

1 **Novel endochin-like quinolones exhibit potent *in vitro* activity against *Plasmodium***
2 ***knowlesi* but do not synergise with proguanil**

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15 Running title: New quinolones show cross-species antimalarial potency

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20 **Abstract**

21 Quinolones, such as the antimalarial atovaquone, are inhibitors of the malarial
22 mitochondrial cytochrome *bc*₁ complex, a target critical to the survival of both liver and
23 blood stage parasites, making these drugs useful as both prophylaxis and treatment.
24 Recently, several derivatives of endochin have been optimised to produce novel
25 quinolones that are active *in vitro* and in animal models. While these quinolones exhibit
26 potent *ex vivo* activity against *Plasmodium falciparum* and *P. vivax*, their activity against
27 the zoonotic *P. knowlesi* is unknown. We screened several of these novel endochin-like
28 quinolones (ELQs) for their activity against *P. knowlesi in vitro*, and compared this with
29 their activity against *P. falciparum* tested under identical conditions. We demonstrate that
30 ELQs are potent against *P. knowlesi* (EC₅₀ values <117 nM), and equally effective against
31 *P. falciparum*. We then screened select quinolones and partner drugs using a longer
32 exposure (2.5 life cycles), and show that proguanil is 10-fold less potent against *P.*
33 *knowlesi* when compared with *P. falciparum*, while the quinolones demonstrate similar
34 susceptibility. Finally, we used isobologram analysis to compare combinations of the ELQs
35 with either proguanil or atovaquone. We show that all quinolone combinations with
36 proguanil are synergistic against *P. falciparum*. However, against *P. knowlesi*, no evidence
37 of synergy between proguanil and the quinolones was found. Importantly, combining the
38 novel quinolone ELQ-300 with atovaquone, was synergistic against both species. Our data
39 identify potentially important species differences in proguanil susceptibility and its
40 interaction with quinolones, and support the ongoing development of novel quinolones as
41 potent antimalarials that target multiple species.

42 **Introduction.**

43 Malaria continues to exert a significant burden on humanity with around 228 million
44 infections estimated in 2018, an increase from the 217 million infections estimated for
45 2014 (1). The World Health Organisation currently recommends artemisinin-based
46 combination therapies (ACT) as the first-line treatment of uncomplicated malaria. These
47 are composed of a potent, but short-lived artemisinin derivative combined with a long-
48 acting partner drug (2). By using drugs in combination with different targets the intention is
49 to delay the emergence of resistance to the individual components. However, recent
50 evidence has emerged in the Greater Mekong subregion of resistance to both artemisinin
51 (3, 4) and current partner drugs (5, 6). There is, therefore, an urgent need to develop new
52 drugs and novel combination regimens before reduction in ACT efficacy occurs more
53 widely.

54 Quinolones have been investigated as potential antimalarial agents since the Second
55 World War (7). However, the only successful candidate from this class to emerge from
56 these studies as an antimalarial has been atovaquone. Atovaquone targets the
57 mitochondrial cytochrome *bc*₁ complex (8, 9) and is highly potent against *Plasmodium*
58 species. Unfortunately, recrudescence after atovaquone monotherapy occurs rapidly.
59 Atovaquone is therefore used in combination with a synergistic partner drug, proguanil
60 (10), but even this combination is vulnerable to mutations in *pf**cytb* especially in areas of
61 cycloguanil resistance (11, 12). Although the target of proguanil is not currently known,
62 proguanil has recently been shown to increase in potency against *Plasmodium falciparum*
63 after a longer *in vitro* exposure (13). Considering that the mitochondrial targeting
64 atovaquone-proguanil combination differs in its mechanism of resistance to the
65 components of current ACT, this combination has been investigated as an alternative
66 treatment of multidrug resistant malaria infections (14), though it should be noted that the
67 atovaquone-proguanil combinations tested performed poorly (only 90-92% effective at 42

68 days post-treatment) in that region of Cambodia, and are unlikely to be useful as
69 treatment. Furthermore, drugs targeting the mitochondria kill both liver- and blood-stage
70 malaria infections, and so can be used for both prophylaxis and treatment.

71 New quinolones based on endochin, a compound shown to be active at clearing avian
72 malaria (15), have recently been synthesised and tested against malaria parasites (16-18).
73 These endochin-like quinolones (ELQ) are equally effective in *ex vivo* blood stage screens
74 against *P. falciparum* and *P. vivax* clinical field isolates, and are also effective against
75 exoerythrocytic forms of rodent and monkey (*P. cynomolgi*) malaria (16). However, the
76 activity of ELQs against the zoonotic *P. knowlesi*, an increasingly important cause of
77 human malaria in Southeast Asia, is unknown. Importantly, recent articles have identified
78 differences in *in vitro* (19, 20) and *ex vivo* (21) susceptibility between *P. knowlesi* and *P.*
79 *falciparum* to established and experimental antimalarial agents. In particular, *in vitro*
80 studies demonstrated that *P. knowlesi* is up to 8-fold less susceptible than *P. falciparum* to
81 inhibitors of dihydroorotate dehydrogenase (e.g., DMS265 (20)), 6-fold less susceptible to
82 ATP4 inhibitors (e.g., cipargamin, SJ733 (19)), around 3-fold less susceptible to
83 cladosporin and pentamidine, and 66-fold less susceptible to the oxaborole AN13762 (19).
84 Conversely, *P. knowlesi* was shown to be 10-fold more susceptible to dihydrofolate
85 reductase inhibitors (e.g., pyrimethamine, cycloguanil (20)), around 4.5-fold more
86 susceptible to ganaplacide (KAF156), and over 3-fold more susceptible to halofantrine
87 (19). In spite of the reduced susceptibility of *P. knowlesi* compared with *P. falciparum*,
88 many antimalarials remain potent against *P. knowlesi in vitro* (e.g., 6 nM for cipargamin),
89 and any clinical significance of these reported species differences is yet to be established.

