MOLECULAR ANALYSIS OF MEMBRANE TRANSPORTERS
IMPLICATED IN DRUG RESISTANCE

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Jacqueline K. Lekostaj, B.S.

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Jacqueline K. Lekostaj
Thesis Advisor: Paul D. Roepe, PhD

ABSTRACT

Resistance to cancer chemotherapeutics and antimalarial drugs is a major obstacle to the successful treatment of these diseases. Membrane transporters have been identified as contributing to this drug resistance. The HuMDR1 protein is thought to reduce accumulation of chemotherapeutics by ATP-dependent transport of the toxic compounds out of the cell. Though previously believed to be a major component of clinical cancer drug resistance, HuMDR1 is now accepted as playing only a minor role, secondary to other mechanisms. The *Plasmodium falciparum* protein homologue, PfMDR1, may be acting in a similar manner within the malarial parasite, affecting the partitioning of antimalarial drugs amongst cellular compartments. However, the evolving picture of quinoline antimalarial drug resistance may point to a mere modulatory role for PfMDR1 in comparison to another membrane protein, PfCRT, which has been proven to be causative of some drug resistance phenotypes but through an unknown mechanism.

Since the protein is native to a subcellular organelle within an intracellular parasite, molecular level analysis of PfMDR1 would benefit from heterologous expression in a simpler system. This thesis reports the successful inducible overexpression of PfMDR1 in *Pichia pastoris* yeast. The tagged protein can be purified by affinity chromatography and functionally reconstituted in proteoliposomes. ATPase
assays of many PfMDR1 variants show the protein to have high basal activity, with very little drug-induced responsiveness. These results support a model in which PfMDR1 acts to modulate the drug resistance profiles determined by PfCRT or to compensate for fitness losses incurred by mutation of PfCRT.

All current hypotheses for the molecular mechanism by which PfCRT confers quinoline antimalarial drug resistance entail the direct interaction of the drug molecule with the protein, but evidence for these theories is inferential. This thesis reports the labeling of PfCRT with a photoactivatable chloroquine analogue. The probe is shown to be specific and labeling is efficiently competed with other antimalarial drugs, suggesting a single drug binding site is present in the protein. The photolabeling site is mapped to within 11 amino acids, and a model is proposed in which PfCRT transmembrane helices 1, 9 and 10 form a drug binding pocket.
DEDICATION

For my Mother;

no dedication I write could compare to that which you have shown.
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LIST OF ABBREVIATIONS

ABC, ATP binding cassette; AOX, alcohol oxidase; AQ, amodiaquine; ARM, artemether; ART, aremisinin; ARU, artesunate; ATP, adenosine triphosphate; AzBCQ, azido biotinylated chloroquine; BAD, biotin acceptor domain; bp, basepairs; BSA, bovine serum albumin; CDC, Centers for Disease Control and Prevention; CFTR, cystic fibrosis transmembrane conductance regulator; CHO, Chinese hamster ovary; CIP, calf intestinal phosphatase; CM, crude membrane; CMC, critical micellar concentrations; CQ, chloroquine; CQR, chloroquine resistant / resistance ; Da, Daltons; ΔpH, pH gradient; DHA, dihydroartemisinin ; DHFR, dihydrofolate reductase; DHPS, dihydroopterate synthase; DM, dodecylmaltoside; DV, digestive vacuole; ECL, enhanced chemiluminescence; FPIX, ferriprotoporphyrin IX; Hb, hemoglobin; HF, halofantrine; Hz, hemozoin; IAD, iminodiacetic acid; IMAC, immobilized metal affinity chromatography; ISOV, inside out vesicle; kb, kilobases; kDa, kiloDaltons; LB, Luria broth; LDH, lactate dehydrogenase; LF, lumefantrine; LPC, lysophosphatidylcholine; MDR, multidrug resistance; MGM, minimal glycol medium; MMM, minimal methanol medium; MQ, mefloquine ; MS, mass spectrometry; MW, molecular weight; MWCO, molecular weight cutoff; NBD, nucleotide binding domain; NIH, National Institutes of Health; NTA, nitrilitriacetic acid; OD, optical density; OG, octylglycoside; PABA, para-aminobenzoic acid; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PEG, polyethylene glycol; PEP, phosphoenolpyruvate; PfATPase 6, Plasmodium falciparum sarcoplasmic
endoplasmic reticulum calcium adenosine triphosphatase; PfCRT, *Plasmodium falciparum* chloroquine resistance transporter; PFIC, progressive familial intrahepatic cholestasis; PfMDR1, *Plasmodium falciparum* multidrug resistance protein; PfNHE, *Plasmodium falciparum* sodium-proton exchanger; Pi, inorganic phosphate; PG, proguanil; PK, pyruvate kinase; PL, proteoliposome; PM, plasma membrane; PVDF, polyvinylidene difluoride; PY, pyrimethamine; QD, quinidine; QN, quinine; QTL, quantitative trait locus; RBC, red blood cell; SDS, sodium dodecyl sulfate; 6His / 6H, hexa-histidine; SNP, single nucleotide polymorphism; SP, sulfadoxine-pyrimethamine; SUR, sulphonylurea receptor; SX, sulfadoxine; Tm, melting temperature; TM, transmembrane; TMD, transmembrane domain; UV, ultraviolet; VBL, vinblastine; VPL, verapamil; XLSA/A, X-linked sideroblastic anemia and ataxia.
CHAPTER 1: BACKGROUND

1.1 Malaria

1.1.1 Epidemiology and pathology

Each year, there are approximately 300-500 million cases of malaria, an infectious disease caused by protozoan parasites of the genus *Plasmodium* [1]. Of the more than 120 species of *Plasmodium*, five infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. Infections with *P. falciparum* are typically the most severe and have the greatest potential for lethality, causing 700,000 – 2.7 million deaths per year globally, mostly in young African children [2]. Infection is transmitted through an insect vector, female *Anopheles* mosquitoes, which require blood for egg production. Although there are more than 400 species of *Anopheles*, less than 50 transmit the infection. In humans, common symptoms include ague (fever, sweating, and chills), nausea and vomiting, and headache. Additionally, physical signs of anemia, jaundice, and splenomegaly may be present. If left untreated, the disease may progress to coma, renal failure, pulmonary edema, and death.

1.1.2 History

Evolutionary biologists estimate that parasitic ancestors of *Plasmodia* existed at least 500 million years ago, and became parasitic to vertebrates about 150 million years ago [3]. One of the first written documentations of the signs and symptoms of malaria is found in the *Nei Ching, The Canon of Medicine*, dated as early as 2700 BC [3]. Serious
scientific study of malaria (from the Italian for “bad air”) began in the late 19th century. While the disease was previously thought to be caused by miasma (the stench from decaying matter), in 1880 Charles Louis Alphonse Laveran identified parasites in the blood of a patient; he won the Nobel Prize for this discovery in 1907. In 1886, Camillo Golgi distinguished between the forms of malaria caused by the different species based on the periodicity of symptoms. In 1897, Ronald Ross demonstrated that parasites could be transferred from infected people to mosquitoes and also (working with avian *Plasmodium* species) that mosquitoes could transfer parasites from bird to bird; this work led to a Nobel Prize in 1902. [4]

1.1.3 Geographic distribution of malaria

The geographic distribution of malaria depends on the presence of *Anopheles* mosquitoes and the ability of parasites to grow within them, which in turn mainly depends on climatic factors, including temperature, humidity, and rainfall. Temperature is particularly critical because *P. falciparum* cannot complete its incubation in the mosquito below 20°C (68°F), and thus cannot be transmitted. The highest disease prevalence is found in sub-Saharan Africa, where swampy areas that favor mosquito growth are widespread. Malaria eradication has been achieved through economic development and public health measures in many temperate areas, such as Western Europe and the United States, but *Anopheles* mosquitoes are present in most of these areas, so resurgence of the disease may be a risk. Global warming also threatens to expand the region of the globe where conditions allow for successful malaria transmission. [5]
1.1.4 *P. falciparum* life cycle

During a blood meal, sporozoites are released from the salivary glands of the mosquito into the bloodstream of a human. The sporozoites migrate to the liver and invade hepatocytes, where they replicate and develop into schizonts. Mature schizonts burst, releasing merozoites into the bloodstream. The merozoites invade erythrocytes (red blood cells, RBCs) and develop progressively into ring stage parasites and then trophozoites. Immature trophozoites can differentiate into either gametocytes (male and female gametes) or, more commonly, merozoites. Upon RBC lysis, the progeny parasites are released into the bloodstream. This massive loss of RBCs is responsible for many of the signs and symptoms of infection (*e.g.*, anemia, jaundice). The merozoites go on to infect a new RBC, while the gametocytes may be ingested by another mosquito in a
blood meal. Within the mosquito midgut, a male and a female gametocyte fuse to form a zygote, which develops into an ookinete and then an oocyst. Mature oocysts burst, releasing sporozoites that migrate to the salivary glands of the mosquito where they can be injected into another human.

**Figure 1.2** Life cycle of *Plasmodium falciparum*. Reprinted by permission from Macmillan Publishers Ltd: *Nature* 419(6906): 495-496, Copyright 2002 [6]

1.1.5 *Intraerythrocytic growth*

One of the primary physical markers of progression through the intracellular cycle is the development of the parasite digestive vacuole (DV). The DV is an acidic organelle analogous to a human lysosome. Within the DV, the parasite degrades hemoglobin (Hb) it ingests from the RBC cytoplasm. From the Hb, it 1) acquires amino acids for protein synthesis [7] 2) creates physical space for growth within the cell [7] and 3) helps maintain osmotic stability [8]. However, the digestion of Hb produces free heme (or ferriprotoporphyrin IX, FPIX, Fe₃O₄) as a toxic byproduct. The parasite detoxifies
heme by converting it into hemozoin (Hz), an insoluble black crystal also known as malarial pigment, which is visible by light microscopy. The crystal structure has been solved, and shows that the molecules dimerize through reciprocal iron–carboxylate bonds to one of the propionic side chains of each porphyrin ring, and these dimers crystallize by forming chains linked by hydrogen bonds [9] (Figure 1.3).

![Figure 1.3 Hemazoin structure and formation. FPIX (left) forms head-to-tail dimers through hydrogen bonds between atoms Fe1 and O41. The dimers (right) then link by hydrogen bonds between O36 and O37 to form an elongating crystal. [Reprinted by permission from Macmillan Publishers Ltd: Nature. 404(6775): 307-10, Copyright 2000]]

1.2 Antimalarial Drugs

The liver stage is relatively short and asymptomatic, resulting in a scarcity of available treatment options and a narrow treatment window. Therefore, drug therapy is generally directed at the erythrocytic stage.
1.2.1 Anti-folates

Antifolate drugs kill parasites by blocking parasite DNA replication. The sulfonamide class of antifolates (including sulfadoxine, SX), compete with para-aminobenzoic acid (PABA) as substrates for dihydropteroate synthase (DHPS), which normally converts PABA to folic acid. Sulfonamides are often combined synergistically with inhibitors of dihydrofolate reductase (DHFR), another enzyme in the purine nucleotide biosynthesis pathway. Pyrimethamine (PY) and other drugs in this class are folic acid antagonists that competitively inhibit DHFR, thereby preventing the reduction of dihydrofolic acid to tetrahydrofolic acid. Tetrahydrofolate is the electron acceptor / methyl donor for the thymidylate synthase-catalyzed conversion of dUMP to dTMP, which is then phosphorylated to dTTP. Without sufficient purine nucleotides, the parasites cannot replicate their DNA for daughter cells.

Figure 1.4 Chemical structures of common anti-folate drugs. Left to right: pyrimethamine (PY), and sulfadoxine (SX), and proguanil (PG).
Sulfadoxine-Pyrimethamine (SP) used to be a mainline treatment for malaria, but resistance to the treatment developed rapidly, currently rendering the drugs almost completely useless. Resistance is ultimately due to point mutations in the \( dhfr \) and \( dhps \) genes that result in amino acid substitutions (N51I, C59R, S108N, I164L in \( dhfr \) and S436A/F, A437G, K540E, A581G and A613S/T in \( dhps \)) [10].

1.2.2 Quinolines

Historical accounts of the use of the bark of the cinchona tree to treat fevers date back to the 1600s. In 1817, Pelletier and Caventou extracted the compound responsible for the medicinal properties of the bark and named it quinine (QN). Woodward and Doering published a formal chemical synthesis in 1944 [11], and a stereoselective synthetic scheme (which distinguishes QN from its diastereomer, quinidine (QD)) was subsequently reported [12], but artificial production is far too costly in comparison to isolation of the natural compound. Drug toxicities and shortage of cinchona plants eventually lead to the search for other 4-amino quinolines with antimalarial activity. Chloroquine (CQ), first produced by Hans Andersag at Bayer laboratories in 1934, was the first fully-synthetic antimalarial drug to be mass produced. CQ was the therapy of choice for decades due to its low production costs and relative lack of side effects, but rampant global resistance has lead to the search for replacement drugs. Another synthetic 4-amino quinoline, mefloquine (MQ), was introduced by the Walter Reed Army Institute of Research in the 1970s, but toxicities limit its use in some patient populations (\( e.g. \), children, pregnant women) [13].
Figure 1.5 Chemical structures of common quinoline drugs. Left to right: chloroquine (CQ), quinine (QN), mefloquine (MQ)

It is currently thought that the quinolines act selectively against stages of the parasite in which hemoglobin is degraded and hemozoin formed, but this tenet has recently been disputed [14]. In 1965, it was proposed that CQ acted by inhibiting DNA and RNA synthesis through direct binding to the nucleic acids [15], but this hypothesis has since been completely rejected. Another hypothesis held that CQ inhibits the heme polymerase responsible for polymerizing heme monomers into hemozoin in the DV. However, the X-ray structure of heme shows that it is a crystal (not a polymer) consisting of dimeric FPIX oriented in a head-to-tail configuration [9] (see Section 1.15). The currently prevailing mechanistic model is that CQ binds heme (Figure 1.5) and the drug-heme complex caps the growing crystal [16], preventing further crystallization. This theory is supported by solution and solid state $^{13}$C and $^{15}$N NMR studies, which have shown that CQ forms a covalent complex with FPIX [17-18]. This could prevent Fe-O bond formation, and thereby prevent heme crystallization. It is thought that all quinolines act in a similar manner to CQ.
The mechanisms for resistance to quinoline drugs will be discussed in depth at a later point (see section 1.5).

### 1.2.3 Aryl alcohols

Halofantrine (HF, Figure 1.7) is a phenanthrene methanol first developed in 1972 that has vague structural similarities with QN. Significant cardiotoxicity can occur even at standard doses, but it has a major advantage in its effectiveness against chloroquine resistant (CQR) malaria. Lumefantrine (LF) is a related drug that does not cause cardiac side effects. The mechanism of action of aryl alcohols is currently unknown, although it is generally thought that they act in a manner similar if not identical to quinolines.
Figure 1.7 Structure of common aryl alcohol drugs. Left to right: halofantrine (HF) and lumefantrine (LF)

1.2.4 Endoperoxides

Artemisinin (ART, Figure 1.8) was first extracted from *Artemisia annua* (the sweet wormwood plant) by Chinese scientists in 1971 [20-21] and was shared with the rest of the world in 1979. The compound has an unusual 15-carbon (sesquiterpene) peroxide structure, the synthesis of which has been achieved [22] but (like QN) is too expensive for mass production [21]. ART is metabolized in the liver to its active form, dihydroartemisinin (DHA). Endoperoxides are extremely effective, killing nearly all the asexual stages of parasites in the blood as well as the gametocytes. These drugs are also very fast-acting, but they have very short half-lives in the body and therefore must be used in combination with other drugs to insure clinical efficacy.
Figure 1.8 Chemical structures of common endoperoxide drugs. Left to right: artemisinin (ART), artemether (ARM), artesunate (ARU), and dihydroartemisinin (DHA).

The mechanism of action of this class of drugs remains under debate. The endoperoxide bridge is necessary (but not sufficient) for activity. One group has proposed that the primary drug target is the sarcoplasmic endoplasmic reticulum calcium adenosine triphosphatase (PfATPase 6) [23]. Ferrous iron-dependent alkylation is another hypothesis [24]. Finally, a mechanism similar to that of CQ, including binding to heme and interfering with hemozoin crystallization, has been put forth [25]. High levels of resistance to ART have not yet been reported. Several isolates with somewhat decreased ART susceptibility were found in French Guiana [26] and their gene encoding for PfATPase 6 contained point mutations, but these findings have not yet been reproduced.

1.2.5 Chemoreversal agents

Verapamil (VPL, Figure 1.9) is a calcium channel blocker used clinically to treat a variety of cardiac disorders. *In vitro*, VPL has the property of re-sensitizing cells to the drugs to which they are resistant. VPL is a monoprotic base with a pKa that is highly
temperature sensitive (8.9 at 25°C). In neutral lipid membranes, VPL undergoes a pKa-shift of 1.2 units and the percent of noncharged species increases from 5% to 45% [27].

![Chemical structure of verapamil (VPL)](image)

**Figure 1.9** Chemical structure of verapamil (VPL)

### 1.3 Transporters Involved In Multidrug Resistance

#### 1.3.1 ABC transporters

ATP binding cassette (ABC) proteins comprise a ubiquitous family, members of which have been found in every organism so far examined. ABC proteins are defined by the presence of the ABC unit, which is a 200-250 amino acid sequence that harbors two short, highly conserved peptide motifs (the Walker A and Walker B), which are involved in ATP binding. A third conserved sequence, the ABC signature, which is diagnostic for the ABC unit, is located between the Walker A and B sequences and is necessary for ATP hydrolysis. As membrane transporters, this class of molecules contains membrane-embedded, transmembrane domains (TMDs), which are usually composed of six transmembrane (TM) helices. The minimal structural requirement for a functional ABC transporter seems to be two TM domains and two ABC units (also known as nucleotide binding domains, NBDs). These may be present within a single polypeptide chain (full
transporters) or within a multiprotein complex. The x-ray crystallographic structure is available for at least 16 NBDs from ABC transporters (as well as for some single-cassette bacterial transporters, such as MsbA [28]), and these indicate that NBDs likely dimerize to form a functional ATP binding site [29].

![Figure 1.10 General structure of full ABC transporters. Grey rectangles represent TMDs, circles represent NBDs containing the Walker A (horizontally striped square) and Walker B (vertically striped square) motifs.](image)

**Figure 1.10** General structure of full ABC transporters. Grey rectangles represent TMDs, circles represent NBDs containing the Walker A (horizontally striped square) and Walker B (vertically striped square) motifs.

Although a large number of the known ABC proteins are active pumps (that is, they use cellular energy to transport against the electrochemical gradient of the substrate), there are several examples deviating from this type of function. The human ABC50 associates with initiation factors and ribosomes and thus might be involved mRNA translation [30]. The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel, and the sulphonylurea receptors (SUR1 and SUR2) regulate the permeability of potassium channels.

Mutations of many human ABC proteins are known to be causative in inherited diseases. The most well-known and well-studied is the CFTR, but other examples
include MDR3, mutation of which causes progressive familial intrahepatic cholestasis (PFIC), and ABC7, whose mutation results in X-linked sideroblastic anemia and ataxia (XLSA/A).

1.3.2 HuMDR1

In 1976, a group working with Chinese hamster ovary (CHO) cells noted that cell lines selected for resistance to colchicine displayed an upregulation of a large cell surface glycoprotein. Since this glycoprotein appeared unique to mutant cells displaying altered drug permeability, it was named the “P” glycoprotein [31]. A corresponding protein was then looked for, and found, in anti-cancer drug resistant mouse and human cells [32]. The human P-glycoprotein is called the Human MultiDrug Resistance protein, or HuMDR1. Encoded by the mdr1 gene on chromosome 7q21 [33], HuMDR1 is 1280 amino acid, 130-170 kDa protein that is variably glycosylated. HuMDR1 is primarily localized to the plasma membrane, generally on the apical (or luminal) surface of polarized epithelial cells. These include the brush border membrane of intestinal cells, the biliary canalicular membrane of hepatocytes, and the luminal membrane of the proximal tubules in the kidney [34]. HuMDR1 is also present at the pharmacological barriers of the body, such as the blood-brain barrier.

HuMDR1 was the first human ABC protein cloned [35]. In an attempt to determine the physiologic function of P-glycoproteins, knockout mice were made. The mouse genome contains three homologues (mdr1, mdr2, and mdr3) but overexpression of mdr2 is not associated with the multidrug resistance phenotype [36]. The only
abnormality observed in mdr1, mdr3, and mdr1/mdr3 knockouts was a hypersensitivity to xenobiotic compounds, including a 100-fold increased sensitivity to ivermectin, a centrally neurotoxic pesticide [37-38]. This led to the hypothesis that HuMDR1’s physiologic function is protection against toxic compounds. Based on the sequence homology between HuMDR1 and known bacterial transport proteins, a model for P-glycoprotein was proposed in which HuMDR1 actively effluxes drugs through a pore or channel in the membrane formed by the transmembrane domains of one or more HuMDR1 molecules [39-41]. The protein was found to bind ATP [42] and exhibit ATPase activity [43], which was taken as evidence of thermodynamically active transport. Using site directed mutagenesis and photoreactive drug analogues, TM helices 5 & 6 and 11 & 12 were defined as contributing to the protein’s drug binding site(s) [44-48]. Some studies suggest that there are as many as 4 different drug binding sites, three of which may be cooperative sites for transport and one of which is for regulation of the transport [49].

Another important observation was that the altered cellular transport drugs by HuMDR1 could be inhibited by certain drugs called chemoresidual agents, named for their ability to re-sensitize drug resistant cells. Some identified chemoresidual agents include calcium channel blockers [50], calmodulin inhibitors [50-51], the anti-estrogen tamoxifen [52], and the immunosuppressant cyclosporin A [53]. However, most investigators have proposed that these drugs do not exert their effects through ion modulation or signal transduction, but rather through competitive binding to and blocking of HuMDR1.
In the mid-90s, evidence began accumulating that drug resistance could not be fully attributed to HuMDR1. In 1996, a paper was published in *Cancer Research* that reported the results from a workshop attended by 15 global institutions. It was found that there is no consistent correlation between HuMDR1 expression and disease progression or outcome [54]. (The conclusion of this workshop was an agreement on the need to standardize HuMDR1 detection methodology. Their failure to find a correlation was assumed to be due to inadequate testing, and it was apparently never considered that the correlation just might not actually exist.) Furthermore, enzymatic analyses of HuMDR1 found that transport kinetics are not correlated with drug toxicity [55]. This paper concluded that a drug efflux model cannot adequately explain their results. Then, it was shown that in HuMDR1-overexpressing cell lines, which were not obtained by drug pressure but were stably transfected with HuMDR1 driven by the CMV promoter, the maximum amount of resistance achieved was 2- to 5-fold for a panel of chemotherapeutic drugs [56]. This is nowhere near the degree of resistance seen in drug-selected lines (which can reach hundreds-fold), which implies that the drug selection must be affecting other cellular processes aside from just the upregulation of HuMDR1. In addition, it was shown that chemoreversal agents induce changes in membrane 'viscosity' and therefore the mechanism of chemoreversal may proceed independently of HuMDR1 [57]. Finally, it is important to note that all HuMDR1 theories of drug resistance evolved before much was known about apoptosis and its role in tumor survival [58].

