The Journal of Experimental Medicine

Radical cure of experimental babesiosis in immunodeficient mice using a combination of an endochin-like quinolone and atovaquone

Lauren A. Lawres,[1]* Aprajita Garg,[1]* Vidya Kumar,[1]* Igor Bruzual,[3] Isaac P. Forquer,[3] Isaline Renard,[1] Azan Z. Virji,[1] Pierre Boulard,[1] Eduardo X. Rodriguez,[1] Alexander J. Allen,[1] Sovitj Pou,[3] Keith W. Wegmann,[3] Rolf W. Winter,[3] Aaron Nilsen,[3] Jialing Mao,[2] Douglas A. Preston,[3] Alexia A. Belperron,[2] Linda K. Bockenstedt,[2] David J. Hinrichs,[3] Michael K. Riscoe,[3] J. Stone Doggett,[3] and Choukri Ben Mamoun[1]

[1]Department of Internal Medicine, Section of Infectious Diseases and [2]Department of Internal Medicine, Section of Rheumatology, Yale School of Medicine, New Haven, CT 06520
[3]Veterans Affairs Medical Center, Portland, OR 97239

Human babesiosis is a tick-borne multisystem disease caused by *Babesia* species of the apicomplexan phylum. Most clinical cases and fatalities of babesiosis are caused by *Babesia microti*. Current treatment for human babesiosis consists of two drug combinations, atovaquone + azithromycin or quinine + clindamycin. These treatments are associated with adverse side effects and a significant rate of drug failure. Here, we provide evidence for radical cure of experimental babesiosis in immunodeficient mice using a combination of an endochin-like quinolone (ELQ) prodrug and atovaquone. In vivo efficacy studies in mice using ELQ-271, ELQ-316, and the ELQ-316 prodrug, ELQ-334, demonstrated excellent growth inhibitory activity against the parasite, with potency equal to that of orally administered atovaquone at 10 mg/kg. Analysis of recrudescent parasites after ELQ or atovaquone monotherapy identified genetic substitutions in the Q_i or Q_o sites, respectively, of the cytochrome bc_1 complex. Impressively, a combination of ELQ-334 and atovaquone, at doses as low as 5.0 mg/kg each, resulted in complete clearance of the parasite with no recrudescence up to 122 d after discontinuation of therapy. These results will set the stage for future clinical evaluation of ELQ and atovaquone combination therapy for treatment of human babesiosis.

Human babesiosis is a worldwide emerging infectious disease caused by protozoan parasites of the genus *Babesia* (Vannier and Krause, 2012; Vannier et al., 2015). Most *Babesia* infections in humans are caused by *B. microti* and are transmitted by the same *Ixodes* ticks that transmit Lyme disease spirochetes. The disease is endemic in the northeastern and northern midwestern United States and has been reported in Canada, Australia, Europe, Asia, Africa, and South America (Kjemtrup and Conrad, 2000; Krause et al., 2008). The first endemic site in the United States was identified in the 1960s in Nantucket, Massachusetts. Since then, the geographic distribution of *Babesia*-infected ticks has expanded and there has been a concomitant increase in babesiosis case incidence, particularly in the last decade (Vannier et al., 2015).

Babesiosis can present with clinical manifestations that range from a mild malaria-like illness to severe anemia, multiorgan system failure, and death. Age is a risk for more severe disease, with young children and elderly people the most susceptible. Severe disease, however, can occur in any age group that has a suppressed immune system due to HIV or conditions associated with the use of immunosuppressive therapies (Hatcher et al., 2001; Tonnetti et al., 2009).

The current treatment for babesiosis consists of combination therapies with atovaquone + azithromycin or clindamycin + quinine (Krause et al., 2000). *B. microti* parasites resistant to atovaquone have been shown to emerge rapidly in both animal and human studies (Wittner et al., 1996; Gray and Pudney, 1999; Wormser et al., 2010) and recrudescent infection or rising parasitemia can occur in humans despite therapy (Weiss et al., 1993; Gupta et al., 1995; Falagas and Klempner, 1996; Wittner et al., 1996; Marley et al., 1997; Krause et al., 1998, 2000, 2008; Shih and Wang, 1998; Dorman et al., 2000; Matsui et al., 2000; Hatcher et al., 2001; Vyas et al., 2007; Zhao et al., 2009; Wudhikarn et al., 2011; Raffalli and Wormser, 2016). Furthermore, several of the drugs presently used to treat babesiosis are associated with significant side effects. In one clinical trial, 15% of subjects receiving atovaquone + azithromycin and 72% of those receiving clindamycin + quinine for babesiosis experienced adverse events, including tinnitus, hearing loss, vertigo, diarrhea, and skin rash (Krause et al., 2000). One-third of patients receiving clin-

---

*L.A. Lawres, A. Garg, and V. Kumar contributed equally to this paper.

Correspondence to Choukri Ben Mamoun: choukri.benmamoun@yale.edu

Abbreviations used: ELQ, endochin-like quinolone; iRBC, infected RBC; PDR, postdrug removal.

damycin + quinine required either a decrease in dosage or discontinuation of the drugs (Krause et al., 2000). These limitations in efficacy and in tolerability emphasize the need for more effective therapies with fewer adverse events.

In the absence of a long-term ex vivo culture system, mice deficient in B and T cells (e.g., SCID and *rag1*⁻/⁻ mice) have served as excellent models to study the pathophysiology of *B. microti* (Li et al., 2012; Terkawi et al., 2015). The mouse model of babesiosis, however, is not amenable to large-scale chemical screens to identify new drugs or drug combinations with desirable efficacy and pharmacological properties for the treatment of human babesiosis. To circumvent this deficiency, we adapted a short-term ex vivo growth assay to identify new *B. microti* agents. These studies identified endochin-like quinolones (ELQs) as a novel class of anti–*B. microti* drugs. In vivo efficacy studies and molecular analyses highlighted the potency of ELQ derivatives, helped to unravel their novel mode of action, and identified a combination therapy regimen that rapidly eliminates bloodstream parasites and prevents relapse in *B. microti*-infected immunodeficient mice.

RESULTS

Combining short-term ex vivo growth and flow cytometry for screening of antiparasitic drugs

To develop an assay for rapid and possibly large scale screening of chemical libraries for new anti-*Babesia* drugs, we examined the intraerythrocytic development of *B. microti* ex vivo under different culture conditions, including those previously described for this parasite and *Plasmodium falciparum* (Trager and Jensen, 1976; Chen et al., 2000; Moitra et al., 2015). Our analyses indicated that optimal short-term ex vivo growth can be achieved when infected RBCs are collected from mice during the exponential phase of infection and immediately mixed with uninfected mouse erythrocytes in complete RPMI medium supplemented with 10% fetal bovine serum. Flow cytometry (not depicted) and microscopic analysis of thin blood smears performed at 48 h of cell culture showed an increase in parasitemia from ~1 to 6% (Fig. 1 A and B), demonstrating ex vivo growth of the parasites.

Using this short-term in vitro culture system, we assessed growth inhibition of *B. microti* exposed to four drugs currently used in the treatment of human babesiosis: quinine, clindamycin, azithromycin, and atovaquone. We also examined the effect of endochin and artesunate, which are known for their potent activity against other apicomplexan parasites. Potent anti–*B. microti* activity was measured for atovaquone and endochin, moderate anti–*B. microti* activity was measured for artesunate, and no activity could be measured for quinine, clindamycin, or azithromycin at a 10-μM concentration (Fig. 1 A).

Because of the impressive ex vivo activity of endochin, four ELQs analogues belonging to the 4(1*H*)-quinolone-3-diarylether series (Winter et al., 2011; Doggett et al., 2012; Nilsen et al., 2014; Stickles et al., 2015) were examined for activity against *B. microti*. ELQs are highly effective in
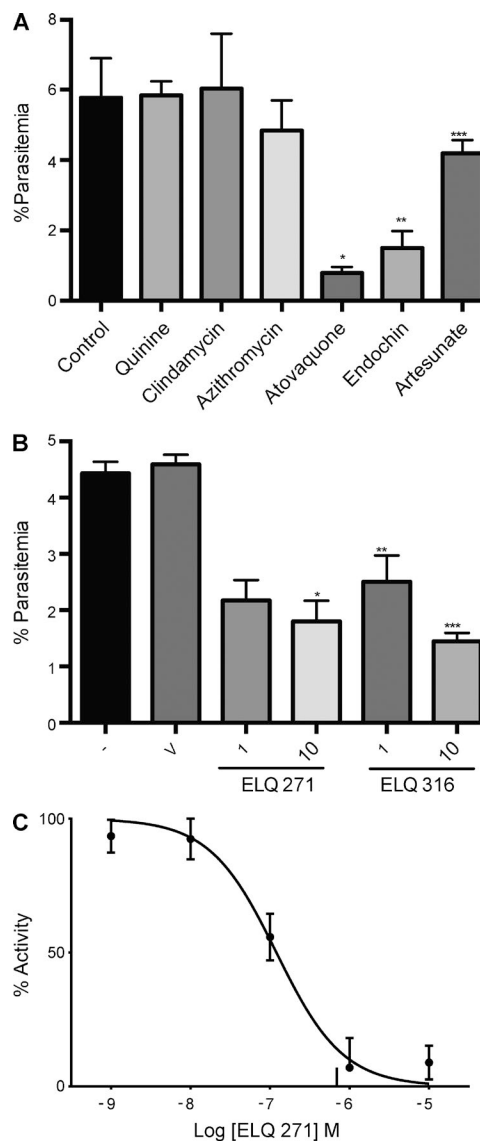

