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Subtle Changes in Endochin-Like Quinolone Structure Alter the Site of Inhibition within the Cytochrome bc_1 Complex of *Plasmodium falciparum*

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The cytochrome bc_1 complex (cyt bc_1) is the third component of the mitochondrial electron transport chain and is the target of several potent antimalarial compounds, including the naphthoquinone atovaquone (ATV) and the 4(1*H*)-quinolone ELQ-300. Mechanistically, cyt bc_1 facilitates the transfer of electrons from ubiquinol to cytochrome *c* and contains both oxidative (Q_o) and reductive (Q_i) catalytic sites that are amenable to small-molecule inhibition. Although many antimalarial compounds, including ATV, effectively target the Q_o site, it has been challenging to design selective Q_i site inhibitors with the ability to circumvent clinical ATV resistance, and little is known about how chemical structure contributes to site selectivity within cyt bc_1. Here, we used the proposed Q_i site inhibitor ELQ-300 to generate a drug-resistant *Plasmodium falciparum* clone containing an I22L mutation at the Q_i region of cyt *b*. Using this D1 clone and the Y268S Q_o mutant strain, *P. falciparum* Tm90-C2B, we created a structure-activity map of Q_i versus Q_o site selectivity for a series of endochin-like 4(1*H*)-quinolones (ELQs). We found that Q_i site inhibition was associated with compounds containing 6-position halogens or aryl 3-position side chains, while Q_o site inhibition was favored by 5,7-dihalogen groups or 7-position substituents. In addition to identifying ELQ-300 as a preferential Q_i site inhibitor, our data suggest that the 4(1*H*)-quinolone scaffold is compatible with binding to either site of cyt bc_1 and that minor chemical changes can influence Q_o or Q_i site inhibition by the ELQs.

Malaria is a devastating parasitic disease that affects >200 million people every year and is a leading cause of mortality in the developing world (1). Malaria is caused by *Plasmodium* parasites, which are transmitted by the bites of infected *Anopheles* mosquitoes and progress through a series of biologically distinct stages within the hepatocytes and red blood cells of the human host. The most lethal malarial parasite, *Plasmodium falciparum*, has developed resistance to many frontline antimalarials, including the quinolines quinine, chloroquine, and mefloquine, as well as the antifolates pyrimethamine and sulfadoxine. In many regions of the world, the treatment of multidrug-resistant malaria relies on the use of artemisinin-based combination therapies (ACTs), such as artesunate-amodiaquine and dihydroartemisinin-piperaquine. Unfortunately, artemisinin resistance has emerged in regions of Southeast Asia (2), which threatens to derail the ACT treatment strategy and further restrict therapeutic options for patients in malarious regions. As a result, there is a pressing need for new antimalarial compounds, particularly those that effectively inhibit drug-resistant parasites and function throughout multiple stages of the parasite life cycle to provide combined treatment, prophylaxis, and transmission-blocking activity against malaria (3).

Complex III of the mitochondrial electron transport chain, also known as the cytochrome bc_1 complex (cyt bc_1), is a validated target for multistage antimalarial therapy. Structurally, there are two known binding sites for antimalarial compounds within cyt bc_1: an oxidative (Q_o) site and a reductive (Q_i) site. Inhibition at either site is sufficient to block the catalytic cycle of cyt bc_1 (Q cycle), which ultimately leads to pyrimidine starvation and cell death in *P. falciparum* (4, 5). To date, pyridones (6, 7), naphthoquinones (8, 9), acridones (10), quinolones (11–13), and benzene sulfonamides (14) have been identified as potent inhibitors of *Plasmodium* cyt bc_1 (15). This includes atovaquone (ATV), which targets the Q_o site of the *P. falciparum* cytochrome bc_1 complex (16).