Another major shortcoming in the HuMDR1 literature has been the inability to translate *in vitro* work to *in vivo* results. Attempts at modifying clinical drug resistance
by inhibiting HuMDR1 with chemoreversal agents have for the most part been unsuccessful. The first clinical trial with a chemoreversal agent took place in 1985, and found no increase in cancer drug efficacy [59]. Further investigations varied dosing schedules, mode of administration, and the chemoreversal agent used, but to no avail [60-64]. Two reviews, both published in 1993, reported failure to successfully modulate resistance by targeting HuMDR1 [65-66], and a meta-analysis conducted a decade later came to the same conclusion [67]. It is believed that blood plasma concentrations of reversal agent sufficient to affect HuMDR1 cannot be reached without incurring unacceptable toxicity. Nevertheless, clinical trials still continue, much with the same results [68-69].

It is now generally accepted that HuMDR1 alone cannot fully account for the spectrum of drug resistance found clinically. Since efflux pumps are localized to the plasma membrane, and are therefore one of the first components of the cell to interact with drugs, reduced accumulation may be a first line of defense. Although two more ABC transporters, ABCC1 (MRP1) and ABCG2, are proposed to contribute to the efflux phenomenon, the majority of the MDR phenotype is determined by altered drug metabolism, p53 mutations, DNA repair capacity, a hostile intratumor microenvironment, and modified apoptotic pathways [29].

1.3.3 PfMDR1

It was noted in the late 1980s (just after human HuMDR1 was identified) that there were several similarities between anti-malarial and anti-cancer multidrug resistance,
including decreased drug accumulation (which raised the possibility of drug efflux), patterns of pleiotropic resistance (resistance to more compounds than just the selecting agent), and reversibility [70]. Additionally, some antineoplastic drugs (such as doxorubicin and vincristine) share chemical properties with the quinoline-based antimalarial drugs: they are polyaromatic, amphipathic, and weakly basic with at least one titratable nitrogen that has a pK\textsubscript{a} between 6 and 9. In light of these parallels, a search of the malarial genome was performed looking for homologues to HuMDR1, and two candidate genes were found.

The smaller gene was named *Plasmodium falciparum* multidrug resistance 2 (*pfmdr2*) [71] and was localized to chromosome 14 [72]. Its protein product was predicted to have 10 TMDs and one ABC [73], which is somewhat different from canonical drug transporters, and indeed numerous studies failed to find an association between *pfmdr2* and drug resistance [72-74]. However, it was noted that *pfmdr2* is similar to the yeast *hmt1* gene [73], which is involved in cadmium resistance, and PfMDR2 has recently been implicated in protection from heavy metals [75].

The larger gene identified in the homology search had 54% similarity to HuMDR1 [76], and was designated *Plasmodium falciparum* multidrug resistance 1 (*pfmdr1*). *pfmdr1* is a 4257 basepair gene on chromosome 5 that encodes a protein (PfMDR1) with a predicted topology of 12 TMDs and 2 cytoplasmic ATP binding sites organized in two identical tandem cassettes, thereby placing it in the ABC superfamily. PfMDR1 looked to be a very promising candidate for mediating drug resistance, as it was localized to the membrane of the parasite DV [72], but the orientation of the molecule
was found to be with the ATPase domains facing the cytoplasm, which via conventional models suggests that it would act to translocate drugs into the DV, towards the primary drug target (FPIX or Hz, see Section 1.2.2). Initial work suggested that PfMDR1 might be acting in a similar manner to HuMDR1, which is a pretty remarkable finding considering the evolutionary distance between the two organisms. It was found that sensitive and resistant strains released different percentages of pre accumulated CQ, which hinted at a possible increased rate of efflux in the resistant strains [77]. Then it was found that some resistant cell lines had amplified pfmdr1 gene copy numbers as well as increased amounts of pfmdr RNA [76]. Finally, one group was able to use pfmdr1 mutational status as a predictor of drug resistance status [78].

1.3.4 PfCRT

In 1990, a paper was published in Nature documenting a genetic cross of a CQ sensitive (CQS) malaria strain with a CQ resistant (CQR) strain [79]. From the progeny of this cross, it was determined that CQ resistance does not segregate with inheritance of the pfmdr1 locus. Further analysis revealed that the resistance locus maps to a segment on chromosome 7 [80]. Finally, in 2000, the critical gene within this region was identified as the Plasmodium falciparum Chloroquine Resistance Transporter gene, or pfcrtn [81]. Pfcrt is a 13 exon gene spanning 36 kb of chromosome 7 that encodes a 424 amino acid protein with 10 transmembrane helices. Although not an ABC transporter, PfCRT is a polytopic integral membrane protein localized to the membrane of the DV
and it belongs to a drug and metabolite transporter superfamily [82-84]. More importantly, it has been shown that mutations in PfCRT are causative of CQR.

CQR took a relatively long time to develop (>20 years between the introduction of the drug in 1934 and the first reports of resistance in the late 1950s), which implies either a multigenic cause or a requirement for multiple mutations in a single gene. Genetic analysis has revealed that CQR arose geographically in at least five independent events: Cambodia, the Thai-Cambodian border, Papua New Guinea [85], Peru [86] and the Philippines [87]. The African focus first observed in 1983 is predicted to have been derived from a Southeast Asian focus [88].

There are at least 21 single nucleotide polymorphisms (SNPs) in pfcrt that distinguish CQR strains from CQS. Of these, the 12 positions usually used to define a haplotype lie within codons for amino acids 72, 73, 74, 75, 76, 77, 97, 220, 271, 356, 356, and 371. CQR strains of African and Southeast Asian origin (Old World) carry the
haplotype CVIETIHSEST/II, while those from South America (New World) generally have SVMNTIHSQDLR [90]. Both field studies and allelic exchange experiments have confirmed that the lysine to threonine mutation at position 76 (K76T) is critical for CQR [91]. However, this one mutation alone is not sufficient to confer resistance; it must be present within a background of at least 4 other amino acid substitutions [87].

1.3.5 PfNHE

Quantitative trait loci (QTL) analysis has implicated a region of chromosome 13 (as a partner with pfcrt) in conferring resistance to QN [92]. This chromosomal segment harbors a putative sodium / proton exchanger. The encoded protein, PfNHE, is 1920 amino acids in length with 12 predicted TM helices and a signal peptide cleavage site [92-93]. High PfNHE activity has been found to correlate with high levels of QNR [94]. It has been proposed that the ratio between the number of C-terminal DNNND repeats and the number of histidine residues in the adjacent polyhistidine rich region (both of which vary amongst isolates [95]) changes the physiologic set point of the protein, which in turn alters cytosolic pH and modulates levels of QNR.

1.4 Mechanisms of Drug Resistance

1.4.1 HuMDR1

The most popular model for the action of HuMDR1 is active drug efflux. HuMDR1 is thought to bind to polar drugs that have passively diffused through the lipid bilayer of the membrane into the cell and actively pump them out, even against a
concentration gradient. Similarly, HuMDR1 is also believed to interact with nonpolar drugs that segregate within the membrane bilayer and pump them out of the cell. The only difference between these two scenarios is the relative hydrophobicity of the substrate, which largely determines its partitioning within cellular compartments. A variation of the efflux pump model is the “flippase” model, which posits that HuMDR1 transports drugs from the inner leaflet of the plasma membrane to the outer leaflet or the external medium [96]. Most of the evidence for flip-flop activity comes from studies using lipids and lipid derivatives as HuMDR1 substrates [97], and its validity has been disputed by data showing equivalent unidirectional flux of substrate in HuMDR1-positive and -negative cells [98].

An alternative hypothesis accounting for the decreased accumulation of drugs in MDR cells is that the altered partitioning of the drugs between the extracellular and intracellular compartments is due to a perturbation in the chemical environment and/or the number of drug binding sites. MDR cells overexpressing HuMDR1 have shown altered intracellular pH homeostasis and decreased membrane potential [56, 99-100], which cause significant variations in the rates of passive diffusion of chemotherapeutic drugs, thereby affecting the efficiency of their retention within MDR cells. As mentioned earlier, CFTR and SUR1/2 are ABC proteins that control ion permeability, so it is possible that huMDR1 is either an ion transporter itself or indirectly regulates an ion transport process.
1.4.2 PfCRT

There is currently disagreement in the literature as to the exact mechanism by which PfCRT confers CQR. One hypothesis is that the protein mediates active drug efflux (similar to that of HuMDR1), using energy to transport CQ out of the DV and thus away from its targets [101]. Via this model, mutations in the protein may alter its substrate specificity, leading to greater CQ affinity for mutant isoforms.

Another suggestion is that PfCRT facilitates diffusion of the charged drug species (often referred to as the “charged drug leak” hypothesis; [102-103]). Within the acidic DV, a greater proportion of the drug molecules present is charged compared to that outside the DV, which (neglecting drug binding) sets up a concentration gradient oriented outwards. Since charged molecules generally cannot pass through the hydrophobic environment of a membrane, they require some sort of carrier. One particular advantage of this model is that it provides an explanation for the importance of the K76T mutation: the lysine in wildtype CQS isoforms has a basic side group whose positive charge repels protonated CQ, while the neutral threonine allows for an open pore through which charged CQ may pass.

An additional proposed mechanism is based upon alterations in the pH of the DV, which may be directly or indirectly influenced by PfCRT. Measurements of DV pH have shown that CQR parasites have a more acidic DV than CQS parasites [104], which is unexpected because weak base partitioning would predict increased drug accumulation at low pH. However, the rates of hematin aggregation and hemozoin formation are increased at acidic pH, which would reduce the amount of target available for CQ
binding [81]. The excess unbound drug could alter the equilibrium of passive drug accumulation [103] or could be transported out of the digestive vacuole by mutant PfCRT [105].

1.4.3 PfMDR1

PfMDR1 is hypothesized to work by the same mechanisms as those put forth for HuMDR1. However, there is an added layer of confusion in that the gene is not only overexpressed in MDR parasites, but also mutated.

Multiple field studies have found that increased pfmdr1 copy number is associated with decreased clinical response to several antimalarial drugs, including MQ [106-108], QN [107], ART [107-108], and HF [106]. These correlations have been supported by laboratory experiments. Parasite strains pressured with MQ resulted in amplification of the pfmdr1 locus, as well as concomitant decreased sensitivity to QN and HF [109-110]. Conversely, exposure of parasites with increased pfmdr1 copy number to increasing CQ led to pfmdr1 deamplification and increased MQ sensitivity [111]. The genetic disruption of one of the two copies of pfmdr1 in a CQR line, with confirmed knockdown of mRNA and protein, resulted in a 3-fold reduction in MQ resistance as well as increased susceptibility to LF, HF, QN, and ART [112]. In addition to these changes at the DNA level, pfmdr1 mRNA transcript levels are also responsive to drug treatment; parasites exposed to CQ, MQ, and QN had increased pfmdr1 transcript levels, while those treated with PYR did not change [113].
Five mutations in \textit{pfmdr1} have been identified and implicated in multidrug resistance: N86Y and Y184F in the N-terminal cassette and S1034C, N1042D, and D1246Y in the C-terminal cassette (Figure 1.10). The Y184F mutation seems to be globally distributed, while the N86Y is common in Africa and Asia [114], and combinations of the 3 C-terminal mutations are more prevalent in South America [115]. Transfection experiments suggest that PfMDR1 mutations may make very small contributions towards relative sensitivity to QN, MQ, HF and ART [116-117]. Using allelic exchange, one group reported that introduction of the three C-terminal mutations into a CQS strain conferred resistance to QN and mildly increased susceptibility to MQ, HF and ART; the MQ and HF effects were even more apparent in a strain bearing only the 1246Y mutation. Reversion of the same three mutations back to wildtype in a CQR (strain 7G8) background resulted in the opposite phenotype (MQ, HF, and ART resistance and QN sensitivity) [116]. These results were later confirmed in additional transfection studies by another group [117], which also suggested the 1042D mutation was particularly influential. Finally, analysis of the progeny from a genetic cross between two CQS strains (which have identical PfCRT sequences but differ in PfMDR at two amino acids, 184 and 1042) observed a complete association between mutant \textit{pfmdr1} and increased sensitivity to MQ, HF and ART [118]. Clinical isolates in both Thailand [119] and The Gambia [120] specifically associate the 86Y mutation with MQ sensitivity.
1.5 Heterologous Expression of Proteins

The complexity of natural biological systems often makes the study of protein structure and function difficult in the native environment. For example, PfCRT and PfMDR1 are found in the DV membrane, which itself is enclosed by the parasite’s plasma membrane and parasitophorous vacuole, all of which is encircled by the plasma membrane of the RBC. Isolation of intact, functional DVs is problematic. Detailed determination of the molecular properties of some proteins is therefore better accomplished via heterologous expression in simpler systems. Such systems are especially helpful when the expression level of the protein of interest is low or when there are no known methods by which to assay its function \textit{in vivo}. 
1.5.1 Expression in bacteria

*E. coli* are by far the most popular host for heterologous protein expression. Much is already known about the genetics (including the entire genomic sequence) and physiology of the organism, which facilitates gene cloning and cultivation. *E. coli* have a high growth rate and can grow to high densities in simple, inexpensive media. Protein production is also very high, with the recombinant product comprising up to 30% of total cellular protein [121]. However, there are also some serious disadvantages to prokaryotic systems, the most significant being the lack of post-translational modifications (including proper folding, glycosylation, phosphorylation, and disulfide bridging). *E. coli* can neither N- nor O-glycosylate proteins, and other bacteria that do O-glycosylation (such as *Neisseria meningitidis*) add a different trisaccharide than that found in eukaryotes. Another disadvantage is that many highly overexpressed proteins aggregate into inclusion bodies that must be solubilized under denaturing conditions and then refolded. A final drawback is that bacteria often produce endotoxins or toxic cell wall pyrogens that might need to be thoroughly removed depending upon the downstream use of the expressed protein (*e.g.*, vaccine immunogen).

1.5.2 Expression in yeast

Yeast have become the favored alternative to bacteria for expression of eukaryotic proteins requiring post-translational modifications. Like bacteria, yeast grow rapidly to high cell density in simple media and a great deal is known about their genetics. Expression in yeast is faster and less expensive than other eukaryotic expression systems
(insect baculovirus, *Dictyostelium* slime mold, or mammalian cells). Inducible systems are also available for regulating the protein expression.

### 1.5.3 Heterologous expression of *Plasmodium* proteins

A major limitation to heterologous expression of foreign genes is the difference in codon usage and codon bias between the originating organism and the host [122-123]. A dramatic example is exemplified by the work in this thesis; the *P. falciparum* genome is extremely AT-rich (~82% overall, [124]), which can result in premature termination of transcription in eukaryotes [125-126]. Low levels of expression for some endogenous *P. falciparum* cDNAs (encoding smaller soluble proteins or soluble domains of larger proteins, [127]) have been reported, but the combination of unusual gene structure, large size, and other features has prevented routine high level heterologous overexpression of large malarial polytopic integral membrane proteins. One previous study reported expression of the native *pfmdr1* cDNA in yeast on the basis of indirect evidence [128], but this paper was subsequently retracted [129]. There was no western blot confirmation of PfMDR1 expression, and the published retraction suggests that inadvertent false positive transfection with the yeast homologue STE6 was likely responsible for some, if not all, of the phenotypic features analyzed in the selected clones. The problem of codon mismatch can be overcome through the replacement of cDNA with synthetic genes constructed using the preferred codons of the host system [130]. A further advantage to the use of synthetic genes is the ability to engineer convenient restriction sites, which facilitates
subcloning and the creation of mutants. The Roepe laboratory has previously used this strategy to express PfCRT in *Pichia pastoris* yeast [131].

1.6 Purification and Reconstitution of Membrane Proteins

Biological membranes are complex systems containing a great number of proteins, which greatly complicates any effort to separate discrete qualities and accurately ascribe them to a single source. The investigation of transport by and/or catalytic properties of any individual membrane protein can be accomplished by its isolation and reconstitution into a closed lipid vesicle (proteoliposome, PL), which removes the interference of other proteins found in the natural environment. Membrane proteins are known to exert or retain full activity only when incorporated in the correct orientation within a lipid bilayer [132].

Purification of recombinant membrane proteins can easily be achieved by affinity chromatography if the gene sequence is fused in-frame with an epitope tag. HuMDR1 has previously been tagged with a biotin acceptor domain (BAD) to enable purification via avidin chromatography [99-100] or either the hexahistidine or decahistidine tag to enable purification via Ni\(^+\)NTA chromatography [133]. A single fusion with both epitope tags can enable a two-step purification [134].

1.6.1 Biotin-avidin chromatography

Avidin is a tetrameric glycoprotein with a molecular weight of \(~67\) kDa (each monomeric unit is comprised of 128 amino acids, with a molecular weight of 16 kDa).
Biotin (cis-hexahydro-2-oxo-1H-thieno [3,4] imidazole-4-pentanoic acid, also known as vitamin H, vitamin B7, and coenzyme R) is a cofactor necessary for cell growth, the production of fatty acids, and the metabolism of fats and amino acids. Avidin’s extraordinary binding capacity for biotin was first noted in 1941 [135-136] and its crystal structure was deduced a year later [137].

Avidin and its subunits have been immobilized to a matrix and used for affinity purification since 1973 [138]. The avidin-biotin interaction has a $K_a = 10^{15} \text{M}^{-1}$ [139-142], which is the strongest known noncovalent biological interaction between a protein and a ligand. The presence of detergents in the binding buffer may weaken the interaction, but very harsh conditions are required to break it [142]. An alternative to avidin is streptavidin, a tetrameric biotin-binding protein isolated from *Streptomyces avidinii*. It binds 4 moles of biotin per mole of protein, but is not glycosylated and therefore has less nonspecific binding compared to avidin. Furthermore, its $K_a$ is an order of magnitude lower ($10^{14} \text{M}^{-1}$), so 2-4 mM free d-biotin is sufficient to competitively elute bound biotinylated proteins. Monomeric avidin is another substitute for avidin in affinity chromatography as it also has a reduced $K_a$ ($10^8 \text{M}^{-1}$) and operates under the same mild elution conditions as streptavidin.

Propionic acid bacteria possess a unique biotin-containing enzyme, a transcarboxylase that catalyzes the reversible transfer of a carboxyl group from methymalonyl CoA to pyruvate. The isoform from *Propionibacterium shermanii*, methylmalonyl CoA-oxalacetate transcarboxylase, has a molecular weight of 67 kDa and is composed of 30 polypeptides of three different types: 1.3S, 5S, and 12S. The enzyme
core contains six 12S subunits, and there are three pairs of 5S subunits on each side, with each pair attached to the core by two 1.3S subunits. The 1.3S subunit (MW ~12 kDa) contains all the biotin of the active enzyme (6 moles biotin per mole enzyme, [143]), which is attached to lysine-89 by another enzyme, biotin holoenzyme synthetase [144]. The sequence of the 1.3S subunit can be fused to another protein so that it acts as a BAD, thereby tagging the protein of interest with biotin.

1.6.2 Immobilized metal affinity chromatography

Immobilized metal affinity chromatography (IMAC) is based on the specific coordinate covalent binding of amino acids (particularly histidine) to metals. This technique was first described in 1975 [145]. In these first experiments, iminodiacetic acid (IAD) was charged with a transition metal ion such as Ni\(^{2+}\) or Zn\(^{2+}\). However, the presence of only three chelating sites led to weak binding of loaded proteins. Nitrilitriacetic acid (NTA) has four chelation sites, which binds metal ions more tightly, preventing ion leaching and resulting in greater protein binding capacity and higher purity.

While some proteins have sufficient unmodified surface amino acids to allow binding, most must be engineered to express a tag consisting of at least six consecutive histidine residues (6His or polyHis), which bind metal-charged NTA with an affinity of \(10^{-13}\) M [146]. One major advantage to the polyHis tag is its very small size, which has minimal effects on the structure and function of the fusion protein [147]. Affinity purification using a polyhistidine tag usually results in relatively pure preparations when
the recombinant protein was expressed in prokaryotic host, but purification from higher organisms may require a tandem affinity purification. Polyhistidine tag columns retain several well known proteins as impurities, including the \textit{E. coli} FKBP-type peptidyl prolyl isomerase (SlyD, [148]). In most IMAC applications, imidazole is used to both increase selectivity for poly-histidine tagged proteins as well as to elute the protein from the metal.

1.6.3 \textit{Purification of membrane proteins}

The purification process begins with the lysis of host cells, followed by harvesting of the membrane fraction. The content of integral membrane proteins can be enriched through the use of a mild chaotrope, such as high concentrations of sodium chloride or moderate concentrations of urea, which serves to remove peripheral membrane proteins that are not strongly embedded within the lipid bilayer. Chaotropic agents are ions which favor the transfer of apolar groups to water, increasing its lipophilicity and thereby increasing the aqueous solubility of some hydrophobic proteins [149]. The integral membrane proteins must then be removed from their lipid environment, which is usually accomplished through solubilization with mild nonionic detergents with low critical micellar concentrations (CMCs) such as octylglycoside (OG) or dodecylmaltoside (DM). The solubilized protein can then applied to an affinity purification column, washed to prevent contaminants, and eluted. To reconstitute the pure protein into PLs, the column eluate is first mixed with rehydrated isolated lipids, and then dialyzed, diluted, or adsorbed onto polystyrene beads (Bio-Beads®) to remove the detergent [150]. Detergent
removal causes growth of the lipid-protein micelles with detergent concentrating at the edges. At a critical detergent-phospholipid ratio (~0.2), the micelles fuse to form permeable bilayer sheets. The protein is incorporated into the bilayer, and these unstable sheets fragment into small unilamellar vesicles [151].

1.7 Functional analysis of MDR proteins

1.7.1 ATP hydrolysis

The main assay used to determine activity of an ABC transporter relies upon the protein’s ability to hydrolyze ATP. ATP hydrolysis can be indirectly calculated through the quantification of the catalysis products, inorganic phosphate (Pi) or ADP. Many assay procedures utilize $\gamma^{32}\text{P}$ ATP, in which the cleaved radiolabeled Pi can be quantified by thin layer chromatography followed by scintillation counting. Disadvantages to this method include the use of radioactivity and the high background due to impure ATP. With colorimetric ATPase assays, based on the early work of Fiske and Subbarow [152], the amount of Pi released can be determined spectrophotometrically through the detection of a blue molybdate-orthophosphate complex (sometimes enhanced by malachite green, [153]). Finally, ATPase activity can be evaluated by a coupled enzyme assay [154], in which the conversion of phosphoenolpyruvate (PEP) to pyruvate by pyruvate kinase (PK) is coupled to the conversion of pyruvate to lactate by lactate dehydrogenase (LDH). The second reaction requires the oxidation of NADH (which absorbs strongly at 340 nm) to NAD$^+$ (which does not). Since every molecule of NADH oxidized to NAD$^+$ corresponds
to the production of one molecule of ADP by the ATPase, the decrease in OD\textsubscript{340} can be converted into ATPase activity.

In addition to ATP hydrolysis, a related characteristic of an ABC transporter is its ability to bind ATP, which can also be assayed using radiolabeled ATP. Some ABCs, including HuMDR1, display a phenomenon known as vanadate trapping, in which vanadate forms a complex with the protein that traps ADP in the active site [155]. With the use of a radiolabeled or photoactive ATP analogue, the rate of ATP hydrolysis is proportional to the intensity of the trapped labeled ADP.