Figure 1. **Ex vivo activity of different classes of antiparasitic drugs against *B. microti* LabS1 strain.** (A) *B. microti* LabS1-infected RBCs at 1% parasitemia were maintained in short-term ex vivo culture for 48 h in the absence or presence of the compounds at 10 μM. Cells were then fixed and stained for flow cytometric analysis with YoYo-1 (Borggraefe et al., 2006), a sensitive DNA dye that labels intracellular parasites. Results indicate the percent of RBCs found to stain positive for *B. microti*. (B) Susceptibility of *B. microti* LabS1 strain to ELQ-271 and ELQ-316 at 1 and 10 μM using the short-term ex vivo culture assay in mouse RBCs. In both A and B, parasitemia was determined by microscopic analysis of Giemsa stained preparations. At least 5,000 RBCs per smear were counted. (C) Cytochrome *bc₁* complex inhibition by ELQ-271. Dodecyl β-D maltoside dispersed mitochondria isolated from parasites were used to measure cytochrome *c* reductase activity using decylubiquinol as an electron donor. *, P = 0.0809; **, P = 0.0809; ***, P = 0.0809. P-values comparing parasitemia between untreated and treated cultures at 48 h were determined using the Mann-Whitney nonparametric *U* test.

vivo against malaria and toxoplasmosis. Microscopy analyses of ELQ-treated *B. microti* parasites demonstrated significant inhibition of parasite growth ex vivo with 41 and 32% growth inhibition achieved using ELQ-271 and ELQ-316, respectively (Fig. 1 B). Similar results were obtained using flow cytometry (unpublished data).

ELQ–271 inhibits *B. microti* cytochrome *bc₁* activity

ELQs inhibit mitochondrial cytochrome bc_1 complex activity, but have different selectivity for specific organisms with changes in the substituents at the fifth, sixth, and seventh positions of the benzenoid ring of the quinolone core (Q_i site). For example, the chlorine atom at position 6 of ELQ-300 significantly decreases potency against *T. gondii,* whereas the unsubstituted quinolone core of ELQ-271 is associated with increased activity against cytochrome bc_1 complex from *S. cerevisiae* (Doggett et al., 2012). To assess the inhibitory activity of ELQs against *B. microti* cytochrome bc_1 complex, *B. microti* mitochondrial membranes were isolated from purified infected RBCs and used to measure cytochrome *c* reductase activity in the absence or presence of ELQ-271 (Fig. 1 C). Dispersed *B. microti* mitochondria yielded cytochrome bc_1 complexes with a V_{max} of 57.2 µM cytochrome *c* reduced s^{-1} mg^{-1} protein and a K_m of 4 µM. The EC_{50} of ELQ-271 was 120 ± 12 nM, and the fit curve revealed a hill slope of 1.1, indicative of a single binding site. By comparison, the EC_{50} of ELQ-271 for inhibition of *T. gondii* and *P. falciparum* cytochrome bc_1 complexes are 30.6 and 8.9 nM, respectively (Doggett et al., 2012; Nilsen et al., 2014).

Efficacy of ELQ compounds against *B. microti* in mice

We next evaluated the ability of selected ELQ derivatives to eliminate *B. microti* infection in SCID mice, which develop persistent, high–level parasitemia. Groups of five mice each were inoculated with 10^7 infected RBCs (iRBCs), and then treated for 1 wk with either the indicated ELQ or vehicle only, beginning on day 4 of infection. Blood samples were obtained at the onset of treatment and every 4 d thereafter to measure parasitemia by microscopic examination of Giemsa-stained smears (Fig. 2). Parasitemia reached ∼30% by day 12 after infection (pi) in control mice that received vehicle only (Fig. 2 A), as well as those treated with ELQ-300 (Fig. 2 C) or ELQ-400 (Fig. 2 E); this parasitemia persisted for the duration of the experiment (50 d). In contrast, mice treated with ELQ-271 (Fig. 2 B) and ELQ-316 (Fig. 2 D) exhibited very low to undetectable parasitemia levels by day 8 pi, and parasites remained undetectable until day 22. To compare the efficacy of ELQ-271 and ELQ-316 to drugs currently used for treatment of human babesiosis, in vivo efficacy studies were also performed in parallel on groups of five mice each using quinine, azithromycin, clindamycin and atovaquone monotherapy at similar doses (10 mg/kg; Fig. 2, F and D). Of these four drugs, only atovaquone showed potent activity against *B. microti* in vivo (Fig. 2 F). A second independent study was also performed using both SCID and *rag*1$^{-/-}$ mice and

showed similar results (Fig. 2 B–I, gray lines). Treatment with clindamycin up to 50 mg/kg, azithromycin up to 50 mg/kg, or quinine up to 100 mg/kg had no significant effect on parasite development in mice (Fig. 2, J–M).

Genetic evidence for the *B. microti* cytochrome *bc₁* complex as a target of ELQ and atovaquone action

Having demonstrated that ELQ-271 and ELQ-316 at 10 mg/kg exhibit potent antibabesiosis activity through 22 d of infection (Fig. 2, B and D), we followed these mice treated with ELQs for reemergence of parasitemia after a more extended period post–drug removal (PDR). By 16 d PDR (26 PDI), *B. microti* iRBCs could be detected in mice treated with ELQ-271 and ELQ-316, and parasitemia levels in these mice ranged between 0 and 1% (Fig. 3 A). By day 29 PDR, however, the parasitemia in two mice (IL271-2 and IL271-3) treated with ELQ-271 rose to 37%, whereas in the third mouse (IL271-1) parasitemia reached 0.06% (Fig. 3 B). However, all three mice treated with ELQ-316 (isolates: IL316-1, IL316-2, and IL316-3) reached a mean parasitemia of roughly 40%. We found similar reemergence and persistence of parasites in mice treated with ELQ-271 and ELQ-316 (Fig. 2, B and D).

To assess whether recurrent parasitemia was associated with mutations in the Q_i site of the cytochrome bc_1 complex, genomic DNA was isolated from blood collected from each of the six treated mice, as well as from two vehicle-only mice, and used to amplify and sequence the *BmCytb* gene. In mice treated with ELQ-271 or ELQ-316, a single mutation was found to alter codon 218 (alanine to valine) in the *BmCytb* gene, changing GCT to GTT and resulting in an amino acid change from alanine to valine in the encoded protein (Fig. 3 C).

To further examine whether recrudescent infections in ELQ-271– and ELQ-316–treated mice were the result of resistance of the parasites to ELQ-271 and/or ELQ-316, iRBCs collected from mice carrying one resistant isolate LabS1-R (isolate IL271-2) were purified and injected into SCID mice. After 3 d of infection, mice were subjected to the same treatment regimen, with either vehicle alone or ELQ-316 at 10 mg/kg daily for 7 d. As shown in Fig. 3 D, whereas the LabS1 parental isolate remained fully sensitive to ELQ-316, the LabS1-R strain was resistant to the drug.

