1 **Novel endochin-like quinolones exhibit potent *in vitro* activity against *Plasmodium***

2 ***knowlesi* but do not synergise with proguanil**

3 Donelly A. van Schalkwyk[a], Michael K. Riscoe[b,c], Sovitj Pou[b], Rolf W. Winter[b], Aaron

4 Nilsen[b], Maëlle Duffey[d], Robert W. Moon[a], Colin J. Sutherland[a,e]

5 [a] Department of Infection Biology, Faculty of Infectious & Tropical Diseases, London

6 School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK

7 [b] Veterans Affairs Medical Center, 3710 Southwest U.S. Veterans Hospital Road, Portland,

8 OR 97239, USA

9 [c] Department of Molecular Microbiology and Immunology, 3181 Sam Jackson Boulevard,

10 Portland, OR 97239, USA

11 [d] Medicines for Malaria Venture, 20 Route de Pré Bois, Geneva CH 1215, Switzerland

12 [e] Department of Clinical Parasitology, Hospital for Tropical Diseases, Mortimer Market

13 Centre, Capper Street, London WC1E 6JB, UK

14

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

16 #Address correspondence to Colin J. Sutherland, colin.sutherland@lshtm.ac.uk

17

18 Key words: *Plasmodium falciparum*, *Plasmodium knowlesi*, drug susceptibility, *in vitro*,

19 isobolograms

Abstract

Quinolones, such as the antimalarial atovaquone, are inhibitors of the malarial

mitochondrial cytochrome bc_1 complex, a target critical to the survival of both liver and

blood stage parasites, making these drugs useful as both prophylaxis and treatment.

Recently, several derivatives of endochin have been optimised to produce novel

quinolones that are active *in vitro* and in animal models. While these quinolones exhibit

potent *ex vivo* activity against *Plasmodium falciparum* and *P. vivax*, their activity against

the zoonotic *P. knowlesi* is unknown. We screened several of these novel endochin-like

quinolones (ELQs) for their activity against *P. knowlesi in vitro*, and compared this with

their activity against *P. falciparum* tested under identical conditions. We demonstrate that

ELQs are potent against *P. knowlesi* (EC_{50} values <117 nM), and equally effective against

P. falciparum. We then screened select quinolones and partner drugs using a longer

exposure (2.5 life cycles), and show that proguanil is 10-fold less potent against *P.*

knowlesi when compared with *P. falciparum*, while the quinolones demonstrate similar

susceptibility. Finally, we used isobologram analysis to compare combinations of the ELQs

with either proguanil or atovaquone. We show that all quinolone combinations with

proguanil are synergistic against *P. falciparum*. However, against *P. knowlesi*, no evidence

of synergy between proguanil and the quinolones was found. Importantly, combining the

novel quinolone ELQ-300 with atovaquone, was synergistic against both species. Our data

identify potentially important species differences in proguanil susceptibility and its

interaction with quinolones, and support the ongoing development of novel quinolones as

potent antimalarials that target multiple species.

Introduction.

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

Quinolones have been investigated as potential antimalarial agents since the Second World War (7). However, the only successful candidate from this class to emerge from these studies as an antimalarial has been atovaquone. Atovaquone targets the mitochondrial cytochrome bc_1 complex (8, 9) and is highly potent against *Plasmodium* species. Unfortunately, recrudescence after atovaquone monotherapy occurs rapidly. Atovaquone is therefore used in combination with a synergistic partner drug, proguanil (10), but even this combination is vulnerable to mutations in *pfcytb* especially in areas of cycloguanil resistance (11, 12). Although the target of proguanil is not currently known, proguanil has recently been shown to increase in potency against *Plasmodium falciparum* after a longer *in vitro* exposure (13). Considering that the mitochondrial targeting atovaquone-proguanil combination differs in its mechanism of resistance to the components of current ACT, this combination has been investigated as an alternative treatment of multidrug resistant malaria infections (14), though it should be noted that the atovaquone-proguanil combinations tested performed poorly (only 90-92% effective at 42

3

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

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

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

4

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.

Results and Discussion.

