Inhibition of Cytochrome bc_1 as a Strategy for Single-Dose, Multi-Stage Antimalarial Therapy

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Abstract. Single-dose therapies for malaria have been proposed as a way to reduce the cost and increase the effectiveness of antimalarial treatment. However, no compound to date has shown single-dose activity against both the blood-stage *Plasmodium* parasites that cause disease and the liver-stage parasites that initiate malaria infection. Here, we describe a subset of cytochrome bc_1 (cyt bc_1) inhibitors, including the novel 4(1*H*)-quinolone ELQ-400, with single-dose activity against liver, blood, and transmission-stage parasites in mouse models of malaria. Although cyt bc_1 inhibitors are generally classified as slow-onset antimalarials, we found that a single dose of ELQ-400 rapidly induced stasis in blood-stage parasites, which was associated with a rapid reduction in parasitemia in vivo. ELQ-400 also exhibited a low propensity for drug resistance and was active against atovaquone-resistant *P. falciparum* strains with point mutations in cyt bc_1 . Ultimately, ELQ-400 shows that cyt bc_1 inhibitors can function as single-dose, blood-stage antimalarials and is the first compound to provide combined treatment, prophylaxis, and transmission blocking activity for malaria after a single oral administration. This remarkable multi-stage efficacy suggests that metabolic therapies, including cyt bc_1 inhibitors, may be valuable additions to the collection of single-dose antimalarials in current development.

INTRODUCTION

Malaria is a global parasitic disease that is highly endemic in tropical and subtropical regions, where more than 200 million clinical cases and half a million deaths are reported every year.¹ Although many well-tolerated and potent antimalarial drugs exist, treatment is often complicated by the complexity of the *Plasmodium* parasites that cause malaria and the difficulty of delivering uninterrupted drug therapy in areas where medical resources are limited.^{2–4} Interruptions in antimalarial dosing, including missed or incorrectly timed doses, can cause wide fluctuations in drug exposure, which puts patients at high risk for medication overdose,^{5,6} parasite recrudescence,⁷ or the development of drug-resistant *Plasmodium* infections.⁸

The pursuit of single-dose antimalarial therapies is one leading strategy to simplify antimalarial treatment, reduce cost, and ensure compliance.^{9,10} Several potential single-dose cure antimalarials are currently in the developmental pipeline. The ozonide OZ439,¹¹ the aminopyridine MMV390048,¹² and the spiroindolone NITD609¹³ all effectively clear asexual, blood-stage parasites in mouse models of malaria with a single oral dose and also inhibit gametocyte development, which reduces the potential for malaria transmission. In contrast, several imidazolopiperazines,^{14,15} 8-aminoquinolines,¹⁶ and anti-respiratory compounds^{17,18} have been identified as single-dose causal prophylactics, which inhibit the sporozoite and liver-stage parasites that are responsible for the earliest stages of *Plasmodium* infection. Although each of these antimalarials is highly potent at specific stages of the parasite life cycle, it has not yet been possible for a single agent to provide simultaneous cures for blood, liver, and transmissive stage malaria after a single oral dose.

A key step in developing a multi-stage, single-dose antimalarial is to identify a biological target that is essential throughout the parasite life cycle. One such target is Complex III of the mitochondrial electron transport chain, which is also known as the cytochrome bc_1 complex (cyt bc_1) and is essential for pyrimidine biosynthesis in *Plasmodium*.^{19,20} Several cyt bc_1 inhibitors, including the naphthoquinone atovaquone¹⁷ and the 4(1*H*)-quinolone ELQ-300,¹⁸ are potent single-dose prophylactics, with remarkable multi-dose efficacy against blood-stage malaria parasites.^{18,21} Although it has not yet been possible to formulate these compounds as single-dose, blood-stage therapies, it is likely that this limitation is related to poor solubility, which restricts the plasma concentrations that can be achieved after a single oral dose. Practically, this exposure barrier could be overcome in one of two ways: 1) by increasing solubility or 2) by increasing intrinsic potency so that a lower plasma concentration is required for maximal effect. Here, we specifically focus on maximizing the potency of the 4(1*H*)-quinolones to obtain a multi-stage, single-dose cure for malaria.

