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Atovaquone and ELQ-300 Combination Therapy as a Novel Dual-Site Cytochrome bc_1 Inhibition Strategy for Malaria

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Antimalarial combination therapies play a crucial role in preventing the emergence of drug-resistant *Plasmodium* parasites. Although artemisinin-based combination therapies (ACTs) comprise the majority of these formulations, inhibitors of the mitochondrial cytochrome bc_1 complex (cyt bc_1) are among the few compounds that are effective for both acute antimalarial treatment and prophylaxis. There are two known sites for inhibition within cyt bc_1: atovaquone (ATV) blocks the quinol oxidase (Q_o) site of cyt bc_1, while some members of the endochin-like quinolone (ELQ) family, including preclinical candidate ELQ-300, inhibit the quinone reductase (Q_i) site and retain full potency against ATV-resistant *Plasmodium falciparum* strains with Q_o site mutations. Here, we provide the first *in vivo* comparison of ATV, ELQ-300, and combination therapy consisting of ATV plus ELQ-300 (ATV:ELQ-300), using *P. yoelii* murine models of malaria. In our monotherapy assessments, we found that ATV functioned as a single-dose curative compound in suppressive tests whereas ELQ-300 demonstrated a unique cumulative dosing effect that successfully blocked recrudescence even in a high-parasitemia acute infection model. ATV:ELQ-300 therapy was highly synergistic, and the combination was curative with a single combined dose of 1 mg/kg of body weight. Compared to the ATV:proguanil (Malarone) formulation, ATV:ELQ-300 was more efficacious in multiday, acute infection models and was equally effective at blocking the emergence of ATV-resistant parasites. Ultimately, our data suggest that dual-site inhibition of cyt bc_1 is a valuable strategy for antimalarial combination therapy and that Q_i site inhibitors such as ELQ-300 represent valuable partner drugs for the clinically successful Q_o site inhibitor ATV.

Malaria is a devastating tropical disease which disproportionately affects children and pregnant women and is responsible for more than 400,000 deaths each year (1). Although many antimalarial drugs exist, treatment is complicated by the rapid emergence of drug resistance among the *Plasmodium* parasites that cause disease. Mutations in the *Plasmodium falciparum* chloroquine resistance transporter (*Pf*CRT) heralded resistance to chloroquine in the late 1950s, and since that time, resistance has developed against compounds in every major antimalarial class (2, 3). Even artemisinin-based combination therapies (ACTs), which are the current mainstay of treatment in regions of malaria endemicity, are slowly losing activity in Southeast Asia (4).

Although the parasitology community continues to develop novel antimalarial compounds, combinations of existing therapies have also been embraced as a way to prevent or overcome drug resistance. The majority of drugs in current phase III clinical trials are ACTs, which typically partner a fast-acting artemisinin derivative with a long-duration antimalarial such as piperaquine or mefloquine (5). In contrast, many antibacterial and anticancer combination therapies prioritize the use of synergistic compounds targeting multiple steps of the same biochemical pathway in an effort to minimize drug resistance (6, 7). In malaria, a promising target for such an approach is the cytochrome bc_1 complex (cyt bc_1), which has recently been identified as a target for single-dose blood and vector-stage antimalarial therapy and is capable of initiating rapid parasite clearance *in vivo* (8).

Cyt bc_1 is the known site of action of several potent inhibitors of *P. falciparum*, including atovaquone (ATV) (9) and the endochin-like quinolones (ELQs) (10). Biologically, cyt bc_1 plays a vital role in *de novo* pyrimidine biosynthesis for *P. falciparum* by facilitating the activity of type II dihydroorotate dehydrogenase (DHODH) (11). Because malaria parasites lack a functional pyrimidine salvage pathway, inhibition of cyt bc_1 (and, by extension, DHODH) is cidal and provides both treatment and prophylactic protection against malaria (12). Currently, ATV is the only cyt bc_1 inhibitor in clinical use and is a key component of the antimalarial combination therapy Malarone, which consists of ATV and proguanil hydrochloride (13).

Although cyt bc_1 can be effectively inhibited at either its quinol oxidase (Q_o) or quinone reductase (Q_i) site, ATV is a selective Q_o site inhibitor (14), and ATV resistance is associated with point mutations at this site, including the clinically relevant Y268S substitution that reduces sensitivity to ATV by more than 1,000-fold (15, 16). Our group has recently shown that current preclinical candidate ELQ-300 potently inhibits the Q_i site of cyt bc_1 rather than the Q_o site exploited by ATV (17). Because of this site specificity, ELQ-300 is fully active against ATV-resistant *P. falciparum* parasites containing Y268S mutations (18). Similarly, ELQ-300-

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resistant *P. falciparum* parasites are fully sensitive to ATV *in vitro* (17). Because all known clinical cases of ATV resistance are attributable to point mutations within the cytochrome *b* gene, this suggests that ATV and ELQ-300 would be mutually protective against the generation of drug resistance and would therefore represent an optimal choice for antimalarial combination therapy.

Combination therapy consisting of ATV plus ELQ-300 (ATV:ELQ-300) is also positioned to overcome several well-established shortcomings of the ATV:proguanil formulation utilized in Malarone. In circulation, proguanil is rapidly metabolized to cycloguanil, while ATV has a significantly longer half-life (19). Although cycloguanil is an effective dihydrofolate reductase (DHFR) inhibitor, there have been reports of Malarone resistance involving simultaneous mutations in DHFR and cytochrome *b* (20, 21), and only unmetabolized proguanil works synergistically with ATV to collapse the mitochondrial membrane potential (11, 22, 23). Consequently and more concerningly, the Y268S mutation associated with high-grade ATV resistance also eliminates ATV:proguanil synergy at the level of the mitochondria (24). Therefore, despite the clinical success of Malarone as a prophylactic agent, there are significant concerns that resistance would compromise its use in acute infections or mass drug administration efforts (25).

