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Surface antigenic changes in *P.falciparum* infected erythrocytes following treatment with Syk inhibitors and Artemisinin

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Surface antigenic changes in P.falciparum
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Syk inhibitors and Artemisinin

Carlo Zuddas

PhD thesis

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ABBREVIATIONS

WHO: World Health Organization

EMA: European Medicines Agency

P. falciparum: Plasmodium falciparum

PBMC: Peripheral blood mononuclear cells

RBCs: Red Blood Cells

PRBC: Parasitized red blood cell

NPRBC: Not parasitized red blood cell

TRPBC: Trophozoite stage parasite

TESs: therapeutic efficacy studies

ACTs: Artemisinin based Combination Therapies

ART: Artemisinin

DHA: Dyhidroartemisinin

PQ: Piperaquine

SYK: Spleen tyrosine kinase

PTK: Protein tyrosine kinase

K13: Kelch 13 propeller

IC50: Inhibitory Concentration of 50%

Hb: Hemoglobin

GSH: Glutathione

MDR: Multidrug resistance

GM: Growth medium

SAG: Saline-adenine-glucose medium

Nabs: Naturally antibodies

ROS: Reactive oxygen species

MFI: Mean fluorescence intensity

RLU: Luminescence signals

M-SFM: Macrophage Serum Free Medium

PBS-G: Phosphate buffered saline – supplemented with Glucose

IP: Immunoprecipitation

SDS: Sodium dodecyl sulfate

TEMED: Tetramethylenthylene-diamine

SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

BSA: Bovine Serum Albumin

FCS: Fetal calf serum

SUMMARY

The human Plasmodium falciparum (*P.falciparum*) parasite, currently infects more than 200 million people annually, causing about 500 000 deaths a year and imposes considerable morbidity on the surviving population. Since 2001, the WHO has recommended Artemisinin based combination therapies (ACTs) as treatment of choice for falciparum malaria. However the WHO has observed foci of suspected artemisinin resistance in South- east Asia. Because strains of *P. falciparum* are rapidly emerging that are resistant to all known antimalarial drugs, including artemisinin, quinine, chloroquine, piperaquine, and mefloquine and their derivatives, emphasis is currently laid on comprehension of new therapies with novel mechanisms of action that includes also the patient's immune response.

Delayed parasite clearance (DPC) has been identified as an useful indicator of artemisin resistance but it has been shown that parasite clearance suffers interindividual variability and reactivity to antimalarials may depend on host immunity.

Recently, studies have demonstrated Syk Inhibitors (R406) as potentially useful new class of antimalarial drugs reducing parasitemia by two ways i) delaying *P.falciparum* growth and ii) suppressing merozoite egress. The latter is caused by interfering of Syk inhibitors with the membrane of the parasite harboring host RBC.

Aim of this study is to understand whether the efficacy of new antimalarial combinations of Syk inhibitors and artemisinins (ARTs) is paralleled by enhanced immune responses of the host. I tried to identify a role of antimalarial drug treatment in the parasites clearance by host's innate immunity. To reach the goal, I studied the activating effect of Syk inhibitor R406, dihydroartemisinin (DHA) and the combination of both on cellular immune functions in *in vitro* experiments with human monocytes.

First line defense mechanism against the malaria parasite, such as phagocytosis and oxidative burst were assessed in cultured primary phagocytes using ring-stage parasitized RBC as phagocytosis target without and with previous DHA and R406 treatment. The molecular basis for observed functional changes was investigated studying DHA- and R406-dependent opsonin-binding to ring-stage pRBCs. by flow cytometry, Western blotting and immune-precipitation.

Monocytes show an increased phagocytosis level after treatment of parasitized Ring-PRBC with DHA and R406 and highest phagocytosis values when DHA and R406 were supplemented together at concentrations of 0.1uM and 0.5 uM, respectively. Membrane-bound autologous IgG and C3c complement factor were remarkably increased on Ring-PRBC surface after treatment with DHA and R406 as judged by flow cytometry. Immunoprecipitation confirmed Band 3 as main protein that is labelled by IgG in Syk-inhibitor treated pRBC and the decreased IgG/band 3 ratio in treated cells vs. untreated ones supports the band 3 aggregation model as signal for IgG flagging.

Enhanced phagocytosis of PRBCs may represent the common mechanism for innate malaria protection in nonimmune individuals. Modifications on band 3 of host cell membranes accumulate by the oxidative challenge of the growing parasite accompanied by binding of haemichromes to the cytoplasmic tail of band 3. At the moment when a threshold of modifications is exceeded mainly at trophozoite stage PRBC are recognized by phagocytes and ingested. We hypothesize Syk kinase inhibitors to anticipate the moment of recognition by an early accumulation of modified band 3 and bound haemichromes already at ring stage. Syk inhibitors are described to specifically inhib phosphorylation used by PRBC to shed off band 3 –rich microparticles from their membrane. Consequently, DHA as radical producing molecule enhances the oxidative challenge in PRBC.

In conclusion, my data support the hypothesis that Syk inhibitors are a promising class of antimalarial drugs that can suppress parasitemia by increasing also the antiparasitic immune defense. Particularly, R406 should not lead to the selection of resistant strains, as it targets host cell molecules and will likely avoid immunosuppressive effects of hemozoin due to the anticipated phagocytosis of Ring stage-PRBC. Therefore, Syk inhibitors may represent a strategic partner drug for artemisinin therapies for counteracting artemisinin resistance.

1. INTRODUCTION

1.1 Background and epidemiology of Malaria

Malaria is a parasitic disease caused by a protozoa hemosporidae belonging to the genus of plasmodium. This infective disease is transmitted by the bite of female Anopheles mosquito, which lives mainly in the region with temperate to hot climate. Among all studied mosquitoes species, only few of them are responsible for malaria disease in men, whereas the others are harmless, as they prefer animal blood compared to human blood. The main vector responsible for malaria in the Afrotropical region is Anopheles Gambiae specie (Fig.1).

Fig. 1. *Female Anopheles mosquito*

In the past few years, the disease has spread again through the western African continent even though it has been eradicated in these regions few years ago. The migrant's fluxes have been claimed as a possible cause of this unexpected event.

Malaria is an infective disease whose severe courses cause a high level of deaths in the world. According the 2018 WHO Malaria report 219 million cases of malaria. and 435 000 deaths from malaria were estimated globally. This high incidence of mortality is due to the considerable percentage of population, particularly in rural remote areas, without access to prompt diagnosis and effective treatment of malaria. More than 77% of children with severe cerebral malaria and convulsion die under 5 years of age, for this reason malaria is also known as "disease of childhood" [1]. The sub-Saharan region

is the most malaria endemic area, infact, WHO African Region accounted for 93% of all malaria deaths in 2017. Although the WHO African Region was home to the highest number of malaria deaths in 2017, it also accounted for 88% of the 172 000 fewer global malaria deaths reported in 2017 compared with 2010.

In Italy 3600 cases of malaria have been registered in the period 2011-2016 mainly due to the migratory fluxes.

Although the number of Plasmodium falciparum malaria cases has rapidly decreased in the last five years [2], malaria remains one of the most devastating infectious diseases in the world, thereby the main effort consists in eradicating it.

This disease is endemic in 103 nations therefore many people are exposed to this kind of pathology. Malaria affects also pregnant women and infants, who encounter the pathology directly from the mother, through blood exchange [3,4].

Annually around 100 thousand of infants die for malaria infected from their mother [5] and 25 million of pregnant women are at risk of infection around the world [6].

Exist five protozoa species able to cause Malaria in humans all belonging to *Plasmodium (P.)* genus:

1. *P. falciparum*
2. *P. vivax*
3. *P. ovale*
4. *P. malariae*
5. *P. knowlesi* (humans and macaques)

These species of Plasmodium cause different malaria pathologies and it is important to differentiate them due to differences in mortality, incidence and distribution.

Among the Plasmodium species, two are most important for clinical and severe malaria, *P. vivax* (endemic region) and *P. falciparum* (Africa, Asia, Latin America). *Plasmodium falciparum* represents the most pathogenic [7] with the highest rates of complications and mortality [8]

It is the most prevalent malaria parasite in the WHO African Region, accounting for 99.7% of estimated malaria cases in 2017, as well as in the WHO regions of South-East

Asia (62.8%), the Eastern Mediterranean (69%) and the Western Pacific (71.9%). *P. vivax* is the predominant parasite in the WHO Region of the Americas, representing 74.1% of malaria cases.

The immune system plays an important role in the defense against illness. The first time that our organism has contact with an infective agent is crucial, dangerous and potentially harmful, because the appropriate immune response is not yet prepared to prevent the infection. For this reason, people who are physiologically immunosuppressed such as kids are more prone to die. Next chapter are going to describe life cycle, pathophysiology and diagnosis of Malaria.

1.1.1 Life cycle of Malaria parasite

The malaria parasite life cycle involves two hosts. As shown in figure 3, during a blood meal, a malaria-infected female Anopheles mosquito inoculates sporozoites into the human host (1). The sporozoites infect liver cells (2) and mature into schizonts (3), with consequent rupture of membrane and release of merozoites (4). In *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.

After this initial replication in the liver (exo-erythrocytic schizogony (A)), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony (B)). Merozoites infect red blood cells (5). The ring stage (1-24 hours) and trophozoites (24-36 hours) mature into schizonts (36-48 hours), with lysis of red blood cells (RBCs) membrane (Fig.2) and release of merozoites (6) to further infect red blood cells. Blood stage parasites are accountable for the clinical manifestations of the disease.

During the erythrocytic cycle some parasites differentiate into sexual erythrocytic stages (gametocytes) (7).

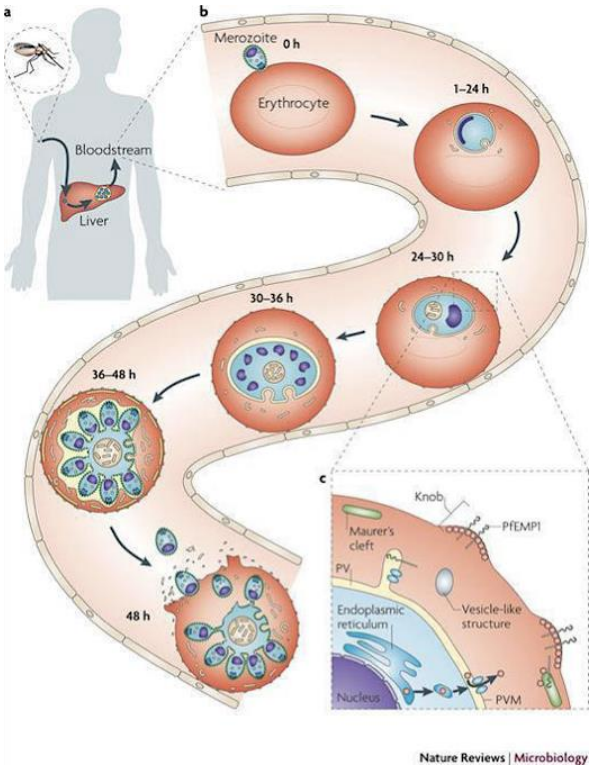

***Fig. 2**. Intraerythrocytic cycle of parasite*

The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an Anopheles mosquito during a blood meal (8). The parasites' fertilization and proliferation in the mosquito is known as the sporogonic cycle (C). In the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes 9. The zygotes in turn become motile and elongated (ookinetes) 10 which invade the midgut wall of the mosquito where they develop into oocysts 11. The oocysts grow, rupture, and release sporozoites 12, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites 1 into a new human host perpetuates the malaria life cycle (Fig.3) [9]

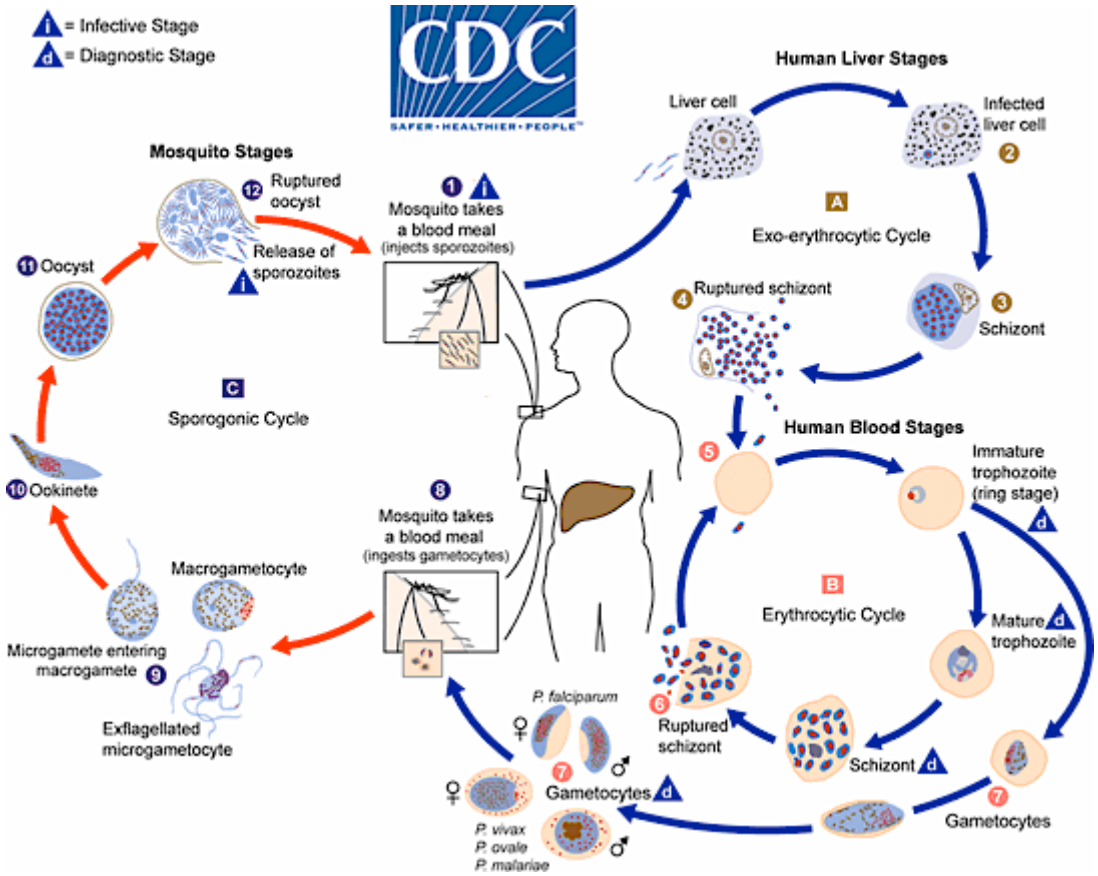

Fig. 3. *Sexual and asexual life cycle of plasmodium*

The activation of parasite metabolic processes inside the RBCs, triggers alterations of host cell energetic metabolism, proteins and membrane structural changes. An example of functional importance for *P. falciparum*- induced changes of the infected RBC is the expression of proteins on the erythrocytes external surface that mediate the infected RBCs process of adhesion to the endothelial cells in capillaries of some organs.

This phenomenon, known as "adhesion", represents an essential pathogenic mechanism in *P. falciparum* severe malaria and avoids spleen passages of the mature parasite and its phagocytic removal. Malaria parasites degrade host cell haemoglobin (hereafter referred to as Hb) as amino acid source for protein synthesis in the intraerythrocytic stage, during which the heme group is converted to hemozoin (hereafter referred to as Hz), which is essential for parasite survival [10]. Mature trophozoites digest haemoglobin and metabolize glucose, through the anaerobic glycolysis. An infected RBC increases of 50 - 100 fold the consumption of glucose with the production of lactic acid compared to uninfected red blood cells. The degradation of Hb occurs in a specialized parasite organelle called the food vacuole. Previously studies have suggested that Hb degradation is a cooperative process that involves proteases of multiple catalytic classes, including cysteine, aspartic, and metalloproteases [11]. These proteases produce short peptides that are further degraded to amino acids, probably by aminopeptidases [12]. During the process of Hb degradation, heme groups released in the food vacuole is toxic to *Plasmodium*, as it induces oxygen-derived free radical formation, lipid peroxidation and protein and DNA oxidation. Organisms such as *Plasmodium*, *Schistosoma*, and *Rhodnius*, which use Hb as a nutrient source, have evolved different strategies to detoxify free heme. Plasmodium spp. converts the heme group to β-hematin, which is a dark brown core of malarial pigment also known as Hz, through a process that is essential for the life cycle of these organisms [13,14]. The beta-hematin core of HZ is a cyclic dimer of ferriprotoporphyrin IX [Fe(III)PPIX] in which the propionate group of each Fe(III)PPIX molecule coordinates the Fe(III) centre of its partner. Dimers form chains linked by hydrogen bonds in the beta-hematin crystal [14]. The formation of mature schizonts containing a variable number of merozoites (24-32), entails their release and the lysis of erythrocytes. This process occurs every 48h corresponding with the parasite life cycle. During this event, the first clinic symptoms

of the disease are shown: the characteristic fever ‘malignant tertian’ in *P. falciparum*, ‘benign tertian’ in *P. vivax* and *P. ovale* and ‘quartan’ in *P. malariae* (Fig 4).

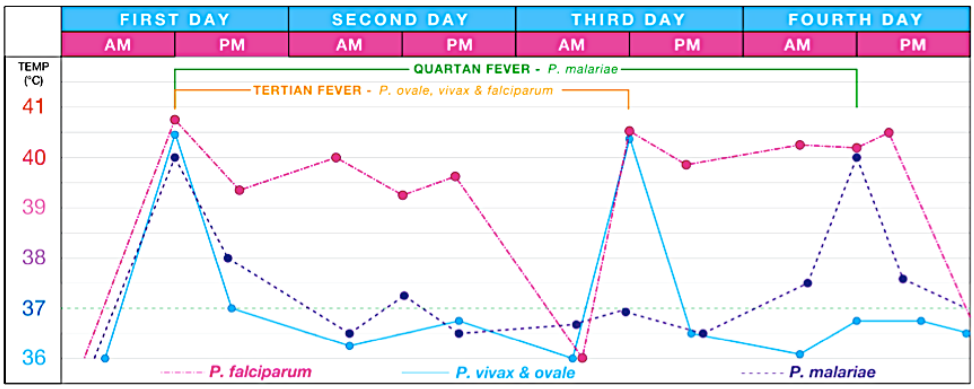

***Fig. 4**. Fever cycle in in different kind of plasmodium*

1.2 Diagnosis of malaria

The signs and symptoms of malaria are similar to those of many other febrile illnesses. In non-immune individuals, malaria typically presents with fever, sometimes accompanied by chills, sweats, headache or other symptoms that may resemble other illnesses. Consequently, fever is the main basis for suspicion of malaria, and a trigger for diagnostic testing of the patient in most malaria endemic settings. No combination of signs or symptoms that reliably distinguishes malaria from other causes of fever exists. For this reason, in malaria-endemic areas, malaria should be suspected in an patient presenting with a history of fever or temperature of 37°C and no other obvious cause. In areas in which malaria transmission is stable, malaria should also be suspected in children with palmar pallor or a haemoglobin concentration of <8 g/dL. In settings where the incidence of malaria is low, health workers should be trained to identify patients who may have been exposed to malaria. In all cases, patients with suspected malaria should have prompt parasitological confirmation of diagnosis, with either microscopy or rapid diagnostic tests (RDT), before antimalarial treatment is started.

Depending on the malaria parasites, it may be present a variety of symptoms [13], ranging from absent or very mild symptoms to severe disease and even death. For this reason, malaria disease can be classified as uncomplicated or complicated (severe) [14].

1.2.1 Uncomplicated malaria

The classical malaria attack lasts 6-10 hours. It consists of a series of events: a cold stage (sensation of cold), hot stage (fever, headaches, vomiting), and finally a sweating stage (sweats, return to normal temperature). Usually attacks occur every second day with the “tertian” parasites (*P. falciparum*, *P. vivax* and *P. ovale*) and every third day with the “quartan” parasite *(P. malariae*). More commonly, the patient presents a combination of the following symptoms: fever, chills, sweats, headaches, nausea and vomiting.

1.2.2 Severe malaria

Severe malaria occurs when infections are complicated by serious organ failures or abnormalities in the patient's blood or metabolism [15]. Most of the severe malaria complications occur in non-immune subjects with falciparum malaria and involve central nervous system (cerebral malaria), pulmonary system (respiratory failure), renal system (acute renal failure) and/or hematopoietic system (severe anaemia) [14].

These complications can be rapid and considering that severe malaria is a potentially fatal disease, any patient with malaria symptoms must be assessed and treated rapidly. The patients also, must be kept under observation to identify early signs of systemic complications.

A parasitological diagnosis should be obtained whenever possible, but the relevance of parasitaemia to the current illness must always be considered carefully. In the absence of diagnostic facilities, antimalarial treatment should not be delayed if the patient is severely ill.

The manifestations of severe malaria include:

1. Cerebral malaria, with abnormal behaviour, impairment of consciousness, seizures, coma, or other neurologic abnormalities
2. Severe anaemia due to haemolysis (destruction of the red blood cells)
3. Haemoglobinuria (haemoglobin in the urine) due to haemolysis
4. Acute respiratory distress syndrome (ARDS), an inflammatory reaction in the lungs that inhibits oxygen exchange, which may occur even after the parasite counts have decreased in response to treatment
5. Abnormalities in blood coagulation
6. Low blood pressure caused by cardiovascular collapse
7. Acute kidney failure
8. Hyperparasitemia, where more than 5% of the red blood cells are infected by malaria parasites
9. Metabolic acidosis (excessive acidity in the blood and tissue fluids), often in association with hypoglycaemia

10. Hypoglycaemia (low blood glucose). Hypoglycaemia may also occur in pregnant women with uncomplicated malaria, or after treatment with quinine.

1.2.3 Cerebral Malaria

Despite decades of research, cerebral malaria remains one of the most serious complications of Plasmodium infection [16]. If left untreated, cerebral malaria is probably nearly always fatal. Even when treated, cerebral malaria has an approximate 20% of mortality rate in adults and 15% in children. Among subjects who survive, the recovery is relatively rapid with complete reversibility of neurological signs and symptoms.

Erythrocytes infected by parasite in the microcirculation, especially the cerebral one have a tendency to accumulate, based on plasmodium antigens present on the RBC surface that bind to endothelial receptors and to form rosettes with non-parasitized RBC causing the formation of clots, blocking the bloodstream.

This process is not a classic thrombosis, because is caused by infected RBCs. Since the brain and the hematic microcirculation cannot be infected, immediately the immune system induces an acute inflammatory response leading to a degeneration of cerebral tissue. The process involves the release of the cerebral TNF and INF-γ, causing the increase of fever and definitely the death. The overwhelming cytokine release is likely due to the release of hemozoin during rupture of schizonts adhered to endothelia. Hemozoin has been shown to be a very potent short-term activator of pro-inflammatory responses, such as ROS production and pro-inflammatory cytokine release, such as TNF or MCP-1.

