ANÁLISE DO CATABOLISMO DA HEMOGLOBINA DE *PLASMODIUM FALCIPARUM*

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Células altamente proliferativas, como o parasita da malária *Plasmodium falciparum* exigem um nível acelerado de macromoléculas, tais como proteínas, para o fornecimento de energia para manutenção de processos intracelulares. Os aminoácidos necessários para a síntese das proteínas podem ser conseguidos por síntese *de novo*, importação a partir do plasma da célula hospedeira e pela digestão de hemoglobina. Durante a digestão da hemoglobina, o heme vai ser lançado, que precisa ser desintoxicado em hemozoiná. Os seres humanos que abrigam doenças eritrocitárias relacionadas com a arquitetura da hemoglobina, como anemia falciforme, ganham uma vantagem protetora quando infectados com o patógeno da malária. O objetivo desta tese é obter ideias sobre o modo de ação do crescimento do parasita dentro de eritrócitos geneticamente diferentes, concentrando-se na via catabólica da hemoglobina plasmodial usando parasitas transgênicos. Todos os construtos clonados (12 das 13 enzimas originais) foram transfetados com sucesso para *P. falciparum* 3D7 e foram realizados ensaios de crescimento em eritrócitos geneticamente modificados. Surpreendentemente, a Dipeptidil amino peptidase 1 (DPAP1) mostrou um efeito negativo em ensaios de proliferação no sangue de células falciformes. Curiosamente, a DPAP1 foi co-localizada no vacúolo alimentar e no citosol assim como outras estruturas, presumivelmente vesículas assumindo que a DPAP1 poderia desempenhar um papel fora do catabolismo da hemoglobina. Por outro lado, a cisteína protease FP2 que participa nas duas primeiras etapas de degradação da hemoglobina, prolifera três vezes mais elevada no sangue de células falciformes do que o Mock, a célula de controle. Adicionalmente, estudos de inibidores indicam que FP2 é uma proteína essencial para o parasita sendo que a sua inibição bloqueia a hidrólise da hemoglobina e o desenvolvimento do parasita. Uma vez que os detalhes estruturais poderiam realçar o modo de ação destas proteínas e, consequentemente, poderiam ser exploradas para a descoberta de medicamentos, a cristalização de uma APP truncada e optimizada para códons foi realizada no DESY em Hamburgo, Alemanha que resultou uma estrutura cristalina difratando até 1,7 Å. Afim de analisar o perfil de atividade da amino peptidase cristalizada, foi estabelecido um ensaio de atividade em cooperação com o CEFAP e mostrou proteína ativa. O metabolismo de nutrientes do parasita abriga um alto potencial para o desenvolvimento de novos alvos de drogas. Portanto, é essencial uma compreensão da ocorrência da digestão da hemoglobina e da natureza protetora das variantes da hemoglobina.

**Palavras-chave:** *Plasmodium falciparum*. Hemoglobina. Protease. Anemia falciforme.
Abstract

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Highly proliferating cells such as the malaria parasite *Plasmodium falciparum* necessitate an accelerated level of macromolecules, such as proteins, for the maintenance of their cellular structure and function. The required amino acids for the synthesis of the proteins can be achieved by *de novo* synthesis, import from host cell plasma and digestion of haemoglobin. During haemoglobin digestion heme is going to be released which needs to be detoxified into hemozoin. Humans who harbours erythrocytic diseases related to the haemoglobin architecture such as sickle cell disease gain a protective advantage during malaria infections. The aim of this thesis is to get insights into the mode of action of parasite growth within genetically different erythrocytes by focussing on the plasmodial haemoglobin catabolic pathway using transgenic parasites.

All cloned constructs (12 from original 13 enzymes) were successfully transfected into *P. falciparum* 3D7 and growth assays in genetically modified erythrocytes were carried out. Surprisingly, the Dipeptidyl aminopeptidase 1 (DPAP1) showed a negative effect in proliferation experiments in sickle cell blood. Interestingly, DPAP1 was co-localised to the food vacuole and the cytosol as well as other structures, presumably vesicles assuming that DPAP1 could play a role outside of the haemoglobin catabolism. On the other hand, the cysteine protease FP2 which participates in the first two steps of haemoglobin degradation, proliferated three times higher in sickle cell blood than the Mock control cell line. Additionally, inhibitor studies indicate that FP2 is an essential protein to the parasite given that its inhibition blocks haemoglobin hydrolysis and parasite development.