Our group has developed an extensive library of 4(1*H*)-quinolones based on the general structure of the cyt bc_1 inhibitor, endochin.²² Within the endochin-like quinolone (ELQ) library, the most potent in vitro inhibitor is the 5,7-difluoro compound, ELQ-121, which inhibits pan-sensitive, *P. falciparum* parasites at picomolar concentrations.²³ Although ELQ-121 is poorly active in vivo, recent work with ELQ-300 has suggested that the 3-diarylether side chain may be instrumental for improving metabolic stability and extending the potency of ELQ-121 to animal models.¹⁸ In this work, we investigate a subset of highly potent 4(1*H*)-quinolone-3-diarylethers that provide single dose cures in murine models of malaria across various stages of parasite development, with a specific focus on the ELQ-121 analog, ELQ-400.

MATERIALS AND METHODS

Chemicals. The chemical structures of ELQ-400, ELQ-404, and ELQ-428 are shown in Figures 1 and 2. Synthetic methods are described in the Supplemental Information.

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FIGURE 1. Chemical structures of ELQ-121, ELQ-300, and ELQ-400.

SYBR Green I assay. In vitro antimalarial activity was assessed using a SYBR Green I fluorescence-based method described previously.²⁴ Drugs were added to 96-well plates using 2-fold, serial dilutions in HEPES-modified RPMI. Asynchronous *P. falciparum* parasites were diluted in uninfected red blood cells (RBCs) and added to wells to give a final volume of 200 μ L, at 2% hematocrit and 0.2% parasitemia. Plates were incubated for 72 hours at 37°C in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂. Parasites were then lysed using SYBR Green I lysis buffer (0.2 μ L SYBR Green I/mL MSF) and incubated in the dark for 30–60 minutes. SYBR Green I signal was quantified using a SpectraMax® Gemini-EM (Molecular Devices, Sunnyvale, CA) plate reader with excitation and emission bands centered at 497 and 520 nm, respectively. Fifty percent (50%) inhibitory concentrations (IC₅₀) were determined by non-linear analysis using GraphPad Prism® (La Jolla, CA) software. All final IC₅₀ values represent averages from at least three independent experiments, with each compound run in triplicate. *Plasmodium falciparum* parasites were cultured in human erythrocytes by standard methods as described in the Supplementary Information.

In vivo blood-stage assays. Mice (6-week, female, CF-1, Charles River Laboratories, Wilmington, MA) were infected with 2.5×10^5 *P. yoelii* (Kenya strain, MR4 MRA-428) parasitized RBCs by tail vein injection. For suppressive tests, drug was administered to animals beginning 24 hours post-inoculation for either 1 or 4 consecutive days. Orally treated animals received 100–200 μ L of drug solution (dissolved in PEG-400) by oral gavage, whereas transdermally treated animals had 10 μ L of drug solution (in biological grade DMSO) applied to the inner surface of each ear. Beginning on post-inoculation Day 5, daily blood samples were collected from the tail vein and parasitemia was determined microscopically using Giemsa stain and NIS-Elements cell-counting software (Nikon, Melville,

NY). Fifty percent (50%) effective dose (ED₅₀) values were defined as the dose required to reduce parasite burden by 50% relative to drug free controls on post-inoculation Day 5. Animals were considered cured if no parasites were detectable on post-inoculation Day 30. For onset of action studies, oral treatment was initiated on post-inoculation Day 5, but dosing and parasite monitoring were as described previously. All protocols were approved by the Portland VA Medical Center Institutional Animal Care and Use Committee.

In vivo prophylactic activity assay. Swiss Webster mice (female, 6–8 weeks, Charles River Laboratories) were intravenously infected with 2,000 luciferase-expressing *Plasmodium yoelii* YM strain sporozoites (PyLuc²⁵) isolated from the salivary glands of infected *Anopheles stephensi* mosquitoes. One hour after sporozoite infection, mice were treated with ELQ-400 (dissolved in PEG-400) by oral gavage. Forty-four hours after sporozoite infection, mice were intravenously injected with 80 mg/kg D-luciferin (Gold Biotechnology, St. Louis, MO), anesthetized with isoflurane, and imaged for liver stage development. Imaging was repeated 72 hours after infection to assess blood-stage parasite development. Bioluminescent imaging of mice was performed using IVIS® Spectrum system (Perkin Elmer, Waltham, MA) and images were analyzed using the Living Image® 4.4 software (Caliper Life Sciences, Hanover, MD).

