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Investigation of Dual Stage Acridones as a Potent Malaria Treatment

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Investigation of Dual Stage Acridones as a Potent Malaria Treatment

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in
Biology

By
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San Rafael, California
April, 2015

Certification of Approval

I certify that I have read Investigation of Dual Stage Acridones as a Potent Malaria Treatment by Stephanie J. Huezo, and I approved this thesis to be submitted in partial fulfillment of the requirements for the degree: Master of Sciences in Biology at Dominican University of California.

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ABSTRACT

The need for potent antimalarials to prevent the emergence of drug resistant *Plasmodium falciparum* is urgent. Discovery of novel acridone chemotypes have shown promise for a new antimalarial drug treatment. Presently, two acridone chemotypes have intrinsic antimalarial potency against chloroquine sensitive and multidrug resistant parasites. Acridones lacking an N10 side chain are known as chemotype I acridones, whereas, chemotype II acridones are defined as having an alkyl side chain at the same position. The N10 substitution of chemotype II acridones is thought to target heme and inhibit hemozoin formation within the parasite's digestive vacuole, and is known to provide synergistic effects with other antimalarials such as quinine. Currently, the molecular target(s) of chemotype I acridones are unknown; therefore, we sought to identify the target of these acridones. Using the chloroquine resistant *P. falciparum* Dd2 line and the chemotype I compound, T13, we selected stable chemotype I acridone resistance by using multiple rounds of 24 hour intermittent pressure and incremental continuous pressure. Control parasites exhibited an IC_{50} value of ~40 nM T13, while cloned resistant parasite lines showed IC_{50} values of ~1300-1700 nM T13. Cross-resistance was seen with other chemotype I acridones, but not chemotype II acridones, indicating the importance of the N10 substitution in avoiding the resistance mechanism. Slight cross-resistance to atovaquone and ELQ-400 was seen in T13-resistant parasites, indicating that T13 may target the component of the electron transport chain pathway that inhibits the binding of ubiquinone to cytochrome *b*. Two previously reported mutations have been identified in the Q_O site of cytochrome *b*, M133I and V259L, which confer low level (IC_{50} = 242 ± 18 nM T13) and high level (IC_{50} = 1439 ± 49) T13 resistance,

respectively, *in vitro*. A third, novel mutation has been identified in the Q_O site, A138T, which accompanies the M133I mutation, and confers high-level T13 resistance (1678 ± 78). Selection of acridone resistance has led to identification of cytochrome *b* as the main target of chemotype I acridones and the mechanism of action within *P. falciparum*.

DEDICATION

This thesis is dedicated to my mother and father, Diane and Victor, who always believed I could achieve anything I set my mind to. To my sister Melissa, and brothers, Karlo and Eric, who supported me through the good times and bad. I would also like to dedicate this thesis to my grandmother, Nana, my second parents, Tia Laura and Uncle Oscar, my cousins, Ashley, Brittany, and Tiffany, and boyfriend, Shane W. Adams, for their constant love and support. I am truly blessed to have you all in my life. Lastly, I would like to dedicate this thesis to all those that may benefit from reading this work.

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Introduction

Malaria is a worldwide burden. In 2013, the World Health Organization (WHO, 2013) reported 207 million malaria cases and 627 thousand malaria-related deaths. Most cases of malaria occur in sub-Saharan Africa where prevalence of antimalarial drug resistance is extremely high. However, the first reported cases of resistance to newly introduced antimalarials are usually observed in Southeast Asia (Dondorp *et al*., 2009). Due to the emergence of drug resistant parasites to artemisinin (ART) analogs used as monotherapies, in the mid 2000s, the WHO recommended implementation of combination therapies for treatment of malaria. Although malaria mortality rates have dropped 45% since the year 2000, it is still one of the most economically damaging parasitic diseases in the world (Centers for Disease Control and Prevention [CDC], 2012). The high morbidity and mortality rates caused by malaria have contributed to the constant impoverished state of many nations, especially in sub-Saharan Africa (White, 2004).

Malaria results from the infection of host erythrocytes by an intracellular protozoan parasite of the phylum Apicomplexa, genus *Plasmodium*. The most lethal of its five human-infecting species is *Plasmodium falciparum*, which is responsible for the majority of reported clinical cases and deaths; with over half of those cases seen in children under the age of five and pregnant women. The lethal reputation of falciparum malaria is attributed to its ability to induce coma, severe anemia, respiratory distress, and cause cytoadhesion of infected erythrocytes (CDC, 2012; WHO, 2013). *P. falciparum* is also well known to become multi-drug resistant (MDR) (White, 2004). The cause of drug resistance are single nucleotide polymorphisms (SNPs) within genes such as *pfcrt, cytb,*

pfdhfr, the recently discovered *K13,* as well as mutations and alterations in *pfmdr1* gene copy number (Echeverry *et al*., 2007; Korsinczky *et al*., 2000; Peterson *et al*., 1990; Ariey *et al*., 2014; Price *et al*., 2004).

Plasmodium falciparum

Malaria undergoes both a sexual and asexual lifecycle, where two hosts are necessary for successful transmission and replication (Figure 1). Transmitted by the female *Anopheles* mosquito, malaria infects not only humans, but other vertebrates as well. *P. falciparum* infection in humans begins as the mosquito takes her blood meal, injecting sporozoite-containing saliva into the host. Sporozoites travel to the liver where they invade hepatic cells. Within the liver, *Plasmodium spp*. undergo their first round of asexual reproduction through schizogony, resulting in newly formed merozoites capable of infecting erythrocytes.

About 14 days after the asymptomatic liver stage, the merozoites burst from hepatocytes and enter the host bloodstream, invading erythrocytes. Here, they undergo three developmental stages; the ring stage, the metabolically active trophozoite stage, and the schizont stage, where the newly formed merozoites prepare to invade other erythrocytes. At the end of *P. falciparum's* 48-hour intraerythrocytic developmental cycle, the newly formed merozoites (ranging from 8-32) rupture the erythrocyte and enter a new, uninfected erythrocyte. The rupturing of the erythrocyte is responsible for clinical symptoms such as fever and chills (Cowman *et. al*, 2012).

Some of the merozoites that invade erythrocytes develop into macro- and microgametocytes which, when ingested by the *Anopheles* mosquito, undergo sexual

reproduction, allowing the transmission of malaria to persist (CDC, 2012). All antimalarials used clinically, with the exception of liver stage-targeting primaquine, target the intraerythrocytic stage (CDC, 2012).

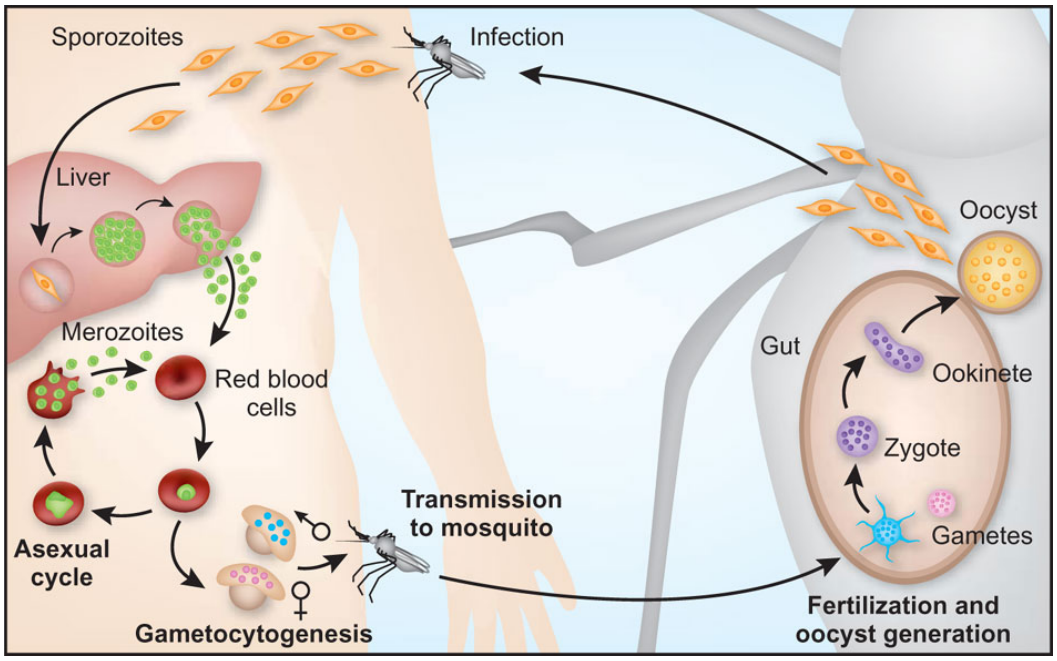


Figure 1: Life cycle of *Plasmodium falciparum*. Image taken from Pasvol, 2010.

Antimalarial Combination Therapy

MDR forms of *P. falciparum* can survive treatment of some of the most widely used and potent antimalarials (Cheeseman *et al.,* 2012). In order to combat MDR malaria, patients are given chemotherapies that combine more than one antimalarial drug. The most commonly used multi-drug treatments for malaria are the ART-based combination therapies (ACTs), which combine a highly potent, short half-life artemisinin analog with a longer acting partner drug to clear malarial parasites (White & Olliaro, 1996). The utilization of two antimalarials allows for a greater likelihood of complete removal of

malaria from the patient (White & Olliaro, 1996). Although this method of malaria treatment has proven highly effective, there is a constant concern with the possibility of if and when parasites become resistant to the combination therapies used. In combination therapy, the shorter acting, highly potent antimalarial rapidly kills the majority of parasites, while the second antimalarial, with a longer half-life, acts upon the last surviving parasites after cessation of treatment (Figure 2; Nosten & White, 2007). However, there are cases where parasites may recrudesce after treatment has been administered, due to the ability of surviving the low levels of the second drug in the patient. Another concern regarding the low levels of drug is the instance of new infections occurring during this period of drug treatment, allowing newly infecting parasites to survive and adapt to the drug at tolerable levels (White, 2004). To maintain the effectiveness of combination therapy and malaria treatment, as well as possibly circumvent the phenomenon of drug resistance, identification of new and potent antimalarials are needed.

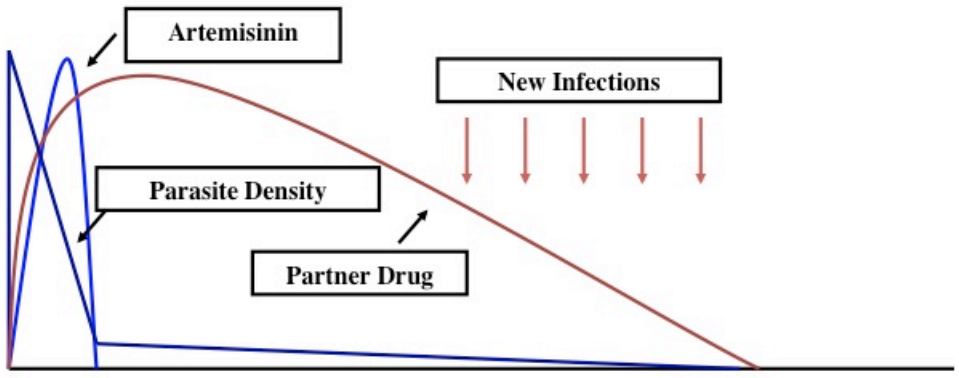


Figure 2: Proposed mechanism of ACTs and parasite clearance. ART serves to kill the majority of malarial parasites while the partner drug serves to protect ART as well as to kill the remaining parasites. Image adapted with permission from Philip J. Rosenthal (UCSF).

pfcrt *and* pfmdr*1: Markers of Resistance*

In the late 1940's, chloroquine (CQ) was the first-line antimalarial and cheapest treatment available for uncomplicated malaria; however, widespread resistance followed in the late 1950s and early 1960s, first arising in the Cambodia-Thailand border and Colombia. In the 1960s, resistance spread across South America and Asia, and by the late 1970s, resistance spread to Africa, which led to the abandonment of CQ as an antimalarial in malaria endemic countries (Figure 3; Payne, 1987; Wellems & Plowe, 2001). The emergence of CQ resistance occurred in several distinct foci over the 30-year span. The long period of time it took for resistance to appear indicates the genetic complexity of resistance and is consistent with the rarity of emergence (Wellems & Plowe, 2001). The wide CQ susceptibility variations of *P. falciparum* resulted in the classification of parasites as "mutant", or CQ-resistant (CQR), and "wild-type", or CQ-sensitive (CQS). CQR is conferred by mutations seen in *pfcrt (Plasmodium falciparum* chloroquine resistance transporter*)*, the gene that encodes for a putative transport protein responsible for the efflux of CQ out of the parasite digestive vacuole (DV) (Cooper *et al.,* 2002; Ecker *et al*., 2012; Fidock *et al.,* 2000; Sidhu *et al.,* 2002). The most studied *pfcrt* SNP in codon 76 results in replacement of lysine (K) with a threonine (T); this mutation is seen in parasites not only resistant to CQ, but other antimalarials as well (Johnson *et al*., 2004; Ecker *et al*., 2012). Multiple polymorphisms in *pfcrt* are needed for a parasite to become CQR; however, only the K76T point mutation has been universally found in CQR lab and field isolates (Cooper *et al.,* 2005). Point mutations in genes such as *pfcrt* and *pfmdr1* can completely change parasite susceptibility to antimalarials (Cooper *et al.,*

2002; Price *et al*., 2004). Polymorphisms within *pfcrt* serve as causal markers of resistance to multiple antimalarials.

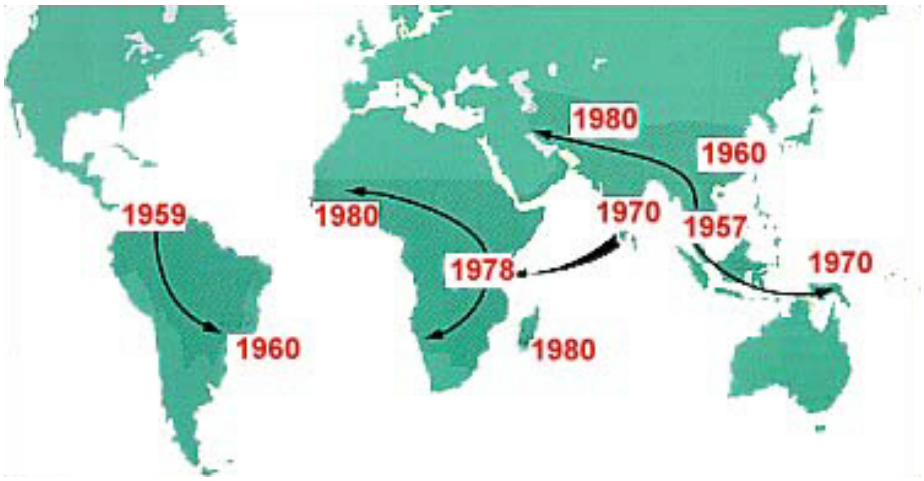


Figure 3: Foci of CQ resistance emerging as independent events in South America and Asia in the late 1950s. Resistance spread from Asia to Africa in the 1970s and by the 1980s it had spread throughout Africa (Wellems & Plowe, 2001).

Initially, evidence suggested that point mutations and amplification of *Plasmodium falciparum* multidrug resistance 1 gene (*pfmdr1*), encoding a P-glycoprotein homolog localized on the parasite's DV membrane, was a key contributor to CQR (Foote *et al*., 1990; Cowman *et al*., 1991). However, a genetic cross, performed by Wellems *et al*. (1990), showed that *pfmdr1* was not responsible for CQ efflux nor was it the key gene responsible for CQ resistance in the progeny. Copy number changes and point mutations in *pfmdr1* have shown to modulate parasite drug susceptibility to multiple antimalarials; however, *pfmdr1* confers resistance to one drug, mefloquine (MEF) (Smrkovski *et al*., 1982; Price *et al*., 2004).

Another antimalarial effective in both CQS and CQR parasites is the quinoline amodiaquine (AQ). AQ is a currently recommended partner drug in ACT treatment of

uncomplicated malaria (Olliaro *et al*., 1996; Rosenthal, 2013). Used as a monotherapy, resistance to AQ is likely and is therefore paired with the artemisinin derivative artesunate. Parasites resistant to CQ are cross-resistant to AQ *in vitro* and *ex vivo* (Olliaro *et al*., 1996; Happi *et al*., 2006). AQ is an analog of CQ and may possibly have the same or similar modes of action; therefore, *pfcrt* and *pfmdr1* mutations seen in CQR parasites resulted in decreased susceptibility to AQ and its metabolite monodesethyl amodiaquine (Sidhu *et al*., 2002; Happi *et al*., 2006; Echeverry *et al*., 2007).

Atovaquone

The only antimalarial currently in clinical use that targets the *P. falciparum* mitochondrial electron transport chain (ETC) is the hydroxynapthoquinone atovaquone (ATQ). Developed in the 1990s to combat malaria, resistance to this drug was rapidly selected when used as a monotherapy (Looareesuwan *et al*., 1996). However, when used in combination with the antimalarial proguanil, it serves as an effective partner drug in prophylaxis and treatment of malaria (Looareesuwan *et al*., 1996; Gassis & Rathod, 1996; Srivastava & Vaidya, 1999; Painter *et al*., 2007). ATQ directly affects the ETC by targeting the cytochrome bc_1 complex (Complex III) and collapsing the mitochondrial membrane potential (Fry & Pudney, 1992; Srivastava, *et al*., 1997, 1999). Korsinczky *et al*. (2000) found a Y268S mutation (A to C mutation at nucleotide 803) and a M133I mutation (nucleotide 399 G to A mutation) in the protein sequence of cytochrome *b* (CYT*b*) that conferred ATQ resistance in *P. falciparum*. The mutations were found within the Q_O site of the cytochrome *b* gene (*cytb*), where ubiquinol is reoxidized to ubiquinone (Figure 4). The Q_O site is a putative binding region for ATQ within *cytb*, found to have a highly conserved motif in amino acid residues around positions 120-150

and 260-280. This also encompasses the well-conserved PEWY motif, which corresponds to a critical binding location of ATQ (Korsinczky *et al*., 2000; Valliéres *et al*., 2013).

