Chapter 4: Examination of the role of single nucleotide polymorphisms in an exonuclease protein and mitochondrial carrier protein in piperaquine resistance

4.1 Declaration of work

The following chapter includes data obtained from plasmids designed by Dr. Marcus Lee and edited parasite lines obtained from Dr. Manuela Carrasquilla. Dr. Lee provided all plasmids used for CRISPR-Cas9 editing in this study. Dr. Carrasquilla generated Dd2-edited parasite lines that contained the wildtype and mutant exonuclease SNPs. Both Drs. Lee and Carrasquilla are cited accordingly in the relevant sections of this chapter for their generous contributions to this work.

4.1.1 Significance and purpose of study

Advances in genetic studies and technologies have greatly enhanced our current understanding of malaria parasites. Whole genome sequencing (WGS) technologies allow for real-time monitoring and detection of genetic changes in parasite populations throughout widespread endemic areas. These technologies enable us to track genetic changes as they occur and provide useful information for both control efforts and the potential elimination of the disease (Ariey et al., 2014, Miotto et al., 2015, Manske et al., 2012, Wootton et al., 2002, Dondorp et al., 2009). With the use of genome wide association studies (GWAS), we can identify potential molecular markers of drug resistance by comparing single nucleotide polymorphisms (SNPs), copy number variations, insertion and deletion polymorphisms (indels), and other genetic changes in parasite populations. However, the identified molecular markers of resistance may have no phenotypic relevance or causal relationship to the resistance observed. Alternatively, the molecular marker could have a more indirect impact on resistance, for example to compensate for the fitness cost of another mutation. In order to understand how drug resistance develops, and definitively test whether a marker is necessary and sufficient to confer resistance, it is necessary to perform functional studies. We can harness the clues GWAS provides and use them as a roadmap for assessing the mechanisms of resistance of various antimalarial drugs. The purpose of this study was to use CRISPR-Cas9 gene editing to investigate the role of two nonsynonymous SNPs associated with piperaquine resistance: a putative exonuclease protein SNP, PF3D7_1362500 (*exo-E415G* SNP), and a putative mitochondrial carrier protein. SNP, PF3D7_1368700 (*mcp-N252D*

SNP) (Amato et al., 2017). This study fills a knowledge gap in the lack of functional data for molecular markers of piperaquine resistance by examining phenotypic and functional relevance of the genotypes observed.

4.1.2 Introduction

The ability of the *P. falciparum* parasite to adapt to the selective pressure of antimalarial drugs is a major impediment to treatment regimens. The use of specific antimalarial drugs in distinct geographic regions can also have an immense effect on the evolution of parasite populations in different areas. It is therefore necessary to have a means to detect and track these genetic changes as they occur. Whole genome sequencing (WGS) of *P. falciparum* was first published in 2002 (Gardner et al., 2002, Hall et al., 2002) and such technologies have revolutionized malaria control and treatment programs by enabling widespread surveillance of malaria parasite populations throughout the globe. When combined with clinical studies and *in vitro* drug susceptibility profiles, genome wide association studies (GWAS) enable identification and genetic tracking of molecular markers of resistance (Ariey et al., 2014, Miotto et al., 2015, Manske et al., 2012, Wootton et al., 2002, Dondorp et al., 2009, Amato et al., 2017, Witkowski et al., 2017, Imwong et al., 2017). This information can provide clues for uncovering mechanisms of resistance, though molecular markers may eventually be found to play little to no causal role in the drug-resistant phenotype. As artemisinin and partner drug resistance continue to spread throughout Southeast Asia (SEA), it is crucial to elucidate and understand mechanisms of antimalarial drug resistance in order to aptly inform current and future malaria treatment strategies.

As detailed in Chapter 1, artemisinin combination therapies (ACTs) are the first line treatment for *P. falciparum* malaria and function by combining a potent short-acting artemisinin derivative with a long-acting partner drug with a different mechanism of action (World Health Organization, 2018). Over the last decade, dihydroartemisinin-piperaquine (DHA-PPQ) has been the ACT commonly used in many countries in SEA, including Cambodia, Thailand, Myanmar, and Vietnam, among others (World Health Organization, 2015). However, both artemisinin and piperaquine resistance have been reported at multiple sites in Cambodia and neighboring countries (Cui et al., 2015), with the most recent reports from 2015-2018 documenting an even further decline of DHA-PPQ efficacy, with efficacy rates of 12%, 38%, and 47% in Northeastern Thailand, Western Cambodia, and Southwestern Vietnam, respectively (van der Pluijm et al., 2019).

In order to monitor and investigate the causes of DHA-PPQ resistance, multiple studies have reported independent molecular markers of resistance for both artemisinin and piperaquine resistance. Several mutations in the *kelch 13* (K13) gene have been accepted as markers of artemisinin resistance (Ariey et al., 2014, Miotto et al., 2013) and multiple studies (outside the scope of this doctoral thesis) have focused on determining the mechanism of artemisinin resistance (Meshnick et al., 1991, Tilley et al., 2016, Bridgford et al., 2018, del Pilar Crespo et al., 2008, Klonis et al., 2013, Heller and Roepe, 2019, Mok et al., 2015, Mok et al., 2011, Rocamora et al., 2018, Sa et al., 2018, Straimer et al., 2015), which is a complex and unfinished story. Molecular markers of piperaquine resistance were first reported in 2017 (Amato et al., 2017, Witkowski et al., 2017) and have emerged on a K13 mutant background (Imwong et al., 2017, Amato et al., 2018). As discussed in Chapter 3, amplification of the *plasmepsin 2* and *plasmepsin 3* (*PM2-3*) genes on chromosome 14 associates with DHA-PPQ failures in patients (Witkowski et al., 2017, Amato et al., 2017) and the amplification is currently used as a reliable marker of piperaquine resistance (Chapter 3) (Jacob et al., 2019, van der Pluijm et al., 2019, Hamilton et al., 2019). Additional *in vitro* studies (Agrawal et al., 2017, Dhingra et al., 2017, Ross et al., 2018a) have reported mutations in the *chloroquine resistance transporter* gene (*pfcrt*) that confer variable degrees of piperaquine resistance. Following these reports, more recent genetic studies (Hamilton et al., 2019) have identified an increased frequency of previously reported (Agrawal et al., 2017, Ross et al., 2018a) and novel mutations in PfCRT that have arisen on an amplified *PM2-3* background that significantly associate with decreased piperaquine susceptibility. However, before these PfCRT mutations were prevalent, piperaquine treatment failures and parasites with decreased *in vitro* piperaquine susceptibility were present on PfCRT backgrounds lacking these mutations (Amato et al., 2017) (**Chapter 2, Table 2.1**). Consequently, many questions remain in the pursuit to discover the genetic determinants of piperaquine resistance.

At the time this thesis commenced, a genome-wide association study (GWAS) (Amato et al., 2017) of 297 parasite isolates exposed to DHA-PPQ identified SNPs that strongly associate with reduced PPQ susceptibility *in vitro* and DHA-PPQ failures in patients detailed in Table 4.1. The top most significant nonsynonymous SNPs are located on chromosome 13 in a putative exonuclease (*exo-E415G*) and a mitochondrial carrier protein (*mcp-N252D*) (**Table 4.1**). Unlike the above-mentioned markers of piperaquine resistance, no *in vitro* work has been reported on the role of the *exo-E415G* or *mcp-N252D* SNPs in the piperaquine-resistant phenotype.

Table 4. 1 Top GWAS candidate SNPs associated with increased piperaquine IC_{50} values

Chr.	Position	Gene ID	Gene Description	N/S	Alteration	p value
13	2,504,560	PF3D7_1362500	exonuclease, putative	N	p.Glu415Gly	2.69×10^{-9}
12	418,346	PF3D7_1208900	conserved *Plasmodium* protein, unknown function	S	p.981Pro	4.80×10^{-8}
13	2,728,402	PF3D7_1368700	mitochondrial carrier protein, putative	N	p.Asn252Asp	2.33×10^{-7}
13	2,512,415	PF3D7_1362700	conserved *Plasmodium* protein, unknown function	S	p.687Asn	6.25×10^{-7}
13	2,519,091	PF3D7_1362800	conserved *Plasmodium* protein, unknown function	N	p.Gly202Asp	2.12×10^{-6}
13	2,447,146	PF3D7_1361000	arginine methyltransferase 5, putative (PRMT5)	N	p.Asn40Ser	3.96×10^{-6}
4	904,088	PF3D7_0420000	zinc finger protein, putative	S	p.213Leu	7.67×10^{-6}
14	2,411,942	PF3D7_1458700	conserved *Plasmodium* protein, unknown function	N	p.Arg25Lys	1.27×10^{-5}
14	2,395,752	PF3D7_1458300	conserved *Plasmodium* protein, unknown function	N	p.Ile301Phe	2.03×10^{-5}
13	2,542,366	PF3D7_1363300	mitochondrial ribosomal protein L9 precursor, putative	N	p.Thr28Ile	2.48×10^{-5}

Table 4.1, adapted from Amato *et al.* 2017 (Amato et al., 2017), displays the top ten GWAS candidate SNPs associated with increased piperaquine IC_{50} values in order of increasing p-value. Each gene is listed with: chromosome number (chr.); nucleotide position (position); gene ID and description; type of SNP: nonsynonymous (N) or synonymous (S); the amino acid difference (alteration); and p-value.

The nonsynonymous SNP in the putative exonuclease protein, PF3D7_1362500, encodes a glutamic acid (E) to glycine (G) substitution at position 415 and the prevalence of this SNP has increased along with the *PM2-3* amplification in Cambodia from the time period of 2012-2015 (**Figure 4.1**) (Amaratunga et al., 2019). Though sparse information is known about this protein in *P. falciparum*, the putative exonuclease contains domains with sequence similarity to 3'- 5' exonucleases from other organisms including both prokaryotes and eukaryotes (PlasmoDB; NCBI BLAST). However, sufficient data is currently not available for generating a proposed model for the domain structures of the putative exonuclease, PF3D7_1362500.