Endochin and six endochin-like quinolones (ELQ) were screened under identical *in vitro* conditions across one complete asexual erythrocytic life cycle against both the *P. knowlesi* A1-H.1 and the *P. falciparum* 3D7 lines (Table 1). All but one (ELQ-271) of the ELQ compounds were potent against the *P. knowlesi* line with EC_{50} values under 100 nM. The potency of endochin and the ELQ compounds was similar against both *P. knowlesi* and *P. falciparum*, with a less than two-fold difference observed between species. With the exception of ELQ-300 all the quinolones screened were more active against *P. falciparum* (Table 1), though for endochin and ELQ-331 the differences were not significant (p = 0.6233 and p = 0.5014, respectively; Table 1). ELQ-400 and ELQ-480 are both active at under 10 nM making them more potent than chloroquine but not as active as dihydroartemisinin (Table 1).

P. falciparum exhibits significantly enhanced susceptibility to proguanil when incubated for more than one life cycle (13). Therefore, in preparation for *in vitro* combination analysis (isobolograms), we screened ELQ-300 and ELQ-400 as well as proguanil and atovaquone using a longer incubation time (2.5 life cycles). We had previously found no activity for proguanil at 10 µM (the highest concentration we tested) after a single life cycle exposure against either *P. knowlesi* or *P. falciparum* (data not shown). However, with a longer exposure (2.5 cycles), we observed an EC_{50} value for proguanil of 2461 ± 236 nM for *P. knowlesi*, over ten-fold higher than the EC_{50} value that we observed for *P. falciparum* 3D7 clone (228 ± 29 nM; Table 2). We expect natural variability within our EC_{50} values as our assays are run using asynchronous parasite populations, and because the parasites have different life cycle lengths, meaning drugs are exposed longer to *P. falciparum* per life cycle than for *P. knowlesi*. Hence, we only consider a greater than three-fold change in EC_{50} between species as a potentially important species difference (19).

6

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　bc_1 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.

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

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

In conclusion, novel endochin-like quinolones exhibit strong antimalarial activity (EC_{50} values <117 nM) against *P. knowlesi in vitro*, and are equipotent against *P. falciparum*. We demonstrate for the first time that quinolone combinations with proguanil lack synergy against *P. knowlesi in vitro*, suggesting distinct mechanisms of action in the malaria parasites. In contrast, combinations of inhibitors targeting the cytochrome bc_1 complex at the Q_o site (e.g., atovaquone) with those targeting the Q_i site (e.g., ELQ-300) show moderate synergism against both species.

Materials and Methods.

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

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

5-fluoro-7-methoxy-2-methyl-3-(4-(4-(trifluoromethoxy)phenoxy)phenyl)quinolin-4(1*H*)-one (ELQ-480): ELQ-480 was synthesized according to the methods described by Nilsen et al. in 2014 (17). Purity of ELQ-480 was assessed as >95% by proton NMR. ^{1}H-NMR spectra were obtained using a Bruker AMX-400 NMR spectrometer operating at 400.14 MHz in DMSO D_6. The NMR raw data were analyzed using the iNMR Spectrum Analyst software. Proton chemical shifts were reported in parts per million units (ppm), (δ) relative to the residual proton at 2.54 ppm in deuterated DMSO D_6. J coupling constants values are in Hertz (Hz). Coupling constants for ^{19}F NMR operating at 376 MHz were also obtained for compounds containing fluorine elements for additional validation of structure. NMR spectrum of ELQ-480: ^{1}H-NMR (400 MHz; DMSO-d_6): δ 11.55 (s, 1H), 7.42 (d, *J* = 8.1 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, 1H), 6.63 (d, *J* = 13.3 Hz, 1H), 3.85 (s, 3H), 2.18 (s, 3H).

Parasite Culture. *P. knowlesi* parasites (clone A1-H.1) and *P. falciparum* parasites (clone 3D7) were grown in RPMI 1640 supplemented with 25 mM HEPES, 25 mM Na_2HCO_3, 10

223 mM D-glucose, 2 mM L-glutamine, 50 mg/L hypoxanthine, 25 mg/L gentamicin sulphate, 5

224 g/L Albumax II and 10% (v/v) donor horse serum (Pan Biotech, P30-0702). All parasites

225 were grown in human A^+ red blood cells (National Health Blood and Transplant, UK).

226 Parasites were incubated in sealed flasks at 37ºC under a culture gas mixture of 96% N_2,

227 3% CO_2 and 1% O_2.

