Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

Study of antimalarial activity of new endoperoxides: potential parasite targets and mechanisms of action

Lis Tavares Coelho Lobo

DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS BIOMÉDICAS, ESPECIALIDADE BIOLOGIA CELULAR E MOLECULAR

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Resumo

Estudo da atividade antimalárica de novos endoperóxidos: potenciais alvos parasitários e mecanismos de ação

Lis Tavares Coelho Lobo

A malária é uma das doenças parasitárias mais prevalentes no mundo, causando um grande impacto no desenvolvimento socioeconómico dos países afetados. Esta doença afeta principalmente a África Subsaariana, mas continua presente em 87 países de regiões tropicais e subtropicais da América do Sul e Sudeste da Ásia. O surgimento e propagação de resistências do *Plasmodium falciparum* à terapia combinada à base de artemisinina (ACT) no sudeste da Ásia acentuou a necessidade de desenvolver novos antimaláricos e/ou medicamentos baseados no farmacóforo endoperóxido.

Neste contexto, uma biblioteca quimicamente diversa de endoperóxidos foi projetada e sintetizada. Foram analisados 36 compostos relativamente à atividade antimalárica *in vitro* e *in vivo*, utilizando, estirpes de *P. falciparum* e modelos murinos resistentes e sensíveis a antimaláricos. Foram explorados possíveis mecanismos de ação através de ensaios de ação específica do estágio do parasita, de alteração do potencial de membrana mitocondrial, e de medida de espécies reativas de oxigênio (ROS), contra estirpes de *P. falciparum* resistentes e sensíveis à artemisinina. A citotoxicidade foi avaliada em linhas celulares de mamífero V79 e HepG2 e a genotoxicidade foi avaliada em linhas celulares V79, utilizando o ensaio cometa. Também foram avaliadas a atividade gametocitocida e o risco de desenvolvimento de resistência dos novos endoperóxidos.

O presente trabalho descreve a avaliação da atividade antimalárica dos 36 novos compostos endoperóxidos dos quais o farmacóforo é parte de uma porção trioxolano (ozonídeo) ou tetraoxano, flanqueados por um grupo adamantilo e um anel ciclohexilo substituído. Oito compostos exibiram atividade antimalárica sub-micromolar (IC_{50} < 71,1 nM), ausência de resistência cruzada com artemisininas (ARTs) ou antimaláricos de base quinolínica, mostrando citotoxicidade e genotoxicidade negligenciáveis em células de mamífero. Estes compostos evidenciaram um impacto relevante no potencial de membrana mitocondrial do parasita e induziram a formação de ROS. Destes, seis produziram sobrevida em estádio de anel <1% contra a estirpe resistente IPC5202 e três

deles suprimiram totalmente a parasitémia por *P. berghei* em ratinhos, após administração oral. Estes três compostos apresentaram também elevada atividade gametocitocida.

Dentre os compostos investigados, os conjugados trioxolano-tetrazol LC131 e LC136 emergiram como potenciais candidatos antimaláricos por apresentar: toxicidade negligenciável em relação às células de mamíferos, atividade sub-micromolar sobre estádios assexuados e gametócitos de *P. falciparum*, supressão total da parasitémia *in vivo* (modelo murino *P. berghei*) e baixo risco de seleção de parasitas resistentes.

Palavras-chave (3-5): *Plasmodium*, Resistências, Endoperóxidos, Atividade antimalárica, Mecanismos de ação.

Abstract

Study of antimalarial activity of new endoperoxides: potential parasite targets and mechanisms of action

Lis Tavares Coelho Lobo

Malaria is one of the most feared parasitic diseases in the world, causing a major impact on the socio-economic development of the affected countries. This disease affects mainly the sub-Saharan Africa but remains in 87 countries from tropical and subtropical regions of South America and Southeast Asia. The emergence and spread of *Plasmodium falciparum* resistance to artemisinin-based combination therapy (ACT) in Southeast Asia prompted the need to develop new endoperoxide-type drugs.

A chemically diverse library of endoperoxides was designed and synthesized. 36 compounds were screened for *in vitro* and *in vivo* antimalarial activity using, respectively, the SYBR Green I assay and a mouse model. Possible mechanisms of action were identified, by using parasite-stage specific action assays, investigating alterations of mitochondrial membrane potential with rhodamine 123 and measuring reactive oxygen species (ROS) with CM- H_2DCFDA. The studies were conducted with artemisinin-resistant and artemisinin-sensitive *P. falciparum* strains. Cytotoxicity was evaluated against mammalian cell lines V79 and HepG2, using the MTT assay, and genotoxicity was evaluated against V79 cell lines, using the comet assay. The gametocytocidal activity and the evaluation of risk for resistance were also evaluated.

The synthesis and antimalarial activity of 36 new endoperoxide-derived compounds is reported, where the peroxide pharmacophore is part of a trioxolane (ozonide) or a tetraoxane moiety, flanked by adamantyl and a substituted cyclohexyl ring. Eight compounds exhibited sub-micromolar antimalarial activity (IC_{50} 0.3–71.1 nM), no cross-resistance with artemisinin or quinolone derivatives and negligible cytotoxicity and genotoxicity towards mammalian cells. These compounds had an impact on the mitochondrial membrane potential of the parasite and induced the formation of ROS. From these, six produced ring stage survival < 1% against the resistant strain IPC5202 and three of them totally suppressed *Plasmodium berghei* parasitaemia in mice after oral administration. These three compounds have also shown high gametocytocidal activity.

From the compounds investigated, trioxolane–tetrazole conjugates LC131 and LC136 emerged as potential antimalarial candidates; they show negligible toxicity towards mammalian cells while exhibiting ability to kill intraerythrocytic asexual and sexual stages of *P. falciparum*, low risk to developing resistance and capacity to totally suppress *P. berghei* parasitaemia in mice.

Key words (3-5): *Plasmodium*, Resistance, Endoperoxides, Antimalarial activity, Mechanisms of action.

Abbreviations

ACTs – Artemisinin-based Combination Therapies

AE – Arteether

AMQ – Amodiaquine

ART – Artemisinin

ART-R – Artemisinin resistant strain

ART-S – Artemisinin sensitive strain

ATM – Artemether

ATQ – Atovaquone

CHO-9 – Chinese hamster ovary cell line

CQ – Chloroquine

CQR – Chloroquine resistance

DCF – 2′,7′- dichlorofluorescein

DHA – Dihydroartemisinin

dhfr – Dihydrofolate reductase

dhps – Dihydropteroate synthase

DHODH – Dihydroorotate dehydrogenase

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DTP – Diphtheria, tetanus and pertussis (vaccine)

DV – Digestive vacuole

EDTA – Ethylenediamine tetra-acetic acid

EMA – European Medicines Agency

ETC – Electron transport chain

FBS – Fetal bovine serum

FDA – Food and Drug Administration

FP – Ferriprotoporphyrin IX

FV – Food vacuole

G6PD – Glucose-6-phosphate dehydrogenase

GFP – Green fluorescent protein

GHIT – Global Fund for Health Innovation Technology

GLU – glutaraldehyde

GMS – Greater Mekong Subregion

GPx – Glutathione peroxidase

GSH – Glutathione

GSK – GlaxoSmithKline

GST – Glutathione S-transferases

GWS – Genome-wide studies

H_2O_2 – Hydrogen peroxide

Hb – Hemoglobin

HDP – Heme detoxification protein

HEPES – N-(2-Hydroxyethyl) piperazine-N-(2-ethanesulfonic acid)

HepG2-A16 – Hepatocellular carcinoma cells

HZ – Hemozoin

IC_{50} –Inhibitory dose for 50% of the parasites

iRBCs – Infected red blood cells

IPT – Intermittent preventive treatment

IPTp – Intermittent preventive treatment in pregnancy

LD_{50} – Lethal dose for 50% of the cells

LDH – L-lactate dehydrogenase

LU – Lumefantrine

MDA – Malondialdehyde

MDR – Multidrug resistance proteins

MEF – Mefloquine

MIR – Minimum inoculum for compound resistance

MMV – Medicines for Malaria Venture

MSA – Mature-stage survival assay

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAC – N-acetyl cysteine

NBCS – Newborn calf serum

NK – Natural killer cells

PBS – Phosphate buffered saline

$PC_{1/2}$ – Parasite clearance half-life

PF – Paraformaldehyde

pfcrt – P. falciparum transporter gene

Pfgdv1 – P. falciparum gametocyte development 1

pfmdr1 – P. falciparum multidrug- resistance gene

Pkrx – Plasmoredoxin

PPQ – Piperaquine

PQ – Primaquine

Prx –Peroxiredoxins

PV – Parasitophorous vacuole

PVM – Parasitophorous vacuole membrane

PYR – Pyrimethamine

QN – Quinine

RBCs – Red blood cells

RDT – Rapid diagnostic test

Rh123 – Rhodamine 123

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

RPM – Revolutions per minute

RPMI 1640 – RPMI medium for *P. falciparum*

RSA – Ring-stage survival assay

SCGE – Single cell gel electrophoresis

SI – Selectivity index

SMC – Seasonal malaria chemoprevention

SNPs – Single nucleotide polymorphisms

SOD – Superoxide dismutase

SP – Sulfadoxine-pyrimethamine

SP-IPTi – Intermittent preventive treatment in infancy with sulfadoxine-pyrimethamine

SP-IPTp – Intermittent preventive treatment in pregnancy with sulfadoxine-pyrimethamine

SYTO^{TM} 61 – Red fluorescent nucleic acid stain

TI – Therapeutic index

TESs – Therapeutic efficacy studies

TGR – Trx- glutathione reductase

Trx – Thioredoxin

TrxR – Thioredoxin reductase

UPR – Unfolded protein response

uRBCs – uninfected red blood cells

V79 – Chinese hamster lung fibroblast cells

WHO –World Health Organization

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I – INTRODUCTION

I.1 – Malaria

Malaria is one of the most important parasitic diseases in the world, causing a major impact on the socio-economic development of the affected countries. This disease affects mainly sub-Saharan Africa but continues to be present in 91 countries from tropical and subtropical regions of South America and Southeast Asia. Due to global warming, there is a possibility of its resurgence in regions where it has been controlled or eliminated (WHO, 2017).

Human malaria is caused by five species of parasites of the genus *Plasmodium*: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* (with two distinct subspecies, *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri*), *Plasmodium malarie* and *Plasmodium knowlesi*). However, a recent outbreak of human malaria caused by *Plasmodium simium*, originating from monkeys, occurred in the Atlantic Forest of South and Southeastern Brazil between the years 2015 and 2016 (Brasil *et al.*, 2017). Human malaria is transmitted by the bite of an infected female mosquito of the genus *Anopheles*. There are about 400 different species of *Anopheles* mosquitoes, but only 30 of these are vectors of major importance. *P. falciparum* and *P. vivax* malaria pose the greatest public health challenge (WHO, 2015). *P. falciparum* is the most prevalent species in sub-Saharan Africa and is responsible for more than 90% of malaria-related deaths (WHO, 2017), worldwide and in sub-Saharan Africa. Outside of Africa, *P. vivax* is the predominant parasite in the Americas, causing 64% of malaria cases, whereas in South-East Asia and the Eastern Mediterranean regions it only accounts for 30% and 40% of malaria cases, respectively (WHO, 2017).

Malaria symptoms include high fever, headache, chills, sweating, general malaise and vomiting, symptoms that usually appear between 7 and 15 days after the mosquito bite. *P. falciparum* is responsible for the most severe forms of the disease, including severe anemia, coma, and death (Idro, Jenkins and Newton, 2005). However, *P. vivax* infection is far from benign, recent studies have reported life-threatening consequences, including acute respiratory distress syndrome, cerebral malaria, multi-organ failure, dyserythropoiesis[1] and anemia (Dayananda, Achur and Gowda, 2018). *P. vivax* and *P.*

[1] Dyserythropoiesis is the defective development of erythrocytes.

ovale may retain latent forms in the liver - hypnozoites, with the possibility of later recurrence of the disease, and therefore require specific treatment with tissue schizontocides such as primaquine, tafenoquine and atovaquone (Orjuela-Sánchez *et al.*, 2009; Maneeboonyang *et al.*, 2011).

P. vivax has a wider geographical distribution than *P. falciparum.* Although *P. vivax* can occur throughout Africa, the risk of infection with this species is quite low in the region because of the absence in many African populations of the Duffy gene, codding a protein necessary for invasion of red blood cells by *P. vivax* (Mendes *et al.*, 2011; WHO, 2015).

In addition to high mortality rates, malaria is also responsible for high levels of morbidity among affected populations and is closely associated with weakening of economic development in endemic countries (Sachs and Malaney, 2002). The main factors contributing to the difficulty of malaria control are related to: development of antimalarial drug resistance by *Plasmodium* spp., namely *P. falciparum*; limitations to the use of insecticides; and economic and social factors, which are also occurring with *P. vivax* (Hupalo *et al.*, 2016; Dayananda, Achur and Gowda, 2018).

I.1.1 – Global epidemiology of malaria

Of the 91 countries and territories with malaria transmission in 2015, 40 are estimated to have achieved a reduction in incidence rates of 40% or more, between 2010 and 2015. Malaria mortality rates are estimated to have declined by 62% globally, between 2000 and 2015, and by 29% between 2010 and 2015 (Figure I.1) (WHO, 2016). These reductions are mainly due to the control of the mosquito vector and the use of fast-acting artemisinin-based combination therapies (ACTs) (Haldar, Bhattacharjee and Safeukui, 2018).

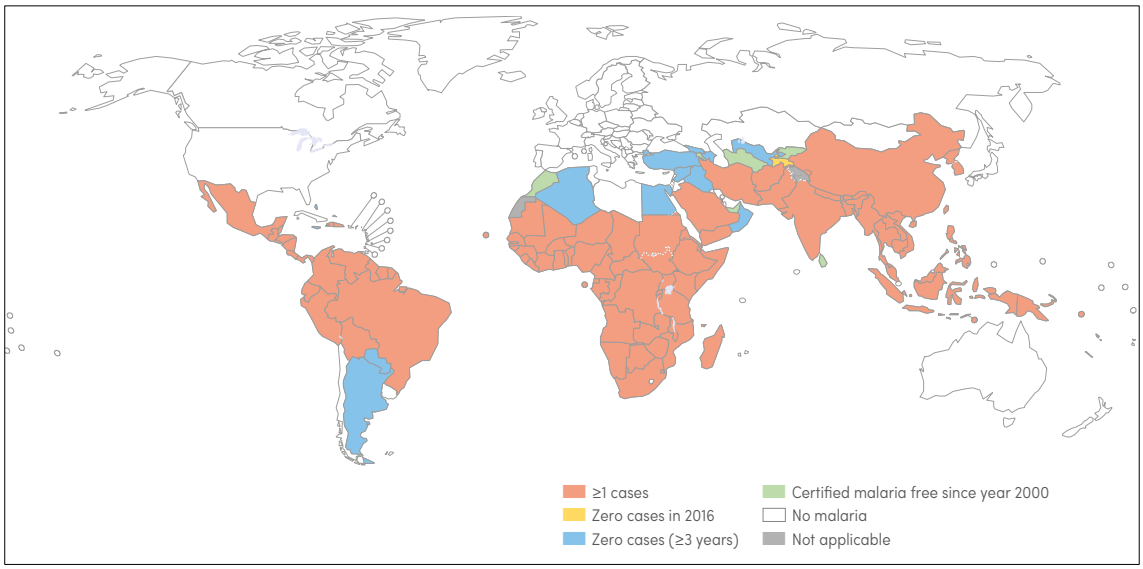


Figure I.1: Countries and territories with indigenous cases in 2000 and their status by 2016. Countries with zero indigenous cases over at least the past 3 consecutive years are eligible to request certification of malaria free status from World Health Organization (WHO). All countries in the WHO European Region reported zero indigenous cases in 2016. Kyrgyzstan and Sri Lanka were certified malaria free in 2016 (Adapted from (WHO, 2017)).

Despite the overall reduction observed between 2014 and 2016, there was a substantial increase in the incidence of malaria cases in the American region, and marginally in the regions of Southeast Asia (namely the Western Pacific) and Africa. Factors that may have contributed to this increase include: worldwide economic downturn, climate change, difficulties in elimination of *P. vivax* malaria, development of pyrethroid resistance by anopheline mosquitoes, and the emergence of artemisinin (ART) resistance by *P. falciparum* in Southeast Asia (White *et al.*, 2014; WHO, 2017; Woodrow and White, 2017).

In 2016, there were an estimated 445 000 deaths from malaria worldwide, compared to 446 000 estimated deaths in 2015. 91% of these occurred in Africa, followed by the Southeast Asian region (6%). 80% of global malaria deaths in 2016 occurred mainly in sub-Saharan Africa, and marginally in India (WHO, 2017).

Between 2010 and 2016, all regions recorded reductions in mortality, except the Eastern Mediterranean region, where mortality rates remained almost unchanged over the same period (WHO, 2017).

Malaria was eradicated from Europe in the 1970s through a combination of insecticide spraying, drug therapy, and environmental engineering. Since then, it has been mostly imported into the continent by international travelers and immigrants from endemic regions (Piperaki and Daikos, 2016). However, after several decades of endemic malaria eradication, some European countries such as Italy, Germany, Spain, and France, have reported cases of autochthonous transmission (Alten, Kampen and Fontenille, 2007). More recently, in 2012, Greece faced its first outbreak of malaria in this century. Therefore, despite the absence of indigenous cases of transmission in 2015, the possibility of a resurgence of malaria outbreaks in Europe remains (Gomes *et al.*, 2016; Salvi, 2016).

In Portugal, the status of malaria before the 1950s is well documented in the studies that identified 6 malariologic regions. These regions revealed different levels of endemicity, which are largely associated with the suitability of the habitat to the mosquito vector *An. atroparvus* (Cambournac, 1942; Capinha *et al.*, 2009). In 1973, after an intensive work of the national authorities, the indigenous strains of malaria parasites were considered eradicated from Portugal by the WHO. Entomological studies on *An. atroparvus*, a widely dispersed native mosquito, has demonstrated that it has competence in transmitting imported strains of *P. falciparum* (Sousa, 2008). Moreover, due to strong historical and cultural affinities, Portugal has a close relationship with an important number of malaria-endemic countries such as Angola, Mozambique or Brazil (Santos *et al.*, 2012). Travelers arriving from these regions provide a regular number of imported infections, ultimately contributing to the risk of indigenous malaria transmission.

I.1.2 – *Plasmodium spp.* life cycle

Plasmodia are unicellular and haploid protozoans during most of their life cycle. The diploid phase occurs for a brief period of the cycle in the invertebrate host: the mosquito.

The new taxonomic classification of *Plasmodium* genus places it in the Alveolate group, which joins a recently recognized group of unicellular eukaryotes, including different protists such as Dinoflagellates, Ciliates and Apicomplexa parasites, in which it is inserted (Adl *et al.*, 2012).

The taxonomic classification of *Plasmodium falciparum*: Group: Alveolata, Subgroup: Apicomplexa, Class Hematozoa, Order Haemosporida, Family Plasmodiidae, Genus *Plasmodium*.

Plasmodium spp. life cycle includes two phases: the sporogonic phase, with multiplication in the definitive host, invertebrate (genus *Anopheles*); and the schizogonic phase, with multiplication in the intermediate host, vertebrate (*Homo sapiens*) (White *et al.*, 2014; Mahmoudi and Keshavarz, 2018).

The cycle is initiated when an anopheline mosquito, taking its blood meal, injects *Plasmodium* sporozoites into the skin of the host. After minutes, sporozoites invade liver cells via the bloodstream, where they replicate and divide as merozoites inside the hepatocytes. At the end of their developmental process in the liver, *Plasmodium* parasites differentiate into merozoites, which are contained inside host cell-derived vesicles called merosomes that are transported away of the hepatocytes and eventually rupture in the lung microvasculature (Sturm *et al.*, 2006; Silvie *et al.*, 2008). The merozoites enter the bloodstream, where they invade the red blood cells and initiate the asexual stages as rings, trophozoites and schizonts (schizogony), which is the symptomatic stage of the disease. *P. vivax* and *P. ovale* may develop latent hepatic forms, the hypnozoites (Delves *et al.*, 2012; Mahmoudi and Keshavarz, 2018). The replication cycle of the merozoites within the red blood cells lasts between 24 to 72 hours, depending on the species. In the case of *P. falciparum,* it lasts for 38 to 48 hours.

For the purpose of the present work, we will focus on *P. falciparum*.

Merozoites released from red blood cells invade other red blood cells and, at some point, they differentiate into male or female gametocytes that are then taken up by female mosquitoes during a blood meal. Inside the mosquito midgut, female gametocytes mature into a macrogamete and male gametocytes undergo exflagellation[2] to release the microgametes (Baker, 2010). The male and female gametocytes fuse, forming the diploid invasive zygote – ookinete, that traverses midgut epithelial cells and transforms into the

[2] Exflagelation is the extrusion of rapidly waving flagellum-like microgametes from microgametocytes; in human malaria parasites, this occurs in the blood meal taken by the anopheline vector within a few minutes after ingestion of the infected blood by the mosquito.

- oocyst. Sporozoites mature inside the oocysts, and move from the mosquito midgut to the salivary glands (Kori, Valecha and Anvikar, 2018). Thus, 10 to 14 days after the feed on blood containing gametocytes, the mosquito is, in theory, able to transmit malaria parasites to another human host (Figure I.2) (Josling and Llinás, 2015; Phillips *et al.*, 2017).

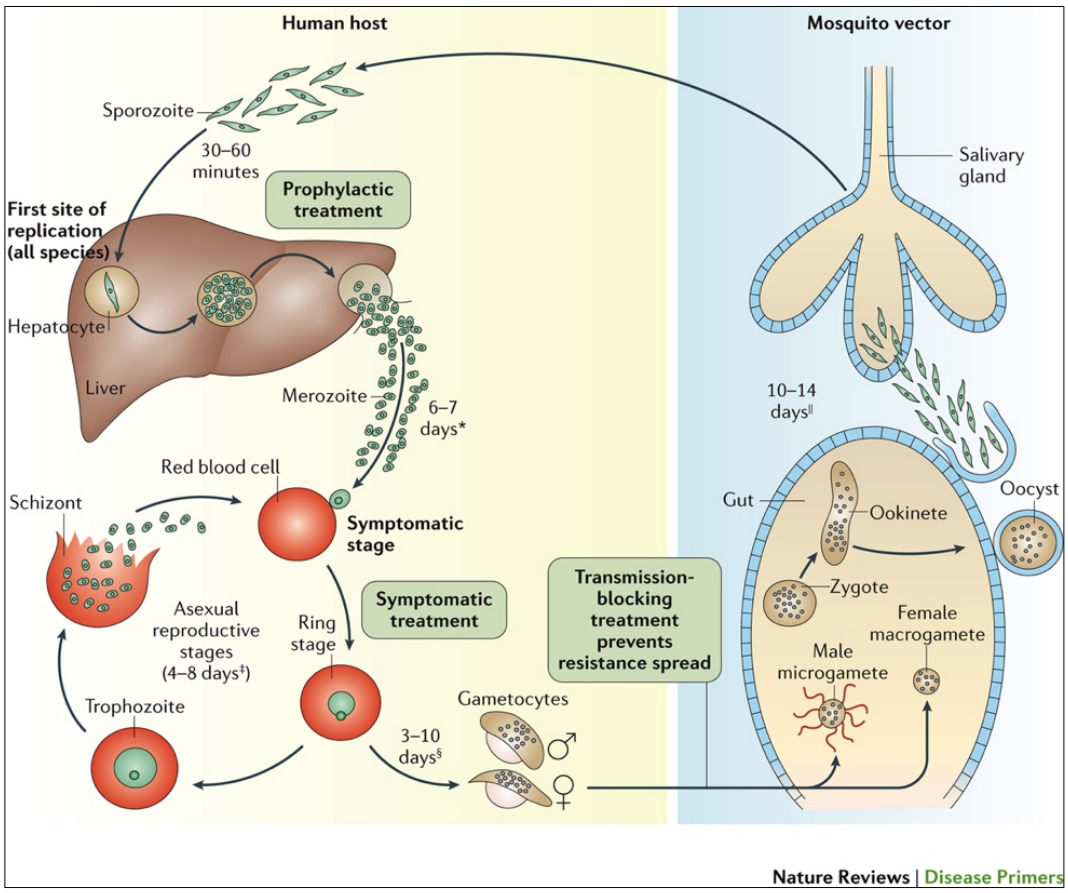


Figure I.2: The *Plasmodium* spp. life cycle. (Adapted from (Josling and Llinás, 2015)).

I.1.3 – Biological features of *Plasmodium falciparum* with implications in drug development

a) Intraerythrocytic asexual stages

When released into the blood, merozoites invade the erythrocytes. Invasion of the erythrocyte by merozoites occurs rapidly (≅ 2 min) (Weiss *et al.*, 2015). Once inside the

erythrocyte, the parasite develops within the parasitophorous vacuole (PV), the membrane surrounding the merozoite that is originated from the erythrocyte and designated the parasitophorous vacuole membrane (PVM) (Cowman *et al.*, 2016, 2017).

Once inside the host erythrocyte, the parasite digests most of the erythrocyte's cytoplasm (highly rich in hemoglobin) in the digestive vacuole (DV) (Figure I.3). The digestion process begins with the formation of the cytostome, an invagination of the PVM, forming vesicles of pinocytosis which carry the erythrocyte cytoplasm. These vesicles fuse with the DV, where they release their contents (mostly hemoglobin). In the DV, proteases degrade hemoglobin (Rosenthal and Meshnick, 1996). Hemoglobin consumption provides a source of amino acids, releases space for growth, and generates osmolytes that maintain osmotic pressure of the erythrocyte (Lew, Tiffert and Ginsburg, 2003; Bakar *et al.*, 2010). Most of this proteolysis occurs during the trophozoite stage, when parasites ingest large quantities of hemoglobin from the erythrocyte into the acidic DV (Bakar *et al.*, 2010; Rosenthal, 2011). However, hemoglobin degradation is a metabolic process that generates large amounts of ferroprotoporphyrin IX (FP) and reactive oxygen species (ROS) (Fu *et al.*, 2010).

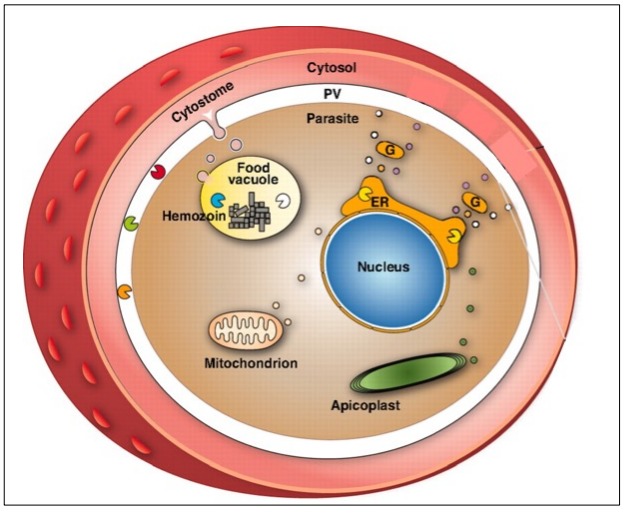


Figure I.3: Structure of the merozoite inside the host erythrocyte. ER: endoplasmic reticulum; PV: parasitophorous vacuole; G: Golgi complex (Adapted from (Kappe *et al.*, 2010)).

