Biochemical Characterisation of The *Plasmodium falciparum* Chloroquine Resistance Transporter

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ABSTRACT

The emergence of resistance to commonly used antimalarials significantly hindered global efforts in eliminating malaria and cost the human race losses of lives in millions. *Plasmodium falciparum* parasites are the most accountable for morbidity and mortality compared to the other species that infect humans. At the present time, artemisinin combination therapies is the approach used in the field to treat malaria infected people and has shown tremendous success. However, resistance to these combinations recently emerged and the pattern of progression and spreading is alarming. Chloroquine once was the first-line drug for treatment of malaria infected people, however, it became in-effective due to the spread of chloroquine resistant strains. Many attributes of chloroquine, at the time when it was effective, were desired and as such the field took on different approaches to revive it. Some people took an approach to withdraw the use of chloroquine for a significant period of time resulting in the emergence of chloroquine sensitive strains. Others looked into modifying the structure of chloroquine in order to make derivatives that would be an improvement on the original. Additionally, others went on to investigate the molecular mechanism by which the parasite confers resistance to chloroquine. Presently, it is well known that mutations in the chloroquine resistance transporter (PfCRT), expressed on the membrane of a lysosome-like organelle in the parasite, the digestive vacuole (DV), are the primary determinants of chloroquine resistance. The physiological role and normal substrates are still matters of speculation but the protein seems to be important for the parasite survival because knockout-PfCRT clones could not be established. The crystal structure was resolved showing the spatial arrangement of the polypeptide chain relative to the juxtaposition of the transmembrane domains forming the central cavity where drugs would interact with PfCRT.

Given PfCRT's role in chloroquine resistance, we thought if chloroquine was slightly modified it would bypass PfCRT resistance mechanism. The first experimental manuscript thesis, we examined the antimalarial activity of 16 novel chloroquine derivatives against chloroquine-sensitive and -resistant *Plasmodium falciparum* strains. Only two compounds (e.g., AQ-13 and AQ-129) showed effects that surpassed chloroquine's effect on chloroquine resistant strains that were examined previously but not to the extent of their relationship with PfCRT. Our results demonstrate that AQ-13 and AQ-129 are poor substrates of PfCRT and thus more effective against chloroquine resistant parasites.

In the 2nd manuscript, we describe the high resolution characterisation of an antiserum raised against the full-length C-terminal domain of PfCRT. An IgG pool that recognises a de-phosphorylated Ser411 epitope was extracted and used as a tool to monitor the phosphorylation status of residue Ser411. This pool of IgG`s identified the presence of an Ser411 de-phosphorylated homodimer form of PfCRT that does not localise to the DV membrane as does the monomer PfCRT. We also show that PfCRT monomer in chloroquine-sensitive strain (3D7) is significantly more phosphorylated than in chloroquine-resistant strain (Dd2-H) at Ser411, suggesting a possible functional role for this residue in drug resistance.

In the last manuscript, we describe the adoption of mammalian HEK-293F cells as a heterologous system to study PfCRT function. Using HEK-293F cells stably expressing PfCRT wild-type and mutants, we show mutant-PfCRT to cause a significant acidification of the lysosomes, relative to wild-type PfCRT. We also provide direct evidence that acidification was mediated through mutant-PfCRT, since using a proline-165-modified mutant-PfCRT clone restored the acidification

of lysosomes to wild-type PfCRT levels. Thus, results of this study show for the first time the role of Pro165 in mutant-PfCRT function.

ABRÉGÉ

L'émergence d'une résistance aux antipaludiques couramment utilisés a considérablement entravé les efforts mondiaux visant à éliminer le paludisme et a coûté des millions de vies à la race humaine. Les parasites de l'espèce *Plasmodium falciparum* sont les plus responsables de la morbidité et de la mortalité par rapport aux autres espèces qui infectent l'humain. À l'heure actuelle, les thérapies combinées à l'artémisinine sont l'approche utilisée sur le terrain pour traiter les personnes infectées par le paludisme et ont connu un énorme succès. Cependant, une résistance à ces combinaisons a récemment émergé et le schéma de progression et de propagation est alarmant. La chloroquine était autrefois le médicament de première intention pour le traitement des personnes infectées par le paludisme, mais elle est devenue inefficace en raison de la propagation de souches résistantes à la chloroquine. De nombreux attributs de la chloroquine, au moment où elle était efficace, étaient souhaités et, à ce titre, la science a adopté différentes approches pour la faire revivre. Certaines personnes ont adopté l'approche de retirer l'utilisation de la chloroquine sur le terrain pendant un bon bout de temps, entraînant l'émergence de souches sensibles à la chloroquine. D'autres ont envisagé de modifier la structure de la chloroquine afin de fabriquer des dérivés qui seraient une amélioration par rapport à l'original. De plus, d'autres ont poursuivi leurs recherches sur le mécanisme moléculaire par lequel le parasite confère une résistance à la chloroquine. Actuellement, il est bien connu que les mutations du transporteur de résistance à la chloroquine (PfCRT), exprimées sur la membrane d'un organite de type lysosome chez le parasite, la vacuole digestive, sont les principaux déterminants de la résistance à la chloroquine. Le rôle physiologique et les substrats normaux de cette protéine sont encore des sujets de spéculation mais la protéine semble être importante

pour la survie du parasite car les clones de *Plasmodium* déficient en PfCRT n'ont pas pu être établis. La structure cristalline de la protéine PfCRT a été résolue montrant l'arrangement spatial de la chaîne polypeptidique par rapport à la juxtaposition des domaines transmembranaires formant la cavité centrale où les médicaments interagiraient avec PfCRT.

Étant donné le rôle des protéines PfCRT dans la résistance à la chloroquine, nous pensions que si la chloroquine était légèrement modifiée, elle contournerait le mécanisme de résistance à la protéine PfCRT. Dans la première partie de cette thèse, nous avons examiné l'activité antipaludique de 16 nouveaux dérivés de la chloroquine contre les souches de *Plasmodium falciparum* sensibles et résistantes à la chloroquine. Seuls deux composés (par exemple, AQ-13 et AQ-129) ont montré des effets supérieurs à l'effet de la chloroquine sur les souches résistantes à la chloroquine qui ont été examinés, mais pas dans la mesure de leur relation avec PfCRT. Nos résultats démontrent que l'AQ-13 et l'AQ-129 sont de mauvais substrats de la protéine PfCRT et donc plus efficaces contre les parasites résistants à la chloroquine.

Dans le chapitre 3, nous avons décrit la caractérisation haute résolution d'un antisérum dirigé contre le domaine entier C-terminal de PfCRT. Un ensemble d'IgG qui reconnaît un épitope Ser411 déphosphorylé a été extrait et utilisé comme outil pour surveiller l'état de phosphorylation du résidu Ser411. Cet ensemble d'IgG a identifié la présence d'une forme homo-dimère Ser411 déphosphorylé de PfCRT qui ne se localise pas à la membrane de la vacuole digestive comme le fait le monomère PfCRT. Nous avons montré également que le monomère PfCRT dans la souche sensible à la chloroquine (3D7) est significativement plus phosphorylé que dans la souche résistante à la chloroquine (Dd2-H) à la position Ser411, suggérant un rôle fonctionnel possible pour ce résidu dans la résistance aux médicaments.

Dans le chapitre 4, nous avons décrit l'utilisation des cellules HEK-293F de mammifères comme système hétérologue pour étudier la fonction de PfCRT. En utilisant des cellules HEK-293F exprimant de manière stable les protéines PfCRT originale et mutante, nous avons montré que la protéine PfCRT mutante provoque une acidification significative des lysosomes, par rapport à la protéine PfCRT originale. De manière très significative, nous avons fournis également des preuves directes que l'acidification a été médiée par la protéine PfCRT mutante, car l'utilisation d'un clone mutant-PfCRT qui a été modifié par la proline 165 a restauré l'acidification des lysosomes au même niveau que celui de la protéine PfCRT originale. Ainsi, les résultats de cette étude montrent pour la première fois le rôle du résidu Pro165 dans la fonction de la protéine mutante-PfCRT.

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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 2), Benita Kapuku synthesized the chloroquine derivatives and Julia Hageman worked with Benita Kapuku only on the synthesis of compounds AQ-13 and AQ-129 used in the study under the supervision of Dr. David Scott Bohle from the Department of Chemistry, McGill University.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

The following aspects described in this thesis are considered original contributions to our knowledge of malaria drug resistance.

MANUSCRIPT I

Fadi Baakdah, Benita Kapuku, Julia Hageman, David Scott Bohle and Elias Georges.

Characterization of 4-Aminoquinoline Derivatives against Chloroquine Resistant Strains of Plasmodium falciparum. (Manuscript in preparation).

This manuscript examined 16 novel chloroquine derivatives modified at 3rd position of the quinoline moiety/ the side-chain of chloroquine that included two previously demonstrated effective compounds on chloroquine resistant strains, AQ-13 and AQ-129. The study found that AQ-13 and AQ-129 were the most effective against chloroquine resistant strains and showed for the first time that they are poor substrates of PfCRT using PfCRT isogenic clones. Selecting for resistant mutants to AQ-13 and AQ-129 for a period of 3 months was not established suggesting that resistance is not an easy task to achieve to these compounds by the parasites. We also demonstrate AQ-13 and AQ-129 inhibit beta-hematin formation *in-vitro* as in the case of chloroquine. Taken together, this work demonstrates that AQ-13 and AQ-129 are upgrade chloroquine derivatives better than original chloroquine.

MANUSCRIPT II

Fadi Baakdah and Elias Georges.

High resolution mapping of PfCRT antiserum identifies a phosphorylated PfCRT at Ser411 in the parasite digestive vacuole. (Manuscript in preparation).

Resistance to chloroquine via mutant-PfCRT was shown to involve phosphorylation of Ser33 on the N-terminal of PfCRT. Here we extracted and characterized an anti-Ser411 pool of IgG`s from a PfCRT antibody raised against synthetic de-phosphorylated PfCRT C-terminal peptide. This special pool of IgG`s showed the presence of homodimer-PfCRT not localized to the DV membrane and is de-phosphorylated on Ser411 in contrast with the monomer form. It also showed that chloroquine sensitive strain 3D7 is significantly more phosphorylated than chloroquine resistant strain Dd2-H at Ser411 suggesting a possible functional role. Taken together, we have a novel tool to monitor the status of residue Ser411 in PfCRT.

MANUSCRIPT III

Fadi Baakdah and Elias Georges.

Substitution of Pro165 in transmembrane 4 of chloroquine resistance transporter PfCRT abolishes lysosome acidification function in stably transfected HEK-293F Cells. (Manuscript in preparation).

Studying PfCRT in the parasite is challenging. Therefore, we stably expressed wt-PfCRT^{ETSE} and mut-PfCRT^{NKAQ} in HEK-293F cells and selected clones for each one. We show that the PfCRT protein in all the clones localised to the lysosomal membrane. Mut-PfCRT^{NKAQ} clones demonstrated significant acidification relative to wt-PfCRT^{ETSE} clones measured by accumulation of pH sensitive dyes Acridine orange and LysoOrange Indicator reagent. We also demonstrate the abolishment of the acidification function mediated by mut-PfCRT^{NKAQ} using our proline mutated clone mut-P165A-PfCRT^{ETSE}. This showed that acidification was directly mediated through mutant PfCRT. Moreover, we demonstrate an essential role for Pro165 in mutant PfCRT lysosomal acidification function.

LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ABCG2	ATP binding cassette member G2
ACT	Artemisinin based Combination Therapies
ART	Artemisinin
ATP	Adenosine Triphosphate
BCRP	Breast Cancer Resistance Protein
CQ	Chloroquine
CQR	Chloroquine Resistance
CQS	Chloroquine Sensitive
DAPI	4',6-diamidino-2-phenylindole
DDT	DichloroDiphenylTrichloroethane
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleotide adenosine
DV	Digestive Vacuole
EDTA	Ethylenediaminetetraacetic acid
GSH	Glutathione
GFP	Green fluorescent protein

HF	Halofantrine
IC_{50}	Inhibitory concentration for 50%
kDa	Kilo Dalton
MDR1	Multi Drug Resistance 1
MQ	Mefloquine
MW	Molecular weight
NBD	Nucleotide Binding Domain
PBS	Phosphate Buffer Saline
P. falciparum	*Plasmodium falciparum*
PfCRT	*Plasmodium falciparum* Chloroquine Resistance Transporter
PfMDR1	*Plasmodium falciparum* Multi Drug Resistance 1
PfMRP1	*Plasmodium falciparum* Multidrug Resistance Protein 1
PfPNP	*Plasmodium falciparum* cytosolic purine nucleoside phosphorylase
Pfkelch13	*Plasmodium falciparum* Kelch propeller protein 13
Pgp	Pglycoprotein
QN	Quinine
RBC	Red blood cell
TM	Transmembrane
TMD	Transmembrane Domain
VP	Verapamil

WHO	World Health Organization

LIST OF FIGURES

CHAPTER 1.

Literature review

CHAPTER 2.

Characterization of 4-Aminoquinoline Derivatives against Chloroquine Resistant Strains of *Plasmodium falciparum*

CHAPTER 3.

High resolution mapping of PfCRT antiserum identifies a phosphorylated PfCRT at Ser411 in the parasite digestive vacuole

CHAPTER 4.

Substitution of Pro165 in transmembrane 4 of chloroquine resistance transporter PfCRT abolishes lysosome acidification function in stably transfected HEK-293F Cells

LIST OF TABLES

Chapter 2.

Characterization of 4-Aminoquinoline Derivatives against Chloroquine Resistant Strains of *Plasmodium falciparum*

CHAPTER 4.

Substitution of Pro165 in transmembrane 4 of chloroquine resistance transporter PfCRT abolishes lysosome acidification function in stably transfected HEK-293F Cells

TABLE OF CONTENTS

GENERAL INTRODUCTION

Malaria is a blood-borne disease that shortened the life-span of millions of people especially in endemic regions. *Plasmodium* parasites are of many species, however, only a few cause the human malaria disease with the *falciparum* species being the most problematic. To survive in humans, the parasites made the host red blood cells their normal living space where they evolve through different life-stages of their erythrocytic cycle. Malaria treatment requires the use of established antimalarial drugs that exert their effects on different targets in the parasite. Based on their chemical structure and mode of action these compounds can be divided into five groups: quinolines, antifolates, antimicrobials, hydroxynaphthaquinones and artemisnins [1, 2]. Quinoline based drugs are an important group of antimalarials that possess the heterocyclic aromatic quinoline ring. Quinine was the first antimalarial that was used for treatment replaced later by its derivative chloroquine [3]. Chloroquine, was the first-line antimalarial drug to treat malaria -infected individuals by virtue of its efficacy, safety and cost of synthesis and affordability until the parasites formulated a mechanism to resist its toxic effects [4]. Currently, it is recommended by the WHO that artemisinin combination therapies or ACTs are to be used for treatment as first-line which showed great success in alleviating the malaria burden and saved many lives [5]. However, resistance has emerged already to these combinations starting from Cambodia in the east and has now traveled west to the African continent [6-8]. Drug resistance is a continuous problem in the malaria field and the only means of defence against these pathogens is either blocking transmission of the parasite by controlling the distance between people and the female *Anopheles* mosquito vector using insecticidal bed nets or the use of effective antimalaria drugs. Additionally, an effective vaccine is still not ready to be used in the

field yet [9]. As limited as our arsenal to take on malaria may be, the antimalaria drugs still remain our best means to treat and prevent malaria infections and the field is always exploring new compounds that can tackle current and future resistance mechanisms. Investigating the molecular resistance mechanisms is crucial to our understanding of molecular drug targets to design better drugs. It is well established that mutations in PfCRT are the primary determinants of chloroquine resistance [10]. The physiological substrate(s) as well as the native role of this transporter are still matters of speculation. The crystal structure was recently resolved but left us with questions about how this protein dynamically moves and function [11].

Therefore, the main objective of this thesis was to biochemically characterize PfCRT and its interactions with novel chloroquine derivatives. This will be illustrated in this thesis in an organised manner. Briefly, Chapter one describes the current knowledge of malaria drug resistance, antimalaria drugs used in the field and the different proteins involved in such mechanisms. Chapter 2 addresses the antimalarial activity of novel chloroquine derivatives and their relationship to PfCRT. Chapter 3 describes the high resolution mapping and characterisation of antibodies raised against PfCRT C-terminal domain and testing their ability to recognise a specific de-phosphorylated epitope to PfCRT at serine-411 that suggests to having a functional role. Chapter 4 addresses the possible functions PfCRT may have in the parasite by stably expressing PfCRT, wild-type and mutant forms, heterologously in HEK-293F cells. In addition to exploring the functional role of pro165 residue in transmembrane domain 4.

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Chapter 1

LITERATURE REVIEW

1. **History and prevalence**

Malaria is a blood borne human disease threatening the lives of millions of people, most of whom are children under five years of age. Periodic fever and chills are symptoms that reveal the onset of infection with malaria. These symptoms have been documented by the early Chinese approximately 2700BC and in early Greek writings, evidence showing that it is a very old disease that still is prevalent today [1, 2]. The name malaria stems from the Italian term mal` aria, which literally translates to bad air because it was thought that swamp fumes caused the illness. It wasn't until French physician, Alphonse Lavern, observed crescent-like bodies in the blood of an infected soldier seen for the first time under light microscopy in the 18th century [2]. These bodies were later designated as parasites of the *Plasmodium* species. To date, there are five species that infect humans: *Plasmodium vivax*, *Plasmodium malariae, Plasmodium ovale* and *Plasmodium falciparum (P. falciparum)* and *Plasmodium knowlesi* (which infects macaque monkeys but has now been established as a zoonotic human pathogen as well [3]). Indeed, the most fatal species of *Plasmodium* that causes human disease is the *falciparum* species. This parasite species is responsible for approximately 90% of the total malaria cases in the African region. The latest World Malaria Report of 2018 estimated 216 million infections and ~445,000 deaths (figure 1) [4]. The most affected parts of the world are African countries, India, Southeast Asian and Latin American countries. North American countries and Europe do have some malaria cases; however, these cases are now called "airport malaria" because they are due to immigration from traveling through endemic countries with malaria [5].

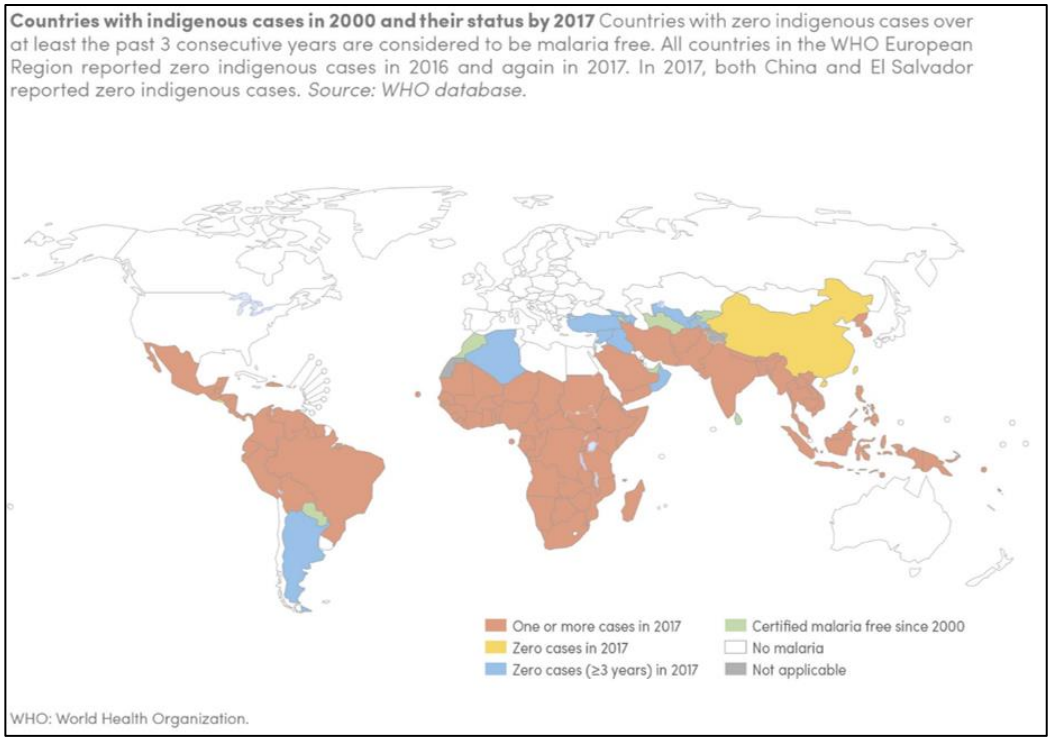

Figure 1. Malaria global distribution map (adopted from World Malaria Report 2018 [4]).

2. Life-cycle of *P. falciparum*

The eukaryotic intracellular protozoan *P. falciparum* parasite is transmitted mainly through the bite of an infected female *Anopheles* mosquito species. This route of transmission was discovered by Ronald Ross who demonstrated the cycle by allowing mosquitoes to feed on infected patients with malaria [6]. The life-cycle involves three stages between the human host and the mosquito vector. When a female *Anopheles* mosquito takes a blood meal from an infected person, gametocytes start their sexual cycle by forming ookinetes that eventually develop into oocysts. The oocysts produce over 10,000 sporozoites that migrate to the salivary glands of the mosquito. The injection of the sporozoites into the human host marks the beginning of the exoerythrocytic stage. The sporozoites migrate to the liver and develop into schizonts. Later, merozoites burst

out of these schizonts into the blood stream. When merozoites infect a red blood cell, it marks the start of the erythrocytic phase.

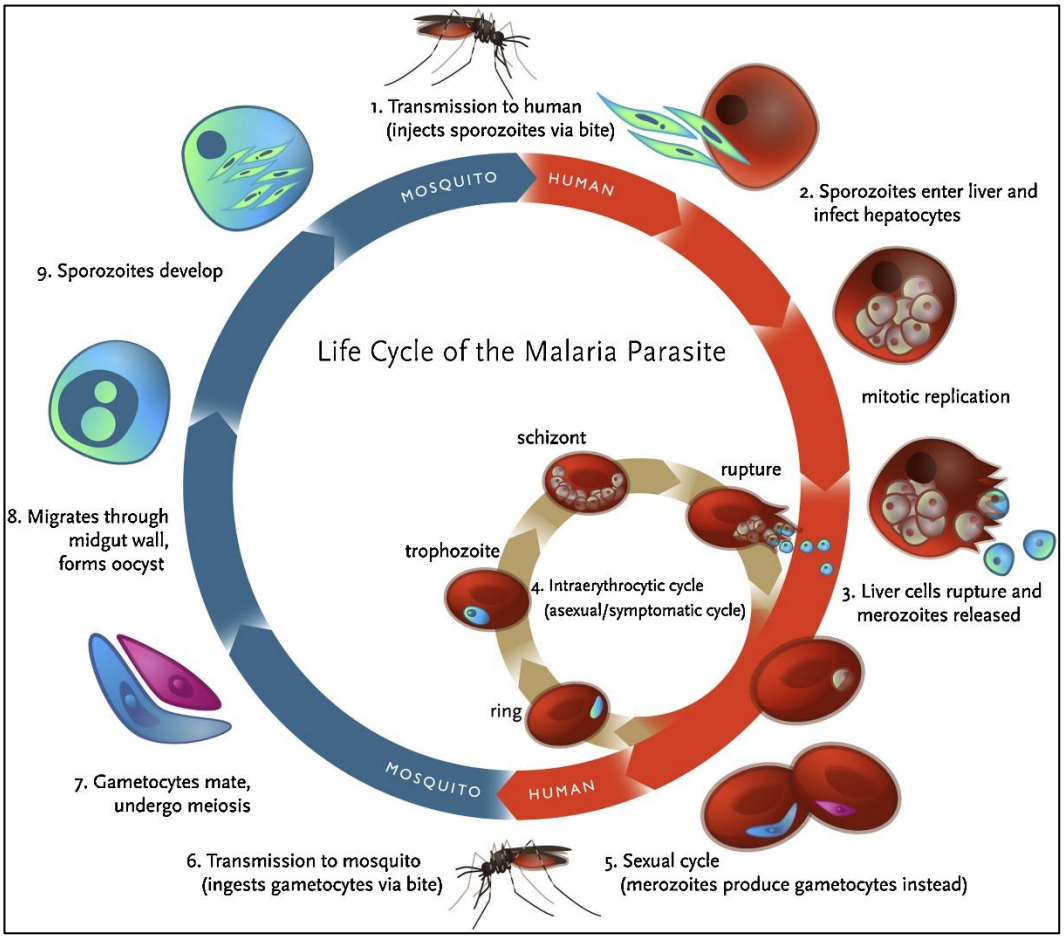

Figure 2. The Life-cycle of the human malaria parasite (adopted from Klein 2013 [7]).

The first stage is the ring stage, characterised by a chromatin dot with a light cytoplasm. Rings mature to trophozoites that have a distinctive important organelle, the DV, where hemoglobin metabolism occurs, resulting in the formation of the malaria pigment hemozoin, which is visible with light microscopy. Later in the life cycle, trophozoites develop into schizonts that generate multiple merozoites. The merozoites are then released and invade other host red blood cells (RBCs). However, not all merozoites that invade the host RBCs go from rings to schizonts; some

develop into sexual stages, macro- and micro- gametocytes in a process called gametocytogenesis. This stage, when taken up by a female *Anopheles* mosquito continues the sexual life cycle [8].

3. The malaria human disease and its clinical manifestations

Proper laboratory diagnosis of infected individuals, especially the most vulnerable as children under five years of age, pregnant women and the immunocompromised, is key to accelerating their treatment and recovery. Suspected cases of malaria are usually diagnosed via blood tested in two different ways: a) under light microscopy examination by screening for the presence of parasites and estimating the intensity of infection and, b) a rapid diagnostic test (RDT) which is an antigen based detection system detecting antigens for *Plasmodium* that are genus or species specific. A commonly detected antigen in RDTs is PfHRP2 (Histidine-rich protein 2) [9].

The erythrocytic phase of the parasite life cycle is where clinical symptoms begin and it is the target of many antimalarials. Basically, the release of the toxic by-products that arise from the rupture of the red blood cell (RBC) membrane with merozoites into the blood stream is the reason for the high fever in malaria infections [10]. The disease is characterised mainly by fever, chills, nausea and sweating. Serious clinical manifestations result in acute renal failure, severe anemia, hemoglobinuria and comas. Moreover, *P. falciparum*, is referred to as the most lethal species of its kind because it can cause cerebral malaria which is a result of blocking of small blood vessels with *P. falciparum*-infected RBC`s [11]. Furthermore, in severe malaria cases, if left untreated, death of the infected person is almost inevitable.

All *Plasmodium* species that cause human disease can have a recrudescence event. It is an event where asexual stages rise again in the blood stream of the host after incomplete treatment with antimalarial drugs. Relapse is a different case from recrudescence [12]. It is a special feature of *Plasmodium ovale* and *Plasmodium malariae,* where the asexual stages show up in the blood stream of the host after eliminating the parasite because they have the dormant hypnozoites in the host liver. These hypnozoites, after weeks or months of malaria elimination, form hepatic schizonts that release merozoites into the blood stream.