228 *Growth inhibition assays and isobologram testing.* Drug susceptibility was assessed

229 precisely as described previously with parasites exposed to the drugs for one complete life

230 cycle (27 h for *P. knowlesi* and 48 h for *P. falciparum*) or 2.5 life cycles (68 h for *P.*

231 *knowlesi* and 120 h for *P. falciparum*) (19). Drug combination studies were performed as

232 described previously (19, 23), with the exception that parasites were exposed to drugs for

233 2.5 cycles instead of one life cycle, and the starting parasitaemia was reduced to 0.5%

234 while maintaining the haematocrit at 1%. The Fractional Inhibitory Concentrations (FICs)

235 were calculated as described previously (33). The SYBR green I method was used to

236 determine parasite viability (19, 34).

Competing interests

Author contributions

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

Acknowledgements

References

1. WHO (World Health Organization), 2019. World Malaria Report.
   https://www.who.int/malaria/publications/world-malaria-report-2019/en/

2. WHO (World Health Organisation), 2015. Guidelines for the treatment of malaria. Third
   Edition. Third Edition. http://www.who.int/malaria/publications/atoz/9789241549127//en/

3. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, Kim S, Duru V,
   Bouchier C, Ma L, Lim P, Leang R, Duong S, Sreng S, Suon S, Chuor CM, Bout DM,
   Menard S, Rogers WO, Genton B, Fandeur T, Miotto O, Ringwald P, Le Bras J, Berry A,
   Barale JC, Fairhurst RM, Benoit-Vical F, Mercereau-Puijalon O, Menard D. 2014. A
   molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. Nature 505:50-5.

4. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong
   W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS,
   Yeung S, Singhasivanon P, Day NP, Lindegardh N, Socheat D, White NJ. 2009.
   Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 361:455-67.

5. Hamilton WL, Amato R, van der Pluijm RW, Jacob CG, Quang HH, Thuy-Nhien NT, Hien
   TT, Hongvanthong B, Chindavongsa K, Mayxay M, Huy R, Leang R, Huch C, Dysoley L,
   Amaratunga C, Suon S, Fairhurst RM, Tripura R, Peto TJ, Sovann Y, Jittamala P,
   Hanboonkunupakarn B, Pukrittayakamee S, Chau NH, Imwong M, Dhorda M, Vongpromek
   R, Chan XHS, Maude RJ, Pearson RD, Nguyen T, Rockett K, Drury E, Goncalves S, White
   NJ, Day NP, Kwiatkowski DP, Dondorp AM, Miotto O. 2019. Evolution and expansion of
   multidrug-resistant malaria in southeast Asia: a genomic epidemiology study. Lancet Infect
   Dis 19:943-951.

6. van der Pluijm RW, Imwong M, Chau NH, Hoa NT, Thuy-Nhien NT, Thanh NV, Jittamala P,
   Hanboonkunupakarn B, Chutasmit K, Saelow C, Runjarern R, Kaewmok W, Tripura R, Peto
   TJ, Yok S, Suon S, Sreng S, Mao S, Oun S, Yen S, Amaratunga C, Lek D, Huy R, Dhorda
   M, Chotivanich K, Ashley EA, Mukaka M, Waithira N, Cheah PY, Maude RJ, Amato R,
   Pearson RD, Goncalves S, Jacob CG, Hamilton WL, Fairhurst RM, Tarning J, Winterberg
   M, Kwiatkowski DP, Pukrittayakamee S, Hien TT, Day NP, Miotto O, White NJ, Dondorp

284   AM. 2019. Determinants of dihydroartemisinin-piperaquine treatment failure in *Plasmodium*

285   *falciparum* malaria in Cambodia, Thailand, and Vietnam: a prospective clinical,

286   pharmacological, and genetic study. Lancet Infect Dis 19:952-961.

287 7.   Hudson AT. 1993. Atovaquone - a novel broad-spectrum anti-infective drug. Parasitol

288   Today 9:66-8.

289 8.   Birth D, Kao WC, Hunte C. 2014. Structural analysis of atovaquone-inhibited cytochrome

290   bc_1 complex reveals the molecular basis of antimalarial drug action. Nat Commun 5:4029.

291 9.   Fry M, Pudney M. 1992. Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-

292   4-(4'-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80). Biochem

293   Pharmacol 43:1545-53.

294 10.   Canfield CJ, Pudney M, Gutteridge WE. 1995. Interactions of atovaquone with other

295   antimalarial drugs against *Plasmodium falciparum in vitro*. Exp Parasitol 80:373-81.

296 11.   Sutherland CJ, Laundy M, Price N, Burke M, Fivelman QL, Pasvol G, Klein JL, Chiodini PL.