1.3 Erythrocytes plasmatic membrane

The RBCs plasmatic membrane is constituted of 50% protein (mainly intrinsic), 40% lipids and 10% carbohydrates. The erythrocyte is different from the other cells since the cytoskeleton forms a shell to support the plasmatic membrane.

Red blood cells plasmatic membrane structure

Protein Band 3 (Anion Exchanger 1) is the main protein (25%) in the red blood cells membrane, involved in different cell process. Its molecular weight is 95 KDa and it can exist in monomeric, dimeric, tetrameric form or aggregates [17]. In the red blood cells, the Band 3 mediates the anionic exchange between the bicarbonate ion (HCO3-) presents in cytoplasm and ion chloride (Cl-) presents in the plasma (Fig. 5).

The bicarbonate ion is involved in the following reaction catalysed from the carbonic anhydrase.

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

The presence of bicarbonate ion activates Band 3 that through an anion exchange allows the diffusion of chloride ion (chloride shift). Exported bicarbonate leaves a proton 'in excess' behind which decreases the cytoplasma pH. Acidic cytoplasm decrease the affinity between haemoglobin and oxygen in order to facilitate the transfer of it to the tissues.

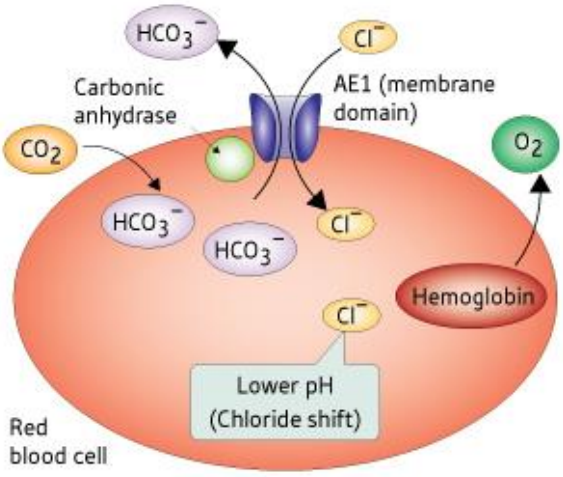

Fig.5 *Schematic showing the exchange of bicarbonate ($HCO3^-$) and chloride (Cl^-) ions via AE1*

Band 3, being the most abundant protein in the erythrocyte plasma membrane do not work only as anionic exchange, but also serves as a protein anchor, connecting the soluble cytoplasmic proteins and components of the cytoskeleton to the membrane. [18].

The protein Band 3 (Fig. 6) is composed of a transmembrane domain and two cytoplasmic domains [19]. Each protein domain has precise and different cellular functions:

- **The transmembrane hydrophobic domain** is a region of 52KDa and includes the amino acid residues 360-878. It is dipped in the bilayer of phospholipids forming a series of 12-14 foldings. This domain lead to the formation of an anionic channel that allows the exchange of Cl - against HCO3 – between the external and internal of cell.

- **The N-terminal** hydrophilic domain (cytoplasmic domain) of 43KDa, includes the amino acid residues 1-359, known as cytosolic domain of Band 3 (cdb3). It penetrates in the cytosol playing a role for anchorage of the cytoskeleton and several cytosolic proteins such as haemoglobin and aldolase. but also Ankyrin, Band 4.1 and 4.2 that define the erythrocyte shape. At the N-terminal domain can bind different kind of proteins including the ankyrin and Band 4.1 and 4.2 that characterize the erythrocyte shape.

- **The C-terminal** hydrophilic domain includes the last 33 aminoacidic residues directed towards the cytoplasm.

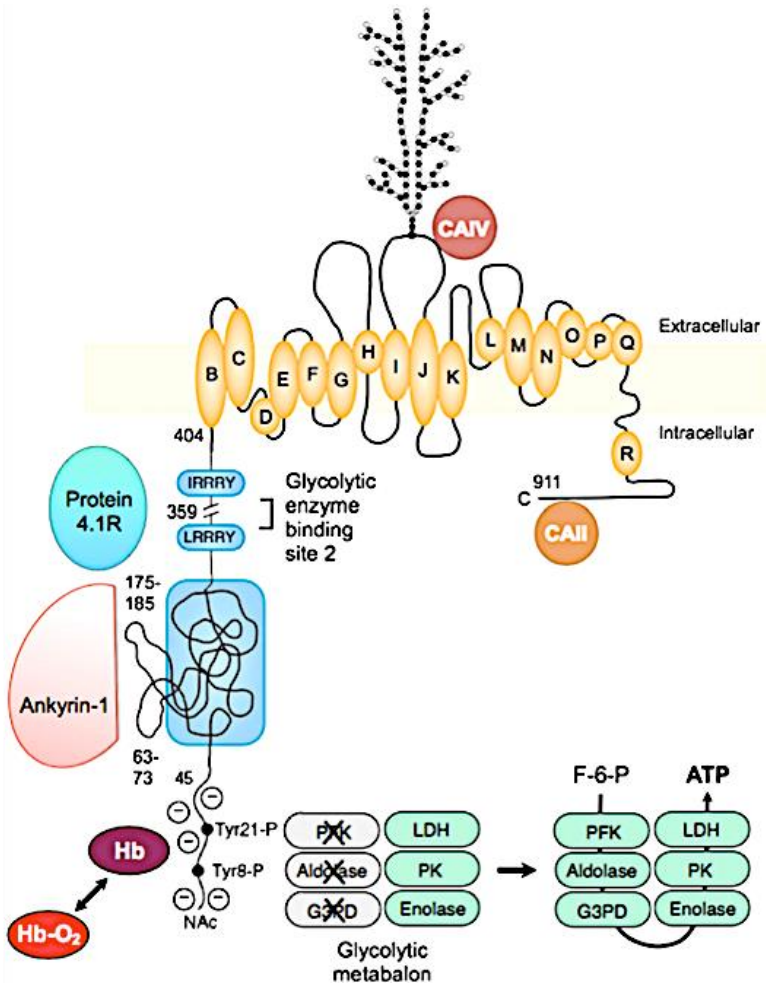

***Fig. 6**. Model of human erythrocyte band 3. The protein contains 2 structurally and functionally distinct domains: a cytoplasmic binding domain (amino acids 1-359) and a transmembrane domain (amino acids 360-878) that forms the anion-exchange channel*

The cytoskeleton is constituted of the Ankyrin complex and Actin junctional complex (Fig.7). The cytoskeletal proteins are situated in the internal surface of erythrocyte membrane and it forms a fibrillar skeleton with the function of holding the red blood cell structure.

The Ankyrin protein binds Band 3 to high molecular weight proteins as α and β spectrins, belonging to the erythrocytes membrane [20]. These proteins create few anchorage points between the β subunit of spectrin and the integral proteins of

membrane as Band 3 and glycophorin. The spectrin dimers associate in tetramers, forming with other proteins as actin, adducin, 4.9 and 4.1 protein and tropomyosin, a fibrillary net that contribute to stabilize the erythrocytes structure. Furthermore, three other proteins are present in the junctional complex, namely adducin, P55 and dematin [21,22].

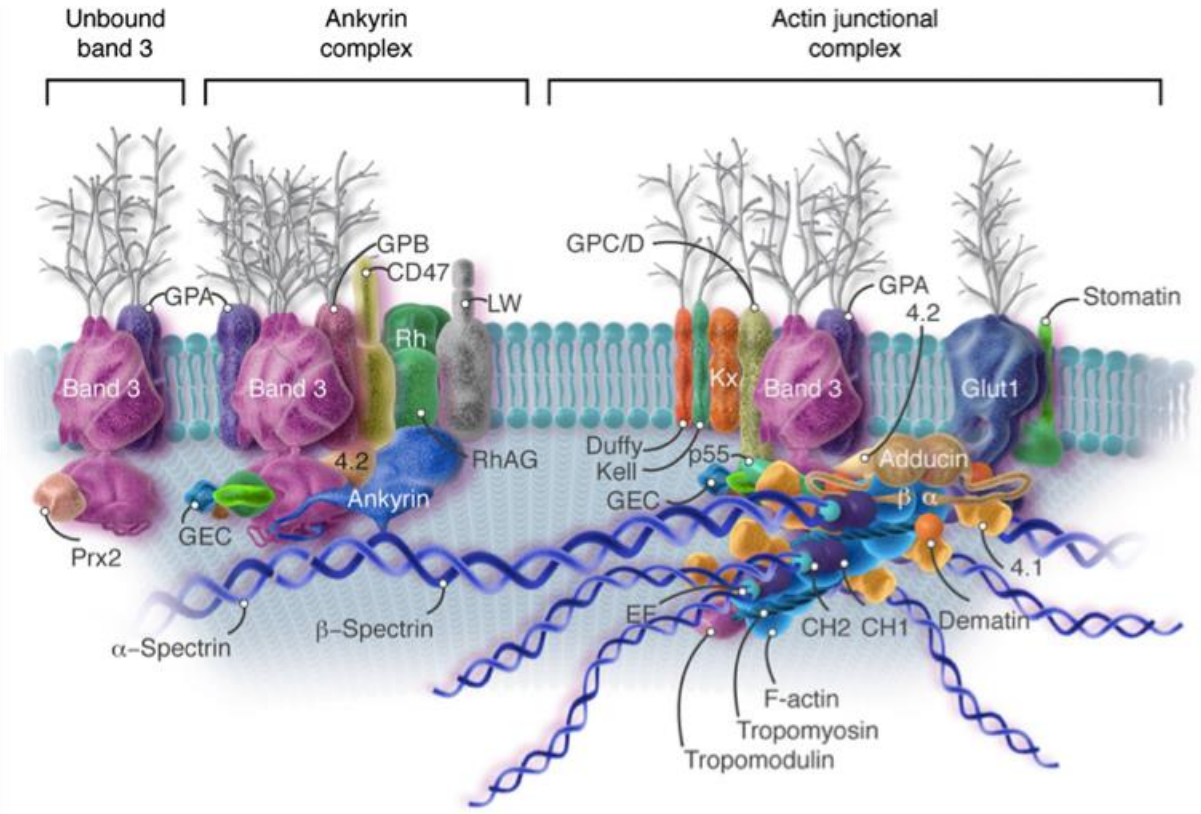

***Fig. 7**. Ankyrin and Actin junctional complex representation*

1.4 Treatment of Malaria

The World Health Organization (WHO) is annually publishing guidelines for the treatment of Malaria. The primary objective of treatment is to ensure complete cure that is the rapid and full elimination of the *Plasmodium* parasite from the patient's blood, in order to prevent progression of uncomplicated malaria to severe disease or death, and to chronic infection that leads to malaria-related anemia [23].

The principal core for the treatment of Malaria according with guidelines of WHO are:

Early diagnosis and prompt effective treatment of malaria.

Uncomplicated falciparum malaria can progress rapidly to severe forms of the disease, especially in people with no or low immunity, and severe *falciparum* malaria is almost always fatal without treatment. Therefore, programs should ensure access to early diagnosis and prompt, effective treatment of malaria within 24-48h of onset of symptoms.

Rational use of antimalarial agents

To reduce the spread of drug resistance, limit unnecessary use of antimalarial drugs and better identify other febrile illnesses in the context of changing malaria epidemiology, antimalarial medicines should be administered only to patients who truly have Malaria. Adherence to a foul treatment course must be promoted.

Combination therapy

Preventing or delaying resistance is essential for the success of both national and global strategies for control and eventual elimination of Malaria. To help protect current and future antimalarial medicines all episodes of malaria should be treated with at least two effective antimalarial medicines with different mechanisms of action (combination therapy).

Appropriate weight-based dosing

To prolong their useful therapeutic life and ensure that all patients have an equal chance of being cure, the quality of antimalarial drugs must be ensure and antimalarial drugs must be given at optimal dosages. Treatment should maximize the likelihood of rapid clinical and parasitological cure and minimize transmission from the treated infection. To achieve this, dosage should be based on the patient weight and should provide effective concentrations of antimalarial drugs for a sufficient time to eliminate the infection in all target population.

Treating uncomplicated P.falciparum malaria.

Following guidelines of WHO is appropriate treat children and adults with uncomplicated *P. falciparum* malaria (expect pregnant women in their first semester) with one of the following recommended artemisinin-based combination therapies (ACT):

Artemether + Lumefantrine

Artesunate + Amodiaquine

Artesunate + Mefloquine

Dihydroartemisinin + Piperaquine

Artesunate + Sulfadoxine-Pyrimethamine

ACT regimens should provide 3 days treatment with an artemisinin derivative. Children < 25kg treated with Dihydroartemisinin + piperaquine should receive a minimum of 2.5 mg/Kg body weight (bw) per day of Dihydroartemisinin and 20mg/kg bw per day of piperaquine daily for 3 days]23]. In low transmission areas, give a single dose of 0.25mg/kg bw primaquine with ACT to patients with *P.falciparum* malaria (expect pregnant woman, infants aged < 6 months and women breastfeeding infants aged < 6 months) to reduce transmission. Testing for glucose-6-phosphate dehydrogenase (G6PD) deficiency is not required.

Treating uncomplicated P.vivax, P. ovale, P. malariae, P. knowlesi malaria.

Following good practice statement, if the malaria species is not known with certainty, treat as for uncomplicated *P.falciparum* malaria.

In areas with chloroquine-susceptible infections, treat adults and children with uncomplicated *P.vivax, P. ovale, P. malariae* or *P. knowlesi* malaria with either ACT (expect pregnant women in their first trimester) or chloroquine.
In areas with chloroquine-resistant infections, treat adults and children with uncomplicated *P.Vivax, P. ovale, P. malariae* or *P. knowlesi* malaria with either ACT malaria (expect pregnant women in their first trimester) with ACT. Treat pregnant women in their first trimester who have chloroquine- resistant *P.vivax* malaria with quinine.

1.4.1 Artemisinin and artemisinin derivates.

The discovery of artemisinin for malaria therapy by Chinese scientists in the 1970s was one of the greatest discoveries in medicine in the 20th Century [24]. The project leading to the discovery of artemisinin was initiated in response to a request from North Vietnamese leaders suffering heavy losses of soldiers due to malaria during the Vietnam War.
Artemisinin is isolated from the plant *Artemisia annua* (Fig. 8), sweet wormwood, an herb employed in Chinese traditional medicine. The drug was brought into modern medicine by Tu Youyou. For her discoveries, Tu received the 2015 Nobel Prize for Physiology or Medicine.

***Fig. 8**. Artemisia Annua*

From Tu's discovery, Artemisinin (ART) and its semi-synthetic derivates, are a group of drugs used against *P. falciparum* malaria [25]. Treatments containing an artemisinin derivates actually represent the standard therapies worldwide for *P. falciparum.*

Chemically, artemisinin is a sesquiterpene lactone containing an unusual peroxide bridge. This peroxide (Fig.9) is believed to be responsible for the drug's mechanism of action. Because the parent drug of artemisinin is poorly soluble in water or oil, the carbonyl group of artemisinin was reduced to obtain DHA and its derivatives such as the water-soluble artesunate and oil-soluble artemether and arteether, which also show greater antimalarial activity. Few other natural compounds with such a peroxide bridge are known [26].

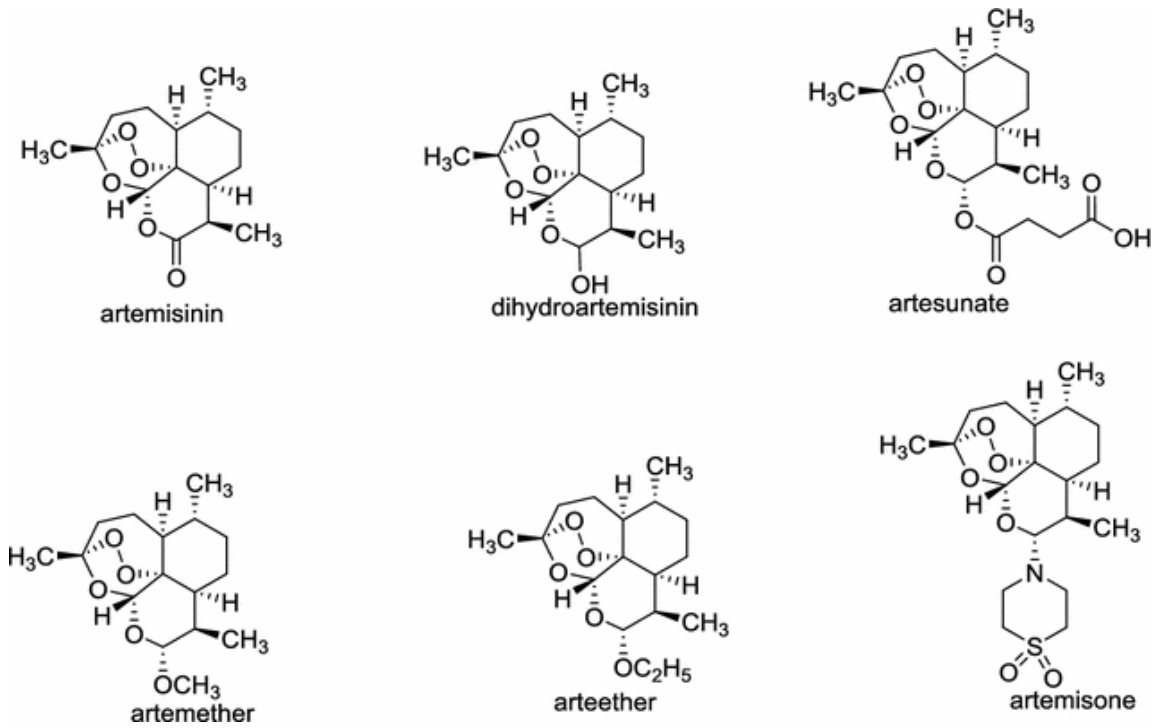

Fig. 9 *Artemisinin (ART), dihydroartemisinin (DHA) and its derivatives arteether, artemether and artesunate, artemisone.*

Therapies that combine artemisinin or its derivatives with some other antimalarial drug are the preferred treatment for malaria and are both effective and well tolerated in patients.

Mode of action & potential cellular targets

The mechanism of action of artemisinins is not known. Despite tremendous research efforts on artemisinin since its discovery, there is still considerable debate concerning its mode of action on malaria parasites. Artemisinins are considered prodrugs that are activated to generate carbon-centered free radicals or reactive oxygen species (ROS). As peroxides are known sources of ROS, earlier studies suggest that artemisinins modulate parasite oxidative stress and reduce the levels of antioxidants and glutathione (GSH) in the parasite. Thus, the most widely accepted theory is that they are first activated through cleavage after reacting with heme and iron (II) oxide, which results in the generation of free radicals that in turn damage susceptible proteins, resulting in the death of the parasite [27,28].

Another hypothetical mechanism considers the heme as target of drug action. Heme, generated from digestion of hemoglobin in the food vacuole of the parasite, is toxic to the parasite and must be detoxified through polymerization to form 'hemozoin' (malaria pigment) [29]. Artemisinin-derived radicals readily react with free hemin, heme present in the hemozoin and hemoglobin to form heme–artemisinin adducts in vitro. These adducts can be isolated from P. falciparum culture and *Plasmodium vinckei*-infected mice after artemisinin treatment. Similar heme adducts are observed with synthetic antimalarial trioxanes, suggesting an analogous mode of action for these compounds. In 2016 artemisinin was shown to bind to a large number of targets suggesting that it acts in a promiscuous manner [30].

Dihydroartemisinin (DHA)

Because the physical properties of artemisinin itself, such as poor bioavailability, limit its effectiveness, semisynthetic derivatives of artemisinin have been developed. Among derivates, dihydroartemisinin (DHA) is the active metabolite of all artemisinin compounds (artemisinin, artesunate, artemether, etc.) and is available as drug itself. It is a semi-synthetic derivate of artemisinin and is widely used as an intermediate in the preparation of other artemisinin-derived antimalaria drugs [31]. The proposed

mechanism of action of artemisinin involves cleavage of endoperoxide bridges by iron, producing free radicals (hypervalent iron-oxo species, epoxides, aldehydes, and dicarbonyl compounds), which damage biological macromolecules causing oxidative stress in the cells of the parasite [32]. Malaria is caused by apicomplexans, primarily Plasmodium falciparum, which largely reside in red blood cells and itself contains iron-rich heme- groups (in the form of hemozoin) [33]. In 2015 artemisinin was shown to bind to a large number targets suggesting that it acts in a promiscuous manner. Recent studies discovered that artemisinin targets a broad spectrum of proteins in the human cancer cell proteome through heme-activated radical alkylation [34].

1.4.2 Artemisinin-based combination therapies (ACTs) and resistance to antimalarial drugs.

Artemisinin-based combination therapy is a combination of a rapidly acting artemisinin derivative with a longer-acting partner drug. Artemisinin-based combination therapies (ACTs) are recommended by WHO as the first-and second-line treatment for uncomplicated *P. falciparum* malaria as well as for chloroquine-resistant *P. vivax* malaria. ACTs combine an artemisinin derivative with a partner drug. The role of the artemisinin compound is to reduce the number of parasites during the first three days of treatment (reduction of parasite biomass), while the role of the partner drug is to eliminate the remaining parasites (cure). The artemisinin component rapidly clears parasites from the blood (reducing parasite number by a factor of approximately 10.000 in each 48-h asexual cycle) and is also active against the sexual stages of parasite that mediate onward transmission to mosquitos [23]. However, the efficacy of ACTs is threatened by the emergence of both artemisinin and partner drug resistance. Partial resistance to artemisinin causes delayed parasite clearance following treatment with an ACT. Such resistance does not usually lead to treatment failure; however, if the artemisinin component is less effective, the partner drug has to clear a greater parasite mass, jeopardizing the future efficacy of the partner drug.

In addition, partner drug resistance can arise independently of artemisinin resistance. Given that an effective partner drug is essential for clearing all remaining parasites, partner drug resistance carries a high risk of treatment failure. Because of their different roles, the efficacy of the artemisinin and the partner drug must be monitored concomitantly but separately.

WHO currently recommends five different ACTs. However, WHO is considering the use of artesunate-pyronaridine, a new ACT that has received a positive scientific opinion from the European Medicines Agency (EMA), in areas where other ACTs are failing. In the absence of resistance, all six partner drugs would be highly efficacious as monotherapies at the dose used in the ACT. Two injectable treatments, artesunate and artemether, are recommended for the treatment of severe malaria and should be followed by an ACT once the patient can tolerate oral therapy.