90 Here we test the *in vitro* activity of endochin and an ELQ series for activity against *P.*
91 *knowlesi*, and compare this to the activity of a quinolone-sensitive, reference *P.*
92 *falciparum* line (3D7) under identical experimental conditions, exposed for a single asexual
93 erythrocytic parasite life cycle (i.e., 27 h for our *P. knowlesi* A1-H.1 clone (22) and 48 h for

94 *P. falciparum* 3D7 clone). We then assess the impact of longer exposures to proguanil and
95 select ELQs on the susceptibility of our *P. knowlesi* and *P. falciparum* lines. Finally, we use
96 isobologram analysis *in vitro* to test for evidence of synergy between proguanil, or
97 atovaquone, and ELQ compounds against both species.

98 **Results and Discussion.**

99 Endochin and six endochin-like quinolones (ELQ) were screened under identical *in vitro*
100 conditions across one complete asexual erythrocytic life cycle against both the *P. knowlesi*
101 A1-H.1 and the *P. falciparum* 3D7 lines (Table 1). All but one (ELQ-271) of the ELQ
102 compounds were potent against the *P. knowlesi* line with EC₅₀ values under 100 nM. The
103 potency of endochin and the ELQ compounds was similar against both *P. knowlesi* and *P.*
104 *falciparum*, with a less than two-fold difference observed between species. With the
105 exception of ELQ-300 all the quinolones screened were more active against *P. falciparum*
106 (Table 1), though for endochin and ELQ-331 the differences were not significant ($p =$
107 0.6233 and $p = 0.5014$, respectively; Table 1). ELQ-400 and ELQ-480 are both active at
108 under 10 nM making them more potent than chloroquine but not as active as
109 dihydroartemisinin (Table 1).

110 *P. falciparum* exhibits significantly enhanced susceptibility to proguanil when incubated for
111 more than one life cycle (13). Therefore, in preparation for *in vitro* combination analysis
112 (isobolograms), we screened ELQ-300 and ELQ-400 as well as proguanil and atovaquone
113 using a longer incubation time (2.5 life cycles). We had previously found no activity for
114 proguanil at 10 μM (the highest concentration we tested) after a single life cycle exposure
115 against either *P. knowlesi* or *P. falciparum* (data not shown). However, with a longer
116 exposure (2.5 cycles), we observed an EC₅₀ value for proguanil of 2461 ± 236 nM for *P.*
117 *knowlesi*, over ten-fold higher than the EC₅₀ value that we observed for *P. falciparum* 3D7
118 clone (228 ± 29 nM; Table 2). We expect natural variability within our EC₅₀ values as our
119 assays are run using asynchronous parasite populations, and because the parasites have
120 different life cycle lengths, meaning drugs are exposed longer to *P. falciparum* per life
121 cycle than for *P. knowlesi*. Hence, we only consider a greater than three-fold change in
122 EC₅₀ between species as a potentially important species difference (19).

123 Atovaquone, ELQ-300, and ELQ-400 were all more potent after the longer exposure.
124 Atovaquone potency increased around three-fold from 2.5 nM (20) to 0.7 nM (Table 2),
125 and was not significantly different between species. ELQ-300 and ELQ-400 were also
126 more potent after longer *in vitro* exposures (Tables 1 and 2). Both compounds were now
127 more active against *P. knowlesi* than *P. falciparum* ($p < 0.0072$), though the fold difference
128 between species was small (< 3-fold).

129 Based on these data, combination studies were then designed to explore the *in vitro*
130 interactions between the compounds. These experiments were also run over multiple life
131 cycles to take into account the increased potency of proguanil after longer exposures (13).
132 As has been shown previously (10, 13, 23), atovaquone is synergistic in combination with
133 proguanil against *P. falciparum* (Fig. 1A, Table 3). The investigational quinolones ELQ-300
134 and ELQ-400 were also synergistic when combined with proguanil against our *P.*
135 *falciparum* line (Fig. 1B, 1C), confirming previous observations for ELQ-300 (16).
136 Surprisingly, neither atovaquone, nor the ELQ compounds demonstrated a synergistic
137 interaction in combination with proguanil when tested against *P. knowlesi*. Instead, all
138 interactions were additive/indifferent (Fig. 1D-F, Table 3). Without knowing the target of
139 proguanil or understanding its mechanism of action, it is not possible to speculate on the
140 reason for this species difference. Clearly, the ten-fold lower proguanil activity against *P.*
141 *knowlesi* (not observed with quinolone activity) coupled with the lack of synergism with
142 quinolones suggest a species difference in the inhibitory activity of this biguanide.