297   2008. Mutations in the *Plasmodium falciparum* cytochrome *b* gene are associated with

298   delayed parasite recrudescence in malaria patients treated with atovaquone-proguanil.

299   Malar J 7:240.

300 12.   Staines HM, Burrow R, Teo BH, Chis Ster I, Kremsner PG, Krishna S. 2018. Clinical

301   implications of Plasmodium resistance to atovaquone/proguanil: a systematic review and

302   meta-analysis. J Antimicrob Chemother 73:581-595.

303 13.   Skinner-Adams TS, Fisher GM, Riches AG, Hutt OE, Jarvis KE, Wilson T, von Itzstein M,

304   Chopra P, Antonova-Koch Y, Meister S, Winzeler EA, Clarke M, Fidock DA, Burrows JN,

305   Ryan JH, Andrews KT. 2019. Cyclization-blocked proguanil as a strategy to improve the

306   antimalarial activity of atovaquone. Commun Biol 2:166.

307 14.   Wojnarski M, Lon C, Vanachayangkul P, Gosi P, Sok S, Rachmat A, Harrison D, Berjohn

308   CM, Spring M, Chaoratanakawee S, Ittiverakul M, Buathong N, Chann S,

309   Wongarunkochakorn S, Waltmann A, Kuntawunginn W, Fukuda MM, Burkly H, Heang V,

310   Heng TK, Kong N, Boonchan T, Chum B, Smith P, Vaughn A, Prom S, Lin J, Lek D,

311   Saunders D. 2019. Atovaquone-proguanil in combination with artesunate to treat multidrug-

312   resistant *P. falciparum* malaria in Cambodia: An open-label randomized trial. Open Forum

313   Infect Dis 6:ofz314.

314 15. Kikuth W, Mudrow-Reichenow L. 1947. Über kausalprophylaktisch bei Vogelmalaria

315   wirksame Substanzen. Z Hyg Infektionskr 127:151-65.

316 16. Nilsen A, LaCrue AN, White KL, Forquer IP, Cross RM, Marfurt J, Mather MW, Delves MJ,

317   Shackleford DM, Saenz FE, Morrisey JM, Steuten J, Mutka T, Li Y, Wirjanata G, Ryan E,

318   Duffy S, Kelly JX, Sebayang BF, Zeeman AM, Noviyanti R, Sinden RE, Kocken CHM, Price

319   RN, Avery VM, Angulo-Barturen I, Jimenez-Diaz MB, Ferrer S, Herreros E, Sanz LM, Gamo

320   FJ, Bathurst I, Burrows JN, Siegl P, Guy RK, Winter RW, Vaidya AB, Charman SA, Kyle

321   DE, Manetsch R, Riscoe MK. 2013. Quinolone-3-diarylethers: a new class of antimalarial

322   drug. Sci Transl Med 5:177ra37.

323 17. Nilsen A, Miley GP, Forquer IP, Mather MW, Katneni K, Li Y, Pou S, Pershing AM, Stickles

324   AM, Ryan E, Kelly JX, Doggett JS, White KL, Hinrichs DJ, Winter RW, Charman SA,

325   Zakharov LN, Bathurst I, Burrows JN, Vaidya AB, Riscoe MK. 2014. Discovery, synthesis,

326   and optimization of antimalarial 4(1H)-quinolone-3-diarylethers. J Med Chem 57:3818-34.

327 18. Winter R, Kelly JX, Smilkstein MJ, Hinrichs D, Koop DR, Riscoe MK. 2011. Optimization of

328   endochin-like quinolones for antimalarial activity. Exp Parasitol 127:545-51.

329 19. van Schalkwyk DA, Blasco B, Davina Nunez R, Liew JWK, Amir A, Lau YL, Leroy D, Moon

330   RW, Sutherland CJ. 2019. *Plasmodium knowlesi* exhibits distinct *in vitro* drug susceptibility

331   profiles from those of *Plasmodium falciparum*. Int J Parasitol Drugs Drug Resist 9:93-99.

332 20. van Schalkwyk DA, Moon RW, Blasco B, Sutherland CJ. 2017. Comparison of the

333   susceptibility of *Plasmodium knowlesi* and *Plasmodium falciparum* to antimalarial agents. J

334   Antimicrob Chemother 72:3051-3058.

335 21. Fatih FA, Staines HM, Siner A, Ahmed MA, Woon LC, Pasini EM, Kocken CH, Singh B,

336   Cox-Singh J, Krishna S. 2013. Susceptibility of human *Plasmodium knowlesi* infections to

337   anti-malarials. Malar J 12:425.

