DISSERTATION

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Characterisation
of the drug transport properties of the
Plasmodium falciparum
chloroquine resistance transporter
through expression
in *Xenopus laevis* oocytes

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Datum Anurag Dave

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Abbreviations

3D	3 Dimesional
A	Adenine or Alanine
Å	Armstrong
AG	Aktiengesellschaft
Amp	Ampicillin
AmpR	β-Lactamase gene for Ampicillin Resistance
Ap	Apicoplast
APS	Ammonium persulphate
AQ	Amodiaquine
ATP	Adenosine Triphosphate
BCE	Before common era
Bp	Base pairs
BSA	Bovine Serum Albumin
C	Cytosine or Cysteine
$CaCl_2$	Calcium Chloride
cDNA	complementary DNA
cm	Centimeter
C-terminus	Carboxy terminus
CQ	Chloroquine
CQR	Chloroquine resistance
CQS	Chloroquine sensitive
D	Aspartic acid
Da	Dalton
dd H_2O	double distilled water
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleoside triphosphate
dsDNA	double stranded DNA
DV	Digestive vacuole
E	Glutamic acid
E. coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
EDTA	Ethylene Diaminotetraacetate
EM	Electrone microscopy
EMP1	Erythrocyte membrane protein1
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
Fig.	Figure
FV	Food vacuole
FP	Forward primer
G	Glycine or Guanine
g	Gram
GmbH	Gesellschaft mit beschränkter Haftung
GNP	Gross National Product
GTP	Guanidine triphosphate

h	hour
H	Histidine
H_2O	Water
HEPES	N-(2-Hydroxylethyl)piperacin-N´-(2-ethylsulphonacid)
I	Isoleucine
IC_{50}	Half of maximal inhibitory concentration
k	Kilo
K	Lysine
KAHRP	Knob Associated Histidine Rich Protein
Kb	Kilobasepair
KCl	Potassium Chloride
KOH	Potassium Hydroxide
L	Leucine
l	Liter
LB	Luria Bertani
LSC	Liquid scintillation counting
m	Milli or Meter
M	Molar or Methionine
MC	Maurer's Cleft
$MgCl_2$	Magnesium Chloride
min	Minute
$MnCl_2$	Manganese Chloride
mRNA	messenger RNA
MSP-1	Merozoite surface protein-1
n	Nano
N	Asparigine
NaAc	Sodium Acetate
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
nm	Nanometers
NPP	New Permeation Pathway
Nu	Nucleus
O_2	Oxygen
°C	degree Celsius
O.D.	Optical density
OR_2	Ooocyte Ringer solution
P	Proline
p	Plasmid
P.	*Plasmodium*
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PBST	Phosphate buffered Saline supplemented with 0.1% Tween-20
PCR	Polymerase Chain Reaction
Pf	*Plasmodium falciparum*
PfiRBC	Plasmodium falciparum infected red blood cells
pfcrt	*Plasmodium falciparum* chloroquine resistance Transporter coding sequence
PfCRT	Plasmodium falciparum chloroquine resistance

	Transporter
pfmdr1	Plasmodium falciparum multidrug resistance protein 1 coding sequence
Pfmdr1	Plasmodium falciparum multidrug resistance protein 1
pH	Potential hydrogenii
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
pmol	picomoles
POD	Peroxidase
PPM	Parasite plasma membrane
PV	Parasitophorous vacuole
PVDF	Polyvinyldifluoride
PVM	Parasitophorous vacuolar membrane
Q	Glutamine
QD	Quinidine
QN	Quinine
R	Arginine
RP	Reverse primer
RIFIN	Repetitive Intersped family
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	revoluations per minute
RT	Room Temperature
S	Serine
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium dodecyl sulphate
sec	Second
SEM	Standard error of measurement
SP	Signal peptide
T	Thymine or Threonine
T_4	Bacteriphage T_4
TAE	Triacetate/EDTA
Taq	*Thermus aquaticus*
TE	Tris/EDTA
TEMED	triethylmethylethyldiamine
TMD	Transmembrane domain
Tris	tris (hydroxymethyl)-aminomethane
U	Units
US	United States
UTR	Untranslated region
UV	Ultraviolet
V	Volt or Valine
v/v	volume to volume
vol	volume
W	Tryptophan
w/v	weight to volume
WHO	World Health Organization
x	times
X	any amino acid
X-ray	Roentgen ray
X .laevis	*Xenopus laevis*

Y	Tyrosine
α	Anti
μ	Micro
φ	Hydrophobic amino acid

Contents

Summary

With an annual mortality of around a million people, most of whom are children, malaria remains a major health hazard in our times. Amongst the drugs used to treat malaria, compounds such as chloroquine (CQ) and quinine (QN) are no longer the first line antimalarials in use because of the spread of drug resistant *Plasmodium falciparum*, in particular that of the chloroquine resistant (CQR) strains.

Central to CQR malaria is the *Plasmodium falciparum* chloroquine transporter (PfCRT), a trans-membrane protein located in the digestive vacuolar membrane of the parasite. It has been shown in the past that mutations in this protein are linked to an enhanced efflux of CQ from the digestive vacuole, which is the basis of CQR in *P. falciparum*. As part of this study, PfCRT was expressed in oocytes of *Xenopus laevis*, in order to understand the relationship between mutant *pfcrt* and transport of quinoline drugs. A number of naturally occurring and lab-constructed *pfcrt* mutants were expressed in oocytes, and transport measured for CQ, QN and its stereoisomer quinidine (QD). The data obtained showed that apart from being a carrier for CQ, mutant PfCRT exhibited saturable and verapamil-sensitive uptake of QN and QD, suggesting that PfCRT is a carrier for QN and QD as well. Using polymorphic *pfcrt* alleles, it was observed that mutations in *pfcrt* can influence the apparent Michaelis-Menten constant for CQ. While all mutant *pfcrt* alleles showed uptake of CQ albeit with differences, only the Dd2 and GB4 alleles showed transport for QN and QD, indicating that *pfcrt* mutations may also influence the substrate specificity. Mutants generated for the Ecu1110 alleles showed that only three mutations can suffice for CQ transport. Amino acid substitution in the Dd2 allele revealed a role for residue 326 in quinoline substrate selectivity. Taken together, the data argue in favour of a model where PfCRT acts as a carrier for quinolines such as CQ and QN, whose kinetic parameters are dependent on the actual combination of mutant residues present.

Zusammenfassung

Mit einer jährlichen Sterblichkeit von rund einer Million Menschen, von denen die meisten Kinder sind, bleibt Malaria eine der bedeutendsten Infektionskrankheiten unserer Zeit. Unter den Medikamenten die zur Bekämpfung von Malaria eingesetzt werden sind Chloroquin (CQ) und Chinin (QN) nicht mehr die erste Wahl. Der Grund dafür ist die sich immer weiter ausbreitender Resistenz von *Plasmodium falciparum* gegen antimalaria Medikamente, insbesondere gegen CQ.

Die Zentrale Rolle bei der CQ Resistenz spielt der *Plasmodium falciparum* Chloroquin resistance transporter (PfCRT), ein in der Nahrungsvakuole des Parasiten lokalisiertes transmembranes Protein. Es wurde gezeigt, dass Mutationen innerhalb dieses Proteins mit einem erhöhten Efflux von CQ aus der Nahrungsvakuole einhergehen. Um die Zusammenhänge zwischen mutiertem *pfcrt* und dem Transport von Chinolinen zu verstehen, wurde PfCRT in Oozyten von *Xenopus laevis* exprimiert. Es wurden natürlich vorkommende sowie im Labor hergestellte Varianten von *pfcrt* in Oozyten exprimiert, anschließend wurde der Transport von CQ, QN und seines Isomers Quinidine (QD) gemessen. Die erhaltenen Daten zeigen, dass mutierte PfCRT Varianten außer CQ auch QN und QD transportieren. Es konnte auch beobachtet werden, dass Mutationen in PfCRT auch einen Einfluss auf die Michaelis-Menten Konstante für CQ haben. Während alle mutierten PfCRT Allele, wenn auch mit Unterschieden, Aufnahme von CQ zeigten, war nur im Fall von Dd2 and GB4 Allelen Transport von QN und QD zu beobachten, was darauf hindeutet, dass Mutationen in PfCRT auch einen Einfluss auf die Substratspezifität haben könnten. Versuche mit Ecu1110 Mutanten zeigen, dass nur drei Mutationen für den CQ Transport ausreichend sind. Die Ersetzung der Aminosäure an Position 326 im Dd2 Allel bewirkt eine Substratselektivität der Chinoline. Zusammenfassend sprechen die Daten für ein Modell, in dem PfCRT als Transporter für Chinoline wie CQ und QD fungiert, deren kinetische Parameter von der jeweiligen Kombination der mutierten Aminosäuren abhängen.

1 Introduction

1.1 Malaria

1.1.1 Global impact

In the entire history of mankind, humanity has suffered much from diseases and the pain they inflict. But few maladies could be blamed for the scale of suffering and mortality that malaria has unleashed upon us. In an age where some diseases, which once meant certain death, have not only become treatable but curable, malaria presents us with a grim challenge. Of the entire world population, it is estimated that 2.37 billion live in areas of any risk of *Plasmodium falciparum* transmission – in Africa, South America, South asia and South east asia (Guerra *et al.*, 2008). Fig 1.1 shows the distribution of *P. falciparum* malaria risk across the world. As for the actual number of cases pertaining to malaria, the World health orgnization has reported that an estimated 243 (range 190-311) million cases of malaria occurred in 2008, of which about 863,000 (range 708-1003 thousand) cases were lethal. 85 % of these deaths occurred in children under 5 years of age. Malaria accounted for 20% of all childhood deaths that occurred in Africa, where it is believed that a child dies every 45 seconds of this disease (WHO, 2009). But the burden of malaria is not limited to mortality alone. It has a detrimental economic effect in areas of incidence; estimations made in the past suggested that the annual gross national product (GNP) grew 2% less in countries where malaria is endemic when compared to countries with similar economic background but without a major malaria burden (Chima *et al.*, 2003, Sachs & Malaney, 2002).

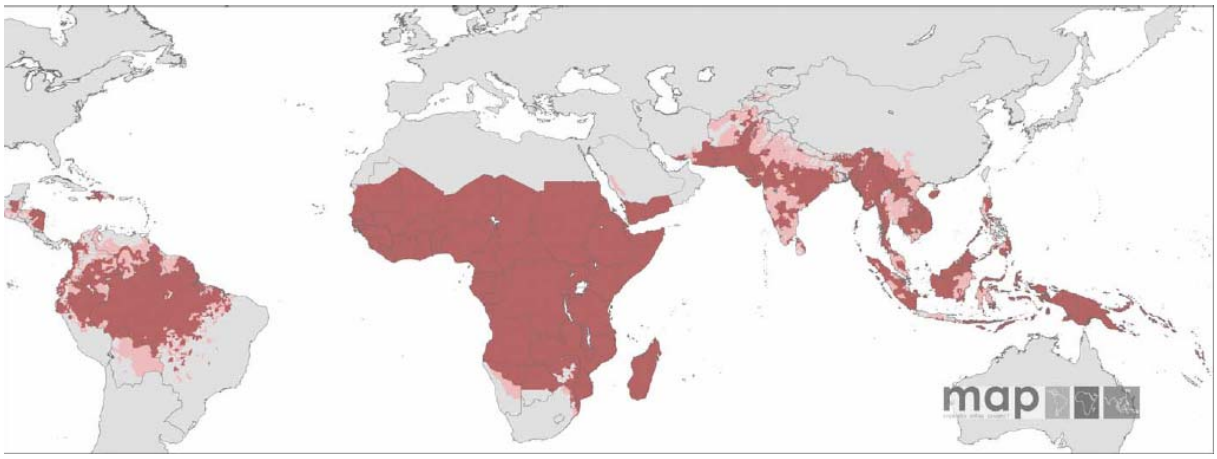


Fig 1.1: Global distribution of Plasmodium falciparum malaria risk (Guerra *et al.*, 2008)

1.1.2 Origin and history

The description of what were most certainly cases of malaria have been found in Mesopotamian clay tablets from 2000 BCE, Egyptian papyri from 1570 BCE and hindu texts from the 6th century BCE (Cox, 2010). Hippocrates observed that "tertian" malaria fevers were more severe than "quartan" fevers. The world malaria is derived from the latin "Malus Aria" meaning bad air, because malarial fevers were associated with swampy marshes in italy where the disease was observed (Haldar *et al.*, 2007). For a large part of recorded history, miasmas arising from marshes were seen as the cause of malaria. This belief was first questioned when scientists such as Louis Pasteur and Robert Koch showed that disease aetiology can be linked to micro-organisms. The then new theories proposed two routes of transmission - either through inhalation i.e. air borne or through contaminated water. In fact, Corrado Thomasi-Crudelli and Theodor Klebs even claimed to have identified *Bacillus malariae* from the Pontine marshes. Charles Laveran questioned this and followed his observation that a pigment appeared to grow and eventually fill the corpuscles of malaria patients. His perseverance led to the discovey of the malaria parasite in 1880. The next breakthrough in understanding causative agents behind malaria came in 1987 when Ronald Ross showed that female *Anopheles* mosquitoes act as vectors for transmission (Cox, 2010).

The malaria parasite is actually a broad term for what are different species of the genus *Plasmodium*. They belong to the Phylum-Apicomplexa; Class-Sporozoa, Order-Coccidia, Suborder-Haemosporidiae, Family-Plasmodiidae. The genus Plasmodium contains as many as 120 species, of which only five infect humans. Four of these are *P. vivax*, *P. malariae*, *P. ovale*, and *P. falciparum* (Greenwood *et al.*, 2008). The fifth one, *P. knowlesi,* infects the long-tailed macaques *Macaca fascicularis*, but there has been evidence in the recent past that it can infect humans too, especially in Malaysia (Singh *et al.*, 2004). Apart from humans, *Plasmodium* species infect a many reptiles, birds and mammals. For instance, *Plasmodium berghei* is a mouse malaria parasite whereas *Plasmodium yoelli yoelli* causes malaria in chicken. Of the human malaria parasites, almost 80% of all malaria cases in Africa are caused by *P. falciparum*, whereas *P. vivax* accounts for 95% of malaria cases in Asia (Carter & Mendis, 2002). *P. vivax* is highly disabling although not as deadly as *P. falciparum*. Hypnozoites of *P. vivax* and *P. ovale* can survive for years in the liver. *P. malariae* can remain for a long period of time as an asymptomatic infection blood stage infection, even if it does not form hypnozoites (Greenwood *et al.*, 2008).

It is believed that the malaria parasite evolved from a free-living protozoan ancestor which had chloroplasts (Wilson & Williamson, 1997). Around 200 million years ago, when early Dipterans appeared, these adapted to a life inside the gut of aquatic insect larvae. Some of these parasites got adapted to a life-cycle divided between two separate hosts, facilitated by the blood-feeding habits of their insect hosts (Carter & Mendis, 2002). Human malaria parasites alternatively grow in humans and their mosquito hosts i.e. the female *Anopheles* mosquitoes. Of 400 species *Anopheles* species found across the world, 60 have been identified as vectors for malaria parasites under natural conditions and of these 30 are of major importance (Tuteja, 2007). In Africa, where malaria mortality is highest, *Anopheles gambiae* and *Anopheles funestus* act as the main vectors.

1.1.3 Clinical manifestations

Symptoms that characterize malaria are typically periodic fibrile episodes accompanied by chills, rigors and sweating. Other general symptoms such as body ache, nausea, weakness and prostration may also be observed. Splenomegaly is observed in untreated patients. *P. falciparum* infections are the deadliest of the human malaria parasites and is life threatening if undiagnosed and untreated. *P.falciparum* may be uncomplicated, and then progress to severe malaria. Severe malaria caused by *P. falciparum* infection causes dysfunctioning of kidneys, lungs and liver, and especially the sequestration of parasites in blood capillaries in the brain. *P. falciparum* malaria can progress from uncompliciated to severe malaria within a few days and the disease outcome is fatal in 10-40% of all severe malaria cases (Schlitzer, 2007). A fatal nephritic syndrome can result due to chronic infection of *P. malariae* (Carter & Mendis, 2002).

While malaria can certainly be a fatal disease, it is not necessarily so. A number of factors such as multiplication rate of the parasites, cytoadherence, invasion pathways, host immunity to the malaria parasite, age of the infected patient, genetic traits of the host, as well as other variables such as availability of and access to treatment can determine the course of development of malaria after an infectious bite by the mosquito vector (Miller *et al.*, 2002). However, when it does develop, disease progression in malaria happens in various stages which in all last 6-10 hours. The first is a cold stage, where the patient feels cold and may shiver. It is followed by hot stage where fever develops, along with headache and vomiting.

Seizures are commonly observed in children at this stage. A sweating stage follows the hot stage and as its name suggests, the patient now sweats and feels tired, whereas the body temperature returns to normal. The periodicity of attacks depends on the actual *Plasmodium* species with which the patient is infected; every second day for *P. falciparum*, *P. vivax* and *P. ovale* ("tertian" parasites) and every third day for *P. malariae* ("quartan" parasites). The intervals at which symptoms occur can be correlated with the release of TNF-α from macrophages as a response to rupturing erythrocytes (Kwiatkowski *et al.*, 1989). On the other hand, parasite egress from erythrocytes is often unsynchronized in case of *P. falciparum*, which leads to a persistent fever or fibril paraoxyms (Rasti *et al.*, 2004).

Severe malaria is a condition described by a *P. falciparum* infection which eventually leads to organ failure and metabolic disequilibrium. Severe anaemia, coma and respiratory distress constitute the clinical spectrum of malaria in African children (Marsh *et al.*, 1995). Anaemia is defined by a 5g/L of haemoglobin in a patient (Warrell, 1989b), and is an outcome of erythrocyte destruction and suppression of erythropoesis (Clark & Chaudhri, 1988). Coma, which occurs in cerebral malaria, is the effect of infecting erythrocytes adhering to the brain microvasculature. Such cytoadherence is the product of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family of proteins (Baruch *et al.*, 1995, Kirchgatter *et al.*, 2005, Warrell, 1989a). Alternately, lactic acidosis can be a cause of severe malaria (English *et al.*, 1996). Respiratory distress is most likely the result of lowered oxygen delivery caused by severe anemia and tissue perfusion impaired by parasite sequestration. It can thus be argued that the clinical outcome of malaria is determined by a number of factors and is in itself a complex process.

1.1.4 Life cycle

The life cycle of malaria parasites consists of a mosquito stage and two human stages – one in the liver and the other in blood. Female *Anopheles* mosquitoes feeding on humans inject around 100 sporozoites during their blood meal (Medica & Sinnis, 2005, Jin *et al.*, 2007). They stay at the site of infection about 30 minutes, where a majority remain confined while some are released in the peripheral circulation. Those sporozoites which do make it into the blood stream reach the liver where they infect hepatocytes (Amino *et al.*, 2008, Vanderberg & Frevert, 2004, Yamauchi *et al.*, 2007).

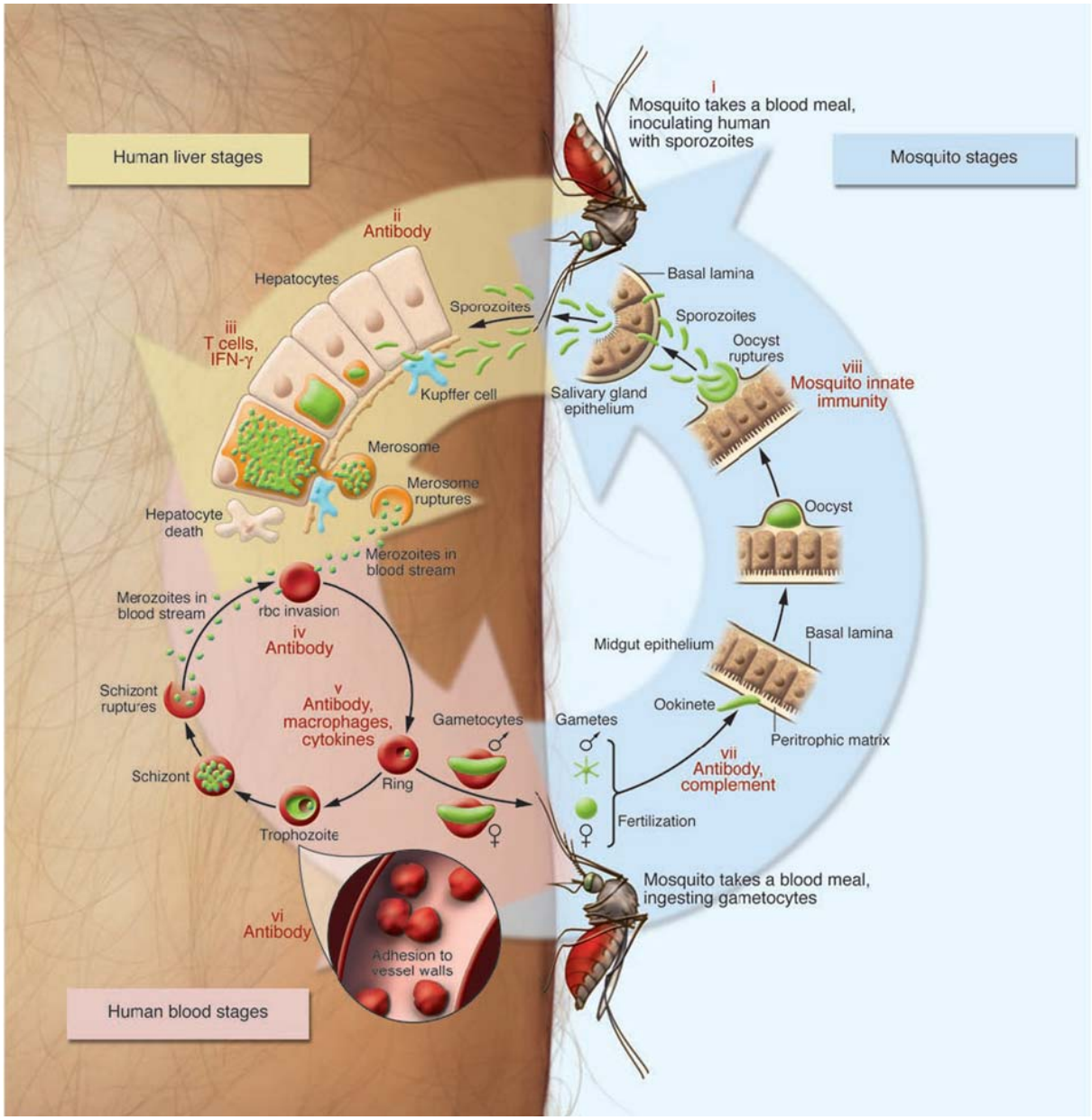


Fig 1.2: Life cycle of the malaria parasite (Greenwood *et al.*, 2008)

In order to gain access to hepatocytes so as to be able to infect them, sporozoites need to cross liver sinusoids which are composed of fenestrated endothelial cells and Kupffer cells. Since the enthothelial cell fenestrae are too small for the sporozoite to cross them, they have to migrate through the sinusoidal cells to access the liver parenchyma. Hepatocyte invasion by sporozoites requires proteins such as the circumsporozoite protein and thrombospondin-related adhesions, which contain a thrombospondin domain (Ejigiri & Sinnis, 2009). These domains interact with their host – cell receptors such as heparin sulphate proteoglycans present on the hepatocyte surface (Frevert *et al.*, 1993). *P. falciparum* sporozoites undergo

asexual replication in the hepatocyte, culminating in the formation of thousands of merozoites. This replication cycle is also called Exo-erythrocytic sporogeny, and a single infected hepatocyte may contain upto 3000 merozoites (Ejigiri & Sinnis, 2009). Merozoites eventually rupture the host hepatocytes and subsequently invade erythrocytes. The liver stage of *Plasmodium* infection, during which merozoites are formed in hepatocytes, is asymptomatic, lasts around 6 days and is also termed the prepatent period (Greenwood *et al.*, 2008). The exact duration of the prepatent phase, however, differs between *Plasmodium* species. It lasts between 8-27 days for *P. vivax*, 9-17 days for. *P. ovale* and 15-30 days for *P. malariae*. With *P. vivax* and *P. ovale* infections, some of the sporozoites that have invaded hepatocytes can form hypnozoites, which develop into exo-erythrocytic schizonts at a later time point. The signal that triggers the conversion of hypnozoites, however, is unknown (Cogswell, 1992).

Within minutes of their release from rupturing hepatocytes, merozoites invade erythrocytes where they begin the second cycle of asexual replication within the infected host. The invasion process is a highly co-ordinated event and occurs in a step-wise manner. The initial contact between the host cell and the merozoite is a low affinity interaction (Bannister & Dluzewski, 1990), involving host cell surface receptors and proteins on the merozoite surface such as the associated protein complex and MSP-1 (Chitnis & Blackman, 2000). Following the initial attachment, the parasite undergoes a reorientation in order to juxtapose its apical end with the erythrocyte membrane (Cowman & Crabb, 2006). Apical membrane antigen-1 (AMA1) is a protein that gets translocated to the merozoite surface before invasion begins and is required for the apical reorientation, although not for the initial interaction between the merozoite and the erythrocyte (Mitchell *et al.*, 2004). The reorientation allows for the formation for a tight junction between the parasite and host surface, which moves from the apical to the posterior end of the invading merozoite through a series of complex events which involve the actin-myosin motor of the invading merozoite (Keeley & Soldati, 2004). Micronemes, which are organells located apically in merozoite, release a serine protease named SUB2 which cleaves off proteins coating the merozoite surface (Harris *et al.*, 2005). The invading merozoite pushes itself into the erythrocyte and forms a parasitophorous vacuole that envelopes the parasite. The invasion process forms a major target for anti-malarial vaccine development, and blocking this process through antibodies against MSP-1 and AMA-1 is being actively pursued (Matuschewski & Mueller, 2007).

Once inside the erythrocyte, the parasite undergoes a second cycle of asexual replication, marked by a series of intraerythrocytic stages of development namely the ring, the trophozoite and the schizont stage (Bannister *et al.*, 2000). The ring stage is marked by low metabolic activity and only ring stage *P. falciparum* parasites are found circulating in the bloodstream of the infected host, as in the later stages the parasite modifies the erythrocyte surface leading to sequestration of the infected erythrocytes (Chen *et al.*, 2000, Baruch, 1999). In contrast to the ring stage, the trophozoite stage is metabolically highly active, as marked by high rates of glycolysis and haemoglobin proteolysis (Miller *et al.*, 2002). The trophozoite stage is followed by multiple rounds of cell division albeit without cytokinesis, leading to the formation of schizonts. Erythrocytes rupture following the schizont stage, and each individual schizont can produce 8-24 merozoites which are then released into the blood stream from the lysed erythrocytes. The release of merozoites coincides with the spiking of fever observed in malaria patients. Since merozoite release is synchronous and they are released approximately at the same of the day, the resulting fever is also periodic (Tuteja, 2007). The erythrocytic stage of *Plasmodium* infection is thus a symptomatic stage. The duration of a single erythrocytic cycle differs among various *Plasmodium* species, with the time taken to complete one such cycle being 48 hours for *P. falciparum* and *P. vivax* and 72 hours for *P. malariae*.