In contrast, ELQ-300 and ATV have similar (~70 h) predicted half-lives in humans (18), eliminating the potential for ATV monotherapy and resultant ATV resistance (ATV^r) mutations. Because both ATV and ELQ-300 act on a single protein, the development of simultaneous Q_o and Q_i site mutations would be exceedingly rare, and the fact that the mitochondrial genome is carried in only female gametocytes would prevent any dual-site resistance secondary to genetic recombination at the sexual stage. Even if rare ATV^r/ELQ-300^r mutants were to arise, recent work suggests that these parasites would likely be incapable of transmission by mosquitoes; the McFadden group has recently shown that multiple ATV^r mutations (including Y268C and Y268N) are associated with arrested oocyst development within the mosquito midgut and with a failure to produce the infectious sporozoites necessary for transmission to mammalian hosts (26). Ultimately, we believe that ATV and ELQ-300 could be ideal partner drugs with a low propensity for drug resistance and with the remarkable potency characteristic of cyt bc_1 inhibitors. In this article, we characterize the single-dose and multidose efficacy of ATV, ELQ-300, and combinations of ATV:ELQ-300 and ATV:proguanil in *P. yoelii* murine models. In addition, the propensity for the development of drug resistance in the various treatment arms is described.

MATERIALS AND METHODS

***In vitro* synergy of ATV and ELQ-300.** The *in vitro* activity of ELQ-300 and ATV was assessed by isobolar analysis using a fixed-ratio strategy (27). In each case, the 50% inhibitory concentration (IC_{50}) data were generated using a published SYBR green I fluorescence-based method (28) and fractional inhibitory concentrations (FICs) were then calculated using the following equations: FIC (A) = IC_{50} of drug A in combination/IC_{50} of drug A alone; FIC (B) = IC_{50} of drug B in combination/IC_{50} of drug B alone; FIC index = FIC (A) + FIC (B). Experiments were performed in duplicate, and isobolograms were constructed by plotting FIC means plus standard errors of the means (SEM) for each combination of ATV and ELQ-300. A straight diagonal line (FIC index = 1) indicates an additive effect of the two drugs, a concave curve (FIC index < 1) indicates synergy of the combination, and a convex curve (FIC index > 1) indicates antagonism.

Compounds and formulation. ELQ-300 was synthesized and purified using previously published methods (10). ATV was obtained from Sigma and recrystallized from DMF (*N*,*N*-dimethyl formamide) and methanol before use. All drug stocks were prepared as stock solutions (10 mg/kg of body weight) in polyethylene glycol (PEG) 400 and were serially diluted such that the desired concentration could be delivered in 100-μl aliquots. For combination therapies, each drug was stored separately, and animals received doses via sequential gavage. Reported doses represent total drug present (e.g., a 20 mg/kg ATV:ELQ-300 dose would represent either 10 mg/kg ATV plus 10 mg/kg ELQ-300 in the 1:1 formulation or 15 mg/kg ATV plus 5 mg/kg ELQ-300 in the 3:1 formulation).

Peters suppressive test. Six-week-old, female CF-1 mice were obtained from Charles River and infected with 2.5×10^5 blood-stage *P. yoelii* (Kenya strain; MR4 MRA-428) parasites via tail vein injection ($n = 4$ animals/group). Drug stocks were administered to animals once daily for 1 or 4 days, beginning 24 h postinfection. For the ATV, ATV:ELQ-300, and ATV:proguanil groups, tested doses were 0.001, 0.01, 0.1, 1, and 10 mg/kg. Single-dose studies performed with ELQ-300 included an additional 20 mg/kg dosing group, and multidose ELQ-300 studies included additional doses at 0.003, 0.03, and 0.3 mg/kg. In all groups, the total dose was delivered via two 100-μl boluses. After treatment, daily blood samples were collected from the tail vein, beginning on postinfection day 5, and parasitemia was determined microscopically using Giemsa stain and NIS-Elements cell-counting software (Nikon, Melville, NY). Animals were treated in accordance with IACUC guidelines and were sacrificed when parasitemia exceeded 30%. The 50% effective dose (ED_{50}) value was calculated as the dose that effectively reduced day 5 parasitemia by 50% relative to control animals. Animals were considered cured if no parasitemia was detectable at postinfection day 30.

Acute infection model. Mice were infected as described above, and blood was monitored daily via the use of thin smears until the parasitemia level reached 15% to 20%. At that time, drugs were administered via oral gavage as solutions in PEG-400, and the level of parasitemia was monitored daily via the use of thin smears and plotted as a function of time posttreatment. Smears were collected daily for the first 60 days postinfection and then weekly/intermittently for at least 4 additional months. For animals that recrudesced posttreatment, resistance was assessed by retreating animals with a second 20 mg/kg drug dose (at 10% to 25% parasitemia) and comparing parasite responses to initial kinetics, lag time, and clearance time. Resistant parasites were collected via cardiac puncture and frozen in 30% glycerol, and the cytochrome *b* gene was sequenced as described below.

Cytochrome *b* sequencing. DNA was isolated from parasites in the middle-to-late trophozoite stage. Parasites were saponin lysed with 0.02% saponin–phosphate-buffered saline (PBS). Pellets were resuspended to a volume of 200 μl with PBS plus 20 mM EDTA, and DNA was isolated using a QIAamp DNA Blood minikit (Qiagen) following the blood protocol. PCR was performed using Herculase II Fusion enzyme (Agilent Technologies). The sequence of Primer1 was 5′-CCAGACGCTTTAAATGGATG-3′; the sequence of Primer2 was 5′-GTTTGCTTGGGAGCTGTAATC-3′. PCR cleanup was performed using an SV gel and PCR cleanup kit (Promega). Direct sequencing was performed by Genewiz.