338 22. Moon RW, Hall J, Rangkuti F, Ho YS, Almond N, Mitchell GH, Pain A, Holder AA,

339   Blackman MJ. 2013. Adaptation of the genetically tractable malaria pathogen *Plasmodium*

340   *knowlesi* to continuous culture in human erythrocytes. Proc Natl Acad Sci U S A 110:531-6.

341   23.   Fivelman QL, Adagu IS, Warhurst DC. 2004. Modified fixed-ratio isobologram method for

342   studying *in vitro* interactions between atovaquone and proguanil or dihydroartemisinin

343   against drug-resistant strains of *Plasmodium falciparum*. Antimicrob Agents Chemother

344   48:4097-102.

345   24.   Stickles AM, de Almeida MJ, Morrisey JM, Sheridan KA, Forquer IP, Nilsen A, Winter RW,

346   Burrows JN, Fidock DA, Vaidya AB, Riscoe MK. 2015. Subtle changes in endochin-like

347   quinolone structure alter the site of inhibition within the cytochrome bc_1 complex of

348   *Plasmodium falciparum*. Antimicrob Agents Chemother 59:1977-82.

349   25.   Stickles AM, Smilkstein MJ, Morrisey JM, Li Y, Forquer IP, Kelly JX, Pou S, Winter RW,

350   Nilsen A, Vaidya AB, Riscoe MK. 2016. Atovaquone and ELQ-300 Combination Therapy as

351   a Novel Dual-Site Cytochrome bc_1 Inhibition Strategy for Malaria. Antimicrob Agents

352   Chemother 60:4853-9.

353   26.   Carrington HC, Crowther AF, Davey DG, Levi AA, Rose FL. 1951. A metabolite of paludrine

354   with high antimalarial activity. Nature 168:1080.

355   27.   Helsby NA, Ward SA, Howells RE, Breckenridge AM. 1990. *In vitro* metabolism of the

356   biguanide antimalarials in human liver microsomes: evidence for a role of the mephenytoin

357   hydroxylase (P450 MP) enzyme. Br J Clin Pharmacol 30:287-91.

358   28.   Rathod PK, McErlean T, Lee PC. 1997. Variations in frequencies of drug resistance in

359   *Plasmodium falciparum*. Proc Natl Acad Sci U S A 94:9389-93.

360   29.   Salzer W, Timmler H, Andersag H. 1948. Über einen neuen, gegen Vogelmalaria

361   wirksamen Verbindungstypus. Chemische Berichte 81:12–19.

362   30.   Doggett JS, Nilsen A, Forquer I, Wegmann KW, Jones-Brando L, Yolken RH, Bordon C,

363   Charman SA, Katneni K, Schultz T, Burrows JN, Hinrichs DJ, Meunier B, Carruthers VB,

364   Riscoe MK. 2012. Endochin-like quinolones are highly efficacious against acute and latent

365   experimental toxoplasmosis. Proc Natl Acad Sci U S A 109:15936-41.

366   31.   Frueh L, Li Y, Mather MW, Li Q, Pou S, Nilsen A, Winter RW, Forquer IP, Pershing AM, Xie

367   LH, Smilkstein MJ, Caridha D, Koop DR, Campbell RF, Sciotti RJ, Kreishman-Deitrick M,

368   Kelly JX, Vesely B, Vaidya AB, Riscoe MK. 2017. Alkoxycarbonate Ester Prodrugs of

369   Preclinical Drug Candidate ELQ-300 for Prophylaxis and Treatment of Malaria. ACS Infect

370   Dis 3:728-735.

371   32.   Stickles AM, Ting LM, Morrisey JM, Li Y, Mather MW, Meermeier E, Pershing AM, Forquer

372   IP, Miley GP, Pou S, Winter RW, Hinrichs DJ, Kelly JX, Kim K, Vaidya AB, Riscoe MK,

373   Nilsen A. 2015. Inhibition of cytochrome bc_1 as a strategy for single-dose, multi-stage

374   antimalarial therapy. Am J Trop Med Hyg 92:1195-201.

375   33.   van Schalkwyk DA, Priebe W, Saliba KJ. 2008. The inhibitory effect of 2-halo derivatives of

376   D-glucose on glycolysis and on the proliferation of the human malaria parasite *Plasmodium*

377   *falciparum*. J Pharmacol Exp Ther 327:511-7.