A small number of merozoites that invade erythrocytes do not undergo asexual replication culminating in schizont formation, but instead differentiate into male and female gametocytes (Bruce *et al.*, 1990). The factors triggering gametoyctogenesis are not clearly understood, and may include interplay between a number of host factors as well as parasite signalling pathways (Baker, 2010). In case of *P. falciparum*, hepatic merozoites cannot transform into gametocytes, although this has been observed for other species (Talman *et al.*, 2004). Gametocytogenesis involves five stages; stage I-IV gametocytes get sequestered in the bone marrow and spleen whereas stage-V gametocytes are released into peripheral circulation (Talman *et al.*, 2004) and get ingested into the midgut of a a mosquito biting an infected host. Within 10 minutes after their entry into the midgut, male gametes undergo three rounds of DNA replication, followed by exflagellation, thus producing motile microgametes. A single microgamete is composed of a plasma membrane enclosing an axoneme and a nucleus. Exflagellation involves binding of the newly formed microgametes to erythrocytes ingested as part of the blood meal forming a cluster known as the exflagellation center. Individual motile microgametes are subsequently released to seek out and fertilize macrogametes. Fertilization of non-motile macrogametes by motile microgametes forms a zygote, which transforms into a

motile ookinete (Eksi *et al.*, 2006). Ookinetes are mature diploid zygotes, and peak ookinete production for *P. falciparum* takes place 24-30 after the bloodmeal (Beier, 1998). Ookinetes are motile and traverse the epithelial layer lining the mosquito midgut (Siden-Kiamos & Louis, 2004), after which they get arrested in the basal lamina and transform into oocysts. Sporoblasts are formed from oocytes after several rounds of mitotic division. Budding of sporozoites from sporoblasts takes place 10-14 days after the blood meal. Sporozoite egress from oocytes through proteolytic activity and enter the mosquito haemolymph. Sporozoites passing the salivary glands of the mosquito adhere to the basal lamina in salivary glands, invade and exit acinar cells to eventually accumulate in the salivary duct. The arrival of sporozoites in the salivary duct completes the mosquito vector stage of the *Plasmodium* life cycle (Matuschewski, 2006). When such a mosquito vector bites a human host, some sporozoites get injected into the human host, thus beginning another round of the *Plasmodium* life cycle.

1.1.5 Remodelling of *P. falciparum* infected erythrocytes

The host erythrocyte invaded by a *P. falciparum* parasite undergoes extensive remodelling, such that new parasite-induced structures appear on the erythrocyte surface as well as within the host cell cytoplasm (Cooke *et al.*, 2004b). Such remodelling is not found in case of infection with the other human malaria parasites (Miller *et al.*, 2002). These modifications contribute to the virulence and pathogenecity of *P. falciparum* (Cooke *et al.*, 2004a). They include the appearance of knob-like extrusions on the erythrocyte surface, establishment of a membranous network of parasite origin called the Maurer's clefts as well as new permeation pathways (NPPs).

It has been known for a long time that *P. falciparum* infected red blood cells (PfiRBCs) have knob-like structures on their surface (Luse & Miller, 1971). These are observed in the trophozoite stage of the parasite life cycle, and can be seen in EM as electro-dense punctuate protrusions underlying the erythrocyte membrane, which are 30-40 nm in height and 90 nm in diameter (Tilley & Hanssen, 2008, Tilley *et al.*, 2008). At the molecular level, knobs are responsible for the cytoadherence property of PfiRBCs which plays an important part in sequestration of parasitized erythrocytes in the microvasculature, which in turn has been implicated in the fatal nature of *P. falciparum* malaria, as manifested by cerebral & placental

malaria and rossetting & clumping of PfiRBCS (Miller *et al.*, 2002). Knobs are associated with proteins of parasite origin such as the Knob associated histidine rich protein 1 (PfKARHP1) and the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). Knobs also modify physical properties of the host cell through their interaction with cytoskeletal components of erythrocytes such as spectrin, ankyrin and actin (Pei *et al.*, 2005, Kilejian *et al.*, 1991a, Kilejian *et al.*, 1991b).

PfEMP1 appears on the erythrocyte surface in the trophozoite stage of the parasite life cycle about 14-18 post merozoite invasion (Baruch *et al.*, 1996) and plays a crucial role in the pathophysiology of *P. falciparum* malaria (Pasternak & Dzikowski, 2009). For instance, PfEMP1 is involved in pregnancy associated malaria as it binds chondroitin sulphate A present in the placental lining (Reeder *et al.*, 2000, Viebig *et al.*, 2007). It also mediates the rosetting of uninfected erythrocytes to PfiRBCs (Rowe *et al.*, 1997). PfEMP1 can bind to host receptors such as intracellular adhesion molecule-1 (Berendt *et al.*, 1989, Ochola *et al.*, 2010), CD36 (Barnwell *et al.*, 1989, Ockenhouse *et al.*, 1989), P-selectin (Ho *et al.*, 1998), PCAM/CD31 (Heddini *et al.*, 2001), and the interaction between PfEMP1 and its host receptor also influences the disease outcome (Ochola *et al.*, 2010). PfEMP1 is encoded by the *var* gene family, and 60 members of this family have been identified in *P. falciparum*. The parasite expresses only a single allele at a time, while maintaining other alleles in a transcriptionally silent state, and expresses different *var* alleles during its life cycle (Scherf *et al.*, 2008). Erythrocytes are terminally differentiated, metabolically dead cells which lack subcellular organelles and a de-novo lipid/protein synthesis mechanism (Mohandas & Chasis, 1993), and thus are an ideal hiding place for the parasite to shield itself from the host immune system. Why then, does the parasite take the risk of revealing its presence in infected erythrocytes by expressing adhesion molecules such as PfEMP1? A widely accepted view is that the sequestration of PfiRBCs in microvasculature allows infected erythrocytes to escape destruction in the spleen (Dondorp *et al.*, 1999). Another hypothesis suggests that PfEMP1 expression helps in illiciting an immune response to PfiRBCs, thereby controlling blood-stage pararasitaemia, which can otherwise rise to levels that threaten host survival (Saul, 1999).

P. falciparum parasites possess components of the classical protein trafficking machinery found in eukaryotes such as the endoplasmic reticulum Sec61 translation complex (Couffin *et al.*, 1998), a signal peptidase complex (Sharma *et al.*, 2005), proteins required for vesicular transport (Adisa *et al.*, 2002, Ayong *et al.*, 2007) as well as a Golgi network (Struck *et al.*,

2005). The parasite can therefore synthesis proteins and traffick them upto the parasitophorous vacuole membrane (PVM). Taking proteins beyond the PVM and expressing them at the host cell surface is complicated by the fact that erythrocytes lack subcellular organelles and a protein trafficking machinery (Mohandas & Chasis, 1993). Maurer's clefts, which are parasite derived membranous structures comprising of one or more lamellae and bordered by a single membrane & an electron-dense coat (Aikawa, 1971, Atkinson & Aikawa, 1990, Atkinson *et al.*, 1988), aid in the trafficking of parasite proteins to the host cell surface (Wickert *et al.*, 2003). Named after Georg Mauer who first decribed them in 1900 (Lanzer *et al.*, 2006, Wickert & Krohne, 2007), Maurer's clefts contain proteins such as the skeletal binding protein-1 (Blisnick *et al.*, 2000, Saridaki *et al.*, 2009), ring exported protein-2 (Spielmann *et al.*, 2006) and members of protein families such as the subtelomeric variable open reading frames (Kaviratne *et al.*, 2002, Przyborski *et al.*, 2005) and repetitive intersped family (Khattab & Klinkert, 2006). Apart from a protein synthesis machinery, human erythrocytes also lack a *de-novo* lipid synthesis mechanism as well as a number of solutes required for parasite survival (Baumeister *et al.*, 2006). The parasite is thus forced to uptake nutrients from the blood plasma. It achieves this through the induction of new permeability pathways (NPPs) in the host cell (Staines *et al.*, 2007, Kirk & Saliba, 2007, Kirk *et al.*, 1999, Decherf *et al.*, 2004). NPPs are induced 12 to 15 hours after invasion, and dramatically increase erythrocyte permeability to low molecular solutes such as amino acids, sugars, nucleosides, vitamins, organic and inorganic ion (Saliba & Kirk, 2001). The molecular composition of NPPS, however, remains unclear. It has been proposed that they may consist of two kinds of channels – one present in small numbers and the other that is in lesser numbers but which is charge and size selective (Ginsburg & Stein, 2004).

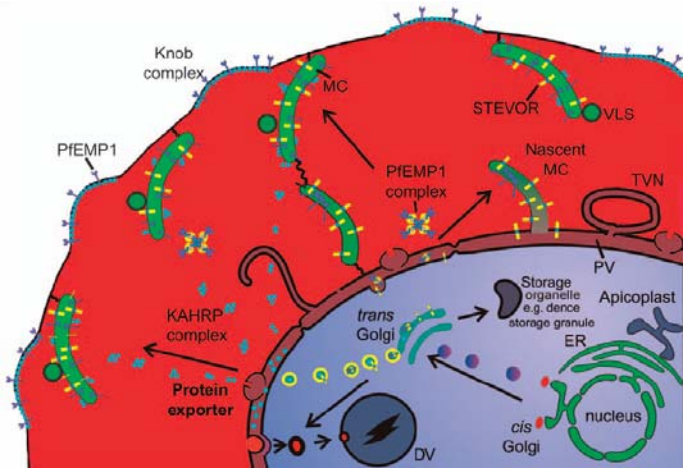


Fig 1.3: Remodelling of *P. falciparum* infected erythrocytes (Tilley *et al.*, 2008b)

1.2 Chemotherapy for malaria

Chemotherapeutic agents used to treat malaria can be classified as per the stage of the *Plasmodium* life cycle they act upon. Blood schizonticides act on the symptomatic asexual intraerythrocytic stage of *Plasmodium* development, and constitute the major class of antimalarial in use – more so since they help in releaving malaria symptoms. Tissue schizonticides, on the other hand, kill hepatic schizonts and therefore are prophylactic in nature as these prevent *Plasmodium* parasites from progressing to the blood stages. As against tissue schizonticides, hypnozoiticides kill the hypnozoites of *P. vivax* and *P. ovale* and are used to prevent a relapse of malaria. Hypnozoiticides may be used in combination with tissue schizonticides to cure the infection and simultaneously prevent relapse in *P. vivax* or *P. ovale* malaria (Kumar *et al.*, 2003). Gametocytocides kill the sexual forms of the malaria parasite, thus blocking parasite transmission from the human to host to the mosquito vector (Schlitzer, 2007). Since *P. falciparum* accounts for the lion's share in malaria mortality, the main focus of malaria chemotherapy is to cure *P. falciparum* infections. This can be achieved with compounds that are classified as antifolates, antibiotics, inhibitors of the parasite respiratory chain, artemisinin derivatives, aminoquinolines and arylaminoalcohols.

1.2.1 Antifolates

Antifolates include the drugs sulfadoxine, dapsone, pyrimethamine and proguanil. The term antifolate describes that these compounds target the folic acid metabolism. Folic acid is required for DNA synthesis and is essential for the malaria parasite (Metz, 2007). The biosynthesis of tetrahydrofolate, the biologically active form of folic acid, involves enzymes such as the dihydropteroate synthase (DHPS) and the dihydrofolate reductase (DHFR). Sulfadoxine is a sulphonamide that acts as a competitive inhibitor of DHPS, whereas dapsone is a sulfone that acts as a pseudosubstrate for this enzyme. Pyrimethamine and proguanil, which is the active metabolite of cycloguanil, inhibit DHFR (Nzila, 2006). DHSP inhibitors have a weak antimalarial activity, and are not used alone but rather with DHFR inhibitors as the two types of compounds have a synergistic effect when used together. Fansidar® is the commercially available combination of sulfadoxine/pyrimethamine (S/P), whereas dapsone/chlroproguanil (D/C) combination is sold as LapDap® (Schlitzer, 2007). Resistance to antifolates involves mutation in *dhfr*, the gene coding for DHFR. S/P was once used as a

first-line antimalarial (Baird, 2005), but the spread of resistant strains carrying dhfr mutations has more or less ended the use of S/P to treat *P. falciparum* infections. D/C is active against strains carrying triple mutations in *dhfr*, which are found in Africa, but not against the quadruple mutants found in Asia and South America (Wilairatana *et al.*, 1997).

1.2.2 Antibiotics

Antibiotics such as doxycyclin, clindamycin and azithromycin have been reported to have antimalarial activity. They interact with and inhibit the protein synthesis machinery of the parasite mitochondrion or apicoplast (Vaidya, 2004, Goodman *et al.*, 2007). One of the hallmarks of their mechanism of action is the so-called "delayed death phenotype", whereby parasites are killed in the second cycle of intraerythrocytic replication (Dahl *et al.*, 2006, Ramya *et al.*, 2007). Consequently, time required for fever and parasite-clearance is higher for antibiotics than classical antimalarials (Lell & Kremsner, 2002). Antibiotics are therefore only used in combination with faster acting drugs such as quinine, artesunate or fosfidomycin. Doxycyclin is the most commonly used in combination with quinine or artesunate for the treatment of uncomplicated and severe malaria (Ashley & White, 2005). It may be replaced with clindamycin, which has a better safety profile in pregnant women and young children (Lell & Kremsner, 2002). Azithromycin is less effective than doxycyclin against *P. falciparum* malaria (Taylor *et al.*, 2003), and has been used in trials in combination with dihydroartemisinine (Krudsood *et al.*, 2002).

1.2.3 Inhibitors of the respiratory chain

Atovaquone is a hydroxynapthoquinone that breaks down the mitochondrial membrane potential in malaria parasites. Its mechanism of action at the molecular level involves binding to the ubiquinone binding site of the cytochrome bc_1 complex in the parasite mitochondrion, thus blocking the movement of an iron-sulfur cluster protein that take part in mitochondrial electron transport (Korsinczky *et al.*, 2000). Resistance against atovaquone is quick to emerge when it is used in monotherapy, because a single amino acid mutation in the Q_o site alters the binding between atovaquone and the cytochrome bc_1 complex by as much 1000 fold (Vaidya & Mather, 2000, Srivastava *et al.*, 1999). It is therefore used in combination with proguanil

and sold under the brand name of Malarone®. Proguanil and atovaquone have a synergistic effect in combination; when atovaquone inhibits the mitochondrial electron transport, an alternative pathway involving ATP/ADP transporter get activated which is inhibited by proguanil (Painter *et al.*, 2007, Painter *et al.*, 2010). Malarone® is used as a prophylactic agent against *P. falciparum* malaria and in the treatment of uncomplicated malaria (Patel & Kain, 2005).

1.2.4 Artemisinin derivatives

Artemisinin is a sesquiterpene lactone that was first isolated from the extracts of the plant *Artemisia annua* in China in 1971 (Haynes & Vonwiller, 1994). Artemisinin has a very good antimalarial activity, as evidenced by mean IC_{50} values in the range of 12 to 20 nM for *P. falciparum* field isolates (Ramharter *et al.*, 2002, Tanariya *et al.*, 2000). It is, however, poorly soluble in water and oil which is why synthetic derivatives of artemisinin are used. These include artemether, artesunate and dihydroartemisinin (Schlitzer, 2007). Artemisinines reduce parasite biomass by upto 10,000 fold in a single asexual cycle of parasite replication and have a short plasma half-life of about an hour in humans making them the fastest acting antimalarials available (White, 1997, Dondorp *et al.*, 2010). It is therefore no surprise that artemisinin compounds have been the drug of choice to treat *P. falciparum* malaria, especially cases of severe malaria, and more so since the global spread of chloroquine and S/P resistant malaria (Krishna *et al.*, 2008). Combination of artemisinin derivatives with other partner drugs, also called Artemisinin combination therapy (ACT), is recommended by the WHO as the first line of treatment against *P. falciparum* infections (WHO, 2009). Artemether-lumefantrine, artesunate-mefolquine, artesunate-amodiaquine, dihydroartemisinin-piperaquine as well as artesunate-pyronaridine are the combinations in use as ACTs (Eastman & Fidock, 2009). It has been established that the key to the antimalarial activity of artemisinin compounds is an endoperoxide linkage, which gets cleaved by iron-II sources of the parasite. This cleavage results in carbon centered radicals which kill the malaria parasite (Schlitzer, 2008). The exact cellular target of artemisinines remains unclear. While some claim that PfATP6, a calcium pump localized in the endoplasmic reticulum membrane, is inhibited by artemisinin derivatives (Eckstein-Ludwig *et al.*, 2003), others have presented the digestive vacuole and the heme detoxification occuring in this parasite organelle as the targets (del Pilar Crespo *et al.*, 2008, Pandey *et al.*, 1999). While the molecular details of artemisinin mode of

action remain to be better established, it is widely accepted that the biggest threat facing global malaria control is potential therapeutic failure of ACTs. Clinically relevant resistance to artemisinines is yet to be reported from the field, but cases of decreases clinical efficiency and susceptibility have already been reported at the border of Cambodia and Thailand (Dondorp et al., 2010, O'Neill *et al.*, 2010).

1.2.5 Aminoquinolines and arylaminoalcohols

Powdered bark of the chinchona tree (*Cinchona pubescens*) was reportedly the first ever chemotherapeutic agent used to treat malaria, and was found to contain quinine and quinidine. Efforts aimed at *in vitro* synthesis of quinine inadvertently led to the production of synthetic dyes, of which methylene blue was found to stain malaria parasites. Paul Ehlrich's use of methylene blue as an antimalarial led to efforts aimed at modifying the chemical structure of methylene blue so as to yield novel compounds with antimalarial activity. Such endeavours eventually led to the synthesis of primaquine and resochin, with resochin being renamed as chloroquine through the course of history (Schlitzer, 2007).

Chloroquine (CQ) and amodiaquine (AQ) belong to the family of 4-aminoquinolines, and currently the only 8-aminoquinoline in use is primaquine. CQ has been the most successful drug in the history of malaria treatment, as well as being one of the cheapest and safest antimalarials to have been used till date (Sanchez & Lanzer, 2000). Against these plus points, CQ has a narrow therapeutic index - the dose is 10 mg/kg; 20 mg/kg is toxic and 30 mg/kg can be lethal (Taylor & White, 2004). Widespread use of CQ, however, led to the emergence of CQ resistant *P. falciparum* which later spread across the world (Wellems & Plowe, 2001). This has resulted in the discontinuation of CQ as the preferred drug to treat *P. falciparum* malaria. AQ is very similar to CQ in its structure, except that it contains an aromatic ring in its side chain. Although structurally similar to CQ, AQ is effective against low-level CQ resistant strains of *P. falciparum*, but not against highly CQ resistant parasites (Sa *et al.*, 2009). AQ is in use mainly as a partner drug in artemisinin combination therapy (Eastman & Fidock, 2009). Both CQ and AQ act against the intraerythrocytic stage of the *Plasmodium* life cycle. In contrast to CQ and AQ, primaquine acts against the liver and sexual stages, and is especially used to treat *P. vivax* malaria (Hill *et al.*, 2006).

Quinine, mefloquine, halofantrine and lumefantrine are classified as arylaminoalcohols (Schlitzer, 2008). Quinine is particularly used to treat severe malaria, mainly through intramuscular or intraperitoneal application. Its half-life of 8-12 hours, which means that the dosage has to be delivered 3 times daily (Okombo *et al.*, 2011). Side effects can be severe, as it causes cardiac arrhythmia and insulin-induced hypoglycaemia (Taylor & White, 2004). This calls for careful monitoring of the patient when quinine is being administered, and due to such reasons quinine usage is not recommended except in cases of severe malaria.. Mefloquine is structurally similar to quinine and is used in combination with artesunate (Eastman & Fidock, 2009). It is also effective against most CQ resistant strains of *P. falciparum* (Ringwald *et al.*, 1999), but can cause dose-related neuropsychiatric toxicity (AlKadi, 2007). Like mefloquine, halofantrine too is functional against CQ resistant strains, but the high risk of cardiac arrhythmia associated with halofantrine usage has meant that its use as an antimalarial is not recommended (Touze *et al.*, 2002). Lumenfantrine, which is structurally similar to halofantrine albeit less potent, does not have the side effects of halofantrine. Lumefantrine is commercially available as a partner drug with artemether as Riamet® (Omari *et al.*, 2004).

1.2.6 New antimalarials undergoing development

Emergence of resistance to existing antimalarial drug regimens not only compromises the fight against malaria, but also highlights the need to develop new compounds to be used to treat malaria. Different laboratories across the world are currently pursuing a number of compounds as potential antimalarial drugs. Some of these are AQ-13, tert-butyl isoquine and ferroquine which belong to the 4-aminoquinoline family of antimalarial drugs, and therefore are thought to share their mechanism of action with better known aminoquinolines such as chloroquine and amodiaquine (Schlitzer, 2008). A non-aminoquinoline drug which targets the parasite phospholipids metabolism is T3, and is also under development (Wengelnik *et al.*, 2002, Caldarelli *et al.*, 2010). Apart from these, research on a guaianolide-endoperoxide 3 as an antimalarial agent has also been reported (Sun *et al.*, 2010).

1.3 Quinolines - mechanism of action and resistance

1.3.1 Haemoglobin degradation

The capacity of the malaria parasite to synthesize amino acids *de novo* is limited (Sherman, 1977). It complements this lack through the uptake of amino acids from its extracellular medium, as well as by deriving amino acids from haemoglobin degradation (Sherman *et al.*, 1969, McCormick, 1970). Hemoglobin, which is present at a concentration of 5 mM in the erythrocyte, accounts for 95% of total erythrocyte protein content (Francis *et al.*, 1997). Inhibition of enzymes involved in haemoglobin degradation suggests that this process is essential for parasite survival (Rosenthal, 1995). Between 60-80% of the host cell haemoglobin is consumed by parasites during their intraerythrocytic proliferation (Rosenthal & Meshnick, 1996, Francis *et al.*, 1997). Not only does this release amino acids require by the parasite, but it also creates space for its proliferation and generates osmolytes that prevent host cell lysis (Lew *et al.*, 2003). Ingestion of the host cytoplasm occurs through endocytic structures called cytostomes, resulting in invaginations that get surrounded by the parasite plasma membrane and the parasitophorous vacuole membrane (Slomianny, 1990). There is some difference in opionion about the details of this process. Studies conducted in the past suggested that haemoglobin was delivered to the acidic digestive vacuole of the parasite in form of single membrane vesicles, after ingestion with a cytostome (Yayon *et al.*, 1983). A more recent publication has put forward another explaination – that ring stage parasites engulf hemeglobin in a “big gulp” and haemoglobin uptake continues as the parasite matures, through the action of small cytostome-derived haemoglobin containing vesicles (Elliott *et al.*, 2008).

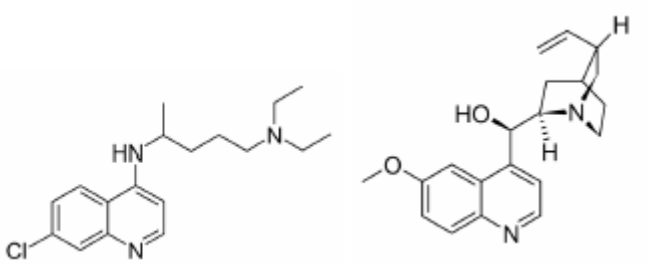
Hemoglobin degradation occurs in the digestive vacuole (DV) of the parasite, although it has been observed that this process may already have started in vesicles bringing haemoglobin to the DV (Hempelmann *et al.*, 2003). A number of proteases are involved in this process - the aspartic proteases Plasmepsin I, II, IV and a histo-aspartic protease along with the cystein proteases Falcipain – 1, 2, 2’ and 3. They convert large globin peptides to smaller fragments. The oligopeptides so formed are then converted by falcilysin and dipeptidylaminopeptidase –I (DRAP1) to dipeptides. Such dipeptides then get transported to the parasite cytoplasm where they are hydrolysed to individual amino acids (Ersmark *et al.*, 2006). Heme is also released during haemoglobin degradation. Ferriprotoporphyrin IX (FPIX), which is the oxidized form

of heme, is highly toxic for the parasite as it damages biological membranes (Fitch, 1998). The parasite also lacks a hemoxygenase that is required for degrading FPIX (Pagola *et al.*, 2000). It therefore sequesters heme into inert hemozoin crystals which appear as refractile, dark brown crystals and have been known to biologists as the "malaria pigment" (Egan *et al.*, 2000, Fitch, 1998). Hemozoin crystals are made of ß-Hematin, which is a head to tail dimer of FPIX (Fitch, 1998, Roepe, 2009). Hemoglobin loaded endocytic vesicles are composed of two membranes which originate from the DV and the plasma membrane. During the maturation of these vesicles, their lumen gets acidified and one of the two membranes is degraded, releasing lipids in this process. The dimerization of FPIX requires an acidic environment, as well as unsaturated lipids such as linolic acid. Thus, both these conditions get fulfilled in the environment where haemoglobin degradation occurs, allowing the formation of hemozoin (Fitch, 2004).

1.3.2 Mechanism of action of chloroquine and quinine

Chloroquine (CQ) is a diprotic weak base that can diffuse through parasite membranes. This permits entry of CQ into the DV, which is the target of CQ (Yayon *et al.*, 1984). The DV has an acidic pH (Kuhn *et al.*, 2007) which causes diprotonation of CQ, and diprotonated CQ can no longer cross membranes with ease. CQ is thus "trapped" in the DV, which is where haemoglobin degradation takes place (Sanchez *et al.*, 2007b). The mechanism of action of chloroquine (CQ) is thought to involve inhibition of heme polymerization to hemozoin (Fitch, 2004). CQ can bind to FPIX, which prevents the dimerisation of FPIX to hemozoin crystals (Gligorijevic *et al.*, 2006). CQ-FPIX complex also inhibits the maturation of endosomes containing haemoglobin, which leads to haemoglobin accumulation in immature endosomes (Fitch & Russell, 2006). Studies carried out using mouse erythrocytes infected with *Plasmodium berghei* have shown that parasites lose upto 80% of their ability to produce ß-hematin in the presence of CQ. Furthermore, CQ also prevents the interaction of lipids involved in ß-hematin formation with FPIX, an effect which some have termed "lipid masking" (Fitch *et al.*, 2003). The effect of CQ is therefore the accumulation of FPIX and the CQ-FPIX complex in the DV (Fitch *et al.*, 2000). The presence of free FPIX damages membranes, either because CQ-FPIX complex directly destroys membranes or because its engenders a release of Ca^{2+} ions, which make vesicle membranes fuse too early with the DV,

thereby disrupting and orderly degradation of haemoglobin. Whatever the exact mechanism, the effects are lethal for the parasite (Fitch, 2004).



Chloroquine Quinine

Fig 1.4: Chemical structures of chloroquine and quinine (Adapted from Schlitzer *et al.*, 2008)

Quinine is believed to share the mechanism of action of CQ to a large part, except another target for QN apart from FPIX is thought to exist (Fitch, 2004). A study in the past used EM to observe the morphology of infected erythrocytes treated with CQ and mefloquine, which is structurally similar to QN. The authors of this study proposed that while different quinolines appear to share the same targets, they led to morphologically different features in infected erythrocytes (Olliaro *et al.*, 1989). Furthermore, the masking effect of CQ on lipids required for ß-hematin formation appears not to happen with QN (Fitch & Chou, 1997). Unlike CQ, QN also seems to inhibit the docking of haemoglobin loaded vesicles (Fitch, 2004). Mefloquine can directly bind to membranes and phospholipids whereas CQ itself did not show a high binding affinity in this study (Chevli & Fitch, 1982). QN, and its stereoisomer quinidine, can also form salt bridges with heme (de Villiers *et al.*, 2008).

1.3.3 Chloroquine and quinine resistance in *P. falciparum*

The World Health Organization (WHO) defines antimalarial drug resistance as the ability of a parasite strain to survive and/or multiply despite the administration and adsorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject. The first cases of CQ resistance appeared in Thailand in 1957, and in Venezuela in 1960. Until 2005, only the island of Hispaniola and coutnries in Central America, among the malaria endemic countriesof the world, remained free from CQ resistance (WHO, 2005). CQ resistant (CQR) strains of *P. falciparum* have originated independently from at least six

locations across the world – one in South East Asia which spread to Africa, one in Papua New Guinea, two in South America, one in Java and one in the Philippines (Wootton *et al.*, 2002, Chen *et al.*, 2003). *In vitro* resistance is measured through a right-ward shift in the IC_{50} values, which correspond to a concentration which kills 50% of the parasitemia (White & Pongtavornpinyo, 2003). CQ IC_{50} values differ amongst isolates of geographically distinct origin. While susceptibility to CQ can be influenced by the parasite's genetic background (Valderramos *et al.*, 2010), *pfcrt* alleles from different geographical origins show variations in CQ IC_{50} when transfected in the same genetic background. Their response to verapamil, a chemosensitizer of CQ resistance, is also different (Sidhu *et al.*, 2002, Henry *et al.*, 2006, Lakshmanan *et al.*, 2005). Apart from K76T, amino acid substitutions harboured by *pfcrt* from different geographical origins also change (Cooper *et al.*, 2005, Chen et al., 2003, Sa *et al.*, 2009). Withdrawal of CQ treatment in Malawi led to a reversal of CQR (Laufer *et al.*, 2006), although it was later shown that this was due to CQS parasites re-expanding rather than CQR parasites reverting to the CQS phenotype (Laufer *et al.*, 2010). However, this does indicate that CQ resistance inflicts a fitness cost for the parasite. A recent study has shown that parasites harbouring the South American 7G8 *pfcrt* allele offer a selection advantage in competitive mosquito infections as compared to the wild-type parasites (Ecker *et al.*, 2011). Thus, it is possible that fitness costs of CQ resistance may be linked to *pfcrt* alleles.