Since structural details could highlight the mode of action of these proteins and consequently could be exploited for drug discovery, crystallisation of a truncated and codon optimised APP was performed at the DESY in Hamburg, Germany and resulted in a solved crystal structure diffracting up to 1.7 Å. In order to analyse the activity profile of the crystallised aminopeptidase, an activity assay has been established in cooperation with the CEFAP and showed active protein. Parasite’s nutrient metabolism shelters a high potential for the development of novel drug targets. Therefore, a clear understanding of the occurring haemoglobin digestion and the protective nature of haemoglobin variants is essential.

**Keywords:** *Plasmodium falciparum*. Haemoglobin. Protease. Sickle cell trait.
1. INTRODUCTION

1.1 Malaria

1.1.1 Short history of Malaria

Malaria is one of the oldest and most devastating parasitic diseases in humans which afflicted human being already 3000 years before Christ in advanced civilizations such as Egypt, India and China. Nevertheless, there have also been several malaria epidemics in Southern and Central Europe in the middle Ages. The cause of the as the "intermittent fever" or "marsh fever" known disease was suspected in the bad air (Old Italian "mal 'aria"). This expression arose from the idea that the pathogens are transmitted in swamps through the air. Therefore, the draining of swamps was an important step in the fight against malaria (WESTHEIDE; RIEGER, 1996). Only in 1880 the French doctor Charles Louis Alphonse Laveran discovered in the blood of deceased malaria patients the malaria parasite Plasmodium (CALMETTE, 1922). However, the association to carriers could only be formed 17 years later by the British physician Ronald Ross (LUCIUS; LOOS-FRANK, 2008).

The origin of malaria in South America is controversial. On the one hand, a recent introduction is suspected by the European colonization and the transatlantic slave trade (CONWAY et al., 2000; CONWAY, 2003; NEAFSEY et al., 2008). On the other hand, other archaeological and genetic studies suggested a much older origin (CALDAS DE CASTRO; SINGER, 2004; JOY et al., 2003; TAYLOR et al., 2013). Brazilian malaria was first reported as "tertian and quartan fevers" affecting the Tupinambá Indians in 1587 (COURA et al., 2006; DEANE 1986).

1.1.2 Distribution

Globally, an estimated 3.2 billion people in 95 countries and territories are living in malaria endemic areas (Figure 1). This devastating parasitic disease is widespread in Africa, Asia and South America, but also occurs with much lower frequency in the Middle East and in some areas of Europe.

There were approximately 214 million cases (uncertainty range 149–303 million) of malaria worldwide and approximately 438 000 malaria deaths (range 236 000–635 000), where children in the African region aged under 5 years accounted two thirds of all death (WORLD HEALTH ORGANISATION, 2015).
2.1.3 Types of malaria

Since the disease progress can vary a lot, it may be distinguished between the falciparum malaria, the tertian malaria and quartan malaria. Tertian malaria is caused by the two parasites \textit{P. ovale} or \textit{P. vivax}. This disease leads to fever attacks that recur classically in a rhythm of 48 hours. Due to the regularity of these bouts of fever and low parasitaemia (up to 2%), this form of malaria can be treated well. However, both types form dormant stages in the liver (so called hypnozoites) as opposed to the following ones. Thereby, even after month- or yearlong asymptomatic intervals new malaria relapses may be triggered. Infection with \textit{P. malariae} causes quartan malaria which is characterized by a 72-hour fever rhythm. The mortality for this form of the disease with a low parasitaemia of up to 1% is very low. Indeed, current research indicates that so far as \textit{P. malariae} infections diagnosed, fatal malaria cases were often caused by \textit{P. knowlesi} (COX-SINGH et al., 2008). The falciparum malaria is considered the most dangerous of the known types of malaria, caused by \textit{P. falciparum}. Due to the occurrence of irregular bouts of fever and with a parasitaemia of up to 20% the infection takes a rapid and severe course. The only type of human-pathogenic
Plasmodium inducing the formation of small, in the erythrocyte membrane lying structures (knobs) is *P. falciparum*. They are composed of proteins that mediate cytoadherence. Thereby, infected erythrocytes can easily adhere to endothelia of capillaries and other erythrocytes (rosetting) impairing or preventing the blood flow. The resulting circulatory disorders can cause serious damage to the brain and other organs (MEHLHORN; PIEKARSKI, 2002). The described cerebral form of malaria is difficult to treat and often leads to death (CENTER FOR DISEASE CONTROL AND PREVENTION, 2005; KAYSER et al., 1997), which is also one of the causes of high mortality among infection with falciparum malaria. Malaria is presently undergoing resurgence and the fight against *P. falciparum* - the most virulent species accounting for over 90% of deaths - has become a significant problem (GREENWOOD et al., 2008).