***In vivo* assessment of ATV^r and ELQ-300^r mutants.** Mice (Charles River; 6 weeks old, female, CF-1) were infected with 2.5×10^5 *P. yoelii* wild-type (Kenya strain; MR4 MRA-428), *P. yoelii*-ATV^{r}B$_{Y268C}$, or *P. yoelii* ELQ-300$^r_{I22L}$-parasitized red blood cells (RBCs) by tail vein injection, and 4-day Peters suppressive tests were conducted as described above ($n = 4$ animals/group). On postinfection day 5, samples were collected from the tail vein and parasitemia was determined by flow cytometry after blood sample incubation with SYBR green I, allophycocyanin (APC)-labeled anti-CD45, and dihydroethidium (29). The 50% effective dose (ED_{50}) was defined as the dose required to reduce the parasite burden by 50% relative to control levels on postinfection day 5. Giemsa-stained blood smears were used to determine the presence or absence of parasites if flow cytometry indicated parasitemia less than 0.3%, and parasite-free mice were maintained and monitored semiweekly. Animals were consid-

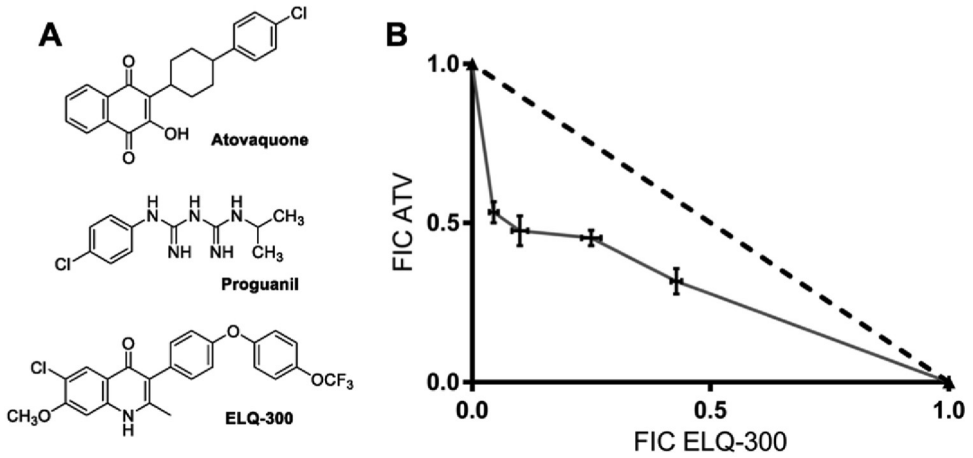

FIG 1 Structures of test compounds (A) and isobolar analysis of ATV and ELQ-300 *in vitro* (B). ATV and ELQ-300 are moderately synergistic *in vitro*, with a mean FIC index of 0.65. Data represent mean FICs of ATV and ELQ-300 as assessed in D6 *P. falciparum* parasites. Data were derived from two independent experiments; error bars, SEM. The dashed line represents a comparative FIC index of 1.

ered cured if they remained parasite free by microscopy until postinfection day 30. The Portland VA Medical Center Institutional Animal Care and Use Committee approved all protocols involving animals used in this study.

RESULTS

***In vitro* synergy of ATV and ELQ-300.** In order to determine if ATV and ELQ-300 (Fig. 1A) acted synergistically *in vitro*, we tested these compounds against wild-type (D6) *P. falciparum* parasites, using a fixed-ratio, isobolar strategy (27). As illustrated in Fig. 1B, parasites were more sensitive to the combination of ATV:ELQ-300 than to higher concentrations of either drug alone, with a mean FIC index of 0.65.

Peters suppressive test. We next used *in vivo* suppressive models to test the activity of ATV, ELQ-300, and ATV:ELQ-300 combinations in both 4-day and 1-day dosing tests (Table 1). For these studies, mice were infected with *P. yoelii* parasites and received the first oral drug dose 24 h postinfection. In the 4-day test, we found that ATV:ELQ-300 combination therapy increased the potency and efficacy of treatment relative to ATV monotherapy, with ED_{50} values of 0.02 mg/kg and 0.01 mg/kg for the 1:1 and 3:1 formulations, respectively. In both ATV:ELQ-300 groups, four daily doses of 1 mg/kg were fully curative. Comparison values for ATV and ELQ-300 were consistent with previous reports.

TABLE 1 Comparative *in vivo* activities of ELQ-300, ATV, and combination therapies in the murine Peters suppressive test[a]

	4-day dosing (mg/kg)		1-day dosing (mg/kg)	
Treatment	ED_{50}	NRD	ED_{50}	NRD
ELQ-300	0.02	0.3	0.03	>20
ATV	0.1	10	0.08	10
(1:1) ATV:300	0.02	1	0.1	10
(3:1) ATV:300	0.01	1	0.14	1
(5:2) ATV:PG	0.14	1	0.5	1*

[a] Coformulation of ATV with either ELQ-300 or proguanil increased *in vivo* efficacy in both 4-day and 1-day studies. ATV, ATV:ELQ-300, and ATV:proguanil were all capable of clearing parasites with a single oral dose. ED_{50}, 50% effective dose (the dose required to suppress day 5 parasitemias by 50% relative to untreated controls); NRD, nonrecrudescence or curative dose (dose required to clear parasites from bloodstream for 30 days following infection); ATV, atovaquone; PG, proguanil; 300, ELQ-300. *, 3 of 4 were animals cured with 1 mg/kg ATV:PG. $n = 4$ animals/group.

In the 1-day test, we also found evidence for increased activity of the combination therapy regimen relative to single-compound treatment. In this model, the 3:1 ratio was most effective and cured mice with a single dose of 1 mg/kg, while the 1:1 combination was curative at a higher, 10 mg/kg dose. Although ELQ-300 monotherapy did not prevent recrudescence in this test at any dose up to its solubility limit (20 mg/kg), ATV demonstrated unanticipated single-dose curative activity at a dose of 10 mg/kg. More surprisingly, the 1-day ED_{50} and nonrecrudescence values for ATV closely matched those of the 4-day treatment study, suggesting that the maximum effect of this compound was obtained following a single oral dose.

In order to determine how ATV:ELQ-300 combination therapy compared to the current standard of care, we next evaluated an ATV:proguanil coformulation at the 5:2 ratio that is used in Malarone (Fig. 1A). In both 1-day and 4-day suppressive tests, ATV:proguanil was less potent than ATV:ELQ-300 combination therapy (Table 1), although it demonstrated single-dose curative activity similar to that of the 3:1 ATV:ELQ-300 formulation, with a nonrecrudescence dose of 1 mg/kg. Like ATV monotherapy, there was little observed benefit of multiday dosing in this model.

