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EXPERIMENTAL THERAPEUTICS

Effective Therapy Targeting Cytochrome bc_1 Prevents *Babesia* Erythrocytic Development and Protects from Lethal Infection

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Joy E. Chiu and Isaline Renard contributed equally to this work. Joy E. Chiu conducted initial *in vitro* and *in vivo* studies. Isaline Renard conducted subsequent *in vivo* efficacy studies using an optimized mouse model.

ABSTRACT An effective strategy to control blood-borne diseases and prevent outbreak recrudescence involves targeting conserved metabolic processes that are essential for pathogen viability. One such target for *Plasmodium* and *Babesia*, the infectious agents of malaria and babesiosis, respectively, is the mitochondrial cytochrome bc_1 protein complex, which can be inhibited by endochin-like quinolones (ELQ) and atovaquone. We used the tick-transmitted and culturable blood-borne pathogen *Babesia duncani* to evaluate the structure-activity relationship, safety, efficacy, and mode of action of ELQs. We identified a potent and highly selective ELQ prodrug (ELQ-502), which, alone or in combination with atovaquone, eliminates *B. microti* and *B. duncani* infections *in vitro* and in mouse models of parasitemia and lethal infection. The strong efficacy at low dose, excellent safety, bioavailability, and long half-life of this experimental therapy make it an ideal clinical candidate for the treatment of human infections caused by *Babesia* and its closely related apicomplexan parasites.

KEYWORDS *Babesia duncani*, *Babesia microti*, babesiosis, endochin-like quinolone, therapy, apicomplexan parasites, parasitology, red blood cells

The rapid increase in the number of tick-borne diseases reported over the last decade, including human babesiosis, highlights the need for developing new and effective therapies (1, 2). *Babesia microti*, *Babesia duncani*, *Babesia divergens*, and *Babesia venatorum* are the main species associated with human babesiosis (1–3). Symptoms of severe cases of human babesiosis include acute respiratory distress syndrome, hemolytic anemia, multiple organ failure, and, possibly, death (2, 3). Although transmission of the parasite to human occurs mainly through a bite from an infected tick, increasing numbers of human-to-human transmission cases through blood transfusion have been reported (4, 5). In 2009, *Babesia* was added to the list of infectious agents identified as a potential threat to the safety of blood supplies, and babesiosis has become a nationally notifiable disease since 2011 (6). Today, *B. microti* is one of the most commonly reported transfusion-transmitted pathogens and is the leading infectious cause of transfusion-related deaths, with one in five cases of babesiosis acquired through blood transfusion resulting in fatal outcome (7, 8).

Due to its shared clinical features with malaria, current treatments for babesiosis are based on the use of combinations of known antimalarial drugs. These include the combination of atovaquone plus azithromycin and the combination of clindamycin plus quinine for moderate and severe babesiosis, respectively (1, 9). However, these therapies are often associated with adverse side effects and may have a role in the emergence of drug-resistant strains (1). As a result, there is a growing need to develop treatments or combination

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therapies that specifically target *Babesia*, have minimal side effects, and are relatively refractory to changes associated with acquired drug resistance in the parasites.

Recent genomic and phylogenetic analyses of several blood-borne pathogens have offered unique insights into the biology of some closely related organisms and revealed conserved metabolic pathways that could be exploited for the development of such targeted therapies. For instance, our group previously identified endochin-like quinolones (ELQs) as a novel class of anti-*B. microti* drugs that target the quinone reduction (Q_i) site of the parasite's cytochrome *b* (Cytb) protein complex (10). Because of the conserved nature of the *Cytb* gene between various apicomplexan parasites, ELQ-based drugs have also shown efficacy against other species, such as *Plasmodium falciparum* and *Toxoplasma gondii*, the infectious agents of malaria and toxoplasmosis, respectively (11–15). However, the poor bioavailability of these compounds due to high crystallinity and low aqueous solubility was a limiting factor to their *in vivo* evaluation, precluding administration of doses higher than 10 mg/kg of body weight. To improve the oral bioavailability of this class of compounds, a prodrug strategy was developed whereby the carbonyl of the quinolone core was esterified, resulting in decreased crystallinity of the prodrug and increased plasma concentration of the corresponding active drug following cleavage by the host esterase (16). With this improved bioavailability, a new prodrug candidate, ELQ-334, was evaluated *in vivo* against *B. microti*. Although single-drug treatment resulted in the emergence of resistant parasites associated with a mutation in the Cytb Q_i site, a combination of ELQ-334 plus atovaquone at doses as low as 5 plus 5 mg/kg resulted in elimination of *B. microti* infection in 100% of mice (10). However, due to the lack of an *in vitro* culture system for *B. microti*, structure-activity relationship (SAR) analyses to improve the potency and pharmacological properties of ELQ-334 and its parent drug, ELQ-316, were limited. The recent breakthrough in the continuous *in vitro* propagation of *B. duncani* in human red blood cells (RBCs) (17), together with the availability of suitable animal models of parasitemia and lethal infection, provided a unique opportunity to screen ELQ derivatives for *in vitro* potency and safety and to evaluate their efficacy in mouse models of babesiosis (17).

Here, we report successful ELQ SAR studies using this new model system and the identification of ELQ-502 as a highly potent, safe, and effective drug, which, alone or in combination with atovaquone, achieves complete elimination of parasite burden and avoids lethal infection.