As implied by their name, exonucleases are enzymes that cleave nucleotides from DNA at the 3' or 5' ends by hydrolyzing the phosphodiester bonds between nucleic acids (Shevelev and Hübscher, 2002, Lovett, 2011). This is a common feature of some polymerases that have "exo activity" or proofreading activity and is distinguished from endonuclease activity because exonucleases excise from the end of the DNA chain as opposed to internally (Lovett, 2011). Prior research has shown that 3'-5' exonuclease activity is also needed in double-stranded break repair and in non-homologous end joining (NHEJ) showing a role for exonucleases not only in DNA replication but also in distinct repair processes (Shevelev and Hübscher, 2002).

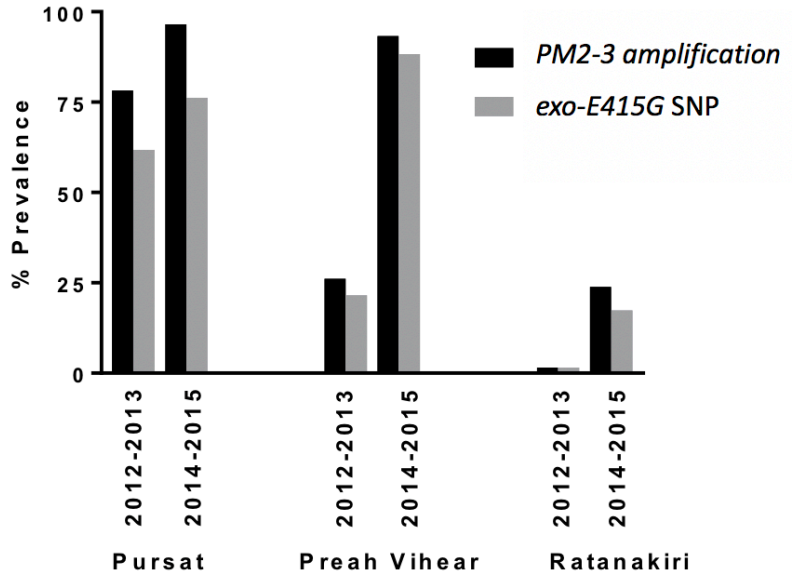


Figure 4. 1. Prevalence of the *PM2-3* copy number amplification and *exo-E415G* SNP in three provinces in Cambodia from 2012-2015. (Adapted from Amaratunga *et al.* (Amaratunga et al., 2019) with *PM2-3* CNV data obtained from Chapter 3.)

This study hypothesized that the *P. falciparum* putative exonuclease plays a 3'- 5' proofreading role during DNA replication and perhaps even DNA repair. It is uncertain what role, if any, the *exo-E415G* SNP plays in piperaquine-resistant *P. falciparum*, however one possibility is that the SNP affects the proofreading or repair ability of the putative protein, leading to significant differences (increases or decreases) in the overall mutations in the parasite, which might aid in the resistant phenotype. Studies by Lee and Fidock (2016) examined mutator rates of Cambodian isolates with mutations in repair genes in comparison to reference laboratory strains without mutations and showed only a mild increase in overall mutations in Cambodian isolates possessing the mutant repair genes (Lee and Fidock, 2016). The role of the *exo-E415G* SNP could thus be directly related to survival or fill a milder, indirect role in resistance via fitness or compensatory measures. This thesis therefore hopes to provide insight into the role the *exo-E415G* SNP plays in the parasite response to piperaquine.

Similar to the exonuclease, little is currently known about the putative mitochondrial carrier protein (MCP). The nonsynonymous SNP in the putative MCP, PF3D7_1368700, encodes an asparagine (N) to aspartic acid (D) substitution at position 252 (*mcp-N252D*). Mitochondria are vital organelles in all eukaryotic organisms, serving key roles in metabolism, apoptosis, the breakdown of amino acids, and many other processes. MCPs are a large family of

proteins that mediate transport of molecules across the mitochondrial membranes. MCPs thus enable mitochondrial activities and connect processes in the cytosol with those in the mitochondrial matrix (Wohlrab, 2009). MCPs are nuclearly encoded and contain inner targeting signals that direct them to the inner mitochondrial membrane, where MCPs transport molecules such as adenosine triphosphate (ATP) or protons (H+) to establish membrane gradients (Kunji, 2004). Sequence analysis of the PF3D7_1368700 putative MCP using PlasmoDB and BLAST, shows that this molecule shares amino acid sequence similarity with other MCPs, specifically with the yeast ADP/ATP carriers, Aac2p and Aac3p (Ruprecht et al., 2014). Conserved motifs for MCPs consist of a PX(D/E)XX(K/R) motif (Ferramosca and Zara, 2013, Robinson and Kunji, 2006, Kunji, 2004), called the carrier signature and sequence analysis by this study suggests the motif is present in the PF3D7_1368700 MCP as PGDTVR. Similar to the putative exonuclease protein, not enough information is available to generate a model for the domain structures of the MCP, PF3D7_1368700.

Antimalarial drug resistance in *P. falciparum* has long been associated with genetic variation in malaria transporters (PfCRT, PfMDR1, PfMDR2) (Ecker et al., 2012, Eastman et al., 2011, Kirk, 2001, Sauvage et al., 2009) but no studies have yet reported a role for the putative MCP PF3D7_1368700 in resistance. However, studies by Cowell *et al*. (Cowell et al., 2018) have identified mutations in another mitochondrial protein on chromosome 1, PF3D7_0108400, after *in vitro* drug selection experiments with several different compounds. Due to the significant correlation of DHA-PPQ failures and the presence of this *mcp-N252D* SNP, I decided to test if the putative MCP, PF3D7_1368700, could also have a functional role in facilitating piperaquine resistance. It should be stated that like the *exo-E415G* SNP, it is possible the *mcp-N252D* SNP could have a modest role in resistance and instead serve a compensatory role in modulating parasite fitness. It is also entirely plausible that both SNPs are unrelated to piperaquine resistance. Furthermore, the putative proteins could behave very differently in *P. falciparum* when compared to the homologous proteins in other organisms from which they inherited their putative names due to sequence similarities. Thus, further genetic studies are imperative to shed light on these questions and postulations.

The goal of this study is to evaluate the potential role of the resistance-associated SNPs, *exo-E415G* and *mcp-N252D*, in susceptibility to piperaquine. To examine these genetic associations, I used CRISPR-Cas9-based genome editing to dissect out the potential individual contributions of each of these candidates in an isogenic parasite background, the

Southeast Asian lab-adapted line Dd2 and in piperaquine-sensitive and resistant Cambodian isolates that contain the wildtype and mutant SNPs, respectively. I then examined if there were any phenotypic differences in the transgenic parasites by analyzing the drug susceptibility profiles of each edited line in response to piperaquine and other commonly used antimalarial drugs.

4.1.3 Objectives

Investigate the role of the putative exonuclease (PF3D7_1362500) and mitochondrial carrier protein (PF3D7_1368700) single nucleotide polymorphisms (SNPs) in piperaquine resistance

(1) Investigate whether the exonuclease nonsynonymous SNP on chromosome 13, *exo-E415G* SNP, confers piperaquine resistance and characterize any additional phenotypes
    a. Use CRISPR-Cas9 to insert the *exo-E415G* SNP into the lab strain, Dd2 and Cambodian field isolates
    b. Characterize the *exo-E415G* SNP in phenotypic assays
(2) Evaluate the role of the putative mitochondrial carrier protein (PF3D7_1368700) nonsynonymous SNP on chromosome 13, *mcp-N252D*, in piperaquine resistance
    a. Use CRISPR-Cas9 to insert the *mcp*-N252D SNP into the lab strain, Dd2 and Cambodian field isolates
    b. Characterize the *mcp*-N252D SNP in phenotypic assays

4.2 Materials and methods

4.2.1 CRISPR-Cas9 mediated genome editing: two-plasmid system

Initial attempts at CRISPR-Cas9-based gene editing (Jiang and Doudna, 2017, Sander and Joung, 2014) of the exonuclease SNPs were made using a two-plasmid system (**Figure 4.2**). All plasmids were designed and generously provided by Dr. Marcus Lee.

4.2.1.1 *Exonuclease exo-415* SNP editing plasmids

For the exonuclease *exo-415* wildtype (E) and mutant (G) SNP two-plasmid editing system, one plasmid (**Figure 4.2A**, plasmid one) contained the gRNA and Cas9 (pDC2-cam-Cas9-U6-h*dhfr*, 11.9 kb) and the second plasmid (plasmid two) contained a 1 kb donor region with either the *exo-E415* wild-type SNP or with the *exo-G415* mutant SNP (pDC2-cam-e*gfp*-BSD-attP, 5.602 kb) (**Figure 4.2B**, plasmid two). Briefly, identification of an appropriate gRNA was performed using the online resources at Benchling (benchling.com) and Cas-OFFinder (http://www.rgenome.net/cas-offinder) to find a unique sequence of 20 base pairs followed

by the NGG motif, located near the SNP at the amino acid position 415 in the *exonuclease* gene, PF3D7_1362500, near the SNP location at amino acid position 415 followed by NGG. The Cas9 endonuclease encoded by plasmid one (**Figure 4.2A**) is from *Streptococcus pyogenes* which recognizes and will create a double stranded DNA break at the protospacer adjacent motif (PAM) sequence, 5'-NGG-3' (Anders et al., 2014). The exonuclease donor sequence in the donor plasmid (plasmid two) (**Figure 4.2B**) was synthesized to include several synonymous SNPs at the gRNA recognition site (**Table 4.2**, lowercase letters) to prevent Cas9 from cleaving the plasmid and the edited genome. It should also be noted that for the *exo*-415 guide, the SNP of interest (415) is located within the gRNA sequence. For the wildtype codon, the sequence "GAG" encodes a glutamic acid (E) at position 415 (**Table 4.2**, in bold), for the mutation, the sequence, "GgG" encodes the mutant glycine (G) (**Table 4.2**, in bold).

