Full title: Efficacy of Lumefantrine against piperaquine resistant *Plasmodium berghei* parasites is selectively restored by probenecid, verapamil, and cyproheptadine through ferredoxin $NADP^+$-reductase and cysteine desulfurase

Short title: Mechanisms of Lumefantrine resistance and reversal in *Plasmodium berghei* ANKA

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Abstract

The ability of the human malaria parasite, *Plasmodium falciparum* to develop resistance against mainstay drugs remains a public health problem. Currently, the antimalarial drugs, lumefantrine (LM), and piperaquine (PQ) are essential components of the mainstay artemisinin-based therapies used for the treatment of malaria globally. Here, we used a model parasite *Plasmodium berghei,* to investigate the mechanisms of LM and PQ resistance. We employed known resistance reversing agents (RA): probenecid, verapamil, or cyproheptadine to study the mechanisms of LM and PQ resistance in the standard 4-day suppressive test. We then employed reverse genetics to assess the impact of deleting or over-expressing plausible genes associated with the metabolism and transport of drugs. We show that only, cyproheptadine at 5mgkg^{-1} restored LM activity by above 65% against LM-resistant parasites (LM^{r}) but failed to reinstate PQ activity against PQ-resistant parasites (PQ^{r}). Whereas the PQ^{r} had lost significant susceptibility to LM, the three RA, cyproheptadine verapamil, and probenecid restored LM potency by above 70%, 60%, and 55% respectively against the PQ^{r}. We thus focused on the mechanisms of LM resistance in PQ^{r}. Here we show the partial deletion of the cysteine desulfurase (SUFS) and overexpression of the Ferredoxin NADP+ reductase (FNR) genes in the PQ^{r} parasite achieved two results; i) abolished the impact of RA on LM activity; ii) restored the susceptibility of PQ^{r} to LM alone. Our findings associated SUFS and FNR protein with the action of LM and RA action in *P. berghei.* We demonstrate that the incorporation of any of the RA into an antimalarial combination that comprises LM would augment LM activity and concomitantly antagonize the emergence of LM resistance derived from PQ pressure. The impact of RA, deletion of SUFS, and overexpression of FNR on LM activity need to be tested in *Plasmodium falciparum*.

Keywords: Malaria; Lumefantrine, Resistance; Reversal; *Plasmodium berghei*; Ferredoxin NADP+ reductase, cysteine desulfurase

Author summary

Lumefantrine (LM) and piperaquine (PQ) are essential drugs for the treatment of malaria globally. Here, we used *Plasmodium berghei,* a model parasite that infects rodents to study how parasites escape killing by PQ and LM. We first used a second drug: probenecid, verapamil, or cyproheptadine to enhance the activity of LM or PQ. We show that cyproheptadine restores LM activity against LM-resistant parasites (LM^r) but failed to reestablish PQ activity against PQ-resistant parasites (PQ^r). Since PQ^r is resistant to LM, combining LM with either cyproheptadine, verapamil, or probenecid reinstates LM activity against PQ^r. We then focused mainly on LM resistance in PQ^r. After genetically manipulating the PQ^r, we reveal that cysteine desulfurase (SUFS) and ferredoxin NADP+ reductase (FNR) regulate LM capacity to kill parasites. Decreasing the level of SUFS or increasing FNR levels in the PQ^r makes the parasites susceptible to LM but abolishes the impact of probenecid, verapamil, and cyproheptadine on LM activity. Overall, we provide clues on the link between SUFS and FNR in the action of LM and RA in *P. berghei.* This study provides a basis for an in-depth analysis of how LM mediates parasites kill and how the parasite escapes LM action in *Plasmodium falciparum*.

60 **Introduction**

61 Malaria disease affects more than one billion people worldwide (1). Presently, five malaria
62 parasites species infect humans. *Plasmodium falciparum* remains a significant contributor to the
63 global disease burden, with an estimated 200 million cases and 500 000 deaths annually (1). An
64 enormous proportion of this burden affects children under five years of age and pregnant women.
65 In Kenya, over 70% of the population is still at risk of infection by malaria parasites (2). To date,
66 the use of drugs is central to the control and management of malaria. However, this approach is
67 hampered by the ability of the parasite to develop resistance against antimalarial drugs rapidly.
68 Currently, the artemisinin-based combination therapies (ACTs) are the mainstay drugs for the
69 treatment and management of uncomplicated *P. falciparum* malaria. The ACTs comprise a short-
70 acting artemisinin derivative and a long-acting partner drug (2). The long-acting partner drugs
71 reduce the remaining parasite biomass after artemisinin clearance and simultaneously protect
72 against reinfection, especially in high transmission settings (3). Thus, the long-acting drug
73 components within the ACTs are of primary importance in the control of subsequent malaria
74 infection in sub-Saharan Africa. Over the last two decades, extensive use of ACTs has correlated
75 with the reduction of mortality associated with malaria worldwide (1). Despite the widespread use
76 of the ACTs, in which the partner drugs are predicted to act on different molecular targets of the
77 parasite, *P. falciparum* has consistently evolved complex resistance mechanisms that have
78 conferred resistance or reduced efficacy to all mainstay antimalarial drugs meant for the treatment
79 and management of malaria (4,5). The emergence of resistance to the ACTs in South-East Asia
80 (6,7) demonstrates the growing need not only to understand the mechanisms of resistance to the
81 long-acting partner drugs but also the importance of seeking alternative approaches to circumvent
82 the drug resistance

83 In many African countries, the long-acting antimalarial drugs: lumefantrine (LM) and
84 piperaquine (PQ) are essential components in the mainstay ACTs; Coartem™ and Artekin™,
85 respectively (8). Unfortunately, the high transmission of malaria in endemic regions coupled with
86 the long half-life portend intense selection pressure (9), a recipe for the rapid emergence of LM
87 and PQ resistant parasites. It is imperative to understand how parasites may evade LM and PQ
88 action. Polymorphisms in two genes, primarily mediate resistance to the 4-aminoquinolines and
89 chemically related drugs such as PQ. The *chloroquine resistance transporter (crt)*, the actual
90 determinant of CQ resistance in *P. falciparum,* which can carry Lys76Thr mutation (10) and
91 *multidrug-resistant 1 (mdr1)* gene that encodes a P-glycoprotein homolog 1 (*Pgh-1*) which
92 modulate resistance to CQ and other quinolines drugs such as AQ and PQ (11,12). In recent
93 studies, Cys101Phe mutation in *chloroquine resistance transporter* (*crt*) conferred resistance to
94 both PQ and CQ in *P. falciparum in vitro* (13). Also, using field isolates, a nonsynonymous
95 mutation, Glu415Gly in the exonuclease (PF3D7_1362500), and amplification of Plasmepsin II
96 and III, proteases involved in the heme degradation within the digestive vacuole are linked with
97 PQ resistance (4). LM, on the other hand, is an aryl alcohol antimalarial drug, chemically related
98 to mefloquine and quinine. Although, like the 4-aminoquinoline, LM is predicted to inhibit heme
99 detoxification, several studies have associated reciprocal resistance between CQ and LM (14),
100 suggesting potentially different mechanisms of resistance and action. To date, many questions
101 regarding how LM exerts its antimalarial action and how the parasites may evolve to evade such
102 action remain unanswered.

103 A recent analysis of *P. falciparum* from The Gambia has associated LM resistance with
104 cysteine desulfurase (15), an enzyme involved in Fe-S biogenesis within the apicoplast. In other
105 studies, V-type H+ pumping pyrophosphatase 2 (*vp2*) and Ca^{2+}/H^{+} antiporter (*vcx1*), putative drug

106 transporter genes have been associated with reducing the biological costs of acquiring the mutation
107 in both CQ and LM resistance (16,17). Our recent investigation identified for the first time the
108 association of *vp2* with PQ resistance and gamma glutamyl-cysteine synthetase (*ggcs*),
109 glutathione-S transferase (*gst)*, and *vcx1* increased transcript levels with PQ and LM resistance in
110 *P. berghei* ANKA (18). These studies suggest a complex multi-gene association of PQ and LM
111 resistance.

112 With only a few promising chemical compounds in the malaria drug discovery pipeline
113 (19–21), there is a need for alternative approaches to antagonize the emergence of resistance and
114 extend the therapeutic lifespan of the currently available drugs. One archetypical method of
115 studying the mechanisms by which parasites evade drug action and simultaneously generate new
116 drugs is the use of chemosensitizer (a drug with the ability to enhance/restore activity) in
117 association with antimalarial drugs for which the parasite is resistant (22). This approach has been
118 utilized previously to generate prophylactic drugs against malaria parasites (23,24). The
119 chemosensitizers have no intrinsic antimalarial potency but reverse resistance mechanisms by
120 either enhancing drug uptake or inhibiting the efflux of drugs from the target site (25–27). Three
121 chemosensitizers have been used extensively to study the resistance mechanisms and design next-
122 generation drugs in both malaria and cancer (28–31). In malaria, verapamil, calcium (Ca^{+2})
123 channel blocker partially reverses CQ resistance in *P. falciparum in vitro* and clinical isolates via
124 increasing net CQ within the infected erythrocyte parasites (32). Probenecid, anion transporter
125 inhibitor, reverses methotrexate resistance in cancer (33), CQ, and antifolates resistance in *P.*
126 *falciparum* (24). Antihistamine compounds such as cyproheptadine and chlorpheniramine restore
127 CQ sensitivity in CQ resistant *P. yoelii* and *P. falciparum in vitro* (34,35).

128 Using model malaria parasite, *Plasmodium berghei* ANKA as a surrogate of the human
129 malaria parasite *P. falciparum*; we previously selected PQ- and LM-resistant parasites. Further
130 analysis of the PQ-resistant parasites showed that the parasite had lost significant susceptibility to
131 LM, AQ, primaquine (PMQ), and dihydroartemisinin (DHA). On the other hand, although LM-
132 resistant parasites retained sensitivity to PQ, the parasites lost significant susceptibility to DHA,
133 PMQ, and CQ (36,37). Also, the resistant parasites acquired significant growth fitness cost
134 probably associated with the changes within the genomes that affect the essential gene necessary
135 for the growth of the asexual blood-stage parasites (38). Here we designed to study the molecular
136 mechanisms of PQ and LM resistance and reversal in *P. berghei* ANKA. We utilized known
137 resistance reversing agents (RA): probenecid, verapamil, or cyproheptadine to restore the
138 susceptibility of the resistant parasite to LM or PQ. Using the resistant lines and drug-sensitive
139 parasite as a reference, we evaluated the differential expression of a panel of plausible genes
140 associated with the metabolism and transport of quinoline and aryl alcohol drugs. Among the gene
141 evaluated are; *crt, mdr1, multidrug resistance-associated protein 2 (mrp2), pantothenate kinase*
142 *(pank), ferredoxin NADP*$^{+}$*-reductase (fnr), cysteine desulfurase (sufs), Plasmepsin IV (pmiv),*
143 *Plasmepsin IX (pmix), Plasmepsin X (pmx) or phosphatidylinositol 3-kinase (pi3k).* We employed
144 a reverse genetics approach, using highly efficient gene knockout and overexpression,
145 *PlasmoGEM* vectors for *P. berghei*, we partially deleted and over-expressed SUFS and FNR
146 respectively, then measured the impact on drug susceptibility and reversal of resistance. Finally,
147 we used *in silico* approaches to predict binding profiles of the SUFS and FNR with the antimalarial
148 drug; LM and the reversing agents.

149 Here we describe a potential novel combination of therapy between LM and
150 cyproheptadine that antagonizes LM resistance in *P. berghei* ANKA. As highlighted, the selection

151 of PQ resistance is accompanied by a significant loss in LM activity (18,36). We report two critical
152 aspects regarding LM cross-resistance in PQ-resistant *P. berghei* ANKA; First, using LM and
153 cyproheptadine, verapamil, or probenecid, we highlight new combination therapies that restore
154 LM activity against the PQ-resistant parasites. Second, using reverse genetics, we provide
155 evidence that mRNA alteration of SUFS and FNR genes in the PQ-resistant parasites restores the
156 activity of LM. The mRNA alteration also abolishes the action of RA on LM activity, suggesting
157 that the reversal of LM activity is via SUFS and FNR proteins. We provide the essential basis for
158 the design and synthesis of novel inhibitors for FNR and SUFS able also to circumvent LM
159 resistance in PQ resistant parasites.

