# Chapter 5: Discovery of molecular markers of piperaquine resistance by *in vitro* drug-pressure of a hypermutator *Plasmodium falciparum* line

## 5.1 Declaration of work

The hypermutator parasite line used in this study was generated by Dr. Krittikorn Kumpornsin. All bioinformatic analyses were performed by Dr. Frank Schwach. Both Drs. Kumpornsin and Schwach are cited in the relevant sections of this chapter for their invaluable contributions to this work.

### 5.1.1 Significance and purpose of study

The current frontline treatments for *Plasmodium falciparum* malaria, artemisinin combination therapies (ACTs), are designed to increase treatment efficacy and prevent the development of drug resistance. These therapies combine potent, short-acting artemisinin derivatives with partner drugs with different mechanisms of action and longer half-lives to clear any remaining parasites. However, the development of artemisinin resistance in Southeast Asia has placed increased pressure on partner drugs. Continued treatment failures of the ACT dihydroartemisinin-piperaquine (DHA-PPQ) have prompted multiple reverse genetic studies that have identified several candidate molecular markers of piperaquine resistance (Amato et al., 2017, Witkowski et al., 2017, Hamilton et al., 2019). Further studies, including work in this doctoral thesis, have performed functional analyses to assess the phenotypic relevance of these genetic markers (Ross et al., 2018a, Loesbanluechai et al., 2019, Bopp et al., 2018) (Chapters 3-4). With limited drug alternatives available, it is imperative to have diverse approaches to study the malaria parasite biology and the molecular mechanisms through which drug resistance can emerge. In comparison to reverse genetics approaches, forward genetics methods in *P. falciparum* have been largely hindered due to the slow spontaneous mutation rates of *P. falciparum* (Bopp et al., 2013). Recent elegant studies in the rodent malaria parasite, *Plasmodium berghei* (Honma et al., 2014, Honma et al., 2016) have designed "hypermutator" parasites that are able to rapidly develop mutations which can be used to obtain resistant phenotypes for drug-pressured lines. Studies by Kumpornsin *et al.* (*in preparation*) used this work as a model to generate a hypermutator parasite line in *P. falciparum*. The purpose of this thesis study was to use a forward genetics approach to elucidate genetic determinants of piperaquine resistance. I was able to select for

piperaquine-resistant parasites in a feasible experimental timespan by utilizing a hypermutator *P. falciparum* parasite line. Through whole genome sequencing (WGS) analyses of these piperaquine-resistant parasites this study identifies several gene candidates that have already been reported to play a role in piperaquine resistance and resistance to other antimalarial compounds. This study also identifies multiple new gene candidates that may play a causal role in piperaquine resistance. These include mutations never identified before in transmembrane domain (TMD) 10 of PfCRT, a domain that is thought to have a critical role in the homo-dimer structure of the trans-membrane channel (Summers et al., 2012). By combining forward genetics tools with the reverse genetics approaches detailed in the previous chapters, this study aims to combine functional and genomic approaches to further drive the quest to uncover the mechanism of piperaquine resistance.

### 5.1.2 Introduction

The ability of the malaria parasite, *Plasmodium falciparum*, to develop resistance to nearly all approved treatments remains a severe challenge for disease prevention and elimination efforts. For almost two decades, ACTs have been the standard treatment for *P. falciparum* malaria because they maximize therapeutic efficacy by combining two drugs with different mechanisms of action while also reducing the potential for resistance to develop (World Health Organization, 2018, World Health Organization, 2015, World Health Organization, 2001, Arrow, 2004). The ACT dihydroartemisinin-piperaquine (DHA-PPQ) combines the fast-acting artemisinin compound with a half-life of approximately one hour with a partner drug with a half-life of around 23 days (Hoglund et al., 2017, Batty et al., 1998). The inherent contradiction in this combination is that the mismatched pharmacokinetic profiles of the drugs means that any recrudescent parasite or new infection is exposed to suboptimal levels of piperaquine monotherapy. With the failures of two different ACTs in Southeast Asia, artesunate-mefloquine (AS-MQ) in 2004 (Denis et al., 2006) and most recently, DHA-PPQ (Leang et al., 2013, Saunders et al., 2014, Spring et al., 2015, Amaratunga et al., 2016), the question is no longer *if* resistance will develop, but *when* and *how*? To inform and improve treatment methods, it is essential to understand the mechanisms through which malaria parasites can respond and adapt to the diverse pressures in their environment.

Similar to many other diseases, antimalarial drug resistance can be caused by a plethora of complex environmental and molecular interactions. Thus, multiple approaches may be needed to identify mechanisms of resistance. Both reverse (target-based) and forward genetics (phenotype-based) approaches are useful methods for identifying mechanisms of

resistance (Mitchison, 1994, Schenone et al., 2013). In previous chapters of this thesis (Chapters 3-4), I made use of genetic information provided by genome wide association studies (GWAS) to examine potential genetic determinants of piperaquine resistance. In addition to these reverse genetics approaches, I also employed a forward genetics approach to complement these studies in *P. falciparum* by using a "hypermutator" parasite line to select for a piperaquine-resistant phenotype. Combining forward and reverse genetics has the potential to provide insight into both the mechanism of resistance and the potential mechanism of action of piperaquine.

In many eukaryotic organisms, forward genetics studies are hindered by the slow occurrence of spontaneous mutations. The estimated mutation rate of human cells is $1 \times 10^{-8}$ per base pair (bp) per generation (Genomes Project et al., 2010). In *P. falciparum*, the mutation rate of asexual *in vitro* cultures is approximately $1.0–9.7 \times 10^{-9}$ per bp per generation (Bopp et al., 2013). For the parasite, this slow mutation rate is desirable as it reduces the potential for deleterious mutations that may negatively affect survival. However, for the researcher performing *in vitro* drug-selection experiments—where obtaining a phenotype of interest is the primary goal—the ability to generate mutations rapidly under selective pressure is advantageous.

The low spontaneous mutation rate in eukaryotes is largely due to the proofreading functions of the high fidelity replication and repair enzymes, DNA polymerases (Shevelev and Hübscher, 2002). There are four nuclear DNA polymerases in eukaryotic cells, α, β, δ, and ε. Both DNA polymerases δ and ε have 3'→5' exonuclease activity and these proofreading domains are highly conserved amongst prokaryotes and eukaryotes (Hubscher et al., 2002, Shevelev and Hübscher, 2002, Swan et al., 2009). Studies in yeast have shown that disruption of the exonuclease domain of polymerase δ has a strong effect on proofreading ability, causing a "hypermutator" phenotype characterized by at least a 10-100-fold increase in spontaneous mutations (Morrison and Sugino, 1994, Fortune et al., 2005). Interestingly, the proofreading domain of polymerase δ is also highly conserved in *Plasmodium* (Ridley et al., 1991). Recent studies in *P. berghei*, the causative agent of rodent malaria, have mutated two highly conserved residues in polymerase δ (D311A and E313A) to obtain a hypermutator parasite line with 86 to 90-fold higher mutation rates than the wildtype parasite (Honma et al., 2014, Honma et al., 2016).

