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1 # Transgenic pyrimethamine-resistant *P. falciparum* reveals transmission blocking
2 # potency of P218, a novel antifolate

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10
11 **Abstract**

12 Antimalarial drug which target more than one life stage of the parasite are valuable tools in the fight
13 against malaria. Previous generation of antifolate drugs are able to inhibit replicative stages of drug-sensitive,
14 but not resistant parasites in humans, and mosquitoes. The lack of reliable gametocyte-producing, antifolate
15 resistant *P. falciparum* hindrance the development of new antifolate compounds against mosquito stages. We
16 used CRISPR-Cas9 technology to develop transgenic gametocyte producing *P. falciparum* with quadruple
17 mutations in *dhfr* gene, using NF54 as a parental strain. The transgenic parasites gained pyrimethamine
18 resistance while maintaining the gametocyte producing activity. In contrast to pyrimethamine that cannot inhibit
19 exflagellation of the quadruple *dhfr* mutant parasite, the novel antifolate P218 showed a good potency for
20 exflagellation inhibition (exflagellation IC_{50} 10.74 ± 4.22 nM). The exflagellation IC_{50} was 5.3 times lower than
21 erythrocytic IC_{50} suggesting that the human to mosquito transmission poses as a strong barrier to prevent
22 P218 resistant parasite among population. This study demonstrates that P218 can be considered as a highly
23 potent tool to prevent the spread of antifolate resistant parasites.
24

25 **Keywords:** antifolate; drug resistance; malaria; dihydrofolate reductase; transmission blocking; CRISPR-
26 Cas9

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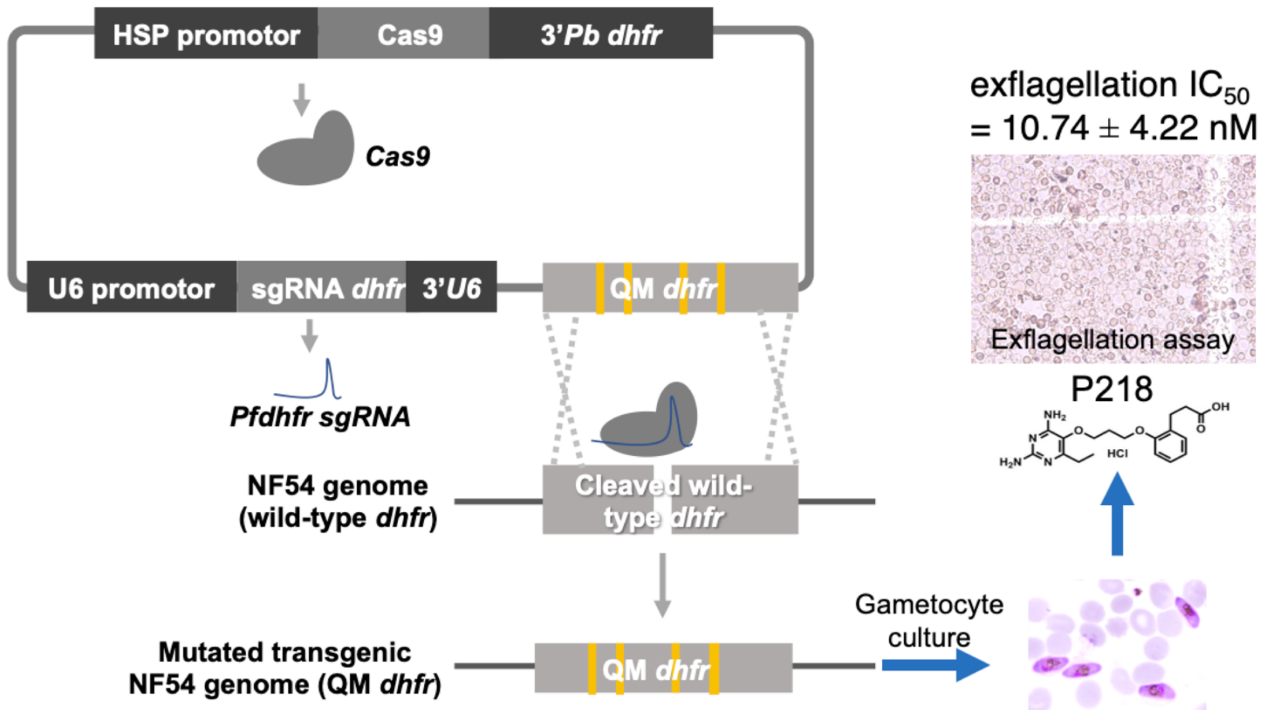
27 **Graphical Abstract**



28

29 **Research Highlights**

30 - Transgenic gametocyte producing pyrimethamine resistant *P. falciparum* was generated.

31 - P218 asexual stage IC_{50} in NF54-4mut*Pfdhfr* was 56.94 ± 15.69 nM.

32 - P218 exflagellation IC_{50} in NF54-4mut*Pfdhfr* was 10.74 ± 4.22 nM.

33 - P218 exflagellation IC_{50} in NF54-4mut*Pfdhfr* is 5.3 times lower than erythrocytic IC_{50}.

34 - P218 is an invaluable tool for malaria treatment and transmission control.

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35 ***1. Introduction***

36 Malaria is a major public health problem that impact many areas of the world causing health, social, and
37 economic impact. The disease is the deadliest mosquito-borne disease causing more than 400,000 deaths from
38 over 200 million cases per year, and most of the death from malaria occur in children under age 5 (World Health
39 Organization, 2019). While effective vaccines are anticipated, antimalarial drugs are still the major means to
40 reduce burden of malaria infection in humans. However, emergence of resistant parasites to the current
41 antimalarials in clinical use has highlighted the importance of antimalarial drug discovery.