1.4.3 Antimalarial drug resistance

Resistance and treatment failures to antimalarial medicines can be defined as follows:

Antimalarial resistance is defined as the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject;

Artemisinin partial resistance is defined as delayed parasite clearance following treatment with an artesunate monotherapy or with an ACT – this represents partial resistance;

Multidrug resistance (MDR) is resistance to more than 2 antimalarial compounds of different chemical classes. This term usually refers to *P. falciparum* resistance to chloroquine, sulfadoxine-pyrimethamine, and a third antimalarial compound;

Treatment failure is the inability to clear parasites from a patient's blood or to prevent their recrudescence after the administration of an antimalarial. Many factors can contribute to treatment failure, including incorrect dosage, poor patient compliance, poor drug quality, and drug interactions and resistance. Most of these factors are addressed by therapeutic efficacy studies [35].

In reporting the findings of therapeutic efficacy studies, the term "ACT resistance" is imprecise. ACT treatment failure (defined as treatment failure following treatment with an ACT, regardless of the presence of artemisinin partial resistance) is a more appropriate term that notes the specific ACT and the nature of the resistance if confirmed (i.e. artemisinin partial resistance or partner drug resistance, or both). The problem of antimalarial drug resistance is compounded by cross resistance, in which resistance to one drug confers resistance to other drugs that belong to the same chemical family or which have similar modes of action.

Clinical artemisinin resistance is defined as delayed parasite clearance; it represents a partial/relative resistance that has thus far only affected ring-stage parasites. Delayed parasite clearance following treatment with an ACT is of paramount concern to WHO.

Possible future consequences of slow parasite clearance, or partial resistance, include: a) the development of total artemisinin resistance; b) the loss of artemisinin as an effective treatment for severe malaria; and c) increased de novo resistance to the partner drug, particularly in patients with high parasitaemia at admission, and/or greater selection of partner drug resistance. If resistance to partner drugs increases, treatment failures are likely to increase in parallel. Nevertheless, the majority of patients who have delayed parasite clearance following treatment with an ACT are still able to clear their infections, as long as the partner drug remains effective.

The identification of the PfKelch13 (K13) mutations has allowed for a more refined definition of artemisinin resistance that includes information on the genotype. However, we have yet to fully understand which specific mutations within the K13 domain are most associated with artemisinin resistance. The current definition of artemisinin resistance is subject to change based on new evidence.

The presence of artemisinin resistance is generally first evaluated during therapeutic efficacy studies (TESs) in which patients receive treatment with an ACT. It can also be evaluated in special clinical studies designed to evaluate artemisinin resistance; for such studies, patients receive artesunate monotherapy alone or before receiving a partner drug. The following definitions apply to both study types:

Suspected endemic artemisinin resistance is defined as:
≥ 10% of patients with a half-life of the parasite clearance slope ≥ 5 hours after treatment with ACT or artesunate monotherapy; or
≥ 5% of patients carrying K13 resistance-confirmed mutations; or
≥ 10% of patients with persistent parasitaemia by microscopy at 72 hours (± 2 hours; i.e., day 3).

Confirmed endemic artemisinin resistance is defined as:

≥ 5% of patients carrying K13 resistance-confirmed mutations, all of whom have been found to have either persistent parasitaemia by microscopy on day 3 or a half-life of the parasite clearance slope ≥ 5 hours after treatment.

Evaluations of artemisinin resistance has to take into consideration several confounding factors, such as the effect of partner drugs, host immunity, insufficient levels of drug in the blood, and non-validated K13 mutations.

Artemisinin resistance alone rarely leads to treatment failure. However, resistance of malaria parasites to ACT partner drugs can lead to treatment failure (regardless the presence of artemisinin partial resistance). As a consequence, WHO in the 2018 Malaria report showed the maps of several ACTs that are failing (Artesunate-Amodiaquine) in Greater Mekong area (Fig. 10)

The Greater Mekong Subregion (GMS) has long been the epicenter of antimalarial drug resistance, and currently *P. falciparum* resistance to artemisinin is present in five countries of the subregion: Cambodia, Lao People's Democratic Republic, Myanmar, Thailand and Viet Nam.

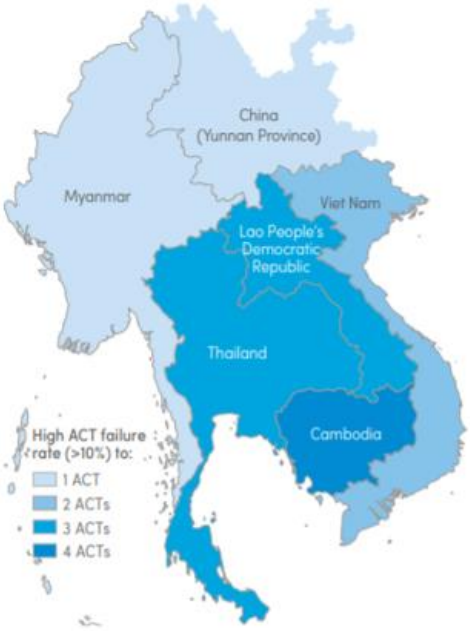

***Fig. 10**. Number of ACTs with high failure rates in the treatment of P. falciparum infections Source: Data were derived from the WHO global database on antimalarial drug efficacy and resistance*

Taking in consideration the increasing resistance, monotherapy with artemisinin and ART derivatives is strongly not recommended and is substituted with ACT as standard therapy.

1.4.4 Syk Inhibitors as a treatment for malaria

The development and research of new antimalarial drugs is necessary to avoid increasing parasite resistance. A promising approach in the antimalarial therapy consists in utilization of Syk kinase inhibitors with the purpose to block the expulsion of denatured haemoglobin and its accumulation inside the parasitized erythrocytes [38] and not allow parasite growth, proliferation and egress from host cell.

Notably, resistance is emerging in areas of low immunity given that *P. falciparum* transmission is declining in many areas including the Greater Mekong Subregion because of the scale-up of artemisinin resistance containment programs and malaria control programs to achieve national malaria elimination targets [36].

As decreasing *P. falciparum* transmission will be accompanied by decreasing immunity, it will be important to understand the contribution of the immune system in the efficacy of the novel antimalarial drug.

Spleen Tyrosine Kinase

Spleen tyrosine kinase (Syk) is a cytosolic non-receptor protein tyrosine kinase (PTK) which was discovered in 1990 and belongs to the Src family [37]. The Syk gene was found to be localized on chromosome 9q22 and is abundantly expressed in hematopoietic cells, such as mast cells, basophils, B-cells, T-cells, neutrophils, dendritic cells, macrophage, monocyte, erythrocytes, and platelets [38]. Syk is highly homologous to ZAP-70, which is present in the cytoplasm and is closely related to SYK in both homology and function. However, ZAP-70 expression is limited to T lymphocytes and natural killer (NK) cells.

SYK and ZAP-70, possessing tandem N-terminal Src homology-2 domains (SH2) referred to as N-SH2 and C-SH2 domains. These are separated by interdomain A, and

the C-SH2 domain is followed by interdomain B which precedes the C-terminal kinase domain (fig 11).

Fig. 11 *Structural sequence of SYK kinase*

A tandem SH2 module (N-SH2 and C-SH2), has an important role in protein-protein interaction and serves as a docking platform for immune receptor tyrosine-based activating motifs (ITAMs) which are displayed on the cytosolic side of the plasma membrane. Importantly, the tandem SH2 domains also help to maintain SYK family kinases in an inactivated state via intramolecular interactions in resting immune cells. The interdomain linker SH2, between N-SH2 and C-SH2 constituted of 50 amino acids represents the most conserved region in family kinase. The interdomain linker of 80-100 amino acids located between C-SH2 and catalytic domain is important to regulate the kinase activity and contains phosphotyrosine residues.

The catalytic domain or SH1 constituted of 300 amino acids follows the interdomain linker. It contains the binding sites for ATP and two autophosphorylation sites (Tyr525 and Tyr526). Finally, a C – terminal tail with unidentified function yet.

The comparison of inactive and phosphorylated Syk structures reveals significant movement of the tandem SH2 domains region that could disrupt the interaction with the kinase domain characteristic of the inactive state. These data with that reported by different authors permitted the proposal of a model for the regulation of Syk kinase.

Phosphorylation of erythrocyte membrane proteins has been previously documented following infection and intracellular growth of the malarial parasite, Plasmodium falciparum in red blood cells. Much of this data dealt with phosphorylation of serine residues. Recently, instead, has been characterized of phosphorylation of serine and tyrosine residues of red cell membrane proteins following infection by *P. falciparum* [39]. Tyrosine phosphorylation of band 3 represented the earliest modification observed

during parasite development. Band 3 tyrosine phosphorylation observed at the ring stage appears to be under the control of Syk kinase. Identification of tyrosine phosphorylation of band 3, band 4.2, catalase and actin suggests new potential regulatory mechanisms that could modify the functions of the host cell membrane.

Syk Inhibitor (R406) as a novel drug against Malaria.

Band 3 serves not only to catalyze exchange of anion across the membrane but several function: to nucleate a complex of glycolytic enzymes on the membrane; provide a binding site for multiple kinases and phosphatases and anchor the spectrin-actin cytoskeleton to the bilayer [40]. All these functions contribute critically to the biology of red blood cells. In particular, its role in connecting the cytoskeleton to the membrane is extremely critical, in fact, the rupture of the band 3-ankyrin bridge or the band 3-adducin bridge to the membrane skeleton based on spectrin leads to destabilization and fragmentation of the membrane [41].

Band 3 has been shown to been important for the malaria parasites *Plasmodium falciparum* and *Plasmodium vivax.* As depicted in the figure 12, the parasite co-opts erythrocyte tyrosine kinase and activates the process of band 3 phosphorylation for its own benefit; the resultant destabilization of the membrane allows for egress of its merozoites and has been implicated in the ability of *P.falciparum* to form knobs and adhere to the microvasculature [42]. Recently, Pantaleo et al, showed a progressive increase in band 3 phosphorylation from ring to trophozoite to schizont stage. This is accompanied by an increase in band 3 containing microparticle formation their shedding and hemolysis. Band 3 phosphorylation is very plausibly due to oxidative stress exerted by the parasite growth because it is capable of activating the docking of Syk to band 3 and to inhibit Tyr phosphatases. Because this process eventually culminates in the rupture of the host cell membrane and release of the newly developed merozoites into circulation, Pantaleo and colleagues wondered whether selective inhibition of the tyrosine phosphorylation of band 3 might inhibit the phosphorylation-induced membrane destabilization sufficiently to prevent parasite egress and thereby

terminate parasitemia. Indeed, Syk tyrosine kinase inhibitors were shown not only to prevent tyrosine phosphorylation of band 3 in *P. falciparum* pRBCs but also to decrease the subsequent release of membrane-derived MPs, to mitigate the loss of band 3 and Syk from infected cells, and to block the egress of mature merozoites from pRBCs.

Syk inhibitors like R406 are under development at various stages and trials are currently underway investigating Syk inhibition in a wide range of disorders from chronic immune disorders such as immune thrombocytopenic purpura to malignancies such as retinoblastoma. Given their general safety in these initial trials, a case can be made for a clinical translational trial of a Syk TKI in malaria.

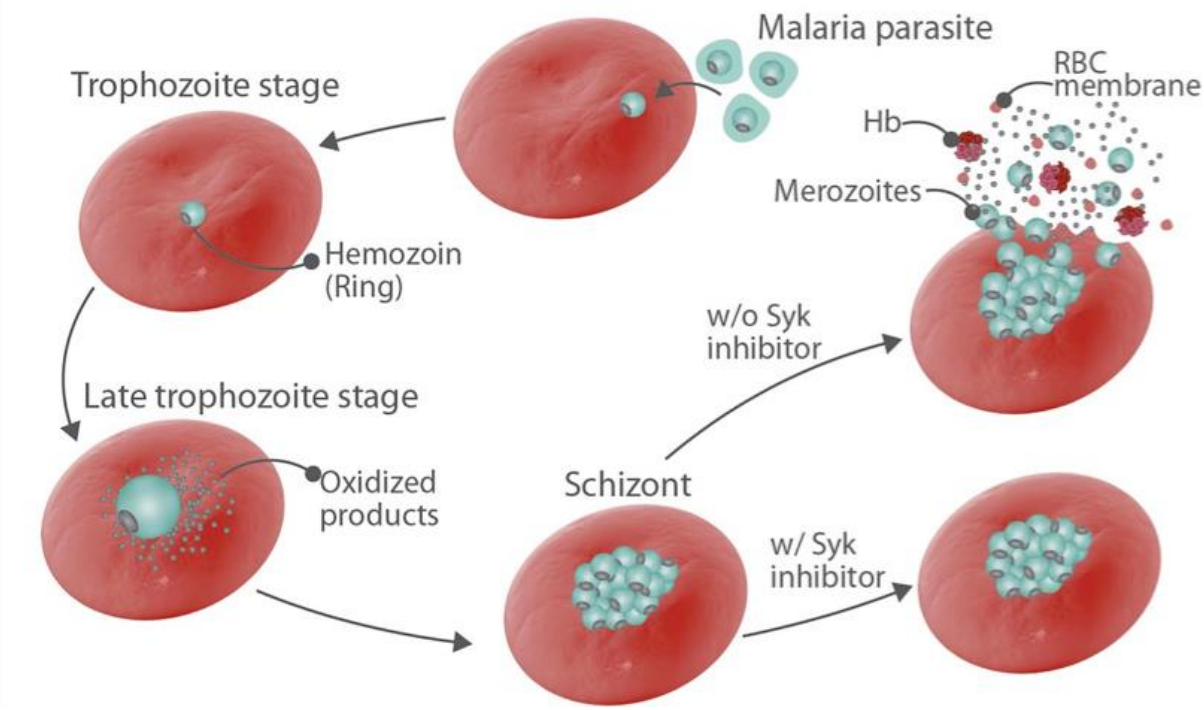

***Fig. 12**. The biological activity of Syk inhibitors in infected erythrocytes.*

Syk inhibitor and their molecular synthesis

Imatinib: (4-[(4-Methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridin-yl)-2-pyrimidinyl]amino]-phenyl]benzamide (Fig.13 A). It is known as Gleevec in United States and Glivec in Europe, commercially available from Novartis. It is a drug non-specific for Syk. Imatinib effectively suppressed parasitemia in culture, with essentially complete inhibition achieved at ~5 μM [41]. This Drug is already used for treating different cancer types, such as Chronic Myeloid Leukaemia (CML), Acute Lymphoblastic Leukaemia (ALL) and gastrointestinal stromal tumor (GIST).

R406(tamatinib):6-(5-fluoro-2-(3,4,5-trimethoxyphenylamino)pyrimidin-4-ylamino)-2,2-dimethyl-2H-pyrido[3,2-b][1,4]oxazin-3(4H)-on (Fig.13 B). It is an active metabolite of prodrug R788 (fostamatinib) and a specific, ATP-competitive inhibitor of spleen tyrosine kinase (Syk), which plays a key role in signaling of Fc- and the B-cell receptor activation. R406 has an IC50 of 41 nM [43] and has already been used in clinical trials for rheumatoid arthritis [44] autoimmune thrombocytopenia [45], autoimmune haemolytic anaemia, IgA nephropathy [46] and lymphoma [47].

P505-15:4-((3-(2H-1,2,3-triazol-2-yl)phenyl)amino)-2-(((1R,2S)-2-amino-cyclohexyl)amino)pyrimidine-5-carboxamide-hydrochloride (Fig.13 C). It is a novel, highly selective Syk inhibitor with an IC50 of 1 nM in cell-free assays. This drug candidate has already been used in *in vivo* studies in mice for rheumatoid arthritis , non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukaemia (CLL) treatment [48,49].

Syk-inhibitor-II: 2-(2-Aminoethylamino)-4-(3-trifluoromethylanilino)-pyrimi- dine-5-carboxamide. It is a cell-permeable compound that acts as a potent, selective, reversible, and ATP-competitive inhibitor of Syk with IC50 41 nM (Fig.13 D)

Syk-inhibitor-IV:2-(7-(3,4-Dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5- ylamino)-nicotinamide (Fig.13 E). It is a cell permeable imidazopyrimidine compound that acts as potent ATP-competitive, reversible, and highly selective inhibitor of Syk tyrosine kinase activity (IC50 = 10 nM)

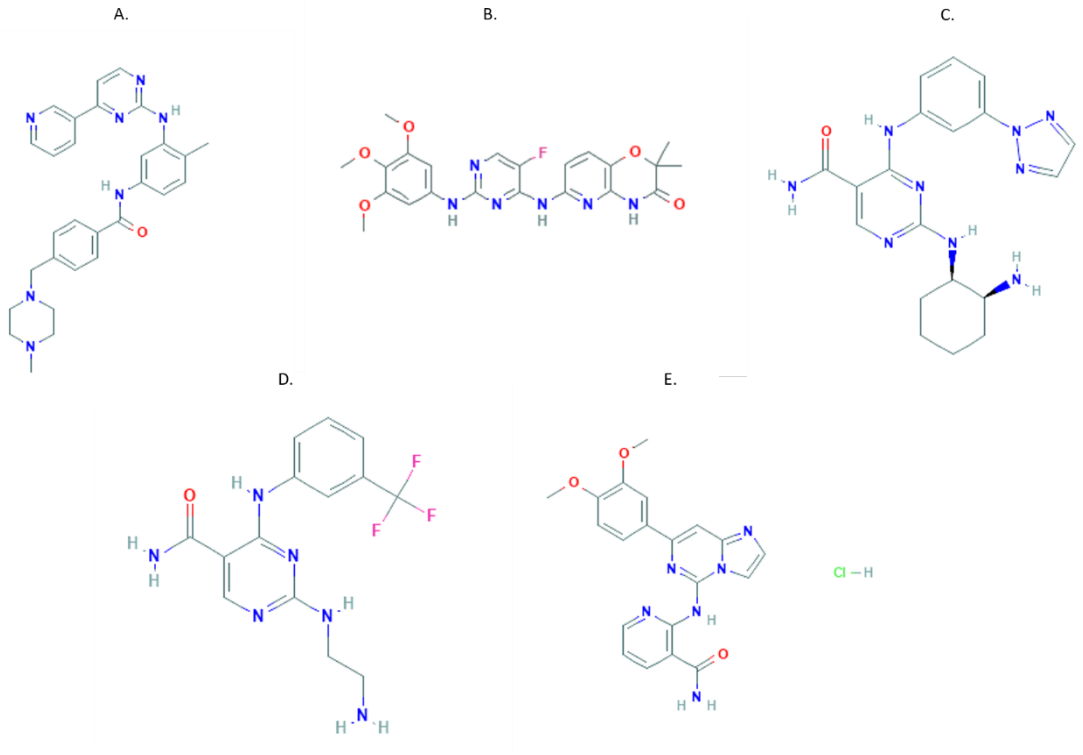

Fig. 13 *Molecular structure of different Syk inhibitors such as Imatinib (A), R406 (B), P505-15 (C), Syk II (D) and Syk IV (E)*

1.5 Malaria parasite clearance

1.5.1 Spleen activity

A key role in the control and clearance of intraerythrocytic infections is played by the spleen [50]. Its physiological function is to remove senescent red cells, malaria parasites, and circulating extracellular cells such as bacteria or cellular debris from the blood. As parasites grow and alter the surface of the host red blood cell, the spleen and its surveillance cells identify infected cells and prevent them from further circulation. The spleen consists of two overlapping blood circulations—a rapid flow by-pass, called the fast closed circulation, which typically takes 90% of the splenic blood flow (100–300 mL/min in a healthy adult), and a slow-open circulation in which the blood is filtered through narrow inter-endothelial slits. This slow filtration allows the blood elements to be assessed for antibody coating and deformability. Abnormal cells which fail inspection and other particulate material are retained. In malaria, the spleen enlarges rapidly, and is often palpable (i.e. ≥3 times enlarged), and clearance function increases. Examined spleen of fatal human malaria showed marked accumulation of parasitized erythrocytes of all stages [50]. The "activated" spleen retains parasitized red cells (including ring stage infected cells) and it removes parasites and parasitized cells. Splenectomy and splenic dysfunction increase the risk of severe malaria [50]. Recent study in fact, has demonstrated that the splenic filtration of parasitized red blood cells is drastically reduced when the ability of splenic macrophages to phagocytose is blocked, with the consequence of rapidly achieving high total parasitemias in the body, comparable to that observed after splenectomy. Thus, macrophage dysfunction may explain the high burdens of asexual parasites in HIV-positive patients [51].

The spleen also removes intraerythrocytic particles such as nuclear remnants (Howell-Jolly bodies), denatured hemoglobin (Heinz bodies) or iron granules (in siderocytes) from intact erythrocytes without destroying the cells, a process so called, pitting. The "pitting" capability of the spleen is substantial. From 1960, Crosby and his research group showed that siderocytes could be pitted of their iron granules with a half-life of

80 min in healthy subjects suggesting that pitting rates were close to removal rates for abnormal erythrocytes. Through the same mechanism the spleen also removes damaged circulating intraerythrocytic malaria parasites without destroying the red cells [52]. This is the main mechanism of ring stage parasite clearance following treatment with artemisinin derivatives in non-immune patients [53]. As to the clinical aspect, it was noted that patients treated with artemisinins for *P. falciparum* malaria clear their parasitemias faster that those treated with quinine or other antimalarial drugs and that pitting contributes substantially to artemisinin-induced parasite clearance [53].

Furthermore, a recent study has been suggested that human immunity is the primary determinant of clearance rates, unless or until artemisinin killing has fallen to near-ineffective levels [54].

1.5.2 Antibody

Human RBC show a very well-defined lifespan of 120 days. At the end of their life, RBC are removed from the circulation by splenic macrophages. In absence of the classical apoptotic pathway, RBC must possess a specific mechanism which triggers their recognition by macrophages at a defined stage of their life. A large number of modification were described in senescent RBC. They are smaller, denser, their surface is partially desialylated, haemoglobin is denatured and bind to the inner face of their membrane, and, finally, senescent RBC binds naturally occurring antibodies (NAbs).

Band 3, the most represented RBC integral membrane protein, appears to be the major target of NAbs. Natural antibodies directed against modified band 3 ("senescent antigen") bind to old erythrocytes resulting in their clearance from the circulation [55]. NAbs appears to bind only to modified band 3 (neoantigen), in particular, evidences indicate that band 3 clusters induced by hemichromes are always associated to intense NAbs binding [56]. Hemichrome binding to band 3 also causes the oxidative cross-linking of their cytoplasmic domains. The band 3/hemichrome complex is found in many pathological conditions characterized by intense RBC splenic trapping

accompanied by anti-band 3 NAbs binding to RBC membrane, for example during intra-erythrocytic malaria parasites growth.

