Accelerating antimalarial drug discovery with a new high-throughput screen for fast-killing compounds

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Abstract

The urgent need for rapidly acting compounds in the development of antimalarial drugs underscores the significance of such compounds in overcoming resistance issues and improving patient adherence to antimalarial treatments. The present study introduces a high-throughput screening (HTS) approach using 1536-well plates, employing *Plasmodium falciparum* lactate dehydrogenase (PfLDH) combined with nitroreductase (NTR) and fluorescent probes to evaluate inhibition of the growth of the asexual blood stage of malaria parasites. This method was adapted to efficiently measure the parasite reduction ratio (PRR) in a 384-well plate format, streamlining the traditionally time-consuming screening process. By successfully screening numerous compounds, this approach identified fast-killing hits early in the screening process, addressing challenges associated with artemisinin-based combination therapies. The high-throughput PRR method is expected to be of value in continuously monitoring fast-killing properties during structure-activity relationship studies, expediting the identification and development of novel, rapidly acting antimalarial drugs within phenotypic drug discovery campaigns.

Keywords: malaria, drug discovery, high-throughput screening, lactate dehydrogenase, nitroreductase, fast-killing,

Introduction

Malaria is a parasitic disease caused by *Plasmodium* spp. infection, and typically is seen in tropical and subtropical areas. In 2021, over 420,000 deaths, predominantly among children under 5 years old, were reported worldwide, along with more than 200 million cases[1]. Artemisinin-based combination therapies (ACTs) are common treatments for malaria in endemic areas. Unfortunately, artemisinin-resistant parasites have spread across Southeast Asia and Africa, highlighting the need for new antimalarial drugs to combat this life-threatening parasitic disease[2–4]. In the last decade, the combination of phenotypic screening, and *in vitro* evolution and whole-genome analysis (IVIEWGA) has played a significant role in antimalarial drug development. This approach has contributed to the identification of several novel drug targets for malarial parasites, facilitating target-based HTS campaigns [5,6]. Despite the strong efforts in target-based drug discovery being conducted by the Medicines for Malaria Venture (MMV) and collaborating researchers, the development of novel antimalarial medicines often has been hampered by resistance of the malarial parasites to the drug candidates. For example, recrudescent parasites emerged in Phase II clinical trials of both of DSM265 and cipargamin that targets dihydroorotate dehydrogenase (DHODH) and the *Plasmodium falciparum* Na^+ pump PfATP4, respectively[7–9]. Although target-based drug discovery is an effective method for generating lead compounds, the targets identified by IVIEWGA carry the risk of potential

57 resistance, a major concern in the later stages of drug development. Therefore, there has been
58 renewed interest in phenotypic screening, with several of the resulting candidates now listed in
59 the current drug development pipeline of MMV.

60 Single-exposure radical cures (SERCs) are highly appealing, since this strategy
61 prevents parasite resistance and simplifies patients' treatment. However, SERCs
62 characteristically require fast-killing, as seen in next-generation lead compounds such as
63 MMV688533, INE963, ZY19489, and GSK484, the subjects of ongoing clinical trials[10–13]. The
64 standard method employed to determine the speed of killing of parasites, the parasite reduction
65 ratio (PRR), first was described in 2012; since then PRR has been utilized widely to assess the
66 killing properties of candidate compounds[14,15]. The conventional PRR assay has a relatively low
67 throughput due to the complexity of the protocol, requiring at least one month to yield results.
68 This aspect means that one cannot determine whether a given hit compound is a fast killer until
69 the candidate selection step in the drug development pipeline. Despite the crucial importance of
70 the PRR properties of next-generation lead compounds, no scalable assay method for
71 determining the PRR values of multiple compounds has been reported to date.

72 Phenotypic screening of the asexual blood stage (ABS) of malaria parasites often
73 employs the *P. falciparum* lactate dehydrogenase (PfLDH) assay to quantify parasite growth[16].
74 Specifically, PfLDH (in the lysate of a parasite culture) is coupled to the *Clostridium kluyveri*

75 diaphorase; the bacterial enzyme catalyzes a redox reaction that converts nitroblue tetrazolium
76 (NBT) to nitroblue formazan (NBF) via 3-acetyl pyridine adenine dinucleotide ($APAD^+$), a
77 nicotinamide adenine dinucleotide (NAD^+) analogue that exhibits cofactor specificity for PfLDH.
78 In the past, GlaxoSmithKline (GSK) employed the PfLDH assay to screen approximately 2 million
79 compounds in a 384-well plate format. However, of late, the SYBR green I-based fluorescence
80 assay, which detects parasite DNA, has become more commonly used for phenotypic HTS of the
81 ABS of malaria parasites. This shift occurred because the fluorescence assay was the only
82 method available for use in the 1536-well format[17,18]. Nevertheless, the simple principle of a
83 PfLDH assay that involves a redox enzyme and a detection probe coupled with the LDH reaction,
84 allows for easy tuning of the assay protocol depending on the specific purpose of the experiment.
85 In the present report, we describe the development of a modified PfLDH assay using
86 the nitroreducatase (NTR) encoded by the *Escherichia coli nfsB* gene. NTR, a flavoprotein, uses
87 NAD(P)H as an electron donor to reduce a variety of nitroaromatics[19]. To enhance the
88 conventional PfLDH assay's performance, we incorporated into the assay system "turn-on"
89 fluorescence probes that include a nitrobenzyl moiety[20,21] that is reduced by NTR. The use of a
90 near-infrared (NIR) probe with an extended wavelength range (excitation/emission
91 wavelengths ($\lambda_{ex/em}$) = 615/690 nm) compared to haemoglobin absorption (~600 nm)
92 effectively decreased interference from haemoglobin absorption in the lysate, resulting in an

93 increased signal window. This innovative approach using the NIR probe enabled us to conduct
94 phenotypic screening of the ABS of malaria in a 1536-well format, and improved the assay
95 robustness compared to that of the conventional SYBR green I assay. Moreover, the use of the
96 optimized PfLDH-NTR assay system streamlined the time-consuming protocol of conventional
97 PRR. A feasibility study with representative antimalarial drugs showed results consistent with
98 those obtained using the conventional PRR method. Our high-throughput PRR (HT-PRR) assay
99 therefore is expected to serve as a powerful tool for identifying, in the early stages of drug
100 discovery, novel antimalarial compounds with fast-killing profiles.
101
102 **Results**
103 **Coupling of NTR reaction with PfLDH**
104 Histidine-tagged NTR expressed in *E. coli* was purified successfully using conventional nickel-
105 nitrilotriacetic acid (Ni-NTA) agarose purification, yielding a specific activity in the range of 3–5
106 μmol/min/mg (Fig. 1a). The recombinant NTR converted nitrobenzyl-umbelliferone 1 (NCOU1)
107 to umbelliferone, such that the fluorescent signal of the product ($\lambda_{ex/em}$ = 315/455 nm) exhibited
108 a time-dependent increase during room temperature incubation (Fig. 1b, c). For our assay, PfLDH
109 was coupled with the purified NTR, rather than with the bacterial diaphorase used in the
110 conventional PfLDH assay, and parasite growth was quantified by monitoring the fluorescence

111 signal of umbelliferone (Fig. 2a). As anticipated, the NTR quantitatively reduced NCOU1 by using
112 the reducing equivalents from the APADH produced by the PfLDH enzyme present in the parasite
113 lysate. A titration of cultured *P. falciparum* using the PfLDH-NTR assay demonstrated that the
114 fluorescent signal increased in a parasitemia-dependent manner, with the highest signal
115 intensity seen under the condition of 1% haematocrit (Fig. 2b and Supplementary Fig. 1).