42 During development in the erythrocytic stage, a small percentage of the malaria parasites differentiate
43 into male and female gametocytes, the mosquito-infective stage, which are taken up by mosquitoes during blood
44 meal. Once inside the mosquito gut, changes in environment triggers gametocytes activation (Josling & Llinás,
45 2015). Male gametocytes undergo exflagellation, which consists of three rounds of DNA replication and
46 differentiation to generate motile parasites called microgametes that can swim through blood meal in the
47 mosquito gut to find and mate with activated female gametocytes (macrogamete). Fertilized diploid zygotes then
48 undergo meiosis and cellular differentiation to generate motile and invasive ookinetes, which then invade the
49 mosquito gut epithelium to form oocysts at around 24 hours after infectious blood meal. The number of parasites
50 that survive these processes in mosquito infection is extremely low and can be considered as a major bottleneck
51 in malaria transmission (Smith et al., 2014). Therefore, the control measures targeting these transmission
52 bottlenecks will be highly effective for malaria control. *Plasmodium* oocysts in the mosquito gut undergo
53 endomitosis to generate thousands of sporozoites over the course of two weeks. Mature sporozoites later emerge
54 from the oocysts then migrate and reside in the mosquito salivary glands before a small proportion of them are
55 injected into human hosts when the infected mosquitoes take a new blood meal. For the goal of malaria
56 elimination, ideal drugs should, in addition to being effective in the pre-erythrocytic and erythrocytic stages, also
57 be able to block the transmission from infected humans to mosquitoes (transmission-blocking) and prevent
58 infection from infectious mosquito bites in human (prophylaxis).

59 The folate pathway, involving in the one-carbon metabolism, is required for DNA and RNA synthesis as
60 well as other metabolic processes. It is essential for parasite development in both human and insect hosts.
61 Antifolate compounds that target dihydrofolate reductase (DHFR) such as cycloguanil (CG) and pyrimethamine
62 (PYR) have long been used in combination with sulfa drugs for treatment and prophylaxis against malaria since
63 1940s-1950s (A Gregson, 2005). However, the malaria parasites soon developed resistance to these drugs in
64 1960s and it was shown that the mutation in DHFR gene is a major contributing factor to the parasite's resistance
65 to DHFR inhibitor (Aric Gregson & Plowe, 2005; Müller & Hyde, 2013; Yuthavong et al., 2006). The crystal
66 structures for the wild-type bifunctional DHFR-TS and the highly PYR-resistant quadruple mutant enzyme (QM)

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67 from *P. falciparum* (Yuvaniyama et al., 2003) gave the structural basis of the lower binding affinity of PYR to
68 quadruple and other mutants (Cowman et al., 1988; Foote et al., 1990; Peterson et al., 1988, 1990; Sirawaraporn
69 et al., 1997). This information was used to design P218, a 2,4-diaminopyrimidine with flexible side chain of 2'-
70 carboxyethylphenyl group (Yuthavong et al., 2012). Although the effectiveness of P218 in antifolate-resistant
71 malaria has been established, the ability of the compound to inhibit mosquito stages of antifolate resistant *P.*
72 *falciparum* has been much less studied. One of the obstacles for such study was that the laboratory maintained
73 quadruple *dhfr* mutant parasites such as the V1/S strain cannot develop into gametocytes.

74 With the advance in *Plasmodium* genetic manipulation, it is possible to generate drug resistant parasite
75 using molecular techniques. Previous study showed that NF54 strain *P. falciparum* could preserve an ability to
76 develop into gametocytes even after half a year of asexual stage culture, while the 3D7 strain quickly lose the
77 gametocyte producing (Delves et al., 2016). Because the generation of transgenic parasite is a long process,
78 the ability of the NF54 strain to preserve gametocyte producing capability is suitable for the generation of
79 transgenic parasite to study extraerythrocytic stages.

80 In the present study, we developed transgenic gametocyte producing *P. falciparum* with quadruple
81 mutation in *dhfr* gene to use as a model for antifolate drug testing in the mosquito stage. With CRISPR-Cas9
82 technology, the wild-type *dhfr* gene of gametocyte producing NF54 strain P*. falciparum* was replaced by
83 quadruple *dhfr* mutant gene with amino acid mutations at N51I, C59R, S108N, and I164L,the same as the V1/S
84 strain. The transgenic parasite gained antifolate resistance as confirmed in the asexual blood stage while
85 maintaining the gametocyte producing activity. Finally, the activity of P218 in transmission blocking was
86 determined by male gametocyte exflagellation assay.

87

88 ***2. Materials and Methods***

89 ***2.1 Ethic statement***

90 Human serum and erythrocytes from donors used for *P. falciparum* culture were obtained under the
91 regulation of the Ethics Committee for Human Research, National Science and Technology Development Agency
92 (NSTDA), following an approved protocol (document number 0021/2560).

93

94 ***2.2 P. falciparum strains and asexual stage culture***

95 *P. falciparum* strain NF54 (Patient Line E) MRA-1000 was used as a parental strain for transgenic parasite
96 construction. The parasites comprise a gametocyte-producing strain with wild-type *dhfr* gene.

97 Asexual blood stage of *P. falciparum* was cultured *in vitro* in human O+ erythrocytes and RPMI medium
98 (Gibco Cat. no. 11875) supplemented with 5% heat-inactivated human serum, 0.125% Albumax I (Gibco Cat.

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99 no. 11020), 5.94 g/L HEPES (Sigma Cat. no. H4034), 2 g/L glucose (Sigma Cat. no. G7021), 5 g/L hypoxanthine
100 (Sigma Cat. no. H9377), and 40 mg/L gentamycin sulfate at 37 ºC under atmosphere of 94% N_2 + 5% CO_2 + 1%
101 O_2.

