Characterization of Plasmodium falciparum resistance to novel drugs: A study of PfCRT, PfMDR1 and PfABCG mediated drug resistance

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I dedicate this thesis in memory to my beloved father Mahoulé Edayé who taught me how to persevere to achieve my goal, to my mother Espérance Fortunato, to my husband Thérence Houngbadji and my sweetheart daughter Sèdali Thonia who both patiently endure the completion of this thesis.

"The most certain way to succeed is trying one more time" – Thomas Edison

ABSTRACT

Plasmodium falciparum is the deadly protozoan parasite responsible for malaria. Malaria is one of the most important infectious diseases that has been raging for millennia and affecting almost half of the world's population. The treatment regimen that was based on quinoline drugs such as chloroquine (CQ), was efficient for decades. Nowadays, the use of this class of drugs is doomed to failure due to the emergence of quinoline-resistant parasites. Today, artemisinin-based combination therapies (ACTs) are the first-line drugs for uncomplicated falciparum malaria treatment. ACTs improve the cure rate of malaria and thus are seen as efficient treatment against uncomplicated forms of the disease. Despite their efficiency, these drugs are currently facing the development of resistance. PfCRT and PfMDR1, which are membrane transporters, have been shown to be involved in malaria parasites drug resistance. To tackle the inefficiency of existing drugs in regard to the development of resistance, alternative therapies must be discovered. In this thesis, antimalarial activity of novel potential drugs against *P. falciparum* is assessed and the interaction of these drugs with PfCRT and PfMDR1 is determined. Furthermore, because many ABC transporter genes play a key role in drug resistance, the characterization of an ABC transporter member of the ABCG family in Plasmodium is addressed and its role in drug resistance investigated.

In the first part of this thesis, MK571 (a quinoline analogue) activity against *P. falciparum* parasites is investigated. MK571 is found to be more toxic to most of the CQ-resistant strains than to the CQ-sensitive strains. In addition, we determine that MK571 is not a substrate of PfCRT as are other quinoline drugs, but is instead a substrate of PfMDR1. Therefore, it can be a good complement to existing quinoline drugs in the treatment of uncomplicated malaria.

In the second part, novel compound analogues of chloroquine are tested for their antimalarial activity against CQ-sensitive and -resistant parasites. Although chloroquine analogues tested possess the quinoline ring structure of chloroquine, they are less efficient than chloroquine and are not substrates of PfCRT. One of the analogues (3-ICQ) reverses the resistance of CQ-

resistant strains to chloroquine and therefore, could be used in combination with chloroquine in cases of CQ-resistant malaria.

In the third part of the thesis we conduct the characterization of PfABCG, the sole member of the *P. falciparum* ABCG family. The characterization study demonstrates that PfABCG is localized on the parasite plasma membrane and is expressed throughout the asexual life cycle of the parasite. In addition, PfABCG is differentially expressed in various Plasmodium strains. This expression does not correlate with the resistance to chloroquine but to the sensitivity of the parasite to an antihistaminic drug named ketotifen. Overall, this thesis sheds light on challenges and understanding of the complex resistance machinery deployed by the *P. falciparum* parasite from novel drug discovery to characterization of proteins.

ABRÉGÉ

Plasmodium falciparum est le parasite protozoaire mortel responsable du paludisme. Le paludisme est l'une des maladies infectieuses les plus importantes qui a fait rage pendant des millénaires et affecte près de la moitié de la population mondiale. Le régime de traitement qui était basé sur les médicaments à base de quinoléine tels que la chloroquine (CQ) a été efficace pendant des décennies. De nos jours, l'utilisation de cette classe de médicaments est vouée à l'échec en raison de l'émergence de parasites résistants aux médicaments à base de quinoléine. Aujourd'hui, les thérapies combinées à base d'artémisinine (ACTs) sont les médicaments de première intention pour le traitement du paludisme simple à *P. falciparum*. Les ACTs ont amélioré le taux de guérison du paludisme et sont donc considérés comme un traitement efficace contre les formes non compliquées de la maladie. Malgré leur efficacité, ces médicaments sont actuellement confrontés à l'apparition d'une résistance. Les protéines PfCRT et PfMDR1, qui sont des transporteurs membranaires, ont été montrés comme étant impliqué dans la résistance des parasites du paludisme aux médicaments. Pour s'attaquer à l'inefficacité des médicaments existants en ce qui concerne le développement de la résistance, des thérapies alternatives doivent être découvertes. Dans cette thèse, l'activité antipaludique de nouveaux médicaments potentiellement efficace contre *P. falciparum* est évaluée et l'interaction de ces médicaments avec PfCRT et PfMDR1 est déterminée. En outre, parce que de nombreux gènes de transporteurs ABC jouent un rôle clé dans la résistance aux médicaments, la caractérisation d'un membre des transporteurs ABC de la famille ABCG chez Plasmodium est adressée et son rôle dans la résistance aux médicaments étudié.

Dans la première partie de cette thèse, l'activité de MK571 (un analogue de la quinoléine) contre *P. falciparum* est étudiée. MK571 s'est révélée plus toxique pour la plupart des souches résistantes à la CQ que pour les souches sensibles à la CQ. En outre, nous avons déterminé que MK571 n'est pas un substrat de la protéine PfCRT comme le sont les autres médicaments à base de quinoléine, mais est plutôt un substrat de la protéine PfMDR1. Par conséquent, il peut être

un bon complément thérapeutique aux médicaments à base de quinoléine, dans le traitement du paludisme simple.

Dans la deuxième partie, de nouveaux composés analogues de la chloroquine sont testés pour leur activité antipaludique contre les parasites sensibles et résistants à la CQ. Bien que les analogues de la chloroquine testés possèdent la structure cyclique de la quinoléine comme la chloroquine, ils sont moins efficaces que la chloroquine et ne sont pas des substrats de la protéine PfCRT. L'un des analogues (3-ICQ) réverse la résistance à la chloroquine des souches résistantes et donc, pourrait être utilisé en combinaison avec la chloroquine dans les cas de paludisme due aux parasites chloroquino-résistants.

Dans la troisième partie de la thèse, nous avons effectué la caractérisation de la protéine PfABCG, seul membre de la famille ABCG de *P. falciparum*. L'étude de caractérisation a démontré que la protéine PfABCG est localisée sur la membrane plasmique du parasite et est exprimée dans tout le cycle de vie asexuée du parasite. En outre, PfABCG est exprimée de manière différentielle dans les différentes souches de Plasmodium étudiées. Cette expression n'est pas en corrélation avec la résistance à la chloroquine, mais corrèle plutôt avec la sensibilité du parasite à un médicament antihistaminique nommé kétotifène. Dans l'ensemble, cette thèse met en lumière les défis et la compréhension des mécanismes de résistance complexe déployés par le parasite *P. falciparum* de la découverte de nouveaux médicaments à la caractérisation des protéines.

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CONTRIBUTION OF AUTHORS

The experimental work presented in this thesis was designed, executed and performed by the author under the supervision of Dr. Elias Georges, who was involved in experimental design, data presentation and editing of this thesis and the manuscripts included.

In the first manuscript (chapter II) the contributions of the co-authors were very important for the completion of this study. Sarah J. Reiling conducted the experiments, analyzed the data and produced the graphs in Figure 5 of the manuscript. Juliane Wunderlich performed the experiments and analyzed the data in Figure 6 of the manuscript. Mara L. Leimanis (previous student of Dr. Elias Georges) contributes in the experiments in Figure 2. Dr. Petra Rohrbach participates in the edition of the manuscript.

In the second manuscript (chapter III), Dagobert Tazoo synthesizes the analogues used in the study under the supervision of Dr. Scott Bohle in the Department of Chemistry, McGill University.

STATEMENT OF ORIGINALITY

The following aspects are considered original contributions to the knowledge of drug discovery and antimalarial drug resistance.

Manuscript I: Sonia Edayé, Sarah J. Reiling, Mara L. Leimanis, Juliane Wunderlich, Petra Rohrbach and Elias Georges. 2014. A 2-amino quinoline, 5-(3-(2-(7-Chloroquinolin-2yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid, Interacts with PfMDR1 and Inhibits its Drug Transport in *Plasmodium falciparum*. Molecular & Biochemical Parasitology, 195; 34-42.

In this manuscript, we determine the potential antimalarial activity of the quinoline analogue MK571 and its interaction with Plasmodium drug resistance transporters. The study shows that MK571 is more toxic to CQ-resistant strains and interestingly, is not a substrate for the chloroquine resistance transporter, PfCRT. We found that MK571 is rather a substrate of the wild-type and mutant type PfMDR1. As MK571 was already clinically tested and used in the treatment of asthma, we suggest that it can be used in combination therapy with existing antimalarial drugs to potentiate their activity.

Manuscript II: Sonia Edayé, Dagobert Tazoo, Scott Bohle and Elias Georges. A Novel Chloroquine Derivative, 3-Iodo-Chloroquine, is a Potent Inhibitor of PfCRT-mediated Drug Resistance in *Plasmodium falciparum*. (Manuscript in preparation).

This manuscript aims to characterize novel potential antimalarial drug analogues to chloroquine with a modification at the 3 position in the quinoline ring. The study in this manuscript demonstrates that the quinoline ring modification in chloroquine changes the specificity of these new drugs to PfCRT. In addition, one of the drugs (3-ICQ) acts as a chloroquine-reversing agent by blocking PfCRT-mediated drug efflux. This work provides a better understanding of the structure-function relationship between drugs and transporters.

Manuscript III. Sonia Edayé and Elias Georges. Expression and Subcellular Localization of the Only Member of ABCG Subfamily in *Plasmodium falciparum*. (Manuscript submitted to Molecular & Biochemical Parasitology).

In this manuscript we characterize the unique ABCG transporter in *P. falciparum*. The study shows that the protein is localized at the parasite plasma membrane. Furthermore, PfABCG is expressed at asexual stages while being differentially expressed in diverse *P. falciparum* strains. We also demonstrate that PfABCG expression correlates with parasite sensitivity to ketotifen and not to chloroquine. To our knowledge, this is the first study on the characterization of the endogenous expression of PfABCG in Plasmodium.

LIST OF ABBREVIATIONS

ABC	ATP Binding Cassette transporter
ABCG1	ATP binding cassette member G1
ABCG2	ATP Binding Cassette (ABC) transporter, member G2
ABCG2	ATP binding cassette member G2
ACT	Artemisinin based Combination Therapies
ATP	Adenosine Triphosphate
BCRP	Breast Cancer Resistance Protein
CCR4	C-C chemokine receptor type 4
cDNA	Complementary DNA
CQ	Chloroquine
CQR	Chloroquine Resistance
CQS	Chloroquine Sensitive
DAPI	4',6-diamidino-2-phenylindole
DDT	DichloroDiphenylTrichloroethane
DHA	DiHydroArtemisinin
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleotide adenosine
DV	Digestive Vacuole
EDTA	Ethylenediaminetetraacetic acid
FDA	Food Drug Administration
FeS	Iron-sulphur
FP	Ferriprotoporphyrin
GSH	Glutathione
GST	Glutathione-S- transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	Halofantrine

IC50	Inhibitory concentration for 50%
MDR1	MultiDrug Resistance 1
MK571	5-(3-(2-(7-Chloroquinolin-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid
MQ	Mefloquine
MSD	Multiple spanning domain
NBD	Nucleotide Binding Domain
PBS	Phosphate Buffer Saline
PfCRT	Plasmodium falciparum Chloroquine Resistance Transporter
PfCRT mut	PfCRT mutant
PfCRT wt	PfCRT wild-type
PfMDR1	Plasmodium falciparum MultiDrug Resistance 1
PfMRP1	Plasmodium falciparum Multidrug Resistance Protein 1
Pgh-1	Pglycoprotein-1
qPCR	Quantitative Polymerase Chain Reaction
RBC	Red Blood Cells
RNA	Ribonucleotide Adenosine
ROI	Region Of Interest
SNARF-4F	Seminaphtharhodafluor-4F
SNP	Single Nucleotide Polymorphism
SufC	Sulfur assimilation protein C
TAP	Transporter associated with Antigen Processing
TM	Transmembrane
TMD	TransMembrane Domain
UTR	Untranslated Region
Vp	Verapamil

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INTRODUCTION

Plasmodium is an obligate intracellular protozoan parasite responsible for malaria, one of the most important parasitic diseases because of its repartition worldwide. Four of the most common species that infect humans are *Plasmodium vivax* (*P. vivax*), *P. ovale*, *P. malariae* and *P. falciparum*. The latter causes the most severe form of the disease and is responsible for over 90% of deaths, mainly in Sub-Saharan Africa. In 2004, *P. knowlesi*, another species *that* generally infects monkeys, was related to many cases of malaria in Southeast Asia, namely Thailand and the Philippines [1]. Since the launching of the program Roll Back Malaria in 2000, four countries located in the Middle East were declared safe of malaria, namely, the United Arab Emirates in 2007, Morocco and Turkmenistan in 2010 and Armenia recently in 2011 [2]. However, malaria is still endemic in Latin America, Asia and for most of Sub-Saharan Africa.

Several tools are available to eliminate malaria, such as insecticides to control the vector, development of effective vaccines and the use of antimalarial drugs to eliminate the parasite. The first tool, vector control, is hindered by the development of resistance in mosquitoes to available insecticides. Many vaccines were developed, including the famous RTS, S/AS01, which is still in phase 3 clinical trials. Even though this vaccine is protective at just 50%, it is projected to save the lives of many children [3]. Lastly, the most important tool to eliminate malaria is the treatment of sick individuals with efficient antimalarial drugs. Chloroquine (a quinoline-based drug) was used to treat malaria until the emergence of resistant parasites in the early 60s [4]. It is believed that chloroquine targets the hemoglobin degradation pathway of the parasite, which occurs inside the digestive vacuole.

The popularity of chloroquine led to the introduction of many other members of the quinoline family (mefloquine, amodiaquine, halofantrine and quinine), which are also efficient antimalarials. However, they quickly became ineffective against chloroquine-resistant parasites. Today's popular drugs are artemisinins. One efficient way of using artemisinins is in combination therapy with long-lasting drugs (mefloquine, halofantrine, lumefantrine, among others) in order

to delay the appearance of drug resistance [5]. Although combination therapy is successful, it does not completely prevent resistance from emerging. In fact, the introduction of any new antimalarial drug is accompanied by the appearance of parasites resistant to that drug [6]. One of the derivatives of artemisinin, namely artesunate, already shows signs of decreased sensitivity on the Thai-Cambodian border, the cradle of antimalarial drug resistance [7]. This aggressive development of drug resistance is hampering elimination of malaria.

It is now clear that resistance to antimalarial drugs by *P. falciparum* is due to point mutations and/or overexpression of membrane-associated proteins. Extensive studies have been conducted to link the function of transporter proteins PfCRT and PfMDR1 to drug resistance. The role of PfCRT (a digestive vacuole membrane protein) in chloroquine resistance is now well established [8]. Point mutations in PfCRT are primary determinants of chloroquine resistance [9, 10]. The PfCRT mutation K76T in particular, ubiquitous in all chloroquine-resistant strains, is now used as a molecular marker for chloroquine resistance *in vivo* and *in vitro* [11]. Many studies identify that the K76T mutation in PfCRT is present together with the N86Y mutation in PfMDR1 in cases of chloroquine resistance [12, 13].

PfMDR1 is found on the parasite digestive vacuole (DV) membrane and pumps drugs inside the DV [14]. Many other point mutations in PfMDR1 have been associated with decreased susceptibility of the parasite to several antimalarial drugs. Not only point mutations but also gene amplification in the *pfmdr1* gene plays a role in plasmodium drug resistance [15, 16]. A better understanding of these mechanisms of drug resistance will help in the design of new antimalarial drugs in the future. Yet, to overcome resistance, new avenues must then be explored, such as the synthesis of new drugs and the identification of their target (s), as well as the determination of their mechanism of action.

The main objective of this thesis is to characterize drug resistance genes in *P. falciparum* and to determine their interaction with potential antimalarial drugs. Briefly, the first chapter gives an overview of the current knowledge of malaria drug resistance as well as that of different drug-resistance genes. The mechanism of action of the quinoline-based drugs and the description of

ABC transporters in *P. falciparum* are also detailed. Given the issues related to drug resistance, there is a need to investigate the role of PfCRT and PfMDR1 in the mechanism of action of novel antimalarial drugs as outlined in the second and third chapters. In chapter four, a new gene member of the ABC transporter family is characterized and its role in malarial drug resistance is assessed.

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CHAPTER 1

Literature review

1. Global burden of malaria

For centuries malaria has been and still is a devastating disease in poor and war-afflicted countries around the world. Malaria stands for “Mala” and “Aria”, which mean “bad” and “air”, respectively. Indeed the symptoms of this disease (including fever, vomiting and muscular pain) occur in individuals living in or next to marshlands where the vapour given off from swamps was thought to be the cause of malaria. After identification of the parasite responsible for the disease in the 18th century by French physician Charles Louis Alphonse Laveran, many species from these apicomplexan parasites were discovered and today five of them are identified as infectious for humans. *Plasmodium vivax* (*P. vivax*), which is present in the tropics and subtropics (South America, India and Southeast Asia), has the widest distribution. *P. ovale* is present in Central and West Africa while *P. malariae* is present in Sub-Saharan Africa and Southeast Asia. Recently, *P. knowlesi*, a zoonotic parasite that is responsible for malaria in monkeys, has been identified as infectious for humans in Southeast Asia [1]. Among the five species responsible for malaria, *P. falciparum* is the most dangerous and is responsible for the death toll associated with malaria. Malaria is the most prevalent and debilitating disease afflicting humans. Although cases of malaria have diminished by 18.5% since the time I began working on this thesis in 2009 (243 million versus 198 million cases), WHO in its report released in 2014 estimates the number of deaths due to malaria to be around 584,000 with an estimated uncertainty range of 367,000–755,000 in the year 2013 [2].

Malaria occurs in 97 countries on three continents mainly in Asia, South America and Africa (Figure 1). It is estimated that 90% of these deaths occur in Sub-Saharan Africa with 78% being children under age five. The loss of economic growth due to malaria is more than US$12 billion per year [2]. Although malaria is often regarded as a tropical disease, it is not confined to the tropics. Some malaria cases called "airport malaria" have been reported in Europe and North America and were due to immigration [3, 4]. Symptoms of malaria resemble those of the flu and malaria is often misdiagnosed in North America. Doctors should exercise caution when diagnosing a patient presenting with flu-like symptoms. There are several routes of transmission of this protozoan. Some of the routes resemble those of HIV transmission, such as mother to

baby through the placenta, blood transfusion and tattooing or sharing dirty needles. However, these routes account for 1% of the disease's transmission and are not as common. The main mode of transmission remains a bite from a female Anopheles mosquito, which leads to infection.

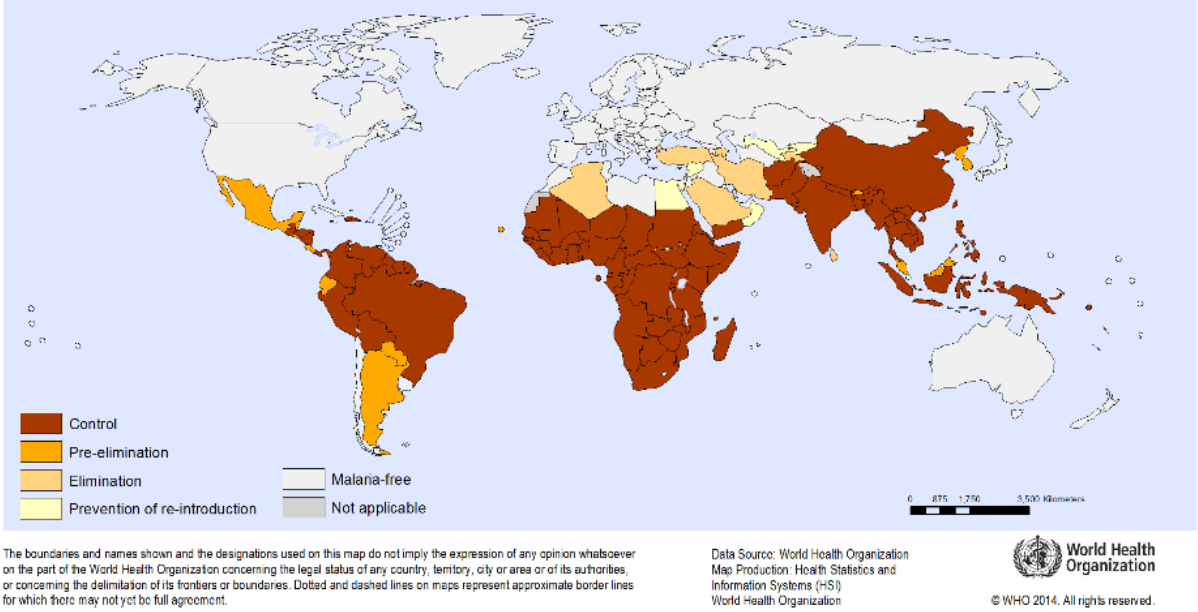

Figure 1. Global distribution of malaria and classification by stage of malaria elimination in 2013 Source: WHO map gallery.

2. Biology and Life cycle of Plasmodium

The life cycle of *Plasmodium* requires the participation of two hosts. The first is the intermediate host (human) in which the asexual development of the parasite occurs, and the second is the definitive host (female Anopheline mosquito), which hosts the sexual development of the parasite (Figure 2). During the asexual development in humans, *Plasmodium* infects both liver and red blood cells (RBCs). The cycle begins when an infected female mosquito (*Anopheles spp*) takes a blood meal. Before the blood uptake, the mosquito inoculates the parasites, in the form of sporozoites, with saliva into the human skin. The motile sporozoites migrate to the liver and infect hepatocytes, where they replicate into schizonts containing merozoites. This phase of parasitic development is called the exoerythrocytic cycle or hepatocytic stage. One sporozoite is able to generate up to 30,000 merozoites in one hepatic schizont. At maturation, the schizont

bursts and releases the merozoites into the blood stream where they infect RBCs, marking the beginning of the erythrocytic cycle. The merozoites invade the RBCs and develop into erythrocytic schizonts that burst and release thousands of new merozoites (for *P. falciparum,* 16–32 merozoites are released per erythrocytic schizont) into the bloodstream. The released merozoites infect new RBCs and the erythrocytic cycle repeats.

It is not well understood what triggers the differentiation of asexual to sexual parasites, but at one point during the cycle, part of the merozoites differentiate into gametocytes (sexual stages of the parasite). Gametocytes circulate in the peripheral blood of the human host and during another blood meal on infected humans, the mosquito ingests blood containing gametocytes. In the lumen of the mosquito's stomach, the gametocytes mature into male and female gametes leading to the beginning of the sexual developmental stage. The gametes crossover produces a zygote that elongates into a motile ookinete, which penetrates the mosquito's gut wall and develops into an oocyst. After a period of growth, the oocyst releases thousands of sporozoites that reach the salivary gland of the mosquito (Figure 2). There, the sporozoites wait for another bite of the mosquito to be released in another human host [5]. The journey of the *Plasmodium* parasite in the human body causes several symptoms such as fever, muscular pain, anemia, etc.

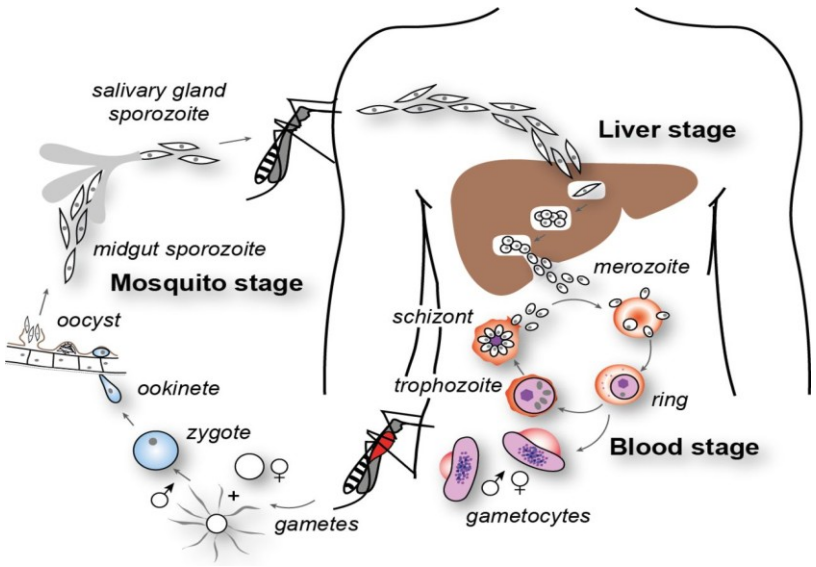

Figure 2. *Plasmodium falciparum* life cycle. Source [6]

3. Erythrocytic development of the parasite and hemoglobin degradation

The invasion of RBCs by merozoites is essential for the survival of the parasite and the continuation of the life cycle. Following entry into RBCs, merozoites develop into rings, trophozoites and schizonts. During parasitic development inside RBCs, the hemoglobin content of RBCs is degraded. Hemoglobin degradation constitutes the source of nutrients and amino acids for the parasite. The peak of hemoglobin degradation appears at the trophozoite stage, where the degradation occurs in the parasite's digestive vacuole (DV). Hemoglobin is degraded by proteases into heme and globin. The latter is then digested in small peptides and finally into amino acids [7]. The remaining heme, also known as ferriprotoporphyrin IX, is toxic to the parasite because it can damage the cellular membranes and inhibit activity of the parasite's proteases. To avoid the toxic effect of heme, the parasite has developed a detoxification mechanism in which heme is polymerized into an inert pigmented crystal known as hemozoin or malaria pigment. It was reported that the pigment responsible for the colour of hemozoin is derived from β-hematin [8]. Heme can be converted into β-hematin *in vitro* in a solution containing a high acetate concentration with an acidic pH [9, 10]. The synthetic β-hematin was proven to be chemically and spectroscopically identical to hemozoin [11]. Hemozoin is stored in the parasite's DV until it matures into schizonts. Then, following the rupture of schizonts, hemozoin crystals are released into the blood circulation of the host. Hemozoin is then rapidly engulfed by macrophages for further elimination.

4. Eradication of malaria: tools and strategies

Malaria remains a threat for children, who pay a huge price every year, since there is no commercially available vaccine to date. Recently the world's most promising vaccine candidate, RTS, S/AS01, was developed. A phase II clinical trial with this vaccine conducted on adults and children has shown a reduction in clinical malaria [12-15]. However, the efficiency of this vaccine decreases over time, as noticed three years post-vaccination [12]. Nevertheless, the phase III clinical trial has already been launched at eleven sites in seven countries located in West, East and Central Africa [16]. The results are expected to be reported by the end of 2014. However, preliminary results show that the co-administration of RTS, S/AS01 with other

vaccines provides a relatively modest protection against malaria in infants [17]. Despite the fact that the vaccine is not 100% protective against malaria, WHO expects to launch its distribution in a few years. The eradication of malaria still needs continuous efforts, and this new vaccine constitutes the first step in the eradication of malaria.

To this day, control of malaria was made by strategies used in the Western World decades ago to eliminate malaria. Indeed, during the mid-1950s, the "Global Malaria Eradication Program" launched by WHO applied two simultaneous strategies. The first consisted of controlling the parasite's vector (*Anopheles* mosquito) with indoor spraying of insecticides such as DTT (dichlorodiphenyltrichloroethane) or the use of larvicides in stagnant water, while the second focused on the killing of parasites by treating humans with antimalarial drugs (e.g. chloroquine). These strategies were very efficient and the countries under this program were declared malaria-free after 15 years [18]. Unfortunately, most of the underdeveloped countries located in tropical and subtropical regions of the world were forgotten. At the time that the elimination program started in the underdeveloped countries, the eradication of the disease had become problematic with the rise of insecticide-resistant mosquitoes and drug-resistant parasites [19, 20]. Drug-resistant parasites appear due to drug misuse and selection pressure [21].

5. Antimalarial drugs: mode of action and resistance

Several drugs are used for the treatment of malaria. They belong to different structural families and have different modes of action. The quinoline drugs are the most common antimalarial drugs used. They have recently been replaced by artemisinin derivatives. Among the quinoline drugs we have the methanolquinoline family, which includes quinine and mefloquine, and the aminoquinolines family, which includes chloroquine.

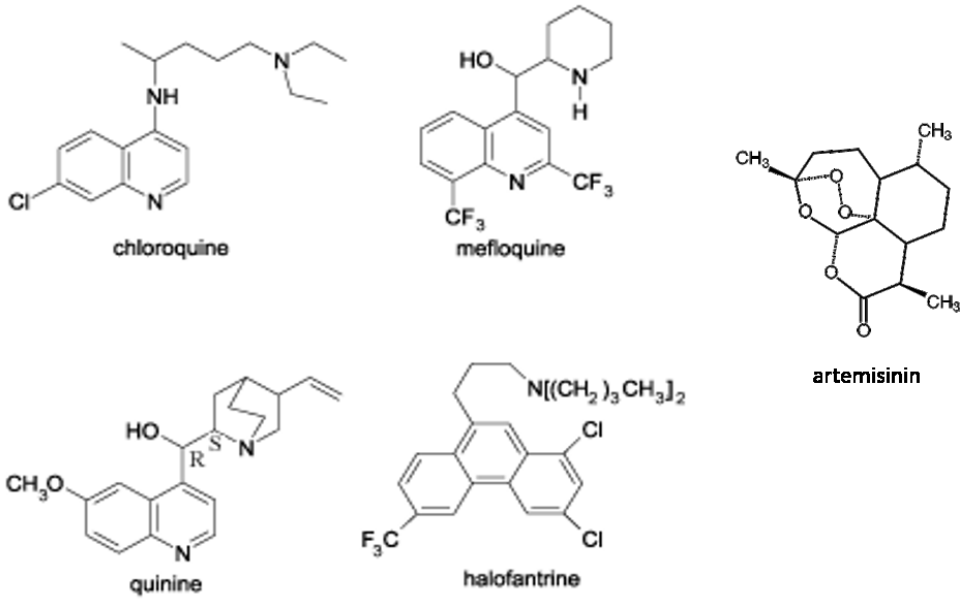

Figure 3. Structures of antimalarial drugs: quinoline-containing drugs (chloroquine, mefloquine, quinine and halofantrine) and artemisinin. Modified from [22].

6. Quinoline drugs and development of resistance

6.1. Quinine

Quinine (Figure 3) was the first natural alkaloid product extracted from the bark of cinchona tree (a tree found in South America). It was used until the 1940's, and was then replaced by chloroquine during World War II. Indeed during that period the complexity of quinine synthesis made it difficult to produce in large quantities [23]. Nowadays quinine is used in severe and chloroquine-resistant malaria cases. Evidence of *P. falciparum* resistance to quinine has been reported in Brazil, Thailand, Senegal and many other countries [24-28]. However, quinine is still a good candidate for the treatment of severe malaria cases. It is also used in combination with antibiotics as a second-line treatment after artemisinin derivatives, for resistant malaria cases. Quinine accumulates inside the parasite's acidic vacuole and is believed to inhibit heme polymerization, which is similar for most of the quinoline drugs [29].

6.2. Chloroquine

Chloroquine (Figure 3), a synthetic analogue of quinine, belongs to the 4-aminoquinoline family. It was the gold standard for many years in the treatment of uncomplicated malaria. Chloroquine was more effective, cheaper, and safer than quinine. Because of its safety, chloroquine was prescribed even to pregnant women. It was also used as a prophylactic drug because of its long half-life (around 60 days). It is believed that this long half-life contributes to the development of resistance because the parasite is exposed for a long time to lower doses of chloroquine [30]. The mode of action of chloroquine is through targeting of the erythrocytic stage of parasitic development. Chloroquine acts by blocking the development of the parasite from the ring to the schizont stage. It is a weak base that diffuses freely across the parasitic membrane and accumulates inside the acidic DV. Chloroquine gets di-protonated and trapped inside the DV, making it unable to cross back through the membrane. Chloroquine blocks the detoxification function of the parasite through its binding to heme. The binding of chloroquine to heme leads to the accumulation of heme monomers in the parasite that permeabilize the DV membrane, resulting in death of the parasite [31].