143 An alternative drug combination strategy for quinolones is suggested by recent data
144 indicating that quinolones can inhibit the cytochrome *bc*₁ complex (cyt *bc*₁) at either the
145 quinol oxidase (*Q*_o) or quinone reductase (*Q*_i) site (24). Atovaquone and ELQ-400 are *Q*_o
146 site inhibitors (8, 24), while ELQ-300 was shown to target the *Q*_i site (24). Isobolograms
147 combining a *Q*_o site inhibitor (atovaquone) with a *Q*_i site inhibitor (ELQ-300) have
148 previously demonstrated a moderately synergistic interaction against *P. falciparum* strain

149 D6 *in vitro* (25). We confirm this moderately synergistic interaction between atovaquone
150 and ELQ-300 against our *P. falciparum* 3D7 line (Fig. 2A) and show also a moderately
151 synergistic interaction with this combination against our *P. knowlesi* line (Fig. 2C and Table
152 3). Combinations of atovaquone with ELQ-400, both inhibitors of the Q_o site, were
153 additive/indifferent against *P. falciparum* (Fig. 2B) and *P. knowlesi* (Fig. 2D and Table 3).
154 Therefore, a more appropriate combination partner for the Q_o site inhibitor, atovaquone,
155 should perhaps be a Q_i site inhibitor (such as ELQ-300) which (a) is considerably more
156 potent than proguanil, and (b) demonstrates moderate synergism in combination with
157 atovaquone against both *P. falciparum* and *P. knowlesi* species *in vitro*, unlike
158 combinations with proguanil.

159 *In vivo*, proguanil is metabolized to cycloguanil by the liver cytochrome P450 (CYP2C19)
160 (26, 27). Cycloguanil is an inhibitor of the enzyme dihydrofolate reductase (DHFR), a
161 component of the folate pathway in malaria parasites. Thus, the drug combination
162 atovaquone-proguanil actually serves as a triple drug therapy of atovaquone (cytochrome
163 *bc₁* inhibitor), proguanil (target unknown), and its metabolite cycloguanil (DHFR inhibitor).
164 Cycloguanil, like atovaquone, has been shown to be highly potent against *P. knowlesi in*
165 *vitro* (20). Therefore, even though antagonistic interactions between atovaquone and
166 cycloguanil have been described *in vitro* (13), the low nanomolar potency of both
167 cycloguanil and atovaquone (20) should still support this combination for *P. knowlesi*
168 infections, despite the reduced activity of proguanil, and its lack of synergy reported here.
169 In light of the above-mentioned data, the recent strategy proposed to block the cyclization
170 of proguanil, thereby reducing its metabolism to cycloguanil, ought to be approached with
171 caution (13). In the absence of cycloguanil, and with the reduced activity of proguanil,
172 atovaquone may be exposed as a monotherapy against *P. knowlesi* infections. It will
173 therefore be critical to screen the cyclization blocked tert-butyl proguanil (13) for its activity
174 against *P. knowlesi*, and to test it in combination studies with quinolones in this species.

175 To our knowledge this is the first study to demonstrate differences in drug interactions
176 between two human malaria species. We have reported previously that compounds in
177 human trials for malaria (e.g., DSM 265, cipargamin) exhibit reduced *in vitro* susceptibility
178 to *P. knowlesi* (19, 20), similar to our observations here for proguanil. Considering all new
179 malaria treatments will likely comprise of combinations of drugs, it will be critical to ensure
180 that new combinations involving compounds with reduced susceptibility against *P.*
181 *knowlesi*, interact similarly across species.

182 Resistance to 10 nM atovaquone ($5 \times EC_{50}$) is induced readily *in vitro* after exposure to
183 only 10^5 parasites of the *P. falciparum* clone W2, or exposure to 10^6 parasites of the 3D7
184 or FCR3 clones (28). Furthermore, exposure of 10^8 parasites of the *P. falciparum* Dd2
185 clone to 10 nM atovaquone ($10 \times EC_{50}$) also selected resistant parasites, but no resistant
186 parasites emerged to 150 nM ELQ-300 (also $10 \times EC_{50}$) at the same inoculum (16). This
187 suggests that the new endochin-like quinolones demonstrate a lower propensity to induce
188 resistance in that parasite clone (16). Similar tests should now be performed on the *P.*
189 *knowlesi* A1-H.1 line and other newly adapted *P. knowlesi* lines to explore the propensity
190 of this species to develop resistance to the various quinolones.

191 In conclusion, novel endochin-like quinolones exhibit strong antimalarial activity (EC_{50}
192 values <117 nM) against *P. knowlesi in vitro*, and are equipotent against *P. falciparum*. We
193 demonstrate for the first time that quinolone combinations with proguanil lack synergy
194 against *P. knowlesi in vitro*, suggesting distinct mechanisms of action in the malaria
195 parasites. In contrast, combinations of inhibitors targeting the cytochrome *bc₁* complex at
196 the Q_o site (e.g., atovaquone) with those targeting the Q_i site (e.g., ELQ-300) show
197 moderate synergism against both species.