We have also evaluated the recrudescence of parasitemia with suboptimal dosing with ELQ-271 (0.1 mg/kg). A nude mouse was inoculated intraperitoneally with 5×10^7 iRBCs (*B. microti* ATCC PRA-99 strain) and treated with 0.1 mg/kg of ELQ-271 via oral gavage on days 13, 14, and 15. When parasitemia levels were >5%, ELQ-271 was again administered on days 25, 26, and 27, as well as days 33 and 34. This treatment regimen had no effect on the persistence of parasitemia (unpublished data). DNA was extracted from the blood of these mice on day 53, and the *BmCytb* gene of the parental strain and the ELQ-271–resistant strain was sequenced (Fig. 3 C). Results revealed that the ELQ-271–resistant strain had an adenine substituted for a cytosine that results in the serine 219
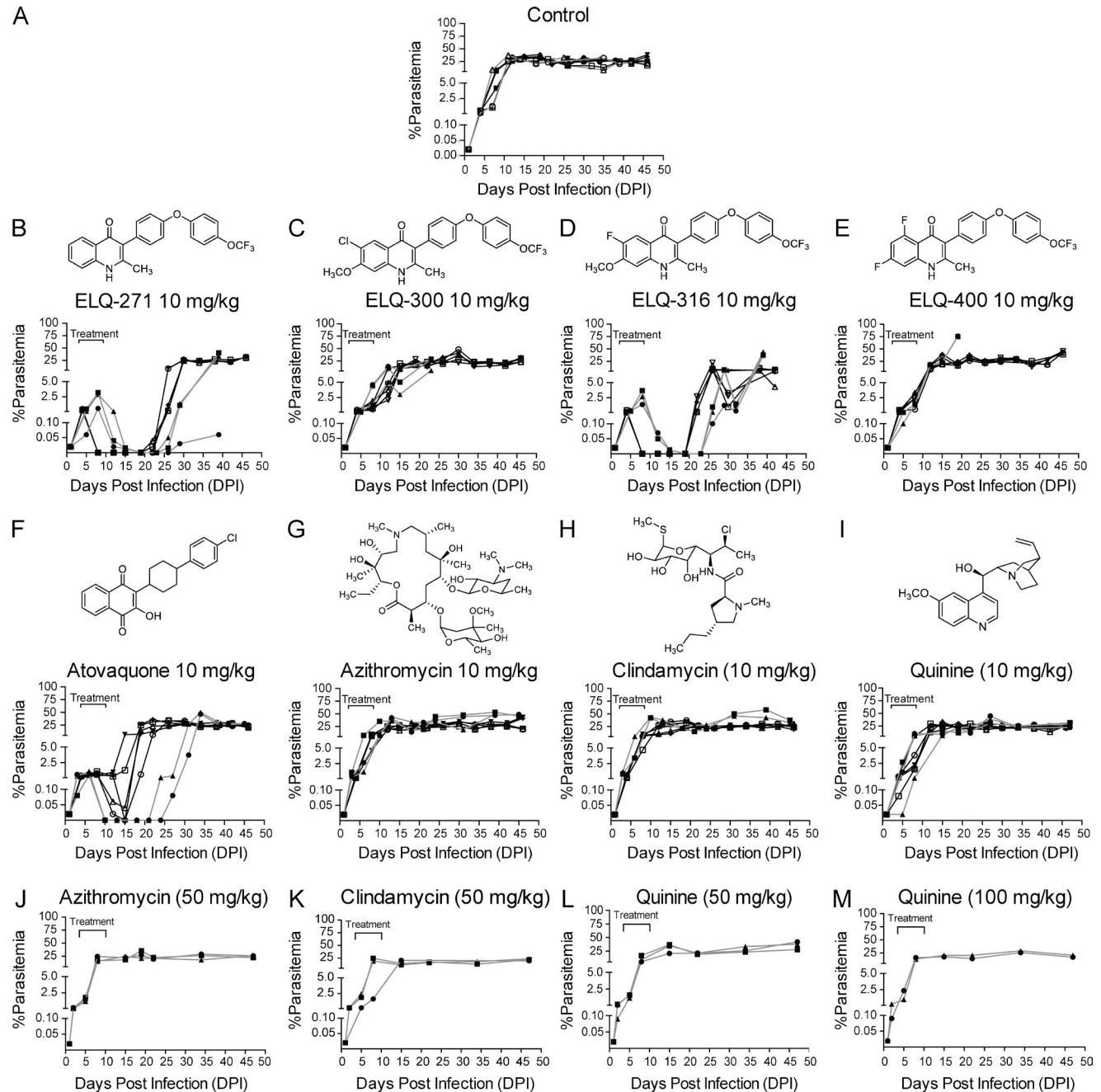
Figure 2. Efficacy of endochin–like quinolones and current therapies against *B. microti* in SCID mice. In the two independent studies, mice were injected with 10[7] iRBCs and treated with a single daily dose of vehicle alone or drugs from day 4 to 10 after infection. Parasitemia in individual mice was determined by microscopic counting of Giemsa-stained blood smears. In the first study (black lines), nine groups of five female SCID mice each were treated with either vehicle alone (A) or one of the compounds (B–I). In the second study (gray lines), groups of three mice each were treated with either vehicle alone or one of the drugs. Drugs used were as follows: ELQ-271 (B), ELQ-300 (C), ELQ-316 (D) or ELQ-400 (E), atovaquone (F), azithromycin (G), clindamycin (H), or quinine (I) at 10 mg/kg. P-values comparing parasitemia between untreated and treated mice at days 8 and 22 after infection were determined using the Mann-Whitney nonparametric *U* test (P_{D8} = 0.0034 and P_{D22} = 0.0010 for ELQ-271 vs. control; P_{D8} = 0.0066 and P_{D22} = 0.0014 for ELQ-316 vs. control; and P_{D8} = 0.0013 and P_{D22} = 0.3062 for Atovaquone vs. control). (J–M) Groups of three mice each were treated with azithromycin at 50 mg/kg (J), clindamycin at 50 mg/kg (K), quinine at 50 mg/kg (L), and quinine at 100 mg/kg (M).
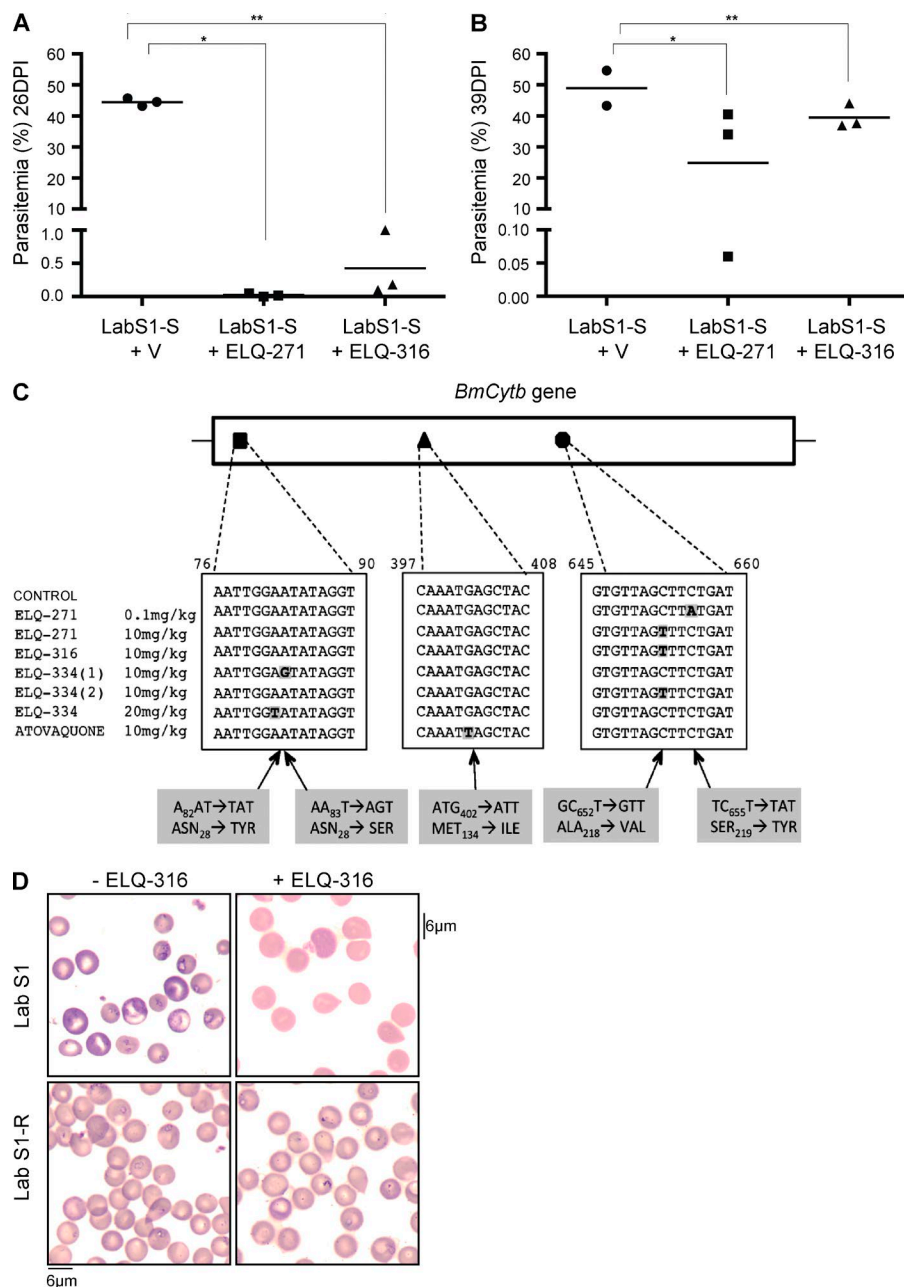
Figure 3. **Mutations in *B. microti* cytochrome *b* gene are associated with resistance to atovaquone and ELQs.** Blood from mice treated with either vehicle, ELQ-271, or ELQ-316 was analyzed on days 26 (A) and 39 (B) after infection. Whereas <1% parasitemia was seen in ELQ-271– and ELQ-316–treated parasites by 26 d after infection, the parasitemia increased to ~35% in five drug-treated mice, with one drug-treated mouse (IS271-1) still presenting low parasitemia. Parasitemia in individual mice was determined on the indicated days by microscopic analysis of Giemsa-stained blood smears. Each symbol represents an individual mouse. P-values comparing parasitemia between untreated and treated mice at days 26 (* = 0.0809 and ** = 0.0809) and 39 (* = 0.1489 and ** = 0.3865) after infection were determined using the Mann-Whitney nonparametric *U* test. (C) Genomic DNA was prepared from resistant parasites isolated from three different mice undergoing independent selections. Each genomic DNA was used in three independent PCR reactions to amplify the *BmCytb* gene. Three independent PCR products were purified and sent for Sanger sequencing at the Yale Keck Sequencing Facility. The sequences were analyzed using Geneious Version 8[1]. Results are shown for resistant parasites isolated from SCID mice infected with the parental LabS1 (from the second independent experiment) or from a nude mouse infected with *B. microti* ATCC PRA-99 strain and suboptimally treated with low-dose ELQ271 (0.1 mg/kg). (D) Test of susceptibility of ELQ-resistant parasites to ELQ-316. Representative images of blood smears prepared from blood collected on day 16 from mice infected with the parental LabS1 and the resistant isolate LabS1-R and treated with either vehicle alone or ELQ-316.

being changed to a tyrosine (S219Y; Fig. 3 C). This finding is unique to the ELQ-271–resistant isolate when compared with the parental strain and the previously known *BmCytb* sequence for the *B. microti* ATCC PRA-99 strain. It is noteworthy that both A218V and the S219Y mutations occur in the Q_i site of the *BmCytb* (see Fig. 5 A). Similarly, treatment with atovaquone at 10 mg/kg also resulted in recrudescence by 14 d PDR. Analysis of the *BmCytb* gene from these parasites identified a single mutation in codon 134 changing ATG to ATT, resulting in substitution of amino acid residue Met_{134} to Ile in the predicted Q_o site of the *B. microti* cytochrome b protein (Figs. 3 C and 5 A).

