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Mechanism of synergic interaction of Syk inhibitors on antimalarial artemisinin activity

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Coordinator: Prof. Lucia De Franceschi
Signature: ________________________

Tutor: Prof. Francesco Michelangelo Turrini
Signature: ________________________

Co Tutor: Prof. Antonella Pantaleo
Signature: ________________________

Ph.D. candidate: Dot. Ioannis Tsamesidis
Signature: ________________________
Mechanism of synergic interaction of Syk inhibitors on antimalarial artemisinin activity

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"Άθλοφόρε ἄγιε, καὶ ιαματικὲς Παντελεήμονι, πρέσβευε τῷ ελεήμονι Θεῷ, ἵνα παισμάτων ἁφεσίν, παράσχῃ ταῖς ψυχαῖς ἡμῶν. Θείον τρόπον σου, τῇ επιστήμῃ, νέμεις ἁμισθὸν, τὸν θεραπείαν, τῶν ψυχῶν καὶ τῶν σωμάτων εἰς Πνεύματι. ὅθεν ἡμᾶς πάσης νόσου απάλλαξον, Παντελεήμονι εἰλέους θησαυρίσσας. Μάρτυς ἐνδοξῆς, Χριστὸν τὸν Θεόν ἴκετε, δωρήσασθαι ἡμῖν τὸ μέγα ἔλεος."

(Ἀπολυτίκιο Αγίου Παντελεήμων)
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ABBREVIATIONS

**WHO**: World Health Organization

**RBCs**: Red Blood Cells

**ACTs**: Artemisinin based Combination Therapies

**ART**: artemisinin

**DHA**: dihydroartemisinin

**AS**: Artesunate

**ATH**: Artemether

**ARTs**: Artemisinin derivatives (DHA, AS and ATH)

**CQ**: Chloroquine

**MQ**: Mefloquine

**PPQ**: Piperaquine

**LMF**: Lumefantrine

**AMQ**: Amodiaquine

**K13**: Kelch 13 propeller

**SYK**: Spleen Tyrosine Kinase

**IC₅₀**: Inhibitory Concentration of 50%

**P. falciparum**: *Plasmodium falciparum*

**HMC**: Hemicromes

**hRBCs**: hemicromes in Red blood cells

**Hb**: Hemoglobin

**metHb**: methemoglobin

**Trx**: thioredoxin

**Digestive vacuole**: DV

**Ferrisprotoporphyrin IX**: FP

**RNS**: Reactive nitrogen species

**SOD**: Superoxide dismutase

**Fig.**: Figure
SUMMARY

The current therapy used for the treatment of malaria is based on the administration of a series of artemisinin derivatives combined with some quinolinic antimalarial drugs that, due to their prolonged action, compensate for the short life of artemisinins. Many of which are affected by diffuse resistance. The efficacy of the artemisinin combined therapies (ACTs) therefore rely on the very fast action of artemisinins, however the available ACTs are designed to compensate for its incomplete antiplasmodial effect. The combinations aimed to synergize the action of artemisinins are not yet available and this opportunity could assist in counteracting the emerging resistance to artemisinin.

The aim of this study is to propose new antimalarial combinations utilising Syk inhibitors and artemisinins (ARTs). The in vitro experiments showed great synergy between all the tested Syk inhibitors and artemisinins, especially between P505-15 and DHA with a combination index of 0.42 at 24 hours of incubation. In fact, Syk inhibitors enhance artemisinins’ anti-plasmodial activity leading to a 3-5 fold decrease of their IC₅₀ values. Furthermore we investigated the mechanism of action between Syk inhibitors and artemisinins in P. falciparum strains.

The mechanism of action of artemisinins is basically unknown but its efficacy is known to rely on the availability of reactive iron which is needed to generate a pharmacologically active radical. Erythrocytes appear to be ideal candidates for the activation of artemisinin because of the exceptionally high iron content. However, no free iron is practically available in erythrocytes and the iron caged in hemoglobin is not useful for catalyzing the artemisinin activation. It has already been described that hemichromes are spontaneously formed in parasitized erythrocytes and band 3 becomes heavily phosphorylated from the earlier stages of parasite development. It is plausible that denatured forms of hemoglobin that are formed in parasitized erythrocytes may provide the reactive iron required for artemisinin activation, but direct evidence is unavailable.

In the present study, we observed that Syk inhibitors determine a massive accumulation of hemichromes in the parasitized erythrocytes. Hemichromes may be responsible for the activation of artemisinin after reduction to Fe²⁺ (artemisinin being insensitive to FeCl₃ or hemichromes in absence of RBC). Hence, an accumulation of Fe³⁺ via accumulation of hemichromes induced by Syk inhibitors would potentiate the artemisinin activity.

In conclusion, considering their demonstrated tolerability, Syk inhibitors represent a new class of antimalarial drugs that possess a unique mechanism of action on a non-parasite target. Furthermore,
should not lead to the selection of resistant strains and may therefore represent a strategic partner drug for artemisinin therapies for counteracting artemisinin resistance.
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ABSTRACT

The primary objective of malaria treatment is to eradicate the disease, which involves rapid and complete elimination of the *Plasmodium* parasite from the patients’ blood. Artemisinin combination therapies (ACTs) are the mainstay of recommended treatment for *P. falciparum* malaria and as no alternatives to artemisinin derivatives are expected to enter the market for several years, their efficacy must be preserved. Unfortunately, the resistance to ACTs, is a major thread for the control and elimination of malaria due to *P. falciparum*. The need for new antimalarial drugs is more urgent than ever before, with emerging strains of the parasite now showing resistance against the best available drugs. Recently it has been demonstrated that Syk inhibitors represent a new class of antimalarial drugs which suppress merozoite egress by inhibiting the host target, a factor that cannot be mutated by the parasite to evolve drug resistance. In this study, our aim is to evaluate the *in vitro* combination activity of Syk inhibitors with artemisinins and understand their mechanistic interaction. The results showed a high synergistic combination activity between all tested Syk inhibitors with artemisinins against *P. falciparum*. The combination index analysis indicated the following Syk inhibitors as the most synergistic with Artemisinins: P505-15, R406 and Imatinib. Furthermore, we observed that Syk inhibitors produce a massive accumulation of hemichromes in the parasitized erythrocytes, this in turn triggers the activation of artemisinins and causes a marked synergistic effect when administered in combination. The synergic effect of Syk inhibitors is based on a novel mechanism of action acting selectively on the parasitized erythrocytes which allows the artemisinins to be activated quicker and more efficiently. The concentrations of Syk inhibitors needed to synergize artemisinins is 5-20 fold lower than the IC$_{50}$ measured as a direct effect on parasite growth. These concentrations correspond to very well tolerated dosages and can be rapidly reached after oral administration. In conclusion, our results potentially suggest Syk inhibitors as a strategic class of partner drugs for a new ACT.
INTRODUCTION

1.1. Chapter Synopsis

The chapter begins with a brief background on the discovery and the history of Malaria, the lifecycle of Plasmodium, their pathophysiology and the effects of the disease. In addition, the oxidative alterations in host induced by Plasmodium are going to be presented.

Since the objective of this thesis is the proposal of new antimalarial combinations, it's important to analyze the principle antimalarials of this decade and the reason why WHO chose to use them in combination. Furthermore, the factors of the antimalarial drug resistance and specifically the artemisinin resistance are going to be described.

Afterwards, an overview of the RBC modifications in pro-oxidant mutations (Band 3 tyrosine phosphorylation and Hemichromes release) will be discussed. Also, the methodologies for double and triple combinations will be analyzed. At the end of the chapter, Syk inhibitors as new potential antimalarial drugs and their hypothetical mechanism of action in parasitized red blood cells will be presented.
1.2. Background of Malaria

Malaria or a disease resembling malaria has been noted for more than 4,000 years. From the Italian for "bad air," mal'aria has probably influenced to a great extent human populations and human history. The early Greeks were also aware of the characteristic poor health and malarial fevers seen in people living in waterlogged places. Nowadays, Malaria remains one of the most important parasitic diseases. In 2016 were estimated 429 000 malaria deaths (range 235 000 – 639 000) worldwide. Most of these deaths occurred in the African Region (91.8%), followed by the South-East Asia Region (6.1%) and by the eastern Mediterranean region (2.1%)². Children under 5 are particularly susceptible to malaria illness, infection and death. In 2015, malaria killed an estimated 303 000 under-fives globally, including 292 000 in the African Region. Between 2010 and 2015, the malaria mortality rate among children under 5 fell by an estimated 35%. Nevertheless, malaria remains a major killer of under-fives, claiming the life of 1 child every 2 minutes. Specifically, malaria is a disease caused by Plasmodium spp, which are microscopic protozoa of the phylum Apicomplexa. P. falciparum, P. vivax, P. malariae and P. ovale are the human malaria species. Among the four species, Plasmodium falciparum represents the most pathogenic³ with the highest rates of complications and mortality⁴. Next chapters are going to describe the life cycle, Pathophysiology and Diagnosis of Malaria.
1.2.1. Life cycle of Malaria parasites

The life cycle of Plasmodium (Fig.3) is similar to several other species in the Haemosporidia. All the Plasmodium species causing malaria in humans are transmitted by mosquito species of the genus Anopheles (Fig.1). The infection starts when an infected mosquito releases sporozoites into the skin of a human during a blood meal. The sporozoites travel to the liver, where they enter hepatocytes and subsequently divide for approximately 10 days, after which they differentiate into merozoites. At the end of their developmental process in the liver, Plasmodium parasites differentiate into infective merozoites. The life cycle of Plasmodium (Fig.3) is similar to several other species in the Haemosporidia. All the Plasmodium species causing malaria in humans are transmitted by mosquito species of the genus Anopheles (Fig.1). The infection starts when an infected mosquito releases sporozoites into the skin of a human during a blood meal. The sporozoites travel to the liver, where they enter hepatocytes and subsequently divide for approximately 10 days, after which they differentiate into merozoites. At the end of their developmental process in the liver, Plasmodium parasites differentiate into infective merozoites. 

Eventually, these liver merozoites are released into the lung capillaries, where they reach the bloodstream and begins the blood-stage of infection. Inside erythrocytes (Fig.2), the parasite undergoes a 48-hour-long developmental process that starts with the ring stage (0–24 hours), followed by DNA replication and parasite growth during the trophozoite stage (24–36 hours) and, ultimately, the schizont stage (36–48 hours), during which infectious merozoites are formed. A small percentage of the parasites undergo differentiation into gametocytes (not shown), which can be taken up by a mosquito during a blood meal and can then mate inside the mosquito gut, export of parasite proteins also occurs at this stage. During the intracellular stages in the vertebrate host, the parasite actively remodels the host erythrocyte. An outline of the export pathway of a protein is shown, starting with secretion through the secretory pathway (initiating in the endoplasmic reticulum) into the parasitophorous vacuole (PV). Transmembrane domain-containing proteins may
be transported through vesicle-like structures, exported proteins, including Plasmodium falciparum erythrocyte membrane protein 1 (PfeMP1), are part of the knobs and the Maurer’s clefts.

1.2.2. Pathophysiology and Diagnosis of Malaria

Figure 3. Life cycle of Malaria parasite at Mosquito, Human liver and Blood stages. (CDC)
Malaria is caused by the direct effects of parasitized red cells and destruction by the asexual parasites, and the host’s reaction to this process. In *P. falciparum* blood-stage infections, protuberances appear on the surface of the erythrocyte 12–15 hours after cellular invasion. These membrane «knobs» extrude a high molecular weight, strain-specific adhesive protein (PfEMP1) that mediates cytoadherence, or attachment of the parasitized erythrocyte to endothelial surface receptors in veins and capillaries, resulting in the sequestration of parasitized erythrocytes in these vessels. Under febrile conditions, which enhance PfEMP1 expression, cytoadhesion begins at approximately 12h of parasite development. Febrile temperatures induce cytoadherence of ring-stage *Plasmodium falciparum*-infected erythrocytes.

Once adherent, the infected red cells do not detach until schizont rupture. There is some ring-stage adherence via separate mechanisms. The infected erythrocytes adhere to the vessel walls and eventually cause heterogeneous blockage of the microcirculation. Parasitized erythrocytes may also adhere to each other (agglutination) and to uninfected erythrocytes (rosetting). These adherence processes are central to the pathogenesis of falciparum malaria, resulting in the sequestration of red cells containing mature parasites in vital organs (including the brain), where they interfere with microcirculatory flow and metabolism and the function of vascular endothelium. Only the younger ring form *P. falciparum* parasites circulate in falciparum malaria, and so the peripheral parasite count may substantially underestimate the actual total parasite biomass. As the intraerythrocytic parasites mature, they make the infected red cells more spherical and rigid.