The Electron Transport Chain

Within the ETC, ubiquinone, or Coenzyme Q (CoQ), serves as an electron carrier, moving through the ETC complexes while generating a protonmotive force (Figure 4a; Vaidya & Mather, 2009). Of the four complexes (including ATP synthase) present in *Plasmodium spp*., Complex III is the most critical component of the ETC. Here, CoQ is reduced to ubiquinol in the Q_i site and re-oxidized back into CoQ by the Q_O site (Figure 4b; Vaidya & Mather, 2009). The re-oxidized CoQ leaves Complex III via cytochrome *c* (CYT*c*), which serves as the carrier from Complex III to Complex IV. As CoQ travels through Complexes II, III, and IV, five dehydrogenases aid in supplying electrons to the ETC (using CoQ). The most important of these is dihydroorotate dehydrogenase (DHODH). This enzyme catalyzes the conversion of dihydroorotate to orotate, which serves as the only source of the precursor for pyrimidine synthesis. Malaria parasites are unable to salvage pyrimidines; consequently, the role of DHODH is crucial to parasite survival (Painter *et al*., 2007). Without a functional ETC, the parasites die; therefore, the ETC is a highly sought after target in drug discovery and synthesis (Vaidya & Mather, 2009; Barton *et al*., 2010).

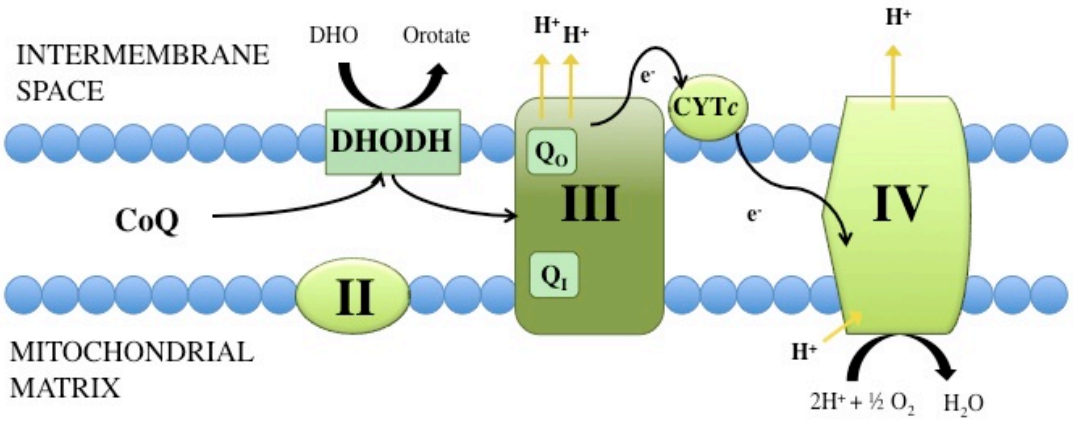
The mitochondrial DNA (mtDNA) of Plasmodium *spp*. is a very short, highly conserved 6 kb long tandemly arranged DNA segment (Vaidya *et al*., 1987, 1989). The mtDNA does not contain introns and encodes only three proteins, the cytochrome oxidase proteins COX1 and COX3, and cytochrome *b* (CYT*b*), as well as some fragmented

rRNA, while the rest of this pathway is encoded by nuclear DNA (Vaidya & Mather, 2009). The *P. falciparum* mitochondria does not contribute much to the ATP pool, as is seen in humans, but serves more as an electron disposal system for DHODH, the fourth enzyme in the ETC (Srivastava *et al*., 1997). This may be due to the ETC lacking a complete ATP synthase complex needed for maintaining the membrane potential.

Although DHODH is essential to *Plasmodium spp*., Painter *et al*. (2007) replaced the ubiquinone-dependent DHODH with a yeast ubiquinone-independent DHODH through transfection. In many bacteria, DHODH is independent of CoQ, utilizing fumarate instead of ubiquinone as an electron acceptor. Painter *et al*. (2007) found that the DHODH transgenic parasites were no longer sensitive to ATQ and that pyrimidine synthesis and membrane potential generation could continue in the presence of CYT*b* inhibitors. The discovery of functional parasites with an independent DHODH and the identification of the critical role of this dehydrogenase for parasite survival indicates that the ETC could essentially cease to exist in *P. falciparum* with an independent DHODH, thus making this enzyme in the ETC an attractive target for drug therapy.

Hydroxynapthoquinones, acridones, acridines, quinolones, and pyridones are five classes of antimalarial compounds proposed to target the ETC and are currently being studied as potential antimalarial treatment and prophylaxis (Kelly *et al*., 2009; Vaidya & Mather, 2009; Barton *et al*., 2010). From this group, we study the antimalarial effects of acridones, which have shown promise as antimalarial treatment for malaria.

a.



b.

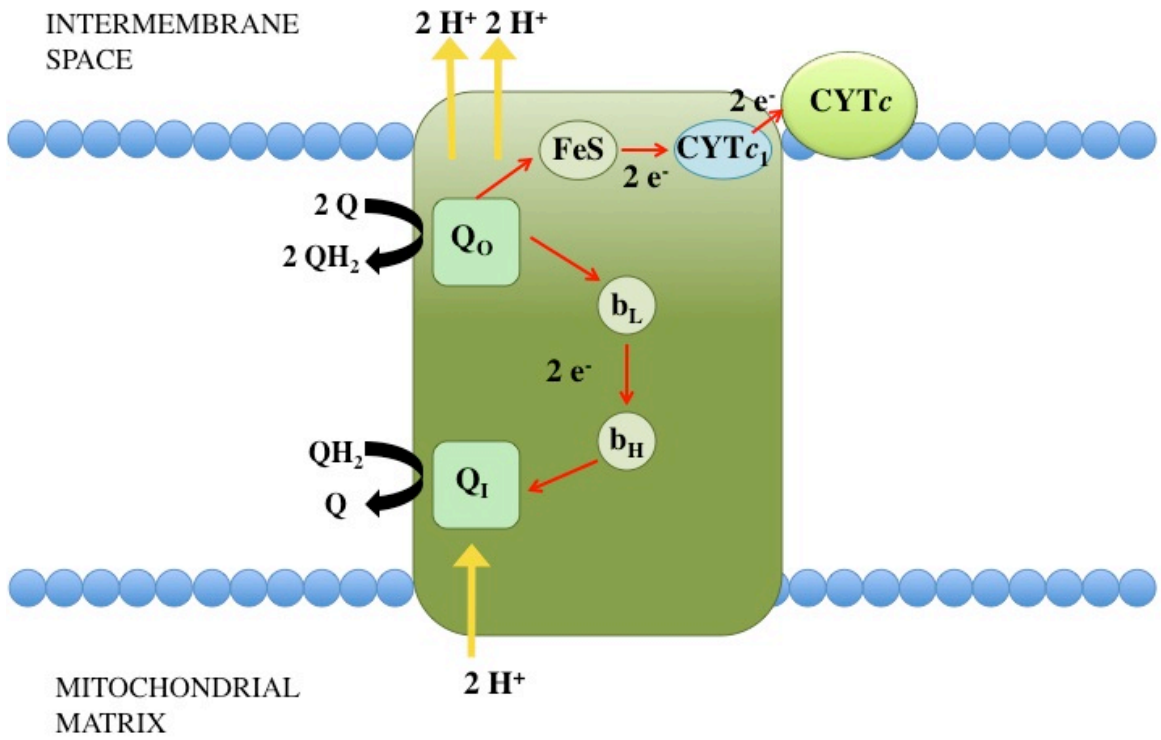


Figure 4: The electron transport chain in *Plasmodium falciparum*. a) Electrons flow from Complex II through IV in the ETC via ubiquinone (CoQ). CoQ reduction occurs by various dehydrogenases embedded within the mitochondrial membrane, specifically, DHODH, which is the essential enzyme in this process. b) Complex III serves to reoxidize ubiquinone as well as transfer electrons to CYT*c*, which results in the transfer of protons across the membrane, thus creating an electrochemical gradient.

Acridones

Acridones are based on the organic compound known as xanthone, which possesses anti-malarial abilities characteristic of its tricyclic structure (Figure 5; Ignatushchenko *et al*., 2000; Kelly *et al*., 2002). Improvements to the xanthone structure by the addition of alkyl side chains with terminal amines at positions 3 and 6 improved heme affinity and inhibition of parasite hemozoin formation. The newly synthesized xanthone derivatives were highly potent against CQS and CQR *P. falciparum* parasites *in vitro*, showing 50% inhibitory concentrations (IC_{50}) in the low nanomolar range (Kelly *et al*., 2002). To make a more potent antimalarial similar to xanthones, Winter *et al*. (2006) synthesized haloalkoxyacridones, which possess the tricyclic xanthone mainframe, but replaced the oxygen with nitrogen at the 10^{th} position (Figures 5-6). Synthesizing haloalkoxyacridones was a fortuitous discovery, as this compound was an intermediate during the process of synthesizing acridones with combined antimalarial ability and resistance-reversal activity that targeted heme. The chemical intermediate found possessed distinctly potent antimalarial activity *in vivo* and *in vitro*, but the drug target was different than the original acridones synthesized (heme targeting) and was therefore termed chemotype I acridones. Chemotype I acridones are dual-stage, broad-spectrum antimalarials shown to be effective against both blood and liver stage malaria, but little is further known about this compound. However, previous reports suggest that chemotype I acridones may inhibit the CYT*b* complex because of the inhibitory effects studied in beef heart mitochondria and the photosynthetic bacteria *Rhodospirillum rubrum* (Oettmeier *et al*., 1994a, 1994b; Winter *et al*., 2006).

In 2007, Kelly *et al*. found that xanthones could be further modified to create a more potent molecule to combat malaria infection. Changing a xanthone to an acridone facilitated the antimalarial potency and supported their aim to combine two drug design strategies; the development of a tricyclic heme targeting molecule possessing antimalarial abilities, as well as a molecule with resistance reversal activity to re-sensitize parasites resistant to quinolines such as CQ. The goal was to synthesize an antimalarial that allows for the reintroduction of CQ and possibly serve as a partner drug in combination therapies.

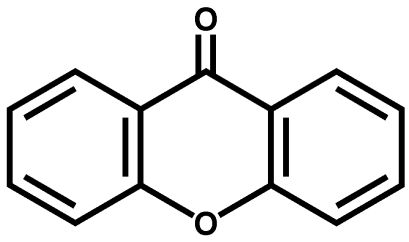


Figure 5: Chemical structure of xanthone.

Further work with acridones by Kelly *et al*. (2007, 2009) found that the addition of an alkyl side chain to the nitrogen at the 10th position on the tricyclic core furthered antimalarial abilities. This addition was key to the acridone resistance reversal activity, or chemosensitization, as well as digestive vacuole targeting (Figure 6). Chemotype II acridones are known to target the parasite digestive vacuole, where the drug can inhibit hemozoin formation (Kelly *et al*., 2007, 2009). Kelly *et al*. (2009) confirmed chemotype II acridones target the DV by finding a decrease in hemozoin production when the parasites were exposed to a chemotype II analog both *in vivo* and *in vitro*, as well as by confocal microscopy, revealing the accumulation of chemotype II acridones in the DV. Chemotype II acridones are also synergistic with quinolines such as CQ, piperaquine

(PIP) and quinine (QN), and are also seen effective in CQR parasites. Altogether, intrinsic antimalarial abilities and chemosensitization, created by the N10 side chain, formed a highly potent, dual-functioning acridone.

Acridones were previously studied for their abilities to treat multiple diseases such as cancer, viral infections, and multiple parasitic diseases (Fujioka *et al*., 1989; Winter *et al*., 2006). Acridones may represent a new, highly potent antimalarial, more potent than quinolines and ATQ (Winter *et al*., 2006; Kelly *et al*. 2007, 2009), which targets a critical process within *P. falciparum*. Introduction of acridones into the clinical setting may result in the reintroduction and sustained effectiveness of old and new quinoline antimalarials.

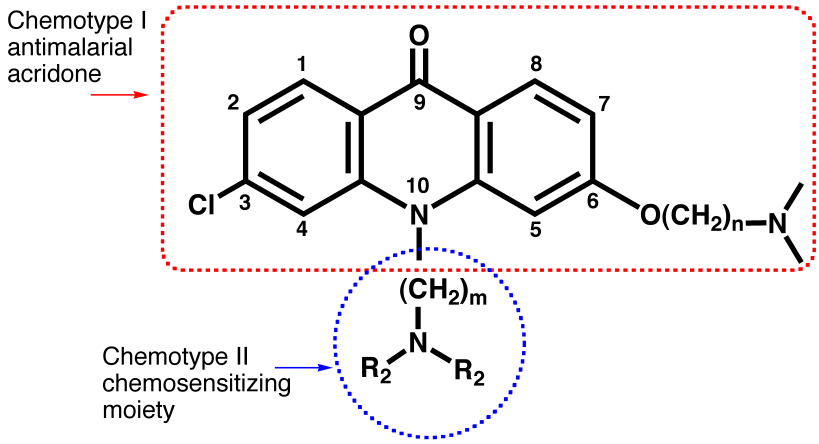


Figure 6: General chemical structure of antimalarial acridones. Dual-stage chemotype I acridones, outlined in red, have nitrogen at the 10th position of the tricyclic mainframe and a side chain at the 6th position for drug uptake and stability. An electron withdrawing substituent (chlorine) is placed at the 3rd position. In chemotype II acridones, the N-10 position has a side chain that allows for acid-trapping, chemosensitization, and targeting the DV.

Hypothesis and Rationale:

Proper functioning of *P. falciparum's* ETC to synthesize pyrimidines and create a membrane potential is essential for parasite survival (Srivastava *et al*., 1997; Painter *et al*., 2007). Halting this process through the inhibition of *cytb* gene results in the collapse of the electrochemical proton gradient. Acridones are thought to target CYT*b*, thus resulting in parasite death; nevertheless, the acridone target has not been demonstrated in *P. falciparum* (Oettmeier *et al*., 1994b; Winter *et al*., 2006).

The goal of our study was to identify molecular targets of chemotype I acridones in *P. falciparum*. We hypothesized that selection of acridone resistant parasites *in vitro* would allow us to identify these molecular target(s). To test our hypothesis, we selected drug resistant *P. falciparum* parasites to chemotype I acridones, investigated the structure activity relationship (SAR) of chemotype I and II acridones, verified the gene target of chemotype I acridones, and determined the relative cost-of-fitness of acridone resistance in parasites.

Methods and Materials

Culturing

P. falciparum was cultured by modified methods of Trager and Jensen (1976) in either AB^+ or O^+ human red blood cells (BioChemed Services; Winchester, VA) in complete medium consisting of RPMI 1640 (Cellgro; Manassas, VA), 0.5% Albumax I (Life Technologies; Carlsbad, CA), 29.8 mM sodium bicarbonate, 25 mM HEPES, 0.37 mM hypoxanthine, and 0.01 mg/ml, and gentamicin sulfate (MP Biomedicals; Solon, OH). Packed red blood cells underwent differential centrifugation and were washed three times with RPMI to remove the buffy coat. Cultures were maintained at 37°C under a gas atmosphere of 5% O_2, 5% CO_2, and 90% N_2. Parasite cultures were monitored by light microscopy at 100x oil with Giemsa-stained (Sigma-Aldrich; St. Louis, MO) thin blood smears. All parasite strains used in the selection assays, unless otherwise stated, had no previous exposure to T13. CQR *P. falciparum* lab strain Dd2 was provided by Dr. Thomas Wellems (NIAID, Rockville, MD).

Selection of T13 Resistant P. falciparum *Parasites*

(a) 24-h On-off Pressure:

Parasites of the CQR Dd2 line, standardized to 2% parasitemia and 5% hematocrit, were exposed to sequential 24-hour exposures to T13, with increasing drug concentration increments at each 24-hour selection. Drug-pressured parasites were maintained in either a 50 mL or a 25 mL culture volume. The 24-hour on-off selections were performed with 3 independent cultures. A Dd2 control culture was maintained

parallel to the drug exposed cultures for the duration of the selections. At 2% parasitemia, Dd2 was exposed to an initial dose of 100 nM T13 for a 24-hour period. At 24-hour exposure, cultures were washed free of drug with pre-warmed RPMI and diluted 90% with fresh erythrocytes and complete media. All cultures were checked 48 hours after drug removal to observe parasite growth. Parasite recovery time was scored as time to ≥ 2% parasitemia following T13 removal. Once recovered, parasites were exposed to stepwise increases in T13 concentration by 100 nM, which continued throughout the selection process (Figure 7).