A combination of ELQ–334 and atovaquone eliminates *B. microti* infection in immunodeficient mice

The finding that ELQs and atovaquone target different sites of the cytochrome bc_1 complex involved in the electron transport chain suggested that a combination of these drugs could be synergistic and prevent the emergence of resistance. Because of the low oral bioavailability of ELQs (Miley et al., 2015), we synthesized a prodrug form of ELQ-316, ELQ-334 (Fig. 4 A), and examined its anti–*B. microti* activity in vivo in groups of five mice each. Similar to the parent drug, treatment with ELQ-334 at 10 mg/kg resulted in rapid clearance of *B. microti* in mice injected with 10^7 iRBCs (Fig. 4 D). We

subsequently assessed the potency and recrudescence profile of different combinations of ELQ-334 and atovaquone in mice infected with *B. microti*. As shown in Fig. 4 (C and D), mice treated with atovaquone or ELQ-334 alone at 10 mg/kg showed recrudescence (by 16 d PDR for ELQ-334 and 9 d PDR for atovaquone, respectively). Note that similar results were obtained in a second independent experiment and at 10 and 20 mg/kg of ELQ-334 or atovaquone monotherapy (Fig. 4, C and D). Recrudescence in these mice after ELQ-334 or atovaquone monotherapy was associated with single mutations in the *BmCytb* gene (M134I in the Q_o site of the BmCytb protein for atovaquone and N28S, N28Y, and A218V in the Q_i site of the BmCytb protein for ELQ-334; Figs. 3 C and 5 A). Reduced parasitemia was achieved at a combination dose of 1 mg/kg of ELQ-334 and atovaquone. However, resistant parasites emerged by day 25 PI, and recrudescence after this combination was associated with single mutations in the *BmCytb* gene (either S25N or A218V in the Q_i site of the BmCytb protein). Notably, in two independent studies, mice treated with combinations of ELQ-334 and atovaquone at doses of 2.5, 5, and 10 mg/kg of each drug showed no parasitemia by the end of therapy, and recrudescence was not detected in mice followed for up to 95 d after infection (Fig. 4, F–H). At day 133 after infection, radical cure was achieved in all 10 mice receiving combination therapies with either 5 or 10 mg/kg of each drug, and in four out of the five mice receiving combination of atovaquone and ELQ-334 at 2.5 mg/kg each. Blood collected 58 d after infection from mice treated with 5 and 10 mg/kg drug combinations tested negative for *B. microti* DNA by PCR and failed to cause infection when injected into uninfected SCID mice (unpublished data).

DISCUSSION

Alternative chemotherapeutic options against *Babesia microti* are needed in the wake of the increasing number of treatment failures using current combination therapies. The current treatment options for mild and severe human babesiosis using quinine, clindamycin, azithromycin, and atovaquone were initially investigated because of their antimalarial activity and are associated with significant side effects and drug failures (Weiss et al., 1993; Gupta et al., 1995; Falagas and Klempner, 1996; Wittner et al., 1996; Marley et al., 1997; Krause et al., 1998, 2000, 2008; Shih and Wang, 1998; Dorman et al., 2000; Matsui et al., 2000; Falagas and Hatcher et al., 2001; Vyas et al., 2007; Zhao et al., 2009; Wudhikarn et al., 2011). Furthermore, the mechanisms by which most of these drugs inhibit intraerythrocytic development of *B. microti* have not been investigated. Cell biological studies, as well as genome and metabolic reconstitution analyses, have indicated that unlike *Plasmodium* parasites, *B. microti* does not degrade hemoglobin and lacks most of the cysteine, aspartic, and metalloproteases known to be required for hemoglobin degradation in malaria parasites (Cornillot et al., 2012). Additionally, no hemozoin-like structures can be seen in *B. microti*–infected RBCs or detected in the liver or spleen of mammals infected with this parasite.

Although these findings question the rationale for using quinine in babesiosis therapy, it is possible that considering the high clinical dose recommended for this drug, it might function by targeting other parasite proteins, such as K^+ channels or methyltransferases (Bobenchik et al., 2010; Kuum et al., 2012; Garg et al., 2015). In the case of clindamycin and azithromycin, the mode of action in *B. microti* remains unknown; although based on knowledge from other parasites, it is believed that these drugs may target the parasite apicoplast (Camps et al., 2002; Sidhu et al., 2007). The fourth compound used in babesiosis therapy is atovaquone, a drug with potent antimalarial effects. However, resistance to atovaquone monotherapy emerges rapidly in malaria–treated patients leading to the formulation of the combination therapy atovaquone-proguanil also known as Malarone. Biochemical and structural studies have demonstrated that atovaquone targets the cytochrome bc_1 complex by specifically binding to the Q_o site of the *Cytb* subunit (Vaidya and Mather, 2000; Mather and Vaidya, 2008).

In vitro drug sensitivity studies of *B. microti* have thus far been hampered by the absence of a continuous culture assay. In this study, we used a short-term ex vivo screening assay to examine different classes of drugs for their activity against the parasite. Of the four drugs presently used in babesiosis therapy, atovaquone was the only compound that showed significant activity against *B. microti*. Interestingly, this assay identified a different class of drugs, endochin, and its analogues as effective anti–*B. microti* inhibitors. ELQs are analogues of endochin, a compound whose antiparasitic activity was described nearly 70 yr ago (Salzer et al., 1948; Nilsen et al., 2013). Because endochin exhibits metabolic instability in liver microsomes from a variety of mammalian species (Winter et al., 2011), analogues with better metabolic stability profiles and enhanced activity against drug-sensitive and –resistant malaria parasites were developed (Nilsen et al., 2014). These analogues have potent activity against other intracellular pathogens, including *Toxoplasma gondii* (Doggett et al., 2012). Interestingly, although these analogues are structurally similar, they have demonstrated different selectivity for specific organisms with changes in the substituents at the fifth, sixth, and seventh positions of the quinolone ring, whereas the chlorine atom at position 6 of ELQ-300 significantly decreases activity against *T. gondii*, compared with ELQ-316, which has a smaller fluorine atom in this position. Moreover, the unsubstituted fifth, sixth, and seventh positions of ELQ-271 broaden activity to include *S. cerevisiae* and *T. gondii*, while retaining activity against *P. falciparum* (Doggett et al., 2012; Nilsen et al., 2013).

In vivo efficacy studies showed that of the four endochin analogues examined in this study, ELQ-271 and ELQ-316, were found to be most effective against *B. microti* after oral administration at 10 mg/kg (Fig. 2, B and D). The other two compounds, ELQ-300 and ELQ-400, were not effective at this dose (Fig. 2, C and E).