Although atovaquone is a potent and well-tolerated antimalarial drug, its clinical utility is limited by the rapid emergence of resistant parasites when used as monotherapy (17). For this reason, atovaquone is coformulated with proguanil (Malarone) to counter the emergence of resistance and improve clinical efficacy. In the *P. falciparum* clinical isolate Tm90-C2B, a point mutation at the Q_o site of cytochrome *b* (i.e., Y268S) results in a 3,000-fold loss of ATV sensitivity. As a result, this parasite line has been used

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as a screening tool for the development of new cyt bc_1 inhibitors able to circumvent ATV resistance mutations. Our group has developed an extensive library of compounds based on the general structure of the cyt bc_1 inhibitor endochin (11, 12, 18, 19). As was previously reported, several of these endochin-like quinolones (ELQs), including the preclinical candidate ELQ-300 (12), demonstrate remarkable potency against Tm90-C2B parasites, which may suggest preferential Q_i site inhibition.

To date, very few Q_i site inhibitors of cyt bc_1 have been identified, and it has been especially difficult to isolate Q_i-selective compounds with activity against *P. falciparum*. Sequencing studies have shown that the *P. falciparum* Q_i site is structurally distinct from that of other species (20). As a result, even antimycin A, the prototype picomolar inhibitor of the Q_i site in bacteria, yeast, and mammalian cells (21), demonstrates decreased activity against *P. falciparum*, with an *in vitro* 50% inhibitory concentration (IC_{50}) in the nanomolar range (22). The uniqueness of the *P. falciparum* Q_i site may confer several therapeutic advantages. In addition to retaining potency against ATV-resistant Q_o site mutant parasites, Q_i site inhibitors may be uniquely selective for parasite cyt bc_1 and would thus constitute a novel class of antimalarial compounds.

A major obstacle to the development of Q_i site inhibitors for *Plasmodium* spp. is the lack of effective screening tools to identify Q_i-selective compounds. Although studies in yeast have suggested that the quinolone compounds ELQ-271 (23) and HDQ [1-hydroxyl-2-dodecyl-4(1*H*)-quinolone] (24) function as Q_i site inhibitors, no *P. falciparum* Q_i site mutants have been available for verification. Furthermore, with such a small group of effective Q_i-targeting antimalarials, it has not yet been possible to make any consistent associations between chemical structure and Q_i site preference. In this paper, we introduce a new *P. falciparum* clone containing a mutation at the cyt bc_1 Q_i site, and we used this mutant in combination with Tm90-C2B to conduct a detailed assessment of Q_i versus Q_o targeting within the ELQ library. We identify several structural features that are associated with preferential Q_i site activity and provide insight into the ongoing development of new multistage antimalarial inhibitors of cyt bc_1.

MATERIALS AND METHODS

Chemicals and chemistry methods. Unless indicated otherwise, all chemicals and reagents used in this study were from Sigma-Aldrich (St. Louis, MO). The ELQs described in this paper were described previously (12, 18, 19) or were synthesized by methods developed in our laboratory and published elsewhere (11, 25).

***In vitro* selection of ELQ-300-resistant *P. falciparum* clones.** A clonal population of *P. falciparum* Dd2 parasites was maintained at 5% final hematocrit in an atmosphere of 90% N_2, 5% CO_2, and 5% O_2 at 37°C in complete culturing medium (10.4 g $liter^{-1}$ RPMI 1640 with 2.1 mM glutamine, 5.94 g $liter^{-1}$ HEPES, 5 g $liter^{-1}$ AlbuMAX II, 50 mg $liter^{-1}$ hypoxanthine, 2.1 g $liter^{-1}$ sodium bicarbonate, and 43 mg $liter^{-1}$ gentamicin). On day 0 of selection, an initial inoculum of 10^9 parasites was cultured in the presence of drug at 25 nM. On days 4, 5, and 7, the drug concentration was increased to 32, 40, and 70 nM, respectively, until the cultures were cleared of parasites. The medium was changed daily until the parasites were microscopically undetectable (as assessed by an examination of Giemsa-stained slides) and subsequently every 2 days for the remainder of the experiment. Upon recrudescence, the population of parasites was cloned by limiting dilution (0.8 infected red blood cell [RBC]/well) at 1.8% hematocrit in a 96-well flat-bottom tissue culture plate in the presence of 70 nM ELQ-300. On day 21 of cloning, 5 μl of parasite culture from each well was mixed with a solution containing 0.1 μl/ml SYBR green I and 0.1 μM MitoTracker Deep Red (Life Technologies) and incubated for 20 min prior to an analysis of parasitemia on an Accuri C6 flow cytometer (26).