RESULTS

Structure-activity relationship analysis of ELQ compounds. To identify new ELQ compounds with improved efficacy and selectivity, structure-activity relationship studies were conducted using *B. duncani* propagated *in vitro* in human red blood cells (17). A set of 28 ELQ analogs were synthesized and tested for their ability to inhibit the development of *B. duncani* in human red blood cells (Fig. 1a and b). Of these drugs, 20 compounds inhibited growth of *B. duncani* by more than 80% at 1 μM. Seven of these compounds inhibited growth of the parasite by more than 80% at 100 nM (Fig. 1b). ELQ-447 and its respective prodrug, ELQ-502, were further selected based on their *in vitro* efficacy and desirable safety profile. Both parent drug (ELQ-447) and prodrug (ELQ-502) showed excellent potency *in vitro* (Fig. 1c). With a 50% inhibitory concentration (IC_{50}) of 165 $\pm$ 1 nM, ELQ-447 displayed a potency similar to that of the previously reported ELQ-316 and ELQ-334, whose IC_{50} values were 136 $\pm$ 1 nM and 193 $\pm$ 56 nM, respectively (Fig. 1c and d). Interestingly, the *in vitro* potency of ELQ-502 (IC_{50} = 6 $\pm$ 2 nM) was 28$\times$ higher than its parent drug (Fig. 1c). We further evaluated the safety profile of ELQ-502 by measuring its toxicity profile in HeLa, HepG2, and HCT116 cell lines under conditions permissive for glycolysis (glucose-based media) or for mitochondrial oxidative phosphorylation (galactose-based media) (Table 1). Under all these conditions, the calculated IC_{50} of ELQ-502 was greater than 5 μM and its therapeutic index (IC_{50} human cells/IC_{50} *B. duncani*) was greater than 833 (Table 1), making it an ideal lead candidate for further evaluation in animal models.

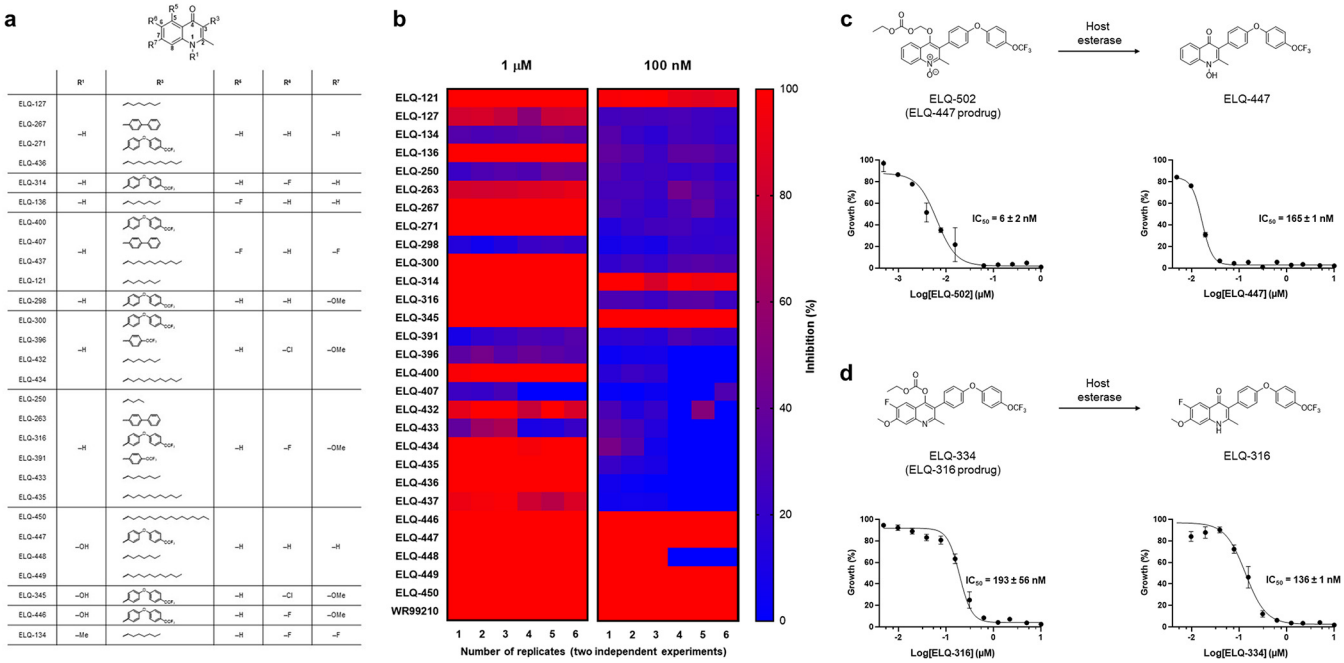

FIG 1 *In vitro* efficacy of ELQ analogs against *B. duncani* in human RBCs. (a) Chemical structure of ELQs used in this study. (b) Twenty-eight ELQ derivatives were evaluated *in vitro* against *B. duncani* at 1 μM and 100 nM. WR99210, a potent antiparasitic drug (25, 26), was used as a positive control and used to determine 100% growth inhibition. (c and d) Structure and potency of ELQ drugs and prodrugs against *B. duncani* and IC_{50} determination using ELQ-447 and ELQ-502 (c) and ELQ-316 and ELQ-334 (d).

