Universidade Nova de Lisboa

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Biological characterization of de-ubiquitylating enzymes (UBPs/UCHs) in *Plasmodium spp* as potential drug targets

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DISSERTAÇÃO PARA OBTENÇÃO DO GRAU DE DOUTOR EM

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1.Neto Z, Machado M, Lindeza A, Gazzarini M, do Rosário V, Lopes D. Treatment of *Plasmodium chabaudi* parasites with curcumin: drug interaction and implications in the ubiquitin proteosome pathway (UPS). *Journal of parasitology Research* (2013). Article ID 429736.

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RESUMO

A malária ainda constitui um grande problema de saúde pública e a resistência aos antimaláricos ameaça todos os esforços efectuados com vista ao combate e controle desta doença. Existe uma grande necessidade de se identificar novos compostos de preferência que actuem em novos alvos terapêuticos. A via da ubiquitinação/proteosoma já foi identificada como um alvo terapêutico interessante. Mutações nas enzimas de des-ubiquitilação (DUBs) que catalizam a remoção da ubiquitina estão associadas ao desenvolvimento de doenças infecciosas e não infecciosas.

Neste projecto quatro DUBs foram identificadas no genoma do parasita *Plasmodium falciparum* e foram caracterizadas. A expressão dos genes que codificam estas enzimas ao longo do ciclo de vida do parasita na presença e ausência de fármaco foi efectuada por RT-PCR.Anticorpos policlonais obtidos a partir de ratinhos foram utilizados para a deteção da abundancia das proteínas ao longo do ciclo de vida do parasita. Utilizou-se ainda a tecnica de transfeção com o objectivo de criar uma linha knockout para determinar se estas proteínas são essências para o parasita. Proteínas recombinantes foram expressas em células de *E.coli* e actividade enzimática das mesmas foi testada usando um substrato específico para as DUBs. O inibidor das DUBs com actividade antimalarica, curcumina foi usado quer *in vitro* para testar a sua actividade sobre as proteínas recombinantes, mas também *in vivo* no modelo de malaria roedora de *Plasmodium chabaudi* em associação com cloroquina e artemisinina.Um ensiao de proteomica foi também usado para ver que proteínas estão alteradas em resposta ao tratamento com curcumina.

Os resultados demonstram que em *P. falciparum* os genes *pfuch-l1*, *pfuch-l3*, *pfuch-l54* e *pfubp-8* são diferencialmente expressos ao longo do ciclo de vida do parasita e as respectivas proteínas são mais abundantes no estadio de trofozoito e esquizonte. O tratamento dos parasitas com artemisinina, cloroquina, curcumina induziu um aumento temporário na expressão dos genes seguido de um declínio. Não foi possível obter uma linha parasitária knockout *pfuch-l1* e *pfuch-l3* viável. As proteínas recombinantes foram expressas com sucesso em células de *E. coli* excepto a *Pfuch-l54*. As *Pfuch-l1*, *Pfuch-l3*, *Pfubp-8* demonstraram actividade enzimática e interagiram com o susbstrato Ub-AMC. Os IC50 da curcumina nas proteínas recombinantes foram: *Pfuch-l1* 15µM, *Pfuch-l3* 25.4µM, *Pfubp-8* 10µM e para a proteina recombinante humana USP2, 5µM. A Curcumina quando testada nas células HepG2 apresenta alguma toxicidade *in vitro*, mas não apresenta uma alta toxicicidade em ratinhos e quando utilizada em associação com a cloroquina apresenta um efeito de sinergismo.Enquanto a associação da curcumina com artemisinina o resultado é antagónico.Os ensaios de proteomica em culturas de *P. falciparum* tratadas com curcumina revelaram 10 proteinas que se encontraram alteradas em resposta ao tratamento. Estas proteínas estão envolvidas no metabolismo do sulfato, tradução e degradação de proteínas, ciclo celular e organização celular.

Em conclusão, este trabalho demonstra que estas enzimas são potenciais alvos terapeuticos, mas será necessário mais estudos moleculares, bioquímicos e farmacológicos para aumentar a selectividade dos inibidores das DUBs para as enzimas do parasita e minimizar os danos nas proteínas do hospedeiro humano.

Palavra chaves: malária, resistência, enzimas de des-ubiquitilação, alvos terapêuticos.

ABSTRACT

Malaria continues to be a major public health concern. Drug resistance continues to threaten all efforts made to control the disease. Hence there is a race to identify new antimalarial drugs that act on newer targets, in order to minimize the spread of drug resistance. The ubiquitin/proteasome pathway has been idientified as a potential drug target. Mutations in de-ubiquitylating enzymes (DUBs), which catalyze the removal of ubiquitin, have been associated with the development of infectious and non infectious diseases. In this project four DUBs namely *pfuch-l1*, *pfuch-l3*, *pfuch-l54* and *pfubp-8* were identified in the *Plasmodium falciparum* genome and were characterized.

The expression profile of genes encoding DUBs throughout the parasite´s life cycle with and without drug treatment was carried out by RT-PCR. Polyclonal antibodies raised in mice were used to detect protein abundance in different stages of the parasite´s life cycle. An attempt was made to produce a DUB knockout line and determine whether they are essential for the parasite. Recombinant proteins were expressed in *E. coli* cells and their de-ubiquitylating activity was tested using a specific substrate for DUBs. The activity of curcumin (a Dub inhibitor) was evaluted *in vitro* on the recombinant proteins and its antimalarial activity was tested in association with chloroquine and artemisinin in an *in vivo* rodent malaria model, *Plasmodium chabaudi*. A proteomics approach was also used to determine what proteins were deregulated in response to curcumin treatment.

The results show that *P. falciparum* genes *pfuch-l1*, *pfuch-l3*, *pfuch-l54* and *pfubp-8* are differentially expressed throughout the parasite´s life cycle and those proteins are more abundant at the trophozoite and schizont stages of the parasite. Treatment of parasites with artemisinin, chloroquine, and curcumin induced a transient increase in the expression of those genes, followed by a steady decrease in the gene expression pattern. No viable *pfuch-l1* and *pfuch-l3* gene knockout lines were obtained. Recombinant proteins were successfully expressed in *E. coli* cells with the exception of *Pfuch-l54*.*Pfuch-l1*, *Pfuch-l3*, *Pfubp-8* demonstrated de-ubiquitylating activity by cleaving the substrate Ub-AMC. *In vitro* IC50 of curcumin towards recombinant *Pfuch-l1* was 15µM, for recombinant *Pfuch-l3* was 25.4µM and for *Pfubp-8* was 10µM and for human USP2 was 5µM.

Curcumin displayed some toxicity to the HepG2 cell lines, but the *in vivo* antimalarial activity assays in the rodent model of malaria *Plasmodium chabaudi* showed that curcumin is non toxic to mice and the association of curcumin with chloroquine displayed synergism whereas the association of curcumin with artemisinin showed antagonism. The proteomics assay performed in *P. falciparum* cultures treated with curcumin revealed 10 deregulated proteins.The proteins identified were involved in sulfur metabolism, protein translation and degradation, cell cycle and cellular organization. In conclusion, the present study showed that *P. falciparum* DUBs are indeed potential drug targets. However further molecular, biochemical and phamacological studies will be required in order to turn the inhibitors more specific towards the parasite´s enzymes and minimise damage to the host´s proteins.

Key words: malaria, drug resistance, de-ubiquitylating enzymes (DUBs), drug targets.

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ACTs	Artemisinin combination therapy
ATG8	Autophagy related protein 8
ATP	Adenosine-5-triphosphate
AU	Absorbance units
Bti	*Bacillus thurigiensis var israeliensis*
Bs	*Bacillus sphaericus*
BSA	Bovine serum albumin
CFA	Complete freunds adjuvant
CP	20S Core protein
CSP	*Plasmodium falciparum* circunsporozoite protein
Cy2	Cyanine dye 2
Cy3	Cyanine dye 3
Cy5	Cyanine dye 5
DDT	Dichloro-diphenyl-trichloroethane
Dhfr	Dehydrofolate reductase enzyme
Dhps	Dehydrofolate pteoroate synthase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DUBs	De-ubiquitylating enzymes
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzymes
E3	Ubiquitin ligase
pfEBL-1	*Plasmodium falciparum* erythrocyte-binding ligand
EDTA	Ethylenediamine tetratacetic acid
ED50	Concentration of drug able to reduce parasitaemia to 50%
ER	Endoplasmic reticulum
GDP	Gross Domestic product
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HUB-1	Homologous to ubiquitin
IC50	Concentration needed to achive 50% inhibition
IEF	Isoelectric focusing
IFA	Incomplete freunds adjuvant
IPG	Immobalized pH gradrient
IPTG	Isopropyl β-D-1thiogalactopyranoside
IRS	Indoor residual spraying
IS	Internal standard
ITNs	Insecticide treated nets
kDa	kiloDaltons
KO	Knockout
LB	Luria broth medium
LD50	Lethal dose to achieve 50% inhibition
mRNA	messenger RNA
MS	Mass spectrometry
MSP-1	Merozoite surface protein

MW	Molecular weight
NEM	N-ethylmaleimide
NEDD8	Neural precursor cell expressed developementally down
NI-NTA	Nickel nitrilotriacetic acid
OUT	Ovarian tumour domain containing protease
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pfatp6	*Plasmodium falciparum* Ca^{2+} depending SERCA type ATPase protein
pfcrt	*Plasm odium falciparum* chloroquine resistance transporter
pfHRD-1	*Plasmodium falciparum* ubiquitin ligase
pfmdr-1	*Plasmodium falciparum* multidrug resistance 1
pftctp	*Plasmodium falciparum* translationally controlled tumor gene
pfUB	Ubiquitin gene
pfUBA-1	*Plasmodium falciparum* ubiquitin activating enzyme 1
pfUBC	*Plasmodium falciparum* uiquitin conjugating enzyme 2
pfuchl-1	*Plasmodium falciparum* ubiquitin carboxyl hydrolase
pfRpn6	*Plasmodium falciparum* proteosome lid subunit 6
PI	Isoelectric point
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RT-PCR	Real time PCR
SDS	Sodium dodecyl sulphate
SUMO	Small ubiquitin like modifier
Ub	Ubiquitin
Ub-AMC	Ubiquitin-7-amino-4-methylcoumarin
UbLp	Ubiquitin like proteins
UBPs	Ubiquitin proteases
UCHs	Ubiquitin C-terminal hydrolases
UN	United Nations
UPS	Ubiquitin/proteasome system
URM-1	Ubiquitin related modifier -1
USD	United states/American Dollar
WHA	World health assembly
WHO	World health organization

Three letter amino acid code

Alanine	ala
Asparagine	asp
Cysteine	cys
Glycine	gly
Histidine	his
Lysine	lys
Serine	ser
Threonine	thr

CHAPTER 1-INTRODUCTION

1.1 An introduction to malaria

1.1.1 A Historical overview on the discovery of malaria

Malaria is one of the oldest human parasitic diseases (Alonso & Tanner, 2013). Historical accounts refer to the existence of malaria back in Chinese documents (The canon of medicine) dating back to 2700 BC (Aikwa, 1971; Cowman, 2006; Cox, 2010). In 400 BC in the Greek era of Hippocrates there were also symptoms of malaria associated with swamps and poor sanitation (Aikwa, 1971). With the advancement of science, in 1886, the French military surgeon Alphonse Louis Laveran was responsible for the observation of *Plasmodium* parasites in the blood of soldiers with malaria symptoms working in Constantine, Algeria (Bruce-Chwat, 1982; Cox, 2010). In 1897, the scientist William H Welch named *Plasmodium falciparum* specie (Coatney,1971) and this name became widely used in the literature. Between 1882 and 1899, the English scientist Sir Ronald Ross allowed mosquitoes to feed on birds infected with *Plasmodium relictum* and through the dissection of mosquito stomach demonstrated that mosquitoes were responsible for malaria transmission (MacCallum, 1897). Since then it has become clear that malaria is transmitted to humans by the bite of female *Anopheles* mosquitoes (Coatney, 1971; Baird 2009; Eade *et al*, 2009).

The emergence of chloroquine in 1934 and its introduction in the clinic in 1947 (Coatney, 1963) in malaria prophylaxis and treatment sparked an interest in malaria eradication. In 1942, DDT (dichloro-diphenyl-trichlorethane) was discovered during World War II and WHO launched a global campaign for malaria elimination between 1940 and 1960 (Greenwood *et al*., 2008). Indoor Residual Spraying (IRS) was carried using DDT and its use as well as other public health measures, such as draining swamps and the use of mosquitoe nets interrupted transmission and raised the possibility of global malaria eradication (Greenwood *et al*., 2008; Mills *et al*., 2008). Europe, North America, the Caribbean region and some areas of South and Central America as well as parts of Asia, benefited from this initial attempt at malaria eradication. However, eradication in sub-Saharan Africa was far from being achieved due to political and financial constraints which did not allow control measures to be implemented successfully (Mills *et al*., 2008). Resistance to DDT as well as its impact on the environment and parasite resistance to

chloroquine (CQ) began to emerge in the 1950´s in South East Asia and contributed to the lack of success at the first attempt of global eradication (Overgaard and Angstreich, 2007).

1.1.2 An overview on the current malaria dissemination

Today malaria affects mainly populations in Sub-Saharan Africa, South East Asia and certain regions of South America and the Middle East (figure 1). According to the WHO in 2010 there were 219 million cases of malaria and 66000 deaths (WHO report, 2013), 91% of deaths are estimated to have occurred in the African region. The most affected groups are pregnant women, vulnerable HIV/AIDS patients and young children under five years of age who have not developed protective immunity against the most severe form of the disease. Malaria has a huge economic impact on endemic countries (Sachs and Malaney, 2002) and the disease accounts for approximately 22% of childhood deaths and it can decrease gross domestic product (GDP) by 1.3% (Sachs and Malaney, 2002).

The health costs of malaria in high transmission areas accounts for 40% of public expenditure and 50% of hospitals admissions, contributing to the poverty often seen in malaria endemic countries (Chima *et al*., 2003; Onwujekwe *et al*., 2010). The 6th United Nations (UN) development goal established in 2000 declares a combat against malaria, tuberculosis and HIV/AIDS. With the establishment of those goals, new mechanisms were introduced to finance the fight against those diseases. The latest WHO report (2013) indicates that funding for malaria control was 100 million USD back in 2000 and has risen since to 1.71 billion USD in 2010 and increased to 1.94 billion USD in 2012 and 1.97 billion USD in 2013 which has contributed to a 51% reduction in the malaria mortality rate in young children under five years of age (WHO, 2013).

Strategies such as the introduction of artemisinin based combination therapy (ACT) and vector control measures such as insecticide treated nets (ITNs) and indoor residual spraying (IRS) has fired up a new optimism in the fight against malaria (Alonso, 2012). Of the 104 countries considered endemic (Figure 1), 79 are in the malaria control phase, 10 are in the pre-elimination phase, 10 in the elimination phase and 5 are in the prevention of re-introduction phase, all together contributing for a renewed hope of a possible eradication.

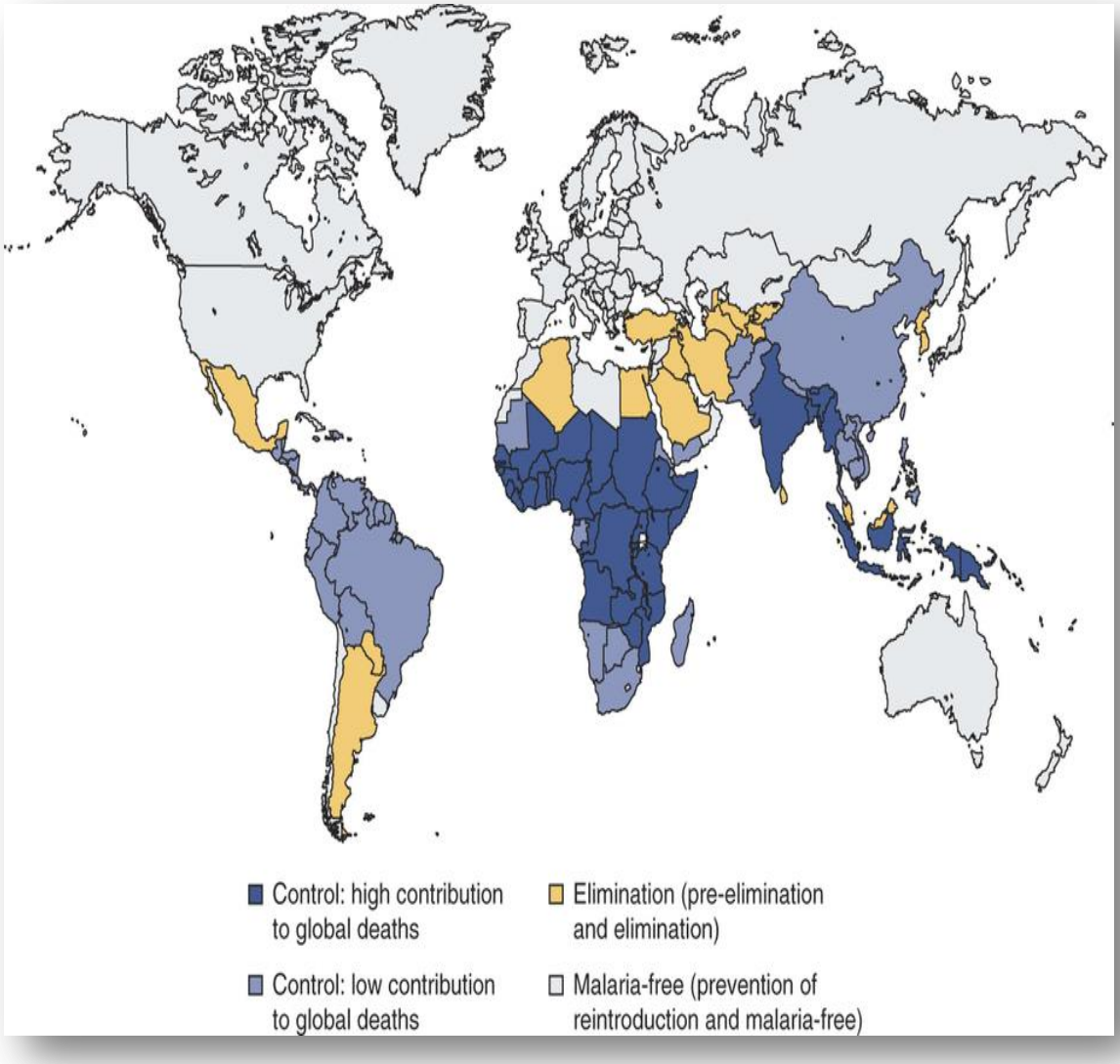


Figure 1. Malaria distribution around the world according to the WHO Report 2012. In dark navy blue: countries with high contribution to global death. In light navy blue: countries with low contribution to global deaths. In light brown: countries which have implemented a pre-elimination and elimination plan. In baby blue colour: countries that are certified as malaria free and/or are considering prevention of re-introduction. Adapted from (Alonso & Tanner, 2013)

1.1.3 The malaria parasite and its life cycle

Plasmodium is a eukaryotic organism that belongs to the phylum *Apicomplexa* (Antinori *et al*., 2012). There are five species of *Plasmodium* responsible for human malaria and these are: *falciparum, vivax, ovale, malaria* and *knowlesi*. *P. knowlesi* predominantly infects *Macaca fascicularis* by the bite of *Anopheles leucosphyrus* mosquitoes but it can also infect humans (Rosenberg *et al*., 1999; Antinori *et al*., 2012). In general the life cycle of human malaria parasites is divided into two phases: an exogenous sexual phase (sporogony) which occurs in the stomach of the female *Anopheles* mosquitoes (figure 2) and an endogenous asexual phase (schizogony) which occurs in the vertebrate host (figure 2).

During a blood meal the malaria infected female *Anopheles* mosquito inoculates sporozoites into the human host (Mackinnon *et al*., 2004). Sporozoites are injected subcutaneous under the skin and from there, the sporozoite travel and enter the parenchymal cells of the liver, (Rosenberg *et al.*, 1999; Amino *et al*., 2006). Once the sporozoites have entered the parenchymal cells of the liver, the circulating sporozoites are first taken up by the Kupffer cells and then they enter the hepatocytes through the thrombosponding domain of the circumsporozoite protein (CSP) on the sporozoite and the heparin sulphate proteoglycan receptor on the hepatocyte (Pradel *et al*., 2002).

Once inside the liver cells, the parasite undergo asexual division and becomes a tissue schizont that contains thousands of merozoites (exo-erythrocytic schizogony) in what is known as the liver stage of the infection (Collins *et al*., 2005). *Plasmodium falciparum* can mature within 7 days and each sporozoite produces 40.000 merozoites, in *P. vivax* and *P. ovale* dormant stages of the parasite, called hypnozoites, can persist in the liver causing relapses characterized by the appearance of parasitemia in the blood. *Plasmodium falciparum* and *P. malarie* do not develop hypnozoites and therefore lack the capacity to relapse (Cowman *et al*., 2006).

Once the hepatocytes rupture, they release merozoites that can invade the erythrocytes and initiate the intraerythrocytic phase of the infection also known as the blood stage (figure 2). Invasion of erythrocytes by the merozoites involves the interaction between protein ligands on the surface of the merozoite such as the merozoite surface protein (MSP-1) or the *Plasmodium falciparum* erythrocyte binding ligand-1 (EBL-1) to the red blood cell receptors such as the glycophorin family of receptors (Mayer *et al*., 2009). Once inside the erythrocyte, trophozoite maturation occurs over a period of 24-72 hours depending on the species (Collins *et al*., 2005). The surface area of the young trophozoite (figure 2) begins to enlarge giving rise to a mature trophozoite (Collins *et al*., 2005; Antinori *et al*., 2012). Further mitotic divisions allow the parasite to mature into a schizont. The mature schizont can contain between 12-16 merozoites, the blood cell ruptures releasing those merozoites which invade new red blood cells maintaining the asexual life cycle. This phase of the infection is associated with malaria symptoms which is characterized by rapid rise of temperature, skin vasodilation and headaches (Collins *et al*, 2005; Cowman *et al*., 2006).

Within the erythrocyte, some parasites undergoe gametocytogenesis producing male (microgametocyte) or female (macrogametocytes) gametocytes (figure 2). If the female mosquitoes bite a malaria infected individual, gametocytes will be ingested with the blood meal and the recombination can occur in the mosquito stomach (sporogonic cycle) (Collins *et al*., 2005). Once ingested by a mosquito, malaria parasites can develop during a period that may range between 10 to 21 days depending on the parasite species and environmental conditions such as temperature. In the mosquito stomach, gametocytogenesis will lead to the production of a zygote (figure 2), which will further develop into elongated ookinetes which invade the midgut wall of the mosquito, developing into large oocystis. The oocysts will develop and after their maturation the rupture occurs and the sporozoites are released (figure 2). The sporozoites make their way into the mosquitoes salivary glands (Collins *et al*., 2005) and they are ready to be inoculated into a new host through the bite of the infected female mosquito, perpetuating the malaria transmission cycle (figure 2).

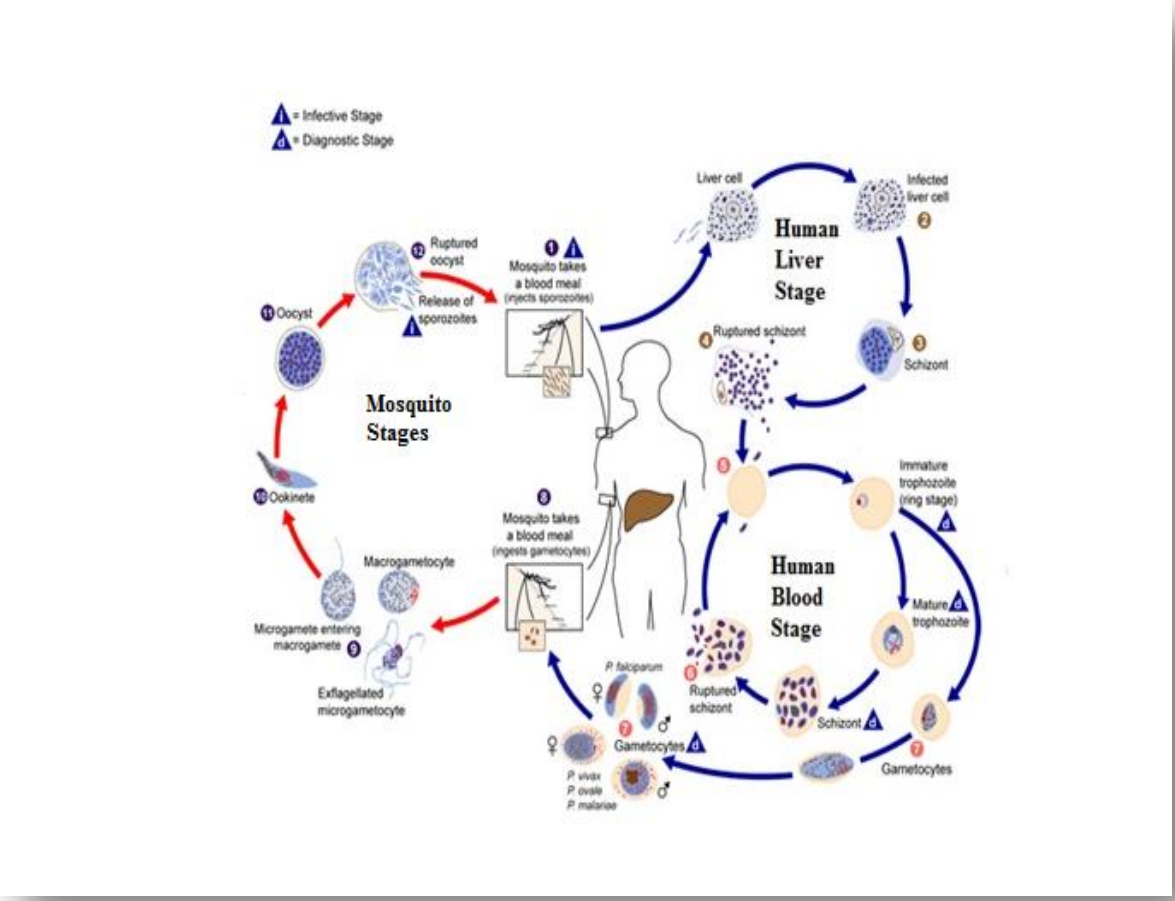


Figure 2. The life cycle of the malaria parasite. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host **(1)**. Sporozoites infect liver cells **(2)** and mature into schizonts **(3)**, which rupture and release merozoites (human liver stage). After this initial replication in the liver, the parasites undergo asexual multiplication in the erythrocytes (human blood stage). Merozoites infect red blood cells **(5)**. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites **(6)**. Some parasites differentiate into sexual erythrocytic stages (gametocytes) **(7)**. The gametocytes, male and female, are ingested by an *Anopheles* mosquito during a blood meal **(8)**. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes **(9)**. The zygote becomes an ookinetes **(10)** which invade the midgut wall of the mosquito where they develop into oocysts **(11)**. The oocysts rupture, and release sporozoites **(12)**, which make their way to the mosquito's salivary glands ready to inoculate the next human host. Image adapted and modified from Centre for disease control (CDC)**.** http://dpd.cdc.gov/dpdx/html/Malaria.htm.

1.2 Malaria control measures

1.2.1. Vector control measures

1.2.1.1. Chemical vector control measures

Vector control can be used in many levels in order to disrupt the parasite´s life cycle and block transmission. In many countries where malaria is endemic IRS has now become an important tool in the combat against malaria. The aim of IRS is to kill female mosquitoes with endophilic behavior, resting inside the house, thereby blocking transmission of disease (WHO report, 2013). Usually IRS is applied three times per year in areas of moderate and high transmission and a wide range of insecticides is available such as DDT, malathion, fenitrophion, alpha-cypermethrin just to name a few. However resistance to insecticides has already been reported in 27 countries (Abilio *et al*., 2011) and threatens the goals that have been achieved thus far. Another anti-vectorial measure is the use of ITNs impregnated with insecticides (WHO report,2013) and according to the WHO report, between 2004-2010, the number of ITNs rose from 5.6 million to 145 million in Sub-Saharan Africa contributing to the drop in the number of malaria cases (WHO report, 2013).

1.2.1.2. Non chemical vector control measures

Non chemical control measures are also seen as an alternative approach to insecticide use. Microorganisms such as *Bacillus thurigiensis var israeliensis* (Bti) and *Bacillus sphaericus* (Bs) biolarvicide can be placed in mosquitoe breeding sites (Lingenfelser *et al*., 2010), they have shown activity against *Anopheles* larvae without affecting the vertebrate host. The use of these microrganisms may help reduce the constant exposure of mosquitoes to insecticides which tends to exacerbate insecticide resistance (Mwuangangi *et al*, 2011). Predator fish such as *Tilapia guineeensis* have also shown efficacy in the removal of late stage *Anopheles* larvae in the Gambia Republic (Louca *et al*., 2009) reducing once again the excessive use of insecticides.

1.2.2. Malaria Vaccines

Several potential malaria vaccine candidates are currently being studied. The *Plasmodium falciparum* circumsporozoite protein (CSP) is one of the candidates (Plassmeyer *et al*.,

2009). This protein is involved in the adhesion of the sporozoite to the hepatocyte and during the liver stage of the disease. Anti-CS antibodies have been shown to inhibit parasite invasion and are also associated with a reduced risk of clinical malaria (Plassmeyer *et al*., 2009). The vaccine induced anti-CS responses and the fact that CS is the predominant surface antigen of sporozoites, makes CS a very good antigen for use in pre-erythrocytic vaccines (Audran *et al*., 2005; Schwartz *et al*., 2012). At the present time, the most promising vaccine is the vaccine candidate RTS,S /AS01E (which contains part of the CSP protein of *P. falciparum* fused to hepatitis B antigens and the adjuvant ASO2A). RTS,S/AS01E is the most advanced malaria vaccine candidate produced thus far (Enosse *et al*., 2006) and has shown 50% efficacy against clinical malarial cases in children ages between 5-17 months after administration of three doses, in a study carried out in Mozambique (Enosse *et al*., 2006; Alonso, 2012). Trials are now being extended to other African countries to assess the efficacy of the vaccine.

1.2.3. Antimalarial drugs

1.2.3.1. Chloroquine

Chloroquine is a blood schizonticide and acts on the parasite food vacuole (Fidock *et al*., 2000; Daily, 2006; Aguiar *et al*., 2012). As the parasite digests hemoglobin, large amount of heme is formed as a byproduct, which is toxic for the parasite. The parasite detoxifies this product in its food vacuole by forming an inert crystal called hemozoin (Fidock *et al*., 2000; Daily, 2006; Aguiar *et al*., 2012). CQ acts by inhibiting the detoxification process, resulting in accumulation of millimolar levels of heme in the digestive vacuole of the parasite, causing their death. The heme-chloroquine complex may permeabilize membranes, interfere with free radical detoxification and block protein synthesis (Fidock *et al*., 2000). Mutation in the gene *Plasmodium falciparum* multidrug resistance 1 (*pfmdr-1*), asparagine (asn) to tyrosine (tyr) at position 86 was shown to contribute to chloroquine drug resistance (Reed, 2000). It has also been shown that *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt)* gene located on chromosome 7 (Plowe, 2003) is associated with CQ resistance phenotype. This gene harbours a mutation lysine (lys) to threonine (thr) at position 76 which confers to the parasite the capacity to support higher levels of CQ (Plowe, 2003). It is believed that resistance of *P. falciparum* to CQ is related to an increased capacity of the parasite to expel the drug at a rate that does not allow CQ to

reach levels required for inhibition of hemozoin formation (Fidock *et al*., 2000; Plowe, 2003; Farooq *et al*., 2004). Transfection assays (Fidock *et al*., 2000) demonstrated that the mutation in the (*pfcrt)* gene referred before was sufficient to induce a chloroquine resistance phenotype. Chloroquine resistance emerged first in South East Asia and it is believed that (CQ) resistance is the result of massive CQ pressure (Fidock *et al*., 2000; Farooq *et al*., 2004). CQ was widely used as the first line of antimalarial therapy for more than 50 years, because it was cheap to produce and had a good safety profile (Fidock *et al*., 2000). However due to the spread of chloroquine resistance worldwide (figure 3) CQ in many countries is no longer used in the clinic. Derivatives of quinine such as amodiaquine and mefloquine have also been synthesized for the treatment of *P. falciparum* infections. It is thought that the mode of action of amodiaquine and mefloquine is similar to chloroquine (Price *et al*., 2004). Resistance to mefloquine (figure 3) has been associated with an increase in the expression of the *pfmdr-1* gene or increase in gene copy number (Price *et al*., 2004; Daily *et al*., 2006).

1.2.3.2 Sulphadoxine/Pyrimethamine

Antifolate drugs, such as sulphadoxine/pyrimethamine act through sequential inhibition of two key enzymes which are dehydrofolate reductase *(Dhfr)* and Dehydropteroate synthase *(Dhps)* involved in folate synthesis (Gatton *et al*., 2004). The parasite uses the host´s purines but has to synthesize its own pyrimidines which are essential for parasite survival (Gatton *et al*., 2004). Sulfonamides such sulphadoxine work by inhibiting the conversion of dehydropteroate diphosphate into dehydropteroic acid (Kiara *et al*., 2009). Pyrimethamine works by inhibiting the conversion of dehydrofolic acid into tetrahydrofolic acid (Kiara *et al*., 2009). Drug resistance to sulphadoxine/pyrimethamine is now well disseminated around the world (figure 3). Studies on field isolates have shown that gene mutations are involved Sulphadoxine/Pyrimethamine resistance. Specific combinations of these mutations have been associated with varying degrees of resistance to this antifolate combination. The serine (ser) to asparagine (asn) substitution at position 108 in *pfdhfr* gene is the principal mutation associated with resistance to pyrimethamine whereas the mutation alanine (ala) to glycine (gly) at position 437 in the gene encoding the enzyme *pfdhps* is mainly associated with resistance to sulphadoxine (Mbugi *et al*., 2006).

1.2.3.3. Atovaquone/Proguanil

Atovaquone is a competitive inhibitor of the Quinol oxidation (Qo) site of the mitochondrial cytochrome b complex (Baggish and Hill, 2002). Atovaquone acts by inhibiting the parasite´s mitochondrial electron transport at the cytochrome b complex (Kroodsood *et al*., 2007). Although resistance to atovaquone develops very rapidly when used alone, when combined with proguanil, which is converted in the liver to cycloguanil (the active compound) which is an inhibitor of *Dhfr* enzyme, enhances the activity of atovaquone (Baggish and Hill, 2002). The combination is commercially known as "malarone". Atovaquone is expensive compared to chloroquine and mefloquine and therefore it is used mainly for prophylactic purposes for travelers visiting endemic areas. However, resistance to malarone has also emerged (Baggish and Hill, 2002) and it is conferred by single point mutations tyrosine (tyr) to serine (ser) at codon 268 in the cytochrome-b gene (Baggish and Hill, 2002).

1.2.3.4. Antibiotics

Doxycycline and other powerful antibiotics such as azithromycin, tetracycline and clindamycin are also being used in malaria chemotherapy (Tan *et al*., 2011). Doxycycline is a derived from oxytetracycline and it is thought to act by binding to ribosome subunits and inhibiting protein synthesis (Tan *et al*., 2011). Clindamycin can also be used in the treatment of malaria as a blood schizonticide and it is thought that it interferes with the apicoplast which is a chloroplast like organelle of algae origin, thought to be acquired by endosymbiosis (Lell and Kresmner, 2002). New formulations of ACTs combined with antibiotics are now emerging in order to boost the pipeline of new ACT combinations (Batwala *et al* 2011).

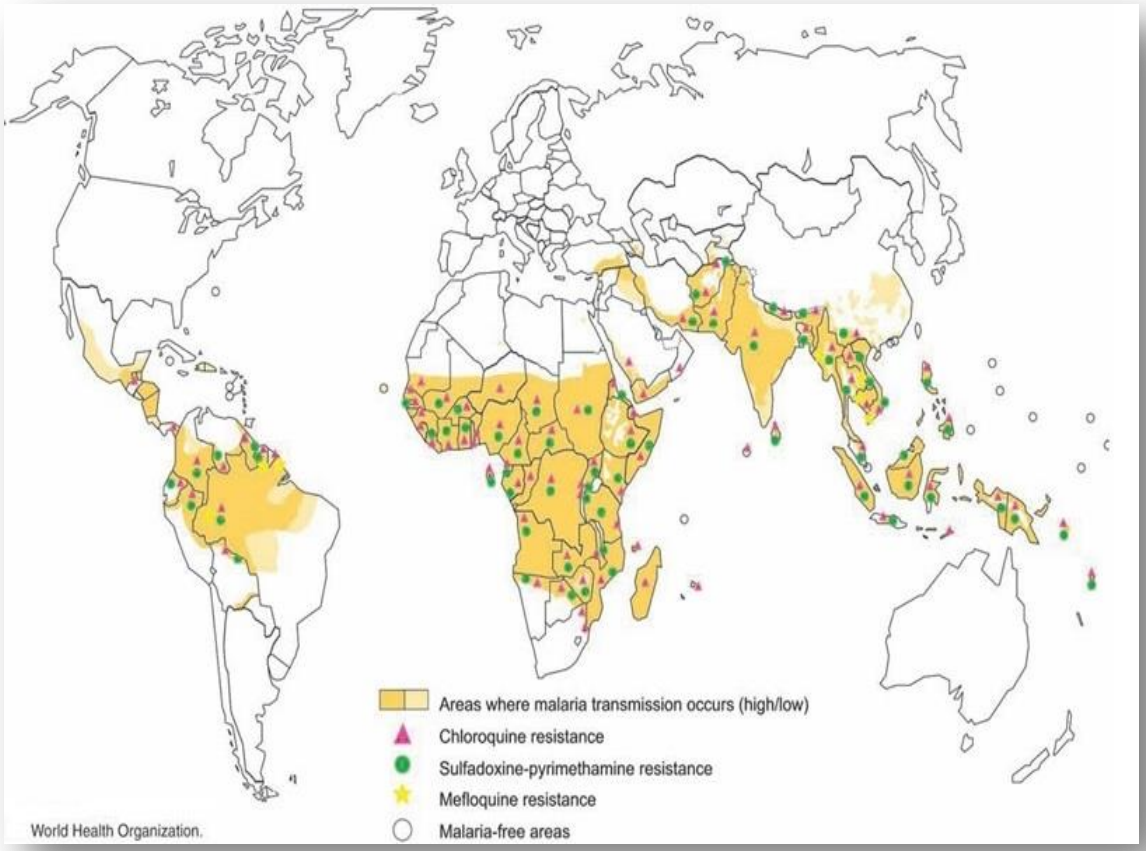


Figure 3. The spread of antimalarial drug resistance around the world. Antimalarial drug resistance remains a major obstacle in malaria control and eradication. The map illustrates the areas where chloroquine drug resistance, sulphadoxine/pyrimethamine drug resistance and mefloquine drug resistance has already occurred. Adapted from the WHO world malaria report 2005.

1.2.3.5. Artemisinin and its derivatives

Artemisinin is an active compound derived from the Chinese plant *Artemisia annua* (Klayman *et al*., 1985). At present artemisinin and its derivatives are the best antimalarial drugs available for the treatment of malaria. Artemisinin is a sequisterpene containing a peroxide bridge (Meshnick *et al*., 1996) this structure is unique to this compound and it is vital for its antimalarial activity (Woodrow *et al*., 2005; O´Neill *et al*., 2010). Evidence suggests that artemisinin´s mode of action is the result of interaction of artemisinin´s endoperoxide bridge with heme group present in the parasite´s digestive vacuole forming highly reactive species which leads to destruction of the parasite´s membranes and lysis of infected erythrocytes (Ellis *et al*., 1985; Meshnick *et al*., 1996).

The only disadvantage shown by artemisinin is that it has a short half life (Krishna *et al*., 2004). Now, several derivatives of artemisinin with better pharmacokinetic profile have been developed, namely artesunate, artemether, arteether, dihydroartemisinin. Those derivatives have between two to eight hours, meaning that not all parasites will be eliminated within that time frame thus resulting in a high risk of recrudescence (Krishna *et al*., 2004). In order to avoid that, new policies regarding artemisinin in the treatment of malaria were endorsed by the World Health Assembly (WHA) in 2007, where the use of artemisinin alone was discouraged and current treatment of malaria is based on the association of artemisinin or its derivatives with other traditional antimalarial drugs such mefloquine, amodiaquine and others forming what is now known as artemisinin combination therapy (ACT).

According to the WHO report 2013, resistance to ACTs has been confirmed in Cambodia although it is not clear what are the mechanisms behind ACTs drug resistance (Wang *et al*., 2011). It is thought that ACT resistance may be attributed to the fact that some of the partner drugs used in the ACT combination such as mefloquine is no longer eficaccious (figure 3). It is not clear which genes are potentially involved in artemisinin drug resistance. However, potential candidate genes were identified and sequenced in field isolates. Those were: *P. falciparum* Ca^{2+} depending SERCA type ATPase (*pfATP6*) (Valderramso *et al*., 2010), *pfcrt* gene (Fidock *et al*., 2000) *pfmdr1* gene (Price *et al*., 1999), translationally controlled tumor gene *(pftctp*) (Eckstein-Ludwig *et al*., 2003) and the *P. falciparum* ubiquitin carboxyl hydrolase-1 gene (*pfuch-l1*) (Hunt *et al*., 2007) have all

been sequenced for mutations related to artemisinin resistance. However, so far no mutations were identified in those candidate genes, which can be linked to drug resistance to ACTs (Wang *et al*., 2011; Zakeri *et al*., 2012). The reality is that resistance to artemisinin and its derivatives has emerged and the failure to develop new antimalarials that preferentially act on new targets would only contribute to the spread of drug resistance and would jeopardize all the efforts that have been made thus far to control the disease.

1.3 *Plasmodium* genome and its potential drug targets

1.3.1 Identification of potential drug targets

Sequencing of the *Plasmodium* genome was a big land mark in the history of malaria (Gardner *et al*., 2002) and revealed 5.403 nuclear genes identified, but only 1.800 genes encode proteins with known function (Gardner *et al*., 2002) which means that the *Plasmodium* parasite may have interesting potential drug targets which must be identified and validated especially now that resistance to ACTs has become evident (Imwong *et al*., 2010). Most of the proteins with known or partially known function are involved in post-translational modifications (Chung *et al*., 2009). *Plasmodium* drug targets, have been identified by looking at proteins in the human host which are drug targets in cancer therapy or looking at proteases which are drug targets in other microorganisms (bacteria, virus) and then identifying whether those targets are also present in the *Plasmodium* genome (Fidock *et al*., 2004).

Other approaches include, the identification of inhibitors against human phosphorylation/dephosphorylation, ubiquitin/proteasome (UPS), methylation, acetylation pathways (Brumlik *et al*., 2011; Fidock *et al*., 2004; Prudhomme *et al*., 2008; Sumanadasa *et al*., 2012) which have been developed for the treatment of human diseases but have shown antimalarial activity. Differences between the *Plasmodium* and the human enzymes involved in those processes are now being exploited speeding up the process of antimalarial drug discovery. In order for any enzyme to be considered as a good drug target, It must be essential for the parasite, the active site must be easily accessed by inhibitors and there must be differences between the parasite´s enzymes and its human counterpart (Fidock *et al*., 2004). In this project the main focus will be on the UPS as a potential drug target in *Plasmodium* spp. Research carried out on the UPS has shown that

this system is responsible for most of the protein regulation inside eukaryotic cells (Aminake *et al*., 2012).Therefore deficiencies in the UPS can lead to the development or progression of metabolic, neurodegenerative and oncogenic diseases (Aminake *et al*., 2012), which has prompted the development of specific inhibitors against those enzymes involved in the UPS.Since the UPS or its components are present in many parasitic protozoa responsible for human diseases such as *Plasmodium spp, Trypanosomes spp, Leishmania spp, Giardia spp, Cryptosporidum spp* and *Theileria spp* (Ponder and Bogyo, 2007) there is a huge interest in the characterization and validation of the UPS as a potential drug target in parasitic protozoa. Development of drugs against components of the UPS in one parasite may be efficient in the killing of another parasite making it easier to treat diseases caused by protozoa and reducing the costs of drug development. In the next section the components/enzymes involved in the UPS in *Plasmodim falciparum* shall be discussed in detail.