Sequencing of cytochrome *b*. DNA was isolated from the parasites at the mid- to late trophozoite stage. The parasites were saponin lysed with 0.02% saponin in 1× phosphate-buffered saline (PBS). The pellets were resuspended to a volume of 200 μl with PBS-20 mM EDTA, and DNA was isolated using the QIAamp DNA blood minikit (Qiagen), according to the blood protocol. PCR was performed using the Herculase II fusion enzyme (Agilent Technologies). Primer 1 was 5′-CCAGACGCTTTAAATGGATG-3′, and primer 2 was 5′-GTTTGCTTGGGAGCTGTAATC-3′. The PCR products were purified using SV gel and the PCR cleanup kit (Promega). DNA sequencing was performed by Genewiz.

***P. falciparum* culture.** Laboratory strains of *P. falciparum* were cultured in human erythrocytes by standard methods under a low-oxygen atmosphere (5% O_2, 5% CO_2, and 90% N_2) in an environmental chamber. The parasites were maintained in fresh human erythrocytes suspended at 2% hematocrit in complete medium at 37°C. The stock cultures were subpassaged every 3 to 4 days by transferring infected RBCs to a flask containing complete medium and uninfected RBCs.

SYBR green I assay. *In vitro* antimalarial activity was assessed using a published SYBR green I fluorescence-based method (27). The drugs were added to 96-well plates using 2-fold serial dilutions in HEPES-modified RPMI (described above). Asynchronous *P. falciparum* parasites were diluted in uninfected RBCs and added to the wells to give a final volume of 200 μl at 2% hematocrit and 0.2% parasitemia. The plates were incubated for 72 h at 37°C. The parasites were then lysed using SYBR green I lysis buffer containing 0.2 μl/ml SYBR green I in 20 mM Tris (pH 7.5), 5 mM EDTA, 0.008% (wt/vol) saponin, and 0.08% (vol/vol) Triton X-100. The plates were incubated in the dark for 30 to 60 min, and the SYBR green I signal was then quantified using a SpectraMax Gemini EM plate reader with excitation and emission bands centered at 497 and 520 nm, respectively. The 50% inhibitory concentrations (IC_{50}) were determined via nonlinear analysis using the GraphPad Prism software. All final IC_{50} values represent the averages from at least three independent experiments, with each compound run in triplicate.

Molecular modeling. The 1KYO (Q_o) and 1EZV (Q_i) crystal structures of *Saccharomyces cerevisiae* cyt bc_1 were obtained from the Protein Data Bank and modified using the Protein Data Bank Viewer, as follows: Q_i site, F225L and K228L, and Q_o site, C133V, C134L, V135P, Y136W, H141Y, and L275F. The adjusted Q_o residues were selected because of their importance in sensitizing *S. cerevisiae* to known antimalarial inhibitors, including ATV and the quinolone compound RCQ06 (28). The Q_i site modifications were made to account for the reduced sensitivity to the Q_i site inhibitor antimycin A that is observed in *P. falciparum* (20). The ELQ models were created using ChemDraw 3D, and docking was performed using the CLC Drug Discovery Workbench software. The Q_o and Q_i binding pockets were centered on the crystal-bound substrates stigmatellin (Q_o) and coenzyme Q6 (Q_i). One thousand docking iterations were run per compound at each site.

RESULTS

Development and characterization of the ELQ-300-resistant *P. falciparum* D1 clone. Drug-resistant *P. falciparum* strains provide a wealth of information about the functions of antimalarial compounds and the adaptive mechanisms that allow parasites to avoid their effects. *In vitro*, drug resistance is generally developed through one of two standard methods, (i) high-concentration drug pressure or (ii) incremental drug pressure, which begins at low concentrations and increases in response to parasite growth over time. Biologically, the first method is used as an indication of resistance propensity, while the second is primarily a tool to generate resistant parasites for further study.