116 Next, we compared the screening performance, in a 384-well plate format, of the
117 PfLDH-NTR assay compared to the conventional PfLDH-diaphorase assay. Specifically, we used
118 the two assays to assess the growth inhibition of ABS parasites by a test set of 3,837 compounds
119 from Shionogi & Co., Ltd., using a compound concentration of 2.5 μM. A correlation (r^2 = 0.387)
120 was observed between the results obtained by the two methods, as depicted in Fig. 2c. While the
121 average signal-to-background (S/B) ratio for the NTR method (5.02) was lower than that of the
122 diaphorase method (8.31), the two assays demonstrated similar Z'-factor values (0.71 and 0.76,
123 respectively) (Table 1). Notably, the diaphorase method produced a higher number of hits (261
124 hits) than did the NTR method (123 hits). The PfLDH-diaphorase method may have selected for
125 a larger number of pseudo-positive hits, given that this assay's percent inhibition data did not
126 exhibit a Gaussian distribution and did not correlate with data obtained by other methods
127 (Supplementary Fig. 2, 3, 4). These results indicated that our fluorescence-based PfLDH assay,
128 which incorporates NTR and NCOU1, is more robust than the conventional PfLDH assay using

129 diaphorase.

130

131 **NIR probe with PfLDH-NTR assay improves screening robustness**

132 The PfLDH-NTR-NCOU1 assay was not suitable for a 1536-well format due to the low S/B ratio.
133 Consequently, our next objective was to improve this assay method using another probe with a
134 different wavelength of fluorescence emission. Given that the parasite lysate contains a
135 substantial amount of haemoglobin from red blood cells, we conjectured that the absorption of
136 haemoglobin (~600 nm) interferes with the fluorescent signal of umbelliferone[20]. Therefore, we
137 introduced another NIR probe, one that exhibits longer excitation and emission wavelengths
138 after reduction by NTR (Fig. 3a). We observed that, following reduction of the NIR probe by NTR
139 in the assay solution, the signal intensity of the reduced species (NIR-P^{red}), increased in a time-
140 dependent manner, permitting quantification of the malaria parasites (Fig. 3b, c). Given the read-
141 time need for measurement of output in the 1536-well format, we quenched the PfLDH reaction
142 by adding a high concentration of pyruvate (0.9 M), resulting in the complete inhibition of
143 NCOU1 reduction (Fig 3c). For reactions run in the 1536-well format, the parasite growth
144 inhibition detected by the PfLDH-NTR-NIR assay showed a strong correlation with that obtained
145 by the SYBR green I-based assay (Fig. 3d). Notably, the PfLDH-NTR-NIR assay achieved a higher
146 S/B ratio and Z'-factor (11.2 and 0.72, respectively) than those measured by the SYBR green I

147 assay (5.89 and 0.61), indicating that PfLDH-NTR-NIR assay is more robust than the existing

148 method when employed in a 1536-well format.

149

150 **High-throughput PRR (HT-PRR) assay**

151 In the ABS, LDH oxidizes NADH and provides NAD^+ to maintain the flux of anaerobic glycolysis

152 required for the synthesis of ATP, a function that is essential for parasite survival[22,23]. We

153 conceived the idea of using PfLDH activity as an indicator of parasite viability and applied the

154 PfLDH-NTR-NCOU1 method in a 384-well format to quantify the PRRs of test compounds. Mixed-

155 stage (non-synchronized) parasites at 1.5% parasitemia were incubated at 37 °C for 0, 24, 48,

156 72, and 96 hours with test compounds at concentrations of 10 μM. Live parasite signals were

157 quantified by the PfLDH-NTR-NCOU1 method and the PRR values were calculated for each

158 compound. The values then were normalized (as a percentage) to those of dihydroartemisinin

159 (DHA; defined as 100%), a representative fast-killing compound, and ELQ300 (defined as 0%),

160 an inhibitor of the electron transport chain (ETC) that is classified as a slow-killing compound

161 (Supplementary Fig. 5). At 24 hours, fast-killing compounds like ACT-451840, artemether, and

162 cipargamin exhibited PRR values exceeding 70%; chloroquine, MMV048, and ganaplacide,

163 categorized as moderate-speed-killing compounds, yielded PRR values in the approximate range

164 of 40–70%. Other known slow-killing ETC inhibitors, DSM265 and atovaquone, yielded lower

165 PRR values (16.9 and 8.6%, respectively). These results are consistent with the data obtained by
166 the conventional PRR method. The sole exception was DDD107498, a compound known to be a
167 slow-killing inhibitor by conventional PRR[24], show high PRR value. Given that this compound is
168 an inhibitor of the eukaryotic translation elongation factor 2 (eEF2)[24]; we hypothesize that the
169 interruption of protein synthesis by DDD107498 may directly impact PfLDH synthesis, leading
170 to lowered enzymatic activity, which our assay detects as an elevated PRR.

171 Subsequently, we sought to optimize the level of parasitemia in the assay by employing
172 our method to assess the effects, on parasites at various densities, of 24- and 48-hour exposure
173 to the known inhibitors atovaquone, DHA, and cipargamin (Supplementary Fig. 6). We observed
174 that when the starting parasitemia exceeded 2%, the signal for vehicle (dimethylsulfoxide;
175 DMSO)-treated parasites achieved a virtual plateau after 48 hours of incubation. Therefore, we
176 fixed the starting parasitemia at 1.5% for further experiments.