198 **Materials and Methods.**

199 *Drugs and experimental compounds.* Proguanil hydrochloride (product no. G7048) was
200 purchased from Sigma-Aldrich UK. Atovaquone was obtained from the Medicines for
201 Malaria Venture. Endochin-like quinolones were synthesised as described below.

202 *Chemical Synthesis.* The chemical synthesis of endochin was performed as originally
203 described by Andersag and others in 1948 (29), while methods for ELQ-271 and ELQ-300
204 were described by Nilsen *et al.* in 2014 (17). Methods for preparing ELQ-316 were described
205 by Doggett *et al.* in 2012 (30). Preparation of ELQ-331 was described previously by Frueh
206 *et al.* (31). Chemical synthesis of ELQ-400 proceeded by the methods of Stickles and
207 coworkers in 2015 (32). Synthesis and characterization of ELQ-480 are described below.

208 **5-fluoro-7-methoxy-2-methyl-3-(4-(4-(trifluoromethoxy)phenoxy)phenyl)quinolin-**

209 **4(1H)-one (ELQ-480):** ELQ-480 was synthesized according to the methods described by
210 Nilsen *et al.* in 2014 (17). Purity of ELQ-480 was assessed as >95% by proton NMR. ¹H-
211 NMR spectra were obtained using a Bruker AMX-400 NMR spectrometer operating at
212 400.14 MHz in DMSO D₆. The NMR raw data were analyzed using the iNMR Spectrum
213 Analyst software. Proton chemical shifts were reported in parts per million units (ppm), (δ)
214 relative to the residual proton at 2.54 ppm in deuterated DMSO D₆. J coupling constants
215 values are in Hertz (Hz). Coupling constants for ¹⁹F NMR operating at 376 MHz were also
216 obtained for compounds containing fluorine elements for additional validation of structure.
217 NMR spectrum of ELQ-480: ¹H-NMR (400 MHz; DMSO-d₆): δ 11.55 (s, 1H), 7.42 (d, *J* = 8.1
218 Hz, 2H), 7.25 (d, *J* = 8.3 Hz, 2H), 7.15 (d, *J* = 8.7 Hz, 2H), 7.06 (d, *J* = 8.2 Hz, 2H), 6.76 (s,
219 1H), 6.63 (d, *J* = 13.3 Hz, 1H), 3.85 (s, 3H), 2.18 (s, 3H).

220 *Parasite Culture.* *P. knowlesi* parasites (clone A1-H.1) and *P. falciparum* parasites (clone
221 3D7) were grown in RPMI 1640 supplemented with 25 mM HEPES, 25 mM Na₂HCO₃, 10
222 mM D-glucose, 2 mM L-glutamine, 50 mg/L hypoxanthine, 25 mg/L gentamicin sulphate, 5

223 g/L Albumax II and 10% (v/v) donor horse serum (Pan Biotech, P30-0702). All parasites
224 were grown in human A⁺ red blood cells (National Health Blood and Transplant, UK).
225 Parasites were incubated in sealed flasks at 37°C under a culture gas mixture of 96% N₂,
226 3% CO₂ and 1% O₂.

227 *Growth inhibition assays and isobologram testing.* Drug susceptibility was assessed
228 precisely as described previously with parasites exposed to the drugs for one complete life
229 cycle (27 h for *P. knowlesi* and 48 h for *P. falciparum*) or 2.5 life cycles (68 h for *P.*
230 *knowlesi* and 120 h for *P. falciparum*) (19). Drug combination studies were performed as
231 described previously (19, 23), with the exception that parasites were exposed to drugs for
232 2.5 cycles instead of one life cycle, and the starting parasitaemia was reduced to 0.5%
233 while maintaining the haematocrit at 1%. The Fractional Inhibitory Concentrations (FICs)
234 were calculated as described previously (33). The SYBR green I method was used to
235 determine parasite viability (19, 34).

236 **Competing interests**

237 MD is employed by the Medicines for Malaria Venture, who partly funded the study.

238 All other authors: no competing interests to declare.

239 **Author contributions**

240 CJS, MKR, RM, DAvS conceived and designed the study. DAvS performed the parasite
241 susceptibility screens. SP, RWW, and AN synthesized the ELQs. DAvS and CJS analysed
242 the data and wrote the paper. All authors read and approved the final manuscript.

