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Trabalho efetuado sob a orientação da
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e da
Professora Doutora Maria Manuel da Cruz Silva

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SUMMARY

Genetic determinants of malaria therapeutics' cross resistance with novel compounds

In 2019, malaria caused half a million deaths worldwide, being elimination hampered by the ability of *Plasmodium falciparum* to evolve antimalarial resistance. The efficacy of artemisinin-based combination therapies (ACT) helped to reduce malaria mortality, however, resistance is a reality. Being ACT efficacy threatened, efforts to define the molecular basis of multidrug resistance and the search for new compound, ideally with new mechanisms of action, are urgently in need. Within the parasite, most of the available antimalarials act at the host's intraerythrocytic stage. Here, many drugs are housed in the digestive vacuole of the parasite with flux promoted by transporter proteins, such as the *Plasmodium falciparum* multidrug resistance protein 1 (PfMDR1), a well-known ACT resistance player.

We explored the interplay of known *pfmdr1* resistance markers, namely, gene copy number variation with N86Y and Y184F single nucleotide polymorphisms to unravel the complex traits that might serve to maximize ACT resistance. Using genomic epidemiology, a global prevalence and temporal changes of *pfmdr1* polymorphisms were assessed and, taking into account the information from this database, through a gene editing approach, we create *in vitro* edited parasite lines to evaluate the impact of these polymorphisms in the kinetics of the transporter. This data provided evidence of specific multicopy PfMDR1 with N86/184F haplotype, geographic selection and expansion in Southeast Asia. The genetic tools created could help on finding drugs with potential of reverting a multidrug resistance phenotype, as the herein explored synthetic compounds derived from steroids, a class of molecules with relevant biological activities. Structure–activity relationship led to the synthesis of steroid derivatives with promising antimalarial activity against the blood stage of the parasite's life cycle with high selectivity and independent of PfMDR1. Exploring possible mechanisms of action of the best compound, revealed induction of oxidative stress inside the parasite and interference with the metabolic process that leads to hemozoin formation inside the digestive vacuole of the parasite.

Overall, the findings presented could help tailor and optimize present antimalarial drug usage by taking into account the regional prevalence of *pfmdr1* polymorphisms and highlights the high potential of the newly developed compounds, thereby underscoring the possibility to develop new antimalarial drugs based on steroids.

Key words: Malaria, Membrane transporter proteins, *Plasmodium falciparum*, Resistance, Synthetic steroids

RESUMO

Determinantes genéticos de resistência cruzada entre a terapêutica da malaria com novos compostos

Em 2019, a malária causou meio milhão de mortes mundialmente, sendo a eliminação dificultada pela capacidade do *Plasmodium falciparum* desenvolver resistência aos antimaláricos atuais. A eficácia da terapia de combinação baseadas em artemisinina (ACT) ajudou a reduzir a mortalidade provocada pela malária, no entanto, a resistência é uma realidade. A eficácia do ACT está comprometida, as tentativas para definir as bases moleculares da multirresistência e a procura por novos compostos, idealmente com novos mecanismos de ação, são urgentes. No parasita, a maioria dos antimaláricos atua no estadio intra-eritrocítico. Muitos são alojados no vacúolo digestivo do parasita através do fluxo promovido por proteínas transportadoras, como a "*Plasmodium falciparum* multidrug resistance protein 1" (PfMDR1), um fator envolvido na resistência aos ACTs. Neste trabalho foi explorada a interação de marcadores de resistência conhecidos no *pfmdr1*, ou seja, variação do número de cópias do gene com polimorfismos no nucleótido N86Y e Y184F para tentar descobrir as características que ajudam a maximizar a resistência aos ACTs. Usando epidemiologia genómica, a prevalência e alterações temporais dos polimorfismos no *pfmdr1* foram avaliadas tendo em consideração a informação obtida desta base de dados, através de edição de genes, geramos *in vitro* parasitas editados para avaliar o impacto desses polimorfismos na cinética do transportador. Isto desvendou evidências acerca do haplótipo específico de multicópias com N86/184F, sobre a seleção geográfica e expansão no Sudeste Asiático. As ferramentas genéticas criadas podem auxiliar na descoberta de fármacos com potencial para reverter o fenótipo de resistência, como os compostos sintéticos derivados de esteróides explorados nesta tese, uma classe de moléculas com atividade biológica relevante. A relação estrutura-atividade levou à síntese de esteróides com uma atividade promissora contra o estadio intra-eritrocítico do ciclo de vida do parasita com grande seletividade e independência do PfMDR1. Os mecanismos de ação do melhor composto foi também explorados, revelando indução de stress oxidativo no parasita e uma interferência no processo metabólico que leva à formação de hemozoína. Concluindo, os resultados apresentados podem ajudar a adaptar e otimizar o uso dos antimaláricos atuais, tendo em consideração a prevalência regional dos polimorfismos no *pfmdr1* e, destaca também o grande potencial dos compostos recentemente desenvolvidos, demonstrando a possibilidade de desenvolver novos antimaláricos baseados em esteróides.

Palavras chave: Esteróides sintéticos, Malária, *Plasmodium falciparum*, Proteínas transportadoras de membrana, Resistência

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LIST OF ABREVIATIONS

ABC transporters - ATP-binding cassete transporters

ADMET – Absorption, distribution, metabolism, excretion, toxicity

ADQ - Amodiaquine

ART - Artemisinin

ACT - Artemisinin-based combination therapy

AS – Artesunate

AS-ADQ - Artesunate-Amodiaquine

AS-MFQ - Artesunate-mefloquine

AS-PND - Artesunate-pyronaridine

AS-SP - Artesunate-sulfadoxine-pyrimethamine

ATM – Artemether

ATM-LMF - Artemether-lumefantrine

ATQ – Atovaquone

CNV – copy number variation

CQ – Chloroquine

DHA - Dihydroartemisinin

DHA-PPQ - Dihydroartemisinin-piperaquine

DHFR - Dihydrofolate reductase

DHODH - *Plasmodium* dihydroorotate dehydrogenase

DHPS - Dihydropteroate synthase enzyme

DMAPP - Dimethylallyl diphosphate

DV - Digestive vacuole

ELC - Elacridar

Fe(II)PPIX - Protoporphyrin IX

FIC – Fractional inhibitory concentration

FPP - Farnesyl pyrophosphate

G6PD - Glucose-6-phosphate dehydrogenase

GFP – Green fluorescent protein

GSK – GlaxoSmithKline

Grx1 – Glutoredoxin-1

Hb – Hemoglobin

hDHFR - human dihydrofolate reductase

Hz - Hemozoin

H_2O_2 - Hydrogen peroxide

HF – Halofantrine

HO - Hydroxyl radicals

IC_{50} - half-maximal in vitro growth inhibitory concentration

IPP - Isopentenyl diphosphate

LMF – Lumefantrine

LogP – partition coefficient

MFQ – Mefloquine

MMV - Medicines for Malaria Venture

NBD - Nucleotide binding domain

PfAtp4 - P-type ATPase 4

PfCRT - *P. falciparum* chloroquine resistance transporter

PfGDV1 - P. falciparum gametocyte development 1

PfK13 - *P. falciparum* kelch 13 protein

PfMDR1 - *P. falciparum* multidrug resistance transporter 1

PfPI4K - Phosphatidylinositol 4-kinase

PG - Proguanil

PMQ - Primaquine

PND - Pyronaridine

PPQ – Piperaquine

PSA – Polar surface area

PYR – Pyrimethamine

QN – Quinine

SAR - Structure-activity relationship

SDX - Sulfadoxine

SNP - single nucleotide polymorphism

TACTs - Triple artemisinin-based combination therapies

TMD - Transmembrane domain

VP - Verapamil

WHO - World Health Organization

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CHAPTER I: GENERAL INTRODUCTION

1. Malaria epidemiology

Malaria is a major cause of illness worldwide, being responsible for approximately half a million deaths per year. According to the World Health Organization (WHO) in 2019, an estimated 229 million cases of malaria occurred worldwide compared with 228 million cases in 2018. Most malaria cases in 2019 were in African regions (215 million or 94%), followed by the Southeast Asia regions with 3% of the cases, the Eastern Mediterranean region with 2% and the American region with 0.4% (Figure 1). Twenty-nine countries accounted for 95% of the global malaria burden. Five countries accounted for more than half of all malaria cases worldwide: Nigeria (27%), the Democratic Republic of Congo (12%), Uganda (5%), Mozambique (4%) and Niger (3%). Concomitantly, in 2019 there were an estimated 409 000 deaths from malaria globally, compared with the 405 000 estimated deaths in 2018, and the 585 000 in 2010 (Figure 2). Children aged under 5 years are the most vulnerable group affected by malaria. In 2019, they accounted for 67% of all malaria deaths worldwide.

Despite these numbers, a great progress in the malaria incidence rate has been achieved in recent years. The Southeast Asia region continued to see its incidence rate falling from 17 cases per 1000 people at risk in 2010 to 4 cases in 2019 (a 78% decrease). In the African region, case incidence levels also declined from 294 in 2010 to 225 in 2019, representing a 22,5% reduction. Also, the African and the Southeast Asia regions showed reductions in malaria deaths compared with 2010. The WHO African region had the largest absolute reduction in malaria deaths, from 542 000 in 2010 to 384 000 in 2019. Additionally, in Southeast Asia a reduction from 38 000 in 2010 to 9 000 malaria deaths was observed (1). This observed decline is mainly attributed to the introduction of artemisinin (ART)-based combination therapies (ACTs) as well as insecticide-treated nets. However, the decreased incidence rate in the last 5 years slowed considerably (1), with past gains being threatened by drug resistance and other diseases such as the COVID-19 pandemic leading to more mortality in 2020 and potentially leading to even greater increases in subsequent years (2).

The disease thrives mainly in tropical and subtropical areas, where climatic conditions for its propagation are optimal and coincide with countries where poverty is prevalent (3), leading to a major threat to the economic and social development of these regions, with nearly US$ 3.0 billion invested in malaria control and elimination in 2018 (1). Poverty can greatly affect the malaria impact due to hampering the availability of treatment and preventive measures, and also making treatment compliance harder.

Early diagnosis and prompt treatment are two important factors to control malaria. However, emerging insecticide resistance, climate change, and widespread antimalarial drug resistance have been slowing the progress in malaria endemic countries.

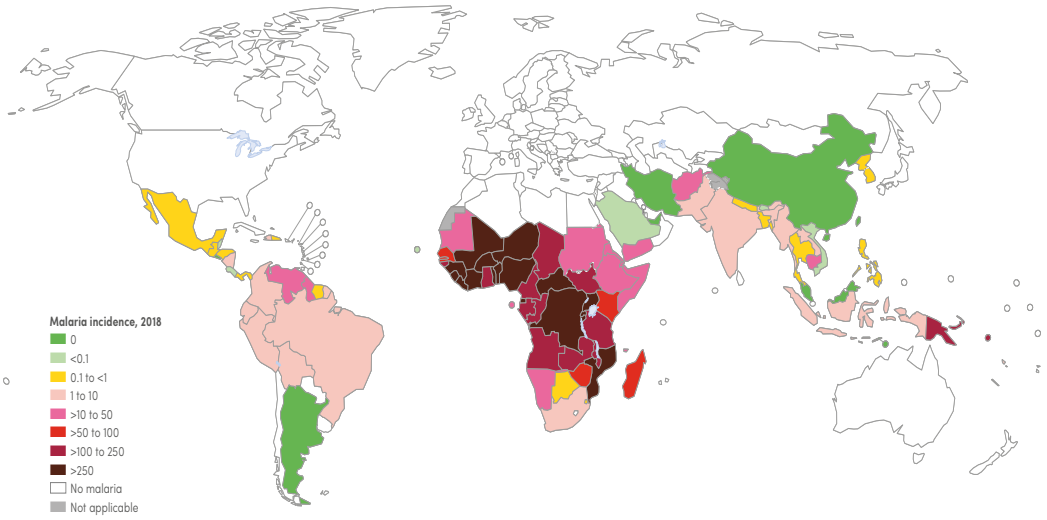


Figure 1: Map of malaria case incidence rate (cases per 1000 population at risk) by country, 2019. (With Permission from WHO report 2020 from (1)).

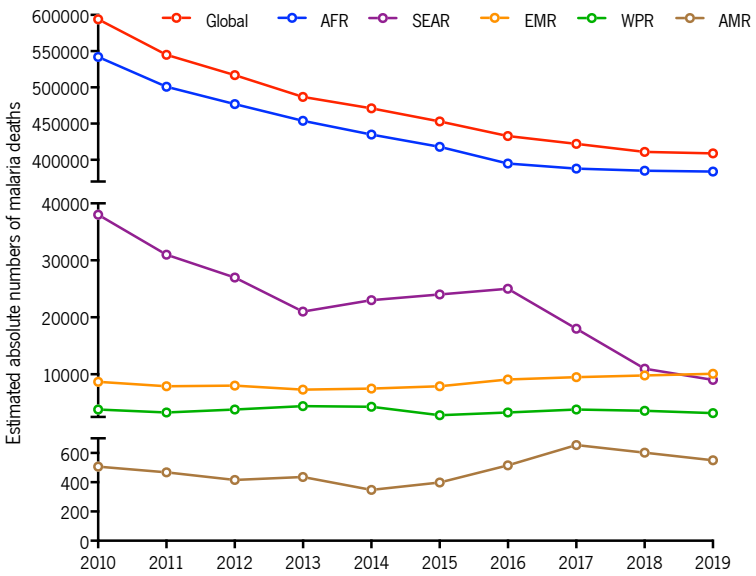


Figure 2: Estimated absolute number of malaria deaths in the WHO regions from 2010 to 2019. The data was extracted from the WHO's World Malaria Report 2020 (1).

AMR: WHO Region of the Americas; EMR: WHO Eastern Mediterranean Region; SEAR: WHO Southeast Asia Region; WHO: World Health Organization; WPR: WHO Western Pacific Region, AFR: WHO Africa Region.

1.1. Malaria infection and transmission

Malaria is a vector-borne infectious disease caused by the protozoan, apicomplexan parasite from the genus *Plasmodium*, which is transmitted between humans through the bites of infected female *Anopheles* mosquitoes. There are several *Plasmodium* species that can infect a wide variety of organisms but only six species are documented to cause disease in humans: *P. falciparum*, *P. knowlesi*, *P. malariae*, *P. ovale*, *P. vivax* and *P. simium*. The majority of infections are caused by *P. falciparum* and *P. vivax,* being *P. falciparum* the main responsible for mortality and the most associated with malaria drug resistance (4, 5). *P. vivax* and *P. ovale* have a latent form called the hypnozoite, residing in the liver and can be reactivated months or years later to initiate a relapse infection (6). *P. knowlesi* infects macaques and is primarily a zoonosis (4).

1.1.1. *Plasmodium falciparum* life cycle

Malaria infection results from a complex interplay between its human host, characterized mainly by a pronounced asexual multiplication, and the *Anopheles* mosquito vector, where the sexual stage of development occurs (Figure 3) (7). The dynamics between these elements is an important factor affecting malaria transmission. In the human host, the infection starts when the sporozoites transmitted from a mosquito blood meal travel through the skin into the bloodstream. These sporozoites migrate into the liver invading hepatocytes. After 1 week of development in the liver, the sporozoites produce thousands of mononucleated merozoites that are released into the bloodstream where they rapidly invade erythrocytes and start an intraerythrocytic 48-hour replication cycle. The merozoites, in the erythrocytes, pass through different morphological forms, from ring (early trophozoites), late trophozoites and schizonts containing multiple merozoites. The infected erythrocytes ultimately lyse at the schizont stage, releasing the merozoites that are ready to infect new erythrocytes. This rupture causes the clinical manifestations of malaria which comprise chills, fever, and anemia and in the most severe cases characterized by metabolic acidosis, respiratory distress, cerebral malaria, coma and death (4). One to two% of the merozoites do not undergo the intraerythrocytic replication cycle, but instead, differentiate into gametocytes. Gametocytes, which do not cause clinical symptoms, can be taken by a mosquito during a blood meal. Once in the mosquito's mid-gut, the male and female parasite gametes form the zygote, which develops into an ookinete that penetrates the gut wall, differentiating into an oocyst. Inside the oocyst, through mitotic division, multiple sporozoites are produced. When the oocyst ruptures, sporozoites are released and migrate to the mosquito salivary glands, ready to infect a new human host and re-starting the parasite's life cycle (5).

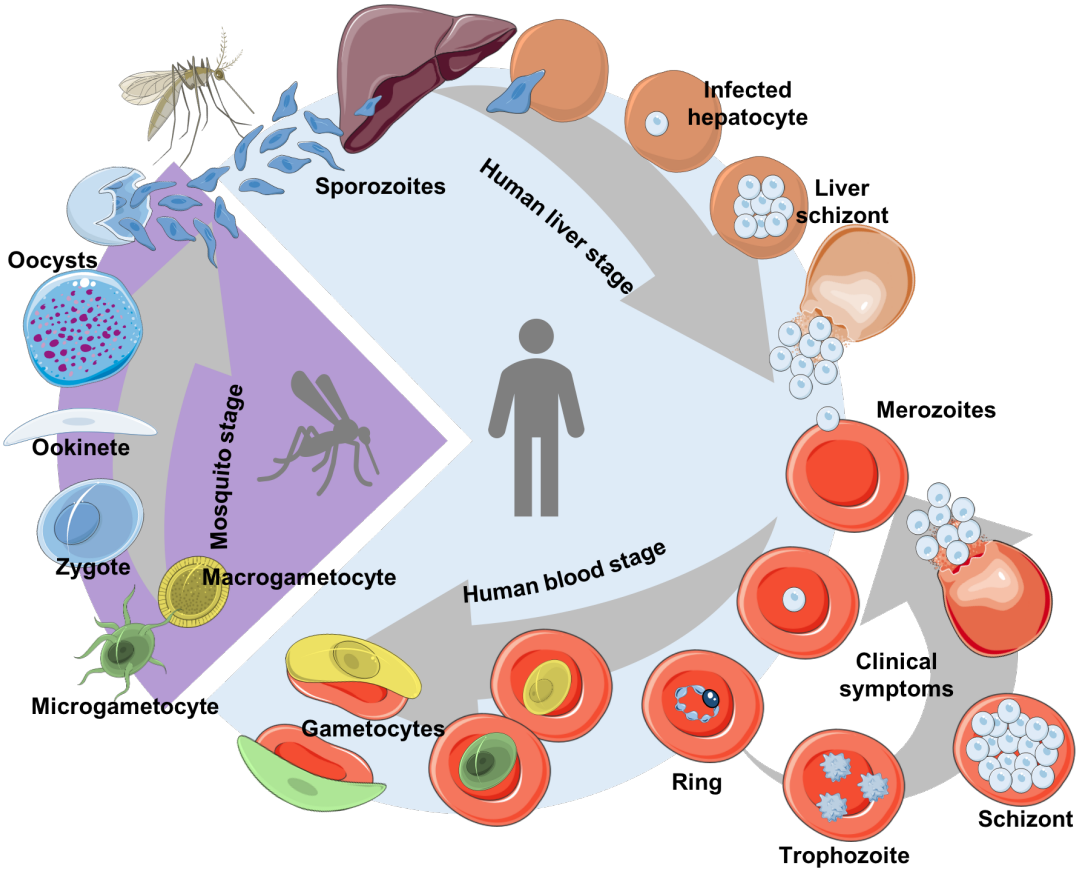


Figure 3: *Plasmodium falciparum* life cycle. The main phases of the parasite's life cycle: liver and blood stages, both in the human host, and mosquito stages (in the gut and in the salivary glands of the mosquito) are illustrated. (Own authorship)

1.2. Strategies for malaria control and elimination

Malaria control and elimination programs already implemented aim to target the *P. falciparum* propagation and reduce malaria-related morbidity and mortality. Globally, in the last years there has been a substantial investment in malaria control and elimination by the governments of malaria endemic countries and international partners. The development of resistance and non-biological factors including affordability and distribution logistics, counterfeit or low-quality drugs, patient non-compliance and inappropriate use of monotherapies, are some of the factors that complicate treatment and compromise the eradication goals (7).

Control and elimination of malaria comprises several strategies that act against the mosquito vector and the human host. The mosquito vector is essential for malaria transmission and thus, trying

to control and eliminate this vector is an essential tool in fighting malaria. Nowadays, vector control is achieved by limiting propagation, using long lasting insecticide treated bed nets and indoor residual spraying (1). As referred, this control and elimination also includes actions targeting the human host such as prompt diagnosis, early chemotherapeutic treatment and intermittent preventive treatment of risk groups (e.g. pregnant women) (1).

Malaria chemotherapy is an important tool in the control of malaria and focuses mainly on the blood stage of the parasite's life cycle, which is the symptomatic stage responsible for the mortality associated with malaria. However, the emergence and spread of resistance to almost all antimalarial drugs used in treatment, greatly challenges current malaria control and elimination strategies (4). Therefore, the discovery and development of new therapeutic tools, with clinical effectiveness, are extremely important and will allow the replacement of antimalarials drugs rendered ineffective by increased parasite resistance.

1.3. Drugs for malaria treatment

1.3.1. Quinoline drugs

The application of chemotherapy to treat malaria dates back to the 17th century with the use of natural extracts from the *Cinchona* bark for the treatment of fevers. Later in the 19th century the most active compound present in these extracts was isolated and named quinine (QN). This compound has been used as a basic structure for the synthesis of related compounds, such as chloroquine (CQ), piperaquine (PPQ), amodiaquine (ADQ), mefloquine (MFQ) and pyronaridine (PND) (8). All these compounds are designed as quinoline antimalarial drugs and have in common the presence of a quinoline ring system. Halofantrine (HF) and lumefantrine (LMF) have less related structures but still belong to this class of compounds (9, 10) (Figure 4).

All these compounds can be divided into two subclasses of quinolines: the aminoquinolines – CQ, ADQ, PND and PPQ – and the aryl-aminoalcohols – MFQ, QN, HF and LUM. Due to the related structures present in all these compounds, it is thought that they have similar mechanisms and act on similar pathways (8, 10) (Figure 4). It has been proposed that some of these compounds such as CQ, ADQ and PPQ act on the parasite's heme detoxification system (8, 10, 11). During the blood stage of the parasite life cycle, the parasite ingests large quantities of hemoglobin (Hb) through an endocytosis process. These vesicles containing Hb are transported to the digestive vacuole (DV), an acidic lysosome, and in the DV the parasite degrades the Hb as a source of aminoacids required for its maturation and growth, since it has a limited ability to synthetize its own (12, 13). A group of aspartic

proteases (plasmepsins) and cysteine proteases (falcipains) are involved in Hb hydrolysis leading to the release of globin moieties that are further degraded into peptides to provide amino acids for protein synthesis. However, Hb degradation leads to the release of toxic iron protoporphyrin IX [Fe(II)PPIX] which is quickly oxidized forming reactive Fe(III)PPIX. Reactive free heme is toxic for the parasite (14, 15), since it has the ability to cause lipid peroxidation, destabilize membranes by a colloid osmotic mechanism and induce oxidative stress (leading to the production of reactive oxygen species including hydroxyl radicals (HO·) and hydrogen peroxide (H_2O_2)). To mitigate this toxicity, Fe(III)PPIX is biomineralized by the parasite, into an inert crystal known as hemozoin (Hz) (16). Quinolines such as CQ and ADQ are weak bases and are deprotonated at the physiological pH levels. However, inside the DV, where the environment is acidic, CQ becomes di-protonated and accumulates (15, 17). Therefore, CQ binds to the reactive free heme disrupting the detoxification process involved in the formation of inert Hz (11, 15, 18). However, how quinolines antimalarial drugs target heme to disrupt Hz formation remains unclear. It is also not well understood whether the different quinolines inhibit Hz via similar or different pathways. As recently reviewed, some studies provide data that demonstrated that quinolines possibly inhibit Hz crystallization by the interaction with one or more forms of uncrystallized Fe(III)PPIX. At least three heme forms exist as Hz precursors of the Fe(III)PPIX monomer, the μ-oxo dimer, or Fe-O41 reciprocal "head-to-tail" dimers and multiple drug-heme binding events occurred. In the μ-oxo dimer the O (from H_2O) bridges two monomers via a nearly linear Fe-O-Fe bond and a "head-to-tail" dimer is formed by linking the proprionate carboxyl group to the ferric iron of the adjacent heme. The heme dimer is then stacked by hydrogen-bonding interactions into the crystal (19–21). Resistance to quinoline antimalarials arises from mutations in membrane transporter proteins, present in the DV membrane, and not from changes in the Hz formation. Therefore, this target remains extremely valuable for the development of novel antimalarials (20).

The mechanism of action of the LUM, MFQ and QN (the aryl-aminoalcohols group), is not well understood as that of the 4-aminoquinolines. However, this type of compounds can not only interfere with Hz formation but also with detoxification of Hb degradation by-products (7, 22) (Table 1).

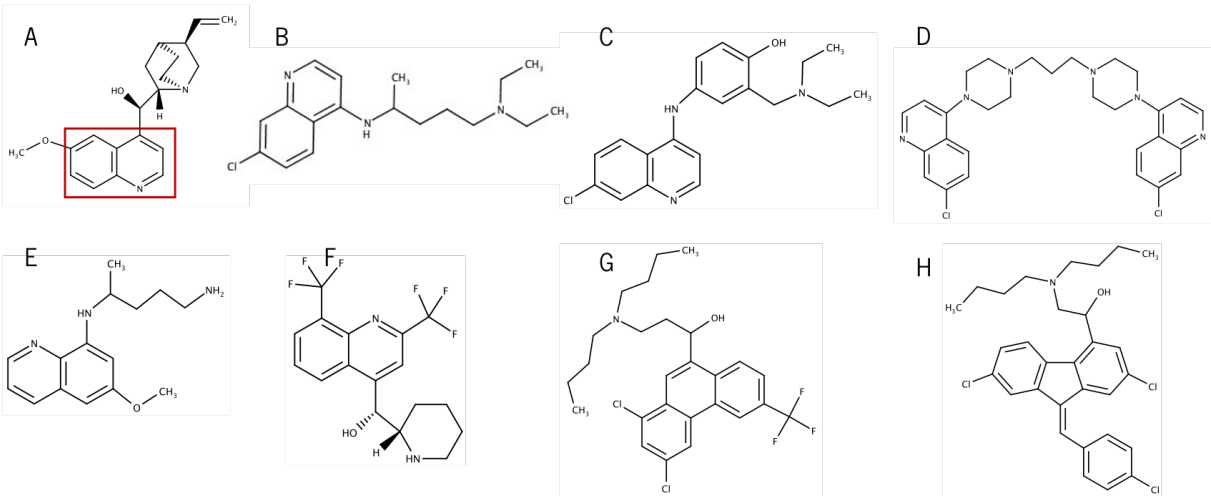


Figure 4: Structures of the aminoquinoline and aryl-amino alcohols used as antimalarials. A- Quinine; B- Chloroquine; C- Amodiaquine; D- Piperaquine; E- Primaquine; F- Mefloquine; G- Halofantrine; H- Lumefantrine. The quinoline ring is highlighted with a red rectangle.

Also, inside the quinoline family there is another class of compounds, the 8-aminoquinolines, that also contain quinoline ring.

High-throughput screening assays led to the identification and development of compounds that act on mature gametocytes to prevent the transmissibility of the disease without exerting selective pressure on the blood stage of the parasite's life cycle that could culminate in the development of resistance (8, 23). Example of this type of drugs is the 8-aminoquinoline primaquine (PMQ) (Figure 4) and Tafenoquine, a next-generation 8-aminoquinoline. Nowadays PMQ is an approved antimalarial drug that prevents relapse in *P. vivax* and *P. ovale* infections and also has potency against gametocytes in *P. falciparum* infections. A single low dose of primaquine with current therapies have been applied in low-transmission areas in order to reduce the transmissibility of drug-resistant parasites. However, the use of this drug has been limited since the risk of toxicity in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency due to the hemolytic toxicity is high (24). Tafenoquine, a long-acting analogue of PMQ, has proved activity against the liver and blood stages forms as well as gametocytes of *Plasmodium* species including *P. vivax* and *P. falciparum*. Results from clinical trials revealed that this drug has an efficacy similar to that of PMQ but interestingly, could be administrated in a single dose, possibly increasing patient compliance (4, 25).

1.3.2. Antifolates, naphthoquinone and antibiotics

Another class of antimalarial drugs already in use to treat malaria are antifolates. These compounds inhibit and interfere with important enzymes involved in the parasite's folate biosynthesis

pathway. This pathway is relevant for several biological processes including cell division and DNA synthesis and, this feature has been crucial for the development of antifolate compounds. Nowadays, there are some drugs such as sulfadoxine and pyrimethamine mainly used in the combination, sulfadoxine-pyrimethamine. Sulfadoxine targets the dihydropteroate synthase (DHPS) enzyme and pyrimethamine targets dihydrofolate reductase (DHFR) both involved in the folate biosynthesis pathway (26). The inhibition of these enzymes culminates in a decreased formation of tetrahydrofolate, an important cofactor involved in the production of folate precursors. The reduced levels of tetrahydrofolate leads to decreased methionine synthesis and lower thymidylate levels culminating in a disturbed in the biosynthesis of nucleotides and subsequent DNA synthesis (26, 27). Currently, sulfadoxine-pyrimethamine treatment is largely used in intermittent preventive treatments during pregnancy and with infants (28).

The cytochrome *bc*1 complex is a relevant enzyme of the respiratory electron transfer chain being a validated drug target for malaria control. This electron transport-chain is extremely important in *Plasmodium* sp., since parasite does not have the enzymatic components required to salvage pyrimidines from their metabolism and, therefore, the need to perform *de novo* pyrimidine synthesis in order to survive (29, 30). Currently, atovaquone, a naphthoquinone, is a competitive inhibitor of cytochrome *bc*1 (31). Emergence of resistance to this drug appeared rapidly and, in an attempt to improve its efficacy, atovaquone is currently used in combination with the antifolate proguanil Malarone™ (32). This combination has been used as a prophylactic treatment for travelers to malaria-endemic regions. Furthermore, this type of treatment is efficacious to treat children and adults containing uncomplicated malaria. However, the treatment's high cost has limited its use in malaria endemic countries. When used alone, proguanil has lower activity and no effect on the membrane potential. However, when combined with ATQ, a synergistic interaction leads to the collapse of the mitochondrial membrane potential (8).

Among the compounds potentially used against *Plasmodium*, antibiotics, including clindamycin and doxycycline, have also been studied. Clindamycin is one example and is currently used in prophylaxis and in combination with quinine (33, 34). Clindamycin inhibits protein translation inside the apicoplast through its binding with ribosomal 23S (34). Furthermore, doxycycline, a synthetically derived tetracyclin, has been used in prophylaxis for non-imunne travelers also in combination with quinine. This slow acting blood schizontocidal agent is highly effective for the prevention of malaria (35). Similar to clindamycin, doxycycline also has the ability to interfere with protein synthesis by binding to the 16S rRNA (8).

1.3.3. Artemisinin and derivatives

The plant *Artemesia annua* sweet wormwood, has been used for more than 2000 years against fever in traditional Chinese medicine. Youyou Tu and her team performed extraction of components from this plant, leading to the discovery of ART. This achievement was recognized with the 2015 Nobel Prize in Physiology and Medicine. ART is a sesquiterpene lactone containing an endoperoxide bridge that appears to be important for its antimalarial activity (36). ARTs do not have a good pharmacokinetic profile, presenting low solubility as well as a very short *in vivo* half-life of approximately 1 hour. Because of these characteristics, semi-synthetic derivatives were synthesized including artesunate (AS), artemether (ATM) and dihydroartemisinin (DHA), with improved bioavailability and efficacy (Figure 5) (37, 38) and all these derivatives are converted into DHA *in vivo*. These class of drugs act rapidly effecting up to 10 000- fold reductions in parasite burden every 48 h and are effective at early phases of the blood stage of the parasite's life cycle (37).

The mechanism of action of ART remains unclear but seems to involve the activation of the endoperoxide bridge by free iron present inside the parasite, Fe(II)PPIX, as a side product of Hb digestion. After ART activation, this drug alkylates several biomolecules including lipid and proteins culminating in the formation of more reactive oxygen species (ROS) that eventually leads to parasite's death (37) (Table 1).

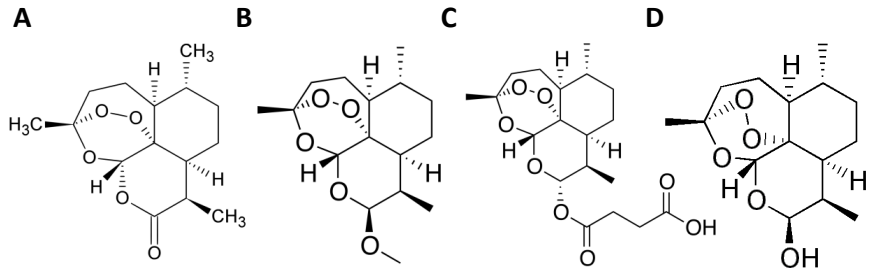


Figure 5: Chemical structure of artemisinin family of antimalarial drugs. A - Artemisinin (ART), B – Arthemether (ATM); C – Artesunate (AS); D – Dihydroartemisinin (DHA).

1.4. Artemisinin-based combination therapy

As described above ARTs are extremely fast-acting drugs against the intraerythrocytic stage of parasite's life cycle, however they also have a very short *in vivo* half-life of approximately 1 hour. Therefore, ARTs are nowadays administrated in combination with longer half-life partner drugs that have unrelated structures, are more slowly eliminated *in vivo* and a different mechanism of action, being designed as Artemisinin-based combination therapy (ACT) (39). These combination therapies work due to the ability of artemisinin to rapidly reduce the parasite biomass (up to 10 000-fold every 48 hours)

leading to a rapid clinical response and a rapid relief of symptoms, leaving the partner drug with a relative low number of remaining parasites to eliminate (40) (Figure 6). The poor pharmacokinetic proprieties of ART and its derivatives, mainly the short half-life, leads to substantial treatment failure rates when used as monotherapy and, because of this, combining ART with a longer-partner drug ensures a continuous pressure after the plasma concentration of ART have decreased below therapeutic levels. This helps prevent recrudescence, which can occur in ART monotherapy (40, 41) and reduces the selective pressure on the partner drug since they encounter less parasites in the patient (39, 42, 43).

Therefore, in 2005, ACTs were implemented as the treatment for malaria in all endemic countries, a policy recommended by the WHO in order to increase the efficacy of malaria chemotherapy, while delaying the emergence of drug resistance. The most used ACTs are: artesunate-amodiaquine (AS-ADQ), artemether-lumefantrine (ATM-LMF), artesunate-sulfadoxine-pyrimethamine (AS-SP), artesunate-mefloquine (AS-MFQ), dihydroartemisinin-piperaquine (DHA-PPQ) and artesunate-pyronaridine (AS-PND) (1).

In low-transmission areas, a single dose of primaquine combined with ACT is provided to patients with *P. falciparum* in order to reduce transmission. This combination could be particularly important in highly endemic regions, where *P. falciparum* infections are often asymptomatic or untreated and, the clinical symptoms tend to develop late in the course of infection, allowing gametocytes to mature and continue the transmission cycle (5).

In severe cases of malaria, adults and children are treated with intravenous or intramuscular AS during 24h. After this time, when patients can tolerate, an oral therapy the treatment is followed with 3 days of ACT (1, 44).

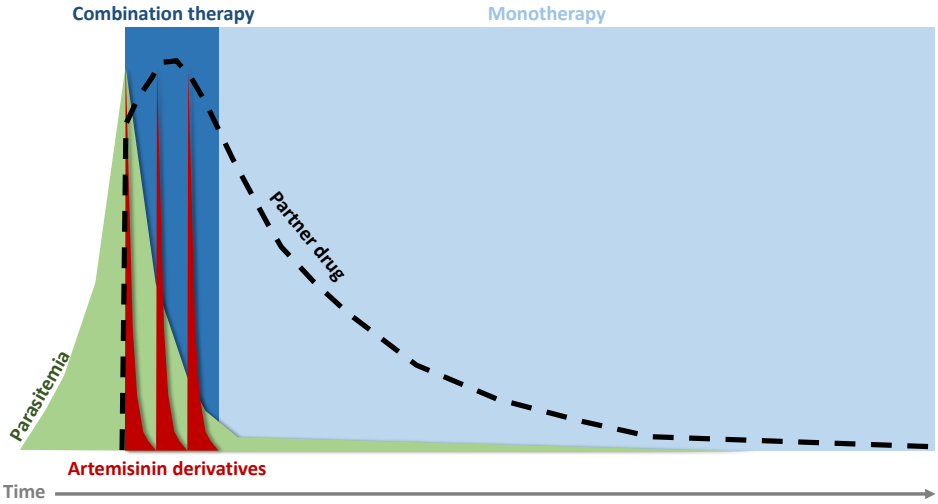


Figure 6: Schematic representation of parasite clearance dynamics and drug elimination dynamics in Artemisinin-based combination therapy. Following 3 days of treatment, the parasitemia (green) is rapidly reduced predominantly by the highly potent yet rapidly eliminated artemisinins (plasma level represented in red). The remaining parasites that have not been cleared by ART will be cleared by the long half - life partner drug (dashed black line representing plasma level) remaining as monotherapy (light blue) during an extended period, that could enable the selection of partner-drug-resistant parasites.

1.5. Development and mechanisms of drug resistance

When CQ was discovered, its implementation into the clinics led to an increased expectation in malaria eradication. The use of CQ, a fast-acting and inexpensive compound, along with the insecticide dichlorodiphenyltrichloroethane (DDT) culminated in a drastic decrease in the incidence of malaria worldwide. However, resistance to CQ emerged causing a higher decline in its therapeutic efficacy. Subsequently, different drugs were adopted, like, the antifolates (sulphadoxine– pyrimethamine), as the treatment to replace CQ (9, 39). In some areas, this combination was replaced with MFQ or QN, resulting in the appearance of multidrug-resistant parasites mainly in Southeast Asia. Also, resistance to PPQ and ADQ as monotherapy was reported (8) (Figure 7). As a result, mortality and morbidity increased and in the middle of 1990 ACTs were introduced because an imminent possibility of untreatable malaria was presented itself in Southeast Asia since resistance to all antimalarial drugs had already been detected (45) (Figure 7). Despite all the efforts made to introduce ACT as antimalarial treatment, resistance emerged in some endemic regions threatening the global malaria control and elimination. Resistance to ART was first reported in Southeast Asia, mainly in Cambodia, where patients with *P. falciparum* exhibited prolonged parasite clearance rates following AS or ACT treatment (46, 47). Treatment of patients with sensitive parasite strains to ART or its derivatives results in a 10 000 times reduction in parasite biomass every 48 hours. In artemisinin-resistant infections there is only 100-fold

decrease in parasitemia every cycle (48). Artemisinin resistance manifests as increased survival and a delayed clearance of young ring-stage parasites (47). Clinical resistance to artemisinin has been defined clinically by an increased parasite clearance time and recrudescence and *in vitro* by parasites having longer ring state survival after 3 hours of artemisinin exposure (49, 50). Since then, the spread of resistance to other regions of Southeast Asia has been found in the Western border of Thailand (51), Western Cambodia (52), Southern Myanmar (53) and Vietnam (45, 54). Since the parasites already developed resistance to several partner drugs, treatment failure has been observed with combination therapies, ACTs. Although still clinically effective, failure rates for ACTs are rising. In the Cambodia-Thailand area, a delay in parasite clearance and treatment efficacy rates of less than 90% has been reported (1). In the Asian region, AS-MFQ treatment was replaced by DHA-PPQ since higher treatment failure rates were detected, leading to this policy change. However, currently the most problematic situation is the rapid increase in failure rates for this regimen, DHA-PPQ, which is the most applied therapy in the Southeast Asian region (51, 55–60). The rise of DHA- and PPQ-resistant parasites threatens the positive progress observed nowadays in malaria reduction, highlighting the need for new interventions (22) (Figure 7).

Most malaria cases occur in Sub-Saharan Africa, but South America has historically been considered as a hotspot outside Southeast Asia for the evolution of antimalarial drug resistance. The Guiana Shield region of South America, including Guyana, French Guiana, Suriname and parts of Brazil is important as a potential source of emerging antimalarial drug resistance. In 2013, one research group reported a delay in the parasite clearance in patients participating in a therapeutic efficacy study in Suriname. This study raised concerns about the efficacy of ART in the Guiana Shield Region. Although ACTs remains effective, ART tolerance has been detected in Guyana. In this region ATM-LMF plus AMQ was introduced as antimalarial treatment. One study demonstrated that mutant *pfK13* has emerged independently in this region, with genomic analysis suggesting an evolutionary origin different from Southeast Asia. These evidences suggested that, besides Southeast Asia, South American should also be considered for the evolution of development and spread of antimalarial resistance (1).

In the African region, ART partial resistance has not yet been confirmed and the treatments used in most African countries, ATM-LMF, AS-ADQ and DHA-PPQ, still exhibited high efficacy rates (higher than 95%) (1, 61). Concerning other regions, American and Eastern Mediterranean regions, ATM-LMF treatment still remains successful (1).

The emergence of multidrug resistance is a reality and forces a change in the treatment policies but fewer options for *P. falciparum* treatment are available for implementation in endemic malaria

countries. Nevertheless, new compounds will not be available within the next few years (62). Nowadays the major concern is the spread of resistance to the Indian subcontinent and sub-Saharan Africa, since in the past, the CQ and sulfadoxine-pyrimethamine resistance that emerged in Southeast Asia spread to sub-Saharan Africa leading to millions of deaths (63–66). In order to prevent a worldwide spread of ACT resistance, constant monitoring of treatment efficacy is required. Furthermore, there is an urgent need to disclose and understand the genetic factors that underline the emergence and spread of resistance.

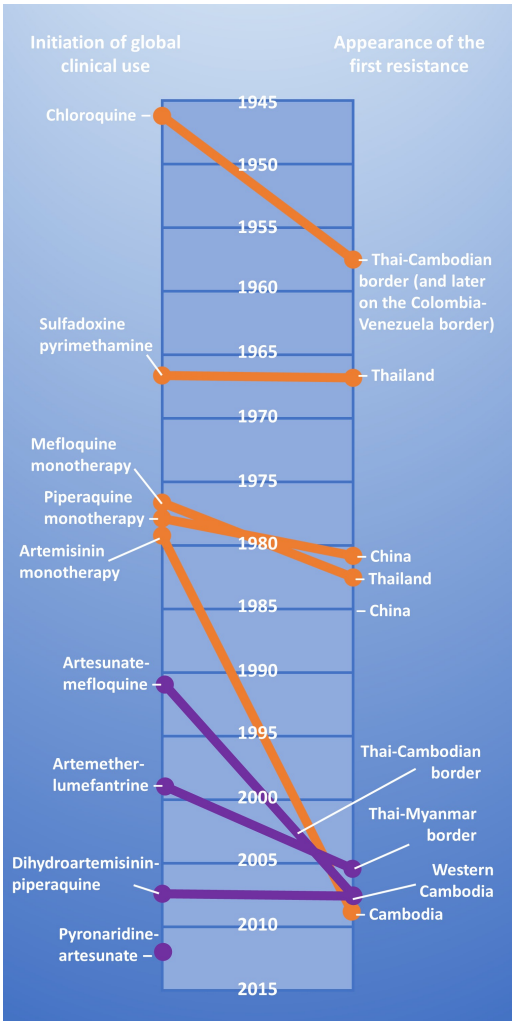


Figure 7: The timeline of introduction of the main antimalarials implemented to treat malaria and the first emergence of resistance in the field. Monotherapy- represented by orange lines; combined therapy represented by purple lines. The left side indicates when antimalarials were firstly implemented in the field and the right side indicates the year and place where resistance first appeared (8). (Own authorship)

1.5.1. Molecular modulators of malaria parasite resistance

Multiple factors lead to the emergence of parasite resistance mainly due to the complex interaction between the mosquito, human, parasite and antimalarial treatment. The human population

has an important role and factors such as treatment compliance, low pharmacokinetic properties associated with antimalarial drugs and the complexity of the human immune system can result in the emergence of resistance and affect the efficacy of the treatment (7). Also, parasite-related factors that lead to the emergence of resistance are their mutation rates, the infection load and the fitness cost linked with these mutations. Also, an inadequate drug exposure due to improper dosing or counterfeit drugs can lead to the development of drug resistance. In this situation, the parasites exposed to suboptimal drug pressures decrease the prevalence of sensitive parasites, but more tolerant parasites will survive and through one or more mechanisms they acquire the ability to adapt to this stress and overcome the drug's effect, thus becoming resistant (7, 67, 68).

Several molecular mechanisms have been associated with drug resistance phenotypes. In *P. falciparum*, a well-known mechanism involved in resistance is the elimination of the antimalarial drug from its place of action. This is achieved by promoting drug elimination or by preventing drug accumulation, either by augmented export or reduced import, a process mediated by membrane transporter proteins. Furthermore, alterations in the drug target and development of compensatory cellular mechanisms (69) can also contribute to the appearance of resistant parasites (70).

The movement of solutes across the different compartments in the *P. falciparum* is mediated by membrane transporter proteins, which have been considered important targets since they participate in obtaining crucial nutrients and eliminate toxic waste. Due to their biological functions, membrane proteins have also been associated with antimalarial drug resistance since, for instance, the parasite could acquire mutations that decrease drug affinity and efficacy (if the transporter is the target) and, could acquire mutations that decrease drug levels in the site of action (if the transporter works as a delivery route) (71).

So, to define their substrate's specificity, subcellular localization as well as the effect of modifications on their sequence or expression levels is crucial to obtain a complete understanding of the role of these transporter proteins, in order to overcome drug resistance. In *P. falciparum* two important transporter proteins involved in drug resistance are the *P. falciparum* multidrug resistance transporter 1 (PfMDR1) and *P. falciparum* chloroquine resistance transporter (PfCRT).

PfMDR1 and PfCRT are both localized in DV of the parasite and are respectively responsible for the intrusion and extrusion of solutes within this organelle, including drugs. The DV is an important organelle during the development of the parasite's life cycle since it is the place where the degradation of host-derived Hb occurs (an important step as a nutrient source). Furthermore, it is the site of action of some antimalarial drugs implemented to treat malaria, being a proven therapeutic target and could

also be a site where the parasite accumulates the drug removing it from its place of action, leading to resistance (72). Therefore, it is relevant to remain focused on these transporters that promote this flux and are long know players in antimalarial multidrug resistance. Therefore, studies that allow the verification of their impact on the antimalarials in use, but also on the formulation of new compounds is extremely relevant.

PfMDR1 belongs to the ATP-binding cassete (ABC) transporters family. These transporters are constituted by two transmembrane domains (TMD), each with six transmembrane helices (TM), and two nucleotide binding domains (NBD). ATPase subunits present in NBD domains hydrolyze ATP in order to promote conformational changes and provide the energy for the transport of solutes across membranes (either uptake or export) (73). PfMDR1 was discovery by Foote and collaborators in 1989 (74) and was identified during the search for a homolog of the mammalian P-glycoprotein (P-gp), a protein associated with drug resistance in mammalian tumor cells and with ability to mediate the transport of a several chemotherapeutic agents (69). Due to its similarity with P-gp, it was initially hypothesized that PfMDR1 was the major player involved in CQ resistance (74–76). This hypothesis emerged from the evidences that verapamil, a calcium channel blocker compound, was capable of reversing CQ resistance as observed previously in cancer cells (77, 78). However, this hypothesis was proved not to be consensual and seems that the PfMDR1 protein is able to modulate the response to CQ depending on the parasite's genetic background (76, 79, 80). The gene *pfmdr1* is localized in chromosome 5 and encodes an ATP-binding cassette protein with 1419 amino acid and 162-kDa, that resides in the parasite's DV membrane (74, 81). This protein contains two homologous halves, each with 6 transmembrane helices (74) with its ATP-binding domain facing the cytosol (81, 82) and transport is predicted to be inwardly directed toward the DV (Figure 8).

The other important mediator referred above is the PfCRT. The discovery of PfCRT transporter was achieved by the analysis of a genetic cross between a CQ-sensitive and a CQ-resistant clone in order to identify the molecular mechanism involved in CQ resistance (83). PfCRT is a transmembrane transporter with 424 amino acids containing ten transmembrane helices and also localized on the membrane of the DV of the parasite (84). PfCRT has been identified as a secondary active transporter, depending on a proton gradient and the membrane potential generated by the V-ATPase in the DV membrane (85) (Figure 8).