***In vivo* efficacy of ELQ-502 in *B. duncani*-infected mice.** The high potency and low toxicity of ELQ-502 against *B. duncani in vitro* led us to investigate the efficacy of this prodrug in mice infected with *B. duncani* WA-1 isolate, both as a single therapy or in combination with atovaquone (Fig. 2). These assays were conducted in C3H/HeJ mice, which show a mortality rate of 100% 10 to 11 days postinfection with 10^3 *B. duncani*-infected red blood cells. Whereas oral administration of the vehicle alone (polyethylene glycol 400 [PEG 400]) resulted in 100% mortality by day 11 postinfection (DPI 11), with parasitemia reaching ~6% (Fig. 2a and e), oral administration of atovaquone, ELQ-502, or ELQ-502 plus atovaquone at 10 mg/kg each for 10 days (DPI 1 to 10) resulted in elimination of parasitemia in most, if not all, infected animals (Fig. 2b to d). Complete elimination of *B. duncani* infection with no recrudescence was achieved with ELQ-502 and ELQ-502 plus atovaquone (Fig. 2c and d). No animals succumbed to lethal *B. duncani* infection following treatment with ELQ-502 or ELQ-502 plus atovaquone (Fig. 2e). To confirm that animals treated with ELQ-502 monotherapy have cleared *B. duncani* infection beyond the endpoint of the experiment (DPI 45), the animals were monitored for an additional 46 days (until DPI 91) and their blood examined. No parasites could be detected following examination of Giemsa-stained thin-blood smears (see Fig. S1 in the supplemental material). The absence of parasites in the ELQ-502-treated animals was further confirmed by real-time quantitative PCR (qPCR) using SYBR green I from genomic DNA extracted from blood samples obtained at DPI 91. Quantification cycle (C_q) values obtained for samples

TABLE 1 Activity of ELQ-502 in *B. duncani*-infected erythrocytes, HeLa, HepG2, and HCT116 cells

Compound	IC_{50} (nM) *B. duncani*	IC_{50} (μM) HeLa H-Glc	HeLa L-Glc	HeLa Gal	HepG2 H-Glc	HepG2 L-Glc	HepG2 Gal	HCT116 H-Glc	HCT116 L-Glc	HCT116 Gal	Therapeutic index
ELQ-502	6 ± 2	>10 μM	>5	>10	>10	>10	>10	>10	>10	>5	>833
Atovaquone	72 ± 6	>5	>5	>5	>5	>5	>5	>5	>5	>5	>69

