Heterologous Expression and Functional Characterization of *Plasmodium falciparum* ABCG in Mammalian Cells

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List of abbreviations

ABC	ATP Binding Cassette
ABCP	ABC placenta
BCRP	Breast cancer resistance protein
BCA	bicinchoninic acid
CQS	Chloroquine sensitive strains
CQR	Chloroquine resistance strains
CQ	Chloroquine
DV	Digestive vacuole
DHPS	Dihydropteroate synthase
DHFR	Dihydrofolate reductase
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's medium
G-6PD	Glucose-6-phosphate dehydrogenase
GSH	Glutathione
G418	Geneticin
HEK293	Human Embryonic Kidney 293 cells
ITNs	Insecticide-treated nets
LLINs	Long-lasting insecticidal bed nets
MXR	Mitoxantrone resistance
MDR	Multidrug resistance
WR	Working Reagent
NBD	Nucleotide binding domains
PfCRT	*Plasmodium falciparum* chloroquine resistance transporter
PfABCG	*Plasmodium falciparum* ATP Binding Cassette G

PfMDR1/ PfMDR2	*P. falciparum* multidrug resistance transporter 1/2
PBS	Phosphate-buffered saline
RIPA	Radioimmunoprecipitation assay buffer
RBCs	Red blood cells
PQ	Primaquine
PVDF membrane	Polyvinylidene Difluoride
SRB	Sulforhodamine B dye
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S-P	Sulfadoxine-Pyrimethamine
TCA	Trichloroacetic acid
TMD	Transmembrane domain
Tg	*Toxoplasma gondii*
DIC	Differential interference contrast
ΔgABCG2	Knock out PfABCG

List of word definition

SDS-PAGE	A very common method called SDS-PAGE for separating proteins by electrophoresis uses polyacrylamide gel and SDS to denature the protein.
RIPA buffer	Lysis buffer uses to lyse cells and tissue for radio immunoprecipctation assay.
SRB dye	An anionic dye that binds with proteins electrostatically under mild acetic condition.
pCruz GFP-PfABCG	Cloned PfABCG into pCruz-GFP vector.
Transfection	Inserting DNA or RNA into mammalian cells
Subcellular localization	Prediction of where a protein resides in a cell.

Abstract

Malaria affects more than a quarter of this planet's population with 214 million infections and 438,000 deaths annually. In the absence of an effective malaria vaccine, the rise and spread of drug resistant strains coupled with the slow development of new antimalarials is a potential human tragedy. Research to date has led to the identification of several proteins that can mediate parasite resistance to most antimalarials. Two of the latter proteins (e.g., PfMDR1 and PfMRP1) are members of a large and evolutionary conserved family of ATP-dependent membrane transporters (e.g., ABC transporters). The *P. falciparum* genome encodes 16 different ABC transporters, including one member of the ABCG subfamily (e.g., PfABCG). By contrast, the human genome encodes 48 members of the ABC transporters, including five members of the ABCG subfamily (huABCG1, G2, G4, G5 and G8). With the exception of huABCG2, which transports many anti-cancer drugs and some normal cell metabolites, huABCG1, G4, G5 and G8 mediate the transport of cholesterol and other sterols. Earlier studies using PfABCG-knockout clones of *P. falciparum* have suggested that PfABCG may play a role in the parasite's sensitivity to ketotifen (an anti-histamine drug), and in the accumulation of neutral lipids in PfABCG-knockout clones. Moreover, we have shown that PfABCG shares 24.3 % and 26.5 % amino acid sequence identity with huABCG1 and huABCG2, respectively. Hence, it is presently not clear if PfABCG is functionally more like huABCG1, huABCG2, or both. In an effort to characterize the functions of PfABCG, it was of interest to compare its substrate specificity and subcellular localization to that of huABCG1 and G2 in the same expression system, using mammalian HEK-293 cells. Our results show the stable expression of PfABCG in HEK-293, as a fusion protein with GFP sequence linked to PfABCG N-terminal. In addition, relying on the fluorescence of

GFP in PfABCG-HEK-293 transfectants, we have demonstrated the localization of GFP-PfABCG to the endosomal membranes, likely the endoplasmic reticulum. We also show that HEK-293 cells stably transfected with GFP-PfABCG are more sensitive to ketotifen in the presence of reseripine, a calcium channel blocker and an inhibitor of huABCB1 and huABCG2 expressed at low levels in HEK-293 cells. Efforts are on going to further characterize the functions of PfABCG and its substrate specificity and how these functions relate to those of huABCG1 and G2.

Résumé

Le paludisme affecte plus d'un quart de la population de cette planète avec 214 millions d'infections et 438 000 décès chaque année. De plus, en l'absence d'un vaccin efficace contre le paludisme, l'augmentation et la propagation de souches résistantes aux médicaments associées au développement lent de nouveaux antipaludiques est une tragédie humaine potentielle. La recherche à ce jour a permis d'identifier plusieurs protéines qui peuvent servir de médiateur à la résistance des parasites à la plupart des antipaludiques. Deux de ces protéines (par exemple, PfMDR1 et PfMRP1) sont des membres d'une importante famille évolutivement conservée de transporteurs membranaires dépendant de l'ATP (par exemple, des transporteurs ABC). Le génome de *P. falciparum* code pour 16 transporteurs ABC différent, y compris un seul membre de la sous-famille ABCG (par exemple, PfABCG). En revanche, le génome humain code pour 48 membres des transporteurs ABC, dont cinq membres de la sous-famille ABCG (huABCG1, G2, G4, G5 et G8). À l'exception de huABCG2 qui transporte de nombreux médicaments anti-cancéreux et certains métabolites cellulaires normaux, huABCG1, G4, G5 et G8 font la médiation du transport du cholestérol et d'autres stérols. Des études antérieures utilisant des clones de PfABCG-knock-out de *P. falciparum* ont suggéré que PfABCG peut jouer un rôle dans la sensibilité du parasite au ketotifène (un médicament anti-histaminique) et à l'accumulation de lipides neutres dans des clones PfABCG-knock-out. En outre, nous avons montré que PfABCG partage 24,3% et 26,5% de séquence d'acides aminés identique avec huABCG1 et huABCG2, respectivement. Par conséquent, il n'est présentement pas encore clair si PfABCG du point de vue fonctionnelle est comme huABCG1, huABCG2 ou les deux. Dans le but de caractériser les fonctions de PfABCG, il était intéressant de comparer sa spécificité de substrat et sa localisation à celle de huABCG1 et G2 dans le même système d'expression, en utilisant des cellules HEK-

293 de mammifères. Nos résultats montrent une expression stable de PfABCG dans HEK-293, en tant que protéine de fusion avec une séquence de la GFP liée à une portion N-terminale de PfABCG. De plus, en prenant avantage de l'auto fluorescence de la GFP dans les transfectants PfABCG-HEK-293, nous avons démontré la localisation de GFP-PfABCG aux membranes endosomales, probablement le réticulum endoplasmique. Nous avons montré également que les cellules HEK-293 transfectées de manière stable avec GFP-PfABCG sont plus sensibles au kétotifène en présence de la réserpine, un bloqueur des canaux calciques et un inhibiteur de huABCB1 et huABCG2 qui sont exprimés à des niveaux faibles dans les cellules HEK-293. Des expériences sont en cours pour caractériser davantage les fonctions de PfABCG et sa spécificité de substrat ainsi que la façon dont ces fonctions se rapportent à celles de huABCG1 et G2.

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Contribution of author

The experimental work presented in this thesis was performed by the author, and Sonia Edaye under the supervision of Dr. Elias Georges.

Chapter One: Introduction and Objectives

1.1 Introduction

1.1.1 Malaria

Malaria, the most common disease worldwide, is named from the Italian words "mal" and "aria" which mean "bad" and "air". Initially, people thought that malaria was caused by foul air, essentially by vapor given off from swamps. The causative agent of malaria was identified by the French physician Charles Louis Alphonse Laveran; he was the first to notice the parasite in the blood of patients suffering from the disease (Sequeira, 1930). As we know today, being bitten by *Plasmodium* infected mosquitoes is the number one cause of malaria (Maier et al., 2009). In addition, infected blood transfusion, transplantation and infected needles are other routes of transmission; however, they occur less frequently than infection through an infected mosquito bite. Malaria is caused by members of genus of *Plasmodium* and there are more than 100 species (Bahl et al., 2002) that infect birds, reptiles and mammals. However, only five common species of *Plasmodium* are recognized to infect humans: *P. falciparum, P. vivax, P. ovale and P. malariae* (Cserti & Dzik, 2007), in addition to a new species named *P. knowlesi* (Yasin et al., 2014a). *P. falciparum and P. vivax* are the most prevalent in the world. *P. ovale* is widespread in West Africa (Yasin et al., 2014b) while *P. malariae* has a low rate of prevalence (Yasin et al., 2014a). In 2015, malaria was the fourth highest cause of deaths (WHO, 2015). The World Health Organization (WHO) estimated 438, 000 deaths and 214 million cases of malaria worldwide (WHO, 2015). *P. knowlesi*, discovered in 2004 as infectious to humans, is a common species in macaque monkeys in South Asian countries such as Malaysia, Thailand, Myanmar and Philippines (Yasin et al., 2014a). *P. knowlesi* is spread between macaques, female *Anopheles*

mosquito and human (WHO, 2015). Malaria most severely affects the population of tropical and sub-tropical countries. This includes the regions of Sub-Saharan Africa, Latin America and Southeast Asia (WHO, 2015)(**Figure 1**). According to the WHO, malaria remains a major killer of children under the age of five, mainly in sub-Saharan Africa (WHO, 2015).