(b) Continuous Pressure:

Dd2 parasites exposed up to 1400 nM T13 in on-off selections that remained T13 sensitive were subjected to continuous drug pressure, initially at 10 nM T13, maintained at 5% hematocrit and initially 1% parasitemia. After observation of healthy parasite growth (recovery to 1% parasitemia) for at least three 48-hour life cycles, T13 concentration was increased in 10 nM increments until 100 nM T13, at which point the concentration was increased by 100 nM increments until 2200 nM T13, followed by 200 nM T13 increments until the culture was ended (Figure 7). Throughout the selection, drug susceptibility tests were performed to monitor for changes in T13 susceptibility. T13-resistant parasites that emerged from drug pressure were cloned according to methods by Rosario *et al*. (1981) and placed into drug susceptibility assays. Genomic DNA from resistant and control parasites was extracted and purified with either the QIAamp DNA extraction kit (QIAGEN; Redwood City, CA) or ZYMO Quick gDNA Miniprep kit (ZYMO Research; Irvine, CA).

The continuous selection process was also performed on parasites that did not undergo previous 24-hour selection. Wild-type Dd2 parasites were continuously pressured for 14 drug cycles, with drug concentrations from 10 nM T13 to 100 nM T13 (10 cycles), increasing by 10 nM increments. Concentrations of T13 following 100 nM were increased by 100 nM increments (4 cycles). An aliquot was taken for cloning when resistance was observed, allowing the parasites to continue drug pressure.

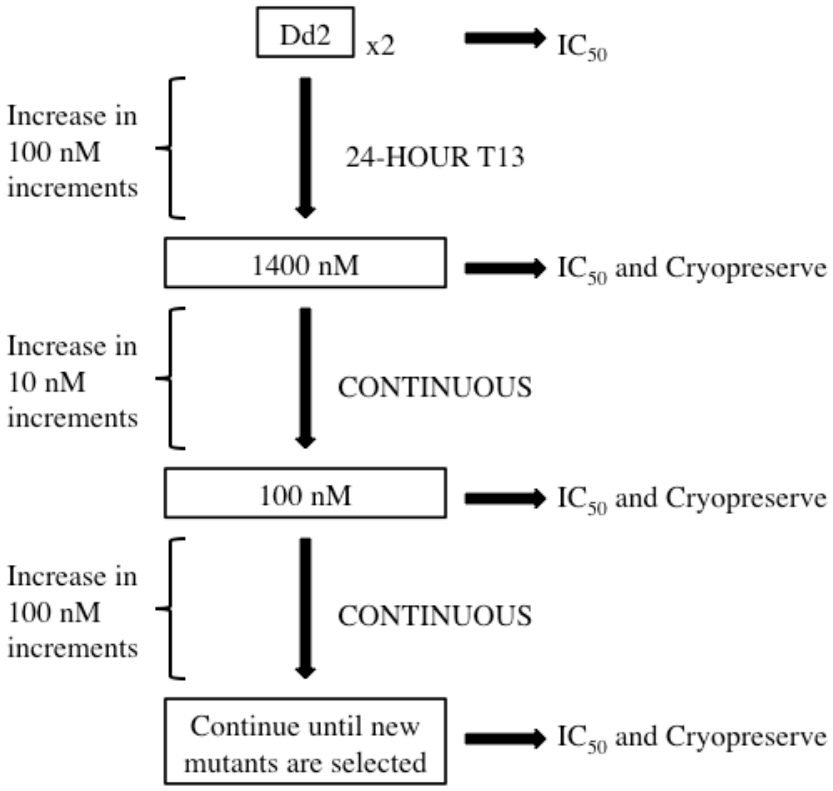


Figure 7: Stepwise selection of T13-resistant parasites by 24-hour on-off pressure and continuous pressure with chemotype I acridone T13. Parasites were pressured incrementally with T13, starting at 100 nM and increasing every 100 nM until 1400 nM T13, afterwhich continuous pressure followed starting at 10 nM. Parasites were assessed by IC_{50} and aliquots were taken for cryopreservation throughout the selection process.

Drug Susceptibility Testing by in vivo *and* ex vivo *Growth Inhibition (IC_{50}) Assays*

Drug stocks were prepared at 10 mM in DMSO, sterile culture grade water, or 70% ethanol. CQ, ATQ, PIP, MEF, QN, quinacrine (QC), dihydroartemisinin (DHA) and lumefantrine (LUM) were purchased from Sigma Aldrich Chemical Co. Chemotype I acridones; T2, T2.2, T13, T13M, T14, T17, T19, T20, T22, T26, T27, T31, T35, and T36 and chemotype II acridones T16.5 and T26.5 were kindly provided by Dr. Jane X. Kelly (Portland State University, Portland OR) and endochin-like quinolones (ELQ) 300 and 400 were kindly provided by Dr. Michael Riscoe (OHSU, Portland OR).

Drugs were serially diluted in complete media 2-fold across wells 1-10 of a Costar® 96 well flat bottom microplate. The last two wells of each row served as drug-free controls. The initial drug concentration was intended to center the IC_{50} in the drug assay plate. Prior to introduction into the IC_{50} assay, culture parasitemia was determined by Giemsa-stained thin smears and light microscopy from a count of at least 1000 red blood cells. Cultures were used in the assay if the parasites were determined to be at least 60% ring stage. All wells within the IC_{50} assay plate contained 150 µL of parasite culture and 50 µL of drug-containing or drug-free media for a final composition of 0.2% parasitemia and 2% hematocrit. Plates were placed in a modular chamber and maintained under blood gas atmosphere of 5% O_2, 5% CO_2, and 90% N_2 at 37°C, for 72 hours. For *in vitro* IC_{50} assays, this method was repeated every 48 hours for at least three independent assays, unless otherwise stated.

IC_{50} values for standard *in vitro* cultures was collected using the SYBR Green I assay based on previously published methods by Smilkstein *et al*. (2004). At 72 h (± 2

hours), plates were completely frozen at -20°C and thawed immediately prior to incubation with SYBR Green I®. 100 µL of the cell lysate from each well was transferred to a Costar® 96 well black flat bottom plate, which already contained an equal volume (100 µL/well) of SYBR Green lysis buffer (20 mM Tris buffer, 5 mM EDTA, 0.008% Saponin, 0.08% Triton X-100, 0.2 µL/mL SYBR Green) at pH 7.4-8.0. The lysate-buffer mixture was mixed well under low light conditions and incubated at room temperature in the dark for a 1-hour period. Measurement of fluorescence was performed with a FLUOstar Omega plate reader (BMG LabTech Inc; Cary, NC) with an excitation wavelength set at 485 nm and emission wavelength at 530 nm. Dose-response data was fit to a non-linear sigmoidal regression function allowing for a variable slope (GraphPad Prism 5.0) to estimate mean IC_{50} values ± standard error of the mean (S.E.M.), which was based on three independent assays. For statistical comparisons, One-way analysis of variance (ANOVA) with a Dunnett's Multiple Comparision test was performed on all T13 resistant parasites and Dd2 control. All plates were processed within a week after being placed into the freezer.

For *ex vivo* studies, *P. falciparum* samples were collected from children enrolled in a longitudinal cohort study for malaria chemoprevention in Tororo, Uganda. Details of the study can be found elsewhere (Wanzira *et al*., 2014). Parasites were placed into a single *ex vivo* drug assay (0.05% parasitemia and 2% hematocrit) within 24 hours after removal of parasitized blood from patients and refrigeration at 4°C. The IC_{50} values were collected for CQ and chemotype I compounds T13, T31, and T35. Growth inhibition was measured using a histidine-rich enzyme protein 2 (HRP2) based ELISA (Noedl *et al*., 2006). Plates used in the ELISA were pre-coated with a primary antibody against

PfHRP2. The lysed *P. falciparum* samples were added to the pre-coated plates, incubated and washed, and then incubated with the secondary antibody (antibody conjugate of IgG1 isotype). 3, 3', 5, 5'-tetramethylbenzidine chromogen (Thermo Scientific; Waltham, MA) substrate was added to each well followed by the stopping solution (10% sulfuric acid) 5-10 minutes later. Absorbance values were collected with a microplate spectrophotometer at 450 nm and data was plotted and analyzed with GraphPad Prism 5.0f to obtain IC_{50} values. All values were based off a single assay plate per sample. Only data with complete sigmodial curves were included in the dataset.

Whole Genome Sequencing

Whole genome sequencing of Dd2 control and T13-resistant parasite DNA was performed at the University of Notre Dame by Dr. John C. Tan. The sequencing process was as follows; 1 mg gDNA was used to generate libraries with the Illumina TruSeq DNA library prep kit following manufacturer's instructions. DNA libraries were sequenced on an Illumina MiSeq using 250 base pair paired-end reads with a 2% PhiX spike-in. The PhiX spike-in served as the sequencing control when libraries were generated. Reads were trimmed to remove adapter sequences using Cutadapt v1.2.1 prior to read mapping. Reads were aligned to the *P. falciparum* PlasmoDB v9.3 3D7 reference genome using the bwa-mem algorithm of bwa v0.7.5a-r405. varScan v2.3.2 was used to identify candidate SNP loci with ≥ 8x read coverage with ≥ 87.5% of the reads supporting the SNP genotype.

cytb *Gene Sequencing*

DNA extraction with the QIAamp DNA extraction kit or ZYMO Quick gDNA Miniprep kit and direct sequencing of the *cytb* gene was performed on cloned T13 resistant parasites selected during drug selections 2-4. PCR was performed with primer 1: 5'-TTCCTGATTATCCAGACGCT-3' and primer 2: 5'-TGTTCCGCTCAATACTCA GA-3' (Appendix VI), which were designed to amplify the flanking regions of the *cytb* gene. PCR cleanup was performed by enzymatic cleanup with ExoSAP-IT (Affymetrix; Santa Clara, CA) or by 1.2% agarose gel electrophoresis, ran at 90V for 50 minutes, and QIAquick PCR purification kit (QIAGEN). Internal primer 5'-CACTCACAGTATATCCTCCACA-3' was paired with primer 1 and a second internal primer 5'-GAGTTATTGGGGTGCAA CTG-3' was paired with primer 2 for direct Sanger sequencing (performed by Genewiz, South Plainfield, NJ). PCR conditions were performed starting at 95°C for 2 minutes followed by 30 cycles of denaturing at 95°C for 30 seconds, annealing at 42°C for 1 minute, and extension at 65°C for 2 minutes. Sequences were assembled and aligned with MacVector 9.5.2.

Determination of pfmdr1 *Copy Number*

Copy number of *pfmdr1* was assessed by quantitative real time PCR with a Life Technologies ViiA7 Real Time PCR System and TaqMan Fast Advanced Mastermix following previously published methods (Price *et al*., 2004). Reactions were performed as a multiplex PCR in a MicroAmp 48 or 96 well plate (Life Technologies; Carlsbad, CA). Calibration of *pfmdr*1 copy number was performed with reference strains Dd2, (3 copies) (Patel *et al*., 2010), and CQS 3D7 (1 copy) (Appendix II) (Happi *et al*., 2009; Ferreria *et*

al., 2006). The *pfmdr1* probe was FAM labeled at the 5' end and β-tubulin was VIC labeled (Appendix V). TAMRA served as the 3' end quencher. The TaqMan Fast Advanced Mastermix (Life Technologies; Carlsbad, CA) contained ROX as the passive reference dye. Reactions were performed in quadruplicate and assays were repeated if data did not conform to exponential kinetics. Data was excluded if threshold values (C_T) were greater than 36 or if copy number was between 1.3-1.6. Quadruplicate samples were run three times for further validation and accuracy.

Statistical analyses were conducted using GraphPad Prism 5.0f for Mac. The distribution of *pfmdr1* gene copy number data was found to be non-normal. Data were log transformed for determination of significance between control and T13 resistant clones by ANOVA using Bartlett's test of equal variances to evaluate homogeneity of variance between groups. Bonferroni Multiple Comparison post hoc tests were performed to compare all groups. All tests were conducted using a significance level of 0.05.

Measurement of Parasite Growth Dynamics

Dd2-T13 mutant clone and Dd2 control parasite were co-cultured in drug-free media to observe the relative fitness of T13 resistant parasites compared to controls (Figure 8). Parasites were placed into flasks containing either a 9:1, 1:1, or 1:9 mixture of both control and mutant parasites at an initial, total parasitemia of 0.5% (0.25% mutant and 0.25% control for 1:1, 0.05% and 0.45% for 9:1 and 1:9). Control and mutant parasites were also maintained in separate control cultures. Cultures were synchronized using 5% D-sorbitol 48 hours prior to beginning the assay, and a 1500-2000-cell count was performed before cultures were mixed. Cultures were maintained in 25 mL volumes

at 5% hematocrit and 1-2% parasitemia for ~10 erythrocytic life cycles (20 days). Every 24 hours (for 1:1 mixtures) and 96 hours, 3 mL aliquots were taken from each culture flask, treated with 0.01% saponin and PBS to lyse red blood cells, centrifuged at 3000 rpm for 7 minutes and washed with PBS. The pellet was stored at -20°C. DNA was isolated as described above with a ZYMO DNA kit.

cytb allele-specific qPCR was used to determine the relative abundance of the control *vs*. mutant parasites. The samples were run in triplicate on a ViiA7 Real Time PCR System with TaqMan Fast Advanced Mastermix and TaqMan MGB probes (Applied Biosystems; Carlsbad, CA) to observe which parasite predominated in each culture by using primers targeting wild-type or mutant *cytb*. VIC and FAM labeled probes allow for distinction between the mutant *cytb* probe and control *cytb* probe (Appendix VII). Primers used in this assay were common primers; forward (5' TCACATCCTGATAATGCTATCGTAGT 3') and reverse (5' GCTGGTTTACTT GGAACAGTTTTTAA 3') while the probes were allele specific. MGB-conjugated probes were used to target the wild-type *cytb* sequence (VIC-CATCTCAAATTGTA CCTGAA-MGB) or the mutated sequence (FAM-TACTCCATCTCAAATTTTACCT-MGB). Standard curves were generated at 1:0, 9:1, 7:3, 1:1, 3:7, 1:9, and 0:1 ratios of wild-type:mutant for determination of primer and probe specificity. All 1:1 mixed cultures were run in duplicate in three independent competition assays and each DNA sample collected was ran in triplicate with the SNP genotyping assay. 9:1 and 1:9 cultures underwent an independent competition assay and each DNA sample was run in triplicate with the SNP assay.

Percentages of parasite composition (wild-type *vs*. Dd2-T13^{V259L}) per day were determined by ΔRn mean values. Values were corrected using Dd2 (FAM) or Dd2-T13^{V259L} (VIC) control's ΔRn mean value, followed by dividing each mean (1:1, 9:1, and 1:9) ΔRn value by the corrected control value to obtain the percentage.

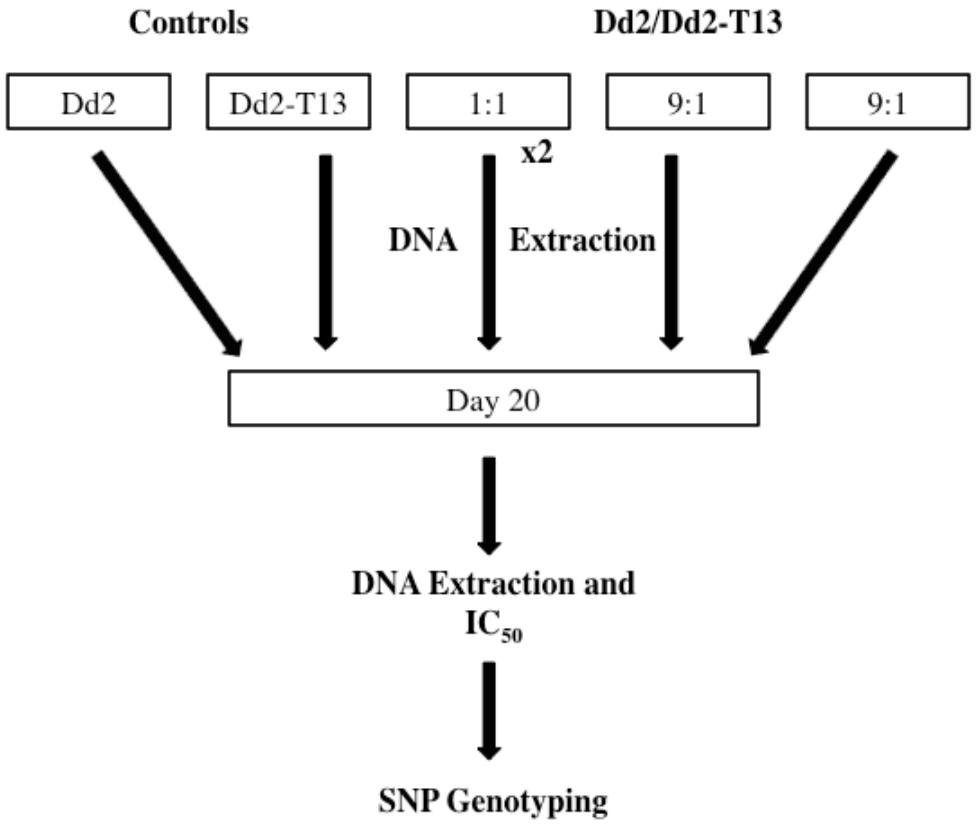


Figure 8: Competition assay procedure. Dd2:Dd2-T13 flasks were set to 9:1, 1:1, and 1:9 mixtures. DNA extraction was performed every two days or four days as well as on the final day (Day 20). SNP genotyping was performed with all DNA collected during the 20 day period. At the end of the 20-day period, all cultures were placed into a single IC_{50} assay and cryopreserved.