There has been extensive analysis of the ATPase activity of HuMDR1. An early study working with protein from adriamycin-resistant leukemia cells found that HuMDR1 ATPase activity was not affected by the antineoplastics adriamycin and vincristine, but was stimulated 50% by 100 µM concentrations of the chemoreversal agents verapamil and trifluoperazine [156]. Similarly, up to 50% activation was produced by colchicine, progesterone, nifedipine, verapamil, and trifluoperazine with protein partially purified from a colchicine-selected line of CHO cells [157]. However, protein purified from these cells and reconstituted into PLs displayed ATPase activity that increased 2-fold with verapamil and trifluoperazine, but was unaffected by colchicine, daunomycin, and vinblastine [158]. These results were for the most part replicated in a different CHO cell line, with the exception that verapamil increased activity over 10-fold [159]. HuMDR1 purified from vinblastine-resistant nasopharyngeal carcinoma cells was stimulated 1.5- to 3-fold by doxorubicin, vinblastine, daunomycin, actinomycin D, verapamil, and colchicine [160]. The ATPase activity of HuMDR1
expressed in insect cells increased 2-3-fold with the addition of anti-cancer drugs (including vinblastine, vincristine, daunomycin, and colchicine), and up to 5-fold with the chemoreversants verapamil and nifedipine [161]. Sf9 insect cells were also used to express the individual ABC cassettes of HuMDR1, both individually and together (the two cassettes in the same cell, but not linked as a full-length protein; [162]). In this system, the half-transporters exhibited ATPase activity, but their activities were not stimulated by drug substrates. Conversely, the ATPase activity of the coexpressed cassettes was found to be drug-stimulatable, which suggests that the coupling of drug binding to ATPase activity requires an interaction between the two different halves of HuMDR1.

1.7.2 Protein photolabeling

Photoaffinity labeling is a well-established technique for probing the proximity of components within biological systems, particularly the nature of protein-ligand interactions. A traditional photolabeling reagent is a biological compound chemically linked to a photoactive moiety. When irradiated, carbonyl, azide, and diazirine groups produce extremely reactive species (excited carbonyl groups, nitrenes, and carbenes, respectively) that are capable of crosslinking to proximate amino acid functional groups. The success of the photolabeling reaction thus depends upon the formation of a covalent bond between the probe and the target. Aryl azides are a very popular photolabeling agent, but the activated nitrene will only react with a nucleophile, such as a free amine, and thus will only insert into the target protein if a lysine or arginine residue is found
within the binding pocket. Perfluorophenyl azides are much more reactive, and offer improved carbon–hydrogen insertion efficiency [163].

The conventional photolabeling approach usually relies on radioactive tags to identify the labeled fragments, but isolation of these fragments by HPLC is difficult due to the co-elution of a relatively large ratio of unlabeled fragments [164]. One solution to this problem is the replacement of radiolabeled isotopes with a biologically functional tag, such as biotin [165]. After photolabeling and proteolysis, the biotinylated fragments can be recovered by avidin chromatography and/or detected by avidin-HRP conjugates.

Photolabeling with subsequent proteolysis and peptide mapping by mass spectrometry (MS) has been used to successfully characterize the substrate binding sites of a number of diverse proteins. However, integral membrane proteins pose a particular challenge in that the hydrophobicity of the amino acid sequences found within transmembrane domains (coupled with the interference of the detergents often used to solubilize the proteins out of their native membrane environments) prevents the proper ionization required for MS analysis [166]. Nevertheless, substantial progress has been made in the field, and the MS mapping of several large membrane proteins has been achieved [166-168].

1.7.3 CQ binding

Most of the data supporting a direct interaction of PfCRT and antimalarial drugs is inferential. The Sudan-106 P. falciparum strain has a PfCRT sequence identical to Dd2 except for the critical K76T, and displays a CQS phenotype. Upon selection with CQ,
resistant clones were obtained containing a 76I mutation [169]. These parasites exhibit a st
stereoselectivity in their drug resistance profiles (hypersensitivity to QN and reduced sensitivity to its diastereomer QD), which suggests drug-protein binding is involved in the resistance mechanism. Further selection of this 76I line on QN led to the generation of second-site suppressor mutations [170]: C72R, Q352K, and Q352R all resulted in QNR / CQS clones. As amino acid position 72 lies within TM domain 1 and 352 within TM 9, these two segments may constitute important structural components of a substrate recognition site. Another study, which obtained CQS strains from CQR lines through amantadine or halofantrine pressure, identified S163R in TM 4 as another putative component of the drug interaction domain [171].

A previous attempt to detect CQ-binding proteins utilized an azido-based radioiodinated probe [172], and obtained labeling of 33- and 42 kDa proteins, the former of which was later identified as the parasite lactate dehydrogenase [173]. However, these experiments were performed using intact parasite cultures in which the relative abundance of CQ target proteins is unknown. A molar ratio of the amount of photolabel to the amount of protein can therefore not be calculated. The photolabeling of the two bands was reduced by 75-85% in the presence of 1000-2000 times as much unlabeled CQ, but a careful titration of competition was not done.

Previous work in the Roepe laboratory [103] using equilibrium centrifugation assays found that yeast-optimized heterologously expressed PfCRT binds $^3$H-CQ. Some small differences were observed in affinity between HB3 (CQS) and Dd2 (CQR) PfCRT isoforms ($K_d$ of 385 vs. 435 nM, respectively), but the propagation of error involved in the
calculations did not result in a statistical distinction. In addition, equilibrium binding experiments are tedious, expensive, and do not easily allow for examination of multiple isoforms or competition studies (e.g., determination of relative affinity for CQ vs. QN vs. MQ, etc.).

1.8 Objectives of this study

1.8.1 Analysis of the molecular function of PfMDR1

The malarial parasite is a very complex system in which to attempt to study membrane proteins. The DV membrane that harbors PfMDR1 is a subcellular compartment within the confines of the parasite plasma membrane, which is itself contained within the parasitophorous vacuole, and of course the whole parasite resides within the RBC host. There are several other ATPases in these membranes that severely complicate any measurement of this activity. Purification of native protein from intact parasites is nearly impossible and certainly cannot be achieved on a scale conducive to detailed analysis in multiple experiments. Indirect information can be obtained through genetic manipulation of parasites, but transfection in Plasmodium cultures is an extremely difficult process that has only been successfully attempted by a few laboratories. Detailed molecular analysis of PfMDR1 would therefore be greatly aided by heterologous expression in a simpler system.

Currently, the main controversy regarding PfMDR1 in the malaria literature concerns whether its effects on drug resistance phenomena are due to mutations in or overexpression of the protein. If its behavior is similar to the homologue HuMDR1, then
PfMDR1 ATPase activity will be modulated by the drugs to which it is believed to influence resistance. Compounds may either stimulate or inhibit activity, seemingly without any dependence on whether the protein confers resistance or hypersensitivity. Since the protein is oriented in such a way as to translocate its substrates into the DV (towards the major target of the quinoline class of drugs), increased ATPase activity should theoretically result in sensitivity while decreased ATPase activity should correlate with resistance. Previous work [106-113] has linked PfMDR1 expression with MQ resistance and QN sensitivity, but the protein is not thought to be a major contributor to CQ response. Thus, it is hypothesized that if PfMDR1 is a significant contributor to drug resistance, MQ at a chemically relevant dose will significantly inhibit PfMDR1 ATPase activity, QN will stimulate ATPase activity, and CQ will have no effect on the protein. On the other hand, allelic exchange experiments have indicated that the three observed C-terminal PfMDR1 mutations result in MQ sensitivity and QN resistance [116-117]. Therefore, it is predicted that relative to wildtype, mutant variants of the protein will display increased ATPase activity in the presence of MQ and decreased activity with QN treatment. Additionally, it has been proposed that mutations in PfMDR1 influence the parasite’s susceptibility to the toxic effects of VPL [174]. Dd2 is a P. falciparum strain that is CQR and VPL-reversible while 7G8 is a parasite strain that is CQR but VPL-irreversible. It is hypothesized that the drug effects on the ATPase activity of the Dd2 PfMDR1 variant will be inverted in the presence of VPL, while the 7G8 variant will be unaffected. Despite all these possible scenarios, if the relative contribution to drug
resistance in comparison to PfCRT is minor, it is expected that the overall drug effects on PfMDR1 will be small.

1.8.2 Characterization of the binding of CQ to PfCRT

Despite the fact that PfCRT has been proven to be the indisputable cause of CQR, the mechanism by which various PfCRT isoforms confer various resistance phenotypes is still unknown. Several hypotheses have been suggested (see Section 1.5.2), all of which somehow involve binding of CQ to the PfCRT protein. However, with one exception [103], all of the evidence for a direct interaction between the drug and the protein is circumstantial, having been inferred from the drug resistance profiles of various mutant strains in vitro. In order to confirm the proposed binding of CQ to PfCRT, purified heterologously expressed protein will be photolabeled with a candidate CQ analogue synthesized by Roepe laboratory colleague Dr. JK Natarajan. Since all the models of PfCRT-mediated CQR depend upon decreased drug accumulation within the DV, and it is known that mutant PfCRT confers CQR, it is expected that the drug-protein interaction will be stronger between the analogue and mutant variants, thus resulting in “better” photolabeling (defined several ways) compared to the CQS variant. If the probe is specific and acts similarly to the parent compound (i.e., occupies the same binding site), the labeling should be efficiently competed by excess amounts of “cold” (unlabeled) drug. Since PfCRT is linked to resistance of CQ and other quinoline antimalarials, such as QN, the presence of these drugs should decrease the amount of photolabeling observed if the drug binding sites overlap. Conversely, drugs from other classes of antimalarials to
which PfCRT is not supposed to confer resistance (e.g., endoperoxides), should not efficiently compete the probe’s ability to photolabel the protein. Furthermore, since some CQR parasite strains are VPL-reversible and some are not, and this difference may possibly be attributable to PfCRT [175], it is expected that VPL may compete the photolabeling of VPL-sensitive variants of the protein but will not affect labeling of VPL-insensitive variants. These photolabeling studies will provide insight into the mechanism of CQR mediated by PfCRT. Finally, if labeling is found to be specific, it should be possible to map the drug binding site via proteolysis and mass spectrometry.

1.8.3 Summary

Hypotheses:

- Mutations in PfMDR1 affect the protein’s ability to confer resistance to MQ and QN, but not CQ.
- PfCRT protein binds CQ directly.

Specific Aims:

- Heterologous expression of PfMDR1
- Analysis of basal and drug-stimulated ATPase activity of multiple PfMDR1 isoforms
- Purification of PfCRT
- Analysis of photolabeling of PfCRT variants representing multiple parasite strains
- Definition of the PfCRT CQ binding site
CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

PfuUltra, QuikChange Multi Site-Directed and Quikchange II XL Site-Directed Mutagenesis Kits were from Stratagene (La Jolla, CA). Streptavidin HRP, ECL detection reagents and His GraviTrap columns were from Amersham Biosciences / GE Healthcare (Piscataway, NJ). Prestained and biotinylated SDS-PAGE molecular markers were from Bio-Rad (Hercules, CA). Yeast growth media components were from Difco (Sparks, MD). The PentaHis-HRP conjugate, 6xHis Protein ladder, QIAquick gel extraction kit, QIAquick PCR purification kit and QIAquick spin miniprep kits were from Qiagen (Valencia, CA). *Pichia* strains and expression plasmids, EasyComp transformation kit and the SilverQuest silver staining kit were from Invitrogen (Carlsbad, CA). Factor Xa and all restriction enzymes were from New England Biolabs (Ipswich, MA) Oligonucleotides were from MWG Biotech (High Point, NC) and Genscript (Piscataway, NJ). *E. coli* polar lipids were from Avanti Polar Lipids (Alabaster, AL). UltraLink immobilized monomeric avidin was from Pierce (Rockford, IL). Mefloquine and halofantrine were a kind gift from Dr. Michael Ferdig (University of Notre Dame, South Bend, Indiana), and anti-PfCRT antibody was thoughtfully provided by Dr. Roland Cooper (Old Dominion University, Virginia). All other materials were reagent grade or better and purchased from Sigma (St. Louis, MO).
2.2 Methods

2.2.1 Strains and Growth Conditions

The *Escherichia coli* strain DH5α (F- φ80lacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1λ-) was used for all bacterial subcloning work. Transformed strains were selected on Luria broth (LB) containing ampicillin and grown at 37°C.

*Pichia pastoris* strain KM71 (his4, aox1::ARG4, arg4) from Invitrogen was used for all heterologous expression. Yeast strains transformed with the expression plasmid pPIC3.5 (Figure 2.1) were selected for growth on minimal glycol medium (MGM) lacking histidine, while those transformed with pPICZc (Figure 2.1) were selected for growth on MGM supplemented with histidine and 100 mM zeocin. Protein expression was induced in minimum methanol medium (MMM). All yeast were cultured at 30°C.

![Plasmid maps of expression vectors](image)

*Figure 2.1* Plasmid maps of expression vectors. pPIC3.5 is used for *pfcrt*, while the smaller pPICZc is used for *pfmdr1* in order to accommodate the larger sized gene.
2.2.2 Optimized pfmdr1 gene design

The wildtype pfmdr1 gene sequence (strain 3D7) was obtained from GenBank (www.ncbi.nlm.nih.gov, accession #AL929353) and translated. The amino acid sequence was entered into CODOP (generously provided by Dr. Elisabeth P. Carpenter, Division of Protein Structure, National Institute for Medical Research, London, UK [130]), a software program that uses a pre-defined codon preference table to back-translate amino acid sequences into “optimized” nucleotide sequences. Both strands of one obtained sequence that had a beneficial distribution of unique restriction sites were split into 40-mer oligonucleotides which were further analyzed for poly-AT stretches and corrected. Silent mutations were also inserted to engineer additional unique restriction sites, and all melting temperatures (Tms) of the oligos were adjusted by judicious AT:GC substitutions to fall within the range of 56-64°C. Additionally, a Kozak sequence was engineered 5’ to the start codon, and a polyHistidine tag and Factor Xa cut site were appended to the 3’ end.

Due to the large size of the gene and previous observations that 12 helix-2 NBD ABC transporters can be expressed as half transporter cassettes (N- or C-terminal half 6 helix-1 NBD polypeptides; [160]), “yeast optimized” PfMDR1 was first designed and constructed in two parts and then later fused.

2.2.3 Synthetic pfmdr1 gene construction

N-terminal cassette (Figure 2.2): A total of 114 40-mer overlapping oligos (57 upper and 57 lower) encoding both strands of the first 2250 nucleotides of the pfmdr1
gene sequence were ordered from MWG. Equal volumes of all 40-mers were combined (1.5 μM each), and the resultant mixture was diluted 25-fold in PfuUltra buffer supplemented with 0.5 mM each dNTP, 2 mM Mg^{2+}, and 1.25 U PfuUltra. The initial assembly PCR program was a 1 min denaturation at 95°C followed by 40 cycles of (denaturation at 95°C for 45 sec, annealing at 52°C for 45 sec, and elongation at 72°C for 3 min), and a final elongation for 10 min at 72°C. The assembled product was separated from excess dNTPs and oligos by using the Qiagen PCR purification kit. 1 μL of the clean product was then amplified in similar buffer with 1 μM of oligos #1 and #58 using the amplification PCR program of one denaturation at 95°C for 1 min, followed by 30 cycles of (denaturation at 95°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 5 min), and a final elongation at 72°C for 10 min. The PCR product was inserted into the EcoRI and NotI sites of the pPICZc vector. After transformation and propagation in E. coli, several PCR product clones were fully sequenced, and the one with the fewest errors was selected for further work. All spurious PCR errors were corrected using the multisite-directed mutagenesis kit (Stratagene) and the completed gene was confirmed by sequencing in both directions.
Figure 2.2  Diagram of N-terminal *pmdr1* gene construction. A pool of all 114 overlapping oligos was mixed and allowed to anneal. The assembled product was then amplified using the first 5’ sense primer (#1) and the first 3’ antisense primer (#58).

C-terminal cassette: The optimized gene sequence encoding the last 2115 nucleotides of the *pfmdr1* gene was ordered from the custom gene synthesis service offered by Genscript. The product was received cloned into the pUC57 vector, which was treated with *Eco*RI and *Xma*I to liberate the *pfmdr1* fragment, which was then inserted into the multiple cloning region of pPICZc.

Full-length fusion: pPICZc/N-terminal was treated with *Not*I to excise the 6H-BAD tags (vector) and dephosphorylated with calf intestinal phosphatase (CIP) while pPICZc/C-terminal was treated with *Not*I to liberate the ABC2 cassette fused with the 6His and BAD tags (fragment). The vector and fragment were ligated in using T4 DNA ligase and transformed into XL Gold competent cells (Stratagen). Two good constructs were sequenced bi-directionally.
This fusion strategy resulted in a construct containing 3 extra alanine residues in the region linking the N- and C-terminal cassettes. A pair of complementary primers was created to “loop out” these 3 A using the QuickChange XL site-directed mutagenesis kit. The primer sequence was:

Linker: 5’-AACTGCAAGAACACTGCTGAGAACGAGAAGGAGGAG-3’

### 2.2.4 Creation of mutants

All amino acid substitution mutants were created using the QuikChange Multi Site-Directed and Quikchange II XL Site-Directed Mutagenesis Kits from Stratagene according to manufacturer’s instructions. The primers used (with mutation bolded and underline) were:

**PfDMR1:**

N86Y: 5’-GGTTATCTTTGAAGAACATG**T**ACCTTGGGGACGATATCAAC-3’

Y184F: 5’-GTCTTTTCCTTGGGCTGT**T**CATCTGGTCCCTGATCAAGAAC-3’

S1034C: 5’-CGCTGCACCTTTGGGATTC**T**GCCAATCGGCAAACTGTTC-3’

N1042D: 5’-AATCGGCAACAATGTTCTCACTCGTTCGTTACTGTT-3’

D1246Y: 5’-TGGCAGACTACAACCTTAGG**T**ACCTACGAAACCTCTTCCTCAA-3’

**PfCRT:**

T76K: 5’-GTCAGTGTTCGATC**A**GAAAGATCTTCGCCAAGAGAACC-3’

T76I: 5’-CAGTTTTGCGTATCGAAA**T**CATCTTGGCCGAAGAGAAG-3’

T76N: 5’-GTCAGTTTTGCGTATCGAAA**A**CATCTTGGCCGAAGAGAACC-3’
2.2.5 Insertion of 6His tag and Factor Xa site into pfcrt plasmids

A DNA sequence encoding for six consecutive histidine residues (6H), a Factor Xa sequence (IDGR) and a SacII site was designed as two complementary 45bp primers with sticky ends simulating NotI and XmaI cuts (Figure 2.3).

**Figure 2.3** Sequence of the 6His tag. SacII site is in italics, 6H sequence is underlined, and Factor Xa site is bolded. The 5’ end of this sequence simulates the 3’ end of a NotI cut while the 3’ end simulates the 5’ end of an XmaI cut. This fragment can thus be inserted directly into a plasmid with a NotI site upstream of an XmaI site.

The primers were annealed to each other by heating a mixture of 50 pmol of each at 98°C for 5 minutes and slowly letting it return to room temperature. The annealed fragment was ligated into the NotI and XmaI sites of pYKM77/pfcrt-HB3-BAD (previously created in the laboratory, [131]) to create pYKM77/pfcrt-HB3-6H-BAD (Figure 2.4).

**Figure 2.4** Insertion of the 6H tag. The fragment on the left gives the amino acid sequence of the 6H tag and the Factor Xa cleavage site, later condensed to 6H in the full plasmid representation on the right. Diagram is not drawn to scale.

A large scale preparation of the 6H-BAD fragment was obtained by treating 80µg of pYKM77/pfcrt-HB3-6H-BAD with NotI. pPIC3.5/pfcrt-HB3-BAD and other CRT-BAD mutants in the pPIC3.5 vector were digested with NotI to liberate the BAD tag and
then dephosphorylated with CIP. The appropriate vectors and fragment (Figure 2.5) were ligated at 16°C overnight and transformed into *E. coli*.

![Diagram](image)

**Figure 2.5** Transfer of 6H-BAD from pYKM77 to PPIC3.5-based plasmids. Both plasmids on the left of the arrow were treated with *Not*I. The 6H-BAD fragment from pYKM77 was inserted into the vector from pPIC3.5. Diagram is not drawn to scale.

### 2.2.6 Removal of BAD from pfcr constructs

A pair of complementary primers was designed to destroy the *Not*I site at the 3’ end of the BAD in pPIC3.5-PfCRT-6HBAD (rightmost diagram in Figure 2.5) and create an *Xma*I site in its place through site-directed mutagenesis. The sequence was:

*Xma*I upper: 5’-GCAGGCATGCAAGCGGCCCCGGGTTAATTCGCTTAGACATG-3’

Colonies transformed by the reaction product were screened by restriction digest: positive colonies had one *Not*I site and two *Xma*I sites. One good construct from each mutant plasmid was digested with *Xma*I. The fragment representing the BAD tag was discarded, and the vector was circularized by the addition of quick ligase for 5 min at room temperature.
2.2.7 DNA sequencing

All DNA samples were sequenced using the Sanger method of dideoxynucleotides (BigDye terminator). A 10 μl reaction mixture containing 0.75 μg dsDNA, 2.5 μl BigDye ready mix and 2.5 pmol primer was cycled in a GeneAmp 2400 PCR system (Perkin Elmer) using the parameters of 25 cycles at 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. Extension products were purified from unincorporated dye terminators using a BioMax Spin-50 mini-column. The recovered sample was lyophilized and sent to the Lombardi Cancer Center DNA sequencing facility for the gel run. Alternatively, DNA samples were sent to the Northwestern University sequencing facility to be sequenced via similar methods.

2.2.8 Yeast transformation

KM71 cells were grown in YPD to an OD_{600nm} of 0.8, harvested by centrifugation at 1600 x g at room temperature for 5 minutes, and made competent using the lithium chloride protocol from Invitrogen. Briefly, 50 μg of salmon sperm DNA, 100 mM LiCl, 35% polyethylene glycol (PEG), and 5 μg of linearized plasmid DNA were added to freshly prepared competent cells. Cells were incubated at 30°C for 1 hr and then heat shocked at 42°C for 10 min. To allow for cellular recovery and/or expression of the zeocin resistance gene, 1 mL YPD was added to each heat shocked sample and incubated at 30°C for 1 hr. The cells were pelleted by centrifugation 3000 x g for 5 min at room temperature and resuspended in 500 μL sterile water. Various amounts were then plated on agar media containing the appropriate selection markers. Alternatively, the Pichia
EasyComp Transformation Kit from Invitrogen was used according to manufacturer’s instructions.

2.2.9 Screening for yeast protein expression

100 mL of MGM was inoculated with cells picked from a single colony; at least three colonies from each transformation were selected for screening. Cultures were grown at 30°C with shaking to an OD_{600nm} >2. The cells were harvested by centrifugation at 1600 x g for 5 min at room temperature and resuspended in 20 mL of MMM to induce protein expression. 1 mL samples were removed after 0, 8, and 24 hr of induction and centrifuged. The pellets were resuspended in ice cold breaking buffer (50 mM sodium phosphate / 1 mM EDTA / 5% glycerol / pH 7.4) and 0.5 mm glass beads were added. The tubes were vortexed for 10 min at 4°C and centrifuged at 16,000 x g for 10 min to pellet the glass beads and unlysed cells. The supernatant (containing the membrane fraction) was removed and analyzed for protein expression by SDS-PAGE and Western blotting.