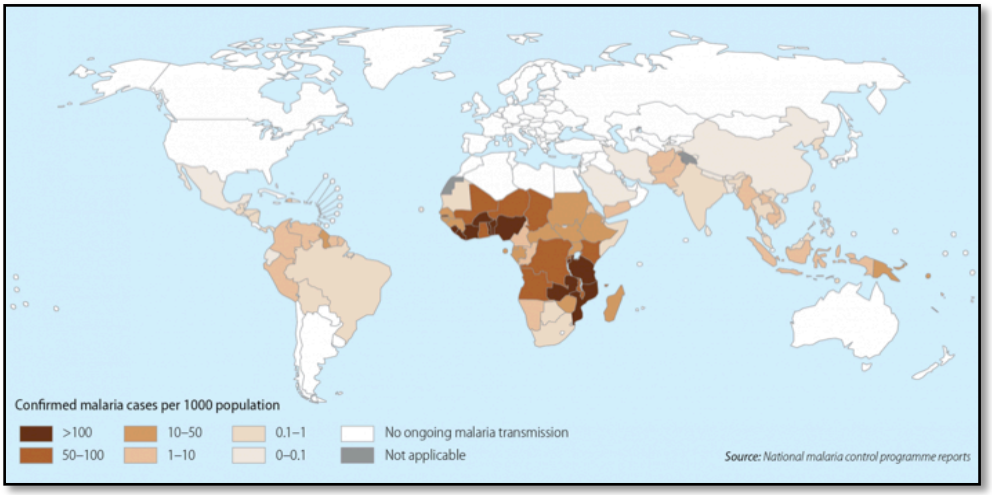

Figure 1: The distribution of malaria in the world (WHO, 2014)

Infected persons usually have a fever for 10 to 15 days; fever temperature can reach as high as 41.5°C (Shahinas et al., 2013). Other common symptoms include chills, nausea, vomiting and headaches (Yasin et al., 2014a). *P. falciparum* is the deadliest species of *Plasmodium* given that the parasite can replicate quickly in the blood stream and destroy red blood cells (RBCs). This destruction due to the infection of RBCs by *P. falciparum* makes the RBCs stick to the capillaries of organs (Egbuche, 2012), preventing blood circulation and causing oxygen deficiency. The occurrence of liver, lung, kidney and/or brain problems can cause coma or cerebral malaria which may lead to death (Egbuche, 2012). *P. falciparum* can also cause anemia (Malarial anemia), which in pregnant women is also associated with anemia in the fetus and increases the risk of infant mortality (Menendez et al., 200).

Life cycle of *Plasmodium falciparum*

Plasmodium life cycle is complex and it depends on the species of *Plasmodium,* for example *P. falciparum, P. vivax, and P. ovale* is approximately 48h, and *P. malaria* is 72h,while for *P.knowlesi* has the shortest cycle is approximately 24h to complete one full asexual replication cycle in the blood (Shahinas et al., 2013). The life cycle follows three consecutive stages: human infected with sporozoites, asexual reproduction and sexual reproduction. The first two stages take place in the human body and the third stage is completed in the mosquito's organism. The life cycle begins when the infected female *Anopheles* mosquito injects sporozoites from their salivary gland into human skin (infection stage) (**Figure 2)** (Thera & Plowe, 2012). The sporozoites travel to the bloodstream and enter the liver. They invade the hepatocytes, where they multiply into merozoites (C. Otoikhian et al., 2014). The merozoites are released into the bloodstream, where they infect RBCs and develop into rings, trophozoites and schizonts. The schizonts rupture and release 16 to 24 merozoites which then infect new RBCs and the asexual life cycle repeats (Otoikhian et al., 2014). At some point during the cycle, some merozoites differentiate and become either male (microgametes) or female (macrogametes) gametocytes (sexual stages of the parasite). During another blood meal on infected humans, mosquitos may ingest blood containing these gametocytes. In the lumen of the mosquito's stomach, the gametocytes mature into male and female gametes (*P. falciparum* needs 8-10 days to mature) resulting in the initiation of the sexual development stage. Oocysts develop in the mosquito's mid gut after the zygote has developed into movable ookinete (Baer et al., 2007). After a period of growth, the oocyst releases thousands of sporozoites that reach the salivary gland of the mosquito. There, the sporozoites wait until another blood meal to be released into another human host (Aly et al., 2009).

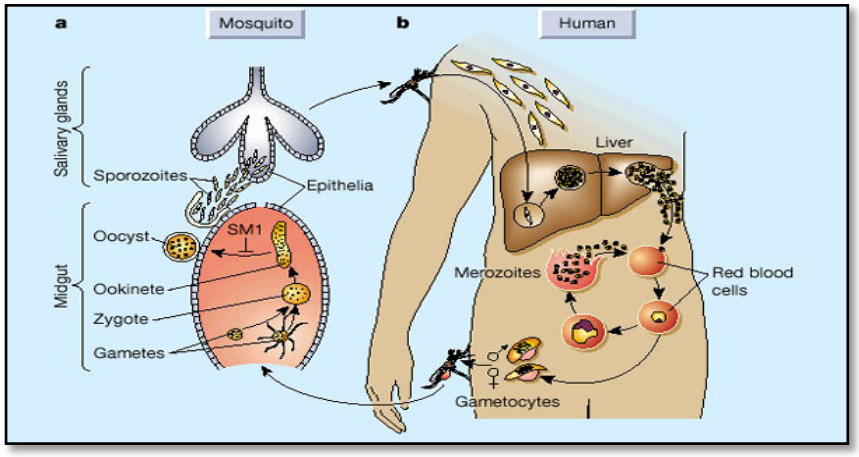

Figure 2: *Plasmodium falciparum* life cycle (Lycett & Kafatos, 2002)

Malaria Control Measures

Two methods have been used to control malaria: First, vector control methods: long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) are the most important methods to protect people from the bites of mosquitoes, however, they are not enough to eradicate the malaria parasite. Second, drug therapies: several antimalarial drugs are available with different mechanisms of action. However, a number of factors such as the mutation rate of the parasite, the strength of drug selection and treatment compliance make *P. falciparum* resistant to antimalarial drugs (Petersen et al., 2011) such as chloroquine (CQ), which has been widely used as an antimalarial drug. Moreover, *P. falciparum* shows resistance to two other families of drugs, anti-folate drugs and, recently, artemisinin drugs. Because of the rise of drug resistance to current antimalarial drugs there is constant pressure to develop new and effective treatment options. Furthermore, RTS/AS01, a candidate vaccine of malaria has been developed to treat infants between 10 -14 weeks and young children between 5-17 months of age living in endemic regions. The vaccine is designed to prevent *P. falciparum* from entering the bloodstream and infecting the RBCs, and more recently it has been approved for use in Africa

(Morrison, 2015).

Antimalarial Drugs

Quinine, the oldest antimalarial drug comes from the cinchona trees in Peru and has been used to treat fever and chills (Butler et al., 2010). It was discovered in 1820 and used to treat a sever malaria up until World War II (Packard, 2014). Quinine has been demonstrated to accumulate in the parasite's digestive vacuole and can inhibits the detoxification of heme (Fitch, 2004). However, there are three genes have been associated with altered quinine response: *P. falciparum* chloroquine resistance transporter (PfCRT), *P. falciparum* multidrug resistance transporter1 (PfMDR1) (Sidhu et al., 2005) and *P. falciparum* multidrug resistance protien1 (PfMRP1).

Chloroquine was synthesized by Hans Andersag in 1934 and was the most widely used antimalarial drug (Pou et al., 2012). Introduced in the 1940s, chloroquine was an efficient and safe drug for use during pregnancy (Levy et al., 1991) and it is easier and cheaper to produce than quinine (Petersen et al., 2011). Chloroquine was recommended to treat all species of *Plasmodium* (*P. falciparum, P. vivax, P. ovale* and *P. malarie*) (Mushtaque, 2015). Chloroquine was introduced in the Thai-Cambodian region and in Colombia. However, chloroquine resistance appeared nearly 10 years after its introduction (Petersen et al., 2011). One explanation for *P. falciparum* resistance to chloroquine is the correlation with a mutation in PfCRT protein (Lehane et al., 2012).

Anti-folate drugs, such as Sulfadoxine-Pyrimethamine (S-P), were once highly effective and inexpensive antimalarial drugs and were used to treat pregnant women infected with malaria (Petersen et al., 2011). However, mutation in the DHFR (dihydrofolate reductase) and DHPS

(dihydropteroate synthase) genes of *P. falciparum* has been associated with decreased parasite sensitivity to anti-folate drugs (Sridaran et al., 2010).