378   34.   Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. 2004. Simple and

379   inexpensive fluorescence-based technique for high-throughput antimalarial drug screening.

380   Antimicrob Agents Chemother 48:1803-6.

381

382

383 **Table 1:** Comparison of the *in vitro* susceptibility of *Plasmodium knowlesi* (clone A1-H.1)

384 and *Plasmodium falciparum* (clone 3D7) exposed to novel endochin-like quinolones for

385 one complete life cycle.

Compound	EC$_{50}$ values (nM)		Fold Difference (Pk/Pf)	P value$^{\Psi}$
	P. knowlesi A1-H.1 27 h exposure	*P. falciparum* 3D7 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

386 EC$_{50}$ data are presented as mean ± SEM from at least 4 experiments each performed in

387 duplicate. $^{\Psi}$ p values are calculated by comparing EC$_{50}$ values for *P. knowlesi* versus *P.*

388 *falciparum* using Student's two-tailed paired t-test.

389

390 **Table 2:** Comparison of the *in vitro* susceptibility of *Plasmodium knowlesi* (clone A1-H.1)

391 and *Plasmodium falciparum* (clone 3D7) exposed to proguanil and select quinolones for

392 two and a half life cycles.

Compound	EC_{50} values (nM)		Fold Difference (Pk/Pf)	P value[Ψ]
	P. knowlesi A1-H.1 68 h exposure	*P. falciparum* 3D7 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

393 EC_{50} data are presented as mean ± SEM from at least 3 experiments each performed in

394 duplicate. [Ψ] p values are calculated by comparing EC_{50} values for *P. knowlesi* versus *P.*

395 *falciparum* using Student's two-tailed unpaired t-test.

396

397 **Table 3:** Mean fractional inhibitory concentrations (FICs) for the drug combinations tested

398 in this study (Fig. 1 and Fig. 2).

Combination tested	Mean FIC[a]	
	P. knowlesi A1-H.1	*P. falciparum* 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

399 [a] The mean FIC is calculated from all FICs within each experiment, and for all experiments

400 performed. The mean is reported, with 95% confidence intervals in parentheses.

401 [b] SYN = synergistic interaction, M-SYN = moderately synergistic interaction, and ADD =

402 additive / indifferent interaction.

403 **Figure 1:** Comparison of the *in vitro* interaction of proguanil with select quinolones against

404 *P. falciparum* (clone 3D7; Panels A-C) and *P. knowlesi* (clone A1-H.1; Panels D-F).

405 Fractional Inhibitory Concentration (FIC) data are averaged from at least three

406 independent experiments, each run in triplicate. Error bars show standard error of the

407 mean (SEM). FIC values < 1.0 are considered synergistic, while FIC values = 1 are

408 considered additive/indifferent.

409

410 **Figure 2:** Comparison of the *in vitro* interaction of atovaquone with two endochin like

411 quinolones against *P. falciparum* (clone 3D7; Panels A-B) and *P. knowlesi* (clone A1-H.1;

412 Panels C-D). Fractional Inhibitory Concentration (FIC) data are averaged from three

413 independent experiments, each run in triplicate. Error bars show standard error of the

414 mean (SEM). FIC values < 1.0 are considered synergistic, while FIC values = 1 are

415 considered additive/indifferent.

416

Figure 1.