2.2.10 Yeast crude membrane (CM) preparation

A starter culture consisting of a one yeast glycerol backup in 30 mL of MGM was grown at 30°C with shaking to an OD_{600nm} of 2. The starter culture was then bulked up into a 4L flask containing 2.5 L of MGM. After ~24 hr of growth (OD_{600nm} >4) the cells were pelleted and resuspended in 500 mL of MMM. After ~20 hr of methanol induction, the cells were harvested by centrifugation at 3000 x g for 5 min at 4°C. The cells were kept on ice from this point forward. The cells were washed three times with 5-fold (v/w)
Harvest Buffer (100 mM glucose / 50 mM imidazole / 5 mM DTT / pH 7.5), resuspended to 2 mg per mL in Breaking Buffer (100 mM glucose / 50 mM imidazole / 250 mM sucrose / 1 mM MgCl$_2$ / 5 mM DTT / pH 7.5), and finally lysed by physical disruption with 0.5 mm glass beads [176]. For cell lyses, 50 mL conical tubes were filled with ~15 mL cell suspension and an equal volume of glass beads. The tubes were snap frozen in a dry ice / ethanol bath and either vortexed for 1 hr or disrupted for six 1 min cycles in a BeadBeater. The resulting cell suspension was decanted from the glass beads. The glass beads were washed with excess Breaking Buffer and the wash added to the decanted cell suspension. Unbroken cells and cell wall debris were removed from the suspension by two 10 min centrifuge spins at 1500 x g followed by another 10 min spin at 4000 x g. The cleared supernatant was centrifuged at 100,000 x g for 1 hr. The CM pellet from this high speed spin was resuspended in Suspension Buffer (10 mM imidazole / 1 mM MgCl$_2$ / pH 7.5). The CMs were either stored at -80°C or used immediately for plasma membrane (PM) preparations or affinity purification.

2.2.11 Preparation of purified yeast PMs

Plasma membranes were prepared via acid precipitation using a protocol adapted from [177]. CMs were resuspended to 3 mg per mL in Precipitation Buffer (10 mM imidazole / 1 mM MgCl$_2$ / pH 5.2) and adjusted to pH 5.2 with 100 mM HCl. The suspension was centrifuged at 7500 x g for 5 min. The supernatant was decanted and the pH was immediately readjusted to 7.5 using 100 mM NaOH. The PMs were pelleted by
centrifugation at 100,000 x g for 1 hr, resuspended in Suspension Buffer, and stored at –80°C.

2.2.12 Preparation of yeast inside out plasma membrane vesicles (ISOVs)

The method used to prepare ISOVs was an adaptation of [178]. Yeast cells were grown, induced, and harvested as described above for preparation of CMs. 10-12 g of cells were washed twice in Buffer A (10 mM Tris / 700 mM sorbitol / 1 mM DTT / pH 7.5) with centrifugation at 3000 x g for 5 minutes. The washed pellet was resuspended in 5 ml Buffer A / 1 g cells and gently mixed with 5 mg zymolase-20T / g wet cell. The suspension was incubated at 30°C while shaking at 100 rpm for 1 hr. Spheroplasts were collected by centrifugation at 3000 x g for 5 min, and zymolase was removed by two washes with Buffer A. The pellets were resuspended in 1.5 mL Buffer B (15 mM Mes-Tris / 500 mM sorbitol / 100 mM glucose / pH 6.5) / 1 g cells and then incubated at 30°C for 10 minutes to induce ATPase activity. All subsequent steps were performed at 4°C. The spheroplast suspension was osmotically lysed by dilution with two volumes of ice cold Buffer C (25 mM Mes-Tris / 5 mM EDTA / 0.2% (w/v) BSA / 0.2% (w/v) casein hydrolysate / 1 mM DTT / 1 mM PMSF / 1 µg/µL aprotinin / 2 µg/mL leupeptin / 1 µg/mL pepstatin A / pH 6.5) and homogenized with eight strokes of a Potter homogenizer. Cell wall fragments were removed by centrifugation at 300 x g for 15 min, and the supernatant was centrifuged at 35,000 x g for 15 min to yield a cell membrane pellet. The membranes were then resuspended in 9 g Buffer D (5 mM potassium phosphate, dibasic / 330 mM sucrose / 1 mM DTT / pH 7.8). 27.0 g of Buffer E (5 mM potassium phosphate,
dibasic / 330 mM sucrose / 7.6% (w/w) Dextran 500,000 / 7.6% (w/w) polyethylene glycol 3350 / 1.4 mM EDTA / 1 mM DTT / pH 7.8) was added to the suspension and the tubes were vigorously shaken. The mixture was allowed to phase separate on ice at 4°C for 2 hr. The ISOV-enriched lower phase and the inter-phase were collected, diluted with 2 volumes of Buffer F (15 mM Mes-Tris / 330 mM Sucrose / pH 6.5), and centrifuged at 60,000 x g for 30 min. The pellets were resuspended in Buffer F and stored in aliquots at -80°C.

2.2.13 Determination of protein concentration

The amido black assay is a reliable method for the determination of protein content in high lipid and detergent environments. Samples and bovine serum albumin (BSA) standards were denatured by heating at 30°C in 1 mM Tris / 1% (w/v) SDS / pH 7.5 for 10 min. Proteins were precipitated in 11.5% TCA at room temperature for 15 min and vacuum filtered through a 0.45 µm Protran membrane. The membrane was stained in 0.1% Amido Black in 45% methanol / 10% acetic acid and destained in 90% methanol / 2% acetic acid. Stained protein spots were cut out of the membrane and dissolved in 25 mM NaOH / 50 µM Na₂EDTA in 50% ethanol. The absorbance of the resulting blue solutions was read at 630 nm and quantified by extrapolation from a curve constructed from the known BSA standards (Figure 2.6).
Figure 2.6  Representative amido black standard curve.

2.2.14 Protein purification

Integral membrane proteins were enriched and peripheral membrane proteins were
removed with a chaotrophic wash. Crude membranes were resuspended to 2 mg per ml in
Wash Buffer (50 mM Tris-Cl / 250 mM sucrose / 20% (v/v) glycerol / 1 mM MgCl₂ / pH 7.5) containing either 3 M urea or NaCl. The samples were rotated on a rotisserie mixer
for 20 min and the washed membranes were recovered by centrifugation at 100,000 x g
for 1 hr. The resulting pellet was resuspended at 2 mg/mL in Solubilization Buffer (Wash
Buffer supplemented with 500 mM NaCl and 1% DM) and mixed for 30 min.
Unsolubilized materials were removed by centrifugation at 100,000 x g for 1 hr. The
detergent extract was applied to a pre-equilibrated affinity chromatography column.

IMAC: Pre-packed 1 mL bed volume His GraviTrap columns were equilibrated
with 10 bed volumes Column Wash Buffer (Solubilization Buffer supplemented with 20
mM imidazole). The column was filled with detergent extract, washed twice with 10 bed
volumes Column Wash Buffer and eluted with 4-6 bed volumes Elution Buffer (Solubilization Buffer supplemented with 500 mM imidazole).

Avidin chromatography: Disposable polypropylene columns were packed with 2 mL 50% monomeric avidin slurry (1 mL bed volume) and equilibrated overnight with Solubilization Buffer. The column was loaded with detergent extract, washed twice with 10 bed volumes Solubilization Buffer and eluted with 6 bed volumes Elution Buffer (Solubilization Buffer supplemented with 5 mM d-biotin).

Both types of column were eluted directly into *E. coli* lipid reconstituted in 50 mM Tris-Cl / 50 mM NaCl / 0.01% DM / pH 7.5 to a final concentration of 1.4% lipid. The protein/lipid/detergent was gently rotated for 30 min on a rotisserie shaker and then transferred into a pretreated dialysis membrane or Float-A-Lyzer (MWCO 12,000-14,000). Dialysis was carried out against at least 50 volumes of Dialysis Buffer (50 mM Tris-Cl / 250 mM sucrose / 1 mM EDTA / pH 7.5) for >16 hr with one change in buffer after the first 4 hr. The dialyzed proteoliposomes (PLs) were transferred into pre-cooled ultracentrifuge tubes and spun for 1 hr at 100,000 x g. The PL pellet was resuspended in Dialysis Buffer and small aliquots were snap frozen and stored at -80°C.
Figure 2.7 Flow chart of protein purification procedure.

2.2.15 ATPase assay

The ATPase activity of purified PM fractions was measured by the colorimetric determination of orthophosphate released through ATP hydrolysis as described previously [179, 100] but optimized for high throughput on a microliter scale in a 96-well plate format (Figure 2.8). Plates were set up on ice: assay buffer (180 mM NH₄Cl / 100 mM Mes-Tris / 10 mM MgCl₂ / 0.01% NaN₃) was added to each well, followed by relevant drug solutions, and finally 2 µg membrane protein to a total volume of 100 µl. The plate was shaken at 650 rpm on an Eppendorf MixMate for 1 min, warmed to 37°C in a water bath for 1 min, and ATP was added to each well. The plate was then shaken at 165 rpm in a 37°C incubator for 15 min (except when performing a time course assay). The reaction
was stopped with 50 µl of 1% sodium molybdate / 12% SDS / 4% 12 N HCl followed immediately by an equal volume of 6% sodium ascorbate in 1 N HCl. The reaction was stabilized 10 min later by addition of 100 µl 2% sodium citrate / 2% sodium arsenate / 2% glacial acetic acid. Absorbance at 720 nm was read 30 min later on a Victor 3V plate reader. Solutions of 6% sodium ascorbate in 1 N HCl and assay buffer at various pH were made daily, while molybdate / SDS and stabilizing solutions were made weekly and stored at 4°C. Stock aliquots of ATP and drugs stored at -20°C were thawed on ice, used once, and then discarded.

![Figure 2.8 Schematic of typical plate set-up for ATPase assay. Three replicates for each of three PfMDR1 strains as well as negative control membranes at seven different drug concentrations plus two standard curves can be fit on the same plate.](image)

Negative control membranes from zeocin-resistant yeast transformed with empty vector (pPICZc) were included in all assays. Absorbance readings were converted to nmol Pi using a standard curve generated with K$_2$HPO$_4$ samples. Values for PM samples were then also scaled for total amount of protein and relative PfMDR1 content (see Section 3.2.1). Replicates were averaged, and the average control value under each assay...
condition was subtracted from that of each PfMDR1 sample. When comparing activity of the half transporter constructs to those of full length PfMDR1, data were also normalized for relative number of NBD.

2.2.16 Saponin isolation of parasites

Dd2 and HB3 strains of *P. falciparum* were cultured using standard techniques at 2% hematocrit in phenol-red free RPMI-1640 (Sigma R8755) supplemented with NaHCO₃ (23.8 mM), d-glucose (10 mM), hypoxanthine (730 µM), HEPES (25 mM), gentamicin sulfate (20 µg/mL), and serum (10%). Cultures were triply synchronized with sorbitol (5%, w/v) as described in detail elsewhere [180].

A 5mL iRBC pellet of ~7.5% parasitemia at the trophozoite stage was washed twice in 50 mL PBS and then resuspended in 50 mL PBS containing 0.5 % (w/v) saponin. The isolated parasites were collected by centrifugation at 1500 x g for 10 min, and then washed twice in 1 mL of ice-cold PBS. The trophozoites pellets were stored at -80°C.

2.2.17 AzBCQ binding

0.1 nmol of PLs were diluted in 50 mM Mes-Tris buffer at varying pH. The mixture was aliquotted into wells of a 96-well plate and appropriate drugs were added (Figure 2.9). All future steps were performed in the dark. AzBCQ was added to each well, and the plate was shaken on a MixMate at 650 rpm for 30 sec and incubated at 37°C for 10 min. The plate was then exposed to short wave ultraviolet radiation (220-280 nm, 254 nm peak) from a hand-held lamp (Spectroline Model ENF-280C, 115 V, 60 Hz, 200
mA) for 10 min. The lamp was placed directly upon the plate so that the samples were 0.8 cm from the light source. The reaction was stopped by mixing equal volumes of the sample and 2X Laemelli buffer. After a 10 min incubation at 37°C, the samples were split in half and equal amounts were loaded onto two 12% polyacrylamide gels and electrophoresed at a constant voltage of 200 V for 1 hour. Each gel contained one well loaded with PfCRT-his-BAD as a positive control. The proteins were then transferred to two polyvinylidene difluoride (PVDF) membranes at a constant current of 350 mAmp for 1 hour. One membrane was immunoblotted for biotin to detect the presence of AzBCQ, while the other was blotted for the polyHis tag to detect total levels of protein.

Figure 2.9 Schematic of typical set-up for AzBCQ binding assay.
2.2.18 Gel purification and trypsin digestion

A large-scale photolabeling reaction was performed as described above. The samples were run in large-well gels with prestained MW ladders (BioRad) on either end. The section of the gel containing the protein of interest was excised, minced into ~1mm x 1 mm cubes, and transferred to an Eppendorff tube. The gel pieces were then ground using the plunger of a 1 mL syringe as a pestle. 1% acetonitrile was added to just cover the acrylamide, and the tube was incubated at 37°C with vigorous shaking for 1-3 hours. The acrylamide was pelleted by a short centrifugation at 16,000 x g and the supernatant was removed.

Sequencing-grade trypsin (Sigma) was reconstituted in molecular grade water at a concentration of 0.1 µg/µL. 0.5 µg from this stock were activated in 15 µL of 25 mM ammonium bicarbonate, and 10 µL of this mixture (0.25 µg trypsin) was added to the protein eluted from the gel. The reaction was allowed to continue at room temperature for various timepoints and was stopped by the addition of 5X Laemmelli buffer. The samples were heated at 37°C for 10 min and then run on a 15% Tris-Glycine gel. Some gels were silver stained using the MS-compatible SilverQuest kit (Invitrogen) and bands of interest were excised, destained, and given to the Lombardi Cancer Center Proteomics Core Facility (Georgetown University Medical Center) for further processing.
2.2.19 Mass spectrometry and peptide identification

The gel slices were washed with 100 mM ammonium bicarbonate and incubated with 50 mM ammonium bicarbonate and 10 µl of 10 mM DTT at 60°C for 30 min. The tubes were cooled to room temperature and 10 µl of 55 mM iodoacetamide was added and the sample incubated further for 30 min in the dark at room temperature. The solvent was discarded and the gel slice was washed in 50% acetonitrile / 100 mM ammonium bicarbonate. Subsequently, the gel slice was transferred onto a 96-well Montage plate (Millipore) and destained with 50% acetonitrile in 25 mM ammonium bicarbonate, dehydrated with acetonitrile for 5 min, and vacuum dried. Gel pieces were then rehydrated with 15 µl of ammonium bicarbonate:acetonitrile (25 mM:10%) supplemented with trypsin (5 ng/µl, Promega) at 37 °C for 16 h. Afterwards, tryptic peptides were extracted in 0.1% TFA / 50% acetonitrile and mixed with an equal volume of 5mg/ml CHCA (Acros Organics). Mass spectra were recorded with a matrix assisted laser desorption/ionization–time of flight, time of flight (MALDI-TOF-TOF) spectrometer (4800 Proteomics Analyzer) set in reflector positive mode by spotting the samples onto a MALDI plate. The samples were ionized with a fixed LASER intensity of 3800J and 1000 LASER shots were collected per subspectrum and were shot randomly with uniform bias. The detector voltage was 2.1 kV, the bin size was set at 0.5 ns and the Signal/Noise threshold was set at 15. The spectra were collected with a specified mass range of 700-4000 Da with a focus mass of 2100 Da.
Once the mass spectra were obtained from the Proteomics Core, tryptic digest maps were generated using the PeptideMass program available on the ExPASy proteomics server (http://ca.expasy.org/tools/peptide-mass.html), and the predicted fragment masses were compared to the experimentally-derived ones with the FindPept tool (http://ca.expasy.org/tools/findpept.html), also available from ExPASy.

2.2.20 Protein detection

Hand-poured 7.5% (for PfMDR1) or 12% (for PfCRT) Tris-Glycine SDS-PAGE gels were run on a Mini-Protean II apparatus (BioRad) and transferred onto PVDF membranes.

Biotin detection: The PVDF membranes were washed once for 5 min with PBS-T (20 mM phosphate / 150 mM NaCl / 0.1% Tween-20 / pH 7.4), incubated for 1 hr in 10% milk (in PBS-T), and washed again for 5 min. The blocked membranes were probed with 10 µL streptavidin-HRP in 20 mL PBS-T for 1 hour, and washed three times for 20 min in PBS-T.

PolyHis detection: The PentaHis detection kit from Qiagen was used according to manufacturer’s instructions. Briefly, the PVDF membrane was washed twice for 10 min in TBS (10 mM Tris-Cl / 150 mM NaCl / pH 7.5), and incubated for 1 hr in proprietary blocking buffer. The membranes were washed for 10 min twice in TBS-T/T (20 mM Tris-Cl / 500 mM NaCl / 0.05% (v/v) Tween 20 / 0.2% (v/v) Triton X-100 / pH 7.5) and once in TBS, then probed with 5 µL anti-pentaHis-HRP in blocking buffer, and washed again.
All immunoblots were developed using the Enhanced ChemiLuminescent (ECL) system from Amersham according to manufacturer’s instructions. Equal volumes of Reagents A and B were mixed and applied to the PVDF membranes for 1 min in the dark. Membranes were then exposed to either film or a CCD camera for varying times.

For total protein detection, gels were either silver or Coomassie stained. Gels were fixed in 40% ethanol / 10% acetic acid. Invitrogen’s SilverQuest MS-compatible kit was used following the manufacturer’s protocol. Alternatively, fixed gels were stained for 1 hr in Coomassie Brilliant Blue R250 stain or 1 hr in BioSafe Coomassie and destained in 50% methanol / 10% acetic acid or water, respectively.

2.2.21 Data analysis

All densitometry was performed using the gel analysis function of Image J (software available from the NIH, http://rsb.info.nih.gov/ij). Data were plotted, curve-fit, and analyzed for statistical significance in either Microsoft Excel or GraphPad Prism 4.0.
3.1 Heterologous Expression of PfdMR1

3.1.1 Design of yeast-optimized pfmdrl gene

The strain 3D7 (designated wildtype in the malaria literature) pfmdrl gene sequence was obtained from GenBank (accession #AL929353) and translated. The amino acid sequence was then entered into the CODOP computer program to generate a yeast-optimized nucleotide sequence (Table 3.1 and Appendix).

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<tr>
<th>Amino Acid</th>
<th>Codon</th>
<th>P. falciparum usage (% codon used per amino acid)</th>
<th>P. pastoris usage (% codon used per amino acid)</th>
<th>NCBI pfmdrl gene (exact number used)</th>
<th>Synthetic N-terminal pfmdrl gene (exact number used)</th>
<th>Synthetic C-terminal pfmdrl gene (exact number used)</th>
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Table 3.1 Comparison of codon usage and base composition for the synthetic *pfmdr1* gene with the those of *P. falciparum* and *P. pastoris*. *P. falciparum* shows a strong AT bias which is corrected in the synthetic gene to closer resemble the *P. pastoris* usage pattern. For the complete linear sequence of the synthetic gene, please see Appendix A.

### 3.1.2 Construction of gene for N-terminal cassette

The yeast-optimized gene sequence for both strands of the N-terminal cassette of *pfmdr1* was ordered as a series of 114 overlapping 40-mer oligonucleotides (#1-57 sense 66
and #58-114 antisense). These were assembled into the full construct in a series of nested
PCRs (Figure 3.1A), and the proper sequence was then inferred by restriction digest
(Figure 3.1B, Table 3.2) and confirmed by DNA sequencing.

![Image](image1.png)

**Figure 3.1** Agarose gels of N-terminal *pfmdr1* construct. (A) PCR amplification of N-
terminal *pfmdr1* as either half or full length gene fragments. Lane 1, EZ Load 100 bp
PCR Molecular Ruler (BioRad); lane 2, 1.25 kb fragment obtained using oligos #1 & #89
for amplification of the 5’ half of the fully assembled N-terminal construct (middle
arrow); lane 3, 1.1 kb fragment corresponding to the 3’ half of the full construct, obtained
from amplification of the assembly solution with oligos #27 & #58 (bottom arrow); lane 4,
2.35 kb full length N-terminal *pfmdr1* construct, obtained by amplification of the
assembly solution with oligos #1 & #58 (top arrow). (B) Diagnostic restriction digests of
the N-terminal *pfmdr1* gene inserted into the pPICZc vector. Lane 1, EZ Load 1 kb
Molecular Ruler (BioRad); lane 2, EZ Load 100 bp Molecular Ruler (BioRad); lane 3,
*EcoRI* & *NotI*; lane 4, *EcoRV* & *NotI*; lane 5, *BglII* & *SacI*; lane 6, *BamHI* & *XhoI*.

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<th>Expected fragments (bp)</th>
<th>Actual fragments (bp)</th>
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<td><em>EcoRV</em> &amp; <em>NotI</em></td>
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<td>~3800, ~1900, ~500</td>
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<td><em>BglII</em> &amp; <em>SacI</em></td>
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<td>~6000, ~2300, ~800, ~350, ~200</td>
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<td><em>BamHI</em> &amp; <em>XhoI</em></td>
<td>8426, 1407, 166</td>
<td>&gt;8000, ~1500</td>
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</table>

**Table 3.2** Analysis of diagnostic digests pictured in Figure 3.1B. The experimentally
derived restriction patterns correspond well to the predicted values.

67
3.1.3 Design and construction of gene for C-terminal cassette

The yeast-optimized C-terminal half of the pfmdr1 gene was designed by the same general methodology. However, by the time the N-terminal gene construction was completed, the price of custom gene synthesis had dropped to the point where it was more time- and cost-effective to simply order the intact C-terminal construct instead of assembling individual oligos. The received pPUC57/C-terminal plasmid was rehydrated and transformed into competent DH5α cells, and colonies were screened by restriction analysis to confirm sequencing results (Figure 3.2, Table 3.3). The C-terminal gene was excised from the pUC57 vector with EcoRI and XmaI and ligated into a pPIC vector cut with the same enzymes (Figure 3.2B).

![Figure 3.2](image-url)

**Figure 3.2** Agarose gels of C-terminal pfmdr1 construct. (A) Diagnostic restriction digests of the C-terminal pfmdr1 gene in its native pPUC vector as received from Genscript. Lane 1, EZ Load 1 kb Molecular Ruler (BioRad); lane 2, EZ Load 100 bp Molecular Ruler (BioRad); lanes 3-5, three colonies treated with NotI & NruI; lanes 6-8, same three colonies treated with AfeI & SacII; lanes 9-11, same three colonies treated with SpeI & NdeI. Colony #3 (lanes 5, 8, & 11) did not contain the gene insert. (B) EcoRI & XmaI digest of pPIC and pPUC57/C-terminal. Lane 1, 1 kb Molecular Ruler; lane 2, pPIC vector; lane 3, pUC57/C-terminal. The vector (top arrow) from lane 2 and the fragment (bottom arrow) from lane 3 were ligated.
<table>
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<th>Enzymes</th>
<th>Expected fragments (kb)</th>
<th>Actual fragments (kb)</th>
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<td><em>NotI</em> &amp; <em>NruI</em></td>
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</table>

Table 3.3 Analysis of digests pictured in Figure 3.2. The experimentally derived restriction patterns correspond well to the predicted values.