Due to the emergence of resistance to quinolone and anti-folate drugs in *P. falciparum*, artemisinin have been used as a replacement in antimalarial chemotherapy worldwide (Petersen et al., 2011). Artemisinin was isolated from the *Artemisia annua* plant in China (Eastman & Fidock, 2009). The drug is active against all species of *Plasmodium* by clearing all parasite stages from the blood (Burrows et al., 2014). Artemisinin combination therapies (ACTs) were originally safe and efficient drugs because these combination therapies (ACTs) such as artesunate-mefloquine, artesunate-sulfadoxin-pyrimethamine or artesunate-amodiaquine decrease the selection pressure and consequently the emergence of antimalarial resistance (Eastman & Fidock, 2009). However, malaria parasite resistant to combination drugs with artemisinin has been recently reported (Dondorp et al., 2011).

Primaquine (PQ) (also known as primaquine phosphate) is an antimalarial drug that prevents relapse in vivax and ovale malaria (Ashley et al., 2014b). It has been shown to be the only drug that kills gametocytes (Butterworth et al., 2013). However, it has not been used widely to treat malaria because of its side effects on glucose-6-phosphate dehydrogenase (G-6PD) deficient individuals an effect, which causes hemolytic anemia (Ashley et al., 2014a).

Ketotifen as antimalarial drug

Ketotifen is an anti-histamine drug, and it has been approved for human and animal use to relieve eye irritation and prevent asthma attacks (Tran et al., 2014). Histamine, stored in mast cells, is released in response to a foreign stimulus and binds to H1 receptors. Ketotifen is thought to prevent the release of histamine from mast cells and blocks H1 receptors. By blocking the

action of histamine, ketotifen prevents the body from reacting to foreign allergen that causes allergic symptoms (Craps & Ney, 1984). In addition, it has been reported by Eastman that ketotifen blocks *P. falciparum* oocyst development (Eastman et al., 2013). Their results also showed that ketotifen has some effect on the killing of trophozoites and schizonts in the liver stage (Eastman et al., 2013). In addition, ketotifen appears to have some activity against relapse of *P. cynomolgi* in monkeys. Ketotifen, in combination with other antimalarial drugs such as CQs, was also successful in the treatment of patients infected with *P. falciparum* (Ibrahim et al., 2000). Moreover, it has been shown that ketotifen and another anti-histamine drug as cyproheptadine and azatadine reverse CQ resistance in *P. falciparum* (Quan H & Tang LH, 2008, Basco et al., 1991).

Reserpine as a transporter inhibitor

Reserpine is a second line treatment drug against hypertension. It is an indiol alkaloid from the root of Rauwolfia serpentine (Abdelfatah & Efferth, 2015). Reserpine works by blocking the neurotransmitters at the nerve ending in the body leading to the relaxation or dilatation of blood vessel walls, which lowers the blood pressure and heart rate. The present investigation has been shown that reserpine inhibits the transport function of P-gp and ABCG2 *in vitro* and it kills tumour cells due to its profound cytotoxicity (Abdelfatah & Efferth, 2015).

1.1.2 ATP Binding Cassette (ABC) Transporters

ABC Transporters

The ABC transporters are one of the largest transporter families of proteins that are present in both eukaryotes and prokaryotes (Ford et al., 2009). ABC transporters have the ability to transport metabolites across the cell membrane in an energy-dependent manner (Davidson & Chen, 2004). ABC transporters are well represented in many organisms. For example, the

Escherichia coli chromosomes encode ≈70 ABC transporters (Linton & Higgins, 1998), yeast genome encodes 30 ABC transporters and 48 are found in humans. It is interesting to know that antimalarial drugs can affect parasites proteins, including ATP binding cassette (ABC) transporters (Hung et al., 1998). In 1982, Giovanna Ames' laboratory was the first to clone an ABC transporter from *Salmonella typhimurium* (Higgins et al., 1982). ABC transporters are important because they are involved in a number of genetic diseases and have the ability to pump cytotoxic molecules out from the cells (Linton, 2007). These transporters are known to play a role in drug and sterol transport, and metabolism in organisms including bacteria, parasites, yeasts, plants, flies, fish and humans. Among the genetic diseases, Tangier disease is caused by mutation in the ABCA1 transporter (Oram, 2000), cystic fibrosis is due to mutation of ABCC7 (Riordan et al., 1989) while ABCG2 is associated with gout disease (Wang et al., 2010). ABC transporters exist either as full transporters that consist of two transmembrane domain (TMD) and have a total 12 transmembrane helices per monomer and two NBDs or as half transporters consisting of one TMD that has six transmembrane helices, and one NBD (Tarling & Edwards, 2011) (**Figure 3).** The conformational changes in TMDs induced by ATP hydrolysis are responsible for the opened or closed states of these transmembrane structures (Glavinas et al., 2004). ABC transporters are grouped into two classes: exporters and importers (Hollenstein et al., 2007). ATP binds at the NBDs of ABC transporters and uses the energy from ATP hydrolysis to translocate different substrates such as sugar, lipids, ions, cholesterols, proteins, peptides, and fatty acid across cell membrane.

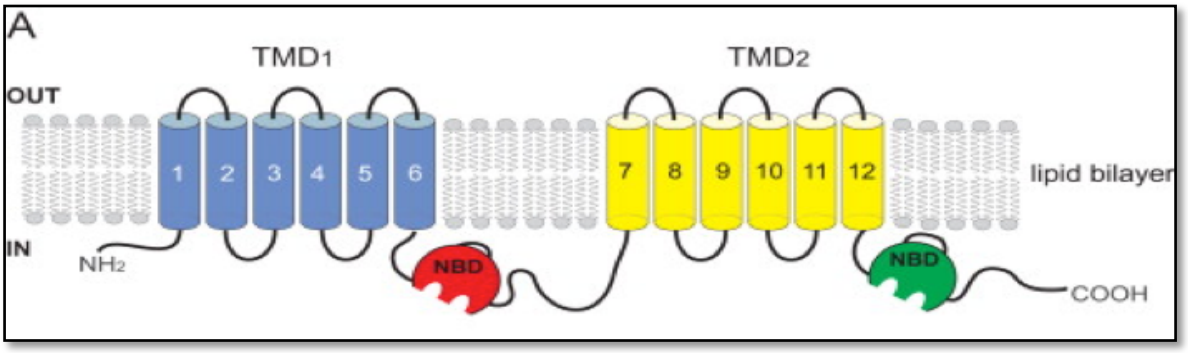

Figure 3: Typical structure of an ABC transporters (Dermauw & Van Leeuwen, 2014)

ABC Transporters in Humans

In humans, 48 ABC transporters are grouped into seven subfamilies that are arranged as ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG (Kerr et al., 2011) based on amino acid sequence homology of the ATP-binding domains (Mo & Zhang, 2012) and the similarity in gene structure (Glavinas et al., 2004). The ABCG subfamily plays a role in drugs and cholesterol transport. In humans, the ABCG family is divided into five subfamilies: ABCG1, ABCG2, ABCG4, ABCG5 and ABCG8 (Dean et al., 2001). Members of the ABCG subfamily are half-transporters containing one NBDs located at the N-terminal, and one TMDs located at the C-terminal (Glavinas et al., 2004). ABCG5 and ABCG8 act as heterodimers, and ABCG2 as a homodimer (Cserepes et al., 2004). ABCG1 and ABCG4 are functional as homodimers and heterodimers (Cserepes et al., 2004). Recently, a study has shown that ABCG1, ABCG5 and ABCG8 play a significant role in the regulation of cholesterol homeostasis (Li et al., 2013).

ABCG1 and Cholesterol Transport

ABCG1 is an intracellular cholesterol transporter that plays a role in cholesterol homeostasis, especially in the liver (Ito, 2003). It has been reported that huABCG1 homodimerize with another huABCG1 and heterodimerize with huABCG4 to transport sterol

(Kerr et al., 2011). ABCG1 is expressed in lung, spleen, brain, and kupffer cells of the liver. In addition, ABCG1 is concentrated in the granules of pancreatic beta cells (Tarling & Edwards, 2011) and is highly expressed in macrophages.

ABCG5/ABCG8 and Cholesterol Transports

ABCG5 and ABCG8 are expressed exclusively in the intestine and the liver. ABCG5 and ABCG8 transport sterol across membranes (Hubacek et al., 2001). Mutations in ABCG5 and ABCG8 cause heritable disorders such as sitosterolemia (Woodward et al., 2011).

