assay (PSA)**

165 The piperaquine survival assay (PSA) was performed as previously described to evaluate the
166 ability of ring-stage parasites to survive piperaquine exposure.³⁰ Briefly, 0-3 h old ring-stage
167 parasites (obtained after a percoll-sorbitol treatment) were exposed to 200 nM of piperaquine
168 or culture medium alone (control) for 48 h in duplicate. After drug exposure, parasites were
169 washed with RPMI-1640 medium and then cultivated for the next 24 h without drug. Survival
170 rates were measured similarly to the RSA (ratio of viable parasites in piperaquine-exposed

171 relative to DMSO-exposed samples). The strains with a survival rate above 10 % in PSA were
172 referred to as piperazine-resistant.³⁰

173

174 **Data Analysis**

175 Where relevant, a statistical analysis (log-rank test) was carried out on data from recrudescence
176 assay and QSA by performing Kaplan-Meier survival analyses considering censored data. The
177 outcome was defined as the time necessary for cultures to reach initial parasitemia.
178 Observations were considered as censored if no recrudescence was observed within the
179 experiment timeframe (30 days).

180

181 **RESULTS AND DISCUSSION**

182 *The QSA (quiescent-stage survival assay), a relevant method to specifically evaluate*
183 *chemosensitivity of quiescent parasites*

184 Quiescence-based artemisinin resistance is a new challenge to consider when proposing
185 relevant antiplasmodial treatments. In this context, we designed a novel assay, the QSA, to
186 evaluate drug activity against artemisinin-resistant parasites in a quiescence state. The QSA
187 interpretation is based on a delay in recrudescence time between cultures exposed to
188 (dihydroartemisinin 6 h / (dihydroartemisinin + molecule) 48 h) and cultures exposed to
189 (dihydroartemisinin 6 h / dihydroartemisinin 48 h) (Fig. 1A). A delayed recrudescence (Fig.
190 1B) or a lack thereof indicates that most or all quiescent parasites are killed by the partner drug
191 tested. Therefore, it is suggested that the more potent the drug against quiescent parasites, the
192 longer the delay because the fewer quiescent parasites, the slower the culture will grow back to
193 initial parasitemia. According to the different results obtained and in order to select relevant
194 compounds, we considered a molecule to be active against quiescent parasites if its
195 recrudescence delay was above a 6-day threshold (Fig. 1B). Below this threshold, the difference

196 could be statistically significant but not necessarily biologically relevant since this could be due
197 to variations in culture parameters independent of the number of surviving parasites. Therefore,
198 from one experiment to the other, there can be a two to three-day variation in recrudescence
199 days, which is why, by setting a 6-day confidence interval, we made sure that the differences
200 were meaningful. To develop the QSA model, chloroquine and atovaquone were used because
201 of their different and well-documented mechanisms of action. The highly artemisinin-resistant
202 *P. falciparum* strain F32-ART5 harboring none of the genotypic markers associated with
203 chloroquine, mefloquine or atovaquone resistance was used (Table 1).^{10,20} Results were then
204 confirmed on artemisinin-resistant isolates from the field.

205

206 ***Chloroquine cannot kill quiescent parasites***

207 In the absence of dihydroartemisinin pre-treatment, F32-ART5 proliferative parasites appeared
208 sensitive to a single dose of 200 nM chloroquine for 48 h leading to parasite elimination with
209 no recrudescence at day 30 (Fig. 2, Table S1). This chloroquine sensitivity of F32-ART5 is in
210 accordance with the obtained IC₅₀ values below the chloroquine-resistance cut-off of 100 nM
211 (Table S2). The combination dihydroartemisinin 6 h / chloroquine 48 h delays recrudescence
212 time of resistant parasites by 7 days compared to dihydroartemisinin 6 h (Table S1). This
213 delayed recrudescence time could be interpreted as the ability of chloroquine to either be active
214 against quiescent parasites or to repress cell cycle recovery of quiescent parasites. To ensure
215 the targeting of quiescent parasites, the quiescent state was maintained with dihydroartemisinin
216 all over the chloroquine treatment. Surprisingly, the 2 conditions (dihydroartemisinin 6 h /
217 (dihydroartemisinin + chloroquine) 48 h) and (dihydroartemisinin 6 h / dihydroartemisinin
218 48 h) showed similar recrudescence profiles (8 and 6 days, respectively) (Fig. 2, Table S1),
219 which is interpreted as a lack of chloroquine activity against quiescent parasites. The
220 mechanism of action of chloroquine, through the inhibition of hemozoin formation, could

221 explain this result since such a mechanism is absent at the quiescent ring-stage.³¹ A recent
222 study showed that chloroquine is active against both early ring stages and trophozoites,
223 suggesting that hemoglobin digestion starts early on in the asexual cycle.³² Hence, this
224 showcases that the efficacy of a drug against quiescent parasites cannot be deduced from its
225 efficacy against proliferating parasites. The QSA condition (dihydroartemisinin 6 h /
226 (dihydroartemisinin + drug to be tested) 48 h) appears thus highly selective for identification of
227 compounds genuinely active against quiescent forms. These results evidenced that drug activity
228 differs according to the proliferating or quiescent state of parasites. Therefore, QSA appears as
229 a global chemosensitivity assay that is able to evaluate drug activity against both artemisinin-
230 resistant proliferating and quiescent parasites.