1.1.3 Comprehensive life cycle of Plasmodium falciparum

The life cycle of the various *Plasmodium* species is very similar in many respects. An obligate host alternation between the final host, the female Anopheles mosquito, and the intermediate host, the human in the case of *P. falciparum*, is essential for proliferation. Two asexual proliferation steps (schizogonies) in the intermediate host held specifically in the liver and in the erythrocytes, are realized, whereas the sexual reproduction (Sporogony) takes place in the mosquito. During a blood meal by a female Anopheles mosquito approximately 8-15 sporozoites enter the bloodstream or lymph of the human host quickly invading hepatocytes. During the next 6 days, the liver stage parasites differentiate and undergo asexual multiplication resulting in about 30000 merozoites that burst from the hepatocyte (exoerythrocytic schizogony) (GREENWOOD et al., 2008). After their release from the merosomes (merozoites-filled vesicles) individual merozoites infect erythrocytes and undergo an additional round of multiplication producing 12-16 merozoites within a schizont (erythrocytic schizogony) (STURM et al., 2006). The length of this erythrocytic stage of the parasite lifecycle depends on the parasite species: irregular cycle for *P. falciparum*, 48 hours for *P. vivax* and *P. ovale*, and 72 hours for *P. malariae*. Whereas the exoerythrocytic multiplication only occurs once, the subsequent erythrocytic schizogony is a permanently repeating process (LUCIUS; LOOS-FRANK, 2008). Upon penetration of the merozoite in the erythrocyte that is enclosed by the host membrane.
Within the resulting parasitophorous vacuole (PV) the parasite evolves from the ring stage over the young and old trophozoites to mature schizonts. During lysis of the erythrocytes proteins and other metabolites of the parasite are released. These antigens are recognized by the immune system causing the clinical manifestations of malaria: fever and chills. The released merozoites go on to invade further erythrocytes. Not all merozoites divide into schizonts, some differentiate into sexual forms, male and female gametocytes (exflagellation). These gametocytes are taken up by the female Anopheles mosquito during another blood meal. Within the mosquito midgut, the male gametocyte undergoes a rapid nuclear division producing eight flagellated microgametes that fertilize the female macrogamete. The resulting zygote become motile and elongated, so called ookinete, and traverses the mosquito gut wall and encysts on the exterior of the gut wall as an oocyst. Sporogony is taking place in the oocyst and subsequently, the oocyst ruptures releasing hundreds of sporozoites into the mosquito body cavity where they eventually migrate to the mosquito saliva glands and the life cycle can start again (LUCIUS; LOOS-FRANK, 2008; MEHLHORN; PIEKARSKI, 2002).
1.1.4 Haemoglobin digestion

For proliferation, the malaria parasite requires amino acids for the synthesis of its proteins. There are three sources of amino acids: *de novo* synthesis, import from host cell plasma, and digestion of haemoglobin. Haemoglobin is an extremely abundant protein in the erythrocyte cytoplasm and serves as the major source of amino acids for the parasite. Malaria parasites degrade 65 - 75% of host haemoglobin (SKINNER-ADAMS et al., 2009). During the initial stage of erythrocyte infection, known as the ring stage, haemoglobin is taken up by pinocytosis resulting in double membrane vesicles where the digestion of haemoglobin takes place during the early trophozoite stage.