Our studies in mice infected with *B. microti* have shown that recrudescence of parasitemia after treatment with ELQ-271 or ELQ-316 occurs 12 to 15 d after drug removal.
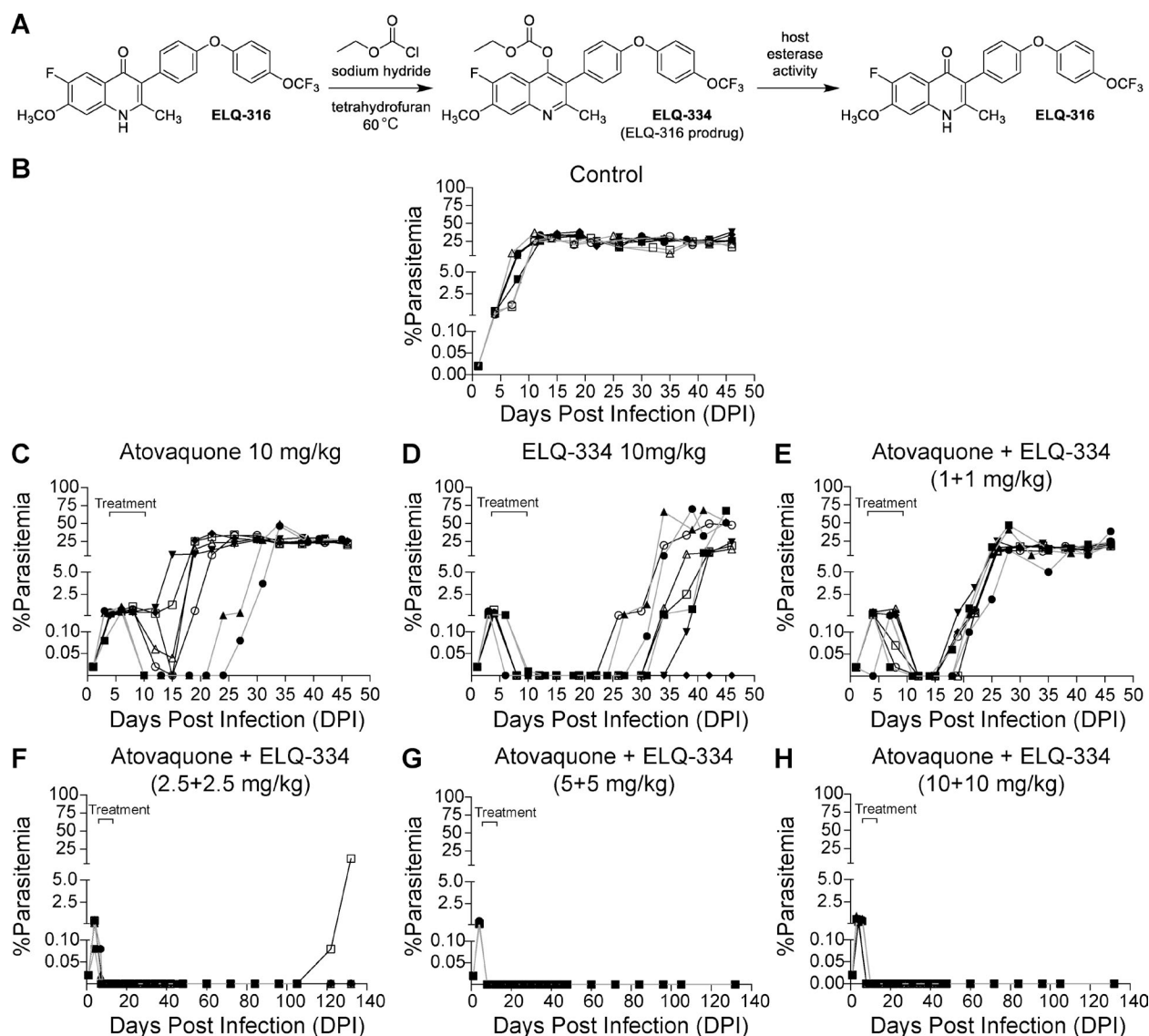
Figure 4. **A combination of ELQ–334 and atovaquone results in radical cure of experimental babesiosis.** (A) Synthesis of the prodrug ELQ-334. (B–H) Groups of five female SCID (black lines) or three mice (gray lines) each were injected with 10^7 iRBCs and treated with either a single daily dose of vehicle alone (B) or the indicated drugs on days 4 to 10 (C–H). Parasitemia in individual mice was determined by microscopic counting of Giemsa-stained blood smears. Drug treatment include atovaquone at 10 mg/kg (C), ELQ-334 at 10 mg/kg (D), and four drug combinations atovaquone + ELQ-334 at 1 mg/kg (E), 2.5 mg/kg (F), 5 mg/kg (G), and 10 mg/kg (H) each. Untreated SCID and $rag1^{-/-}$ were combined in a single graph. No significant differences were seen between these mice independent of the gender. P-values comparing parasitemia between untreated and treated mice at days 8 and 22 after infection were determined using the Mann-Whitney nonparametric U test ($P_{D8} = 0.0007$ and $P_{D22} = 0.0006$ for ELQ-334 vs. control; $P_{D8} = 0.0009$ and $P_{D22} = 0.0006$ for ELQ-334+Atovaquone [1+1 mg/kg] vs. control; $P_{D8} = 0.0007$ and $P_{D22} = 0.0006$ for ELQ-334+Atovaquone [2.5+2.5 mg/kg] vs. control); $P_{D8} = 0.0004$ and $P_{D22} = 0.0006$ for ELQ-334+Atovaquone (5+5 mg/kg) versus control; and $P_{D8} = 0.0008$ and $P_{D22} = 0.0006$ for ELQ-334+Atovaquone (10+10 mg/kg) versus control.

Although the SCID and nude mouse models are more likely to have recrudescent babesiosis than an immunocompetent human host due to the profound immunocompromised condition of the mice, they proved valuable for understanding the mode of action of these drugs, substantiating the observation of direct inhibition of the *B. microti* cytochrome bc_1 by ELQ-271 (Fig. 1 C). Sequencing analyses of drug-resistant parasites from two different strains of *B. microti* (LabS1 and

ATCC PRA-99 strain) treated with either 10 or 0.1 mg/kg identified two independent mutations in the *Cytb* gene that are responsible for the resistant phenotype. The two mutations affect adjacent residues in the Q_i site of the parasite cytochrome bc_1 complex (Fig. 5 A). A known K→M substitution in the analogous residue of the *S. cerevisiae* (228) *Cytb* gene causes resistance to 1-hydroxy-2-dodecyl-4(1*H*) quinolone (HDQ) and antimycin A (Vallières et al., 2012).
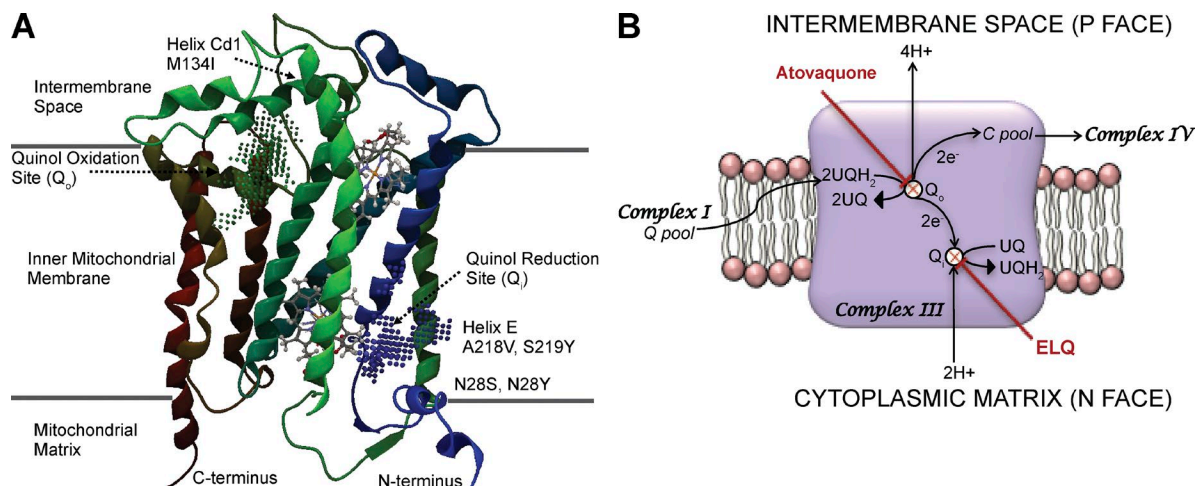
A

Helix Cd1
M134I

Intermembrane
Space

Quinol Oxidation
Site (Q_o)

Inner Mitochondrial
Membrane

Quinol Reduction
Site (Q_i)

Helix E
A218V, S219Y

N28S, N28Y

Mitochondrial
Matrix

C-terminus   N-terminus

B

INTERMEMBRANE SPACE (P FACE)

4H+

Atovaquone

C pool → Complex IV

2e⁻

Complex I
Q pool $2UQH_2$ Q_o
                 $2UQ$

2e

UQ
Q_i UQH_2

Complex III

ELQ

2H+

CYTOPLASMIC MATRIX (N FACE)

Figure 5. **Mode of action of ELQ and atovaquone.** (A) Location of atovaquone and ELQ-316 resistance mutations in the Q_o and Q_i sites of the cytochrome *b*. *Saccharomyces cerevisiae* cytochrome *b* is used to illustrate the approximate location of the corresponding *B. microti* drug-resistance mutations, which are denoted with *B. microti* amino acid numbering. ELQ-316 resistance mutations A218V and S219Y correspond to the E α helix, whereas N28S and N28Y correspond to the N-terminal region of the *S. cerevisiae* Q_i site. The M134I atovaquone-resistance mutation corresponds to the M139 position in *S. cerevisiae*, located on the cd1 helix of the Q_o site. The structure and the Q_o and Q_i sites in this image were generated using the protein structure 4PD4.pdb and Molegro software. (B) Quinol oxidation takes place at the Q_o site and ubiquinone reduction takes place at the Qi site of complex III (cytochrome bc_1 complex) in the mitochondrial membrane of *B. microti*. Atovaquone inhibits the Q_o site where as ELQs inhibit the Q_i site of BmCytb protein (Doggett et al., 2012).