231 In order to better mimic the antimalarial drug regimens, the impact of repetitive chloroquine
232 treatments was tested on quiescent parasites. Because a repeated (dihydroartemisinin 6 h /
233 dihydroartemisinin 48 h) treatment did not yield any recrudescence (data not shown), the 48 h-
234 exposure was thus reduced to 24 h in each cycle. In these conditions, two successive treatments
235 (dihydroartemisinin 6 h / (dihydroartemisinin + chloroquine) 24 h / dihydroartemisinin 6 h /
236 (dihydroartemisinin + chloroquine) 24 h) led to the same recrudescence time of 12 days as a
237 single 60 h exposure to dihydroartemisinin alone (Table S3). This result was consistent with
238 the total absence of chloroquine activity against quiescent parasites, independent of the
239 treatment duration.

240

241 ***Atovaquone targets both quiescent and proliferating parasites***

242 The delayed parasite recrudescence time of 12 days for the combination (dihydroartemisinin
243 6 h / (dihydroartemisinin + atovaquone) 48 h) compared to dihydroartemisinin alone (Fig. 2,
244 Table 2 and Table S4) evidenced that atovaquone is not only highly active against quiescent
245 parasites but also against proliferating artemisinin-resistant parasites (Fig. 1, control condition

246 e). This is in accordance with previously published atovaquone activity towards the
247 mitochondrial electron transport chain,^{33,34} whose activity seems maintained during
248 quiescence.^{10,12,35}

249

250 *The newly-designed QSA specifically targets quiescent parasites*

251 The induction of quiescence by ARTs had already been demonstrated.¹⁰ Here, two sorbitol
252 treatments were performed during the QSA to eliminate any potential non ring-stage and thus
253 non-quiescent parasites or awaking parasites (Fig. 3A). Regardless of the treatment conditions
254 and the drugs used, sorbitol treatment did not lead to any delay in the recrudescence times
255 compared to the no-sorbitol condition (Fig. 3B, Table S5). These data confirm that the QSA
256 allows the specifically evaluation of the activities of drugs on *P. falciparum* quiescent parasites.

257

258 *QSA applied to artemisinin-resistant field isolates confirms its stage specificity*

259 The QSA was also performed on artemisinin-resistant isolates from the field to confirm its
260 potential as a new screening tool for antimalarial development. Chloroquine and atovaquone
261 were tested on four *P. falciparum* Cambodian isolates all harboring the C580Y mutation on the
262 *kelch13* gene and having survival rates above 1 % in RSA, confirming their artemisinin
263 resistance (Table 1). Notably, all the isolates used harbored a chloroquine-resistant phenotype
264 with IC₅₀ values superior to 100 nM (Table S2). This phenotype was confirmed by the fast
265 parasite recrudescence after 48-h chloroquine treatment (2-day recrudescence time for
266 IPC8262, IPC6938, IPC6388, and 6-day recrudescence time for IPC8317, compared to no
267 recrudescence of the chloroquine-sensitive F32-ART strain) (Fig. 2, Table S1). Independent of
268 the isolate used, the QSA confirmed the lack of chloroquine activity towards quiescent parasites
269 with no significant delay between drug combination and the dihydroartemisinin control (Table
270 2, Table S1).

271 The efficacy of atovaquone was confirmed regardless of the field isolate and the combination
272 tested, with delays in recrudescence times all superior (at least 6 days) to the control condition
273 (dihydroartemisinin 54 h) supporting atovaquone activity against both quiescent (conditions a-
274 b) and artemisinin-resistant proliferating parasites (condition e) from the field (Fig. 2, Table 2,
275 Table S4).

276 By validating the QSA protocol on both laboratory and field-isolated artemisinin-resistant
277 strains, we evidenced that this newly designed protocol was able to evaluate the activity of
278 compounds against artemisinin-resistant parasites both in the specific artemisinin-induced
279 quiescent state and in proliferating forms.

280

281 ***ACT partner drugs display different activities against artemisinin-resistant quiescent***
282 ***parasites***

283 After standardizing the QSA protocol, the activity of WHO-recommended ACT partner drugs,
284 namely *N*-desethyl amodiaquine (the active metabolite of amodiaquine), piperaquine,
285 lumefantrine, and mefloquine were evaluated on quiescent parasites.

286 Although active towards proliferative parasites (Table S6, Fig. 1A condition e), *N*-desethyl
287 amodiaquine showed a very weak activity against quiescent parasites from the laboratory strain
288 F32-ART5 and the field isolate IPC8317 with a delay of 3-4 days (Fig. 2, Table S6), which is
289 below the 6-day delay threshold. This lack of efficacy towards quiescent parasites could be
290 explained by the mode of action of the molecule which, like chloroquine,^{36,37} targets the
291 polymerization of heme into hemozoin, a mechanism absent during quiescence. This is further
292 corroborated by the stage specificity of *N*-desethyl amodiaquine, which is mostly active against
293 trophozoites.³⁸

294 In a similar fashion, a weak piperaquine activity was observed in QSA against quiescent
295 parasites with a delay in recrudescence time below the 6-day threshold for F32-ART5,

296 IPC8317, IPC8262 and IPC6388, (Fig. 2, Table 2, Table S7). These strains harbor a single copy
297 of the *pm2* gene (except for IPC8262 with 1.58 *pm2* copies) and a PSA survival rate below
298 10 % (Table 1). Firstly, the absence of delay between F32-ART and F32-TEM in piperazine
299 recrudescence assay and PSA data confirm that the partial lack of piperazine activity against
300 quiescent parasites is not linked with any piperazine resistance or cross-resistance with
301 artemisinin (Table S8). Secondly, these results are in accordance with the purported mode of
302 action of piperazine which is thought to interfere with heme detoxification in a similar fashion
303 and with similar stage specificity than chloroquine.^{32,39}