Acute infection model. In order to determine if ATV:ELQ-300 combination therapy was capable of clearing established infections, we next tested these compounds in an acute infection model (Fig. 2 and 3). The experimental setup was identical to that used in the suppressive tests, but treatment was delayed until the initial parasitemia level ranged from 15% to 20%. Animals received either a single oral drug dose at the maximum soluble concentration (20 mg/kg) or sequential 10 mg/kg doses delivered daily for 4 days. Parasite clearance was monitored via daily blood smear. Although none of the tested compounds were reliably curative in the single-dose groups, we found that all formulations containing ELQ-300 effectively prevented recrudescence in the 4-day dosing studies (Fig. 2D and 3D and E). In contrast, animals treated with ATV or ATV:proguanil recrudesced, and, as in the suppressive tests, there was little discernible difference between single-day and multiday dosing results for these compounds (Fig. 2A and C and 3C and F).

Resistance propensity. Due to the high parasite burden at the

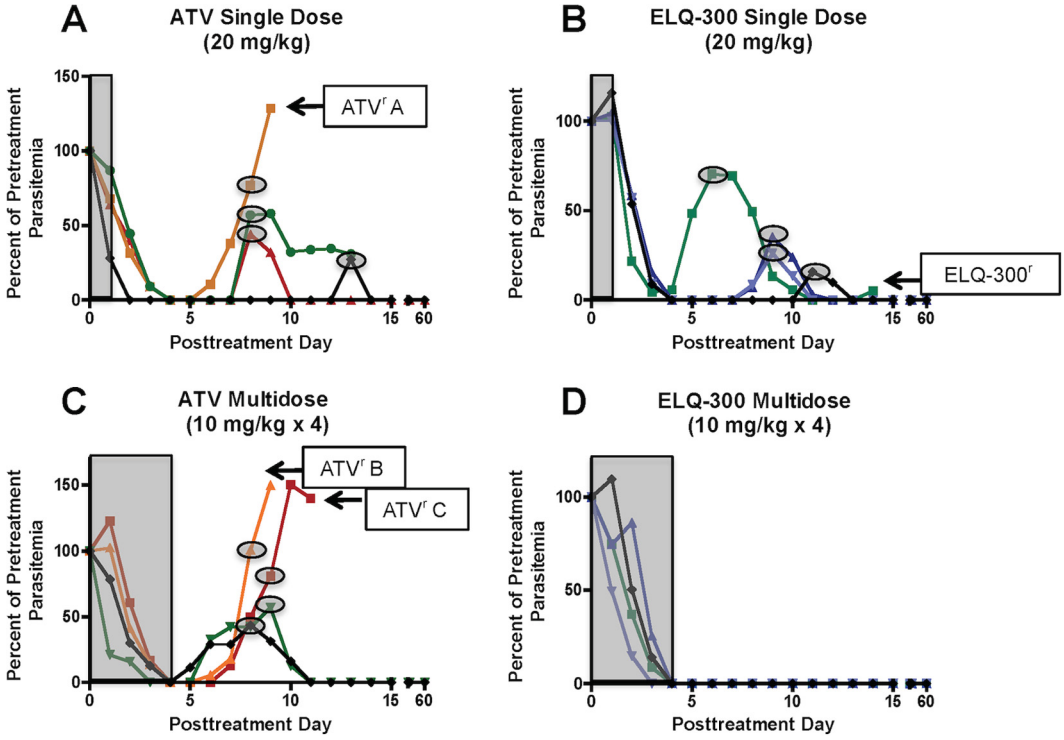

FIG 2 Efficacy of ATV and ELQ-300 in murine acute infection models. In both 1-day (A) and 4-day (C) treatment models, ATV therapy was associated with rapid parasite clearance but with high resistance propensity. In contrast, while ELQ-300-treated animals recrudesced in 1-day models (B), 4-day treatment universally prevented recrudescence (D). Gray bars/circles indicate drug administration. Arrows indicate animals sacrificed for blood collection and parasite gene sequencing. Each trace represents a single animal. $n = 4$ animals/group.

time of drug treatment, our acute infection model also served as a measure of *in vivo* resistance propensity. In order to determine the susceptibility of recrudescent parasites to the treatment compounds, all animals were rechallenged with a 20 mg/kg drug dose when recrudescent parasitemia reached 15% to 25%. If there was any delay in parasite clearance compared to that seen with the initial drug exposure, animals were sacrificed and parasites were collected for cytochrome *b* sequencing. We found that three ATV-treated animals harbored resistant infections (Fig. 2A and C), each with Y268C mutations corresponding to the Q_o site of cyt bc_1 (16) (Fig. 4A and C). Similarly, one ELQ-300-treated animal failed to respond to rechallenge (Fig. 2B) and sequencing revealed an I22L mutation at the cyt bc_1 Q_i site (Fig. 4B and C), which was consistent with known ELQ-300^r mutations in *P. falciparum* (17). No resistant parasites were identified in animals receiving either ATV:proguanil or ATV:ELQ-300 combination therapy (Fig. 3A and F). To determine the long-term potential for recrudescence, animals were intermittently monitored by blood smear for at least 6 months following initial infection; with the exception of the animals harboring resistant parasites (described above), the rechallenge dose effectively prevented recrudescence across all treatment groups.

Although ATV^r and ELQ-300^r strains have been well characterized in *P. falciparum in vitro*, we next wanted to assess the degree of *in vivo* resistance conferred by these *P. yoelii* mutants. We used the ELQ-300^r and ATV^{r}B strains to infect naive mice and then performed 4-day suppressive tests as described above. Although both the ATV^r and ELQ-300^r strains were highly resistant to their respective compounds, we found that ATV^r parasites remained fully sensitive to ELQ-300 and vice versa (Table 2).

DISCUSSION

The therapeutic combination of ATV and ELQ-300 is the first example of dual-site, antimalarial inhibition of a single protein target. In *P. yoelii* murine models, this coformulation was more effective than ATV alone in both 1-day and 4-day suppressive tests, and the 3:1 ratio of ATV:ELQ-300 was especially useful as a single-dose, blood-stage therapeutic. In comparison to ATV and proguanil, which are the active components of Malarone, the ATV:ELQ-300 formulation demonstrated superior multidose activity in an acute infection model and was equally effective at preventing the emergence of drug-resistant parasites.