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255 **References**

- 256 1. WHO (World Health Organization), 2019. World Malaria Report.
257 <https://www.who.int/malaria/publications/world-malaria-report-2019/en/>
- 258 2. WHO (World Health Organisation), 2015. Guidelines for the treatment of malaria. Third
259 Edition. Third Edition. <http://www.who.int/malaria/publications/atoz/9789241549127/en/>
- 260 3. Ariev F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, Kim S, Duru V,
261 Bouchier C, Ma L, Lim P, Leang R, Duong S, Sreng S, Suon S, Chuor CM, Bout DM,
262 Menard S, Rogers WO, Genton B, Fandeur T, Miotto O, Ringwald P, Le Bras J, Berry A,
263 Barale JC, Fairhurst RM, Benoit-Vical F, Mercereau-Puijalon O, Menard D. 2014. A
264 molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. Nature 505:50-5.
- 265 4. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariev F, Hanpithakpong
266 W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS,
267 Yeung S, Singhasivanon P, Day NP, Lindegardh N, Socheat D, White NJ. 2009.
268 Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 361:455-67.
- 269 5. Hamilton WL, Amato R, van der Pluijm RW, Jacob CG, Quang HH, Thuy-Nhien NT, Hien
270 TT, Hongvanthong B, Chindavongsa K, Mayxay M, Huy R, Leang R, Huch C, Dysoley L,
271 Amaratunga C, Suon S, Fairhurst RM, Tripura R, Peto TJ, Sovann Y, Jittamala P,
272 Hanboonkunupakarn B, Pukrittayakamee S, Chau NH, Imwong M, Dhorda M, Vongpromek
273 R, Chan XHS, Maude RJ, Pearson RD, Nguyen T, Rockett K, Drury E, Goncalves S, White
274 NJ, Day NP, Kwiatkowski DP, Dondorp AM, Miotto O. 2019. Evolution and expansion of
275 multidrug-resistant malaria in southeast Asia: a genomic epidemiology study. Lancet Infect
276 Dis 19:943-951.
- 277 6. van der Pluijm RW, Imwong M, Chau NH, Hoa NT, Thuy-Nhien NT, Thanh NV, Jittamala P,
278 Hanboonkunupakarn B, Chutasmith K, Saelow C, Runjarern R, Kaewmok W, Tripura R, Peto
279 TJ, Yok S, Suon S, Sreng S, Mao S, Oun S, Yen S, Amaratunga C, Lek D, Huy R, Dhorda
280 M, Chotivanich K, Ashley EA, Mukaka M, Waithira N, Cheah PY, Maude RJ, Amato R,
281 Pearson RD, Goncalves S, Jacob CG, Hamilton WL, Fairhurst RM, Tarning J, Winterberg
282 M, Kwiatkowski DP, Pukrittayakamee S, Hien TT, Day NP, Miotto O, White NJ, Dondorp

- 283 AM. 2019. Determinants of dihydroartemisinin-piperaquine treatment failure in *Plasmodium*
284 *falciparum* malaria in Cambodia, Thailand, and Vietnam: a prospective clinical,
285 pharmacological, and genetic study. *Lancet Infect Dis* 19:952-961.
- 286 7. Hudson AT. 1993. Atovaquone - a novel broad-spectrum anti-infective drug. *Parasitol*
287 *Today* 9:66-8.
- 288 8. Birth D, Kao WC, Hunte C. 2014. Structural analysis of atovaquone-inhibited cytochrome
289 *bc₁* complex reveals the molecular basis of antimalarial drug action. *Nat Commun* 5:4029.
- 290 9. Fry M, Pudney M. 1992. Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-
291 4-(4'-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80). *Biochem*
292 *Pharmacol* 43:1545-53.
- 293 10. Canfield CJ, Pudney M, Gutteridge WE. 1995. Interactions of atovaquone with other
294 antimalarial drugs against *Plasmodium falciparum* *in vitro*. *Exp Parasitol* 80:373-81.
- 295 11. Sutherland CJ, Laundry M, Price N, Burke M, Fivelman QL, Pasvol G, Klein JL, Chiodini PL.
296 2008. Mutations in the *Plasmodium falciparum* cytochrome *b* gene are associated with
297 delayed parasite recrudescence in malaria patients treated with atovaquone-proguanil.
298 *Malar J* 7:240.
- 299 12. Staines HM, Burrow R, Teo BH, Chis Ster I, Kremsner PG, Krishna S. 2018. Clinical
300 implications of Plasmodium resistance to atovaquone/proguanil: a systematic review and
301 meta-analysis. *J Antimicrob Chemother* 73:581-595.
- 302 13. Skinner-Adams TS, Fisher GM, Riches AG, Hutt OE, Jarvis KE, Wilson T, von Itzstein M,
303 Chopra P, Antonova-Koch Y, Meister S, Winzeler EA, Clarke M, Fidock DA, Burrows JN,
304 Ryan JH, Andrews KT. 2019. Cyclization-blocked proguanil as a strategy to improve the
305 antimalarial activity of atovaquone. *Commun Biol* 2:166.
- 306 14. Wojnarski M, Lon C, Vanachayangkul P, Gosi P, Sok S, Rachmat A, Harrison D, Berjohn
307 CM, Spring M, Chaoratanakawee S, Ittiverakul M, Buathong N, Chann S,
308 Wongarunkochakorn S, Waltmann A, Kuntawunginn W, Fukuda MM, Burkly H, Heang V,
309 Heng TK, Kong N, Boonchan T, Chum B, Smith P, Vaughn A, Prom S, Lin J, Lek D,
310 Saunders D. 2019. Atovaquone-proguanil in combination with artesunate to treat multidrug-

