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

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### ► 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-02948398>**

Submitted on 6 Oct 2021

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

3  
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17  
18          **Running title:** Compounds active against quiescent artemisinin-resistant malaria parasites

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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.

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## 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. <sup>1</sup> 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. <sup>2</sup> However, *Plasmodium* resistance to ARTs emerged in 2006  
54 and could now jeopardize malaria elimination programs. <sup>3-7</sup> ARTs-resistance is linked to a  
55 mutation on the *kelch13* gene, <sup>8</sup> which encodes for a protein involved in different mechanisms,  
56 among which hemoglobin uptake. <sup>9</sup> ARTs-resistance is mediated by a quiescent state upon  
57 ARTs exposure, <sup>10</sup> 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, <sup>11,12</sup> and subsequent up-regulation to enable parasite recrudescence after drug  
61 elimination. <sup>10,13</sup> 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. <sup>7,14-19</sup> 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. <sup>20</sup> 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. <sup>21,22</sup> 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,<sup>8,10,20</sup> 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 % CO<sub>2</sub>  
83 in a humid atmosphere at 37°C.<sup>10,20,23,24</sup> Artemisinin-resistant field isolates (from Pasteur  
84 Institute of Cambodia) IPC8262, IPC8317, IPC6388 and IPC6938 were cultivated under 5 %  
85 O<sub>2</sub>, 5 % CO<sub>2</sub>, and 90 % N<sub>2</sub> (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).<sup>25</sup> 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  $\mu$ M atovaquone, 200 nM piperaquine, 964 nM mefloquine, 800 nM *N*-  
97 desethyl amodiaquine, 5  $\mu$ M lumefantrine), equal or close to their plasma peak concentrations  
98 in patients.<sup>26</sup> 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.<sup>27</sup> The antiplasmodial activities were evaluated  
105 using the SYBR Green I method.<sup>28</sup> 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  $\mu$ L of  
108 each well were transferred into a black 96-well plate. 100  $\mu$ 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 ( $\lambda_{\text{excitation}} = 485 \text{ nm}$ ,  $\lambda_{\text{emission}} = 528 \text{ nm}$ ). IC<sub>50</sub> 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  $\mu$ L of fresh red blood cells were added once a week. If no recrudescence occurred  
132 after 30 days, the experiment was stopped.<sup>10,20</sup> 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  $\mu$ 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.<sup>10,20</sup> 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.<sup>10,20</sup>

152

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

154 The Ring-stage Survival Assay (RSA) was carried out as previously reported.<sup>29</sup> 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  $\geq 2$  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.<sup>29</sup>

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.<sup>30</sup> 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.<sup>30</sup>

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).<sup>10,20</sup> 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 IC<sub>50</sub> 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.<sup>31</sup> 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.<sup>32</sup> 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,<sup>33,34</sup> whose activity seems maintained during  
248 quiescence.<sup>10,12,35</sup>

249

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

251 The induction of quiescence by ARTs had already been demonstrated.<sup>10</sup> 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 IC<sub>50</sub> 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), piperazine,  
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,<sup>36,37</sup> 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.<sup>38</sup>

294 In a similar fashion, a weak piperazine 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.<sup>32,39</sup>

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.<sup>32</sup> 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.<sup>40</sup>

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 IC<sub>50</sub> 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.<sup>32</sup> It cannot be explained by its  
328 ability to impair hemoglobin uptake,<sup>41</sup> hemozoin biosynthesis,<sup>42</sup> or even inhibit protein  
329 synthesis by targeting the 80S ribosome,<sup>43</sup> 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,<sup>44</sup> 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.<sup>45,46</sup>

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.<sup>11,47-49</sup> 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.<sup>26</sup> 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.<sup>50</sup>  
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