One of the early reports on CQ resistance showed that altering pH of the DV can change the parasite susceptibility to CQ (Yayon *et al.*, 1985). It was thought that CQR parasites may have a different digestive vacuolar pH (pH_{DV}) than CQS parasites, which could led to CQ resistance. Some studies even backed this theory (Dzekunov *et al.*, 2000, Ursos *et al.*, 2000). However, it is known now that pH_{DV} is around 5.2 units and is similar in both CQS and CQR parasites (Hayward *et al.*, 2006, Kuhn *et al.*, 2007). An alternative to this theory is that CQ accumulation is reduced in CQR than in CQS parasites, and that CQ resistance involves removal of the drug from its target. This has been backed by many studies (Krogstad *et al.*, 1992, Krogstad *et al.*, 1987, Sanchez *et al.*, 2003, Bray *et al.*, 2006) and is now the consensus view. The involvement of ATP-binding cassete transporters, which mediate multidrug resistance in tumor cells(Baguley, 2010), was once implicated in CQR (Foote *et al.*, 1990), although this has been found to be untrue (Wellems *et al.*, 1990).

Unlike CQ resistance, quinine (QN) resistance is not widespread, but found at low levels in Africa and South East Asia. Moreover, efficacy of QN has not changed much in malaria

endemic areas, which have otherwise become highly resistant to chloroquine (Okombo et al., 2011). The mechanisms of CQ and QN resistance, however, do have something in common. Quantitative trait loci analyses have shown that *pfcrt* appears to be the single major genetic determinant of CQ resistance, whereas QN resistance involves the *Plasmodium falciparum* multidrug resistant transporter (PfMDR1) and the *Plasmodium falciparum* sodium proton exchanger (PfNHE1) in addition to PfCRT (Ferdig *et al.*, 2004). *pfmdr1* mutations and copy number have been linked to QN resistance, whereby a decrease in Pfmdr1 copy number increases QN susceptibility (Sidhu *et al.*, 2006, Sidhu *et al.*, 2005). PfNHE contains 3 microsatellite regions, one of which contains DNNND repeats. The copy number of these repeats is in an inverse relationship with QN susceptibility (Ferdig et al., 2004). However, the interplay between different QN resistance markers is not fully understood.

1.3.4 PfCRT

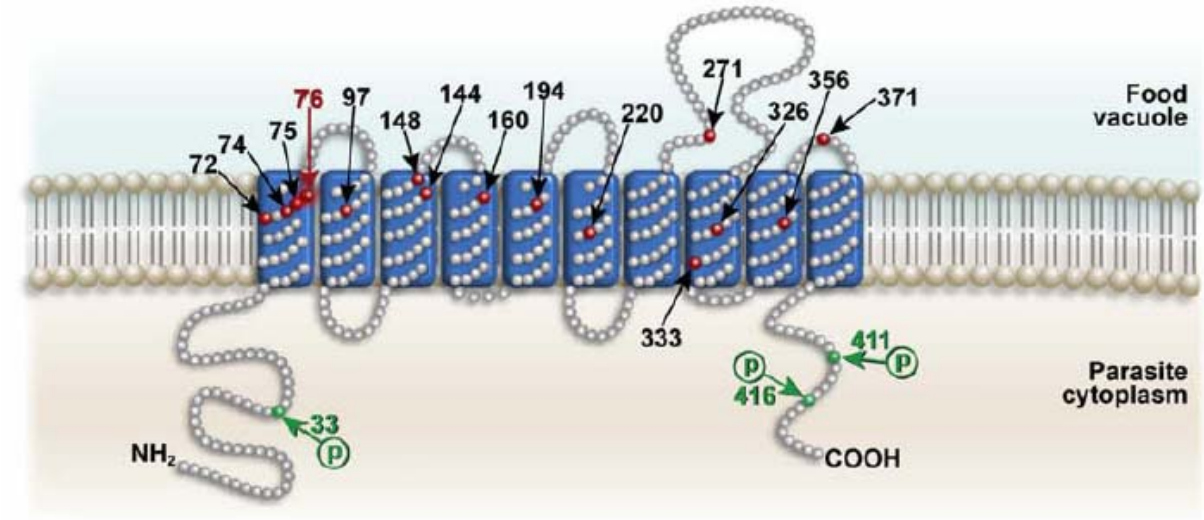


Fig 1.5: Predicted topological model of PfCRT (Adapted from Sanchez *et al.*, 2010)

Transmembrane topology of PfCRT is shown, where blue coloured blocks indicate trans-membrane domains. Polymorphic residues are shown in red and numbered arrows indicate their numeric position in PfCRT sequence. Green arrows denote residues that may be phosphorylated.

Using progenies of a cross between the chloroquine sensitive (CQS) HB3 strain and the chloroquine resistant (CQR) Dd2 strain, Wellems and co-workers identified a locus on chromosome 7 of the parasite that segregated with the CQR phenotype (Wellems *et al.*, 1990). This locus was found to contain the *Plasmodium falciparum* chloroquine resistance

transporter gene (*pfcrt*), which encodes a transmembrane protein that localizes to the DV membrane (Fidock *et al.*, 2000). Transfection of mutant *pfcrt* into a wild-type background resulted in a moderate level of CQ resistance, thus confirming that *pfcrt* is central to CQR (Sidhu *et al.*, 2002, Durand *et al.*, 2001). Sequence analysis of *pfcrt* showed that CQR parasites possess a mutated copy of the gene as compared to the CQS strains. Among such mutations, the change of a lysine to threonine residue at position 76 (K76T), was found to be conserved (Fidock *et al.*, 2000). When the reverse T76K replacement was introduced to CQR parasites, CQ and QN IC_{50} values to CQ dropped to CQS levels (Lakshmanan *et al.*, 2005), suggesting that K76T is crucial for CQ resistance. The same report also proposed that the differences in verapamil reversibility of CQR, seen between South-American parasites such as 7G8 and South-East Asian parasites such as Dd2, are linked to the amino acid residues preceding K76T.

It is known that CQR parasites accumulate less CQ than CQS parasites (Krogstad *et al.*, 1992, Krogstad *et al.*, 1987, Bray *et al.*, 2006), and that most of the CQ within the parasite is contained in the DV (Bray et al., 2006). Because this organelle forms the target for CQ (Yayon *et al.*, 1984), mutant PfCRT can mediate the removal of CQ from the DV, which forms the basis of CQ resistance (Roepe, 2009). However, two opposing schools of thought have tried to explain the *pfcrt* linked mechanism of CQ removal from the DV. One view postulates that PfCRT is a channel. Supporters of the channel model have claimed that K76T leads to loss of a positive charge, which in case of CQS PfCRT repels the diprotonated CQ found in the DV, but its loss in CQR PfCRT allows positively charged CQ to flow out of the vacuole along the proton gradient across the DV membrane (Warhurst *et al.*, 2002, Bray et al., 2006, Zhang *et al.*, 2004). In this scheme, verapamil restores the loss of positive charge, thereby blocking CQ outflow from the DV and sensitizing CQR parasites to CQ. Opponents of the channel hypothesis have presented evidence in favour of a PfCRT linked, energy dependent, verapamil sensitive and substrate specific efflux mechanism for CQ (Sanchez *et al.*, 2005, Sanchez *et al.*, 2004, Sanchez *et al.*, 2007b). These authors argue that PfCRT is a carrier protein, and mutations change the affinity of the carrier for its substrate. Trans-stimulation experiments aimed at differentiating between channels and carriers have demonstrated that PfCRT linked CQ efflux can be trans-stimulated with CQ as well as quinoline substrates such as QN, QD and AQ (Sanchez *et al.*, 2007a, Sanchez *et al.*, 2003). Other reports also support the carrier model. These include mathematical analyses of the CQ transport data pertaining to the two opposing models (Chinappi *et al.*, 2010) as well as PfCRT

expressing oocytes that show saturation kinetics for CQ uptake (Martin *et al.*, 2009b, Summers & Martin, 2010). Another set of studies has revealed the presence of a CQ linked leak of protons from the DV which is more enhanced in CQR parasites than in CQS parasites, and which can be reversed using CQR sensitizers (Lehane & Kirk, 2010, Lehane & Kirk, 2008). Taken together, these data sets support a model where PfCRT acts as a carrier for CQ, and transports the drug from the DV in association with protons (Sanchez et al., 2007b).

PfCRT resides in the DV membrane, has a molecular weight of 45 KDa and is composed of 424 amino acid residues (Sanchez *et al.*, 2010). Kuhn *et al.* have shown that the trafficking of PfCRT to the DV membrane involves the phosphorylation of a threonine residue at position 416 (Kuhn *et al.*, 2010). Bioinformatic analyses predict PfCRT to possess 10 transmembrane domains, with the N and the C-terminal facing the parasite cytoplasm, and it to be a member of the drug metabolite transporter superfamily (Martin & Kirk, 2004). Transmembrane domains of PfCRT show pseudosymmetry; such an internal symmetry is a characteristic of many carrier proteins (Sanchez *et al.*, 2010). While there is increasing agreement for PfCRT to be a CQ carrier, knowledge about its natural substrate remains a holy grail. Martin *et al.* successfully inhibited CQ transport in oocytes expressing the PfCRT Dd2 allele with a range of oligopeptides, whereas no inhibition was observed with amino acids, di- or tri-peptides. They also showed uptake for one tetrapeptide that could inhibit CQ uptake (Martin *et al.*, 2009a). This raises the possibility that PfCRT may transport peptides from the DV to the parasite cytoplasm, and this fits with the DV being an organelle for hemoglobin degradation. However, this claim remains on shaky ground as peptide uptake in this report was seen only for the CQR allele (Dd2) and not for the CQS allele (CQS). Another group has expressed *clt* (CRT like transport) - an *Arabidopsis thaliana* homologue of PfCRT in *X. laevis* oocytes (Maughan *et al.*, 2010). These authors show that *clt* mediates glutathione uptake upon expression in oocytes. No evidence showing glutathione transport by PfCRT is yet published, although glutathione metabolism and CQ resistance have been linked in the past (Meierjohann *et al.*, 2002).

But the role of PfCRT is not limited to CQ transport alone. PfCRT has been implicated in altered suscepitibilites to amodiaquine, quinine, quinidine, amantadine and halofantrine (Fidock et al., 2000, Ferdig *et al.*, 2004, Sidhu et al., 2002, Cooper *et al.*, 2002, Cooper *et al.*, 2007, Sa *et al.*, 2009). Martin *et al.* have confirmed that PfCRT can interact with antimalarial drugs other than CQ. They were able to inhibit PfCRT Dd2 associated CQ transport in

X. laevis oocytes with amodiaquine, quinine, quinidine, quinacrine, verapamil at low concentrations and with primaquine & mefloquine at higher concentrations (Martin *et al.*, 2009a). It is thought that chemical structures require a hydrogen bond acceptor and two hydrophobic aromatic rings to be able to interact with PfCRT (Bhattacharjee *et al.*, 2002, van Schalkwyk & Egan, 2006). More recently, N-benzyl-N-methyl-1-phenylmethanamine derivatives have been shown to inhibit CQ transport in PfCRT expressing oocytes (Zishiri *et al.*, 2011). Understanding the interaction between PfCRT and compounds other than CQ is all the more important because modified CQ analogues and many other compounds are being pursued as potential antimalarial drugs (Schlitzer, 2007). Moreover, drugs such as amodiaquine and quinine, which share the mechanism of action of CQ, are still in use. Thus, studying drug transport properties of PfCRT remains a relevant issue. One way of measuring an interaction between PfCRT and compounds such as CQ, quinine and quinidine would be to express the protein heterologously. This would allow a determination of substrate transport kinetics without being influenced by non-PfCRT parasite factors; these can complicate such determinations if performed *in situ*.

1.4 Use of *Xenopus laevis* oocytes to study membrane proteins

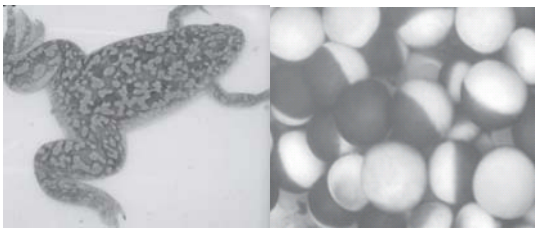


Fig 1.6: An Adult female *Xenopus laevis* (left) and its oocytes after surgical removal (right)

Heterologous expression of PfCRT has been achieved in the past, in the yeast *Pichia pastoris (Zhang et al., 2002, Zhang et al., 2004)*, in *Dictyostelium discoideum (Naude et al., 2005, Sa et al., 2006)* and also in *X. laevis* oocytes (Nessler *et al.*, 2004). PfCRT containing vesicles and proteoliposomes have also been reconstituted from yeast (Tan *et al.*, 2006, Paguio *et al.*, 2009). In a more recent piece of work, Martin *et al.* have expressed a modified form of PfCRT in *X. laevis* oocytes and shown time dependent, saturable, verapamil sensitive and quinoline inhibitable uptake of CQ (Martin et al., 2009a).

Oocytes of the South African clawed frog *Xenopus laevis* have been used to study proteins since John Gurdon introduced their application to biological sciences in late 1950s (Brown, 2004, Kashiwagi *et al.*, 2010). The oocyte is a relatively large cell; stage V-VI oocytes have an average diameter of 1 to 1.3 mm (Kashiwagi et al., 2010, Weber, 1999). This facilitates injection of foreign RNA into the oocyte cytoplasm. Gurdon and his colleagues successfully synthesized rabbit globin by injecting its mRNA in cytoplasm of *X. laevis* oocytes (Gurdon *et al.*, 1971). This technique got further enhanced when Douglas Melton established a method to synthesize synthetic RNA and express it in oocytes (Krieg & Melton, 1984). Since then, *X. laevis* oocytes have found their way as an experimental system in a wide variety of biological disciplines (Brown, 2004, Miller & Zhou, 2000). *X. laevis* oocytes have been particularly useful in studying membrane transport processes, by expressing membrane carriers and channels in these cells. This is largely because an extended range of techniques such as two-electrode voltage clamp, cut-open technique, giant patch clamp, ion selective electrodes to measure intracellular ion concentrations, tracer efflux and influx experiments, concentration jump technique, confocal microscopy, binding assays and volume

measurements can be applied to oocytes (Weber, 1999). It is therefore no surprise that *X. laevis* oocytes have also been employed in investigating membrane transport proteins of the malaria parasite. These include the digestive vacuolar *Plasmodium falciparum* multidrug resistance protein 1 (Sanchez *et al.*, 2008a), PfATP6 which is the homologue of the sarco-endoplasmic reticulum Ca^{2+} ATPase (Eckstein-Ludwig *et al.*, 2003) , the mitochondrial $Ca2^{+}$ /H^{+}exchanger PfCHA1 (Rotmann *et al.*, 2010), a hexose transporter PfHT1 (Krishna & Woodrow, 1999) and a sodium dependent transporter of inorganic phosphate PfPiT(Saliba *et al.*, 2006). Expression of PfCRT in *X. laevis* oocytes was thus an attractive choice for a method to answer the questions asked as part of this study.

1.5 Aim of the study

Understanding the role of PfCRT in mediating resistance to quinolines and arylaminoalcohols has been an area of considerable interest in malaria research. A number of studies mentioned in the previous sections have done so and advanced our knowledge of drug resistant malaria. However, such attempts have traditionally concentrated on three aspects of quinoline transport in the malaria parasite – one is the relationship between mutant *pfcrt* and resistance to a particular drug measured in terms of IC_{50} values (Fidock et al., 2000, Sidhu et al., 2002), second is the extent to which loci other than *pfcrt* as well as the genetic background of the parasite modulate resistance to quinolines (Ferdig et al., 2004, Mu *et al.*, 2003), and thirdly the actual mechanism and kinetics of PfCRT linked quinoline transport in the parasites (Sanchez et al., 2004, Sanchez et al., 2007a, Sanchez et al., 2003). The influence of point mutations has been studied mainly for the K76T mutation (Lakshmanan et al., 2005, Cooper et al., 2002). Cooper and colleagues have shown that mutations in transmembrane domains 1,4 and 9 alter susceptibility to quinolines (Cooper et al., 2007). However, this study concentrated on amino acid substitutions selected under *in vitro* drug pressure, and not the point mutations found in *pfcrt* isolates from the world over. The objective of this project was to investigate role of mutant PfCRT polymorphisms in transport of quinolines. Another aim was to try and establish if PfCRT can transport substrates other than chloroquine. It was proposed, as part of this study, to express a number of *pfcrt* alleles in *X. laevis* oocytes and subsequently carry out uptake measurements with radiolabelled substrates, so as to be able to answer the questions being raised.

2 Materials and methods

2.1 Materials

2.1.1 Equipments

Analytical scales	Sartorius, Göttingen
Autoclave	Tuttnauer Systec 2540, Wettenberg
Camera, DC 120 Zoom Digital	Intas, Germany
Centrifuges	
Biofuge fresco	Heraeus Instruments, Hanau
Biofuge pico	Heraeus Instruments, Hanau
J2-MC	Beckman, Krefeld
L-60 Ultracentrifuge	Beckman, Krefeld
Megafuge 1.0 R	Heraeus Instruments, Hanau
RC5BPlus	Sorvall, Langenselbold
Computer Software	
Adobe Photoshop ®5.0	Adobe Systems Inc, USA
EndNote 8.0.	ISI Research Soft, CA, USA
Internet Explorer	Microsoft Corporation, USA
MS Powerpoint	Microsoft Corporation, USA
MS Word 2000	Microsoft Corporation, USA
SigmaPlot 11.0	Systat Software Inc.
DNA-electrophoresis apparatus	Biorad, München
Transjector 5246	Eppendorf, Germany
Freezer -80°C, UF85-300S^	Heraeus GmbH, Hanau
Freezers -20°C	Liebherr, Biberach
Fridges	Liebherr, Biberach
Gas burner gasprofi 1 micro	WLD-TEC
Glass capillaries GB100F10	Scientific products GmBH, Germany
Ice machine AF 30	Scotasman, Milano, Italy
Incubator (ooyctes)	Memmert
Liquid nitrogen tank	Air Liquide, Ludwigshafen

TR-CARB 2100 TR	Packard
Liquid scintillation counter	
Magnetic stirrer	Heidolph, Schwabach
Microscopes	
Light optical microscope	Zeiss, Jena
Axiolab Leica DMII	Leica
Light microscope	
Microwave oven	AEG, Nürnberg
PCR machine	
T gradient Thermocycler	Biometra, Göttingen
pH-Meter pH 537	WTW, Weilheim
Pipetman Gilson P10, P20, P1000	Abimed, Langenfeld
Pipetus-akku	Hirschman labortechnik, Eberstadt
pipetus® standard	Hirschman labortechnik, Eberstadt
Power supply : Power Pac 300	Biorad, München
Rotor JA20.1	Beckman instruments, USA
Spectrometer UVIKON 923	Kontron Instruments
Sterile work bench Herasafe	Heraeus Instruments, Hanau
Stopwatch	Roth, Karlsruhe
Tweezers	Dumont, Switzerland
UV-table UV –Transilluminator	Gibco BRL, Karlsruhe
Vortex Genie 2	Roth, Karlsruhe
Watherbath Julabo 7A	Julabo Labortechnik, Seelbach

2.1.2 Disposables

Aluminium foil	Roth, Karlsruhe
Centrifuge tubes, Polypropylene	Greiner Bio-one, Frickenhausen
18/95	
Clingfilm Saran	Dow Chemical Company, Schwalbach
Cuvettes	Saarstedt, Nümbrecht, Germany
Eppendorf tubes	Saarstedt, Nümbrecht, Germany
Falcon tubes (15 ml, 50 ml)	Corning incorporation, Bodenheim
Gloves	Harmann, Heidenheim
Immersion oil	Zeiss, Jena

Kimwipes lite 200	Kimberly Clark
Parafilm	Americal International CanTM, USA
PCR softtubes 0.25 ml	Biozym Scientific GmbH
Petri dishes (10 ml diameter)	Greiner Bio-one, Frickenhausen
Petri-dishes (25 ml diameter)	Greiner Bio-one, Frickenhausen
Pipette Tipps	Corning Inc, Bodenheim
Plastic pipettes	Corning Inc. Bodenheim
(1 ml; 2 ml; 5 ml; 10 ml; 25 ml)	
Radioactive Vials	
Mini PolyQ vials 6 ml	Beckman Instruments Inc., USA
Sterile filters (0,2 µm)	Millipore GmbH, Ashburn
Stiches	
Sterile filtration devices	Corning incorporation, Bodenheim
Thermowell PCR tubes	Corning incorporation, Bodenheim
X-ray film	Kodak

2.1.3 Chemicals

2.1.3.1 Non-radioactive chemicals

The non-radioactive chemicals for the purpose of this study were purchased from the firms Roth, Merck, Sigma, Serva and Applichem. These were either ordered directly or through the chemicals facility of the University of Heidelberg medical school.

Non-radioactive quinoline drugs used in uptake experiments were purchased from following sources

Chloroquine	Chloroquine Disphosphate, Sigma, Germany.
Quinine	Quinine Hydrochloride, Sigma, Germany.
Quinidine	Quinidine Sulphate, Sigma, Germany.
Verapamil	Verapamil Hydrochloride, Sigma, Germany.

2.1.3.2 Radioactive chemicals

[^{3}H]-chloroquine	25 Ci mmol^{-1}	Amersham Radiolabelled chemicals
[^{3}H]-quinine	20 Ci mmol^{-1}	Amersham Radiolabelled chemicals
[^{3}H]-quinidine	20 Ci mmol^{-1}	Amersham Radiolabelled chemicals
[^{3}H]-amodiaquine	09 Ci mmol^{-1}	Amersham Radiolabelled chemicals

2.1.4 Kits

Gel extraction kit	QIAGEN®
PCR purification kit	QIAGEN®
High pure plasmid miniprep Kit	Roche, Mannheim
Plasmid maxiprep kit	QIAGEN®
in vitro RNA transcription kit (mMessage mMachine SP6)	Ambion , U.S.A.
Enhanced Chemiluminescence kit	Pierce® , U.S.A.

2.1.5 Biological materials

2.1.5.1 Size Markers and loading buffer

6 x DNA loading buffer	Fermentas, Germany.
GeneRuler 1 Kb DNA ladder plus	Fermentas, Germany.
2x Protein loading buffer	
PageRuler Plus Prestained protein ladder (SM1811)	Fermentas, Germany.

2.1.5.2 Enzymes

Collagenase Type IA	Sigma, Mannheim, Germany.
EuroTaq polymerase	BioCat GmbH, Heidelberg, Germany.
Phusion Polymerase	Fermentas, Germany.
Restriction Enzymes (XhoI, AvrII, BamHI, PstI, BglII)	New England Biolabs
Shrimp Alkaline Phosphatase	Promega
T_4 DNA Ligase	Invitrogen

2.1.5.3 Plasmids

pfcrt alleles used in the study were cloned into the pSP64T vector (Krieg & Melton, 1984). For facilitating cloning, XhoI and AvrII sites were first introduced in the pSP64T vector and this vector was used for all subsequent cloning steps.

P. falciparum coding sequences, which harbour AT rich sequences and therefore display a codon bias, are optimized to a yeast codon usage for facilitating heterologous expression

(Zhang *et al.*, 2002, Nessler *et al.*, 2004, Birkholtz *et al.*, 2008). To this end, *pfcr*t haplotypes from the strain HB3, Dd2, 7G8 and Ph1 were synthesized by GENEART AG (Regensburg) and then subcloned into the pSP64T vector using XhoI and AvrII sites. All the other alleles, which used for the purpose of this study, were synthesized through mutagenesis by using the appropriate template from the above mentioned *pfcr*t coding sequences.

2.1.5.4 Oligonucleotides

All oligonucleotides were purchases from Thermo Electron GmbH (Ulm).

Sequencing primers

SP6-66 FP	5'GCTTGTACATATTGTCGTTAGAACG 3'
PfCRT+731 FP	5'GATTGAACGCTATGGTTTC 3'
pSP64T RP	5'GTAAGTTGGGTATTATGTAGC 3'

Primers for megaprimer synthesis

5'Globin FP	5'GCAGAAGCTCAGAATAAACGCTCAAC 3'
3' Globin RP	5' GTAGCTTAGAGATCCCATTCG 3'
PfCRT 7G8 S72C FP	5' CTTGTCTGTTTGCGTCATGAAC 3'
PfCRT 7G8 S72C RP	5' CGTGTTAATGACGCAAACGGAC 3'
PfCRT Ph1 C72S FP	5' CTTGTCTGTTAGCGTCATGAAC 3'
PfCRT Ph1 C72S RP	5' CGTGTTAATGACGCTAACGGAC 3'
PfCRT HB3 K76T FP	5' CACAAACGCAGTACTTGTGCTAGAAG 3'
PfCRT HB3 K76T RP	5' GAAGATCGTTTGAATGACGCAAACAC 3'
PfCRT Ecu1110 T76K FP	5' GTCTGTTTGCGTCATGAACAAGATCTTCG 3'
PfCRT Ecu1110 T76K RP	5' GAAGATCTTGTTCATGACACGAACAC 3'
PfCRT S220A FP	5' GATTTCCGCATTGATTCCAGTTTGTTTCTCC 3'
PfCRT S220A RP	5' GGAGAAACAAACTGGAATCAATGCGGAAATC 3'
PfCRT S326D FP	5' CTCCTTCTTCGACATTTGCGATAACTTG 3'
PfCRT S326D RP	5' GTGATCAAGTTATCGCATAAGTCAAGAAGG 3'
PfCRT D326N FP	5' CTCCTTCTTCAACATCTGCGATAAC 3'
PfCRT D326N RP	5' GTTATCGCAGATGTTGAAGAAGAAG 3'
PfCRT L356I FP	5' GTCCAGCTATTGCTATTGCCTAC 3'
PfCRT L356I RP	5' GCAATAGCAATAGCTGGACCTTG 3'
PfCRT T356I FP	5' GTCCAGCTATTGCTATTGCCTAC 3'
PfCRT T356T RP	5' GCAATAGCAATAGCTGGACCTTG 3'

2.1.5.5 Bacteria

E. coli strain XL1 Blue was used for cloning and propagation of the plasmid.

2.1.5.6 Antibodies

Antigen	Isotype	Raised in	Company
Anti-PfCRT	Polyclonal	Guinea pig	
Anti-alpha Tubulin	Monoclonal	Mouse	Sigma, Germany
Anti-mouse conjugated with peroxidase	Monoclonal	Donkey	Jackson Immunoresearch
Anti-guineapig conjugated with peroxidase	Monoclonal	Donkey	Jackson Immunoresearch

2.1.5.7 *Xenopus laevis* frogs

Adult female *Xenopus laevis* frogs were purchased from NASCO, U.S.A. and maintained in the animal facility of the Interfakultär Biomedizinisches Forschungszentrum (IBF) of the University of Heidelberg medical school. Frogs were maintained in water tanks at a temperature of 18°C and fed thrice a week with food pellets. Frogs were 2 years of age at the time of purchase.