4. Efforts in malaria control

Malaria remains one of the 21st century public health challenges of vector-borne diseases. The right strategy, cost, proper application and execution are key elements in a successful program to eliminate a pathogen. For the parasite to stay alive, it can only be in humans or mosquitoes. If the parasite was in the human host, it would have to be eliminated to remove the burden. However, if the parasite is in the vector, then the vector must be controlled. According to the Centers for Disease Control and Prevention, adult male *Anopheles* mosquitoes do not transmit malaria because they satisfy their appetite by feeding on nectar and sugars and they live for approximately a week. Female *Anopheles* mosquitoes (the responsible vectors for malaria transmission) do feed on sugar sources for energy but they also require blood meals for eggs development and live up to 2 weeks. There are approximately 70 species of the *Anopheles* mosquitoes that are vectors for the human malaria parasites, the main being: *Anopheles gambiae* (known to be out-door biters) and *Anopheles funestus* (known to be in-door biters). Current methods of vector control include [13, 14]:

1- **Insecticidal-treated mosquito nets (ITN):** It is a large net with pores that are small enough to not allow mosquitoes in, treated with insecticides that last for a certain period of time, and it works as a barrier between the human host and the vector. This method is cost-effective, especially for endemic areas where it has to be affordable otherwise it cannot be purchased or used, and is also very effective in blocking transmission. In fact, this application reduced malaria transmission in Sub-Saharan Africa where malaria was endemic by more than 50% [14]. However, a few shortcomings are: 1) Some adults, and especially children under five years of age, do not apply the ITNs [15, 16]; 2) Some people cannot afford them or cannot obtain them; and 3) Some mosquitoes have developed resistance to the insecticides [17, 18].

2- **Indoor residual spraying:** This is the best method to eliminate in-door mosquitoes. In fact, it was the principle method for the Global Malaria Eradication Campaign [19, 20]. The main deficit of this strategy is that dichlorodiphenyltrichloroethane (DDT) is environmentally unfriendly. It was used in the past and now banned in many endemic countries because of its environmental impact is [21, 22].

3- **Larval source management:** It is the management of mosquito breeding sites. These areas are usually small aquatic habitats that play a role in malaria transmission [23]. This strategy can block the life-cycle of the vector completely because, if these sites were controlled, there would be no new generation of mosquitoes to arise. Only a few show that this strategy contributed to reducing in-door and out-door biting and out-door breeding of the vector [24]. There are multiple ways of managing breeding sites: 1- Draining of surface water. 2- Adding predators that would live and feed on the larvae for

example a fish called *Gambusia affinis,* a larvivorous fish. 3- The addition of an insecticide would be able to eliminate the larvae [25].

Ultimately, to treat malaria in humans, the antimalarial drugs are the best way. However, many efforts are underway to develop a protective vaccine especially for children under the age of 5 and pregnant women, who are at greater risk of infection [26].

5. Malaria vaccine

Small pox and polio have been eradicated by effective vaccines. Indeed, it would be ideal to have a malaria vaccine to add to the antimalaria arsenal [27]. Many approaches have been conducted by different groups in order to make an effective vaccine against malaria. One of these approaches was the RTS,S/AS01 vaccine which targets PfCSP (*P. falciparum* circumsporozoite protein), it was predicted to lead to the death of the parasite in the pre-erythrocytic stage [26, 28].

6. Hemoglobin degradation

Plasmodium parasites are dependent on the host red blood cells as a normal habitat. The asexual erythrocytic phase of the life-cycle begins with merozoites invading host RBC's developing into rings then trophozoites then to schizonts. In order to develop and morphologically change the parasites require the products of hemoglobin degradation because it is a source of nutrition and amino acids to the parasites. Parasite invasion of host RBC's results in the ingestion of large amounts of hemoglobin into the DV organelle. Within the DV, hemoglobin is degraded with proteolytic enzymes to globin moieties that are degraded further into smaller peptides that are important for protein synthesis [29]. The heme part is ~20mM Fe^{2+} and is toxic to the parasite if not dealt with. Fe^{2+} rapidly oxidizes to Fe^{3+} (also known as: ferriprotoporphyrin IX or FPIX) which

is very insoluble and as such can disrupt membrane integrity and initiate reactive oxygen species that include H_2O_2 and OH radicals. Therefore, to avoid serious threats to its survival, the parasite converts heme into beta hematin that biomineralize to form non-toxic inert crystals known as hemozoin or malaria pigment in the DV that are visible using light microscopy [30, 31]. Once merozoites rupture from the schizonts and host RBCs, hemozoin crystals are released in the blood circulation and later pursued by macrophages for further clearing.

7. Antimalaria drugs

Many drugs have been used in the field in order to treat and eliminate malaria. The majority of these antimalarials are active against the erythrocytic stages of the parasite development. The most common group of antimalarial drugs are quinolines that include quinine (QN), quinidine, chloroquine (CQ), amodiaquine, primaquine, piperaquine, mefloquine (MQ), halofantrine (HF) and lumefantrine. Antifolates include sulphonamides, pyrimethamine and proguanil. Hydroxynaphthaquinones includes atovaquone. Antimicrobials that include tetracycline, doxycycline and clindamycin. Lastly, artemisinin include artemisinin, dihydroartemisinin, artesunate artemether and arteether [32, 33]. Quinolines are a family of compounds that exert their effects against the asexual stages of the parasite life-cycle. These compounds are weak bases and as such accumulate in the acidic DV compartment and are suggested to interfere with hemozoin formation as observed with QN and CQ [32]. Other quinolines possess properties to eliminate liver dormant stages hypnozoites and sexual stages gametocytes, like primaquine [33-35]. Antifolates exert their effects on the erythrocytic schizont stage. They are a class of compounds that interfere with an essential metabolic pathway that produces tetrahydrofolate that is crucial for DNA synthesis. The enzymes targeted by these compounds are dihydrofolate

reductase (DHFR) and dihydropteroate synthase (DHPS) [36]. Atovaquone is a broad spectrum antiprotozoal drug that targets electron transport in the mitochondria by binding cytochrome bc1 complex and as such disrupting the membrane potential in *Plasmodium* parasites. This compound is always used in a synergistic combination with the antifolate proguanil [37]. Antimicrobials are inhibitors for prokaryotic microorganisms but it was shown that they do display anti-*Plasmodial* activity. Studies suggest that they act on the apicoplast. These compounds are slow acting and are usually used in combination with a fast acting antimalarial drug e.g. artemisinin [38]. Artemisnins are plant-derived peroxides and are the fastest acting antimalarials that kill blood stages and suppress gametocytes. Their combination with longer half-life compounds are used in the field as the first-line of defense against malaria [39].

The following section will discuss a few of the most common antimalarials in more detail.

7.1. Quinine

Quinolones are a group of compounds that contain a quinolone ring. The earliest known quinolone is an aryl-amino alcohol extracted from the bark of the cinchona tree (South America) named quinine (Qualaquin™). It was introduced to Europe in the 17th century [28]. QN and its enantiomer quinidine are used in severe malaria cases and chloroquine-resistant malaria cases [40]. QN is believed to act on the heme-polymerization pathway, which is mainly the target of quinolone-based drugs. However, it was recently suggested that QN acts by binding a critical component of the purine salvage pathway: *P. falciparum* cytosolic purine nucleoside phosphorylase (PfPNP) [41]. Synthesis of QN is expensive because of its complex structure. So for now, the cinchona bark remains the main source of QN for treatment and commercial use [42].

7.2. Chloroquine

This synthetic analogue of QN was synthesised in the 1930s by German chemist Hans Andersag. It was later taken by the United States of America after World War II [43]. Quinine therapy was replaced with CQ (commercial name Resochin®) in the 1940s in efforts to eradicate malaria by the WHO [44]. Many features accompanied this drug including: 1- It was very effective. 2- It was low in toxicity. 3- It was cheap to make, which made it available in low income areas. Moreover, it was effective against all *Plasmodium* species that cause human disease. Moreover, the half-life of CQ is approximately 60 days [45]. For this reason it was used as a prophylactic drug [46]. It was also prescribed for pregnant women [47].

Structurally, CQ is a diprotic weak base and once protonated, it accumulates in the parasite the DV, the site of hemoglobin degradation [48, 49]. The mechanism by which CQ works is still not clear, however, it is thought that CQ acts by binding heme and preventing heme conversion into inert crystals known as hemozoins. It was believed that this would lead to the accumulation of heme monomers that later causes parasites to die [50]. Currently, CQ it is not prescribed to treat *P. falciparum* infected patients as the first-line of treatment and some countries withdrew the drug for a significant period of time [51]. A few years later, the malaria field saw the re-emergence of CQ-sensitive parasites. Malawi, Kenya, Cameroon and Senegal are countries that have reported a decline in CQ resistance due to the discontinuation of CQ use and/or withdrawal of CQ as the first-line of treatment [52, 53]. This pattern of re-emergence of CQS strains speculates that CQR is accompanied with a price of fitness to the parasites [54].

7.3. Mefloquine and Halofantrine

In response to CQ resistance cases that affected American troops in the 1960`s, the Walter Reed Army Institute of Research in Washington DC researched drugs that were rejected as candidates after WWII [55]. This work led to the discovery of MQ and HF. MQ (Lariam®), 4-methanolquinoline, is a drug approved for clinical use by the Food and Drug Administration in 1989 [56]. MQ was used as a prophylactic drug because its half-life was 10 – 23 days. The mechanism of action of MQ is not yet understood, however like quinolone-based drugs, it is believed to act on the heme detoxification pathway [57]. MQ is now used in effective combinations with artesunate in treating uncomplicated malaria infections in adults and children [58].

HF, an aryl-amino-alcohol, is an effective antimalaria drug acting against CQR and CQS *P. falciparum*. Unfortunately, due to HF`s associated cardiotoxicity, the drug was withdrawn from international guidelines in treating malaria-infected individuals [59].

7.4. Artemisinin

Chinese traditional herbal medicine treated cases of fever, then named “qinghaosu”, a sweet wormwood plant, now called *Artemisia annua*, in early 340 A.D. Western research confirmed artemisinin`s potent antimalarial activity in the 1990`s [59]. Artemisinin is now a major player in malaria elimination programs [60]. The best source of artemisinin is from the plant, however, this is not ideal due to the dependence of the plants on climate [61]. Unfortunately, chemical production has seen drawbacks due to the complicated structure of the compound [62]. However, genetically modified yeast has been reported to increase production of the drug [63]. Furthermore, artemisinin`s original form is not well absorbed, therefore, multiple easily absorbed

derivatives have since been produced, such as artemether, artesunate and dihydroartemisinin [64].

The mode of action of artemisinin's is not clear; some researchers suggest it acts on targets in the DV such as plasmepsin II, which is one of the enzymes involved in hemoglobin degradation [65-67]. The half-lives of artemisinin derivatives is relatively short [64, 68]. Although this is thought to reduce the likelihood of resistance, there are cases of resistance to artemisinin derivative monotherapy [69]. The WHO provided directions in 2001 on artemisinin's drug administration, by pairing artemisinin derivatives with other effective antimalarial drugs with a different mode of action (e.g. MQ, amodiaquine, sulfadoxine-pyrimethamine, piperaquine and lumefantrine (artemether-lumefantrine is commercially known as Coartem®) in a double-barrel approach known now as Artemisinin Combination Therapy or ACT [70]. Artemisnins are administered as a 3-day regimen in combination with a longer half-life partner drug [71]. The ACT approach reduces the probability of drug resistance because the parasite has to avoid lethal effects of both agents at the same time. Unfortunately, a few cases of emerging resistance to ACTs in the Thailand-Cambodian border and in some African countries have been reported [72, 73]. Many proteins are being investigated to uncover the molecular mechanism of resistance to ART led to the discovery of mutations in k13 propeller region thought to be important in ART inactivation [74]. Be that as it may, ACT are still the first-line of treatment of uncomplicated malaria from *P. falciparum* in endemic regions by WHO guidelines [75-77].

8. Resistance in *P. falciparum*

P. falciparum is a parasite of medical importance. While the DV organelle is one target, a few other targets worthy of exploiting associated with the DV include the DV pH, redox-cycle, DV

enzymes and DV integral membrane proteins. Moreover, efforts are ongoing to bring CQ back into the field. Therefore, an investigation on CQR and the possible members responsible for this process is required.

9. Chloroquine resistance

CQ was effective and efficient in curing malaria for many years [78]. Its low toxicity levels and low production cost are some of the desired features for drugs used in the field. Like many effective antimalarial drugs used against *P. falciparum*, resistant phenotypes emerged [79, 80]. After approximately 20 years of its usefulness in the field, cases of CQR emerged in Thailand in 1957 and by the 1970s resistance spread through the African region (sub-Saharan Africa) [7, 81]. Antimalarial drugs with similar features or superior to CQ are urgently needed. For this, understanding the molecular mechanism of CQR became a priority for many researchers in the field. CQ is thought to act on the heme biocrystallisation pathway [31, 82]. Verapamil, an L-type calcium channel blocker, also known as an inhibitor to P-glycoprotein (or P-gp, an ABC transporter associated with drug resistance in cancer cells) was shown to reverse CQR in CQ resistant strains [83]. ABC transporters are known for their ability to export many anti-cancer drugs. This suggested that CQR could be due to a homolog of MDR in *P. falciparum* which was proven otherwise with a genetic-cross between Dd2 and HB3 [84, 85].

10. Transporters implicated in drug resistance

10.1. Pfkelch13

Artemisinin combination therapies (ACT) are the first-line of treatment recommended by the WHO against *P. falciparum* parasites [86]. Current successes with ACT is now threatened by the emergence of resistance seen in the malaria field [87]. Resistance emerged in western Cambodia

and now has spread to Southeast Asia and South China [69, 88-90]. Using whole genome sequencing of an ART sensitive parasite line pressured for approximately 5 years with ART uncovered a gene designated PF3D7_1343700 (k13) also known as Kelch protein or Pfkelch13 and mutations in this intron-less gene were linked to ART resistance [74]. There are over a hundred different k13 mutations but the most frequent are R539T, C580Y and Y493Y [91-93]. Pfkelch13 is found to localise in vesicles that reside close to the parasite produced erythrocyte-cytosol containing structures known as cytostomes (cell mouth). Using human KEAP1 as a template, the structure of Pfkelch13 by homology modeling is composed of three functional domains: a *Plasmodium*-specific localisation sequence on the N-terminus, BTB/POZ domain that is known to facilitate ubiquitin-mediated degradation and a six blade carboxyl-terminal Kelch propeller repeat domain predicted to function as a scaffold for protein-protein interactions [74, 94, 95]. Based on the Kelch/BTB/POZ family of adaptors, the protein is speculated to function as a regulatory protein [96, 97]. It was shown that Pfkelch13 proteins in such compartments were required for hemoglobin endocytotic uptake [94]. Interestingly, parasites with inactivated PfKelch13 or resistance-conferring mutation of PfKelch13 showed lower hemoglobin uptake and enhanced ring stage resistance to ART when exposed to high concentrations of ART [94, 95]. A model proposed that when the ability of Pfkelch13 to assist in hemoglobin engulfment was impaired due to mutations in the propeller domain resulted in less hemoglobin breakdown and as such less heme is available for ART activation [98]. This process slows the parasites growth because of the reduced amino acid pools derived from hemoglobin degradation that is needed for synthesis. This in turn leads to cellular stress relieved by unfolded protein responses [98-100]. ART inhibition of hemoglobin engulfment suggests a possible target to explore for future

antimalarials [101]. Resistance to ART is not limited to the Pfkelch13 domain, other proteins have are being investigated in this spectrum including PfMDR1, AP-2μ, coronin, falcipain 2, UBP1 and PfPI3K [102-111].

10.2. ABC transporters in *Plasmodium spp.*

There are 48 ABC transporter genes in humans, divided into 7 subfamilies from A to G [112]. By contrast, the *Plasmodium* species encode only 16 ABC transporters, divided into 6 subfamilies: B, C, E, F, G and I [112]. These proteins require adenosine-tri-phosphate (ATP) as fuel for their transport functions. ABC transporters are found in humans as well as many parasites [113]. They are composed of two transmembrane domains with 6 alpha-helices each (the number of alpha helical domains can vary between different members of the ABC-transporters) and two nucleotide binding domains (NBD) in the cytosol [114]. Each NBD contains Walker A and Walker B consensus sequences separated by approximately 90 - 120 amino acids. The NBDs share high sequence homology between the different members of the ABC transporters. In contrast, the transmembrane domains share little or no sequence homology [114]. Both P-gp and MRP have been shown to transport a large array of substrates and have been implicated in drug resistance in tumor cells [115, 116]. In addition, the overexpression of P-gp and/or MRP in tumor cells results in reduced intracellular drug concentration through active drug efflux [115, 116]. Homologues of P-gp and MRP have been described in *P. falciparum* (e.g., PfMDR1, PfMRP1 and PfMDR2 [117, 118]).

10.2.1. PfMDR1

Early evidence suggested that the chloroquine-heme complex formation, in the DV acidic organelle in the parasite, was responsible for the anti-*Plasmodial* activity of CQ [119, 120]. It was

also observed that there was less CQ accumulating in CQR strains [121, 122]. Moreover, reversal of CQ resistance with VP and reduced accumulation of CQ in resistant parasites resembled behavior of multidrug resistance in mammalian cells similar to P-gp [84, 121, 123, 124]. This striking resemblance to P-gp prompted the discovery of transporter homologs of mammalian drug resistance in *P. falciparum* genes [125, 126]. The *P. falciparum* genome encodes 7 members of the ABCB subfamily [117]. *P. falciparum* multidrug resistance protein 1 or PfMDR1 (also known as P-glycoprotein homolog 1 or P-gh1) gene is located on chromosome 5 and is intronless. It encodes a single protein composed of 1419 amino acids and a molecular mass of 162.25kDa and is expressed throughout the asexual life stages of the parasite localising to the DV membrane [125, 127-129]. Like a typical ABC transporter, the protein structure is predicted to be of two domains consisting of six predicted transmembrane alpha helixes with two NBDs (the NBDs are on the cytosolic side of the DV membrane [129, 130]) joined together with a linker domain [128, 131]. PfMDR1 is expressed in CQS strains and overexpressed in some CQR strains; additionally, point mutations in PfMDR1 of CQR strains have been associated with chloroquine resistance [125, 132]. Another study showed that mutations in PfMDR1 can modulate the levels of CQR in cells that possess mutant-PfCRT but cannot confer resistance to CQ by themselves [133]. Selecting mutants pressured in high concentrations of CQ caused de-amplification of PfMDR1 and increased sensitivity to MQ [134]. However, selecting mutants pressured in high concentrations of MQ caused gene amplification of PfMDR1 which correlates with the increase of PfMDR1 transcripts and protein levels in MQ resistant strains [135, 136]. Indeed, the key determinant of MQ, QN and HF is the amplification of PfMDR1 [135]. Five polymorphisms in PfMDR1 appear to contribute to CQR N86Y, S1034C, N1042D, D1246Y and Y184F [132, 133, 137].

Moreover, heterologous expression of wild-type PfMDR1 in CHO cells increased CQ accumulation in the cells making them more sensitive to CQ. This lead to the hypothesis that PfMDR1 may function as an importer of CQ into the DV of the parasite [138]. However, when mutant PfMDR1 containing mutations S1034C and N1042D that were thought to be associated with CQR did not make the cells more sensitive to CQ which suggest that some mutations alter the import of CQ via PfMDR1 [138]. Similarly, expression of wild-type PfMDR1 in frog system *Xenopus laevis* oocytes showed increased sensitivity to CQ and less accumulation of CQ when mutant form was expressed and this suggests that resistance to CQ conferred by PfMDR1 would be reduced or abolished import of CQ into the DV [139, 140]. The normal substrate of PfMDR1 is un-known but it has been shown to import a variety of synthetic compounds such as Fluo-4, Fluo-4AM, CQ, AQ, QN, MQ, HF and ART [129, 133, 140-142]. Moreover, it was suspected that PfMDR1 is involved in parasite sensitivity to ACT partner drugs and artemisinin derivatives [143-145]. *In-vitro* selection of parasites incubated with artelinic acid and artemisinin resulted in parasites less sensitive to ART in addition to MQ, QN, HF and Lumefantrine [102]. Recently, a study involving 37 malaria positive patients in Nigeria detected rare mutations in PfMDR1 speculated to be involved in resistance to antimalarials used in the field (N504K, N649D, F938Y and S967N) that demonstrates the everlasting resistance that comes with treating with antimalaria drugs [146].

10.2.2. PfMRP1

Other members of the ABC transporters superfamily implicated in drug resistance in *P. falciparum* are from the ABCC sub family; members included: PfMRP1 and PfMRP2 [147]. Topology of PfMRP1 suggests a composition of 12 membrane-spanning alpha helices with 2 NBDs extending into the cytosol [148]. These proteins are expressed on the plasma membrane and

membrane-bound vesicles throughout the asexual life stages of the parasite, but not on the DV membrane such as PfMDR1. Some PfMRP1 single nucleotide polymorphisms were linked to decreased sensitivity of the parasite to CQ and QN [117, 149]. Indeed, mammalian MRP1 was reported to transport the antioxidant GSH as well as leukotriene C_4. Similarly, studies suggested that PfMRP1 actively transports GSH in addition to GSH-conjugates, CQ, QN and ART in an efflux manner [147]. Moreover, studies have shown a defect in the fitness of the parasite when the function of the gene encoding PfMRP1 was disrupted *in-vivo*, showing more GSH accumulation as well as the inability of the parasites to survive beyond 5% parasite density in addition to increased sensitivity to CQ, QN and ART [150]. All of this could be due to the possible role of PfMRP1 to export toxic waste and support a healthy parasite life-cycle.

Even though PfMRP1 influences CQR to a certain degree, it was not expressed on the DV. Therefore, it was not the target of verapamil that had the major influence on CQR. Moreover, in the case of human MRP1 (ABCC1), verapamil increases the transport activity of MRP1 for GSH [151] which can result in increased sensitivity of tumor cells to oxidative stress [152].

10.2.3. PfABCG

Unlike many ABC transporters that are usually composed of approximately 12 transmembrane domains with two NDB`s in a single polypeptide chain, the members of the G subfamily consist of 6 membrane spanning alpha helices with a single NBD, but they function as homo- or hetero-dimers [117]. Interestingly, while mammalian cells encodes 5 members of the G subfamily, the parasite encodes only one ABCG protein, known as PfABCG [117]. Moreover, a protein BLAST with a local alignment suggests that PfABCG shares sequence Identity with human ABCG1 and human ABCG2. It has been shown that PfABCG is expressed in all parasite blood stages (rings,

trophozoites, schizonts and gametocytes)[153]. Interestingly, it has also been shown that PfABCG localises to the plasma membrane as well as to a special unidentified organelle in the parasite [153]. Furthermore, human ABCG1 was shown to transport cholesterol and other sterols, while human ABCG2, known as BCRP because of its involvement in breast cancer drug resistance, was shown to transport many anti-cancer drugs, as well as GSH, uric acid and heme [154]. However, the normal function of PfABCG is still a matter of speculation but some studies suggest that it transports ketotifen (an anti-histamine)[155]. The predicted molecular weight of PfABCG is approximately 75kDa.

10.3. PfCRT

The evidence that was pointing to the primary determinant of CQ resistance in *P. falciparum* was that resistance can be reversed significantly in the presence of verapamil in CQR strains. Later, a genetic-cross between Dd2 (CQR) and HB3 (CQS) parasites showed that there was no link between *mdr*-like genes and CQR. Instead, the examination of chromosome 7 of the same genetic cross revealed a unique gene sequence of 13 exons that encodes a highly polymorphic transporter, then named the *P. falciparum* CQ resistance transporter, abbreviated PfCRT [156, 157].

PfCRT is an integral membrane protein localising to the DV membrane of *P. falciparum* [156]. The recently resolved structure of PfCRT-CQR-7G8 strain (UNIPROT W7FI62) at 3.2Å resolution shows that PfCRT is composed of ten membrane-spanning alpha-helices with the N- and C- termini extending into the cytosol [158]. Indeed, it was shown that the key determinant for CQR is a mutation in TMD1 at position 76 [85, 159]. Moreover, the amino acid substitution of the positively charged lysine (K) with the non-charged threonine (K76T) is a conserved mutation in

CQR strains [85, 159]. To date, there are three phosphorylation sites reported in PfCRT, all in the cytosolic domains: N-terminal Ser33 and C-terminal Ser411 and Thr416. Moreover, phosphorylation of Thr416 was demonstrated to be a possible deciding signal for trafficking of PfCRT to its location on the DV [160]. A later report demonstrated with an inducible expression of PfCRT-GFP fusion protein, where it showed expression starts at the ring stage in pre-DV compartments, then the expression peaks at the trophozoite stage at mature DV stage [161]. Bioinformatics analyses suggest PfCRT is a member of the drug/metabolite transporter superfamily of electrochemical potential-driven transporters [162, 163].

10.3.1. PfCRT polymorphisms and chloroquine resistance

PfCRT has been shown to encode 4 to 10 mutated residues in addition to the K76T mutation in CQR strains, with a few exceptions to some strains that have a K76A variant or K76I or K76N that are both laboratory strains that have undergone drug pressure with CQ [156, 164, 165]. To date, there are 32 polymorphisms associated with this protein. The number of mutations PfCRT can possess and how it acquires them is unknown. However, it could be due to different drug pressure selections depending on the geographical region of the parasite. Moreover, the K76T mutation is the most conserved mutation in CQR strains. Interestingly, restoring K76T mutation back to lysine reversed CQ resistance demonstrating that the latter amino acid at position 76 in PfCRT is a key determinant for CQ resistance [159]. Heme (Fe^{2+}) is a by-product of hemoglobin digestion that is rapidly oxidised to form insoluble Fe^{3+} (ferriprotoporphyrin IX or FPIX), which is lethal because it can disrupt membrane function and release reactive oxygen species [166]. To survive in such conditions, the parasite bio-crystallises heme into inert hemozoin crystals which are visible using light microscopy [29-31, 167]. The mechanism of CQ resistance is still not entirely

clear [168, 169], however, it is thought that the weak base, CQ, enters the DV mainly via passive diffusion, becomes protonated and accumulates in the organelle [170]. The mode of action of quinoline based drugs, such as CQ, is believed to cause interference in the heme detoxification pathway where CQ would bind alpha-hematin (ferriprotoporphyrin IX) before it's converted into inert non-toxic hemozoin crystals. Thus, the reduced accumulation of CQ in the DV is the major cause of CQ resistance. Reduced CQ accumulation in CQR was demonstrated with radiolabelled CQ studies [171-173]. Lesser accumulation of CQ in CQS vs CQR was also observed in CQ uptake kinetic studies [174]. It seems that the loss of the positive charge at position 76 allows mutant PfCRT to transport protonated CQ [156]. The ability of PfCRT to transport protonated weak bases is also consistent with the finding that PfCRT can leak or efflux protons into the cytosol [175, 176].

10.3.2. PfCRT and chloroquine analogs

QN is a fast acting schizonticidal drug against malaria parasites. Unfortunately, due to its toxicity levels, it is currently used to treat severe malaria cases only if artemisinin is not available [40]. Later, CQ became the mainstay drug for malaria treatment. Today, the use of CQ is restricted after widespread parasitic resistance in endemic regions and only to be used in some uncomplicated malaria cases according to CDC`s guidelines for clinicians.