To generate the D1 clone, we cultured *P. falciparum* Dd2 parasites in the presence of a low concentration of ELQ-300 (25 nM,

TABLE 1 Sensitivities of the *P. falciparum* Dd2, Tm90-C2B, and D1 strains to ATV, ELQ-300, CQ, and the canonical Q_i and Q_o site inhibitors antimycin A and myxothiazol[a]

Compound	IC_{50} value (nM) or ratio for[b]:				
	Dd2	Tm90-C2B	C2B/Dd2	D1	D1/Dd2
ATV	0.4	>2,500	>7,800	0.7	1.7
ELQ-300	6.6	4.6	0.7	160	24
CQ	71	91	1.3	110	1.5
Antimycin A	72	39	0.5	35	0.5
Myxothiazol	1.7	320	190	3.5	2.0

[a] ATV, atovaquone; CQ, chloroquine.
[b] IC_{50} values were averaged from ≥3 independent experiments run in triplicate. The standard deviations were <10%.

approximately 4× the IC_{50}). We monitored parasitemia daily and gradually increased the ELQ-300 concentration until parasite growth was fully inhibited and parasitemia dropped below the limit of detection. After 2 weeks at this final concentration of 70 nM, parasites were detected, and three clones were isolated by a limiting dilution (29). DNA sequencing of the cytochrome *b* gene revealed an I22L mutation in all clones, which mapped to the *P. falciparum* Q_i site. The D1 clone was used for subsequent studies and was found to be 25-fold less sensitive to ELQ-300 than was the parental Dd2 strain. As would be expected for a Q_i site mutant, no loss of potency was observed for the Q_o site inhibitors ATV and myxothiazol (22) or for the non-cyt bc_1-targeting compound chloroquine (CQ) (Table 1).

***In vitro* Q_o versus Q_i preference of ELQs.** To explore how chemical structure contributed to Q_i versus Q_o site preference within the ELQ series, we conducted a large-scale assessment of our ELQ library using the *P. falciparum* D1 and Tm90-C2B drug-resistant clones. We used the fluorescence-based SYBR green I assay (27) to determine the IC_{50} for each compound and created cross-resistance indices by normalizing D1 and Tm90-C2B activities against that of the Dd2 parental strain. Because the D1 and Tm90-C2B clones contain Q_i and Q_o site point mutations, respectively, we used D1 cross-resistance as an indicator of preferential Q_i site activity and Tm90-C2B cross-resistance as an indicator of preferential Q_o site activity.

ELQ 6 and 7 positions. We assessed the roles of the 6-position and 7-position groups (Fig. 1) in Q_o/Q_i preference by first evaluating a set of ELQs containing the 3-position alkyl chain of endochin (Table 2). At the 7 position, all chemical groups were associated with an increase in Tm90-C2B cross-resistance relative to that of the undecorated compound ELQ-127. Electron-withdrawing substituents had the most dramatic effect, especially for the 7-Cl compound ELQ-109, which was >25 times less potent against the Tm90-C2B strain. In contrast, the effect of 6-position groups was dependent on electrostatic character. Electron-donating groups (e.g., SCH_3, OH, and OCH_3) dramatically reduced potency and increased cross-resistance in Tm90-C2B parasites, while electron-withdrawing groups (e.g., halogens and NO_2) were universally associated with D1 cross-resistance and Q_i targeting. Overall, the most Q_i-selective compound was ELQ-130, which contained a 6-chloro substituent and was undecorated at the 7 position.

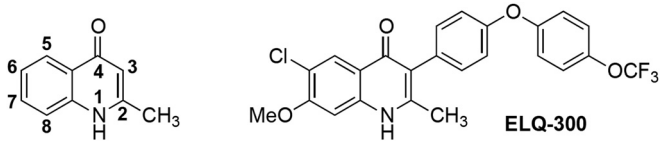

FIG 1 Left, general structure of 4(1*H*)-quinolones with numbered chemical positions. Right, structure of ELQ-300, the compound used to generate the Q_i mutant D1 clone.

