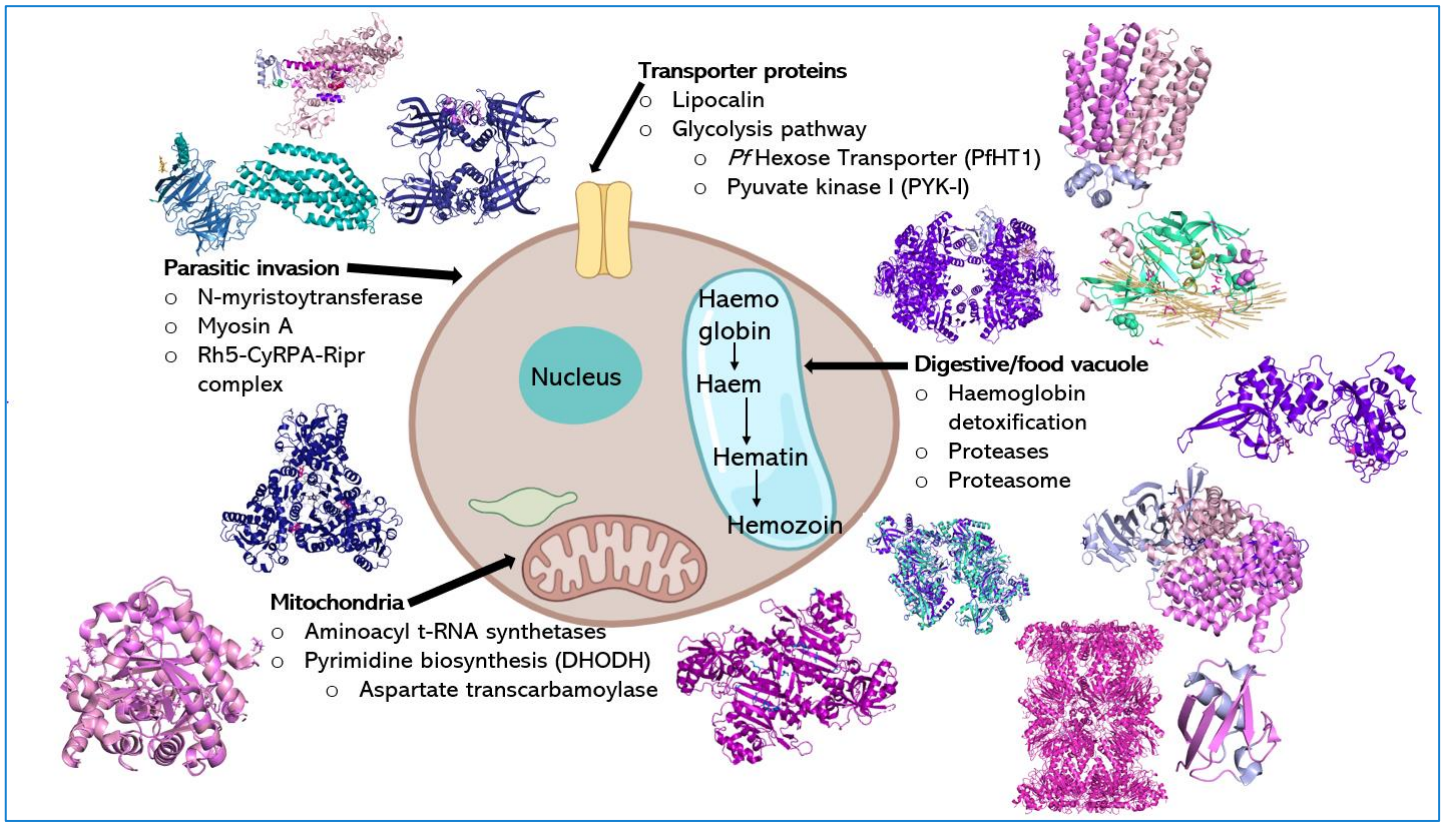
3D-visualization of proteins to get an insight into novel drug targets to treat malaria

Achieving an excellent interpretation of enzymes involved in *Plasmodium* parasite survival pathways, to develop advanced highly selective drugs targeted against malaria-induced parasites: a summary of antimalarial drug discovery



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Abstract

Did you know that approximately 3.5 billion people live at risk of getting infected by apicomplexan parasites, causing the much-discussed mosquito-transmitted disease malaria (1)? And that, despite the usage of several prevention-tools, such as particular mosquito nets treated with insecticide (ITN) and the method of indoor residual spraying (IRS), malaria remains a big killer? This protozoan disease is one of the most critical infectious diseases worldwide and responsible for 405000 deaths/year globally, with 92% of the cases affecting Africa. Apicomplexan parasites originating from *Plasmodium falciparum (Pf)* and *Plasmodium vivax (Pv)* cause the largest burden (2). Although prophylaxis medicines are available and targeted against various proteins in the life-cycle of the parasite, the decisive mode of action is incomplete. Severe malaria is treated with higher doses of prophylaxis medicines, correlated with inconvenient side effects and drug-resistance (3). Therefore, more specific and effective drugs are desired to battle this disease. One of the methods to get a better perception of *Plasmodium* species its survival pathway is structural biology, which images the three-dimensional structure of enzymes imperative for parasitic survival (4). In that way, a better understanding of the parasite its life cycle and therefore identification of highly effective novel anti-malarial drugs can be developed. In this essay structures of proteins involved in haemoglobin degradation, protein synthesis, host-cell invasion and energy production of *Plasmodium* species will be visualized, looking at, for instance, its global structure, atomic interactions and even more!

Keywords

Apicomplexan parasite | malaria | *Plasmodium* species | life-cycle | drug-resistance | structural biology | three-dimensional structures | drug targets

Abbreviations

insecticide-treated mosquito nets (ITN) | indoor residual spraying (IRS) | *Plasmodium falciparum (Pf)* | *Plasmodium vivax (Pv)* | *Plasmodium species* (*P.* species)

Introduction

It is almost unbelievable, nevertheless, Anopheles mosquitoes are responsible for 405000 deaths/year and more than 200 million reported cases in sub-Saharan Africa, Asia, and South-America. Overwhelming vulnerable populations in Africa with 93% of the cases (figure 1). Although improved measures against these mosquitoes through personal protection and mosquito control are taken, still many people suffer from the disease. The disease spreads by five species *Plasmodium* parasites which are carried by the Anopheles mosquitoes; *Plasmodium falciparum (Pf), P. vivax (Pv), P. malariae (Pm), P. ovale (Po)* and *P. knowlesi (Pk)*, of which *P. falciparum* and *P. vivax* as the lethal species (2).

Malaria is one of the most extremely complicated and dangerous infectious diseases in the world and no convenient drug in the treatment of severe malaria has been successful. Although chemoprophylaxis drugs are on the market, malaria remains a big killer. Antimalarials are on the market as single used therapies and combination therapies, exhibited in table 1 (3). One of the main reasons why malaria remains a hazardous disease is the increasing problem of drug-resistance (5). Therefore, new drug compounds managing innovative drug targets need to be discovered, in which structural biology plays an exclusive role.

Structural biology is becoming indispensable in the understanding of biological mechanisms of diseases (4). Therefore, this article will explain the use of structural biology to understand the complex disease mechanism of malaria. A coherent perception of crucial factors of parasitic survival, for instance, host cell invasion, parasitic survival and receptor binding will be discussed later in this review, using three-dimensional protein structures. Furthermore, the problems experienced with malaria disease and how these can be revised and resolved to apply structural biology will be defined.

The first part of this review will clarify currently prescribed drugs in the Netherlands, including the novel drug DSM265 (1). An overview of these prevailing prescribed drugs will be given, including the issues of these drugs towards resistance. Further, multi-target drug discovery will be explained and its positive effect on anti-malarial therapy. Three-dimensional protein structures will be used to visualize possible new drug molecules and targets in the treatment of malaria. Insight into new drug targets will be explained comprehensively. Simultaneously, the complications correlated with the development of new drugs will be mentioned. The last element of this article will clarify the various methods of drug development strategies.

Mechanism of malaria: pathogenesis and diagnosis

Anopheles mosquitoes are carriers of the protozoan disease malaria, caused by five hazardous species: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*, at which *P. falciparum* and *P. vivax* are accountable for serious complications and in worst cases, death (2). Only female Anopheles mosquitoes are accountable for transmitting the parasite because female mosquitos need human blood to produce eggs. Male mosquitos, on the other hand, are fulfilled with nectar from flowers (6). The parasitic life cycle is very complex, yet not fully elucidated (3). After successful gamete fusion, meiosis and recombination of the parasite into the female *Anopheles* mosquitoes, the parasitic invader is transferred into the human body during a bite of an infected mosquito. The parasite, in the form of a sporozoite, is transferred into the human body, into the liver and eventually to the central bloodstream, leading to anaemia, low flow of oxygen and blockage of the immune system when not treated within 24hours (7)(8).

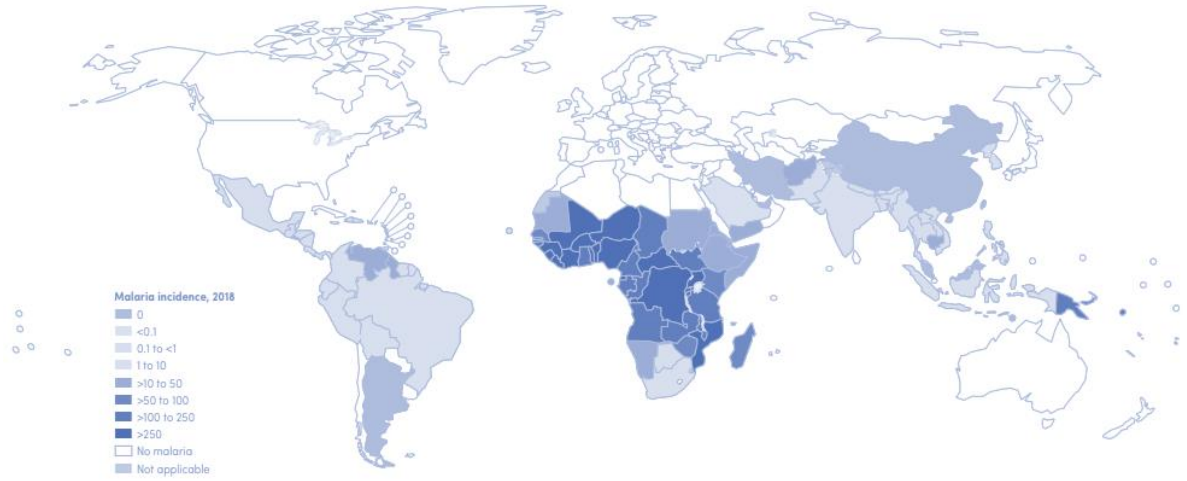

Figure 1. Map of the world demonstrating the malaria case incidence rate in 2018, reported by World Health Organization.

The life cycle of *Plasmodium* species

The malaria parasite is part of the protozoa, which is a heterogeneous group of unicellular eukaryotic organisms (9). Its life cycle consists of two stages: trophozoites and cysts (figure 2). The trophozoite phase is known as the proliferation stage, where schizonts are formed. Slumbered cysts have the characteristic to live in solid circumstances. The life cycle of *Plasmodium* species is very complex and consists of three stages: the pre-erythrocytic stage, the asexual erythrocytic stage and the sexual mosquito stage. The pre-erythrocytic stage starts as a result of an infected mosquito bite. Sporozoites, which are located in the salivary glands of the Anopheles mosquitoes, are released into the human blood circulation. Sporozoites are targeted to the liver where they infect liver cells and exo-erythrocytic mature schizonts are formed. These exo-erythrocytic schizonts are released as merosomes, which contain thousands of merozoites. In the asexual erythrocytic cycle, merosomes are disrupted, causing merozoites to invade healthy erythrocytes. To be specific, the merozoite forms a tight junction connected to the actomyosin motor, forming the parasitic vacuole and the membrane (10). Some of these merozoites will evolve into gametocytes and can remain in the bloodstream for several days. Another mosquito bite will cause the mosquito to be infected by gametocytes and the same life cycle will continue repeatedly (1).