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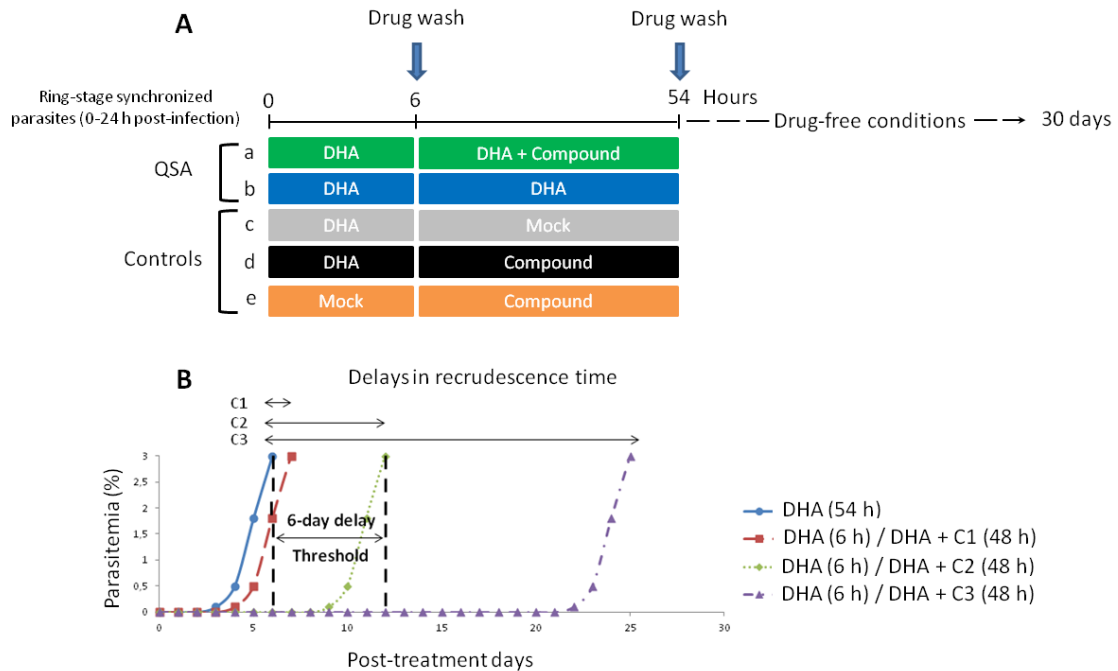
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## Figures & Tables