160 **Results**

161 **Cyproheptadine selectively restores LM activity against lumefantrine and piperaquine**
162 **resistant parasites**

163 We first determined the activity profile of cyproheptadine alone against PQ^s, PQ^r, LM^s, and
164 LM^r parasites to select the appropriate dosage for combination with either LM or PQ. Interestingly,
165 30mg/kg of cyproheptadine killed above 90% and 40% of the LM-resistant and PQ resistance
166 parasites, respectively (Figure 1). However, at a lower concentration of 5mg/kg, cyproheptadine
167 had no significant activity against either sensitive or resistant parasites. On the other hand,
168 100mg/kg of LM or 75mg/kg of PQ could not suppress parasite growth above 20% in LM^r and
169 PQ^r phenotypes, respectively. We, therefore, selected 5mg/kg or 2.5mg/kg of cyproheptadine for
170 combination with 100mg/kg of LM or 75mg/kg of PQ. Using a combination of 5mg/kg of
171 cyproheptadine and 100mg/kg of LM, the activity of LM was significantly restored from 18% to
172 70% ($p<0.001$) (Figure 2A). Encouragingly, four of the five mice treated with this combination
173 had no patent parasitemia 28 days post-infection, suggesting that the combination continued to
174 suppress the parasite growth beyond the day four post parasite infection. We thus, associate this
175 combination with a 60-80% protection range. We then reasoned that the activity recorded in a
176 combination of LM (100mg/kg) and cyproheptadine (5mg/kg) was an additive effect of both drugs.
177 We assayed cyproheptadine and LM at lower dosages of 2.5mg/kg and 25mg/kg, respectively;
178 these concentrations, when administered alone, had recorded zero (0%) parasite killing (Figure
179 2B). The combination yielded 66%, a significant parasite killing ($p<0.001$) suggesting a lack of
180 additive effect. We then hypothesized that the restoration of LM activity was dose-dependent. To
181 dissect this hypothesis, cyproheptadine at 2.5mg/kg and 5mg/kg with 25 or 50mg/kg of LM was
182 evaluated. The chemo-sensitization power in the different combinations was not significantly

183 different ($p<0.08$) (Figure 2B), signifying that the restoration of LM activity was not dose-
184 dependent. To evaluate the selectivity of cyproheptadine on the resistant parasites, 2.5 and 5mg/kg
185 of cyproheptadine was combined with 0.375mg/kg of LM against the sensitive parasites (Table
186 1b). Lumefantrine at 0.375mg/kg against sensitive drug parasite was below 20% parasite
187 suppression (Table 1b), thus no significant activity. Neither of the cyproheptadine concentration
188 increased the susceptibility of the sensitive parasites to LM ($p<0.09$), meaning that the potentiation
189 was selective only in the resistant parasites. We were, however, surprised that 5mg/kg of
190 cyproheptadine failed to potentiate 75mg/kg of PQ against PQ^r (Table 2). As mentioned earlier,
191 PQ^r parasites are also highly resistant to LM (36). We, therefore, sought to investigate whether
192 cyproheptadine would restore LM activity against PQ^r. As expected, 50mg/kg of LM alone had no
193 measurable antimalarial activity. Surprisingly, LM activity was re-established by 5mg/kg of
194 cyproheptadine to above 70% ($p<0.001$) (Figure 3). From these results, the study concluded that
195 cyproheptadine selectively reverses LM resistance in LM^r parasites but does not restore PQ activity
196 against PQ^r phenotypes. However, it selectively restores LM activity against PQ^r parasites.

197 **Probenecid reinstates the activity of LM against the piperaquine resistant but not against**
198 **the lumefantrine resistant parasites**

199 By screening the effect of probenecid on the growth of the asexual blood-stage parasites,
200 we found that 400mg/kg of probenecid had no significant antimalarial effect on LM and PQ
201 resistant parasites ($p<0.07$) (Table 1a). A combination of 400mg/kg of probenecid and 100mg/kg
202 of LM or 75 mg/kg of PQ against LM^r and PQ^r parasites respectively failed to restore the activity
203 of either LM or PQ (Figure 2A; Table 2). We then sought to evaluate whether probenecid could
204 restore LM activity against PQ^r parasites. To our surprise, probenecid at 400mg/kg reinstated the
205 activity of LM (50mg/kg) against PQ^r parasites to above 60% ($p<0.0001$) (Figure 3).

206 **Verapamil re-establishes LM activity against the piperaquine resistant but not against**
207 **lumefantrine resistant parasites**

208 We next queried the effect of verapamil on PQ^r and LM^r parasites. 50mg/kg of verapamil
209 lacked any antimalarial activity against both resistant parasites (Table 1a). Similarly, a
210 combination of verapamil at 50mg/kg and 100mg/kg of LM or 75 mg/kg of PQ against LM^r and
211 PQ^r parasites respectively failed to enhance the activity of either drug (Figure 2A; Table 2). In a
212 peculiar but familiar trend with other reversing agents, 50mg/kg of verapamil significantly re-
213 established the activity of LM (50mg/kg) against PQ^r parasites to above 55% ($p<0.0001$) (Figure
214 3).

215 **Essential drugs transporters and enzymes are differentially expressed between drug-**
216 **resistant and sensitive parasites**

217 To dissect the possible molecular mechanisms of PQ and LM resistance and reversal, we
218 hypothesized that major transporters or enzymes involved in drug transport and metabolism could
219 mediate LM and PQ resistance through altered mRNA amounts; these genes are; *crt, mdr1, mrp2,*
220 *pank, fnr, pmiv, pmix, pmx, sufs, and pi3k*. Only one gene, the *pmiv,* had high mRNA levels with
221 a 1.78-fold ($p<0.001$) in LM^r parasites (Figure 4B). In PQ^r parasites, two genes, *sufs,* and *pmix* had
222 0.67-fold ($p<0.0001$) and 0.36-fold ($p<0.0001$) increase in the mRNA amount (Figure 5B and 5C).
223 Overall, we recorded a low expression of mRNA in *crt, mdr1, mrp2, pank, fnr, pmx,* and *pi3k*
224 genes in both LM^r and PQ^r parasites.

225 **Transfection of PQ^r parasites with SUFS knockout and FNR over-expression vectors yielded**
226 **transgenic parasites**

227 To gain insight into whether reduced expression of the FNR or increased expression of the
228 SUFS reduced the susceptibility of PQ^r parasites to LM or the two genes associated with restoring

229 the activity of LM potency against the PQ^r parasites, two transgenic parasite lines; one with a
230 deleted SUFS gene (PQ^R_SUFS_KD) and the other with an overexpressed FNR gene
231 (PQ^R_FNR_OE) were generated (Figure 6). For the FNR transfection assays, the three mice
232 infected with the transfected schizont and treated with pyrimethamine from day one (D1) post-
233 infection onwards had an average parasitemia of above 5% on day seven post parasite infection.
234 For SUFS, all the three mice yielded parasitemia of between 6-8% on day six post parasite
235 infection. After isolation of parasitic DNA, we confirmed the genotype of the SUFS knockdown
236 (PQ^r_SUFS_KD) and FNR over-expression (PQ^r_FNR_OE) parasites by PCR amplification using
237 three sets of primers; QCR2/GW2, GT/GW1, and QCR1/QCR2. As expected, using the vector-
238 specific QCR2 and the standard GW2 primers, we obtained a fragment of 0.9kb, and 1.5kb,
239 respectively, in PQ^r_SUFS_KD and PQ^r_FNR_OE parasites (Figure 6). PCR amplification
240 analysis using vector-specific quality control primers, the QCR1, and QCR2 amplified in both PQ^r
241 parasites and PQ^r_FNR_OE parasites as expected. The QCR1/QCR2 quality control primers also
242 amplified in both PQ^r and PQ^r_SUFS_KD parasites showing that although the vector used in
243 deleting SUFS gene is a knock out vector (39), the vector does not cover the entire gene thus a
244 possibility of generating a knockdown transgenic parasite. The mRNA expression was, however,
245 measured to assess the partial disruption of the SUFS gene in PQ^r_SUFS_KD parasites resulted in
246 reduced expression of the SUFS mRNA amounts, as shown in section 3.6. To confirm integrated
247 into the correct chromosome and position, we utilized the standard primers GW1 and the vector-
248 specific primers GT, and as expected, we obtained a PCR product of 1.9kb and 3.9kb for
249 PQ^r_SUFS_KD and PQ^r_FNR_OE parasites, respectively (Figure 6).

250 **Disruption of the SUFS and FNR genes' expression confirms the successful generation of the**
251 **PQ^r_FNR_OE and PQ^r_SUFS_KD transgenic lines.**

252 To confirm the partial deletion and overexpression of the SUFS and FNR, respectively, we
253 quantified the mRNA amount in PQ^r_SUFS_OE and PQ^r_FNR_OE relative to the PQ^r parasites.
254 The results indicated that the mRNA amount of the FNR gene in the PQ^r_FNR_OE parasites
255 increased by 5-fold ($p<0.0001$). The mRNA amount of SUFS in the PQ^r_SUFS_KD parasites was
256 2-fold less as compared to the PQ^r, suggesting downregulation of the SUFS gene ($p<0.0001$)
257 (Figure 7). Overall, these results affirmed the successful generation of the transgenic parasites.
258 The transgenic parasites were then utilized to measure the impact of deleting SUFS or
259 overexpressing FNR genes on drug responses relative to the PQ^r and sensitive parasites.

260 **The alteration of SUFS and FNR genes results in significant attenuation of the growth rate**
261 **of the asexual blood-stage parasites.**

262 Here we hypothesized that alteration of a gene in parasites might concomitantly alter the
263 growth rates of the asexual blood-stage parasites. To evaluate this hypothesis, we assessed the
264 growth rates of the transgenic parasites. As expected, the drug-sensitive wildtype parasite grew
265 normally with parasitemia reaching an average of 15% on Day four post parasite inoculation (D4
266 PI). Similarly, the PQ^r parasite had an average parasitemia of 13% on D4 PI. However, the growth
267 rate of the transgenic parasite was significantly low, for instance, PQ^R_FNR_OE and
268 PQ^R_SUFS_KD yielded an average parasitemia of 8% and 10% respectively suggesting a fitness
269 cost on the growth of the asexual blood-stage parasites (Figure 8). After quantifying the growth
270 fitness cost, the study revealed that alteration of FNR and SUFS significantly reduces normal
271 parasite growth rate phenotype by 47% and 33%, respectively.

272 **The PQ^r_SUFS_KD and the PQ^r_FNR_OE lines gained significant susceptibility to LM**
273 **alone or in combination with the RA.**

274 To query the potential role of SUFS and FNR in mediating LM and RA activity, we
275 evaluated the activity of LM alone or in combination with the RA against the transgenic and PQ^r
276 parasites Surprisingly and interestingly, the LM alone at 50mg/kg killed above 64% and 67% of
277 the PQ^r_FNR_OE and PQ^r_SUFS_KD parasites respectively as compared to 0% parasite reduction
278 in the PQ^r parasites (Figures 9-10). A combination of LM at 50 mg/kg with either of the RA
279 eliminated between 53% and 73% of the PQ^r_FNR_OE parasites. On the other hand, LM at
280 50mg/kg, in combination with any of the RA significantly reduced the PQ^r_SUFS_KD parasites
281 by above 73%. As expected, similar combinations against PQ^r yielded percentage drug killing of
282 above 61%, while 5mg/kg of LM yielded 100% activity against the parent sensitive parasites
283 (PQ^S). Overall, these results indicate that the partial deletion of SUFS or overexpression of FNR
284 in PQ^r parasites restores parasite susceptibility to LM; however, this alteration seems to abolish
285 the action of RA on LM activity in the transgenic parasites.

286 **Lumefantrine and cyproheptadine exhibited higher binding affinities for both FNR and**
287 **SUFS proteins than verapamil or probenecid.**

288 We used *in silico* approaches to confirm *in vivo* drug profiles results. For all the structures
289 modeled, the Z-scores from the PROSA-web server that indicates the degree of correctness showed
290 scores of -8.49 and -8.67 for FNR and SUFS, respectively (Table 3). The results of the Z-scores
291 obtained imply that the modeled protein structures were within the range of scores typical of the
292 experimentally defined proteins. As expected, LM yielded high but equal binding affinities of -
293 7.9kcal/mol on both SUFS and FNR., indicating a better binding between LM and the proteins.
294 Surprisingly, cyproheptadine showed the highest binding affinity to SUFS (-9.5kcal/mol). The
295 lowest binding affinity of -6.3 kcal/mol was between FNR and verapamil (Figure 11).

296 **Discussion**

297 Using both traditional and high-end technologies, we studied the mechanisms of LM and
298 PQ resistance in *P. berghei*. We provide evidence that cyproheptadine, verapamil, or probenecid
299 selectively restore LM activity against PQ^r parasites through the ferredoxin $NADP^+$-reductase and
300 cysteine desulfurase. At 10mg/kg, cyproheptadine appears to have two functions; first, and as
301 expected, is the parasite killing ability against both the LM^r and the sensitive parasites, possibly
302 by inhibition of heme polymerization as previously elaborated (34). Second, cyproheptadine
303 enhances parasite kill by selectively restoring LM activity against both the LM^r and PQ^r parasites.
304 Inhibition of heme polymerization is the predicted mechanism of action for PQ, and therefore, the
305 low susceptibility of the PQ^r parasites to cyproheptadine compared to the LM^r and the sensitive
306 parasites suggest that PQ and cyproheptadine also share common resistance mechanisms. The fact
307 that cyproheptadine failed to restore PQ activity against PQ^r parasites suggests that the PQ^r
308 parasites possess at least two types of resistance mechanisms, one which code for PQ resistance
309 and is cyproheptadine insensitive and second, which mediates LM resistance and is cyproheptadine
310 sensitive. It is, however, not clear whether the genes associated with cyproheptadine modifiable
311 mechanisms in both LM^r and PQ^r are similar or different. A lower dose of 5mg/kg had only one
312 biological function of restoring LM activity against both LM^r and PQ^r parasites.