102 Asexual blood stage synchronization was performed by sorbitol treatment. Briefly *P. falciparum* infected
103 erythrocytes were incubated with 5 volumes of 5% sorbitol then incubated at 37 ºC for 15 minutes. After
104 incubation, infected erythrocytes were harvested by centrifugation at 1,200 x *g* for 3 minutes. Pelleted
105 erythrocytes were washed with 5 mL of prewarmed RPMI medium before sub-culturing in fresh erythrocytes and
106 complete RPMI to desired hematocrit and parasitemia.

107

108 ***2.3 Construction of transgenic gametocyte-producing P. falciparum with mutations on dhfr gene***

109 The *P. falciparum* NF54 strain (MRA-1000) was used as a parental strain for transgenic parasite
110 production as it is an efficient gametocyte-producing strain (Figure 1). The pCas.SgDHFR.HR.V1S generated in
111 previous study consist of Cas9 expression cassette under a control of heat shock promotor and *Pbdhfr* terminator
112 from pUF1-Cas9 plasmid (Ghorbal et al., 2014), single-guide RNA (sgRNA) specific to *P. falciparum dhfr* (*Pfdhfr*)
113 under a control of *P. falciparum* U6 promotor and terminator from pL7 plasmid (Ghorbal et al., 2014), and
114 homology repair quadruple *dhfr* mutant from V1/S strain with mutated PAM site to prevent Cas9 cleavage in the
115 transgenic parasite. Sequence of the pCas.SgDHFR.HR.V1S was in the supplementary information 1.
116 Synchronized ring stage of NF54 strain parasite was transfected with 100 µg of pCas.SgDHFR.HR.V1S plasmid
117 as previously described (Somsak et al., 2011). The transfected parasite was then selected using 1 µM
118 pyrimethamine from day 2 for 14 days, then cloned by limiting dilution method in 96-well plates. The *dhfr* gene
119 from the cloned parasite was then amplified with PfDT Nhe Forward primer (5'
120 GATGCTAGCATGATGGAACAAGTCTGCG 3') PfDT3 Hind Reverse primer
121 (5'GCAAGCTTTTAAGCAGCCATATCCATTG 3') then sent for sequencing with PfD779-pCasR primer (5'
122 GTGACACTATAGAATACTCAAGCTTTGACATGTATCTTTGTCATC 3'). The mutations of DHFR gene were
123 confirmed by DNA sequencing. The resulting transgenic parasite was called NF54-4mut*Pfdhfr*. The schematic
124 representation of this plasmid is given in Figure 1.

125

126 ***2.4 Assessment of susceptibility of the transgenic NF54-4mutPfdhfr to antimalarial drugs in asexual***
127 ***blood stage***

128 To confirm that the introduction of DHFR mutations to the transgenic NF54-4mut*Pfdhfr* confers antifolate
129 resistance, the transgenic NF54-4mut*Pfdhfr* was tested for its susceptibility to antifolate drugs: pyrimethamine
130 and P218 using Malaria SYBR Green I-base fluorescence (MSF) assay (Johnson et al., 2007).

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131 Dihydroartemisinin (DHA) was used as an unrelated drug control. Drug susceptibility of the transgenic parasite
132 was compared with the parental NF54 and V1/S *P. falciparum* parasites. Briefly, 90 µl of 1% ring-stage
133 synchronized parasites at 2% hematocrit were transferred into 96-well flat bottom microtiter plate and treated
134 with 10 µl of serial dilution of each drug prepared in DMSO. The plates were then incubated at 37 °C under
135 atmosphere of 94% N_2 + 5% CO_2 + 1% O_2 for 48 hours followed by an addition of 100 µl of SYBR Green I dye
136 (Invitrogen, Cat. no. S7563) in lysis buffer (20 mM Tris, 5mM EDTA, 0.008% w/v saponin, 0.08% v/v Triton X-
137 100, pH 7.5). Fluorescence signal was measured with a microplate reader (excitation at 435 nm and emission at
138 535 nm). The SYBR Green I signal from drug-treated parasites were normalized to untreated (DMSO) control
139 parasite in the same experiment.
140

141 ***2.5 P. falciparum gametocyte culture***

142 *P. falciparum* gametocytes were cultured following previously published protocol with slight modifications
143 (Delves et al., 2016; Gupta et al., 1985). The culture was started with 1% parasitemia of synchronized ring stage,
144 4% hematocrit and the medium was changed daily for 16 days. To prevent reinvasion of asexual stage parasite,
145 N-acetyl-glucosamine (NAG, Sigma, Cat. No. A3286) was added to the culture to a final concentration of 50 mM
146 from day 6 to day 11 of the culture. *P. falciparum* gametocyte development was monitored using Giemsa-stained
147 thin blood smear.
148

149 ***2.6 Male gamete activation (exflagellation) assay in P. falciparum***

150 The male gamete activation assay was performed following a slight modification of an established
151 protocol (Delves et al., 2013, 2016). In brief, *in vitro* gametocyte culture was set up as described above. On day
152 13 of the culture, the gametocytes were treated with P218 or other antimalarial compounds at 37 °C for 48 hours.
153 Half of the media supplemented with antimalarial compounds was changed and then incubated for another 24
154 hours before exflagellation readout. The male gametocyte exflagellation was induced using ookinetes culture
155 medium (RPMI 1640 with 25 mM HEPES, 2 mM glutamine (Sigma, Cat no. G7513), supplemented with 100 µM
156 xanthurenic acid (Sigma, Cat. no. D120804) and observed with hemocytometer under a light-contrast
157 microscope. For each replicate, eight fields of view were recorded with Olympus video camera system (Model
158 DP71) then the total number of exflagellation sites/1,000 red blood cells was calculated then exflagellation
159 inhibition was compared to the DMSO control.
160

161 ***2.7 Statistical analyses***

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162 Dose response analyses were performed using 4-parameter log-logistic regression model using the *drc*
163 package (Ritz et al., 2015) in the R program (R Core Team, 2020).