Results

Generation of in vitro *Acridone Resistance in* P. falciparum

Previous studies performed to select drug resistant *P. falciparum* have utilized single step selection or incremental drug pressure (Cooper *et al*., 2002; Korsinczky *et al*., 2000; Witkowski *et al*, 2009). To investigate the potential for resistance and identify mechanisms of resistance in *P. falciparum* to chemotype I acridones, we utilized an *in vitro* on-off selection technique to select parasites resistant to the chemotype I analog T13. T13 resistance was selected in the MDR laboratory strain Dd2; Dd2 is resistant to CQ, QN and AQ and has a proclivity for drug resistance, but is sensitive to T13 (IC_{50} = 40 ± 5.5 nM).

In the first selection experiment, 24-hour on-off T13 pressure continued for 81 days and 14 drug pressure cycles, up to 1400 nM T13, with no resistance detected. The culture was split into two flasks, where one continued to undergo 24-hour exposure cycles while the other culture was introduced to continuous pressure starting at 10 nM T13. The on-off selection continued for another 177 days, at which time T13 concentration had increased to 3.8 μM (~190-fold higher than Dd2 control IC_{50}); however, there was no increase in the T13 IC_{50} value compared to controls, resulting in termination of the culture at this concentration. In contrast, after 65 days (7 drug cycles; 70 nM T13), resistance was detected in the line that was split off for continuous T13 pressure. IC_{50} tests demonstrated a 37-fold shift in IC_{50}, from 40 ± 5.5 nM T13 in controls to 1483 ± 73 nM in the resistant line (designated Dd2-T13a). The IC_{50} results were verified with three independent assays after the parasites were pressured at 90 nM T13

(Table 1). Seven independent clonal lines were derived from the resistant culture by limiting dilution (Rosario, 1981). IC_{50} assays were performed on three randomly chosen clonal lines D1, F1, and H12. All three clones exhibited ~33-39-fold decrease in susceptibility to T13 with IC_{50} values of 1548 ± 26 nM, 1324 ± 49 nM, and 1370 ± 47 nM, respectively (N = 3, $p < 0.05$ Dd2-T13 *vs*. control).

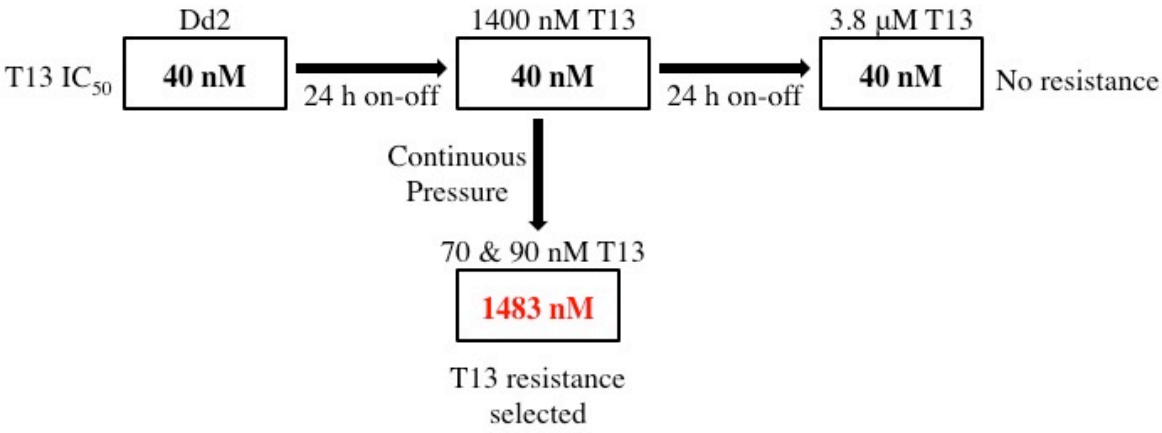


Figure 9: Schematic of selection of T13-resistant *P. falciparum*. The CQR lab strain Dd2 was used in selection experiments. Wild-type Dd2 underwent 24-hour (h) on-off pressure followed by both continuous pressure and on-off pressure at 1400 nM T13. Values in boxes indicate T13 IC_{50} measured from parasites under pressure at the T13 concentration indicated above the value.

To attempt selection of additional parasites resistant to T13, we repeated the on-off experiment with two independent wild-type Dd2 cultures (Dd2-T13b and Dd2-T13c). Both Dd2 cultures tested resistant by IC_{50} after parasites recovered from 1300 nM, 24-hour T13 pressure, with T13 IC_{50} values of 1407 nM and 796 nM (N=1) (Table 1). However, subsequent cloning resulted in mixed sensitive and resistant parasites. Therefore, to kill remaining wild-type parasites, both cultures underwent continuous T13 pressure from 10 to 20 nM T13 over a period of approximately 3 weeks. Two subsequent

clonal lines from Dd2-T13b and Dd2-T13c (clones H9 and D3) had T13 IC_{50} values of 1578 ± 79 nM and 1528 ± 70 nM (N = 3; $p < 0.0001$), respectively (Table 3).

The days to recovery after each 24-hour drugging cycle in the three independent cultures were examined for an association with T13 dose. Following drug washout, the T13-pressured Dd2 cultures grew similar to controls (parasitemia ≥ 2% within 48 hours) throughout the selection experiment, including T13 doses ~95-fold greater than the wild-type Dd2 IC_{50}. The average days of on-off recovery for Dd2-T13a, pressured up to 3.8 μM T13, was 2.4 days (Figure 10). Dd2-T13a parasites recovered at an average rate of 1.3 days after 24-hour pressure from 100 nM to 600 nM T13. Parasite recovery declined slightly during T13 pressure from 700 to 2800 nM, averaging 2.8 days. 24-hour pressure from 2800 nM to 3800 nM T13 had a recovery rate of 1.6 days, similar to the initial recovery time (data not shown). During on-off pressure in Dd2-T13b and Dd2-T13c, average recovery rates up to 1400 nM T13 (35-fold greater than Dd2 IC_{50}) were 2.8 and 2.9 days, respectively (Figure 10). Bivariate correlation analyses indicated there was no association between growth pattern and drug concentration prior to selection of T13-resistance, suggesting that the parasites remaining in the culture after drug removal and 90% cut did not die, but instead, recovered.

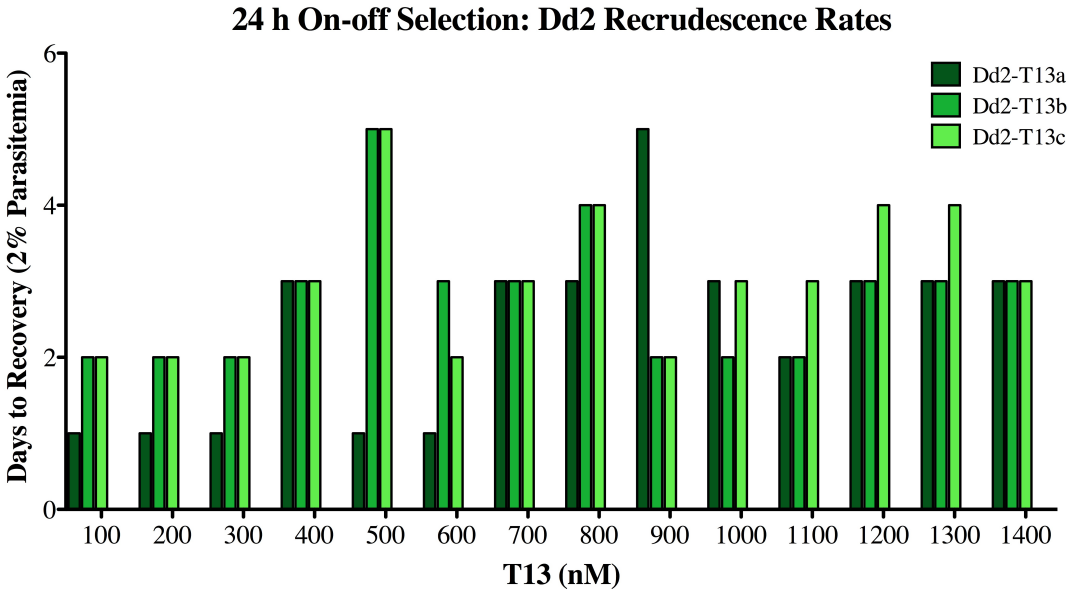


Figure 10: Dd2 recrudescence rates during 24-hour on-off selection. Days to recovery from 100 nM to 1400 nM T13 for three independent Dd2 cultures that underwent 24-hour on-off pressure. All days to recovery values are based on the time it took the culture to reach at least 2% parasitemia after drug removal. Each color represents a Dd2 culture.

To determine the stability of the T13 resistance phenotype in long-term culture, we cultured clone Dd2-T13^{H12} in the absence of T13 pressure for 82 days. T13 resistant parasites grew at a rate comparable to controls during the period without drug exposure, and remained T13 resistant at the end of the experiment when tested by IC_{50} assay (Dd2-T13^{H12} IC_{50} = 900 nM T13, N = 1).

We next investigated if T13 resistance could be selected with only continuous pressure. A Dd2 culture with no prior exposure to T13 was maintained under continuous, increasing T13 pressure for 144 days. This experiment generated parasites with a moderate level of resistance compared to earlier resistant lines, with an IC_{50} of 290 ± 17 nM (~ 6-fold increase relative to parent line, $p < 0.05$) following 136 days of pressure and drug increased to 500 nM T13 for 18 days (Table 1). Cloning of the T13-resistant culture

(Dd2-T13d) resulted in recovery of five clonal lines, with IC_{50} values of 257 nM-337 nM T13. Continued drug pressure on Dd2-T13d for 39 days further decreased parasite susceptibility, with T13 resistant parasites emerging at 900 nM T13 pressure exhibiting a T13 IC_{50} of 1065 nM (N = 1), similar to T13 resistant parasites selected earlier (Table 1).

Table 1: *In vitro* antimalarial response of pre-clonal T13 resistant *P. falciparum* cultures Dd2-T13a-d.

Drug	Parasite line (IC_{50} in nM)					
	Dd2	Dd2-T13a	Dd2-T13b	Dd2-T13c	Dd2-T13d	
					500 nM	900 nM
CQ	217 ± 10	208 ± 13	285[a]	309[a]	254 ± 8.6	191[a]
T13	40 ± 5.5	1483 ± 73****	1407	796	290 ± 17 **	1065
ATQ	0.50 ± 0.07	------	3.4	1.7	5.3 ± 0.14***	1.2

IC_{50} values ± SEM in nM. N = 3. 72 h microplate test using Sybr Green detection.

[a] N = 1 for values without SEM.

------ IC_{50} was not taken with the specified drug during this time.

Two-tailed t-test of resistant *vs*. Dd2 control where $p < 0.0001$ [****]

Two-tailed t-test of resistant *vs*. Dd2 control where $p < 0.001$ [***]

Two-tailed t-test of resistant *vs*. Dd2 control where $p < 0.01$ [**]

Whole Genome Sequencing for SNP Identification

The genomes of two Dd2 control clones, $Dd2^{H5}$ and $Dd2^{G4}$, as well as two Dd2-T13 resistant clones, Dd2-$T13a^{F1}$ and Dd2-$T13a^{H12}$, were sequenced by the Illumina MiSeq to identify putative single nucleotide polymorphisms (SNPs) associated with T13 resistance. 15 nonsynonymous SNPs were identified in the coding regions of the Dd2-T13a parasites genome (Table 2). We have identified a SNP from a G to T amino acid change at position 775 in *cytb* codon 259 that confers a valine (V) to leucine (L) change (Figure 11a) in both clonal lines, relative to controls. These results support previous observations of acridones targeting CYT*b* (Oettmeier, 1994b), and therefore were considered the most likely cause of T13 resistance in the selected parasite lines.

Protein sequences from six *Plasmodium spp*. and three other eukaryotic organisms (Toxoplasma, human and chicken) were aligned and compared to the mutant clones Dd2-$T13a^{F1}$ and Dd2-$T13a^{H12}$. The aligned sequences have a high degree of conservation between all species, especially in the 5 different species of *Plasmodia*. The V259L mutation in CYT*b* was in the highly conserved region of the Q_O site, specifically next to the "pewy" loop, which is conserved across eukaryotic species (Figure 11a; Vaidya & Mather, 2009).

Table 2: SNPs identified within *P. falciparum* Dd2-T13a^{F1} and ^{H12} nuclear and mitochondrial genomes.

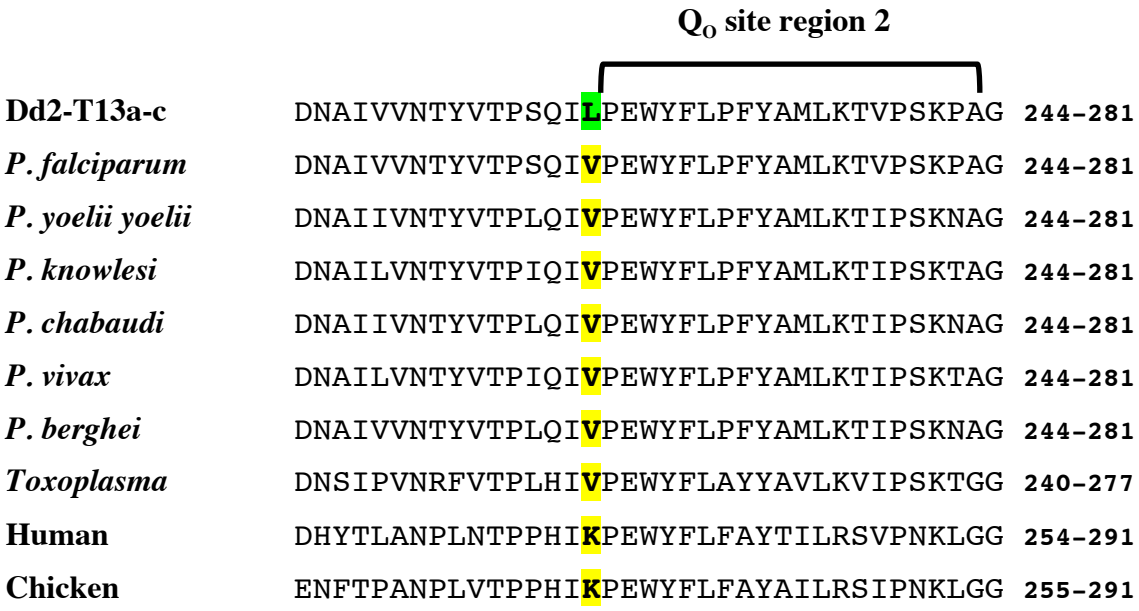
Chromosome	Position	3D7 (Ref)	Dd2	Mutant	Gene	Gene Description
Mitochondrial	4266 (775)	G	G	T	mal_mito_3	apocytochrome b (*cytb*)
6	629354	A	A	G	PF3D7_0615300	Conserved *Plasmodium* membrane protein, unknown function
7	1298098	A	C	A	PF3D7_0730300	transcription factor with AP2 domain(s) (ApiAP2)
7	192738	C	T	C	PF3D7_0704200	tRNA m5C-methyltransferase, putative
7	778333	G	A	G	PF3D7_0718000	dynein heavy chain, putative
8	261401	C	A	C	PF3D7_0804500	Conserved *Plasmodium* membrane protein, unknown function
8	544091	A	A	T	PF3D7_0810600	RNA helicase, putative
11	218599	A	C	A	PF3D7_1104900	Calcium/calmodulin-dependent protein kinase, putative
11	24858	G	C	G	PF3D7_1100100	Erythrocyte membrane protein 1, PfEMP1 (VAR)
11	330499	C	C	T	PF3D7_1107800	Transcription factor with AP2 domain(s) (ApiAP2)
12	1670672	C	T	C	PF3D7_1239800	Conserved *Plasmodium* protein, unknown function
12	2246731	C	C	A	PF3D7_1255200	Erythrocyte membrane protein 1, PfEMP1 (VAR)
13	1259510	T	C	T	PF3D7_1329600	Conserved *Plasmodium* protein, unknown function
14	1225767	T	C	T	PF3D7_1431200	Conserved *Plasmodium* protein, unknown function
14	1319520	G	A	G	PF3D7_1433400	Conserved *Plasmodium* membrane protein, unknown function

Highlighted rows indicate SNP in T13-resistant mutant differs from both the reference strain (3D7) and the parental line (Dd2). Data provided by John C. Tan from University of Notre Dame.

cytb *Sequencing from additional T13 Resistant Parasites*

To ascertain whether a *cytb* mutation was the probable source of T13 resistance in *P. falciparum* parasites, Sanger sequencing of *cytb* was performed with four other independently selected T13 resistant parasites. The samples sequenced were eight Dd2-T13 resistant clones: Dd2-T13b^{A3} and ^{H9}, Dd2-T13c ^{D3} and ^{A2}, Dd2-T13d^{F5} and ^{G9}, all exhibiting IC_{50} values greater than 1300 nM T13, and Dd2-T13d clones E4 and H11, which produced IC_{50}s of 242 ± 17.6 and 216 ± 14.3, respectively. IC_{50} values for each independent mutant clone have been reported in table 3. Sequence alignment with MacVector revealed a common V259L mutation among Dd2-T13a-c parasites with the higher level of T13 resistance. All Dd2-T13d clones (F5, G9, E4 and H11) had an M133I polymorphism in CYT*b* (G to T change at nucleotide 399) while retaining the wild-type V259. The former mutation is known to play a role in ATQ resistance (Figure 11b) (Korsinczky *et al*., 2000). Dd2-T13d clones F5 and G9 had a secondary G to A mutation at nucleotide position 412 (A138T amino acid change), which has not been previously reported.