Besides the role of these two transporters explored in this thesis, there is not only other transporter proteins, but also other crucial molecular markers that modulate the parasite's response. One of the most relevant markers associated with ART resistance, defined by a delayed parasite

clearance time, is the *P. falciparum* kelch 13 protein (PfK13) (86, 87). Several hypotheses implicated the PfK13 as a responder to downstream effects of ART activation, especially in up-regulation of pathways involved in the cellular stress response (88). PfK13 is localized in vesicles close to cytostomes, which are erythrocyte-cytosol containing structures used by the parasite in the uptake of Hb (86). Recently, it has been described that PfK13 regulates the degradation, uptake of Hb and DV biogenesis leading to an enhanced ring-stage survival (89). Parasites with mutated or inactivated PfK13 were found to exhibit reduced Hb endocytosis and thereby ART activation, culminating in parasite resistance since ARTs are activated by byproducts derived from Hb degradation (90). Furthermore, one study comprising patients treated with ATM-LMF demonstrated that reduced PfK13 transcriptional levels is associated with longer parasite clearance time, culminating in an altered parasite response (91).

1.5.2. Polymorphisms associated with drug resistance related to PfMDR1 and PfCRT

Due to their relevance and role for the malaria parasite, several studies have been performed in order to unveil the polymorphisms associated with the transporter proteins focused throughout this thesis, PfMDR1 and PfCRT.

PfMDR1 has been associated with drug resistance phenotypes and originally gene copy number amplification was the first polymorphism detected in this gene (74, 75). Over the years, *pfmdr1* copy number was established as an important molecular marker in resistance against MFQ both *ex vivo* (92) and *in vivo* where a study performed in Thailand demonstrated treatment failure with MFQ and AS+MFQ (93). Furthermore, one study has also shown a high risk of treatment failure after a short-term treatment with ATM-LMF in patients with increased *pfmdr1* copy number (94). *In vitro* studies complement these *in vivo* observations with knock-down experiments where one of the two *pfmdr1* gene copies was disrupted showing increased susceptibility to MFQ, LMF, halofantrine, QN and ART (95). *pfmdr1* amplification has also been implicated in PPQ drug resistance since one genetic marker associated with PPQ resistance was decreased copy number in the *pfmdr1* (59, 96, 97). A bimodal response of resistance to high concentrations of PPQ revealed that there is a prevalence of a single copy of *pfmdr1* in association with increased amplification of plasmepsins (another genetic factor associated with PPQ resistance) (98). Recently, an *in vitro* study using transgenic parasite lines also

corroborated these findings, showing that a de-amplification of *pfmdr1* is also a genetic marker for PPQ resistance (99).

pfmdr1 amplification is frequent in Southeast Asia and South America. In African regions, the *pfmdr1* amplification is rare which could be related with the known fitness cost associated with these amplifications, displaying a decreased survival in the absence of drug pressure (100, 101). One study has shown that *pfmdr1* amplification circulates in Africa mainly in Gabon upon analysis of recurrent infections after treatment with MFQ (102). After the full sequence of the *pfmdr1* gene, a set of five canonical point mutations were identified: N86Y, Y184F, S1034C, N1042D and D1246Y (103). The N-terminal mutations (N86 and Y184F) are more prevalent in Asian and African parasites while the C-terminal mutations (S1034C, N1042D and D1246Y) are found more often in South American parasites (76).

These mutations are associated with altered drug susceptibility, both *in vivo* and *in vitro*, to a wide range of antimalarial drugs. *In vivo*, the N86 allele is selected upon treatment with ATM+LMF suggesting that this polymorphism might be used as molecular marker for LMF resistance (104, 105). Additionally, the 86Y allele has been selected after CQ (106) and ADQ treatments (107, 108). Recently, through allelic exchange *in vitro* the role of positions 86 and 184 of PfMDR1 were discriminated. It was observed that the N86 allele increases the parasite's susceptibility to MFQ, LMF and DHA and decreases its susceptibility to ADQ and CQ, corroborating the *in vivo* associations (109). Additionally, this study also suggested that position 184 induced a decrease of susceptibility to PPQ (109). The C-terminal mutations, S1034C, N1042D and D1246Y, of *pfmdr1* have been involved in resistance to MFQ, QN, HF and ART, depending on the parasite's genetic background (79, 95). Furthermore, functional expression studies of PfMDR1 performed in *Xenopus laevis* oocytes have shown that it has the ability to transport antimalarial drugs. The wildtype PfMDR1 transports QN and CQ while mutant PfMDR1 transports only halofantrine but not QN or CQ (110). Another study demonstrated that PfMDR1 might also transport MFQ and ART into the DV where these may be less harmful for the parasite (111).

Additionally, to *pfmdr1*, several polymorphisms have also been found for the *pfcrt*. Field isolates have been found with polymorphisms in this gene and associated with CQ resistance (112) but between all the polymorphisms, position 76 seems to be crucial in CQ resistance since a threonine in this position (76T) instead of the wild-type's lysine (K76) was identified in all CQ resistant field isolates (70). Studies using resistant field isolates suggested that the K76T mutation is critical for resistance to CQ *in vitro* (113). Regarding PfCRT transport, studies with CQ uptake kinetics demonstrated that CQ-resistant parasites accumulate less CQ than CQ-sensitive parasites (112, 114), an observation also obtained with

isolated DVs (115). Additionally, the differences in accumulation have been associated with mutant PfCRT (116, 117). Allelic exchange studies have shown that in the wild-type parasite (containing the K76 allele) the positively charged lysine residue may prevent the trafficking of CQ through PfCRT. However, in mutant parasites containing the 76T allele, there is a loss of the positive charge at this position allowing the flux of CQ into the cytosol, resulting in a reduction of CQ inside the DV and conferring resistance (118). However, the genetic background and additional factors present in the parasite determines the degree of resistance and are important for a full resistant phenotype (119). After the discovery of the K76T mutation, other non-synonymous mutations were identified. Several patterns of genetic mutations have been identified mainly occurring at codons 72, 74, 75, and 76 resulting in different genotypic sequences mainly, CVMNK, CVIET, and SVMNT, leading to different levels of CQ resistance. CVMNK is present in CQ susceptible parasites while both CVIET (more prevalent in Asian and African regions) and SVMNT (more prevalent in South America) are characteristic of CQ resistant parasites (120). Most of the mutant *pfcrt* confers to the parasite a fitness cost, since a decrease in their prevalence and a return to the wild-type allele in the absence of CQ pressure occurs in the field (121–123). Besides CQ, this transporter also modulates the parasite's susceptibility to other antimalarial drugs *in vitro* and *in vivo*, mainly ADQ, QN (124, 125) and ATM-LMF where it seems to select for the wild-type K76 allele (105). Also, *in vitro* studies with genetically modified strains demonstrated that mutant PfCRT can improve the parasite's susceptibility to ARTs (119, 125). Therefore, *pfcrt* mutations were also associated with PPQ resistance. As described above, in addition to other genetic markers of resistance to PPQ, it has been found that mutations in the *pfcrt*, mainly T93S, H97Y F145I and I218F, were associated with treatment failure after DHA-PPQ (58, 126). Some mutations presented mainly in Southeast Asia (H97Y, F145I, G353V, T93S and I218F) have been identified and validated through gene edited parasite strains *in vitro*, to confer resistance to PPQ (127, 128).

All of this data highlights that polymorphisms in *pfmdr1* and *pfcrt* can induce different parasite responses depending on the antimalarial drug and are molecular markers of resistance. Table 1 describes the major antimalarial drugs in use to treat malaria and the associated molecular markers of resistance, where the impact and relevance of PfMDR1 and PfCRT proteins is well evidenced, since mutations in these transporters were detected for almost all approved antimalarials (Table 1).

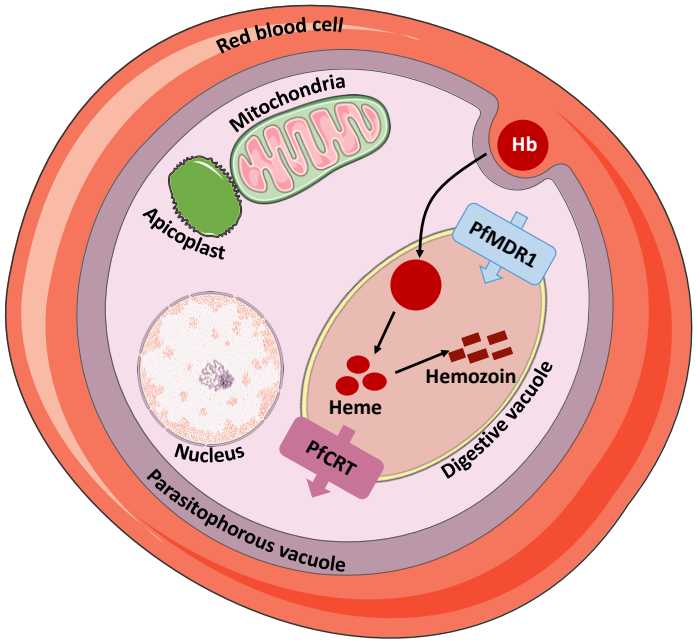


Figure 8: Cellular localization of *P. falciparum* multidrug resistance transporter 1 (PfMDR1) and *P. falciparum* chloroquine resistance transporter (PfCRT). PfMDR1 and PfCRT resides in the parasite's digestive food vacuole membrane and is responsible for the influx or efflux, respectively of substrates from the DV. (Own authorship)

Table 1: Major antimalarial drugs and associated molecular markers of resistance in *Plasmodium falciparum.* The antimalarial drugs (Dihydroartemisinin (DHA); Artemether (ATM); Artesunate (AS); Chloroquine (CQ); Amodiaquine (ADQ); Piperaquine (PPQ); Pyronaridine (PND); Primaquine (PMQ); Mefloquine (MFQ); Lumefantrine (LMF); Quinine (QN); Atovaquone (ATQ); pyrimethamine (PYR); proguanil (PG); sulfadoxine (SDX)), its clinical use, the mechanism of action and polymorphisms associated to resistance (8, 22, 61).

Antimalarial classes	Antimalarial drug	Clinical use	Pathway or mechanism of action	Polymorphisms associated to resistance	Situation in Asia	Situation in Africa
Endoperoxides	Dihydroartemisinin (DHA)	Used in combination with PPQ	Oxidative stress by the formation of reactive oxygen species and protein damage	K13 mutations	Partial resistance in Greater Mekong subregion (Southeast Asia)	Highly effective
	Artemether (ATM)	Used in combination with LMF				
	Artesunate (AS)	Used in combination with MFQ, AMQ or PND; intravenous in severe malaria				
4-aminoquinolines	Chloroquine (CQ)	Treatment of non-*P.falciparum* malaria	Heme detoxification	Mutations in *pfmdr1* and *pfcrt*	Widespread resistance	Resistance has been widespread. Reversion to sensitive parasites has been observed in many regions
	Amodiaquine (AMQ)	Used as partner drug with AS	Heme detoxification	Mutations in *pfmdr1* and *pfcrt*	Widespread resistance	AS+AMQ ACT higly efficacious. Detected cross-resistance with chloroquine but artesunate-amodiaquine highly efficacious
	Piperaquine (PPQ)	Used as partner drug with DHA	Heme detoxification	Amplification of plasmepsins 2 and 3; *pfmdr1* deamplification; Mutations in *pfcrt*	Effective in combination with DHA	Highly effective in combination with DHA
Mannich base	Pyronaridine (PND)	Used as partner drug with AS	Heme detoxification	Resistance not documented	Effective in combination with AS	Effective in combination with AS
8-aminoquinolines	Primaquine (PMQ)	Gametocytocidal drug for *P. falciparum;* prophylaxis; elimination of dormant stages *P. vivax* and *ovale*	Unknown	Resistance not documented	Effective as single dose	Rarely used
Aryl-amino alcohols	Mefloquine (MFQ)	Prophylaxis as monotherapy or in combination with ATS	Unknown	Mainly *pfmdr1* amplification	Resistance detected when combined with AS	Effective in combination with AS
	Lumefantrine (LMF)	Used as partner drug with ATM	Unknown		Effective when combined with ATM	Effective in combination with ATM
	Quinine (QN)	Used in uncomplicated malaria in first trimester of pregnancy, or severe malaria	Unknown		Applied in pregnancy and in cases of severe malaria	Highly efficacious
Naphthoquinones	Atovaquone (ATQ)	Used in combination with PG	Electron transport chain	Mutation in cytochrome *bc* 1	Remains effective	Resistance has been reported
Antifolates	Pyrimethamine (PYR)	Used in combination with SDX mainly for preventive treatment	Folate biosynthesis	Mutations in dihydrofolate reductase (*dhfr*)	Widespread resistance	Widespread resistance
	Proguanil (PG)	Prophylaxis and treatment, both used in combination with ATQ			Widespread resistance	Widespread resistance
	Sulfadoxine (SDX)	Used in combination with PYR		Mutations in dihydropteroate synthase (*dhps*)	Widespread resistance	Widespread resistance

1.6. The need for novel compounds against malaria

While some researchers deliberate about the relevance of ACT resistance in the fight against malaria, there are others that are always looking for alternatives.

The emergence of drug resistance has already been found in the field and in anticipation to ACT failure, it becomes extremely important to develop novel antimalarial drugs to replace ACTs. As the parasite has the ability to evolve and adapt to basically all current drugs implemented to treat malaria, this urges research to identify novel compounds as well as novel targets for antimalarial therapy.

Furthermore, emergence and worldwide spread of resistance to approved antimalarial drugs also has an impact in the global public health and higher economic costs. For instance, patients whose treatment has failed need to have constant consultations culminating in the loss of work/school days and have greater health care costs. Additionally, the implementation of novel national treatment policies require more costs and additional training of health staff (129). In 2014, a research work evaluated the potential impact of the human and economic cost using a hypothetical scenario. In this analysis it was predicted that US$ 32 million per year needed to be applied to cover the medical costs for re-treatment of patients and for the management of severe malaria. In this scenario, the model predicted to have an annually increase of US$ 22 million, with treatment failures resulting in 116 000 deaths. An estimated economic cost of around US$ 130 million was calculated to change the treatment policy in all endemic countries where ACT failure is 30% and treatment of severe malaria is reverted to QN instead AS. This study gives an idea of the impact in global human health and associated economic costs that could appear if all ACTs lose their efficacy in all endemic countries (130).

According to the WHO, the highest proportion of investment in malaria endemic countries is spent on malaria prevention, diagnosis and treatment. Funding for drug research and product development for malaria increased over the years and the highest level was reached in 2018 (US$ 663 million). These investments from the private sector industry in several phase II clinical trials of new chemical compounds with the potential for single-exposure radical cure has been increasing and shows a clear tendency to continue to grow (1) (Figure 9).

The eradication agenda for malaria contains a series of requirements that should be considered to understand if a compound can be considered as a new therapeutic drug to control and eliminate malaria disease. First, there is a need to find novel antimalarials with new mechanisms of action in order to overcome the ACTs resistance with no cross-resistance to current antimalarial drugs. Also, it is desirable that new compound possess pharmacokinetic proprieties that allow for single-dose administration in order to increase compliance and delay the development of resistance. Preferably,

new compounds should also have activity against the asexual and sexual stages of the parasite's life cycle, blocking the transmission of the disease from human to mosquito, decreasing the number of new infections. Ideally new molecules should have activity against the sporozoitic and the exo-erythrocytic liver stages of the parasite including dormant hypnozoites, the relapsing form of malaria caused by *P. vivax* and *P. ovale*, which is a major health issue in some endemic malaria areas. Third, there is a needed to find new medicines that provide increased protection from re-infection as a result of their pharmacokinetic profiles (post-treatment prophylaxis). According to the eradication agenda, it is expected that all these measures together with the increased use of bed nets and insecticides and the promise of vaccine trials leads to a significant reduction in the malaria disease burden (17, 62, 131, 132). Although, some progress have been performed in developing a vaccine, the best candidate RTS,S/AS01 only provides partial immunity, may be age dependent (protection was lower in infants 6–12 weeks of age than in young children 5–17 months old) and some severe complications were observed after vaccination (133). These limitations in protective efficacy and the need to continuously update vaccine formulations due to antigenic polymorphism highlight even more the current dependence on antimalarial chemotherapy (134).

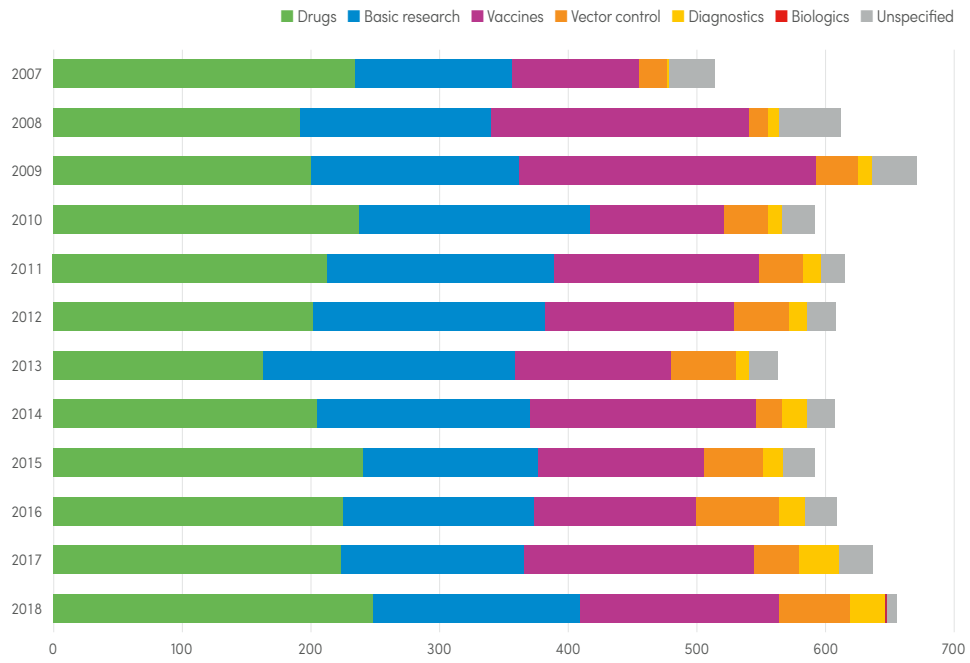


Figure 9: Funding for malaria basic research and drug development between 2010 and 2018. The figure shows the investment in drug discovery and development, basic research, vaccines, vector control and diagnostics (1). (Permission from (1)).

1.7. New strategies and drugs for overcoming resistance

The most complete antimalarial discovery library has been developed by the not-for-profit product development partnership Medicines for Malaria Venture (MMV) in collaboration with its partners in both the academia and the pharmaceutical industry (Figure 10). In the next sections it will be described some compounds present in this library, resulting from different approaches (Figure 10).

Promising compounds from different origins have been discovered mainly from four approaches: (i) new combinations and reformulation of current antimalarial drugs; (ii) modification of existing antimalarials; (iii) target-based screening and (iv) phenotypic screening (4, 62, 132).

The target-based screenings need to identify an essential pathway, receptor or enzyme that is crucial for parasite survival (132). After screening, the most promising structure is identified by informatic analysis based on the potency, toxicity and novelty. Further selection, additional derivatives are generated and consequently tested against the parasite life cycle or against the specific protein target. This type of analysis can reveal structure-activity relationships (SARs) which are relevant to predict the effects of structural modifications on the properties of the molecule including solubility and bioavailability. Furthermore, this type of approach can render potent compounds with known mechanisms of action. This type of process can be improved if the crystal structure of the target molecule is available since this can support computational studies and guide the discovery of novel inhibitors with increased selectivity against the parasite. Over the last years, several therapeutic agents have been developed including dihydrofolate reductase and cytochrome bc1, both targets inhibited by the antifolate class (PYR and PG) and ATQ. While this type of approach has been providing compounds that inhibit malaria parasite survival, the absence of selectivity for some molecules between parasite and host human enzymes/proteins has limited drug discovery (135–137).

Phenotypic screening can be defined as an approach to identify compounds or classes of compounds with activity against the whole parasite. Although the mechanism of action is unknown, the compounds identified from this type of screening can be considered toxic for the parasite. Indeed, this approach involves the exposure of the parasite to the test compounds and after the incubation period the parasites are examined to determine if compounds are capable of killing parasites at the studied developmental stage. One advantage of these phenotypic screenings is that, after identification of a potent compound, subsequent molecular biology studies can lead to the identification of novel and multiple targets. However, the challenge of this type of approach is related with the reproducibility and cost-effectiveness when a large number of compounds need to be tested (135–137). The advances in technology have made possible the screening of several libraries of compounds that allows the

identification of unknown chemical compounds that can become novel antimalarial candidates. Among the new classes of antimalarial drugs identified using phenotypic-based approaches are the spiroindolones which will be further described.

1.7.1. Novel combinations and reformulations of current antimalarial drugs

Regarding novel combinations and reformulations of current antimalarial drugs, these may help to overcome the drug resistance to a particular component and, reformulation may assist in the delivery of the drug, allowing it to be more effective. The triple artemisinin-based combination therapies (TACTs) are a good example of new combinations that have been evaluated to treat malaria and overcome the high treatment failure rates (i.e. recrudescent infections). This treatment derives from the molecular knowledge of each of individual drug, while also using drugs with different mechanisms of action, and resistance knowledge in order to promote a better disease outcome. The PfMDR1 and PfCRT transporter proteins are molecular markers that are considered when TACTs are designed since they have a preponderant role in the development of resistance to most antimalarial drugs, as evidenced in Table 1. TACTs combine an artemisinin derivative with two slowly partner drugs. After the action of artemisinin in TACTs, there are two more slowly partner drugs to kill the remaining parasites providing a mutual protection for the partner drugs (138). This type of combination is not yet available since its tolerability and safety still needs to be evaluated. Recent results with two potential candidate combinations (ATM–LMF plus ADQ and DHA-PPQ plus MFQ) demonstrated that these combinations are safe, efficacious and well tolerated (139). More studies need to be conducted before their application but it is expected that TACTs will extend the useful life of the few available and still effective antimalarial drugs (138).

The reformulation of current antimalarial drugs, as mentioned above, is a strategy implemented by some pharmaceutical industries in order to improve the therapeutic efficacy of a particular antimalarial drug. Thus, improving the drug's bioavailability, lowering the total daily intake and decreasing pharmacokinetic variability can extend the lifetime of antimalarial drugs already implemented to treat malaria (140). An example of this strategy is the reformulation of ATM+LMF tablets, where a faster dispersible formulation to be applied in infants and children in Africa was developed (141).

Alternatively, drugs already in use for other diseases may act as antimalarial agents and consequently be repurposed as a new antimalarial treatment. In fact, drug repurposing is an interesting

approach since these compounds have already been studied in terms of safety and pharmacokinetics and may also display novel mechanisms of action within the parasite, being a time and cost-efficient strategy. The methylene blue, used in the treatment of methemoglobinemia is already in Phase II trials in combination with PMQ, fosmidomycin is an antibiotic and is currently in Phase II trials as a combination with PPQ, rosiglitazone is an antidiabetic in clinical trials as an adjunctive therapy for severe malaria, imatinib is a cancer drug in Phase II trials in triple combination with DHA-PPQ, and sevuparin a drug for sickle cell disease already in Phase I/II trials in combination with ATQ-PG are examples of novel antimalarials in development as a result of drug repurposing (62).

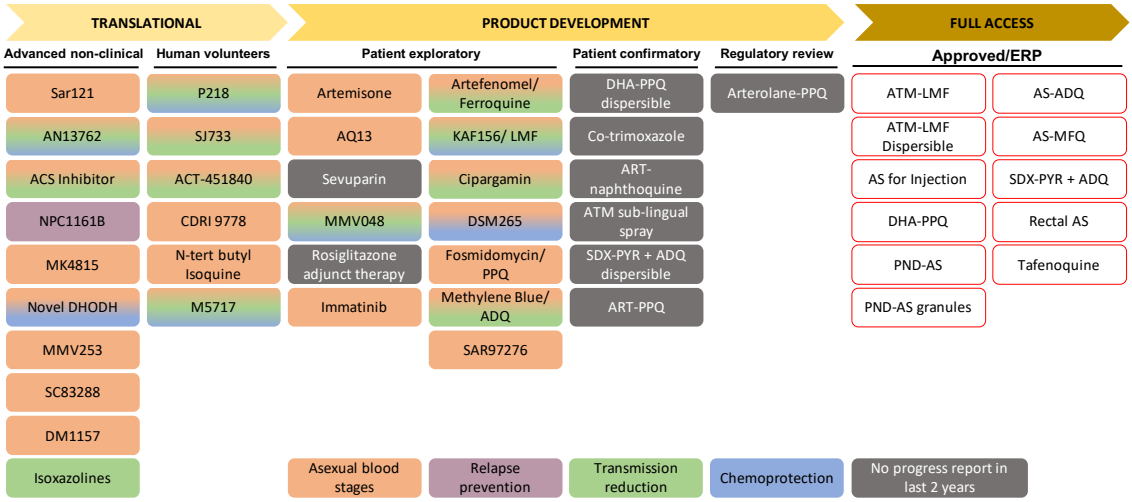


Figure 10: Clinical progression of new antimalarial drug candidates over the past 5 years according to MMV. Schematic representation of the all novel antimalarial drugs and its development progress on the market, from pre-clinical development to approved or marketed drugs. (Dihydroartemisinin (DHA); Artemether (ATM); Artesunate (AS); Amodiaquine (ADQ); Piperaquine (PPQ); Pyronaridine (PND); Mefloquine (MFQ); Lumefantrine (LMF); pyrimethamine (PYR); sulfadoxine (SDX)).

1.7.2. Modification of existing antimalarial drugs

Current antimalarials have been used as staring points for drug development aiming to improve their bioavailability, potency and pharmacodynamics. ART and its derivatives are the main components for the current treatments and are a good example of compounds that have been used as starting materials for drug development. The main relevant feature of all ARTs is the 1,2,4-trioxene moiety, the endoperoxide. This substructure is considered of extreme importance for the antimalarial activity of these compounds and its activation is thought to involve the interaction with heme iron, that breaks the endoperoxide bridge creating carbon-centered radicals that react with susceptible groups in parasites, such as proteins and other biomolecules (37). Taking into account the advantage of these compounds

several fully synthetic endoperoxides compounds were obtained. One good example is the ozonides, which are synthetic peroxides, with an endoperoxide bridge giving a potent blood-stage activity, but they also contain a bulky amantadine ring, which increases their stability in the plasma. OZ439, also known as artefenomel, is a compound in Phase IIb trials, being as potent as AS *in vitro* and displayed higher activity in mouse models. Furthermore, sustained plasma exposure is obtained after a single oral dose in humans. Due to these characteristics the hope is that this compound could replace the three doses required for the already implemented ART derivatives. OZ439 it is being tested in combination with PPQ (132) (Figure 11).

The 4-aminoquinolines scaffold are being pursued in drug discovery programs and few compounds have been generated based on this scaffold. Ferroquine is an example of such a candidate and is a CQ analog containing a ferrocene moiety coupled to a 4-aminoquinoline and has the ability to kill parasites faster due to the capacity of ferrocene to generate parasite-killing radicals (142). It is already in Phase II clinical trials for the treatment of uncomplicated malaria. It has been described that ferroquine is potent against CQ-, ADQ- and MFQ-resistant strains (143). Another example is AQ-13, which is a short-chain compound with increased pharmacokinetic profile than CQ. It has shown low toxicity and high efficacy and is already in Phase II trials. ELQ-300 is a new quinoline containing a diarylether moiety targeting the *P. falciparum* bc1 complex. P218, resulted from structure optimization of pyrimethamine and is an inhibitor of the *P. falciparum* dihydrofolate reductase. This compound has shown high selectivity for malarial DHFR, leading to reduced toxicity (62) (Figure 11).

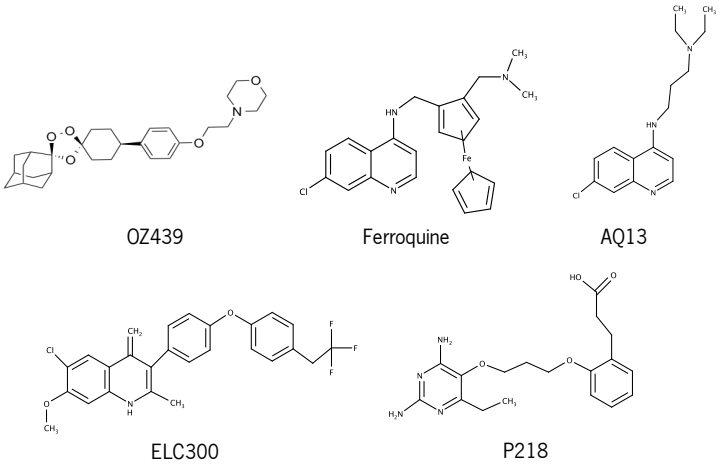


Figure 11: Chemical structures of some experimental antimalarial compounds already in clinical trials and derived from modifications of existing antimalarial drugs. Chemical structure of OZ439, ferroquine, AQ13, ELQ300 and P218.

1.8. New drug candidates

While most current therapies in development exhibited only some chemical alterations in the structure of present antimalarials, it would be desirable to discover novel chemotypes aiming to encounter different mechanisms of action and resistance. Although significant advances in understanding the *Plasmodium* genome have been achieved, the identification and validation of novel drugs have proven challenging. Until now only ACTs were approved for the treatment of malaria. All compounds in more advanced stages of development for malaria treatment in Figure 10, derived from novel combinations of the approved antimalarials and new reformulations. Molecules with completely new chemotypes or chemical modifications of current antimalarial drugs are in early stages of clinical trials (Figure 10). In the following section some of the most promising compounds in this library (MMV) with completely new chemical entities will be described.

The phenotypic screening, as described above, represents one of the most successful approach to date to identify novel molecular targets and consequently to design novel molecules through the sequencing of resistant mutants (4). As a consequence of development of drug resistance, there is an urgent need for new compounds, ideally with new chemotypes acting on non-explored parasite targets. Nowadays there are several libraries, for instance from Novartis, St. Jude Children's Research Hospital and GlaxoSmithKline (GSK), that use phenotypic screening with *P. falciparum* sensitive and resistant strains to discover new compounds (144, 145).

Among the new classes of antimalarial drugs identified using phenotypic-based approaches are the spiroindolones that were discovered from a screening of natural products or synthetic compounds with structural characteristics found in natural products. This screening revealed that this class of compounds has a promising bioactivity profile and was used as a chemical starting material in order to improve the potency and bioavailability. KAE609 (Cipargamin) (Figure 12), currently in Phase IIb trials, results from this strategy and demonstrated potent schizonticidal activity against resistant *Plasmodium* strains. It possesses a rapid capability to clear parasitemia in adults with uncomplicated *P. falciparum* or *P. vivax* (146, 147). Furthermore, KAE609 has gametocytocidal activity and potent transmission-blocking potential (147). It is assumed that this compound targets the outer membrane transporter P-type ATPase 4 (PfAtp4), an important player for maintaining parasite sodium homeostasis. SJ733, currently in phase I clinical trials, also inhibits PfATP4 (62).

The imidazolopiperazine class of compounds was also discovered by phenotypic high-throughput screening. One example of this class is the KAF156, also known as GNF156 that is already in Phase II trials and is administrated in combination with LMF (62). This compound has shown potent activity

against the blood stage of sensitive and resistant parasite strains and a potent therapeutic activity in mouse models of malaria and also has activity against gametocytes, blocking parasite transmission (148). The mechanism of action remains unknown although drug resistant parasites allow to identify mutations in three genes: *P. falciparum* Cyclic Amine Resistance Locus (PfCARL), UDP-galactose and Acetyl-CoA transporters (62, 148) (Figure 12).

Another candidate that has been discovered through phenotypic screening is MMV390048, that belongs to the 3,5-dia-ryl-2-aminopyridine class. This compound, currently in Phase IIa clinical trials in Ethiopia, shows high potency against all stages of the parasite's life cycle, apart from late hypnozoites in the liver and with a single dose being capable to completely cure *P. berghei*-infected mice (62, 149). Phosphatidylinositol 4-kinase (PfPI4K) was identified as the molecular target of MMV390048 (Figure 12).

On the other hand, as mentioned above, target-based high-throughput screenings have also been performed as a tool for drug discovery and to identify new lead compounds to eradicate malaria. One example of this approach is the high-throughput enzyme screen against *Plasmodium falciparum* dihydroorotate dehydrogenase (PfDHODH), a mitochondrial enzyme involved in the *de novo* pyrimidine biosynthesis pathway. From this screening, DSM265 which belongs to the triazolopyrimidines family, was discovered and then described as a selective inhibitor of PfDHOH, with high activity against blood and liver stages of *P. falciparum,* being also active against resistant parasite strains (150, 151). This compound displayed a promising safety profile, could be applied on a single oral dose, presented a low clearance rate and a long half-life when applied in humans (150). This compound completed Phase IIa trials in Peru in patients with *P. falciparum* and *P. vivax* infections (4, 62, 132) (Figure 12).

Besides these described compounds, extensive screening efforts have been made and new antimalarial drugs with novel modes of action unrelated to current ACTs have been unveiled through the research work developed by several companies and laboratories (Table 2).

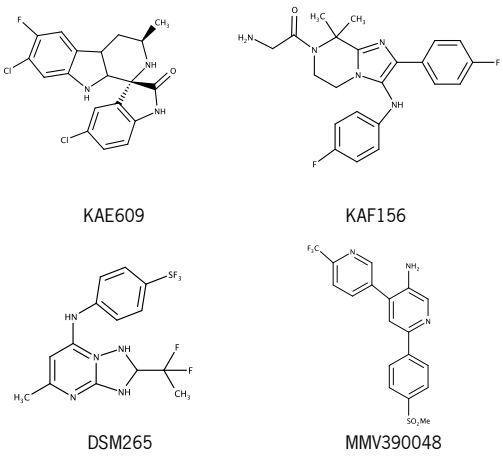


Figure 12: Chemical structures of antimalarial drugs currently in Phase II clinical trials. Chemical structures of KAE609, KAF156, MMV390048 and DSM265 compounds.

Table 2: Some examples of new antimalarial compounds in development against *P. falciparum* life cycle. The target localization, mechanism of action, target molecule and resistance genes associated to each compound are described (4, 7).

Compound in development	Target localization	Mechanism or pathway affected	Target molecule	Resistance gene
KAE609[191,192], SJ733[193] and 21A092	Plasma membrane	Ion homeostasis	*P. falciparum* ATPase 4 (PfATPase4)	*pfatp4*
AZ412[194]	Plasma membrane	pH regulation	V-type H+-ATPase	*pfVATPase-D*
DDD107498 [195]	Cytoplasm	Protein synthesis	Eukaryotic elongation factor 2 (eEF2)	*pfeEF2*
AN6426[196]			Leucine tRNA ligase (LeuRS)	*pfcLeuRS*
Thiaisoleucine[197]			Cytoplasmic isoleucine tRNA ligase (Cyto-IleRS)	*pfcIleRS*
Halofuginone[197]			Proline tRNA ligase (ProRS)	*pfcProRS*
Cladosporin[198]			Lysine tRNA ligase (LysRS)	*pfKrs1*
BRD1095[199]			Phenylalanine tRNA ligase (PheRS)	*pfcPheRS*
ELQ-300[200]	Nucleous (mitochondrion)	Electron transport	Cytochrome b	*pfcytb*
Decoquinate[201]				
DSM265[202, 203]		Pyrimidine biosynthesis	Dihydroorotate dehydrogenase (DHODH)	*pfdhodh*
KAF156[204]	Endoplasmatic reticulum and Golgi apparatus	Unknown	Unknown	*P. falciparum cyclic amine resistance locus (pfcarl), P. falciparum UDP-galactose transporter (pfugt), P. falciparum acetyl-CoA transporter (pfact)*
KAI407[205]	Endomembrane system	Membrane trafficking	Phosphatidylinositol 4-kinase (PI4K)	*pfpi4k*
MMV390048[206, 207]				
Fosmidomycin[208, 209]	Apicoplast	Isoprenoid synthesis	1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR)	*pfdxr*

1.9. Natural products with antimalarial activity

Nature has been a major source of drugs throughout history, including the most efficient antimalarial drugs. Malaria treatment has a strong historical relationship with natural products. The most successful antimalarial drugs, QN and ART, have their origins in plant-derived metabolites. QN was the first original natural product isolated from *Cinchona* tree bark (historically used to treat fever) and was purified in 1820. The effort to synthesize QN led to the development of methylene blue and from this came the classical 4-aminoquinolines and amino-alcohols as described above. Likewise, ART is a sesquiterpene lactone and was isolated from *Artemisia annua* leaves in 1971. The endoperoxide group formed the basis for a range of fully synthetic ART compounds, and they are the most important therapeutics applied to treat malaria and it has been used as the basis of the next generation of therapies (152).

As malaria has been historically treated with herbal medicinal products, the strategy of selecting extracts with desirable biological activity could lead to the discovery of new drugs (132). Due to complex structural characteristics, multiple stereocenters, flexible conformations, presence of heteroatoms, it is expected that natural compounds can interact with several targets and/or with new targets compared with synthetic compounds (153). Therefore, exploring natural products provides the opportunity to discover novel bioactive molecules with unique structures and improved activity and selectivity which can be further optimized using chemical modifications (152, 154).

Some reviews summarized multiple natural compounds with antimalarial activity isolated from plants and non-plant sources comprising different species mainly marine sponges, fungi, alga, cyanobacteria, actinobacteria, ascidians and nudibranch. These natural compounds were organized in classes comprising terpenes (sesquiterpenes, diterpenes, steroids, terpenoid benzoquinones, quassinoids), flavonoids, alkaloids, xanthones, phenylalkanoids, chromenes, benzophenones and others (153, 155). All this research demonstrated that natural compounds are indeed a source of potential molecules with good antimalarial activity and can be used as a basis for drug discovery.

Among all of the above-mentioned compounds, we will focus on the steroid class as interesting natural bioactive compounds and their potential as antimalarial drugs.

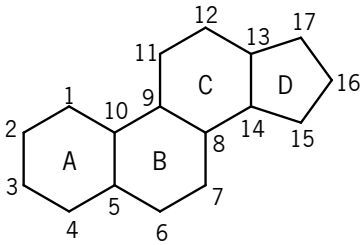
2. Steroids as natural compounds

Lipids have been regaining a higher attention in recent years mainly due to a new insight on the role of membranes in cellular function. Lipids are known to be structural builders of the cell and an energy source for cell functioning. Furthermore, these compounds are crucial for controlling several

biological functions as cell signaling, receptor function and regulation of protein sorting and trafficking (156, 157).

Steroids are a group of lipids comprising a higher repertoire of structurally related natural compounds with important functions *in vivo* acting as physiological regulators, hormones and provitamins (158).

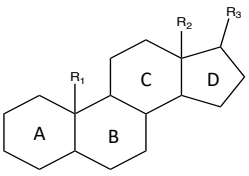
Steroids are composed by a tetracyclic carbon skeleton composed by three fused cyclohexane rings (A, B and C) and a fourth cyclopentane ring (D) (Figure 13). Different classes of steroids resemble closely because all have the same structure and slight variations in the nucleus or in lateral chain. Such variations can occur at R_1, R_2 and in the lateral chain R_3 leading to the formation of diverse steroids including estratrienes, androstanes, pregnanes, cholestanes and cholane (159) (Table 3).

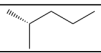
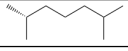
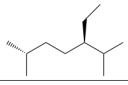


Steroid skeleton

Figure 13: Steroid skeleton. The steroid structure comprise a tetracyclic carbon skeleton composed by fused three cyclohexane rings (A, B and C) and a fourth cyclopentane ring (D) and 17-carbon.

Table 3: Hydrocarbon skeleton with variations in the tetracyclic framework and/or lateral chain leading to the formation of different series of steroids. Variations at positions R1, R2 and/ or R3 leads to the formation of gonane, estranes, androstane, pregnane, cholane, cholestane and stigmastane steroids.



Substituent			Number of C	Steroid Class
R_1	R_2	R_3		
H	H	H	17	Gonanes
H	CH_3	H	18	Estranes
CH_3	CH_3	H	19	Androstane
CH_3	CH_3	CH2CH3	21	Pregnanes
CH_3	CH_3		24	Cholane
CH_3	CH_3		27	Cholestane
CH_3	CH_3		29	Stigmastane

Steroids are synthesized via the mevalonate pathway which takes place in the cytosol and mitochondria in all higher eukaryotes and many bacteria. Despite their structural complexity, steroids arise from two non-complex 5-carbon precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These molecules of IPP and DMAPP can be converted into farnesyl pyrophosphate (FPP) which in turn can be converted into squalene-by-squalene synthase (SQS). The oxidized form of squalene (2,3-oxidosqualene) is a precursor for the synthesis of sterols (leading to the production of lanosterol in fungi and animals, or cycloartenol in plants) (160) (Figure 14).

Nowadays many natural steroids produced endogenously are widely used in the therapy for several pathologies. For instance, endogenous steroid hormones including progesterone (a progestin steroid) and estradiol (an estrogen steroid) have been used as contraceptive drugs from the end of the 1950s and applied for hormone therapies. Progesterone controls events during pregnancy, and estradiol regulates female characteristics. Both of these steroid hormones are components of birth control pills and prevent ovulation (161).

Another example are the anabolic steroids, which are steroidal androgens naturally produced by the body in order to maintain male characteristics and promote muscle mass growth (162). Although this type of molecules are sometimes illegally used in sports, these steroids are relevant therapeutic agents in many pathological conditions mainly, HIV-related muscle wasting and neuromuscular disorders (163).

One natural steroid produced by the adrenal gland in response to stress is cortisol. The anti-inflammatory properties of cortisol makes its application as therapeutic compound possible (164).

Neurosteroids are synthesized in the central nervous system from cholesterol and play an important role as allosteric modulators for neurotransmitter receptors. Synthetic neurosteroids have been described as potent anticonvulsant and anxiolytic agents (165, 166). Finally, bile acids, which are synthesized from cholesterol are important physiological compounds for intestinal nutrient absorption and biliary secretion of lipids, xenobiotics and toxic metabolites. They also are signaling molecules and metabolic regulators responsible for maintaining metabolic homeostasis. Bile acids are used in the treatment of liver diseases and in the dissolution of cholesterol gallstones (167, 168).

Therefore, steroids constitute a family of compounds with great therapeutic value. In this way, beyond the effort to design and synthetize new steroidal compounds, there is also a great interest in the isolation and identification of natural steroids with undisclosed bioactivities.

Due to the hydrophobic character, steroid hormones are able to enter by diffusion across the plasma membrane. After steroid hormones are transported to their target tissues and cells, they bind

with the corresponding hormone receptors. Steroid hormone receptors are found in the nucleus, cytosol and also in the plasma membrane of target cells. They are generally intracellular receptors (typically cytoplasmic or nuclear) and initiate signal transduction for steroid hormones which lead to changes in gene expression (169–171). The glucocorticoid receptor, mineralocorticoid receptor, progesterone receptor and androgen receptor are classic members of the nuclear receptor superfamily (172).

One subclass of steroids are the sterols. These molecules contain, comparatively with steroids, a hydroxyl group at the 3-position of the A-ring and an aliphatic lateral chain at the 17β-position (159). The presence of an hydroxyl group confers an amphiphilic character that allows it to readily incorporate into phospholipid bilayers, extensively modulating their structure and behavior (173). In fact, sterols are required for diverse cellular functions, including binding to sterol-sensing domains to regulate protein function, participating in the formation of lipid rafts and sustain the structure of cell membranes, being essential for the formation, maintenance and fluid characteristics of these membranes (174). Cholesterol is an example of a major sterol of vertebrates and is composed by an eight-carbon sidechain in the 17-position on the steroid nucleus comprising a total of 27 carbons (175). Cholesterol present in our body arises from two different sources. It can be either synthesized *de novo* within our cells or obtained through nutritional sources.

In addition to be a structural component of membranes, cholesterol also acts as a precursor for the biosynthesis of some compounds such as bile acids, vitamin D, and steroid hormones that are produced by the adrenal gland and by the male and female sex glands (175). All steroid hormones are synthesized from cholesterol by a common precursor steroid, pregnenolone, which is formed by the enzymatic cleavage of the lateral chain of the 27-carbon cholesterol, a reaction catalyzed by the cytochrome P450 side-chain cleavage enzyme (P450scc, CYP11A1) (161) (Figure 15).

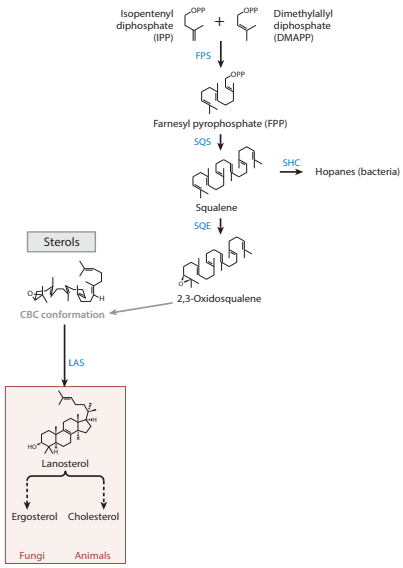


Figure 14: Representative scheme of the biosynthetic pathways to sterols formation. Sterols are synthesized via the mevalonate pathway. The enzymes that catalyze the several steps are indicated. Enzyme abbreviations: FPS, farnesyl pyrophosphate synthase; SQS, squalene synthase; SQE, squalene monooxygenase or epoxidase; SHC, squalene-hopene cyclase; LAS, lanosterol synthase; BAS, β-amyrin synthase. Other abbreviations: CBC, chair-boat-chair; CCC, chair-chair-chair (adapted from (160)).

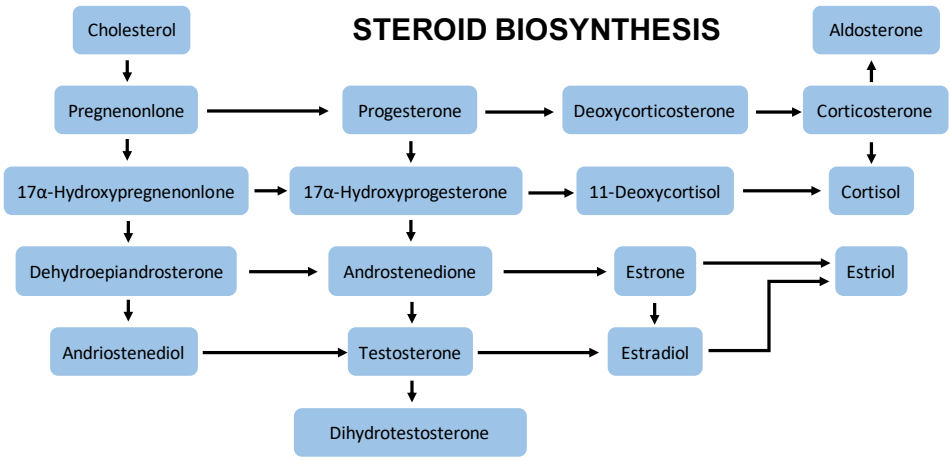


Figure 15: Major steps involved in the biosynthesis of several steroid hormones. All steroids in the figure are synthesized from cholesterol by a common precursor, pregnenolone (161).

2.1. Steroids as antimalarial compounds

As mentioned above, a valuable strategy to overcome resistance and to control and eliminate malaria is the discovery and development of new drugs with different structural features from current ACTs. For this reason, steroids have been used in the last years as a source of novel antimalarial drugs. These compounds have great advantages compared with other natural compounds making them very

attractive in medicinal chemistry. Firstly, steroids have completely different structures when compared with current ACTs. Also, as described above, there is a great structural variability of the tetracyclic framework, including cholestanes, pregnanes, estratrienes and androstanes, and functional groups can be attached in diverse positions of the framework or in the lateral chain. In addition, ring junctions (5α/5β stereochemistry at the A/B rings for instance) can influence considerably the resulting bioactivities.

The delivery of molecules to cells could be mediated for instance through lipids, polymers and nanosystems (176) or by changing the membrane's permeability (177). Being a group of lipids and considering their degree of lipophilicity, steroids could be considered to influence cellular uptake and intracellular transport. It has been described that steroid compounds could enter into cells either via membrane passive diffusion (171), through endocytic processes (178) or via steroid binding sites as described for steroid hormones. Taking this into account molecules containing this type of framework may display advantages compared, not only with other natural compounds, but also with current ACTs since they possess lipophilic characteristics and a higher bioavailability, passing lipophilic barriers much more efficiently.