Table 4. 2 gRNA sequences in the donor plasmids for the *exo-415* and *mcp-252* CRISPR-Cas9 plasmids

Gene	SNP position	Guide RNA (5' - 3') in donor template
exonuclease, E415	415	AA**GAG**GAAGTgAACAAcCAc
exonuclease, G415	415	AA**GgG**GAAGTgAACAAcCAc
mitochondrial carrier protein	252	CGATATTTTATTGATGCtTC

4.2.1.2 Two-plasmid system: cloning of the gRNA and donor plasmids

To clone the gRNA into plasmid one, pDC2-cam-Cas9-U6-h*dhfr*, two complementary primers including the specific gRNA sequence and overhangs for ligation into the BbsI site were generated (**Figure 2A**). The pDC2-cam-Cas9-U6-h*dhfr* plasmid was digested with BbsI followed by dephosphorylation of the vector using an alkaline phosphatase (NEB). At the same time, the complementary guide oligos were resuspended to 100 μM in water and phosphorylated in 10 μL total using: 1 μL of each oligo, 1 μL 10X ligation buffer (NEB) and 0.5 μL T4 polynucleotide kinase (NEB), and 6.5 μL water. Guide oligos were then incubated in a thermocycler for 30 minutes at 37°C, followed by denaturation at 94°C for 5 minutes, then annealing in decreasing steps of 5°C every 5 minutes to 25°C. After annealing, the oligos were ligated into the BbsI digested pDC2-cam-Cas9-U6-h*dhfr* vector. A 1:200 dilution of the oligo mix was made and 1 μL of oligos was added to 1 μL of digested plasmid (50 ng), 1 μL 10x ligase buffer (NEB) and 1 μL T4 ligase (NEB) in water up to 10 μL total. The ligation mix was incubated for 10 minutes at room temperature then transformed into XL10 gold competent cells (**section 2.8.1**) and plated on LB-ampicillin plates. Donor regions were cloned into plasmid two, the pDC2-cam-e*gfp*-BSD-attP plasmid (**Figure 2B**), following a

similar procedure using the enzymes AatII and EcoRI to digest the plasmid at the donor site. Following confirmation of the correct insertion of the guide and donor sequences, the plasmids were prepared by midi or maxi preps (**section 2.8.2**) and transfected into field isolates.

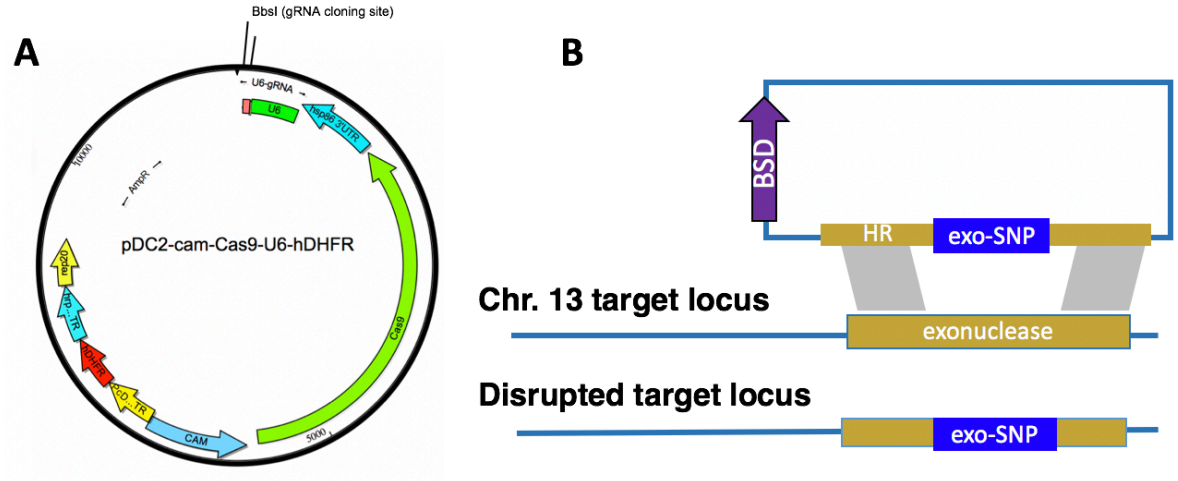


Figure 4. 2. Two-plasmid system for CRISPR-Cas9 editing of the *exonuclease* SNP. A.) Plasmid one: the pDC2-cam-Cas9-U6-h*dhfr* plasmid with Cas9 expression cassettes. The gRNA cloning site is flanked by BbsI digestion sites (labeled) **B.)** Plasmid two: the pDC2-cam-e*gfp*-BSD-attP donor plasmid containing the homology region (HR) and the desired exonuclease SNP for editing parasites to either the wild-type (E) or mutant (G) exonuclease SNP on Chromosome 13. The donor cloning site outside of the homology region (gold) includes EcoRI and AatII digestion sites.

4.2.2 CRISPR-Cas9 mediated genome editing: single plasmid system

The pDC2-cam-coCas9-U6-h*dhfr* single plasmid editing system (**Figure 4.3**) designed by Dr. Marcus Lee was also used in this study. In this vector, Cas9 has been codon optimized for expression in *P. falciparum*, and in addition to the gRNA cassette, contains cloning sites for insertion of the donor region (**Figure 4.3**). This enabled the pDC2-cam-coCas9-U6-h*dhfr* plasmid (~11 kb, depending on donor size) to be used as a backbone for generating both the exo-E415G and mcp-N252D editing plasmids, both of which were also generated by Dr. Marcus Lee for use in this study. The procedure for inserting both the gRNA and donor sites was similar to the methods described above in **section *4.2.1.2*.** The gRNA sequences used for the *exo-415* single plasmid system were the same gRNAs used for the two-plasmid system and listed in **Table 4.2**.

The *mcp-252* editing plasmids for the mitochondrial carrier protein, PF3D7_1368700, were generated in a similar manner to the above *exo-415* two-plasmid system (**section 4.2.1.2**).

Selection of gRNA (**Table 4.2**) and molecular cloning of the donor and gRNA regions into the vector plasmids was performed as described for the *exo-415* plasmids. The gRNA sequence in the donor region (**Figure 4.3**) was modified to introduce a synonymous SNP (**Table 4.2**, lowercase letters) to prevent Cas9 from cutting the plasmid and the edited region in the genome. The SNP was located 87 bp from the gRNA site and the mutant N252D plasmid changes the wildtype "AAT" (N) sequence to the mutant "gAT" codon (D), whereas the silent N-252N plasmid maintains the wildtype (N) sequence.

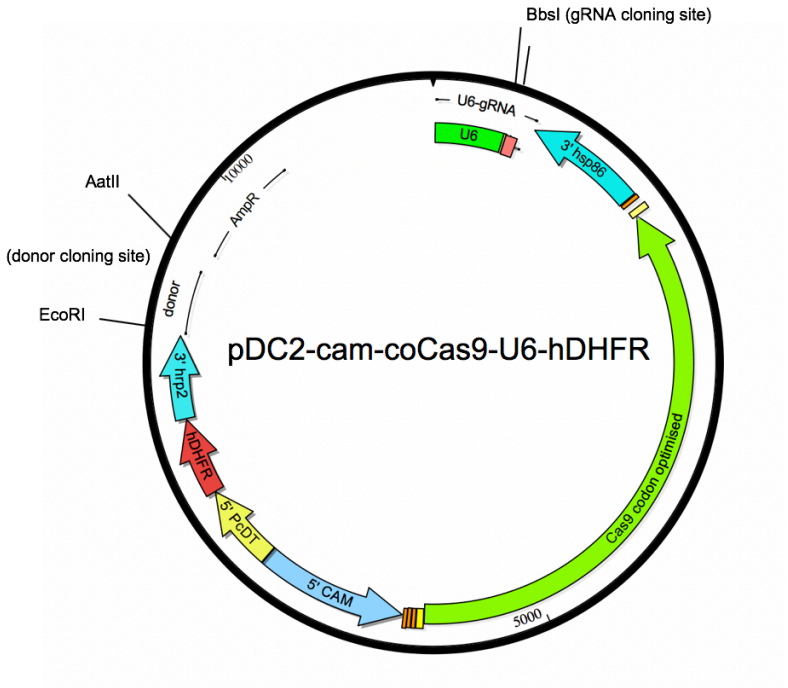


Figure 4. 3. All-in-one pDC2-cam-coCas9-U6-h*dhfr* plasmid. Schematic of the all-in-one pDC2-cam-coCas9-U6-h*dhfr* plasmid (~11 kb) design for CRISPR-Cas9 editing contains codon optimized Cas9 for expression in *P. falciparum*, and includes the cloning sites for inserting the donor (using EcoRI and AatII) and gRNA (using BbsI).

4.2.3 Transfection of CRISPR-Cas9 *exo-415* and *mcp-252* plasmids

The plasmids were transfected into field isolates and Dd2 parasites (**Chapter 2, Table 1**) following the protocols detailed in Chapter 2, **sections 2.3.1-2.3.2**. Both pre-loaded red blood cells (RBCs) and ring stage transfections were performed. As mentioned in Chapter 2, the pre-loaded RBC transfection method was preferred for field isolates because it does not involve direct electroporation of the parasites and as a result, is likely to be less harsh to these parasites. Ring stage transfections were always performed for the laboratory strain, Dd2, but were also attempted with field isolates. Both the PPQ-sensitive and resistant isolates (**Chapter 2, Table 1**) were transfected with the plasmids containing the SNP they already contain (i.e. only introducing the silent gRNA site mutations) to serve as an editing control, ensuring that the CRISPR-Cas9 system did not cause any additional genome disruption.

For the two-plasmid system (**Figure 4.2**), both the gRNA/Cas9 plasmid and donor plasmid were transfected at the same time using 50 μg of each plasmid. The pDC2-cam-Cas9-U6-h*dhfr* contains a human dihydrofolate resistance gene (h*dhfr*) so transfectants were selected with 2 nM WR99210, a DHFR inhibitor. The pDC2-cam-e*gfp*-BSD-attP donor encodes a blasticidin (BSD) resistance gene and 2 μg/mL of blasticidin (BSD) was used to select for this plasmid (**Figure 4.2**). Drug selection was started on day 1 or 2 post-transfection depending on parasitemia on day 1 and parasites were maintained on drug for at least 8 days and up to 2 weeks following transfection.