Modelled after the novel studies with hypermutator *P. berghei* parasite lines, Kumpornsin *et al.* (*in preparation*) generated a mutant *P. falciparum* polymerase δ parasite line by mutating the two conserved residues (D308A and E310A) in the exonuclease domain reported to affect proofreading ability (Morrison and Sugino, 1994, Honma et al., 2014, Honma et al., 2016). Based on the similarity of the *P. falciparum* polymerase δ to its homologues in other eukaryotes (Ridley et al., 1991), it is hypothesized that mutations of the conserved exonuclease domain will also have a profound effect on mutation rates, as was observed in *P. berghei* (Honma et al., 2014, Honma et al., 2016). Though measurements of the spontaneous mutation rate of the *P. falciparum* polymerase δ mutant compared to the wildtype parasite are in progress, preliminary data by Dr. Kumpornsin indicated that the mutant parasite line had a higher propensity to yield drug resistance, observations that stimulated my use of this line for drug-pressure experiments with piperaquine.

Hypermutator lines can rapidly generate mutations under various environmental conditions, which can be useful in determining if certain conditions produce measurable phenotypes. The use of hypermutator lines enable forward genetics approaches to gather data in more feasible experimental timelines. Such lines also enable the possibility of examining whether genetic changes observed *in vivo* can be observed in *in vitro* settings. As discussed in the previous chapters, multiple independent studies (Agrawal et al., 2017, Ross et al., 2018a, van der Pluijm et al., 2019, Hamilton et al., 2019) have reported a role for mutations in the chloroquine resistance transporter (PfCRT) in the piperaquine-resistant phenotype. Early *in vitro* drug-pressure studies by Eastman *et al.* found a SNP in *pfcrt* (encoding a C101F mutation) and an amplification event upstream of the *pfmdr1* gene on chromosome 5 as well as a deamplification of *pfmdr1* (also on chromosome 5) to be associated with piperaquine-resistant parasites (Eastman et al., 2011), though the same mutations have not been identified in field isolates, to date. Recent studies by Agrawal *et al.*, van der Pluijm *et al.*, and Hamilton *et al.* have identified *pfcrt* SNPs in patient isolates from Southeast Asia that strongly associated with DHA-PPQ failures in patients (Hamilton et al., 2019, van der Pluijm et al., 2019, Agrawal et al., 2017). Ross *et al.* have used genetic editing techniques to demonstrate a role for several of these *pfcrt* SNPs in piperaquine resistance *in vitro (Ross et al., 2018a).* The ability to gather multiple lines of evidence for the role of various genes in resistant phenotypes greatly aids in surveillance of drug resistance. Such extensive confirmation also influences current treatment regimens and the development of new drugs.

As mentioned in Chapter 4, piperaquine treatment failures and reduced PPQ susceptibility *in vitro* was observed in patient isolates lacking the more recently reported mutations, for example the recently prevalent PfCRT T93S mutation (Hamilton et al., 2019) (**Chapter 2, Table 2.1**) (Amaratunga et al., 2016, Amato et al., 2017, Witkowski et al., 2017). However, most of the isolates used by our study from 2012-2013 (Amaratunga et al., 2016) contained at least one PfCRT mutation (H97Y, M343L, and one isolate with no PfCRT mutations) (**Chapter 2, Table 2.1**), though the GWAS study by Amato *et al.* did not find an association of mutations in PfCRT and the piperaquine resistant phenotype (Amato et al., 2017, Witkowski et al., 2017). The role of the other molecular markers of piperaquine resistance (*PM2-3* copy number amplification) in the resistant-phenotype also remains unclear. It is therefore necessary to combine forward and reverse genetic approaches to gain further insight into the mechanisms of piperaquine resistance.

The aim of this study was to elucidate the genetic determinants of piperaquine resistance by using *in vitro* drug selection methods. I used a hypermutator *P. falciparum* line to select for piperaquine-resistant parasites. Through WGS analyses of these piperaquine-resistant parasites, this study identifies nonsynonymous SNPs in multiple gene candidates that have not been previously linked to piperaquine resistance, including the *formate-nitrite transporter* (*FNT*), the *multidrug resistance protein 1* (*pfmdr1*), and the *multidrug resistance-associated protein 2* (*mrp2*). The study also identifies mutations in PfCRT, including an A366T mutation that is present in a piperaquine-resistant isolate used in this study (**Chapter 2, Table 2.1**) (Amaratunga et al., 2016). Additionally, this study identifies a *pfcrt* SNP resulting in a point mutation at position G353C, which is at the same position as a G353V mutation observed in field isolates and reported to demonstrate a piperaquine-resistant phenotype *in vitro* (Ross et al., 2018a). By combining these forward genetics tools with the reverse genetics approaches detailed in the previous chapters, this study aims to combine functional and genomic studies to investigate the mechanism of piperaquine resistance.

### 5.1.3 Objectives

Use a hypermutator *P. falciparum* line to select for a piperaquine-resistant phenotype *in vitro*

(1) Examine the phenotype of piperaquine-pressured parasites in comparison to the parental hypermutator line

(2) Use whole genome sequencing analysis to compare genetic differences between the parental hypermutator parasites and the piperaquine-pressured parasites

   a. Identify potential gene candidates that may play a role in the piperaquine-resistant phenotype

## 5.2 Materials and methods

### 5.2.1 Hypermutator parasites: *P. falciparum* Dd2-polymerase δ mutants

All experiments were performed with a hypermutator *P. falciparum* line generated by Dr. Krittikorn Kumpornsin (*manuscript in preparation*). The generation of this hypermutator was based on studies in *Saccharomyces cerevisiae* (yeast) and *P. berghei* that have shown that mutation of the conserved aspartic acid (D) and glutamic acid (E) residues in the exonuclease domain of DNA polymerase δ (**Figure 5.1**) significantly disrupts proofreading ability, causing a hypermutator phenotype (Morrison and Sugino, 1994, Fortune et al., 2005, Ridley et al., 1991, Honma et al., 2014, Honma et al., 2016).