Considering all these advantages, one approach to discover new biologically active compounds is to combine a steroid skeleton with structural components possessing appropriate biological activities. Advances in synthetic chemistry over the last years made possible the discovery and semisynthetic optimization of these natural compounds.

On the other hand, over the years multiple compounds with steroid skeleton have been isolated from several sources and their antimalarial activity disclosed.

There are several studies demonstrating that steroids extracted from plants, some of them already in use in traditional medicine, exhibited a promising antimalarial activity.

Sarachine (3β-amino-22,26-epiminocholest-5-ene) (Figure 16, compound 1) is an example of an alkaloid with an amino steroid structure isolated from the leaves of *Saraca punctate* and it demonstrated a potent activity against *P. falciparum* (half-maximal *in vitro* growth inhibitory concentration (IC_{50}) value of 25nM against a CQ-sensitive strain and IC_{50} value of 176nM against CQ-resistant strain) (179). Taking this compound as starting material, recently several amino steroids were prepared. Among all the synthetic library, there was a promising derivative, the 1o compound (Figure 16, compound 2), a steroid structure linked to a hydroxyarylmethylamino group that exhibited potent antimalarial activity (3D7 IC_{50} value of 4.1nM and Dd2 IC_{50} value of 1nM) (180). Furthermore, it demonstrated *in vivo* activity in *P. berghei*-infected mice and a decrease of parasite transmission to

mosquitoes, which could be as a result of the compound's ability to interfere with gametocyte formation. It is important to note that in this study other compounds were synthesized, however, those that have a steroid skeleton afforded a higher antimalarial activity. The results indicated that the lipophilic steroid framework could facilitate membrane permeation and bioavailability whereas the functional group added at C-17 exert its antimalarial activity (180).

Other example of steroids isolated from plants and that exhibited antimalarial activity are steroidal alkaloids from *Holarrhena pubescens* roots (181). Irehline (Figure 16, compound 3) and Mokluangin A (Figure 16, compound 4) demonstrated a potent antimalarial activity against the *P. falciparum* K1 strain with the IC_{50} values of 1.2 and 2.0 μM, respectively, with high selectivity index.

A crude extract from *Galtonia regalis,* a native plant in South Africa, exhibited moderate antimalarial activity (IC_{50} value of 1.25 μg/mL). One of the isolated steroids from this crude extract demonstrated a higher antimalarial activity with an IC_{50} value of 0.214 μM against a drug resistant Dd2 *P. falciparum* strain and did not induce *in vitro* hemolysis after treatment (182) (Figure 16, compound 5).

Also, plants used in traditional Colombian medicine such as *Solanum nudum* (Solanaceae) were studied as a source of antimalarial candidates. Five steroids extracted from the stems and leaves demonstrated a significant respective decrease in parasite growth of 56% and 21% against FCB-2 parasite strain (183). Also, a reduction of 47% and 39% was observed in hepatic *P. vivax* trophozoites, respectively (184).

The design and synthesis of hybrid compounds with antimalarial activity can be an excellent strategy to overcome drug resistance ACTs (185–187) and this type of approaches has also been applied to treat other diseases (188). Taking this into account, some research works have been applying this strategy. For instance, a hybrid containing an ATS linked to a steroid isolated from *Solanum nudum,* tumacona B, was less cytotoxic than artesunate alone and demonstrated an activity with the same degree as artesunate and a higher activity than tumacona B alone (189) (Figure 16, compound 6).

A hybrid compound combining an artesunate covalently linked to a C-16 amine derived from 3-methoxy-estradiol exhibited potent antimalarial activity (IC_{50} value of 3.8nM) and is about 2 times more active than its parent compound ATS (IC_{50} value of 8.9nM) as well as CQ (IC_{50} value of 9.8nM). However, no information regarding the cytotoxicity was revealed (Figure 16, compound 7).

Tetraoxanes have been largely studied since they are synthetic analogues of ART. This type of compounds might overcome the high cost and bioavailability issues related with ART and its derivatives.

Recently, two steroidal compounds exhibited a potent antimalarial activity against the blood stage of both *P. falciparum* strains with IC_{50} values between 4 and 40nM (190). *In vivo* experiments were performed with these compounds revealing a respective reduction of 60% and 91% of the *P. berghei* liver load. Furthermore, these compounds have a moderate *in vitro* activity against stage IV-V *P. falciparum* gametocytes (IC_{50} value of 1.16 μM). The authors did not explore the mechanism of action, but they speculated that peroxide-generated oxidative radicals could contribute to parasite elimination (190) (Figure 16, compound 8 and 9).

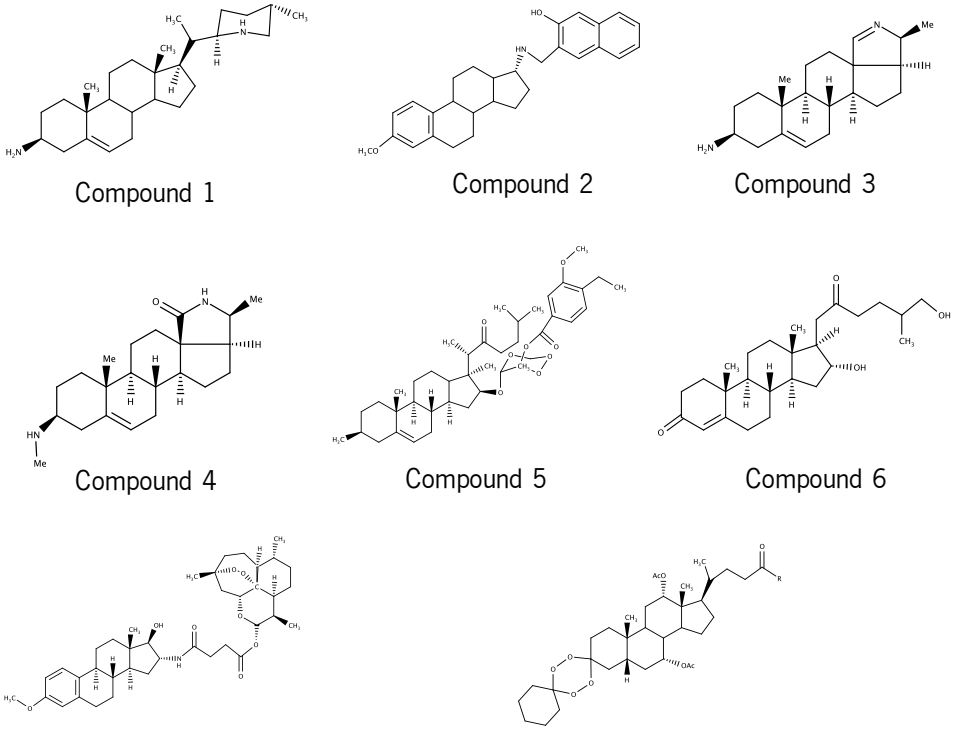


Figure 16: Examples of synthetic steroids explored as antimalarial compounds. Chemical structures of synthetic steroid derivatives where the antimalarial activity was displayed.

3. AIMS OF THE THESIS

P. falciparum multidrug resistance is a relevant event that contributes for the failure of present antimalarial treatment based on ACTs. Development and spread of resistance to ACTs is a real threat to recent global malaria control and elimination achievements. Therefore, it is fundamental to fully understand the mechanisms involved in resistance to improve the longevity of the current ACTs. Unveil these molecular factors is also important not only to circumvent the expansion and spread of malaria but it will further contribute for the formulation and development of new antimalarial candidates, urgently in need since no new drugs are yet available to replace the current ACTs. To overcome this problem, the most important strategies include rational drug design and exploitation of natural compounds either as a source of antimalarial agents or as a starting point to design novel bioactive derivatives.

Taking this into account, the specific objectives of this thesis were:

Aim 1: To probe the interplay of known *pfmdr1* resistance markers, namely, gene copy number variation with N86Y and Y184F single nucleotide polymorphisms, we aim to explore the natural worldwide prevalence and temporal changes of this polymorphism's combination. Furthermore, through gene editing approach, create tools that enable to evaluate their impact in the kinetics of the transporter helping to explain the differential antimalarial response and aid on drug development strategies.

Aim 2: To design, synthetize and exploit compounds based on steroid scaffold for their antimalarial properties in regards to parasite multistage activity, potency against multidrug resistance strains and synergism with present antimalarials. We also aim to unveil possible mechanisms of action thereby underscoring the possibility to find and develop new antimalarial drugs based on steroids.

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CHAPTER II

EXPANSION OF A SPECIFIC *PLASMODIUM FALCIPARUM* PfMDR1 HAPLOTYPE IN SOUTHEAST ASIA WITH INCREASED SUBSTRATE TRANSPORT

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Manuscript sum-up

In vitro data combined with public database alerted us about the geographic selection and expansion of a *pfmdr1* gene amplification with N86F184 haplotype with higher digestive transport efficacy associated with multidrug resistance in Southeast-Asia.

Highlights

- The public database MalariaGEN project, demonstrated that *pfmdr1* gene amplification is especially common in Southeast Asia, with amplifications being selected along with the N86 and 184F alleles.

- Using the genetic tool zinc-finger nucleases, the PfMDR1 was modified at amino acids 86 and 184, to obtain two gene-editing parasites comprising the most prevalent polymorphisms found in Southeast Asia, *pfmdr1* amplified with N86Y184 and N86F184 haplotypes.

- Parasites expressing the N86F184 haplotype possess a higher digestive transport capacity.

- This feature directly affects the parasite's response since parasites containing the N86F184 haplotype have a decreased susceptibility to LMF compared with parasites harboring the N86Y184 haplotype.

- The data here presented could help us optimize and personalize antimalarial usage to different malaria endemic regions taking into account the prevalence of associated *pfmdr1* polymorphisms.

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RESEARCH ARTICLE
Molecular Biology and Physiology

Expansion of a Specific *Plasmodium falciparum* PfMDR1 Haplotype in Southeast Asia with Increased Substrate Transport

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ABSTRACT Artemisinin-based combination therapies (ACTs) have been vital in reducing malaria mortality rates since the 2000s. Their efficacy, however, is threatened by the emergence and spread of artemisinin resistance in Southeast Asia. The *Plasmodium falciparum* multidrug resistance protein 1 (PfMDR1) transporter plays a central role in parasite resistance to ACT partner drugs through gene copy number variations (CNV) and/or single nucleotide polymorphisms (SNPs). Using genomic epidemiology, we show that multiple *pfmdr1* copies encoding the N86 and 184F haplotype are prevalent across Southeast Asia. Applying genome editing tools on the Southeast Asian Dd2 strain and using a surrogate assay to measure transporter activity in infected red blood cells, we demonstrate that parasites harboring multicopy N86/184F PfMDR1 have a higher Fluo-4 transport capacity compared with those expressing the wild-type N86/Y184 haplotype. Multicopy N86/184F PfMDR1 is also associated with decreased parasite susceptibility to lumefantrine. These findings provide evidence of the geographic selection and expansion of specific multicopy PfMDR1 haplotypes associated with multidrug resistance in Southeast Asia.

IMPORTANCE Global efforts to eliminate malaria depend on the continued success of artemisinin-based combination therapies (ACTs) that target *Plasmodium* asexual blood-stage parasites. Resistance to ACTs, however, has emerged, creating the need to define the underlying mechanisms. Mutations in the *P. falciparum* multidrug resistance protein 1 (PfMDR1) transporter constitute an important determinant of resistance. Applying gene editing tools combined with an analysis of a public database containing thousands of parasite genomes, we show geographic selection and expansion of a *pfmdr1* gene amplification encoding the N86/184F haplotype in Southeast Asia. Parasites expressing this PfMDR1 variant possess a higher transport capacity that modulates their responses to antimalarials. These data could help tailor and optimize antimalarial drug usage in different regions where malaria is endemic by taking into account the regional prevalence of *pfmdr1* polymorphisms.

KEYWORDS malaria, *Plasmodium falciparum*, *pfmdr1*, antimalarial drug resistance, copy number variation, Y184F mutation

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In 2018, an estimated 228 million cases of malaria, predominantly in sub-Saharan Africa, resulted in 405,000 deaths worldwide, mostly in children less than five years old. *Plasmodium falciparum* is responsible for most of the global malaria burden. Over the past 2 decades, impressive gains have been made in the reduction of global malaria morbidity and mortality rates, which stood at an estimated one million deaths per year in the early 2000s (1). The widespread adoption of artemisinin (ART)-based combination therapies (ACTs) and increased population-level coverage of insecticide-treated bed nets to protect against infective *Anopheles* mosquito vectors have been important contributors in the fight against malaria (2, 3). Unfortunately, the emergence and spread of ART- and ACT partner drug-resistant *P. falciparum* lineages in the Greater Mekong subregion of Southeast Asia (4–9) and in South America (10, 11) during the past decade pose a significant threat to malaria control and elimination. Efforts are under way to contain the spread of ART resistance into Africa and India (12–14) where it is predicted to have devastating results (15), as happened earlier with the emergence and global spread of chloroquine (CQ) resistance (8, 16, 17).

One important mediator involved in multidrug resistance to ACTs is the ATP-binding cassette (ABC) transporter *P. falciparum* multidrug resistance protein 1 (PfMDR1). PfMDR1 is localized in the membrane of the digestive vacuole (DV) of the parasite and possesses the ability to influx antimalarial drugs toward the lumen of this organelle (18–20). Transport studies using heterologous expression systems have shown that PfMDR1 variants have the ability to modulate the transport of the antimalarial drugs halofantrine and quinine (QN) (21). A surrogate assay of PfMDR1 activity using the fluorochrome Fluo-4 revealed its subcellular distribution and showed that PfMDR1-mediated solute import into the parasite DV is modulated by at least three C-terminal codon variants (S1034C, N1042D, and D1246Y) (22, 23). The *pfmdr1* N-terminal allelic variants N86Y and Y184F have also been implicated in multidrug-resistant phenotypes. In Africa and Asia, the N86 allele is relevant for *in vivo* and *in vitro* parasite antimalarial responses against aryl-amino alcohols such as mefloquine (MFQ) (24, 25) and lumefantrine (LMF) (26–28). In clinical trials of artemether-LMF or artesunate plus amodiaquine (ADQ), an increased risk of posttreatment recrudescent *P. falciparum* infections was associated with parasites harboring the N86 wild-type or 86Y mutant allele, respectively (29). These *in vivo* observations have been supported by recent allelic exchange approaches (30). Mutations in *pfmdr1* have a drug-specific resistance contribution being "pro and con" depending on the antimalarial. Compared to *pfmdr1* N86Y, the impact of the Y184F mutation on parasite responses to ACTs remains less studied (31). This mutation is one of the five canonical PfMDR1 mutations that was earlier considered to be associated with chloroquine resistance (32) and is now widespread in Southeast Asia and Africa (33–36). The capacity of 184F to influence parasite antimalarial responses is thought to be related to alterations in protein structure through allosteric effects that reduce drug binding or alter transport kinetics (20).*pfmdr1* copy number variation (CNV) is another important factor that impacts parasite susceptibility to several antimalarial drugs (18). *P. falciparum* parasites harboring *pfmdr1* amplifications are widely distributed in South America (37) and Southeast Asia (24, 25). Amplification of *pfmdr1* is associated with an increased risk of treatment failure with therapies combining an aryl-amino alcohol such as MFQ or LMF with an ART derivative (25, 38, 39).

Using data from the updated MalariaGEN *Plasmodium falciparum* Community Project (40), we explored the prevalence and distribution of *pfmdr1* copy numbers encoding specific haplotypes. We show that *pfmdr1* gene amplification is especially common in Southeast Asia, with amplifications being selected along with the N86 and 184F alleles. Applying genome editing tools on the 86Y- and Y184-harboring Southeast Asian Dd2 strain, we demonstrate that the N86/184F haplotype in the context of amplified *pfmdr1* imparts a higher transport capacity that directly affects parasite antimalarial responses.

RESULTS

Temporal distribution of PfMDR1 haplotypes and copy number variants in Southeast Asia. We determined the worldwide frequencies of *pfmdr1* N86Y and Y184F mutations and *pfmdr1* copy number using a collection of 7,113 parasite genomes from the MalariaGEN *P. falciparum* Community Project version 6 (https://www.malariagen.net/resource/26) (40). Genomic data were available from 73 countries where malaria is endemic with information on *pfmdr1* N86Y and Y184F mutations, as well as *pfmdr1* copy number, in a subset of 5,003 samples. From that subset, 650 had *pfmdr1* amplifications. Of these, 53 were excluded from further frequency analysis due to the presence of multiple alleles at codon positions 86 and/or 184, indicating the presence of mixed infections (see Table S1 in the supplemental material).*pfmdr1* allele frequency data were extracted from the MalariaGEN Community Project during the period from 2002 to 2015, revealing that the *pfmdr1* N86Y mutation frequency has remained relatively constant in Southeast Asia, with the N86 allele nearly fixed at a frequency of 96 to 98% (N86 allele frequency, 98% between 2002 and 2007, 98% between 2008 and 2010, 99% between 2011 and 2013, and 96% between 2014 and 2015). In Africa, the N86 allele frequency has increased, whereas 86Y has decreased over the same time period (see Fig. S1 in the supplemental material). In contrast to the fixation of the N86 allele in Southeast Asia, the frequency of the 184F variant has increased within the same time frame (21% between 2002 and 2007, 30% between 2008 and 2010, 40% between 2011 and 2013, and 59% between 2014 and 2015), leading to the selection of the N86/184F haplotype in Southeast Asia between 2002 and 2015 (Fig. S1).

The MalariaGEN Community Project data also showed that *pfmdr1* amplifications are the most prevalent in Southeast Asia, with only one African isolate reported in 2009 from Ghana harboring two *pfmdr1* copies encoding a N86/Y184 haplotype (Table S1). In Southeast Asia, infections with *P. falciparum* isolates harboring *pfmdr1* amplifications were most prevalent in Thailand (52%), followed by Cambodia, Myanmar, and Vietnam (15%, 14%, and 6%, respectively) (Fig. 1A). In Cambodia, the frequency of isolates with *pfmdr1* amplifications decreased progressively from 48% (12/25 isolates) during 2002 to 2007 to 25.6% (69/269 isolates) during 2008 to 2010 and 9.6% (55/572 isolates) between 2011 and 2013) (41, 42). In contrast to Cambodia, the frequency of isolates with *pfmdr1* gene amplifications in Thailand showed an increasing trend over the same period (45% [104/229] during 2002 to 2007, 47% [144/306] during 2008 to 2010, and 61% [168/275] during 2011 to 2013). Genomes containing *pfmdr1* amplifications encoding the N86 allele predominated (97.8%), with only three isolates identified in Cambodia carrying amplified copies of the *pfmdr1* 86Y variant (3/136 genomes) (Fig. 1B and Table S1).

In Cambodia, the 184F allele frequency in isolates also carrying multiple copies of the *pfmdr1* N86 allele has increased (25% [3/12] between 2002 and 2007% to 62% [43/69] between 2008 and 2010 and 85% [47/55] between 2011 and 2013). In contrast, in Thailand, the 184F allele frequency has remained stable in isolates containing multicopy *pfmdr1* over the years (8% [8/104] between 2002 and 2007% to 6% [8/144] between 2008 and 2010 and 10% [17/168] between 2011 and 2013). Limited or no genome data were available from Vietnam and Myanmar between 2002 and 2010, precluding the analysis of haplotype selection over time in these countries (Fig. 1B and Table S1).

Together, these results show that *pfmdr1* amplification is especially common in Southeast Asia, with amplifications encoding the N86 and 184F residues being selected.

Amplified *pfmdr1*-edited parasites at codons 86 and 184. We developed a targeted gene editing approach to study the impact of haplotype-specific increases in *pfmdr1* copy number on *P. falciparum*. Parasite lines derived from Southeast Asian *P. falciparum* isolates from the 1980s, like Dd2, characteristically contain the 86Y allele. To investigate the advantage of the predominant genotypes observed in Southeast Asia described above (*pfmdr1* amplifications containing haplotypes N86/184F or N86/Y184), we performed gene editing in all *pfmdr1* gene copies present in Dd2 parasites. Our Dd2 line represents a MFQ-resistant clone carrying four *pfmdr1* copies isolated from the

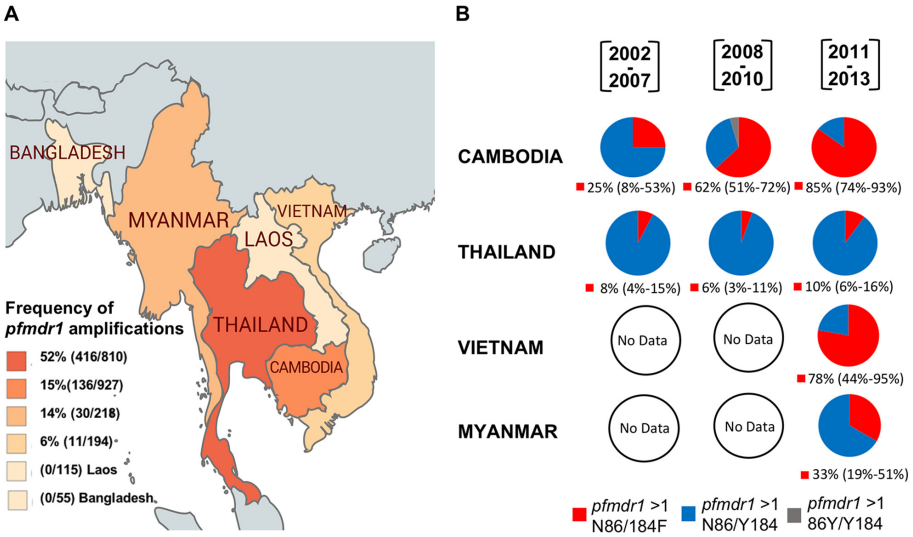

FIG 1 Geographical distribution of amplified *pfmdr1* gene copies and haplotype selection in Southeast Asia. Genetic analysis used data generated by the recently updated MalariaGEN *Plasmodium falciparum* Community Project (version 6; https://www.malariagen.net/resource/26) (40), comprising genomes with information on *pfmdr1* copy number, single nucleotide polymorphisms (SNPs), and no evidence of mixed infections. (A) Country shadings show the frequency of genomes with amplified *pfmdr1* copies (number of amplified *pfmdr1* copies/total number of genomes) collected between 2002 and 2015. (B) Pie charts represent different PfMDR1 haplotypes present in genomes containing multiple *pfmdr1* gene copies (per country data are shown in Table S1 in the supplemental material) and stratified into three intervals of time (2002 to 2007, 2008 to 2010, and 2011 to 2013). No samples were available for the years 2014 and 2015. The N86/184F haplotype frequency is shown with 95% confidence intervals in parentheses below each pie chart. Empty pie charts represent time periods in countries where no data were available.

W2mef line, originally derived from the Southeast Asian CQ-resistant parasite strain W2 (harboring a single copy of *pfmdr1*) upon extended culture with MFQ for 96 weeks (43). Dd2 and W2 parasites express the PfMDR1 86Y/Y184 haplotype and the CQ resistance-conferring CVIET haplotype of the *P. falciparum* chloroquine resistance transporter (PfCRT) (Table 1) (44, 45).

Using the previously described zinc-finger nuclease (ZFN) strategy to engineer *pfmdr1* mutations at codons 86 and 184, we successfully generated the Dd2 edited lines, NF^{Dd2} and NY^{Dd2}, each maintaining four copies of *pfmdr1* encoding the N86/184F and N86/Y184 haplotypes, respectively (Table 1 and Fig. 2A and B). These edited lines represent the two major geographic variants of *pfmdr1* present in Southeast Asia and include gene amplifications. The presence of pure N86/184F and N86/Y184 haplotypes in NF^{Dd2} and NY^{Dd2} lines, respectively, was confirmed by Sanger sequencing (Fig. 2C). Quantitative PCR analysis demonstrated that the edited NF^{Dd2} and NY^{Dd2} lines maintained four copies of *pfmdr1* as present in the unedited Dd2 parental strain (Fig. 2D). Attempts to engineer parasites expressing the 86Y/184F haplotype failed. We verified that this haplotype combination was not found among the 597 parasite genomes harboring amplified copies of *pfmdr1* in the MalariaGEN Community Project data set

TABLE 1 *pfmdr1* and *pfcrt* polymorphisms of gene-edited and parental *P. falciparum* laboratory strains

		pfmdr1 polymorphisms						*pfcrt* polymorphisms				
Parasite	Plasmid	CNV	86	184	1034	1042	1246	72	73	74	75	76
NF^{Dd2}	$pmdr1^{NF}$[a]	4	N	F	S	N	D	C	V	I	E	T
NY^{Dd2}	$pmdr1^{NY}$[a]	4	N	Y	S	N	D	C	V	I	E	T
Dd2		4	Y	Y	S	N	D	C	V	I	E	T
W2		1	Y	Y	S	N	D	C	V	I	E	T

[a]Plasmids were previously constructed (30). CNV, copy number variation.

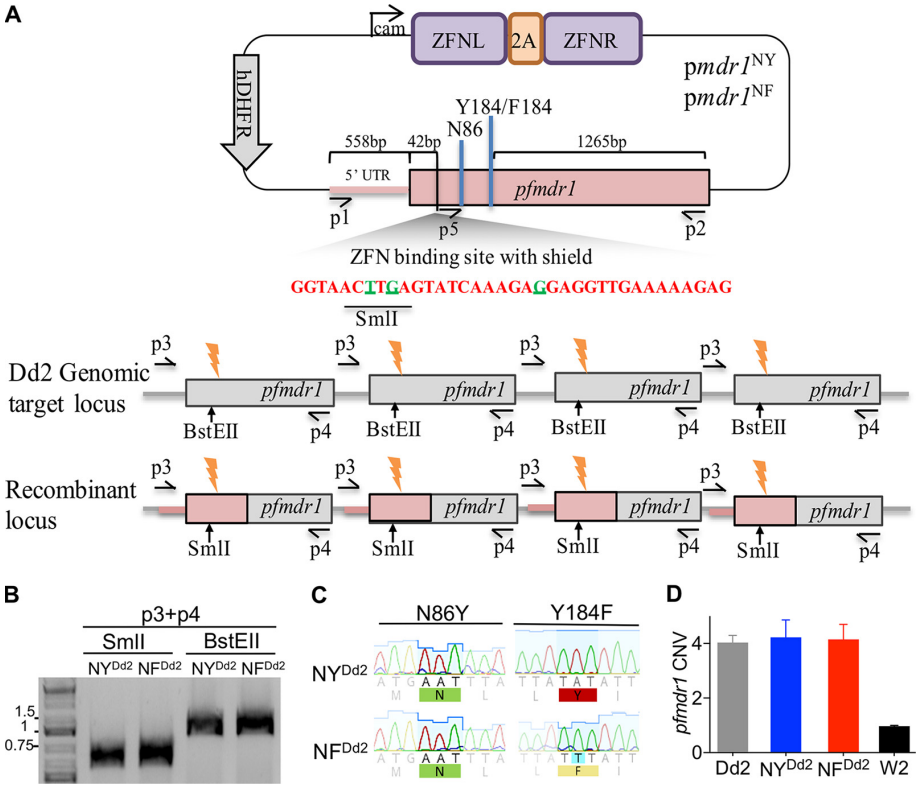

FIG 2 Schematic representation of the ZFN-based *pfmdr1* editing strategy. (A) The plasmids p*mdr1*^{NY} and p*mdr1*^{NF} (30) harbor a pair of *pfmdr1*-specific ZFNs (ZFN left [ZFN L] and ZFN right [ZFN R]) separated by a 2A skip peptide that drives their polycistronic expression from the calmodulin promoter (cam), and the human dhfr (hdhfr) selectable marker. The ZFNs induce a double-strand break (orange thunderbolt) 42 bp downstream of the *pfmdr1* start codon, which is repaired by homologous recombination with the 2.4-kb *pfmdr1* donor sequence present on the plasmid. The *pfmdr1* donor sequence from p*mdr1*^{NY} and p*mdr1*^{NF} plasmids contains the N86/Y184 or N86/184F genotypes, respectively, and carries three synonymous mutations (nucleotides colored in green) at the ZFN binding site to prevent ZFNs from cleaving the plasmids or the *pfmdr1*-edited sequences. Two of the three synonymous mutations create a SmlI restriction site and abolish a BstEII restriction site present in the nonedited (genomic) template. (B) PCR-RFLP genotyping confirming the presence of *pfmdr1*-edited lines NY^{Dd2} and NF^{Dd2}. The silent mutations introduced into the donor sequence created a SmlI restriction site, which was used to screen PCR products (yielding a 1.3-kb band with P3 and P4 primers, which formed a doublet at 667/669 bp in edited parasites upon SmlI digestion). BstEII cuts the P3 and P4 PCR product in nonedited parasites and was used as a control; the observed 1.3-kb band was not cut, indicating the presence of correctly edited parasites. (C) Sanger sequencing chromatograms of DNA samples from the NY^{Dd2} and NF^{Dd2} edited lines at the *pfmdr1* locus, confirming correct editing at codons 86 and 184. (D) Plot showing means plus standard errors of the means (SEM) (error bars) from three independent assays of *pfmdr1* copy number, analyzed through quantitative PCR using β-tubulin as a control gene and the 3D7 strain as a calibrator with a single *pfmdr1* copy.

(Table S1). These findings suggest that the presence of the 86Y/184F haplotype in amplified *pfmdr1* copies might render the parasites nonviable or might confer a survival disadvantage to the parasite population.

Fluo-4 transport into the parasite DV is altered by amplified PfMDR1 haplotypes. We evaluated the influence of *pfmdr1* polymorphisms at codons 86 and 184 on PfMDR1 functional activity by measuring the accumulation of Fluo-4 in the DVs (22) of the gene-edited parasite lines (NF^{Dd2} and NY^{Dd2}) compared to the Dd2 parental control strain.

Accumulation of Fluo-4 in the DVs of NF^{Dd2}, NY^{Dd2}, and Dd2 parasites was observed by fluorescence microscopy and measured by flow cytometry (Fig. 3). Fluo-4 accumulation in the DV of the parasite was detectable after 120 min of incubation, prior to which time the probe was distributed throughout the infected red blood cell (RBC) and parasite cytosol (Fig. S2).

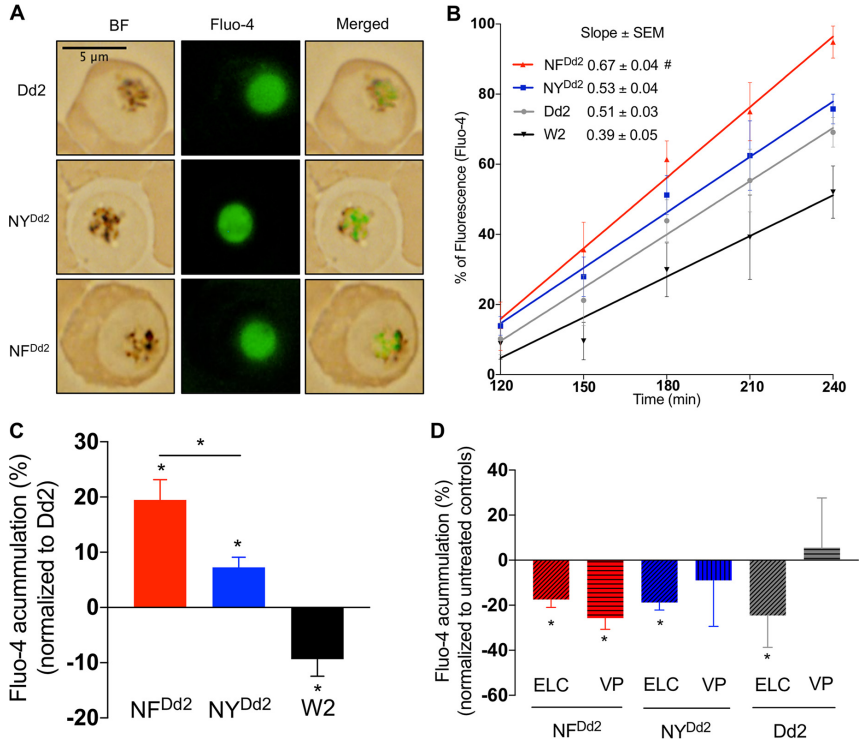

FIG 3 Evaluation of Fluo-4 accumulation. (A) Representative images of Dd2 and *pfmdr1*-edited lines (NY^{Dd2} and NF^{Dd2}) stained with Fluo-4 AM (green). BF, Bright field. The bar measures 5 μm. (B) PfMDR1-associated dynamic transport between the strains Dd2, NF^{Dd2}, NY^{Dd2}, and W2, as determined by linear regression of the Fluo-4 probe signal over time. Data are shown as comparative mean ± SEM (error bars) (as a percentage) from four independent assays. Linear regression and slopes extracted from each equation. # indicates a significant difference in the rate of Fluo-4 accumulation in NF^{Dd2} parasites compared with the rates obtained in NY^{Dd2} and Dd2 parasites ($P = 0.04$ and $P = 0.007$, respectively). (C) Normalized Fluo-4 AM intensities (as a percentage) in all parasite strains measured at 240 min. The fluorescence intensities exhibited by NF^{Dd2} and NY^{Dd2} and the W2 strain were normalized to the parental Dd2 parasite strain (indicated in the graph as the baseline [$y = 0$]). Values below or greater than zero indicate a decrease or increase, respectively, in the percentage of accumulated fluorescence compared with Dd2 parasites. Mann-Whitney U tests were used to assess statistically significant differences between the NF^{Dd2}, NY^{Dd2}, Dd2, and W2 parasites in panels B and C (*, $P < 0.05$). (D) Normalized Fluo-4 intensities (as a percentage) in the absence (untreated control) and presence of elacridar (ELC, 0.1 μM) and verapamil (VP, 0.8 μM). Results were normalized to untreated controls (indicated in the graph as the baseline [$y = 0$]). Values below or greater than zero indicate a decrease or increase, respectively, in the percentage of accumulated fluorescence compared with untreated controls. The means plus SEM of four independent measurements are shown. Mann-Whitney U tests were used to assess statistically significant differences between untreated and treated parasites (*, $P < 0.05$).

Time variances in Fluo-4 transport associated with the PfMDR1 N86/184F and N86/Y184 haplotypes in the edited parasite lines were measured by applying a linear regression model from 120 min to 240 min (Fig. 3B). We observed a higher positive slope in the edited NF^{Dd2} parasites that express the 184F variant (slope = 0.67 ± 0.04), compared with NY^{Dd2} ($P = 0.04$) or the Dd2 parental strain (86Y/Y184) ($P = 0.007$) (slope = 0.53 ± 0.04 and 0.51 ± 0.03, respectively), suggesting a nonnegligible influence of amino acid position 184 in PfMDR1 transport capacity (Fig. 3B).

To determine whether there were PfMDR1 haplotype-specific differences in the accumulation of Fluo-4, we measured the Fluo-4 intensity of the *pfmdr1*-edited lines and the W2 comparator strain after normalizing to the fluorescence intensity of Dd2 parasites after 240 min. The results showed that the N86 allele has an important role in the modulation of the PfMDR1 transporter since N86 edited parasites accumulated more Fluo-4 compared with the Dd2 parental strain containing the 86Y allele ($P = 0.03$). Furthermore, edited NF^{Dd2} parasites containing the N86/184F haplotype accumulated

more Fluo-4 in their DVs than edited NY^{Dd2} parasites (haplotype N86/Y184) ($P = 0.03$). W2 parasites, which harbor a single genomic copy of the *pfmdr1* gene with the 86Y/Y184 haplotype, accumulated less Fluo-4 ($P = 0.03$) relative to the parental Dd2 control strain that expresses four copies with the same 86Y/Y184 haplotype (Fig. 3C). The influence of *pfmdr1* copy number on the import of Fluo-4 into the parasite DV observed herein confirms previous reports where a parasite strain harboring only one *pfmdr1* copy accumulated less Fluo-4 compared with parasites expressing two copies (31).

To determine whether the changes in Fluo-4 accumulation were related to the PfMDR1 haplotype, we used elacridar (ELC) as a tool, since it is a well-established inhibitor of P-glycoprotein type ABC transporters (46). We measured the fluorescence intensity of Fluo-4 in NF^{Dd2}, NY^{Dd2}, or Dd2 parasites in the presence of ELC. Data were normalized to the respective untreated controls (NF^{Dd2}, NY^{Dd2}, or Dd2; indicated in Fig. 3D as the baseline [$y = 0$]). We observed a significantly decreased Fluo-4 accumulation in ELC-treated parasites compared with untreated controls ($P = 0.03$). The addition of verapamil (VP), a calcium ion channel blocker that reverses parasite resistance to CQ, led to a significant reduction of Fluo-4 accumulation only in the edited NF^{Dd2} line compared to untreated NF^{Dd2} ($P = 0.03$), suggesting a possible interplay between the PfMDR1 and PfCRT transporters in 184F-expressing parasites (Fig. 3D). The addition of VP produced a trend toward more Fluo-4 accumulation in NY^{Dd2} and Y184-harboring Dd2 parasites compared with NF^{Dd2}. Nevertheless, despite having a different trend, the quite large standard error of the mean (SEM) made these results inconclusive.

Amplified *pfmdr1* with the N86 allele decreases parasite susceptibility to antimalarials. We determined the impact of the N86 allele in the genomic context of multicopy *pfmdr1* on parasite susceptibility to antimalarial drugs. Replacement of the 86Y variant present in Dd2 with N86 in each of the four *pfmdr1* copies (edited parasite line NY^{Dd2}) induced a significant increase in the half-maximal *in vitro* growth inhibitory concentration (IC_{50}) values for MFQ (2.5-fold, $P = 0.02$) and LMF (2.4-fold, $P = 0.02$), as well as for dihydroartemisinin (DHA) (2.3-fold, $P = 0.05$) (Fig. 4 and Table 2). A reduction in the IC_{50} values for MFQ (6.1-fold, $P = 0.05$) in W2 (a single *pfmdr1* copy strain with the 86Y/Y184 haplotype) compared with Dd2 parasites was observed. These data show a clear role of *pfmdr1* gene amplifications, particularly those encoding the N86 allelic variant, in the multidrug resistance phenotype.

In the case of QN, the replacement of 86Y to N86 in multicopy *pfmdr1* resulted in a decrease of IC_{50} values in the NF^{Dd2} and NY^{Dd2} lines (1.6-fold, $P = 0.02$) relative to the parental Dd2 strain.

Our assays revealed no differences in susceptibilities to the quinoline drugs CQ, monodesethyl-ADQ (md-ADQ), piperaquine (PPQ), and pyronaridine (PND) in the context of multicopy *pfmdr1* containing N86.

The N86/184F PfMDR1 haplotype decreases parasite susceptibility to LMF. Analysis of the MalariaGEN *P. falciparum* Community Project data predicted a prevalence of the *pfmdr1* 184F allele in Africa of 58.6% (184F, 1,438/2,454 genomes sampled between 2002 and 2015) and 36% (184F, 858/2,403 between 2002 and 2015) in Southeast Asia, regardless of the number of *pfmdr1* copies (Table S1 and Fig. S1). The frequency of the 184F allele increased worldwide over the years analyzed (Table S1 and Fig. S1). Here, in the context of amplified *pfmdr1* gene copies, we observed up to 1.7-fold ($P = 0.002$) decreased susceptibility to LMF in edited parasites expressing the N86/184F haplotype compared with isogenic parasites expressing the N86/Y184 haplotype (Fig. 4 and Table 2). This suggests a specific effect of the 184F allele in conferring resistance to LMF in parasites that harbor amplified copies of *pfmdr1*.

PfMDR1-mediated resistance phenotypes are modulated by VP and ELC. We investigated the potential interplay between PfMDR1 and PfCRT transporters in modulating parasite susceptibility to antimalarials using the PfCRT and P-type ATPase transporter inhibitors VP and ELC, respectively. All the parasite strains used in this study express the CVIET variant at amino acid positions 72 to 76 of PfCRT (Table 1). In the

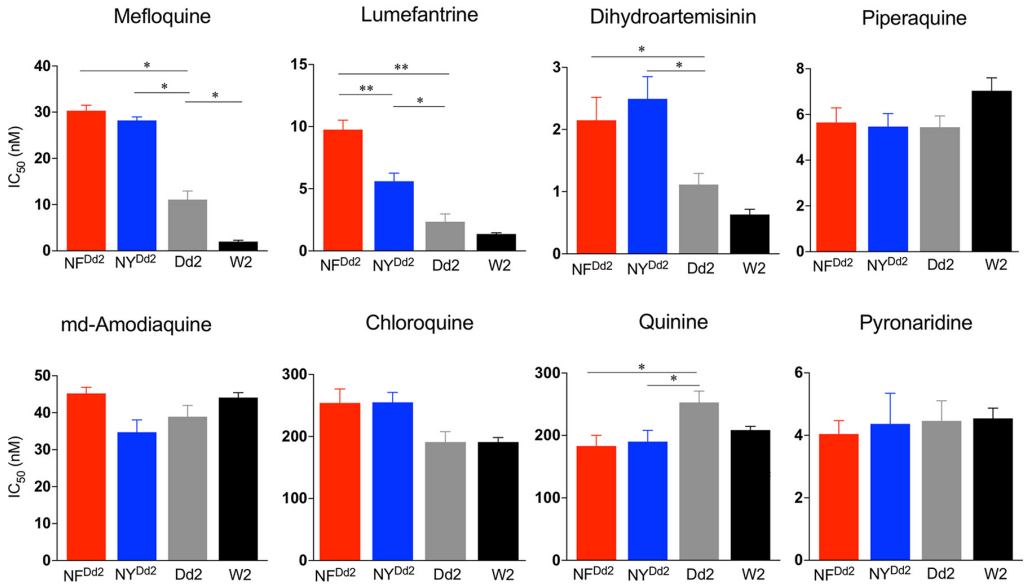

FIG 4 *In vitro* IC_{50} antimalarial response of NF^{Dd2}, NY^{Dd2}, Dd2, and W2 parasite strains. The antimalarial drugs (mefloquine [MFQ], lumefantrine [LMF], dihydroartemisinin [DHA], piperaquine [PPQ], monodesethyl-amodiaquine [md-ADQ], chloroquine [CQ], quinine [QN], and pyronaridine [PND]) were serially diluted, and after 72 h, the parasitemias were determined by flow cytometry after staining the parasites with Mito Tracker Deep Red FM and SYBR green. IC_{50} values were calculated using nonlinear regression analysis. Data are means plus SEM of at least three independent assays. Mann-Whitney U tests were used to assess statistically differences between Dd2 parental strain and NF^{Dd2}, NY^{Dd2}, and W2 strains. *, $P < 0.05$; **, $P < 0.01$.

presence of VP, the multicopy *pfmdr1* Dd2 strain and the edited parasites lines NF^{Dd2} and NY^{Dd2} exhibited increased susceptibility to the 4-aminoquinolines CQ and md-ADQ compared with the respective untreated parasites, with 5-fold and 2- to 3-fold changes, respectively. Increases in susceptibility were also obtained with QN and MFQ in the presence of VP compared with untreated parasites, with N86 producing higher fold changes than 86Y (QN, NF^{Dd2} = 2.8-fold, NY^{Dd2} = 3.1-fold, and Dd2 = 2.2-fold; MFQ, NF^{Dd2} = 4.1-fold, NY^{Dd2} = 5.0-fold, and Dd2 = 2.9-fold) (Table 2). The differences in fold changes observed in the presence of VP can be explained by the differential basal levels of susceptibility to QN and MFQ in the absence of VP in NF^{Dd2}, NY^{Dd2}, and Dd2 parasites (Fig. 4). For LMF, VP sensitized NF^{Dd2} and NY^{Dd2} parasites up to twofold with no effect observed on unedited Dd2 parasites compared with untreated parasites (Table 2).

Comparing the IC_{50} values between the three haplotypes in the presence of VP, a significant increase in QN susceptibility was observed in NF^{Dd2} and NY^{Dd2} parasites compared with the Dd2 parental control (NY^{Dd2} IC_{50} = 60.6 nM versus Dd2 IC_{50} = 111.5 nM, $P = 0.01$; NF^{Dd2} IC_{50} = 65.3 nM versus Dd2 IC_{50} = 111.5 nM, $P = 0.02$). For MFQ and LMF, there was an increase in IC_{50} values for NF^{Dd2} in the presence of VP compared with VP-treated Dd2 parasites (MFQ NF^{Dd2} IC_{50} = 7.5 nM versus Dd2 IC_{50} = 4.2 nM, $P = 0.005$; LMF NF^{Dd2} IC_{50} = 4.4 nM versus Dd2 IC_{50} = 2.5 nM, $P = 0.01$).

In the presence of the P-type ATPase transporter inhibitor ELC, the parasite lines NF^{Dd2} and NY^{Dd2} exhibited 2.8-fold and 2.5-fold increased susceptibility to MFQ, respectively, compared with the corresponding untreated parasites. It is interesting to observe that ELC treatment reverted the MFQ IC_{50} values of NF^{Dd2} and NY^{Dd2} parasites to the same level as that of Dd2 in the absence of ELC, validating the impact of the N86 *pfmdr1* variant. ELC treatment did not significantly impact the susceptibility of Dd2, NY^{Dd2}, or NF^{Dd2} parasites to any of the other aryl-amino alcohols tested (i.e., QN and LMF) or the 4-aminoquinolines (CQ and md-ADQ).

TABLE 2 Parasite responses to antimalarials in the presence of verapamil or elacridar[a]

	NF^{Dd2}		NY^{Dd2}		Dd2	
Antimalarial drug(s)	Mean IC_{50} (± SEM)	*P* value	Mean IC_{50} (± SEM)	*P* value	Mean IC_{50} (± SEM)	*P* value
MFQ	30.3 (±1.1)		28.2 (±1.0)		11.1 (±1.8)	
MFQ + VP	7.5 (±0.4)	0.03	5.6 (±0.8)	0.03	4.2 (±0.1)	0.03
MFQ + ELC	10.9 (±0.7)	0.03	11.1 (±1.5)	0.03	7.7 (±0.8)	NS
LMF	9.7 (±0.8)		5.6 (±0.6)		2.3 (±0.6)	
LMF + VP	4.4 (±0.3)	0.01	3.0 (±0.2)	0.008	2.5 (±0.3)	NS
LMF + ELC	6.8 (±1.7)	NS	5.5 (±0.8)	NS	3.6 (±0.4)	NS
DHA	2.1 (±0.4)		2.5 (±0.4)		1.1 (±0.2)	
DHA + VP	1.2 (±0.1)	NS	1.3 (±0.1)	NS	1.0 (±0.1)	NS
DHA + ELC	1.5 (±0.1)	NS	1.8 (±0.09)	NS	1.2 (±0.1)	NS
PPQ	5.7 (±0.6)		5.5 (±0.6)		5.4 (±0.5)	
PPQ + VP	4.8 (±0.9)	NS	4.7 (±0.2)	NS	4.9 (±0.5)	NS
PPQ + ELC	5.9 (±1.2)	NS	5.1 (±0.5)	NS	5.0 (±0.7)	NS
md-ADQ	45.2 (±1.7)		34.7 (±3.4)		38.9 (±3.0)	
md-ADQ + VP	21.7 (±1.0)	0.05	15.0 (±2.8)	0.05	14.3 (±1.2)	0.05
md-ADQ + ELC	39.6 (±3.9)	NS	35.4 (±3.2)	NS	43.3 (±1.4)	NS
CQ	254.1 (±15.8)		255.1 (±22.6)		191.2 (±16.6)	
CQ + VP	45.8 (±2.7)	0.008	47.2 (±2.8)	0.004	37.1 (±2.6)	0.004
CQ + ELC	211.9 (±18.4)	NS	224.7 (±19.2)	NS	169.2 (±6.6)	NS
QN	183.1 (±16.9)		190.0 (±18.1)		252.8 (±18.3)	
QN + VP	65.3 (±22.8)	0.003	60.6 (±33.3)	0.003	111.5 (±3.3)	0.003
QN + ELC	198.1 (±39.6)	NS	211.2 (±57.6)	NS	263.2 (±19.5)	NS
PND	4.1 (±0.4)		4.4 (±0.9)		4.4 (±0.6)	
PND + VP	2.5 (±0.5)	NS	3.4 (±0.2)	NS	3.0 (±0.3)	NS
PND + ELC	2.6 (±0.6)	NS	2.7 (±0.3)	NS	2.6 (±0.2)	0.05

[a]NF^{Dd2}, NY^{Dd2}, and Dd2 parasites were incubated with mefloquine (MFQ), lumefantrine (LMF), dihydroartemisinin (DHA), piperaquine (PPQ), monodesethyl-amodiaquine (md-ADQ), chloroquine (CQ), quinine (QN), and pyronaridine (PND) in the presence or absence of verapamil (VP) or elacridar (ELC). When incubated with verapamil or elacridar, the antimalarial drugs used in study were serially diluted in the presence of 0.8 μM VP or 0.1 μM ELC for the 72-h *in vitro* growth inhibition assays. Parasitemias were determined by flow cytometry after staining the parasites with Mito Tracker Deep Red FM and SYBR green. Mean ± SEM IC_{50} values (nanomolar) were derived from three to five independent drug assays. Mann-Whitney U tests were used to assess statistically significant differences between the untreated control parasites (Dd2, NY^{Dd2}, and NF^{Dd2}) and VP- or ELC-treated parasites. NS, not significant.