CQ is a 4-aminoquinoline compound. Because of the level of satisfaction with this compound from its use in the past, researchers are now focusing their efforts into modifying the structure of CQ to create effective and acceptable analogs for treatment that hopefully are not transported by PfCRT. Moreover, CQ structure is composed of a hetero-dimeric quinolone ring, which exists in all quinolone antimalarials, with a chlorine atom at the 7^{th} position and a penta-di-amino side chain. It is important to note that the quinolone ring is crucial for alpha-haematin binding and

the 7-chloro- is associated with higher antimalarial activity [48, 49]. Furthermore, the amine in the hetero-aromatic ring and the tertiary amine in the amino alkyl section are weak bases that cause CQ to accumulate in the DV via pH trapping [48]. With this information in mind, modification of different parts of CQ has generated meaningful insights on where changes can be made on the CQ compound structure. Some studies have demonstrated, by reducing the carbon linker alkyl side chain to 2 carbons instead of 4, the compound still retains its lethal profile to the parasite [177-179].

The best example was the modification of the side-chain with a phenol group that culminated in one of the most well-known CQ analogs, amodiaquine. The drug was widely used and acts in a similar way as CQ [180]; however, amodiaquine's use was limited due to toxicity in patients receiving long-term treatment [181, 182]. Furthermore, important to mention was the synthesis in the 1960's of bis-quinoline dimers, with the best example being piperaquine. Due to its dimer like structure of CQ, it was thought that it would be too bulky to be recognised by PfCRT and it would be trapped in the DV because it would have acquired four positive charges once in the target organelle; now it is a part of the WHO recommendations of utilisation for ACT. Unfortunately, clinical resistance was recently reported for this antimalaria combination [71, 183, 184]. . Recently, the resolved structure of PfCRT showed that most of the residues involved in piperaquine resistance line the negatively charged central cavity of the protein [158]. Their biochemical studies showed that CQ and piperaquine can bind to PfCRT, but only CQ is transported. This shows that PfCRT requires more than just K76T mutations to confer resistance to piperaquine. Another example of CQ analogs was the modification of the third position of the heterodimeric ring with one of three halogen atoms: chlorine, bromine and iodine. Out of all

three compounds, 3-ICQ scored the lowest IC_{50} value with respect to CQR strains (~600nM) while CQS strains IC_{50} were half of that value (~300nM). Moreover, none of these compounds were reversed by verapamil, suggesting that they may not be substrates of CQR PfCRT. However, when isogenic clones ($C2^{GC03}$ and $C4^{DD2}$) and lab CQR and CQS strains were exposed to a fixed concentration of CQ in the presence of increasing concentration of 3-ICQ there was a significant decrease in the IC_{50}. These results, in combination with other experiments, suggested that 3-ICQ was behaving like a classical chemosensitizer, such as verapamil [153]. Interestingly, this very minute modification of CQ at the third position of the quinoline moiety made the drug not only unrecognisable to wildtype or mutant PfCRT but also a possible blocker of the transporter. These result also suggest that PfCRT has a limited substrate specify towards quinolone based drugs. The slightest change to the quinolone structure can restore the anti-*Plasmodial* activity and may or may not give the compound special features.

10.3.3. PfCRT normal substrates and its essential role in the parasite

The normal function of PfCRT and the physiological substrate(s) are still a matter of speculation. Efforts to generate a knockout-PfCRT strain or clonal isolate resulted in a non-viable phenotype [185]. This suggests that PfCRT is transporting something of importance for the survival of the parasite. So far, it has been suggested that PfCRT is capable of transporting short peptides and hemoglobin degradation products, as seen in heterologous expressions of PfCRT in *Xenopus laevis* oocytes [186]. There is some evidence suggesting that PfCRT could act as a glutathione (GSH) transporter [187]. A homolog of PfCRT in plant *Arabidopsis thaliana* was shown to transport Glutathione (GSH) [188]. More recently, it was demonstrated that iron can be a substrate of PfCRT in *Xenopus laevis* oocytes [189]. In another heterologous system, HEK293F

cells, CQR PfCRT displayed an increase in lysosomal acidity, where mutant PfCRT was expressed, suggesting that PfCRT may act as a proton importer [190].

10.3.4. Structure and predicted MW of PfCRT

PfCRT is composed of 13 exons encoding an integral membrane protein localising to the DV membrane of *P. falciparum*. It is composed of 424 amino acids and shown to form 10 membrane spanning helices with the N and C termini extending into the cytosol [158]. There are three phosphorylation sites that have been identified with PfCRT divided between the cytosolic domains [160]. Residue Thr416 on the C-terminal which was found to be a possible deciding signal to traffic PfCRT to its location on the DV membrane [160]. The other two residues are of unknown function yet, one on the N-terminal and the other on the C-terminal respectfully: Ser33 and Thr411, but a recent study suggests that Ser33 can have a role in modulating CQR [191]. PfCRT belongs to the Drug/metabolite transporters and the EamA-like superfamily of proteins. The closest members to PfCRT in a phylogenetic tree are proteins from *Plasmodium spp., parasite Cryptosporidium parvum,* yeast slime *Dictyostelium discoideum and* plant *Arabidopsis thaliana* [162]. These members share some sequence homology in addition to having similar patterns in a hydropathy plot. Moreover, members of the Drug/metabolite transporters are known to transport organic cations, amino acids and weak bases. The fact that the Drug/metabolite transporters can transport weak bases puts more strength on the hypothesis that PfCRT can transport CQ [162]. The most known protein in the malaria field when resistance is mentioned is PfCRT because it consists of the primary determinant key for CQR, the K76T mutation as well as other polymorphisms in the protein that contribute to CQR. This mutation in TMD1 was thought to be involved in substrate recognition and selectivity. PfCRT may also possess the ability to form

oligomers due to the presence of motif GXXXXXXG in TMD`s 5 and 10 [162], although one of the glycine residues in TMD 5 is substituted with a threonine residue. Indeed the structure of PfCRT has been resolved to a 3.2Å resolution using cryo-electron microscopy [158]. Moreover, PfCRT is a small protein to be studied with this technique, so the authors added an antibody fragment to stabilize PfCRT through the process. As a result, the protein was resolved in locked conformation that is open to the DV because of the antibody binding [158]. However, this structure shows PfCRT transmembrane domains are split in to two groups of 5 forming a negatively charged central cavity that is large enough to fit CQ. Furthermore, wild type PfCRT possessing K76 would repel protonated CQ because of the positive charge. Moreover, a multiple sequence alignment of the primary structure of PfCRT with orthologues shows a number of interesting locations of some residues [162]. Interesting conserved residues were: Pro165 in TMD 4 and Pro354 (it is more conserved than Pro165) in TMD 9. Similar citing of prolines central to the alpha helix have demonstrated a kink-like function driving the function of some proteins which could be important for function [192, 193]. Also interesting, there are only two tryptophanes in the whole sequence: W280 (conserved in *P. falciparum*) and W316 (conserved in *P. falciparum* and some orthologues).

Furthermore, the predicted molecular weight of PfCRT is 48.67kDa [156]; however, the apparent molecular weight is a topic of debate. Many investigators show that the protein migrates with molecular weights between 40kDa and 50kDa [141, 156, 194]. Antibodies to PfCRT from certain labs recognise the N-terminal domain, while others recognise the C-terminal domain [156, 194]. This variance in molecular weight was noticed by some groups working on PfCRT, which lead them to construct a florescent fusion protein (example: GFP) for precise and easy detection because they thought that current antibodies to PfCRT are not trust worthy [161]. Earlier

characterization of two antisera against the N- and C-termini, generated in this lab, bound to one common polypeptide band in *P. falciparum* protein extract migrating with an apparent molecular mass of ~42kDa [195]. An additional polypeptide band migrating with an apparent molecular mass of 52kDa was detected with the antisera against the N-terminal of PfCRT, but not with the antisera against the C-terminal. Further work revealed that the lack of C-terminal antisera binding to the 52kDa polypeptide was due to phosphorylation of residues Ser411 and Thr416 in PfCRT C-terminal masking the epitopes. To uncover the identity of both polypeptides, one must determine the masses these reactive polypeptides by spectrometry. Moreover, PfCRT primary structure predicted that the protein may oligomerize [162]. In fact, Martin and Kirk predicted that PfCRT may function as a dimer in a similar fashion to some members of the DMT family [162]. Consistent with this, our anti-C does detected a band at ~90kDa (Baakdah un-published results) [195]. The sum of the estimated molecular weight proposes a possibility of a homodimer form of PfCRT that could shed some light on the function of the protein and the mechanism of CQ resistance.

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Connecting statement 1

Chapter 1 provided a review of current knowledge on *P. falciparum* drug resistance and the antimalaria drugs that are used to defend the host against such life-threatening parasites. We mentioned that PfCRT is implicated in CQR. PfCRT was shown to have a limited substrate specificity; for example: a modest modification at 3rd position of CQ, generating 3-ICQ, affected its transport via mutant-PfCRT, in addition to some loss of antimalarial activity. This suggested that modifications of CQ chemical structure can allow such modified derivatives to escape mutant PfCRT-mediated drug resistance while maintaining their antimalarial activity. The next chapter describes the antimalarial activity of novel CQ derivatives against CQS and CQR strains of *P. falciparum,* in addition to two previously tested CQ derivatives AQ-13 and AQ-129. Furthermore, we investigated the effects of AQ-13 and AQ-129 on mutant-PfCRT relative to CQ.

Chapter 2

Manuscript I

Characterization of 4-Aminoquinoline Derivatives against Chloroquine Resistant Strains of *Plasmodium falciparum*

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Abstract

The human malaria disease is becoming progressively more difficult to treat due to continuous rise of resistant *Plasmodium* species, in particular, *Plasmodium falciparum,* the leading cause of morbidity and mortality in endemic areas. Presently, chloroquine, a 4-aminoquinoline, fails to deliver appropriate toxicity to the malaria causing parasites due to internal resistance mechanisms primarily mediated by mutant-PfCRT. This study examined the cytotoxic effects of 16 novel chloroquine derivatives that included two compounds that previously demonstrated a significant efficacy towards chloroquine resistant strains, AQ-13 and AQ-129. Our interest in understanding the relationship of these compounds to mutant-PfCRT suggested: a) Both are more lethal than CQ towards chloroquine resistant strains; b) Isogenic clones of PfCRT show that they are poor substrates to mutant-PfCRT; c) Prolonged exposure of Dd2-H to both compounds in anticipation of novel point mutations in PfCRT resulted in non-viable parasites, indicating that resistance to these compounds is not easily established by the parasites ;and 4) *In-vitro* beta hematin formation assays suggest that the mechanism of action seems to be similar to chloroquine in inhibiting heme polymerisation. Taken together, AQ-13 and AQ-129 are more effective than chloroquine against chloroquine resistant strains and are less efficient substrates of mutant-PfCRT than chloroquine.

Introduction

The spread of resistance to current antimalarials has prompted investigators and the pharmaceutical industry to search for new and effective antimalarial drugs. *Plasmodium falciparum* (*P. falciparum*) is the most problematic species of its kind to humans, responsible for almost 90% of the malaria mortality cases[1]. As fragile as these protozoan parasites may be and their dependence on the intracellular habitat within the host red blood cells, their main route of transmission is via the female *Anopheles* mosquito. Chloroquine (CQ) was the gold standard drug for treatment and elimination of the malaria from burdened areas of the world [2]. However, the emergence of CQ-resistant parasites and more recently that of multidrug resistant parasites to certain artemisinin combination therapies (ACT) drug combinations, highlights the urgent need to develop new antimalaria drugs in an effort to prevent human catastrophe [3, 4]. While many factors may contribute to CQ resistance, it was confirmed that the primary determinant for CQ resistance was the *P. falciparum* CQ resistance transporter (PfCRT) [5]. A mutation in PfCRT substitutes a positively charged lysine side-chain with a polar uncharged threonine at position 76 in transmembrane domain 1 (TMD1). This mutation appears to be a key determinant of CQ resistance and it is conserved in the majority of malaria CQ-resistant strains [5]. Additional mutations in this transporter play a role in the level of resistance to CQ [6]. Indeed, in some parts of the world, as in Malawi, the use of CQ was abstained for several years to implement a drug cycling strategy [7, 8]. This led to the re-emergence of CQ sensitive parasites.

More efforts into reviving CQ back into the field led to the synthesis of CQ analogs or derivatives. The ideal qualities anticipated from a modified CQ compound is that it would be equally lethal against CQ-sensitive (CQS) and CQ-resistant (CQR) strains, thus bypassing PfCRT-mediated drug

resistance, safe for humans use and inexpensive to manufacture. Chloroquine, is a 4-aminoquinoline with a chlorine atom at the 7th position and a penta-di-amino side-chain. Most attempts to modify the structure of CQ maintained the 7-chloro-4-aminoquinoline structure intact, because this part of the structure was deemed crucial for heme binding as well as some antimalarial activity [9, 10]. For this reason, changes around the quinolone ring, such as the side chain, seemed to be the most promising sites to manipulate [11].

Moreover, early reports screened CQ derivatives with varying lengths of the diaminoalkane side chain, which culminated in the discovery of AQ-13 (diethylaminopropyl CQ derivative), also known as Ro 47-0543, an effective antimalarial drug against CQR parasites and not reversed by verapamil (VP) unlike native CQ [12, 13]. In addition, AQ-13 is now a top candidate antimalarial drug advancing through various stages of clinical trials [14]. Another interesting compound that showed significant activity against CQR *Plasmodium* parasites was AQ-129 [15]. The latter CQ derivatives` diethyl tertiary amine was replaced with a pyrrolidine ring and connected to the CQ secondary amine at 4th position with only two carbon spacers; however, this compound was not studied further by the authors [15].

In this study, we have screened 16 novel CQ derivatives with modifications on either the 3rd position of the quinoline moiety or the side-chain of CQ. Initial screening suggested that modifications at 3rd position of the quinoline moiety reduced the antimalarial activity of the drugs, contrary to some of the screened derivatives modified at the side-chain of CQ that show better antimalarial activity. We also re-examined the antimalarial effects of AQ-13 and AQ-129 against multiple *P. falciparum* lab adapted strains to understand the relationship between AQ-

13 and AQ-129 towards mutant-PfCRT activity. Our data suggests that both compounds are more active than CQ on CQR *P. falciparum* and they are poor substrates for mutant-PfCRT.

Materials and methods

Materials used - Chloroquine diphosphate (Sigma; cat: C-6628), verapamil hydrochloride (Sigma; cat: V4629-1G), hemin (Sigma; cat: H9039-1G) and SYBR® Green I nucleic acid gel stain (Life technologies™; cat: S7563). All other chemicals were of the highest grade available. The CQ derivatives were made either with or without di-phosphate and were characterized with NMR. All compounds were solubilised in DMSO (Sigma; cat: D8418-100ML) or double distilled water, diluted in RPMI 1640 supplemented with 0.5 % Albumax I (Life technologies™; cat: 11020021) and 0.1 mM hypoxanthine (Sigma; cat: H9377).

Parasite strains **-** *P. falciparum* strains used in this study included several CQS strains (e.g., 3D7, HB3 and D10) and CQR strains (e.g., Dd2-H, 7G8 and K1) from MR4 Unit of the American Type Culture Collection (ATCC, Manassas, VA). Isogenic clones used were: $C2^{GCO3}$ and $C4^{Dd2}$ (A kind gift from Dr. David Fidock at Columbia University Medical Center, New York, USA).

In-vitro parasites cultivation **-** A modified Trager and Jensen protocol [16] was used to maintain the culture of *P. falciparum* strains used in this work. Briefly, parasites were cultivated in A^+ human erythrocytes (Interstate Blood Bank Inc.) in RPMI 1640 (supplemented with L-Glutamine and 25mM HEPES) (Life technologies™; cat: 22400-105), 100µM hypoxanthine and 0.5% Albumax I. Cultures were maintained in a 37^{o}C incubator under atmospheric conditions of 3% O_2, 5% CO_2 and 92% N_2. The parasites were maintained at 2% hematocrit.

Parasite cytotoxicity assays - All parasite proliferation assays were performed in the presence of the various compounds were done in triplicates, alone or in combination with other compounds. Briefly, each assay began with a 0.5% parasitemia (mostly ring stage synchronised using 5% L-sorbitol) and a 2% hematocrit in black, clear bottom 96 well plates (Corning Inc.; ref: 3603). After 72hrs of incubation at 37^{o}C in an incubator under atmospheric conditions of 3% O_2, 5% CO_2 and 92% N_2, plates were frozen at -80^{o}C. Frozen plates were later thawed at RT in lysis-developing solution (Tris, 20mM pH 7.5, 5mM EDTA; 0.008% w/v saponin, 0.08% v/v Triton X-100, 0.2µL /ml of 10,000x SYBR® Green I Nucleic Acid gel stain dye) and kept away from light. After an hour incubation, the fluorescence was measured using the Synergy H4 plate reader with E_X 485nm / E_m 535nm. The resulting data were analyzed using GraphPad Prism version 8.4.0 (671) to obtain the 50% and 90% inhibitory concentration (IC_{50}). All the graphs represent the mean ± SD of three independent experiments done in triplicate.

In-vitro selection for resistance to AQ-13 and AQ-129 - Selecting for resistant clones to AQ-13 and AQ-129 in *P. falciparum* was conducted an approach based on a previously reported method by Oduola *et al.* [17]. Briefly, *P. falciparum* Dd2-H strain was cultured *in-vitro* in the presence of increasing AQ-13 or AQ-129 drug concentrations. The drug pressure was maintained for 3 months at 120 nM, 360 nM and 720 nM of AQ-13 or AQ-129. The drug exposure cycle started with asynchronous cultures with 4.4% parasitemia. Media changes were made daily. The IC_{50} concentrations were calculated via GraphPad Prism version 8.4.0 (671) by non-linear regression analysis of the inhibition of uptake values and log-transformed concentration values. However, no viable parasites were observed.

Statistical analysis – Non-linear regression plots, IC_{50} estimation and student *t* tests were done using GraphPad Prism version 8.4.0 (671).

Results

Screening of 16 CQ-derivatives. We have examined the lethal effects of 16 novel CQ-derivatives against *P. falciparum* field isolates. The compounds synthesis strategy is discussed in a separate report (Kapuku, B. and Bohle, S., in preparation). To get a better understanding of the effectiveness of the modifications done to CQ, we divided the derivatives into 2 groups based on the site of modification of the quinolone ring. As displayed in figure 1, the compounds were divide into: group A: CQ-derivatives modified at the side-chain of the quinolone moiety; and group B: CQ-derivatives modified at 3rd position of the quinolone moiety (figure 1 group B). We first screened the antimalarial toxicity of all 16 novel compounds alongside CQ against CQS strain (e.g., 3D7) and CQR strain (e.g., Dd2-H), and estimated the IC_{50} concentration for each derivative. The values obtained were compared to CQ IC_{50} values to see if any of the compounds were an improvement on CQ cytotoxic effects, especially against CQR parasites.

Group A compounds. It was expected that CQ would be more lethal to 3D7 than Dd2-H with IC_{50} values of 12nM and 170nM, respectively (~ 14.2 fold difference in sensitivity) (figure 2). While two compounds from this group, BK-2085 and BK-2094, showed very high IC_{50}`s on the parasites, the other three compounds, BK-435, AQ-13 and AQ-129 had more lethal effects on 3D7 and Dd2-H. Moreover, BK-435 was more lethal than CQ on 3D7 at IC_{50} 1.972nM; however, Dd2-H parasites displayed a slightly higher IC_{50} with this compound (IC_{50} 220nM) than CQ (170nM) (figure 2). When comparing all the compounds in group A with respect to their effective antimalarial IC_{50} values, compounds AQ-13 and AQ-129 were very efficient and more lethal than CQ on Dd2-H.

Both compounds showed very similar IC_{50} values relative to each other when used on 3D7 and Dd2-H (i.e. 10.56nM – 13.68nM for 3D7 verses 38.35nM – 33.45nM for Dd2-H) (figure 2). The results for these two compounds show that modifications to the side-chain of CQ caused roughly a 4.8 fold decrease in IC_{50} relative to native CQ at least against Dd2-H. In addition, the IC_{50}s of both compounds were very similar to CQ against 3D7 (i.e. 12nM for CQ, 10.56nM for AQ-13 and 13.68nM for AQ-129; table 1 and figure 2).

Group B compounds. The 11 compounds in this group had varying IC_{50} values relative to each other, ranging from 8.7nM to 1298nM when tested on 3D7 (~ 150 folds difference) and 205.5nM to 3597nM when tested on Dd2-H parasites (~17.5 folds) (figure 3, group A and group B). Moreover, 6 compounds had a halogen, chloride atom at 4th/ 3rd/ 2nd position, respectively: BK-699, BK-692 and BK-601; or fluoride in compounds: BK-592, BK-603 and BK-602, at different positions of the benzamide moiety`s benzene ring (figure 1).

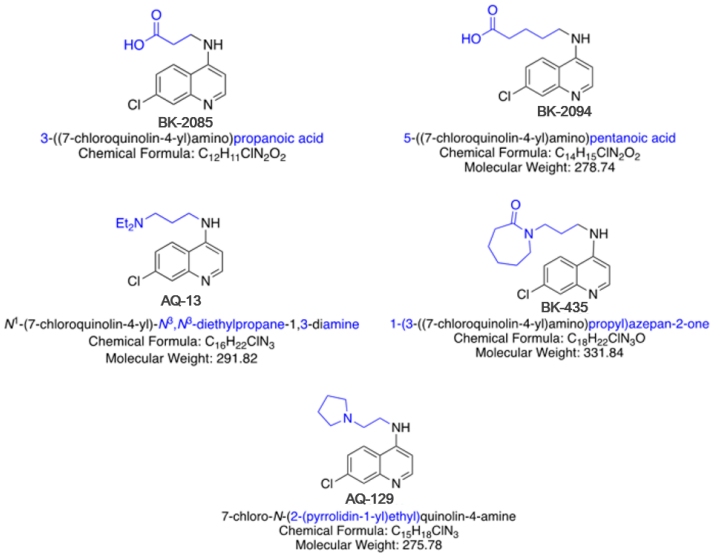
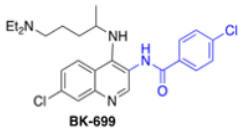
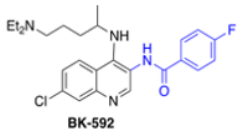


Figure 1. Group A CQ-derivatives. CQ-derivatives modified at the side chain of chloroquine. In blue are the modifications done to chloroquine`s original structure.



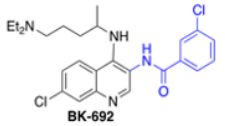
BK-699

4-chloro-*N*-(7-chloro-4-((5-(diethylamino)pentan-2-yl)amino)quinolin-3-yl)benzamide
Chemical Formula: $C_{25}H_{30}Cl_2N_4O$
Molecular Weight: 473.44



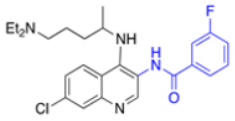
BK-592

N-(7-chloro-4-((5-(diethylamino)pentan-2-yl)amino)quinolin-3-yl)-4-fluorobenzamide
Chemical Formula: $C_{25}H_{30}ClFN_4O$
Molecular Weight: 456.99



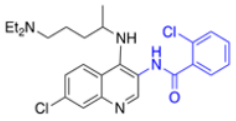
BK-692

3-chloro-*N*-(7-chloro-4-((5-(diethylamino)pentan-2-yl)amino)quinolin-3-yl)benzamide
Chemical Formula: $C_{25}H_{30}Cl_2N_4O$
Molecular Weight: 473.44



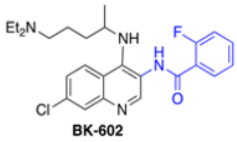
BK-603

N-(7-chloro-4-((5-(diethylamino)pentan-2-yl)amino)quinolin-3-yl)-3-fluorobenzamide
Chemical Formula: $C_{25}H_{30}ClFN_4O$
Molecular Weight: 456.99



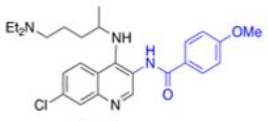
BK-601

2-chloro-*N*-(7-chloro-4-((5-(diethylamino)pentan-2-yl)amino)quinolin-3-yl)benzamide
Chemical Formula: $C_{25}H_{30}Cl_2N_4O$
Molecular Weight: 473.44



BK-602

N-(7-chloro-4-((5-(diethylamino)pentan-2-yl)amino)quinolin-3-yl)-2-fluorobenzamide
Chemical Formula: $C_{25}H_{30}ClFN_4O$
Molecular Weight: 456.99

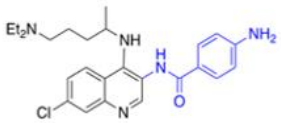


BK-593

N-(7-chloro-4-((5-(diethylamino)pentan-2-yl)amino)quinolin-3-yl)-4-methoxybenzamide
Chemical Formula: $C_{26}H_{33}ClN_4O_2$
Molecular Weight: 469.03

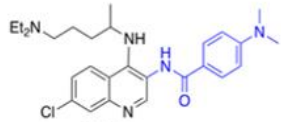
BK-689

N-(7-chloro-4-((5-(diethylamino)pentan-2-yl)amino)quinolin-3-yl)-4-(methylthio)benzamide
Chemical Formula: $C_{26}H_{33}ClN_4OS$
Molecular Weight: 485.09



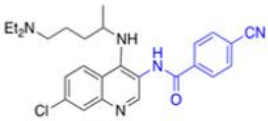
BK-605

4-amino-*N*-(7-chloro-4-((5-(diethylamino)pentan-2-yl)amino)quinolin-3-yl)benzamide
Chemical Formula: $C_{25}H_{32}ClN_5O$
Molecular Weight: 454.02



BK-691

N-(7-chloro-4-((5-(diethylamino)pentan-2-yl)amino)quinolin-3-yl)-4-(dimethylamino)benzamide
Chemical Formula: $C_{27}H_{36}ClN_5O$
Molecular Weight: 482.07



BK-709

N-(7-chloro-4-((5-(diethylamino)pentan-2-yl)amino)quinolin-3-yl)-4-cyanobenzamide
Chemical Formula: $C_{26}H_{30}ClN_5O$
Molecular Weight: 464.01

Figure 1. Group B CQ-derivatives. CQ-derivatives modified at 3rd position of chloroquine. In blue are the modifications done on chloroquine`s original structure.