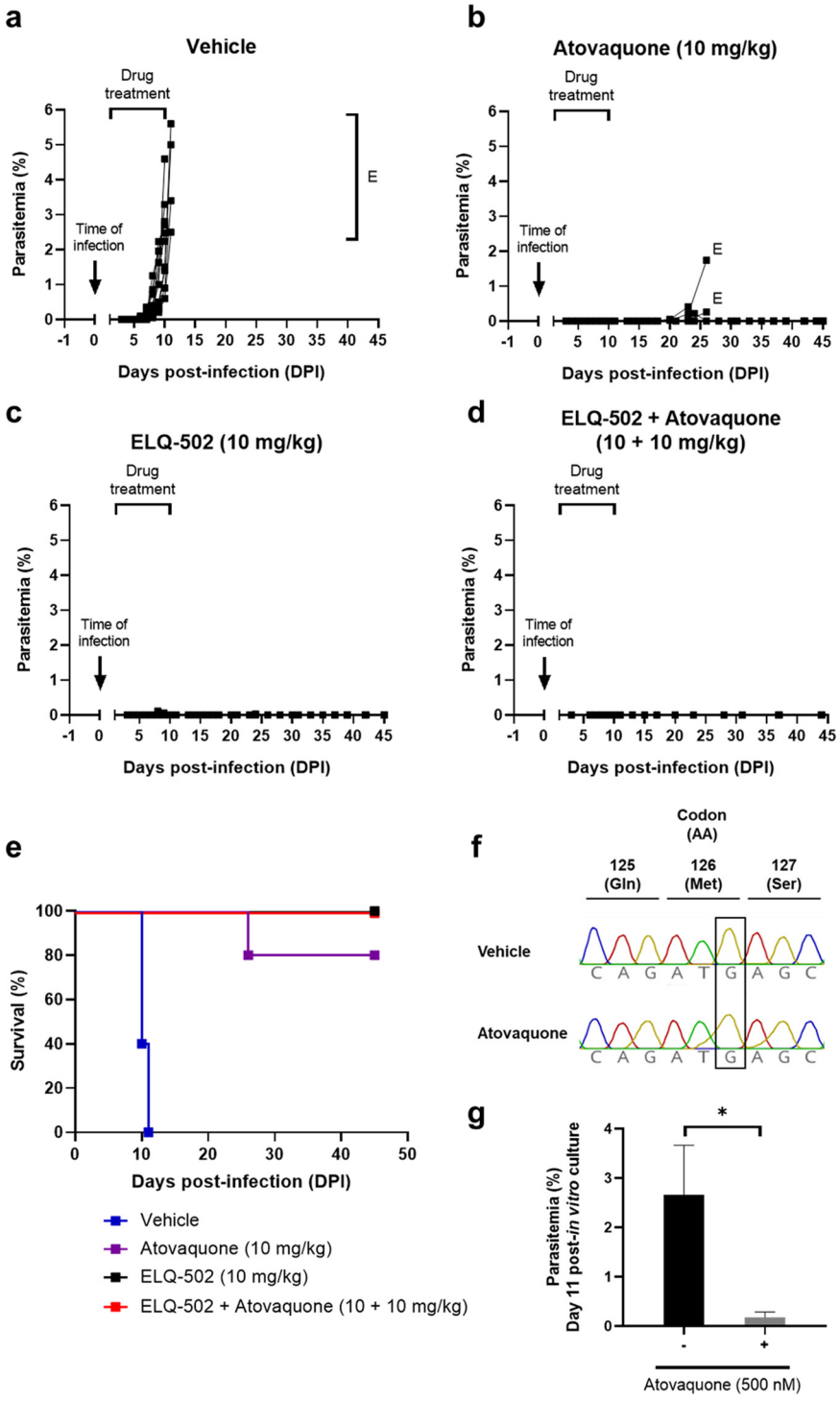

FIG 2 Efficacy of atovaquone and ELQ-502, alone or in combination, in a mouse model of lethal *B. duncani* infection. (a to e) Female C3H/HeJ mice were infected i.v. with 10^3 *B. duncani*-infected red blood cells. Animals were treated daily (DPI 1 to 10) by oral gavage with the vehicle alone (PEG 400) (a), atovaquone at 10 mg/kg (b), ELQ-502 at 10 mg/kg (c), or a combination of ELQ-502 plus atovaquone at 10 plus 10 mg/kg (d). The letter E indicates when an individual mouse was euthanized. (e) Survival rate of *B. duncani*-infected mice in the absence or following treatment with atovaquone, ELQ-502, or atovaquone plus ELQ-502. (f) Representative sequencing chromatogram of recrudescent parasites from atovaquone-treated *B. duncani*-infected mice. Genomic DNA was extracted from recrudescent parasites and used to amplify the *B. duncani Cytb* gene, followed by sequencing. As a control, the chromatogram of wild-type parasites (obtained from control animals) is shown. No mutation in the *B. duncani Cytb* gene could be found. (g) *In vitro* drug susceptibility of recrudescent parasites from atovaquone-treated *B. duncani*-infected mice. Parasites were cultured *in vitro* in human erythrocytes in the absence or presence

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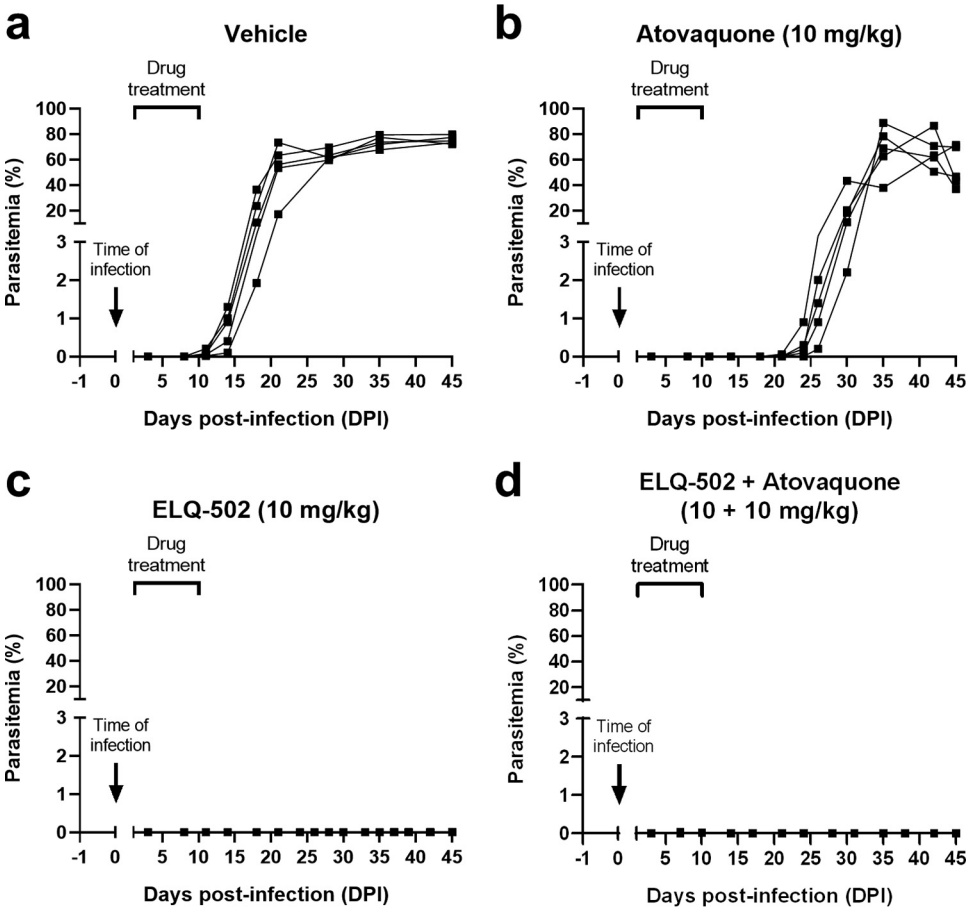
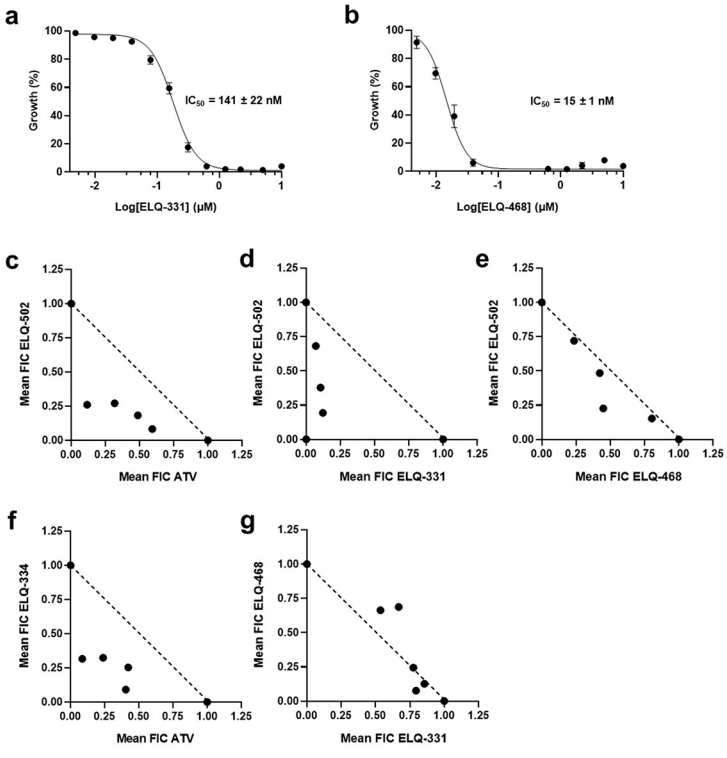