One of our most intriguing results was that ATV monotherapy effectively cured *P. yoelii*-infected animals in our 1-day suppressive tests. Although ATV has been studied in rodents since the

TABLE 2 Susceptibility of ATV^r and ELQ-300^r mutants isolated from the acute treatment model to ELQ-300 and ATV in 4-day suppressive tests[a]

	Atovaquone (mg/kg/day)		ELQ-300 (mg/kg/day)	
P. yoelii strain	ED_{50}	NRD	ED_{50}	NRD
Wild type	0.03	1.0	0.02	1.0
ATV^r_{Y268C}	>10	>10	0.02	1.0
$ELQ\text{-}300^r_{I22L}$	0.03	1.0	1.0	>10

[a] In both cases, mutations conferred high-grade resistance to the parent compound, but parasites remained fully sensitive to inhibition at the alternate site of cyt bc_1. The concentration of 10 mg/kg represents the solubility limit of ELQ-300 in 100 μl of the PEG-400 vehicle. $n = 4$ animals/group.

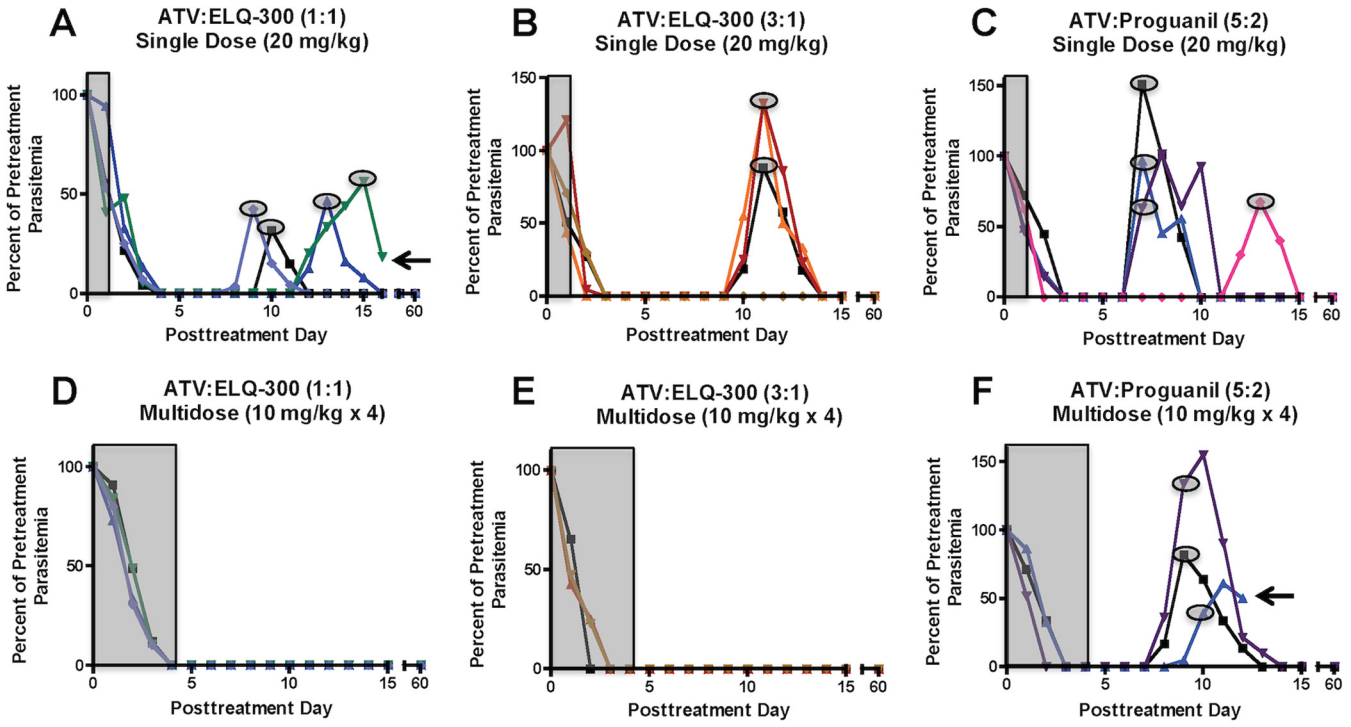

FIG 3 Comparison of ATV:ELQ-300 and ATV:proguanil combination therapies in murine acute infection models. In 1-day studies, ATV:ELQ-300 combination therapy (A and B) delayed recrudescence compared to the results seen with ATV or ELQ-300 comparison groups, and one animal in the 3:1 ATV:ELQ-300 group (B) was fully cured with a single 20 mg/kg oral dose. In 4-day tests, ATV:ELQ-300 combination therapy delivered as either a 1:1 (D) or a 3:1 (E) coformulation effectively prevented recrudescence. No resistant parasites were detected in any ATV:ELQ-300 treatment group. In contrast, while ATV:proguanil treatment effectively prevented resistance, it did not delay the onset of recrudescence in either the 1-day treatment group (C) or the 4-day treatment group (F) compared to ATV monotherapy. Gray bars/circles represent drug administration. Arrows indicate animals sacrificed for blood collection and parasite gene sequencing. Each trace represents a single animal. $n = 4$ animals/group.

time of its discovery in the late 1980s, the only hint of this single-dose activity in the literature comes from the landmark paper by Hudson et al., where it was shown that 1-day and 7-day ATV dosing models had similar ED_{50} values in *P. yoelii* suppressive tests (30). Our data build on that work to show that ATV is also single-dose curative in the suppressive model and that this property is retained by ATV-containing combination therapies, including ATV:proguanil and ATV:ELQ-300. Although none of our tested therapies was an effective single-dose cure in the acute infection model, recrudescent animals rechallenged with a second 20 mg/kg dose of ELQ-300, ATV:proguanil, or the ATV:ELQ-300 combination therapy were permanently cleared of parasites, suggesting that these formulations may be effective with a reduced or weekly dosing schedule, even in acute infections. While recent clinical studies have explored the potential for weekly prophylactic dosing with Malarone (31), none have yet evaluated nondaily therapy in an acute setting. If effective, such regimens could dramatically reduce treatment cost and make cyt bc_1 inhibitors more accessible for use in areas of malaria endemicity.