Several authors have proposed mechanisms for recognition and phagocytic removal of senescent or damaged RBCs based on oxidative and non-oxidative clustering of band 3 as the starting event with subsequent opsonization and phagocytic removal by circulating or resident phagocytes [55]. Marguerite Kay, was the first to show that dense human RBC bound increased amounts of autologous IgGs. After her data, band 3 centered removal model has received great attention. According to Low's model, aggregation of band 3 induces clustering of potential antibody-binding sites and promotes deposition of autologous IgGs. This mechanism does not imply covalent modifications of band 3 but simply assumes that antibodies with affinities too weak to bind to band 3 monovalently would react avidly with band 3 aggregates due to enhanced affinity of the bivalent interaction. Lutz et al, proposed another model, sharing element with Low's model. Lutz demonstrate increased binding of anti-band 3 antibodies to band 3 oligomers in senescent RBC, a correlation between cross-linkability of band 3 and anti-band 3 binding, and enhanced binding of anti-band 3 to aggregated band 3 on immunoblots. The fundamental difference respect to the Low model is the crucial role firstly described by Lutz of both anti-band 3 antibodies and complement deposition as mediators of opsonization and phagocytosis [55].

Membrane-bound anti–band 3 antibodies partially activate complement resulting in red-cell membrane deposition of C3 fragments. The antibody-C3 complex is then readily recognized by phagocyte CR1 complement receptors [55]. This process may be accelerated in malaria infected red cells. The affinity for C3 may render these antibodies a preferential site of binding of the short-lived C3b and may result in a preferential C3b-anti-band 3 complex formation. The role of immune haemolysis in the pathogenesis of malaria anaemia has been controversial. However, it is clear that the threshold for splenic recognition of erythrocyte bound antibody is lowered markedly in malaria, although there is substantial inter-individual variability. Thus, red cells with low antibody coating, which would normally escape clearance, are removed in malaria. As

with mechanical clearance, immune clearance usually increases after anti-malarial treatment has started.

The effectiveness of innate immunity in malaria response has been documented. Response to band 3 neoantigens in subjects living in an area of intense malaria transmission is associated with lower parasitemia and improved haematological symptoms. Infected RBC shows a stage dependent hemichrome binding to the membrane and a parallel opsonization by anti-band 3 antibodies and C3b. Hemichromes formation, band 3 clusterisation and its oxidation were proved to be essential factors to allow the opsonization by Nabs and complement. These events allow the involvement of autologous IgG with anti-band 3 specificity and complement, which are identified as the opsonins responsible for phagocytic recognition at ring-stage, the early parasite forms. At this stage, phagocytosis is modest and almost totally mediated by complement deposition and recognition by the phagocyte complement receptor type 1. Arese's group research demonstrated also that at trophozoite stage and schizont stage, the phagocytosis was strongly increased and the role of complement in phagocytic recognition was reduced.

Different research groups work on hypothetical mechanism to support the hypothesis that a common mechanism involving anti-band 3 Nabs appears to determine RBC removal in different physiological and pathological situations (Fig. 14). Oxidative denaturation of hemoglobin lead to hemichromes formation **1**. Hemichromes, lead to band 3 clustering and to exposure of band 3 neo-antigens. Band 3 and several membrane proteins undergo phosphorylation in response to different stimuli. Band 3 cytoplasmatic domain, appears to be the substrate of RBC tyrosine kinases activated by oxidative stress. The linkage between hemichromes and band 3 cytoplasmic domain cause their oxidative cross-linking through disulfide bonds and their Tyr phosphorylation **2**. Band 3 dissociation from cytoskeletal proteins and its clusterisation **3**. Formation of large band 3/hemichromes clusters and opsonization by Nabs and C3b **4**. These signal leads to recognition by immune cells.

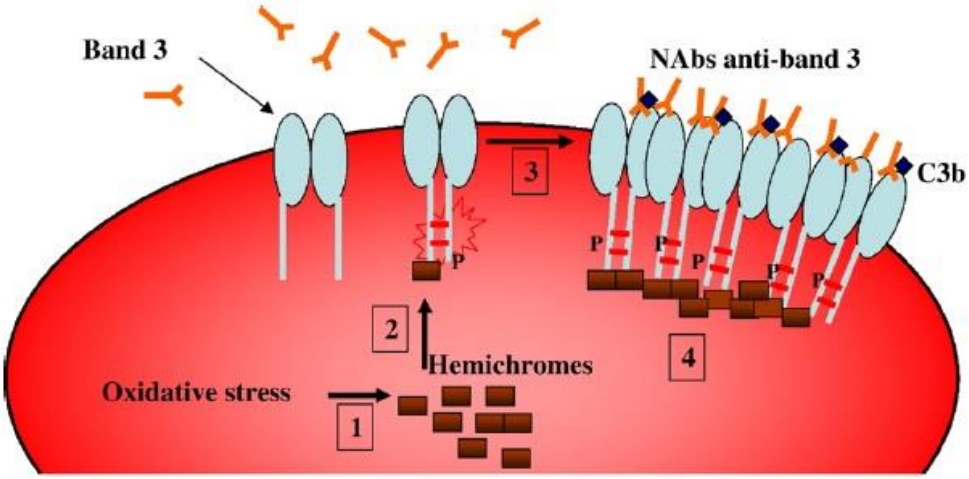

Fig. 14. *Proposed pathway involvement of anti-Band 3 Nabs. The band 3 – IgG/Complement RBC removal model. Sequence of events after oxidant damage of the RBC leading to opsonization with anti-band 3 IgG and complement, recognition and removal by the phagocyte. 1) After oxidant insult, denaturation of hemoglobin leads to hemicrome formation. 2) Hemicromes binding to band 3 cytoplasmic domain cause their tyrosine phosphorylation. 3) Band 3 dissociation from cytoskeletal proteins and its clusterisation. 4) Formation of large band 3/hemicromes clusters and opsonization by Nabs and C3b. This signals leads to recognition by immune cells.*

1.5.3 Acquired immunity and parasite clearance

Naturally acquired immunity to malaria develops after repeated exposure to parasites, is acquired faster in high- compared with low-transmission areas [36] and is lost after relative short time without exposure to the parasite. *P. falciparum* antibodies are an important component of immunity and can target the sporozoite stage, reducing transmission and infection, and blood-stage parasites (merozoites, infected erythrocytes), reducing parasite multiplication and increasing parasite clearance rates, thereby suppressing parasite densities and clinical symptoms. Despite enormous research investment and effort immunity to malaria is still poorly understood. Immunity may confound the interpretation of parasite clearance measures in drug-efficacy studies. The main implications of an effect of host immunity on parasite clearance measures are that in populations with high levels of immunity and faster parasite clearance, early signs of multidrug resistance could be undetected, and conversely, in populations with

lower immunity and slower parasite clearance, a false impression of reduced drug efficacy could arise.

In general terms, the acute malaria infection is contained by non-specific host-defence mechanisms including splenic activation and fever (which inhibits schizogony). Later more specific humoral and cellular immunity control and finally eliminate the infection.

In malaria-endemic areas, where people are infected frequently, most infections are controlled at densities causing little or no symptoms because after repeated malaria infections a person may develop a partially protective immunity (Fig.15) [57]. Such "semi-immune" persons have acquired clinical immunity and can still be infected by malaria parasites, which they tolerate without showing typical clinical symptoms of malaria . These persons can be considered as asymptomatic malaria parasite carriers The illness instead, results from infections to which the individual has insufficient immunity to control parasite multiplication. In areas of higher transmission illness most likely occurs in young children who have had few or no previous infections. In older children and adults rapid mobilization of both non-specific and specific host-defense mechanisms usually results in prompt control of the infection—even without anti-malarial treatment. As a result "immunity" complements the effects of anti-malarial drugs, accelerating parasite clearance and augmenting cure rates or, drugs could enhance immune recognition, thus favoring parasite clearance. Acquired immunity explains why cure rates are always higher in adults and older children in endemic areas and why anti-malarial treatment efficacy assessments in high transmission settings should always include young children. Infact, in areas with high *P. falciparum* transmission (most of Africa south of the Sahara), newborns will be protected during the first few months of life presumably by maternal antibodies transferred to them through the placenta. As these antibodies decrease with time, these young children become vulnerable to disease and death by malaria. In high transmission areas, young children are at major risk and are targeted preferentially by malaria control interventions. It's possible that variations in host immunity between- and within-population influence parasite clearance after artemisinin treatment, confounding the current WHO working definitions of artemisinin resistance, and consequently the

interpretation of the geographical spread of artemisinin resistance. Several researchers question the role of the immune system that remains complex to understand. Uhlemann and Fidock [58], for example, sustained that the shift in parasite clearance rates with time could be caused by drop of immunity as interventions reduced exposure of patients to parasites and so the increasing failure rates of other drugs can be due to decreased immunity rather. Greenhouse and colleagues in their report [59] sustained that the increasing drug failure were due to decreasing levels of immunity rather than changes in parasites drug resistance levels. Thus, a consequence of an incomplete eradication of malaria by malaria control measures in a population results in a decrease of acquired immunity and increase of vulnerability for clinical malaria in the population. In this scenario a malaria drug that strengthens the innate immune responses is desirable. Surely, the optimal *P.falciparum* malaria drug would satisfy three demands, firstly to kill asexual parasite stages in the blood, secondly not to block but sustain the immune cell response to mount an efficient but not host-damaging immunity and thirdly, to attack gametocytes to inhibit transmission.

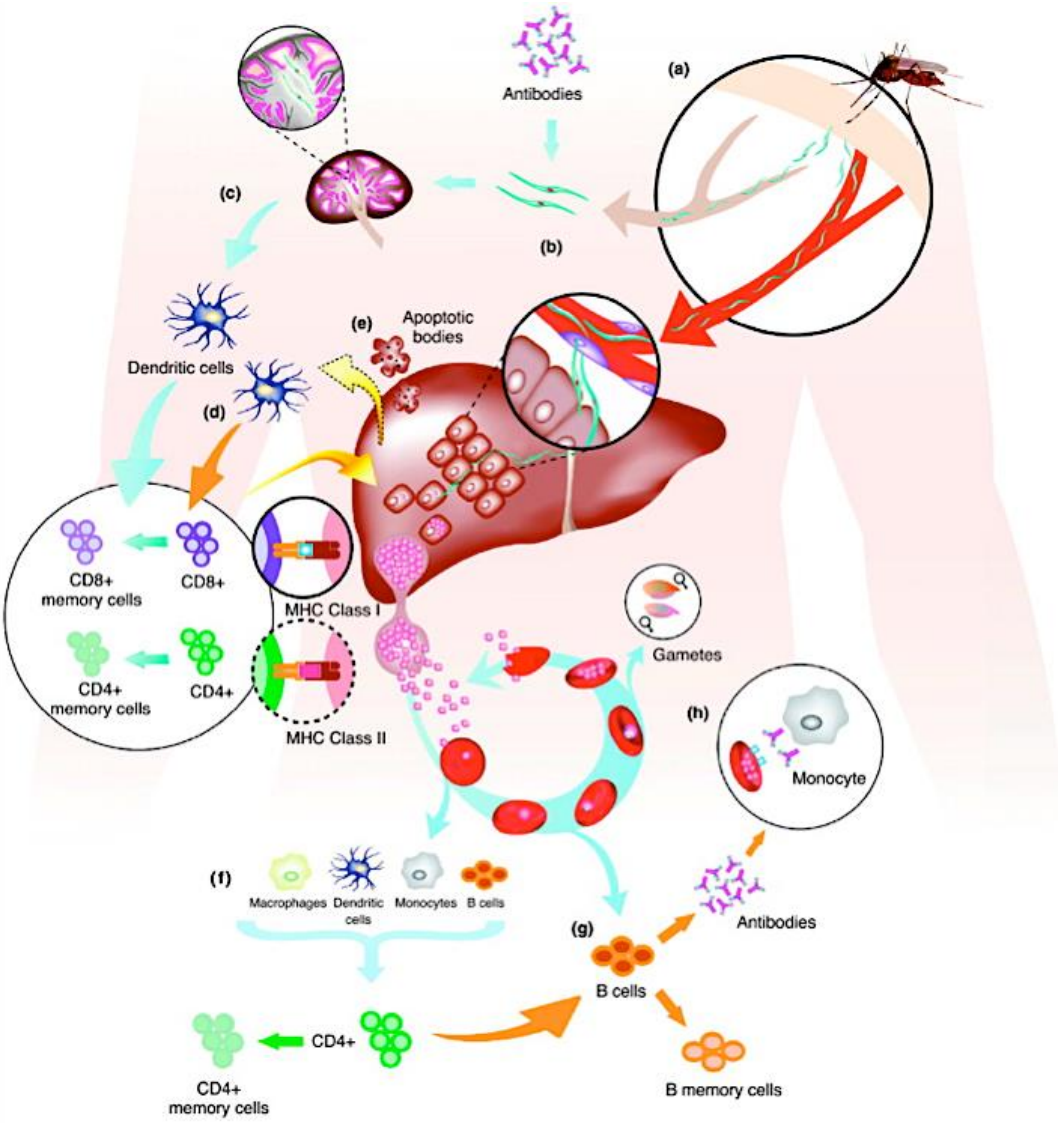

Fig. 15 *Immune response with P. falciparum infection*

1.6 Innate immune functions of the monocyte-macrophage

1.6.1 Phagocytosis and oxidative burst

Phagocytosis of parasitized RBC is triggered by IgG and Complement factors on the surface of RBC. Most studied IgGs are either autoantibodies against band 3 clusters, above defined as neo-antigens (innate immunity) or against parasite-specific antigens (acquired immunity). Specialized phagocytic cells express high levels of specific receptors for immunoglobulins (Ig), the Fc receptors CD 16, CD 32, CD 64, which recognize and bind the Fc portion of IgGs with different affinities, as single IgG or as immune cluster. Binding the Fc domain to its receptor activates phagocytic engulfment of IgG-labelled particles, e.g. an IgG-flagged pRBC, its intracellular transport and fusion with the lysosome and consequent degradation. Suitable antigen peptides are load up on MHC class II protein groove and transported for exocytosis and insertion into the membrane of the antigen-presenting cell. These last steps are crucial for linking innate immune response to acquired immunity and mounting the latter. Syk-dependent phosphorylation is crucially involved in the process of Fc-receptor signaling. In human, the molecular features of Fc receptors for IgG (FcγRs) have been extensively studied. FcγRIIA and FcγRIIC are known to possess intramolecular ITAM in the cytoplasmic region. Cross-linking of these receptors induces tyrosine phosphorylation of ITAM through Src-type kinases such as Hck, Lyn and Fgr, leading to the recruitment of Syk for activation. Activation of Syk is critical for production of cytokines and chemokines in response to cross-linking of FcγRs. In addition to FcγRs, Syk is critical for immune responses mediated by various antigen receptors such as the B-cell receptor (BCR) and high-affinity IgE receptor (FcεRI).

Recently, the role of an adaptive protein, c-Abl Src omology (SH) 3 domain binding protein-2 (3BP2), has been studied. The 3BP2 protein was originally identified as an Abl-binding protein of unknown function but it was suggested that 3BP2 plays an important role in FcRγ-mediated phagocytosis Syk-dependent tyrosine phosphorylation of 3BP2 (P) is critical for optimal FcRγ-mediated phagocytosis. In fact, phosphorylation

of Tyr174, 183 and 448 by Syk kinase in 3BP2 is a requirement for 3BP2 to induce FcRγ-mediated phagocytosis [60].

Similar to IgG, complement factor 3b (C3b) is deposed on the pRBC membrane and elicits phagocytosis by monocytes and monocyte derived macrophages when recognized by complement receptor 1 (CR1).

Both receptors Fc-gamma as well as CR1 are able to elicit oxidative burst, a mean of the monocyte and granulocyte to destroy the parasite in the circulation followed by the appearance of crisis forms that have to be removed in the spleen by pitting or phagocytosis. The INF-gamma seems to play a key role due to FcR and CR expression. Recent studies have focused on the role of IFN-γ in regulating the phagocytic capacity of macrophages, as its role remains to be considered controversial. It has been reported that IFN-γ improves the phagocytic activity of macrophages, also leading to the up-regulation of FcRI (or CD64, the high affinity Fc receptor for IgG) and CR expression with an increase in phagocytosis of opsonized pathogens. In fact, *in vivo* treatment of normal human subjects with IFN-γ led to increased expression of FcγRI by their phagocytes and a related improvement in FcγR-mediated phagocytosis by neutrophils. Futhermore, IFN-γ treatment is associated with increased expression on monocytes of the opsonic receptors FcγRII, FcγRIII and C3. However, it was also observed that IFN-γ significantly inhibits non-opsonized phagocytosis of macrophages. "Activation" of macrophages induced by IFN-γ for opsonized particles and inhibition for non-opsonized particles, seems to be sought in the down-regulation in the expression of MARCO, the main surface-binding macrophage receptor for non-opsonized particles. Thus, INF-γ plays distinct roles in opsonized and nonopsonized phagocytosis [61].

The secretion of IFN-γ by human and natural killer (NK) T cells induced by synergistic costimulation with interleukin (IL) -12 and IL-18 is well established [62]. The contribution of macrophages to the release of IFN-γ could be a significant factor in understanding mechanisms that induce phagocytosis but a similar activity in macrophages is still controversial. It has been shown that human macrophages derived from monocytes in vitro through stimulation with a combination of IL-12 and IL-18 or with macrophage colony-stimulating factor (M-CSF) were able to produce IFN-γ when

further stimulated with a combination of IL-12 and IL-18. The release of IFN-γ results in a high level of proinflammatory cytokines and a low level of anti-inflammatory cytokines to promote the immune response, promoting the phagocytic capacity of macrophages to eliminate the pathogen.

Based on these considerations understanding the contribution of the immune system, especially innate immunity in the treatment of malaria is of fundamental importance in an age where great efforts are concentrated to identify new antimalarial drugs, which could, with the aid of immune system, promote parasite clearance.

1.7 Antimalarials and immunity

Innate and adaptive immune functions assaulting malaria parasites include a network of cytokines, ROS production and involve immune cells, comprising neutrophils, activated macrophages, natural killer (NK) cells, dendritic cells, and T-cells. Both cell-mediated and humoral components of immune system serve their actions and activities to prevent malaria infection by promoting phagocytosis, elimination of infected red blood cells and subduing merozoite intrusion of erythrocytes through antibody-mediated neutralization.

To understand how drugs affect the immune system is very important in order to treat a disease successfully leading to improvement of clinical symptoms and reducing unwanted side effects. Drugs of all types and classes affect the immune system, but the mechanisms responsible for these effects are often misinterpreted [63]. Some drugs target immune cells specifically for the treatment of immunological diseases, such as B-cell lymphomas, while other drugs for the treatment of rheumatoid arthritis have immunosuppressive or anti-inflammatory effects [64]. Artemisinin and its derivates are currently considered the most effective drug in the treatment of cerebral malaria and chloroquine-resistant *P.falciparum* malaria. The peroxide group is essential for the medicines of the artemisinin family to exert antimalarial effects. In addition to their excellent clinical antimalarial effects, recent studies promoted the drugs of this family as potent anti-inflammatories and able to regulate innate and adaptive immunity. Macrophages, have a double-faced role in cell-mediated (act as antigen presenting and effector cells) as well as in humoral immunity (phagocytose opsonized microbes) and can produce both pro-inflammatory cytokines, such as IL-12/ and TNFα, and anti-inflammatory cytokines, including IL-10. Several research group found that Artemisinin family drugs inhibit the secretion of macrophage-derived proinflammatory cytokines, particularly TNF. Results of recent study manifested that artemisinin can abolish NF-kB mediated release of TNF and IL-1beta proinflammatory cytokines in human adherent monocytes [65]. Artemisinin family drugs could also induce the anti-inflammatory cytokine production, such as IL-10, whereas decrease IL-12 production

in primary peritoneal macrophages after IFN-γ stimulation *in vitro* or *in vivo*. These studies suggest that artemisinin family drugs are able to suppress the activation of macrophage. Looking from the perspective of early innate immunity against malaria, in which proinflammatory cytokines themselves and their capacity to mediate activation of phagocytic macrophages is of great importance, the prevailingly suppressant nature of artemisinins against cytokines release, macrophages viability, and phagocytic capacity might raise highly relevant and important questions about their apparent immunosuppressant capability. However, data from *in vitro* and *in vivo* studies on immune modulatory effects of Art are often inconsistent. Initial studies with highly-dosed Artesunate showed a significantly decreased phagocytosis of peritoneal macrophages and phagocytic index *in vivo*, reducing the weight of thymus in Artesunate treated mice. Treatment of *Plasmodium berghei* infected mice with Artesunate, enhanced the serum C3 level. Clinical trials showed that serum IgM and IgG levels were increased but serum C3 contents were reduced in malarial patients. These data led to attribute an inhibitory effect on the humoral immunity to Art and the regulating the C3, which was beneficial to form immune complexes in patients suffering from malaria or autoimmune and immune complex disease [66].

In vitro experiments with Art showed inhibited lymphocyte proliferation induced by T cells mitogens.

T lymphocytes play a fundamental role in adaptive immune responses to guide cellular and humoral immunity. Following binding to the antigen, T cells are activated and secrete IL-2. Subsequently, through the autocrine / paracrine proliferative cycle, IL-2 induces clonal expansion, promotes the survival of activated T cells; after the correct elimination of pathogens, activated T cells undergo apoptosis to maintain immune homeostasis [67]. Artemisinin family drugs can suppress T cell activation both *in vitro* and *in vivo*. Artemether was reported to suppress T cell proliferation and IL-2 production in response to TCR engagement or mitogens in vitro.

B lymphocytes are essential for mounting adaptive immunity and play a critical role in the humoral immune response by secreting antibodies. Artemisinin family drugs have been shown to preferentially affect antibody production upon antigen immunization anda significant inhibition of LPS-induced B cell proliferation has been demonstrated.

In contrast, studies conducted in children and adults with uncomplicated Malaria in endemic areas as Burkina Faso, have demonstrated that use of ACT based therapies have no influence on the antibodies response against experimental malaria antigens. The different dosages and different assays applied in *in vivo* mice and clinical studies may explain conflicting results. In mouse artemisinin derivatives were given daily for 7 days while in children three doses were prescribed. It is very likely that drug treatment and antibodies are clearing parasites in synergy since elderly children resolve the infection in shorter time.

Immune pharmacologic actions of artemisinin drug family deserve further investigation to understand also the enigmatic relationship between malarial parasite and host immunity that allows a sustained persistence of parasites in otherwise efficient immune system. Perception of several aspects of host immune responses to malarial parasites is essential, not only for novel drug development but also for understanding the interference of existing anti-malarial drugs with host's anti-malarial immunity.

2. AIM OF THE PROJECT

Since 2001, the WHO has recommended ACTs for treatment of uncomplicated malaria caused by *P. falciparum*. ACTs have been an integral part of the recent success in global malaria control. However the WHO has observed foci of suspected artemisinin resistance in South-East Asia and emergence of resistance to both artemisinin and the partner drugs endangers the future ACT efficacy. Because strains of *P falciparum* are rapidly emerging that are resistant to all known antimalarial drugs, including artemisinin, quinine, chloroquine, piperaquine, and mefloquine and their derivatives, emphasis is currently laid on comprehension of new therapies with novel mechanisms of action that includes also the patient's immune response. The primary objective of research efforts is to reduce the risk of recrudescence. A new pharmacological treatment for malaria should lead to a faster elimination of parasites from blood with consequent improvement of symptoms and clinical prognosis and to interrupt transmission.