3.1.4 Construction of the full-length *pfmdr1* gene

Plasmids containing N-terminal and C-terminal *pfmdr1* were each treated with *NotI* (Figure 3.3A). This enzyme is unique to the 3’ end of the N-terminal sequence (yielding a linear plasmid) and cuts at both ends of the C-terminal sequence (liberating this fragment). Following ligation, the full length construct was transformed into XL-Gold ultra-competent cells. Colonies were screened for fragment insertion (Figure 3.3B) and directionality (Figure 3.3D).
Figure 3.3 Construction and analysis of full-length pfmdr1. (A) *NotI* digest of plasmids harboring N-terminal and C-terminal genes. Lane 1, N-terminal construct, lane 2, PCR Molecular Ruler; lane 3, C-terminal plasmid. (B) *NotI* digest of 4 colonies screened for full-length pfmdr1 construct. Lane 1, 1 kb Molecular Ruler; lanes 2-5 plasmid isolated from four different colonies. All colonies contained the insert, but directionality is uncertain. (C) Plasmid map of full-length pfmdr1 showing some important restriction sites and their positions marked in bp. (D) Diagnostic restriction digest of colony #1 (lane 2 in (B)). Lane 1, 1 kb Molecular Ruler; lane 2, 100 bp Molecular Ruler; lane 3, *Xmal*; lane 4, *EcoRI* & *SacII*; lane 5, *SpeI*; lane 6, *Pmel*. Analysis confirmed that colonies #1 and #2 (not shown) contained the C-terminal section in the correct orientation.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Expected fragments (kb)</th>
<th>Actual fragments (kb)</th>
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<tr>
<td><em>Pmel</em></td>
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Table 3.4 Analysis of digests pictured in Figures 3.3B & D.
3.1.5 Removal of extra alanines in linker region

The fusion of the N- and C-terminal halves resulted in a construct containing 3 extra alanine residues in the region linking the N- and C-terminal cassettes. A pair of complementary primers was created to “loop out” the 9 basepairs encoding these 3 Ala through site-directed mutagenesis. This removal also caused the destruction of the \textit{NotI} site between the two halves (compare Figure 3.3B to Figure 3.4). At least one construct was sequenced all the way through to ensure that no spurious errors had been introduced by the PCR-based mutagenesis.

![Figure 3.4 NotI digest of colonies screened for removal of 3A. Lane 1, 1 kb Molecular Ruler; lanes 2-9, eight colonies from transformation with “-3A” constructs. Plasmids from colonies in lanes 2-5 and 7 only had one \textit{NotI} site, indicating that the mutagenesis reaction was successful.](image)

3.1.6 Creation of \textit{pfmdr1} mutants

Site-directed mutagenesis was used on the N- and C-terminal cassettes as well as the full-length construct to create strains harboring various combinations of the 5 mutations associated with drug resistance (Tables 3.5-3.6).
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<td>C</td>
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**Table 3.5** List of all created full length isoforms

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**Table 3.6** List of all created half-cassette isoforms

### 3.1.7 Expression in yeast

Expression vectors (Figure 3.5) harboring different alleles (both naturally occurring and artificial) encoding for *pfmdr1* full-length or half transporter isoforms were
transformed into *Pichia pastoris* yeast. The genes are fused in-frame to hexaHis
tidine (6H) and biotin acceptor domain (BAD) sequences, enabling protein detection by anti-His or avidin blotting, respectively (Figure 3.6). The genes are under control of the alcohol oxidase promoter, so transcription is controlled by a metabolic shift from glycerol to methanol utilization (Figure 3.7). A consensus Kozak sequence was engineered upstream of the start codon to improve initiation of translation.

**Figure 3.5** Schematic of *pfmdr1* yeast construct. The positions of the alcohol oxidase promoter (AOX), the Kozak sequence (italics) and start codon, restriction sites unique to the open reading frame, predicted 6 transmembrane helix domains (TMDs), nucleotide binding domains (NBDs), and fused hexa histidine (6His) and biotin acceptor domain (BAD) tags are labeled. Stars indicate the locations of the 5 mutations present in some isoforms.

Since the individual half transporter genes were cloned into two vectors with different selection markers (histidine anabolism for pPIC3.5 and zeocin resistance for pPICZc), it was possible to transfect a single yeast strain with both plasmids and thus obtain coexpression of the unfused N-terminal and C-terminal halves (Figure 3.6, lanes 4 & 9). The same methodology was used to create yeast strains coexpressing full length PfMDR1 and PfCRT (Figure 3.8).
Figure 3.6 Expression of wildtype PfMDR1. (A) Avidin and (B) polyHis blot of CMs (lanes 1-4) and PMs (lanes 6-9) harboring full length (lanes 1 & 6), N-terminal (lanes 2 & 7), C-terminal (lanes 3 & 8), or coexpressed N- and C-terminal PfMDR1 protein. The full length P/MDR1 protein migrates at 161.69 kDa (upper arrow; predicted mass = 171.81 kDa), N-terminal cassette migrates at 87.44 kDa (middle arrow; predicted mass = 94.69 kDa), and the C-terminal cassette migrates at 77.03 kDa (bottom arrow; predicted mass = 86.94 kDa). Lane 5 shows molecular weight standards, with sizes indicated in kDa.

Figure 3.7 Time course of methanol induction of full-length PfMDR1 expression. Lane 1, high range biotinylated molecular weight standards (BioRad); lanes 2-7, yeast membranes harvested at 0, 3, 6, 9, 12, and 24 hours post-induction. Mutants and half-transporters show similar membrane insertion kinetics (not shown).
3.1.8 Localization of heterologously expressed protein

PfMDR1 does not contain any recognizable signal sequences that determine its subcellular localization. In *P. falciparum* parasites, the protein is found in the membrane of the DV, but it was unclear where the protein would be targeted when expressed in yeast. Therefore, yeast cells were fractionated by differential centrifugation. No PfMDR1 was found in the cytosolic fraction 24 hr post-induction, and crude membrane fractions or purified PM did not release PfMDR1 upon washing with high salt (Figure 3.9).
Figure 3.9 Analysis of relative PfMDR1 abundance in various cell fractions and assessment of membrane integration. Lane 1, cytosolic fraction; lane 2, 18,000 x g pellet; lane 3, CM fraction; lane 4, 4 M NaCl wash of CM; lane 5, PM fraction; lane 6, 4 M NaCl wash of PM; lane 7, polyHis molecular weight standards (Qiagen).

3.1.9 PfMDR1 purification

As an integral membrane protein, the purification of PfMDR1 begins with the removal of peripheral membrane proteins by a chaotropic wash (Figure 3.10). Moderate concentrations of sodium chloride (1-4 M) effectively stripped peripheral membrane proteins without affecting PfMDR1. Some PfMDR1 was lost in urea washes, which also failed to remove as many undesirable proteins as NaCl. Therefore, 3M NaCl was chosen as the standard chaotrope for all purifications.
Figure 3.10 Silver stains of the optimization of the chaotropic wash. Full length PfMDR1 (arrow) CMs were washed with increasing amounts (from 1M to 5M in 1M increments) of NaCl (A) or urea (B). Lanes 1 & 13 are the original CM, lanes 2-6 are the washed membrane pellets, and lanes 8-12 are the wash supernatants. The molecular weight markers in lane 7 are labeled in kDa.

Following chaotropic extraction, PfMDR1 is solubilized out of its native membrane environment with a mild detergent (Figure 3.11). Dodecyl maltoside (DM) is a nonionic detergent with low critical micellar concentration (CMC) that has been used extensively for the purification of integral membrane proteins [181]. In general, up to 50% of total membrane protein was lost during this step. Lysophosphatidylcholine (LPC)
and octyl-β-D-glucopyranoside (OG) were also investigated as potential detergents, but they were not found to selectively solubilize PfMDR1 (not shown).

**Figure 3.11** Coomassie blue stain of the optimization of detergent solubilization. Full-length PfDMR1 CMs were treated with varying concentrations of DM at varying pH. Lane 1, molecular weight markers (kDa); lanes 2-4, 0.25% DM at pH 6.5, 7.0, and 7.5; lanes 5-7, 0.5% DM at pH 6.5, 7.0, and 7.5; lanes 8-10, 0.75% DM at pH 6.5, 7.0, and 7.5.

Detergent-extracted proteins are next applied to an affinity chromatography column. The 6H and BAD fused to the C-terminal end of PfMDR1 allow for purification via either an immobilized avidin (Figure 3.12) or nickel (Figure 3.13) column. Avidin chromatography allowed for purification almost to homogeneity, while nickel columns always contained a greater number of contaminants.
Figure 3.12  Avidin purification profile of full-length PfMDR1. Silver stain (A) and avidin blot (B) of various fractions in the purification process. Lane 1, MW standards (labeled in kDa); lane 2, negative control CM; lane 3, PfMDR1 CM; lane 4, chaotropically washed pellet; lane 5, detergent extract; lane 6, column flow through; lane 7, first column wash; lane 8, second column wash; lane 9, column eluate; lane 10, PL.
Figure 3.13  Nickel purification profile of full-length PfMDR1. Silver stain of various fractions in the purification process. Lane 1, MW standards (labeled in kDa); lane 2, negative control CM; lane 3, PfMDR1 CM; lane 4, chaotropically washed pellet; lane 5, detergent extract; lane 6, column flow through; lane 7, first column wash; lane 8, second column wash; lane 9, column eluate; lane 10, PL.

The individual as well as the coexpressed N- and C-terminal cassettes could also be purified by affinity chromatography. Interestingly, a band at the approximate size of full length PfMDR1 could be seen upon reconstitution of the coexpressed halves (Figure 3.14), suggesting that the two halves can self-associate.
Figure 3.14  Purification of coexpressed N- and C- terminal cassettes.  (A) Silver stain and (B) avidin blot of various fractions of the purification process.  Lane 1, negative control membranes; lane 2, CM expressing both PfMDR1 halves; lane 3, membrane pellet from chaotropic wash; lane 4, chaotropic wash supernatant; lane 5, residual unsolubilized pellet; lane 6, detergent extract; lane 7, column flow through; lane 8, column wash 1; lane 9, column wash; lane 10, column eluate; lane 11, reconstituted PL; lane 12, MW standards.  Note the appearance of a high MW band in the PLs at the appropriate size for full-length protein (uppermost arrow in B).
3.2 Functional Analysis of PfMDR1

[Reproduced in part with permission from Amoah LE, Lekostaj JK, and Roepe PD. Heterologous expression and ATPase activity of mutant versus wild type PfMDR1 protein. 46(20): 6060-73. Copyright 2007 American Chemical Society] [182]

[Reproduced in part with permission from Lekostaj JK, Amoah LE, and Roepe PD. A single S1034C mutation confers altered drug sensitivity to PfMDR1 ATPase activity that is characteristic of the 7G8 isoform. 157(1): 107-11. Copyright 2008 Elsevier] [183]

For the initial survey of PfMDR1 activity, the designated “wildtype” isoform from a CQS strain (3D7) was compared to mutant isoforms from verapamil-reversible and -irreversible CQR strains (Dd2 and 7G8, respectively). Follow-up studies were then conducted on other mutants to more precisely characterize the 7G8 isoform.

<table>
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Table 3.7 PfMDR1 amino acid identities at residues associated with MDR. The 3D7 PfMDR1 sequence has been designated wildtype in the field, while the Dd2 variant possess one mutation and the 7G8 variant possesses 4 of the 5 identified mutations.

3.2.1 Determination of membrane protein content

The level of PfMDR1 expression varied slightly among different strains, and the induction times between membrane preparations also varied from 18-24 hours. Before quantitative ATPase assays could be performed on purified PM fractions, the percentage of membrane protein constituted by PfMDR1 had to be determined. This was done by comparing PfMDR1 membranes to polyHis tagged standards that were run on the same gel and developed together (Figure 3.15, Figure 3.16, Table 3.8).
**Figure 3.15** Densitometry standardization of PfMDR1 membrane content. HexaHis molecular mass standards (Qiagen) were serially diluted (inset, lanes 1-5) to yield variable nanograms of protein per lane [150, 120, 90, 60, and 30 ng for the 100 kDa band (top); 120, 96, 72, 48, and 24 ng for the 75 kDa band (middle); and 100, 80, 60, 40, and 20 ng for the 50 kDa band (bottom)]. Densitometry of each band was obtained using ImageJ and plotted vs. moles protein. A linear fit was applied to the data, yielding a straight line with equation $y = 1802.8x + 1201.7$, $R^2=0.81$.

**Figure 3.16** Representative polyHis blot of the quantification of protein in PM preparations. Lanes 1 & 2, two dilutions of 3D7 PfMDR1 PMs; lanes 3 & 4, two dilutions of Dd2 PfMDR1 PMs; lanes 5-9, serial dilutions of polyHis ladder as shown in Figure 3.15.

83
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<th>Isoform</th>
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<tr>
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<tr>
<td>7G8</td>
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Table 3.8 PfDMR1 percentages of total PM protein. The equation of the line from Figure 3.15 was applied to the densitometry of the bands from Figure 3.16 to extrapolate nmol PfMDR1 present. After converting to mass (using the predicted MW of 171.81 kDa), the values were divided by total amount of protein loaded in each lane (based upon amido black quantification) to determine PfMDR1 content. The values from several such trials were averaged.

3.2.2 Assay validation

The ATPase assay protocol previously utilized in the Roepe laboratory [100] employs borosilicate glass test tubes and requires individual reading of samples in a spectrophotometer. This is not an efficient method for testing a wide spectrum of drugs against a large number of samples. Therefore, a 96-well plate-based assay was developed to evaluate the ATPase activity of PfMDR1 (see Section 2.2.15). The original protocol ended with a total volume of 1 mL, and 96-well plates have a maximum capacity of 350 µL per well, so it was decided to scale down by 75%. Different forms of the reducing agent were investigated and it was found that sodium ascorbate yields more consistent results than ascorbic acid. Potassium phosphate standards were also found to be superior to sodium phosphate. Experience with the assay taught that thorough mixing of the solutions was necessary at each step, but that vigorous pipetting resulted in too many bubbles from the SDS-based stopping solution that interfered with the spectrophotometric reading. A good indication of the success of the high throughput ATPase assay was the linearity of the data obtained from the phosphate standard curve (Figure 3.17), which was
linear to an OD$_{720\text{nm}} > 1.0$. The standards were not appreciably affected by temperature or pH of the reaction buffer (not shown).

**Figure 3.17** Representative phosphate standard curve. Standards were prepared by serial dilution and subjected to the procedure for colorimetric detection of orthophosphate-molybdate (see Section 2.2.15). The optical density at 720 nm of each standard was read by an automated plate reader and plotted vs. known phosphate content.

### 3.2.3 Basal ATPase activity

To test whether the expressed proteins were functional, ATPase activity was analyzed as a function of time, pH, ATP and magnesium concentration, and ATPase inhibitors (Figure 3.18). Activity exhibited inhibition at high [ATP] with an optimum near 5 mM (Figure 3.18A), was linear with time for at least 15 minutes (Figure 3.18B), was clearly Mg$^{++}$ dependent (Figure 3.18C), had high activity at neutral-to-alkaline pH (Figure 3.18D), and had relatively low sensitivity to vanadate (Figure 3.18E) but high sensitivity to concanamycin (Figure 3.18F). Interestingly, the Dd2 PfMDR1 isoform was significantly more active under basal conditions relative to 3D7, yet 7G8 (the other drug resistance-associated isoform) was less active (p<0.001 by ANOVA).
Figure 3.18 Basal ATPase activity of recombinant PfMDR1 isoforms. PfMDR1 ATPase plate assay as a function of (A) [ATP], (B) time, (C) [Mg$^{++}$], (D) pH, (E) vanadate and (F) concanamycin for 3D7 (square symbols, solid lines), Dd2 (triangles, dashed lines) and 7G8 (circles, dotted lines) PfMDR1 isoforms. Each point is an average of at least 6 determinations (3 replicates in each of 2 independent experiments) and error bars show standard deviation.

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<td>Concanamycin IC$_{50}$ (nM)</td>
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<td>69.7</td>
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Table 3.9 Kinetic parameters and inhibitor sensitivities of PfMDR1. A summary of the results from Figure 3.16 is shown. For row 1, activity was tested at pH = 5.5 to 8.0 in 0.5 unit increments and the value at which the highest activity was measured is reported. $V_{\text{max}}$ and $K_m$ were calculated from double-reciprocal plots of data such as that in Figure 3.16A. The vanadate and concanamycin IC$_{50}$s were calculated from the equations of the best-fit curves of the data in Figures 3.16E and F, respectively.

3.2.4 Drug-stimulated ATPase activity

A hallmark feature of most ABC transporters involved in drug resistance phenomena is that drugs to which they confer resistance either stimulate or inhibit their ATPase activity. Reported effects are usually stimulatory, can be mild (2-5 fold) or robust (10-20 fold), and are often, but not always, further modulated by VPL and other chemoreversal agents. However, the range of drug concentrations used in such assays varies widely and is often non-physiologic. In the case of PfMDR1, definition of the “physiologically relevant” drug concentration that should be examined is somewhat ambiguous. For example, in vitro IC$_{50}$ data suggest 10-20 nM CQ is physiologically relevant for CQS strains (e.g., 3D7), whereas 100-200 nM is relevant for CQR (e.g., those expressing Dd2 and 7G8 PfMDR1 alleles or overexpressing the 3D7 allele).
Parasite cytosolic concentrations for CQ and other quinolines are predicted to be near growth medium concentrations [184], so if drugs interact with PfMDR1 via the cytosolically disposed face (that harboring the NBDs), then concentrations near IC$_{50}$ would be physiologically relevant with respect to possible PfMDR1 interactions. However, the DV membrane maintains a high ΔpH that acts to strongly concentrate weak base antimalarial drugs within the DV. If the drug is effectively diprotic (e.g., CQ) this effect varies as the square of the net pH gradient (since the pH scale is logarithmic), whereas if the drug is effectively monoprotic (e.g., MQ, QN) its concentration is linearly related to the pH scale [184].

Therefore, PfMDR1 ATPase activity +/- MQ, QN, and CQ in either the absence (Figure 3.19) or presence (Figure 3.20) of VPL was analyzed over two concentration ranges that reflect reasonable anticipated equilibrium cytosolic (lower range, left side of each Figure panel) and DV (higher range, right side of each Figure panel) concentrations. CQ, MQ and QN were chosen for this initial analysis based on data with field isolates and $P. falciparum$ transfectants, which indicate that mutation and/or over-expression of PfMDR1 has the greatest effect on responsiveness to MQ and QN, but has no appreciable effect on susceptibility to the related drug CQ (see section 1.5.3).

It had been hypothesized (see section 1.8.1) that MQ would inhibit ATPase activity in 3D7 PfMDR1 but stimulate the 7G8 variant. Surprisingly, none of the isoforms tested were stimulated more than 1.5-fold at any concentration tested (Figure 3.19A). It had also been anticipated that QN would stimulate the 3D7 protein and inhibit 7G8 PfMDR1. Again unexpectedly, a mild QN stimulation was found for all three
isoforms, but only at very high dosages that correspond to the upper limit of what is expected within the DV (right of Figure 3.19B). At 200 µM QN, the stimulatory effect was approximately 75% (1.75-fold), 30% (1.3-fold), and 50% (1.5-fold) for the 3D7, Dd2 and 7G8 isoforms, respectively. A t-test of these values compared to baseline (no drug) showed significant differences at the p<0.05 level for Dd2 and 7G8 and p<0.01 for 3D7. Interestingly, the largest effects on ATPase activity were seen for CQ (Figure 3.19C), to which PfMDR1 is not believed to confer resistance [79, 116-117]. CQ very weakly (non-significantly) stimulated ATPase activity (10-20%) for the 3D7 and Dd2 isoforms at the higher range of anticipated cytosolic concentrations, but then very strongly inhibited all isoforms at anticipated DV concentrations to less than 10% of their basal activities (p<0.001 for all points ≥1.25 mM compared to no drug by ANOVA for all three isoforms).

In some cases, VPL alone, and/or non-lethal doses of VPL along with drugs to which the ABC transporter confers resistance, further stimulates ATPase activity of the transporter. Since some strains of CQR parasites can be resensitized by VPL, but the mechanism of this chemoreversal is unknown, it was hypothesized that VPL would affect the ATPase activity of Dd2 PfMDR1 but not 7G8. Indeed, physiologic (non-toxic) dosages of VPL (1-2 µM) had a significant effect on Dd2 PfMDR1 ATPase activity (p<0.001 by t-test) but not 3D7 and 7G8, which appeared insensitive over this range (Figure 3.19D). The Dd2 isoform was inhibited by approximately 2-fold at 2 µM VPL, and ~60% stimulation from this value was then seen at progressively higher (toxic)
concentrations. The 7G8 variant was particularly unresponsive to this drug, with activity fluctuating by only a couple of percent across a wide range of [VPL].
Figure 3.19 Effects of drugs on PfMDR1 ATPase activity. Various concentrations of (A) MQ, (B) QN, (C) CQ, and (D) VPL were tested for their ability to modulate the ATPase activity of the 3D7 (squares, solid lines), Dd2 (triangles, dashed lines), and 7G8 (circles, dotted lines) PfMDR1 isoforms. The left half of each graph corresponds to drug concentrations present in the parasite cytoplasm, while the right half of each graph corresponds to drug concentrations predicted to accumulate within the DV. Each point is an average of at least 6 determinations (3 replicates in each of 2 independent experiments) and error bars show standard deviation.

Since VPL is capable of reversing the drug resistance phenotype, and may be exerting this effect through PfMDR1, it seems reasonable that this compound might also reverse any drug-induced effects on the ATPase activity of this protein. While some
variants tested showed a slight activation in the presence of MQ alone, the addition of 2 µM VPL did not further stimulate as has been seen in HuMDR1, but in fact negated or shifted the concentration dependence for MQ-induced stimulation (Figure 3.20A). 3D7 and 7G8 variants displayed almost no response to the drug combination, while the Dd2 protein was initially inhibited at cytosolic concentrations of MQ and then later recovered its basal level of activity at DV concentrations. In the case of QN combined with 2 µM VPL (Figure 3.20B), again 3D7 PfMDR1 was unaffected across the entire range of [QN]. The Dd2 isoform exhibited a biphasic profile, with up to 40% stimulation at cytosolic concentrations of QN followed by 25% inhibition at DV concentrations. Somewhat similarly, the 7G8 variant was stimulated up to 50% in the low drug range and then brought back to basal activity by high drug concentrations. Finally, when 2 µM VPL was added to various amounts of CQ, an almost complete reversal of the inhibition previously found at DV concentrations of CQ alone was achieved (Figure 3.20C). The Dd2 protein variant was even mildly stimulated under these conditions.