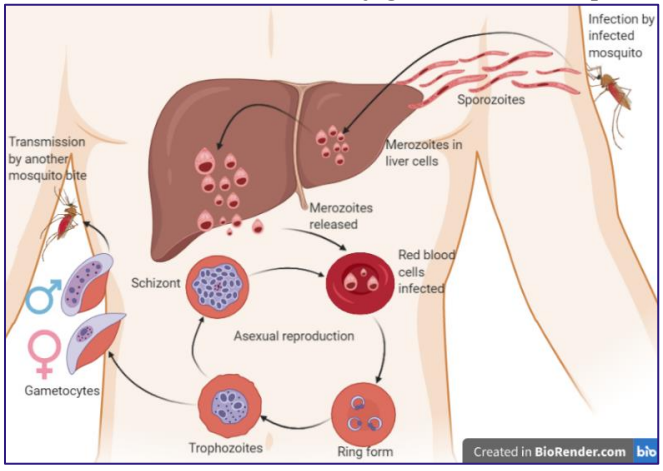

Figure 2. Schematic representation of Plasmodium species life cycle including three stages: pre-erythrocytic stage, asexual erythrocytic stage and sexual mosquito stage. Figure is generated with Biorender.

Currently used anti-malaria medicine in the Netherlands

Table 1. Summary of prescribed prophylaxis antimalarials in the Netherlands.

Compound	Chemical class	Mechanism of action	Targeted against
Artesunate	Sesquiterpene lactone	Targeted against blood schizonticides: production of free radicals by disrupting mitochondrial function and inhibition of calcium-ATP-ases in the endoplasmic reticulum	Chloroquine-sensitive (3D7) *Pf* strains
Hydroxychloroquine	Quinoline derivative	Targeted against blood schizonticides: inhibition of haem crystallization	Chloroquine sensitive (T996) *Pf* stains & chloroquine-resistant (K1) *Pf* strains
Quinine	Amino alcohol	Targeted against blood schizonticides: inhibition of haem crystallization	Chloroquine-sensitive (3D7A) *Pf* strains
Mefloquine	Amino alcohol	Targeted against blood schizonticides: inhibition of haem crystallization	Chloroquine-sensitive (3D7) Pf strains & chloroquine-resistant (W2) *Pf* strains
Pyrimethamine	Diaminopyrimidine	Targeted against blood schizonticides: DHFR inhibitor	Chloroquine-sensitive (3D7) *Pf* strains
Artemether	Sesquiterpene lactone	Inhibition of nucleic acid synthesis: production of free radicals	Chloroquine-sensitive (3D7) *Pf* strains
Lumefantrine	Amino alcohol	Inhibition of haem crystallization	Chloroquine-sensitive (3D7A) *Pf* strains
Piperaquine	Quinoline derivative	Inhibition of haem crystallization	Chloroquine-resistant *Pf* isolates
Artenimol	Sesquiterpene lactone	Weaken parasitic membrane by disrupting mitochondrial function and inhibition of calcium-ATP-ases in the endoplasmic reticulum	Chloroquine-sensitive (3D7) *Pf* strains
Proguanil	Biguanide derivative	DHFR inhibitor: blockage of the folic acid metabolism	*Pf* isolates
Pyrimethamine	Diaminopyrimidine	DHFR inhibitor: blockage of the folic acid metabolism	Chloroquine-sensitive (3D7) *Pf* strains
Atovaquone	Naphthoquinone	Inhibition of electron transport in parasitic mitochondria	Chloroquine-sensitive (3D7) Pf strains
Doxycycline	Antibiotic	Inhibition of parasitic protein synthesis	Multi-resistant *Pf* isolates
Artemether/lumefantrine	Sesquiterpene lactone/Quinoline derivative	Targeted against blood schizonticides: Inhibition of nucleic acid synthesis: production of free radicals and inhibition of haem crystallization	Chloroquine-sensitive (3D7) *Pf* strains & chloroquine-sensitive (3D7A) *Pf* strains
Artenimol/piperaquine	Sesquiterpene lactone/Quinoline derivative	Targeted against blood schizonticides: Weaken parasitic membrane by disrupting mitochondrial function and inhibition of calcium-ATP-ases in the endoplasmic reticulum and inhibition of haem crystallization	Chloroquine-sensitive (3D7) *Pf* strains & Chloroquine-resistant *Pf* isolates
Atovaquone/proguanil	Naphthoquinone/Biguanide derivative	Targeted against blood schizonticides: Inhibition of electron transport in parasitic mitochondria and DHFR inhibitor: blockage of the folic acid metabolism	Chloroquine-sensitive (3D7) *Pf* strains & *Pf* isolates

Various prophylaxis medicines against malaria are prescribed in The Netherlands when travelling to a precarious country (3). Most of these drugs are targeted against diverse stages of the life cycle of the parasites. Unfortunately, most of these mechanisms of actions are not understood completely. Accordingly, adequate knowledge of the mechanism is mandatory to develop an exceptional drug. Structural biology is a great technique to get an appropriate visualization of the three-dimensional protein structure (4). As a result, further development of new drugs will evolve smoother. In the present day, a new drug is in clinical trials: DSM265, which is an encouraging single-dose antimalarial drug (11). In this paragraph, the currently prescribed drugs in the Netherlands will be elucidated in Table 1. Also, the new drug DSM265 will be explained. As a consequence of increasing drug-resistance, some of the prophylaxis medicines are prescribed as a combination (5). Presently used antimalarials are targeted against blood-schizonticides, tissue-schizonticides and gametocides. Although considerably drugs are available, drug-resistance and toxic side-effects are disturbing the effectiveness of these therapies (3). Similarly, it is highly recommended to develop a single-dose drug, associated with fewer side effects and great compliance of which DSM265 is an example (1)(11)(12)(13).

An introduction to novel drugs to treat malaria: DSM265

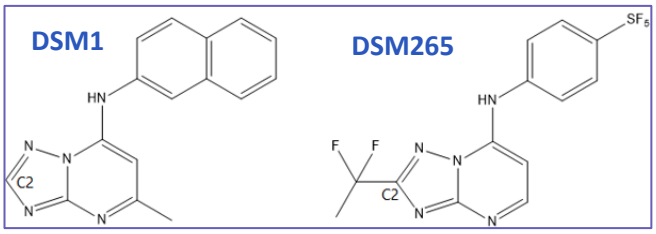

Figure 3 Linear structures of potential anti-malarial compounds DSM1 and DSM265. DSM1 is identified through high-throughput screening and does not contain a substituent in C2 position, while DSM265 does contain a substituent in C2 position. Structures are exhibited using Chemdraw Professional 18.1 (PerkinElmer).

An important enzyme involved in protein synthesis in *Plasmodium* species is Dihydroorotate dehydrogenase (DHODH) (12). DHODH is located in the inner mitochondrial membrane of *Plasmodium* species where it functions as a catalyst in the *de novo* pyrimidine synthesis (11)(12). It verifies the conversion of dihydroorotate (DHO), to orotic acid (11). Pyrimidine synthesis is indispensable to form DNA and RNA. This pathway plays an important role in parasite replication in the early trophozoite stage. Henceforth, a *pf*DHODH inhibitor is a considerable antimalarial, which targets liver- and blood-stage parasites (3). DSM265 is an encouraging compound because of its aspect to actively inhibit schizont development (11). To be specific, DSM265 is a DHODH inhibitor (1)(12). The antimalarial activity of DSM265 is encouraging. First of all, great activity towards asexual *Pf* strains was achieved. Unfortunately, it did not show any inhibitory effects towards gametocytes, which implies it cannot be used as a transmission-blocking agent. Also, liver cell invasion of sporozoites was not affected by DSM265. Equally important, full protection against *Plasmodium falciparum* was provided, if 400 mg, similar to a single dose, was given to the patient. A lower dose led, as purposed, to a shortened cure rate (1). An exceptional finding is the discovery of DSM265 mutations leading to resistance. Two mutations were found: C276Y (13) and C276F (1)(11)(13). Slightly changes in surrounded residues causing diminishing of DSM265 binding pocket, and therefore reduced enzyme binding which eventually will cause drug-resistance. *Pf*DHODH C276F mutant bound with DSM1 is shown in figure 4. The reason why DSM1 has been used, instead of DSM265, is because it does not contain a substituent in the C2 position (figure 3). By all means, it showed similar characteristics and inhibitory activity as DSM265 (13). The following is determined using Ligplot: the protein itself consists of three β-sheets, 20 α-helices and 17 helix-helix interactions, defining stability. Correspondingly, the active site residues include Arg265 and His185 respectively interacting utilizing hydrogen bonds of 2,4Å and 2,2Å. To summarize,

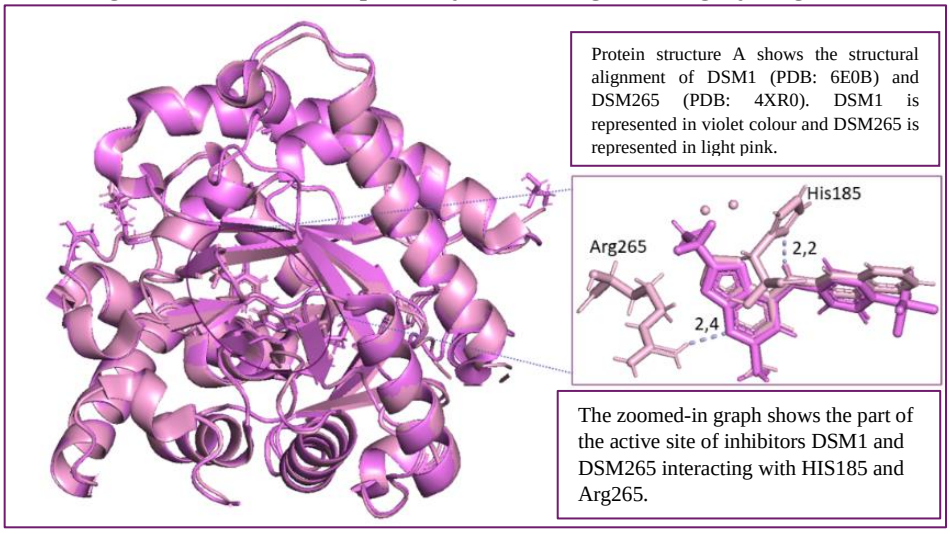

Figure 4 Structure alignment of DSM1 bound to C276F PfDHODH (violet) and DSM265 bound to Wild Type (WT) PfDHODH (light pink) by means of X-ray structure analysis. Hydrogen bonds of respectively 2,2Å interacting with His185 and 2,4Å interacting with Arg265 were revealed. Protein Data Bank (PDB) was used to visualize protein structures. Images are generated with PyMol Molecular Graphics System (Delano Scientific).

DSM265 seems to be a great drug because it only has to be administrated once weekly, leading to fewer side effects and toxicity, high compliance and in a lower degree sensitivity towards drug-resistance. Additional research has been done to establish novel DHODH inhibitors, due to potential success of DSM265 but unfortunately showed off-target toxicity (11).