313 Resistance to quinoline antimalarials is associated with acidification of the digestive
314 vacuole (DV) (22). Verapamil mediates reversal by replacement of positive charge and
315 normalization of pH, blocking efflux of quinoline drugs from digestive vacuole (DV) via the
316 mutated PfCRT protein (40). The mechanism of action of PQ and LM is predicted to be similar to
317 that of standard quinoline; chloroquine (41). Thus, these drugs may share some resistance
318 mechanisms. However, *PbCRT* in PQ^r or LM^r parasites were found not to carry mutations (18).
319 We have confirmed that resistance mechanisms mediating the loss of LM activity against the PQ^r

320 parasites are verapamil sensitive, but the loss of LM activity against LM^r parasites are independent
321 of verapamil action suggesting a complex emergence of LM resistance. We provide clues that the
322 reversal of LM resistance in PQ^r suggests that verapamil may bind to other protein(s) apart from
323 the traditional CRT protein to restore and enhance LM activity in PQ^r parasites. Currently, LM
324 and PQ are used in respective ACTs combination therapies in many African countries (42). We
325 show that verapamil selectively restored LM activity against PQ^r but not in LM^r parasites. If
326 mechanisms of resistance to LM are similar in *P. berghei* and *P. falciparum*, then the emergence
327 of LM resistance may proceed through two possible mechanisms; first is through the indirect PQ
328 selection pressure via the PQ/DHA combination. The second mechanisms are by the direct LM
329 pressure via the LM/ATM combination. The fact that the deletion of the SUFS gene and
330 overexpression of FNR abolished the impact of verapamil on LM activity against PQ^r parasites
331 suggest a potential interaction between SUFS and FNR proteins with both verapamil and LM.
332 However, the molecular mechanisms of how verapamil enhances LM activity via SUFS or FNR
333 is not understood and requires further studies.

334 We also show organic anion inhibitors, probenecid chemo-sensitizes LM against PQ^r
335 parasites. Through a direct or indirect action on the *Pfcrt* gene associated with CQ resistance in *P.*
336 *falciparum*, probenecid selectively sensitizes chloroquine-resistant isolates by increasing the level
337 of chloroquine accumulation *in vitro* (24). Similarly, probenecid unselectively potentiates the
338 activities of antifolates agents against both antifolates resistant and sensitive *P. falciparum* (24).
339 Reversal of antifolates resistance is associated with inhibition of multi-resistance-associated
340 proteins (43) and inhibition of endogenous folate transport (24). Multi-resistance associated
341 proteins are implicated in resistance to quinoline antimalarial drugs in both *P. falciparum* and *P.*
342 *berghei* (44). We have previously confirmed PQ^r parasites as multidrug-resistant parasites; thus,

343 probenecid mediated inhibition of multi-resistance proteins in re-establishing LM activity against
344 PQ^r parasites is a possible alternative. Indeed, we confirm that altering the expression of SUFS
345 and FNR, two proteins linked with different parasite pathway blocks the impact of probenecid on
346 LM activity against the PQ^r parasites.

347 The next generation of antimalarial drugs has envisioned the incorporation of three drugs
348 comprising of two long-acting drugs and a short-acting artemisinin drug (45). Currently,
349 cyproheptadine, an H-1 antagonist, at 4mg/kg, is recommended for the treatment of allergic
350 reactions and improvement of appetite (www.medscape.com). We have shown that 2.5mg/kg of
351 cyproheptadine is a potent modulator of lumefantrine resistance in both PQ^r and LM^r parasites, at
352 least in *P. berghei*. In humans, the recommended dosage for verapamil and probenecid is 80mg/kg
353 and 500mg/kg, respectively (www.medscape.com). Here, we have used a lower dosage to restore
354 the activity of LM against PQ^r parasites. Taken together, we demonstrate that incorporation of
355 cyproheptadine into antimalarial combination therapy that comprises LM would achieve two aims;
356 first is to augment LM activity and possibly delay the emergence of LM resistance and; second,
357 an optimized cyproheptadine concentration would offer an added advantage in the combination by
358 exerting extra antimalarial. The data also confirms the existence of at least more than one controls
359 channels for PQ resistance, one associated with LM resistance and is verapamil, probenecid, or
360 cyproheptadine inhibitable channel only in the presence of LM and the other linked with PQ
361 resistance which is verapamil, probenecid or cyproheptadine insensitive channel. The selective
362 potentiation of LM activity against PQ resistance by probenecid, verapamil, and cyproheptadine
363 is appealing since PQ^r parasites are resistant to CQ, dihydroartemisinin (DHA), and amodiaquine
364 (AQ) (36). Therefore, incorporation of any of the reversing agent into an antimalarial combination

365 therapy that comprises LM as one of the components would concomitantly antagonize the
366 emergence of LM resistance derived from PQ selection pressure.

367 In the malaria parasite, drug resistance may arise as a result of a change in the expression
368 levels of genes associated with transport or metabolism of drugs (17,46). Amplification of PMII
369 and III is associated with PQ resistance (4). Further interrogation of other plasmepsins IX and X
370 confirmed druggability and the role in the growth of the asexual blood parasite (47). *Plasmodium*
371 *falciparum* expresses a total of ten plasmepsins (aspartyl protease) during the asexual and sexual
372 stage of the parasites, four of the seven proteases; the PMI, PMII, histoaspartic protease (HAP)
373 and PMIV localized within the digestive vacuole and digest hemoglobin in the red blood cells (48).
374 The digestive vacuole is the predicted site of action for PQ and LM. PMIV is the only protease
375 found in all *Plasmodium* species (48), and in *P. berghei,* it is the only Plasmepsin localized within
376 the DV vacuole (49), suggesting a sole potential role of heme metabolism. We hypothesized that
377 PQ^r and LM^r parasites have unique expression pattern for the PMs IV, IX and X. However, we
378 found no apparent pattern in the expression profiles of the PMs between PQ^r and LM^r parasites
379 (Figure 4 and 5) suggesting that PQ and LM selection pressure follow independent patterns.
380 Nonetheless, the PMIV, PMIX, and PMIV were differentially expressed in the PQ^r and $LM^{r,}$
381 suggesting a possible association between LM and PQ resistance in PQ^r and LM^r. The actual link
382 between PQ and LM resistance in *P. berghei* and the differential expression of PMIV, PMIX and
383 PMX need further interrogation through forward and reverse genetics in both *P. berghei* and *P.*
384 *falciparum* using CRISPR/Cas9 approach.

385 Enzymes are essential to drug activators and a potential target for the drug metabolites
386 (50,51), Here, we evaluated four enzymes. Enzymes involved in energy generation, potential drug
387 activators, and Fe-S biogenesis pathways such as SUFS (15), FNR (52), PANK (53), and PI3K

388 (54) have been proposed as targets for the development of specific new anti-malarial agents.
389 Indeed, one of the ways of identifying and validating a drug target is by dissecting polymorphism
390 after drug selection pressure. For instance, a mutation in one of the cysteine desulfurases, NFS, is
391 associated with resistance to LM in *P. falciparum* (15). In the current study, the expression of
392 SUFS in PQ^r and LM^r relative to the sensitive parental parasites was antagonistic, suggesting that
393 the high expression of SUFS in PQ^r may be one of the mechanisms for mediating LM cross-
394 resistance. On the contrary, the low expression of SUFS in the LM^r may suggest adaptive
395 mechanisms for the parasites to reduce potential binding targets for the LM. Indeed, the adaptive
396 parasite mechanism is supported by the low growth profiles of the parasite observed in this study
397 after partial deletion of the SUFS in the PQ^r parasites.

398 Next, we proceeded to assay the other three enzymes, FNR, PI3K, and PANK, which were
399 downregulated in both PQ^r and LM^r parasites. The FNR is involved in the generation of reducing
400 equivalents for the synthesis of isoprenoid within the apicoplast compartment (55). The interaction
401 between FNR and the ferredoxin (Fd) proteins has been exploited to design new chemical
402 compounds that kill the parasite by inhibiting the generation of reducing equivalent for powering
403 biochemical pathways in the apicoplast (52). Further validation of the FNR redox system as a
404 potential target and resistance marker for the artemisinins was revealed by the nonsynonymous
405 background mutation (Asp193Tyr) in the *Fd* protein in parasite exposed to the ACTs(56). This
406 study associates reduced FNR expression with LM resistance in both PQ^r and LM^r. For instance,
407 overexpression of FNR in the PQ^r parasites was accompanied by increased sensitivity to LM and
408 the loss of RA action, suggesting a link between the FNR protein, LM, and RA. Further study on
409 the modes of action of LM and RA action in the *P. falciparum* may be investigated through the

410 editing of the FNR and its interacting partner Fd by editing the expression profiles using the
411 CRISPR/Cas9 followed by the assessment of drug response profiles.

412 We then focused on a unique protein; the Pantothenate kinase (PANK). PANK though not
413 essential for the growth of the asexual blood-stage parasite, catalyzes the phosphorylation of
414 pantothenic acid in the first step of the biosynthesis of essential cofactor coenzyme A (53,57).
415 Analogs of pantothenic acid and pantothenamides inhibited the growth of the malaria parasite via
416 competitive inhibition of PANK and inhibition of PANK activity, respectively (58). The
417 nonessential genes may not be critical drug targets; however, such proteins are likely drug
418 activators or detoxifiers (59). Coenzyme A biosynthetic enzymes, including PANK, convert
419 pantothenamides into coenzymes A analogs (60). Drug resistance studies show reciprocal
420 resistance between LM and CQ despite their predicted similar modes of action (61). This means
421 that LM may have a different molecular target; PANK may be one of the targets. It is likely that
422 PANK may be an activator for LM; thus, the reduced expression of PANK in both PQ^r and LM^r
423 may be one of the ways the parasite reduces activation of LM, thus reducing the concentration of
424 drug metabolites available for action.

425 A previous study on the cross-resistance profile of LM^r revealed that the acquisition of LM
426 resistance is also accompanied by loss of DHA activity (36), suggesting that the LM^r also possess
427 gene expression changes that also mediate DHA and by extension artemisinin resistance. One such
428 gene is the phosphatidylinositol-3-kinase (PI3K), in which increased expression decreases parasite
429 susceptibility to the artemisinin. This study thus hypothesized that changes in gene expression in
430 PI3K occur in both PQ^r and LM^r parasites. In agreement to prediction, but contrary to expectation,
431 PI3K was found to be downregulated, suggesting an antagonistic resistance mechanism between
432 the artemisinins and bisquinoline-PQ and aryl alcohol- LM in *P. berghei*. Chemically, LM, and

433 PQ are different from the artemisinin; thus, it would be expected that they may involve different
434 mechanisms of resistance. However, the actual link between reduced expression of the PI3K
435 enzyme, PQ, and LM resistance in *P. berghei* requires further studies.

436 Transporters are vital mediators of drug entry for antimalarial action and efflux in the case
437 of drug resistance (44). Findings in this study associate reduced expression of *crt*, *mrp2,* and *mdr1*
438 genes in PQ^r and LM^r parasites. Different studies have revealed differing expressions of essential
439 transporters with mediating reciprocal drug susceptibility to antimalarial drugs. For instance, an
440 increase in the copy number variation in the *mdr1* gene does not alter parasite susceptibility to PQ
441 (13). On the contrary, a decrease in the copy number variation is associated with an increase in the
442 LM susceptibility in both *P. falciparum* and *P. chabaudi* (11,62). These studies highlight
443 reciprocal resistance mechanisms between the quinoline drugs such as PQ and aryl alcohol, such
444 as LM. In the acquisition of drug resistance, an increase rather than a decrease in the expression
445 of major transporters is the most prevalent occurrence. Also, changes in the expression of *mdr1*
446 and *mrp2* maybe act as compensatory resistance mechanisms (46). Thus, we argue that the
447 decreased expression of *mrp2*, *mdr1,* and *crt* may act as compensatory mechanisms in both PQ^r
448 and LM^r parasites. Overall, this study has revealed that PQ^r and LM^r parasites have differential
449 expression patterns in key drug transporters and enzymes associated with metabolism or potential
450 targets.

451 Using PQ^r parasites and a reverse genetics approach, the study dissected the mechanisms
452 of LM resistance by assessing the link between gene expression of two genes; SUFS and FNR,
453 LM activity, and reversal of resistance by RA. The essentiality of the sulfur mobilization
454 (SUF) pathway for apicoplast maintenance and parasite survival in erythrocytic stages, as recently
455 confirmed from disruption of the *P. falciparum sufC* gene (63), identifies it as a leading potential

456 target for antimalarial drug discovery. Recent studies have provided evidence that the cysteine
457 desulfurase is involved in the initial stages of sulfur trafficking within cells. The cysteine
458 desulfurase decomposes L-cysteine to L-alanine and sulfane sulfur via the formation of an enzyme-
459 bound persulfide intermediate. The persulfide sulfur is then incorporated into the biosynthetic
460 pathways of sulfur-containing biofactors (64). We confirm a significant attenuation of the growth
461 rates of the asexual blood-stage in the transgenic parasites providing evidence that SUFS and FNR
462 are essential for the growth of asexual blood-stage parasites. Second, SUFS is associated with LM
463 activity in *P. berghei* since partial deletion in PQ^r parasites resulted in a significant increase in LM
464 susceptibility. Third, SUFS protein is associated with mediating reversal of LM resistance in the
465 PQ^r parasites since partial deletion of the SUFS abolished the impact of cyproheptadine, verapamil,
466 and probenecid. On the other hand, this study finds increase expression of SUFS as predictive of
467 LM activity.