Recently, Pantaleo et al, has demonstrated that Syk Inhibitors could represent a new class of antimalarial drugs reducing parassitaemia by delay of *P.falciparum* growth and suppression of merozoite egress. Anti-parasitic activity of Syk inhibitors was shown in *in vitro* cultures of *P. falciparum* laboratory strains and field isolates. A crucial point for the anti-parasitic activity of Syk inhibitors is the persistence of anchorage protein band 3 in the membrane of maturing blood stage parasites. On the other hand band 3 is well known as platform for autoantibody binding to RBCs, the widely accepted mechanism for recognition of senescent RBC, RBC in several hereditary anemias and PRBC by spleen phagocytes.

These facts led us to focus on the role of immune processes in the decrease of parasitemia of *P. falciparum* after treatment with Syk inhibitors.

The main goal of my research was to test new effective antimalarial combinations that possess the capacity to potentiate artemisinin effect, and to investigate the mode of action of R406 hypothesizing an involvement of host immunity.

Specific aims of my studies were firstly, to verify the improvement of innate immune mechanisms, such as phagocytosis of young ring-stage PRBC; by artemisinin alone and in combination with a Syk-inhibitor

Secondly to study the involved immune mechanisms that lead to an improved recognition by phagocytes of ring-stage PRBC if treated with Syk inhibitors and thirdly to demonstrate RBC membrane changes induced by Syk inhibitors, that cause increased binding of opsonins. Finally, first field studies will evaluate in malaria patients in Vietnam the efficiency of the innovative combination therapy with Syk-inhibitors.

In order to achieve above objectives:

- phagocytosis of ring-stage parasitized RBC (PRBC) by primary human monocytes was assessed after dose–dependent treatment of RBC with the active derivative of artemisinin DHA or Syk inhibitor R406 or the combination of both.
- levels of opsonins, IgG and complement factor C3c on ring-PRBC was assessed after treatment of RBC with DHA, R406, or both.
- Syk-inhibitor dependent accumulation and aggregation of band 3 proteins in the membrane of PRBC were tested.

Parasite clearing was assessed in malaria patients treated with established ACTs vs. Syk-inhibitor/DHA combination. The results of this study may hopefully contribute to the establishment of an urgently needed innovative artemisinin-based combination therapy for malaria.

3. MATERIAL AND METHODS

3.1 *Plasmodium falciparum (P.f.) in vitro* cultures, separation, synchronization and ring stage-specific stage-enrichment procedures.

3.1.1 Permanent *P.f.* cultures

Human red blood cells parasitized with *Plasmodium falciparum* (Palo Alto strain, Mycoplasma free) were cultivated in growth medium (GM), consisting of RPMI 1640, HEPES modification from Sigma (Saint Louis – Missouri - USA), supplemented with 20mM glucose, 2mM glutamine, 25 uM adenine, gentamicin 25 mg/ml, 1% (w/v) Albumax I. The cultures were permanently kept at 1% of hematocrit, in an air/ CO_2atmosphere of 95%/5% vol/vol) at 37 °C. GM was changed daily and parasitemia maintained between 5-10 % by adding washed RBCs from healthy donors.

Heparinized blood samples from healthy adult donors of both sexes were obtained from the local blood bank (A.S.L TO 4, Ivrea, Italy). Red blood cells (RBCs) were separated from plasma, platelets and white blood cells by three washes at 1800 rpm for 10 minutes in wash medium (RPMI 1640, HEPES modification), and stored at 4°C after resuspension in WM at 50% hematocrit and supplementation of 2% (v/v) saline-adenine-glucose medium (SAG: 150 mM NaCl, 1.25 mM adenine, 45 mM glucose).

3.1.2 Synchronization and infection of not-parasitized donor RBCs

One day ahead experiments with ring-parasitized RBCs, preferentially late trophozoite and schizont stage *P.f.* cultures were loaded on the top of a 10, 40, 80 % (v/v) discontinuous Percoll gradient containing 6 % (w/v) mannitol and centrifuged for 30 minutes at 3800 rpm and 30 C without brake. The mature parasites were collected from the 40/80 % interphase, washed once with RPMI 1640 and the re-infection of fresh washed RBCs from a healthy donor was performed by inoculating mature trophozoite/schizont stages into a RBC suspension in GM at hematocrit of </= 1 %.

Parasitemia was adjusted to 5-7.5 % and culture kept overnight at 37 °C in an air /CO_2 cell incubator.

3.1.3 Enrichment and harvesting ring-stage parasitized RBC

All experiments were conducted after opportune synchronization of cultures, carried out the same day of experiment with two aims: 1) elimination of not-Ring stage parasites and expelled hemozoin and 2) increase of ring-stage parasites by separation from not-parasitized RBC and to obtain ring stage parasites, useful for DHA and R406 incubation experiments. For synchronization of cultures by parasite stage, schizont, trophozoite and ring stage parasites were collected after passing a synchronized culture at predominantly ring stages through a discontinuous Percoll ™ (GE Healthcare) density gradient. Precisely, solutions with different concentrations of Percoll (90, 80, 40 and 10% (v/v)), each supplemented with 6 % mannitol (w/v), were stratified at 3, 2, 2 and 1ml, respectively, into a 15 ml tube from bottom to top. Approx 1 ml cells of the *P.f.* - cultures were loaded at a hematocrit between 15-30 % on top of one Percoll density gradient and centrifuged at 3800 rpm for 30 minutes (no brake). After centrifugation, different life cycle stages of parasite enrich in different fractions. Hemozoin enriches in the 10% to 40% interface, trophozoites/schizonts in the 40% to 80% interface. Rings at nearly 100% parasitemia were collected from the 90 % percoll cushion above the bottom fraction and ring-parasitized RBC at lower parasitemia from the bottom fraction for stage-specific incubation with DHA and R406 (Schwarzer et al, Blood 2003). Collected parasites were washed twice in RPMI 1640, HEPES modification (Sigma) and incubated for metabolic recovery after separation in GM for 1 hour at 37°C before incubation with DHA and R406.

3.1.4 Handling of not-parasitized control RBC (npRBC)

TRBC of the same donor that we used for reinfection were treated in parallel cultures including separation on percol.

3.2 Phagocytosis assay

3.2.1 Preparation of adherent human monocytes

For phagocytosis experiments I isolated monocytes from buffy coats obtained from 450ml blood donations which were provided by the local blood bank (A.S.L TO 4, Ivrea, Italy). One buffy coat from one blood donation of about 50 ml was transferred into two 50 ml tubes (Greiner) under sterile conditions and each one filled up to 50 ml with RPMI 1640 and resuspended.

Resuspended buffy coat cells were deprived of platelets by centrifugation at 1000 rpm (no brake) for 20' with subsequent removal of the platelet-enriched supernatant. Sedimented cells were resuspended with pre-warmed RPMI 1640 to obtain 120ml cell suspension. Thereafter, 30ml of suspension were carefully stratified on 15ml of Biocoll (Biochrom GMBH), isotonic cell separation solution with a density of 1.077g/ ml, avoiding mixing of the two solutions. After centrifugation at 1700 rpm for 20' without brake the fraction of peripheral blood mononuclear cells (PBMC), i.e. lympho- and monocytes, were found at the interphase between the transparent orange to red colored supernatant and the underlying transparent colorless Biocoll (fig.16). The supernatant consists of plasma diluted by cell culture medium. Erythrocytes and granulocytes pass Biocoll 1.077g / ml and sediment at the bottom.

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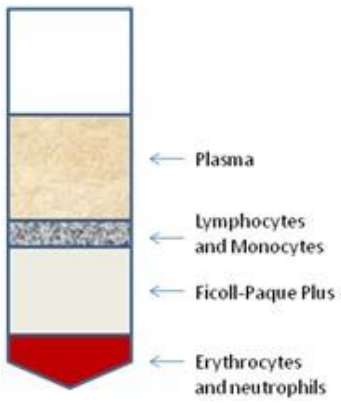


Fig. 16 *The separation of cells after centrifuge by Ficoll*

The cells were sedimented by centrifugation at 1700 rpm for 7'and the supernatant was completely removed by careful aspiration to avoid the presence of Biocoll in the cell pellet. The monocyte-enriched cell pellet was resuspended in 25 ml RPMI 1640. An aliquot of suspension was used for manual cell counting in a haemocytometer (Bürker chamber).

PBMCs were suspended at 5 million cells/ml RPMI 1640 and plated in 24 well plates (Falcon) at 10 million per well [68]. A short adherence period was chosen to minimize lymphocyte adherence and after only 35 min incubation in a humidified incubator (5% CO2, 95% air, 37°C), non-adherent cells were removed by three washes with RPMI 1640. Thereafter, adherent monocytes were re-incubated in 1 ml of M-SFM (Macrophage Serum Free Medium, GIBCO) / well for 5-10h until beginning phagocytosis experiments. The medium was changed immediately before adding red blood cells for the phagocytosis by monocytes.

3.2.2 Treatment of not-parasitized RBC (NPRBC) and parasitized RBC (PRBC) for phagocytosis

All inhibitors were solubilized in anhydrous DMSO at 10 mM stock concentration and diluted in anhydrous DMSO prior to addition to malaria cultures avoiding excessive light exposure as indicated by the manufacturer

Washed npRBC and PRBC resuspended in GM at hematocrit of 1%, and incubated for one hour for recovery after synchronization by Percoll centrifugation, were treated with different concentrations of inhibitors, i) Syk inhibitor R406 (Calbiochem), ii) dihydroartemisinin (DHA) (Selleckchem) or iii) both at variable concentrations and incubated at 37°C in an air/CO_2 cell-incubator under routine cell culture conditions for indicated time. The handling of the substances and exposed cells was performed in the dark as explained above After incubation, RBCs were washed three time in 10 vol of Phosphate buffered saline – supplemented with 5mM Glucose (PBS-G). For all protocols described. Untreated controls were processed identically and run in parallel with the same final concentration of DMSO as the drug-treated cultures.

3.2.3 Opsonization of NPRBC and PRBC

Immediately before phagocytosis, washed Rh-positive NPRBC resuspended in PBS-G (50% hematocrit) were incubated with 25ug anti-D IgG/ml packed RBC for 30’ at 37°C as a positive phagocytosis control. NPRBC and PRBC treated with DHA, R406 or both were opsonized either with autologous fresh serum whenever possible or with human AB normal serum. For this, one volume of packed washed NPRBC or PRBC was supplemented with an equal volume of PBS and two volumes of serum, mixed and incubated for 30’ at 37°C. Thereafter, RBC were washed three times in 10 vol PBS-G and resuspended in PBS-G to obtain 10% hematocrit.

3.2.4 Phagocytosis of RBC

About 10 million serum-opsonized or anti-D IgG-opsonized RBCs (10 ul of a 10% hematocrit suspension) were added (gentle mixing) to each well of a 24 well plate containing 10^6 monocytes at most. The plates were moved horizontally in all directions, making sure that the RBCs were well distributed in the well and equally cover the adherent cell layer. For a correct phagocytosis a ratio RBC to monocyte of approximately 100 should be respected [71]. After 3h of incubation at 37°C in 5% CO_2/ 95% air atmosphere, non-ingested RBC were removed from the wells by washing twice with PBS, followed by lysing not ingested but adherent RBCs by the addition of ice-cold distilled water for 20 seconds and an additional washing step with PBS to remove extracellular hemoglobin. This treatment didn't compromise the integrity of monocytes. Thereafter, adherent monocytes were solubilized by adding to each well 0,5 ml solubilizing solution (0,1 N NaOH, 0,025% Triton X-100, 3mM EDTA) for later heme quantification. Plates were stored overnight at 4°C. Phagocytosis was quantified by measuring ingested hemoglobin by a luminescence method using a Sirius Single Tube Luminometer (Titertek-Berthold) [72]. Heme-associated peroxidase activity catalyzed the electron transfer from tert-butylhydroperoxide to luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) at alkaline pH, which results in photon emission by reduced luminol. The amount of emitted light is proportional to the heme concentration in the tested sample [71]. In our assay, chemiluminescence was elicited by injecting 280 uL of a tert-butylhydroperoxid/EDTA solution (containing 3,7mM tert-butylhydroperoxide and 3mM EDTA dissolved in 0,1 N NaOH) as electron donor and in parallel 280 ul of 0.1N NaOH/3mM EDTA supplemented with 19 mM luminol as electron acceptor into the test tube that contains an aliquot of the solubilized phagocytes. The injection of tert-butylhydroperoxide triggered photon emission by luminol proportional to the amount of heme present in the sample and allows counting during 2 seconds. Photon counts per well were transformed into a number of RBC ingested by using a calibration curve constructed for each experiment with known amounts of cells of the same sample utilized for the phagocytosis experiment. Parallel to PRBC phagocytosis, opsonized NPRBC were added to monocytes in parallel and treated in the same way [73].

The luminometer has two high precision reagent injectors set at 280 ul for each injector. The system simultaneously injects the two reactants that trigger the reaction (luminol and tert-butylhydroperoxide) providing the RLU values that will be used for heme quantification and statistical analysis.

3.3 Measurement of Oxidative burst

The oxidative burst of monocytes is the enzymatic production of the superoxide radicals by NADPH oxidase which is assembled in membrane toward appropriate stimuli of receptors like Fc – complement or Toll-like receptors, but also after non-specific protein kinase C activation e.g. by phorbol ester.

The oxidative burst of monocytes was quantified by measuring ROS-elicited luminol-based chemiluminescence by using Sirius Single Tube Luminometer (Titertek-Berthold). The experiments were conducted using monocytes isolated from buffy coat, as described above. Human monocytes at 1,5 x 10^5 /ml M-SFM were incubated in suspension with different concentrations of R406 at 37°Cin a 95% air/ 5% $C0_2$-incubator). At indicated times 10^5 to 10^6 untreated control monocytes or treated monocytes were transferred into a luminometer cuvette containing 500 ul of PBS-G supplemented with 2mM Ca2+, 2mM Mg2+ and 10uM luminol [60]. Luminescence emitted by cells was monitored as RLU each 30 seconds until achievement of peak or plateau which indicates the basal ROS production of monocytes under study without further stimuli. At this time the total capacity of the cell to build up oxidative burst due to agonists was proven by adding phorbol 12-myristate 13-acetate PMA (100 nM). PMA elicits oxidative burst by a maximal and immediate activation of PKC independently of any receptor and RLU measurement each 30 seconds after PMA addition allows to determine the peak luminescence which is indicative for the maximal ROS production of cells at this time point considering the rather short luminol-based luminescence with a half-life of 8-9 seconds. This does not allow a real accumulation of luminescence signals (RLU) during the measuring time, as the precedent measurement adds just about 10% of its real value to the measurement after 30 seconds and nil after 60 seconds. All obtained data were collected for statistical analysis.

3.4 Immune precipitation

Assuming R406 to favor binding of immunoglobulins to RBC [40], by band 3 clustering, I decided to evaluate the amount of band 3 protein labelled with human immunoglobulins after treatment of cells with R406. Immunoprecipitation (IP) is a procedure designed to precipitate a single antigen from a complex mixture using a specific antibody that recognizes the antigen and binds with its Fc portion to protein G attached to sepharose beads (fig.17). In my study I intended to precipitate and identify the membrane protein(s) of pRBCs that were recognized and bound by immunoglobulins of autologous serum. For this extracted RBCs' membrane proteins were incubated with the beads on which immunoglobulin –tagged proteins were bound by an IgG binding protein such as Protein G capturing the antibody-antigen complex. After binding antigen, the beads are washed extensively, and the antigen eluted from the support using an appropriate elution buffer.

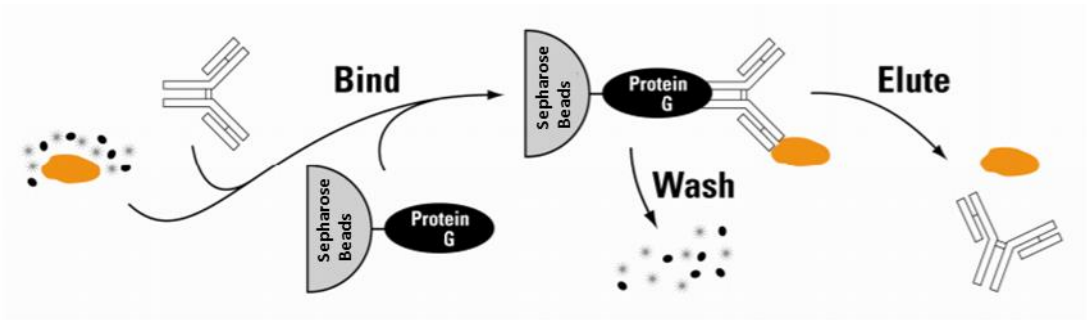


Fig. 17 *Scheme of immunoprecipitation (IP) assay.*

More precisely, the method consists of the following steps:

Firstly, synchronized ring stage pRBCs were incubated under previously described culture conditions in presence of 1 uM R406 at a hematocrit of 1%, and 37°C for 6 hours. After incubation, NPRBC and PRBC were washed with PBS-G at 0°C. Secondly, washed RBCs were than resuspended in wash medium and fresh autologous serum at 33% hematocrit and a final serum concentration of 33% (vol/vol) for opsonization at 37°C for 30 minutes [68]. Thereafter, RBC were washed three times with PBS-G at 4°C and RBCs were subjected to membrane preparation.

3.4.1 Preparation of Red Blood Cells (RBC) membranes

RBC and parasitized RBCs (PRBC) from control and R406 treated PRBCs were lysed in hypotonic buffer (KH_2PO4 10mM; pH=8, supplemented with EDTA 1mM) under non- reducing conditions in presence of Complete© protease inhibitor cocktail).
I prepared the RBC membranes strictly on ice with ice-cold solutions and centrifugation was performed at 4°C.
RBC lysis was performed by adding lysis buffer at ten-fold volume excess to the cell-suspension keeping it on ice and shaking several times for 5 minutes. Thereafter, the lysate was distributed in microtubes of 1.5 ml, centrifuged for 3 min at 12500g and supernatant was discarded. The membrane – containing pellet was washed at least 5 times at 12500g for 1 minute at 4°C. Wash steps were considered enough when a) the ghosts did no longer change color and b) the supernatant did not show any trace of hemoglobin. After washes pellets were unified and resuspended. Aliquots (1ul in triplicate) were taken for protein quantification, 10 ul were solubilized with Laemmli sample buffer as a ghost protein reference for western blot analysis. The remaining membranes (200 ul) were used to extract proteins in 3 volumes of extraction buffer (50 mM Tris HCl, pH 8 supplemented with 150mM NaCl, 1% NP40, 1% Triton X100) for 20 min at 4°C under gentle mixing. Not extracted material was sedimented by centrifugation for 5min in a refrigerated Eppendorf microfuge at 15000 rpm and 4 °C with slow deceleration. Supernatant was collected in a new Eppendorf tube 1,5 ml, while pellet was discarded. Aliquots (1 ul in triplicate) were taken from supernatant for protein quantification and a second aliquot of 10 uL was solubilized with Laemmli sample buffer 5 times concentrated for Western blot analysis. The remaining part were incubated with protein G – Sepharose (30 ul for 500 ug of protein) (*GE Healthcare – Protein G Sepharose ™ 4 fast flow*) for 5 hours at 4°C (gentle mixing). Thereafter the beads were washed three times adding 500 ul of extraction buffer (see above) and centrifuging for 1’ at 15000 rpm (no brake) in a refrigerated Eppendorf microfuge, adding 500 ul of and once adding 500 ul of 50 mM Tris HCl, pH 8 supplemented with 150mM NaCl without NP40 and Triton X100. Laemmli sample buffer (5-times concentrated) containing 2% (w/v) DTT was added to packed beads at 1:1 ratio

(vol/vol), that were heated a 95°C for 5’ and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE) and Western blot analysis with mouse anti-band 3 and goat anti human IgG antibodies.

3.4.2 Protein quantification

RBC ghost proteins were quantified according the Bradford method. Bradford BioRad Dye Reagent Concentrate (BioRad, Hercules, California, United States), was added to the diluted aliquots of ghosts, as indicated by the manufacturer, vortexed, incubated for 10 minutes at RT and placed in the cuvette for the measurement of absorbance at 595 nm in a spectrophotometer (Ultrospec® 2000, Amersham Pharmacia Biotech Italia, Cologno Monzese (MI), Italy). The standard curve with Bovine Serum Albumin (BSA, BioRad 2mg/ml) was obtained by measuring solutions of 0.5, 1.0 and 2.0 mg/ml in triplicate, obtained by serial dilution of the original standard. The protein concentration of samples was calculated based on the standard curve. Results were expressed in ug/ul and inserted in database.

3.4.3 Electrophoretic separation of proteins and Western blot

The levels of Band 3 protein and the levels of IgG bound on the antigens surface were measured by SDS PAGE after transfer to a nitrocellulose membrane (Thermo Scientific, Waltham, Massachusetts) by western blot.

Before electrophoretic run the solubilized protein samples were supplemented with beta – mercapto–ethanol 5% (v/v) final to reduce sulfhydryl groups of cysteine residues, braking down sulfur bridges and with 0,01% Bromophenol Blue to monitor the progress of polyacrylamide gel electrophoresis. The samples were boiled for 5 minutes to complete the denaturation. In parallel to protein samples, molecular weight markers (Amersham Full-Range Rainbow 12-225 kDa) were loaded in the wells of the stacking gel prepared for SDS-PAGE.

Running gels were prepared at 8% of acrylamide by mixing H_20, 30% (w/v) acrylamide: N', N'-methylene-bis-acrylamide solution (37.5:1), 1.5 M Tris (pH 8,8, final buffer conc.), 10 % sodium dodecyl sulfate (SDS), 10% ammonium persulfate (APS; SIGMA, St Louis, Missouri), 0,04 % tetramethylenthylene-diamine (TEMED). After solidification of the running gel, stacking gel of x % acrylamide was prepared by mixing H_2O, 30% (w/v) acrylamide: N',N'-methylene-bis-acrylamide solution (37.5:1), , stacking buffer 1,5 M Tris (pH 6,8; final buffer concentration), 10 % SDS, 10 % APS and 0,04% TEMED, layered on top of the running gel and a 10- well comb inserted before solidification.

Proteins solubilized in Laemmli buffer were load at 5-20 ug/ well of the stacking gel.