Plasmodium falciparum
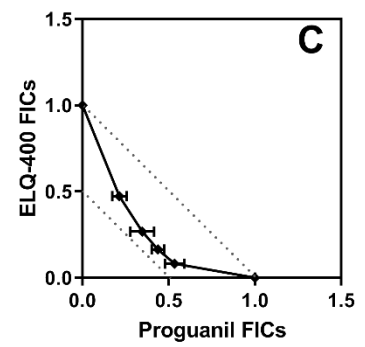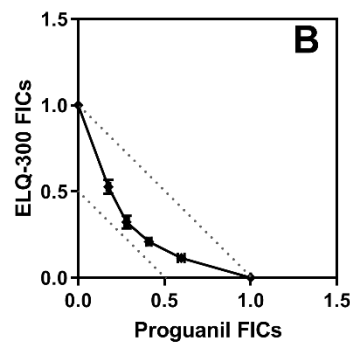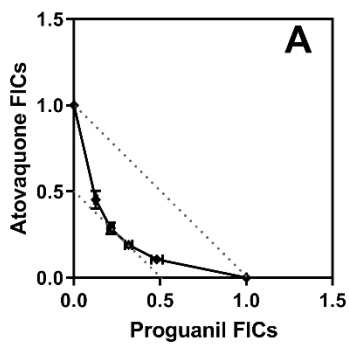
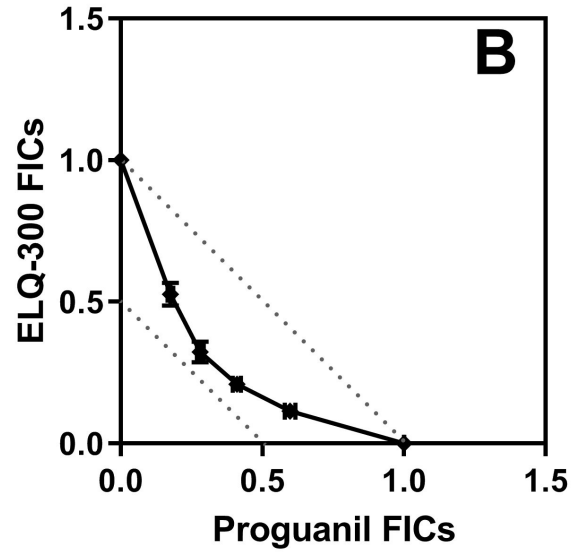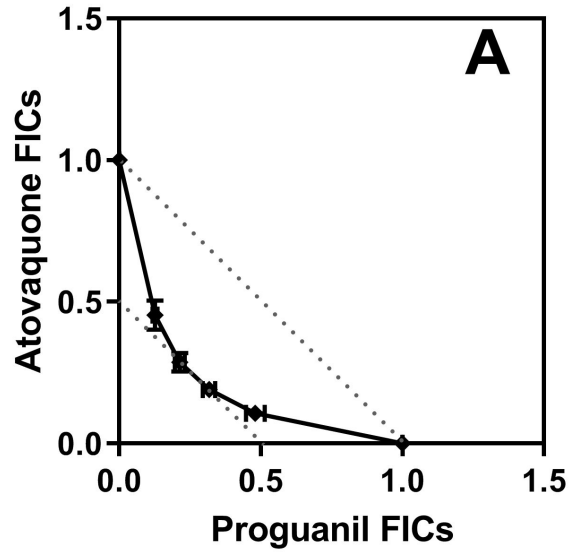*Plasmodium knowlesi*
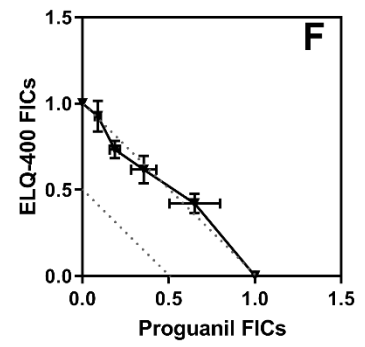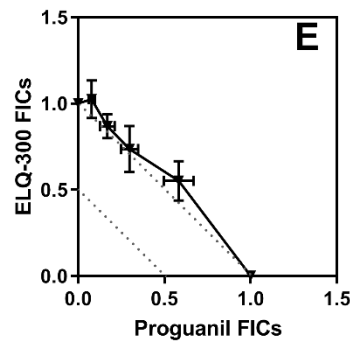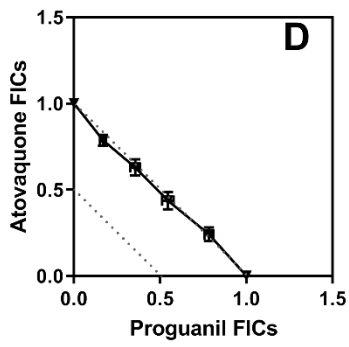
420   **Figure 2.**