DISCUSSION

The propensity of *P. falciparum* to develop antimalarial drug resistance underscores the importance of characterizing its genetic basis. The amplification of *pfmdr1* was the first candidate mechanism implicated in CQ resistance (CQR) (47). Although we now know that PfCRT is the primary determinant of CQR (48), *pfmdr1* can modulate the degree of resistance (49, 50). Several other studies have also shown that increased *pfmdr1* copy number decreases the parasite's susceptibility to other antimalarials such as MFQ, LMF, and ART (25, 38, 39). The worldwide spread of the *pfmdr1* N-terminal 86Y and 184F mutations have also been linked with differential responses to multiple quinoline-based antimalarials and ART, both *in vitro* and *in vivo* (33, 36, 51–54). The conjugation of these two types of polymorphisms (gene copy number and specific sequence haplotypes) could modulate the *P. falciparum* response to these antimalarials.

Using data from the MalariaGEN *P. falciparum* Community Project, we determined the worldwide prevalence and temporal changes of *pfmdr1* copy number with specific *pfmdr1* N-terminal mutations. Furthermore, we measured the impact of these polymorphisms on solute kinetics of this transporter. We note that the MalariaGEN data from a given period (2002 to 2015 in our case) relates to samples whose locations were dependent on which partner studies were operative at the time (40). Temporal trends in aggregated data should therefore be interpreted with caution. From 73 countries where malaria is endemic with information on *pfmdr1* amplifications and mutations at amino acid positions 86 and 184, between 2002 and 2015, *pfmdr1* amplification was found almost exclusively in Southeast Asia, where we also observed a near fixation of the N86 allele (Fig. 1; see also Table S1 and Fig. S1 in the supplemental material). Considering the previous strong association of *pfmdr1* amplifications and the N86 allele with treatment failures that included MFQ or LMF (24, 25, 39), the frequencies observed could be explained by the extensive use of artesunate plus MFQ and artemether plus LMF in this region during that time period. Thailand, for example, is the country with

the highest frequency of *pfmdr1* amplifications, possibly reflecting the extensive use of artesunate-MFQ as a first-line antimalarial treatment. Its failure during the following years led to changes in treatment policy, with the adoption of dihydroartemisinin (DHA)-PPQ as first-line therapy in 2008 (1, 2). This combination was associated with a rapid decline in *pfmdr1* copy number (55, 56), which could have resulted from PPQ-mediated selection against multicopy *pfmdr1* and/or selective pressure against multicopy *pfmdr1* parasites that show some loss of fitness and can therefore be outcompeted by single-copy *pfmdr1* parasites (2, 57, 58). In Cambodia, the loss of DHA-PPQ efficacy led to the resumption of artesunate-MFQ use beginning in 2011, and this combination retains good efficacy with *pfmdr1* copy number generally remaining at one (59).

In contrast, the MalariaGEN data illustrate *pfmdr1* amplifications as a rare event in Africa. The substantial use of CQ in the past and the current use of the ACT combination artesunate-ADQ possibly contribute to this fact by selecting for deamplifications (60, 61). Furthermore, although artemether-LMF has been used for many years in Africa (62), the lack of MFQ usage could partially explain the lack of *pfmdr1* amplifications. Nevertheless, it is conceivable that *pfmdr1* amplifications may increase in prevalence in Africa due to the widespread use of artemether-LMF (63, 64). The presence of the 86Y allele in Africa could also contribute to the low prevalence of *pfmdr1* amplifications as this allele is present at a very low frequency worldwide when combined with *pfmdr1* amplifications (6/597 genomes). Another factor could be related to the known fitness cost associated with *pfmdr1* amplifications, which manifests as decreased parasite survival in the absence of drug pressure (58, 65). This feature is of particular relevance in high transmission areas such as in sub-Saharan Africa. An interesting observation is that within the MalariaGEN data, *pfmdr1* amplifications with the haplotype 86Y/184F were not present and despite several attempts to engineer this specific parasite line, we were unsuccessful, possibly related to a genetic background that is detrimental for survival.

Over time in Southeast Asia, we observed a clear selection of the 184F allele in combination with *pfmdr1* amplifications containing the N86 allele. Cambodia was the country with the highest observed selection (25% [3/12] between 2002 and 2007% to 62% [43/69] between 2008 and 2010 and 85% [47/55] between 2011 and 2013) (Fig. 1). It is important to note that at the same time that this takeover of the 184F occurs, the prevalence of *pfmdr1* duplications was actually shrinking due to the switch in first-line treatment to DHA-PPQ. To better understand this selection of the N86/184F haplotype in the context of amplified *pfmdr1*, we generated two edited lines representing the two major geographic variants from Southeast Asia and studied the impact of the specific haplotypes on antimalarial responses. The impact of these polymorphisms on PfMDR1 functional transport was also measured through accumulation of Fluo-4 in the DV of the parasite (Fig. 3). This approach has been previously used for *pfmdr1* variants and revealed an important impact of the C-terminal amino acid 1042 on Fluo-4 transport (22, 23). In these studies, although they used strains encompassing different haplotypes in regard to the *pfmdr1* N-terminal mutations, their direct impact was not evident. In our Fluo-4 accumulation study with the Dd2 strain and isogenic lines differing only in their *pfmdr1* N-terminal residues at positions 86 and 184, we observed that parasites expressing the mutations 86Y and 184F were capable of transporting Fluo-4 into the DV (Fig. 3A). The N86 allele by itself impacted Fluo-4 transport capacity, with significant differences obtained between the parental Dd2 strain and NY^{Dd2} (Fig. 3C). Transport studies in Xenopus oocytes have also provided evidence of the impact of polymorphisms at position 86 on the ability of PfMDR1 to transport different drugs. A single amino acid alteration from N86 to 86Y in PfMDR1 resulted in loss of the ability to transport QN and CQ (21). Consistent with this observation, the presence of the N86 allele induced a significant impact on antimalarial responses (Fig. 4). We were able to confirm the involvement of residue 86, specifically in the context of multicopy *pfmdr1*, in modulating parasite susceptibility to multiple antimalarials. Gene-edited parasites with amplified *pfmdr1* harboring the N86 allele displayed decreased susceptibility to

MFQ, LMF, and DHA as well as increased susceptibility to QN, compared to the unedited Dd2 parental line possessing the 86Y allele (Fig. 4). These findings are consistent with our previous allelic exchange work that used ZFN-edited parasites expressing the 86Y variant in the context of single-copy *pfmdr1* (30). This observation explains the selection of the N86 allele after treatment with artemether-LMF (26–28) and corroborates with N86 being a risk factor for recrudescence following treatment with artemether-LMF compared with *P. falciparum* infections containing 86Y (29).

The *pfmdr1* 184F mutation adds to the role of polymorphisms at position 86 in modulating parasite drug resistance and transport. We found that edited parasites harboring the N86/184F haplotype accumulated more Fluo-4 inside the DV compared to edited parasites with the N86/Y184 haplotype, accompanied by faster kinetics of PfMDR1-mediated transport activity (Fig. 3B). A previous in silico PfMDR1 model proposed that the 184F allele alters the parasite's response by an allosteric effect on transport kinetics, independent from drug-binding capacity (20). Our gene-edited parasites expressing amplified *pfmdr1* with the N86/184F haplotype displayed a mild but significant decreased susceptibility to LMF (1.7-fold) compared to those harboring the PfMDR1 N86/Y184 haplotype or to the 86Y/Y184-bearing parental Dd2 parasites (Fig. 4). The mild but significant fold difference herein observed was not detected in our previous allelic exchange work for the same haplotype, possibly due to the single *pfmdr1* copy present in the NF10 and KC5 strains precluding sufficient expression to display this phenotype (30). *In vivo* selection of the 184F allele has been reported, typically along with other *pfmdr1* alleles, after artemether-LMF treatment. Most notably, the N86/184F haplotype was selected upon the regular six-dose therapy with artemether-LMF (26, 27). In their analysis of two clinical trials after artemether-LMF treatment, Malmberg et al. demonstrated that recrudescent infections harboring the PfMDR1 N86/184F haplotype, in the context of a single *pfmdr1* copy, were able to endure higher blood LMF concentrations (28). Together with our results that examine the role of copy number, these *in vivo* data suggest that although the *pfmdr1* 184F allele might not be the primary determinant for parasite resistance, it could provide a complementary genetic background that facilitates the acquisition of multidrug-resistant phenotypes, possibly leading to the specific haplotype selection observed in Southeast Asia (Fig. 1).

The interplay between PfMDR1 and PfCRT transporters in modulating parasite susceptibility to antimalarials was also evident in this work (Table 2). Heightened susceptibilities of NF^{Dd2}, NY^{Dd2}, and Dd2 *P. falciparum* lines to MFQ, md-ADQ, CQ, and QN were affected by VP as previously observed in other genetic backgrounds (48, 66). The role of PfMDR1 in the parasite's response to QN remains unclear, but mutant PfCRT is known to contribute to QN resistance (67). Our results with VP also suggest a role for PfCRT. We note that VP increased susceptibility to LMF only in the edited parasites and not in the unedited Dd2 control. Prior data showed selection of the *pfcrt* CQ-sensitive wild-type K76 allele occurs upon artemether-LMF treatment, as confirmed *in vitro* using allelic exchange (68). In the case of LMF, VP might have a greater effect on PfMDR1 in the context of N86, since we observed that VP decreased Fluo-4 accumulation in the parasite's DV in N86 parasites (Fig. 3D). This observation would explain the increased parasite susceptibility to LMF upon VP exposure.

In the presence of ELC, decreased MFQ IC_{50} values were observed in NF^{Dd2} and NY^{Dd2} edited parasites, reinforcing the hypothesis that not only are *pfmdr1* amplifications associated with MFQ resistance (24, 25, 39) but also that the *pfmdr1* N86 allele is a key mediator of MFQ susceptibility. These findings are in line with the MalariaGEN data showing that almost all parasites that contain *pfmdr1* amplifications are in the context of the *pfmdr1* N86 allele.

In conclusion, our *in vitro* parasite drug susceptibility data using *pfmdr1* gene-edited parasites reinforce the role of both *pfmdr1* CNV and haplotype variation in modulating parasite responses to multiple first-line antimalarial drugs. Our investigation highlights the steady geographic expansion of a parasite population harboring a *pfmdr1* gene amplification with the N86/184F haplotype in Southeast Asia, which we show has a

proven higher DV transport efficacy. These data could help tailor and optimize antimalarial drug usage in different areas where malaria is endemic by taking into account the regional prevalence of *pfmdr1* polymorphisms.

MATERIALS AND METHODS

Geographical distribution of *P. falciparum* isolates carrying *pfmdr1* copy number amplifications and the mutations N86Y and Y184F. Genome analysis used data generated by the Wellcome Sanger Institute as part of the MalariaGEN *Plasmodium falciparum* Community Project version 6 (https://www.malariagen.net/resource/26) (40). Sample metadata were obtained from https://www.malariagen.net/resource/26 and were combined with the genotype data for *pfmdr1* polymorphisms at codons 86 and 184. *pfmdr1* copy number was determined using a combination of a coverage-based approach and a method based on position and orientation of reads near discovered duplication breakpoints as described elsewhere (41). The map presented in Fig. 1 was created using mapchart.net, geo-referencing the *pfmdr1* gene copy number prevalence in the region.

Parasite culture. The Dd2 strain (MRA-156, MR4-Malaria Resources) was maintained at ~4% hematocrit with human red blood cells in RPMI 1640 medium supplemented with 2 mM l-glutamine, 200 μM hypoxanthine, 0.25 μg/ml gentamicin, 25 mM HEPES, 0.2% NaHCO3, and 0.25% Albumax II (Invitrogen; ThermoFisher Scientific). Red blood cells were isolated either from whole blood or from buffy coat samples provided by healthy donors of all blood types. Parasite cultures were maintained at 37°C under a humidified controlled atmosphere of 5% O2/5% CO2/90% N2. Parasite growth was monitored by inspecting Giemsa-stained blood smears. Parasite synchronization was performed with 5% sorbitol for 15 min at 37°C. To obtain highly synchronous cultures, sorbitol was added to the culture to eliminate trophozoites. This was repeated 20 h and 44 h after the initial treatment to obtain 4-h postinvasion ring stages that then mature into trophozoites, which were used to measure Fluo-4 fluorescence.

***P. falciparum* transfections.** The previously designed plasmids p*mdr1*^{NF} and p*mdr1*^{NY} (30) were used to transfect Dd2 parasites. Briefly, we introduced the desired mutations into the endogenous *pfmdr1* locus of Dd2 parasites using ZFN-mediated genome editing. These customized ZFNs bind on opposite strands of *pfmdr1*, producing a double-stranded break 42 bp downstream of the start codon. DNA repair proceeds via homologous recombination, as *P. falciparum* lacks the nonhomologous end-joining pathway (69). Our homology-driven repair template consisted of a 2.4-kb *pfmdr1* fragment that encompassed codons 86 and 184 (30).

Ring-stage cultures at 5% parasitemia were electroporated with ~50 μg of plasmid DNA diluted in Cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM HEPES [pH 7.6]). The plasmids express the human dihydrofolate reductase (hDHFR) selectable marker, which confers resistance to the antifolate drug WR99210. To allow transient expression of the ZFNs, WR99210 selection was applied to transfected cultures beginning 24 h postelectroporation and maintained for 6 days to select for edited parasites. Parasites were visible microscopically 6 weeks postelectroporation and screened for editing events via blood PCR using the primers p3 and p4. Positive bulk cultures were cloned by limiting dilution in 96-well plates. After 15 days, cloning plates were screened and parasite-positive wells were screened for ZFN-mediated editing by blood PCR amplification using a supreme NZYTaq II 2× Green Master Mix (NZYTech, Lisbon, Portugal) and the p3 and p4 primers (see Table S2 in the supplemental material). The donor sequence includes silent mutations that create a SmlI restriction site, which was used to screen the PCR products by restriction fragment length polymorphism (RFLP) analysis (a 1.3-kb band obtained with primers P3 plus P4 [Table S2] that forms a doublet at 667/669 bp with SmlI in edited parasites). The BstEII restriction site is present only in the nonedited genomic *pfmdr1* sequence and was therefore used as a control in the PCR-RFLP assay (Fig. 2B). Editing events at codons 86 and 184 were confirmed by Sanger sequencing using the p5 primer (Table S2). Successfully edited parasite lines (NF^{Dd2} and NY^{Dd2}) were expanded and cryopreserved.

In this work, we have compared the edited NF^{Dd2} and NY^{Dd2} lines to an unedited Dd2 parental control harboring the 86Y/Y184 haplotype. An edited Dd2 control was not performed in this study, since the plasmids used herein to edit Dd2 parasites were the same as those used previously by our group (30). In that study, we demonstrated that gene-edited control parasites carrying only silent ZFN binding site mutations did not alter the parasite's response to antimalarial drugs compared with unedited parental lines.

Copy number analysis of *pfmdr1*. Genomic DNA from parental control Dd2 and *pfmdr1*-edited parasites (NY^{Dd2} and NF^{Dd2}) were extracted using the NZY Blood gDNA isolation kit (NZYTech, Lisbon, Portugal) with RNase treatment. Copy numbers of the *pfmdr1* gene were determined using a NZYSpeedy Green Master Mix (2×) (NZYTech, Lisbon, Portugal). The amplification reactions were carried out in 20-μl volumes in a 96-well plate using 10 ng of genomic DNA, 400 nM each forward and reverse primer, and the master mix. The *P. falciparum* β-tubulin gene was used as the housekeeping control gene. The *pfmdr1* and pfβ-tubulin forward and reverse primers (Table S2) were previously designed (70). For each run, 3D7 was used as a single-copy-number control for *pfmdr1*. Thermal cycling was performed at 98°C for 3 min, followed by 44 cycles with 1 cycle consisting of 98°C for 15 s, 59°C for 30 s, and 72°C for 25 s using the Bio-Rad CFX96 real-time system C1000 thermal cycler. Results were analyzed by the $2^{-\Delta\Delta Ct}$ (threshold cycle) method of relative quantification. The $\Delta\Delta Ct$ calculation was used as follows $\Delta\Delta Ct = (Ct \text{ of } pfmdr1 - Ct \text{ of Pf}\beta\text{-tubulin})_\chi - (Ct \text{ of } pfmdr1 - Ct \text{ of Pf}\beta\text{-tubulin})_y$, where χ is sample and y is *P. falciparum* 3D7 strain. The average gene copy number was calculated from three biological replicates, each with technical replicates run in duplicate.

Live-cell imaging of Fluo-4 accumulation in the DV. Fluorescence microscopy was used to evaluate the accumulation site of Fluo-4 AM (Invitrogen; ThermoFisher Scientific) in different parasite strains in trophozoite stages. Synchronized trophozoite cultures, at ~2% parasitemia and 2% hematocrit, were washed with RPMI 1640 medium and loaded with 5 μM Fluo-4 diluted in RPMI 1640 medium. Parasites were incubated for 240 min at 37°C in a humidified controlled atmosphere of 5% O2/5% CO2/90% N2. After 240 min, the parasites were washed twice with 1× phosphate-buffered saline (PBS) and transferred onto a slide. Fluorescence microscopy was performed using an Olympus BX61 microscope equipped with a visible light laser, and the images were recorded with a digital camera (DP70). The parasites incubated with Fluo-4 were excited at 488 nm with the emission in the green channel (505-nm filter), and an exposure time of 457 ms was applied to obtain the images. Single images were obtained using a 100× objective lens. Regions of interest within the infected RBC, including the parasite cytosol and the parasite DV, were recorded with Cell^P software (Electro optics, UK) and the image overlays were obtained using Image J software version 1.52a.

Quantification of Fluo-4 accumulation by flow cytometry. Flow cytometry was used to quantify Fluo-4 accumulation in NF^{Dd2}, NY^{Dd2}, Dd2, and W2 parasites. Parasite labeling and evaluation of Fluo-4 accumulation were performed as described previously (22) with minor modifications. Briefly, synchronized trophozoites were incubated with 5 μM Fluo-4 AM (Invitrogen; ThermoFisher Scientific) for 240 min at 37°C in an airtight environment flushed with 5% O2/5% CO2/90% N2. The level of fluorescence in infected RBCs was recorded by flow cytometry every 15 min until 120 min, and every 30 min thereafter up to a total incubation period of 240 min (see Fig. S2 in the supplemental material). This assay was followed by live-cell imaging at all time points described above to visualize when the Fluo-4 probe accumulated in the parasite DV (Fig. S2). Fluo-4 accumulation in the DV, presented in Fig. 3, was confirmed by fluorescence microscopy before flow cytometry. Thirty minutes before the incubation ended, parasites were loaded with 1.6 μM Mitotracker Deep Red FM (Invitrogen; ThermoFisher Scientific). For flow cytometry, labeled parasites were excited at 488 nm and 633 nm to detect Fluo-4 and Mitotracker Deep Red FM, respectively. Forward (FSC) and side scatterplots (SSC) were used to define the RBC populations followed by gating double-positive populations for Fluo-4 and Mitotracker Deep Red FM. Approximately 200,000 events were read per experimental condition, and the mean Fluo-4 intensity was calculated for each parasite strain. Due to intrinsic variability observed in the different replicate assays, the fluorescence intensity of NF^{Dd2}, NY^{Dd2}, and W2 was normalized to the Dd2 parental control (i.e., Dd2 corresponding to 100%; indicated in the graph as the baseline [$y = 0$]). Values below zero indicate a decrease in the percentage of accumulated fluorescence compared with Dd2 parasites, whereas values above zero indicate an increase.

Fluo-4 accumulation in NF^{Dd2}, NY^{Dd2}, and Dd2 parasites was also measured in the presence or absence of ELC, an inhibitor of P-type ATPase transporters, including PfMDR1 (and others, e.g., ABCI3), or VP, a calcium channel blocker that functions as a CQ resistance reversal agent. Briefly, synchronized trophozoites were washed with RPMI 1640 medium and preincubated for 10 min with ELC (0.1 μM) or VP (0.8 μM). Sample preparation and flow cytometry were performed as described above. The fluorescence intensities of NF^{Dd2}, NY^{Dd2}, and Dd2 in the presence of VP or ELC were normalized to the respective untreated controls (indicated in the graph as the baseline [$y = 0$]). Values below zero indicate a decrease in the percentage of accumulated fluorescence compared with untreated controls, while values above zero indicate an increase.

***In vitro* antimalarial drug assays.** Drug susceptibility assays using CQ, DHA, MFQ, QN, LMF, md-ADQ, PPQ, and PND in the absence or presence of ELC (0.1 μM) or VP (0.8 μM) (48) were run for the edited parasite lines NF^{Dd2} and NY^{Dd2}, the parental control strain Dd2, and the W2 strain. These assays were performed using a published flow cytometry-based method (30). Briefly, synchronized ring-stage parasites at 0.2% starting parasitemia and 1% hematocrit were incubated in the presence of different concentrations of drug (across a dilution range of twofold; 12-point dilution series). After 72 h of incubation at 37°C, samples were stained with 1.6 μM Mitotracker Deep Red FM (Invitrogen; ThermoFisher Scientific) and 2× SYBR green (for DNA staining) (Invitrogen; ThermoFisher Scientific) in 1× PBS for 30 min and analyzed by flow cytometry to determine the parasite growth rates. Approximately 100,000 events were captured per well. *In vitro* IC_{50} values were calculated using nonlinear regression analysis performed with GraphPad Prism 6 software.

Statistical analysis. Nonparametric, two-tailed Mann-Whitney U tests were used to assess IC_{50} antimalarial responses in the presence or absence of VP or ELC and Fluo-4 accumulation differences between the NF^{Dd2} and NY^{Dd2}, Dd2, and W2 parasite strains (normal distribution not assumed; performed with GraphPad Prism Software).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.7 MB.

FIG S2, TIF file, 0.4 MB.

TABLE S1, XLSX file, 0.02 MB.

TABLE S2, XLSX file, 0.01 MB.

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SUPPLEMENTARY MATERIAL

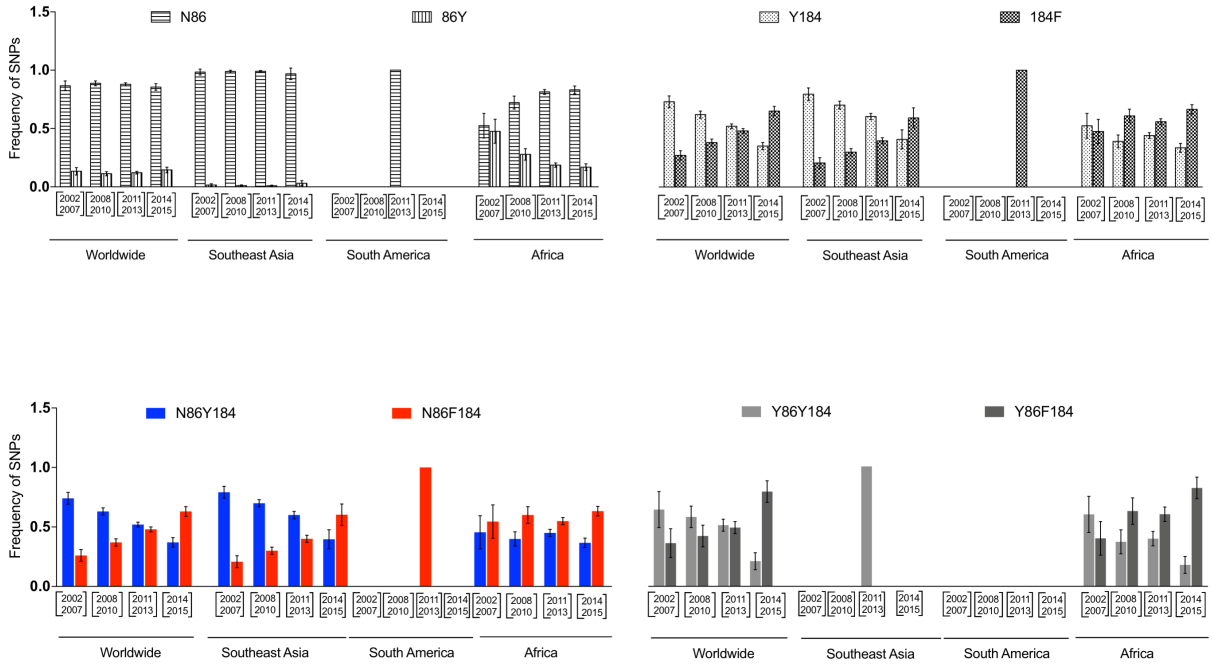


FIG S1 Worldwide frequencies of *pfmdr1* N86Y and Y184F mutations. Genetic analysis used data generated by the recent updated MalariaGEN *Plasmodium falciparum* Community Project (version 6; https://www.malariagen.net/resource/26) (40) and comprised genomes with information on *pfmdr1* gene copy number, SNPs, and showed no evidence of mixed infections. Bars represent the allelic frequency calculated for each allele or combination of alleles and are stratified into four intervals of time (2002 to 2007, 2008 to 2010, 2011 to 2013, and 2014 to 2015). Allele frequencies were determined for the worldwide sample set and were then stratified into three regional categories: Southeast Asia, South America, and Africa. Error bars are 95% confidence intervals. Time intervals without information or below 10 genomes are not represented.

TABLE S1 Prevalence of PfMDR1 haplotypes at amino acid positions 86 and184 and copy number variation. Genetic analysis used data from the MalariaGEN *P. falciparum* Community Project (version 6) (https://www.malariagen.net/resource/26) (40).

Region	Countries	Number of isolates*	Time	Copy Number Variation <1.5-fold					Copy Number Variation > 1.5-fold				
				NY	NF	YY	YF	TOTAL	NY	NF	YY	YF	TOTAL
Southeast Asia	Cambodia	927	2002-2007	7	6	0	0	791	9	3	0	0	136
			2008-2010	85	115	0	0		23	43	3	0	
			2011 - 2013	224	293	0	0		8	47	0	0	
			2014- 2015	22	39	0	0		0	0	0	0	
	Indonesia	84	2002-2007	0	0	0	0	84	0	0	0	0	0
			2008-2010	0	1	0	0		0	0	0	0	
			2011 - 2013	3	41	3	0		0	0	0	0	
			2014- 2015	1	33	1	1		0	0	0	0	
	Laos	115	2002-2007	0	0	0	0	115	0	0	0	0	0
			2008-2010	37	0	0	0		0	0	0	0	
			2011 - 2013	75	3	0	0		0	0	0	0	
			2014- 2015	0	0	0	0		0	0	0	0	
	Myanmar	218	2002-2007	0	0	0	0	188	0	0	0	0	30
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	116	38	1	0		20	10	0	0	
			2014- 2015	27	4	2	0		0	0	0	0	
	Thailand	810	2002-2007	86	35	4	0	394	96	8	0	0	416
			2008-2010	130	30	2	0		136	8	0	0	
			2011 - 2013	72	34	1	0		151	17	0	0	
			2014- 2015	0	0	0	0		0	0	0	0	
	Vietnam	194	2002-2007	0	0	0	0	183	0	0	0	0	11
			2008-2010	71	12	0	0		1	1	0	0	
			2011 - 2013	79	20	1	0		2	7	0	0	
			2014- 2015	0	0	0	0		0	0	0	0	
	Bangladesh	55	2002-2007	0	0	0	0	55	0	0	0	0	0
			2008-2010	11	3	3	0		0	0	0	0	
			2011 - 2013	25	6	7	0		0	0	0	0	
			2014- 2015	0	0	0	0		0	0	0	0	
South America	Colombia	15	2002-2007	0	0	0	0	15	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	0	15	0	0		0	0	0	0	
			2014- 2015	0	0	0	0		0	0	0	0	
	Peru	20	2002-2007	0	0	0	0	19	0	0	0	0	1
			2008-2010	1	9	0	0		0	0	0	0	
			2011 - 2013	0	9	0	0		0	0	0	0	
			2014- 2015	0	0	0	0		0	0	1	0	
Oceania	Papua New Guinea	111	2002-2007	0	0	1	0	109	0	0	0	0	2
			2008-2010	5	1	31	0		0	0	0	0	
			2011 - 2013	16	2	53	0		0	0	2	0	
			2014- 2015	0	0	0	0		0	0	0	0	

Sub-Saharan Africa	Benin	59	2002-2007	0	0	0	0	59	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	0	0	0	0		0	0	0	0	
			2014- 2015	17	21	4	17		0	0	0	0	
	Burkina Faso	20	2002-2007	0	0	0	0	20	0	0	0	0	0
			2008-2010	7	6	0	7		0	0	0	0	
			2011 - 2013	0	0	0	0		0	0	0	0	
			2014- 2015	0	0	0	0		0	0	0	0	
	Cameroon	174	2002-2007	0	0	0	0	174	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	56	93	1	24		0	0	0	0	
			2014- 2015	0	0	0	0		0	0	0	0	
	Congo DR	219	2002-2007	0	0	0	0	219	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	62	40	56	26		0	0	0	0	
			2014- 2015	14	10	7	4		0	0	0	0	
	Ethiopia	24	2002-2007	0	0	0	0	24	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	0	14	0	0		0	0	0	0	
			2014- 2015	0	7	0	3		0	0	0	0	
	Ghana	579	2002-2007	0	0	0	0	578	0	0	0	0	1
			2008-2010	45	85	10	31		1	0	0	0	
			2011 - 2013	72	153	5	31		0	0	0	0	
			2014- 2015	35	100	0	11		0	0	0	0	
	Guinea	92	2002-2007	0	0	0	0	92	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	26	33	8	25		0	0	0	0	
			2014- 2015	0	0	0	0		0	0	0	0	
	Côte d'Ivoire	60	2002-2007	0	0	0	0	60	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	20	21	0	5		0	0	0	0	
			2014- 2015	7	5	0	2		0	0	0	0	
	Kenya	75	2002-2007	6	6	23	0	75	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	0	0	0	0		0	0	0	0	
			2014- 2015	24	16	0	0		0	0	0	0	
	Madagascar	21	2002-2007	0	0	0	0	21	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	2	4	2	9		0	0	0	0	
			2014- 2015	0	4	0	0		0	0	0	0	
	Malawi	201	2002-2007	0	0	0	0	201	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	110	89	1	1		0	0	0	0	
			2014- 2015	0	0	0	0		0	0	0	0	
	Mali	320	2002-2007	14	18	1	16	320	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	68	116	4	36		0	0	0	0	
			2014- 2015	14	21	2	10		0	0	0	0	
	Mauritania	62	2002-2007	0	0	0	0	62	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	0	0	0	0		0	0	0	0	
			2014- 2015	21	30	1	10		0	0	0	0	
	Mozambique	0	2002-2007	0	0	0	0	0	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	0	0	0	0		0	0	0	0	
			2014- 2015	0	0	0	0		0	0	0	0	
	Nigeria	31	2002-2007	0	0	0	0	31	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	2	2	0	3		0	0	0	0	
			2014- 2015	4	10	1	9		0	0	0	0	
	Senegal	76	2002-2007	0	0	0	0	76	0	0	0	0	0
			2008-2010	0	0	0	0		0	0	0	0	
			2011 - 2013	29	20	0	4		0	0	0	0	
			2014- 2015	10	10	0	3		0	0	0	0	
	Tanzania	264	2002-2007	0	0	0	0	264	0	0	0	0	0
			2008-2010	7	8	17	0		0	0	0	0	
			2011 - 2013	107	87	36	2		0	0	0	0	
			2014- 2015	0	0	0	0		0	0	0	0	
	The Gambia	169	2002-2007	0	0	0	0	169	0	0	0	0	0
			2008-2010	17	20	1	11		0	0	0	0	
			2011 - 2013	6	9	0	5		0	0	0	0	
			2014- 2015	26	63	2	9		0	0	0	0	
	Uganda	8	2002-2007	0	0	0	0	8	0	0	0	0	0
			2008-2010	4	3	1	0		0	0	0	0	
			2011 - 2013	0	0	0	0		0	0	0	0	
			2014- 2015	0	0	0	0		0	0	0	0	

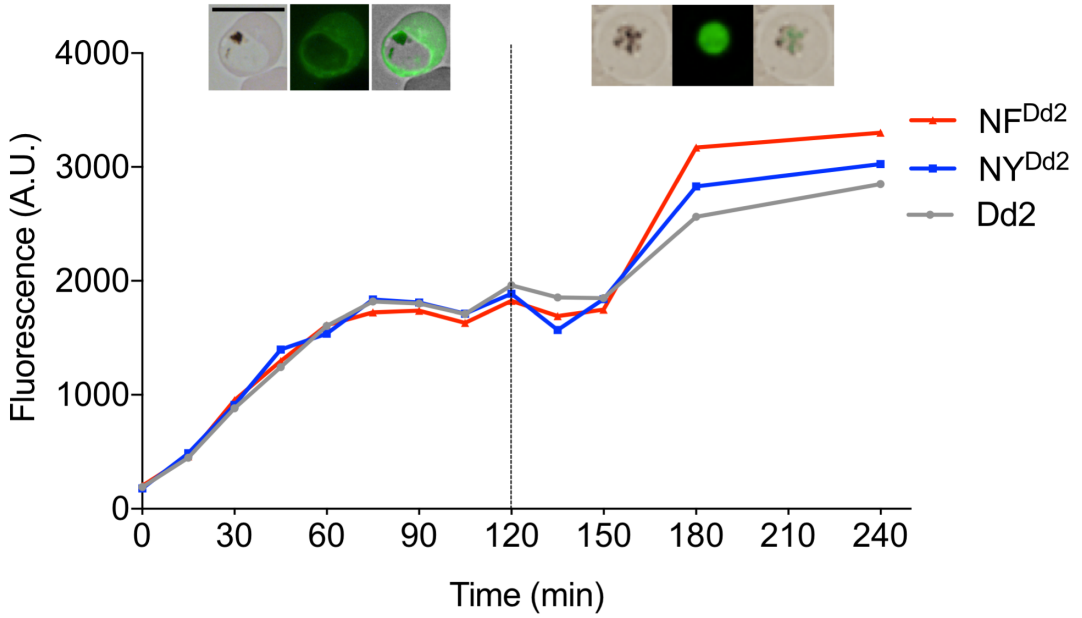


FIG S2 Accumulation of Fluo-4 in arbitrary units of fluorescence over time measured by flow cytometry. Parasites were incubated with Fluo-4 AM (5 μM) for 240 min at 37°C under a humidified controlled atmosphere recording fluorescence by flow cytometer, every 15 min until 120 min and then every 30 min until 240 min. Prior to flow cytometry, live-cell imaging was performed at all time points described above to visualize when the Fluo-4 probe entered the parasite DV. Images presented are representative of the time points. For the flow cytometry assays, the parasites were also loaded with Mitotracker Deep Red FM 30 min before the incubation ended. The scale bar measures 10 μm.

TABLE S2 List of primers used in this study. Primers p1 and p2 were used to perform site-directed mutagenesis at amino acid positions 86 and 184 of *pfmdr1* to obtain the desired sequence. Primers p3 and p4 were used to detect the *pfmdr1*-modified parasites. Primers *pfmdr1* and the reference gene β-tubulin were used to determine copy number variation at the *pfmdr1* locus.

Primers	Sequence (5'–3')
p1_Fw	GTTTGGTGTAATATTAAAGAACATGTtTTTAGGTGATGATATTAATCC
p2_Rv	GGATTAATATCATCACCTAAAaACATGTTCTTTAATATTACACCAAAC
p3_Fw	CATTTTTACTGATTCCTTTAAAAGGC
p4_Rv	CCTCTTCTATAATGGACATGGTATTG
p5	AGAGTACCGCTGAATTATTTAG
***pfmdr1*_Fw$^{(70)}$**	TGCATCTATAAAACGATCAGACAAA
***pfmdr1*_Rv$^{(70)}$**	TCGTGTGTTCCATGTGACTGT
***β-tubulin*_Fw$^{(70)}$**	TGATGTGCGCAAGTGATCC
***β-tubulin*_Rv$^{(70)}$**	TCCTTTGTGGACATTCTTCCTC

CHAPTER III

SYNTHETIC ANTIMALARIAL STEROIDS PRESENT A PROMISING MULTISTAGE ACTIVITY AGAINST *PLASMODIUM FALCIPARUM* THROUGH OXIDATIVE STRESS AND HEME INTERFERENCE

Manuscript in preparation

Manuscript sum-up

Synthetic steroids, with completely unrelated chemical structures to current ACTs, exhibited a promising antimalarial activity. The steroid scaffold has the potential to generate novel bioactive compounds and with appropriate functionalization of these compounds can lead to an encouraging antimalarial activity.

Highlights

- All the synthetic steroids obtained displayed micromolar activity against CQ-sensitive and CQ-resistance parasites (3D7 and Dd2 strains, respectively).

- Low cytotoxicity against human hepatic cell line was revealed for all synthetic steroids displaying a higher selectivity.

- Among these, one compound containing acetate and phthalate functional groups attached to steroid scaffold displayed the most promising antimalarial activity.

- Mechanistic studies suggested that induction of oxidative stress and interference with heme detoxification process could be possible mechanisms of action.

- Synthetic steroids seem to be independent from well-known *P. falciparum* resistance players, such as PfMDR1 and PfCRT.

- A synergistic effect was detected between synthetic steroid compound and MFQ as well as LMF.

ABSTRACT

In 2019, malaria caused near half a million deaths worldwide, being control and elimination hampered by the ability of the malaria parasite to develop drug resistance. Indeed, resistance has been reported even to the present treatment based on artemisinin combination therapies (ACTs). In anticipation of an eventual global ACT failure, there is an urgent need to discover new antimalarial drug candidates. Here we studied the antimalarial potency of synthetic steroids. Steroids comprise a class of molecules involved in several cell biology pathways and pathophysiology systems. Seventeen synthetic steroids were synthesized and screened against the blood stage of CQ-resistant and -sensitive *Plasmodium falciparum* strains. Promising antiplasmodial activities were revealed, with IC_{50} values ranging from 400 nM to 3 μM and a negligible cytotoxicity against human hepatocytes. Preliminary results suggest that these compounds also impact the sexual stage of the parasite life cycle, revealing low micromolar activity against late-V-stage gametocytes.

To understand the possible mechanisms of action in the malaria parasite, several pathophysiological studies were performed. These compounds affected the redox state of the parasites, and inhibit the formation of inert hemozoin, led to accumulation of free heme, as observed in heme fractionation assays. Hemozoin formation takes place in the DV of the parasite. Therefore, the roles of two known transporters players involved in drug resistance (PfMDR1 and PfCRT) located in this organelle, were evaluated to understand cross-resistance with current antimalarials. Our synthetic steroids revealed not to be dependent on these transporters potentiating their value for combination therapies. Indeed, these compounds have been shown to have a synergistic interaction when combined with MFQ and LMF.

Our results suggest that the steroid scaffold is valuable for antimalarial drug discovery and opens the way for optimized functionalization towards novel bioactive steroids.

INTRODUCTION

The increasing occurrence of drug resistance to the currently implemented antimalarial drugs and the lack of effective alternative therapies highlight the urgent need for continuous research in order to discover novel antimalarials and transmission-blocking agents to tackle malaria (1, 2). Nowadays, there is a dramatic decline in the number of new antimalarials entering in the market. Drugs with completely new chemical structures and ideally with new modes of action are rarely becoming available. New developments are usually based on derivatives of existing antimalarials (1–3).

To promote the eradication of malaria, the Malaria Eradication Research Agenda (4) propose that an ideal new antimalarial compound would demonstrate activity against asexual and sexual stages of the parasite life cycle. Preferably, such compound would block transmission from human to mosquito (decreasing the number of new infections), and would be active against multidrug resistant parasites and would be safe in vulnerable populations (children, pregnant women, and immune-compromised patients). Furthermore, the ideal new antimalarial compound should possess pharmacokinetic proprieties that allow for single-dose administration, for better compliance of the treatment (1, 4). Even though the new antimalarial drugs would be used in combination therapies, to delay the development of resistance, each active molecule should be able to kill a large amount of the parasites itself. Nevertheless, novel molecules would need to be active during for at least three or four parasite life cycles to ensure a complete cure. Therefore, they need to have a fast kill profile and additionally be capable to maintain a plasma concentration above the minimal inhibitory concentration (1, 5, 6).

To achieve these goals, the most important strategies include rational drug design and exploitation of natural bioactive compounds. Natural compounds display unique structural features that can enable the discovery of new bioactive chemotypes and can be further exploited as starting materials to develop novel semisynthetic libraries of compounds with improved biological activities (7). It is well-recognized that compounds of natural origin represent a source of new antimalarial drugs being QN and ART good examples of this (8, 9).

One class of organic molecules involved in diverse cell biology pathways are the steroids, a group of lipids that contain a tetracyclic carbon skeleton, composed by three cyclohexane rings and a fourth cyclopentane ring (10, 11). Depending on the lateral chain, steroids are divided in subclasses named cholestanes, cholanes, pregnanes and androstanes. Loss of the C19 methyl group of androstanes, affords estranes and aromatization of the A ring gives place to estratrienes. Sterols are a

subclass of steroids with a 3β-hydroxyl group and a lateral chain at the 17β-position, being cholesterol the most well-known sterol.

The structural characteristics of steroids make them amphiphilic molecules and this is crucial for their incorporation into the cytoplasmic membrane bilayer. Functional groups can be attached at diverse positions in the framework or on lateral chain allowing to modulate the biological activity of these compounds (12). Over the years, innumerous physiological functions of steroids have been revealed (13–17) and the therapeutic potential of steroids has been highlighted in several pathologies like cancer (10). Moreover, some studies have shown that natural occurring steroids and some synthetic compounds containing the steroid scaffold have a great potential as antimalarial candidates displaying low cytotoxicity against human cells (18–21). Furthermore, the steroid scaffold has been used to develop hybrid compounds and a promising antimalarial activity was afforded when ART or its derivatives were covalently linked to a steroid (22, 23). Noteworthy, steroids are inexpensive starting materials for semisynthetic optimization, and allow chirality control of the reactions due to their diastereomeric nature, thus simplifying syntheses and purifications. These are important advantages of steroids in the context of malaria treatment, since novel antimalarials must be inexpensive (24).

A screening assay of a library of steroids synthesized in the Faculty of Pharmacy of the University of Coimbra (25–27) revealed that some steroids possessing ester moieties at diverse positions of the tetracyclic framework were active against the blood stage of malaria (results not published).

Taking into account the structural characteristics of steroids and the bioactivities observed in previous studies, our approach to discover new biologically active compounds was to combine a steroid skeleton with additional structural components conferring appropriate biological activities.

In this work, a library of synthetic steroids was prepared and screened against blood stage *Plasmodium falciparum* strains. Promising antiplasmodial activity were revealed with negligible cytotoxicity against human hepatocytes. The synthetic compounds were active against multidrug resistance parasites. To gain insights into the mechanisms of action of these compounds, additional studies were performed to evaluate the ability to induce oxidative stress and to inhibit heme detoxification process (intrinsic to malaria parasite metabolism). Our data demonstrates the high potential of synthetic steroids as antimalarials, detailing promising functional groups into the scaffold leading to useful information for the structural optimization of a new generation of antimalarial compounds.

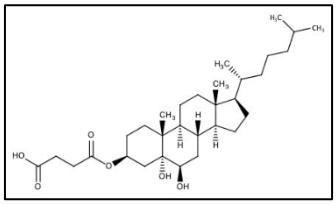
MATERIAL AND METHODS

Chemical synthesis and characterization

General methods

Cholesterol, pregnenolone, diosgenin, sitosterol and dehydroepiandrosterone were purchased from Sigma-Aldrich. All reagents and solvents were purchased from Sigma-Aldrich Co. Reactions were controlled by thin layer chromatography (TLC) using silica gel 60 F254 aluminum sheets (Kieselgel 60HF254, Merck). Preparative TLC experiments were performed on glass plates coated with a mixture of silica gel 60 F_{254} aluminum sheets (Kieselgel 60HF254, Merck). Column chromatographic separations were done using silica gel 60 (Kieselgel, Merck). The melting points for all the compounds were determined using open capillary tubes on a Buchi Melting Point B-540 and are uncorrected. ^{1}H, ^{13}C and DEPT NMR spectra were recorded in a Bruker Avance 400 MHz and sample were prepared in deuterated chloroform ($CDCl_3$), deuterated methanol (MeOD) or DMSO-*d*6. High resolution mass spectrometry (ESI-HRMS) was performed by the microanalysis service on a QSTAR XL instrument (Salamanca, Spain).

Compound CC2 -5α,6β-Dihydroxycholestan-3β-yl hemisuccinate



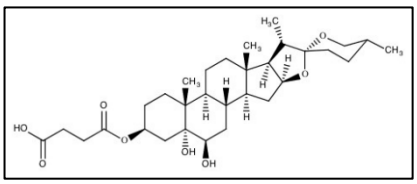
To the solution of cholesterol (386 mg, 1 mmol) in dichloromethane (13 mL), pyridine (3 mL) and succinic anhydride (700 mg) were added. Reaction was stirred at reflux temperature for 7 h and monitored through thin layer chromatography (TLC). After completion, methanol was added to stop the reaction. Then the mixture was dissolved in ethyl acetate and washed sequentially with HCl (5% aqueous solution), $NaHCO_3$ (saturated aqueous solution) and water, dried with anhydrous Na_2SO_4, filtrated and evaporated under vacuum, affording a white solid (390 mg, 80 % yield).

To the solution of cholest-5-ene-3β-yl hemisucinate (150 mg, 0.308 mmol) in acetone (6 ml) at reflux temperature, magnesium bis (monoperoxyphthalate) hexahydrate (MMPP) (250 mg, 1.6 eq.) was added. The reaction mixture was stirred during 2 h and then cooled to room temperature (RT). The reaction was filtered to remove the MMPP and the filtrate washed with acetone (5 mL).

To the resulting solution, bismuth(III) trifluoromethanesulfonate ($Bi(OTf)_3$) was added (25 mg, 0.12 eq). After 2 h at RT, the reaction mixture was stopped by evaporation under vacuum and the solid dissolved in ethyl acetate. The organic phase was washed with water and dried with anhydrous Na_2SO_4, filtrated

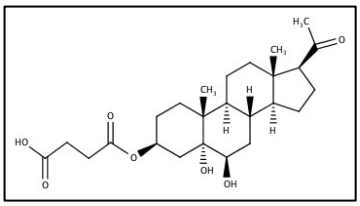
and evaporated under vacuum. The product was subjected to flash column chromatography with gradient elution of petroleum ether/ethyl acetate from 30:1 to 10:7 (v/v), affording 5α,6β-dihydroxycholestan-3β-yl hemisuccinate (**CC2**) as a white solid (104.2 mg, 65 % yield). 52% overall yield from cholesterol. Mp 229-230 °C; IR (film): 3526, 3306, 2932, 2868, 1719, 1708, 1687, 1423, 1279, 1186, 1155, 988,867, 805 cm^{-1}.^{1}H NMR (400 MHz, MeOD) δ 5.21 (1H, s, 3β-H), 3.69 (1H, s, 6β-H), 3.48 (1H, s, 5α-OH), 2.59 (4H, m, $HOOC(CH_2)_2$), 2.19 (1H, t, *J* = 12.0 Hz), 2.04 (1H, d, *J* = 11.7 Hz), 1.19 (3H, s, 19-CH_3), 0.95 (3H, s, 21-CH_3), 0.90 (6H, m, 26-CH_3 and 27-CH_3), 0.73 (s, 3H, 18-CH_3); ^{13}C NMR (101 MHz, MeOD) δ 176.1 (C=O acid), 174.0 (C=O ester), 76.6 (C-5), 76.4 (C-6), 73.2 (C-3), 57.7, 57.5, 46.5, 43.9, 41.5, 40.7, 39.4, 38.0, 37.4, 37.2, 35.3, 33.2, 31.6, 30.5, 29.8, 29.4, 29.2, 27.9, 25.2, 25.0, 23.2, 22.9, 22.2, 19.2, 17.2, 12.6.