*Figure 2 - Comprehensive lifecycle of Plasmodium falciparum* (according to DELVES et al., 2012).
Lysosomal vesicles transfer haemoglobin to acidic digestive vacuoles in an actindependent process that is regulated by Rab5 and PfPI3K proteins (ELLIOTT et al., 2008; VAID et al., 2010) which subsequently fuse with the food vacuole. The inner membrane (originally the PVM) is lysed and the haemoglobin is released into the food vacuole (SLOMIANNY, 1990; YAYON et al., 1984). The food vacuole is an acidic compartment (pH 5.0 - 5.4) that contains acidic protease and phosphatase activities for the digestion of proteins, in particular haemoglobin, and dephosphorylation of nutrients (GLUZMAN et al., 1994, MÜLLER et al., 2010). During haemoglobin digestion heme is going to be released. Free heme is toxic due to its ability to form reactive oxygen species (ROS) and thereby destabilizes the food vacuole membrane as well as other membranes which results in the death of the parasite. Heme can be detoxified within the parasite by polymerisation. Currently, three mechanisms by which heme could be detoxified have been identified. One suggestion how heme detoxification could take place is that H2O2 oxidizes the porphyrin ring which leads to its opening and subsequent breakdown. Another possibility is that some of the heme translocates across the food vacuole membrane into the host cytoplasm where it is oxidized by reduced glutathione. Currently, a new protein, HDP (heme detoxification protein) was identified. JANI et al (2008) investigated that HDP binds heme with a high affinity and converts it rapidly to hemozoin, also known as the malaria pigment. HDP then delivers the produced hemozoin dimers to the lipid nanospheres and it is proposed that interaction between HDP and lipids form the hemozoin crystals. Furthermore, HDP is also expressed at mosquito and liver stages suggesting that the protein has more than one function.

Several distinct protease activities, representing three of the four major classes of proteases, have been identified in the food vacuole (multiple plasmepsins, falcipains and falcilysins). Haemoglobin is sequentially digested by aspartic proteases, cysteine proteases, metalloproteases, and aminopeptidases by a semi-ordered process involving the sequential action of different proteases (GOLDBERG et al., 2005). Several plasmepsin genes have been identified in the genome of P. falciparum and four of these appear to function in the food vacuole (BANERJEE et al., 2002). Plasmepsin-1 and Plasmepsin-2 are the best characterised and initiate the degradative process by cleaving non-denatured haemoglobin between phenylalanine and leucine residues. Cleavage at this site presumably causes the globin subunits to dissociate and partially unfold exposing additional protease sites within the globin polypeptide.
chains. The other plasmepsins and falcipains are then able to further degrade these large globin fragments. It has been suggested that falcipain-2 (SHENAI et al., 2000), and possibly falcipain-3 (SIJWALI et al., 2001), are capable of digesting either native haemoglobin. The proposed pathway of haemoglobin digestion involves an initial cleavage by plasmepsin-1 (and possibly falcipain-2) followed by the combined actions of several plasmepsins and falcipains (Figure 3). The peptide fragments produced by these digestions (up to 20 amino acids) are then digested into smaller peptides by falcilysin leading to the formation of 6-8 amino acids in length. Initially no food vacuole associated exopeptidase activity could be identified within the food vacuole (KOLAKOVICH et al., 1997). However, two further amino peptidases (PfAPP and PfAAP) were identified within the food vacuole (DALAL; KLEMBÁ, 2007), which are able to convert peptides into amino acids. In addition, a dipeptidyl aminopeptidase (PfDPAP1) activity was found in the food vacuole (KLEMBÁ et al., 2004). Moreover, a neutral aminopeptidase activity has been identified in cytoplasm of several Plasmodium species (CURLEY et al., 1994; FLORENT et al., 1998). This implies that the digestion of the small peptides also takes place in the parasite cytoplasm, and therefore must be transported across the membrane of the food vacuole into the parasite’s cytoplasm. Pfmdr-1, a member of the ATP-binding cassette (ABC) transporter superfamily, has been localized in the food vacuole membrane. Some ABC transporters function to translocate polypeptides across membranes as it has also been suggested for complementation assays for Pfmdr-1 (VOLKMAN et al., 1995) (Figure 3).
The critical importance of haemoglobin digestion is illustrated by the fact that inhibition of the aspartic and cysteine as well as some aminopeptidases prevents parasite development (DALAL; KLEMBBA, 2007; DEU et al., 2010; FRANCIS; SULLIVAN; GOLDBERG, 1997; JIN et al., 2014; PANDEY et al., 2006; SKINNER-ADAMS et al., 2009). These unique enzymes within the haemoglobin degradation may represent new drug targets.