Novel antimalarial drug targets

Crucial enzymes in the haemoglobin degradation pathway of *Plasmodium sp.*

To survive, the *Plasmodium* parasite produces different enzymes to synthesize proteins from amino acids. One of these class enzymes is plasmepsins (PMs), which are culpable in malaria disease. PMs, which are also called aspartic proteases or haemoglobinase's, are indispensable in the complete life cycle of *Plasmodium.* These enzymes are located in the food vacuole of the parasite, where host intraerythrocytic haemoglobin degradation takes place (14). It has been shown that pepsin-like aspartic proteases play a key role in the degradation of human haemoglobin, making it a favourable drug target to exploit the parasite (15). Four types of plasmepsins provide intraerythrocytic haemoglobin degradation, namely plasmepsin I (PMI), plasmepsin II (PMII), histo-aspartic protease (HAP) and plasmepsin IV (PMIV). Inactive plasmepsins are called zymogens or pro-plasmepsins (pro-PM) because only its mature form is active. Stabilization of zymogens happens when the prosegment interacts with the enzyme its C-terminal domain. Pro-PM consists of a prosegment, which is on 124 residues long, attached to the N-terminal. When residues 77 to 123 have been removed, the protein is activated. Still, the conversion mechanism of a pro-plasmepsin into an active plasmepsin has not been fully elucidated. However, inhibition of this maturity mechanism would be a possible anti-malarial target (14). In figure 5, three crystal potential structures of Plasmodium falciparum histo-aspartic protease (HAP) zymogen are shown: HAP-zymo1 (PDB code: 6KUB), HAP-zymo2 (PDB code: 6KUC) and HAP-zymo3 (PDB code: 6KUD) demonstrated in the figure as A, B and C respectively.

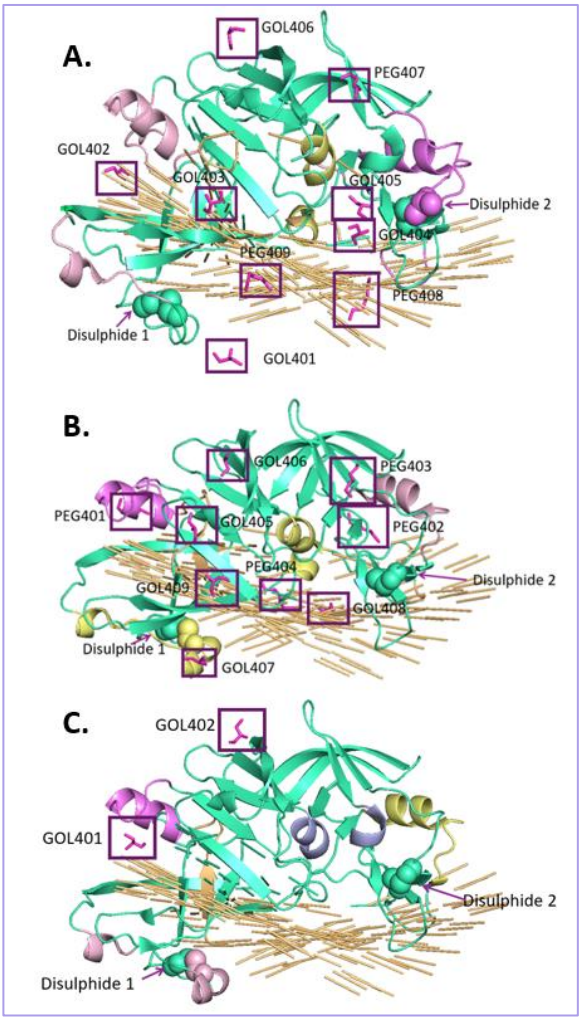

Figure 5 Crystal structures of Plasmodium falciparum histo-aspartic proteases (A) HAP-zymo1, (B) HAP-zymo2 and (C) HAP-zymo3. Images are developed with PyMol Molecular Graphics System (Delano Scientific).

HAP-zymo1, HAP-zymo2 and HAP-zymo3

HAP-zymo1 consists of nine ligands; six GOL ligands and three PEG ligands (enclosed in a squared manner). Its structure contains three β-sheets coloured in green cyan, ten α-helices (coloured in violet, light blue, pale-yellow and light-pink) and four helix-helix interactions. Besides, two disulphides are present (showed as spheres). α-helices are important features in protein-protein interactions. HAP-zymo2 is compared to HAP-zymo1 a larger protein, which consists of four PEG ligands and 5 GOL ligands. It is composed of four β-sheets, ten α-helices and three helix-helix interactions. It also consists of two disulphides. The smallest protein is HAP-zymo3, which consists of the same structure as HAP-zymo2, but has only two ligands, see figure 5. Root-mean-square deviations were obtained from 0.42 A, 0,44A and 0,42A, resulting in minimal anatomical differences in the mature regions. However, differences in the pro-mature region were significant.

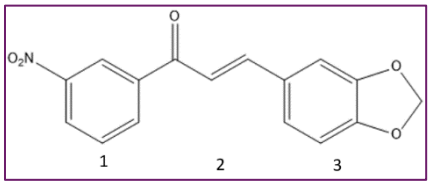

Figure 6. Linear structure of papain-type protease falcipain-2 inhibitor, EC48. 1, 2 and 3 represent fundamental chemical groups, respectively nitrobenzene, ketone and benzodioxol. Structure is exhibited using Chemdraw Professional 18.1 (PerkinElmer).

As mentioned before, the haemoglobin degradation pathway is an encouraging pathway to look into, due to its preservation of managing parasite's activity. Next to plasmepsins, papain-type protease falcipain-2 is another auspicious class of enzymes involved in the haemoglobin degradation pathway. This protease (figure 6) also influences the conversion of lethal haem to hemozoin, which is nontoxic. Although present drugs are on the market targeted against the haemoglobin pathway, no straightforward blockage of papain-type protease falcipain-2 has been evolved. Novel high selective non-peptide drugs functioning as antagonists against falcipain-2 are aimed due to their ability to cleave haemoglobin into tiny peptides. In figure 7, the structure of Plasmodium falciparum falcipain-2 protease aggregated with a small molecule (figure 6), an (E)-chalcone inhibitor (EC48) is demonstrated (PDB code: 6SSZ) (16). Chalcones are generally essential compounds, used for forever and a day to treat cancer, infectious diseases and preventing many other disorders. Chalcones originates from flavonoids consisting of interesting aspects to diminish various disease states and are accessible to synthesize (17).

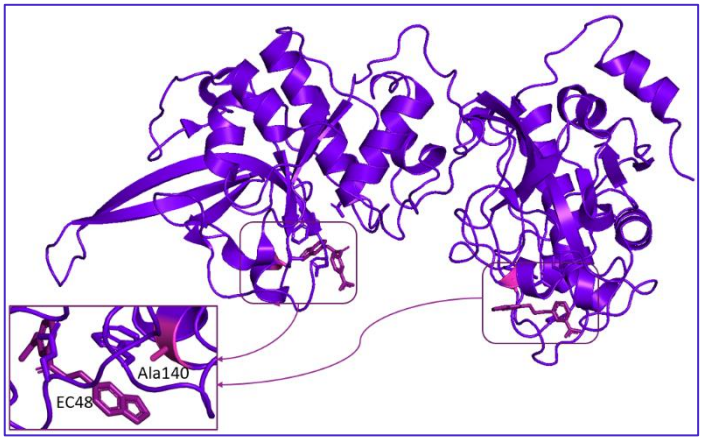

Figure 7 X-ray crystallographic structure of EC48 inhibitor bound to falcipain-2 protease. Inhibitor EC48 is coloured in dark-purple, while amino acid Ala140 is coloured in purple. Two identical copies are shown. Images are developed with PyMol Molecular Graphics System (Delano Scientific).

Accordingly, it is presumable that a combination of a chalcone attached to an inhibitor and falcipain-2 protease is a promising target to treat malaria. This is the first time visualization of a small molecule inhibitor EC48 bound to the backside of the substrate-binding cleft of falcipain-2 is available due to improved structural biology techniques. When falcipain-2 is evolved, it consists of a 27 kDa papain-type protease. The molecule shows two identical copies, which is demonstrated in figure 7. Furthermore, (E) – Chalcone is bound to both ligands. Thus, two ligands both interacting hydrophobically with alanine. It is suggested that hydrophobic interactions, as well as π-stacking interactions between benzodioxol (the upper part of the EC48 structure), shown in figure 7, and the side-chain of FP2, mediate strong binding(16). Also, the protein includes one β-sheet and eight α-helices together with six helix-helix interactions. The fundamental substrate-cleft is located between the two domains (α-helices and β-sheets).

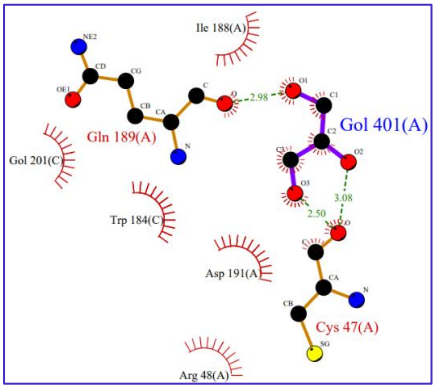

Figure 8 Ligplot figure of Plasmodium Falciparum lipocalin (PfLCN) (Pf3d7_0925900). Image developed using Ligplot.

Other small groups of proteins, such as the lipocalin family have been discovered as potential antimalarials because they play an essential role in various important processes, such as transportation of lipids, hormones and fatty acids. Thus it can be suggested that lipocalin in *Pf* is crucial for parasite survival within the erythrocytes (18). Also, it is an essential compound to control cell regulation and it plays a role in the defence mechanism of oxidative stress, which is one of the main causes of malaria infection. *Plasmodium* species are highly dependent on human haemoglobin, after digestion of haemoglobin, free haem is released, which is toxic to the parasite. To withstand, the parasite is polymerizing the haem to nontoxic hemozoin causing the parasite to continue its proliferation.

This protection system has an enormous effect on the human body, forming superoxide high active radicals. These radicals cause lipid peroxidation, leading to inflammation and severe symptoms (19). Thus, inhibitory effects of lipocalin in the human malaria parasite *Plasmodium falciparum,* will reduce inflammation and so, lipocalin is a potential drug target. In this research, the structure of *Pf* lipocalin (*Pf*LPN*)* is identified (figure 8&9). The X-ray crystallography resulted in a 2,85Å resolution (18). Furthermore, a hydrophobic binding pocket has been formed of eight anti-parallel β-barrel strands which is a typical feature for lipocalins.
Consequently, apolar ligands are sufficient for attachment. The positive surface charges of *Pf*LPN are mainly located on the bottom. Furthermore, two disulphide bonds are located nearby each other. The Gol ligand is attached to two amino acids: Gln189 and Cys47. This binding assessment takes place through hydrogen bonds. To point out, Gln189 is attached through one H-bond of 3,4Å, whilst Cys47 is attached by two H-bonds of respectively 3,1Å and 2,5Å. Furthermore, several hydrophobic interactions are involved in this molecule. Ile118, Gol201 and Trp184 interact in a hydrophobic manner to Gln189 when Asp191 and Arg48 interact hydrophobically with Cys47.

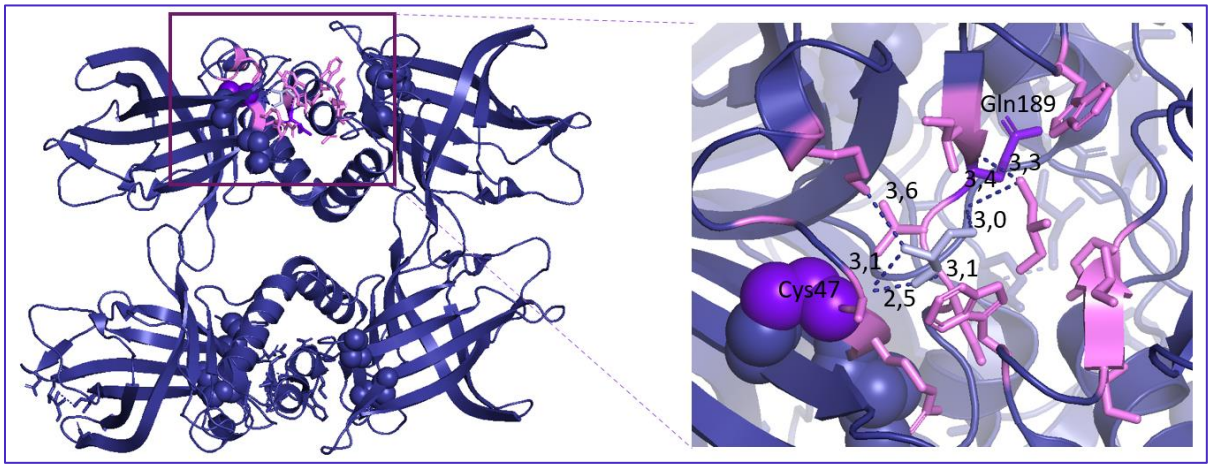

Figure 9. Tetrameric structure of PFLPN together with its zoomed-in active site (shown on the right). Hydrophobic interacting proteins are visualized in pink, the active ligand GOL401 is coloured in light blue, hydrogen bonds are coloured in density blue and disulphides are visualized as spheres in purpleblue. Image is developed with PyMol Molecular Graphics System (Delano Scientific).