304 The field isolate IPC6938 showed no recrudescence after treatment with piperazine alone
305 whereas this line harbors a PSA survival rate of 10.7 % and 3 copies of the *pm2* gene consistent
306 with piperazine resistance (Table 1). These conflicting results could be explained by the age
307 of ring-stage parasites during drug exposure in the different assays. Indeed, when PSA was
308 performed on ring-stage parasites older than 6 hours (condition similar to the QSA), the survival
309 rate dropped to 0.16 %. Unlike the other parasite lines, preliminary data obtained with this
310 isolate showed a delayed recrudescence with piperazine above 6 days in QSA (Table 2, Table
311 S7). Further experiments should be conducted in order to clarify the impact of piperazine
312 resistance on quiescent parasites.

313 In contrast, lumefantrine appeared active against quiescent parasites both on the laboratory
314 strain F32-ART5 and on the parasite isolate from the field IPC8317 with a recrudescence time
315 delay above 9 days (Fig. 2, Table 2, Table S9). Recently, lumefantrine has been shown to be
316 active against all but the schizont stage.³² These data, in addition to our results which also
317 showed its activity against quiescent parasites, seem to indicate that lumefantrine targets other
318 pathway(s) apart from the inhibition of hemozoin formation.⁴⁰

319 Mefloquine also showed an activity against quiescent parasites on all the tested strains with
320 very high recrudescence delays (Fig. 2, Table 2, Table S10). One field isolate, IPC6388, showed

321 a faster recrudescence after treatment with mefloquine alone compared to the other strains,
322 correlating with a higher IC₅₀ value. Consistently, this isolate harbors 3 copies of the *mdr1* gene
323 which is associated with mefloquine resistance (Table 1). Interestingly, its recrudescence delay
324 was of 6 days in QSA. Therefore, these results suggest that although mefloquine-resistant
325 parasites in quiescent state are less sensitive to the drug than mefloquine-sensitive parasites,
326 they can still be killed by mefloquine treatment. Its activity against quiescent parasites is
327 surprising considering its specificity towards trophozoites.³² It cannot be explained by its
328 ability to impair hemoglobin uptake,⁴¹ hemozoin biosynthesis,⁴² or even inhibit protein
329 synthesis by targeting the 80S ribosome,⁴³ since these different pathways are supposed to be
330 inactive during quiescence. Although the effect of dihydroartemisinin treatment on metacaspase
331 transcripts remains to be determined,⁴⁴ the mefloquine activity against quiescent parasites
332 could be due to its capacity to trigger an apoptotic process by *Plasmodium* metacaspase
333 activation leading to parasite death.^{45,46}

334

335 ***Targeting quiescent parasites can be achieved with different treatment schedules***

336 In order to optimize the QSA protocol, different quiescence-inducing drugs and different drug
337 schedules were tested against F32-ART5 in combination with 4 antimalarials namely
338 chloroquine, atovaquone, piperazine and mefloquine. Similar activities were obtained
339 regardless of the artemisinin derivative used (3 μM of artemisinin or 700 nM of
340 dihydroartemisinin) or the treatment duration (24 h or 48 h; Table S11) except when treating
341 quiescent parasites with piperazine. Indeed, a 24-h exposure to piperazine has no effect on
342 quiescent parasites as shown by the lack of delay between the combination and the control
343 regardless of the drug used to induce quiescence (Table S11), whereas a 48-h exposure to
344 piperazine led to a 5-day delay (Table 2). Because ACT partner drugs have half-lives of
345 several days, we reasoned that lengthening drug exposure to 48 hours (corresponding to a

346 hypothetical complete erythrocytic cycle) allowed better approximation of physiological
347 conditions.

348 Tests of molecules against quiescent parasites had already been reported.^{11,47-49} However, our
349 results underlined the importance of i) pre-treatment by ARTs to induce quiescence, ii)
350 maintaining quiescence throughout the whole drug evaluation, and iii) comparing the
351 combination dihydroartemisinin / (dihydroartemisinin + drug to be tested) to
352 dihydroartemisinin / dihydroartemisinin in order to be able to conclude on the genuine efficacy
353 of the drug towards quiescent parasites.

354

355 *Utility and limitations of the QSA*

356 Standard chemosensitivity assays are proliferation-based and hence, cannot allow the
357 evaluation of the missing variable of partner drug activity against quiescent parasites. Because
358 of its experimental design, the QSA presented herein can overcome these limitations. This assay
359 appears as a highly relevant tool to study artemisinin-induced quiescence in *P. falciparum*
360 parasites. It also constitutes an *in vitro* model that tries to grasp the pharmacological complexity
361 of ACTs by triggering quiescence before exposing parasites to the partner drug. Compounds
362 tested were used at high concentrations over 48 hours to approximate their physiological
363 conditions since ACT partner drugs have half-lives of several days and high plasma peak
364 concentrations.²⁶ Therefore, these parameters could be fine-tuned when evaluating the activity
365 of other compounds. The assay was not designed to compare the activity of different drugs on
366 quiescent parasites but rather to determine whether a drug efficiently kills quiescent parasites.
367 Moreover, it does not allow direct assessment of parasite death for obvious quantitation
368 limitations. The outcome of the assay is thereby indirect as it relies on a delayed parasite
369 recrudescence, correlated with the ability of a drug to kill quiescent parasites.