164 ## 3. Results

165 ### 3.1 Generation of gametocyte-producing P. falciparum with mutations on DHFR gene

166 The pCas.SgDHFR.HR.V1S plasmid was successfully transfected into the gametocyte producing NF54
167 strain. The sequencing results confirmed that the nucleotide at the amino acid position 51, 59, 108, and 164 of
168 the NF54-4mut*Pfdhfr* parasite was successfully mutated (Figure 2).

169 Because the main goal of generating the transgenic NF54-4mut*Pfdhfr* was to obtain the gametocyte
170 producing parasites with quadruple mutations on the *dhfr* gene, the parasite was then checked for its ability to
171 develop into gametocyte. The NF54-4mut*Pfdhfr* gametocyte was cultured alongside with the NF54 parental and
172 V1/S strain. The result showed that the Nf54-4mut*Pfdhfr* maintain the gametocyte-producing phenotype of the
173 NF54 parental strain with the gametocytemia yield of ~4-10%, which is comparable to the wild-type NF54 strain
174 (Figure 3A). Male gametocytes of the NF54-4mut*Pfdhfr* was highly active and could be further used for
175 exflagellation assay to screen for transmission blocking antifolate compounds (Figure 3B).

176

177 ### 3.2 Asexual erythrocytic stage of the transgenic NF54-4mutPfdhfr has similar antimalarial drug
178 ### sensitivity compared to the quadruple mutant V1/S strain

179 After the transgenic parasite was validated and cloned, we then compared its asexual stage
180 antimalarial drug susceptibility to those of the NF54 and V1/S strain (Figure 4, Table 1). While the wild-type
181 NF54 strain is susceptible to PYR with IC_{50} at 126.56 nM, the quadruple mutant V1/S strain had PYR IC_{50} at
182 65.57 μM or 518-fold higher than that of the wild-type NF54. The replacement of wild-type *dhfr* with quadruple
183 mutant *dhfr* in the transgenic NF54-4mut*Pfdhfr* increased PYR IC_{50} to 87.59 μM or 692-fold increase.

184 Similar to a previous study (Yuthavong et al., 2012), our result demonstrated that P218 has better
185 asexual stage antimalarial activity against both wild-type and quadruple mutant parasites compared to PYR.
186 IC_{50} of P218 in NF54, V1/S, and NF54-4mut*Pfdhfr* were 6.15, 100.69, and 56.94 nM, respectively (Figure 4,
187 Table 1). The increase in IC_{50} correspond to 16- and 9-fold increase for the V1/S, and NF54-4mut*Pfdhfr*
188 strains. These increases are far lower than those for pyrimethamine, with ratios of 32 for V1/S and 75 for
189 NF54-4mut*Pfdhfr*, validating P218 efficacy against pyrimethamine-resistant parasites.

190 When treated with DHA, an antimalarial compound in different class, the wild-type and transgenic
191 parasites had similar IC_{50} (Figure 4, Table 1). These results demonstrated that the genetic modification
192 specifically increased antifolate resistance.

193

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194 ***3.3 P218 effectively inhibits male gametocyte exflagellation of the transgenic antifolate resistant***
195 ***parasite***
196 The exflagellation of transgenic and original NF54 strain *P. falciparum* was tested with various
197 antimalarial compounds including PYR, P218, DHA, and methylene blue (MB, an antimalarial compound with
198 confirmed exflagellation inhibition activity) (Figure 5). The exflagellation inhibition of DHA and MB were similar
199 between the NF54 and NF54-4mut*Pfdhfr* parasites suggesting that the replacement of the wild-type *dhfr* to the
200 quadruple mutant *dhfr* only had impact on the parasite's susceptibility to antifolate compounds (Figure 5, Table
201 2).
202 As expected, the quadruple *dhfr* mutation substantially increased parasite's susceptibility to previous
203 generation of antifolate compound such as PYR. PYR exflagellation IC_{50} of the NF54-4mut*Pfdhfr* parasite was
204 99.89 μM, a 3,626 fold-increase from 27.55 nM in the wild-type *dhfr* NF54 parasite (Figure 5, Table 2). In contrast,
205 exflagellation IC_{50} of P218 in the transgenic quadruple *dhfr* mutant, was 10.74 nM, a 3-fold increase from 4.06
206 nM in the wild-type (Figure 5, Table 2). Interestingly, the exflagellation IC_{50} of the antifolate compounds were
207 lower than asexual stage IC_{50} except for the PYR IC_{50} in NF54-4mut*Pfdhfr*. This suggests that male gametocyte
208 exflagellation might be more susceptible to antifolate than the asexual blood stage.
209
210 ***4. Discussion***
211 In addition to vector control, antimalarial drugs with activity against multiple stages of the parasite life
212 cycle form another key component to achieve the goal of malaria elimination and eradication. Our research group
213 developed novel, rationally-designed antifolate compounds that can inhibit asexual stage of both wild-type and
214 quadruple *dhfr* mutant *P. falciparum* at nanomolar level, with P218 as a lead compound (Yuthavong et al., 2012).
215 However, the activity of P218 against mosquito stage of antifolate resistant parasite, especially for the quadruple
216 mutant parasite, has up to now only been implied without experimental evidence. The laboratory strain of
217 quadruple mutant parasite (V1/S strain) cannot develop into gametocyte stage, thus limiting the transmission
218 blocking activity screening pipeline against quadruple *dhfr* mutant parasite.
219 To develop a model to determine transmission blocking activity of P218 and other compounds on
220 quadruple *dhfr* mutant parasite, we successfully generated transgenic NF54 strain of *P. falciparum* harboring the
221 *dhfr* gene with quadruple mutations identical to the V1/S strain. The resulting transgenic parasite showed an
222 increased resistance to antifolate drugs in asexual blood stage at a similar level to that of the V1/S parasite while
223 preserving the ability to develop into gametocytes, which can be used for male gametocyte exflagellation assay.
224 The ability of NF54 parasite to maintain gametocyte producing property even after a long transfection and cloning
225 process (up to 2-3 months from transfection until the cloned parasites were obtained) was evident in our study.