FIG 3 *In vivo* evaluation of atovaquone and ELQ-502, as single drugs or in combination in *B. microti*-infected mice. Female SCID mice were injected with either 10^4 i.v. or 10^6 i.p. *B. microti*-infected red blood cells. Animals were treated daily (DPI 1 to 10) by oral gavage with the vehicle alone (PEG 400) (a), atovaquone at 10 mg/kg (b), ELQ-502 at 10 mg/kg (c), or ELQ-502 plus atovaquone at 10 plus 10 mg/kg (d).

showing no parasitemia by light microscopy were above the lower limit of detection (C_q, ~32) and were similar to those obtained for samples from uninfected mice and from the no-template control (NTC) (Fig. S3). In contrast, samples collected at DPI 10 and 11 had C_q values of ~22, consistent with the presence of infected red blood cells, as demonstrated by Giemsa staining (Fig. 2a and Fig. S3). Together, these data demonstrate that treatment with ELQ-502 results in complete elimination of infection in animals. Similar to previous analyses of relapse following drug treatment of *B. microti*-infected mice (10), treatment with atovaquone was accompanied by recrudescence in 2 out of 10 mice (Fig. 2b) starting at DPI 20, whereas no parasitemia could be detected in the remaining 8 mice up to DPI 45 (end of study). Surprisingly, analysis of the *Cytb* gene from reemerging parasites showed a lack of mutations in this gene, and *in vitro* culture of parasites that emerged from atovaquone-treated mice showed that they remained susceptible to the drug (Fig. 2f and g).

***In vivo* efficacy of ELQ-502 in *B. microti*-infected mice.** Following the promising results observed in the *in vivo* model of *B. duncani*, ELQ-502 was subsequently evaluated alone or in combination with atovaquone in *B. microti*-infected mice using the SCID mouse model of *B. microti* infection. Whereas parasitemia in vehicle-administered control animals reached high levels by DPI 20 and leveled out at 60 to 80% parasitemia for the remainder of the study (Fig. 3a), treatment with atovaquone at 10 mg/kg was

FIG 2 Legend (Continued)
of atovaquone at 500 nM. At day 11 after *in vitro* culture, parasites cultured in the absence of drug reached ~2.5% parasitemia, whereas parasites treated with atovaquone remained at 0.18% parasitemia. *, $P < 0.02$.



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Drug combination	Mean ΣFIC$_{50}$	Interaction
ELQ-502 + Atovaquone	0.6	Synergistic
ELQ-334 + Atovaquone	0.5	Synergistic
ELQ-502 + ELQ-331	0.4	Synergistic
ELQ-502 + ELQ-468	0.9	Synergistic
ELQ-468 + ELQ-331	1.2	Additive

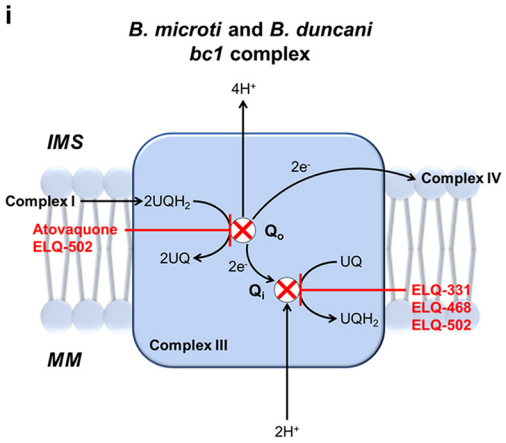

FIG 4 Atovaquone-ELQ and ELQ-ELQ drug-drug interactions. (a and b) Potency of two other promising ELQ prodrugs against *B. duncani* and IC_{50} determination, ELQ-331 (a) and ELQ-468 (b). (c to g) Isobolograms depicting the activity of ELQ-502 in combination with atovaquone (c), ELQ-331 (d), and ELQ-468 (e). For reference, isobolograms of combination of ELQ-334 plus atovaquone (f) and ELQ-331 plus ELQ-468 (g) are shown. Each point in the graph represents the mean FIC_{50} value from a fixed drug ratio as described in Materials and Methods. A dotted line plotted between the individual mean FIC_{50} value of each drug serves as additive line. (h) Summary of drug interactions. (i) Schematic representation of the *B. microti* and *B. duncani* bc_1 complex and proposed mode of action of atovaquone, ELQ-331, ELQ-468, and ELQ-502. IMS, intermembrane space; MM, mitochondrial matrix.

accompanied by the emergence of recrudescent parasites by DPI 24 (Fig. 3b). Interestingly, *B. microti*-infected mice treated with ELQ-502 alone (10 mg/kg) or in combination with atovaquone (10 plus 10 mg/kg) showed no sign of infection for at least 35 days after completion of the drug treatment (Fig. 3c and d). These mice were further monitored until at least DPI 91, and no parasites could be detected by examination of Giemsa-stained thin-blood smears (Fig. S2a). qPCR analysis of samples collected at DPI 91 showed C_q values of ~33, similar to those obtained for samples from uninfected mice and from the no-template control (NTC), whereas samples collected at DPI 21 or DPI 24 showed C_q values of ~18, suggesting the samples obtained from treated animals at DPI 91 were free of parasites (Fig. S4). Mice treated with a combination of ELQ-502 and atovaquone were monitored for up to 120 days (110 posttreatment), and no parasites could be detected (Fig. S2b).