370

371 In conclusion, thanks to the QSA, we highlighted that i) drug activity against quiescent parasites
372 depends on the metabolic status of its target throughout quiescence, regardless of the drug's
373 stage specificity in asexual replication, ii) because of quiescence-based mechanism, drug
374 efficacy against artemisinin-induced quiescent forms of the *falciparum* malaria parasite is
375 unrelated to its activity against proliferating parasites, and iii) some molecules active against
376 ARTs-induced quiescent parasites can be identified and can shed light on pathways that remain
377 active within this dormant state, potentially guiding the search for new antimalarials. Custom
378 ACTs through the addition of a third compound are already evaluated in clinical trials.⁵⁰
379 However the added value of QSA-based drug design would be to enable the design of novel
380 ACTs that effectively target dormant parasites. This strategy could greatly enhance ACT
381 clinical efficacy and drastically limit the emergence of resistant parasites. Ultimately, this
382 approach could also confer an extended lifespan to ACTs even in a drug resistance context.

383

384 **Acknowledgements**

385 We would like to thank Chinedu O. Egwu for proofreading the article and we would also like
386 to acknowledge the WorldWide Antimalarial Resistance Network (WWARN) Reference
387 Standards Program for reference drug providing.

388

389 **Funding**

390 This study was supported in part by the Centre National de la Recherche Scientifique and the
391 French Agence Nationale de la Recherche (ANR-13-BSV3-0018 & ANR-13-CE35-0003). T.
392 R. was funded by a Graduate Fellowship Program from the University Paul Sabatier of
393 Toulouse. The funding sources of the study had no role in the study design, data collection, data
394 analysis, data interpretation or writing of the report.

395

396 **Transparency declarations**

397 None to declare.

398

399 **References**

- 400 1. White N. Antimalarial drug resistance and combination chemotherapy. *Philos Trans R Soc B Biol*
401 *Sci* 1999; **354**: 739–49.
- 402 2. World Health Organization. *World Malaria Report 2019*. 2019.
403 <https://www.who.int/publications-detail/world-malaria-report-2019>
- 404 3. Noedl H, Se Y, Schaecher K *et al*. Evidence of artemisinin-resistant malaria in Western Cambodia.
405 *N Engl J Med* 2008; **359**: 2619–20.
- 406 4. Dondorp AM, Nosten F, Yi P *et al*. Artemisinin Resistance in *Plasmodium falciparum* Malaria. *N*
407 *Engl J Med* 2009; **361**: 455–67.
- 408 5. Ashley EA, Dhorda M, Fairhurst RM *et al*. Spread of Artemisinin Resistance in *Plasmodium*
409 *falciparum* Malaria. *N Engl J Med* 2014; **371**: 411–23.
- 410 6. Ménard D, Khim N, Beghain J *et al*. A worldwide map of *Plasmodium falciparum* K13-propeller
411 polymorphisms. *N Engl J Med* 2016; **374**: 2453–64.
- 412 7. World Health Organization. *Status report on Artemisinin and ACT resistance (April 2017)*. 2017.
413 <http://www.who.int/malaria/publications/atoz/artemisinin-resistance-april2017/en/>
- 414 8. Ariey F, Witkowski B, Amaratunga C *et al*. A molecular marker of artemisinin-resistant
415 *Plasmodium falciparum* malaria. *Nature* 2014; **505**: 50–5.
- 416 9. Birnbaum J, Scharf S, Schmidt S *et al*. A Kelch13-defined endocytosis pathway mediates
417 artemisinin resistance in malaria parasites. *Science* 2020; **367**: 51–59.
- 418 10. Witkowski B, Lelièvre J, Barragán MJL *et al*. Increased tolerance to artemisinin in *Plasmodium*
419 *falciparum* is mediated by a quiescence mechanism. *Antimicrob Agents Chemother* 2010; **54**: 1872–7.
- 420 11. Chen N, LaCrue AN, Teuscher F *et al*. Fatty Acid Synthesis and Pyruvate Metabolism Pathways
421 Remain Active in Dihydroartemisinin-Induced Dormant Ring Stages of *Plasmodium falciparum*.
422 *Antimicrob Agents Chemother* 2014; **58**: 4773–81.
- 423 12. Paloque L, Ramadani AP, Mercereau-puijalon O *et al*. *Plasmodium falciparum*: multifaceted
424 resistance to artemisinins. *Malar J* 2016; **15**: 149.
- 425 13. Witkowski B, Khim N, Chim P *et al*. Reduced artemisinin susceptibility of *Plasmodium*
426 *falciparum* ring stages in western cambodia. *Antimicrob Agents Chemother* 2013; **57**: 914–23.