2.1.6 Buffers, media and solutions

Ampicillin stock, 100 x	50 mg/ml in ddH_2O
Anesthetic solution	0.1% (w/v) Ethyl 2-aminobenzoate methane sulfonate in tap water
APS	10% (w/v) APS in ddH_2O
Blocking solution	5% (w/v) skimmed milk in PBS
Coomassie Destaining solution	5% methanol 10% acetic acid
Coomassie Staining solution	5% methanol 10% acetic acid 0.0.5% Coomassie Brilliant Blue R-250
DNA loading buffer, 6 x	9 mg Bromophenol blue 9 mg Xylene Cyanol FF Dissolve in 8.8 ml of 60% Glycerol and add 1.2 ml of 0.5 M EDTA
LB broth	10 g Tryptone 5 g yeast extract 5 g NaCl Dissolve in 1 l ddH_2O and autoclave
LB agar	10 g Tryptone 5 g yeast extract 5 g NaCl 15 g Agar Dissolve in 1 l ddH_2O and autoclave
ND96, pH 7.5	96 mM NaCl 2 mM KCl 1 mM $MgCl_2$ 1.8 mM $CaCl_2$ 10 mM HEPES Set pH to 7.5 with NaOH and autoclave
ND96, pH 6.0	96 mM NaCl 2 mM KCl 1 mM $MgCl_2$ 1.8 mM $CaCl_2$ 10 mM Tris 10 mM HEPES, pH 7.5 Set pH to 6.0 with HCl

OR_2	96 mM NaCl 2 mM KCl 1 mM $MgCl_2$ 10 mM HEPES Set pH to 7.5 with NaOH and autoclave
RIPA buffer	100 mM Tris.HCl pH 7.4 150 mm NaCl 1 mM EDTA 1% Triton X-100 1% Sodium Deoxycholate 0.1% SDS
RNA gel running buffer, 20 x	20 mM MOPS 2 mM Sodium Acetate 0.25 mM EDTA
SDS loading buffer, 2 x	8 M Urea 5 % (w/v) SDS 40 mM Tris.HCl pH 6.8 0.1 mm EDTA 0.4 mg/ml Bromophenol blue
SDS-PAGE running buffer	25 mM Tris 192 mM Glycine 0.1% SDS
Semi-dry transfer buffer	48 mM Tris 39 mM Glycine 0.38% (w/v) SDS
SOB	20 g Tryptone 5 g Yeast extract 0.5 g NaCl 0.186 g KCl Dissolve in 1 l ddH_2O and autoclave
SOC	SOB with 20 mM Glucose
Stripping buffer	50 mM Tris pH 6.8 2 % SDS 100 mM 2-Mercaptoethanol
Super broth	35 g Tryptone 30 g yeast extract 5 g NaCl Add 1 l ddH_2O

TAE	40 mM Tris-acetate 1 mM EDTA (pH 8.0)
TB Buffer	10 mM PIPES 15 mM $CaCl_2$ 55 mM $MnCl_2.4H_2O$ 250 mM KCl Dissolve all components except $MnCl_2$. Set pH to 6.7 with KOH Then add $MnCl_2$, mix and filter sterilize by passing through 0.2 mm filter.

2.2 Methods

2.2.1 Microbiological methods

2.2.1.1 Preparation of chemocompetent *E. coli* cells

Bacterial cells that are able to take up foreign DNA molecules such as plasmids are termed "Competent cells". Chemically competent cells were prepared with a method involving DMSO (Inoue *et al.*, 1990). *E. coli* XL1 blue cells were streaked on a L.B. Agar plate and grown overnight at 37°C. Thereafter a single colony was used to inoculate 5 ml SOB medium and the culture allowed to grow overnight at 37°C, shaking at 230 rpm. The following day the 5 ml overnight culture was used as a started culture to inoculate 250 ml of SOB medium, grown in a 2 liter flask at 37°C and shaking at a rotational speed of 230 rpm, till a $O.D._{600}$ value of 0.6 units was attained. The culture was then chilled on ice for 10 minutes, and subsequently centrifuged at 6000 rpm, 4 °C for 15 minutes. Supernatant was discarded, the pellet re-suspended in 80 ml of ice-cold CC buffer and chilled on ice for 10 minutes, after which it was centrifuged at 6000 rpm, 4 °C for 15 minutes. The supernatant was discarded and pellet re-suspended in 20 ml of ice-cold CC buffer, after which 1.4 ml of DMSO was added to the re-suspended cells while stirring gently. Cells were chilled on ice for 10 minutes and then aliquoted in 50 µl aliquots in microcentrifuge tubes placed in a bath of dry ice and ethanol. Immediately after aliquoting cells, the tubes were closed and stored at -80 °C for further use.

2.2.1.2 Transformation of competent *E. coli*

Transformation of competent bacteria refers to the process by which DNA molecules are inserted in the cells, and was carried out using a heat-shock (Inoue *et al.*, 1990). Briefly, 50 µl of chemocompetent *E. coli* XL1 Blue cells (stored at -80 °C) were thawed on ice, to which 10 µl of a ligation sample or a desired amount of a supercoiled DNA sample was added. After mixing by gently tapping the base of the tube the mixture was incubated on ice for 30 minutes. The samples were thereafter placed in a water bath at 42 °C for 45 seconds, and then immediately placed on ice for 2 minutes, after which 1000 µl of SOC medium, pre-warmed at 37 °C ,was added. This mixture was incubated at 37 °C for 1 hour, while shaking at 230 rpm. 100 µl of this transformed medium was plated on L.B.Agar plates containing 100 µg/ml

Ampicillin, and the plates were incubated overnight at 37 °C. Plates were checked the following days for growth of bacterial colonies, where each colony represented growth from a single clone.

2.2.1.3 Glycerol-stocks of Bacteria

In order to maintain bacterial cells for long term storage, they are routinely stored at -80 °C in form of glycerol-stocks. These were prepared by mixing 800 µl of bacterial culture with 200 µl of 100% sterile glycerol. For further use, such a stock was not thawed but cells were simply scratched off the surface with a pipette tip and subsequently used to inoculate a starter culture.

2.2.2 Molecular biology methods

2.2.3.1 Photometric determination of DNA/RNA concentration

Nucleic acids can absorb UV light with an absorption maximum at 260 nm. The amount of light absorbed is related to the concentration of the absorbing molecule as per Beer Lambert's law, using which an extinction co-efficient of 0.020 $(\mu g/ml)^{-1}$ cm^{-1} for double-stranded DNA and of 0.020 $(\mu g/ml)^{-1}$ cm^{-1} for single-stranded RNA have been calculated. Thus, an $O.D._{260}$ of 1 Unit corresponds to 50 µg/ml of DNA and 40 µg/ml of RNA.

To measure concentration in DNA samples, a UVIKON 923 spectrophotometer (Kontrol Instruments) was used. DNA or RNA samples were diluted 1:100 and absorbance at 260 nm was measured for the same. The concentration of DNA or RNA sample was calculated from the O.D.at 260 nm.

2.2.3.2 Agarose gel Electrophoresis of nucleic acids

Agarose, a linear polymer of agarobiose, exhibits the property of melting at 85°C and solidifying at 32 - 40°C. It can therefore be mixed with a buffer, boiled, poured into a mould and then allowed to solidify. Migration of nucleic acids in an electric field is dependent on size and conformation. The sizes of pores within such an agarose gel can manipulated by increasing or decreasing the amount of agarose added. Low concentration of agarose is used to separate large nucleic acid fragments, whereas high concentration is used when isolating

small fragments. DNA fragments ranging between 0.1 and 25 kb in size can be separated in agarose gels.

2.2.3.2.1 Gel Electrophoresis of DNA

1% agarose gels were prepared by adding appropriate amount of solid agarose powder to TAE buffer, and the mixture was boiled in microwave till the agarose dissolved. After the temperature of this mixture was below 60°C, Ethidium bromide was added to a final concentration of 1 µg/ml and the mixture poured in a gel cast. Ethidium bromide gets degraded above 60°C, hence the need to cool down the gel. It intercalates with nucleic acids and fluoresces under UV illumination. 6 x DNA loading buffer was added to DNA samples to a final concentration of 1 x and samples were then loaded on the gel. 1 Kb Plus DNA Ladder™ was run alongside the samples as a size marker. Electrophoresis was carried out for 60 minutes at a constant voltage of 90 V for a small gel and 120 V for a big gel. Samples were photographed under UV illumination using a DC120 Zoom Digital camera (Kodak).

2.2.3.2.2 Gel electrophoresis of RNA

Agarose gel electrophoresis of RNA was carried out slightly differently than that for DNA, in that the agarose used was of special grade so as to minimize endosmosis. RNA can form secondary structures due to its single stranded nature. Formaldehyde was therefore used in the gels to maintain it in a denatured condition, so that the migration pattern would mainly be due to differences in its length. To this end, gels were prepared as under:

Agarose	0.28 g
H2O	30 ml
20 x RNA gel running buffer	2 ml

Boiled in autoclave, cooled down to 50-60 °C, then 0.5 µl of EtBr added and poured in gel mould. After gel solidified, 8 ml of 37% formaldehyde was added on its surface, kept for 1 min and then removed. Electrophoresis was carried out using RNA gel running buffer for 60 min at 60 V, after which a photograph was taken under UV illumination.

2.2.3.4 Restriction digestion of DNA

DNA can be enzymatically cleaved by restriction endonucleases. These enzymes recognize short, often palindromic sequences and catalyze a break in the sugar-phosphate backbone of DNA by hydrolysis. The resulting fragments have either "blunt" or "sticky" ends depending on the type of enzyme used. Sticky ends refer to the single-stranded overhang of a few nucleotides produced as a result of the cleavage, whereas in case of blunt ends no such overhang is found. Sticky ends are particularly useful since these anneal specifically to a complimentary overhang, and a ligation between two different fragments of DNA can thus be catalyzed in a desired orientation.

Restriction digestion was carried out to prepare vector and insert fragments for cloning, for checking plasmid DNA obtained from bacterial colonies and for linearizing plasmid DNA to be used for *in vitro* RNA transcription. Digests were set up as under:

Preparation of vector DNA

H_2O	as needed
Plasmid DNA	3 µg
10 x Buffer	5 µl
10 x BSA	5 µl
XhoI	1 µl
AvrII	2 µl
Total Volume	50 µl

Incubated overnight at 37°C

Preparation of insert DNA

H_2O	12 µl
PCR insert	30 µl
10 x Buffer	5 µl
10 x BSA	5 µl
XhoI	1 µl
AvrII	2 µl
Total Volume	50 µl

Incubated overnight at 37°C

Analysing plasmid minipreps

H_2O	11 µl
Plasmid miniprep	4 µl
10 x Buffer	2 µl
10 x BSA	2 µl
XhoI	0.5 µl
AvrII	0.5 µl
Total Volume	20 µl

Incubated 60 min at 37°C

Linearizing plasmid

H_2O	as needed
Plasmid DNA	20 µg
10 x Buffer	10 µl
10 x BSA	10 µl
BamHI	4 µl
Total Volume	100 µl

Incubated overnight at 37°C

2.2.3.5 Extraction and purification of DNA

2.2.3.5.1 Agarose gel extraction

Vector and insert DNA fragments as well as PCR samples were fractionated by subjecting the samples to agarose gel electrophoresis. This enabled isolation of specific fragments of required length, and the DNA fragment therein was purified using QIAGEN Gel extraction kit. The kit involved solubilizing the agarose gel slice by melting at 50°C, after which the sample was allowed to flow through a QIAquick™ membrane. This is a silica membrane which adsorbs DNA in presence of high concentrations of salt whereas the contaminants flow through. After washing steps, DNA was eluted in low salt conditions with Tris buffer or water provided in the kit.

2.2.3.5.2 PCR Column purification

This method was used to purify restriction digested PCR products, using QIAGEN purification kit. The principle behind the method involved adsorption of DNA onto a silica membrane in presence of high salt concentration and elution with a low salt buffer or water.

2.2.3.5.3 Phenol-Chloroform precipitation

Concentration, recovery and desalting of nucleic acids is routinely done using alcohol. Proteins in a reaction mixture can be denatured using Phenol-Chloroform and the nucleic acid can eventually be precipitated using high concentration of salt in presence of an alcohol.

Plasmid DNA was precipitated after linearization using this protocol. After digestion, 100 µl of the digest was mixed with 100 µl of Phenol:Chloroform:Isoamylalcohol::23:24:1. The sample was vortexed and centrifuged at 8000 rpm for 5 minutes in a microcentrifuge. The aqeous layer lying on the top was extracted with a pipette and transferred to a fresh tube. To it a 1/10 x volume of 3 M Sodium Acetate, pH 5.2 and 2 x volume of Isopropanol were added. The sample was vortexed, maintained at -20°C for 60 min and then centrifuged for 30 min at 13000 rpm in a microcentrifuge. DNA formed a pellet at the bottom of the tube, while the supernatant was removed. The pellet was washed with 1 ml of ice-cold 70% Ethanol (v/v) and then with 1 ml of 100% Ethanol, after which pellet was air dried and dissolved in 20 µl of water.

2.2.3.6 Dephosphorylation of DNA ends

When a ligation reaction between two DNA fragments is attempted, three possibilities arise: (1) Ligation between two ends of the vector fragment (2) Ligation between two ends of the insert fragment and (3) Ligation between one end of insert with another end of vector fragment. Scenario no.3 represents the desirable outcome, whereas scenario no.2 would not lead to viable colonies with bacterial cells after transformation as long as it lacks a selection marker. On the contrary re-ligation of vector fragment, that contains a selection marker, can lead to colonies. Moreover, the likelihood that two ends of the same DNA fragment collide with each other is much higher than that for meeting of two ends from two different fragments. To avoid this, use is made of the fact that ligation of two DNA ends requires a 5'phosphate and a 3'hydroxyl group. Re-ligation of vector ends can thus be avoided by removing its phosphate groups. This is done by treatment with an alkaline phosphatase which can be heat inactivated so that it does not interfere in subsequent ligations. The reaction set-up used was as under:

Vector digest	50 µl
10 x SAP buffer	6 µl
SAP	1 µl

Incubated at 37°C for 30 minutes and then heat inactivated at 65°C for 15 minutes.

2.2.3.7 Ligation of DNA fragments

Replication and repair of DNA in cells involves the action of DNA ligases. One such enzyme is the T_4 DNA ligase isolated from the T_4 bacteriophage. It catalyzes an ATP-dependent formation of a phosphodiester bond between 5'-phosphate and 3'-hydroxyl groups present at DNA termini. This enzyme can join both blunt and cohesive ends (Rossi *et al.*, 1997). It therefore allows ligation of different DNA fragments and is extensively used in cloning. The plasmid DNA where another DNA fragment is inserted is called "vector", whereas the inserted fragment is termed "insert". It has been observed that ligation efficiency is optimal when the insert is in molar excess to the vector. Ligation reactions were set up as below:

Dephosphorylated DNA vector	0.5 µl
Insert fragment	6.5 µl
5 x Ligase buffer	2.0 µl
T_4 DNA Ligase (1U/µl)	1.0 µl
Total Volume	10.0 µl

Incubated at RT for 2 hours, then transformed into competent *E.coli.*

Similarly, a vector control was set up where H_2O was added instead of insert.

2.2.3.8 Polymerase chain reaction

DNA sequences can be amplified *in vitro* using a Polymerase chain reaction (PCR) which involves a temperature dependent DNA polymerase, a template DNA sequence and oligonucleotide primers that bind to the the template (Saiki *et al.*, 1985). PCR was used to amplify *pfcrt* sequences for cloning as well, for screening bacterial colonies as well as to introduce mutations at specific positions within the coding sequence.

2.2.3.8.1 Site-directed mutagenesis via megaprimer synthesis

In order to mutate specific amino acid residues in the PfCRT sequence, megaprimers were first synthesized by employing oligonucleotides with the desired mutation. This PCR was set up as follows:

Reaction set-up for megaprimer PCR

H_2O	10.5 µl
5 x Phusion HF Buffer	5.0 µl
50 mM MgCl2	1.5 µl
10 mM dNTP mix	2.0 µl
Template (1 ng/µl)	10.0 µl
2.5 µM FP	10.0 µl
2.5 µM RP	10.0 µl
Phusion Polymerase	1.0 µl
Total Volume	50.0 µl

Cycling conditions

Initial denaturation	:	98°C 5 min
Denaturation	:	98°C 30 sec
Annealing	:	62°C 15 sec
Extension	:	72°C 20 sec
Final extension	:	72°C 5 min
No. of cycles	:	30

PCR reactions were then subjected to agrose gel electrophoresis and bands of appropriate length were excised and DNA purified. Using these DNA fragments as megaprimers, another set of PCR was performed as follows:

Reaction set-up for second PCR

H_2O	10.5 µl
5 x Phusion HF Buffer	5.0 µl
50 mM MgCl2	1.5 µl
10 mM dNTP mix	2.0 µl
Megaprimer-1	2.0 µl
Megaprimer-1	2.0 µl
2.5 µM 5’ Globin FP	10.0 µl
2.5 µM 3’ Globin RP	10.0 µl
Phusion Polymerase	1.0 µl
Total Volume	50.0 µl

Cycling conditions

Initial denaturation	:	98°C 5 min
Denaturation	:	98°C 30 sec
Annealing	:	62°C 15 sec
Extension	:	72°C 30 sec
Final extension	:	72°C 5 min
No. of cycles	:	30

Full length *pfcrt* coding sequence was yielded in this PCR. Ater agarose gel purification, it was digested with XhoI and AvrII restriction enzymes. The digested fragment was used for cloning after DNA purification achieved with QIAGEN PCR purification kit.

2.2.3.8.2 Colony PCR

Colonies obtained after transformation were screened with a PCR to fish out positive cloness. Colonies were picked from the plate under sterile conditions, streaked into a 0.5 ml PCR tube and sequentially on a new LB Agar plate supplemented with the appropriate antibiotic as a selection marker. Primers were designed such that the forward primer annealed in the vector, upstream of the insert, and the reverse primer annealed in the insert itself. PCR reactions were analysed on a 1% Agarose gel to find the colonies for which a positive PCR result was seen under UV illumination. The LB agar plate, where colonies had been streaked, was incubated overnight at 37°C, and used on the following day to inoculate cultures for positive colonies identified during the screen.

Reaction set-up for Colony PCR

H_2O	10.25 µl
10 x Euro Taq buffer	2.50 µl
50 mM $MgCl_2$	1.25 µl
10 mM dTPs mix	0.50 µl
2.5 µM FP	5.00 µl
2.5 µM RP	5.00 µl
Euro-Taq DNA Polymerase	0.50 µl
Total Volume	50.00 µl

2.2.3.9 Isolation of plasmid DNA from bacteria

2.2.3.9.1 Small scale isolation – "minipreps"

After identifying positive clones through colony PCR, 10 ml LB cultures (supplemented with µg/ml Ampicillin) were inoculated and grown overnight at 37°C with shaking. Plasmid DNA was isolated from the bacteria using a High Pure Plasmid isolation Kit (Roche, Mannheim). This system is based on an alkaline lysis of cells followed by adsorption of DNA onto special glass fibers. DNA was eluted using Tris-HCl buffer and and was aliquot sent for sequencing.

2.2.3.9.2 Large scale isolation-"maxipreps"

Large scale preparations of plasmid DNA were carried out using a QIAGEN Plasmid Maxi kit. A colony containing the plasmid of choice was used to inoculate 400 ml Super broth containing 100 µg/ml Ampicillin, and grown for 12-16 hours at 37°C with shaking. This kit too uses alkaline lysis of cells but involves binding of DNA to an anion-exchange resin under appropriate pH and low salt conditions. Contaminants are removed using a medium salt wash, DNA eluted using high salt buffer followed by precipitation and desalting with isopropanol.

2.2.3.10 Sequencing of DNA

DNA samples were sent to GATC, (Konstanz) for sequencing. The Sanger dideoxynucleotide method (Sanger *et al.*, 1977) was used. BioEdit software was used to analyze the sequences obtained.

2.2.3.11 in vitro synthesis of RNA

cRNA to be injected in *X. laevis* oocytes was transcribed *in vitro* using a mMessage mMachine SP6 kit (Ambion). It involved the use of a SP6 promotor binding RNA polymerase to transcribe RNA using a linear DNA template, after which the DNA template was degraded through the action of a DNase. Linearised plasmid (dissolved in DEPC treated H_2O) was used as template to avoid formation of cRNA molecules that vary in their length, which would happen if a circular plasmid were used since RNA polymerase would fall off the template at arbitrary points. Reactoins were set up as below:

<u>cRNA Transcription</u>

Nuclease-free H2O	as required
10 x Reaction buffer	2 µl
2 x dNTP	10 µl
Linearized plasmid (1 µg)	as required
Enzyme mix	2 µl
Total Volume	20 µl

Incubated at 37 °C for 2 hours.
+ 1 µl of DNAse, and incubated at 37 °C for 15 min.
Then, +30 µl of LiCl and +30 µl Nuclease-free H_20
Incubated overnight at -80°C.

DNase was used to degrade the template DNA whereas RNA was precipitated with LiCl. After overnight incubation, reactions were centrifuged at 13000 rpm, 4°C for 30 min in a microcentrifuge. The pellet was washed once with 70% Ethanol to remove contaminating salts, centrifuged and again washed with 100% Ethanol. Pellet was dissolved in 10µl of nuclease-free H_2O after air drying. RNA concentration was measured with a UV spectrophotometer (UVIKON) and an aliquot analyzed on agarose gel to check for integrity of RNA.

2.2.4.1 Isolation of total protein from *X. laevis* oocytes

Oocytes injected with the desired RNA were collected 3 days after injection in RIPA buffer containing Complete™ Protease inhibitor cocktail (Roche, Mannheim). 10 oocytes were washed 3-4 times with RIPA buffer before adding to 100 µl of RIPA buffer with protease inhibitor. Samples were placed on ice and oocytes crushed manually with the help of a pipette tip and by pipetting up and down. Lysed oocytes were maintained on ice for 30 min. while vortexing intermittently, after which lysates were centrifuged at 13000 rpm, 4°C for 30 minutes. The supernatant was pipetted out and placed in a fresh tube, while taking care to avoid the frothy top layer. The centrifugation step was repeated two more times and the supernatant thus cleared, after which lysates were stored at -20°C.

2.2.4.2 SDS-PAGE electrophoresis

Proteins can be separated through Polyacrylamide gel electrophoresis (PAGE) according to their electrophoretic mobility. Loading buffer and gel contain SDS, an anionic detergent which denatures secondary and non-disulfide linked tertiary structures and applies a negative charge to each protein. This enables separation based on charge to mass ratio of each protein. Before loading protein samples are heated in presence of β-mercaptoethanol, a reducing agent, so that any disulfide linkages present in the protein get reduced. PAGE gels are formed by polymerization of Acrylamide and N,N'-methylbisacrylamide. The reaction is inititated by ammoniumpersulfate (APS) and catalyzed by tetramethylethylenediamide (TEMED). Manipulating the polyacrylamide content changes the pore size which influences the degree of separation of proteins. The gel itself is made up of two parts - a stacking gel and a running gel. Stacking gel contains the loading pockets and has larger pores to concentrate the sample

before separation, whereas the running gel has smaller pores for dispersion of proteins (Laemmli, 1970). Composition of each gel was as described:

Component	Stacking gel	Running gel
H_2O :	3.46 ml	3.35 ml
1M Tris pH 6.8 :	0.63 ml	----------
1.5 M Tris pH 8.8 :	---------	2.50 ml
10% SDS :	0.05 ml	0.10 ml
30 % Acrylamide :	0.83 ml	4.00 ml
10% APS :	0.05 ml	0.10 ml
TEMED :	5 µl	6 µl

Prior to loading, protein samples were diluted 1:1 (v/v) with 2 x SDS loading buffer and heated at 70°C for 3 minutes. Electrophoresis was carried out at 60 mA constant current for 75 minutes in SDS gel running buffer till bromophenol blue present in samples ran out of the gel.

2.2.4.3 Coomassie staining of proteins

Proteins immobilized in SDS-PAGE gel were detected by Coomassie staining after gel electrophoresis. This was done to control loading of similar amounts of protein in different samples. SDS-PAGE gels were quickly washed with deionised water after electrophoresis and then stained with Coomassie staining solutions for 20 minutes at room temperature. Destaining was carried out by incubating in Coomassie destaining solution till bands were detectable.

2.2.4.4 Western blotting

Western blotting is a method for detection of proteins where the samples are immobilized on a membrane support. Protein samples are first run on a SDS-PAGE gel, after which they are transferred to a membrane such as Polyvinyldifluoride (PVDF) or Nitrocellulose. The membrane is subsequently subjected immunochemical analysis whereby antibodies against a specific protein or polypeptide are used to detect the band of interest.

Transfer of proteins from SDS-PAGE gel to PVDF membrane was carried out through a semi-dry transfer. After electrophoresis, the gel was rinsed 3-4 times with deionised water and

incubated in semi-dry transfer for 20 minutes. PVDF membrane was cut to the size of gel and activated by dipping in methanol for 30 seconds, and subsequently incubated in semi-dry transfer for 20 minutes at RT. 6 pieces of Whatman filter paper were also cut to the size of gel and kept in transfer buffer. After lapse of the incubation period, 3 pieces of filter paper were laid on the electrode of transfer chamber, while being careful not to trap air bubbles between filter papers. The membrane was laid over filter papers, and then slight amount of buffer was layered over. The gel was placed on top and then covered with 3 piece of filter paper. Transfer was carried out at 15V, 230 mA constant current for 60 minutes.

After transfer, blots were incubated overnight with the blocking solution and subsequently incubated with a 1:1000 dilution of primary antibody (α-PfCRT or α-Tubulin) prepared in 1% BSA in PBS. Blots were then washed with PBST, 3 times 10 min each, after which they were incubated with secondary antibody coupled with POD diluted 1:10000 in blocking solution at RT for 30 min, followed by 3 x 10 min. washes with PBST and 1 x 10 min. with PBS.

Blots were developed with the help of Enhanced Chemiluminescence kits (ECL). Perodixase substrate from the kit was applied to the blot for one minute at RT and then drained Peroxidase antibody attached on the blot acts on the substrate and the reaction produces luminescenbce, which was used to expose an X-Ray film in a dark room. X-Ray films were developed using a Hyperprocessor developing machine (Amersham Pharmacia Biotech).

2.2.4.5 Stripping western blots

Membranes used for western blotting with one antibody were reused to detect another protein such as Tubulin. This was achieved by stripping the blot of the antibody sandwich and immunochemical detection was subsequently carried out. After exposure of X-ray films, membranes were washed 4 x 10 min with PBS. Stripping was carried out by incubating membrane in stripping buffer at 55°C for 30min. Membranes were washed 5 x 10 min with PBS after washing, and then blocked overnight with blocking solution. Rest of the immunochemistry was carried out subsequently.

2.2.5 Xenopus laevis oocytes

2.2.5.1 Surgical isolation of ovaries from *Xenopus laevis* frogs

Adult female *X. laevis* frogs, purchased from NASCO, U.S.A., were anaesthesized with 0.1% (w/v) solution of Ethyl 3-amino benzoate methanesulfonate (Sigma, Mannheim) for 15-20 min. Frogs were then laid on a moist tissue paper placed on ice, and an incision made with a scalpel to cut the outer skin. After slitting open the abdominal muscle layer, oocytes lobes were pulled out with the help of tweezers. Each cut was individually stitched together and the frog was revived by placing in a water tank. A glass support was placed below the frogs so as to prevent drowning; the support was removed once frogs' movement was observed to be normal. The following day frogs were returned to the animal facility. Individual frogs were operated upto four times, while giving at least 4 weeks recovery time between successive operations.

2.2.5.2 Collagenase treatment

Oocytes are wrapped in a collagenous membrane that envelopes individual oocytes as well as oocyte lobes. Oocytes separated from lobes still retain collagen on their surface, which proves to be an impediment in injecting RNA. For this purpose, collagenase was enzymatically removed using collagenase extracts from *Clostridium histolyticum*. Collagenase calatalyzes hydrolysis of –R-Pro-8-X-Gly-Pro-R- sequence in polypeptides where R is most often a neutral amino acid.

Surgically isolated oocytes lobes of *X. laevis* were manually separated into groups of 8-10 oocytes in OR_2 buffer. After washing 4-5 times with OR_2 buffer, 2-3 ml volume of separated oocytes were placed in a 50 ml tube and volume made upto 30 ml with OR_2. Collagenase I A (Sigma, Mannheim) was added to a final concentration of 0.3 FALGPA units/ml, and the mixture gently rotated on a vertical shaker at RT. Oocytes were checked for removal of collagene after 90, and then at 15 min. intervals. The treatment lasted 2 hours on average, after which oocytes were washed 10-15 times with OR_2 and 3-5 times with ND96 (supplemented with Penicillin-Streptomycin and Sodium Pyruvate).

2.2.5.3 Selection and culture of *X. laevis* oocytes

Oocytes endure enzymatic as well as mechanical stress during collagenase treatment, and hence many rupture or get apoptotic. Healthy oocytes were, therefore, selected visually under microscope and separated from the main batch. These, like the treated oocytes, were maintained in ND96 buffer supplemented with Penicillin-Streptomycin and Sodium pyruvate at 18°C. Selected oocytes were injected the following day. After injection oocytes were checked daily. Apoptotic or demorphed oocytes, as identified by loss of colouration in the animal hemisphere or loss of shape, were selected out. ND96 buffer was replaced daily.