Ten years after its introduction, evidence of chloroquine resistance emerged and twenty years after, it became ineffective. The first cases of chloroquine resistance were documented in the late 1950s in Southeast Asia (Thai-Cambodian border) and then spread to Sub-Saharan Africa during the 1970s. At the same time, other chloroquine resistance cases had appeared in Columbia and spread throughout South America (Figure 4) [32, 33]. Interestingly, a decade after chloroquine withdrawal in Malawi (East African country), where around 85% of infections were due to chloroquine-resistant parasites, chloroquine made a comeback with a clinical trial demonstrating efficiency of about 99% for the treatment of uncomplicated malaria [34, 35]. Chloroquine is still used in non-endemic areas such as Egypt, to treat *P. falciparum* malaria, but it is also efficient against *P. malariae*, *P. vivax* and *P. knowlesi,* since no evidence of chloroquine resistance with these species has been reported [36]. With the success of chloroquine, other synthetic antimalarial drugs such as mefloquine and halofantrine have been produced.

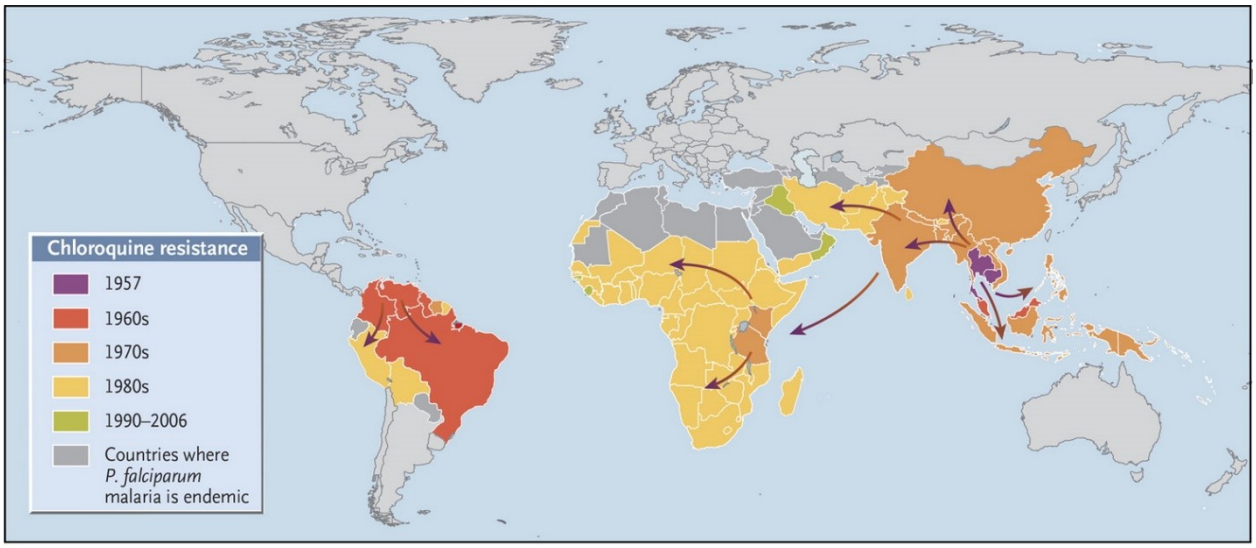

Figure 4. History of chloroquine resistant malaria. From [33].

6.3. Mefloquine and Halofantrine

These drugs were produced by the US army in the 1970s shortly after the Vietnam War to supply the demand for efficient antimalarial drugs, to face the proliferation of chloroquine resistant parasites. Mefloquine (Figure 3) is a 4-methanolquinoline introduced in Thailand in the mid-80s and used as a prophylactic drug. It is also used alone for the treatment of chloroquine-resistant malaria. In 1989, at the time mefloquine had just been approved by the Food and Drug Administration (FDA) for use in chloroquine-resistant regions, cases of resistance were reported in Thailand [37]. The quick development of a parasite's resistance to mefloquine is due to the long half-life of the drug (10–23 days). Although mefloquine provides protection against reinfection, the residual drug can select parasites resistant to mefloquine [38]

The mechanism of action of mefloquine is still unclear. In previous studies, mefloquine was believed to act in the DV by inhibiting the polymerization of heme into hemozoin [39]. However, a recent study suggested that mefloquine acts in the cytosol on an unknown target [40]. Chloroquine and mefloquine resistance are related because parasites resistant to chloroquine are sensitive to mefloquine and vice versa [41]. Recently, a second life was given to mefloquine for its use in combination with artemisinin.

Knowing that parasites develop resistance to any produced antimalarial, a backup drug for mefloquine was created in the early 90s and named halofantrine (Figure 3). Halofantrine is an aryl-amino-alcohol that was produced by the Walter Reed Army Institute of Research in collaboration with WHO and approved for use by the FDA. Halofantrine is used in the treatment of chloroquine-sensitive, -resistant and multidrug-resistant *P. falciparum* malaria [42]. Although it has some side effects like cardiotoxicity, it appears to be less toxic than quinine and mefloquine.

6.4. Artemisinins

Artemisinin (Figure 3) belongs to *Artemisia annua*, a Chinese medicinal plant that was used for more than 1000 years to treat fever in China [43]. The production of artemisinin is not consistent because it relies on the cultivated *Artemisia annua*. To solve this production issue, genetically engineered yeast is now used to increase the yield of artemisinin [44]. Artemisinin is not well absorbed, therefore fast-acting artemisinin derivatives (dihydroartemisinin, artesunate, artemether, etc.) are produced and used in the treatment of drug-resistant and severe malaria [45]. The potency and the action of artemisinin on the various stages of the Plasmodium life cycle make it the central drug for antimalarial chemotherapy.

The mechanism of action of artemisinin is controversial. While some authors believe that artemisinin targets DV, others propose that artemisinin acts in the cytoplasm [40, 46-48]. Although artemisinin derivatives have short half-lives (a few hours) that reduced the possibility of resistance, a decrease in artemether sensitivity has been reported *in vitro* in French Guyana and Senegal parasite isolates [49]. Furthermore, studies conducted in Asia and Africa have shown that there is a reduction in *P. falciparum* susceptibility to artesunate *in vivo* [50-52]. This led to the prohibition of artemisinin monotherapy by WHO, which recommends the use of artemisinin-based combination therapies (ACTs) as first–line treatment of uncomplicated malaria in endemic regions [53, 54]. ACTs are based on the combination of artemisinin derivatives and some long-lasting antimalarial drugs (mefloquine, sulfadoxine-pyrimethamine, amodiaquine, piperaquine or lumefantrine). Several other combinations also exist, namely:

artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine, artesunate-amodiaquine, artemether-lumefantrine, dihydroartemisinin-piperaquine, etc. Until recently, no resistance to ACTs has been observed in the field. However, there is evidence of resistance to ACTs on the Thailand-Cambodia border, considered to be the origin of chloroquine resistance [55, 56]. With the emergence of resistance to artemisinin derivatives and the evidence of resistance to ACTs, research should focus on effective antimalarial drug production. There are new promising antimalarial drugs that exhibit novel mechanisms of action and can potentially replace artemisinins [57]. Since the drug resistance phenomenon is still rampant, one strategy that can be pursued to reduce the prevalence of malaria is to "reverse" CQ resistance chemically.

7. Drug resistance reversal

The use of reversing agents, also known as chemosensitizers, for drug resistance started with tumour cells in cancer research. Studies show that different chemosensitizing agents can reverse drug resistance mediated by the ABC transporter P-glycoprotein 1 in cancer cells. One of the first chemosensitizers discovered is the calcium channel blocker verapamil [58]. Due to its inefficiency *in vivo,* a second and then a third generation of chemosensitizers replaced this first generation. The first and second generation failed in clinical trials because of their toxicity (high dose was required to achieve efficacy) and their inhibition of drug metabolism in patients. Most third generation chemosensitizers are efficient in the nanomolar range and have successfully passed phase III clinical trials. Such success in cancer research has prompted the malaria research community to pursue the development of *Plasmodium* drug resistant chemosensitizers.

To this end, some studies revealed that verapamil also reverses chloroquine resistance in plasmodium-resistant strains *in vitro* [59, 60]. Following the same path, several groups have discovered many chemosensitizing agents that belong to different chemical groups and have variable effects on drug-resistant parasites. Compounds such as tricyclic antidepressant drugs [61-63] and antihistaminic drugs [64-67] have shown promising capabilities to reverse CQ resistance in parasite isolates *in vitro*, in animal models and in human malaria. However, these

chemosensitizing agents are effective only at doses toxic to humans, thus limiting their use *in vivo*. Luckily, the antihistaminic drugs desipramine and chlorpheniramine have successfully reached the clinical trial step in malaria patients but chlorpheniramine failed to be efficacious [67]. The fact that chemosensitizers that are able to reverse drug resistance in cancer cells were also able to reverse drug resistance in malaria suggests the involvement of drug transporters [68]. To support this hypothesis, studies done in the Wirth lab have identified two homologues of human multidrug resistance transporters in *Plasmodium,* namely PfMDR1 (*P. falciparum* multidrug resistant transporter 1) and PfMDR2. These transporters were initially deemed to be involved in chloroquine resistance [69]. However, it was later found that the primary determinant of chloroquine resistance is a transporter named PfCRT (*Plasmodium falciparum* chloroquine resistance transporter). The mechanism of resistance reversal by verapamil is related to the presence of point mutations in PfCRT, mainly the K76T mutation [70, 71].

8. Drug resistance gene and resistance mechanism

Chloroquine resistance lies in the difference of CQ accumulation levels between sensitive and resistant parasites. Indeed, resistant parasites accumulate less drug (CQ) than sensitive parasites [72]. The complexity of drug resistance in *Plasmodium* is a daunting issue, given the involvement of various transporters such as drug/metabolite transporters, ABC transporters, etc.

8.1. The drug/ metabolite transporter PfCRT

In a recent study, Krogstad and colleagues demonstrated that CQ resistant parasites efflux CQ fifty times faster than CQ-sensitive parasites [72]. Therefore, in an attempt to identify the gene behind CQ resistance, a genetic-cross experiment between Dd2 (CQR) and HB3 (CQS) clones was performed. The haploid progeny was found to encode a putative transporter, later designated *Plasmodium falciparum* chloroquine resistant transporter (PfCRT), as being responsible for CQ resistance [73]. PfCRT is member of the drug/metabolite transporter superfamily composed of 10 transmembrane domains. It is localized to the DV membrane of the parasite [74]. It is now generally accepted that PfCRT is one of the most important genes conferring resistance to CQ.

Chloroquine-resistant parasites harbour multiple mutations in the *pfcrt* gene (mutant PfCRT). Mutant PfCRT acts by causing the efflux of CQ out of the DV, while wild-type PfCRT does not.
The number of these mutations varies depending on the parasites' origin [75]. Currently, several mutations associated with CQ resistance have been identified in the *pfcrt* gene. Some of these mutations were created in laboratories after drug pressure selection studies [75, 76]. Drug selection experiments also created new mutations in PfCRT that were never found in field isolates [76]. The key point mutation associated with CQ resistance *in vitro* and *in vivo* is the substitution of the lysine residue at position 76 with a threonine residue (K76T) in the PfCRT protein sequence [70, 77]. This mutation is never found alone in mutant PfCRT, although it is a required mutation for CQ resistance. Martin et al. offer an explanation on how this point mutation confers CQ resistance when they experimented on oocytes expressing wild-type and mutant PfCRT. It is believed that the occurrence of the positively charged lysine residue at position 76 in the substrate binding site of PfCRT prevents the protonated CQ from binding to the transporter, and thus not resulting in efflux. In contrast, the substitution with a non-charged amino acid residue as threonine in mutant PfCRT allows the interaction of CQ with PfCRT, leading to the trafficking of the protonated form of CQ outside the DV. This study shows the direct transport of CQ by mutant PfCRT, whereas wild-type PfCRT does not [78].

Moreover, it has been shown that CQ transport is inhibited by other antimalarial drugs such as quinine, primaquine and amodiaquine, which are very active against CQ-resistant parasites [79]. Although this is known about PfCRT, the way this protein behaves in terms of transport is unclear. Some studies suggest a transporter model and others suggest a channel model. Nonetheless, it is yet to be determined [80-83].

8.2. ABC transporters

The ABC transporter family is one of the largest existing gene families found in all cells. By using the energy derived from ATP hydrolysis, ABC proteins transport various ions and molecules across membranes. Those molecules include drugs, lipids, peptides, toxins, nucleotides, heavy metals, etc. ABC transporters can function as importers, allowing the influx of nutrients inside

the cell or as exporters mediating efflux of metabolites and other toxic compounds out of the cell. While exporters are found in all forms of life, importers are more restricted to prokaryotic organisms [84].

In humans, ABC transporters have been classified into seven subclasses according to their sequence and structural homologies. ABC proteins encode two main domains: an ATP-binding domain, also known as a nucleotide-binding domain (NBD), which is located in the cytoplasm, and a transmembrane domain (TMD) localized to the lipid bilayer of the cell membrane. To be functional, an ABC transporter requires two NBDs and two TMDs that can be encoded by the same or different genes. The NBD has three major conserved motifs: two characteristic motifs (Walker A and B) common in all ATP-binding proteins and a third motif called “ABC signature”, located just upstream of the walker B site [85]. The ABC signature motif is exclusive to ABC transporters. The NBD uses the energy generated by ATP hydrolysis to transport the substrate across the membrane. The TMD contains five or six membrane-spanning α-helices, which encode the transporter substrate specificity.

In eukaryotes, there are two classes of ABC transporters: the “full transporters" containing two TMDs and two NBDs, and the “half transporters” that consist of one TMD and one NBD (Figure 5) [85]. A functional “half transporter” must form either a homodimer or heterodimer. ABC genes are widely dispersed in eukaryotic genomes and are highly conserved between species [86]. In humans, the genes are divided into seven subfamilies (ABCA to ABCG) based on the similarity in their gene structures (half versus full transporters), the order of the domains and the sequence homology in the NBDs and TMDs. In eukaryotes, some ABC proteins do not have TMDs and are probably involved in other biological processes not related to transport [87].

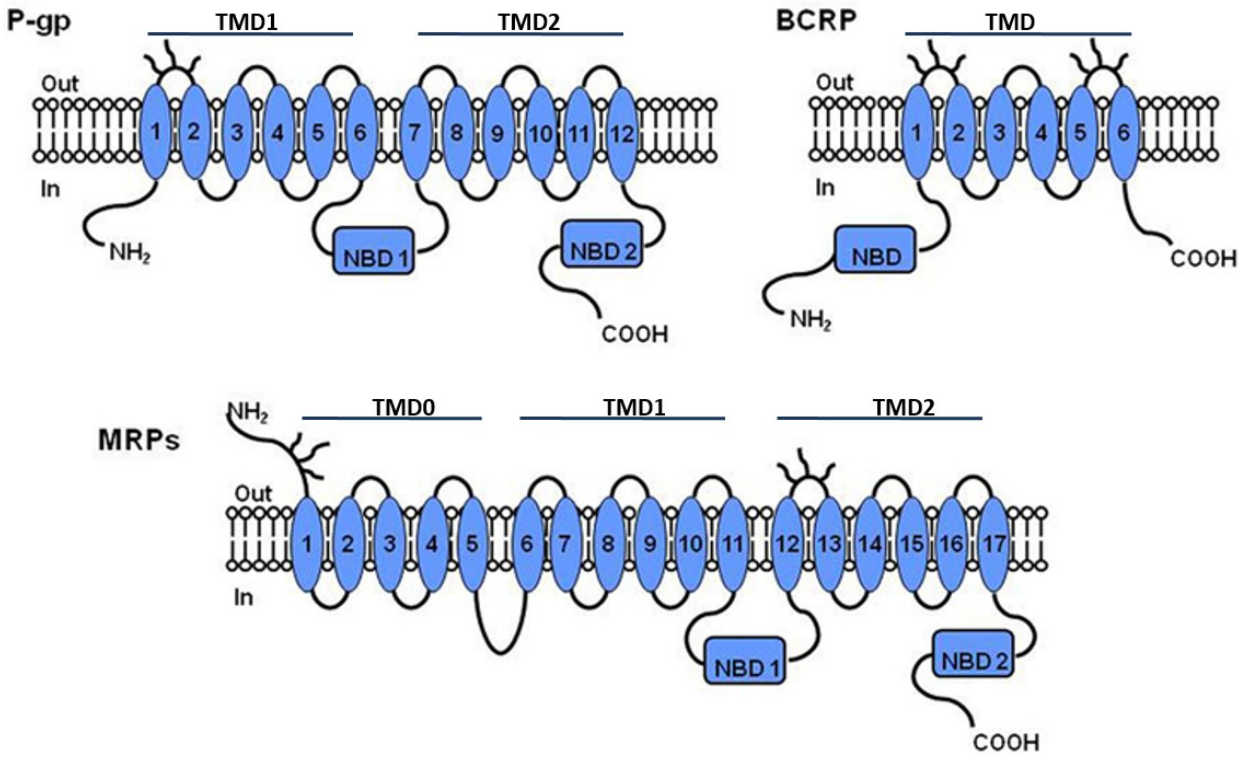

Figure 5. Topologies of three ABC transporters. Two full transporters P-gp and MRPs and one half transporter BCRP. Modified from [88].

8.3. ABC transporters in Plasmodium

Since the discovery of P-glycoprotein1 (Pgp-1) 38 years ago as a transporter responsible for drug resistance in tumour cells, an important advance has been made in understanding the structure, the function and the mechanism of action of the ABC superfamily. The number of ABC transporters varies with the organism. As in many protozoans, ABC transporter proteins are also present in *P. falciparum*. The availability of the *P. falciparum* genome [89] has helped in the identification of members of the ABC transporter family [90]. The annotation of the genome revealed fifteen predicted genes encoding putative ABC transporters in *Plasmodium*. These genes are classified into six subfamilies, namely ABC-B, C, E, F, G and ABC-H [91, 92]. There is neither ABCA nor ABCD in the *Plasmodium* genome, while the ABCH subfamily exists in *Plasmodium* but not in humans. Table 1 gives a description of the ABC transporter families in *P. falciparum*. To date, eight *Plasmodium* ABC transporter proteins have been studied. The two most studied are PfMDR1 (*Plasmodium falciparum* multidrug resistance 1) and PfMRP1

(*Plasmodium falciparum* multidrug resistance protein 1), and both are involved in *P. falciparum* drug resistance.

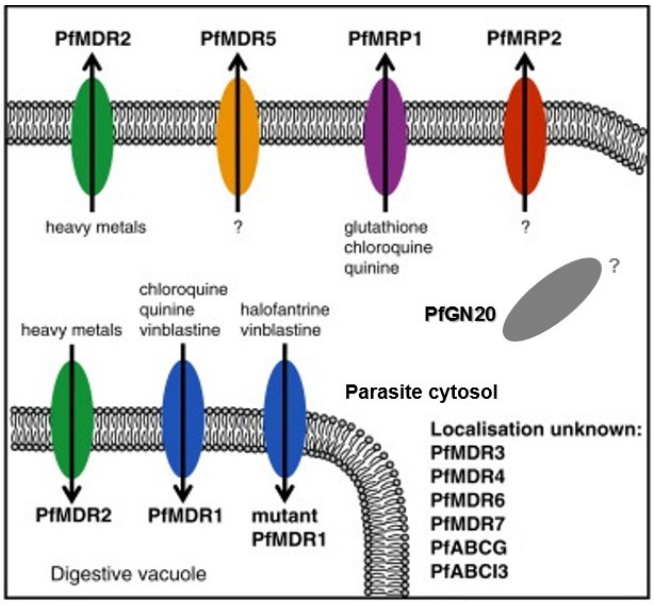

Figure 6. Schematic representation of the localization of *P. falciparum* ABC transporters. Modified from [93]).

Table 1. *Plasmodium falciparum* ABC transporters family

ABC Subfamily	PlasmoDB identification	Alias	Domain organization	Product description	Amino acids
ABCB	PF3D7_0523000	PfMDR1	$(TMD\text{-}NBD)_2$	Multidrug resistance protein	1419
	PF3D7_1447900	PfMDR2	$(TMD\text{-}NBD)_2$	Multidrug resistance protein 2 (heavy metal transporter family)	1024
	PF3D7_1145500		TMD-NBD	Transporter putative	872
	PF3D7_0302600		TMD-NBD	ABC transporter (TAP family), putative	1365
	PF3D7_1339900	PfMDR5	TMD-NBD	ABC transporter (MDR family)	925
	PF3D7_1352100	PfMDR6	TMD-NBD	ABC transporter (heavy metal transporter family)	1049
	PF3D7_1209900		TMD-NBD	ABC transporter (TAP family), putative	855
ABCC	PF3D7_0112200	PfMRP1	$(TMD\text{-}NBD)_2$	Multidrug resistance-associated protein 1	1822
	PF3D7_1229100	PfMRP2	$(TMD\text{-}NBD)_2$	ABC transporter (CT family)	2108
ABCE	PF3D7_1368200		(NBD)2	RNase L inhibitor protein, putative	619
ABCF	PF3D7_1121700	PfGCN20	(NBD)2	Protein GCN20	815
	PF3D7_0813700		(NBD)2	ABC transporter, putative	1419
ABCG	PF3D7_1426500	PfABCG2	NBD-TMD	ABC subfamily G member 2	660
ABCH	PF3D7_1413500	PfSufC	NBD	FeS assembly ATPase SufC	347
	PF3D7_1434000	PfCAF16	NBD	CCR4-associated factor 16, putative	257

8.4. Most characterized Plasmodium ABC transporters

8.4.1. PfMDR1

PfMDR1 is a full transporter consisting of two NBDs and two TMDs. The identification of MDR homologues in *Plasmodium* was a breakthrough in the understanding of drug resistance in Plasmodium. PfMDR1 and PfMDR2 are expressed in drug-sensitive parasites but only PfMDR1 is overexpressed in drug-resistant parasites. Point mutations in the *pfmdr1* gene have been associated with CQ resistance, given that CQ-sensitive parasites have identical sequences of *pfmdr1* while CQ-resistant strains possess one or multiple mutations in the *pfmdr1* sequence [94]. However, another study found that an increase in CQ resistance level does not affect the mutations in *pfmdr1*, indicating an absence of correlation between PfMDR1 and CQ resistance [95]. Gene amplification of *pfmdr1* is associated with resistance to mefloquine, when parasites were allowed to grow under high concentrations of mefloquine. In addition, there is an increase in PfMDR1 transcripts and protein levels in the mefloquine-resistant strains [96, 97]. PfMDR1 overexpression also correlates with parasitic resistance to halofantrine and quinine but not to amodiaquine, suggesting that PfMDR1 overexpression is drug-dependent [96]. *Pfmdr1* amplification is a key determinant for both *in vivo* and *in vitro* resistance to mefloquine, quinine and halofantrine [96, 98, 99].

Similar effects were also observed in the study conducted by Sidhu and colleagues [100]. The mutation N86Y in PfMDR1 has been demonstrated to contribute to CQ resistance [101, 102], whereas another mutation, N1042D, contributes to quinine resistance [100]. The triple PfMDR1 mutations S1034C, N1042D and D1246Y induce hypersensitivity to mefloquine, halofantrine and artemisinin but do not confer resistance to quinine [103]. A recent study demonstrated that point mutations in PfMDR1 modify its substrate specificity to several antimalarial drugs such as chloroquine, quinine and halofantrine. Indeed, heterologous expression of PfMDR1 in frog oocytes revealed that the wild-type PfMDR1 protein can transport chloroquine and quinine but not halofantrine, while the mutated PfMDR1 protein can transport halofantrine but not quinine

or chloroquine [104]. This study suggests that alternative drugs can be used to counter drug resistance.

There is also some evidence suggesting a possible link of PfMDR1 with resistance to artemisinin. An *in vitro* selection study performed by Kyle et al. exposed different strains of parasites to artelinic acid (a semi-synthetic derivative of artemisinin) and artemisinin *in vitro.* The study indicates that the parasites become less sensitive to artemisinin but also to mefloquine, quinine, halofantrine and lumefantrine. This sensitivity is due not only to gene amplification, but also to an overexpression of the PfMDR1 transcript and protein [105]. These results were corroborated by a genetic modification study in which one of the two copies of the *pfmdr1* gene present in a drug-resistant parasite FCB was disrupted. The disruption leads to an increase in the sensitivity of the parasite to mefloquine, halofantrine, lumefantrine, quinine and artemisinin [40]. A recent study has also associated the failure of artesunate/mefloquine treatment on the Thai-Cambodian border with an increase in the *pfmdr1* copy number [106].

PfMDR1 is localized on the parasite DV membrane (Figure 6) with the NBD facing the cytosol of the parasite [107, 108]. Following the drug efflux model of ABC transporters, PfMDR1 may transport solutes and drugs inside the parasite's DV. This assumption has been addressed by Rohrbach et al. in their study related to the PfMDR1 modulation of fluorescent dye Fluo-4 accumulation inside the DV [109]. Overall, the presence of point mutations and the overexpression of PfMDR1 are not directly responsible for CQ resistance, but modulate the effects of different drugs including CQ [99, 110].

8.4.2. PfMRPs

In *P. falciparum*, two MRP homologues have been identified namely PfMRP1 and PfMRP2 [111]. They are both members of the ABCC family and are categorized as full transporters, as presented in Table 1.

A number of studies were conducted on PfMRP2 (PF3D7_1229100), and the latest one had shown that the protein is localized in the plasma membrane of the parasite (Figure 6) [92, 112].

Although an *in vitro* study has shown that *pfmrp2* gene expression increases in cultured parasites under mefloquine or chloroquine pressure, the role of this protein in *P. falciparum* remains to be elucidated [113]. A recent study provided some evidence regarding the involvement of PfMRP2 in drug resistance. The study was performed on two isogenic clones (from the CQ-sensitive parasite 3D7), which display different sensitivities to drugs. The results show that a deletion in the *pfmrp2* promoter correlates with a decreased sensitivity of the mutant parasite to quinine, mefloquine and chloroquine, along with an increase in the PfMRP2 protein level [114]. There is still a need to investigate whether genetic polymorphism at the *pfmrp2* promoter could be associated with drug resistance in the field.

Like PfMRP2, PfMRP1 (PF3D7_0112200) is localized in the plasma membrane of the parasite (Figure 6) [92, 111]. Most studies on PfMRP1 are related to the involvement of this protein in *P. falciparum* drug resistance. Mu et al. have linked PfMRP1 single nucleotide polymorphisms (SNPs) to a decrease in the response of *P. falciparum in vitro* to quinine and chloroquine [112]. This was confirmed by another study that found an association between SNPs in *Pfmrp* (*Pfmrp1*) and parasite response to quinoline drugs [115]. In the study conducted by Raj et al., the disruption of the *pfmrp1* gene in a chloroquine-resistant parasite shows an increased susceptibility of the parasites to several antimalarial drugs (chloroquine, quinine, piperaquine, primaquine and artemisinin), suggesting a possible role of PfMRP1 in drug efflux [116]. Recently, the presence of the 1466K allele in PfMRP1 was associated with recrudescent infections in children, following a sulfadoxine-pyrimethamine treatment. In addition, the same authors also correlate the I876V mutation with recurrent infections after artemether-lumefantrine treatment in East Africa [117, 118]. Another study has also identified a possible association between different mutations in PfMRP1 and the resistance to multiple drugs, implying that PfMRP1 is an important candidate for monitoring drug resistance [119]. The direct role of PfMRP1 in the resistance of parasites to these drugs remains an open issue and should be investigated.

8.5. Other ABC transporters characterized in Plasmodium

8.5.1. PfMDR2

When screening *P. falciparum* the genome for a human multidrug resistance protein 1 (MDR1) homologue, Wilson et al. have identified, in addition to PfMDR1, another transporter designated PfMDR2 *(Plasmodium falciparum* Multidrug resistance protein 2) [69]. *Pfmdr2* (PF3D7_1447900) belongs to the ABC transporter superfamily, but in contrast to *Pfmdr1*, *Pfmdr2* is present as a single copy gene in both chloroquine-sensitive and -resistant parasites. The PfMDR2 protein is located in the DV membrane of the parasite (Figure 6) [120, 121]. In contrast to PfMDR1, PfMDR2 is a half transporter containing one TMD and one NBD. Although the *pfmdr2* transcript is more highly expressed in chloroquine-resistant parasites than in sensitive ones, the analysis of the protein encoded by the *pfmdr2* gene has shown no differential expression between chloroquine-sensitive and -resistant parasites [121, 122]. To date, there is no evidence of PfMDR2 involvement in *Plasmodium* drug resistance. However, the PfMDR2 protein is assumed to have a role in metal homeostasis in the parasite. This assumption was confirmed by the work of Rosenberg et al., which shows that the PfMDR2 protein acts as an efflux pump of heavy metals [123]. Furthermore, the role of PfMDR2 in a heavy metal (cadmium) efflux is demonstrated in a recent study in which cadmium-resistant parasites accumulate less cadmium than sensitive parasites [124].

8.5.2. PfMDR5 and PfMDR6

PfMDR5 and PfMDR6 are both half transporters with one NBD and one TMD. Like many ABC transporters, PfMDR5 (PF3D7_1339900) is localized in the plasma membrane of the parasite (Figure 6) [92]. A correlation was recently observed *in vitro* between an increase in IC_{50}s of the antimalarial drug lumefantrine and the presence of allelic repeats in the *pfmdr5* gene 5'UTR region [125]. This latter study is to our knowledge the only one associating PfMDR5 with *Plasmodium* drug resistance.

The first report on *pfmdr6* (PF3D7_1352100) occurred early in the year 2000 in a study done by Mu et al. using a genome-wide association approach [112]. Several transporter genes were

positively selected in different parasite populations for their association with the parasite's response to CQ and/or quinine. One of them is the gene designated "G7", which was strongly associated with the CQ response and was later identified as PfMDR6 [112]. Thus far, few studies have been performed to determine the role of the *pfmdr6* gene in drug resistance. Okombo et al. demonstrate an association between the presence of asparagine repeats in the 5'UTR of the *pfmdr6* gene and the susceptibility of parasites to piperaquine [125]. In a recent study, polymorphisms in a microsatellite region of the *pfmdr6* gene affected parasite sensitivity to dihydroartemisinin (DHA) [126].

8.5.3. PfGCN20

The fact that some ABC transporters are associated with multidrug resistance, mainly PfMDR1 in *P. falciparum,* encourages some authors to pursue the identification of a novel ABC transporter that may play a role in *Plasmodium* drug resistance. To this end, Bozdech et al. have identified the *pfgcn20* gene (PF3D7_1121700), which encodes the PfGCN20 protein [127]. The closest homologue of PfGCN20 is the yeast Gcn20 protein, which plays a role in the translation initiation pathway in starved yeast, mainly in the induction of amino acid synthesis. The PfGCN20 protein contains two NBDs, including an ABC signature sequence, Walker A and B regions, but no transmembrane domain [127]. In contrast to the characterized ABC transporter proteins in *P. falciparum* (PfMDR1 and PfMDR2), PfGCN20 is localized in membrane and non-membrane compartments of both the infected erythrocytes and the parasite [128]. These observations raise several hypotheses about the possible biological function of the PfGCN20 protein. The erythrocytic localization of PfGCN20 suggests that the protein functions as an ATP-binding subunit of a multimeric ABC transporter. In the case of the cytosolic localization of PfGCN20 in the parasite, an involvement of PfGCN20 in the translational regulation pathway in the parasite is proposed [128]. Indeed, by acting as part of the yeast translation regulatory pathway, PfGCN20 complements the activity of its yeast homologue in a Gcn20 deleted yeast mutant [129]. This function is far from that of *P. falciparum* drug resistance, and to date there are no data supporting the function of PfGCN20 in *P. falciparum* drug resistance.