- 427 14. Na-Bangchang K, Muhamad P, Ruaengweerayut R *et al.* Identification of resistance of
428 *Plasmodium falciparum* to artesunate-mefloquine combination in an area along the Thai-Myanmar
429 border: integration of clinico-parasitological response, systemic drug exposure, and in vitro parasite
430 sensitivity. *Malar J* 2013; **12**: 263.
- 431 15. Saunders DL, Vanachayangkul P, Lon C. Dihydroartemisinin-piperaquine failure in Cambodia. *N*
432 *Engl J Med* 2014; **371**: 484–5.
- 433 16. Spring MD, Lin JT, Manning JE *et al.* Dihydroartemisinin-piperaquine failure associated with a
434 triple mutant including kelch13 C580Y in Cambodia: An observational cohort study. *Lancet Infect Dis*
435 2015; **15**: 683–91.
- 436 17. Dondorp AM, Fairhurst RM. Artemisinin-Resistant *Plasmodium falciparum* Malaria. *Microbiol*
437 *Spectr* 2016; **4**: 409–29.
- 438 18. Amaratunga C, Lim P, Suon S *et al.* Dihydroartemisinin-piperaquine resistance in *Plasmodium*
439 *falciparum* malaria in Cambodia: a multisite prospective cohort study. *Lancet Infect Dis* 2016; **16**:
440 357–65.
- 441 19. Amato R, Pearson RD, Almagro-Garcia J *et al.* Origins of the current outbreak of multidrug-
442 resistant malaria in southeast Asia: a retrospective genetic study. *Lancet Infect Dis* 2018; **18**: 337–45.
- 443 20. Ménard S, Ben Haddou T, Ramadani AP *et al.* Induction of multidrug tolerance in *Plasmodium*
444 *falciparum* by extended artemisinin pressure. *Emerg Infect Dis* 2015; **21**: 1733–41.
- 445 21. Putaporntip C, Kuamsab N, Kosuwin R *et al.* Natural selection of K13 mutants of *Plasmodium*
446 *falciparum* in response to artemisinin combination therapies in Thailand. *Clin Microbiol Infect* 2016;
447 **22**: 285.e1-8.
- 448 22. WHO. *Status report on artemisinin resistance*. 2014.
449 <https://www.who.int/malaria/publications/atoz/status-rep-artemisinin-resistance-sep2014.pdf>
- 450 23. Benoit-Vical F, Lelièvre J, Berry A *et al.* Trioxaquinones are new antimalarial agents active on all
451 erythrocytic forms, including gametocytes. *Antimicrob Agents Chemother* 2007. **51**: 1463-72
- 452 24. Wenger RH, Kurtcuoglu V, Scholz CC *et al.* Frequently asked questions in hypoxia research.
453 *Hypoxia (Auckland, NZ)* 2015; **3**: 35–43.
- 454 25. Niaré K, Paloque L, Ménard S *et al.* Multiple phenotypic and genotypic artemisinin sensitivity

455 evaluation of malian *Plasmodium falciparum* isolates. *Am J Trop Med Hyg* 2018; **98**: 1123–31.

456 26. Nosten F, White NJ. Artemisinin-based combination treatment of falciparum malaria. *Am J Trop*
457 *Med Hyg* 2007; **77**: 181–92.

458 27. Lambros C, Vanderberg JP. Synchronization of *Plasmodium falciparum* Erythrocytic Stages in
459 Culture. *J Parasitol* 1979; **65**: 418–20.

460 28. Smilkstein M, Sriwilaijaroen N, Kelly JX *et al.* Simple and Inexpensive Fluorescence-Based
461 Technique for High-Throughput Antimalarial Drug Screening. *Antimicrob Agents Chemother* 2004;
462 **48**: 1803–6.

463 29. Witkowski B, Amaratunga C, Khim N *et al.* Novel phenotypic assays for the detection of
464 artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: In-vitro and ex-vivo drug-response
465 studies. *Lancet Infect Dis* 2013; **13**: 1043–9.

466 30. Duru V, Khim N, Leang R *et al.* *Plasmodium falciparum* dihydroartemisinin-piperaquine failures
467 in Cambodia are associated with mutant K13 parasites presenting high survival rates in novel
468 piperaquine in vitro assays: Retrospective and prospective investigations. *BMC Med* 2015; **13**: 305.

469 31. Fitch CD. Ferriprotoporphyrin IX, phospholipids, and the antimalarial actions of quinoline drugs.
470 *Life Sci* 2004; **74**: 1957–72.

471 32. Murithi JM, Owen ES, Istvan ES *et al.* Combining Stage Specificity and Metabolomic Profiling to
472 Advance Antimalarial Drug Discovery. *Cell Chem Biol* 2020; **27**: 158-171.e3.

473 33. Nixon GL, Pidathala C, Shone AE *et al.* Targeting the mitochondrial electron transport chain of
474 *Plasmodium falciparum*: New strategies towards the development of improved antimalarials for the
475 elimination era. *Future Med Chem* 2013; **5**: 1573–91.

476 34. Siregar JE, Kurisu G, Kobayashi T *et al.* Direct evidence for the atovaquone action on the
477 *Plasmodium* cytochrome bc1 complex. *Parasitol Int* 2015; **64**: 295–300.

478 35. Peatey CL, Chavchich M, Chen N *et al.* Mitochondrial Membrane Potential in a Small Subset of
479 Artemisinin-Induced Dormant *Plasmodium falciparum* Parasites in Vitro. *J Infect Dis* 2015. **212**: 426-
480 34

481 36. Bray PG, Mungthin M, Ridley RG *et al.* Access to Hematin: The Basis of Chloroquine Resistance.
482 *Mol Pharmacol* 1998; **54**: 170–9.

483 37. Ginsburg H, Famin O, Zhang J *et al.* Inhibition of glutathione-dependent degradation of heme by
484 chloroquine and amodiaquine as a possible basis for their antimalarial mode of action. *Biochem*
485 *Pharmacol* 1998; **56**: 1305–13.

486 38. Wilson DW, Langer C, Goodman CD *et al.* Defining the timing of action of antimalarial drugs
487 against *Plasmodium falciparum*. *Antimicrob Agents Chemother* 2013; **57**: 1455–67.

488 39. Sachanonta N, Chotivanich K, Chaisri U *et al.* Ultrastructural and real-time microscopic changes
489 in *P. falciparum*-infected red blood cells following treatment with antimalarial drugs. *Ultrastruct*
490 *Pathol* 2011; **35**: 214–25.