Since VPL is monobasic, it will also accumulate in the DV. Therefore, the effect of the quinoline drugs on PfMDR1 ATPase activity was also examined at 20 µM VPL (Figure 3.20D-F). Again, very little stimulation or inhibition was seen with MQ, with the exception that the activity of the 3D7 isoform was raised to Dd2-like levels across the board. This was also true for the QN-VPL and CQ-VPL combinations. With the co-treatment with CQ and VPL, Dd2 PfMDR1 displayed a particularly complex behavior, with an initially marked inhibition at low cytosolic concentrations that was then returned to baseline at mid-range concentrations, and finally re-inhibited at very high [CQ].
Figure 3.20 Effects of drugs on PfMDR1 ATPase activity in the presence of verapamil. Various concentrations of (A, D) MQ, (B, E) QN, and (C, F) CQ were tested for their ability to modulate the ATPase activity of the 3D7 (squares, solid lines), Dd2 (triangles, dashed lines), and 7G8 (circles, dotted lines) PfMDR1 isoforms in the presence of 2 µM (A-C) or 20 µM (D-F) VPL. The left half of each graph corresponds to drug concentrations present in the parasite cytoplasm, while the right half of each graph corresponds to drug concentrations predicted to accumulate within the DV. Each point is an average of at least 6 determinations (3 replicates in each of 2 independent experiments) and error bars show standard deviation.

Finally, the ATPase activity of the 3D7 “half transporter” constructs expressed either alone (N-terminal, C-terminal) or together (coexpressed) was compared to the behavior of the full length 3D7 isoform (Figure 3.21). As expected [162], ATPase activity of the N-terminal and C-terminal halves was lower than that of the full length, even after normalization to number of NBDs, yet exhibited similar [ATP] dependency. The two half transporters expressed together did not show maximum ATPase activity until higher [ATP]. The pH dependency for ATP hydrolysis was lost for the half transporters, yet was steeper for the coexpressed pair. N-terminal PfMDR1 was found to have similar activity relative to C-terminal, and at optimum pH, the coexpressed halves’ activity was 100% that of the full length. Based on these data, it is likely that communication between the two halves is important with regard to optimal activity.
Figure 3.21 Basal ATPase characterization of N- and C-terminal half constructs compared to full-length PfDMR1. (A) ATP dependence and (B) pH profile of wildtype full length (solid squares, solid line), N-terminal (open triangles, dashed line), C-terminal (open circles, dotted line), and coexpressed N- and C-terminal (closed diamonds, thatched line) PfMDR1. Each point is an average of at least 6 determinations (3 replicates in each of 2 independent experiments) and error bars show standard deviation.

QN and CQ drug stimulatory effects observed for the full length PfMDR1 were lost when the protein was expressed as N-terminal or C-terminal forms (Figure 3.22). However, mild MQ stimulation, but at higher dose (corresponding to predicted DV concentrations) was preserved for the N- and C-terminal half-transporters, as were inhibitory effects seen at high doses of CQ.
Figure 3.22 Drug effects on ATPase activity of wildtype half transporters in comparison to full length PfMDR1. Two concentrations of (A) MQ, (B) QN, and (C) CQ (representative of one cytosolic and one DV concentration) were tested. Each value is an average of at least 6 determinations (3 replicates in each of 2 independent experiments) and error bars show standard deviation.

3.2.5 Further definition of 7G8 PfMDR1 ATPase activity

Among the differences found between 3D7, Dd2, and 7G8 isoforms of PfMDR1, the disparity in basal activity was particularly intriguing. The Dd2 (VPL-reversible CQR phenotype) variant exhibited higher ATPase activity relative to that of 3D7 (CQS), while
activity of the 7G8 (VPL-irreversible CQR) isoform was by far the lowest of the three. Also, in contrast to Dd2 and 3D7 isoforms, ATPase activity of 7G8 PfMDR1 was largely unaffected by quinoline antimalarial drugs, except at very high [CQ]. Since Dd2 PfMDR1 possesses a single amino acid change relative to 3D7 (N86Y), some of the observed changes in activity may be assigned to this substitution. However, 7G8 PfMDR1 contains four additional substitutions relative to wild type: Y184F, S1034C, N1042D, and D1246Y. In order to investigate which of these mutations is responsible for the unusual 7G8 isoform ATPase activity (i.e., low $V_{\text{max}}$, high $K_m$, conspicuous loss of drug response), yeast strains expressing PfMDR1 harboring various combinations of these mutations were created. This is the first step in identifying regions of the PfMDR1 molecule that may or may not interact with specific drugs.

Initial data indicated that a mutant containing the three C-terminal substitutions (called “triple mutant” or TM) behaved similarly relative to 7G8 PfMDR1. Attention was therefore focused on the effects of the 1034C, 1042D, and 1246Y substitutions, both alone and in various possible combinations. Three yeast strains expressing PfMDR1 with each single mutation as well as 3 strains with each possible double mutation (see Section 3.1.6) were created. Plasma membrane fractions were purified from each strain (Figure 3.23) and quantified as previously described (Table 3.10; see Section 3.2.1).
Figure 3.23 PolyHis blot of C-terminal mutant plasma membranes. There is approximately equal inducible expression of each mutant isoform. Lane 1, negative control membranes (transformed with empty pPICZc plasmid); lane 2, 1034C; lane 3, 1042D; lane 4, 1246Y; lane 5, 1034C/1042D; lane 6, 1034C/1246Y; lane 7, 1042D/1246Y; lane 8, 1034C/1042D/1246Y triple mutant.

<table>
<thead>
<tr>
<th>Isoform</th>
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<tr>
<td>1034</td>
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<tr>
<td>1042</td>
<td>2.45%</td>
</tr>
<tr>
<td>1246</td>
<td>2.71%</td>
</tr>
<tr>
<td>1034/1042</td>
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<td>1042/1246</td>
<td>2.05%</td>
</tr>
<tr>
<td>TM</td>
<td>2.46%</td>
</tr>
</tbody>
</table>

Table 3.10 Percent of total membrane protein constituted by PfMDR1.

All mutants tested had an optimum pH near 7.0 or 7.5 and all but one showed optimal activity near 5.0 or 7.5 mM ATP (Table 3.11). In terms of basal activity, the S1034C mutant closely approximates 7G8 PfMDR1, whereas N1042D is more similar to the Dd2 isoform, and D1246Y exhibits the highest ATPase activity of any variant yet tested (with a $V_{\text{max}}$ identical to that of Dd2). However, $K_m$ for S1034C is conspicuously higher relative to 7G8. That is, no single C-terminal substitution recapitulates both $V_{\text{max}}$ and $K_m$ seen for the 7G8 isoform. Two or more mutations must therefore act in concert to produce an enzyme with 7G8 PfMDR1 kinetic characteristics. Interestingly, the 1034/1042 and 1034/1046 double mutant isoforms both show elevated $V_{\text{max}}$ and lower $K_m$ relative to the S1034C. The 1042/1246 double mutant is similar to the 3D7 variant, and
shows higher $K_m$ relative to the single-site 1042 and 1246 mutants. Thus the relatively lower $V_{max}$ and higher $K_m$ that is seen for TM and 7G8 PfMDR1 is not seen in any of the double mutants; all three C-terminal substitutions are required for this behavior. The remaining mutation that converts the TM variant to a full 7G8 haplotype (Y184F) must produce a small effect that then mildly raises $V_{max}$ and lowers $K_m$ but does not alter drug response (consistent with previous suggestions from analysis of transfectants [116-117]).

<table>
<thead>
<tr>
<th></th>
<th>3D7</th>
<th>Dd2</th>
<th>1034C</th>
<th>1042D</th>
<th>1246Y</th>
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<tr>
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<td>70.08</td>
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<td>69.59</td>
<td>56.94</td>
<td>45.34</td>
<td>41.07</td>
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<tr>
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<td>7.00</td>
<td>3.65</td>
<td>4.29</td>
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</table>

**Table 3.11** Kinetic parameters and inhibitor sensitivities of PfMDR1 isoforms. Basal ATPase activity +/- VPL (rows 1,2) is measured under optimum conditions (temp, [ATP], pH) in the absence of antimalarial drugs, and numbers shown are the average of at least 6 measurements +/- standard deviation. For row 3, activity was tested at [ATP] = 2.5, 5.0, 7.5 and 10.0 mM; for row 4, activity was tested at pH = 6.0 to 8.0 in 0.5 unit increments. For other rows, values were computed after curve-fitting the relevant plotted variables. N/A denotes not analyzed, as the measured activity in the presence of vanadate was too low to yield a statistically reliable calculation.

Earlier, it was shown that the ATPase activity of 3D7 PfMDR1 is mildly stimulated (maximal of ~1.5-fold) over a wide range of estimated cytoplasmic and
digestive vacuolar concentrations of MQ (Figure 3.19A). Amongst the C-terminal mutants, the N1042D and D1246Y single mutants as well as the 1034C/1042D double mutant are similarly stimulated by MQ, but only at cytoplasmic concentrations of the drug (Figure 3.24A). A decrease in ATPase activity was then seen at DV concentrations for almost all the variants tested. This inhibition was generally around 50%, but reached almost 75% for the double 1042D/1246Y isoform (p<0.001 by t-test). In contrast, the S1034C single mutant showed very little response in either direction upon addition of any concentration of MQ, remarkably similar to the behavior of TM and 7G8. A significant QN stimulatory effect was previously seen for 3D7 and Dd2 isoforms, but only at very high concentrations that correspond to the very upper range of what can be expected within the DV. None of the C-terminal mutants display this stimulation, and indeed some (particularly the 1034C/1246Y and 1042D/1246Y) even seem to be inhibited by high amounts of QN (Figure 3.24B). However, 1246Y and 1034C/1042D were stimulated (up to 50% and 70% respectively, corresponding to a p<0.01 by t-test) at much lower concentrations. Considering the fact that CQ is generally not believed to interact with PfMDR1, it was surprising that the earlier studies found CQ had the greatest effect on ATPase activity: high DV-compatible concentrations of CQ severely inhibited both 3D7 and 7G8 isoforms. Under similar conditions, all C-terminal mutants exhibit some degree of inhibition, but only S1034C and TM are rendered almost completely inactive (Figure 3.24C). In sum, of all mutants tested, only the S1034C variant exhibited a nearly identical “drug profile” to 7G8 PfMDR1. Also, interestingly, S1034C and 7G8 are the only two isoforms that were not affected at all by VPL (Table 3.11).
Figure 3.24 Drug effects on C-terminal mutants. Cytosolic (left) and DV (right) concentrations of MQ (A), QN (B), and CQ (C) were investigated for their effects on a panel of mutant PfMDR1 isoforms. A visual legend has been provided to assist in the deciphering of the congested graphs. Each point is the average of at least 6 determinations (3 replicates in 2 independent experiments) and error bars indicate standard deviation.

3.3 CQ binding to PfCRT

3.3.1 Creation of “BAD-less” pfcr1 constructs

In anticipation of a biotinylated photoligand (see Section 3.3.3), site-directed mutagenesis was used to removed the BAD (see Section 2.2.6) from plasmids previously created in the laboratory [131].
Figure 3.25  Screening for removal of BAD from PfCRT. (A) Avidin and (B) polyHis blots of yeast colonies transformed with HB3 (lanes 3-8) and Dd2 (lanes 9-14) BAD-less constructs. Lane 1, MW standards labeled in kDa; lane 2, control CRT-HB membranes; odd-numbered lanes 3-13, CMs from 0 hours of induction; even-numbered lanes 4-14, CMs harvested 24 hours post-induction. The removal of the BAD domain decreases the size of PfCRT from ~54 kDa (only arrow in A, upper arrow in B) to ~48 kDa (lower arrow in B).

3.3.2 Purification of PfCRT

The purification of PfCRT was accomplished using a similar procedure to that for PfMDR1 (Section 3.1.9). As found previously, avidin chromatography resulted in much
purer PLs, while nickel IMAC produced PLs with more contaminating proteins (Figure 3.26).

![Image](image.png)

**Figure 3.26** Purification of PfCRT. Silver stain of various fractions from the purification process. Lane 1, broad range MW standards; lane 2, CRT-His-BAD crude membrane; lane 3, supernatant from chaotropic wash; lane 4, residual pellet from detergent solubilization; lane 5, detergent extract; lane 6, column flow through; lane 7, column wash; lane 8, column eluate; lane 9, PL; Lane 10, CRT-His Nickel-purified PL.

*In vivo*, PfCRT is oriented with its N- and C-terminal tails in the cytoplasm [83]. The directionality of the protein within the reconstituted lipid system was determined by cleaving the BAD from the rest of the molecule with Factor Xa (Figure 3.27). Assuming a certain degree of “tightness” to the PLs that excludes the enzyme from accessing the internal environment, only proteins that mimic the native orientation within the DV membrane will be susceptible to Factor Xa digest. Comparison of the amount of fully cleaved protein to the original sample shows that the majority of PfCRT is oriented correctly.
Figure 3.27 Determination of PfCRT orientation. (A) Avidin (upper) and polyHistidine (lower) immunoblots of Dd2-His-BAD digested with Factor Xa. Lane 1, biotinylated MW standards; Lane 2, original undigested PL; lanes 3-7, PL digested for 30, 60, 90, 120, and 150 min, respectively. Note that the presence of the BAD seems to interfere with the binding of the anti-His antibody (compare lanes 2 and 3, which contain equal amounts of protein). (B) Plot of amount of full-length CRT-His-BAD remaining over time. Image J (NIH) was used to find the densitometry of the band corresponding to CRT-His-BAD in each of the blots in (A). The values were converted to percent of Lane 2, and the results for both blots averaged. ~76% of the initial protein is cleaved, indicating that ¾ of the molecules are oriented with the C-terminal tail on the outside of the lipid.
3.3.3 Synthesis of AzBCQ

A photoreactive analogue of CQ was designed by Dr. Paul Roepe and Dr. Christian Wolf and synthesized by Dr. Jayakumar Natarajan (all from the Department of Chemistry, Georgetown University). As the molecule contains a perfluoro phenyl azide and a biotin tag, it has come to be called Azido-Biotinylated-Chloroquine (AzBCQ; Figure 3.28). The essential pharmacophore of the drug is preserved, and the pKa of the side chain remains unaltered. The aryl azide forms a reactive nitrene upon UV irradiation, which can undergo C-H insertion with proximal amino acid side groups, thus covalently attaching to the protein. The biotin tag can be quantified by avidin-HRP detection and can also be used to purify protein-drug conjugates. The azide and biotin groups are both attached by flexible linkers whose lengths can be easily modified.

![Synthetic scheme for Azido-Biotinylated-Chloroquine (AzBCQ). Yields are marked in percentages underneath each product. The final compound was assessed by $^1$H and $^{13}$C-NMR spectroscopy as well as HPLC and was found to be ~97% pure.](image)

Figure 3.28 Synthesis of AzBCQ. Yields are marked in percentages underneath each product. The final compound was assessed by $^1$H and $^{13}$C-NMR spectroscopy as well as HPLC and was found to be ~97% pure.
3.3.4 Photolabeling of PfCRT in native membranes

Repeated attempts to photolabel PfCRT in several yeast membrane preparations (CMs, PMs, and inside-out vesicles (ISOVs)) were unsuccessful due to the high background caused by the relatively large number of endogenously biotinylated proteins. *Plasmodium* has no naturally occurring biotinylation, so efforts were then made to photolabel parasites directly. HB3 and Dd2 strains of *P. falciparum* were maintained in culture by Mynthia Cabrera (Department of Chemistry, Georgetown University) and grown to ~10% parasitemia. The prepared culture was harvested and the parasites were released from their red blood cell hosts by gentle treatment with saponin [185]. The intact parasites were then incubated with AzBCQ and photolabeled. The protein extracts were analyzed by immunoblotting with avidin (to detect proteins bound to probe) as well as anti-CRT antibody (Figure 3.29). The specificity of the label was demonstrated through competition of labeling with “cold” (non-azido-biotinylated) CQ.

As shown in Figure 3.29A, endogenous wildtype (HB3) PfCRT protein is efficiently labeled with 10 µM AzBCQ, and labeling is competed by “cold” CQ. Figure 3.29B compares PfCRT labeling for approximately equal amounts of HB3 (CQS) and Dd2 (CQR) parasites and shows that competition of labeling by cold CQ is qualitatively similar for both isoforms. It is noted that isolation of saponin-treated parasites routinely yields suspected partially proteolyzed PfCRT fragments and that some of these apparently include the AzBCQ binding site. Other immunoreactive bands may represent
proteolyzed PfCRT domains that do not contain the AzBCQ binding site or nonspecific reactivity of the polyclonal antibody.

![Image](image1.png)

**Figure 3.29** AzBCQ labeling of saponin-isolated parasites. (A) Competition of AzBCQ labeling of native HB3 PfCRT. Avidin (lanes 1-6) and anti-PfCRT (lanes 7-12) blots of HB3 parasites reacted with 10 µM AzBCQ in the absence or presence of unlabeled CQ. Lanes 1 & 7, no competitor; lanes 2 & 8, 0.8 mM CQ; lanes 3 & 9, 1.6 mM CQ; lanes 4 & 10, 2.4 mM CQ; lanes 5 & 11, 3.2 mM AzBCQ; lanes 6 & 12, 4.0 mM CQ. (B) Comparison of competition of photolabeling of HB3 and Dd2 PfCRT. Avidin (lanes 1-6) and anti-PfCRT (lanes 7-12) blot of HB3 (lanes 1-3 & 7-9) and Dd2 (lanes 4-6 & 10-12) parasites reacted with 10 µM AzBCQ in the absence or presence of competing unlabeled CQ. Lanes 1, 4, 7, & 10, no competitor; lanes 2, 5, 8, & 11, 2 mM CQ; lanes 3, 6, 9, & 12, 4 mM CQ. Arrow indicates major immunoreactive band with anti-PfCRT probe, while asterisk is presumably a degradation product that retains the AzBCQ binding site.

3.3.5 Photolabeling of purified heterologously expressed PfCRT

Extensive photolabeling of intact parasites requires complicated, expensive culture conditions (e.g., human blood, specialized gas mixtures) and such extremely large volumes of culture as to make it impractical. It takes over a week of growth to obtain enough material for only a few photolabeling reactions. In addition, there is no commercial source for anti-PfCRT antibody and the supply is severely limited. Detailed
analysis was therefore carried out on yeast-expressed PfCRT reconstituted in PLs. Nickel-column purified PfCRT-H PLs were used in these experiments for two reasons: first, the BAD attached to PfCRT-His-BAD would obviously compete for avidin-HRP detection with the biotin moiety attached to AzBCQ, making quantation impossible (and residual biotin would remain even after Factor Xa cleavage, as in Figure 3.27); second, even though the PfCRT-His PLs are only partially purified (Figure 3.26) the presence of a number of contaminants represents an additional way to gauge specificity of the photolabeling reaction. Meaning, if reaction conditions were too robust, additional crosslinking to contaminants would be seen, whereas if the conditions generated PfCRT-specific binding, a single band would be observed.

Each photolabeling experiment was analyzed using a normalization procedure (Figure 3.30) involving finding the ratio of AzBCQ binding (determined by avidin detection) to total PfCRT present (determined by polyHis detection). Also, fortuitously, a naturally biotinylated yeast protein contaminant is carried through via the nickel chelation purification strategy (Figure 3.30A, band migrating near 30 kDa). This band provides convenient verification in avidin-HRP blots that equal amounts of AzBCQ-reacted PfCRT-H PLs are loaded in each lane. This is confirmed by the companion anti-his-HRP western blot (Figure 3.30B). Membranes expressing PfCRT-6H-BAD were always run as a positive control, remembering that the presence of the BAD causes the protein to run at a slightly larger MW.
Figure 3.30 Illustration of photolabeling analysis methodology. (A) avidin blot (B) polyHis blot and (C) normalized densitometry of CQ competition of HB3 labeling at different concentrations of AzBCQ. pH of the reactions was 5.2 and UV illumination time was 10 minutes. The densitometry of the PfCRT band in (A) was divided by that in (B) and averaged with the values from another set of gels to obtain the plot shown in (C). For both western blots: lane 1, MW standards; lanes 2-7, 50x AzBCQ; lanes 8-13, 100x AzBCQ; lane 14, HB3-His-BAD crude membrane (upper arrow). Lanes 2 & 8, no competing CQ; lanes 3 & 9, 10-fold CQ relative to AzBCQ; Lanes 4 & 10, 20-fold CQ; Lanes 5 & 11, 40-fold CQ; Lanes 6 & 12, 60-fold CQ; Lanes 7 & 13, 80-fold CQ. For (C), 50x AzBCQ, solid squares, solid line; 100x AzBCQ, open squares, dashed line. Nonlinear regression analysis of the data points fit the curves to an equation for one-phase exponential decay, \( y = \text{span} \times e^{-Kx} + \text{plateau} \). \( R^2 \) values were 0.96 and 0.94 for HB3 and Dd2, respectively.

Initial photolabeling experiments were conducted to optimize reaction conditions. First, the time of UV radiation was varied (Figure 3.31) to ensure complete lysis and maximum insertion of the photolabel. Some binding can be seen in less than one minute of activation, consistent with the highly reactive nature of the perfluoro phenyl azide. Plateau is reached within 10 minutes of UV exposure, so this time was chosen for all future reactions.
Figure 3.31 AzBCQ labeling as a function of UV illumination time. HB3 (squares, solid line) and Dd2 (triangles, dashed line) were labeled with 50-fold molar AzBCQ relative to protein at pH 5.2. For avidin blots in (A): Lane 1, 0 min; lane 2, 30 sec; lane 3, 1 min; lane 4, 5 min; lane 5, 10 min; lane 6, 15 min. Data were curve-fit to a hyperbolic line with equation $y = (B_{\text{max}} \times x)/(K_d + x)$. For HB3, $R^2=0.99$ and for Dd2, $R^2=0.95$.

Next, the concentration of probe was titrated relative to the amount of protein present (Figure 3.32). For both HB3 and Dd2 isoforms, labeling plateaus by 200-fold molar excess of AzBCQ. A concentration of 50x AzBCQ was chosen for all future work so as to avoid saturation, which might mask potential effects or differences between isoforms. Densitometry reveals that half-maximal labeling (50% of the plateau value determined by nonlinear regression) occurs at a probe/protein ratio of $\sim$34 and 102 for HB3 and Dd2, respectively. Although photolabeling efficiencies of probes that are believed to be specific to transporters or channels varies widely [44, 186-188], these results are at the lower end of the range of apparent affinities for azido-drug probes and
suggest that AzBCQ photolabeling is quite efficient and specific. Using known amounts of biotinylated protein standards (e.g., lane 1, Figure 3.30A) and quantitation of purified PfCRT protein, it is estimated that the stoichiometry of labeling is 0.14 mol AzBCQ per mol PfCRT when illumination time is fixed at 10 min and the molar ratio of AzBCQ:PfCRT in the reaction is set at 50. Since stoichiometry is less than 1:1, this again suggests (but does not prove) that photolabeling under these initial plateau conditions is quite specific and involves a single drug binding site.