Normally, most of the released FP is polymerized into crystalline hemozoin (≅ 90%) (Egan *et al.*, 2002), but a significant amount escapes polymerization and has to be detoxified in the cytoplasm (Zhang, Krugliak and Ginsburg, 1999). Free FP can interact with phospholipid membranes, causing structural defects due to the reactivity of its attached Fe^{3+} with unsaturated membrane lipids. This can lead to increased membrane permeability for ions, cell swelling, and lysis (Famin, Krugliak and Ginsburg, 1999; Zhang, Krugliak and Ginsburg, 1999).

Once the erythrocyte infection is established, over the subsequent 48h cell division (schizogony) results in 16 to 32 merozoites that egress when mature, resulting in destruction of the erythrocyte membrane and explosive release of parasites to access new host cells for invasion (Figure I.4) (Cowman *et al.*, 2016).

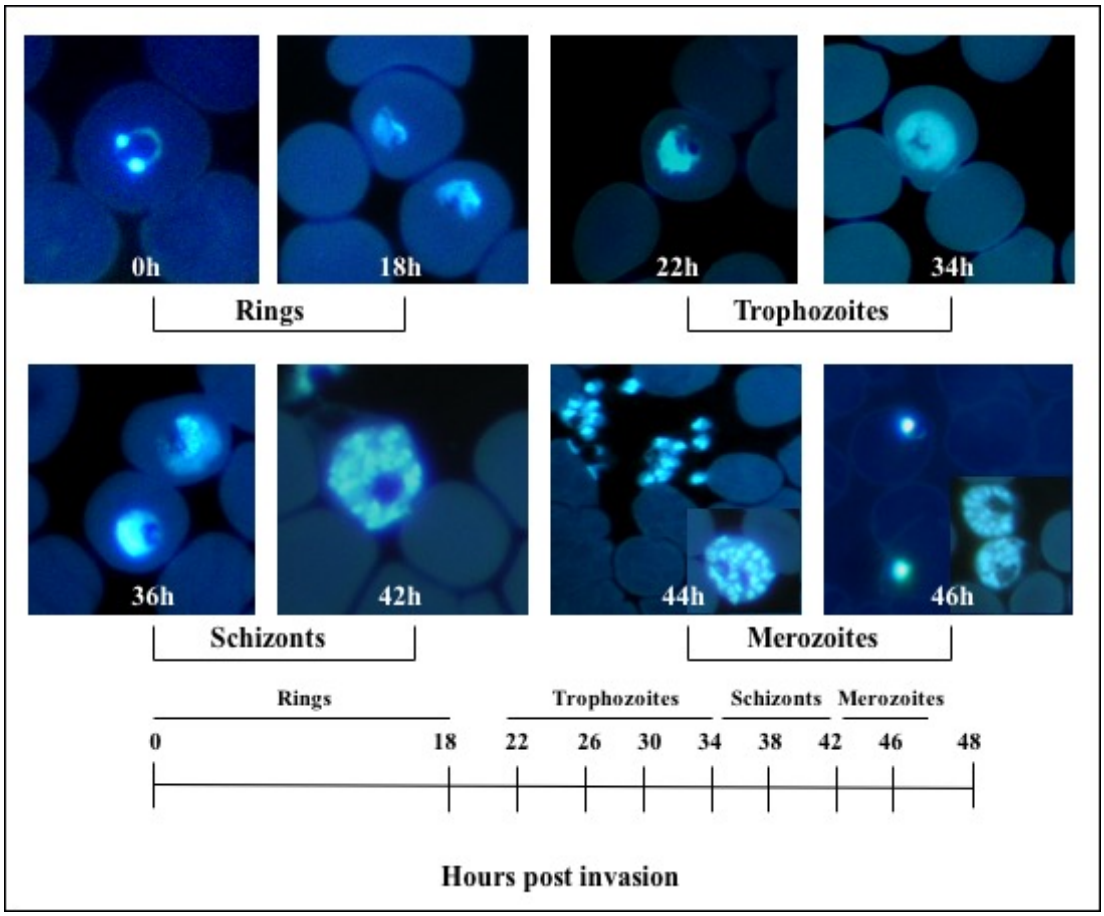


Figure I.4: Intraerythrocytic asexual stages of *P. falciparum*. Parasites were stained with DAPI (4', 6-diamidino-2-phenylindole) and photographed under UV light by fluorescence microscopy (magnification 100X with immersion oil) (Adapted from (Nogueira and Rosário, 2010)).

b) Sexual stages - Gametocytes

The gametocyte is the specialized cell in the transmission between the human host and the anopheline mosquito. For adaptation in such different environments, many changes occur in their cell biology, gene expression, metabolism, and protein synthesis (Talman *et al.*, 2004). Within each replication cycle, a small proportion (0.1% – 5%) of asexual parasites develop into male and female sexual stages - gametocytes (Meibalan and Marti, 2017).

Gametocytogenesis and modulation of gametocyte production in a natural infection is influenced by host environmental factors, including stress induced by host immunity, high parasitaemia, antimalarial drug treatment, or anemia, as well as host genetic factors such as human hemoglobin variants, indicating that parasites may somehow sense their environment (Bousema and Drakeley, 2011). In addition, cell-to-cell communication is also known to increase production of gametocytes (Mantel *et al.*, 2013). *P. falciparum* gametocytes take 8 to 10 days to mature into five morphologically distinct phases (stages I – V) (Meibalan and Marti, 2017) (Table I.1). *P. falciparum* stage I gametocytes are round compact forms containing hemozoin. Thus, microRNAs from sickle cell erythrocytes have been associated with increased gametocyte numbers (LaMonte *et al.*, 2012) while on the parasite side, genes such as *P. falciparum* gametocyte development gene 1 (*Pfgdv1*) have been implicated in the control of sexual differentiation (Eksi *et al.*, 2012).

Table I.1: Morphology of gametocytogenesis. (Adapted from (Talman *et al.*, 2004)).

	Stages				
	I	II	III	IV	V
Time of appearance (days)	0 – 2	1 – 4	2 – 8	6 – 10	9 – 23
Shape (light microscopy)	-Indistinguishable from the small round trophozoite -Larger round shape, distinguished by the granular distribution of pigment in food	-Elongates within the erythrocyte -D shaped	-D shaped, slightly distorted erythrocyte -The pink/blue distinction of the male/female	-Elongated and thin parasite, distorted red cell -Male: pigment tends to be scattered -Female: denser pigment	-Sausage shaped parasite with rounded extremities -Male: pigment scattered, pink -Female: dense pigment, light violet

Immature *P. falciparum* gametocytes, stages I - IV (Figure I.5), are sequestered away from the bloodstream to the bone marrow (Joice *et al.*, 2014). Once mature gametocytes are released into the bloodstream, stage V (Figure I.5), they are accessible to mosquitoes through a blood meal (Bousema and Drakeley, 2011; Cowman *et al.*, 2016).

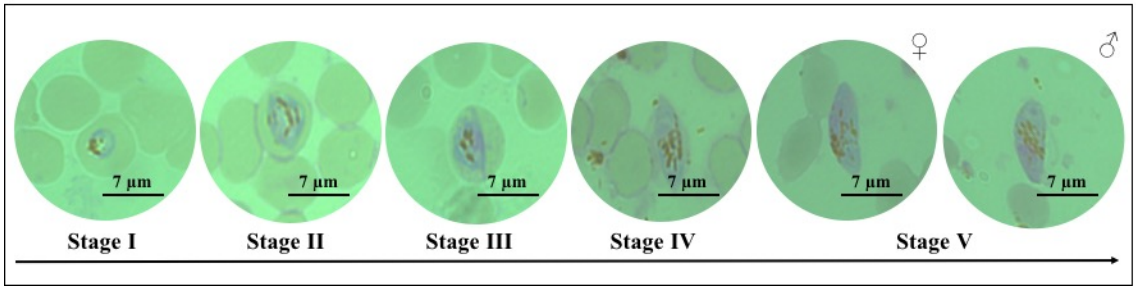


Figure I.5: Gametocytes of *P. falciparum* produced and maintained in culture. The panels show the morphology of gametocytes (stages I - V). Gametocytes were stained by Giemsa (magnification 100X with immersion oil).

I.2 – *P. falciparum* antimalarial drug resistance: its origin and spread

In general terms, drug resistance to antimalarials has been described for two species of *Plasmodium* that cause human malaria, *P vivax* and *P. falciparum*, and the last has demonstrated resistance to all antimalarials in use (Table I.2), including artemisinin-based combination therapies (ACTs) (WHO, 2017b).

Over the past five decades, the emergence of *P. falciparum* resistance to the successively introduced antimalarials (Table I.1) has substantial and significant implications for malaria control programs and global public health (Trape, 2001). The emergence and spread from Asia to Africa of chloroquine (CQ) resistance by *P. falciparum*, followed by anti-folate resistance, led to the loss of millions of lives (Lwin *et al.*, 2015).

Table I.2: Dates of introduction and first reports of resistance of available antimalarials for the treatment of *P. falciparum* malaria.

Antimalarial drug	Introduced	First reported resistance	Half-life time (hours)
Amodiaquine	1960	> 1960	216 – 432
Artemisinins	1980	2008	< 1 – 11
Chloroquine	1945	1960	720 – 1440
Lumefantrine	1970	n.d.	72 – 144
Mefloquine	1985	1992	336 – 504
Piperaquine	1978	1990	336 – 672
Primaquine	1950	n.d.	4 – 9
Pyronaridine	1980	n.d.	288 – 336
Quinine	1632	1910	16
Sulphadoxine-pyrimethamine	1967	1967	72 – 240

Adapted and updated from (Wongsrichanalai *et al.*, 2002).

n.d.- not determined.

Historically, the Thailand-Cambodia border has been the geographical origin of resistance to most of the antimalarials: CQ, then sulfadoxine-pyrimethamine (SP), then mefloquine (MEF), and, more recently, ART (Wongsrichanalai *et al.*, 2002; Dondorp *et al.*, 2009; Noedl, Socheat and Satimai, 2009; Arjen M Dondorp *et al.*, 2010). CQ resistance was first reported in the 1960s in the Thailand-Cambodia border (Eyles *et al.*, 1963; Young *et al.*, 1963; Harinasuta, Suntharasamai and Viravan, 1965), and spread across South Asia, South America and to East Africa by 1978 (Hong *et al.*, 2014), then

subsequently across the continent, leading to catastrophic increases in child morbidity and mortality in sub-Saharan Africa (WHO, 2013).

In 1973 CQ was substituted by SP (Chin *et al.*, 1973; Segal *et al.*, 1975; Doberstyn *et al.*, 1976, 1979; Na-Bangchang and Congpuong, 2007), but by 1980 SP cure rate had dropped unacceptably (Hurwitz, Johnson and Campbell, 1981; Dixon *et al.*, 1982; Pinichpongse *et al.*, 1982). Eventually, SP became ineffective in Thailand (Johnson, Roendej and Williams, 1982; White, 1992; Na-Bangchang and Congpuong, 2007). MEF was introduced in 1985, but clinical resistance to it emerged soon after, in 1992 (Wongsrichanalai *et al.*, 2001).

In 1995, an ACT consisting of artesunate (ATN) plus MEF was introduced as the first-line treatment against uncomplicated *P.falciparum* malaria across Thailand (WHO, 2010). ACTs provided a rapid, effective and well-tolerated antimalarial regimen, with sustained efficacy, and by 2006 WHO recommended ACTs as standard treatment for *P. falciparum* uncomplicated malaria worldwide (Thwing, Eisele and Steketee, 2011; WHO, 2017b).

Recently, there have been signs that the efficacy of ACTs and artesunate monotherapy have declined in western Cambodia (Alker *et al.*, 2007; Noedl *et al*., 2008; Dondorp *et al.*, 2009).

I.2.1 – Multiresistance

The designation of multiresistance to antimalarials refers to the phenotype of resistance to two or more drugs, a phenomenon that has been observed in *P. falciparum*. Resistance to several antimalarials simultaneously results from their frequent and simultaneous use, causing a selective pressure that culminates in the appearance of the phenomenon of multiresistance; the cross-resistance between antimalarials is related to the common aspects of their mechanisms of action, as well as the mechanisms of resistance associated with them (Le Bras and Durand, 2003). *P. falciparum* has developed resistance to virtually all antimalarials in use, including ACTs (WHO, 2017a).

I.3 – Available antimalarials for *P. falciparum* malaria treatment

WHO recommends that all suspected cases of malaria should have a parasitological test (microscopy or rapid diagnostic test (RDT)) to confirm the diagnosis (WHO, 2015a).

Since 2006, WHO recommends the use of artemisinin-based combination therapies (ACTs) (Table I.3), for 3 consecutive days, for the treatment of uncomplicated *P. falciparum* malaria. ART is a sesquiterpene lactone extracted from the Chinese medicinal herb *Artemisia annua* (White, 2008; Tilley, Straimer, Gnädig, *et al.*, 2016) which, in combination with other antiplasmodial drugs, led to significant declines in malaria morbidity and mortality (Barnes and White, 2005; Arjen M. Dondorp *et al.*, 2010; WHO, 2016).

Table I.3: ACTs recommended by the WHO for the treatment of uncomplicated *P. falciparum* malaria. (Adapted from(WHO, 2015a, 2018c)).

ACTs
Artemeter + Lumefantrine
Artesunate + Amodiaquine
Artesunate + Mefloquine
Dihydroartemisinin + Piperaquine
Artesunate + Sulfadoxine-pyrimethamine
Artesunate + Pyronaridine[3]

ACT is the combination of a fast-acting ART derivative with a longer acting partner drug (more slowly eliminated from the blood) (Figure I.6) (Ljolje *et al.*, 2018). The ART component has a good safety profile, provides rapid and effective reduction of parasite biomass, including multidrug-resistant parasites, rapid resolution of fever and is also

[3] Artesunate-pyronaridine, has been given a positive scientific opinion by the European Medicines Agency under article 58 and is being considered by WHO in areas where other ACTs are failing.

active against the parasite's sexual stages (White, 2008). The longer-acting partner drug eliminates much more slowly the remaining parasites and prevents recrudescence, providing mutual augmentation of efficacy and protection against the development of resistance to the ART derivative. Partner drugs with longer elimination half-lives also provide a period of post-treatment prophylaxis (Chotivanich *et al.*, 2014; WHO, 2015a).

ART is poorly soluble in water as well as in oil and is rapidly reduced into dihydroartemisinin (DHA), the active metabolite of all ART derived compounds (Gunjan *et al.*, 2018). In particular, ART derivatives have short plasma elimination half-lives, ranging from <1 to 3 hours for the water-soluble artesunate (ATN) and DHA, and from 3 to 11 hours for the oil-soluble artemether (ATM) and arteether (AE) (Kavishe, Koenderink and Alifrangis, 2017). This contrasts with the partner drugs, chosen to have considerably slower elimination times, persisting over several days to several weeks; in particular, lumefantrine (LU) and sulfadoxine-pyrimethamine (SP) range from 3 to 8 days while mefloquine (MEF), piperaquine (PPQ) and pyronaridine (PYR) range from 2 to 3 weeks (Figure I.6 and Table I.2). Hence, ACTs are the frontline therapies generally used by all malaria control programs around the world (Rocamora *et al.*, 2018).

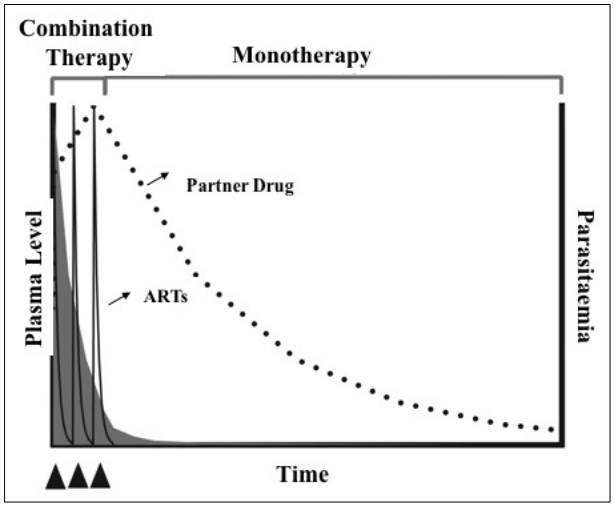


Figure I.6: Schematic representation of malaria parasite killing following artemisinin combination therapy, typically administered in 3 daily doses. The theoretical plasma levels for the ART derivative (solid lines) and partner drug (dotted line) are shown (Adapted from (Taylor and Juliano, 2014)).

Currently, WHO recommends that in low-transmission areas a single dose of primaquine (PQ) should be given together with ACT to patients with *P. falciparum* malaria (except to pregnant women, infants aged < 6 months and women breastfeeding) to reduce transmission since PQ has excellent gametocytocidal activity against *P. falciparum*. Testing for glucose-6-phosphate dehydrogenase (G6PD) deficiency is not required (Global Malaria Programme, 2015).

For the treatment of severe malaria, it is recommended that adults and children (including infants, pregnant women in all quarters and infants) should be treated with injectable ATN or ATM for at least 24 hours and until they can tolerate oral medication, they must complete the 3-day treatment of ACT (plus a single dose of PQ in the low-transmission areas) (Global Malaria Programme, 2015; WHO, 2015a, 2018a).

I.3.1 – *P. falciparum* drug resistance to ACTs

Resistance to ACTs can involve ART derivatives resistance, partner drug resistance, or both (WHO, 2017a, 2018a). In 2008 the first reports of confirmed ART resistance came from the Mekong region (Dondorp *et al.*, 2009; Noedl, Socheat and Satimai, 2009; Arjen M Dondorp *et al.*, 2010), placing once again the Greater Mekong Sub-region (GMS), which encompasses Cambodia, China (Yunnan Province), Laos, Myanmar, Thailand and Vietnam, as the epicentre of ART resistance. Loci of resistance have been identified along the Thailand-Myanmar, Thailand-Cambodia, Vietnam-Cambodia, and Vietnam-Laos borders over the years (Figure I.7) (WHO, 2013; Dondorp *et al.*, 2017).

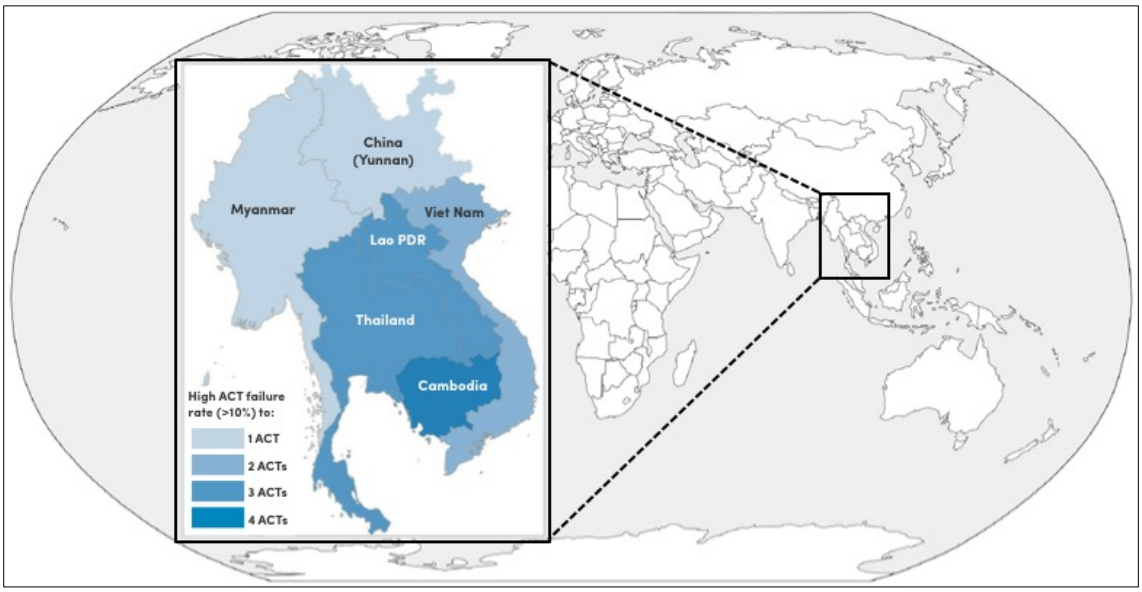


Figure I.7: Numbers of ACTs failing in the Greater Mekong Sub-region. (Adapted from (WHO, 2018a)).

There have been some reports conducted in Africa of delayed parasite clearance during routine therapeutic efficacy studies (TESs) of ACTs (Hong *et al.*, 2014; WHO, 2018a), the emergence of indigenous ART-resistant *P. falciparum* (Lu *et al.*, 2017) and an increasing resistance to multiple partner drugs also, including PPQ (Leang *et al.*, 2015). Resistance to partner drugs of ACTs has historically manifested before that of ARTs, whose short half-lives result in the exposure of residual parasites to sub-therapeutic levels of the partner drug alone. Response to the partner drug is, therefore, a key component of overall ACT efficacy (Venkatesan *et al.*, 2014; WHO, 2018a).

Therapeutic failure to ACTs (as well as to other drugs) may or may not be due to drug-resistant parasites. The WHO has a particular protocol to identify and classify a therapeutic failure to ACTs due to parasite resistance (WHO, 2017a). Regarding *P. falciparum* uncomplicated malaria treatment, ART resistance is defined as the delayed parasite clearance (after an adequate dose has been administered and the recommended plasma concentration has been attained) (WHO, 2017a). The reduced *in vivo* susceptibility to ART derivatives is manifested by the prolongation of parasite clearance times from patient's blood (Alker *et al.*, 2007; Stepniewska *et al.*, 2010), as reflected in Figure I.8.

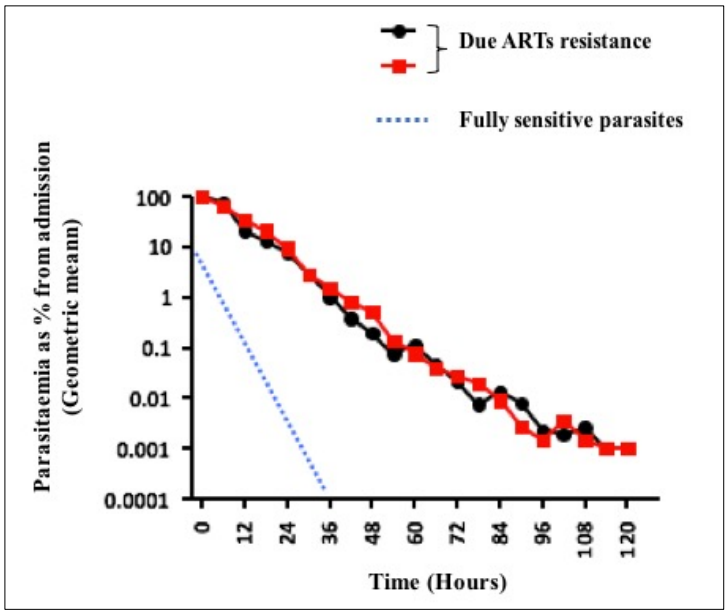


Figure I.8: Dynamics of artemisinin activity to kill sensitive and resistant parasites. (Adapted from (White, 2011)).

Currently, the WHO classifies ACTs drug resistance in two levels, suspected and confirmed, each including several parameters, as follows (WHO, 2017a):

Suspected endemic ART resistance is defined as:

1. ≥ 10% of patients with a half-life of the parasite clearance slope ≥ 5 hours after treatment with ACT or ATN monotherapy; or

2. ≥ 5% of patients carrying K13 resistance-confirmed mutations (listed in Table III.4); or

3. ≥ 10% of patients with persistent parasitaemia by microscopy at 72 hours (± 2 hours; i.e., day 3).

Confirmed endemic ART resistance is defined as:

1. ≥ 5% of patients carrying K13 resistance-confirmed mutations, all of whom have been found to have either persistent parasitaemia by microscopy on day 3 or a half-life of the parasite clearance slope ≥ 5 hours after treatment.

ART resistance in *P. falciparum* can be assessed by a range of phenotypic and genotypic parameters: a) Proportion of positive cases by microscopy on day 3 of follow-up; b)

Parasite clearance half-life; c) *pfk13* mutations and d) Ring-stage survival rate (Chotivanich *et al.*, 2014; WHO, 2015b, 2017a; Woodrow and White, 2017).

I.3.2 – New parameters for evaluation of ARTs

In a recent study, in 2014, Chotivanich *et al.* demonstrated that, despite obvious resistance *in vivo*, conventional 48h *in vitro* tests (IC_{50}) had not shown corresponding reductions in *in vitro* susceptibility (Dondorp *et al.*, 2009), and so they are not useful as an epidemiological tool for monitoring ART resistance. Thus, it was assumed that a reduction in susceptibility in the ring-stage, without a corresponding reduction in the susceptibility in the more mature parasite stages, could explain this apparent discrepancy. Therefore, a simple adaptation of the standard WHO *in vitro* 48h antimalarial drug susceptibility test was done, focusing on the ring-stage development of the parasite, in order to predict resistance to ART *in vivo*.

a) Proportion of cases positive by microscopy on day 3 of follow-up

The proportion of patients with parasitaemia (positive slide by microscopy) on day 3, is relatively easy to measure and is reported widely. Although influenced by starting parasitaemia (and partner drug), it still provides useful information (Stepniewska *et al.*, 2010; Bethell *et al.*, 2011). If more than 10% of patients are parasitemic on day 3, resistance is present, hence further and more definitive clinical and laboratory studies are recommended (Stepniewska *et al.*, 2010; WHO, 2017b). Day 3 positivity rate, is very useful and has been used to detect delayed parasite clearance at an early stage (White *et al.*, 2015).

b) Parasite clearance half-life

Clinical efficacy surveys designed to dissect study of ART resistance in detail, with frequent quantitation of parasitaemia (Stepniewska *et al.*, 2010; Fairhurst *et al.*, 2012), confirmed that despite adequate drug levels in blood, parasite clearance rates were higher

in some Cambodian provinces than in the Thai–Myanmar border (Dondorp *et al.*, 2009; Noedl *et al.*, 2010; Amaratunga *et al.*, 2012). Slow clearing infections were subsequently found to be common across the GMS (Hien *et al.*, 2012; Kyaw *et al.*, 2013; Ashley *et al.*, 2014). The slow parasite clearance, which is assessed from the slope of the log-linear phase of parasitaemia reduction or parasite clearance half-life ($PC_{1/2}$) (Flegg *et al.*, 2011, 2013; White *et al.*, 2015), reflects the known reduced ring stage susceptibility (Witkowski, Amaratunga, *et al.*, 2013; Witkowski, Khim, *et al.*, 2013; Fairhurst and Dondorp, 2016). $PC_{1/2}$ is a reliable marker for the assessment of parasite clearance rate that takes into account differences in initial parasitaemia (Flegg *et al.*, 2011), currently representing one of the parameters inscribed in the WHO definition of ACT drug resistance (WHO, 2017a). Even though, it requires starting parasitaemia >10000 parasites/µl and parasitaemia measurements every 6 to 8h (Flegg *et al.*, 2013).

c) *pfk13* mutations

Part of the current WHO classification of drug resistance to ACTs (WHO, 2017a) is the presence of certain single nucleotide polymorphisms (SNPs) in the gene coding for the Kelch-like protein K13 – the *pfK13*, PF3D7_1343700 (Ariey *et al.*, 2014; Straimer *et al.*, 2015, 2017; Ménard *et al.*, 2016). In Cambodia, where these polymorphisms were first described, the presence of *pfK13* mutations are correlated to *in vitro* parasite survival rates and *in vivo* parasite clearance rates. A series of genome-wide studies (GWS) confirmed that the slow parasite clearance observed in clinical efficacy studies was hereditary (Anderson *et al.*, 2010; Amaratunga *et al.*, 2012; Miotto *et al.*, 2013; Takala-Harrison *et al.*, 2013). The GWS allowed the detection of a region on *P. falciparum* chromosome 13 strongly associated with slow parasite clearance and lead to the identification of *pfk13* as a marker of drug resistance (Cheeseman *et al.*, 2012; Takala-Harrison *et al.*, 2013).