Compound CC5 - 5α,6β-Dihydroxyspirostan-3β-yl hemisuccinate



Prepared according to the general procedure described above for the synthesis of CC2 however diosgenin was used as starting substrate (414.6 mg, 1 mmol). 5α,6β-dihydroxyspirostan-3β-yl hemisuccinate (**CC5**) was obtained as a white solid in 49 % overall yield. Mp 247-251 °C; IR (film): 3491, 2959, 2937, 2867, 1719, 1700, 1277, 1259, 1064, 979, 867 cm^{-1}; ^{1}H NMR (400 MHz, MeOD) δ 5.23 (1H, dt, *J* = 16.4, 5.5 Hz), 4.43 (1H, dd, *J* = 14.2, 7.7 Hz,), 3.48 (2H, m, 6β-H and 26-H), 2.60 (4H, m, $HOOC(CH_2)_2$), 2.20 (1H, m), 1.23 (3H, s, 19-CH_3), 0.99 (3H, d, *J* = 7.1 Hz, 27-CH_3), 0.85 (3H, s, 18-CH_3), 0.83 (3H, d, *J* = 6.4 Hz, 21-CH_3); ^{13}C NMR (101 MHz, MeOD) δ 174.0 (C=O), 171.9 (C=O), 108.6 (C-22), 80.2 (C-16), 77.2, 74.5 (C-5), 74.3 (C-6), 71.1(C-3), 65.8 (C-26), 61.7, 55.1, 44.5, 40.9, 39.8, 39.2, 37.5, 35.9, 33.4, 31.2, 30.6, 30.4, 29.4, 29.2, 28.5, 27.8, 25.8, 20.0, 15.5, 15.2, 15.1, 12.9; *m/z* 571.3236 $[M + Na]^+$ (calcd for $C_{31}H_{48}O_8Na$, 571.3241), Δ = -0.88 ppm.

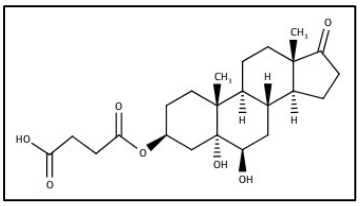
Compound CC8 - 5α,6β-Dihydroxy-20-oxopregnan-3β-yl hemisuccinate



Prepared according to the general procedure described above for the synthesis of CC2 however pregnenolone was used as starting material (316.5 mg, 1 mmol). 5α,6β-Dihydroxy-20-oxopregnan-3-yl hemisuccinate (**CC8**) was obtained as a white solid in 55 % overall yield. Mp 240-242 °C; IR (film): 3551, 3447, 2941, 2876, 1734, 1705, 1700, 1423, 1355, 1172,

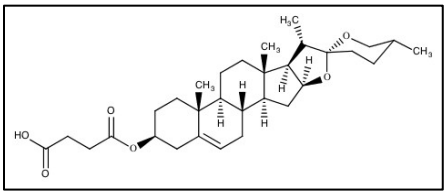
1163, 1072, 805 cm^{-1}; ^{1}H NMR (400 MHz, MeOD) δ 5.26 – 5.18 (1H, m, 3β-H), 3.49 (1H, d, J = 2.8 Hz, 6β-H), 2.67 (1H, t, J = 8.9 Hz, 17-H), 2.59 (4H, m, $HOOC(CH_2)_2$), 2.13 (3H, s, 21-CH_3), 1.19 (3H, s, 19-CH_3), 0.64 (3H, s, 18-CH_3); ^{13}C NMR (101 MHz, MeOD) δ 212.5 (C-20), 176.1 (C=O acid), 173.9 (C=O ester), 76.5 (C-6), 76.3 (C-5), 73.1 (C-3), 64.8 (C-17), 57.5 (C-14), 46.4, 45.5, 40.2, 39.4, 38.0, 35.2, 33.2, 31.7, 31.6, 30.5, 29.8, 27.9, 25.4, 23.8, 22.3, 17.1, 13.9. *m/z* 473.2505 $[M + Na]^+$ (calcd for $C_{25}H_{38}O_7Na$, 473.2510), Δ = -1.06 ppm.

Compound CC14 - 5α,6β-Dihydroxy-17-oxoandrostan-3β-yl hemisuccinate



Prepared according to the general procedure described above for the synthesis of CC2 however dehydroepiandrosterone was used as starting substrate (288.4 mg, 1 mmol). 5α,6β-Dihydroxy-17-oxoandrostan-3β-yl hemisuccinate (**CC14**) was obtained as a white solid in 58 % overall yield. Mp 163-166 °C; IR (film): 3490, 2939, 2866, 1687, 1669, 1585, 1401, 1267, 1154, 1070, 899, 796 cm^{-1}. ^{1}H NMR (400 MHz, MeOD) 5.25 – 5.15 (1H, m, 3β-H), 3.67 (1H, s, 5α-OH), 3.51 (1H, t, J = 2.8 Hz), 2.59 (2H, s), 2.58 – 2.55 (4H, m, $HOOC(CH_2)_2$), 1.20 (3H, s, 18-CH_3), 0.89 (3H, s, 19-CH_3). ^{13}C NMR (101 MHz, MeOD) δ 224.1 (C-17), 176.0 (C=O acid), 173.9 (C=O ester), 76.5 (C-5), 76.1 (C-6), 73.0 (C-3), 52.4, 52.2, 49.0, 46.7, 39.6, 37.9, 36.7, 34.1, 33.2, 32.9, 31.3, 30.5, 29.8, 29.8, 27.8, 22.7, 21.5, 17.1, 14.3; *m/z* 445.2192 $[M + Na]^+$ (calcd for $C_{23}H_{34}O_7Na$, 445.2197), Δ = -1.12 ppm.

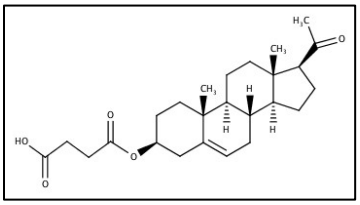
Compound CC4 - Spirost-5-en-3β-yl hemisuccinate



To the solution of diosgenin (414.6 mg, 1 mmol) in dichloromethane (13 mL), pyridine (3 mL) and succinic anhydride (700 mg) were added. Reaction was stirred at reflux temperature for 7 h and monitored through thin layer chromatography (TLC). After completion, methanol was added to stop the reaction. Then the mixture was dissolved in ethyl acetate and washed sequentially with HCl (5% aqueous solution), $NaHCO_3$ (saturated aqueous solution) and water, dried with anhydrous Na_2SO_4, filtrated and evaporated under vacuum. The product was subjected to flash column chromatography with gradient elution of petroleum ether/ethyl acetate from 30:1 to 10:7 (v/v). Spirost-5-en-3β-yl hemisuccinate (**CC4**) (400 mg, 78 % yield) was obtained as a white solid.Mp 206-208 °C; IR (film): 3509, 2967, 2955, 2926,

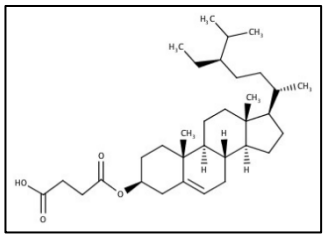
2837, 1735, 1709, 1453, 1382, 1299, 1165, 1048, 981, 900, 795 cm^{-1}; ^{1}H NMR (400 MHz, MeOD) δ 5.37 (1H, d, *J* = 4.9 Hz, 6-H), 4.66 – 4.58 (1H, m, 3-H), 4.41 (1H, dd, *J* = 15.0, 7.4 Hz, 16-H), 3.48 (1H, m, 26-H), 2.67 and 2.60 (each 2H, 2m, $HOOC(CH_2)_2$), 2.32 (2H, d, *J* = 7.0 Hz), 1.03 (3H, s, 19-CH_3), 0.97 (3H, d, *J* = 6.9 Hz, 27-CH_3), 0.78 (6H, m, 18-CH_3 and 21-CH_3; ^{13}C NMR (101 MHz, MeOD) δ 177.6 (C=O acid), 171.7 (C=O ester), 139.7 (C-6), 122.6 (C-7), 109.5 (C-22), 81.0 (C-3), 74.6 (C-26), 67.0, 62.2, 56.6, 50.1, 41.8, 40.4, 39.9, 38.1, 37.1, 36.9, 32.2, 32.0, 31.53, 31.52, 30.4, 29.4, 29.1, 28.9, 27.8, 21.0, 19.5, 17.3, 16.4, 14.7.^{13}C NMR (101 MHz, $CDCl_3$) δ 177.42, 171.54, 139.59, 122.47, 109.34, 76.70, 74.46, 66.85, 62.06, 56.44, 49.93, 41.63, 40.27, 39.73, 38.00, 36.93, 36.73, 32.05, 31.83, 31.39, 30.30, 29.24, 28.92, 28.80, 27.67, 20.82, 19.33, 17.14, 16.29, 14.52; *m/z* 537.3180 $[M + Na]^+$ (calcd for $C_{31}H_{46}O_6Na$, 537.3187), Δ = 1.30 ppm.

Compound CC7 - 20-Oxopregn-5-en-3β-yl hemisuccinate



Prepared according to the general procedure described above for the synthesis of CC4 however pregnenolone was used as starting substrate (316 mg, 1 mmol). 20-Oxopregn-5-en-3β-yl hemisuccinate (**CC7**) was obtained as a white solid in 82 % yield. Mp 152-154 °C; IR (film): 2962, 2953, 2847, 1737, 1724, 1706, 1686, 1427, 1404, 1359, 1196, 1164, 837, 799 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (1H, d, *J* = 4.8 Hz, 6-H), 4.63 (1H, m, 3β-H), 3.70 (1H, s), 2.53 (1H, t, *J* = 8.9 Hz, 17-H), 2.32 (2H, m), 2.12 (3H, s, 21-CH_3), 1.01 (3H, s, 19-CH_3), 0.63 (3H, s, 18-CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 209.9 (C-20), 178.0 (C=O), 171.7 (C=O), 139.7 (C-5), 122.6 (C-6), 74.5 (C-3), 63.8 (C-17), 57.0, 52.1, 50.0, 44.2, 38.9, 38.1, 37.1, 36.7, 32.0, 31.9, 31.7, 29.4, 29.1, 29.0, 27.8, 24.6, 23.0, 21.2, 19.4, 13.4; *m/z* 439.2449 $[M + Na]^+$ (calcd for $C_{25}H_{36}O_5Na$, 439.2455), Δ = -1.37 ppm.

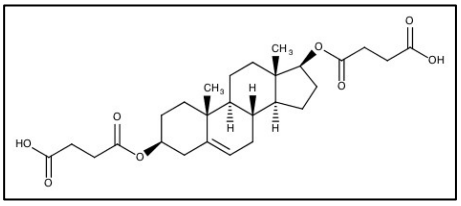
Compound CC18 - Sitost-5-en-3β-yl hemisuccinate



Prepared according to the general procedure described above for the synthesis of CC4 however sitosterol was used as starting substrate (414,7 mg, 1 mmol). Sitost-5-en-3β-yl hemisuccinate (**CC18**) was obtained as a white solid in 85 % yield. Mp 146-148 °C; IR (film): 3547, 2964, 2937, 2859, 1702, 1585, 1221, 1171, 1001, 836 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38 (1H, d, *J* = 4.4 Hz, 6-H), 4.67 – 4.59 (1H, m, 3β-H), 2.69 – 2.58 (4H, m, $HOOC(CH_2)_2$), 2.32 (2H, d, *J* = 7.7 Hz), 1.02 (3H, s, 19-CH_3), 0.92 (3H, d, *J* = 6.5 Hz, 21-CH_3), 0.84 (3H, t, *J* = 7.8

Hz, 24-CH_3), 0.82 (3H, d, *J* = 7.0 Hz, 26-CH_3), 0.81 (3H, d, *J* = 6.8 Hz, 27-CH_3), 0.68 (3H, s, 19-CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.2 (C=O acid), 171.7 (C=O ester), 139.7 (C-5), 122.9 (C-6), 74.7 (C-3), 56.8 (C-14), 56.2 (C-17), 50.2, 46.0, 42.5, 39.9, 38.2, 37.1, 36.7, 36.3, 34.1, 32.1, 32.0, 29.4, 29.3, 29.0, 28.4, 27.9, 26.2, 24.5, 23.2, 21.2, 20.0, 19.5, 19.2, 18.9, 12.1, 12.0; *m/z* 537.3912 $[M + Na]^+$ (calcd for $C_{33}H_{54}O_4Na$, 537.3914), Δ = -0.37 ppm.

Compound CC17 - Androst-5-ene-3β,17β-diyl dihemisuccinate



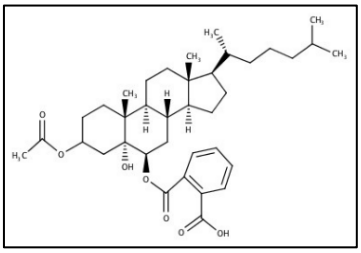
To the solution of androstenediol (290,4 mg, 1 mmol) in dichloromethane (13 mL), pyridine (3 mL) and succinic anhydride (500 mg) were added. Reaction was stirred at reflux temperature (60°C) for 7 h and monitored through TLC. After completion methanol was added to stop the reaction. Then the mixture was dissolved in ethyl acetate and washed sequentially with HCl (5% aqueous solution), $NaHCO_3$ (saturated aqueous solution) and water, dried with anhydrous Na_2SO_4, filtrated and evaporated under vacuum. The product was subjected to flash column chromatography with gradient elution of petroleum ether/ethyl acetate from 30:1 to 10:9 (v/v). Androst-5-ene-3β,17β-diyl dihemisuccinate (**CC17**) (376 mg, 76 % yield) was obtained as a white solid. Mp 125-127 °C; IR (film): 2988, 2895, 2849, 1745, 1723, 1437, 1365, 1249, 1240, 1170, 1155, 1032, 1002, 903, 799 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (1H, d, *J* = 5.2 Hz, 6-H), 4.65 – 4.55 (2H, m, 3β-H and 17-H), 2.72 – 2.60 (8H, m, $HOOC(CH_2)_2$), 1.02 (3H, s, 19-CH_3), 0.80 (3H, s, 18-CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.0 (C=O acid), 177.8 (C=O acid), 172.7 (C=O ester), 172.2 (C=O ester), 139.9 (C-5), 122.4 (C-6), 83.4 (C-17), 74.0 (C-3), 51.1, 50.1, 42.6, 38.2, 37.1, 36.9, 36.8, 31.8, 31.6, 29.3, 29.2, 29.0, 28.8, 27.9, 27.6, 23.7, 21.6, 20.6, 19.5, 12.1. *m/z* 489.2483 $[M - H]^-$ (calcd for $C_{27}H_{37}O_8$, 489.2488), Δ = -1.02 ppm.

General procedure for the synthesis of sterol 3β-acetate:

To a solution of cholesterol (386 mg, 1 mmol) or diosgenin (414 mg, 1 mmol), in tetrahydrofuran (THF, 10 mL), pyridine (1 mL) and acetic anhydride (3 mL) were added. The reaction was stirred at room temperature for 5 h and monitored through TLC. After completion, methanol was added to stop the reaction and then dissolved in ethyl acetate. The resulting organic phase was washed sequentially with HCl (5% aqueous solution), $NaHCO_3$ (saturated aqueous solution) and water, dried with anhydrous Na_2SO_4, filtrated and evaporated under vacuum. The product was subjected to flash column chromatography with gradient elution of petroleum ether/ethyl acetate from 30:1 to 10:7 (v/v).

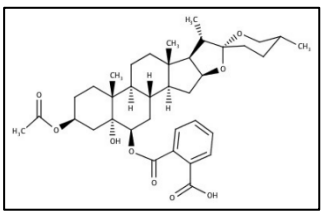
General procedure for synthesis of sterol 6β-hemiphthalates:

Compound CC1 - 5α-Hydroxycholestane-3β-yl acetate 6β-yl hemiphthalate



To a solution of cholesterol acetate (250 mg, 0.583 mmol) in acetonitrile (20 mL) under reflux at 82 °C, MMPP (1 mmol) was added. The reaction mixture was stirred at reflux temperature for 24 h and then stopped by evaporation under vacuum. When epoxidation of cholesterol is performed with MMPP in acetonitrile at reflux temperature, only 10 minutes are needed to completely consume the substrate. After 24 h, a considerable amount of a product with very low Rf was observed by TLC and the reaction was stopped by evaporation of the solvent at reduced pressure. The crude solid was dissolved in ethyl acetate, washed with water and dried with anhydrous Na_2SO_4, filtrated and evaporated under vacuum. The product was subjected to flash column chromatography with chloroform and then a gradient elution of chloroform/ethanol from 50:1 to 1:1 (v/v). 5α - hydroxycholestan-3β-yl acetate 6β-yl hemiphthalate (**CC1**) was recovered as a white solid (125 mg, 30 % yield). Mp 148-151°C; IR (film): 70,2945, 2867, 1715, 1576, 1466, 1365, 1280, 1129, 1074, 758 cm^{-1}; ^{1}H NMR (400 MHz, MeOD) δ ppm 7.73 (1H, d, *J* = 7.5 Hz, Ar), 7.50 (2H, m, Ar), 7.40 (1H, m, Ar), 5.19 (1H, tt, *J* =11.2, 5.6 Hz, 3α-H), 4.93 (1H, br s, 6α-H), 1.98 (3H, s, 3β-CH_3COO), 1.26 (3H, s, 19-CH_3), 0.93 (3H, d, *J* = 6.5 Hz, 21-CH_3), 0.88 (3H, d, *J* = 6.6 Hz, 27-CH_3), 0.87 (3H, d, *J* = 6.6 Hz, 26-CH_3), 0.70 (3H, s, 18-CH_3); ^{13}C NMR (101 MHz, MeOD) δ ppm 177.0 (C=O), 172.7 (C=O), 168.2 (C=O), 144.0, 132.7, 129.9, 129.4, 128.7, 128.5, 79.5, 79.2, 78.9, 78.1, 75.6, 72.5, 57.6, 57.3, 46.1, 43.9, 41.4, 40.6, 39.5, 37.7, 37.3, 37.0, 32.9, 32.3, 32.1, 29.3, 29.1, 27.8, 25.1, 24.9, 23.2, 22.9, 22.2, 21.3, 19.2, 17.5, 12.6. *m/z* 633.3755 $[M + Na]^+$ (calcd for $C_{37}H_{54}O_7Na$, 633.3762), Δ = -1.11 ppm.

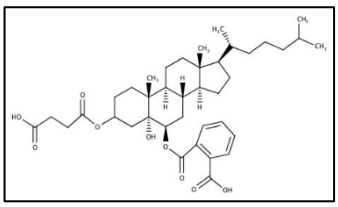
Compound CC6 - 5α-Hydroxyspirostan-3β-yl acetate 6β-yl hemiphthalate



Prepared according to the general procedure described above for the synthesis of CC1 but using diosgenin 3β-acetate (250 mg, 0.55 mmol) as starting material. 5α - Hydroxyspirostan-3β-yl acetate 6-yl hemiphthalates (**CC6**) was obtained as a white solid, in 48 % overall yield. Mp 178-180 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 – 7.40 (4H, m, Ar), 5.20 (1H, s, 3β-H), 5.02 (1H, s, 6β-H), 4.36 (1H, s, 16-H), 3.45 (1H, m), 3.36 (1H, m, 26-H), 2.17 (4H, m, HOOC(CH_2)$_2$), 2.00 (3H, s, $COCH_3$), 1.15 (3H, s, 19-CH_3), 0.95 (3H, s, 27-CH_3), 0.78 (6H, m, 18-CH_3 and 26-CH_3); ^{13}C NMR (101

MHz, MeOD) δ 177.2 (C=O), 172.9 (C=O), 168.2 (C=O), 144.3, 132.7, 129.9, 129.4, 128.6, 128.5, 110.6, 82.2, 79.6, 79.2, 78.9, 77.9, 75.6, 72.5, 67.8, 63.7, 61.6, 57.1, 49.9, 46.2, 42.9, 41.8, 41.2, 39.7, 37.6, 32.9, 32.5, 31.8, 31.4, 29.8, 27.8, 22.0, 21.3, 17.5, 17.1, 14.8. *m/z* 661.3342 $[M + Na]^+$ (calcd for $C_{37}H_{50}O_9Na$, 661.3347), Δ = -0.76 ppm.

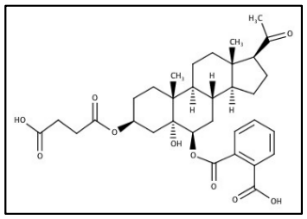
Compound CC3 - 5β-Hydroxycholestane-3β-yl hemisuccinate 6β-yl hemiphthalate



Prepared according to the general procedure described above for the synthesis of CC1 but using cholesterol 3β- hemisuccinate (250 mg, 0.51 mmol) as starting material. 5α -hydroxycholestan-3β-yl hemisuccinate 6β-yl hemiphthalate (**CC3**) was obtained as a white solid, in 52 % overall yield.

Mp 212-214 °C; IR (film): 3349, 2934, 2867, 1714, 1684, 1586, 1559, 1464, 1410, 1278, 1075, 1007, 740 cm^{-1}; ^1H NMR (400 MHz, DMSO) δ 7.92 (d, *J* = 6.6 Hz, 1H, Ar), 7.55 – 7.30 (m, 2H, Ar), 7.13 (d, *J* = 6.6 Hz, 1H, Ar), 5.11 (s, 1H, 3-H), 4.76 (s, 1H, 6-H), 4.41 (s, 1H, 5-OH), 2.46 – 2.18 (m, 4H, $HOOC(CH_2)_2$), 0.90 (s, 3H), 0.89 (d, *J* = 6.7 Hz, 3H), 0.86 (d, *J* = 6.6 Hz, 3H), 0.85 (d, 3H), 0.64 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$/DMSO) δ ppm 11.8, 16.0, 18.4, 20.4 (CH2), 22.3, 22.6, 23.2 (CH2), 23.7 (CH2), 26.2 (CH2), 27.3, 27.7 (CH2), 30.3 (CH2), 30.4, 30.7 (CH2), 30.9 (CH2), 31.2 (CH2), 35.1, 35.6 (CH2), 35.9 (CH2), 37.8 (C), 38.9 (CH2), 39.5 (CH2), 42.2 (C), 44.0, 55.3, 55.6, 70.3, 73.2 (C5), 75.8, 126.0, 128.5, 128.7, 129.0, 134,0 (C), 137.9 (C), 168.7 (C=O), 169.1 (C=O), 171.8 (C=O), 174.0 (C=O); *m/z* 691.3807 $[M + Na]^+$ (calcd for $C_{39}H_{56}O_9Na$, 691.3816).

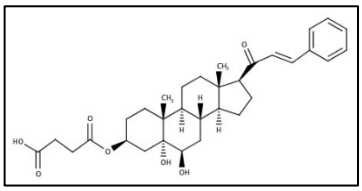
Compound CC9 - 5α-Hydroxy-20-oxopregnan-3β-yl hemisuccinate 6β-yl hemiphthalate



Prepared according to the general procedure described above for the synthesis of CC1 but using pregnenolone 3β- hemisuccinate (250 mg, 0.6 mmol) as starting material. 5α-Hydroxy-20-oxopregnan-3β-yl hemisuccinate 6β-yl hemiphthalate (**CC9**) was obtained as a white solid, in 48 % overall yield. Mp 208-209 °C; ^1H NMR (400 MHz, DMSO) δ 7.90 (d, *J* = 6.1 Hz, 1H), 7.58 – 7.28 (m, 2H), 7.16 (d, *J* = 6.3 Hz, 1H), 5.11 (s, 1H), 4.77 (s, 2H), 4.53 (s, 1H), 2.59 (t, *J* = 8.8 Hz, 1H), 2.51-2.05 (m, 6H), 2.06 (s, 3H), 2.01 – 1.10 (m, 10H), 0.90 (s, 3H), 0.52 (d, *J* = 7.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 208.58 (C-20), 168.7 (C=O), 169.1 (C=O), 171.8 (C=O), 174.0 (C=O), 129.63 (Ar), 128.66 (Ar), 125.56 (Ar), 79.19 (C-7), 75.87 (C-6), 73.22 (C-3), 69.75 (C-17), 62.67, 56.03, 55.25,

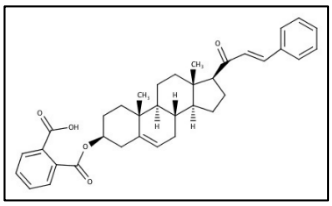
44.11, 43.62, 40.15, 38.19, 38.06, 35.84, 31.52, 31.17, 30.88, 30.49, 26.44, 24.03, 22.28, 20.52, 18.56, 16.35, 13.13. *m/z* 621.2665 $[M + Na]^+$ (calcd for $C_{32}H_{42}O_7Na$, 621.26674)

Compound CC10 - 21*E*-Benzylidene-5α,6β-dihydroxy-20-oxopregnan-3β-yl hemisuccinate



Pregnenolone (316 mg, 1 mmol) was dissolved in absolute ethanol (5 mL), and KOH (10%, 10 mL) and benzaldehyde (1 mmol) were added. The reaction was stirred at room temperature for 5 h. After completion, the mixture was dissolved in ethyl acetate and washed sequentially with HCl (5% aqueous solution), $NaHCO_3$ (saturated aqueous solution) and water, dried with anhydrous Na_2SO_4, filtrated and evaporated under vacuum. After, to this crude product it was subjected to esterification with succinic anhydride and epoxidation with MMPP, as described for compound CC2. The product was subjected to flash column chromatography with gradient elution of petroleum ether/ethyl acetate from 30:1 to 10:7 (v/v). 21*E*-Benzylidene-5α,6β-dihydroxy-20-oxopregnan-3β-yl hemisuccinate (**CC10**) was obtained as a white solid, in 22 % overall yield. Mp 164-165 °C; IR (film): 3482, 2940, 2751, 2650, 1700, 1678, 1605, 1576, 1449, 1402, 1155, 1101, 1071, 865, 755 cm^{-1}; ^1H NMR (400 MHz, MeOD) δ 7.61 (1H, m, 22-H), 7.58 – 7.50 (2H, m, Ar), 7.42 – 7.38 (3H, m, Ar), 6.87 (1H, d, *J* = 16.1 Hz, 21-H), 5.20 (1H, m, 3β-H), 3.66 (1H, s, 6β-H), 3.48 (1H, m, 5α-OH), 3.01 (1H, t, *J* = 8.9 Hz, 17-H), 1.16 (3H, s, 19-CH_3), 0.62 (3H, s, 18-CH_3); ^{13}C NMR (101 MHz, MeOD) δ 203.0 (C-20), 176.0 (C=O acid), 173.9 (C=O ester), 143.3 (C-22), 136.1 (Ar), 132.0, 131.6, 130.07, 130.05, 129.48, 129.45, 128.1 (C-21), 76.5 (C-5), 76.3 (C-6), 73.1 (C-3), 62.9 (C-17), 57.7 (C-14), 46.6, 46.5, 40.4, 39.4, 38.0, 35.3, 33.2, 31.8, 30.5, 29.8, 27.8, 25.6, 23.7, 22.3, 17.1, 14.2. *m/z* 561.2818 $[M + Na]^+$ (calcd for $C_{32}H_{42}O_7Na$, 561.2823), Δ = -0.89 ppm.

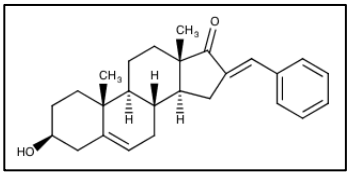
Compound CC11 - 21*E*-Benzyliden-20-oxopregn-5-en-3β-yl hemiphtalate



Pregnenolone (316.5 mg, 1 mmol) was dissolved in absolute ethanol (5 mL), 10% KOH (10 mL) and benzaldehyde (1 mmol) were added. The reaction was stirred at room temperature for 5 h. After completion, the mixture was dissolved in ethyl acetate and washed sequentially with HCl (5% aqueous solution), $NaHCO_3$ (saturated aqueous solution) and water, dried with anhydrous Na_2SO_4, filtrated and evaporated under vacuum. After, this crude product underwent a new reaction to add the hemiphthalate group. For this, to the product (150 mg) in pyridine (20 ml), 4-dimethylaminopyridine

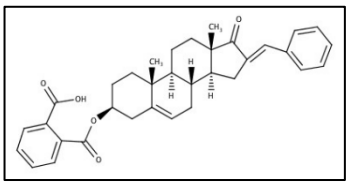
(DMAP) (50 mg) and phthalic anhydride (100 mg) were added. Reaction was stirred at reflux temperature (110 °C) for 48 h and monitored through TLC. After completion, the mixture was dissolved in ethyl acetate and washed sequentially with HCl (5% aqueous solution), $NaHCO_3$ (saturated aqueous solution) and water, dried with anhydrous Na_2SO_4, filtrated and evaporated under vacuum. The product was subjected to flash column chromatography with gradient elution of chloroform: ethanol from 50:1 to 10:1 (v/v). 21*E*-Benzyliden-20-oxopregn-5-en-3β-yl hemiphtalate (**CC11**) was obtained as a white solid (138 mg, 25 % yield). Mp 131-133 °C; IR (film): 2941, 2851, 1718, 1600, 1576, 1449, 1288, 1256, 1126, 1064, 957, 766, 688 cm^{-1}; ^1H NMR (400 MHz, MeOD) δ 7.80 – 7.32 (10H, m, Ar and 22-H), 6.91 (1H, d, *J* = 16.1 Hz, 21-H), 5.46 (1H, t, *J* = 6.0 Hz, 6-H), 4.81 – 4.74 (1H, m, 3β-H), 1.07 (3H, s, 19-CH_3), 0.65 (3H, s, 18-CH_3); ^{13}C NMR (101 MHz, MeOD) δ 203.1 (C-20), 177.0 (C=O acid), 169.6 (C=O ester), 143.6 (C-22), 141.5 (C-5), 136.1, 132.3, 131.6, 130.7, 130.09, 130.07, 129.7, 129.50, 129.47, 128.6, 128.4, 128.1, 123.2 (C-21), 76.5, 62.7, 58.3, 51.5, 46.2, 40.1, 38.9, 38.3, 37.9, 33.4, 33.0, 28.5, 25.7, 23.7, 22.2, 19.8, 13.9; *m/z* 575.2763 [M + Na]+ (calcd for C36H40O5Na, 575.2768), Δ = -0.869 ppm.

Compound CC15 - 3β-Hydroxy-16-benzyliden-androst-5-en-17-one



Dehydroepiandrosterone (288.4 mg, 1 mmol) was dissolved in absolute ethanol (5 mL), 10% KOH (10 mL) and benzaldehyde (1 mmol) were added. The reaction was stirring under reflux (50°C) for 7 h. After completion, the mixture was dissolved in ethyl acetate and washed sequentially with HCl (5% aqueous solution), $NaHCO_3$ (saturated aqueous solution) and water, dried with anhydrous Na_2SO_4, filtrated and evaporated under vacuum. The product was subjected to flash column chromatography with gradient elution of dichloromethane/methanol from 30:1 to 15:1 (v/v). 3β-Hydroxy-16-benzyliden-androst-5-en-17-one (**CC15**) was obtained as a white solid (233 mg, 62 % yield). Mp 186-189 °C; IR (film): 3185, 12965, 2935, 28330, 1714, 1631, 1493, 1061, 771, 694 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.54 (2H, d, *J* = 7.5 Hz, Ar), 7.45 – 7.34 (4H, m, Ar), 5.41 – 5.38 (1H, m, 6-H), 3.58 – 3.50 (1H, m, 3β-H), 2.89 (1H, ddd, *J* = 16.0, 6.7, 1.7 Hz, 15-Ha), 2.45 (1H, ddd, *J* = 15.7, 12.7, 2.8 Hz, 15-Hb), 1.07 (3H, s, 18-H), 0.98 (3H, s, 19-H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 209.9 (C=O), 141.3 (C-14), 136.1, 135.8, 133.3, 130.46, 130.45, 129.4, 128.82, 128.81, 121.0 (C-6), 71.7 (C-3), 50.5, 50.0, 47.8, 42.3, 37.3, 36.9, 31.70, 31.69, 31.3, 31.1, 29.5, 20.6, 19.6, 14.4; *m/z* 399.2289 [M + Na]$^+$ (calcd for $C_{26}H_{32}O_2Na$, 399.2295), Δ = -1.50 ppm.

Compound CC16 - 16*E*-Benzylidene-3β-hydroxy-17-oxoandrost-5-en-3β-yl hemiphthalate



Dehydroepiandrosterone (288.4 mg, 1 mmol) was dissolved in absolute ethanol (5 mL), 10% KOH (10 mL) and benzaldehyde (1 mmol) were added. The reaction was stirring under reflux (50°C) for 7 h. After completion, the mixture was dissolved in ethyl acetate and washed sequentially with HCl (5% aqueous solution), $NaHCO_3$ (saturated aqueous solution) and water, dried with anhydrous Na_2SO_4, filtrated and evaporated under vacuum. The product was subjected to a new reaction to add the 3β-hemiphthalate group. For this, to the solution of 3β-hydroxy-16*E*-benzylidenandrost-5-en-17-one (150 mg) in pyridine (20 ml), DMAP (50 mg) and phthalic anhydride (100 mg) were added. Reaction was stirred at reflux temperature (110 °C) for 48 h and monitored through TLC. After completion, the mixture was dissolved in ethyl acetate and washed sequentially with HCl (5% aqueous solution), $NaHCO_3$ (saturated aqueous solution) and water, dried with anhydrous Na_2SO_4, filtrated and evaporated under vacuum. The product was subjected to flash column chromatography with gradient elution of chloroform: ethanol from 50:1 to 10:1 (v/v). 16*E*-Benzylidene-3β-hydroxy-17-oxoandrost-5-en-3β-yl hemiphthalate **(CC16)** was obtained as a white solid in 30 % overall yield. Mp 288-293 °C; IR (film): 3083, 2947, 2842, 1735, 1713, 1629, 1321, 1299, 12223, 1136, 1073, 771, 737, 694 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$/ MeOD) δ 7.85 – 7.32 (10H, m, Ar and 20-H), 5.42 (1H, m, 6-H), 4.88 – 4.79 (1H, m, 3β-H), 1.03 (3H, s, 19-CH_3), 0.96 (3H, s, 18-CH_3); ^{13}C NMR (101 MHz, $CDCl_3$/ MeOD) δ 211.9 (C-17), 172.3 (C=O acid), 169.3 (C=O ester), 141.1 (C-16), 137.0, 136.5, 135.7, 134.5, 133.2, 131.9, 131.4, 131.35, 131.33, 130.4, 129.68, 129.66, 129.5, 129.4, 122.9 (C-6), 76.5 (C-3), 51.5, 51.0, 48.5, 38.7, 37.9, 37.8, 32.5, 32.2, 31.8, 30.2, 28.3, 21.3, 19.8, 14.7. *m/z* 547.2449 $[M + Na]^+$ (calcd for $C_{34}H_{36}O_5Na$, 547.2455), Δ = -1.10 ppm.

Biological evaluation

Cytotoxicity against HepG2 human cell line

Cytotoxicity of the synthetic steroids was assessed against the HepG2 human hepatic cell line (kindly provided by Prof. Teresa Cruz, FFUC), maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic under a humidified atmosphere with 5% CO_2 at 37°C. Cells was seeded into 96-well plates at a density of 2,000 cells per well. The compounds were serially diluted and added to the plates to generate a dose-response with a maximal concentration of 100 μM. After 72 h of incubation, the resazurin-based metabolic assay was performed to determine cell viability as previously described (28). Briefly, the resazurin compound was

added to each well and the plates incubated during 4 h under standard conditions and absorbance was read at 570/620nm. The number of viable cells was estimated, and the results expressed as percentage of viable cells relative to control (which was defined as 100% of cell viability). The concentration of a compound reflecting the IC_{50} was determined from the concentration-response curve applying non-linear regression. Data are presented as mean ± standard error of the mean (SEM) from three independent assays.

Parasite culture

The 3D7, Dd2 (MRA-102 and MRA-156, from MR4/BEI Resources, NIAID, respectively) and NF10 gene edited lines ($NF10^{NF}$, $NF10^{NY}$, $NF10^{YF}$, $NF10^{YY}$) (29) were maintained at ~ 4% hematocrit with human red blood cells (RBCs) (isolated either from whole blood or from buffy coat samples provided by healthy donors of all blood types) in RPMI-1640, supplemented with 2 mM L-glutamine, 200 μM hypoxanthine, 0.25 μg/mL gentamycin, 25 mM HEPES, 0.2% $NaHCO_3$, and 0.25% Albumax II (Invitrogen; ThermoFisher Scientific). Parasite cultures were maintained at 37°C at 5% O_2/ 5% CO_2/ 90% N_2. Parasite growth was monitored through Giemsa-stained blood smears. Parasite synchronization was performed with 5% sorbitol for 15 min at 37°C (30).

For the stage-specific activity experiments highly synchronized cultures of Dd2 were used. To obtain highly synchronous cultures, sorbitol was added to the culture to eliminate trophozoites. This was repeated 20 h and 44 h after first treatment to obtain 4 h-post-invasion ring stages. The drug susceptibility assays were performed at four specific *P. falciparum* blood stages: ring (4h post-invasion), early trophozoites (20h post-invasion), late trophozoites (32h post-invasion) and mature schizonts (40h post-invasion).

***In vitro* hemolysis assay**

The toxic effect of the best synthetic compound (CC1, of the 16 compounds synthesized in this work) was evaluated on uninfected RBCs by hemolysis assay as described previously (31). The hemolysis assay was performed at 2% hematocrit with a range of concentrations (from 0.1 to 100 μM) of synthetic compound in a final volume of 0.2 mL, followed by incubation at 37°C for 24 h. Saponin (0.05%) was used as a positive control, whereas uninfected RBCs cultured in RPMI medium (as described in the section above) was used as a negative control. After incubation, all the wells incubated with the different conditions were centrifuged at 1000 xg for 10 min and the supernatant recovered. The absorbance of supernatants was determined over a wavelength range of 300 to 700 nm in order

to measure the amount of Hb released upon RBC lysis. The results are expressed as arbitrary units of absorbance. Data are presented as mean ± SEM from three independent assays.

In vitro IC_{50} drug susceptibility assays

Drug susceptibility assays for all the compounds in study were determined using the 3D7, Dd2 strains and NF10 gene edited lines, $NF10^{NF}$, $NF10^{NY}$, $NF10^{YF}$, $NF10^{YY}$ using a flow cytometry-based method as previously described (32). Briefly, parasites highly synchronized at the four different stages were incubated at 37°C with 0.2% starting parasitemia and 1% hematocrit across a range of compound concentration with two-fold dilutions (11-points). After 72 h, to determine the parasite growth, the parasites were stained with 1.6 μM MitoTracker Deep Red (Invitrogen, ThermoFisher Scientific) and 2x SYBR Green (Invitrogen, ThermoFisher Scientific) in PBS 1x for 30 min and then analyzed by flow cytometry (BD LSR II Flow Cytometer). Approximately, 100000 events were read per well. The IC_{50} values was calculated using nonlinear regression analysis. Data are presented as mean ± SEM from three independent assays.

To evaluate the interaction with well-known drug resistance player transporters, the PfMDR1 and PfCRT (33), the synthesized compound was incubated in the presence of elacridar (ELC, 0.1 μM) and verapamil (VP, 0.8 μM) (34) using the same conditions described above.

Two-tailed *t*tests were used to assess statistically significant differences between untreated and treated parasites and also to assess differences between parasite strains, 3D7 and Dd2 (*, $p < 0.05$).

Gametocyte formation and compounds screening

To obtain a gametocyte rich-culture, the Dd2 parasite line was transfected with a plasmid containing the *P. falciparum* gametocyte development 1, (*pfgdv1*), tagged in frame with green fluorescent protein (GFP) followed by the immunophilin protein- folding chaperone FK506 binding protein (FKBP)-destabilization domain (DD) (kindly provided by Till Voss, Swiss Tropical and Public Health Institute (35)). Proteins tagged with FKBP-DD are proteolytic degraded allowing the track protein expression (36, 37). Ring-stage cultures at 5% parasitemia were electroporated with ~50 μg of plasmid DNA diluted in Cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/KH^2PO^4, 25 mM HEPES [pH 7.6]). The transfected parasites were maintained in the presence of 2.5 nM of WR99210 since the plasmids express the human dihydrofolate reductase (hDHFR) selectable marker, which confers resistance to this drug. Then, to obtain the edited parasites and to

promote the induction of gametocytes, a synchronous ring stage cultures at 2% parasitemia at 4% hematocrit were cultured in the presence of a small ligand stabilizer, the Shield-1 (final concentration of 312 nM), which allows the stabilization of the GDV1-GFP-DD fusion protein expression. When mature stage IV-V gametocytes (11-13-days-old) were observed in Giemsa-stained thin blood smears, drug susceptibility assays were performed. Briefly, 1% of gametocytemia at 1% hematocrit were incubated in the presence of different concentrations of the synthetic compound, PMQ and DHA (across a dilution range of twofold; 7-point dilution series being the last well as negative control without drug). Reference compounds PMQ and DHA were used in this assay as positive controls. After 72 h incubation, under controlled atmosphere (37°C at 5% O_2/5% CO_2/90% N_2), gametocytes were stained with 1.6 μM MitoTracker Deep Red FM (Invitrogen; ThermoFisher Scientific) in 1x PBS 30 min. For flow cytometry, the gametocytes were excited at 488 nm, since they are GFP+ cells, and 644 nm to detect MitoTracker Deep Red FM and the double-positive population were considered to determine the gametocyte viability. Approximately 100 000 events were read per experimental condition. As described above *in vitro* IC_{50} values were calculated using nonlinear regression analysis. These results are preliminary, representing a single assay.

Cytosolic redox potential assays

To evaluate the compound potential to disturb the parasite redox balance, we used a genetically encoded sensor (Grx1-roGFP) episomally expressed in Dd2 *att*B *P. falciparum* as previously described (38). Briefly, dual plasmid system for gene integration on *P. falciparum* genome were used to obtain the genetically modified parasites. pINT plasmid express mycobacteriophage Bxb1 integrase which mediates recombination between *att*P site at pDC2 plasmid (provided by David Fidock, Columbia University) and the *att*B site integrated on the parasite genome. The pDC2 plasmid was modified to contain a copy of Grx1- roGFP (obtained from Addgene, plasmid number #64975) under selective market gene that confers resistance to blastidicin (BSD). The system readily results in the integration of grx1-roGFP into parasite genome (39). The effect of synthetic compound on *P. falciparum* was investigated in mid incubation experiments (2 h and 4 h) using trophozoites and measured through flow cytometry as described previously (38). Trophozoite-stage parasites at 1% hematocrit and 3% parasitemia were treated with the synthesized compound at 10, 20, 30 and 40x IC_{50} under controlled atmosphere (37°C at 5% O_2/ 5% CO_2/ 90% N_2). All experiments included untreated parasites as control, and fully reduced and oxidized parasites (treated with 10 mM dithiothreitol (DTT) and 1 mM hydrogen peroxide (H_2O_2) respectively). The starting material used to obtain the synthesized compound CC1,

cholesterol, was tested at concentration of 50 μM during 4 h. At the flow cytometer (BD LSR II Flow Cytometer), the cells were excited with 405 and 488 nm laser wavelengths, and the ratio of emissions (405/488 nm) in the green channel (500–530 nm) analyzed. When roGFP is oxidized, the excitation peak at 405 nm increases, while the 488 nm peak decreases. The extent of oxidation was determined, by calculating the ratio of 405/488 nm. Approximately, 100000 events were read per condition. The ratio values were normalized in each condition in relation to the control value (untreated parasites) indicated in the graph as the baseline [y<0]. Values greater than zero indicate an increase in the percentage ratio fluorescence compared with untreated parasites. Data are presented as mean ± SEM from three independent assays. Two-tailed *t*-test were used to determine statistical differences between compound-treated and untreated parasites.

Heme fractionation assays

The heme fractionation assays were performed as described previously (40). Briefly, *P. falciparum* Dd2 strain synchronized at ring-stage was exposed at 4x IC_{50} values of each compound. In all experiments, CQ and ATQ were used as positive and negative controls, respectively, as well as no drug control (untreated parasites). After 32 h, to quantify the parasitemia of each sample, the parasites were stained with 1.6 μM MitoTracker Deep Red (Invitrogen, ThermoFisher Scientific) and 2x SYBR Green (Invitrogen, ThermoFisher Scientific) in PBS 1x for 30 min and measured by flow cytometry. After this, infected RBCs were harvested and the trophozoites were isolated with 0.05% (wt/vol) saponin and washed with 1x PBS (pH 7.5) to remove traces of the RBC Hb. The contents present on trophozoite pellet were released by hypotonic lysis and sonication. Following centrifugation, the supernatants corresponding to Hb (fraction 1) were treated with 4% (wt/vol) sodium dodecyl sulfate (SDS) and 2.5% (vol/vol) pyridine. Pellets were again treated with 4% SDS, 2.5% pyridine, sonicated, and centrifuged and the resulting supernatants (corresponding to the free heme fraction (fraction 2)) were recovered. The remaining Hz pellets (fraction 3) were then solubilized in 4% SDS, 0.3 M NaOH and neutralized with 0.3 M HCl, sonicated, and treated with 25% pyridine. The UV-visible spectrum of each heme fraction as a Fe(III) heme-pyridine complex was measured using a multiwell plate reader (Spectramax 340PC; Molecular Devices). The maximum absorbances (405 nm) of the Fe(III)heme pyridine complex in each condition were used to calculate the percentage of each heme species in each fraction by following formulas: Abs_{Total}= Abs max HB+ Abs max free heme + Abs max Hz. The % for each fraction= (Abs_{max} fraction/ Abs_{Total})x100. Data are presented as mean ± SEM from four independent

assays. Two-tailed *t*-test were used to determine statistical differences between compound-treated parasites and untreated parasites.

Assessing compound synergism with present antimalarials

Determination of synergy/additivity of the synthetic steroid compound with the currently used antimalarial drugs CQ, monodesethyl-amodiaquine (md-ADQ), DHA, MFQ, LMF, PND and PPQ, was performed based on isobologram analysis. Briefly, *P. falciparum* Dd2 strain, synchronized at ring-stage with 0.2% starting parasitemia and 1% hematocrit was incubated, at 96-well plates, in the presence of different drug concentrations mixture. The antimalarial drugs were added vertically in the plate, across a twofold dilution where line A contained the highest concentration and G line the lowest, leaving the H line without drug. The concentration of the antimalarial drug used at C line corresponded to the IC_{50} value when measured alone. Compound CC1 was added horizontally in the same plate, across a twofold dilution where column 1 corresponded to the highest concentration and column 11 to the lowest, leaving column 12 without compound. The concentration of the compound used at column 5 corresponded to the IC_{50} values when measured alone.

This experimental design allows that each 96 well plate corresponds to different pairs of concentration of the antimalarial drug with the synthetic steroid, reflected in isobologram curves. The IC_{50} of the drugs alone or in combination was calculated using nonlinear regression analysis. The determination of drug interaction was based on previously described criteria (41) where the calculation of the sum of the fractional inhibitory concentrations ($\sum$FIC) was obtained using the following equation: FICs = (IC_{50} of drug A in the combination/IC_{50} of drug A when tested alone) + (IC_{50} of drug B in the combination/IC_{50} of drug B when tested alone). Isobologram curves were constructed by plotting FIC (antimalarial drug) *vs* FIC (synthetic compound). $\sum$FIC <1 indicate synergism, $\sum$FIC ≥ 1 and <2 indicate additive interaction, FIC ≥ 2 and <4 denote antagonism. Data are presented as mean ± SEM from four independent assays.