In *S. cerevisiae*, lysine 228 forms a water-mediated hydrogen bond with ubiquinone and plays an important role in the Q_i site proton–uptake pathway (Hunte et al., 2003). Recently, in silico modeling of ELQ-300 docking to bovine cytochrome bc_1 predicts binding of the quinolone carbonyl group in the Q_i pocket where the conserved 228 position aspartic acid residue (position 220 in *B. microti*, 229 in *S. cerevisiae*) has previously been shown to interact via a water molecule with the carbonyl oxygen of ubiquinone (Gao et al., 2003; Capper et al., 2015). It is possible that the A218V and the S219Y mutations interfere with the interaction between ELQs and the conserved aspartic acid at position 220. Mutations at the N28 position of the Q_i site of ELQ-treated *B. microti* (N28S and N28Y) correspond to the N31 position in *S. cerevisiae*, where it is known that an asparagine to lysine mutation causes resistance to the herbicide, 3-(3,4–dichlorophenyl)–1,1–dimethylurea (Fig. 5 A; Fisher and Meunier, 2008). Mice infected with one of the resistant isolates showed increased parasitemia over time after treatment with ELQ-316, indicating that the parasite isolate was stably and fully resistant to the drug. Q_i site inhibition is a new mechanism of action for an antiparasitic drug that provides a promising strategy for combination therapy with the current Q_o site inhibitor, atovaquone.

Higher doses or longer duration of ELQs may prevent recrudescence in the SCID mouse model, however the challenging physicochemical properties of these drugs, i.e., high degree of crystallinity and poor aqueous solubility, preclude administration of doses >10 mg/kg (Nilsen et al., 2013; Miley et al., 2015). To solve this problem, a prodrug, ELQ-334, was designed to deliver higher concentrations of ELQ-316 to the bloodstream. In vivo experiments were performed to assess the efficacy of ELQ-334 against *B. microti* and compare it to other drugs in clinical use against *B. microti* infections: azithromycin, clindamycin, quinine, and atovaquone. The results showed that ELQ-334 is highly effective against the parasite at concentrations up to 20 mg/kg. Unfortunately, the 7-d treatment regimen resulted in recrudescence by day 27 after infection. Sequencing of genomic DNA from mice presenting recrudescent infection showed different mutations depending on the dose of the compound, all of them located in the Q_i site of the *Cytb* complex, thereby providing genetic validation of selective drug targeting in *B. microti*.

A critical finding of this study is that a combination of atovaquone + ELQ-334 at doses as low as 5.0 mg/kg of each drug resulted in apparent cure, as all immunodeficient mice showed no detectable parasitemia after treatment. The radical cure of experimental babesiosis was confirmed in two independent studies after examination of peripheral blood smears up to 122 d PDR, as well as by PCR and reinfection studies. As this cure was achieved in severely immunodeficient mice, it demonstrates the superior efficacy of the combination in eliminating infection even in immunocompromised hosts. It is possible that this enhancement of in vivo efficacy is a result of synergism between atovaquone and ELQ-316 or a prolonged half-life of one or both compounds; however, this result also suggests that *B. microti* has a limited capacity to generate mutations at both the Q_o and the Q_i sites when exposed to simultaneous drug pressure. The elevated barrier to resistance is possibly caused by intolerable fitness costs in the enzyme complex that may result from having mutations at both active sites.

In summary, our data demonstrate that ELQs effectively treat experimental babesiosis by inhibiting the Qi site of cy-

tochrome bc_1 complex. Moreover, a combination of atovaquone and ELQ-334 is effective at eliminating parasitemia without demonstrable relapse after drug discontinuation in experimentally infected immunodeficient mice. This combination therapy is predicted to effect a complete blockage of the electron transport chain through complex III (Fig. 5 B), leading to ATP depletion, pyrimidine starvation, and inhibition of heme biosynthesis (Sarewicz and Osyczka, 2015). We propose that this combination is a promising treatment option for human babesiosis that may eliminate clinical *B. microti* recrudescence, including in immunocompromised hosts.

MATERIALS AND METHODS

Mouse strains. C.B17 scid C.B-Igh-1b/IcrTac-*Prkdc^scid* and CB17/Icr-*Prkdc^scid*/IcrIcoCrl mice were obtained from Taconic and Charles River. The C57BL/6 *rag*1^−/− knockout mice were a gift from R. Medzhitov (Yale, New Haven, CT) and were bred in our animal facility. Nude mice were obtained from The Jackson Laboratory. All animal experimental protocols followed Yale University and Portland Veterans Affairs (VA) Medical Center institutional guidelines for care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committees (IACUC) at Yale University and Portland VA Medical Center. Rules for ending experiments in mice were to be enacted if mice showed any signs of distress or appeared moribund. This, however, was not the case for any animals in the study. Parasitemia was determined using standard methods for collecting a drop of blood from the tail vein and using this blood to perform Giemsa staining and flow cytometry. Randomization of mice to the various groups was performed by randomly picking animals to one group or another.

Chemicals. The chemical structures of ELQ-271, ELQ-300, ELQ-316, and ELQ-400 are shown in Fig. 2. The compounds were synthesized as previously described (Nilsen et al., 2014). The chemical structure of ELQ-334 and its synthesis are described in Fig. 4 A.

Cell culture and materials. *B. microti* LabS1 (ELQ sensitive strain is referred to as LabS1-S) and ATCC PRA-99 strains were maintained in SCID (CB17/Icr-*Prkdc^scid*/IcrIcoCrl; Charles River) or *rag*1^−/− knockouts, where they reached high parasitemia. Short-term ex vivo culture of *B. microti* LabS1 was performed as follows: SCID or *rag*1^−/− mice were infected with *B. microti* LabS1 strain parasites by i.p. inoculation of ∼10^7 iRBCs. Infected blood was collected by retroorbital bleeding when the parasitemia in the mice was ∼10%. An uninfected syngeneic mouse was sacrificed for blood, which was used for dilution of infected blood to achieve a final parasitemia of ∼1%. The blood was washed three times with RPMI. A 96-well flat-bottom plate was used for drug screening. Parasites were grown at 10% hematocrit. Complete medium, used for propagation of *B. microti* culture, consists of RPMI medium 1640 supplemented with 30 mg/l hypoxanthine (Sigma-Aldrich), 25 mM Hepes (Sigma-Aldrich), 0.225% NaHCO₃ (Sigma-

Aldrich), 10% FBS (HI FBS; Gibco; 10082–139), Pen Strep (Gibco; 15140–122), 100 µg/ml Kanamycin, and 10 µg/ml gentamycin (Life Technologies). The cultures were grown using a gas mixture of 3% O_2, 3% CO_2, and 94% N_2.

Mitochondrial isolation and enzyme inhibition assays. Infected mouse blood was diluted 1:1 in PBS and passed over a microcrystalline cellulose (Avicel PH-101; Sigma-Aldrich) column to remove white blood cells. The RBCs were then lysed in 0.075% (wt/vol) saponin at 37°C for 1 h. Whole parasites were pelleted at 1,000 *g* for 20 min. The parasites were resuspended in 10 ml of ice-cold PBS (containing 1 mM PMSF) and passed through a French press at 20,000 psi. The resulting mixture was then centrifuged at 20,000 *g* for 45 min to pellet mitochondria. The mitochondria were resuspended in 1 ml of cold PBS with 10% glycerol (vol/vol.) The aliquots were stored at −80°C until needed. To measure cytochrome *c* reduction, an aliquot of mitochondria was allowed to thaw on ice, and n-dodecyl β-D maltoside was added to a final concentration of 6 mg/ml. The mixture was allowed to rest on ice for 1 h, and then was centrifuged at 10,000 *g* to remove insoluble material. The clarified supernatant was used for enzyme assays. Reactions were initiated by adding dispersed mitochondria (∼5 ng/ml protein) to reaction buffer (50 mM Tricine, 100 mM KCl, 2 mM sodium azide, 50 µM equine cytochrome *c*, and 50 µM decylubiquinol). The background reduction of cytochrome *c* by decylubiquinol was subtracted from the initial rate of cytochrome *c* reduction after the addition of mitochondria. Cytochrome *c* reduction was monitored at 550 nm minus 542 nm. The reactions were conducted at 30°C. Final DMSO concentrations introduced from ELQ-271 solutions were <0.1%.

In vivo efficacy assays. Two independent studies were conducted to evaluate the efficacy of compounds against *B. microti* in vivo. The first study included groups of five female SCID mice each (C.B17 scid C.B-Igh-1b/IcrTac-*Prkdc^scid*; Taconic). In the second study, in vivo drug efficacy studies were performed in SCID, *rag*1^−/−, or nude mice depending on availability in the laboratory. *B. microti* infection of each of these immunodeficient mice results in persistent parasitemia in mice (Clark and Allison, 1974). Untreated mice from the same genetic background were included as controls. Mice were infected with 10^7 *B. microti* (LabS1) iRBCs via i.p. injection. Drugs were administered to animals by gavage beginning 4 d after infection and continued for 7 consecutive days. Treated animals received 100 µl of drug solution in PEG-400 via oral gavage. Blood samples were collected every 4 d until parasitemia in control mice treated with vehicle alone reached 50% for at least two blood collections. Parasitemia was determined using light microscopy and confirmed with Giemsa staining of thin blood smears and by flow cytometry using YOYO-1 (Borggraefe et al., 2006). Recrudescence was monitored by measuring parasitemia for a minimum of 45 d after infection. In the low-dose ELQ-271 treatment experiment, two nude mice were inoculated i.p. with 5 × 10^7 iRBCs (*B.*

microti ATCC PRA-99 strain). One mouse was treated with 0.1 mg/kg of ELQ-271 in PEG-400 via oral gavage on days 13, 14, and 15. Parasitemia was determined using light microscopy after Giemsa staining of thin blood smears. When parasitemia levels >5% were detected, ELQ-271 was administered on days 25, 26, 27, 33, and 34. Despite the subsequent treatments, parasitemia persisted and blood was collected on day 53. Blood was obtained from the untreated mouse after parasitemia reached >50%.