For the single plasmid system (**Figure 4.3**), the pDC2-cam-coCas9-U6-h*dhfr* transfectants were selected with 5-10 nM WR99210 on day 1 post-transfection and parasites were maintained with drug for 10-12 days. The Cambodian isolates have a moderate level of resistance to DHFR inhibitors, due to the quadruple pyrimethamine-resistance mutations in *dhfr*, so 5-10 nM WR99210 was used for the transfected parasites while 5 nM WR99210 was used for Dd2.

To increase transfection efficiency in field isolates, some of the transfected isolates were cultured in parasite-conditioned media **(Tables 4.4-4.5**), consisting of complete media (CM) supplemented with drug (WR or BSD) and 25-50% of spent media from the specific parasite line (called conditioned media), prepared as described in **section 2.4**.

4.2.4 Screening for integration of the *exo*- and *mcp*-SNPs in transfected parasites

As soon as the parasites recrudesced after transfection, an aliquot of the culture was saved for genomic DNA extraction as described in **section 2.5**. The parasites were screened for integration of the *exo-E415G* and *mcp-N252D* SNPs using the primers listed in **Table 4.3**. PCR amplification and sequencing protocols were performed as described in **sections 2.8.3-2.8.4**. At least one primer was designed outside of the donor homology region to ensure that the region amplified was from the genome and not the episomal donor plasmid (**Figure 4.4**).

After initial screening, the parasites with confirmed edits were cloned by limiting dilution (**section 2.4**) and collected for sequencing (**section 2.8.4**) to confirm the genotype in each clone. Following confirmation, clones were cryopreserved (**section 2.1.3**; at least two vials per edited line) and phenotypes were examined in parasite survival assays.

Figure 4. 4. Primers for PCR screening of transfectants for integration of the desired locus on chromosome 13 (blue line) to capture the *exo-415* or *mcp-252* SNPs. The gold HR1 and HR2 regions represent the homology regions surrounding the SNP included in the donor region. The forward (Fwd) primer includes the region with the SNP and the reverse (Rev) primer is outside of the donor homology region so that only regions integrated into the genome will be amplified.

Table 4. 3 Primers used to screen for integration of SNPs

Gene	SNP	Plasmid system	Primer name	Primer (5' - 3') sequence	Description
exo	exo-415	two	exo_fwd1	CTGACCCCTTTATAGGAATGTGC	fwd inside donor region; for sequencing
			exo_rev1	CTTGGTTAAGTGGTCTTATATTCCAGTTAGC	rev flanking donor region
mcp	mcp-252	single	p1580	GATGACAAAACAAAGTTACGCTTCC	fwd flanking donor; for sequencing
			p1581	CTTAATAAATCTAATGGATATGAGAGAG	rev flanking donor

4.2.4 Drug sensitivity assays

Drug susceptibility profiles of the wildtype and mutant *exo-415* and *mcp-252* edited parasite lines were assessed according to the protocols detailed in Chapter 2 (**section 2.6**). Standard IC_{50} assays (**section 2.6.2**) were performed using piperaquine (PPQ) and other commonly used antimalarial drugs including: dihydroartemisinin (DHA), lumefantrine (LUM), chloroquine (CQ), and mefloquine (MQ). Piperaquine survival assays (PSAs) (**section 2.6.3**) were also performed on the wildtype and mutant edited *exo-415* and *mcp-252* parasite lines. All IC_{50} assays and PSAs were performed in duplicate and triplicate, respectively, with three independent experiments (biological replicates) performed for each assay.

4.3 Results

4.3.1 CRISPR-Cas9 editing of the *exonuclease-415* SNPs: low transfection efficiency in field isolates

CRISPR-Cas9 gene editing of the exonuclease, *exo-415* SNP, to the wildtype (E) and mutant (G) SNPs observed in piperaquine-sensitive (PPQ-S) and resistant (PPQ-R) isolates from Cambodia was attempted in culture-adapted Cambodian field isolates (**Ch. 2, Table 2.1**). All field isolate transfections for both plasmid systems (two-plasmid and single-plasmid) were

performed using pre-loaded RBCs. The two-plasmid was used at the commencement of this study because this system had been used successfully by the Lee Lab and many other labs for CRISPR-Cas9 editing (Ghorbal et al., 2014). During the course of this study, Dr. Marcus Lee designed the all-in-one CRISPR-Cas9 plasmid and due poor transfection efficiencies with the two-plasmid system, I switched to the single-plasmid system.

Transfection efficiency for all isolates was low with only five transfections out of 158 total transfections recrudescing post-drug selection, two of which were cultured with parasite-conditioned media (**Table 4.4**). Amplified PCR products from transfected parasites that recrudesced were screened for edits and sequencing data showed that none of these transfectants contained the desired edits (**Figure 4.5**). The parasite lines 033 and 056 are PPQ-S isolates that should have been edited to the mutant G amino acid codon ("GGG") but they remained the wildtype ("GAG"). The parasite line 021 is a PPQ-R isolate that should have been edited to the wildtype E amino acid codon ("GAG") but also remained unedited ("GGG") (**Figure 4.5**).

Table 4. 4 CRISPR-Cas9 transfections and outcomes of *exo-415* SNP editing experiments in Cambodian field isolates

				exo-SNP transfections			
Parasite line	**PPQ status**	**Plasmid system**	**SNP**	**No. of transfections**	**Recrudescence**	**Edited SNP**	**Conditioned media***
163-KH2-033 (PH1097-C)	PPQ-sensitive	two	*exo-E415E (wt)*	10	0	0	Y (5)
			exo-E415G (mutant)	16	2*	0	Y (6)
		single	*exo-E415E (wt)*	4	0	0	N
			exo-E415G (mutant)	6	0	0	N
163-KH3-056 (PH1310-C)	PPQ-sensitive	two	*exo-E415E (wt)*	10	0	0	N
			exo-E415G (mutant)	14	1	0	Y (6)
		single	*exo-E415E (wt)*	4	0	0	N
			exo-E415G (mutant)	4	0	0	N
163-KH3-005 (PH0971-C)	PPQ-sensitive	two	*exo-E415E (wt)*	0	N/A	N/A	
			exo-E415G (mutant)	0	N/A	N/A	
		single	*exo-E415E (wt)*	0	N/A	N/A	
			exo-E415G (mutant)	6	0	0	Y (4)
163-KH1-081 (PH1224-C)	PPQ-resistant	two	*exo-E415E (wt)*	14	0	0	Y (6)
			exo-E415G (mutant)	10	0	0	
		single	*exo-E415E (wt)*	0	N/A	N/A	
			exo-E415G (mutant)	0	N/A	N/A	
163-KH2-021(PH1387-C)	PPQ-resistant	two	*exo-E415E (wt)*	16	2	0	Y (6)
			exo-E415G (mutant)	10	0	0	Y (5)
		single	*exo-E415E (wt)*	0	N/A	N/A	
			exo-E415G (mutant)	0	N/A	N/A	
163-KH1-060RME (PH1265-C)	PPQ-resistant	two	*exo-E415E (wt)*	12	0	0	Y (6)
			exo-E415G (mutant)	10	0	0	N
		single	*exo-E415E (wt)*	6	0	0	N
			exo-E415G (mutant)	6	0	0	N
			Total	158	5	0	

Table 4.4 Details of the field isolate transfections with *exo-415* SNP plasmids, categorized by parasite line (field isolate name), PPQ susceptibility status (sensitive or resistant), and the plasmid system used for CRISPR-Cas9 gene editing (two-plasmid or single-plasmid). The transfections are further sorted based on whether conditioned media was used (Y= yes N= no) with the number of transfections exposed to conditioned media indicated in parentheses. Recrudescent transfections are highlighted in yellow and are denoted with an asterisk (*) if the transfections that recrudesced were cultured with conditioned media. All transfections were performed using pre-loaded RBCs.

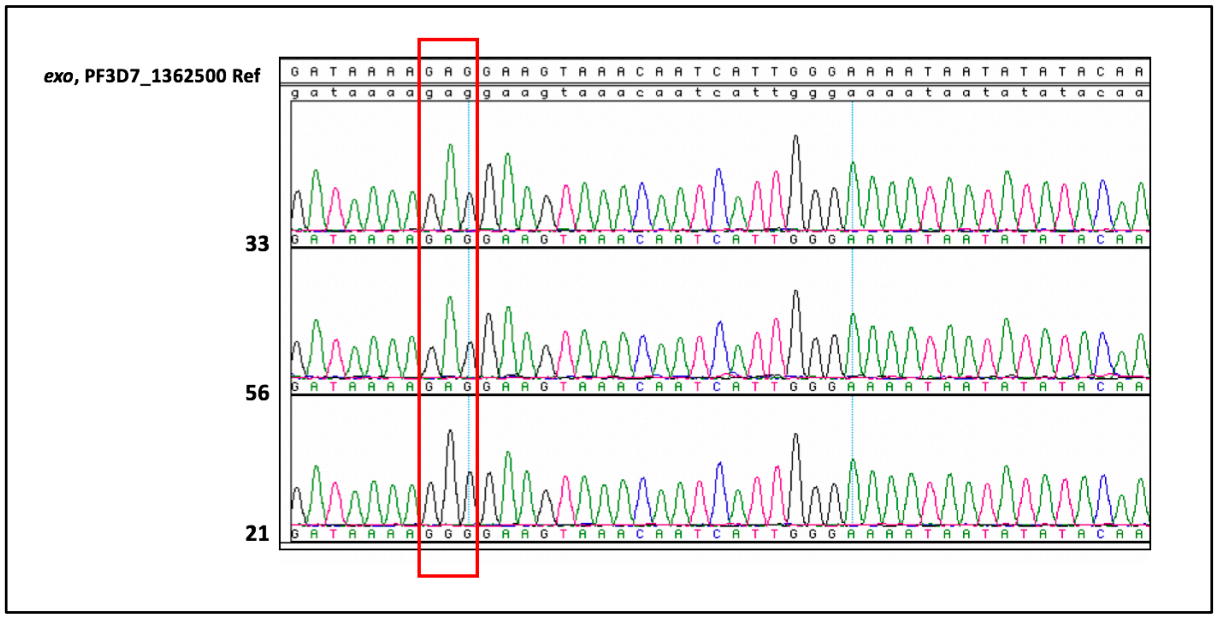


Figure 4. 5. **Sequencing analysis of the *exo*-SNP field isolate transfections that recrudesced.** The top *exo*, PF3D7_1362500 Ref is the wildtype reference sequence. The red box highlights the location of the codon 415 SNP in the reference and isolates. Parasite isolates 033 and 056 are PPQ-S *exo-E415* isolates that should have been edited to G415. Parasite isolate 021 is a PPQ-R *exo-G415* isolate that should have been edited to the wildtype E415 SNP. Primers used to screen the *exo*-SNP two-plasmid transfectants are listed in Table 4.3.