1.3.2. Discovery of the ubiquitin molecule in *Plasmodium* genome

The ubiquitin molecule (Ub) was discovered in 1970 and its name reflects the fact that is a ubiquitous molecule meaning that it is found in various organelles inside the cell (Goldstein *et al*., 1975) and it is a key component in the UPS. Addition of this molecule to target proteins is called ubiquitylation (Horrocks and Newbold, 2000). The Ub molecule has special features such as the C terminal glycine (gly) 76 residue and also has on its sequence seven lys residues (lys6, lys11, lys29, lys33, lys48 and lys63) which play a crucial role in the activity of this molecule (figure 4).

Once proteins are tagged with an Ub molecule they will either be involved in other cellular activities such as cell cycle regulation, signal transduction, apoptosis, DNA repair whereas short lived proteins, damaged or abnormal proteins with an ubiquitin tag will be degraded by the proteasome (Ponder and Bogyo, 2007; Ponts *et al*., 2008). Inside the cell, ubiquitylation/de-ubiquitylation of proteins often occurs in the cytoplasm where it regulates protein degradation, endocytosis and cell signaling (Horrocks and Newbold, 2000; Le Roch *et al*., 2003). However it can also occur in the nucleus where it is involved in chromatin remodeling, DNA repair and regulation of proteins involved in transcription (Dantuma *et al*., 2006) making the UPS responsible for most of the intracellular protein regulation (Ponder and Bogyo, 2007; Ponts *et al*., 2008).

The *Plasmodium* genome encodes an ubiquitin gene, whose official symbol is *pfUB* (Horrocks and Newbold, 2000; Ponts *et al*., 2008) found on chromosome 12. Gene expression studies in *P.falciparum* confirmed that *pfUB* is expressed in all life cycle stages of the parasite especially the late trophozoite stage (Horrocks and Newbold, 2000; Le Roch *et al*., 2003) which means that ubiquitylation is an ongoing process which appears to be vital for the parasite (Ponts *et al*., 2008). *PfUB* was cloned and the protein was expressed and showed to be an 8.5 Kda protein on SDS-PAGE and its activity confirmed by ubiquitylation assays (Horrocks and Newbold, 2000). The human Ub sequence shares 98% similarity with *Plasmodium pfUB* and yeast Ub sequence meaning that Ub is very much conserved amongst eukaryotic organisms.

The *Plasmodium* genome also has ubiquitin like proteins/ubiquitin like modifiers (UbLps) these are: SUMO, NEDD8, HUB-1, URM1 (Ponts *et al*., 2011) which appear to be expressed in all stages of the *Plasmodium* life cycle. These molecules share similar tertiary structure with Ub and attachment of UbLps occurs in a similar mechanism as ubiquitin as explained below. *PfSUMO* an ubiquitin like molecule has also been characterized (table 1) and it was 40% identical to human SUMO-1 and found mainly in the nucleus and the cytoplasm of the parasite *Plasmodium falciparum* (Issar *et al*., 2008). Proteins tagged with (UbLPs) usually function in regulatory activities rather than being tagged for degradation (Frickel *et al*., 2007; Aminake *et al*., 2012).

Inhibitors of both Ub and UbLPs (SUMO) have been developed (table 1) and are now being tested for the treatment of neurological, microbial diseases and cancer (Edelmann *et al*., 2011). So far those inhibitors have not been tested for their antimalarial activity. However, since the ub molecule shares 98% similiarity with human and yeast ub molecule, inhibiting the ub molecule is no longer seen as a rational approach to interfere with the UPS system. Instead inhibition of specific enzymes involved in the UPS is now being considered as a more rational approach.

Table 1. Characterization of ubiquitin (Ub) and ubiquitin like proteins (UbLps) in *Plasmodium falciparum.*

Component of the UPS	Putative biological role	Biological characterization	Inhibitors available	References
Ubiquitin	Post translational modification	*pfUB was* characterized involved in ubiquitylation of proteins	Synthetic compound Ubiquitin aldehyde and ubiquitin vinyl sulfone	Hershko and Rose 1987; Horrocks and Newbold, 2000
SUMO	Post translational modification	*pfSUMO* was characterized as an ubiquitin like molecule	Anacardic acid isolated from cashew nut plant *Anacardium occidentale* Ginkgolic acid isolated from *Ginkgo biloba* leaves	Issar *et al*., 2008 Fukuda *et al*.,2009
Nedd8	Post translational modification	Not available		
URM-1 and HUB-1	Post translational modification	Not available		

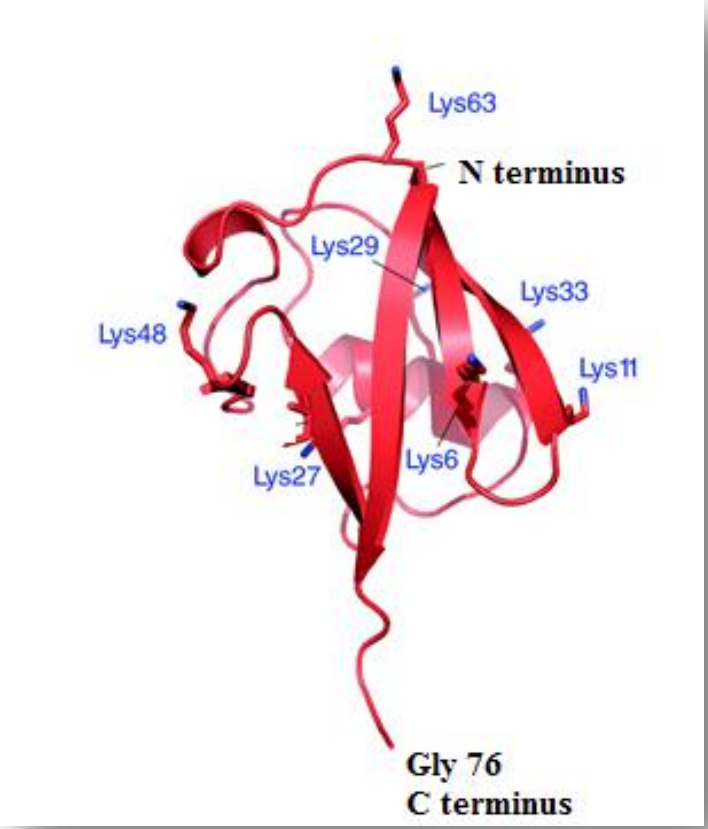


Figure 4 A general structure of the ubiquitin molecule and its main features. The main features of the molecule are the C terminal gly 76 residue, the 7 lysine residues and its N terminal. Addition of the C terminal gly 76 residue to the lysine residue of the substrate protein occurs through a process known as ubiquitylation. Once ubiquitin has been conjugated to the substrate protein, more Ub molecules can be linked to one another Ub-Ub forming polyubiquitin chains. The Ub molecule has on its sequence seven lys residues (lys6, lys11, lys27, lys29, lys33, lys48 and lys63) which can participate in the formation of polyubiquitin chains which will then determine the fate of the target protein. Adapted and modified from (Traub and Lukcas, 2007).

1.3.4 Ubiquitin Ligases

Attachment of ubiquitin molecules (figure 4) to target proteins is catalyzed by the action of ubiquitin activating enzymes also known as ubiquitin carrier protein (E1), Ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3) altogether known as ubiquitin ligases (Ponder and Bogyo, 2007; Ponts *et al*., 2008). The ubiquitylation cascade initiates when (E1) adenylates the ubiquitin molecule in an ATP dependent reaction (figure 5). Then ubiquitin is transferred to the active site cysteine (cys) residue on the E1 enzyme forming an E1-Ub thiolester complex, thereby activating the Ub molecule. The next step requires the action of another enzyme known as Ubiquitin conjugating enzymes (E2) (Ciechanover *et al*., 2000; Glickman and Ciechanover, 2002).

E2 is responsible for the transfer of the activated Ub molecule from E1 via a high energy thiolester intermediate forming an E2-Ub complex at the active site cys residue of the enzyme. The E2-ubiquitin complex is then transferred to the active site cysteine residue on the E3 enzyme where the substrate protein binds directly to the E3 ligase (figure 5) via NH_2 terminal residue (Glickman and Ciechanover, 2002; Ponts *et al*., 2008). Hence the E3 ligases are responsible for the last step in the reaction cascade and the E3-ubiquitin complex is transferred to the lysine (lys) residue on the target protein via an isopeptide bond which is formed between the glycine (gly) 76 amino acid (figure 4) on the Ub molecule and the lys residue on the target protein (Ciechanover *et al*., 2000; Glickman and Ciechanover, 2002).

Once Ub has been conjugated to the substrate protein, more Ub molecules can be linked to one another forming linear or branched polyubiquitin chains (Glickman and Ciechanover, 2002). The Ub molecule has on its sequence seven lys residues (figure 4) which can participate in the formation of polyubiquitin chains. Monoubiquitylation (figure 5) is involved in regulation of the cell cycle, endocytosis, mitochondrial inheritance, ribosome function, and post replicative DNA repair, transcriptional regulation, regulation of the immune system and many other biological processes (Mullaly and FitzPatrick, 2002; Amerik and Hochstrasse, 2004; Nijman *et al*., 2005). Whereas polyubiquitylation formed via lys6, lys27, lys29 or lys48 on the Ub molecule targets the protein for degradation by the proteosome (Glickman and Ciechanover , 2000).

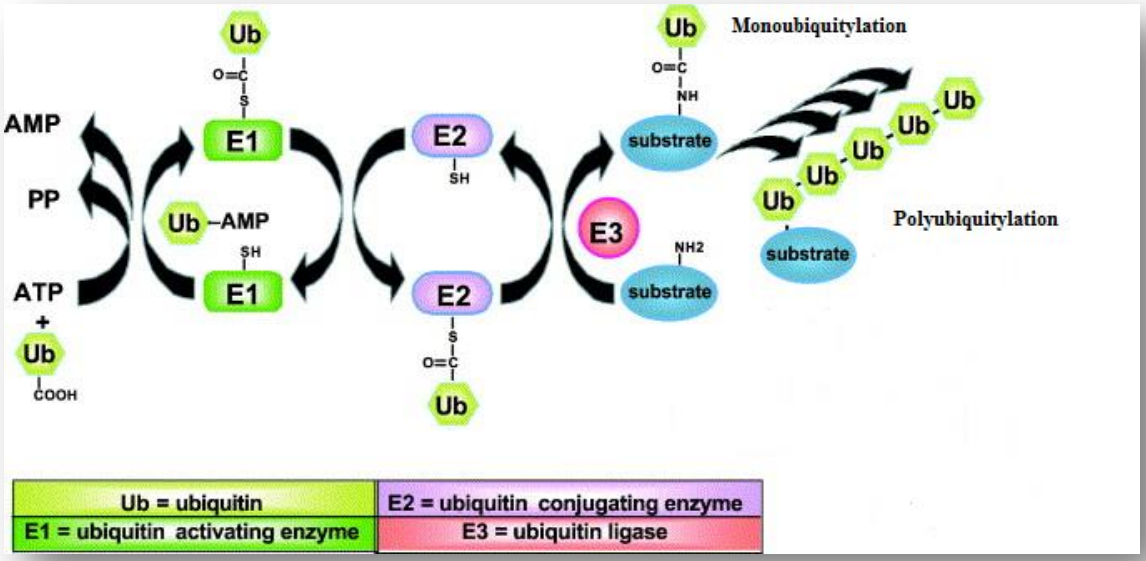


Figure 5. A simplistic overview of the activation of the ubiquitin molecule. Ubiquitin molecule is first activated by activating enzyme, (E1). Activation of ubiquitin initiates with the adenylation of ubiquitin a process which involves ATP. Once ubiquitin is activated a complex is formed known as Ub-E1 complex. This complex is then transferred to E2, forming an Ub-E2 complex. The Ub-E2 complex is then transferred to the E3 ligase. E3 ligases catalyzes the final step in the cascade by transferring the E3-Ub complex to a lysine residue on the substrate protein. Polyubiquitylation, depending on which lys residue is used to form the chain, targets the protein for degradation by the proteosome. Whereas monoubiquitylation targets the protein for other biological processes such as: regulation of the cell cycle, endocytosis, mitochondrial inheritance, ribosome function, transcriptional regulation. Image adapted and modified from (Pray *et al.,* 2002).

1.3.5 Ubiquitin Ligases in the *Plasmodium* genome

In *Plasmodium falciparum* genome, at least 8 putative E1 genes encoding enzymes have been identified. At least 14 putative genes encoding E2 enzymes (Ponts *et al*., 2008) have also been identified and were detected at the trophozoite and schizont stages of the parasite. Finally 54 putative E3 ligases have been identified and were detected at the ring, trophozoite and schizont stages of the parasite (Ponts *et al*., 2011). Making a total of 76 putative genes encoding ubiquitin ligases. The E3 family of enzymes is very diverse, the large number of E3 is due to the fact that there are different domain families of E3 ligases (Ring finger domain E3s, HECT domain E3s and U-box domain E3 ligases).

The Ring finger family represents the largest group of enzymes in eukaryotes (Ponts *et al*., 2008) and they contain a cystein/histadine/zinc domain involved in protein-protein interaction. A total of 68 putative ub ligases have also been identified in *Plasmodium vivax*, 62 putative ubiquitin ligases have been identified in *Plasmodium yoelli* but so far no biochemical characterization has been done (Ponts *et al*., 2008). Of the predictive 76 putative Ub ligases identified in the *Plasmodium falciparum* genome only three ubiquitin ligases have so far been characterized (table 2): The E1 *Plasmodium falciparum pfUBA1*, the E2 *Plasmodium falciparum pfUBC*, the E3 enzyme *Plasmodium falciparum pfHRD1*, which are involved in ubiquitylation of proteins in the endoplasmic reticulum (ER) (Chung *et al*., 2012) and its ubiquitylating enzyme activity was confirmed by ubiquitylation assays. Several inhibitors that were discovered in the field of cancer research are now being tested for their antimalarial activity (table 2) (Chung *et al*., 2012).

In table 2, a summary of those inhibitors is shown and those with an asterisks have already shown antimalarial activity either against the parasite itself or by inhibiting the activity of Ub ligases (table 2) (Chung *et al*., 2012).

Table 2. Characterization of ubiquitin ligases in *Plasmodium falciparum.*

Component of the UPS	**Putative biological role**	**Biological characterization**	**Inhibitors available**	**References**
Ubiquitin activating enzymes (E1)	Activation of ubiquitin	*pfUBA-1* characterized as an E1 and found in the ER	Synthetic compounds, Benzothiazole derivatives and PYR-41	Guedat and Colland, 2007; Yang *et al*., 2007
Ubiquitin conjugating enzymes (E2)	Conjugation of ubiquitin	*pfUBC* characterized as an E2 found in the ER	Leucettamol A Isolated from marine sponge *Leucetta aff.microraphis*	Edelmann *et al*., 2011
Ubiquitin ligase (E3)	Ligation of ubiquitin	*pfHRD-1* characterized as an E3 found in the ER	Synthetic compound Eeyarestatin*	Fiebiger *et al*., 2004; Chung *et al*., 2012

1.3.6 The Proteosome

The proteosome is also known as the 26S proteasome, it is a complex with many subunits (figure 6) involved in the regulated degradation of ubiquitylated proteins (Ciechanover *et al*., 2000). The proteosome has a barrel like 20S core protein (CP) where proteolysis occurs and a multiprotein regulatory particle (RP) also known as the 19S (figure 6) that is responsible for the recognition and preparation of substrates for degradation by CP (Hochstrasser, 1996; Ciechanover *et al*., 2000). Once the protein substrate has been recognized and anchored to the proteosome proteins are unraveled, unfolded and translocated by the 19S (RP) into the proteolytic core particle 20S where hydrolysis of proteins into short peptides will take place in an ATP-dependent manner (Glickman and Ciechanover, 2002). The byproduct of proteolysis is the polyubiquitin chain which will be processed by proteases present inside the proteasome (Eytan *et al*., 1993) and will recycle ubiquitin molecules thus restoring the pool of free ubiquitin which can then be attached to other proteins perpetuating the process (figure 6) (Nijman *et al*., 2005; Ashiwaza *et al*., 2012).

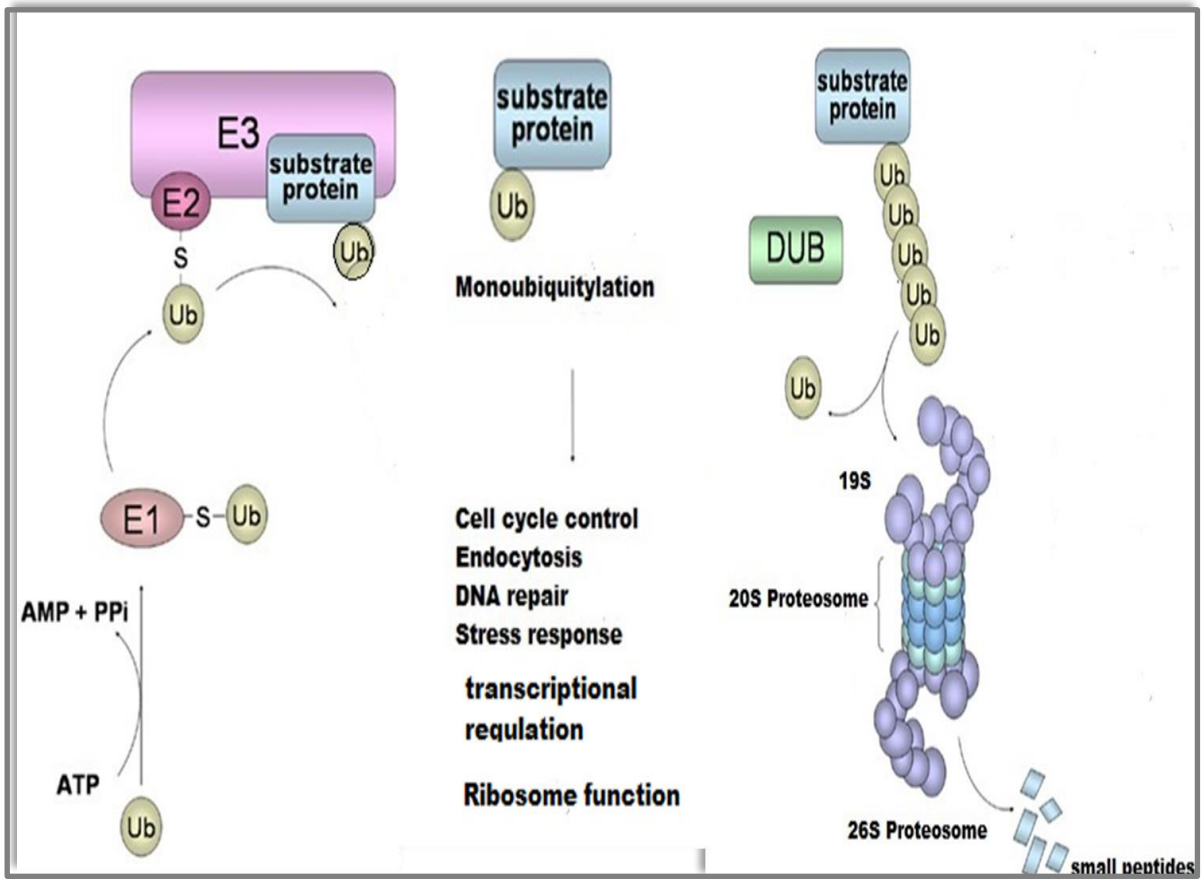


Figure 6.A general representation of the Ubiquitin/Proteosome system (UPS). Addition of Ub to proteins involves a cascade of reaction catalyzed by E1, E2 and E3. Proteins can either be monoubiquitylated or polyubiquitylated. Polyubiquitylated proteins are marked for degradation by the proteosme which is a complex subunit of enzymes which will degrade the protein into small peptides. Whereas monoubiquitylation can activate the target protein and allow the protein to participate in a various cellular processes such as: endocytosis, DNA repair, stress response, transcriptional regulation and ribosome function. Here only the 20S and the 26S proteasome subunits are shown for the sake of simplicity. Adapted and modified from (Ashiwaza *et al*., 2012).

1.3.7 The proteasome in the *Plasmodium* genome

In *P. falciparum* genome, 14 putative proteins, homologous to the yeast 20S CP subunit of the proteasome, were identified (Mordmuller *et al*., 2006). 20S CP subunits were shown to be present in the cytoplasm and nucleus of blood stage *Plasmodium* parasites, particularly in trophozoites and schizonts where there is a peak of ubiquitylated proteins (Aminake *et al*., 2012; Ponts *et al*., 2011) and the 26S CP subunit is expressed at the trophozoite and schizont stages of the parasite´s life cycle (Kreidenweiss *et al*., 2008).

Another component of the proteasome of *P. falciparum* is the protein RPn6 which is found in the lid of the protesome, this protein was characterized in *P. falciparum* and found in the cytosol of the parasite and biochemical assays indicated that this protein is an important part of the proteosome specially for the degradation of ubiquitylated proteins (Muralidharan *et al*., 2011). Thus, confirming the existence of an active UPS in the malaria parasite. Research carried out in cancer in the last decade has identified many proteasome inhibitors with antimalarial activity marked with an asterisk in the (table 3).

The proteasome inhibitor epoxomicin for example has antimalarial activity against chloroquine sensitive strains with an IC50 of 6.8 nM and it also has activity against field isolates from Gabon with an IC50 of 8.5 nM (Kredeinweiss *et al*., 2008). Even though many proteasome inhibitors with antimalarial activity have been developed, proteosome inhibitors are also known to be very toxic (Reynolds *et al*., 2007), hence it is thought that targeting individual enzymes involved in the UPS may be a more viable alternative chemotherapy for both infectious and non-infectious diseases.

Another proteasome inhibitor used in the treatment of multiple myeloma known as Bortezomib (table 3) is a well known proteosome inhibitor able to inhibit the intraerythrocytic developmental stages of *P. falciparum* (Reynolds *et al*., 2007). Salinosporomide A is a proteasome inhibitor, now in clinical trials phase I for the treatment of multiple myeloma and has antimalarial activity against *P. falciparum* and *P. yoelli* (table 3). Biochemical assays showed that salinosporomide A induced the accumulation of polyubiquitylated proteins which indicates that this compound affects UPS mediated processes (Prudhomme *et al*., 2008)(table 3).

Other inhibitors have also been developed as seen in (table 3) and those with an asterisk are the ones whose antimalarial activity has been confirmed. Those encouraging findings indicate that the UPS is an interesting drug target that needs to be further explored especially now, when resistance to ACTs has emerged.

Table 3. Characterization of the proteosome in *Plasmodium falciparum*.

Component of the proteosome	Putative biological Role	Biological characterization	Inhibitors Available	References
20S proteasome subunit and Rpn6 subunit	Protein degradation	Found to be expressed in the trophozoite and schizont stages and localized in the cytosol	Lactacystin* isolated from Streptomyces	Gantt *et al*., 1998
			Synthetic compound bortezomib*	Reynolds *et al* 2007
			Salinosporomide A* isolated from marine bacteria *Salinospora tropica*	Prudhomme *et al*., 2008
			Synthetic compounds	Kreidenweiss *et al.*,
			MG132*and Epoxomicin* isolated from Actinomycetes	2008;Cszesny *et al.*,2009

1.3.8 De-ubiquitylating enzymes (DUBs)

Protein ubiquitylation is a reversible process; the removal of ubiquitin molecules is carried out by de-ubiquitylating enzymes also found in the literature as de-ubiquitinases or de-ubiquitinating enzymes, but in the present study they shall be referred to as de-ubiquitylating enzymes (DUBs) (Mullaly and FitzPatrick 2002; Amerik and Hochstrasse, 2004; Nijman *et al*., 2005), which are responsible for the generation of free ubiquitin molecules (figure 6) and the disassembly of mono or polyubiquitin chains on substrate proteins.

DUBs are classified as proteases (Nijman *et al*., 2005) depending on their mechanism of catalysis they have been divided into: cysteine proteases and zinc dependent mettalloproteases which can be further subdivided into distinct subfamilies (Nijman *et al*., 2005; Mukhopadhyay and Riezman, 2007) these are: The Ubiquitin C terminal Hydrolases (UCHs) (figure 7), the Ubiquitin proteases (UBPs) or USPs (Ubiquitin specific proteases) from now on referred to as UBPs. The Machado Joseph Disease protein domain proteases (MJDs). The Outbains (OTUs), JAMM (motif mettallo proteases) which are classified as zinc dependent proteases. In addition to this major group of DUBs, there is also three distinct families of de-ubiquitylating like enzymes (DUBLs) these are the SUMO specific proteases, the autophagins proteases and the WLM (weak suppressor mettalloproteases) family of zinc dependent proteases (Nijman *et al*., 2005; Ponder and Bogyo, 2007) which will not be discussed further as they are beyond the scope of this project and in this project the focus will be mainly on UCHs and UBPs.

In general Dubs have in their catalytic site three key amino acids (aa) cysteine (cys), histidine (his) (figure 7) and an aspartate residue (asp) known as the catalytic triad (Nijman *et al*., 2005) and their folding resembles the papain family of proteases (Reyes-Turcu *et al*., 2009). During the process of de-ubiquitylation, with the help of the (his) residue which contributes to a low PKa, the cys residue launches a nucleophilic attack on the isopeptide bond that is between the ubiquitin C terminal and the lysine (lys) residue on the target protein (Reyes-Turcu *et al*., 2009). The result of the reaction is the release of the target protein and formation of a covalent intermediate with the Ub moiety. The reaction of this intermediate with water results in the release of the free enzyme and ubiquitin molecules (Nijman *et al*., 2005; Ponder and Bogyo, 2007; Reyes-Turcu *et al*., 2009).

In general UCHs are 20-50 kDa proteins whereas UBPs can have up 100 kDa. UCHs cleave small protein substrates from Ub tagged proteins. UCHs are known to release Ub molecules from inappropriately labeled proteins whereas UBPs are known to process large polyubiquitin chains because they are able to hydrolyse the isopeptide bond linking Ub-Ub molecules and peptide bond between ubiquitin molecule and the target protein (Reyes-Turcu *et al*., 2009). Since DUBs are proteases their activity has to be very well regulated to avoid proteolytic activity towards other substrates, studies conducted in the past have shown that DUBs can themselves be covalently modified by phosphorylation which affects their activity, localization and half life (Wilkinson, 2009) thus switching off the activity of these enzymes. Upon binding of the Ub molecule those enzymes undergo a conformational change in the catalytic triad (figure 7), provoking an active site rearrangement and exposing the active site residues which must occur in order for hydrolysis to take place (Reyes-Turcu *et al*., 2009).

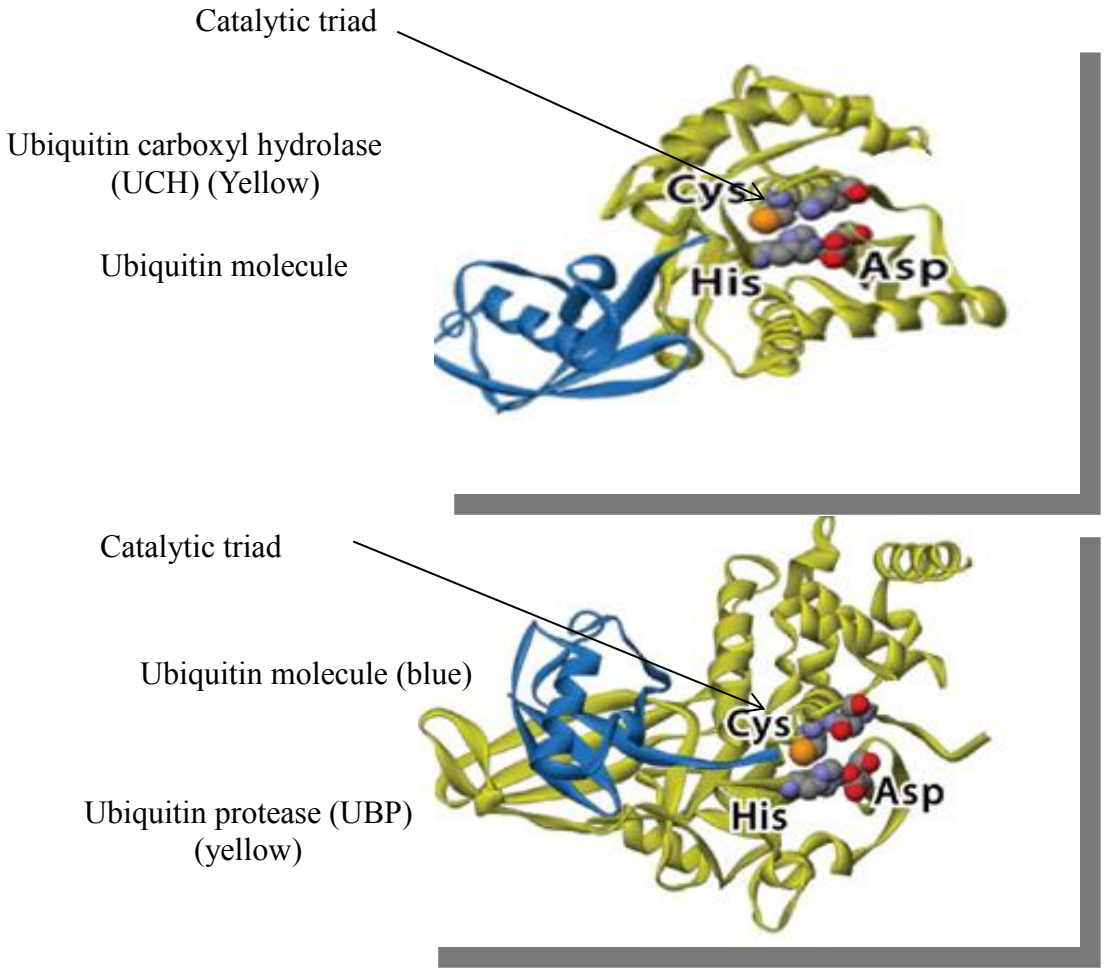


Figure 7. A general structure of the catalytic domain of ubiquitin carboxyl hydrolase (UCH) and ubiquitin protease (UBP). DUBs are highlighted in yellow and interacting with the ubiquitin molecule highlighted in blue. In general DUBs have in their catalytic site three key amino acids cysteine (cys), histidine (his) and an aspartate residue (asp) known as the catalytic triad. De-ubiquitylation occurs when the cysteine residue launches a nucleophilic attack on the isopeptide bond that is between the ubiquitin C terminal and the lysine (lys) residue on the target protein. The reaction results in the release of the target protein and free ubiquitin molecules. Adapted and modified from (Nijman *et al*., 2005).

1.3.9 De-ubiquitylating (DUBs) enzymes in the *Plasmodium* genome

An *in silico* study has shown that the *Plasmodium* genome encodes at least 40 putative DUBs whereas the human genome contains approximately 95 putative DUBs (Nijman *et al*., 2005; Ponder and Bogyo, 2007; Ponts *et al*., 2008; Wilkinson, 2009). In spite of the large number of DUBs in the *Plasmodium* genome, very few proteins have been characterized. The first *Plasmodium* DUB to be characterized was *Plasmodium falciparum ubiquitin carboxyl hydrolase* 54 (*Pfuch-l54*) (Artavanis-Tsakonas *et al*., 2006). The crystal structure of this enzyme was determined and it was found that *Pfuch-l54* protein has 54 KDa and has moderate sequence identity with human ubiquitin carboxyl hydrolase 1 (*huch-l1*) and (*huch-l3*) but the active site residues were conserved amongst those proteins (Artavanis-Tsakonas *et al*., 2006).

No biochemical assays, gene knockout assays and localization studies were carried out in order to clarify the role and the location of this protein in *Plasmodium´s* life cycle. The second DUB whose crystal structure was determined was *Plasmodium falciparum ubiquitin carboxyl hydrolase*-3 (*Pfuch-l3*) (table 4), this protein is 30% identical to *huch-l3* (Artavanis-Tsakonas *et al*., 2006). The recombinant protein appeared on the SDS-PAGE as a 30-32Kds band but in the literature is known as *Pfuch-l3* (Artavanis-Tsakonas *et al*., 2011) and site directed mutagenesis assays showed that *Pfuch-l3* gene product might be essential for parasite survival as substitution of a cys residue by an alanine (ala) in the active site resulted in the death of mutant parasites (Artavanis-Tsakonas *et al*., 2011). Furthermore, there is only moderate identity between the human and *Plasmodium Pfuch-l3* which means that these enzymes may be selectively targeted in malaria chemotherapy without damaging the host´s enzyme (Artavanis-Tsakonas *et al*., 2011) generating an interest in the characterization of these proteins as future drug targets.

The genome of *Plasmodium berghei* has about 24 putative genes encoding DUBs, *Plasmodium yoelli* has about 27 putative genes encoding DUBs and *Plasmodium chabaudi* has about 21 putative genes encoding DUBs (Ponst *et al*., 2008) but none of the proteins encoded by those genes has been characterized yet. All together this data shows that the ubiquitin/proteosome pathway represents a new avenue in drug discovery that needs to be further explored. Hence DUBs are now seen as a potential group of interesting enzymes for antimalarial drug targeting. Throughout the years it has been difficult to develop drugs

against important parasites responsible for human diseases.In order to develop new drugs several parameters must be taken into account such as: drug efficacy, pharmacology, general toxicity and potential side effects.Developing anti-malarial drugs from scratch is time consuming and often results is drug development projects being abandoned halfway due to the lack of return on investments (Fidock *et al*., 2004; Chung *et al*., 2009).

Recent approaches to speed up the discovery and development of new compounds with antimalarial activity involve screening of compounds which have shown therapeutical potential in the treatment of other diseases, but also display antimalarial activity. The natural compound curcumin is a polyphenolic compound with anti-cancer, anti-inflammatory, anti-viral and antimalarial activity. Curcumin is widely used in traditional Indian medicine in the treatment of cancer. Now several studies have shown that curcumin is active against coxsackie viral infections, prevents mycocardial infarction, rheumatoid arthiritis, multiple sclerosis and Alzheimer disease (Anand *et al*., 2007; Mimche *et al*., 2011).There is a growing body of evidence indicating that curcumin has potent antimalarial activity both *in vivo* and *in vitro* (Mullaly and Fitzpatrick *et al*., 2002, Nandakumar *et al*., 2006, Martinelli *et al*., 2008).

Curcumin has proved to be potent against other parasitic organisms including: *Schistosoma mansoni* adult worms (Magalhães *et al*., 2009), *Cryptosporidum parvum* (Shahiduzamman *et al*., 2009) and *Trypanosome cruzi* (Nagajyothi *et al*., 2012). Recent reports have shown that curcumin´s mode of action may be attributed to inhibition of de-ubiquitylating enzymes (DUBs). Given the diverse nature of DUBs, the characterization of DUBs and the identification of inhibitors against DUBs is now being pursued has new avenue in antimalarial drug discovery.It can be argued that inhibitors developed against *Plasmodium* DUBs may also affect Human Dubs, but the use of molecular docking and other sophisticated pharmacology software may allow the development of specific inhibitors which target with high efficacy the parasite´s enzyme with minimal damage to the host.

Table 4. Characterization of de-ubiquitylating enzymes (DUBs) and de-ubiquitylating like enzymes (DUBLs) in *Plasmodium falciparum.*

Component of the UPS	Putative biological Role	Biological characterization	Inhibitors available	References
DUBs & DUBLs				
Ubiquitin carboxyl hydrolase (UCH)	de-ubiquitylation	*Pfuch-l3* & *Pfuch-l54* characterized as DUBs	Synthetic compounds Cyclopentenone and DBA	Guedat and Colland, 2007; Artavanis-Tsakonas *et al*., 2011
Ubiquitin proteases (UBP)	de-ubiquitylation	Not available	Curcumin* isolated from the plant *Curcuma longa* Shikoccin isolated from the plant *Rabdosia shikokiana occidentalis*	Mullaly and FitzPatrick 2002; Reddy *et al*., 2005; Nandakumar *et al*., 2006
Machado Joseph disease (MJD)	de-ubiquitylation	Not available		
JAMM motif metalloprotease (JAMM)	de-ubiquitylation	Not available		

SUMO specific proteases (SENPs)	de-ubiquitylation	*pfSENP-1* and *pfSENP-2* characterized and SUMO cleavage activity confirmed	Synthetic compound JCP-666*	Ponder *et al*.,2011
Ovarian tumour proteases (OTU)	de-ubiquitylation	Not available		

Given the fact that antimalarial drug resistance is emerging at a much faster rate then antimalarial drug development, the enzymes involve in the UPS and its inhibitors represent a promising avenue in antimalarial chemotherapy. In this project the main focus will be on de-ubiquitylating enzymes (DUBs) as previous study carried out by our laboratory identified a mutation in a gene encoding a ubiquitin carboxyl hydrolase-1 enzyme in *Plasmodium chabaudi* (*pcuch-l1*) strains resistant to artemisinin and artesunate (Afonso *et al*., 2006; Hunt *et al*., 2007). In light of the previous research that was carried out in our laboratory, there is now a major interest in characterizing DUBs not only because of their possible involvement in drug resistance (Hunt *et al*., 2007) but also because of their involvement in the development and the progression of infectious and non infectious diseases (Le Negrate *et al*., 2008; Luise *et al*., 2011).

Given the large number of DUBs in the *Plasmodium* genome (section 1.1.3.9) four of them were identified in the *Plasmodium* genome using the PlasmoDB and the Protein Data Bank (APPENDIX A and APPENDIX B and APPENDIX I) to be characterized in this project, these are: *Plasmodium falciparum* ubiquitin carboxyl hydrolase-1 (*Pfuch-l1*) *Plasmodium falciparum* ubiquitin carboxyl hydrolase-3 (*Pfuch-l3*) *Plasmodium falciparum* Ubiquitin carboxyl hydrolase 54 (*Pfuch-l54*) and *Plasmodium falciparum* Ubiquitin protesase 8 (*Pfubp-8*).Those genes were chosen based on previous work published by others and their relevance in other biological systems.The human homologue of *Pfuch-l1* has been implicated in kidney carcinomas (Luise *et al*., 2011) and point mutations in the human gene *huch-l1* are associated with Parkinson´s disease (Liu *et al*., 2002). This gene also appears to be mutated in *Plasmodium chabaudi* parasites resistant to artesunate (Hunt *et al*., 2007).The protein sequence of *Pfuch-l1* was used to interrogate the protein data bank (PDB) the protein query indicated that human ubiquitin carboxyl hydrolase 8 (*huch-l8*) is the human homologue of *Pfuch-l1*.

Pairwise sequence alignment performed by CLUSTALW2 revelaed that both protein sequences only have 18% sequence identity (APPENDIX A and B). *Pfuch-l3* protein sequence was also used to interrogate PDB and the closest human homologue was ubiquitin carboxyl hydrolase 3 (*huch-l3*). Pairwise alignment performed by CLUSTAL W2 confirmed 36% sequence identity between the two protein sequences (APPENDIX A and B) as previously published (Artavanis-Tsakonas *et al*., 2011). In mice, the gene

uch-l3 regulates the apical membrane recycling of epithelial sodium channels (Butterworth *et al*., 2007) however its function in *Plasmodium spp* is unkown. In humans the enzyme encoded by *huch-l5* gene is known to be associated to the proteosome and might be involved in TGF-ß signaling (Nijman *et al.*, 2005), enquirying PDB and performing pairwise sequence alignment performed by CLUSTALW2 revelead *Pfuch-l54* and *huch-l5* share 31% sequence identity. *Ubp-8* gene in yeast regulates transcription mechanisms and it is responsible for the de-ubiquitylation of histone H2B which is involved in DNA replication (Henry *et al*., 2003) however, in *Plasmodium spp* no function has been assigned so far. Enquirying PDB and performing pairwise alignment revelead that the catalytic domain of human ubiquitin specific protease 2 (USP-2) is the closest human homologue of *Pfubp-8* and both protein sequences share only 25% sequence identity, sequences were retrieved from PlasmoDB and used to carry out the objectives of the project. The major objective of this project is to identify DUBs in the *Plasmodium falciparum* genome, characterize them and evaluate their potential as future drug targets.

The specific aims of this project are:

1) Analyze the expression profile of genes encoding DUBs throughout the life cycle of *Plasmodium falciparum* parasites in the presence and absence of drugs.

2) To knockout *pfuch-l1* and *pfuch-l3* genes in *P. falciparum* through a gene disruption technique with the aim of understanding whether DUBs are essential for the parasite survival.

3) Express recombinant DUBs in *E.coli* cells, purify them and determine their *in vitro* activity using a fluoremitric based assay.

4) Determine the *in vivo* efficacy of curcumin alone and in combination with chloroquine and artemisinin in *Plasmodium chabaudi* parasites, a murine model of malaria.

5) Perform proteomic assays in *P. falciparum* parasites exposed to curcumin in order to determine the parasite´s response to curcumin and identify othet potential drug targets.

CHAPTER 2-MATERIALS AND METHODS

2.1. Biological Material

***Plasmodium falciparum* strain 3D7:** was originally isolated in Amsterdam and is susceptible to chloroquine, amodiaquine, mefloquine and quinine (Miller *et al*., 1993).

***Plasmodium falciparum* strain Dd2:** was first collected in Indochina and its phenotype is chloroquine and mefloquine resistant (Wellems *et al*., 1988).

***Plasmodium chabaudi* strain AS-3CQ:** AS-3CQ (resistant to chloroquine) and selected from the clone AS-Pyr which was subjected to six daily doses of chloroquine (CQ) at 3 mg/kg. This parasite line was cloned and named AS-3CQ (Do Rosário *et al* 1976).

***Plasmodium chabaudi* strain AS-ART:** The AS-ART clone resistant to artemisinin was obtained from a clone known as AS-30CQ which tolerated 300mg/kg/day of artemisinin obtained by serial passages in the presence of increasing subcurative doses of artemisinin, this parasite line was cloned and named AS-ART (Afonso *et al*., 2006).

Pet28a+ cloning Vector: Protein expression vector carrying an N Terminal and C Terminal optional Histidine (his) tag. It also harbours a kanamycin resistance gene for bacteria selection in agar plates provided by Novagen.

BL21 Codon plus cells: Competent cells derived from stratagene BL21 Gold competent cells widely used in efficient high level expression of proteins in *E. coli* cells.

***Plasmodium falciparum* PHH transfection vector:** The pHH vector is a single cross over vector provided by MR4 normally used for gene disruption and contains a Human *dhfr* mutated that encode resistance to the drug WR99210 for selection of transformed parasites.

Hepatocellular carcinoma cells (HepG2): Cell line derived from hepatocellular carcinoma of epithelial morphology with adherent properties widely used in drug cytotoxicity studies provided by MR4.

2.2. Methods

2.2.1. Expression profile study of genes encoding DUBs in *Plasmodium falciparum* strains 3D7 and Dd2, in the presence and absence of drug pressure

Since *Plasmodium falciparum* is the parasite responsible for most cases of human malaria in endemic aeas and very few studies have been carried out focusing on *P. falciparum* DUBs, most of the work carried out in this project will focus on *P.falciparum* DUBs. Gene expression studies have been a helpful tool in understanding what genes are being expressed and at what stage, giving an indication of when that gene product may be needed by the parasite.