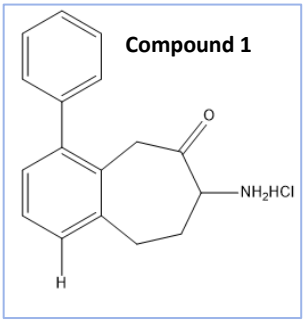

Figure 10 Linear structure of compound A, a aminobenzosuberone hydrochloride derivative. Structure is exhibited using Chemdraw Proffesioanl 18.1. (PerkinElmer).

Host cell erythrocytic invasion, haemoglobin degradation and egress are crucial in parasitic survival, in which proteases play a key role. Another protease, M1 aminopeptidase originated from *Plasmodium falciparum* (*Pf*A-M1) is involved in the breakdown of dipeptides into necessary amino-acids, by a process called hydrolysis. It has been suggested that this enzyme is a vital component in parasite growth and replication. Additionally, positive effects have been observed when antagonizing PfA-M1 in adolescents using small molecular inhibitors, without the occurrence of toxicity. Numerous PfA-M1 inhibitors have been investigated and only a few studies have shown inhibitors demonstrating repression in parasite durability, which is not within the scope of this review. An aminobenzosuberone derivative showed favourable profiles. In figure 11, the racemic substituted aminobenzosuberone hydrochloride derivative is demonstrated (PDB code: 6SBQ), which is especially expressing strong attachment and high selectivity to *Pf*A-M1. *Pf*A-M1 is an aminopeptidase dependent on zinc-complexes whereby it is an indispensable assembly in cleaving amino acids at the N-terminus of peptides. Various aminobenzosuberone derivatives were synthesized and the most efficacies compound (figure 10) is further analysed. The structural study exposed a crystal structure of PfA-M1 together with compound 1 (figure 11) with a 1.5Å resolution. Out of five present ligands, one ligand 7ML is the aminobenzosuberone core (20).

This conformation is stacked between Tyr580, His496, Glu497 and Glu463. The primary amine of the 7ML core plays an important role in salt bridge interactions, electrostatic interactions and hydrogen bonds interactions.

Hydrogen bonds are formed between side-chain oxygen atoms of 7ML and Glu463 (2,8Å), Glu497 (2,7Å and 3,1Å), His496 (3,4Å) and Tyr580 (2,7Å). The salt bridge interaction of 2,7Å is formed between the zinc atom and 7ML. Numerous hydrophobic interactions have also been reported. On the rear side of 7ML, hydrophobic interactions are formed with Glu519, Glu 319, Tyr575, Val459, Ala461 and Val393. Also, hydrophobic interaction is formed between Glu463 and Glu497, namely interaction with His500. A network of hydrogen bonds keeps the active site together. Important interactions are determined using Ligplot and visualized on the right in figure 11.

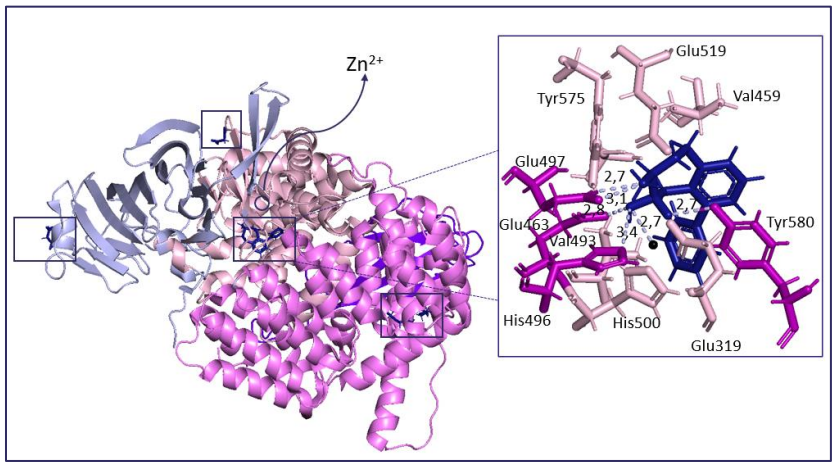

Figure 11. Crystal structure of compound A complexed with PfA-M1 (PDB code: 6SBQ). Compound A is shown in deepblue, the zinc ion is shown as a black sphere and the interacting proteins are shown in light pink (hydrophobic interactions) and purple (hydrogen-bond interactions). Pi-pi interactions have occurred between aromatic centroids and are light blue (same as hydrogen bonds). Figure on the right shows hydrogen bonds and distances are also marked. Image is developed with PyMol Molecular Graphics System (Delano Scientific).

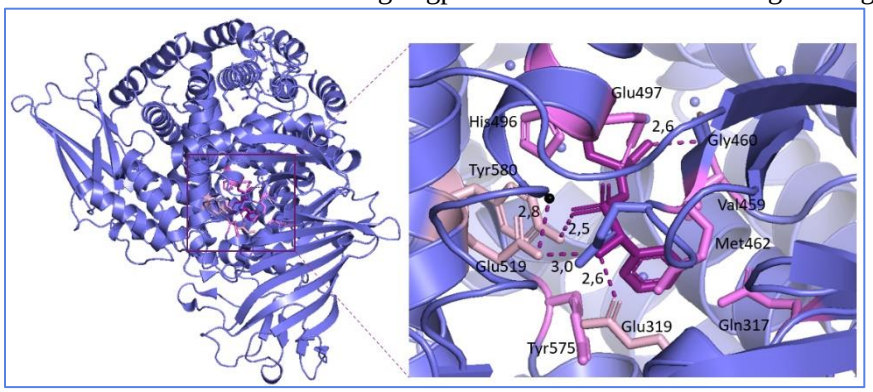

Figure 12. Predicted binding mode of PfA-M1 with a virtual ligand inhibitor. Zink metal is visualized as a black sphere, the ligand is coloured in dark-purple and its interacting amino acids in light pink. Hydrophobic interactions are visualized in violet. Image is developed with PyMol Molecular Graphics System (Delano Scientific).

As mentioned before, Red blood cell (RBC) haemoglobin is an attractive drug target due to the involvement of different enzymes in the catalysis of haemoglobin by *Plasmodium* species. Accordingly, several various potential targets have been suggested.

Aminopeptidases are a great target because they play a crucial role in the abolishment of N-terminal amino acids which are part of small peptides. This process occurs at the ultimate stage of haemoglobin degradation. Alanyl aminopeptidase, *Pf*A-M1, and leucyl aminopeptidase, *Pf*A-M17, are two promising drug targets, containing zinc-ions in the active centre. These zinc-ions are indispensable for the catalytic mechanism of haemoglobin digestion. As can be seen in figure 12, *Pf*A-M1 contains a single zinc-ion, whereas *Pf*A-M17 contains two zinc-ions representing two binding sites (figure 13). Inhibitory compounds will be targeted to the zinc-ions through a zinc-binding group (ZBG) (21). The structure of PfA-M1 exposes a total of seven hydrophobic patches: Met462, Tyr575, Val459, Gln317, Ala461, Glu463, Glu497 and His496. Additionally, the protein contains three binding sites. However, the compound will bind to the active binding site shown in figure 12. Out of these interactions, Met462, Tyr575, Val459 and Gln317 were positioned at the hydrophobic cleft. Furthermore, hydrogen bonds were also observed, involving the acceptor residues Glu319, Glu519 and the donor residue Tyr580.

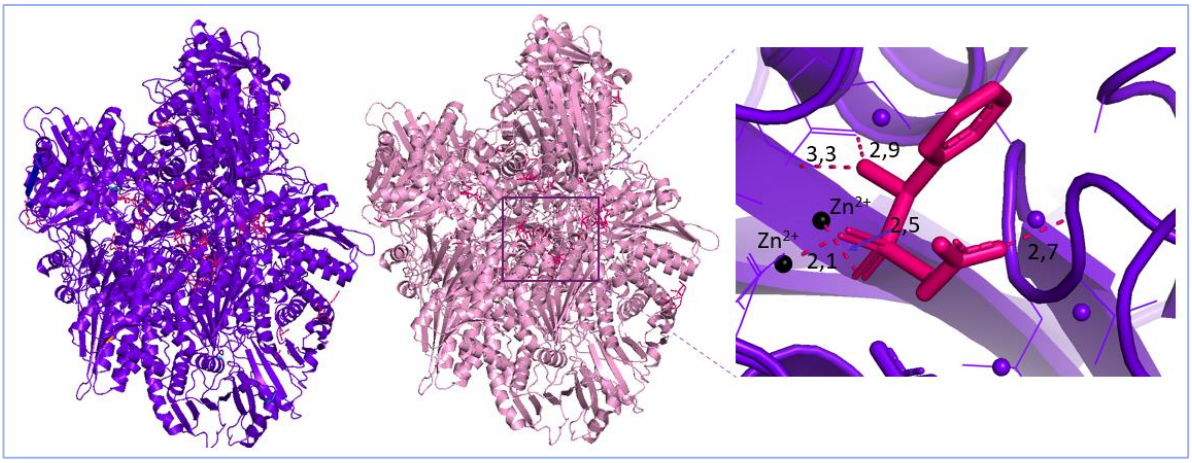

Figure 13. 3D-visualization of PfA-M17. This aminopeptidase is composed of two identical proteins with two active sites, visualized in blue-purple and light-pink, respectively. The two zinc ions are visualized as black spheres and the ligand in hot-pink. Image is developed with PyMol Molecular Graphics System (Delano Scientific).

Protein translation mechanisms

Aminoacyl t-RNA synthetases

Here a look will be given into innovative crucial enzymes involved in protein translation of the genetic code of bacteria, fungi and parasites: aminoacyl tRNA synthetases (aaRSs). Furthermore, a specific look into structural changes and their influence on active site binding is visualized through three substitutions: methylcyclohexane for methyl tetrahydropyran moiety, cyclohexane for methylcyclohexane moiety and lactam for lactone moiety. This is a summary of the research accomplished by Zhou et al (22). The original publication elucidated more structural changes and their effects on active site binding. Aminoacyl t-RNA presents an influential role in living cells such as mitochondria in humans and the apicoplast in parasitic apicomplexan. Additionally, it is involved in the protein synthesis of plants, where it is located in the chloroplast. Below, the possibility of aminoacyl t-RNA synthetase as a potential drug target to treat malaria will be discussed. At first, the role of aaRSs in protein synthesis is explained, followed by an analysis of potential antimalarial drug targets against protein synthesis of *Plasmodium.* species.

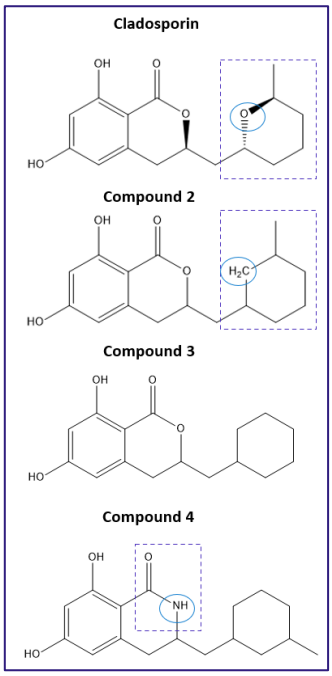

Figure 14. Linear structures of cladosporin and derivatives 2, 3 and 4. Structure is exhibited using Chemdraw Professional 18.1 (PerkinElmer).