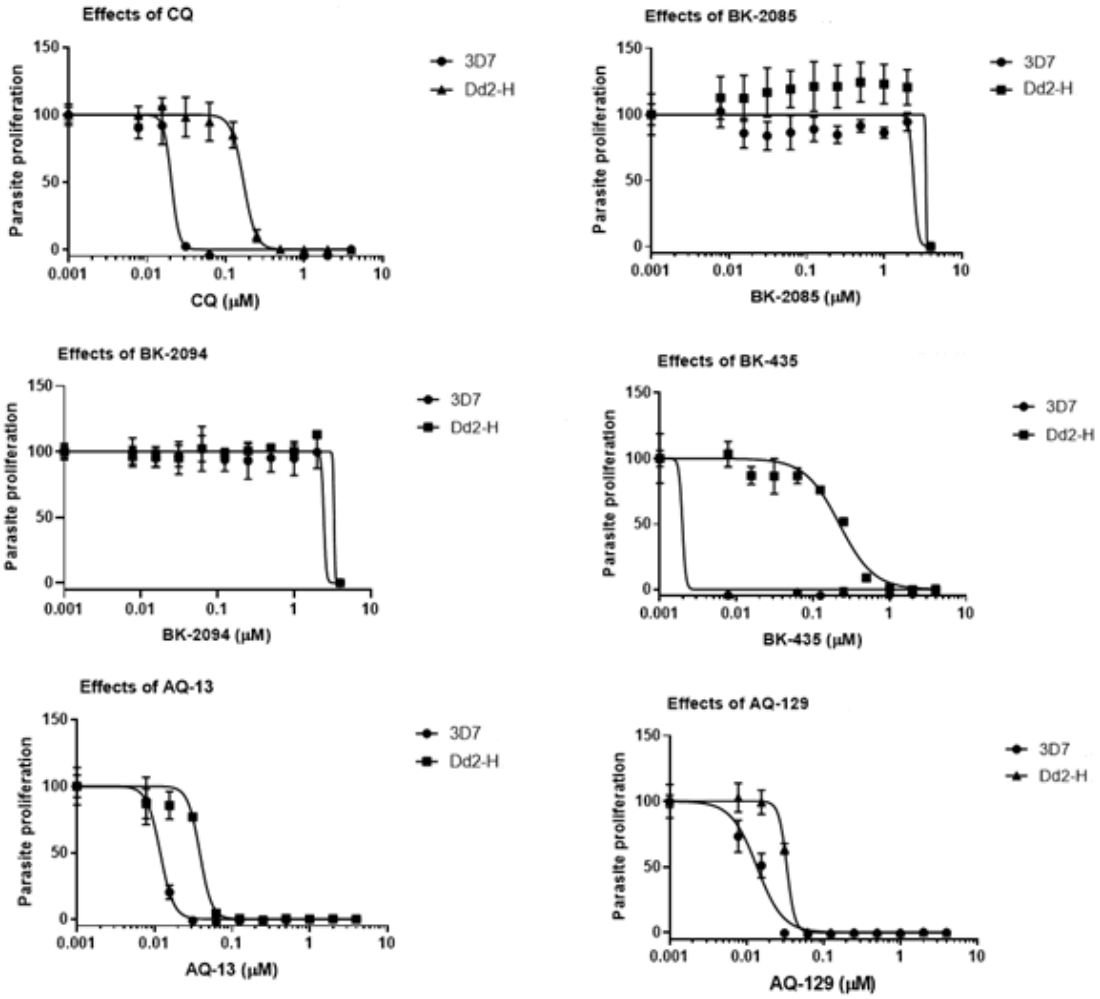


Figure 2. *Plasmodial* activity of Group A CQ-analogs. 3D7 and Dd2-H were incubated in increasing concentration of CQ, BK-2085, BK-2094, BK-435, AQ-13 and AQ-129 and plates were developed after 72hrs of incubation.

Interestingly, shifting the chloride atom location on the benzene ring (2nd, 3rd and 4th positions) of the benzamide moiety showed a dramatic shift in IC_{50} values against 3D7 and Dd2-H (i.e. BK-699 IC_{50} 1289nM to BK-601 IC_{50} 69.82nM is ~ 18.5 folds for 3D7; BK-699 IC_{50} 3597nM to BK-692 IC_{50} 497.2 is ~ 7 folds) (figure 3, Panel 1 and 2). Similarly, shifting the fluoride atom location on the benzene ring (2nd, 3rd and 4th positions) of the benzamide moiety showed a dramatic shift in IC_{50} values with 3D7 and Dd2-H (i.e. BK-592 IC_{50} 8.7nM to BK-602 IC_{50} 502.9nM is ~ 58 folds for 3D7; BK-592 IC_{50} 667nM to BK-602 IC_{50} 1010 is ~ 1.5 folds) (figure 3, part 1 and part 2). It is

interesting to note that the chloride at 4th position in BK-699 reduced the compound activity or toxicity against both 3D7 and Dd2-H; whereas the other two, BK-692 and BK-601, had better toxicity against both parasites. Moreover, a similar pattern was seen as well with the fluoride relocated derivatives where in BK-602 the fluoride is at 2nd position and showed reduced activity with 3D7 and Dd2-H compared to the other two (BK-592 and BK-603). The rest of the compounds in group B were modifications of the 4th position of the benzamide moiety`s benzene ring, the additive modification in CQ 3rd positon (figure 1 group B). The functional groups were, methoxy group in BK-593, amino group in BK-605, a cyanide atom in BK-709, di-methyl amino group in BK-691 and methyl-thio group in BK-689. The difference in IC_{50} values between the most effective compound and the least effective compound for these five compounds was: for 3D7 ~ 38 folds and for Dd2-H ~ 21 folds (figure 3; Table 1). Taken together, the introduction of the different halogens or other functional groups onto the benzamide ring did not improve the activity of the new derivatives significantly. The most active compound in this group against CQR Dd2-H was BK-689, however it was not significantly more active than CQ. Because none of these AQs were more active than CQ on CQ-resistant *P. falciparum*, they were not studied further. All 16 compounds had different IC_{50}s, some were active against 3D7; while others were active against Dd2-H. However, only two compounds were very active against both 3D7 and Dd2-H. Interestingly, both compounds were also more active than CQ against CQR strain Dd2-H. Consequently, these compounds, AQ-13 and AQ-129, were further characterized with respect to their activity or toxicity against different strains of *P. falciparum* in an effort to gain a better understanding of their mode of action.

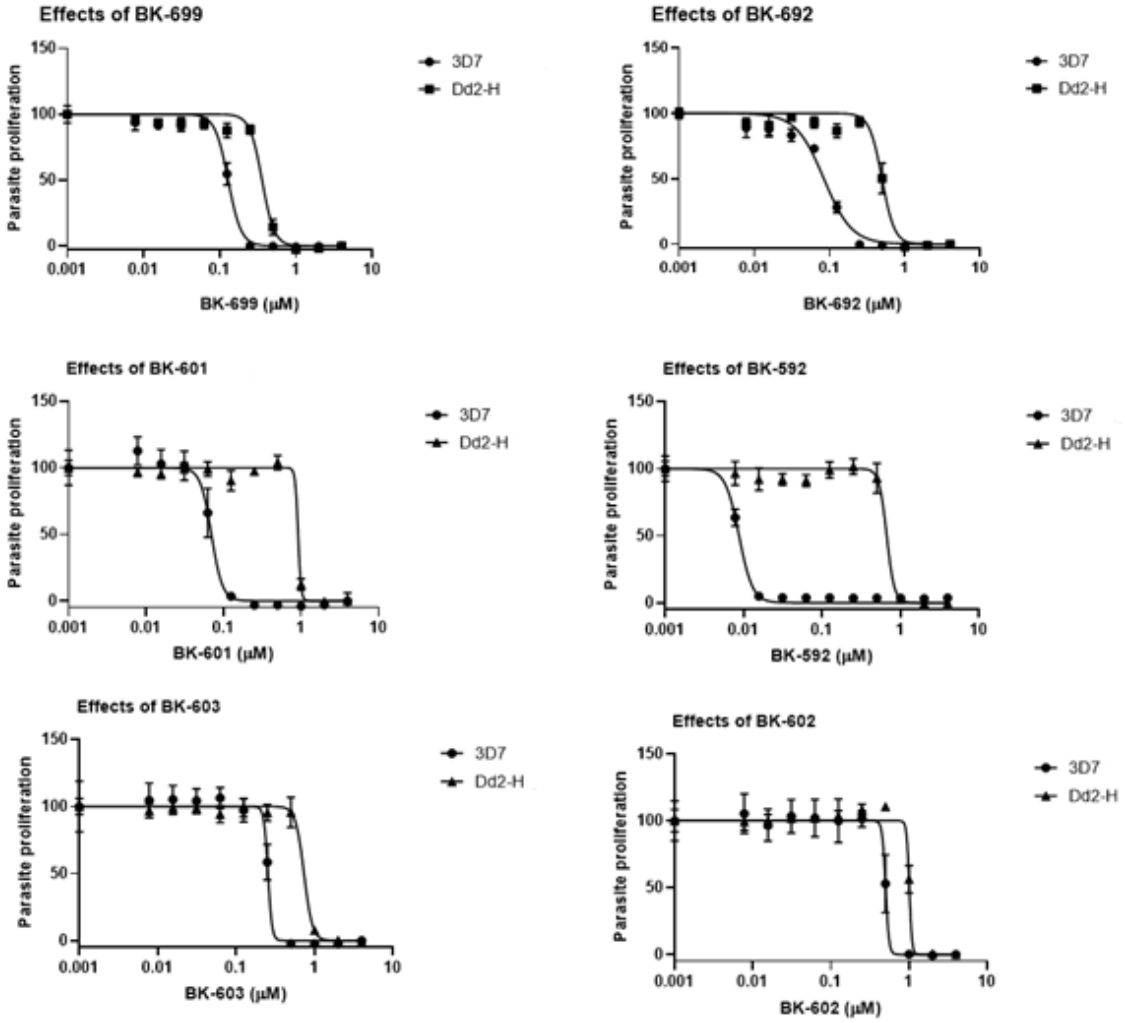


Figure 3. Anti-*Plasmodial* activity of Group B CQ-analogs (part1). 3D7 and Dd2-H were incubated in increasing concentration of BK-699, BK-692, BK-601, BK-592, BK-603 and BK-602 plates were developed after 72hrs of incubation. Statistical analysis and graphs were made using GraphPad Prism.

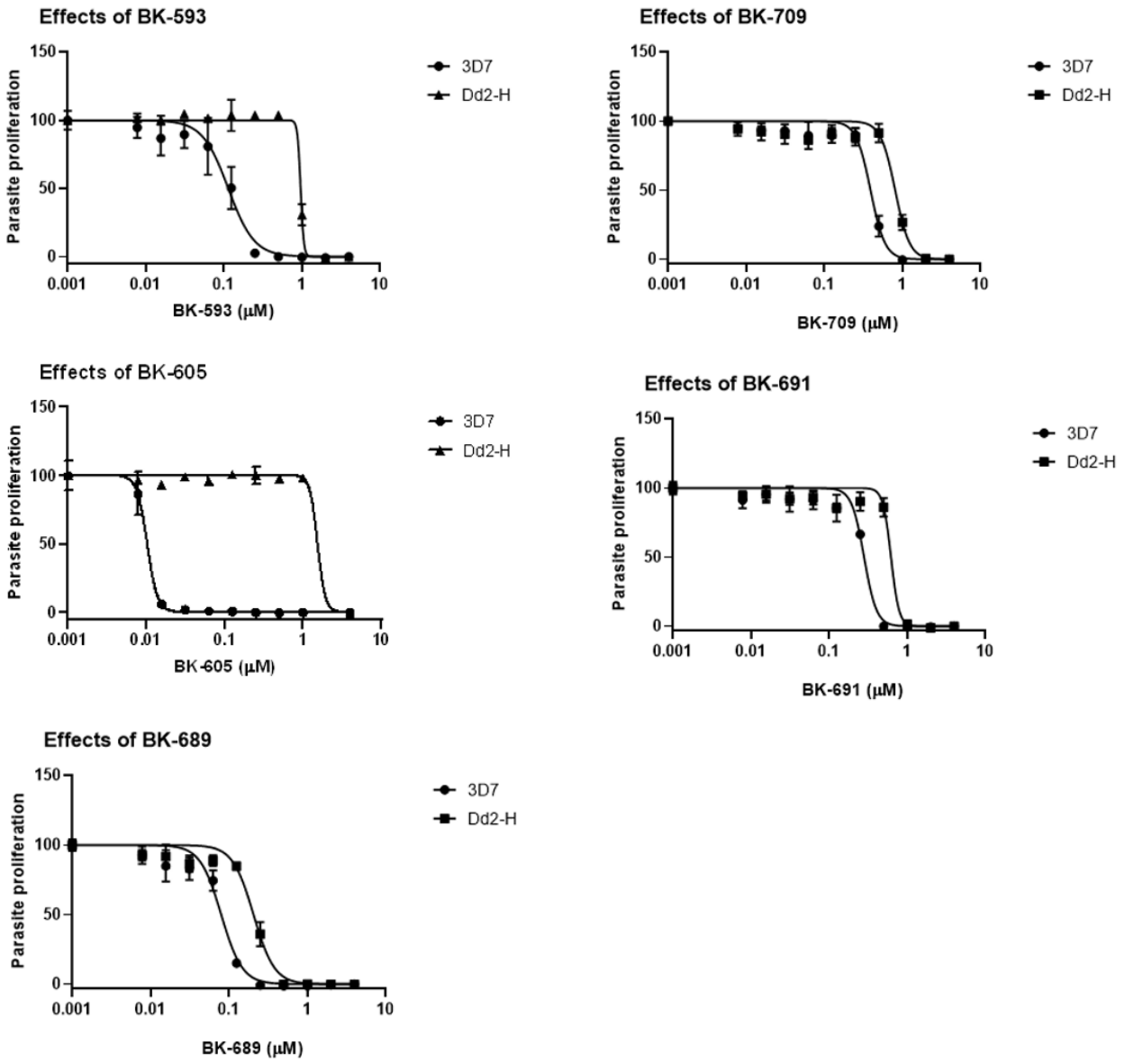


Figure 3. Anti-*Plasmodial* activity of Group B CQ-analogs (part2). 3D7 and Dd2-H were incubated in increasing concentration of BK-593, BK-709, BK-605, BK-691 and BK-689 and plates were developed after 72hrs of incubation. Statistical analysis and graphs were made using GraphPad Prism.

Drug	3D7	Dd2-H
	IC_{50} ± SD (nM)	IC_{50} ± SD (nM)
CQ	12nM ± 0.59	170nM ± 11.60
Group A derivatives		
BK-2085	2410 ± 48.11	3483 ± 43.41
BK-2094	2447 ± 99.14	3338 ± 32.40
BK-435	1.972 ± 9.01	220.9 ± 35
AQ-13	10.56 ± 0.63	38.35 ± 3.8
AQ-129	13.68 ± 1.65	33.45 ± 2
Group B derivatives		
BK-699	1289 ± 7.3	3597 ± 32.5
BK-692	84.01 ± 9.7	497.2 ± 36.7
BK-601	69.82 ± 8.33	918 ± 3.03
BK-592	8.7 ± 0.4	667 ± 60.38
BK-603	255.1 ± 2.0	733.9 ±79
BK-602	502.9 ± 19.86	1010 ± 11.84
BK-593	114.2 ± 18.6	963 ± 8.51
BK-605	10.38 ± 0.8	1564 ± 53.01
BK-709	391.3 ± 37.2	808.4 ± 85
BK-691	280.4 ± 25.4	619.9 ± 89
BK-689	79.51 ± 9.11	205.5 ± 21.6

Table 1. Summary of screened compounds IC_{50} values. The table shows the IC_{50} values obtained from 3D7 and Dd2-H when treated with CQ, the 5 compounds from group A and the other 11 compounds from group B. The experiments here were done once in triplicates for screening purposes. Statistical analysis and IC_{50} values were estimated via Graph Pad prism.

Effects of AQ-13 and AQ-129 on 3D7 and Dd2-H with and without VP. CQ resistance in *P. falciparum* is primarily determined by mutations in PfCRT [5, 6]. VP, a weak base, has been shown to interact with mutant-PfCRT and speculated to block the transport of CQ via mutant-PfCRT, and other quinolone-based compounds, from the DV of the parasite into its cytosol [18]. Consequently, in an effort to determine if mutant-PfCRT is responsible for resistance towards a given compound, the use of VP could address this question [18, 19].

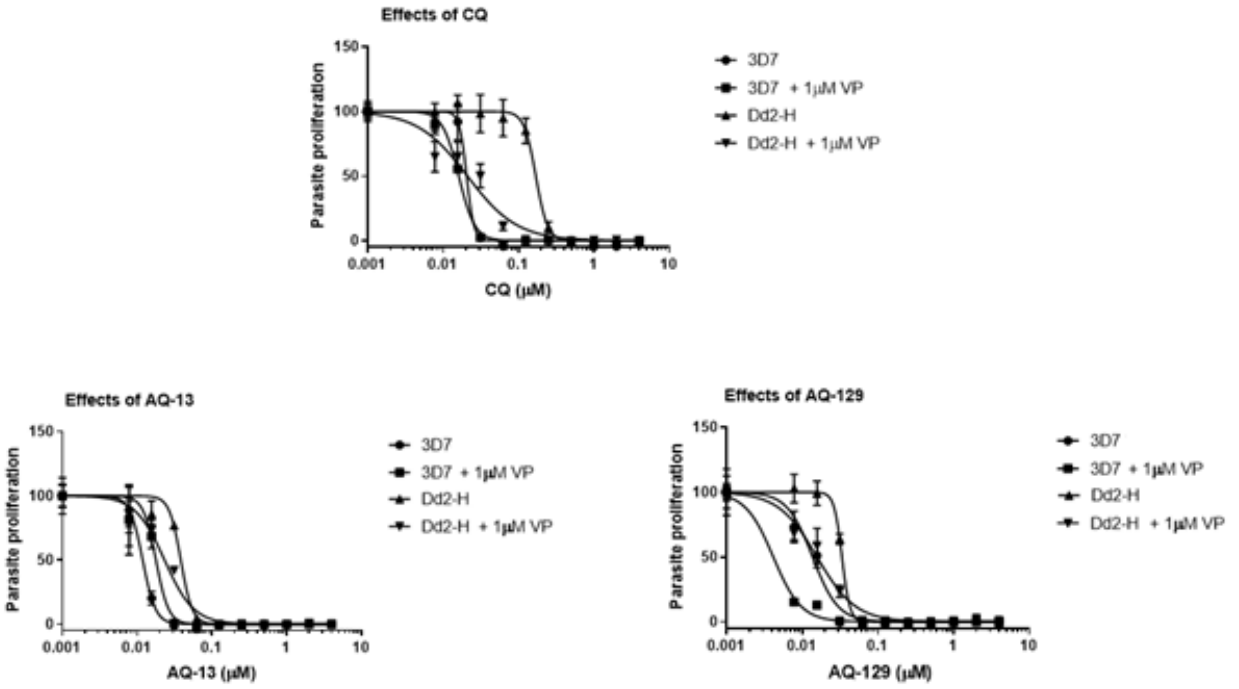


Figure 4. Effects of AQ-13 and AQ-129 on proliferation of CQS and CQR strains (3D7 and Dd2-H) of *P. falciparum* without/with VP. 3D7 and Dd2-H were incubated in increasing concentration of CQ, AQ-13 and AQ-129; plates were developed after 72hrs of incubation. Graphs were made using GraphPad Prism. The graphs show the mean ± SD of three independent experiments done in triplicates.

Since AQ-13 and AQ-129 were CQ derivatives modified on the side-chain of the quinolone moiety, it was of interest to study their interactions with mutant-PfCRT. Figure 4 shows the proliferation of CQR Dd2-H strain with increasing molar concentrations of AQ-13 or AQ-129 alone and in the presence of 1μM VP. Surprisingly, the presence of VP showed a modest reversal of resistance in Dd2-H for AQ-13 and no reversal with AQ-129 by contrast to CQ (i.e., ~ 9.3 folds in Dd2-H parasites, a drop from 170nM to 18.26nM, while it was ~ 1.4 folds for AQ-13 and ~ 2.1 folds for AQ-129 (from 38.35nM to 23.24nM for AQ-13 and from 33.45nM to 15.65nM for AQ-129) (figure 4; table 2).

Drug	1µM VP	3D7	Dd2-H
		IC_{50} ± SD (nM)	IC_{50} ± SD (nM)
CQ	-	12 ± 0.59	170 ± 11.6
	+	14.14 ± 1.83	18.26 ± 4.55
AQ-13	-	10.56 ± 0.63	38.35 ± 3.8
	+	9.78 ± 0.6	23.24 ± 3.67
AQ-129	-	13.68 ± 1.65	33.45 ± 2
	+	4.1 ± 1.26	15.65 ± 2.1

P<.0001, by Student *t* test, for comparison between CQ and AQ-13 on Dd2-H

P<.0001, by Student *t* test, for comparison between CQ and AQ-129 on Dd2-H

P<.0001, by Student *t* test, for comparison between CQ effects +/- VP on Dd2

P<.0049, by Student *t* test, for comparison between AQ-13 effects +/- VP on Dd2

P<.152, by Student *t* test, for comparison between AQ-129 effects +/- VP on Dd2

Table 2. Summary of AQ-13 and AQ-129 effects on the proliferation of CQS and CQR strains (3D7 and Dd2-H) of *P. falciparum* without/with VP. The table summarizes the IC_{50} values obtained from graphs in figure 4. Statistical analysis was made using GraphPad Prism. The table shows the mean ± SD of three independent experiments done in triplicates.

Furthermore, the IC_{50}`s of AQ-13 and AQ-129 with 3D7 parasites were very similar to CQ effects on 3D7 (12nM for CQ, 9.78nM for AQ-13 and 13.68nM for AQ-129) (figure 4; table 2). Taken together, AQ-13 and AQ-129 seem to be as effective as CQ against 3D7 parasites but more potent than CQ on Dd2-H parasites.

Effects of AQ-13 and AQ-129 on CQS HB3 and D10 and CQR 7G8 and K1 with and without VP.

The K76T mutation in PfCRT amino acid sequence was shown to be consistently associated with CQ resistance in *P. falciparum* strains. Dd2-H parasites possess this and additional mutations in PfCRT [20], while other CQR parasites possess different mutations in PfCRT that would modulate their responses to CQ and other compounds [21]. To examine the possibility that mutations other than K76T amino acid change are responsible for Dd2-H response to AQ-13 and AQ-129, we tested the effects of AQ-13 and AQ-129 on four additional *P. falciparum* strains, two CQS strains

(HB3 and D10) and two CQR strains (7G8 and K1). The results in figure 5 show that AQ-13 is more lethal towards two CQR strains (7G8 and K1) than CQ, with differences in IC_{50} between AQ-13 or AQ-129 and CQ for 7G8 of ~ 5.3 folds and K1 of ~ 6.8 folds (figure 5; table 3). In both cases when the parasites were treated with AQ-13 in the presence of VP, the reversal of resistance was not as efficient as that for CQ (i.e. 7G8 CQ IC_{50} -/+VP: 117.6nM / 90.83nM; 7G8 AQ-13 IC_{50} -/+VP: 22.12nM / 18.39nM; K1 CQ IC_{50} -/+VP: 207.3nM / 40.85nM; K1 AQ-13 IC_{50} -/+VP: 30.57nM / 22.73nM) (figure 5; table 3).

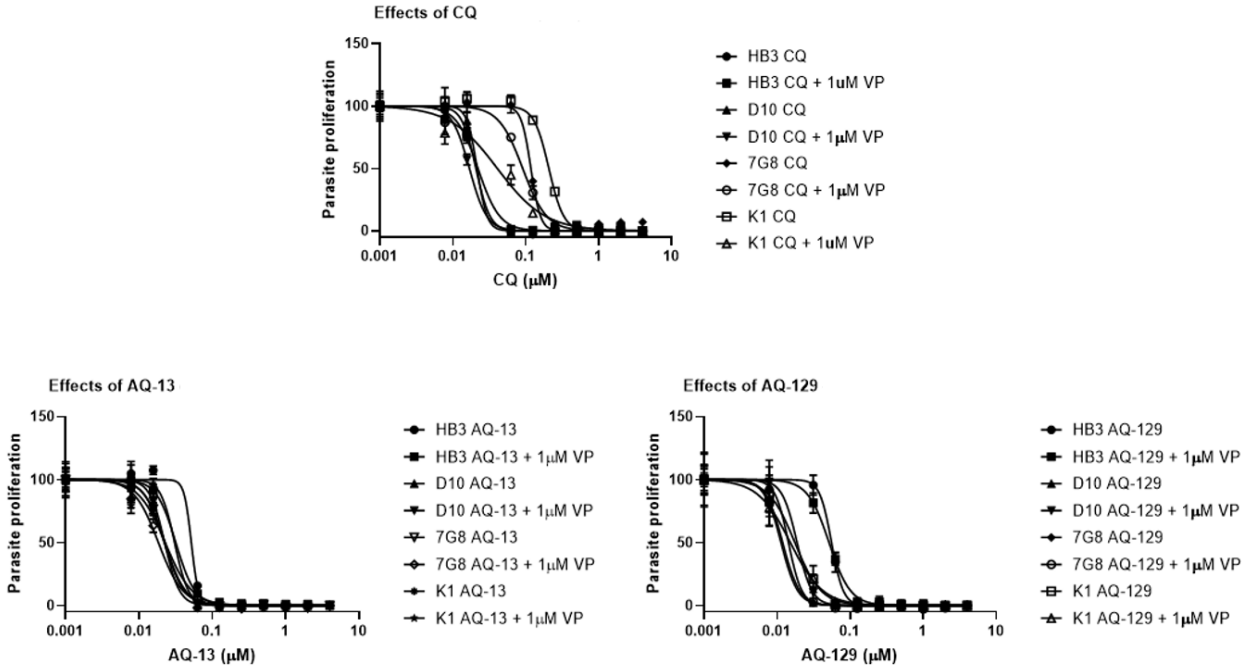


Figure 5. Effects of AQ-13 and AQ-129 on proliferation on additional CQS (HB3, D10) and CQR (7G8 and K1) strains of *P. falciparum* without/with VP. 3D7 and Dd2-H were incubated in increasing concentrations of CQ, AQ-13 and AQ-129; plates were developed after 72hrs of incubation. Graphs were made using GraphPad Prism. The graphs show the mean ± SD of three independent experiments done in triplicates.

AQ-129 effects against CQR strains (e.g. 7G8 and K1) were more lethal than CQ (figure 5; table 3). The difference between AQ-129 and CQ IC_{50}`s for 7G8 and K1 was ~10.4 folds (figure 5; table 3). Moreover, when 7G8 and K1 CQR strains were treated with AQ-129 in the presence of VP, the

reversal of resistance was less efficient than for CQ (i.e. 7G8 CQ IC_{50} -/+VP: 117.6nM / 90.83nM; 7G8 AQ-129 IC_{50} -/+VP: 11.30nM / 11.89nM; K1 CQ IC_{50} -/+VP: 207.3nM / 40.85nM; K1 AQ-129 IC_{50} -/+VP: 17.47nM / 15.16nM) (figure 5; table 3). These results suggest that AQ-13 and AQ-129 are more lethal than CQ against all CQR strains tested (Dd2-H, 7G8 and K1) and both compounds are poorly transported by mutant-PfCRT compared to CQ.

Drug	1µM VP	HB3	D10	7G8	K1
		IC_{50} ± SD (nM)	IC_{50} ± SD (nM)	IC_{50} ± SD (nM)	IC_{50} ± SD (nM)
CQ	-	20.78 ± 3.4	21.54 ± 5.72	117.6 ± 8.6	207.3 ± 10
	+	22.12 ± 2.28	17 ± 0.37	90.83 ± 14	40.85 ± 8.3
AQ-13	-	51.7 ± 11.17	22.61 ± 3.33	22.12 ± 1.6	30.57 ± 6.17
	+	31.7 ± 3.21	22.38 ± 5.1	18.39 ± 1.61	22.73 ± 3.33
AQ-129	-	55.5 ± 3.7	14.28 ± 2.66	11.30 ± 1.58	17.47 ± 5
	+	51.33 ± 7.74	18.74 ± 3.76	11.89 ± 1.07	15.16 ± 1.11

P<.0041, by Student *t* test, for comparison between CQ and AQ-13 effects on 7G8

P<.0025, by Student *t* test, for comparison between CQ and AQ-129 effects on 7G8

P<.0010, by Student *t* test, for comparison between CQ and AQ-13 effects on K1

P<.0009, by Student *t* test, for comparison between CQ and AQ-129 effects on K1

P<.194, by Student *t* test, for comparison between CQ effects +/- VP on 7G8

P<.788, by Student *t* test, for comparison between AQ-13 effects +/- VP on 7G8

P<.921, by Student *t* test, for comparison between AQ-129 effects +/- VP on 7G8

P<.0021, by Student *t* test, for comparison between CQ effects +/- VP on K1

P<.0158, by Student *t* test, for comparison between AQ-13 effects +/- VP on K1

P<.1140, by Student *t* test, for comparison between AQ-129 effects +/- VP on K1

Table 3. Summary of AQ-13 and AQ-129 effects on the proliferation on additional CQS (HB3, D10) and CQR (7G8 and K1) strains of *P. falciparum* without/with VP. The table summarizes the IC_{50} values obtained from graphs in figure 5. Statistical analysis was made using GraphPad Prism. The table shows the mean ± SD of three independent experiments done in triplicates.