491 40. Warhurst DC, Adagu IS, Beck H-P *et al.* Mode of action of artemether/lumefantrine
492 (COARTEM): The sole, fixed, oral ADCC and its role in combatting multidrug-resistance. *Southeast*
493 *Asian J Trop Med Public Health* 2001; **32**: 4–8.

494 41. Famin O, Ginsburg H. Differential effects of 4-aminoquinoline-containing antimalarial drugs on
495 hemoglobin digestion in *Plasmodium falciparum*-infected erythrocytes. *Biochem Pharmacol* 2002; **63**:
496 393–8.

497 42. Sullivan DJ, Matile H, Ridley RG *et al.* A common mechanism for blockade of heme
498 polymerization by antimalarial quinolines. *J Biol Chem* 1998; **273**: 31103–7.

499 43. Wong W, Bai XC, Sleebs BE *et al.* Mefloquine targets the *Plasmodium falciparum* 80S ribosome
500 to inhibit protein synthesis. *Nat Microbiol* 2017; **2**: 17031

501 44. Shaw PJ, Chaotheing S, Kaewprommal P *et al.* *Plasmodium* parasites mount an arrest response to
502 dihydroartemisinin , as revealed by whole transcriptome shotgun sequencing (RNA-seq) and
503 microarray study. *BMC Genomics* 2015; **16**: 830.

504 45. Gunjan S, Singh SK, Sharma T *et al.* Mefloquine induces ROS mediated programmed cell death in
505 malaria parasite: *Plasmodium*. *Apoptosis* 2016; **21**: 955–64.

506 46. Meslin B, Beavogui AH, Fasel N *et al.* *Plasmodium falciparum* metacaspase PfMCA-1 triggers a
507 z-VAD-fmk inhibitable protease to promote cell death. *PLoS One* 2011; **6**: e23867.

508 47. Teuscher F, Gatton ML, Chen N *et al.* Artemisinin-Induced Dormancy in *Plasmodium falciparum* :
509 Duration, Recovery Rates, and Implications in Treatment Failure. *J Infect Dis* 2010; **202**: 1362–8.

510 48. Dembele L, Gupta DK, Yi-Xiu Lim M *et al.* Imidazolopiperazines Kill both Rings and Dormant

511 Rings in Wild-Type and K13 Artemisinin-Resistant *Plasmodium falciparum* in vitro. *Antimicrob*
512 *Agents Chemother* 2018; **62**: e02235-17.

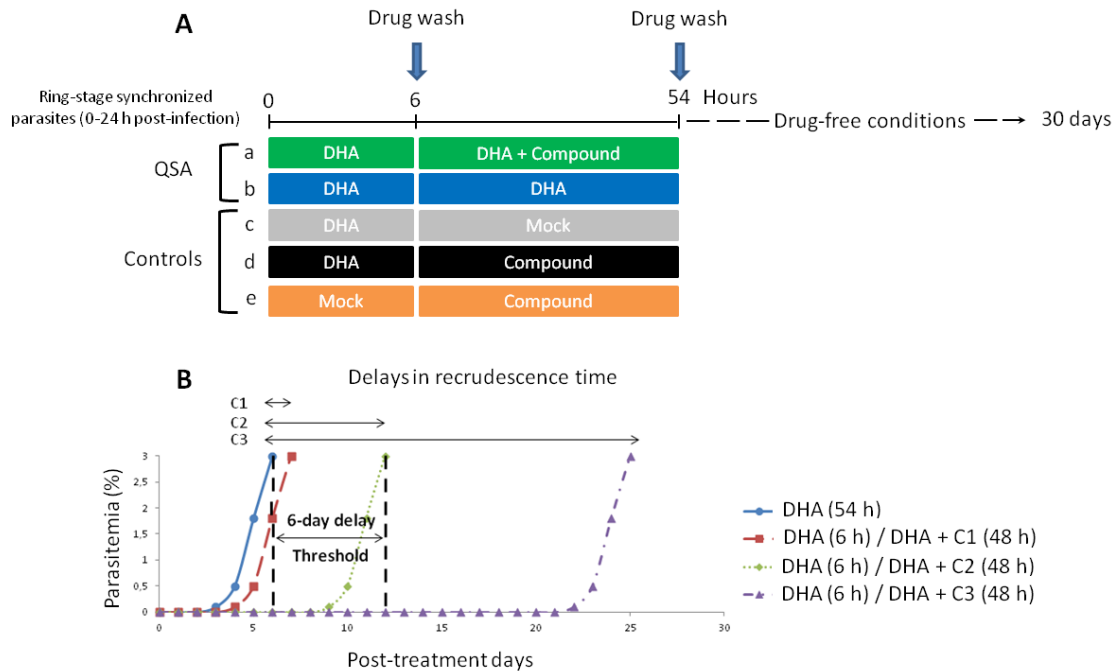
513 49. Clements RL, Strelva V, Dumoulin P *et al.* A novel antiparasitic compound kills ring-stage
514 *Plasmodium falciparum* and retains activity against artemisinin-resistant parasites. *J Infect Dis* 2020;
515 **221**: 956-62

516 50. Thu AM, Phyo AP, Landier J *et al.* Combating multidrug-resistant *Plasmodium falciparum*
517 malaria. *FEBS J* 2017; **284**: 2569–78.