1.2.2.1. Diagnosis of uncomplicated Malaria

The signs and symptoms of malaria are non-specific. Malaria is suspected clinically primarily on the basis of fever or a history of fever. No combination of signs or symptoms that reliably distinguishes malaria from other causes of fever exists. In this way, diagnosis based only on clinical features has a low specificity and results in overtreatment. The focus of malaria diagnosis should be to identify patients truly suffering from malaria, to guide rational use of antimalarial medicines. In malaria-endemic areas, malaria should be suspected in any patient presenting with a history of fever or temperature 37.5 °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 very low, parasitological diagnosis of all cases of fever may result in considerable expenditure to detect only a few patients with malaria. In these settings, health workers should be trained to identify patients who may have been exposed to malaria (e.g. recent travel to a malaria-endemic area without protective measures) and have fever or a history of fever with no other obvious cause, before they conduct a parasitological test.
In all settings, suspected malaria should be confirmed with a parasitological test. The results of parasitological diagnosis should be available within a short time (< 2 h) of the patient presenting. In settings where parasitological diagnosis is not possible, a decision to provide antimalarial treatment must be based on the probability that the illness is malaria. The benefit of parasitological diagnosis relies entirely on an appropriate management response of health care providers. Light microscopy and immunochromatographic RDTs are the two methods used routinely for parasitological diagnosis of malaria. Both must be supported by a quality assurance program. Antimalarial treatment should be limited to cases with positive tests, and patients with negative results should be reassessed for other common causes of fever and treated appropriately. In almost all cases of symptomatic malaria, examination of thick and thin blood films by a competent microscopist will reveal malaria parasites. Malaria RDTs should be used if quality-assured malaria microscopy is not readily available. RDTs for detecting PfHRP2 can be useful for patients who have received incomplete antimalarial treatment, in whom blood films can be negative. This is particularly likely if the patient received a recent dose of an artemisinin derivative. If the initial blood film examination is negative in patients with manifestations compatible with severe malaria, a series of blood films should be examined at 6-12h intervals, or an RDT (preferably one detecting PfHRP2) should be performed. If both the slide examination and the RDT results are negative, malaria is extremely unlikely, and other causes of the illness should be sought and treated. In patients with suspected severe malaria and in other high-risk groups, such as patients living with HIV/AIDS, absence or delay of parasitological diagnosis should not delay an immediate start of antimalarial treatment. At present, molecular diagnostic tools based on nucleic-acid amplification techniques (e.g. loop-mediated isothermal amplification or PCR) do not have a role in the clinical management of malaria. Where P. vivax malaria is common and microscopy is not available, it is recommended that a combination RDT be used that allows detection of P. vivax (pLDH antigen from P. vivax) or pan-malarial antigens (Pan-pLDH or aldolase).
1.2.2.2. Diagnosis of severe Malaria

Severe malaria is clinically similar to other severe febrile illnesses. Each clinical syndrome of severe malaria (coma, severe anaemia, acidosis) can have other causes (meningitis, sickle cell disease, septicaemia). Obtaining a parasitological diagnosis does not resolve the diagnostic problem, especially in high transmission areas, where asymptomatic parasitemia is common and may be incidental in any severe illnesses\(^\text{10}\).

Overdiagnosis constitutes another possibility in countries where malaria is common, diverting attention from other infectious causes of severe febrile illness. Overdiagnosis of malaria, which is commonly due to neglect of a negative blood film or to failure to obtain a blood film or rapid diagnostic test (RDT), has been shown to contribute to mortality. 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. Yet, it should be started based on a clinical suspicion while other diagnoses are also considered. Microscopy remains the reference standard for the diagnosis of falciparum malaria, but this requires the availability of a good microscope, significant technical skills, good-quality reagents and clean slides. The diagnostic quality of microscopy is very variable in routine hospitals in sub-Saharan Africa\(^\text{11}\).

1.2.3. Oxidative alterations in the host induced by Plasmodium

It has been shown that oxidative stress is related to a protective role in malaria patients, as possible agents capable of destroying the Plasmodium. Thus, H_2O_2 and O_2•− can operate independently as cytotoxic agents or form other toxic molecules, including radical OH•, hypochlorous acid (HOCl) and peroxynitrite (ONOO−) in the presence of NO. In fact, it is generally accepted that ROS, including O_2•− and ONOO−, can destroy the parasite intraerythrocytically. Specifically, in response to infection caused by Plasmodium parasites, the natural host defense mechanism is activated with the involvement of phagocytes (macrophages and neutrophils). These, in turn, generate large amounts of ROS and RNS, causing an imbalance between the formation of oxidizing species and the activity of antioxidants. This imbalance is what triggers oxidative stress, which is an important
mechanism of human hosts in response to infections and, in the case of malaria, can lead to the death of the parasites.

In vitro studies have demonstrated the ability of oxidative stress to promote the killing of parasites. Incubation of *Plasmodium yoelii* species in the presence of glucose and glucose oxidase-generated H2O2, a reactive oxygen species, capable of killing the parasite. Likewise, when incubated in the presence of xanthine and xanthine oxidase, it generated free radical superoxide (O2•⁻) and a subsequent burst of other oxidative products, with consequent destruction of parasites.12

Finally, there are biomarkers to indicate oxidative stress in malaria, as malondialdehyde (MDA) a product of lipid peroxidation. Other biomarkers of oxidative stress include human SOD-1 (Cu/Zn-SOD), TNF-a, catalase and glutathione peroxidase, while C-reactive protein has been related to severe malaria in children12-14.

### 1.2.3.1. Haemoglobin metabolism of *P.falciparum*

During intra-erythrocytic development, *P.falciparum* ingests a large amount of hemoglobin to meet its nutrient requirement15 and to maintain osmotic stability within the host cell. Specifically, the malaria parasite ingests 25 to 80% of total hemoglobin content16. Haemoglobin molecules, taken up by endocytosis undergo hydrolysis in the parasite’s digestive acidic vacuole called the food vacuole. Cysteine and aspartic proteases are involved in hemoglobin proteolysis and have reported pH optimums in the range of 4.5-5.016. A pH homeostasis plays an important role in the pathophysiology of falciparum malaria, such as host cell exploitation and responses to antimalarial drugs. Accordingly, baseline pH values and the mechanisms underpinning pH homeostasis in different parasite compartments have been of interest for several decades17.

Endogenous production of reactive oxygen species (ROS) in parasitized erythrocytes are triggered following the digestion of haemoglobin and subsequent biochemical reactions in the parasites. Haemoglobin that is taken up by the parasites into their acid food vacuole leads to the spontaneous oxidation of haem iron from Fe²⁺ to Fe³⁺ (haemin) and the formation of superoxide radicals (O₂•⁻). The combination of O₂•⁻ and haemin inevitably leads to the generation of hydrogen peroxide (H₂O₂) and subsequently, hydroxyl radicals (•OH), which are highly reactive and cytotoxic oxygen intermediates18. Furthermore, toxic (ferroporphyrin IX; containing Fe²⁺) and hemin/hematin (ferriporphyrin IX; containing Fe³⁺) (FPIX) that are released upon haemoglobin digestion must be detoxified within the acid food vacuole to prevent downstream toxicity. Most of the released FPIX is biomineralized (up to 90%); to form inert hemozoin. However, there are reports that 18...
substantial amount of FPIX (even as much as 50%) escapes bio-mineralization and has to be degraded or sequestered by other means to prevent membrane damage and parasite death.

However, some free FPIX (up to 50% according to from the food vacuole pass into the parasite compartment. The $\text{O}_2$ $^\cdot$− resulting from the oxidation of haem-iron of haemoglobin are either detoxified by superoxide dismutase (SOD) to yield $\text{H}_2\text{O}_2$ or in a spontaneous reaction with $\text{H}_2\text{O}_2$, lead to the formation of $^\cdot$−OH. In addition, ferric iron ($\text{Fe}^{3+}$) reacts with molecular oxygen via the Fenton reaction pathways to generate $^\cdot$−OH$^{13}$ (Equation 1)$^{18}$.

$$\begin{align*}
\text{O}_2 + \text{Fe}^{3+} & \rightarrow \text{O}_2 + \text{Fe}^{2+} \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{OH} + \cdot\text{OH}
\end{align*}$$

Equation 1: The Fenton reaction

These radicals are highly reactive and cause, for instance, lipid peroxidation and DNA oxidative damage. Additionally, $\text{H}_2\text{O}_2$ generated by the SOD activity has to be detoxified by reduction reaction to produce water. In *P. falciparum*, thioredoxin (Trx)-dependent peroxidase pathway plays this role because the parasites lack catalase and glutathione peroxidase$^{19}$, which initially, raised doubts about the relevance of reduced glutathione (GSH) in detoxification of ROS in Plasmodium.
1.3. Treatment of Malaria

World Health Organization (WHO) every year is 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 anaemia. From a public health perspective, treatment is meant to reduce transmission of the infection to others, by reducing the infectious reservoir and by preventing the emergence and spread of resistance to antimalarial medicines\(^2\).

<table>
<thead>
<tr>
<th>Drug class</th>
<th>Example drugs</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemisinins (or artemisinin</td>
<td>Artesunate,</td>
<td>The most widely used of the anti-malarial drugs with very short half-lives.</td>
</tr>
<tr>
<td>derivatives)</td>
<td>artemether and</td>
<td>These are sub-curative in standard 3 day regimens if used as monotherapies</td>
</tr>
<tr>
<td></td>
<td>dihydroartemisinin</td>
<td></td>
</tr>
<tr>
<td>Antifolates</td>
<td>Pyrimethamine,</td>
<td>The combination sulphadoxine-pyrimethamine (SP; also known by its trade name</td>
</tr>
<tr>
<td></td>
<td>chlorproguanil,</td>
<td>'Fansidar') is widely used for therapy. Both constituents have long half-lives so it</td>
</tr>
<tr>
<td></td>
<td>proguanil,</td>
<td>was given as a single-dose regimen but resistance quickly evolved. Its use is now</td>
</tr>
<tr>
<td></td>
<td>sulphadoxine and</td>
<td>primarily restricted to treatment/prophylaxis in intermittent treatment</td>
</tr>
<tr>
<td></td>
<td>dapsone</td>
<td>programmes</td>
</tr>
<tr>
<td>4-aminoquinolines</td>
<td>Chloroquine,</td>
<td>Chloroquine was used in huge quantities as a monotherapy for over 30 years.</td>
</tr>
<tr>
<td></td>
<td>amodiaquine,</td>
<td>Resistance occurred only infrequently and Africa never developed its own</td>
</tr>
<tr>
<td></td>
<td>piperazine,</td>
<td>resistance instead it was aquired by immigrations from South-East Asia[^42].</td>
</tr>
<tr>
<td></td>
<td>pyronaridine and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>naphthoquine</td>
<td></td>
</tr>
<tr>
<td>Arylamino-alcohols</td>
<td>Quinine, mefloquine,</td>
<td>Quinine was the first anti-malarial to be identified. A long treatment duration</td>
</tr>
<tr>
<td></td>
<td>lumefantrine and</td>
<td>and its safety profile means it is now mainly used in early pregnancy or as a</td>
</tr>
<tr>
<td></td>
<td>halofantrine</td>
<td>(parenteral) second-line treatment either alone or in combination in</td>
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<tr>
<td></td>
<td></td>
<td>uncomplicated or severe malaria. Lumefantrine with artemether is currently the</td>
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<tr>
<td></td>
<td></td>
<td>most widely used anti-malarial combination therapy; it has low-level antagonistic</td>
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<tr>
<td></td>
<td></td>
<td>resistance with chloroquine[^43].</td>
</tr>
</tbody>
</table>

Table 1. Drug class of antimalarial drugs

Core Principles

The following are the core principles for the treatment of Malaria according to the latest update of WHO\[^20\]:

- 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, programmes should ensure access to early diagnosis and prompt, effective treatment within 24-48 hours of the onset of Malaria 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
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

Duration of ACT treatment
ACT regimens should provide 3 days treatment with an artemisinin derivative.

Revised dose recommendation for Dihydroartemisinin + piperaquine in young children
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.
Reducing the transmissibility of treated *P.falciparum* infections

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 or P. knowlesi* malaria Blood stage infection**

If the malaria species is not known with certainty, treat as for uncomplicated *P.falciparum* malaria. *Good practice statement*

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 malaria (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.

1.3.1. Quinine and its synthetic derivatives

The first class of compounds used for the successful treatment of malaria and drugs of choice for the present time were Aminoquinolines derivatives. The first attempt of successful treatment of malaria was made in the 18th century by using the bark of cinchona trees. Gomes et al. in 1810 extracted the cinchona bark, but only after a decade an active ingredient of quinine was isolated and made Malaria the first disease for which a pure compound was used for the treatment. The structure elucidation and different synthetic routes have come up in near 19th century.

In 1856, chemist William Henry Perkins, set out to synthesize quinine. His efforts resulted in the first synthetic textile dye called “mauve” and not in quinine. Paul Ehrlich noticed that methylene blue was particularly effective in staining malaria parasites. He rationalized that this dye
might also be selectively toxic to the parasite. In 1891, Ehrlich and Guttmann cured two malaria patients with methylene blue, which became the first synthetic drug ever used in therapy. Thus, methylene blue constituted the basis for the development of synthetic antimalarials, even it has not been used further at that time.

![Chemical structure of Methylene Blue](image)

Mode of action of aminoquinoline classes of compounds is still a matter of debate despite the overwhelming importance. Various theories have been proposed and reviewed and they all agree that CQ interacts with the parasite’s ability to digest hemoglobin. During its erythrocytic stages, the parasite consumes large quantities of hemoglobin from its host cell, either for the purpose of amino acid supply, or simply to create space inside the erythrocyte. Hemoglobin is shuttled by vesicles to a specialized organelle called digestive vacuole (DV). Early biochemical studies demonstrated that CQ was able to inhibit DNA and RNA synthesis. However, the interaction of CQ with DNA does not explain the antimalarial activity and the selective toxicity of this compound.

The mechanism of action of CQ is active only against the erythrocytic stages of malaria parasites. During the feeding process of the parasites, CQ was able to inhibit the hemoglobin degradation. The 4-aminoquinoline derived drugs have been proposed that selectively target the hemoglobin degradation which is specific to parasites.