8.5.4. PfABCG

This protein does not arise much interest until the beginning of this thesis. The only information available was the sequence of the gene in 3D7 CQ-sensitive parasites, genomic [112], microarray [130] and proteomic data [131]. In their attempt to identify additional genes involved in *Plasmodium* chloroquine resistance, Mu et al. searched for the presence of SNPs in different putative *Plasmodium* transporter genes. Their results demonstrate that there are no SNPs in the PfABCG gene sequenced from 97 isolates and consequently, there is no association of PfABCG with decreased sensitivity of the parasites to chloroquine and quinine [112].

P. falciparum proteomes from different stages of the *Plasmodium* life cycle (sporozoites, merozoites, trophozoites and gametocytes) were analyzed in a study conducted by Florens et al., which identified PfABCG as highly expressed at the gametocyte stage [131]. This result was confirmed with DNA microarray experiments done by Leroch et al., who determined that PfABCG is differentially expressed in the blood stages of *P. falciparum,* with a higher expression observed at the gametocyte stage [130]. More information on PfABCG is revealed by two recent studies wherein the possible function of PfABCG was assessed [132, 133]. In the Eastman et al. paper, PfABCG (named PfABCG2) was disrupted in 3D7 (CQ-sensitive parasites) and the disrupted parasites were screened against more than 2000 drugs. Their findings demonstrate that the presence of PfABCG in CQ-sensitive parasites is associated with the sensitivity of the parasites to ketotifen (an antihistamine drug), but not with chloroquine and dihydroartemisinin [132]. However, in the study performed by Tran et al., knock-out of PfABCG in Plasmodium is associated with neutral lipid regulation since the production of di- and triacylglycerols in knockout parasites are significantly reduced [133].

Many transporters exist in malaria parasites but only few have been studied. Therefore, challenges related to the characterization of Plasmodium ABC transporters still need to be tackled.

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Connecting statement 1

In the previous chapter we reviewed the current knowledge on malaria and its causative agent (Plasmodium). We briefly described the characterized ABC transporters in plasmodium and we gave an overview on the genes responsible for drug resistance. We also presented some antimalarial drugs belonging to the quinoline family and their use to eradicate malaria. Field studies demonstrate that these drugs were effective for a short period of time and then exhibited reduced efficacy caused by the development of resistance. Furthermore, the most promising drugs known as ACTs present some signs of decreased sensitivity *in vitro* and *in vivo*. Many studies associate the action of two protein transporters, PfCRT and PfMDR1, with the development of drug resistant malaria. In order to evade the drug resistance mechanism of the parasite, these two transporters are usually investigated when any novel antimalarial drug is developed. In the next chapter we investigate the efficiency of a quinoline-based drug, MK571, on CQ-sensitive and CQ-resistant parasites. We also study the involvement of the two well-known drug resistance genes in the mode of action of MK571.

CHAPTER 2

A 2-amino quinoline, 5-(3-(2-(7-Chloroquinolin-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid, Interacts with Pgh1 and Inhibits its Drug Transport in Plasmodium falciparum

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ABSTRACT

Malaria is a major disease in the tropics where chemotherapy remains the main mode of treatment and as such the rise and spread of drug-resistant malaria can lead to human tragedy. Two membrane transport proteins, PfMDR1 (*Plasmodium falciparum* multidrug resistance protein 1) and PfCRT (*Plasmodium falciparum* chloroquine resistance transporter), have been shown to cause resistance to several antimalarials. Both PfMDR1 and PfCRT are localized to the digestive vacuolar membrane and appear to regulate the transport of drugs and physiological metabolites. In this study we have used MK571, a 2-amino quinoline, to explore its interaction with PfMDR1 and PfCRT in chloroquine-sensitive and -resistant strains of *Plasmodium falciparum*. Our results show that chloroquine-resistant strains (e.g., K1, Dd2, and 7G8) are consistently more sensitive to MK571 than chloroquine-sensitive strains (e.g., 3D7, 106/1 and D10). This association, however, was not maintained with the chloroquine-resistant strain FCB which IC_{50} value was similar to chloroquine-sensitive strains. Moreover, the susceptibility of chloroquine-sensitive and -resistant strains to MK571 does not correlate with mutated PfCRT, nor is it reversible with verapamil; but correlates with mutations in PfMDR1. Furthermore, MK571 appears to target the parasite's digestive vacuole (DV), as demonstrated by the ability of MK571 to: 1) block the accumulation of the fluorescent dye Fluo-4 AM, a PfMDR1 substrate, into the digestive vacuole; 2) reduce the transvacuolar pH gradient; and 3) inhibit the formation of β-hematin *in vitro*. Moreover, the presence of non-toxic concentrations of MK571 sensitized both chloroquine-sensitive and -resistant parasites to mefloquine and halofantrine, likely by competing against PfMDR1-mediated sequestering of the drugs into the DV compartment and away from the drugs'

cytosolic targets. Our data, nevertheless, found only a minimal decrease in MK571 IC_{50} value in FCB parasite which second pfmdr1 copy was inactivated via gene disruption. Taken together, the findings of this study suggest that MK571 interacts with native and mutant PfMDR1 and modulates the import of drugs or solutes into the parasite's DV and, as such, MK571 may be a useful tool in the characterization of PfMDR1 drug interactions and substrate specificity.

INTRODUCTION

Malaria remains a major cause of death in many parts of the world, especially Africa, with ~216 million cases of infection and 665,000 to 1.2 million deaths annually [1, 2]. Moreover, global efforts to control malaria have been hampered by the emergence and spread of drug resistant parasites [3]. Previously effective antimalarials, such as chloroquine (CQ), pyrimethamine, and sulfadoxine, have lost their efficacy in most endemic regions, while the emergence of resistance to mefloquine and quinine threatens the use of these affordable antimalarials. In an effort to curb the rise of resistance to single-agent treatment, current guidelines for malaria treatment recommend artemisinin-based combination therapy (ACT) that includes a fast-acting drug (e.g., artemisinin or artemether) together with a slower or longer half-life drug (e.g., quinoline or antifolate) [4]. Although the rationale for this latter strategy is sound, it carries the risk of selecting for "pleiotropic" or multi-drug resistant parasites.

Efforts over the past two decades have focused on resolving the molecular mechanisms of chloroquine resistance in laboratory and field strains. Chloroquine resistance is largely attributed to two membrane transporters expressed in the digestive vacuolar membrane of the parasite [5, 6]. These transporters are thought to decrease the effective chloroquine concentration in the parasite's digestive vacuole (DV) [7]. One of the two transporters, the chloroquine resistance transporter, or PfCRT, is a member of the drug-metabolite transporter superfamily [8] and was shown to be responsible for the reduction of chloroquine accumulation in the DV of CQ-resistant parasites. Although multiple mutations in PfCRT have been demonstrated to modulate PfCRT-mediated resistance to CQ, a point mutation in the first transmembrane domain ($76^{Lys\text{-}Thr}$) was

shown to correlate consistently with CQ and other quinoline-based drugs resistance [5, 9]. It is now believed that a mutant form of PfCRT (mut^{76} PfCRT) is responsible for the release or transport of chloroquine, and possibly other 4-aminoquinolines, from the DV of resistant parasites [10-12]. Moreover, the calcium channel blocker verapamil, which reverses chloroquine resistance, has been suggested to bind to PfCRT and to inhibit the passage of chloroquine by restoring the positive charge of lysine76 onto PfCRT, thus preventing drug transport out of the DV due to charge repulsion [13]. The second membrane transporter PfMDR1, encoded by *pfmdr1*, initially thought to be causative of chloroquine resistance, is now believed to modulate the level of resistance to chloroquine [14]. PfMDR1 (*Plasmodium falciparum* multi-drug resistance protein 1) or Pgh-1 (P-glycoprotein homologue 1), is a member of a large family of ATP-Binding Cassette (ABC) transporters shown to confer resistance to anti-cancer drugs in tumor cells [15]. Unlike mammalian P-glycoprotein (or ABCB1), which localizes to the cell surface, PfMDR1 localizes to the DV membrane and displays more restrictive substrate specificity [6, 14]. PfMDR1 has been shown to reduce the sensitivity of *P. falciparum* to mefloquine and chloroquine through enhanced or reduced drug influx, respectively [16, 17]. Consequently, PfMDR1-mediated drug resistance has been attributed to gene amplification and point mutations. Foote *et al.* [18] described four codon polymorphisms in PfMDR1 that are strongly associated with chloroquine-resistance, presumably affecting its drug influx activity. Sidhu *et al*. [19] reported the occurrence of three point mutations in PfMDR1 (1034$^{\text{Ser-Cys}}$, 1042$^{\text{Asn-Asp}}$ and 1246$^{\text{Asp-Tyr}}$) that increase the sensitivity of the parasite to mefloquine but has no effect on the sensitivity to CQ. Interestingly another study by Reed *et al.* [14] showed that altering these latter residues in PfMDR1 (1034$^{\text{Ser-}}$

^{Cys}, 1042$^{Asn-Asp}$ and 1246$^{Asp-Tyr}$), in the 7G8 lab strain, results in increased sensitivity to chloroquine through enhance drug influx. Furthermore, PfMDR1 encoding (86^{Tyr}184^{Tyr}1034^{Ser}1042^{Asn}1246^{Asp} or mut1-PfMDR1) has been implicated in the transport of quinine, mefloquine and halofantrine, while PfMDR1 encoding (86^{Asn} 184^{Phe} 1034^{Cys} 1042^{Asp} 1246^{Tyr} mut2-PfMDR1) did not allow the import of these drugs into the DV. More recently, Rohrbach *et al.* [20] have shown a PfMDR1-mediated transport of the fluorescent dye, Fluo-4, into the DV of chloroquine-resistant strains (Dd2, K1, FCB with (mut1-PfMDR1) encoding 86^{Tyr} 184^{Tyr} 1034^{Ser} 1042^{Asn} 1246^{Asp}) and to a much lesser extent in chloroquine-sensitive strains (D10, HB3 and NF54). Furthermore, Fluo-4 accumulation in the DV was inhibited by mefloquine and quinine but not chloroquine. Taken together, the resistance phenotype to quinoline-based drugs appears to be mediated by a complex mechanism involving at least two membrane transporters (e.g., PfCRT and PfMDR1), in addition to the genetic background of the parasite. However, it is well accepted now that PfCRT is more dominant in parasite's susceptibility to CQ, while resistance to mefloquine is mediated mostly by PfMDR1. In this report, we have examined the effects of a 2-amino quinoline drug, MK571, on the proliferation of several chloroquine-sensitive and -resistant strains of *P. falciparum*. The findings of this study show MK571 interacts with PfMDR1 and blocks its ability to transport Fluo-4 and several antimalarial drugs into the DV. Moreover, MK571 is not a substrate for native or mutant PfCRT. Interestingly, chloroquine-resistant strains show increased sensitivity to MK571, likely due to enhanced PfMDR1-mediated transport of MK571 in the DV of resistant parasites or due to blocking of physiologic metabolites transport into the DV. Furthermore, we show that MK571 decreases the pH gradient between the DV lumen and the parasite cytosol *in*

situ and inhibits hemin polymerization *in vitro*. The ability of MK571 to interact with and inhibit PfMDR1-mediated drug transport without affecting PfCRT provides a valuable tool to dissect the transport mechanism of PfMDR1.

MATERIALS AND METHODS

Materials- Chloroquine diphosphate, verapamil hydrochloride, mefloquine hydrochloride, halofantrine hydrochloride and hemin were purchased from Sigma-Aldrich, MK571 (3-[[[3-[(1*E*)-2-(7-Chloro-2-quinolinyl)ethenyl]phenyl][[3-(dimethylamino)-3-oxopropyl]thio]methyl] thio]propanoic acid) from Enzo Life Sciences and concanamycin A (ConA) from Santa Cruz Biotechnology Inc. *SYBR Green I* nucleic acid gel stain and Fluo-4 AM were purchased from Life technologies. All other chemicals were of the highest grade available.

Parasite Cultures- *P. falciparum* strains (e.g., 3D7, 106/1, D10, K1, Dd2, 7G8 and 7G8mdr^{D10}) were obtained from the Reference Reagent Resource Center (MR4) (Manassas, VA). The other strains (FCB, KD1^{mdr1}, GCO3, C2^{GCO3} and C4^{Dd2}) were a kind gift from Dr. David Fidock (Columbia University Medical Center, New York). All strains were maintained in culture in RPMI-1640 medium supplemented with L-glutamine (Gibco, Life technologies), 25 mM HEPES, 100 µM hypoxanthine and 10% human serum (A+ type pooled) as described previously, with modifications [21]. The parasite cultures were grown using type A+ human erythrocytes (Interstate Blood Bank) and incubated at 37°C under conditions of 3% oxygen, 5% carbon dioxide and 92% nitrogen. The culture was routinely synchronized with 5% sorbitol and the parasitemia was determined microscopically from blood smears stained with 5% Giemsa solution.

Drug Survival Assays- The SYBR Green I assay was conducted as described previously [22], with some modifications. The parasite culture was synchronized at ring stage using 5% sorbitol and diluted to 0.5% parasitemia and 2% hematocrit in 96-well plates containing serial dilutions of the appropriate drug. Parasites were allowed to proliferate in the absence or presence of drugs for 72

h at 37°C. The plates were frozen at -80°C and thawed at room temperature prior to the addition of lysis buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.008% saponin, 0.08% Triton X-100 containing 0.2 µL/ml of 10 000x SYBR nucleic gel stain dye). The lysates were incubated for 1 h at room temperature and the fluorescence was read using the microplate reader SYNERGY H4 hybrid (BIOTEK) with excitation and emission wavelengths of 485 nm and 535 nm, respectively. The data was analyzed using Prism 5.0 (GraphPad Software) to obtain the 50% inhibitory concentration (IC_{50}). All graphs shown represent the mean ± SD of two or three independent experiments done in triplicate.

In vitro β-hematin polymerization- β-hematin inhibitory activity assay was used to determine the effects of MK571 and CQ on the formation of β-hematin, as described previously [23, 24]. A total of 50 µL of an 8 mM solution of hemin dissolved in DMSO was distributed in 96-well U-bottom microplate (0.4 µmol/well) containing increasing concentrations of CQ or MK571. β-hematin formation was initiated by the addition of 100 µL of 8 M acetate buffer (pH 5.0). The plates were incubated at 37°C for 18 h and then centrifuged at 3000 rpm for 15 min. The pellet was washed once with DMSO (200 µL) to remove excess hemin; the remaining β-hematin precipitate was dissolved in 0.1 M NaOH solution (200 µL/well). The solubilized aggregates were transferred to a second 96-well flat bottom plate and diluted 1:5 with NaOH (0.1 M). The relative absorbance was measured at 405 nm using the multi-mode microplate reader (SYNERGY H4 hybrid, BIOTEK). The results are expressed as percentage of inhibition of β-hematin formation, given by the equation: % inhibition = (1 - (OD drug) / (OD control)) * 100. The drug concentration required to inhibit β-hematin formation by 50% (IC_{50}) was determined for each compound.

Live Cell Imaging of Fluo-4 Labeled Parasites- Trophozoite stage parasites (Dd2 and K1) were washed twice with Ringer's solution (122.5 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 11 mM D-glucose, 10 mM HEPES, 1 mM NaH_2PO_4, pH 7.4) and incubated for 30 min with 100 µM MK571 at 37°C prior to loading with 5 µM Fluo-4 AM for 30 min at 37°C. After incubation, the parasites were washed with Ringer's solution to remove excess dye and placed in a microperfusion chamber containing warm Ringer's solution, as described previously [20, 25]. Confocal laser scanning microscopy was performed using a Zeiss LSM710 (Carl Zeiss, Jena, Germany). Fluo-4 was excited at 488 nm with 1% transmission (argon laser, 25 mW) and fluorescence emission was detected at 493-622 nm. Single images were obtained using a 63x objective (C-APO, N.A. = 1.2), a 5-fold software zoom and a frame size of 512 × 512 pixels. A pinhole of 1.2 µm (1.34 Airy units) was chosen to ensure that measured fluorescence was mainly from within the DV and not regions lying above or below this compartment.

Image analysis – Images were obtained with the ZEN 2010 software and processed using ImageJ 1.47q. Regions of interest (ROI) were determined for the parasite's cytosol and DV. Background fluorescence was subtracted from the fluorescence intensity for each ROI. At least 70 parasites per strain were quantified. The fluorescence ratio between DV and cytosol was calculated and displayed as box plots. Absolute fluorescence and S.E.M. are shown as RFU.

pH measurements- Erythrocytes infected with Dd2 parasites at trophozoite stage (28–36 h post invasion) were washed twice with Ringer's solution (122.5 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 11 mM D-glucose, 25 mM Hepes, 1 mM NaH_2PO_4, pH 7.4) and seeded on poly-L-lysine-coated round coverslips (diameter 30 mm, No. 1.5 thickness, Harvard Apparatus, Holliston,

MA, USA) in an open chamber [20, 25, 26]. Cells were loaded with 6 µM SNARF-4F *5*-(and-6)-carboxylic acid (Life Technologies Corp., Eugene, OR, USA) and 0.05% pluronic acid (Sigma-Aldrich Co.) for 40 min and the fluorescence was monitored using an LSM710 confocal microscope system (Carl Zeiss GmbH, Jena, Germany) with a 63x water-corrected lens. A 543 nm laser was used for excitation and the emission was detected at 587 nm and 650 nm. As in previous experiments [27], ROI were defined in the parasite's cytosol and DV. For the quantification of the fluorescence intensities of the ROI, the average pixel intensity was calculated, the background fluorescence subtracted and the ratio $R_{650/587}$ evaluated using ImageJ 1.47g (National Institutes of Health, http://rsb.info.nih.gov/ij). The fluorescence ratios were measured before and 2 min after the addition of 50 µM MK571 or 100 nM concanamycin A. For each cell, a ratio of the DV and cytosolic $R_{650/587}$ ratios was calculated to estimate the pH difference between these compartments (ratio = 1 stands for identical pH in DV and cytosol, ratio < 1 for a more acidic DV and ratio > 1 for a more alkaline DV compared to the cytosol).

RESULTS

Two membrane transporters (PfCRT and PfMDR1) have been shown to mediate or modulate the sensitivity of *P. falciparum* to chloroquine and other quinoline-based drugs [14, 28]. To determine if CQ-resistant parasites are cross-resistant to the 2-amino quinoline MK571 (figure 1), CQ-sensitive and -resistant strains were allowed to proliferate in the presence of increasing concentrations of CQ or MK571.

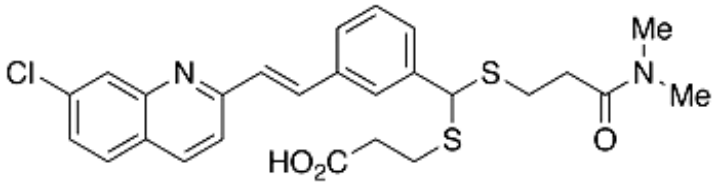

Figure 1. Organic structure of MK571 (5-(3-(2-(7-Chloroquinolin-2-yl) ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid)

Figure 2 shows the survival curves for CQ-sensitive (3D7, 106/1 and D10) and -resistant (K1 and Dd2) strains in the presence of CQ or MK571. The IC_{50} values derived from the survival curves in figure 2 for CQ-sensitive and -resistant strains for CQ are between 13 nM – 42 nM and 126 nM – 151 nM, respectively. The IC_{50} values for CQ-sensitive and -resistant strains for MK571 are between 46 µM – 55 µM and 20 µM – 23 µM, respectively. Although the results in figure 2 for CQ are as expected, those for MK571 are unexpected, where CQ-sensitive strains show a 2-fold higher sensitivity to MK571 than CQ-resistant strains (Table 1). In addition, MK571 was clearly

less toxic than CQ to the parasite by a factor of 3 logs. However, MK571 is well tolerated in humans with safe plasma levels of the drugs as high as 300 µM [29].

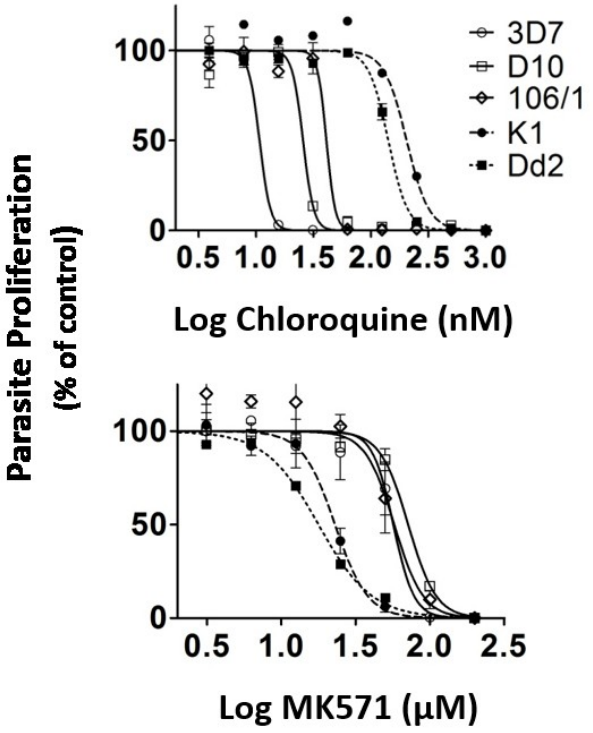

Figure 2. The proliferation of CQ-sensitive and -resistant P. falciparum in increasing concentrations of CQ and MK571. CQ-sensitive (3D7, 106/1, D10) and -resistant (K1, Dd2) strains of *P. falciparum* were cultured in the presence of increasing concentrations of CQ or MK571. The results show the effects of CQ or MK571 on proliferation of the parasite cultures relative to no drug. The graphs show the mean ± SD of three independent experiments done in triplicates.

Table 1. *In vitro* activity of MK571 and CQ on *P. falciparum* strains – effect of drugs on parasite proliferation (IC_{50}) and mutations in PfMDR1 and PfCRT.

	PfMDR1 mutations					PfCRT	IC_{50} [mean ±SD (nM)][a]	IC_{50} [mean ±SD (μM)][b]
Strains	**86**	**184**	**1034**	**1042**	**1246**	**76**	**CQ**	**MK571**
3D7	N	Y	S	N	D	K	12.85±4.3	46.25±10.0
D10	N	Y	S	N	D	K	24.98±2.2	55.09±3.7
106/1	Y	Y	S	N	D	K	42.5±5.6	48.06±5.9
K1	Y	Y	S	N	D	T	151.5±21	23.42±3.5
Dd2	Y	Y	S	N	D	T	125.92±11.1	19.75±5.4
7G8	N	F	C	D	Y	T	104.42±3.6	19.42±0.8

[a,b] Values represent mean ± SD of three independent experiments, each of which was performed in triplicate. Chloroquine (CQ).

Given the results in figure 2, it was of interest to determine if the differential sensitivity of CQ-sensitive and -resistant strains to MK571 are mediated by wild-type or mutant PfCRT. To examine this possibility, the proliferation of different parasites was determined in the presence of CQ or MK571, with or without verapamil, shown previously to block PfCRT-mediated transport of CQ [30-32]. The results in figure 3 show that the presence of 1 μM verapamil causes a significant increase in the sensitivity of CQ-resistant strains (K1 and Dd2) to CQ but had no effect on the sensitivity of CQ-sensitive strains (3D7, 106/1 and D10). These results are consistent with earlier observations, where CQ is a substrate for mutant but not wild-type PfCRT and verapamil was shown to block PfCRT-mediated transport of CQ [33]. In contrast, verapamil did not modulate the sensitivity of CQ-sensitive nor -resistant strains to MK571 (figure 3).

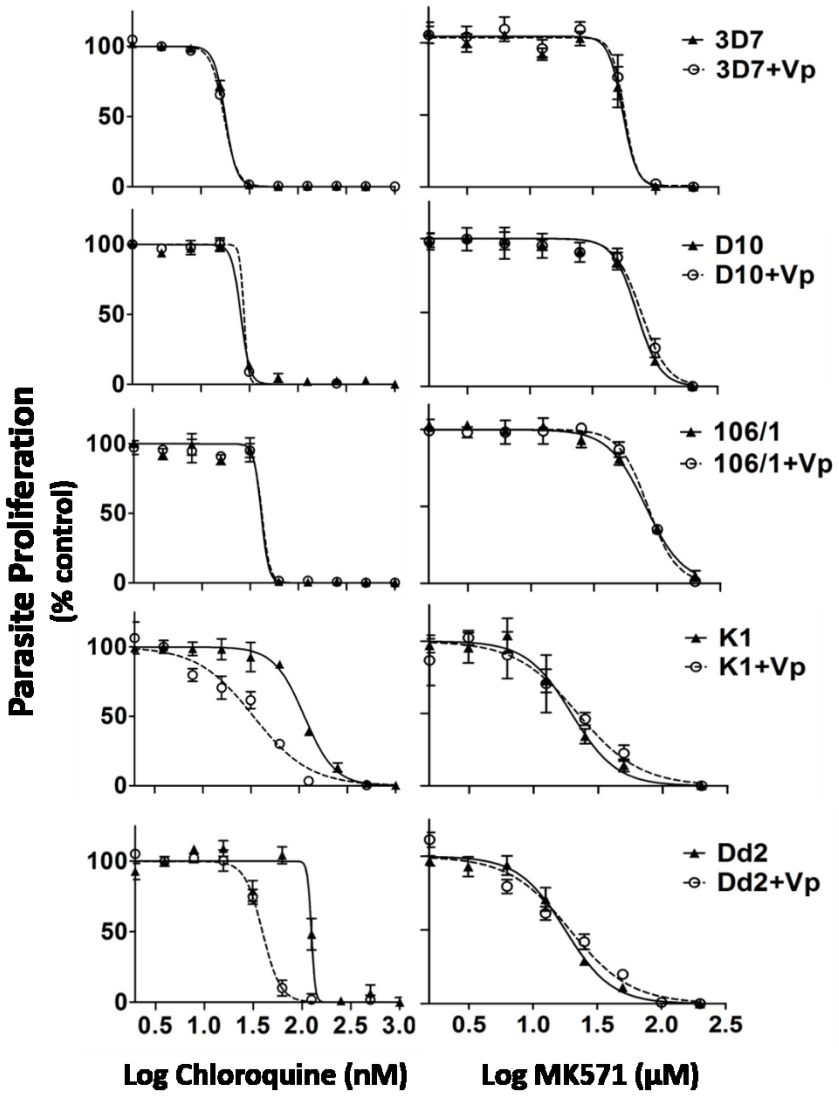

Figure 3. The effects of verapamil on the proliferation of CQ-sensitive and -resistant parasites. CQ-sensitive (3D7, 106/1 and D10) and -resistant (K1 and Dd2) *P. falciparum* were incubated in increasing concentrations of CQ or MK571 without or with 1 µM verapamil. The results show the effects of CQ plus verapamil or MK571 plus verapamil on the proliferation of parasite cultures relative to CQ or MK571 alone. The graphs show the mean ± SD of three independent experiments done in triplicates.

However, given the effects of other membrane transporters or genetic differences between the different parasites on their susceptibility to CQ and other quinoline drugs, we examined the

susceptibility of two recombinant clones encoding the wild-type and Dd2 mutant *pfcrt*-alleles in GC03 parental strain (e.g., $C2^{GC03}$ and $C4^{Dd2}$ clones, respectively) to CQ and MK571 [28]. The results in figure 4 show that the presence of Dd2 *pfcrt*-allele in GCO3 caused a significant decrease in the sensitivity of $C4^{Dd2}$ clone to CQ (IC_{50} 96.5 nM) relative to the wild-type allele in clone $C2^{GC03}$ or parental GC03 (IC_{50} 18.8 nM and 20.5 nM, respectively). However, by contrast, the presence of Dd2 *pfcrt* allele in GC03 background (e.g., $C4^{Dd2}$) did not cause a significant change in the sensitivity of the parasites to MK571 relative to the presence of the wild-type *pfcrt*-allele (e.g., $C2^{GC03}$) or parental GC03 strain ((IC_{50} 44.0 µM, 42.4 µM, 34.5 µM, respectively; figure 4 and Table 2).

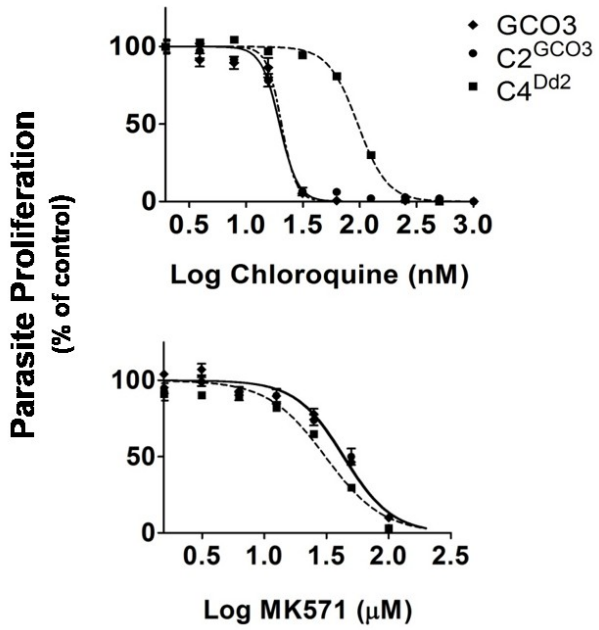

Figure 4. The proliferation of pfcrt-modified P. falciparum clones in increasing concentrations of CQ and MK571. GCO3 *P. falciparum* strain encoding wild type *pfcrt*-allele ($C2^{GCO3}$) and Dd2 *pfcrt*-allele ($C4^{Dd2}$) as GCO3 parental strains were cultured in the presence of increasing concentrations of CQ or MK571. The results show the effects of CQ or MK571 on proliferation of the parasite cultures relative to no drug. The graphs show the mean ± SD of three independent experiments done in triplicates.

Table 2. In vitro activity of MK571 and CQ on *P. falciparum* encoding wild-type *pfcrt-allele* ($C2^{GCO3}$), Dd2 *pfcrt-allele* ($C4^{Dd2}$) and parental GCO3 parasites.

	PfCRT mutations						IC_{50} [mean ±SD (nM)][a]	IC_{50} [mean ±SD (µM)][b]
Strains	**72**	**74**	**75**	**76**	**220**	**271**	**CQ**	**MK571**
GCO3	C	M	N	K	A	Q	20.91±1.9	43.77±3.1
$C2^{GCO3}$	C	M	N	K	A	Q	18.81±0.90	42.4±6.84
$C4^{Dd2}$	C	I	E	T	S	E	96.5 ±0.98	34.54±5.0

[a,b] Values represent mean ± SD of three independent experiments, each of which was performed in triplicate. Chloroquine (CQ).