2.2.5.4 Injecting RNA in *X. laevis* oocytes

In vitro transcribed cRNA was diluted to 0.6 µg/µl final concentration and dispensed into aliquots of 3 µl and stored at -80°C. These aliquots were taken out prior to injection. Transjector 5246 (Eppendorf, Germany) was used to inject RNA aliquots into collagenase treated oocytes. Glass capillaries GB 100 F10 (Scientific products GmbH, Germany) were pulled to yield capillaries with a tapered end. These were filled with RNA aliquot to be injected and affixed onto a microinjector. The tip of the capillary was broken with the help of fine tweezers (Dumont, Switzerland) and the parameters of injector manipulated till a drop of 50 nL was obtained, as observed under the microscope (LEICA). The drop volume was computed by measuring the diameter of drop under 1.6 x magnifications. The injection capillary was inserted into individual oocytes lined up in a injection Petri dish with the help of manipulator. Oocytes were observed for slight swelling caused by injection, after which the capillary was carefully taken out of the oocyte, and the procedure continued. After injection, oocytes were maintained in ND96 with Penincillin-Streptomycin and Sodium pyruvate at 18°C.

2.2.6 Measurement of radioisotope accumulation

X. laevis oocytes injected with the appropriate cRNA were used for uptake experiments 2-3 days after injection. Experiments were carried out by incubating groups of 10 ooyctes in 2 ml tubes containing 200 µl of uptake assay buffer. Uptake assay buffer was ND96 pH 6.0 containing non-labelled drug and the radioactively labelled form of the drug; the latter was used at a final concentration of 50 nM. Oocytes were first washed with ND96 pH 6.0 before

adding to the uptake assay buffer. Uptakes were carried out at 25°C, and after the desired amount of incubation time, oocytes were taken out add washed 3 times with ice-cold ND96 pH 6.0 containing only the non-radioactive substrate, in a 24-well cell culture plate. After washing, oocytes were individually placed in 2 ml liquid scintillation counting vials (Beckman, Germany) and lysed with 200 µl of 5% SDS. Having homogenized the lysed oocytes by vortexing, 2 ml of liquid scintillation counting cocktail was added, vials closed with screw-caps and vortexed before proceeding to counting. Counting was done with a Liquid scintiallation counter (Beckman, Germany) which measured the amount of radioactive label incorporated in ocytes in counts per minute (cpm). Using the specific activity of the radiolabelled compound and the amount of cold substrate in the uptake buffer, total amount of the compound incorporated was calculated in picomoles.

2.2.7 Data analysis

Data were analyzed with Prism 3.0 (GraphPad Software) and SigmaPlot 11.0 (Systat Software Inc.). Graphs were drawn using SigmaPlot 11.0.

3 Results

3.1 *X. laevis* oocytes as a system to measure CQ transport

PfCRT expressed in *X. laevis* oocytes has been shown to have saturable Michaelis-Menten kinetics for chloroquine transport (Martin *et al.*, 2009a). It was, however, shown in this work that PfCRT being a vacuolar membrane protein harbours a number of putative targeting motifs in its N and C-termini, which target the protein to cellular compartments other than the plasma membrane upon expression in oocytes. The same study showed that mutating the putative motifs to alanine allows efficient plasma membrane expression, leading to increased accumulation of CQ in oocytes. This approach was used here to express PfCRT in *X. laevis* oocytes. Fig. 3.1 shows in detail the changes made in the terminal portions of PfCRT. The motif free form of the protein has been termed PfCRT*.

N-terminus

```
                1         10        20        30        40        50
                |....|....|....|....|....|....|....|....|....|....|
PfCRT           MKFASKKNNQKNSSKNDERYRELDNLVQEGNGSRLGGGSCLGKCAHVFKLI
PfCRT*          MKFASKKNNQKNSSKNAERARAADNAAQEGNGSRLGGGSCLGKCAHAAKAA
```

C-terminus

```
                     410       420
                |....|....|....|....
PfCRT           NEENEDSEGELTNVDSIITQ
PfCRT*          NEENADSAGALTNVDSAATQ
```

Figure 3.1: Mutating putative trafficking motifs in PfCRT.

Putative membrane trafficking motifs in the N and C termini of PfCRT were mutated to alanine residues (highlighted in grey) to ensure targeting to the oocyte plasma membrane. PfCRT refers to the motif-replete form whereas PfCRT* is the motif-free version.

Table-1: Differences in the amino acid sequence of PfCRT HB3 and Dd2 alleles

Resistance	Allele	Origin	Amino acid position of mutations in PfCRT										
			72	**74**	**75**	**76**	**144**	**160**	**220**	**271**	**326**	**356**	**371**
CQS	**HB3**	Honduras	C	M	N	K	A	L	A	Q	N	I	R
CQR	**Dd2**	Indochina	C	I	E	T	A	L	S	E	S	T	I

CQS = Chloroquine sensitive, CQR = Chloroquine resistant

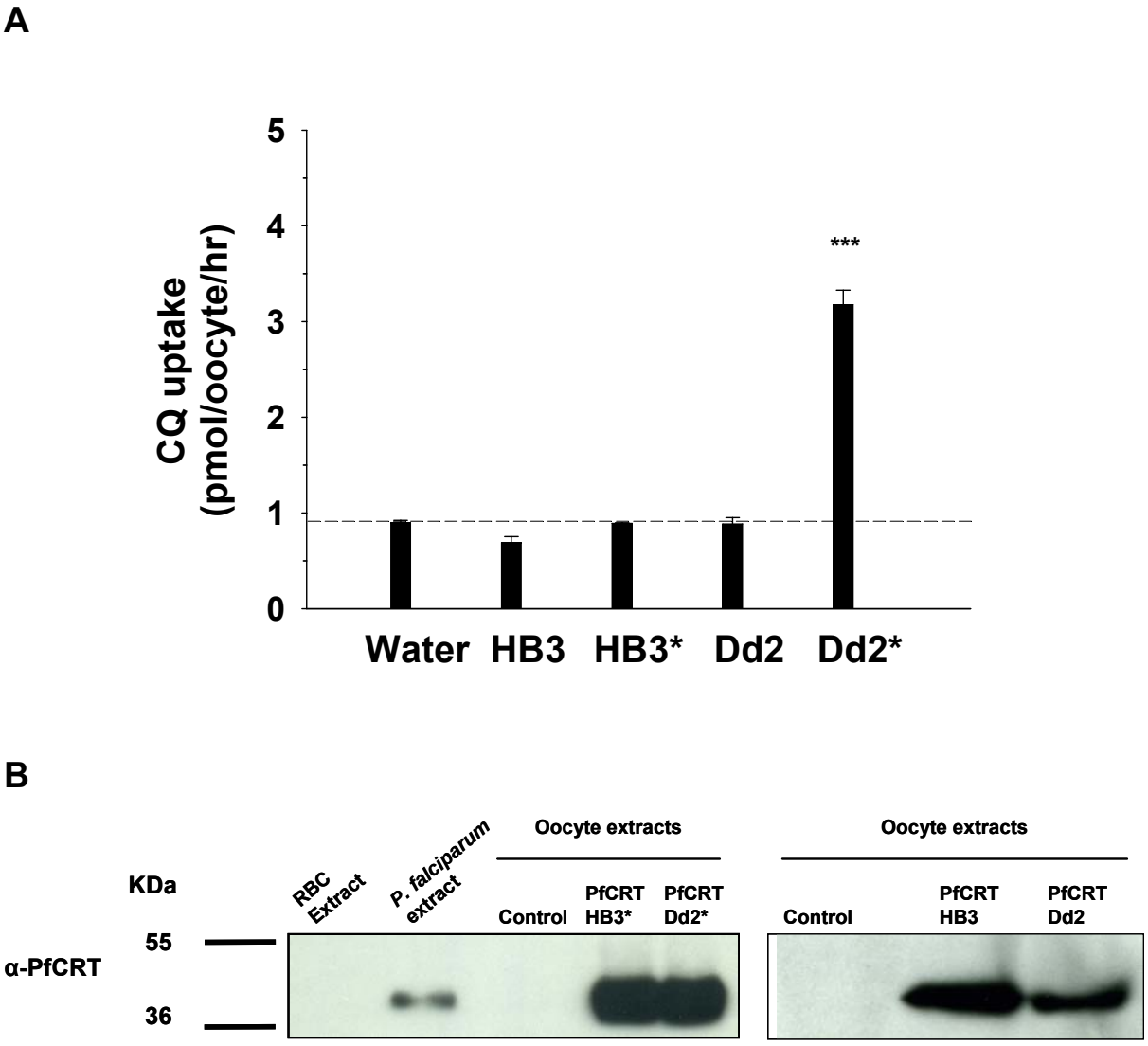


Figure-3.2: Expression and activity of trafficking motif free PfCRT in PfCRT

A. Uptake of chloroquine in PfCRT injected oocytes. cRNA for HB3 (motif replete), Dd2 (motif replete), HB3* (motif free) and Dd2* (motif free) was injected. Only the motif free PfCRT Dd2* showed significant accumulation of CQ as compared to water injected control (*** = $p < 0.001$). Data are presented as mean ± SEM from three independent measurements.

B. Western blots showing expression of PfCRT alleles in *X. laevis* oocytes. PfCRT lacking putatitve targeting motifs has been shown as PfCRT mut. RBC and *P. falciparum Dd2* infected RBC (iRBC) were used as negative and positive controls respectively for PfCRT antibody.

Yeast-codon optimized PfCRT HB3 and Dd2 coding sequences were cloned in the pSP64T vector as described in materials and methods. As for the motif free HB3 and Dd2, these were synthesized by GeneART AG and subsequently cloned into pSP64T vector. *In vitro* RNA to

be injected in oocytes was transcribed from these constructs as per the protocol given in materials and methods.

CQ accumulation was measured in oocytes injected with both motif free and motif replete forms of PfCRT, as a proof of principle (Fig 3.2). Table-1 shows the differences between the amino acid sequences of the HB3 (chloroquine sensitive) and Dd2 (chloroquine resistant) alleles. *X. laevis* oocytes were injected with RNA for the HB3 and Dd2 alleles of PfCRT in both motif-replete and motif-free forms. Uptake of CQ was measured 3 days post injection in pH 6.0 buffer containing 10 μM chloroquine and 50 nM [^{3}H] CQ. Only oocytes injected with motif-free Dd2 allele showed significant increase ($p < 0.001$, t-test) in chloroquine uptake as compared to water injected controls. Uptake in case of HB3 injected oocytes was similar to control irrespective of the motif-replete or free version (Fig 3.2A). A Western analysis carried out to check expression of the injected cRNA verified that in all cases PfCRT was expressed (Fig 3.2B). Moreover, the anti-PfCRT antibody identified a band specific to infected erythrocytes, and of a similar molecular weight, showing that the protein being expressed in oocytes is indeed PfCRT. All PfCRT alleles mentioned hereafter refer to the motif free form.

3.2 CQ uptake mediated by PfCRT Dd2 is time dependent, saturable and sensitive to verapamil inhibition

To ascertain the dependence of CQ uptake on time, a time-course was carried out as shown in Fig 3.3. Oocytes injected with water, PfCRT HB3 and Dd2 were incubated for 3 days after injection with 10 μM CQ and 50 nM [^{3}H] CQ at pH 6.0 in groups of 8-10 oocytes. Drug accumulation in oocytes was measured at different time points, and plotted as a function of time. Data points were fitted using an exponential rise to maximum function.

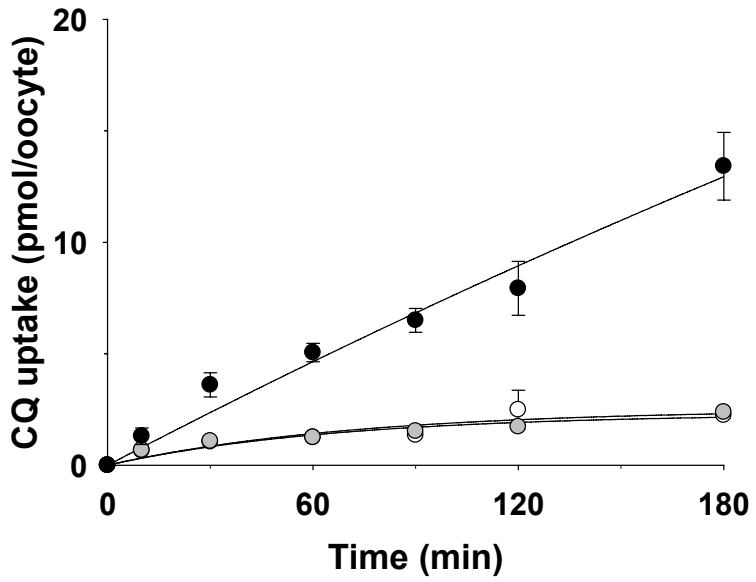


Fig 3.3: Time course of chloroquine uptake in PfCRT injected oocytes.

Accumulation of chloroquine (CQ) was measured at varying time points for water-injected control (open circles), PfCRT HB3 injected (grey-filled circles) and PfCRT Dd2 injected oocytes (black-filled circles). Each data point represents mean ± SEM. (n = 3, with 10 oocytes per time point in each experiment).

Fig 3.3 shows that with increasing time, the difference of uptake between PfCRT Dd2 injected and water injected control oocytes increased. Accumulation in PfCRT HB3 injected oocytes was similar to water-injected control oocytes at all time points. Dd2 mediated CQ uptake remained linear over time upto 180 minutes.

After measuring the dependence on time, the relationship between uptake and substrate concentration was tested (Fig 3.4). Accumulation was measured at 60 minutes time point as this lay within the linear phase of CQ uptake, as demonstrated by the time course in Fig 3.3. Because PfCRT HB3 injected oocytes did not show increase an CQ accumulation as

compared to controls, the substrate dependence was measured only for PfCRT Dd2. Oocytes injected with PfCRT Dd2 were incubated for 60 minutes in pH 6.0 buffer containing increasing amounts of unlabelled CQ, along with 50 nM of [^{3}H] CQ. Accumulation measured in controls was substracted from that in Dd2 injected oocytes, and plotted as a function of the substrate concentration (Fig 3.4). Data points were fitted with the Michaelis-Menten equation $V = V_{max}*S/K_m+S$ (Atkins, 2010) where V is the uptake, V_{max} is maximum velocity of uptake, K_m is the Michaelis-Menten constant and S is the substrate concentration. These curves also yielded the apparent maximum velocity of uptake V_{max} and the Michaelis-Menten constant K_m. Fitting the points with a modified version of the Michaelis-Menten equation gave V_{max}/K_m values.

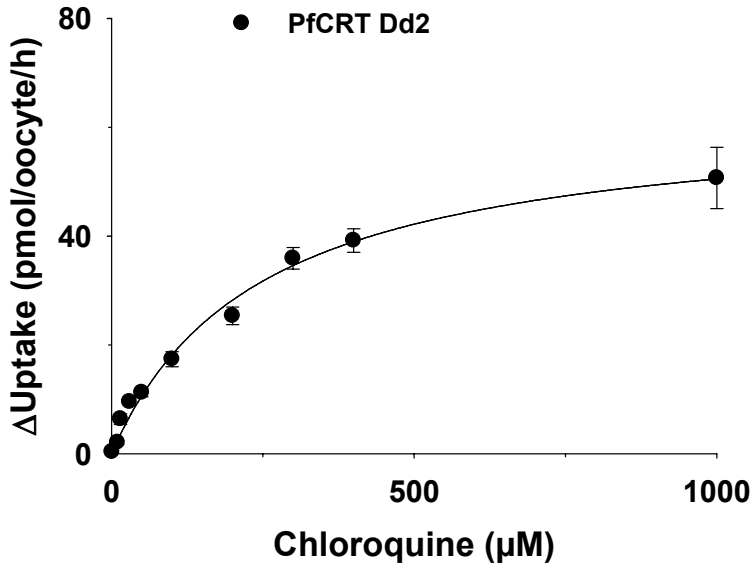


Fig 3.4: Concentration dependence of chloroquine uptake in PfCRT injected oocytes.

Accumulation of chloroquine (CQ) measured at increasing concentration for water-injected control (open circles), PfCRT HB3 injected (grey-filled circles) and PfCRT Dd2 injected oocytes (black-filled circles), and their difference plotted as a function of substrate concenration. Each data point represents mean ± SEM. (n = 3, with 10 oocytes per time point in each experiment).

CQ uptake mediated by PfCRT Dd2 initially increased with increasing amounts of CQ, and eventually approached saturation (Fig 3.4). Apparent K_m and V_{max} values of 245 ± 32 μM and 63 ± 3 picomoles oocyte^{-1} hour^{-1} were obtained from the curve fit. These were similar to previously measured values for these parameters (Martin *et al.*, 2009b). A V_{max}/K_m value of 0.256 ± 0.022 μl oocyte^{-1} hour^{-1} was also obtained.

In order to confirm that the CQ accumulation was a result of PfCRT transport activity, uptake was inhibited with verapamil, a known chemosensitizer of CQ resistance and a blocker of PfCRT (Henry *et al.*, 2006). PfCRT Dd2 injected and water injected control oocytes were incubated in pH 6.0 buffer containing 10 μM CQ with 50 nM [^{3}H] CQ. In another group, both control and Dd2 oocytes were incubated in a buffer containing the same amount of labelled and un-labelled CQ, but supplemented with 100 μM of Verapamil. Each group contained 10 oocytes and was incubated for 60 minutes in the uptake buffer, after which accumulation was measured.

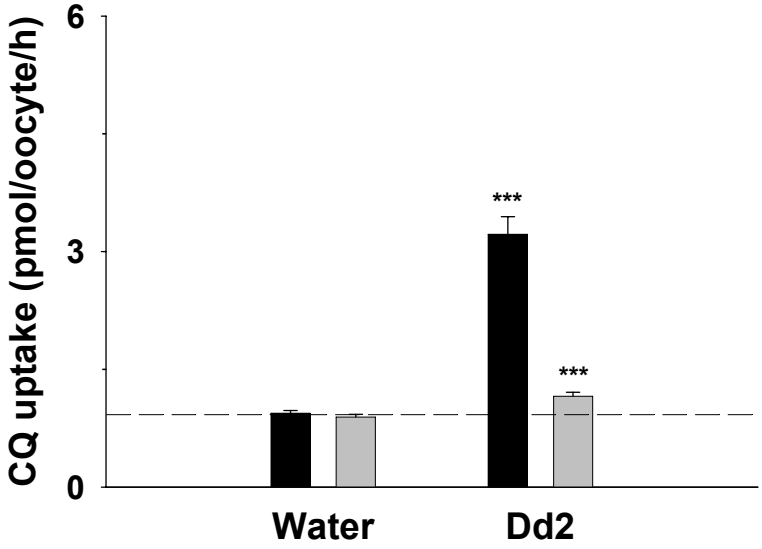


Fig 3.5: Inhibition of PfCRT Dd2 mediated CQ transport with Verapamil

Accumulation of chloroquine (CQ) was measured with 10 μM CQ, in absence (black bars) and presence (grey bars) of 100 μM verapamil. Bars represent means ± SEM. from 4 independent experiments with 10 oocytes per group in each. Stars stand for p-values obtained from a t-test (*** = $p < 0.001$).

Fig 3.5 shows the result of CQ uptake performed in presence of verapamil. Uptake of CQ in water injected control oocytes was not influenced by verapamil. PfCRT Dd2 injected oocytes accumulated significantly more CQ than controls ($p < 0.001$, t-test). However, uptake of CQ in Dd2 injected oocytes incubated with verapamil was significantly less in comparison to oocytes without verapamil ($p < 0.001$, t-test). This showed that the CQ uptake mediated by PfCRT Dd2 is specific and inhibitable with verapamil.

3.3 Quinine and quinidine are substrates for mutant PfCRT

Time-course, concentration dependence and verapamil inhibition of CQ uptake were carried out to establish a PfCRT transport system in *X. laevis* oocytes, as shown in a previous study (Martin *et al.*, 2009b). Having achieved this, the next question was whether other aminoquinolines such as quinine (QN) and its stereoisomer quinidine (QD) are also substrates of PfCRT mediated drug transport. In order to answer this question, a time-course was first performed for quinine and quinidine. Water injected, PfCRT HB3 injected and PfCRT Dd2 injected oocytes were incubated at room temperature in pH 6.0 buffers with 10 μM of unlabelled and 50 nM of [^{3}H]-labelled quinine and quinidine each. Incubation was carried out for increasing time points, after which drug accumulation was measured. These time-courses are shown in Fig 3.5 (on the following page).

Similar to chloroquine, the accumulation of both QN and QD increased in PfCRT Dd2 injected oocytes over time, in comparison to water injected and PfCRT HB3 injected oocytes. In both cases, uptake was linear upto at least 2 hours. Uptake of both substrates in PfCRT HB3 injected oocytes was similar to that for control. This showed that both quinine and quinidine are substrates for PfCRT Dd2 mediated transport.

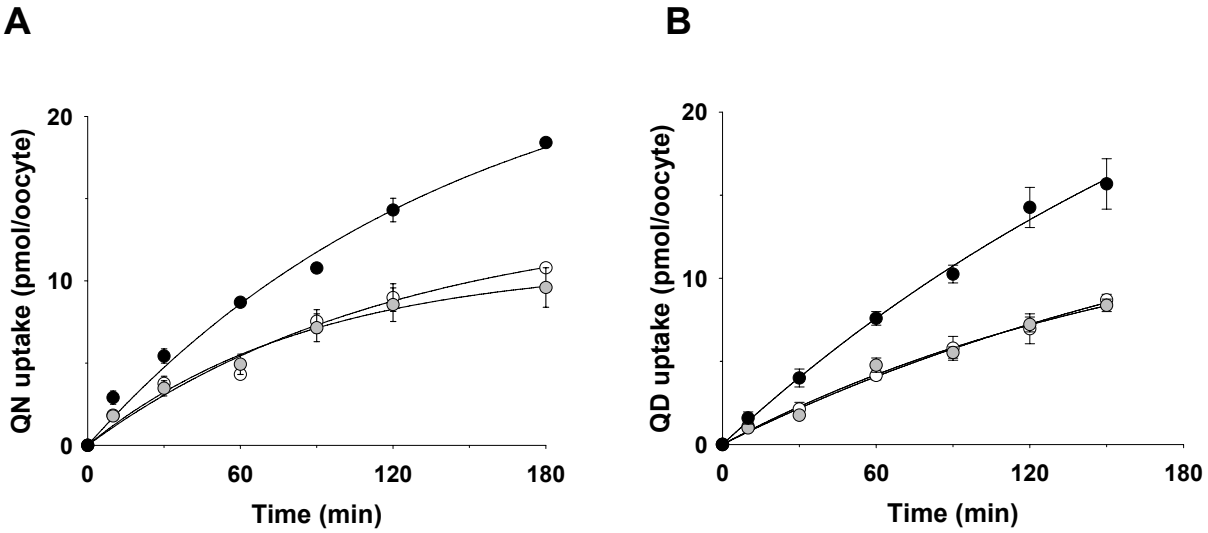


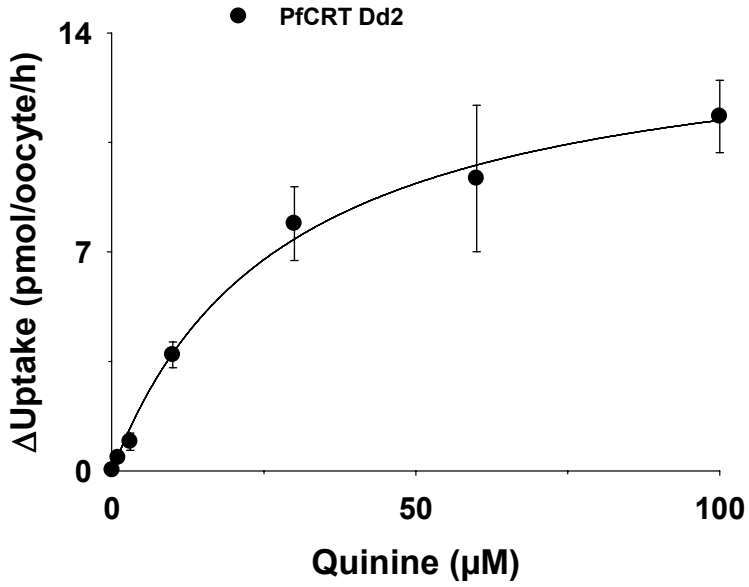
Fig 3.6: Time course of quinine and quinidine uptake in PfCRT injected oocytes.

A. Accumulation of quinine (QN) measured at varying time points for water-injected control (open circles), PfCRT HB3 injected (grey-filled circles) and PfCRT Dd2 injected oocytes (black-filled circles). Each data point represents mean ± SEM. (n = 3, with 10 oocytes per time point in each experiment).

B. Uptake of quinidine (QD) measured in water-injected control (open circles), PfCRT HB3 (grey circles) and PfCRT Dd2 (black filled circles) injected oocytes. Data represents mean ± SEM. from 3 independent experiments where 10 oocytes per time point were used.

The next step was to see if quinine and quinidine accumulation was saturable or not. For this purpose, water injected control oocytes and PfCRT Dd2 injected oocytes were incubated with increasing amounts of unlabelled QN and QD, along with 50 nM each of [^{3}H]-labelled QN and QD. This was carried out at room temperature in pH 6.0 buffer. Accumulation was measured at 60 minutes of incubation as this time point fell within the linear phase of uptake.

A



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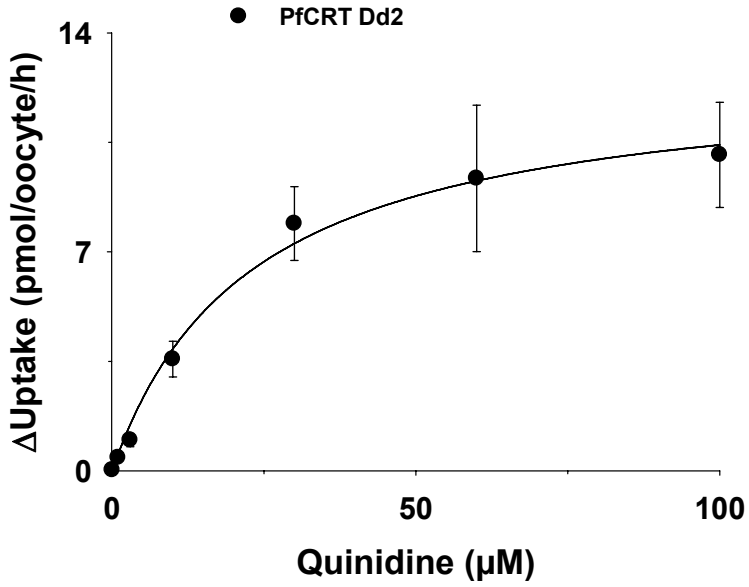


Fig. 3.7: Substrate dependence of QN and QD uptake in PfCRT injected oocytes.

Difference in uptake between water-injected control oocytes and PfCRT Dd2 injected oocytes has been plotted as a function of substrate concentration. Each point represents mean ± SEM from 3 independent experiments, where 8-10 oocytes were used per data point in each experiment.

PfCRT Dd2 mediated QN and QD transport was plotted as a function of substrate concentration, after substracting the control values from those for PfCRT Dd2 (Fig 3.7). Data points were fitted using a Michaelis-Menten equation. Accumulation of both QN and QD

increased with increasing substrate concentration, culminating in a plateau phase. This was indicative of saturation of transport by increasing QN and QD concentrations. PfCRT Dd2 showed transport of QN and QD quite similar to that for CQ, thus indicating that PfCRT is also a carrier for these two substrates.

Curve fitting yielded kinetic parameters which have been summarized below, along with those for obtained for CQ transport:

Table-2: Apparent maximum velocity, Michaelis-Menten constant and V_{max}/K_m for chloroquine, quinine and quinidine transport

Substrate	PfCRT	Apparent V_{max} (pmol/oocyte/hr)	Apparent K_m (μM)	Apparent V_{max}/ K_m	Goodness of fit (R^2)
Chloroquine	Dd2	63 ± 3	245 ± 34	0.256 ± 0.022	0.9888
Quinine	Dd2	14.53 ± 0.94	31.4 ± 5.4	0.509 ± 0.051	0.9936
Quinidine	Dd2	12.65 ± 0.55	21.3 ± 2.9	0.560 ± 0.071	0.9954

To check for inhibition of QN accumulation with verapamil, oocytes were incubated with and without 100 μM Verapamil. The result of such an experiment is shown in Fig 3.8.