In the first part of this project, the purpose was to analyze the basal expression of *P. falciparum* genes encoding DUBs throughout the parasite´s life cycle. This was done by collecting blood samples at different time points throughout the parasite life cycle (48h) the first blood collection (0h) represents the control. The protocol involved the extraction of RNA and analysing the expression of the genes at the ring, trophozoite and schizont stage in the absence of any drug by RT-PCR.

The second part of this study was to evaluate the expression profile of the same genes but in parasites that were subjected to the drugs: chloroquine (CQ); artemisinin (Art) and the DUB inhibitor curcumin (Curc) which has been shown to interefere with the UPS (Si *et al*., 2007). For that part of the project the IC50 of each individual drug as well as the cytotoxicity levels were determined using a commonly used technique based on the DNA staining dye SYBR GREEN for *in vitro* drug susceptibility assays and the cytotoxicity of each drug was determined using HepG2 cell line and the MTT (3 – 4.5 – dimethylthiazol-2-yl) – 2.5 diphenyltetrazolium bromide) colorimetric based assays.

2.2.1.1 Culture of *Plasmodium falciparum* parasite strains 3D7 and Dd2

For the *in vitro* study we used the following clones: 3D7, which is sensitive to all known antimalarials (Miller *et al*., 1993), and Dd2 highly resistant to chloroquine and mefloquine (Wellems *et al*., 1988; Bacon *et al*., 2007). The *P. falciparum* strains were maintained in continuous culture in human erythrocytes as previously described by others (Trager and Jensen, 1976) with minor modifications. Prior to the experiments, the cultures were maintained in fresh human erythrocytes suspended at 5% hematocrit in RPMI 1640 containing 10% albumax, 25 mM Hepes, 3 g of glucose per liter, 45 g of hypoxanthine per liter and 5% $NaHCO_3$. The medium was changed daily and the cultures were incubated at 37°C under an atmosphere of a certified gas mixture containing 5% CO2, 5% O2, and 90% N2.

2.2.1.2 Determination of *in vitro* IC50 of chloroquine, artemisinin and curcumin with SYBR GREEN based method

Chloroquine and artemisinin were chosen as the standard antimalarial drugs and curcumin was also introduced as it is known as a potential inhibitor of DUBs. A 10 mM stock solution of chloroquine, artemisinin and curcumin was prepared by dissolving the drugs in DMSO, except chloroquine which was dissolved in water and subsequently diluted to working concentrations in RPMI 1640 culture medium. Stock solutions of each drug were diluted in 96 well plates with concentrations ranging from 0.001-10 µM using 50 µl volume of each drug. The parasites development stage was synchronized with 5% sorbitol (Lambros and Vanderberg, 1979). The percentage of synchronization and parasitaemia was evaluated by light microscopy on Giemsa-stained thin blood smears. The cultures were then diluted with complete medium and non-infected human erythrocytes to a final hematocrit of 2% and 1% of parasitaemia and 50 µl of this parasites suspension were added to which well. Plates were incubated at 37º C, under an atmosphere of a certified gas mixture containing 5% CO2, 5% O2, and 90% N2 for 48 hours.

After this incubation period lyses buffer with SYBR Green was added to each well using a protocol previously described by others with minor modifications (Smilkstein *et al*., 2004) for fluorescence measurements: 100 µl of lyses buffer containing Tris-20 mM (Sigma- Aldrich); pH 7.5, EDTA (Sigma-Aldrich) 5mM, saponin (Sigma - Aldrich) 0.008 %; wt/vol, Triton X-100 (Sigma- Aldrich) 0.08 %; vol/vol, and 0.2 µl of SYBR Green I (Invitrogen) per ml of lyses buffer were added to each well, and the contents were mixed until no visible erythrocyte sediment remained. After 1 hour of incubation with shaking in the dark at room temperature, fluorescence was measured with a fluorescence multiwell plate reader, Anthos zenyth 3100 (Alfagene) with excitation and emission wavelength bands centered at 485 and 530 nm, respectively, and a gain setting equal to 50. The data was analyzed by HN-Non lineV1.1 software used for *in vitro* drug susceptibility assays. The half maximal inhibitory concentration (IC50) for each compound was determined from Log dose-response curves. Assays were repeated three times.

2.2.1.3 Cytotoxical evaluation of artemisinin, chloroquine and curcumin in Hepatocellular carcinoma cells (HepG2)

2.2.1.3.1. HepG2 culture

Hepatocellular carcinoma cells (HepG2) cells were cultured in William´s medium supplemented with 10% bovine serum albumin (BSA) and kept at 37ºC under an atmosphere of a certified gas mixture containing 5% CO2, 5% O2, and 90% N2 and the medium was changed every two days. The monolayer of cells was passaged by rinsing the cells with 1x PBS (phosphate buffered solution) and cells were removed from the bottom of the flask with trypsin solution (0.05%) and incubating the flasks at 37º C for a period of 5 minutes.

2.2.1.3.2. Cytotoxicity assay

A suspension of 200µl of HepG2 cells at $1x10^3$ cells/well were seeded in 96 well plates and cultured at 37 ºC overnight. Curcumin, Chloroquine, Artemisinin was added to the culture plates in concentrations ranging from 1-300µg/ml in triplicate and the cells were incubated for 48h. The medium with a new dose of drug was changed after 24 hours. After 48 hours under drug action, MTT (3-4,5-dimethylthiazol-2-yl)-2,5

diphenyltetrazolium bromide) was added to each well at a final concentration of 5 mg/ml dissolved in RPMI medium and incubated for 3 hours at 37º C. The medium was then replaced by a solubilization solution (5% SDS, 200 µl of acetic isopropanol and isopropanol and HCL (0.04-0.1 N HCl) em isopropanol absoluto and incubated for 5 minutes under rotation at room temperature to solubilize the formazan crystals which emits a purple colour. Readings were taken using a spectrophotometer with filters 550-630 nm range the 50% cytocixity values were obtained from GraphPad Prism4.The selectivity index of artemisinin, chloroquine and curcumin was calculate in the following manner:

$$\text{Selectivity Index (SI)} = \frac{\text{Cytoxicity value (HepG2cells)}}{\textit{in vitro}\ \text{IC50}}$$

2.2.1.4 *Plasmodium falciparum* parasite culture for gene expression studies in the absence and presence of drugs

For the gene expression profile assays the cultures were maintained in 24 well plates, the assays were initiated with parasites synchronized at ring stage, 2% parasitaemia, 5% of haematocrit and a concentration of drug corresponding to the IC50 of each individual drug; chloroquine (CQ) Artemisinin (Art) and Curcumin (Curc). A group of plates were included without drug in order to study the basal expression of genes encoding de-ubiquitylating enzymes (*pfuch-l1*, *pfuch-l3*, *pfuch-l54*, *pfubp*-8). Plates were incubated at 37º C for 48 h and samples were collected at different time points (0h, 3h, 6h, 9h, 12 h, 15h, 18h, 21h, 24h, 27h, 30h, 33h, 36h, 39h, 42h, 45h and 48h). Giemsa stained smears were also prepared to assess the development and morphology of the parasites throughout the assay. The blood samples were centrigured 700 g for 5 minutes to eliminate the supernatant and pellets were stored at -80º C until RNA extraction.

2.2.1.5 *Plasmodium falciparum* RNA extraction and cDNA synthesis

The RNA was extracted from the obtained pellet using Trizol (Sigma-Aldrich) and following the manufacturer's instructions. The RNA (~50 ng) was mixed with 5 µl of DNase I buffer and 1 µl of DNase I (Promega) and incubated at 37 ºC for 15 min. DNase I was inactivated by adding 1 µl of EDTA (Promega) and incubated at 65 ºC for

5 min. RNA (50 ng) previously treated with DNase I (Promega), was used as template and was mixed with the Maxima first strand cDNA synthesis kit for RT-PCR (Fermentas) and water to a final volume of 25 µl according to the manufacturer's instructions. Samples were incubated for 10 min at 25 ºC followed by 30 min at 50 ºC and the reaction of cDNA synthesis was terminated by incubation at 85 ºC for 5 min.

2.2.1.6 Real time PCR conditions

Real time PCR(figure 8) using the iQ SYBR green supermix (Bio-RAD) was carried out in triplicates using microAmp 96 well plates (Applied Biosystems), with a 25 µl final volume containing IQ SYBR green supermix dye (Bio-RAD) 0.025 U/µl iTaq DNA polymerase (Promega), 200 µM dNTPs (Promega), 3.5 mM $MgCl_2$ (Promega) and each individual mixture contained 300nM of each primer, forward and reverse (StabVida) specific for the genes (*pfuch-l1*, *pfuch-l3*, *pfuch-l54*, *pfubp-8*) (figure 8, table 5) finally 2 µl of cDNA corresponding to each time point diluted 1:100, was added to each individual primer mixture. *PfβActinI* was used as the endogenous control (Ferreira *et al*., 2004). In order to determine PCR efficiencies for each individual gene, samples were diluted in serial 10 fold ranges and the CT value at each dilution was measured. A curve was then constructed for each gene from which efficiency was determined. Real-time PCR efficiencies (E) were calculated from given slopes, according to the equation: E = 10(-1/slope), where E = 2 corresponds to 100% efficiency (Pfall *et al*., 2001; Ferreira *et al*., 2004). At the end of each PCR run each melting curve was analyzed to make sure that there were no contaminated products (APPENDIX C).

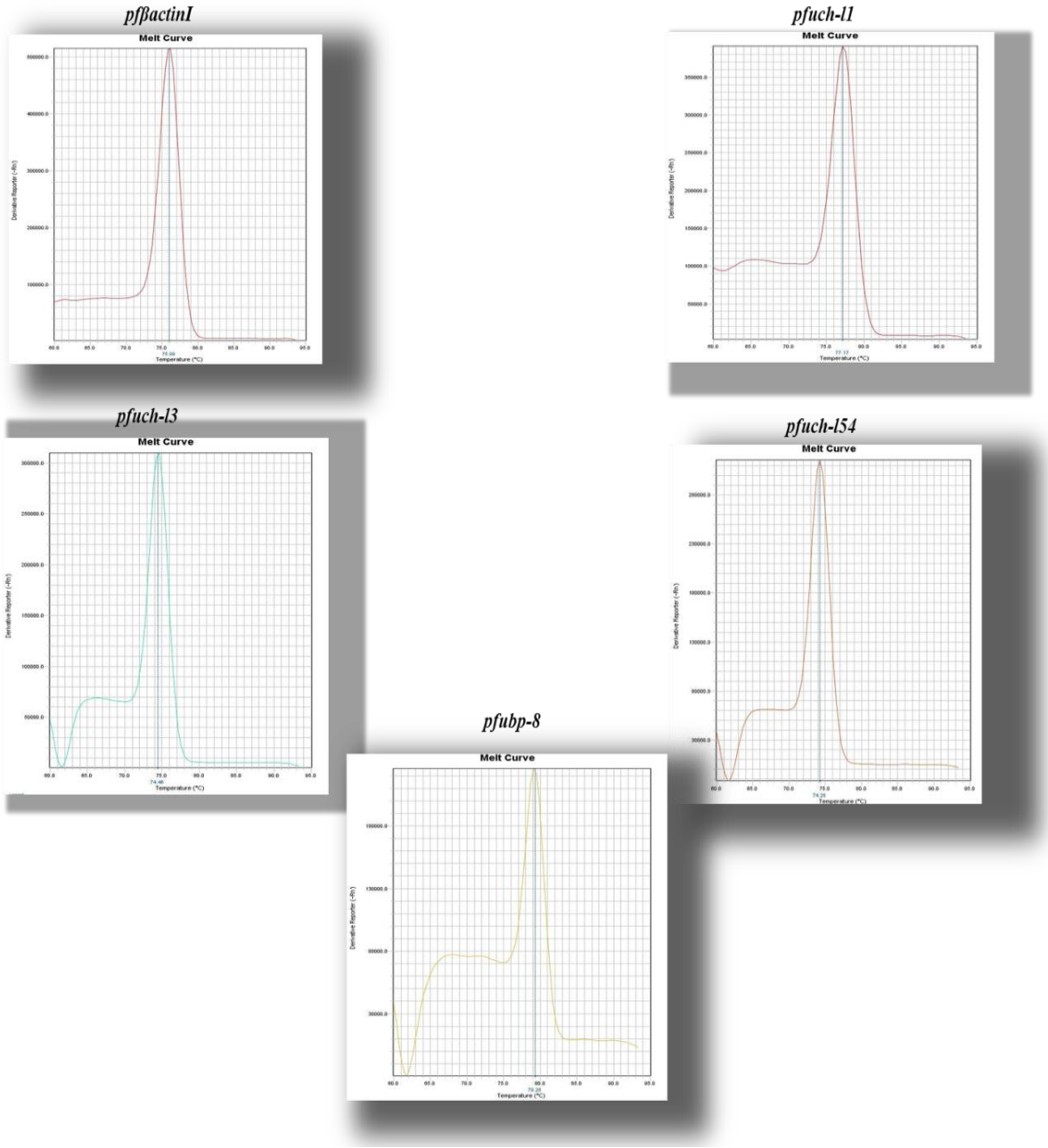


Figure 8. Illustration of the melting curves obtained by RT-PCR. The primers used were *pfβactin*, *pfuch-l1*, *pfuch-l3*, *pfuch-l5*, *pfubp-8*. Real time PCR was performed in a 7500 Applied Biosystems device. The RT-PCR reaction was performed in a 7500 fast RT-PCR thermocycler (Applied Biosystems).

Table 5. *Plasmodium falciparum* RT-PCR primers designed from mRNA sequence.

Gene name	Sequence	Amplicon size (bps)	Reaction conditions
pfβactinI	Forward: TGTTGACAACGGATCAG Reverse: GGAACGAGGTGCATCAT	77	
pfuch-l1	Forward: CTTTCTTTGGAGCGACCAATAT Reverse: GACGATTTCTCCATA AGGGGTG	138	95ºC 10 secs
pfuch-l3	Forward: GATTCCACAACCTGTTCAAGCG Reverse: GGCTATGGTTCCACATGAGTTT	157	95ºC 10 secs 60ºC 60 secs
pfuch-l54	Forward: CAGACGAGCAAAATAAACCCA Reverse: TTCTATCCAATCTTTTCCATTCAT	170	40 cycles
pfubp-8	Forward: GTGGATAATAATGGAAATGTAG Reverse: CATATTTTCGTTGTTGTCTACAT	129	

2.2.1.7 Analysis of relative expression using the $2^{-\Delta\Delta ct}$ method

The $2^{-\Delta\Delta ct}$ method was used to calculate the relative quantification of target gene (Pfall, *et al*., 2001). The N - fold difference was calculated in the following manner ΔΔct = (Ct *pfuch-l1* – Ct *PfβActin*)A - (Ct *pfuch-l1* – Ct *PfβActin*) B, where A = sample treated with Chloroquine, Artemisinin or Curcumin and B corresponds to a sample collected at time point (0h) which was not exposed to drug.

2.2.1.8 Statistical analysis

All gene expression assays were analysed using a T-test, statistical significance of gene expression levels compared with control values was * $P \leq 0.05$ (n=3 assays) using a paired T-test provided by SPSS software version 9.0.

2.2.2 Evaluating the importance of de-ubiquitylating enzymes in *Plasmodium falciparum* by generating a transgenic parasite line by homologous recombination

In order to determine whether DUBs are good drug targets, it is important to know whether these enzymes are essential for the intraerythrocytic stage of the parasite or not and whether they are essential for parasite growth and development. An attempt was made to knockout two genes encoding DUBs by homologous recombination. *Pfuch-l1* gene was selected since this gene appears to be mutated in *Plasmodium chabaudi* parasites resistant to artemisinin (Hunt *et al*., 2007) and also point mutations in the *huch-l1* are associated with Parkinson´s disease (Liu *et al*., 2002).

In a second attempt the strategy was to knockout the gene *pfuch-l3* which has been shown in mice to be involved in the regulation of apical membrane recycling of epithelial sodium channels. Genomic DNA from *Plasmodium falciparum* strain 3D7 was amplified by PCR using primers containing restriction sites for directional cloning into the disruption transfection vector pHH. Both the vector and the PCR products were restricted with the same enzymes followed by a ligation reaction. Competent *E. coli* cells were transformed and cultured in appropriate medium in order to uptake the plasmid and maximize plasmid DNA. Circular plasmid DNA was then cleaned up and used to transfect *P. falciparum* ring stage parasites

A control vector known as PARL-2 vector expressing the GFP gene was also used to verify whether the technique works. Parasites transfected with success will express the GFP gene throughout its life cycle. GFP tagged parasites can be esasily visualized by fluorescent microscopy.Transfected parasites that did take up the plasmid DNA contain the *hdhfr* selection cassette which will confer resistant to the drug WR99210 whose mode of action is similar to pyrimethamine, allowing the selection of transfected versus non transfected parasites. Transformed parasites would then be analyzed to see whether their growth curves and response to drug treatment and biochemical activity differs from non transformed parasites.

2.2.2.1 pHHpfuch-l1 and pHHpfuch-l3 knockout construction

Genomic DNA was extracted from *Plasmodium falciparum* cultures using a DNA extraction kit (Qiagen) and the *pfuch-l1* DNA fragment was amplified using the primers and conditions described on (table 6). Primers contain restriction sites BgLII/XhoI forward/reverse which are underlined. For *pfuch-l3* a DNA fragment was amplified using the following primers containing SpeI/AflIII sites for directional cloning into the vector pHH (MR4).

To yield a PCR products of 618 bps and 573 bps respectively with a premature stop codon introduced at the anti-sense primer, the PCR components and conditions for the amplification of the fragments for transfection was carried out as described in the table 7. PCR product was cleaned with (Qiagen PCR purification kit) and both the PCR product and the vector were digested with the same enzymes for directional cloning. The vector/insert construct was used for transformation of *E. coli* cells in order to increase plasmid DNA for transfection assays.

A control vector was transfected in parallel to ensure that the transfection technique is working. The control vector named PARL-2 vector bearing the GFP (green fluorescent protein) which is under the control of EF-1 alpha promoter and the *P.berghei dhfr* 3UTR gene (MR4).The PARL-2 vector (figure 9) bears a 910 bps sequence belonging to the gene *Pfs47* which will recombine with the *Pfs47* gene sequence on *Plasmodium falciparum* chromosome 13. *Pfs47* is known to be, not essential for the parasite (Talman *et al*.,2010) therefore interuption of this gene sequence will not affect parasite viability. Sucessful transfection would result in parasites expressing GFP gene which can be easily visualized under a fluorescent microscope, thus confirming that the technique is working. The culture and preparation of ring stage 3D7 *Plasmodium falciparum* parasites used for transfection was carried out as described earlier in section 2.2.1.1.

Table 6. Amplification of *pfuch-l1* and *pfuch-l3* PCR products for transfection

PCR components	**Final concentration**	**PCR conditions**
Distilled ultrapure water		
Buffer with $MgCl_2$ (10x)	1x	95ºC: 5mins 94ºC: 3mins
$MgCl_2$ (25mM)	1.5mM	55ºC: 55 secs
DNTPs	1.5mM	72ºC: 1min
Forward primer	250 µM	72ºC: 1min
Reverse primer	250 µM	
DNA polymerase (pfu)	2.5U	25 cycles
cDNA	20ng	
***Pfuch-l1* restriction sites BgLII/XhoI**		
Forward: CCTAGATCTCGGAAGCTTAGGACAAGATG		
Reverse: GGACTCGAGGTTACAACGATAAAACAGA		
***Pfuch-l3* restriction sites SpeI/AflII**		
Forward: GGCACTAGTATGGCAAAGAATGATATTT		
Reverse: CCGCTTAAGTTA GGTAAAACAGTGAACA		

2.2.2.2 PCR product purification

PCR product was cleaned with Qiagen PCR purification kit. Both the PCR product and the vector were digested with the same enzymes for cloning. The ligation product resulting from (PCR prodcut and vector) was used for transformation of *E. coli* cells in order to increase plasmid DNA. The bacteria cells were grown over night at 37º C within a 250 ml Erlenmeyer flask in the presence of ampicillin 0.5 µg/ml. Plasmid DNA

was then cleaned using a mini-prep kit (Sigma-Aldrich). DNA was eluted in TE buffer (10 mM Tris-HCL pH 7.5 and 1 mM EDTA) and was used for transfection assays.

2.2.2.3 Transfection of parasites by electroporation

Infected red blood cells, obtained from the culture, were pelleted by centrifugation (Eppendorf) 5 mins 500 g. Infected Red blood cells were resuspended in cytomix transfection buffer, to the same cuvette 0.2 cm (BioRAD) was added 50µg of the control vector PARL-2 bearing the GFP gene. Transfection was carried out in Gene Pulser (BioRAD) with 310 voltage and 950µF, this set of conditions was previously established by others (Talman *et al*., 2010) and was shown to be adequate for transfection of PARL-2 vector. Transfection of pHHpfuch-l1 KO and pHHpfuch-l3 KO was carried out with various quantities of plasmid DNA to maximize the chances of transfection. To the same cuvette, was added ring stage parasites (2%) and cytomix transfection buffer (120 mM Kcl, 0.20 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/KH_2PO_4 and 25 mM Hepes pH 7.5). Electroporation (table 7) was carried out in a gene pulser II under the following conditions:

Table 7. Electroporation settings for transfection of *P. falciparum* parasites

Construct name	Voltage (Kv)	Capacitance (µF)	DNA amount (µg)	Number of Trials
pHHpfuch-l1 KO	2.5	25	150	6
	0.31	960	150	6
	2.5	25	50	6
	0.31	960	50	6
pHHpfuch-l3 KO	2.5	25	150	4
	0.31	960	150	4

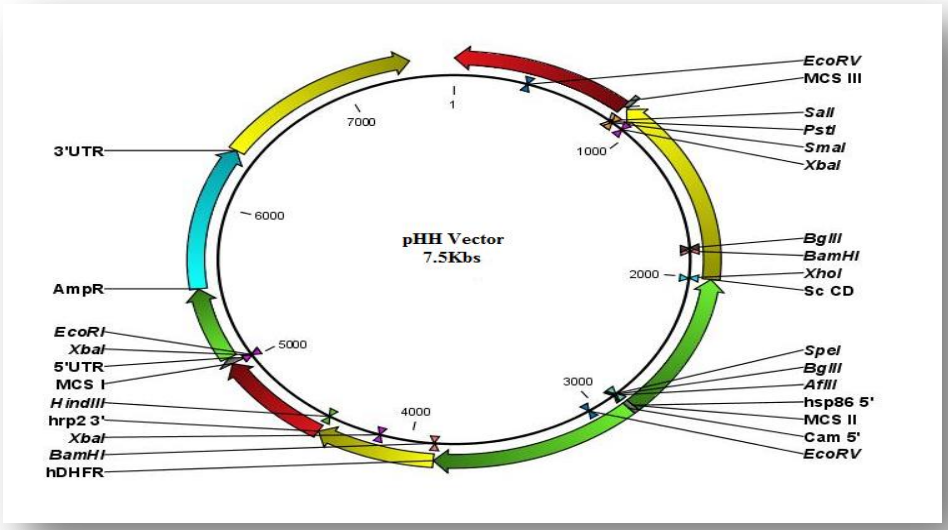
	2.5	25	50	4
	0.31	960	50	4

After electroporation samples were immediately mixed with 25 ml of RPMI culture (Skinner-Adams *et al*., 2003) medium containing fresh blood to give a 5% haematocrit. Smears were prepared every day and stained with freshly prepared Giemsa 20% (Sigma-Aldrich) to monitor the growth of the transfected parasites. After selection of transfected parasites, a diagnostic southern blot or PCR was performed to verify integration. PARL-2 GFP parasites were visualized using a fluorescent microscope (Zeiss).

2.2.2.4 Selection of transfected parasites

The pHHpfuch-l1 KO and the pHHpfuch-l3 KO lacks the ATG start codon 5´ and has a premature stop codon at the 3´ region. Upon recombination of the plasmid by a single cross over two non functional incomplete gene copies would be generated. The first copy retains the endogenous promoter and the start codon but it contains a premature stop codon and the second copy would be without the promoter and start codon but retains its natural stop codon. The pHH vector is a single cross over vector normally used for gene disruption (Fidock *et al*., 2000; Skinner-Adams *et al*., 2003) and contains a Human *dhfr* mutated that encode resistance to the drug WR99210 (Jacobus Pharmaceuticals) (Koning-Ward *et al*., 1999). The Human *dhfr* cassette is comprised of the Human *dhfr* gene 0.56 kb which is under the control of the calmodulin gene 5´(CAM) untranslated region (UTR) and hrp2 3´(UTR) (figure 9).

Successfully transfected parasites would be selected with 5 µM of WR99210 (Jacobus Pharmaceuticals) which was added to the culture medium 48 h after the transfection. Parasites that did not uptake the construct die in the presence of drug treatment. Whereas transfected parasites that did successfully uptake the plasmid will survive the drug treatment. Giemsa stained smears from the culture were prepared every day for a period of 60 days to watch out for the re-appearance of transfected parasites.



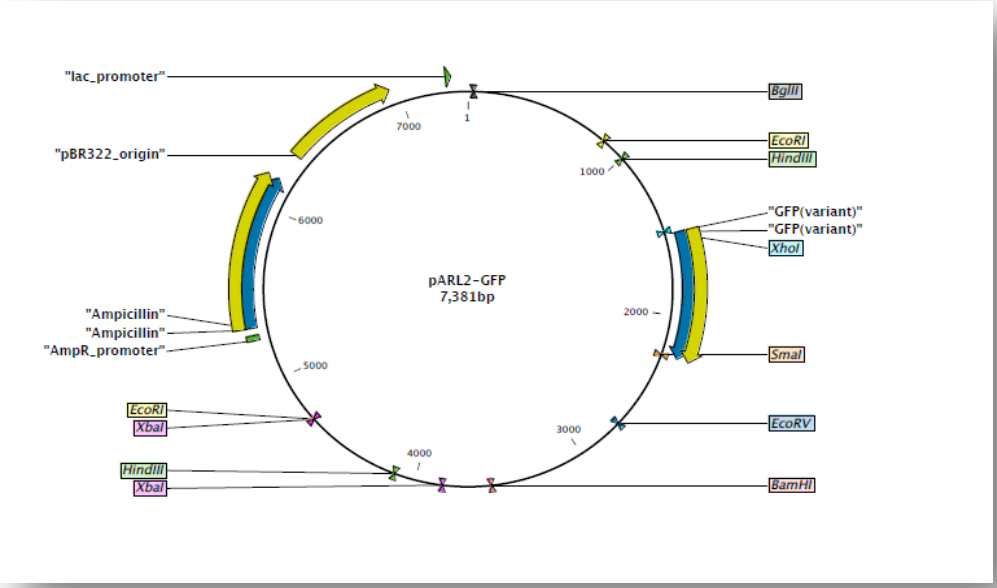


Figure 9. Structure of the single cross over vector pHH. The pHH vector (top panel) is commonly used for gene disruption. The bottom panel represents the PARL-2 vector bearing the GFP gene for GFP tagging of parasites www.mr4.org.

2.2.3 Recombinant protein expression and *in vitro* activity of curcumin towards recombinant DUBs

It was important to confirm the de-ubiquitylating activity of the recombinant proteins produced here. For protein expression, gene sequences were amplified by PCR and cloned into the protein expression vector pET28a+. After restriction and ligation of the PCR product to the vector, competent BL21 cells were transformed using the vector insert construct. Transformed cells were plated in agar plates in the presence of kanamycin for selection of colonies. A single colony was then picked and grown in LB medium in the presence of antibiotics and this starter culture was used to inoculate 2 L cultures for the expansion of the cells.

Protein expression was induced at the appropriate time and recombinant proteins were harvested, eluted and tested for DUB activity using the typical substrate for DUBs Ubiquitin-7-amino-methyl-coumarin (Ub-AMC). Attempts were also made to test recombinant DUB activity in the presence of curcumin, the plant derived DUB inhibitor with antimalarial activity.

Part of the recombinant protein was used to immunize Balb/C mice for the production of polyclonal antibodies. Mice were immunized with each antigen dissolved in PBS and complete Freund´s adjuvant to stimulate the immune system. The protocol had a total duration of 39 days and mice received boosters on appropriate dates. During the protocol serum was being collected to monitor the titres of antibody. On day 39, mice were euthanized and total blood was collected by cardiac puncture. The serum was separated and the polyclonal antisera were used for western blot protein detection in different stages of the parasite´s life cycle.

2.2.3.1 Amplification of PCR products for production of recombinant proteins

Sequences of the proteins (APPENDIX A) encoding the catalytic domain of *Pfuch-l1*, *Pfuch-l3* (full length sequence), *Pfuch-l54* (full length sequence) and *Pfubp-8* (catalytic domain)(table 10) were amplified by the polymerase chain reaction (PCR) using a proof reading enzyme *Pfu* (Fermentas) to minimize errors in the final PCR product. Primers had restriction sites incorporated into them, as described in (table 8) for directional cloning of the proteins *Pfuch-l1* (NdeI/XhoI), *Pfuch-l3* (EcoRI/XhoI), *Pfuch-l54* (EcoRI/BamHI), *Pfubp-8* (SacI/NdeI) to be in frame with the N terminal his-6 tag and reaction was carried out as shown in the tables 9 and 10.

Table 8. Primers designed for amplification of PCR products for recombinant protein production.

Gene name	Sequences with restriction sites underlined	Product size (bps)
pfuch-l1	F:CGCCATATGGTGAGCCGCATGAA R:CCGCTCGAGCCTAGACATCCCCT	1.149
pfuch-l3	F:CCGGAATTCATGGCAAAGAATGATA R:CCCCTCGAGTATAATATCAAAGTTATC	696
pfuch-l54	F:GCCGAATTCATGGCGAGGGATAATGAA R:CCCGGATCCATTTTTCTTTTTGATAAGC	1.375
pfubp-8	F:CCCGAGCTCGATACATACAACTGGTAT R:GGCCATATGCATTCCTGTCCATATTTTC	1.285

Table 9. PCR reaction components and conditions for the amplification of the gene sequences of *pfuch-l1, pfuch-l3, pfuch-l54* and *pfubp-8*.

PCR components	Final concentration	PCR conditions
Distilled Ultrapure water		
Buffer with $MgCl_2$(10x)	1x	95ºC: 5mins
DNTPs	150µM	94ºC: 3mins
Forward primer	0.1 µM	62ºC: 45 secs
Reverse primer	0.1 µM	72ºC: 1min
DNA polymerase (*pfu*) 2.5 U/µl	2.5 U	72ºC: 5 mins
cDNA	50ng	25 cycles

2.2.3.2 PCR product purification

For removal of primers and other PCR components, amplified products were purified using the Qiagen Qiaquick PCR purification kit (Qiagen). DNA was sequenced by Macrogen. Following confirmation of the sequences, PCR products were restricted with the appropriate enzymes for cloning into the expression vector pET28a+ (figure 10).

2.2.3.3 Cloning of PCR products into the protein expression vector pET28a+

Aliquots of plasmid DNA were individually digested with the following enzymes NdeI/XhoI (Fermentas), EcoRI/XhoI (Fermentas), BamHI/EcoRI (Fermentas) and NdeI/SacI (Fermentas). PCR fragments were digested with the same enzymes and incubated at 37º C. Restriction products were purified again with a Qiagen Qiaquick PCR purification kit (Qiagen) and the plasmid DNA and PCR product were ligated using the Rapid DNA ligation kit (Fermentas). The ligation mixture was made up of 10 X T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, pH 7. 5). Vector DNA 50 ng insert DNA 150 ng, 20 µl of nuclease free water, followed

by addition of 1 µl of T4 DNA ligase enzyme (Fermentas). To maximize the chances of ligation, ligations were kept at 4º C overnight.

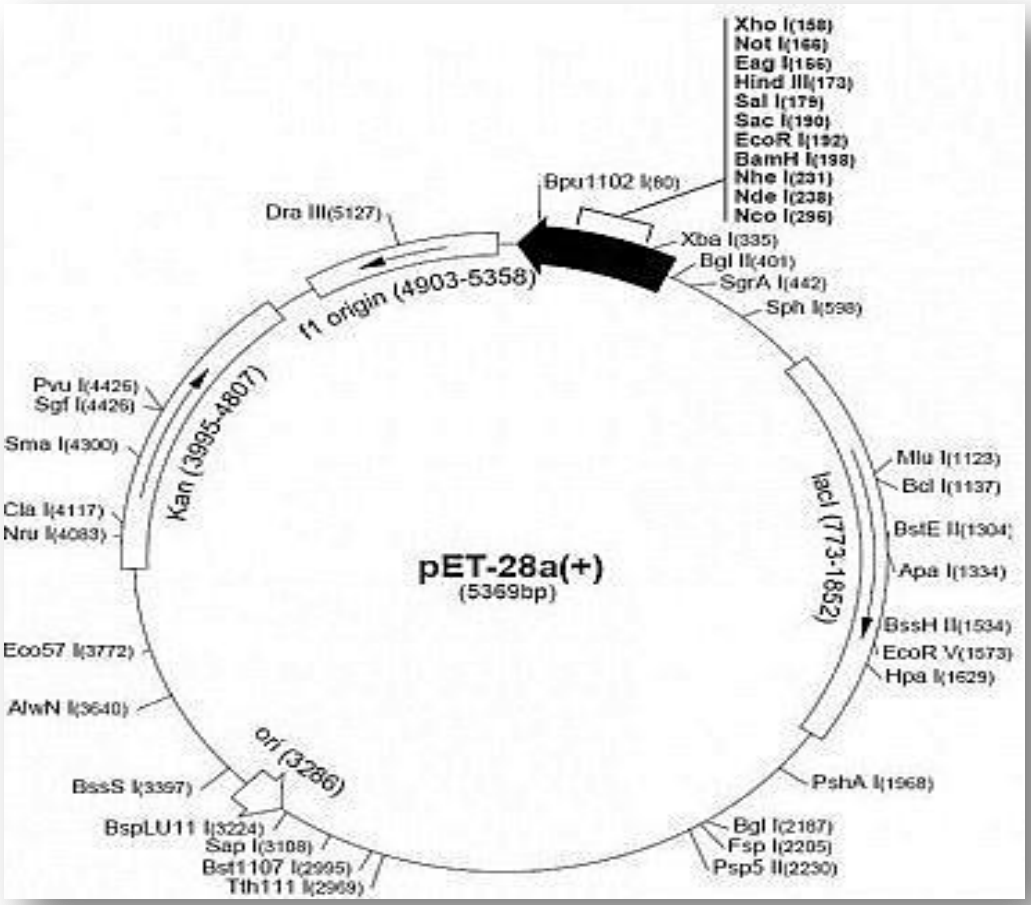


Figure 10. pET28a+ protein expression vector. Several restriction sites are available for cloning (black bold). The vector bears the kanamycin resistance gene for bacterial selection in liquid and solid media. The vector also has a special his tag sequence which means that recombinant proteins will express the histidine (his) sequence which facilitates protein purification using the his-select system. The map of the vector was provided by EMD Millipore www.emdmillipore.com

2.2.3.4 Transformation of BL21 DE3 RIL Codon Plus cells

Competent BL21 DE3 RIL codon plus cells (Stratagene) were transformed with 1 µl of the ligation reaction (PCR product from each gene and vector pET28a+)(figure 10). Transformation reaction was placed on ice for 20 minutes. Cells were heat shocked in water bath warmed at 42º C for 30 seconds. The transformation reaction was placed back on ice for 2 minutes. The entire content of the reaction was mixed with LB medium and placed in an Erlenmeyer flask which was placed in a shaking incubator at 37º C for 1 hour. After that 0.5 ml of cultures was removed under sterile conditions and placed in an agar plate containing kanamycin 100 µg/ml. Bacterial cells were spread evenly on the agar plate and were allowed to dry at room temperature. Inverted agar plates were placed in an incubator at 37º C overnight for colony growth.

2.2.3.5 Expression of the recombinant proteins in BL21 DE3 RIL Codon Plus cells

A colony of the transformed bacteria was used to inoculate 1L of LB medium containing 100 µg/ml of kanamycin and 50 µg/ml of chloramphenicol and cultivated at 37º C, overnight. The culture was carried out in 2L Erlenmayer flasks in a shaking incubator with a speed of 250 rpm (Becton-Dickinson). The pH and the optical density (OD) at A600 absorbance units (AU) of the culture were monitored using a spectrophotometer (BioRad) by taking culture samples every 30 minutes. When de OD reached 0.6-0.8, 1 mM of IPTG (isoprophyl-b-d-thiogalactopyranoside) was added to 1 L of culture to induce expression of the recombinant proteins. The expression of the catalytic region of recombinant *Pfuch-l1*, *Pfubp-8* (table 10) was carried out for three hours at 35º C. Recombinant protein *Pfuch-l3* and *Pfuch-l54* was expressed at 30º C and bacteria were harvested afterwards by centrifugation (Becton-Dickinson) at 15.000g for 25 mins.

Table 10. Illustration of *Plasmodium falciparum* DUBs studied and their respective predictive active site obtained from the database Pfam (http://pfam.sanger.ac.uk). For the recombinant proteins full lengthp *Pfuch-l3* (30 kDa) and *Pfuch-l54* (54 kDa) were expressed. Whereas *Pfuch-l1* and *Pfubp-8* proteins have a predictive size of 416 kDa and 207 kDa, respectively, due to their large size, sequences were chosen encompassing the catalytic region of those proteins highlighted in green. The expected sizes of the proteins are shown on the table.

Protein	Active site residues	predictive full size	size of recombinant proteins
Pfuch-l1	3170-3490	416kDa	42kDa
Pfuch-l3	73-120	30kDa	30kDa
Pfuch-l54	72-119	54kDa	54kDa
Pfubp-8	1415-1770	207kDa	50kDa

2.2.3.6 Purification of the recombinant proteins

Purification of the recombinant proteins was carried out in his-Select spin Ni-NTA columns suitable for proteins with the 6 his-tag (Qiagen). The columns contain 20 µm spherical silica particles made up of 10 nm pores the silica particles are charged with Nickel (Ni^{2+}) ions which is selective for recombinant proteins expressing a 6 his-tag. Briefly, bacterial cells were centrifuged at 15.000 g for 20 mins, the supernatant was discarded and the pellet frozen at -80º C. The pellet was then resuspended in lysis buffer (50 mM Tris pH 8.0, 10% glycerol, 0.1% Triton-X 100, 1 mM PMSF, Dnase 2U) and incubated on ice for 30 mins. The his-select columns were first equilibrated with 600 µl of equilibration buffer (50 mM NaH_2PO_4 and 0.3 M NaCl pH 8.0). The columns were span at 2000 g for 2 mins centrifuge (Eppendorf). The crude lysate was then passed through the his-select spin columns and columns were centrifuged for 2 mins at 2000 g as mentioned before. The columns were washed with wash buffer (50 mM NaH_2PO_4 and 0.3 M NaCl and 0.5 mM Imidazole pH 8.0) to reduce non specific binding.

The recombinant his tagged protein was then eluted using a solution made up of 50 mM sodium phosphate, 0.3 M sodium chloride and 250 mM Imidazole, pH 8.0. Recombinant proteins were concentrated and desalted using an Amicon Ultra-15 centrifugal filter device (Millipore) the solution was centrifuged at 4000 g, 25º C for 20 mins. Approximately 300 µg/ml of proteins (*Pfuch-l1*, *Pfuch-l3*, *Pfubp-8*) was recovered from 1L bacterial cultures and stored in aliquots in buffer A (50 mM Tris-Hcl, 100 mM NaCl, 25% glycerol, 1 mM DTT) at -80ºC for mice immunization for production of polyclonal antibodies. Small aliquots of fresh purified recombinant protein were immediately used for enzyme activity assays.

Most of the recombinant *Pfuch-l54* formed insoluble aggregates, therefore no enzymatic assays were carried out for this protein. Instead, the protein was solubilized and used to immunize mice, a procedure that has been carried out by others successfully (Yang *et al*., 2011) for western blot applications. Bacterial cell lysate were first centrifuged and the pellet was frozen at -80º C. Bacterial cells were sonicated in lysis buffer (50 mM Tris-Hcl, 100 mM Nacl, 5 mM EDTA, 0.1% NAN_3, 0.4% Triton X-100, 0.1 mM PMSF and 1 mM DTT). After sonication 5 mM $MgSO_4$ was added to chelate EDTA. Lysozyme (0.01 mg/ml) and DNAse (0.01 mg/ml) was also added and the mixture was

incubated at 30 mins at room temperature. The sample was centrifuged at 10.000 g for 20 mins. The pellet was further resuspended and sonicated in lysis buffer. After another round of centrifugation the pellet was resuspended in buffer without Triton X (50 mM Tris-Hcl, 100 mM Nacl, 5 mM EDTA and 0.1% NaN_3). Samples were run on SDS-PAGE gel and then a representative band was cut from the gel and used to and used to immunize mice for polyclonal antibody production (Yang *et al*., 2011).

2.2.3.7 Determination of the enzymatic activity of recombinant DUBs by cleavage of the fluoregenic substrate Ub-AMC

Ub-AMC is a fluoregenic substrate (figure 11) for DUBs and is made by the conjugation of the C-terminus of Ub with 7 amino-methylcoumarin (AMC), which is a fluorescent labeling reagent (Dang *et al*., 1998). The addition of a DUB to the assay mixture causes the Ub to be cleaved, releasing AMC resulting in a fluorescence increase. The purified his-tagged catalytic domain of *Pfuch-l1*, of *Pfubp-8* and full length *Pfuch-l3* were incubated in DUB buffer made up of 50 mM Tris-Hcl, 150 mM Nacl, pH7.5, 0.1 mg/ml BSA, 1 mM DTT at room temperature pH 7.5 (Artavanis-Tsakonas *et al*., 2011). Recombinant proteins approximately 300 pmol of each recombinant protein were placed in a 384 wells plate and incubated for 30 minutes in the presence of 2 µM of substrate Ub-AMC (Biomol). Cleavage of the substrate by the recombinant proteins was monitored every minute over a period of 30 mins on a spectrophotometer (Molecular devices). The Ub-AMC was also tested alone in the absence of recombinant protein in order to make sure that it did not autofluoresce. The release of AMC was monitored by fluorescence spectrophotometer Spectra Max Gemini (Molecular devices) at excitation 400 nm and emission 505 nm and all assays were carried out in triplicates. In order to confirm the presence of cysteine residues on the active site of DUBs, N-ethylmaleimide NEM an irreversible inhibitor of cysteine proteases commonly used in DUB assays to interfere with DUB activity (Hjerpe *et al*., 2009; Kapuria *et al*., 2010) (Sigma-Aldrich) was also used in this assay.

2.2.3.8 Screening and determination of the IC50 of curcumin on recombinant de-ubiquitylating enzymes (DUBs)

Black flat bottom 96 well plates were incubated with 12.5 µl of curcumin solution, in concentrations ranging from 0.01mM-0.1 mM. To the same plate was added 25 µl of the substrate Ub-AMC in a concentration of 2 µM and 12.5µl of recombinant proteins in DUB buffer made up of 50 mM Tris-Hcl, 150 mM NaCl, pH7.50, 0.1 mg/ml BSA, 1 mM DTT (Artavanis-Tsakonas *et al*., 2011) to give a final volume of 50 µl. Plates were shaken vigorously for 30 seconds and incubated at 25º C for 5 mins and assays were initiated thereafter.

To determine whether curcumin, is a specific inhibitor of *Plasmodium falciparum* DUBs or whether curcumin also displays activity towards human DUBs. In parallel a similar assay was carried out with 100 pmol/µl of Human recombinant ubiquitin protease 2 (USP2) (Biomol) incubated with various concentrations of curcumin ranging from 0.005 mM-0.1mM for each assay a negative control made up of assay buffer, substrate but lacking the enzyme was used.