To obtain a hypermutator *P. falciparum line*, Kumpornsin *et al.* used CRISPR-Cas9 gene editing to mutate two conserved residues (D308A and E310A, **Figure 5.1**) in the proofreading domain of DNA polymerase δ (PF3D7_1017000) in the laboratory line, Dd2. After confirmation of the desired edits in a clonal population of Dd2-polymerase δ D308A-E310A parasites, Dr. Kumpornsin kindly provided the line to be used in this study, referred to as "Dd2-hypermutator." Any description of a "parental line" in this study refers to the Dd2-hypermutator without drug-pressure.

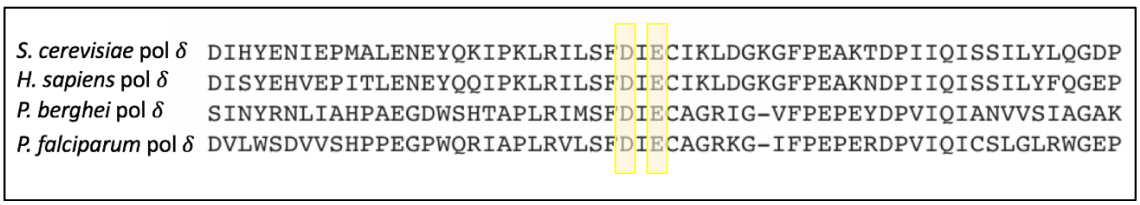



**Figure 5. 1**. **Alignment of the DNA polymerase δ amino acid sequence highlighting the conserved residues in the 3'→ 5'exonuclease domain.** Yeast (*S. cerevisiae*), human (*H. sapiens*), *P. berghei*, and *P. falciparum* all share the aspartic acid (D) and glutamic acid (E) residues in DNA polymerase δ exonuclease active site. The alignment was generated using the CLUSTAL O (1.2.4) multiple sequence alignment tool.

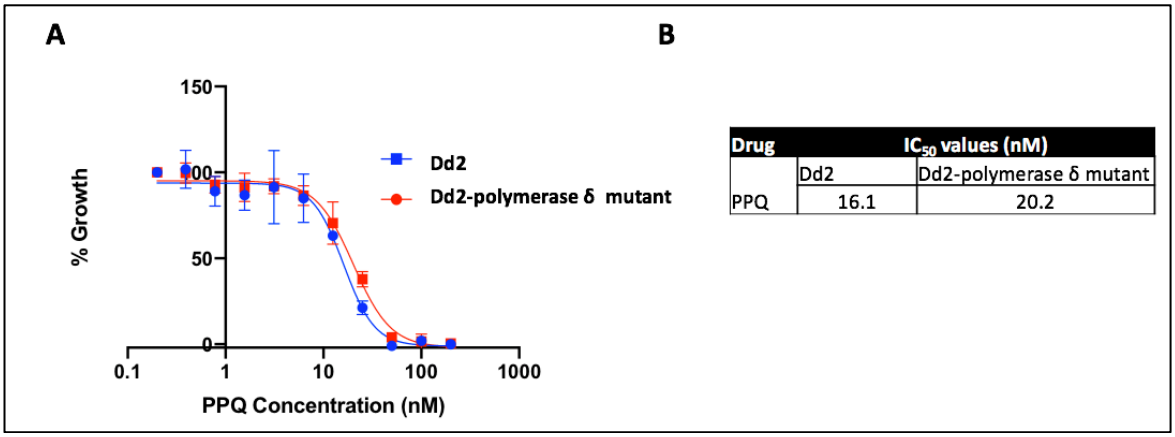
### 5.2.2 Experimental design for selection of piperaquine resistant Dd2-hypermutator parasites: $IC_{50}$ assays and selection procedures

Previous work has selected for PPQ-resistant parasites using a clone of the Dd2 parasite line (Eastman et al., 2011). Eastman *et al*. pressured the parasite with 47 nM and 140 nM PPQ based on the peak plasma levels of PPQ following a low-fat or high-fat meal, respectively (Ashley et al., 2004, Sim et al., 2005, Tarning et al., 2005). They observed that no parasites recrudesced after 80 days of continuous 140 nM PPQ-pressure but did observe one flask (of triplicate flasks) that recrudesced under 47 nM PPQ-pressure at day 54 (Eastman et al., 2011).

Based on this prior work, my study was designed to avoid a PPQ concentration that would be too high to obtain recrudescent parasites. In order to tip the drug-selection balance in our favor, I decided to base the PPQ concentration on the standard PPQ $IC_{50}$ values of the Dd2-hypermutator line. $IC_{50}$ assays were performed as previously described (**Chapter 2, section 2.6.2**) to assess the PPQ half maximal inhibitory concentration for the Dd2-hypermutator line in comparison to the Dd2 wildtype line (Dd2). Significance was determined using Mann-Whitney U tests comparing the $IC_{50}$ values of wildtype Dd2 with the Dd2-hypermutator line and no significant differences were observed between the $IC_{50}$ values for the two lines ($p>0.05$) (**Figure 5.2**). As seen in Figure 5.2B, the PPQ $IC_{50}$ values were between 16 nM-20 nM for both Dd2 and the Dd2-hypermutator line. I therefore decided to start PPQ-pressure at various increments of the lower of the two $IC_{50}$ values, 16 nM.

Two PPQ-selection attempts were made during the course of this study. The initial selection attempt was set up with $5 \times 10^8$ parasites per flask (in triplicate) at 3xPPQ $IC_{50}$ (48 nM) in 50 mL. As mentioned above, since spontaneous mutation rates of *P. falciparum* are quite low, in the second selection attempt, we increased the number of parasites to $1 \times 10^9$ parasites per flask (in triplicate) at 2xPPQ $IC_{50}$ (32 nM) in 100 mL. Parasites were grown as described previously (**Chapter 2, section 2.1.1**) in complete media (CM) supplemented with the desired concentration of PPQ at 3% hematocrit. During the first week of drug pressure, media was changed daily and fresh red blood cells (RBCs) were added as needed for cutting the flasks, since the parasitemia was quite high and piperaquine clearance of parasites took several days. Parasitemia was maintained below 8% and preferably maintained around 3% until the parasites cleared (no parasites were observed by smear). Continuous drug pressure was maintained for at least 21 days unless otherwise noted.