Although the presence of Dd2 *pfcrt*-allele caused ~19% increase in the sensitivity of clone $C4^{Dd2}$ to MK571 relative to wild-type *pfcrt*-allele in $C2^{GCO3}$ clone, this was a modest change relative to the sensitivity of Dd2 strain (IC_{50} 19 µM versus 34.5 µM). Moreover, based on the known mechanism of PfCRT, one would have expected a decrease rather than an increase in the sensitivity of $C4^{Dd2}$ to MK571, as MK571 is expected to be transported outside the DV compartment. Although the role of PfMDR1 in CQ transport has been controversial, less debated is the direction of substrate transport across the DV membrane of the parasite. PfMDR1 is thought to mediate the transport of substrates from the parasite cytosol into the DV space [20]. Moreover, it has been suggested that the mutant but not wild-type PfMDR1 transports quinine, and mefloquine [20]. In addition, the transport function of PfMDR1 was revealed through the accumulation of the fluorescent compound Fluo-4 into parasite's DV [20]. To determine if MK571 interacts with PfMDR1, we examined the effect of MK571 on the accumulation of Fluo-4 in the CQ-resistant strains K1 and

Dd2. Figure 5 shows the accumulation of Fluo-4 as a ratio of mean fluorescence between DV and cytosol as well as the mean absolute fluorescence of Fluo-4, in Dd2 (panel A) and K1 (panel B) parasites in the absence and presence of MK571 (100 μM). The results demonstrate that the addition of MK571 to Dd2 and K1 parasites causes a large drop in the accumulation of Fluo-4 in the DV. The reduced Fluo-4 accumulation in the DV by MK571 does not lead to a significant change in Fluo-4 levels in the cytosol (Figure 5A and 5B). Figure 5C shows single cell images of K1 and Dd2 strains of *P. falciparum*-infected erythrocytes revealing visible accumulation of Fluo-4 dye in the DV in the absence of MK571, while the addition of MK571 completely inhibits the accumulation of Fluo-4 in the DV of both K1 and Dd2 strains. The difference in Fluo-4 accumulation between K1 and Dd2 could be due to different *pfmdr1* copy numbers, one and three respectively [20]. Of importance, the data demonstrates that MK571 inhibits the accumulation of Fluo-4, which suggests it to be a substrate for PfMDR1.

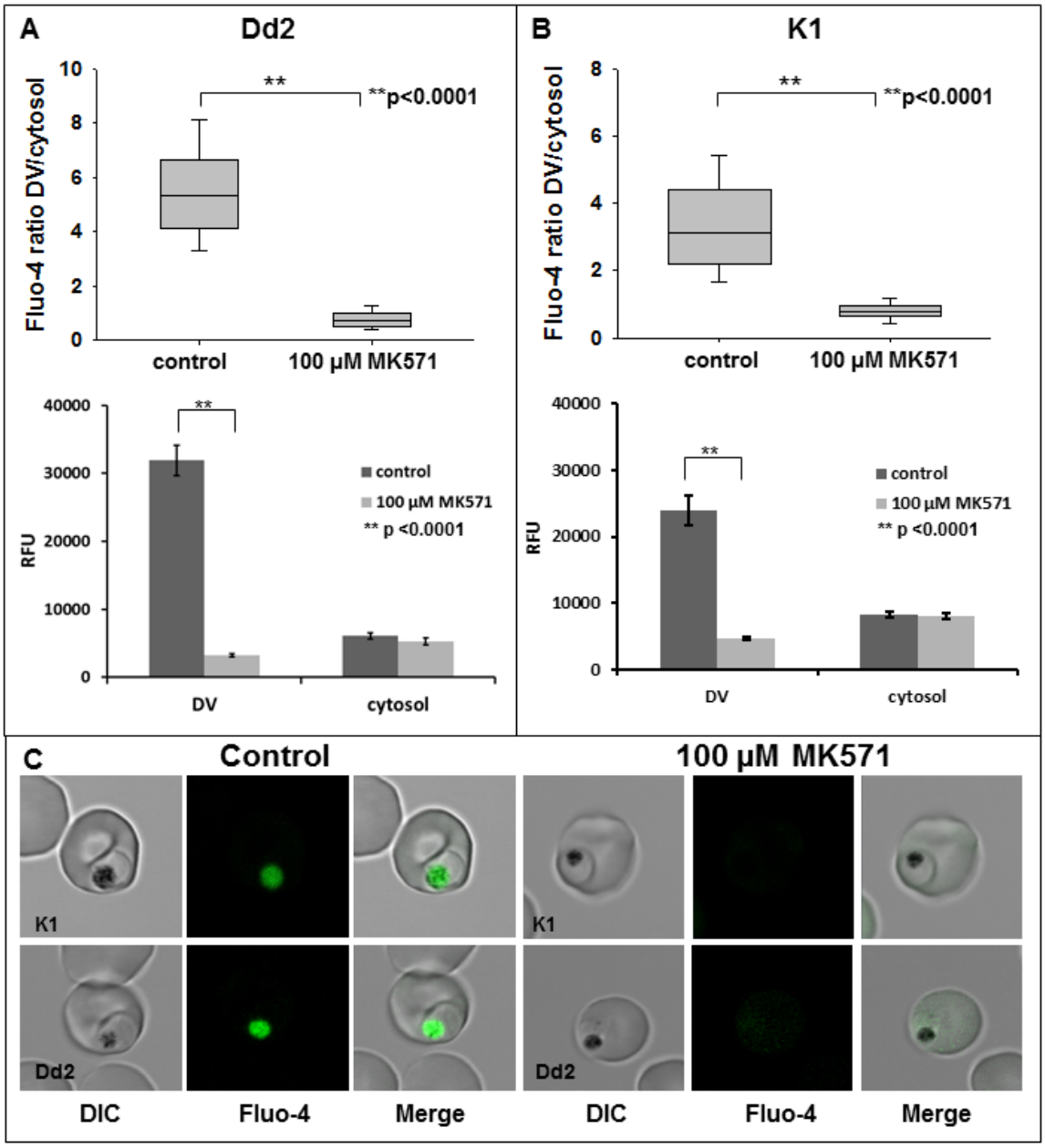

Figure 5. Fluo-4 fluorescence in Dd2 and K1 P. falciparum strains. (A and B) Mean of Fluo-4 fluorescence quantified from the digestive vacuole and the cytoplasmic regions. The first panel represents the ratios of the mean Fluo-4 fluorescence signals in the digestive vacuole and cytoplasmic regions (ratio DV/cytosol) and the second panel represents the mean of the absolute Fluo-4 fluorescence in the separate compartments. $^{**}p < 0.0001$, comparing Fluo-4 fluorescence in the control and the MK571 treated parasites. (C) Single images of *P. falciparum*-infected erythrocytes (K1 and Dd2 strains) stained with Fluo-4 AM (green) in absence (control) or presence of 100 µM MK571.

To determine if MK571 accumulates in the DV, we made use of the weak-base property of MK571 and determined its effect on the pH of the DV using a pH-sensitive dye, SNARF-4F ([34]; Wunderlich, J. *et al.,* in preparation). Figure 6 shows the effect of MK571 accumulation on the pH of the DV, relative to control (absence of drug) or in the presence of ConA. After incubation with 50 µM MK571 or 100 nM ConA for 2 min, the fluorescence ratios DV/cytosol were higher compared to untreated cells (0.71 ± 0.04, 0.83 ± 0.04 and 0.60 ± 0.02, respectively, Figure 6). These results indicate that the addition of 50 µM MK571 causes a significant reduction in the pH gradient across the DV membrane consistent with the accumulation of a weak base and trapping of the protonated MK571, which in turn leads to an increase in vacuolar pH.

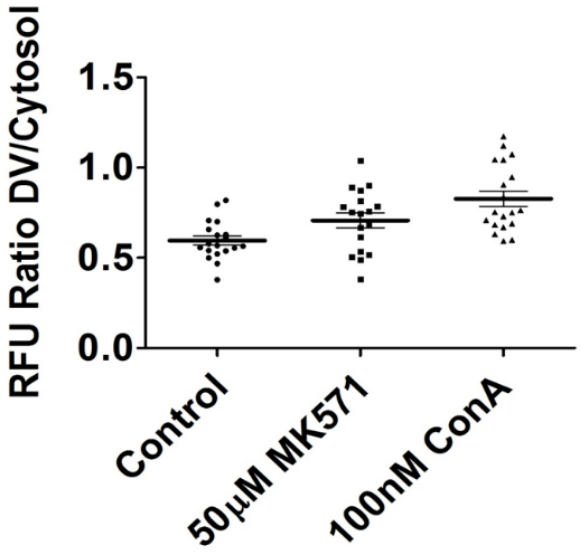

Figure 6. MK571 affects the intracellular pH of *P. falciparum*. Live cell imaging measurements show that the DV/cytosol fluorescence ratios were higher in Dd2 treated parasites as compared to untreated parasites, indicating that these drugs caused a reduction in the pH gradient between the DV and the parasite cytosol (ratio = 1 stands for identical pH in DV and cytosol and a ratio < 1 for a more acidic DV lumen compared to the cytosol). The mean ± SEM of 18 - 19 independent measurements are shown.

Taken together, the results in figures 5 and 6 suggest that MK571 accumulates in the DV and, as such, its toxicity may be due in part to the inhibition of hemozoin formation. To determine if MK571 inhibits the formation of β-hematin, we examined the effect of MK571 on the formation of β-hematin *in vitro,* as described previously [23, 24]. The results in figure 7 show dose-dependent inhibition of β-hematin with increasing concentrations of MK571 or CQ. Figure 7 shows CQ to be more effective than MK571 at lower drug concentrations. However, at higher concentrations both CQ and MK571 equally inhibited β-hematin formation *in vitro*. These results suggest that one possible mode of action of MK571 on parasite survival may be due to inhibition of β-hematin (hemozoin) formation in the DV similar to CQ and other quinoline-based drugs [35].

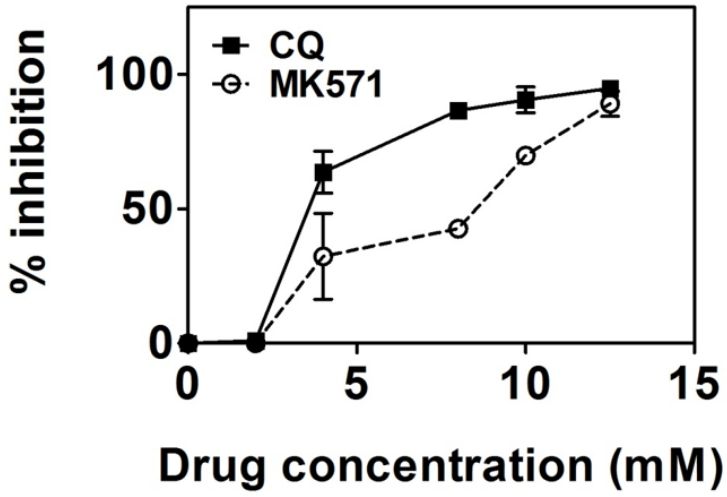

Figure 7. Effects of increasing concentrations of MK571 and CQ on β-hematin formation. The formation of β-hematin was initiated with the addition of acetate buffer to a solution of hemin in the absence and presence of increasing concentrations of MK571 or CQ. Following an overnight incubation, β-hematin aggregates were quantified relative to control, in the absence of drug, by measuring the absorbance of solubilized aggregates at 405 nm. The mean ± SD of three independent experiments done in triplicates are shown.

To determine if the differential sensitivity to MK571 between CQ-sensitive and -resistant strains is due to polymorphism in PfMDR1, we examined the effects of increasing concentrations of CQ and MK571 on the growth of 7G8 and 7G8mdr^{D10} mutant parasites. The results in figure 8A show 7G8mdr^{D10} more sensitive to CQ relative to 7G8 (IC_{50} 78.8 nM versus 104.4 nM), while the reverse was true for MK571, whereby 7G8mdr^{D10} was less sensitive to MK571 (IC_{50} 39.0 µM versus 19.4 µM; figure 8B). Although the results in figure 8 suggest that PfMDR1 polymorphism is responsible for the enhanced toxicity of MK571 with a decrease of IC_{50} from 39.0 µM to 19.4 µM for 7G8mdr^{D10} and 7G8 respectively, it is interesting that 7G8mdr^{D10} is significantly more sensitive to MK571 than the D10 strain (IC_{50} 39.0 µM versus 55.1 µM, respectively; see Table 3).

Taken together, the above results suggest that MK571 is transported more efficiently into the DV by 7G8 *pfmdr1* allele (86^{Asn} 184^{Phe} 1034^{Cys} 1042^{Asp} 1246^{Tyr}) or by K1 and Dd2 allele (86^{Tyr} 184^{Tyr} 1034^{ser} 1042^{Asn} 1246^{Asp}) than the D10 *pfmdr1* allele (86^{Asn} 184^{Tyr} 1034^{ser} 1042^{Asn} 1246^{Asp}).

Table 3. *In vitro* activity of MK571 and CQ on *P. falciparum* encoding wild-type (D10 and 7G8mdr^{D10}) and mutant *pfmdrl-allele* (7G8).

	PfMDR1 mutations					IC_{50} [mean ±SD (nM)][a]	IC_{50} [mean ±SD (µM)][b]
Strains	**86**	**184**	**1034**	**1042**	**1246**	**CQ**	**MK571**
D10	N	Y	S	N	D	24.98±2.2	55.09±3.7
7G8	N	F	C	D	Y	104.42±3.6	19.42±0.8
7G8mdr^{D10}	N	Y	S	N	D	78.8±9.8	39.02±4.6

[a,b] Values represent mean ± SD of three independent experiments, each of which was performed in triplicate. Chloroquine (CQ) and MK571.

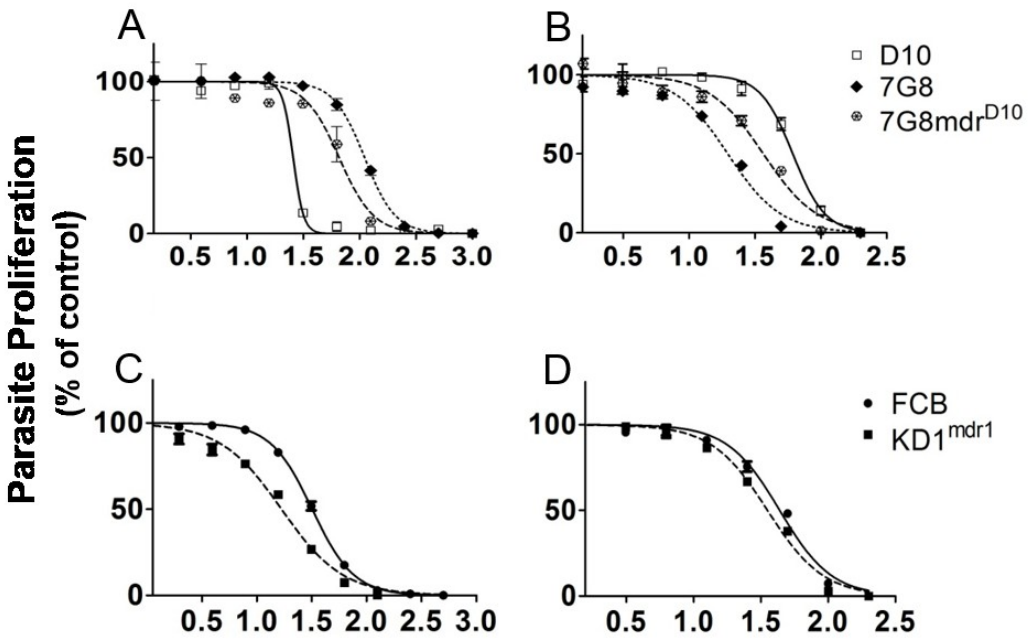

Figure 8. Effects of PfMDR1 mutations and copy numbers on the proliferation of parasites in increasing concentrations of CQ and MK571. CQ-sensitive (D10), -resistant (7G8) and mutant (7G8mdr^{D10}) parasites were allowed to proliferate in the presence of increasing concentrations of CQ or MK571 (panels A and B, respectively). FCB parental strain (two PfMDR1 gene copies) and PfMDR1 knockdown strain KD1^{mdr1} (one copy of PfMDR1) were allowed to proliferate in the presence of increasing concentrations of MQ and MK571 (panels C and D, respectively). The graphs show the mean ± SD of three independent experiments done in triplicates.

A previous study has identified a correlation between PfMDR1 copy number and the sensitivity of plasmodium strains to antimalarial drugs [36]. To assess whether PfMDR1 copy number affect the sensitivity to MK571, we compared the effects of mefloquine and MK571 on the proliferation of PfMDR1 knockdown strain KD1^{mdr1} (one copy of PfMDR1) and the parental strain FCB (two PfMDR1 gene copies). The results in figure 8C show that knockdown of one copy of PfMDR1 decreases the sensitivity of KD1^{mdr1} to mefloquine versus FCB parental strain (30.8 nM versus

18.9 nM) as previously demonstrate [36]. Interestingly, PfMDR1 knockdown in FCB caused only a small drop in the sensitivity of $KD1^{mdr1}$ to MK571 (IC_{50} 52.9 µM to 43.1 µM, respectively). Moreover, IC_{50} of the FCB CQ-resistant strain for MK571 was considerably higher (e.g., 52.9 µM) than the other CQ-resistant strains tested in this study (e.g., 7G8, K1 and Dd2 with IC_{50} (19 µM - 23 µM). The lower sensitivity of FCB strain to MK571 relative to the other CQ-resistant strains (e.g., 7G8, K1 and Dd2) is presently not clear.

Earlier studies have demonstrated that both wild-type and mut-PfMDR1 can mediate the transport of mefloquine and halofantrine across the parasite DV membrane, while only mut-PfMDR1 can mediate the transport of CQ [14, 37]. Given the results in figure 8, it was important to determine if MK571 modulated the effects of these drugs in parasites with wild-type (3D7) and mut-PfMDR1 (Dd2). Figure 9 shows the proliferation of CQ-sensitive and -resistant parasites in the presence of increasing concentrations of chloroquine, mefloquine or halofantrine in the absence or presence of non-toxic concentration of MK571. The results in figure 9 show that 10 µM of MK571 did not affect the sensitivity of 3D7 to chloroquine, while Dd2 showed a small but significant decrease in sensitivity (IC_{50} 198 nM to 236 nM). By contrast, the addition of MK571 caused a considerable increase in sensitivity to mefloquine and halofantrine in 3D7 and Dd2 strains (Table 4).

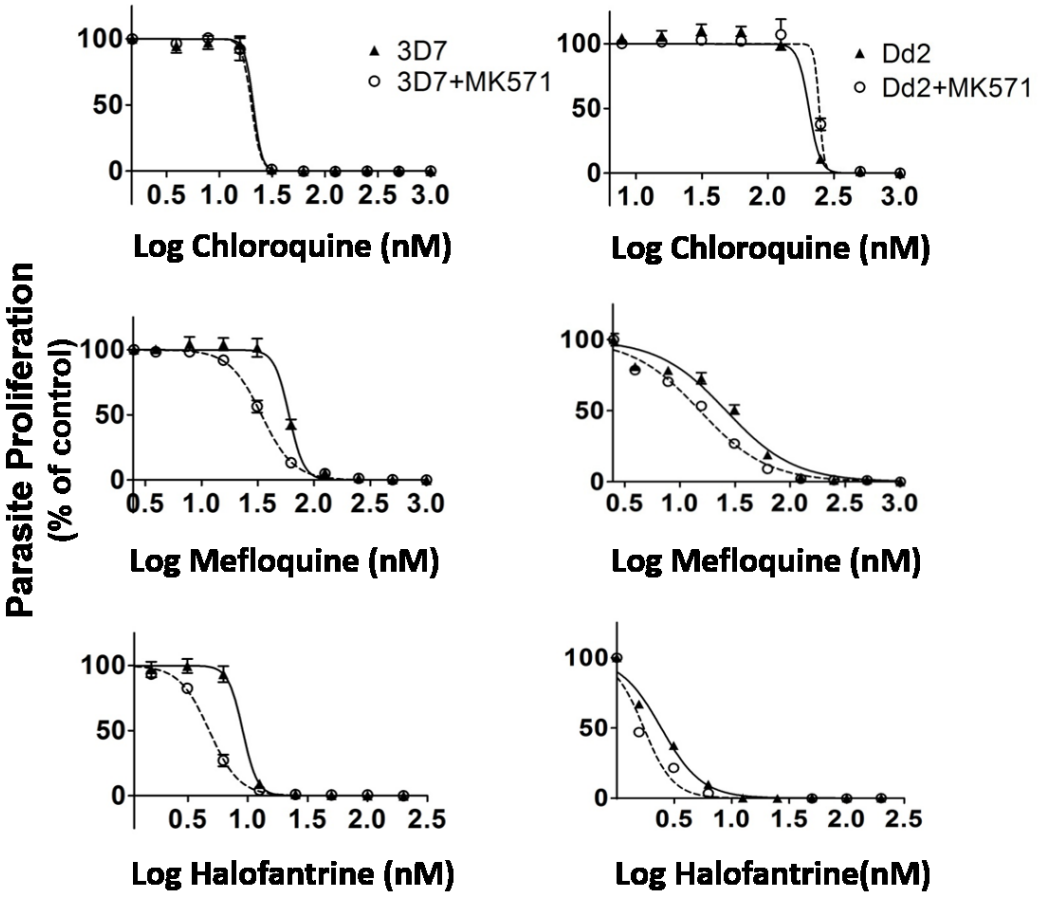

Figure 9. Effect of MK571 in combination with CQ, mefloquine or halofantrine on the proliferation of P. falciparum. Parasites (3D7 and Dd2) were allowed to proliferate in the presence of fix concentration of MK571 without or with increasing concentrations of CQ, mefloquine and halofantrine. The graphs show the mean ± SD of two independent experiments done in triplicates.

Table 4. *In vitro* activity of CQ, MQ and HF alone and in combination with MK571 for 3D7 and Dd2 strains

	IC_{50} [mean ± SD (nM)][a]	
Drug combination	3D7	Dd2
CQ	21.22±0.9	198.2±11.2
CQ+MK571	22.78±3.3	236.4±12.1
MQ	57.33±4.0	29.07±5.5
MQ+MK571	31.88±4.3	14.72±0.5
HF	8.62±1.5	2.2±0.2
HF+MK571	4.57±0.6	1.32±0.03

[a] Values represent mean ± SD two independent experiments, each of which was performed in triplicate. Chloroquine (CQ), mefloquine (MQ), halofantrine (HF).

These results suggest that MK571 interacts with both wild-type and mut-PfMDR1 and as such decreases the influx of mefloquine and halofantrine into the DV compartment causing an increase in their cytosolic drug concentrations at or near their cytosolic drug targets.

DISCUSSION

In this study we show MK571 to inhibit the proliferation of CQ-sensitive and -resistant strains of *P. falciparum*. Interestingly, MK571 was more toxic to CQ-resistant than -sensitive strains, with an average two-fold IC_{50} decrease. The toxicity of MK571, unlike that of CQ, was not reversible with verapamil; hence MK571 is unlikely to be efficiently transported by wild-type or mut-PfCRT. The observed difference between wild-type and mut-PfCRT transfected GC03 strain (e.g., $C2^{GC03}$ and $C4^{Dd2}$, respectively) to MK571 is not entirely clear, since $C4^{Dd2}$ clone showed ~19% decrease rather than the expected increase in sensitivity to MK571, if the drug was a substrate for the mutant PfCRT. Given the results in figure 4, it is likely that MK571 is a poor substrate for wild-type PfCRT, whereby the modest decrease in the sensitivity of $C2^{GC03}$ to MK571 may be due to its transport out of the DV by wild-type PfCRT. However, the lack of increase in the sensitivity of 3D7, D10 or 106/1 strains (encoding wild-type PfCRT) to MK571 in the presence of verapamil, suggests that MK571 is also unlikely to be transported by wild-type PfCRT. Although, the possibility that MK571 is a weak substrate for native PfCRT cannot be entirely ruled out based on the lack of verapamil response, as this may be due to different binding sites on PfCRT. It is worthy to note that in addition to the $76^{lys\text{-}Thr}$ PfCRT polymorphism, 3D7 and D10 which show less sensitivity to MK571 differ from K1 and Dd2 at positions $74^{Met\text{-}Ile}$ and $75^{Asn\text{-}Glu}$ and these polymorphisms may be responsible for the differential sensitivity of these strains to MK571. Alternatively, and supported by evidence described in this study, MK571 differential toxicity may be mediated by PfMDR1, and MK571 may be a better substrate for mutant compared to wild-type PfMDR1. The latter possibility is supported further by results obtained with the effects of

MK571 on the growth of 7G8 and 7G8mdr^{D10} mutant strains, which show that the decrease in CQ resistance correlates inversely with the insensitivity or increase resistance to MK571. Although, these latter results point to mut-PfMDR1-mediated influx of MK571, they are in contrast with the results obtained with PfMDR1 knockout parasites (KD1^{mdr1} clone) in figure 8. For example, if MK571 is transported into the DV via PfMDR1, then the knockout of one of two copies of PfMDR1 in FCB should lead to lesser MK571 accumulation in KD1^{mdr1} clone and consequently to a measurable decrease in the sensitivity of KD1^{mdr1} clone to MK571 relative to FCB. Interestingly, only a small decrease in the sensitivity of KD1^{mdr1} clone to MK571 was observed. This latter observation is presently not clear and is in contrast with the results obtained with 7G8 versus 7G8mdr^{D10} and those with CQ-resistant (K1, 7G8 and Dd2) versus CQ-sensitive (3D7, D10 and 106/1) strains. Moreover, the finding that FCB, a CQ-resistant parasite, is less susceptible to MK571, than all other CQ-resistant parasites tested with MK571 (e.g. K1, 7G8 and Dd2, in addition to FCR3, W2; data not shown) was unexpected. One possibility to explain the reduced sensitivity of FCB and its PfMDR1 knockout clone (KD1^{mdr1}) is the reduced accumulation of MK571 due to enhanced efflux by PfMRP1 localized to the parasite cell membrane [38]. Although the latter is a matter of speculation, it is interesting that FCB encodes two point mutations in PfMRP1 not reported to be associated to drug resistance in PfMRP1 gene (e.g., 37$^{Pro\text{-}Ser}$, 202$^{Lys\text{-}Glu}$) but are not found in 3D7, 7G8 and Dd2 strains. Hence, we speculate that mutate PfMRP1 in FCB could mediate the efflux of MK571 that may explain the reduced sensitivity of FCB to MK571 and consequently would also negate the effect of PfMDR1 knockdown in KD1^{mdr1} clone. Although the mechanism of action of MK571 on the parasite is not clear, our results show that MK571 inhibits

heme polymerization *in vitro*, albeit less efficiently than CQ. These results are not surprising given that MK571 contains the 7-chloro-4-aminoquinoline nucleus that has been shown to be essential for the inhibition of heme polymerization and consequently the growth of the parasite [39]. Moreover, the presence of the basic side chain is thought to be required for the accumulation of the drug within the DV of the parasite [35]. These structural properties of MK571 (figure 1) are consistent with our results, where we demonstrate MK571 accumulates in the DV and reduces the pH gradient across the DV membrane. MK571 might reach the DV through active transport by PfMDR1 as well as passive diffusion along the pH gradient and consequently lead to a rise in the digestive vacuolar pH via its weak-base properties. The reduced toxicity of MK571 in comparison to CQ may be related to its reduced capacity to inhibit heme polymerization.

MK571 is a leukotriene D receptor antagonist previously shown to inhibit the multidrug resistance associated protein (MRP1 or ABCC1) drug efflux function [40, 41]; however it is not known if MK571 also interacts with and inhibits the function of MRP1 homologs in *P. falciparum* (e.g., PfMRP1 and PfMRP2 [38, 42]). The results in this study suggest that MK571 interacts with PfMDR1 and inhibits the accumulation of Fluo-4 in the DV of the parasite. Furthermore, similar to Fluo-4, MK571 appears to be a better substrate for mut-PfMDR1 (86^{Tyr} 184^{Tyr} 1034^{Ser} 1042^{Asn} 1246^{Asp}) but is also transported by wild-type PfMDR1. The latter is supported by the fact that: a) MK571 is toxic to both CQ-sensitive and -resistant *P. falciparum*, and b) the presence of non-toxic concentrations of MK571 decrease the IC_{50} of CQ-sensitive strains to mefloquine and halofantrine, likely by competing against PfMDR1-mediated influx of the drugs into the parasite

DV compartment and away from its cytosolic target site(s) [14]. Taken together, the findings in this report demonstrate the interplay between PfMDR1 and PfCRT with respect to quinoline-based drugs, whereby mutations in one transporter (PfMDR1) allow for increased accumulation of MK571 and other quinoline-based drugs into the DV compartment while the limited substrate specificity of mutant or wild-type PfCRT can lead to two possibilities, a resistant phenotype (e.g., CQ) or increased sensitivity (e.g., MK571) (figure 10).

It is interesting that several structurally similar drugs to chloroquine, namely the 8-aminoquinolines (primaquine and pamaquine), are more potent against chloroquine-resistant *P. falciparum* [43]. Although earlier studies suggested that this is likely due to enhanced drug activity against chloroquine drug targets, we propose that the increased sensitivity of CQ-resistant parasites to the 8-aminoquinolines is consistent with our model in figure 10. It would be of interest to further elucidate the mechanism of action of MK571 by identifying its interaction site(s) on PfMDR1 and its potential interaction with other proteins, including PfMRP1 and PfMRP2 in CQ-sensitive and -resistant parasites. Earlier clinical trials using MK571 as an antagonist of LTD_4 receptor demonstrated that plasma drug concentrations as high as 300 µM were well tolerated in patients [29, 44]. These plasma level concentrations are higher than the levels used for CQ-resistant parasites. Therefore the addition of MK571 to other antimalarials, especially those that include mefloquine or halofantrine, could be an alternative combination therapy for the future. The presence of MK571 in drug combination would be more effective against mefloquine resistant parasites by competing against PfMDR1-mediated influx of

mefloquine into the DV and by its enhanced accumulation in the DV of resistant parasites that encode mut-PfMDR1.

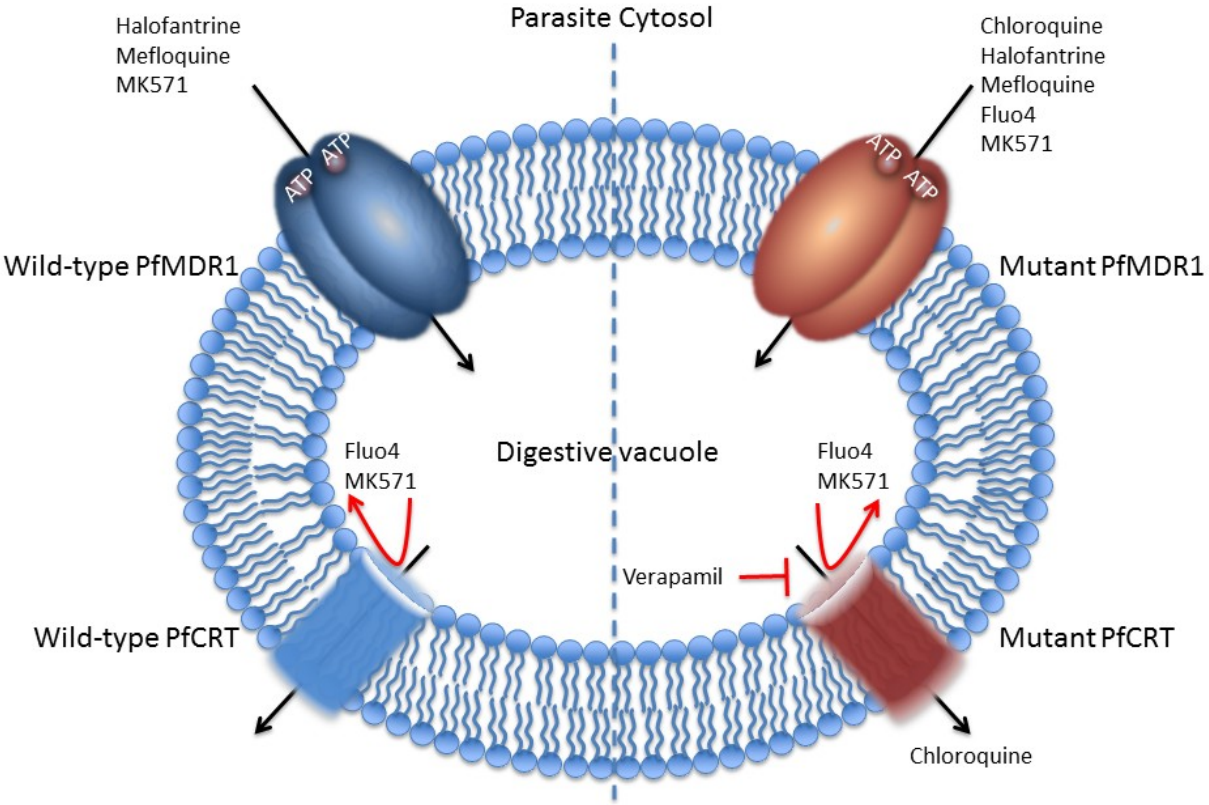

Figure 10. Schematic diagram showing substrate specificities of wild-type and mutant-PfCRT and -PfMDR1. The directions of the arrowheads across each transporter indicate the direction of substrate movement between the parasite cytosol and the digestive vacuole. The wild-type PfCRT and PfMDR1 are represented by blue figures while the mutant PfCRT and mutant PfMDR1 are represented by dark red figures. Verapamil is shown to inhibit mutant PfCRT, while Fluo-4 and MK571 are not substrates for either wild-type or mutant PfCRT. MK571, mefloquine and halofantrine are substrates for wild-type PfMDR1, while mutant PfMDR1 can transport chloroquine, Fluo4, MK571, mefloquine, halofantrine into the digestive vacuole. Possible passive diffusion of the drugs across the digestive vacuole membrane is not indicated.