177 Having completed the optimization of HT-PRR, we conducted PRR assays in an 8-point
178 dose-response experiment. The dose-response PRR experiment was implemented because
179 during the test run, we observed that the shape of PRR dose curves differed among the
180 compounds (Supplementary Fig. 7). The experiment assessed PRR at intervals of 0, 24, 48, and
181 72 hours; and determined the 50% effective concentration to reduce the PRR values (PRR_{50}) of
182 the representative test compounds, in addition to the 72-hour cultures for standard growth

183 inhibition (EC_{50}) assays (Fig. 4a). A comparison of the inhibition curves revealed that Hill slopes
184 and plateau levels of the PRR curves varied among compounds and time points. To quantify the
185 shape of the dose-response curve and utilize these data as an indicator of PRR, we calculated for
186 each compound the ratio of area under the curve (AUC) between the growth inhibition curve
187 and the PRR curve, yielding a parameter that we refer to as the "AUC%" (Table 2). We observed
188 that the AUC% values of the fast-killing compounds exceeded 80%. In contrast, the slow-killing
189 compound exhibited lower AUC% values (of approximately 50%); furthermore, the % inhibition
190 by slow-killing compounds did not reach 100%, even at the highest tested concentration (1 μM).
191 These data suggested that robust AUC% values, calculated using multiple data points, permitted
192 effective classification of compounds according to their killing-rate properties.
193
194 **Discussion**
195 In the present work, we established a novel PfLDH-based assay method for detecting
196 antimalarial candidates targeting the ABS by using NTR and "turn-on" fluorescent probes with a
197 nitrobenzyl moiety. The new method provides an assay with robustness superior to that of
198 existing screening methods, and we successfully conducted HTS with a small compound library
199 in a 1536-well format. Our method also was able to assess the parasite survival rate, as assessed
200 by PfLDH activity. The PfLDH-NTR assay permits quantification of the PRR for many compounds

201 in a 384-well format, significantly decreasing experimental time. The PRR classification of
202 known antimalarial drugs by our HT-PRR method was consistent with the data obtained by
203 conventional assays. Moreover, HT-PRR permitted the determination of the dose-dependency of
204 a compound's PRR, yielding a new parameter (the AUC%) indicating the speed of killing. Our
205 novel PfLDH-NTR-based assay is expected to be valuable for determining the PRR values of many
206 hit compounds in the early stages of drug discovery, facilitating the development of next-
207 generation fast-killing antimalarial drugs.

208 In the field of cancer research, NTR is widely used for the *in vivo* detection of tumour
209 cells and the activation of prodrugs, reflecting the fact that NTR expression is specifically
210 increased in tumour cells growing under hypoxic conditions[25–27]. Additionally, NTR plays a
211 crucial role in the treatment of Chagas disease, a condition caused by *Trypanosoma cruzi*
212 infection; benznidazole and nifurtimox, front-line drugs used against Chagas, are activated by
213 NTR activity. Resistance to these drugs is determined primarily by loss of function mutations in
214 the *T. cruzi* NTR-encoding gene (including homozygous nonsense mutations and the loss of one
215 copy) that lead to decreased levels of the trypanosomal enzyme[28,29]. Given NTR's biological
216 significance and diverse applications in biomedical research, we envisioned using NTR to
217 establish innovative assays for the discovery of antimalarial drugs. Moreover, the tissue
218 transparency of the $\lambda_{ex/em}$ of NIR, which fall within the bio-optical window (650–1100 nm),

219 makes these wavelengths useful in biomedical research, especially for *in vivo* imaging, due to
220 lower haemoglobin absorption[30]. This property of the NIR probe fluorophore mitigates
221 interference from the abundant haemoglobin in red blood cells, greatly enhancing the signal
222 strength of the PfLDH assay. Through the introduction of a coupling enzyme and probes, we
223 improved the performance of the conventional PfLDH assay for detecting growth inhibition of
224 the malaria parasite at the ABS. In the future, it will be interesting to introduce modifications to
225 existing assay systems, thereby enhancing robustness and reducing cost. This initiative has the
226 potential to pave the way for the creation of advanced HTS systems applicable to other infectious
227 diseases, including *Trypanosoma* spp. and *Mycobacteria* spp.; indeed, revisions to facilitate
228 screens for these systems are currently in progress.

229 For HTS of the ABS of the malaria parasite in a 1536-well format, the commonly used
230 assay employs SYBR green I, a fluorescent dye with excitation and emission wavelengths of 485
231 nm and 528 nm, respectively. Our PfLDH-NTR assay with NIR^{ox} would be (to our knowledge) the
232 first system using NIR wavelengths to screen for antimalarial compounds. A noteworthy finding
233 is that a small number of hit compounds of the SYBR green I-based assay was not common with
234 that of the PfLDH-NTR with NIR^{ox} assay (Supplementary Fig. 4). The optical properties of small
235 organic compounds in chemical libraries may impact the readout signal, resulting in false-
236 positives and -negatives[31,32]. Indeed, a small number of compounds showed less than 40%

237 inhibition as assessed by the SYBR green I-based assay, possibly reflecting the optical properties
238 of these compounds (Supplementary Fig. 2). The PfLDH-NTR with NIR^{ox} assay may serve as a
239 viable alternative screening method to prevent the loss of promising compounds as pseudo-
240 negatives in future phenotypic HTS of the malaria parasite at the ABS.

241 To enhance the throughput in assessing PRR, we developed the PfLDH-NTR assay
242 system, enabling the HTS evaluation of antimalarial compounds based on PRR values. Notably,
243 we observed that the dose dependent PRRs of fast-killing compounds typically demonstrate
244 curves that resembled the growth inhibition curve of the same chemicals. However, disparities
245 between these two types of curves are more pronounced for moderate- and slow-killing
246 compounds. To quantify these differences, and to classify multiple test compounds according to
247 the PRR value, we defined the AUC%. This parameter provides a succinct description of the
248 parasite killing rate of test compounds and is expected to accelerate structure-activity
249 relationship (SAR) studies, particularly for fast-killing compounds. At the same time, we note
250 that a limitation of HT-PRR is that PRR values for compounds targeting protein synthesis (e.g.,
251 DDD107498) may be overestimated due to the dependence of the assay on PfLDH activity.
252 However, this limitation can be addressed by cross-referencing results with conventional PRR or
253 classical Giemsa staining at later stages of drug discovery.

254

255 **Conclusion**

256 We developed, in-house, NTR-based methods for the identification of compounds with
257 activity against the ABS of malaria parasites, and successfully implemented one such assay for
258 HTS in a 1536-well format. The use of the PfLDH-NTR method for PRR determination represents
259 a novel assay of the parasite-killing rate of drug candidates. These innovative new HTS methods
260 are expected to permit the identification of fast-killing compounds with novel scaffolds and
261 properties, paving the way for the discovery of next-generation antimalarial drugs that may find
262 use in combatting drug resistance in malaria-endemic areas.
263

264 **Methods**

265 **Preparation of recombinant enzymes**

266 Prior to assay development, we prepared two coupling enzymes, *Clostridium kluyveri* diaphorase
267 and *Escherichia coli* NAD(P)H nitroreductase NfsB (NTR), as previously described[19,33]. Briefly,
268 codon-optimized genes were synthesized to encode proteins with N-terminal His10-SUMO tags
269 and C-terminal His10 tags. These genes with C-terminal His10 were cloned separately into the
270 pET101-D-TOPO expression vector (Thermo Fisher Scientific), and the resulting plasmids were
271 transformed into *E. coli* BL21™ Star (DE3) cells according to the manufacturer's protocol. The
272 resulting *E. coli* strains were pre-cultured (overnight at 37 °C with shaking at 200 rpm) in Luria–
273 Bertani medium supplemented with 100 μg/mL carbenicillin. The resulting cultures were used
274 as inocula for 600-mL volumes of Terrific-Broth medium supplemented with 100 μg/mL
275 carbenicillin and 0.4% glycerol, and the resulting cultures were incubated at 37 °C with shaking
276 at 200 rpm. When the optical density at 600 nm (OD_{600}) reached 0.4 to 0.6, protein expression
277 was induced by adding 50 μM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma) and 1
278 mg/mL riboflavin (Sigma). After incubation for 16 h at 20 °C, the cells were harvested by
279 centrifugation at 7,000 × *g* for 10 min at 4 °C and re-suspended at a density of 0.4 g cell pellet/mL
280 in cold lysis buffer (50 mM potassium phosphate buffer, pH 8.0, 300 mM NaCl, 0.5 mM
281 ethylenediaminetetraacetic acid (EDTA) and 0.25 mM phenylmethylsulfonyl fluoride (PMSF)).
282 The suspended cells were lysed using a French press (Ohtake) at 180 MPa, and the supernatant

