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

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Running title: New quinolones show cross-species antimalarial potency

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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

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 exoerythocytic 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*. *falciparum*
92 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

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

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

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

An alternative drug combination strategy for quinolones is suggested by recent data indicating that quinolones can inhibit the cytochrome bc_1 complex (cyt bc_1) at either the quinol oxidase (Q_o) or quinone reductase (Q_i) site (24). Atovaquone and ELQ-400 are Q_o site inhibitors (8, 24), while ELQ-300 was shown to target the Q_i site (24). Isobolograms combining a Q_o site inhibitor (atovaquone) with a Q_i site inhibitor (ELQ-300) have previously demonstrated a moderately synergistic interaction against *P*. *falciparum* strain

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

In vivo, proguanil is metabolized to cycloguanil by the liver cytochrome P450 (CYP2C19) (26, 27). Cycloguanil is an inhibitor of the enzyme dihydrofolate reductase (DHFR), a component of the folate pathway in malaria parasites. Thus, the drug combination atovaquone-proguanil actually serves as a triple drug therapy of atovaquone (cytochrome bc_1 inhibitor), proguanil (target unknown), and its metabolite cycloguanil (DHFR inhibitor). Cycloguanil, like atovaquone, has been shown to be highly potent against *P*. *knowlesi in vitro* (20). Therefore, even though antagonistic interactions between atovaquone and cycloguanil have been described *in vitro* (13), the low nanomolar potency of both cycloguanil and atovaquone (20) should still support this combination for *P*. *knowlesi* infections, despite the reduced activity of proguanil, and its lack of synergy reported here.

In light of the above-mentioned data, the recent strategy proposed to block the cyclization of proguanil, thereby reducing its metabolism to cycloguanil, ought to be approached with caution (13). In the absence of cycloguanil, and with the reduced activity of proguanil, atovaquone may be exposed as a monotherapy against *P*. *knowlesi* infections. It will therefore be critical to screen the cyclization blocked tert-butyl proguanil (13) for its activity 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 × 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 × EC_{50}) also selected resistant parasites, but no resistant parasites emerged to 150 nM ELQ-300 (also 10 × 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

mM D-glucose, 2 mM L-glutamine, 50 mg/L hypoxanthine, 25 mg/L gentamicin sulphate, 5 g/L Albumax II and 10% (v/v) donor horse serum (Pan Biotech, P30-0702). All parasites were grown in human A^+ red blood cells (National Health Blood and Transplant, UK). Parasites were incubated in sealed flasks at 37ºC under a culture gas mixture of 96% N_2, 3% CO_2 and 1% O_2.

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

Competing interests

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

All other authors: no competing interests to declare.

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.

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

Compound	EC_{50} values (nM)		Fold Difference (Pk/Pf)	P value[Ψ]
	***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

EC_{50} data are presented as mean ± SEM from at least 4 experiments each performed in duplicate. [Ψ] p values are calculated by comparing EC_{50} values for *P. knowlesi* versus *P. falciparum* using Student's two-tailed paired t-test.

Table 2: Comparison of the *in vitro* susceptibility of *Plasmodium knowlesi* (clone A1-H.1) and *Plasmodium falciparum* (clone 3D7) exposed to proguanil and select quinolones for 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

EC_{50} data are presented as mean ± SEM from at least 3 experiments each performed in duplicate. [Ψ] p values are calculated by comparing EC_{50} values for *P. knowlesi* versus *P. falciparum* using Student's two-tailed unpaired t-test.

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

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

[b] SYN = synergistic interaction, M-SYN = moderately synergistic interaction, and ADD = additive / indifferent interaction.