- 311 resistant *P. falciparum* malaria in Cambodia: An open-label randomized trial. Open Forum
312 Infect Dis 6:ofz314.
- 313 15. Kikuth W, Mudrow-Reichenow L. 1947. Über kausalprophylaktisch bei Vogel malaria
314 wirksame Substanzen. Z Hyg Infektionskr 127:151-65.
- 315 16. Nilsen A, LaCrue AN, White KL, Forquer IP, Cross RM, Marfurt J, Mather MW, Delves MJ,
316 Shackleford DM, Saenz FE, Morrisey JM, Steuten J, Mutka T, Li Y, Wirjanata G, Ryan E,
317 Duffy S, Kelly JX, Sebayang BF, Zeeman AM, Noviyanti R, Sinden RE, Kocken CHM, Price
318 RN, Avery VM, Angulo-Barturen I, Jimenez-Diaz MB, Ferrer S, Herreros E, Sanz LM, Gamo
319 FJ, Bathurst I, Burrows JN, Siegl P, Guy RK, Winter RW, Vaidya AB, Charman SA, Kyle
320 DE, Manetsch R, Riscoe MK. 2013. Quinolone-3-diarylethers: a new class of antimalarial
321 drug. Sci Transl Med 5:177ra37.
- 322 17. Nilsen A, Miley GP, Forquer IP, Mather MW, Katneni K, Li Y, Pou S, Pershing AM, Stickles
323 AM, Ryan E, Kelly JX, Doggett JS, White KL, Hinrichs DJ, Winter RW, Charman SA,
324 Zakharov LN, Bathurst I, Burrows JN, Vaidya AB, Riscoe MK. 2014. Discovery, synthesis,
325 and optimization of antimalarial 4(1H)-quinolone-3-diarylethers. J Med Chem 57:3818-34.
- 326 18. Winter R, Kelly JX, Smilkstein MJ, Hinrichs D, Koop DR, Riscoe MK. 2011. Optimization of
327 endochin-like quinolones for antimalarial activity. Exp Parasitol 127:545-51.
- 328 19. van Schalkwyk DA, Blasco B, Davina Nunez R, Liew JWK, Amir A, Lau YL, Leroy D, Moon
329 RW, Sutherland CJ. 2019. *Plasmodium knowlesi* exhibits distinct *in vitro* drug susceptibility
330 profiles from those of *Plasmodium falciparum*. Int J Parasitol Drugs Drug Resist 9:93-99.
- 331 20. van Schalkwyk DA, Moon RW, Blasco B, Sutherland CJ. 2017. Comparison of the
332 susceptibility of *Plasmodium knowlesi* and *Plasmodium falciparum* to antimalarial agents. J
333 Antimicrob Chemother 72:3051-3058.
- 334 21. Fatih FA, Staines HM, Siner A, Ahmed MA, Woon LC, Pasini EM, Kocken CH, Singh B,
335 Cox-Singh J, Krishna S. 2013. Susceptibility of human *Plasmodium knowlesi* infections to
336 anti-malarials. Malar J 12:425.
- 337 22. Moon RW, Hall J, Rangkuti F, Ho YS, Almond N, Mitchell GH, Pain A, Holder AA,
338 Blackman MJ. 2013. Adaptation of the genetically tractable malaria pathogen *Plasmodium*
339 *knowlesi* to continuous culture in human erythrocytes. Proc Natl Acad Sci U S A 110:531-6.

- 340 23. Fivelman QL, Adagu IS, Warhurst DC. 2004. Modified fixed-ratio isobologram method for
341 studying *in vitro* interactions between atovaquone and proguanil or dihydroartemisinin
342 against drug-resistant strains of *Plasmodium falciparum*. *Antimicrob Agents Chemother*
343 48:4097-102.
- 344 24. Stickles AM, de Almeida MJ, Morrisey JM, Sheridan KA, Forquer IP, Nilsen A, Winter RW,
345 Burrows JN, Fidock DA, Vaidya AB, Riscoe MK. 2015. Subtle changes in endochin-like
346 quinolone structure alter the site of inhibition within the cytochrome *bc₁* complex of
347 *Plasmodium falciparum*. *Antimicrob Agents Chemother* 59:1977-82.
- 348 25. Stickles AM, Smilkstein MJ, Morrisey JM, Li Y, Forquer IP, Kelly JX, Pou S, Winter RW,
349 Nilsen A, Vaidya AB, Riscoe MK. 2016. Atovaquone and ELQ-300 Combination Therapy as
350 a Novel Dual-Site Cytochrome *bc₁* Inhibition Strategy for Malaria. *Antimicrob Agents*
351 *Chemother* 60:4853-9.
- 352 26. Carrington HC, Crowther AF, Davey DG, Levi AA, Rose FL. 1951. A metabolite of paludrine
353 with high antimalarial activity. *Nature* 168:1080.
- 354 27. Helsby NA, Ward SA, Howells RE, Breckenridge AM. 1990. *In vitro* metabolism of the
355 biguanide antimalarials in human liver microsomes: evidence for a role of the mephenytoin
356 hydroxylase (P450 MP) enzyme. *Br J Clin Pharmacol* 30:287-91.
- 357 28. Rathod PK, McErlean T, Lee PC. 1997. Variations in frequencies of drug resistance in
358 *Plasmodium falciparum*. *Proc Natl Acad Sci U S A* 94:9389-93.
- 359 29. Salzer W, Timmler H, Andersag H. 1948. Über einen neuen, gegen Vogelmalária
360 wirksamen Verbindungstypus. *Chemische Berichte* 81:12-19.
- 361 30. Doggett JS, Nilsen A, Forquer I, Wegmann KW, Jones-Brando L, Yolken RH, Bordon C,
362 Charman SA, Katneni K, Schultz T, Burrows JN, Hinrichs DJ, Meunier B, Carruthers VB,
363 Riscoe MK. 2012. Endochin-like quinolones are highly efficacious against acute and latent
364 experimental toxoplasmosis. *Proc Natl Acad Sci U S A* 109:15936-41.
- 365 31. Frueh L, Li Y, Mather MW, Li Q, Pou S, Nilsen A, Winter RW, Forquer IP, Pershing AM, Xie
366 LH, Smilkstein MJ, Caridha D, Koop DR, Campbell RF, Sciotti RJ, Kreishman-Deitrick M,
367 Kelly JX, Vesely B, Vaidya AB, Riscoe MK. 2017. Alkoxycarbonate Ester Prodrugs of