283 was collected by centrifugation at 40,000 × *g* for 30 min at 4 °C. The His-tagged proteins were
284 purified using Ni-NTA (Qiagen) following the manufacturer's protocol and eluted using lysis
285 buffer containing 200 mM imidazole. The eluate was concentrated using an Amicon Ultra
286 Centrifugal Filter 10 kDa Molecular Weight Cut-off (Merck) according to the manufacturer's
287 protocol, and the resulting purified enzyme was mixed with an equal volume of cold glycerol and
288 stored frozen at -30 °C. The protein concentrations in the final enzyme stocks were determined
289 by the Bradford assay using a Bio-Rad kit, and the specific activities of the enzymes were
290 quantified by measuring the reduction of 2,6-dichlorophenolindophenol (DCIP; Sigma) using a
291 UV760 spectrophotometer (Jasco). To confirm the purity of the enzymes, proteins were
292 separated by sodium dodecyl sulfate-polyacrylamide gel electrophoreses (SDS-PAGE) and
293 stained using GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific). The specific activities
294 of diaphorase and NTR were typically in the range of 100–150 and 3–5 μmol/min/mg,
295 respectively.
296
297 **Quantification of the reduction of fluorescence probes by NTR**
298 To test whether the purified NTR was capable of reducing fluorescence probes, we initially
299 measured NTR activity prior to using the enzyme for parasite quantification. Using a cuvette, 20
300 μM NCOU1 (Enamine) was combined with a mixture containing 5 μg/mL NTR, 50 μM NADH, and

301 0.25 % Triton X-100 in 30 mM Tris-HCl (pH 8.0), and the fluorescence of the umbelliferone

302 ($\lambda_{ex}/_{em}$ = 315/455 nm) generated by the reaction was measured. In addition, we synthesized the

303 NIR^{ox} probe according to the previously described route[21]. For the NIR reaction, 50 μM $NIR\text{-}P^{ox}$

304 was combined with a mixture containing 0.5 μg/mL NTR and 50 μM NADH in 30 mM Tris-HCl

305 (pH 8.0), and the fluorescence of $NIR\text{-}P^{red}$ ($\lambda_{ex}/_{em}$ = 615/670 nm) was measured. The

306 fluorescence kinetics of both reduced probes were monitored using a FP-6300

307 spectrofluorometer (Jasco).

308

309 **Parasite cultures**

310 *Plasmodium falciparum* 3D7 parasites were maintained in RPMI1640 medium supplemented

311 with 25 mg/L gentamicin, 50 mg/L hypoxanthine, 23.8 mM sodium bicarbonate, and 0.5% (w/v)

312 Albumax II (Gibco). The culture was maintained at 2% haematocrit using O^+ human erythrocytes

313 obtained from The Japanese Red Cross Society. As per established protocols[34,35], the incubation

314 temperature was set at 37 °C and the environment consisted of mixed gas (5% O_2, 5% CO_2, 90%

315 N_2). Parasitemia was determined using either Giemsa staining or an XN-30 automated

316 haematology analyser (Sysmex)[36]. For plate assays, ring-stage synchronized parasites were

317 prepared using sorbitol treatment[37]. In the growth inhibition assay, the compound solvent DMSO

318 and a mixture of 1 μM artemisinin/atovaquone served as negative and positive controls,

319 respectively.

320

321 **Diaphorase-NBT and NTR-NCOU1 assay in 384-well plates**

322 Test compounds were pre-dispensed at a volume of 100 nL/well into 384-well plates. For the
323 diaphorase-NBT assay, ring-stage parasites at 0.3% parasitemia and 2% haematocrit were
324 dispensed into 384-well clear plates (Corning); for the NTR-NCOU1 assay, ring-stage parasites
325 at 0.3% parasitemia and 1% haematocrit were dispensed into 384-well black plates (Greiner).
326 Parasites then were dispensed at 25 μL/well into the compound-containing plates using a
327 Multidrop Combi with a small metal cassette (Thermo Fisher Scientific). The parasite cultures in
328 the assay plates then were incubated in a moist chamber filled with mixed gas for 72 hours and
329 subsequently frozen overnight (or longer) at -30 °C. The resulting frozen plates were thawed at
330 room temperature for at least 2 hours before the assay was performed. For the diaphorase-NBT
331 assay, the reaction mix was prepared in modified PfLDH buffer (100 mM Tris-HCl, pH 8.0, 150
332 mM lithium L-lactate, and 0.25% Triton X-100) supplemented with 75 μM $APAD^+$, 0.2 mg/mL
333 NBT, and 1 U/mL diaphorase (one unit was defined as the amount of enzyme required to reduce
334 1 μmol of APADH per minute). For this assay, the reaction mixture was dispensed into the assay
335 plates at 70 μL/well, and the plates then were incubated at room temperature with agitation at
336 650 rpm for 20 minutes. On the other hand, the reaction mixture for the NTR-NCOU1 assay

337 consisted of modified PfLDH buffer supplemented with 100 μM $APAD^+$, 100 μM NCOU1, 0.05
338 U/mL NTR, and 0.015% (v/v) KM-70 defoaming agent (Shin-Etsu). For this assay, the reaction
339 mix was dispensed into the assay plates at 35 μL/well, and the plates then were incubated under
340 the same condition as those used for the diaphorase-NBT assay. The absorption of nitro blue
341 formazan (650 nm; for the diaphorase-NBT assay) or the fluorescence of umbelliferone ($\lambda_{ex/em}$ =
342 360/465 nm; for the NTR-NCOU1 assay) was measured using a SpectraMax® Paradigm
343 spectrophotometer (Molecular Devices, Inc.).
344
345 **NTR-NIR^{ox} assay in 1536-well plates**
346 For the HTS in 1536-well format, ring-stage parasites at 0.3% parasitemia and 1%
347 haematocrit were dispensed at 4 μL/well into 1536-well black plates (Greiner) containing 20
348 nL/well of test compounds. Parasite-dispensed assay plates were maintained under the same
349 conditions as described above for the 384-well plate assays. The NIR^{ox} probe was initially
350 suspended at 1 mM in 90% acetonitrile. The reaction mixture for the NTR-NIR^{ox} assay was
351 prepared by supplementing modified PfLDH buffer with 500 μM $APAD^+$, 200 μM NIR^{ox} probe,
352 and 0.05 U/mL NTR. For this assay, the reaction mixture was dispensed into the assay plates at
353 3 μL/well, and the contents of each well were mixed at 1,200 rpm. The plates then were
354 incubated at room temperature for 3 minutes. The enzymatic reactions were quenched by