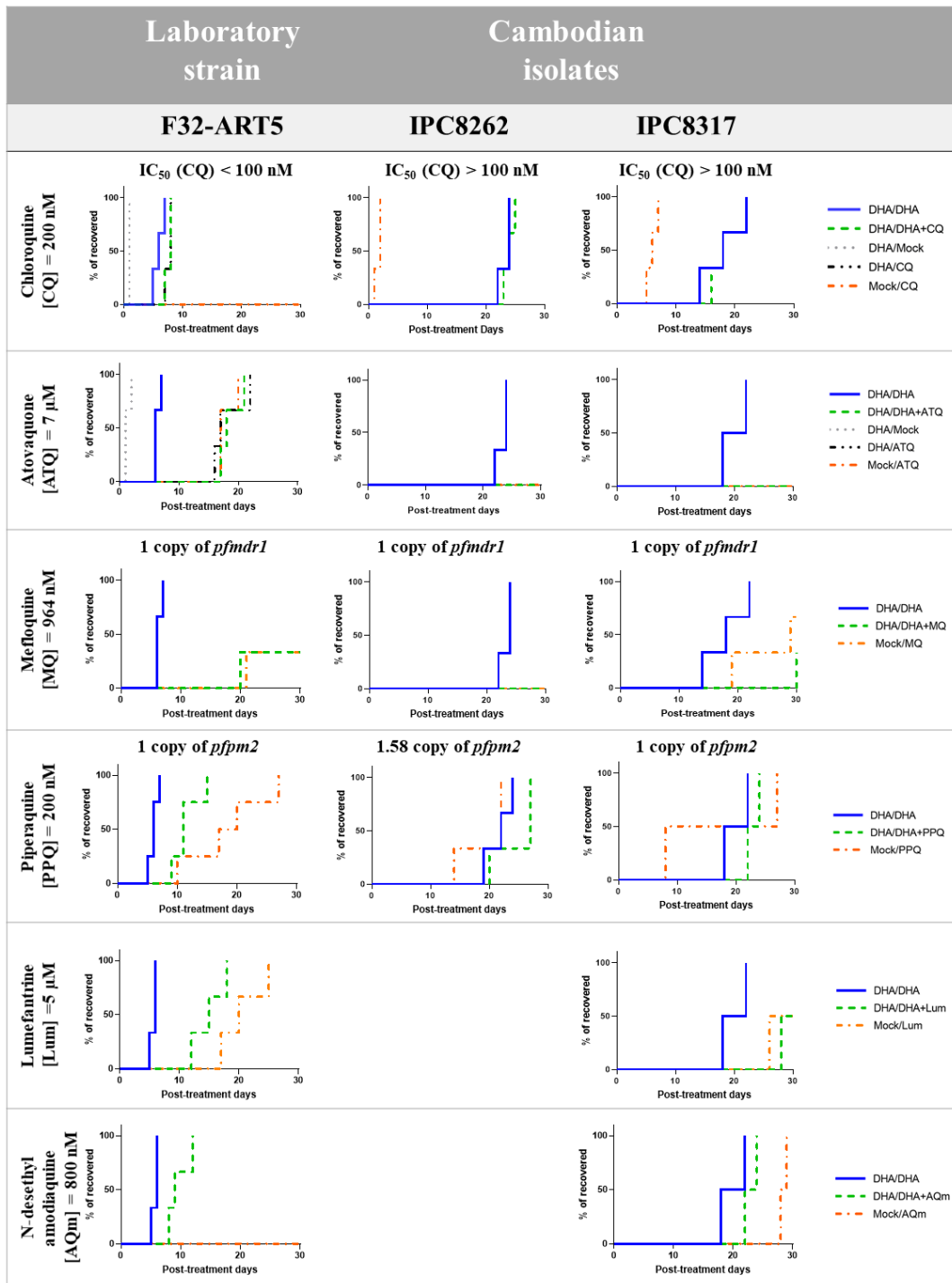


522

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

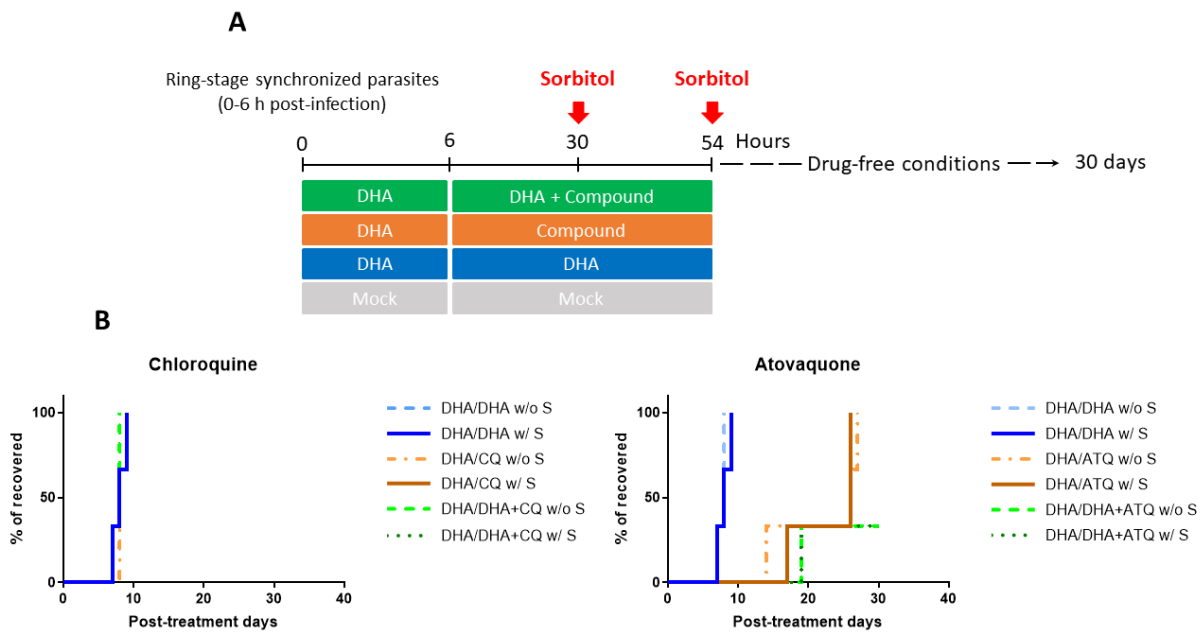




534

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

557

558 ND: Not Determined; WT: Wild Type

559 <sup>a</sup> n=3

560 <sup>b</sup> n=2

561 <sup>c</sup> 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
<b>F32-ART5</b>	2	12	4	5	10	> 24
<b>IPC8262</b>	0	> 6		5		> 6
<b>IPC8317</b>	0	> 10	3	3	> 9	> 12
<b>IPC6938</b>	3	> 6		> 6		> 6
<b>IPC6388</b>	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

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