518

519
520
521

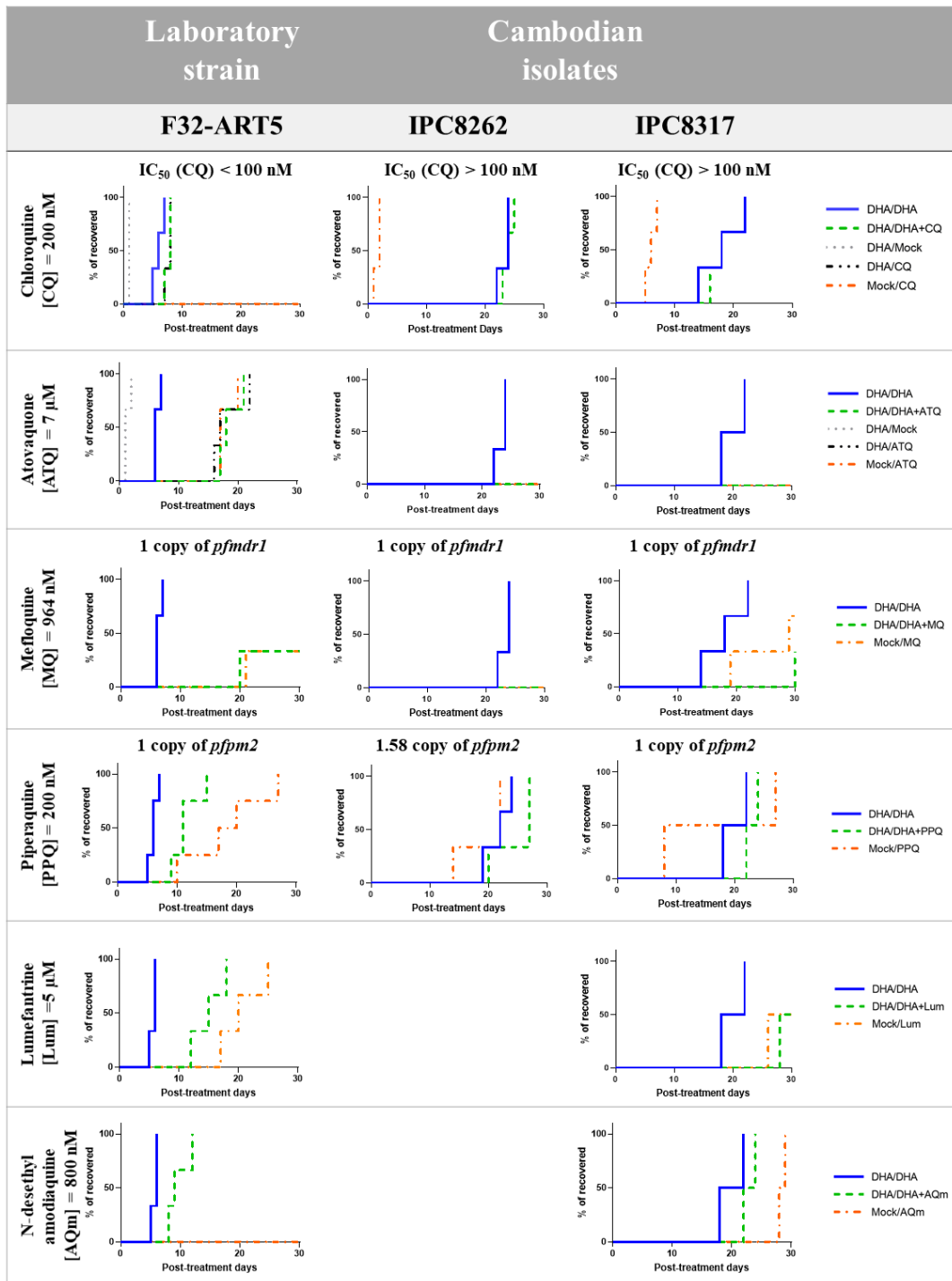
Figures & Tables



522

523 **Figure 1.** QSA (Quiescent-stage Survival Assay) design and interpretation. **A:** Treatment
524 schedule of the different drug combinations studied (DHA: dihydroartemisinin); a-b) conditions
525 to assess activity of the molecule on quiescent parasites; c-e) control conditions. **B:** Schematic
526 curves of recrudesence profiles of artemisinin-resistant parasites to reach the initial 3 %
527 parasitemia obtained *via* the QSA after 54-h treatment with DHA 700 nM alone or in
528 combination with different compounds tested (C1, C2 and C3). C1 appears inactive, C2 is active
529 with a 6-day delay (corresponding to the designated threshold) between **a** and **b** conditions and
530 C3 is highly active against quiescent parasites with a delay largely higher than the 6-day
531 threshold. Double-headed arrows highlight delay in recrudesence times between combination
532 treatments (DHA / DHA + C1, C2 or C3) and DHA (54 h) alone.