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226 This demonstrates the possibility to use this approach with other drug targets to study the effect of genetic
227 variations in drug susceptibility of extraerythrocytic stages in the future.

228 In this study male gametocyte exflagellation was used as a proxy to determine transmission blocking
229 efficiency of P218 because 1) antifolate compounds only target male but not female gametocyte activation
230 (Delves et al., 2013), 2) the exflagellation assay was performed in multi well format *in vitro* thus suitable for testing
231 multiple concentrations for dose response analysis. Our results showed that the exflagellation of male
232 gametocytes are more sensitive to antifolates when compared to the asexual blood stage, which is in
233 concordance with the previously published data of PYR treatment, which reported asexual stage IC_{50} at 17 nM
234 and exflagellation IC_{50} at 8.7 nM (Delves et al., 2013).

235 Transmission from human to mosquito is a major bottleneck for *P. falciparum* life cycle (Smith et al.,
236 2014). Previous experimental infection of *Anopheles gambiae* mosquitoes with blood *from P. falciparum*-infected
237 patients carrying high gametocytemia (an average of 433.5 gametocytes in each mosquito midgut) resulted in
238 an average of 5.5 ookinete (91.9% prevalence) on day 1, and two oocyst (37.8 % prevalence) on day 7 (Gouagna
239 et al., 1998). With this low number of ookinete and oocyst able to establish infection in the mosquito midgut, the
240 highly potent P218 should drastically prevent malaria transmission from P218-treated patients to mosquito even
241 at nanomolar plasma concentration. This transmission blocking potential remains even for pyrimethamine-
242 resistant parasites, where the exflagellation of which is still effectively inhibited.

243 Another important aspect from this study is that P218 will be very effective in preventing geographical
244 expansion of drug resistant parasites. Our previous study suggested that additional mutation on *dhfr* gene will
245 impose strong fitness cost to the parasite because the enzyme will have less catalytic activity to natural substrate
246 (Yuthavong et al., 2012). Additionally, even if the parasite gains higher resistance to P218, the higher sensitivity
247 of male gametocyte compared to the erythrocytic stage (exflagellation IC_{50} was 5.3 times lower than erythrocytic
248 IC_{50}) will pose a second stronger barrier to prevent the spread of the P218 resistant parasite from that individual
249 to other humans through mosquito. This is a very important aspect for the malaria elimination effort because the
250 compound can prevent the geographical expansion of antifolate resistant parasites.

251 In summary, this study used CRISPR-Cas9 technology to generate a transgenic gametocyte producing
252 *P. falciparum* with quadruple *dhfr* mutant replacing the wild-type *dhfr*. The parasite was then used to demonstrate
253 that P218, a novel antifolate compound, is highly active against erythrocytic and mosquito stages of *P. falciparum*
254 with quadruple mutations on *dhfr* gene. The compound is thereby an invaluable tool for malaria treatment and
255 transmission control with the goal of malaria elimination.

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259 Line E) MRA-1000 contributed by Megan G. Dowler was obtained through BEI Resources, NIAID, NIH.