Figure 3.32 AzBCQ labeling as a function of photoprobe concentration. HB3 (squares, solid line) and Dd2 (triangles, dashed line) were labeled at pH 5.2 with 10 min of UV irradiation. For avidin blots: Lane 1, no AzBCQ; lane 2, 20-fold AzBCQ relative to PfCRT; lane 3, 40-fold excess AzBCQ; lane 4, 60-fold excess AzBCQ; lane 5, 80-fold excess AzBCQ; lane 6, 100-fold excess AzBCQ. Data were curve-fit to an equation for one-phase exponential association, \( y = y_{\text{max}}(1-e^{-K_s}) \). \( R^2 \) values were 0.98 and 0.97 for HB3 and Dd2, respectively.
Once these experimental parameters had been optimized, the photolabeling studies were expanded to include a third PfCRT isoform, 7G8 (VPL-insensitive CQR). The pH inside the DV is a topic of great debate in the malaria literature. The Roepe laboratory has previously measured the pH of CQR parasites to be 0.4 – 0.5 units lower than that of CQS [104], but this result has been disputed [189]. For all three isoforms examined, binding of AzBCQ was markedly better at low pH (Figure 3.33). However, at pH 5.0 both CQR-conferring variants (Dd2 and 7G8) are less efficiently labeled than the HB3 (CQS) isoform. Since absolute efficiency in these reactions is a function of several variables, both absolute densitometry for the three isoforms (Figure 3.33B) as well as percent labeling (Figure 3.3C), normalized to maximal labeling for each isoform, are plotted.
Figure 3.33 AzBCQ labeling as a function of pH. HB3 (squares, solid line), Dd2 (triangles, dashed line), and 7G8 (circles, dotted line) were labeled at 50-fold molar excess AzBCQ with 10 min of UV irradiation. For avidin blots in (A): Lane 1, pH 5.5; lane 2, pH 6.0; lane 3, pH 6.5; lane 4, pH 7.0; lane 5, pH 7.5; lane 6, pH 8.0. In (B) and (C), nonlinear regression analysis fit the data to a one-phase exponential decay with equation \( y = \text{span}e^{(-Kx)} + \text{plateau} \) and \( R^2 \) values of 0.98, 0.99, and 0.95 for HB3, Dd2, and 7G8, respectively.
Calculating the affinity of AzBCQ for PfCRT is difficult because the irreversible nature of the binding makes equilibrium conditions impossible. However, an apparent affinity can be estimated through competition studies with unlabeled quinoline drugs. AzBCQ appears to be functionally comparable to CQ, as labeling is readily diminished by increasing concentrations of CQ (Figure 3.34). In terms of maximum binding, the competition is similar for both HB3 and Dd2 isoforms, with 50% knockdown occurring at 22.2- and 24.9-fold molar excess of unlabeled CQ. In contrast, labeling of 7G8 PfCRT seems particularly sensitive to competition with CQ, with half-maximal binding at only 6.54-fold competing CQ. Although the range of successful competition ratios for “cold” substrates vs. azido probes varies widely in the literature, it is noted that these ratios are very much at the low end of the range, particularly for the 7G8 variant. For example, efficient competition for azido analogues of vinblastine (VBL) or VPL photolabeling of HuMDR1 is seen anywhere from 200 – 10,000-fold molar excess cold VBL or cold VPL vs. the corresponding azido derivatives [44, 186-187].
Figure 3.34 Competition of AzBCQ binding with unlabeled CQ. HB3 (squares, solid line), Dd2 (triangles, dashed line), and 7G8 (circles, dotted line) were labeled at 50-fold molar excess AzBCQ at pH 5.2 with 10 min of UV irradiation. For avidin blots in (A): Lane 1, no CQ competitor; lane 2, 8-fold unlabeled CQ relative to AzBCQ; lane 3, 16-fold excess CQ; lane 4, 24-fold excess CQ; lane 5, 32-fold excess CQ; lane 6, 40-fold excess CQ. Data were curve-fit to a one-phase exponential decay with equation $y = span*(e^{-Kx}) + plateau$. HB3 $R^2=0.97$, Dd2 $R^2=0.95$, and 7G8 $R^2=0.99$.

While not strictly correlated, CQR is often found concomitantly with QNR. Competition studies were therefore also performed with unlabeled QN (Figure 3.35). The
competition profiles are more uniform for QN than CQ, with half the binding lost at 18.6, 15.7, and 10.1-fold molar excess unlabeled drug for HB3, Dd2, and 7G8, respectively.

**Figure 3.35** Competition of AzBCQ binding with unlabeled QN. HB3 (squares, solid line), Dd2 (triangles, dashed line), and 7G8 (circles, dotted line) were labeled at 50-fold molar excess AzBCQ at pH 5.2 with 10 min of UV irradiation. For avidin blots in (A): Lane 1, no QN competitor; lane 2, 8-fold unlabeled QN relative to AzBCQ; lane 3, 16-fold excess QN; lane 4, 24-fold excess QN; lane 5, 32-fold excess QN; lane 6, 40-fold excess QN.
excess QN. Data were curve-fit to a one-phase exponential decay with equation $y = \text{span} \cdot (e^{-kx}) + \text{plateau}$. HB3 $R^2=0.98$, Dd2 $R^2=0.97$, and 7G8 $R^2=0.98$.

In order to exclude the possibility that AzBCQ binding could be nonspecifically competed by an excess of any exogenous compound, labeling was assayed in the presence of ART (Figure 3.36), a drug to which PfCRT does not confer resistance. ART failed to reduce AzBCQ binding of any isoform tested, and even appeared to mildly increase labeling of the Dd2 and 7G8 variants at higher concentrations, perhaps by reducing nonspecific labeling of contaminating proteins in the PLs.
Figure 3.36 Competition of AzBCQ binding with unlabeled ART. HB3 (squares, solid line), Dd2 (triangles, dashed line), and 7G8 (circles, dotted line) were labeled at 50-fold molar excess AzBCQ at pH 5.2 with 10 min of UV irradiation. For avidin blots in (A): Lane 1, no ART competitor; lane 2, 8-fold unlabeled ART relative to AzBCQ; lane 3, 16-fold excess ART; lane 4, 24-fold excess ART; lane 5, 32-fold excess ART; lane 6, 40-fold excess ART. Data were analyzed by linear regression to generate a straight line with equation $y = mx + b$. $R^2$ values were 0.03, 0.53, and 0.93 for HB3, Dd2, and 7G8, respectively.
In live parasites, the CQR conferred by the Dd2 \textit{pfcr}t allele is reversed by VPL through an unknown mechanism. One possibility is that VPL associates with PfCRT and blocks it from transporting CQ out of the DV. HB3 and Dd2 photolabeling with AzBCQ is competed by VPL (Figure 3.37), but at higher concentrations compared to CQ or QN (35.4-fold excess for HB3 and 39.7-fold for Dd2), which is consistent with data obtained in parasite culture, where CQ IC\textsubscript{50} values are in the nM range, while 1-2 µM VPL is required for resensitization. Remarkably, VPL failed to outcompete AzBCQ binding to the 7G8 isoform, consistent with its conferral of a VPL-irreversible phenotype in live parasites.
Figure 3.37  Competition of AzBCQ binding with unlabeled VPL. HB3 (squares, solid line), Dd2 (triangles, dashed line), and 7G8 (circles, dotted line) were labeled at 50-fold molar excess AzBCQ at pH 5.2 with 10 min of UV irradiation. For avidin blots in (A): Lane 1, no VPL competitor; lane 2, 20-fold unlabeled VPL relative to AzBCQ; lane 3, 40-fold excess VPL; lane 4, 60-fold excess VPL; lane 5, 80-fold excess VPL; lane 6, 100-fold excess VPL. Data were curve-fit to a one-phase exponential decay with equation $y = \text{span} \cdot (e^{-Kx}) + \text{plateau}$. HB3 $R^2=0.91$, Dd2 $R^2=0.91$, and 7G8 $R^2=0.66$. 
3.3.6 Identification of the AzBCQ binding site

Since AzBCQ labels PfCRT so well, the next logical step was to define where in the protein the probe binds. As a first step, PfCRT was reacted with AzBCQ and then partially digested with trypsin (Figure 3.38), which selectively cleaves at amino acid residues C-terminal to arginine or lysine. The major initial degradation product was ~17 kDa and must be the C-terminus of the protein as it retains the polyHis tag (Figure 3.39). Further digestion did not allow for resolution of smaller labeled fragments, although a ~6.5 kDa unlabeled and non anti-his reactive fragment was identified on companion silver-stained gels (not shown).

![Avidin blot of trypsin digest of PfCRT. Lane 1, MW standards; lane 2, trypsin; lane 3, undigested PfCRT; lanes 4-9, PfCRT digested with trypsin for 2.5, 5, 10, 15, 20, and 30 min respectively.](image)

**Figure 3.38** Avidin blot of trypsin digest of PfCRT. Lane 1, MW standards; lane 2, trypsin; lane 3, undigested PfCRT; lanes 4-9, PfCRT digested with trypsin for 2.5, 5, 10, 15, 20, and 30 min respectively.
Figure 3.39 Identification of the major tryptic fragment as the C-terminus. (A) silver stain, (B) avidin blot, and (C) polyHis blot of tryptic digest of PfCRT. Lane 1, MW standards; lane 2, trypsin; lane 3, undigested PfCRT; lane 4, PfCRT digested with trypsin for 2.5 min.
The labeled protein fragment was excised from the silver stained gel (Figure 3.39A) and sent to the Georgetown Proteomics Core Facility for mass spectrometry analysis. The peaks from the resulting mass spectrum (Figure 3.40) were examined for their amino acid composition and compared to the predicted masses of the expected tryptic fragments from the terminal ~17 kDa of PfCRT (Table 3.12). A few peaks corresponded to keratin (a ubiquitous contaminant from human skin) and one to a trypsin autolytic fragment. Peptides corresponding to PfCRT amino acids 285-317 and 405-424 were also identified (Figure 3.41, italics). Additionally, one low-mass peak could represent either a tripeptide from keratin or PfCRT amino acids 372-374. Since their masses corresponded exactly to the observed MS peaks +/- 0.5 Da, these fragments cannot contain the AzBCQ tag.

<table>
<thead>
<tr>
<th>Mass (Da)</th>
<th>Amino Acid Position</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1768.9380</td>
<td>271-284</td>
<td>QLHLPYNEIWTNIK</td>
</tr>
<tr>
<td>1097.5560</td>
<td>285-294</td>
<td>NGFACLFLGR</td>
</tr>
<tr>
<td>1335.6395</td>
<td>295-307</td>
<td>NTVVENCGLGMAK</td>
</tr>
<tr>
<td>1125.4339</td>
<td>308-317</td>
<td>LCDDCDGAWK</td>
</tr>
<tr>
<td>2585.2996</td>
<td>318-339</td>
<td>TFALFSFFNIDNLITYIYIYDK</td>
</tr>
<tr>
<td>2688.3452</td>
<td>340-363</td>
<td>FSTMTTYTIVSCIQGAIAIAYYFK</td>
</tr>
<tr>
<td>876.4937</td>
<td>364-371</td>
<td>FLAGDVVR</td>
</tr>
<tr>
<td>401.2143</td>
<td>372-374</td>
<td>EPR</td>
</tr>
<tr>
<td>2137.1732</td>
<td>375-392</td>
<td>LLDFVTLFGYLFGSIYR</td>
</tr>
<tr>
<td>913.5465</td>
<td>393-400</td>
<td>VGINLIER</td>
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<tr>
<td>147.1128</td>
<td>401-401</td>
<td>K</td>
</tr>
<tr>
<td>147.1128</td>
<td>402-402</td>
<td>K</td>
</tr>
<tr>
<td>306.1594</td>
<td>403-404</td>
<td>MR</td>
</tr>
<tr>
<td>2235.9735</td>
<td>405-424</td>
<td>NEENEDSEGELTNVDSITQ</td>
</tr>
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</table>

Table 3.12 Predicted tryptic fragments of the C-terminal 17 kDa of PfCRT.
Figure 3.40 Mass spectrum of PfCRT tryptic fragment.
Upon UV activation, the AzBCQ molecule loses N\textsubscript{2}, resulting in an adduct with MW 766.25 Da. Subtraction of this value from the masses of all peaks displayed in the peptide m/z spectrum yielded only one peak that corresponds to a predicted tryptic fragment residing with the C-terminal region of HB3 PfCRT. That peak has m/z of 2024.64, which is within 0.30 Da of that expected for the peptide defined by residues 364 – 374 (Figure 3.41, bold). The AzBCQ must therefore be attached to one of these amino acids. Moreover, the last three amino acids of this segment are those that may correspond to the low-mass unlabeled fragment mentioned earlier, thereby further narrowing the possible positions for the label. These residues are believed to comprise the intra-digestive vacuolar loop connecting putative helices 9 and 10 (Figure 3.41, underlined) of PfCRT protein, and contained within this sequence is position 371, which is known to be mutated in some CQR strains (e.g., Dd2 R371I).

Figure 3.41 C-terminal sequence of PfCRT containing the AzBCQ binding site. Shown are amino acids 271-424, corresponding to the last 18.6 kDa of the protein. Unlabeled peptide fragments identified by MS are in italics, predicted transmembrane domains are underlined, and the AzBCQ-bound fragment is in bold.
3.3.7 Preliminary photolabeling of PfMDR1

Initial experiments have indicated that AzBCQ also binds to PfMDR1. Half maximal labeling of the TM isoform occurs at 58.8 µM AzBCQ, and CQ inhibits half of the labeling at a probe/competitor ratio of 41.68 (Figure 3.42). This is roughly twice the ratio needed to compete away half of HB3 and Dd2 PfCRT labeling, which indicates a greater affinity of the probe for PfCRT than PfMDR1. Labeling is also competed by QN (with half the binding lost at 45.15-fold excess competitor) but is not affected by VPL (Figure 3.43).
Figure B: Gel electrophoresis showing biotin/his densitometry.

Figure C: Graph of biotin/his densitometry against [AzBCQ] uM.
Figure 3.42  AzBCQ labeling of PfMDR1.  (A) avidin and (B) polyHis blots of TM PfMDR1 labeled with increasing AzBCQ (lanes 2-7) and competition of the labeling with CQ (lanes 8-13).  Lane 1, MW standards; lanes 2-7, 20, 40, 60, 80, and 100 µM AzBCQ respectively; lanes 8-13, 0, 16, 32, 48, 64, and 80-fold unlabeled CQ relative to AzBCQ; lane 14, PfMDR1-His-BAD PL.  Note that the small difference in MW between PfMDR1-His-BAD and PfMDR1-His is not detectable (unlike that for PfCRT) due to the relatively large size of the protein and the resolving power of the gels.  (C) normalized densitometry of PfMDR1 photolabeling as a function of [AzBCQ] from lanes 2-7 in A and B.  Nonlinear regression analysis fit the data points to a hyperbolic curve of the equation $y = (B_{max} \times x)/(K_d + x)$ with $R^2$ of 0.90.  (D) normalized densitometry of competition of AzBCQ photolabeling by CQ from lanes 8-13 in A and B.  Data were curve-fit to a one-phase exponential decay with equation $y = span \times (e^{-ks} + plateau)$ and had an $R^2$ of 0.94.
Figure 3.43  Competition of AzBCQ labeling of PfMDR1. TM PfMDR1 was labeled with 50 μM AZBCQ at pH 5.2 and 10 min UV illumination. Lanes 1-6: 0, 16, 32, 48, 64, and 80-fold unlabeled QN or VPL relative to AzBCQ, respectively. For QN, nonlinear regression fit the data to an equation for one-phase exponential decay with equation $y = \text{span} \times (e^{-kx}) + \text{plateau}$ ($R^2$ of 0.89) and for VPL, the data were fit to a straight line with equation $y = mx + b$ ($R^2$ of 0.03).
CHAPTER 4: DISCUSSION

4.1 Functional analysis of PfMDR1

[Reproduced in part with permission from Amoah LE, Lekostaj JK, and Roepe PD. Heterologous expression and ATPase activity of mutant versus wild type PfMDR1 protein. 46(20): 6060-73. Copyright 2007 American Chemical Society] [182]

[Reproduced in part with permission from Lekostaj JK, Amoah LE, and Roepe PD. A single S1034C mutation confers altered drug sensitivity to PfMDR1 ATPase activity that is characteristic of the 7G8 isoform. 157(1): 107-11. Copyright 2008 Elsevier] [183]

4.1.1 ATPase activity of HB3, Dd2, and 7G8 PfMDR1

Although a range of characteristics can be noted in the literature for ABC proteins, in general, PfMDR1 ATPase activity is similar to other 12-helix ABCB transporters. However, at high [ATP] the ATPase activity of PfMDR1 is particularly robust relative to human or mouse P-glycoproteins, yeast PDR5, and other ABC-Bs involved in drug resistance phenomena, and is instead more similar to basal activity previously measured for prokaryotic ABC transporters. Using densitometry to rigorously quantify PfMDR1 in the yeast PM, apparent $K_m$ and $V_{\text{max}}$ for 3D7 PfMDR1 were calculated to be 2.14 mM and 62.9 µmol Pi released / mg PfMDR1 / min, respectively (at pH 7.5 and 10 mM Mg$^{++}$). Other eukaryotic 12-helix ABC transporters have been reported to exhibit $K_m$ for basal ATPase activity that range from 0.5 – 2.5 mM, and $V_{\text{max}}$ of 0.5 – 5 µmol / mg / min. However, relatively few eukaryotic ABC $V_{\text{max}}$ have previously been quantified using integral native membrane preparations, and are instead more often calculated using purified detergent-extracted enzyme that could conceivably
have lost some level of activity. Also, unlike PfMDR1, other ABC proteins exhibit up to 20-fold stimulation in the presence of various drugs. Thus, basal ATPase activity of heterologously expressed PfMDR1 is commensurate with the higher end of previously measured optimized \((i.e.,\) drug-stimulated) activity for various ABC transporters.

Definition of the molecular mechanism behind any ABC-B transporter-mediated drug resistance phenomenon remains elusive. In the particular case of PfMDR1 and antimalarial drug resistance, there is some disagreement in the relevant literature. First, any antimalarial resistance mediated \textit{in vivo} by mutated or alternately expressed PfMDR1 appears to be dependent upon the simultaneous expression of CQR-conferring mutant PfCRT [81]. That is, mutation and/or increased expression of PfMDR1 likely does not promote appreciable drug resistance in and of itself, as shown recently for strain GC03 parasites that express wildtype PfCRT [116]. The nature of this PfCRT-PfMDR1 interaction \((i.e.,\) whether it is direct or indirect) is not known, but obviously merits additional study using heterologous expression and purification methods. Second, the relative importance of PfMDR1 overexpression vs. mutation (as well as overexpression of wildtype vs. overexpression of mutant PfMDR1 isoforms) in contributing to antimalarial drug resistance is currently a topic of debate. Notwithstanding these two complexities, the data in this thesis do help to further define an emerging model for the role of PfMDR1 in antimalarial drug resistance.

From prokaryotes to humans, there are multiple examples wherein overexpression or mutation of 6-helix or 12-helix ABC proteins contribute to pleiotropic resistance phenomena (resistance to multiple classes of antibiotics, heavy metals, antiparasitic
drugs, or anticancer drugs depending on the host organism). In reviewing all of this, it is difficult and perhaps dangerous to attempt to draw too many general conclusions. However, there are important comparisons that are relevant for interpreting these data for PfMDR1. PfMDR1 is a 12 helix, 2 NBD member of the ABC-B subfamily that is believed to confer resistance to hydrophobic weakly basic compounds. These drugs are in some respects structurally reminiscent of cancer chemotherapeutics, to which overexpression of the human ABC-B protein HuMDR1 confers resistance. A similar parallel could be drawn using the yeast ABC-B protein PDR5 which is known to be similar to HuMDR1 in many respects [190]. Reports of how HuMDR1 or PDR5 ATPase activities are altered by the drugs to which these proteins confer resistance vary (e.g., [133, 162, 191-196]), but with a few exceptions they are stimulatory and typically at least 2-3 fold, occasionally even as high as 10-20 fold. Notably, PfMDR1 differs from these related resistance conferring ABC-B proteins in that the drug to which it is currently believed to most influence the parasite’s response (MQ) stimulates ATPase activity by at most only 30-50%. Also, again in contrast to HuMDR1 and PDR5, the chemoreversal agent VPL does not further stimulate within the relevant concentration range. This suggests that compared to the relationship between HuMDR1 or PDR5 and the drugs to which they confer resistance, MQ interacts more weakly with PfMDR1, and VPL may interact in a different fashion altogether. This would be consistent with PfMDR1 playing only a small role in conferring resistance to MQ and other quinoline-based antimalarials, as has been recently suggested by several laboratories based on QTL analysis or transfection results [92, 116-117]. ATPase stimulation is somewhat greater at very high
levels of QN, but even in this case is below what has typically been measured for HuMDR1 or PDR5. Overall then, drug stimulation of PfMDR1 ATPase activity is unlike that for any previously characterized drug-resistance associated ABC-B protein, and is instead curiously much more consistent with recent results for Cdr1p, a member of the architecturally distinct ABC-G sub-family that is involved in antifungal drug resistance [197].

The PfMDR1 ATPase profile in response to CQ, differences in this profile between 3D7 and Dd2 isoforms, and the effects of VPL on these profiles are intriguing. The “biphasic” (mild stimulatory following by strongly inhibitory) profile observed across the wide range of [CQ] inspected is unusual but is again somewhat reminiscent of HuMDR1, which has been observed to exhibit biphasic profiles for several compounds to which it mediates resistance [192]. But since the prevailing hypothesis is that PfMDR1 does not mediate resistance to CQ, these data are surprising. The nearly complete inhibition of PfMDR1 by high “DV” concentrations of CQ could be quite important for interpreting complex patterns of active or passive drug transport proposed to be linked to the CQR phenotype [198-199].

In the case of HuMDR1, VPL typically further stimulates ATPase activity when combined with stimulatory weak base chemotherapeutics. In the case of PfMDR1, 2 µM VPL reverses both the mild stimulatory effect seen at 200 nm MQ as well as the drastic CQ inhibition caused by mM concentrations. These effects could be due in part to the fact that unlike HuMDR1, PfMDR1 does not appear to be simulated by VPL alone to any appreciable extent. A weak interaction with VPL is consistent with the recent
conclusions of Cooper et al. [169] that link VPL chemoreversal of quinoline drug resistance in *P. falciparum* to PfCRT mutations, and not to mutation or altered expression of PfMDR1. Conversely, Kirk and colleagues [174] have recently suggested that PfMDR1 mutations influence the intrinsic antiplasmodial activity of VPL (which is toxic to *P. falciparum* above 2 µM).

Although the effects are mild, there are differences in how the three PfCRT variants examined respond to VPL. ATPase activity of the 7G8 isoform is not affected by VPL alone and data in [174] suggest mutations found in the 7G8 isoform confer increased sensitivity to VPL. Thus, stimulation of PfMDR1 ATPase by toxic levels of VPL may be linked to decreased sensitivity to VPL. This might be consistent with 3D7 and Dd2 PfMDR1 acting to concentrate VPL within the DV, away from targets elsewhere in the cell. How the other ATPase effects measured correspond to patterns of drug resistance believed to be partially mediated by PfMDR1 in vivo is less clear. As mentioned, for *P. falciparum*, low levels of resistance that may be mediated by PfMDR1 require the presence of mutated PfCRT, and the relative importance of PfMDR1 overexpression vs. mutation in contributing to this resistance is currently a topic of debate. Recent field studies [108] suggest that for isolates harboring CQR-conferring *pfcrt* mutations, gene duplication of 3D7 *pfmdr1* is associated with increased resistance to MQ and QN but does not further influence the level of CQR. *In vitro*, some increased resistance to MQ and QN was seen for strains expressing the N86Y (Dd2) isoform [108], but no *in vivo* correlation was observed. Relatedly, recent gene disruption studies using a strain exhibiting *pfmdr1* gene duplication presumably due to CQ selection [112] have
shown the converse \textit{(i.e.,} that decreased expression of PfMDR1 confers increased susceptibility to MQ and QN).