RESULTS

Chemistry

A library of synthetic steroids (>100 compounds) (25, 26, 42) has been screened against the blood stage of malaria and the results obtained revealed that steroids bearing ester groups at different positions afforded the best activities (unpublished results).

A total of 16 steroid derivatives with a wide range of substituents at rings A, B and D were synthesized to explore the potential of this scaffold as a platform to design new antimalarial agents. The functional groups acetate, hemiphthalate and hemisuccinate were used in the derivatization of the 3β-, 6β-, and/or 17β-hydroxy positions of the steroid structure. Chalcone derivatives were prepared at the 16 and 21 positions. Steroids with different side chains were used as starting materials, namely cholesterol, dehydroepiandrosterone, sitosterol, diosgenin and pregnenolone, to understand the importance of the steroid side chain in parasite toxicity (Figure 1). To generate the desired dihydroxysteroids (5α,6β-dihydroxysteroids) for the subsequent addition of the above functional groups, a two-step procedure was performed as previously described (25) using MMPP as a convenient solid oxidant reagent, in acetone at reflux during 2 h and followed by *trans*-diaxial epoxide opening with $Bi(Otf)_3$ in a two-step one-pot sequential procedure (Figure 1C).

As previously described (26) a one-step procedure (Figure 1A) was performed in order to obtain the 5α-hydroxy 6β-hemiphthalate derivatives directly from the olefin (compounds CC1, CC3, CC6 and CC9).

The compounds containing the chalcone group (compounds CC10, CC11, CC15 and CC16) were obtained by the aldol condensation of pregnenolone or dehydroepiandrosterone with benzaldehyde in the presence of basic catalysis (KOH) (Figure 1D and E), in an adaptation of published procedures (43).

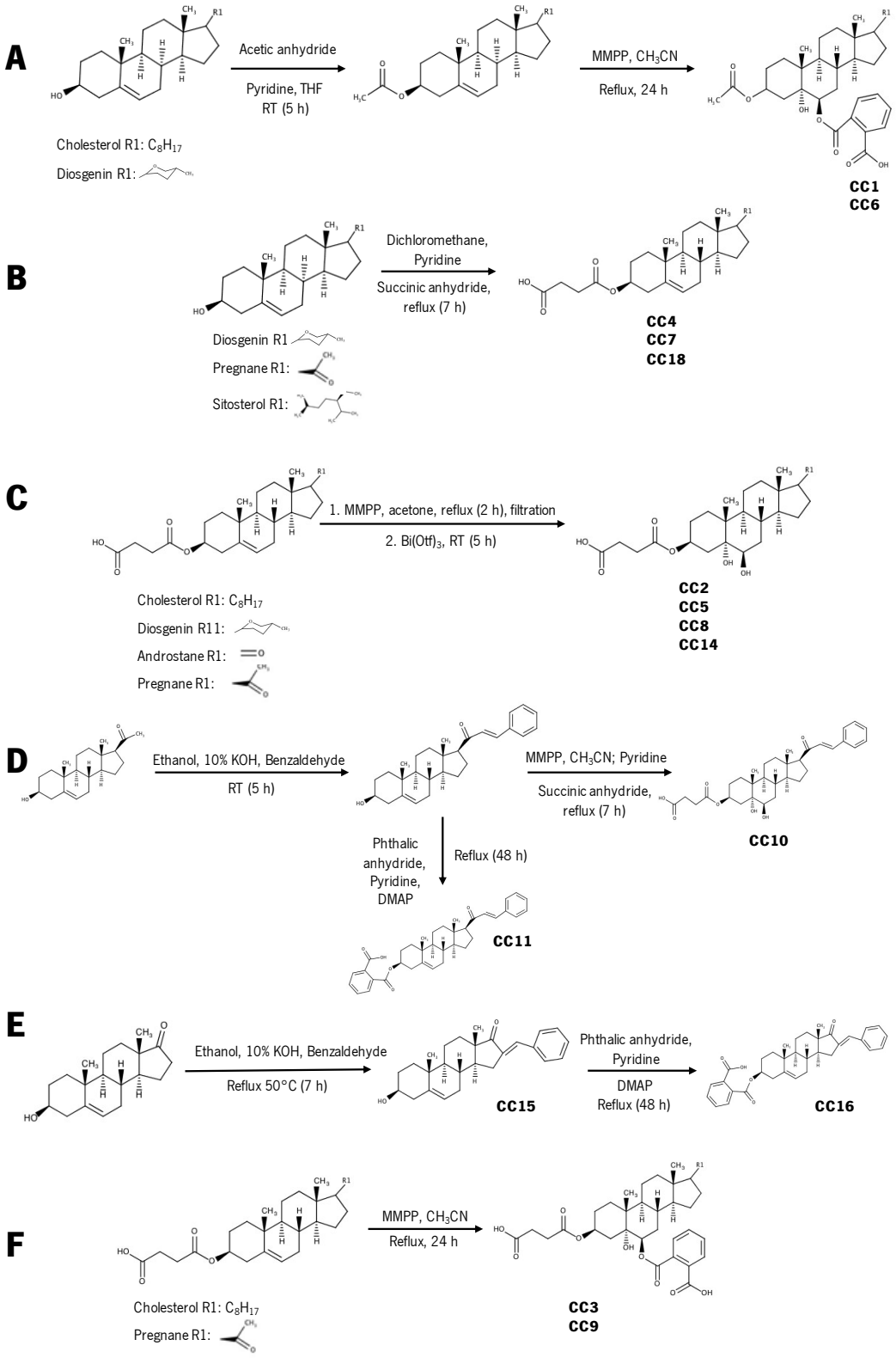


Figure 1: Chemical reactions and structures of the steroid derivatives grouped by reaction type and starting material. Different chemical reactions (A-F) were implemented with cholesterol, diosgenin, pregnenolone, dehydroepiandrosterone and sitosterol, which were used as starting materials for the synthesis of the 16 compounds in study.

Synthetic steroids derivatives display a selective and nontoxic antiplasmodial activity

All synthesized compounds were assayed for their toxicity against HepG2 human liver cell line and for their antimalarial activity against the asexual blood stages of a CQ sensitive- and resistant- *P. falciparum* strains, 3D7 and Dd2, respectively. From the 16 compounds, all presented negligible cytotoxicity, with IC_{50} values against human cells ranging from 23.4 μM to higher than 100 μM, with 11 of them displaying antimalarial activity with IC_{50} values ranging from 0.3 μM to 10 μM (Table 1). In general, all the compounds presented selectivity indexes (SI= IC_{50} (HepG2)/IC_{50} (Dd2 or 3D7)) higher than 10, indicating that our compounds are selective for parasites and nontoxic to the human cells used. Between these 10 compounds with relevant antimalarial activity no significant differences were detected when the compounds were tested against a sensitive 3D7 parasite strain or multi-resistant Dd2 parasite strain (Table 1). As shown in Table 1, compound CC1 presented the best antimalarial activity with an IC_{50} value of 0.39 μM (±0.03) on Dd2 and 0.46 μM (±0.01) on 3D7 parasite strains. The second most promising compound found in this library was compound CC6 with an IC_{50} value of 1.08 μM (±0.05) on Dd2 and 2.47 μM (±0.05) on 3D7 parasite strains.

Table 1: Cytotoxic and antimalarial activities of synthetic steroids. The cytotoxicity was evaluated against a human cell line, HepG2, and the antimalarial activity evaluated against two parasite strains (Dd2 and 3D7). Mean ± SEM IC_{50} values (μM) were derived from three independent experiments. The selectivity indexes (SI=IC_{50} (HepG2)/IC_{50}(Dd2) for Dd2 or SI=IC_{50} (HepG2)/IC_{50}(3D7) for 3D7) were calculated. Also, the predictive partition coeficient (LogP) values for the 16 synthetic sterols was calculated. These values were obtained using the swissADME software (http://www.swissadme.ch/index.php).

Synthetic compounds	Mean IC_{50} (μM±SEM) HepG2 cell line	Mean IC_{50} (μM±SEM) Dd2	Mean IC_{50} (μM±SEM) 3D7	Selectivity index for Dd2	Selectivity index for 3D7	LogP
CC1	23.4±1.5	0.39±0.03	0.46±0.01	60	51	5.62
CC2	34.6±1.8	2.54±0.5	3.85±0.3	14	9	4.33
CC3	60.4±1.4	2.69±0.5	3.32±0.4	22	18	5.13
CC4	>100	>10	>10	-	-	4.64
CC5	>100	4.01±0.4	7.9±0.2	-	-	3.15
CC6	58.5±0.9	1.08±0.05	2.47±0.05	54	24	4.46
CC7	>100	>10	>10	-	-	3.77
CC8	>100	8.79±0.4	6.78±0.3	-	-	2.25
CC9	>100	>10	>10	-	-	3.22
CC10	49.7±3.1	3.24±0.2	4.09±0.2	15	12	3.28
CC11	43.4±0.9	1.85±0.1	2.44±0.2	23	18	5.86
CC14	>100	>10	>10	-	-	1.83
CC15	53.4±1.9	3.61±0.1	4.67±0.3	15	11	5.00
CC16	39.3±2.1	1.54±0.3	1.40±0.2	26	28	5.50
CC17	>100	>10	>10	-	-	3.32
CC18	>100	>10	>10	-	-	6.25

In order to obtain a more detailed *in vitro* study about the toxicity and specificity of the best compound (CC1), its hemolytic capacity was evaluated using uninfected RBCs (Figure 2). For that, hemolysis was monitored by measuring the release of Hb from uninfected RBCs in absence or

presence of several concentrations of our compound (from 0.1 to 100 μM). As observed at Figure 2A, the absorbance of the supernatant of the parasites treated with the higher concentration of our synthetic compound (100 μM) remained constant and lower along the analyzed wavelengths (from 300 to 700nm) compared with uninfected RBCs treated with saponin (0.05%) which was used as positive control in this experiment. A peak at 405 nm was observed after incubation with saponin and the values at this wavelength were plotted to all conditions (untreated parasites (control), parasites treated with saponin 0.05% and parasites treated with synthetic steroids at all range of concentrations (0.1 to 100 μM) (Figure 2B). As observed at Figure 2B, compound CC1 did not induced any significant hemolysis at concentrations of 0.1 to 100 μM comparatively with untreated RBCs (control condition).

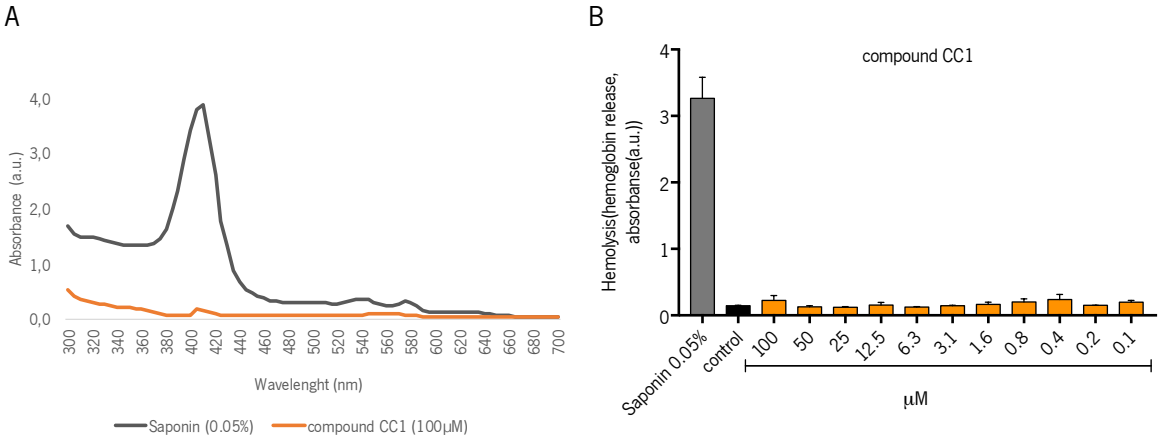


Figure 2: RBC lysis was measured in the presence of compound CC1 at several concentrations (0 to 100 μM) at 24 h. (A) Example of spectra (in the range of 300 to 700 nm) of supernatant of red blood cells incubated with saponin (0.05%) (gray line) and compound CC1 (A, orange line) at concentration of 100 μM at 24 h. (B) Hemoglobin release measured at peak, 405 nm, in untreated parasites (control bar) and after incubation with saponin (0.05%), compound CC1 (B) at 24 h incubation with a range of concentrations (0.1 to 100 μM). Data are mean ± SEM of three independent assays.

Structure–activity relationship (SAR) analysis of the library of compounds

Taking into account the results obtained for cytotoxicity and the potency against the blood stage of the parasite life cycle it is possible to perform a SAR analysis.

Analyzing Table 1, it was found that the steroids containing the 3'- acetate and 6'-hemiphthalate functional groups present improved antimalarial activity when compared with the other combinations of functional groups. The most active, compound CC1, a cholesterol derivative, has an IC_{50} value of 0.39 μM (±0.03) on Dd2 and 0.46 μM (±0.01) on 3D7 parasite strains with selectivity index of 60 for Dd2 and 51 for 3D7. The second most active compound CC6, a diosgenin derivative, showed an IC_{50} value of 1.08 μM (±0.05) on Dd2 and 2.47 μM (±0.05) on 3D7 parasite strains with a selectivity index of 54

for Dd2 and 24 for 3D7. These two synthetic steroids bearing a 3β-acetate and a 6β-hemiphthalate differ in the lateral chain indicating that this structural component affects the antimalarial activity.

Other evidences suggesting that the lateral chain of the steroid can affect the antimalarial activity can be seen between cholestane derivative CC3 and pregnane derivative CC9, both bearing a 3β-succinate and a 6β-hemiphthalate, since a loss of activity was observed for the pregnane side chain (compound CC9; IC_{50} values higher than 10 μM) was used as starting material (Table 1).

Additionally, steroids containing an aromatic enone (chalcone) at C21 (compounds CC10 and CC11) exhibited antimalarial activity in low micromolar range. Compound CC10 displayed IC_{50} values of 3.24 μM (±0.2) for Dd2 and 4.09 μM (±0.2) for 3D7 and compound CC11 displayed an IC_{50} value of 1.85 μM (±0.1) for Dd2 and 2.44 μM (±0.2) for 3D7. Interestingly, CC16 displays similar activities when compared to CC11, indicating that the positions of the enone and aromatic moieties attached to ring D are not critical.

The addition of succinate group to C3 position of the steroid does not confer antimalarial activity as observed for compounds CC4, CC7 and CC18. On the other hand, functionalization of the B-ring of compounds CC4 or CC7 into the corresponding dihydroxy derivatives CC5 or CC8 affords toxicity against the parasite.

The 3β-succinate ester had a detrimental effect on antimalarial activity compared to the 3β-acetate, as can be observed for CC1 and CC3.

Introduction of a 6β-hemiphthalate afforded highantimalarial activities (CC1, CC3 and CC6).

Comparing compounds referred above CC10, CC11, CC15 and CC16, the presence of phthalate group attached to the steroid framework enhanced, not only, the antimalarial activity of these compounds but also the selectivity to parasite.

Steroids with benzylidene functional group at C16 were also prepared, corresponding to compounds CC15 and CC16, and their antimalarial activity evaluated. Compound CC15 has micromolar activity with an IC_{50} value of 3.61 μM (±0.1) for Dd2 and 4.67 μM (±0.3) for 3D7. Compound CC16 has a better antimalarial activity with IC_{50} values of 1.54 μM (±0.3) for Dd2 and 1.40 μM (±0.2) for 3D7. Compound CC15 contains a hydroxyl group in C3 of steroid while compound CC16 has a phthalate group attached in this position. This modification could explain the difference obtained between these two compounds. Altogether the results evidenced that the lateral chain possibly modulate the parasite susceptibility to these compounds and the presence of acetate and phthalate groups attached in the same steroid molecule leads to a promising antimalarial compound.

Compound CC1 is more active at *P. falciparum* ring stage of the parasite life cycle

During the blood stage of the parasite life cycle, parasites assume different forms. The parasite inside the red blood cell passes through ring, trophozoite and schizont stages. It has been described that during these stages the gene and protein expression patterns alter greatly from one stage to another (44). Therefore, the stage-specific activity of compound CC1 was evaluated (Figure 3). The data revealed that the compound is more effective at ring-stage since a lower IC_{50} value (0.33 μM ±0.01 for compound CC1 (Figure 3A)) was obtained compared with the other stages tested. For compound CC1, there is an increasing IC_{50} trend, specifically a 1.2 (0.43 μM ±0.03), a 2.4 (0.79 μM ±0.08) and a 3.1-fold (1.05 μM ±0.009) increase, respectively, for early trophozoites, trophozoites and schizonts when compared with ring stage. These findings suggested that our compound may interact with target molecules that are expressed at specific parasite stage.

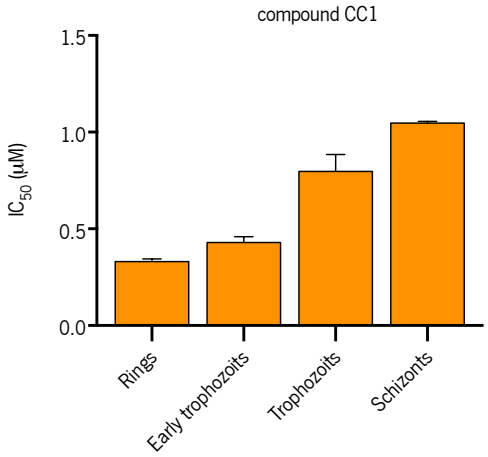


Figure 3: *In vitro* stage-specific activity of compound CC1 measured using a Dd2 parasite strain. The synthetic compound CC1, was serially diluted and incubated at different blood-stages of the parasite life cycle: rings (4h post-invasion) early trophozoites (20h post-invasion), trophozoites (32h post-invasion) and schizonts (40h post-invasion). After 72 h of incubation, parasitemias were determined by flow cytometry after staining the parasites with MitoTracker Deep Red FM and SYBR Green. IC_{50} values (μM) were calculated using nonlinear regression analysis. Data are means ± SEM of three independent assays.

Compound CC1 has a promising gametocidal activity

Parasite gametocytes are the only stage that is the vehicle for disease transmission. To determine whether compound CC1 has any activity on sexual stage, late (IV-V) *P. falciparum* gametocytes were exposed to different concentrations during 72h (Figure 4). Preliminary results suggested a promising late-stage gametocytocidal activity of the compound CC1 (orange bar). PMQ

(red bar) was used as control, since it is an approved drug presenting gametocytocidal activity against late stages gametocytes (45). In agreement with previous results (46, 47) gametocytes exposed to this antimalarial drug exhibited a IC_{50} value of 19.9 μM.

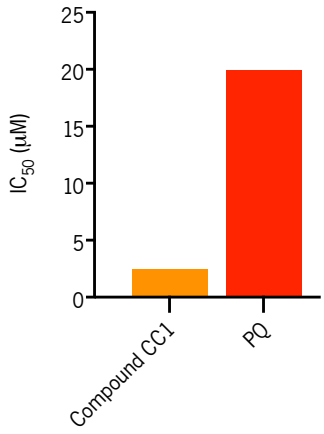


Figure 4: *In vitro* gametocytocidal activity of compound CC1. Preliminary IC_{50} values (μM) are represented in the bars. The compounds CC1 (orange) and primaquine (PMQ, red) were serially diluted and after 72 h of incubation the parasitemias were determined by flow cytometry after staining the parasites with MitoTracker Deep Red FM. IC_{50} values were calculated using nonlinear regression analysis. Data represents only one experimental assay.

Compound CC1 induce redox imbalance

Trying to understand the mode of action of synthetic compounds, a *P. falciparum* Dd2 strain that express episomally a glutathione biosensor linked to a redox sensitive GFP (Grx1-roGFP2 sensor) was used in order to measure redox changes into the parasite in the presence of the compound CC1. The parasites were exposed to a range of compound IC_{50} (10x, 20x, 30x and 40x of IC_{50} value) and the redox imbalance measured after 2 h and 4 h of exposure.

Compound CC1 induced a significant increase of redox ratio compared with untreated control parasites after 2 h of incubation at 40x IC_{50} values and after 4 h incubation in all conditions (Figure 5) pointing to increased oxidation and alterations in the intracellular redox potential. These results indicate a potential influence of synthetic steroids on the glutathione redox homeostasis.

Additionally, in order to understand if redox imbalance is due to the presence of functional groups added to steroid scaffold, starting material without any chemical modification were also investigated. The reported redox imbalance of compound CC1 is specific as no significant differences were detected between control and parasites incubated with starting material, cholesterol, used as scaffold to generate the compound CC1 (Figure 5).

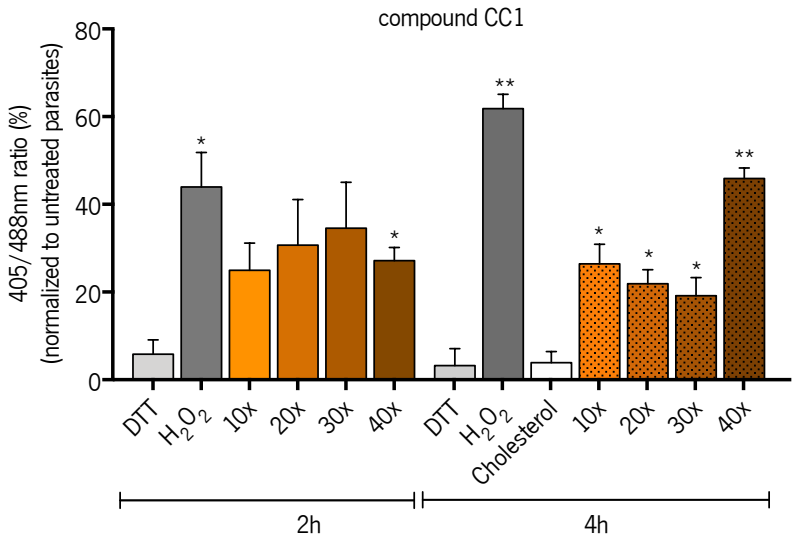


Figure 5: Mid-term effects of compound CC1 on the redox ratio of *P. falciparum* Dd2-*att*B-roGFP-grx1 transfected parasites. A trophozoite-stage culture of Dd2 strain of *P. falciparum* expressing grx1-roGFP were treated with compound CC1 at concentrations of 10x, 20x, 30x and 40x IC_{50}values for 2 and 4 h. Following the incubation time, the parasites were excited with 405 and 488 nm laser wavelengths and the ratio of emissions (405/488 nm) was calculated. Normalized fluorescence ratio (405/488nm, as a percentage) for the treated conditions and for starting material used to generate the derivative compound CC1, cholesterol, was evaluated. All experiments included untreated parasites as control, and fully reduced and oxidized parasites with 10mM dithiothreitol (DTT) and 1mM hydrogen peroxide (H_2O_2), respectively. The fluorescence ratio for all treated conditions referred above were normalized to the untreated control (indicated in the graph as the baseline [y<0]). Values greater than zero indicate an increase in the percentage ratio fluorescence compared with untreated parasites. Data are mean ± SEM of three independent assays. Two tailed *t*-tests were used to assess statistically differences between control and treated-parasites. *$p< 0.05$, ** $p< 0.01$.

Heme detoxification process inhibited by CC1 compound

One of the possible internal sources of oxidative stress inside parasite is the degradation of host Hb through the release of reactive free heme that is toxic for parasite and for instance triggering oxidative stress. To investigate further on the mode of action of compound CC1, its impact on Hz formation process was assessed. Using a pyridine-labeled heme fractionation assay (40, 48), we determined the effect of CC1 on Hz biocrystallization. CQ was used as positive control since it has been described that CQ concentrate in the DV, where this drug can directly bind to the Fe(III) center present in reactive free heme (Fe (III)heme) via a deprotonated hydroxyl group preventing the Hz formation (49). Atovaquone was used as negative control since it is thought that this drug inhibits the cytochrome *bc*1 complex in the parasitic electron transport chain disrupting the mitochondrial membrane potential (50). In the presence of 4x IC_{50} value of CC1, we observed that the parasite's

ability to catabolize Hb was compromised, with significant increase in the percentage of undigested Hb between treated and untreated parasites (Figure 6A). The results also showed a significant increase in the percentage of free heme compared with untreated parasites (Figure 6B) and that corresponded to a significant decrease in Hz (Figure 6C), indicating that free heme is accumulating inside the DV of the parasite and is not being converted to Hz. The pattern obtained for free heme and Hz was also observed in CQ-treated parasites in agreement with previous results (40, 48). No significant differences were detected between untreated and ATQ-treated parasites in all fractions measured (figure 6A, B and C) indicating that the free heme was sequestered in the form of Hz.

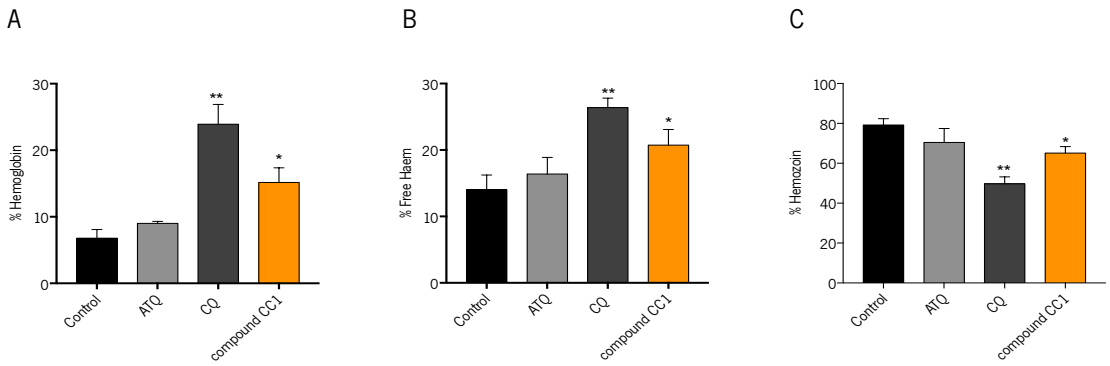


Figure 6: Percentage of heme species found in compound CC1-treated trophozoites. Percentage of hemoglobin (A) free heme (B) and hemozoin (C) present in Dd2 parasite strain were measured after 32h incubation with 4x IC_{50} of compound CC1. Statistical comparisons of the drug-treated parasites compared to its untreated control were performed using two-tailed *t*tests. (*$p < 0.05$, ** $p < 0.01$).

Compound CC1 in not dependent on well know *P. falciparum* resistance players, PfMDR1 and PfCRT transporters

Since the Hz detoxification process occurs inside the DV, we tried to understand the compounds interplay with two important players associated with antimalarial resistance, the PfMDR1 and PfCRT, both located at the DV membrane of the parasite.

Compound IC_{50} values was measured in *P. falciparum* NF10 isogenic parasite lines solely differing at the PfMDR1 aminoacid positions 86 and 184, well known molecular markers of antimalarial drugs (29, 51–53). No significant differences were detected between NF10 parasite lines containing the haplotypes Y86Y184, Y86F184, N86Y184 and N86F184 (Figure 7), suggesting that these mutations do not have a role in parasite response to synthetic steroid compound.

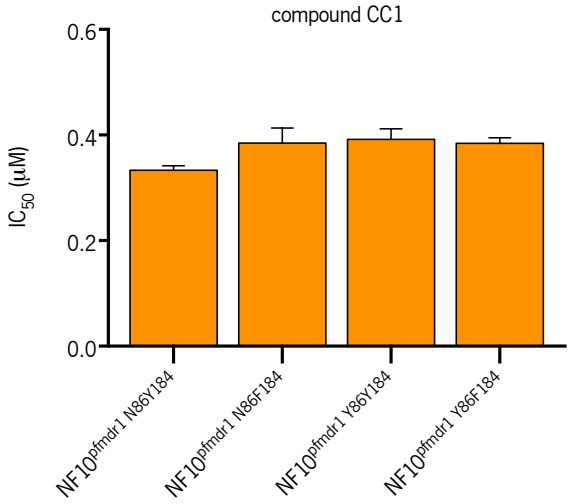


Figure 7: *In vitro* IC_{50} steroid antimalarial response of isogenic parasite lines: $NF10^{N86Y184}$, $NF10^{N86F184}$, $NF10^{Y86Y184}$ and $NF10^{Y86F184}$. IC_{50} values (µM) are presented as mean ± SEM of at least three replicates. The compound CC1 was serially diluted and after 72 h of incubation the parasitemias were determined by flow cytometry after staining the parasites with MitoTracker Deep Red FM and SYBR Green. IC_{50} values were calculated using nonlinear regression analysis.

Additionally, the role of PfMDR1 as possible modulator of compounds susceptibility was measured using a well-established inhibitor of P-glycoprotein type ABC transporters, elacridar (ELC) (Figure 8A). The antimalarial MFQ was used as positive control, since PfMDR1 is considered the main molecular marker of resistance (54). No significant differences on the IC_{50} values were detected between parasites treated with CC1 in the presence or absence of ELC suggesting that our steroids containing cholesterol as the starting material are not dependent on PfMDR1 protein (Figure 8A). As expected, a significant increase in the parasite susceptibility when treated with MFQ+ELC was observed compared with the parasite incubated with MFQ only.

The same approach was performed to study the role of PfCRT as possible modulator of compounds susceptibility using verapamil, a calcium ion channel blocker that reverses parasite resistance to CQ (Figure 8B). In the presence of verapamil, no significant differences in the IC_{50} values of CC1 were obtained from *P. falciparum* Dd2 strain between parasites treated with our compound in the presence or absence of VP suggesting independency on PfCRT.

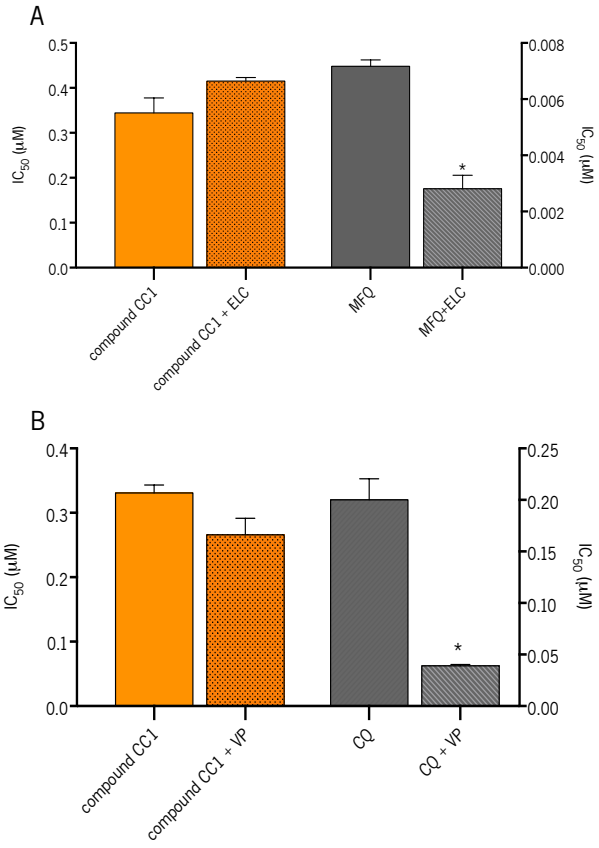


Figure 8: *In vitro* IC_{50} antimalarial response of Dd2 parasite strain in the presence or absence of elacridar (ELC) and verapamil (VP). The Dd2 parasite strain were incubated with compound CC1 in the presence or absence of elacridar (ELC, A) or verapamil (VP, B). When incubated with ELC or VP, the compound CC1 was serially diluted in the presence of 0.1µM ELC or 0.8µM of VP for the 72 h *in vitro* growth inhibition assays. Mefloquine (MFQ, A) and Chloroquine (CQ, B) were used as positive control of the experiment and also incubated in the presence or absence of ELC or VP. Parasitemias were determined by flow cytometry after staining the parasites with MitoTracker Deep Red FM and SYBR Green. Mean (µM) ±SEM IC_{50} values were derived from three independent experiments and calculated using nonlinear regression analysis. Two tailed *t*-tests were used to assess statistically differences between the untreated control parasites and VP or ELC-treated parasites. *, $p<0.05$.

Compound CC1 is synergic with MFQ and LMF

Determination of synergy of the CC1 with current antimalarial drugs CQ, md-ADQ, DHA, MFQ, LMF, PND and PPQ, was performed based on *in vitro* IC_{50} isobologram analysis, with determination of drug interaction based on previously described criteria (41). To determine the drug combination interactions between our synthetic steroid and ACT-partner drugs in asexual parasites, FICs were used to construct isobolograms (Figure 9). The isobolograms showed that compound CC1 has a synergistic interaction *in vitro* with the aryl-aminoalcohols, MFQ and LMF. Furthermore, interaction with DHA and compound CC1 was found to be additive in three combinations where the three highest concentrations

of each compound are present in combination, while synergistic interaction was observed in the other six combinations. Interaction of the 4-aminoquinolines, CQ and md-ADQ with compound CC1 was found to be antagonist, independent on the different concentration proportions used. Additionally, an additive interaction was detected when combined our compound, CC1, with 4-aminoquinoline PND.

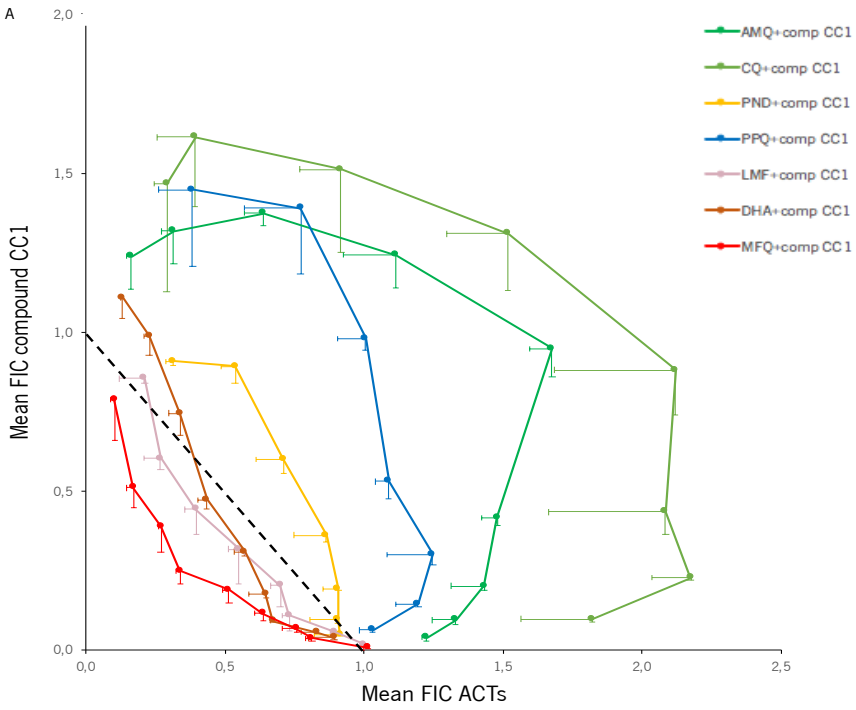


Figure 9: IC_{50}-based isobologram curves against Dd2 parasite line showing the interaction between some approved antimalarial drugs and synthetic steroid compound in study. The mean of fractional inhibitory concentration (FIC) of our synthetic compound is plotted with the mean of FIC of monodesethyl-amodiaquine (md-ADQ), chloroquine (CQ), pyronaridine (PND), piperaquine (PPQ), lumefantrine (LMF), dihydroartemisinin (DHA) and mefloquine (MFQ). The compounds were serially diluted and, after 72 h, the parasitemia was determined by fluorimetry after staining the parasites with SYBR Green. Data are means ± SEM of at least three independent experiments.

DISCUSSION

Nature has been a major source of pharmacological drugs throughout history and the most efficient antimalarial drugs have a strong historical relation to natural products (7–9). Steroids isolated from natural sources or obtained by semi-synthetic modifications have been shown potential for the development of new candidates for malaria treatment (18–21). Since this type of molecules has great advantages compared with other natural products, applying appropriate SAR can lead to combine the steroid scaffold with functional groups conferring optimized biological activities. Previously, in our group, several polyfunctional steroids with diverse oxygenated groups were designed and tested against a panel of human cancer line lines with high selectivity demonstrating the promising potential of these polyfunctional steroids (26, 42). These steroids were tested against the blood stage of malaria and the results of that preliminary screening allowed us to design new steroid derivatives bearing different ester groups.

Therefore, taking into consideration the SAR obtained from the screening, we designed and synthesized a set of new steroid derivatives aiming to explore the Importance of different ester groups and their locations. In the steroid framework and the importance of the side chain. The synthetic steroids obtained were evaluated in terms of the selectivity index towards the parasite, revealing high *in vitro* antimalarial activity. Our studies aimed to unveil some parasite biological pathways disturbed in the presence of these compounds. Among the 16 synthesized compounds we showed that the highest biological activity was found among cholestane derivatives followed by diosgenin derivatives, while the androstane, pregnane and sitosterol derivatives were less potent. Noteworthy, a benzylidene moiety at the side chain or at the D ring allows retention of antimalarial activity if the best functional groups are present at rings A and B (Table 2) (Figure 10). Recently, by synthesis and SAR studies, it was demonstrated that the highest antimalarial activity was found on steroid derivatives as compared to non-steroid analogs (20) indicating that the steroid moiety is crucial for the biological activity of the compounds. This observation can give rise to some key considerations since the delivery of small molecules to the cells can be mediated by lipids, polymers, nanoparticles(55) or by changing the membrane's permeability (56). In our study, the presence of the lipophilic steroid moiety, present in all synthesized compounds, may be important to mediate cellular uptake and intracellular transport. Steroid compounds are able to enter cells by diffusion across the plasma membrane, via membrane association or through endocytic processes (57, 58). Therefore, compounds containing this scaffold may present advantages compared, not only with other natural compounds, but also with ACTs, since

these have lipophilic characteristics and a higher bioavailability, passing lipophilic barriers much more efficiently. These characteristics may direct the molecule to its' desired target, and if an optimized functional group were to be added to these scaffolds, this could improve even more their antimalarial activity.

The 16 synthesized compounds exhibited low cytotoxicity against a human hepatic cell line suggesting that these compounds have high selectivity against parasite. Complementarily, hemolysis studies showed that the integrity of uninfected RBCs membrane was not affected by the synthetic steroids, reinforcing not only their low cytotoxicity for human host but also their specificity for the parasite.

Taking into account that steroid-based compounds as antimalarial agents are being proposed, the intrinsic biological activity of the steroid should be considered. In most bioactive steroids, the presence at C3 position of either a hydroxyl group, or a ketone or an enone are critical for enzyme or receptor binding and consequent biological activities (59–62). In our work, the best compound, CC1, has the C3 position impaired by the presence of an acetate group. Therefore, potential side effects based on steroid activity at other human molecular targets are unlikely to occur.

Notably, the presence of both acetate and phthalate linked to the same steroid skeleton conferred a higher antimalarial activity compared with other functional groups tested, along with high selectivity, higher than 50 for compound CC1 and 24 for compound CC6 (Table 1). The presence of an ester group in a molecule such as steroid could be useful to introduce large polar groups. Also, the presence of polar substituents at ring B is important to turn the molecule more polar and induce destabilization of cellular membranes (Figure 10). Indeed, the presence of these functional groups, like the phthalate at the 6β axial position, turn the synthetic steroid more polar, passing more efficiently the membrane becoming more bioavailable compared with steroids without this type of chemical modifications.

In drug development, lipophilicity is an important feature to determine solubility and absorption when the compounds are tested *in vivo* (63). Regarding our results, using diosgenin as starting material to attach functional groups could be more interesting since the partition coefficient (logP) values are relatively lower compared, for instance, with cholesterol (from which the compound with the highest antimalarial activity was obtained, CC1) (Table 1) and therefore further optimizations can be targeted for the use of diosgenin as starting material.

The results also demonstrated that the lateral chain altered the antimalarial potency of synthetic compounds since the IC_{50} values increased when the same functional groups were used in different

starting steroids (IC_{50} value for compound CC1: Dd2: 0.39 μM±0.03; 3D7: 0.46 μM±0.01; IC_{50} values for compound CC6: Dd2:1.08 μM±0.05; 3D7: 2.47 μM±0.05). Also, comparing the compounds CC2, CC5, CC8 and CC14, which have only differences regarding the lateral chain, it was observed that the best result was obtained for cholestane CC2 confirming that lateral chain impacts the parasite's response to steroids.

The best synthesized compound, CC1, was deeper explored to understand the possible parasite biological pathways that compounds could act on.

The delineation of the timing of action of a compound, namely the parasite stage specific activity, provides important insights into the development, growth and clinical clearance of the parasite. Our compound exerts a higher antimalarial activity in the ring blood stage (Figure 3). This pattern was similar to ART and AS, the most potent and faster drugs applied in malaria treatment nowadays, since they exert a higher impact on parasite's ring stage (64).

It is desired that novel compounds exert activity in several stages of the parasite life cycle (1, 65) including sexual stages. Gametocytes circulate in the blood stream and although does not influence disease progression they are the vehicle for disease transmission being extremely important therapeutic targets. The first step of the production of gametocytes is marked by expression of the regulator PfAP2-G, a transcription factor of the ApiAp2 family. In asexual parasites the *pfap2-g* gene is epigenetically silenced by the heterochromatin protein 1 (HP1). Therefore, the activation of *pfap2-g*, which triggers the sexual development, requires the removal of HP1 by the gametocyte development 1 (GDV1) protein (35, 66). Therefore, in this work, the effects on gametocytes were evaluated using a genetically modify parasite line containing the *pfgdv1* gene fused with a destabilization domain and being proteolytically degraded unless the parasites were cultured in the presence of a small ligand stabilizer, shield-1 (35–37). Preliminary results, using this approach, suggested that our synthetic compound CC1 has a promising late-stage gametocytocidal activity evidenced the multistage applicability of our compound (Figure 4).

It has been described in the literature that synthetic steroids can lead to reactive oxygen species overproduction (18, 20). Taking this into account the approach applied here (Figure 5) directly addresses this process using a genetic encoded biosensor to measure in real-time the redox status of the cell upon drug exposure (38). Reduction-oxidation-sensitive green fluorescent protein (roGFP) present in the biosensor, detects oxidative changes by the presence of dithiol-disulfide pair in the structure of GFP, allowing to detect fluorescence in response to oxidation states. Our data shown that compound CC1 induced an increase in oxidation and consequently alterations in the intracellular redox

potential pointing that these compounds modulate the glutathione redox homeostasis suggesting that this could be one of the mechanisms of action of this type of compounds.

One of the internal sources of oxidative stress is the degradation of Hb by the parasite, a process that occur inside the DV, as a source to obtain essential aminoacids for their growth and survival (67). However, Hb degradation leads to the release of toxic iron protoporphyrin IX [Fe(II)PPIX] which are immediately autooxidized to reactive Fe(III)PPIX (68, 69). This product has the ability to cause lipid peroxidation, to destabilize membranes by a colloid osmotic mechanism and induce oxidative stress (leading to the production of reactive oxygen species including H_2O_2 and O_2^{2-}. On way for the parasite to detoxify this reactive Fe(III)PPIX is try to convert it into an inert biocrystal, the Hz. Disruption of Hz formation appears to be an attractive target in killing the parasite as the process is indispensable for the survival of parasite (70). Importantly, several antimalarial drugs such as quinoline class including CQ and PPQ have been suggested to interact with Fe centers present in reactive Fe(III)PPIX preventing the Hz formation (40, 49). Using heme fractionation assays, we provide compelling evidence that our synthetic steroid acts by preventing Hz formation (Figure 6C) inducing a buildup of reactive free heme (Figure 6B). Also, in the presence of our synthetic compound the ability of the parasite to degraded Hb was compromised (Figure 6A). Given the lipophilicity of this compound it is most likely that they access passively the DV of the parasite. Compound CC1 contains a bidentate O binding site at B-ring (5' and 6'). At the pH of the organelle (5.0), the OH-group at position 5´ is protonated and the carboxyl group of the hemiphthalate is de-protonated. It is possible that the COO^- group can interact by electrostatic forces with Fe(II) and Fe(III) present in the heme or hematin molecules, while the aromatic moiety may establish π-π-interactions with the heme group, thus disturbing the formation of hemozoin. These interactions may induce the accumulation of free heme inside the DV of the parasite causing impaired parasite growth and survival.

In the parasite DV membrane, two transporter proteins, PfMDR1 and PfCRT, are well-known modulators of the parasite response to almost all antimalarial drugs used to treat malaria (29, 53, 71–74). These transporters belong to the ATP binding cassette (PfMDR1) and drug/metabolite transporter superfamily (PfCRT), and regulate the flux across DV membrane of the parasite, being therefore, capable of eliminating the drug from the place of action, promoting the development of resistance. Some studies demonstrated that ABC transporters have the ability to efflux endogenous steroids (75). However, reviewing the literature there is no information regarding the role of ABC transporters and synthetic steroids. Our data suggested that our best compound CC1 is not dependent on PfMDR1 and PfCRT transporters since no significant differences were detected between the isogenic strains and in

the presence or absence of inhibitors of PfMDR1 or PfCRT, ELC or VP, respectively (Figure 7 and 8). As referred above, the best lipophilicity obtained for CC1 comparatively to steroids without any chemical modification could help to explain in part these results. Our compound is more polar and soluble passing more efficiently the lipophilic membranes passively (76, 77). The non-dependence of these two membrane transporters studied here must be taken into account when formulating new therapies for malaria since it is desirable to obtain molecules that do not interact with these transporters. The results presented here highlight the promising features of our compounds and could suggest a delay in the appearance of resistance.

The inhibition of Hz formation is already a validated drug target and is considered a valuable target for the design of novel antimalarial drugs since no changes in the formation of free heme and Hz has been reported (78). Indeed, the resistance of antimalarials currently in use to treat malaria is directly related to mutations in the membrane transporters and not to the formation of Hz (78). In this way, as demonstrated here, our compound appears to interfere with this process that occurs in DV (Figure 6) without depending on the transporters and this demonstrates once again the relevance and applicability of our synthetic compounds.

Understanding the potency of combining this type of steroid compounds with present antimalarials was also studied. The drug combination for the treatment of malaria achieved better therapeutic effects comparatively with monotherapy for several reasons including the delayed emergence of resistant parasites and dose reduction while maintaining potency and avoiding adverse effects (79). With the current decline of efficacy of partner drugs in the approved ACTs, there is an important need to evaluate if novel compounds could be used in combined therapies. The *in vitro* results (Figure 9) suggest that CC1 could be combined with the aryl-aminoalcohols MFQ and LMF since synergistic interactions were obtained. Until now the mechanism of action of MFQ and LMF remains unknown (33, 80) but alterations in the PfMDR1 transporter protein modulates the parasite's response to these antimalarial drugs (33, 54) since the parasite, through this transporter, may be moving these drugs out of their primary site of action and placing them into the DV where they are less toxic (80, 81). The combination with CC1, that seem to be independent of the PfMDR1 transporter, appears to be beneficial and can possibly prevent the parasite from moving these antimalarials away from their place of action. Concerning DHA, the concentration of the combination needs to be taken into consideration since only in some concentrations a synergistic interaction was observed. ART and its derivatives have been reported to bind to several proteins and affect cellular processes including hemoglobin endocytosis and protein synthesis. This occurs due to the cleavage of the endoperoxide

bridge by the presence of Fe(II)heme released from the Hb digestion process. This activates the ART, culminating in the alkylation of multiple proteins and lipids, triggering oxidative stress (82, 83). As evidenced, CC1 also has an impact in the redox balance and this could exacerbate even more this imbalance inside the parasite when the two compounds are combined.

Interestingly, there was a consistent antagonistic interaction between synthetic steroids with drugs that belong to the quinoline class, CQ and md-ADQ. As referred above, this antimalarial drugs strongly bind to Fe(II)PPIX molecule and interfere with its detoxification (84, 85). The results from combination assays can suggest that these two classes of compounds can be competing for the same target inside the parasite. Another hypothesis is that the synthetic steroid is establishing intermolecular interactions with the quinoline drugs tested, preventing there binding with Fe(III) heme groups affecting the Hz formation.

More studies need to be conducted to address the *in vivo* pharmacokinetic and pharmacodynamic profiles for these synthetic compounds. As described, the higher potency against the ring stage, the induction of oxidative stress and the interference with the metabolism of Hb are similar mechanisms to ART and its derivatives. Thus, possibly when steroids are administered *in vivo* they may behave similarly to ARTs. A pharmacokinetic study, has described that synthetic steroids displayed moderate clearance with a long half-life (48 h) after intravenous application. Furthermore, the authors found that a single dose administration of the compound was able to reduce parasitemia by 98% and enhanced the lifespan from 6 to 14 days (20). These results are promising, since formulating an antimalarial treatment based in a single dose will enhance the patient compliance (6, 65).