a)



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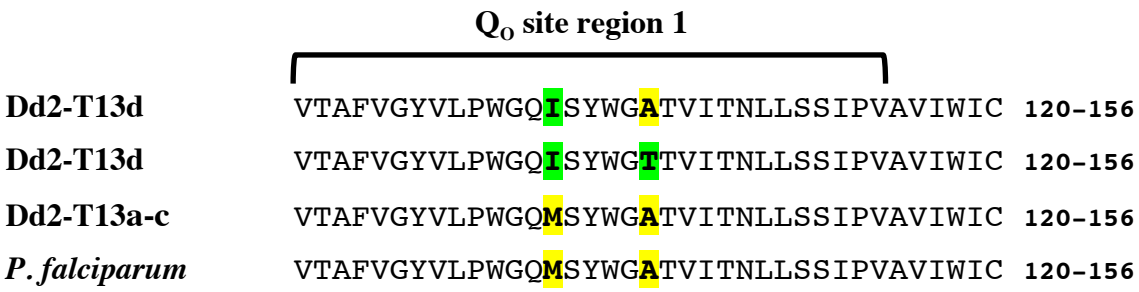


Figure 11: CYT*b* amino acid sequence of Dd2-T13 resistant parasites. **a)** Dd2-T13a-c resistant clones revealed a SNP in *cytb* corresponding to a V259L amino acid change, lying in or near the Q_O site region 2 of the CYT*b* complex (Valliéres *et al*., 2013). This SNP differs from mutations seen in ATQ-resistant parasites (Korsinczky *et al*., 2000; Mather, Henry & Vaidya, 2007). b) Dd2-T13d clones revealed a SNP in *cytb* that corresponds to a M133I mutation, lying within putative Q_O site region 1 of the CYT*b* complex (Valliéres *et al*., 2013). This mutation was previously reported by Korsinczky *et al*. (2000) to play a role in ATQ resistance. The Dd2-T13d clones also revealed a novel A138T mutation accompanying the M133I mutation in Q_O site region 1.The Dd2-T13d clones did not acquire the V259L mutation.

pfmdr1 *Copy Number Variation*

pfmdr1 copy number variation is associated with parasite resistance to multiple antimalarials, such as CQ, PIP, and MEF (Reed *et al*., 2000; Eastman *et al*., 2011; Price *et al*., 2004). Therefore we tested the Dd2-T13^{V259L} mutant parasites for *pfmdr1* copy number variation by qPCR to identify whether gene copy changes were associated with T13 resistance. Three independent analyses for *pfmdr1* copy number with three Dd2-T13a clones (F1, D1, and H12) indicated an increase in *pfmdr1* copy number from 3 to 3.78 (SD = 0.39), 3.76 (SD = 0.42), and 4.0 (SD = 0.23), respectively, when compared to the controls 3D7 (N = 1) and Dd2 (N = 3) (Figure 12).

Bartlett's test of equal variances indicated that copy number did not differ significantly between controls and Dd2-T13 clones ($B = 4.91$, $p = 0.30$) and satisfied the assumption of homogeneity of variance. A one-way ANOVA indicated that there was a statistically significant difference between Dd2 and Dd2-T13 clones *pfmdr1* copy number (F [4, 55] = 517.6; $R^2 = 0.97$; $p < 0.0001$). Bonferroni post hoc tests revealed that Dd2 was significantly less than all three Dd2-T13 clones ($p < 0.05$). The increase in *pfmdr1* copy number that accompanies CYT*b* V259L may be a compensatory adaptation related to parasite fitness (Price *et al*., 2004), or the selection of increased copies was due to a mixed Dd2 population.

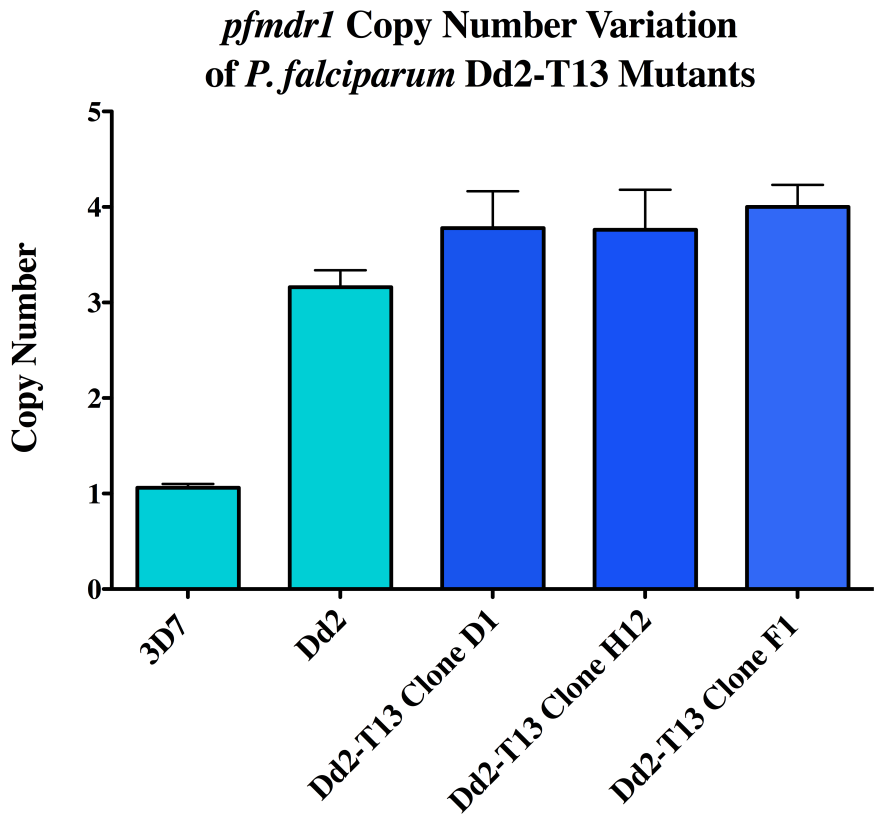


Figure 12: *pfmdr1* copy number variation. Copy number (CN) analysis of reference strain 3D7 (CN = 1.01, SD = 0.04), Dd2 parental (CN = 3.16, SD = 0.18), and Dd2-T13a-resistant clones (CN = 4.0, SD = 0.23; 3.76, SD = 0.42; and 3.78, SD = 0.39). Samples were run in quadruplicate on a Viia7 Real Time PCR instrument (Life Technologies Inc.) for three independent assays. Error bars indicate standard deviation.

Structure Activity Relationships of Acridones in T13-resistant P. falciparum

To determine whether T13 resistance affected parasite susceptibility to other chemotype I acridones, we performed growth inhibition assays on a Dd2-T13^{V259L} clone against a panel of 13 chemotype I compounds. Chemotype I acridone analogs were potent antiplasmodials *in vitro* against control *P. falciparum* line Dd2 (Tables 3 and 4), with IC_{50} values ranging from 0.29 nM to 616 nM, with six out of the 13 chemotype I acridones tested exhibiting IC_{50} values below 100 nM. All chemotype I compounds, except for T2.2, exhibited varying degrees of cross-resistance to T13 in the Dd2-T13^{V259L} parasites, with IC_{50} values 3-fold to 26-fold higher than control values ($p < 0.05$). Conversely, cross-resistance was not observed when Dd2-T13^{V259L} was tested against chemotype II acridones T16.5 and T26.5 (Table 3), indicating the importance of the N10 side chain in prevention of cross-resistance.

Chemotype I acridones T2, T14, T26, and T27 have the same alkoxyamine side chain as T13 at the 7th position of the acridone core (Appendix III). All 4 of these acridone analogs have significantly higher IC_{50} values in Dd2-T13^{V259L} compared to Dd2 from 6-fold to 26-fold higher (Table 3). The Cl substituents at positions 2 and 3 on T14 may be the cause for the lowest fold change in IC_{50}. T19, T20, T31 and T13M have Cl substituents at the 1 and 2 positions on the acridone core, as does T13 (Appendix III). The four analogs had IC_{50} values 2.9-10 fold higher than the control, with the highest shift from 14 ± 1.4 nM to 140 ± 4.5 nM to T31 ($p < 0.0001$). The side chain on T31 is the most structurally similar to T13, which may be the cause for the highest shift in susceptibility from this group. T17 had a 25-fold shift in IC_{50} from 22 ± 1.5 nM to 551 ± 13 nM T17 ($p < 0.0001$), which may result from the Cl substituent at the same position as

seen on T13. These results suggest that most of the chemotype I acridones tested have the same or similar drug target as T13.

Table 3: Structure activity relationship (SAR) of chemotype I and II acridones demonstrate varying degrees of cross-resistance in chemotype I acridones when compared to Dd2 control.

Class	Drug	IC_{50} ± SEM (nM) *P. falciparum*		Index of Resistance
		Dd2	Dd2-T13a^{F1}	
4-aminoquinoline	**CQ**	217 ± 9.9	260 ± 17	1.2
Hydroxynapthoquinone	**ATQ**	0.50 ± 0.043	4.1 ± 0.15	8.2****
Chemotype I Acridone	**T2**	117 ± 12	2122 ± 83	18****
	T2.2	366 ± 14	355 ± 7.9	1.0
	T13	39 ± 1.1	1439 ± 49	37****
	T13M	186 ± 7.3	843 ± 31	4.5****
	T14	212 ± 7.6	1321 ± 58	6.2****
	T17	22 ± 1.5	551 ± 13	25****
	T19	390 ± 15	1103 ± 48	2.8**
	T20	616 ± 17	3113 ± 81	5.1****
	T22	139 ± 3.9	2426[a]	18
	T26	20 ± 1.6	525 ± 20	26****
	T27	31 ± 1.4	668 ± 32	22****
	T31	14 ± 1.4	140 ± 4.5	10****
	T35	9.0 ± 0.98	216 ± 8.2	24****
	T36	0.29 ± 0.030	5.5 ± 0.15	19****
Chemotype II Acridone	**T16.5**	231 ± 5.4	248 ± 7.0	1.1
	T26.5	91 ± 5.5	95 ± 2.2	1.0

*IC_{50} values ± SEM in nM. N = 3. 72 h microplate test using Sybr Green detection.

Refer to Appendix III for structures of antimalarials.

[a] Value based on a single assay.

Two-tailed t-test of mutant *vs*. Dd2 control where $p < 0.0001$ [****]

Two-tailed t-test of mutant *vs*. Dd2 control where $p < 0.01$ [**]

Drug Susceptibility of cytb *Mutants to Other Antimalarials*

To test the susceptibility of Dd2-T13 resistant parasites to mechanistically diverse antimalarials, we screened the parasites with CQ, QN, MEF, LUM, PIP, DHA, QC, ATQ, ELQ-300, and ELQ-400 (Table 4; Appendix IV). The *cytb* mutants IC_{50} values presented no change in susceptibility except to CYT*b* Q_O site targeting antimalarials ATQ and ELQ-400. There was an 8.8-fold shift in ATQ IC_{50} in Dd2-T13^{V259L} mutants (3.8 ± 0.26 nM) relative to Dd2 (0.43 ± 0.038 nM; $p < 0.0001$), 17-fold increase in Dd2-T13^{M133I} mutants (6.9 ± 0.95 nM ATQ; $p < 0.0001$) and a 3.3-fold increase in ATQ IC_{50} to Dd2-T13$^{M133I-?}$ (1.4 ± 0.052 nM, $p < 0.05$). When tested against the Q_O site targeting compound ELQ-400, Dd2-T13^{V259L} mutants exhibited a 10-fold shift in susceptibility (Dd2 3.4 ± 0.11 *vs*. Dd2-T13^{F1} 33 ± 2.2; $p < 0.001$), while the Dd2-T13^{M133I} mutants had a 4.7-fold increase in ELQ-400 IC_{50}. However, the Dd2-T13$^{M133I+A138T}$ mutant had no change in ELQ-400 susceptibility (Table 4). The lack of cross-resistance observed in the CYT*b* Q_I-site targeting ELQ-300 (Stickles *et al*., 2015a) and cross-resistance in Q_O site targeting antimalarials strongly suggest T13 is also a CYT*b* Q_O site targeting antimalarial.

Table 4: Drug susceptibility results of cloned *P. falciparum* Dd2-T13 mutants.

Drug	Dd2	Dd2-T13 Resistant Mutants; IC_{50} ± SEM (nM)		
		$T13^{V259L}$	$T13^{M133I}$	$T13^{M133I+A138T}$
CQ	181 ± 4.3	166 ± 7.1	182 ± 5.6	186 ± 8.0
QN	254 ± 9.8	215 ± 7.5	197 ± 3.5	199 ± 13
MEF	18 ± 1.3	23 ± 0.95	14 ± 0.71	13 ± 0.61
LUM	5.7 ± 0.21	6.5 ± 0.32	3.6 ± 0.19	3.6 ± 0.26
PIP	9.0 ± 0.17	7.1 ± 0.25	10 ± 0.31	7.4 ± 0.27
ATQ	0.43 ± 0.038	3.8 ± 0.26****	6.9 ± 0.95****	1.4 ± 0.052**
T13	40 ± 5.5	1439 ± 49****	242 ± 18**	1678 ± 78****
QC	29 ± 1.3	18 ± 0.74*	23 ± 0.59	29 ± 0.67
ELQ-400	3.4 ± 0.11	33 ± 2.2***	0.72 ± 0.035	3.1 ± 0.14

IC_{50} values ± SEM in nM. N ≥ 3. 72 h Microplate test using Sybr Green detection.

Parasites with the V259L mutation underwent both 24-hour and continuous pressure. IC_{50}s represent values collected from three independent cultures that selected for the V259L mutation in CYT*b*. Parasites with the M133I and M133I+A138T mutation underwent only continuous pressure.

Two-tailed t-test of average IC_{50} of mutant *vs*. Dd2 control where $p < 0.0001$ [****]

Two-tailed t-test of average IC_{50} of mutant *vs*. Dd2 control where $p < 0.001$ [***]

Two-tailed t-test of average IC_{50} of mutant *vs*. Dd2 control where $p < 0.01$ [**]

Two-tailed t-test of average IC_{50} of mutant *vs*. Dd2 control where $p < 0.05$ [*]

ex vivo *Data Indicate High Potency of Chemotype I Acridones*

Drug sensitivity data was collected from 20 *P. falciparum* isolates from Tororo, Uganda against CQ and chemotype I acridones T13, T31, and T35 in 2013. The chemotype I acridones were highly potent against clinical isolates, exhibiting *ex vivo* IC_{50} values similar to *in vitro* values against lab strain Dd2 (40 ± 5.5 nM T13; 14 ± 1.4 nM T31; 9.0 ± 0.98 T35). Table 5 shows IC_{50} values in median and range due to the variability in isolate susceptibility to each antimalarial tested. The maximum IC_{50} values observed in clinical isolates for T13, T31, and T35 were 20-fold, 19-fold, and 9.4-fold lower in IC_{50}, respectively, than for the *in vitro* selected T13 resistant line, Dd2-T13^{V259L} (Tables 3 and 5). From the data collected, no clinical isolate appeared resistant to the chemotype I acridones tested.

Table 5: *ex vivo* data collected from *P. falciparum* Ugandan isolates against CQ and Chemotype I acridones.

***ex vivo* IC_{50}s from *P. falciparum* Ugandan Isolates. Median and Range (nM).**				
Drug	**CQ (n = 17)**	**T13 (n = 20)**	**T31 (n = 20)**	**T35 (n = 18)**
Median	124	28	3.4	6.5
Range	18 – 1152	11 – 72	0.54 – 7.4	2.3 – 23

IC_{50} values; N = 1. 72 h microplate test using HRP2 ELISA (Noedl *et al.,* 2006).

n = number of isolates tested per drug.

Fitness Cost of the V259L Mutation

Acquired mutations in drug resistant *P. falciparum* come at a fitness cost (Rosenthal, 2013). Therefore, we hypothesized that the T13 resistant parasites with the CYT*b* V259L mutation would incur a fitness cost relative to the Dd2 control. The long-term competition experiment with wild-type Dd2 and Dd2-T13^{V259L} was performed for a 20-day period (~10 generations) in mixed cultures at 1:1, 9:1, and 1:9 ratios. DNA was harvested every 48 or 96 hours and culture composition was quantified by TaqMan qPCR using probes specific to the wild-type *cytb* allele (V259) and the mutant *cytb* allele (V259L) to determine allele abundance over time from the mixed cultures. The competition experiment was performed three times for the 1:1 mixtures and once for the 9:1 and 1:9 mixtures. The 1:1 mixed populations became predominantly T13 resistant over the 20-day period for all three experiments. In the 1:1 *in vitro* cultures, the results showed that the Dd2-T13^{V259L} line consistently outcompeted the wild-type control. In the 9:1 culture (90% wild-type to 10% mutant), parasite composition was unchanged by day 20, with 93% of the culture being wild-type Dd2 parasites. In contrast, the 1:9 culture (90% mutant) became 99% Dd2-T13^{V259L} mutant at the end of the 20 days. The single IC_{50} assay ran at the end of the 20-day period for each culture reflected the proportion of mutant or wild-type alleles measured by qPCR and the standard curve (Table 6 and Figure 13).

Table 6: Competition assay IC_{50} values on day 20-22 and standard curve [a].