Genomic DNA isolation and sequencing. Blood was collected in EDTA tubes (Medtronic; 8881311149). The blood was passed through a cellulose column (Thermo Fisher Scientific; S25241A), PBS was added, and then the blood was collected and centrifuged to obtain a blood pellet. Genomic DNA was isolated using DNeasy Blood and Tissue kit (QIAGEN; 69504). The isolated DNA was amplified using KOD DNA Polymerase (Novagen; 71085–3). PCR reactions to amplify the cytochrome *b* gene were performed using the following primers: F4302, 5′-AGATACAGCGAG AAGGGAAT-3′ and R5809, 5′-AACCTTTCCTTTTCC TTACG-3′. The PCR products were then sent for Sanger sequencing at Keck Sequencing Facility (Yale University), using the previous primers in addition to R5190, 5′-TACTTGAGTTTAGATGTAGG-3′.

Flow cytometry. Blood samples were fixed in 0.025% glutaraldehyde (Sigma-Aldrich; #G5882) and stored at 4°C. For staining, the cells were washed with PBS and permeabilized with 0.25% Triton X-100 for 10 min. The cells were washed again with PBS and treated with 0.25 mg/ml RNase (Invitrogen; #12091–021) for 1 h at 37°C. After RNase treatment, cells were stained with 20 nM YOYO-1 (Invitrogen; #Y3601) for 1 h at room temperature and in the dark. The cells were washed and resuspended in PBS for flow analysis.

Statistical analyses. Statistical analyses were performed using an unpaired Student's *t* test, as well as the Mann-Whitney nonparametric *U* test. Differences were considered statistically significant when P < 0.05.

REFERENCES

Bobenchik, A.M., J.Y. Choi, A. Mishra, I.N. Rujan, B. Hao, D.R. Voelker, J.C. Hoch, and C.B. Mamoun. 2010. Identification of inhibitors of Plasmodium falciparum phosphoethanolamine methyltransferase using an enzyme-coupled transmethylation assay. *BMC Biochem.* 11:4. http://dx.doi.org/10.1186/1471-2091-11-4

Borggraefe, I., J. Yuan, S.R. Telford III, S. Menon, R. Hunter, S. Shah, A. Spielman, J.A. Gelfand, H.H. Wortis, and E. Vannier. 2006. Babesia microti primarily invades mature erythrocytes in mice. *Infect. Immun.* 74:3204–3212. http://dx.doi.org/10.1128/IAI.01560-05

Camps, M., G. Arrizabalaga, and J. Boothroyd. 2002. An rRNA mutation identifies the apicoplast as the target for clindamycin in Toxoplasma gondii. *Mol. Microbiol.* 43:1309–1318. http://dx.doi.org/10.1046/j.1365-2958.2002.02825.x

Capper, M.J., P.M. O'Neill, N. Fisher, R.W. Strange, D. Moss, S.A. Ward, N.G. Berry, A.S. Lawrenson, S.S. Hasnain, G.A. Biagini, and S.V. Antonyuk. 2015. Antimalarial 4(1H)-pyridones bind to the Qi site of cytochrome bc1. *Proc. Natl. Acad. Sci. USA.* 112:755–760. http://dx.doi.org/10.1073/pnas.1416611112

Chen, D., D.B. Copeman, G.W. Hutchinson, and J. Burnell. 2000. Inhibition of growth of cultured Babesia microti by serum and macrophages in the presence or absence of T cells. *Parasitol. Int.* 48:223–231. http://dx.doi.org/10.1016/S1383-5769(99)00022-7

Clark, I.A., and A.C. Allison. 1974. Babesia microti and Plasmodium berghei yoelii infections in nude mice. *Nature.* 252:328–329. http://dx.doi.org/10.1038/252328a0

Cornillot, E., K. Hadj-Kaddour, A. Dassouli, B. Noel, V. Ranwez, B. Vacherie, Y. Augagneur, V. Brès, A. Duclos, S. Randazzo, et al. 2012. Sequencing of the smallest Apicomplexan genome from the human pathogen Babesia microti. *Nucleic Acids Res.* 40:9102–9114. http://dx.doi.org/10.1093/nar/gks700

Doggett, J.S., A. Nilsen, I. Forquer, K.W. Wegmann, L. Jones-Brando, R.H. Yolken, C. Bordón, S.A. Charman, K. Katneni, T. Schultz, et al. 2012. Endochin-like quinolones are highly efficacious against acute and latent experimental toxoplasmosis. *Proc. Natl. Acad. Sci. USA.* 109:15936–15941. http://dx.doi.org/10.1073/pnas.1208069109

Dorman, S.E., M.E. Cannon, S.R. Telford III, K.M. Frank, and W.H. Churchill. 2000. Fulminant babesiosis treated with clindamycin, quinine, and whole-blood exchange transfusion. *Transfusion.* 40:375–380. http://dx.doi.org/10.1046/j.1537-2995.2000.40030375.x

Falagas, M.E., and M.S. Klempner. 1996. Babesiosis in patients with AIDS: a chronic infection presenting as fever of unknown origin. *Clin. Infect. Dis.* 22:809–812. http://dx.doi.org/10.1093/clinids/22.5.809

Fisher, N., and B. Meunier. 2008. Molecular basis of resistance to cytochrome bc1 inhibitors. *FEMS Yeast Res.* 8:183–192. http://dx.doi.org/10.1111/j.1567-1364.2007.00328.x

Gao, X., X. Wen, L. Esser, B. Quinn, L. Yu, C.A. Yu, and D. Xia. 2003. Structural basis for the quinone reduction in the bc1 complex: a comparative analysis of crystal structures of mitochondrial cytochrome bc1 with bound substrate and inhibitors at the Qi site. *Biochemistry.* 42:9067–9080. http://dx.doi.org/10.1021/bi0341814

Garg, A., T. Lukk, V. Kumar, J.Y. Choi, Y. Augagneur, D.R. Voelker, S. Nair, and C. Ben Mamoun. 2015. Structure, function and inhibition of the phosphoethanolamine methyltransferases of the human malaria parasites Plasmodium vivax and Plasmodium knowlesi. *Sci. Rep.* 5:9064. http://dx.doi.org/10.1038/srep09064

Gray, J.S., and M. Pudney. 1999. Activity of atovaquone against Babesia microti in the Mongolian gerbil, Meriones unguiculatus. *J. Parasitol.* 85:723–728. http://dx.doi.org/10.2307/3285750

Gupta, P., R.W. Hurley, P.H. Helseth, J.L. Goodman, and D.E. Hammerschmidt. 1995. Pancytopenia due to hemophagocytic syndrome as the presenting manifestation of babesiosis. *Am. J. Hematol.* 50:60–62. http://dx.doi.org/10.1002/ajh.2830500113

Hatcher, J.C., P.D. Greenberg, J. Antique, and V.E. Jimenez-Lucho. 2001. Severe babesiosis in Long Island: review of 34 cases and their complications. *Clin. Infect. Dis.* 32:1117–1125. http://dx.doi.org/10.1086/319742

Hunte, C., H. Palsdottir, and B.L. Trumpower. 2003. Protonmotive pathways and mechanisms in the cytochrome bc1 complex. *FEBS Lett.* 545:39–46. http://dx.doi.org/10.1016/S0014-5793(03)00391-0

Kjemtrup, A.M., and P.A. Conrad. 2000. Human babesiosis: an emerging tick-borne disease. *Int. J. Parasitol.* 30:1323–1337. http://dx.doi.org/10.1016/S0020-7519(00)00137-5

Krause, P.J., A. Spielman, S.R. Telford III, V.K. Sikand, K. McKay, D. Christianson, R.J. Pollack, P. Brassard, J. Magera, R. Ryan, and D.H. Persing. 1998. Persistent parasitemia after acute babesiosis. *N. Engl. J. Med.* 339:160–165. http://dx.doi.org/10.1056/NEJM199807163390304

Krause, P.J., T. Lepore, V.K. Sikand, J. Gadbaw Jr., G. Burke, S.R. Telford III, P. Brassard, D. Pearl, J. Azlanzadeh, D. Christianson, et al. 2000. Atovaquone and azithromycin for the treatment of babesiosis. *N. Engl. J. Med.* 343:1454–1458. http://dx.doi.org/10.1056/NEJM200011163432004

Krause, P.J., B.E. Gewurz, D. Hill, F.M. Marty, E. Vannier, I.M. Foppa, R.R. Furman, E. Neuhaus, G. Skowron, S. Gupta, et al. 2008. Persistent and relapsing babesiosis in immunocompromised patients. *Clin. Infect. Dis.* 46:370–376. http://dx.doi.org/10.1086/525852

Kuum, M., V. Veksler, J. Liiv, R. Ventura-Clapier, and A. Kaasik. 2012. Endoplasmic reticulum potassium-hydrogen exchanger and small conductance calcium-activated potassium channel activities are essential for ER calcium uptake in neurons and cardiomyocytes. *J. Cell Sci.* 125:625–633. http://dx.doi.org/10.1242/jcs.090126

Li, Y., M.A. Terkawi, Y. Nishikawa, G.O. Aboge, Y. Luo, H. Ooka, Y.K. Goo, L. Yu, S. Cao, Y. Sun, et al. 2012. Macrophages are critical for cross-protective immunity conferred by Babesia microti against Babesia rodhaini infection in mice. *Infect. Immun.* 80:311–320. http://dx.doi.org/10.1128/IAI.05900-11

Marley, S.E., M.L. Eberhard, F.J. Steurer, W.L. Ellis, P.B. McGreevy, and T.K. Ruebush II. 1997. Evaluation of selected antiprotozoal drugs in the Babesia microti–hamster model. *Antimicrob. Agents Chemother.* 41:91–94.