The ART resistant phenotype is currently associated with *pfk13* SNPs (Ariey *et al.*, 2014; Li *et al.*, 2016), which potentially serve as molecular markers, allowing rapid assessment of the presence of ART resistance, particularly in remote locations where phenotypic studies are challenging (Roper *et al.*, 2014; Tun *et al.*, 2015).

However, not all *pfk13* SNPs are associated with ART resistance, the ones identified and

those already validated (correlated with drug resistance) are described in Table I.4 (WHO, 2018a). The most predominant and the best-characterized mutation is C580Y (Venkatesan *et al.*, 2014). Its prevalence across all of Southeast Asia ranges from 26% to 70%, being the highest on the border between Thailand and Myanmar (Imwong *et al.*, 2015; MalariaGEN Plasmodium falciparum Community Project, 2016; Ménard *et al.*, 2016). The R539T mutation (present in the IPC5202 strain) confers high levels of *in vitro* resistance and has been associated with delayed parasite clearance in patients; however, it is less prevalent than C580Y, with 3.5% of K13 mutations in Cambodia-Vietnam-Lao People's Democratic Republic and 0.3% in Thailand-Myanmar-China (Ariey *et al.*, 2014; Straimer *et al.*, 2015, 2017; Takala-Harrison *et al.*, 2015; Ménard *et al.*, 2016). Similarly, the I543T mutation (present in IPC4912 strain) has been associated with an increased survival *in vitro* and a longer parasite clearance half-life in patients, but its prevalence was recently reported to reach only about 2% in Southeast Asia (MalariaGEN Plasmodium falciparum Community Project, 2016).

Table I.4: Candidate[a]/associated and validated[b] resistance mutations in the K13 propeller domain. This list is updated regularly. (Adapted from (WHO, 2018a)).

VALIDATED		CANDIDATES/ASSOCIATED	
F446I	**P553L**	P441L	G538V
N458Y	**R561H**	G449A	V568G
M476I	**C580Y**	C469F	P574L
Y493H		A481V	F673I
R539T		P527H	A675V
I543T		N537I	

[a] Correlated with delayed parasite clearance

[b] By *in vivo* and *in vitro* data

Surveys have provided reassuring evidence that K13 mutations have not reached high prevalence outside Southeast Asia so far (Ménard *et al.*, 2016). K13 mutations are found in Africa at low prevalence, consistent with background variation rather than selection (Kamau *et al.*, 2015; Taylor *et al.*, 2015; MalariaGEN Plasmodium falciparum Community Project, 2016; Ménard *et al.*, 2016).

d) Ring-stage survival rate

P. falciparum reduced susceptibility *in vivo* to ART is characterized by slow parasite clearance but does not correspond to reductions on conventional *in vitro* susceptibility testing, like the 50% inhibitory concentration (IC_{50}) (Dondorp *et al.*, 2009; Chotivanich *et al.*, 2014; Hastings, Kay and Hodel, 2015). The IC_{50} is evaluated by exposing parasites *in vitro* to serial dilutions of drug (Smilkstein *et al.*, 2004; Machado *et al.*, 2016). The delayed parasite clearance phenotype is not associated with a substantial shift in ART IC_{50} (Dondorp *et al.*, 2009; Chotivanich *et al.*, 2014; Hastings, Kay and Hodel, 2015) but it is correlated with reduced ring-stage susceptibility (Witkowski, Amaratunga, *et al.*, 2013; Witkowski, Khim, *et al.*, 2013; Fairhurst and Dondorp, 2016). Based on this, an alternative method for measuring ART sensitivity was developed (Witkowski, Amaratunga, *et al.*, 2013), the ring-stage susceptibility assay $RSA_{0\text{-}3h}$ (See session II.3.3.2).

I.3.3 – The mechanisms of action of artemisinins

Despite its wide use, the mechanisms by which ART and its derivatives (from this point onward, we will use ARTs to refer to ART and its derivatives collectively) exert their antimalarial action is not fully understood (O'Neill, Barton and Ward, 2010; Ding, Beck and Raso, 2011). ART, a natural product extracted from the plant *Artemisia annua* (White, 2008; Tilley, Straimer, Gnädig, *et al.*, 2016), and its derivatives (Figure I.9) are central to all current first-line antimalarial treatments, the ACTs (Table I.3). ARTs belong to the class of sesquiterpene trioxane lactones, bearing an endoperoxide bridge which is essential for their antimalarial activity (Meshnick, 2002; O'Neill, Barton and Ward, 2010). ARTs-mediated parasite death requires bioactivation of the peroxide structure (Li and Zhou, 2010). Inside the infected erythrocyte, ARTs undergo reductive cleavage of the endoperoxide bridge by Fe^{2+}-heme (Krungkrai and Yuthavong, 1987; Maeno *et al.*, 1993; Stocks *et al.*, 2007; Hartwig *et al.*, 2009; J. Wang *et al.*, 2015). The abundance of Fe^{2+}-heme becomes accessible upon hemoglobin degradation inside the parasite DV (Bakar *et al.*, 2010; Rosenthal, 2011). Unlike most other antimalarials, ARTs are active against all stages of the intra-erythrocytic cycle, highly active against trophozoites, in

which Hb catabolism reaches its peak (Klonis *et al.*, 2013), and also active against early ring-stage parasites (Xie *et al.*, 2016). Parasite endocytosis and host Hb degradation begin in very early ring-stages, hence providing a potential source of Fe^{2+}-heme for ARTs activation (Klonis *et al.*, 2013; Xie *et al.*, 2016). The cleavage of the endoperoxide generates reactive oxygen species (ROS) that subsequently damage several cellular targets such as nucleic acids, lipids and key target proteins, killing the parasite (O'Neill, Barton and Ward, 2010; Tilley, Straimer, Gnadig, *et al.*, 2016; Kavishe, Koenderink and Alifrangis, 2017). ROS also mediate depolarization of the membrane potential, both in the mitochondrial (Wang *et al.*, 2010) and the plasma membrane (Antoine *et al.*, 2014). Recent proteomic data suggests that there are probably numerous parasite targets for ARTs, therefore, parasite inactivation may be due to a generalized degeneration of cellular proteins (J. Wang *et al.*, 2015; Ismail *et al.*, 2016), the so-called proteopathy (Bhattacharjee *et al.*, 2018; Haldar, Bhattacharjee and Safeukui, 2018).

In cancer cells, the apoptosis has also been commonly reported as mechanism of ARTs cytotoxic action (Chen *et al.*, 2009; Hamacher-Brady *et al.*, 2011; Gao *et al.*, 2013). Besides this, induction of autophagy (Wang *et al.*, 2012), cell cycle arrest (Jiang *et al.*, 2012), ROS generation (Hamacher-Brady *et al.*, 2011) and, involvement of iron (Singh and Lai, 2001; Handrick *et al.*, 2010; Jiang *et al.*, 2012) have also been demonstrated.

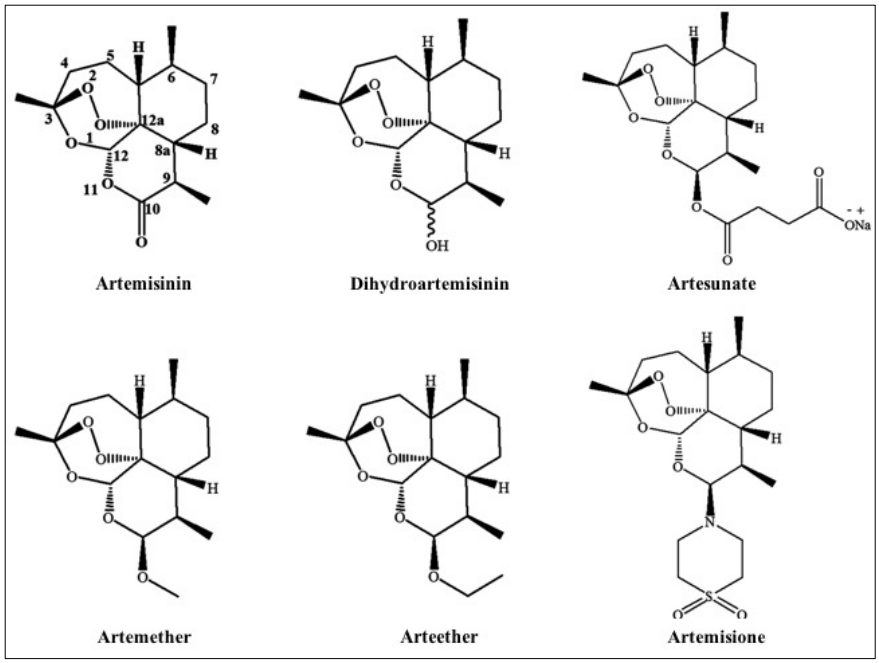


Figure I.9: Representation of the chemical structures of ART and derivatives used as antimalarial drugs. (Adapted from (Z. Li *et al.*, 2016)).

I.3.4 – Mechanisms of ARTs resistance

Mathematical modeling of the prolonged parasite clearance rates predicted that ARTs resistance was a result of ring-stage parasites becoming insensitive to drug action (Saralamba *et al.*, 2011). This correlates with $RSA_{0\text{-}3h}$ results from slow-clearing patient isolates, which are higher than those from drug-sensitive parasites (Witkowski, Amaratunga, *et al.*, 2013). A whole-genome sequencing analysis (WGS) of normal- and slow-clearing patient isolates implicated the gene *pfk13* (coding for the *P. falciparum* Kelch-like protein- PfK13) in the genetic basis for ARTs resistance (Ariey *et al.*, 2014). Later, gene-editing and epidemiological studies established a strong association between slow-clearing infections (with parasite clearance half-lives of > 5 h) and SNPs in the correspondent K13 propeller domain of *PfK13* (Ashley *et al.*, 2014; Ghorbal *et al.*, 2014; Miotto *et al.*, 2015; Straimer *et al.*, 2015; Takala-Harrison *et al.*, 2015; MalariaGEN Plasmodium falciparum Community Project, 2016; Ménard *et al.*, 2016; Phyo, Ashley, *et al.*, 2016; Anderson *et al.*, 2017; Imwong *et al.*, 2017). In fact, certain *pfk13* SNPs (See session I.4.2 - iii and Table I.4) are currently used as major ARTs-resistance molecular

markers (Ariey *et al.*, 2014; Straimer *et al.*, 2015, 2017; Ménard *et al.*, 2016).

Resistance to ARTs is due to mutations of the PfK13 propeller domain which confer an increased ability to enter a quiescent/dormant state during ring-stage (Hoshen *et al.*, 2000; Witkowski *et al.*, 2010; Daignan-Fornier and Sagot, 2011; Witkowski, Khim, *et al.*, 2013; Ariey *et al.*, 2014).

ARTs toxicity is potentiated by Hb degradation inside the DV, generating heme and Fe(II) that react with ARTs and cause oxidative stress and irreversible damages (J. Wang *et al.*, 2015). Hb endocytosis and degradation begins in the mid-ring-stage and increases throughout the trophozoite-and schizont-stage. Hence, low levels of hemoglobin degradation in younger rings could explain their reduced sensitivity to ARTs on these stages (Hott *et al.*, 2015).

Accordingly, *pfk13* mutations do not seem to protect trophozoite-stages against ARTs (Witkowski, Khim, *et al.*, 2013). At trophozoite-stage, the higher potency of ARTs probably results from higher cellular levels of the heme activator, possibly overpowering the mutant K13-mediated defenses (Klonis *et al.*, 2011; Dogovski *et al.*, 2015).

Apart from *pfk13* mutations, the genetic background of the parasites seems to play an important role in ARTs tolerance. The introduction of the wild-type allele into resistant parasites produced a very low $RSA_{0–3h}$ survival rate (0.3 - 0.7 %). Conversely, the introduction of one resistant allele (C580Y) into wild-type ART-sensitive parasites conferred varying degrees of *in vitro* resistance, suggesting an additional contribution from the genetic background (Straimer *et al.*, 2015).

The enhanced *P. falciparum* quiescence capacity of ART-resistant parasites results from an increased ability to manage oxidative damage and an altered cell cycle gene regulation involving the unfolded protein response (UPR), the dysregulation of phosphatidylinositol-3-kinase (PfPI3K) pathway and other yet unidentified targets (Haldar, Bhattacharjee and Safeukui, 2018).

PfKelch13, predictably a regulator of protein quality control (Gupta and Beggs, 2014), has mammalian orthologues that confer resistance to cancer drugs, inducing proteopathy (death from global abnormal protein-toxicity) (Nikesitch and Ling, 2016). There is

evidence that ARTs kill by targeting hundreds of proteins (J. Wang *et al.*, 2015; Ismail *et al.*, 2016; Bhattacharjee *et al.*, 2018), suggesting that parasite death is caused by proteopathy. This supports the idea that the PfKelch13-mediated resistance may be linked to the capacity of restoring complex protein functions in the parasite - the so-called proteostasis (Bhattacharjee *et al.*, 2018; Suresh and Haldar, 2018), hence, promoting its survival from ART-induced proteopathy.

In *P. falciparum*, two major *PfK13* effector mechanisms have been proposed to overcome ARTs-induced proteopathy and death. One involves the (i) upregulation of parasite oxidative stress response and protein damage pathways via the unfolded protein response (UPR) (Dogovski *et al.*, 2015; Mok *et al.*, 2015) and the other results from the (ii) elevation of parasite phosphatidylinositol-3-phosphate (PI3P) caused by proteostatic dysregulation of phosphatidylinositol-3-kinase (PfPI3K) (Mbengue *et al.*, 2015). Both pathways are linked to *PfK13*.

Bioactivation of the peroxide structure in the ARTs, leads to generation of reactive ROS, that rearrange to form carbon-centred radicals, which subsequently damage proteins, lipids and nucleic acids (Meshnick, 2002; O'Neill, Barton and Ward, 2010; Gopalakrishnan and Kumar, 2015), and alkylate heme (Robert, Coppel and Meunier, 2002), hampering hemozoin polymerization (Pandey *et al.*, 1999) and eventually killing the parasite. ARTs also induce the ROS-mediated depolarization of both the mitochondrial (Wang *et al.*, 2010; Antoine *et al.*, 2014) and plasma membranes (Antoine *et al.*, 2014), leading to parasite death. Free radicals do not have a specific cell target (J. Wang *et al.*, 2015; Ismail *et al.*, 2016) but rather indiscriminately damage proteins, resulting in proteopathy (Nikesitch and Ling, 2016).

Recovery from proteopathy requires multiple cellular functions that include removal of misfolded, aggregated toxic proteins and their replacement to return to proteostasis. ARTs resistant parasites show increased transcription of the reactive oxidative stress complex linked to the UPR. UPR is one of the multiple proteostasis mechanisms used to maintain quality control of protein folding in eukaryotes, responsible for triggering a transcriptional response to restore redox conditions. One of the downstream effector components of the UPR is the elongation initiation factor 2A (eIF2A), suggesting a mechanism of translational repression (as an adaptive response to misfolded proteins) that

transiently delays maturation and morphology of apparent cell cycle arrest of mutant ring-stage parasites (Cheng, Kyle and Gatton, 2012; Zhang *et al.*, 2012).

Certain *PfK13* SNPs in the Kelch domain, show reduced binding to and ubiquitination of *PfPI3K*, increasing the levels of the kinase itself and of its lipid product PI3P (Mbengue *et al.*, 2015). Those mutations are expected to increase substrate levels, thus redox sensing by PfK13 is also expected to increase substrate levels and their associated mechanisms of resistance.

PfK13 may act as an adapter of PfPI3K to control PfPI3K protein levels. Wild-type PfK13 protein bound *P. falciparum* PfPI3K catalysed kinase ubiquitination and lowered production of the phospholipid signaling molecule PI3P (Mbengue *et al.*, 2015), while *PfK13* mutants failed to bind, increasing levels of PfPI3K and elevating its lipid product PI3P. PI3P is a phospholipid found in cell membranes that help to recruit proteins (Gillooly, Simonsen and Stenmark, 2001). It has been implicated in intracellular trafficking events, including protein export and hemoglobin endocytosis (Bhattacharjee, Stahelin and Haldar, 2012). Furthermore, elevation of PI3P alone efficiently conferred ART resistance (Mbengue *et al.*, 2015).

This suggests that a proteasome-engaging cell stress response is involved in protecting both sensitive and resistant parasites from the action of ARTs, which seems to be more effective in the K13 mutant parasites.

I.3.5 – Partner drugs of artemisinins in ACTs

a) Lumefantrine

Lumefantrine (LU) belongs to the group of amino alcohols and is an antimalarial agent used to treat acute uncomplicated malaria (Figure I.10). It is administered in combination with artemether (ATM) for improved efficacy. The exact mechanism by which LU exerts its antimalarial effect is unknown. However, available data suggest that LU inhibits the formation of β-hematin by forming a complex with hemin and inhibits nucleic acid and protein synthesis, acting in blood stages (trophozoite and schizont) (Haldar, Bhattacharjee and Safeukui, 2018).

In Africa, ATM-LU (Coartem®), the most common ACT (Kavishe, Koenderink and Alifrangis, 2017), has demonstrated consistently high efficacy and safety for over a decade. Currently deployed as a first- or second-line treatment in most sub-Saharan African countries, its extensive use is endorsed by a lack of reported parasite resistance in Africa, to date (Ogbonna and Uneke, 2008; Hamed and Grueninger, 2012).

Polymorphisms in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) and *P. falciparum* multidrug resistance 1 (*pfmdr1*) genes are associated with decreased sensitivity to LU, but effects of these polymorphisms on therapeutic responses to ATM-LU have not been clearly defined (Venkatesan *et al.*, 2014). The *pfmdr1* haplotypes of N86, F184 and D1246 (NFD) were selected in recrudescence samples after ATM-LU treatment, suggesting that this haplotype conferred a fitness advantage upon ATM-LU pressure (Happi *et al.*, 2009; Mbaye *et al.*, 2016). The introduction of ATM-LU in Tanzania and Mozambique led to an increasing trend in NFD prevalence, which supports the observed ATM-LU pressure. In addition, the *pfmdr1* N86 and *pfcrt* K76 wild-type alleles are also selected by ATM-LU treatment (Menard *et al.*, 2012).

The *Pfmdr1* protein located on the vacuolar membrane has the function to pump substrates into the DV. Thus, for drugs whose primary targets are in the DV, alterations that favor less accumulation of the drug in the DV may be favored (Cowman *et al.*, 1991).

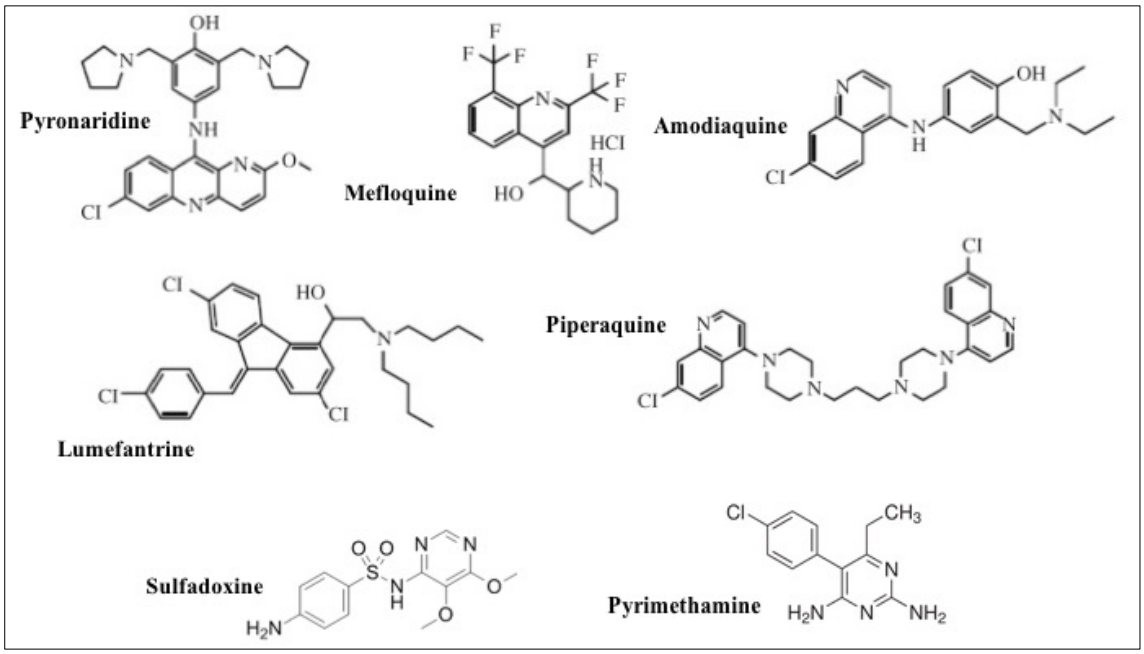


Figure I.10: Representation of the chemical structures of partner drugs of ARTs used for the artemisinin-based combination therapy of malaria (ACTs). (Adapted from (Krungkrai and Krungkrai, 2016)).

b) Mefloquine

Mefloquine (MEF) is a quinoline derivative (Figure I.10) belonging to the group of amino alcohols with antimalarial activity (Haldar, Bhattacharjee and Safeukui, 2018) that bind to FP and inhibit its polymerization into hemozoin, causing accumulation of the oxidatively toxic PF in the parasite's DV (Foley and Tilley, 1997). MEF also binds to cysteine proteases, inducing apoptosis and ROS production (Gunjan *et al.*, 2016) and acting in blood stages of the parasite (trophozoite and schizont) (Haldar, Bhattacharjee and Safeukui, 2018).

MEF has been widely used previously in the treatment and prophylaxis of malaria and is currently administered in combination with artesunate (ATN-MEF). This combination is commonly associated with neuropsychiatric side effects and cardiotoxicity, attributed to MEF (Mawson, 2013; Jain, Nevin and Ahmed, 2016).

ATN-MEF resistance is very high in most of East Asia, where this combination has been extensively used, and resistance against MEF monotherapy had reached alarming levels

before it was combined with ATN (Nosten *et al.*, 2000; Kavishe, Koenderink and Alifrangis, 2017). This combination is rarely used in Africa (Ogbonna and Uneke, 2008).

Mutations in and/or amplification of *pfmdr1* confer resistance to partner drugs such as MEF and LU and thus limit effective treatment with ACTs (Price *et al.*, 2004; Sidhu *et al.*, 2006; Veiga *et al.*, 2016). The *pfmdr1* S1034C and N1042D mutations were reported to reduce or abolish resistance to MEF (Duah *et al.*, 2013).

In Southeast Asia, MEF will be added to DHA-PPQ to generate triple ACTs as an elimination strategy (WWARN, 2017). This needs careful monitoring because MEF can affect heart rhythms and has neurological side effects (Haldar, Bhattacharjee and Safeukui, 2018).

c) Amodiaquine

Amodiaquine (AQ) is a 4-aminoquinoline (Figure I.10) used in combination with ATN (ASAQ) that has remained effective against uncomplicated malaria even in areas where CQ resistance marker is very high (Shayo, Buza and Ishengoma, 2015).

Like other quinolines, AQ acts by interfering with hemoglobin digestion in the blood stages of the malaria life cycle (Foley and Tilley, 1997).

Some studies of AQ have reported reduced *in vivo* response and increased IC_{50} values *in vitro*, in association with the presence of *pfmdr1* 86Y and *pfcrt* 76T alleles (Venkatesan *et al.*, 2014; Ljolje *et al.*, 2018). Selection of these alleles in recurrent parasites, after treatment with AQ alone or in combination with ATN, has been observed in a number of studies (Humphreys *et al.*, 2007). The *pfmdr1* D1246Y and N86Y mutations predict resistance and recrudescence to AQ in Uganda (Nsobya *et al.*, 2010).

Resistance to partner drugs has also been linked to polymorphisms in certain genes: single-nucleotide polymorphisms (SNPs) in *pfcrt* and *pfmdr1* for AQ.

In Southeast Asia, AQ will be added to ATM-LU to generate triple ACTs as an elimination strategy (WWARN, 2017).

d) Piperaquine

Piperaquine (PPQ), a bisquinone derivative of 4-aminoquinoline (Figure I.10), also inhibits hemozoin formation, leading to oxidative stress (Kavishe, Koenderink and Alifrangis, 2017). PPQ acts on trophozoites and schizonts (Haldar, Bhattacharjee and Safeukui, 2018).

It was reported that the efficacy of the current first-line drug DHA-PPQ in Cambodia has decreased, leading to a drop of about 50% in cure rates in some parts of the country. An increased number of copies of the *plasmepsin 2–plasmepsin 3* gene, associated with PPQ resistance, has been implicated. Amplification of *Pfplasmepsin* 2 and *Pfplasmepsin* 3 almost invariably appears on parasites carrying the marker for ART resistance, *PfKelch13*, in particular the C580Y allele (Amato *et al.*, 2016; Witkowski *et al.*, 2017).

The amplification of *plasmepsin 2–3* encodes hemoglobin-digesting proteases, regardless of the location. It was discovered that the *plasmepsin 2* gene copy amplification correlates to DHA-PPQ failure rates at day 42 of treatment follow-up, and this resistance is attributed to PPQ rather than DHA (Witkowski *et al.*, 2017). The *pfmdr1* N86Y mutation increases sensitivity to DHA-PPQ (Pillai *et al.*, 2012) and the *pfcrt* gene is also associated with the PPQ resistance (Agrawal *et al.*, 2017; Dhingra *et al.*, 2017).

It has been shown that the *in vitro* piperaquine survival assay (PSA) (Duru *et al.*, 2015) with *in vitro* culture-adapted parasites and freshly collected *ex vivo* patient isolates, to detect PPQ resistance and treatment failure, is more reliable than classic dose-response assays. The *in vitro* PSA was therefore proposed as a reliable tool to identify molecular signatures associated with resistance SNPs and copy number variations (Witkowski *et al.*, 2017).

e) Sulfadoxine-pyrimethamine

Sulfadoxine (S) is an ultra-long-lasting sulfonamide used in combination with pyrimethamine (P), a diaminopyrimidine, which belongs to the antifolates class (Figure I.10) and is used for the treatment of uncomplicated malaria. The combination SP (Fansidar®) presents low toxicity, acting in blood and liver schizonts and in the mosquito

stages (oocysts) (Haldar, Bhattacharjee and Safeukui, 2018), but is no longer used for the treatment of clinical malaria in Africa because of resistance emergence. However, it continues to be used as prophylaxis and is administered routinely as an intermittent preventive treatment (IPT) for malaria in pregnancy and in infants (See I.4.7) (Naidoo and Roper, 2013; WHO, 2017b).

In Northeast India, ATN-SP became redundant due to high resistance to SP, which lowered the efficacy of ATN-SP to unacceptable levels (Mishra *et al.*, 2014). The resistance to SP has been attributed to point mutations in the parasite genes encoding their target enzymes, dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*). These mutations accumulate at several sites in the *dhfr* and *dhps* genes (Roper *et al.*, 2003). The quintuple mutant, a combination of the *dhfr* triple mutant (51I + 59R + 108N) and the *dhps* double mutant (437G + 540E), is found throughout sub-Saharan Africa and is associated with SP treatment failure (Naidoo and Roper, 2013; Warsame *et al.*, 2017).

