

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

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22 ABSTRACT

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

Objectives: To thwart this multi-resistance, the quiescent state of artemisinin-resistant parasites 28 must be taken into consideration from the very early stages of the drug discovery process. 29 30 Methods: We designed a novel phenotypic assay named Quiescent-stage Survival Assay (QSA) to assess the antiplasmodial activity of drugs on quiescent parasites. This assay was first 31 validated on quiescent forms from different artemisinin-resistant parasite lines (laboratory 32 33 strain and field isolates), using two reference drugs with different mechanisms of action: chloroquine and atovaquone. Furthermore, the efficacies of different partner drugs of 34 artemisinins used in ACTs were investigated against both laboratory strains and field isolates 35 from Cambodia. 36

Results: Our results highlight that because of quiescence-based mechanism and the respective
pharmacological targets of drugs, their efficacies on artemisinin-resistant parasites may be
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**

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

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

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78 MATERIALS AND METHODS

79 Parasite culture

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

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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).

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102 Standard chemosensitivity assay

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

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114 Quiescent-stage Survival Assay (QSA)

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

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

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145 **Recrudescence assay**

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

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

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

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164 **Piperaquine survival assay (PSA)**

The piperaquine survival assay (PSA) was performed as previously described to evaluate the ability of ring-stage parasites to survive piperaquine exposure. ³⁰ Briefly, 0-3 h old ring-stage parasites (obtained after a percoll-sorbitol treatment) were exposed to 200 nM of piperaquine or culture medium alone (control) for 48 h in duplicate. After drug exposure, parasites were washed with RPMI-1640 medium and then cultivated for the next 24 h without drug. Survival rates were measured similarly to the RSA (ratio of viable parasites in piperaquine-exposed relative to DMSO-exposed samples). The strains with a survival rate above 10 % in PSA were
 referred to as piperaquine-resistant. ³⁰

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174 Data Analysis

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

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181 **RESULTS AND DISCUSSION**

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

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

could be statistically significant but not necessarily biologically relevant since this could be due 196 197 to variations in culture parameters independent of the number of surviving parasites. Therefore, from one experiment to the other, there can be a two to three-day variation in recrudescence 198 days, which is why, by setting a 6-day confidence interval, we made sure that the differences 199 were meaningful. To develop the QSA model, chloroquine and atovaquone were used because 200 201 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 chloroquine, mefloquine or atovaquone resistance was used (Table 1). ^{10,20} Results were then 203 confirmed on artemisinin-resistant isolates from the field. 204

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206 Chloroquine cannot kill quiescent parasites

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

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

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

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241 Atovaquone targets both quiescent and proliferating parasites

The delayed parasite recrudescence time of 12 days for the combination (dihydroartemisinin 6 h / (dihydroartemisinin + atovaquone) 48 h) compared to dihydroartemisinin alone (Fig. 2, Table 2 and Table S4) evidenced that atovaquone is not only highly active against quiescent parasites but also against proliferating artemisinin-resistant parasites (Fig. 1, control condition e). This is in accordance with previously published atovaquone activity towards the mitochondrial electron transport chain, 33,34 whose activity seems maintained during quiescence. 10,12,35

249

250 The newly-designed QSA specifically targets quiescent parasites

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

The QSA was also performed on artemisinin-resistant isolates from the field to confirm its 259 potential as a new screening tool for antimalarial development. Chloroquine and atovaquone 260 were tested on four P. falciparum Cambodian isolates all harboring the C580Y mutation on the 261 kelch13 gene and having survival rates above 1% in RSA, confirming their artemisinin 262 263 resistance (Table 1). Notably, all the isolates used harbored a chloroquine-resistant phenotype with IC_{50} values superior to 100 nM (Table S2). This phenotype was confirmed by the fast 264 parasite recrudescence after 48-h chloroquine treatment (2-day recrudescence time for 265 IPC8262, IPC6938, IPC6388, and 6-day recrudescence time for IPC8317, compared to no 266 recrudescence of the chloroquine-sensitive F32-ART strain) (Fig. 2, Table S1). Independent of 267 the isolate used, the QSA confirmed the lack of chloroquine activity towards quiescent parasites 268 with no significant delay between drug combination and the dihydroartemisinin control (Table 269 2, Table S1). 270

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

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

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

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

Although active towards proliferative parasites (Table S6, Fig. 1A condition e), N-desethyl 286 287 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 below the 6-day delay threshold. This lack of efficacy towards quiescent parasites could be 289 explained by the mode of action of the molecule which, like chloroquine, ^{36,37} targets the 290 polymerization of heme into hemozoin, a mechanism absent during quiescence. This is further 291 corroborated by the stage specificity of N-desethyl amodiaquine, which is mostly active against 292 trophozoites. ³⁸ 293

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

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

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

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

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

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

334

335 Targeting quiescent parasites can be achieved with different treatment schedules

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

346 hypothetical complete erythrocytic cycle) allowed better approximation of physiological347 conditions.

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

354

355 Utility and limitations of the QSA

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

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

383

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Transparency declarations

397 None to declare.

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



Figure 2. QSA (Quiescent-stage Survival Assay) results represented by Kaplan-Meier survival
curves showing recrudescence days of synchronous ring-stage parasites of the laboratory
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
- rates > 1 %), piperaquine-sensitive (PSA survival rates < 10%) and mefloquine-sensitive.



Figure 3. Quiescence specificity of the Quiescent-stage Survival Assay. A: Sorbitol treatments 542 schedule applied on parasites submitted to QSA conditions. In this experiment, two consecutive 543 sorbitol treatments were conducted at 24-h interval to make sure that dihydroartemisinin-544 treatment maintained parasites in a quiescent state, i.e. that parasites did not proceed with their 545 cell cycle. Treating parasite cultures with a 5 % sorbitol solution selectively eliminates all 546 possible forms older than 24 h post-invasion preventing any recrudescence. Comparing the 547 effect of sorbitol treatment for a given treatment schedule will thus reveal if the QSA protocol 548 549 is indeed adequate to target quiescent forms of the parasite.

B: Kaplan-Meier survival curves on F32-ART5 showing days needed for cultures to reach initial parasitemia with or without sorbitol treatment in different conditions. Raw data are presented in supplementary Table S5.

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

554

Stuain	Description			Genotype		RSA
Strain	Description	nfkølch 13	nfcrt	<i>nfnm</i> ² conv number	<i>nfmdr1</i> copy number	- ra

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

Strain	Description			RSA survival	PSA survival		
Stram		pfkelch13	pfcrt	<i>pfpm2</i> copy number	<i>pfmdr1</i> copy number	rate (%)	rate (%) ^b
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

ND: Not Determined; WT: Wild Type

^a n=3

^b n=2

^c n=1

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

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578