Cladosporin, a very small compound, is known as a natural product in the treatment of malaria. It functions as an antibiotic and is originated from *Cladosporium cladosporioides* and *Aspergillus flavus.* It targets lysyl-tRNA synthetase of the parasite *P. falciparum*, affecting blood- and liver-stage parasites (22). The genome of malaria parasite encodes two different lysyl-tRNA synthetases (23). Since humans and parasites include aaRSs to synthesize amino acids and thus proteins, it is challenging to achieve great selectivity towards the parasitic aaRSs. Besides, the side chain of *P. falciparum* lysyl-tRNA synthetase (*Pf*LysRS) demonstrates rotameric changes as well as backbone dynamics at the ATP binding site. For this reason, high selectivity against *Pf*LysRS had been observed, leaving the human LysRS unattached. At the same time, cladosporin shows poor oral bioavailability, which discourages practical use (22). With this in mind, the following derivatives were designed in favour of developing powerful antibiotics to combat malaria:

The substitution of methylcyclohexane for methyl tetrahydropyran moiety: compound 2 (PDB code 6KA6)

The oxygen atom, which plays a crucial role in the selectivity of cladosporin, is attached to the Gly556 residue employing a van der

Waals interaction. The protein structure with PDB code 6KA6 (figure 15) was changed in such a way that this oxygen was replaced by carbon. In this way, the protein structure differs from cladosporin. L-lysine together with compound 2 showed considerable effects against *Pf*LysRS.

The substitution of cyclohexane for methylcyclohexane moiety: compound 3 (PDB code 6KAB)

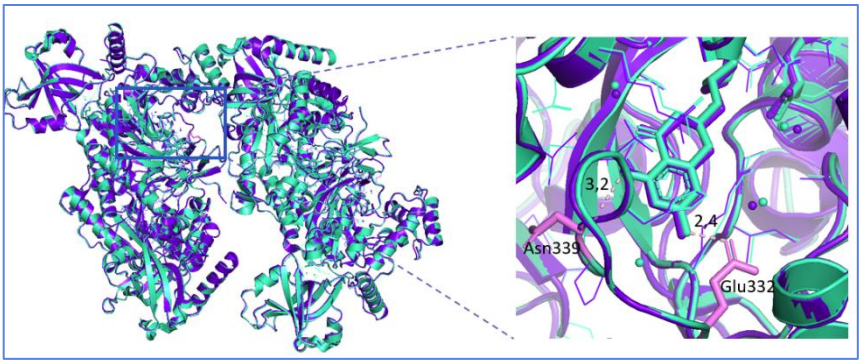

Figure 15. Three-dimensional structural homology protein structure of compound 2 (PDB code 6KA6) (greencyan) and compound 3 (PDB code 6KAB) (bluepurple) their binding mode. Residues Glu332 and Asn332 interact with both compounds via hydrogen bonds. Image is developed with PyMol Molecular Graphics System (Delano Scientific).

The methyl-tetra pyran moiety plays an important role in cladosporin its selectivity. Hydrophobic interactions with de Cβ-atom of the Ser344 residue were formed by both compound 3 and the methyl group in cladosporin. The effect of this methyl group was further investigated by substituting the starting material; methylcyclohexane-carbaldehyde was substituted with cyclo-hexane-carboxaldehyde. Here, the structure revealed a resolution of 2,89Å. As a result of substituting the starting material with cyclo-hexane-carboxaldehyde, the methyl group was removed. Therefore, the hydrophobic interaction with Ser344 vanished, leading to higher specificity and affinity of the target protein (22).

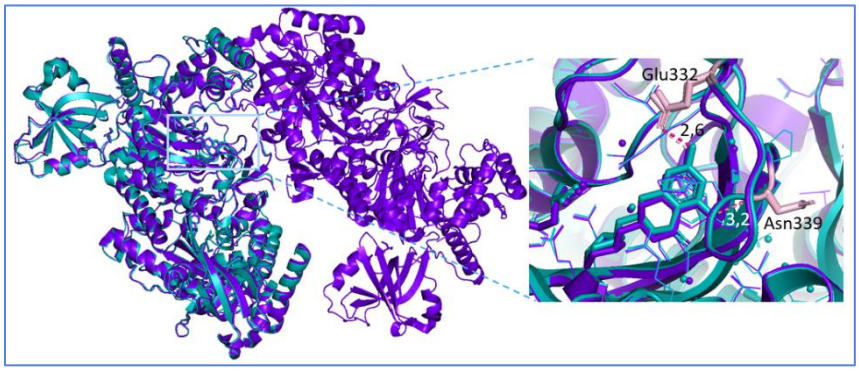

Figure 16. Three-dimensional structural homology protein structure of compound 2 (blue-purple) and compound 4 (PDB code 6KBF) (deep-teal) their binding mode. Residues Glu332 and Asn339 interact with both compounds via hydrogen bonds. Image is developed with PyMol Molecular Graphics System (Delano Scientific).

The substitution of lactam for lactone moiety: compound 4 (PDB code 6KBF)

Earlier research reported that substitution of the ester with amide resulted in higher plasma stability of substances without decreasing its biological activity. The only strong interaction of the enzyme interferes the bond between the singly-bound oxygen atom of the lactone to the side chain of Arg559 (3,3 Å). So, a compound was synthesized in which the lactone group has been substituted to a lactam group (figure 16), resulting in a highly potent optimized compound (22).

Further elucidation of lysyl-tRNA synthetase as an anti-malarial drug target has been observed by Baragańa et al (2018) (23). Compound 5 (figure 17) showed great oral bioavailability, meaning that it could be excellent patient-friendly therapy. Another great accomplishment of this compound was that it turned out to be active against both bloodstream *Pf* and to *Pf*LysRS (23).

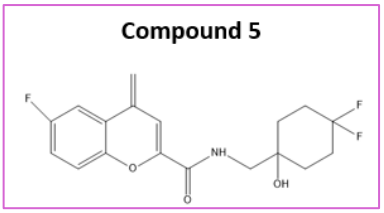

Figure 17 Linear structure of cladosporin derivative 5, approached from screening hit. Structure is exhibited using Chemdraw Professional 18.1 (PerkinElmer).

Compound 5 bound to *Pf*LysRS is shown in figure 18 (PDB protein structure 6HCU). The overall protein structure consists of four β-sheets and 18 α-helices. The protein is bound to Asn339 and Glu500 and is enclosed with ten hydrophobic interactions. Hydrogen bonds of respectively 3,1Å and 2,8Å were formed to the active site (23).

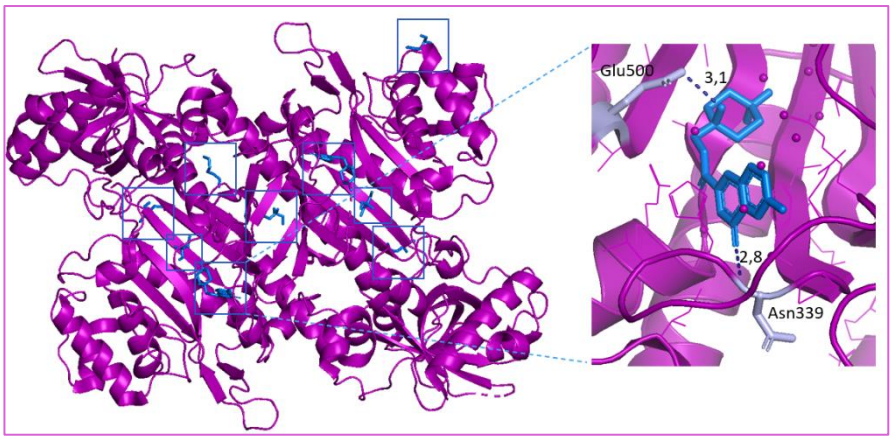

Figure 18. Binding status of ligand (coloured in blue) to PfLysRS (coloured in purple). Several binding modes are marked as squares, with a zoomed-in view on the active binding status of which interacting residues are coloured in light-blue. Image is developed with PyMol Molecular Graphics System (Delano Scientific).

Role of parasitic proteasome in anti-malarial therapy

In *P*. species, protein degradation is fundamental for a fast replication in the human host and mosquito vector. Proteasomes are crucial in the annihilation of proteins. The proteasome consists of two external cavities and a proteolytic chamber. When a protein is not folded properly due to mistakes in transcription, RNA splicing and translation and when the help of chaperones is not valuable, the protein is modified by ubiquitination and proteasome is activated. Only ubiquitin-tagged proteins are designated to the proteasome. The external cavities of the proteasome recognize the ubiquitinated proteins. Proteasomes can unfold the protein and break the peptide bonds, which is called proteolysis. This leads to the formation of small peptides. Accordingly, proteases will break down short polypeptides in amino acids. Therefore, the proteasome is crucial in parasitic survival (24). To activate the proteasome, shuttle proteins are needed, which is elucidated below.

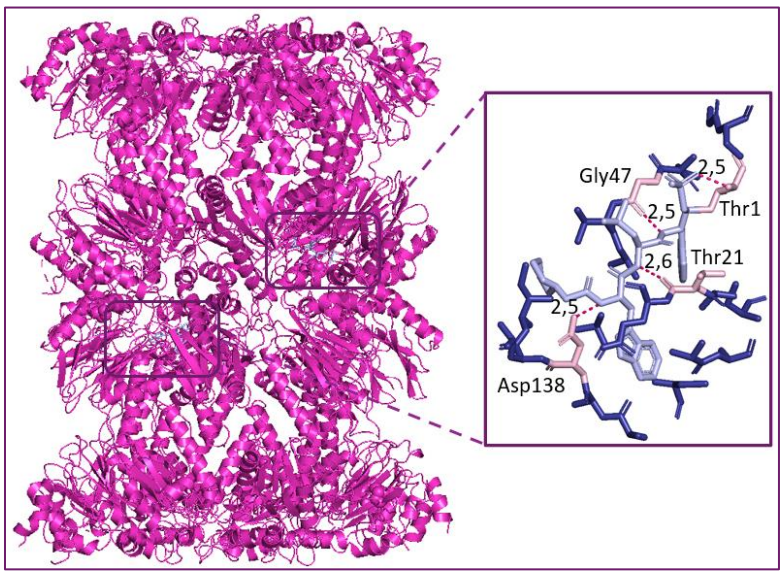

Figure 19. High resolution cryo-electron microscopy (Cryo-EM) structure of Pf 20S proteasome bound to inhibitor WLW-vs (light blue). Image is made with PyMol Molecular Graphics System (Delano Scientific).

The previous investigation showed that proteasome inhibitors play a crucial role in the destruction of the malaria parasite. Unfortunately, these compounds also affect the human proteasome which limits their usage as a therapeutic agent. For this reason, a better understanding of the parasitic proteasome, including necessary proteins, is required in which structural biology is of fundamental importance (4). In the research of Li et al (2016) (24), specific inhibitors targeted against the parasitic proteasome are designed. The results of this particular study demonstrated that these inhibitors preferentially interact with the β2-subunit in an inhibitory manner. The structure of a Plasmodium-selective proteasome inhibitor (PDB code: 5FMG) is shown in figure 19 (24). The following is determined using Ligplot. Four proteins play a crucial role in interacting with proteasome its active site, namely Asp138, Thr1, Gly47 and Thr21. It is an X-modelled structure containing two identical binding sites. WLW-vs is a compound synthesized after substitution

of Leu residues to Trp residues. This compound showed great activity and therefore inhibitory features of β_2 and β_5 activities (24).