In Somalia, a treatment failure rate as high as 12% was recently reported for ATN-SP, with SP-resistance molecular markers (quintuple mutations) reported in the majority of patients (89%) (Warsame *et al.*, 2017)

f) Pyronaridine

Pyronaridine (PYR) belongs to the family of 4-aminoquinolines (Figure I.10) and was developed in China as one of the earliest synthetic antimalarial drugs in the late 1970s (Zheng *et al.*, 1982). It was initially used in a monotherapy regimen (Ringwald *et al.*, 1996) but is nowadays formulated as a fixed-dose with ATN (ATN-PYR) (Pyramax®), a new and highly effective ACT that has earned a positive scientific opinion from the European Medicines Agency under article 58 and is now being considered by the WHO for administration in areas where other ACTs are failing (WHO, 2018a). PYR acts in rings, trophozoites and schizont stages of malaria parasites (Haldar, Bhattacharjee and Safeukui, 2018) and has also been shown to possess an *in vitro* gametocytocidal effect in *P. falciparum* (Chavalitshewinkoon-Petmitr *et al.*, 2000).

The mechanism of action of PYR remains unclear but it has already been demonstrated

that PYR acts similarly to chloroquine with regard to the inhibition of hematin formation *in vitro*, through formation of a drug-hematin complex, inhibition of glutathione (GSH)-dependent degradation of hematin, and enhancement of hematin-induced lysis of red blood cells (Auparakkitanon *et al.*, 2006).

In areas of low transmission, the WHO recommends that the combination ATN-PYR should be administered with primaquine (PQ) in *P. falciparum* malaria, to reduce the transmissibility of the treated infection, and also in Southeast Asia, to decrease the risk of spreading ART resistance (WHO, 2015a).

However, *P. falciparum* reduced susceptibility to the PYR component was detected in western Cambodia. Notably, the parasite clearance rate was significantly extended with both ATN-PYR and ATN-MEF in Cambodia, versus other countries, suggestive of ART resistance despite high efficacy elsewhere in Asia and Africa (Rueangweerayut *et al.*, 2012; Leang *et al.*, 2016). In a recent study in Africa, it was shown that *ex vivo* reduced susceptibility to PYR, i.e., $IC_{50} > 60$ nM, was associated with the K76T mutation in the *pfcrt* gene (Agrawal *et al.*, 2017).

I.3.6 – Other antimalarial drugs of interest

a) Chloroquine

Chloroquine (CQ) is a 4-aminoquinoline (Figure I.11) that has been extensively used in the prevention and treatment of malaria. The studies carried out in the different phases of the intra-erythrocyte life cycle of *P. falciparum* have indicated the DV (a lysosome-like organelle) as the site of action of this antimalarial (Ecker *et al.*, 2012). CQ, as well as other chemically similar drugs, acts by accumulation at high concentrations (~85%) in the DV of the parasite, reaching concentrations many times higher than plasma concentration (Bray *et al.*, 2006; Gligorijevic *et al.*, 2006). CQ becomes toxic due to its binding to FP, helping to retain the drug at high concentrations within the parasite and making it impossible to detoxify the heme. In essence, the parasite is poisoned by its own waste (Roepe, 2009; Ecker *et al.*, 2012).

Mutations in the gene encoding the *P. falciparum* CQ resistance transporter (*pfcrt*), on

chromosome 7, are associated with chloroquine resistance (Fidock *et al.*, 2000); a substitution from lysine to threonine at codon 76 in *pfcrt* predicts responses of parasites to CQ (Djimdé *et al.*, 2001; Venkatesan *et al.*, 2014). This mutation is the most reliable molecular marker of resistance to CQ among the several identified mutations (72, 74, 75, 97, 152, 163, 220, 271, 326, 356 and 371 (Djimdé *et al.*, 2001; Ibrahim *et al.*, 2009).

Mutations in the gene that encodes the *P. falciparum* multidrug resistance transporter 1 (*pfmdr1*) on chromosome 5, linked to antimalarial drug resistance occur at codons 86, 184, 1034, 1042 and 1246 (Foote *et al.*, 1990; Reed *et al.*, 2000; Venkatesan *et al.*, 2014). However, the mutation that occurs as a result of the substitution of asparagine for tyrosine at position 86 is linked with CQ resistance (Wellems *et al.*, 1990). Additionally, multiple *pfmdr1* SNPs have been associated with susceptibility profiles of many antimalarial drugs. The Y184F, N1042D and D1246Y mutations are associated with the CQ resistance phenotype from samples in Africa, Asia and South America (Menard *et al.*, 2006).

The *pfmdr1* gene expression levels have been considered in the etiology of the parasite resistance to some antimalarial drugs and it is being explored in epidemiological studies. Increase in *pfmdr1* gene copy numbers has been linked to *P. falciparum* diminished susceptibility to antimalarial drugs, such as MEF, ATN-MEF, and ATM-LU combinations (Price *et al.*, 2004). Although there is no reported correlation between *pfmdr1* gene copy numbers and treatment failure, this marker is important for the prediction of recrudescence with the use of the antimalarials mentioned above. There is an assertion that *pfmdr1* gene copy numbers rather than the SNPs, exert greater influence in mediating antimalarial drug resistance to some compounds. This was reported to be due to the fact that many transporter proteins mandate concerted complementary attention to copy number variations in mediating antimalarial activity (Duah *et al.*, 2013).

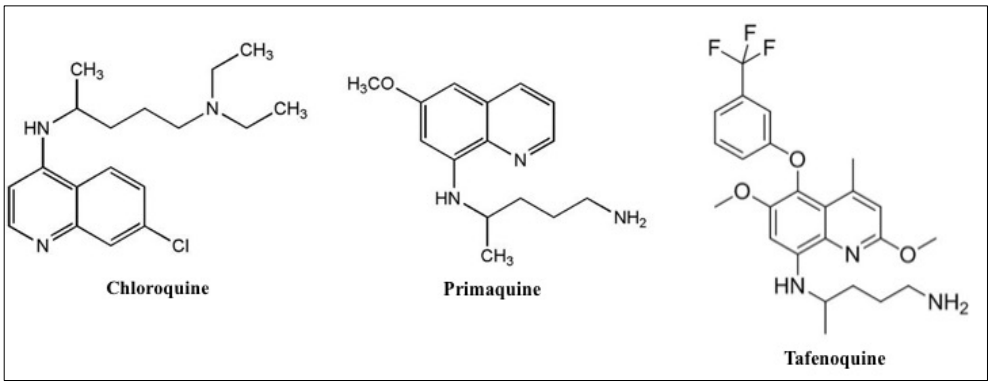


Figure I.11: Representation of the chemical structures of other relevant quinoline-based antimalarial drugs.

b) Primaquine

Primaquine (PQ), an important 8-aminoquinoline (Figure I.11), is metabolized into 5-hydroxyprimaquine, which reacts to form ROS in erythrocytes, leading to cytoskeletal and membrane lipid peroxidation and hemolysis (Bowman *et al.*, 2005; Bowman, Jollow and McMillan, 2005; Kavishe, Koenderink and Alifrangis, 2017). PQ is well known for its high ROS production, leading to rapid depletion of glutathione in erythrocytes; hence, it is contraindicated in patients with G6PD

deficiency (Beutler, 1994).

PQ has been used as an antimalarial drug for more than 50 years. It has excellent gametocytocidal activity against *P. falciparum*, thus reducing transmission, and has hypnozoitocidal activity against *P. vivax* and *P. ovale* infections (Galappaththy, Tharyan and Kirubakaran, 2013). For these reasons, the WHO recommends the use of a single dose of PQ in combination with ACTs for *P. falciparum* malaria treatment (WHO, 2015a). PQ is not suitable as a single drug for malaria chemotherapy because it is not effective against intra-erythrocytic forms of Plasmodia. Thus, PQ must be co-administered with blood schizontocides (Baird and Hoffman, 2004).

Currently, PQ is the only treatment available to prevent relapses of *P. vivax* malaria and is the only licensed drug that has proven gametocytocidal activity *in vivo* (Gebru, Mordmuller and Heldb, 2014). Because PQ has a half-life of 4 to 9 hours (Vale, Moreira and Gomes, 2009), the WHO recommends that it is administered once a day, for 14 days,

for the treatment of *P. vivax* malaria. It is difficult for patients to follow such a regimen, which leads to treatment withdrawal (MMV, 2018).

The exact mechanism by which PQ so effectively eliminates Plasmodia hypnozoites and gametocytes is still under investigation but it has been proposed that PQ selectively destroys the inner structure of mitochondria, eventually by interference with the ubiquinone function, as an electron carrier in the respiratory chain (Pukrittayakamee *et al.*, 2004; Vale, Moreira and Gomes, 2009; Leliévre *et al.*, 2012). The biotransformation pathways important for PQ therapeutic and toxic effects are unclear, but recent evidence suggests that cytochrome CYP2D6 plays a crucial role in generating highly reactive intermediate metabolites that generate intracellular oxidative potentials, which provides the antimalarial activity of PQ (Vale, Moreira and Gomes, 2009). A key safety issue for PQ is its capacity to induce hemolysis in G6PD-deficient patients (Llanos-Cuentas *et al.*, 2014).

Resistance to PQ by blood stages of *Plasmodium* parasites (Arnold, Alving and Clayman, 1961) is of little clinical consequence. Resistance in tissue stages dominates public health concern, and the absence of such resistance after 50 years seems incredible. The resistance to PQ is notably low and hardly noteworthy, a fact that is still not understood (Baird and Hoffman, 2004; Vale, Moreira and Gomes, 2009).

c) Tafenoquine

Tafenoquine, a 8-aminoquinoline and a synthetic analogue of PQ (Figure I.11), is a long-acting antihypnozoite drug (Bousema and Drakeley, 2011), developed for *P. vivax* radical cure that must be co-administered with a blood schizontocide, either CQ or ACT (Green *et al.*, 2016). Tafenoquine has a half-life of 2 to 3 weeks, raising the possibility of single-dose treatment and directly observed therapy (Llanos-Cuentas *et al.*, 2014).

Tafenoquine (Krintafel®), developed by the pharmaceutical company GlaxoSmithKline (GSK), has completed phase III studies. In July 2018, GSK and Medicines for Malaria Venture (MMV) announced that: "*the United States Food and Drug Administration (FDA) has approved, under Priority Review, single-dose Krintafel for the radical cure*

(prevention of relapse) of Plasmodium vivax malaria in patients aged 16 years and older who are receiving appropriate antimalarial therapy for acute P. vivax infection" (MMV, 2018).

To reduce the risk of hemolysis, GSK is working on the development of a quantitative G6PD point-of-care diagnostic test, so that patients with G6PD status can be tested to determine if tafenoquine or PQ can be safely administered (MMV, 2017).

The antimalarial drugs currently in use and the respective resistance markers are listed in Annex XI.

I.3.7 – Chemoprophylaxis and chemoprevention with antimalarials

The WHO recommends preventive therapies for vulnerable groups, such as: travelers during potential exposure to malaria; pregnant women, infants (< 12 months of age) and children (< 5 years of age) living in endemic areas. However, resistance has already developed against drugs approved for this indication (i.e., chloroquine and proguanil), in most endemic areas (WHO, 2017b).

The chemoprophylaxis recommended for travelers during potential exposure to malaria depends on local patterns of susceptibility to antimalarials and on the likelihood of Plasmodial infection. Since 2016, 36 African countries have adopted a policy of providing three or more doses of intermittent preventive treatment in pregnancy (IPTp) with sulfadoxine-pyrimethamine (SP) to all pregnant women in endemic areas, to provide continuous preventive effects as part of antenatal care (WHO, 2017b). Resistance to SP is increasing in Africa, and therefore alternative drugs are being investigated for its use in preventive treatment during pregnancy (WHO, 2017b). In areas of moderate-to-high malaria transmission in Africa where SP is still effective, intermittent preventive treatment with SP should be provided to infants (< 12 months of age) (SP-IPTi) at the time of the second and third rounds of vaccination against diphtheria, tetanus and pertussis (DTP) and vaccination against measles (WHO, 2017b). Seasonal malaria chemoprevention (SMC) with SP + AMQ is recommended to children, but only in areas of highly seasonal transmission, across the Sahel subregion of Africa (WHO, 2017b,

2018b).

I.4 – New endoperoxides as antimalarial candidates

The widespread application of ART and its derivatives leads to difficulties in maintaining supply. The parent pharmacologically active compound, ART, is obtained by large-scale extraction from shrubs of *Artemisia annua* and ART derivatives are generated semi-synthetically, with growth, harvest, and production processes taking about 18 months (Wells, Huijsduijnen and Voorhis, 2015). In addition to this limitation, recent findings of resistance to ACTs stimulated further efforts towards the development of the next generation of potent antimalarial endoperoxides, equally effective against ART-susceptible and -resistant strains of *P. falciparum,* as well as safer and cheaper than ARTs. Thus, there is an urgent need for wholly synthetic endoperoxides that are as effective as the currently used ARTs but are cheaper and more easily available (Yang *et al.*, 2016).

The core structure of ARTs comprises a 1,2,4-trioxane incorporating an endoperoxide linkage that is essential for activity (Tang, Dong and Vennerstrom, 2004; O'Neill, Barton and Ward, 2010). The endoperoxide pharmacophore has stimulated the development of several different classes of synthetic endoperoxides including trioxolanes (Vennerstrom *et al.*, 2004) and tetraoxanes (Vennerstrom *et al.*, 1992; Fontaine *et al.*, 2015), which are particularly promising in the context of antimalarial chemotherapy, exhibiting similar activity to the ARTs (O'Neill *et al.*, 2017).

The first synthetic ozonide developed for use as an antimalarial drug, OZ277 (arterolane maleate; also called RBx-11160), proved to be well tolerated by humans (Gautam *et al.*, 2011; Wells, Huijsduijnen and Voorhis, 2015) and a combination of OZ277 and PPQ, known as Synriam^{TM}, was registered in India for use in antimalarial chemotherapy (Tilley, Straimer, Gnädig, *et al.*, 2016). OZ277 has good activity against all asexual blood stages of *P. falciparum*; however, the half-life of OZ277 is only 2- to 3-fold longer than DHA (Gautam *et al.*, 2011) and it is reported to have lower plasma exposure in malaria patients than in uninfected volunteers (Saha *et al.*, 2014).

OZ439 (artefenomel), the second synthetic ozonide that advanced to the stage of clinical

candidate selection, appears to be more promising, exhibiting a much longer *in vivo* elimination half-life (46 to 62 hours) (Moehrle *et al.*, 2012; Phyo, Jittamala, *et al.*, 2016). OZ439 is currently being assessed, either as monotherapy or as combination therapy, in Phase II human clinical trials (Wells, Huijsduijnen and Voorhis, 2015; Phyo, Jittamala, *et al.*, 2016; Rosenthal, 2016). The extended exposure profile (Moehrle *et al.*, 2012) raises the possibility that it might be effective, in combination with a second antiplasmodial agent, as a single-dose oral cure for malaria (Charman *et al.*, 2011; Moehrle *et al.*, 2012; Wells, Huijsduijnen and Voorhis, 2015; Phyo, Jittamala, *et al.*, 2016; Yang *et al.*, 2016) (Figure I.12).

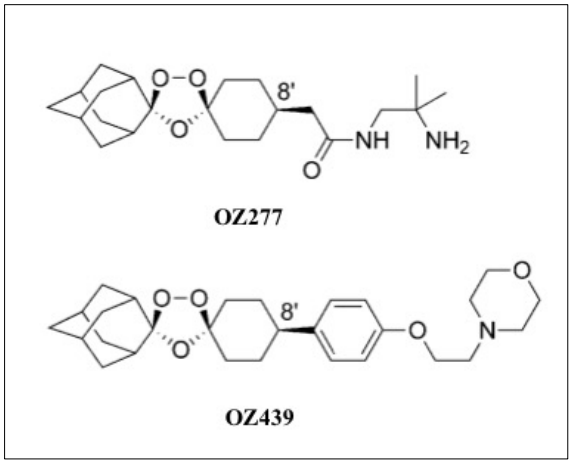


Figure I.12: Representation of the chemical structures of trioxolanes OZ277 and OZ439, advanced as antimalarial candidates. (Adapted from (Charman *et al.*, 2011)).

As proposed for the ARTs, the peroxide bond of the ozonides appears to be instrumental to the antimalarial activity (Tang, Dong and Vennerstrom, 2004; Fontaine *et al.*, 2015), consistent with the observation that Fe^{2+} availability is required for bioactivation and subsequent antimalarial activity. The involvement of carbon-centred radicals as the toxic species is supported by the observation that nitroxide radical spin trap compounds antagonize the activity of both ARTs and OZ277 (Fugi *et al.*, 2010).

Thus, the accessibility (relatively easy and inexpensive preparation) and stability of 1,2,4-trioxolanes allows the synthesis of derivatives with various structures, extending the

possibility of developing potentially more effective drugs. The tolerance of the 1,2,4-trioxolane moiety to various synthetic conditions allows the synthesis of a significant number of chemically diverse derivatives (Tang, Dong and Vennerstrom, 2004).

1,2,4,5-tetraoxanes, which differ from the structure of ARTs, are readily prepared in two steps from substituted cyclohexanones, and have good antimalarial efficacy, exhibiting pronounced activity against both CQ-sensitive and CQ-resistant strains of *P. falciparum*, (Rudrapal and Chetia, 2016) even though some are less active than semi-synthetic ARTs (Dong *et al.*, 2007).

E209 and RKA182 (O'Neill *et al.*, 2010) are known as the next-generation tetraoxane-based antimalarial drugs. TDD E209 meets key requirements of MMV for drug candidate profile. It presented a potent nanomolar inhibitory activity against multiple strains of *P. falciparum* and *P. vivax in vitro*, was efficacious against *P. falciparum in vivo* in rodent models, produced parasite reduction ratios equivalent to DHA, and evidenced pharmacokinetic and pharmacodynamic characteristics compatible with a single-dose cure. Therefore, it is currently under preclinical phase of development (Vennerstrom *et al.*, 1992, 2000; Dong *et al.*, 2007; Opsenica and Šolaja, 2009; Rudrapal and Chetia, 2016; O'Neill *et al.*, 2017).

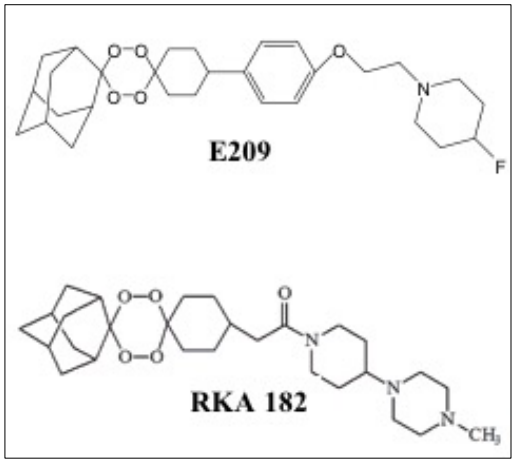


Figure I.13: Representation of the chemical structures of tetraoxanes TDD E209 and RKA182, advanced as antimalarial candidates. (Adapted from (Rudrapal and Chetia, 2016).

In a recent study performed by us, three synthetic trioxolanes prepared by Cristiano's group were tested *in vitro* and *in vivo* against a mouse model infected with ART-resistant (ART-R) parasites, and the compounds exhibited high efficacy in clearing the infection (Lobo *et al.*, 2016). These results inspired the expansion of the library of compounds and further investigation of their efficacy against ART-R *P. falciparum* strains. This thesis reports the antimalarial activity *in vitro* and *in vivo* of a library of 36 endoperoxide-type compounds. From these, the synthesis and antimalarial activity of a library of 21 compounds (trioxolanes and tetraoxanes) have already been disclosed (Lobo *et al.*, 2018).

I.5 - Oxidative stress in *P. falciparum*

During the intra-erythrocytic part of the parasite's life cycle, malaria parasites degrade ~75% of the host hemoglobin in the DV(Bakar *et al.*, 2010). This provides a necessary source of aminoacids, releases space for growth and maintains the osmotic balance of the infected erythrocyte (Lew, Tiffert and Ginsburg, 2003; Bakar *et al.*, 2010). However, hemoglobin degradation is a metabolic process that generates large amounts of ROS, since it occurs in the presence of oxygen and Fe(II) or heme, the key prerequisite for the formation of ROS via the Fenton reaction[4] (Fu *et al.*, 2010). In fact, the main source of ROS in *P. falciparum* is the digestion of hemoglobin in the DV of the parasite (Lew, Tiffert and Ginsburg, 2003; Becker and Kirk, 2004), that produces high amounts of heme, a highly reactive molecule that generates ROS (Gluzman *et al.*, 1994). Unlike mammals, which detoxify the heme groups by opening the ring enzymatically and by glucuronidation, the parasite uses an enzymatic mechanism of cell detoxification by polymerizing the heme groups, producing a crystalline non-toxic matrix, hemozoin, stored in parasite's DV (Fu *et al.*, 2010).

In order to survive and develop in such environment, *P. falciparum* relies on a robust antioxidant system to minimize the damage caused by ROS and reactive nitrogen species (RNS) (Kavishe, Koenderink and Alifrangis, 2017; Sussmann *et al.*, 2017). Several enzymes of the glutathione system have been described in *Plasmodium* species. These include glutathione synthase (Meierjohann, Walter and Müller, 2002) and glutathione

[4] $O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$ / $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^-$

reductase (Farber *et al.*, 1996), superoxide dismutase (Bécuwe *et al.*, 1993), glutamate dehydrogenase (Krauth-Siegel *et al.*, 1996), and G6PD. Additionally, the parasite has a functional thioredoxin system with thioredoxin reductase (Kanzok *et al.*, 2000), thioredoxin (Krnajski *et al.*, 2001), thioredoxin peroxidase (Krnajski, Walter and Müller, 2001), 1 cys-peroxiredoxin (Kawazu *et al.*, 2005), and α-tocopherol (Sussmann *et al.*, 2011).

The oxidative stress induced by antimalarial agents is known to interfere and disrupt the antioxidant-system balance and is important in malaria parasite clearance, both in quinoline-based antimalarial drugs and in endoperoxides: ARTs (Pandey *et al.*, 1999; Kannan, Sahal and Chauhan, 2002; Cui and Su, 2009; Hartwig *et al.*, 2009; Antoine *et al.*, 2014; J. Wang *et al.*, 2015); 4-aminoquinolines: chloroquine (Foley and Tilley, 1997; Ridley *et al.*, 1997; Srivastava *et al.*, 1999; Olafson *et al.*, 2015)**,** amodiaquine (Foley and Tilley, 1997; Ridley *et al.*, 1997), piperaquine (Davis *et al.*, 2005) and pyronaridine (Auparakkitanon *et al.*, 2006; Kritsiriwuthinan *et al.*, 2011); 8-aminoquinolines: primaquine (Beutler, 1994; Bowman *et al.*, 2005; Bowman, Jollow and McMillan, 2005; Kavishe, Koenderink and Alifrangis, 2017); arylamino alcohols: mefloquine (Sullivan *et al.*, 1998; Gunjan *et al.*, 2016) and lumefantrine (Sullivan *et al.*, 1998) (Kavishe, Koenderink and Alifrangis, 2017).

I.6 - Effects of endoperoxides on mitochondria of *P. falciparum*

The mitochondria of malaria parasites differ from that of other eukaryotic cells. They are present in all stages, including the ring stage, and share a close association with the apicoplast (Hopkins *et al.*, 1999). The proximity of these organelles has been hypothesized as necessary for metabolic interaction (Aikawa, 1966; Hopkins *et al.*, 1999). The parasite´s mitochondria is involved in several metabolic pathways, including pyrimidine biosynthesis, iron-sulfur cluster and heme biogenesis, the biosynthesis of ubiquinone, and tricarboxylic acid metabolism (Painter *et al.*, 2007; Painter, Morrisey and Vaidya, 2010; Peatey *et al.*, 2015).

The electron transport chain (ETC) of intraerythrocytic malaria parasites is believed to contain five dehydrogenases, namely NADH: ubiquinone oxidoreductase (*Pf*NDH2),

succinate: ubiquinone oxidoreductase (Complex II or SDH), glycerol-3-phosphate dehydrogenase, the malate quinone oxidoreductase (MQO) and dihydroorotate dehydrogenase (DHODH) (Nixon *et al.*, 2013b). The ETC helps to maintain a proton gradient, an evolutionarily conserved prerequisite for protein and metabolite transport across the inner mitochondrial membrane (Eckers *et al.*, 2012). However, the major function of the ETC in asexual blood-stage cultures of *P. falciparum* is to regenerate ubiquinone as the electron acceptor of the mitochondrial DHODH, which catalyzes a key step in pyrimidine biosynthesis (Painter, Morrisey and Vaidya, 2010; Ehrhardt *et al.*, 2013).

Mitochondrial dysfunction can induce apoptotic pathways (Kataoka *et al.*, 2005). The mitochondria control apoptosis acts at several levels, such as maintenance of ATP production and mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondrial membrane permeability, to release certain apoptogenic factors from the intermembrane spaces into the cytosol (Ly, Grubb and Lawen, 2003). Thus, $\Delta\Psi_m$ is a governing parameter of mitochondrial functions and cell health (Kataoka *et al.*, 2005). Changes in $\Delta\Psi_m$ are observed by staining with rhodamine 123 (Rh123), a permeant cationic fluorescencent dye that is selectively accumulated by the mitochondria of living cells. Mitochondria-specific interaction of such molecules is apparently dependent on the high trans-membrane potential (inside negative) maintained by functional mitochondria. Thus, the tests carried out in this work were performed using Rh123 to detect mitochondrial damage (Johnson *et al.*, 1981; Divo *et al.*, 1985).

I.7 – General objectives

A library of new trioxolanes and tetraoxanes was proposed and synthesized at Centro de Ciências do Mar, Faculdade de Ciências e Tecnologia, Universidade do Algarve under the guidance of Professor Maria de Lurdes Cristiano. The main goals were to i) identify newly synthesized trioxolanes and tetraoxanes with potent antimalarial activity and ii) elucidate their mechanisms of action.

I.7.1 – Specific objectives

1. Evaluation of the cytotoxicity of new endoperoxides against mammalian cells.
2. Characterization of antimalarial activity of the new endoperoxides *in vitro*, against strains of *P. falciparum* with different susceptibilities to antimalarials.
3. Determination of the antimalarial activity of new endoperoxides *in vivo*.
4. Evaluation of the genotoxicity of new endoperoxides against mammalian cells.
5. Exploration of the gametocytocidal activity of the new endoperoxides.
6. Identification of stage-specific response of *P. falciparum* to new endoperoxides.
7. Determination of the minimum inoculum for new endoperoxides resistance.
8. Evaluation of mitochondrial membrane potential after treatment with the new endoperoxides.
9. Evaluation of the new endoperoxides role on the increase of reactive oxygen species in *P. falciparum*.

II – MATERIALS AND METHODS

In a recent study conducted by our groups, three synthetic trioxolanes were tested *in vitro* and *in vivo* against a mouse model infected with artemisinin-resistant parasites and the compounds showed high efficacy in clearing the infection (Lobo *et al.*, 2016). These results inspired the expansion of the compounds library and further investigations of their efficacy against artemisinin-resistant *P. falciparum* strains.