In light of these observations, interestingly, the basal ATPase activity we measure is conspicuously higher for Dd2 and 3D7 isoforms than 7G8, with Dd2 being the highest. Although allele-specific overexpression trends have not been examined in any detail, based on this result it could be predicted that selection for overexpression of 7G8 PfMDR1 would be less common than overexpression of 3D7 or Dd2 isoforms in drug resistant \textit{P. falciparum}. Furthermore, the data suggest that if the net level of PfMDR1 ATPase activity is the relevant factor in conferring resistance, overexpression of Dd2 PfMDR1 under CQ selection pressure would be more “efficient” than overexpression of 3D7. Meaning, in the presence of CQS IC\textsubscript{50} levels of CQ (the clinically relevant level of CQ selection pressure) 2 copies of Dd2 PfMDR1 would provide similar increased PfMDR1 ATPase activity relative to 4 copies of 3D7 (whereas nearly 10 copies of 7G8 would be required to obtain the same level of ATPase activity). Therefore, overexpression of 7G8 or 7G8-like variants yet to be discovered will be probably be less well correlated with drug resistance (relative to 3D7 and Dd2), since reasonable increases in copy number will not impart similar levels of drug-stimulated PfMDR1 ATPase activity.

Interestingly, Dd2 CQR parasites have elevated cytosolic pH \cite{184} relative to 3D7, which according to the data presented earlier (Figure 3.18D) might act to further stimulate PfMDR1 in this strain. That is, the difference in ATPase activities for 3D7 and Dd2 PfMDR1 may be even greater in their respective native environments than when
both are examined under identical \textit{in vitro} conditions. In further prediction of relative effects of these PfMDR1 isoforms, precise quantification of cytosolic [ATP] and pH for the malarial parasite will be crucial. Only a handful of measurements have been reported, but available data [200] suggest that due to anaerobic metabolism, ATP concentrations are lower than for other eukaryotes, perhaps as low as 1-2 mM.

A lingering mystery has always been that since ABC-B proteins act to translocate drug in some fashion from the NBD-disposed side of the membrane (cytosolic for PfMDR1) to the opposite (DV interior), and since the DV is believed to be the primary site of action for quinoline-based antimalarials, how does PfMDR1 concentrating more drug within the DV lead to resistance? That is, in all other examples of ABC-mediated drug resistance, the proteins function to lower accumulation of drug on the NBD side of the membrane (\textit{e.g.}, the cytosol for a drug resistant tumor cell) because the NBD side is disposed towards the drug target (\textit{e.g.}, cytosolic tubulins in the case of HuMDR1-mediated resistance to vinca alkaloids). Alternatively, resistance-conferring ABC proteins expressed in vacuolar membranes (again with NBD disposed to the cytosol) act to concentrate drug into the vacuole in order to again sequester drug away from the drug target (which is cytosolically or nuclearly localized).

In the case of PfMDR1, the simple prediction is that the protein would act to concentrate drugs \textit{at} their site of action (inside the DV where the heme target lies), not \textit{away}. Higher levels of PfMDR1 (as in drug resistant field isolates over expressing \textit{pfmdr1}), or drug stimulation of PfMDR1 ATPase shown here would be predicted to concentrate even \textit{more} drug at the site of action, not less. If the DV is indeed where the
primary drug target (FPIX heme) resides, as is generally accepted [201], this would act to encourage drug-target interactions, which is the converse of what is typically promoted by any drug resistance mechanism.

One possible explanation involves the previous observation that quinoline drugs act to prematurely precipitate heme dimers from solution [201]. These curious nucleation phenomena, which occur at different rates as drug is varied from vastly sub-stoichiometric to high molar excess, would lower drug target availability. If pH, volume (concentration of heme), and ionic strength were manipulated in certain ways (as indeed seems to be the case for drug resistant malaria [180]), synergistic (and quinoline drug-specific) heme aggregation would occur. Alternatively, although the concept has not been as extensively explored as has the drug interaction concept, it is also true that drug resistance-conferring members of the ABC transporter family have been observed to mediate movement of ions under various conditions [e.g., 100, 202]. This possibility for PfMDR1 merits additional scrutiny, since the pH gradient across the DV membrane is very large and directly or indirectly controls accumulation of CQ, MQ, QN and other drugs within the DV, as well as biomineralization of the heme drug target. Even subtle effects on this parameter and others closely linked to it (e.g., DV volume and other ion activities, see [180]), which via this model could then be influenced by drug effects on PfMDR1 ATPase activity, would contribute to resistance in interesting and drug specific ways [201].

Along with a better understanding of the nature of quinoline-heme interactions [180, 201], many additional molecular questions remain. The perfected heterologous
expression of PfMDR1 will be extremely useful in addressing these. For example, it will be interesting to dissect out the ATPase effects observed in naturally occurring (e.g., Dd2 and 7G8) as well as artificial [116] PfMDR1 isoforms. Some of these amino acid substitutions lie within homologous regions that are believed to be involved in drug binding for other ABC-B proteins (see Section 4.1.2). Also, how PfCRT and PfMDR1 might interact to further modify either these PfMDR1 properties, or PfCRT properties will be important to examine.

In conclusion, the data showing higher basal ATPase and shifted pH optimum for the Dd2 PfMDR1 variant, mild stimulation of PfMDR1 ATPase by physiologically relevant doses of MQ and QN, and VPL reversal (not simulation) of these effects, are in general consistent with the evolving picture of PfMDR1 as contributing toward clinically relevant drug resistance [108, 116-117]. Assuming these drug effects on PfMDR1 are germane to the resistance mechanism, overexpression of 3D7 PfMDR1 will confer many of the same effects, regardless of mutation, as Dd2 or 7G8 isoforms, and in some cases, higher levels of 3D7 would be predicted to be more effective than increased expression of some CQR-associated isoforms (i.e., 7G8).

4.1.2 ATPase activity of C-terminal mutants

Some parallels can be drawn between the PfMDR1 mutation sites and corresponding positions in the huMDR1 sequence. Topologically (see Figure 1.12), the N86Y mutation (which defines the Dd2 variant) is found in the DV-disposed loop between the first and second predicted TM domains. Similar to the ATPase results seen
for Dd2, deletions in this region of HuMDR1 lead to altered verapamil sensitivity [203]. The 1034 and 1042 mutations lie in the middle of predicted TM helix 11, which has been hypothesized to be part of a drug-binding site in HuMDR1 [44-46]. TM11 has also been implicated in the release of drug during ATP hydrolysis [204]. Thus, the finding that the S1034C substitution has the most significant effect on PfMDR1 ATPase drug stimulation is consistent with previous drug interaction domain analyses for other ABC proteins. The D1246Y mutation is situated within the C-terminal NBD. While there are no direct comparisons to HuMDR1 currently in the literature, it seems likely that any mutation within the NBD is liable to affect ATP binding or hydrolysis. How this domain then interacts with others predicted to interact with drug (e.g., TM 11) to further modify $V_{\text{max}}$ and $K_m$ remains to be determined, but ongoing crystallographic analyses of other ABC proteins (e.g., [205]) will be helpful in this regard.

Since mutations in PfCRT originated in at least four independent foci worldwide [85], PfMDR1 mutations may have arisen in similar fashion, against a backdrop of selective drug pressure and fitness adaptation in response to different PfCRT mutations. In evolutionary terms, a number of scenarios are possible since it is unclear which quinoline drugs (if any) selected for Dd2 and 7G8 PfMDR1. But based on inspection of $V_{\text{max}}$, $K_m$, and drug stimulation of ATPase for all the variants tested, and assuming quinoline exposure was the ultimate driving force for PfMDR1 mutation, it may be speculated that the 7G8 strain first acquired the S1034C mutation in order to bias against drug effects on the enzyme, followed by the other substitutions, which merely act to “fine tune” $V_{\text{max}}$ and $K_m$. Regardless the order, the final consequence is an enzyme that has
reduced catalytic efficiency and that is insensitive to quinoline-based antimalarial drugs, both of which would seem to be advantageous based on conventional drug transport models for MDR proteins and the DV localization of both PfMDR1 and the quinoline target (see section 4.1.1). That is, both features would presumably act synergistically to lower quinoline drug accumulation into the DV via an ABC transporter with cytosolicly disposed NBD.

However, in a comparison of the behavior of Dd2, 7G8 (both CQR strains) and 3D7 (CQS) PfMDR1 isoforms, the conclusion can be drawn that the level of basal ATPase activity is not necessarily relevant for quinoline drug resistance. Dd2 has higher $V_{\text{max}}$ relative to 3D7, whereas 7G8 has lower. The degree of QN and MQ stimulation could be related to resistance to these specific compounds, since although the differences between Dd2 and 3D7 isoforms are quite small, 3D7 PfMDR1 showed the greatest proportional drug stimulation of ATPase activity. Interestingly, strain 7G8 exhibits VPL-insensitive CQR, whereas the strain Dd2 CQR phenotype is VPL-reversible. Perhaps correspondingly, Dd2 PfMDR1 ATPase is inhibited by VPL, but the 7G8 isoform is not. Interestingly, the S1034C mutation is responsible for this loss of VPL sensitivity along with reduced catalytic efficiency. However, most importantly, these results indicate no simple pattern among quinoline drug effects on ATPase activity for the PfMDR1 variants found in these strains and the level or pattern of quinoline drug resistance exhibited by the strain. Also, PfMDR1 function is clearly quite different from that of its close homologue HuMDR1, since VPL has the strongest effects of any compound on HuMDR1 ATPase [206], whereas effects are minor to nonexistent for PfMDR1 isoforms.
associated with resistance. Taken together, these data indicate that the relative contribution of PfMDR1 to drug resistance is likely different for various CQR strains (e.g., Dd2 vs. 7G8).

The PfMDR1 isoforms that are currently found in CQR isolates may illustrate symbiotic relationships between mutant PfCRT and mutant PfMDR1 that confer either preferred resistance patterns or fitness adaptations, or perhaps both. Perhaps the different \( V_{\text{max}} \) and \( K_m \) for Dd2 and 7G8 PfMDR1 isoforms, along with overexpression levels (gene copy number), reflect fitness adaptations relevant to distinct Dd2 and 7G8 PfCRT mutations, whereas decreased quinoline drug stimulation of PfMDR1 ATPase activity reflects selection to further subtly modify quinoline drug resistance conferred by PfCRT.

Experiments with purified membranes allow precise and accurate quantification of PfMDR1 ATPase activity and other molecular characteristics (e.g., perhaps binding of some drugs). However, obviously only transfection with PfMDR1 alleles into various parasite strains can provide precise quantification of their minor role in modulating drug resistance profiles. Put together, the two approaches are synergistic; transfection of some of the mutants constructed in the present work may offer one convenient way in which to test some concepts. Also, although specific PfMDR1 codons are often sequenced for CQR isolates, more complete sequencing of PfMDR1 alleles as well as additional quantification of the frequency of 7G8 and Dd2 allele overexpression in CQS and CQR isolates [108] will eventually be required to distinguish between current models generated by \textit{in vitro} work.
4.2 CQ binding to PfCRT


4.2.1 Photolabeling PfCRT with a CQ analogue and definition of its binding site

Photolabeling of transporters, channels or receptors with azido substrate analogues is a well known approach for defining general features of binding as well as mapping of binding site domains. Perfluoroazido probes have not been used as extensively as other azido moieties but are nonetheless valuable in a number of settings (e.g., [207-208]). Only a handful of investigators have engineered non-radioactive, biotinylated, azido probes for labeling studies (e.g., [209-210]), and none of these have incorporated use of the more convenient perfluoroazidophenyl group, which upon UV illumination efficiently inserts at C-H [163].

In this thesis, a perfluoroazido, biotinylated chloroquine analogue was utilized to analyze the reactivity of that probe with both endogenous and recombinant, partially purified PfCRT protein. The pH dependency of photolabeling was quantified for three different PfCRT isoforms (CQS, CQR conferring/VPL sensitive [Dd2], and CQR conferring/VPL insensitive [7G8]), as was how “cold” CQ, QN, ART, and VPL compete for the AzBCQ photolabeling under fixed “early plateau” conditions. It was determined that there is a single covalent attachment site for AzBCQ within the C-terminal tail region of PfCRT which maps the AzBCQ binding site for HB3 PfCRT to 8 – 11 residues that define the predicted loop between putative helices 9 and 10 of the PfCRT protein.
Previously, Tilley and colleagues [172] used an $^{125}$I-labeled azidosalicylate CQ analogue in an attempt to identify CQ binding proteins in *P. falciparum*. Aside from using azido phenyl instead of perfluorophenyl azido, the probe design also required loss of the chlorine atom at position 7 of the CQ pharmacophore. Other work has shown that the Cl atom is crucial for activity of 4-amino quinoline drugs [211], so some differences in results between the two probes are to be expected. Also, the photo-activatable group used in [172] was attached to the quinoline ring system (not to the end of the CQ side chain via a flexible linker as in AzBCQ). This bulky addition to the quinoline ring system pharmacophore would be predicted to negatively affect binding of the pharmacophore to PfCRT.

It is tempting to interpret photolabeling efficiencies for the different PfCRT isoforms in terms of binding affinities (meaning, more efficient photolabeling indicates more efficient binding). However, this is overly simplistic since environmental effects that are unrelated to probe affinity can significantly influence net efficiency of the photolabeling reaction. Nonetheless, quantifying competition of the photolabeling reaction under conditions wherein labeling is just achieving saturation likely yields relative affinities for a competitor, assuming binding sites for probe and competitor overlap. Based on calculated stoichiometries and the similar, extremely efficient competition of cold CQ for the AzBCQ reaction for all three protein variants, it seems reasonable to suggest that a single AzBCQ and a single CQ binding site overlap considerably for PfCRT. Furthermore, competition by CQ at low-fold molar excess
suggests that AzBCQ photolabeling is mimicking physiologically relevant binding of CQ to the PfCRT isoforms.

A shared single-site model is also supported by other indirect evidence: 1) Most dramatically, a single attachment site for the probe is mapped to residues 364 – 374 and is likely to lie within the segment F_{364} – R_{371}. This loop is predicted by multiple algorithms [81] to reside within the DV environment, which is known to have an acidic pH between 5.2 and 5.6, depending on whether the parasite is CQS or CQR [104]. 2) Importantly then, binding and/or subsequent photolabeling of AzBCQ is strongly activated by acidic pH. 3) Earlier, initial equilibrium binding studies with ^3H-CQ [103] calculated $K_d$ for PfCRT that corresponded to concentrations of CQ that are predicted for the acidic DV, but that are far higher than concentrations predicted for the cytosol. Taking these three observations together, one obvious conclusion is that AzBCQ binding is indeed mimicking CQ binding, at an acidic, DV-disposed binding site that is in part defined by the helix 9-helix 10 domain.

The loop connecting helices 9 and 10, to which AzBCQ is found covalently attached, is predicted by multiple algorithms to lie within the DV interior [81]. Again perhaps not coincidentally, residue 371 lying within this loop is mutated in a number of well-known CQR *P. falciparum* strains and isolates, including Dd2, W2, FCB, 2300 (Indonesia) and 742 (Cambodia). Poorly understood patterns of mutations that include other nearby residues 356 (helix 9), 326 (helix 8) and 271 (loop 7) are also found in most known CQR strains and isolates. Based on predicted models for PfCRT function [81,
it is logical to assume that mutations in PfCRT associated with CQR might cluster within or near a CQ binding site.

Another line of evidence that strongly supports the notion that AzBCQ photolabeling is revealing physiologically meaningful (“CQ-like”) drug binding comes from the recent work of Cooper *et al* [170]. In these studies, “second site suppressor” mutations were found when pressuring Sudan 106/1-derived CQR clones harboring K76I mutations with QN. These clones had been unusually sensitive to QN, but upon selection with the drug became QNR and concomitantly reacquired sensitivity to CQ. The suppressor site was found to be Q352; introduction of a positive charge (either K or R) at this residue conferred the unusual QNR/CQS phenotype to these K76I clones. The authors interpreted these results to suggest that putative helix 9 (within which Q352 is predicted to lie) participates with helix 1 (site of the well-known K76T, K76I or K76N mutations that confer CQR [81, 170]) to form a quinoline drug binding pocket. The results with AzBCQ strongly support this hypothesis. Furthermore, by finding that the single attachment site for AzBCQ likely lies within residues F364 – R371, and knowing the geometry and size of the AzBCQ molecule, it is suggested that helix 10 must also be proximal to this binding pocket (Figure 4.1).

Other drug selection results suggest some possible interaction with helix 4 [170], but it is difficult to construct a viable model that places helices 1, 9, 10, loop 9 (between helices 9 & 10) and helix 4 together to form one pocket such that PfCRT residues found to be mutated in CQR are proximal to the CQ pharmacophore within AzBCQ. It may be possible that multiple PfCRT conformations exist, that this leads to somewhat different
drug binding domains, and that CQ (or “CQ-like”, i.e., AzBCQ) binding biases towards one involving helices 1, 9 and 10. Additional studies with AzBCQ and similar probes with altered geometries may be able to test this idea (see Section 4.3.3).

**Figure 4.1** Ribbon diagram of proposed PfCRT drug binding pocket. The CQ pharmacophore is predicted to lie within the lower third of the membrane, while the bulky biotin and perfluorophenyl azido groups stretch into the DV, with the latter making contact with (and inserting into) the interhelix loop of amino acids. Asterisks mark sections of helices that contain mutations associated with CQR.

It is notable that the requirement for higher amounts of VPL (relative to CQ) to compete labeling is consistent with the need for relatively high VPL concentrations (1-2 µM) for chemoreversal in culture. The fact that AzBCQ binding to Dd2 PfCRT (found in VPL-reversible CQR strains) is efficiently reversed by similar levels of VPL but 7G8 PfCRT (found in VPL-insensitive CQR strains) is not is again very strong evidence that
the binding site revealed by AzBCQ photolabeling is physiologically relevant. Also, it is very interesting that VPL efficiently competes for AzBCQ labeling of HB3 PfCRT. It seems reasonable to suggest that mutations which distinguish the Dd2 from the HB3 protein variant may not be relevant for VPL binding but that mutations that distinguish the 7G8 isoform from both the HB3 and Dd2 isoforms (72S, 326D and 356L) destroy the VPL binding site. Perhaps not coincidentally, two of these reside near the mapped AzBCQ attachment site, and all three lie within or near the proposed CQ binding pocket.

Photolabelling efficiencies are a product of probe binding as well as the chemical environment within which the photochemistry is generated. The identified attachment site is within a loop disposed outside of the membrane, such that it can be reasonably supposed that the chemical environment nearby the reactive perfluorophenyl azido group is similar for all three isoforms. Thus, labeling differences may reflect different binding affinities of the pharmacophore, and these will be the product of on- vs. off-rates of the probe. This then leads to the speculatation that pH either increases the CQ off-rate or decreases the CQ on-rate for Dd2 and 7G8 PfCRT variants compared to the HB3 isoform. Such pH-dependent substrate interaction is indirect evidence for PfCRT acting as a pH-dependent facilitative CQ transporter (perhaps a channel specific to CQ^{2+}), as has been proposed [102-103].

4.3 Future directions

The results described in this thesis have provided the basis for a number of projects to be continued by other members of the Roepe laboratory.
4.3.1 Heterologous expression

Since the Roepe laboratory has now successfully heterologously expressed two *P. falciparum* polytopic integral membrane proteins using yeast-optimization procedures [131, 182], it might be worth considering applying the same methodology to the PfNHE, which has been linked to one pathway of QN resistance (see Section 1.4.5). Unlike PfCRT, the endogenous function of the protein is known and there is a simple test for activity of the protein. These constructs could be transformed into yeast strains deficient in endogenous sodium/proton exchange to test for complementation. Transformed strains could then be assayed for their ability to modulate cellular accumulation of antimalarial drugs, particularly QN [92-95].

4.3.2 ATPase assays

While the ATPase activity of an extensive number of PfMDR1 variants has been achieved, the possibilities for subtly changing experimental parameters are endless. Even though the first studies of HuMDR1 ATPase activity were conducted over 20 years ago, similar investigations continue to this day (e.g., [212-215]), persistently testing more compounds that may prove to be a substrate for the protein. The data presented here looked at the effects of three quinoline antimalarials on PfMDR1, so there are still several drugs that may merit investigation, such as HF or ART. However, since all the drug-induced effects on PfMDR1 ATPase activity were less than 2-fold, perhaps time and resources would be better spent on different assays that may provide superior insight into
the behavior of the protein. For example, fluorescent or radioactive drug accumulation and/or efflux could be measured in yeast vesicles.

### 4.3.3 Photolabeling

Another obvious first step in the continuing work with PfMDR1 would be the detailed examination of AzBCQ photolabeling of the protein. The major unexpected result of the ATPase studies was the dramatic inhibition of activity at DV-compatible concentrations of CQ, which indicates that (against expectations) this drug probably does interact with PfMDR1. Photolabeling and subsequent mapping of the substrate binding site may help interpret previous results. Additionally, plans are underway to synthesize AzB- versions of QN and MQ. Data obtained with these compounds might distinguish whether PfMDR1 contains a single or multiple drug binding sites.

Further photolabeling studies of PfCRT must also continue. A number of isoforms of this protein differ in their ability to confer resistance to various compounds and/or to be reversed in this ability by non-antimalarial drugs. For example, similar to the interesting example set by Dd2 and 7G8 PfCRT and VPL, a set of experiments can be done with the PfCRT variants identified by the CQ/QN second site suppressor mutants (see Section 1.7.3) that, based on resistance patterns, may interact very differently with CQ vs. QN or QN vs. QD.

It is very well known in the antimalarial drug resistance community that CQ analogues with either shorter (number of carbons < 4) or longer (n > 4) side chains are more active in CQR strains with mutated PfCRT, relative to CQ (n = 4) [216]. For CQS
strains, the activity of all compounds is the same, regardless of the side chain length. The general supposition is that some CQR-associated PfCRT isoforms recognize CQ better than shorter or longer chain CQ analogues. This theory can be tested with quantitative photolabeling experiments using AzB-CQs with different side chain lengths, coupled with competition with “cold” short and long chain CQs (non-AzB analogues that are otherwise identical).

Another project of great potential importance would be more exact definition of the AzBCQ binding site. The model in this thesis proposes that the PfCRT drug binding pocket is formed by the lower (toward the DV) third of helices 1, 9, and 10 such that the azide moiety protruding from the CQ pharmacophore inserts into the loop between TMs 9 & 10. This could be probed further in several ways. The amino acids comprising the loop region could be mutated to show that their identity does not affect binding of the probe. The residues within the helices could also be mutated, with the expectation that key residues within drug recognition site would perturb the photolabeling. Finally, a series of photolabels could be synthesized similar to the short and long chain analogues described above, but varying the length of the biotin and perfluoroazide linkers instead of the CQ side chain. By systematically moving the photoreactive group an increasing number of carbon bonds away from a given position on the pharmacophore, a 3-dimensional picture of the binding pocket (which may be discontinuous with respect to the primary sequence) can be obtained.
## APPENDIX A

Oligos to create N-Terminal *pfmdr1*

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155
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156
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