The release of AMC fluorescence by DUB enzymes was monitored at 400 nm excitation and 505 nm emission wavelengths using a Spectramax Gemini EM fluorescence fluorometer (Molecular Devices).The fluorescence values, were used to determine the IC50 of curcumin for each enzyme was using a log Dose *vs* response curve (Hill slope) GraphPad version 4.0 according to the equation:Y=Bottom+(Top-Bottom)/(1+10^((LogIC50-X)*HillSlope))(APPENDIX F). The result is the mean of 3 independent experiments.

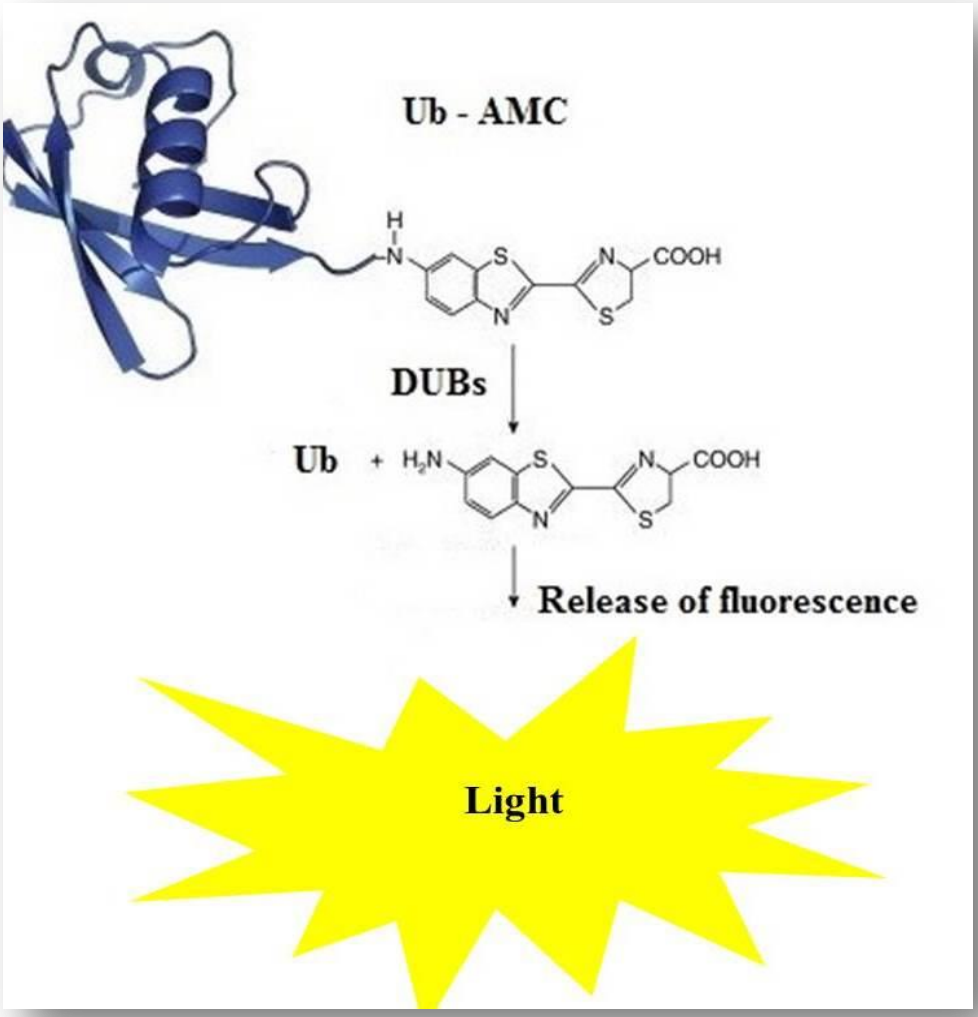


Figure 11. Determination of the enzymatic activity of recombinant DUBs by cleavage of the fluoregenic substrate Ub-AMC. Ub-AMC is a fluoregenic substrate for DUBs and is made up via the conjugation of the C-terminus of Ub with 7 amino-methylcoumarin (AMC) which a fluorescent labeling reagent. The addition of a DUB to the assay mixture causes the Ub to be cleaved, releasing AMC which emits fluorescence which can be measured using a fluorescent spectrophotometer. Adapted and modified from (Dang *et al*., 1998).

2.2.3.9 Immunization procedure for the production of polyclonal antibodies

Five Balb/C male 6 weeks old mice were used for polyclonal antibody production of each protein fragment (*Pfuch-l1*, *Pfuch-l3*, *Pfubp-8*). Mice were immunized with 100 µg/ml of antigen dissolved in 0.5 ml of PBS and 0.5 mg/ml of complete Freund´s adjuvant (CFA) (Sigma-Aldrich) and administered via intraperitoneal to stimulate the immune system (Shimizu *et al*., 2007). For *Pfuch-l54* protein mice were immunized with an emulsion made up of 300 µg/ml of solubilized inclusion body in complete Freund´s adjuvant (0.5 ml) and PBS (0.5 ml). The rest of the protocol was similar.Before immunization, approximately 0.2 ml of pre-immune serum was collected to ensure that mice had no naturally occurring antibodies against *Plasmodium falciparum* DUBs (negative control). The protocol had a total duration of 39 days (APPDENDIX D). Mice received boosters on day 14, day 21 and day 35. Again the antigen was prepared in the same manner, but this time in Incomplete Freund's adjuvant (IFA) which is less toxic than CFA (Sigma-Aldrich) (Marikar *et al*., 2006).Polyclonal antisera were collected 7-14 days after each booster was given, to test by ELISA the detection of antibodies (APPENDIX D). Once sufficient antibody titres were detected by western blot, on day 39 mice received general anesthesia and each mice group were bled for collection of total serum. After the serum samples were incubated for 1 hour at 37º C, the serum was placed at 4º C overnight. On the next day the serum was centrifuged for 5000 g, 10 mins at 4º C and the supernatant was stored in aliquots at -20º C for western blot detection of the proteins in different stages of the parasite´s life cycle.

2.2.3.10 Western blots for the detection of DUBs at different stages of the parasite´s life cycle (ring stage, trophozoite and schizonts)

Proteins were extracted from separate *Plasmodium falciparum* synchronized cultures at a different parasite stages (Rings, trophozoites and schizonts). Parasites were lysed in 0.2% saponin (Simga-Aldrich) for 15 mins, centrifuged at 10.000 g for 5 mins and washed with PBS three times. Parasites were then lysed in a lysis buffer made up of 25 mM Hepes (Sigma-Aldrich), 5 mM EDTA (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), 1 mM DTT (Sigma-Aldrich) and 1 mM cocktail of protease inhibitor (Roche) on ice for 30 min. The obtained lysates were centrifuged at 15.000 g at 4 º C for

10 min and the pellet was discarded. Protein concentration was determined using the Bradford assay (BioRAD). Protein lysate (100µg/ml) was run on 12.5% SDS-PAGE gel and then transferred to PVDF membrane (Thermo Fisher Scientific). Membranes were blocked for 1 hour with 5% non fat dry milk solution. Membranes were then incubated for 2 hours with the polyclonal antibody sera dilutions: *Pfuch-l1* 1:500; *Pfuch-l3* 1:250; *Pfuch-l54* 1:1000 and *Pfubp-8* 1:500 which were found to be the most appropriate diluitons after several optimisations of the western blot.Membranes were subsequently washed and incubated with secondary antibody conjugated to horseradish peroxidase (HRP) 1:2000 dilution and proteins were detected with super signal chemiluminescence kit (Thermo Fisher Scientific). *Plasmodium falciparum* heat shock protein 70 (*Pfhsp70*) which is constitutively expressed in the cytosol (Pesce *et al*., 2008) was used as loading control. Band intensity was measured using BioRAD versa doc Image software (BioRAD) which measures the band intensities of *Pfuch-l1, Pfuch-l3, Pfuch-l54, Pfubp-8* relative to the control reference sample (*Pfhsp70*)(MR4). The intensity value was determined by measuring the density of the band of interest for 500 seconds to display the intensity value. The relative density can be calculated by dividing the densitometric value of the test protein by the densitometric value of the reference sample *Pfhsp70*. The reference sample always has a relative density of 1 allowing a comparison to be made.

2.2.3.11 *Pfuch-l1* protein quantification in response to drug treatment

Recombinant *Pfuch-l1* whose homologue in *P. chabaudi* is putatively involved in artemisinin and artesunate drug resistance (Hunt *et al*., 2007) was analyzed here, in order to verify whether this protein suffers any alteration in abundance in response to drug treatment. Parasites were treated for 18 h, 24 h, 33 h and 48 h with chloroquine, curcumin and artemisinin, the cultures were centrifuged and proteins were extracted from the pellets as previously described in section 2.2.3.10. Proteins (100 µg/ml) were subsequently run on SDS-PAGE page gel 12.5% and then transferred to PVDF membrane (Thermo Fisher Scientific) as before and incubated with polyclonal anti-sera *Pfuch-l1* (1:500) and mouse secondary antibody conjugated to Horseradish peroxidase. HRP (1:2000) and proteins were detected with super signal chemiluminescence kit (Thermo Fisher Scientific).

2.2.4 *In vivo* efficacy of curcumin as antimalarial drug in *Plasmodium chabaudi* parasites, a murine model of malaria

As previously mentioned in the introduction (section 1.1.3.9) curcucmin has anti-cancer,anti-inflammatory,anti-viral and antimalarial activity. After confirmation of antimalarial *in vitro* activity, the *in vivo* activity of this plant derived compound was tested in *Plasmodium chabaudi* clones resistant chloroquine and artemisinin. The assays were carried out in Balb/C mice caged in appropriate conditions. An acute toxicity test was done in order to deterimine the *in vivo* toxicity of curcumin. Curcumin was either tested alone or in association with piperine which has no antimalarial activity (Martinelli *et al*., 2008) but has been reported *in vivo* as an enhancer of curcumin uptake (Suresh and Srinivasan, 2007). Given the emergence of ACT drug resistance, associations of curcumin/piperine/chloroquine and curcumin/piperine/artemisinin were also tested as this information will be relevant to evaluate the potential of curcumin in combination with current antimalarial drugs.

2.2.4.1 Selection of *Plasmodium chabaudi* parasite clones

The *P. chabaudi* clones available in our laboratory and used in this study were AS-3CQ (resistant to chloroquine) (Rosario *et al*., 1976) and AS-ART clone resistant to artemisinin (Afonso *et al*., 2006). The clones displayed a stable phenotype even after freeze/thawing, serial blood passages through mice in the absence or presence of drug treatment and transmission through the mosquito vector *Anopheles stephensi*.

2.2.4.2 Acute toxicity of curcumin

BALB/c male mice 6-7 weeks old were purchased from the animal house facility at the IHMT (Institute of Hygiene & Tropical Medicine, Lisbon, Portugal). The LD50 (lethal dose to achieve 50% inhibition) of curcumin in BALB/c mice was determined by oral administration of the drug to five groups with differents doses (table 11) to each individual mouse after four hours of fasting. Five grams per kilogram of body weight (kg/bw), is the concentration reported by others (Lorke *et al*., 1983; Chandel *et al*., 2012) to be the highest dose known administered to mice for the acute toxicity test of any drug. Animals were observed for 14 days for any physical signs of toxicity including trembling, lethargic behavior and impaired body movements.

Table 11. Acute toxicity for curcumin. Mice were allowed to fast 4 hours. After 4 hours a single dose of Curcumin was orally administered in different concentrations to BALB/c infected mice. Mice were observed for 14 days for any physical signs of toxicity.

LD50 cytotoxicity test	Animals tested	Number of animals tested	Weight (g)
2.0 g of curcumin	Balb/c mice	4	15
2.5 g of curcumin	Balb/c mice	4	15
3.0 g of curcumin	Balb/c mice	4	15
3.5 g of curcumin	Balb/c mice	4	15
5.0 g of curcumin	Balb/c mice	4	15

2.2.4.3 *In vivo* four day suppressive test of curcumin, curcumin/piperine, curcumin/piperine/chloroquine and curcumin/piperine/artemisinin

In the present study the *in vivo* efficacy and the interaction of curcumin/ piperine in combination with artemisinin and chloroquine was assayed using the 4 day suppressive test (Knight and Peters, 1980). Curcumin with 94% cucuminoid content (Sigma - Aldrich) and artemisinin (Sigma-Aldrich) were dissolved in DMSO (Sigma-Aldrich) and Chloroquine (Sigma-Aldrich) was dissolved in water. The parasites kept in liquid

nitrogen were thawed and mice were inoculated with 1×10^6 infected red blood cells. Parasitemia was allowed to evolve and once parasitemia reached 30% infected blood was collected and diluted with citrate saline solution. An intraperitoneal injection of 1×10^6 infected red blood cells was administered to individual mice. Cages contained a maximum of 5 mice each and were kept in a light-dark cycle and mice had food and water *ad libitum*. All animal experiments were carried out according to the guidelines of the animal facility of the Institute of Hygiene and Tropical Medicine (IHMT), Portuguese law and according to the FELASA guidelines.

Three hours after mice were infected with *P. chabaudi* parasites, they were administered by oral gavage chloroquine alone, curcumimin alone, artemisinin alone or the combination of curcumin/piperine/chloroquine and curcumin/piperine/artemisinin. Drugs were administered orally for 4 days (Day 0, 1, 2, 3). Parasitemia was monitored everyday post drug treatment for a period of 7 consecutive days. Thin blood smears were prepared and stained with 20% Giemsa/PBS solution (Sigma-Aldrich) pH 7.2 and microscopic slides were analyzed by light microscopy. The same procedure was carried out in mice infected with AS-ART, resistant parasites clone. All experiments included a drug free control group and were carried out in triplicate.

2.2.4.4 *In vivo* drug interaction studies and Isobolograms

The ED50 values (the concentration of drug that produces 50% reduction of parasitemia) was calculated by plotting the log dose versus relative percentage inhibition using GraphPad Prism 4 Software using non linear regression dose response curve according to the 4 parameter logistic equation (Hill slope). From the ED_{50} and the Hill slope the ED90 values were calculated using the formula: $LogED_{50} = LogED_{90} - (1/HillSlope) \times \log(9)$ and the equation Y = Bottom + (Top-Bottom)/(1+10^(($LogED_{50}$-X) x Hillslope)) (Vivas *et al*., 2007). The ED_{90} were then used to calculate the isobolar equivalent (IE) values (Chawira *et al*., 1987):

$$\text{Isobolar equivalent (IE)} = \frac{ED_{90} \text{ drug combination}}{ED_{90} \text{ drug alone}}$$

Isobolograms were designed to include a diagonal line which represents the line of additivity. Isobolograms allowed the visualization of additivity/synergism or

antagonism. If the IE values are below 1 it produces an isobologram that skews below the additivity line indicating synergism (the total effect is greater than the effect of the individual drug). When the IE values are equal or close to 1 most values will lie closely to the additivity line indicating addivity (the total effect is improved by addition of another drug) (Chawira *et al*., 1987; Vivas *et al*., 2007).If most IE values are above 1 indicates antagonism (the total effect is less when the individual drugs are used together) (Chawira *et al*., 1987; Vivas *et al*., 2007).

2.2.4.5 Statistical analysis

A student T-test and the ANOVA test were used for statistical analysis using GraphPad Prism 4 software.

2.2.5 A proteomics (2DE) approach for the identification of *Plasmodium falciparum* schizont stage proteins altered in response to curcumin treatment

Two dimensional electrophoresis (2DE) is a widely used technique that allows a quick snapshot of the proteome at a specific time under specific conditions. In this part of the project the aim was to analyze the parasite´s response to curcumin treatment in order to understand what are the intracellular changes occurring inside the parasite in response to curcumin treatment and gain a glimpse of the potential pathways or targets affected by curcumim. A group of cultures was allowed to grow without any drug treatment whereas the other group was allowed to grow in the presence of curcumin. Parasites were centrifuged the supernatant was discarded and the red blood cell pellet (RBC) was washed and lysed with lysis buffer for protein extraction. Protein samples were labeled with specific 2DE dyes, namely Cy2, Cy3 and Cy5 dyes allowing the protein to be quantified through the release of fluorescence. The mixture of labeled proteins was separated by 2DE and images were acquired using a scanner typhoon (GE Healthcare). Images were cropped with IamgeQuant software and DeCyder software (GE Healthcare). After image acquisition protein spots were manually digested with trypsin and analyzed by mass spectrometry. Peptides belonging to the parasite were then identified using appropriate databases (APPENDIX I).

2.2.5.1 Preparation of *Plasmodium falciparum* parasites for proteomic analysis

Plasmodium falciparum parasites 3D7 at the schizont stage were cultured in RPMI as described before in section 2.2.1.1. Schizont stage parasites were chosen as curcumin is known to act at the trophozoite and schizont stage (Mimche *et al*., 2011) and schizonts occupies most of the red blood cell, therefore more proteins belonging to the parasite can be extracted at this stage (Artavanis-Tsakonas *et al*., 2006; Prieto *et al*., 2011). Parasites were incubated for 8 hours with the concentration corresponding to the IC50 value of curcumin (5 µM). Control and treated groups consisted of three biological replicates. Thin smears of the parasites untreated and submitted to curcumin treatment were prepared every 4 hours to ensure that the parasites were not dying and that proteins altered were due to curcumin treatment and not due to parasite death. After the incubation period parasites were lysed with 0.2% saponin (Simga-Aldrich) for 5 mins,

centrifuged at 10.000 g for 5 mins, washed with PBS three times followed by three washes with 10 mM Tris-HCl pH 7.4 to remove excess β-haematin. Samples were then lysed with lysis buffer (7 M Urea, 30 mM Tris pH 8.5, 1% Triton X, 1 mM PMSF, 2% CHAPS (cholamidopropyl dimethylamonio-1-propanesulfone) (Prieto *et al*., 2008) for 30 mins on ice with vigorous shaking. This was followed by three cycles of freezing and thawing 5 minutes at 80º C and 5 minutes at 37º C. After this period, the samples were centrifuged and supernatant containing proteins was collected into a fresh tube and the pellet at the bottom of the tube was also kept for DIGE analysis. Lipids and other contaminants were removed by treating protein samples with 100% acetone and tubes were kept at -80º C for 10 mins and then placed overnight at -20º C. Next day samples were centrifuged for 15 mins at 4º C acetone was removed by pipetting and remainder evaporated by air drying the samples. Samples were eluted in lysis buffer and proteins were quantified using the Bradford assay (BioRad).

2.2.5.2 2D-DIGE and Protein labeling

Pooled protein samples (300 µg) were used for each DIGE experiment. Control samples were labeled with 400 pmol of Cy5 and curcumin treated samples were labeled with 400 pmol of Cy3 dye (figure 12). The internal standard sample (IS) was a pooled sample that contains a mixture of the (control and the treated sample) usually used in 2DE to avoid gel to gel variability (Varga, 2004) was labeled with Cy2. Three biological replicates from each group of samples control, soluble, membrane samples were used for the DIGE experiment including a dye swap which minimizes experimental bias (Briolant *et al*., 2010).All the cyanine dyes were reconstituted and dissolved in N, N-dimethylformamide (DMF). For each 50 µg of proteins used 400 pmol, of CyDyes in 1 µl of DMF was used. After 30 min of incubation on ice in the dark, the reaction was quenched with 10 mM lysine for 10 min on ice. Cy3, Cy5, Cy2 labeled samples were then pooled and 2X sample IPG buffer pH gradrient 3-10 soluble samples, IPG buffer pH gradient 4-7 and IPG buffer pH gradient 6-11 for insoluble samples was added to each tube. IPG strips were rehydrated with 7M urea, 2M thiourea, 4% CHAPS, 2% IPGP buffer for the loading process. The mixture of labeled proteins was then separated by two dimensional gel electrophoresis (2DE) which separates proteins based on their molecular weight (MW) and isoelectric point (PI).

2.2.5.3 2DE Image acquisition and analysis

Isolectrofocusing was performed at 20º C using the following program: 120 V for 1 h; 500 V for 2 h; 500-1000 V in gradient for 2 h; 1000 – 5000 V in gradient for 6 and 5000 V for 10 h (APPENDIX G). After that the strips were equilibrated for second dimension separation first in a solution made up of 6 M Urea, 50 mM Tris-HCL pH 6.8, 30% glycerol, 2% SDS and 2% DTT. This was followed by equilibration in an alkylating solution similar as before but with 2% iodoacetamide. Second-dimension SDS-PAGE separation was performed on 12.5% polyacrylamide gels overnight at 18º C. Images was acquired using a Typhoon 9400 scanner (Amersham Biosciences). The wavelengths for the dyes were:

Cy3 green	532 nm excitation	580 nm emission
Cy5 red	633 nm excitation	670 nm emission
Cy2 blue	488 nm excitation	520 nm emission

Images were acquired with a 100 μm pixel size, were cropped with Image Quant software and analyzed using DeCyder version 6.5 (GE, Healthcare) a differential analysis software for 2DE gels, used for gel alignment and spot averaging.

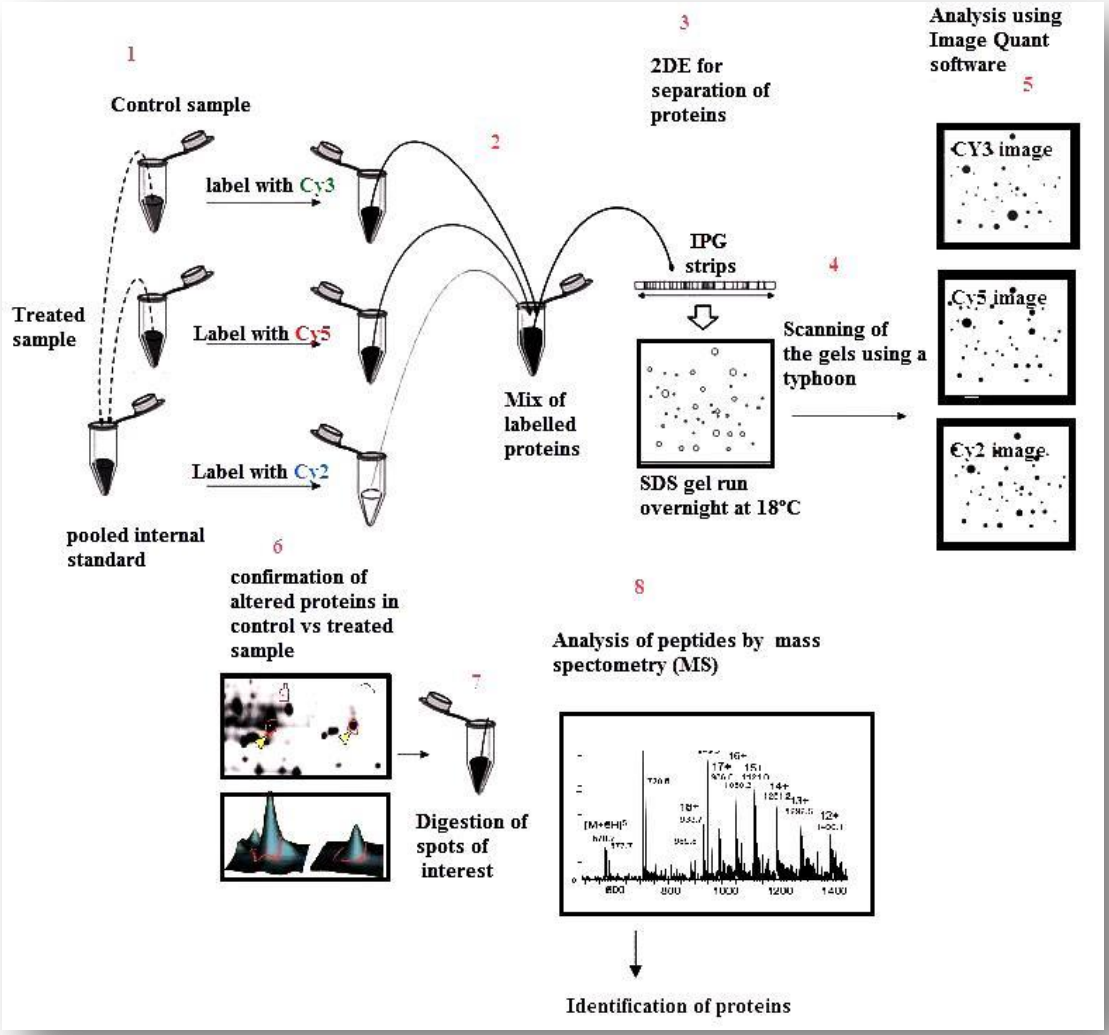


Figure 12. A simple overview of the 2DE gel electrophoresis process. Proteins from the control and proteins from the *P. falciparum* treated sample are individually labeled with the cyanine dyes Cy3 and Cy5 **(1)**. An aliquot from each tube is placed in a new tube which will be the internal standard which will be labeled with Cy2 **(2).** All samples including the internal standard are combined in a unique tube and run on the IPG strips and separated on SDS gel **(3)**. The gel is the scanned and gel images are acquired using a fluorescent scanner in order to visualize differences in the treated vs the control sample **(4)(5).** Spots of interest were digested with trypsin **(6).** The resulting peptides are analyzed **(7)** by mass spectrometry **(8)** followed by protein identification using appropriate databases. Adapted and modified from (Rozanas and Loyland, 2008).

2.2.5.4 Trypsin digestion of spots of interest

After imaging, the gels were stained with Comassie brilliant blue G-250 (Sigma-Aldrich) and spots of interest were manually cut (Briolant *et al*., 2010). Protein spots were digested (figure 12) overnight at 37º C with trypsin (Sigma-Aldrich). The peptides of interest were dehydrated in a solution of acetone nitrile and incubated with 5% formic acid for 10 mins under shaking. Samples were once again dehydrated in acetonenitrile and completely dried until no acetone could be observed in the tubes. Samples were then analyzed by mass spectrometry (MS).

2.2.5.5 Bioinformatics and protein database analysis

For protein identification, peptide sequences were entered into a database known as Mascot. Search parameters allowed for one missed tryptic cleavage, the carbamidomethylation of cysteine and the possible oxidations of methionine, the precursor and product mass error was < 0.2 Da. All identified peptides that had a Mascot score of 70 or above were considered significant meaning that a Mascot score greater than 70 indicaties that the protein identified by mass spectrometry matches those protein sequences found in the databases.

NCBI GI (gene identifier) and PlasmoDB (APPENDIX I) were used to confirm the proteins identified. The database PANTHER (protein analysis through evolutionary relationships) part Gene Ontology Project (GO) which facilitates the identification of proteins across different database. PANTHER was used to assign proteins according to their molecular function; biological process, which refers to the network or the wider process in which the protein is involve; family, referring to the group of proteins which are evolutionary related; and pathway (referring to potential proteins that may interact with the identified protein). A student t test and ANOVA test was used to determine which protein spots changed in abundance in response to curcumin treatment. The number of spots showing a difference with a P-value > 0.005 was determined. The cut off point was set as < 0.55 was applied for proteins considered as downregulated and > 1.50 for proteins considered as upregulated.

CHAPTER 3-RESULTS & DISCUSSION

3.1 Expression profile of genes encoding DUBs in *Plasmodium falciparum* strains 3D7 and Dd2 and detection of protein abundance in different stages of the parasite´s life cycle

The gene expression profile of DUBs in *P. falciparum* was carried out by collecting blood samples at different time points as fully described in chapter 2 (2.2.1.1 -2.2.1.5) and analyzing the expression of the genes at the ring, trophozoite and schizont stage in the absence and presence of drugs, then polyclonal antibodies were raised in mice and the anti-sera were used for western blot detection of DUB abundance at the intraerythrocytic stages of the parasite´s life cycle. The results are presented and discussed simultaneously and are presented here in tables and in bar graphs with error bars. A T test was applied to analyze the results of the gene expression N fold at given time point, always relative to the control sample and P values < 0.05 were considered as statistically significant.

3.1.1 Determination of the *in vitro* IC50 of artemisinin, chloroquine and curcumin

The IC50 of chloroquine in *P. falciparum* 3D7 strain was 12nM and Dd2 was 290nM those values are in agreement with previous data obtained by others (Akoachere *et al*., 2005) and for curcumin the IC50 was much higher at 5µM for 3D7 and 5.5µM for Dd2 which is in agreement with previous work (Reddy *et al*., 2005) which indicates that a large quantity of curcumin is necessary just to achieve 50% of the inhibitory concentration. The *in vitro* antimalarial activity of curcumin was confirmed in the present study and it did not differ between *Plasmodium falciparum* sensitive and resistant strains. Others have obtained IC50 values for a curcumin like compound (licochalcone A) of 3.21µM for Chloroquine sensitive strain 3D7 and 4.21µM for chloroquine resistant strain RKL 303 (Mishra *et al*., 2008). This variation in results may reflect the diferencies associated with the method used to carry out IC50 assays. For artemisinin the IC50 value determined for this study was 3D7 was 4nM and for Dd2 was 5.3nM which is also in agreement with previous work (Akoachere *et al*., 2005). In the present sudy the *in vitro* cytoxicity test showed that curcumin and chloroquine seemed to be slightly toxic to HepG2 cell line with a selectivity index of 1.3 for 3D7, 0.05 for Dd2 (SI) and 1.4 for 3D7 strain and 1.27 for Dd2 strain (SI) (Table 12) which was already reported by others (Jassabi *et al*., 2011). The cytoxicity values for artemisinin are in agreement with previous data which indicate that artemisinin is non toxic to HepG2 cell line, whereas small doses of chloroquine and and curcumin are enough to produce a cytotoxic effect on HepG2 cells, perhaps an indication that HepG2

cell line is very sensitive to both chloroquine and curcumin (table12) (Chaijaroenkul *et al*., 2011). A summary of results is also present in (APPENDIX E).

Table 12. Determination of the *in vitro* IC50 of artemisinin, chloroquine and curcumin.

Drugs	IC50 3D7 (nM)	IC50Dd2 (nM)	Cytoxicity (HepG2) µg/ml	Selectivity Index (SI) 3D7	Selectivity Index(SI) Dd2
Artemisinin	4 ± 2.0	6.3 ± 1.9	250	62.5	39.6
Chloroquine	12 ± 1.5	290 ±0.5	16	1.3	0.05
Curcumin	5000 ± 0.8	5500 ±0.8	7	1.4	1.27

3.1.2 Expression profile of gene *pfuch-l1* in *Plasmodium falciparum* strains

With regards to the basal gene expression pattern observed in the absence of drugs, in general genes encoding DUBs are being expressed throughout the parasite´s life cycle, with some genes showing a differential pattern in their expression as the parasite transitions from the ring stage to the trophozoite stage and from trophozoite to schizont stage (figure 13).

Basal gene expression levels of *pfuch-l1* are very steady between 0h-18h (figure 14) and at this stage the parasites are mainly evolving from ring stage to mature trophozoites (figure 13, figure 14, APPENDIX C-table 1). The western blot confirms this result and indicates that this protein is actively translated and is more abundant at the ring and trophozoite stages (figure 15A). Between 21h-33h the expression of this gene begins to reduce and remains low which coincides with the transition of the parasite from early to late schizont stage (figure 13), indicating that this gene product may not be necessary at this stage and the western blot confirms that there is indeed less protein abundance at this stage (figure 15 A). The expression of this gene goes up again between 36h-48h hours when the schizont contains merozoites and is ready to burst releasing the merozoites which will invade new red blood cells (figure 14). Those periodic variations in the expression pattern of this gene indicates that *pfuch-l1* gene product may be necessary for the parasite at the ring, early trophozoite to mature trophozoite and

merozoite stages of the parasite. This is in agreement with a study of the transcriptome of the *P. falciparum* parasite which showed that transition of the parasite from young trophozoite to mature trophozoite coincides with the induction of genes involved in transcription/translation, metabolic synthesis, DNA replication and protein degradation (Bozdech *et al*., 2003). The results also indicate that the UPS is active and is important at the merozoite stage which is in agreement with previous studies of the parasite which are responsible for parasite re-invasion (Ponts *et al*., 2011).

When exposed to the IC50 of drugs artemisinin, chloroquine and curcumin a general transient increase in the expression pattern was observed. Here the IC50 previously determined above, was used as previous studies have shown that doses lower than the IC50 do not show any alterations in the gene expression pattern whereas higher doses quickly arrest and kill the parasite, allowing no conclusions to be drawn (Kritsiriwuthinan *et al*., 2011). In the present study treatment of parasites with artemisinin caused an increase in the expression of *pfuch-l1* gene which was more pronounced between 15h-33h where the N fold went up gradually to 2.50, the expression of this gene appeared to decrease and returned to normal basal levels 48 hours later (figure 14, APPENDIX C-table 1). Gene expression study led us to analyze whether the increase observed at the mRNA level was also happening at the protein level, given that the same protein is mutated in *P. chabaudi* parasites resistant to artemisinin and artesunate (Hunt *et al*., 2007) a western blot was performed on samples collected between the 18h-48h time points in order to verify whether alterations were also occurring at the protein level (figure 15B).

When *Plasmodium falciparum* parasites 3D7 and Dd2 were subjected to artemisinin treatment and the proteins analyzed by western blot, an increase in band intensity was observed between the 18h-33h time points. With the intensity of the band decreasing between the 33h-48h (figure 15B) indicating that the changes induced by drug treatment are indeed transient. An analysis of this gene was performed after exposure to drug treatment with chloroquine there was a gradual increase in the expression of this gene (figure 14). This increase began 9h hours after drug exposure and reached a peak between 21h-33h where the N fold increased by 2 fold relative to the control sample ($P < 0.03$). In these samples an analysis of the protein bands indicated a strong band

intensity between the 24h-33h time points with the 48h time point displaying less band intensity. This is in agreement with previous studies carried out by others which have shown that alterations caused by chloroquine treatment are only observed more than 6h hours after drug exposure (Gunasekera *et al*., 2003; Kritsiriwuthinan *et al*., 2011).

Treatment with curcumin also induced an increase in the expression of *pfuch-l1*. Significant changes were observed after 3 hours of drug exposure where the N fold was 1.22 and continued to increase up to 2.50 fold 33 hours after drug treatment relative to the control sample (P = 0.001) (figure 14). After which there is a gradual decrease in the expression of the gene under study returning to normal levels. The changes at the mRNA level were also reflected at the protein level (figure 15B). Although individual bands were not quantified due to time constraints, nevertheless, the bands indicate a more intense signal between 18h-33h time points (figure 14, figure 15B) confirming that curcumin is more active against the trophozoite and schizont stages of the parasite. Most of the significant changes seem to last up to 30 hours indicating that drug exposure for more than 33 hours probably induces cell cycle arrest, hence the parasites may no longer be responding well to the drug treatment as they did at the beginning of the study. This is not the first study in which components of the UPS are altered in response to drug treatment.

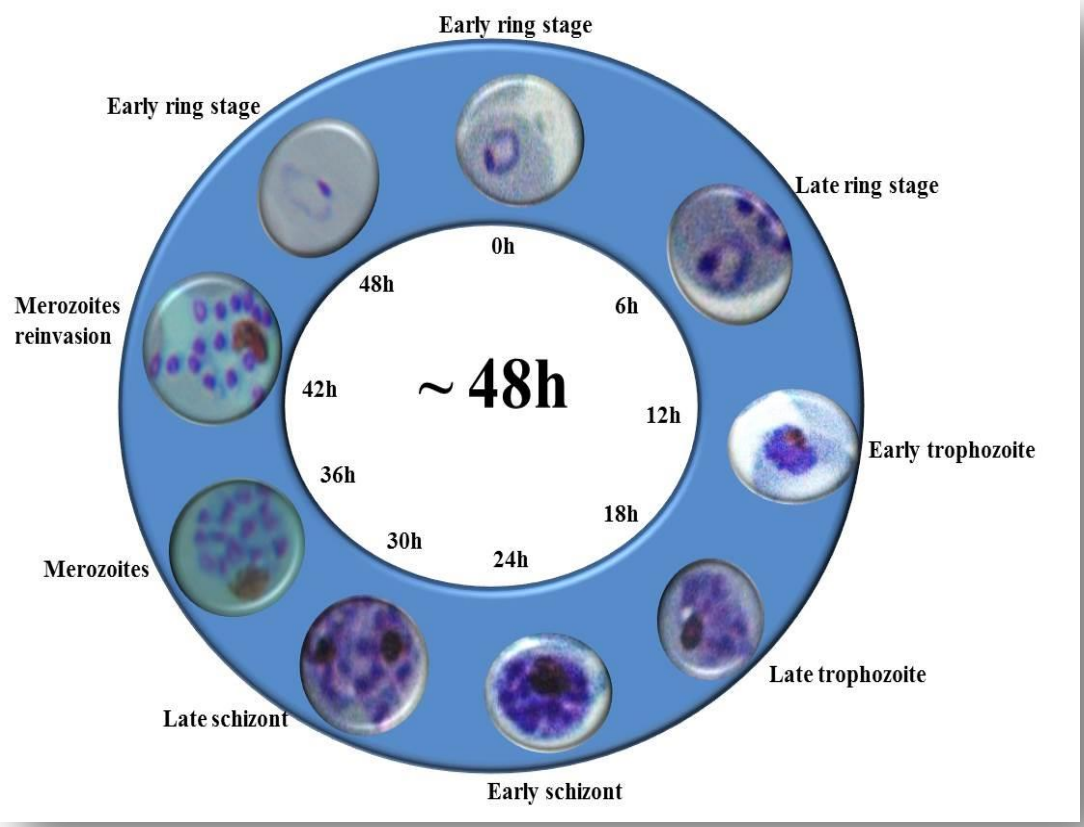


Figure 13. A simple representation of *Plasmodium falciparum* life cycle. Samples were taken every 6 hours and stained with Giemsa and parasites were visualized using a light microscope in order to follow parasite development and evaluate any morphological changes observed in the parasites throughout the gene expression study.

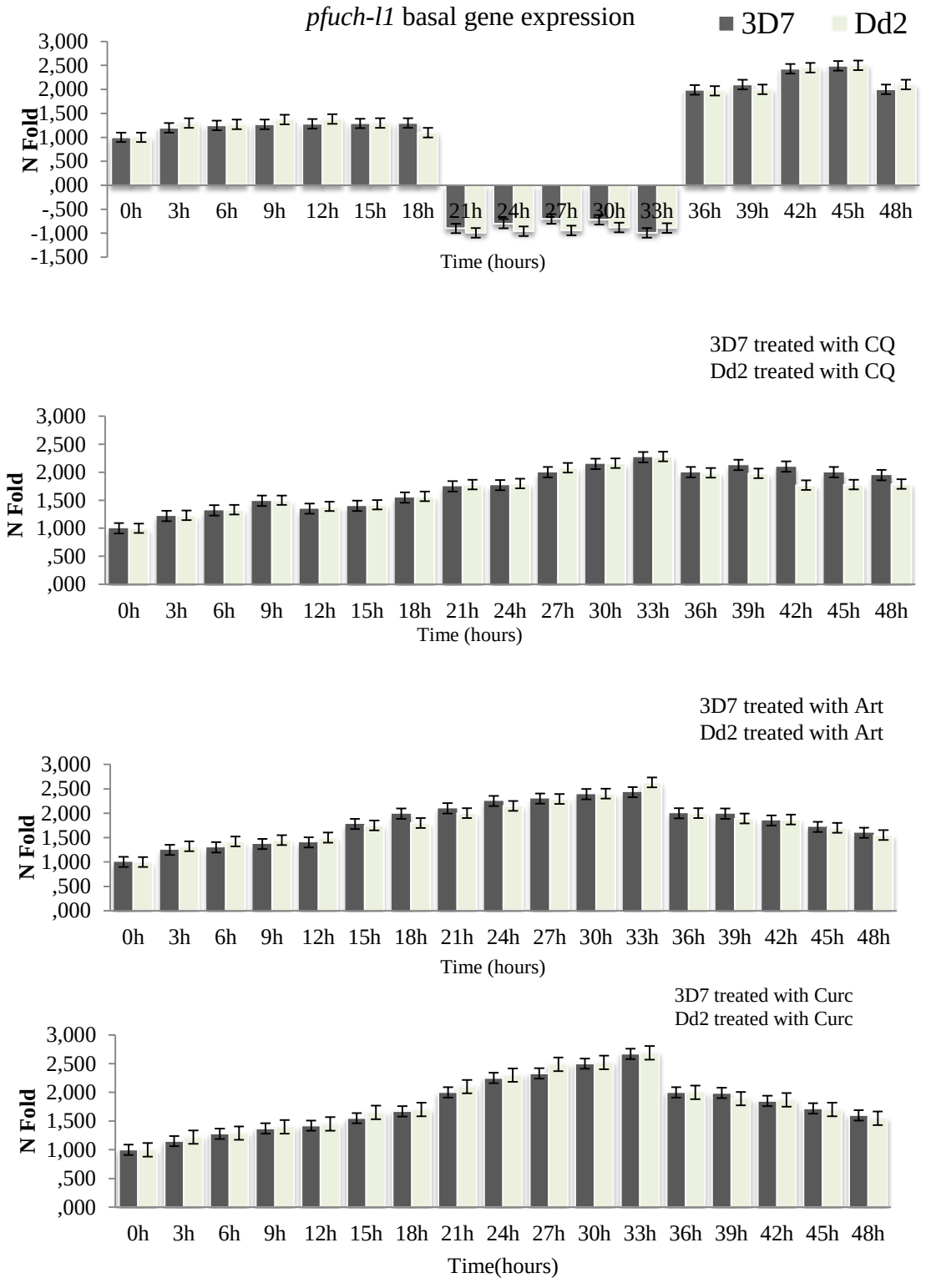


Figure 14. Expression profile of gene *pfuch-l1* in the absence and in the presence of drug treatment in *Plasmodium falciparum* clones 3D7 and Dd2 (n = 3 experiments)(P value < 0.05) is considered as significant.

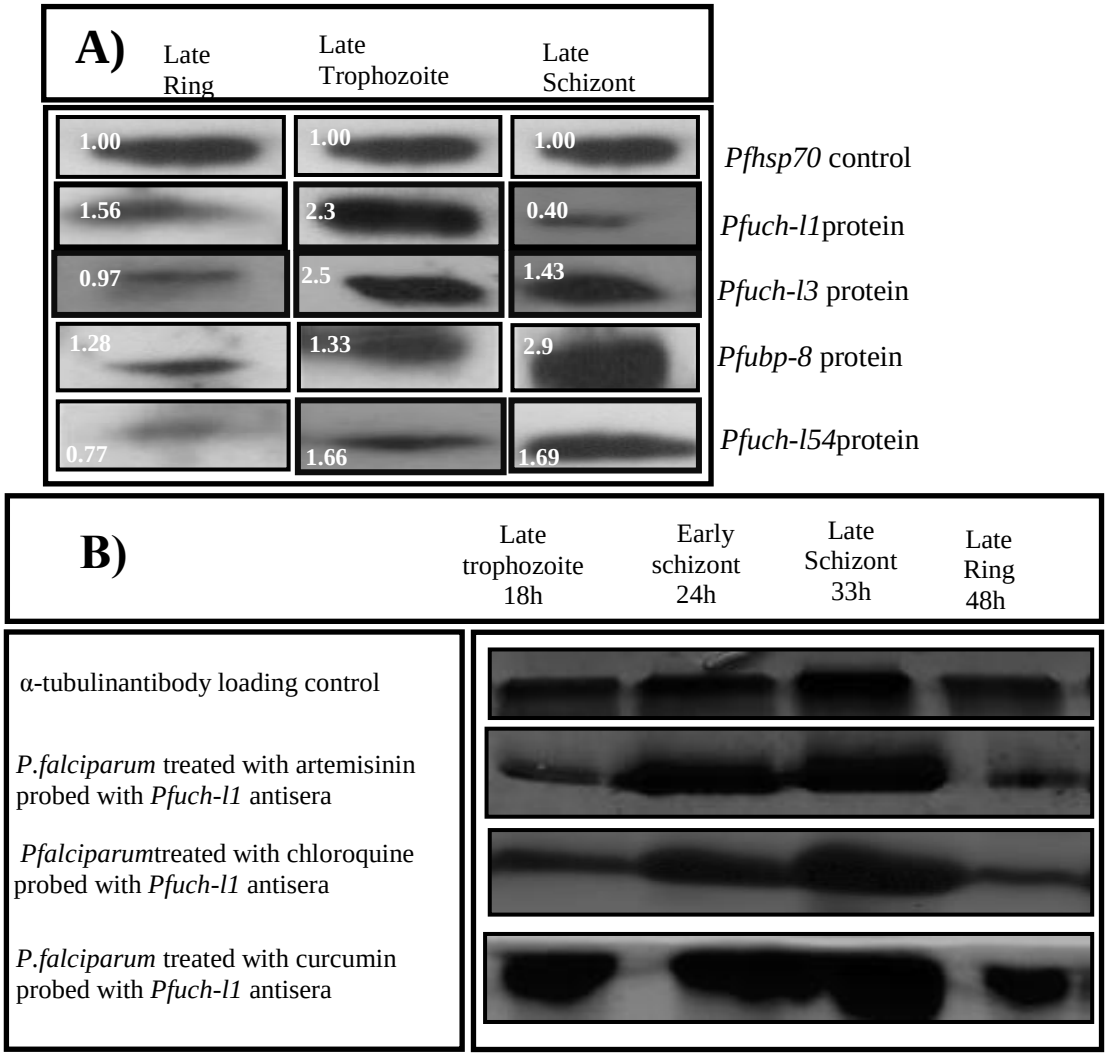


Figure 15. Confirmation of protein abundance at ring trophozoite and schizont stage parasite lysates and parasite response to drug treatment. **A**) Parasite lysates were run on a 12.5% SDS - PAGE gel and western Blot probed with anti *Pfuch-l1*, *Pfuch-l3*, *Pfuch-l54* and *Pfubp-8* polyclonal antisera raised in mice and bands detected by chemiluminescence and band intensity was measured by BioRAD Versa Doc image software. **B**) Representative samples of *P. falciparum* cultures subjected to drugs and collected between 18h-48h time points were used for western blot analysis with *Pfuch-l1* antisera to verify protein abundance in response to drug treatment.