The research described in this thesis focused on 4 complementary lines of work. In the first line, a study was carried out to characterize cytotoxicity effects of the new endoperoxides against mammalian cells. In the second line, the antimalarial activity of endoperoxides was characterized *in vitro* in 4 strains of *P. falciparum* with different susceptibilities to antimalarials. In the third line of this investigation, the antimalarial activity against *P. berghei* was evaluated *in vivo*. Finally, in the fourth line we aimed at identifying possible mechanisms of action of these endoperoxides in *P. falciparum*. We evaluated these by performing ring and mature-stages assays, by investigating genotoxicity, gametocytocidal activity, stage-specificity, the effect on mitochondria membrane potential, generation of reactive oxygen species (ROS), and by evaluation of resistance. In Figure II.1 an overview of the studies carried out in this work is presented.

Note: The Materials and Methods chapter is presented in sessions: 1) Biological material used in research; 2) description of the techniques, with laboratory details; 3) research methodology, completing point 2.

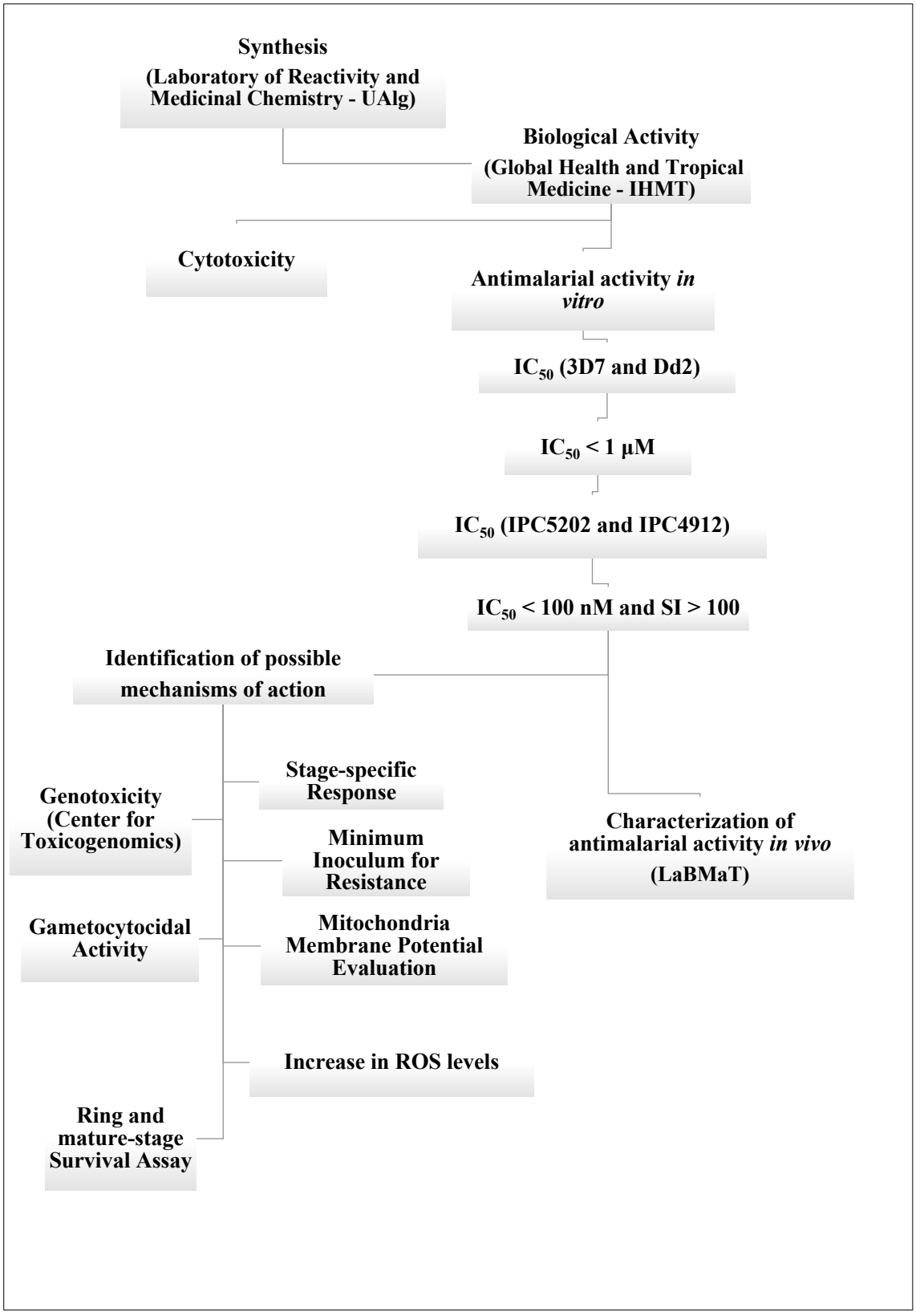


Figure II.1: Flowchart with an overview of the studies carried out in this work.

II.1 – Biological Materials

II.1.1 – Mammalian cells

Two mammalian cell lines were used: (a) a malignant cell line, HepG2-A16; (b) a line of normal cells, V79-2 (Table II.1).

1. **HepG2-A16** cells were stored in the cryo-preserved collection of the UEI Malaria Laboratory/ IHMT;
2. **V79-2** cells were kindly provided by Professor António Sebastião Rodrigues; Centre for Toxicogenomics and Human Health, Genetics, Oncology and Human Toxicology, Nova Medical School/ Faculdade de Ciências Médicas, Universidade Nova de Lisboa.

Table II.1: Characteristics and origins of mammalian cell lines HepG2-A16 and V79.

	HepG2-A16	V79-2
Organism	*Homo sapiens*, human	*Cricetulus griseus*, Chinese hamster
Tissue	Liver	Lung
Morphology	Epithelial	Fibroblast
Culture properties	Adherent	Adherent
Disease	Hepatocellular carcinoma	Not associated
Culture Medium	Williams E (Annex I)	Ham's F-10 Nutrient Mixture (Annex I)

II.1.2 – *P. falciparum* strains

1. 3D7 (Rosario, 1981), sensitive to chloroquine and mefloquine; a cryo-preserved collection of the UEI Malaria Laboratory/ IHMT;

2. Dd2 (Oduola *et al.*, 1988), resistant to chloroquine and mefloquine; a cryo-preserved collection of the UEI Malaria Laboratory/ IHMT;

3. IPC5202 (MRA-1240, MR4, ATCC® Manassas Virginia); provided by Malaria Research and Reference Reagent Resource Center (MR4) for distribution by BEI Resources. It was isolated in 2011 from a human patient with malaria in Battambang province, western Cambodia. This strain has previously shown resistance to artemisinin and the presence of the R539T mutation in the K13 gene. When exposed to dihydroartemisinin (DHA), it demonstrated a ring-stage survival assay (RSA_{0-3h}) value of 88.2%. Deposited by Didier Ménard, Institut Pasteur du Cambodge;

4. IPC4912 (MRA-1241, MR4, ATCC® Manassas Virginia); provided by MR4 for distribution by BEI Resources. It was isolated in 2011 from the blood of a human patient with malaria in the Mondulkiri province, southeastern Cambodia. This strain has previously shown resistance to artemisinin and the presence of the I543T mutation in the K13 gene. When exposed to DHA, it demonstrated (RSA_{0-3h}) value of 49.3%. Deposited by Didier Ménard, Institut Pasteur du Cambodge;

5. 3D7HT-GFP (MRA-1029, MR4, ATCC® Manassas Virginia); provided by MR4, for distribution by BEI Resources. It is a genetic recombinant constructed by integration of green fluorescent protein (GFP) under control of the EF1 promoter into the *Pf*47 gene. This strain constitutively expresses GFP throughout the parasite life cycle, including the mosquito stages. Gametocytes are competent for mosquito infection. Deposited by Andrew Talman and Robert Sinden.

II.1.3 – *Plasmodium berghei* strain

1. ***Plasmodium berghei*** **NK65** was kindly provided by Professor Valter Andrade Neto from Laboratório de Biologia da Malária e Toxoplasmose, Departamento de Microbiologia e Parasitologia, Universidade Federal do Rio Grande do Norte, Brasil. This strain produces lethal infection, which causes high levels of parasitaemia and severe pathogenesis in mice.

II.2 – Description of techniques

II.2.1 – *In vitro* cultures of V79 and HepG2 cells

The mammalian cells were thawed from the liquid nitrogen, washed with phosphate buffered saline (PBS) (Annex II) and seeded into 75 cm^2 tissue culture flasks. After this procedure, cells were allowed to adhere overnight and were subsequently observed under an inverted microscope. The culture media was changed daily until the cultures were confluent and the assays could be initiated.

The HepG2-A16 cells were maintained in Williams medium, supplemented with fetal bovine serum (FBS) (10%), penicillin-streptomycin (1%) and $NaHCO_3$ (0.2%), under a gas mixture containing 5% CO_2, 5% O_2, and 90% N_2, at 37 ºC, in an incubator.

Under the same conditions, the V79 cells were maintained in Ham's F-10 medium, supplemented with newborn calf serum (NBCS) (10%) and penicillin-streptomycin (1%) (Annex I).

II.2.1.1 – Trypsinization of adherent mammalian cells

Trypsinization was performed when cell cultures were confluent. The culture flask medium was removed and 5 ml of trypsin (1x) were added. After 5 minutes, the flask was shaken vigorously so that all the cells were detached from it. After this period, the cell solution was placed in a 15 ml falcon tube for further centrifugation (2500 rpm/ 5min). After centrifugation, the supernatant was removed and 5 ml of sterile PBS were added. Further centrifugation was performed under the same conditions and the supernatant was removed. Cells were then resuspended in 1 ml of sterile PBS. 20 µl of this cell solution were placed in a *neubauer* chamber for cell counting. After counting the cells, 200 µl of culture medium containing 1×10^6 cells/well were added into a flat bottomed 96 well plate and left overnight for adherence. Treatment with the compounds under investigation was started in the following day.

II.2.2 – *In vitro* culture of *P. falciparum*

II.2.2.1 – Defrosting of *Plasmodium spp.*

The cryogenic vials were withdrawn from the liquid nitrogen and maintained at 37° C until complete thawing. The ampoule volumes were measured and transferred to 15 ml falcons. To each falcon, containing 1 ml of culture, 0.2 ml of solution A were added and the mixture was incubated for 3 min. After this incubation, 10 ml of solution B were added. The falcon was centrifuged at 2500 rpm for 5 min. The supernatant was removed and 10 ml of solution C were added to the pellet (Annex I). The culture was centrifuged again, and the supernatant was removed. Finally, whole RPMI was added to the erythrocyte pellet, under the same culture maintenance conditions.

II.2.2.2 – Cryopreservation of *Plasmodium spp.*

Cultures were frozen in liquid nitrogen when they presented high parasitemia and were predominantly at the ring stage. For this, cultures were centrifuged at 2500 rpm for 3 min and the supernatants were discarded. The remaining pellets were resuspended in equal volumes of the cryopreservation solution (Annex I), and finally homogenized. The cultures were transferred to identified cryogenic vials and frozen at -80º C.

II.2.2.3 – Preparation of non-parasitized erythrocytes

In the clinical analysis laboratory of the IHMT, 20 ml of venous blood from healthy donors, preferably of the blood type 0+, were collected with a syringe. The blood was divided between three vacuum tubes containing ethylenediamine tetraacetic acid (EDTA). In the malaria culture laboratory, the blood was then divided into two 15 ml hawk tubes and centrifuged at 2500 rpm for 3 min, in order to remove the plasma and the fraction of white blood cells. 10 ml of sterile PBS were added to each tube. This procedure was repeated 5 times, until the white blood cells were completely withdrawn from our blood sample. Following the blood washing procedure, the same volume of complete sterile RPMI (Annex I) was added to the red blood cells fraction. This erythrocyte

suspension (50% hematocrit) was stored at 4° C and used within a period of up to 15 days. The entire procedure was performed in a laminar flow chamber to maintain sterile conditions.

II.2.2.4 – Culturing of *P. falciparum*

In vitro cultures of *P. falciparum* were prepared and maintained, following methods previously described (Trager & Jensen, 1997), with some modifications. When necessary, *P. falciparum* was thawed from liquid nitrogen (-80ºC) and maintained in human erythrocytes (blood group O+) in RPMI 1640 supplemented with Hepes, AlbuMAX™ I, $NaHCO_3$ and hypoxanthine (Annex I). Cultures were grown under a gas mixture containing 5% CO_2, 5% O_2, and 90% N_2 at 37 ºC, in an incubator. The parasites were diluted with 9.5 ml of culture medium containing 0.5 ml of sufficient non-infected human erythrocytes to yield a final hematocrit of 5% and a parasitaemia of 0.5%, in 75 cm^2 flasks.

Parasite development was evaluated daily by optical microscopy, using thin blood smears stained with 20% Giemsa. Standard *in vitro* cultures were maintained at a parasitaemia below 5%.

II.2.2.5 – Parasitaemia determination

Thin blood smears were prepared, air dried, methanol fixed, and stained with 20% Giemsa (Annex II). 4000 erythrocytes were counted per slide in order to determine the respective parasitaemia, using the following formula:

(Nº of infected erythrocytes/ Nº total of erythrocytes counted) x 100.

II.2.2.6 – Sincronization of *P. falciparum* with D-sorbitol

This method is based on the fact that erythrocytes infected with parasites with more than ± 20 hours of intra-erythrocyte development have a higher osmotic fragility and are lysed due to the action of D-sorbitol (Lambros and Vandemberg, 1979).

Cultures (> 8% parasitaemia) with predominance of ring stages were centrifuged (2500 rpm/3 min). The culture medium was discarded, and 10 ml of 5% autoclaved D-sorbitol were then added to the pellet (Annex II).

After 10 min incubation at 37°C, the culture was centrifuged and washed with sterile PBS. The erythrocytes recovered after washing were incubated under optimum culture conditions.

II.2.2.7 – Sincronization of *P. falciparum* with Percoll®

This method separates schizonts from trophozoites by a density gradient. The medium was removed from the cultures (> 10% parasitaemia) by centrifugation (2500 rpm/ 3 min) and the pellet was slowly added to 10 ml of 70% percoll® (Annex II) pre-heated at 37ºC. The culture was centrifuged at 2300 rpm for 11 min. The phase containing the ring and schizont stages was collected and transferred to a falcon with 40 ml of washing buffer (RPMI 1640 without AlbuMAX I), and then centrifuged at 1800 rpm for 6 min. The erythrocytes recovered after washing were placed under standard culture conditions for reinvasion to occur.

II.2.2.8 – Gametocytes production

P. falciparum 3D7-GFP cultures were used to seed gametocyte cultures at 1-2% parasitemia and 5% hematocrit. Two cultures of 10 ml were synchronized (initial parasitaemia > 10%) with 5% D-sorbitol. After 24 hours, the cultures were divided into four flasks of 10 ml and maintained for more 48 hours (37ºC, 5% O_2, 5% CO_2 and 90% N_2) The percoll® synchronization was performed on day 0 to separate the schizonts, which were maintained for another 24 hours in culture. After this period, treatment with

endovenous heparin (20U/ml) was started for 2 consecutive days and the hematocrit was adjusted to 2.5%. The cultures were maintained for more 24 hours without heparin (Stage II) (Figure II.2) and, at this stage, the compounds toxicity test could be initiated. The RPMI medium was replaced daily (Onambele *et al.*, 2015).

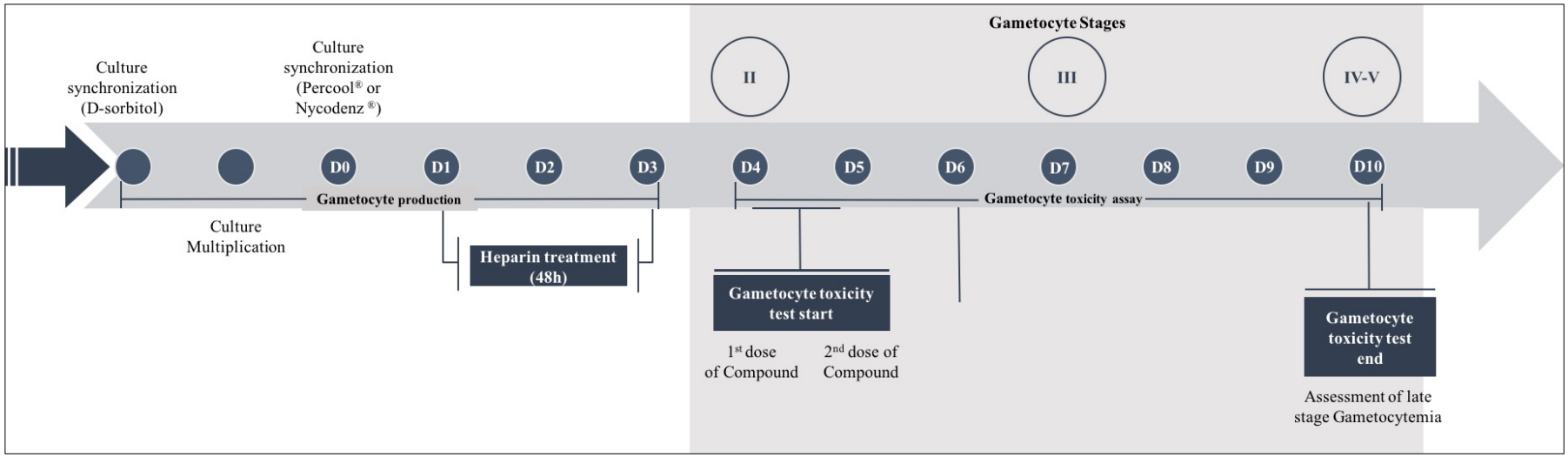


Figure II.2: Optimized protocol for gametocytes production and the gametocytes toxicity assay.

II.3 – Methodology

II.3.1 – Synthesis of endoperoxides with potential antiplasmodial activity

In line with the objectives of the investigation, a chemically diverse library of endoperoxides was designed and synthesized in the laboratory of Organic Reactivity and Medicinal Chemistry of the Centre of Marine Sciences (CCMar), located at the Faculty of Sciences and Technology of Universidade do Algarve, under the supervision of Maria de Lurdes Cristiano. The structures of the novel compounds were chosen keeping in mind the need to build a chemically coherent and diversified library, which would be submitted to evaluation of activity and safety, and from which a small selection of the best performing compounds, with potential broad range antimalarial activity, could be issued. The synthesis of the endoperoxides was carried out by the following researchers: Lília Lameirinhas Cabral (who prepared most of the compounds), Maria Inês Sena, and Bruno Guerreiro. After synthesis, purification and characterisation, the compounds were sent for biological evaluation at the Global Health and Tropical Medicine, Unidade de Ensino e Investigação de Parasitologia Médica, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa. In total, 36 endoperoxides were tested for their cytotoxicities on mammalian cells V79 and HepG2 and screened against 3D7 and Dd2 *P. falciparum* strains. The compounds which exhibited undetected or neglectable cytotoxicity at the range of concentrations tested and showed sub-micromolar antimalarial activity were chosen to be part of the library under study. The references of all the endoperoxides studied are detailed in Annex III and their structures are presented in Figure II.3, Schemes 1 and 2.

Some of the trioxolanes (compounds LC28, LC50, LC92, LC95, LC129, LC130, LC131, LC132, LC136, LC142, MIS13, MIS14, MIS15, and MIS16; Figure II.3, Scheme 1), and some of the tetraoxanes (compounds LC140 and LC163; Figure II.3, Scheme 2) were prepared using the synthetic approaches depicted in Figure II.3, Schemes 1 and 2. Some of the procedures for the preparation of the trioxolane and tetraoxane building blocks were adapted from the literature (Dong *et al*., 2006; Fugi *et al*., 2010). Generally, the endoperoxides LC50, LC67 and LC140 were used as intermediate building blocks for the preparation of other endoperoxide-based compounds, introducing chemical diversity through modification of the cyclohexyl functionality. The synthesis of the trioxolane-

tetrazole, tetraoxane-tetrazole, and trioxolane-saccharyl conjugates were achieved using a convergent approach whereby the endoperoxide and tetrazole or saccharyl building blocks were separately prepared and then linked to form the desired endoperoxide-based targets.

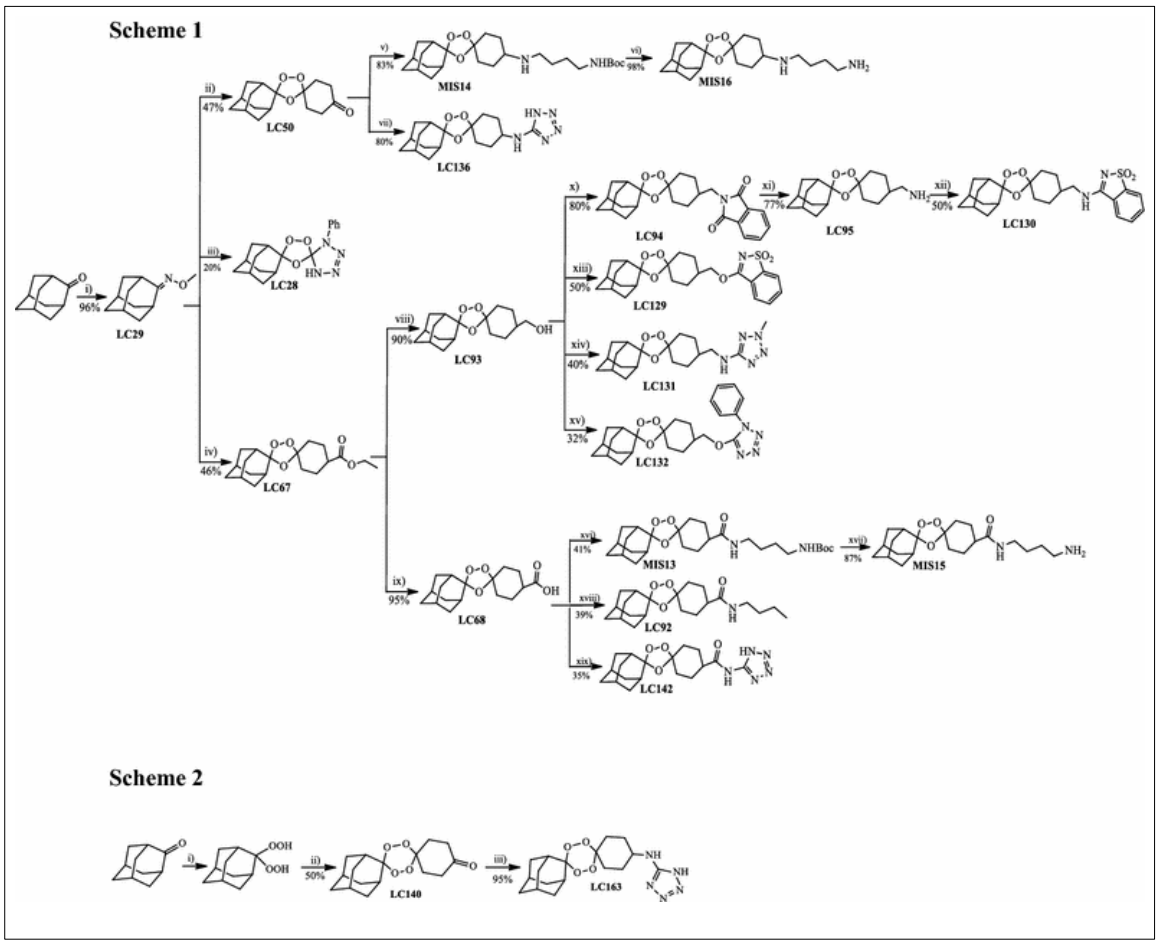


Figure II.3: Representation of the chemical structure of some trioxolanes and some tetraoxanes investigated in this work. Scheme 1: Representation of the synthetic routes to the trioxolanes prepared; reagents and conditions: (i) Pyridine, $MeONH_2$, MeOH, r.t; (ii) 1,4-Cyclohexadione, O_3, DCM/pentane, − 78 °C; (iii) LC133, O_3, DCM/pentane, − 78 °C; (iv) Ethyl 4-oxocyclohexanecarboxylate, O_3, DCM/pentane, − 78 ºC; (v) LC64, AcOH, DCE, $NaBH(OAc)_3$, r.t.; (vi) Trichloroacetic acid, DCM, H_2O, r.t.; (vii) 5-Aminotetrazole, DCE, AcOH, $NaBH(OAc)_3$, r.t.; (viii) $LiBH_4$, Et_2O, $LiBH(Et)_3$, r.t.; (vi) KOH (3M), MeOH, 60 °C; (x) Phthalimide, Ph_3P, DIAD, THF, 0 ºC; (xi) Hydrazine hydrate, chloroform/MeOH, 60 °C; (xii) 3-Chloro-1,2-benzisothiazole-1,1-dioxide, THF, 60 °C; (xiii) 3-Chloro-1,2-benzisothiazole-1,1-dioxide, TEA, toluene, 45 °C; (xiv) 2-Methyl-2H-tetrazole-5-amine, TEA, mesyl chloride, THF, 60 ºC; (xv) 5-Chloro-1-phenyl-tetrazole, TEA, mesyl chloride, THF, 60 °C; (xvi) tert-Butyl(4-aminobutyl)carbamate, EDC, HOBt, N-methylmorpholine, DCM, r.t.; (xvii) Trichloroacetic acid, DCM, H_2O, r.t.; (xviii) Butylamine, EDC, HOBt, N-Methylmorpholine, DCM, r.t.; (ix) 1-Aminobutane, EDC, HOBt, N-Methylmorpholine, DCM, r.t.; Scheme 2: Representation of the synthetic route to tetraoxanes LC140 and LC163; reagents and conditions: (i) HCO_2H, CH_3CN, H_2O_2 50%, 0 °C; (ii) 1,4-Cyclohexadione, DCM, HBF_4, 0 °C; (iii) 5-Aminotetrazole, DCE, AcOH, $NaBH(OAc)_3$, r.t.

II.3.2 – Evaluation of the cytotoxicity against mammalian cells using the MTT assay

Cytotoxicity of the compounds was assessed on the mammalian cell lines HepG2-A16 and V79, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich). For the cytotoxicity assays, 1 x 10^6 cells/well were seeded in a flat-bottomed 96-well plate and allowed to adhere overnight. After removing the medium, the compound under study was added to the seeded cells. Tests were conducted for each compound at concentrations ranging from1 mM to 1.372 μM, in triplicate (concentration of DMSO < 1%). Three independent assays were performed *per* tested compound. A negative control was also included by adding drug-free medium with 1% of DMSO. V79 and HepG2 cells were incubated for 24 h and 48 h, respectively (medium with drugs was changed at 24 h interval). After finishing the treatment, 20 μl of MTT (5 mg/mL in PBS) were added to each well and the plates were incubated for 3 h under standard culture conditions. Supernatant was then removed and 200 μl DMSO were added to each well. Absorbance was read at 570 nm on a multi-mode microplate reader (Triad, Dynex Technologies) to produce a log dose-dependence curve. The LD_{50}, dose required to kill half of the cells, was estimated for each compound by non-linear interpolation of the dose-dependence curve (GraphPad Software).