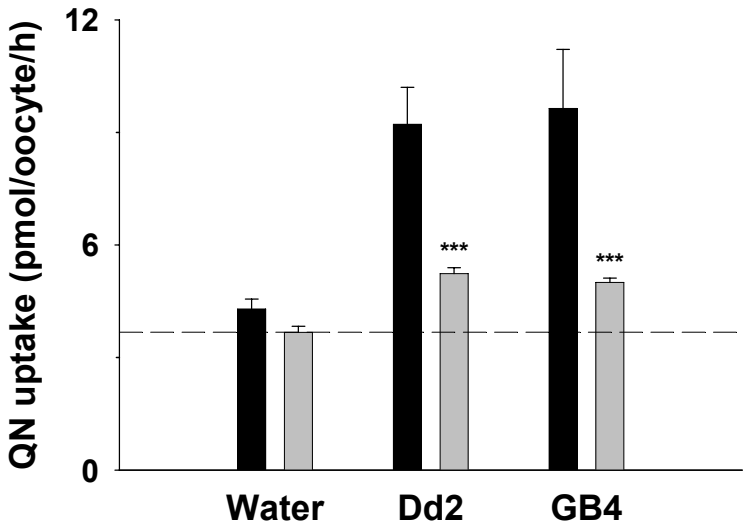


Fig. 3.8: Inhibition of QN uptake with Verapamil

Bars represent accumulation of 10 µM QN in oocytes, with 100 µM Verapamil (grey bars) and without verapamil (black bars). Each bar represents mean ± SEM. from 2 independent experiments, where 8-10 oocytes were used per group. (*** = $p < 0.001$).

It is evident from Fig 3.8 that accumulation of verapamil in Dd2 and GB4 PfCRT injected oocytes decreased significantly ($p < 0.001$, t-test) as compared to oocytes without verapamil. Uptake in water injected control oocytes remained unaffected. Thus, similar to CQ, PfCRT mediated QN transport is sensitive to verapamil.

3.4 Naturally occurring mutant pfcrt alleles transport CQ

How does CQ transport by PfCRT compare to that by other mutant *pfcrt* alleles? In order to answer this question, five other variants of mutant *pfcrt* were cloned in the oocyte expression vector pSP64T. These were the, 7G8 (Brazil), Ecu1110 (Ecuador), Ph1 & Ph2 (Phillippines) and GB4 (Ghana) alleles (Chen *et al.*, 2003, Fidock *et al.*, 2000, Sa et al., 2009). Countries indicated in brackets stand for the places from which these strains had been isolated. They represent distinct origins of chloroquine resistance, and the differences in amino acid sequence they encode for are summarized in table-3.

Table-3: Mutant *pfcrt* variants from different geographical origins

Allele	Origin	Amino acid position of mutations in PfCRT										
		72	**74**	**75**	**76**	**144**	**160**	**220**	**271**	**326**	**356**	**371**
Dd2	South-east asia	C	I	E	T	A	L	S	E	S	T	I
GB4	S.E.Asia/Africa	C	I	E	T	A	L	S	E	N	I	I
Ecu1110	South-america	C	M	N	T	A	L	S	Q	D	L	R
7G8	South-america	S	M	N	T	A	L	S	Q	D	L	R
Ph1	Phillippines	C	M	N	T	T	Y	A	Q	D	I	R
Ph2	Phillippines	S	M	N	T	T	Y	A	Q	D	I	R

Yeast-codon optimized coding sequences for PfCRT 7G8 and Ph1 were synthesized by GeneART AG and subsequently cloned into pSP64T vector. Ecu1110 and Ph2 coding sequences were constructed by mutating cysteine at position 72 to a serine. Oocytes were injected with mRNA transcribed *in vitro* from these plasmids. PfCRT Dd2 injected and water injected oocytes served as positive and negative controls respectively. 3 days after injection, oocytes injected with all these alleles were subjected to a CQ uptake experiment. Oocytes were incubated for 60 minutes at room temperature in pH 6.0 buffer containing 10 µM CQ and 50 nM [^{3}H CQ]. Results of this CQ screen have been shown in Fig 3.9.

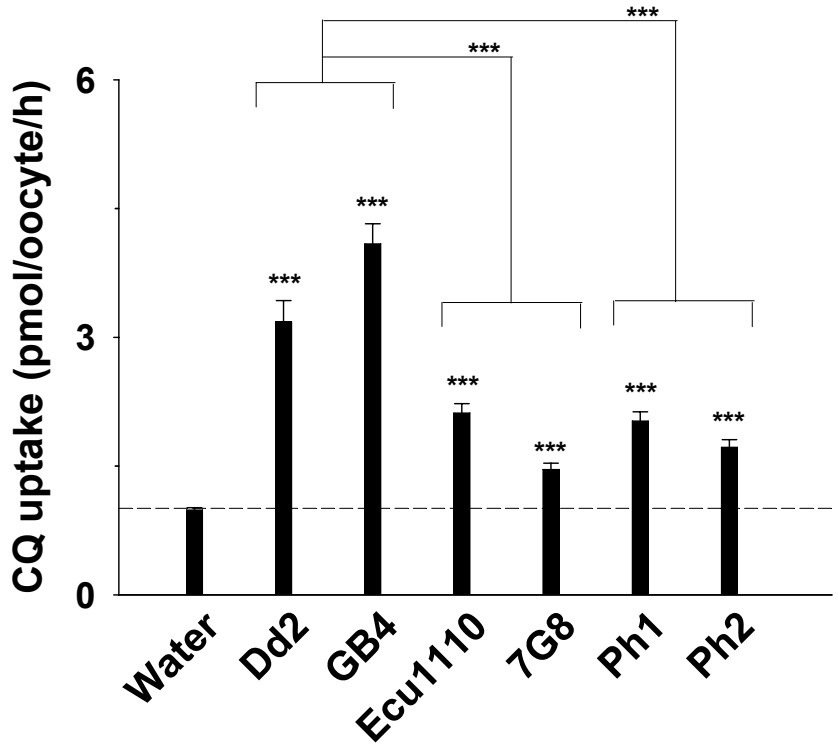
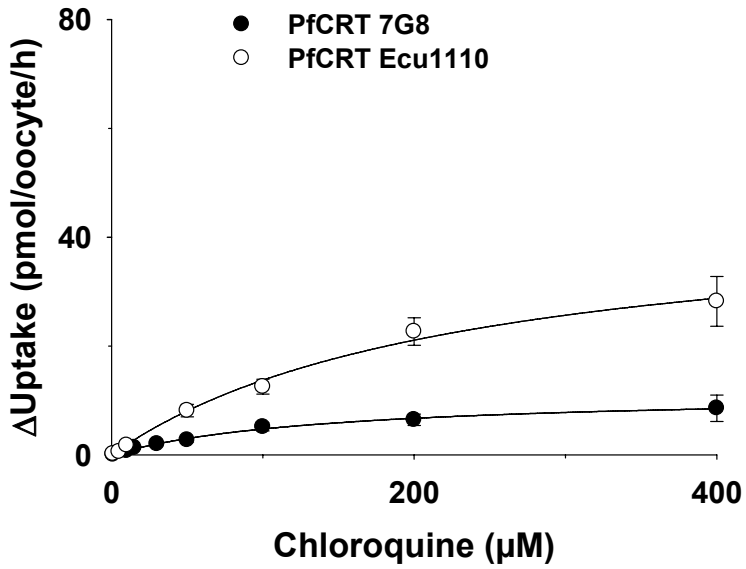


Fig 3.9: Screening naturally occurring mutant *pfcrt* alleles for chloroquine uptake

Oocytes injected with different PfCRT alleles were checked for chloroquine (CQ) uptake after one hour incubation in pH 6.0 buffer containing 10 µM unlabelled and 50 nM [^{3}H]CQ respectively. The dotted line in the middle represents the mean uptake obtained in water injected control oocytes. Data represents means ± SEM. from 4-5 independent experiments with 10 oocytes per allele in each. (*** = $p < 0.001$).

Fig 3.9 shows that injection of all the six mutant PfCRT variants led to increased uptake of CQ in comparison to water injected control oocytes ($p < 0.001$, t-test). The South-East Asian/African alleles (Dd2, GB4) accumulated significantly more CQ than did oocytes injected with South-American (7G8, Ecu1110) or Phillipine (Ph1, Ph2) alleles ($p < 0.001$, t-test). Thus, this experiment demonstrated that different alleles of mutant *pfcrt* can mediate CQ uptake upon expression in oocytes. However, the differences in the levels of accumulation suggest that differences in the mutated amino acid residues may influence the kinetics of CQ transport. To investigate this point further, concentration dependence of CQ uptake was measured for these alleles. The method employed was similar to that for PfCRT Dd2, where accumulation of CQ was measured at increasing concentrations of unlabelled CQ. Uptake was measured at the 60 minut incubation time point, and values obtained for water injected control oocytes were substracted from those for PfCRT injected oocytes. The resultant accumulation values were plotted as a function of the CQ concentration, and have been featured in Fig 3.19 and 3.11. Data points were fitted using the Michaelis-Menten equation.

A



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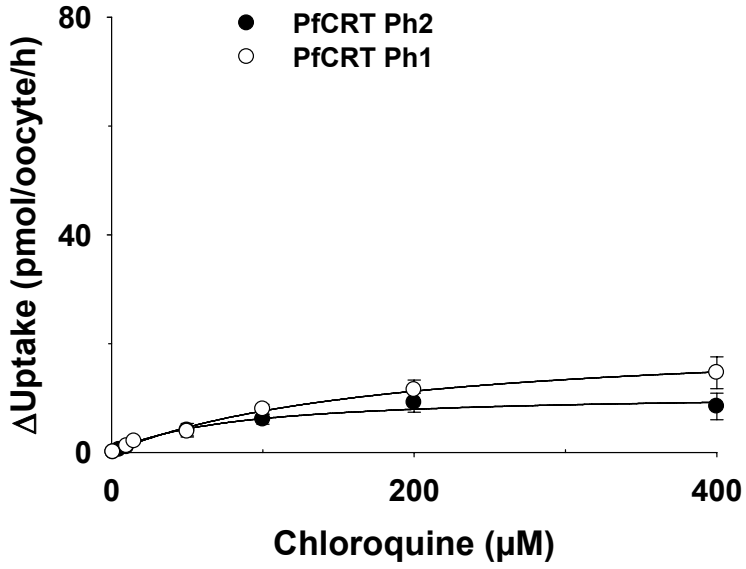


Fig 3.10: Dependence of CQ uptake on substrate concentration for mutant PfCRT alleles

CQ accumulation in water injected control oocytes was substrated from that in PfCRT injected oocytes, and plotted against concentration. Data represents means ± SEM from 3 independent experiments with 10 oocytes per allele per data point in each.

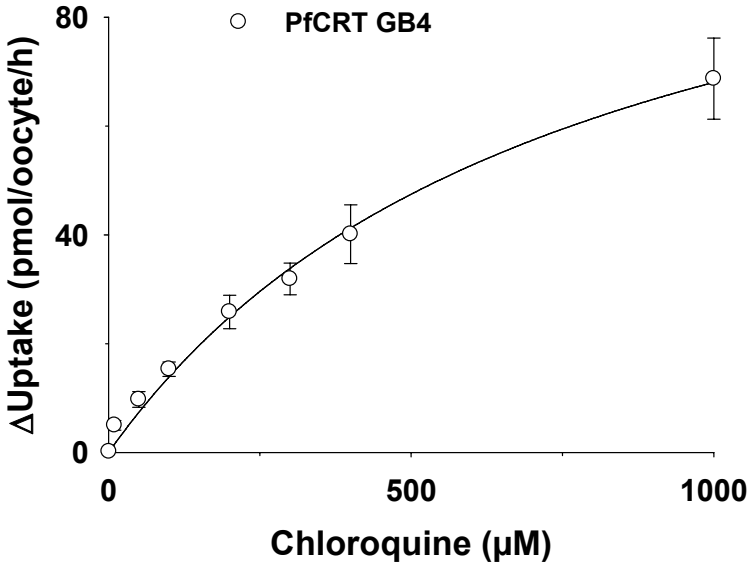


Fig 3.11: Dependence of CQ uptake on substrate concentration for PfCRT GB4

CQ accumulation as a function of concentration, plotted for PfCRT GB4 after background substraction. Data represents means ± SEM. from 3 independent determinations with 10 oocytes per data point in each.

Curves in Fig. 3.10 and 3.11 yielded apparent V_{max}, K_m and V_{max}/K_m values. These, along with those obtained earlier for PfCRT Dd2, are shown in Table-3 below.

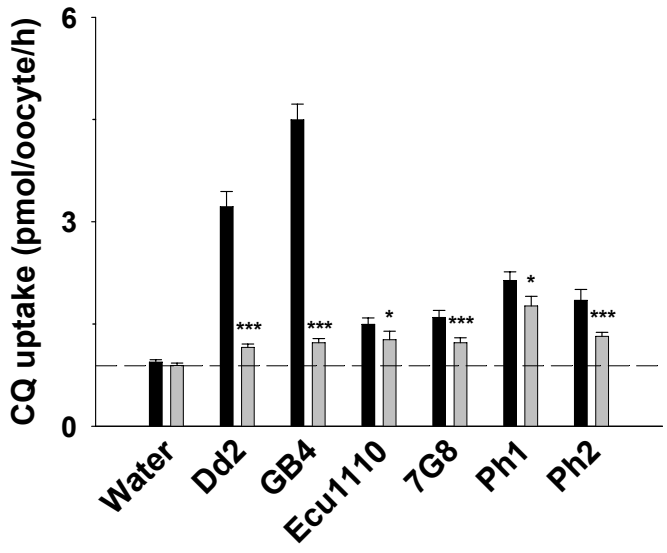
Table-4: Apparent V_{max}, K_m and V_{max}/K_m obtained for mutant PfCRT.

Allele	Apparent CQ V_{max} [pmol/oocyte/h]	Apparent CQ K_m [μM]	Apparent V_{max}/K_m for CQ	Goodness of fit (R^2)
Dd2	63 ± 3	245 ± 32	0.256 ± 0.022	0.9902
GB4	120 ± 10	763 ± 118	0.157 ± 0.013	0.9926
Ecu1110	45 ± 4	231 ± 40	0.197 ± 0.018	0.9943
7G8	11.5 ± 0.6	141 ± 16	0.081 ± 0.005	0.9941
Ph1	21.5 ± 1.4	182 ± 26	0.118 ± 0.001	0.9948
Ph2	11 ± 1	76 ± 23	0.144 ± 0.031	0.9746

K_m is independent of the amount of carrier protein, whereas V_{max} is not (Atkins, 2010).The apparent Michaelis-Menten constants for different PfCRT variants could therefore be compared with a t-test without taking into account individual expression level of these alleles in the oocyte. This showed that Dd2 and GB4 ($p < 0.01$), Ph1 and Ph2 ($p < 0.05$), Ecu1110 and Ph2 ($p < 0.05$) K_m values were significantly different. GB4 K_m was found to be significantly different for that of Ecu1110 ($p < 0.001$), 7G8 ($p < 0.01$), Ph1 ($p < 0.001$) and Ph2 ($p < 0.001$). While the same result was not seen when Ecu1110 and 7G8 PfCRT were compared ($p = 0.1049$), this may have been due to the comparatively higher experimentally error in the 7G8 measurements. Ecu1110 and Ph1 too did not differ significantly in their CQ K_m. These K_m comparisons highlight that single (as between Ph1 and Ph2), or at the most two amino acid substitutions (as between Dd2 and GB4) could significantly alter the half-saturation constant for CQ. The significant difference in K_m between Ecu1110 & Ph2 and that between Ph1 & Ph2, but not between Ph1 & Ecu1110 and 7G8 & Ph2 suggests that having the amino residue serine at position 72 implies a PfCRT with lower K_m for CQ, as compared to a PfCRT with cysteine at the same position.

To confirm that CQ accumulation observed in Fig. 3.9 was indeed because of transport through PfCRT, a verapamil inhibition experiment was performed. This was carried out in a similar way as done earlier for PfCRT Dd2. Oocytes injected with a particular allele were divided into two groups and incubated for 60 minutes in the uptake assay buffer. Both groups contained 10 µM of unlabelled and 50 nM of ^{3}H CQ, whereas only had 100 µM Verapamil in addition. Water injected and PfCRT Dd2 injected oocytes were used as negative and positive controls respectively. The results obtained are shown in Fig 3.12.

A



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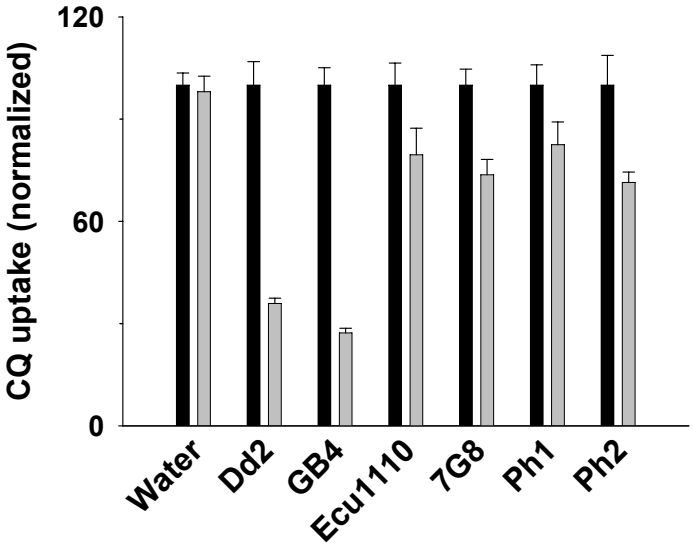


Fig 3.12: Inhibition of CQ uptake in PfCRT injected oocytes with Verapamil

A. CQ accumulation in oocytes was measured with (grey bars) and without (black bars) 100 μM Verapamil. Stars represent p-values from t-tests performed to measure significance of the Verapamil induced decrease in CQ uptake (* = $p<0.05$, *** = $p<0.001$). Bars represent means ± SEM. from 4 independent experiments with 8-10 oocytes in each.

B. shows data from Panel A in a normalized format. For each group, uptake with CQ alone has been normalized to converted to 100% (black bars). CQ uptake in presence of verapamil (grey bars) is thus expressed as the fraction of uptake without Verapamil.

Fig 3.12A shows that Verapamil inhibited CQ uptake in PfCRT injected oocytes, whereas in water injected control oocytes it did not have an effect. This is consistent with uptake in control being a result of CQ diffusing through the oocyte plasma membrane. The elevated uptake in PfCRT injected oocytes, on the other hand, was mediated by PfCRT and could therefore be inhibited. However, the significance of this reduction varied amongst PfCRT variants. Inhibition was highly significant ($p < 0.001$, t-test) for the Dd2, GB4, 7G8 and Ph2 alleles, but only slightly significant ($p < 0.05$, t-test) for PfCRT Ecu1110 and Ph1. To highlight these differences, data from Panel A of Fig. 3.12 have been expressed as percentage of uptake with CQ alone in Fig. 3.12B. For each group of each injected oocytes, accumulation with CQ alone is shown as 100%. The inhibition of CQ uptake by verapamil is thus the percentage of uptake with CQ alone. It is clear that for the Dd2 and GB4 alleles uptake with verapamil was about 25-30% of uptake without verapamil. This figure was around 75 % for Ecu1110, 7G8, Ph1 and Ph2 PfCRT. Thus, the South-American and Phillipine PfCRT variants showed only slight reduction in CQ uptake when verapamil was used, whereas such a reduction in the South-East Asian/African PfCRT was much more pronounced.

3.5 Mutant PfCRT with only three amino acid changes can still transport CQ

The data presented so far argue that mutant PfCRT alleles can transport CQ, although with different kinetic properties. On the other hand wild-type PfCRT does not lead to accumulation of CQ when expressed in oocytes. So what then is the minimum change required to convert the wild-type PfCRT into a mutant PfCRT capable of CQ uptake? A look at Table-2 shows that both Ph1 and Ecu1110 alleles are mutated at only 4 positions in comparison to the wild-type, although only two of them, namely K76T and D326N are present in both. Replacing the mutated residues with wild-type amino acids would show which of the substitutions are necessary for CQ transport. The Ecu1110 was chosen for this purpose because the susbtitutions it contains are found more commonly among mutant *pfcrt* than those from Ph1. The constructs that were used in this experiment have been shown in Table-5.

Table-5: Single amino acid replacement constructs made with PfCRT HB3 and Ecu1110.
Residues shaded that differ from the wild-type PfCRT HB3 have been shaded in grey.

PfCRT	Amino acid position of mutations in PfCRT										
	72	74	75	76	144	160	220	271	326	356	371
HB3	C	M	N	K	A	L	A	Q	N	I	R
HB3^{K76T}	C	M	N	T	A	L	A	Q	N	I	R
Ecu1110	C	M	N	T	A	L	S	Q	D	L	R
Ecu1110^{T76K}	C	M	N	K	A	L	S	Q	D	L	R
Ecu1110^{S220A}	C	M	N	T	A	L	A	Q	D	L	R
Ecu1110^{D326N}	C	M	N	T	A	L	S	Q	N	L	R
Ecu1110^{L356I}	C	M	N	T	A	L	S	Q	D	I	R

Mutant constructs shown above were synthesized by megaprimer synthesis method, and cloned into the pSP64T vector. PfCRT HB3, HB3^{K76T} and Dd2^{T76K}have earlier been shown not to transport CQ (Martin *et al.*, 2009b). Thus, only the lysine to threonine change cannot account for CQ transport even if it is necessary. HB3^{K76T} and Ecu1110^{T76K} were hence used as negative controls, whereas Ecu1110 functioned as a positive control. Uptake in oocytes injected with PfCRT Ecu1110^{S220A}, Ecu1110^{D326N} and Ecu1110^{L356I} was compared to those injected with Ecu1110. In each case, one amino acid had been mutated back to the wild-type counterpart, thus creating a PfCRT with only 2 more mutations apart from K76T.

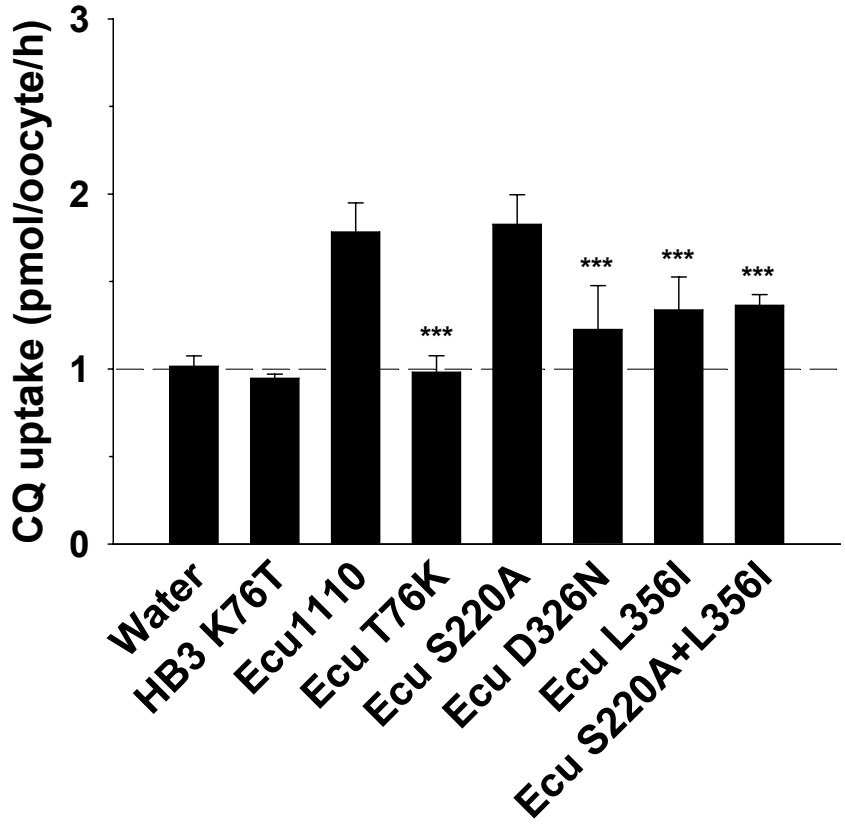


Fig 3.13: Uptake of chloroquine in PfCT HB3 and Ecu1110 mutant constructs

Data represent means with SEM obtained from 3 independent accumulation experiments where 10 oocytes per group were used. Stars represent p-values from t-test (*** = $p<0.001$).

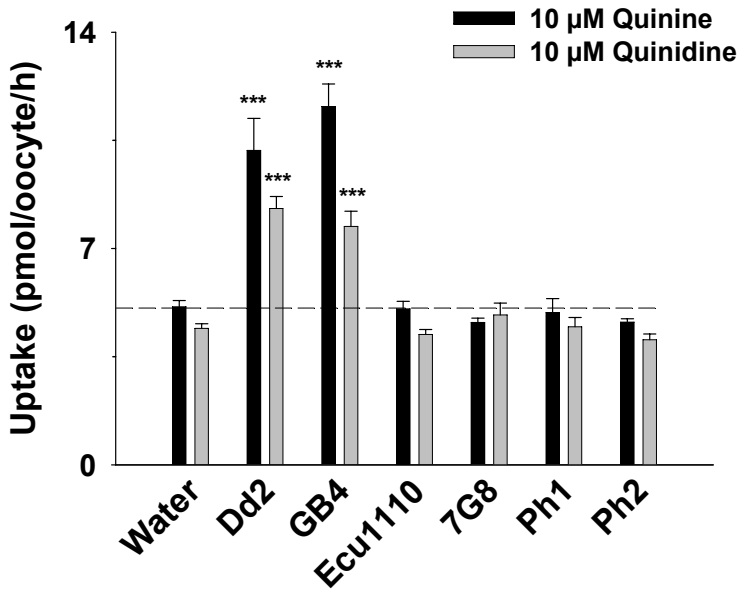
Fig 3.13 features the result of an accumulation experiment performed with 10 μM of CQ. It shows that oocytes injected with PfCRT Ecu1110 accumulated significantly more CQ than those injected with water (i.e. control) or $HB3^{K76T}$. The same figure also shows that introduction of T76K, D326N and L356I substitutions reduced CQ uptake. This reduction was to the level of water injected control oocytes in case of T76K, but not as much for D326N or L356I. In each case, reduction in comparison to Ecu1110 was significant (t-test, $p < 0.001$). $Ecu1110^{S220A}$ on the other hand showed no significant difference in CQ accumulation with reference to Ecu1110. It can be concluded from this experiment that only 3 amino acid substitutions – K76T, D326N and L356I can still account for CQ uptake in oocytes.

3.6 PfCRT mutations influence transport of quinine and quinidine

Experiments listed previously establish that mutant PfCRT alleles had saturable kinetics for CQ accumulation. Of these, PfCRT Dd2 showed accumulation of QN and QD that could be saturated and inihibited. In that case, how do other mutant *pfcrt* alleles fare in relation to QN and QD transport? To answer this question, an uptake screen for QN and QD was carried out. The results of such a screen have been shown in Fig 3.12. for which both QN and QD were used individually at a concentration of 10 μM along with 50 nM of [^{3}H] labelled drug. Drug accumulation at 60 minutes time point was measured.

Fig 3.14A indicates that oocytes injected with the South American and Philippine isolates namely Ecu1110, 7G8, Ph1 and Ph2 accumulated similar QN and QD as compared to water injected controls. PfCRT Dd2 and GB4, on the contrary, took up significantly more ($p < 0.001$, t-test) substrate than the aforementioned alleles, as well as water-controls. QN and QD uptake for PfCRT GB4 were significantly different from each other whereas no such difference was observed in case of PfCRT Dd2. Fig. 3.14B shows the fraction of uptake corresponding to PfCRT alone, which was obtained by substracting control values from PfCRT injected oocytes, corresponding to the data in Fig. 3.14A. Uptake of QN between Dd2 and GB4, and that of QN and QD for GB4 were compared with a t-test, which yielded a p-value of $p<0.05$. Thus, the mutations present in Dd2, but absent in GB4, seem to have a positive effect on quinine uptake, since quinidine uptake between Dd2 and GB4 was similar.