Shuttle proteins maintain the transfer of disabled proteins to the proteasome by interacting with proteasome receptors. One of these essential proteins is Dsk2, this protein cooperates with early transmembrane protein 5 (ETRAMP-5), which are indispensable in the blood stage survival of parasites. Dsk2 is part of ubiquitin domains. On the one hand, ubiquitin-like domains are located at the N-termini (UBL), when on the other hand, ubiquitin-associated domains are located at the C-termini (UBA) (15). Parasitic proteasome receptors include Rpn10/S5a, Rpn13/Adrm1 and Rpn1. Although the function of plasmodium shuttle protein and their complete interaction mechanism of the parasitic proteasome is still not elucidated fully. Based on human proteasome knowledge, it has been observed that shuttle proteins maintain the transfer of disabled proteins to the proteasome for degradation of ubiquitin-like and ubiquitin-associated domains (UBL-UBA). Plasmodium falciparum Dsk2 (*Pf*Dsk2) contains 388 residues and maintains a UBL-UBA domain, at N-termini and C-termini, respectively. It was discovered that the UBL domain retains two active binding sites.

The crystal structure of *Pf*Dsk2-UBL reveals two core-binding surfaces, where interactions between the proteasome receptor and the residues occur (figure 20). The first core-binding surfaces (CBS) consists of four β-loops formed by residues Ile48, Phe49, Lys50, Gly51, Lys52, Ile53 and Leu54. The second CBS is formed of five amino acids Asp69, Thr70, Met71, His72 and Val74. HumanDsk2-UBL is characterized by slightly different amino acids numbers. Yet, the same charged surface as *Pf*Dsk2-UBL has been recognized. To gain selective proteasome inhibitors against *Pf,* differences in core binding surfaces are fundamental. Luckily, a dissimilar feature has been observed. When comparing α-helices of humanDSK2-UBL and *Pf*Dsk2-UBL to each other, differences between its surface electrostatic potential were observed. In *Pf*Dsk2-UBL the surface of the α-helix is formed by acidic residues, establishing a net negative surface charge towards the exposed solvent. On the contrary, in humanDSK2-UBL this region is creating a net positive charge because of the α-helix displays basic residues towards the solvent. Enthusiastically, another extensive diversity between humanDSK2-UBL and *Pf*Dsk2-UBL has been detected. Where Asp39 and Glu36 are based in *Pf*Dsk2-UBL, two different amino acids were located at the same spot in humanDSK-2 (Lys63 and Lys66) (25).

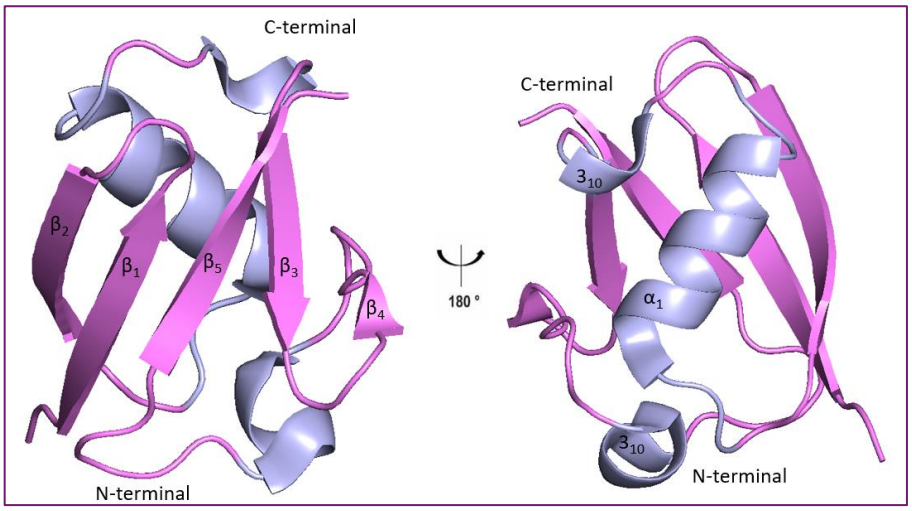

Figure 20. Monomeric structure of PfDsk2-UBL and first core-binding surface. Image is made with PyMol Molecular Graphics System (Delano Scientific).

Parasitic invasion in host cells

Here, a look into enzymes involved to achieve a favourable invasion into human host cells is discussed. At first, the role of myosin-A will be explained, followed by an enzyme important in membrane targeting: N-myristoytransferase. Additionally, the heat stress response of the malaria parasite-induced by Hsp70x will be reviewed. Last but not least, Rh-5-CyRPA-Ripr invasion complex will be deliberated.

In this publication, the role of *Plasmodium falciparum* myosin-A (*Pf*MyoA) in cell-invasion is studied. It showed to be an essential enzyme involved in red blood cell (RBC) invasion by merozoites, which contains parasitic sources for survival. Commonly, myosin-A is a crucial enzyme participating in

muscle-cell contraction in humans, but remarkably also in parasites. One of the advantageous characteristics of *Plasmodium* parasites is its ability to replicate and move very quickly. To move most efficiently, the parasite utilized a substrate-dependent mechanism which is called gliding motility using the protein glideasome and consists of both myosin and actin. *Pf*MyoA only consists of two domains, a motor domain and a light chain binding domain, respectively. The N-terminal of the protein has been named after its consisting protein, namely: myosin tail interacting protein (MTIP). This protein plays a key role in the binding of myosin to a complex of glideosome-proteins (GAP45-GAP60-GAP40). Recent studies showed that genetic ablation of GAP45 resulted in less invasion in merozoite stages. However, the structural function of GAP45 in apicomplexan cells is still unresolved, which is not within the scope of this review and therefore will not be discussed further. In short, since glideasome (composed of both actin and myosin) is such a fundamental protein, it is an attractive target for new antimalarial drugs.

The motor domain of *Pf*MyoA has been visualized with a resolution of 2,82Å (PDB code 6I7E). One part of the motor domain is located in the Rigor-like state (RLS), while the other three motor domains are located in the Post-rigor state (PRS). The *Pf*MyoA RLS maintains a perfectly closed cleft, meaning it could be a great model of myosin XIV. Various myosin classes are existing, with *Pf*MyoA belonging to the class myosin XIV. Switch-2 is fundamental in causing movement adjoining the active site. Further, the 'Wedge' is also involved in the interaction to Switch-2, which is indispensable in motor activity. The motor domain of *Pf*MyoA consists of two ligands (ADP801 and VO4803) and one metal (magnesium). The ligand ADP801 is attached to Thr198, Lys197, Glu199, Glu196, Asn244, Gly194 and the other ligand (VO4803). VO4803 is composed of hydrogen bonds between Ser246, Asn242, Ser245, Ser193, Lys197 and Adp801, forming a large complex (figure 21) (10).

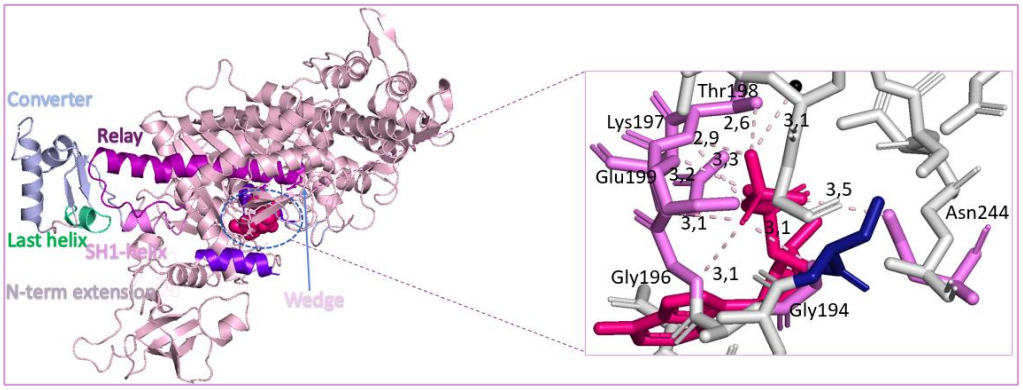

Figure 21. Overall view of Plasmodium myosin A generating protein (PfMyoA), pre-powerstroke. Relay is characterized as purple, converter as light blue, last helix as green, N-term extension as light grey and wedge is pointed with the arrow. Image is made with PyMol Molecular Graphics System (Delano Scientific).

N-myristoytransferase (NMT) is a key protein involved in membrane targeting, which is essential in various eukaryotic species. Research already concluded that more than 30 NMT substrate proteins are involved in membrane targeting and thus membrane invasion of erythrocytic stage *Pf* species. NMT is a catalysator of myristoylation of protein substrates, which is a central compartment of parasitic host cell membrane invasion. To be specific, it catalyses the relocation of the myristate compartment from myristoyl coenzyme A (Myr-CoA) towards the N-terminal of the glycine residue, where cleavage of the initiator methionine happens. This is essential for protein stability and localization of proteins to membranes.

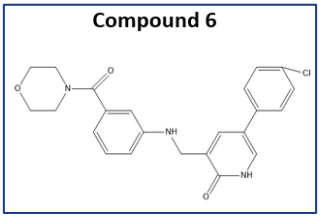

Figure 22. Linear structure of prioritized compound cluster compound 6, a NMT inhibitor. Structure is exhibited using Chemdraw Professional 18.1 (PerkinElmer).

With this in mind, inhibiting NMT could be a potential anti-malarial drug target. Accordingly, this was elucidated and encouraging results were obtained. First of all, NMT inhibitors showed poisonous activity against blood-stage and liver-stage *P*. species, which happily means it could target multiple life-cycle stages. On the contrary, NMT is not only expressed in parasites but also humans. Two forms of NMT (NMT-1 and NMT-2) are expressed in the human genome, while the parasite genome only expresses one isoform. Consequently, extraordinarily specific small molecule inhibitors are required with affinity over *Plasmodium falciparum* NMT (*Pf*NMT), then over humans NMT isoforms. Here, selective inhibitors against *Pf*NMT and *Pv*NMT are visualized. Compound 6 (figure 22) is bound to *Pv*NMT and myristoyl-CoA (ternary structure). The inhibiting peptide-binding pocket of *Pv*NMT (Tyr334) differs from *Pf*NMT (Tyr211) (figure 23). Also, compound 6 its C-terminal is composed of amino acids Val96, Phe103, Phe105, Tyr211 and Leu388 where next to a hydrophobic pocket is located. Furthermore, a direct hydrogen bonding interaction between the N atom of Asn365 and the O atom of the morpholine group (Tyr211) has been detected (26).

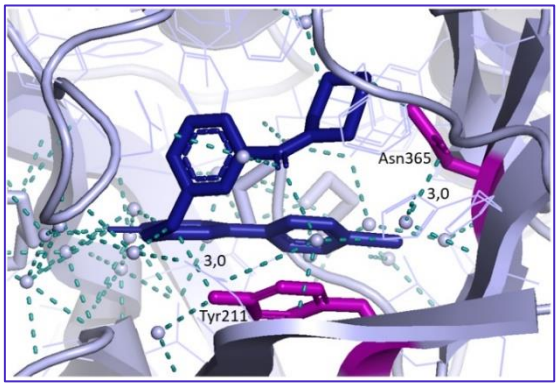

Figure 23. Ternary complex of the active ligand binding site of PfNMT with myristoyl-CoA and compound 6 (blue). Hydrogen atoms are visualized as light blue spheres. Image is made with PyMol Molecular Graphics System (Delano Scientific).

Host cell remodelling is mediated by various parasitic proteins, including *Plasmodium falciparum* heat shock protein chaperone: Hsp70 (*Pf*Hsp70). *Pf*Hsp70 plays an influential role in the appearance of *Plasmodium* its life-cycle. It reinforces the growth of the parasite at the beginning in its life-cycle. It has been suggested that *Pf*Hsp70 is similar to human-Hsp70 chaperones. Nevertheless, selective inhibition of this protein may result in shrinking parasitic growth. Given these points, antagonizing *Pf*Hsp70 is a hopeful drug target in the defence mechanism of malaria. The *Pf* genome encodes numbers of chaperones, which are meaningful to protein quality control. As a result, *Pf* uses this protein to expand its survival likelihood by correct protein folding. The genomic locus is located at chromosome 8 (*Pf3d7-0831700*), of which a cochaperone PFA0660w (*Pf3d6-0113700*) can enhance Hsp70 its activity. In figure 25, the catalytic domain of *Pf*Hsp70 has been shown, as well as the chaperone-enhancing domain of PFA0660w. Three different crystallographic structures were visualized in figure 24, 25 and 26: *Pf*Hsp70 in complex with AMP-Pnp (*Pf*Hsp70-A) (PDB code 6RZQ), *Pf*Hsp70 in complex with ADP (*Pf*Hsp70-B) (PDB 6S02) and PFA0660w (PDB code 6RZY). *Pf*Hsp70-A consists of 5867 proteins, 112 ligands and 357 water molecules, *Pf*Hsp70-B is composed of 5671 proteins, 79 ligands and 287 water molecules and PFA0660w consists of 1140 proteins, zero ligands and 124 water molecules(27).