The IC_{50} of CQ sensitive strain D10 treated with AQ-13 or AQ-129 was similar to CQ (i.e. 21.54nM, 22.61nM and 14.28nM, respectively) (figure 5; table 3). Interestingly, HB3 CQS strain showed an

elevated IC_{50} of 51.7nM and 55.5nM with AQ-13 and AQ-129, respectively versus 20.78nM for CQ (figure 5; table 3).

Effects of AQ-13 and AQ-129 on isogenic clones $C2^{GCO3}$ and $C4^{Dd2}$ with and without VP. Given the above results with respect to the effect of AQ-13 and AQ129 on CQS and CQR strains, it was of interest to isolate these differences to wild-type and mutant-PfCRT using isogenic clones (i.e. $C2^{GCO3}$ and $C4^{Dd2}$, respectively) that share the similar genetic backgrounds but differ only in PfCRT sequence [6]. The results in figure 6 show the proliferation of $C2^{GCO3}$ and $C4^{Dd2}$ parasites in increasing molar concentrations of AQ-13 or AQ-129 in the absence or presence of 1µM VP. For $C2^{GCO3}$ treated with AQ-13 or AQ-129 without and with VP, the IC_{50} values were similar to CQ (i.e. IC_{50} for CQ -/+ VP: 21.33nM/19.15nM; IC50 for AQ-13 -/+ VP: 18.97nM/ 16.11nM; 29.48nM/ 23.15nM) (figure 6; table 4).

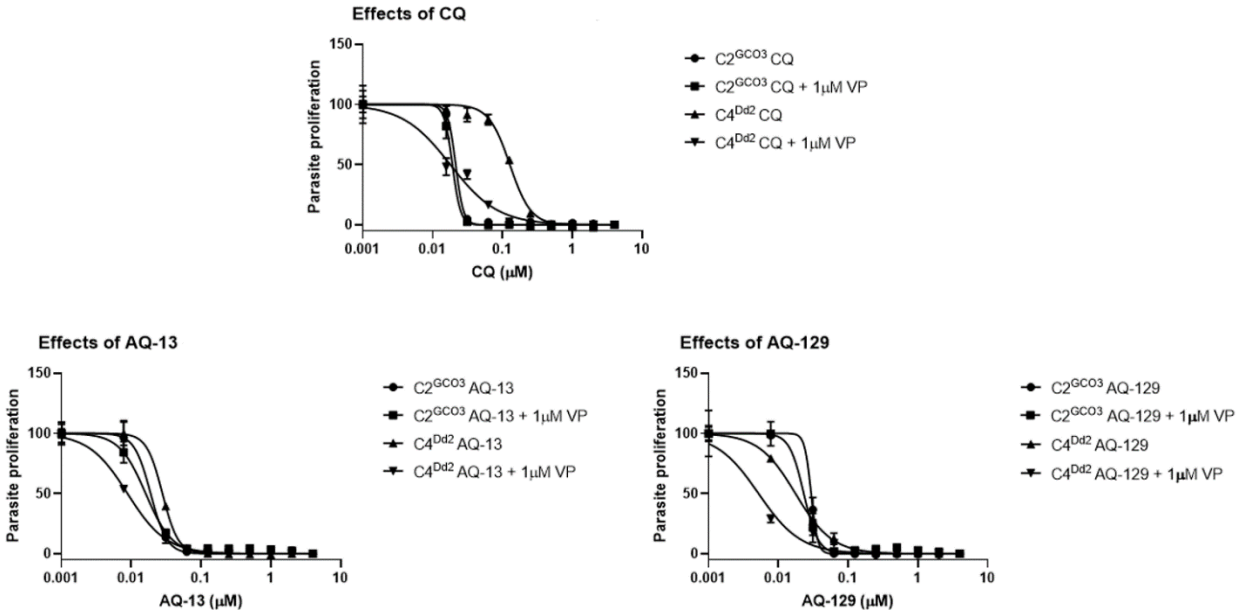


Figure 6. Effects of AQ-13 and AQ-129 on proliferation on isogenic clones $C2^{GCO3}$ and $C4^{Dd2}$ of *P. falciparum* without/with VP. $C2^{GCO3}$ and $C4^{Dd2}$ were incubated in increasing concentration of CQ, AQ-13 and AQ-129 plates were developed after 72hrs of incubation. Statistical analysis and

graphs were made using GraphPad Prism. The graphs show the mean ± SD of three independent experiments done in triplicates.

However, the IC_{50} values extracted from the proliferation curves for clone $C4^{Dd2}$ treated with AQ-13 or AQ-129, without and with 1µM VP were different from those for CQ, consistent with results obtained with other CQR strains, Dd2-H, 7G8 and K1 (figure 4; figure 5). Moreover, while CQ IC_{50} was 127.2nM, the IC_{50} values for AQ-13 and AQ-129 were 28nM and 17.6nM, respectively (figure 6; table 4). Furthermore, while the reversal of CQ resistance in the presence of VP for $C4^{Dd2}$ was very strong (from IC_{50} 127.2nM to 17.88nM ~ 7 folds); VP was very weak at reversing AQ-13 and AQ-129 resistance in $C4^{Dd2}$ (from IC_{50} 28nM to 8.85nM ~ 3.2 folds and from IC_{50} 17.6nM to 5.205nM ~ 3.3 folds, respectively) (figure 6; table 4). Collectively, the results using these isogenic clones suggest that both compounds are more active than CQ on CQR strains and are poor substrates for mutant-PfCRT.

Drug	1µM VP	$C2^{GCO3}$	$C4^{Dd2}$
		IC_{50} ± SD (nM)	IC_{50} ± SD (nM)
CQ	-	21.33 ± 1.57	127.2 ± 6.8
	+	19.15 ± 1.67	17.88 ± 2.65
AQ-13	-	18.97 ± 3	28 ± 1.78
	+	16.11 ± 1.8	8.85 ± 0.45
AQ-129	-	29.48 ± 3.01	17.6 ± 2.76
	+	23.15 ± 3.22	5.205 ± 1.145

P<.0001, by Student *t* test, for comparison between CQ effects +/- VP on $C4^{Dd2}$

P<.0913, by Student *t* test, for comparison between AQ-13 effects +/- VP on $C4^{Dd2}$

P<.062, by Student *t* test, for comparison between AQ-129 effects +/- VP on $C4^{Dd2}$

Table 4. Summary of AQ-13 and AQ-129 effects on the proliferation on isogenic clones $C2^{GCO3}$ and $C4^{Dd2}$ of *P. falciparum* without/with VP. The table summarizes the IC_{50} values obtained from graphs in figure 5. Statistical analysis was made using GraphPad Prism. The table shows the mean ± SD of three independent experiments done in triplicates.

Taken together, the data so far suggests that AQ-13 and AQ-129 are superior compounds to CQ against CQR strains. Moreover, AQ-13 and AQ-129 appear to be poorly transported by mutant-PfCRT suggesting that these modifications to CQ hindered their recognition by mutant-PfCRT.

Discussion

Current effective antimalarials, such as artemisinin combination therapies, are under threat of emerging resistance [22, 23]. Other drugs are risks with benefits because while they`re effective, treatment doses can be toxic to the patient [24]. Therefore, safe and affordable effective antimalarials against resistant *Plasmodium* parasites is an urgent public health priority. Moreover, it has been shown that drugs that interfere with the formation of hemozoin crystals are some of the most successful antimalarials. CQ was the drug of choice before resistance emerged primarily due to mutations in PfCRT that is expressed on the DV membrane [5]. Also, it is well known that the quinoline ring is an integral part of CQ and 4-aminoquinolines derivatives because it plays a crucial role in inhibiting hemozoin formation. Additionally, modifications to the aromatic rings can be detrimental to the compound activity [9, 25].

In this study, all 11 CQ derivatives in group B were modified at 3rd position of the quinoline ring with a benzamide functional group modified with either a halogen (chloride atom or fluoride) or other functional groups (methoxy group, amino group, cyanide atom, di-methyl amino group and methyl-thio group). The introduction of these functional groups at the 3rd position of the 7-chloro-4-aminoquinoline moiety did not improve the activity of these derivatives towards CQS and CQR lab adapted strains displaying reduced activity in varying folds compared to CQ. Moreover, the reduced activity of these CQ derivatives is consistent with earlier reports whereby modifications to the quinoline moiety reduces its antimalarial activity [9, 10, 25]. Overall, it seems

that modifications at 3rd position of the quinoline moiety, although accessible, do not increase the activity of the CQ derivatives towards CQR strains. It is possible that the size of the compound may reduce the beta-hematin inhibition because it may not form a strong association with heme. Therefore, the compounds from this group were not further investigated for their antimalarial activities or their mechanism of action against the parasite.

By contrast, some modifications on the side-chain connected to the secondary amine at the 4th position of CQ showed increased antimalarial activity towards CQR strains. Moreover, in BK-435, the shortening of the aliphatic chain to three carbons and adding an azepan ring did show improvement towards CQS 3D7 but the effects on Dd2-H were close to that of CQ. It was independently reported in an earlier study that AQ-13 is a possible compound to use for future treatment of malaria infected patients [14]. Here, our results indicate that it is as toxic to CQS *P. falciparum* strains as CQ, but more lethal to CQR strains than CQ. However, it was reported previously that this compound was not reversed by VP [13] which was not the case with some of the strains used in this study. The fact that strains Dd2-H and K1 did show a very slight reversal effect compared to almost no reversal in 7G8 would suggest that different backgrounds and PfCRT mutations may be modulating the effects of the compound. As such, we eliminated these possibilities with respect to PfCRT using isogenic clones of wild-type and mutant-PfCRT sequences. The results show that AQ-13 is more lethal than CQ against $C4^{Dd2}$and reversal with VP was not significant. Moreover, fairly similar observations were seen with AQ-129 as well. This isogenic clone ($C4^{Dd2}$) shows that mutant-PfCRT is probably involved in transporting AQ-13 and AQ-129 outside the DV but is not the only transporter implicated. Moreover, we also tested the capability of AQ-13 and AQ-129 to inhibit beta hematin formation *in-vitro* and our initial data

suggest both derivatives have the potential to act in the same way as CQ in inhibiting beta-hematin formation (data not shown). Furthermore, our efforts to isolate Dd-2-H resistant clones to AQ-13 and AQ-129, through continuous drug pressure that encode novel mutations in PfCRT, were unsuccessful. It is presently not clear if our drug pressure protocol was at fault or that mutations in PfCRT that leads to resistance to AQ-13 and AQ-129 inactivated PfCRT and are consequently lethal to the parasite, as PfCRT was shown to be an essential gene for the survival of the parasite [26]. Taken together, our results shed some light on the relationship of AQ-13 and AQ-129 with mutant-PfCRT. Even though they are very toxic to CQR strains compared to CQ, they seem to be poorly recognised by mutant-PfCRT and VP does not affect the transport via mutant-PfCRT as it does with CQ. Furthermore, experiments should be done to investigate the role of other membrane proteins in trafficking of these compounds as PfMDRI to understand the level of involvement of these transporters to get a clearer picture of the compound`s activity in the parasites.

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Connecting statement 2

In the previous chapter we showed mutant-PfCRT`s involvement in CQR. We also showed that AQ-13 and AQ-129 are CQ derivatives that are significantly more lethal than CQ on CQR tested lab-adapted strains. The data supported that both compounds would be potentially better than CQ. It was recently reported that serine residues in PfCRT are involved in modulating the levels of CQR. This next chapter describes the isolation and characterization of a novel pool of antibodies that can distinguish between phosphorylated and de-phosphorylated Ser411 on the C-terminal of PfCRT. A tool that enabled the monitoring of CQS and CQR strains with respect to the phosphorylation status of Ser411 and the potential role of post-translational modification in PfCRT function.

Chapter 3

Manuscript II

High resolution mapping of PfCRT antiserum identifies a phosphorylated PfCRT at Ser411 in the parasite digestive vacuole

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(Manuscript in preparation)

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Abstract

Mutated *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) has set back global malaria eradication efforts in endemic countries. The crystal structure of this protein was determined but the normal function(s) is still unknown. PfCRT structural residues, serine-33/threonine-416, have been reported to play phosphorylation-mediated physiological roles as modulation of chloroquine resistance or trafficking the protein to the digestive vacuolar membrane, respectively. Here we report the high resolution mapping of PfCRT antiserum raised to its C-terminal cytoplasmic sequence. Characterization of the binding specificity of PfCRT antiserum lead to the identification of three pools of immunoglobulin-G (IgG), each recognizing a different epitope. IgGs from pool 2 bound a heptapeptide epitope in the PfCRT C-terminus containing an un-phosphorylated Ser411. Using the latter purified pool of IgG, we show the presence of a homodimer of PfCRT that contains un-phosphorylated Ser411 and migrates as a 90kDa polypeptide on SDS-PAGE. Moreover, immuno-fluorescence staining of *Plasmodium falciparum* reveals the localization of the PfCRT homodimer outside the parasite digestive vacuole. By contrast, PfCRT monomer was localized to the digestive vacuole and was phosphorylated at Ser411. Interestingly, a chloroquine sensitive strain (3D7) showed PfCRT to be significantly more phosphorylated than chloroquine resistant stain (Dd2-H), suggesting a possible role for Ser411 phosphorylation in PfCRT function. Taken together, the findings of this study describe the isolation of a specific IgG pool that allows for monitoring the phosphorylation status of Ser411 in PfCRT.

Introduction

Malaria is an ancient disease, yet it still exists in the 21st century. The organisms responsible for this human disease are parasites from five *Plasmodium* species. *Plasmodium falciparum* (*P. falciparum*) is the most deadly, causing the most severe clinical symptoms including cerebral malaria and if left un-treated, death. Research over the past two decades have revealed that mutations in the *P. falciparum* Chloroquine Resistance Transporter (PfCRT) is the primary determinant for chloroquine resistance [1]. PfCRT is an integral membrane protein localising to the digestive vacuole (DV) of the parasite. The crystal structure of PfCRT shows that it is arranged in a 10 transmembrane domain organisation with cytosolic N and C termini [2]. The physiological role as well as its normal substrates are still matters of speculation. Indeed, research on PfCRT has focused mainly on its role in drug resistance, with little focus on the biochemistry of PfCRT and its physiological functions in the parasite. The predicted molecular mass of PfCRT is 48.67kDa, however multiple forms of PfCRT with respect to its mobility on SDS-PAGE have been cited using antibodies raised to either the N- or C-cytosolic domains [3-5]. The variation in molecular masses could be conformational and/related to post-translational modifications. The PfCRT amino acid sequence has at least three phosphorylation sites localized to its N- (Ser33) and C-termini (Ser411 and Thr416 [6]). Phosphorylation of PfCRT at Ser33 was suggested to play a role in modulating responses to chloroquine and quinine resistance [7]. Moreover, phosphorylation at Thr416 was shown to affect PfCRT trafficking from the endoplasmic reticulum to the DV membrane [6]. These findings reveal a role for PfCRT phosphorylation that modulate its transport and cellular trafficking functions. Another phosphorylation site in PfCRT was recently mapped to Ser411 in 3D7 and Dd2, however no function has been described for this site [6, 7]. In this report, and in

the process of characterizing the binding specificity of one anti-serum raised against the PfCRT C-terminal sequence to a resolution of single amino acids, we isolated and purified a pool of IgGs that bound only dephosphorylated Ser411. Using this purified pool of IgG we show for the first time the presence of the PfCRT homodimer migrating with an apparent molecular mass of 90 kDa and contains an unphosphorylated Ser411; while in contrast the monomer PfCRT migrated as a 42kDa polypeptide and was phosphorylated in the DV. Moreover, PfCRT in 3D7 parasites are more phosphorylated at Ser411 than Dd2-H, a difference that could be related to the mechanism of chloroquine resistance in chloroquine resistant strains.

Material and methods

Malaria culture and protein extraction **-** A continuous parasite culture of 3D7 *P. falciparum* was maintained using the previously described method of Trager and Jensen [8]. Briefly, parasites were allowed to proliferate on 2% packed human (A^+) erythrocytes in RPMI 1640 medium containing L-glutamine and HEPES. The medium was supplemented with 50mg/L (w/v) hypoxanthine and 5% (v/v) human serum. The parasite culture was maintained in 10cm tissue culture dishes, incubated at 37°C under a gas mixture of 3% O_2, 5% CO_2 and 92% N_2. The culture medium was changed every 12hrs and the parasitemia was determined via stained blood smears using 5% Giemsa stain. Synchronous parasites were obtained using a previously described protocol [9]. For protein extractions, 500 µl of infected erythrocytes with 3D7/ HB3/ Dd2-H parasites of the trophozoite stage were washed with cold phosphate buffered saline (PBS) and incubated in PBS containing 0.05% (w/v) saponin on ice for 10min. After centrifugation, the proteins from the resulting pellets were extracted for 20min with CHAPS extraction buffer, containing 10mM HEPES pH 7.3, 150mM NaCl, 1mM $CaCl_2$, 10mM CHAPS and protease inhibitors

cocktail (Thermo scientific). The extracted parasite lysate was collected after brief centrifugation at 13000rpm at 4°C. Parasite protein lysates were kept at -70^{o}C if not used immediately.

Antigen preparation and immunization - Antiserum against the C-terminal of PfCRT was raised in rabbits using a 26 amino acid synthetic peptide encoding the entire exposed C-terminal cytoplasmic domain (Ac-CG-ERKKMRNEENEDSEGELTNVDSIITQ; Canpeptide Inc.). The peptide was cross-linked through its N-terminal cysteine to ovalbumin and used for rabbit immunization (McGill comparative medicine and animal resources center).

SDS-PAGE and immune-blotting- Total parasite lysate or transiently transfected HEK-293F cells lysate and erythrocyte ghost proteins were resolved on SDS-PAGE [10] and transferred onto PVDF membrane in Tris-glycine buffer in the presence of 20% (v/v) methanol as previously described [11]. After the transfer, membranes were blocked in 3% (w/v) non-fat milk phosphate buffered saline containing 0.05% Tween-20 (PBST). Following several washes with PBST, membranes were probed with anti-C-PfCRT or purified anti-Ser411 antibodies, alone or in the presence of molar excess of the C-terminal peptide or transient transfected HEK-293F cells lysate with human codon optimized wt-PfCRT or empty vector pCDNA3.1+ in addition to an irrelevant recombinant protein or peptide, respectively. Membranes were then washed and incubated with horseradish peroxidase (HRP)-conjugated goat-anti-rabbit antiserum at 1:5000 (v/v) dilution (Bio-Rad, Inc.) and the signals were developed using a chemiluminescent kit according to the manufacturer protocol (Thermo Scientific®).

Peptide synthesis and epitope mapping - Overlapping heptapeptides encoding the entire C-terminal domain of PfCRT were synthesized on pre-derivatized polypropylene plastic pins

(Mimotopes Ltd.) or filter paper using F-moc protected amino acids as previously described [12]. For epitope mapping, pins were incubated in PBS for 30min at room temperature and blocked in 3% non-fat-milk-PBST plus 0.02% (w/v) sodium azide for 2hrs. After washing in PBST for 1hr, the primary antibody, anti-C full-serum (anti-C terminal for PfCRT), was prepared in milk at 1:1000 (v/v). Binding of the antibody to the peptides was allowed to take place overnight at 4°C. Un-bound antibodies were removed by washing the pins four times in PBST for 45min. Post washing, the pins were incubated in the presence of the secondary antibody, goat anti-rabbit (Bio-Rad, Inc.), in 3% (w/v) milk minus sodium azide, at a 1:3000 (v/v) dilution for 30min at room temperature, followed by a second 1.5-hour incubation at 4°C. The pins were washed again with PBST four times for 45min. For signal development, the pins were incubated in a solution of 100mM sodium dihydrogen orthophosphate, 80mM citric acid pH 4.0, 0.009% (v/v) hydrogen peroxide and 0.5mg/ml ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) in 96 well plate for 40min. The absorbance was read at 410nm.

Dephosphorylation of PfCRT on PVDF - Malaria lysates (10µg CHAPS buffer-extracted 3D7 or Dd2-H proteins) were resolved on 10% SDS-PAGE and transferred to PVDF membrane according to the method of Towbin [11]. The lanes on the membrane were separated followed by blocking the control lanes in 3% (w/v) non-fat milk in PBST. Lanes to be dephosphorylated were washed with TBST for 10min and subsequently incubated, in dephosphorylation buffer containing 20U (20U/µl) calf intestinal alkaline phosphatase (CAP) (Invitrogen™; cat: 18009019) and protease inhibitors, for 3hrs 20min at 37°C. Post incubation, the membranes were treated with their respective antibodies (anti-PfCRT-C/ anti-Ser411 and goat anti mouse at 1:5000 (v/v) dilution (Bio-Rad, Inc.), respectively).

Immunofluorescence staining - The parasite culture was collected at the trophozoite stage and processed for fluorescence microscopy. Briefly, infected erythrocyte pellets were fixed with 4% (v/v) paraformaldehyde in PBS for 30min at room temperature. Fixed cells were spread on individual cover slips (Fisher Scientific®), air dried for 2–5min and incubated in 0.15% (w/v) glycine in PBS for 10min. The cover slips were gently washed twice with PBST, permeabilized with 0.1% (v/v) Triton X-100/PBS for 30min, washed twice with PBST and blocked in 1% (v/v) goat serum/PBS for 2hrs at 4°C. Following two washes with PBST, the cover slips were incubated with anti-C PfCRT full-serum and anti-Ser411 antibodies (1:25 (v/v) dilutions) in blocking buffer overnight at 4°C. The slides were washed three times with PBST and incubated with the secondary antibodies Alexa-594 goat anti-rabbit diluted 1:2000 (v/v) in blocking buffer for 45min at room temperature. Immediately after incubation, the cover slips were washed three times with PBST and immersed in 4', 6-Diamidino-2-Phenylindole (DAPI)/PBS for 4min at room temperature. Cover slips were washed five times with PBST, followed by a final wash with PBS. The cover slips were dipped once in water, air-dried in dim light, mounted on microscopy slides and viewed under confocal microscopy (Carl Zeiss GmbH, Jena, Germany).

Cloning of codon optimized PfCRT and C-terminal mutants - The sequence of wt-*PfCRT* (3D7) that encodes a chloroquine sensitive gene was obtained from plasmo DB (gene ID PF3D7_1133400), human codon optimised and commercially synthesized (Genscript®, USA). The full-length gene was ligated to pCDNA3.1+ (Invitrogen™) between two restriction sites, BamHI and EcoRI (New England Biolabs Inc.; BamHI-HF cat: R3136S and EcoRI-HF cat: R3101S), with a Kozak consensus sequence at 5`end just before the ATG.

To establish wt-PfCRT-C-terminal-mutants, wt-PfCRT-(Ser411Ala+Thr416Asp) and wt-PfCRT-(Ser411Asp+Thr416Ala) the Gibson assembly approach was used [13]. The primers and reaction conditions are provided in table 1. The fragments were put together and inoculated into TOP10 chemically competent cells via heat shock. Bacterial clones were selected and the plasmids extracted were sequenced by Genome Quebec, Montreal, CA.

Tissue culture and PfCRT transient transfection in HEK-293F cells - HEK-293F cells were cultivated and maintained in DMEM culture media (Gibco™, cat: 11965118) containing 10% fetal bovine serum (FBS) in 37°C incubator with environmental O_2 and 5% CO_2. Cells were grown to 70-80% confluency prior to transfection. Transfections, using pCDNA3.1+ (Invitrogen™) empty plasmid, full-length wt-PfCRT, wt-PfCRT-(Ser411Ala+Thr416Asp) and wt-PfCRT-(Ser411Asp+Thr416Ala) containing plasmids, all were transfected into HEK-293F cells using Lipofectamine 2000 (Invitrogen™; ref: 11668-030), following manufacturer instructions. After 72hrs incubation, cells were removed, washed in PBS and extracted by CHAPS extraction buffer, containing 10mM HEPES pH 7.3, 150mM NaCl, 1mM $CaCl_2$, 10mM CHAPS and protease inhibitors cocktail (Thermo Fisher Scientific).

Extraction of anti-Ser411 from anti-C full-serum using overlapping peptides to PfCRT C-terminal - Overlapping peptides, pins sequences: ^{408}NEDSEGE^{214}, ^{403}MRNEENE^{409} and ^{415}LTNVDSI^{421} were washed in PBS containing 0.05% (w/v) NaN_3 at 4°C. Pins were blocked in 5% (w/v) non-fat milk PBS containing 0.05% (w/v) NaN_3 for 1hr at 4°C, and incubated in 5% (w/v) non-fat milk PBS with 0.05% (w/v) NaN_3 and 1:500 (v/v) dilution of anti-C full-serum overnight at 4°C. The pins were subjected to several rounds of washing in PBS containing 0.05% (w/v) NaN_3 for 5hrs at 4°C. The peptide bound antibodies were eluted by incubating the pins in elution buffer (200mM Glycine-

HCL pH 2.8) for 4min at room temperature and immediately neutralized by adding 3M Tris-HCl pH 8.8 and stored at 4°C in storage buffer (0.1% (w/v) BSA, 0.05% (w/v) NaN_3 in PBS) until use.

Extraction of anti-Ser411 from anti-C full-serum using dimer PfCRT as bait - PVDF membranes containing chloroquine sensitive strain 3D7 resolved proteins were washed in PBS containing 0.05% (w/v) NaN_3 at 4°C. Strips containing dephosphorylated dimer PfCRT (~90kDa) were excised from all the PVDF membranes and blocked in 5% (w/v) non-fat milk PBS containing 0.05% (w/v) NaN_3 for 1hr at 4°C. PVDF strips were then incubated in 5% (w/v) non-fat milk PBS containing 0.05% (w/v) NaN_3 and 3:1000 (v/v) dilution of anti-C full-serum for ~72hrs at 4°C. After incubation, the strips were subjected to several rounds of washing in PBS containing 0.05% (w/v) NaN_3 for 48hrs at 4°C. The bound antibodies were eluted by incubating the strips in elution buffer (200mM Glycine-HCL pH 2.8) for 4min at room temperature and the pH was neutralized immediately with addition 3M Tris-HCl pH 8.8. The antibodies were concentrated using size exclusion chromatography Amicon® Ultra-15 Centrifugal Filter Unit tubes with 50kDa cut off (Millipore Sigma; cat: UFC905008). The concentrated anti-Ser411 was stored at 4°C in storage buffer (0.1% (w/v) BSA, 0.05% (w/v) NaN_3 in PBS) until use.