To account for spontaneous mutations that could be generated in the PPQ-pressured lines that are the result of the hypermutator line and not PPQ-pressure, the Dd2-hypermutator line was maintained in continuous culture for the duration of all experiments and was never pressured with PPQ.




| Drug | $IC_{50}$ values (nM) | |
|---|---|---|
| | Dd2 | Dd2-polymerase δ mutant |
| PPQ | 16.1 | 20.2 |

**Figure 5. 2. Determination of PPQ concentration to use for selection of PPQ-resistant Dd2-polymerase δ mutant parasites. A.)** Dose-response curve for Dd2 (wildtype) and Dd2-polymerase δ mutant (Dd2-hypermutator) parasites after exposure to PPQ. Error bars represent SD (n=2 biological replicates). **B.)** Table showing the average $IC_{50}$ values for Dd2 and the Dd2-polymerase $\delta$ mutant calculated using GraphPad Prism Version 8.0.2. Significance was determined using Mann-Whitney U tests comparing the $IC_{50}$ values of wildtype Dd2 with the Dd2-hypermutator line. No significant differences were observed between the two lines ($p>0.05$).

### 5.2.3 Preparation of PPQ-pressured parasites for whole genome sequencing analysis: cloning and genomic DNA extraction

Any parasites that recrudesced following PPQ-selection were expanded and the lines were cloned by limiting dilution (**Chapter 2, section 2.4**). Genomic DNA (gDNA) from the bulk cultures and the clones was extracted as described previously and all lines were cryopreserved (**Chapter 2, sections 2.1.3 and 2.5**). The control parental parasite line (Dd2-hypermutator parent) at the start of the experiment was also sent for sequencing analysis. The DNA concentration and quality were measured using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). DNA from the bulk PPQ-pressured cultures, clones, and the two parental controls was sent for WGS analysis at the Wellcome Sanger Institute.

### 5.2.4 Whole genome sequencing analysis

All WGS analyses were performed by Dr. Frank Schwach. Illumina sequence reads were mapped to *P. falciparum* genome version 3.1 using the Burrows-Wheeler Aligner (Li and Durbin, 2009) and the Picard toolkit. Local realignment by assembly as well as variant data calibration on known variants was performed using the GATK toolkit, following published

recommendations for the GATK toolkit (DePristo et al., 2011), and the MalariaGEN project (https://www.malariagen.net/). Known *P. falciparum* variants were obtained from the MalariaGEN project. The SNPeff software (Cingolani et al., 2012) was used to annotate functional implications of variants and variants that did not have an impact on a protein product were filtered out. In addition, a minimum coverage of 30 reads was required for the final set of high quality variants. Variants that were observed in the parental line were disregarded in order to exclude differences between Dd2 and the 3D7 reference genome. Variants in known highly variable gene families were also excluded from further analysis. The Dd2-hypermutator parental line was used as the reference sequence for identifying variations present in the PPQ-pressured parasite lines.

## 5.3 Results

### 5.3.1 Selection of piperaquine-resistant Dd2-hypermutator parasites

Two PPQ-selection experiments using the Dd2-hypermutator line were performed as outlined in **Figures 5.3-5.4**. The first selection experiment (Exp. 1, Figure 5.3), was started at 3xPPQ $IC_{50}$ and when parasitemia was still high on day 4, the parasites were brought up to 60 nM PPQ and then to 90 nM on day 6. By day 9 the parasites were starting to clear and no parasites were visible by microscopy on day 12. On day 22, the cultures were split and one set of flasks was maintained at 90 nM PPQ and the other set was taken off drug. No recrudescent parasites were observed after 65 days (**Figure 5.3**). We hypothesized that the lack of recrudescence was due to the number of starting parasites or the rapid ramping up of the concentration of PPQ.

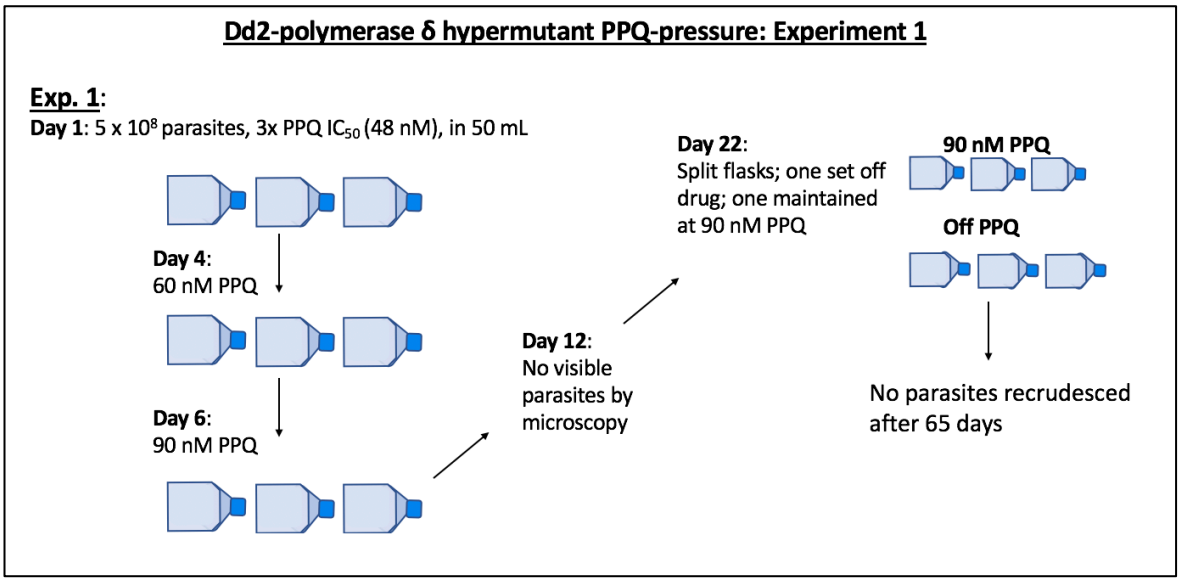



**Figure 5. 3. Initial attempt (experiment 1) for selection of piperaquine (PPQ)-resistant Dd2-hypermutator parasite lines.** No recrudescent parasites were observed.