468 To further understand the link between LM resistance in PQ^r parasites, restoration of LM
469 activity by verapamil, cyproheptadine, and probenecid and the expression profiles, we focused on
470 FNR protein. Several studies have established that the malaria parasite plant-type ferredoxin (Fd),
471 and ferredoxin $NADP^+$ reductase (FNR), a plastidic-derived organelle called the apicoplast,
472 possesses metabolic pathways, which is not found in the human host (65). The Fd/FNR redox
473 system, which potentially provides reducing power for essential biosynthetic pathways in the
474 apicoplast (66), has been proposed as a target for the development of specific new anti-malarial
475 agents (52). We provide a clear link between LM activity, RA action, and overexpression of the
476 FNR gene. Thus two possible assumptions exist; First, FNR may be a target for LM. Reduced
477 expression of FNR in the PQ^r parasites may potentially result in reduced binding targets for LM.
478 The overexpression of FNR in the transgenic parasites PQ^r_FNR_OE seems to restore the

479 concentration of the FNR, thus more binding targets for LM and a consequent high susceptibility
480 of PQ^r_FNR_OE to LM. Second, the FNR is a potential activator for LM, thus reduced expression
481 of FNR in the PQ^r is accompanied by reduced active metabolites from LM, thus less activity. The
482 increase in the FNR, as confirmed in the PQ^r_FNR_OE transgenic parasites, seems to reinstate the
483 activator; thus, more of the LM metabolite is generated restoring LM activity in the PQ^r_FNR_OE
484 transgenic parasites as compared to PQ^r parasites. The fact that all the RA lost the ability to
485 potentiate LM activity in the PQ^r_FNR_OE parasites confirms the interaction between FNR and
486 LM and RA. Also, we show that the amount of FNR is predictive of LM activity. The reversing
487 agents restore the activity of antimalarial drugs by enhancing the accumulation of drugs within the
488 target site (27). It is likely that in the event the FNR amount is low, the threshold concentration for
489 LM action is not achieved, the threshold may be enhanced by an RA possibly by increasing the
490 uptake of the few active LM metabolites.

491 Overall, this study highlights two critical aspects regarding LM resistance in PQ resistant
492 *P. berghei* ANKA; First, using LM and cyproheptadine, verapamil, or probenecid, the study
493 reports new combination therapies that restored LM activity against the PQ^r parasites. Second,
494 using reverse genetics, the study shows that alteration of two essential parasite enzymes (SUFS
495 and FNR) restores LM activity against PQ^r parasites. Thus, mRNA expression of the two enzymes
496 in PQ-resistant parasites is predictive of LM activity against the PQ resistant *P. berghei* ANKA,
497 and the alteration of their expression profiles alter the parasite to LM. Taken together, this study
498 provides essential clues of SUFS and FNR as potential resistance markers and targets for LM in
499 *P. berghei* ANKA. The role of SUFS and FNR and the impact of differential expression in drug
500 susceptibility should be investigated in the human malaria parasite *P. falciparum* in vitro using the

501 CRISPR/Ca9 technique. Also, the expression profiles of SUFS and FNR may be used as a potential
502 monitor for the emergence of LM resistance in the *P. falciparum* field isolates.

503 **Materials and methods**

504 **Parasites, Host, and Compounds**

505 We used two drug-sensitive parasite reference lines *of P. berghei* ANKA, the MRA-865
506 and MRA-868 reference lines obtained from MR4, ATCC® Manassas, Virginia. Also, two
507 multidrug-resistant parasite lines; the PQ-resistant (PQ^r) and LM-resistant (LM^r) *P. berghei*
508 ANKA parasites previously selected from MRA-865 and MRA-868 lines, respectively (36) were
509 studied. We utilized male Swiss albino mice weighing 20±2g out-bred at KEMRI, Animal house
510 Nairobi. Experimental mice were kept in standard polypropylene cages clearly labeled with
511 experimental details and fed on commercial rodent food and water *ad libitum*. The knockout (KO)
512 vector (PbGEM-018972), as well as the overexpression (OE) vector (PbGEM-456502), were
513 kindly provided by the *Plasmo*GEM project under an agreement of a material transfer (PG-MTA-
514 0093). Verapamil, probenecid, and cyproheptadine were purchased from Carramore international
515 limited (UK). LM and PQ were donated from Universal Corporation, Kikuyu, Kenya. All drugs;
516 PQ, LM, verapamil, probenecid, and cyproheptadine were freshly prepared by dissolving them in
517 a solvent consisting of 70% Tween-80 (d=1.08g/ml) and 30% ethanol (d=0.81g/ml) and
518 subsequently diluted 10-fold with double distilled water which was used as a vehicle and control
519 for the drug profile assays.

520 **Ethics statement**

521 This study was conducted at KEMRI and JKUAT. All mouse experiments were carried out
522 as per relevant national and international standards (The ARRIVE Guidelines) and as approved by

523 KEMRI-Animal Use and Care Committee. This study was permitted and ethically approved by
524 KEMRI under the SERU No 3914.

525 **Drug sensitivity assays**

526 The activity profiles of probenecid, verapamil, or cyproheptadine alone and in combination
527 with PQ, or LM against the drug-sensitive, resistant, or the transgenic parasites were assessed in
528 the standard 4-day suppressive test (4-DT) in which the parasite is exposed to four daily drug doses
529 (67). Infection in mice was by intraperitoneal inoculation of approximately 1×10^6 parasites per
530 mouse. The infected mice were then randomly allocated to the test groups and the control group
531 (at least three mice per group). Treatment was performed orally starting on day 0 (4 h post-
532 infection) and continued for four (4) days, days 0–3 (24, 48, and 72 hours post-infection). Parasite
533 density was estimated microscopically (×100) on day 4 (96 hours) post parasite inoculation using
534 thin blood films made from tail blood. Parasite growth was then followed for at least 11 days post-
535 infection to assess the recrudescence of the parasites after cessation of drug treatment. Percentage
536 (%) activity (parasite reduction) was determined using the following equation: [(A-B)/A] x 100]
537 = % Chemo-suppression; where A is the mean parasitemia in the negative control group and B is
538 the parasitemia in the test group.

539 **Extraction of RNA, synthesis of cDNA, and qRT-PCR assays**

540 The quantification of mRNA transcripts of *Pbcrt, Pbmdr1, Pbmrp2, Pbpank, Pbfnr,*
541 *Pbsufs, Pbpm4, Pbpm9, Pbpm10,* and *Pbpi3k* was carried out after cDNA synthesis from mRNA
542 extracted from sensitive parental parasites, LM^r, PQ^r, and transgenic parasites. All buffer and
543 solutions for parasite preparation and mRNA extraction were treated with 0.1% (v/v) of
544 diethylpyrocarbonate (DEPC). Total RNA was extracted from approximately 1×10^6 fresh parasites
545 pellets. The parasite pellet was prepared as per the previous protocol (68). Total RNA was purified

546 using Quick-RNA™ MiniPrep (Zymo Research™) following instructions form the manufacturer.
547 The first-strand cDNA synthesis was performed in a final volume of 20µl using RevertAid First
548 Strand cDNA synthesis kit and Oligo$(dT)_{18}$ as primers. The mix contained 4µl Reaction buffer
549 (5×), 2µl of dNTPs (10mM), 1µl of RevertAid RT (200U/µl), 1µl RiboLock RNase Inhibitor
550 (U/µl), 1µl of oligo-DT and 5µg of total RNA. To synthesize cDNA, the reaction mix was
551 incubated at 42°C for 60min, then at 70°C for 10min and finally chilled on ice. The cDNA
552 synthesized was later used as a template for qRT-PCR assays. Gene expression levels were
553 measured in a total reaction of 20µl using Maxima SYBR Green/ROX qPCR Master Mix (Thermo
554 Scientific™). Oligonucleotides for *Pbcrt, Pbmdr1, Pbmrp2, Pbpank, Pbfnr, Pbsufs, Pbpm4,*
555 *Pbpm9, Pbpm10,* and *Pbpi3k* were designed for cycling using similar conditions relative to the
556 housekeeping gene; *Pbβ-actin I* (Table S1). The reaction mix contained 3µl water, 2.0µl (0.25µM)
557 of forward and reverse primers each and 12µl of Maxima SYBR mix. The mix was cycled for pre-
558 treatment step at 50°C, for 2 min; initial denaturation of 95°C for 10 min; denaturation of 95°C for
559 15 secs; and annealing at 60°C for 60 secs for 45 cycles.

560 **Generation of the transgenic parasites**

561 **Isolation, digestion, and purification of the vector DNA**

562 Using the QIAfilter Plasmid Midi Kit (Qiagen™), Plasmid DNA for each of the vectors
563 was isolated from cultures of *E. coli* (vector hosts) in terrific broth (TB) supplemented with
564 kanamycin after overnight culture. Following a standard protocol, the extracted plasmid DNA was
565 restricted using Not I enzyme to release the vector backbone, followed by concentration using
566 conventional ethanol precipitation protocol (69). The vector was dried for 5 min at 65˚C and then
567 dissolved in 10µl of PCR water. Besides Not I restriction digest, a diagnostic PCR was performed

568 using standard primer pair (GW2 and a vector-specific QCR2) (39) to verify the isolated vector of
569 interest

570 **Purification of the *P. berghei* schizonts for transfection**

571 Using standard protocols as described by (70), we collected *P. berghei* schizonts for
572 transfection. Briefly, at least three mice were used for schizont culture for each of the vectors.
573 Propagation of *P. berghei* parasites was done by intraperitoneal (IP) injection into the mice, and
574 parasites were harvested using cardiac puncture for schizont culture at 3% parasitemia. For
575 schizonts propagation, rings stage parasites (the asexual blood-stages) were incubated at 37˚C in
576 vitro as reported previously (71,72). A 100ml schizont, culture medium contained 25ml freshly
577 thawed FBS, 72 ml RPMI1640, 2ml 0.5M NaHC03, and 1ml of antibiotic Pen/Strep (1:100
578 Penicillin/ streptomycin, and 2ml of parasitized mice blood. The mixture was gassed with malaria
579 gas (3% oxygen, 5% carbon dioxide, and 92% Nitrogen) for 2 minutes, tightly sealed, and
580 incubated at 37°C for with minimal shaking. After 22 hours, the schizonts were harvested and
581 purified by Nycodenz density gradient centrifugation, and each pellet was re-suspended in schizont
582 culture media, ready for transfection.

583 **Transfection and selection of transgenic parasite lines**

584 A SUFS specific knockout vector (PbGEM-018972) and an FNR overexpression vector
585 (PbGEM-456502) were separately transfected in the PQ^r parasites. Each of the transfection mixes
586 contained, 20µl of schizonts, 10µl (5µg) of plasmid DNA, and 100ml of supplemented
587 nucleofector solution (Amaxa™). Exactly 100µl of the transfection mix in Amaxa cuvette was
588 electroporated in a Nucleofector 2B Device (Lonza™) using the program U33. A naïve mouse was
589 intravenously injected with the electroplated parasites. Three transfections were performed for
590 each of the vectors to increase the chance of recovering transgenic parasites. Pyrimethamine drug

591 at 7 mg/mL, for the selection of transgenic parasites, was provided in drinking water, 24hours post-
592 parasite inoculation for a total of 12 days. Freshly prepared pyrimethamine drug was provided
593 every 72 hours. After the recovery of transgenic parasites, genetically homogenous parasite lines
594 were generated by dilution cloning protocol (18,70).

595 **Genotyping of transgenic parasites**

596 Genomic DNA (gDNA) was extracted from the transgenic parasite lines using the QIAamp
597 DNA Blood Mini Kit (Qiagen™) following manufacturer instructions. Three pairs of primers were
598 used for diagnosis and genotyping, as detailed in Table S2; i) QCR2/GW2 pair ii) QCR1/QCR2
599 and iii) GT/GW1 or GW2. Each of the reaction mixes contained 1µl of gDNA, 12.5µl of 2xGoTag
600 Green master mix (Promega™), 6.5µl of water and 2.5µl of each of the primers. The PCR reactions
601 were optimized, as detailed in Table 4. The PCR products were resolved on a 1% agarose gel.

602 **Molecular docking**

603 **Homology modeling**

604 The 3D structures of the SUFS; PBANKA_0614300, and FNR; PBANKA_1122100, were
605 predicted by selecting as templates, the best structures with the highest similarity to their sequences
606 available in NCBI protein database http://ncbi.nlm.nih.gov and *Plasmodium* database
607 http://plasmodb.org/. SWISS-MODEL (73) available at https://swissmodel.expasy.org/ was used
608 to predict the homology models. The SWISS-MODEL was a preferred modeling server because it
609 annotates essential cofactors and ligands as well as quaternary structures, allowing for modeling
610 of complete structures with less complicated software packages (73). The structures were then
611 downloaded and saved in PDB format.

612 **Structure validation**

613 The models were analyzed using PROCHECK (74) and PRoSA-web (75) to determine the
614 quality of the modeled structures. PROCHECK was used to evaluate the general stereochemistry
615 of the protein, while PRoSA-web was employed to evaluate for potential errors in the 3D
616 structures. Verify_3D was used to determine the compatibility of an atomic model (3D) with its
617 amino acid sequence (1D), (76).

618 **Ligand selection**

619 To perform docking experiments, the most potent RA, as well as LM, were selected as test
620 ligands. The molecular structures of the four selected ligands were downloaded from the
621 ChemSpider (http://www.chemspider.com/), an online database to access unique chemical
622 compounds (77). For compatibility with the docking software
623 (https://cactus.nci.nih.gov/translate/), we converted the chemical structures from the Mol2 format
624 into PDB format using the CADD Group's Chemoinformatics Tools and User Services

625 **Binding site analysis and ligand docking**

626 PyMOL version 1.6.x (Delano, 2002) was used to visualize the modeled structures and
627 binding sites. The grid box parameters of the ligands and the receptors optimization were executed
628 using scripts within AutoDock Tools (ADT). The files were then saved in PDBQT format, and
629 their corresponding coordinates rewrote into a configuration file used for docking. The
630 configuration file specified the pdbqt files for both the ligand and the proteins as well as the
631 docking parameters (dimensions and spacing angstrom). Optimization of the inputs involved the
632 removal of water molecules from the receptor and the addition of missing hydrogen ions. Docking
633 of a single receptor and a single ligand was carried out using the Autodock Vina (78,79). Autodock
634 Vina employs the Lamarckian genetic algorithm (LGA). FNR and SUFS, whose 3D structures

635 were available in the protein data bank (PDB;
636 http://www.rcsb.org/pdb/explore.do?structureId=2hvp) were also included. The binding energies
637 and the positional root-mean-square deviation (RMSD) of the proteins and the ligands were
638 presented on an output file. A ligand orientation with low binding energy signifies a better affinity
639 towards a receptor.