![Chemical structures of Aminoquinolines](image)

Figure 5. Chemical structure of Methylene Blue

Figure 6. Chemical structure of Aminoquinolines
Aminoquinoline derived drugs (Fig. 6) are known to inhibit the hematin formation by complexing with ferriprotoporphorin IX (FPIX) thereby prevents its polymerization into hemozoin, which results in parasite death. Most NMR and molecular modeling studies\textsuperscript{23} show a face-to-face $\pi$ staggering of the porphyrin and quinoline systems, although a structure showing an edge-to-face complex with the ring nitrogen atom sitting above the ring iron center has also been reported. Very recently, structure determination by NMR spectroscopy showed CQ sitting in a central position over the outermost porphyrin rings of a FPIX–CQ 4:2 complex. Most researchers assume that the buildup of noncrystalline FPIX, either in its free form or as a FPIX–CQ complex, finally kills the parasite. Considerable evidence has accumulated in recent years that antimalarial drugs such as CQ act by forming complexes with FP, the hydroxo or aqua complex of Ferriprotoporphyrin IX (Fe(III) FP), derived from parasite proteolysis of host haemoglobin. Remarkable data supports the hypothesis that hematin is the target of 4-aminoquinoline class of compounds. 4-AQ are weak bases and are expected to accumulate in an acidic food vacuole to many folds\textsuperscript{23}.

1.3.2. Artemisinin and its synthetic derivatives

During the latter part of the Vietnam War, in the late 1960s to early 1970s, malarial infections, combined with drug resistance to common anti-malarial drugs, resulted in huge losses of military personnel on both combating sides. Responding to a request by the North Vietnamese, the Chinese government engaged in a national effort involving more than 50 institutes to develop improved anti-malarial drugs\textsuperscript{24}.

The most important discovery of these efforts is Artemisinin (ART). Artemisinin also called qinghaosu, is derived from the sweet wormwood plant, Artemisia annua (Fig. 7). Sweet wormwood has been used by the Chinese as a herbal medicine for centuries and the drug was brought into modern medicine by Tu Youyou, who was awarded the Nobel prize for Medicine in 2015. Artemisinin is a sesquiterpene lactone with an endoperoxide bridge (essential for its action). After the initial discovery of ART, medicinal chemists made great strides in improving the efficacy of 24
ART. A set of ART derivatives including dihydroartemisinin (DHA), artemether, artesunate and arteether, all modified at the C10 position, were produced (Fig. 8). These ART derivatives, together with the ART prototype, are collectively called artemisinins (ARTs).

Second generation derivatives, which structurally deviate much more from the ARTs but all contain the crucial endoperoxide bridge, were later developed. Although original applications of ARTs were reported in anti-malarial treatment, activities against cancer, viruses and other parasites such as schistosoma, clonorchis, Toxoplasma and Leishmania have now also been documented. Therefore, it appears ARTs possess inhibitory activities against an array of different maladies\(^{24}\).

**Figure 8.**: Artemisinin (ART) and some of its derivatives. The endoperoxide bond constitutes the pharmacophore for the action of ARTs. Direct ART derivatives are usually modifiers of ART at the C10 position. Some other endoperoxides differ greatly structurally but also manifest potent anti-malarial activities.

1 Artemisinin. 2a Dihydroartemisinin (DHA). 2b Artemether. 2c Arteether. 2d Artesunate. 3, 4 An analogue with close structure to artemisinin. 5a, 5b Enantiomers with similar activities against malaria parasites. 6 An antimalarial tetraoxane. 7 OZ439 (in clinical trial).
1.3.2.1. Mechanism of action of artemisinin, different Hypothesis

Understanding the mode of action of Artemisinin is important for designing artemisinin derivatives with better antiparasitic activity and predicting mechanisms of resistance. Despite tremendous research efforts on artemisinin since its discovery, there is still considerable debate about the mode of action on malaria parasites. Over the years a lot of researchers tried to understand the mode of action of Artemisinin and four are the main hypotheses.  

- The C-radical Hypothesis  
- The Heme Hypothesis  
- The SERCA Hypothesis  
- The Co-factor Hypothesis

Main Hypothesis
Artemisinins are considered prodrugs that are activated to generate carbon-centered free radicals or reactive oxygen species (ROS). As the O-centered radical formed upon cleavage is unable to oxidatively cycle, ROS is less likely important for the action of artemisinins. Because Fe$^{2+}$ is a catalyst that can generate free radicals from peroxides and large amounts of iron accumulate in the food vacuole, hemoglobin, heme, and free iron has been studied as key molecules participating in the initial artemisinin interaction and cleavage event.
‘The C-radical Hypothesis’
Fe2+ induces reductive cleavage of the peroxide, referred to generally as the ‘Fenton reaction’ to form O-radicals that in turn interact with hydrocarbon scaffold encapsulating the peroxide to generate C-radicals. **Those radicals are held to be the toxic agents for parasites.** In any case, the nature of the target, and whether indeed an intracellular alkylation takes place, remain undefined. By definition, as spin unpairing in the generation of the radical has taken place, a penultimate step in the hypothetical scheme requires an electron transfer, presumably from whatever the radical product is, back to the initiating metal such that a caution is produced. The caution may be quenched by a nucleophile such as water or endogenous thiol. Alternatively, an endogenous redox-active substrate, such as the reduced conjugate of a flavin, may donate an electron to generate an incipient carbanion that may me protonated. Alternatively, dioxygen in principle may form a peroxo radical that may undergo further SET to generate a hydroperoxide. In any event, such downstream events have not been considered within the framework of this hypothesis27.

‘The Heme Hypothesis’
Heme has been intensively studied as an 'activator' of artemisinins and other antimalarial peroxides, and indeed the hypothesis seemingly has become firmly embedded in the underlying brickwork of the scientific edifice. The locus of activity of the peroxides interacting with the heme is considered to be the parasite digestive vacuole. The basis for the nanomolar activities of artemisinins and synthetic peroxides is variously ascribed to heme-Fe2+-mediated generation of C-radicals from the peroxides, formation of heme-artemisinin adducts that are held either to engage in redox cycling with concomitant **generation of ROS** or to inhibit the formation of hemozoin. In the last case, just like the aminooquinolines and arylmethanols, the peroxides are not the active agents, but exert their parasitical effects through allowing the build-up of free heme-Fe3+, the ultimate cytotoxic entity27. Wang and his group published in nature communication (2015) that artemisinin activation is mainly heme dependent, which they believe resolve previous arguments regarding the source of iron required for the drug activation. Artemisinin activation at the early ring stage seems to rely on parasite’s heme biosynthesis, whereas drug activation depends on hemoglobin digestion as the main heme source at the latter parasite stages, which determines the high specificity of the drug towards the parasite13.
‘The SERCA Hypothesis’
Modulation by artemisinin and synthetic peroxides of operation of the Plasmodium falciparum sarco-endoplasmic reticulum calcium pump SERCA Ca\(^{2+}\)-ATPase ATP6 is held to occur by binding of the artemisinin or synthetic peroxide into a cleft on the cytosolic side of transporter. However, it is important to recognize that Fe\(^{2+}\) is regarded as essential pre-requisite for this mechanism that is, the bound peroxide is ‘‘fixed’’ covalently by an SET reaction with Fe\(^{2+}\)\(^{27}\).

‘The Co-factor Hypothesis’
Artemisinin and its synthetic peroxides rapidly oxidize reduced conjugates of flavin cofactors, or those of flavin cofactor precursors such as riboflavin, and other similarly susceptible endogenous substrates that play a crucial role in maintaining intraparasitic redox homeostasis. Given that the means by which the parasite is able to control the generation of cytotoxic reactive oxygen species (ROS) is suppressed, the consequence will be rapid build-up of ROS. Overall, redox homeostasis is overwhelmed. This proposal differs fundamentally to the other three in that the peroxide overall acts as a two-electron oxidant, in common with a vast family of related reactions involving peroxides. So in this sense, artemisinins and synthetic peroxides are considered to act as oxidant drugs. Iron does not react with the peroxide, but its presence markedly, not least because its redox cycling contributes to generation of ROS, and effects the hezose monophosphate shunt involved in the generation of NADPH\(^{27}\).
1.3.3. Artemisinin based combination therapies (ACTs)

Artemisinin-based combination therapy (Table 2) is a combination of a rapidly acting artemisinin derivative with a longer-acting partner drug. The artemisinin component rapidly clears parasites from the blood (reducing parasite numbers by a factor of approximately 10 000 in each 48-h asexual cycle) and is also active against the sexual stages of parasites that mediate onward transmission to mosquitoes.

The longer-acting partner drug clears the remaining parasites and provides protection against development of resistance to the artemisinin derivative. Partner drugs with longer elimination half-lives also provide a period of post-treatment prophylaxis.\textsuperscript{2,28} The basic ACTs are presented below and are the following: Artemether + Lumefantrine, artesunate + Amodiaquine, artesunate + mefloquine, Dihydroartemisinin + piperaquine and artesunate + sulfadoxine-pyrimethamine\textsuperscript{20}.

**Dosing of ACTs**

ACTs regimens must ensure optimal dosing to prolong their useful therapeutic life in order to maximize the likelihood of rapid clinical and parasitological cure, minimize transmission and retard drug resistance. It is essential to achieve effective antimalarial drug concentration for a sufficient time (exposure) in all target populations in order to ensure high cure rates. The dosage recommendations below are derived from understanding the relationship between dosage and the profiles of exposure to the drug (pharmacokinetics) and resulting therapeutic efficacy (pharmacodynamics) and safety. Some patient groups, notably younger children, are not dosed optimally with the ‘dosage regimens’ recommended by manufacturers, which compromises efficacy and fuels resistance. In these guidelines when there was pharmacological evidence that certain patient groups are not receiving optimal doses, dose regimens were adjusted to ensure similar exposure across all patient groups.
Weight-based dosage recommendations are summarized below. While age-based dosing may be more practical in children, the relation between age and weight differs in different populations. Age-based dosing can therefore result in under-dosing of some patients, unless large, region-specific weight-for-age databases are available to guide dosing in that region\textsuperscript{20}.

**Artemether + Lumefantrine**
Comments: An advantage of this ACT is that Lumefantrine is not available as monotherapy and has never been used alone for the treatment of malaria. Absorption of Lumefantrine is enhanced by co-administration with fat. Patients or caregivers should be informed that this ACT should be taken immediately after food or fat containing drink, particularly on the second and third days of treatment\textsuperscript{20}.

**Artesunate + Amodiaquine**
Comments: No significant changes in the pharmacokinetics of Amodiaquine or its metabolite desethylamodiaquine have been observed during the second and third trimesters of pregnancy; therefore, no dosage adjustments are recommended. No effect of age has been observed on the plasma concentration of Amodiaquine, so no dose adjustment by age is indicated. Few data are available on the pharmacokinetics of Amodiaquine in the first year of life\textsuperscript{20}.

**Artesunate + mefloquine**
Comments: Mefloquine was associated with increased incidences of nausea, vomiting, dizziness, dysphoria and sleep disturbance in clinical trials, but these symptoms are seldom debilitating, and, where this ACT has been used, it has generally been well tolerated. To reduce acute vomiting and optimize absorption, the total mefloquine dose should preferably be split over 3 days, as in current fixed-dose combinations.

**Artesunate + sulfadoxine-pyrimethamine**
Comments: The disadvantage of this ACT is that it is not available as a fixed-dose combination. This may compromise adherence and increase the risk of contribution of loose artesunate tablets, despite the WHO ban on artesunate monotherapy. Resistance is likely to increase with continued widespread use of Sulfacline-pyrimethamine and cotrimoxazole. Fortunately, molecular markers of resistance to antifols and sulfonamides correlate well with therapeutic responses. These should be monitored in areas in which this drug is used\textsuperscript{20}.
**Dihydroartemisinin + piperaquine**

Comments: Piperaquine (PQ) prolongs the QT interval by approximately the same amount as chloroquine but by less than quinine. It is not necessary to perform an electrocardiogram before prescribing Dihydroartemisinin + piperaquine, but this ACT should not be used in patients with congenital QT prolongation or who have a clinical condition or are on medication that prolong the QT interval.
Proportion of antimalarial treatments from 2010-2015

Regarding the latest report (2016) of WHO\textsuperscript{2}, based on nationally representative household surveys, the proportion of antimalarial treatments that are ACTs (for children with both a fever in the previous 2 weeks and a positive RDT at the time of survey) increased from a median of 29\% in 2010–2012 (IQR: 17–55\%) to 80\% in 2013–2015 (IQR: 29–95\%) (Fig.10). However, the ranges associated with the medians are wide, indicating large variation between countries, and the number of household surveys covering anyone 3-year period is comparatively small. Antimalarial treatments are more likely to be ACTs if children seek treatment at public health facilities or via community health workers than if they seek treatment in the private sector\textsuperscript{20}.

1.3.3.1. ACTs not currently recommended for general use

According to the latest guideline for the treatment of malaria of WHO for Malaria, the pipeline for new antimalarial drugs is healthier than ever before, and several new compounds are in various stages of development. Some of them are still in the pre-registration phase and are not discussed in the Malaria report. The following are some novel antimalarial combinations registered in some countries.

- **Artesunate + Pyronaridine**
- **Arterolane + piperaquine**
- **Artemisinin + naphtoquine**

A systematic review of **artesunate + Pyronaridine** included six trials with a total of 3718 patients. The mentioned combination showed good efficacy as compared with Artemether + Lumefantrine and artesunate + mefloquine in adults and older children with P.falciparum malaria, but the current
evidence for young children is insufficient to be confident that the drug is as effective as currently recommended options. Further studies to characterize the risk for hepatotoxicity I needed. Preliminary data from repeat-dosing studies are reassuring.