The presence of SDS (sodium dodecylsulfate) in the Laemmli loading buffer is an anionic detergent that denatures proteins and gives them an uniform negative charge density and therefore allows proteins to be separated exclusively on basis of their molecular weight and not their intrinsic charge. Glycerol has the task of increasing the density of the sample to facilitate loading it into the gel well. Bromophenol blue is the tracer of electrophoretic run. Solubilized proteins (5-20 ug) were separated by SDS – PAGE in a gel with a thickness of 1mm and an acrylameide concentration of 8% (w/v) using Tris-Glycine running buffer (Tris base 0.25mM, glycine 0.192 M, SDS 0.1%)

using a MiniProtean II cell vertical apparatus (Bio-Rad). Electrophoresis was performed at 50 V for 15 minutes and subsequently at 150 V until the bromophenol blue tracer reached the lower edge of the gel.

After completed electrophoretic run, separated proteins were transferred from the gel to nitrocellulose membranes (previously activated in transfer buffer containing Trizma base, glycine, SDS, and methanol 15%) in a BioRad submarine transfer apparatus Mini-Protean® system. Transfer condition were 70 V for at least 3 hours at 4°C. To visualize protein bands the membrane was incubated with Ponceau Red solution (0.2 % (w/v) Ponceau Red in 6% (w/v) trichloroacetic acid) Ponceau Red binds NH_3 groups of lysine and arginine and colors the transferred proteins red, while the gel was incubated with Coomassie Blue (Coomassie Blu R 0,2% (w/v), methanol 50% (v/v), acetic acid 7% (%), H_20) to check whether the transfer was efficient.

3.4.4 Assessment of proteins by immunochemistry after Western Blotting and Analysis of data

Membranes were blocked with Bovine Serum Albumin (BSA 5% in PBS – Tween 0,1 %) for 60 min to prevent antibody binding to the membrane and then incubated with primary monoclonal antibodies i) mouse anti-Band 3 protein antibody (Sigma, 1:25000 diluted in 1% (w/v) BSA in PBS, supplemented with 0.1 %(w/v) tween) and ii) goat anti-human IgG (Sigma 1:20.000 diluted in 1% (w/v) BSA in PBS, supplemented with Tween 0,1 % (w/v) (PBS-Tween) for 2 hours at 4°C. Thereafter, membranes were washed and incubated for 1 hour at RT with a secondary goat anti-mouse horseradish peroxidase (HRP) – conjugated antibody, diluted 1:40.000 (Santa Cruz Biotechnology) in PBS-Tween, supplemented with 1% (w/v) BSA for Band 3 evaluation, while with a secondary mouse anti goat horseradish peroxidase (HRP) – conjugated antibody, diluted 1:20.000 (Santa Cruz Biotechnology) in PBS-Tween, supplemented with 1% (w/v) BSA.

protein bands recognized by antibody were detected using Enhanced Chemiluminescence (ECL) based on electron transfer from Hydrogen Peroxide to

luminol in presence of HRP, which is conjugated to the secondary antibody. Hence, visualizes band 3 protein and IgG bound to the aggregated surface antigens by luminescence. To collect and elaborate quantitative data of protein conjugates the luminescence intensity was acquired and transformed in optical density OD) by in a Chemidoc apparatus (Chemidoc™ Touch Imaging System, BioRad, Hercules, California) and densitometry analysis was performed by ImageLab™ (Biorad).

3.5 Flow Cytometry Analysis, Fluorescence-activated cell sorting (FACS)

To understand the molecular causes of enhanced phagocytosis of PRBC after treatment with R406, DHA and their combination, I analyzed the level of IgG and C3c complement factor bound to the cell surface by flow cytometry.

Synchronized ring stage PRBC were incubated under previously described culture conditions with different concentration of DHA and R406 at hematrocrit of 1% for 6 hours at 37°C. Each reaction was terminated by three washes with PBS-glucose distributing PRBC in microtubes of 1,5 mL and any centrifugation step was performed at 4°C. Thereafter, PRBCs were adjusted to a hematrocrit of 50%, resuspended and opsonized with fresh autologous serum (ratio 1:1) for 30 minutes at 37°C. Opsonized PRBC were than washed three times with PBS-G.

Preparation of Sample

For each test sample 2uL of packed PRBC were sedimented by centrifugation at 15.000 rpm for 1 min and re-suspended in 80 ul PBS-G, supplemented with 2% (v/v) fetal calf serum (FCS) and distributed in four Eppendorf tubes to obtain 0.5 ul of packed PRBC suspended in 20 ul.

PRBC aliquots were incubated with the antibody as follows:

Goat anti-human IgG F(ab')2 FITC conjugated (Thermofisher) at a final diluition of 1:50

Goat Anti-human C3c FITC conjugated (Dako) final dilution 1:200

Goat anti-human Ker FITC conjugated (Abcam) final diluition 1:200 (isotype control of 1.)

Goat anti-rabbit IgG (whole molecule) FITC conjugated (Sigma) at a final dilution of 1:100.

In parallel other tubes with 1 ul of packed NPRBCs from the same donor were similarly treated to use it as negative control. Incubation was conducted for 30' at RT in the dark.

Thereafter, PRBCs and NPRBCs were washed once, and resuspended in 400ul of PBS-G FCS 2%. After washing the fluorescence of PRBCs was measured by FACS in an Easy Cyte Guava cytofluorimeter (Millipore, Burlington, Massachusetts).

Statistical analysis

To understand the effect of major antimalarial drugs as DHA and R406 (as a new candidate to cause an antimalarial effect) I try to evaluate the level of bound IgG and C3c complement factor on the surface of red blood cells. The obtained fluorescence data were analysed with InCyte Guava Software and presented as mean fluorescence intensity (MFI) [69, 70]

4. RESULTS

4.1 Chapter synopsis

The result chapter consists of four main sections. Firstly, the effect of Syk inhibitor R406 on phagocytic activity of human adherent monocytes is described. In this section data are presented from experiments with three different concentrations of Syk inhibitor to evaluate their effect on phagocytic activity of the monocyte. Secondly, I show the assessment of oxidative burst of monocytes after their incubation with R406. Thirdly, the effect of Syk inhibitor on parasitized red blood cells respective their phagocytosis by the monocyte is presented. In this section, three different concentration of R406 (0.1 uM, 0.5 uM, 1 uM) and their combinatory effect with different concentrations of the bioactive artemisinin derivate dihydroartemisinin (DHA) are evaluated. In the last and fourth section the modification elicited by R406 and DHA on the red blood cell membrane is presented as evaluated by measuring bound opsonins by flow cytometry. Furthermore, I present data obtained by immunoprecipitation experiments on clusterization of band 3 protein in the RBC membrane after R406 treatment obtained.

4.2 Impairment of phagocytic activity of monocyte after treatment with R406

In order to reach our objectives, I tried as first approach to functionally characterize monocytes, involved in the PRBC phagocytosis, identifying the factors that may favor or inhibit their activity and studying their action in the presence of antimalarial drugs. The effect of Syk inhibitor R406 on phagocytic activity of human primary monocytes was studied after their incubation for 5 hours (37°C, 95% atmosphere, 5% CO2) at increasing concentrations (0; 0.05; 0.5; 1; 2.5 uM) with adherent phagocytes. Phagocytosis was measured after feeding monocyte with anti-D IgG treated Rh-positive RBCs by quantifying ingested hemoglobin.

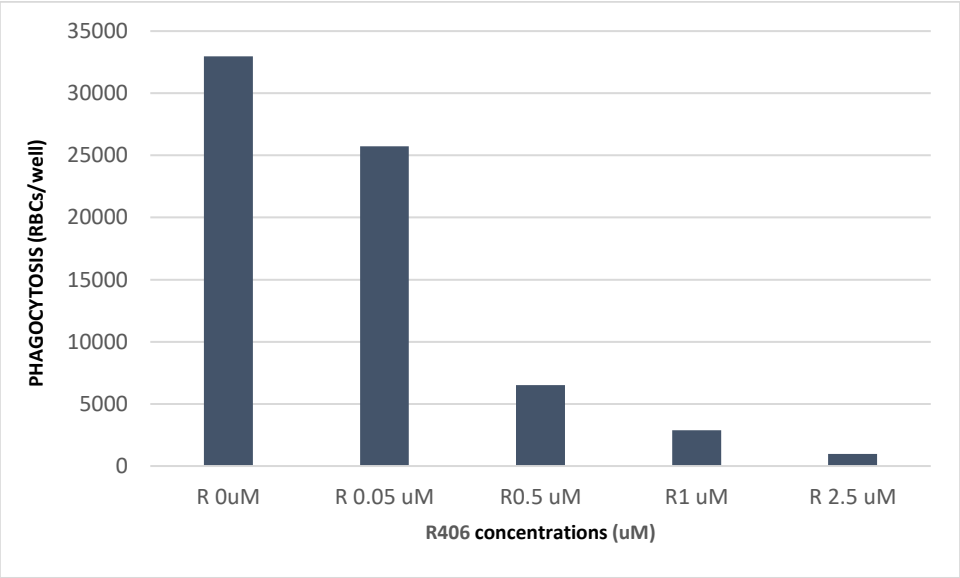


Figure 18: Impairment of RBC phagocytosis by human adherent monocytes after their pretreatment with Syk Inhibitor (R406). A) The figure represents the level of phagocytosis of human adherent monocytes, fed with anti-D IgG treated RBCs, after a previous treatment of monocyte with indicated concentrations of R406 for 5 hours (37°C, 95% atmosphere, 5% CO2). Approximately 0.1-1 x 10^6 monocytes were fed with 10^8 RBC. After 3h of phagocytosis non-ingested RBC were removed by washings and osmotic lysis. Ingested RBC were quantified as described in Materials and Methods. Luminescence data were transformed in number of RBC phagocytosed in each well.

As shown in Figure 18, monocytes without pretreatment were highly responsive and phagocytosed anti-D IgG treated RBCs avidly while a significant and increasing impairment of phagocytic activity was observed after pretreatment of monocytes with R406 from 0.5 to 2.5 uM.

To evaluate whether priming of monocytes modulates the inhibitory effect exerted by Syk-inhibitor R406 on monocyte phagocytosis, cells were pre-treated for 16 hours with IFN-γ (Thermofisher Scientific, Waltham, Massachussets) at 30 ng/ml (50 U/ml) under standard cell culture conditions.

Despite the incubation of human adherent monocytes with INF-ɣ, a decrease in the phagocytic activity of monocytes is observed with increasing R406 concentrations (Fig.19). However, higher concentrations of R406 are necessary to exert the same inhibition observed in unprimed cells and a significant inhibition is seen only at 1uM and higher in IFN-gamma primed monocytes (Fig.19). Even at the highest concentration tested, 2.5 uM, the phagocytosis rate is still 3-5 times higher compared to unprimed cells, i.e. approximately 10 % of control phagocytosis in IFN-gamma treated cells vs. 2.5% of respective control phagocytosis in naïve phagocytes.

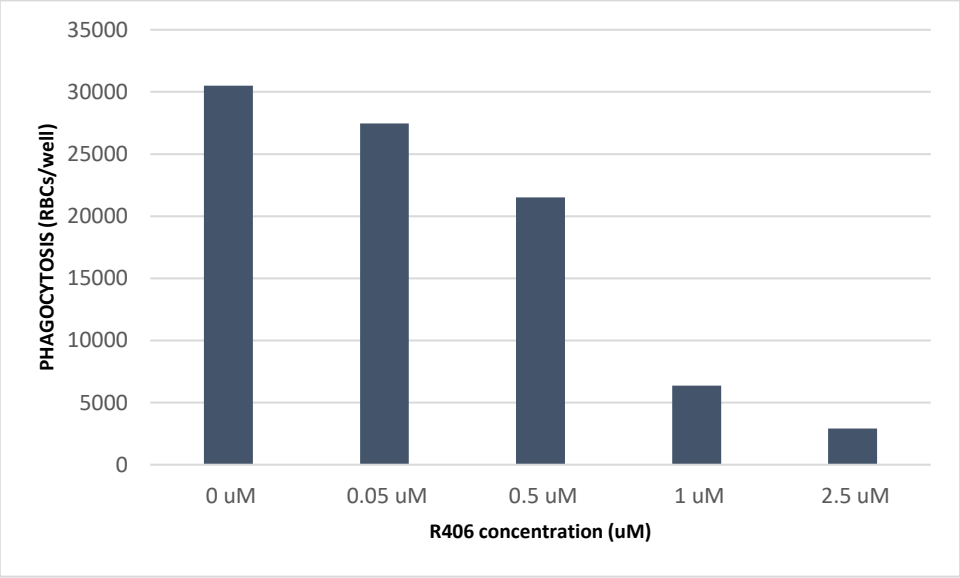


Figure 19: Impairment of Phagocytic activity of human adherent monocytes after their pretreatment with Syk Inhibitor (R406) in INF-ɣ-primed monocytes. The figure represents the level of phagocytosis of human adherent monocytes, fed with anti-D IgG treated RBCs, after previous treatment of monocyte with INF-ɣ (30ng/ml) for 16 hours and subsequently with R406 at indicated concentrations for 5 hours at 37°C, in a 95:5% air: CO2 atmosphere).

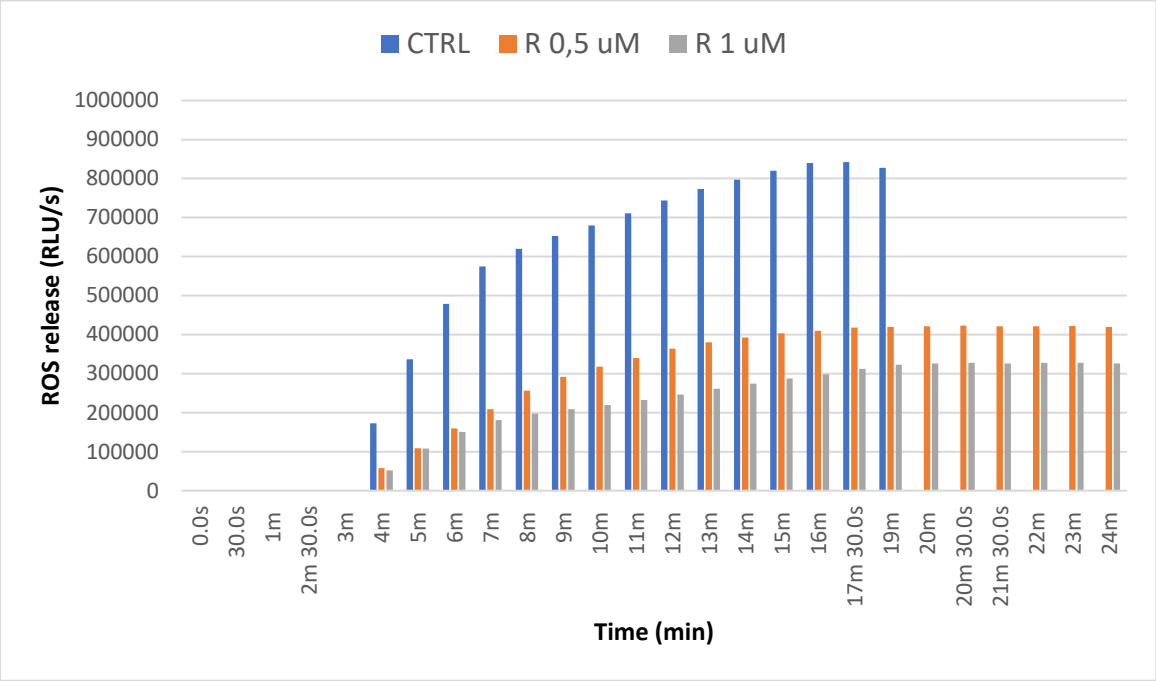
4.3 ROS release (oxidative burst) by human monocyte after treatment with Syk inhibitor (R406)

In order to verify modifications in the first line cellular response of human monocytes by R406, I analyzed the basal and phorbol-ester-elicited ROS release from cultured monocytes after treatment with R406 at different times of incubation by measuring luminol dependent chemiluminescence. Addition of R406 (0,5 uM – 1 uM) to adherent monocytes induced a decrease of ROS release (equivalent to oxidative burst). The capacity of monocyte to generate oxidative burst after PMA stimulation declined sharply after their treatment with R406.

As shown in figure 20A, ROS release by untreated human monocytes increase immediately after adding PMA (3 minutes after starts the experiment) and reach a peak value 15 minutes after PMA stimulation. ROS released from human monocytes pretreated with 0.5 uM R406 mount slower, reaching peak production later and remains at lower levels than untreated control monocytes. Human monocytes pretreated with 1uM R406 show ROS release values significantly lower than both control and 0.5 uM pretreated cells. R406 at concentration of 1uM reduce the ability of monocytes to respond to PMA, reaching less then 40% of control values only after 20 minutes of measurement. ROS release levels was reduced by 48% and 65% after 60 minutes incubation with 0.5 uM and 1uM, respectively.

Figure 20B shows the dependency of PMA-elicited ROS release from human monocytes on the exposure time of cells to 0.5 uM - 1 uM R406. Incubation periods of 30, 60 and 120 minutes with 0-1 uM R406 were studied. and oxidative burst declined progressively with an increase of incubation time with R406.

A)



B)

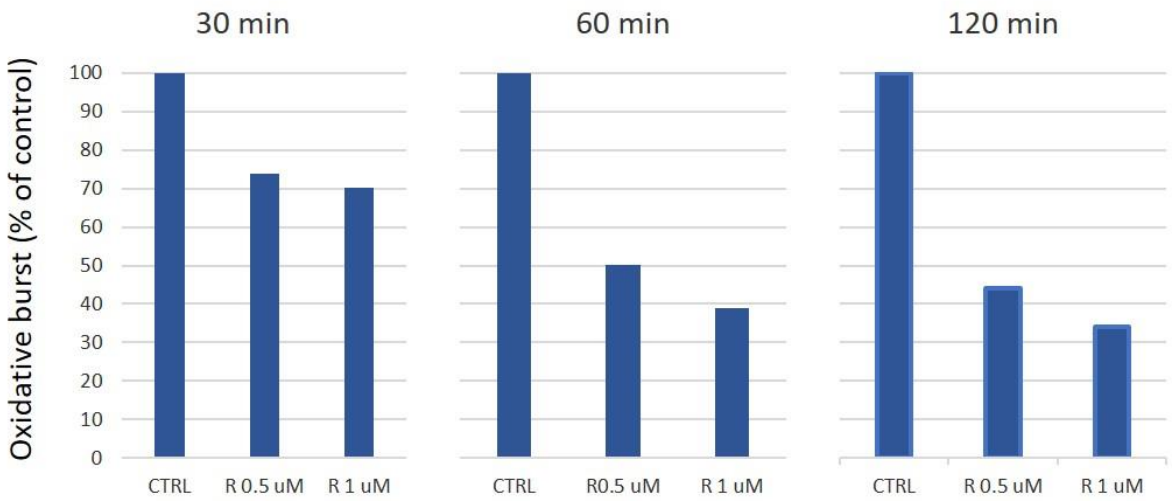


Figure 20: Inhibition of PMA-elicited oxidative burst activity in monocytes pretreated with R406. A) basal and PMA-elicited ROS release (oxidative burst) from monocytes after treatment for 60 minutes with different concentration of R406. **B)** Time course of **PMA-elicited** OS release from monocytes treated with R406. Immediately after isolation of PBMC from healthy donors, monocytes were kept as control, while aliquots were incubated with R406 (0,5 uM – 1 uM) at 37°C. Aliquots of 3,5 x 10^5 monocytes were taken at indicated times and ROS quantified by luminol enhanced luminescence. After measuring the basal luminescence in the presence of 10uM luminol, oxidative burst was elicited by addition of 100 nM PMA at 3 min. Panel represent results of one typical out of two separate experiments.

4.4 The levels of in vitro phagocytosis of PRBC before and after treatment with R406 and DHA

The first line of defense against the intrerythrocytic parasite is provided by phagocytic cells, which recognize PRBC as non-self cells and attack them by the same response adopted against invaders. Progressively, from ring-stage on, parasite-harboring RBC are transformed into non-self cells, opsonized and phagocytosed by circulating and resident phagocytes. Opsonization of parasitized RBC occurs in nonimmune autologous serum. Several investigators explain phagocytosis of senescent and oxidatively damaged RBC and under malaria conditions by following removal model. Modification of band 3 and deposition of anti-band 3 antibodies are the first in a series of events, eventually leading to activation of alternative complement pathway and C3b deposition on the RBC membrane. The growing *P. falciparum* exerts oxidative challenge and induces profound modification in the host RBC membrane. In fact, band 3 conformational changes were described in malaria PRBC, and band 3 clustering was further demonstrated to be determinant for the exposure of band 3 neo-antigens in the host RBC membrane.

Thus, PRBC are recognized as non-self cells and phagocytosed. Deposition of immunoglobulin G (IgG) and complement on band 3 protein produce recognition signals for phagocytosis.

In this section I evaluate the capacity of human monocytes to recognize and remove PRBC efficiently as principal line of defense in non-immune individuals and show changes in efficacy of phagocytosis after treatment of pRBC with R406 and DHA. The hypothesis is that R406 and DHA, alone and in combination, lead to increased binding of IgG and C3b complement factor on the RBC membrane, promoting the clearance of the parasite and suggesting an immune-modulating role of R406 as potential antimalarial drug.

Firstly, I tested the phagocytic activity of monocyte with PRBC at different life cycle stages including malaria pigment hemozoin (HZ). Non synchronous cultures of PRBC were fractionated to obtain non parasitized RBC (NRBC), ring stage parasite (RPRBC),

trophozoite stage parasite (TRPBC) and malaria pigment hemozoin. Serum-opsonized PRBC were phagocytosed more intensely than NPRBC and the level of phagocytosis depended on the parasite maturation stage. As shown in figure 21 phagocytosis increases with parasite maturation and is maximal with trophozoite stage parasite. As expected, malaria pigment hemozoin was most phagocytosed by human adherent monocytes. In this experiment I used human monocytes fed with malaria pigment as a positive control of phagocytosis and the stage-dependent phagocytosis was expressed as a percentage of control.