The second selection experiment (Exp. 2) was set up with a higher number of parasites and a lower concentration of PPQ (**Figure 5.4**). As seen in Figure 5.4, the parasites were still surviving well on 2xPPQ $IC_{50}$ on day 6, so the flasks were split to 50 mL and one set was maintained at 2xPPQ $IC_{50}$ and the other set was brought up to 3xPPQ $IC_{50}$ (called 3x-day 6). On day 9, the 2xPPQ $IC_{50}$ parasites were still not clearing, so they were also brought to 3xPPQ $IC_{50}$ (called 3x-day 9). On day 13, there were no visible parasites by microscopy in any flasks but flasks were maintained and checked every 2-3 days for parasites (**Figure 5.4**). On day 21, recrudescence was observed in a 3x-day 9 flask (PPQ#1) (**Figure 5.4**). On day ~24 all flasks were taken off drug pressure including the PPQ#1 flask for which recrudescence was already observed. On day ~45, another 3x-day 9 flask (PPQ#2) recrudesced and on day 59 a 3x-day 6 flask recrudesced. The remaining flasks with no observed parasites were maintained in CM until days 65-70, but no parasites were observed.

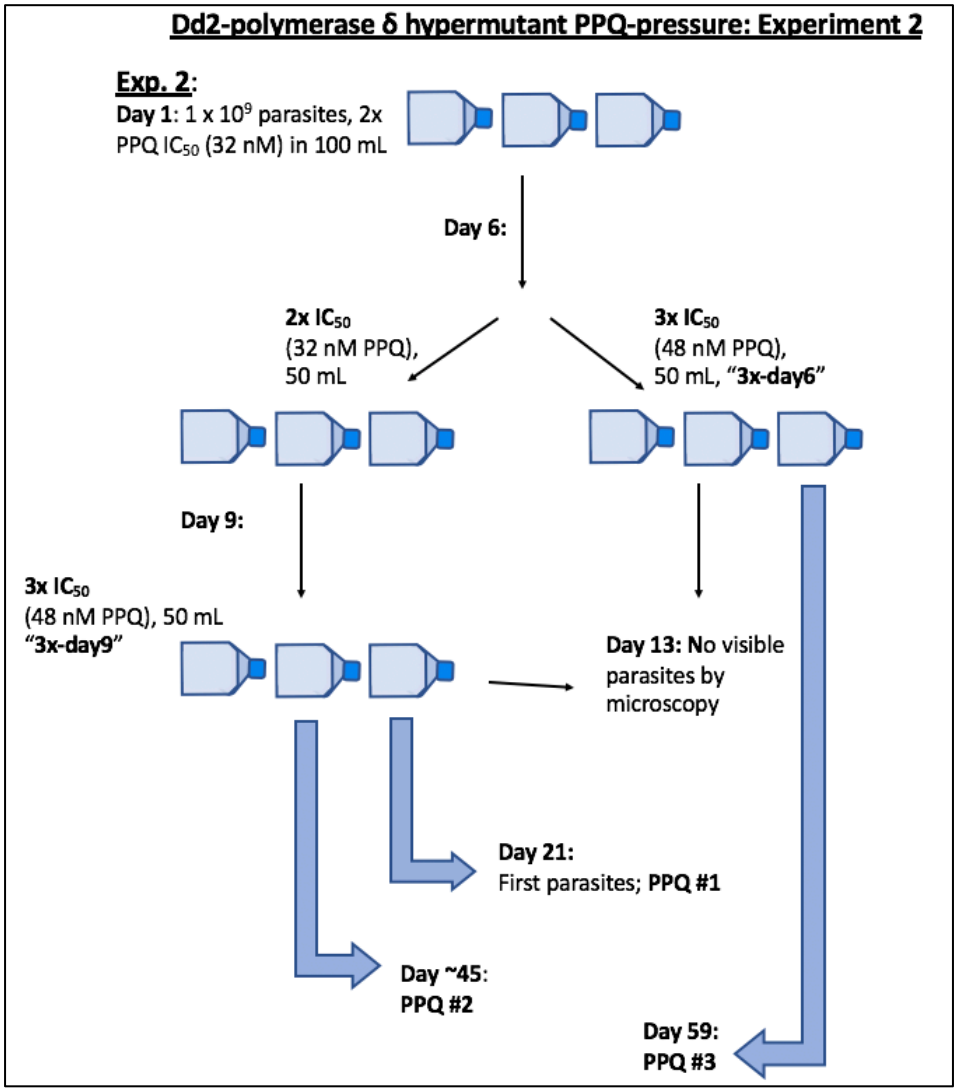


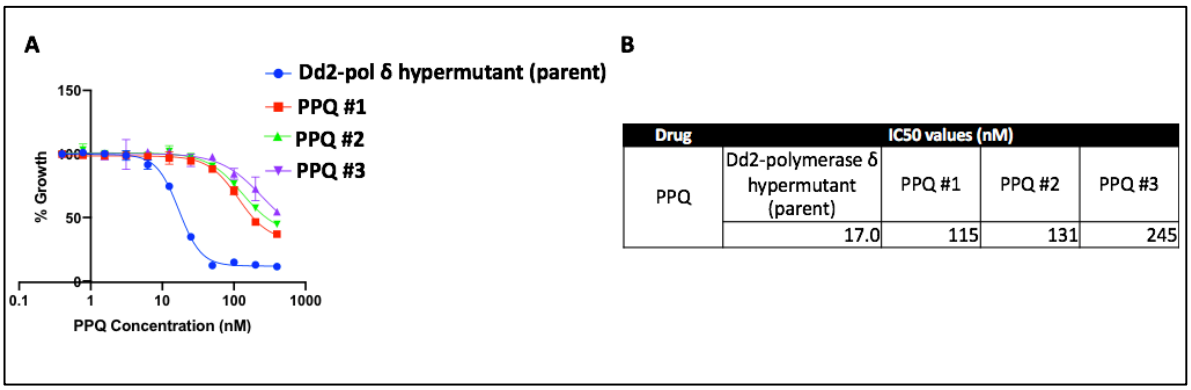

**Figure 5. 4. Second attempt (experiment 2) for selection of piperaquine (PPQ)-resistant Dd2-hypermutator parasite lines.**

Of the three parasite lines that came up on PPQ-pressure (PPQ#1-3), PPQ#1 and PPQ#3 were cloned and a total of 23 clones from PPQ-pressured parasites and all three bulk cultures (PPQ#1-3), were whole genome sequenced. As described by Honma *et al*., the clones were also referred to as "PPQ-pressured lines" since presumably, these isogenic clones would also acquire mutations while in continuous culture (Honma et al., 2014).