ABCG2 and Drug Transport

ABCG2 is one of few human ABC transporters that have been implicated in multidrug resistance (MDR) in cancer chemotherapy (Mo & Zhang, 2012). HuABCG2 is localized to the plasma membrane (Tarling & Edwards, 2011) and is known as the breast cancer resistance protein (BCRP), ABC placenta protein (ABCP) (Xu et al., 2004) or mitoxantrone resistance (MXR)(Maier et al., 2009). ABCG2 plays a role in multidrug resistance by effluxion of anticancer drugs (Xu et al., 2004). It has been reported that huABCG2 homodimerize to transport anticancer drugs, and other normal cell metabolites, such as uric acid and heme (Kerr et al., 2011). Moreover, huABCG2 is highly expressed in the placenta, the blood-brain barrier (Mo et al., 2012), the large and small intestine (Clark et al., 2006), the liver (Massey et al., 2014), the colon (Mo & Zhang, 2012) and in red blood cells (Leimanis & Georges, 2007).

Table 1: The human ABCG transporters (Kerr et al., 2011).

Name	Dimerization	Substrate
ABCG1	Homo; possibly hetero with ABCG4	Lipids
ABCG2	Homo	Urate, heam, drugs
ABCG4	Homo; possibly hetero with ABCG1	Lipids
ABCG5	Obligate hetero with G8	Plant sterols
ABCG8	Obligate hetero with G5	Plant sterols

1.1.3 Transporters involved in resistance to antimalarial drugs

ABC transporters are also present in many protozoans such as *Leishmania*, *Trypanosoma*, *Toxoplasma*, *Entamoeba* species and *Plasmodium* (Sauvage et al., 2009). In *P. falciparum,* there are 16 ABC transporters (Koenderink et al., 2010), which are grouped into six sub-families: ABCB, ABCC, ABCE, ABCF, ABCG and ABCI (Koenderink et al., 2010). *P. falciparum* does not possess ABCA and ABCD transporters, which are present in humans. However, *P. falciparum* expresses ABCI, which does not exist in humans.

Table 2: ABC transporters selected from *Plasmodium falciparum* (Sauvage et al., 2009)

Sub-family	*Leishmania spp.*	*T. brucei*	*T. cruzi*	*P. falciparum*	*T. gondii*	*C. parvum*	*E. histolytica*	*G. lamblia*
ABCA	10	2	5	0	0	0	2	15
ABCB	4	2	2	7	6	2	7	1
ABCC	8	3	4	2	2	14	6	4
ABCD	3	3	3	0	0	1	0	0
ABCE	1	1	1	1	1	1	1	1
ABCF	3	3	3	2	1	1	2	1
ABCG	6	4	4	1	5	2	1	0
ABCH	3	1	3	-	4	0	2	0
ABCI	-	-	-	3	-	-	-	-
Total	42	22	28	16	20	21	21	22

Two transporters have been shown to cause resistance to antimalarial drugs in *Plasmodium falciparum*: the chloroquine resistance transporter (PfCRT), a member of the drug metabolite transporters and the P-glycoprotein homologue (Pgh-PfMDR1), a member of the ATP-binding cassette (ABC) transporters. The normal substrate of PfCRT and PfMDR1 are not known. In *P. falciparum* the B-subfamily contains seven members includes PfABCB1 and PfABCB2 or PfMDR1 and PfMDR2 associated with drug resistance; PfMDR1 and PfMDR2 localized in the digestive vacuole (DV) and plasma membrane of the parasite, respectively (Cowman et al., 1994; Rosenberg et al., 2006). In addition to the ABCB transporters, PfABCC1 and PfABCC2 or (PfMRP1 and PfMRP2) have been implicated in conferring resistance to quinolone drugs. They are localized to the plasma membrane of the parasite and mediate the efflux of glutathione (Kavishe et al., 2009). The function and normal substrate of PfABCG are currently not known. The objective of this study is to characterize the expression of the only member of the G-subfamily i.e. PfABCG and determine the subcellular localization and substrate specificity in heterologous expression system.

ABCG Transporter in *P. falciparum*

PfABCG is the only member of G-subfamily in *P. falciparum* while humans encode five ABCG transporters. The amino acid sequence of PfABCG encodes a half transporter with one N-terminal nucleotide-binding domain (NBD) and a transmembrane domain with six helices (Edaye & Georges, 2015). PfABCG shows nearly 24.3% and 26.5% amino acid sequence identity with the human orthologues ABCG1 and ABCG2, respectively (Edaye & Georges, 2015). PfABCG sequence identity with human (ABCG1, 2, 4, 5 and 8) is shown in **Table 3.**

Table 3: Sequence homology and identity between PfABCG and human ABCG1, 2,4,5 and 8

PfABCG alignment	Global alignment	
	Identity	Similarity
HuABCG1	24.3 %	42.1 %
HuABCG2	26.5 %	46.6 %
HuABCG4	23.2 %	41.9 %
HuABCG5	22.4 %	42.6 %
HuABCG8	21.2 %	37.9 %

In an earlier study by Tran and colleagues, it was demonstrated that a knock out of the PfABCG transporter in *P. falciparum* 3D7 parasites **(ΔgABCG2)** caused an increase in gametocyte production and a decrease in neutral lipid accumulation (Tran et al., 2014).

Moreover, the same author showed that neutral lipids (cholesterol ester, diacylglycerol and triacylglycerol) isolated from the knockout gametocytes (ΔgABCG2) decreased from 24% to 13% compared to the wild type (Tran et al., 2014). By contrast, there was an increase in the amount of Phosphatidylethanolamine (PE) in ΔgABCG2 gametocytes compare to the wild type 3D7 parasites. The lipid content is important for the production and integrity of the parasite's cell membrane. Moreover, lipids play a role in haemozoin formation in the parasite's digestive vacuole. Both neutral lipids and PE are associated with lipid bodies suggesting a possible role of PfABCG in lipid transport in *P. falciparum* during gametocyte production (Tran et al., 2014).

In another report by Eastman et al., more than 2000 drugs were screened against wild type and PfABCG knockout of 3D7 parasite strain. In this study, PfABCG knockout parasites showed resistance to a drug named ketotifen while there was no difference in IC_{50} for

chloroquine and dihydroartemisin (Eastman et al., 2013). However, it was not determined if this effect was because of PfABCG or other cellular changes that occurring in the parasite due to the deletion of PfABCG (Eastman et al., 2013). From Eastman's report, PfABCG might play a role in ketotifen sensitivity and may behave as huABCG2 by transporting drugs.

More recently, Edaye et al. have shown that PfABCG is expressed at all stages of the *Plasmodium* life cycle and in several lab strains independent of the strains sensitivity to chloroquine. In this study, Edaye et al. showed that PfABCG expression is lower in the HB3 chloroquine sensitive strain relative to other chloroquine-sensitive and resistant strains (**Figure 4).**

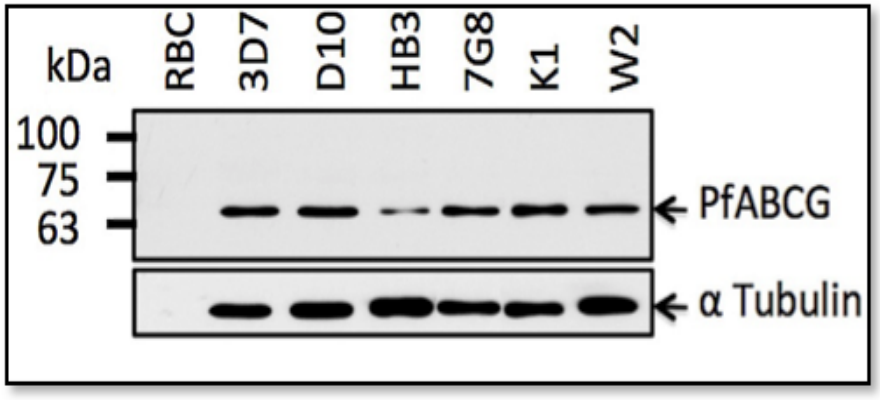

Figure 4: PfABCG expression in different chloroquine-sensitive (3D7, D10 and HB3) and resistant strains (7G8, K1 and W2) of *P. falciparum* (Edaye & Georges, 2015)

Interestingly, HB3 a (CQS strain) that shows lower expression of PfABCG was also less sensitive to ketotifen, while 3D7 and D10 (also CQS strains) expressed higher levels of PfABCG were more sensitive to ketotifen (**Figure 5 and Table 3)** (Edaye & Georges, 2015).

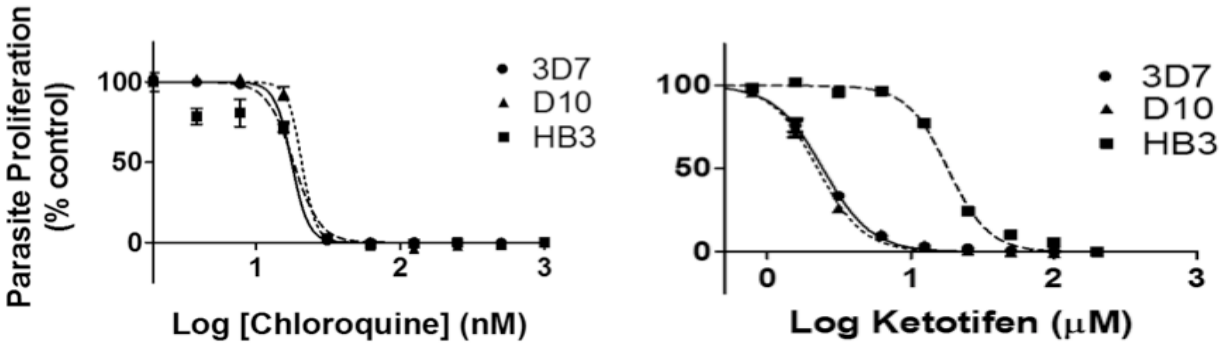

Figure 5: The proliferation of chloroquine-sensitive parasites with increasing concentration of ketotifen and chloroquine (Edaye & Georges, 2015)

Table 4: IC50 ± SD of ketotifen and chloroquine concentration on three chloroquine-sensitive parasites.