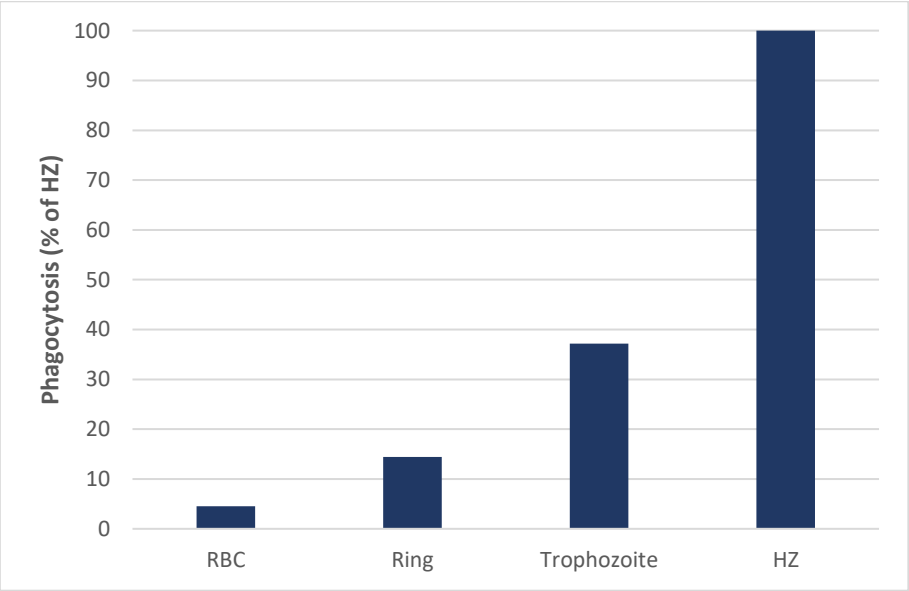


Figure 21: Phagocytosis of noninfected and malaria-infected RBC as function of parasite development. Noninfected and infected RBC were fractionated from synchronous cultures by Percoll density gradient. After washing, RBC were opsonized with fresh non-immune serum and subjected to phagocytosis assay. (Parasitemia of 100% for rings and trophozoite stage). For details, see Materials and Methods. Monocytes fed with HZ were used as a positive control of phagocytosis and the levels of stage-dependend phagocytosis were expressed as percentage of positive control.

To evaluate the effect of antimalaria drug on phagocytosis of PRBC, I pretreated PRBC with different concentration of DHA (Figure 23A) and R406 (figure 23B). PRBC were fractionated from synchronous cultures by Percoll density separation. The phagocytosis experiments were conducted using the fraction of Ring stage collected from the 90 % percoll cushion above the bottom fraction and ring-parasitized RBC at lower parasitemia from the bottom fraction and incubated with DHA and R406. Figure 23A shows increasing phagocytosis levels by increasing DHA concentrations until 120% of control at 1uM DHA. While figure 23B shows an increasing phagocytosis level until 0.5 uM R406 (117% of control) while there isn't significant differences on phagocytosis levels with R406 1 uM. Observing smear of PRBC R406 treated, immediately before phagocytosis experiments we observed a delay of growth of parasite compared to control. The delay of growth of the parasite with the reduction of the parasitemia after treatment with R406 0,5 uM, helped us to consider R406 0.5 uM the optimal concentration for studies on the synergistic effect of a combination between R406 and DHA. Morphology of human adherent monocytes before and after fed with anti D – IgG opsonized RBC has shown in figure 22.

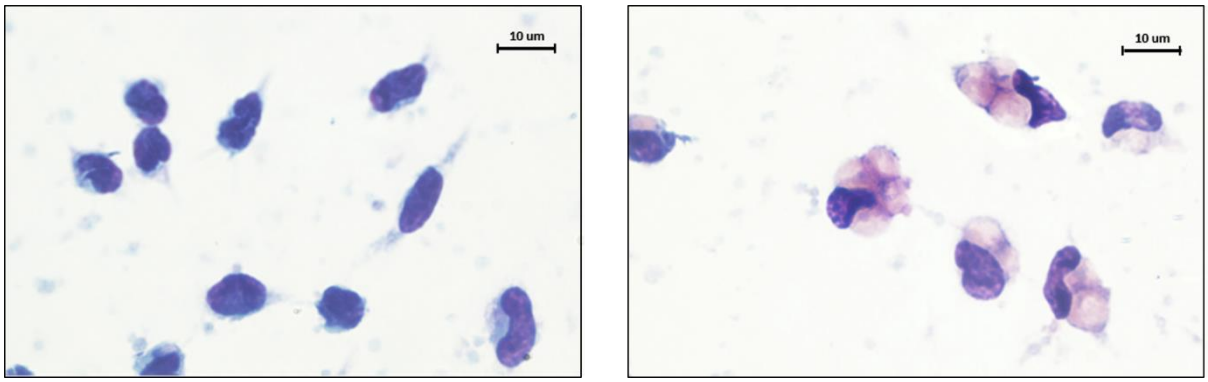
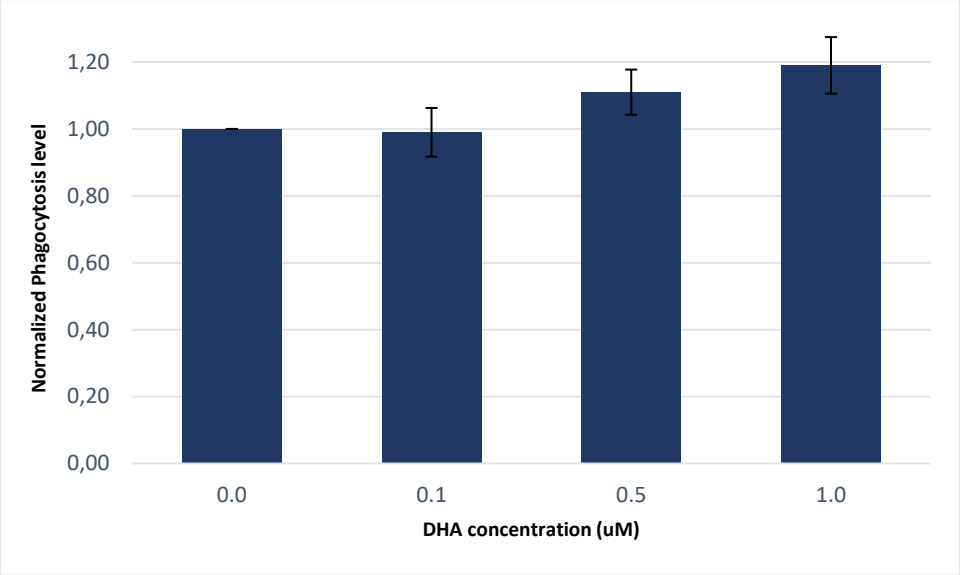


Figure 22: Erytrophagocytosis by human adherent monocytes. Human adherent monocytes were isolated from buffycoat of healthy donor in a multiwell plate. Gimsa-stained of human adherent monocytes as control (panel left) and monocytes after incubation for 3 hours with anti D – IgG opsonized RBC (panel right).

A)



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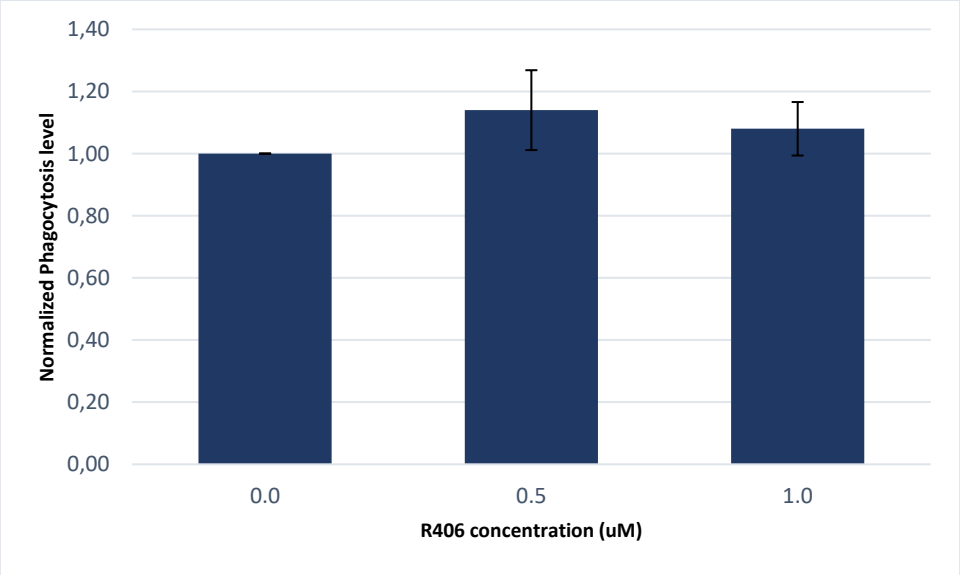


Figure 23: Effect of DHA and R406 on phagocytosis of PRBC. PRBC were fractionated from asynchronous cultures by Percoll methods. After 5 hours of incubation with DHA (panel A) and R406 (panel B), PRBC were opsonized with fresh serum and subjected to phagocytosis assay. After 3 hours of phagocytosis, monocytes were lysed and phagocytosis was measured by the assay of heme-derived luminescence. For details, see Materials and Methods. The phagocytosis levels for DHA/R406-treated RBC of each donor were normalized by setting the phagocytosis value for untreated RBC of the same donor one. The not-normalized phagocytosis value for not-treated RBCs was 30.000 fmol/well hemin +/- SE. Normalized phagocytosis values are plotted. Mean values ±SD (N=20)

4.5 Synergistic effect of DHA and R406 on phagocytosis of RING-parasitized RBCs by adherent primary human monocytes.

In order to analyze the *in vitro* complementary activity of Syk inhibitors (R406) on artemisinin efficacy, *P. falciparum* cultures were treated with different concentrations of DHA and R406 as described above (materials and methods).

Figure 24 shows the synergic interaction between DHA 0,1 uM and R406 0.5uM after 5 hour of incubation determining an increase of phagocytosis level. This result indicates very different mechanism of action and effects on plasmodium of Syk inhibitors as single agents, and in combination with DHA: a) at 0.5 uM concentration, R406 show measurable effect on phagocytosis of the parasite and when used in combination with DHA they potentiate the artemisinin activation and leads an a significant increase of phagocytosis level; b) higher concentration of R406 (1uM), in reverse, leads a impairment of phagocytosis level, presumably due to monocyte toxicity or red blood cell death.

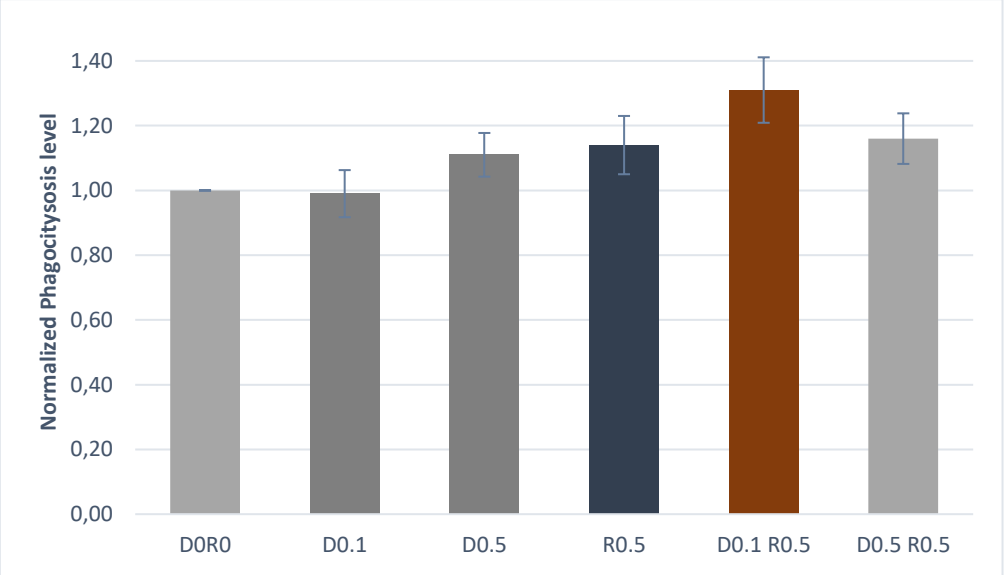


Figure 24: Sinergistic effect of DHA and R406 combination on phagocytosis of PRBC. PRBC were fractionated from asynchronous cultures by Percoll methods. After 5 hours of incubation of DHA and R406 PRBC were opsonized with fresh serum and subjected to phagocytosis assay. After 3 hours of phagocytosis, monocytes were lysed and phagocytosis was measured by the assay of heme-derived luminescence. For details, see Materials and Methods. The phagocytosis levels of PRBC- DHA/R406 treated were expressed as arbitrary units after set the PRBC untreated as value of one. Mean values ±SD (N=8)

4.6 Analysis by FACS of bound IgG and C3 on RBC antigens surface before and after treatment with Syk inhibitor

In order to understand the molecular cause of enhanced phagocytosis, I have characterized the surface of RBC, PRBC, and PRBC treated with DHA and R406 measuring the membrane bound IgG and C3c complement factor.

The capture of microorganisms are modified cells is a necessary prerequisite for their killing and intracellular degradation. IgG class antibodies cover microorganisms or flag modified autologous cells such as the parasitized RBC, favoring phagocytosis by binding FcR receptors on phagocytic cells. The phagocytic cells express on their surface a series of receptors that, by directly binding the microorganisms, favor their capture even in the absence of antibodies. The expression of specific receptors for the Fc portion of the IgG however, that cover the microorganisms (fig.25). represents the main effector mechanisms of innate immunity. The efficiency of this process is significantly enhanced if the phagocyte binds the microorganism with greater affinity.

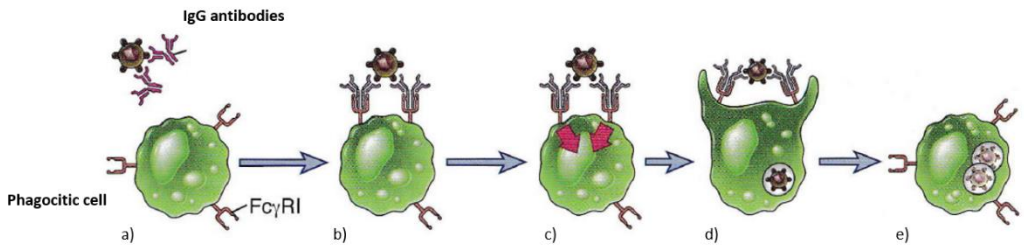


Figure 25: Opsonization and phagocytosis mediated by antibodies. a) Opsonization by IgG; b) Binding to receptors FcɣR on the surface of monocyte; c) activation of monocyte by FcɣR transduction; d) phagocytosis of microorganism; e) killing of phagocytosed microorganism.

Another way to recognize, no-self cells can be mediated by a product of the activation of the complement system. The complement system therefore includes proteins that collaborate with each other in order to opsonize microbes, promote phagocyte recruitment and, in some cases, directly kill the microbe. The recognition of non-self cells by one of the three pathways of complement activation leads to the recruitment and assembly of additional complement proteins to form complexes with protease activity. The biological effects of the complement system are due to the binding of its components and its fragments generated during activation with the receptors expressed on the membrane of various cell types. The best characterized receptor is the CR1 receptor which has the function of promoting phagocytosis also on monocytes.

In this section of results, the aim of the experiments was to evaluate the mechanism by which DHA and R406 can lead to an increase of phagocytosis and to identify the role played by IgG and complement system activation, respectively, on enhanced phagocytosis.

In order to reach our objectives, *P.falciparum* cultures, treated with R406, DHA and their combination, were opsonized with fresh human serum and, membrane deposition of C3c complement fragments and autologous IgG, two strong opsonins that play a fundamental role in inducing phagocytosis, was evaluated by flow cytometry. Analysis by flow cytometer allows to rapidly and reproducibly analyze a high number of cells, through the analysis of physical parameters and fluorescence. The principle of this essay is simple: the flow cytometer analysis of a parasitized erythrocyte population previously incubated with a fluorescent antibody provides a fluorescence value (see Material and methods). The fluorescence value of untreated parasitized red blood will be compared to the fluorescence of parasitized red blood cells treated with DHA and R406.

In figure 26, I show the effect of different concentrations of DHA and R406 on membrane bound IgG. The results show that the level of membrane-bound IgG were increased after treatment with 0.1 uM DHA while decreased with higher concentrations (1 uM). Membrane bound autologous IgG increased by 26 and 18 % for 0.1 uM and 0.5 uM DHA, respectively. Furthermore, IgG binding increased with increasing R406

concentration. A significant increase of membrane bound IgG by 26 % was observed after incubation 0.5 uM R406.

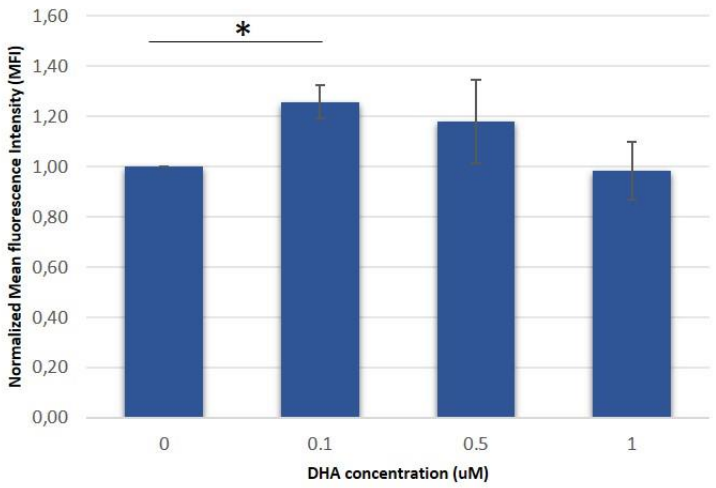
Figure 27 shows the effect of different concentrations of DHA and R406 on membrane bound C3c. Similar to IgG, DHA augments also the C3c binding to the membrane. C3c increases with increasing concentration of DHA until 0,5 uM.

Membrane bound C3c was significantly increased by 17 and 35% after RBC treatment with 0,5 and 1 uM DHA, respectively, while 0.5uM R406 provoked an increase of 29%. Not significant increase was detectable at lower concentrations of R406 (see 0.1 uM).

Neither DHA nor R406 had effects on IgG or C3c binding to the membrane of NPRBC at any concentration tested.

The observed increase of both C3c and IgG deposition on PRBC provoked by DHA and R406 suggests a role for opsonin-dependent PRBC phagocytosis in the antimalarial action of DHA and R406.

A)



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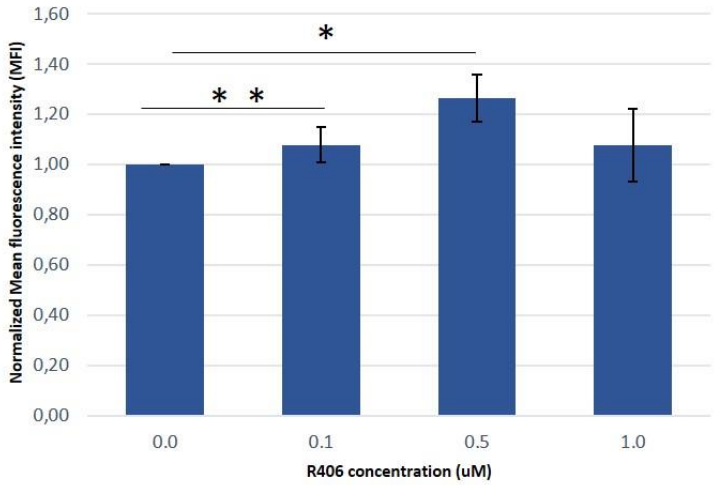

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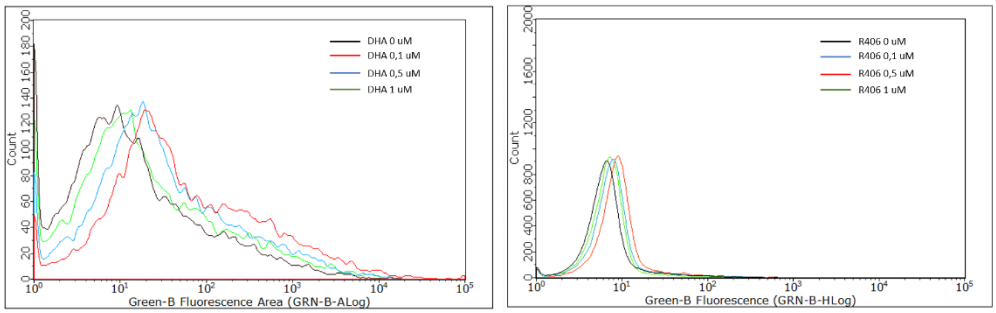
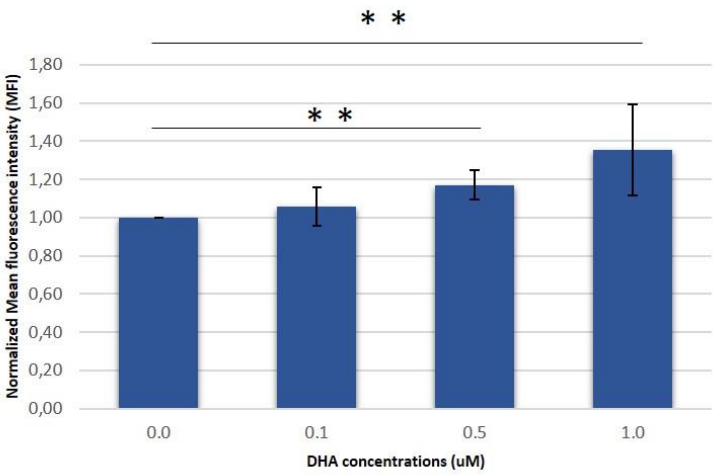


Figure 26: Effect of DHA and R406 on membrane bound autologous IgG. The level of membrane bound IgG after 5 hours of incubation with DHA (panel A) and R406 (panel B) was measured in PRBC after opsonization with fresh serum by FACS analysis. Histogramm (panel C) shows a fluorescence curve for PRBC treated with DHA (left) and R406 (right) One representative experiment out of eight with similar results. For details, see Materials and Methods. The level of membrane bound IgG of PRBC- DHA/R406 treated were expressed as relative mean fluorescence intensity (MFI) after setting the MFI value for each untreated PRBC sample one. Mean values ±SD (N=8) (* = P<0,005; ** = P<0,05)

A)



B)

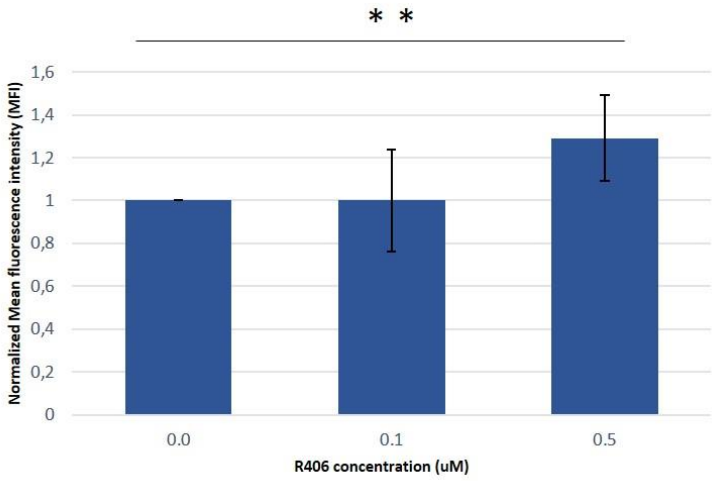


Figure 27: Effect of DHA and R406 on membrane bound autologous C3c. The level of membrane bound C3c after 5 hours of incubation with DHA (panel A) and R406 (panel B) was measured in PRBC after opsonizion with fresh serum by FACS analysis. For details, see Materials and Methods. The level of membrane bound IgG of DHA/R406 treated PRBC is expressed as relative mean fluorescence intensity (MFI) after setting the value for untreated PRBC one for each donor. Mean values ±SD (N=8) (** P<0,05)

4.7 Synergistic effect of DHA and R406 combination on membrane bound autologous IgG and C3c.

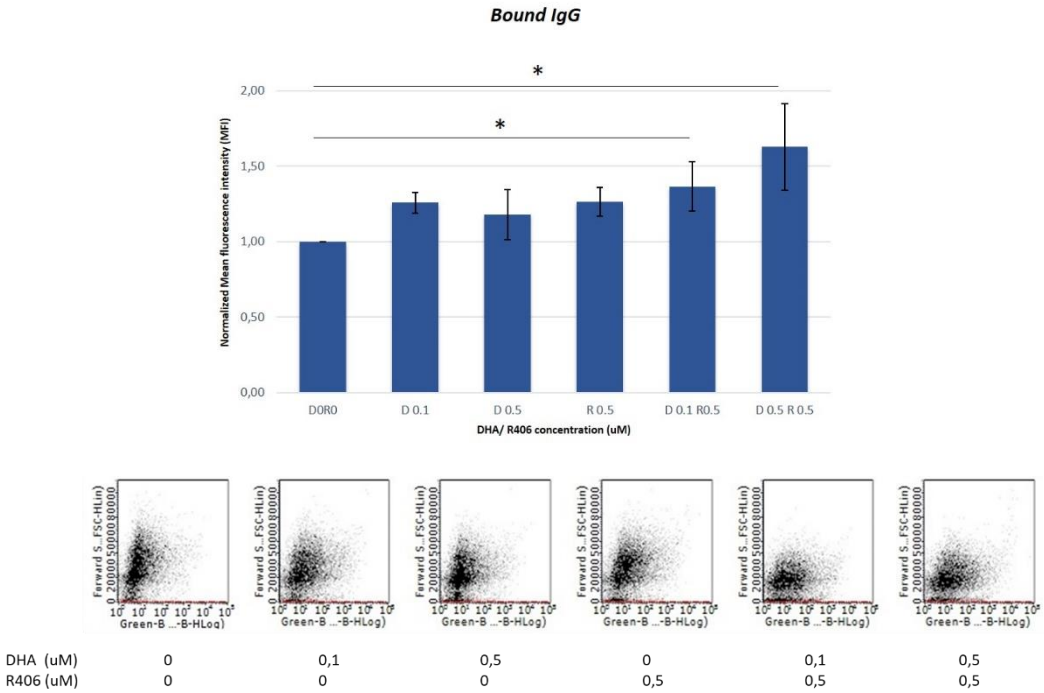
The promising results obtained by cytofluorimetry on increased C3c and IgG binding to the membrane of parasitized RBC after treatment either with DHA or R406, led us to investigate the combination of DHA and R406 by flow cytometry.