TABLE 2 Comparative activities of 3-alkyl ELQs with various 6- and 7-position substituents

ELQ	Substituent at position:			IC_{50} value (nM) or ratio for[a]:		
	3	6	7	Dd2	C2B/Dd2	D1/Dd2
131	C_7H_{15}	F	H	47	1.9	2.8
130	C_7H_{15}	Cl	H	29	0.9	9.7
162	C_7H_{15}	NO_2	H	260	1.4	4.6
150	C_7H_{15}	OCH_3	H	440	3.4	0.9
133	C_7H_{15}	SCH_3	H	280	5.9	1.5
127	C_7H_{15}	H	H	39	1.7	2.4
120	C_7H_{15}	H	F	9.6	19	1.0
109	C_7H_{15}	H	Cl	7.1	26	1.4
118	C_7H_{15}	H	CN	38	14	0.8
110	C_7H_{15}	H	NO_2	27	15	1.7
100	C_7H_{15}	H	OCH_3	5.2	6.1	0.9

[a] IC_{50} values were averaged from ≥3 independent experiments run in triplicate. The standard deviations were <10%.

In a second set of compounds consisting of 3-diarylether ELQs, 6-position halogens had an even more dramatic effect on D1 cross-resistance and Q_i site preference (Table 3). While ELQ-130 was approximately 10-fold less active against the D1 clone, the corresponding 6-chloro 3-diarylether ELQ-296 was 15-fold less active, and a similar loss of activity was observed for the 6-fluoro comparators ELQ-131 and ELQ-314. Intriguingly, the combination of 6-halogen and 7-methoxy groups produced an unanticipated increase in Q_i site preference among the 3-diarylether ELQs (Table 3). Compared to the 6-halogen compounds ELQ-314, ELQ-296, and ELQ-339, the corresponding 7-methoxy analogs (ELQ-316, ELQ-300, and ELQ-340, respectively) demonstrated both increased potency and more pronounced D1 cross-resistance, which correlated with halogen size.

ELQ 3-position side chains. The difference in D1 cross-resistance observed between the 3-alkyl and the 3-diarylether ELQs suggested that the 3-position side chain might play an integral role in Q_o versus Q_i targeting for the ELQs. To assess this relationship,

TABLE 3 Comparative activities of 3-diarylether ELQs containing 6-position halogens and 7-position OCH_3 groups

ELQ	Substituent at position:			IC_{50} value (nM) or ratio for[a]:		
	3	6	7	Dd2	C2B/Dd2	D1/Dd2
271	DAE[b]	H	H	12	2.8	0.2
298	DAE	H	OCH_3	5.5	0.7	0.8
314	DAE	F	H	33	1.3	3.4
316	DAE	F	OCH_3	3.1	1.1	3.6
296	DAE	Cl	H	29	0.9	16
300	DAE	Cl	OCH_3	6.6	0.7	24
339	DAE	Br	H	170	1.1	14
340	DAE	Br	OCH_3	25	0.4	65

[a] IC_{50} values were averaged from ≥3 independent experiments run in triplicate. The standard deviations were <10%.
[b] DAE, para-OCF_3 diaryl ether side chain, as present in ELQ-300.

TABLE 4 Comparative activities of 6-chloro ELQs with various 3-position side chains

ELQ	Substituent at position[a]: 3	6	7	IC_{50} value (nM) or ratio for[b]: Dd2	C2B/Dd2	D1/Dd2
220	Benzyl	Cl	H	510	4.1	2.7
200	C_4H_9	Cl	H	121	1.6	5.1
130	C_7H_{15}	Cl	H	29	0.9	9.7
296	DAE	Cl	H	29	0.9	16
317	Het- DAE	Cl	H	58	0.5	15
269	Biphenyl	Cl	H	42	0.3	110

[a] DAE, para-OCF_3 diaryl ether side chain, as present in ELQ-300. Het-DAE, para-OCF_3 diaryl ether analog, containing a pyridyl inner ring.
[b] IC_{50} values were averaged from ≥3 independent experiments run in triplicate. The standard deviations were <10%.

we next analyzed a series of 6-chloro ELQs containing various 3-position side chains (Table 4). Among these compounds, the most dramatic D1 cross-resistance was observed for the 3-biphenyl compound ELQ-269, while the lowest degree of cross-resistance was associated with ELQ-200, which contained a truncated 3-alkyl side chain. Generally, D1 cross-resistance correlated with side-chain length and rigidity but was not noticeably altered by the presence of additional charged groups or heterocycles, as was demonstrated by the comparison between ELQ-296 and ELQ-317.