Drugs	3D7	D10	HB3
Ketotifen (μ M)	2.4 ± 0.4	2.2 ± 0.4	18.7 ±1.07
Chloroquine (nM)	17.7 ± 0.9	20.9 ± 2.2	18.3 ±1.3

This agrees with Eastman's work, where there is a positive correlation between PfABCG expression and the sensitivity to ketotifen. It confirms that parasites expressing PfABCG are more sensitive to ketotifen. Moreover, PfABCG was found to be expressed in all stages of the parasite in the plasma membrane in addition to intense staining below the plasma membrane. The nature of the latter stained structure is presently unknown (Edaye & Georges, 2015).

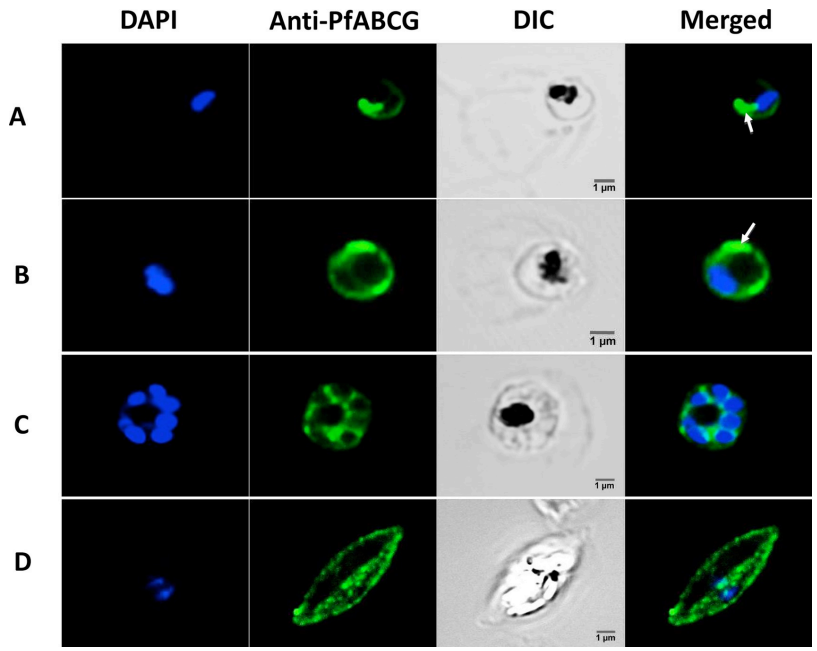

Figure 6: PfABCG localization in different *P.falciparum* stages (Edaye & Georges, 2015)

1.2 Rational and Objectives

P. falciparum encodes only one ABCG protein that shares ~24.3% and 26.5% amino acid sequence identity with human ABCG1 and ABCG2, respectively (Edaye & Georges, 2015). It is not entirely clear if PfABCG is more like ABCG1 or ABCG2. Members of the human ABCG subfamily (e.g., ABCG1, ABCG4, ABCG5 and ABCG8) transport the same substrate (e.g., cholesterol), while human ABCG2 transports chemotherapeutic drugs and other normal cell metabolites. Additionally, human ABCG1 and ABCG2 are differentially localized to the endosome and plasma membrane, respectively. Furthermore, one study (Tran et al., 2014) has suggested that PfABCG might play a role in lipid transport and thus behaves as ABCG1, while Eastman et al have suggested that PfABCG might play a role in drug transport (Eastman et al., 2013).

1.2.1 Objectives of the project

The overall objective of this project is to determine if PfABCG is a drug transporter (e.g., like human ABCG2) or a sterol transport (e.g., like human ABCG1). To achieve this objective PfABCG was expressed in a heterologous expression system in mammalian HEK-293 cells due to for their ease growth and transfection in an effort to better characterize its substrate specificity and subcellular localization.

The aim of the dissertation was:

1- Characterize PfABCG in a heterologous expression system, in HEK-293 mammalian cells.

2- Determine the subcellular localization of PfABCG in mammalian cells.

3- Determine the substrate specificity of PfABCG in mammalian cells.

1. 3 Reference

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Chapter Two

Draft Manuscript

Characterization of PfABCG in a heterologous expression system in HEK-293 mammalian cells

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1. Abstract

In *P. falciparum*, mutations and altered expression of PfMDR1 (PfABCB1) and PfMRP1 (PfABCC1) have been implicated in the parasite's resistance to several antimalarials, including chloroquine and anti-folates. Previous studies have identified the ATP Binding Cassette (ABC) transporters as drug targets and modulators of resistance to diverse drugs. In an earlier report, we have demonstrated the expression and subcellular localization of PfABCG in different stages of the parasite. Moreover, we also showed that PfABCG expression correlates with the sensitivity of parasite to ketotifen, an anti-histamine drug. In this report, we demonstrate the expression and subcellular localization of codon optimized GFP-PfABCG in HEK-293 cells. The expression of GFP-PfABCG in HEK-293 cells was confirmed by Western blot using anti-GFP antibodies. Using GFP-PfABCG transfectants, we also show that the protein localizes to the endoplasmic reticulum. In addition, and consistent with earlier observation, we demonstrate that HEK-293 clones stably transfected with GFP-PfABCG are more sensitive to ketotifen, when endogenous P-glycoprotein (ABCB1) activity is inhibited with reserpine. Together, our results demonstrate for the first time the functional expression of PfABCG in a heterologous system and provide an assay system to further investigate its substrate specificity.

2.Introduction

Malaria is a deadly infectious disease in many tropical and subtropical countries with more than 438.000 deaths and 214 million cases annually (WHO, 2015). Previous efforts to eradicate malaria have failed, due to the emergence of drug-resistant parasites, insecticide-resistant mosquitoes and lack of drugs or vaccines to block parasite transmission. In the last decades resistance to quinoline-based drugs have been attributed to the action of molecular efflux pumps that reduce the toxicity of such antimalarials drugs (Bray & Ward, 1993). Two transporters have been shown to cause resistance to antimalarial drugs in *P. falciparum*: the chloroquine resistance transporter (PfCRT) (Ecker et al., 2012), a member of the drug metabolite transporter and the P-glycoprotein homolog (PfMDR1 or Pgh1), a member of the ATP-binding cassette proteins (Reed et al., 2000). ABC transporters are organized either as full transporters containing two TMDs and two NBDs (e.g.PfMDR1) or half transporters containing one TMD and one NBD that homo or hetero dimerize to form a functionally active transporter *P. falciparum* encodes 16 members of the ABC family grouped into 6 subfamilies ABCB, ABCC, ABCE, ABCF, ABCG and ABCI (Sauvage et al., 2009). It is interesting that while in humans there are five members in the ABCG subfamily (ABCG1, 2,4, 5 and 8), *P. falciparum* encodes one ABCG (Sauvage et al., 2009). *P. falciparum* ABCG (PfABCG) protein shares 24% and 26% amino acid sequence identity with human ABCG1 and G2, respectively (Edaye & Georges, 2015). It is not entirely clear if PfABCG substrate specificity is more like ABCG1 or ABCG2. Members of the human ABCG subfamily (ABCG1, G4, G5 and G8) transport cholesterol (Kerr et al., 2011) while human ABCG2 transport chemotherapeutic drugs and other normal cells metabolites (Tarling & Edwards, 2011). Additionally, human ABCG1 and ABCG2 are differently localized to the endosome and plasma membrane, respectively (Kerr et al., 2011;

Tarling & Edwards, 2011). To date, the function and normal substrate of PfABCG is not known. The objective of this study is to demonstrate the expression of GFP-PfABCG protein and determine its subcellular localization in a heterologous expression system, in HEK-293 mammalian cells.

3. Material and Methods

3.1 Cloning of PfABCG gene into pCruz-GFP vector

The full-length PfABCG cDNA previously cloned from *P. falciparum* (ref. Edaye S & Georges) was used to codon optimize PfABCG for mammalian expression. Codon optimized PfABCG was synthesized and cloned into pUC57/pBlueScript II SK (+) (GenScript Biotech Co. USA). Full length, codon optimized PfABCG, was cut out from PfABCG/pUC57 and inserted into a mammalian expression vector pCruz-GFP vector (Santa Cruz Biotechnologies) at Knp1-Hpa I restriction sites. The resultant pCruz-GFP-PfABCG construct generated full length PfABCG protein with the green fluorescent protein (GFP) fused to the N-terminal side of PfABCG protein. GFP-PfABCG sequence integrity was confirmed by DNA sequencing using vector compatible primers (McGill DNA sequencing faculties).