1.1.5 Treatment of malaria

There is no efficient vaccine available at this moment, but several studies are ongoing in this regard. RTS,S/AS01 is the most advanced vaccine candidate (phase 3 of clinical trials) against the deadliest form of malaria, *P. falciparum* (WHO, 2016). It targets the pre-erythrocytic stage of *P. falciparum* inducing humoral and cellular immune responses to the circumsporozoite protein (CSP) present on the surface of sporozoites and liver stage schizonts. There was detected a protection against clinical episodes of malaria in the range of 30% - 60%. In a large African phase 3 trial, this vaccine had an efficacy against clinical malaria of 55.8% (50% – 60%) in children aged 5 – 17 months.

![Suggested haemoglobin catabolism pathway in P. falciparum](image-url)
and 31.3% (23.6% – 38.3%) in infants aged 6 – 12 weeks over the first year after vaccination. Furthermore, protection against severe malaria was noted, but protection wanes over the time in both age categories (OLOTU et al., 2016; RTS,S CLINICAL TRIALS PARTNERSHIPS, 2012; RTS,S CLINICAL TRIALS PARTNERSHIPS, 2015). Recently, a combination of the vaccine candidate RTS,S/AS01 with Chimpanzee Adenovirus 63 and modified Vaccinia Ankara vectored vaccines expressing ME-TRAP (ChAD63/MVA/ME-TRAP) yield in a vaccine efficacy of 78.7% (75 - 82.4%). Whereas, the individual vaccine candidates lose their efficacy over the time, vaccine candidates remain immunogenic when the regimens are combined (85.4% (83.3 - 87.5%) after six months) (RAMPLING et al., 2016).

Treatment and Prophylaxis of malaria is based on a small number of drugs. The most important drugs currently in use are focused either on the food vacuole - a special organelle for the digestion of host haemoglobin - of ring-stage and trophozoites of blood-stage parasites or on enzymes in the trophozoite folic acid biosynthesis pathway (WILSON et al., 2013). Only a few drugs, including artemisinin, Artemisinin-based Combination Therapies (ATCs), methylene blue, primaquine and thiostrepton target the sexual stages of Plasmodium falciparum life cycle (BEAUDOIN; AIKAWA, 1968; DELVES et al., 2012; DELVES et al., 2013; KISZEWESKI 2011). Primarily, the antimalarial drugs can be divided into eight main classes namely the endoperoxides, 4-aminoquinolines, 8-aminoquinolines, antifolates, sulphonamides, antibiotics, amino alcohols and others (Figure 4). Currently, most potent antimalarials against asexual blood stages are the natural, semi-synthetic and synthetic endoperoxides: artemisinin, DHA, artesunate, OZ277 and OZ439 (DONDORP et al., 2010; WHITE 1997; WILSON et al., 2013). These antimalarials likely act by alkylating heme and other vital biomolecules of the parasite (KLONIS et al., 2011; O’NEILL et al., 2010), such as PITCTP (BHISUTTHIBHAN et al., 1998; CALDERON-PEREZ et al., 2014), and degrading phospholipids in parasite membranes (KUMURA et al., 2009). Furthermore, 4-aminoquinolines are also highly active against asexual blood-stages, while 8-aminoquinolines are not. The latter class of antimalarials is known to be active on the hypnozoite liver form of P. vivax. Additionally, amodiaquine inhibits haemoglobin digestion and exflagellation/ gametocyte maturation. Besides asexual blood stages atovaquone targets efficiently the electron transport chain in liver and vector (DELVES et al., 2012). In addition to known antimalarials, trichostatin A which acts by inhibiting the histone deacetylase (HDAC) by modifying gene expression (ANDREWS; TRAN;
FAIRLIE, 2012) and cycloheximide, an antibiotic inhibiting the protein translation, are promising compounds for treatment of malaria (GEARY; JENSEN, 1983).