Arterolane + piperaquine is a combination of a synthetic ozonide and piperaquine phosphate that is registered in India for use only in adults. There are general insufficient data to make recommendations.

Artemisinin + naphtoquine is also a combination of two relatively old compounds that are currently being promoted as a single dose regimen, contrary to WHO advise for 3 days of the artemisinin derivative. There are currently insufficient data from rigorously conducted randomized controlled trials to make general recommendations.20

1.3.4. Antimalarial drug resistance

The exact definition of an antimalarial drug resistance is the ability of parasite strain to survive and/or multiply despite administration and absorption of an antimalarial drug given in doses equal to or higher than those usually recommended, provided the drug exposure is adequate. Resistance to antimalarial drugs arises because selection of parasites with genetic changes (mutations or gene amplifications) that confer reduced susceptibility.

Unfortunately, resistance has been documented to all classes of antimalarial medicines, including the artemisinin derivatives, which is the major threat to malaria control. Widespread inappropriate use of antimalarial drugs exerts a strong selective pressure on malaria parasites to develop high levels of resistance. It can be prevented or its onset slowed considerably by combining antimalarial drugs with different mechanisms of action and ensuring high cure rates through full adherence to correct dose regimens. If different drugs with different mechanisms of resistance are used together, the emergence and spread of resistance should be slowed28,29.

Clinical and parasitological assessment of therapeutic efficacy should include:

- Confirmation of the quality of the antimalarial medicines tested;
- Molecular genotyping to distinguish between re-infections and recrudescence and to identify genetic markers of drug resistance;
- Studies of parasite susceptibility to antimalarial drugs in culture; and
- Measurement of antimalarial drug levels to assess exposure in cases of slow therapeutic response or treatment failure
Recurrent Falciparum Malaria

Recurrence of *P. falciparum* malaria can result from re-infection or recrudescence (treatment failure). Treatment failure may result from drug resistance or inadequate exposure to the drug due to sub-optimal dosing, poor adherence, vomiting, unusual pharmacokinetics in an individual or substandard medicines. It is important to determine the patient’s history whether he or she vomited the previous treatment or did not complete a full of treatment.

When possible, treatment failure must be confirmed parasitologically. This may require referring the patient to a facility with microscopy or LDH-based RTDs, as *P. falciparum* histidine-rich protein-2 (PfHRP2)-based tests may remain positive for weeks after the initial infection, even without recrudescence. Referral may be necessary anyway to obtain second line-treatment. In individual patients, it may not be possible.

1.3.4.1. Definition of artemisinin resistance

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. 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. There is no evidence that higher levels of artemisinin resistance (full resistance) have emerged.
1.3.5. Antimalarial drugs and Oxidative stress

The pharmacological therapy currently used is based on the susceptibility of the genus *Plasmodium* to free radicals and oxidants, as well as the interference or inhibition of a metabolic synthesis pathway of a molecule essential to the parasite. The ROS and iron byproducts present in the pRBCs, which are rare in healthy RBCs, remain the typical pRBC targets. Aminoquinolines such as chloroquine (CQ) and ARTs are thought to target these redox-active heme byproducts generating an oxidative stress fatal to the parasite. Considering that pRBC are selectively damaged by oxidants, the interest for new oxidant drugs with antimalarial properties began more than 30 years ago\textsuperscript{31,32}.

At this point, it is important to clarify that there are two types of oxidants, some are intrinsically oxidizing (e.g., quinones and quinoid compounds, peroxides), while others do not have apparent redox properties but indirectly induce oxidation reactions by their mechanisms of action (e.g., aminoquinolines). In this view, artemisinin-like endoperoxides are oxidant while the oxidative stress generated by chloroquine is more a downstream event arising from its strong interaction with heme. This effect may be due to the drug’s ability to promote the direct production of free radicals or by inhibiting molecules with antioxidant activity.

1.4. RBC membrane modifications in pro-oxidant mutations

A number of hemolytic disorders are known to damage the RBC membrane increasing the production of free radicals originating from denatured hemoglobin species (hemichromes), invariably present in thalassemia, sickle cell disease or with a decreased ability of RBCs to deal with extracellular oxidants as in G6PD deficiency. It is noteworthy that approximately 7% of world population is affected by those mutations which have been selected by malaria and protect patients from its severe manifestations. The tyrosine phosphorylation of band 3 is increased in hemoglobinopathies even in the absence of high reticulocyte count\textsuperscript{33-37}.

At least two mechanisms might be involved in the increased tyrosine phosphorylation of band 3 in these hemoglobin disorders, probably related to the endogenous reactive oxygen intermediates generated by the abnormal erythrocyte: an inhibition of protein tyrosine phosphatase activity or an
activation of the protein tyrosine kinase p72syk. The mechanisms leading to its phosphorylation and its pathophysiological significance have been partially defined. Recently Ferru and her colleagues described that band 3 phosphorylation appears to be increased in intermediate thalassemia and that this phenomenon is closely related to the formation of hemichromes. Band 3 phosphorylation and hemichromes formation have been also described in malaria-infected RBC\textsuperscript{38}. In both pathological situations, band 3 phosphorylation appears to play a permissive role in the release of membrane microparticles\textsuperscript{37}.

Current knowledge appears to be still insufficient to explain the molecular details of the underlying mechanism, although some recent findings clearly indicate a role of band 3 phosphorylation in the regulation of metabolism mediated by the binding of deoxygenated hemoglobin (Hb) and in the modification of the affinity between band 3 and ankyrin following oxidative stress. In most instances, those mutations do not seem to directly affect the parasite growth but seem to unbalance the complex relationships that undergo between the erythrocytes and their intracellular parasites.

1.4.1. Band 3 tyrosine phosphorylation

Band 3 is the most abundant RBC membrane protein and represents one of the major components of the junctional complexes that connect the lipid bilayer to the cytoskeleton. It has been previously found that the oxidation of two cysteine residues in the band 3 cytoplasmic domain leads to the docking of Syk kinase. RBC appears to possess a mechanism able to recruit Syk kinase to a fraction of less glycosylated band 3 molecules capable of forming disulfide dimers. It has been previously demonstrated that HMC bind to band 3 causing free iron accumulation and free radical production.

RBCs also respond by activating tyrosine kinases determining the tyrosine (Tyr) phosphorylation of band 3, the major linkage between the cytoskeleton and the lipid bilayer. In RBCs, hyperphosphorylation of band 3 has been constantly reported in all the prooxidant hemolytic disorders and in malaria\textsuperscript{39-42}. 
### 1.4.2. Hemichromes (HMC)

![Figure 11. Confocal image of pRBC with hemicromes bounded to the red cell membrane](image)

The autoxidation of Hb (Fe$^{II}$) occurs spontaneously in normal RBCs. MetHb can also be produced by oxidants and ROS. In normal conditions the reaction is reversible and production of metHb is in balance with the conversion of metHb back to Hb. For this reason, Hemichrome is rarely found in erythrocytes in situ, even though the reaction dynamics of Hb with molecular oxygen (O$_2$) make it a particularly suitable O$_2$ carrier. Hb can bind O$_2$ in ferrous form to carry out its physiological functions$^{43}$.

During this reversible O$_2$ binding, the oxygenated form of Hb (HbO$_2$) is known to be oxidized by the bound oxygen to the ferric met form (metHb), which cannot be oxygenated, and is thus physiologically inactive. Although metHb is reduced back to the ferrous state by an intraerythrocytic nicotinamide adenine dinucleotide (NADH)-dependent reducing system, it has been suggested that its oxidation (autoxidation) process can be followed by transformation of the oxidized molecule (high-spin Fe$^{3+}$) into a species absorbing as a low-spin compound, i.e., hemichrome, the formation of which can result in the accumulation of soluble and insoluble hemichromes and precipitation.

Despite these findings, direct evidence of hemichrome formation in normal erythrocytes is lacking. On the contrary, in pathological conditions, increased oxidative stress and/or impaired antioxidant defence enhance the production of metHb, the generation of ROS in consequently the regeneration of unstable Hb and irreversible Hb oxidation products, giving hemichrome forms$^{44}$.

The first area in which hemichrome formation plays a role, although perhaps a negative role, is in the formation of Heinz bodies and the subsequent destruction of red cells.

It has been shown that HMC bind to the cytoplasmic portion of band 3 of the erythrocyte membrane. The binding of hemoglobin to band 3 of the erythrocyte membrane involves insertion of the N-terminal peptide of band 3 into the central cavity of hemoglobin between the β chains$^{45}$. 37
This binding is enhanced for deoxyhemoglobin, in which the distance between the β chains is greater, and is blocked by cross-linking the β chains. The binding of HMC to band 3 also involves the same N-terminal cytoplasmic peptide. However, the binding is much stronger than for hemoglobin and it does not involve insertion into the central cavity. Instead, it involves the interaction of two hemoglobin molecules with each band 3 chain\textsuperscript{46}.

HMC rapidly copolymerize with the soluble cytoplasmic domain of the erythrocyte membrane band 3 and form an insoluble copolymer. It has been suggested that in the intact cell the same binding of HMC to band 3 causes a clustering of band 3 and results in the formation of insoluble Heinz bodies. This hypothesis is supported by several studies of abnormal haemoglobins that show that the formation of Heinz bodies correlates with the presence of HMC bound to the membrane. The cells with Heinz bodies are rapidly taken out of circulation. Thus in splenectomized patients increased levels of Heinz bodies are detected. It is clear, at least with certain abnormal haemoglobins, that the hemichrome formation and subsequent Heinz body formation are part of the etiology of hemolytic anemia. Finally, in pRBCs HMC release their heme into the erythrocyte membrane with consequent lipid peroxidation, biomolecule oxidation and membrane destabilization. A chain of events is induced by the generation of HMC during the intra-erythrocyte parasite development\textsuperscript{44}. 

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1.5. Syk inhibitors as treatment for Malaria

Figure 12. The role of band 3 in the life of the malarial parasite with (w/) and without (w/o) Syk tyrosine kinase inhibition. Hb, hemoglobin; p, phosphorylation of band 3; RBC, red blood cell. Professional illustration by Somersault18:24.¹

AE1 or Band 3 is the most abundant erythrocyte membrane protein which constitutes the major attachment site of the spectrin-based cytoskeleton to the erythrocyte’s lipid bilayer and thereby contributes critically to the stability of the red cell membrane⁴⁷. Recently, Pantaleo et all 39
demonstrated that during the intra-erythrocytic stage of *Plasmodium falciparum*’s life cycle, band 3 becomes tyrosine phosphorylated in response to oxidative stress, leading to a decrease in its affinity for the spectrin/actin cytoskeleton and causing global membrane destabilization (Fig.12). According to this hypothesis, this membrane weakening facilitates to parasite egress and the consequent dissemination of released merozoites throughout the bloodstream.

Syk kinase inhibitors both prevent parasite-induced band 3 tyrosine phosphorylation and inhibit parasite-promoted membrane destabilization. Indeed, the same Syk kinase inhibitors suppress merozoite egress near the end of the parasite’s intra-erythrocytic life cycle. Because the entrapped merozoites die when prevented from escaping their host erythrocytes and since some Syk inhibitors have displayed long term safety in human clinical trials, they are a promising class of anti-malarial drugs that can suppress parasitemia by inhibiting a host target that cannot be mutated by the parasite to evolve drug resistance. Syk inhibitors like R406 are in various stages of development 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\(^{48-51}\).

1.5.1. Syk inhibitors and their molecular synthesis

Syk inhibitors act as ATP-competitive inhibitors of Syk. These compounds have already used in several inflammatory and allergic diseases. Specifically, **R406 (Fig.13A)**, showed its long-term safety in large studies to cure rheumatoid arthritis, and is currently in clinical trial (phase 2); furthermore the pyrimidine carboxamide derivatives, as Syk inhibitor II (Syk II) and IV (Syk IV) (Fig.13E and 13D) are used to treat allergic diseases. While **Imatinib (Fig.13F)**, marked as Gleevec by Novartis, acts as tyrosine kinase inhibitor and is a well-tolerated drug for long-term treatment of several cancerous diseases, most notably Philadelphia chromosome-positive (Ph+) chronic myelogenous leukemia\(^{48}\) (CML).

**Piceatannol (Fig.13B)** (3, 4’, 3’, 5-trans-trihydroxystilbene) is a resveratrol analogue with antioxidant, anticancer and anti-inflammatory activities. **Entospletinib (Fig.13C)** (GS-9973) is an oral, selective inhibitor of spleen tyrosine kinase. This multicenter, phase 2 study enrolled subjects with relapsed or refractory chronic lymphocytic leukemia (CLL; \(n = 41\)) or non-Hodgkin lymphoma.
P505-15 (Fig.13G) (also known as PRT062607) is a novel, highly selective, and orally bioavailable small molecule SYK inhibitor (SYK IC$_{50}$ = 1 nM) with anti-SYK activity that is at least 80-fold greater than its affinity for other kinases. There are findings supporting the ongoing development of P505-15 as a therapeutic agent for B-cell malignancies.