3.2 Transfection of HEK-293 cells

pCruz-GFP and pCruz-GFP-PfABCG were stably transfected in HEK-293 cells. One day before transfection, 600,000 cells / 2 ml of HEK-293 cells were seeded into 6 well plate. After 24 hours, the media was removed and fresh free serum DMEM media was added. Transfection was performed using 2.5 μg of each plasmid in a serum-free OPTI-MEM media and Lipofectamine 2000 transfection reagent (Invitrogen). On the day of transfection, each of the DNA sample were diluted with 250 μn of a serum-free OPTI-MEM media (Invitrogen). The transfection reagent was prepared by mixing 10 µl of Lipofectmaine 2000 (Invitrogen) in 250 µl of OPTI-MEM media. The mixture was incubated for 30 min prior to its addition (dropwise) onto HEK293 cells with constant rocking to distribute the mixture evenly over the surface of the dish. Cells were incubated at 37^{o}C for 48 hours and the transfection efficiency was assessed by microscopy and

Western blotting. Transfected cells were then sorted using flow cytometry, cloned and selected with G418 (400mg/ml).

3.3 Cell culture and Western blotting

GFP-PfABCG stably transfected HEK-293 cells and, control (non-transfected) HEK-293 cells were grown in DMEM media containing 10% FBS, 5% CO_2 at 37^{o}C. The media was removed and the cells were washed twice with serum-free DMEM media. Cells were collected with 4 ml of cold PBS and then washed 3 times with cold PBS. The cell pellets (~ 8x 10^6 cells) were extracted with 100-150 µl of freshly prepared Radio-immunoprecipitation assay (RIPA) buffer containing 1 μ l (of protease inhibitor per 1 ml RIPA buffer (Thermo Scientific). Protein extractions were quantified using BCA protein assay according to the manufactured protocol (Thermo Fisher).

For Western blotting, protein extracts (20 μ g) were resolved on 8% polyacrylamide gels (Reference: ·Refer. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227 (1970), pp. 680–685) and transferred onto PVDF membrane (Towbin et al., 1979). The membrane was blocked with 5 % milk prepared in PBS-Tween 20 overnight at 4 ^{o}C. After blocking, the membrane was probed with anti-GFP monoclonal primary antibody at dilution 1:500 at 4^{o}C, overnight (Life technologies). After incubation with the primary antibody, the PVDF membrane was probed with goat anti-rat, as secondary antibody at 1:5000 dilution for 3 hours. The bands were visualized with an enhance chemiluminescence detection kit (Thermo Fisher scientific) Pico.

3.4 Subcellular localization of GFP-PfABCG transfected in HEK-293 cells by confocal fluorescence microscopy

The subcellular localization of GFP and GFP-PfABCG was determined in HEK-293 cells. Cells were fixed with 1% paraformaldehyde in PBS for 15 min at room temperature and then washed with PBS. Cells were then permeabilized with 0.1 % Triton X-100 in PBS for 2 min and washed with PBS. Finally, the cells were stained with 1x DAPI (4',6-diamidino-2-phenylindole), which was prepared by mixing 1 μl of DAPI (10000X) in 10 ml PBS. After staining, the coverslips were washed 5 times with PBS, mounted on slides and visualized using a fluorescent microscope. For live microscopy, 48h after transfection, the cells were incubated with 300 nM MitoTracker® Orange CM-H2TMRos (Life technologies) or 1 μM ER-Tracker™ Red (Life technologies) for 30 min followed by 10 min incubation with Hoechst 33342. After the incubation, the cells were visualized with confocal laser scanning microscopy using a Zeiss LSM710 (Carl Zeiss, Jena, Germany). Images were obtained using 20x and 60x objectives.

3.5 Cell proliferation assay

HEK-293/GFP-PfABCG transfected and HEK-293 untransfected cells (3000 cells per well) were seeded at density of 3000 cells in 100 μl media (α-MEM containing 10% FBS; Gibco, Life technologies) per well of 96-well plates. Cells were kept overnight at 37^{o}C, prior to the addition of increasing concentration of ketotifen (0 - 400 μM) with or without 20 μM reserpin in 100 μl/well. Cells were allowed to proliferate for 72 hours at 37^{o}C, then fixed with the addition of 100 μl of 10 % TCA into each well of 96-well plates. Cells were incubated for 1 hour at 4^{o}C, then washed 3 times with distilled water. Fixed cells were stained with Sulfo-rhodamine (SRB; 100 μl/well of 0.057% SRB/1% acetic acid) dye for 30 min at room temperature. Plates were washed with 3X times with 1% acetic acid/distilled water. The dye was solubilized with the

addition of 100 µl/well of 10 µM Tris base (pH 10.5) and plates were mixed for 5-10 minutes to prior to measuring fluorescence intensity at 510 nm using the micro-plate reader SYNERGY H4 hybrid (BIOTEK). Data analysis was performed with Prism 6.0 (GraphPad Software) to obtain the 50% inhibitory concentrations (IC_{50}). Graphs show the mean ± SD of a representative experiment done in triplicate.

4. Results and Discussion

To determine the expression of GFP-PfABCG in stable transfectant clones, GFP antibody was used. The Western blot results in (**Figure 1)** show the presence of a unique band migrating around 75 kDa in the GFP-PfABCG samples while no band is in the HEK-293 sample. Moreover, the lysate of GFP transfected cells show a band around 27 kDa corresponding to the GFP protein (**Figure 1)**. Although GFP migrated with its expected molecular mass of 27 kDa, the apparent molecular mass of GFP-PfABCG was lower than the calculated mass of 104 kDa. This observed difference between apparent and calculated molecular mass is due to PfABCG mobility on SDS-PAGE reported earlier (Edaye & Georges, 2015). Moreover, the latter is consistent with published reports on the apparent molecular mass of human ABCG2 (60-65 kDa) versus its calculated molecular mass of 72 kDa (Ni et al., 2010; Pal et al., 2007). The rational for constructing the GFP tag on the N-terminal of PfABCG rather than the C-terminal was based on earlier reports of human ABCG2, where C-terminal fused GFP (huABCG2-GFP) was non-functional (Orbán et al., 2008; Takada et al., 2005).

Given the results obtained for the expression of GFP-PfABCG in HEK-293 cells, it was of interest to determine the subcellular localization of the fusion protein. The subcellular localization study was based on the fluorescence emitted by GFP associated with PfABCG. It is important to note that the GFP fluorescence was stable in HEK-293 cells transfected with the GFP-PfABCG plasmid. (**Figure 2)** shows the immunofluorescence staining of HEK-293 transfected cells. The signal obtained for GFP transfected cell was diffused and spread all over the cells denoting a cytosolic location for GFP. In the GFP-PfABCG transfected cells, the fluorescence signal is surrounding the nucleus and is localized in part of the cytosol.

To determine if GFP-PfABCG localized to the ER or to the mitochondria, the GFP-PfABCG transfected cells were stained with ER tracker red or Mito tracker orange. The results in (**Figure 3 and 4)** show that GFP-PfABCG signal overlapped with ER tracker signal appearing as red signal (**Figure 3**) but not with Mitotracker orange signal (**Figure 4**) suggesting that the subcellular localization of GFP-PfABCG protein is in the endoplasmic reticulum and does not localized in the mitochondria. Since GFP-PfABCG is localized to the ER it was of interest to determine the function of the fusion protein. Using the SRB assay, ketotifen effect was tested on HEK-293 cells and HEK-293 transfected with GFP-PfABCG. The results show that there is a slight difference in the IC50 between the two cells lines with GFP-PfABCG transfected cells being more resistant to ketotifen than HEK-293 alone (**Figure 5**). The reason for not seeing a major difference between HEK-293 and HEK-293 with GFP-PfABCG with ketotifen alone may be due to the efflux activity of the endogenous ABC proteins specifically P-gp and possibly ABCG2. It has been shown that P-gp, ABCG2 and MRP1 are expressed at low levels in HEK-293 cells (Scharenberg et al., 2002). In order to test this possibility, we used reserpine, which can inhibit the efflux function of two ABC transporters (P-gp and ABCG2). The results obtained with the combination of increasing concentration of ketotifen with 20 μM reserpine show that GFP-PfABCG transfected cells are more sensitive to ketotifen compare to HEK-293 cells (**Figure 6**). 20 μM of reserpine caused a significant decreased in the IC50 of ketotifen (55.1 ± 17.07,13.76 ± 1.30) versus ketotifen alone (56.04 ± 4.78, 74.23 ± 2.3) in HEK-293 and HEK-293 transfected with GFP-PfABCG respectively **(Table 1)**.