A



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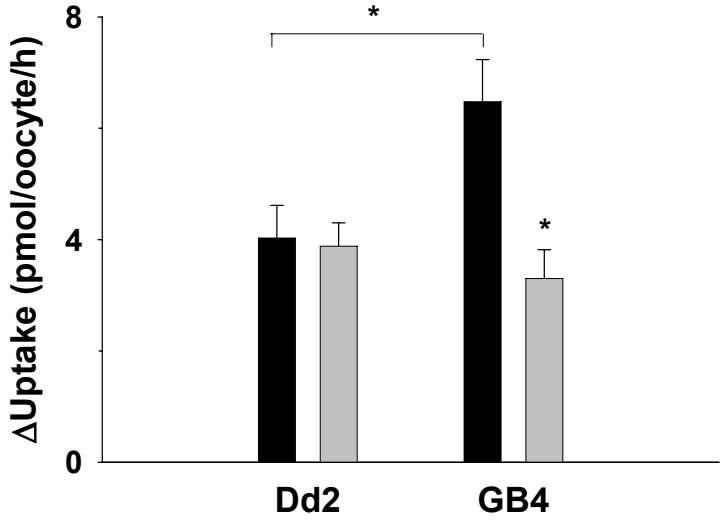


Fig 3.14: Screening pfcrt alleles for quinine and quinidine uptake

A. Oocytes injected with different PfCRT alleles were checked for quinine (QN) and quinidine (QD) uptake Water injected oocytes were used as control.. Data represents means ± SEM. from 3-4 independent experiments with 10 oocytes per allele in each. Stars indicate p-values obtained from a t-test performed to measure the significance of uptake (*** = $p < 0.001$). Black bars represent QN uptake whereas grey bars stand for QD.

B. QN and QD uptake mediated by PfCRT Dd2 and GB4. Accumulation of QN and QD obtained for controls was substracted from values of uptake in Dd2 and GB4 injected oocytes. Data represents means ± SEM. Black and grey bars stand for QN and QD respectively. Star shows p-value corresponding to a test (* = $p<0.05$).

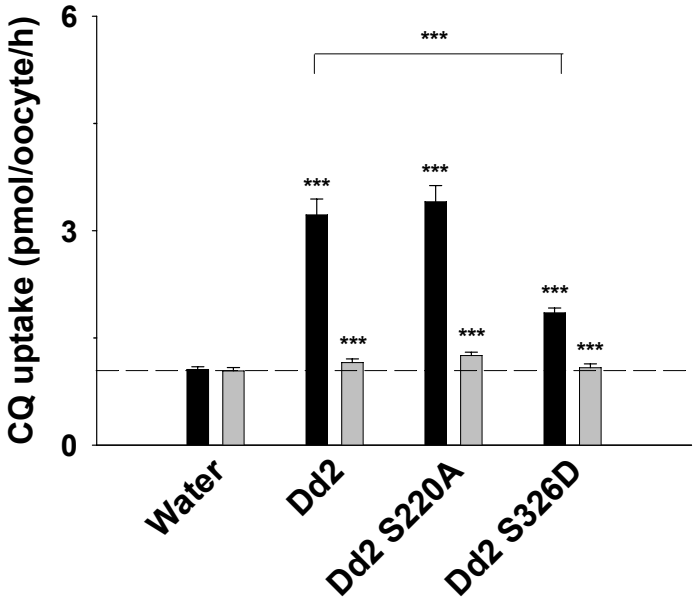
Data featured in Fig 3.14 suggests that mutations influence the substrate specificity of PfCRT mediated transport. A look at Table-3 indicates that many differences underly the set of mutations harboured by *pfcrt* alleles. But could single amino acid replacements influence the ability of PfCRT to transport different quinolines? For this purpose, attention was focused on two particular amino acid substitutions found in mutant *pfcrt* – A220S and N326D. The change from alanine to serine at position 220 (A220S) has been observed in all *pfcrt* isolates sequenced so far except in those originating in Philippines such as Ph1 and Ph2 On the contrary they have two other substitutions unique to the Philippine variants – A144T and L160Y. It is therefore possible that these are in some way compensating for the lack of A220S (Bray *et al.*, 2005, Chen et al., 2003). Another interesting change is at position 326, where arginine residue at position 326 in the wild-type pfcrt gets replaced by either a serine (N326S) or aspartate (N326D). This particular variation also depends on the geographical origin of the isolate, in that South-East Asian/African alleles have N362S as against the south American, papua new guinea and Philippine isolates which harbour the N326D replacement (Cooper *et al.*, 2005). In order to understand how they may influence quinoline transport, two mutants were constructed in the Dd2 background by megaprimer synthesis method and their coding sequenced cloned into pSP64T vector. One of these was a PfCRT Dd2^{S220A} where the serine was mutated back to alanine. Another construct was a PfCRT Dd2^{S326D} where serine at position 326 was replaced by an aspartate. These have been shown in Table-6.

Table-6: Single amino acid replacements in PfCRT Dd2

Allele	Amino acid position of mutations in PfCRT										
	72	**74**	**75**	**76**	**144**	**160**	**220**	**271**	**326**	**356**	**371**
Dd2	C	I	E	T	A	L	S	E	S	T	I
Dd2^{S220A}	C	I	E	T	A	L	A	E	S	T	I
Dd2^{S326D}	C	I	E	T	A	L	S	E	D	T	I

These two constructs were compared to PfCRT Dd2 for their ability to transport CQ, QN and QD. Results from such a comparison have been shown in Fig 3.15.

A



B

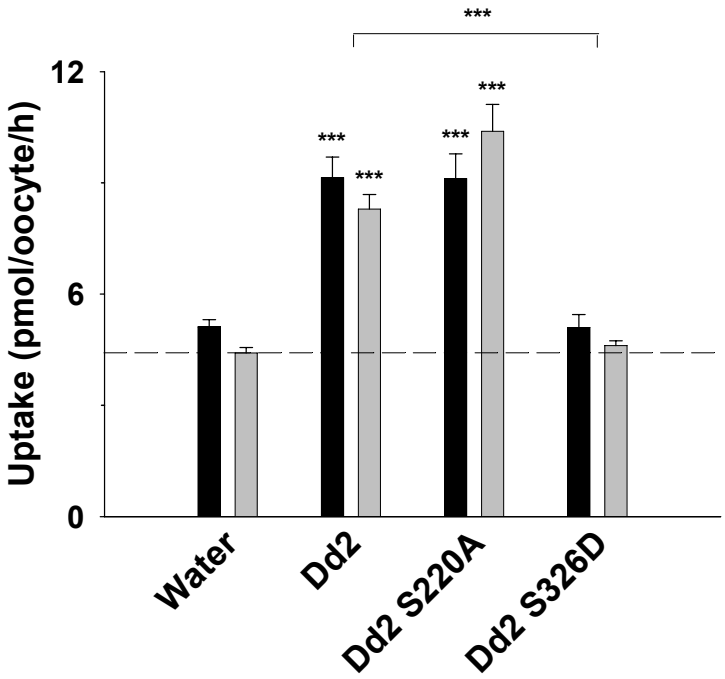


Fig 3.15: Accumulation of chloroquine, quinine and quinidine in PfCRT Dd2 mutants

A. Chloroquine (CQ) uptake mediated byPfCRT Dd2 mutant constructs, with water injected oocytes as negative control. Both black (10 µM CQ) and grey (10 µM CQ+100 µM Verapamil) bars represent means ± SEM. from 4 independent experiments. (*** = $p < 0.001$, t-test).

B. Uptake with 10 µM each of quinine (black bars) and quinidine (grey bars). Data are means means ± SEM from 4 independent determeniations with 8-10 oocytes per group in each experiment.

Fig 3.15A shows accumulation of CQ measured at a concentration of 10 µM and 60 minutes of incubation. PfCRT Dd2 and water injected oocytes were used as positive and negative controls respectively. It shows that oocytes expressing PfCRT Dd2 mediated significant accumulation of CQ as compared to water injected controls ($p < 0.001$, t-test). CQ uptake was not influenced by introduction of the S220A mutation in Dd2. The S236D substitution, however, led to a significant reduction in CQ uptake as compared to the Dd2 positive control ($p < 0.001$, t-test). These mutations did not affect verapamil inhibition, as verapamil caused a significant reduction ($p < 0.001$ for all, t-test). of CQ uptake for each PfCRT. Fig 3.14B demonstrates that that uptake of QN and QD in Dd2 and Dd2^{S220A} injected oocytes was significantly higher than in water injected controls, but not in oocytes injected with Dd2^{S326D}. The reduction in QN and QD accumulation seen on introduction of the S326D replacement was significant ($p < 0.001$, t-test). Thus, QN and QD transport mediated by PfCRT Dd2 was abrogated by the S326D replacement but not by the S220A mutation. Since both the Dd2 mutant constructs showed accumulation of CQ, a dose response curve was plotted in order to measure the Michaelis-Menten constant. This has been shown in Fig 3.16. The procedure followed was similar to that for other alleles described in previous sections.

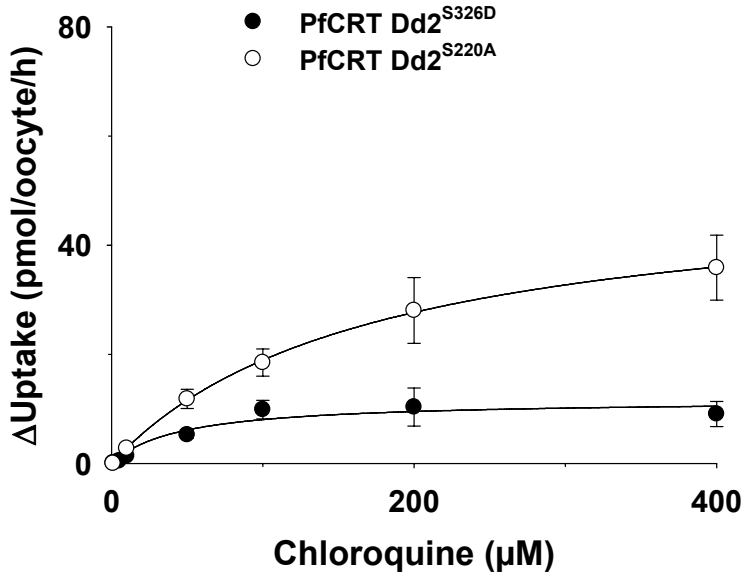


Fig 3.16: Substrate dependence of chloroquine upake for PfCRT Dd2^{S220A} and Dd2^{S326D}

Graph shows difference in uptake between PfCRT injected and water injected oocytes was plotted as a function of concentration. Data points are means means ± SEM from 3 independent determinations with 8-10 oocytes per group in each experiment.

Difference of uptake between PfCRT injected and water injected oocytes has been plotted as a function of chloroquine concentration in Fig 3.16. Data points were fitted with a hyperbolic Michaelis-Menten equation, as done for other PfCRT alleles. V_{max}, K_m and V_{max}/K_m values obtained from the same are shown below in Table-8.

Table-7: Apparent V_{max} and K_m for PfCRT Dd2 replacement mutants

PfCRT	R^2	Apparent CQ K_m [µM]	Apparent CQ V_{max} [pmol/oocyte/h]	Apparent V_{max}/K_m for CQ
Dd2^{S220A}	0.9995	172 ± 8	51.5 ± 1	0.299 ± 0.008
Dd2^{S326D}	0.9378	45 ± 21	11.7 ± 1.5	0.259 ± 0.097

Comparing the K_m values for Dd2 to those mentioned in Table-8 showed that the S220A replacement did not lead to a significant change in the K_m whereas the S326D change caused a significant decrease in the Km ($p < 0.01$, t-test).

3.7 Western blot shows expression of PfCRT alleles in oocytes

Fig 3.1 shown earlier confirmed that *X. laevis* oocytes express a full-length PfCRT protein upon injection with the appropriate RNA. This figure also confirmed that the PfCRT* (HB3 and Dd2) version is recognized by an antibody raised against PfCRT. But are all alleles used in this study expressed in oocytes to the same level? To confirm this, a Western blot analysis was carried out using oocytes injected with all the PfCRT constructs described in previous sections. Oocytes were collected 3 days after injection with 30 ng of RNA for each variant and total cell lysates prepared as per the method described in the materials and methods section. This blot is shown below in Fig 3.16.

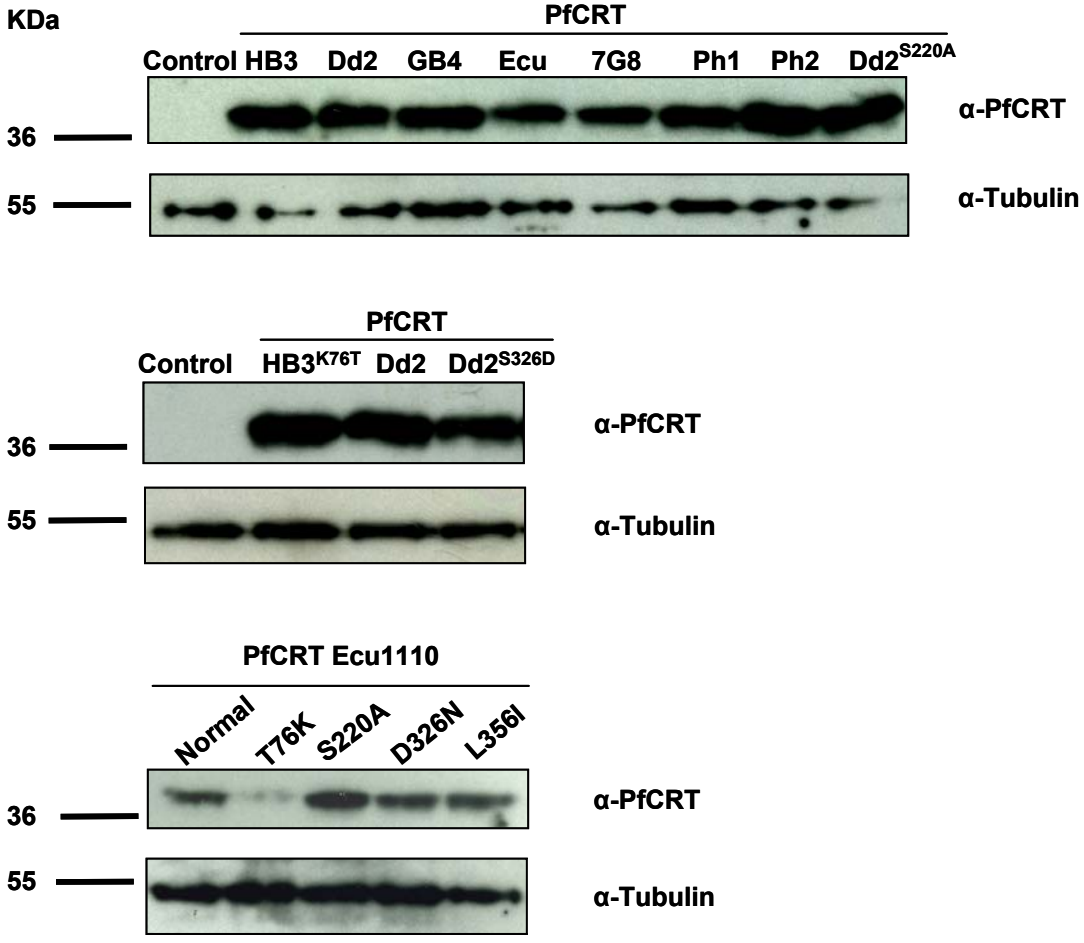


Fig 3.17: Western blot showing expression of PfCRT variants.

10 µl of total cell lysates prepared from PfCRT injected oocytes and water injected oocytes (control) were electrophoresed on a 12% agarose gel, and subsequently transferred onto a membrane support to immunochemically detect expression of PfCRT. Ant-tubulin antibody was used as a loading control.

Fig 3.17 shows that proteins migrating around 36 KDa were detected with an anti-PfCRT antibody in each lane were PfCRT injected oocyte lysates were run. The molecular size is close to the predicted molecular weight of full length PfCRT which is 45 kDa. No protein could be detected in control lysates prepared from water injected oocytes. This shows that the signal is specific to PfCRT and that each PfCRT variant gets expressed upon injection of the corresponding RNA in oocytes. After detecting PfCRT, the same membrane was stripped of the antibodies. This was followed by use of an anti-alpha tubulin antibody to detect Tubulin in the oocyte lysates. With this procedure a band co-migrating with the marker at 55 KDa was detected in each lysate, including in the control samples. This is in order because tubulin is a constitutive protein of the oocyte and hence should be detectable in all the lysates. Amongst these, it appears that the signal for PfCRT Ph2 and Ecu1110 is stronger than that for Ph1 and Ecu1110 respectively. This allows the use of tubulin as a loading control for the oocyte lysates. A visual comparison of the band intensity for the HB3, Dd2, GB4, Ecu1110, 7G8, Ph1, Ph2 and $Dd2^{S220A}$ bands suggests differences in expression, when the tubulin loading control is taken into account. Amongst $HB3^{K76T}$, Dd2 and $Dd2^{S220A}$ samples, the tubulin band intensity varies only slightly. As for the PfCRT bands for these samples, only the Dd2S326D band appears a little smaller than the Dd2 signal, suggesting a potential difference in the expression of these alleles. In the panel with Ecu1110 mutants, the $Ecu1110^{T76K}$ sample shows a signal substantially weaker than other lysates on the same blot. However, the normal Ecu1110 as well as other three mutants had similar signals for both the PfCRT and the Tubulin antibodies. Of the oocytes that were used to prepared the lysates, 10 oocytes for each group were employed for an accumulation experiment with 10 μM CQ, the result for which did not differ from the screens features in previous sections.

4 Discussion

Three questions were raised about PfCRT mediated drug transport in this work– i) Is PfCRT a carrier of substrates other than chloroquine ii) Do mutant pfcrt alleles differ in their transport of quinolines? and iii) With reference to drug transport, what role is played by amino acid substitutions in PfCRT? It was attempted to answer these by expressing a number of naturally occurring and laboratory constructed PfCRT alleles in *Xenopous laevis* oocytes. A Western blot analysis confirmed the successful expression of individual PfCRT variants. Accumulation experiments performed for chloroquine (CQ), quinine (QN) and quinidine (QD) showed that PfCRT corresponding to distinct geographical origins transports CQ. While PfCRT Dd2 showed carrier-like transport of QN and QD, not all alleles expressed as part of this study presented evidence of QN and QD transport functions. Amino acid substitutions at positions 72, 326 and 356 were shown to influence quinoline transport, and only three amino acid replacements in the wild-type PfCRT were sufficient for CQ transport.

4.1 PfCRT as a transporter of CQ, QN and QD

While CQ resistant malaria has been linked to mutant *pfcrt* (Lakshmanan *et al.*, 2005, Fidock et al., 2000, Sidhu *et al.*, 2002), a genetic analysis of QN resistance (QNR) in *P. falciparum* parasites also identified its involvement in QNR (Ferdig *et al.*, 2004). *pfcrt* polymorphisms have also been associated with reduced QN accumulation in *P. falciparum* parasites (Sanchez *et al.*, 2008b), as well as to stereospecific responses to QN and QD (Cooper *et al.*, 2002, Cooper *et al.*, 2007). Kinetics of CQ accumulation in chloroquine sensitive (CQS) and chloroquine resistant (CQR) parasites have pointed to PfCRT being a carrier of CQ (Sanchez *et al.*, 2005, Sanchez *et al.*, 2004, Sanchez *et al.*, 2007a). It was therefore natural to examine if PfCRT can independently transport CQ, QN and QD. Through heterologous expression of PfCRT in *Xenopus laevis* oocytes, a PfCRT mediated, verapamil sensitive and saturable uptake of CQ has been demonstrated (Martin *et al.*, 2009b). The same publication also showed inhibition of PfCRT Dd2 mediated CQ uptake with a large number of compounds, including QN and QD. However, the authors of this study did not present any direct evidence of QN and QD transport by PfCRT. The uptake of QN and QD in PfCRT Dd2 expressing oocytes, shown as part of this study, directly links PfCRT to transport of these two

compounds. Transport of QN from the extracellular buffer to the oocyte cytosol indicates a transport from vacuole to cytosol in case of the parasites. PfCRT associated QN transport thus suggests removal of QN from the vacuole, and fits into a mechanism for QNR where removal of QN from its purpoted target can lead to decreased QN sensitivity.

Time courses for CQ, QN and QD (Fig 3.3, 3.6) showed that uptake of QN and QD at 60 minutes was higher than that for CQ in water injected control oocytes, even though 10 μM of each compound was used. This difference can be explained by protonation and solubility of these compounds, which have been described in Table-8.

Table-8: Protonation state and distribution co-efficient for quinoline compounds

Compound	% of total amount protonated at pH 6.0			Log P	Log D at pH 6.0
	Non-	Di-	Mono-		
Chloroquine	0.00005	99.20	0.79	4.72	-1.84
Quinine	0.26	0	99.74	3.17	0.58
Quinidine	0.26	0	99.74	2.84	0.26

Protonation values given in Table-8 have been calculated from the Handerson-Hasselbach equation using pK_a values for CQ, QN and QD, whereas the distribution co-efficient Log D has been calculated from the partition co-efficient Log P values and the equation describing relationship between Log P, pH and pK_a (Sanchez *et al.*, 2007b, Warhurst *et al.*, 2003). Log D describes the solubility of a compound in an octanol/water suspension at a particular pH. By definition, a Log D value close to zero describes equal preference between octanol and water, whereas negative and positive values describe a tendency for the aqeous and alcohol phases respectively. It is evident from Table-6 that both QN and QD are only mono-protonated at pH 6.0 and are more hydrophobic than CQ which is diprotonated and strongly hydrophilic. This explains the difference in background uptake levels of CQ, QN and QD in oocytes.

Similar to CQ, QN and QD transport through PfCRT Dd2 was found to approach saturation when substrate concentration was increased (Fig 3.4, 3.7). These, along with the verapamil inhibition data (Fig 3.5, 3.8), point towards a carrier-based transport as the likely mechanism for QN and QD accumulation. The mean apparent K_m values obtained for QN and QD were much lower than that for CQ. Thus, saturation of the carrier occurs at a much lower

concentration for QN and QD than for CQ, indicating that the affinity of PfCRT Dd2 for QN and QD may be higher than that for CQ. Kinetics for transport of QN and that for its stereoisomer QD did not differ (Fig 3.7). Another kinetic parameter, namely V_{max}/K_m, represents the rate constant of the transport process at substrate concentration far less than the K_m. These were measured to be 0.509 ± 0.051 µl oocyte^{-1} hour^{-1} for QN, 0.560 ± 0.071 for QD and 0.256 ± 0.022 for CQ. Since all three measurements were for PfCRT Dd2 and were repeated independently a number of times, it could be assumed that expression levels were similar between measurements for the three drugs. In that case, taking ratios of apparent V_{max}/K_m for the different drugs gives the ratio of the efficiency of transport for each compound, where efficiency $\varepsilon = V_{max} / K_m * E_o$ (Atkins, 2010). Such a ratio for QN/CQ is 1.988 whereas that for QN/QD is 2.18, suggesting that efficiency of QN and QD transport by PfCRT Dd2 is twice that for CQ. As indicated by Table-8, QN and QD carry a single positive charge at pH 6.0 whereas a molecule of CQ carries two. This means that under the conditions of measurement, the net displacement of charge remained the same for these three quinolines. As for the binding sites for CQ and QN/QD, the data presented here do not allow any speculation in that regard. Measurement of CQ transport in the presence of QN, and vice-versa, would throw more light on the nature of CQ and QN/QD binding sites in PfCRT. Concerning stereoisomerism between QN and QD, kinetics for PfCRT Dd2 linked transport of these compounds did not seem to differ (Fig 3.7).

Apart from the *pfcrt* locus on Chromosome 7 of *P. falciparum*, two other polymorphic loci have been identified with a possible role in QNR. These include the transporters Pfmdr1 and PfNHE1(Ferdig *et al.*, 2004). *pfmdr1* mutations are known to be associated with QNR (Reed *et al.*, 2000, Sidhu *et al.*, 2005) and QN is amongst the substrates transported by Pfmdr1 (Sanchez *et al.*, 2008a, Rohrbach *et al.*, 2006). QN gets pumped into the food vacuole by the action of Pfmdr1, but it has been shown that the N1042D mutation in this protein abrogrates QN transport (Rohrbach *et al.*, 2006) and decreases IC_{50} values for QN in parasites (Sidhu *et al.*, 2005). This is in accordance with the observed QN transport by PfCRT, as reducing entry of QN into the food vacuole as well as increasing its efflux from this organelle can both contribute to the QNR phenotype. As for PfNHE, it has been proposed that polymorphisms in this sodium proton exchanger contribute to an altered cytosolic pH, which in turn influences QNR (Bennett *et al.*, 2007). While the exact involvement of PfNHE in QNR is not yet fully understood, it cannot be ruled out that QNR is a complex phenomenon involving factors other than mutant *pfcrt*.

4.2 Influence of *pfcrt* polymorphisms on drug transport

Mutant *pfcrt* allele exhibits polymorphism, and a number of combinations of amino acid substitutions are found to occur in *pfcrt* harboured by CQR *P. falciparum* strains (Cooper *et al.*, 2005). This raised the question that if the mutant Dd2 allele can transport CQ, QN and QD, can pfcrt polymorphisms influence transport of the aforementioned drugs?

Uptake with fixed and varying concentrations of CQ, QN and QD showed some differences in transport with reference to the PfCRT alleles used. Amongst the mutant PfCRT variants tested, Dd2 and GB4 were found to transport QN and QD, whereas Ecu1110, 7G8, Ph1 and Ph2 showed uptake similar to that in controls (Fig 3.14). Verapamil-sensitive uptake of CQ was observed for all the mutant alleles (Fig 3.11). Expression of the sensitive HB3 allele did not lead to uptake for either of the drugs tested. As the only difference between the mutant alleles was in the polymorphisms they carried, this suggested that polymorphisms within PfCRT can influence substrate specificity to quinoline compounds. If so, how do they influence drug transport within the parasite? Genetically altered parasites carrying *pfcrt* alleles Dd2 and 7G8 in the same genetic background show decreased accumulation of CQ and QN as compared to parasites carrying wild type *pfcrt* (Sanchez et al., 2008b, Sanchez et al., 2005). In the same studies, however, parasites harbouring wild-type *pfcrt* too showed CQ efflux from the vacuole, although the kinetics of such an efflux were different compared to parasites with mutant *pfcrt*. Then why does the wild-type HB3 allele not exhibit uptake for CQ, QN and QD when expressed in oocytes? And why does 7G8 show accumulation of CQ but not that for QN when expressed heterologously? A possible explanation is that alleles such as HB3 and 7G8 do interact with CQ, QN and QD when expressed, but that the release of the substrate from the binding site is very poor and therefore the amount of substrate translocated across the oocyte plasma membrane is much below detection levels. *P. falciparum* parasites within their host are at a temperature of 37°C, whereas the transport assays shown here were performed at 25°C. Since protein conformation is influenced by temperature, it may be that the conformation of those alleles, for whom no QN or QD transport was detected, is not optimal at the assay temperature. Another scenario is that QN transport within *P. falciparum* requires factors in addition to PfCRT which are absent in the oocytes system, and hence some alleles showed no uptake for QN or QD when expressed in oocytes. Supporting this hypothesis is a report describing that the genetic background of *P. falciparum* parasites in which mutant *pfcrt* is replaced does play a role in determining QN IC_{50} values (Valderramos *et al.*, 2010).

Moreover, another report shows that QN response differs amongst parasites with different genetic backgrounds but same alleles of *pfcrt* and *pfmdr1* (Mu *et al.*, 2003). The absence of QN transport, however, does not rule out an interaction between the south-american and philippine alleles and QN/QD. This can be checked by measuring CQ accumulation in PfCRT expressing oocytes in the presence of QN and QD. An inhibition of CQ uptake, if observed, can then investigate in further detail by comparing inhibitions constants for different alleles.