355 dispensing the "stop" mixture (0.9 M sodium pyruvate containing 0.015% KM-70) at 3 μL/well,
356 and the assay plates then were centrifuged at 90 × *g* for 1 min at room temperature. The
357 centrifuged plates were incubated for 1 hour at room temperature in a moist chamber, at which
358 point the NIR^{red} signal ($\lambda_{ex/em}$ = 615/690 nm) was measured using a PHERAstar Plus microplate
359 reader (BMG LABTECH).
360
361 **SYBR green assay in 1536-well plates**
362 For the SYBR green assay, ring-stage parasites at 0.3% parasitemia and 3% haematocrit were
363 dispensed at 4 μL/well into 1536-well black plates (Greiner) containing 20 nL/well of test
364 compounds. Parasite-dispensed assay plates were maintained under the same conditions as
365 described above for the 384-well plate assays. The reaction mixture for the SYBR green I assay
366 consisted of lysis buffer (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 0.1% Triton X-100)
367 supplemented with 0.02% SYBR green I and 0.015% KM-70. This reaction mixture was
368 dispensed into the assay plates at 4 μL/well, and the contents of each well were mixed at 1,200
369 rpm. The plates then were incubated at room temperature for 1 hour. The fluorescence of SYBR
370 green I ($\lambda_{ex}/_{em}$ = 485/528 nm) was measured using a SpectraMax® Paradigm
371 spectrophotometer.
372

373 **Data analysis**

374 Hits of a test set of compounds were chosen by activity criteria [>3 standard deviation (SD)
375 cutoff of DMSO controls] that were calculated using Spotfire software (version 11.4.3; TIBCO). Z'
376 was calculated as $1 - (3 \times SD_{100\%} + 3 \times SD_{0\%})/(Mean_{100\%} - Mean_{0\%})$. The correlations
377 of %Inhibition and histograms were visualized with R (version 4.0.2). The inhibition curves and
378 EC_{50} values were determined using GraphPad Prism (version 8.4.3. GraphPad Software, Inc., San
379 Diego, California, USA) and Spotfire. Data visualization for the other figures was performed using
380 GraphPad Prism. The area under the curve (AUC) for both the HT-PRR assay and growth
381 inhibition assay in the 384-well plates was calculated using Spotfire software, and the AUC% was
382 calculated as 100 × [(AUC of HT-PRR assay) / (AUC of NTR-NCOU1 assay)]. Venny (version 2.1)
383 was used to make a Venn diagram.

384

385 **HT-PRR assay in 384-well plates**

386 Test compounds were pre-dispensed at 100 nL/well in 384-well plates, and replicate plates were
387 prepared. Non-synchronized parasites at 1.5% parasitemia and 1% haematocrit were dispensed
388 into five 384-well black compound-containing plates at 25 μL/well using a Multidrop Combi
389 with a small metal cassette. One of the plates was frozen at -30 °C right immediately following
390 dispensing of parasites (control plate; 0 hour), while the other plates were incubated in a moist

391 chamber filled with mixed gas for 24, 48, 72, or 96 hours; at the indicated time points, growth
392 was terminated by freezing overnight (or longer) at -30 °C (PRR plates). Additional plates for the
393 generation of the growth inhibition curve were prepared and subjected to the NTR-NCOU1 assay
394 using the procedure described in the previous section, with a 72-hour incubation period. The
395 fluorescence of umbelliferone ($\lambda_{ex/em}$ = 360/465 nm) was measured using a PHERAstar
396 microplate reader, and the relative LDH activity was calculated by dividing the fluorescence of
397 the PRR plates by that of the respective control plate. The PRR inhibition percentage was
398 calculated by normalizing the primary values to the LDH activity obtained in the negative and
399 positive control reactions (compound solvent DMSO and 1 μM dihydroartemisinin, respectively).
400 In addition, the concentration of each compound to reduce the PRR value to 50% (PRR_{50}) was
401 calculated in each time point.
402

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407

408 **Abbreviations Used**

409 PfLDH, *Plasmodium falciparum* lactate dehydrogenase; NTR, nitroreductase; PRR, parasite
410 reduction ratio; ACTs, artemisinin-based combination therapies; IVIEWGA, *in vitro* evolution and
411 whole-genome analysis; MMV, Medicines for Malaria Venture; DHODH, dihydroorotate
412 dehydrogenase; SERCs, single-exposure radical cures; ABS, asexual blood stage; NBT, nitroblue
413 tetrazolium; NBF, nitroblue formazan; $APAD^+$, 3-acetyl pyridine adenine dinucleotide; NIR, near-
414 infrared; NCOU1, nitrobenzyl-umbelliferone 1; ETC, electron transport chain; DHA,
415 dihydroartemisinin; eEF2, elongation factor 2; PMSF, phenylmethylsulfonyl fluoride; DCIP, 2,6-
416 dichlorophenolindophenol
417

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Author contributions

D.K.I., T.K., and K.K. directed the work. T.S., R.I., and E.Y. performed the experiments. T.S., R.I., and E.Y. analysed the data. T.S., R.I., E.Y., and D.K.I. wrote the paper. All authors directly participated in this work, including preparation of the manuscript and approval of the final version. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

RI and TK are employees of Shionogi & Co., Ltd..

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536 **Tables**

537 **Table 1: Parameters for each assay method**

Method	Plate format	S/B	CV (%)		Z'	Number of hits	Hit rate (%)
			Negative	Positive			
Diaphorase-NBT	384	8.31	8.1	2.6	0.71	261	6.8
NTR-NCOU1	384	5.02	5.6	3.9	0.76	123	3.2
SYBR green	1536	5.89	9.6	6.2	0.61	66	1.7
NTR-NIR	1536	11.2	8.0	6.2	0.72	70	1.8

538 NBT: nitroblue tetrazolium, NTR: nitroreductase, NCOU1: nitrobenzyl-umbelliferone 1, NIR:

539 near-infrared, S/B: signal/background ratio, CV: coefficient of variation

540

541 **Table 2: Activity and AUC% of test compounds**

Compound	PRR_{50} (nM)			EC_{50} (nM)	AUC%		
	24 h	48 h	72 h		24 h	48 h	72 h
ACT-451840	2.77	2.72	4.07	1.95	89.2	95.6	90.6
Artemether	16.5	16.4	35.2	6.34	87.1	88.2	77.3
DHA	3.36	4.13	11.8	2.05	94.2	93.9	81.8
Cipargamin	1.61	1.17	1.15	1.29	88.6	98.2	100.1
DDD-107498	2.70	3.12	3.67	0.84	76.1	84.0	85.0
Chloroquine	16.9	11.3	10.4	4.85	78.6	86.6	89.1
Ganaplacide	117.5	30.0	15.9	7.05	55.1	71.9	80.0
Lumefantorine	24.7	15.3	15.0	7.28	67.9	67.4	66.7
Mefloquine	52.9	54.5	66.7	15.9	76.1	72.3	71.7
MMV048	276.9	209.6	23.5	23.9	62.6	72.1	78.3
Atovaquone	124.9	15.0	20.8	0.57	49.5	51.4	46.9
DSM265	1186	211.5	332.4	5.84	50.6	51.7	50.2
ELQ-300	9990	139.3	298.5	24.8	40.3	56.9	48.5
Pyrimethamine	9990	9990	9990	15.3	10.1	21.5	0.00
Cytochalasin D	9990	15.9	24.1	20.5	24.7	65.3	86.8