3.3.3 Expression profile of gene *pfuch-l3* in *Plasmodium falciparum*

Pfuch-l3 gene seems to be expressed throughout the parasite´s life cycle and a peak in N fold expression was observed between 24h-36 h when the parasite has transitioned from early schizont to mature schizont stage, this was also confirmed by the western blot (figure 15A) which indicates that the protein is being translated and is more abundant at the trophozoite and schizont stages (figure 15A). The gene expression levels gradually decreased between 42h-48h hours (figure 16) (APPENDIX C-table 1) as the parasite enters the ring stage. Indicating that this gene product although expressed throughout the parasite´s life cycle, is probably needed by the parasite only at the trophozoite and schizont stages.

With regards to treatment with artemisinin the general trend observed was an increase in expression between 9h-36h where the N fold increased from 2 to 2.27 relative to the control sample where the N fold was 1 (P=0.03). After 36 h of drug treatment, expression levels begin to go down (figure 16) indicating that the increase in gene expression was transient. In response to treatment with chloroquine (figure 16) this gene also increased its expression starting at 9h after drug exposure all the way to 33h relative to the control N fold was 1.00(P=0.02). From 33h time point the gene expression gradually begin to go down and returned to basal levels. *Pfuch-l3* also responded to treatment with curcumin (figure 16) a drug which is known to interfere with the UPS (Si *et al*., 2008) this increase was evident from 9h-33h relative to the control sample where the N fold was 1.00 (P =0.01).

The gene expression levels achieved a maximum N fold of 2.38, 33h after drug exposure, from there onwards expression gradually decreasing to basal levels (APPENDIX C-table 1). It is not clear yet what the function and specific interacting partners of *Pfuch-l3* protein are. Mouse *uch-l3* is known to regulate epithelial sodium channels (Butterworth *et al*., 2007). *Pfuch-l3* protein can interact with Ub and Nedd8 a Ub like molecule (Frickel *et al*., 2007) and both molecules are expressed throughout the parasite´s life cycle. Meaning that *Pfuch-l3* protein can de-ubiquitylate as well as de-neddylate target proteins. Clearly the gene expression study results shown here indicate that this gene may be relevant for the parasite.

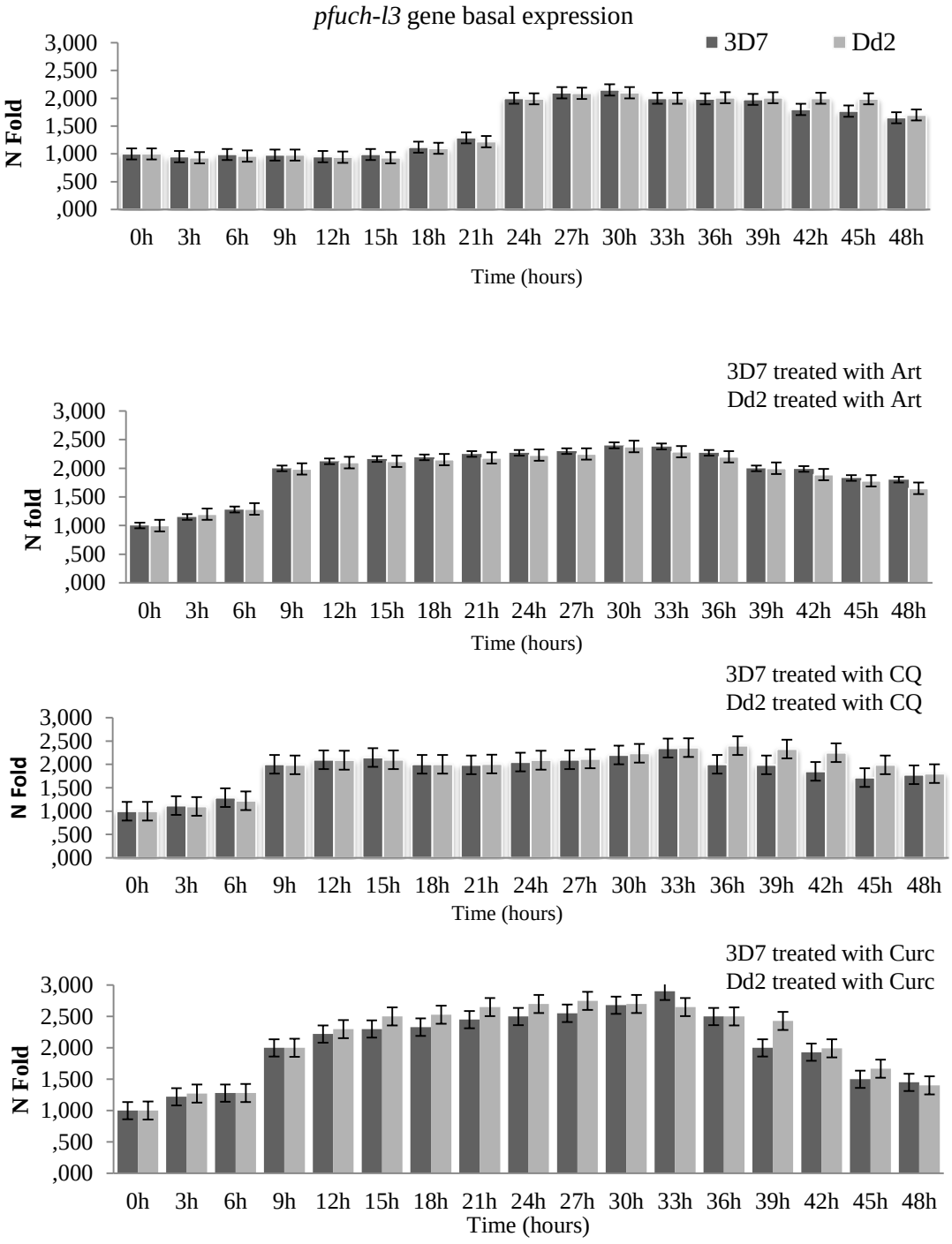


Figure 16. Expression profile of gene *pfuch-l3* in the absence and in the presence of drug treatment in *Plasmodium falciparum* clones 3D7 and Dd2 (n= 3 experiments) (P value < 0.05) is considered as significant.

3.3.4 Expression profile of gene *pfuch-l54* in *Plasmodium falciparum*

The expression of *pfuchl-54* gene is very low at the early stages of parasite development when the parasite is mostly at the ring stage when there is little metabolic cellular activity (Ponts *et al*., 2011). This result can also be confirmed by low protein abundance at this stage (figure 15 A). Between 18h-39h the N fold increases gradually reaching 2.50 relative to the control sample (P=0.01) again which coincides with a more mature phase of the parasite (late trophozoite to late schizont with merozoites) and a gradual decrease is observed between 39h- 48h (figure 17, APPENDIX C-table 1) at this stage the schizonts have burst releasing merozoites which will reinvade new blood cells.

This is a very interesting result as human homologue of *Pfuch-l54* protein and *Trichinella spiralis* (TsUCH37) protein have been shown to be associated with the proteasome (Sowa *et al*., 2009; White *et al*., 2011). Studies in the past have shown that not all Dubs are located in the cytosol, some DUBs are associated with subunits of the proteasome lid such as human USP14, RPN11 proteins (Lee *et al*., 2011) possibly ensuring that all Ub molecules have been removed before the protein is committed to destruction. Since there is little cellular activity at the ring stage of the parasite the rate of protein turnover would be very low hence it is understandable that *Pfuch-l54* protein would only be expressed at the trophozoite and schizont stages of the parasite, when metabolic activity occurring inside the parasite is at its highest and is often accompanied by several rounds of DNA replication (Ponts *et al*., 2011).

This is more evident, when the parasites are exposed to drug treatment. Treatment of parasites with artemisinin which acts against all stages of the parasite increased the expression of *pfuchl-54* gene (figure 17, APPENDIX C-table 2) three hours after drug treatment and continued to increase up to 36 h relative to the control sample collected at time point 0h (P = 00.01). Expression levels gradually decreased from 36h time point onwards. *Pfuchl-54* gene also responded to treatment with the schizonticidal chloroquine (figure 17, APPENDIX C-table 3). The increase was more pronounced up to 36h (P=0.01) when the parasite is a late schizont with merozoites. Expression levels decreased from 42 h time point onwards. Treatment with curcumin which is known to act on trophozoites and schizonts (Mimche *et al*., 2011) induced an increase in the expression of this gene, with expression levels increasing steadily up to 36h N fold was

2.60 when the parasite has reached a mature schizont stage, after which a general decrease is observed (APPENDIX C-table 2, 3,4).

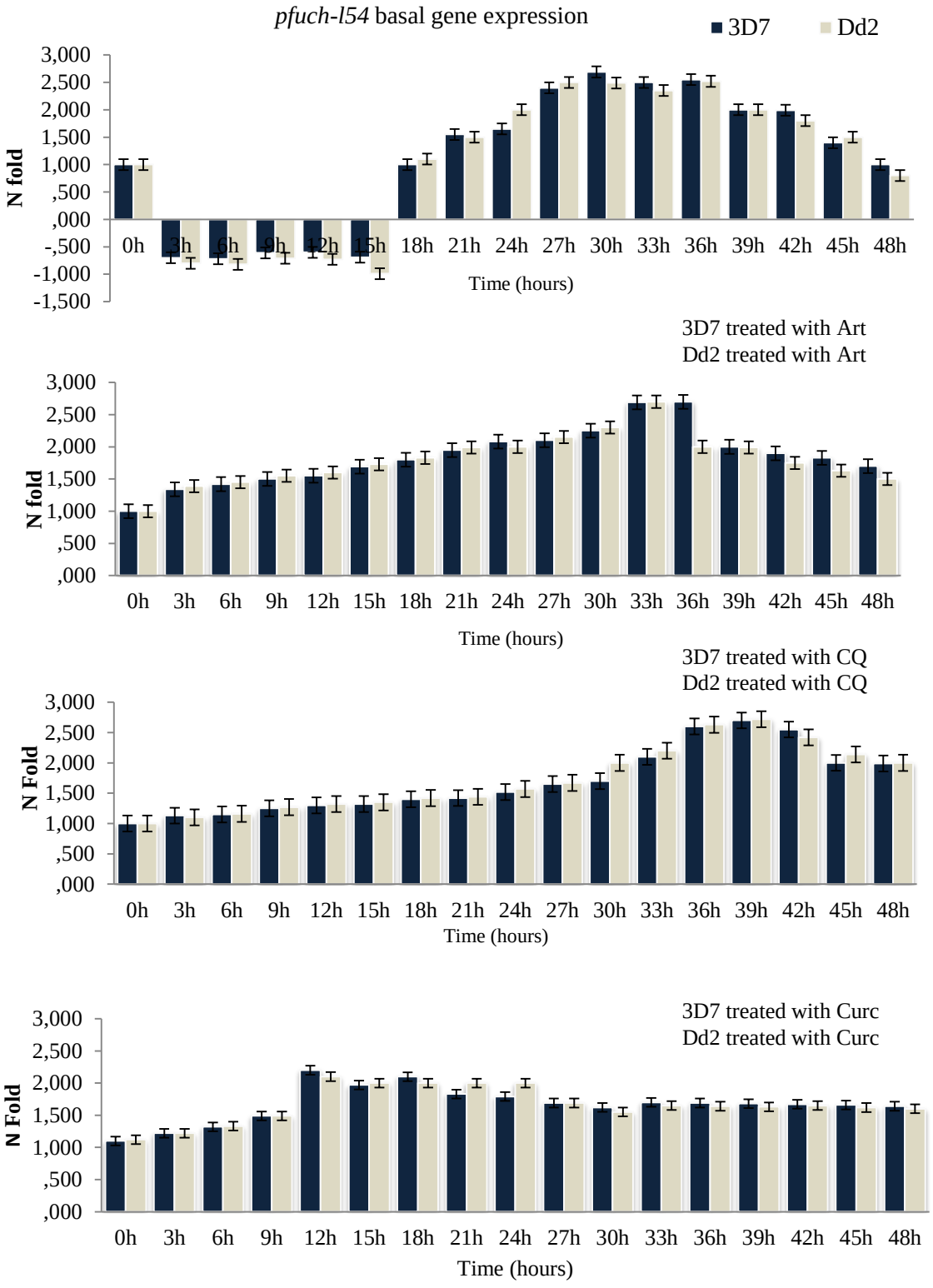


Figure 17. Expression profile of gene *pfuch-l54* in the absence and in the presence of drug treatment in *Plasmodium falciparum* clones 3D7 and Dd2 (n= 3 experiments) (P value < 0.05) is considered as significant.

3.3.5 Expression profile of gene *pfubp-8* in *Plasmodium falciparum*

Pfubp-8 basal gene expression seems to be expressed throughout the parasite´s life cycle with a steady state level being observed with no major increase or decrease in its expression profile pattern throughout the 48 hour period. The western blot also confirms this finding showing evidence of protein existence at the three intraerythrocytic stages (figure 15A). It is not clear what is the role of *pfubp-8* in *Plasmodium spp*, but in the yeast *Saccharomyces cerevisiae* it is responsible for the de-ubiquitylation of histone H2B an enzyme involved in chromatin remodeling (Henry *et al*., 2003).

During the schizont stage parasite undergoes several rounds of DNA replication and DNA repair which requires the use of several histones involved in chromatin remodeling and packaging. Histones themselves undergo post translational modifications such acetylation and ubiquitylation (Cui *et al*., 2007). Assuming that *pfubp-8* is indeed involved in the regulation of transcription, it is likely that it will be expressed constitutively in order to de-ubiquitylate its target proteins (figure 18)(APPENDIXC).

Exposure to artemisinin (figure18) induced an increase in the expression levels of this gene, which was evident from 3h-36h relative to the control sample (P=0.001). Treatment with chloroquine caused an increase in the expression of this gene which was more evident at 30h after drug exposure and treatment with curcumin also induced an increase which was evident from 3h-30h relative to the control sample (P=0.001). From the 30h time point onwards expression levels begin to go down returning to basal levels.

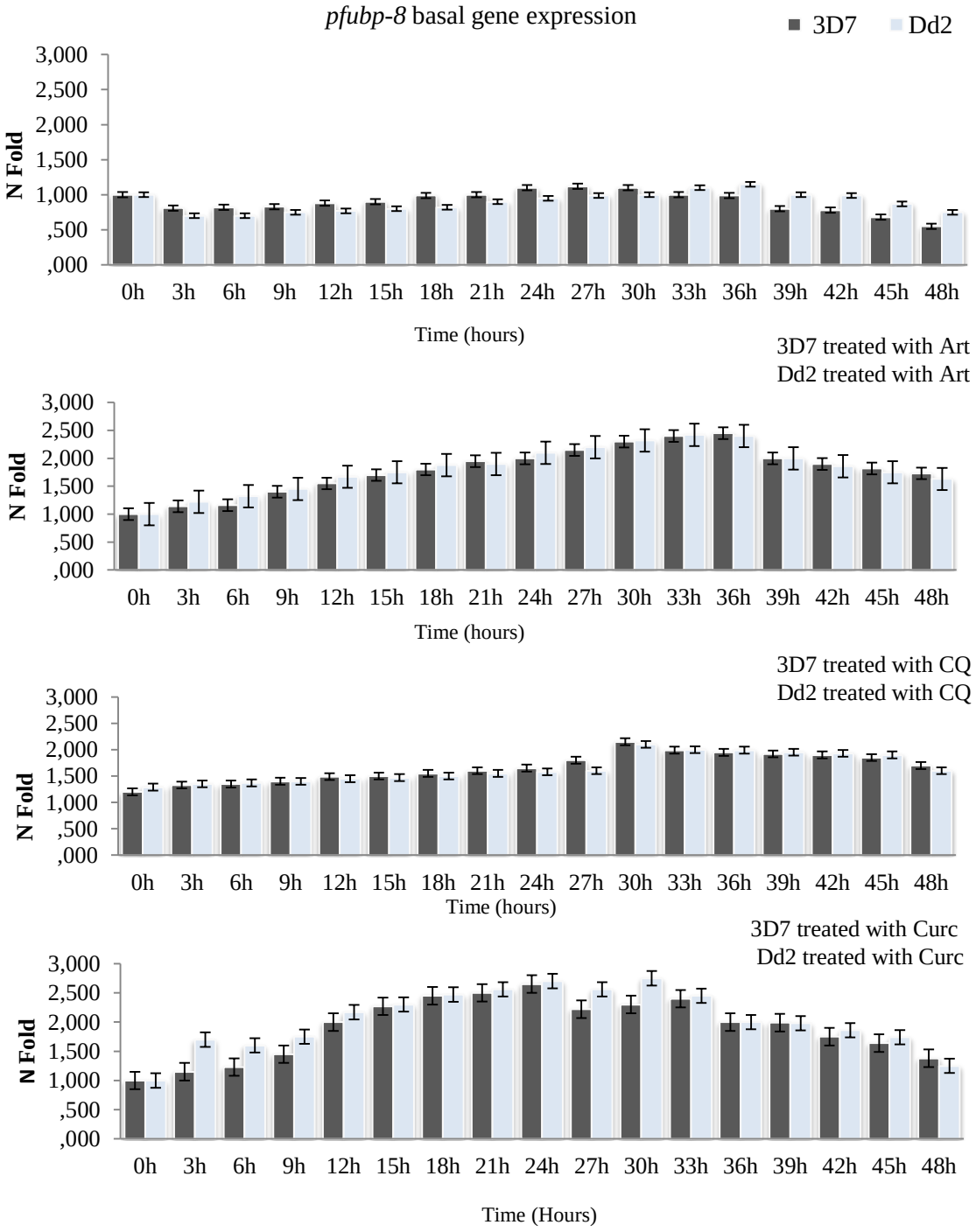


Figure 18. Expression profile of gene *pfubp-8* in the absence and in the presence of drug treatment in *Plasmodium falciparum* clones 3D7 and Dd2 (n= 3 experiments) (P value < 0.05) is considered as significant.

Work done in the past has shown that the activity of DUBs can be induced by DUB drug treatment. Examples from previous publications have shown that the activity of human DUB known as DUB-3 can be induced by interleukin (IL-4) and (IL-5) stimulation (Burrows *et al*., 2004). Another study had already established that *Plasmodium* parasites subjected to heat shock showed an increase in the mRNA levels of the gene encoding Ub *(pfUB)* and also an increase in ubiquitylated proteins (Horrocks and Newbold, 2000). This was further confirmed by a study carried out in 2006 in which cells treated with heat, there was an accumulation of ubiquitylated proteins inside the cell, these proteins must be rapidly polyubiquitylated so that they can be degraded by the proteasome (Dantuma *et al*., 2006).This type of induced cellular stress tends to increase the cell´s need for free Ub and increase the activity of the DUB enzymes responsible for the generation of free Ub molecules (Dantuma *et al*., 2006). A study performed using SAGE (Serial analyses of gene expression) technique, has shown that in chloroquine treated *P. falciparum* cultures there was a 5.5 fold increase in a gene encoding an ubiquitin specific protease and a 5.5 fold increase in a gene encoding a proteosome subunit α, indicating that the UPS is probably one of the key pathways used by the parasite in response to drug and other types of stress response (Gunasekera *et al*., 2003).

Therefore, it can be suggested that treatment of parasites with artemisinin, chloroquine or curcumin induced alterations in the expression of the genes under study, indicating that drug treatment is likely to interfere with all ubiquitin dependent processes such as DNA replication, cell cycle control, ribosome function, and post replicative DNA repair, transcriptional regulation and many others (Ponder and Bogyo, 2007; Ponts *et al*., 2008). Hence in the presence of drugs it is likely that the activity of DUBs will increase in order to compensate for all the intracellular damage caused by drug treatment. It is likely that drug treatment causes an accumulation of damaged proteins inside the cell resulting in a "proteotoxic environment" (Mullaly and FitzPatrick 2002), which the parasite must quickly get rid of. In terms of drug action artemisinin, curcumin and chloroquine are known to have a pleiotropic effect meaning that they interfere with several targets at the same time (Cui *et al*., 2007; Cooper and Magwere, 2008; Mimche *et al*., 2011).

Artemisinin is known to induce oxadivative stress via the formation of free radicals or it can also be involved in the alkylation of lipids, proteins and haem (Mok *et al*., 2011). Curcucmin for example is known to interefere with histones acetyltransfereases, it can also induce the production of reactive oxygen species (Cui *et al*., 2007) or interfere with many signaling pathways including: the mitogen activated protein kinases (MAPKs), casein kinase II (CKII) and the COP9 signalosome (CSN) as well as the ubiquitin proteosome pathway (UPS) (Si *et al*., 2007). Treatment of parasites with curcumin induced changes which could be observed as early as 3 hours after drug exposure (figure 14,16,17,18) meaning that changes in the gene expression pattern observed in the present study after drug treatment, may also be a reflection of the pleiotropic nature of the drugs. Hence it would explain the need for an increase at the protein level as DUBs would be necessary in order to respond to intracellular stress. It is well known that antimalarial drug treatment can interfere with ion homeostasis, which would result in alterations in the intracellular pH of several organelles which are crucial for parasite survival, thereby interfering with enzyme activity and function (Gazarini *et al*., 2007).

Overall, the present study confirms what other *in silico* studies have found. A bioinformatics and data mining study carried out in families of proteases in the *Plasmodium* genome had already predicted that *Pfuch-l1*, *Pfuch-l3*, *Pfuch-l54* and *Pfubp-8* proteins are being expressed in at least one stage of the parasite´s life cycle (Wu *et al*., 2003). A global microarray study had also predicted that the DUBs under study were being expressed in at least one stage of the parasite´s life cycle (Le Roch *et al*., 2003).In the present thesis we confirm and present evidence that DUBs are indeed being transcribed and translated in a stage specific manner.

Overall there was no difference between the *P. falciparum* sensitive and resistant strain meaning that although both clones have different phenotypes the basal gene expression and the gene expression after drug treatment was similar. Since the gene expression pattern indicates that those genes under study are being expressed with different periodic variations, this result prompted us to analyze to what extent are DUBS essential for the parasite. To answer this question an attempt was carried out to generate a transgenic parasite line in which genes encoding DUBs were knocked out. The results and discussion are shown in the next chapter.

CHAPTER 4-RESULTS & DISCUSSION

4.1 Evaluating the importance of de-ubiquitylating enzymes in *Plasmodium falciparum* by generating a transgenic parasite line by homologous recombination.

In order to understand whether DUBs are essential for the parasite survival and determine whether these enzymes are good drug targets classical assays such as gene disruption, by homologous recombination was employed in order to generate a knockout line of parasites. In this part of the project the classical vector for *Plasmodium falciparum* gene disruption pHH1 (Skinner-Adams *et al*., 2003) was used. The genes of choice to knockout were *pfuch-l1* and *pfuch-l3* as fully explained in section 2.2.2.1 – 2.2.2.4, a schematic diagram is shown in (figure 19). Transfection of the PARL-2 vector bearing the GFP gene was successful and resulted in the appearance of GFP parasites 30 days after transfection. Analyses of the parasites using a fluorescent microscope allowed us to visualize the presence of transformed parasites bearing the GFP gene throughout the parasite´s life cycle, confirming that the technique was working (figure 19).

However, after several attempts at transfection no viable stable pHHpfuchl1 KO and pHHpfuch-l3KO parasite lines were obtained. Once the parasites were transfected by electroporation, they were immediately placed back in the culture medium. Giemsa stained smears were prepared every day for a period of 60 days as previous work has shown that transfected parasites can emerge between 26-52 days (Fidock *et al*., 2000) after electroporation, however that did not happen, there may be several reasons why this has happened and they shall be discussed.

Electroporation of *Plasmodium falciparum* parasites is a very common way of transfecting parasites although it is known that stable transfection frequencies are close to ~10^{-6} to 10^{-9} (Carvalho and Menard, 2005) meaning that the rate of success using this technique is very low. It could be the case that genes encoding DUBs are essential for the parasite hence they are difficult to disrupt (Carvalho and Menard, 2005). Transfection experiments published (Triglia *et al*., 2000) have shown that some important genes in *P. falciparum* are difficult to transfect.

For example the *Plasmodium falciparum* membrane antigen-1 (*pfAMA*) gene which encodes a protein which is expressed on the surface of merozoites during parasite invasion was impossible to disrupt using knockout plasmids (Triglia *et al*., 2000). In this study the authors have shown that no integration of the plasmid into the desired locus had occurred possibly because the plasmid used was incapable of targeting the

pfAMA gene locus (Triglia *et al*., 2000). Another frequent problem often reported in the literature is the richness of A/T bases found in the *Plasmodium* genome (Carvalho and Menard, 2005) the A/T bases can easily recombine with other elements in the construct such as regulatory sequences or even bacterial DNA making it difficult to transfect (Carvalho and Menard, 2005). On the other hand, attempts to knock out the *Plasmodium falciparum* cysteine protease falcipain-1, which was once thought to be one of the most promising drug targets for antimalarial chemotherapy due to its involvement in haemoglobin degradation have been successful. Falcipain-1 has been shown not to be essential for normal parasite development (Sijiwali *et al*., 2004). In this study the authors suggest that up regulation of other falcipain family members may have compensated for the loss of falcipain-1 in the knockout parasites (Sijiwali *et al*., 2004). That is the reason why transfection assays remain crucial, in order to decipher which proteins are actually essential for the parasite.

In yeast *Saccharomycese cerevisiae* disruption of YUH1 an ubiquitin carboxyl hydrolase has failed to show a discernible phenotype (Jonhston *et al*., 1999). Also deletion of a gene encoding the mammalian *uch-1l* protein which is highly expressed in the brain caused very few visible effects (Wilkinson, 2009) an effect that can be attributed to the fact that loss of one DUB can be compensated by another (Wilkinson, 2009).However, biochemical assays in KO parasites still needs to be carried out as a complement to the transfection assays, to show what proteins on the inside of the cell are not being de-ubiquitylated properly. Another approach at knocking out DUBs is site directed mutagenesis which has resulted in the successful mutagenesis of *pfuch-l3* (Artavanis-Tsakonas *et al*., 2011) and this study concluded that *pfuch-l3* gene product might be essential for parasite survival as substitution of a cys residue by an ala in the active site resulted in the death of mutant parasites (Artavanis-Tsakonas *et al*., 2011). In future approaches this technique will be considered.

Perhaps in the future approaches such as the spontaneous uptake of DNA (Deitsch *et al*., 2001) could be used. This approach involves electroporating a mixture containing non infected red blood cells, the plasmid DNA containing the constructs and the transfection solution. After electroporation the mixture is immediately placed in a culture flask containing infected red blood cells, hoping that when the parasites invade

the new red blood cell they will spontaneously up take the plasmid DNA which is already found inside the cell. It has been suggested that the electroporation of red blood cells with no parasite inside, reduces the chances of interfering with parasite viability (Deitsch *et al*., 2001).

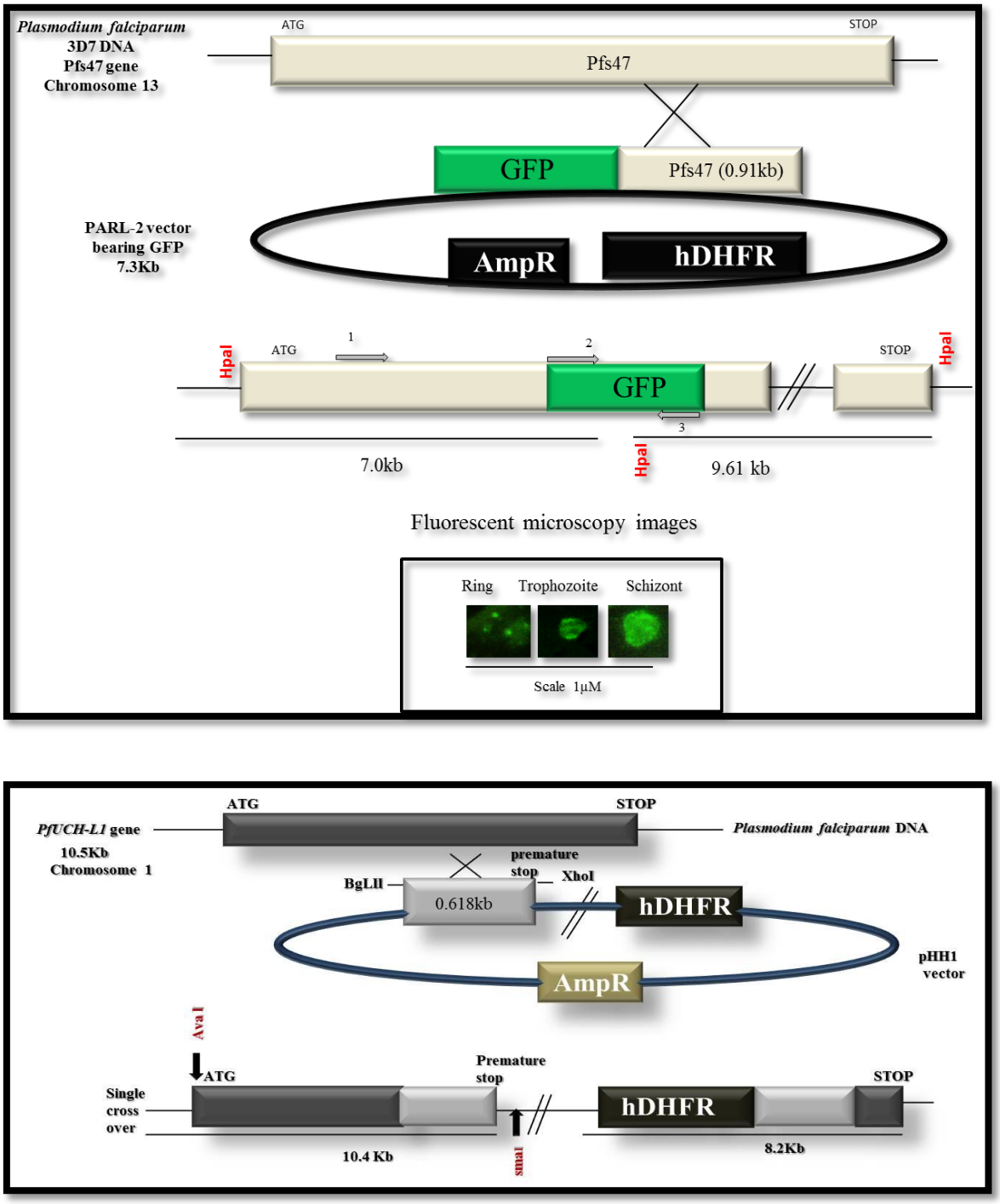


Figure 19. *Plasmodium falciparum pfuch-l1* gene knockout strategy. Transfection plasmid pHH-1Pfuch-l1 KO was designed to disrupt the locus by a single cross over homologous recombination. The PARL-2 vector was used as a control to verify that the technique is working.

Other approaches such as RNAi could have been used however, RNAi is controversial and is not considered as the most appropriate method for gene knock out in *Plasmodium* (Baum *et al*., 2009). Several reports have indicated that it is possible to introduce double stranded small RNA (dsRNAs) by electroporation. Other studies have shown small interfering RNA (siRNA) can be injected into mice infected with *P. berghei* parasites (Mohmmed *et al*., 2003). However, it is believed that RNAi leads to a global down regulation of expression of multiple genes, making it impossible to analyze the effects of RNAi on a specific gene, this has been confirmed by the fact that falcipain -1 gene knock out assays (Sijiwali *et al*., 2004; Baum *et al*., 2009) have shown, that the gene is not essential using gene disruption techniques whereas the RNAi assays have shown that falcipain-1 is essential for the intraerythrocytic stages of the parasite (Malhotra *et al*., 2002). Since there is no consensus amongst the scientific community with regards to RNAi, future studies will have to stick to transfection by electroporation using the traditional gene disruption techniques and site directed mutagenesis, widening the variety of plasmids and different strains of *P. falciparum* to increase the chances of a successful transfection.

CHAPTER 5-RESULTS AND DISCUSSION

5.1 Recombinant protein expression and *In vitro* activity of curcumin on recombinant proteins

Having studied the expression pattern of genes encoding DUBs and detected their stage specific protein abundance, it was important to verify whether recombinant proteins being expressed did have de-ubiquitylating activity. To answer this question, recombinant proteins expressed in *E. coli* cells were tested for their activity using the fluorogenic substrate Ub-AMC described in section 2.2.3.1–2.2.3.6. Cleavage of Ub-AMC by recombinant DUBs either alone or in the presence of NEM, is a classical way of confirming DUB activity (Dang *et al*., 1998). The results are presented here in graphs (mean ± SD) and SDS-PAGE gels and discussed.

5.1.1 Recombinant protein expression in *E.coli* cells BL21 DE3 codon Plus

The catalytic domain of *Pfuch-l1*, *Pfubp-8* and full length *Pfuch-l3* were easily expressed in *E. coli* cells (figure 20). However, *Pfuch-l54* either was expressed as a truncated form or it formed aggregates (figure 20) known as inclusion bodies and it was not possible to express this protein in the soluble form. Inclusion bodies were used anyway to immunize mice for antibody production but no activity based assays were carried out for this protein. It is not clear why this has happened although reports have shown that proteins containing long consecutive repetition of amino acids can lead to protein aggregation (Ravikuma *et al*., 2002). The protein sequence of *Pfuch-l54* does have a long stretch of asparagine repeats (Artavanis-Tsakonas *et al*., 2006) which might have contributed to protein aggregation. Perhaps in future attempts other expression systems such as baculovirus or the yeast *Saccharomyces cerevisiae* could be used as they have been reported to be good expression systems for *Plasmodium* proteins (Flick *et al*., 2004). There have also been reports that some *Plasmodium* proteins such as the duffy binding domain of *pfEMP-1* protein was easily collected in the soluble fraction if IPTG was added with an OD of 2 (Flick *et al*., 2004). Clearly future attempts will have to explore different times of induction, pH and temperature in order to facilitate the expression of this protein.

5.1.2 Enzymatic activity of recombinant *Plasmodium falciparum* de-ubiquitylating enzymes (DUBs)

In terms of DUB activity, the results show that recombinant *Pfuch-l1*, *Pfuch-l3* and *Pfubp-8* possess genuine de-ubiquitylating activity. In the presence of Ub-AMC these

enzymes were able to cleave the substrate with *Pfubp-8* showed the most activity and released a maximum of 300 ± 1.0 (figure 21) relative fluorescence intensity units which is often represented as relative fluorescence units (RFUs) over a period of 30 minutes. *Pfuch-l1* released a maximum fluorescence of 280 ± 0.8 before plateau and *Pfuch-l3* released a maximum of 210 ± 0.9 RFUs before plateau was achieved (figure 21). The irreversible cysteine protease inhibitor NEM was able to abrogate enzyme activity indicating the presence of cysteine residues in the active site of the proteins (figure 21).

The results obtained here are in agreement with others (Artavanis-Tsakonas *et al.*, 2006). When the recombinant proteins were incubated with various concentrations of curcumin, it became evident that curcumin was able to inhibit DUBs in a dose dependent manner. A dose response curve was plotted in which percentage inhibition was plotted against the log concentration of curcumin (APPENDIX F). From the dose response curve the IC50 (50% inhibition) was determine using GraphPad software version 4. The IC50 for recombinant *Pfuch-l1* was 15 µM , for recombinant *Pfuch-l3* was 25,4 µM. For *Pfubp-8* was 10 µM and for human USP was 5 µM (APPENDIX F). The results clearly show that only a small concentration of curcumin is necessary to inhibit the human enzyme USP2 activity by 50%. Indicating that curcumin may not be an ideal inhibitor for antimalarial chemotherapy as it also displays activity towards a human DUB.

Pfuch-l1 and *Pfuch-l3* required very large amounts of curcumin in order to inhibit 50% of its activity when compared to *Pfubp-8* perhaps an indication that curcumin may be more selective towards UBPs rathen than UCHs. A compound similar to curcumin known as WP1130 which is a Janus kinase 2 (JAK2) inhibitor with anti-tumoral activity has also shown more activity towards human ubiquitin specific protease nine (USP9) and human ubiquitin specific protease five USP5 compared to human UCH37 (Kapuria *et al.*, 2012; Love *et al.*, 2007) maybe an indication of a particular feature of this class of inhibitors. It is not clear what the mode of action/ inhibition of curcumin towards *Plasmodium falciparum* DUBs is, but based on structural similarity with other well known inhibitors such as WP1130, DBA, shikoccin they all have an unsaturated β carbon group which reacts with cysteine residues (Mullaly and Fitzpatrick et al., 2002; Kapuria *et al*., 2010).

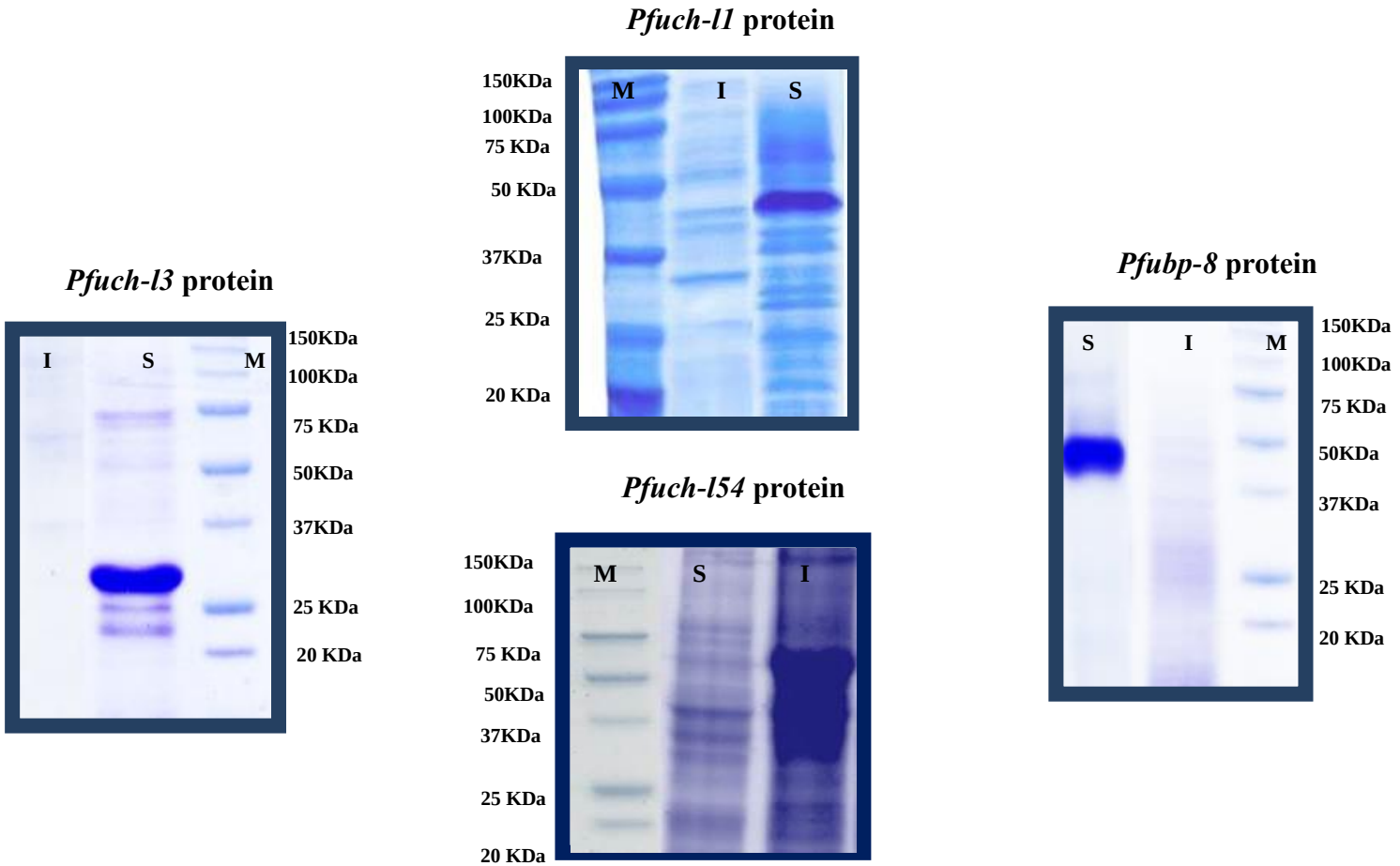


Figure 20. Expression of recombinant proteins in *E.coli* cells BL21 DE3 Codon Plus cells. *Pfuch-l1* (44 Kds) and *Pfuch-l3* (30Kds). *Pfuch-*l54 (54Kds) and *Pfubp-8* (50Kds) on 12.5% SDS-PAGE gels. M (marker), S (soluble fraction), I (insoluble fraction).

The ideal scenario would be to test a wider pannel of inhibitors of DUBs in order to identify the best inhibitors for the different classes of DUBs (UBPs/UCHs) and determined the mode of inhibition of each compound as well as their kinetic paramerters. Although different inhibitors of Ub molecules, DUBs, DUBLs, Ub ligases and the protesome itself are availbale not all of them are cell permeable (Love *et a*l., 2007; Kapuria *et al*., 2010) which may limit the therapeutical application of those inhibitors. This is one of the measure drawbacks in identifying cysteine protease inhibitors with good pharmacological characteristics. Fist the human genome has approximatelly 553 genes that encode proteases of which 143 are cysteine proteases (Puente *et al*., 2003) that in itself indicates that inhibitors against cystein proteases have to be very specific otherwise they will also affect other proteins within the cell. In spite of this major setback, there now more than 30 new protease inhibitors in the marketplace (Ratia *et al*., 2008) used in the treatment of many diseases; the most successuful are the HIV protease inhibitors directed against viral proteases (Ratia *et al*., 2008) indicating that protease inhibitors can be used in the treatment of human diseases.