While the single-dose efficacy of ATV may simply be a reflection of its exceptional intrinsic potency, which exceeds that of ELQ-300 by approximately 10-fold, these findings could also be explained by the mechanics of the cyt bc_1 complex. In yeast and bacterial model systems, numerous bypass reactions partially compensate for Q_i site blockade by passing electrons to alternative carriers such as oxygen (32). Because similar pathways play a more minor role in Q_o site inhibition, it is possible that Q_o site blockade more rapidly disrupts mitochondrial electron transport and hence pyrimidine biosynthesis. In contrast, the superior multiday efficacy of ELQ-300 may be attributable to the lower resistance propensity associated with this compound (17). Because we sequenced cytochrome *b* only in highly ATV-resistant parasites, it is possible that our cases of ATV recrudescence actually represented the emergence of parasites with low-grade ATV resistance similar to those previously reported both *in vitro* and *in vivo* (16, 33).

One lingering issue is whether there is any inherent clinical risk in utilizing partner drugs with the same biological target. Although this is a novel strategy for malaria, it has been used successfully in anticancer therapies (6). With respect to resistance, although our group has in fact identified resistant *P. falciparum* strains with near-complete resistance to all cyt bc_1 inhibitors *in vitro* (34), such mutations have never been detected in animal models or humans. These resistant isolates also remain susceptible to ATV:proguanil and ELQ-300:proguanil therapy *in vitro*, suggesting that ATV:ELQ-300:proguanil triple therapy could be a second-line strategy in the unlikely event of ATV:ELQ-300 resistance. In light of the recent finding that ATV^r mutations prevent parasite transmission at the mosquito stage (26), we are also evaluating the transmissibility of $ELQ\text{-}300^r$ parasites to determine if impaired replication within the mosquito midgut is a generalizable feature of cytochrome *b* mutations.

Obviously, another limitation of this work is the presumption that our observed effects on *P. yoelii* parasites may be generalized to *P. falciparum* and, by extension, to human disease. ELQ-300 has

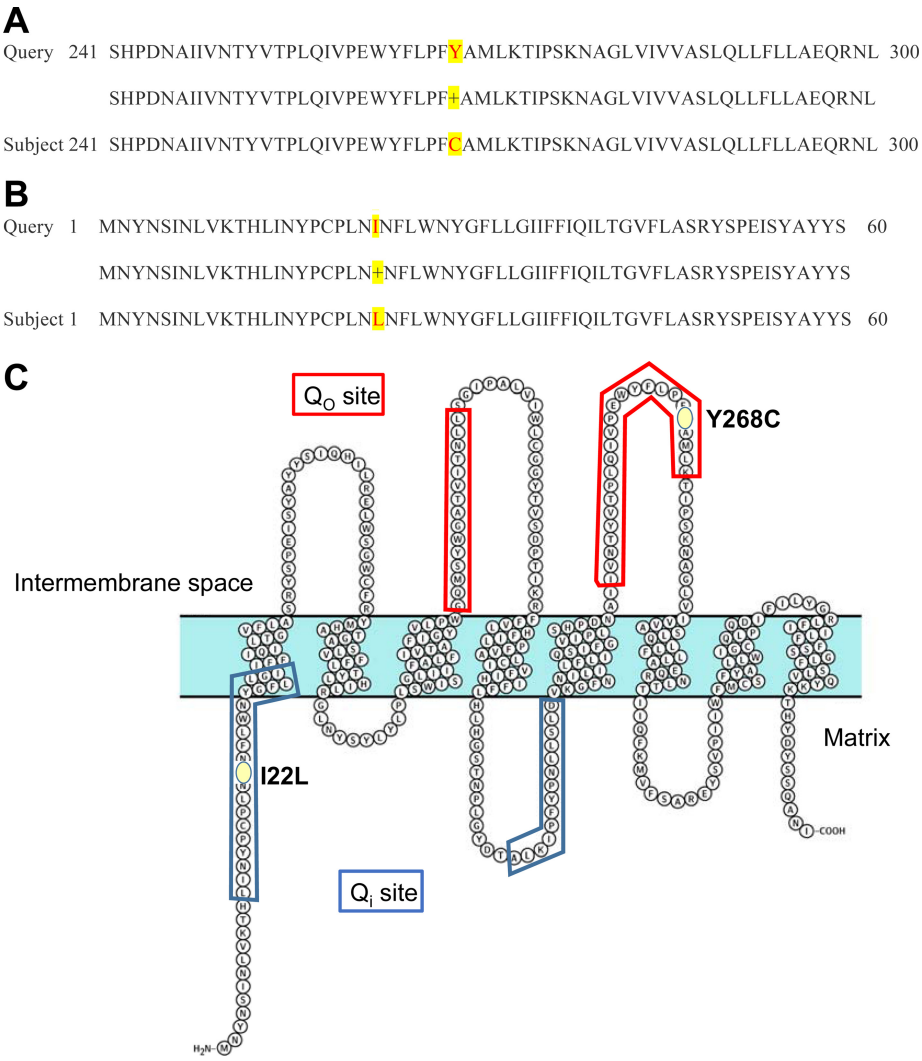

FIG 4 Sequence alignments of cytochrome *b* in ELQ-300^r and ATV^r *P. yoelii* parasites. Three strains collected from ATV-treated animals in the 1-day acute treatment model (ATV^r strain A, ATV^r strain B, and ATV^r strain C) contained Y268C mutations at the cyt bc_1 Q_o site (A), while parasites isolated following ELQ-300 exposure contained an I22L mutation at the cyt bc_1 Q_i site (B). Sequences of parasites from ATV:ELQ-300 and ATV:proguanil-treated animals matched the wild-type sequence. The schematic in panel C shows relevant Q_o and Q_i site residues as well as the Y268C and I22L mutations characteristic of ATV and ELQ-300 resistance.