5. Conclusion

GFP-PfABCG fusion protein with GFP at the N-terminus of PfABCG was functionally expressed in HEK-293 cells. GFP fusion PfABCG showed protein expression at 75 KDa in the cultured mammalian HEK-293 cells. GFP-PfABCG migrates with a lower mass on SDS-PAGE consistent with earlier observation of PfABCG in *Plasmodium falciparum* (Edaye & Georges, 2015). Fluorescent microscopy suggested that the subcellular localization of GFP-PfABCG protein expressed in HEK-293 cells is in the endoplasmic reticulum since it colocalizes with ER tracker red, and does not localize in the mitochondria. Finally, the preliminary SRB assay suggested the possible role of PfABCG in drug transport, however it is not entirely clear why PfABCG-mediated sensitivity to ketotifen is not seen in the absence of reserpine. Further research is required to confirm the results described in this manuscript and PfABCG function.

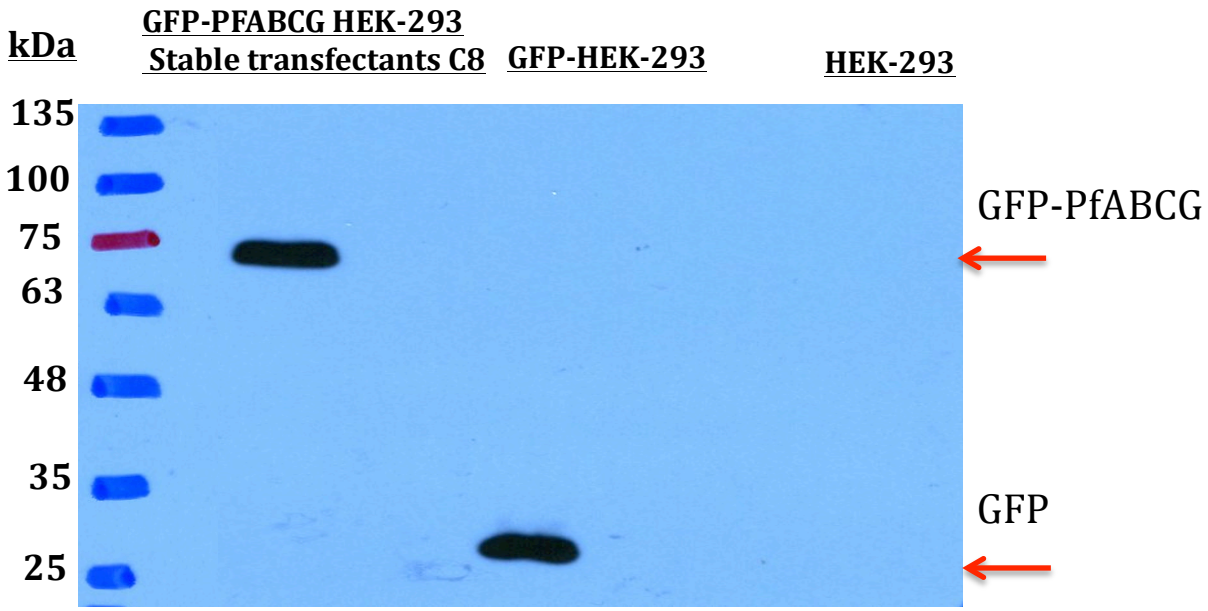

Figure 1: Heterologus expression of GFP-PfABCG in HEK-293 cells -Western blot of total cell extracts from non-transfected (HEK-293), stable transfectant clones of human codon optimized cDNA in pCruz vector containing GFP-PfABCG fusion construct (clone 8) and pCruz-GFP stable transfectant HEK-293 cells resolved on SDS-PAGE, transferred to PVDF membrane and probed with anti-GFP mAb. Molecular markers are to the left of the figure. Migration of GFP-PfABCG and GFP (Green fluorescent protein) on SDS-PAGE are indicated with red arrows to the right of the figure.

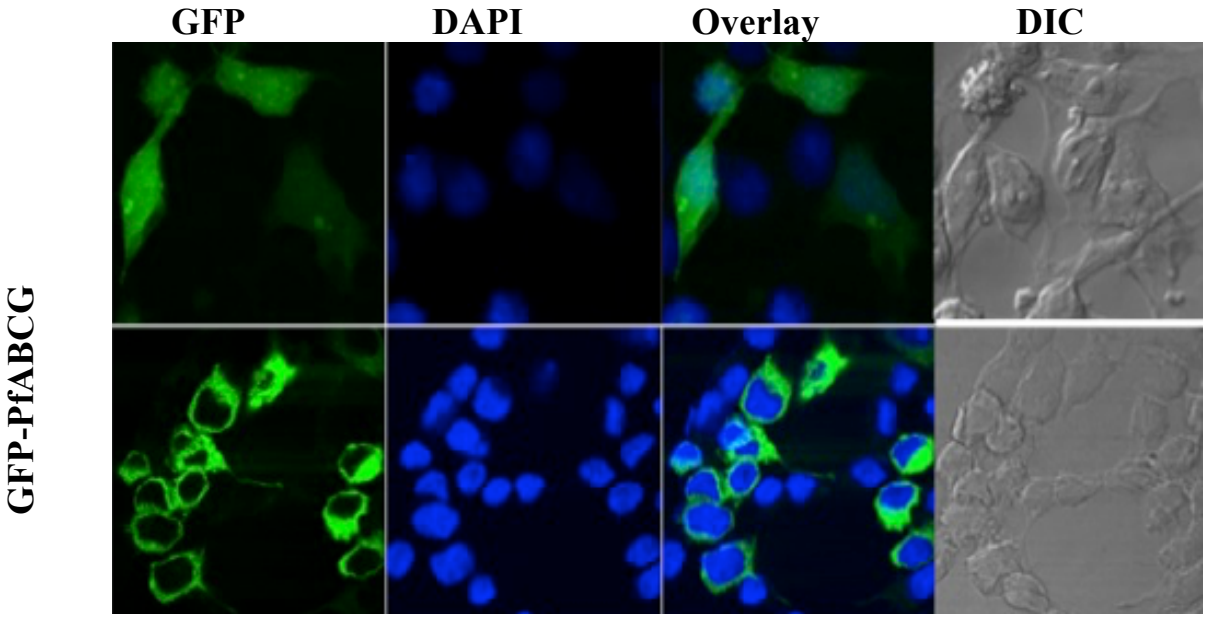

Figure 2: GFP-PfABCG expression in HEK-293 cells: Fluorescent microscopy of GFP and GFP-PfABCG HEK-293 transfectants Columns 1 and 2 show the fluorescence of unstained, and DAPI nuclear DNA stained HEK-293 cells transfected with pCruz-GFP or pCruz-GFP-PfABCG vectors. Column 3 shows the overlay of GFP-fluorescence. Column 4 shows the differential interference contrast of GFP and GFP-PfABCG transfectants.

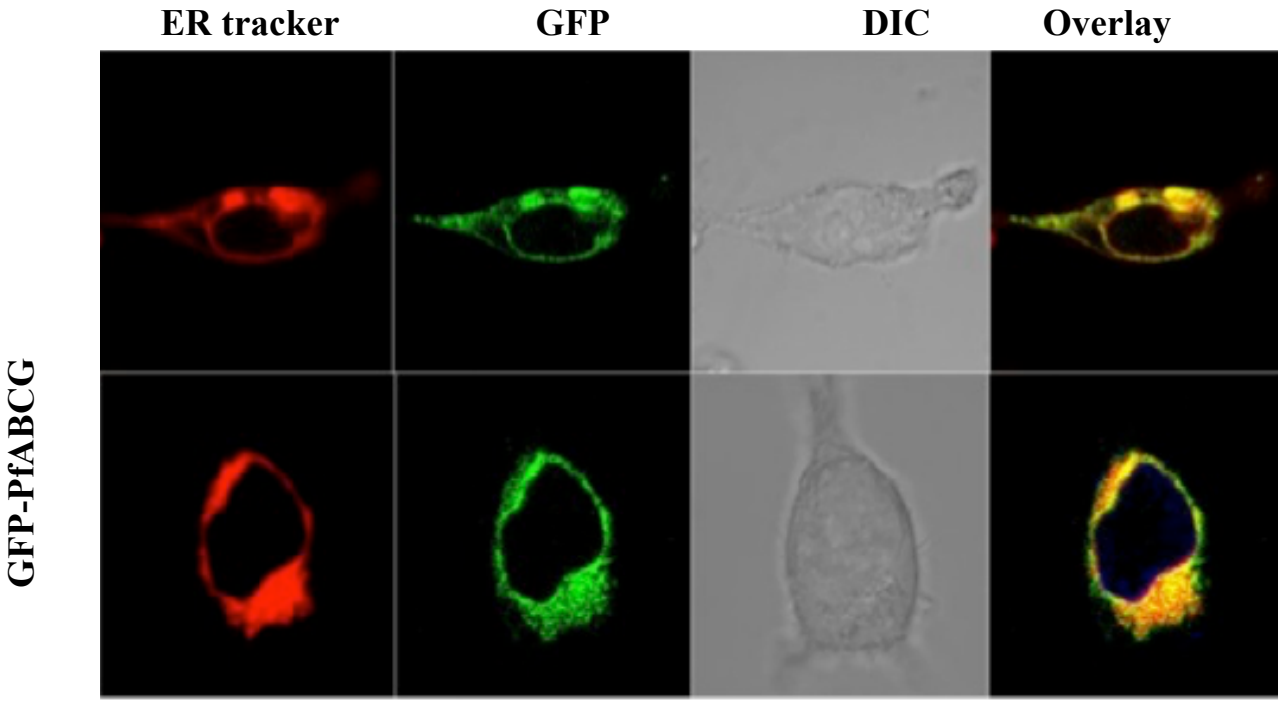

Figure 3: PfABCG localizes to the Endoplasm reticulum- Fluorescent microscopy of GFP-PfABCG HEK-293 transfectants stained with ER tracker red - Columns 1 and 2 show the fluorescence of GFP-PfABCG HEK-293 cells stained with endoplasmic reticulum specific dye (ER tracker red) and GFP fluorescence- Column 4 shows the overlay of GFP-fluorescence and ER tracker red. Column 3 shows the differential interference contrast of GFP-PfABCG transfectants.