Evidence of a dual mode of action of ELQ-502 through drug-drug interaction analyses. The ability of ELQ-502 to achieve cure with no recrudescence in both *B. duncani*- and *B. microti*-infected mice following a 10-day treatment at 10 mg/kg starting at D1 postinfection led us to further investigate its mode of action. This was achieved by examining the possible additive, synergistic, or antagonistic effect of dual combinations of ELQ-502 with drugs known to inhibit either the Q_o site (atovaquone) or the Q_i site (ELQ-331 and ELQ-468) of the cytochrome bc_1 complex (18). Both ELQ-331 and ELQ-468 were found to have potent activity against *B. duncani in vitro*, with IC_{50} values of 141 ± 22 nM and 15 ± 1 nM, respectively (Fig. 4a and b). Interestingly, combinations of ELQ-502 with atovaquone, ELQ-331, or ELQ-468 were found to be synergistic, with a mean fractional inhibitory concentration (ΣFIC_{50}) value of 0.6, 0.4, and 0.9, respectively (Fig. 4c to e and h). As a control, a combination of ELQ-334 and atovaquone (Fig. 4f and h) was also found to be synergistic, consistent with its previously reported efficacy in *B. microti*-infected mice (10). However, unlike the synergistic effects seen with

ELQ-502, a combination of ELQ-331 and ELQ-468 was mainly additive ($\Sigma FIC_{50} = 1.2$) (Fig. 4g and h). Together, these data suggest that ELQ-502 targets not only the Q_i site of the *cytochrome* bc_1 complex but also its Q_o site, and this dual activity may account for its high potency (Fig. 4i).

DISCUSSION

Our results show that ELQ-502 is a potent and highly effective endochin-like quinolone against *Babesia microti* and *Babesia duncani in vitro* and in mouse models of high parasitemia and lethal babesiosis infection, either as a monotherapy or in combination with atovaquone. ELQs have been shown to target the Q_i site or the Q_o site of cytochrome *b* of multiple apicomplexan parasites, such as *Plasmodium* spp. (15, 16, 19–21), *Toxoplasma gondii* (11), and *Babesia microti* (10). Whereas the ability to maintain *P. falciparum* and *T. gondii* in culture made it possible to conduct structure-activity relationship studies to identify inhibitors suitable for each pathogen, detailed structure-activity relationship evaluation could not be conducted in *B. microti* due to the lack of an *in vitro* culture system. The recent development of a continuous *in vitro* culture system for *B. duncani* in human red blood cells (17) provides a means to probe different moieties that define the selective potency of ELQs both *in vitro* and in a small-animal model.

With an IC_{50} value of 6 ± 2 nM, ELQ-502 was found to be more active than its parent compound, ELQ-447 ($IC_{50} = 165 \pm 1$ nM), and more active than the reference compounds ELQ-316 and ELQ-334 ($IC_{50} = 136 \pm 1$ nM and $IC_{50} = 193 \pm 56$ nM, respectively). This enhanced efficacy most likely can be attributed to the higher aqueous solubility of ELQ-502. Moreover, the lack of activity of ELQ-502 in HepG2, HeLa, and HCT116 human cell lines at concentrations of $\leq 5\ \mu$M resulted in a highly desirable therapeutic index (>833).

Unlike the previously examined antibabesial prodrug ELQ-334, the prodrug moiety selected to synthesize ELQ-502 was a methoxy ethyl carbonate. The two derivatives share a position 3 diarylether side chain replaced in *para* of the external ring with a trifluoromethoxy functional group. The use of this type of side chain was previously reported to improve potency and metabolic stability when assessed against *Plasmodium* parasites (13). One notable difference between ELQ-502 and ELQ-334 is the replacement of the amine with a hydroxyl functional group in the case of ELQ-502, potentially resulting in increased hydrogen bonding and/or electrostatic interactions.

Our studies in both *B. duncani*- and *B. microti*-infected mice showed strong *in vivo* efficacy of ELQ-502 as a monotherapy or in combination with atovaquone, resulting in complete parasite elimination with no recrudescence, and, in the case of *B. duncani*, 100% survival of animals from lethal infection. To determine whether the success of the ELQ-502 plus atovaquone combination therapy *in vivo* can be attributed to synergy between the two drugs, combination studies were carried out *in vitro* in *B. duncani* and identified synergistic interactions between ELQ-502 and atovaquone. To further understand the mode of action of ELQ-502, drug-drug interaction studies were carried out with two ELQ derivatives, ELQ-331 and ELQ-468, with potent activity against *B. duncani in vitro* ($IC_{50} = 141 \pm 22$ nM and $IC_{50} = 15 \pm 1$ nM, respectively). These studies showed synergy between ELQ-502 and either ELQ-331 or ELQ-468, whereas only additive interactions were observed between ELQ-331 and ELQ-468. One possible explanation for the unique potency of ELQ-502 is that this drug targets both the Q_i and Q_o sites of the cytochrome bc_1 complex. This ability of some ELQ compounds to target both sites was previously demonstrated for ELQ-400 in yeast (22). Nevertheless, a 5-day treatment with ELQ-502 starting at day 3 resulted in the emergence of resistant parasites carrying a mutation in the Q_i site. This emphasizes the importance of a combination therapy as an ideal strategy for the development of new treatment for human babesiosis.

In conclusion, we propose that a combination of ELQ-502 plus atovaquone offers a superior option to current treatments for human babesiosis, which are associated with adverse events, low efficacy, and resistance occurrence. Future studies aimed at characterizing

the safety of this compound in other mammals and in humans are needed before it can be evaluated for efficacy in human trials.

MATERIALS AND METHODS

Chemistry. Unless otherwise stated, chemicals were purchased from commercial suppliers and used as received. ELQ compounds were synthesized by following methods previously described by Doggett et al. (11), Nilsen et al. (13), and Frueh et al. (18), identified by proton nuclear magnetic resonance (^{1}H NMR), and determined to be ≥95% pure by reversed-phase high-performance liquid chromatography (RP-HPLC).