In vivo “bite back” transmission-blocking assay. Swiss Webster mice were intravenously infected with 5×10^5 PyLuc blood stage parasites. Three days post-infection, PyLuc-infected mice were treated with ELQ-400 (dissolved in PEG-400) by oral gavage. One or 24 hours after drug treatment, mice were used to feed female *Anopheles stephensi* mosquitoes for 20 minutes and the mosquitoes were subsequently maintained under 80% humidity at 24°C. Seven days after the infected blood meal, 10 mosquitoes per group were dissected to evaluate the development of parasites by counting the numbers of oocysts per midgut. Fourteen days after the infected blood meal, naive mice ($N = 5$ per group; three experiments) were anesthetized and placed on the feeding cages containing infected mosquitoes for 15 minutes. Forty-four hours or 72 hours after bite back, in vivo imaging was performed as described previously. Prophylactic and bite-back protocols were approved by the Institutional Animal Care and Use Committees of Albert Einstein College of Medicine.

Measurement of cytochrome *bc*₁ complex inhibition. Mitochondria were isolated from HEK-293 cells (Supplemental Methods) and permeabilized in 2 mg/mL n-dodecyl β -D-maltoside. Enzymatic activity was measured in reaction buffer containing 50 mM Tricine, 100 mM KCl, 4 mM KCN, 50 μ M

FIGURE 2. Characterization of the ELQ-400 analogs, ELQ-404 and ELQ-428. (A) Chemical structures of ELQ-404 and ELQ-428. (B) IC₅₀ curves for ELQ-400, ELQ-404, and ELQ-428 against HEK-derived cytochrome *bc*₁. Values represented as percentage of uninhibited activity (mean $\pm$ SE, $N = 2$ replicates per point).

cytochrome *c* (horse heart, Sigma-Aldrich, St. Louis, MO), 0.1 mg/mL n-dodecyl β -D-maltoside, 50 μ M decylubiquinol, pH = 8.0. Measurements were made at 550–542 nm at 30°C and initiated by the addition of decylubiquinol. Baseline readings were collected for ~20 seconds to account for the non-enzymatic reduction of cytochrome *c*, and then enzyme was added to the mixture. For analysis, the baseline was subtracted from the initial rate of enzymatic activity. The activity of each kinetic trace is reported as the fraction of activity with respect to control uninhibited enzyme activity under identical conditions. *Plasmodium falciparum* assays were performed similarly as described in the Supplemental Methods.

For information regarding cell culture, resistance propensity, sequencing, parasite reduction ratio, toxicity, and microscopy studies, please see supplemental information.

RESULTS

In vitro activity against *P. falciparum*. We used a SYBR Green-based fluorescence assay²⁴ to compare the in vitro activity of ELQ-400, ELQ-121, and ELQ-300 against various *P. falciparum* strains (Figure 1). In pan-sensitive and chloroquine-resistant parasites, ELQ-400 was only slightly less active than the parent compound ELQ-121 and was ~10-fold more potent than ELQ-300 (Table 1). Like atovaquone and the parent ELQs, ELQ-400 was inactive against transgenic parasites expressing yeast dihydroorotate dehydrogenase (DHODH), showing that its predominant mechanism of action involved pyrimidine starvation secondary to *cyt bc₁* inhibition.¹⁹ However, in contrast to ELQ-121, which showed a nearly 1,000-fold loss of potency against atovaquone-resistant Tm90-C2B parasites (which contain a Y268S point mutations in *cyt b*) ELQ-400 retained substantial activity against this strain with an IC₅₀ of 35nM.

In vivo activity against blood-stage *Plasmodium yoelii*. In vivo, ELQ-400 was highly active against blood-stage parasites. In these experiments, we used a modified Peter's test in which CF-1 mice were intravenously injected with blood-stage *P. yoelii*, treated orally with drug beginning 24 hours after infection, and monitored for parasitemia by thin smear. Although ELQ-121 was inactive in vivo, ELQ-300 and ELQ-400 were both highly effective after 4 days of treatment, with 50% effective doses (ED₅₀) of 0.02 and 0.01 mg/kg and curative doses of 0.3 and 0.1 mg/kg, respectively (Table 2).