640 **Data presentation and statistical analysis**

641 The percentage parasitemia and the percentage of drug killing were analyzed using the
642 Nonparametric Mann-Whitney U Test in the R statistical software with a *p*-value set at 0.05. The
643 means for the gene expression levels from three independent experiments and triplicate assays
644 were compared using the Nonparametric Mann-Whitney U test with a p-value set at 0.05. The
645 relative expression level data were normalized using β-actin I as the housekeeping based on the
646 formula $2^{\Delta\Delta CT}$ by (Livak and Schmittgen 2001).

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Table 1a: Effect of verapamil (Ver) and probenecid (Prob) alone against piperaquine (PQ) and lumefantrine (LM) resistant Plasmodium berghei ANKA assessed in the 4-Day Test. Results presented as percentage (%) parasite killing ± SD (Standard Deviation) of five mice per dose.

Reversing agent	*Dose (mg/kg)*	*% chemo-suppression ± SE (LM^r)*	*% chemo-suppression± SE (PQ^r)*
Probenecid (Prob)	400	8.44±3.07	8.03±1.86
	200	0.00	4.06±2.75
Verapamil (Ver)	50	0.00	0.81±1.31
	25	0.00	0.78±1.56

Table 1b: Effects of cyproheptadine (Cyp) alone or in combination with lumefantrine (LM) against drug-sensitive Plasmodium berghei ANKA assessed in the 4-day Test. Results presented as percentage (%) parasite killing ± SD (standard deviation) of five mice per dose

Drug	*Dose (mg/kg)*	*% chemo-suppression ± SE*
Cyp	5	4.44±1.65
Cyp	2.5	5.32±4.12
LM	0.375	17.12±4.12
LM + Cyp	0.375 + 5	20.77±5.25
LM + Cyp	0.375 + 2.5	5.93±2.57

Table

Table 2: Lack of potentiation effects of cyproheptadine (Cyp), verapamil (Ver), and probenecid (Prob) in combination with PQ against PQ-resistant *Plasmodium berghei* ANKA assessed in the 4-day Test. Results presented as percentage (%) parasite killing ± SD (standard deviation) of five mice per dose

Drugs	*Dose (mg/kg)*	*% chemo-suppression ± SE*
PQ	75	8.39±3.85
Prob + PQ	400 + 75	0.00
	200 + 75	0.00
Ver + PQ	50 + 75	0.00
	25 + 75	0.00
Cyp + PQ	5.0 + 75	14.18±1.77
	2.5 + 75	8.71±3.85

Table

Table 3: Validation of the modeled 3D structures of the *Plasmodium berghei* ferredoxin NADP+ reductase (*PbFNR*) and *Plasmodium berghei* cysteine desulfurase (*PbSUFS*) proteins as determined using PROSA-web server.

Protein	*Gene ID*	*Z-Score*	*score Averaged 3D-1D score > = 0.2 (%)*	*Residues in most favored regions (%)*	*Residues in additional allowed regions (%)*	*Residues in disallowed regions (%)*
PbFNR	PBANKA_112210	-8.49	86.21	85.9	13.2	0.4
PbSUFS	PBANKA_061430	-8.67	75.11	82.2	14.1	1.6

Table

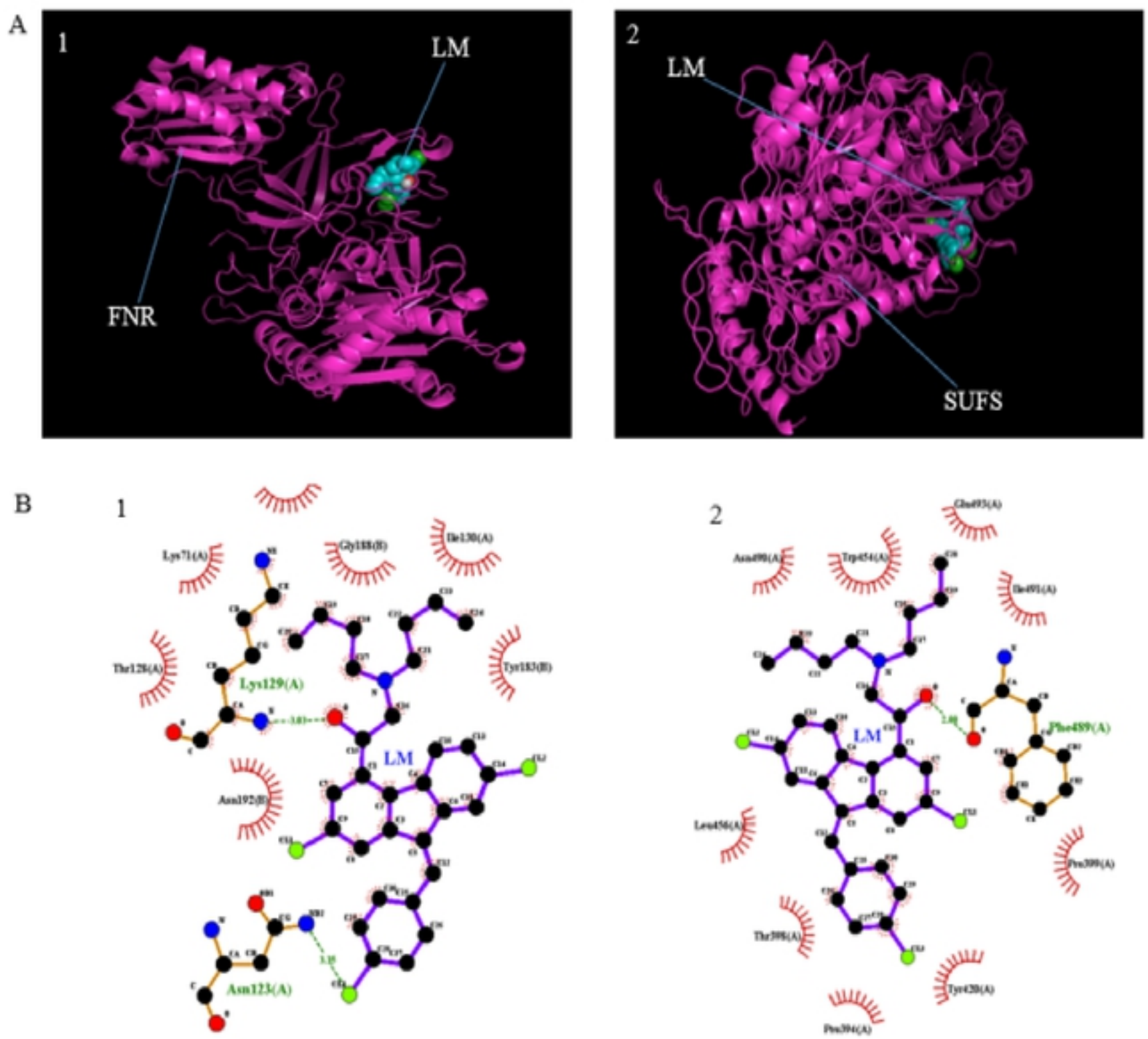


Fig 12: Interaction of Lumefantrine (LM) with the Ferredoxin NADP+ reductase (FNR) and Cysteine desulfurase (SUFS) (A) The crystallographic structure of (1) FNR and (2) SUFS in complex with LM as visualized using PyMOL. (B) 2D: (1) LM-FNR and (2) LM-SUFS interaction diagram with the oxygen atoms shown in red, nitrogen atoms shown in blue, while hydrogen bonds are shown as olive-green dotted parasites. The interaction plots were generated using LigPlot+.

Figure

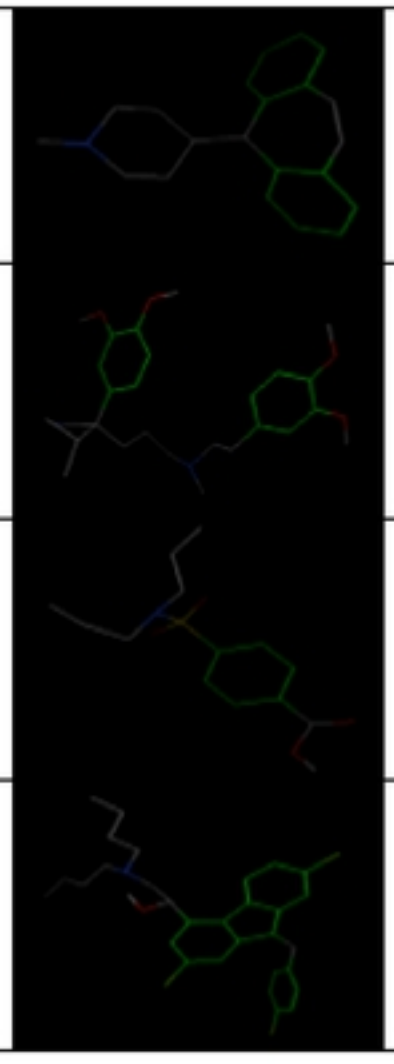
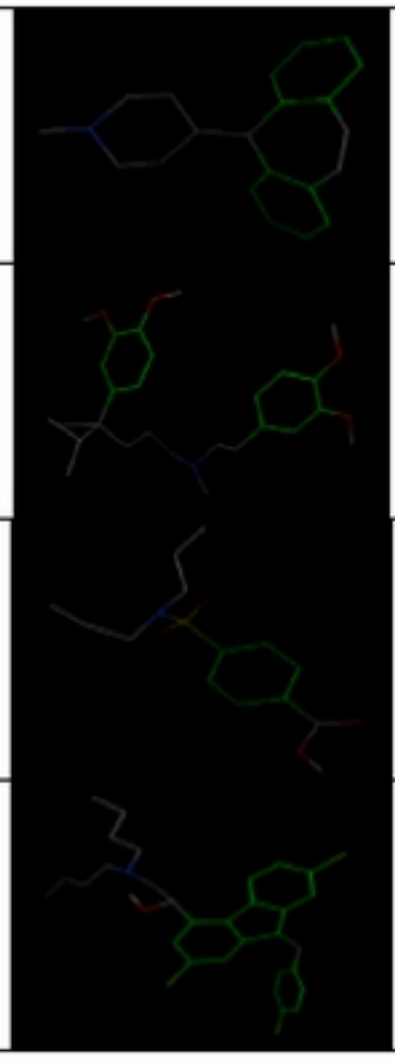
LIGAND	PROTEIN					
	Ferredoxin NADP-reductase (FNR)			Cysteine desulfurase (SUFS)		
	Ligand mode		Binding affinity (kcal/mol)	Ligand mode		Binding affinity (kcal/mol)
Cyproheptadine	1		-7.8	1		-9.5
Verapamil	1		-6.3	1		-7.4
Probenecid	1		-7.1	1		-6.5
Lumefantrine	1		-7.9	1		-7.9

Fig 11: Ligand best docking modes with respective binding energies towards FNR and SUFS proteins. The binding geometries for docking of a single ligand with a single receptor as modeled using the Autodock Vina. The figure portrays low binding affinities for cyproheptadine and lumefantrine to the FNR and SUFS proteins.