II.3.3 – Evaluation of the *in vitro* antimalarial activity against erythrocyte stages

II.3.3.1 – Half maximal inhibitory concentration (IC_{50}) determination using whole cell SYBR Green I assay

Antimalarial activity of the compounds was determined by using the SYBR Green I assay, as previously described (Machado *et al.*, 2016). Early ring stage parasites (> 80% of rings, 3% hematocrit and 1% parasitaemia) were tested in triplicate in a 96-well plate and incubated in standard culture conditions with the compounds for 48h. Parasite growth was assessed with SYBR Green I (100 μl of a solution 0.001% v/v in PBS to each well). Each compound was tested with concentrations ranging from 10000 nM to 0.169 nM and at least three independent tests were conducted for each concentration. Fluorescence intensity was measured (multimode microplate reader; Dynex Triad) with excitation and emission wavelengths of 485 and 535 nm, respectively, plotted to generate dose-response

curves and analysed by nonlinear regression using GraphPad Prism to determine IC_{50} values. The IC_{50}, half maximal inhibitory concentration, is a measure of the effectiveness of a compound in inhibiting parasites growth.

II.3.3.2 – Adapted Ring-stage survival assay

Parasite cultures were synchronized twice (6 h interval window) with 5% D-sorbitol (See II.2.3.6). Ring-stage survival assays (RSA) were carried out as described previously (Witkowski *et al*., 2013a), with some modifications. Ring-stage parasite cultures (<10 h post-reinvasion) contained in a 96-well plate with 1% parasitaemia and 2% hematocrit, were exposed during a 6 h pulse to 700 nM of dihydroartemisinin (DHA) or to each of the tested compounds at a concentration corresponding to ≅ 10x the respective IC_{50}.

Subsequently, plates were centrifuged, and the supernatant was replaced by drug free medium. After additional incubation (66 h), susceptibility was assessed microscopically on Giemsa stained thin blood smears by estimating the percentage of viable parasites that had developed into a second generation of rings (Figure II.4).

These assays provide the first robust correlation between reduced clearance of clinically resistant parasites and reduced killing by DHA *in vitro* (Haldar, Bhattacharjee and Safeukui, 2018).

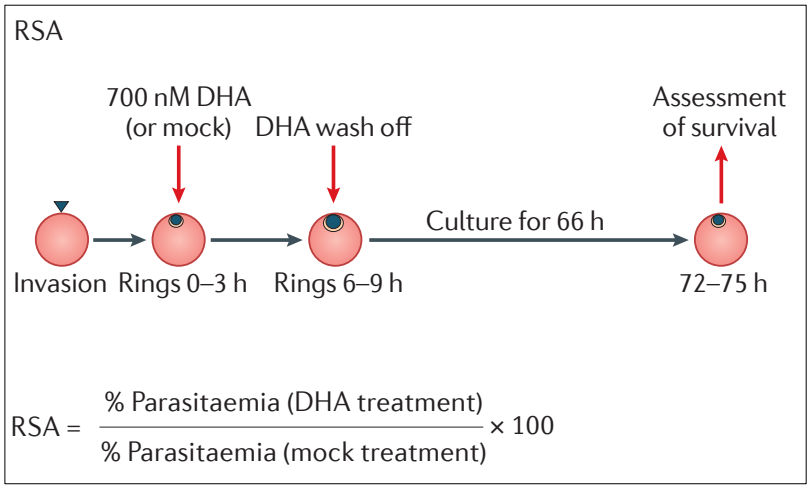


Figure II.4: Correlating a clinical phenotype and artemisinin-resistant proliferation in the laboratory. RSA, ring-stage survival assay. (Adapted from (Haldar, Bhattacharjee and Safeukui, 2018)).

II.3.3.3 – Adapted Mature-stage survival assay

Parasite cultures were synchronized twice (6 h interval window) with 5% D-sorbitol (See II.2.3.6). Mature-stage survival assays (MSA) were carried out as described previously (Witkowski *et al*., 2013a), with some modifications. Mature-stage parasite cultures (>36h post-reinvasion), contained in a 96-well plate with 1% parasitaemia and 2% hematocrit were exposed during a 6 h pulse to 700 nM of DHA or to each of the tested compounds, at a concentrations corresponding to ≅ 10x the respective IC_{50}.

Subsequently, plates were centrifuged, and supernatant replaced by drug free media. After additional incubation (42 h), susceptibility was assessed microscopically on Giemsa stained thin blood smears by estimating the percentage of viable parasites that had developed into a second generation of schizonts.

II.3.3.4 – Gametocytocidal activity of the new endoperoxides

P. falciparum 3D7-GFP parasites were cultured at high parasitaemia to favor gametocyte formation, as described above (Figure II.2). Upon the appearance of stage II gametocytes, treatment with the compounds was initiated (Figure II.3).

1 ml of gametocyte culture was aliquoted (parasitemia 0.5 - 1% and 1% hematocrit) in duplicate on a 24-well plate and treated with the new endoperoxides, DHA and PQ, at their respective IC_{50s} (to asexual and sexual stages), previously determined for 3D7 strain (Table III.1; Annex IV). An extra concentration, equivalent to 10x IC_{50} to sexual stages of the PQ was also used as a control (Table II.2) (Leliévre *et al.*, 2012; Gebru, Mordmuller and Heldb, 2014).

Table II.2: Respective IC_{50s} of the new endoperoxides, DHA and PQ used in the gametocytocidal activity assay.

Compounds	Equivalent IC_{50}	Concentrations
PQ	1 x IC_{50} asexual stages	1 µM
PQ	1 x IC_{50} sexual stages	20 µM
PQ	10 x IC_{50} sexual stages	200 µM
DHA	1 x IC_{50} asexual stages	4.7 nM
DHA	1 x IC_{50} sexual stages	4.7 µM
LC131	1 x IC_{50} asexual stages	2.9 nM
LC131	1 x IC_{50} sexual stages	2.9 µM
LC136	1 x IC_{50} asexual stages	2.8 nM
LC136	1 x IC_{50} sexual stages	2.8 µM
MIS13	1 x IC_{50} asexual stages	7.4 nM
MIS13	1 x IC_{50} sexual stages	7.4 µM

During the first 48 hours of the assay, the gametocytes were treated with the test compounds at their respective concentrations. After this incubation, the pre-warmed RPMI medium with no testing compounds was changed daily. After 6 days, Giemsa-stained blood smears were prepared and the gametocytemia was evaluated by counting

the number of stage IV and V gametocytes (Table I.1) in a total number of 4000 erythrocytes. The experiments were performed at least 2 times.

II.3.4 – *In vivo* antimalarial activity

In vivo tests were performed following the Guidelines for Ethical Conduct in The Care and Use of Animals of the Federal University of Rio Grande do Norte - Brazil (CEUA/UFRN/ 46/2013) (Annex X).

The cryopreserved *P. berghei* NK65 strains were thawed and intraperitoneally (inoculum blind) inoculated immediately in swiss albino mice. After four days, the parasitemia for the standard inoculum preparation was checked for 1 x 10^6 parasitized erythrocytes/200 µl for infection of each mice.

Evaluation of antimalarial activity *in vivo* was carried out by using the Peters' 4-day suppressive test (Peters, 1965), with modifications, as previously described (Baptista *et al*., 2010; Carvalho *et al*., 1991). Briefly, adult swiss albino female mice weighing 20 ± 2 g were injected intraperitoneally with infected blood containing 1 x 10^6 *Plasmodium berghei* in PBS (200 µl final volume). Mice were randomly allocated to groups of five animals *per* cage. The compounds were tested using the gavage technique. For oral treatment, compounds were diluted in DMSO solution with PBS (final concentration of DMSO < 1%), and 200 µl of the solution at the concentration of test was administrated orally to each animal in a dose of 50 mg/kg/day, for four consecutive days (Figure II.5). A 1% solution of DMSO in PBS was orally administered to each animal of the untreated control group. On days 5 (D5), 7 (D7) and 10 (D10), after parasite inoculation, tail blood smears were Giemsa stained and examined microscopically to estimate parasitaemia. Acute toxicity testing was carried out to determine adverse effects due to compounds administration (including weight loss, hair appearance, skin wounds and behavioural changes), and recorded daily (de Sena Pereira *et al.*, 2016).

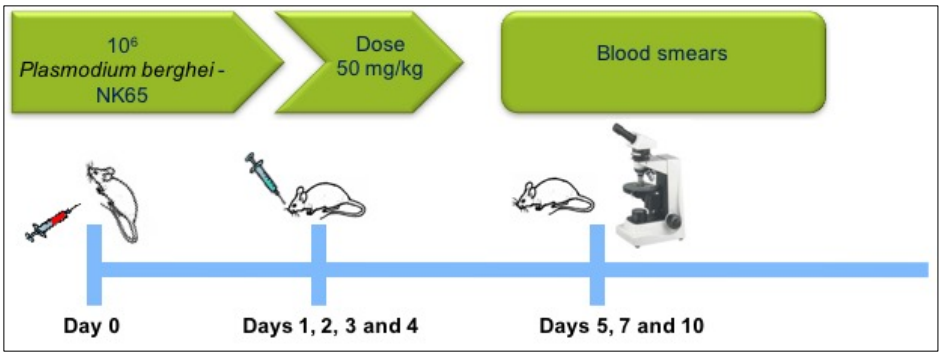


Figure II.5: Evaluation of antimalarial activity *in vivo* using the Peters' 4-day suppressive test, with modifications.

II.3.5 – Evaluation of the genotoxicity against mammalian cells using Comet assay or Single cell gel electrophoresis assay (SCGE)

V79 cells were grown in 75 cm^2 flasks (Corning) at a concentration of 10^6 cells *per* bottle and treated with different concentrations of the tested compounds (at different concentrations; 1, 10, 100, and 500 µM) and with doxorubicin (the positive control, at 20 µM) for 3 h. After treatment, the cells were trypsinized and 30 µl of this cell pellet was homogenized with 150 µl of a low-melting-point agarose (0.5%), spread onto micro-scope slides pre-coated with a normal melting point agarose (1.5%) and covered with a coverslip. After 5 min at 4º C, the coverslip was removed and the slides were immersed in cold lysis solution for 2 h. After lysis, the slides were placed in an electrophoresis chamber and covered with freshly made electrophoresis buffer. The electrophoresis was run for 20 min (25 V). Afterwards, the slides were neutralized by spray neutralization buffer three times, and fixed in ethanol (50º - 70º - 100 ºC) for 5 min in each concentration. Staining of the slides was performed immediately before the analysis using GelRed™ (VWR). Slides were prepared in triplicate, and 1500 cells were screened *per* sample (500 cells from each slide) using a fluorescence microscope. The damage was quantified based on the size, intensity and the percentage of DNA in the comet's tail, using the software Comet Score.

II.3.6 – Characterizations of antimalarial activity

II.3.6.1 – Stage-specificity analysis (susceptibility)

Stage-specific compound treatment effects were elucidated using highly synchronized cultures of 3D7-GFP in 96-well plates with a starting parasitemia of 1% and a 2% hematocrit. Parasites were treated for 24 h with 10x IC_{50} concentrations of compounds LC92, LC129, LC130, LC131, LC132, LC136, MIS13, and LC163 which showed the best antimalarial activity in the *in vitro* and *in vivo* assays, or controls (DHA and atovaquone (ATQ)), during two distinct periods of the intraerythrocytic life cycle - rings and trophozoites (Le Manach *et al*., 2013; Sundriyal *et al*., 2014). After 24 h of treatment, one part of the parasites were washed with pre-heated RPMI 1640 medium and re-cultured without the test compounds for reinvasion analysis (48 h). Another part of the

parasites were washed with RPMI 1640 medium, pre-heated and diluted 1:16 and allowed to grow without compound for another two cell cycles (96 h). Parasitaemias were always monitored at times 0, 48 and 96 hours by Giemsa-stained thin blood smears and by flow cytometry for the fluorescence measured with excitation wave 488 nm and emission 525 nm (green) (CytoFLEX Blue-Red-Violet (B-R-V) (Beckman Coulter).

II.3.6.2 – The minimum inoculum for compound resistance (MIR)

Resistant parasites can be selected by applying drug pressure *in vitro* and *in vivo*. These approaches have successfully identified genes, and sometimes codons involved in naturally occurring resistance.

The simplest approach is *in vitro* selection of resistant parasites, which can be applied on a large number of compounds earlier in drug discovery. *P. falciparum* intraerythrocytic cultures, with starting inocula ranging from 10^5 to 10^9 parasites, are exposed to a concentration of the compound nearing IC_{90} (≅ 10x IC_{50}) and monitored during 60 days for recrudescent parasites.

The minimal inoculum for resistance (MIR) can be determined and is an indirect measure of the likelihood of a resistant genotype to occur and to be selected *in vitro*. In addition, the IC_{50} shift of the resistant mutants as compared to the parental sensitive strain is a measure of the resistance intensity. The MIR is a measure of the resistance selection frequency, while the IC_{50} fold increase measures the level of resistance (Ding, Ubben and Wells, 2012).

Dd2 *P. falciparum* intraerythrocytic cultures have a known resistance profile, showing an increase in the number of copies of *pfmdr1* gene and mutations at several codons. These changes influence sensitivity to multiple drugs, including mefloquine and artemisinin derivatives.

Dd2 *P. falciparum* cultures were used in this study, with inoculum of 10^7 and 10^8 parasites and were exposed to a concentration of the compounds LC131, LC132, LC136, MIS13, and LC163 with the correspondent 10x IC_{50}, and monitored during 60 consecutive days for recrudescence of the parasites (Figure II.6).

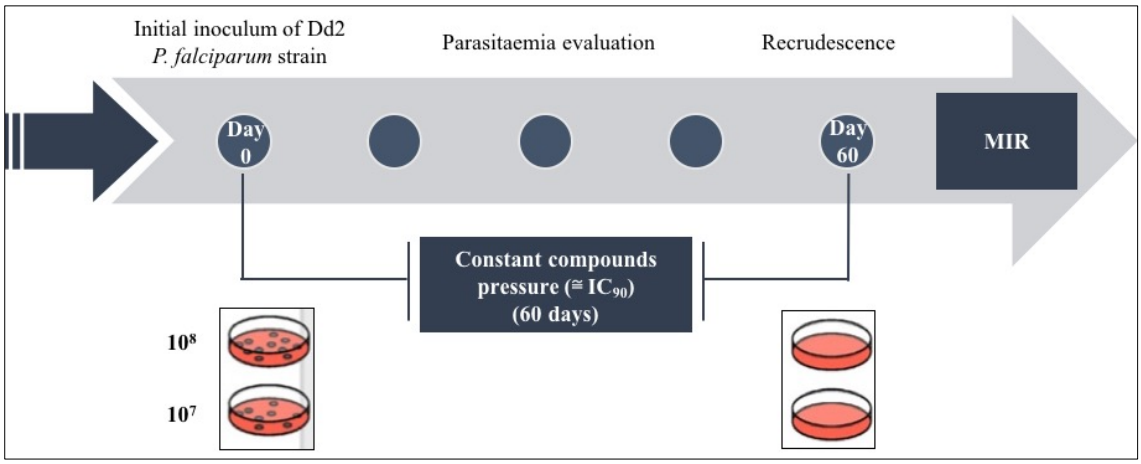


Figure II.6: *In vitro* resistance selection assessment. Initial inoculum of 10^7 and 10^8 parasites of Dd2 *P. falciparum* strain were pressured with a constant level of endoperoxides (10x IC_{50}), for 60 days.

II.3.6.3 – Effects of endoperoxides on mitochondrial membrane potential

Rhodamine 123 (Rh123) (Sigma-Aldrich) was used to monitor the potential of the plasma membrane ($\Delta\psi_p$) and mitochondrial membrane ($\Delta\psi_m$) of malaria-infected red blood cells. Rh123 is cationic and reversibly accumulates inside energized membranes according to the Nernst equation. For experimentation, suspensions (1% parasitemia and 2% hematocrit) of two different cultures of *P. falciparum* 3D7 and IPC5202, not synchronized, were treated for 6 h with the new endoperoxides and DHA (10x IC_{50}), ATQ (10 µM) (Biagini *et al.*, 2006) or deoxy-dihydroartemisinin (Deoxy-DHA) (8 µM) (Wang *et al.*, 2010) in 96-well black plate. Deoxy-DHA, which lacks the endoperoxide bridge, is ineffective in inhibiting malaria parasites and was used as a negative control (Wang *et al.*, 2010). After treatment, the cultures were stained with Rh123, at 5 µM for 5 min, washed, and left for 30 min (37ºC, 5% CO_2).

For imaging, malaria parasite-infected erythrocytes were placed on 8-well chambered coverslips (Ibidi GmbH, Munich, Germany) and the membrane potential-dependent fluorescence responses were monitored in real time. The fluorescence signals from malaria-infected erythrocytes were collected on a Leica TCS SP5 (Leica Microsystems CMS GmbH, Mannheim, Germany) inverted confocal microscope (DMI6000) through a 63x amplification apochromatic water immersion objective with NA=1.2. Excitation of Rh123 was performed using the 488 nm argon laser line (emission collected at 500 –

652nm). All images where collected using the same acquisition parameters. Dye photobleaching during image acquisition was also avoided by always keeping the power of each laser line to a minimum. The captured digital images were analyzed using ImageJ software to calculate the average Rh123 fluorescence intensity from a ROI encompassing each Rh123 labeled-malaria parasites (Sarmento *et al.*, 2016).

After fluorescence microscopy, the fluorescence intensity was measured with a multimode microplate reader (Dynex Triad), with excitation and emission wavelengths of 488 and 530 nm, respectively.

After fluorescence reading, bafilomycin A_1 (200 nM) was added to the cultures, treated for 5 min, washed, and left for 30 min (37 ºC, 5% CO_2). At this stage, the fluorescence intensity was measured again, under the same conditions. Bafilomycin A_1, a V-type H^+ ATPase inhibitor, was used to inhibit the plasma membrane potential and to allow measurement of only the mitochondrial membrane potential (Biagini *et al.*, 2006).

After performing the measurements, cultures were fixed with 4% paraformaldehyde (PF) and 0.0075% glutaraldehyde (GLU) for further fluorescence microscopy visualization. Hochest 58 was also used to stain the DNA.

ATQ, a hydroxynaphthoquinone, was used as a control because it is a known inhibitor of complex III (or *bc*1 complex) and the consequence of this inhibition is the collapse of mitochondrial membrane potential of the parasite. Thus, it was possible to compare the activity of our endoperoxides with ATQ (Antoine *et al.*, 2014).

II.3.6.4 – Reactive oxygen species measurement

Membrane-permeant fluorescent probes such as the CM-H_2DCFDA (Invitrogen™) have been widely used to detect oxidative stress and to measure antioxidant capacity in the cytoplasm of different cell types (LeBel, Ischiropoulos and Bondy, 1992). When the reduced form of CM- H_2DCFDA is added to cells, it diffuses freely across membranes and the diacetate groups are cleaved by intracellular esterases to give a deacetylated nonfluorescent form, 2′,7′- dichlorofluorescin (H_2DCF) (Halliwell and Whiteman, 2014). The CM-H_2DCFDA probe also contains a thiol-reactive chloromethyl group (CM-),

which forms covalent bonds with intracellular components, enhancing the retention of the probe. Oxidation of H_2DCF by ROS yields highly fluorescent 2′,7′-dichlorofluorescein (DCF) which is trapped inside the cell, thus facilitating long-term studies (Fu *et al.*, 2010).

Furthermore, in this work, CM-H_2DCFDA was used to assess oxidative stress in different stages of the intraerythrocytic development of *P. falciparum* in strains 3D7 and IPC5202. To quantify DCF fluorescence in parasite-infected RBCs within a mixed culture, we used the oxidative stress sensor in combination with a nucleic acid-binding dye that can distinguish infected erythrocytes in a flow cytometric format.

The SYTO™ 61 (Invitrogen™), red cell fluorescent nucleic acid staining, was chosen to be used in combination with DCF (Fu *et al.*, 2010).

a) Labeling with Probes

With the objective of analyzing the oxidative stress, trophozoite or ring stage parasites or asynchronous culture (2% hematocrit, 5% parasitemia) were previously treated for 6 h with the controls CQ or DHA (1x IC_{50}, 10x IC_{50} and 100x IC_{50}) or endoperoxides (10x IC_{50}). Deoxy-DHA (8 μM) was also used, as a negative control (Wang *et al.*, 2010). CQ was used as a positive control due to its known mechanism of action as an inducer of oxidative stress (Vallières and Avery, 2017), by the inhibition of heme polymerization.

The same procedure was run in parallel with uninfect and infected erythrocytes incubated with 0.5 μM H_2O_2 (positive control) (Sigma-Aldrich) (Sussmann *et al.*, 2017), for 30 min, before the DCF and SYTO™ 61 addition.

After the treatment, the cultures were washed with pre-warmed PBS and incubated with 0.5 μM SYTO™ 61 for 45 min in the dark, at 37 ºC, and with 2,5 μM DCF for 30 min, in the same conditions, and then washed with PBS. The samples were then maintained at 20 ºC and analyzed as quickly as possible (Klonis *et al.*, 2011; Mohring, Jortzik and Becker, 2016).

b) Flow Cytometry

Flow cytometric measurements were performed on a CytoFLEX Blue-Red-Violet (B-R-V) Flow Cytometer (Beckman Coulter) equipped with 405 nm, 488 nm and 633 nm lasers. We used the following channels and probes: green (DCF; 492 - 495 nm excitation, emission 517 - 527 nm bandpass) and red (SYTO^{TM} 61; 628 nm excitation, emission 645 nm bandpass). Samples were diluted to 0.5 - 1% hematocrit and 10 000 events (encompassing uninfected and infected RBCs) were acquired based on the forward *versus* side scatter profiles. Detector gain settings varied between experiments to optimize signals but were kept constant within individual experiments and no compensation was applied to any of the channels. Analysis was performed using FlowJo Vx0.7.

III – RESULTS AND DISCUSSION

Malaria is one of the most important parasitic diseases in the world, causing a major impact on the socio-economic development of the affected countries, with over 445 000 deaths occurring mainly in children and pregnant women in sub-Saharan Africa (WHO, 2017b). Over the past five decades, the emergence of *P. falciparum* resistance to the successively introduced antimalarials, including artemisinin-based combination therapies (ACTs) (WHO, 2017b), had substantial and significant implications for malaria control programs and for global public health (Lwin *et al.*, 2015; WHO, 2017a).

The recent findings of resistance to ACTs motivated further efforts towards the development of a next generation of potent antimalarial endoperoxides (Copple *et al.*, 2012), equally effective against ART-susceptible and -resistant strains of *P. falciparum,* as well as safer and cheaper than ARTs. (Yang *et al.*, 2016).

The core structure of ARTs comprises a 1,2,4-trioxane incorporating an endoperoxide linkage that is essential for activity (Tang, Dong and Vennerstrom, 2004; O'Neill, Barton and Ward, 2010). The identification of the endoperoxide as pharmacophore has stimulated the development of several different classes of synthetic endoperoxides, including trioxolanes (Vennerstrom *et al.*, 2004) and tetraoxanes (Vennerstrom *et al.*, 1992; Fontaine *et al.*, 2015), which are particularly promising in the context of antimalarial chemotherapy, exhibiting similar activity to the ARTs (O'Neill *et al.*, 2017).

The discovery of novel drugs leads with the potential to become usable medicines is an important component of the drug innovation cycle, but remains a major obstacle in the development of new drugs for infectious tropical diseases (Nwaka and Hudson, 2006). However, the feasibility of efficient medicines, whether synthetic or from natural products, requires investigation of their safety and efficacy prior to their production and release in the market, which refer to different paradigms in drug research and development and scientific controversies (Purves *et al.*, 1995); fact that hinders its production.

The identification of therapeutic and their toxicological effects must be evaluated (Aquino, Perazzo and Maistro, 2011), and serious biological side effects may result in cancellation of drug development or return to basic chemistry to modify structure and to reduce risks. Regulatory authorities around the world require data on the toxic potential

of new compounds as part of the safety assessment process (Jena, Kaul and Ramarao, 2002; Rivera *et al.*, 2014).

Some desirable criteria for the development of new antimalarial drugs were created by the committee coordinated by the Global Fund for Health Innovation Technology (GHIT) (Katsuno *et al.*, 2015) and were followed in this work. The *in vitro* criteria are as follows:

Validated hit:

- *Cellular potency criteria: hits should have an effective concentration for half-maximum response (IC_{50}) <1 μM for sensitive and multiple resistant strains of Plasmodium spp.*
- *Cytotoxicity criteria: hits require a greater than 10-fold selectivity between the half-maximal cytotoxic concentration (LD_{50}) for the mammalian cell line and the IC_{50} for Plasmodium spp.*

Early lead:

- *Cellular potency criteria: a lead requires IC_{50} <100 nM for sensitive and multidrug-resistant strains of Plasmodium spp.*
- *Cytotoxicity criteria: a lead should have a greater than 100-fold selectivity between mammalian cell line LD_{50} and Plasmodium IC_{50}. Frontrunners should be tested across the malaria life cycle and key mechanistic assays so as to ensure an understanding of the phenotype and target candidate profile potential of each series and novel mechanisms of action.*

Synthetic trioxolanes and tetraoxanes have shown promise as next-generation antimalarial drug candidates, particularly in terms of therapeutic efficacy and lack of synthetic constraints (Copple *et al.*, 2012). Like this, in a recent study conducted by our group, three synthetic trioxolanes were tested *in vitro* and *in vivo* against a mouse model infected with artemisinin-resistant parasites and the compounds showed high efficacy in clearing the infection (Lobo *et al.*, 2016). These results inspired the expansion of the compounds library and further investigation of efficacy against artemisinin- resistant *P. falciparum* strains.

The major objectives of this thesis were the characterization of antimalarial activity of newly synthesized trioxolanes and tetraoxanes and the identification of potential mechanisms of action. This research was carried out on 4 complementary lines of work (Figure II.1). In the first line, a study was carried out to characterize cytotoxicity effects of the new endoperoxides against mammalian cells. In the second line, the antimalarial activity of endoperoxides was characterized *in vitro* in 4 strains of *P. falciparum* with different susceptibilities to antimalarials. In the third line of this investigation, the antimalarial activity of selected endoperoxides against *P. berghei* was evaluated *in vivo*. And, finally, in the fourth line it was possible to identify possible mechanisms of action of the endoperoxides in *P. falciparum* were assessed, by evaluating the parasite-stage specific action (against rings and mature-stages), genotoxicity, gametocytocidal activity, effect on mitochondria membrane potential, generation of reactive oxygen species (ROS) and evaluation of potential for resistance development.

The results are presented and discussed according to the proposed objectives.

III.1 – Evaluation of the cytotoxicity of the endoperoxides against mammalian cells

The endoperoxides were evaluated for their potential cytotoxicity against human hepatocellular carcinoma (HepG2) and hamster lung (V79) cell lines (See II.1.1) using an MTT assay (See II.3.2). Generally, all compounds have shown low or undetectable cytotoxicity in both cell lines (Annex III). Of the 36 compounds studied, only LC90 and LC139 presented *in vitro* cytotoxicity at the maximum concentration tested (1 mM), with viability reduction of ≅ 20% in V79 and HepG2 cells. The compounds MIS13 and MIS14 also revealed some *in vitro* reduction of viability at a maximum concentration of 1mM, inducing a decrease in HepG2 survival of ≅ 30%. However, no decrease in viability for V79 was observed upon treatment with MIS13 or MIS14.

In vitro studies using cancer cell lines suggested that ART and some of its derivatives have cytotoxic effects, by altering the cell cycle, inducing apoptosis (Hou *et al.*, 2008; Zhang, Chen and Gerhard, 2010) and proliferation of these cells (Efferth *et al.*, 2001). Genotoxic and cytotoxic effects of ATN *in vitro* in Chinese hamster ovary cells (CHO-9) are also reported (Li *et al.*, 2008; Aquino *et al.*, 2013).