Strikingly, ELQ-400 was also curative after a single oral dose. When administered 24 hours post-inoculation, a 1 mg/kg dose of ELQ-400 prevented recrudescence in all animals, and the single-dose ED₅₀ of 0.01 mg/kg was identical to that obtained from the 4-day test (Table 2). Interestingly, the single-dose efficacy of ELQ-400 was not limited to oral

TABLE 2
Efficacy of ELQ-300 and ELQ-400 against blood-stage *P. yoelii* in mice*

Treatment	4-day ED ₅₀	4-day NRD†	1-day ED ₅₀	1-day NRD
Oral ELQ-300	0.02	0.3	ND‡	> 20§
Oral ELQ-400	0.01	0.1	0.01	1
TD¶ ELQ-400	0.01	1	0.06	1

*Values presented in mg/kg, N = 4 mice per group.

†NRD = non-recrudescence/curative dose.

‡ND = not determined.

§20 mg/kg represents solubility limit of ELQ-300 in PEG-400. Higher concentrations not tested.

¶TD = transdermal.

administration. When applied topically to the surface of the inner ear as a solution in DMSO, ELQ-400 was also fully curative after a single 1 mg/kg dose. As was observed in the oral dosing studies, additional days of ELQ-400 treatment provided little added benefit over single-day therapy, suggesting that *Plasmodium* parasites respond rapidly and maximally to an initial dose of ELQ-400.

Onset of activity against blood-stage parasites. To determine the kinetics of the in vivo response to ELQ-400, we again used the *P. yoelii* model but delayed ELQ-400 treatment until day 5 post-inoculation, when parasitemia ranged from 20–25%. We then followed parasitemia and parasite morphology by thin smear to determine how parasites responded to ELQ-400 over time. A single oral dose of 5, 10, or 20 mg/kg of ELQ-400 effectively reduced parasitemia within 24 hours of treatment, and no observable parasites were present in any group by post-treatment day 5 (Figure 3A). Ultimately, a single dose of 20 mg/kg was sufficient to prevent recrudescence in all animals, whereas single animals recrudesced in both the 5 and 10 mg/kg groups, on post-treatment days 10 and 9, respectively.

Morphologically, ELQ-400-treated parasites showed several obvious abnormalities. In vivo, parasites exposed to ELQ-400 became arrested at the schizont stage, with evidence of pyknosis and red blood cell (RBC) membrane rupture 24–48 hours after treatment (Figure 3B). We were able to mimic this schizont stage arrest in vitro using *P. falciparum* and verified that ELQ-400 exposure resulted in a rapid and long-lasting parasite stasis (Supplemental Figure S1). Although ELQ-400 did show a lag phase and required at least 48 hours to kill parasites in onset-of-action studies in vitro (Supplemental Table S1), it is likely that the drug-induced morphological changes associated with parasite stasis contributed to rapid clearance in our animal model.

Single-dose causal prophylactic and transmission blocking activity. After establishing that single-dose administration of ELQ-400 successfully cleared murine blood-stage malaria infections, we next evaluated this compound's effect on liver stage development and disease transmission by *Anopheles* mosquitoes (Supplemental Figure S2). ELQ-400 was first tested in a causal prophylaxis assay, which evaluated its ability to block the establishment of liver-stage infection by firefly luciferase-expressing *P. yoelii* (PyLuc) sporozoites.²⁵ A single dose of ELQ-400 administered orally 1 hour after sporozoite inoculation completely prevented the development of both liver and blood-stage infections at oral doses as low as 0.08 mg/kg (Figure 4A).