Results and discussion

Characterization of PfCRT anti-C terminal rabbit serum. A rabbit anti-serum to a PfCRT C-terminal polypeptide was developed and its binding specificity to PfCRT was determined. To examine PfCRT anti-C-serum reactivity, membrane extracts from chloroquine sensitive strain (3D7) and erythrocyte ghosts were investigated. Figure 1A, lane 2, shows the results of a Western blot containing SDS-PAGE resolved proteins transferred to PVDF membrane probed with anti-C full-serum. The results in figure 1A show anti-C serum binds three different polypeptides in 3D7

migrating with apparent molecular masses of 90kDa, 42kDa and 32kDa. No cross-reacting polypeptides were observed in membrane extracts form RBC ghosts (figure 1A, lane 1) suggesting that all reactive proteins are of parasite origin. Based on the apparent molecular masses of the three polypeptides, the 42kDa polypeptide is likely to represent the full-length PfCRT given the predicted molecular mass of PfCRT (48.67kDa) and published reports showing a protein with apparent molecular mass between 42kDa – 45kDa (when anti-C-terminal PfCRT was used) [4, 5, 14]. The 90kDa anti-C-PfCRT reactive polypeptide is likely a non-specific reactive polypeptide or a homodimer of PfCRT. A homodimer PfCRT band was seen in an earlier report in a 3D7 *P. falciparum* parasite shown in different cellular fractions than the parasite DV membrane [4]. The origin of the 32kDa polypeptide is likely a degradation product of PfCRT or a cross-reacting parasite protein. To confirm the presence of the 42kDa and 90kDa in other strains of *P. falciparum*, membrane extracts from another drug sensitive strain (HB3) and chloroquine resistant strain (Dd2-H) were examined by Western blotting. Figure 1B shows PfCRT anti-C terminal sera to recognize the same polypeptides (42kDa and 90KDa) in membrane extracts from HB3 and Dd2-H strains.

Interestingly, the mobility of the 42kDa polypeptide appears to vary slightly in HB3 and Dd2-H strains versus 3D7 (figure 1B). To confirm the specificity of anti-C PfCRT antiserum to the potential monomer PfCRT (42kDa) and homodimer PfCRT (90kDa) polypeptides detected in membrane extracts from CQ Sensitive and resistant *P. falciparum* strains, human codon optimized wt-PfCRT was transiently expressed in HEK-293F cells and cell extracts were analysed by western blotting.

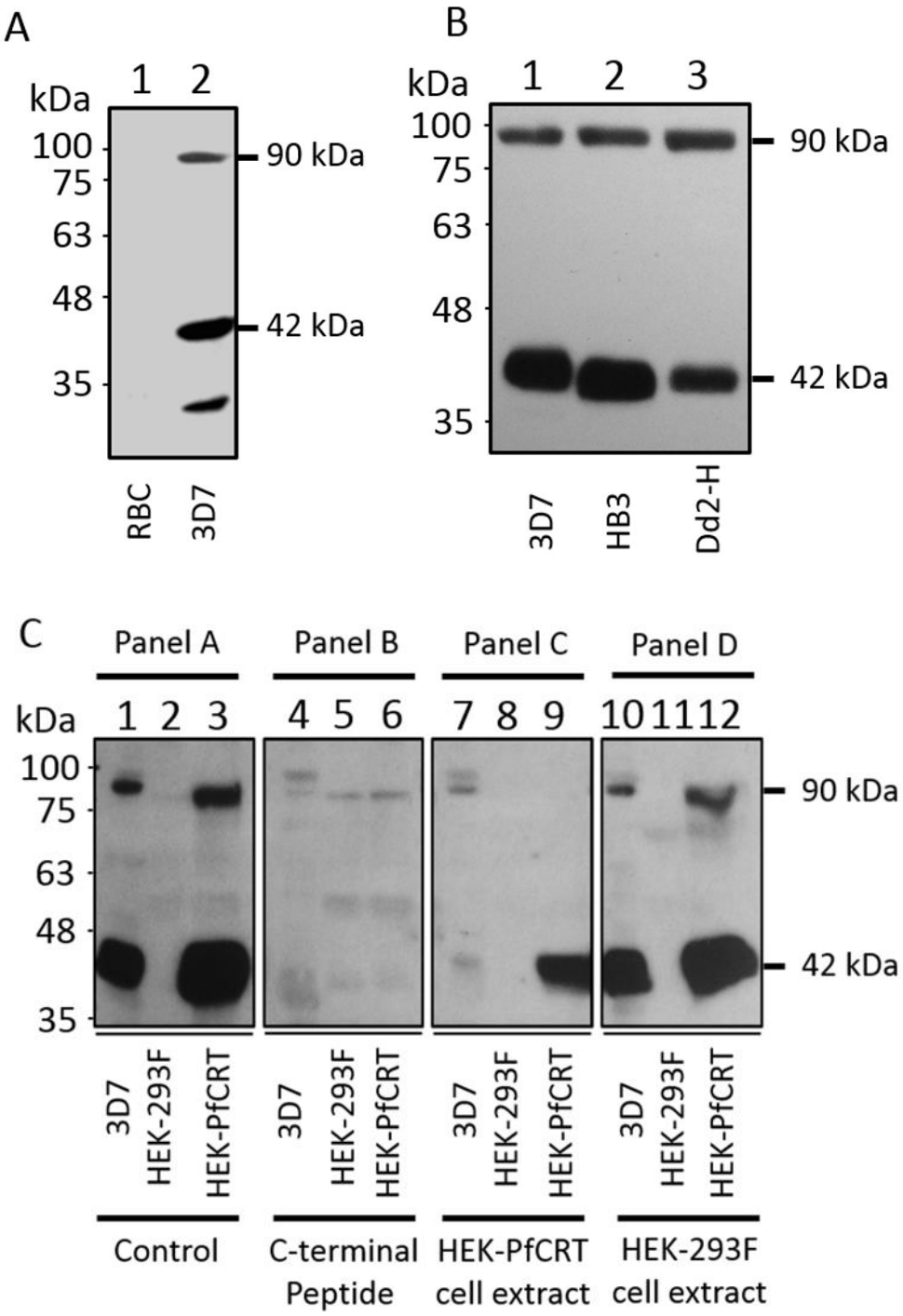

Figure 1. Testing the specificity of anti-C PfCRT. A - Anti-C full-serum reactivity towards 3D7 lysate. The Western blot in lane 1 shows anti-C full-serum has no reactivity towards un-infected human erythrocyte membrane extracted proteins. Lane 2 shows anti-C full-serum cross-reacting with three polypeptides. From highest to lowest molecular weights, the estimated molecular weights were: 90kDa, 42kDa and 32kDa. **B - Anti-C full-serum reactivity towards multiple *P. falciparum* strains from the field lysates.** The western blot shows all three *Plasmodium*

falciparum strains, chloroquine sensitive 3D7 and HB3 and chloroquine resistant Dd2-H express the 90kDa and the 42kDa polypeptides. **C - Anti-C full-serum reactivity towards wt-PfCRT transiently transfected HEK-293F cells and antibody pre-adsorption assays.** The western blot control panel (panel A) shows: anti-C cross-reacting with the 90kDa and the 42kDa polypeptides in 3D7 lysate lane 1 and in wt-PfCRT transiently transfected HEK-293F cells in lane 3 while no reactivity with un-transfected HEK-293F cells extracts in lane 2. Panel B shows almost no reactivity with membrane transferred proteins of: 3D7 lysate in lane 4, HEK-293F lysate in lane 5 and wt-PfCRT transiently transfected HEK-293F cells in lane 6 when the membrane was probed with anti-C pre-incubated with molar excess C-terminal PfCRT peptides. Panel C shows almost no reactivity with membrane transferred proteins of: 3D7 lysate in lane 7, HEK-293F lysate in lane 8 and almost no signal for the 90kDa polypeptide and a reduced signal for the 42kDa polypeptide in the wt-PfCRT transiently transfected HEK-293F cells in lane 9 when the membrane was probed with a anti-C pre-incubated with wt-PfCRT transiently transfected HEK-293F cells lysate. Panel D shows cross-reacting signals with the 90kDa and the 42kDa polypeptides in 3D7 lysate lane 10 and in wt-PfCRT transiently transfected HEK-293F cells in lane 11 while no reactivity with un-transfected HEK-293F cells extracts in lane 12 when the membrane was probed with an anti-C pre-incubated with molar excess HEK-293F cells lysate.

Figure 1C shows PfCRT anti-C serum to recognize 42kDa and 90kDa in cell extracts from 3D7 and wt-PfCRT-HEK-293F transiently transfected cells (panel A lanes 1 and 3, respectively), but not from vector transfected HEK-293F cells (lane 2). The mobility of the 90kDa polypeptide is slightly shifted in PfCRT-HEK-293F extract (lane 3) relative to that from 3D7 extract (lane 1). This slight shift in mobility on SDS-PAGE maybe due to differential post-translational modification of the same protein. It is noteworthy that the relative levels of the 42kDa to the 90kDa polypeptide is similar in the two membrane extracts (3D7 and wt-PfCRT HEK-293F cell) with the 90kDa as the minor form (figure 1C, lanes 1 and 3). To further confirm the binding specificity of PfCRT anti-C serum to the specific C-terminal sequence of PfCRT, membranes containing the same cell extracts from figure 1C (lanes 1-3) were co-incubated in the presence of PfCRT C-terminal peptide (panel B, lanes 4-6), protein extracts from PfCRT-HEK-293F transiently transfected cells (panel C, lanes 7-9) and protein extracts from empty vector transfected HEK-293F cells (lanes 10-12), respectively. The results in lanes 4-6 (figure 1C) show that the presence of excess PfCRT C-

terminal peptide completely inhibited the binding of anti-C serum to the 42kDa and 90kDa polypeptides in 3D7 and PfCRT-HEK-293F cell extracts (lanes 1 and 3, respectively). Similarly, the results in figure 1C (lanes 7 - 9) show that the addition of PfCRT-HEK-293F excess cell extracts also inhibited the binding of PfCRT anti-C serum to the 42kDa and 90kDa polypeptides from 3D7 and PfCRT-HEK-293F cell extracts (lanes 7 and 9, respectively). The presence of residual signal for the 42kDa polypeptide in the PfCRT-HEK-293F cell extract (figure 1C, lane 9) is expected given the strong signal in the absence of competing PfCRT-HEK-293F extract (figure 1C, lane 3). By contrast, the addition of cell extracts from HEK-293F cells (figure 1C panel D, lanes 10-12), which does not express PfCRT, did not significantly inhibit the binding of PfCRT anti-C serum to the 42kDa and 90kDa polypeptides (figure 1C, lanes 10 and 12, respectively). Taken together, our results show that PfCRT anti-C terminal rabbit serum binds specifically to 42kDa and 90kDa polypeptides that represent a monomer and homodimer forms of PfCRT. Moreover, both forms of PfCRT are found in HEK-293F cell lysate transfected with full-length codon optimized PfCRT gene. In addition, the homodimer polypeptide is found in HB3 and Dd2-H, in addition to 3D7 *P. falciparum* strains. The possibility that the homodimer PfCRT polypeptide represent an SDS-PAGE gel artifact due to sample oxidation was ruled out with the addition of excess reducing agent dithiothreitol (DTT) (data not shown).

PfCRT anti-C serum detects three different epitopes in PfCRT. To further characterize the binding specificity of PfCRT anti-C serum, it was of interest to map the binding specificity of this anti-serum to a resolution of a single amino acid using overlapping heptapeptides to cover the entire C-terminal sequence of PfCRT (ERKKMRNEENEDSEGELTNVDSIITQ). Figure 2A shows the relative binding of PfCRT anti-C serum IgGs to each of the overlapping heptapeptides as reflected

by the absorbance signal at 410nm (see methods). The results in figure 2A show three pools of IgGs each recognizing a different epitope sequence in PfCRT C-terminal sequence [epitope 1 ^{401}KKMRNEENED^{410}, epitope 2 ^{407}ENEDSEGE^{414}, and epitope 3 ^{412}EGELTNVDSII^{422}]. In an effort to determine the binding specificity of each antibody pool, each antibody pool was isolated with its respective heptapeptide sequence, [^{403}MRNEENE^{409}, ^{408}NEDSEGE^{414} and ^{415}LTNVDSI^{421} from PfCRT anti-C serum (see methods)], and the binding of each IgG pool was tested against its binding to PfCRT from 3D7 parasite extract by Western blotting. Figure 2B shows Western blot of 3D7 cell extract probed separately with IgG pools against epitope 1, 2 and 3 (lanes 1-3, respectively). Interestingly, IgG pool that recognized epitope 2 sequence (^{408}NEDSEGE^{414}) recognized mainly the 90kDa polypeptide (figure 2B, lane 2), while the other two epitope-specific IgG pools (^{403}MRNEENE^{409}, ^{415}LTNVDSI^{421}), bound mainly to the 42kDa polypeptide (figure 2B, lanes 1 and 3). The differential binding of the three IgG pools to different forms of PfCRT was surprising; however, two IgG pools which bound epitope 2 and 3 sequence contain two previously identified phosphorylation sites in the C-terminal of PfCRT [6]. Consequently we made use of this observation by using the possible homodimer as a ligand to extract the anti-Ser411 population from anti-C full-serum that only binds a dephosphorylated Ser411 epitope.

It was shown earlier that one of the two phosphorylation sites (Thr416) may be implicated in PfCRT trafficking from the ER to the DV membrane, however, the role of Ser411 phosphorylation is not known [6]. Recently, phosphorylation at Ser33 in PfCRT N-terminal was shown to be involved in modulating the levels of chloroquine resistance [7]. Hence, to determine if the binding of pool 2 IgGs to the epitope 2 sequence is modulated by phosphorylation at Ser411, the binding of pool 2 IgGs was tested by substituting Ser411 with Asp (a phospho-mimic) or Ala (un-

phosphorylated) residues in this epitope sequence. Figure 2C shows the binding of pool 2 IgG to synthetic PfCRT-C-terminal overlapping and substituted heptapeptides synthesised directly onto filter paper in a spot manner.

The results in figure 2C show strong reactivity of pool 2 IgGs to peptides 18, 19 and 20. The sequence shared between these peptides is nine residues: ^{406}EENEDSEGE^{414}. Moreover, when Ser411 was substituted with Ala (Ser411Ala) the signal reduced dramatically as shown in spot 21. Similarly, substitution of Ser411 with Asp (Ser411Asp), the binding of pool 2 IgG was completely lost. The results in figure 2C reveal the effects of Ser411 phosphorylation on its binding to pool 2 IgG, highlighting the ability of this IgG pool (pool 2) to predict the phosphorylation status of Ser411 in the C-terminal of PfCRT.

Based on these results, it is possible that pool 2 IgG recognize a dephosphorylated epitope 2 ^{408}NEDSEGE^{414} in the 90kDa polypeptide; while the 42kDa polypeptide is likely to be phosphorylated at Ser411.

To investigate the possibility that pool 2 IgG can identify the phosphorylation status of Ser411, PfCRT codon optimized cDNA was mutated at position Ser411 to Asp411 or Ala411 and transfected transiently into HEK-293F cells. Figure 2D shows a Western blots containing HEK-293F cell extracts from PfCRT-Ser411, PfCRT-Asp411 and PfCRT-Ala411 probed with pool 2 IgG. The results in figure 2D show strong binding to PfCRT-Ser411, followed by a huge drop in the binding to PfCRT-Ser411Asp and PfCRT-Ser411Ala.

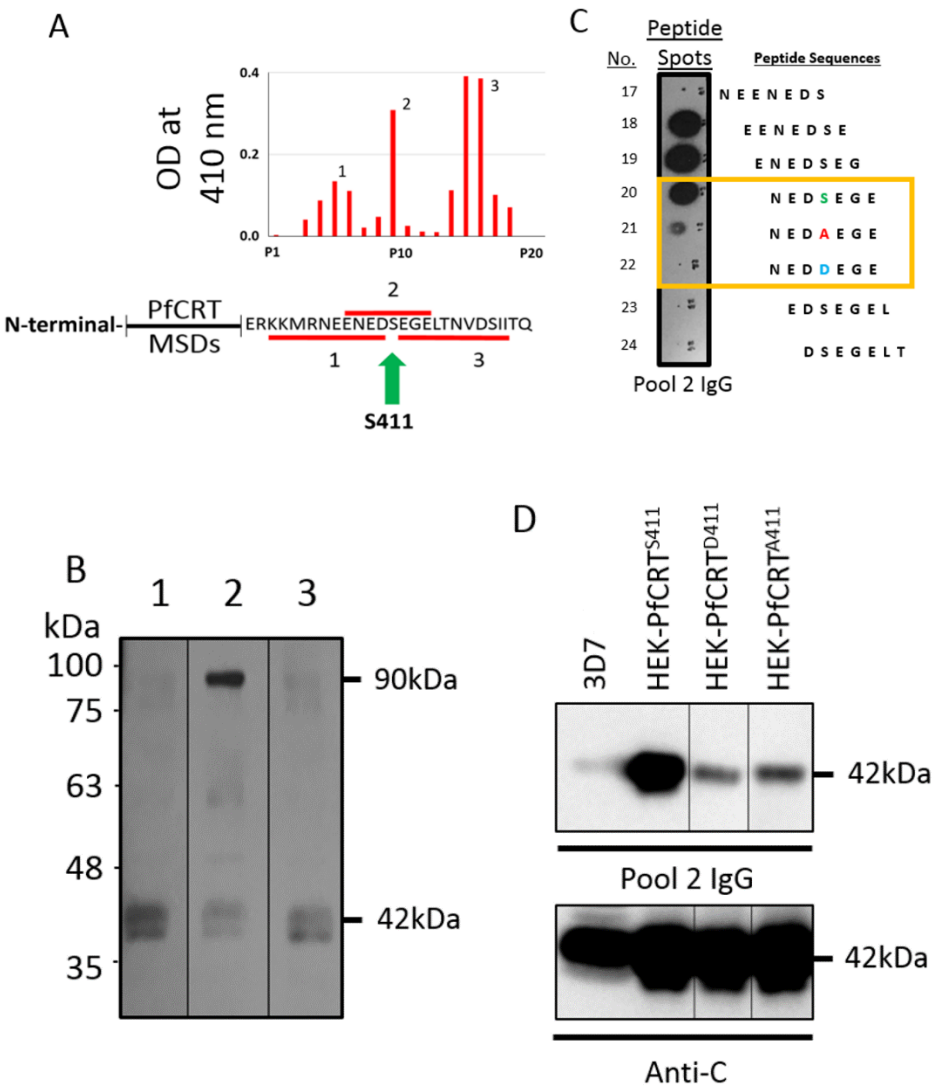

Figure 2. Testing anti-Ser411 PfCRT specificity. A - PfCRT anti-C serum binds three epitopes. The figure shows the binding of anti-C full-serum PfCRT antibodies to each of the heptapeptides with the relative binding intensity to each peptide as measured by the absorbance value (410nm) showing three epitopes. The epitopes are represented in a simplified PfCRT representation with the full-sequence of the C-terminal showing the location of the three epitopes. A green arrow points to the location of Ser411 residue in epitope 2. **B - Reactivity of peptide specific extracted pools of IgG`s from anti-C serum to 3D7 lysate extracts**. The western blot in lane 1 shows the reactivity of anti-C IgG pool extracted by peptide sequence ^{415}LTNVDSI^{421}. The 42kDa seems to be stronger in signal than the 90kDa band. Lane 2 shows the reactivity of anti-C IgG pool extracted by peptide sequence ^{408}NEDSEGE^{214}. The 90kDa seems to be stronger in signal than the 42kDa band. Lane 3 shows reactivity of anti-C IgG pool extracted by peptide sequence ^{403}MRNEENE^{409}. The 42kDa seems to be stronger in signal than the 90kDa band. **C - Dissecting pool 2 IgG epitope.** The membrane shows the reactivity of pool 2 IgG`s to synthetic overlapping peptides of the sequences containing the Ser411 residue on the C-terminal of PfCRT in addition to two spots

containing peptides (21, 22) where Ser411 was substituted with alanine or aspartic acid respectively. Pool 2 IgG`s shows reactivity to peptides in spots 18, 19, 20 with a very weak reactivity with peptides in spot 21 of the substitution Ser411A and loss of reactivity in spot 22 when Ser411 was replaced with aspartic acid. **D - Reactivity of pool 2 IgG`s towards transiently transfected HEK-293F cells with wt-PfCRT compared to native PfCRT in 3D7.** The 3D7 lane shows the reactivity of pool 2 IgG`s to PfCRT in 3D7 lysate. The result shows a very weak signal at 42kDa. Wt-PfCRT transiently transfected HEK-293F cells shows strong reactivity to pool 2. Wt-PfCRT^{D411} and wt-PfCRT^{A411} transiently transfected HEK-293F cells shows weak reactivity to pool 2. Re-probing the membrane with anti-C shows strong signals in all lanes at 42kDa.

These results confirm the findings in figure 2C, showing the binding of pool 2 IgG to heptapeptides containing the Asp and Ala substitution dramatically affect the binding. The remaining signal of pool 2 IgG to PfCRT-Ser411Asp and PfCRT-Ser411Ala is likely due the weak binding of pool 2 IgG to sequences within the epitope beyond the effects of Ser411. When the membrane was re-probed with anti-C we retain very intense signals in all showing that the protein is expressed in great amounts and it's the substitutions made at residue Ser411 that affected the purified antibody binding (figure 2D).

Taken together, all the results so far suggest that: 1- The majority of the 42kDa PfCRT in the chloroquine sensitive strain 3D7 are phosphorylated at Ser411. 2- Phosphorylation or modification of residue Ser411 in PfCRT affects the ability of anti-Ser411 to bind the Ser411 epitope (i.e. ^{406}EENEDSEGE^{414}) probably due to steric hindrance. Furthermore, these results demonstrate the practical use of pool 2 IgG`s as a tool to study phosphorylation of residue Ser411 in PfCRT.

Native 42kDa PfCRT in CQR strain Dd2-H is more dephosphorylated than in CQS strain 3D7. To investigate the functional potential of residue Ser411 in PfCRT we sought to compare the status of residue Ser411 in PfCRT of chloroquine sensitive 3D7 to chloroquine resistant strain in Dd2-H. Figure 3 shows almost equal levels of expression of 42kDa PfCRT and 90kDa possible PfCRT dimer

in 3D7 and Dd2-H (lanes 1 and 2 respectively) using anti-C full-serum. Lane 4 shows a strong signal at 42kDa relative to the 42kDa in lane 3. This result shows a clear difference in the phospho-state of residue Ser411 between 3D7 and Dd2-H. Moreover, lanes 5 and 6 show almost equal signals at 42kDa PfCRT after membrane dephosphorylation with CAP. Although quite large, there does not seem to be a difference in the intensity of the 90kDa possible dimer band pre/post dephosphorylation between the two strains (lanes 3, 4, 5 and 6) relative to the C-terminal full-serum signals (lanes 1 and 2). Taken together, the results show that there is a difference in the phosphorylation state of residue Ser411 between chloroquine sensitive strain 3D7 and chloroquine resistant strain Dd2-H.

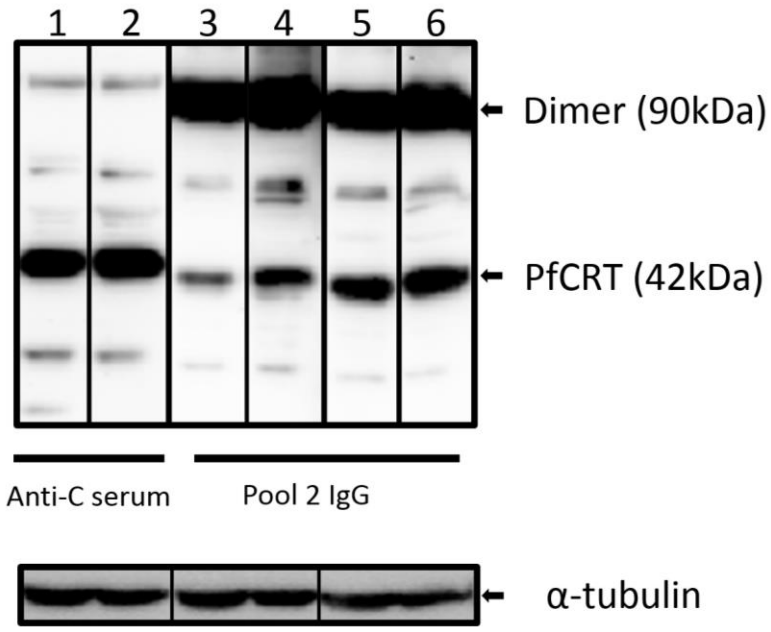

Figure 3. Reactivity of pool2 IgG`s to PfCRT in chloroquine sensitive 3D7 and chloroquine resistant Dd2-H. Lanes 1 and 2 show the reactivity of anti-C to PfCRT in 3D7 and Dd2-H lysates respectively. In both lanes, anti-C full-serum shows a very strong signal with the 42kDa and a weak signal with the 90kDa polypeptide. Lanes 3 and 4 show the reactivity of pool 2 to PfCRT in 3D7 and Dd2-H lysates respectively without CAP treatment. The results show a weaker 42kDa signal in 3D7 compared to a stronger signal of 42kDa in Dd2-H. The 90kDa shows a very strong signal relative to the 42kDa band in both. Lanes 5 and 6 show the reactivity of pool 2 to PfCRT in

3D7 and Dd2-H lysates respectively post-CAP treatment. The results show an almost equally strong 42kDa signal in both lanes relative to the 42kDa bands without CAP treatment in lanes 3 and 4. The 90kDa shows a very strong signal relative to the 42kDa band in both. Anti-tubulin reactivity was used as a loading control.

Moreover, membrane dephosphorylation experiments, showing that there was a phosphorylated population of 42kDa PfCRT (lanes 5 and 6) relative to the dephosphorylated population of the same band (lanes 3 and 4) implies that there are continuous molecular switch events occurring to regulate a certain function that involves residue Ser411.

Also, the presence of phosphorylated and dephosphorylated PfCRT on Ser411 between chloroquine sensitive and resistant strains may imply a role for Ser411 in modulating chloroquine drug resistance.

Potential PfCRT homodimer 90kDa polypeptide is localized outside the DV in *P. falciparum*. In 3D7 lysates, PfCRT anti-C-serum shows a more intense signal at 42kDa compared to the 90kDa band (figure 1). On the other hand, pool 2 IgG purified from the latter antiserum shows stronger reactivity towards the 90kDa homodimer polypeptide than the monomer 42kDa polypeptide, due to differential phosphorylation, whereby the 90kDa polypeptide contains an un-phosphorylated Ser411, while the 42kDa polypeptide contains phosphorylated Ser411. To determine the *in-vitro* subcellular distribution of the monomer and homodimer polypeptides, parasites were fixed, permeabilized and the stained with pool 2 IgG or PfCRT anti-C serum, then visualized by confocal microscopy (figure 3). The results in figure 4 panels A, B, C and D describe 3D7 parasites probed with anti-C serum while panels E, F, G and H describe 3D7 parasites probed with pool 2 IgG.