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Connecting statement 2

In the previous chapter we determined that the quinoline drug MK571 has the ability to inhibit the transport function of PfMDR1. Moreover, it is more efficient against resistant Plasmodium strains compared to sensitive strains. Decades ago, the quinoline drug chloroquine was considered the most important drug for the control and elimination of malaria. Despite the establishment of the parasite's resistance to chloroquine, novel drugs based on the chloroquine structure continue to be considered. In the next chapter, we study the antimalarial potency of three novel chloroquine analogues. In addition, we investigate the mechanism of action of one of the analogues named 3-ICQ.

CHAPTER 3

Novel Chloroquine Derivative, 3-Iodo-Chloroquine, is a Potent Inhibitor of PfCRT-mediated Drug Resistance in Plasmodium falciparum

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Manuscript in preparation

ABSTRACT

Malaria affects millions of people around the world and is a serious public health concern given the development and the spread of antimalarial drug resistance. The antimalarial drug artemisinin has been effective against *Plasmodium falciparum* malaria for many years now. However the recent rise of decreased sensitivity to this drug has once again elevated the urgent need to identify and develop new antimalarial drugs. The 4-aminoquinoline, chloroquine (CQ), was the first cost effective antimalarial drug which is render now useless in some regions due to the spread of chloroquine resistant *P. falciparum*. Given the present understanding of chloroquine mode of action and of the parasite mechanism(s) of drug resistance, it is likely that novel derivatives of this drug may be viable antimalarials drugs. In this study we evaluate the *in vitro* antimalarial potential of three novel chloroquine derivatives namely 3-ClCQ, 3-BrCQ and 3-ICQ against chloroquine-susceptible and -resistant strains of *P. falciparum*. Our results show that the IC_{50}s values for the tested compounds are 10 times higher than those obtained with CQ. The derivatives IC_{50}s values for the CQ sensitive-strain range from 249 nM to 747 nM and for -resistant strain from 623 nM to 1447 nM. Moreover the CQ derivatives effect is not reversed by verapamil and thus are not substrate of $PfCRT^{mut}$. Despite its moderate activity, 3-ICQ is able to inhibit β-hematin formation *in vitro*. Interestingly, the combination of 3-ICQ with CQ results in a synergistic effect on CQ-resistant parasites demonstrating a potential of 3-ICQ to be a reversing agent for CQ resistance. 3-ICQ is a pleiotropic antimalarial agent; 1) it interferes with hemozoin formation in digestive vacuole and 2) it is a selective inhibitor of *Plasmodium falciparum* chloroquine resistance transporter (PfCRT). Our findings suggested that 3-ICQ should be considered for the development of a potential drug combination therapy with CQ.

INTRODUCTION

Malaria continues to be one of most important infectious diseases worldwide, with 135 million to 287 million cases in 2012 and an estimated 627 000 deaths annually [1]. Chloroquine (CQ) used for decades to treat malaria has been all but abandoned due to the rise and spread of CQ resistance in most malaria endemic regions [2]. Efforts to reduce the rise of drug resistance have relied on the use of multiple antimalarial drugs with different drug targets. Such malaria treatment has relied on the fast acting and highly effective antimalarial (e.g., artemisinin) together with a long lasting antimalarial drug combination (e.g., mefloquine, lumefantrine) [3]. However, given recent reports of decrease sensitivity to the latter artemisinin-based drug combination (ACT) there is an urgent need to develop novel and inexpensive antimalarial drugs and/or combination of drugs [4, 5]. Given drug development time-lines and the cost of bringing drugs from bench to bed side, one strategy for antimalarial drug treatment has been the re-introduction of previously effective antimalarial, as has been recently seen with the re-introduction of CQ [6-8].

Chloroquine is believed to inhibit the proliferation of the parasite by accumulating in the digestive vacuole (DV) whereby the protonated drug binds to hemin and interfere with the formation of hemozoin crystals [9]. The ability of CQ to interfere with hemozoin formation is governed by the presence of two chemical moieties, a) the 7-chloro group in the 4-aminoquinoline pharmacophore which plays a crucial role in the inhibition of hemozoin formation [10, 11]; and b) the presence of the basic amino group in the side chain that allows the accumulation of the drug in the parasite DV [10, 12].

Decades of accumulating evidence have established that polymorphisms in the *P. falciparum* chloroquine resistance transporter (PfCRT) are mainly responsible for parasites resistance to chloroquine and other quinoline antimalarials [13]. The presence of key point mutation at position 76 in PfCRT (e.g., K76T) has been consistently associated with chloroquine resistance in *P. falciparum* [14, 15]. Several unrelated drugs have been shown to reverse PfCRT-associated drug resistance. These drugs include, the calcium channel blocker verapamil [16], the antihistamine chlorpheniramine [17], and the tricyclic antidepressant desipramine [18]. Moreover, certain analogues of chloroquine (e.g., 7-chloro-4-aminoquinoline, have also been shown to reverse chloroquine resistance [19]. In addition to PfCRT, a second membrane transporter, the *P. falciparum* multidrug resistance protein or PfMDR1, an ABC transporter, is thought to modulate the parasite's sensitivity to chloroquine and other quinoline-based drugs [20]. Mutations in *pfmdr1* gene have been demonstrated to modulate the level of CQ resistance [21] as well as being partially responsible for the acquired resistance to other drugs such as mefloquine and halofantrine [22].

Efforts to generate new antimalarials have relied on *de novo* drug design and chemical modification of existing antimalarials. Still, 75 years after CQ introduction and despite the resistance of *P. falciparum* to CQ, novel drug candidates based on the structure of CQ continue to be considered [23-26]. Previous studies linking the structure of aminoquinoline drugs to their function have mostly focused on modifications of both the side chain and the quinoline ring [27, 28]. Several aminoquinolines derivatives have been shown to be more or less efficient than chloroquine *in vitro* on chloroquine resistant parasites [29-31]. In this report we describe the characterization of novel 4-aminoquinoline derivatives, modified at the 3rd position (with

iodine, bromine or chlorine) on the quinoline moiety. Our results show that the iodine-modified chloroquine (or 3-ICQ) is the most effective drug among the three 4-aminoquinolines derivatives tested. Interestingly, the sensitivity of the parasite to 3-ICQ is not modulated by verapamil, hence 3-ICQ is not transported outside the DV by wild-type or mutant PfCRT ($PfCRT^{mut}$). However, the presence of 3-ICQ synergizes with CQ against CQ-resistant *P. falciparum*. Possible mechanisms of synergy between 3-ICQ and CQ in resistant parasites are discussed.

MATERIAL AND METHODS

Material- *Chloroquine* diphosphate, verapamil hydrochloride, mefloquine hydrochloride, halofantrine hydrochloride and hemin were purchased from Sigma-Aldrich. SYBR® Green I nucleic acid gel stain was purchased from Life technologies. All other chemicals were of the highest grade available. The CQ derivatives were isolated as di-phosphate or tri-phosphate white solid and were characterized as NMR or IR spectroscopy.

In vitro Plasmodium falciparum parasites culture- *P. falciparum* strains 3D7 and Dd2 were provided by the MR4 Unit of the American Type Culture Collection (ATCC, Manassas, VA). The *pfcrt*-modified *P. falciparum* clones $C2^{GCO3}$ and $C4^{Dd2}$ were a kind gift from Dr. David Fidock (Columbia University Medical Center, New York). The different strains were maintained in culture in RPMI-1640 medium supplemented with L-Glutamine, 25 mM HEPES, 100 µM hypoxanthine, 2.5 µg/ml gentamicin and 10% human serum (A+ type pooled). The parasites were grown on type A-positive human erythrocytes (Interstate Blood Bank, Memphis, TN) at 37°C, with a gas mixture of 92% nitrogen, 3% oxygen and 5% carbon dioxide. Routine synchronization was performed with 5% sorbitol and the parasitemia was determined microscopically from blood smears stained with 5% Giemsa solution.

In vitro drug effect on parasite proliferation- To test the effects of various drugs on the proliferation of parasites *in vitro*, drugs (alone or in combination) were added in triplicate to ring stage parasite cultures at 0.5% parasitemia and 2% hematocrit. Following the 72h incubation at 37°C, the plates were frozen at -80°C until the performance of the assay. Frozen plates were thawed in the presence of a parasite lysis mixture (Tris, 20 mM [pH 7.5]; EDTA, 5

mM; 0.008% saponin; 0.08% Triton X-100; 0.2 µL/ml of 10 000x SYBR® Green I Nucleic Acid gel stain dye) in black plates and incubated at room temperature for an hour in the dark as previously described [32]. The fluorescence was measured using Synergy H4 plate reader with excitation and emission wavelengths of 485 nm and 535 nm, respectively. The data was analyzed using Prism 5.0 (GraphPad Software) to obtain the 50% inhibitory concentration (IC_{50}). All graphs shown represent the mean ± SD of three independent experiments done in triplicate.

In vitro isobologram analysis – The effects of CQ alone and in combination with 3-ICQ on the proliferation of the parasite was assessed using the modified fixed-ratio isobologram analysis protocol. This method is usually adopted to measure the degree of chemosensitization of a given compound and to detect if the effect between two drugs is synergistic, additive or antagonistic [33]. A stock of CQ and 3-ICQ combination was prepared in fixed-concentration ratio of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5 and serially diluted two fold to obtain six concentrations in each case. Chloroquine sensitive and resistant strains (e.g., 3D7 and Dd2, respectively) were incubated with the above drug combination for 72h and a nonlinear regression curve was used to determine the IC_{50} values for each drug alone and in combination. Fractional inhibitory concentrations (FICs) were then calculated using the equations below, as previously described [34, 35].

$$\mathrm{FICCQ} = \frac{\mathrm{IC50\ of\ CQ\ in\ combination}}{\mathrm{IC50\ of\ CQ\ alone}}$$

$$\mathrm{FIC3ICQ} = \frac{\mathrm{IC50\ of\ 3ICQ\ in\ combination}}{\mathrm{IC50\ of\ 3ICQ\ alone}}$$

$$\mathrm{FICindex} = \mathrm{FICCQ} + \mathrm{FIC3ICQ}$$

The isobologram curves were constructed by plotting FIC_{CQ} vs $FIC_{3\text{-}ICQ}$. The drug combination effect is assessed from the graphs but also by calculation. A straight diagonal line or FIC_{index}=1 indicates a clear additive effect between the two drugs, while a concave curve below the diagonal of the graph denotes a synergistic effect between the drugs and demonstrate the presence of chemosensitization. In addition the chemosensitization may be strong or moderate when the means of FIC_{index} values are respectively below 0.5 or between 0.5 and 1. A convex curve above the diagonal indicates an antagonistic effect and demonstrates an absence of chemosensitization with the FIC_{index}> 4. An FIC_{index} between 1 and 4.0 is defined as no interaction [36].

β-hematin formation assay- The BHIA (β-hematin inhibitory activity) assay is used to determine a 50% inhibitory concentration for β-hematin inhibition in equivalents of CQ and 3-ICQ with respect to hemin. The assay was performed as described previously [32] and the results are expressed as percentage of inhibition of β-hematin formation given by the equation:

% inhibition = (1 - (OD drug) / (OD control)) * 100.

The data was analyzed using Prism 5.0 (GraphPad Software) to obtain the 50% inhibitory concentration (IC_{50}). All graphs shown represent the mean ± SD of three independent experiments done in triplicate.

MTT cell viability assay- The human leukemic CCRF-CEM cells and the cervical cancer Hela cells were grown in α-MEM medium supplemented with 10% fetal bovine serum (FBS, Gibco) at 37 °C in the presence of 5% CO_2. The sensitivity of the cells to CQ and 3-ICQ was determined using MTT assay [37].Cells were seeded in triplicate into 96-wells plate at 2.5×10^3 cells/well for HeLa

and 1.5 × 10^4 cells/well for CCRF-CEM cells prior to the addition of drugs. The cells were grown in the absence or presence of 1 - 100 µM of 3-ICQ or CQ for 48h at 37 °C before the addition of MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide]. The MTT dye was added to each well at a final concentration of 0.5mg/mL, and cells were then incubated for another 4h at 37 °C. The reduced formazan dye was dissolved with 100 µL DMSO/Ethanol (V/V), and the effect of the drugs on cell growth was determined from differences in absorbance between drug-treated cells and untreated or solvent control. The results shown represent the mean ± SD of three independent experiments done in triplicate.

RESULTS

The synthesis strategy of the three CQ derivatives, 3-ClCQ, 3-BrCQ and 3-ICQ, will be described elsewhere (Tazoo, D. and Bohle S., in preparation). Figure 1 shows the structures of the CQ derivatives, with the halogen substituent at position 3 on the quinoline ring.

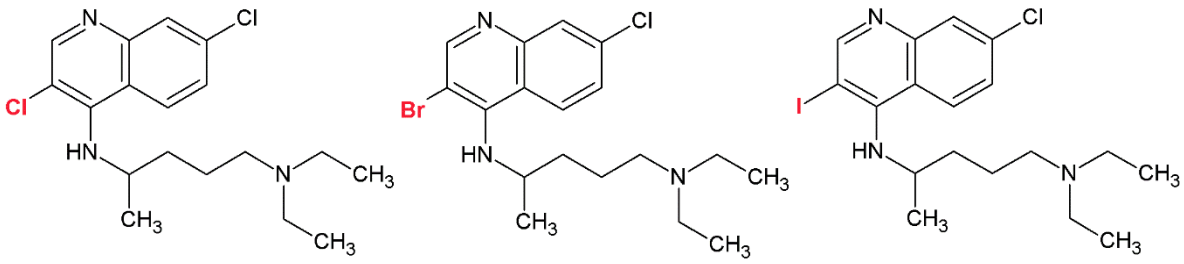

Figure 1. Chemical structure of chloroquine derivatives.

The efficiency of the three derivatives (e.g., 3-ClCQ, 3-BrCQ and 3-ICQ) against drug sensitive and resistant lab strains of *P. falciparum* was evaluated *in vitro* and compared to CQ. Figure 2 shows the effects of drugs on the proliferation of CQ-sensitive (3D7) and CQ-resistant (Dd2) strains in the presence of increasing molar concentrations of CQ and each of the three CQ derivatives. As expected, CQ was less effective at inhibiting the proliferation of CQ-resistant (Dd2) than the CQ-sensitive (3D7) strain with the IC_{50}s values of 178 nM and 21 nM, respectively (~8.5 fold difference in sensitivity). Interestingly, the IC_{50}s for all three CQ derivatives showed 1-2 fold differences for CQ-sensitive relative to -resistant strain (i.e., 367 nM – 747 nM for 3D7 versus 623 nM to 1163 nM for Dd2; see Table 1). Figure 2 also shows that modification at 3rd position of the quinoline ring causes roughly 10-fold drop in the IC_{50} values

of the three derivatives, relative to unmodified CQ, at least with respect to 3D7. Moreover, among the three derivatives, 3-ICQ showed the lowest IC_{50} for CQ-sensitive and –resistant strains (e.g., 367 nM and 623 nM, respectively; Table 1).

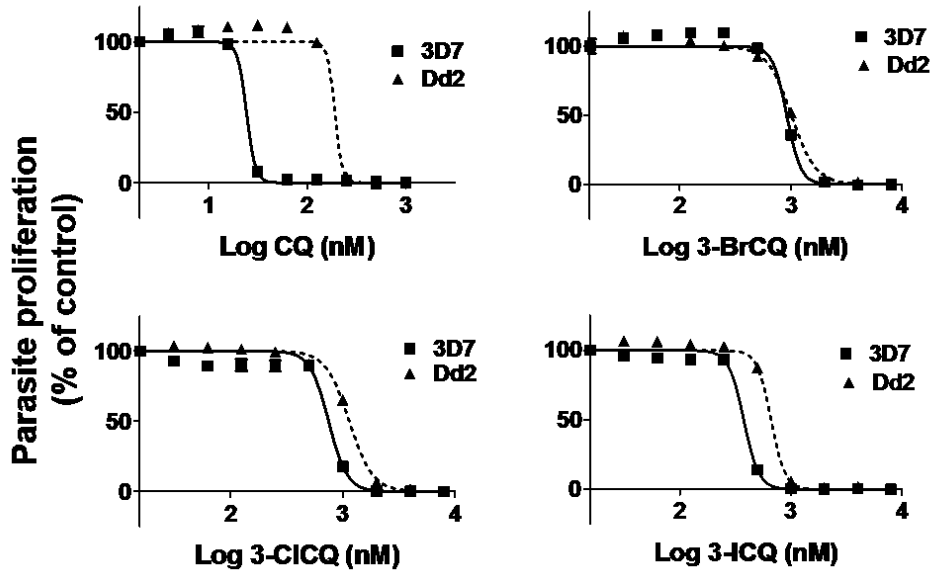

Figure 2. Antiplasmodial activity of CQ and CQ derivatives on *P. falciparum* strains. CQ-sensitive (3D7) and -resistant (Dd2) strains of *P. falciparum* were incubated with increasing concentrations of CQ, 3-ClCQ, 3-BrCQ or 3-ICQ. The graphs show the mean ± SD of three independent experiments done in triplicates. Note that the error bars fall within the boundaries of the symbols.

Table 1. *In vitro* activity of CQ and CQ analogues alone and in combination with 1 µM Vp on 3D7 and Dd2 *P. falciparum* strains.

Drugs		IC50 [mean±SD (nM)][a]	
	VP (1µM)	3D7	Dd2
CQ	-	21.24±1.65	178.27±4.61
	+	21.26±2.04	77.11±2.58
3-ClCQ	-	747.20±65.0	1163.33±46
	+	767.05±90.7	1141.3±20.8
3-BrCQ	-	712.40±89.6	987.77±48.5
	+	647.65±85.3	982.80±47.3
3-ICQ	-	367.00±24	623.47±72.5
	+	335.35±46.1	621.23±23.3

[a] Values represent mean ± SD of three independent experiments, each of which was performed in triplicate. Chloroquine (CQ).

Previous studies have shown that verapamil reverses CQ resistance *in vitro* by blocking $PfCRT^{mut}$-mediated efflux of CQ from the DV [16, 38, 39]. Given the results in figure 2, it was of interest to investigate the ability of verapamil to increase the sensitivity of CQ-resistant parasites to some or all three CQ derivatives. To test the latter possibility, the proliferation of CQ-sensitive and -resistant strains was determined in the presence of CQ and CQ derivatives with or without 1 µM verapamil. The results in figure 3 show that the presence of verapamil significantly increases the sensitivity of the CQ-resistant (Dd2), but not CQ-sensitive (3D7), strain to CQ. Interestingly, verapamil did not modulate the sensitivity of CQ-sensitive or -resistant strains to CQ derivatives (figure 3, Table 1).

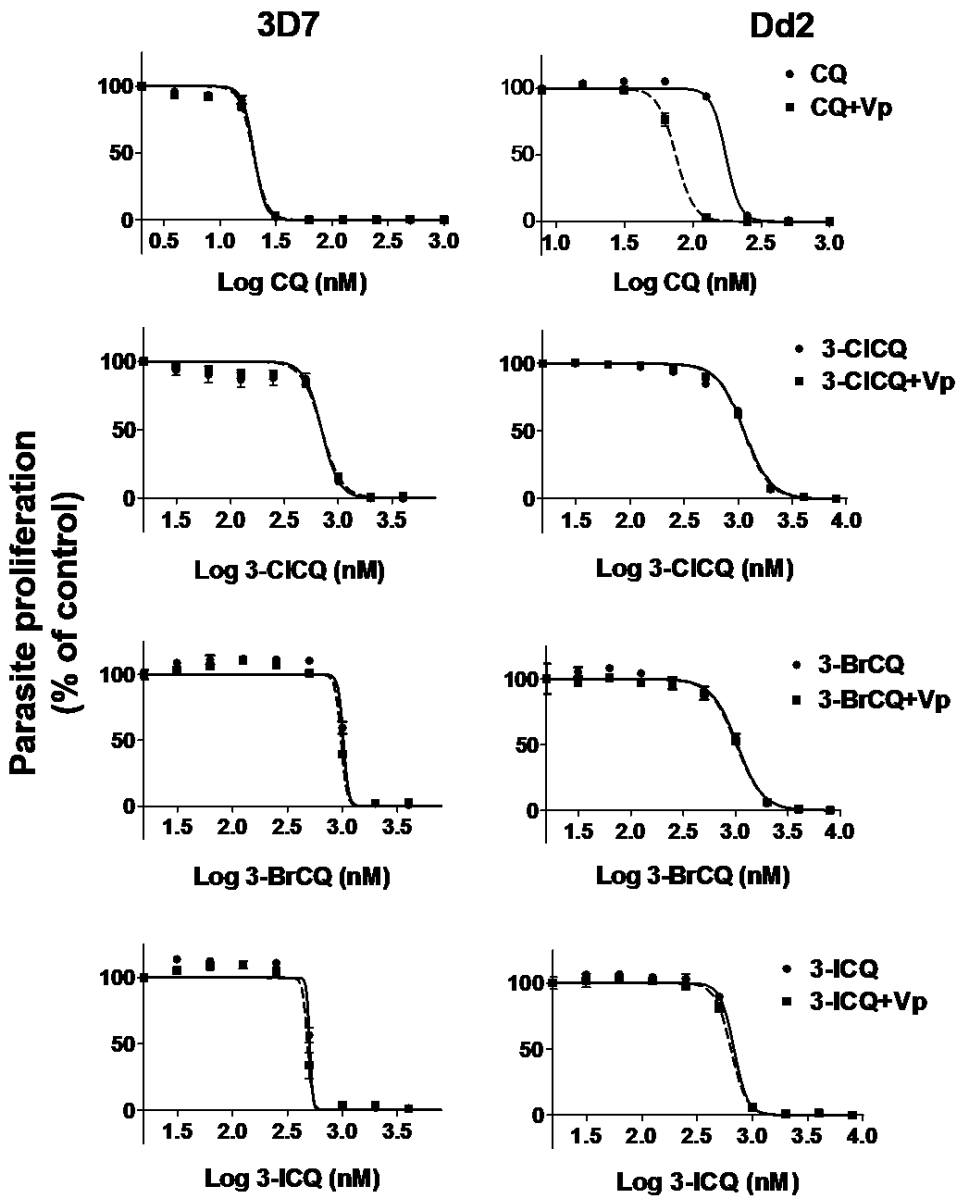

Figure 3. Effects of verapamil on the proliferation of P. falciparum parasites in the presence of CQ and CQ derivatives. CQ-sensitive (3D7) and -resistant (Dd2) strains were incubated in increasing concentrations of CQ, 3-ClCQ, 3-BrCQ and 3-ICQ without or with 1 µM verapamil. The graphs are from a representative experiment done in triplicates.

These results suggest that CQ derivatives are not transported via wild-type or mutant-PfCRT. However given the presence of other genetic differences between the two strains (3D7 and

Dd2), in addition to mutations in PfCRT, it is not possible to rule out the role of PfCRT in modulating the sensitivity of the parasite to CQ-derivatives. To determine if CQ-derivatives are transported by $PfCRT^{mut}$, similar to CQ, we made use of two clones generated from a parental strain GCO3 (CQ-sensitive) which harbour wild-type *pfcrt*-allele ($C2^{GCO3}$) or mutant Dd2 *pfcrt*-allele ($C4^{Dd2}$) [2]. The results in figure 4 show that the presence of Dd2 *pfcrt*-allele in the CQ-sensitive strain GCO3 did not modulate the IC_{50} values of 3-ICQ (174.7 nM and 176.05 nM respectively for $C2^{GCO3}$and $C4^{Dd2}$). Moreover, the presence of verapamil did not modulate the sensitivity of $C4^{Dd2}$ to 3-ICQ, but caused a significant decrease in the sensitivity of $C4^{Dd2}$ clone to CQ (Table 2, figure 4). Taken together, the sensitivity of *P. falciparum* to 3-ICQ is not modulated by wild-type or mutant PfCRT.

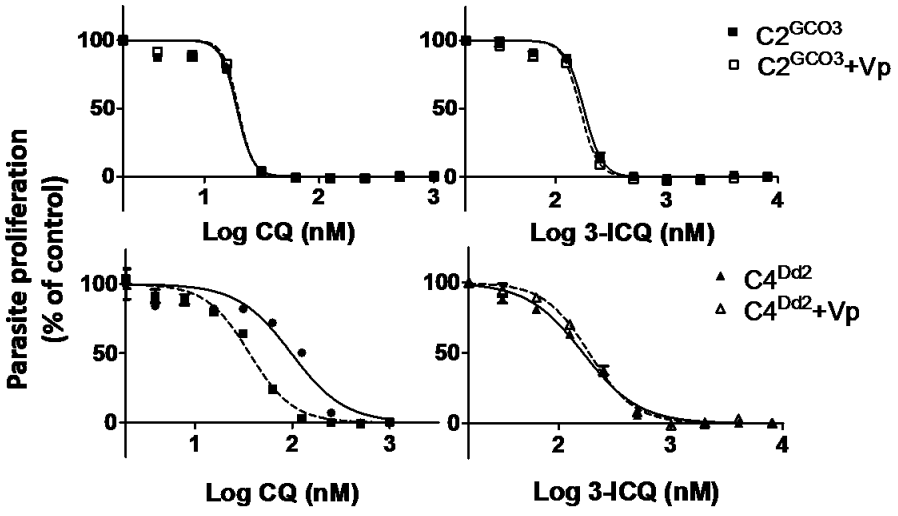

Figure 4. Effects of verapamil on *pfcrt*-modified *P. falciparum* clones in the presence of CQ and 3-ICQ. $C2^{GCO3}$with wild type *pfcrt*-allele and $C4^{Dd2}$ with mutant type Dd2 *pfcrt*-allele were incubated in increasing concentrations of CQ and 3-ICQ with or without 1 µM verapamil. The results show the effects of CQ plus verapamil or 3-ICQ plus verapamil on the proliferation of the parasites relative to CQ or 3-ICQ alone. The graphs are from a representative experiment done in triplicates.

Table 2. *In vitro* activity of 3-ICQ and CQ alone or in combination with 1µM verapamil (Vp) on *P. falciparum* PfCRT-transfectants strains

	PfCRT mutations						IC_{50} [mean ±SD (nM)][a]			
Strains	**75**	**76**	**220**	**271**	**326**	**356**	**CQ**	**CQ +Vp (1µM)**	**3-ICQ**	**3-ICQ+Vp (1µM)**
$C2^{GCO3}$	N	K	A	Q	N	I	18.81±0.9	19.65±0.4	175.33±10.7	166.33±3.5
$C4^{Dd2}$	E	T	S	E	S	T	96.5±0.9	32.44±6.4	168.68±17.5	185.5±9.3

[a] Values represent mean ± SD of three independent experiments, each of which was performed in triplicate. Chloroquine (CQ), Verapamil (Vp).

CQ is thought to bind ferroprotoporphyrin IX (FP-IX) mainly in the DV and interfere with the parasite's ability to detoxify the released hemin into insoluble hemozoin or β-hematin [40, 41]. Given the structural similarities between CQ and 3-ICQ, we examined the ability of 3-ICQ to interact with ferroprotoprophyrin IX (FP-IX) and prevent β-hematin formation *in vitro*, as previously demonstrated for CQ [32, 42]. The results in figure 5 show the percent inhibition of β-hematin formation in the presence of increasing molar concentrations of CQ or 3-ICQ.

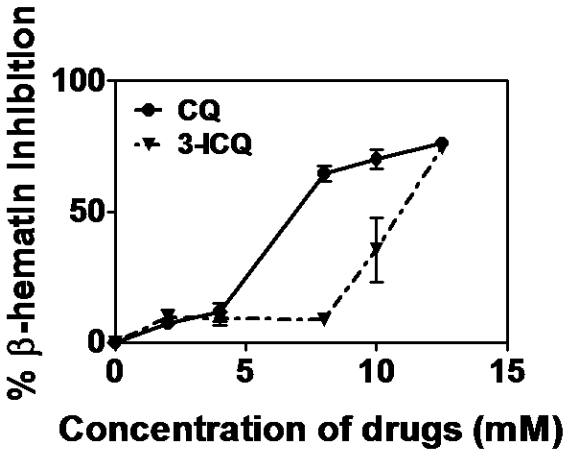

Figure 5. Effect of CQ and 3-ICQ on β-Hematin formation.

Both CQ and 3-ICQ inhibit β-hematin formation, albeit at different molar concentrations, with IC_{50}s for CQ and 3-ICQ at 8 mM and 12 mM, respectively. It should be mentioned that the latter β-hematin inhibitory assay is an *in vitro* approximation of the drug effects in the DV of the parasite; nonetheless these results are supported by differences in the efficacies of the drugs on the proliferation of the parasite (see figure 2 and table 1). Consequently, the results in figure 5 support the mode of action of 3-ICQ and suggest that the presence of the large Iodine atom at 3^{rd} position of the quinoline ring is likely to reduce its binding to hemin.

Earlier reports suggested that mutations in *pfmdr1* gene can modulate the levels of CQ resistance [21] and consequently 3-ICQ could interact with wild-type or mutant PfMDR1. To test for this possibility, we examined the effects of non-toxic concentrations of 3-ICQ on proliferation of 3D7 (wild-type PfMDR1) and Dd2 (mutant PfMDR1) in the presence of increasing concentrations of CQ, MQ and HF. Figure 6 shows that non-toxic concentrations of 3-ICQ increased the sensitivity of CQ in both 3D7 and Dd2, but to greater extend that of Dd2.