Figure

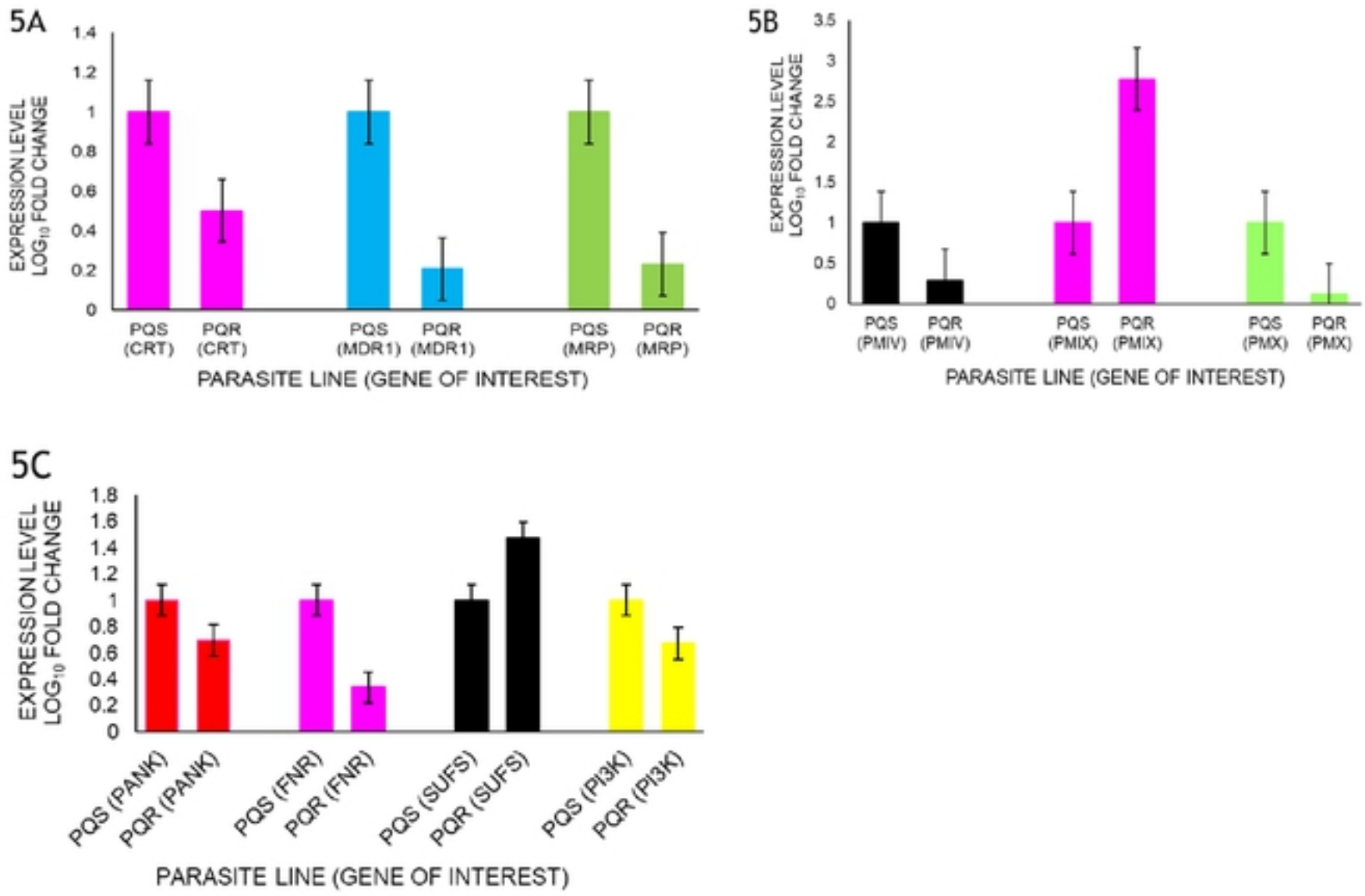


Fig 5: Expression profiles of transporters and enzymes. **(A)** Drug transporters: *chloroquine resistance transporter (CRT), multidrug resistance protein (MDR1), multidrug resistance-associated protein 1 (MRP1),* **(B)** Plasmepsins*: Plasmepsin IV (PMIV), Plasmepsin IX (PMIX), Plasmepsin X (PMX) or phosphatidylinositol 3-kinase (PI3K)* **(C)** Enzymes: *pantothenate kinase (PANK), ferredoxin NADP-reductase (FNR), cysteine desulfurase (SUFS),* as measured from cDNA amount derived from 5µg/µl of total RNA isolated from piperaquine resistant clones relative to their wild type drug-sensitive parental clones piperaquine sensitive (PQ^S). The differential expression from a mean of three independent experiments were significantly different for CRT ($p<0.001$), MDR1 ($p<0.0001$), MRP ($p<001$), PANK ($p<0.0001$), FNR ($p<001$), SUFS ($p<001$), PM4 ($p<001$), PM9 ($p<001$), PM10 and PI3K ($p<001$) after Student's t-test analysis with p-value set at 0.05.

Figure

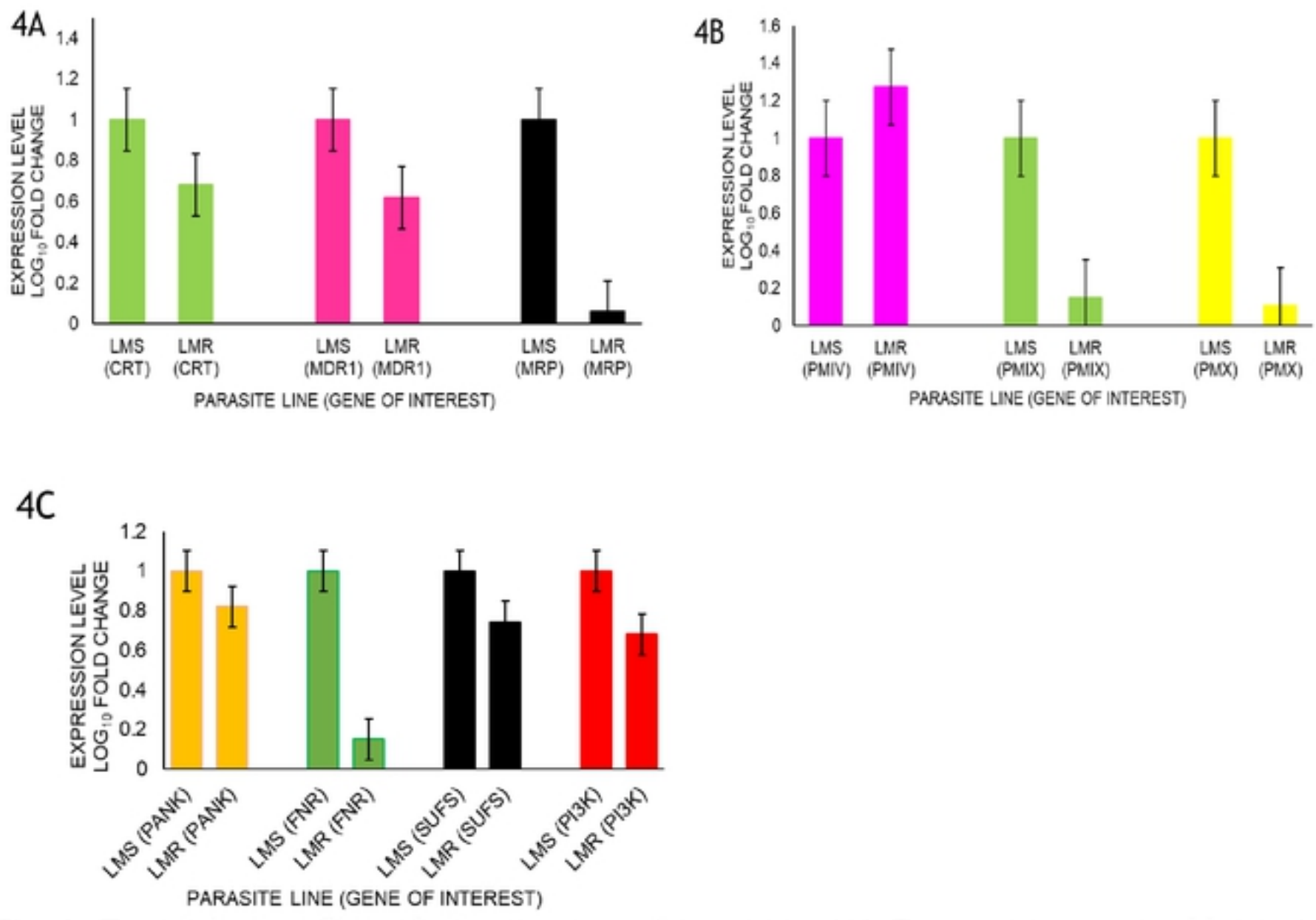


Fig 4: Expression profiles of transporters and enzymes. **(A)** Drug transporters*: chloroquine resistance transporter (CRT), multidrug resistance protein (MDR1), multidrug resistance-associated protein 1 (MRP1),* **(B)** Plasmepsins*: Plasmepsin IV (PMIV), Plasmepsin IX (PMIX), Plasmepsin X (PMX) or phosphatidylinositol 3-kinase (PI3K)* **(C)** Enzymes: *pantothenate kinase (PANK), ferredoxin NADP-reductase (FNR), cysteine desulfurase (SUFS),* as measured from cDNA amount derived from 5µg/µl of total RNA isolated from lumefantrine resistant parasites (LMR) relative to their wild type drug-sensitive parental clones lumefantrine sensitive (LMS). The differential expression from a mean of three independent experiments were significantly different for CRT ($p<0.001$), MDR1 ($p<0.0001$), MRP ($p<001$), PANK ($p<0.0001$), FNR ($p<001$), SUFS ($p<001$), PM4 ($p<001$), PM9 ($p<001$), PM10 and PI3K ($p<001$) after Student's t-test analysis with p-value set at 0.05.

Figure

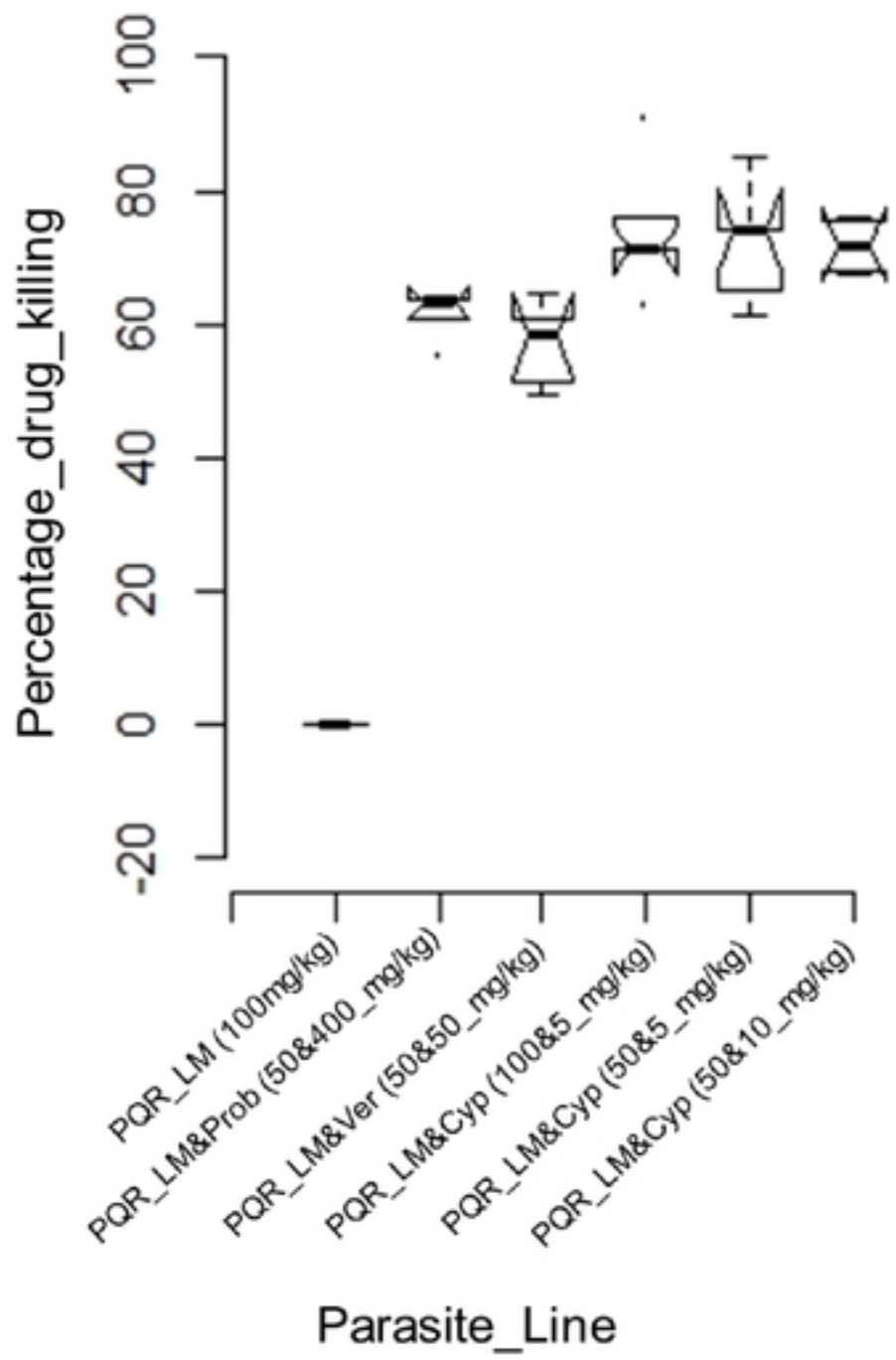


Fig 3: The effect of reversing agents (RA); probenecid (Prob), verapamil (Ver), or cyproheptadine (Cyp) on lumefantrine (LM) activity against piperaquine resistant *Plasmodium berghei* ANKA parasites (PQ^r) as assessed in the 4-day Test. The results are presented as percentage (%) drug killing of parasites in at least five mice per dose.