ELQ 5 and 7 positions. While 6-position halogens and rigid 3-position side chains were associated with the highest degree of Q_i site selectivity, Q_o site inhibition was strongly favored in ELQs containing 5,7-dihalogen groups (Table 5). The 5,7-difluoro compound ELQ-121 was >600 times less potent against Tm90-C2B parasites and was the most Q_o-selective compound within our ELQ library. Structurally, the 5-position fluorine contributed most strongly to this Q_o site preference; Tm90-C2B parasites were more cross-resistant to the 5-fluoro analog ELQ-136 than to the 7-fluoro analog ELQ-120. In contrast to the effect of isolated 7-position halogens, cross-resistance did not increase with 5,7-dihalogen size, and the 5,7-dichloro compound ELQ-124 was less Q_o selective than was the difluoro compound ELQ-121.

Intriguingly, the Q_o-directing effects of the 5,7-difluoro configuration were effectively modulated by the addition of Q_i-directing chemical substituents (Table 5). The combination of a 3-position diarylether chain with 5,7-difluoro groups produced

TABLE 5 Comparative activities of ELQs containing 5- or 7-position halogens, with various Q_i-directing substituents

ELQ	Substituent at position[a]: 3	5	6	7	IC_{50} value (nM) or ratio for[b]: Dd2	C2B/Dd2	D1/Dd2
136	C_7H_{15}	F	H	H	3.1	76	1.3
121	C_7H_{15}	F	H	F	0.5	610	0.6
124	C_7H_{15}	Cl	H	Cl	24	15	0.9
141	Benzyl	F	H	F	38	120	1.4
400	DAE	F	H	F	1.5	23	1.1
140	C_7H_{15}	F	F	F	2.8	34	1.1
404	DAE	F	F	F	4.9	5.8	1.4
428	DAE	F	Cl	F	11	3.4	3.3
429	DAE	F	OCH_3	F	46	26	2.0

[a] DAE, para-OCF_3 diaryl ether side chain, as present in ELQ-300.
[b] IC_{50} values were averaged from ≥3 independent experiments run in triplicate. The standard deviations were <10%.

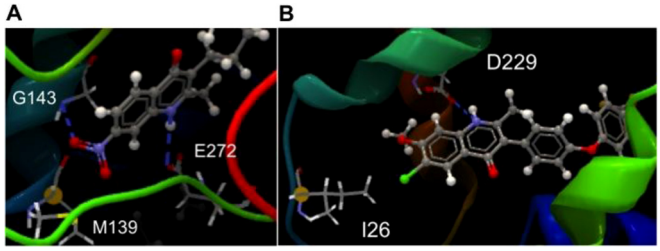

FIG 2 *S. cerevisiae* homology models depicting possible interactions between ELQs and cyt bc_1. Shown are the predicted interactions between the 1-NH group of ELQ-110 and the E272 residue at the Q_o site (A) and hydrogen bonding of the 1-NH group of ELQ-300 to the Q_i site D229 residue (B), showing the 6-position chlorine in close proximity to the isoleucine residue mutated in the *P. falciparum* D1 clone. The dashed blue lines represent predicted hydrogen bonds.

ELQ-400, which was associated with diminished Tm90-C2B cross-resistance relative to that of the 3-alkyl compound ELQ-121. Tm90-C2B cross-resistance was further reduced by the incorporation of halogens at the 6 position, and low levels of both D1 and Tm90-C2B cross-resistance were observed for the 5,7-difluoro 6-chloro analog ELQ-428. Conversely, the addition of a 6-methoxy group between the flanking fluorine atoms increased Tm90-C2B cross-resistance relative to that of ELQ-400, which was consistent with the predicted effect of an electron-donating 6-position substituent.

DISCUSSION

Using a set of mutant *P. falciparum* strains, including the novel D1 clone, we have identified several key ELQ features associated with preferential Q_o or Q_i site inhibition of *Plasmodium* cyt bc_1. The primary contributors to Q_i site preference included (i) electron-withdrawing 6-position substituents (including halogens), (ii) combined 6-halogen 7-methoxy groups, and (iii) aryl 3-position side chains. Alternately, 7-position groups were broadly associated with Q_o site targeting, especially in combination with 5-fluorine moieties, as was observed for the 5,7-difluoro ELQs.