Figure 28 shows a synergistic effect of R406 for DHA-elicited IgG- and C3c- binding to the membrane. The results show that membrane-bound autologous IgG and C3c were remarkably higher after the combined treatment with DHA-R406 vs. R406 alone, confirming the synergism of both substances observed in phagocytosis efficacy.

More precisely, the level of membrane bound IgG increases by 45% and 37% after incubation with the combination of 0.5 uM DHA with 0.5 uM R406 in comparison with 0.5 uM DHA or 0.5 uM R406 alone, respectively.

Very interesting is the substantial increase of DHA-elicited C3c deposits on the membrane by an additional supplementation of 0.5 uM R406 to PRBC. I observed: While 0.1uM DHA increased the C3c level found in untreated PRBC by 10 %, the additional supplementation with 0.5 uM R406 rose the level by 38%, hence 28% due to the Syk- inhibitor. Even in PRBC treated with high concentrations of DHA (0.5 uM) R406 treatment increased the complement binding by further 34 %. From these observations it is possible to hypothesize that i) R406 leads to an activation of DHA favoring the binding of IgG and C3c to antigenic receptors on the surface of the red blood cell and consequently triggering the phagocytosis signals or ii) DHA and R406 act simultaneously triggering a synergistic action in the binding of IgG and C3c to the surface of red blood cells.

A)



B)

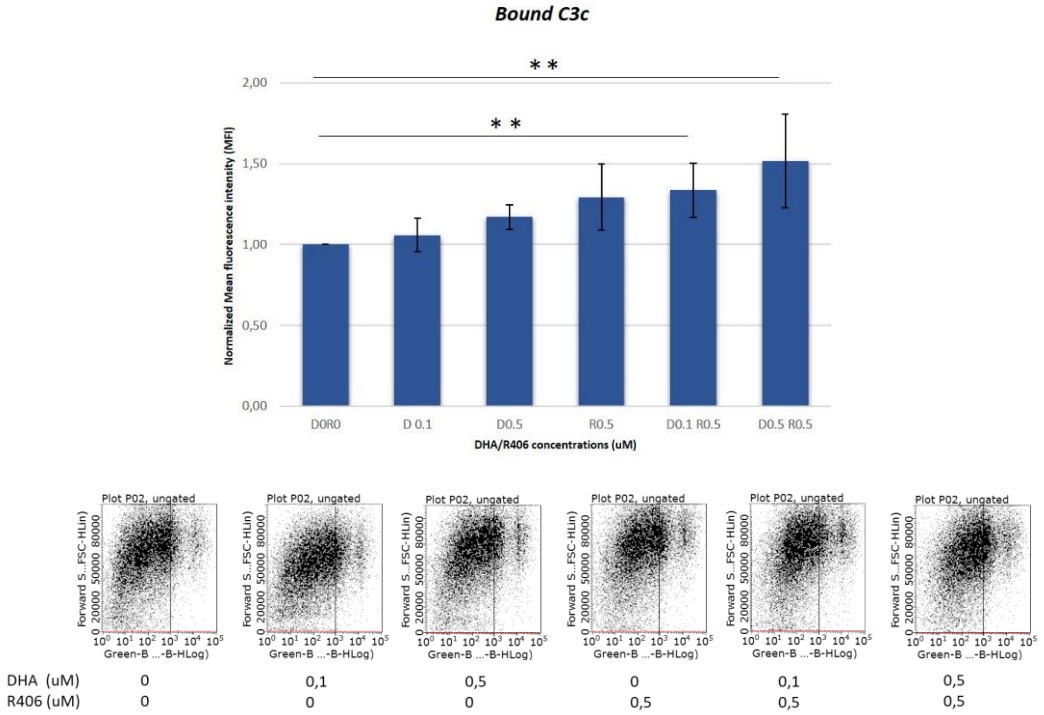


Figure 28: Synergistic action of DHA and R406 combination on membrane bound autologous IgG and C3c. The level of membrane bound IgG (panel A) and C3c (panel B) after 5 hours of incubation with DHA and R406 comabination. PRBC were opsonized with fresh serum and subjected to FACS analysis. I For details, see Materials and Methods. The level of membrane bound IgG of PRBC- DHA/R406 treated were expressed as normalized mean fluorescence after setting the value for control one for each donor.

4.8 Morphological changes induced by Syk inhibitor (R406), DHA and after their combination.

In this embodiment, synchronized cultures were treated with DHA as a single agent and in combination with Syk inhibitors (R406). Selected PRBC were presented after 6 and 18 hours of treatment. The most striking morphological alterations were observed in fixed blood smears of parasite culture treated with Syk inhibitor in combination with DHA after 18 hours, in comparison to the control cultures. A modest reduction in their size was observed at 6 hours of incubation, perhaps suggesting slower parasite growth (cells cycle delay). At 18 hours of incubation, there was evidence of a marked reduction in parasite growth (smaller size) (fig. 30).

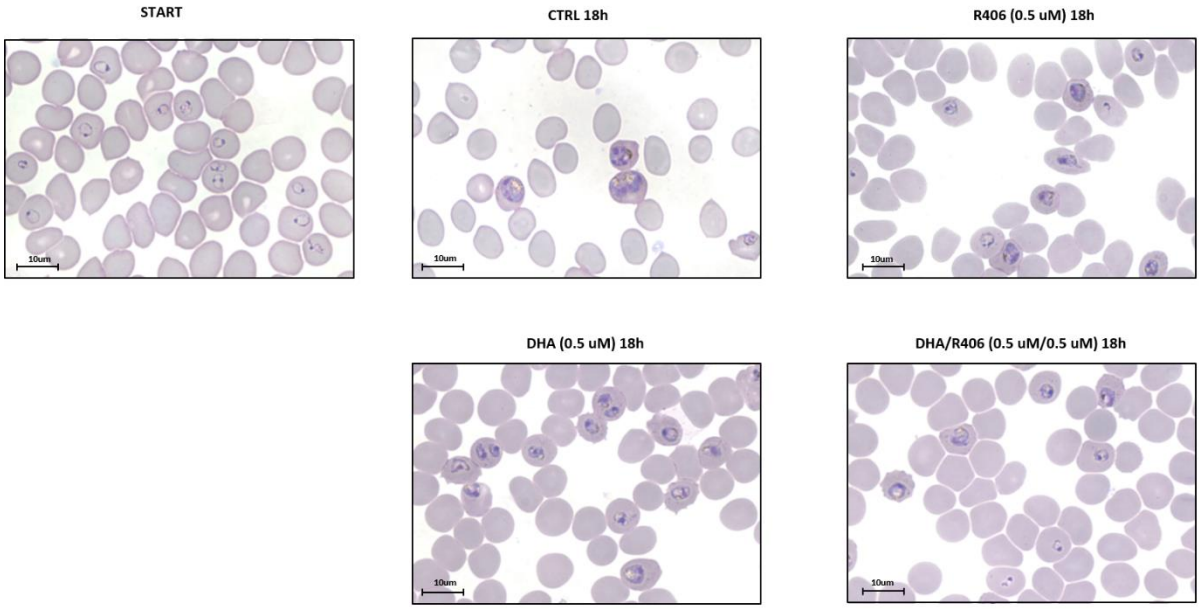


Figure 30: Parasite morphology after 18 hours of drug treatment. Plasmodium falciparum Palo alto parasite cultures were incubated with 0.5 uM DHA as a single agent and in combination with 0.5 uM R406. Giemsa-stained of blood smears were prepared and viewed by microscopy using a 100x objective.

4.9 Immunoprecipitation (IP) of Band 3 protein from R406- treated PRBC

After observing, by flow cytometry, differences between treated and untreated PRBC on the binding of IgG and complement factor C3c on the red blood cells membranes, I assessed the level of band 3 protein and IgG level by immunoprecipitation with protein G and subsequent western blot. I observed that, concentrations of R406 0,1/0,5 and 1 uM leads (as shown previously section) an increase of the level of membrane bound IgG, suggesting that R406 cause a modification on the RBC membrane, favoring recognition antigen-antibody complex and consequently, favoring erytrophagocytosis by human monocyte. To confirm the results seen by flow cytometry and to better understand the hypothetical mechanism of R406, I used IP assay to show the level of IgG-bound band 3 protein and IgG level on erythrocyte membrane of PRBC after treatment with R406 1uM. The results in figure 31 show that there is an increase of Band 3 and IgG level after treatment with R406 1uM versus control. After densitometry analysis the level of Bd 3 is increased by 82% after treatment with R406 1uM. The level of IgG bound on erythrocyte ghost membrane are increased by 32% after treatment with R406 1uM.

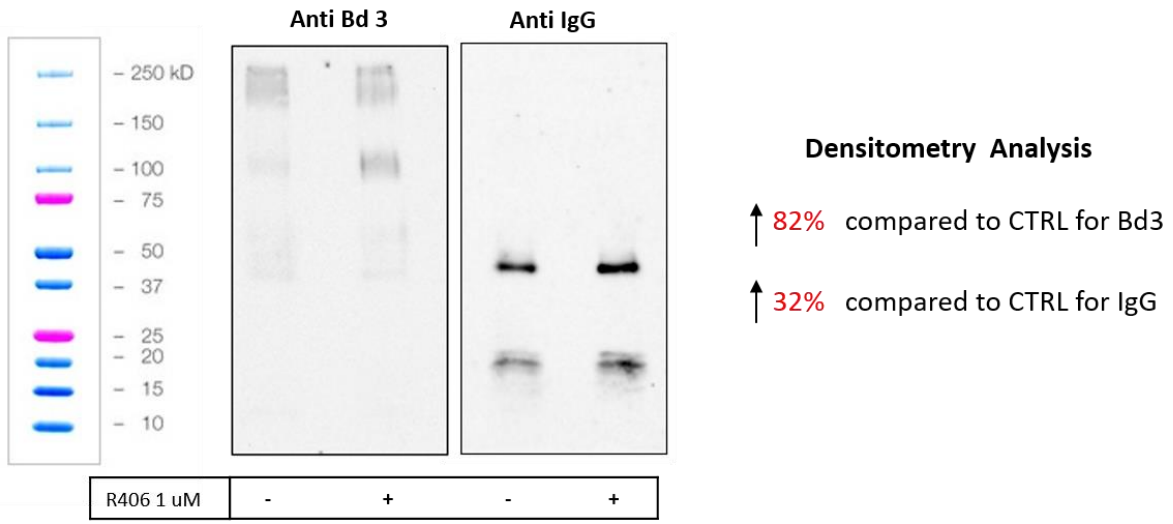


Figure 31: Level of Band 3 protein and IgG, bound to erythrocyte membrane (ghost) evaluated after immunoprecipitation of proteins linked with IgG. The level of Band 3 protein (panel left) and IgG (panel right) after 5 hours of incubation with 1 uM R406. PRBC were opsonized with fresh serum. RBC membranes were prepared , proteins extracted and subjected to IP with protein G Sepharose. For details, see Materials and Methods.

5. Discussion

Currently, the best treatment of *Plasmodium falciparum* malaria is the artemisinin-based combination therapy (ACT), in which derivates of artemisinin are combined with a partner antimalarial drug from another class. It has been demonstrated that Syk inhibitors suppress the capability of the parasites to complete their life cycle and to infect new erythrocytes. Thus, it's possible to consider Syk inhibitors a new promising class of antimalarial drugs. The antiparasitic activity of Syk inhibitors is based on the inhibition of microparticle shedding that removes band 3 protein bound hemichromes from the RBC membrane. As band 3 protein clusters in RBC membranes bind auto anti-band 3 antibodies I hypothesized that Syk inhibitors may provoke an elevated phagocytic removal of pRBC.

The purpose of this work was to investigate the combination activity of Syk inhibitor with ART. In particular, I investigated the role of immune cells on clearance of *P.falciparum* infected RBC before and after treatment with DHA, R406 alone and both combination.

The cell mediated response in nonimmune malaria patients involves circulating and tissue resident phagocytes. The phagocytic system has the capacity to control parasitemia or to annihilate it altogether. Phagocytic cells recognizes parasitized RBC as nonself cells and attack them by the same response adopted against any invader.

We think that the identification of novel drug, capable of promoting the role of the immune system in the clearance of the parasite, represents a goal to counteract artemisinin resistance.

In the present study, I show that human adherent monocytes are able to phagocyte anti-D IgG-opsonized RBC and *P. falciparum* infected red blood cells at different life cycle stage. As expected, the highest phagocytosis levels were observed with monocytes fed with anti-D IgG-opsonized RBC and malaria pigment hemozoin, the latter being 25 times higher than phagocytosis values with NPRBC. Also mature blood stages (trophozoites) were avidly phagocytosed (9.2 times above NPRBC values), while young staged ring-PRBC show rather low phagocytosis rates just 3-fold higher than those with

NPRBC. Note, that the measurable phagocytosis level of NPRBC might be an overestimation due to the homologous phagocytosis model, where monocytes and RBC derived from different donors. Nonetheless, phagocytosis of ring-PRBC remains clearly inferior the later stage trophozoite phagocytosis by about 70%. This differences were described previously and are also due to profound host cell membrane changes elicited by the growing parasite not before late stages, such as the introduction of parasite antigens into the RBC membrane. The avid phagocytosis *in vivo* of trophozoites results in the uptake and consequent persistence of malarial pigment hemozoin in the phagocytes. There is a huge body of literature that describes the immune-modulatory consequences of the presence of this non-degradable material in the lysosome of monocytes, macrophages and dendritic cells. Hemozoin persistence in monocytes leads to the functional incapacitation of the phagocyte, which is no longer able to perform further phagocytic cycles, oxidative burs, migration and differentiation to macrophages and dendritic cells or function as nursery cell in erythropoiesis . On the other, hand hemozoin elicits an immediate strong release of ROS and pro-inflammatory cytokines, such as TNF. Both, immune-suppressive and inflammatory actions of hemozoin had been associated with malaria patho-mechanisms and severe malaria. Thus the stimulation of phagocytosis of young, ring-stage PRBC by Syk inhibitors would be beneficial.

Firstly I had to rule out concerns regarding the effects of Syk inhibitors on phagocyte function as to the known regulatory role of Syk in phagocytosis and oxidative burst.

As assumed, I saw that R406 inhibits the phagocytosis of anti-D IgG-opsonized RBC by human adherent monocytes in a dose dependent manner. A decrease of phagocytic activity was observed when monocytes were pre-incubated with increasing R406 concentrations, with a substantial inhibition of about 70 % at 0.5 uM. Though, in presence of IFN-γ, the agonist that strengthens the Fc- dependent phagocytosis, inhibition by Syk inhibitor R406 was just 25 % at this concentration. Lower concentrations of the inhibitor did not significantly inhibit at all. The presence of interferon meets the malaria condition where the blood level of this cytokine is increased due to activated lymphocytes. Calculating the concentration that a monocyte/macrophage might see in vivo after a single dose administration of R406,

bearing in mind the pharmacokinetics the value remains well below 0.5 uM. Thus a direct effect of Syk inhibitors on phagocytes might be insignificant *in vivo*.

Surprisingly we did not see an increase of phagocytosis by IFN-gamma alone, which had been the case if Fc-receptor dependent phagocytosis would mainly contribute to the overall phagocytosis. One might argue that phagocytes were already primed without exogenous treatment of with interferon as phagocytosis was performed the second day after blood donation and buffy coats were stored overnight. These conditions may mask he increase in FcR-mediated phagocytosis induced by IFN-γ.

I further show that R406 lead a decrease of PMA-elicited oxidative burst with increasing R406 concentrations. These data suggest that some essential functions of monocyte, like phagocytic activity and to produce PMA-elicited oxidative burst, are incapacitated after treatment with R406.

To avoid the incapacitation of monocyte functions by Syk-inhibitors phagocytosis experiments with PRBC were designed that monocytes were not exposed to free R406. To evaluate the effect of Syk inhibitor on phagocytosis of P. falciparum infected red blood cells, different concentrations of R406 (0.1 – 0.5 – 1 uM) have been tested in combination with DHA. I show that Syk inhibitors such as R406 used in combination with DHA exerts a marked synergic effect on phagocytosis of *P. falciparum* infected red blood cells suggesting a novel mechanism of action of this promising antimalarial drug.

In fact, it should be noticed that at 0.5 uM concentration R406 show measurable effect on phagocytosis of the parasite and when used in combination with DHA 0.1 uM, it potentiates the activation seen with artemisinin and leads to a significant increase of phagocytosis level. Not significant increase was detectable at lower concentrations of R406 (0.1 uM) while higher concentration of R406 (1uM), leads to an impairment of phagocytosis. Consistent with the effect of R406 on monocyte activity, it is possible that at the 1uM concentration the phagocytic activity of the monocyte is strongly compromised or, considering its toxicity, concentration higher 0.5 uM leads to red blood cells death. To evaluate the mechanism by which DHA and R406 can lead to an increase of phagocytosis I try to identify the role played by IgG and complement system activation, respectively, on enhanced phagocytosis. IgG and C3c bound to the host cell

membrane has been measured by flow cytometry in presence of increasing concentration of R406, DHA and after their combination. As we know from the literature, the malarial parasite induces an oxidative stress on its host erythrocyte and, despite the antioxidant defense systems, oxidative damage is observed on the erythrocyte membrane with a consequent increase in hemicromes. These alterations lead to the deposition of autologous IgG specific for Band 3 protein with a subsequent deposition of C3c fragments of the complement. All these processes modify the surface of the infected erythrocyte allowing the recognition by the phagocytes. Deposition of autologous IgG and C3c fragments on the erythrocyte membrane was increased by 26% and 29% respectively for IgG and C3c after incubation with 0.5 uM R406. But the very interesting data regards the increased of membrane bound IgG and C3c after combination DHA 0.5 uM and R406 0.5 uM, valued for 37% and 28% respectively for IgG and C3c.

These significant data obtained by flow cytometry are comforting about the role of Syk inhibitor on phagocytosis of parasites, considering that opsonization with pooled human serum (in presence of complement) may mask the increase in FcR-mediated phagocytosis.

Data of increased phagocytosis by monocytes and data about increased deposition of IgG and C3c on the red blood cell membrane after treatment of PRBC with DHA and R406 suggest an important role of Syk Inhibitor in parasite clearance.

The analysis of the role of Syk inhibitor on the immune system gains more emphasis thanks to recent studies showing that R406 leads to an inhibition of the growth of the parasite. In fact, as demonstrated by studies in our laboratory, younger stage parasite are rapidly digested by monocytes and the process can be repeated without loss of efficiency by phagocytes. As showed by Schwarzer et al, phagocytosis of more mature stages of the parasite inhibits the ability of monocytes to repeat the phagocytosis process and to express class II membrane antigens after IFN-γ stimulation and to correctly present antigens.

Observing the data obtained with the flow cytometer, we hypothesized that R406 may induce modification on the red blood cells membrane. This idea has been supported by assessed the level of band 3 protein, flagged by IgG and the IgG level by

immunoprecipitation. I show that, R406 concentrations of 0,1- 1 uM leads to an increase of the level of membrane bound IgG, confirming that R406 cause a modification on the RBC membrane, favoring recognition antigen-antibody complex and consequently, favoring erytrophagocytosis by human monocyte.

Concluding, our research group demonstrated that Syk kinase inhibitors do not promote oxidative toxicity to healthy RBCs as they do not produce appreciable amounts of hemichromes. Since some Syk kinase inhibitors can be taken daily with minimal side effects, we proposed that Syk kinase inhibitors could contribute measurably to the potencies of ACTs.

My data support the hypothesis that Syk inhibitors are a promising class of antimalarial drugs that can suppress parasitemia by increasing also the antiparasitic immune defense. Particularly, R406 should not lead to the selection of resistant strains, as it targets host cell molecules and will likely avoid immunosuppressive effects of hemozoin due to the anticipated phagocytosis of Ring stage-PRBC. Therefore, Syk inhibitors may represent a strategic partner drug for artemisinin therapies for counteracting artemisinin resistance.

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