Figure

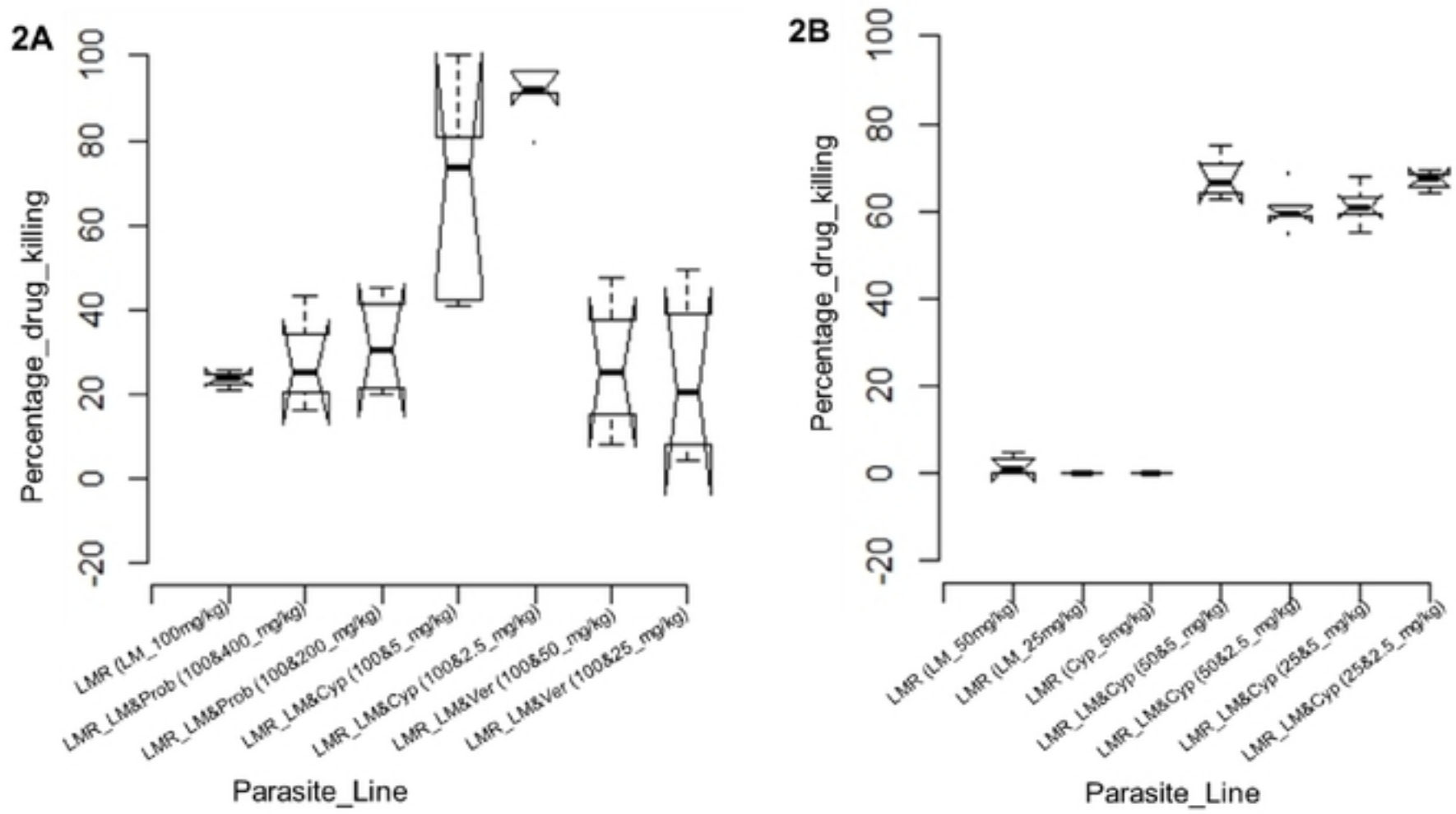


Fig 2: Effect of reversing agents (RA); probenecid (Prob), verapamil (Ver), or cyproheptadine (Cyp) on lumefantrine (LM) activity as assessed in the 4-day Test. **(A)** The effects of LM alone or in combination with the RA against lumefantrine resistant (LM^R) *Plasmodium berghei* ANKA **(B)** Lack of synergistic and additive effects of cyproheptadine in combination with LM against LM^R parasites. The results are presented as percentage (%) drug killing of parasites in at least five mice per dose.

Figure

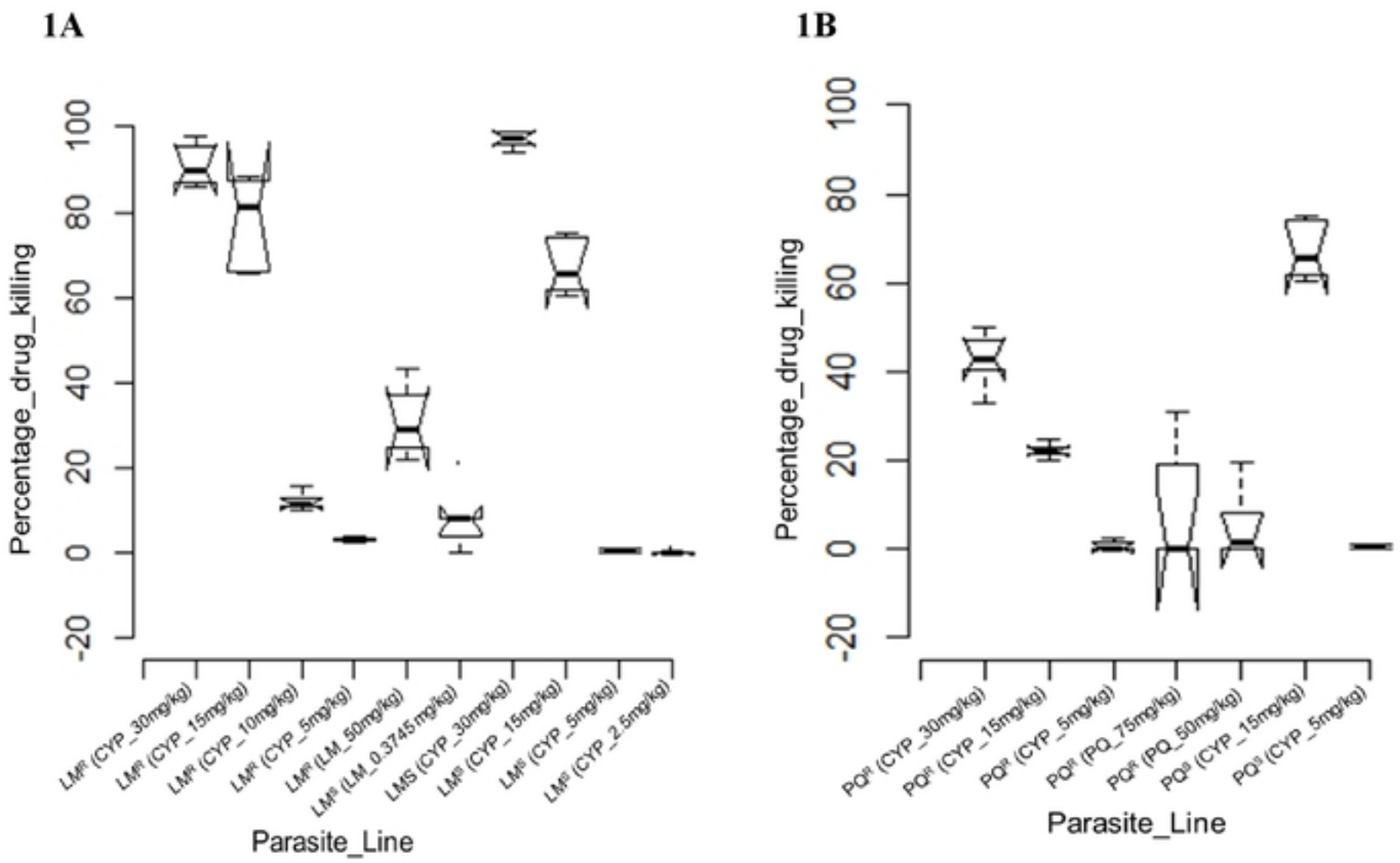


Fig 1: Parasite killing potential of cyproheptadine as assessed in the standard 4-Day suppressive test. **(A)** The activity of cyproheptadine (CYP) and lumefantrine (LM) against the lumefantrine resistant (LM^R) and lumefantrine sensitive *Plasmodium berghei* ANKA parasites (LM^S). **(B)** The activity of cyproheptadine and piperaquine (PQ) against the piperaquine resistant (PQ^R) and piperaquine sensitive *Plasmodium berghei* ANKA parasites (PQ^S). The results are presented as percentage (%) drug killing of parasites in at least five mice per dose

Figure

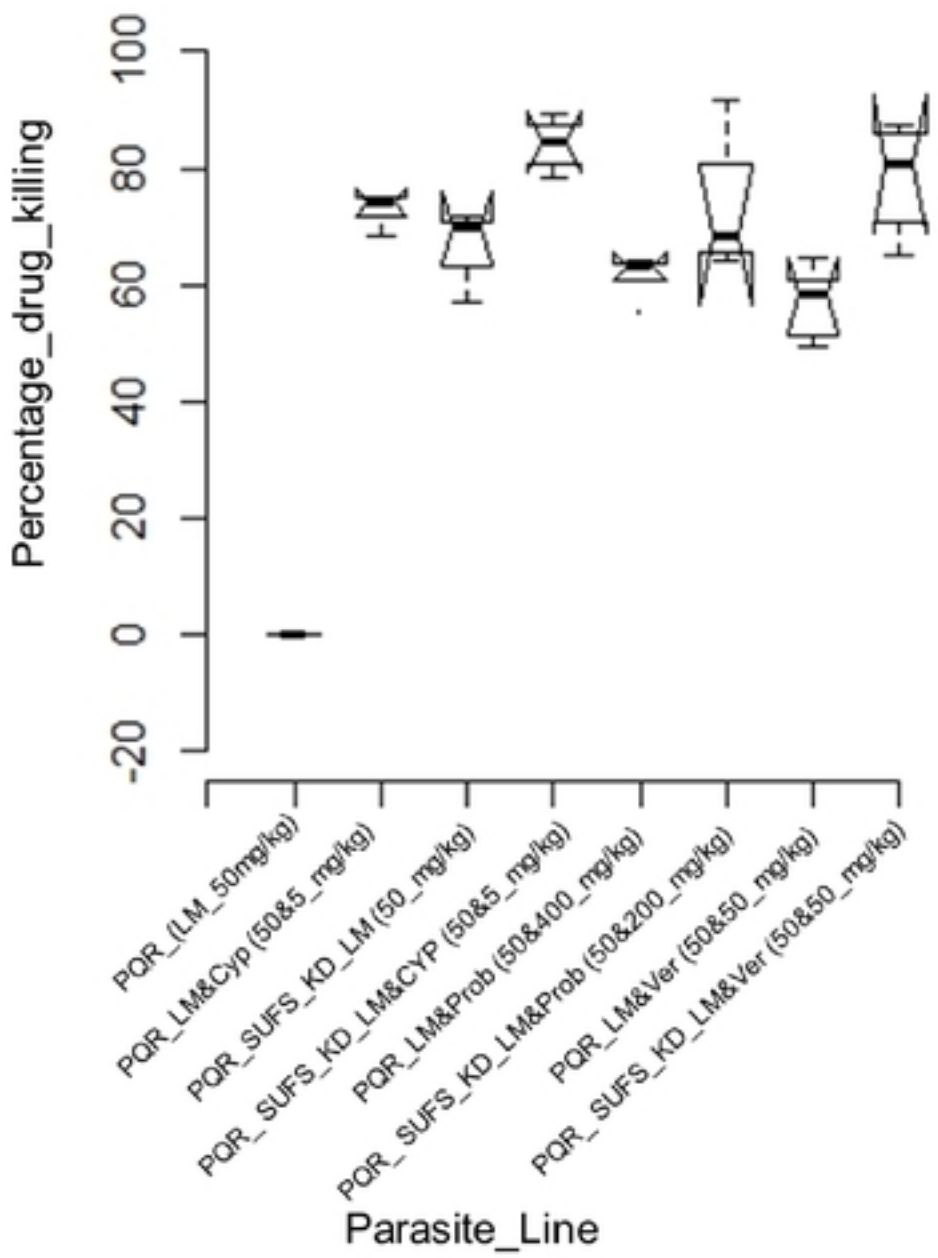


Fig 10: The activity profile of LM alone or in combination with either cyproheptadine (Cyp), verapamil (Ver), and probenecid (Prob) against the transgenic parasites (PQ^R_SUFS_KD) relative to piperaquine resistant parasites (PQ^R) as assessed in the standard 4-Day test. The results are presented as percentage (%) drug killing of parasites from at least five mice per dose.

Figure

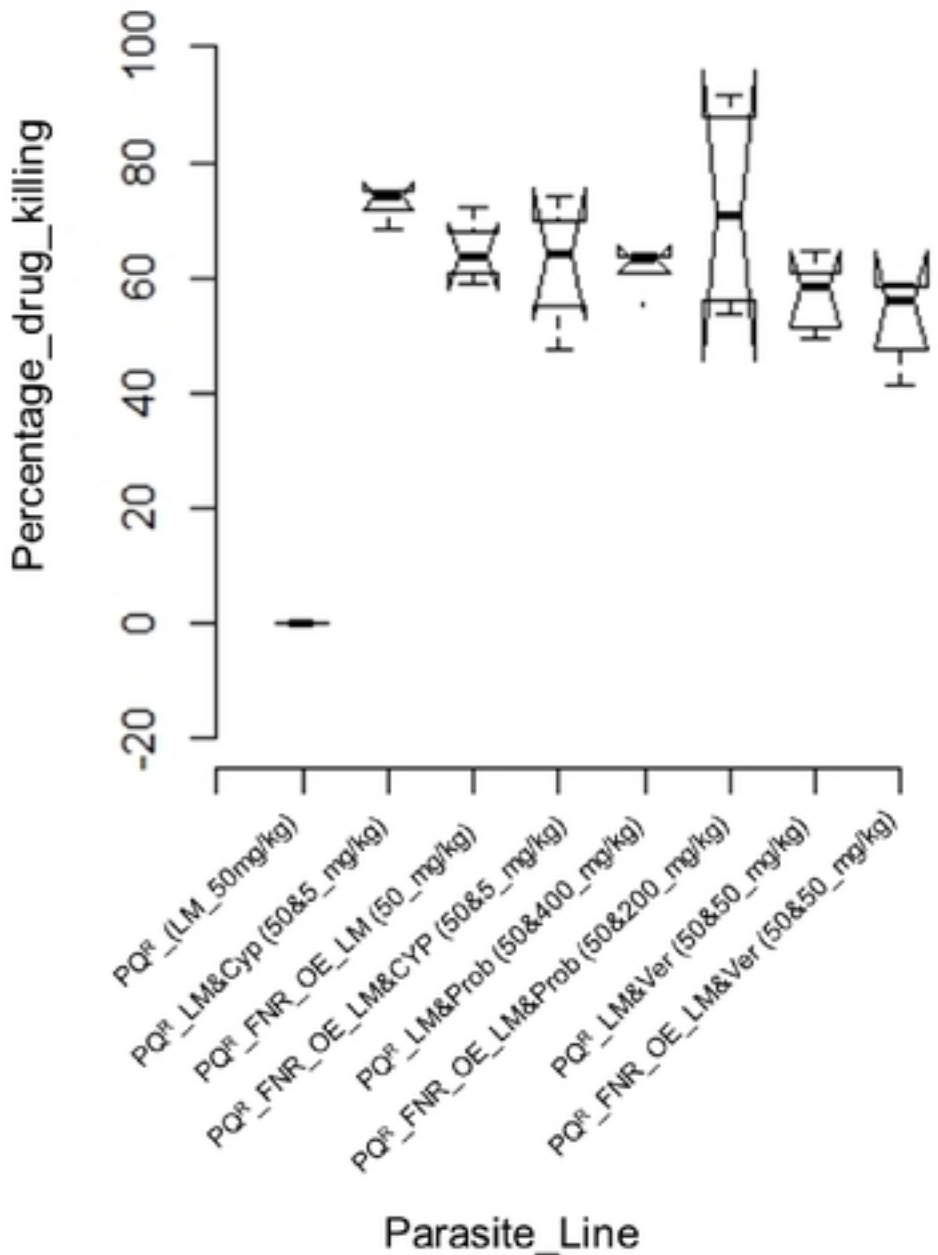


Fig 9: The activity profile of LM alone or in combination with either cyproheptadine (Cyp), verapamil (Ver), and probenecid (Prob) against the transgenic parasites (PQ^R_FNR_OE) relative to piperaquine-resistant parasites (PQ^R) as assessed in the standard 4-Day test. The results are presented as percentage (%) drug killing of parasites from at least five mice per dose.