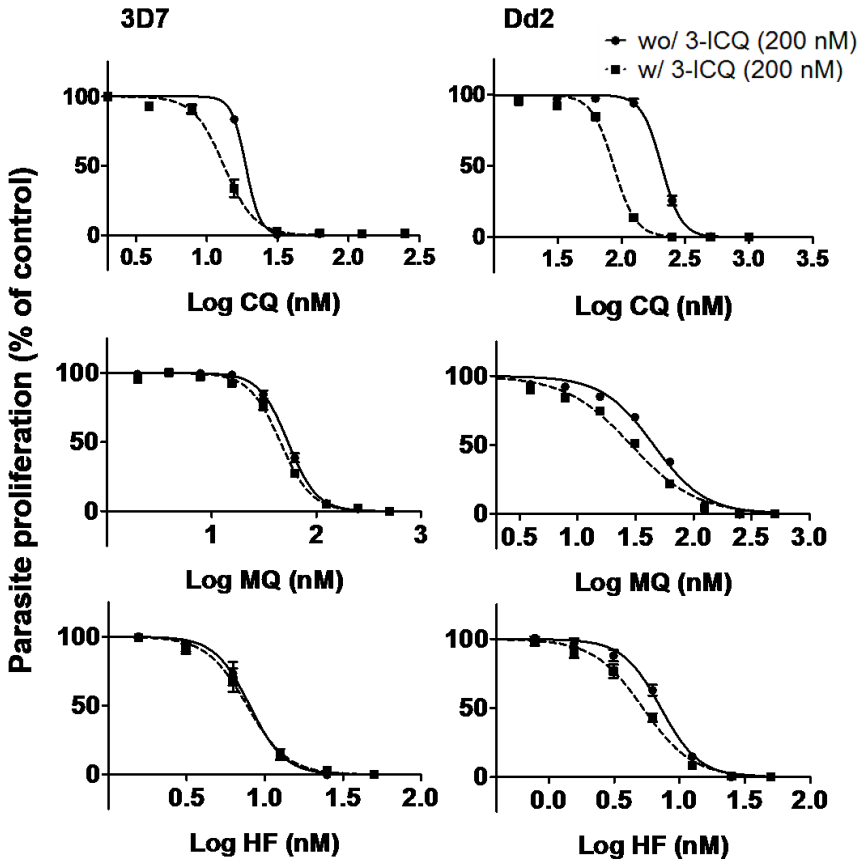

Figure 6. Effect of 3-ICQ in combination with chloroquine (CQ), mefloquine (MQ) or halofantrine (HF) on the proliferation of P. falciparum strains. CQ-sensitive (3D7) and -resistant (Dd2) strains were grown in the presence of an increasing concentrations of CQ, MQ and HF without or with 200 nM 3-ICQ. The graphs show the mean ± SD of three independent experiments done in triplicates. Note that the error bars fall within the boundaries of the symbols. w, with; wo, without.

The latter findings are significant since the potentiation of CQ toxicity in Dd2 is unlikely due to enhanced influx of CQ in the presence of 3-ICQ, but rather due to inhibition of CQ efflux via $PfCRT^{mut}$, similar to the effect of verapamil on CQ toxicity seen in figure 3. The small, but significant increase in the sensitivity of Dd2 to MQ and HF in the presence of non-toxic concentrations of 3-ICQ is suggestive of its interaction with PfMDR1 and consequently

interfering with PfMDR1-mediated influx of MQ or HF away from their cytoplasmic drug targets [43]. To determine if the potentiation of CQ toxicity in the presence of non-toxic concentrations of 3-ICQ is dose-dependent and observed with other strains of *P. falciparum*, we examined the effects of non-toxic concentrations of 3-ICQ (50 – 200 nM) on the proliferation of two CQ-sensitive (3D7 and D10) and CQ-resistant (Dd2 and FCR3) parasites in the presence of increasing concentrations of CQ. The results in figure 7 and table 3 show that 50 nM of 3-ICQ is able to render 3D7, D10, FCR3 and Dd2 more sensitive to chloroquine. Moreover, the sensitivity of all four strains to CQ increased in a dose-dependent manner with increasing concentration of 3-ICQ (50, 100 and 200nM) (Figure 7, Table 3).

Table 3. *In vitro* activity of fixed concentration of 3-ICQ on CQ effect on *P. falciparum* strains

	IC50 [mean±SD (nM)][a]			
Drugs (nM)	**3D7**	**D10**	**Dd2**	**FCR3**
CQ	23.17±1.99	29.64±5.85	185±14.6	122.2±7.6
CQ+3-ICQ (50 nM)	19.35±0.89	23.03±1.77	108±21.1	70.2±0.9
CQ+3-ICQ (100 nM)	18.10±2.28	18.98±0.93	83.3±5.02	41.3±1.8
CQ+3-ICQ (200 nM)	10.05±2.82	5.39±0.79	46±12.3	14.1±5.3

[a] Values represent mean ± SD of three independent experiments, each of which was performed in triplicate. Chloroquine (CQ).

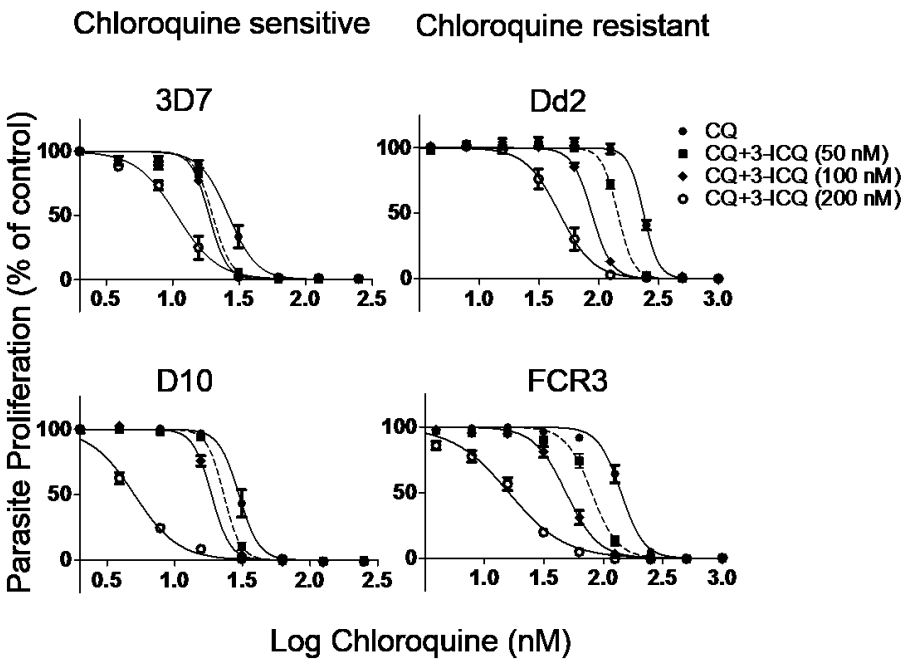

Figure 7. Effect of fixed concentration of 3-ICQ in combination with chloroquine on the proliferation of 3D7, D10, FCR3 and Dd2 plasmodium strains. CQ-sensitive (3D7, D10) and -resistant (FCR3, Dd2) strains were grown in the presence of increasing concentrations of CQ, without or with 50, 100 and 200 nM of 3-ICQ. The graphs show the mean ± SD of three independent experiments done in triplicates.

The fact that the IC_{50} value of both drugs combination is lower than the IC_{50} value of each drug alone suggests a chemosensitization effect. To identify the nature of the interaction between CQ and 3-ICQ, fixed ratios of both compounds were diluted and FIC values derived from IC_{50} values were determined for each of the compound alone and in combination. The results of the FIC-based isobologram analysis are shown in figure 8.

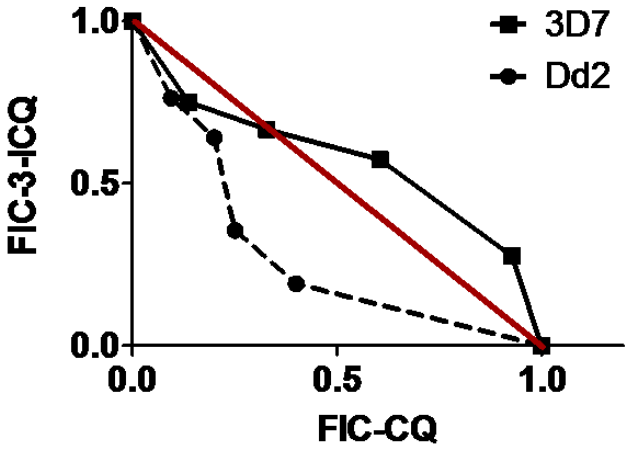

Figure 8. Isobologram of the in vitro interaction between CQ and 3-ICQ against the CQ-resistant Dd2 (circular symbols) and the CQ-sensitive 3D7 (square symbols) P. falciparum strains. The X axis represents the FICs of CQ, and the Y axis represents the FICs of 3-ICQ. Each point represents a FIC of 3-ICQ and its corresponding FIC of CQ. The diagonal red straight line indicates the hypothetical additive drug effect.

One can notice a moderate synergy in the combination of CQ with 3-ICQ against Dd2 (CQR strain) with the mean FIC_{index} =0.7 meaning <1. In contrast there is no synergism between CQ and 3-ICQ against 3D7 (CQS strain) but rather an additive effect as evidenced by the isobologram curve. Indeed, this curve is near the diagonal line with the mean FIC_{index} = 1.06. These results demonstrate that 3-ICQ behaves like the classical chemosensitizer verapamil [16].

The increased sensitivity of 3D7 and D10 to CQ in the presence of non-toxic concentrations of 3-ICQ is interesting and suggests that a) wild-type PfCRT allows the efflux of CQ (Figure 6), or b) 3-ICQ potentiates the toxicity of CQ via another mechanism.

To examine the effect of PfCRT mutations in the ability of 3-ICQ to enhance the potency of CQ, *pfcrt* modified-clones ($C2^{GCO3}$ with wild type *pfcrt*-allele; $C4^{Dd2}$ with mutant type Dd2 *pfcrt*-allele) were chosen for their common isogenic background with the only modification being in the PfCRT allele [2]. Figure 9 shows the proliferation of the parasites in increasing concentration of CQ and fixed concentrations for both 3-ICQ and verapamil (25, 50, 75, and 100 nM).

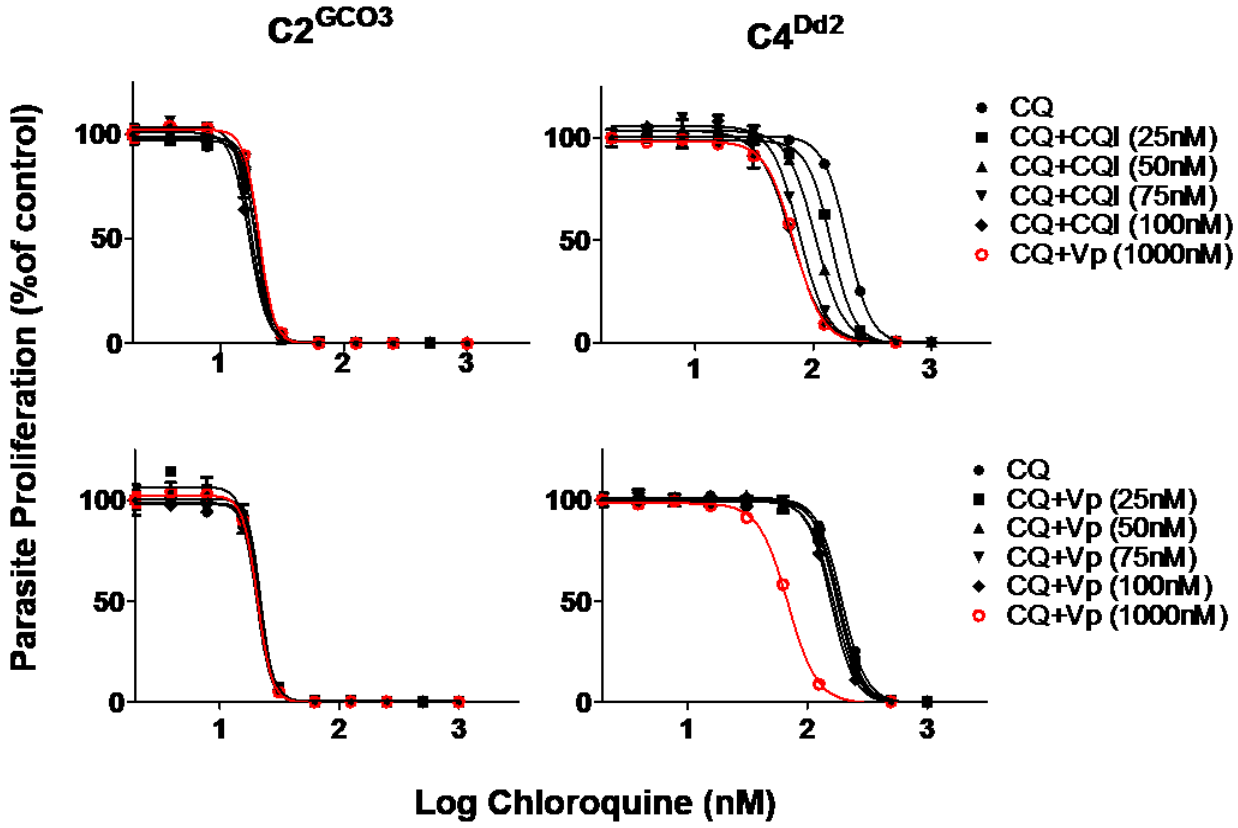

Figure 9. Effect of fixed concentration of 3-ICQ and verapamil in combination with chloroquine on the proliferation of pfcrt-modified clones. $C2^{GCO3}$ and $C4^{Dd2}$ plasmodium strains were grown in the presence of increasing concentrations of CQ, without or with 25, 50, 75 and 100 nM of 3-ICQ and Verapamil. 1µM of verapamil was also used as control. The graphs represent the mean ± SD of three independent assays.

Verapamil was also used at higher concentration (e.g., 1000 nM) to demonstrate clear potentiation of CQ sensitivity in $C4^{Dd2}$ based on earlier observations (Figure 4). The results show that 3-ICQ and verapamil did not potentiate the effect of CQ on the proliferation of $C2^{GCO3}$ clone expressing wild-type PfCRT (see figure 9 and Table 4). However, by contrast, 3-ICQ potentiated the toxicity of CQ in $C4^{Dd2}$ expressing the mutant PfCRT (Figure 9 and Table 4). It is important to note that 100 nM of 3-ICQ showed equal potentiation of CQ toxicity to 1000 nM verapamil (Table 4).

Table 4. IC_{50}s of CQ alone and in combination with 3-ICQ and Verapamil for $C2^{GCO3}$ (CQS) and $C4^{Dd2}$ (CQR) strains

	IC50 [mean±SD (nM)][a]	
Drugs combination (nM)	**$C2^{GCO3}$ (CQS)**	**$C4^{Dd2}$ (CQR)**
CQ	23.34±4.45	198.95±7.42
CQ+3-ICQ-25	19.86±0.18	135.25±8.84
CQ+3-ICQ-50	18.53±1.16	103.00±1.70
CQ+3-ICQ-75	17.80±0.13	74.95±1.76
CQ+3-ICQ-100	15.10±2.06	63.78±2.21
CQ+Vp-25	21.20±1.60	197.60±18.24
CQ+Vp-50	20.49±2.83	176.80±0.71
CQ+Vp-75	20.51±2.34	163.95±2.47
CQ+Vp-100	19.43±5.59	156.75±1.06
CQ+Vp-1000	22.58±4.5	70.13±1.8

[a] Values represent mean ± SD of three independent experiments, each of which was performed in triplicate. Chloroquine (CQ), Vp (verapamil).

Taken together, the above results suggest that 3-ICQ may be clinically useful compound, in combination with CQ. To assess whether 3-ICQ is toxic to mammalian cells, two human cells lines (CEM or T-cell lymphoma and HeLa Ovarian cancer cell lines) were used to compare its toxicity to CQ. Figure 10 shows the effects of CQ or 3-ICQ (0 – 100 uM) on the growth of the two human tumour cell lines. At 50 µM, both CQ and 3-ICQ affect the growth of both cell lines while 10 µM did not show a significant effect on cell growth.

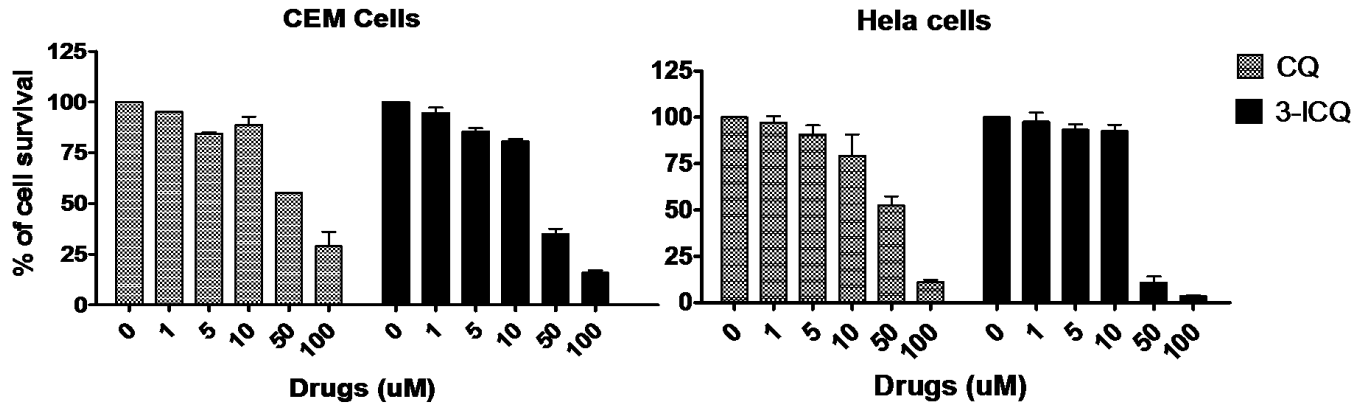

Figure 10. Effect of increasing concentration of CQ and 3-ICQ on the growth of Hela and CEM cells. Hela and CEM cells were grown in the presence of fixed concentrations of CQ and 3-ICQ (1, 5, 10, 50, 100 µM). The graphs represent the mean ± SD of two independent experiments done in triplicate.

DISCUSSION

The antiplasmodial activity of 4-aminoquinolines lies in the presence of an aromatic group and an amino alcohol group in such a way that the amino and alcohol groups are separated by two to three carbon atoms [44]. The introduction of Chlorine, Bromine or Iodine atom between the amino and alcohol group in the 7-chloro-4-aminoquinoline core of CQ reduces the activity of the derivatives towards CQS and CQR strains. This is consistent with earlier studies that have revealed that a structural change of the 7-chloro-4-aminoquinoline ring in CQ reduces its antimalarial activity [10, 45]; whereas changes in the CQ side chain appears to be more promising [46]. Of the CQ derivatives tested, only 3-ICQ has shown the highest activity and this is likely due to the premature break of Carbone-Iodine bound in the 3-ICQ compound. Such break leads to the release of CQ and then triggers the destruction of the parasites. The higher IC_{50}s value can be assimilated to the delay in 3-ICQ bond breakage [47]. Previous studies have shown that the carbon-halogen bound dissociation energies decrease respectively in compound containing chlorine, bromine and iodine group with the latest having the weakest energy bound. Such dissociation can explain the differential activities among the CQ derivatives [47]. The most efficient derivative, 3-ICQ, was able to interact with hemin and inhibit β-hematin formation *in vitro,* as previously demonstrated for CQ [42]. However the inhibition of β-hematin formation solely is not a prediction of a good antimalarial activity as seen in many studies [19, 48]. Many factors explain this weaker activity namely the degree of hydrophobicity of the drug, the differential accumulation of 3-ICQ in the food vacuole, or the involvement of an unidentified transporter. The activity of the three tested compounds on CQR strains is not enhanced by the addition of verapamil, unlike CQ. This non-chemosensitization effect of

verapamil have been reported in previous studies with other aminoquinoline derivatives on CQR strains [46, 49]. The authors demonstrated that the effect of the derivatives (which contain a modification at the quinoline ring) on the parasites is not potentiated by verapamil. Hence, the structural modification of the quinoline ring is able to change the transport capacity of CQ derivatives by $PfCRT^{mut}$ and therefore making them good candidate for further drug development.

While the results obtained for 3-ICQ and CQ for CQS parasite (3D7) suggest that both drug interaction is additive, the results obtained with CQR parasite (Dd2) suggest a synergistic effect. Such difference in drug interaction with sensitive and resistant parasites was observed for other drugs combination such as chloroquine-primaquine (additive and antagonistic), piperaquine-dihydroartemisinin (antagonist and additive) and tafenoquine-methylene blue (additive and synergistic) for respectively CQS and CQR parasites [34, 35]. A likely explanation for this observation with 3-ICQ and CQ is that: 1) in CQS strain, there is an accumulation of both CQ and 3-ICQ in the DV with both drug inhibiting hemozoin formation (3-ICQ less than CQ), leading to heme toxicity and death of the parasite; 2) in CQR strain both drugs inhibit hemozoin formation and accumulate inside the DV but in addition, 3-ICQ inhibits CQ efflux through $PfCRT^{mut}$ leading to an increase in accumulation of CQ in the DV and to the parasite death (Figure 11).

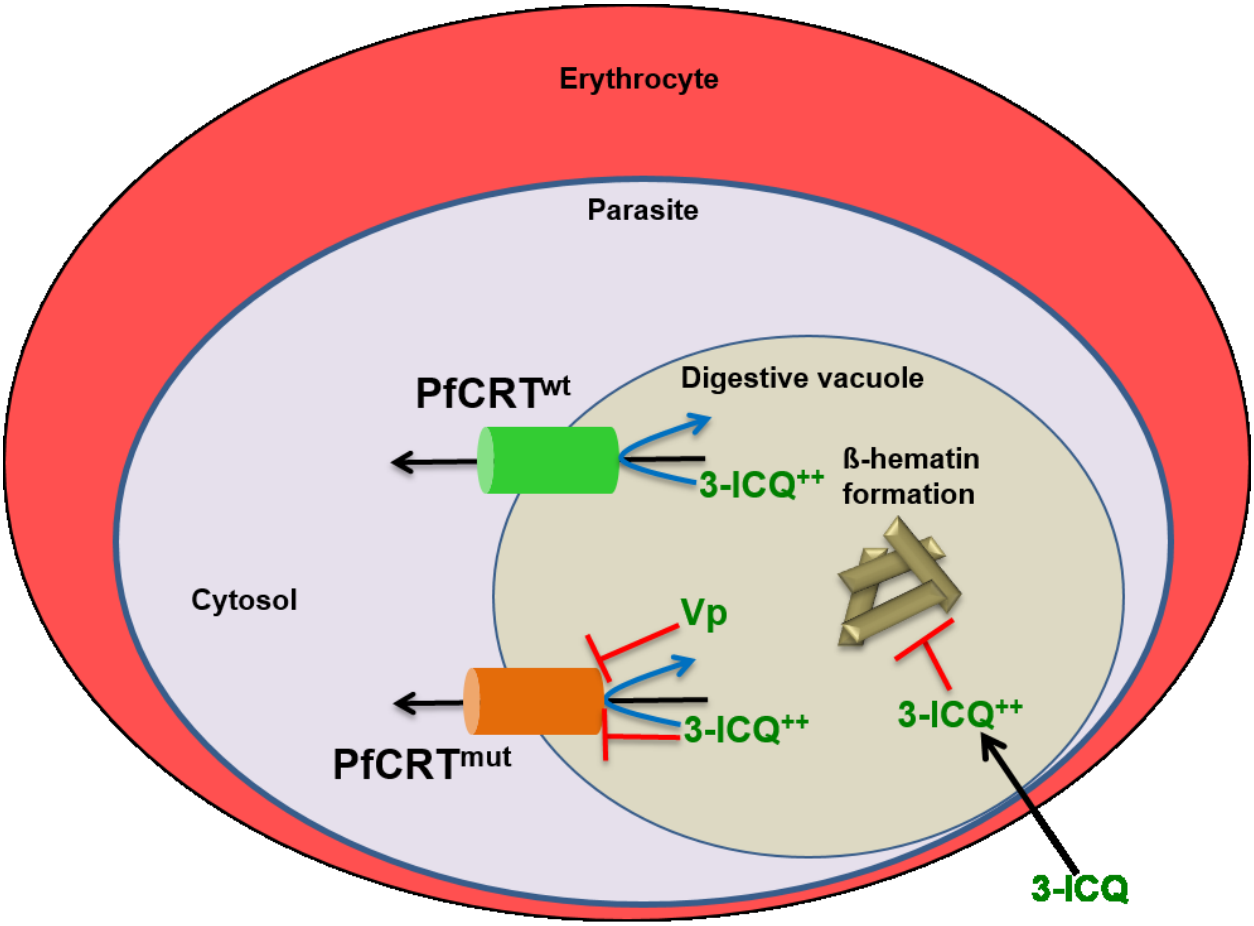

Figure 11. Schematic diagram showing effect of 3-ICQ in the parasite. The directions of the arrowheads indicate the direction of substrate movement between the parasite cytosol and the digestive vacuole. The wild-type ($PfCRT^{wt}$) is represented by a green figure while the mutant PfCRT ($PfCRT^{mut}$) is represented by an orange figure. 3-ICQ gets inside the digestive vacuole by passive diffusion, gets protonated and blocks hemozoin formation. 3-ICQ is not substrate for either $PfCRT^{wt}$ or $PfCRT^{mut}$. Verapamil and 3-ICQ are shown to inhibit $PfCRT^{mut}$. Possible other mechanism involving PfMDR1 is not indicated.

Indeed this dose dependency effect was shown for verapamil (Martin et al. 1987) with a reversal activity at 1 µM and 2 µM. Verapamil effect on CQ resistant strains is well established and this effect is found to be moderate (means FIC=0.4). Unlike verapamil reversibility effect

which is in micromolar range, 3-ICQ effect is in nanomolar range which makes 3-ICQ a better reversal drug than verapamil. Verapamil has a weak intrinsic antimalarial activity and there were no differences in its effect between CQS and CQR strains [39]. However, 3-ICQ has some antimalarial activities when used alone with a significant difference in the IC_{50} value between CQS and CQR strain (Table 1). Our studies show that 3-ICQ is not substrate for both wild-type PfCRT and mutant PfCRT but reverses chloroquine resistance triggered by $PfCRT^{mut}$. In a recent study it was shown that some quinine dimers compounds are not substrate for PfCRT but do inhibit $PfCRT^{mut}$ [50]. To our knowledge 3-ICQ is not the first compound which possesses these features but its combination with chloroquine can be consider as further development to treat drug resistant *P. falciparum* malaria. Several characteristics make 3-ICQ a suitable drug for use in combination therapy: 1) it is as stable as chloroquine and require no special storage conditions, 2) its synthesis is inexpensive enough to be used in endemic countries, 3) it inhibits hemozoin formation and provide a synergistic effect to chloroquine, 4) it inhibits the efflux of chloroquine through $PfCRT^{mut}$ leading to enhanced accumulation of CQ in the digestive vacuole, thus to the parasite death. It is unknown if 3-ICQ directly bind to $PfCRT^{mut}$ but we speculate that 3-ICQ and verapamil have different binding site on $PfCRT^{mut}$.

An earlier study has suggested that the combination of a chemosensitizer with CQ can be used to reverse CQ resistance *in vivo* [51]. It will be of interest to demonstrate that 3-ICQ-CQ combination is more effective *in vivo* than the CQ-Vp combination. Our data show that mammalian cells (Hela and CEM) can tolerate 10 µM of 3-ICQ which is the same concentration as CQ (Figure 10). Hence, the use of 3-ICQ in combination with CQ, due to the low cost of 3-ICQ synthesis, should be considered as an alternative to the use of CQ in region where CQ

resistance is endemic. This strategy will be more suitable than the introduction of a novel antimalarial drug.

In summary, we have investigated the antimalarial activities of three novels chloroquine derivatives. These latter show a moderate antimalarial activity on CQ-sensitive and -resistant strains with iodine modified chloroquine (3-ICQ) showing the best activity. Interestingly, these derivatives are not substrate of PfCRT. One of them, 3-ICQ was able to inhibit β-hematin formation and its combination with CQ on CQR strains reveals a high potency of CQ with a synergistic interaction. Consequently, 3-ICQ inhibits CQ efflux through $PfCRT^{mut}$ and thus increases the sensitivity of CQ resistant parasites to CQ. One has to further investigate the contribution of 3-ICQ as a potential drug for use in combination therapy with chloroquine.

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Connecting statement 3

In the previous chapters (2 and 3) we demonstrate the importance of multidrug resistance protein characterization in drug development. Moreover, existing transporters are characterized relative to their function in Plasmodium drug resistance. Among these transporters is a family that utilizes ATP for energy, ABC transporters, which display diverse functions in plasmodium. They have been reported to play a role in drug and heavy metal resistance and also in parasitic translational regulation. Since most of the ABC transporters have been involved in drug resistance, such involvement arouses our interest in characterizing the unique ABC transporter of subfamily G in Plasmodium, PfABCG. In the following study we characterize PfABCG and assess its potential role in drug resistance.

CHAPTER 4

Expression and Subcellular Localization of the Only Member of ABCG Subfamily in Plasmodium falciparum

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ABSTRACT

The high incidence of malaria and the rise of drug-resistance pose major problems for malaria control. Two membrane transport proteins, PfMDR1 (*Plasmodium falciparum* multidrug resistance 1) and PfMRP1 (*P. falciparum* multidrug resistance protein 1), have been associated with resistance to several antimalarial drugs. The *P. falciparum* genome encodes 15 members of ABC proteins, with one member of the ABCG subfamily (PfABCG). In humans, the ABCG subfamily is composed of five members (hsABCG1, 2, 4, 5, and 8) that mediate the transport of normal cell metabolites and anti-cancer drugs. Analysis of PfABCG amino acid sequence shows equal sequence identity to hsABCG1 and G2. Using N-terminal directed antibody against a recombinant fragment of PfABCG, we show that PfABCG migrates with an apparent molecular mass of 65KDa polypeptide on SDS-PAGE. PfABCG is ubiquitously expressed in all four stages of the parasite erythrocytic life cycle, with lower and higher expression in ring and late trophozoite stages, respectively. The protein localizes to the plasma membrane and a novel round structure beneath the cell membrane, but not to the digestive vacuole, in all stages of the parasite's erythrocytic cycle. Similar localization, is also observed in gametocytes where PfABCG is highly expressed. Analysis of PfABCG genomic sequences for polymorphisms and changes in protein expression between different strains of *P. falciparum* revealed identical nucleotide sequence among the different strains, but variable protein expression. PfABCG expression is least in HB3 chloroquine sensitive strain, while higher expression levels are seen in other chloroquine-sensitive and -resistant strains, with highest levels of expression in 7G8. The differential expression of PfABCG in three chloroquine-sensitive strains (e.g., 3D7, HB3 and D10) predicts the sensitivity of the different strains to ketotifen, an anti-histaminic drug, whereby

low expression is associated with decreased sensitivity to ketotifen. Taken together, the results in this report provide the first description of native PfABCG expression and subcellular localization in asexual stages of the parasite and its localization in gametocytes. It remains to be determined if PfABCG is functionally equivalent to mammalian ABCG1 or ABCG2.

INTRODUCTION

Malaria remains one of the most deadly diseases affecting humanity with 665,000 to 1.2 million deaths annually [1, 2]., In the absence of an effective and lasting vaccine, treatment for malaria infections continues to rely heavily on the use of several antimalarials which often leads to the rise and spread of drug resistant parasites. In the last two decades resistance to quinoline-based drugs has been attributed to the action of molecular efflux pumps that severely reduce the toxicity of such antimalarials. Two membrane transporters have been shown to cause resistance to antimalarials in *P. falciparum*: the chloroquine resistance transporter (PfCRT), a member of the drug metabolite transporters [3, 4], and the P-glycoprotein homologue-1 (Pgh-1 or PfMDR1), a member of the ATP-binding cassette (ABC) proteins superfamily [5]. Although the normal substrates for PfCRT and PfMDR1 are not known, polymorphisms in these drug transporters have been shown to alter the parasite's response to Artemisinin-based Combination Therapy [6].