Figure 4 - The main classes of antimalarial drugs (DELVES et al., 2012).
Due to the high mutational rate of the parasite and its resulting rapid adaptation to environmental changes, drug resistance is increasing. In the mid-1990s, where resistance to all available antimalarial drugs had developed (Figure 5), the artemisinin-based combination therapies (ACTs) were introduced. Artemisinin resistant *P. falciparum* is firmly established in eastern Myanmar, western Cambodia and Thailand and southern Vietnam and is emerging in southern Laos and north-eastern Cambodia. The resistance is triggered by a single nucleotide polymorphism (SNP) in the kelch protein gene on chromosome 13 (*kelch13*) after position 440 and is characterized by a slow parasite clearances (Parasite clearance half-life > 5 hours) which reflects the reduced susceptibility of ring-stage parasites. So far, ACTs are still efficacious in areas where standard three day treatments with artemisinin are failing (ASHLEY et al., 2014).

However, not only the parasite has developed resistance, but also the human in the sense of natural protection. Epidemiological studies revealed that there is a high correlation between abnormalities of erythrocytes and *falciparum* endemic countries (PIEL et al., 2010). Figure 6 and 7 show clearly a co-localization of malaria occurrence and haemoglobin-inherited disorders (Haldane’s malaria hypothesis), such as the sickle cell trait.
1.2 Human haemoglobin

To date, well over 200 haemoglobin variants have been described. Human haemoglobin is a globular metalloprotein transporting oxygen from the lungs to the tissues and facilitating the return transport of carbon dioxide (MARENGO-ROWE,
2006). It consists of four polypeptide subunits, known as 2α-globin chains and 2 β-globin chains, similar in length but differing in amino acid sequence. They are held together by ionic bonds, hydrogen bonds, hydrophobic interactions, and van der Waals forces, as well as four non-protein heme pigments, one in each of the subunits (WAZIR, 2015). These heme groups contain positively-charged iron (Fe$^{2+}$) ions which can reversibly bind to oxygen molecules and transport them to various areas of the body (Figure 8). The binding or release of oxygen is attendant on conformational changes within the entire haemoglobin which alter its affinity for oxygen. Besides the oxygen haemoglobin can also bind other molecules such as carbon monoxide, carbon dioxide and nitric oxide. The binding affinity for carbon monoxide is two hundred and fifty times greater than its affinity for oxygen. To bind oxygen successfully iron must be in the ferrous (Fe$^{2+}$) oxidation state. Oxidation to the ferric (Fe$^{3+}$) state without oxygen converts haemoglobin into methaemoglobin, which cannot bind oxygen (THOM et al., 2013).

![Figure 8 - Molecular haemoglobin modified according to www.shutterstock.com](image)

The alpha chain of all human haemoglobins, embryonic and adult, is the same. The normal adult haemoglobin HbA ($\alpha_2\beta_2$) is the most common with a normal amount over
97%. HbA2 is a normal variant of HbA and consists of 2 α and 2 δ chains. It exists in small amounts in all adult humans (1.5 - 3.5% of all haemoglobin molecules). Albeit its biological importance is not yet known, it may be increased in people with the sickle-cell disease. In the first trimester of intrauterine life, zeta, epsilon, alpha, and gamma chains attain significant levels and in various combinations form Hb Gower I (ζ2ε2), Hb Gower II (α2ε2), Hb Portland (ζ2γ2), and foetal haemoglobin (HbF) (α2γ2) (SCHROEDER et al., 1968). Fetal haemoglobin (HbF) persists in the new-born roughly until 6 months and can bind oxygen with a higher affinity than adult HbA. In adults HbF is restricted to a small portion (5 - 8%) of red blood cells, termed F-cells (BOYER et al., 1975; MARENGO-ROWE, 1971). The level of HbF can be elevated in persons with sickle-cell disease and beta-thalassemia (SERJEANT, 2013).