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

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

417 **Figure 1.**

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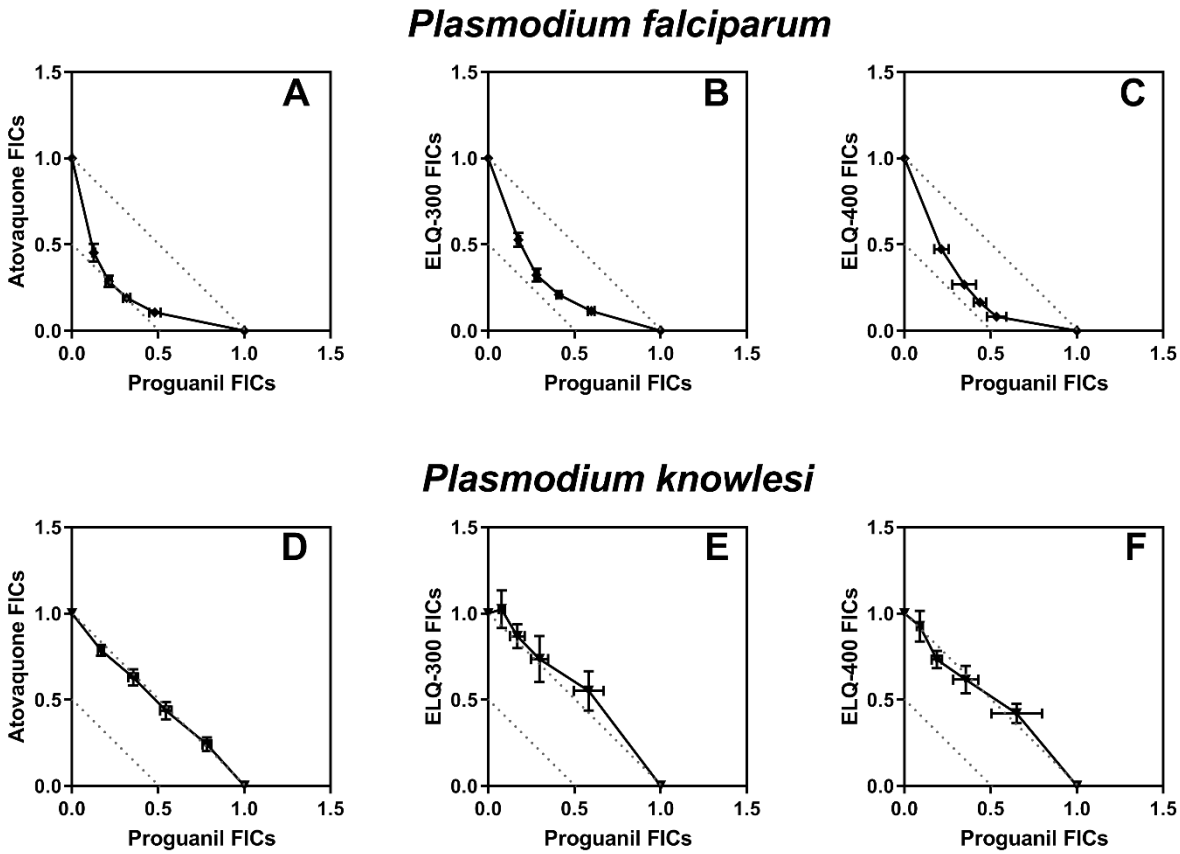