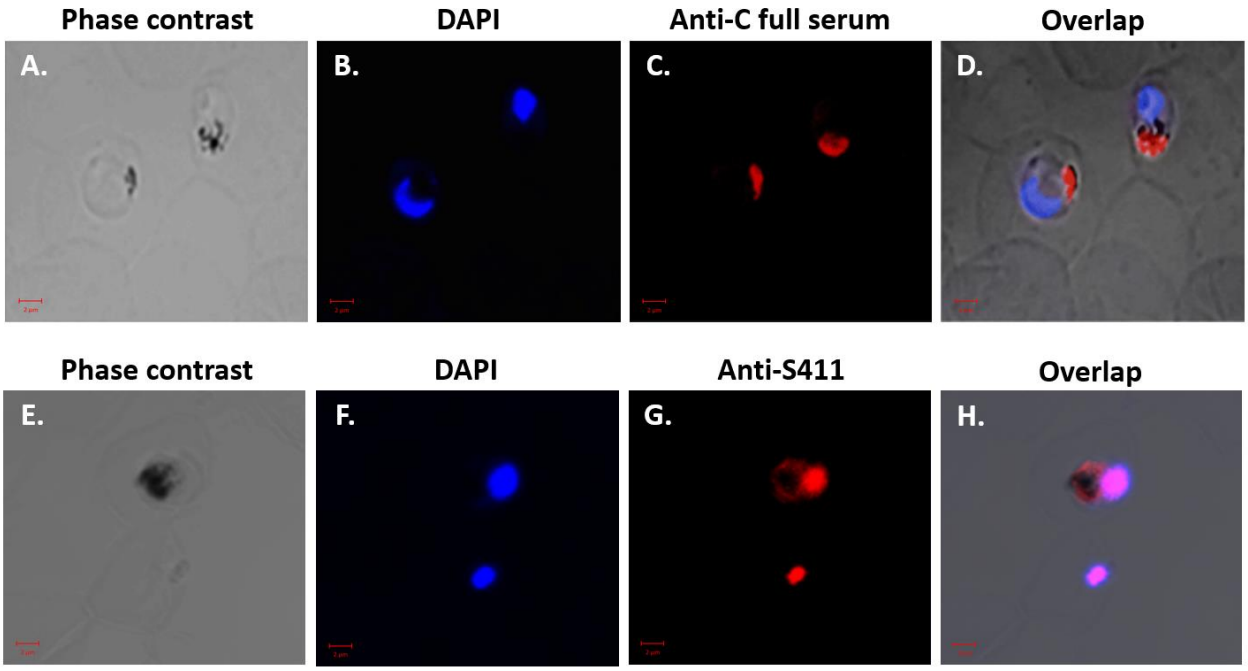

**Figure 4. IFA on 3D7 parasites: anti-C (A, B, C and D) [15] and pool 2 IgG`s (E, F, G and H).** Images A and E show phase contrast. Images B and F nucleus staining with DAPI. Image C shows staining with anti-C and image G shows staining with pool 2 IgG`s. Images D and H show the overlay of DAPI and the PfCRT antibodies. Scale bar reads 2µm.

Moreover, anti-C serum shows red signals (due to 2nd antibody Alexa-594) arising from the DV where PfCRT 42kDa is known to localise [1], marked by the dark hemozoin crystals in the organelle, while DAPI shows a nuclear signal in blue. Interestingly, pool 2 IgG shows a weak red ring-like signal originating from the DV (panels G and H) in addition to a very intense red signal, outside the DV but looks to be around the vicinity of the DAPI stained nucleus (panels F and H) and likely to be an un-identified vacuole.

A strong correlation was observed between the IFA signal intensity and localisation versus their intensities on a Western blot. Meaning, the 42kDa PfCRT seems to be the form localised to the DV membrane because PfCRT anti-C-serum shows very strong 42kDa signal by Western blotting and a very weak signal for the 90kDa band. In support of the latter, pool 2 IgG shows a much

weaker signal from the DV in IFA similar to its image on the Western blot where the 42kDa signal is very weak as well. A similar observation was seen with the 90kDa suggested PfCRT dimer where it seems to be localised outside the DV and was only seen with the purified and concentrated pool 2 IgG and not seen with anti-C serum because there was not enough antibody units in the amount used for IFA to show a localisation. The possibility of a dimer form of PfCRT being expressed outside the DV was reported to the extent of a molecular weight band not seen in isolated DV`s [4]. Bioinformatics analysis of PfCRT suggested that PfCRT may form a dimer as a member of the ligand-metabolite membrane transporter family [16]. One may speculate that the availability of a dimer pool of PfCRT represents an inactive PfCRT that can be easily recruited to the DV via specific phosphorylation (in this case at Ser411). Consistent with the latter speculation, two pools of glucose transporters are normally present either on the cell surface and/ in internalized storage vesicles. Insulin-mediated kinase signaling leads to the trafficking of the intracellular glucose transporters to the cell surface to allow for increased import of glucose [17, 18].

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Conflicts of interest - There are no conflict of interest from any of the authors.

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Connecting statement 3

In previous chapters we looked at PfCRT in the parasite and we saw that PfCRT was clearly involved in CQR and we have characterized the tools to study this protein. Exploring the normal substrate(s) and normal function(s) of this protein is a formidable task in the parasite. Therefore in this final chapter of thesis we demonstrate the functional expression of PfCRT in HEK-293F cells and show that a specific amino acid mutation inhibit its function.

Chapter 4

Manuscript III

Substitution of Pro165 in transmembrane 4 of the chloroquine resistance transporter PfCRT abolishes lysosome acidification function in stably transfected HEK-293F Cells

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Running title: Pro165 in mutant-PfCRT is essential for lysosome acidification of HEK-293F cells.

Keywords: *Plasmodium falciparum*; PfCRT; heterologous expression; lysosome-acidification; malaria.

Abstract

Mutations in the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) are the primary determinants of chloroquine resistance. PfCRT is localised to the parasite digestive vacuole, an organelle that maintains an acidic lumen. Previous attempts to study PfCRT transport function in HEK-293 cells demonstrated the localization of PfCRT to the lysosomal membrane and an increased acidification of this organelle in HEK-293 cells transiently expressing a mutant form of the protein. However, it was not possible to conclude from this earlier study if PfCRT directly caused lysosomal acidification. In this report, we demonstrate the isolation of HEK-293F cells stably expressing wild-type 3D7 PfCRT^{NKAQ} and mutant-PfCRT (N75E, K76T, A220S and Q271E), mut-PfCRT^{ETSE}, with PfCRT localized to lysosomal membrane. Moreover, cells expressing mut-PfCRT^{ETSE} showed greater lysosomal acidification as demonstrated by the significant increase in the accumulation of two weak bases, acridine orange (AO) and CytiPainter LysoOrange Indicator reagent (LO) dyes, relative to HEK-293F cells expressing wild-type 3D7 PfCRT^{NKAQ}. Moreover, using mut-PfCRT^{ETSE} with a single proline mutation, Pro165Ala (mut-P165A-PfCRT^{ETSE}), in transmembrane 4 of PfCRT completely inhibited the accumulation of AO and LO dyes in HEK-293F cells stably expressing mut-PfCRT^{ETSE}. Taken together, our results demonstrate a direct role for mut-PfCRT^{ETSE} in lysosomal acidification and show for the first time a functional role for Pro165 in transmembrane 4 of PfCRT.

Introduction

The human malaria disease is a major public health problem. *Plasmodium falciparum* (*P. falciparum*) accounts for the majority of the total malaria cases according to the World Malaria Report 2019 [1]. The progression of treatment from quinine to chloroquine (CQ) reversed the fates of many people infected with this lethal form of malaria, giving hope to populations for approximately 20 years [2]. Eventually, CQ resistant parasites emerged whereby the mechanism of CQ resistance was thought to be mainly but not exclusively due to mutations in CQ resistant protein *Plasmodium falciparum* Chloroquine Resistant Transporter (PfCRT) [3]. Although several mutations have been identified in CQ resistant isolates of PfCRT [4], a key causative mutation of lysine to threonine at position 76 has been consistently found in CQ resistant strains [5]. PfCRT encodes a 424 amino acid protein with 10 transmembrane (TM) helices localized to the membrane of the digestive vacuole (DV) in the parasite with both of its N- and C-termini in the parasite cytosol [6]. Using single-particle cryo-electron microscopy, the three-dimensional structure of PfCRT, encoding 7G8-variant protein expressed in HEK-293 (human embryonic kidney cells) cells complexed with epitope-specific Fab fragment, was resolved to a resolution of 3.2Å [7, 8]. PfCRT structure revealed 8 TM (TMs 1-4 and 6-9) helices forming the central cavity with a negative net charge, maximum diameter of 25Å and resistance causative mutations localized to TM helices of the central cavity [7]. Moreover, it was suggested that residues in TMs 3 and 4 may contribute to the open and closed conformations of the vacuolar side, while residues in TMs 8 and 9 may play the same role on the opposite side of the membrane in a rocker-switch possible motion [9].

The normal function(s) and substrate(s) of PfCRT are presently a matter of speculation. Efforts to establish PfCRT null *P. falciparum* parasites were unsuccessful suggesting that PfCRT is essential for the survival of the parasite [8]. In addition, studies of drug transport using parasitized red blood cells have proven difficult due to the complex kinetics of drug transport across several biologic membranes. Hence, attempts to establish heterologous expression systems of PfCRT in yeast, *Xenopus laevis* oocytes and mammalian cells have been undertaken over the past several years [10, 11]. PfCRT expression in *Xenopus laevis* oocytes to study drug transport is currently used as a surrogate system to study PfCRT drug transport, in spite of several disadvantages [10]. Moreover, efforts to express yeast codon optimized PfCRT, with tags, transiently in mammalian cells (e.g., HEK-293) have been demonstrated previously, however, PfCRT did not transport CQ [11]. Interestingly, PfCRT localized to the lysosomal membrane in HEK-293 cells, an organelle similar to the DV of the parasite [12, 13], was shown to cause higher levels of lysosomal acidification relative to cells expressing wild-type (wt) PfCRT. However, given the transient nature of PfCRT expression in HEK-293 cells and the absence of CQ transport it was not clear if the observed increase in lysosomal acidification was due to the expression of mutant-PfCRT on the lysosome or due to the activation of an endogenous transporter, as previously suggested [10].

To address the validity of the HEK-293 heterologous expression system to study PfCRT normal and mutant functions, it was of interest to select HEK-293F that stably express wild-type and mutant-PfCRT, together with additional mutations that inactivate its function. Therefore, in this report we examined the effects of the minimum number of point mutations described earlier [4] to confer a CQ resistance phenotype on PfCRT (e.g. mut-PfCRT^{ETSE}). In addition, and in an effort to modulate mutant-PfCRT function, we substituted highly conserved proline residues (Pro165

and Pro354) in TM domains (TMD) 4 and 9 with an alanine. The rationale for substituting the two proline residues (Pro165 and Pro354) in TMD 4 and 9 was based on several factors: a) the recent high resolution PfCRT 3D structure predicted TMDs 3-4 and 8-9 to play a role in open-close conformations of PfCRT [9]; b) the proline residues in TMD 4 and 9 are conserved in all PfCRT orthologues [6, 7]; and c) proline residues in TMDs are helix breakers, and as such can modulate PfCRT substrate specificity and/or functions [14, 15]. In this report, we demonstrate the isolation of HEK-293F cells stably expressing full-length wild-type (3D7) and mutants of PfCRT. Moreover, we show for the first time the effects of specific mutations in PfCRT on its *in-situ* lysosomal acidification function.

Material methods

Cloning of codon optimized PfCRT mutants - Wild-type PfCRT (3D7) encoding the CQ sensitive gene sequence obtained from plasmo DB (gene ID PF3D7_1133400) was human codon optimised and commercially synthesized (Genscript®, USA). Human codon optimized full-length PfCRT was excised from pUC57 vector and re-ligated into pCDNA3.1+ (Invitrogen™) at restriction sites BamHI and EcoRI (New England Biolabs Inc.; BamHI-HF cat: R3136S and EcoRI-HF: R3101S) with a Kozak consensus sequence included. PfCRT gene encoding mutations that confer CQ resistance (mut-PfCRT^{ETSE}), as described earlier by Summers *et al*. [4]; D17 clone or variant) was constructed by ligating 3 fragments, amplified using the Phusion High-Fidelity DNA Polymerase (Life Technologies™; cat: F-530S), possessing 4 point mutations (N75E, K76T, A220S & Q271E) into the humanised 3D7-PfCRT (wt-PfCRT^{NKAQ}) gene sequence using the Gibson assembly approach [16]. Primers and reaction conditions are provided in table 1 of the supplementary part (supplementary information for manuscript III). The latter approach was also used to introduce

a mutation at position Pro165 to Ala (P165A) of mut-PfCRT^{ETSE} gene (or mut-P165A-PfCRT^{ETSE}). Primers and reaction conditions are provided in table 2 of the supplementary part (supplementary information for manuscript III). All generated plasmids were inoculated in TOP10 chemically competent cells and confirmed by sequencing (Genome Quebec, Montreal, CA) then stored at -80°C.

Tissue culture and selection of stable transfectants - HEK-293F cells were cultivated and maintained in DMEM culture media (Gibco™, cat: 11965118) containing 10% (v/v) fetal bovine serum (FBS) in a 37°C incubator with environmental O_2 and 5% CO_2. Cells were grown to 70-80% confluency prior to transfection. Transfections, using pCDNA3.1+ empty plasmid, full-length wt-PfCRT^{NKAQ}, mut-PfCRT^{ETSE}, mut-P165A-PfCRT^{ETSE} and mut-P354A-PfCRT^{ETSE} containing plasmids, all were linearized with MfeI restriction enzyme (New England Biolabs Inc.; cat: R3589S) and transfected into HEK-293F cells using Lipofectamine 2000 (Invitrogen™; Ref: 11668-030), following manufacturer instructions. Clones from several transfections were selected in 800 µg/ml G418 (Gibco™; cat: 11811031) in 10 % (v/v) FBS DMEM (Gibco™; cat: 11965118) and allowed to proliferate for several weeks prior to freezing aliquots from each clone in liquid nitrogen. Two clones for wt-PfCRT^{NKAQ} (clone 2 and 4), two clones for mut-PfCRT^{ETSE} (clones 8 and 9) and one clone for the mut-P165A-PfCRT^{ETSE} were isolated and allowed to proliferate continuously in the presence of G418 prior to characterization for PfCRT (wt or mutant) expression and drug transport studies. Unfortunately, it was not possible to obtain clones for mut-P354A-PfCRT^{ETSE}, after many transfection and selection attempts.

Protein extraction and western blotting - For each clone of HEK-293F, 3 million cells were washed in PBS and extracted in RIPA buffer (50 mM Tris HCL pH 7.4, 1% (v/v) NP40, 0.25% (w/v) sodium deoxycholate in the presence of protease inhibitors). The protein extracts were resolved on 10% SDS-PAGE [17] and transferred on PVDF membranes [18]. Membranes were probed for PfCRT expression using rabbit anti-serum raised against PfCRT N-terminal sequence (1-58 polypeptide) at 1:6000 (v/v) dilution (Baakdah and Georges, unpublished results). All PVDF membranes were also probed with anti-tubulin monoclonal antibody (1 µg/ml) for equal protein loading. Following the latter overnight incubation at 4˚C with primary antibodies, PVDF membranes were washed 4 times with PBS and incubated with goat anti-rabbit or goat anti-mouse HRP conjugated secondary antibodies (1:3000 (v/v) dilution in 3% (w/v) milk/PBS) for 4hrs at room temperature (RT). Following 5 washes with PBS, the membranes were developed with Thermo Scientific™ SuperSignal™ West Pico Chemiluminescent Substrate (cat: 34087) and images were captured using ChemiDoc imaging system from BIO-RAD Inc.

Indirect immunofluorescence assay - Cells were cultivated in tissue culture onto poly L-lysine coated cover slips. The cover slips containing cells were washed with PBS and fixed with 4% paraformaldehyde (Electron microscopy sciences; cat: 15710-S) in PBS for 30min at RT. Fixed cells were washed once in PBS then quenched with 0.15% (w/v) glycine in PBS for 10min at RT. Cells were washed in PBS then permeabilized with 0.1% (v/v) TritonX-100 (Bio Basic Inc.; cat: C34H62O11) in PBS for 10min at RT. Permeabilized cells were washed three times with PBS, then blocked with 1% (v/v) goat serum in PBS for 1hr at 4°C. Cells were then incubated with anti-PfCRT and/or anti-sirtuin1 or anti-LAMP1 monoclonal antibodies (anti-sirtuin1 and anti-LAMP1 monoclonal antibodies; cat: PCRP-SIRT1-1E11, G1/139/5, respectively were obtained from the

Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at the University of Iowa, Department of Biology, Iowa City, IA 52242) at 4°C overnight followed by washing in PBS and probing with secondary antibody Alexa-fluor 594-conjugated goat anti-rabbit (Life Technologies™) for anti-PfCRT and Alexa-fluor 488-conjugated goat anti-mouse for the monoclonal primary antibodies for 45min. The cover slips were washed three times in PBS, dipped once in distilled water, air dried and mounted on microscope slides using fluoromount G mounting medium (Southern Biotech; cat: 0100-01). Images were captured using confocal microscopy (Carl Zeiss GmbH, Jena, Germany).

Dye accumulation assay - Cells were detached using 0.25% (w/v) trypsin in 0.5mM (w/v) EDTA solution. A total of 3 X 10^6 cells, in two sets for each cell line, were washed in HEPES-Hanks Balanced Salt Solution (HBSS) buffer (20mM (w/v) HEPES pH 7.2 (BioShop Inc.; cat: HEP005.500) and 1 X HBSS (Life Technologies™; cat: 14065056), filter sterilized and preheated at 37°C). One set was re-suspended in 1ml 30mM NH_4Cl HHBS buffer pH 7.2, while the second set was re-suspended in 1ml HHBS alone, and were both placed for 30minutes at 37°C. The two dyes (acridine orange (AO) Invitrogen™ cat: A1301 or CytiPainter LysoOrange (LO) Indicator reagent, abcam® cat: ab17682) were added to cells (4µM for AO and 2units/ml for LO), and incubated away from light in the incubator at environmental O_2 and 5% CO_2 while inverting the tubes every 10min to keep cells in suspension. After one hour, tubes were spun at 900rpm in a swing bucket rotor for 5min at 37°C. The resulting cell pellets of set 1 were re-suspended in 10ml 30mM NH_4Cl/HHBS buffer and set 2 were re-suspended in 10ml HHBS buffer for 1hr at 37°C incubator with environmental O_2 and 5% CO_2 while inverting the tubes every 10min. Following 60 minutes incubation, cells were spun at 900rpm in a swing bucket rotor for 5min at 37°C. The supernatant

was removed and pellets of set 1 were re-suspended in 1ml 30mM NH_4Cl/HHBS buffer; set 2 were re-suspended in 1ml HHBS buffer and spun down again; this step was repeated one more time. The final cell pellet was re-suspend in 1ml wash buffers and aliquoted into a round bottom black 96 well plate. Florescence was measured using Synergy H4 hybrid spectrophotometer from BioTek® and Gen5™ microplate reader and image software, according to the dyes respective wavelengths (AO E_X 485/E_m 530 and LO E_X 542/E_m 565). Cells were checked for viability at the end of the experiment with trypan blue. The plotted graphs represent values adjusted for PfCRT expression and equal loading based on tubulin levels minus dye accumulation in vector transfected HEK-293F cells.

Homology Modeling - The SWISS-MODEL tool [19] was used to generate the PDB file for mut-$PfCRT^{ETSE}$-Pro165A- using model-template PDB 6ukj [7] and the homology models were built and edited in PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.

Statistical analysis - Plotted results were analysed by one way ANOVA using GraphPad Prism version 8.4.0 (671).

Results and discussion

PfCRT-mediated drug transport studies in *P. falciparum* have been hampered by the complexity of measuring meaningful drug transport across several membranes in infected erythrocytes. Hence, efforts to establish a simple *in-situ* heterologous expression system for PfCRT in HEK-293F cells stably expressing wild-type and mutant forms of PfCRT would greatly facilitate PfCRT structure-functions studies. Figure 1 shows the amino acid sequences of 3D7 background wild-type PfCRT (encoding N75, K76, A220 & Q217 or wt-$PfCRT^{NKAQ}$), mutant-PfCRT encoding four

mutations shown previously to be sufficient to mimic resistance to CQ (N75E, K76T, A220S & Q217E; or mut-PfCRT^{ETSE}; [4]) and mutant-PfCRT^{ETSE} containing an additional proline to alanine mutation at position 165 (mut-P165A-PfCRT^{ETSE}).

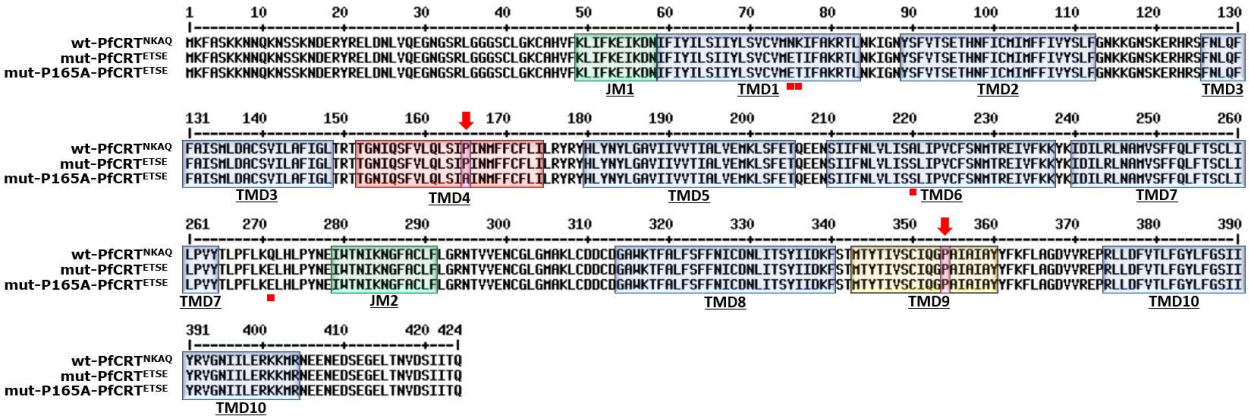

Figure 1. Sequence alignment of wild-type and mutant PfCRT amino acid sequences. The three polypeptide sequence alignment represent the full-length amino acid sequence of wild-type 3D7 PfCRT (wt-PfCRT^{NKAQ}), mutant-PfCRT (mut-PfCRT^{ETSE}) and Pro165Ala substituted mutant-PfCRT (mut-P165A-PfCRT^{ETSE}). The small red squares point to the positions of amino acid substitutions in each of the full-length sequences of PfCRT polypeptides. The blue colored rectangles show TMDs 1, 2, 3, 5, 6, 7, 8 and 10. The green colored rectangles show the juxtamembrane helices. The red and yellow colored rectangles show the positions of TMD 4 and 9, respectively. The red arrows point down to pink blocks showing the location of the proline residues and their substitution by alanine in TMD4. PfCRT amino acid sequences are shown using a single letter code.

Human codon optimized full-length constructs of wt- PfCRT^{NKAQ}, mut-PfCRT^{ETSE} or mut-P165A-PfCRT^{ETSE} were linearized and transfected into HEK-293F cells. Clones were selected with 800µg/ml G418 over a course of approximately 4 weeks, allowed to proliferate and extracted for protein analysis. Figure 2 shows Western blot results of HEK-293F cells extracts from cells transfected with empty plasmid (Vc1), wt-PfCRT^{NKAQ} (clones C2 and C4), mut-PfCRT^{ETSE} (clones C8 and C9) and mut-P165A-PfCRT^{ETSE} probed with PfCRT N-terminal-specific antibody. The results in lanes 2-5 of figure 2 show a 42kDa polypeptide encoding wt-PfCRT^{NKAQ}, mut-PfCRT^{ETSE} and mut-

P165A-PfCRT^{ETSE} to varying levels. Interestingly, consistently, mut-PfCRT^{ETSE} clones expressed significantly less protein than wt-PfCRT^{NKAQ} (lanes 2-3 versus 4-5).

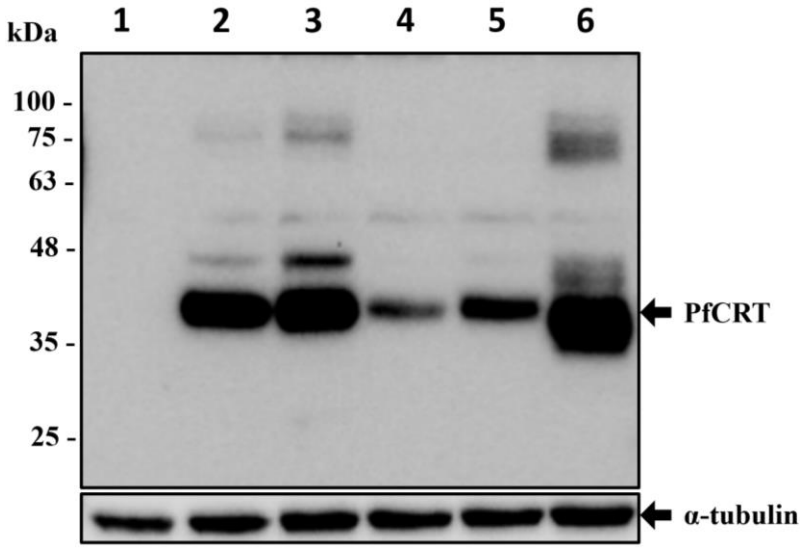

Figure 2. Stable expression of wild-type and mutant-PfCRT in HEK-293F cells. Cell lysates from HEK-293F cells expressing vector transfected (Vc1; lane 1), wt-PfCRT^{NKAQ} (clones C2 and C4; lanes 2 and 3), mut-PfCRT^{ETSE} (clones C8 and C9; lanes 4 and 5) and mut-P165A-PfCRT^{ETSE} (lane 6) were resolved on 10% SDS-PAGE, transferred to PVDF membrane and probed with anti-PfCRT and tubulin antibodies. PfCRT migrating with an apparent molecular mass of 42kDa was seen, to varying levels, in all HEK-293F transfectants (lanes 2-6), except in vector transfected HEK-293F (Vc1; lane 1). The tubulin polypeptide bands, loading control, is detected in all samples (lanes 1-6). The position of wild-type and mutant-PfCRT polypeptides is identified by the red arrow to the right of the figure. The migration of the molecular weight markers are indicated to the left of the figure.

Moreover, mut-P165A-PfCRT^{ETSE} consistently migrates slightly faster on SDS-PAGE than wt-PfCRT^{NKAQ} or mut-P165A-PfCRT^{ETSE} (lanes 2-5 versus 6). For wt-PfCRT^{NKAQ} and mut-PfCRT^{ETSE}, clones 4 and 9 show higher levels of protein expression than clones 2 and 8, respectively. In addition, lanes 2, 3 and 6 show additional polypeptides migrating with molecular masses of ~52kDa and ~90kDa with varying intensities consistent with 42kDa band intensity suggesting that they represent possible post-translationally modified PfCRT (e.g. 52kDa) or homodimer PfCRT (e.g. 90kDa). The latter speculation, is strengthened further in view of the fact that both the

52kDa and 90kDa polypeptides in lanes 2 and 3 (wt-PfCRT^{NKAQ}) appear to migrate slightly faster in lane 6 (mut-P165A-PfCRT^{ETSE}) mimicking the slight increase in mobility of the 42kDa polypeptide. The significance of the 52kDa and 90kDa PfCRT proteins is presently not clear, but appear to be present at lower levels than the 42kDa form of PfCRT, consistent with presence of a faint 52kDa band in lane 4 (clone C9 of mut-PfCRT^{ETSE}).