1.3 Sickle Cell Disease
Sickle cells were first described in the peripheral blood of an anaemic patient from the West Indies by the Chicago physician Robert Herrick in 1910 (HERRICK, 1910). Haemoglobin S (“the sickle cell haemoglobin”) is a structurally variant form of normal haemoglobin (HbA) that result from a single base pair mutation in the gene for the beta-globin chain of adult haemoglobin where glutamic acid at position 6 of the beta chain of HbA is changed to valine (Figure 9) (BUNN, 1997; RAPHAEL, 2005; ROSENTHAL, 2011).

Figure 9 - The sickle cell mutation (www.bbc.vo.uk)
This substitution yields the electrophoretically distinct haemoglobin described by Linus Pauling in 1949 (PAULING et al., 1949). In sufficient concentration, these insoluble polymers give rise to the classical sickle morphology with polymerized HbS strands stretching and distorting the cell shape (Figure 10). This process causes severe damage to the erythrocyte membrane. Sickle red blood cells may adhere to endothelial cells or other normal erythrocytes resulting in aggregates and microvascular obstruction. (NAGEL; PLATT, 2001).

![Figure 10 A - normal haemoglobin](sicklecellanemia2051.wordpress.com)  
![Figure 10 B - polymerised haemoglobin](sicklecellanemia2051.wordpress.com)

The sickle cell disease is characterised by the process of microthrombosis and microembolization which may result in stroke. Sickle cell anaemia exhibits polymorphic clinical complications, such as painful crises, priapism, dactylitis, pulmonary emboli, and osteonecrosis and ultimately damages every organ system including the retinæ, spleen, liver, and kidneys (FARID, 2013; HEBBEL; MOHANDAS, 2001). Currently, the drug hydroxyurea is used as remedy which reduces significantly the clinical severity of SCD, for example by increasing the HbF levels. However, there are serious adverse effects, such as mutagenesis and carcinogenesis. Alternatively, blood transfusions and stem-cell based transplantations are used, though the costs are very high (FARID, 2013; STEINBERG et al., 2003; VERMYLEN; CORNU, 1997).

The occurrence of sickle cell disease has been analysed for more than 50 years (ALLISON, 1954; BEET, 1946) and the respective mode of action has been studied as well. However, a precise mechanism has not been identified yet and all hypothesis relating to a protective role against malaria fall into three main categories. Early work suggested that both erythrocytes containing HbS are less supportive for *P. falciparum* growth under low oxygen tensions as well as a reduced parasite invasion event into
HbS carrying erythrocytes under low oxygen levels (FRIEDMAN, 1978; PASVOL et al., 1978). Further it has been observed that HbS cells deposit oxidised, denatured haemoglobin at the inner site of the erythrocytic membrane (BROWNE et al., 1998), which occurs to a higher extent in HbS- than in HbA-red blood cells (RBC) and is even forced by release of non-haem iron that also binds to the RBC membrane (HEBBEL, 2003; SHENG et al., 1998). Due to these denaturing, pro-oxidative habitat the intracellular proliferation of the malaria parasite might be attenuated (BECKER et al., 2004). Secondly, a higher phagocytosis of parasite-infected sickled erythrocytes by host immune cells is suggested due to different shape facilitating the recognizing by immune cells (ABU_ZEID et al., 1991; LOPEZ et al., 2010). Recently, data have been accumulated which suggest that HbS might be involved in pathophysiological consequences of *P. falciparum* by reducing the quantity of proteins encoded by the var-gene family on the surface of the erythrocyte, such as *PfEMP1*, which leads to a higher level of sequestration (CHOLERA et al., 2008; FAIRHURST et al., 2005). Indeed, in a very recent study by CYRKLAFF et al. (2011) it has been implicated that HbS carrying erythrocytes influence the actin cytoskeleton and the Maurer’s cleft formation and thereby impair the vesicle transport towards the erythrocytic surface (Figure 11). More recently, it has been suggested that HbS is mediating a higher tolerance of the host as shown by a non-reduction of the parasite quantity or virulence (FERREIRA et al., 2011; HAQUE; ENGWERDA, 2011).

![Figure 11](image_url) - Hypothesis for malaria protection (Modified according to BUNN, 2013).