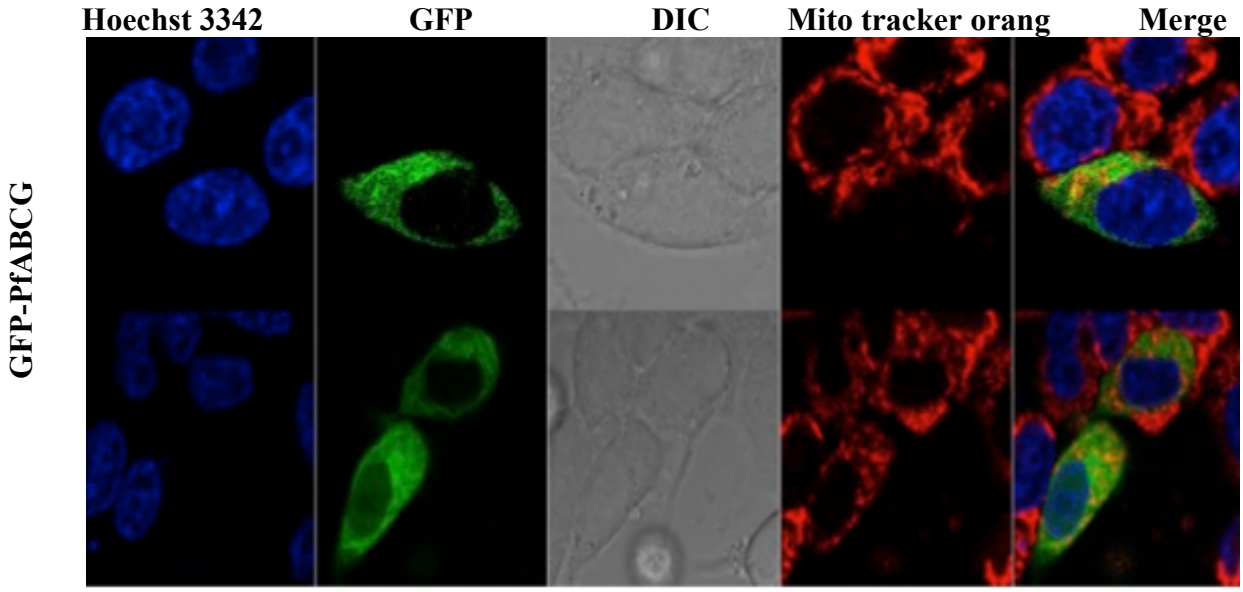

Figure 4: PfABCG does not localize to the mitochondria-Fluorescent microscopy of GFP-PfABCG HEK-293 transfectants stained with Mito tracker orange - Columns 1 and 4 show the GFP-PfABCG HEK-293 cells stained with Hoechst 33342 and the mitochondria specific dye (Mito tracker orange), respectively. Columns 2 and 5 show the GFP fluorescence of GFP-PfABCG cells and overlay of staining and fluorescence, respectively. Column 3 shows the differential interference contrast of GFP-PfABCG transfectants.

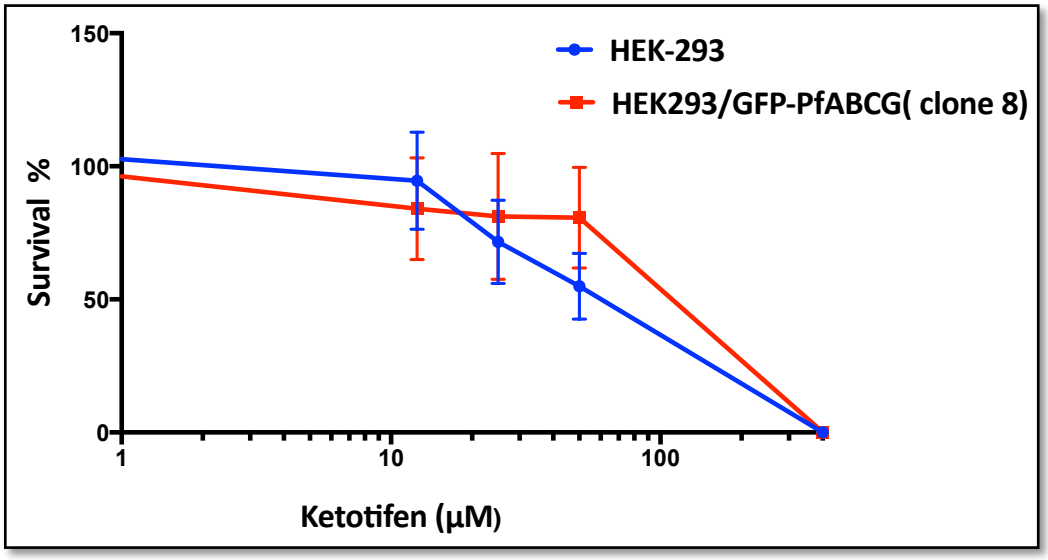

Figure 5: PfABCG mediates sensitivity of HEK-293 cells to ketotifen- Effects of ketotifen on the proliferation of HEK-293 and HEK-293/GFP-PfABCG cells – Cells (3000 cells/well) were grown in the presence of ketotifen (0- 400 µM). The graphs show a representative growth assay done in triplicates.

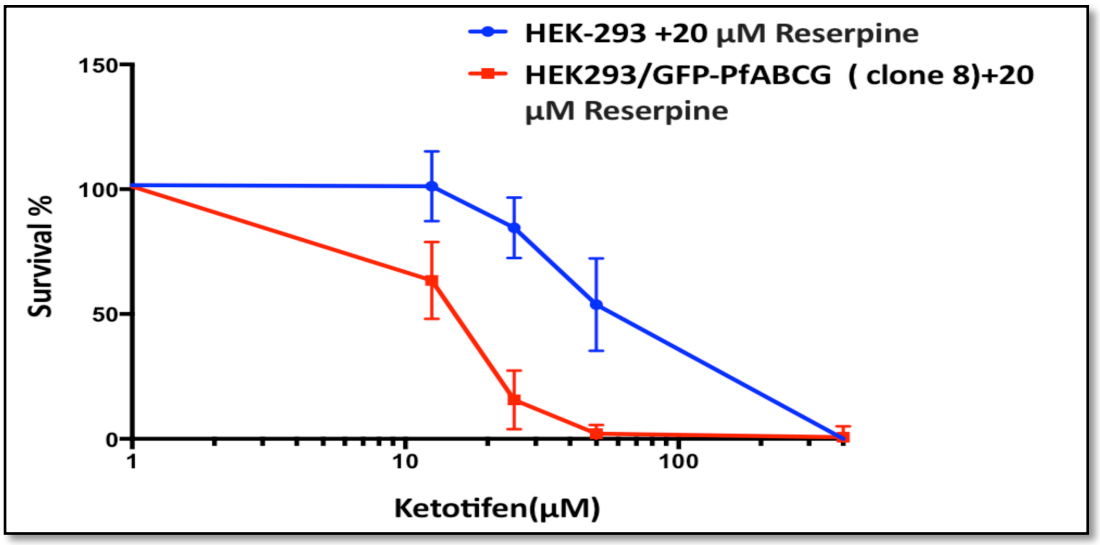

Figure 6: The proliferation of HEK-293 and HEK-293 transfected with PfABCG in increasing concentration of ketotifen (0- 400 µM) with reserpine (20 µM). The result shows the effect of ketotifen in the presence of 20 μ M reserpine on the proliferation of cells culture. The graph shows the mean of ± SD of three experiments done in triplicate.

Table 1: In vitro activity of ketotifen and reserpine on HEK-293 cells transfected with PfABCG: Effects of drugs on cell survival (IC_{50}) and PfABCG expression.

Drugs	HEK-293	HEK-293/GFP-PfABCG
Ketotifen (μ M)	56.04± 4.78	74.23± 2.3
Reserpine (μ M)+ Ketotifen (μ M)	55.1± 17.07	13.76± 1.30

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