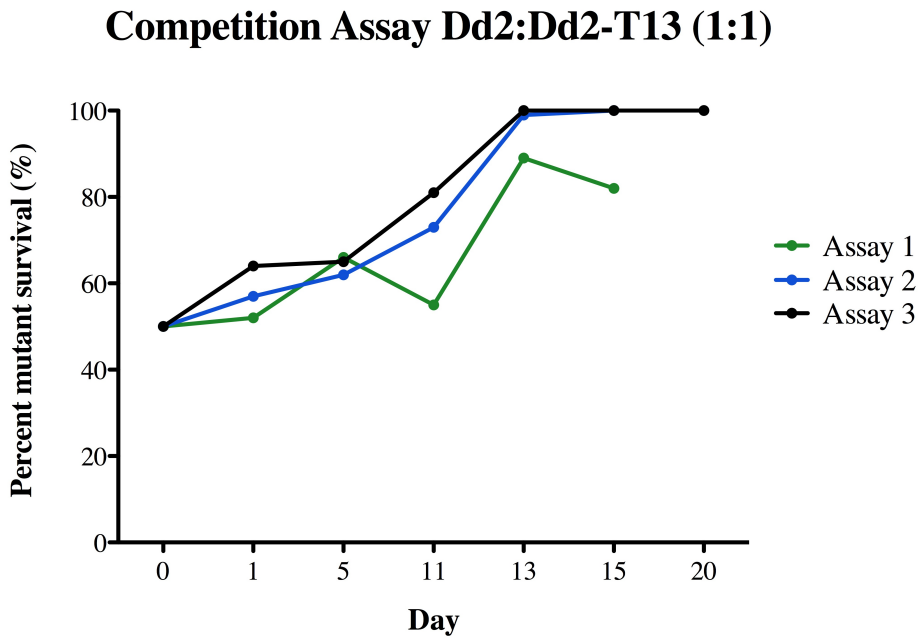
Drug	*P. falciparum* Standard Curve (IC_{50} in nM)						
	Dd2	**9:1**	**7:3**	**1:1**	**3:7**	**1:9**	**Dd2-T13^{V259L}**
CQ	166	168	160	158	160	171	175
T13	27	27	37	641	905	1095	1240
ATQ	0.29	0.42	0.43	0.49	1.3	2.6	2.7

Drug	*P. falciparum* Mixed Ratio Cultures *						
	Dd2	**Dd2-T13^{V259L}**	**9:1**	**1:9**	**1:1 assay 1**	**1:1 assay 2**	**1:1 assay 3**
CQ	252	215	232	225	225	149	223
T13	40	1056	47	1315	1240	1232	1359
ATQ	0.23	2.0	0.15	1.1	3.2	2.6	2.2

* Ratio indicates parasite composition at the beginning of the experiment (Dd2:Dd2-T13^{V259L}). IC_{50} values represent parasite cultures after 20-22 days of culturing.

[a] IC_{50} values where N = 1.

a)



b)

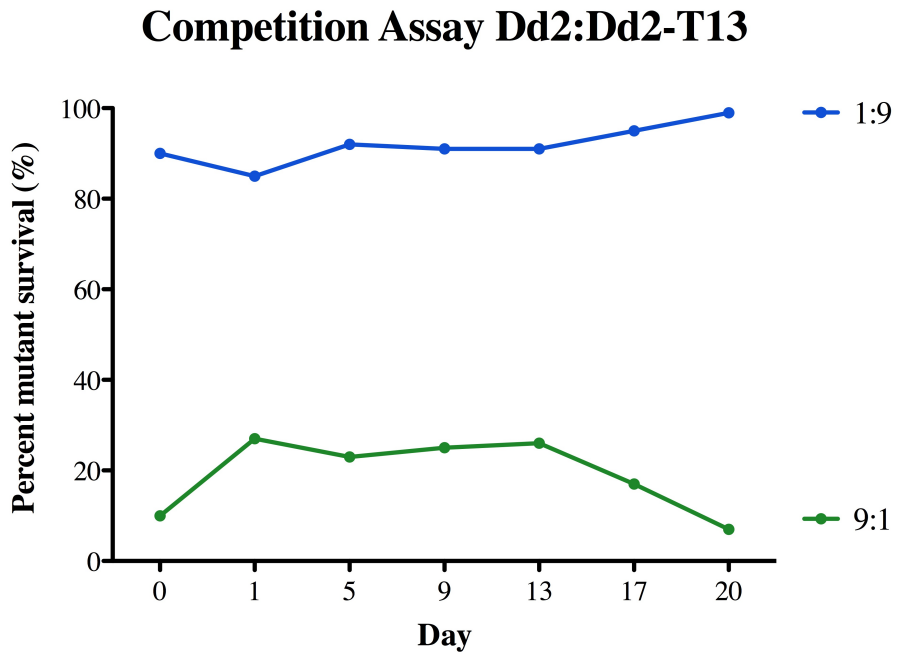


Figure 13: Dd2:Dd2-T13^{V259L} competition assay results collected by qPCR. a) In all three 1:1 competition assays, culture composition shifted from mixed to majority Dd2-T13^{V259L}. b) The culture with majority T13 mutant (1:9) became fully resistant at the end of 20 days while the culture with majority wild-type Dd2 (9:1) became sensitive.

Discussion:

The long-term efficacy of antimalarial treatment is of great importance in eradicating malaria. Acridones are powerful antimalarial compounds and an attractive drug candidate for prophylaxis and treatment of malaria. In murine models, 40 mg/kg of T13 prevented development of liver stage malaria, with no parasites visible in the mouse 24 and 48 hours later (Kelly, J. X., personal communication). Multiple chemotype I acridones are reported to be effective antimalarials *in vitro* and *in vivo* (Winter *et al*., 2006; Kelly, J. X., personal communication).

Although proper chemotherapy is generally effective in clearing the majority of malaria in a patient, the selective pressure created as blood drug levels decrease in malaria-infected patients may result in the selection of drug resistant *P. falciparum* (Malmberg *et al*., 2013). The select few parasites that survive high, lethal doses of drug exposure may result in the eventual replication and spread of drug resistant parasites (White, 2004). *In vitro* and *in vivo* reports of resistant parasites arising from selective drug pressure date back to the late 1950s, with the rise of CQ-resistant parasites (Nguyen-Dinh & Trager, 1978; Payne, 1987). The emergence of resistant parasites by *in vitro* selective drug pressure may occur with both continuous (Korsinczky *et al*., 2000; Johnson *et al*., 2004) and intermittent exposure (Eastman *et al*., 2011).

Acridone Chemotype I (T13) Resistance in P. falciparum

In this study, we report the *in vitro* selection of chemotype I acridone T13 resistance in *P. falciparum* using the CQR parasite Dd2, originating from Indochina (Oduola *et al*., 1988). MDR Dd2 parasites were selected for the drug pressure study

because of their propensity to acquire drug resistance (Rathod *et al.,* 1997). In published methods on selecting drug resistant *P. falciparum*, experiments used incremental, intermittent or continuous pressure. Johnson *et al*. (2004) used intermittent pressure to select drug resistant *P. falciparum* to the antiviral amantadine and antimalarial halofantrine (HF), while simultaneously restoring CQ sensitivity, leading to the discovery of novel *pfcrt* mutations involved in antimalarial cross-resistance and the proposal of a charged drug leak. Utilization of continuous pressure with spiroindolones derived drug resistant parasites after 3-4 months of pressure that suggested the P-type cation-transporter ATPase4 as the drug target (Rottmann *et al*., 2010).

Our study applied 24-hour on-off, intermittent pressure reflective of drug exposure and clearance observed clinically in malaria chemotherapy regimens. In a murine model, T13 had a half-life of 4.7 hours in plasma and 36 hours in the liver after a single dose of 80 mg/kg (Kelly, J. X., personal communication). Using both 24-hour and continuous pressure, we selected parasite lines resistant to chemotype I acridone T13 in four independent experiments. A single nucleotide change in parasites from three of the cultures resulted in a V259L amino acid change in CYT*b*, while the fourth selection resulted in an M133I and A138T double amino acid change in the same protein. These mutations correspond to the ubiquinol oxidation site, or Q_O binding site in CYT*b* (Valliéres *et al*., 2013). The V259L mutation was recently reported from ELQ-400 resistant *P. falciparum* parasites (Stickles *et al*., 2015b), while the M133I mutation was reported from ATQ resistant *P. falciparum* and *P. berghei* (Korsinczky *et al*., 2000; Syafruddin *et al*., 1999); however, the A138T mutation was previously unknown. The V259L mutation conferred a higher level of resistance to T13, with IC_{50} values from

1300-1600 nM T13, while M133I conferred lower levels of T13 resistance, with IC_{50} values ranging from 200-400 nM. The combination of the M133I and A138T mutations resulted in higher levels of T13 resistance compared to the latter single mutant, with IC_{50} values from 1300-1700 nM T13.

Sequencing of *cytb* in the Dd2-T13$^{M133I+A138T}$ mutant selected in the fourth experiment revealed a double mutation in CYT*b*, the M133I mutation and a novel mutation at amino acid position 138, where an alanine was replaced with a threonine. The secondary mutation is the most likely cause for the increase in IC_{50} from ~230 nM T13 to values from 1300-1700 nM T13 (Appendix IV), suggesting the mutation further reduced binding affinity of T13 to the Q_O site. It is unknown whether the A138T mutation can confer resistance on its own; therefore, this mutation may need to be studied further by site-directed mutagenesis or additional selection experiments. The increased susceptibility of Dd2-T13$^{M133I+A138T}$ to ELQ-400 and ATQ indicates that the secondary mutation provided a better binding site for both antimalarials and suggests that the A138T mutation compensated for the M133I mutation, thus resulting in inverse cross-resistance with T13. Korsinczky *et al*. (2000) reported the same event in CYT*b* when M133I was found with mutations P275T, K272R, or G280D that conferred 220-, 537-, and 897-fold the IC_{50} of the control parasites, respectively.

In our first T13 selection experiment, parasites remained sensitive with on-off pressure; however, resistant parasites emerged under subsequent continuous pressure. The emergence of resistant parasites under continuous pressure was most likely due to the resistant parasites not being selected initially during on-off pressure, or perhaps they were present during this time, but too low in number to be detected. In the second and

third selections, a combination of intermittent and continuous pressure produced additional lines of T13 resistant parasites. Although resistant parasites were selected with on-off pressure, the following continuous pressure was necessary to allow the T13 resistant parasites to outcompete the wild-type Dd2. The fourth selection with Dd2 demonstrated that T13 resistant parasites could be selected using only continuous pressure. Altogether, the results indicate there is no pattern associated with drug selection strategies because T13 resistant parasites were selected using both methods combined or alone.

The period of time it took to select resistant parasites with T13 is greater than the time it took to select ATQ resistant and ELQ-400 resistant parasites. ATQ is known to be a highly potent antimalarial; however, ATQ resistance can be rapidly selected when used as a monotherapy (Looareesuwan *et al*., 1996). Rathod *et al*. (1997) selected ATQ resistant *P. falciparum* parasites *in vitro* after a 2-month period of 100 nM ATQ pressure. ATQ resistant parasites were also selected within 14-16 days with continuous 100 nM ATQ (Gassis & Rathod, 1996) and 10 nM ATQ pressure (Stickles *et al*., 2015b), respectively. Stickles *et al*. (2015b) recently reported the emergence of CYT*b* V259L ELQ-400 resistant parasites after 47 days under a continuous drug concentration of 100 nM ELQ-400. Alternatively, our V259L mutants were not selected with T13 until parasites were exposed to continuous, increasing T13 pressure for 65 days, which occurred after the parasites had intermittent exposure to doses of T13 for an 81 day period. The M133I CYT*b* mutation, first reported by Korsinczky *et al*. (2000), was selected within 10 days using 20 nM ATQ pressure; however, it took 144 days to select M133I mutants with continuous T13 pressure. It is unknown why T13 has a longer period

of time to select resistant parasites with the same mutations as ATQ (M133I) (Korsinczky *et al*., 2000) and ELQ-400 (V259L) (Stickles *et al*., 2015b); however, this suggests that T13 does not readily select drug resistant parasites *in vivo*. We acknowledge that without a side-by-side comparison of selection rates with T13 and ATQ (or other CYT*b* inhibitors), as was performed by Stickles *et al*. (2015b) with ELQ400 and ATQ, it is difficult to determine the actual difference in time to select drug resistant parasites.

We believe that T13 has a lower chance of selecting resistant parasites based on several possibilities. First, the binding affinity and chemical nature of each compound suggests there may be fewer mutational options for chemotype I acridones than ATQ. T13 may have a greater affinity to bind to CYT*b* than ATQ, resulting in an increased frequency for SNP selection in ATQ, or perhaps other SNPs may have been selected with T13 but lacked the fitness needed to survive in culture. To determine mutation frequency, side-by-side comparison of T13 and ATQ should be performed, as mentioned earlier, along with continuing T13 pressure on acridone resistant parasites in attempt to select more mutants, as was performed by Korsinczky *et al*. (2000) with ATQ. Secondly, secondary targets may slow down the rate of resistance; however this has not been determined with T13. To resolve this, future studies with yeast DHODH transgenic parasites tested against T13 may elucidate other T13 targets outside of *cytb*, similar to studies by Painter *et al*. (2007) demonstrating that ATQ apparently only targets Complex III in the ETC and is not an effective antimalarial if the critical function of this pathway (pyrimidine synthesis) is removed.

To determine if T13 resistance could be selected in *P. falciparum* parasites with different genetic backgrounds than Dd2, CQS line 3D7 and CQR line 7G8 from Africa

and Brazil (Wootton *et al*., 2002), respectively, are currently being pressured with increasing, continuous T13 pressure. Resistance was recently selected in 3D7, exhibiting a 42-fold shift in T13 IC_{50} after 155 days of continuous T13 pressure (data not shown). Dd2 and 3D7 genetic backgrounds appear favorable in acquiring *cytb* mutations while 7G8 may have a lower frequency of acquiring resistance; however, this relationship must be further investigated. We note that pressuring the 7G8 line with the antiviral amantadine for over a year did not produce detectable changes in drug phenotypes (R. Cooper, unpublished observations). The period of time it took to select (or not select) T13 resistance in all three parasite lines was longer than the time to select ATQ and ELQ-400 resistance; therefore suggesting T13 resistance is harder to select, irrespective of parasite genetic background.

Genome Sequencing and SNPs Identified in T13 Resistant Parasites

Mutations in genes such as *pfcrt, pfatp4,* and *cytb* provide a resource for drug target identification of multiple antimalarials by revealing molecular marker(s) of resistance in *P. falciparum* (Wellems & Plowe, 2001; Rottmann *et al*., 2010; Korsinczky *et al*., 2000). Polymorphisms acquired in the Q_O site of *P. falciparum* CYT*b*, specifically M133I and Y268S (Y268C/N also observed), exhibit a 25-fold and 9,354-fold decrease in ATQ susceptibility, respectively (Korsinczky *et al*., 2000). Research on mitochondrial beef heart extracts and the photosynthetic bacterium *Rhodospirillum rubrum* showed that acridones target Complex III of the ETC, specifically in the Q_O site of CYT*b* (Oettmeier *et al*., 1994a, 1994b); however, an acridone target had not been identified in *Plasmodium spp*. Winter *et al*. (2006) was the first to propose that alkoxy acridones (chemotype I) target the malarial CYT*b* protein due to the structural similarity of their side chain to the

experimental drug floxacrine, which has slight cross-resistance with ATQ (Suswam *et al*., 2001). Winter *et al*. (2006) also proposed CYT*b* as the target of alkoxy acridones because of their structural similarity to ubiquinone, specifically, the long, flexible side chains characteristic of both molecules. Observation of the chemical structure of chemotype I acridones we tested, most notably T19 and T31, show the structural similarity of acridones to ubiquinone (Appendix III).

Our work is the first direct evidence of acridones targeting CYT*b* in *P. falciparum*, thus validating the previous theories proposed for alkoxy acridones (Winter *et al*., 2006). Whole genome sequencing of Dd2-T13 resistant parasites identified a G to T mutation at *cytb* nucleotide 775, resulting in an amino acid change from $V_{(GTA)}$ to $L_{(TTA)}$ at position 259 next to the highly conserved "pewy" loop in the Q_O site (Figures 11 and 14). The elevated T13 IC_{50} value in the V259L *cytb* mutants suggests that position 259 in CYT*b* may be a putative contact residue for T13. The V259L mutation explains the slight cross-resistance observed with Q_O site targeting ATQ and ELQ-400. We note that our V259L CYT*b* mutation differs from mutations acquired in ATQ resistant *P. falciparum*, but is the same mutation recently reported in ELQ-400 resistant parasites (Siregar *et al*., 2014; Stickles *et al*., 2015b). The V to L mutation is a conservative chemical change, where the mutation maintained a nonpolar, hydrophobic amino acid; however, the change resulted in an additional carbon on the functional side chain. The M133I mutation previously reported in ATQ resistant parasites (Korsinczky *et al*., 2000) also maintained hydrophobicity and remained nonpolar; however, the amino acid change was significant, where the straight side chain containing sulfur was replaced with a branched carbon chain.