Due to the lack of editing success in Cambodian field isolates, the exo editing plasmids were transfected into the Southeast Asian lab strain, Dd2, in parallel by Dr. Manuela Carrasquilla. Both the wildtype (silent) and mutant (G) *exo-415* SNPs were edited in the Dd2 parasite using the single-plasmid system (**Figure 4.3**). Dr. Carrasquilla confirmed the edits using PCR and sequencing (data not shown).

4.3.2 CRISPR-Cas9 editing of the *mitochondrial carrier protein*-252 SNPs: low transfection efficiency in field isolates and successful editing in Dd2 parasites

CRISPR-Cas9 gene editing of the mitochondrial carrier protein, *mcp-252* SNP, yielding the wildtype N codon (AAT) or the mutant D codon (GAT), observed in PPQ-sensitive and resistant isolates respectively, was attempted in lab-adapted Cambodian field isolates (**Ch. 2, Table 2.1**) and the Dd2 parasite strain. Both pre-loaded RBC and ring stage transfections were performed and are detailed in **Table 4.5**. Similar to the *exo-415* plasmid transfections, efficiency of field isolate transfections with the *mcp-252* single plasmid was low with one field isolate out of 120 total transfections recrudescing (**Table 4.5**). The line 033-*mcp-N252D* transfectant that recrudesced (**Table 4.5, Figure 4.6**) was screened for edits and sequencing data showed that the SNP remained unedited although the silent mutation in the gRNA donor region was introduced (**Figure 4.6C**).

Transfection efficiency of the Dd2 strain was much higher with two of four total transfections recrudescing and one of the transfections containing the edited SNP (**Table 4.5)**. The two Dd2-*mcp-N252D* transfectants that recrudesced were screened for edits and the sequencing data showed that one of the transfections carried approximately 30% of the edited SNP in the bulk culture (**Figure 4.7A**). The culture was cloned by limiting dilution and clone H5 was shown to contain the edited SNP (**Figure 4.7B**). Clone D2 did not contain the mutant SNP and remained wildtype (N252) (**Figure 4.7C**) but did contain the edit of the silent mutation in the gRNA region (**Table 4.2**). Since this indicates that clone D2 was edited, I used this clone as the *mcp-N252N* silent editing control for subsequent phenotypic assays.

Table 4. 5 CRISPR-Cas9 transfections and editing efficiency of the mcp-252 SNPs in Cambodian field isolates and Dd2

				mcp-SNP transfections				
Parasite line	**PPQ status**	**Plasmid system**	**SNP**	**Type**	**No. of transfections**	**Recrudescence**	**Edited SNP**	**Conditioned media***
163-KH2-033 (PH1097-C)	PPQ-sensitive	single	*mcp-N252N (wt)*	loaded	8	0	0	Y (4)
				ring	4	0	0	N
			mcp-N252D (mutant)	loaded	16	1*	0	Y (8)
				ring	4	0	0	N
163-KH3-056 (PH1310-C)	PPQ-sensitive	single	*mcp-N252N (wt)*	loaded	0	N/A	N/A	
				ring	0	N/A	N/A	
			mcp-N252D (mutant)	loaded	10	0	0	N
				ring	0	N/A	N/A	
163-KH3-005 (PH0971-C)	PPQ-sensitive	single	*mcp-N252N (wt)*	loaded	8	0	0	N
				ring	0	N/A	N/A	
			mcp-N252D (mutant)	loaded	12	0	0	Y (4)
				ring	6	0	0	
163-KH2-021(PH1387-C)	PPQ-resistant	single	*mcp-N252N (wt)*	loaded	0	N/A	N/A	
				ring	0	N/A	N/A	
			mcp-N252D (mutant)	loaded	6	0	0	N
				ring	0	N/A	N/A	
163-KH1-060RME (PH1265-C)	PPQ-resistant	single	*mcp-N252N (wt)*	loaded	14	0	0	Y (6)
				ring	0	N/A	N/A	
			mcp-N252D (mutant)	loaded	10	0	0	Y
				ring	0	N/A	N/A	
163-KH1-001RME (PH1008-C)	PPQ-resistant	single	*mcp-N252N (wt)*	loaded	8	0	0	N
				ring	4	0	0	N
			mcp-N252D (mutant)	loaded	6	0	0	N
				ring	0	N/A	N/A	
Dd2	PPQ-sensitive	single	*mcp-N252N (wt)*	loaded	0	N/A	N/A	
				ring	2	0	0	N
			mcp-N252D (mutant)	loaded	0	N/A	N/A	
				ring	2	2	1	N
			Total		120	3	1	

Table 4.5 Details of the field isolate transfections with *mcp-252* SNP editing plasmids categorized by parasite line (field isolate name), PPQ susceptibility status (sensitive or resistant), and the plasmid system used for CRISPR-Cas9 gene editing (single-plasmid), and the method for transfection, pre-loaded RBCs (loaded) or directed electroporation (rings). The transfections are further sorted based on whether conditioned media was used (Y= yes; N= no) with the number of transfections exposed to conditioned media indicated in parentheses. Recrudescent transfections are highlighted in yellow and are denoted with an asterisk (*) if the transfections that recrudesced were cultured with conditioned media.

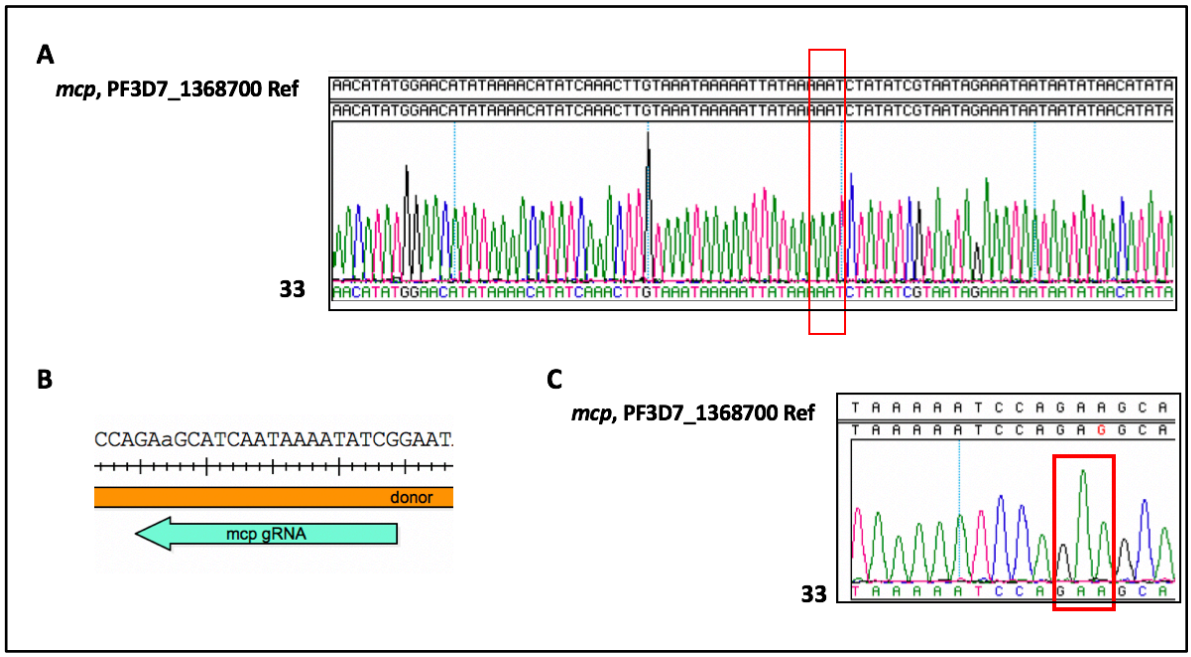


Figure 4. 6. Sequencing analysis of the *mcp*-SNP field isolate transfection post-recrudescence. **A.**) The Ref sequence is the wildtype reference sequence. The red box highlights the location of the *mcp-252* codon in the reference and isolate. Parasite isolate 033 is a PPQ-S *mcp-N252* isolate that should have been edited to D252 ("GAT") but remains the wildtype N ("AAT") sequence. **B.**) The *mcp* gRNA sequence (listed in Table 4.2 and located 87 bp from the SNP) showing a silent mutation (lowercase letter "a") in the donor region. **C.**) Sequence analysis of the transfectant 033-*mcp-N252D* shows that the silent mutation in the gRNA is edited in the parasite compared to the wildtype *mcp*-Ref. Primers used to screen the *mcp*-SNP transfectants are listed in Table 4.3.