### 5.3.2 Phenotypic characterization of the piperaquine-pressured Dd2-polymerase δ mutant parasites

#### 5.3.2.1 $IC_{50}$ survival assays of piperaquine-pressured Dd2-polymerase δ mutant parasites

In order to determine if there were any phenotypic differences between the PPQ-pressured lines (PPQ#1-#3) and the parental Dd2-hypermutator line, $IC_{50}$ assays were performed to compare PPQ susceptibility (**Figure 5.5**). The dose-response curves show differences in susceptibility of the PPQ-pressured lines compared to the parental line. The parental line has an $IC_{50}$ similar to the value observed at the beginning of the drug-selection experiments, as expected (**Figure 5.2**) while the PPQ-pressured lines have $IC_{50}$ values that are shifted approximately 6-fold or more (**Figure 5.5**). Due to time constraints, $IC_{50}$ assays were only performed on the bulk PPQ-pressured lines, but analysis of the cloned PPQ-pressured lines is forthcoming. Also due to time constraints, determination of the optimal concentration of PPQ that results in complete death of the PPQ-pressured lines (PPQ#1-#3) is ongoing, therefore the dose response curves shown in Figure 5.5 are only intended to show the relative differences between the parental hypermutator line and the PPQ-pressured lines. As can be seen in Figure 5.5, without complete killing of the PPQ-pressured lines, the dose-response curves do not plateau and thus accurate $IC_{50}$ values cannot be calculated and statistical significance cannot be determined.




| Drug | IC50 values (nM) | | | |
|---|---|---|---|---|
| PPQ | Dd2-polymerase δ hypermutant (parent) | PPQ #1 | PPQ #2 | PPQ #3 |
| | 17.0 | 115 | 131 | 245 |

**Figure 5. 5**. **Comparison of PPQ susceptibility between the parental Dd2-polymerase δ hypermutator parasite and the PPQ-pressured lines. A.**) Estimated dose-response curve of Dd2-hypermutator (parent) line and PPQ-pressured lines (PPQ#1-#3). Error bars represent SD (n=2 technical replicates). **B.**) Table showing the estimated average $IC_{50}$ values for Dd2-hypermutator parent and PPQ-pressured lines. $IC_{50}$ values were calculated using GraphPad Prism Version 8.0.2. The concentration of PPQ was not high enough to result in complete killing of the three PPQ-pressured lines (PPQ#1-#3) so the dose-response curves and $IC_{50}$ values shown here are only estimates used to show the relative differences between the lines. Accurate $IC_{50}$ values and statistical significance cannot be determined from the data currently available, but continued assays with the PPQ-pressured lines are in progress.

### 5.3.2.2 Morphological differences between the PPQ-pressured parasites and the parental hypermutator line

Further analysis of the PPQ-pressured lines also indicated a starkly different morphological phenotype. Figure 5.6 shows Giemsa stained microscopy slides (**Chapter 2, section 2.1.2**) for both the Dd2-hypermutator parent and a PPQ-pressured line (**Figure 5.6**). As seen in the parental images, the parasites have the normal morphology of a mixed, asynchronous culture, with early ring stages and later trophozoites and schizonts (**Figure 5.6A**). In the PPQ-pressured parasite line, the late stage trophozoites and schizonts strikingly show large, distended digestive vacuoles (**Figure 5.6B**). This phenotype is noted in the presence and absence of drug, and the pictures included in Figure 5.6 are from a parasite that had been off PPQ-pressure for at least two weeks. The parasites have not been synchronized and ongoing work to compare stage-specific images for the parental and PPQ-pressured parasites is necessary.

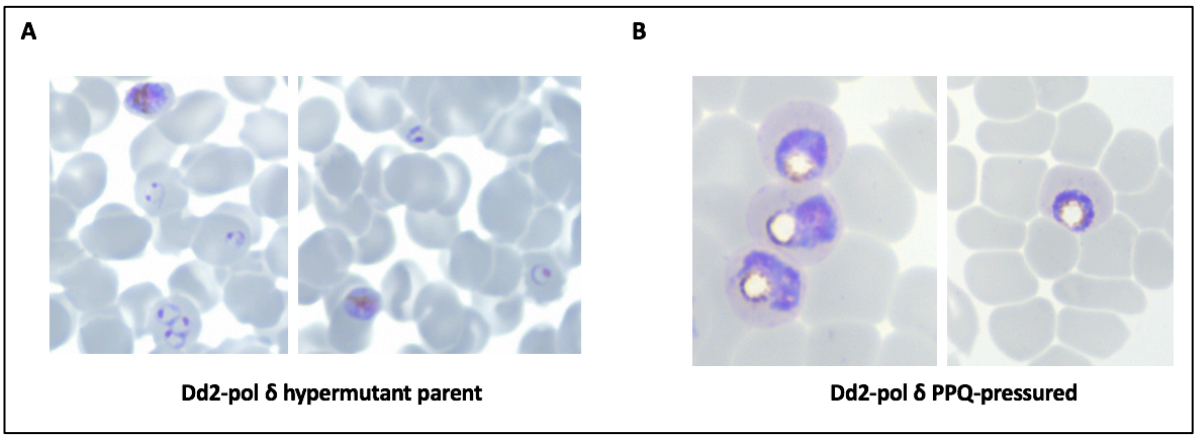



**Figure 5. 6**. **Morphological difference between the parental Dd2-polymerase δ hypermutator parasite and a PPQ-pressured line. A.**) Dd2-polymerase δ hypermutator parental line **B.**) PPQ-pressured line showing large digestive vacuoles in the absence of PPQ. Slides were viewed with a light microscope using a 100X immersion field. Pictures were taken with a Leica EC4 Digital Microscope camera.

### 5.3.3 Whole genome sequencing analysis and genetic characterization of the PPQ-pressured Dd2-hypermutator parasites

Whole genome sequencing was performed for the parental Dd2-hypermutator line, the bulk PPQ-pressured cultures (**Figure 5.4**), and the cloned PPQ-pressured lines (23 total clones) obtained from bulk cultures "PPQ#1" and "PPQ#3" Two controls were sequenced (section 5.2.3), the Dd2-hypermutator line at the beginning for the experiment and the same parent line at the end of the experiment at the time the clones and bulk cultures were collected for sequencing. Preliminary alignments mapped the PPQ-pressured lines to the parental line at the start of the experiment as the reference.

Initial analyses of the 23 PPQ-pressured lines in comparison to the Dd2-hypermutator parent have revealed multiple non-synonymous SNPs in genes previously reported to play a role in antimalarial drug resistance (**Table 5.1**): *pfcrt*, *pfmdr1*, *formate-nitrite transporter* (*FNT*), and *multidrug resistance-associated protein 2* (**Figure 5.7**). The study identified *pfcrt* SNPs that resulted in the following mutations: N295T, G353C, A366T, D377Y, and T380S. The PfCRT A366 and G353 residues have been previously implicated in piperaquine resistance (Ross et al., 2018a). SNPs were found in other genes but due to time constraints, the study was specifically interested in looking at SNPs in transporter genes. Additionally, the SNPs listed in Table 5.1 are not present in each clone and vary by line. Future work dissecting out the various mutations in each clone and analyzing the full read-out of SNPs from the WGS analysis is necessary and planned.