The ABC transporters constitute one of the largest families of proteins that are evolutionarily conserved from bacteria to human. Transmembrane ABC transporters encode at least one multiple spanning domain (MSD), with six transmembrane helices, and one cytoplasmic domain encoding an ATP-binding cassette [7]. Functionally, ABC transporters are organized either as full transporters containing two of each domain, or half transporters that homo- or hetero-dimerize to form a functionally active transporter [8]. In humans, the ABC transporter family consists of 48 members grouped into 7 subfamilies (ABCA to ABCG; [9]); while in *P. falciparum*, the ABC family consists of fifteen members grouped into six subfamilies (ABCB, C, E, F, G, and H; [10-

12]). The B-subfamily of *P. falciparum* encodes seven proteins, with PfMDR1 and PfMDR2 (or PfABCB1 and PfABCB2, respectively) associated with drug resistance. Moreover, PfMDR1 shown to localize to the vacuolar membrane of the parasite digestive vacuole (DV) confers resistance to mefloquine and other related quinolines [13]. PfMDR2 is localized to the parasite plasma membrane and shown recently to confer resistance to heavy metals (e.g., cadmium) presumably through an efflux mechanism [14, 15]. Two additional ABC transporters, PfMRP1 and PfMRP2 (or PfABCC1 and PfABCC2), found at the parasite plasma membrane have been implicated in conferring resistance to quinoline drugs and the transport of glutathione [16, 17]. The objective of this study was to characterize the expression of the only member of the G-subfamily (PfABCG) in different *P. falciparum* strains and to determine its subcellular localization in the parasite.

MATERIALS AND METHODS

Parasite cultures - *P. falciparum* strains (e.g., 3D7, D10, HB3, 7G8, K1 and W2) were obtained from the Reference Reagent Resource Center (MR4) (Manassas, VA, USA) and maintained in culture in RPMI 1640 medium (*Invitrogen*) supplemented with 25 mM HEPES, L-Glutamine, 0.1 mM hypoxanthine (*Sigma*) and 10 % heat-inactivated human plasma (Interstate blood bank, Manassas, USA). Parasites were allowed to proliferate on type A^+ human erythrocytes (Interstate Blood Bank, Manassas, USA) at 37°C under conditions of 3% O_2, 5% CO_2 and 92% N_2. The parasite cultures were routinely synchronized with 5% sorbitol and parasitemia was assessed by blood smears stained with 5% Giemsa solution.

Recombinant expression and antibody production- The predicted DNA sequence encoding PfABCG (PF14_0244 or PF3D7_1426500) was retrieved from PlasmoDB database. PfABCG gene did not contain any introns hence; genomic DNA was used to amplify PfABCG coding sequence from 3D7 (Netherlands lab strain) genomic DNA. A fragment (1-1067 bp, encoding residues 1-356 of PfABCG N-terminal nucleotide binding domain ($PfABCG^{1-356}$)) was amplified with PfABCG-specific forward 5'ATGGATTTGAAAGGGTGGAT3' and reverse 5'TCATTGTGTGGATAATATAGGTG3' primers by PCR using Platinum Pfx DNA polymerase (Invitrogen, Life technologies). The amplified DNA fragment was directionally cloned into pGEX-6P1 vector (Amersham Biosciences, Pittsburgh, PA, USA) at the C-terminal of GST protein and propagated in *E. coli,* strain BL21 (Invitrogen, Life technologies). GST-$PfABCG^{1-356}$ fusion protein was affinity purified on GSH-coupled Sepharose resin according to the manufacturer protocol (Amersham Biosciences, Pittsburgh, PA, USA), and subsequently used to generate polyclonal

antibodies in rabbits (using McGill's SOP; McGill Comparative medicine and animal resources Center).

Parasite protein extraction and Western blotting- A parasite culture of 3D7 strain was synchronized at 5% to 10% parasitemia and harvested at different stages of the parasite asexual life cycle: rings (6-12 hpi), trophozoites (20-26 hpi), late-trophozoites (30-36 hpi) and schizonts (44 hpi) (hpi: hours post-infection). In addition to 3D7, other chloroquine-sensitive (D10, HB3) and -resistant parasite (7G8, K1, W2) cultures were synchronized and harvested at late trophozoites-schizonts stages by percoll gradient centrifugation as described by Victor Fernandez (Methods in Malaria Research, 5^{th} edition, MR4, Manassas). Percoll purified parasites were harvested and washed twice in RPMI-1640, then resuspended in phosphate-buffered saline (PBS). The number of parasites or parasitized RBCs was determined using hemocytometer. The parasites were released from infected RBCs by saponin treatment and the free parasites were lysed in PBS containing 0.25% SDS (sodium dodecyl sulfate) and 0.25% sodium deoxycholic acid, supplemented with a cocktail of protease inhibitors (Thermo Scientific, Rockford, IL). Lysates were cleared by centrifugation at 13,000 rpm at 4°C for 10 minutes. Parasites lysates were mixed with 2X SDS PAGE sample loading buffer and resolved onto 8% acrylamide SDS PAGE. Resolved proteins were transferred to PVDF membranes and probed with PfABCG antiserum (dilution, 1:1000) and horseradish peroxidase-conjugated goat anti-rabbit IgG (dilution, 1:5000 v/v). The reactive polypeptide bands were visualized using the SuperSignal West Pico chemiluminescent kit according to the manufacturer's protocol (Thermo Scientific, Rockford, IL).

RNA extraction and Real time PCR - *P. falciparum* 3D7, HB3 and 7G8 parasite were grown and collected 24h after synchronization. Total RNA was extracted with TriZol reagent (Life technologies) and quantified using NanoDrop^{TM} 1000 (Thermo Scientific). RNA purity was confirmed using PCR amplification of an intron-spanning region of PfCRT gene. Aliquots of purified RNA (1 µg) from different strains of *P. falciparum* were reverse transcribed using QuantiTect reverse transcription kit (QIAGEN). The resultant cDNAs were compared by real time PCR using the Power SYBR® Green PCR master mix (Life technologies). The relative expression of PfABCG transcript in the different strains relative to the reference strain (3D7) was calculated using the $2^{-\Delta\Delta Ct}$ method [18]. The seryl-tRNA synthetase (PF3D7_0717700) gene was used as the internal reference gene [19, 20]. The nucleotide sequences of all qPCR primers used above are shown in Table 1.

Table 1. Primers sequences for QPCR

Primers name	Sens	Sequence 5'-3'
Seryl-t-RNA Synthetase-qPCR	Forward	TATCATCTCAACAGGTATCTACATCTC
	Reverse	TTTGAGAGTTACATCTGGTATCATCTT
PfABCG-qPCR	Forward	TATCTATGAAGGTAACCGAAGG
	Reverse	GACGATGAATCTAATCCACTT

Immunofluorescence staining - Parasitized erythrocytes were washed once with PBS and then fixed with 4% paraformaldehyde for 30 minutes at room temperature. Fixed cells were washed once in PBS and then applied onto poly-L-lysine coated slide cover slips. Slides were air dried for 5 minutes and quenched with 0.15% glycine in PBS for 10 minutes at room temperature. Slides were subjected to a wash step with 0.05% PBS-tween20 (PBST) and then permeabilized with 0.1% tritonX-100 in PBS for 10 minutes at room temperature. After washing three times with PBST, cells were blocked with 1% normal goat serum in PBST for 1hr at room temperature.

Cover slips were then incubated with PfABCG antiserum (1:50 v/v dilution) at 4°C overnight followed by three 10 minutes washes with PBST. Alexa-fluor 488 conjugated goat anti-rabbit (Life Technologies) was added at 1:2000 dilution for 45 minutes. For the double immuno-histochemical staining of infected erythrocytes, anti-PfEXP-1 antibody was used at 1:100 v/v dilution, followed by an incubation with Alexa-fluor 594-conjugated second antibody (1:2000 v/v). After three washes with PBST, the coverslips were incubated with anti-PfABCG or anti-PfCRT at 1:50 v/v dilution and subsequently with Alexa-fluor 488-conjugated second antibody (1:2000 v/v). Cover slips were rinsed three times with PBST, incubated for 5 min with DAPI (1:1000 v/v) and washed further with PBST. The cover slips were mounted on microscope slides using fluoromount G mounting medium (Southern Biotech, USA). Slides were imaged using a confocal microscope (Carl Zeiss GmbH, Jena, Germany) and images were captured and analyzed using Image J software, version 1.43 [21, 22].

Drug survival assays – Three chloroquine-sensitive strains (e.g., 3D7, D10 and HB3) with different expression levels of PfABCG protein were tested for their ability to proliferate, *in vitro*, in the presence of increasing concentrations of chloroquine, ketotifen and primaquine using SYBR Green I assay [23]. Ketotifen and Primaquine were dissolved in DMSO while chloroquine was dissolved in water. Parasite cultures were synchronized at ring stage using 5% sorbitol (Sigma), diluted to 0.5% parasitemia - 2% hematocrit and added to the pre-dosed 96-wells plates. The plates were incubated at 37°C for 72h. The assay was terminated by transferring the plates to -80°C until the performance of the assay. The SYBR Green I dye – parasite lysis mixture (Tris, 20 mM [pH 7.5]; EDTA, 5 mM; 0.008% saponin; 0.08% Triton X-100; 0.2 µL/ml of 10 000x SYBR nucleic gel stain dye) was added to parasites in black plates and incubated at room

temperature for an hour in the dark. The fluorescence in each well was measured using the Synergy H4 plate reader with excitation and emission wavelengths of 485 nm and 535 nm, respectively. The data was analyzed using Prism 5.0 (GraphPad Software) to obtain the 50% inhibitory concentration (IC_{50}). All graphs shown represent the mean ± SD of three independent experiments done in triplicate.

RESULTS

PfABCG is the only member of G-subfamily in *P. falciparum*. Analysis of PfABCG amino acid sequence shows that it encodes a half transporter with one N-terminal nucleotide-binding domain and transmembrane domain with six transmembrane helices as illustrated in Figure 1A.

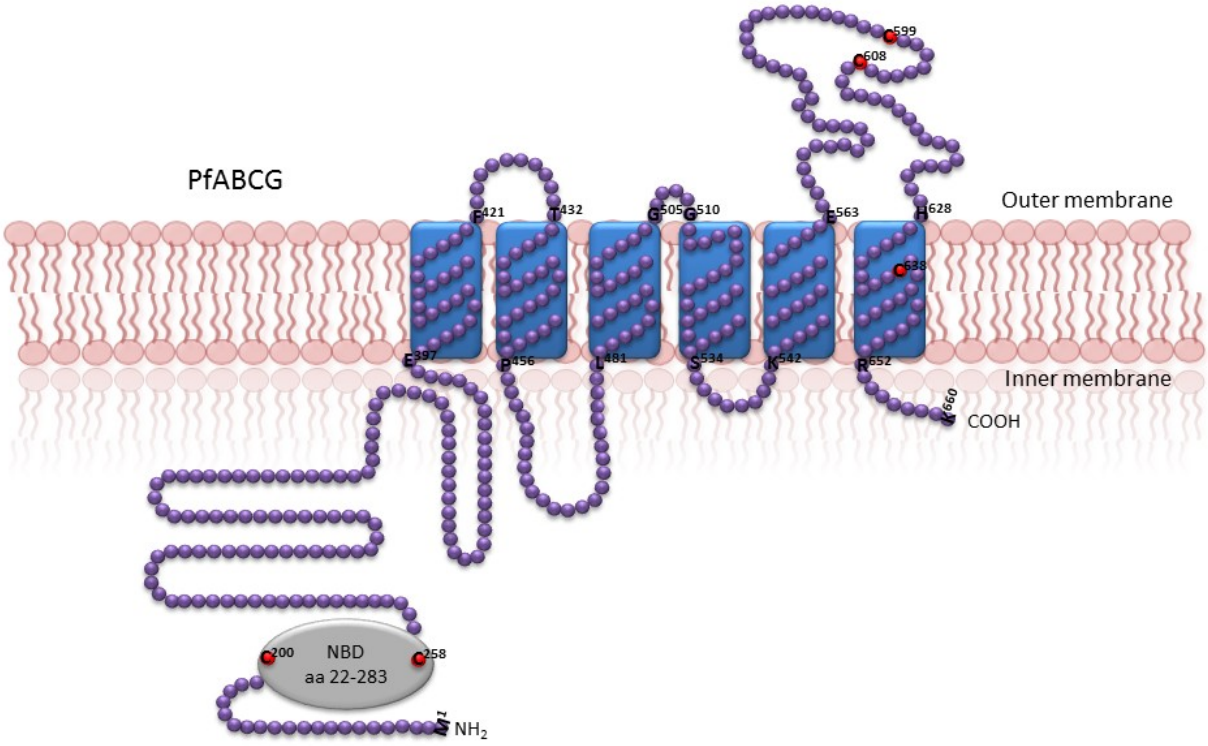


Figure 1A. Predicted secondary structure of PfABCG. A secondary structure schematic based on analysis of PfABCG primary amino acid sequence is shown. PfABCG encodes an N-terminal cytoplasmic domain with nucleotide binding domain, followed by a hydrophobic domain with six transmembrane helices. Each circle represents a single amino acid residue. The transmembrane helices are indicated with blue rectangles. The nucleotide binding domain (or NBD, residues 22 – 283) is represented by the gray sphere. The positions of the five cysteines are indicated on the secondary structure as red circles.

The results of PfABCG sequence alignment with human G-subfamily members (ABCG1, 2, 4, 5 and 8) are shown in Table 2.

Table 2. Sequence homology and identity between PfABCG and human ABCG1-8.

Alignment PfABCG	Global alignment				Local alignment			
	Identity	Similarity	Gaps	Score	Identity	Similarity	Gaps	Score
HuABCG1	24.3%	42.1%	21.4%	583.5	26.0%	45.0%	15.5%	592.0
HuABCG2	**26.5%**	**46.6%**	15.3%	569.0	**28.3%**	**48.9%**	11.1%	575.5
HuABCG4	23.2%	41.9%	19.6%	579.0	25.2%	45.3%	13.4%	590.5
HuABCG5	22.4%	42.6%	19.7%	457.0	24.0%	45.4%	15.9%	472.0
HuABCG8	21.2%	37.9%	23.4%	429.5	23.4%	41.9%	15.2%	436.0

PfABCG shows highest sequence identity and similarity to hsABCG2 and hsABCG1 members (26.5% and 24.3% or 28.3% and 26.0% for global and local alignments, respectively). Figure 1B shows the amino acid sequence alignments between PfABCG and hsABCG1 and G2, whereby PfABCG shows four sequence insertions of 8, 5, 6 and 5 amino acids at positions K^{275}- I^{282}, G^{296} - N^{300}, I^{341} - I^{346} and L^{390}- I^{394} respectively, relative to hsABCG1 and G2.

These amino acids insertions occur in the cytoplasmic domain prior to the multiple spanning domain of PfABCG (figure 1A). The functional role of these amino acid insertions is presently not clear. Earlier studies [24-27] have identified three cysteine residues in hsABCG2 cytoplasmic domain (e.g., Cys^{284}, Cys^{374} and Cys^{438}) and three in the 3rd extracellular loop from the N-terminal (e.g., Cys^{592}, Cys^{603} and Cys^{608}) of hsABCG2. These latter cysteine residues were shown to form intra- and inter-disulphides bridges that affect the transport, membrane sorting, and dimerization of hsABCG2. Analysis of PfABCG amino acid sequence revealed five cysteine residues [Cys^{200} and Cys^{258} in the ATP binding domain, Cys^{599} and Cys^{608} in 3rd extracellular loop and Cys^{635} in transmembrane 6 (figure 1A)].

Figure 1B. Sequence alignment of PfABCG with human ABCG1/G2. The alignment between the *P. falciparum* ABCG (PfABCG), human ABCG2 (hsABCG2), and ABCG1 (hsABCG1) is obtained using Geneious version (7.0.4) created by Biomatters. A single letter code for amino acids is used for all three ABCG sequences. The inserted amino acids are highlighted in blue and the conserved cysteine residues in red. The boxed sequences in gray show the alignment of ABC walker motif consensus sequences. The bars below the aligned sequences point to the positions of the transmembrane domains (TM1-6) of PfABCG.

To further characterize PfABCG protein expression in the parasite, antiserum was generated in rabbits to the recombinant GST-PfABCG fusion polypeptide encoding the first 356 N-terminal amino acid residues (GST-PfABCG$^{1\text{-}356}$). The results in figure 2A show the binding specificity of the pre-immune and the PfABCG antiserum to the recombinant immunogen (GST-PfABCG$^{1\text{-}356}$) and erythrocytes protein lysate (lanes 2, and 1, respectively).

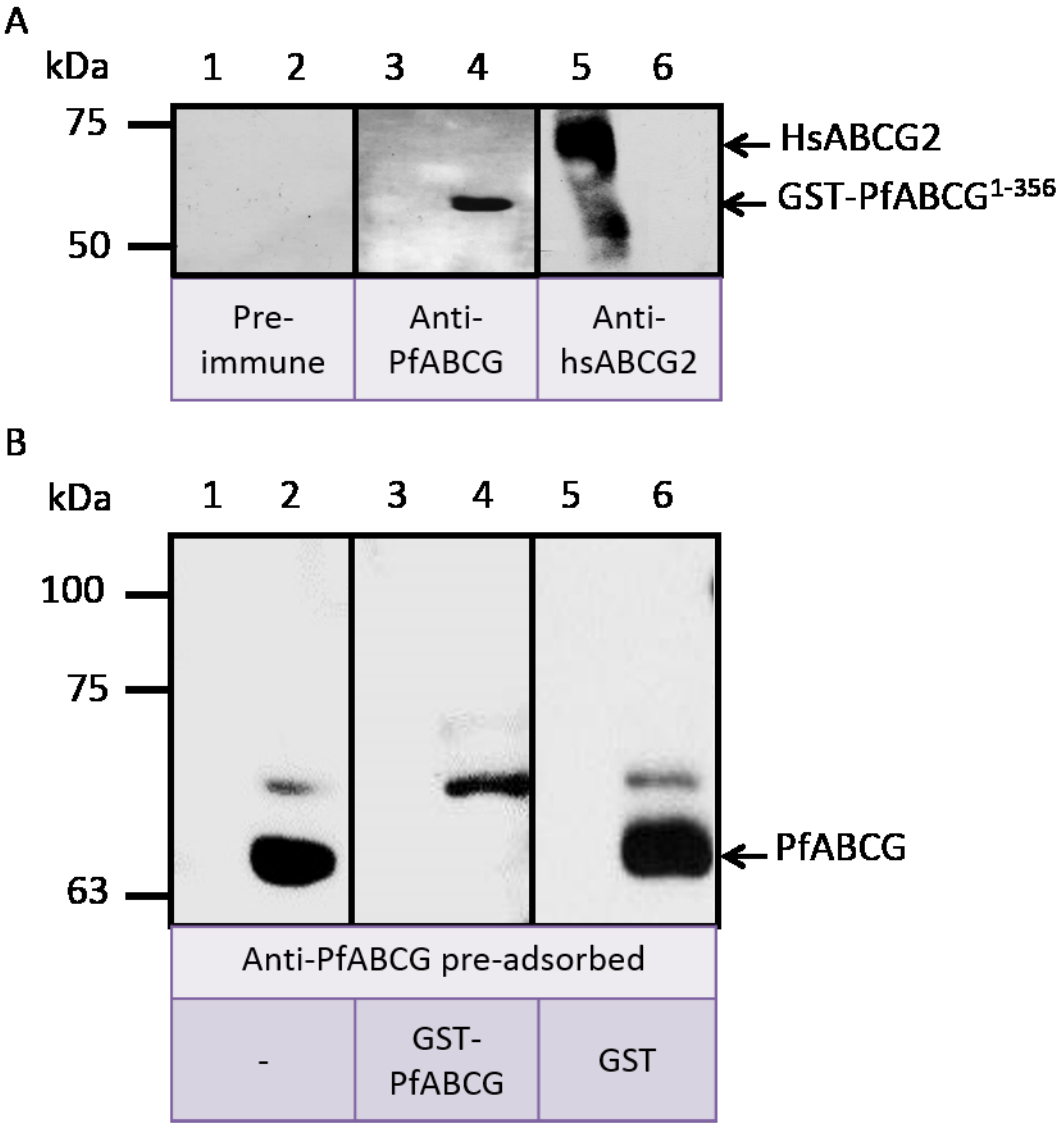


Figure 2. Characterization of PfABCG antiserum binding specificity. Panel A shows Western blotting results using protein extracts from red blood cell ghosts and purified recombinant immunogen (GST-PfABCG$^{1-356}$) probed with pre-immune rabbit serum (lanes 1 and 2), anti-PfABCG (lanes 3 and 4) and anti-human ABCG2 monoclonal antibody (lanes 5 and 6), respectively. Panel B shows Western blot results of protein lysates from red blood cell ghosts and *P. falciparum* (schizont stage) resolved on SDS-PAGE and probed with PfABCG antiserum (lanes 1 and 2), PfABCG antiserum pre-adsorbed with recombinant GST-PfABCG$^{1-356}$-sepharose (lanes 3 and 4) and PfABCG antiserum pre-adsorbed with recombinant GST (lanes 5 and 6), respectively. The mobility of PfABCG, hsABCG2 and GST-PfABCG$^{1-356}$ recombinant protein are indicated with arrows on the right of the figure.

The Western blot shows a specific binding of PfABCG antiserum to a 68kDa polypeptide, not recognized by the pre-immune antiserum. Moreover, PfABCG antiserum did not cross-react with human ABCG2 proteins shown previously to be expressed in erythrocyte membranes [28]. Furthermore, the human ABCG2-specific antibody recognized hsABCG2 in erythrocyte lysate but did not recognize the recombinant GST-PfABCG$^{1-356}$ polypeptide (lanes 5 and 6, respectively). Having established the specificity of anti-PfABCG antiserum to the N-terminal sequence of PfABCG protein, figure 2B shows a Western blot containing total protein extracts from non-infected and *P. falciparum* infected erythrocytes. The results of figure 2B show the binding of PfABCG antiserum to a 65kDa and 70kDa polypeptides found in *P. falciparum* infected but not in uninfected erythrocyte extracts (lanes 2A and 1A, respectively). To confirm the specificity of this antiserum towards either or both polypeptides, Western blots were performed using PfABCG antiserum pre-adsorbed with excess recombinant GST-PfABCG$^{1-356}$ fusion protein or GST protein alone (lanes 3 and 4 or lanes 5 and 6, respectively). Figure 2B (lane 4) shows that pre-adsorption of PfABCG antiserum with excess of purified recombinant GST-PfABCG$^{1-356}$ polypeptide abolished the binding of PfABCG antiserum to the 65kDa polypeptides, but not the 70kDa band. By contrast, pre-adsorption of the antiserum with recombinant GST protein did not affect the binding of the antiserum to either polypeptide (lane 6 of fig. 2B). Interestingly, PfABCG migrates with an apparent molecular mass of 65kDa, relative to its calculated molecular mass of ~75kDa. The reason(s) for this difference in the molecular mass of PfABCG is not clear but have been previously observed with other ABC proteins, including hABCG1 and 2 family members [29, 30]. Taken together, the results in figure 2 demonstrate the specificity of a polyclonal antiserum to the N-terminal cytoplasmic domain of

PfABCG protein migrating as 65kDa polypeptide on SDS PAGE using protein extracts from *P. falciparum*.

Earlier studies of using high density oligonucleotide arrays to examine the expression profiling of malaria parasite life cycle have suggested that PfABCG RNA is expressed at higher levels in the gametocyte stage (http://www.plasmodb.org) [31] [32, 33]. To determine the protein expression levels of PfABCG in the different intra-erythrocytic stages of the parasite's life cycle, protein extracts from tightly synchronized cultures at rings, trophozoites, late-trophozoites, and schizonts stages were prepared for Western blot analysis. Figure 3A shows equal protein extracts from different erythrocytic stages resolved on SDS PAGE and stained with Coomassie brilliant blue as control for equal protein loading and the results in figure 3B show that PfABCG expression is detectable in all stages of the parasite's erythrocytic life cycle, with lowest and highest expression levels in schizont and trophozoite stages, respectively. When PfABCG expression was analyzed based on per parasite expression levels; figure 3C shows ring stage to express the lowest levels of PfABCG per parasite while trophozoites and late trophozoites showed roughly 5 and 15-fold increase in PfABCG expression relative to that of ring stage, respectively. Schizonts showed roughly 4-fold PfABCG expression relative to that of ring stage (Fig. 3C).

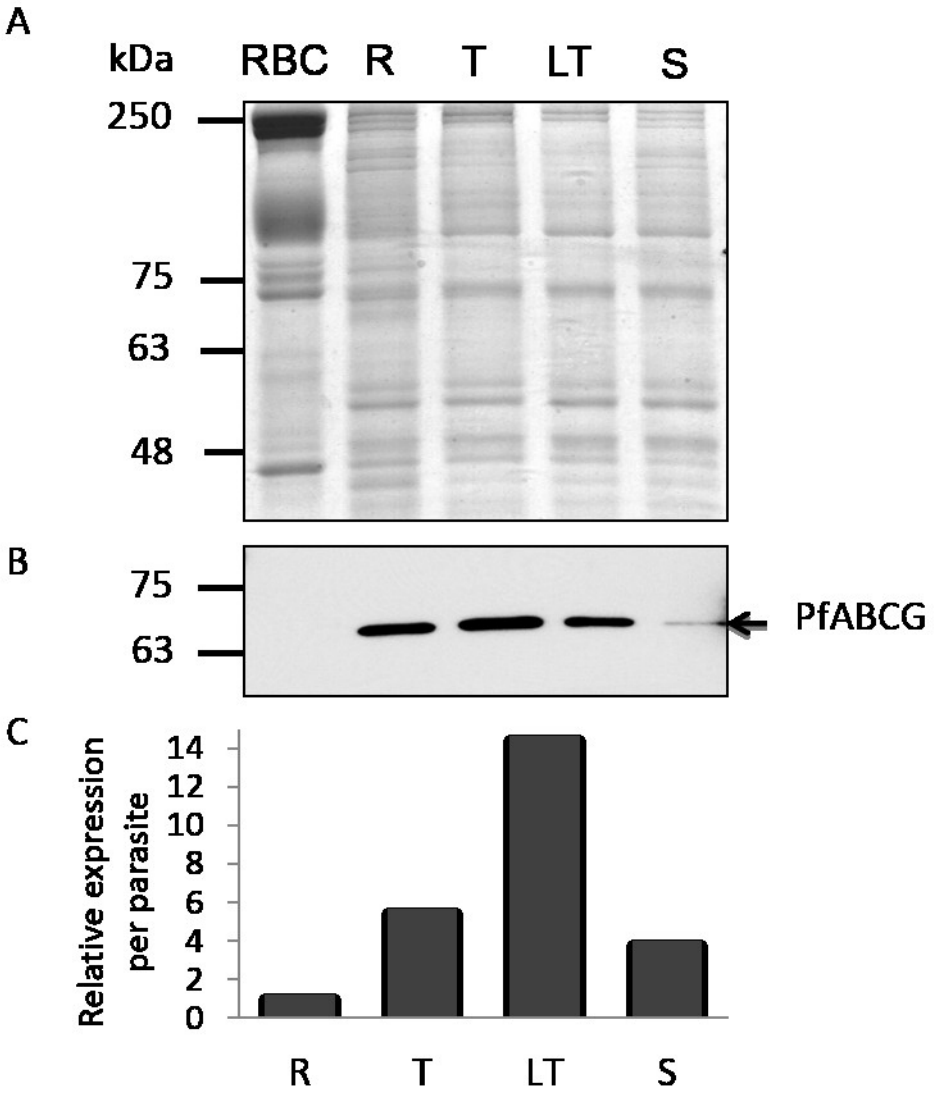


Figure 3. PfABCG protein expression in different erythrocytic stages of *P. falciparum*. Parasite protein extracts from different stages of 3D7 (rings, trophozoites, late-trophozoites and schizonts) were resolved on SDS PAGE and Western blotted. Panel A shows Coomassie blue stained SDS PAGE. Panel B shows a Western blot of a replica of panel A probed with PfABCG antiserum. The different stages of the parasite erythrocytic life cycle isolated are: rings (R) (6-12hpi), trophozoites (T) (20-26hpi); late-trophozoites (LT) (30-36hpi) and schizonts (S) (44hpi) stages (hpi: hours post-infection). Panel C shows quantification of PfABCG expression of a representative Western blot, relative to parasite number from the different stages above.

Given the role of ABC proteins in drug resistance in tumour cells and parasites [25, 34, 35], it was of interest to determine if PfABCG expression correlated with the parasites' resistance to chloroquine in different laboratory strains of *P. falciparum*. To this end, parasite cultures were tightly synchronized and late-trophozoites-schizonts stage parasites were isolated on percoll gradient. The purification efficiency was between 80-90%, (free of non-infected RBC). Equal proteins were loaded onto SDS PAGE and analyzed by Western blotting using PfABCG antiserum. Figure 4A shows the expression of PfABCG in all six different strains of *P. falciparum* tested (3D7, D10, HB3, 7G8, K1, and W2).

The results in figure 4A show that PfABCG is expressed in all six strains independent of the strains' sensitivity to chloroquine. In addition to equal protein loading of the different strains, the same Western blot was also probed for alpha-tubulin expression. Figure 4B shows the relative expression of PfABCG in all six *P. falciparum* strains, normalized to alpha-tubulin expression. The chloroquine sensitive strain (HB3) showed the lowest PfABCG expression relative to the chloroquine resistant strain (7G8) with the highest expression level (Fig. 4B). The expression of PfABCG in the other chloroquine sensitive (3D7 and D10) and –resistant (K1 and W2) strains was roughly similar. To determine if PfABCG protein expression correlates with its RNA levels, figure 4C shows quantitative PCR analysis for 3D7, HB3 and 7G8 strains. There was a strong correlation between RNA and protein levels in these three strains, with HB3 showing the lower RNA levels relative to 3D7 and 7G8. Taken together, the above results show good correlation between protein and RNA expression levels of PfABCG; however the expression level of PfABCG did not predict the parasite sensitivity to chloroquine. Moreover, the QPCR results provide further support for the specificity of anti-PfABCG antiserum.