- 368 Preclinical Drug Candidate ELQ-300 for Prophylaxis and Treatment of Malaria. ACS Infect
369 Dis 3:728-735.
- 370 32. Stickles AM, Ting LM, Morrisey JM, Li Y, Mather MW, Meermeier E, Pershing AM, Forquer
371 IP, Miley GP, Pou S, Winter RW, Hinrichs DJ, Kelly JX, Kim K, Vaidya AB, Riscoe MK,
372 Nilsen A. 2015. Inhibition of cytochrome *bc₁* as a strategy for single-dose, multi-stage
373 antimalarial therapy. Am J Trop Med Hyg 92:1195-201.
- 374 33. van Schalkwyk DA, Priebe W, Saliba KJ. 2008. The inhibitory effect of 2-halo derivatives of
375 D-glucose on glycolysis and on the proliferation of the human malaria parasite *Plasmodium*
376 *falciparum*. J Pharmacol Exp Ther 327:511-7.
- 377 34. Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. 2004. Simple and
378 inexpensive fluorescence-based technique for high-throughput antimalarial drug screening.
379 Antimicrob Agents Chemother 48:1803-6.

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381

382 **Table 1:** Comparison of the *in vitro* susceptibility of *Plasmodium knowlesi* (clone A1-H.1)
 383 and *Plasmodium falciparum* (clone 3D7) exposed to novel endochin-like quinolones for
 384 one complete life cycle.

Compound	EC ₅₀ values (nM)		Fold	
	<i>P. knowlesi</i> A1-H.1	<i>P. falciparum</i> 3D7	Difference (Pk/Pf)	P value ^ψ
	27 h exposure	48 h exposure		
Endochin	18.9 ± 1.2	18.1 ± 0.5	1.04	0.6233
ELQ-271	117 ± 12	64.5 ± 3.1	1.81	0.0081
ELQ-300	15.4 ± 0.9	23.1 ± 1.2	0.67	0.0215
ELQ-316	47.1 ± 2.6	33.5 ± 2.3	1.41	0.0097
ELQ-331	49.0 ± 6.2	45.4 ± 1.6	1.08	0.5014
ELQ-400	6.80 ± 0.26	4.95 ± 0.26	1.37	0.0030
ELQ-480	7.06 ± 0.32	5.81 ± 0.26	1.22	0.0433
Chloroquine	33.1 ± 2.0	17.7 ± 1.3	1.87	<0.0001
Dihydroartemisinin	1.52 ± 0.07	3.64 ± 0.42	0.42	0.0112

385 EC₅₀ data are presented as mean ± SEM from at least 4 experiments each performed in
 386 duplicate. ^ψ p values are calculated by comparing EC₅₀ values for *P. knowlesi* versus *P.*
 387 *falciparum* using Student's two-tailed paired t-test.

388

389 **Table 2:** Comparison of the *in vitro* susceptibility of *Plasmodium knowlesi* (clone A1-H.1)
 390 and *Plasmodium falciparum* (clone 3D7) exposed to proguanil and select quinolones for
 391 two and a half life cycles.

Compound	EC ₅₀ values (nM)		Fold	
	<i>P. knowlesi</i> A1-H.1	<i>P. falciparum</i> 3D7	Difference (Pk/Pf)	P value ^ψ
	68 h exposure	120 h exposure		
Proguanil	2461 ± 236	228 ± 29	10.79	0.0007
Atovaquone	0.71 ± 0.02	0.74 ± 0.09	0.99	0.1211
ELQ-300	5.31 ± 0.3	15.29 ± 1.2	0.35	0.0011
ELQ-400	1.32 ± 0.2	2.66 ± 0.3	0.50	0.0072

392 EC₅₀ data are presented as mean ± SEM from at least 3 experiments each performed in
 393 duplicate. ^ψ p values are calculated by comparing EC₅₀ values for *P. knowlesi* versus *P.*
 394 *falciparum* using Student's two-tailed unpaired t-test.

395

396 **Table 3:** Mean fractional inhibitory concentrations (FICs) for the drug combinations tested
 397 in this study (Fig. 1 and Fig. 2).