An approach that is being used to modify the chemical characteristics of an inhibitor in order to make it more cell permeable and water soluble, less toxic or even to predict whether an inhibitor will interact well with its target is known as molecular docking (Singh and Misra, 2009). Molecular docking is a virtual screening computational tool which allows virtual interactions to be made between an inhibitor of interest and its potential target (Singh and Misra, 2009) the simulation can than be tested *in vivo* and *in vitro*. If curcumin is to join the current list of antimalarial drugs, more *in vitro* and *in vivo* studies are needed in order to evalutate its potential as antimalarial drugs. In the present study, the *in vivo* efficacy of curcumin in a mice model of malaria was tested using *Plasmodium chabaudi* clones resistant to chloroquine and artemisinin giving a good representation of the current drug resistant scneraio observed in the field. The results and the discussion are shown in the next section.

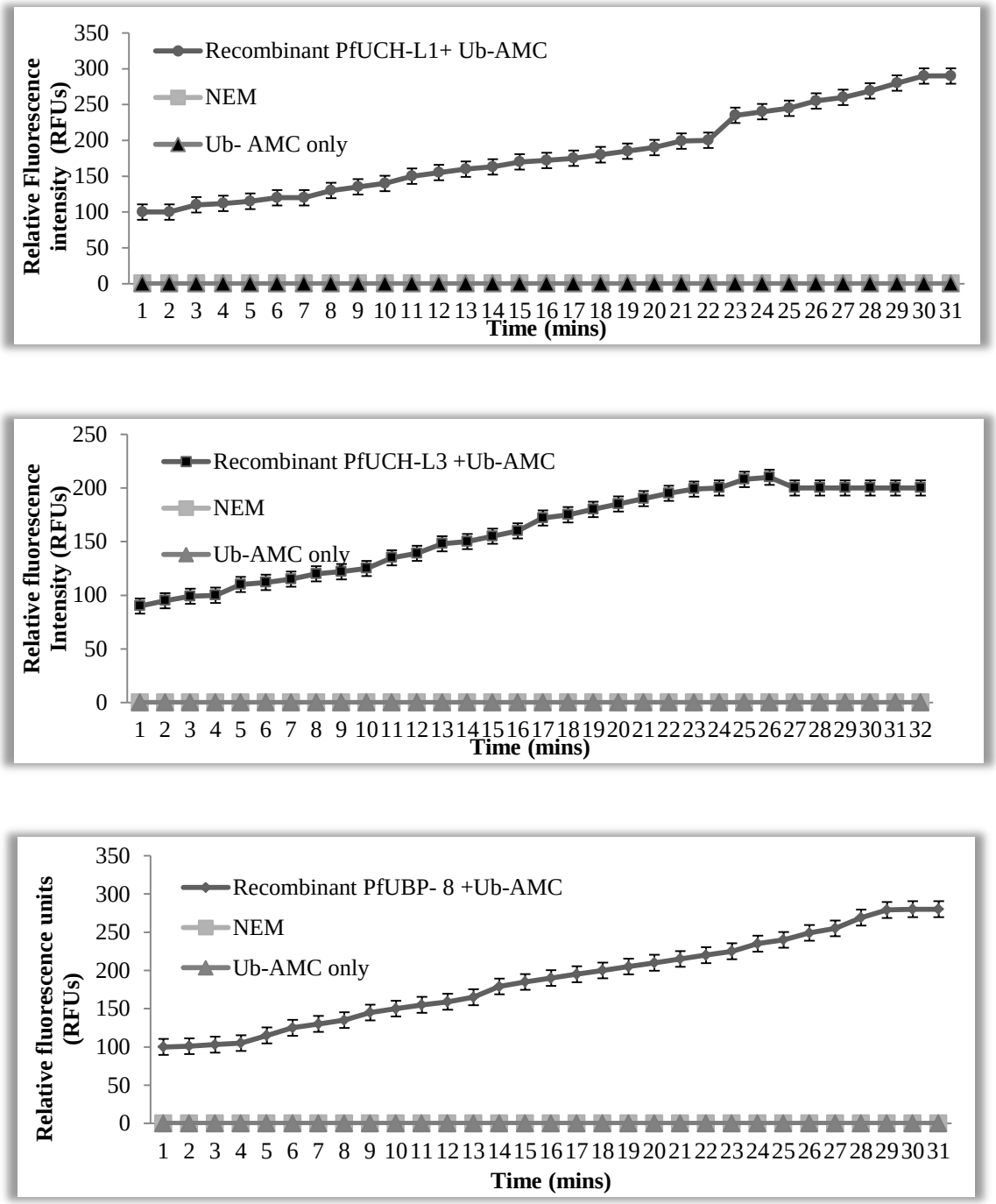


Figure 21. Evaluation of recombinant protein activity. Purified his-tagged recombinant *Pfuch-l1*, *Pfuch-l3*,*Pfubp-8* were added to a DUB buffer and the substrate for DUBs Ub-AMC was added to the mixture. Release of AMC fluorescence was monitored at Ex 400 nm Em 505 nm. The results were presented as mean ± SD (n=3 experiments).

CHAPTER 6-RESULTS & DISCUSSION

6.1 *In vivo* efficacy and acute toxicity test of curcumin in *Plasmodium chabaudi* parasites

Given the emergence of drug resistance to artemisinin combination therapy (ACT) new antimalarial are urgently need. In this part of the study the *in vivo* efficacy of curcumin alone or in combination: curcumin/piperine/chloroquine curcumin/piperine/artemisinin was determined as described fully in section 2.2.4.1-2.2.4.5 tested in order to clarify its potential as an antimalarial drug. The acute toxicity studies revealed that curcumin was non toxic to mice even at 2 g per kilogram of body weight. Mice survived for 14 days with no signs of toxicity (table 13).

Table 13 *In vivo* acute toxicity test of curcumin in Balb/c mice.

LD50 cytotoxicity test (g)	Animals tested	Animal Weight (g)	Survival (days)
2.0 g of curcumin	Balb/c mice	15	14
2.5 g of curcumin	Balb/c mice	15	10
3.0 g of curcumin	Balb/c mice	15	5
3.5 of curcumin	Balb/c mice	15	3
5.0 of curcumin	Balb/c mice	15	1

6.1.1 *In vivo* efficacy of curcumin in chloroquine resistant *Plasmodium chabaudi* parasites

The results show that curcumin alone was able to delay peak parasitemia in a dose dependent manner in both *P. chabaudi* clones (figure 22 and 24). Statistically there was no significant difference ($P > 0.05$) between the control untreated group and the groups treated with (50 mg and 150 mg curcumin alone). Significant results were only observed at 500 mg/kg/bw where parasitemia dropped to 47% in the AS-3CQ clone and 45% in the AS-ART clone compared to the control untreated group which had a parasitemia of 65% and 62%, respectively ($P=0.003$). Curcumin combined with piperine showed a mild antimalarial effect which is in agreement with previous work (Martinelli *et al*., 2008). Again in both clones curcumin/piperine combination was more efficient at

reducing parasitemia at higher doses. The combination of 250 mg curcumin/20 mg of piperine parasitemia dropped to 45% in mice infected with the chloroquine resistance clone (AS-3CQ) and 44% in mice infected with the artemisinin resistance clone (AS-ART) relative to the control where parasitemia was 65% and 62% (P=0.04) (figure 22 and 24). Curcumin at 500 mg/20 mg of piperine parasitemia dropped to 42% in mice infected with the chloroquine resistant parasite line and 40% in mice infected with the artemisinin resistant parasite line with P value significant (P = 0.02). Indicating that the efficacy of the curcumin/piperine combination in *P. chabaudi* clones was also in a dose dependent manner.

For the drug interaction studies four doses of chloroquine were administered orally to mice infected with chloroquine resistant parasite line (AS-3CQ) and of the 4 tested doses 2.5 mg of chloroquine was found to reduce parasitemia to 48% after 7 days post drug treatment. AS-3CQ parasites were treated with 5mg/kg and 10 mg/kg which reduced parasitemia to 15% and 9% respectively (figure 22). Hence a choice was made not to combine these higher doses with curcumin/piperine as they would mask the effect of the combination.When curcumin/piperine was combined with a fixed dose of 2.5 mg/kg of chloroquine, parasitemia reduction was better than when curcumin was used either alone or when it was used in combination with piperine (figure 22).

When curcumin/piperine/chloroquine was administered to mice a significant reduction in parasitemia was achieved, compared to the control group. This reduction was even more evident at higher doses: 150 mg/20 mg/2.5 mg parasitemia 45% (P = 0.033), 250 mg/ 20 mg/ 2.5 mg parasitemia 39% (P = 0.001), 500 mg/ 20 mg/ 2.5 mg parasitemia 37% (P = 0.0001) relative to the control sample (figure 22). Indicating an additive/weak synergistic effect which was confirmed by the Isobologram (figure 23) with most values achieved were below 1.5.

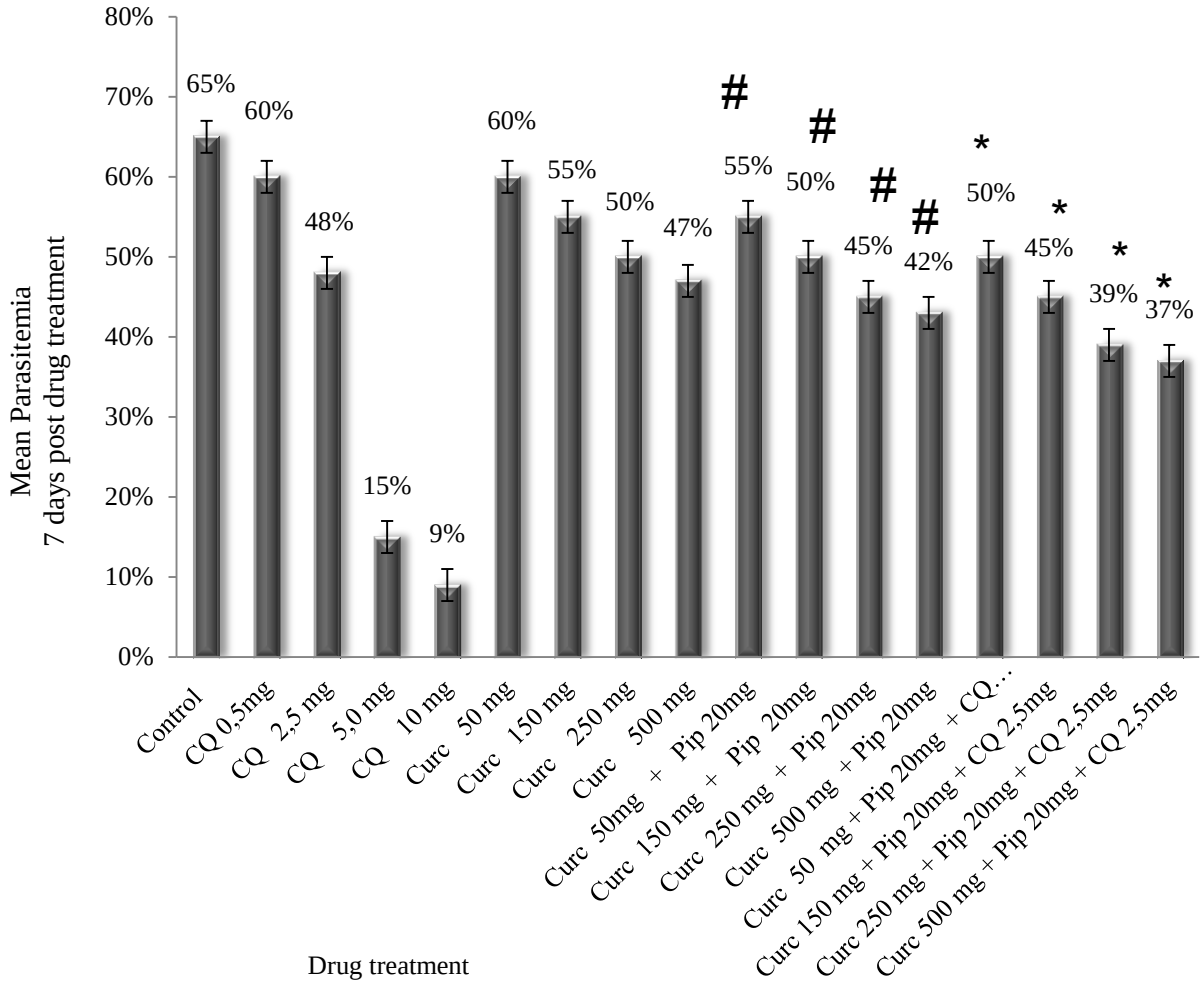


Figure 22. Parasitaemia evolution in mice infected with *P. chabaudi* clone AS-3CQ. Infected mice were treated with chloroquine, curcumin, curcumin/ piperine, and a combination of curcumin/piperine/chloroquine. Results represent the mean parasitemia ± S.D. # Statistical difference ($P < 0.05$) was found between the curcumin/piperine treatment group versus the curcumin only treatment group. A student T test was applied and significant difference * ($P < 0.05$) was found between curcumin/piperine/ chloroquine group versus the curcumin alone, curcumin/ piperine, chloroquine alone treatment groups (2.5 mg/kg).

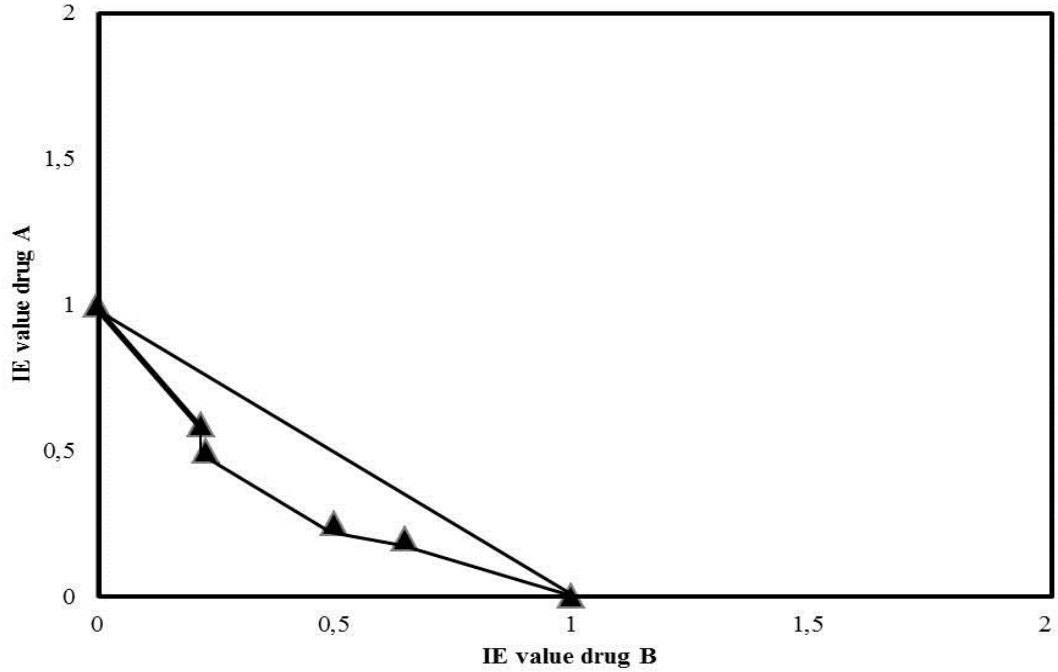


Figure 23. Isobologram illustrating the *in vivo* interaction at the ED90 level between drug A (curcumin) with drug B (chloroquine) in *Plasmodium chabaudi* AS-3CQ chloroquine resistant parasites. The isobolar values fall below the additivy line indicating weak additivity/synergism.

6.1.2 *In vivo* efficacy of curcumin in artemisinin resistant *Plasmodium chabaudi* parasites

The group of mice infected with the artemisinin resistant parasite line AS-ART was also treated with four doses of artemisinin alone (50 mg, 150 mg, 250 mg and 350 mg) and at 350 mg/kg of artemisinin alone parasitemia dropped to 5% compared to the control group 67% (P = 0.0001). A decrease in parasitemia was also observed at (50 mg, 150 mg, 250 mg) (figure 24) and 150 mg/kg was chosen as the dose used to combine with curcumin/ piperine (figure 24) as 350 mg/kg would mask the effect of curcumin.

Again treatment of *Plasmodium chabaudi* resistant AS-ART parasite line with curcumin alone only resulted in a significant parasitemia reduction at higher doses. At 500 mg/kg of curcumin parasitemia reduced to 45% compared to the control group 62% (P = 0.0001) (Figure 24). This reduction was even more evident when curcumin was combined with piperine which resulted in a parasitemia reduction from 67% to 40% (P = 0.0001) (figure 24).

Addition of a fixed dose of 150 mg/kg of artemisinin to curcumin/piperine did not result in a clear difference in parasitemia reduction between the control group and the artemisinin/curcumin/piperine (P=0.08) which is in agreement with previous work (Martinelli *et al*., 2008). In fact, even at higher doses 500 mg of curcumin/20 mg of piperine and 150 mg of artemisinin parasitemia dropped to 50% relative to the control group 62% (P=0.055) (figure 24). It seems that curcumin, curcumin/ piperine and artemisinin alone performed better separately as opposed to when the three compounds are combined (figure 24). The isobologram indicates that most values were bigger than 1.5 (figure 25) which resulted in an isobologram where most of the values are far away from the additivity line, indicating antagonism amongst the components of the drug combination.

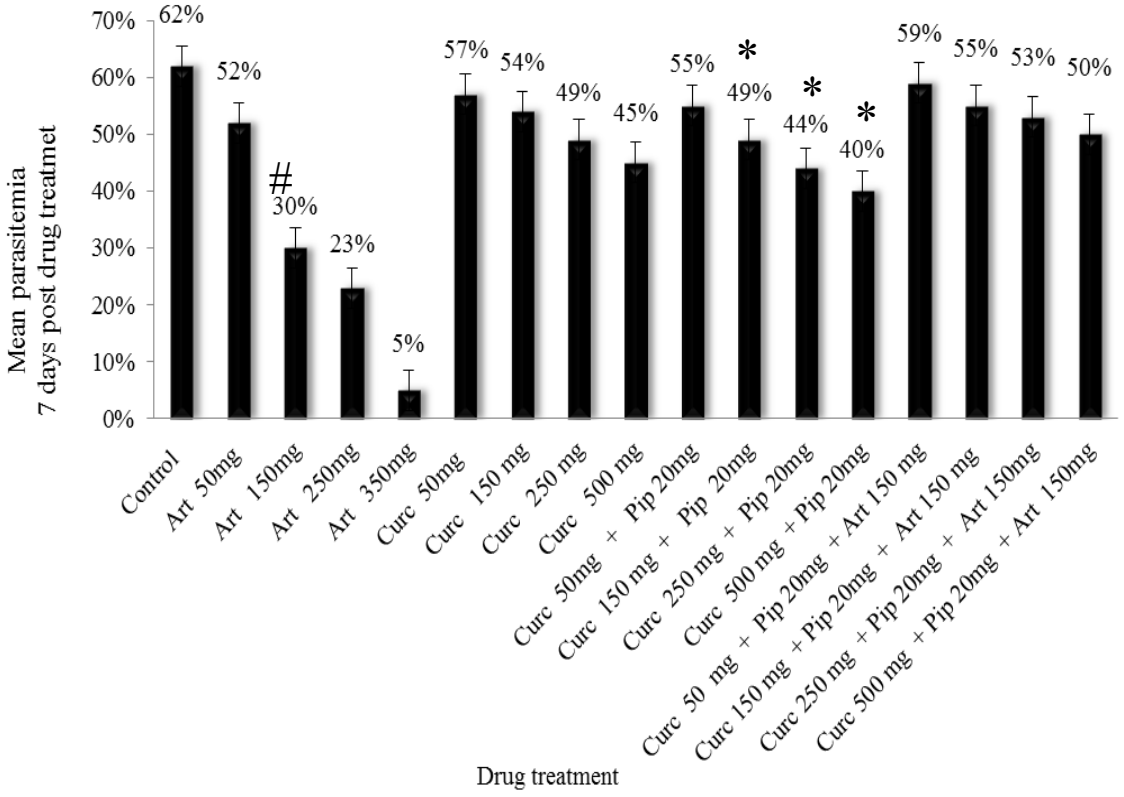


Figure 24. Parasitaemia evolution in mice infected with *P. chabaudi* clone AS-ART treated with: artemisinin, curcumin, curcumin/ piperine and the combination curcumin/ piperine/artemisinin. Results represent the mean parasitemia ± S.D. A student T test was applied and statistical significant # difference was found between the artemisinin alone (150 mg/kg) versus the curcumin, curcumin/ piperine, curcumin/ piperine/ artemisinin treatment groups.* A statistical significant difference was found ($P < 0.05$) between the curcumin/piperine versus curcumin alone and curcumin/piperine/artemisinin treatment groups.

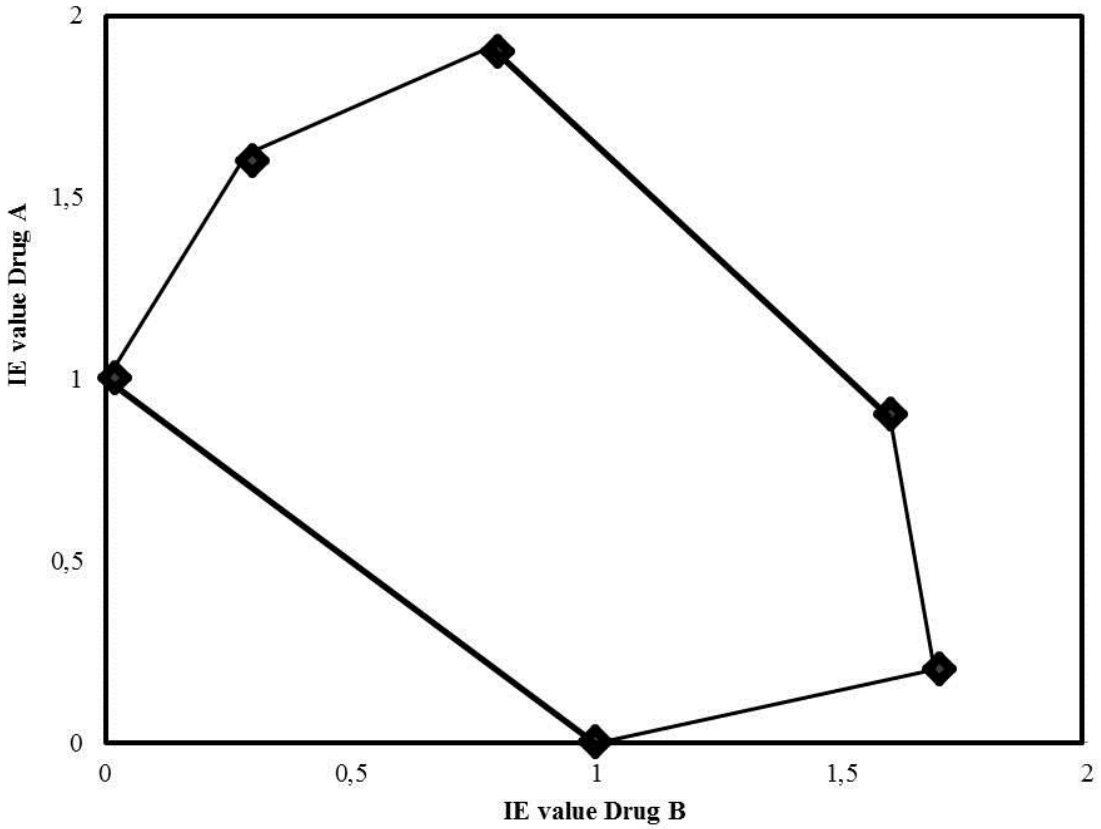


Figure 25. Isobologram illustrating the *in vivo* interaction at the ED90 level between drug A (Curcumin) with drug B (artemisinin) in *Plasmodium chabaudi* AS-ART (artemisinin) resistant parasites. IE values above the addivity line indicate antagonism.

Given the emergence of resistance against ACTs, new alternatives for the treatment of malaria are urgently needed. Curcumin has already shown great potential both *In vitro* and *In vivo* against *Plasmodium spp* (Nandakumar *et al*., 2006; Martinelli *et al*., 2008). However, its poor availability and rapid metabolism are issues to overcome in order to exploit the full benefits of this plant derived compound (Anand *et al*., 2007). Enhancers such as piperine derived from black pepper which is already known to improve the bioavailability of curcumin (Anand *et al*., 2007) were hereby tested as a combination: curcumin/piperine/chloroquine and curcumin/piperine/artemisinin.This part of the thesis work resulted in a publication.

Overall the results show that the interaction between curcumin/piperine/chloroquine was additive and helped in the reduction of the parasite load 7 days after treatment had ended. The results are interesting, although both drugs have different structures and different modes of action they both have anti-inflammatory properties which possibly contribute to parasitemia reduction (Vathsala *et al*., 2012). Curcumin is well known for its immunomodulatory properties which include: activation of TLR2, increase in IL-10 and production of anti-parasite antibodies (Vathsala *et al*., 2012). Chloroquine is well known for its antimalarial schizonticidal activity as well as its anti-inflammatory properties such as inhibition of tumor necrosis factor-α, IL-1ß and IL-6 (Karres *et al*., 1998) making the drug combination interesting in the treatment of other diseases where an excess of pro-inflammatory cytokines is produced. It is believed that curcumin is an attractive compound for adjunctive treatment of cerebral malaria (Mimche *et al*., 2008) which is often treated with quinine, from which chloroquine derives. Hence further pharmacokinetic studies between curcumin and quinine and its derivatives are needed to exploit their potential in antimalarial treatment.

The combination of curcumin/piperine/artemisinin did not show a favourable drug interaction. Although it was able to reduce parasitemia statistically there was no difference between the control untreated group and the curcumin/piperine/artemisinin group. In the present study the mixture of the three compounds administered orally resulted in an unfavourable pharmacodynamic interaction. Recent studies in *P. berghei* infected mice using a combination of artemisinin and curcumin have also shown that although parasites can be cleared from the blood they remain in the spleen and the liver

(Vathsala *et al*., 2012) favouring recrudescence. Artemisinin has a half life of approximately 8-14 hours and curcumin has half life of 8h (Vathsala *et al*., 2012). Studies carried out in rats have reported that only about 0.1%-0.25% of piperine administered orally can be detected in the liver whereas intraperitoneal administration of piperine resulted in 1-2.5% of piperine detection in the liver 6 hours later (Mimche *et al*., 2011). To include curcumin in the current arsenal of antimalarial drugs, more studies are needed including different administration routes and HPLC analysis of mice tissue, after treatment with curcumin/piperine/artemisinin in order to clarify drug distribution and elimination.

CHAPTER 7-RESULTS AND DISCUSSION

7.1 A proteomics (2DE) approach for the identification of *Plasmodium falciparum* schizont stage proteins altered in response to curcumin treatment.

In this part of the work an attempt was made to find out what other proteins are altered in *Plasmodium falciparum* schizont stage parasites in response to curcumin treatment. To answer this question a 2DE proteomic approach was taken. Schizont stage *Plasmodium falciparum* 3D7 parasites were culture and treated with curcumin as described fully in the section 2.2.5.1-2.2.5.5 and proteins were extracted. Proteins were quantified by Bradford reagent and were labeled with fluorescent dyes Cy2, Cy3 and Cy5 run onto 2DE, trypsinized and analyzed by mass spectrometry. The results and discussion are presented here in tables, Pie charts, gels and fluorescent images acquired from the software are also included. A T student test and ANOVA test was used to determine which protein spots were deregulated in response to treatment.

7.1.1 In gel protein identification

In the present study only 13 proteins were found to be deregulated in response to curcumin treatment. Of the total of 13 proteins only 10 proteins were manually excised and analyzed by mass spectrometry (MS) (figure 26,27,28).The remaining three proteins became invisible after gel coloration with comassie brilliant blue those were proteins number: 543, 363, 791 hence they were not subjected to mass spectrometry. The proteins that were successfully identified by mass spectrometry are summarised in (figures 27 and 28) as well as table 14.

The reason why so few proteins were detected is because proteomic analysis of *Plasmodium falciparum* samples is still a very challenging field (Prieto *et al*., 2008). During the process of parasite protein extraction from infected red blood cells the samples are washed several times to reduce β haematin contamination, that in itself reduces the final yield of total protein. The fact that human red blood cell proteins were identified in the mass spectrometry analysis, also indicates that the protein extraction method used in this study was not 100% pure as red blood cell contaminants were still present in the sample. There are now reports that haemoglobin can be removed using nickel affinity chromatography, however, it is thought that this technique can deplete the sample from potential relevant proteins, hence alternative methods are urgently needed (Williams *et al*., 2010). Of the 10 proteins identified some of them belong to red blood cell membrane as expected, those proteins were identified in all the samples. Those were: Human erythrocyte catalase, human S100 calcium binding protein from red

blood cells and human erythrocyte membrane protein band 3. According to the PANTHER database, Human catalase belongs to the peroxidase class of proteins, human S100 calcium binding protein belongs to the signalling molecule class of proteins and membrane protein band 3 belongs to the class of protein transporters (figure 29). According to the PANTHER database the red blood cell membranes identified are involved in the following biological processes: Human S100 calcium binding protein is insvolved in macrophage activation, cell cycle, intracellular signaling cascade, DNA replication, cell motion and cell cycle (figure 29). Human catalase is involved immune system processes, respitarory electron transport chain, oxygen and reactive oxygen species, metabolic processes (figure 29). Human Band 3 protein is involved in biological processes such as ion transport, cellular component and organization. It is interesting to note that *Plasmodium falciparum* interacts with human band 3 protein and can induce changes in this protein which may help infected erythrocytes during cytoadherence (Goel *et al*., 2003). It is interesting to see that this protein was down regulated (-1.78)(table 14) perhaps an indication that curcumin interefering with cell surface red blood cell proteins. The presence of this protein is common in proteomics assays because of the abundance of this protein, band 3 (table 14) is known to be present 1 million copies per red blood cell (Pasini *et al* 2010).

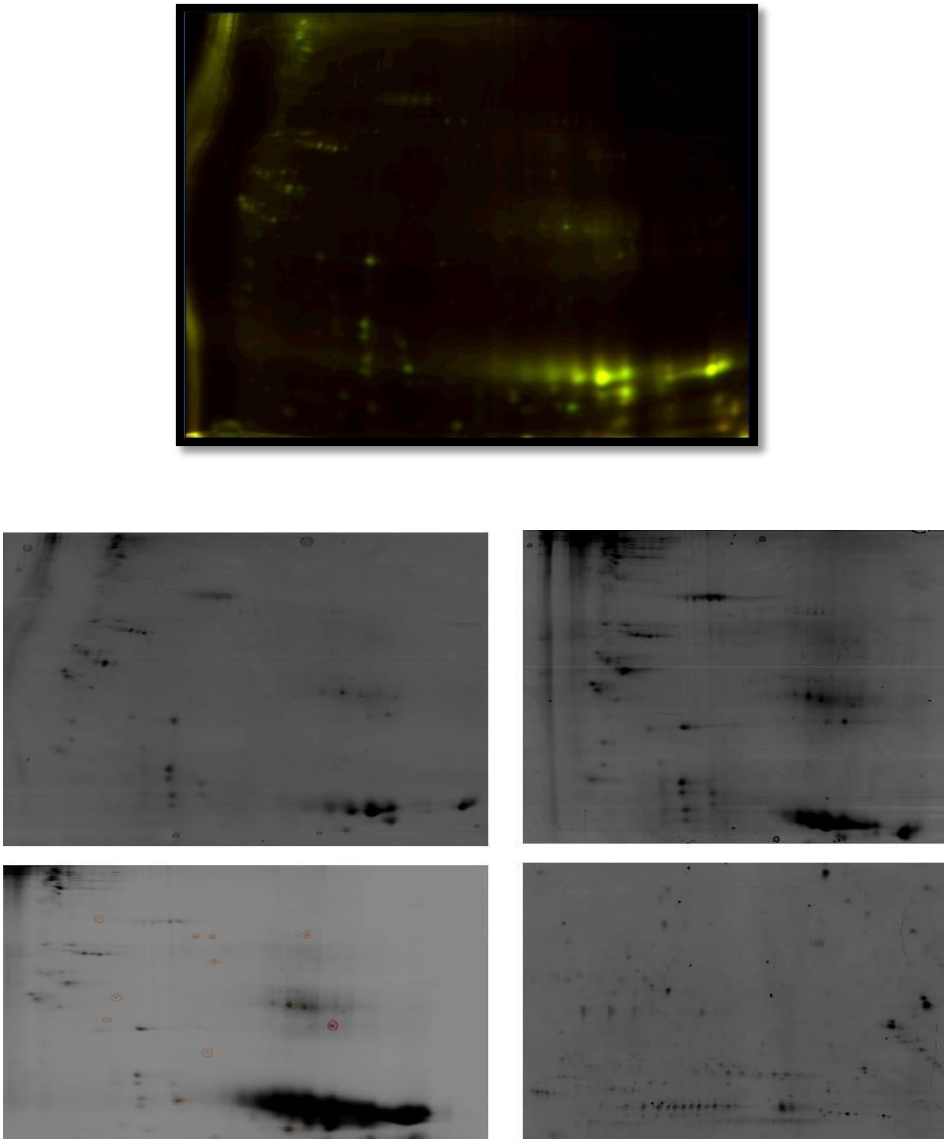


Figure 26. *Plasmodium falciparum* proteins labeled with cyanine dyes (top panel). A representative merged image is shown here, pooled samples labeled with cyanine dyes were then separated based on their isolectric point (PI) and molecular weight (WM (bottom panel). Gel images were acquired with typhoon image scanner. Images were cropped with ImageQuant software and further analysed using DeCyder software version 6.5.

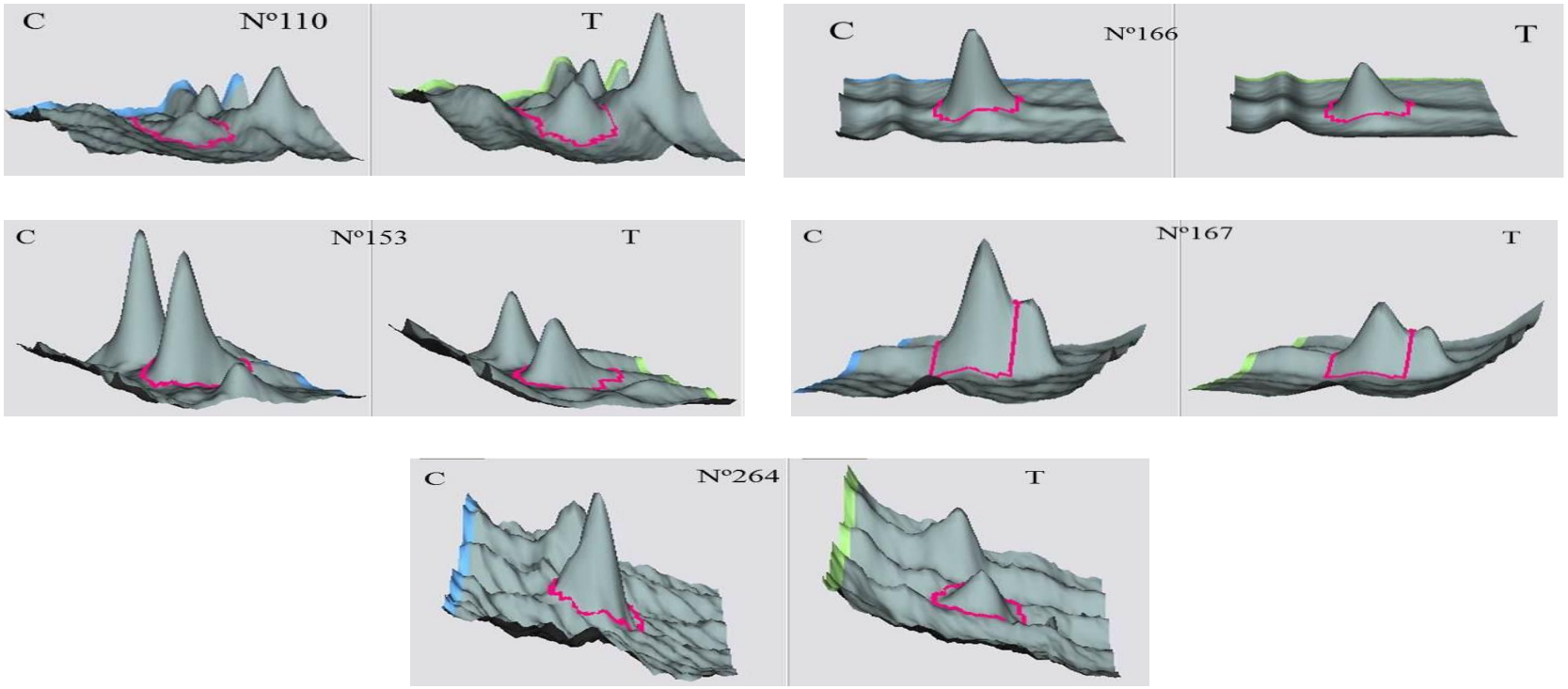


Figure 27 Fluorescence intensity 3D images of spots deregulated in response to curcumin treatment part I. DeCyder software was used to analyze 2D fluorescence difference in protein abundance in control (C) and treated samples (T). The DeCyder software was used to perform gel alignment, spot averaging and normalization.

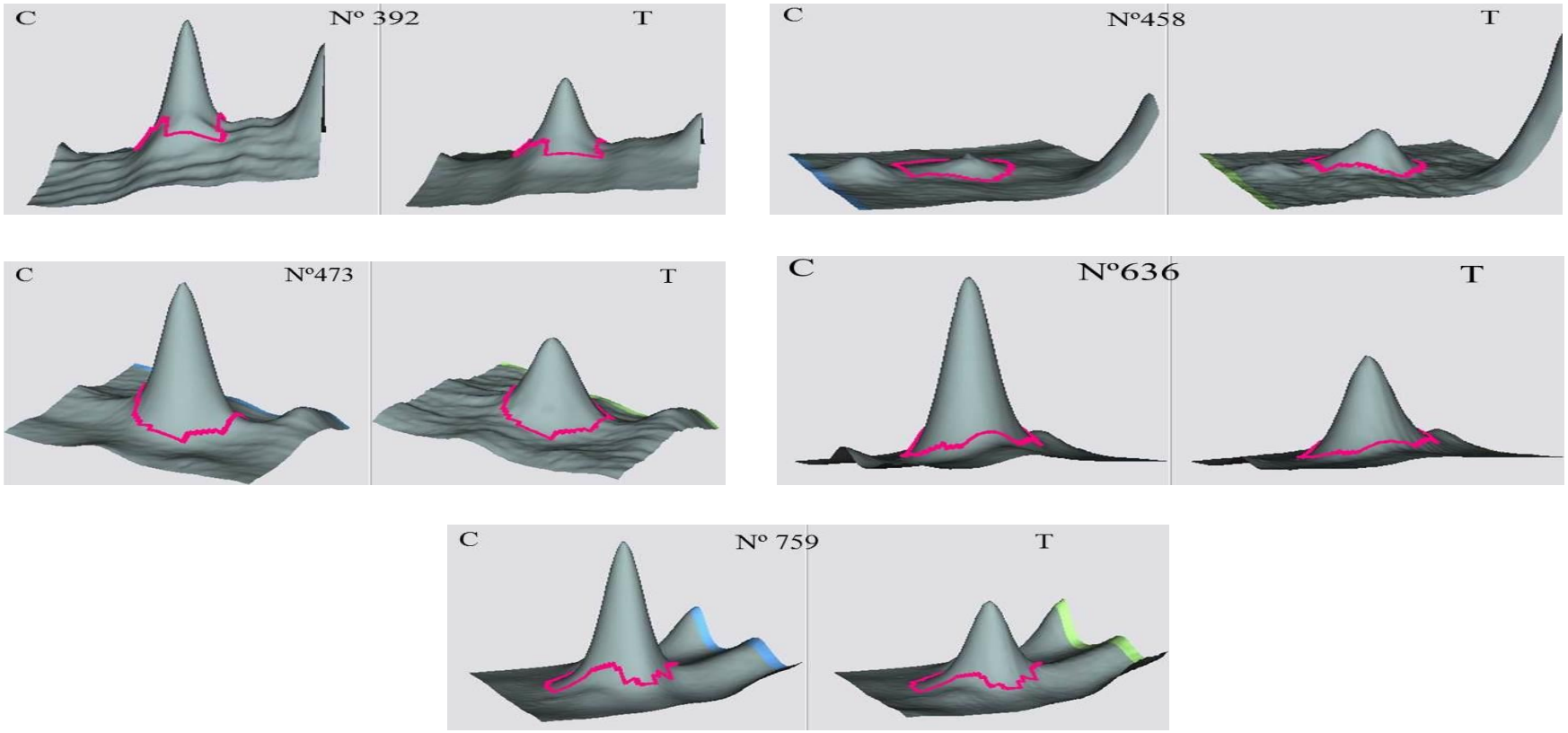


Figure 28. Fluorescence intensity 3D images of spots deregulated in response to curcumin treatment part II. DeCyder software was used to analyze 2D fluorescence difference in protein abundance in control and treated samples. The DeCyder software was used to perform gel alignment, spot averaging and normalization.

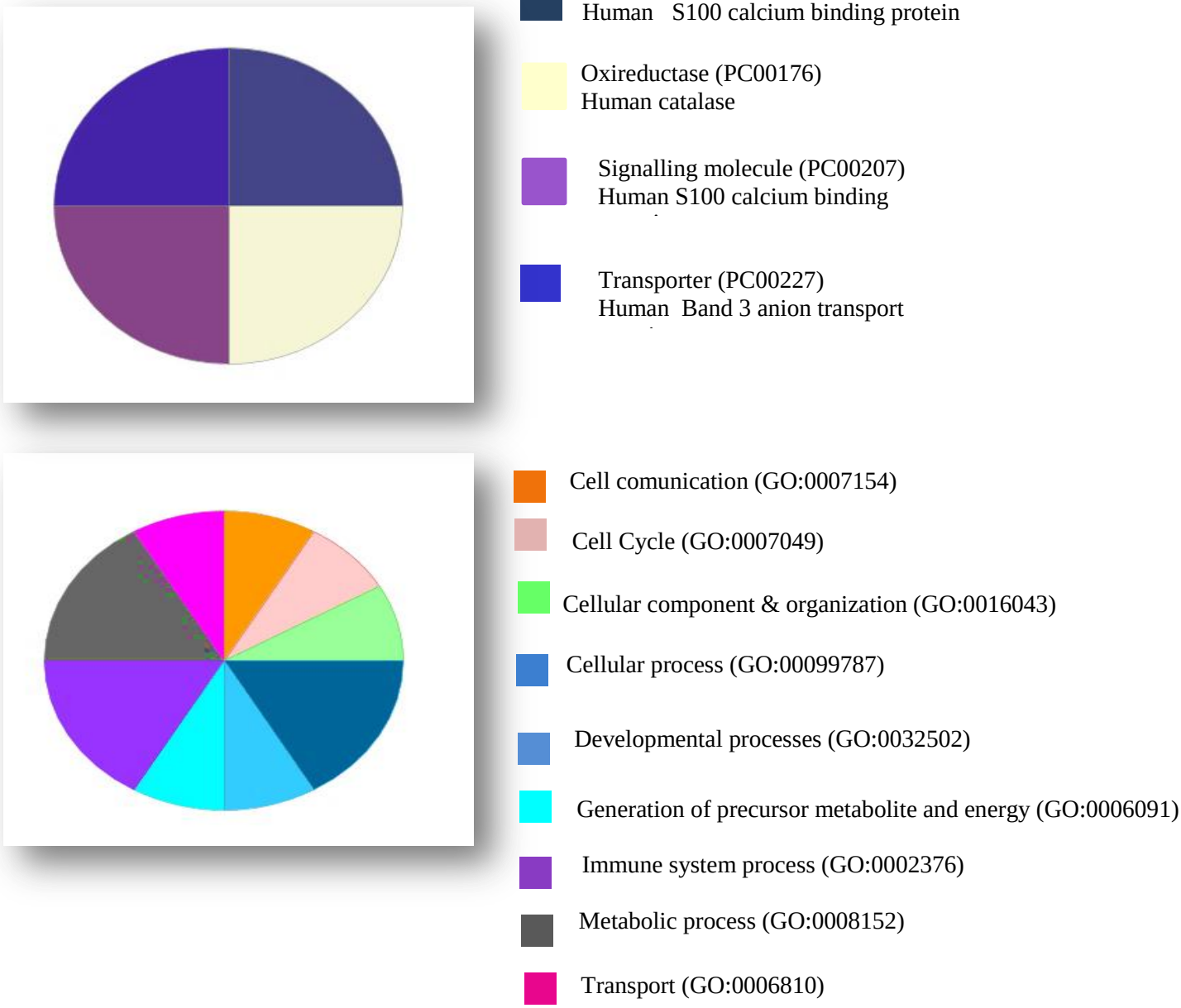


Figure 29. Classification of human proteins identified according to the PANTHER database. Proteins were grouped according to their protein class and biological processes that they might be involved in. The data presente hare was generated by the PANTHER database www.pantherdb.org

7.1.2 *Plasmodium falciparum* proteins deregulated in response to curcumin treatment

A total of 7 *Plasmodium falciparum* protein were found to be deregulated in response to curcumin treatment (table 14). Amongst the proteins that were downregulated the following proteins were identified: *Plasmodium falciparum* heat shock protein found in the membrane fraction, this kind of protein is usually expressed during a stress response. Several members of the heat shock protein family (*Pfhsp40, Pfhsp70, Pfhsp90*) have been very well studied and are thought to be involved in parasite survival in response to stressful conditions (Pesce *et al*., 2008). It is therefore understandable that those proteins would be involved in drug responses. The other protein that was found to be up regulated was histone H3 (tabe 14) found in the membrane fraction. This is an interesting finding since studies have shown that curcumin might be an inhibitor of histone acetyltransferases (HAT) and curcumin can induce the production of reactive oxygen species which may contribute to parasite´s death (Cui *et al*., 2007). Histones are responsible for the regulation of chromatin, gene activation and gene silencing (Salcedo-Amaya *et al*., 2009). In response to drug treatment it is expected that these proteins will be upregulated as they control the activation of genes involved in protein synthesis and protein degradation thus allowing the parasite to cope with intracellular stress (Mok *et al*., 2011).