421

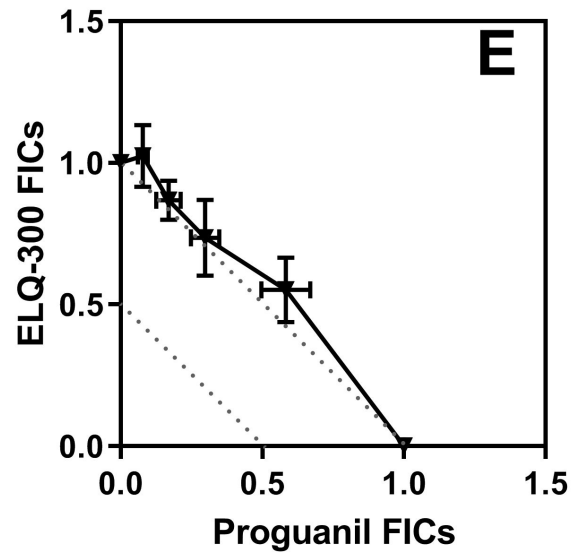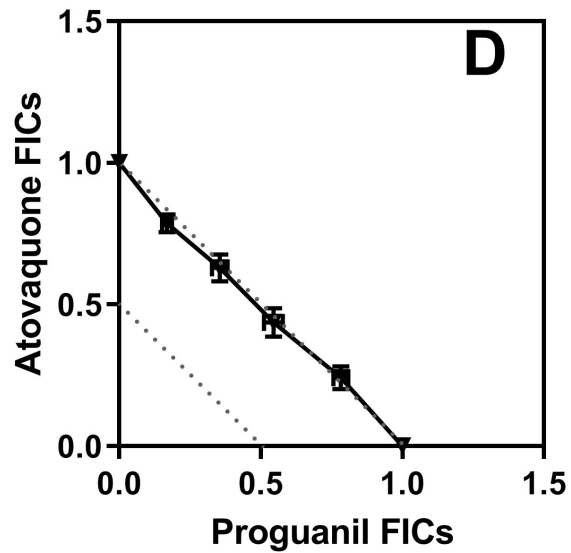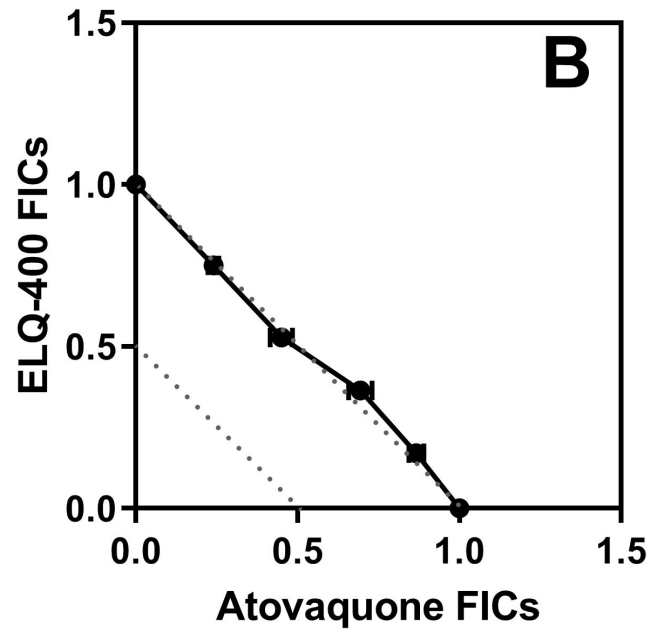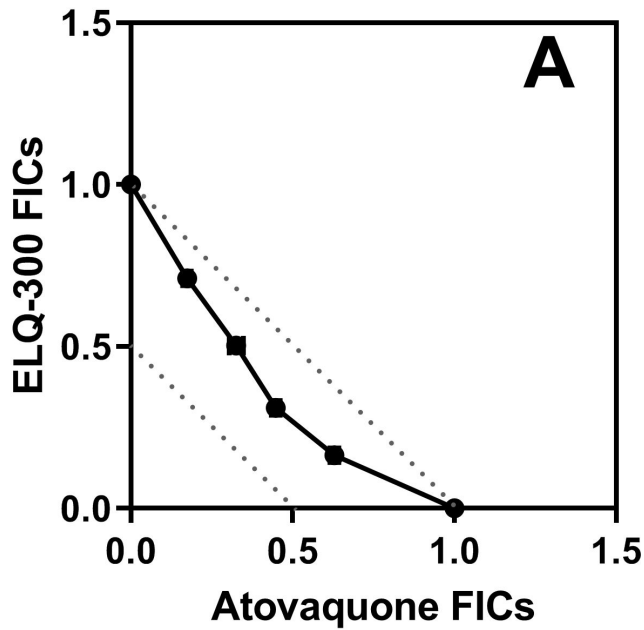*Plasmodium falciparum*

Plasmodium knowlesi

Plasmodium falciparum

Plasmodium knowlesi

Plasmodium falciparum

Plasmodium knowlesi