Mather, M.W., and A.B. Vaidya. 2008. Mitochondria in malaria and related parasites: ancient, diverse and streamlined. *J. Bioenerg. Biomembr.* 40:425–433. http://dx.doi.org/10.1007/s10863-008-9176-4

Matsui, T., R. Inoue, K. Kajimoto, A. Tamekane, A. Okamura, Y. Katayama, M. Shimoyama, K. Chihara, A. Saito-Ito, and M. Tsuji. 2000. [First documentation of transfusion-associated babesiosis in Japan]. *Rinsho Ketsueki.* 41:628–634 (Rinsho ketsueki).

Miley, G.P., S. Pou, R. Winter, A. Nilsen, Y. Li, J.X. Kelly, A.M. Stickles, M.W. Mather, I.P. Forquer, A.M. Pershing, et al. 2015. ELQ-300 prodrugs for enhanced delivery and single-dose cure of malaria. *Antimicrob. Agents Chemother.* 59:5555–5560. http://dx.doi.org/10.1128/AAC.01183-15

Moitra, P., H. Zheng, V. Anantharaman, R. Banerjee, K. Takeda, Y. Kozakai, T. Lepore, P.J. Krause, L. Aravind, and S. Kumar. 2015. Expression, Purification, and Biological Characterization of Babesia microti Apical Membrane Antigen 1. *Infect. Immun.* 83:3890–3901. http://dx.doi.org/10.1128/IAI.00168-15

Nilsen, A., A.N. LaCrue, K.L. White, I.P. Forquer, R.M. Cross, J. Marfurt, M.W. Mather, M.J. Delves, D.M. Shackleford, F.E. Saenz, et al. 2013. Quinolone-3-diarylethers: a new class of antimalarial drug. *Sci. Transl. Med.* 5:177ra37. http://dx.doi.org/10.1126/scitranslmed.3005029

Nilsen, A., G.P. Miley, I.P. Forquer, M.W. Mather, K. Katneni, Y. Li, S. Pou, A.M. Pershing, A.M. Stickles, E. Ryan, et al. 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

Raffalli, J., and G.P. Wormser. 2016. Persistence of babesiosis for >2 years in a patient on rituximab for rheumatoid arthritis. *Diagn. Microbiol. Infect. Dis.*:S0732-8893(16)30029-3.

Salzer, W., H. Timmler, and H. Andersag. 1948. Über einen neuen, gegen Vogelmalaria wirksamen Verbindungstypus. *Chem. Ber.* 81:12–19. http://dx.doi.org/10.1002/cber.19480810103

Sarewicz, M., and A. Osyczka. 2015. Electronic connection between the quinone and cytochrome C redox pools and its role in regulation of mitochondrial electron transport and redox signaling. *Physiol. Rev.* 95:219–243. http://dx.doi.org/10.1152/physrev.00006.2014

Shih, C.M., and C.C. Wang. 1998. Ability of azithromycin in combination with quinine for the elimination of babesial infection in humans. *Am. J. Trop. Med. Hyg.* 59:509–512.

Sidhu, A.B., Q. Sun, L.J. Nkrumah, M.W. Dunne, J.C. Sacchettini, and D.A. Fidock. 2007. In vitro efficacy, resistance selection, and structural modeling studies implicate the malarial parasite apicoplast as the target of azithromycin. *J. Biol. Chem.* 282:2494–2504. http://dx.doi.org/10.1074/jbc.M608615200

Stickles, A.M., M.J. de Almeida, J.M. Morrisey, K.A. Sheridan, I.P. Forquer, A. Nilsen, R.W. Winter, J.N. Burrows, D.A. Fidock, A.B. Vaidya, and M.K. Riscoe. 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

Terkawi, M.A., S. Cao, M.S. Herbas, M. Nishimura, Y. Li, P.F. Moumouni, A.H. Pyarokhil, D. Kondoh, N. Kitamura, Y. Nishikawa, et al. 2015. Macrophages are the determinant of resistance to and outcome of nonlethal Babesia microti infection in mice. *Infect. Immun.* 83:8–16. http://dx.doi.org/10.1128/IAI.02128-14

Tonnetti, L., A.F. Eder, B. Dy, J. Kennedy, P. Pisciotto, R.J. Benjamin, and D.A. Leiby. 2009. Transfusion-transmitted Babesia microti identified through hemovigilance. *Transfusion.* 49:2557–2563. http://dx.doi.org/10.1111/j.1537-2995.2009.02317.x

Trager, W., and J.B. Jensen. 1976. Human malaria parasites in continuous culture. *Science.* 193:673–675. http://dx.doi.org/10.1126/science.781840

Vaidya, A.B., and M.W. Mather. 2000. Atovaquone resistance in malaria parasites. *Drug Resist. Updat.* 3:283–287. http://dx.doi.org/10.1054/drup.2000.0157

Vallières, C., N. Fisher, T. Antoine, M. Al-Helal, P. Stocks, N.G. Berry, A.S. Lawrenson, S.A. Ward, P.M. O'Neill, G.A. Biagini, and B. Meunier. 2012. HDQ, a potent inhibitor of Plasmodium falciparum proliferation, binds to the quinone reduction site of the cytochrome bc1 complex. *Antimicrob. Agents Chemother.* 56:3739–3747. http://dx.doi.org/10.1128/AAC.00486-12

Vannier, E., and P.J. Krause. 2012. Human babesiosis. *N. Engl. J. Med.* 366:2397–2407. http://dx.doi.org/10.1056/NEJMra1202018

Vannier, E.G., M.A. Diuk-Wasser, C. Ben Mamoun, and P.J. Krause. 2015. Babesiosis. *Infect. Dis. Clin. North Am.* 29:357–370. http://dx.doi.org/10.1016/j.idc.2015.02.008

Vyas, J.M., S.R. Telford, and G.K. Robbins. 2007. Treatment of refractory Babesia microti infection with atovaquone-proguanil in an HIV-infected patient: case report. *Clin. Infect. Dis.* 45:1588–1590. http://dx.doi.org/10.1086/523731

Weiss, L.M., M. Wittner, S. Wasserman, H.S. Oz, J. Retsema, and H.B. Tanowitz. 1993. Efficacy of azithromycin for treating Babesia microti infection in the hamster model. *J. Infect. Dis.* 168:1289–1292. http://dx.doi.org/10.1093/infdis/168.5.1289

Winter, R., J.X. Kelly, M.J. Smilkstein, D. Hinrichs, D.R. Koop, and M.K. Riscoe. 2011. Optimization of endochin-like quinolones for antimalarial activity. *Exp. Parasitol.* 127:545–551. http://dx.doi.org/10.1016/j.exppara.2010.10.016

Wittner, M., J. Lederman, H.B. Tanowitz, G.S. Rosenbaum, and L.M. Weiss. 1996. Atovaquone in the treatment of Babesia microti infections in hamsters. *Am. J. Trop. Med. Hyg.* 55:219–222.

Wormser, G.P., A. Prasad, E. Neuhaus, S. Joshi, J. Nowakowski, J. Nelson, A. Mittleman, M. Aguero-Rosenfeld, J. Topal, and P.J. Krause. 2010. Emergence of resistance to azithromycin-atovaquone in immunocompromised patients with Babesia microti infection. *Clin. Infect. Dis.* 50:381–386. http://dx.doi.org/10.1086/649859

Wudhikarn, K., E.H. Perry, M. Kemperman, K.A. Jensen, and S.E. Kline. 2011. Transfusion-transmitted babesiosis in an immunocompromised patient: a case report and review. *Am. J. Med.* 124:800–805. http://dx.doi.org/10.1016/j.amjmed.2011.03.009

Zhao, Y., K.R. Love, S.W. Hall, and F.V. Beardell. 2009. A fatal case of transfusion-transmitted babesiosis in the State of Delaware. *Transfusion.* 49:2583–2587. http://dx.doi.org/10.1111/j.1537-2995.2009.02454.x