The dramatic impact of subtle chemical changes on site specificity suggests that ELQ substituents may divergently interact with key residues of cyt bc_1. Although exact binding interactions cannot be identified without a crystal structure of the *Plasmodium* enzyme, we were able to explain several of our observed structural effects using homology modeling in *S. cerevisiae*. In Q_o site models, we found that the 1-NH group of the quinolone core made a predicted hydrogen bond with the conserved E272 residue, which is important for binding of the natural cyt bc_1 substrate ubiquinol and the potent Q_o site inhibitor stigmatellin (30). In this conformation, 7-position substituents were well tolerated and frequently formed additional predicted bonds with backbone atoms of the nearby M139 and G143 residues (Fig. 2A). Interestingly, this predicted binding alignment was similar to that recently reported for atovaquone (16), which may explain the reduced sensitivity of the ATV-resistant TM90-C2B clinical isolate to several members of the ELQ library, including 5,7-difluoro compounds.

With respect to the Q_i site, we identified a potential hydrogen bond interaction between the 1-NH group of the quinolone core and the nearby D229 residue of the Q_i binding pocket (Fig. 2B). In this conformation, the 6-position chlorine atom of ELQ-300 was in close proximity to I26, which is homologous to the I22 residue

mutated in ELQ-300-resistant D1 parasites. While the involvement of the conserved quinolone core suggests that all ELQs are likely capable of Q_i site interaction, the specific bond with the 1-NH group also provides a potential explanation for the increased Q_i site selectivity of ELQs containing 6-position electron-withdrawing groups. Because the quinolone ring is aromatic in nature, electron-withdrawing groups located *para* to the 1-position nitrogen (e.g., at the 6 position) should draw electron density away from the 1-NH group, making the associated hydrogen more acidic and available for hydrogen bond formation. Alternately, 6-position electron-donating groups would be expected to decrease the potential for hydrogen bonding, which is consistent with our finding that such compounds demonstrate both decreased potency and reduced Q_i site preference.

The predicted binding conformation of ELQ-300 at the Q_i site also suggests that observed D1 cross-resistance is likely a direct reflection of the steric interference between 6-position substituents and the interfacing I22L residue (Fig. 2B). In addition to justifying the correlation between 6-halogen size and D1 cross-resistance, this steric hypothesis provides a compelling explanation for the lack of cross-resistance observed for the known Q_i site inhibitor ELQ-271 (23), which is undecorated at the 6 position. While a more general Q_i site *P. falciparum* mutant may be desirable from a screening perspective, our efforts to generate resistance under ELQ-271 pressure have thus far been unsuccessful. Furthermore, constant exposure to ELQ-300 at 10× its IC_{50} fails to select for resistant parasites (12), suggesting that a significant fitness cost may be associated with mutations that confer high-level resistance to ELQs or that there may simply be limited genetic space for mutations within the cyt *b* Q_i site.

In conclusion, our studies have shown that the *P. falciparum* D1 clone has considerable potential for use in comparative assessments and large-scale structure-activity analyses. Intriguingly, this model demonstrated that the 4(1*H*)-quinolone scaffold was compatible with inhibition at either the Q_o or Q_i site of cyt bc_1. Although it is unknown whether ELQs can simultaneously inhibit both catalytic sites, a dual-site interaction may explain why even the most Q_o-selective compounds in the ELQ library still function as nanomolar inhibitors of Tm90-C2B parasites, and it might also explain the remarkable *in vivo* efficacies of the ELQs relative to those of comparison compounds, such as ATV (12, 31). In theory, dual-site inhibitors of the parasite cyt bc_1 complex may be highly desirable, because high-level drug resistance would require the simultaneous appearance of two independent mutations within a single protein target. Ultimately, it will be necessary to parse the effects of isolated Q_i site inhibition from those of a dual-site interaction so that cyt bc_1 inhibitors can be optimized for clinical use. Until then, the structural insights obtained from the ELQ library and the D1 clone have the potential to guide drug development efforts for several chemical subseries and advance our understanding of how these compounds interact with *P. falciparum* cyt bc_1.

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