ELQ-400 was also highly effective at blocking disease transmission by mosquitoes. In this assay, ELQ-400 was administered orally to mice with established blood-stage

TABLE 1

Antiplasmodial IC₅₀ values (nM) against pan-sensitive (D6, D10), chloroquine-resistant (Dd2, 7G8), and anti-respiratory resistant (Tm90-C2B, D10yDHOD) *P. falciparum* parasites in the SYBR Green I assay*

Compound	D6	D10	Dd2	7G8	Tm90-C2B	D10yDHOD†
ELQ-121	0.2	0.9	0.5	2.0	310	> 2,500
ELQ-300	7.2	20	6.6	33	4.6	> 2,500
ELQ-400	0.6	2.2	1.5	4.2	35	> 2,500

*Mean IC₅₀ values calculated from three independent experiments. SE < 10%.

†D10yDHOD = transgenic D10 strain expressing yeast dihydroorotate dehydrogenase.

FIGURE 3. Onset of action of ELQ-400 against blood-stage *P. yoelii*. (A) Clearance of *P. yoelii* parasites after single-dose oral ELQ-400 treatment. Groups = 5 mg/kg ($N = 3$), 10 mg/kg ($N = 3$), or 20 mg/kg ($N = 6$) ELQ-400. Data represented as average percentage of Day 5 parasitemia. Error bars = SE. (B) Representative Giemsa-stained smears from an animal treated with 20 mg/kg ELQ-400.

PyLuc infections, and mosquitoes were allowed to feed on infected animals either 1 hour or 24 hours post-treatment. Malaria transmission was then measured by determining oocyst number in the midgut of dissected mosquitoes (Figure 4B and Supplemental Figure S3) and by assessing the ability of fed mosquitoes to initiate infections in untreated, immunologically naive mice (Figure 4C and Supplemental Figure S3). A single oral dose of 0.1 mg/kg ELQ-400 was sufficient to inhibit both oocyst formation and transmission to naive mice, whether administered 1 hour or 24 hours before mosquito feeding.

Resistance propensity. One major concern regarding the use of cyt *bc*₁ inhibitors as treatments for malaria is the propensity for *Plasmodium* drug resistance.^{26–28} Although atovaquone is the only cyt *bc*₁ inhibitor in clinical use, atovaquone resistance develops rapidly in the setting of monotherapy. As a result, several highly resistant strains have emerged, including the clinical isolate Tm90-C2B, which contains a Y268S mutation at the cyt *b* Q_o site and is more than 3,000-fold less sensitive to atovaquone.²⁸ To assess the resistance propensity of ELQ-400, we maintained the hyper-

mutable Dd2 strain of *P. falciparum* under sustained drug pressure and monitored parasite outgrowth. In this model, atovaquone-resistant parasites developed from an initial population of 10^8 parasites within 16 days of 10 nM drug pressure, yet ELQ-400 resistance required a higher parasite load (10^9 parasites), a higher concentration of drug (100 nM), and an extended period of time (47 days) to develop (Supplemental Table S2). More importantly, although the V259L mutation that conferred ELQ-400 resistance also mapped to the cyt *b* Q_o site (Supplemental Figure S4), mutant parasites were only 10-fold less sensitive to both ELQ-400 and atovaquone (Supplemental Table S3).

Cytochrome *bc*₁ inhibition and host versus parasite selectivity. In addition to being expressed throughout the *Plasmodium* life cycle, the cyt *bc*₁ complex is highly conserved across eukaryotic species, and selectivity for *Plasmodium* is a desirable characteristic of new cyt *bc*₁ inhibitors. We assessed cyt *bc*₁ inhibition by ELQ-400 in permeabilized mitochondria isolated from *P. falciparum* versus HEK293 cells and determined that ELQ-400 inhibits the parasite and human

FIGURE 4. In vivo causal prophylactic and transmission blocking activity of ELQ-400 against luciferase-expressing *P. yoelii* parasites (PyLuc). (A) Whole-animal bioluminescence of animals infected with PyLuc sporozoites and treated with single oral doses ELQ-400 1 hour post-inoculation. (B) Oocyst counts from mosquitoes fed on blood-stage, PyLuc infected mice 1 hour after ELQ-400 treatment. (C) Whole-animal bioluminescence of animals in the “bite back” assay, bitten by PyLuc exposed mosquitoes fed on PyLuc infected mice 1 hour after ELQ-400 treatment.