The structure of *cytb* is highly conserved across eukaryotic species (Berry, 2000). The *P. falciparum cytb* sequence was aligned to chicken and bovine mitochondrial DNA in multiple studies on ATQ resistance, most notably in the report by Korsinczky *et al*. (2000), showing that the *cytb* gene sequence is highly conserved across eukaryotic species. Molecular and biochemical characterization of the effect of ATQ on ATQ resistant mutants has been studied in a variety of model organisms such as the yeast *Saccharomyces cervisiae* (Kessl *et al*., 2003; 2005), bovine (Kessl *et al*., 2007), and *Rhodobacter capsulatus* bacterial system (Mather *et al*., 2005). Studies by both groups demonstrated the *cytb* sequences use as a model system for *Plasmodium* given the high conservation of this gene, therefore allowing them to describe the binding of ATQ to the ubiquinol binding site (Q_O) and interactions with the Reiske iron-sulfur protein (ISP). By utilizing the model systems, Kessl *et al*. (2003; 2005; 2007) and Mather *et al*. (2005) were able to understand the molecular basis of ATQ resistance and provide further insight into the mode of action of ATQ.

ATQ is a competitive inhibitor of the ubiquinol oxidation pocket (Kessl *et al*., 2003). The naphthoquinone binds where it interacts with the ISP complex by aromatic combinations and hydrogen bonding that mimic binding determinants seen with ubiquinol. ATQ fixes the conformation of the ISP, thus preventing complete hydrogen bonding and immobilizing the ISP structure from docking into the Q_O site (Mather *et al*., 2005; Esser *et al*., 2006), specifically at positions 275 and 278 on CYT*b*, which are proposed to be key residues in ligand binding of the ISP and Q_O site (Kessl *et al*., 2003; 2005). Possible disruption of the *ef* loop and protein structure surrounding the Q_O site by the V259L mutation may have caused instability of the ISP in the *cytb* mutants, thus

weakening hydrogen bonding and creating a greater binding distance, or displacement of the ISP and Q_O site (Esser *et al.,* 2006; Xia *et al*., 2013). This process, in turn, halts work by DHODH and collapses the mitochondrial membrane potential by preventing electron transfer between the ISP and Q_O site (Barton *et al*., 2010). The M133I and novel V259L and A138T mutations have revealed the binding site of chemotype I acridones within the ubiquinol oxidation site. The V259L and A138T mutations identified will contribute to better understanding the ATQ binding site considering that the mutations are mapped to the same region as identified ATQ mutations. Furthermore, our work may also contribute to understanding the method by which other ETC targeting antimalarials bind to *cytb* in *P. falciparum*.

The rare event of a valine to leucine amino acid change has also been investigated in human models. Liu *et al*. (2003) studied the effect of a valine to leucine mutation at residue 382 in cytochrome P450 1A1, an oxidase enzyme that plays a critical role in drug and toxicant metabolism and activation (hydrocarbons), while characterizing substrate binding of alkoxyresorufin derivatives to 1A1. V382 is postulated to interact with the alkoxy side chain of the resorufin substrates; therefore, this residue was identified as an important factor in p450 1A1 activity for docking enzyme substrates to the active site. Liu *et al*. (2003) found that the leucine in the V382L mutant blocked the alkoxyresorufins from proper binding orientation, thus leading to lowered enzyme activity and drastically affecting positioning of the alkoxyresorufin substrates to the active site by creating an unfavorable distance between the oxidation site hydrogen of resorufins and the ferryl oxygen. Moreover, the effect of the CYT*b* V259L mutation in our parasites most likely results in a conformational change that reduced the binding affinity of chemotype I

acridone T13 to the Q_O site, therefore allowing the parasites harboring the V259L mutation to evade membrane potential collapse and provide continual function of DHODH. Changes in the binding affinity that affect the transfer of electrons from the Q_O site to the ISP have been reported in other Q_O site inhibitors such as ATQ, famoxadone, and stigmatellin (Kessl *et al*., 2003; Esser *et al*., 2006; Berry *et al*., 2011).

Although CYT*b* was the proposed target of chemotype I acridones (Winter *et al*., 2006), it was never demonstrated in *Plasmodium spp*. Therefore, we performed whole genome sequencing on the originally selected T13-resistant parasites to determine all possible markers of T13 resistance. Out of the 14 nonsynonymous SNPs identified outside of *cytb*, 8 of the SNPs were in either highly polymorphic *var* genes or in proteins of unknown function (Table 2); therefore, they were not looked at further. Four of the SNPs with identifiable locations (methyltransferase, protein kinase, AP2 domain, and dynein heavy chain), were not further studied as the nucleotide mutation differed from Dd2, but matched the 3D7 reference strain. The 2 remaining SNPs that differed from both 3D7 and Dd2 reference strains were found in a transcription factor in the AP2 domain (ApiAP2) and in a putative RNA helicase. It is unknown whether these mutations are contributory; consequently, the investigation of the ApiAP2 and RNA helicase SNPs identified from whole genome sequencing is a limitation of our study. However, Painter *et al*. (2010) suggested ApiAP2 proteins may serve as important transcription factors in parasite development while Tarique *et al*. (2013) stressed the importance of DEAD box RNA helicases and their critical role in RNA metabolism; therefore the mutations acquired in these two proteins in the T13 mutants may play an important role in parasite survival. Another limitation of the study is that the genome sequencing was performed on

two clones from only one independently selected resistant culture; therefore, further investigation with the remaining Dd2-T13^{V259L} mutants is warranted to ascertain if the mutations outside of *cytb* are present in other independently selected T13 resistant parasites. To make full use of the genome sequencing results, we should perform targeted sequencing of the AP2 domain and of the RNA helicase sequence in all T13 mutants.

The mutations identified in the *cytb* mutants provide a means for a more detailed analysis of acridone binding to CYT*b* in control *vs*. mutant parasites by molecular modeling and activity assays. Sequencing of the *cytb* gene in 3D7-T13 parasites identified the V259L mutation seen in Dd2-T13 parasites as well as a novel $A_{(GCT)}$ to $T_{(ACT)}$ mutation at amino acid position 82. The A82T mutation observed in the 3D7-T13 mutants is the only mutation in *cytb* that does not lie within the Q_O regions. Therefore, we believe the mutation may play a compensatory role in parasite fitness rather than decreasing parasite susceptibility given the T13 IC_{50} has a similar value observed in Dd2-T13^{V259L} parasites. The identification of the $M_{(GTA)}$ 133 $I_{(ATA)}$ and $A_{(GCA)}$ 138 $T_{(ACA)}$ mutations in the *cytb* mutants selected during continuous pressure further strengthens our claim that chemotype I acridones target the Q_O site in CYT*b*, as well as shows that acridones have a similar target, and effect on the ETC, as ATQ. Identification of mutations in the Q_O site provides a resource for future studies on ETC targeting antimalarials and now provides direct evidence of chemotype I acridones target in *P. falciparum*.

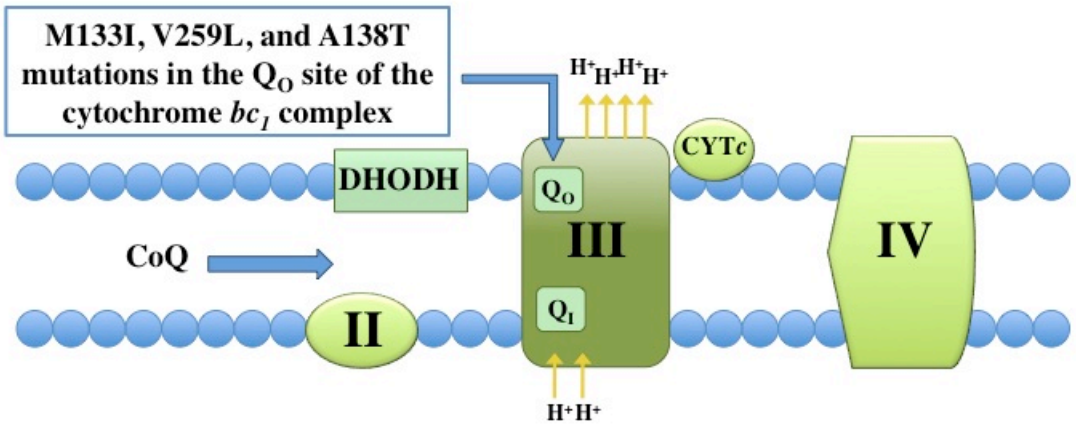


Figure 14: Proposed target and mutations identified in the Q_O site of the ETC in T13-resistant parasites.

pfmdr1 *Copy Number and its Role in T13 Resistance*

pfmdr1 encodes for the pgh1 protein localized on the parasite DV, and is hypothesized to be responsible for the influx of solutes into the DV (Cowman, *et al*., 1991; Rohrbach *et al*., 2006). Variation in *pfmdr1* copy number can mediate resistance to multiple antimalarials with diverse targets and can even confer resistance to the antimalarial MEF (Price *et al*., 2004). Sidhu *et al*. (2006) confirmed the importance of *pfmdr1* copy number in multifactorial resistance and cross-resistant patterns in the antimalarials LUM, QN, ART, and HF. They suggested other factors associated with resistance might be polymorphisms in *pfmdr1* and discussed how increased copy number has resulted in increased drug susceptibility to LUM and QN in some studies, but not others. Amplification of *pfmdr1* in the *cytb* mutants may also serve as a factor in acridone resistance.

We sought to identify whether *pfmdr1* copy number played a role in T13 resistance; therefore, we carried out a multiplex qPCR to look at this gene in the Dd2-T13^{V259L} *cytb* mutants. *pfmdr1* copy number increased by a single copy in the *cytb* mutants tested, from 3 to 4 copies, when compared to wild-type Dd2 (3 copies). Dd2 parasites are known to have 3-4 copies of *pfmdr1* (Patel *et* al., 2010); therefore, the elevated number in *pfmdr1* copies may not have been due to an amplification event, but rather, the parasites harboring 4 copies in wild-type Dd2 outcompeted the parasites with 3 *pfmdr1* copies; however, the mixed copies (3-4) observed in the *cytb* mutants indicates that one did not outcompete the other. Long-term studies would have to be performed with the *cytb* mutants to understand if it is advantageous to harbor 4 copies of *pfmdr1*. Although it appears CYT*b* is the main target of T13 and the major cause for T13 resistance, elevated *pfmdr1* copy number may play a minor role in decreased T13 susceptibility or may compensate for the fitness cost of acquired resistance. Future work should be performed on all *cytb* mutants harboring mutations selected with T13 to see if there is a change in *pfmdr1* copies.

Structure Activity Relationship of Chemotype I Acridones

In-depth structure activity relationship (SAR) analysis will help elucidate the binding relationship of chemotype I acridones with CYT*b* by analyzing cross-resistance patterns in Dd2-T13^{V259L} *cytb* mutant parasites. We have identified varying levels of cross-resistance from a 2.8-26 fold increase in IC_{50} in a panel of 13 chemotype I acridones. No cross-resistance was observed with T2.2, which was the only chemotype I acridone tested with an alkoxy side chain at the 7th position, instead of the 6th position, of the acridone core (Appendix III). The lack of cross-resistance with T2.2 suggests there is

a second chemotype I acridone target, or that T2.2 interacts differently with CYT*b* where it is able to evade cross-resistance. It also suggests that T2.2 could be used in antimalarial treatment where there would be no concern for cross-resistance with ATQ, ELQ-400 or the other chemotype I acridones tested.

The Dd2-T13^{V259L} *cytb* mutants varying degrees of cross-resistance seen with the other 11 chemotype I analogs prompted us to examine their structures in greater detail. The highest levels of cross-resistance were obtained with T2, T26, and T27, implying the CYT*b* Q_O site is also the main target for these three acridones. The fluorine (F) and chlorine (Cl) substituents at positions 1 and 3 of T26 and T27 (Appendix III), respectively, may not be optimal placement for preventing cross-resistance. Winter *et al*. (2006) reported that placement of a highly electronegative nitro group as the electron withdrawing substituent resulted in a significant decrease in antimalarial activity of alkoxy acridones, whereas, reduction of the nitro group to an amine caused a dramatic increase in antimalarial potency. This may also be the case in the effect of the highly electronegative fluorine on the *cytb* mutants, where a high level of cross-resistance was observed in the V259L *cytb* mutant.

Alternatively, the Cl substituent at the 1st position on T2 does not appear to play a role in cross-resistance considering the substituent is in the same position on T2.2, which has no cross-resistance to T13. T14 was the only chemotype I analog with the same side chain as T13 that exhibited a mild degree of cross-resistance. The mild degree of cross-resistance may result from the placement of the Cl substituents at the 2nd and 3rd positions, unlike T13, where the two chlorines are on the 1st and 2nd positions of the acridone. T19, T20, T31 and T13M, with the Cl substituents in the 1st and 2nd positions (same as T13) on

the acridone core, have a minor shift in IC_{50} from 2.8-10 fold (Table 3; Appendix III). The low degree of cross-resistance in these four analogs may be due to the differing side chains at the 7th position. The side chain on T31 is the most similar to T13 between these four analogs, which may have caused the highest shift in susceptibility from this group. Lastly, the 25-fold shift in susceptibility of analog T17 may be the result of the structural similarity of its side chain to T13, except terminating with a Cl, which may not be optimal for evading cross-resistance with T13; however, further testing must be performed with other terminal Cl analogs to determine if this statement is true. The lack of cross-resistance observed with chemotype II acridones T16.5 and T26.5 indicate that chemotype I and II acridones do not share the same drug target. Chemotype II acridones target the *P. falciparum* digestive vacuole; therefore, the digestive vacuole is most likely not a target of T13. Our studies on SAR with chemotype I and II acridones provide insight as to which acridones may have similar or identical target interactions as T13. The varying degrees of cross-resistance indicate which compounds may need to be further optimized to prevent cross-resistance or reduce the degree of resistance.

Cross-resistance of T13 with Common Antimalarials

Multiple types of antimalarials exhibit cross-resistance because of having the same or similar targets within the malaria parasite (Johnson *et al*., 2004; Sidhu *et al*., 2006; Cooper *et al*., 2002 & 2007). CQ and AQ have varying degrees of cross-resistance because of mutations in *pfcrt* and *pfmdr1*, especially K76T and N86Y, respectively (Holmgren *et al*., 2006; Sá *et al*., 2007). Identifying antimalarial cross-resistance patterns not only aids in drug target discovery, such as identifying *cytb* as a chemotype I acridone target based on cross-resistance with ATQ, but it provides insight as to which

antimalarial therapies would be best suited in regions of the world where a drug may be rendered ineffective. Therefore, we tested the *cytb* mutants against an array of antimalarials with diverse drug targets with the goal of identifying to which, if any, antimalarials the mutant parasites may have also acquired resistance.

Winter *et al*. (2006) suggested that acridones target *P. falciparum* Complex III of the ETC; therefore, the Dd2-T13^{V259L}, Dd2-T13^{M133I}, and Dd2-T13$^{M133I+A138T}$ lines were also tested for altered susceptibility to the antimalarials ATQ and ELQ-400, which target CYT*b* (Vaidya & Mather, 2009; Siregar *et al*., 2014; Stickles *et al*., 2015b). Cross-resistance was observed in only ATQ and ELQ-400, while the susceptibility of all other antimalarials remained unchanged. The V259L mutation resulted in only a 10-fold shift in ELQ-400 IC_{50}, unlike the 36-38 fold shift this mutation imparted on T13, indicating that position 259 is most likely a central location in CYT*b* where T13 binds, but not ELQ-400. To note, the 10-fold shift observed with ELQ-400 and ATQ susceptibility in V259L mutants agrees with V259L data reported by Stickles *et al*. (2015b). The G to A mutation at nucleotide 399 (M133I) lying within Q_O site region 1 conferred a 16-18 fold increase in ATQ, agreeing with previous reports about the M133I mutation and susceptibility to ATQ (Korsinczky *et al*., 2000). The 6-fold shift in T13 susceptibility accompanied by the M133I mutation supports the argument that ATQ and T13 share a similar drug target in CYT*b* (Winter *et al*., 2006). The increased sensitivity to ELQ-400 in parasites with the M133I mutation implies that this mutation augments parasite susceptibility to ELQ-400. Surprisingly, the same effect was seen with ATQ in the Dd2-T13$^{M133I+A138T}$ parasites after the acquisition of the A138T mutation, suggesting this mutation increases ATQ susceptibility. Similar work by Sidhu *et al*. (2005) found an

inverse relationship where an N1042D mutation in *pfmdr1* enhanced MEF, ART and HF sensitivity while contributing to QN and QD resistance. The cross-resistance observed with only ATQ and ELQ-400 suggests that CYT*b* is the major target of chemotype I acridones, and that all three drugs share a common binding site in CYT*b*. Therefore, if chemotype I compounds will be used in the clinical setting, it may not be ideal to use in areas where high-level ATQ resistance is observed.

Currently, there are limited drug targets in *P. falciparum*; therefore, it is common for multiple, chemically distinct antimalarials to target the same location. Antimalarials such as CQ or MEF will select for drug resistant parasites when used as a monotherapy (Payne, 1987; Smrkovski *et al*., 1982), initiating the need for combination therapy (Nosten & White, 2007). ATQ, a CYT*b* inhibitor, will never be used as a monotherapy as it rapidly selects for drug resistant parasites (Looareesuwan *et al*., 1996); however, when used in combination with proguanil, such as in Malarone, the treatment is highly effective, with a 98% success rate, and few reports of treatment failure (Wurtz *et al*., 2012; Plucinski *et al*., 2014). Presently, many antimalarials are being developed that target locations in *P. falciparum* where resistance has already been selected. Highly potent endochin-like quinolones show that they can select drug resistance, however the drugs still remain potent (Stickles *et al*., 2015b). Seemingly, no matter where the target, resistance will be selected; therefore, combination therapy is key to protecting antimalarials from becoming ineffective.