420 **Figure 2.**

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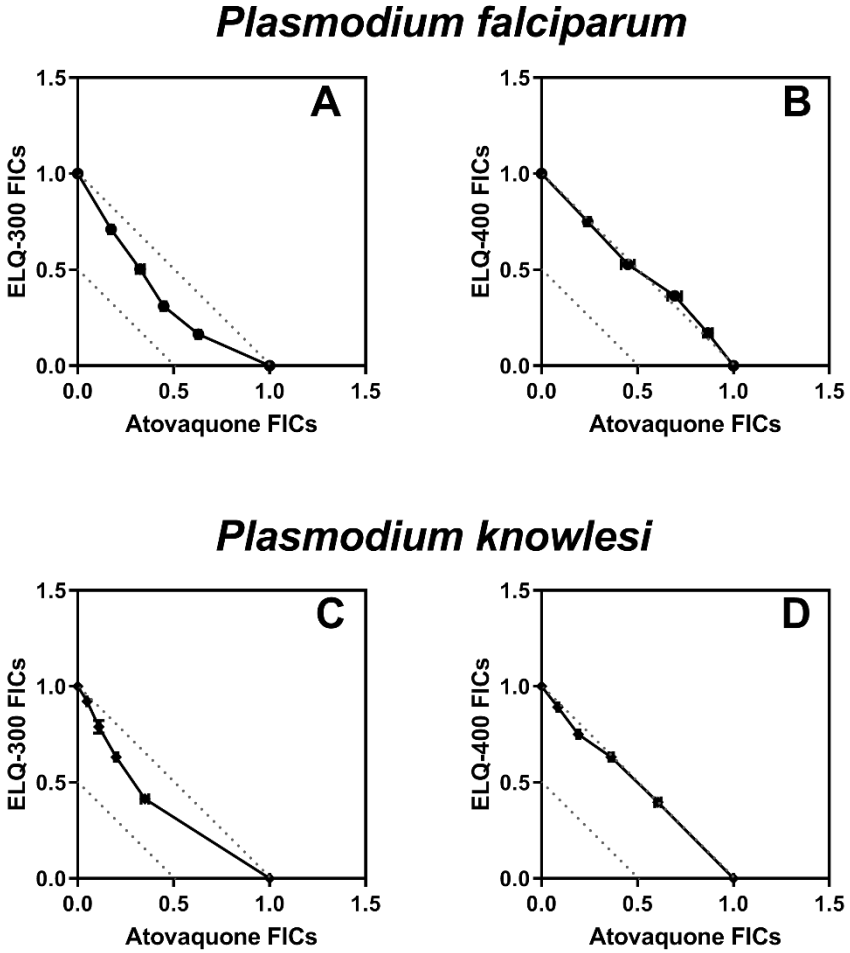
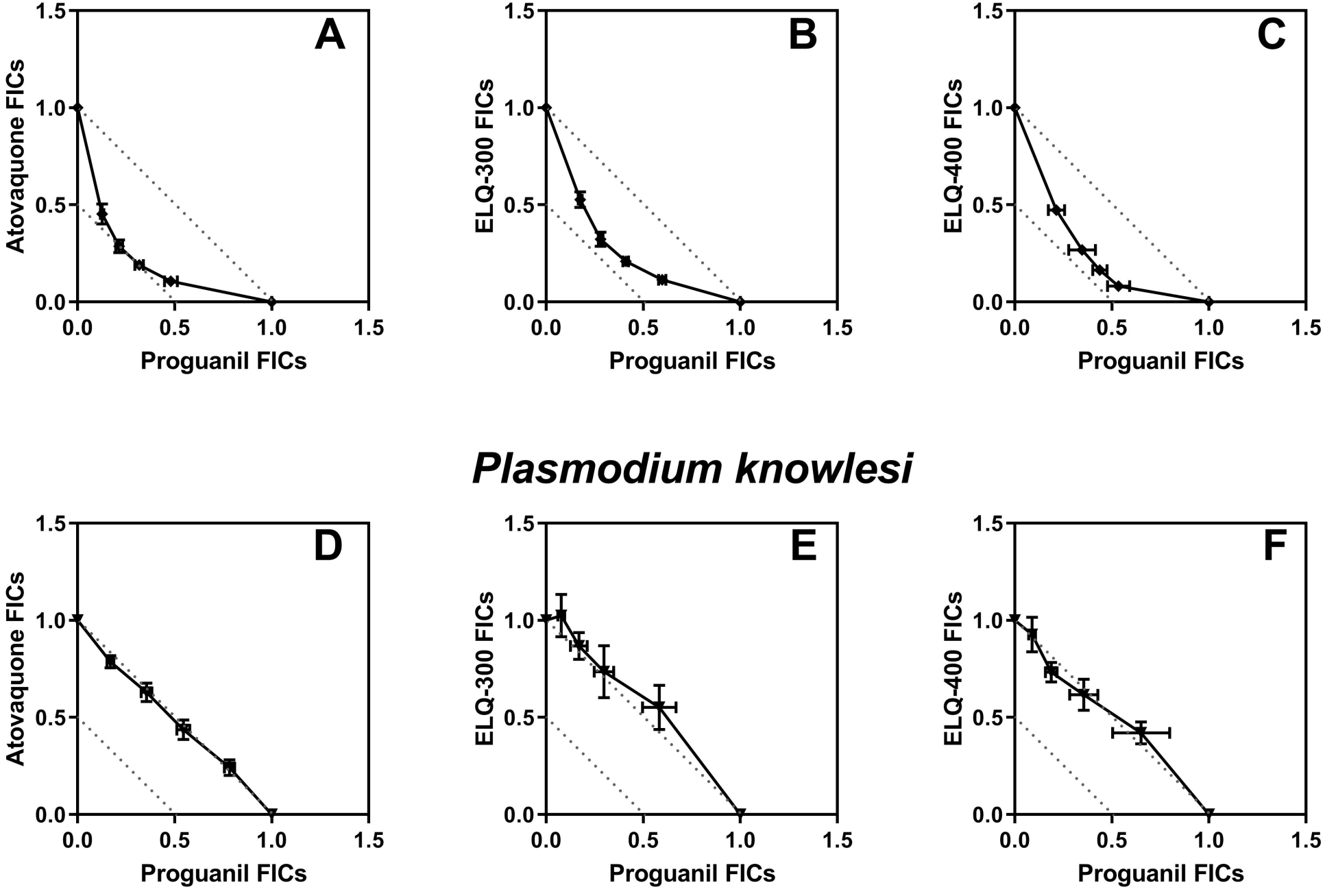
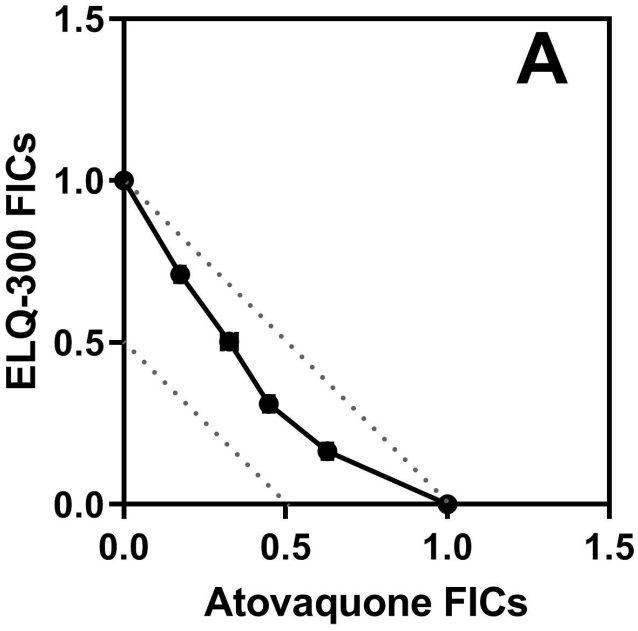