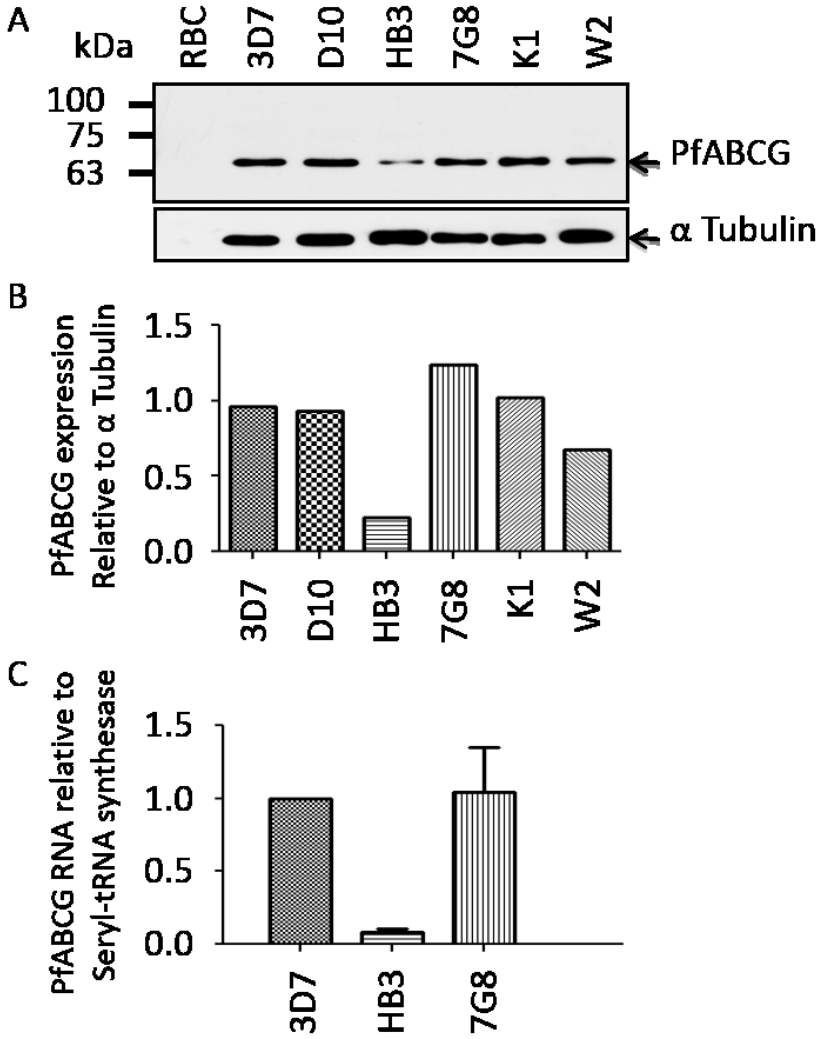
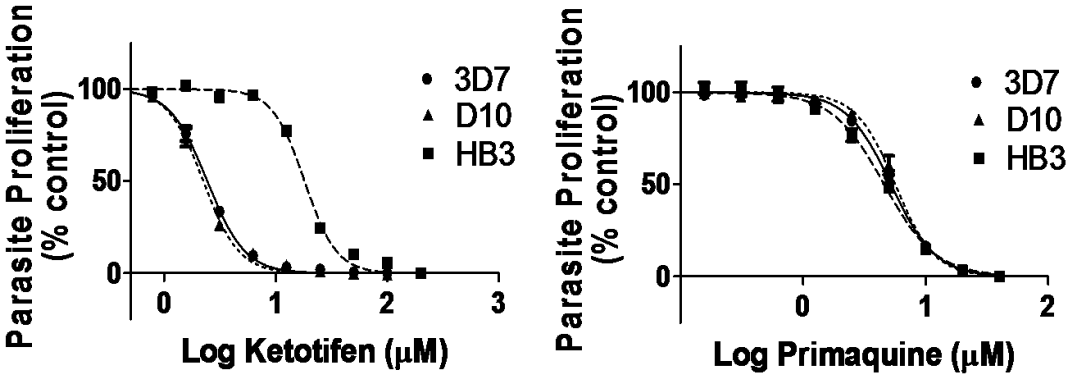


Figure 4. PfABCG expression in different chloroquine-sensitive and –resistant strains of *P. falciparum*. Protein extracts from percoll purified late trophozoites-schizonts of different lab strains were resolved on SDS PAGE and probed for PfABCG expression by Western blotting. Equal proteins from red blood cells (RBC); 3D7, D10, HB3 (chloroquine-sensitive); 7G8, K1, W2 (chloroquine-resistant) were subjected to Western blotting and probed with PfABCG antiserum and alpha-tubulin specific antibody (Panel A). Panel B shows the relative levels of PfABCG expression normalized to alpha-tubulin in late trophozoites-schizonts from different chloroquine-sensitive and resistant lab strains of *P. falciparum*. Panel C shows the relative levels of PfABCG RNA to that of seryl-tRNA synthesase (endogenous reference gene) in different chloroquine-sensitive strains of *P. falciparum* (e.g., 3D7, HB3, and 7G8) as determined by RT-PCR. The graphs show the mean ± SD of three independent experiments done in triplicates.

Using high throughput drug screening of gene knockouts in *P. falciparum*, Eastman et al. [36] have shown recently that knockdown of PfABCG in 3D7 decreased its sensitivity to Ketotifen, a noncompetitive H1-antihistamine drug, relative to wild type parasite. Given the differential expression of PfABCG in different strains of *P. falciparum* (figure 4), it was of interest to compare the sensitivity of three chloroquine sensitive strains (e.g., 3D7, HB3 and D10) to ketotifen and primaquine. Both 3D7 and D10 expressed relatively equal levels of PfABCG, while HB3 shows more than 3 to 8-fold decrease in protein and RNA expression levels, respectively (Figure 4B and 4C). Figure 5 shows the proliferation of 3D7, HB3 and D10 in the presence of increasing concentrations of ketotifen or primaquine.



	IC50 [mean±SD][a]		
Drugs	**3D7**	**D10**	**HB3**
Ketotifen (μM)	**2.4±0.4**	**2.2±0.4**	**18.2±1.07**
Primaquine (μM)	**5.3±1.07**	**5.7±1.6**	**4.6±0.9**

[a] Values represent mean ± SD of four independent experiments, each of which was performed in triplicate.

Figure 5. The proliferation of chloroquine-sensitive parasites in increasing concentrations of ketotifen and primaquine. Three CQ-sensitive strains (3D7, HB3 and D10) of *P. falciparum* were incubated in increasing concentrations of ketotifen or primaquine. The IC50 values for each of the three parasite strains for ketotifen and primaquine are tabulated below the proliferation graphs in this figure.

HB3 with the lowest expression level of PfABCG was less sensitive to Ketotifen with an IC_{50} value of 18.2 µM compare to 3D7 and D10 which express more PfABCG and are more sensitive to Ketotifen (2.4 µM and 2.2 µM respectively). By contrast, increasing concentrations of primaquine showed similar effects for all three strains with IC_{50} values of 4.6 µM to 5.7 µM (Figure 5). Taken together, the above results suggest a possible correlation between PfABCG expression and the parasite sensitivity to ketotifen; however effects of other cellular changes as mediators of ketotifen sensitivity independent of PfABCG expression cannot be ruled out at this point.

Given the results in figure 5, relating to the role of PfABCG in the sensitivity of the parasite to ketotifen, it was of interest to determine the subcellular localization of native PfABCG in different stages of *P. falciparum.* Figure 6 shows an immunofluorescence staining of parasitized erythrocytes at different asexual stages of 3D7 *P. falciparum,* using PfABCG antiserum. No signal was observed in the region of the digestive vacuole as seen by the dark spot appearance of hemozoin crystal (Fig. 6A-6C). Instead the antiserum bound to the plasma membrane of the parasite at early and late trophozoite stages (Fig. 3A and 3B). In the segmented schizonts parasites, the cytoplasmic staining is due to PfABCG expression signal on each individually forming merozoite plasma membrane. In addition to the membrane staining, PfABCG antiserum also stained elongated/round structures beneath the plasma membrane in all stages of the parasite (fig. 6). The nature of this stained body underneath the parasite plasma membrane, not part of the digestive vacuole, in early and late trophozoite stages is unknown, but appears as a single body in early trophozoite (fig. 6A) and two bodies in late trophozoite and merozoite (fig. 6B and 6C).

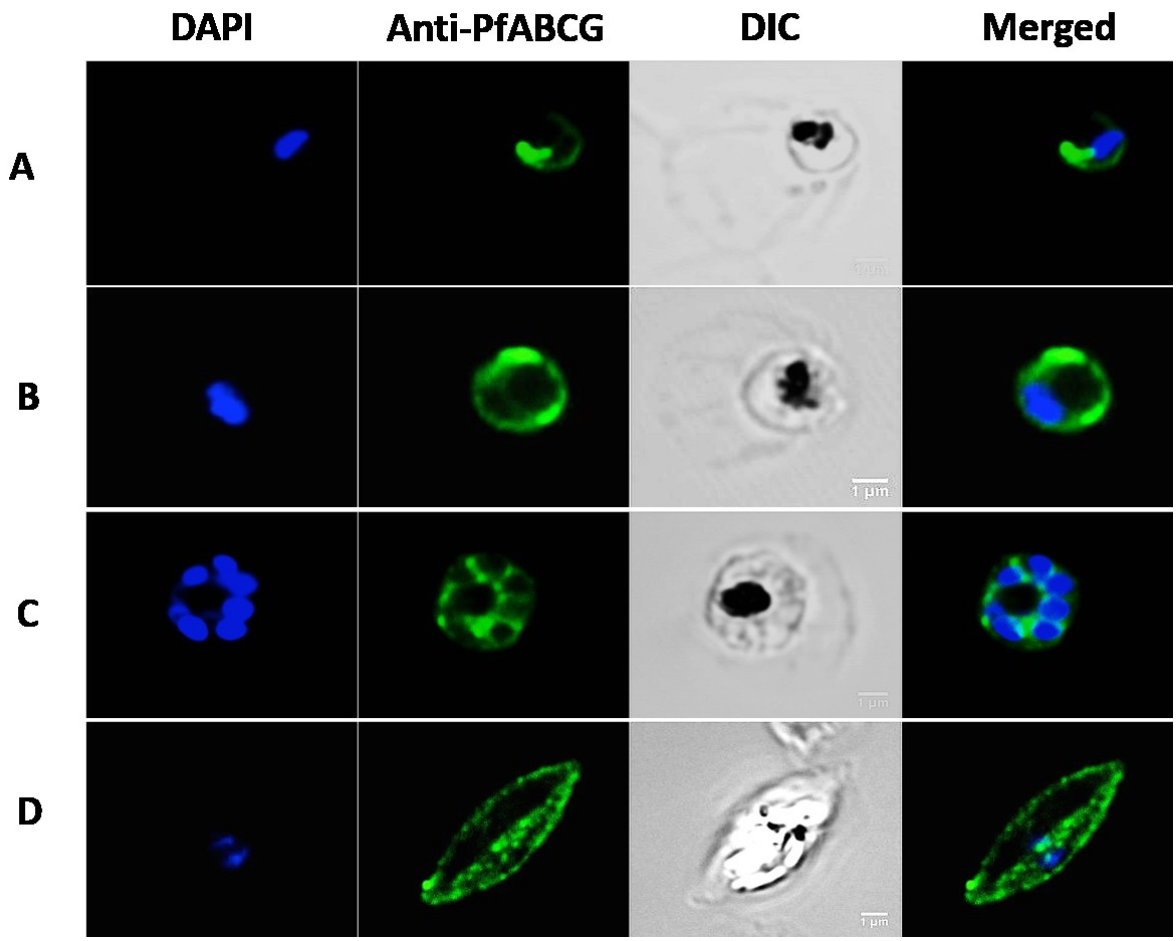


Figure 6. Confocal microscopy of plasmodium stages immuno-stained with PfABCG antiserum. Immuno-histochemical staining of 3D7 cultured parasites with PfABCG antiserum. Columns 1-3 show PfABCG immuno-histochemical staining resolved with Alexa-fluor 488-labeled goat anti-rabbit secondary antibody (Green), counterstaining of nuclear DNA with DAPI (Blue), and differential interference contrast of infected erythrocyte, respectively. Rows A-C show early trophozoites, late trophozoites and schizonts stained with PfABCG antiserum, respectively. Column 4 shows the merge of column 1 and 2, without Differential Interference Contrast (DIC). White arrowheads indicate the intensely stained structures beneath the parasite's plasma membrane.

Of considerable interest is the staining of the plasma membrane of gametocytes, in addition to multiple dark stained bodies throughout the gametocyte (fig. 6D). The expression of PfABCG in

gametocytes is consistent with earlier RNA data, suggesting high levels of PfABCG RNA in gametocytes [31] [32, 33].

To confirm the localization of PfABCG to the parasite plasma membrane, PfEXP-1 (a parasitophorous vacuole membrane marker; [37]) was stained together with PfABCG.

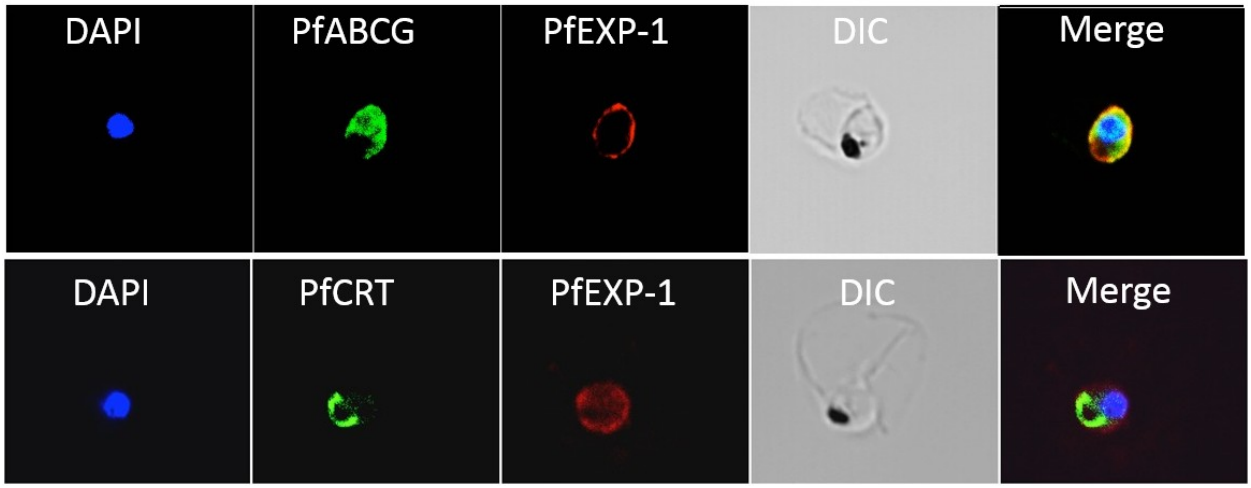


Figure 7. Co-localization of PfExp-1 and PfABCG in *P. falciparum*-infected erythrocytes. Double immuno-histochemical staining of infected erythrocytes at trophozoites stage with antibodies against PfABCG and PfExp-1 (Panel A) and PfCRT and PfExp-1 (Panel B). Column 1 shows the nuclear DNA staining with DAPI (blue). In columns 2 and 3, PfABCG signal (green) (A) and PfCRT signal (green) (B) are resolved with Alexa-fluor 488-labeled goat anti-rabbit secondary antibody, and PfExp-1 signal (red) (A and B) is resolved with Alexa-fluor 594-labeled goat anti-rabbit secondary antibody. Columns 4 and 5 show respectively the differential interference contrast (DIC) of infected erythrocyte and the merger of column 1, 2 and 3 without DIC. The digestive vacuole membrane staining of PfCRT does not overlap with PfExp-1 staining while the plasma membrane staining of PfABCG partially overlaps with the parasitophorous vacuole membrane signal of PfExp-1 as indicated by the arrow.

The results in figure 7A show that PfABCG signal partially overlaps with that of PfEXP-1 (Figure 7A). Figure 7B demonstrate that PfCRT signal does not interfere with PfEXP-1 signal, further confirming the accuracy of PfABCG subcellular localization.

DISCUSSION

Plasmodium falciparum encodes a single ABC transporter of the G-branch of ABC superfamily (PfABCG), versus five members in humans (ABCG1, 2, 4, 5 and 8) [38]. The functions and normal substrates of PfABCG are currently not known; however hsABCG1, G4, G5 and G8 have been shown to transport cholesterol and other sterols [39]. The hsABCG2 (BCRP) originally isolated from mitoxantrone-selected breast cancer cells was shown to transport structurally diverse compounds, including anti-cancer drugs and normal cell metabolites (e.g., heme, uric acid and glutathione [25, 40, 41]). Sequence alignment of PfABCG with all five hsABCGs revealed highest sequence identity to hsABCG2 (26.5% and 46.6%) and hsABCG1 (24.3% and 42.1%; Table 2). Analysis of PfABCG amino acid showed N-terminal ATP-binding domain followed by six multiple spanning domains and a short C-terminal domain. PfABCG encodes 5 cysteine residues, while hsABCG1 and hsABCG2 encode 17 and 12 cysteine residues, respectively. Cys^{200} and Cys^{258} are localized to the ATP binding domain of PfABCG, while Cys^{599} and Cys^{608} are localized to the 3^{rd} extracellular loop and Cys^{638} to the transmembrane 6. Cys^{608} which is conserved among PfABCG, hsABCG1 and hsABCG2 (located at positions 617 in hsABCG1-isoform 2 and at position 603 in hsABCG2) has been shown previously to be responsible for inter-homodimer formation of hsABCG2 and G1 via disulphide bond [42]. However, only PfABCG and hsABCG2 encode a cysteine residue in transmembrane 6 (Cys^{638} and Cys^{635}, respectively). The latter TM6-Cys^{635} is conserved in human, mouse and rat ABCG2, but not in hsABCG1, 4, 5 and 8 [27]. Given the functional role of Cys-residues in protein functions [43-45], one would predict that PfABCG is representative of mammalian ABCG2 isoform. Furthermore, PfABCG shares slightly higher sequence identity with hsABCG2 and hsABCG1 (26.5% versus 24.3%, respectively; Table 2).

However, sequence identity of PfABCG alone, in the absence of direct functional similarities is not sufficient to tag PfABCG as ortholog of mammalian hsABCG2 or G1 of the ABCG family. More recently Tran et al. [46], using gene knockout, have shown PfABCG to play a role in lipid transport consistent with an ABCG1 function. In this respect, PfABCG may be functionally similar to hsABCG1, which mediates the transport of cholesterol and other sterols [39]. Furthermore, PfABCG shows strong homology to one of the six ABCG members in *Toxoplasma gondii*, (e.g., $TgABCG_{77}$) which has been suggested to mediate cholesterol and phospholipid transport [47]. Taken together, we believe that it is premature to label PfABCG as homolog of either hsABCG2 or G1 at the present time in light of the limited functional results available. It would be interesting if PfABCG in *P. falciparum* mediates the substrate specificities of both hsABCG1 and G2 members or is unlike either in terms of substrate specificity, hence a new family member or Gx.

In an effort to study the endogenous expression levels of PfABCG and its subcellular localization in the parasite, rabbit antiserum was made against the N-terminal 356 residues of PfABCG. The generated antiserum was shown to specifically recognize the N-terminal domain of PfABCG and not hsABCG2 or other ABC proteins in erythrocytes [28]. Western blotting, using this antiserum recognized mainly one polypeptide migrating with an apparent molecular mass of 65kDa on SDS-PAGE and a minor band migrating with a molecular mass of 70 kDa. Interestingly, only the 65 kDa but not the 70 kDa band that was completely competed with excess purified specific antigen, but not an irrelevant polypeptide. The observed difference between the apparent and calculated molecular masses of PfABCG (65 kDa and 75 kDa) is presently not clear but has been observed with mammalian ABCG proteins. For example, hsABCG1 with a calculated molecular

mass of ~75kDa has been shown to migrate with an apparent molecular mass of 90kDa [48]; while hsABCG2 was seen to migrate with an apparent molecular mass of 60-65 kDa instead of 72kDa [49, 50]. Alternatively PfABCG reactive polypeptide, migrating with a molecular mass of 65 kDa, may be a cross-reacting protein different from PfABCG. Although this possibility cannot be entirely ruled out at this stage, it is highly unlikely for the following reasons: a) the specificity of the PfABCG antiserum towards the recombinant N-terminal fragment of PfABCG and the absence of other reactive polypeptides in both erythrocytes and *P. falciparum* lysate, save the 70 kDa polypeptide reactivity which could not be competed with excess antigen, b) the correlation between PfABCG protein and RNA expression levels in HB3, 7G8 and 3D7 strains of *P. falciparum* (figure 4), and c) the differential sensitivity of the three chloroquine sensitive strains (e.g., HB3, D10 and 3D7) to ketotifen, a PfABCG associated drug [36]. Another possibility is that the 65kDa polypeptide is a proteolytic fragment of the native polypeptide or a modified PfABCG. Again, although these possibilities cannot be entirely ruled out at this point, the possibility that the 65 kDa is a proteolytic fragment of PfABCG is unlikely given that the same molecular mass polypeptide was consistently observed in different extracts and from different stages of the parasite life cycle. Moreover, GFP-PfABCG construct expressed in heterologous mammalian cell line also migrated with lower apparent molecular mass following a Western blot with anti-GFP monoclonal antibody (74 kDa instead of 102 kDa, Results not shown).

Our results show PfABCG to be differentially expressed in three stages of the parasite erythrocytic life cycle, with lowest expression levels in the ring stage and highest in late trophozoite stage, in addition to its high expression in gametocytes. However, a comparative Western blot of asexual stages and gametocyte lysates was not done. Among the characterized

ABC transporters, only PfGCN20 is similarly expressed throughout the parasite life cycle [51], while PfMDR1 expression was mainly found in trophozoite stages [52] and PfMRP1 in trophozoites and schizonts stages [53]. In our study, the ring stage was overloaded and protein expression was related to the number of parasites per microgram of protein. Attempts to detect PfABCG expression by immunofluorescence microscopy reveal a less intense signal in ring stage. However, as expected a strong fluorescence signal for PfABCG was observed in early, late trophozoites and schizonts. Moreover, the fluorescence signal was abolished when antiserum was pre-absorbed with purified GST-PfABCG$^{1-356}$ but not GST protein (results not shown). Consistent with earlier microarray results [31], gametocytes show strong signal for PfABCG. We are aware of the fact that RNA expression studies have shown PfABCG RNA to be maximally expressed in gametocytes and weaker expression in early ring stage [46]. Other reports [54] measuring RNA levels of PfABCG in different stages of the parasite life cycle have shown other asexual stages of the parasite to express PfABCG RNA, consistent with our findings in this report. Based on our immunofluorescence staining results, it is not clear if the observed high RNA levels in gametocytes is due to increased transcription or RNA stability in gametocytes versus that observed in the asexual stages. To our knowledge, this report is the only study describing endogenous protein expression using a specific antibody to PfABCG and that the expression of both protein and RNA are consistent for the different strains studied in this report (figure 4). Moreover, it is not clear why knockdown of PfABCG expression in 3D7 [36], which is presumably expressed only in gametocytes and not in the asexual stages of the parasite, could affect the sensitivity of the asexual stages of the parasite to ketotifen. The fact that knockdown

of PfABCG in 3D7 leads to decreased sensitivity to ketotifen suggests that the protein (PfABCG) is functionally expressed in the various asexual stages of the parasite.

PfABCG in early and late trophozoites localizes to the parasite plasma membrane but not the digestive vacuolar membrane. In addition to the plasma membrane staining, several bright staining regions beneath the parasite plasma membrane are observed in both early and late trophozoite stages; however the nature of these stained membranous regions is currently not clear. We speculate that such intensely stained regions could represent specialized micro-domains that may reveal the normal substrate specificity of PfABCG. Similar bright regions underneath the plasma membrane of *P. falciparum* were also observed in a study by Tran et al. [46] in early ring and gametocytes using GFP-PfABCG transfectants. Interestingly, no GFP-PfABCG signal was observed in other asexual stages of the parasite in that study, however in this latter study PfABCG expression was not native.

Staining of segmented schizonts, whereby individual membrane staining of each merozoite is observed and is consistent with plasma membrane. Similar staining was observed for PfMRP1, which localizes to the parasite plasma membrane and plays a role in the efflux of quinoline drugs and glutathione [17]. It is not yet clear how the subcellular localization of PfABCG mediates the sensitivity of the parasite to ketotifen, whereby decreased expression of PfABCG ([36], and our results in figure 4) correlates with decreased sensitivity to ketotifen. One possibility is that PfABCG mediates the transport of a normal metabolite similar to hsABCG2 (e.g., uric acid or glutathione; [41, 55]) or hsABCG1 (e.g., cholesterol) whereby ketotifen

activates PfABCG and consequently higher efflux of either of the latter cell metabolites leads to increased sensitivity.

In summary, we provide evidence that the unique *P. falciparum* ABCG transporter (PfABCG) is expressed throughout the life cycle of the parasite and is differentially expressed in different *Plasmodium* strains independent of chloroquine resistance. The identification of HB3 strain that expresses much lower levels of PfABCG RNA and protein that correlates with this strains sensitivity to ketotifen relative to other chloroquine sensitive strains (3D7 and D10) provide a normal system to test possible function of PfABCG. Moreover, we demonstrate that PfABCG expression is localized to the parasite plasma membrane and tubular round structure underneath the cell membrane.

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Addendum to Chapter 4

PfABCG is a 1980 bp gene without intron which is translated in 660 amino acid (see www.plasmodb.org). As most of the proteins in plasmodium, PfABCG gene is AT rich.

PfABCG sequencing and single nucleotide polymorphisms (SNP) in field isolates

Genomic DNA was extracted from different laboratory strains *P. falciparum* and amplified using specific PfABCG primers. There were no point mutations in all the laboratory strains tested (3D7, FCR3, D10, HB3, Dd2). However a recent study in which several genes were sequenced in field isolates show that there are sixteen SNPs in PfABCG with six predominant SNPs as shown in Figure S1 (see "SNP overview" on www.plasmodb.org).

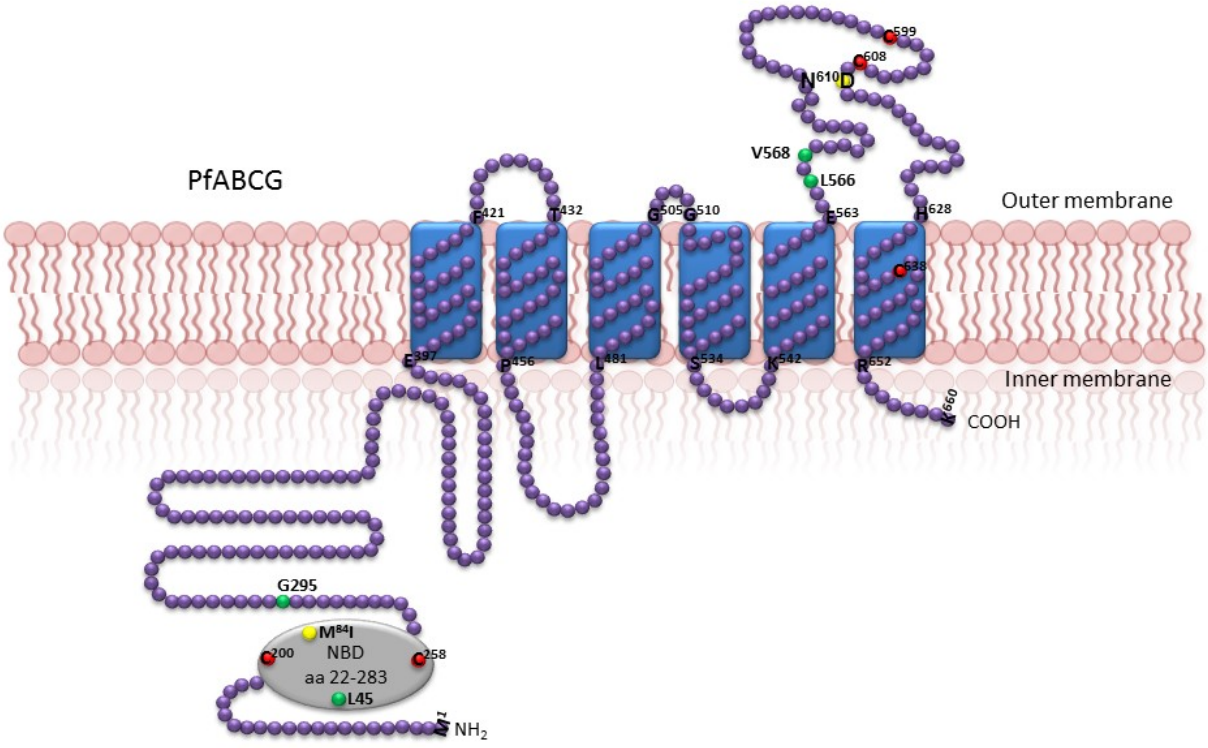

Figure S1. PfABCG topology with the different SNPs. The non-synonymous and synonymous SNPs are indicated respectively with green and yellow circles.

SUMMARY AND CONCLUSIONS

Drug resistance constitutes a bottleneck in the elimination of malaria. Indeed, malaria parasites develop resistance to any existing antimalarial drugs, and even to the ACTs which are nowadays the treatment of choice for uncomplicated malaria [1]. Like in tumour cells, drug resistance in malaria involves transporters such as those from the ABC family [2, 3]. It is well understood now that PfCRT, albeit not an ABC transporter, is the key transporter responsible for chloroquine resistance, while PfMDR1 (an ABC transporter) plays a modulatory role in multidrug resistance [4, 5]. Major efforts have been provided to reduce the rise of drug resistance namely the use of combination therapies (ACTs), the screening of drugs that are clinically used for other diseases and the generation of novel drug candidate based on the modification of existing antimalarials.

In the first part of this thesis, we determine the antimalarial activity of a drug currently used for the treatment of asthma (the LTD4 antagonist MK571), and of novel synthesized molecules (analogues of chloroquine) against CQ-sensitive and -resistant parasites. We also explore their mechanism of action with the involvement of PfCRT and PfMDR1.

The results obtained in chapter two reveal that although MK571 has a structure similar to that of chloroquine, it does not interact with PfCRT and PfMDR1 in the same manner as does chloroquine. MK571 is more efficient against chloroquine-resistant than chloroquine-sensitive strains but overall, this drug is less efficient then chloroquine. Interestingly, MK571 effects cannot be reversed by verapamil. This result excludes PfCRT as being involved in the mechanism of action of MK571. However, we demonstrate that MK571 is a substrate of wild-type and mutant-type PfMDR1. We also notice some similarities between MK571 and chloroquine, namely: 1) an accumulation in the parasite's DV and an alkalization of the DV's pH; 2) an inhibition of β-hematin formation *in vitro*. Moreover, our results suggest that MK571 can be used alone or in combination with other antimalarial drugs because the concentration of MK571 tolerated by humans is higher than the dose used to kill the parasite. With these encouraging results, drug binding studies of MK571 with PfMDR1 are future areas that can be

explored. A framework for clinical use of MK571 with quinoline drugs need to be implemented to include MK571 in the pipeline of combination therapies.

Likewise, the results of the study presented in chapter three reveal that a single modification of chloroquine can completely change its function. Novel synthesized chloroquine analogues are found less effective than chloroquine against sensitive and resistant strains. Moreover, these analogues effect cannot be reversed by verapamil. Thus, the modification introduced at position 3 in the quinoline core changes the specificity of chloroquine to PfCRT. In addition, we highlight analogue 3-ICQ as being the most effective drug among the synthesized analogues. 3-ICQ, like chloroquine, is able to inhibit hemozoin formation. Interestingly, 3-ICQ is able to reverse chloroquine resistance *in vitro* better than the well-known reversing agent verapamil. With the introduction of ACTs as the first-line treatment of malaria, a new drug combination including 3-ICQ and CQ can add a great value to the existing drugs pipeline for the treatment of malaria. To get to this point, more toxicity tests need to be performed on mammalian cells and also in animal model before the approval for human testing.

Given the important role of ABC transporters in Plasmodium drug resistance, it was of interest to biochemically characterize a novel Plasmodium ABC transporter (PfABCG) as a potential actor in drug resistance. Given the scarcity of studies related to level of expression, localization and function of this protein in the parasite, we took the advantage of the availability of the *P. falciparum* genome to characterize PfABCG and contribute to the knowledge of ABC transporters in Plasmodium. To this end, we generate a specific antibody against PfABCG and our results in chapter four reveal that PfABCG is expressed at the asexual stages of the *P. falciparum* life cycle. The protein is localized to the parasite's plasma membrane and its expression correlates with the sensitivity of the parasite to an antihistamine drug named ketotifen. To the best of our knowledge, this is the first study describing the endogenous expression of PfABCG in *P. falciparum* and this study will be beneficial to the research community. To complete the profile of this protein, future work will have to investigate the physiological function of this protein and its ability to transport normal cell metabolites and drugs as hsABCG2 or sterols as hsABCG1. This may give to the malaria community another tool

to block the development of the parasite and thus to stop the transmission of the disease by targeting this protein.

In conclusion, I would like to share with you this quote from the authors Picot et al. [6]: “Most of the people suffering malaria do not know how complex is that disease and how hard researchers are working to fight against it. Some of these people are dying from malaria while still efficient drug combinations should have saved their lives. But these drugs were not available for them when needed, or the diagnosis was wrong, or the drugs were fake, or the doctor was untrained, or the patient had no money. Should this terrible reality preclude the need for continuous research to develop new antimalarial drugs?” In my opinion, not only research must continue with the collaboration of the entire malaria research community (endemic and non-endemic countries) for development of new antimalarial drugs and better diagnostic tools, but also education and information of doctors and the population in endemic countries have to be addressed in order to eliminate malaria once and for all. As scientists we hope that our understanding of plasmodium drug resistance mechanism and the discovery of new drugs whose mechanism of action could bypasses defense mechanisms of the parasite will enable us to see the light at the end of the tunnel.

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