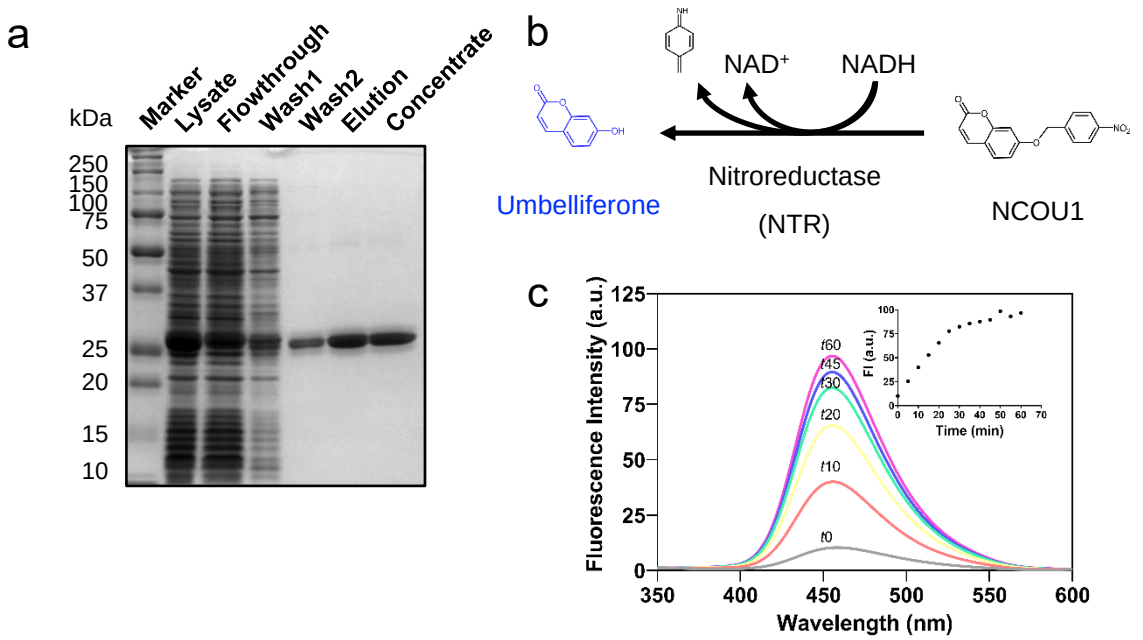
542 PRR_{50}: Concentration of the compounds required to reduce the PRR values to 50%; EC_{50}:

543 effective concentration to reduce the parasite growth to 50%; AUC: area under the curve; DHA:

544 dihydroartemisinin

545

546 **Figures**



547 **Figure 1. Purification of recombinant nitroreductase (NTR) and quantification of**

548 **enzymatic activity. a** Recombinant C-terminally His_{10}-tagged NTR was expressed in *E. coli* BL21

549 Star (DE3) and purified using Ni-NTA agarose. In the lanes denoted as Marker, Lysate,

550 Flowthrough, Wash1 and Wash2, a total of 10 μg protein were loaded. For Elution and

551 Concentrated lanes, 2 μg were loaded. Estimated protein size is 26.7 kDa. **b** Scheme of the

552 enzymatic reaction catalyzed by NTR reducing NCOU1 to umbelliferone ($\lambda_{ex/em}$ = 315/455 nm).

553 **c** The fluorescent spectra of umbelliferone. Time-dependent increases in the fluorescence

554 spectra were recorded for 60 minutes at room temperature in NTR assay buffer (30 mM Tris-

555 HCl, pH 8.0, 0.25 % Triton X-100) containing 5 μg/mL NTR, 20 μM NCOU1, and 50 μM NADH

556 ($\lambda_{ex/em}$ = 315/350–600 nm over 0.5-nm intervals, recorded every 5 minutes). For better

557 visualization, only the spectra from time points at 0, 10, 20, 30, 45, and 60 minutes are shown

558 (grey: 0 min, orange: 10 min, yellow: 20 min, light blue: 30 min, blue: 45 min, purple: 60 min).

559 Fluorescence intensity (FI) over time ($\lambda_{ex/em}$ = 315/455 nm) is plotted in the inset. NCOU1:

560 nitrobenzyl-umbelliferone 1, NAD^+: oxidized nicotinamide adenine dinucleotide, NADH:

561 reduced nicotinamide adenine dinucleotide, a.u.: arbitrary unit.