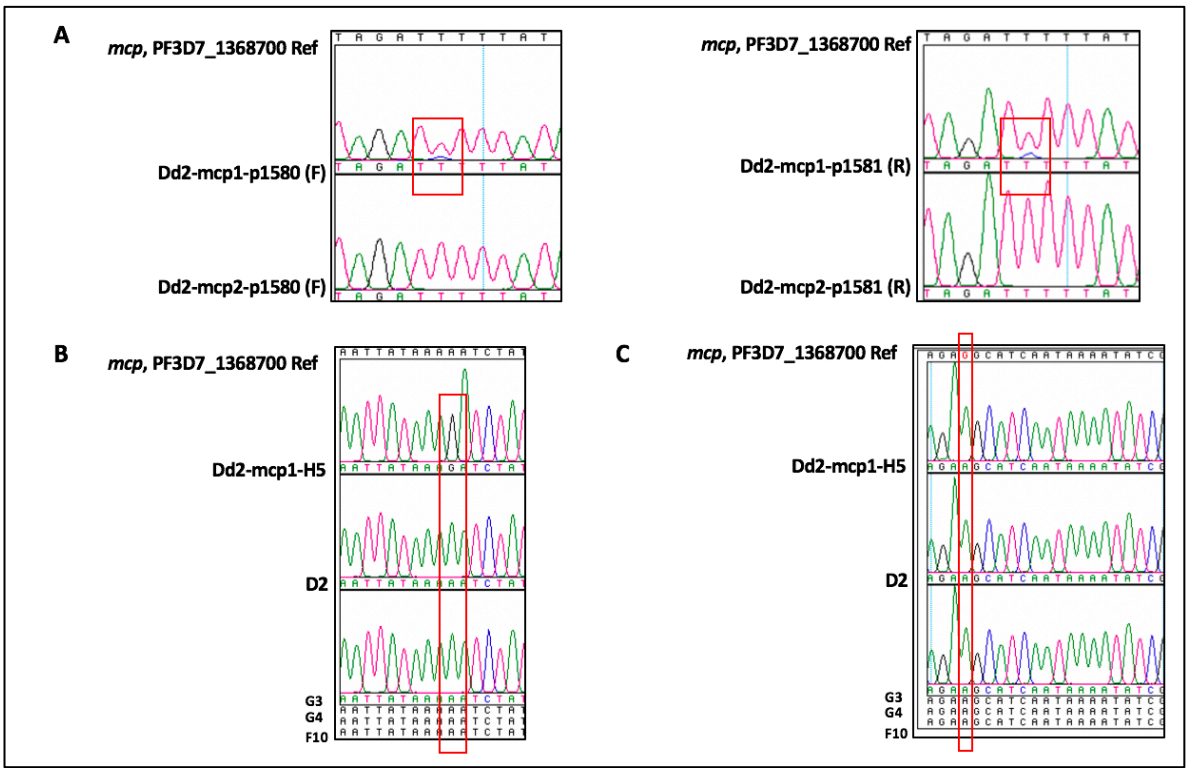


Figure 4. 7. Sequencing analysis of the Dd2-*mcp*-SNP transfections. A.) The top Ref sequence is the wildtype reference sequence. The chromatogram shows the region of the *mcp-252* SNP in the reference and the two Dd2 transfections that recrudesced: Dd2-mcp1 and Dd2-mcp2. The Dd2-mcp1 chromatogram shows a mixed peak (red box) around position 252, displaying both a wildtype "T" and a small blue peak on the chromatogram that is the mutant "C". Chromatograms for both the forward p1580 (F) and reverse p1581 (R) primers are displayed. Dd2-mcp2 did not show mixed peaks or any signs of editing. **B**.) Sequence analysis of Dd2-mcp1-N252D clones. Dd2 clone H5 contains the edited SNP "G" (highlighted in the red box) and the other clones (D2, G3, G4, and F10) are unedited and remain the wildtype "A". Note: the sequence in panel (B) is the reverse sequence of the sequence displayed in panel (A). In the sequence in panel (A), the edited nucleotide is a C and in the reverse sequence in panel (B), the edited nucleotide is a G. **C.**) Sequence analysis of Dd2 clones shows that although the mutant D SNP is not edited in every clone, the silent mutation in the gRNA site is edited in all Dd2-mcp1 clones. Clone Dd2-D2 was used as the silent-edited wildtype line for all drug assays. Primers used to screen the *mcp*-SNP transfectants are listed in Table 4.3.

4.3.3 Drug susceptibility profiles of the edited exonuclease and mitochondrial carrier protein SNPs in Dd2-edited parasite lines

4.3.3.1 The exo-E415G SNP does not alter parasite susceptibility to common antimalarial drugs

The transgenic *exo-E415G* mutant and wildtype (silent *exo-E415E*) parasites were tested in 72-hour survival assays to compare susceptibility to piperaquine (PPQ) and several other antimalarial drugs: chloroquine (CQ), dihydroartemisinin (DHA), mefloquine (MQ), and lumefantrine (LUM). MQ, LUM, and CQ were selected based on their structural similarity to piperaquine, as they are quinolone drugs, and DHA, MQ, and LUM are all currently recommended antimalarial drugs in ACT regimens. The dose response curves for all five

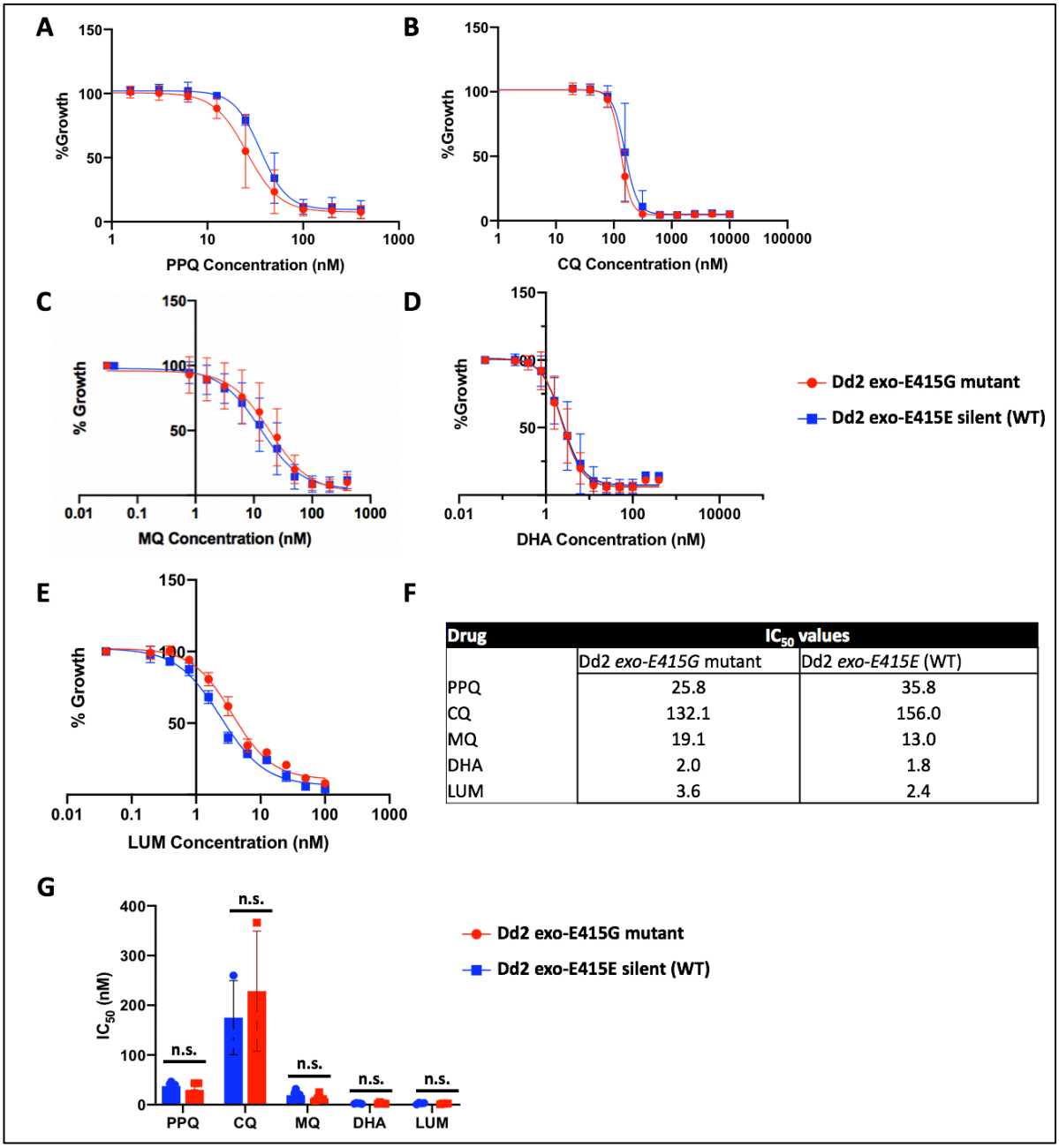
antimalarial drugs showed that the *exo-E415G* mutant SNP had little to no effect on parasite susceptibility to the five drugs compared to the wildtype control (Figure 4.8). This was further supported by the IC_{50} values for each drug condition (Figure 4.8F).

4.3.3.2 The mcp-N252D SNP does not alter parasite susceptibility to common antimalarial drugs

The transgenic *mcp-N252D* mutant and wildtype (silent *mcp-N252N*) parasites were also tested in 72-hour survival assays to compare susceptibility to the same drugs as described for the *exo*-SNP lines: PPQ, CQ, DHA, MQ, and LUM. The dose response curves for all five antimalarial drugs showed that the *mcp-N252D* mutant SNP had little to no effect on parasite susceptibility to the five drugs compared to the wildtype control (**Figure 4.9**). This was further supported by the IC_{50} values for each drug condition (**Figure 4.9F**).