been studied in SCID mouse models, where its efficacy against *P. falciparum* closely matched that seen in our *P. yoelii* studies (18). Furthermore, the resistant mutants isolated from our *P. yoelii* acute infection model consistently demonstrated the presence of ATV^r and ELQ-300^r resistance mutations identified in *P. falciparum*, including the clinically relevant ATV$^r_{Y268C}$ mutation noted in human patients. In contrast to *in vitro* studies, where ELQ-300 resistance requires precise drug titration (17) and ATV resistance fails to replicate the clinically relevant mutations noted in human disease (33), our model generated key resistance mutations in a span of days, suggesting that our acute infection model is both a highly accurate and time-efficient tool for the evaluation of antimalarial drug resistance. Ultimately, our work demonstrates that ATV:ELQ-300 is a promising combination drug therapy that combines the single-dose efficacy of ATV with the low resistance propensity of ELQ-300, while maintaining the remarkable potency and multistage activity that make cyt bc_1 inhibitors an ideal choice for both prophylactic and acute antimalarial therapy.

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REFERENCES

1. **World Health Organization.** 2015. World Malaria Report 2015. World Health Organization, Geneva, Switzerland.

2. **Sibley CH.** 2014. Understanding drug resistance in malaria parasites: basic science for public health. Mol Biochem Parasitol **195:**107–114. http://dx.doi.org/10.1016/j.molbiopara.2014.06.001.
3. **Bloland P.** 2001. Drug resistance in malaria. World Health Organization, Department of communicable disease surveillance and response, Geneva, Switzerland.
4. **Winzeler E, Manary M.** 25 November 2014. Drug resistance genomics of the antimalarial drug artemisinin. Genome Biol http://dx.doi.org/10.1186/s13059-014-0544-6.
5. **Wells TN, Hooft van Huijsduijnen R, Van Voorhis WC.** 2015. Malaria medicines: a glass half full? Nat Rev Drug Discov **14:**424–442. http://dx.doi.org/10.1038/nrd4573.
6. **Jia J, Zhu F, Ma X, Cao Z, Li Y, Chen YZ.** 2009. Mechanisms of drug combinations: interaction and network perspectives. Nat Rev Drug Discov **8:**111–128. http://dx.doi.org/10.1038/nrd2683.
7. **Fischbach MA.** 2011. Combination therapies for combating antimicrobial resistance. Curr Opin Microbiol **14:**519–523. http://dx.doi.org/10.1016/j.mib.2011.08.003.
8. **Stickles AM, Ting LM, Morrisey JM, Li Y, Mather MW, Meermeier E, Pershing AM, Forquer IP, Miley GP, Pou S, Winter RW, Hinrichs DJ, Kelly JX, Kim K, Vaidya AB, Riscoe MK, Nilsen A.** 2015. Inhibition of cytochrome bc1 as a strategy for single-dose, multi-stage antimalarial therapy. Am J Trop Med Hyg **92:**1195–1201. http://dx.doi.org/10.4269/ajtmh.14-0553.
9. **Srivastava IK, Rottenberg H, Vaidya AB.** 1997. Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite. J Biol Chem **272:**3961–3966. http://dx.doi.org/10.1074/jbc.272.7.3961.
10. **Nilsen A, Miley GP, Forquer IP, Mather MW, Katneni K, Li Y, Pou S, Pershing AM, Stickles AM, Ryan E, Kelly JX, Doggett JS, White KL, Hinrichs DJ, Winter RW, Charman SA, Zakharov LN, Bathurst I, Burrows JN, Vaidya AB, Riscoe MK.** 2014. Discovery, synthesis, and optimization of antimalarial 4(1H)-quinolone-3-diarylethers. J Med Chem **57:**3818–3834. http://dx.doi.org/10.1021/jm500147k.
11. **Painter HJ, Morrisey JM, Mather MW, Vaidya AB.** 2007. Specific role of mitochondrial electron transport in blood-stage Plasmodium falciparum. Nature **446:**88–91. http://dx.doi.org/10.1038/nature05572.
12. **Vaidya AB, Mather MW.** 2009. Mitochondrial evolution and functions in malaria parasites. Annu Rev Microbiol **63:**249–267. http://dx.doi.org/10.1146/annurev.micro.091208.073424.
13. **Looareesuwan S, Chulay JD, Canfield CJ, Hutchinson DBA.** 1999. Malarone (atovaquone and proguanil hydrochloride): a review of its clinical development for treatment of malaria. Am J Trop Med Hyg **60:**533–541.
14. **Birth D, Kao WC, Hunte C.** 2014. Structural analysis of atovaquone-inhibited cytochrome bc1 complex reveals the molecular basis of antimalarial drug action. Nat Commun **5:**4029.
15. **Fisher N, Abd Majid R, Antoine T, Al-Helal M, Warman AJ, Johnson DJ, Lawrenson AS, Ranson H, O'Neill PM, Ward SA, Biagini GA.** 2012. Cytochrome b mutation Y268S conferring atovaquone resistance phenotype in malaria parasite results in reduced parasite bc1 catalytic turnover and protein expression. J Biol Chem **287:**9731–9741. http://dx.doi.org/10.1074/jbc.M111.324319.
16. **Vaidya AB, Mather MW.** 2000. Atovaquone resistance in malaria parasites. Drug Resist Updat **3:**283–287. http://dx.doi.org/10.1054/drup.2000.0157.
17. **Stickles AM, de Almeida MJ, Morrisey JM, Sheridan KA, Forquer IP, Nilsen A, Winter RW, Burrows JN, Fidock DA, Vaidya AB, Riscoe MK.** 2015. Subtle changes in endochin-like quinolone structure alter the site of inhibition within the cytochrome bc1 complex of Plasmodium falciparum. Antimicrob Agents Chemother **59:**1977–1982. http://dx.doi.org/10.1128/AAC.04149-14.