Although these experiments were of some controversial nature as already outlined by ROSENTHAL (2011) the focus was on how the parasite is or not proliferating in an elevated oxidative environment. Human who are sickle cell carriers – either homozygote or heterozygote – have higher levels of free, non-protein bound heme in
the blood circulation (MÜLLER-EBERHARD et al., 1968), which is potentially toxic, due to its oxidative nature. It has been suggested that increased levels of human heme oxygenase 1 (HO-1) might detoxifying free heme to CO, biliverdin and iron that binds subsequently to the protein ferritin H chain in HbS blood and thereby renders complicated (cerebral) malaria (FERREIRA et al., 2011). However, it remains questionable whether the protective nature of the increased level of free heme in HbS carriers is related to a higher tolerance to an increased level oxidative stressor mediated by HO-1 or by a higher susceptibility of the parasite by a decreased parasitaemia (ALLISON et al., 1954) within a pro-oxidative environment.
6. CONCLUSION

The cysteine protease Falcipain 2 (FP2) showed an increased proliferation rate (factor 3) in sickle cell blood compared to the corresponding control cell line (empty vector). Additional analyses of aspartic protease-expressing *P. falciparum* strains in sickle cell blood are necessary to be performed to validate the role in haemoglobin hydrolysis. These analytic experiments will shed light on the importance of individual haemoglobin degradative enzymes in *P. falciparum*. Even further comparative proliferation assays of MOCK WR and expression-plasmid transfected *P. falciparum* will lead to the identification which haemoglobin degradative enzymes or enzyme combination (co-transfection) is more favourable for proliferation in sickle cell erythrocytes and thereby shed light on the mode of globin degradation.

Nevertheless, the dipeptidyl aminopeptidase (DPAP1) showed a negative effect in proliferation assays in sickle cell blood. The fusion protein *PfDPAP1*-GFP was localised to either vesicles or/and the food vacuole. These results are consistent with the observations from KLEMBBA et al., 2004 and suggest an alternative trafficking route of DPAP1 in *falciparum*. As outlined above dual trafficking of proteases is occurring in *P. falciparum* (LINDNER et al., 2013). Co-localisation of proteins present in the cytosol as well as in an intracellular organelle bares always the risk of misinterpretations since the cytosolic localisation can dramatically quench the fluorescence signal in the respective organelle. In order to gain direct trafficking to the food vacuole the FP2DE4 (Subramanian et al., 2007) targeting sequence was cloned in front of a pH sensitive variant of GFP (de4GFP) to facilitate single trafficking to the digestive vacuole. Therefore, de4GFP could theoretically be used for ratiometric pH recordings (HANSON et al., 2002; MCANANEY et al., 2002). This construct can be appropriated for further localisation experiments in order to get insights into the food vacuole trafficking pathway in *P. falciparum* as well as for physiologic studies of the food vacuole environment.

The particular problem in the production of proteases in this expression system is that these enzymes do not occur in *E. coli* itself. In addition, proteins having catalytic activity, such as cysteine proteases, which affect the expression cell and an overproduction of this protein can cause toxicity in *E. coli*. A further method for increasing the solubility of recombinant proteins and thus avoiding the formation of inclusion bodies is in the purification by "solubility tags", such as GST (glutathione-S-
transferase) or MBP (maltose-binding protein). JEONG et al. (2006) has managed to purge the MBP-FP2b complex successfully and soluble, however only a 420 bp fragment of FP2b was examined. The MBP could be removed by Factor Xa digestion and separated from FP2b via gelfiltration.

The Heme detoxification protein HDP could only be expressed soluble together with a chaperone. Codon optimisation and/or purification by MBP could improve solubility of the investigated enzyme. Furthermore, re-purification of the pellet is principally possible, but this process is often combined with protein refolding experiments and this process could subsequently hinder the crystallisation.

Finally, highly pure and active APP was purified and crystallised yielding a colourless, rhombic crystal which diffracted up to 1,7 Å. In future, co-crystallisation of APP with a non-cleavable peptide substrate is of great interest in order to get more insides in substrate specificity. This knowledge can be used for the design of new chemotherapeutics.
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