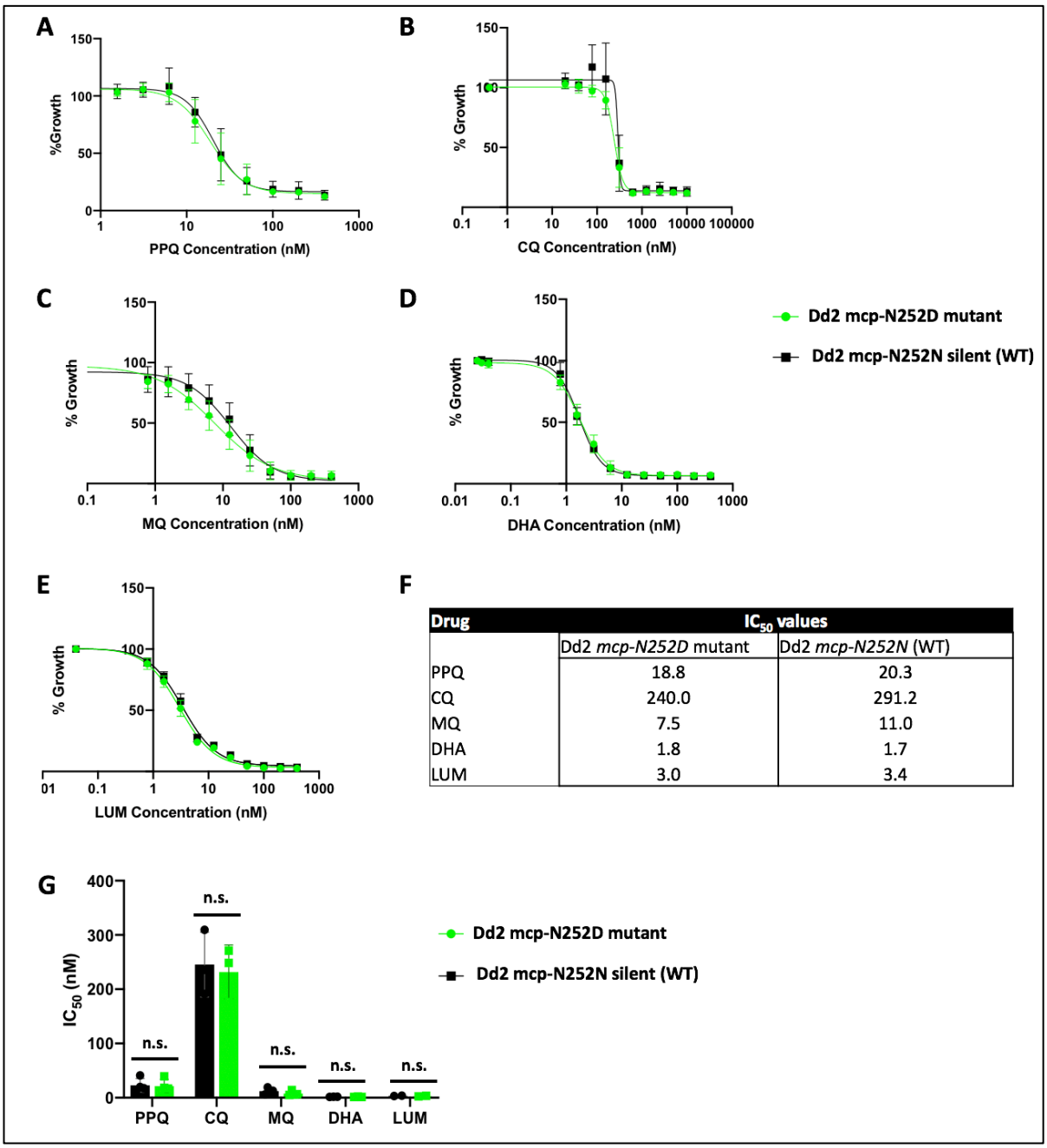
4.3.3.3 The exo-E415G and mcp-N252D SNPs do not alter parasite survival in piperaquine survival assays

I further assessed the phenotype of the transgenic *exo-E415G* and *mcp-N252D* parasites in piperaquine survival assays (PSAs). After 48 hour exposure to piperaquine and then a further incubation period of 24 hours (as described in **section 2.6.3**), little to no differences were again observed between the edited *exo-415* and *mcp-252* SNP edited parasite lines (**Figure 4.10**).



Drug	IC_{50} values	
	Dd2 *exo-E415G* mutant	Dd2 *exo-E415E* (WT)
PPQ	25.8	35.8
CQ	132.1	156.0
MQ	19.1	13.0
DHA	2.0	1.8
LUM	3.6	2.4

Figure 4. 8. The *exo-E415G* SNP has no effect on IC_{50} values or susceptibility to PPQ and other commonly used antimalarial drugs. A.-E.) Dose-response curves for Dd2 edited parasites containing the mutant *exo-E415G* SNP (red) or the wildtype *exo-E415E* control (blue) exposed to: piperaquine (PPQ) (**A**), chloroquine (CQ) (**B**), mefloquine (MQ) (**C**), dihydroartemisinin (DHA) (**D**), and lumefantrine (LUM) (E). Error bars represent SD (n=3 biological replicates). **F.**) Table showing the average IC_{50} values for the mutant and wildtype (WT) *exo-415* SNP transgenic Dd2 lines calculated using GraphPad Prism Version 8.0.2. **G**.) Mean IC_{50} values ± SD for each drug (PPQ, CQ, MQ, DHA, LUM) tested on the *exo-E415G* SNP (red) and the wildtype *exo-E415E* control (blue) lines. Significance was determined using Mann-Whitney U tests comparing the mutant and silent edited lines. n.s. indicates not significant ($p>0.05$). No significant differences were observed between the WT and mutant SNP edited lines for any of the drug treatments.



Drug	IC_{50} values	
	Dd2 *mcp-N252D* mutant	Dd2 *mcp-N252N* (WT)
PPQ	18.8	20.3
CQ	240.0	291.2
MQ	7.5	11.0
DHA	1.8	1.7
LUM	3.0	3.4

Figure 4. 9. The *mcp-N252D* SNP has no effect on IC_{50} values or susceptibility to PPQ and other commonly used antimalarial drugs. A.-E.) Dose-response curves for Dd2 edited parasites containing the mutant *mcp-N252D* SNP (green) or the wildtype *mcp-N252N* control (black) exposed to: piperaquine (PPQ) (**A**), chloroquine (CQ) (**B**), mefloquine (MQ) (**C**), dihydroartemisinin (DHA) (**D**), and lumefantrine (LUM) (**E**). Error bars represent SD (n=3 biological replicates). **F**.) Table showing the average IC_{50} values for the mutant and wildtype (WT) *mcp-252* SNP transgenic Dd2 lines calculated using GraphPad Prism Version 8.0.2. **G**.) Mean IC_{50} values ± SD for each drug (PPQ, CQ, MQ, DHA, LUM) tested on the *mcp-N252D* SNP (green) and the wildtype *mcp-N252N* control (black) lines. Significance was determined using Mann-Whitney U tests comparing the mutant and silent edited lines. n.s. indicates not significant ($p>0.05$). No significant differences were observed between the WT and mutant SNP edited lines for any of the drug treatments.

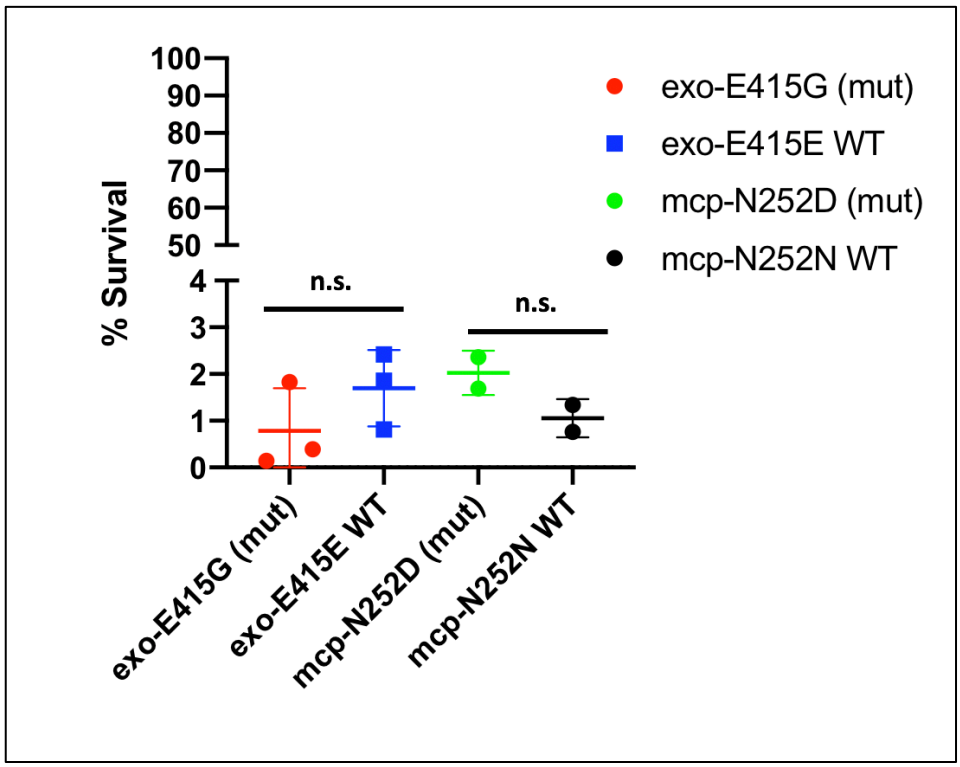


Figure 4. 10. **The Dd2 transgenic *exo-E415G* and *mcp-N252D* edited SNPs have no effect on parasite survival to PPQ in piperaquine survival assays (PSAs).** PSAs comparing parasite susceptibility to piperaquine. Parasites were exposed to 200 nM PPQ for 48 hours and measured at 72 hours. Error bars represent SD (for *exo*, n=3 biological replicates; for *mcp*, n=2). Significance was determined using Mann-Whitney U tests comparing PSA values for the mutant and silent edited lines for each SNP, *exo-415* and *mcp-252*. n.s. indicates not significant (p>0.05).