**Figure 13.** Molecular synthesis of different Syk inhibitors such as R406, Piceatannol, Entospletinib, SYK IV, SYK II, Imatinib and P505-15.
1.6. Drug combinations

Drug combination is most widely used in treating the most dreadful diseases, such as cancer, AIDS and Malaria. The main aims are to achieve synergistic therapeutic effect, dose and toxicity reduction, and to minimize or delay the induction of drug resistance. Toxicity reduction and resistance minimization benefits could also be the outcomes of synergism. The main obstacles to the success of combination treatment in preventing the emergence of resistance will be inadequate treatment (e.g., substandard drugs, incorrect dosing, unusual pharmacokinetics, poor adherence) and use one of the combination partners alone. This is why blister packing has been encouraged and fixed-dose combinations are now being developed and recommended.\(^{52}\)

1.6.1. Terms of Synergy, additivity and antagonism

When the addition of one agent apparently increases the effect of the other, so that the effect of a combination appears to be greater than would be expected; the term “synergism” is used to describe these situations. The term “antagonism” is used when the effect of the combination is less effective than the sum of the individual effects; without synergism and antagonism, the two individual effects are “additive”.\(^{53}\)

1.6.2. Combination index by Chou-Talalay

In 1984 Chou and Talalay jointly introduced a concept called the “Combination Index” (CI) to quantitatively depict synergism (CI). Users of the index (CI) claim that the Chou-Talalay Combination Index is based on the median-effect principle which is derived from the “mass action law” principle. Chou declared that “synergism is basically a physiochemical mass-action issue, not a statistical issue; determine synergism with CI values, not with p-values”\(^{52,54}\).

<table>
<thead>
<tr>
<th>CI</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.1</td>
<td>Very Strong Synergism (VSS)</td>
</tr>
<tr>
<td>0.1–0.3</td>
<td>Strong Synergism (SS)</td>
</tr>
<tr>
<td>0.3–0.7</td>
<td>Synergism (S)</td>
</tr>
<tr>
<td>0.7–0.85</td>
<td>Moderate Synergism (MS)</td>
</tr>
<tr>
<td>0.85–0.90</td>
<td>Slight Synergism (SSI)</td>
</tr>
<tr>
<td>0.90–1.10</td>
<td>Nearly Additive (NAdd)</td>
</tr>
<tr>
<td>1.10–1.20</td>
<td>Slight Antagonism (SIA)</td>
</tr>
<tr>
<td>1.20–1.45</td>
<td>Moderate Antagonism (MA)</td>
</tr>
<tr>
<td>1.45–3.3</td>
<td>Antagonism (A)</td>
</tr>
<tr>
<td>3.3–10</td>
<td>Strong Antagonism (SA)</td>
</tr>
<tr>
<td>&gt;10</td>
<td>Very Strong Antagonism (V2A)</td>
</tr>
</tbody>
</table>

Table 3. Combination index values and its description
Calculation of the combination index: Chou and Talalay defined a “combination index” CI as follows: Using a combined dose, d1 of agent A mixed with d2 of agent B so as to achieve 50% response, then calculating:

\[
CI = \frac{d_1}{EDS0}
\]

After the IC\(_{50}\) concentration of the combination is obtained, the needed amount of each drug and the CI is calculated as follows:

\[
\frac{IC_{50} \text{ of combination}}{IC_{50} \text{ of Drug A}} + \frac{\text{Concentration of Drug B}}{IC_{50} \text{ of Drug B}}
\]

The CI is the natural law–based general expression of pharmacologic drug interactions. It is shown to be the simplest possible way for quantifying synergism or antagonism. Its simplicity in equations, experimental designs, and data analysis features efficiency, economy, and reducing the experimental size of animals used or the number of patients needed for drug combination clinical trials. The general theory of the MEP of the mass-action law, its CI algorithm, and its computerized simulation have paved the way for future drug combination studies, as indicated by the broad acceptance in scientific applications and by the rapid increase in citation numbers.
1.6.3. Isobologram analysis

The isobologram analysis (Fig. 14) provides a graphical presentation of the nature of interaction of two drugs, i.e., drug A and drug B. First, in a two-coordinate plot with one coordinate representing concentration of drug A and the other representing concentration of drug B, the concentrations of drugs A and B required to produce a defined effect x (e.g., IC$_{50}$, A and IC$_{50}$, B when x=50%), when used as single agents, are placed on the x and y-axes, respectively. The line of additivity is constructed by connecting these two points (e.g., (IC$_{50}$, A, 0) and (0, IC$_{50}$, B) for a 50% effect isobologram plot). Second, the concentrations of the two drugs used in combination to provide the same effect x (e.g., x=50%), denoted by point (CA, x, CB, x), are placed in the same plot. Synergy, additivity, or antagonism is indicated when this point is located below, on, or above the line, respectively.

The method is relatively simple and is not limited by whether the dose-effect relationships are hyperbolic or sigmoidal, whether the effects of the drugs are mutually exclusive or nonexclusive, whether the ligand interactions are competitive, noncompetitive or uncompetitive, whether the drugs are agonists or antagonists, or the number of drugs involved. The equations for the two most widely used methods for analyzing synergism, antagonism and summation of effects of multiple drugs. It can be shown that the isobologram is valid only for drugs whose effects are mutually exclusive, whereas the fractional product method is valid only for mutually non-exclusive drugs which have hyperbolic dose-effect curves.

Furthermore, in the isobol method, it is laborious to find proper combinations of drugs that would produce an isoeffective curve, and the fractional product method tends to give indication of synergism, since it underestimates the summation of the effect of mutually nonexclusive drugs that have sigmoidal dose-effect curves. The simplified experimental design proposed for multiple drug-effect analysis has the following advantages: It provides a simple diagnostic plot (i.e., the median-
effect plot) for evaluating the applicability of the data, and provides parameters that can be directly used to obtain a general equation for the dose-effect relation; the analysis which involves logarithmic conversion and linear regression can be readily carried out with a simple programmable electronic calculator and does not require special graph paper or tables; and the simplicity of the equation allows flexibility of application and the use of a minimum number of data points. This method has been used to analyze experimental data obtained from enzymatic, cellular and animal systems\textsuperscript{54}.

1.6.3.1. Isobologram analysis in triple combinations

However, such a generalization to the analysis of more than two drugs does not allow investigation of the contribution of each drug to the whole combination effect. A combination of three drugs (A, B, and C) with a synergistic effect (CI < 1) could result from the synergistic effect between A and B only. A complete understanding of the contribution of each drug to the whole combination effect would require an assessment over all the subcombinations which is generally not feasible in practice. When the combination is based on logic such as the combination of two new investigational drugs (A and B) with a reference treatment (C), the analysis should follow the same logic in order to show here that A and B are synergistic, and that the combination of A + B (considered as a new single agent) with the reference treatment C is also synergistic\textsuperscript{55}. 

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2. AIM OF THE PROJECT

Recently it has been demonstrated that Syk inhibitors represent a new class of antimalarial drugs by suppressing merozoite egress by inhibiting the host target that cannot be mutated by the parasite to evolve drug resistance. It is worth noting that WHO recommends artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated malaria caused from the \textit{P. falciparum} parasite, by combining two active drugs with different mechanisms of action. Unfortunately, ACTs are losing their efficacy in large areas of South East Asia with the appearance of increasing resistance. The resistance to ACTs is a major threat for the control and elimination of malaria due to \textit{P. falciparum}. To reduce the risk of recrudescence and emergence of resistant microbes, a new drug treatment for malaria should determine a more rapid and complete elimination of parasites from the blood, along with rapid amelioration of symptoms. Notably, these requisites are also essential to prevent and treat severe malaria. The main goal of this study is to establish new effective antimalarial combinations that possess the following pre-requisites:

- To potentiate synergically the artemisinins
- To exert a synergic effect also in combination with the most widely used ACT
- To be effective in all stages of the asexual parasite growth
- To be effective on all sexual and asexual development stages of \textit{P. falciparum}
- To have a long plasma half-life

Taking into consideration both the urgent need of new antimalarial combinations and Syk inhibitors as potential antimalarial drugs, this work has two main goals:

1. To test the \textit{in vitro} combination activity of Syk inhibitors with currently used antimalarial drugs such as ART’s and quinolines in order to determine their possible synergy.
2. To understand how Syk inhibitors potentiate the artemisinin activation, whereby ARTs are considered prodrugs that are activated to generate ROS which can destroy the parasite intraerythrocytically.
3. MATERIAL AND METHODS

3.1. Cultivation of *P. falciparum*-infected RBCs.

Freshly drawn blood (Rh+) from healthy adults of both sexes was used. Patients provided written, informed consent before entering the study. The study was conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. Blood anti-coagulated with heparin was stored in citrate-phosphate-dextrose with adenine (CPDA-1) prior to its use. RBCs were separated from plasma and leukocytes by three washings in RPMI 1640 medium. *P. falciparum* laboratory strains Palo Alto, FCB1 and It-G (both mycoplasma-free) were cultivated in RPMI 1640 medium containing HEPES, supplemented with 20 mM glucose, 2 mM glutamine, 0.025 mM adenine and 32 mg/l gentamycin at 2% hematocrit. Parasite cultures were synchronized as described by Lambros and Vanderberg. It was observed that through this procedure *P. falciparum* cultures maintain synchronicity for 2-3 cycles as already described by Ayi K, et al.

3.2. Treatment of parasitized/red blood cells

To stimulate HMC formation, RBC and pRBC were suspended at an hematocrit of 30% and incubated with different concentrations (0, 0.2, 0.4, 0.8, 1 mM) of Phenylhydrazine at 37 °C for 4 hours. Also for the same reason were treated with dihydroartemisinin and different Syk inhibitors for 12 and 24 hours at 37 °C in the dark. Each reaction was terminated by three washes with PBS-glucose. For all protocols described, untreated controls were processed identically except that the stimulant/inducer was omitted from the incubation.

3.3. Drug Susceptibility Assays of Cultured Parasites.

All inhibitors were solubilized in anhydrous DMSO at a 10mM stock concentration and serially diluted in anhydrous DMSO prior to addition to malaria cultures. Untreated cultures were run in parallel with the same final concentration of DMSO as the drug treated cultures. 24 hours after their synchronization (estimated time from the start of the cycle 20-22 hours), cultures and fresh isolates of *P. falciparum* were treated in presence of different concentrations of dihydro-artemisinin (DHA), or artesunate at a variable concentrations (from 0.6 to 10 nM) combined with the Syk inhibitors: P505-15, R406 (Calbiochem, Darmstadt, Germany), Entospletinib, Syk inhibitor II (from now on abbreviated as SYK II), Piceatannol and Imatinib at variable concentrations (from 0.1 to 10 µM).

Experiments were also performed treating *Plasmodium* cultures with fixed concentrations of (0.05
to 2 μM) R406, SYK II or Imatinib in presence of variable concentrations of DHA (from 0.6 to 10 nM) combined with mefloquine (MFQ), Piperaquine (PPQ), Lumefantrine (LMF), Amodiaquine (AMQ) using each of them at a fixed concentration (1 to 10 nM).

3.4. Isobologram preparation and Combination index (CI) measurement.
Parasitemia levels of parasite maturation in wells were measured and nonlinear concentration response curves of drug A, drug B and all the combinations were made in Microsoft excel. Isobolograms were plotted with IC₅₀ concentrations to determine the interaction between drug A, B and C. In plot x there is the IC₅₀ concentration of drug A alone and in plot y the IC₅₀ concentration of drug B alone. The line of additivity is constructed by connecting these two points. The other spots are the IC₅₀ concentrations of the two drugs used in combination. Synergy (CI< 1), additivity (C=1), or antagonism (CI>1) are indicated by the combination index theorem of Chow-Talalay which is offering a quantitative definition as already described above.

3.5. Assessment of parasitemia by Light Microscopy.
To assess total parasitemia, the share of rings, trophozoites, schizonts and merozoites, and to judge on morphology, thin smears were prepared from cultures at indicated times and stained with Diff-Quick stain (Medion Diagnostics, CH) and at least 2000 cells were examined microscopically by 3 observers. The experiments were done in triplicate.

3.6. IC₅₀ measurement.
To calculate the half maximal inhibitory concentration, that inhibits 50% of malaria parasite (IC₅₀), of different Syk inhibitors used as single drugs or in combination with others antimalarial drugs, we used ICEstimator software 1.2 version. The program estimates IC₅₀ through nonlinear regression using a standard function of the R software.

3.7. Preparation of cells for immunofluorescence (Confocal microscopy)
RBCs treated with/without PHZ and pRBCs were pelleted and washed twice in PBS 1× containing 5 mM glucose and then fixed for 5 min in 0.5% acrolein in PBS. Cells were rinsed three times then permeabilized in PBS containing 0.1 M glycine (rinsing buffer) plus 0.1% Triton X-100 for 5 min
and again rinsed 3× in rinsing buffer. To ensure complete neutralization of unreacted aldehydes, the cells were then incubated in rinsing buffer at room temperature for 30 min.

After incubation, all nonspecific binding was blocked by incubation again for 60 min in blocking buffer (PBS containing 0.05 mM glycine, 0.2% fish skin gelatin and 0.05% sodium azide). Staining of fixed, permeabilized RBCs was performed by using specific antibodies diluted in blocking buffer. After labeling, resuspended RBCs were allowed to attach to coverslips coated with polylysine, and the coverslips were mounted by using Aqua-Mount (Lerner Laboratories, New Haven, CT). The auto-fluorescence of HMC was visualized by exciting at 488 nm and observing their emission in the 630–750 nm range. Samples were imaged with a Bio-Rad MRC1024 (Bio-Rad) confocal microscope equipped with a 60×1.4 numerical aperture oil immersion lens.