Glutathione synthetase was found in the soluble fraction. This protein was upregulated (table 14) and it is involved in the maintenance of intracellular redox environment. Attempts to knock out this protein failed indicating that glutathione synthase is essential for the parasite and protection of the parasites against highly reactive oxygen species (Patzewitz *et al*., 2012). Confirming to the suggestion made before by other authors which believe that curcumin maybe in fact kill the parasite by interfering with ROS production (Cui *et al* 2007). The fact that ribosomal 60S (table 14) subunit found in the membrane fraction was upregulated it's an indication that curcumin not only induces major oxidative stress (Khan *et al* 2012), but it might also interfere with protein synthesis which would result in a buildup of damaged proteins inside the cell creating a "proteotoxic" environment for the parasite. Another interesting protein that was up regulated was the proteasome subunit α found in the soluble fraction indicating that the

proteosome maybe one of the many targets of curcumin. The proteosome is where most proteins within the cell are degraded (Amerik and Hochstrasser, 2004) and others have reported that curcumin may interfere with DUBs located inside the proteasome (Si *et al* 2007) possibly indicating that curcumin is an inhibitors of DUBs. Previous studies had already reported upregulation of proteasome regulatory subunit in reponse to treatment with artemisinin (Prieto *et al* 2008) and parasites exposed do the antibiotic doxycycline also show deregulation of proteasome subunit α type 5 (Briolant *et al* 2010), indicating that deregulation of the protesome maybe also be a general primary response to intracellular stress.

Another protein that was identified was *Plasmodium falciparum* replication factor 6 found in the membrane fraction which is probably involved in DNA replication (table 14) and (figure 30 and 31) (Briolant *et al* 2010). Possibly indicating that curcumin is indeed a drug with multiple targets. All of the identified proteins were classified by the PANTHER database as: chaperone referring to *P.falciparum hsp60* hydrolase/protease referring to *P.falciparum* proteosome subunit α; Ligase glutathione synthetase; nucleic acid binding referring to *P. falciparum* replication factor C and histone 3; tranferase referring to *P.falciparum* replication factor C (figure 30 and 31). The only major limitation with 2DIGE experiments is the hypothetical conserved proteins with unknown function. In the present study one protein found in the soluble fraction was identified as *Plasmodium falciparum* protein with hypothetical function, however the PANTHER database could not assign a function to this protein (table 14), these proteins that cannot be assigned to any group or class may be relevant in deciphering new metabolic pathways (Prieto *et al.,* 2008).

According to the PANTHER database the proteins identified in this project were involved in the following biological processes: protein folding, DNA replication, chromatin architecture, protein translation, proteolysis and sulfur metabolism (figure 30 and 31) indicating that curcumin may interefere with all these biological processes thereby giving us a glimpse of curcumin´s potential drug targets. The fact that different groups of proteins were identified here shows that curcumin acts on multiple targets and elicits multiple responses just like chloroquine. Although DIGE analysis is crucial in the identification of new drug targets and metabolic pathways involved in drug response

(figure 26, 27 28), it is a very expensive technique and it also requires between 300 µg – 500 µg of protein for each gel which requires a large number of synchronized *Plasmodium falciparum* cultures and constant culture maintenance to achieve high quantities of parasite protein.

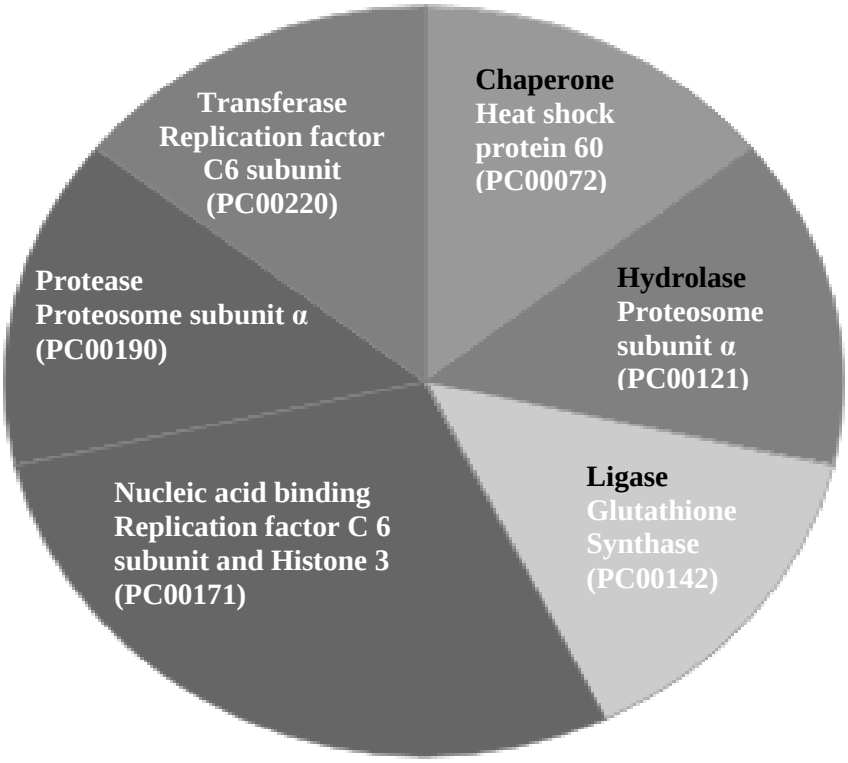


Figure 31. Functional classification of *Plasmodium falciparum* protein class identified by PANTHER database. Identified proteins were grouped according to their class. Classification indicates the widespred distrubtion of proteins. Proteins were classified as chaperone, ligase, transferase, hydrolase and nucleic acid binding (www.pantherdb.org).

Protein number	Gene ID	Sequence coverage	Mascot Score	Treated/Control Average ratio	Peptide number	Anova	T-test	Protein name
110	PF100153	50%	77	1.55	9	0.042	0.042	*Plasmodium falciparum* heat shock protein 60
153	847	40%	73	-1.5	5	0.013	0.013	Human erythrocyte catalase
166	PF140176	42%	82	-1.79	7	0.002	0.002	Proteasome subunit α type I
167	PF130013	53%	100	-1.61	12	0.045	0.045	Conserved *Plasmodium falciparum* hypothetical protein
264	PFE0350	43%	79	-2.23	8	0.023	0.023	*Plasmodium falciparum* 60S ribosomal protein L4
392	PF110117	48%	83	-1.7	9	0.0001	0.0001	*Plasmodium falciparum* replication factor 6
458	PFF0510w	45%	76	2.46	8	0.002	0.002	*Plasmodium falciparum* histone H3
473	PFE0605c	50%	85	-1.59	10	0003	0.003	*Plasmodium falciparum* glutathione synthase

636	6285	54%	103	1.61	15	0.013	0.013	Human S100 calcium binding protein from red blood cells
759	6521	52%	90,0	-1.78	11	0.001	0.01	Human erythrocyte membrane protein band 3

Table 14. Differentially expressed proteins in *Plasmodium falciparum* curcumin treated parasites. Proteins were considered as differentially expressed in response to treatment when P = or < 0.05 were considered as significant. Cut off point for upregulated proteins was > 1.50 and downregulated proteins was < 0.50. Protein number (number attributed on the gel), Gene ID (acession number given on the database) sequence coverage (coverage of the protein sequence) Mascot score (a score greater than 70) indicaties that the protein identified by mass spectrometry matches those protein sequences found in the databases. Peptide number (number of peptides corresponding to that protein identified by mass spectrometry). Treated/Control ration (to quantify the effect of treatment). Anova/T test (Statistical test used to obtain the P values).

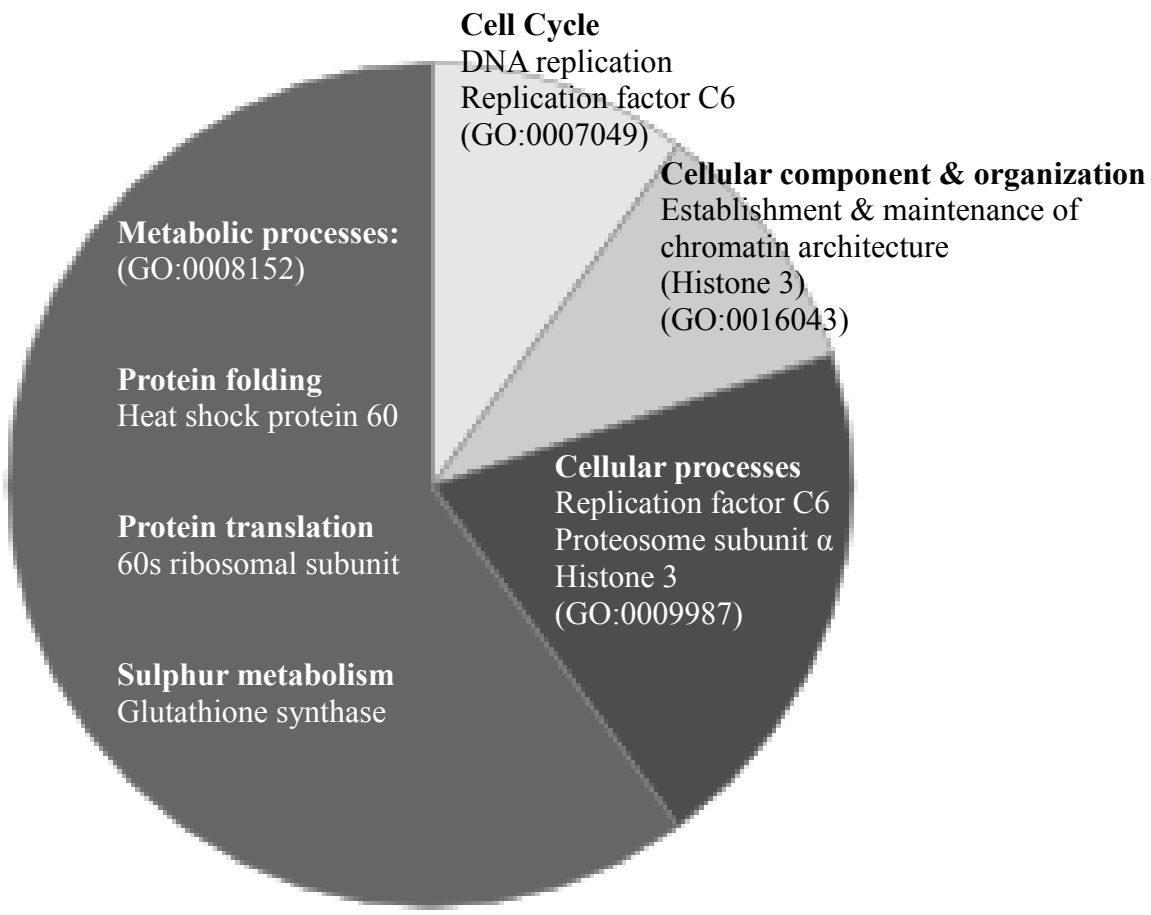


Figure 32. Functional classification of *Plasmodium falciparum* proteins and the biological processes identified by PANTHER database. The classification indicates widsespread distribution of proteins involved in metabolic processes. The proteins identified are involved in metabolic processes, cellular processes, cell cycle, cellular component and organization (www.pantherdb.org).

CHAPTER 8-GENERAL CONCLUSIONS

8.1.1 General Conclusions

In general the study has shown that *Plasmodium falciparum* has an active UPS. Components of the UPS have already been identified in *Plasmodium* spp. In the present study four DUBs namely *pfuch-l1*, *pfuch-l3*, *pfuch-l54* and *pfubp-8* have been studied both at the gene level and the protein level. In general the genes/proteins under study showed differential expression patterns, both at the protein and at the gene level. In the absence of drug *pfuch-l1* gene (figure 14) was found mainly to be expressed at the ring, early trophozoite to mature trophozoite and merozoite stages of the parasite. Western blot performed with *pfuch-l1* antisera confirmed that the protein was actively being translated although with different levels of abundance at the ring, trophozoite and schizont stages. The schizont stage was the stage in which protein abundance diminishes and concides with the results obtained at the mRNA level.

Pfuch-l3 gene is expressed throughout the parasite´s life cycle with a peak increase in expression as the parasite transioned from early to late schizont (figure 16). At the protein level the western blot confirms that the protein is being translated throughout the parasite´s life cycle but band intensity indicates that this protein is more abundant at the trophozoite and schizont stages. *Pfuch-l54* gene expression (figure 17) is very low at the early stages of parasite development (ring stage) this was evident both the mRNA and the protein level (figure 15A) and (figure 17). Upregulation of this gene is observed at 18 h time point as the parasite enters the late trophozoite stage. Western blot confirms that *pfuchl-54* is being translated with increase band intensity at the trophozoite and schizont stages. *Pfubp-8* gene seems to be expressed throughout the parasite ´s life cycle (figure 18) with a steady state level being observed throughout the parasite´s life cycle. At the protein level *pfubp-8* (figure 15A) is being actively translated and band intensity increase at the trophozoite and schizont stages.

When the parasites were exposed to artemisinin, chloroquine and curcumin treatment, the response was gene specific, but a general increase in mRNA levels was observed. Artemisinin is known to act on the ring, trophozoite, and schizont stages of the parasite so treatment with artemsinin induced an increase in the expression of the four genes. This increase was observed as early as 3 hours after drug treatment and continued up to 36h when mRNA levels gradually began to decrease (figure 14, 16, 17, 18). Treatment

with chloroquine which is known to act mainly on the schizont stages of the parasite also induced an increase in the expression of the four genes under study. This increase was more pronounced from 21h time point and remained high even towards the end of the experiment at 48h. Treatment with curcumin which is known to act mainly on trophozoites and schizonts also induced an increase in the expression of the four genes under study (figure 14, 16, 17, 18). This increase was observed as early as 3h after drug exposure and continues to increase up to 36 hours of drug exposure this is followed by gradual decrease. Since *pfuch-l1* is the gene that is mutated in *Plasmodium chabaudi* parasites selected under drug pressure with and artemisinin and artesunate (Hunt *et al*., 2007) a western blot was performed to analyse if there is an association between drug treatment and protein expression. Drug treatment does induce an increase in protein expression as observed in (figure 15B). Treatment with artemisinin, chloroquine and curcumin all resulted in an increase in band intensity, although bands were unable to be quantified due to time constraints, nevertheless it is evident that drug treatment alters protein expression perhaps a strategy employed by the parasite to cope with drug pressure.

The gene knockout strategy employed here to determine whether DUBs are essential for the parasite failed to produce viable knock out lines (figure 19). Indicating either the gene knockout strategy may not have been the most appropriate or that the locus where *pfuch-l1* is located is difficult to disrupt. In future attempts red blood cells loaded with plasmid constructs will be used to maintain *Plasmodium falciparum* infected cultures, although labour intensive, spontaneous DNA uptake may yield better results. The recombinant DUBs that were successfully expressed in *E.coli* cells were *Pfuch-l1* catalytic domain, *Pfuch-l3* full length protein and *Pfubp-8* catalytic domain. These proteins were successfully expressed and showed de-ubiquitylating activity in the presence of the DUB substrate Ub-AMC. NEM, a typical inhibitor of cysteine proteases, was able to abrogate enzyme activity confirming the presence of cysteine residues in their active site. Curcumin was found to inhibit *Plasmodium falciparum* recombinant DUBs with an IC50 of: 15µM for *Pfuch-l1*, for recombinant *Pfuch-l3* was 25,4µM and for *Pfubp-8 was* 10µM. However, curcumin also inhibits human recombinant DUB USP-2 with an IC50 of 5µM.If curcumin is to be used as antimalarial drug caution would have to be taken as it also apperas to interefe with human enzymes.

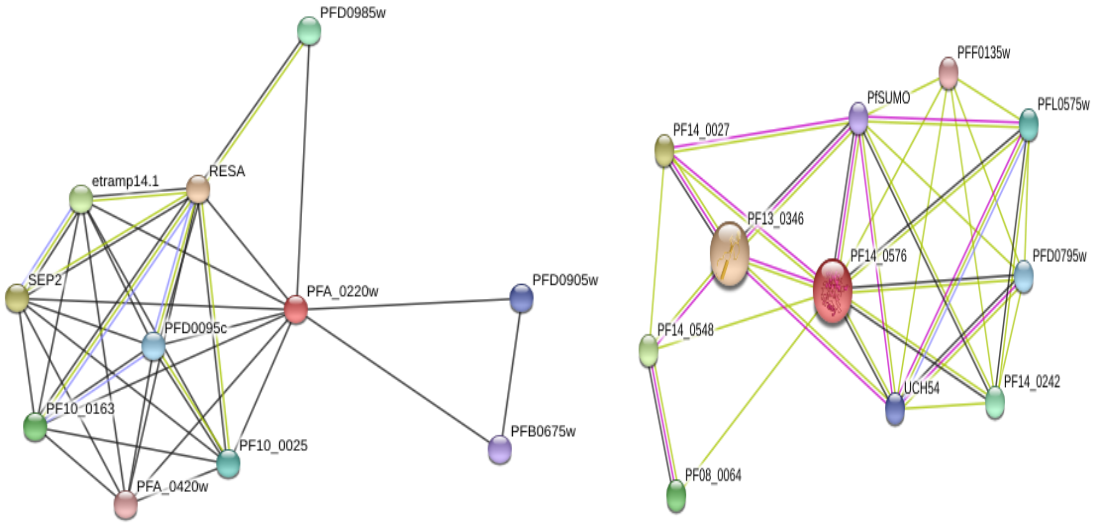
In future studies a wider panel of inhibitors of DUBs will be explored in order to idenyify the ones with better activity towards *Plasmodium* DUBs as opposed to human DUBs. This study highlights the difficulty in working with cyteine proteases, due to the large number of these proteases in the human genome and their wide cellular function it is difficult to find specific inhibitors which will cause minimal damage to the host´s proteins.

The proteomics study in the presence of curcumin revealed a total of 10 proteins that were altered in response to drug treatment. Seven proteins were found to be upregulated and three proteins were down regulated with proteins being involved in a diverse range of biological processes including: sulfur metabolism, protein trnaslation and degradation, cell cycle and cellular organization (figures 29,30,31). The limited number of proteins identified can also be attributed to the fact that only one parasite stage (schizont) was studied. If the ring stage, trophozoite stage and gametocyte stages of the parasite were subjected to DIGE analysis a wider array of proteins would have been identified. Nevertheless, the future of antimalarial drug development is worrying as resistance is becoming widespread and novel pharmacological molecules with antimalarial activity are getting more difficult to find. In the present study we conclude that curcumin is indeed an interesting antimalarial drug with a diverse range of potential targets which deserves to be investigated further.

The *in vivo* study in *P. chabaudi* parasites revelead that curcumin either alone or in combination with chloroquine can reduce parasitemia in *Plasmodium chabaudi* parasites with a resistant phenotype (figure 22,23). The combinantion of curcumin and artemisinin resulted in unfavourable pharamacokinetic interaction the two drugs did not appear to work well together (figure 24,25). This is interesting as current antimalarial drug treatment is based in association of artemisin with other antimalarial drugs in order to maximize the half life of drugs and reduce the risk of drug resistance. In this case the curcumin/piperine/chloroquine combination would be interesting to use in areas where there is no chlorquine resistance or in endemic areas in which chloroquine has been withdrawn and sentivity has returned. This highlights the importance of conducting both *in vitro* and *in vivo* studies in order to understand drug-drug interactions before new formulations of antimalarials can be launched into the clinical

setting, as observed in the present study not everything can be associated with artemsinin and poor pharamacokinetic interactions amongst drugs may facilitate de acquisition of drug resistant.

Pfuch-l1 protein ***Pfuch-l3 protein***



Pfuchl-54 protein ***Pfubp-8 protein***

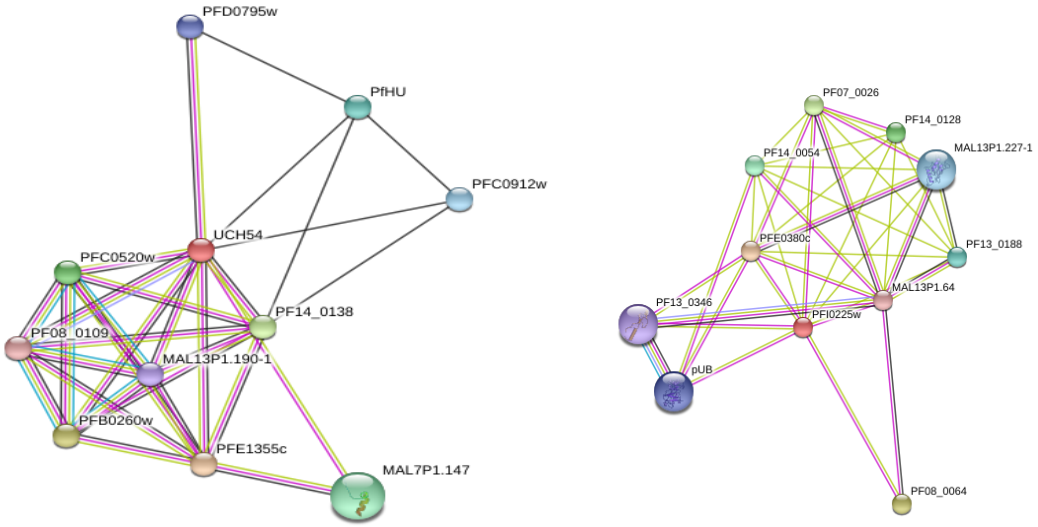


Figure 32. De-ubiquitylating enzymes (*Pfuch-l1*, *Pfuch-l3*, *Pfuch-l54*, *Pfubp-8*) interacting partners.The STRING Database has predicted the proteins inside the cell that may interact with the DUBs analyzed in this project. A complete list of the putative interacting partners of (*Pfuch-l1*, *Pfuch-l3*, *Pfuch-l54*, *Pfubp-8*) can be found in (APPENDIX H).

8.1.2 Future studies

Although DUBS were characterized here, several questions remain unanswered. It would be interesting to know what the potential *in vivo* substrates of *Pfuch-l1*, *Pfuch-l3*, *Pfuch-l5* and *Pfubp-8* gene product are. The STRING Database (protein interaction database) has predicted that *Pfuch-l1*, *Pfuch-l3*, *Pfuch-l5* and *Pfubp-8* have many intracellular interacting partners such as: ubiquitin and RESA(Ring infected erythrocyte surface antigen and many others) for a complete list of those interacting partners see (APPENDIX H, figure 32) Those could be identified by immunoprecipitation assays. Whereby all proteins in a cellular extract attached to a particular anti-DUB antibody would be precipitated and analysed by mass spectometry in order to identify and confirm the proteins which interact with DUBs *in vivo*.

As several DUB inhibitors are being developed by pharmaceutical companies it would be interesting to test a wider panel of DUB inhibitors as reports have shown that some DUB inhibitors are more selective towards UBPs rather than UCHs it would be interesting to know whether this also applies to *Plasmodium* DUBs. Several curcumin derivatives with better pharmacological and bioavailability profile have also been developed. It would be interesting to perform further antimalarial drug association studies in order to further evaluate the potential of this plant derived compound in the treatment of malaria. Future studies should also aim at further characterizing the recombinant proteins catalytic mechanism and kinetic analyses as well as incubation with other ubiquitin substrates in order to identify whether these proteins are capable of cleaving polyubiquitin as well as monoubiquitin chains providing us an indication of the intracellular processes those proteins participate in.

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CHAPTER 10-APPENDIXES

APPENDIX A - Gene sequences

pfuch-l1 gene

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Sequence length: 10500 bps

pfuch-l3 gene

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Sequence Length: 699 bps

pfuchl-54 gene

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Sequence length:1398 bps

pfubp-8 gene

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Sequence Length: 5313 bps

pfactinI gene

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Sequence length:1131 bps

Protein sequences

Pfuch-l1

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Pfuch-l3

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size:30Kds

Pfuch-l54

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Pfubp-8

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APPENDIX B

CLUSTAL 2.1 sequence alignments

Pairwaise alignment between pfuch-l1 and human ubiquitin carboxyl hydrolase 8 (18% sequence identity)

```
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HumanUCH-8      ------------------------------------------------------------

pfuch-l1        NMEKDKKLRLLNNYINMFDKNKNDKVTTNHPSHNIYNRKNNDTYDDQDKDEQYVDTDDSF 120
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        SLSNTKKKINKRDIISYDNYIFEDEDKVSSKYLEYKNDSTSHMKKKKDEGSNRKGNINMD 180
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        SNNNDDNNNNINMKSNTNNNNNNNNNNNKDDDYYDDNNYYHKNHSDSINNSINYNDIHRK 240
HumanUCH-8      ------------------------------------------------------------

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HumanUCH-8      ------------------------------------------------------------

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HumanUCH-8      ------------------------------------------------------------

pfuch-l1        DDSNNNNNDDDSNNNNNDDDSNNNNNDDSNNNNNDDSNNNNNDDSNNTGSFFKNKMIQSH 420
HumanUCH-8      ------------------------------------------------------------

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HumanUCH-8      ------------------------------------------------------------

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HumanUCH-8      ------------------------------------------------------------

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HumanUCH-8      ------------------------------------------------------------

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HumanUCH-8      ------------------------------------------------------------

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pfuch-l1        HEGLSKKMLSYHMDEQEDVQGMKSIEDDNKGGDNKGGDNKGGDKKGGDNKGGDKKGDDKK 780
HumanUCH-8      ------------------------------------------------------------

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HumanUCH-8      ------------------------------------------------------------

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HumanUCH-8      ------------------------------------------------------------

pfuch-l1        NMNSNKNNNDNNMNSNNNNNDNNMNSNNNNNDNNMNSNNNNNNNYNSSSDMKIIESNNIP 960
HumanUCH-8      ------------------------------------------------------------
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```
pfuch-l1        YSPNNNMKKKKKKKKKNLKEKLNNNIKYNEIITKTHIFPTNKIIQHDKGVEYETTNSKHL 1020
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        LHKNINNIYNQSEQNWSLHEDLLKEVLTKEEYNEKLIKKNKNKNSKINKKTVDNKETHLH 1080
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        KQIAEKYDNIHTYIVETRKDKYSPSDHEKQNSFIKERVLHSKKKIKGKKNSKRNIKMVSR 1140
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        NKENKKERTMKSQNDNHLNNHESDDNNSIENSYEESAMYDENNSSIHDDNSKKEKFSDNE 1200
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        KYHERAEEEIVSDDLYQEDDNSDHSNKKIKMNMKSMTSFDKDKRRYTIQNLEEIKKKSKK 1260
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        SINKNENDKYGYNSDYMNDSGDFAVEKKDKKKKTQQENCDNKYGNKYNKCDKDKDKDNYN 1320
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        NKDKFLPSDQAFHYDNRKAKKKNKEDILKDQYNDEHIKEYFYSLIEGQVSKNNKNKKKKN 1380
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        SQRDYSLNKSTKEKGVKKERLLHNKHFKETDSEEDQNNKKNKNNIYLKKNYDQENEKDNE 1440
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        YENEKSYKKSTRPYYEEDHTPYRKQNIQDWSSYTKDKENKLNMDDDINMNKGNDQDVNRT 1500
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        YKNEKNKEEDKYGKNEKNEKYDKYDKYEKYEKYDKYKKDNKNQHDDPLYDNINKNYDNDN 1560
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        KGLEFFSNNFFHIKKFIEKKENENVHMSKIENSQKEEELNHKRNNLNSSGKTEKLEKFLG 1620
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        LYKENNEAMDFYKSVLIEENNSMNISKNKINKNNIIDDRMKDNISKINRYNSDDTYIKVE 1680
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        NNYDNKKEMNNSDELNGNNNNNNNKNNNNNNNNNNNNNNNNNNNNNINNGGDKNRRNNFN 1740
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        NNNIYMCKNVKNIILSLELSNEEKINEVRKILFYSSSDEKKYIMNEILNILYIYPQLYVS 1800
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        CIISLFYLFILDNEIFEKYFNADDLVYLFNEKIDFRYAEWFLKTYLFYKYKYSDNTHTKG 1860
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        SIYYIKKGSPRNSIKREDSNMYADQSVMAENIKKNYLNEGNQKDDDNKNNYDDKENNYDD 1920
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        KENNYDDKENNYDDNKNNYDDNKNNYDDKKNNYDDKKNNYDDNKNNYDYNNNKNDDDDSI 1980
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        NVSSSLKGIHKNTFDPFFEKHSNNSLMDSGDDYLCDMNNLSNNKKDIYILWTYFESSKCV 2040
HumanUCH-8      ------------------------------------------------------------
```

```
pfuch-l1        GYNECKTLLSLCLKNENETCINNISASKLRSLVISIWSNIPSSKPKRSFIKLIFNWINNK 2100
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        KDDLHKKKNLFYLLKSEKKNNKNLSKICFNYFLNYLIKYKDNCSNDIIYILYLIDENELK 2160
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        IYSKNFIQNHKINFNQFISIWNIMCILFWDTDEINNFTFLQKNKYYYYDFMLIFLKTFYD 2220
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        YINVNRDMREIMKMKLKRTFLTGYHHDVEEPSQEHMSLYQEKNNIHNQDNRLSFTYMKKM 2280
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        SLSNSSINNKQDKHEDQNEYLNLFDIENIINNFNFTDFVNNEISRDNYFDSFFGATNMPI 2340
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        PSMSNISLAGNHTTQYEKNTRHNYNSPLTHPLWRNRQEKERDLQRIKDEEERLKRRGGVP 2400
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        LTEAYDIENLIFLGICIKIVICRISNLLNAKSCLQQFHYFLNHKRLGLKIFKYSHIILVY 2460
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        FIPFFKKYYFLWKFIEHEIDKDIMNLIKYIMDHLENMQVENIPLSLCNINNTSNQMLPGV 2520
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        SNQNMNERTYAENLHNMNNIHNNKFCPSSYRHTQNILNMNSTHNNSSVNNNFNKMNHSIS 2580
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        EKMGKNKNDNIFSFLKSTKNNMSFDQNGRLVNSNINYMKNKNLLLCKEEQEKHTSFQSLN 2640
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        CNRTKNNSIQERVVYGKEINNNHNLKDINVFKYKKHEHKHGEFFNLNNMKYPLYGKNKNI 2700
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        MDDDNLGNNIFHPKKKNKDEFIGSFKNNSSYVINDEDDEHYISYDDMFRNYDSDDDSNIS 2760
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        NSKNTSENFNVKDFITNLHFANLDDDNNIISKNFFSTSKKLNDQKGEQKGEQNGEQKCEQ 2820
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        KYEQKYEHQGSSVKIQNNKIINKMKYDPFLSSSESSNYNEDKNIMYMYPNEPNYKDSKKV 2880
HumanUCH-8      ------------------------------------------------------------

pfuch-l1        LSQKKKKKKSTINNFHRINSNGPHTNEEFIEKDQSTSIIGSLGQDDSFDKISHKNTHFDH 2940
HumanUCH-8      ----------------------------------------MGSS-------------HH 6
                                                         :*..             .*

pfuch-l1        HKNNPSDLTNNHMMKNVKHMKNIKQHCSNDDYSTSKYEELVNEHTIRKNTNRRNSLYAYP 3000
HumanUCH-8      HHHHSSGLVP------------------------------RGSPTVTPTVNRENKPTCYP 36
                *:::.*.*.                                .. *:  ..**.*.  .**

pfuch-l1        TQNRISDQMENQKIRKNTSLEKNVHHMNDNYDEINFTEKYFEQEYGSDQHDQRNNSMDAV 3060
HumanUCH-8      KAEIS--RLSASQIRN------------------------LNPVFGGS-----GPALTGL 65
                . :    ::. .:**:                        ::  :*..     . :: .:

pfuch-l1        NSVNHVNRMDGVNHVNRMDGVNHVNRMDGVNRVNRMNHANRVSRMNHANRVSRMNHANRV 3120
HumanUCH-8      RNLGNTCYMNSILQC----------------------LCNAPHLADYFNRNCYQDDINRS 103
                ..:.:.  *:.: :                        .*     :: ** .  :. **

pfuch-l1        SRMNHANRVSPNNIEDIRMGGVKIKKYLMLPINKFTFENMSKRNYPHPPVGLMNLGNTCY 3180
```

```
HumanUCH-8      NLLGHKGEVAEEFGIIMKALWTGQYRYISPKDFKITIGKINDQFAGYSQQDSQ------- 156
                . :.* ..*: :    ::   .   :*:     *:*: ::..:   :.  .

pfuch-l1        LNSLLQALYSTVSFIVNLFLFKINETNNKVRTVPNYEIYKSQMHQENTNSELDYFLEEIK 3240
HumanUCH-8      ----------------ELLLFLMDGLHEDLNKADNRKRYK-----EENNDHLDDFKAAEH 195
                           :*:** ::  ::.:... * : **     *:.*..** *    :

pfuch-l1        SFFKNMLTTDKSYISADRVLNMLPVELNNRNQQDVTEVFRYIFDKLGGSEKEFLRLIFSG 3300
HumanUCH-8      AWQKHKQLNES-----------------------------------------IIVALFQG 214
                :: *:   .:.                                          ::  :*.*

pfuch-l1        VVIQKVQCQKCLFISKKKEIIHDLSFPVPISTNEKLSIQRFFDTFIQKEKIYGNNKYKCS 3360
HumanUCH-8      QFKSTVQCLTCHKKSRTFEAFMYLSLPLASTS--KCTLQDCLRLFSKEEKLTDNNRFYCS 272
                 . ..*** .*   *:. * :  **:*:. ::  * ::*  :  * ::**: .**:: **

pfuch-l1        RCNKKRNALKWNEIISPPCHLILILNRYNWSFSSNEKKKIKTHVKINSKIVVNNFDYKLY 3420
HumanUCH-8      HCRARRDSLKKIEIWKLPPVLLVHLKRFSYDGRWKQKLQTSVDFPLEN-LDLSQYVIGPK 331
                :*. :*::**  ** . *  *:: *:*:.:.   ::* : .... ::. : :.::

pfuch-l1        GAIIHGGISASSGHYYFIGK---KSERQNKKKSSWYQMNDSVVTKANSKMINKISKDLSN 3477
HumanUCH-8      NNLKKYNLFSVSNHYGGLDGGHYTAYCKNAARQRWFKFDDHEVS--------DISVSSVK 383
                . : : .: : *.**  :.    .:  :*  :. *::::*  *:        .** .  :

pfuch-l1        DHTPYVLFYRCKQAPISPDLYF 3499
HumanUCH-8      SSAAYILFYTSLG--------- 396
```

Pairwise sequence alignment between Pfuch-l3 and human uch-l3(36% sequence identity)

```
Pfuch-L3        MAKNDIWTPLESNPDSLYLYSCKLGQS-KLKFVDIYGFNNDLLDMIPQPVQAVIFLYPVN 59
HumanUCH-L3     -MEGQRWLPLEANPEVTNQFLKQLGLHPNWQFVDVYGMDPELLSMVPRPVCAVLLLFPIT 59
                  :.: * ***:**:    :  :**   : :***:**:: :**.*:*:** **::*:*:.

Pfuch-L3        DN---IVSENNTNDKHNLKENFDNVWFIKQYIPNSCGTIALLHLYGNLRNKFELDKDSVL 116
HumanUCH-L3     EKYEVFRTEEEEKIKSQGQDVTSSVYFMKQTISNACGTIGLIHAIANNKDKMHFESGSTL 119
                ::   : :*:: : * : ::  ..*:*:** *.*:****.*:*  .* ::*:.::..*.*

Pfuch-L3        DDFFNKVNEMSAEKRGQELKNNKSIENLHH--EFCGQVENRDDILDVDTHFIVFVQIEGK 174
HumanUCH-L3     KKFLEESVSMSPEERARYLENYDAIRVTHETSAHEGQTEAPSIDEKVDLHFIALVHVDGH 179
                ..*:::  .**.*:*.: *:* .:*.  *.   . **.*  .   .** ***.:*:::*:

Pfuch-L3        IIELDGRKDHPTVHCFTNGDNFLYDTGKIIQDKFIEKCKDDLRFSALAVIPNDNFDII 232
HumanUCH-L3     LYELDGRKPFPINHGETSDETLLEDAIEVCK-KFMERDPDELRFNAIALSAA------ 230
                : ****** .*  *  *..:.:* *: :: : **:*:  *:***.*:*: .
```

Pairwise sequence alignment between pfuch-l5 and human uch-l5(31% sequence identity)

```
pfuch-L54       MARDNENILEEWCLIESNPCIFYDMLKRMGATEISVEDVYSLSYFDDYINNKEIINMNHI 60
HumanuchL-5     MTGNAG----EWCLMESDPGVFTELIKGFGCRGAQVEEIWSLE----------------- 39
                *: :      ****:**:* :* :::* :*.   .**:::**.

pfuch-L54       LGVDTYLGENNKTLDKENNVVDVIELYKNNICMEDKYNKLLKHHSYIYGIIFLFNIG-KH 119
HumanuchL-5     -------PENFEKLKP-----------------------------VHGLIFLFKWQPGE 62
                       ** :.*.                               ::*:****:    .

pfuch-L54       YKNNKYIEHNVPDNLFFAKQVIPNACATQAILSIVLN---KDIELNDEIKNIKTFSLNFD 176
HumanuchL-5     EPAGSVVQDSRLDTIFFAKQVINNACATQAIVSVLLNCTHQDVHLGETLSEFKEFSQSFD 122
                   .. ::..  *.:******* ********:*::**   :*:.*.: :.::* ** .**

pfuch-L54       SSMKGLTLSNCTFLRNIHNSYKPPIYLDKEDVHHDKKKSEDSFHFVSYISFQDKVYLLDG 236
HumanuchL-5     AAMKGLALSNSDVIRQVHNSFARQQMFEFDTKTSAKE--EDAFHFVSYVPVNGRLYELDG 180
                ::****:***. .:*::****:     :: :     *:  **:******:..:.::* ***

pfuch-L54       LQSGPVLINADEQNKPNPNNNNNNKDNDNDNNNNNNNNNNNNNNNNNNNNNNNNNIGMN 296
HumanuchL-5     LREGPIDLGACNQD---------------------------------------------- 194
                *:.**: :.* :*:

pfuch-L54       GKDWIEISREHIKKEIDEICNSQTNNDVRFNIIAVMKDKEYIIQEYINIHRIVKQRVNIK 356
HumanuchL-5     --DWISAVRPVIEKRIQKYS----EGEIRFNLMAIVSDR--------------------K 228
                  ***.  *  *:*.*:: .    :.::***::*::.*:                    *
```

```
pfuch-L54        LINLGENIELSDEINEDEFPLLNDIPSIENLPNNVDTLYNIVNKSTLEINYLQSLLHEQK 416
HumanuchL-5      MIYEQKIAELQRQLAEE-------------PMDTDQGNSMLSAIQSEVAKNQMLIEEEV 274
                 :*   :  **. :: *:              * :.*   .::.    *:   * *:.*:

pfuch-L54        EIKKLWNKELTFKFFNFYPFIMSSLNLMAKHKLLKDAYQKEKLKNATKS 465
HumanuchL-5        QKLKRYKIENIRRKHNYLPFIMELLKTLAEHQQLIPLVEKGK------- 316
                   :  * :: *    : .*: ****. *: :*:*: *     :* *
```

Pairwise sequence alignment between pfubp-8 and human ubiquitin protease 2(25% sequence identity)

```
 pfubp-8         MIKDKKFIIENINNKVISKDNMTKKGKKICELKEFQNINEFNNSVLISNNKYILSDLKKN 60
HumanUSP-2       ------------------------------------------------------------

pfubp-8          DNIIQNNKNVPSSNSAVNFVKDIGQHDFININQDYTNSNDNNNNNNEEYTNNYYPKNIVK 120
HumanUSP-2       ------------------------------------------------------------

pfubp-8          NNMLASQETNTKHTRCNIKHIDDIELNDKIKNSTTIIENNNNNNNIVNINNINNVDNINN 180
HumanUSP-2       ------------------------------------------------------------

pfubp-8          VNNINNVNDLNNLNNINNLNKKDYNHINENFQENINSNSNLKKKKGTYIKNCHAENYNRP 240
HumanUSP-2       ------------------------------------------------------------

pfubp-8          LNDNSNNISKDDIKEKKNNNINSTVNYDNTNTEENITSDHCNIKDDTRLEKDMEEYIKKK 300
HumanUSP-2       ------------------------------------------------------------

pfubp-8          NIYMSNSNKIINELYNNLIYDEYSENILSKKGVKEKDHIEYYEEQNIHMKANEESTNISI 360
HumanUSP-2       ------------------------------------------------------------

pfubp-8          DIPPCCQIIYDNVDDATNEQYDNSQKDTYNWYMQKTNNNKLLYHINKNLIFLKRIQQYFY 420
HumanUSP-2       ------------------------------------------------------------

pfubp-8          QKYINIKFSNDTNDYYYIIHLEWFNKLKKFINNESNDFPGSISNWELYEYTHDEIFKNYN 480
HumanUSP-2       ------------------------------------------------------------

pfubp-8          ISESNYVFSDDKNMNDNIYLKKQCLKKNLKEGKDYICTNKYMWRFLQFLYNGGPCIKRIS 540
HumanUSP-2       ------------------------------------------------------------

pfubp-8          NNIYNTFIPISSNDIMNNNIMYLLESRYIKNLFSLFNYIDHTKFIYNEPKGNEHTLYKNE 600
HumanUSP-2       ------------------------------------------------------------

pfubp-8          YYNDNDKYTHDYILEETNEKKMCAHNYHELLQFYNLKEQEKNIILYIEYDDKHINKEILD 660
HumanUSP-2       ------------------------------------------------------------

pfubp-8          EIKKIKNKNSNNKQNILISNDENFSSDSSNMYNIINAKHNDKLNTQKLFLLENDKICANS 720
HumanUSP-2       ------------------------------------------------------------

pfubp-8          HISSNMNQTEYISLDNFDADYLLNNPHNLSRGFPNSYKLDINTDNNENVDNNGNVDSNEN 780
HumanUSP-2       ------------------------------------------------------------

pfubp-8          VDSNENVDSNENVDNNENVDSNENVDNNENMDRNDNMYNNENVDNSKMFINCNKSQRSNI 840
HumanUSP-2       ------------------------------------------------------------

pfubp-8          KKSNSTNSTRRNYNRNNNNNNNKNNNNNNNNNNNNNNNNNNNSRNNNNNNNNNNNNNNN 900
HumanUSP-2       ------------------------------------------------------------
```

```
pfubp-8          NNNNNNNNNNNNHNNNNKDDNTSDNNNNNINKEEDKKNKTTNNKKKENEKDDENKCKGNLN 960
HumanUSP-2       ------------------------------------------------------------

pfubp-8          GSVEIYELKREFEENNNIIYNDSYNNRNINNVIDDLKKNEEDINKINDRNIYLSPNISAN 1020
HumanUSP-2       ------------------------------------------------------------

pfubp-8          EMNINNFNKSYNSSSNKKNSIPCNSSNGNDIYKSCEEYNNDNEKISSNGYLTTTESQSKG 1080
HumanUSP-2       ------------------------------------------------------------

pfubp-8          TTDGNTERGSIYEYENDNNNNNKNNNKNESNNNRNEKKIYYDSIENLDDVVKKRKHIKNA 1140
HumanUSP-2       ------------------------------------------------------------

pfubp-8          QNNTTNNRVCSSNCGEQQVTEKINNILDNTHLNNIQNKNHNLKNNNSSTIQNGCTIKGNE 1200
HumanUSP-2       ------------------------------------------------------------

pfubp-8          QNVKNTNNINEEDNITNLENHKKDQKKKNHIMKKKLDDIDVKQGDLKSNNHENKNDVEDN 1260
HumanUSP-2       ------------------------------------------------------------

pfubp-8          MEMDETNNNSMDPQQRCNLISVFNKQKNHKNNISNNNNKKDDDDDDQSVYSSNITNTNSS 1320
HumanUSP-2       ------------------------------------------------------------MGSS 4
                                                                               .**

pfubp-8          SLHNSCSSSSSGGNNSLYNENDISKYNIFNNNDNDNLKNLLVPNNNSNNNNNNNNIIIIN 1380
HumanUSP-2       HHHHHHSSG----------------------------------LVPRGSMNSKSAQG------ 27
                   *:  **.                                   ***... *.:. :.

pfubp-8          SNNNNNNNNNNNNFKRDNESSLNYHTSIMTKEQPAGIINYSTTCYINVVMQCLSVFFKLI 1440
HumanUSP-2       ------------------------------------LAGLRNLGNTCFMNSILQCLSN----T 50
                                                      **: * ..**::* ::****

pfubp-8          YTLHNYVTVKYKNVNMSSDENENMNSSFINKNFFTNSIPFNIFGSNNNNNKKKDECLLLT 1500
HumanUSP-2       RELRDYCLQRLYMRDLHHGSN---AHTALVEEFAKLIQTIWTSSPNDVVSPSEFKTQIQR 107
                   *::*   :    ::  ..*     : : ::* .   .:   ..*:  . .: :  :

pfubp-8          FSFKLFQLSKMHNKGKVLCVNKLLNLLNDKYSYLFEYNEQQDCHEFLLLVFDFIHNMVKV 1560
HumanUSP-2       YAPRFVGYNQQD-------AQEFLRFLLDG--------LHNEVNRVTLRPKSNPENLDHL 152
                 :: ::.  .: .       .:::*.:* *          ::: :.. *   .  .*: ::

pfubp-8          IDESVDKNNQIDYYLKKEQSIISDLFLGLIEEKITCSQCEYVNYIYQPVYNLSVNVFKK- 1619
HumanUSP-2       PDDEKGRQ-MWRKYLEREDSRIGDLFVGQLKSSLTCTDCGYCSTVFDPFWDLSLPIAKRG 211
                  *:. .::     **::*:* *.***:* ::..:**::* * . :::*.::**: : *:

pfubp-8          NPENNINDNLIEYFKKEEVN----STCEKCKCKK-MFKYSCVYKQPNILIIHLIRLQEDG 1674
HumanUSP-2       YPEVTLMDCMRLFTKEDVLDGDEKPTCCRCRGRKRCIKKFSIQRFPKILVLHLKRFSE-- 269
                  ** .: * :  : *:: ::    .** :*: :*  :*  .: : *:**:** *:.*

pfubp-8          SKIDKPIKFDMADFTIENVLKKKDNQFIEPIKKYNLCGVIVHRGLNSNCGHYICYTKRKH 1734
HumanUSP-2       SRIRTSKLTTFVNFPLRDLDLREFASENTNHAVYNLYAVSNHSGT-TMGGHYTAYCR--- 325
                 *:* ..    :.:*.:.::  ::  .       *** .*  * *  :  *** .* :

pfubp-8          SNGVNVWYKFDDSTVTSVDVEEVESAKAYCLFYQSQ------ 1770
HumanUSP-2       SPGTGEWHTFNDSSVTPMSSSQVRTSDAYLLFYELASPPSRM 367
                 * *.. *:.*:**:**.:. .:*.::.** ***:
```

"*" ” represents residues or nucleotides in that column are identical in allsequences in the alignment.