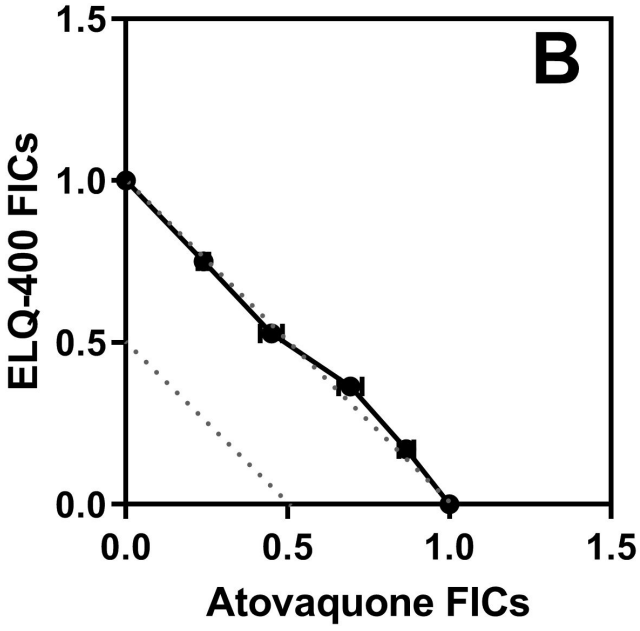



Plasmodium falciparum
A
B
C
Atovaquone FICs
ELQ-300 FICs
ELQ-400 FICs
Proguanil FICs
0.0
0.5
1.0
1.5
Plasmodium knowlesi
D
E
F

Plasmodium falciparum





Plasmodium knowlesi