The SAR analysis of our compounds indicates that acetate and hemiphthalate esters are the best functional groups at C3 and C6, while the cholestane side chain affords the best activities (Figure 10). Further semisynthetic exploitation of these compounds needs to be carried out in order to decrease the lipophilicity of the compounds, possibly manipulating the side chain, and to replace the 3β-acetate moiety by a metabolically more stable group. The 6β-phthalate deserves further investigation in order to disclose other polyoxygenated moieties able to confer antimalarial activity.

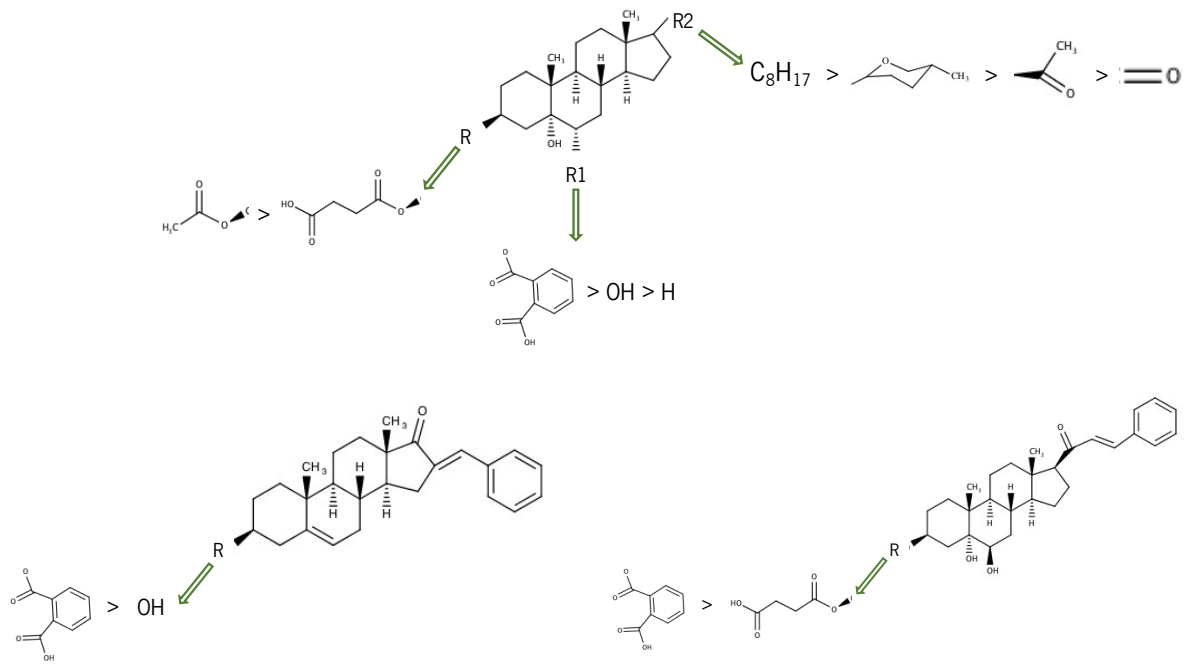


Figure 10: Representative image of SAR analysis for the synthetic steroids in study based on the IC_{50} results on malaria parasite.

Altogether these data reveal that steroids scaffold is valuable to inspire the discovery and development of new antimalarial drugs since these natural compounds have unrelated chemical entities when compared with ACTs and an optimization of functional groups attached to the framework or lateral chain can improve even more the antimalarial potency of these compounds.

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SUPPLEMENTARY MATERIAL

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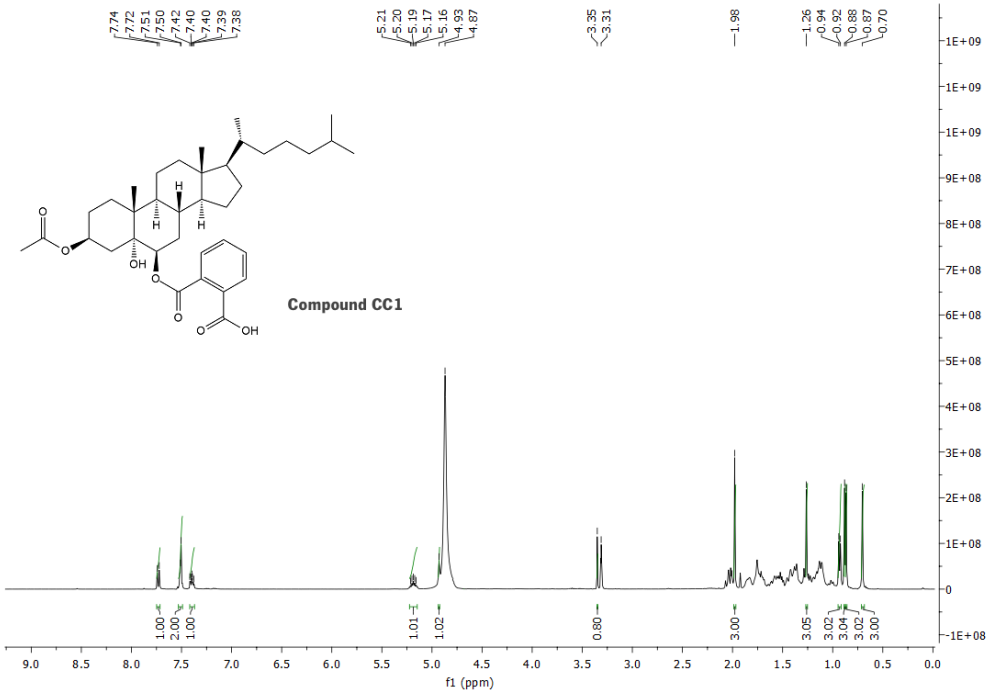

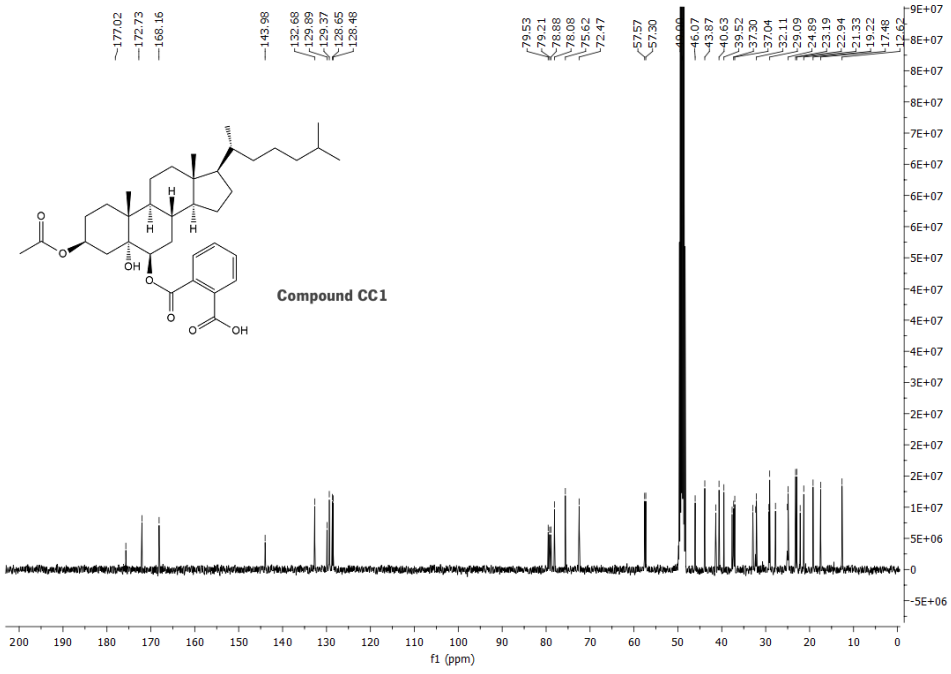

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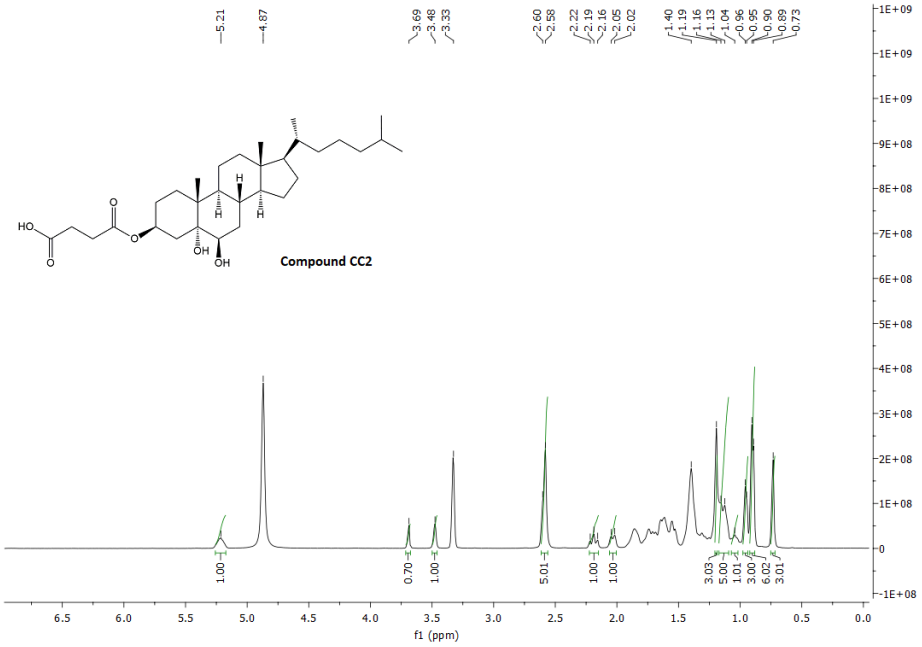

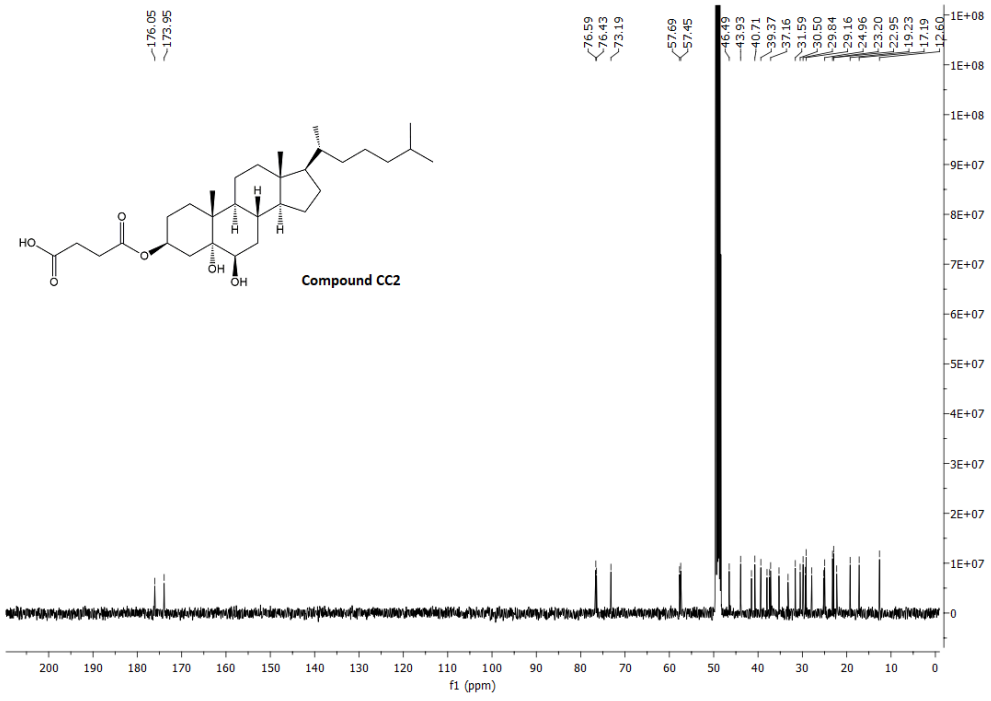

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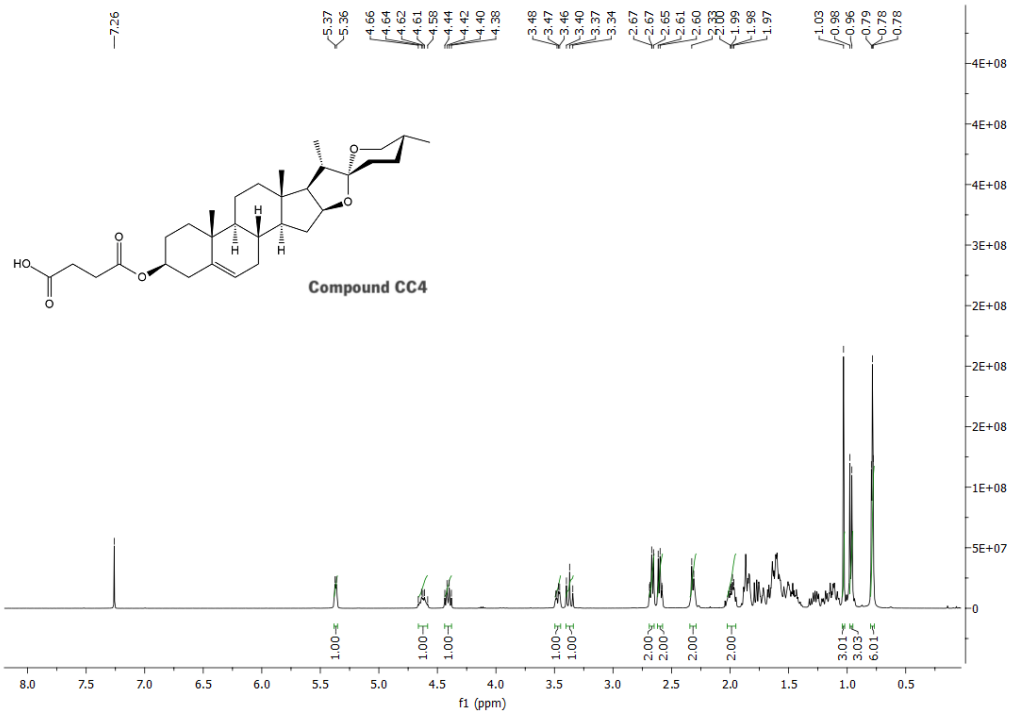

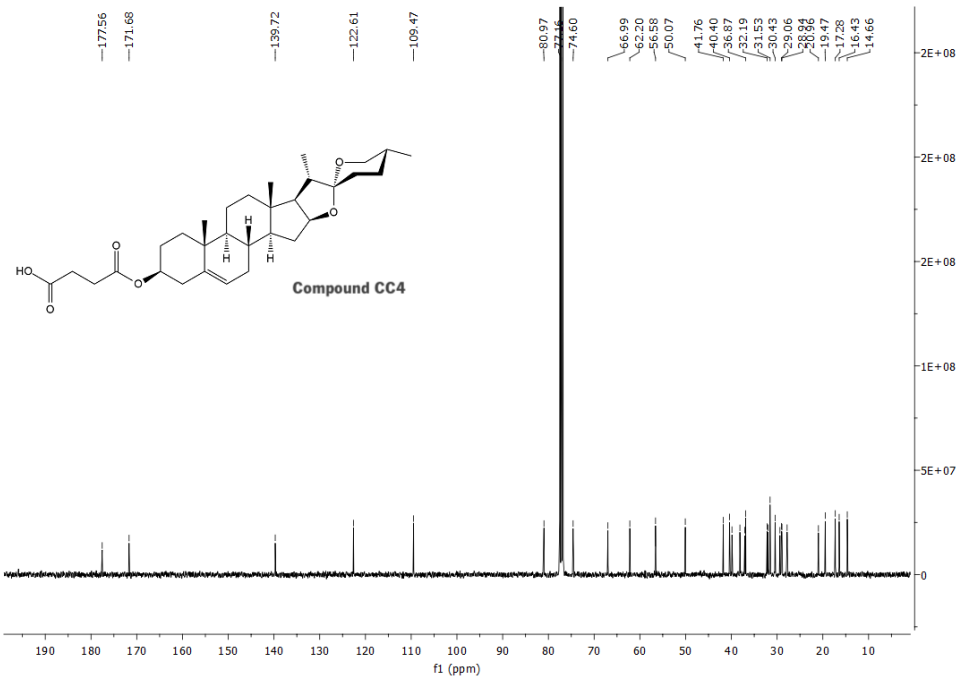

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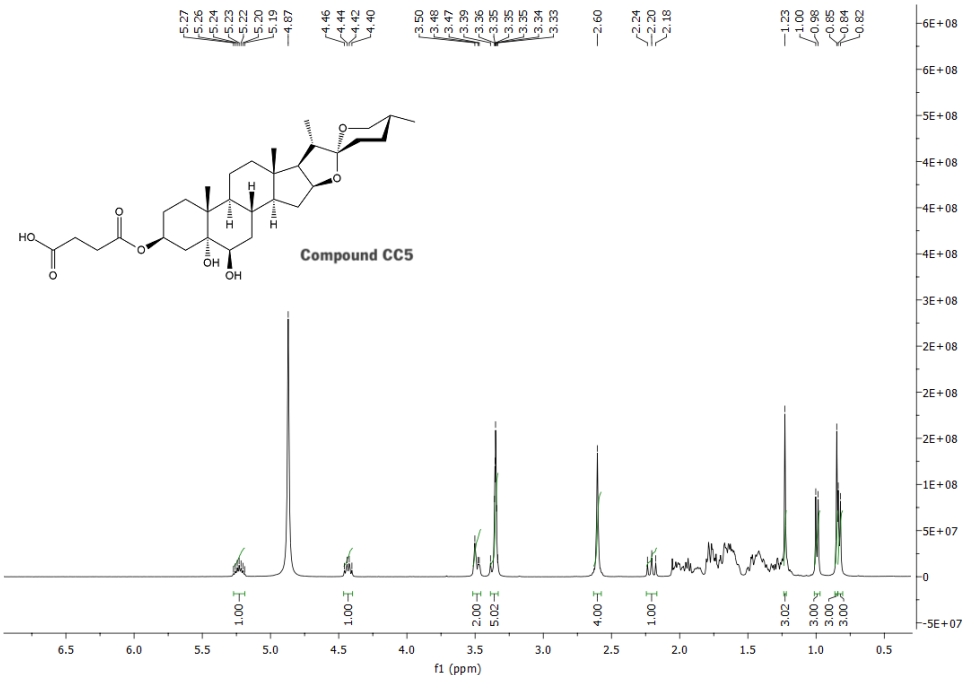

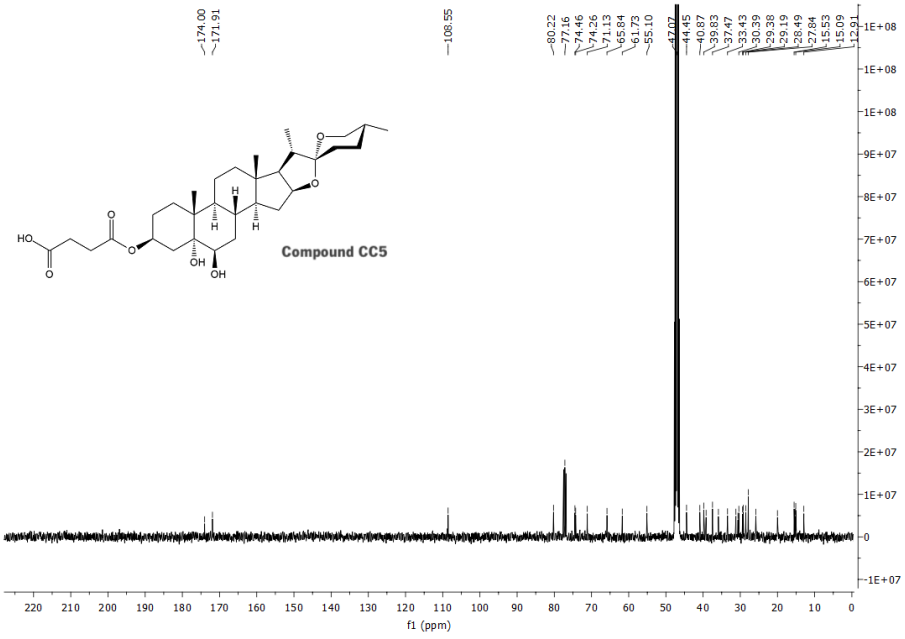

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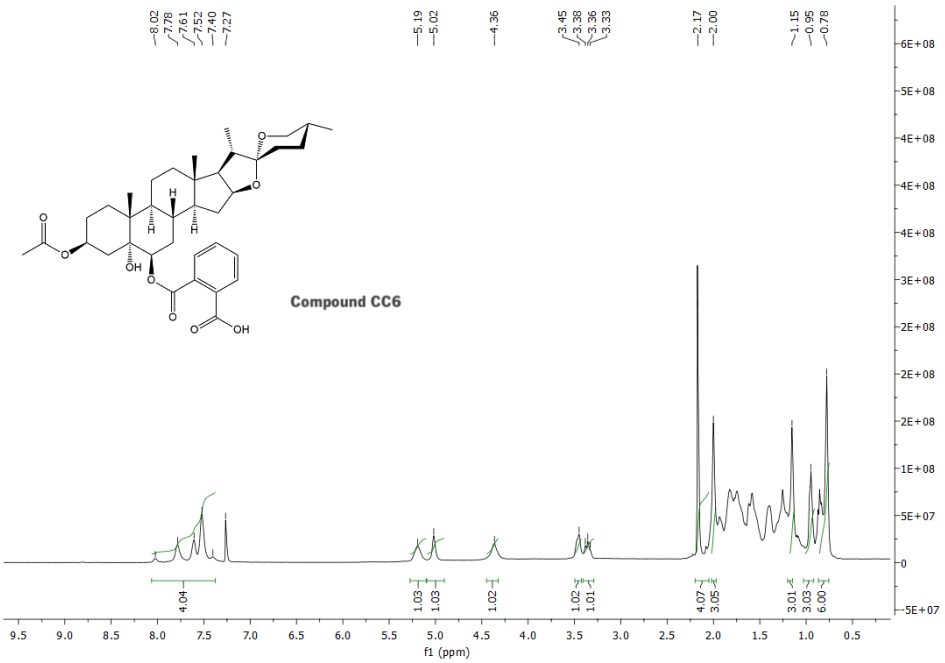

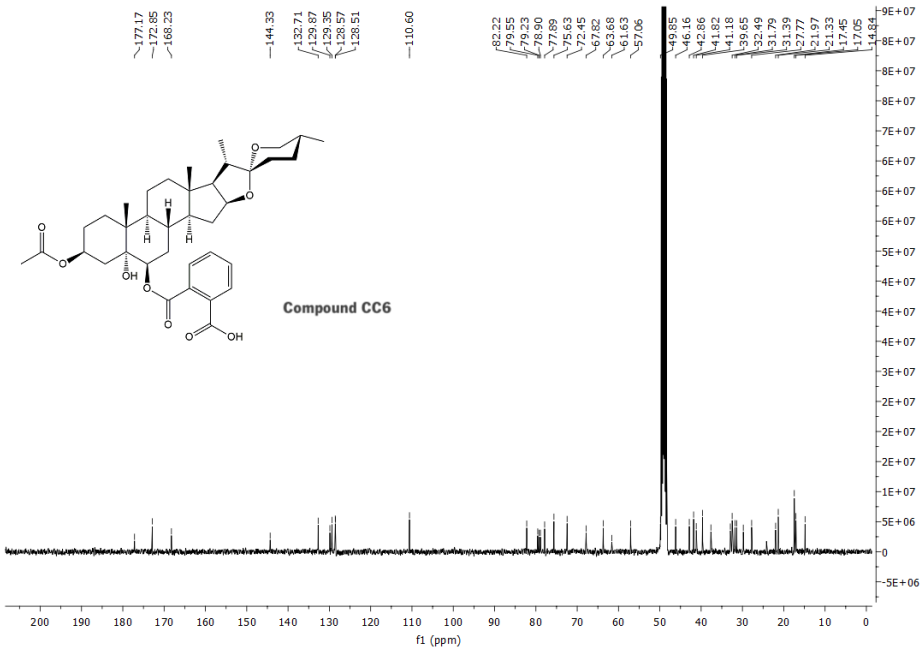

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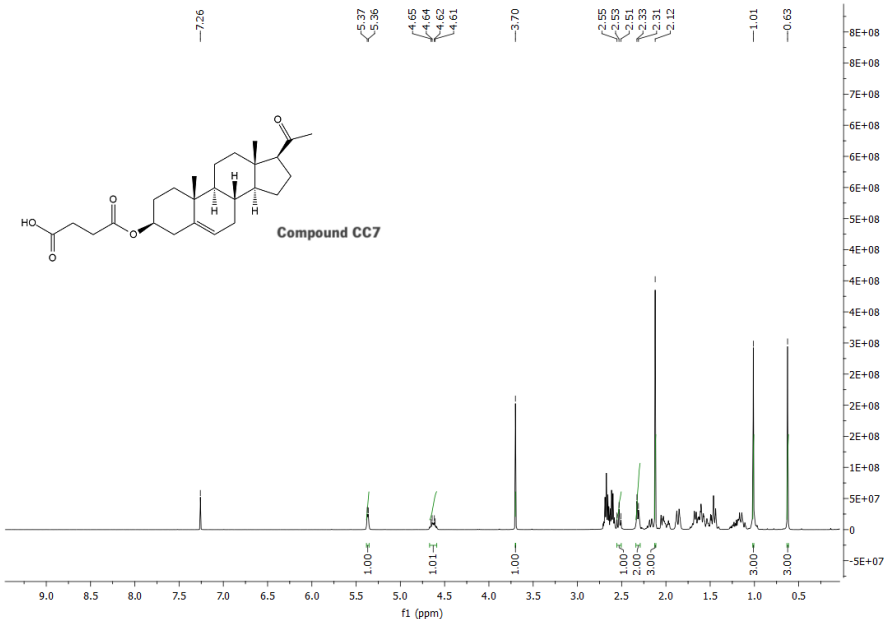

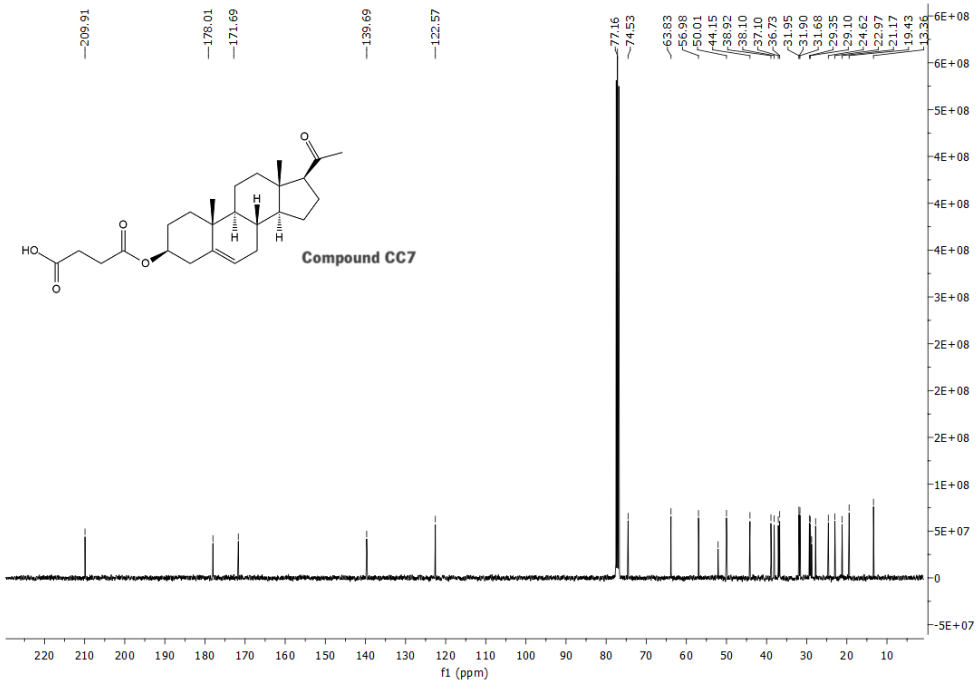

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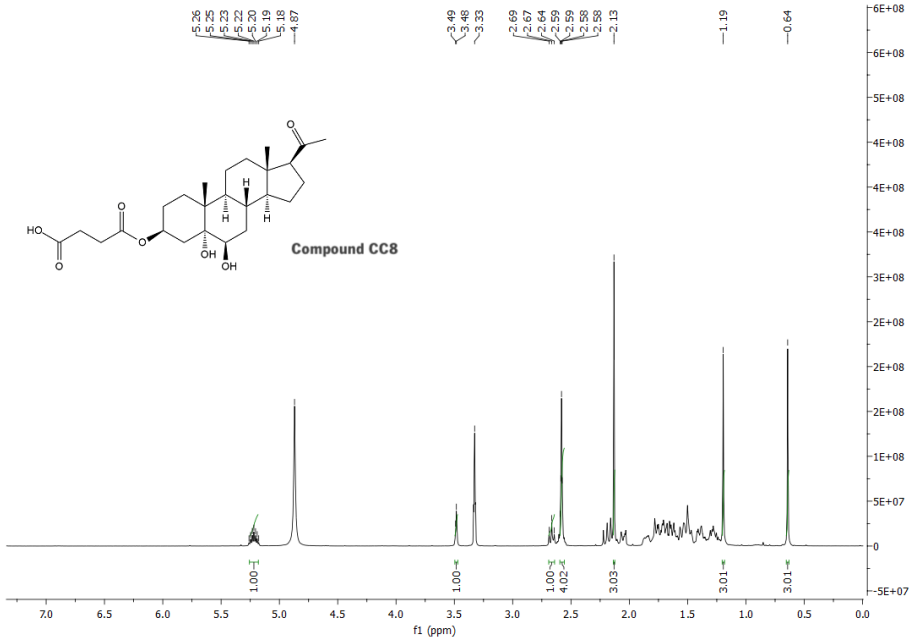

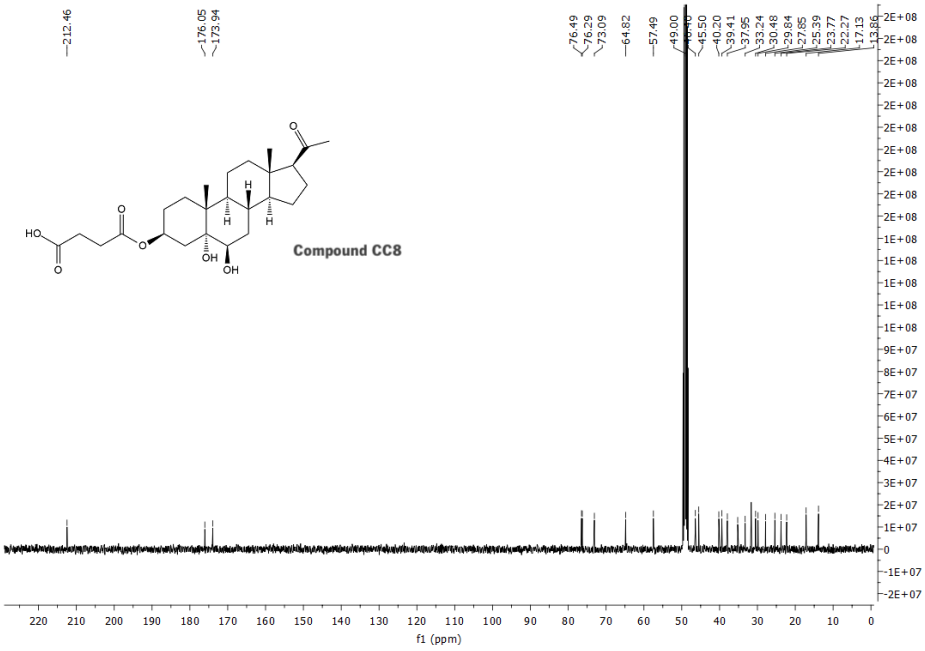

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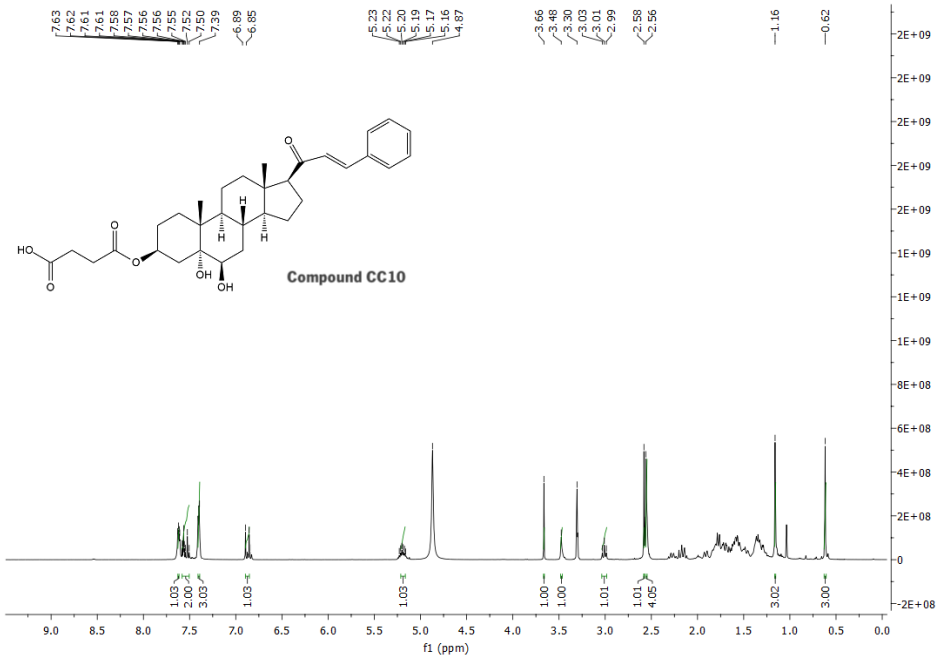

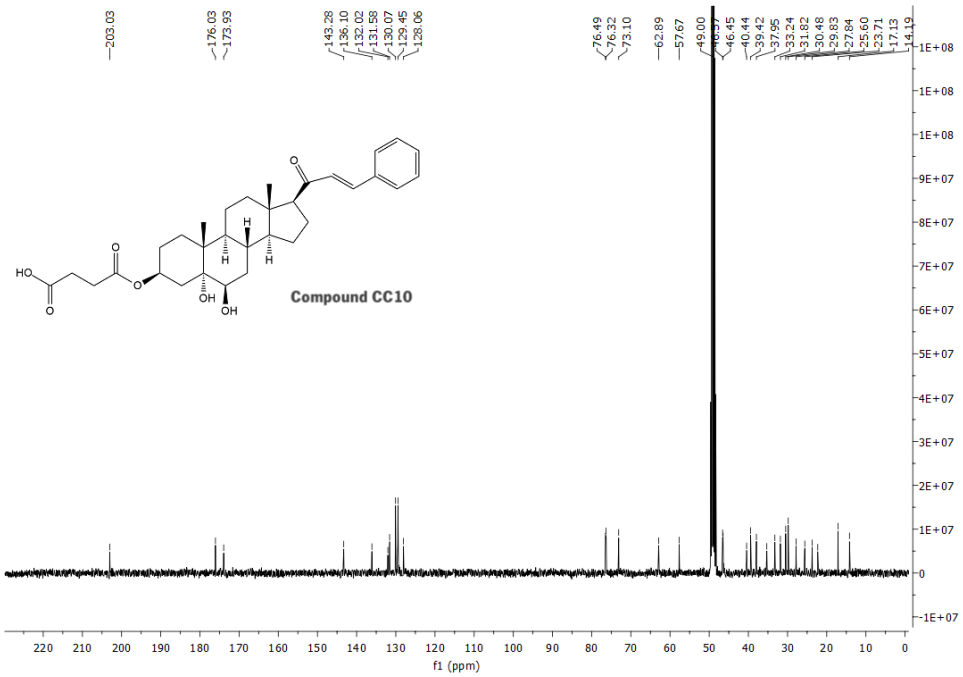

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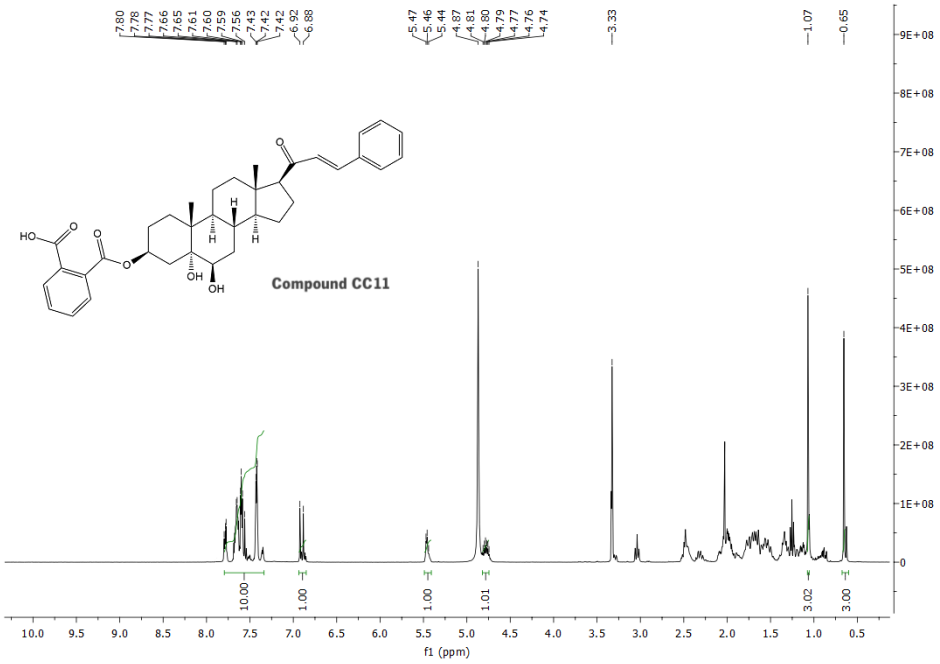

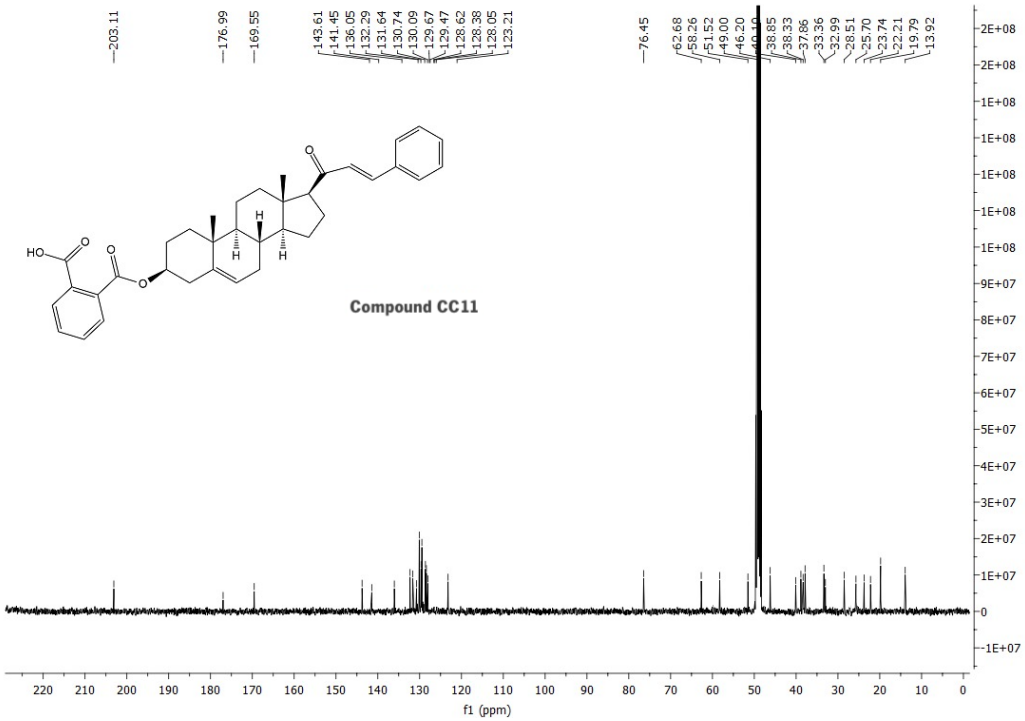

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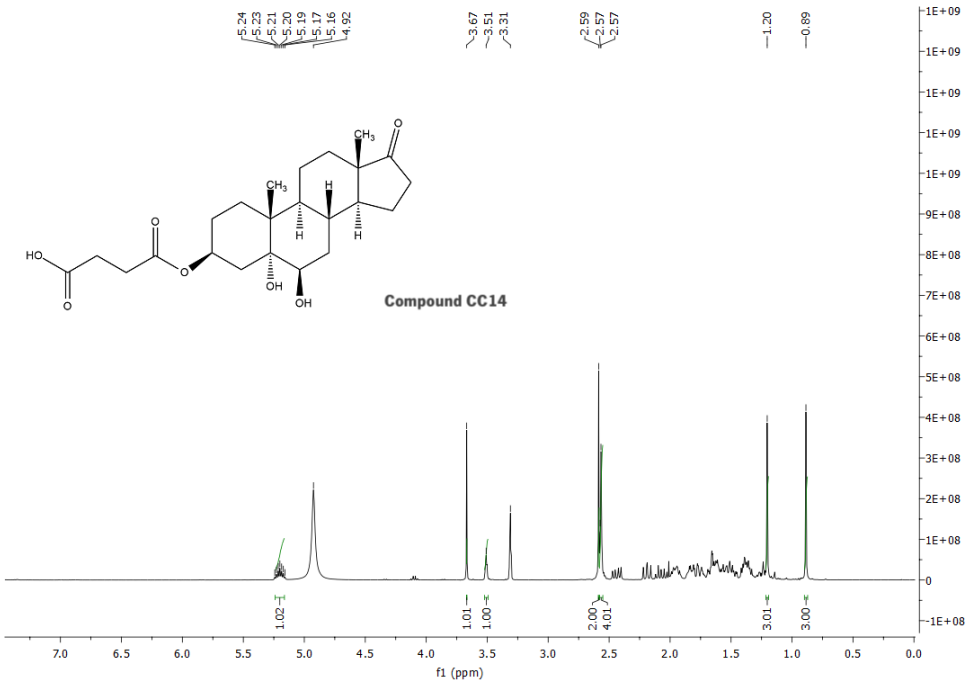

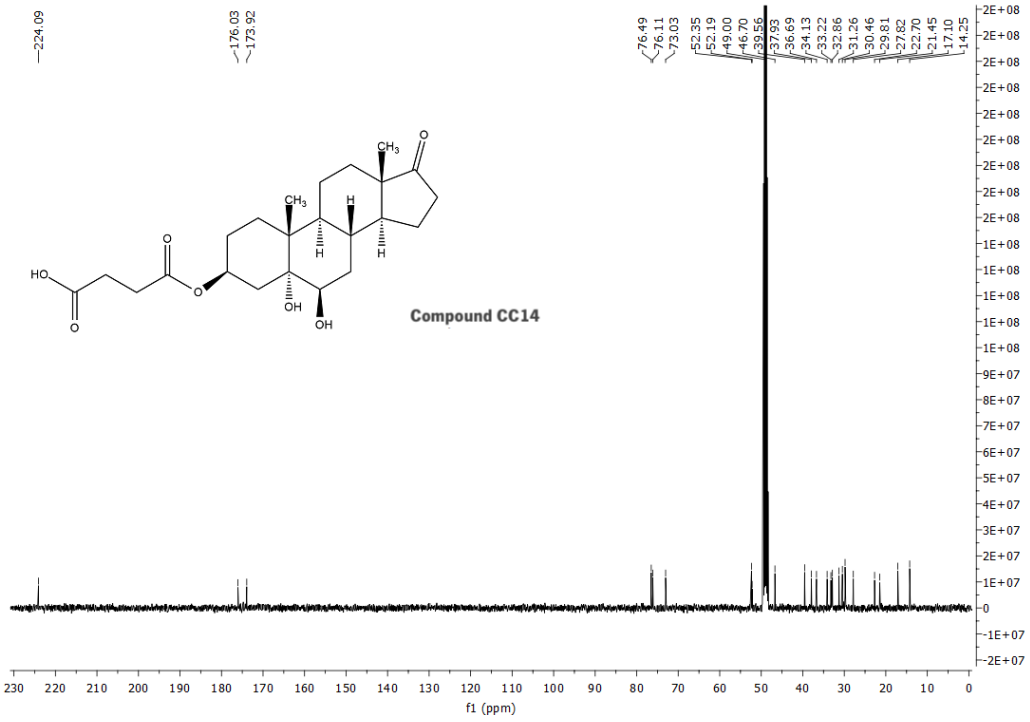

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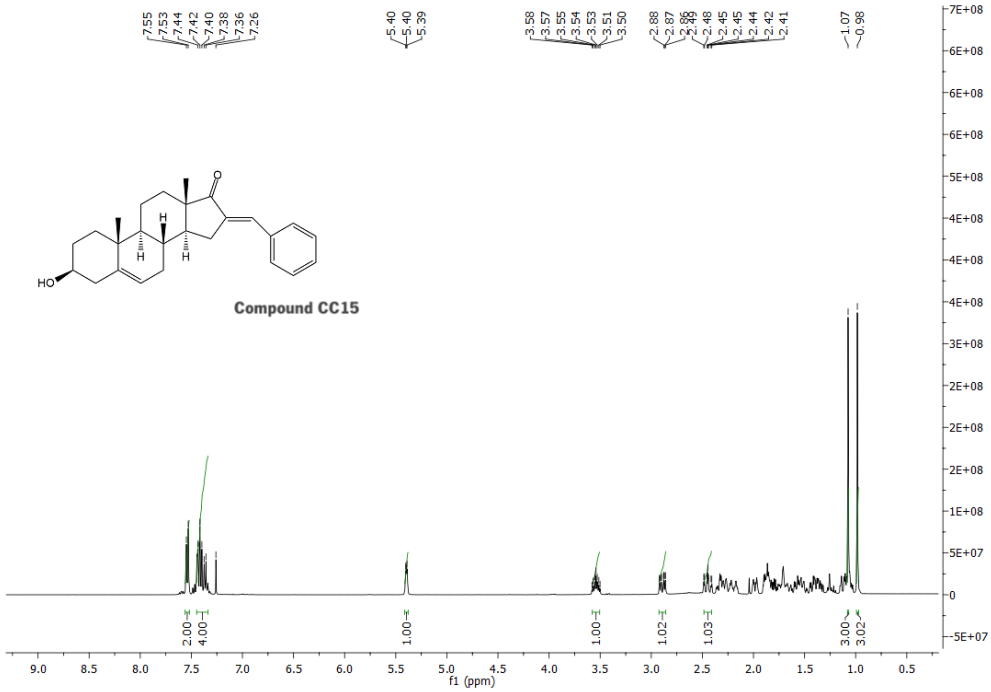