Figure

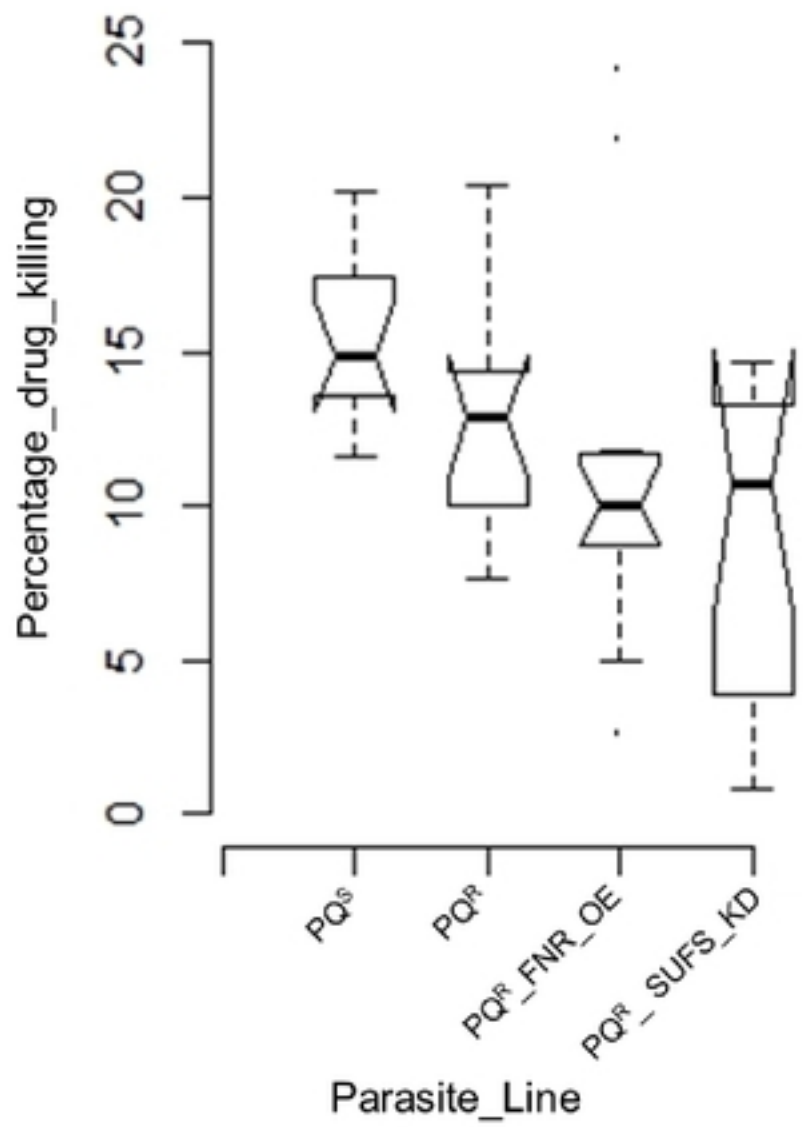


Fig 8: The growth rates of transgenic parasites: Average growth rate profiles of PQ^R_SUFS_KD or PQ^R_FNR_OE relative to the drug-sensitive (PQ^S), and the piperaquine resistant parasites (PQ^R) as determined on day 3, day 4, and day 7 post parasite inoculation. The results are presented as the average percentage (%) parasitemia calculated on day 3, day 4, and day 7 in at least five mice.

Figure

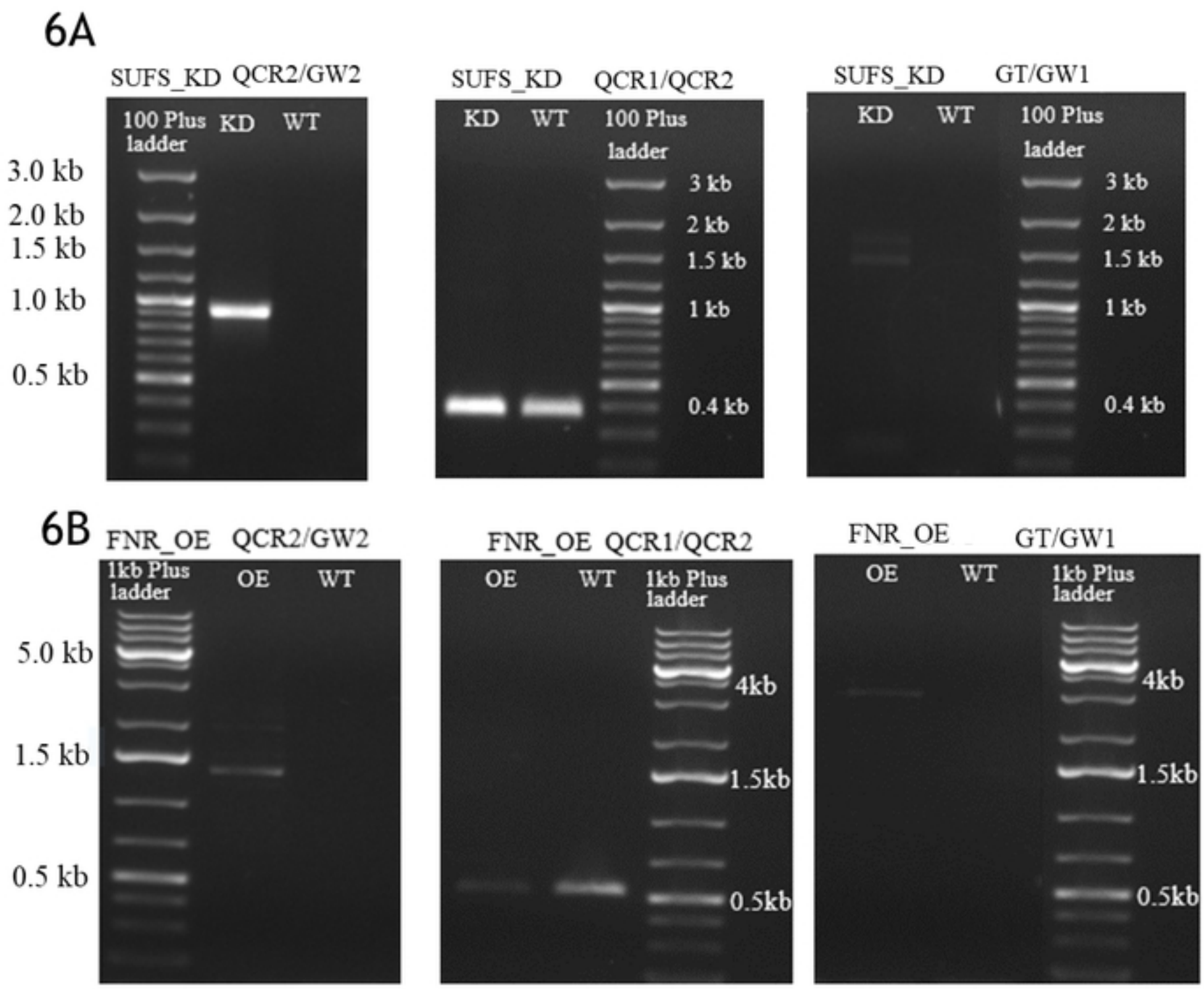


Fig 6: PCR genotyping of the SUFS-knockdown (KD) and FNR over-expression (OE) parasites. **(A)** SUFS_KD, **(B)** FNR_OE, and the parent (PQR) control lines here referred to as WT. The PCR amplification used three sets of primer pairs; QCR2/GW2 to confirm the presence of the vector, GT/GW1, to verify the integration of the vector into the parasite genome and QCR1/QCR2 to confirm the modification of the specific genes. The QCR1, QCR2, and GT are vector-specific primers, while GW2 and GW1 primers are standard primers.

Figure

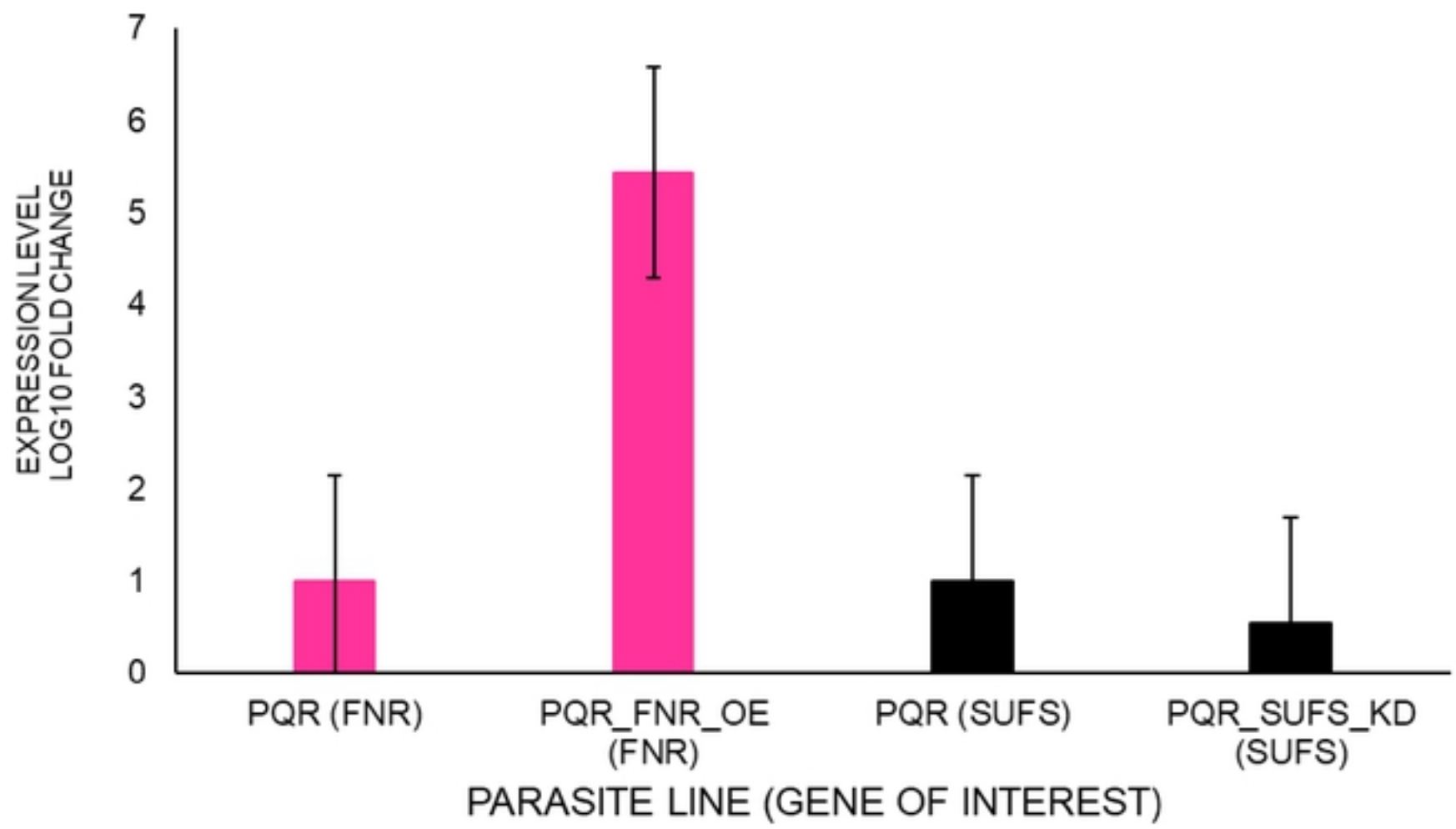


Fig 7: Expression profiles in ferredoxin NADP-reductase (FNR), cysteine desulfurase (SUFS) as measured from cDNA amount derived from 5µg/ul of total RNA isolated from transgenic parasites (PQ^R_FNR_OE and PQ^R_SUFS_KD) relative to the drug-resistant parent piperaquine resistant parasites (PQ^R). The differential expression from a mean of three independent experiments was significantly different for FNR ($p<0.001$), and SUFS ($p<0.0001$) after Student's t-test analysis with p-value set at 0.05.

Figure