260

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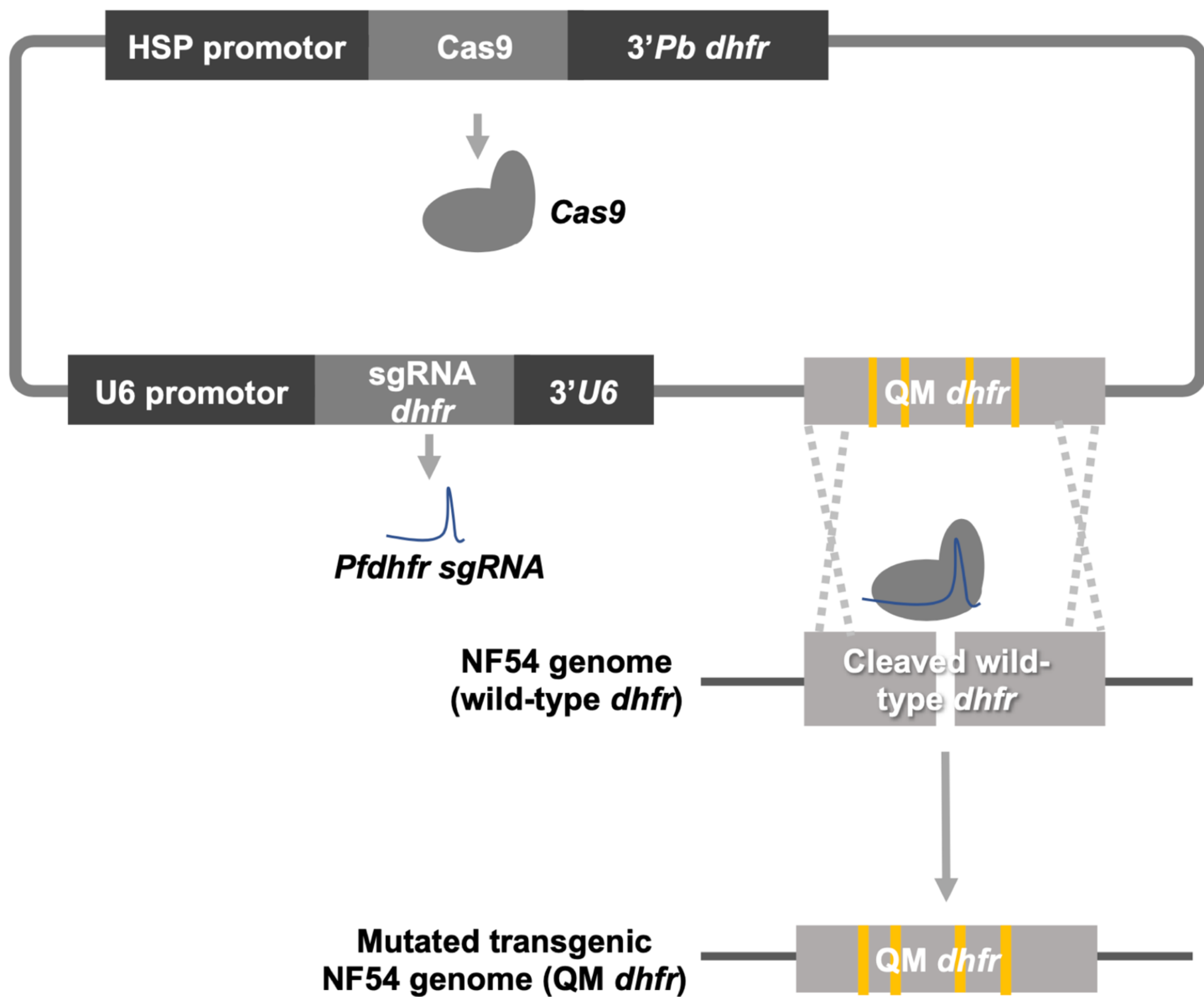

333

334 **Figure 1:** Schematic diagram of the pCas**.**SgDHFR**.**HR**.**V1S plasmid, and the integration of the homology**-**
335 directed repair cassette into the wild-type *dhfr* locus of the NF54 strain *P. falciparum* to introduce quadruple
336 mutations in the *Pfdhfr* gene**.**

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337

338 **Figure 2.** Multiple sequence alignment between the NF54 parental line and the transgenic NF54-4mut*Pfdhfr*
339 parasites**.** Introduced nucleotide mutations resulted in amino acid mutations at N51I, C59R, S108N, and
340 I164L**.** Nucleotide 249**-**258 are synonymous mutations to mutate PAM site for *dhfr* sgRNA**.**

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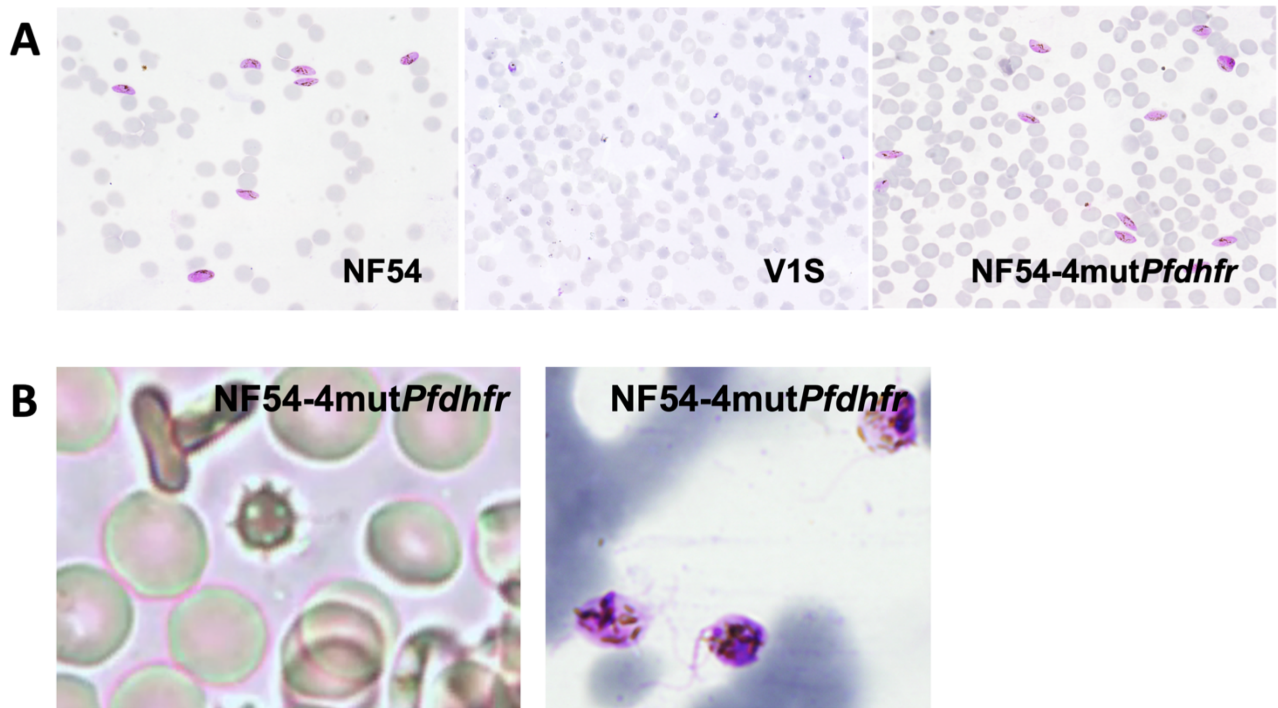