3.8. Electron paramagnetic Resonance (EPR) measurements

The detection of free radicals was carried out using N-tert-Butyl-α-phenylnitronate (PBN) as spin trap. 100 µL of PBN (stock solution 1M in DMSO) was added to 50 µL of normal (RBCs) or parasitized (pRBCs) red blood cells (hematocrit 10 %) and the volume completed to 300 µL with PBS after addition of Dihydroartemisinin (200 µM in DMSO). The solution was then transferred into a flat quartz cell (FZKI160-5 X 0.3 mm, Magnettech, Berlin, Germany) for EPR analyses.

EPR spectra were obtained at room temperature using the X-band on a Bruker EMX-8/2.7 (9.86 GHz) equipped with a gaussmeter (Bruker, Wissembourg, France) and a high-sensitivity cavity (4119/HS 0205). WINEPR and SIMFONIA softwares (Bruker, Wissembourg, France) were used for EPR data processing and spectrum simulation. Typical scanning parameters were: scan number, 5; scan rate, 1.2 G/s; modulation frequency, 100 kHz; modulation amplitude, 1 G; microwave power, 20 mW; sweep width, 100 G; sweep time, 83.88 s; time constant, 40.96 ms; magnetic field 3460-3560 G.

3.9. Fluorescence analyses

For the detection of intracellular ROS level we used the cell-permeable ROS-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA) which becomes fluorescent upon oxidation. The oxidation of H$_2$DCFDA was followed by measuring the fluorescence in 96-well black-walled microplates (Corning®, Sigma Aldrich) using a SAFAS Xenius (Monaco). 8 µL of the stock 49
solution (0.5 mM in DMSO) was added to 100 µL of the RBC solutions and the volume was completed to 200 µL with PBS after addition of dihydroartemisinin (final concentration 200 µM) when needed.
4. RESULTS

4.1. Chapter synopsis

This chapter of the results constitutes of three main sections. Firstly, the IC$_{50}$ concentrations of different Syk inhibitors are presented along with currently used antimalarial drugs in different *P. falciparum* strains. Secondly, the effect of Syk inhibitors on artemisinin efficacy is analyzed. In that section, six different Syk inhibitors and their combinatory effect on different artemisinin derivatives (Dihydroartemisinin, Artesunate and Artemether) are presented by graphical presentations (isobolograms) and their combination index values (Chou-Talalay). Finally, as it is already known that iron (II) activates artemisinin by producing radicals, we present the hypothetical role of HMC [Fe (III)] accumulation by Syk inhibitors to the artemisinin activation.

4.2. *In vitro* study of Syk inhibitors in *Plasmodium falciparum* strains

4.2.1. IC$_{50}$ values of all tested Syk inhibitors in vitro

In order to investigate the action of different Syk inhibitors (P505-15, R406, Entospletinib, Imatinib, SYK II, Piceatannol and SYK IV) on *P. falciparum* cultures and evaluate their IC$_{50}$ concentrations, we measured their activity at varying concentrations (0.05 to 10 μM), durations and on different parasite stages in several strains (Palo alto, FCB-1 and It-G).

*In Tables 4 and 5 there are the* IC$_{50}$ *concentrations of all tested Syk inhibitors after 24 and 48 hours of incubation. This section also presents the parasitemia levels under different drug treatments and their morphological changes in the presence of a specific Syk inhibitor (P505-15) on *P. falciparum* cultures after 24, 48, 72 and 96 hours of treatment. Among all tested strains, the most efficient Syk inhibitor appears to be P505-15, as it presents the lowest IC$_{50}$ concentration (both in 24 and 48 hours) needed to eliminate 50% of parasitemia. The IC$_{50}$ concentration of P505-15 was 0.83 μM (± 0.06) and 0.49 μM (± 0.07) at 24 and 48 hours of incubation respectively.

The mentioned data refers to the Palo alto strain as no significant differences have been observed among the 3 strains regarding their IC$_{50}$ concentrations. Indeed, P505-15 (Syk IC$_{50}$ = 1 nM) is a
highly selective novel inhibitor of Syk with the lowest IC₅₀ concentration from all the tested Syk inhibitors.

Furthermore, R406 appears to be the second most efficient Syk inhibitor regarding its IC₅₀s (2.62 ± 0.83, 0.55 ± 0.19) at 24 and 48 hours of treatment respectively.

Table 4. In vitro susceptibility (IC₅₀ values) of P. falciparum Palo alto strain after 24 and 48 hours of treatment with different Syk inhibitors.

<table>
<thead>
<tr>
<th>Palo Alto strain</th>
<th>24 hours</th>
<th>48hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syk inhibitors</td>
<td>IC₅₀ (µM)</td>
<td>Range (µM)</td>
</tr>
<tr>
<td>P505-15</td>
<td>0.83 ± 0.06</td>
<td>0.78 - 0.9</td>
</tr>
<tr>
<td>R406</td>
<td>2.62 ± 0.83</td>
<td>1.42 - 3.85</td>
</tr>
<tr>
<td>Entospletinib</td>
<td>3.07 ± 0.59</td>
<td>2.21 - 3.86</td>
</tr>
<tr>
<td>Imatinib</td>
<td>3.81 ± 0.55</td>
<td>3.24 - 4.95</td>
</tr>
<tr>
<td>SYK II</td>
<td>5.01 ± 0.44</td>
<td>4.65 - 5.87</td>
</tr>
<tr>
<td>Piceatannol</td>
<td>3.17 ± 0.24</td>
<td>2.95 - 3.55</td>
</tr>
<tr>
<td>SYK IV</td>
<td>7.28 ± 0.40</td>
<td>6.87 - 7.91</td>
</tr>
</tbody>
</table>

Table 5. In vitro susceptibility (IC₅₀ values) of P. falciparum FCB1 and It-G strain after 24 and 48 hours of treatment with different Syk inhibitors.

<table>
<thead>
<tr>
<th>Syk inhibitors</th>
<th>FCB1 strain</th>
<th>ItG1 strain</th>
<th>FCB1 strain</th>
<th>ItG1 strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>P505-15</td>
<td>0.95 ± 0.16</td>
<td>0.89 ± 0.12</td>
<td>0.59 ± 0.09</td>
<td>0.48 ± 0.11</td>
</tr>
<tr>
<td>R406</td>
<td>1.34 ± 0.28</td>
<td>1.54 ± 0.46</td>
<td>0.60 ± 0.32</td>
<td>0.61 ± 0.16</td>
</tr>
<tr>
<td>Entospletinib</td>
<td>2.85 ± 0.48</td>
<td>2.98 ± 0.39</td>
<td>1.28 ± 0.16</td>
<td>1.12 ± 0.17</td>
</tr>
<tr>
<td>Imatinib</td>
<td>3.61 ± 0.62</td>
<td>3.57 ± 0.38</td>
<td>1.62 ± 0.26</td>
<td>1.52 ± 0.21</td>
</tr>
<tr>
<td>SYK II</td>
<td>4.91 ± 0.36</td>
<td>4.83 ± 0.34</td>
<td>0.87 ± 0.45</td>
<td>0.89 ± 0.23</td>
</tr>
<tr>
<td>Piceatannol</td>
<td>3.10 ± 0.14</td>
<td>3.06 ± 0.15</td>
<td>1.15 ± 0.14</td>
<td>1.17 ± 0.26</td>
</tr>
<tr>
<td>SYK IV</td>
<td>7.51 ± 0.32</td>
<td>7.36 ± 0.38</td>
<td>1.56 ± 0.31</td>
<td>1.75 ± 0.31</td>
</tr>
</tbody>
</table>

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4.2.2. Morphological changes induced by a representative Syk inhibitor

**Figure 15.** shows the morphological changes of parasites induced by the high concentration (5μM) of a specific Syk inhibitor (P505-15) at different durations of treatment (24, 48, 72 and 96 hours). Parasites are incapable to accomplish their egress and to finish their life cycle. Indeed, after 24 hours of treatment some trophozoites appeared to be out of the erythrocyte, and compared to the control cultures, they seemed to be more pyknotic. Finally after the 48 hour treatment period, highly shrunken and stained deep purple parasites (presumably dead) became noticeable.

**Figure 15.** Morphological changes in Plasmodium falciparum cultures after treatment with a specific Syk inhibitor (P505-15)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Time of treatment</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
<th>96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P505-15 (5μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.2.3. Effect of P505-15 on inhibition of *P.falciparum* parasitemia

In the third set of experiments, parasitemia was monitored after 24 and 48 hours of treatment in presence of different concentrations (0.2 to 5 μM) of P505-15 (representative Syk inhibitor). After 24 hours of treatment, at the stage of trophozoites with concentrations as low as 1 μM, a two-fold decrease in parasitemia was observed. Finally, in presence of a potent concentration (5 μM) the parasitemia dropped five times in comparison with the control. Interestingly, P505-15 appears to be more potent at 48 hours of treatment (at the ring stage) and almost eliminates all parasites when higher concentrations were used. It has to be mentioned that not only P505-15 shows this effect, but all tested Syk inhibitors have the same behavior regarding the ability to completely eliminate parasites after 48 hours of treatment.

**Figure 16A & B.** Parasitemia (%) after 24 and 48 hours of treatment with P505-15

![Graph A](image1)

**Parasitemia (%) 24 hours after the treatment**

- 0%: 4.3%
- 0.2%: 3.8%
- 0.8%: 3%
- 1%: 1.9%
- 2%: 2%
- 2.5%: 1%
- 5%: 0.76%

**SYK inhibitor P505-15 (μM)**

![Graph B](image2)

**Parasitemia (%) 48 hours after the treatment**

- 0%: 12.5%
- 0.2%: 8.4%
- 0.8%: 5%
- 1%: 4.5%
- 2%: 4%
- 2.5%: 1%
- 5%: 0.10%
4.2.4.  *In vitro* evaluation (IC$_{50}$ values) of different antimalarial drugs

In order to analyze the *in vitro* combination activity of different Syk inhibitors with already in use antimalarials, it was necessary first to test them alone at different concentrations in all the mentioned strains, and obtain their IC$_{50}$ concentrations. In Table 6 all the IC$_{50}$ concentrations of the different antimalarial drugs in Palo alto strain are presented. All ARTs (Dihydroartemisinin, Artesunate and Artemether) present IC$_{50}$ concentrations lower than 3 nM. In agreement with the literature data, artemisinin appears in higher IC$_{50}$ concentrations, as are the tested aminoquinolines. No significant differences have been observed among the 3 strains and for this reason, we will present the isobolograms only in one strain (Palo alto).

*Table 6. IC$_{50}$ values of different antimalarial drugs in Palo alto strain after 24 hours of treatment*

<table>
<thead>
<tr>
<th>Artemisinin Derivatives</th>
<th>IC$_{50}$ (nM)</th>
<th>Range (nM)</th>
<th>IC$_{50}$ (nM)</th>
<th>Range (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemisinin</td>
<td>16.8 ± 1.44</td>
<td>15.1 - 18.6</td>
<td>16.14 ± 1.10</td>
<td>14.8 - 17.5</td>
</tr>
<tr>
<td>Dihydroartemisinin</td>
<td>2.64 ± 0.37</td>
<td>2.2 - 3.3</td>
<td>2.45 ± 0.53</td>
<td>1.43 - 3.69</td>
</tr>
<tr>
<td>Artesunate</td>
<td>2.45 ± 0.26</td>
<td>2 - 2.74</td>
<td>4.25 ± 0.27</td>
<td>3.72 - 4.51</td>
</tr>
<tr>
<td>Artemether</td>
<td>2 ± 0.5</td>
<td>1.5 - 2.5</td>
<td>3.44 ± 0.12</td>
<td>3.35 - 3.58</td>
</tr>
<tr>
<td>Antimalarial drugs</td>
<td>IC$_{50}$ (nM)</td>
<td>Range (nM)</td>
<td>IC$_{50}$ (nM)</td>
<td>Range (nM)</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>18.8 ± 4.6</td>
<td>13-20.5</td>
<td>6.3 ± 0.9</td>
<td>5.5-7.3</td>
</tr>
<tr>
<td>Piperaquine</td>
<td>20.8 ± 4.7</td>
<td>15.39-23.9</td>
<td>19.19 ± 6.9</td>
<td>14.27-24.1</td>
</tr>
<tr>
<td>Lumefantrine</td>
<td>24.5 ± 3</td>
<td>22.4-26.2</td>
<td>13.6 ± 1.6</td>
<td>11.6-15.1</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>26.8 ± 3.5</td>
<td>27.04-30.2</td>
<td>15.37 ± 2</td>
<td>13.5 – 18.2</td>
</tr>
</tbody>
</table>
4.3. *In vitro* activity of Syk inhibitors in combination with ARTs

In order to analyze the *in vitro* combination activity of Syk inhibitors on artemisinin efficacy, *Plasmodium falciparum* was treated with different Syk inhibitors and ARTs as described above (material and methods). The evaluation of the possible synergism has been done based on the method of Chou – Talalay as already described. It is worth noting that synergism is a physicochemical mass-action law issue and not a statistical issue, so we determine synergism with isobolograms and CI values, rather than *P* values. Also, the combination index analysis provides an easy presentation of quantitative synergy data. IC₅₀ values of dihydroartemisinin, artesunate and artemether have been measured at 6, 12, 24 and 48 hours in combination with different concentrations (0.05-10 µM) of different Syk inhibitors (P505-15, R406, Entospletinib, Syk II, Imatinib and Piceatannol. The following, *Figures (17-19)* and *Table 9*, show all the tested combinations with isobolograms and combination index values.
4.3.1. Syk inhibitors in combination with ARTs at 24 hours of incubation

4.3.1.1. Syk inhibitors in combination with Dihydroartemisinin (DHA)

Figure 17 shows the isobolograms at 50% effect level, and Table 9 summarizes the results. In every concentration of each SYK inhibitor combined with DHA, the isobologram analysis showed extensive synergy, with the maximum extent of about 5-fold synergy occurring at a fairly broad range of concentration ratios. In Table 9, there are the concentrations of the Syk inhibitors that determine a synergic effect and are included between 50 and 500 nM, determining approximately a 2-5 fold decrease of the IC$_{50}$ of the combined DHA. In particular, Table 7 shows a fold decrease of the IC$_{50}$ concentration of DHA, based on the use of different Syk inhibitors (250 nM). The highest decrease, 4.8 folds observed with the use of P505-15 and the lower decrease observed with the use of Imatinib (1.5 fold decrease).