TABLE 3
In vitro and in vivo activity of ELQ-400, ELQ-404, and ELQ-428 against blood-stage parasites*

Compound	D6 (nM)	Dd2 (nM)	Tm90-C2B (nM)	4-day ED ₅₀ (mg/kg)	4-day NRD (mg/kg)	1-day NRD (mg/kg)
ELQ-400	0.6	1.5	35	0.01	0.1	1.0
ELQ-404	0.9	6.2	27	0.03	1.0	10†
ELQ-428	5.8	11	34	0.1	1.0	10

*IC₅₀ calculated as the mean of three independent experiments (SE < 10%) in SYBR Green I assay. ED₅₀ and NRD determined from *P. yoelii* suppressive tests, (N = 4 animals per group).
†10 mg/kg ELQ-404 was curative in 3/4 animals.

enzymes with IC₅₀ values of 6.7 nM and 108 nM, respectively (Supplemental Table S4). Despite this limited selectivity, ELQ-400 did not show toxicity in human, whole-cell models. In toxicity screens with proliferating human foreskin fibroblasts and activated murine T-lymphocytes, the 50% toxic dose (TD₅₀) of ELQ-400 exceeded 25 μ M (Supplemental Table S4), suggesting that ELQ-400 may have a pharmacodynamic interaction with human cells that cannot be adequately predicted from assessment of cyt *bc*₁ inhibition in isolated mitochondria.

Because several ELQs lack human cytochrome *bc*₁ inhibition, we next addressed the possibility of chemically modifying ELQ-400 to improve selectivity for the parasite target. We have previously shown that human cyt *bc*₁ inhibition is minimized in ELQs containing large substituents at the 6 or 7 positions, especially 6-position halogens.¹⁸ Consistent with this trend, the 6-fluoro and 6-chloro analogs of ELQ-400, ELQ-404 and ELQ-428 (Figure 2A), showed reduced inhibition of human HEK-derived cytochrome *bc*₁ with IC₅₀ values of 1 μ M and > 10 μ M, respectively (Figure 2B).

Although 6-position modification successfully increased the selectivity of the 5,7-difluoro ELQs, this adjustment was associated with a reduction in antiparasmodial activity and antimalarial efficacy, and ELQ-404 and ELQ-428 were between 5- and 10-fold less active than ELQ-400 both in vitro and in vivo (Table 3). Encouragingly, both compounds were still capable of producing single-dose cures against blood-stage murine malaria in the 1-day Peters test.

DISCUSSION

ELQ-400 is a potent antimalarial that provides prophylactic, treatment, and transmission blocking activity against malaria after administration of a single low dose. In mouse models, ELQ-400 prevented malaria transmission and blocked the development of liver stage infections with single oral doses of 0.1 and 0.08 mg/kg, respectively. More impressively, ELQ-400 also effectively cleared asexual, blood-stage parasites with a single 1 mg/kg dose, making it the most active single-dose therapy to date, with a 20-fold to 100-fold increase in efficacy over other single-dose compounds in the developmental pipeline.^{11–13}

As with other single-dose antimalarials, we also found that ELQ-400 was capable of rapidly reducing blood-stage parasitemia in vivo. This finding was especially striking because cyt *bc*₁ inhibitors such as ELQ-400 have generally been classified as slow-onset antimalarials.²⁹ Although ELQ-400 did require at least 48 hours to initiate cell death in vitro, it triggered a rapid and long-lasting stasis in blood-stage parasites that was associated with clear morphological changes in vivo. These changes likely increased clearance by the spleen and allowed ELQ-400 to rapidly reduce parasite burden even before the onset of parasite death. Although we do not yet

know the kinetics of ELQ-400's interaction with liver and sexual stage parasites, our finding that a mere 1 hour of ELQ-400 exposure was sufficient to maximally inhibit oocyst development in fed mosquitoes suggests that the rapid inhibitory effects of ELQ-400 are likely conserved across multiple stages of *Plasmodium* development.