The WGS analyses also identified a PfMDR1-N86I mutation in the PPQ-pressured lines (**Figure 5.7**) which is the same residue of a N86Y natural variant previously reported by multiple studies to affect susceptibility to various antimalarial drugs (Veiga et al., 2016b, Sisowath et al., 2005, Duraisingh and Cowman, 2005). SNPs were also discovered in two genes coding for transporters that have been implicated in antimalarial drug resistance, *FNT-A224T* and *FNT-S17F* (Hapuarachchi et al., 2017) and *pfmrp2-D1028Y* (Cowell et al., 2018, Veiga et al., 2014).

**Table 5. 1 Non-synonymous SNPs discovered in the PPQ-pressured Dd2-hypermutator lines**

| Chr. | Position | Gene ID | Gene Description | Alteration | Reported in field |
|---|---|---|---|---|---|
| 7 | 405005 | PF3D7_0709000 | chloroquine resistance transporter (CRT) | p.Asn295Thr | N |
| 7 | 405590 | PF3D7_0709000 | chloroquine resistance transporter (CRT) | p.Gly353Cys | Y, G353V |
| 7 | 405629 | PF3D7_0709000 | chloroquine resistance transporter (CRT) | p.Ala366Thr | Y |
| 7 | 405855 | PF3D7_0709000 | chloroquine resistance transporter (CRT) | p.Asp377Tyr | N |
| 7 | 405864 | PF3D7_0709000 | chloroquine resistance transporter (CRT) | p.Thr380Ser | N |
| 5 | 958146 | PF3D7_0523000 | multidrug resistance protein 1 (mdr1) | p.Asn86Ile | Y, N86Y |
| 3 | 669939 | PF3D7_0316600 | formate nitrite transporter | p.Ala224Thr | N |
| 3 | 670824 | PF3D7_0316600 | formate nitrite transporter | p.Ser17Phe | N |
| 12 | 1196133 | PF3D7_1229100 | multidrug resistance-associated protein 2 (mrp2) | p.Asp1028Tyr | N |

**Table 5.1**. Representation of several non-synonymous SNPs discovered in the PPQ-pressured Dd2-hypermutator lines. The SNPs in each of the 23 clones were different and this table shows a general subset of genes with the SNPs discovered in each. Further analysis of the WGS data is planned for assessing and comparing the genotypes of all clones. All genes included in this table have previously been reported to be involved in antimalarial drug resistance. Each gene is listed with: chromosome number (chr.); nucleotide position (position); gene ID and description; type and the amino acid difference (alteration). The last column includes if the SNP has been previously reported in field isolates.

## 5.4 Discussion and future work

The purpose of this study was to employ a forward genetics approach to gain further insight into the mechanisms of piperaquine resistance. This study has utilized a *P. falciparum* hypermutator line to select for a piperaquine-resistant phenotype *in vitro*. I was able to generate piperaquine-resistant parasites in a feasible experimental timespan with the first parasites demonstrating a PPQ-resistant phenotype by day 21 (**Figure 5.4**). The other two PPQ-resistant lines recrudesced within two months from the start of the experiment (days 40 and 59, **Figure 5.4**). The use of the hypermutator *P. falciparum* polymerase $\delta$ mutant parasite appears to have greatly reduced the amount of time it took to generate parasites with a resistant phenotype. Though studies directly comparing the unedited Dd2 line (wildtype polymerase $\delta$) (**Figure 5.2**) to the Dd2-hypermutator under PPQ pressure would be able to confirm if this suggestion holds in practice. Previous work by Eastman *et al.* pressured a Dd2 clone (Dd2 1pa) and 7G8 isolate and obtained only one recrudescent Dd2 flask (of triplicate

flasks) after 54 days (Eastman et al., 2011). This is likely due to the slow spontaneous mutation rate of *P. falciparum* (Bopp et al., 2013) and the concentration of PPQ used. As shown through the studies of Oduola *et al.* in obtaining a mefloquine resistant *P. falciparum* line, generation of a mutant parasite can require continuous culture for over a year, depending on the drug and protocol used (Oduola et al., 1988a). Drug selection experiments often entail a bit of trial and error, based on what seems most appropriate for the particular parasite line and the specific drug of interest. However, hypermutator lines may tip the balance in favor of selecting a resistant-phenotype.

WGS of the piperaquine-resistant lines obtained through these experiments have identified nonsynonymous SNPs in gene candidates that have previously been reported to play a role in antimalarial drug resistance (**Table 5.1**), including SNPs in the transporter genes: *chloroquine resistance transporter* (*pfcrt* PF3D7_0709000), *multidrug resistance protein 1* (*pfmdr1*, PF3D7_0523000, *formate-nitrite transporter* (*FNT*, PF3D7_0316600), and *multidrug resistance-associated protein 2* (*mrp2*, PF3D7_1229100).

Recent studies have discovered SNPs in *pfcrt* that confer piperaquine resistance *in vitro* and strongly associate with DHA-PPQ failures in patients (Agrawal et al., 2017, Ross et al., 2018a, van der Pluijm et al., 2019, Hamilton et al., 2019). Interestingly, two of the *pfcrt* SNPs discovered by this study, *pfcrt A366T* and *pfcrt G353C* have been observed in field isolates and associated with PPQ-resistance *in vitro*. The *pfcrt A366T* SNP discovered in our WGS analysis is present in a PPQ-resistant field isolate from Pursat, Cambodia that has been used by this study (**Chapter 2, Table 2.1**) and has been reported in Cambodian isolates by other studies (Ross et al., 2018a). The *pfcrt G353C* SNP from our study results in a point mutation at residue G353, which has previously been found to harbor a G353V mutation in field isolates. Gene editing of the *pfcrt G353V* SNP was shown to confer PPQ-resistance *in vitro* (Ross et al., 2018a). Further work by Ross *et al*. edited several other *pfcrt* SNPs present in field isolates, *H97Y*, *F145I*, *M343L*, into Dd2 parasites and found that the gene-edited *pfcrt* mutations alone could confer PPQ-resistance *in vitro*, though a fitness cost was noted for all SNPs but the PfCRT M343L variant (Ross et al., 2018a).