Previous heterologous expression of wild-type HB3 and Dd2 mutant-PfCRT into HEK-293 cells showed PfCRT, full-length and N-terminal truncated polypeptide, to localize to lysosomes, equivalent organelles to the DV in *P. falciparum* [11]. To determine the subcellular localization of PfCRT in HEK-293F cells stably expressing wt-PfCRT^{NKAQ} and mut-PfCRT^{ETSE}, cells were subjected to double immunofluorescence staining (IFA) with anti-PfCRT and anti-LAMP1 (established lysosomal protein [20] or anti-sirtuin 1 (a nuclear protein [21]) antibodies. Figure 3 shows punctate staining with anti-PfCRT and anti-LAMP1 in the cytosol and outside the cell nuclei that perfectly overlap. By contrast, and as expected, anti-sirtuin 1 stained cell nuclei and did not overlap with anti-PfCRT staining (fig. 3). Similar results were observed for mut-PfCRT^{ETSE} and mut-P165A-PfCRT^{ETSE} (results not shown). These results are in keeping with previously published report of PfCRT transiently expressed in HEK-293 cells [11] confirming the lysosomal localization in cells stably expressing wild-type and mutant-PfCRT's. In light of the above results, it was interesting that PfCRT expression in yeast and *Xenopus laevis* oocytes the protein was localized to the plasma membrane [10, 22]. The differences in PfCRT intracellular trafficking to lysosomal (or DV) versus plasma membranes in yeast and *Xenopus laevis* oocytes versus the *P. falciparum* and HEK-293F cells is not entirely clear, but possibly due to the absence of specific associated proteins in the different systems or differential post-translational modifications. It was previously

suggested that phosphorylation of threonine 416 in PfCRT C-terminal may play a role as sorting signal to the DV membrane [23]. The development of a functional expression system that stably expresses PfCRT could greatly expand our abilities to study PfCRT structure-function.

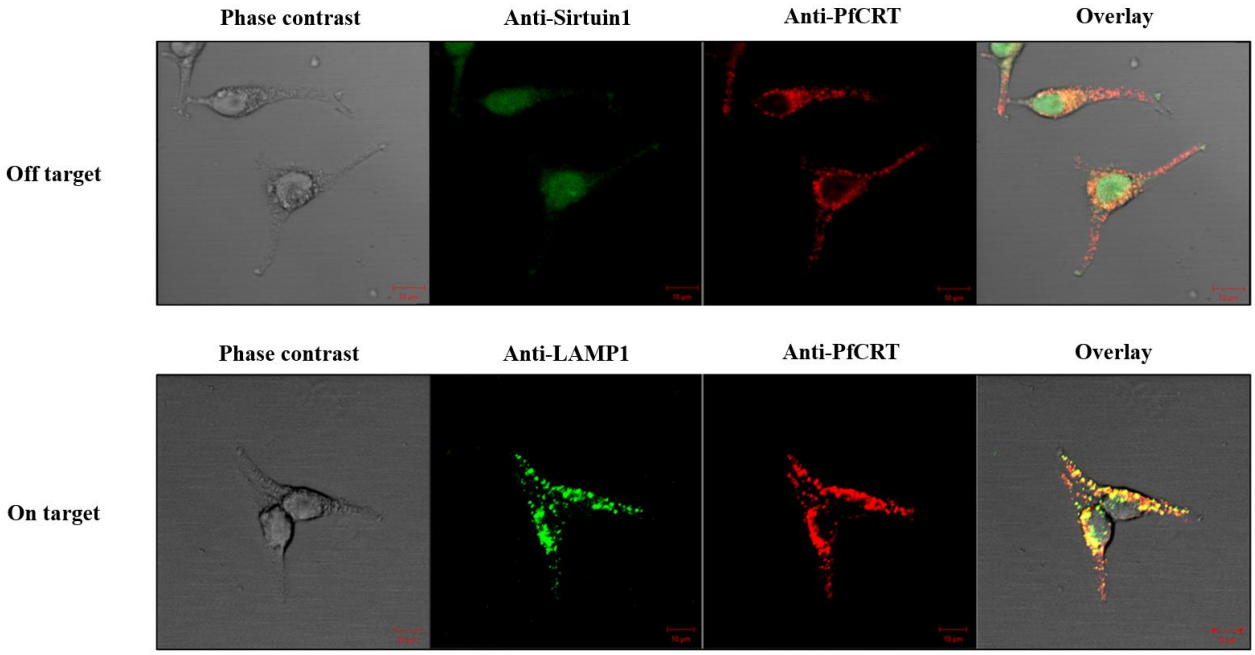

Figure 3. Localization of PfCRT in HEK-293F cells. PfCRT-HEK clones were prepared for IFA and images were taken using confocal microscopy. The off target panel shows PfCRT does not localise to the nucleus as PfCRT signal does not overlap with anti-sirtuin1. However, the on target panel shows PfCRT staining adopting a similar pattern as anti-LAMP1 suggesting a lysosomal localisation. Scale bar reads 10µm.

Given earlier findings [11] relating to increased lysosomal acidification in HEK-293 cells transiently expressing mutant Dd2 PfCRT, we made use of two lysosome fluorescent dyes (i.e. AO and LO) to validate the function of mutant-PfCRT as an enhancer of lysosome acidification [24]. The results in figure 4A shows the relative accumulation of AO in HEK-293F cells stably transfected with wt-PfCRT^{NKAQ} (clones C2 and C4) and mut-PfCRT^{ETSE} (clones C8 and C9) relative to vector transfected cells adjusted to PfCRT protein expression levels (more information on adjustment is provided in supplementary information for manuscript III; Equating the expression

levels of PfCRT clones). The results of figure 4A show a dramatic increase in AO accumulation in clones C8 and C9 expressing mut-PfCRT^{ETSE} relative to clones C2 and C4 which express wt-PfCRT^{NKAQ}. This increased AO accumulation seen in clones C8 and C9 is likely due to the enhanced acidification of the lysosomal vesicles by mut-PfCRT^{ETSE} (fig. 4A). Moreover, short-term pre-incubation of cells expressing both wt-PfCRT^{NKAQ} and mut-PfCRT^{ETSE} in 30mM NH_4Cl completely abolished the accumulation of AO in all cells relative to vector transfected HEK-293F cells, confirming the role of differential proton gradient on AO accumulation (fig. 4A).

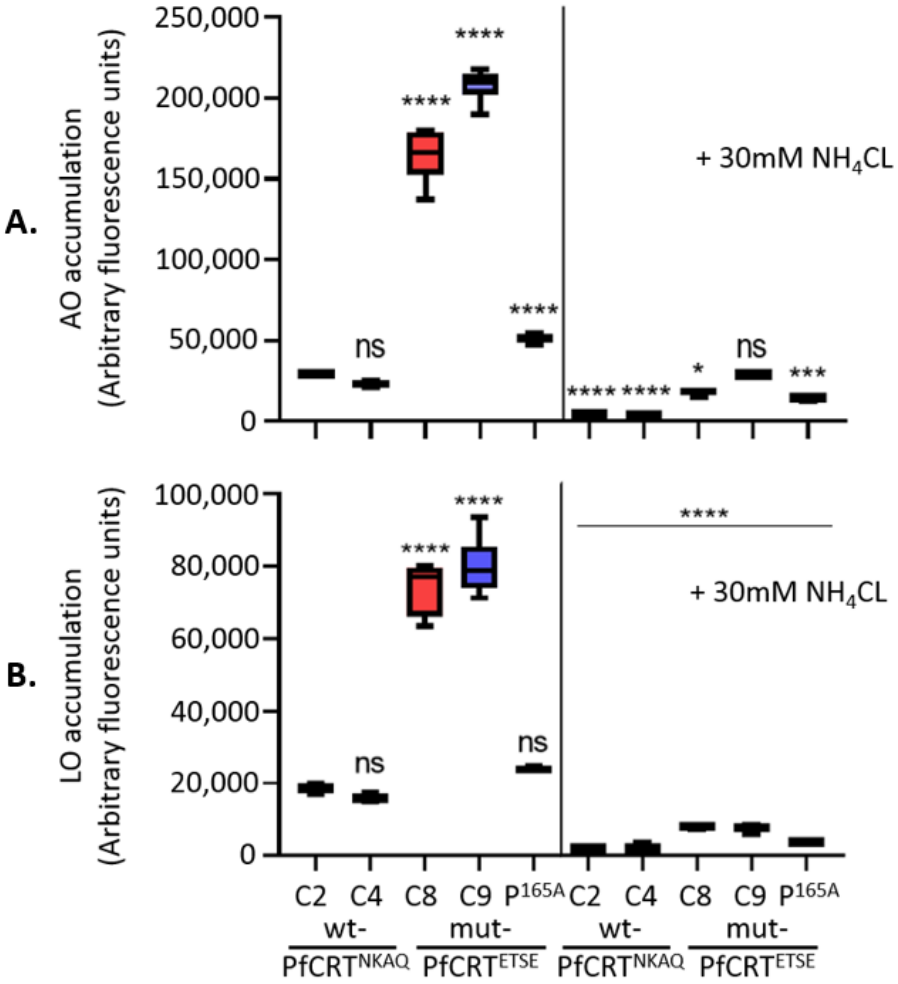

Figure 4. Accumulation of AO and LO in HEK-293F cells stably expressing wild-type and mutant-PfCRT. HEK-293F cells stably expressing wt-PfCRT^{NKAQ} (clones C2 and C4), mut-PfCRT^{ETSE} (clones C8 and C9) and mut-P165A-PfCRT^{ETSE} were incubated with AO (Panel A) or LO (Panel B) without

and with 30mM NH_4Cl. The relative accumulation of AO and LO in arbitrary fluorescence units is shown on the Y-axis. Results represent experiments three independent repeats done in triplicates. Statistical significance (****P value <0.0001) was analysed by one way ANOVA using GraphPad Prism.

Similar results were also seen using a different lysosome targeting dye, LO (fig. 4B), confirming the effect of mut-PfCRT^{ETSE} on the acidification of lysosomes in HEK-293F cells (clones C8 and C9), relative to wt-PfCRT^{NKAQ} expressing clones (C2 and C4). The mutations in PfCRT selected for this study (i.e. mut-PfCRT^{ETSE}) were the same mutations that increased the uptake of CQ in *Xenopus laevis* oocytes, but are fewer mutations than Dd2-PfCRT [4] used in an earlier study to demonstrate lysosomal acidification in HEK-293 cells [11]. Hence, the results of this study suggest a correlation between PfCRT ability to transport CQ into *Xenopus laevis* oocytes (which resembles CQ resistance as PfCRT's orientation on the DV membrane) and lysosome acidification in HEK-293 cells [4]. Similar results were observed in *Dictyostelium discoideum*, whereby mutant-PfCRT expelled CQ and caused a significant intravesicular acidification of the acidic vesicles [25].

Moreover, the results of this study are consistent with earlier findings using HEK-293 cells that transiently express wild-type HB3 and mutant Dd2 PfCRT with respect to the latter conferring increased lysosome acidification onto HEK-293 cells; it was not clear from this earlier study if lysosome acidification was mediated directly by Dd2 PfCRT [11]. To resolve this issue, it was of interest to introduce single point mutations in PfCRT that could specifically affect its function. To this end, two proline residues (Pro165 and Pro354) were identified as potential modulators of PfCRT function given that they are highly conserved in *Plasmodium* orthologues of PfCRT and their localization in TM helices 4 and 9 is likely to affect helical distortion and flexibility of these domains. In addition, proline residues in α-helical TMDs play structural and/or functional roles in polytopic membrane proteins whereby the geometry and the limited hydrogen bonding of this

residue introduce a molecular joint or hinge that could be important for function [14, 15, 26]. Therefore, proline residues in TM helices can have important structural and/or functional roles in membrane proteins. Substitution of proline by alanine, an amino acid with the highest helix propensity, contrary to proline, should have minimal effects on PfCRT structural integrity and would eliminate the possible molecular hinge introduced by proline [27]. The results in figure 4A and 4B show the effect of Pro165Ala on AO and LO accumulation in HEK-293 cells expressing mut-P165A-PfCRT^{ETSE}. Interestingly, substitution of Pro165 for Ala-165 in TMD4 of mut-PfCRT^{ETSE} completely abolished its lysosome acidification function to the same level as wt-PfCRT^{NKAQ} as measured by the accumulation of AO and LO in HEK-293F cells (fig. 4A and 4B, respectively). Moreover, treatment of cells with NH_4Cl, caused a further decrease in AO and LO accumulation, relative to control vector transfected HEK-293F cells (fig. 4A and 4B, respectively). Unfortunately, it was not possible to isolate stable transfectants of Pro354Ala-PfCRT. Although, it is not entirely clear how substituting Pro165 for Ala inhibits mut-PfCRT^{ETSE} ability to acidify the lysosomes, it was suggested that residues in TMD 3 and 4 may contribute to the open and close conformations at the vacuolar side of the DV, while residues in TMD 8 and 9 may play the same role on the opposite side of the membrane in a rocker-switch possible movement [9]. Based on the resolved structure of 7G8 mutant-PfCRT [7], Pro165 is localized midway of TM helix 4 facing outside of the pore cavity formed by TMDs 1-4 and 6-9, but in close contact with TM helix 5 (fig. 5). Similarly, P354 is also localized roughly midway of TM helix 9 facing outside pore cavity, and in close vicinity of TMD 10 (fig. 5). Together, it is likely TMD 4 and 9 affect the transport function of PfCRT through their interactions with TMD 5 and 10, respectively. It would be of interest to test the role of Pro165 substitution on the ability of mutant-PfCRT to confer resistance to CQ in the parasite.

Moreover, given that PfCRT continues to perform its normal function in CQ-resistant strains, it would be equally important to learn if substitution of Pro165 affects both the wt-type and mutant-PfCRT functions in the parasite, and consequently such a mutation would be lethal to the parasite, given that knock-down of PfCRT was shown to be essential for parasite survival [28]. PfCRT is a member of the drug/ metabolite transporter superfamily and may act as a proton-coupled transporter as other members of the family [6]. However, it was reported that parasite strains encoding CQ-resistant mutants of PfCRT allow leakage of protons from the DV [29]. The latter results are at odds with our findings and those by Reeves *et al.*, it is likely that in HEK-293 cells, mut-PfCRT^{ETSE} does not transport the same substrate as that from the parasite DV. Moreover, mut-PfCRT^{ETSE} in HEK-293F is does not seem to mediate the efflux of CQ from lysosomes (our results and [11]) and consequently, proton-leakage from the parasite DV appears to be associated with CQ-efflux. We speculate that mut-PfCRT^{ETSE} expressed in HEK-293F may not undergo similar post-translational modification to that in *P. falciparum* and this could affect its CQ-mediated drug resistance and transport. In line with the latter possibility is the recent report whereby substituting the N-terminal phospho-amino acid Ser-33 in Dd2 with alanine caused 50% reduction in CQ resistance; while substituting the same Ser-33 residue with phospho-mimetic amino acid aspartic acid reconstituted the level of CQ resistance [30].Thorough investigations should be conducted to explore the role of serine's in PfCRT function.

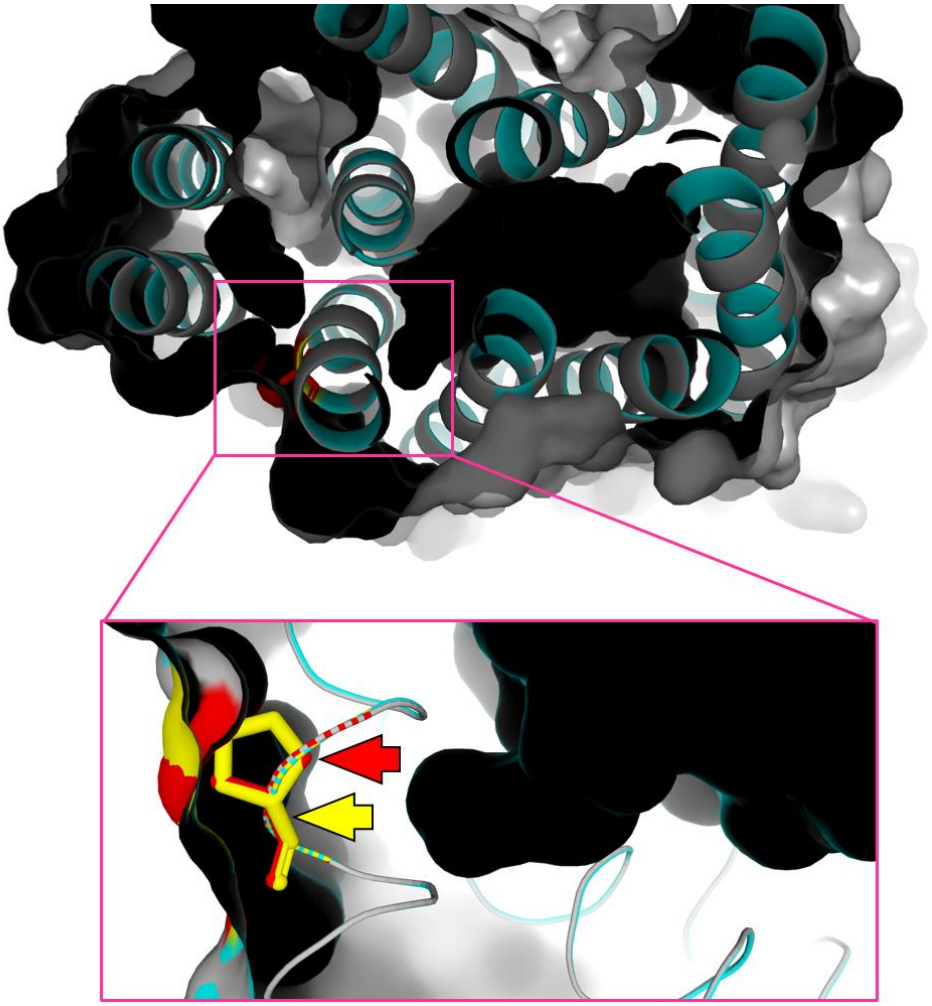

Figure 5. PfCRT homology modeling with P-165-A mutation in PfCRT. The homology modeling of PfCRT was done based on PfCRT-7G8-Pro165 TMD`s crystal structure (PDB 6UKJ). In blue are the TMDs of mut-PfCRT-7G8 overlapping with the gray TMDs of mut-P165A-PfCR^{ETSE}.In yellow is the proline (yellow arrow) and in red is the alanine (red arrow).

Acknowledgments

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Conflicts of interest - There are no conflict of interest from any of the authors.

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Supplementary information for manuscript III

Primer name	Primer sequence	Tm.	Amplification conditions
Dd2.muN75E.K76T.1vec.REV	CGCTTGGCGAAGATGGTCTCCATCACGCACACGCTCAGG	63.53	98°C ^{30sec} - 98°C$^{10\ Sec}$, 65°C^{30sec}, 72°C^{30sec})-72°C^{7min}
Dd2.muQ271E.1vec.FWD	CCCTGCCCTTCCTGAAGGAGCTGCACCTGCCCTACAACGAG	63.86	
Dd2.mu.3.ins.REV	AAACAAACTGGGATCAGGCTGCTGATCAGCACCAGGTTGAAG	62.94	
Dd2.mu.2.ins.FWD	GCGTGTGCGTGATGGAGACCATCTTCGCCAAGCGGACC	62.06	
Dd2.mu.2.ins.REV	GTTGTAGGGCAGGTGCAGCTCCTTCAGGAAGGGCAGGGTGTAC	62.88	
Dd2.mu.3.ins.FWD	CAACCTGGTGCTGATCAGCAGCCTGATCCCAGTTTGTTTTTCGAAC	61.78	

Table 1. Primers and reaction conditions for mut-PfCRT^{ETSE}.

Primer name	Primer sequence	Tm.	Amplification conditions
hu-PfCRT-P354.vec.FWD	TGAGCTGCATCCAGGGCCCCGCCATCGCCATCGCCTAC	75	98°C ^{30sec} - 98°C$^{10\ Sec}$, 65°C^{30sec}, 72°C^{30sec})-72°C^{7min}
hu-PfCRT.P165A.vec.REV	GCAGAAGAACATGTTGATGGCGATGCTCAGCTGCAGCACG	69.4	
hu-PfCRT.P354.ins.REV	GTAGGCGATGGCGATGGCGGGGCCCTGGATGCAGCTCAC	74.8	
hu-PfCRT.P165A.ins.FWD	GTGCTGCAGCTGAGCATCGCCATCAACATGTTCTTCTGCTTCCTGATC	69.7	

Table 2. Primers and reaction conditions for mut-P165A-PfCRT^{ETSE}.

Equating the expression levels of PfCRT clones. Our stable clones show varying degrees of PfCRT expression. So, in order to analyse the data and interpret the results, we equated the expression levels of PfCRT using the following method. All our western blots were probed with anti-N PfCRT for PfCRT protein detection and anti-tubulin as a loading control. The images obtained from the ChemiDoc imaging system BIO-RAD were quantified via Image Lab™ software. Each PfCRT value was divided by its own tubulin value to obtain a PfCRT: tubulin ratio. The highest ratio value was divided by all the PfCRT: tubulin ratios we obtained in the first step to obtain the difference in expression factor. The accumulation values, for AO or LO, obtained for each clone via Gen5™ microplate reader and image software were multiplied by their own difference in expression factor after subtracting the background and the average accumulation of Vc1 values from each one. These values were plotted and the statistical significance (****P value <0.0001) was analysed by one way ANOVA using GraphPad Prism version 8.4.0 (671).

General discussion and concluding remarks

The human malaria disease leads all current parasitic diseases in global death rates and available therapeutics are not sufficient for eradication [1-3]. Apicomplexan *P. falciparum* parasites cause the most severe form of malaria. It is surprising that such fragile parasites managed to cause a substantial toll on human life over the centuries given their reliance on delivery mechanics to make it into the blood stream of an individual. With no effective vaccine, prevention and treatment options rely heavily on insecticidal bed-nets and antimalarial drugs, respectively. The foremost obstacle to a malaria-free world is drug resistance. CQ was used for decades as the "gold standard" for treatment of uncomplicated malaria relieving the malaria burden in endemic areas especially the most vulnerable age groups [4]. Eventually, drug resistance sabotaged chloroquine`s success and is now considered inappropriate to treat *falciparum* caused malaria infections succeeded with front-line ACT [5, 6]. Likewise, resistance to ACT has emerged and is spreading rapidly from Cambodia to Africa [7, 8]. Consequently, the field is in dire need of new and effective antimalarials and appropriate strategies to combat the continuous resistant forms of the parasite.

Mutant-PfCRT is accepted as the primary determinant for CQ resistance by reducing CQ units in the DV [9, 10]. Previous work revealed that little modifications to CQ result in different drug-PfCRT relationships [11]. In the first manuscript we aimed to deceive mutant-PfCRT substrate specificity by modifying CQ at different positions to avoid being effluxed by mutant-PfCRT resistance mechanism, thus, sensitising the parasite. Novel CQ-derivatives were made and tested on CQS and CQR strains, and the results showed that modifications at 3rd position of the quinoline moiety rendered the compounds unworthy of further investigation due to much reduced effects

on CQR strains relative to CQ which is in keeping with published work, in the sense that modifications to the quinoline moiety are un-favoured [12-14]. Alternatively, some modifications made on the side-chain of CQ showed increased antimalarial activity towards CQR strains. Additionally, two side-chain modified CQ derivatives previously independently reported on, were of interest, AQ-13 and AQ-129 and as such were included in this work. The former is now going through several rounds of clinical trials while the latter was only screened without further investigation [15-18]. Here we have confirmed that AQ-13 and AQ-129 are more lethal than CQ towards CQR strains. Moreover, our interest in understanding the relationship between AQ-13 and AQ-129 to mutant-PfCRT showed that both were not reversed efficiently by VP relative to CQ which is contrary to previous results that indicated AQ-13 was not reversed by VP [18]. Using $C4^{Dd2}$ isogenic clones we showed that both AQ-13 and AQ-129 are poor substrates of mutant-PfCRT. We also studied into their relationship with hemozoin formation and our initial data suggest that both compounds can inhibit beta-hematin formation *in-vitro* like CQ. Unfortunately, we were un-successful in isolating Dd2-H AQ-13 and AQ-129 resistant clones which suggests either technical human error in trying to induce resistance or resistance is not easily achieved by the parasites to such compounds within the time limit the experiment was conducted in or the mutation to confer resistance was lethal to the parasites. All and all, this work dissected the relationship between two novel CQ derivatives to mutant-PfCRT. With a few more investigations on the role of other transporters that could be involved in the transport of such compounds in addition to extensive and thorough *in-vivo* testing we would have a better understanding on the workings of these compounds to implement better strategies to increase our limited arsenal of effective antimalarials.

Given the role of PfCRT in drug resistance it was of interest to study how the protein confers resistance to CQ. Here, in the second manuscript we dissected an anti-C terminal to PfCRT we raised against the full-length C-terminal domain because tools are only as good as their characterisation. Our high resolution mapping showed that PfCRT is phosphorylated in CQS strain 3D7 at position Ser411 monomer 42kDa form. This was interesting because it was recently reported that Ser33 on the N-terminal of PfCRT was a modulator of CQR [19]. This suggested that serine residues were important for PfCRT function. Our isolated anti-Ser411 antibodies detected that PfCRT exists in a possible dimer form and is de-phosphorylated at Ser411 relative to the monomer. Moreover, immunohistochemistry showed the dimer to be located away from the DV membrane by contrast to our understanding of the monomer localisation. Even more interesting, anti-Ser411 revealed that CQS strain 3D7 is more phosphorylated on Ser411 compared to CQR strain Dd2-H which suggests a possible role for Ser411 in CQR. It could be that PfCRT is made in dormant dimer forms and located elsewhere until needed to do certain molecular functions that involve transport which has been seen in other systems [20, 21]. This work revealed a new avenue on how CQ resistance could be conferred by resistant strains. As such it would be interesting to see the phosphorylation status of Ser411 using isolated anti-Ser411 post treatment of CQS and CQR strains with CQ.

The third manuscript was intended to explore the normal function of PfCRT. Because such a study was difficult to implement on the parasite itself, heterologous expression of PfCRT in mammalian HEK-293F cells was performed. This system was used previously and the authors suggested that transiently transfected PfCRT in HEK-293 cells could be responsible for acidification of the lysosome it was found to be expressed on [22]. Our study isolated several clones for wt-PfCRT^{NKAQ}

and mut-PfCRT^{ETSE} stably transfected in HEK-293F cells. We show that mut-PfCRT^{ETSE}, possessing only four mutations over wild-type, are better acidifiers than wt-PfCRT^{NKAQ} using pH sensitive dyes AO and LO. Moreover, our mut-P165A-PfCRT^{ETSE} shows that acidification was directly mediated through PfCRT which was not shown before and it seems that Pro165 is an essential amino acid for the function of PfCRT. Furthermore, substitution of Pro165 with alanine did not affect the TMD4 alpha helix spiral. This suggested that Pro165 is a structural residue that is probably important for the function of PfCRT, in this case, acidification of the lysosome in HEK-293F cells.

Taken together, the different studies and experiments displayed in this thesis unveiled important biochemical aspects about the CQ resistant transporter PfCRT. Chloroquine resistance is a complex of many mechanisms that involve many elements. PfCRT is but one piece of this huge network of interactions. Research in malaria drug resistance is probably a never ending process that requires a lot of hard work patience, dedication, team work and paying attention to detail. Our duty is to analyse the problems and find effective solutions and strategies to help relieve the suffering and maintain the wellbeing of people.

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