342

343 **Figure 3**. Transgenic NF54-4mutPfdhfr maintains ability to develop into active gametocytes**.** A**)** Giemsa stain

344 of thin blood smear of gametocyte culture**.** B**)** bright field **(**left**)** and Giemsa stained **(**right**)** images of activated

345 NF54-4mut*Pfdhfr* male gametocytes**.**

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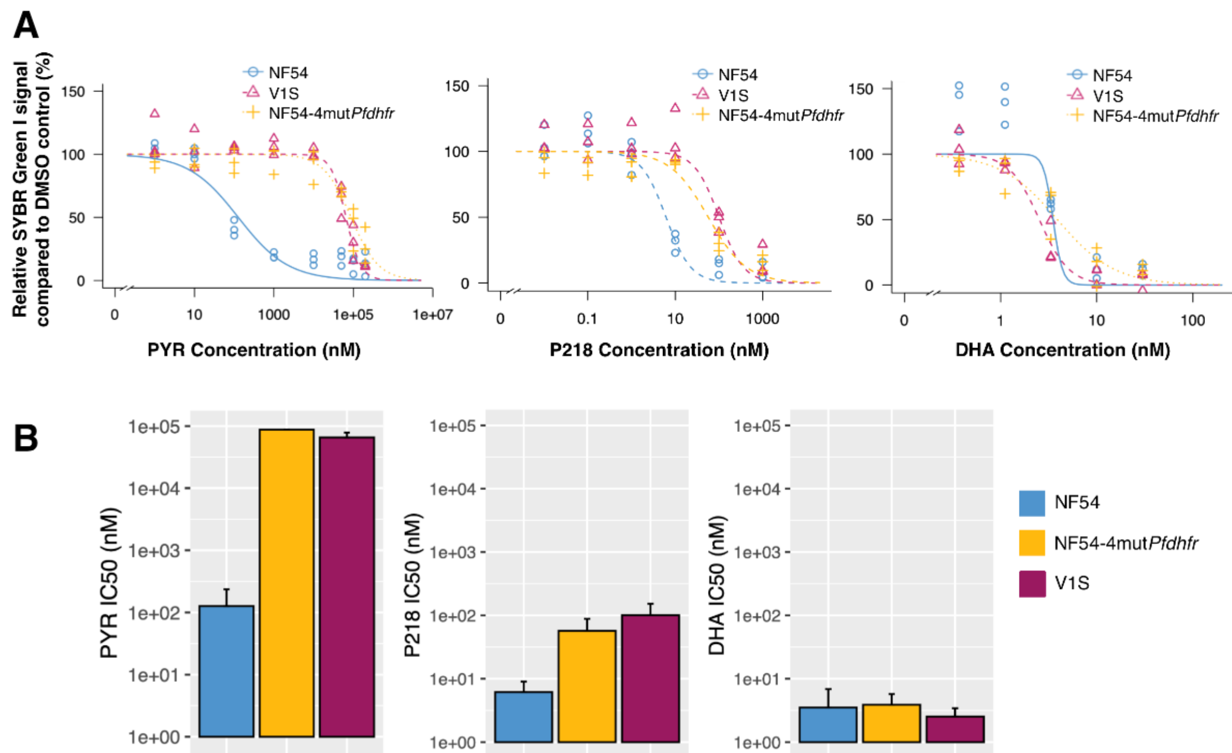

347

348 **Figure 4.** Antimalarial activity of PYR, P218, and DHA in asexual stage of NF54, NF54-4mut*Pfdhfr*, and V1/S
349 strains *P. falciparum*. A) Dose response curves of PYR, P218, and DHA. B) Bar charts representing asexual
350 stage IC_{50} values of of PYR, P218, and DHA in NF54, NF54-4mut*Pfdhfr*, and V1/S strains *P. falciparum* error
351 bars represent 95% confidence intervals (±2SD) of the IC_{50} values.