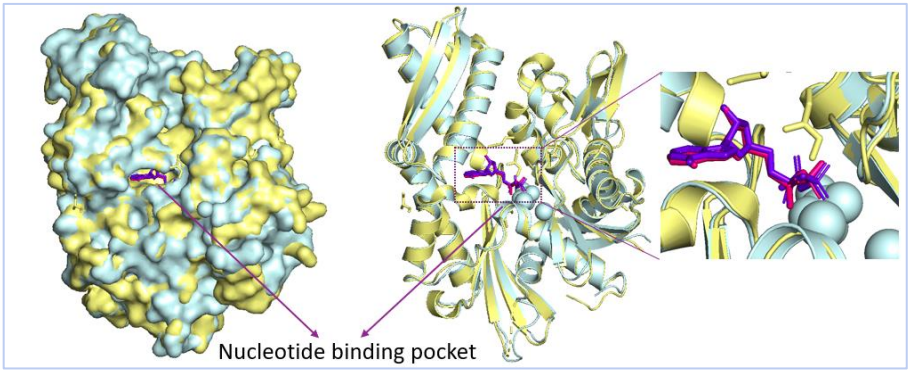

Figure 24. Structural alignment of PfHsp-70A and human Hsp70 showing excessive structural similarities and their nucleotide binding pocket. Image is made with PyMol Molecular Graphics System (Delano Scientific).

***Pf*Hsp70-A**

*Pf*Hsp70-A largest ligand and its interactions involved are resolved using Ligplot. The active site is composed of electrostatic interactions, hydrogen bonds and metal-ion interaction. Hydrogen bonds are formed between Asp182/Arg51, Gln186/Asn52 and Asp182 and Tyr164. Additionally, salt bridges are formed between Glu248/Lys218 and Asp182/Arg51.

***Pf*Hsp70-B**

*Pf*Hsp70-B consists of five β-sheets and 18 α-helices. Further, a less extensive ligand has been formed compared to *Pf*Hsp70-A. Direct hydrogen bonds are formed between ligand Adp450, Tyr44, Thr43, Arg373, Lys302 and Gly299. Furthermore, A magnesium atom plays an important role in the active site. Besides, hydrophobic interactions are formed all over the protein.

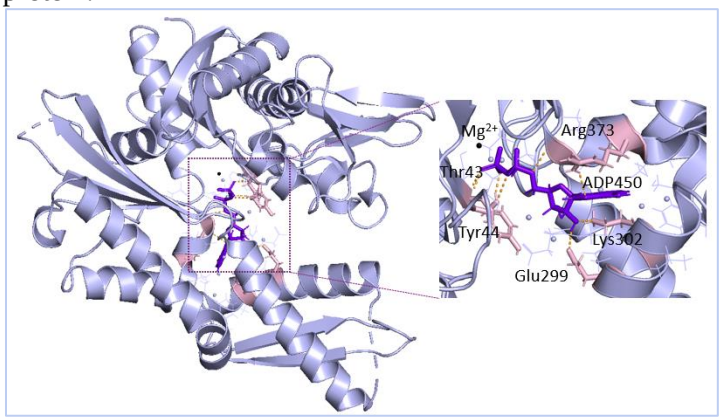

Figure 26 Crystal structure of PfHsp70-B, ligand is coloured in blue-purple, hydrophobic interactions are shown as lines and interacting residues forming hydrogen bonds are visualized in pink. Image is made with PyMol Molecular Graphics System (Delano Scientific).

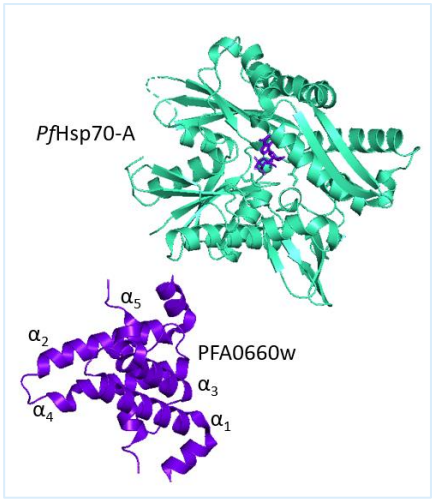

Figure 25. Hsp70 chaperones complex of PFA0660w (bluepurple) and PfHsp70-A (greencyan). Image is made with PyMol Molecular Graphics System (Delano Scientific).

PFA0660w

This protein consists of five α-helices, two β-turns and nine helix-helix interactions. This tiny protein consists of several residues interacting through hydrogen bonds and salt bridges. The core consists of a large negative cleft, surrounded by aromatic residues.

To accomplish erythrocytic cell invasion, many interactions are required between ligands from the parasite and host receptors. Here, a structure essential for erythrocytic host cell invasion is shown: *Pf* Rh5-CyRPA-Ripr complex. Additionally, this complex is of great importance in Ca^{2+} transport. The research by Wong, et al. demonstrated that Rh5-CyRPA-Ripr binds with greater affinity to the erythrocyte cell line JK-1 than Rh5 alone. Furthermore, this binding feature only arises when ligand Rh5 and Ripr are inserted into the host membranes. The interactions between the essential invasion complex, *Pf* Rh5-CyRPA-Ripr, and between members of this complex are visualized in figure 27. As a result, CyRPA established a fundamental mediator of complex assembly. The β-sheet of CyRPA is suited as an active site for Rh5 and Ripr.

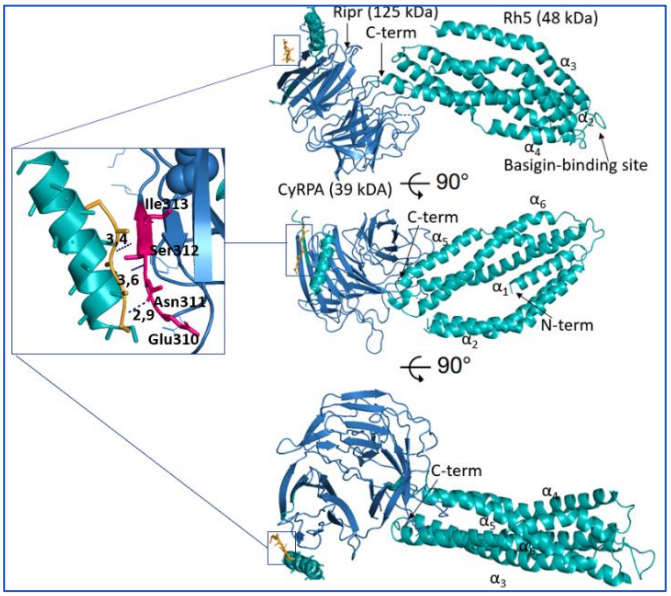

Figure 27. 3D-construction of the organization of Plasmodium falciparum Rh5-CyRPA-Ripr invasion complex. Image is made with PyMol Molecular Graphics System (Delano Scientific. Active ligand is shown in orange and its interactions are coloured in hot-pink. Image is made with PyMol Molecular Graphics System (Delano Scientific).

The complexes Rh5-CyRPA and CyRPS-Ripr are dependent on the separation of Rh5 and Ripr from CyRPA to insert erythrocytic membranes. Additionally, by identifying the crystal structure of Rh5-basigin and cryo-electron microscopy structure of Rh5-CyRPA-Ripr, it was suggested that Rh5 and Ripr are located parallel to the erythrocyte membrane before membrane invasion. This is novel expertise towards *Pf* erythrocytic host-cell invasion. In figure 27, the organization of Rh5-CyRPA-Ripr ternary complex is shown. The protein reflects a nicely rounded shape, composed of five disulphides and seven β-sheets. A strong hydrogen bond of 2,9Å, arranging from Asn312, interacts with the active ligand. Also, three strong hydrophobic interactions of Ile313, Ser312 and Glu310 are of great importance in this structure. A large negative cleft is exhibited in the centre of the protein, which displays the CyRPA region(28).

Inhibition of energy requirements crucial for malaria parasite survival

Just like humans, parasites are dependent on glucose to stay alive. Glucose is of great importance during blood-stage malaria parasites. Henceforth, inhibition of glucose uptake mechanism can function as a potential antimalarial drug target. However, the same physiological process in human beings must not be affected! This is in the development of new drugs, of course, absolutely challenging.

Glucose uptake and transport of *Plasmodium species*

The *Pf* mediates glucose uptake utilizing a hexoses transporter (*Pf*HT1). The genome of the parasite encodes only one single gene: *PF3D7_0204700*. The human glucose transporter in erythrocytes, GLUT1, shares roughly 30% and 50% sequence identity and analogy to the parasitic transporter (29). Even so, very selective inhibitors of *Pf*HT1 may hopefully result in undermining parasite growth (29)(30). *Pf*HT1 is a member of the major facilitator superfamily (MFS), which is categorized as two symmetrical six transmembranes (TM) α-helices packages (30).

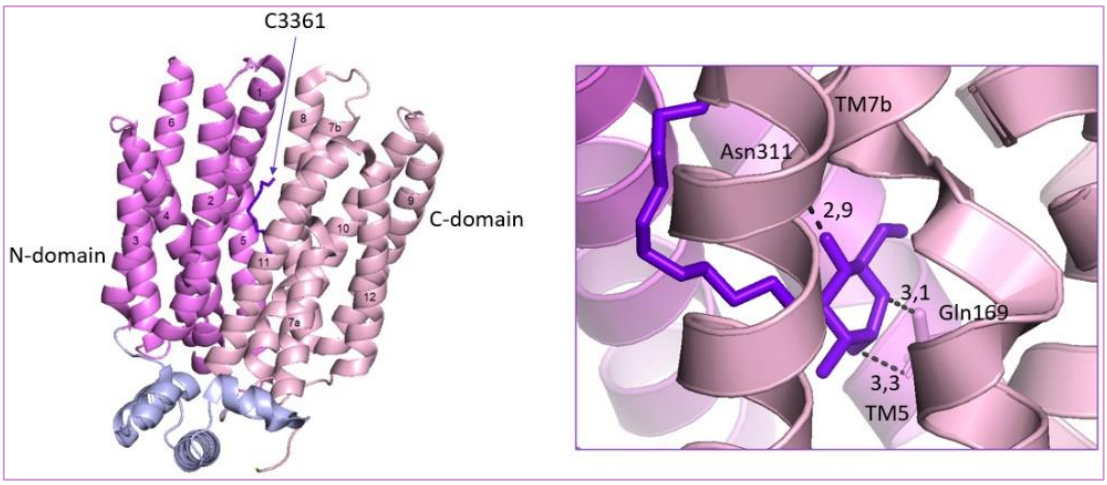

Figure 28. Left: overall protein structure of PfHT1 (3,7Å resolution), together with inhibitor C3361 (blue-purple) which is located right in the middle of protein HT1. The image on the right shows the Inhibitor-Binding-Induced pocket. Image is made with PyMol Molecular Graphics System (Delano Scientific).