":" means that **conserved substitutions** have been observed.

"." means that **semi-conserved** substitutions are observed.

APPENDIX C - RT-PCR

RT-PCR Assays were carried out by Applied Biosystems on cDNA samples collected at:0h,3h,6h,9h,12h,15,18h,21h,24h,27h,30h,33h,36h,40h42h,46h,48h the resulting melting curves from each RT-PCR run were analyzed to make sure that there were no primer dimers and and contaminated products were amplified. Contaminated samples were discared and the RT-PCR assays were repeated where needed.

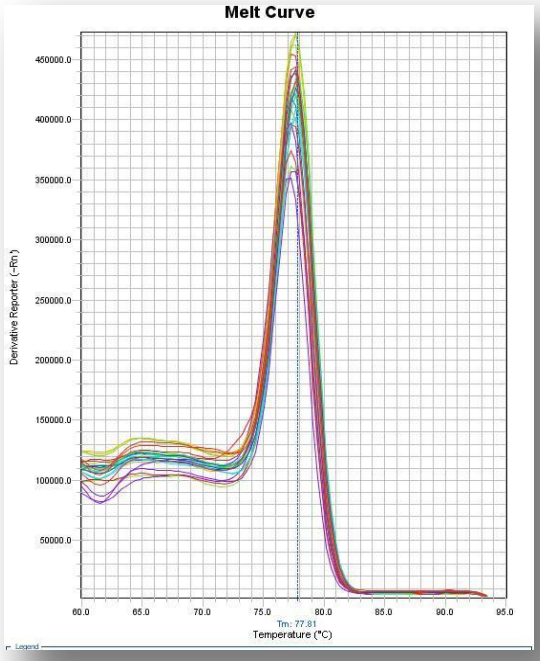


Figure 1. *pfactin* I melting curves corresponding to samples collected from 0h - 48h time point generated by Applied biosystems

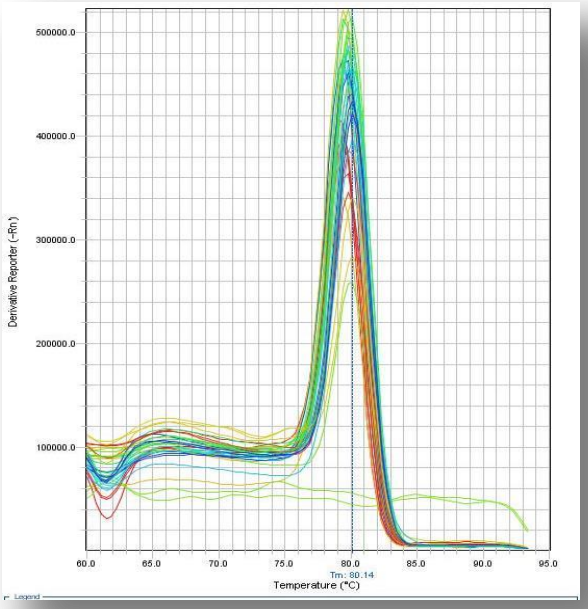


Figure 2. *pfuch-l1* melting curves corresponding to samples collected from 0h-48h time point generated by Applied biosystems.

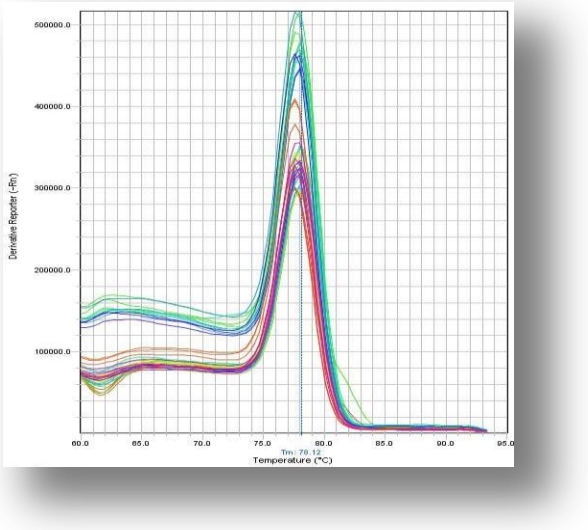


Figure 3.*pfuch-l3* melting curves corresponding to samples collected from 0h-48h time point generated by Applied biosystems.

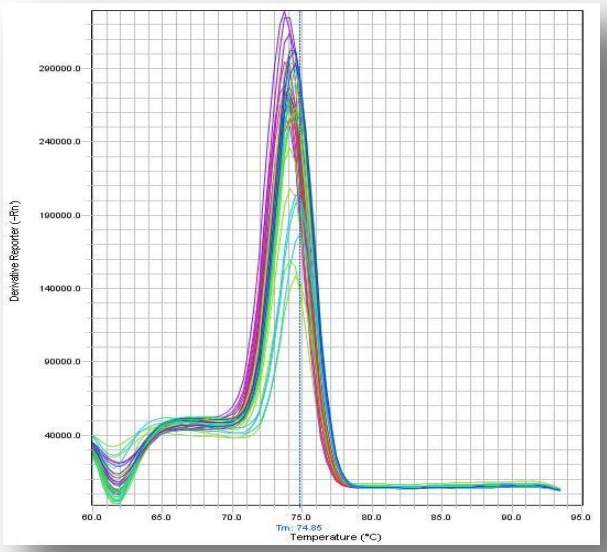


Figure 4. *pfuch-l54* melting curves corresponding to samples collected from 0h-48h time point generated by Applied biosystems.

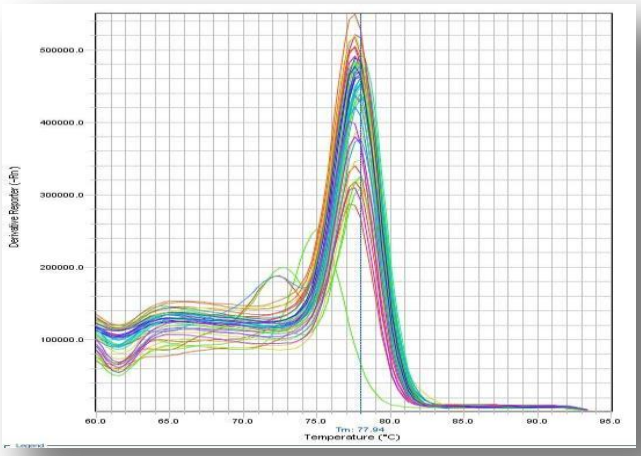


Figure 5. *pfubp-8* melting curves corresponding to samples collected from 0h-48h time point generated by Applied biosystems.

APPENDIX C- (CONTINUED).

Fold change in basal gene expression of *Plasmodium falciparum* strains 3D7 and Dd2 mRNA samples were collected over a period of 48hours. The N fold change was calculated using $2^{-\Delta\Delta ct}$ method.The results were presented in body of the text in graphical form.

Table 1. Fold change in basal expression of genes encoding DUBs in *Plasmodium falciparum* strains 3D7 and Dd2 over a period of 48 hours.

Time	***pfuch-l1***		***pfuch-l3***		***pfuch-l54***		***pfubp-8***	
	3D7	Dd2	3D7	Dd2	3D7	Dd2	3D7	Dd2
0h	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3h	1.20	1.30	0.95	0.93	-0.70	-0.80	0.81	0.70
6h	1.25	1.27	0.96	0.96	-0.72	-0.82	0.82	0.70
9h	1.27	1.37	0.98	0.98	-0.61	-0.71	0.83	0.75
12h	1.28	1.38	0.95	0.94	-0.73	-0.73	0.88	0.77
15h	1.29	1.30	0.99	0.93	-0.69	-0.99	0.90	0.80
18h	1.10	1.10	1.12	1.10	1.00	1.10	0.99	0.82
21h	-0.90	-0.99	1.29	1.22	1.55	1.50	1.00	0.90
24h	-0.80	-0.96	2.00	1.99	1.65	2.00	1.10	0.95
27h	-0.70	-0.94	2.10	2.09	2.40	2.50	0.99	0.99
30h	-0.72	-0.88	2.10	2.10	2.69	2.49	1.10	1.00
33h	-0.99	-0.89	2.00	2.00	2.50	2.35	1.00	1.10
36h	1.97	1.97	2.01	1.99	2.55	2.52	0.99	1.15
39h	2.00	2.00	1.98	2.01	2.00	2.00	0.80	1.00
42h	2.45	2.45	1.80	2.00	1.99	1.80	0.78	0.99
45h	2.50	2.50	1.77	1.99	1.50	1.50	0.66	0.87
48h	2.00	2.00	1.70	1.70	1.00	0.80	0.55	0.75

Table 2. Fold change in basal expression of genes encoding DUBs in *Plasmodium falciparum* strains 3D7 and Dd2 over a period of 48 hours in the presence of artemisinin.

Time	***pfuch-l1***		***pfuch-l3***		***pfuch-l54***		***pfubp-8***	
	3D7	Dd2	3D7	Dd2	3D7	Dd2	3D7	Dd2
0h	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3h	1.25	1.32	1.15	1.20	1.34	1.39	1.14	1.22
6h	1.30	1.42	1.28	1.29	1.42	1.45	0.82	0.70
9h	1.37	1.45	2.00	1.99	1.50	1.55	1.40	1.45
12h	1.50	1.40	2.12	2.10	1.60	1.55	1.55	1.67
15h	1.75	1.78	2.15	2.16	1.69	1.73	0.90	0.80
18h	1.80	1.80	2.15	2.19	1.80	1.83	0.99	0.82

21h	2.10	2.00	2.18	2.18	1.95	1.99	1.00	0.90
24h	2.25	2.15	2.27	2.23	2.08	2.00	1.10	0.95
27h	2.30	2.29	2.30	2.25	2.10	2.15	2.15	2.20
30h	2.39	2.40	2.40	2.38	2.30	2.25	2.30	3.32
33h	2.43	2.63	2.38	2.29	2.69	2.72	2.40	2.42
36h	2.00	2.00	2.27	2.20	2.70	2.00	2.40	2.45
39h	1.99	1.89	2.00	2.00	2.00	1.99	2.00	2.00
42h	1.85	1.87	1.99	1.89	1.90	1.75	1.90	1.86
45h	1.72	1.70	1.83	1.78	1.83	1.63	1.75	1.75
48h	1.60	1.55	1.80	1.65	1.70	1.50	1.63	1.63

Table 3. Fold change in basal expression of genes encoding DUBs in *Plasmodium falciparum* strains 3D7 and Dd2 over a period of 48 hours in the presence of chloroquine.

Time	***pfuch-l1***		***pfuch-l3***		***pfuch-l54***		***pfubp-8***	
	3D7	Dd2	3D7	Dd2	3D7	Dd2	3D7	Dd2
0h	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3h	1.22	1.23	1.12	1.10	1.13	1.10	1.33	1.35
6h	1.32	1.33	1.29	1.22	1.15	1.16	1.35	1.37
9h	1.49	1.50	2.00	1.99	1.25	1.27	1.40	1.40
12h	1.35	1.39	2.10	2.09	1.30	1.32	1.49	1.45
15h	1.42	1.40	2.15	2.10	1.32	1.35	1.50	1.47
18h	1.55	1.57	2.00	2.00	1.40	1.42	1.55	1.50
21h	1.75	1.78	1.99	2.01	1.42	1.44	1.60	1.55
24h	1.77	1.80	2.05	2.09	1.52	1.57	1.65	1.58
27h	2.00	2.08	2.10	2.12	1.65	1.67	1.80	1.60
30h	2.15	2.16	2.24	2.20	1.70	2.00	2.15	2.10
33h	2.27	2.28	2.35	2.36	1.10	2.20	2.00	2.00
36h	2.00	1.99	2.00	2.40	2.60	2.63	1.95	1.99
39h	2.13	1.98	1.99	2.33	2.70	2.72	1.92	1.95
42h	2.10	1.77	1.85	2.25	2.42	2.55	1.90	1.93
45h	2.00	1.78	1.72	1.99	2.00	2.14	1.85	1.90
48h	1.95	1.79	1.78	1.80	1.99	2.00	1.60	1.60

Table 4. Fold change in basal expression of genes encoding DUBs in *Plasmodium falciparum* strains 3D7 and Dd2 over a period of 48 hours in the presence of curcumin.

Time	***pfuch-l1***		***pfuch-l3***		***pfuch-l54***		***pfubp-8***	
	3D7	Dd2	3D7	Dd2	3D7	Dd2	3D7	Dd2
0h	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3h	0.81	0.70	1.22	1.27	1.22	1.22	1.15	1.70
6h	0.82	0.70	1.28	1.28	1.32	1.33	1.23	1.60

9h	0.83	0.75	2.00	2.00	1.49	1.49	1.45	1.75
12h	0.88	0.77	2.22	2.30	2.20	2.10	2.00	2.17
15h	0.90	0.80	2.30	2.50	1.97	2.00	2.27	2.30
18h	1.99	1.99	2.33	2.53	2.10	2.00	2.45	2.47
21h	2.00	2.10	2.30	2.50	1.79	2.00	2.50	2.56
24h	2.50	2.40	2.50	2.70	1.69	1.69	2.65	2.70
27h	2.55	2.65	2.55	2.75	1.69	1.69	2.22	2.56
30h	3.10	3.10	2.68	2.70	1.62	1.55	2.30	2.75
33h	2.80	2.70	2.90	2.65	1.70	1.65	2.40	2.45
36h	2.00	1.98	2.50	2.50	1.69	1.34	2.00	2.00
39h	1.90	1.88	2.00	2.43	1.63	1.68	1.99	1.98
42h	0.78	0.99	1.93	1.99	1.67	1.65	1.75	1.86
45h	0.68	0.87	1.50	1.67	1.66	1.62	1.64	1.74
48h	0.55	0.75	1.45	1.40	1.64	1.60	1.38	1.25

APPENDIX D- Immunization protocol

Immunization protocol and calendar used for the production of polyclonal antibodies.

Calendar	Procedure	Purpose
Day 1	Collect 0.2 ml of blood followed by immunization with CFA	Used as pre-immune serum
Day 14	Collect 0.2 ml of blood followed by second immunization with IFA	Test antibody titres by ELISA and Western Blot
Day 21	Collect 0.2 ml of blood followed by third immunization with IFA	Test antibody titres by ELISA and Western Blot
Day 35	Collect 0.2 ml of blood followed by a fourth immunization with IFA	Test antibody titres by ELISA and Western blot

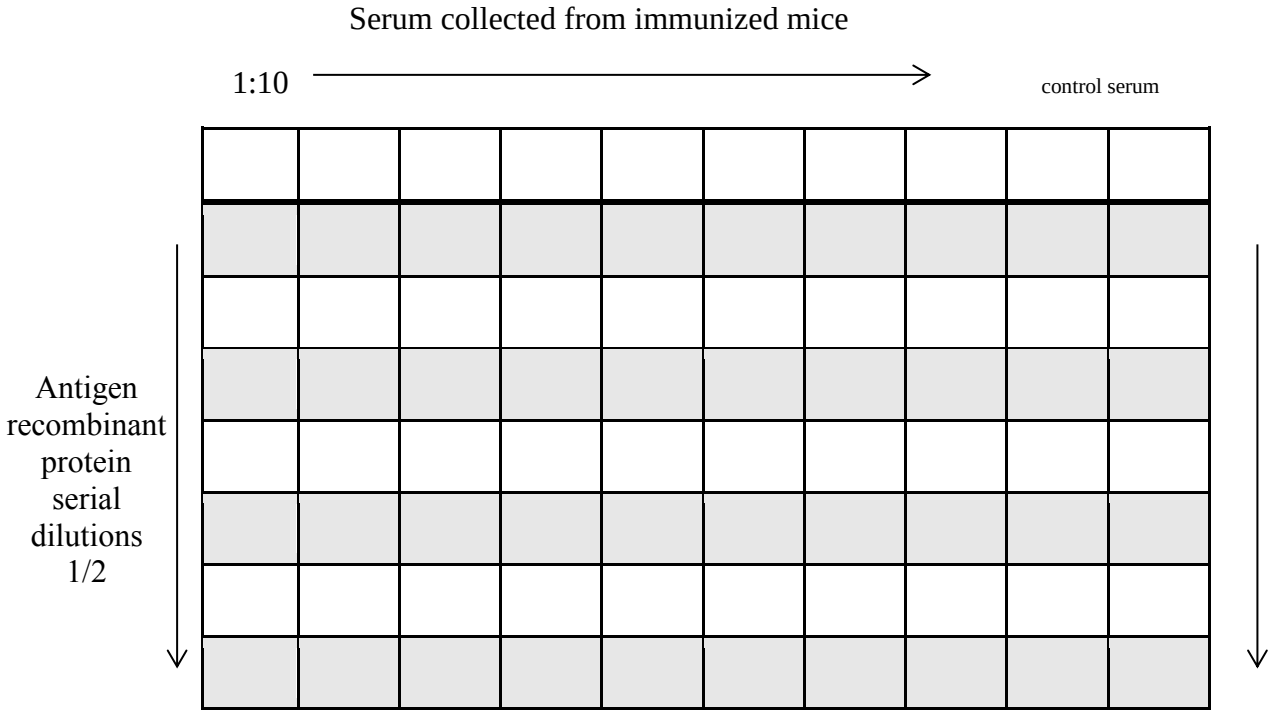
ELISA PROTOCOL

- 2 µg/ml of recombinant proteins dissolved in PBS was used to coat 96 wells plates overnight at 4ºC.
- Plates were blocked with 5% non fat milk in PBS-TWEEN (PBS-T) for 60 mins.
- Plates were washed with PBS-T four times
- Appropriate dilutions of serum collected from mice were made in PBS-T and 3% BSA
- Incubate plates for 60 minutes and 25ºC
- Wash plates four times with PBST

- Add anti mouse IgG HRP conjugate diluted 1/2000 in PBST 3% BSA incubate the plate at 60mins 25ºc degrees.

- Wash plates four times with PBS-T

- After the final wash add 100µl of substrate solutrion (5mM citrate buffer, 4mM 2.2 azinobis (3 ethylbenzthiazoline-6-sulfonic acid) diammonium salt and 1mM H_2O_2 after 10 minutes of incubation absorbance was read at 414nm.



APPDENDIX-E

Plate readings

Determination of the *in vitro* IC50 in *Plasmodium falciparum* strains 3D7 and Dd2 IC50 was determined using the software HN-NON Lin V1.1.

Dd2	Chloroquine			
	log (concentration+1)	Y obs	Y %	Y% pred
A	0,00	10707,963	100,00	88,02
B	0,41	7088,397	43,95	63,47
C	0,62	7043,373	43,25	41,30
D	0,86	6085,941	28,42	26,40
E	1,13	6233,084	30,70	19,34
F	1,41	5012,131	11,79	16,38
G	1,71	4514,023	4,08	11,15
H	2,00	4250,657	0,00	0,00
			Correlation	
EC50	290nM		R^2	0,8949

3D7	Chloroquine			
	Log(concentration+1)	Y obs	Y %	Y% pred
A	0,25	23288,063	100,00	94,30
B	0,41	19314,496	71,91	79,36
C	0,62	18277,991	64,59	61,82
D	0,86	15026,219	41,60	44,00
E	1,13	12558,344	24,16	28,07
F	1,41	12147,305	21,25	15,51
G	1,71	10546,072	9,93	7,11
H	2,00	9141,093	0,00	3,26
			Correlation	
EC50	12,0 nM		R^2	0,9797

3D7	Curcumin			
	log (concentration+1)	Y obs	Y %	Y% pred
A	0,00	22781,000	100,00	94,09
B	0,41	16809,237	57,80	66,06
C	0,62	13717,727	35,95	39,90
D	0,86	12759,950	29,18	21,30
E	1,13	10538,710	13,48	11,87
F	1,41	9541,998	6,44	8,82
G	1,71	9235,994	4,28	6,61
H	2,00	8631,062	0,00	0,00
			Correlation	
EC50	5000 nM		R^2	0,9757

Dd2	Curcumin			
	log (concentration+1)	Y obs	Y %	Y% pred
A	0,00	25782,054	100,00	100,00
B	0,41	23060,911	81,75	77,75
C	0,62	18181,909	49,03	54,76
D	0,86	16508,266	37,81	36,09
E	1,13	14349,871	23,33	23,35
F	1,41	13768,536	19,43	15,18
G	1,71	11338,534	3,14	8,25
H	2,00	10870,643	0,00	0,00
			Correlation	
EC50	5500 nM		R^2	0,9891

3D7	Artemisinin			
	log (concentration+1)	Y obs	Y %	Y% pred
A	0,00	23311,624	100,00	97,40
B	0,41	20966,172	73,56	77,55
C	0,62	19603,704	58,20	59,24
D	0,86	18751,368	48,59	46,00
E	1,13	18095,578	41,19	37,85
F	1,41	16725,159	25,74	31,35
G	1,71	16507,090	23,29	20,95
H	2,00	14441,565	0,00	0,22
EC	Conc		Correlation	
EC50	4,1052		R^2	0,9886

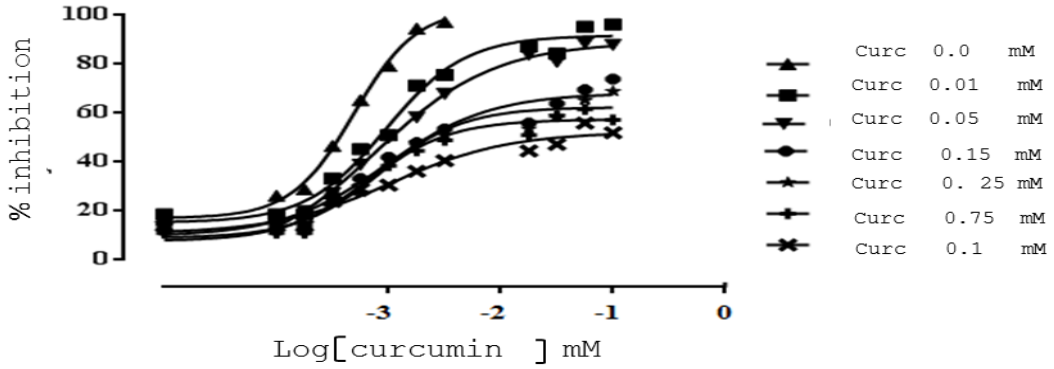
INDIVIDUAL REGRESSION PARAMETERS				
DD2	Artemisinin	Dd2		
	log (concentration+1)	Y obs	Y %	Y% pred
A	0,00	10707,963	100,00	95,31
B	0,41	9816,623	79,95	87,70
C	0,62	9852,974	80,77	76,19
D	0,86	8521,479	50,83	61,06
E	1,13	8868,772	58,64	43,61
F	1,41	7269,572	22,67	25,74
G	1,71	6411,165	3,36	9,54
H	2,00	6261,586	0,00	0,00
EC	Conc		Correlation	
EC50	6,3757		R^2	0,9502

Figure 6. Determination of the in *vitro* IC50 of artemisinin, chloroquine, and curcumin on *Plasmodium falciparum* strains 3D7 and DD2.

APPENDIX F - Determination of Curcumin IC50 on recombinant proteins

Recombinat proteins was incubated with various doses of curcumin, a log dose response curve was plotted where log concentration of curcumin was plotted against % enzyme activity using GraphPad version 4.

Pfuch-l1



Pfuch-l3

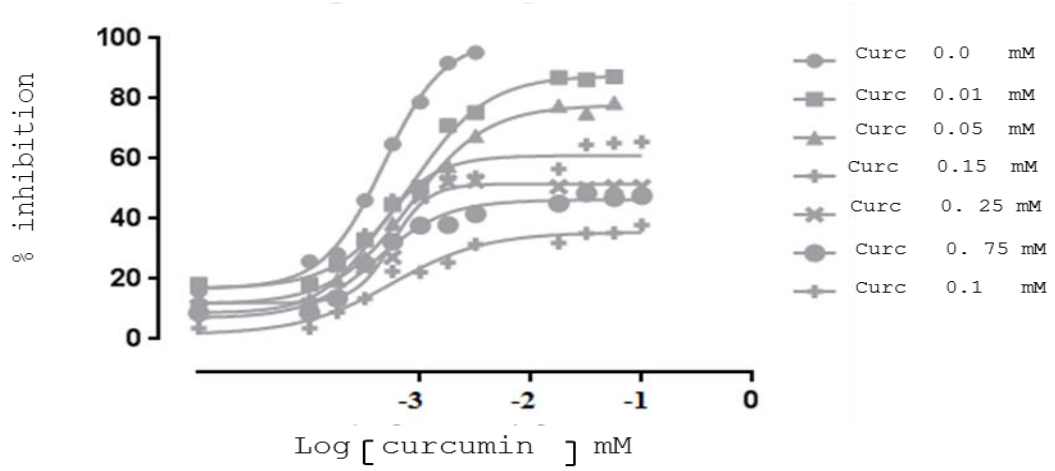


Figure 7. Log vs dose response curve generated by GraphPAD. The log concentration of curcumin was plotted against the % inhibition and the IC50 was determined.

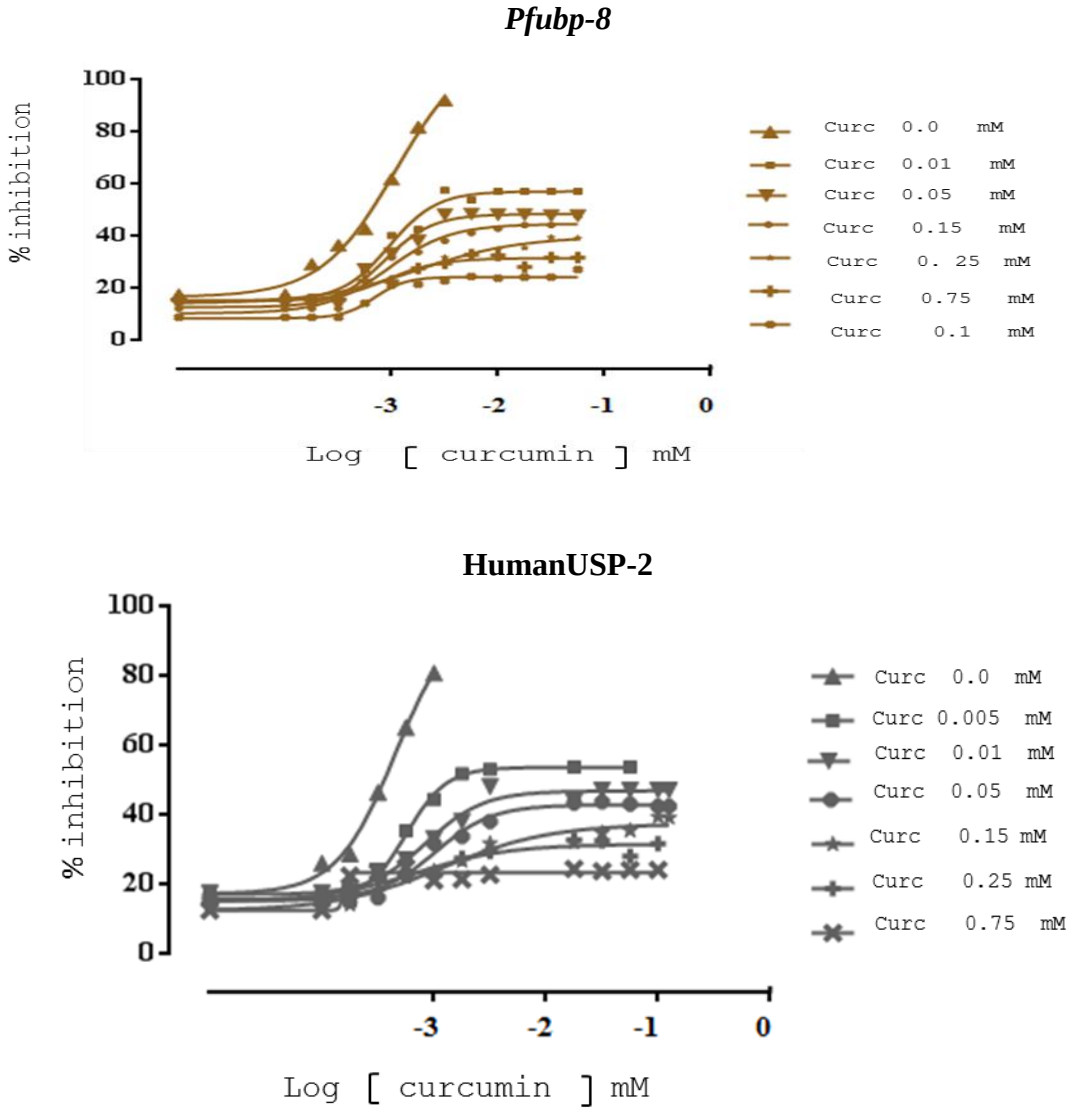


Figure 8. Log vs dose response curve generated by GraphPAD. The log concentration of curcumin was plotted against the % inhibition and the EC50 was determined.

APPENDIX G-Isoelectric Focusing run

Proteomics Isoelectrofocusing run.

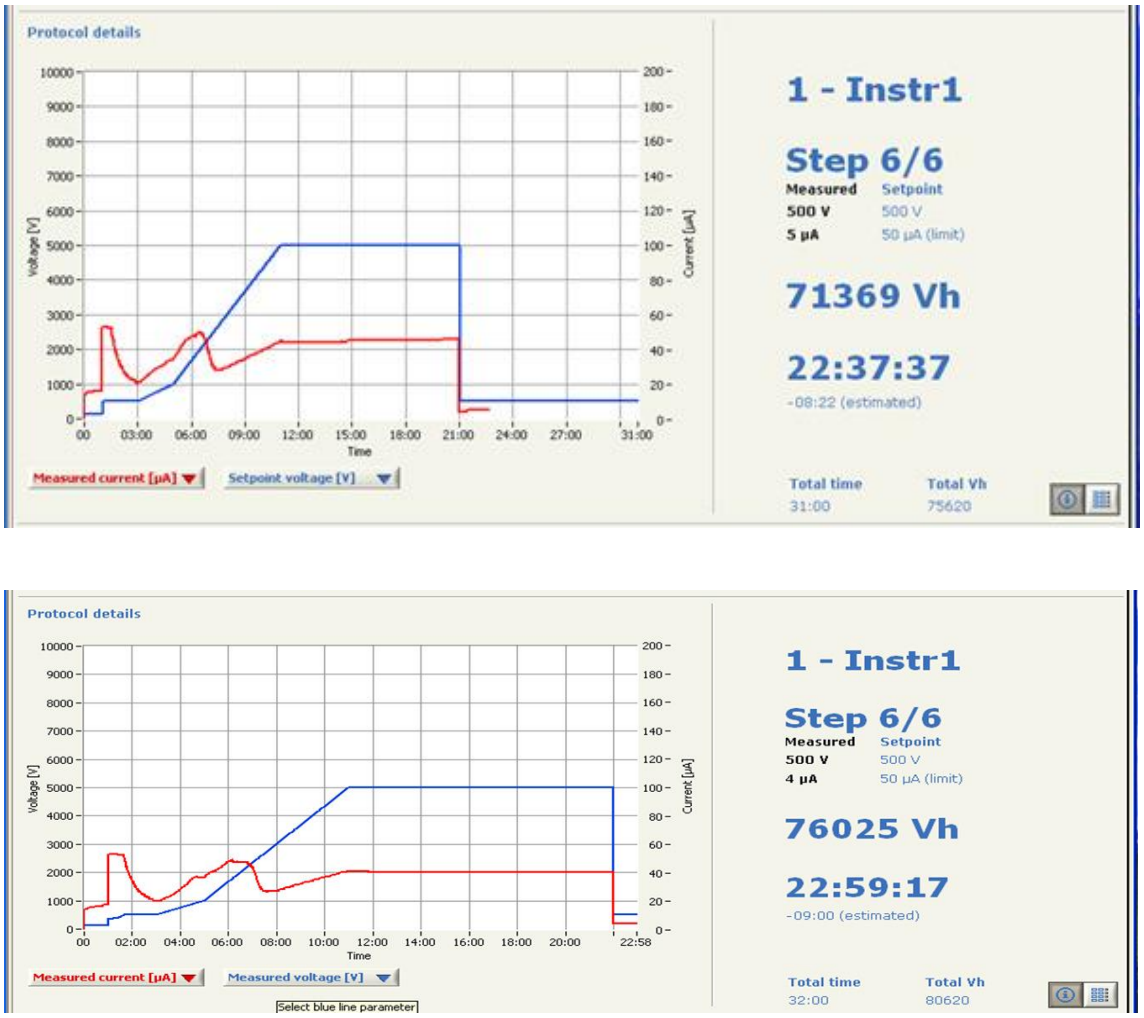


Figure 9. Isoelectric focusing run. Isoelectric focus was performed on a IPGPhor control software which records parameters such as voltage, current and volthours during the run.

APPENDIX H

De-ubiquitylating enzymes interacting partners

STRING Database derived putative interacting partners of the de-ubiquitylating enzymes analyzed in this project. If the score value is above 0.6 it is a strong indication that the identified protein is a real interacting partner.Predictive interaction partners are based on co-expression, homology, co-occurrence, gene fusion, neighborhood,text mining experiments.

PF14_0576 ***Pfuch-l3***

Predicted Functional Partners:

		Neighborhood	Gene Fusion	Cooccurrence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
PF13_0346	Ubiquitin ribosomal fusion protein uba52 homologue, putative (128 aa)					•		•		0.900
PF14_0027	ribosomal S27a, putative (149 aa)							•		0.743
PF14_0548	ATPase, putative (419 aa)							•		0.723
PF08_0064	hypothetical protein, conserved (209 aa)							•		0.720
PF14_0242	arginine n-methyltransferase, putative (401 aa)							•		0.710
PFL0575w	hypothetical protein, conserved (2961 aa)							•		0.706
PFD0795w	hypothetical protein, conserved (1267 aa)							•		0.703
UCH54	ubiquitin C-terminal hydrolase, family 1, putative (465 aa)							•		0.698
PfSUMO	ubiquitin-like protein, putative (100 aa)							•		0.691
PFF0135w	hypothetical protein, conserved (646 aa)							•		0.688

APPENDIX H (CONTINUED)

UCH54 (PF11-0177) ***Pfuch-l54***

Predicted Functional Partners:

		Neighborhood	Gene Fusion	Cooccurrence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
PFE1355c	ubiquitin carboxyl-terminal hydrolase, putative (605 aa)					•		•		0.929
PFB0260w	proteasome 26S regulatory subunit, putative (969 aa)					•		•		0.921
PF14_0138	hypothetical protein (227 aa)				•	•		•		0.919
PFC0520w	26S proteasome regulatory subunit S14, putative (304 aa)					•		•		0.914
MAL7P1.147	ubiquitin carboxyl-terminal hydrolase, putative (3183 aa)							•		0.912
PfHU	bacterial histone-like protein, putative (189 aa)				•					0.861
PFC0912w	hypothetical protein, conserved (179 aa)				•					0.857
PFD0795w	hypothetical protein, conserved (1267 aa)				•	•		•		0.857
MAL13P1.190-1	proteasome regulatory component, putative (503 aa)					•				0.855
PF08_0109	hypothetical protein, conserved (481 aa)					•		•	•	0.851

APPENDIX H (CONTINUED)

PFA_0220w ***Pfuch-L1***

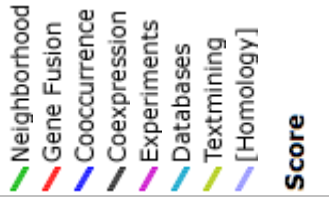
Predicted Functional Partners:

		Neighborhood	Gene Fusion	Cooccurrence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
RESA	ring-infected erythrocyte surface antigen; (1085 aa)				•					0.669
SEP2	early transcribed membrane protein 2, ETRAMP2 (106 aa)				•					0.665
etramp14.1	early transcribed membrane protein 14.1, etramp14.1 (107 aa)				•					0.665
PF10_0163	hypothetical protein (314 aa)				•					0.661
PFD0985w	hypothetical protein, conserved (3473 aa)				•					0.655
PF10_0025	PF70 protein (631 aa)				•					0.655
PFD0095c	hypothetical protein, conserved in *P.falciparum* (575 aa)				•					0.581
PFD0905w	hypothetical protein, conserved (569 aa)				•					0.547
PFB0675w	hypothetical protein (1371 aa)				•					0.547
PFA_0420w	hypothetical protein, conserved (179 aa)				•					0.547

APPENDIX H (CONTINUED)

PFI0225w ***Pfubp-8***

Predicted Functional Partners:



PFE0380c	hypothetical protein, conserved (531 aa)	0.672
PF08_0064	hypothetical protein, conserved (209 aa)	0.662
PF07_0026	ubiquitin-protein ligase E3, putative (961 aa)	0.649
PF14_0128	ubiquitin conjugating enzyme, putative (299 aa)	0.643
PF14_0054	hypothetical protein, conserved (719 aa)	0.643
PF13_0188	hypothetical protein, conserved (445 aa)	0.643
MAL13P1.227-1	ubiquitin-conjugating enzyme, putative (278 aa)	0.643
pUB	PfpUB *Plasmodium falciparum* polyubiquitin (381 aa)	0.626
PF13_0346	ubiquitin%2 Ribosomal fusion protein uba52 homologue, putative (128 aa)	0.626
MAL13P1.64	ubiquitin-like protein nedd8 homologue, putative (76 aa)	0.618

APPENDIX I

DATABASES used during the elaboration of the thesis work.

PlasmoDB

It's a public database that contains the sequence of *Plasmodium* genomes including DNA, mRNA and protein sequences.www.plasmodb.org

ClustalW2

ClustalW2 is an automatic program for multiple sequence alignment for DNA or proteins.www.ebi.ac.uk

NCBI

It's the national centre for biotechnology information belonging to the USA and it provides molecular bilogy information (DNA, RNA, protein sequences) as well as links to other public databases. www.ncbi.nlm.nih.gov

PANTHER

It's a protein analysis through evolution relationship classification system to classify proteins and their genes. Proteins can classified according to family and subfamily, molecular function, biological process and pathway. www.pantherdb.org

Pfam

Is a protein database containing a larg collection of protein families each represented by multiple sequence alignment. www.pfam.sanger.ac.uk.

STRING

Its is a database of known and predicted interactions. The interactions include direct physical and indirect functional associations.

http://string-db.org