Our tested compounds exhibited low cytotoxicity when compared to the ART, which, at concentration of 0.11 mM, lead to a decrease in cell viability of 40% in both cell lines. Thus, from the viewpoint of safety, all of the 36 compounds evaluated performed better than ART showing lower toxicity (Annex III).

Thus, as the endoperoxides have shown low or undetectable cytotoxicity to HepG2 and V79 cells, were further evaluated for antimalarial activity.

III.2 – Evaluation of the *in vitro* antimalarial activity against asexual blood stages of *P. falciparum*

III.2.1 – Determination of the IC_{50} and selectivity index to parasites in culture

According to the criteria above, antimalarial activity tests were performed on several *P. falciparum* strains.

The *in vitro* activity of the 36 newly synthesized endoperoxides was screened against chloroquine-susceptible (3D7) and multidrug-resistant (Dd2) *P. falciparum* strains, using the whole cell Sybr Green I assay (See II.3.3.1) to determine the IC_{50} values. The antimalarials ART, artesunate (ATN), dihydroartemisinin (DHA) and chloroquine (CQ) were used as reference drugs. The values of IC_{50} obtained for the new endoperoxides are available in Annex IV.

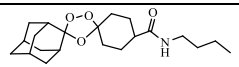
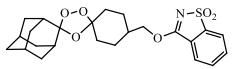
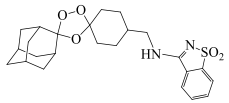
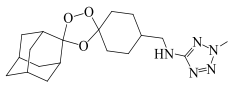
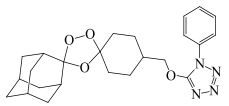
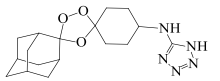
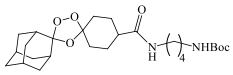
Of the 36 compounds tested, 22 presented sub-micromolar activity ($IC_{50} < 1$ μM) (Fidock *et al.*, 2004) against both 3D7 and Dd2 strains. From these, 9 exhibited $IC_{50} < 100$ nM (LC92, LC129, LC130, LC131, LC132, LC136, LC163, MIS13 and MIS14). These best performing 9 compounds were selected for further testing against two ART-resistant strains (ART-R), IPC5202 and IPC4912; 8 (LC92, LC129, LC130, LC131, LC132, LC136, MIS13 and LC163) exhibited $IC_{50} < 100$ nM (Katsuno *et al.*, 2015) against these strains. In addition to sub-micromolar activity, these eight compounds also demonstrated a resistance index ranging from 0.1 to 2.4 (RI = IC_{50} resistant strain /IC_{50} susceptible strain). Thus, compounds LC92, LC129, LC130, LC131, LC132, LC136, MIS13 and LC163 were selected for further analysis. The RI provides a quantitative measure of the antiplasmodial activity against resistant strains and reveals promising drug discovery

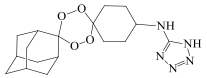
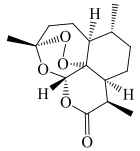
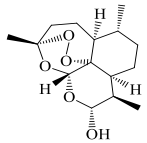
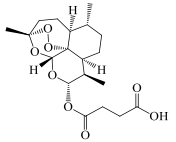
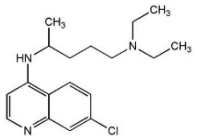
leads. The higher the RI, the higher is the probability of cross resistance with antimalarials in use. Thus, no cross-resistance with quinolone-type antimalarials is foreseeable for our compounds, as RIs between the multidrug-resistant (Dd2) and the chloroquine-susceptible (3D7) are ≅ 1 (Table III.1). Regarding the ART-R strains (IPC5202 and IPC4912), even though the RIs ranged from 0.1 to 2.4 the compounds still performed better, or in the same range, as ART and DHA. As shown in Table III.1, the eight selected compounds were exceptionally active across all four *P. falciparum* strains, with IC_{50} ranging from 0.3 - 71.1 nM. Though LC136 presented RIs > 1 (0.9 - 2.4), the values fall within the range of those exhibited by ART derivatives for the same parasite strains (0.6 - 8.4).

It is generally accepted that, if SI >10, the observed pharmacological activity is not due to cytotoxicity (Weniger *et al.*, 2001; Soh and Benoit-Vical, 2007; Katsuno *et al.*, 2015). Since the SI values calculated for the compounds are considerably higher, then the activity exhibited by the compounds is unlikely due to general cellular toxicity, but rather due to specific antiplasmodial activity.

The selectivity index (SI = LD_{50} in mammalian cells/ IC_{50} *P. falciparum*) for the 36 compounds determined in two mammalian cell lines, V79 and HepG2, is presented in Annex V. The eight best performing compounds (Table III.1) presented SI > 14064.7 (> 1400-fold selectivity between LD_{50} and IC_{50}) (Katsuno *et al.*, 2015). The calculated SI is considerably higher than 10 (Annex V).The eight endoperoxides selected from our library that met the criteria: SI > 100 and IC_{50} < 100 nM (Nwaka and Hudson, 2006; Katsuno *et al.*, 2015) were evaluated for *in vitro* and *in vivo* efficacy, demonstrating IC_{50} values in the range of those exhibited by ARTs. Hence, the tested compounds can be considered to have negligible *in vitro* toxicity against mammalian cells while being highly selective against the parasite (Annex V).

Table III.1: Antiplasmodial activity *in vitro* against sensitive and resistant *P. falciparum* strains

Compounds		*P. falciparum* IC_{50} ± SD (nM)				Resistance Index		
ID	**Structure**	**Dd2**	**3D7**	**IPC5202**	**IPC4912**	**RI[a]**	**RI[b]**	**RI[c]**
LC92		22.6 ± 2.6	26.7 ± 2.4	37.7 ± 9.9	34.9 ± 7.9	0.8	1.4	1.3
LC129		43.2 ± 0.8	42.9 ± 3.9	36.4 ± 0.6	33,7 ± 0.4	1.0	0.8	0.8
LC130		24.8 ± 17.2	23.3 ± 15.2	8.2 ± 1.9	2.4 ± 1.6	1.1	0.3	0.1
LC131		2.1 ± 1.9	2.9 ± 2.4	3.4 ± 2.1	4.8 ± 1.2	0.7	1.2	1.7
LC132		26.6 ± 15.9	24.2 ± 12.8	25.0 ± 7.4	19.7 ± 3.4	1.1	1.0	0.8
LC136		2.5 ± 1.3	2.8 ± 1.2	6.1 ± 1.9	6.8 ± 2.3	0.9	2.2	2.4
MIS13		3.4 ± 3.0	7.4 ± 6.5	1.7 ± 1.1	0.3 ± 0.3	0.5	0.2	0.0

LC163		43.5 ± 3.0	63.0 ± 0.7	71.1 ± 1.1	18.6 ± 16.9	0.7	1.1	0.3
ART		2.5 ± 0.1	4.0 ± 0.1	33.3 ± 8.0	13.3 ± 2.2	0.6	8.4	3.3
DHA		4.2 ± 0.5	4.7 ± 1.5	6.2 ± 1.6	3.7 ± 2.0	0.9	1.3	0.8
ATN		4.6 ± 1.4	5.1 ± 0.1	3.4 ± 0.1	3.9 ± 1.2	0.9	0.7	0.8
CQ		340.1 ± 20.7	15.8 ± 0.8	n.d.	n.d.	21.5	n.d.	n.d.

SD: standard deviation;
n.d.- not determined;
ART: artemisinin; DHA: dihydroartemisinin; ATN: artesunate; CQ: chloroquine;
RI[a]: resistance index = IC_{50} (Dd2)/ IC_{50} (3D7); RI[b]: resistance index = IC_{50} (IPC5202)/ IC_{50} (3D7); RI[c]: resistance index = IC_{50} (IPC4912)/ IC_{50} (3D7);
At least 3 independent assays were performed per compound and strain.

III.2.2 – Determination of the ring and mature-stage survival rates

Structural analogies between DHA, trioxolanes and tetraoxanes may accommodate similar modes of action, hence some level of cross resistance (Tang, Dong and Vennerstrom, 2004; Vennerstrom *et al.*, 2004; J. Wang *et al.*, 2015; Straimer *et al.*, 2017). In the light of this, it was decided to investigate whether parasites expressing variant forms R539T (IPC5202) and I543T (IPC4912) of K13 are cross-resistant to the newly synthesized trioxolanes and tetraoxanes.

The resistance phenotype to ART has been difficult to study and cannot be evidenced by the standard 48 hours *in vitro* (IC_{50}) assay (Witkowski, Amaratunga, *et al.*, 2013; Ismael *et al.*, 2015; Yang *et al.*, 2016; Straimer *et al.*, 2017). Critical for the development of antimalarials is the evaluation of their activity against ART-R parasites, which has been defined as delayed parasite clearance in patients (Dondorp *et al.*, 2009). Slow parasite clearance of *P. falciparum* malaria in patients results from reduced ring-stage susceptibility (Noedl *et al.*, 2008; Dondorp *et al.*, 2009; Amaratunga *et al.*, 2012; Chotivanich *et al.*, 2014), this increasing the need for new compounds with a low RSA. The mutation R539T (present in IPC5202) is one of the k13 mutations that confer high levels of DHA resistance *in vitro* (J. Wang *et al.*, 2015; Straimer *et al.*, 2015, 2017).

Studies with *in vitro* cultured parasites have begun to shed light on the molecular mechanism underlying K13-mediated ART resistance. Assays with very early ring stage parasites (average age 1.2 hr post-invasion with a 1 hr synchronization window) found that the 50% lethal dose of DHA, when given as a 3 hr pulse, was ~70–fold higher in K13 mutant compared to wild-type parasites (Dogovski *et al.*, 2015).

Thus, we explored the susceptibility of the IPC5202 ring and mature-stage parasites (RSA and MSA) to the best performing compounds.

As depicted in Figures III.1 and III.2, the endoperoxides selected demonstrated higher activity than DHA, both against ring and mature stages. As expected, DHA exposure resulted in a higher survival rate of IPC5202 (up to 25%) than for 3D7 (Kite *et al.*, 2016; Thuy-Nhien *et al.*, 2017), though lower than in other reports (Chaorattanakawee *et al.*, 2016). A survival rate RSA < 1% is generally considered as susceptible behaviour. The tested trioxolanes were able to reduce ring survival to less than 1% in both resistant and

susceptible strains (IPC5202 and 3D7), hence no cross- resistance with DHA is apparent, even in parasites carrying the K13 mutation R539T. This notable observation indicates that our compounds have an improved range of activity, compared to other trioxolanes in use, namely the registered drug OZ277, which is compromised by K13 mutations (Straimer *et al.*, 2017). Regarding the tetraoxane LC163, both the K13 wild type (3D7) and mutant (IPC5202) parasites presented RSA>1% (though not significantly different). These observations are in agreement with data recently published showing that a tetraoxane also allowed growth above 1% in RSA assay in a strain carrying K13 R539T mutation (O'Neill *et al.*, 2017). The results indicate therefore that the antimalarial activity of the newly synthesized endoperoxides is not compromised by the K13 mutation R539T, a mutation that confers high levels of *in vitro* resistance and has been associated with delayed parasite clearance in patients (Takala-Harrison *et al.*, 2013, 2015; Ariey *et al.*, 2014; Straimer *et al.*, 2015; Ménard *et al.*, 2016).

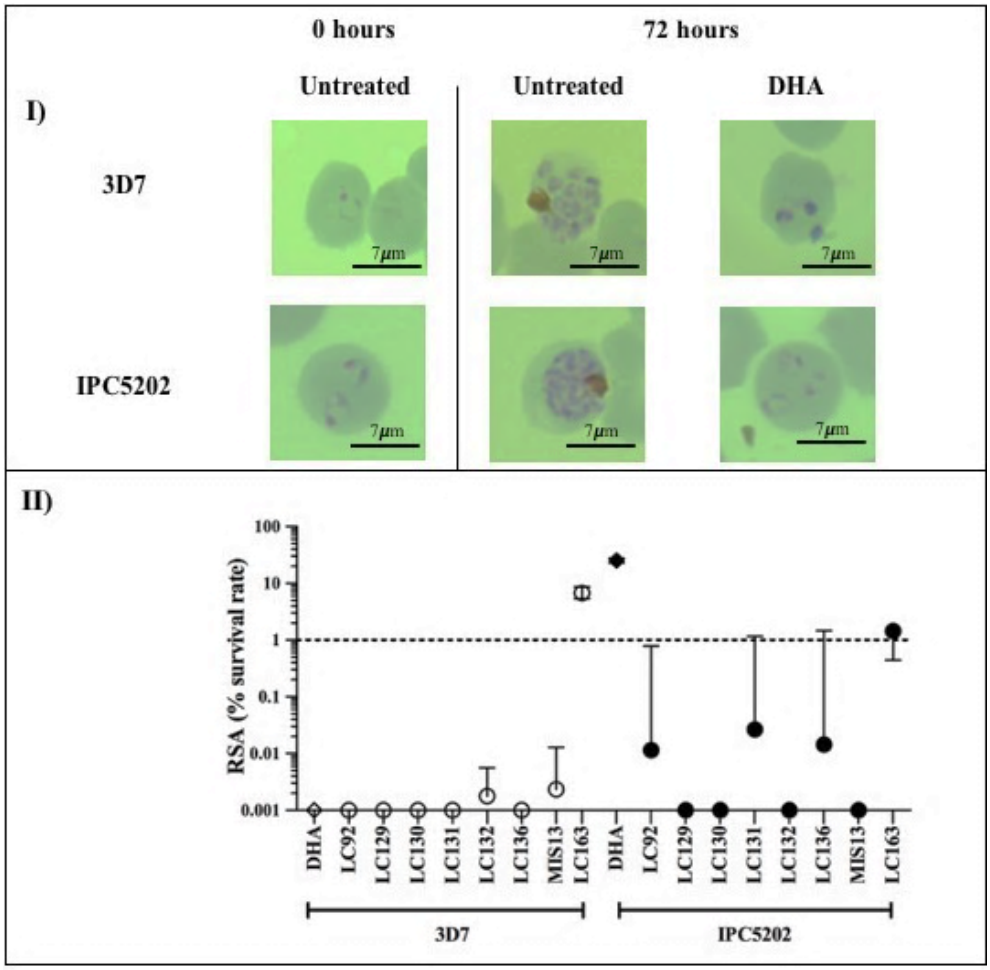


Figure III.1: Ring-stage survival assay. Rings were treated with a pulse of DHA (700 nM) or of new endoperoxides (10x IC_{50}) in 3D7 and IPC5202 strains. The panel I shows the morphology of untreated parasites at time 0 hours and of untreated and treated parasites with DHA at time 72 hours. The panel II shows the ring-stage survival rate, expressed as the percentage of viable parasites at time 72 hours. The parasites were stained by Giemsa (magnification 100X with immersion oil).

The mature-stage assay (MSA) evidenced nearly full susceptibility of the two strains to all of the eight compounds (Figure III.2). Thus, all compounds performed better than DHA. Typically, DHA allows a ≦ 1% of viable mature-stage (Witkowski, Amaratunga, *et al.*, 2013; Yang *et al.*, 2016). ARTs resistance phenotypes are associated with a decreased susceptibility of the ring stage to enter dormancy, a decreased sensitivity of mature-stage parasites and a faster recovery from dormancy (Teuscher *et al.*, 2012; Witkowski, Khim, *et al.*, 2013). Currently these parameters are being addressed in order to gather more information regarding the mode of action of the more promising

compounds.

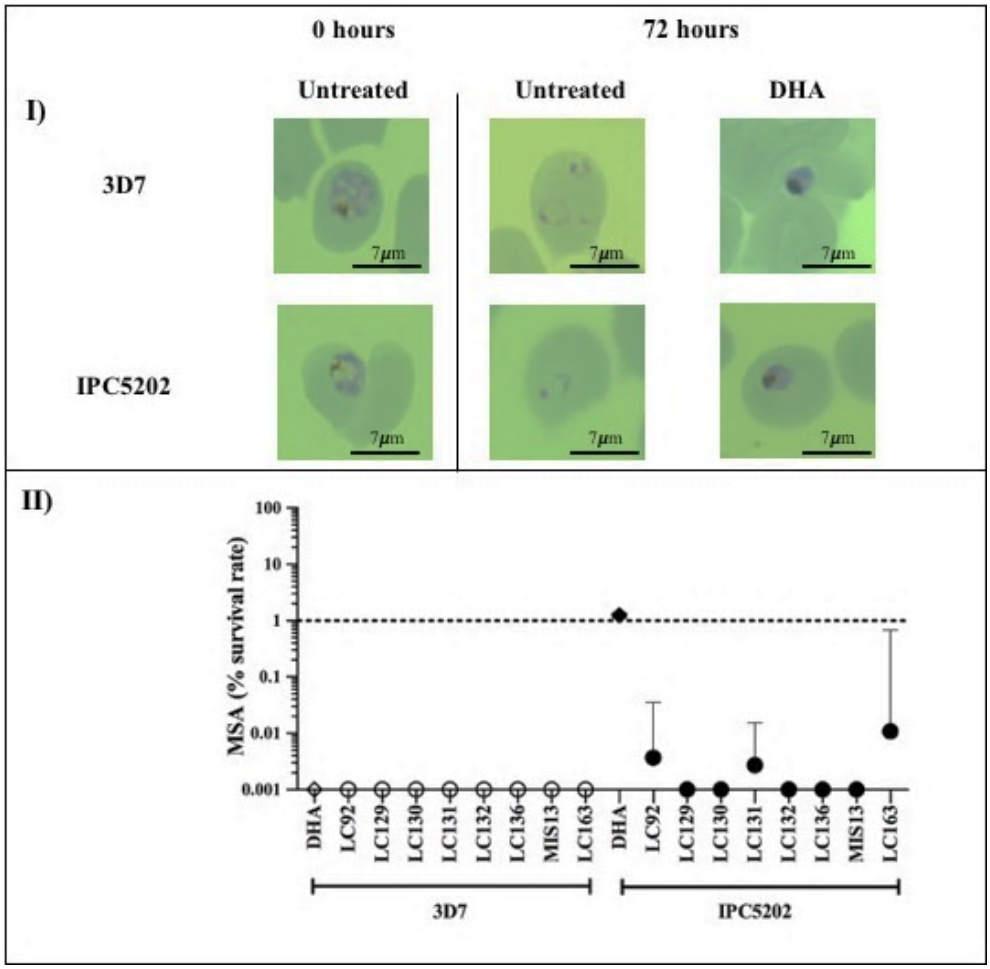


Figure III.2: Mature-stage survival assay. Trophozoites were treated with a pulse of DHA (700 nM) or of new endoperoxides (10x IC_{50}) in 3D7 and IPC5202 strains. The panel I shows the morphology of untreated parasites at time 0 hours and of untreated and treated parasites with DHA at time 72 hours. The panel II shows the ring-stage survival rate, expressed as the percentage of viable parasites at time 72 hours. The parasites were stained by Giemsa (magnification 100X with immersion oil).

As expected, no cross-resistance of the proposed compounds with quinolone-type anti-malarials is foreseeable, as the calculated RIs for Dd2 and 3D7 were ≦ 1 (Table III.1), which is considerably lower than the RI determined for CQ (21.5) (Paulo *et al.*, 2014; Machado *et al.*, 2016).

Mature-stages of both the ART-S and ART-R parasite strains exhibited full susceptibility to all the 8 endoperoxides, exciding DHA performance (Figure III.2).

III.3 – Evaluation of *in vivo* antimalarial activity against *P. berghei*

The 8 best performing compounds, given orally at 50 mg/kg/day, exhibited a high inhibition capacity, with parasitaemia ranging from: 0 to 0.19 ± 0.12% on day 5 post-infection; 0 to 1.76 ± 1.36% on day 7 post-infection; and 0 to 1.32 ± 1.24% on day 10 post-infection (Table III.2). The efficacy difference was very significant ($p < 0.0002$; Mann-Whitney test), compared to the vehicle-treated mice; the untreated control inhibited parasitaemia by 1.51% ± 0.22 on day 5 post-infection (Table III.2). On day 10 post-infection, mice in the untreated control group had developed significant parasitaemia (5.6 - 6.6%), whereas 5 of the tested compounds had led to an appreciable reduction of parasitaemia (0.2 ± 0.13 to 1.32 ± 1.24%) and the remaining 3 compounds had completely suppressed parasitaemia (Table III.2). In the group of mice treated with LC92, all 5 animals developed parasitaemia from day 5 onwards, though at a low level. In the group treated with MIS13 only 1/5 mice presented parasitaemia on day 5, and by day 10 all five mice where cured. This increased activity probably arises from an improvement in pharmacokinetic properties due to: (i) a more substituted amino functionality that increases the overall hydrophilicity and favours protonation in acidic environments; and (ii) to the BOC-protection of the side chain that facilitates the transport of MIS13 through the cell membrane (compared to LC92) (Drag-Zalesińska *et al.*, 2015). Even though MIS13 presented some cytotoxicity *in vitro* against HepG2 cells, the animals were monitored daily during the experimental procedure and no adverse effects were observed for any of the tested compounds. Appreciable *in vivo* antimalarial activity was observed for the two sacharyl substituted compounds (LC129, LC130). LC129 kept parasitaemia at a low level but failed to completely suppress it. On the other hand, LC130 was able to suppress parasitaemia until day 5 but recrudescence occurred on day 7 (2/5 mice were parasitemic) and by day 10 all animals presented parasitaemia (Table III.2), although significantly lower than in the control group ($p = 0.017$; Mann-Whitney test). In the group treated with LC132 and LC163 recrudescence also occurred on day 10. However, a strong *in vivo* antimalarial effect against *P. berghei* was observed with LC131 and LC136, with complete suppression of parasitaemia throughout the experiment (10 days), which may be due to the greater metabolic stability conferred by tetrazole (Singh *et al.*, 1980; Arulmurugan and Kavitha, 2013; Tukulula *et al.*, 2013; Chauhan *et al.*, 2014) and could be improving pharmacological properties or increasing accumulation (due to the electron

withdrawal properties). This may produce a sufficient plasma concentration, indicating promising bioavailability and pharmacokinetic profile. In line with this, a better *in vivo* antimalarial profile for the tetrazole conjugates LC131, LC132, LC136 and LC163, was observed.

Our results suggest that the compounds present a high therapeutic index (TI), that is a ratio that compares the lethal dose of a drug for 50 % of the population divided by the minimum effective dose for 50 % of the population (Tamargo, Heuzey and Mabo, 2015). No animal died or showed any adverse effects during the treatment and future studies should be performed at lower doses with the goal of further increasing TI.

The results indicated that the compounds are very promising and safe since they have a high TI, low IC_{50}s, high SIs, undetectable cytotoxicity, high *in vivo* activity and no adverse effects in animals.

Table III.2: Antimalarial activity of endoperoxides administered orally (*per gavage*).

Compounds	Dose (mg/Kg/day)	Parasitaemia ± SD (% parasite suppression[a])		
		D5	**D7**	**D10**
Untreated		1.51 ± 0.22	3.58 ± 0.81	5.65 ± 0.43
LC92	50	0.02 ± 0.05 (98.34)	0.46 ± 0.60 (86.99)	0.77 ± 0.26 (86.27)
LC129	50	0.19 ± 0.12 (87.60)	1.76 ± 1.36 (50.69)	1.32 ± 1.24 (76.67)
LC130	50	0 (100)	1.4 ± 0.94 (60.83)	1.32 ± 0.24 (76.67)
LC131	50	0 (100)	0 (100)	0 (100)
LC132	50	0 (100)	0 (100)	0.31 ± 0.10 (94.46)
LC136	50	0 (100)	0 (100)	0 (100)
MIS13	50	0.11 ± 0.19 (92.56)	0.05 ±0.10 (98.60)	0 (100)
LC163	50	0 (100)	0 (100)	0.2 ± 0.13 (96.46)

[a] Parasitaemia suppression, compared to untreated control group; SD: Standard deviation.

III.4 – Evaluation of genotoxicity against mammalian cells

Genotoxicity was analysed for the novel endoperoxides LC131, LC132, LC136 and MIS13, that demonstrated > 90% parasitaemia suppression *in vivo* and RSA <1. Only one experiment was performed in duplicate, so the results are preliminary.

In order to evaluate the genotoxicity of the endoperoxides, the comet assay was used, through which it is possible to quantify and distinguish different levels of DNA damage, since the evaluation of the scores (according to the tail size) for each experimental group is highly important (Aquino, Perazzo and Maistro, 2011). Cells were analysed by fluorescence microscopy and DNA damage – genotoxicity, was expressed as the % of DNA in the tail of the comets resulting from V79 cells treated with the new endoperoxides. Genotoxicity was estimated from the % of DNA in the comet's tail and compared to the positive control doxorubicin (represents ≅ 50% of DNA damage) using the software Comet Score (Figure III.3).

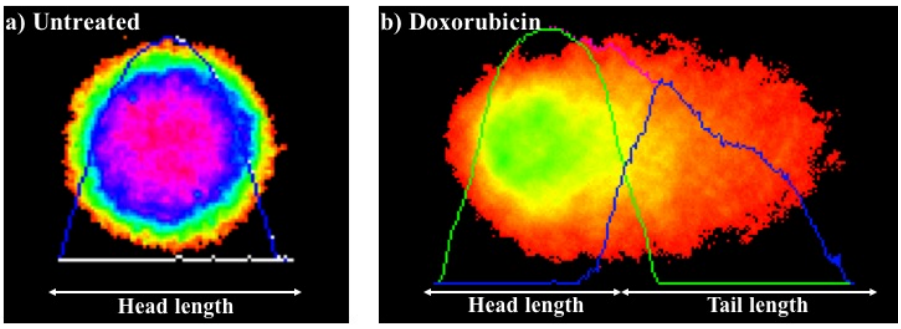


Figure III.3: Analysis of the comet assay using the software Comet Score. Representative images of comets resulted from 3h treatment of V79 cells with a) culture medium - untreated (negative control), shows only head after electrophoresis, without DNA damage and b) doxorubicin 20 µM (positive control), shows head and tail after electrophoresis, with DNA damage. Comets were stained with GelRed™ (magnification 40X). These colours are produced by the software Comet Score.

Except for LC131, which showed a concentration-dependent effect ranging between 9 and 43%, a constant % of DNA in the tail of the comets, ranging between 4 and 13%, was observed for all new endoperoxides evaluated, regardless of the increase in concentrations, showing no damage to the genetic material of V79 cells, indicating that

the compounds tested are quite safe (Table III.3; Figure III.4; Annex VI).

Table III.3: DNA migration in the comet assay for the assessment of genotoxicity of new endoperoxides.

	% of DNA in comet's tail (Mean ± SD)					
	Concentrations					
Compound	0 µM	1 µM	10 µM	20 µM	100 µM	500 µM
Untreated	7.0 ± 0.4	n.d.	n.d.	n.d.	n.d.	n.d.
LC131	n.d.	9.5 ± 0.7	25.3 ± 0.3	n.d.	29.8 ± 0.3	43.8 ± 2.3
LC132	n.d.	7.2 ± 2.1	8.5 ± 2.9	n.d.	8.8 ± 2.3	10.0 ± 1.0
LC136	n.d.	5.4 ± 0.8	4.9 ± 1.0	n.d.	5.7 ± 0.2	13.9 ± 0.1
MIS13	n.d.	5.0 ± 0.5	6.0 ± 1.2	n.d.	6.8 ± 0.1	6.9 ± 0.5
Doxorubicin	n.d.	n.d.	n.d.	46.3 ± 1.2	n.d.	n.d.

n.d. - not determined;

SD: Standard deviation.