562

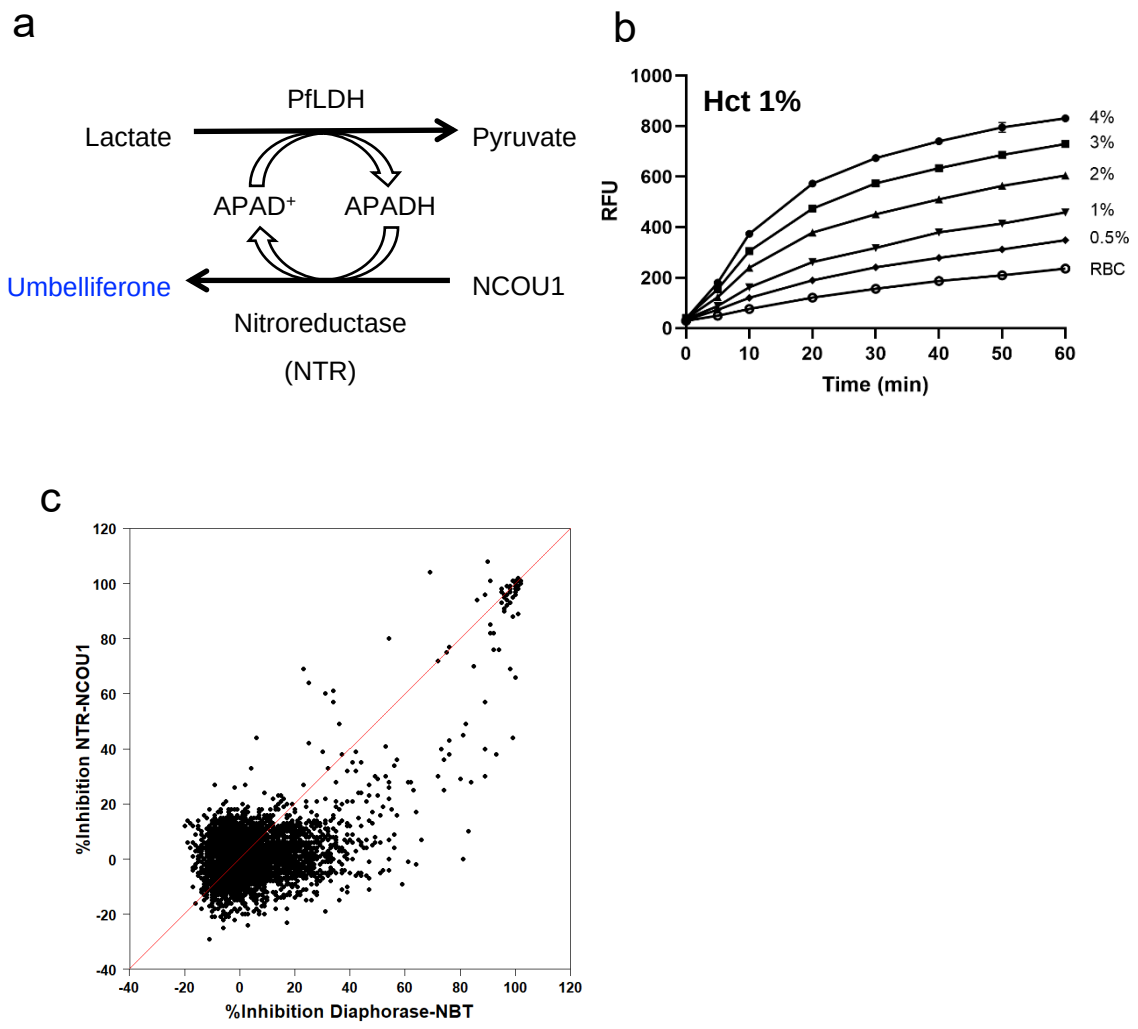


Figure 2. Novel assay using nitroreductase (NTR) coupled with *Plasmodium falciparum* lactate dehydrogenase (PfLDH).

a Scheme of the PfLDH-NTR coupled reaction used for quantification of parasite growth using NCOU1 as the NTR probe. **b** Parasitemia titration measured by the NTR-NCOU1 assay. The umbelliferone signal increased in a parasitemia-dependent manner. Each parasite culture (1% haematocrit (Hct)) was assayed with PfLDH buffer (100 mM Tris-HCl (pH 8.0), 150 mM lithium L-lactate, and 0.25 % Triton X-100) containing 100 μM $APAD^+$, 100 μM NCOU1, and 0.05–0.1

570 U/mL NTR at room temperature. **c** Correlation of %Inhibition between conventional

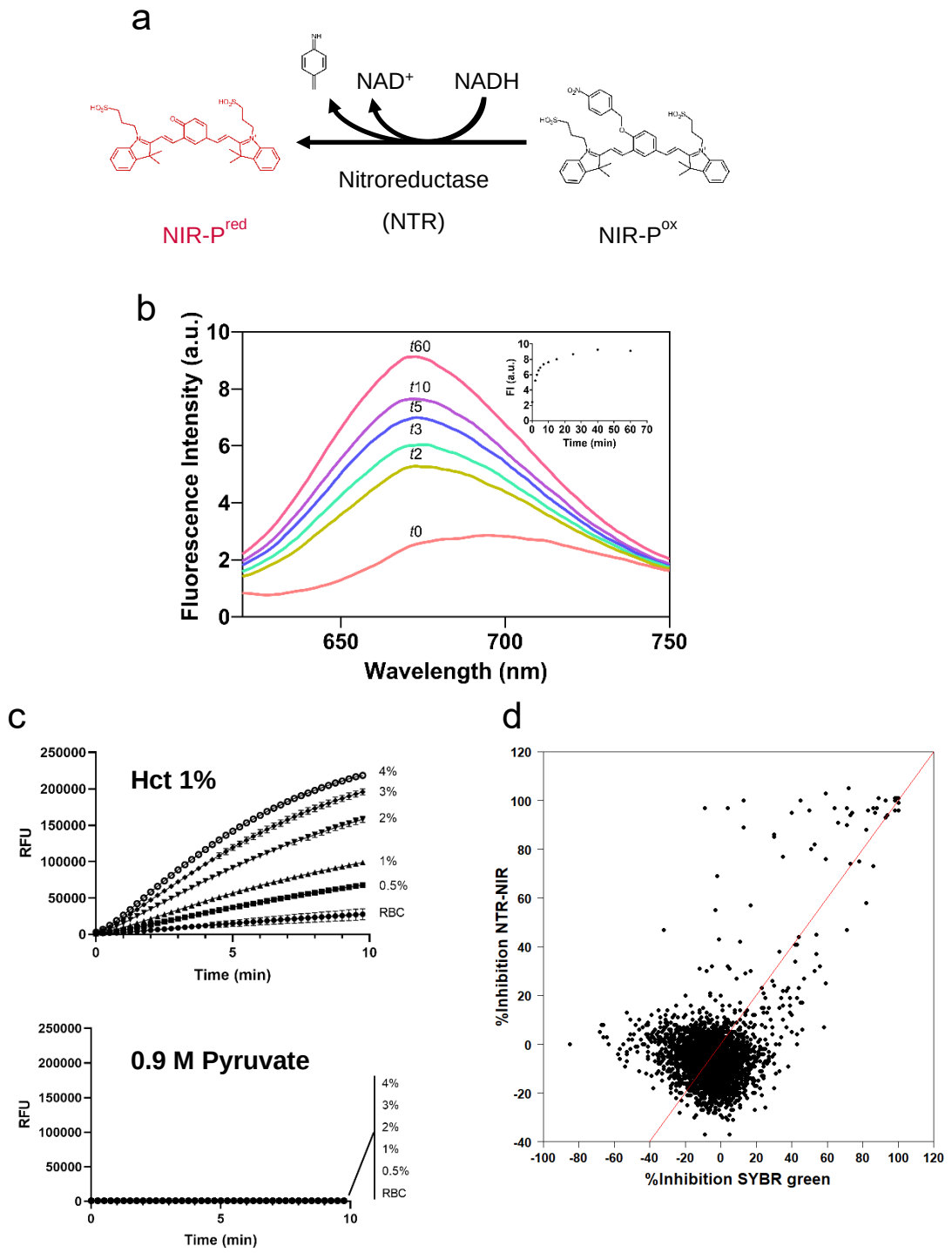
571 diaphorase-NBT and NTR-NCOU1 assays of PfLDH activity. The red line indicates unity (y = x).

572 NCOU1: nitrobenzyl-umbelliferone 1, $APAD^+$: oxidized 3-acetylpyridine adenine dinucleotide,

573 APADH: reduced 3-acetylpyridine adenine dinucleotide, RFU: relative fluorescence units. RBC:

574 red blood cell.