Ex vivo *Studies of Chemotype I Compounds in Clinical* P. falciparum *Isolates*

Ex vivo assays provide a non-invasive method of testing antimalarial potency against parasites taken directly from the clinical setting. Although the testing is not within malaria-infected humans, the data obtained provides insight on drug susceptibility in parasites that have not become culture adapted. In an attempt to monitor drug susceptibility in Burkina Faso, Tinto *et al*. (2014) carried out *ex vivo* studies with 440 malaria-infected patient blood samples 5 years after the implementation of ACT treatment. From the six drugs tested by IC_{50} (CQ, QN, DHA, LUM, monodesethylamodiaquine and PIP), they were able to determine the continual efficacy of artemisinin derivatives while there was a worrisome decline in parasite susceptibility to LUM. Tinto *et al*. (2014) concluded that ACT treatment with DHA-PIP was still highly effective treatment while the treatment with artemether-LUM should be monitored more closely in this region; therefore, they were able to establish that the potency of artemisinins and PIP were unchanged. The potency of chemotype I acridones was determined for analogs T13, T31, and T35 against parasite isolates collected directly from Ugandan children in 2012. The results were consistent with their *in vitro* potency against lab-adapted lines. The *in vitro* and *ex vivo* potency of several chemotype I acridones, such as T31 and T35, have comparable IC_{50} values to highly potent antimalarials in current clinical treatment or prophylaxis, such as LUM (Dd2 $IC_{50} = 5.7 \pm 0.21$) and ATQ (Dd2 $IC_{50} = 0.50 \pm 0.043$). The data shows that clinical isolates are highly sensitive to chemotype I acridones; therefore, there is no observed resistance in the clinical setting and the compounds may serve as effective chemotherapeutics in malaria endemic countries.

*The Effect of CYT*b *V259L Mutation on Parasite Growth*

The fitness of resistant parasites is based on the comparison of growth rates by utilizing competitive growth experiments. Mutations in drug resistant parasites come at a fitness cost (Rosenthal, 2013). However, compensatory mutations may improve fitness for resistant parasites, thus allowing survival without drug pressure or even during competitive growth experiments (Rosenthal, 2013). IC_{50} assays, performed after the final day of the competition assay, and mutation-specific qPCR results indicate that the V259L *cytb* mutants were more fit than Dd2 in mixed cultures. Fitness consequences with acquired drug resistance in *Plasmodium spp*. are studied *in vitro*, in animal models, and in clinical studies. Previous studies performed on parasites harboring high-level resistance to antifolates had increased copy number of the gene GTP-cyclohydrolase 1 (*gch1*) that accompanied the dihydrofolate reductase (*dhfr*) I164L mutation (Nair *et al*., 2008). The combination of the two made the mutant parasites more fit than the parasites that did not have the I164L mutation accompany the elevated *gch1* copy number. If the compensatory change is a similar fitness advantage mechanism in the V259L *cytb* mutants, but with elevated *pfmdr1* copy number or a SNP in another gene as a compensatory adaptation, then there would appear to be no cost of fitness in the T13-resistant parasites.

Genome sequencing of the V259L CYT*b* mutants revealed 14 SNPs outside of *cytb*. It remains unknown whether 6 of the SNPs identified serve a role in parasite fitness considering the mutations are in proteins of unknown function. Two of the SNPs identified are located in highly polymorphic *var* genes, thus were not considered to serve as a fitness advantage. However, a SNP identified in transcription factor proteins ApiAP2

and in an RNA helicase may shed light on the acquired fitness advantage. To determine whether the V259L mutants continually outcompete wild-type parasites, other V259L mutants need to undergo the competition assay experiment. Transfection studies should also be performed to determine if the V259L mutation confers the fitness advantage or if one of the 14 SNPs identified by genome sequencing (or *pfmdr1* copy number) is responsible. Transfection studies by Painter *et al*. (2007) that replaced *Pf*DHODH with the yeast enzyme (*y*DHODH), thus functionally removing the need for CYT*b* to regenerate ubiquinone, revealed that ATQ was not effective without the presence of functional CYT*b*.

Use of the *y*DHODH-transgenic parasites (Painter *et al*. 2007), along with our competition assay, will reveal if the V259L mutation is the sole contributor to T13 *cytb* mutant fitness. The *y*DHODH parasites could serve as a control in transfection studies with V259L mutants to show that parasites with a nonfunctional ETC, and alternative pathway for pyrimidine synthesis, are fit in culture when *cytb* is the sole contributor to resistance. Replacing the *Plasmodium* DHODH in the V259L mutants with *y*DHODH, or transfecting the V259L mutation into a wild-type Dd2 lacking the SNPs identified outside of *cytb* will reveal whether the V259L mutation confers the fitness advantage or if the improved parasite fitness is multigenic. To observe the effect of the transfected T13 mutant parasite growth, the parasites would be placed into the competition assay against Dd2 or *y*DHODH. Highly fit parasites *in vitro* may reflect how well the parasites will survive in the clinical setting; however, the SNPs selected *in vitro* may not reflect what mutations will be observed in clinical isolates.

Conclusion:

The emergence of drug resistance is a continuous threat to antimalarials, old and new. Therefore, the need for antimalarial drugs that can circumvent this event is of great urgency. The *P. falciparum* mitochondrial electron transport chain, specifically the cytochrome bc_1 complex, is an attractive target for drug design due to the undeniable necessity for the biosynthesis of pyrimidines.

Antimalarial acridones show potential in the treatment of malaria. Not only do they have intrinsic antimalarial potency and liver targeting ability (chemotype I), but chemosensitizing capabilities as well (chemotype II). We successfully selected *P. falciparum* parasites resistant to dual stage chemotype I acridones and have identified a V259L mutation within the Q_O site of CYT*b*, recently seen in ELQ-400 resistant parasites, as well as an M133I mutation seen in ATQ resistant parasites. We have also identified novel A to T mutations at positions 138 and 82 in CYT*b*. We can infer that the mitochondrial ETC CYT*b* protein is likely the main target of chemotype I acridones. Further investigation to validate the resistance acquired in our mutant parasites as well as the possible identification of more or all factors attributed to the mechanism of action of chemotype I acridones will need to be performed. Identification of T13 resistance determinants in the *in vitro* selected lines will provide a resource for studies into the pathways that may mediate chemotype I resistance in the clinical setting.

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APPENDICES

APPENDIX I: ABBREVIATIONS

3D7: Chloroquine sensitive parasite harboring a single copy of *pfmdr1*

7G8: Chloroquine resistant parasite

ACT: Artemisinin-based combination therapy

ATQ: Atovaquone

AQ: Amodiaquine

Complex III: cytochrome bc_1 complex of the electron transport chain

CQ: Chloroquine

CQR: Chloroquine Resistant

CQS: Chloroquine Sensitive

CYT*b:* cytochrome *b* protein

cytb: cytochrome *b* gene

Dd2: Chloroquine resistant parasite harboring 3-4 copies of *pfmdr1*

Dd2-T13: T13 resistant selected parasites

DHA: Dihydroartemisinin

DHODH: dihydroorotate dehydrogenase

DV: digestive vacuole

ELQ: endochin-like quinolone

ETC: Electron Transport Chain

h: Hour

I: Isoleucine

IC_{50}: Concentration of drug that inhibits 50% of parasite growth

APPENDIX I: ABBREVIATIONS

ISP: Iron-sulfur protein; transfers electrons from CYT*b* to CYT*c*

K: Lysine

L: Leucine

LUM: Lumefantrine

M: Methionine

MEF: Mefloquine

nM: nanomolar

pfcrt: Plasmodium falciparum Chloroquine Resistance Transporter gene

PFCRT: *Plasmodium falciparum* Chloroquine Resistance Transporter protein

PFMDR1: *Plasmodium falciparum* Multi-drug Resistance Protein 1

pfmdr1: Plasmodium falciparum Multi-drug Resistance gene

PIP: Piperaquine

QC: Quinacrine

QN: Quinine

Q_O site: ubiquinol oxidation site

SNP: Single Nucleotide Polymorphism

T: Threonine

T13: Chemotype I acridone analog

T2.2: Chemotype I acridone analog

T16.5: Chemotype II acridone analog

V: Valine

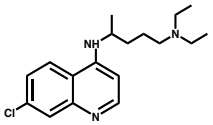
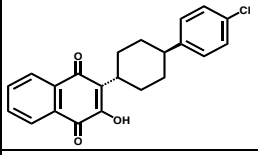
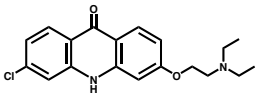
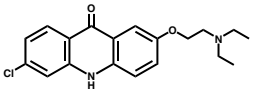
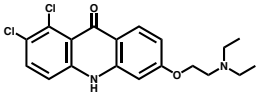
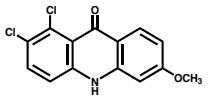
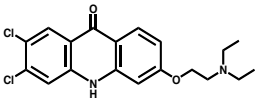
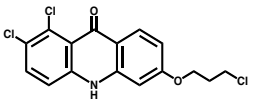
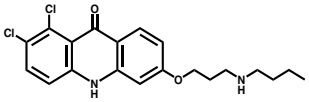
WHO: World Health Organization

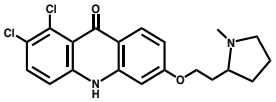
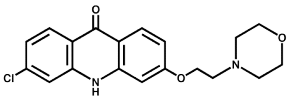
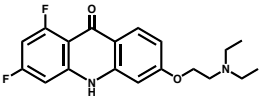
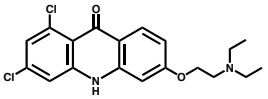
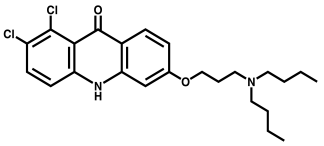
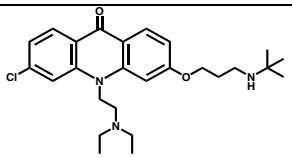
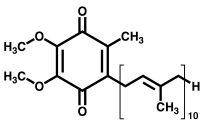
APPENDIX II: ***P. falciparum Laboratory Strains***

Isolate	Origin	CQ Sensitivity	ATQ Sensitivity	*pfmdr1* CN
Dd2	Indochina	CQR	ATQ-S	2-4
7G8	Brazil	CQR	ATQ-S	1
3D7	Africa	CQR	ATQ-S	1

CN: copy number

APPENDIX III: Drug Classes and Structures

Class	Drug	Structure
4-aminoquinoline	**CQ**	
Hydroxynapthoquinone	**ATQ**	
Chemotype I Acridone	**T2**	
	T2.2	
	T13	
	T13M	
	T14	
	T17	
	T19	

Chemotype I Acridone	**T20**	
	T22	
	T26	
	T27	
	T31	
	T35	----
	T36	----
Chemotype II Acridone	**T16.5**	
	T26.5	----
Ubiquinone (CoQ)	**----**	

APPENDIX IV: Drug Susceptibilities of Cloned Dd2-T13 Mutants

Drug susceptibility results of cloned *P. falciparum* Dd2-T13 mutants.

Drug	Dd2	Dd2-T13 Resistant Mutants; IC_{50} ± SEM (nM)				
		T13a^{F1}	T13b^{H9}	T13c^{D3}	T13d^{E4}	T13d^{F5}
CQ	181 ± 4.3	166 ± 7.1	185 ± 4.4	231 ± 11	182 ± 5.6	186 ± 8.0
QN	254 ± 9.8	215 ± 7.5	317 ± 20	254 ± 16	197 ± 3.5	199 ± 13
MEF	18 ± 1.3	23 ± 0.95	21 ± 1.9	21 ± 2.3	14 ± 0.71	13 ± 0.61
LUM	5.7 ± 0.21	6.5 ± 0.32	6.4 ± 2.3	6.4 ± 0.53	3.6 ± 0.19	3.6 ± 0.26
PIP	9.0 ± 0.17	7.1 ± 0.25	11 ± 0.58	9.0 ± 0.31	10 ± 0.31	7.4 ± 0.27
ATQ	0.43 ± 0.038	3.8 ± 0.26****	3.7 ± 0.27***	2.8 ± 0.17**	6.9 ± 0.95****	1.4 ± 0.052**
T13	40 ± 5.5	1439 ± 49****	1578 ± 79 ****	1528 ± 70****	242 ± 18**	1678 ± 78****
QC	29 ± 1.3	18 ± 0.74*	29 ± 1.5	29 ± 1.8	23 ± 0.59	29 ± .67
ELQ-400	3.4 ± 0.11	33 ± 2.2***	24 ± 0.91***	39 ± 1.7***	0.72 ± 0.035	3.1 ± 0.14

IC_{50} values ± SEM in nM. N ≥ 3. 72 h Microplate test using Sybr Green detection.

Dd2-T13a underwent 24-hour and continuous pressure, with resistance selected after continuous pressure. Dd2-T13b-c underwent 24-hour and continuous pressure. Resistance selected during on-off pressure. Dd2-T13d underwent continuous pressure.

Two-tailed t-test of average IC_{50} of mutant *vs*. Dd2 control where $p < 0.0001$ [****]

Two-tailed t-test of average IC_{50} of mutant *vs*. Dd2 control where $p < 0.001$ [***]

Two-tailed t-test of average IC_{50} of mutant *vs*. Dd2 control where $p < 0.01$, $p < 0.05$ [**]

APPENDIX V: qPCR Primers and Conditions for *pfmdr1* Copy Number Analysis

Primers	Sequence	5' end	3' end
pfmdr1–1F	5'-TGCATCTATAAAACGATCAGACAAA		
pfmdr1–1R	5'-TCGTGTGTTCCATGTGACTGT		
pfmdr1–probe	5'-TTTAATAACCCTGATCGAAATGGAACCTTTG	FAM	TAMRA
β-*tubulin*-1F	5'-TGATGTGCGCAAGTGATCC		
β-*tubulin*-1R	5'-TCCTTTGTGGACATTCTTCCTC		
β-*tubulin*-probe	5'-TAGCACATGCCGTTAAATATCTTCCATGTCT	VIC	TAMRA

PCR Amplification Protocol

Temperature	Time (seconds)	Cycles
95°C	20	40
95°C	1	40
60˚C	20	40

APPENDIX V: qPCR Primers and Conditions for *pfmdr1* Copy Number Analysis

qPCR Sample Volumes for *pfmdr1* Copy Number Analysis

	[Final]	Volume (µL)	Multiple	Total (µL)
Taqman Mastermix (2X)	1X	12.5		1200
H_2O		6.5	100	1015
MDR_F 100uM	0.3 uM	0.75	100	7.5
MDR_R 100uM	0.3 uM	0.75	100	7.5
βtubulin_F 100uM	0.3 uM	0.75	100	7.5
βtubulin_R 100uM	0.3 uM	0.75	100	7.5
MDR_pB 100uM	0.1 uM	0.25	100	2.5
βtubulin_pB 100uM	0.1 uM	0.25	100	2.5
TOTAL	----	**22.5**	100	**2250**
DNA per well	~2 ng/µL	2.5	4	**10**
TOTAL PER WELL	----	25		

APPENDIX VI: PCR Primers and Conditions for Sanger Sequencing of *cytb*

Primers used for Sanger sequencing of *cytb*.

Primers	Sequence	Start	Stop	T_m
cytb-F	5'-TTCCTGATTATCCAGACGCT	3380	3399	53.2
cytb-R internal	5'-CACTCACAGTATATCCTCCACA	3978	3957	53.5
cytb-F internal	5'-GAGTTATTGGGGTGCAACTG	3890	3909	53.8
cytb-R	5'-TGTTCCGCTCAATACTCAGA	4670	4651	53.3

PCR Amplification Protocol

Temperature	Time	Cycles
95°C	2 minutes	Pre-read stage
95°C	30 seconds	30
42°C	1 minute	30
65°C	2 minutes	30

PCR Sample Volumes

	[Final]	Volume (µL)
MeanGreen Taq Master mix (2X)	1X	25
H_2O		18
Forward Primer	0.2 µM	1
Reverse Primer	0.2 µM	1
TOTAL	----	**45**
DNA per well	~10 ng/µL	5
TOTAL PER WELL	----	50

APPENDIX VII: PCR Primers and Conditions for SNP Genotyping

Primers	Sequence	5' end	3' end	T_m
cytb-F	5'-TCACATCCTGATAATGCTATCGTAGT	----	----	57
cytb-R	5'-GCTGGTTTACTTGGAACAGTTTTTAA	----	----	58
cytb-probe	5'-CATCTCAAATTGTACCTGAA	VIC	MGB	67
*cytb*T13-probe	5'-TACTCCATCTCAAATTTTACCT	FAM	MGB	67

PCR Amplification Protocol

Temperature	Time (seconds)	Cycles
60°C	30	Pre-read stage
95°C	10 minutes	Hold stage
95°C	15	40
60°C	1	40
60˚C	30	40

qPCR Sample Volumes for SNP Genotyping for Competition Assay

	[Final]	Volume (µL)	Multiple	Total (µL)
Taqman Mastermix (2X)	1X	10		1000
H_2O		7	100	700
SNP Assay Mix (20X)	1X	1	100	100
TOTAL	----	**18**	100	**1800**
DNA per well	~10 ng/uL	2	4	**8**
TOTAL PER WELL	----	20		