As noted in **Figure 5.6**, I observed large, distended digestive vacuoles in the PPQ-pressured lines. Ross *et al*. also reported this phenotype in their *pfcrt*-edited parasites, including the G353V edited line (Ross et al., 2018a). Previous studies by Pulcini *et al*. have also reported distended vacuoles in parasites pressured with amantadine or blasticidin that contain PfCRT

C101F or L272F mutations (Pulcini et al., 2015). Intriguingly, the light microscopy images published by Ross *et al.* and Pulcini *et al.* display striking similarity to the images shown in this thesis, pictured in Figure 5.6 (Pulcini et al., 2015, Ross et al., 2018a). It is acknowledged that the parasites shown in Figure 5.6 are asynchronous and the parental and PPQ-resistant lines are not showing the same stage parasites. However, this preliminary indication of a distinct morphological phenotype will guide future work to demonstrate this vacuole phenotype in synchronised parasites. Intriguingly, Ross *et al.* noted that the distended vacuole phenotype was not observed in the field isolates containing these mutations (Ross et al., 2018a). PPQ-resistant isolates may have compensated for this effect through other genetic pathways and that the observed phenotype may be dependent on the genetic background of the parasite. Future work planned by this study will examine if the *plasmepsin 2-3* (*PM2-3*) copy number amplification has any effect on the PPQ-pressured hypermutator lines. We plan to transfect the PPQ-pressured lines with the *PM2-3* overexpression plasmid generated in Chapter 3. This will enable us to see if the *PM2-3* amplification, and consequent increase in abundance of these vacuolar proteases, has any effect on the PPQ-resistant phenotype and will enable us to see if this amplification may reduce the distended vacuole phenotype.

The other transporters with nonsynonymous SNPs discovered by this study: PfMDR, FNT, and PfMRP2 have all previously been shown to play a role in antimalarial drug resistance and could have a role in PPQ-resistance. The N86Y mutation in PfMDR1 is associated with chloroquine and amodiaquine resistance, but reported to increase susceptibility to lumefantrine, mefloquine, and dihydroartemisinin and moderately to piperaquine (Veiga et al., 2016b). Recent drug-pressure experiments used to screen new compounds with antiplasmodial activity found mutations in the lactate/H+ transporter, FNT, specifically FNT-G107S, to reduce susceptibility to two of these novel, structurally similar compounds, MMV007839 and MMV000972 (Hapuarachchi et al., 2017). The study by Hapuarachchi *et* al. (Hapuarachchi et al., 2017) is the first to report compounds that target FNT, which is located on the parasite cell membrane and functions to remove lactic acid, a waste product from glycolysis (Marchetti et al., 2015, Wu et al., 2015). Prior work assessing mrp2 polymorphisms reported a high frequency of SNPs within the gene as well as microindels, which have been shown to reduce parasite susceptibility to quinolone drugs (Veiga et al., 2014, Mok et al., 2014). It is not clear what role these SNPs could play in PPQ resistance, but ability of *pfmdr1* and *pfmrp2* SNPs to have an effect on quinolone drugs could also apply to piperaquine.

The intriguing results of selection pressure experiments and WGS analyses have clearly demonstrated the need for future studies. Continued examination of the WGS data is in progress. Importantly, analysis of copy number variations (CNVs) is ongoing. It will be interesting to learn if any CNVs are present in the *PM2-3* genes or any other genes. In most PPQ-resistant isolates observed to date, the parasites have a single copy of *pfmdr1* (Amato et al., 2017, Witkowski et al., 2017). The Dd2 wildtype parasite has two copies of *pfmdr1* and presumably the Dd2 polymerase $\delta$ hypermutator line used by this study has two copies of *pfmdr1*. However, it is important to check if the increased copy numbers have been retained in the PPQ-resistant lines. Studies have hypothesized that PfMDR1 could facilitate PPQ entry into the digestive vacuole (Witkowski et al., 2017), which could explain the presence of single *pfmdr1* copy numbers in field isolates from regions where DHA-PPQ has been used. Other *in vitro* studies have reported a deamplification on chromosome 5 in the region with *pfmdr1* after exposure to piperaquine (Eastman et al., 2011), thus there is precedence for further investigation of the role of PfMDR1 in the PPQ-resistant phenotype.

Further analysis of the WGS data will also enable us to determine which gene candidates to target for further study. CRISPR-Cas9 gene editing can be used to evaluate non-synonymous SNPs. It is also necessary to compare growth rates of the parasites and conduct competition assays to determine if there are any fitness effects of the acquired mutations. Cross-resistance studies with different antimalarial drugs will also provide vital insight into the characterization of the PPQ-pressured lines.

It is imperative to discuss that there are inherent caveats when using hypermutator parasites. The ability of the parasite to develop mutations more rapidly would mean that drug-pressured parasites may also rapidly acquire mutations that have nothing to do with the drug pressure. This can add unnecessary noise when trying to filter out the genetic variants that play a causal role in the resistant phenotype. It is also possible that while cloning to obtain isogenic parasites, the parasites are acquiring further mutations that have nothing to do with the resistance observed. However, this can be controlled for by performing multiple independent drug-pressure selections. I have performed the piperaquine pressure experiments in triplicate flasks, thus hits obtained across multiple independent selections adds confidence that the variations identified are due to drug pressure, and are not random. We have also included a control in this study to assuage, not solve, these limitations. The parental-hypermutator line from the start of the experiment was maintained in culture for as long as the other drug-pressured lines, but it was never pressured with PPQ. In addition to sequencing the parental

line at the start of the experiment, we also sequenced this parental line that was grown for the duration of the experiment. As mentioned, due to time constraints, we have only been able to compare the PPQ-pressured lines to the parental line from the start of the experiment. However, comparison of both parental lines parental-start and parental-end will be performed. When we are able to compare the parental-start and parental-end genotypes, we will be able to estimate the spontaneous mutation rate of the parasite and this information can provide insight into how long the PPQ-pressured cloned lines can be kept in continuous culture. We will also be able to compare mutation rates of the PPQ-pressured lines. This information can inform future drug selection experiments.

Through the use of a hypermutator line, this study provides new insights into the PPQ-resistant phenotype while providing experimental evidence that supports previously reported markers of PPQ resistance. This study may also serve as a proof-of-concept study for using a hypermutator line for *in vitro* drug selection. Though the data presented here is preliminary, it offers promising potential and clearly defines future work needed in the quest to understand the mechanisms of PPQ resistance.