Kinetic parameters such as the apparent Michaelis-Menten constant K_m, apparent maximum velocity V_{max} and V_{max}/K_m were measured for CQ transport mediated by PfCRT variants. The definition of apparent V_{max} and V_{max}/K_m values includes the amount of active carrier protein mediating substrate transport (Atkins, 2010). Data shown in this work indicate major differences in the CQ V_{max} values for PfCRT alleles (Table-3). A Western blot (Fig 3.17) showed that the amount of PfCRT HB3 protein was higher than that for Dd2, but yet no net uptake of CQ was seen. The 7G8 allele showed a weaker signal for PfCRT expression than other alleles. Ph2 allele had apparently higher expression than Ph1, and yet uptake levels did not differ significantly between the two. Thus, a more accurate analysis of V_{max} is not possible without carefully comparing the levels of expression of each allele in the oocyte plasma membrane. One way of achieving this is by tagging PfCRT protein with a C-terminal tag such as a Haemagluttinin tag or a GFP moiety, and then biotinylating surface proteins to isolate the plasma membrane fraction. Another option available is to prepare slices of oocytes fixed in a polymer, label the protein of interest with an antibody and use a fluorescent tagged secondary antibody to visualize the protein. There are instances where such methods have been used successfully to demonstrate plasma membrane expression of heterologous proteins in *X. laevis* oocytes (Rotmann *et al.*, 2004, Martin et al., 2009b, Nessler et al., 2004). Such a procedure can be used to normalize V_{max} with respect to expression levels of each protein. K_m, in contrast to V_{max}, is independent of the amount of active carrier protein involved in transport (Atkins, 2010). This allowed a direct evaluation of how *pfcrt* polymorphisms may determine CQ K_m. Table-3 permitted a grouping of PfCRT variants on the basis of CQ K_m – low (7G8, Ph2), medium (Ecu1110, Ph1, Dd2) and high (GB4). In other words, saturation of PfCRT mediated CQ transport would occur at much higher concentration with the GB4 than with the 7G8 allele. This can be of therapeutic significance - if one were to increase the dosage of CQ administered to patients infected with *P. falciparum* strains harbouring a 7G8-like *pfcrt* it would result in an accumulation of CQ in the digestive vacuole as the pfcrt mediated CQ efflux process would be saturated, which in theory can lead to parasite killing.

However, one should be reminded that such a scenario is highly unlikely because of the small therapeutic window for CQ (Hoshen *et al.*, 1998). It is also probable that different alleles may have the same turnover number and transport efficiency for CQ, despite of the differences in their K_m values. Turnover number is the ratio of the V_{max} to the amount of carrier protein and it indicates number of molecules transported per unit binding site. The ratio of the turnover number to the K_m is the efficiency of the enzyme, or in this case the carrier (Atkins, 2010). Even if two enzymes vary in their K_m and V_{max} values for a substrate, their efficiency can be similar. Just as with V_{max} and V_{max}/K_m, an accurate determination of the turnover number and the carrier efficiency relied on V_{max} values normalized with respect to levels of expression of the alleles in question.

Verapamil has been known as a chemosensitizer of CQR for many years (Henry *et al.*, 2006). CQ uptake was hence measured with verapamil to test if *pfcrt* polymorphisms influenced verapamil inhibition of CQ uptake. Verapamil induced decrease in CQ uptake was much more pronounced for Dd2 and GB4 alleles, whereas in comparison to these two the effect was not as strong in the other alleles measured (Fig 3.11). $Dd2^{S220A}$ and $Dd2^{S326D}$ constructs were identical to Dd2 in their verapamil reversibility (Table-3.14A), and same was the observation between Dd2 and GB4. This suggests that mutations in TMD other than TMD no.1 are not related to verapamil inhibition, but that the TMD no.1 is a major determinant. This result was in agreement with previous observations that CQ IC50 of Dd2 strain decreased to CQS levels with verapamil, but only a slight reduction was observed with the 7G8 strain (Mehlotra *et al.*, 2001, Sidhu et al., 2002). In another study, Lehane & Kirk demonstrated a verapamil induced proton leak in CQR but not wild-type parasites, and explained that this suggests verapamil to be a substrate of mutant but not wild-type PfCRT (Lehane & Kirk, 2010). They too observed strain specific differences in the verapamil effect between asian/African and south-american CQR parasites. The data shown in Fig 3.11 confirm that mutations in *pfcrt* influence its interaction with verapamil. Initial transport data suggest that verapamil is a substrate of PfCRT and inhibition kinetics point to mixed inhibition. It could be hypothesized that M74I and N75E substitutions present in Dd2 allele enhance binding of verapamil to PfCRT, and hence either out compete or block interaction between CQ and PfCRT, thus leading to decrease in CQ uptake. Lack of these substitutions in the SVMNT or CVMNT type *pfcrt* alleles would then lead to a much less pronounced inhibition effect with verapamil.

4.3 Role of single amino acid substitutions in PfCRT mediated transport

Looking at the CQ uptake with different *pfcrt* alleles, it is evident that different combinations of amino acid substitutions can account for transport (Table-2, Fig 3.9). For instance Dd2 and Ph1 alleles have only the K76T substitution in common, whereas arginine at position 326 gets replaced with an aspartate in Ph1 and serine in Dd2. Ecu1110, on the other hand, harbours four substitutions as compared to wild-type, and each of these positions is also mutated in the Dd2 allele. And yet oocytes injected with all three accumulated significantly more CQ than the controls (Fig 3.9). While K76T alone does not account for CQ transport in oocytes, introducing T76K into the Dd2 background abrogates uptake (Martin *et al.*, 2009b). Thus, more than one mutation has to be present in the wild-type to account for CQ transport, but different combinations of such mutations are possible. What then is the minimum number of substitutions required by wild-type PfCRT to transport CQ in oocytes? Measuring CQ transport with Ecu1110 back-mutants answered this question (Table-4, Fig 3.12). PfCRT with only three amino acid substitutions, namely K76T, D326N and L356I, can still mediate CQ uptake. That D326N is required was an interesting observation, because the position 32, along with 76 and 220, is found to be mutated in a majority of mutant CQ alleles identified from field isolates of *P.falciparum* (Cooper *et al.*, 2005). But in contrast to K76T and D326N, S220A was expendable as Ecu1110^{S220A} did not differ from Ecu1110 in its CQ uptake (Fig 3.13). This was confirmed with Dd2^{S220A} which too did not differ from Dd2 in its CQ uptake and verapamil reversibility (Fig 3.14A). QN and QD uptake between Dd2 and Dd2^{S220A} was also similar (Fig 3.14B).

GB4 and Dd2^{S326D} alleles allowed a more detailed examination of position 326 and its role in quinoline transport. Ecu1110 mutants suggested that N236D is required for CQ uptake, GB4 has no mutation at this position and retains the arginine residue found in wild-type PfCRT (Table-2), and yet GB4 expression in oocytes caused uptake of CQ, QN and QD. Dd2^{S326D} mutant, on the other hand, had significantly decreased accumulation and K_m for CQ than Dd2 and did not cause uptake of QN and QD (Fig 3.15, Fig 3.16, and Table-5). Screening Ecu1110 mutants CQ uptake showed that introducing D326N significantly reduced CQ accumulation (Fig-3.13). These constructs show that the amino acid present at position 326 may play a role in the substrate selectivity of PfCRT, as serine and arginine appear to favour QN/QD transport as compared to aspartate. This may explain why Ecu1110, 7G8, Ph1 and Ph2 PfCRT

were negative for QN and QD uptake, as they all have an aspartate as the 326th amino acid residue (Table-2). PfCRT GB4 also showed higher uptake for QD than its steroisomer QN (Fig 3.4), thus adding weight to the argument that position 326 plays an important role in quinoline transport by PfCRT. Thus, differential respsonses of PfCRT allele to CQ, QN and QD uptake may be in part explained by the residue occurring at position 326.

Another variant residue is located at position 72 in PfCRT, which is either a cysteine or serine depending on the allele, with Ecu1110-7G8 and Ph1-Ph2 alleles differing only in this one respect. CQ IC_{50} values measured with $C8^{Ph1}$ and $C10^{Ph2}$ allelic exchange *P. falciparum* parasites yielded CQS-like CQ IC50 for Ph1 as against $C10^{Ph2}$ which gave higher or CQR-like IC_{50} values. An analysis of parasite fitness revealed $C8^{Ph1}$ to be fitter than $C10^{Ph2}$, and 7G8 to be fitter than Dd2 (Dr. Ines Petersen, personal communication). However, CQ K_m values were significantly different between Ph1-Ph2 and Ph2-7G8, but not between Ecu1110-7G8. This suggests that while S72 implies a lower K_m for CQ as compared C72, the effect of this substitution is not independent of other mutant residues.

Other authors in the past have pointed out that distinct residues in PfCRT have a bearing on substrate specificity. Cooper *et al.* exposed *P. falciparum*106/1 strain to *in vitro* CQ pressured which yielded 106/1^{K76N} and 106/1^{K76I} strains having mutations at position 76 in *pfcrt* (Cooper *et al.*, 2002). Of these the 106/1^{K76I} was a CQ resistant line showing increased sensitivity to QN but reduced sensitivity to QD. In a separate study the authors exposed 106/1^{K76I} parasites to QN selection pressured, which gave parasites with additional *pfcrt* mutations (Cooper *et al.*, 2007). These were 106/1$^{76I\text{-}352K}$, 106/1$^{76I\text{-}352R}$ and 106/1$^{72R\text{-}76I}$, which were CQ sensitive but QN resistant. The authors argued that these mutations occur in TMD 1, 4 and 9 of PfCRT which are often involved in substrate binding site in carriers of the drug-metabolite transporter superfamily, of which PfCRT is thought to be a member (Martin & Kirk, 2004). Position 326 did not feature in these reports and to this day no other study showing effects of individual or pairs of mutations on substrate selectivity is known to have been published. Such a lack of overlap between this work and the studies by Cooper *et al.* is hardly suprising. The analyses involving oocyte expression of *pfcrt* alleles relied on mutations occuring in the field, where selection landscape involves many other parameters such as frequency of malaria transmission, host-parasite interactions, pharmacological properties of the drug to name a few (Mackinnon & Marsh, 2010).

The finding that the S220A mutation is not required for transport of CQ, QN or QD was hardly surprising. Mutations are selected under drug pressure not only to acquire the drug resistance phenotype, but also to balance the fitness cost incurred by introduction of such changes (Brown *et al.*, 2010). Drug resistant *P. falciparum* strains are known to differ in their fitness as compared to the wild-type, as mutations in *pfmdr1* gene and its amplification in multi-drug resistant *P. falciparum* has shown to contribute to decreased parasite fitness (Hayward *et al.*, 2005, Preechapornkul *et al.*, 2009). As for CQR parasites, discontinuation of CQ use in Malawi led to re-emergence of CQS malaria in Malawi (Kublin *et al.*, 2003), which has been explained by re-emergence of CQS parasites under the lack of drug pressure (Laufer et al., 2010). These reports, along with reports showing seasonal carriage of CQR parasites in malaria-endemic regions (Ord *et al.*, 2007) suggest that CQR parasites have decreased fitness. Within this context, it is not fanciful to hypothesize that the S220A mutation may be a compensatory mutation linked to parasite fitness, and hence its almost ubiquitous presence in CQR *pfcrt* (Cooper *et al.*, 2005).

4.4 Relationship between *pfcrt,* quinoline transport and drug resistant malaria

The relationship between *pfcrt* polymorphisms, geography of the corresponding *P. falciparum* isolate and transport of CQ, QN and QD was examined earlier. But it is also possible to view the substrate specificity of PfCRT for aminoquinolines in the context of drug resistance or reduced susceptibility to these drugs, as observed in the field. Resistance to CQ is widespread across the world, and malaria endemic regions in South-America, Sub-saharan Africa as well as in South-East Asia carry CQR parasite strains (WHO, 2005). The Brazilian Amazon region is malaria endemic, and one study found that 97% of samples collected in this region to be resistant to CQ, as against 3% to QN (Cerutti Junior *et al.*, 1999). Another study found that in isolates pertaining to patients coming from this region, 100% of the isolates tested were CQ resistant whereas 11% QN resistant (Segurado *et al.*, 1997). In Africa, all countries except Djibouti and Swaziland show clinical treatment rate of failure to be higher than 25% for CQ. The same was around 80% in South-America and around 40% in South-East Asia in the year 2002 (WHO, 2005). Quinine efficacy, on the other hand, was not as starkly reduced in South-America, Africa or South-East Asia and observed failure rates with QN treatment remained

beween 5-10% (WHO, 2005). Each naturally occurring *pfcrt* allele tested positive for CQ transport in the oocyte assay, and this fits together with the observed pattern of CQR malaria as a mutant PfCRT transporting CQ out of the DV vacuole is consistent with CQR. However, a similar relationship cannot be established for QN transport and QN resistance. QN resistance is difficult to demonstrate as only low levels of QN resistance are observed across the world (WHO, 2005). Secondly, the South-American alleles which tested negative for QN transport in the oocyte assay, come from a region where reduced QN IC_{50} values for field isolates have been reported (Cerutti Junior *et al.*, 1999). That QN is effective against CQ and mefloquine resistant *P. falciparum* supports such a lack of overlap between observed patterns of CQ and QN resistance. A QTL analysis of progenies from *P. falciparum* HB3 x Dd2 crosses has shown in the past that 95% of CQR phenotype could be ascribed to a chromosomal section containing *pfcrt*, whereas the same QTL showed QN resistance to be a multifactorial trait (Ferdig *et al.*, 2004). In order to better understand the influence of *pfcrt* alone on QN IC_{50}, one could look at allelic exchange parasites that have the same genetic background and differ only in their *pfcrt* alleles. QN IC_{50} values measured for such parasites, namely $C2^{GC03}$, $C4^{Dd2}$ and $C6^{7G8}$, were measured to be 171, 93 and 71.5 nM respectively (Sidhu *et al.*, 2002). Thus, QN IC_{50} values were not drastically different for parasites carrying Dd2 or 7G8 *pfcrt*. This suggests that QN IC_{50} and transport could be influenced by different factors within the parasite. So while PfCRT could transport both CQ and QN out of the DV, there may be factors within the parasite that influence QN but not CQ IC_{50}. It is also tempting to speculate that the different mutant *pfcrt* polymorphisms may have been selected under different drug pressure conditions, such that alleles in one area would have got selected to transport a certain range of compounds that were being used, and in another area another allele got selected to transport say only CQ. There may also be a trade off between parasite fitness and the range of substrates being transporter by a particular *pfcrt* allele. This, however, remains to be experimentally examined.

5. Outlook

The work presented here argues in favour of PfCRT being a carrier for multiple substrates, and that polymorphisms in this protein influence its transport properties for such substrates. It however, also raises some questions that could not be answered within the scope of this study. Apart from chloroquine (CQ), quinine (QN) and quinidine (QD), uptake screens may be performed with more substrates such as verapamil, amodiaquine and lumenfantrine to name a few. It remains to be seen if quinolines share a binding site in PfCRT or interact with distinct sites, which can be ascertained by competition studies where transport of one substrate is measured in the presence of another. The measurement of plasma membrane expression levels for different constructs should offer further insights into the kinetics of PfCRT mediated CQ transport by allowing a determination of the turnover number and transport efficiency. Similar to CQ uptake, it needs to be ascertained as to which are the minimum number of changes required to transport QN, and whether compounds such as amodiaquine too get transported by such minimal constructs. The role of K76T substitution in PfCRT mediated transport needs to be further examined by replacing the threonine residues to see if other presence of other amino acid residues too can account for quinoline transport. Experiments outlined above can all be carried out in the *Xenopus laevis* expression system. Having the knowledge obtained from oocyte experiments, *Plasmodium falciparum* parasites could be engineered such that the endogenous *pfcrt* gets replaced with the construct of interest. This would allow a better understanding of the crucial role this trans-membrane protein plays in both parasite physiology and in conferring drug resistance.

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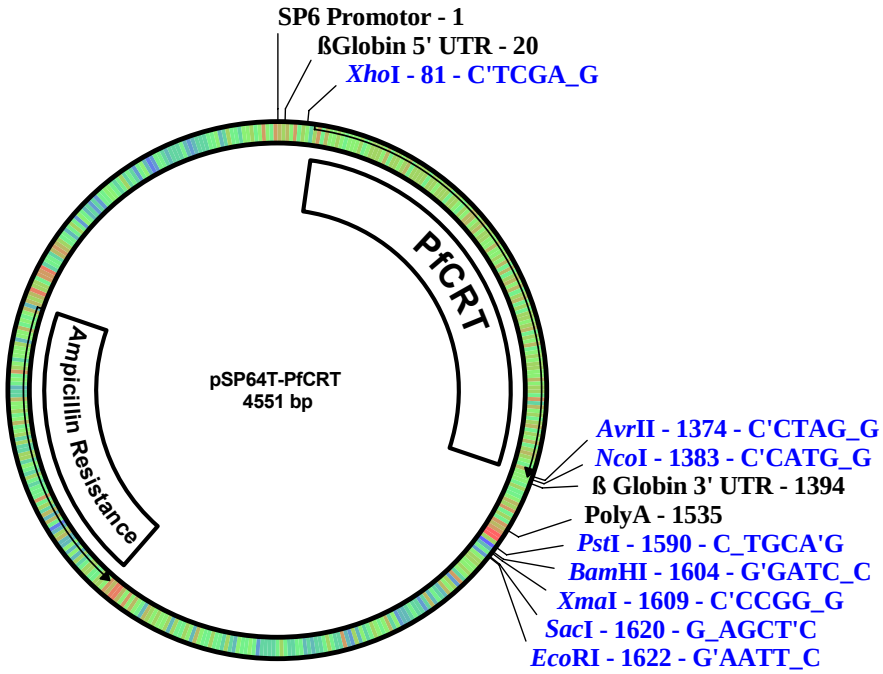
Appendix



Vector map of PfCRT cloned into the pSP64T vector

PfCRT HB3 sequence (MAL7P1.27)

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201  CTACTTGTCT GTTTGCGTCA TGAACAAGAT TTTCGCCAAG AGAACCTTGA
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801  ATTCTTGAAG CAATTGCACT TGCCATACAA CGAAATTTGG ACCAACATCA
851  AGAATGGTTT CGCTTGTTTG TTCTTGGGTA GAAACACCGT TGTTGAAAAC
901  TGTGGTTTGG GTATGGCTAA GTTGTGTGAT GATTGTGATG GTGCTTGGAA
951  AACTTTCGCT TTGTTCTCCT TCTTCGACAT CTGCGATAAC TTGATTACCT
1001 CCTACATCAT CGATAAGTTC TCTACTATGA CCTACACCAT CGTTTCTTGT
1051 ATTCAAGGTC CAGCTCTTGC TATTGCCTAC TACTTCAAGT TTTTGGCCGG
1101 TGATGTTGTT AGAGAACCTA GATTATTGGA CTTCGTCACC TTGTTTGGTT
1151 ACTTGTTCGG TTCCATTATC TACAGAGTCG GTAACATCAT CTTGGAAAGA
1201 AAGAAGATGA GAAACGAAGA AAACGCTGAT TCTGCTGGTG CTTTGACTAA
1251 TGTTGATTCT GCTGCTACTC AACCTAGGTA A

1   MKFASKKNNQ KNSSKNAERA RAADNAAQEG NGSRLGGGSC LGKCAHAAKA
51  AFKEIKDNIF IYILSIIYLS VSVMNTIFAK RTLNKIGNYS FVTSETHNFI
101 CMIMFFIVYS LFGNKKGNSK ERHRSFNLQF FAISMLDACS VILAFIGLTR
151 TTGNIQSFVL QLSIPINMFF CFLILRYRYH LYNYLGAVII VVTIALVEMK
201 LSFETQEENS IIFNLVLISS LIPVCFSNMT REIVFKKYKI DILRLNAMVS
251 FFQLFTSCLI LPVYTLPFLK QLHLPYNEIW TNIKNGFACL FLGRNTVVEN
301 CGLGMAKLCD DCDGAWKTFA LFSFFDICDN LITSYIIDKF STMTYTIVSC
351 IQGPALAIAY YFKFLAGDVV REPRLLDFVT LFGYLFGSII YRVGNIILER
401 KKMRNEENAD SAGALTNVDS AATQPR*
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PfCRT Ph1 sequence

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1    ATGAAGTTCG CCTCTAAGAA GAACAATCAA AAGAACTCCT CCAAGAATGC
51   TGAAAGAGCT AGAGCTGCTG ATAATGCTGC TCAAGAAGGT AACGGTTCTA
101  GATTGGGTGG TGGTTCTTGT TTGGGTAAAT GTGCTCATGC TGCTAAAGCT
151  GCCTTCAAAG AAATCAAGGA CAACATCTTC ATCTACATCT TGTCCATCAT
201  CTACTTGTCT GTTTGCGTCA TGAACACGAT TTTCGCCAAG AGAACCTTGA
251  ACAAGATTGG TAACTACTCT TTCGTTACCT CTGAAACCCA TAACTTCATC
301  TGCATGATCA TGTTCTTCAT CGTCTATTCC TTGTTCGGTA ACAAGAAGGG
351  TAACTCCAAA GAAAGACACA GATCCTTCAA CTTGCAATTC TTCGCCATTT
401  CTATGTTGGA TGCCTGCTCT GTTATTTTGA CTTTCATCGG TTTGACTAGA
451  ACTACCGGTA ACATCCAATC TTTCGTCTAC CAATTGTCCA TTCCAATCAA
501  TATGTTCTTC TGCTTCTTGA TCTTGAGATA CAGATACCAC TTGTACAATT
551  ACTTGGGTGC CGTTATTATT GTCGTTACCA TTGCCTTGGT TGAAATGAAG
601  TTGTCCTTCG AAACCCAAGA AGAAAACTCC ATCATCTTCA ACTTGGTTTT
651  GATTTCCGCC TTGATTCCAG TTTGTTTCTC CAACATGACC AGAGAAATCG
701  TTTTCAAGAA GTACAAGATC GACATCTTGA GATTGAACGC TATGGTTTCC
751  TTCTTCCAAT TATTCACCTC CTGCTTGATT TTGCCAGTTT ACACCTTGCC
801  ATTCTTGAAG CAATTGCACT TGCCATACAA CGAAATTTGG ACCAACATCA
851  AGAATGGTTT CGCTTGTTTG TTCTTGGGTA GAAACACCGT TGTTGAAAAC
901  TGTGGTTTGG GTATGGCTAA GTTGTGTGAT GATTGTGATG GTGCTTGGAA
951  AACTTTCGCT TTGTTCTCCT TCTTCGACAT CTGCGATAAC TTGATTACCT
1001 CCTACATCAT CGATAAGTTC TCTACTATGA CCTACACCAT CGTTTCTTGT
1051 ATTCAAGGTC CAGCTATTGC TATTGCCTAC TACTTCAAGT TTTTGGCCGG
1101 TGATGTTGTT AGAGAACCTA GATTATTGGA CTTCGTCACC TTGTTTGGTT
1151 ACTTGTTCGG TTCCATTATC TACAGAGTCG GTAACATCAT CTTGGAAAGA
1201 AAGAAGATGA GAAACGAAGA AAACGCTGAT TCTGCTGGTG CTTTGACTAA
1251 TGTTGATTCT GCTGCTACTC AACCTAGGTA A
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1   MKFASKKNNQ KNSSKNAERA RAADNAAQEG NGSRLGGGSC LGKCAHAAKA
51  AFKEIKDNIF IYILSIIYLS VCVMNTIFAK RTLNKIGNYS FVTSETHNFI
101 CMIMFFIVYS LFGNKKGNSK ERHRSFNLQF FAISMLDACS VILTFIGLTR
151 TTGNIQSFVY QLSIPINMFF CFLILRYRYH LYNYLGAVII VVTIALVEMK
201 LSFETQEENS IIFNLVLISA LIPVCFSNMT REIVFKKYKI DILRLNAMVS
251 FFQLFTSCLI LPVYTLPFLK QLHLPYNEIW TNIKNGFACL FLGRNTVVEN
301 CGLGMAKLCD DCDGAWKTFA LFSFFDICDN LITSYIIDKF STMTYTIVSC
351 IQGPAIAIAY YFKFLAGDVV REPRLLDFVT LFGYLFGSII YRVGNIILER
401 KKMRNEENAD SAGALTNVDS AATQPR*
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PfCRT Ph2 sequences

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1    ATGAAGTTCG CCTCTAAGAA GAACAATCAA AAGAACTCCT CCAAGAATGC
51   TGAAAGAGCT AGAGCTGCTG ATAATGCTGC TCAAGAAGGT AACGGTTCTA
101  GATTGGGTGG TGGTTCTTGT TTGGGTAAAT GTGCTCATGC TGCTAAAGCT
151  GCCTTCAAAG AAATCAAGGA CAACATCTTC ATCTACATCT TGTCCATCAT
201  CTACTTGTCT GTTAGCGTCA TGAACACGAT TTTCGCCAAG AGAACCTTGA
251  ACAAGATTGG TAACTACTCT TTCGTTACCT CTGAAACCCA TAACTTCATC
301  TGCATGATCA TGTTCTTCAT CGTCTATTCC TTGTTCGGTA ACAAGAAGGG
351  TAACTCCAAA GAAAGACACA GATCCTTCAA CTTGCAATTC TTCGCCATTT
401  CTATGTTGGA TGCCTGCTCT GTTATTTTGA CTTTCATCGG TTTGACTAGA
451  ACTACCGGTA ACATCCAATC TTTCGTCTAC CAATTGTCCA TTCCAATCAA
501  TATGTTCTTC TGCTTCTTGA TCTTGAGATA CAGATACCAC TTGTACAATT
551  ACTTGGGTGC CGTTATTATT GTCGTTACCA TTGCCTTGGT TGAAATGAAG
601  TTGTCCTTCG AAACCCAAGA AGAAAACTCC ATCATCTTCA ACTTGGTTTT
51  GATTTCCGCC TTGATTCCAG TTTGTTTCTC CAACATGACC AGAGAAATCG
701  TTTTCAAGAA GTACAAGATC GACATCTTGA GATTGAACGC TATGGTTTCC
751  TTCTTCCAAT TATTCACCTC CTGCTTGATT TTGCCAGTTT ACACCTTGCC
801  ATTCTTGAAG CAATTGCACT TGCCATACAA CGAAATTTGG ACCAACATCA
851  AGAATGGTTT CGCTTGTTTG TTCTTGGGTA GAAACACCGT TGTTGAAAAC
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901  TGTGGTTTGG GTATGGCTAA GTTGTGTGAT GATTGTGATG GTGCTTGGAA
951  AACTTTCGCT TTGTTCTCCT TCTTCGACAT CTGCGATAAC TTGATTACCT
1001 CCTACATCAT CGATAAGTTC TCTACTATGA CCTACACCAT CGTTTCTTGT
1051 ATTCAAGGTC CAGCTATTGC TATTGCCTAC TACTTCAAGT TTTTGGCCGG
1101 TGATGTTGTT AGAGAACCTA GATTATTGGA CTTCGTCACC TTGTTTGGTT
1151 ACTTGTTCGG TTCCATTATC TACAGAGTCG GTAACATCAT CTTGGAAAGA
1201 AAGAAGATGA GAAACGAAGA AAACGCTGAT TCTGCTGGTG CTTTGACTAA
1251 TGTTGATTCT GCTGCTACTC AACCTAGGTA A

1   MKFASKKNNQ KNSSKNAERA RAADNAAQEG NGSRLGGGSC LGKCAHAAKA
51  AFKEIKDNIF IYILSIIYLS VSVMNTIFAK RTLNKIGNYS FVTSETHNFI
101 CMIMFFIVYS LFGNKKGNSK ERHRSFNLQF FAISMLDACS VILTFIGLTR
151 TTGNIQSFVY QLSIPINMFF CFLILRYRYH LYNYLGAVII VVTIALVEMK
201 LSFETQEENS IIFNLVLISA LIPVCFSNMT REIVFKKYKI DILRLNAMVS
251 FFQLFTSCLI LPVYTLPFLK QLHLPYNEIW TNIKNGFACL FLGRNTVVEN
301 CGLGMAKLCD DCDGAWKTFA LFSFFDICDN LITSYIIDKF STMTYTIVSC
351 IQGPAIAIAY YFKFLAGDVV REPRLLDFVT LFGYLFGSII YRVGNIILER
401 KKMRNEENAD SAGALTNVDS AATQPR*
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