In the research of Jiang et al, it had been discovered that a small molecule inhibitor, compound 3361 (C3361), blocks glucose uptake into parasitic red blood cells (RBC) in a medium composed of D-glucose. In figure 28 (PDB: 6M2L), the crystal structure of transport protein sole hexose transporter in a complex (*Pf*HT) with D-glucose and a selective inhibitor C3361 is visualized. The binding pocket of C3361 is located accurately in the middle between de N- and C-domains, consisting of six transmembranes each (29)(30). Notably, one disulphide bond (between Cys61 and Cys70) is of particular importance in this structure. When Cys61 is substituted for Ser, a remarkable reductive chance in glucose transport activity appeared. Cys61 and Cys70 represent two α-helices through the formation of a disulphide bridge, a coordinated movement between these two helices can be eased. Nevertheless, extracellular loops (coloured in light blue), are expected not to take part in glucose transport (29). Glucose is composed of several hydroxyl groups, each harmonized with partially one hydrogen bond (H-bond).

On the one hand, the N-domain of the protein only exhibits one residue Gln169, which is located on transmembrane 5 (TM5), while on the other hand, the C-domain exhibits four polar residues: Gln305 (TM7a), Asn311 (TM7b), Asn341 (TM8), Trp412 (TM10).

Azia Qureshi et al (2020) (30), also investigated the molecular basis for sugar import in malaria parasites (30). Here, the structure of *Pf*HT1 is shown (PDB: 6RW3) (figure 29). *Pf*HT1 is stabilized by an intracellular salt-bridge network and consists of 24 α-helices and 64 helix-helix interactions. As a result, the protein is stabilized to a great extent. Residues Gln306, Trp412, Gln305, Gln169 and Asn311 were purposeful to be imperative in sugar transport. Hydrogen bonds are formed between amide (N-atom) of the residues and the O-atoms of the sugar-binding site.

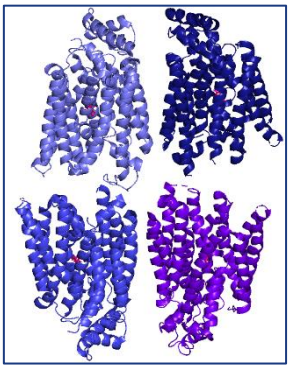

Figure 29. Quaternary complex of PfHT1. Image is made with PyMol Molecular Graphics System (Delano Scientific).

Instantly, the relevance of processes such as glycolysis for the parasite will be discussed. Parasites are dependent on glycolysis to produce energy (ATP). Enzymes involved in this pathway, such as the catalysator pyruvate kinase (PYK), is crucial for ATP production.

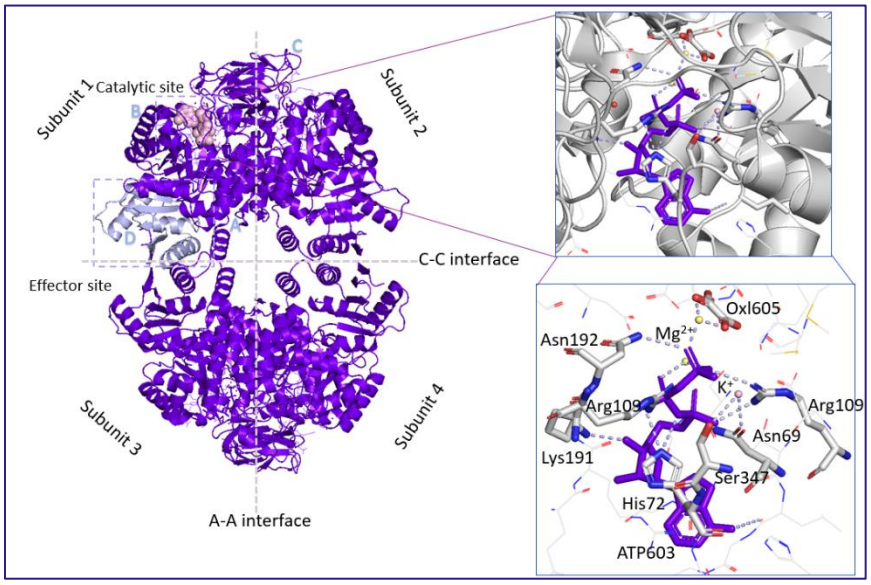

Figure 30. Four monomeric structure of PfPYK-1, including four subunits containing each four domains (A, B, C and D), marked in light blue. Image is made with PyMol Molecular Graphics System (Delano Scientific).

Two isoenzymes are present in *Pf*, namely PYK-I which is primarily participating in the glycolysis pathway and PYK-II which is principally involved in lipid synthesis. Here PYK-I of *Pf* (*Pf*PYK-1) is further elucidated. The structure of PfPYK-1 exists of four monomers (one tetramer), including four domains (A, B, C and D) in each monomer which is shown in figure 30. The small N-terminus (A) is composed of residues 1-28, while the B-domain consists of residues 29-105 and 204-375. Further, domain C is composed of residues 106-203 and D-domain consists of residues 376-511. The B-domain is a closed confirmation and is, therefore, able to hold ligands to the active site. When the active site is not bound, the B-domain will adopt an open conformation (31). Eight magnesium (Mg) atoms and four kalium (K) atoms are involved in this protein structure and interact with several amino acids. Interactions involving metal K include amino acids Asp102, Asn69, Ser71, Thr103, Ser228 and certainly ATP. ATP is undoubtedly also involved in interactions involving metal Mg, as well as residues Asn192, Arg109, Lys191, His72, Ser237, Arg109, As69, Ser347 and ligand oxalate.

De-novo pyrimidine synthesis of Plasmodium falciparum

Aspartate transcarbamoylase (ATC) is a key enzyme involved in *de-novo* pyrimidine synthesis of *Pf*, which is cited as *Pf*ATC. It catalyses the condensation reaction of carbamoyl-phosphate (CP) and L-aspartate to synthesize N-carbamoyl-L-aspartate (CA) and phosphate, which represents the second step of *de-novo* pyrimidine synthesis of *Pf*. With this in mind, enzymes involved in this pathway, such as aspartate transcarbamoylase, might be a potential drug target. In addition, dihydroorotate dehydrogenase originated from *Pf* (*Pf*DHODH) has been approved as a drug target and several clinical trials are currently progressing (DSM265). Here a compound antagonizing *Pf*ATC (2,3-napthalenediol) is shown in complex with crystal structure *Pf*ATC. As shown in figure 31, 2,3-napthalenediol does not bind the active site precisely, but within reach. Furthermore, two 2,3-napthalenediol molecules were bound to the asymmetric unit. Polar contacts are formed of 2,5Å between 2,3-napthalenediol and Glu140. Moreover, the inhibitory compound showed seven hydrophobic interactions (32).

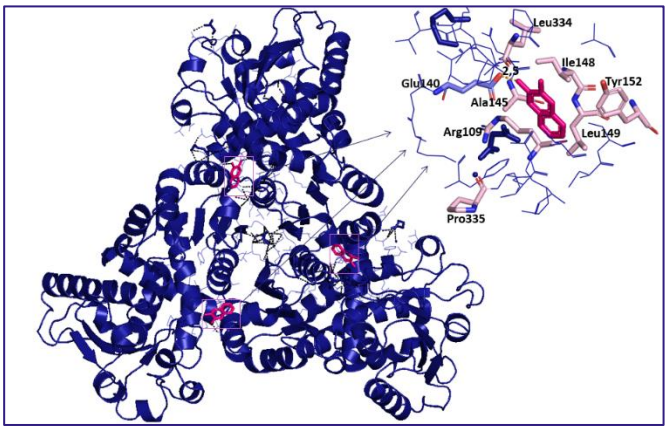

Figure 31. Ternary crystal structure of Plasmodium falciparum aspartate transcarbamoylase (PfATC), composed of three active binding sites (PDB code 6FBA). Image is made with PyMol Molecular Graphics System (Delano Scientific).

Dihydroorotate dehydrogenase DHODH

As mentioned before, dihydroorotate dehydrogenase enzymes are promising antimalarial drug targets. Currently, DSM265, a triazolopyrimidine class inhibitor, is in the progress of clinical development (1)(11)(12)(13). Novel hydroxyazole scaffold-based *Pf*DHODH inhibitors are currently explored. Crystal structures of two most encouraging inhibitors, thiadiazole 7 and hydroxypyrazole 8 have been determined. A resolution of 1,95Å was obtained in terms of inhibitor 7 (PDB 6I55) and inhibitor 8 revealed a 1,98Å resolution (PDB 6I4B).

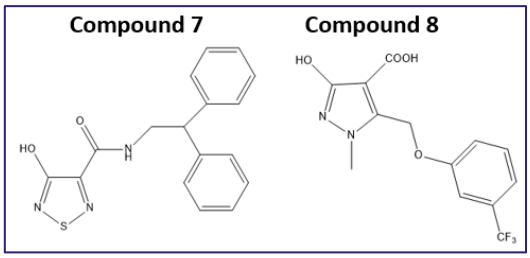

Figure 32. Linear structure of compound 7 (thiadiazole) and 8 (hydroxypyrazole) scaffolds, PfDHODH inhibitors. Structure is exhibited using Chemdraw Professional 18.1 (PerkinElmer).

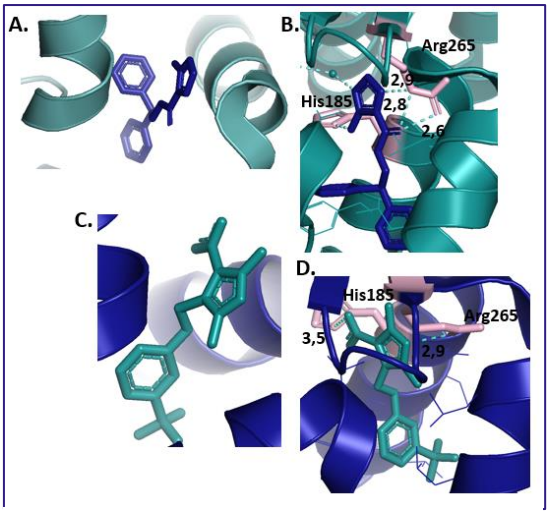

Figure 33. Binding modes of compounds 7 (A & B) and compound 8 (C & D) co-crystallized with PfDHODH. Images are made with PyMol Molecular Graphics System (Delano Scientific).

Decisive amino acid residues that play a crucial role in selectivity are Arg265 and His185 assigning both compounds. Of which the key polar interaction is formed between compound 7 and His185. Also, compound 7 is able to form interactions with the lipophilic region (consisting of Ile272 and Ile263), because this district consists of sulphur in thiadiazole ring which is pointed to these residues. Speaking of compound 8, the free carboxylate is crucial in order to interact with His185 (12).

Drug development strategies

Previously a non-toxic, child-friendly antimalarial drug gets on the market, a lot of research has been done of which discovery of potential drug targets and their affinity and selectivity towards the active site of plasmodium proteins is of indispensable importance. Here, numbers of novel potential drug targets are shown, meaning that great potential agents with new modes of actions are currently investigated. Therefore, still hope for a non-resistance great working, low costs, the anti-malarial drug has elevated! Several strategies to develop new antimalarial drugs are currently used. At first, combination therapies are prescribed to diminish resistance and, in effect improved efficacy (5)(33). Another strategy is the development of analogues of available drugs (33). Table 1 shows the prophylaxis drugs prescribed and used in The Netherlands. An example of structural analogy is the antimalarial drug mefloquine, which is derived from quinine employing chemical modifications. With this in mind, DSM265 is synthesised and derivatives are researched to gain higher selective drugs with fewer side effects. The third strategy is the exploitation of natural products, and of course, their derivatives, compounds used to treat other diseases, including derivatives (33). The last strategy is the strategy this thesis is about: compounds active against novel drug targets. This seems to be the most engaging method to develop new chemotherapy. The development of new protein visualization techniques (cryo-EM, X-ray crystallography and NMR-spectroscopy) makes it clear what the protein is made of, what it looks like and how its shape is important in binding. Additionally, it is a promising technique to visualize the better of worse binding affinity of derivative compounds. Hopefully, a better insight into numbers of new possible drug targets is visualized clearly to get a better understanding of the proteins involved in the life cycle of the malaria parasite, *Plasmodium* species, with a direct ambition to develop new antimalarial drugs!

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