18. **Nilsen A, LaCrue AN, White KL, Forquer IP, Cross RM, Marfurt J, Mather MW, Delves MJ, Shackleford DM, Saenz FE, Morrisey JM, Steuten J, Mutka T, Li Y, Wirjanata G, Ryan E, Duffy S, Kelly JX, Sebayang BF, Zeeman AM, Noviyanti R, Sinden RE, Kocken CH, Price RN, Avery VM, Angulo-Barturen I, Jimenez-Diaz MB, Ferrer S, Herreros E, Sanz LM, Gamo FJ, Bathurst I, Burrows JN, Siegl P, Guy RK, Winter RW, Vaidya AB, Charman SA, Kyle DE, Manetsch R, Riscoe MK.** 2013. Quinolone-3-diarylethers: a new class of antimalarial drug. Sci Transl Med **5:**177ra37. http://dx.doi.org/10.1126/scitranslmed.3005029.
19. **Edstein MD, Kotecka BM, Anderson KL, Pombo DJ, Kyle DE, Rieckmann KH, Good MF.** 2005. Lengthy antimalarial activity of atovaquone in human plasma following atovaquone-proguanil administration. Antimicrob Agents Chemother **49:**4421–4422. http://dx.doi.org/10.1128/AAC.49.10.4421-4422.2005.
20. **Pimentel S, Nogueira F, Benchimol C, Quinhentos V, Bom J, Varandas L, do Rosario V, Bernardino L.** 2006. Detection of atovaquone-proguanil resistance conferring mutations in Plasmodium falciparum cytochrome b gene in Luanda, Angola. Malar J **5:**30. http://dx.doi.org/10.1186/1475-2875-5-30.
21. **Plucinski MM, Huber CS, Akinyi S, Dalton W, Eschete M, Grady K, Silva-Flannery L, Mathison BA, Udhayakumar V, Arguin PM, Barnwell JW.** 2014. Novel mutation in cytochrome b of Plasmodium falciparum in one of two atovaquone-proguanil treatment failures in travelers returning from same site in Nigeria. Open Forum Infect Dis **1:**ofu059. http://dx.doi.org/10.1093/ofid/ofu059.
22. **Jones K, Ward SA.** 2002. Biguanide-atovaquone synergy against Plasmodium falciparum in vitro. Antimicrob Agents Chemother **46:**2700–2703. http://dx.doi.org/10.1128/AAC.46.8.2700-2703.2002.
23. **Srivastava IK, Vaidya AB.** 1999. A mechanism for the synergistic antimalarial action of atovaquone and proguanil. Antimicrob Agents Chemother **43:**1334–1339.
24. **Vaidya A.** 2012. Naphthoquinones: atovaquone and other antimalarials targeting mitochondrial functions, p 127–139. *In* Staines HM, Krishna S. (ed), Milestones in drug therapy. Treatment and prevention of malaria: antimalarial drug chemistry, action and use. Springer, New York, NY.
25. **Maude RJ, Nguon C, Dondorp AM, White LJ, White NJ.** 24 September 2014. The diminishing returns of atovaquone-proguanil for elimination of Plasmodium falciparum malaria: modelling mass drug administration and treatment. Malaria http://dx.doi.org/10.1186/1475-2875-13-380.
26. **Goodman CD, Siregar JE, Mollard V, Vega-Rodriguez J, Syafruddin D, Matsuoka H, Matsuzaki M, Toyama T, Sturm A, Cozijnsen A, Jacobs-Lorena M, Kita K, Marzuki S, McFadden GI.** 2016. Parasites resistant to the antimalarial atovaquone fail to transmit by mosquitoes. Science **352:**349–353. http://dx.doi.org/10.1126/science.aad9279.
27. **Fivelman QL, Adagu IS, Warhurst DC.** 2004. Modified fixed-ratio isobologram method for studying in vitro interactions between atovaquone and proguanil or dihydroartemisinin against drug-resistant strains of Plasmodium falciparum. Antimicrob Agents Chemother **48:**4097–4102. http://dx.doi.org/10.1128/AAC.48.11.4097-4102.2004.
28. **Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe MK.** 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob Agents Chemother **48:**1803–1806. http://dx.doi.org/10.1128/AAC.48.5.1803-1806.2004.
29. **Malleret B, Claser C, Ong AS, Suwanarusk R, Sriprawat K, Howland SW, Russell B, Nosten F, Renia L.** 2011. A rapid and robust tri-color flow cytometry assay for monitoring malaria parasite development. Sci Rep **1:**118.
30. **Hudson AT, Dickins M, Ginger CD, Gutteridge WE, Holdich T, Hutchinson DBA, Pudney M, Randall AW, Latter VS.** 1991. 566C80: a potent broad spectrum anti-infective agent with activity against malaria and opportunistic infections in AIDS patients. Drugs Exp Clin Res **17:**427–435.
31. **Deye GA, Miller RS, Miller L, Salas CJ, Tosh D, Macareo L, Smith BL, Fracisco S, Clemens EG, Murphy J, Sousa JC, Dumler JS, Magill AJ.** 2012. Prolonged protection provided by a single dose of atovaquone-proguanil for the chemoprophylaxis of Plasmodium falciparum malaria in a human challenge model. Clin Infect Dis **54:**232–239. http://dx.doi.org/10.1093/cid/cir770.
32. **Muller F, Crofts AR, Kramer DM.** 2002. Multiple Q-cycle bypass reactions at the Qo site of the cytochrome bc1 complex. Biochemistry **41:**7866–7874. http://dx.doi.org/10.1021/bi025581e.
33. **Schwobel B, Alifrangis M, Salanti A, Jelinek T.** 19 March 2003. Different mutation patterns of atovaquone resistance to Plasmodium falciparum in vitro and in vivo: rapid detection of codon 268 polymorphisms in the cytochrome b as potential in vivo resistance marker. Malaria J http://dx.doi.org/10.1186/1475-2875-2-5.
34. **Smilkstein MJ, Forquer I, Kanazawa A, Kelly JX, Winter RW, Hinrichs DJ, Kramer DM, Riscoe MK.** 2008. A drug-selected Plasmodium falciparum lacking the need for conventional electron transport. Mol Biochem Parasitol **159:**64–68. http://dx.doi.org/10.1016/j.molbiopara.2008.01.002.