533



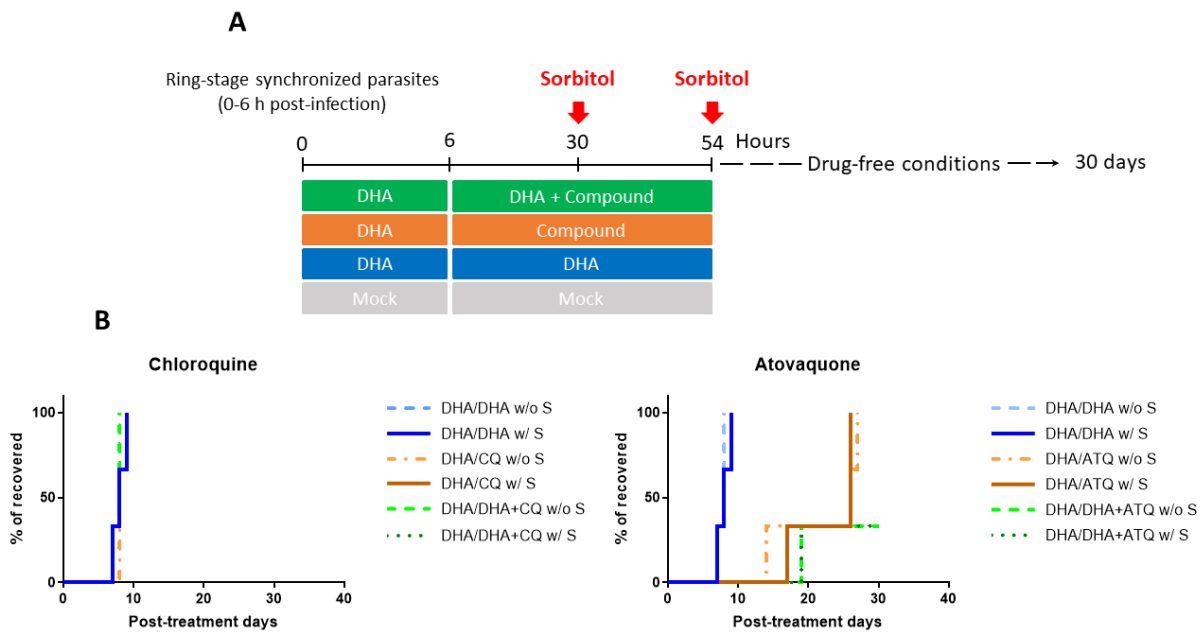
534

535 **Figure 2.** QSA (Quiescent-stage Survival Assay) results represented by Kaplan-Meier survival

536 curves showing recrudescence days of synchronous ring-stage parasites of the laboratory

537 artemisinin-resistant strain F32-ART5 and two artemisinin-resistant Cambodian isolates after

538 different treatments. D0 corresponds to the day when all drugs were removed. Raw data are
539 presented in supplementary tables. These 3 strains are artemisinin-resistant (RSA survival
540 rates > 1 %), piperaquine-sensitive (PSA survival rates < 10%) and mefloquine-sensitive.



541

542 **Figure 3.** Quiescence specificity of the Quiescent-stage Survival Assay. **A:** Sorbitol treatments

543 schedule applied on parasites submitted to QSA conditions. In this experiment, two consecutive

544 sorbitol treatments were conducted at 24-h interval to make sure that dihydroartemisinin-

545 treatment maintained parasites in a quiescent state, i.e. that parasites did not proceed with their

546 cell cycle. Treating parasite cultures with a 5 % sorbitol solution selectively eliminates all

547 possible forms older than 24 h post-invasion preventing any recrudescence. Comparing the

548 effect of sorbitol treatment for a given treatment schedule will thus reveal if the QSA protocol

549 is indeed adequate to target quiescent forms of the parasite.

550 **B:** Kaplan-Meier survival curves on F32-ART5 showing days needed for cultures to reach

551 initial parasitemia with or without sorbitol treatment in different conditions. Raw data are

552 presented in supplementary Table S5.

553 w/ S (with sorbitol), w/o S (without sorbitol).

554

555

556 **Table 1.** Phenotypic and genotypic traits of the *P. falciparum* strains used in this study

Strain	Description	Genotype				RSA survival rate (%)	PSA survival rate (%) ^b
		<i>pfkelch13</i>	<i>pfprt</i>	<i>pfpm2</i> copy number	<i>pfmdr1</i> copy number		
F32-ART5	Laboratory	M476I	WT	1 copy	1 copy	10.7 ^a	0.4
F32-TEM	Laboratory	WT	WT	1 copy	1 copy	0.09 ^a	ND
IPC8262	Field	C580Y	ND	1.58 copy	1 copy	13 ^b	2.7
IPC8317	Field	C580Y	ND	1 copy	1 copy	8.8 ^c	0
IPC6388	Field	C580Y	ND	1 copy	3 copies	6.8 ^c	1.9
IPC6938	Field	C580Y	ND	3 copies	1 copy	6.6 ^c	10.7

557

558 ND: Not Determined; WT: Wild Type

559 ^a n=3

560 ^b n=2

561 ^c n=1

562

563

564

565 **Table 2.** Difference in median recrudescence times from independent experiments obtained for
 566 the artemisinin-resistant strain F32-ART5 and 4 Cambodian ART-resistant isolates following
 567 exposure to different antimalarial drugs in QSA conditions (DHA 6 h / (DHA + compound
 568 48 h)) compared to DHA alone (DHA 6 h / DHA 48 h). Raw data are presented in
 569 supplementary tables.

570

	Delay in recrudescence time (post-treatment days)					
	CQ	ATQ	AQm	PPQ	LUM	MQ
F32-ART5	2	12	4	5	10	> 24
IPC8262	0	> 6		5		> 6
IPC8317	0	> 10	3	3	> 9	> 12
IPC6938	3	> 6		> 6		> 6
IPC6388	0	14		3		6

571

572 CQ (chloroquine), ATQ (atovaquone), AQm (*N*-desethyl amodiaquine), PPQ (piperaquine),
 573 LUM (lumefantrine), MQ (mefloquine).

574 > means that for at least one of the three replicates, no recrudescence was observed up to 30
 575 days, in which case, the value was censored.

576

577

578

579