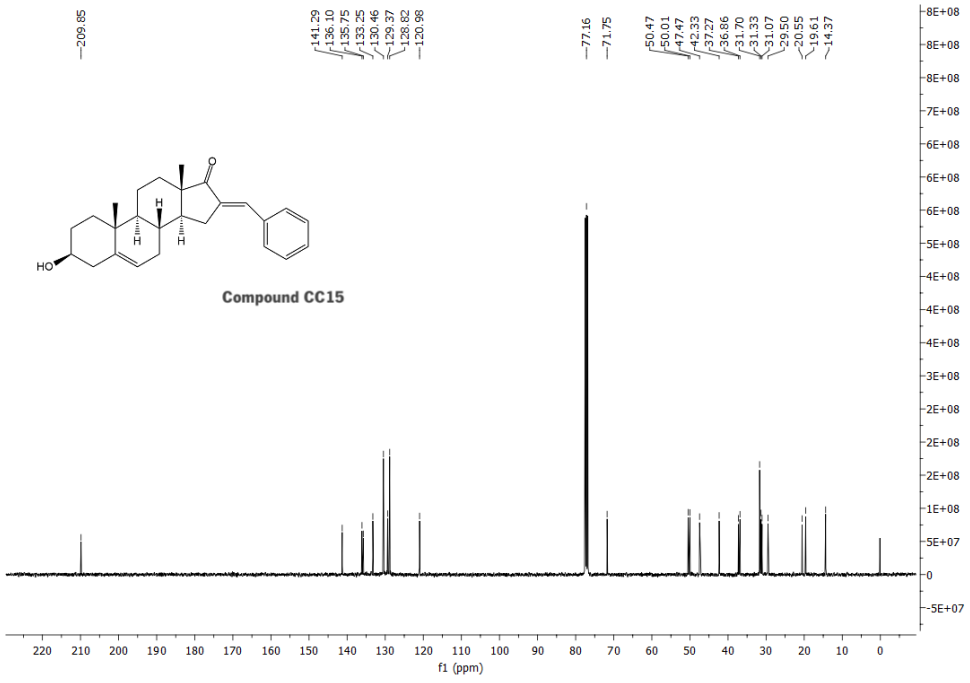

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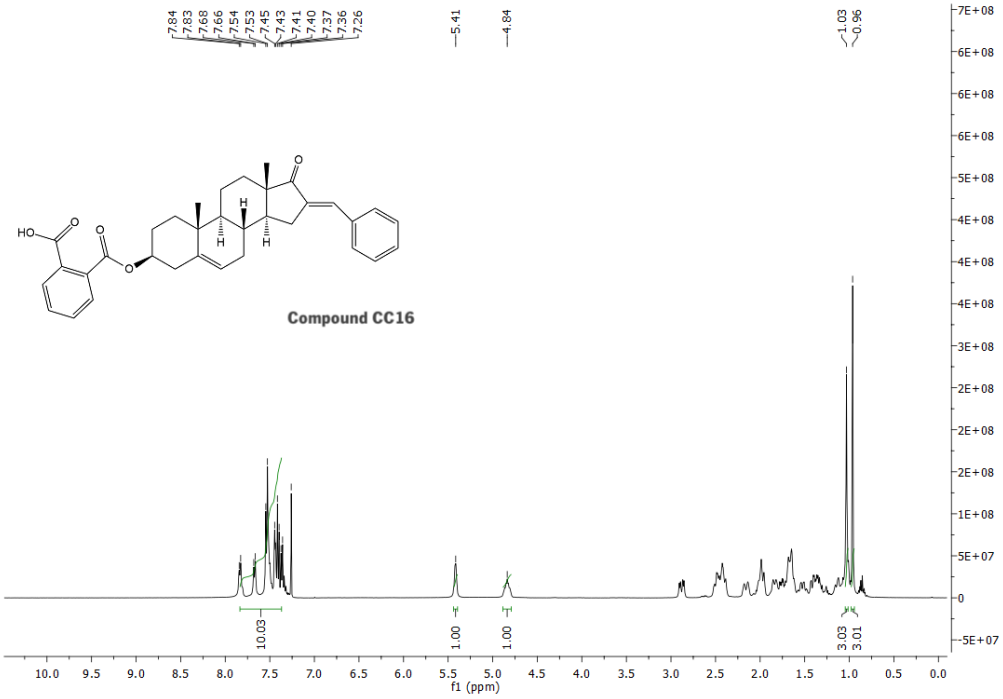

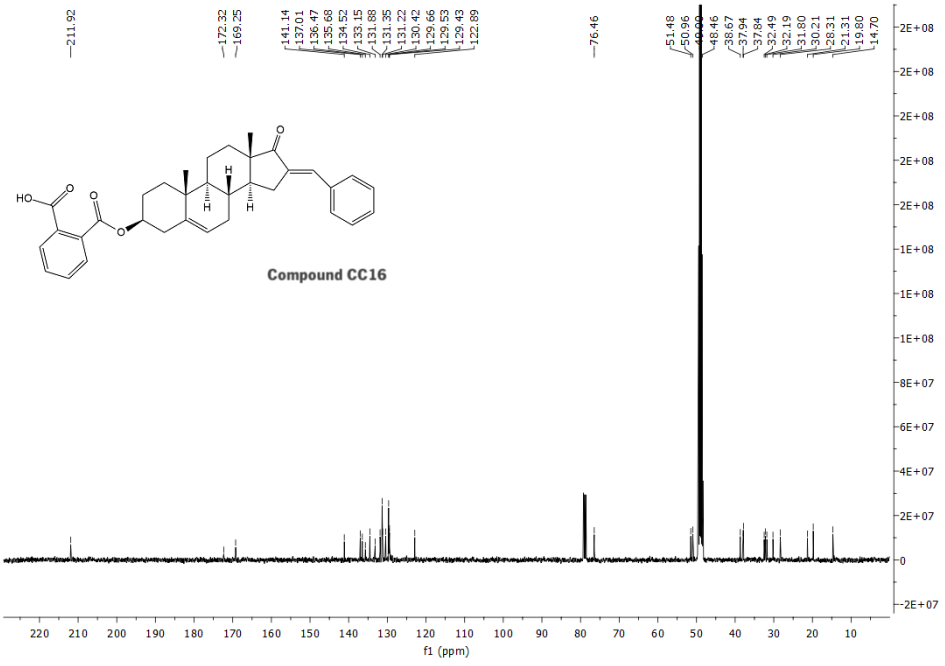

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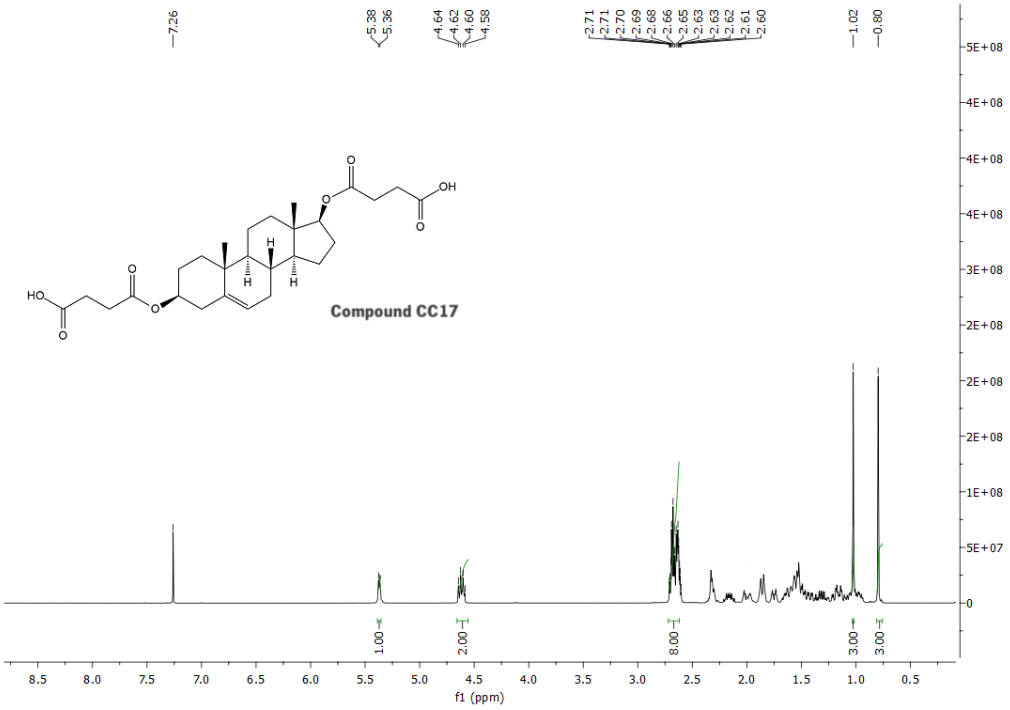

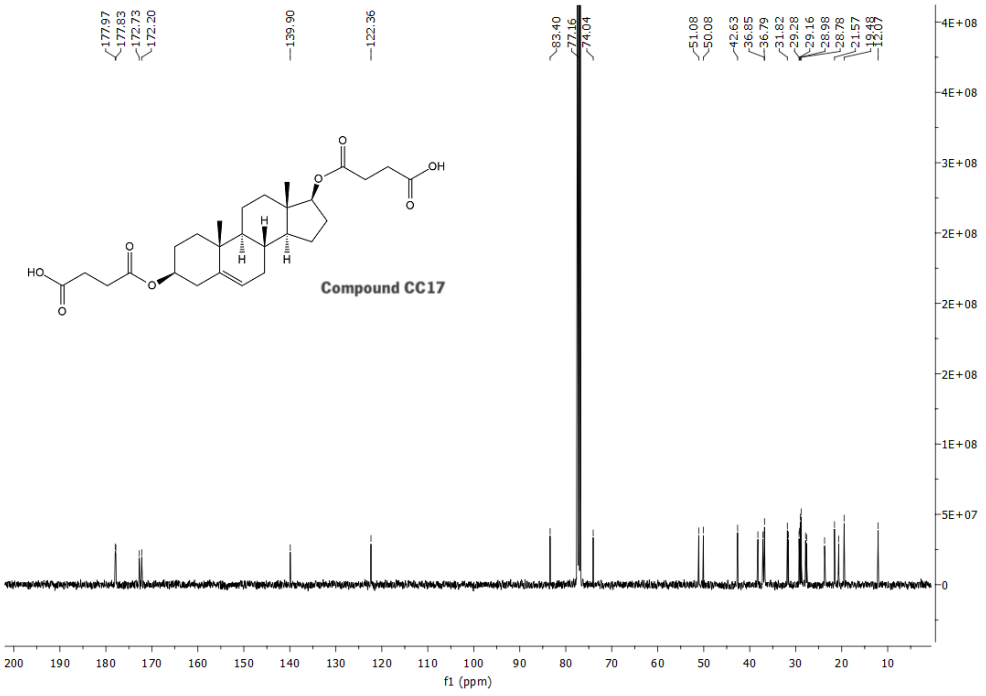

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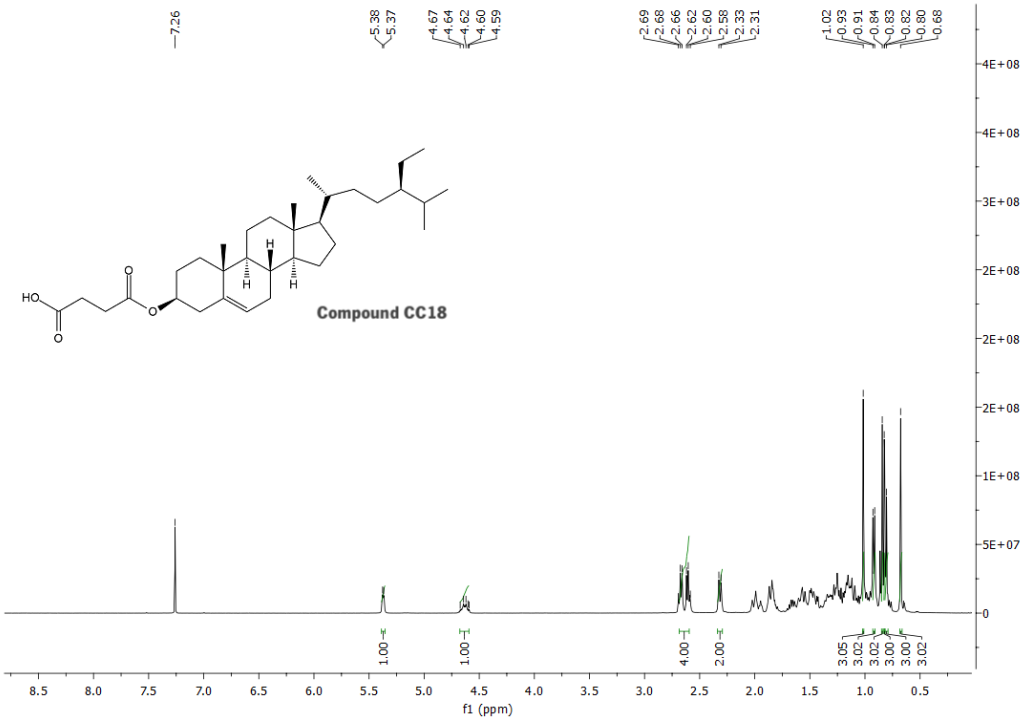

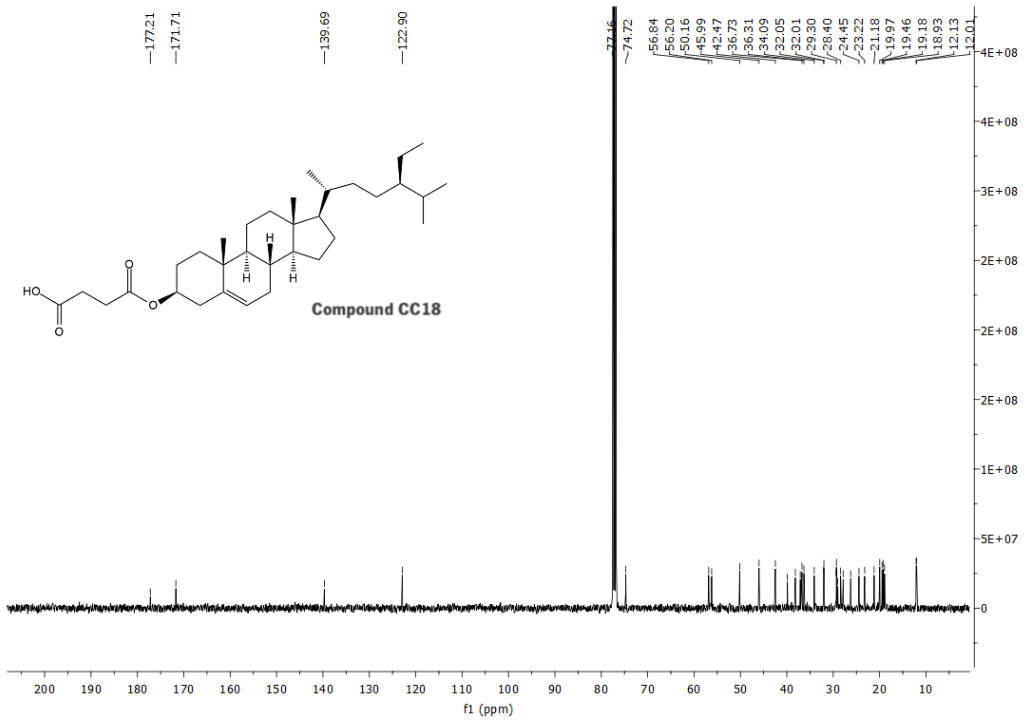

CHAPTER IV: GENERAL DISCUSSION

The data presented in this thesis addresses two complementary topics providing relevant advances to halt the progression of malaria worldwide. The first focuses on unveiling mechanisms involved in parasite drug resistance, leveraging the advances in *P. falciparum* genome editing tools. Via genomic epidemiology, we revealed a geographic selection and expansion of multiple *pfmdr1* copies encoding the N86F184 haplotype in Southeast Asia that, and through *P. falciparum* gene editing, we demonstrated to possess a higher transport capacity, leading to differential drug response. The second topic is based on exploring novel compounds, urgently in need to respond to the present ACT resistance reported in Southeast Asia and that will inevitably reach Africa where malaria exerts its heaviest toll. We explored a new class of promising antimalarial molecules, the steroids, involved in several pathways and with innumerous biological functions in order to unveil their potential to be used for malaria therapy. Our synthetic steroid derivatives revealed promising multistage antimalarial activity with high selectivity and did not demonstrate dependence on two key mediators of multidrug resistance in the parasite, the PfMDR1 and PfCRT. Exploring the underlying mechanisms of action, it was found that the synthetic steroids interfere with redox balance of the parasite. Therefore, this could be a promising strategy to eliminate multi resistant parasite that have different genetic backgrounds.

The topics addressed in this thesis – unveiling molecular mechanisms involved in resistance and the discovery of novel compounds for malaria treatment – have an undeniable relevance in the context of malaria disease. Drug resistance is a serious concern in malaria that hampers all efforts to eliminate the disease. The resilient ability of the parasite to acquire resistance to the antimalarials in current clinical use, led to the current worrying situation of lack of antimalarials ready to replace the already failing ACT. Acknowledging this situation, the global funding and research on this topic has been increasing, nevertheless, the number of new promising compounds is still low.

1. MOLECULAR MECHANISMS INVOLVED IN PARASITE RESISTANCE

Over the last years an impressive reduction of global malaria mortality and morbidity rates have been achieved mainly through the introduction and widespread adoption of ACTs together with other preventive measures including insecticide-treated bed nets (1). Despite this encouraging reduction, the emergence and spread of ART and ACT partner drug-resistant *P. falciparum* lines in Southeast Asia and South America represents a significant threat to malaria control and elimination. Resistance is defined as the ability of the parasite to multiply or survive after properly administration of the therapy. To avoid this, nowadays antimalarials are administrated in drug combination to increase efficacy and delay the development of resistance (2). Drug resistance in *P. falciparum* occurs due to multiple factors and

mechanisms, where the first step in its development has been described as an initial genetic event which is considered spontaneous and thought to be independent of the drug applied. Genetic events could be mutations or changes in the copy number of the genes that are targeted by the antimalarial drug or alterations in influx/efflux transporters proteins that affect the concentration of the drug near the target. In human infections, an average of 10^{9}-10^{13} parasites are present in the blood stream during the intraerythrocytic stage of the parasite life cycle and it is estimated that 1-9.7x10^{9} mutations occurred per base pair per generation (3, 4). Therefore, there is a high probability that a random mutation can occur leading to antimalarial resistance within a few cycles of replication. Subsequent selection of the specific mutation happens mainly due to a survival advantage during drug pressure. Other factors could also favor the selection of resistant parasites including increased levels of parasitemia and lower patient immunity. A repeated exposure of the parasites to residual antimalarial drug concentrations of the ACT partner drugs in areas of frequent infection is an important factor that also leads to the emergence of resistance (4). The levels of malaria transmission also can interfere with the development of resistance since populations in low-transmission settings, where infections are acquired occasionally, are more expected to be symptomatic and treated comparatively to those in high transmission settings. In high transmission areas, such as sub-Saharan Africa, the majority of the infections with *P. falciparum* are asymptomatic resulting in untreated cases and creating a constant reservoir and transmission. Additionally, in lower transmission areas populations have lower immunity and a higher parasite genetic variability has the opportunity to proliferate and also in these areas there is a less inter-parasite competition allowing lower fitness parasites to survive and these parasites could contain relevant mutation for drug resistance. These low fitness parasites have a window of opportunity to expand and be selected leading to an increase in malaria transmission of resistant parasites. On the other hand, in high transmission settings, a single infection is more likely to contain multiple genotypes and thus resistant parasites have to compete with wild-type parasites (4, 5). Taking this into account, identifying the genetic alterations that mediate antimalarial resistance is an important key to understand how the parasites evade treatments.

The emergence and spread of antimalarial drug resistance have been frequently detected, firstly in the Greater Mekong region of Southeast Asia. Initially, it is though that parasites from this region might have a hypermutable phenotype (6). However, *in vitro* studies did not find a higher mutation rate in parasites strains derived from this region (3, 7, 8). One possible explanation for this is that in Southeast Asia malaria transmission is seasonal and intermittent resulting in decreased host immunity. This factor can contribute to an increased propensity for drug resistance to appear. Furthermore, in the

Thai-Cambodia border, there is a significant amount of substandard medication and poor patient compliance leading to the emergence of drug resistance (5).

Therefore, unveil the mechanism involved in drug resistance becomes extremely important to avoid the spread and to guide the development of new therapeutic strategies for malaria.

Membrane transporter proteins are important players not only for the survival and multiplication during the parasite's life cycle but also for the development of parasite resistance. These proteins are important for the uptake of nutrients essential for parasite life cycle and for the removal of metabolic wastes. They also mediate the movement of metabolites between intra-parasitic compartments and are responsible for the regulation of ion composition of the host RBC, the cytosol and parasite organelles. Furthermore, they have been implicated in antimalarial drug resistance since they can provide a path for antimalarials to reach their therapeutic target as well as being potential drug targets (9). The two transporter proteins explored in this thesis, the PfMDR1 and PfCRT, are both present in the membrane of the DV of the parasite and promote the flux of solutes within this compartment and assume a crucial role in the drug resistance phenotypes. It is thought that PfMDR1 transports antimalarial drugs into the DV while PfCRT promotes the transport of drugs out of the DV (10, 11). These transporters are capable to eliminate the antimalarial drug from the place of action by preventing the drug accumulation or promoting drug elimination. In the acidic DV, proteases degrade Hb from the host RBC leading to the formation of reactive free heme which is detoxified through its conversion into the inert biocrystal, the Hz. Several antimalarial drugs act either by binding to this reactive heme or by inhibiting Hz formation, for instance CQ and ADQ, or in the case of ART that seems to be activated by the interaction with heme iron (12, 13).

However, mutations in DV transmembrane transporters, PfMDR1 and PfCRT, have been associated with *in vitro* and *in vivo* parasite susceptibility to these antimalarial drugs probably by affecting the transport (10, 11, 14–17). It has been described that there is a functional relatedness among these two transporters. This is evidenced by the linkage disequilibrium observed between certain combinations of *pfmdr1* and *pfcrt* (18, 19). For instance, association and linkage disequilibrium between PfCRT 76T SNP and PfMDR1 86Y SNP were determined in patients from Nigeria treated with CQ. The study demonstrated that 55 of the 111 *P. falciparum* isolates contained the mutant alleles, 76T and 86Y, and both was associated to *in vivo* CQ resistance. Furthermore, these two alleles were selected in patients who failed treatment and these alleles were in linkage disequilibrium (18). Another study reinforces the linkage through a genetic cross between CQ-resistant from South America (7G8) and Africa (GB4), showing that the South American *pfcrt* and *pfmdr1* alleles combined to exhibite

higher levels of resistance to md-ADQ (19). Indeed, this cross talk has been selected upon different ACT treatments since after ATM-LMF treatment parasites in reinfected patients containing the *pfmdr1* N86 and *pfcrt* K76 alleles and parasites harboring *pfmdr1* 86Y and *pfcrt* 76T alleles reinfected patients after AS-ADQ (20–22). These results indicated that the resistance to certain drugs is highly dependent on the joint contribution of the *pfcrt* and *pfmdr1* alleles found in these strains.

Studies of *pfmdr1* identified five global prevalent amino acids mutations being the N-terminal mutations, N86Y and Y184F, more frequent in Asian and African parasite lines (14). The worldwide spread of these mutations have been linked to differential responses to quinoline-based antimalarial drugs and also ART both *in vitro* and *in vivo* (14, 16, 17, 23, 24). Additionally, the amplification of *pfmdr1* is another relevant factor that impacts parasite susceptibility to several antimalarial drugs including MFQ and LMF (25–27). The *pfmdr1* CNV is a large tandem amplicon and in *P. falciparum* amplicon breakpoints in CNV are found in monomeric tracts of A or T in intergenic regions (28–30). However, some studies demonstrated that amplicons from Thai isolates were smaller, 14Kb, compared with some laboratory strains (100Kb) (31). Since the genome of *P. falciparum* is extremely rich in A/T nucleotides and has frequent A/T monomeric tracts, *pfmdr1* amplification is a crucial mechanism of evolutionary adaptation and resistance (32). Therefore, the conjugation of these two polymorphisms, gene amplification and specific haplotype, can modulate the parasite response to antimalarial drugs. Notwithstanding, in chapter II using data from MalariaGEN *P. falciparum* Community Project, the prevalence and distribution of parasites with *pfmdr1* with specific N-terminal PfMDR1 mutations was explored. Additionally, applying genome editing tools on a parasite strain derived from Southeast Asia we have evaluated the impact of these polymorphisms on solute kinetics of the PfMDR1 transporter. The open access of thousands of *P. falciparum* genomes, through the MalariaGEN *P. falciparum* Community Project, allowed us to explore the regional and temporal distribution of the conjugation of the two *pfmdr1* polymorphisms (CNV and SNPs) in study. We found that *pfmdr1* amplification, between 2002 and 2015, was frequent in Southeast Asia but a rare event in the African region. The extensive use of AS-MFQ and ATM-LMF as antimalarial therapy in Southeast Asia could be an explanation for this frequency during the time analyzed. These types of treatments have been strongly associated with parasites containing multiple copies of *pfmdr1* (25, 26, 33). Due to this extensive use, there was a change in the policy treatment and the adoption of DHA-PPQ as antimalarial treatment in this region (1, 2). This leads to the selection of parasites containing a single copy of *pfmdr1* (34, 35).

On the other hand, the rarity of *pfmdr1* amplification in the African region could be explained by the substantial use of AS-ADQ therapy contributing to its deamplification (36). Furthermore, although

ATM-LMF has been used for many years in Africa (37), the lack of MFQ usage could partially explain the absence of *pfmdr1* amplifications. Another relevant factor for the presence of a single copy of *pfmdr1* could be related with the fitness cost associated with amplifications (38). One of the most convincing demonstrations of the fitness cost associated with antimalarial drug resistance derived from clinical studies demonstrating that a discontinuation of CQ selective drug pressure allowed the reappearance of CQ-sensitive parasites demonstrating that despite the clear virulence associated to CQ-resistant parasites, the sensitive parasites have a strong fitness advantage (39). An example of fitness cost associated with *pfmdr1* amplification was detected *in vitro* in a Thai strain of *P. falciparum*. As described above, and confirmed with this study, decreased susceptibility to MFQ was related with increased *pfmdr1* copy number. Furthermore, when MFQ-resistant parasites (with approximately 2, 3 copies) were co-cultured with a parasite strain expressing a single copy of *pfmdr1* they presented a fitness disadvantage in the absence of MFQ selective drug pressure with an overgrowth of the single copy parasites over 3-4 weeks (40). Modelling predicted that under drug pressure *pfmdr1* amplification is a frequent event, which increases from one to two copies occurring once in every 10^8 parasites and from two to three copies one in every 10^3 parasites (40). Therefore, selective drug pressure is responsible for an increased copy number but this is limited due to fitness disadvantageous associated with gene amplification (38, 40). Since that *pfmdr1* codifies for an ATPase, this could explain that, in the absence of drug pressure, an increase in the expression of this gene will give a less favorable metabolic balance (40). This characteristic is particularly important in high transmission areas such as in sub-Saharan Africa, given that infections with several clones are common compared with low transmission areas like Southeast Asia. It is thought that parasite populations with several gene copies also contain a small fraction of parasites with a single gene copy and in the absence of drug pressure these two fractions will auto-compete and there will be a prevalence of parasite with a single gene copy (38, 40).

Additionally, in combination with *pfmdr1* amplification, a selection of the 184F allele with N86 allele was observed by analyzing the public database in Southeast Asia mainly in Cambodia. In order to understand the selection of this particular N86F184 haplotype in the context of *pfmdr1* amplified, we generated two edited parasite lines representing the major variants presented in Southeast Asia (N86Y184 and N86F184) and, the impact on antimalarial response and kinetic transport was studied.

To explore the PfMDR1 transport associated with each haplotype we used a previous surrogate assay using a Fluo-4 probe, to understand the impact of the N-terminal positions of PfMDR1. Fluo-4 fluorochrome is actively transported by PfMDR1 and can be quantitively evaluated by measuring its

accumulation inside the DV of the parasite. Therefore, this surrogate assay for PfMDR1 is an important approach to evaluate if different haplotypes, that have been associated with antimalarial drug resistance, could modulate the transport capacity (41, 42). This approach has been used previously for *pfmdr1* variants and revealed an important impact of the C-terminal amino acid 1042 on Fluo-4 transport (41, 42). In these studies, although they used strains encompassing different haplotypes regard to the N-terminal mutations, their direct impact was not evident. In our Fluo-4 accumulation study, the Dd2 strain and isogenic lines differing only in PfMDR1 residues at positions 86 and 184. In agreement with previous studies (41, 42), our gene edited lines and parental line had the ability to transport Fluo-4 and N86 allele by itself modulated Fluo-4 transport capacity. Previous studies using *Xenopus* oocytes provide direct evidences of the impact of N86Y mutation on PfMDR1 transport capacity to different antimalarial drugs. An alteration from N86 to 86Y allele in PfMDR1 leads to a loss of the ability to transport CQ and QN (43). Importantly, the 184F mutation also displayed an important role in the modulation of the transporter, since parasites harboring N86F184 haplotype accumulated more Fluo-4 inside the DV of the parasite accompanied by a faster kinetics of PfMDR1 transport activity comparatively with N86Y184 parasites. Indeed, structural models for PfMDR1 demonstrated that the residue 184 resides in the third transmembrane domain and it was hypothesized that 184F allele alters the parasite's response by an allosteric effect on transport kinetics independent from drug-binding capacity (44). These authors also suggested that PfMDR1 variant 86Y/Y184 relates to a PfMDR1 low performance quinoline antimalarial transporter. The inverse variant (N86F184) relates to a higher-performance PfMDR1 transporter (44), being coherently associated with its effects in terms of enhanced resistance to MFQ (25).

Consistent with these observations related with transport and in agreement with previous studies (14) we were able to confirm that edited parasites harboring amplified *pfmdr1* with N86 allele have an important role in the modulation of the parasite's response, since these parasites exhibited decreased susceptibility to MFQ, DHA and LMF and increased susceptibility to QN comparatively with the parental line harboring 86Y allele. This data also confirm genotyping and drug association studies using Asian isolates (16). Also, this is consistent with reports that showed the presence of parasites harboring the N86 allele after treatment with ATM-LMF and in recrudescence infections followed treatment with ATM-LMF (20, 22, 45).

The database analysis showed that in Southeast Asia, during 2002 and 2015, the N86 allele was predominant and remained constant compared with 86Y. One possible explanation for this profile could be related with the widely use of AS-MFQ treatment through Southeast Asia. Some studies already

demonstrated that the N86 allele *in vitro* was associated with higher IC_{50} of MFQ compared with the 86Y allele (14). A study with 54 parasites collected from the Western border of Thailand also demonstrated the same pattern (33), which could explain the overall rarity of the Y86 allele in this region. In Africa, almost all the countries rely on ATM+LMF as a ACT treatment and, according to the literature, this could lead to the selection of parasites with the N86 allele (22, 45, 46).

Furthermore, 184F also impacted the parasite response to antimalarial drugs. Indeed, parasites harboring N86F184 haplotype displayed a decrease in the LMF susceptibility comparatively with N86Y184 parasites. Previous studies demonstrated that selection of 184F has been reported after ATM-LMF treatment along with other *pfmdr1* alleles (22, 45). Despite, the 184F allele may not be the primary determinant of parasite resistance to all antimalarial drugs implemented to treat malaria, it could provide a complementary and advantageous genetic background that could help explain the observed selection in Southeast Asia.

The transport and parasite response results presented here illustrate the utility of the *in vitro* data validating susceptibility changes that could be correlated with alterations in clinical outcomes. Indeed, nowadays TACTs, that combine a triple drug regimen, culminated from in field observations but also from approaches like genome editing *in vitro*. This therapy it has been studied in order to protect and extend the useful therapeutic application of the current generation of antimalarial drugs. Nowadays, two different TACTs containing partner drugs with opposite resistance mechanisms have been studied for the treatment of *P. falciparum*: DHA-PPQ plus MFQ and ATM-LMF plus ADQ (47, 48). *In vitro* studies using gene editing are valuable in assessing if parasites can become resistant to all components and to understand if certain combinations are better than others to enhanced the therapeutic efficacy (49). TACTs approach considers the natural opposite genetic forced that were explored in this work, *pfmdr1* and *pfcrt*. TACTs have beneficial points firstly because ART resistance is not full resistant yet since parasites harboring K13 mutations are eliminated by ART albeit more slowly than wild-type parasites. Second, the partner drugs applied have opposite drug resistance mechanisms. The polymorphism in the transporter that mediate decreased sensitivity for instance to ADQ mediate the increase of susceptibility to LMF and MFQ (47). For example, LMF and ADQ exert opposite pressures on wild-type or mutant variants of both PfCRT and PfMDR1 (2, 20). On the other hand, PPQ and MFQ exert opposite pressures based on the *pfmdr1* amplification since this feature contributes to MFQ resistance and treatment failures.

In conclusion, and as was evidenced throughout this thesis, studies using genome editing could be relevant to help to change the policy treatments taking into account the prevalence of

polymorphisms involved in drug resistance for each endemic country. Moreover, gene editing approach, as the herein used beside dissecting the role of specific mutation in drug resistance, could be relevant to help design and develop novel antimalarial therapeutics that are independent of these multi resistant factors described here.

2. SYNTHETIC DERIVATIVES OF STEROIDS FOR MALARIA TREATMENT

As described in the above topic, resistance to almost all antimalarial drugs is a reality and the need for new drugs and novel approaches to treat malaria has become crucial and urgent. The history of antimalarial resistance allows us to learn lessons that can help the design of future therapies. There are several issues that could be taken into consideration when novel approaches or new future therapies are designed. Resistance appears rapidly when monotherapy is applied and therefore, combination therapies employing at least two drugs with distinct mechanisms of action helps mitigate this. When determining which partner drug to be used, drug pairs with a synergistic interaction should be used while avoiding a combination with unfavorable pharmacokinetics (5, 50). The generation of resistance to fast acting drugs such as ART is less expected to occur comparatively with slower-acting drugs, as already demonstrated for instance to MFQ (51). Drugs with several biological targets present a higher barrier to resistance compared with drugs with a single cellular target such as pyrimethamine (52).

As described in the WHO 2020 report (1), over the years there was an increase in the funding for drug research and development. Several organizations such as MMV joined with industrial and academic laboratories have as aim to identify novel promising compounds as candidates to treat malaria. There are several criteria that these compounds should fulfill in order to obtain the best possible candidates that can replace current ACTs in the future. Some of these criteria include the high potency against more than one *P. falciparum* life cycle stage; they should have high potency against field isolates from areas where resistance was already detected and should not present cross-resistance against adapted parasite strains that are resistant to antimalarial drugs currently in use. Furthermore, it is also important to determine how quickly resistance *in vitro* is acquired and to determine the fitness cost associated to this resistance mutation (53).

Some promising compounds with novel cellular targets have been discovered and identified and they are already in clinical trials (54–56). Nowadays, approaches to obtain *in vitro* resistant parasites combined with whole genome analysis allow the identification of possible cellular targets involved in the activity of new compounds through the comparison of polymorphisms acquired by the resistant clones and the parental clone. Indeed, a systematic analysis of the genetic alterations was conducted in

response to 37 new compounds with potent antimalarial activity (52). The study revealed that, between all of the tested library, 6 compounds acquired not only single nucleotide polymorphisms but also harbored an amplified *pfmdr1*. Additionally, *pfcrt* mutations were also detected after exposure to to these compounds in study (52). Another study screened 50 antimalarial compounds against specific resistant clones comprising pre-existing resistance alleles associated with *pfmdr1* and *pfcrt*. The study demonstrated that 2 small molecules reduced their efficacy when tested in parasite clones containing several copies of *pfmdr1* (51). These results highlight the important role of these two transporters, which were explored in this thesis, in the development of resistance even for new candidates. Indeed, our results suggests that our synthetic compounds are not dependent on PfMDR1 and PfCRT and this could be advantageous in terms of a delay in the resistance development or used in combination with present antimalarials.

Natural compounds have long been used for malaria therapy and two good examples of this include ART and QN which were isolated from natural sources and, after some chemical modifications, they are now part of current therapies (57). However, natural products are often complex structures and exhibit problems regarding supply. Seasonal or environmental changes or loss of the natural source through extinction or legislation restrictions, complexity of the mixtures after fractionation, the isolation of very small quantities of bioactive pure substance and challenging physicochemical properties such as solubility and stability are some drawbacks that this type of compounds shows (58). Target identification strategies, complemented with the availability of *P. falciparum* genome and medicinal chemistry strategies, did not lead to the expected increase in the number of new antimalarial drugs and therefore, it is plausible that new drugs will be identified from natural sources (56, 59). Due to their chemical structures, the presence of multiple stereocenters and flexible conformations, natural products could be considered as good starting materials for the development of novel compounds to treat malaria.

Most antimalarial candidates isolated from natural sources show moderate activity or contain challenging biological or physicochemical properties, being only considered as a starting material rather than the final drug candidate. This type of compounds usually needs to be structurally optimized to obtain a promising compound. For instance, modification of ART to AS allowed a decrease in dose, and more consistent bioavailability. Additionally, the optimization from QN to CQ is another example that gave a significant improvement in the frequency of administration (57). Possibly, optimization processes need to be performed in almost all natural compounds to obtain a promising antimalarial compound.

The potential combination of current approved antimalarial drugs with bioactive compounds derived from natural sources has been described. One example is the combination of febrifugine and isofebrifugine (isolated from the leaves of Hydrangea macrophylla) with CQ that demonstrated to be more potent in *in vivo* assays compared with the individual uses of the compounds (60). More recently, a compound isolated and purified from *Solanum nudum*, the 16α-acetoxy-26-hydroxycholest-4-ene-3,22-dione, had a synergistic effect when combined with CQ and QN (61).

Between all the natural compounds that have been explored over the years, we focused our phenotypic screening in the steroids class. Steroids are a class of tetracyclic chiral natural compounds that occur in animal, fungi and plants having crucial endocrine roles and increasingly recognized therapeutic value (62). The potential of these compounds has been explored over the years and several synthetic steroids displayed relevant activities in other type of diseases such as cancer. They presented potent cytotoxicity against human cancer cell lines with high selectivity (63, 64). Furthermore, some studies have been exploring the potency of natural occurring steroids and synthetic compounds containing the steroid framework to be used in malaria therapy (61, 65–69). There are some reasons that explain why this type of compounds could be attractive scaffolds to formulate novel therapeutic approaches in malaria. They have completely different chemical entities compared with the current ACTs. Additionally, these molecules possess a framework that allows the placement of chemical substituents in different positions of the tetracyclic framework or in the lateral chain. There is a great structure variability since different tetracyclic frameworks, such as androstanes, cholestanes and pregnanes can be found in Nature (70). Furthermore, due to the lipophilic character of the steroid molecule it could be considered to mediate the cellular uptake since these compounds can pass the lipophilic membranes either through free diffusion or endocytic processes (71–74). In agreement with this, recently one study demonstrated that the presence of the lipophilic steroid framework is crucial for the antimalarial activity since a higher antimalarial activity was observed when compared with non-steroid derivatives analogs (68). So, all these features can explain the potential of these compounds and an optimal functionalization could lead to the development of promising antimalarial candidates. Indeed, recently several synthetic arylmethylamino steroids were designed and they displayed a potent antimalarial activity *in vitro* and *in vivo* (68). Reviewing the literature until now, the most potent and the best characterized synthetic steroid, regarding its bioactivity, pharmacokinetic and pharmacodynamics belongs to this library of compounds (68). However, despite their potent *in vitro* and *in vivo* efficacy, these compounds possess a functional group attached to the steroid framework that is also present in a current antimalarial drug implemented to treat malaria, ADQ, and this could lead to a rapid acquisition

of resistance. One advantage of our synthetic compounds compared with the work referred above (68) is that besides having a steroid scaffold they also contain functional groups that have yet to been used in current antimalarial drugs, which could lead to different molecular targets inside the parasite. In chapter III, after phenotypic screening, it was observed that the synthetic compounds with acetate and phthalate functional groups in the same structure (compound CC1 and CC6) afforded the most promising antimalarial activity against the blood stage of the parasite life cycle with a high selectivity for malaria parasites. A change in the lateral chain induced an impact in antimalarial activity since a significant increase in IC_{50} was observed when the same functional groups attached were used in diosgenin (compound CC6) compared with cholesterol (compound CC1). This suggests that the lateral chain itself can modulate the parasite response. In agreement, this evidence was also reported by other authors where estratriene derivatives showed higher antimalarial activity than the cholane derivatives (68).

One disadvantage that our compounds present is related with lipophilicity and solubility since these are two important features to enhance the druggability of the compounds. Lipophilicity is an important physicochemical parameter that plays a relevant role to determine the ADMET (absorption, distribution, metabolism, excretion and toxicity) properties of a compound. Often in the early stages of development the molecules presented high lipophilicity. This results in compounds that have a high rate of metabolism, poor solubility and low absorption compromising the bioavailability (75). Some synthetic compounds designed in this work displayed a high partition coefficient (logP) and this issue needs to be addressed in future optimizations. Polar surface area (PSA), defined as the sum of surface contributions of polar atoms (usually oxygens and nitrogens) in a molecule, is a measure that has been correlated with passive molecular transport through membranes and, therefore, allows the prediction of transport properties of drugs (76). The PSA value was calculated and compound CC1 presented a value lower than 140 indicating that predictably our compound has a good capacity for penetrating cell membranes. In terms of pharmacokinetics parameters, compound CC1 was predicted to have poor absorption in the intestine but interestingly, it is not expected to inhibit some cytochrome P450 enzymes such as cytochrome P450 2D6 (CYP2D6), CYP1A2, CYP2C9 and CYP2C19, that are important enzymes responsible for the drug metabolism (77). This data must be carefully intertwined since it is an *in silico* prevision. As mentioned above, some groups have been studying the antimalarial activity of natural and semisynthetic compounds but little information regarding the pharmacokinetic and pharmacodynamics is available. However, recently a research group evaluated, *in vitro* and *in vivo*, some pharmacokinetic parameters. They found that, although steroidal compounds have a poor

aqueous solubility, they have a promising stability in plasma and moderate microsomal stability (68). All this data can suggest that our compounds are promising drug candidates and have relevant features for their *in vivo* applicability. Further chemical optimization should be done and the addition of optimized functional groups can lead to more potent molecules to treat malaria.

In this study, we also explored possible biological pathways by which our best compound might affect the parasite. We observed that our compound interferes with the glutathione redox homeostasis and with the metabolic pathway that lead to the degradation of hemoglobin and formation of hemozoin. Indeed, previous studies also demonstrated that synthetic steroids and plant steroids could have these two processes as mechanism of action (68, 78). The study concerning synthetic steroids showed that the presence of a steroid moiety favored the physiochemical properties needed to interfere with hemozoin formation, thus increasing the antimalarial activity of the steroid compounds over non-steroid counterparts (68). As referred above the metabolic degradation of Hb and Hz formation occurs inside the DV. Some approved antimalarials to treat malaria also interfere with this metabolic process, which is extremely important for the growth and proliferation of the parasite. This metabolic process has been described as a valuable target since no changes have been described and Hz is a specific molecule to the parasite (79, 80). Rather, as described in chapter I and II, resistance to these antimalarial drugs arises from mutations and alterations in expression levels of two transporters referred in this thesis located in the DV membrane, PfMDR1 and PfCRT (2, 79). Therefore, promising results were obtained for our compound since CC1 also interfere with Hz formation and proved to be possibly independent of these two transporters highlighting the importance of our compounds. The oxidative stress detected after exposure to our compounds has also been considered a relevant mechanism of action since could lead to damage of molecules such as lipids, proteins and nucleic acids (81). Therefore, dissecting the effect of compounds in the parasite biological pathways is a crucial information to formulate combination therapies. The combination therapies should take advantage of synergistic interactions aiming to increase therapeutic efficacy and decrease the risk of resistance emergence. If combination drugs have an antagonist behavior, the efficacy might be compromised and the probability of developing resistance and spread increases, since this antagonism may allow resistant clones to survive and be transmitted (50). It is difficult to predict *in vivo* drug interactions in a human host using only *in vitro* studies, however this could be an important predictor for the potential of our synthetic compounds. Combining our compound with MFQ or LMF led to an *in vitro* synergistic effect independent on the drug concentration mix used, hypothetically revealing a possible future good combination. Interesting, contrarily to MFQ and LMF, where their potency is mediated by PfMDR1 and PfCRT proteins (14, 21,

82), our molecule does not seem to exhibited this dependency, leading possibly to an advantageous combination. Interestingly, our compound exhibited an antagonist effect when combined with 4-aminoquinolines suggesting that both classes of compounds could be possibly competing by the same target, for instance free heme or Hz or, hypothetically, they can establish a chemical interaction with each other and therefore lead to structural changes that prevent their connection to the target.

Overall, the results from chapter III suggested that a steroid moiety is a relevant starting material for the design of a novel class of antimalarial compounds and an optimization of the functional groups attached could lead to the formation of potent compounds that could be used, for instance, in combination therapies.

3. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Altogether, the results obtained in this thesis contribute in a relevant way for the two most important problems related with control and elimination of malaria disease: drug resistance and lack of new therapies to replace the current failing ACTs.

In the chapter II, the approach of reverse genetics led to the development of genetic tools useful for future validation of other possible molecular markers of resistance. Providing this knowledge, ahead of the true development of clinical failure, strengthens the importance of molecular epidemiology studies, giving an opportunity for a change of the policy treatment, based on molecular facts, leading to a rational use of the available ACTs in order to preserve their efficacy and increase the clinical success rate. Furthermore, the PfMDR1 being a well-known multidrug resistance player, the genetic tools developed have also a great potential to support the design of new molecules with antimalarial activity and validate novel targets inside the parasite. Finding new compounds through phenotypic screening, the strategy used in this thesis that led to the disclose of, for instance CC1, will demand future steps to find their cellular target. So, reverse genetics strategies, as the herein developed, will also benefit this phenotypic screening once a hit target is disclosed. Furthermore, for drug discovery through target-based screen, the gene editing has also an important role to validate the target by, for example, designing different parasite lines harboring specific mutations on the target. The results obtained with these specific parasites can also allow future optimization of the desired molecule.

One of the desired characteristics of the next generation of antimalarials compounds is their multistage activity behind the activity at the symptomatic stage of the disease. Over the years novel laboratorial assays have been developed in order to obtain gametocytes *in vitro* since this stage of the

parasite, although asymptomatic, is responsible for the transmission. However, the acquisition of gametocytes *in vitro* revealed to be challenging and time consuming, requiring the application of several stimulant stress factors (a drop in hematocrit, spent media and higher parasitemia) (83). After the unveiling the genes responsible for gametocytogenesis (84), techniques based on genome editing have recently been developed, that have facilitated this differentiation *in vitro* (85, 86). These techniques rely on conditional knockdown of essential proteins involved in the differentiation of the intraerythrocytic parasites to gametocytes. Being the genetic systems previously developed a costly assay, we are presently improving it, making use of a riboswitch-based inducible gene expression system (87), to create a *P. falciparum* line capable to control gametocytogenesis, developing fluorescent-tagged gametocytes that will allow a faster and less costly approach to determine drug potency in gametocytes.

The optimized and developed methods presented in this thesis will thrive future studies in *P. falciparum,* routed to understand changes into the parasite biology and physiology, either due to changes into its genome or due to external factors such as the action of a killing drug as the herein explored steroid based compounds. The data presented in this thesis leaves no doubts that steroid scaffold exploitation could be an attractive field to explore in the design of new interventions to tackle malaria disease. Further studies will be necessary to optimize the functional groups attached in the steroid moiety to enhance the potency of the herein synthesized compounds against the blood stage of the parasite life cycle. To obtain a final molecule for development, extensive SAR is needed and an improvement in lipophilicity and ADME properties including solubility are features to take in consideration. The next step after optimization of our compounds include an in-depth *in vivo* analysis of pharmacokinetics, pharmacodynamics and toxicity. For instance, to determine the metabolic stability *in vitro* microsomal analysis could be applied since the compound could be exposed to species-specific liver microsomes that contained cytochrome P450s, carboxyl-esterases, flavin-monooxygenases and other drug-metabolizing enzymes. Hence, it would be interesting to evaluate the possibility of these compounds to act in other stages of the parasite's life cycle, including the liver stage. Another relevant issue that needs to be addressed is related with acquisition of resistance to these new compounds. One major concern about these new compounds is whether resistant parasites can be obtained. Compounds resulting in rapid resistance acquisition are not ideal for clinical development. In this line, "forcing" the development *in vitro* steroid-resistant parasites will be useful to address this question. Developing such parasite lines, not only will allow to study possible mechanisms of resistance but could also reveal some path towards disclosing the mechanism of action.

Considering the resilient capacity of the *P. falciparum* malaria parasite to acquired resistance, with PfMDR1 being a well-known player of resistance, novel antimalarials with new targets and mechanisms of action, or compounds with ability to inhibit the protein transporters involved in resistance, should be considered to replace or to be added in combination with the current antimalarials. A multiple drug approach is required, to further decrease the changes of new resistance pathways to be activated leading to higher drug efficacy success. As natural compounds possess completely unrelated chemical entities compared with ACTs this could lead to a promising strategy in the future to tackle this worldwide devastating disease.

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