In addition to providing unprecedented, multi-stage, single-dose cures for malaria, lipophilic cyt *bc*₁ inhibitors such as ELQ-400 may be ideally suited for topical formulation. In both 4-day and 1-day dosing tests against blood-stage parasites, transdermal administration of ELQ-400 closely replicated the effects of oral delivery. Although oral therapy is the most common treatment route for uncomplicated malaria, IV drug administration is still the standard of care for infants and patients with severe disease.^{30,31} For children, rectal formulations of artesunate have also been proposed for pre-referral treatment in cases where parenteral therapy will be delayed or oral administration is not possible.³² Our findings with ELQ-400 suggest that topical treatment may be a viable alternative, which could decrease the risk of blood-borne infections, reduce the medical training required to provide treatment, and ultimately provide safer, more accessible care for patients.

Biologically, the impressive single-dose efficacy of ELQ-400 has yet to be fully explained. Although we initially attributed the single-dose activity of ELQ-400 to its remarkable potency against *Plasmodium*, this cannot explain the response to ELQ-404 and ELQ-428, which are less potent both in vitro and in vivo. Therefore, we hypothesize that the 5,7-difluoro substituents play a specific role in the activity of ELQ-400, ELQ-404, and ELQ-428. One possible explanation is that the 5,7-difluoro groups improve the pharmacokinetic properties of the ELQs either by increasing absorption or extending in vivo half-life. It is also possible that the 5,7-difluoro configuration directly influences the interaction with cyt *bc*₁. Fluorine atoms are hydrogen bond acceptors and may interact with cyt *bc*₁ residues to facilitate a more rapid or longer lasting inhibition.³³ This could also explain the moderate cross-resistance that Tm90-C2B parasites exhibit toward the 5,7-difluoro substituted ELQs; the mutated Y268 residue plays a predicted role in hydrogen bonding within the Q_o site of cyt *bc*₁.³⁴

Ultimately, the major obstacle to ELQ-400 development is its limited selectivity for *Plasmodium* cyt *bc*₁. Off-target inhibition of the mammalian enzyme was presented as a possible explanation for the failure of the recent GSK pyridone project, during which a prodrug form of the lead pyridone compound, GSK932121, produced acute toxicity in rats.³⁵ Although the pyridones do inhibit human cyt *bc*₁ at levels comparable to ELQ-400, it is noteworthy that ELQ-400 has never produced toxicity in mice or in several primary cell culture models. Unlike immortalized cells, which rely primarily on glycolysis for metabolism, these primary cells are very sensitive to changes in mitochondrial function, and our observed lack of toxicity suggests that ELQ-400 does not inhibit cyt

bc_1 in intact cells. In the case of atovaquone, which also inhibits human cyt bc_1 but is clinically well-tolerated by patients, drug efflux or reduced uptake by intact cells have been proposed as possible protective mechanisms for human cells, based on the finding that atovaquone is a known substrate for several efflux transporters in yeast.³⁶ It is possible that a similar efflux process reduces the ELQ-400 concentration within host cells, and this could explain the discrepancy between enzyme-level EC_{50} and whole-cell toxicity that we have observed in our HFF and T-cell models. Therefore, we believe that further investigation of ELQ-400 is warranted and that its inhibition of permeabilized, HEK293 mitochondria should not be used as a *de facto* indicator of human toxicity.

It is worthy of note that we observed a relatively low propensity for resistance to ELQ-400 relative to the frequency observed for atovaquone. Although this observation predicts a diminished risk for ELQ-400 resistance developing in *P. falciparum* exposed to the drug, clinical experience has proven the value of a combination strategy to prolong the clinical use of new antimalarial drugs and to delay the emergence of drug resistance. Drug combinations reduce the probability of *de novo* resistance selection; if a parasite that is resistant to one drug occurs, the other should kill it. Combinations therefore protect each of the partner drugs. We are actively engaged in research to identify suitable combination partner drugs for ELQ analogs with compatible pharmacodynamic and pharmacokinetic properties that could be developed for clinical use.

In conclusion, ELQ-400 provides the first evidence that cyt bc_1 inhibitors can function as single-dose, multi-stage antimalarials. In addition to providing several direct options for ongoing development, our work with ELQ-400 also raises the intriguing possibility that other potent single-dose curative compounds may exist within the broader library of anti-respiratory inhibitors. Direct assessment of these compounds, and specific formulation to maximize systemic exposure may be invaluable tools for the further development and evaluation of single-dose treatments for malaria.

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