***In vitro* culture of *B. duncani*.** *In vitro* culture of *B. duncani* was carried out as previously reported by Abraham et al. (17). A cryostock of *B. duncani* was thawed at 37°C. The content of the vial (≈1 ml) was transferred to a 50-ml falcon tube, and 200 μl of 12% (wt/vol) NaCl was added dropwise. The resulting mixture was incubated at room temperature for 5 min; 10 ml of 1.6% (wt/vol) NaCl was slowly added, and the suspension was centrifuged at 1,800 rpm for 5 min. The supernatant was removed, leaving ≈500 μl to resuspend the pellet. Ten milliliters of prewarmed complete HL-1 medium (HL-1 base medium [344017; Lonza] supplemented with 20% heat-inactivated fetal bovine serum [FBS; F4135; Sigma], 2% 50× HT medium supplement Hybri-Max [H0137; Sigma], 1% 200 mM L-glutamine [25030-081; Gibco], 1% 100× penicillin-streptomycin [15240-062; Gibco], and 1% 10 mg/ml gentamicin [15710-072; Gibco]) was added slowly, and the resulting mixture was centrifuged at 1,800 rpm for 5 min. The supernatant was removed, and the pellet was resuspended in 500 μl of complete HL-1 medium. The parasite suspension and 100 μl of 50% hematocrit A$^+$ RBCs (5% final hematocrit) were plated in one well of a 24-well plate. The final volume was made up to 1 ml by adding complete medium. The plate was incubated at 37°C under a 2% O_2, 5% CO_2, 93% N_2 atmosphere in a humidified chamber. Culture medium was changed daily, and parasitemia was monitored by light microscope examination of Giemsa-stained thin-blood smears.

***In vitro* drug efficacy in *B. duncani*-infected human red blood cells.** Experiments were performed to evaluate the effect of ELQs on intraerythrocytic development cycle (IDC) inhibition of *B. duncani* and determine their IC_{50} values. *In vitro* parasite culture (0.1% parasitemia with 5% hematocrit in HL-1 medium) was treated with decreasing concentrations (2-fold dilution starting from 10 μM) of the compound of interest for 60 h in a 96-well plate. After treatment, parasite viability was determined by the SYBR green I method (17). Briefly, to 100 μl of parasite culture, an equal volume of lysis buffer (0.008% saponin, 0.08% Triton X-100, 20 mM Tris-HCl [pH 7.5], and 5 mM EDTA) containing SYBR green I (0.01%) was added and incubated at 37°C for 1 h in the dark. The fluorescent intensity was measured for λ_{ex} = 480 nm and λ_{em} = 540 nm using a BioTek Synergy Mx microplate reader. Background fluorescence (uninfected RBCs in HL-1 medium) was subtracted from each measurement. The half-maximal inhibitory concentration (IC_{50}) was determined from a sigmoidal dose-response curve using GraphPad Prism version 8.1. Data are shown as means ± standard deviations (SD) from two independent experiments performed in biological triplicates.

Drug activity in mammalian cells. HeLa, HepG2, and HCT116 cells were purchased from the ATCC. Cell toxicity studies were performed at the Yale Center for Molecular Discovery using seeding cell densities optimized by the Center: HeLa (400 cells/well), HepG2 (1,500 cells/well), and HCT116 (1,500 cells/well). Each cell line was grown in three different media: high-glucose medium (high-glucose Dulbecco's modified Eagle's medium [11995-065; DMEM; Invitrogen] containing 25 mM glucose, 1 mM sodium pyruvate and supplemented with 5 mM HEPES, 10% FBS, and penicillin-streptomycin [50 U/ml penicillin, 50 μg/ml streptomycin]), low-glucose medium (11966-025; DMEM deprived of glucose; Invitrogen) supplemented with 5.5 mM glucose, 5 mM HEPES, 10% FBS, 1 mM sodium pyruvate, and penicillin-streptomycin (as described above), and galactose medium (11966-025; DMEM deprived of glucose; Invitrogen) supplemented with 10 mM galactose, 2 mM glutamine, 5 mM HEPES, 10% FBS, 1 mM sodium pyruvate, and penicillin-streptomycin (as described above). Cells were seeded into 384-well tissue culture-treated plates (3764; Corning) and allowed to adhere overnight prior to compound treatment. On day 2, compounds and dimethyl sulfoxide (DMSO) were added using an Echo acoustic dispenser (Labcyte), and 72 h later viable cells were detected using CellTiter Glo (Promega). Compounds were tested in a 16-point dose response (10 μM highest final concentration and 2-fold dilutions). Each plate included 16 wells treated with 10% DMSO (positive control for complete toxicity) and 16 wells treated with 0.1% DMSO (negative control or vehicle). Signal-to-background (S/B), coefficient of variation (CV), and Z prime factor (Z′) were calculated for each cell line using mean and standard deviation values of the positive and negative controls to ensure assay robustness. Compound data were normalized to the mean of 10% DMSO wells (set as 100% toxicity) and mean of the vehicle control wells (set as 0% toxicity). Dose-response curves were plotted using GraphPad Prism, version 8.1.

Mouse strains. SCID mice (C.B-17/IcrHsd-Prkdc^{scid}) were obtained from Envigo. C3H/HeJ mice were obtained from The Jackson Laboratory. All animal experiments followed Yale University institutional guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committees (IACUC) at Yale University.