At the higher Syk inhibitors concentration (500 nM), the CI values were consistently below 1, indicating synergy. In general, the plots showed well-spaced concentration points, with several data points near the IC$_{50}$ value. The experimental design used approximately five-fold steps in concentration dilution. Furthermore, maximal synergy of approximately 5-fold at 100 nM of P505-15 was achieved with a combination index of 0.42. Also, the combination with R406 appears a CI value of 0.42 (Table 9). Indeed, at lower concentrations, all data points were below the line of additivity indicating still synergy. CI values decreased at increasing effect levels, indicating increasing degree of synergy at higher effect levels. Synergy of greater than 1.5- fold at the 50% effect level was observed for all the tested Syk inhibitors.
Table 7. Fold decrease of IC$_{50}$ concentration of DHA in presence of Syk inhibitors (the IC$_{50}$ concentration of DHA alone is 2.64 nM).

<table>
<thead>
<tr>
<th>Syk inhibitors (250 nM)</th>
<th>IC$_{50}$ of DHA in combination with Syk inhibitors (nM)</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>P505-15</td>
<td>0.55</td>
<td>4.8</td>
</tr>
<tr>
<td>Entospletinib</td>
<td>1.14</td>
<td>2.3</td>
</tr>
<tr>
<td>R406</td>
<td>0.95</td>
<td>2.8</td>
</tr>
<tr>
<td>SYK II</td>
<td>1.67</td>
<td>1.6</td>
</tr>
<tr>
<td>Imatinib</td>
<td>1.75</td>
<td>1.5</td>
</tr>
<tr>
<td>Piceatannol</td>
<td>1.2</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Figure 17. Isobologram presentation of Dihydroartemisinin (DHA) in combination with different Syk inhibitors after 24 hours of treatment.
4.3.1.2. Syk inhibitors in combination with Artesunate (AS) and Artemether (ATH)

At all concentrations of all Syk inhibitors combined with AS, the isobologram analysis (Fig.18) and the CI showed synergy, with the maximum extent of about 2.13-fold synergy. In Table 9 there are the concentrations of the Syk inhibitors that determine a synergic effect and are included between 50 and 500 nM, determining approximately a 1.82-2.13 fold decrease of the IC\textsubscript{50} of the combined AS. In particular, Table 8 demonstrates a fold decrease of the IC\textsubscript{50} concentration of AS based on the use of different Syk inhibitors (250 nM). The greatest reduction, of 2.13 fold was observed with the use of P505-15, and the lowest decrease observed with the use of Imatinib (1.82 fold decrease).

At the higher Syk inhibitors concentrations (500 nM), the CI values were consistently below 1, indicating synergy. CI values increased in combination with AS, indicating the decreasing degree of synergy in comparison with DHA. Maximal synergy at 250 nM of SYK II was achieved with a combination index of 0.55. Finally, one more derivative of artemisinin, ATH, showed nearly synergism with all the combined Syk inhibitors. The maximal synergy observed at 250 nM of Piceatannol with a CI value of 0.8. In comparison with the other artemisinin derivatives, ATH appears the lower CI values (Table 9) and the lower fold decrease (data not shown).

Table 8. Fold decrease of IC\textsubscript{50} concentration of DHA in presence of Syk inhibitors (the IC\textsubscript{50} concentration of AS alone is 2.45 nM).

<table>
<thead>
<tr>
<th>Syk inhibitors (250 nM)</th>
<th>IC\textsubscript{50} of AS in combination with Syk inhibitors (nM)</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>P505-15</td>
<td>1.15</td>
<td>2.13</td>
</tr>
<tr>
<td>Entospletinib</td>
<td>1.16</td>
<td>2.11</td>
</tr>
<tr>
<td>R406</td>
<td>1.32</td>
<td>1.85</td>
</tr>
<tr>
<td>SYK II</td>
<td>1.23</td>
<td>1.99</td>
</tr>
<tr>
<td>Imatinib</td>
<td>1.34</td>
<td>1.82</td>
</tr>
<tr>
<td>Piceatannol</td>
<td>1.19</td>
<td>2.05</td>
</tr>
</tbody>
</table>
Figure 18. Isobologram presentation of Artesunate (AS) in combination with different Syk inhibitors after 24 hours of treatment.
4.3.2 Syk inhibitors in combination with ARTs at 48 hours of incubation

Finally, regarding 48 hours of incubation of Syk inhibitors and ARTs, the results remain really interesting as there is still a noticeable synergy in all concentrations with all Syk inhibitors. In figure 19 there are the isobolograms of 3 representative Syk inhibitors (R406, SYK II and Imatinib) in combination with DHA and AS. The isobogram analysis presents synergy in all conditions determining approximately a 1.5-4 fold decrease of the IC$_{50}$ of the combinations. In general, the plots showed well-spaced concentration points, with several data points near the IC$_{50}$ value. The experimental design used approximately five-fold steps in concentration dilution for 24 hours. Furthermore, the maximal synergy of approximately 5-fold at 50 nM of R406 was achieved with a combination index of 0.44. Also, the combination with SYK II presented a CI value of 0.46 (table 9). Indeed at higher concentrations all data points were below the line of additivity, indicating synergy. In general, CI values decreased at decreasing effect levels, indicating an increased degree of synergy at lower effect levels. Synergy greater than 1.5- fold at the 50% effect level was observed for all the tested Syk inhibitors with all ARTs.
Figure 19. Isobologram presentation of Dihydroartemisinin (DHA) and Artesunate (AS) in combination with representative Syk inhibitors (R406, SYK II and Imatinib) after 48 hours of treatment.
All the presented data evidence marked a synergic interaction after 24 and 48 hours of incubation on all tested Syk inhibitors (P505-15, Entospletinib, R406, SYK II, Imatinib and Piceatannol) with all the tested ARTs. All the concentrations of Syk inhibitors (50 to 500 nM) have a synergic activity with ARTs determining a decrease of their IC50s. This result indicates very different mechanisms of action and effects on plasmodium of both Syk inhibitors as single agents, and in combination with artemisinins: a) At low concentrations (5-250 nM), Syk inhibitors do not show any measurable effect on the growth of the parasite, however when used in combination with ARTs, they potentiate the artemisinin activation and inhibit parasite growth; b) the appearance of the synergic effect of Syk inhibitors in combination with artemisinins is shown at both 24 and 48 hours of incubation.
4.3.3 Syk inhibitors in combination with other antimalarial drugs

Sets of experiments have been performed in order to identify the possible synergy, or not, of Syk inhibitor representatives (R406 and Imatinib) in combination with different in-use antimalarial drugs such as mefloquine (MFQ), piperaquine (PPQ), lumefantrine (LMF) and amodiaquine (AMQ). In figure 20 and table 10 there are the mentioned isobolograms and their CI values. On one hand, the combination of Syk inhibitors with MFQ present slight antagonism as all the points are located away from the line of additivity for both imatinib and R406. Indeed almost all the CI values are more than 1 indicating additivity or even slight antagonism. On the other hand, PPQ and LMF present slight synergy or additivity with CI values for R406 from 0.61 to 1.04 and 0.78 to 0.92 for imatinib. Finally, regarding the combination with AMQ, the plots showed well-spaced concentration points, with several data points near the IC\textsubscript{50} value. The maximal synergy of approximately 4 fold decrease was observed in combination with imatinib with a CI value at 0.47 (indicating synergy) (table 9). Synergy greater than 2- fold at the 50\% effect level was observed for all the tested Syk inhibitors with AMQ.

| Table 10. Combination index value (CI) 24 and 48 hours after treatment |
|-----------------|-----------|-----------|-----------|-----------|
|                  | MFQ       | PPQ       | LMF       | AMQ       |
| R406 (nM)        |           |           |           |           |
| 500              | 1.02      | 0.80      | 0.79      | 0.70      |
| 250              | 0.99      | 0.61      | 0.77      | 0.62      |
| 100              | 1.08      | 0.82      | 0.98      | 0.67      |
| 50               | 1.09      | 0.85      | 1.04      | 0.74      |
| Imatinib (nM)    |           |           |           |           |
| 500              | 1.21      | 0.81      | 0.79      | 0.47      |
| 250              | 1.14      | 0.90      | 0.78      | 0.62      |
| 100              | 1.21      | 0.84      | 0.82      | 0.69      |
| 50               | 1.27      | 0.90      | 0.92      | 0.71      |
Figure 20. Isobologram presentation of different antimalarial drugs, Mefloquine (MFQ), Lumefantrine (LMF), Piperaquine (PPQ) and Amodiaquine (AMQ) in combination with representative Syk inhibitors (R406, and Imatinib) after 24 hours of treatment.
4.3.4 Triple combinations (DHA + Imatinib + other antimalarial drugs)

A fixed concentration (10 nM) of the anti-malarial drugs MFQ, PPQ, LMF and AMQ (A to D respectively), that are currently combined to artemisinins in ACT, have been added to the synergistic combination of DHA with Imatinib (representative Syk inhibitor). In the case of the following isobolograms in figure21 (A to D), in plot x there is the IC$_{50}$ concentration of DHA in combination with MFQ, PPQ, LMF and AMQ respectively (A-D) and in plot y there is the IC$_{50}$ concentration of Imatinib. Those drugs were used at 10 nM concentration that corresponds to approximately 30-50% of their IC$_{50}$. The results contained in the isobolograms (Fig.21) present that sub-therapeutic concentrations of those drugs enhance the synergic effect of Syk inhibitors on artemisinins. Indeed, all the IC$_{50}$ concentrations are below the line of additivity indicating synergy between them. It should be noticed that even at low concentrations of Imatinib there is synergy with all the tested antimalarials.

Figure 21. Isobologram presentation of triple combination with fix concentration (10 nM) of MFQ, PPQ, LMF and AMQ (A-D respectively) with DHA and a representative syk inhibitor (Imatinib).
4.3.4.1 Cumulative drug effect in triple combinations

In this set of experiments, the cumulative effect of the triple combination is evaluated after repeated administrations every 24 hours with the following compounds: DHA (1 and 2.5 nM) in combination with fix concentrations of Imatinib (500 nM) and PPQ (10 nM). When pRBCs were treated with the triple combination the parasitemia decreased 1.6 folds compared to DHA (2.5 nM) alone. After the second administration a 7.2 and 25 fold reduction of parasitemia was seen. As the concentration of DHA was decreased to 1 nM, following the first administration parasitemia decreased 1.5 fold and then by 2.2 folds after the second. Finally the last administration saw only a 2.5 fold reduction, indicating the need of higher DHA concentrations to provide more effective synergy (Fig.22). The addition of Imatinib and PPQ enhance the action of DHA, when compared as a single agent. Indeed, the triple administration produces effects that are more pronounced than those produced by the first dose.
Figure 22. Cumulative drug effect of DHA (1 and 2.5 nM) as a single agent and also with fix concentrations of PPQ (10 nM) and Imatinib (500 nM) after three administrations every 24 hours.

*add of compounds
Imatinib: 500 nM
PPQ: 10 nM
4.3.5 Morphological changes of *P. falciparum* induced by Syk inhibitors in combination with DHA

In this embodiment (Fig. 23), synchronized cultures were treated with DHA as a single agent and in combination with two representative Syk inhibitors (R406 and P505-15). Selected pRBCs were presented after 6, 12 and 24 hours of treatment. The most striking morphological alterations were observed in fixed blood smears of parasite culture treated with both Syk inhibitors in combination with DHA after 24 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 (i.e. cell cycle delay). At 12 hours of incubation, there was evidence of a marked reduction in parasitic growth (i.e. smaller size). Irregularly shaped and pyknotic (highly shrunken and stained deep purple) parasites (presumably dead) were finally observed at the last time-point (24 hours). On the other hand, DHA treated parasites did not present similar morphological changes at the various time points.

**Figure 23.** Parasite morphology after 6, 12 and 24 hours of drug treatment. *Plasmodium falciparum* Palo alto parasite cultures were incubated with 1.25 nM DHA as a single agent or in combination with representative Syk inhibitors (R406 and P505-15). Giemsa-stained thin blood smears were prepared from drug-exposed and solvent control cultures and viewed by bright-field light microscopy using a 100x oil-immersion objective. In each case, two representative images of damaged parasites are shown.
4.3.6 Stage-dependent activity of R406 in combination with DHA

In the last set of experiments, we measured the stage-dependent activity of a representative Syk inhibitor (R406) in combination with DHA. **Figure 24** shows the stage-dependent activity of pRBCs in presence of R406 (250 nM) in combination with DHA (2 nM) at different stages of parasite development. The maximal activity was observed between 12 and 40 hours post-infection which corresponds to the parasite’s intraerythrocytic stages. This indicates that a long-term inhibition of R406 in combination with DHA markedly affects the capability of the parasite to complete its growth cycle and potentiate the activity of DHA.