4.4 Discussion and future work

The studies by Amato *et al.* and Witkowski *et al.* (2017) were the first reports to independently identify molecular markers of piperaquine resistance that associated with DHA-PPQ failures in patients and decreased PPQ susceptibility *in vitro* (Amato et al., 2017, Witkowski et al., 2017). The top two nonsynonymous SNPs (**Table 4.1**) discovered by the GWAS performed by Amato *et al.*, but not reported by Witkowski *et al.,* were in a putative exonuclease protein, PF3D7_13625000, and a mitochondrial carrier protein, PF3D7_1368700 (Amato et al., 2017). These findings laid the groundwork for this thesis, which aimed to characterize the potential functional roles of the *exo-E415G* and *mcp-N252D* SNPs in piperaquine resistance. In order to investigate the molecular mechanisms of piperaquine resistance and to validate these two molecular markers of resistance, the study began with the initial questions: do these genes play a causal or compensatory role, if any, in the piperaquine resistance phenotype? Could these genes play a role in resistance to other antimalarial drugs?

This study used parasites generated by CRISPR-Cas9 gene editing to elucidate phenotypic effects of the *exo-E415G* and *mcp-N252D* SNPs in response to piperaquine pressure. One of the main objectives was to insert the wildtype and mutant SNPs into both piperaquine-resistant (PPQ-R) and PPQ-sensitive (PPQ-S) isolates to examine if reverting PPQ-R parasites to the wildtype SNP and changing the PPQ-S to the mutant SNP would alter susceptibility of the isolates to piperaquine. Though over a hundred transfections of culture-adapted lines from various Cambodian field sites were attempted for both the *exo*- and *mcp*-SNPs (**Tables 4.4-4.5**), only five *exo-415* SNP transfectants from three different isolates recrudesced; but sequencing showed that none of the desired editing events occurred (**Table 4.4**, **Figure 4.5**). For the *mcp-252* transfections, one transfectant recrudesced, but sequencing showed only a silent mutation present in the donor region of the gRNA and no change in the desired SNP (**Table 4.2**, **Figure 4.6**). Though the gRNA selected for the *mcp*-252 editing plasmid was close to the *mcp*-252 SNP (87 bp downstream) and contained all features of a suitable gRNA candidate with no predicted off-target sites (**Table 4.2**), it is possible that it was too far from the region of the SNP to be edited. It should also be noted that the genomic sequences of the field isolates were compared to the reference sequences of Dd2 from which the gRNA sequences were designed to ensure there were no additional SNPs in the field isolates that may have interfered with the editing processes for both the *exo* and *mcp* genes. Transformation of the Dd2 strain, however, proved successful with both *exo*- and *mcp*-SNPs, raising the intriguing question of other possible differences that might account for the editing successes with Dd2 but not with the more recently lab-adapted Cambodian lines.

Transfections in *P. falciparum* asexual stage parasites are notorious for their low efficiency (de Koning-Ward et al., 2000, Carvalho and Menard, 2005). Prior notable studies have shown that transfections in the rodent malaria parasite, *P. berghei* are much higher than *P. falciparum*, with efficiency rates of 10^{-2} to 10^{-3} (Janse et al., 2006) compared to 10^{-6} to 10^{-9} in *P. falciparum*. Further studies in *P. knowlesi* have demonstrated even higher success rates, reporting 30-40% transfection efficiency (Moon et al., 2013). Due to these known transfection obstacles in *P. falciparum*, this study attempted to increase chances of transfection success in field isolates by favoring pre-loaded RBC transfection methods, which are shown to be ~5-180 fold more efficient than direct parasite electroporation methods (Hasenkamp et al., 2012). Additionally, about half of field isolate transfections were supplemented with parasite-conditioned media (**Tables 4.4-4.5**) to try to reduce parasite stress and promote favorable growth conditions. Several transfectants cultured with conditioned media, did recrudesce to parasitemias greater than 1%, (Tables 4.4-4.5) however,

as seen in the parasite transfection efficiency Tables 4.4-4.5, these additional procedures did not produce edited parasites. Further comparisons of transfection efficiency with conditioned media and without are therefore necessary to determine if the supplemented media has any effect on transfection success. Furthermore, parasite-conditioned media is widely used to promote conversion of asexual stage parasites to gametocytes (Williams, 1999), based on the findings from Carter and Miller (Carter and Miller, 1979) which showed that the environmental culture conditions affect gametocytogenesis. Since field isolates already have a higher proportion of gametocytes and greater likelihood of parasites to commit to sexual stages than lab reference strains, it is possible that rather than promoting parasite growth, the conditioned media used in the field isolate transfections maintained stress in cultures that were already stressed, post-electroporation. However, conditioned media was only used for 28% of the *exo* transfections and 18% of the *mcp* (**Tables 4.4-4.5**) field isolate transfections, so this would not explain why the other transfections did not recrudesce. Continued optimization of transfection efficiency in *P. falciparum* in general, and especially in lab-adapted field isolates, is required.

Successful editing of the lab strain, Dd2, with the single-plasmid system for both the *exo* and *mcp* SNPs was achieved (**Figures 4.- 4**). This indicates that the single-plasmid systems for the *exo*- and *mcp*- were able to produce the desired edits in *P. falciparum*. Since the two-plasmid editing system for the *exo*-SNP was not attempted in Dd2, it is unclear if the low editing success was due to the plasmids or the low transfection efficiency in the field isolates. However, this cannot explain why the single-plasmid system for both the *exo* and *mcp* SNPs worked in Dd2 but not in the field isolates. As mentioned, the Dd2-*exo-415* mutant and silent edited (wildtype) parasites were obtained from Dr. Carrasquilla and no data on transfection efficiency are available for those lines. However, for the Dd2-*mcp-252* mutant and silent parasites, two out of two total Dd2 transfections (100% transfection efficiency) recrudesced with one of the transfections (50% editing success) carrying the desired *N252D* mutation. For the silent editing plasmid, *mcp-N252N*, 0 of 2 transfections recrudesced and further transfections of this plasmid in Dd2 were not performed because field isolate transfections were prioritized. As mentioned in section 4.3.2, one of the clones, Clone D2, transfected with the mutant *mcp-N252D* plasmid did not contain the mutant SNP and remained wildtype (N252) but did contain the edit of the silent mutation that was in the *mcp-252* editing donor plasmid in the gRNA region (**Table 4.2; Figure 4.7**). Since this indicates that clone D2 was edited, it was used as a silent editing control for the wildtype N252N SNP in all phenotypic assays.

Analysis of the drug susceptibility profile of the Dd2 transgenic parasites revealed that neither the *exo-E415G* nor *mcp-N252D* SNPs altered susceptibility to PPQ based on IC_{50} survival assays and PSAs (**Figures 4.8-4.10**). My findings indicate that the mutations observed in the exonuclease and mitochondrial carrier protein are not the primary genetic determinants of PPQ resistance, suggesting, in line with other studies, that mutations elsewhere in the *P. falciparum* genome are responsible for resistance. The Dd2-edited *exo-415* and *mcp-252* lines also showed no difference in susceptibility to other quinolone drugs, CQ and MQ, and additional antimalarial drugs, DHA and LUM in 72 hour IC_{50} survival assays (**Figures 4.8-4.9**). Based on these results, this study further indicates that the *exo-E415G* and *mcp-N252D* SNPs do have a causal role in resistance to several commonly used antimalarial drugs. It remains possible that the SNPs may affect parasite fitness. Drug resistance mutations are often accompanied by fitness costs (Rosenthal, 2013), so the *exo-E415G* and *mcp-N252D* SNPs could serve in these roles. Further studies would be required to compare fitness between the wildtype and mutant SNP transgenic parasites.

It must be noted that Dd2 is lab strain that has been cultivated for many years *in vitro* and although it comes from a Southeast Asian lineage, it is genetically different from the more recently adapted field isolates from Cambodia used in this study (Mackinnon et al., 2009). The phenotypic effects of SNPs observed in the *exo* and *mcp* genes could be dependent on the genetic background of the Cambodian isolates. Therefore continued efforts to edit field isolates with both the *exo-* and *mcp*-SNP plasmids is necessary. If editing of the Cambodian isolates continues to prove difficult, it could also be interesting to further genetically modify Dd2-transgenic parasites obtained by this study. For example, the *PM2-2A-PM3* episomal plasmid generated by this study in Chapter 3 could be transfected into the Dd2 parasites to evaluate if increase *PM2-3* copy numbers are needed for the *exo-* and or *mcp-* SNPs to have a measurable phenotypic effect. Or perhaps a mutant *kelch13* background is necessary for the effects to be observed.

Although Amato *et al*. performed stringent tests in their GWAS analysis to ensure the signal on chromosome 13 was not due to confounding or batch effects (Amato et al., 2017), it is still possible that the *exo-E415G* and *mcp-N252D* SNPs are associated with piperaquine resistance because of linkage to other functional mutations in the Cambodian isolates, such as the *PM2-3* CNV (Amato et al., 2017). Further studies of Cambodian parasites may shed light on these and other possibilities.

Though outside the scope of this study, it would be very interesting to examine the functions of both the exonuclease and mitochondrial carrier proteins in *P. falciparum*.
Aside from preliminary transcriptomics and sequencing work, it is also important to acknowledge that no studies have confirmed or refuted whether the putative exonuclease protein and mitochondrial carrier protein in *P. falciparum* are actually homologous to the proteins to which they share sequence similarity. Therefore, future studies into the exonuclease and mitochondrial carrier protein, including localization, should help to evaluate their potential roles in drug resistance and in the biology of the malaria parasite.

In order to fully understand the causes of drug resistance and inform drug development and treatment strategies, it is necessary to complement genetic studies with functional assays. This study has enabled the possibility of examining whether genetic changes observed in patient isolates can also be investigated and observed in *in vitro* settings. This study successfully edited a molecular marker of piperaquine resistance, an *mcp-N252D* SNP, in Dd2 parasites. Additionally, this work assessed the phenotypes of the resistance-associated *exo-E415G* and *mcp-N252D* SNPs in response to piperaquine and other commonly used antimalarial drugs. The results from this study of transgenic Dd2 parasites indicates that the *exo-E415G* and *mcp-N252D* SNPs are not genetic determinants of piperaquine resistance and likely do not play a significant role in piperaquine resistance. Though future work is needed, this study provides novel insight into the roles of these genetic markers in piperaquine resistance and suggests additional genetic determinants remain to be uncovered.