***In vivo* drug efficacy in *B. duncani*- and *B. microti*-infected mice.** Female C3H/HeJ mice (5 to 6 weeks old, n = 5 to 10 mice/group) were infected intravenously (i.v.) with 10^3 *B. duncani* (WA1)-infected RBCs. Treatment was administered daily for 10 days by oral gavage, beginning on day 1 postinfection (DPI 1). Animals received 100 μl of the vehicle alone (PEG 400), atovaquone (10 mg/kg), ELQ-502 (10 mg/kg), or a combination of ELQ-502 plus atovaquone (10 plus 10 mg/kg).

Female SCID mice (5 to 6 weeks old, n = 5 mice/group) were infected with either 10^4 i.v. or 10^6 intraperitoneally (i.p.) *B. microti* (LabS1)-infected RBCs. Treatment was administered daily for 10 days by oral gavage,

beginning on day 1 postinfection. Animals received 100 μl of the vehicle alone (PEG 400), atovaquone (10 mg/kg), ELQ-502 (10 mg/kg), or a combination of ELQ-502 plus atovaquone (10 plus 10 mg/kg).

Genomic DNA isolation, *Cytb* PCR, and sequencing. Blood was collected and allowed to clot at room temperature for 30 min. Genomic DNA was extracted from the blood pellet using a DNeasy blood and tissue kit (69506; Qiagen). PCR was performed on the extracted DNA samples to amplify the *B. duncani* cytochrome *b* gene. The following primers were used: 1F, 5′-GGATACAGGGCTATAACCAACAA-3′; 2R, 5′-GGAAGTTGGCGTCTAGAGTCACTC-3′. The cycling conditions were 95°C for 5 min, 12 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s, and 33 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s. The PCR products were purified using a QIAquick PCR purification kit (28106; Qiagen) and sent for Sanger sequencing at the Keck Sequencing Facility (Yale University) using the previous primers in addition to 1R, 5′-TATGCAATTTGAAGTGAAATTCC-3′, and 2F, 5′-TTGGGTTGGAGACTTTGTCAG-3′. Sequences were analyzed using Geneious 11.1.5 (https://www.geneious.com).

Parasitemia determination. Parasitemia was monitored by light microscopy evaluation of Giemsa-stained thin-blood smears. In each case, a minimum of 2,000 red blood cells were counted to determine the parasitemia. To further confirm the absence of parasitemia in samples from *B. duncani*- and *B. microti*-infected mice that received ELQ-502 monotherapy, genomic DNA was isolated from samples collected at different times following infection and analyzed by real-time PCR using SYBR green I and a sequence within the internal transcribed spacer (ITS) of nuclear rRNA as the amplification target. Real-time PCR experiments were carried out in a Bio-Rad CFX96 system. Each reaction mixture contained 1× SsoAdvanced universal SYBR green supermix (2×) (1725270; Bio-Rad), 0.5 μM each primer, and 2 μl of DNA. Nuclease-free water was added to achieve a final volume of 20 μl. The following primers were used: BdITS1-F, 5′-GCTTCCTAACCCGAGACCAA-3′, and BdITS1-R, 5′-CACTGGCGGGGTGAAAAGTA-3′, for *B. duncani* and BmITS1-F, 5′-TCCCATTTGGGTTACGCTGG-3′, and BmITS1-R, 5′-CGTGCAGACAAACCCGCCTT-3′, for *B. microti*. The cycling conditions were 98°C for 3 min and 40 cycles of 98°C for 10 s and 60°C for 30 s. Fluorescence was read after each amplification cycle. Each sample was tested in duplicate, and 2 independent experiments were conducted.

***In vitro* evaluation of drug combinations in *B. duncani*-infected human red blood cells.** To understand the type of interaction between the drugs of interest in this study, we followed an adapted protocol of a fixed-ratio drug combination experiment, a well-established assay in the field of *Plasmodium* (23). Combinations of the drugs of interest were performed using six fixed ratios (5:0, 4:1, 3:2, 2:3, 1:4, and 0:5). For each single drug (5:0 and 0:5), the starting concentration was fixed at 8× IC_{50}. For each ratio, a 2-fold dilution series was carried out so that the IC_{50} of each drug falls in either the third or fourth dilution. The experiment was set up in a 96-well plate, where each well contained 200 μl of complete HL-1 medium with or without drug and 0.1% parasitemia with 5% hematocrit. Plates were incubated at 37°C for 60 h. Parasite viability was determined by the SYBR green I method as described above. For each ratio, the half-maximal inhibitory concentration (IC_{50}) was determined from a sigmoidal dose-response curve. The fractional inhibitory concentration (FIC_{50}) of each drug at different ratios was calculated using the following equation:

$$\mathrm{FIC_{50}(drug\ A)} = \frac{\mathrm{IC_{50}\ drug\ A\ in\ combination}}{\mathrm{IC_{50}\ drug\ A\ alone}}$$

The interaction between drug A and drug B was determined by the sum of FIC_{50} (ΣFIC_{50}) using the following formula:

$$\Sigma\mathrm{FIC_{50}} = \frac{\mathrm{IC_{50}\ drug\ A\ in\ combination}}{\mathrm{IC_{50}\ drug\ A\ alone}} + \frac{\mathrm{IC_{50}\ drug\ B\ in\ combination}}{\mathrm{IC_{50}\ drug\ B\ alone}}$$

According to the literature, ΣFIC_{50} of <0.8 represents synergy between the two drugs, $0.8 \leq \Sigma FIC_{50} < 1.4$ suggests additive effect, and mean ΣFIC_{50} of ≥ 1.4 represents antagonistic properties (24). To illustrate these interactions, isobolograms were plotted as FIC(drug A) = f(FIC(drug B)). Data are averaged from at least two independent experiments, each run in triplicates. Analysis was carried out using GraphPad Prism, version 8.1.

Statistical analysis. Data sets were analyzed with GraphPad Prism, version 8.1. Statistical significance was determined using unpaired *t* test.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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