**Figure 24. Stage-dependent sensitivity of parasites to a fixed dosage of R406-DHA.** Relative activity of R406-DHA (250 nM + 2nM) added at 6, 12, 24, 36, 40 and 48 hours post infection. Values are expressed as a percentage of the maximal R406-DHA activity (treatment time 24 hours post infection) measured as % activity. The data is an average of 5 experiments ±SD.
4.4 Role of hemichromes on artemisinin activation

4.4.1 Effect of Syk inhibitors on hemichromes (HMC) accumulation

Ferrous iron and heme are known to be necessary for the activation of artemisinin in parasitized RBCs and its subsequent anti-malarial action. It has also been demonstrated that Syk inhibitors block the efflux of denatured hemoglobin products (hemichromes) from erythrocytes treated with oxidant agents. In order to define the mechanism of action which explains the synergic effect of Syk inhibitors on ARTs, we investigated their effect on the accumulation of denatured hemoglobin (HMC) in parasitized erythrocytes. The accumulation of HMC bound to the host cell has been measured in presence of DHA and P505-15 after 12 and 24 hours of treatment. It is worth noting that the accumulation of HMC is notable 3 hours after treatment with R406 that is coherent with the time needed to observe the synergic effect of Syk inhibitors on DHA.

Figure 25 shows that R406 induced the accumulation of HMC in pRBCs starting from 3 hours of incubation until the following 24 hours of treatment. To better visualize HMC accumulation, we treated normal RBCs and pRBCs with 1 mM of PHZ to induce the formation of HMC through the specific oxidation of hemoglobin and then we observed them with Confocal microscopy (Fig. 26). HMC formation in RBCs and pRBCs are visible in figures 26B and 26E after the treatment with PHZ. Figure 26D confirms the formation of HMC after the treatment of pRBCs with R406.
Figure 25. Hemichromes accumulation after 3, 6, 12 and 24 hours of incubation in parasitized RBCs treated with DHA (0.5 nM) and/or R406 (0.5 μM).

Figure 26. Confocal Images of HMC contained in pRBCs treated 12 hours with R406 (0.5 μM) (C) and 4 hours with PHZ (1 mM) (D). HMC were visualized by their auto-fluorescence at 488 nm (excitation) / 630-750 nm range (emission). Images were acquired using the same magnification with a Leica TCS SP5 X (Leica Microsystems, Germany) confocal microscope equipped with a 6061.4 numerical aperture oil immersion lens.
4.4.2 Free radical production in RBCs treated with PHZ and DHA

To analyze the effect of hemichromes upon the artemisinin activation we first measured the production of ROS using EPR associated with the spin trapping method. The spin trap used was PBN (α-phenyl-N-tert-butyl-nitrone) which allows, whereas it’s not the case for DMPO, intracellular radicals to be trapped due to its greater lipophilicity. The corresponding spectra are presented in Figure 28. The spectra recorded for hRBCs (RBCs with hemichromes) after adding 200 µM Dihydroartemisinin clearly demonstrated the apparition of the six-lined spectrum characteristic of the PBN-adduct formed after radicals trapping (Fig.28d). Simulation yielded to the following parameters: $g = 2.0059$; $a_N = 14.9$ G; $a_H = 3.3$ G indicating that the hydroxyl radical was formed and transformed into $\cdot$CH$_3$ radicals after reaction with DMSO before trapping.

**Figure 27.** pRBCs with PBN in flat cell before EPR reaction

**Figure 28.** EPR spectra (arbitrary unit) recorded with PBN 50 mM for a) RBCs, b) RBCs + DHA (200µM), c) hRBCs, d) hRBCs + DHA (200µM).
To confirm these results the same experiments were performed using the fluorescent probe CM-H$_2$DCFDA, a cell-permeable indicator for the formation of ROS. The incubation of RBCs with increasing concentrations of phenylhydrazine caused a parallel rise of all parameters measured. To support the hypothesis that HMC promote the activation of DHA, we treated RBCs with different concentrations of PHZ for 4 hours in order to have different concentrations of HMC.

**Figure 29** shows that in presence of a fix concentration of DHA (200 μM) as higher as the concentration of HMC is, the higher the production of ROS is. More specific, RBCs containing the higher concentration of HMC present the higher increase of ROS production. Indeed, **figure 29** shows a strong correlation (R: 0.95) between the ROS production and hemichromes accumulation in RBCs. To better understand the role of HMC we treated RBCs with the higher concentration of PHZ (1 mM) to induce the formation of HMC in presence of different concentrations of DHA (10 – 200 μM). **Figure 30** shows that as higher as the concentration of DHA (10 μM- 200 μM) is, the higher the production of ROS is.

**Figure 29.** HMC accumulation and ROS production using the fluorescent probe H$_2$DCFDA in RBCs treated with DHA (200 μM) and different concentrations (200-1000 μM) of Phenylhydrazine (PHZ)
Figure 30. ROS production using the fluorescent probe H$_2$DCFDA in RBCs treated with fix concentration of PHZ (1 mM) and different concentrations (10-200 μM) of DHA

![Graph showing ROS production](image)

4.4.3 Free radical production in pRBCs treated with DHA and Syk inhibitors

In this set of experiments in order to analyze the effect of HMC in pRBCs upon the artemisinin activation, we treated pRBCs with Syk inhibitors (R406 or Imatinib) to induce the accumulation of HMC as already described. We measured the production of ROS using EPR associated with the spin trapping method. The corresponding spectra are presented in Figure 31. Firstly, we recorded spectra for pRBCs (Fig.31b) without any treatment and then with the addition of DHA (200 μM). The addition of DHA clearly demonstrates the apparition of the six-lined spectrum characteristic of the PBN-adduct formed after radicals trapping (Fig.31c). The same experiments carried out after 24 hours incubation of pRBCs with R406 and the addition of DHA (200 μM) that increased the intensity of the peak by 12% (Fig.31d).
Figure 31. EPR spectra (arbitrary unit) recorded with PBN 50 mM for a) pRBCs [Rings/Troph.], b) pRBCs [Rings] + DHA (200µM), c) pRBCs [Troph.] + DHA, d) pRBCs [Troph.] (treated with Syk inhibitors) + DHA (200µM).
4.4.4 Effect of iron chelators to the production of radicals

The activation of artemisinin has been previously described to be inhibited by iron chelators such as deferoxamine\textsuperscript{59}. In the present experiment, we have observed that at 400 $\mu$M of DFX completely abolishes the ROS production (Fig.\textbf{32 and 33}). \textbf{Figure 32} shows the decrease of ROS production in RBCs treated with PHZ at different concentrations of DFX (100-400 $\mu$M). At the same time, the same result observed at pRBCs treated with Syk inhibitors (Fig.\textbf{33}). This observation strongly indicates that the activation of artemisinins induced by Syk inhibitors is due to the accumulation of accessible iron bound to hemichromes. These data provide further evidence for the importance of the iron pool in the mechanism of action of artemisinin even as ferric ions.

\textbf{Figure 32.} Intensity of EPR spectra (arbitrary unit) recorded with PBN 50 mM for a) hRBCs treated with a stable concentration of DHA (200 $\mu$M) b, c, d, e) with different concentrations (100-400 $\mu$M) of Deferasirox (DFX) respectively.
Figure 33. Intensity of EPR spectra (arbitrary unit) recorded with PBN 50 mM for pRBCs untreated with R406 for 24 hours in combination with a fix concentration of DHA (200 μM) and with different concentrations (100-400 μM) of Deferasirox (DFX). Inlet: example of EPR spectra recorded for pRBC ± deferasirox (400 μM).
DISCUSSION

Most current antimalarial drugs are combinations of an artemisinin plus a ‘partner’ drug from another class and are known as artemisinin-based combination therapies (ACTs). They are the frontline drugs in treating human malaria infections\(^\text{20}\). They also have a public-health role as an essential component of recent, comprehensive scale-ups of malaria interventions and containment efforts conceived as part of long-term malaria elimination efforts. Recent reports have shown that resistance has arisen to artemisinins which has caused considerable concern and the need to produce new antimalarials\(^\text{28,29}\). It has been demonstrated that SYK inhibition in erythrocyte membrane suppresses both band 3 phosphorylation and its loss, contextually it also suppresses the capability of the parasites to complete their life cycle and infect new erythrocytes. Syk inhibitors are a new promising class of antimalarials that have an entirely different mechanism of action from all the currently used antimalarials. Syk inhibitors are slow-acting and it will be an advantage if administered in association with a fast-acting antimalarial drug such as an artemisinin derivative, allowing for both a rapid and prolonged anti-plasmodial activity.

The purpose of this work was to investigate the in vitro combination activity of Syk inhibitors with ARTs\(^\text{53}\). Different Syk inhibitors (P505-15, R406, SYK II, SYK IV, Piceatannol, Entospletinib and Imatinib) have been tested in combination with different ARTs and quinolines in P.falciparum infected RBCs. Syk inhibitors, in combination with a set of artemisinin derivatives widely used for the treatment of malaria, exert a marked synergic effect on the growth inhibition of P.falciparum by a novel mechanism of action. Supporting their therapeutic utilization it should be noticed that all the tested Syk inhibitors caused a 3-5 fold increase in the efficacy of artemisinins for the 50-500 nM concentration range.

Interestingly, this range of concentrations is than plasma concentrations measured in subjects treated with well-tolerated dosages of Syk inhibitors for other diseases. In order to define the mechanism of action to explain the synergic effect of Syk inhibitors on ARTs, we investigated their effect on the accumulation of denatured hemoglobin (HMC) in parasitized erythrocytes. Hemichromes bound to the host cell membrane and the augmentation of dihydroartemisinin (DHA) anti-plasmodial activity has been measured in presence of increasing concentrations of Syk.
inhibitors. A strong correlation exists (R: 0.96) between ROS production from the activation of DHA and hemichrome accumulation at fixed concentrations of the Syk inhibitor. In favor of a causal relationship between hemichromes accumulation and DHA activation it should be noted that both phenomena become evident at the concentration of 500 nM for R406. The accumulation of hemichromes is measurable 3 hours after treatment with R406, this result is also coherent with the time needed to observe the synergic effect of Syk inhibitors on DHA.

The activation of artemisinin has been previously described to be inhibited by iron chelators such as DFX. In the present study we have observed that at a concentration of 400 μM, DFX completely abolishes the synergic effect of Syk inhibitors on artemisinins. This observation strongly indicated that the activation of artemisinins induced by Syk inhibitors is due to the accumulation of accessible iron and or heme bound to hemichromes. Indeed, these data provide further evidence for the importance of the iron pool in the mechanism of action of artemisinin, even as ferric ions. The mechanism of action is based on the reductive cleavage of the endoperoxide bridge by ferrous complexes to form highly reactive O-centered radicals, then C-centered radicals after self-arrangement. This activation depends on Hb uptake and digestion. During the hemoglobin digestion process, four equivalents of [Fe\textsuperscript{II}-heme] are released and oxidized into hematin (Fe\textsuperscript{III}). This is detoxified by the parasite into hemozoin, a highly insoluble microcrystalline form of Fe\textsuperscript{III} (protoporphyrinIX), however a significant quantity escapes polymerization. This free hematin may be responsible for the activation of artemisinin after reduction into Fe\textsuperscript{II} (artemisinin being insensitive to FeCl\textsubscript{3} or hemichromes in absence of RBC\textsuperscript{25,60}.

Hence, an accumulation of Fe\textsuperscript{III} via collection of hemichromes induced by Syk inhibitors would potentiate the artemisinin activity. In conclusion, considering their demonstrated tolerability, Syk inhibitors represent a new class of antimalarial drugs that possess a unique mechanism of action on a non-parasite target; they are also less likely to produce resistant strains and may therefore represent a strategic partner drug for ARTs for counteracting artemisinin resistance.
6 CONCLUSIONS AND PERSPECTIVES

In conclusion, Syk inhibitors enhance artemisinins anti-plasmodial activity leading to a 5 fold decrease of their IC₅₀ values by increasing their efficacy in lower concentrations. Syk inhibitors cause a large accumulation of hemichromes in the parasitized erythrocytes triggering the activation of artemisinins by producing high amounts of ROS and disturbing the mitochondrial functions of parasites leading to their death. Therefore, an accumulation of FeIII via accumulation of hemichromes induced by Syk inhibitors, potentiate the artemisinin activity.

Also, considering the results obtained in vitro at all Syk inhibitors concentrations (50-500 nM), the CI values were consistently below 1, indicating synergy. Interestingly, no other compounds associated to ARTs exist that exert this degree of synergy. The concentrations of Syk inhibitors needed to synergize artemisinins is 5-20 fold lower than the IC₅₀ measured to cause a direct effect on parasite growth. Such concentrations correspond to very well tolerated dosages and can be rapidly reached after oral administration.

Furthermore, Syk inhibitors exert an in vitro synergic activity when used in combination with the most widely used ACTs. Such combinations include DHA with PPQ (commercial name Artecan®), and ATH with LMF (commercial name Coartem®).

In addition, taking into consideration that Syk inhibitors are acting on the host cell membrane instead of the parasite, theoretically this should not lead to the selection of resistant strains and may therefore represent a strategic partner drug for counteracting artemisinin resistance.

In the light of these results our future plan will be the conduction of clinical trials in order to test the efficacy of Syk inhibitors (Imatinib) in combination with the most widely used ACTs on malaria patients.
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