Combination tested	Mean FIC ^a	
	<i>P. knowlesi</i> A1-H.1	<i>P. falciparum</i> 3D7
Proguanil: Atovaquone	0.986 (0.949-1.024) ADD ^b	0.545 (0.503-0.586) SYN
Proguanil: ELQ-300	1.077 (0.970-1.184) ADD	0.660 (0.619-0.700) SYN
Proguanil: ELQ-400	0.995 (0.858-1.132) ADD	0.631 (0.571-0.690) SYN
Atovaquone: ELQ-300	0.867 (0.814-0.920) M-SYN	0.816 (0.785-0.848) M-SYN
Atovaquone: ELQ-400	0.980 (0.961-0.998) ADD	1.016 (0.980-1.052) ADD

398 ^a The mean FIC is calculated from all FICs within each experiment, and for all experiments
 399 performed. The mean is reported, with 95% confidence intervals in parentheses.

400 ^b SYN = synergistic interaction, M-SYN = moderately synergistic interaction, and ADD =
 401 additive / indifferent interaction.

402 **Figure 1:** Comparison of the *in vitro* interaction of proguanil with select quinolones against
403 *P. falciparum* (clone 3D7; Panels A-C) and *P. knowlesi* (clone A1-H.1; Panels D-F).
404 Fractional Inhibitory Concentration (FIC) data are averaged from at least three
405 independent experiments, each run in triplicate. Error bars show standard error of the
406 mean (SEM). FIC values < 1.0 are considered synergistic, while FIC values = 1 are
407 considered additive/indifferent.

408

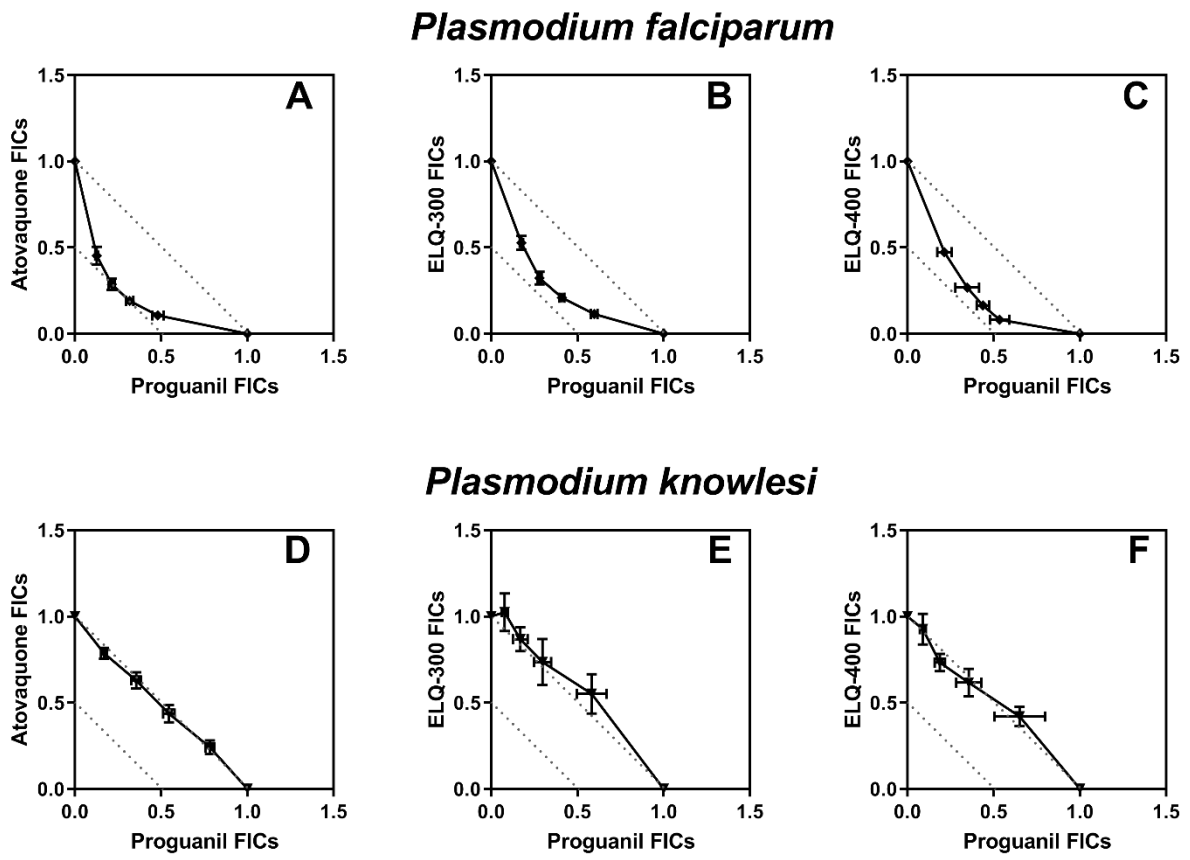
409 **Figure 2:** Comparison of the *in vitro* interaction of atovaquone with two endochin like
410 quinolones against *P. falciparum* (clone 3D7; Panels A-B) and *P. knowlesi* (clone A1-H.1;
411 Panels C-D). Fractional Inhibitory Concentration (FIC) data are averaged from three
412 independent experiments, each run in triplicate. Error bars show standard error of the
413 mean (SEM). FIC values < 1.0 are considered synergistic, while FIC values = 1 are
414 considered additive/indifferent.

415

416 **Figure 1.**

417

418



419 **Figure 2.**

420