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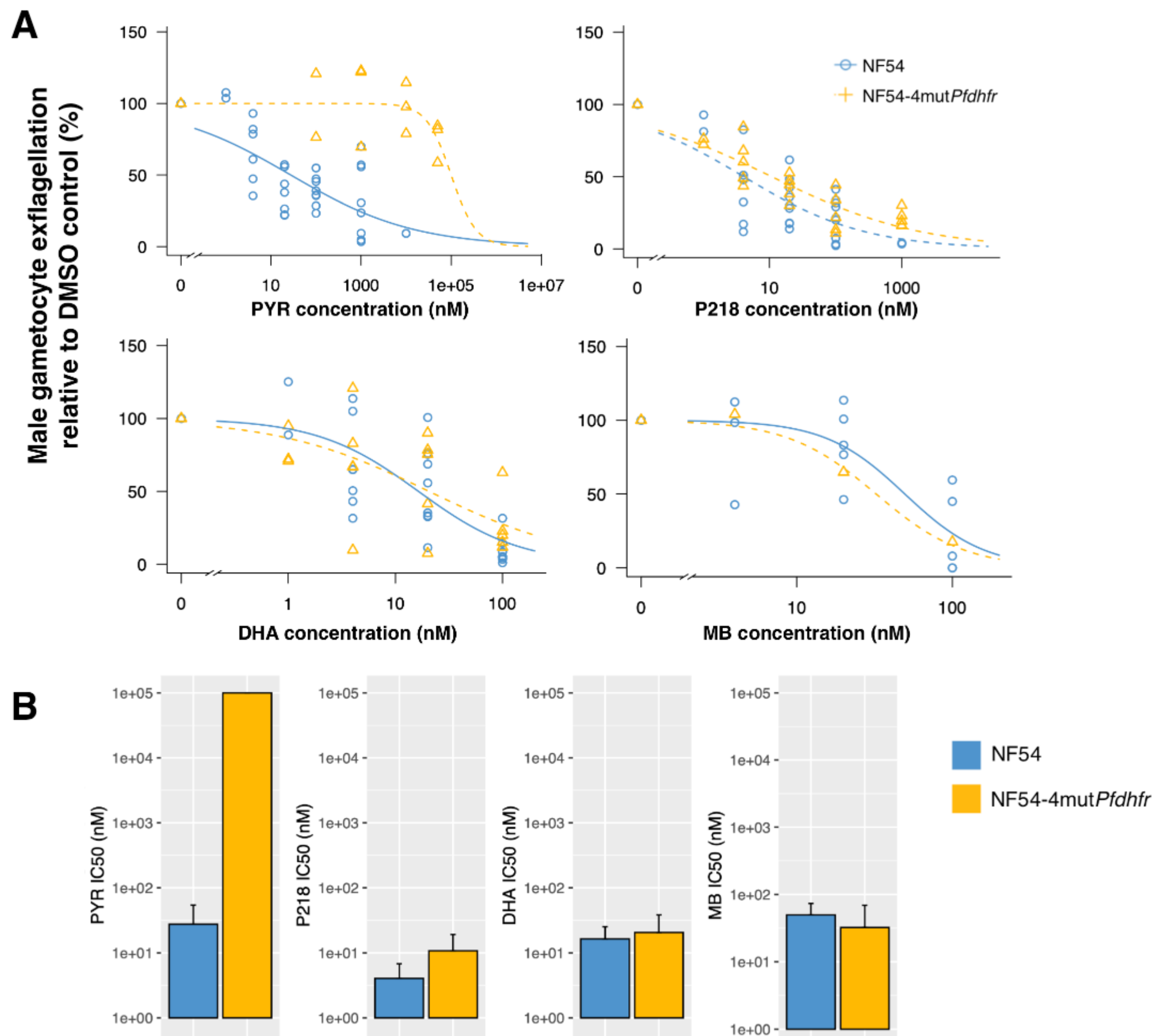

352

353 **Figure 5.** Exflagellation inhibition of PYR, P218, and DHA in NF54 and NF54-4mut*Pfdhfr* strains *P. falciparum*.

354 A) Dose response curve of PYR, P218, and DHA. B) Bar chart representing asexual stage IC_{50} values of PYR,

355 P218, and DHA in NF54 and NF54-4mut*Pfdhfr*. Error bars represent 95% confidence intervals (±2SD) of the IC_{50}

356 values.

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359 **Table 1.** Asexual blood stage IC_{50} of PYR, P218, and DHA in the NF54, NF54-4mut*Pfdhfr*, and V1/S
360 parasites

P. falciparum **strain**	**PYR (nM)**		**P218 (nM)**		**DHA (nM)**	
	Asexual blood stage IC_{50} (±SD)	**Fold of NF54 IC_{50}**	**Asexual blood stage IC_{50} (±SD)**	**Fold of NF54 IC_{50}**	**Asexual blood stage IC_{50} (±SD)**	**Fold of NF54 IC_{50}**
eNF54	126.56 (±54.90)	1	6.15 (±1.42)	1	3.52 (±1.66)	1
V1S	65573.92 (±6499.00)	518.13	100.69 (± 26.07)	16.37	2.53 (±0.44)	0.72
NF54-4mut*Pfdhfr*	87,590.94 (±12,430.96)	692.09	56.94 (± 15.69)	9.26	3.89 (±0.90)	1.11

361

362 **Table 2.** Exflagellation IC_{50} of PYR, P218, and DHA in the NF54 and NF54-4mut*Pfdhfr* parasites

P. falciparum **strain**	**PYR (nM)**		**P218 (nM)**		**DHA (nM)**		**MB (nM)**	
	Exflagellation IC_{50} (±SD)	**Fold of NF54 IC_{50}**	**Exflagellation IC_{50} (±SD)**	**Fold of NF54 IC_{50}**	**Exflagellation IC_{50} (±SD)**	**Fold of NF54 IC_{50}**	**Exflagellation IC_{50} (±SD)**	**Fold of NF54 IC_{50}**
NF54	27.55 (±13.24)	1	4.06 (±1.36)	1	16.39 (±4.43)	1	49.60 (±11.56)	1
NF54-4mut*Pfdhfr*	99,885 (±120,920)	3625.59	10.74 (± 4.22)	2.65	20.56 (±8.84)	1.25	32.42 (±17.74)	0.65

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365 **Authors' Contributions**

366 **Navaporn Posayapisit:** Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft,
367 Writing - Review & Editing
368 **Jutharat Pengon:** Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing -
369 Review & Editing
370 **Parichat Prommana:** Methodology, Validation, Formal analysis, Investigation, Writing - Review & Editing
371 **Molnipha Shoram:** Investigation
372 **Yongyuth Yuthavong:** Resources, Writing - Review & Editing
373 **Chairat Uthaipibull:** Methodology, Resources, Funding acquisition
374 **Sumalee Kamchonwongpaisan:** Methodology, Resources, Writing - Review & Editing, Funding acquisition
375 **Natapong Jupatanakul:** Conceptualization, Methodology, Validation, Formal analysis, Investigation,
376 Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project
377 administration, Funding acquisition
378

379 **Declaration of Conflicting Interests**

380 The generated transgenic parasite has been filed for a Thai petty patent.
381