575



576 **Figure 3. Nitroreductase (NTR) assay using near-infrared probe (NIR-P). a** Scheme of

577 enzymatic reaction of NTR and NIR-P^{ox} producing the fluorescent NIR-P^{red} ($\lambda_{ex/em}$ = 615/670

578 nm**). b** Time-dependent fluorescent spectra of NIR-P^{red} produced by NTR. The enzymatic

579 reaction catalyzed by NTR was monitored using 50 μM NIR-P^{ox} and 50 μM NADH in 30 mM Tris-
580 HCl, pH 8.0, buffer containing 0.5 μg/mL NTR. Fluorescence signals ($\lambda_{ex/em}$ = 605/620–740 nm
581 over 1-nm intervals) were measured over a 60-minute period at room temperature (orange: 0
582 min, yellow: 2 min, light green: 3 min, blue: 5 min, purple: 10 min, red: 60 min). Fluorescence
583 intensity (FI) over time is plotted in the inset. Note that the reduction of NIR-P by NTR is much
584 faster than the reaction observed using the NCOU1 substrate (Fig. 1c). **c** Parasitemia titration
585 measured using the NTR-NIR assay. Each parasite culture (1% haematocrit (Hct)) was assayed
586 in PfLDH buffer (100 mM Tris-HCl, pH8.0, 300 mM lithium L-lactate, and 0.25% Triton X-100)
587 containing 500 μM $APAD^+$, 200 μM NIR probe, and 0.05 U/mL NTR at room temperature. The
588 quenching ("stop") solution consisted of 0.9 M pyruvate. Data are presented as the mean ±
589 standard deviation (SD) (n=4). **d** Correlation of %Inhibition between conventional SYBR green
590 and NTR-NIR assays of PfLDH activity. The red line in the graph represents the line of equality
591 (y = x). a.u.: arbitrary unit, NAD^+: oxidized nicotinamide adenine dinucleotide, NADH: reduced
592 nicotinamide adenine dinucleotide, RFU: relative fluorescence units.
593

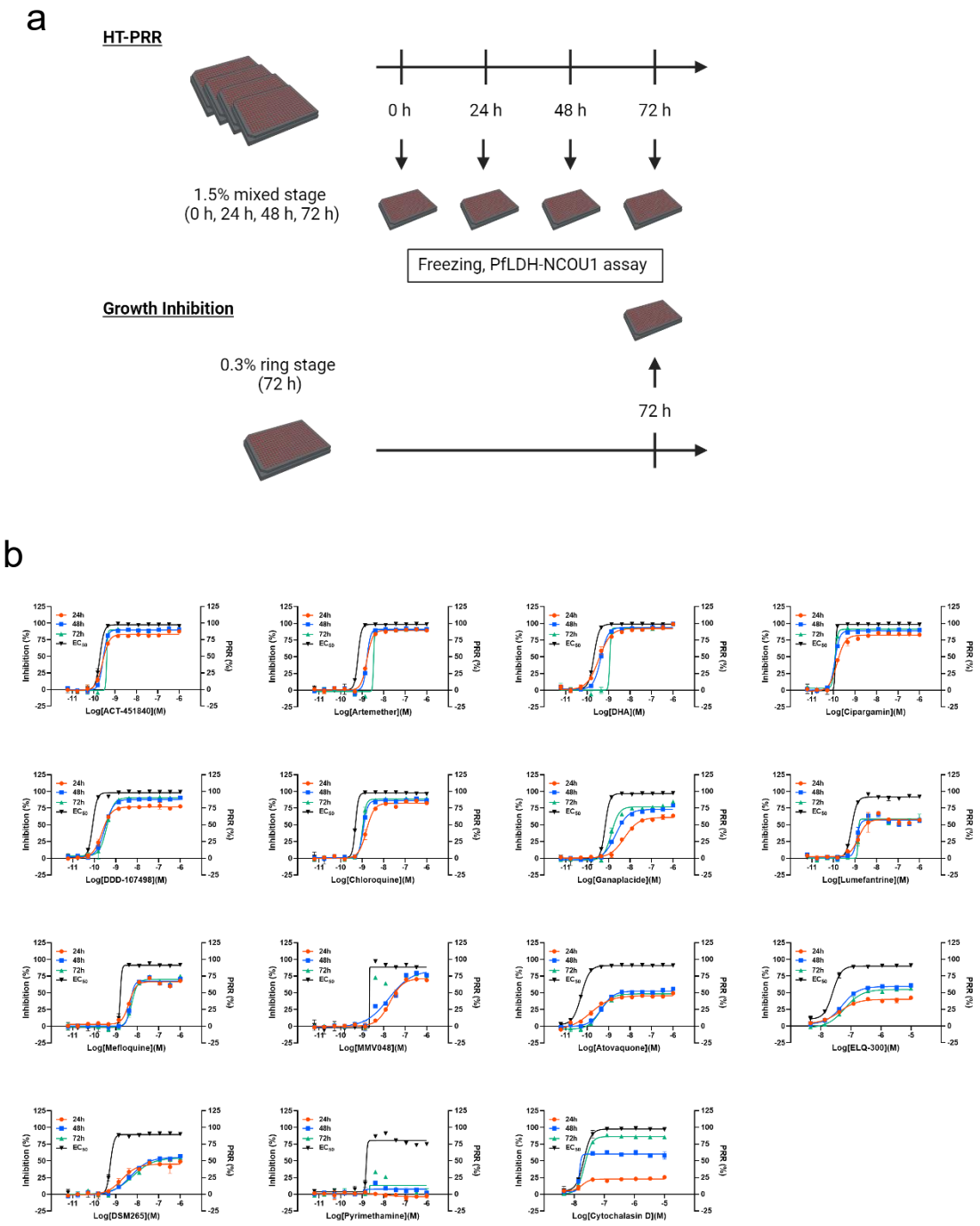


Figure 4. Schematic representation of high-throughput parasite reduction ratio (HT-PRR) and assessment of well-characterized antimalarial agents. a To evaluate the parasite reduction ratio (PRR) by established HT-PRR methods, non-synchronized 3D7 parasites

597 (haematocrit (Hct) 1%, parasitemia 1.5%) were plate-cultured for 0, 24, 48, and 72 hours in the
598 presence of compound. For the growth inhibition assay, 3D7 parasites were synchronized by
599 exposure to 5% sorbitol; ring stage parasites then were plate-cultured for 72 hours in the
600 presence of compound. Growth was terminated at the indicated time point by freezing (at -30 °
601 C) the respective assay plates; the number of live parasites then was quantified via an LDH assay
602 using retororeductase (NTR) and nitrobenzyl-umbelliferone 1 (NCOU1). For the growth
603 inhibition assay, the positive control consisted of a mixture of 1 μM atovaquone and artemisinin;
604 the negative control consisted of 0.4% dimethylsulfoxide (DMSO). For the HT-PRR assay, the
605 positive and negative controls consisted of 1 μM dihydroartemisinin (DHA) and 0.4% DMSO,
606 respectively. **b** Dose-response curves of Inhibition (%) and PRR (%) by test compounds were
607 generated for each assay condition (n =2).