In Figure III.4 it is possible to visualize how the V79 cells react to the 3 h-treatment with the new endoperoxides and their respective lower and higher concentrations. Only the endoperoxide LC131, at the highest concentration, showed an increase of % DNA in the tail of the comets (around 43%), similar to the control doxorubicin (46%) (p = 0.3103, unpaired t test), at the concentration of 20 µM, demonstrating that this compound, in this concentration, can cause DNA damage. No significant DNA damage was observed in mammalian cells induced by pharmacologically relevant concentrations of the new tested endoperoxides. Though there is evidence, of ATN causing DNA damage in *P. falciparum* in a concentration- and time-dependent manner (Gopalakrishnan and Kumar, 2015), induced DNA damage, apoptosis and generated damage through direct or indirect

oxidative stress, resulting in DNA strand breakage in CHO-9 cells and V79 cells (Li *et al.*, 2008). Like this, we chose to priorities our analysis and assess the DNA damage effect of our endoperoxides in a near future.

Aquino *et al.*, 2013 demonstrated that ART and ATN, at concentrations between 5 and 35 μM, caused DNA damage in HepG2 cells. The new endoperoxides evaluated showed no damage in DNA of V79 cells at much higher concentrations (100 to 500 μM) in the same comet assay. Indicating that the compounds tested are quite safe, except for compound LC131 at the concentration of 500 μM.

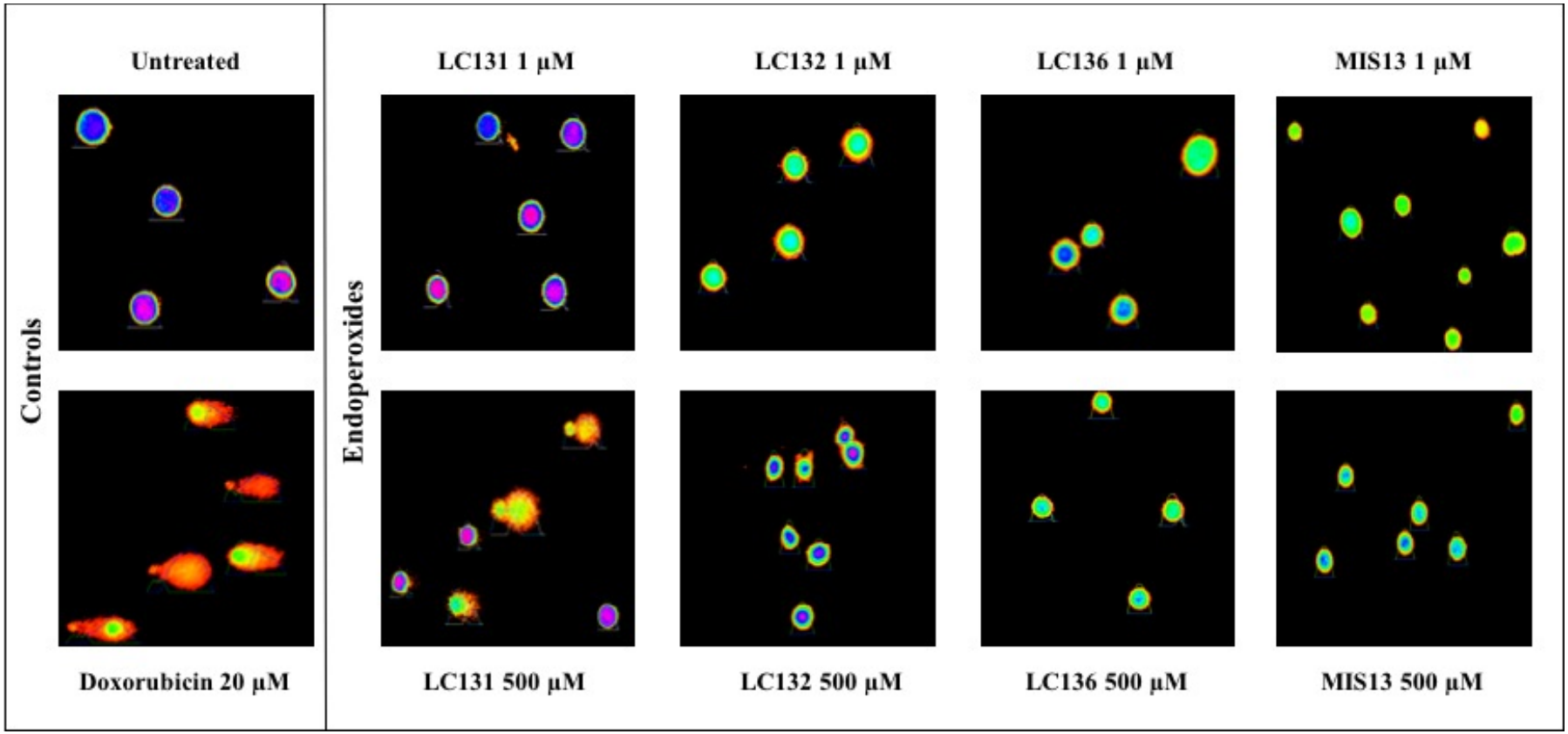


Figure III.4: Comet images from V79 cells treated with the new endoperoxides and doxorubicin. Representative images of comets resulted from 3h treatment with culture media - Untreated (negative control), doxorubicin (positive control) and the new endoperoxides, at concentrations of 1 µM and 500 µM. Comets were stained with GelRed™ (magnification 40X). These colours are produced by the software Comet Score.

III.5 – Evaluation of gametocytocidal activity

We investigated the effect of compounds which led to cure in the *in vivo* tests (LC131, LC136 and MIS13) on the development of gametocytes. PQ and DHA were also evaluated, as controls. Data from the gametocidal assay, expressed as % of healthy gametocytes and inhibition effect, is shown in Table III.4. The inhibition effect was determined in comparison to untreated control (Figure III.5). PQ has long been known to reduce the prevalence of circulating gametocytes and exflagellation of microgametes (Pukrittayakamee *et al.*, 2004; Vale, Moreira and Gomes, 2009; Gebru, Mordmuller and Heldb, 2014; Cabrera and Cui, 2015b). PQ is the only licensed drug available that has proven gametocytocidal activity (stage I - V) *in vivo* (Pukrittayakamee *et al.*, 2004; Gebru, Mordmuller and Heldb, 2014). However, PQ has a half-life of 4-9 hours (Vale, Moreira and Gomes, 2009) and causes haemolysis (Kavishe, Koenderink and Alifrangis, 2017). For *P. vivax* malaria treatment, WHO recommends to *P. vivax* malaria treatment, that it be given once a day for 14 days, a regimen that is difficult for patients to follow, which leads to treatment withdrawal (MMV, 2018). Gametocytocidal activity of DHA against gametocytes stages I – III has also been described previously (Adjalley *et al.*, 2011; Gebru, Mordmuller and Heldb, 2014; Wang *et al.*, 2014). Reference compounds, PQ and DHA (Table III.4), performed within the range normally expected for morphology-based assays and other gametocytocidal assays platforms (Adjalley *et al.*, 2011; Leliévre *et al.*, 2012; Cabrera and Cui, 2015b).

The gametocytes 3D7-GFP were treated with the new endoperoxides, DHA and PQ at their respective IC_{50s} (to asexual and sexual stages), previously determined to 3D7 strain (Table III.1; Annex IV). An extra concentration, equivalent to 10x IC_{50} to sexual stages of the PQ was also used as control of the experiment (See Table II.2) (Leliévre *et al.*, 2012). Gametocytes reduction occurred in a concentration-dependent manner for all drug treatments, except for MIS13, that completely inhibited gametocyte growth in both concentrations. All new endoperoxides had gametocytocidal activity better than PQ at the concentration equivalent to 1x IC_{50} of the asexual stages ($p < 0.05$; Mann-Whitney test) and gametocytocidal activity similar to PQ at the concentration of 1x IC_{50} of the sexual stages ($p > 0.05$; Mann-Whitney test) (Annex VII). All new endoperoxides had better or equal performance than DHA, which ranged from 90% to 96% for the corresponding

concentrations (Table III.4; Annex VII).

The results confirmed that micromolar concentration of PQ was able to inhibit the gametocytes maturation in the culture, which is in agreement with previous findings about its gametocytocidal activity on late-stage gametocytes (Cabrera and Cui, 2015) that may be related to its capacity to selectively destroy the inner structure of *P. falciparum* mitochondria (Leliévre *et al.*, 2012). The new endoperoxides exhibited potent activity against gametocytes, the form transmitted to mosquitoes, as killing of the gametocytes is essential to limit the spread of malaria. With these good results, future studies must be conducted with the aim to evaluate transmission-blocking activities of the new endoperoxides in mice (Adjalley *et al.*, 2011).Therefore, in the future, the use of new endoperoxides for the treatment of malaria could effectively reduce the gametocyte population and, consequently, lower transmission rates.

Table III.4: In vitro activity of the PQ, DHA and new endoperoxides against mature gametocytes of *P. falciparum.*

Compounds	Concentrations	% Gametocytemia (Mean ± SD)	% Gametocytemia Inhibition[a]
Untreated		2.07 ± 1.66	0
PQ	1 µM[b]	0.73 ± 0.09	64.44
	20 µM [c]	0.01 ± 0.01	99.51
	200 µM[d]	0	100
DHA	4.7 nM[b]	0.20 ± 0.19	90.33
	4.7 µM[c]	0.06 ± 0.12	96.82
LC131	2.9 nM[b]	0.01 ± 0.02	99.30
	2.9 µM[c]	0	100
LC136	2.8 nM[b]	0.10 ± 0.08	95.17
	2.8 µM[c]	0.06 ± 0.07	96.86
MIS13	7.4 nM[b]	0	100
	7.4 µM[c]	0	100

SD: Standard deviation;

[a] Gametocytemia inhibition compared to untreated control group;

[b] corresponds to 1x IC_{50} for asexual stages;

[c] corresponds to 1x IC_{50} to sexual stages;

[d] corresponds to 10x IC_{50} to sexual stages.

The experiments were performed at least 2 times, in duplicate

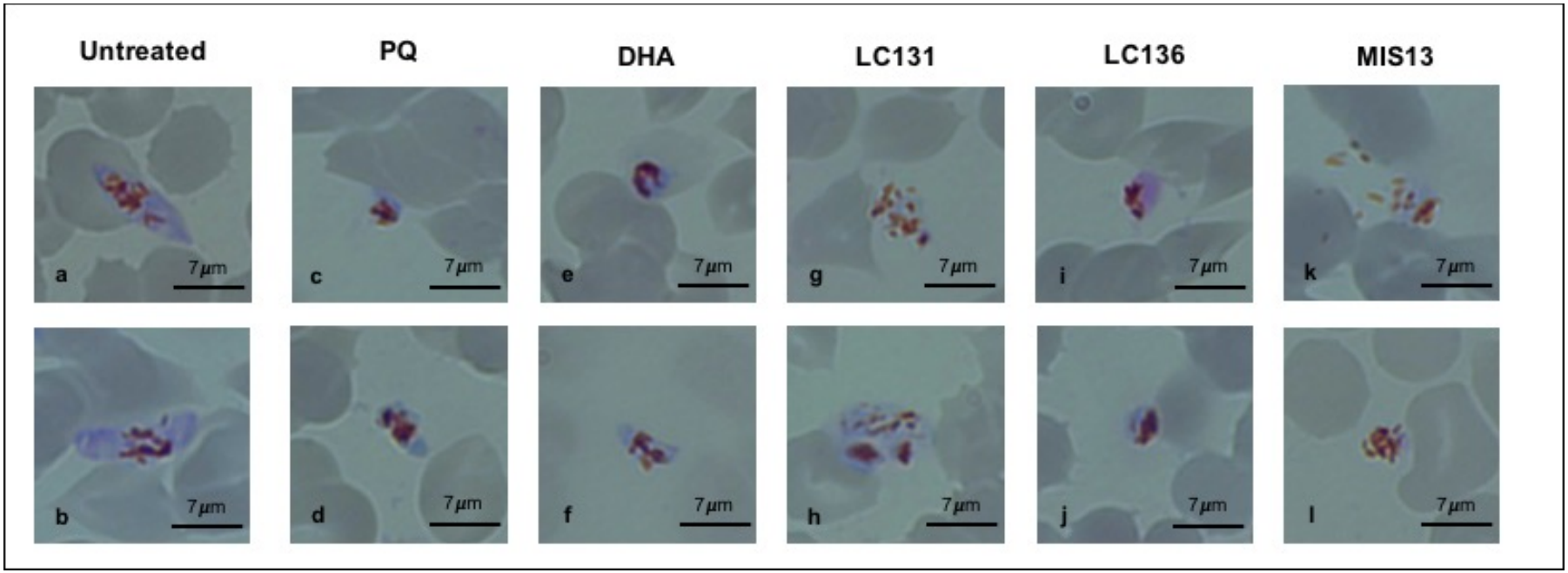


Figure III.5: Effect of PQ, DHA and tested new endoperoxides on gametocytes morphology. The panels show the morphology of a) untreated gametocytes (stage IV), b) untreated gametocytes (stage V), treated gametocytes with PQ (c and d), treated gametocytes with DHA (e and f), treated gametocytes with LC131 (g and h), treated gametocytes with LC136 (i and j) and treated gametocytes with MIS13 (k and l) at equivalent concentrations of 1x IC_{50} to sexual stages. The gametocytes were stained by Giemsa (magnification 100X with immersion oil).

III.6 – Identification of the potential mode of action of the new endoperoxides

The mechanism of action of ARTs is still not conclusively determined, but the role of heme, iron and ROS in the pharmacological and toxicological actions of ARTs has already been described (Copple *et al.*, 2012). It is believed that ARTs are prodrugs that their endoperoxide bridge is cleaved through a mechanism that is thought to be catalyzed by free heme, forming O-centered radicals that self-arranges to form C-centered radicals (Cui and Su, 2009). It has been proposed that such radicals can further react with several cellular targets including proteins, among which are parasitic enzymes that perform crucial functions for parasites survival, and membrane lipids. *In vitro* experiments have shown that ART-heme adducts inhibit heme polymerization into hemozoin leading to the accumulation of the toxic ferroprotoporphyrin IX (FP) (J. Wang *et al.*, 2015). In addition, ART derivatives have been found to accumulate in the parasite's food vacuole, forming ART-FP adducts and increase reactive oxygen species (ROS) that induce membrane damage and, eventually, parasite death (Pandey *et al.*, 1999; Kannan, Sahal and Chauhan, 2002; Hartwig *et al.*, 2009; Kavishe, Koenderink and Alifrangis, 2017).

In a recent study, it was demonstrated that ART and its derivatives, DHA and ATN, act through rapid depolarization of membrane potential of the parasite at pharmacological concentrations and this depolarization was inhibited by ROS scavengers and iron (Fe^{3+}) chelators (Antoine *et al.*, 2014). ROS also mediated depolarization of membrane potential both in the mitochondrial (Wang *et al.*, 2010) and plasma membrane (Antoine *et al.*, 2014). Recent proteomic data suggests that there are probably numerous parasite targets for ARTs, therefore, parasite inactivation may be due to a generalized degeneration of cellular proteins (J. Wang *et al.*, 2015; Ismail *et al.*, 2016), the so called proteopathy (Bhattacharjee *et al.*, 2018; Haldar, Bhattacharjee and Safeukui, 2018).

Structural analogies between DHA, trioxolanes and tetraoxanes may accommodate similar modes of action, hence some level of cross resistance (Tang, Dong and Vennerstrom, 2004; Vennerstrom *et al.*, 2004; Z. Wang *et al.*, 2015; Ismail *et al.*, 2016; Jourdan *et al.*, 2016; Straimer *et al.*, 2017). As proposed for the ARTs, the peroxide bond of the ozonides appears to be instrumental to the antimalarial activity (Tang, Dong and Vennerstrom, 2004; Fontaine *et al.*, 2015), consistent with the observation that a reducing iron source is required for bioactivation and subsequent antimalarial activity (Fugi *et al.*,

2010).

III.6.1 – Stage-dependent activity against *P. falciparum*

We first examined the sensitivity of a tightly synchronized *P. falciparum* culture (3D7-GFP) in rings or trophozoites to a 24 h drug pulse. Parasitaemias and the level of parasite development were verified by Giemsa-stained thin blood smears and by flow cytometry before the beginning of the assays (T0), to verify synchrony (Figure III.6; Annex VIII).

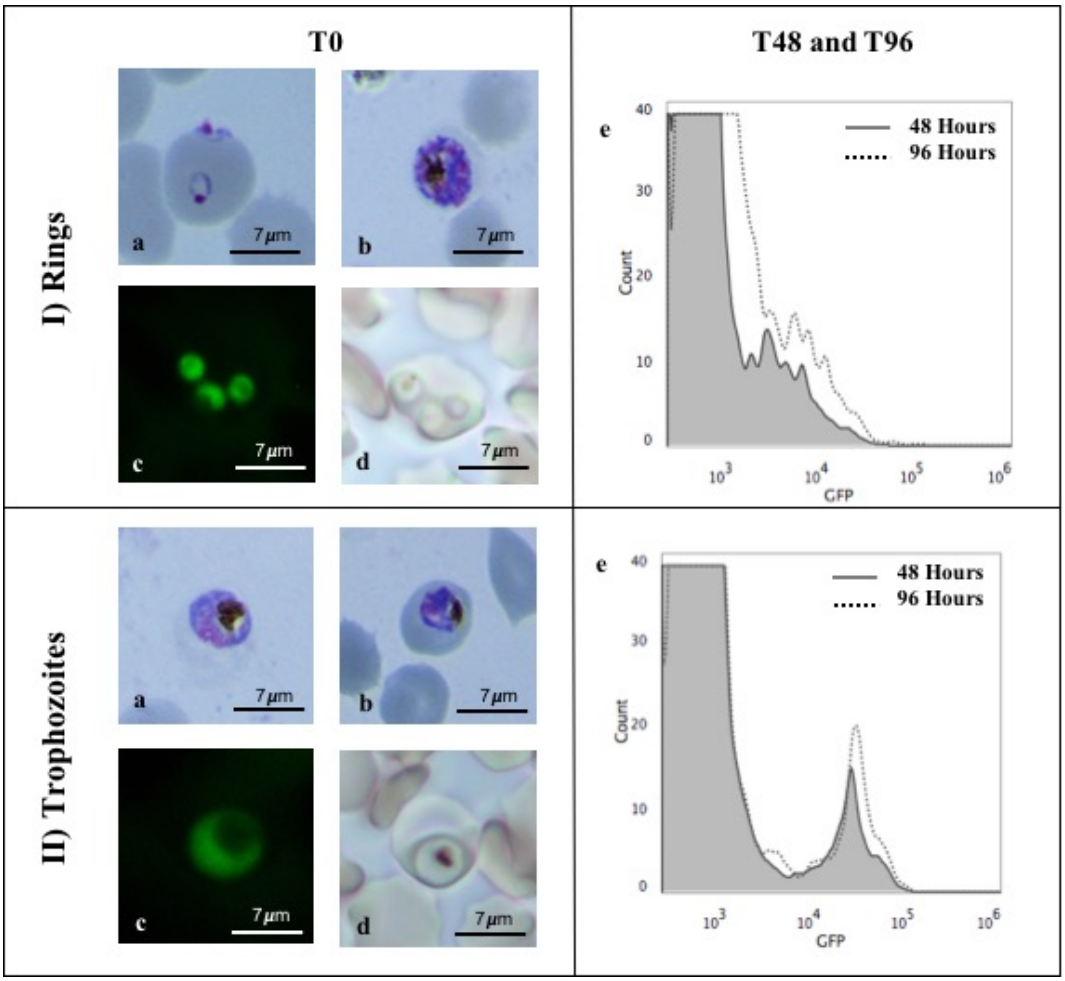


Figure III.6: Synchrony analysis by microscopy and flow cytometry of *P. falciparum* 3D7-GFP culture. Panel I: Rings at time 0 hours – a) and b) Giemsa stained, rings and morula, c) GFP-fluorescence and d) DIC (differential interference contrast); Rings at time 48 and 96 hours – e) histograms obtained by flow cytometry. Panel II: Trophozoites at time 0 hours – a) and b) Giemsa stained, trophozoites, c) GFP-fluorescence and d) DIC; Trophozoites at time 48 and 96 hours – e) histograms obtained by flow cytometry. (magnification 100X with immersion oil).

After this verification, it was observed that the results of the optical microscopy are identical to those of flow cytometry (Annex VIII), thus validating flow cytometry to detect the stage-dependent activity of endoperoxides against *P. falciparum*.

The stage-specificity assay was performed with rings and trophozoites, which were incubated during 24 h with 10x IC_{50} of each new endoperoxide and 1x, 10x and 100x their IC_{50} of the controls (DHA and ATQ) and analysed at 48 h and at 96 h (after incubation without compounds). The experiments were performed at least 3 times, in triplicate.

For the control drugs atovaquone (ATQ) and DHA the activity against trophozoite-stages, was higher than against rings as expected (Figure III.7). This effect was more pronounced in the ATQ than DHA, consistent with ATQ being a slow acting drug (Manach *et al.*, 2013; Stickles *et al.*, 2015). ATQ is a relatively slow acting drug compared with other antimalarials such as ARTs (Manach *et al.*, 2013) possibly due to the drug acting on mitochondria (Antoine *et al.*, 2014) and only on late trophozoites and not on the earlier ring-stages (Klonis *et al.*, 2013; Tilley, Straimer, Gnadig, *et al.*, 2016).

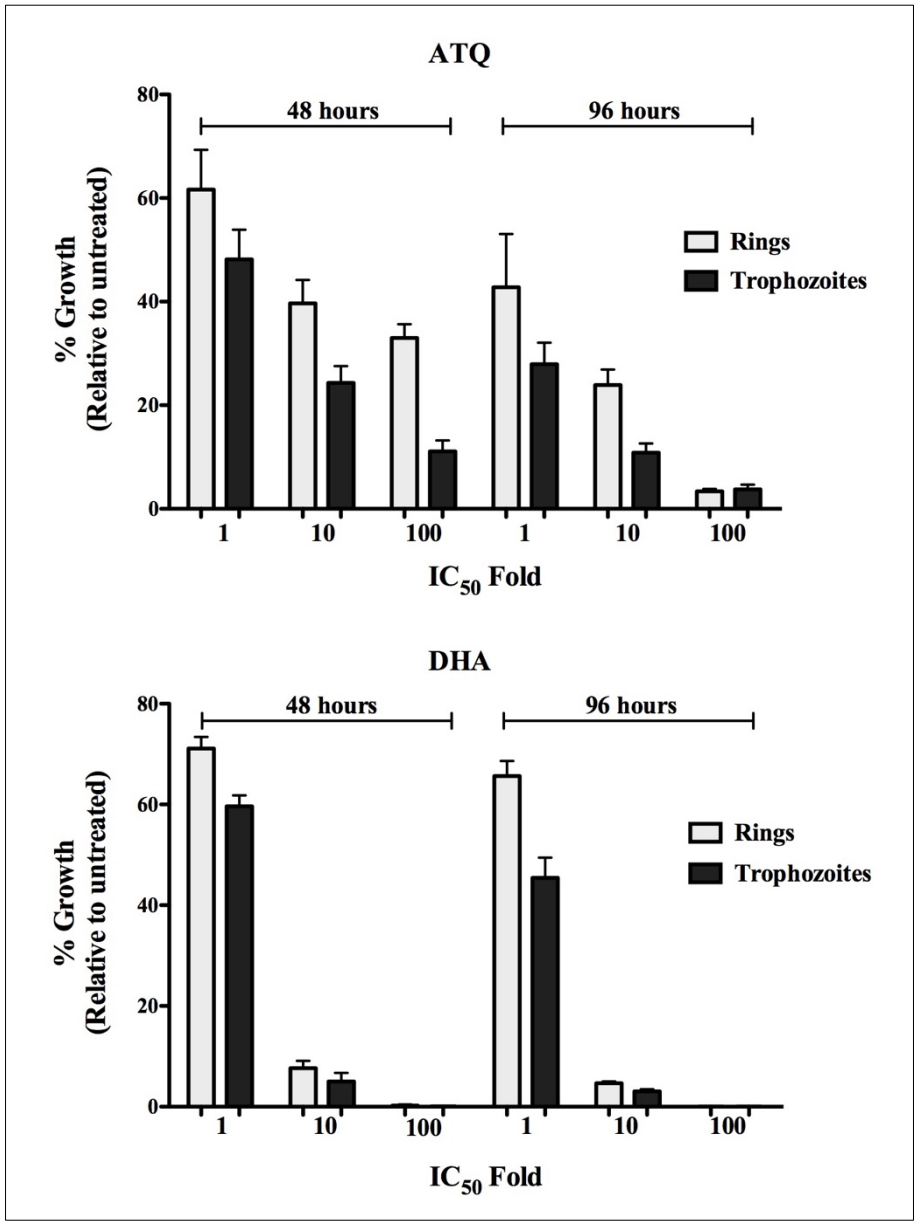


Figure III.7: Stage-dependent effect of ATQ and DHA on *P. falciparum* 3D7-GFP cultures. Cultures were exposed to 3 different concentrations of the compounds (1x, 10x and 100x their IC_{50}) for 24 hours. After removal of the compounds, parasites were incubated for another 24 hours in the absence of compounds. Re-invasion (48 h) and 4-day outgrowth (96 h) was quantified by flow cytometry. Compound effect is expressed as the percentage of growth relative to untreated control. The experiments were performed at least 3 times, in triplicate, and data is expressed as mean values ± SD.

Regarding DHA, our findings are in agreement with what is described in the literature. ART derivatives are known to be more active against trophozoites and less active against ring-stage parasites (Klonis *et al.*, 2013; Tilley, Straimer, Gnadig, *et al.*, 2016; Xie *et al.*,

2016), consistent with the lower hemoglobin digestion at ring-stage (Tilley, Straimer, Gnadig, *et al.*, 2016). Nevertheless, ARTs are considered fast acting drugs, capable of killing very young ring-stage parasites, just after erythrocyte invasion (Klonis *et al.*, 2013; Xie *et al.*, 2016). This is probably due to the ability of early ring-stages to import and digest host hemoglobin, even before formation of the digestive vacuole where most of the hemoglobin is degraded during the trophozoite stage (Xie *et al.*, 2016; Tilley *et al.*, 2016).

Stage specificity experiments, indicate that at time 48 h, all the new endoperoxides were more active against trophozoites than ring-stage parasites ($p < 0.05$, unpaired t test) (Figure III.8; Table III.5). In Table III. 5 it is possible to compare the antiparasitic activity, expressed as the percentage of growth relative to an untreated control, of each compound against ring and trophozoite-stages. At 48 h, the percentage of growth of the parasites treated during the ring stage ranged between 17 and 51%, while they varied between 6 and 23% for the trophozoite stage. Moreover, against trophozoites, LC92, LC129 and LC130 performed better than ATQ and within the range of DHA. This was not evident in ring-stages, since all new endoperoxides where significantly less active than DHA (Figure III.8; Table III.5).

Parasite growth was also determined after two cycles of re-invasion without compounds (96 h outgrowth). At T96 h, only ATQ and two of the new endoperoxides (LC136 and LC163) allowed parasite growth above 10%, in both ring- and trophozoite-stage (Figure III.8; Table III.5).

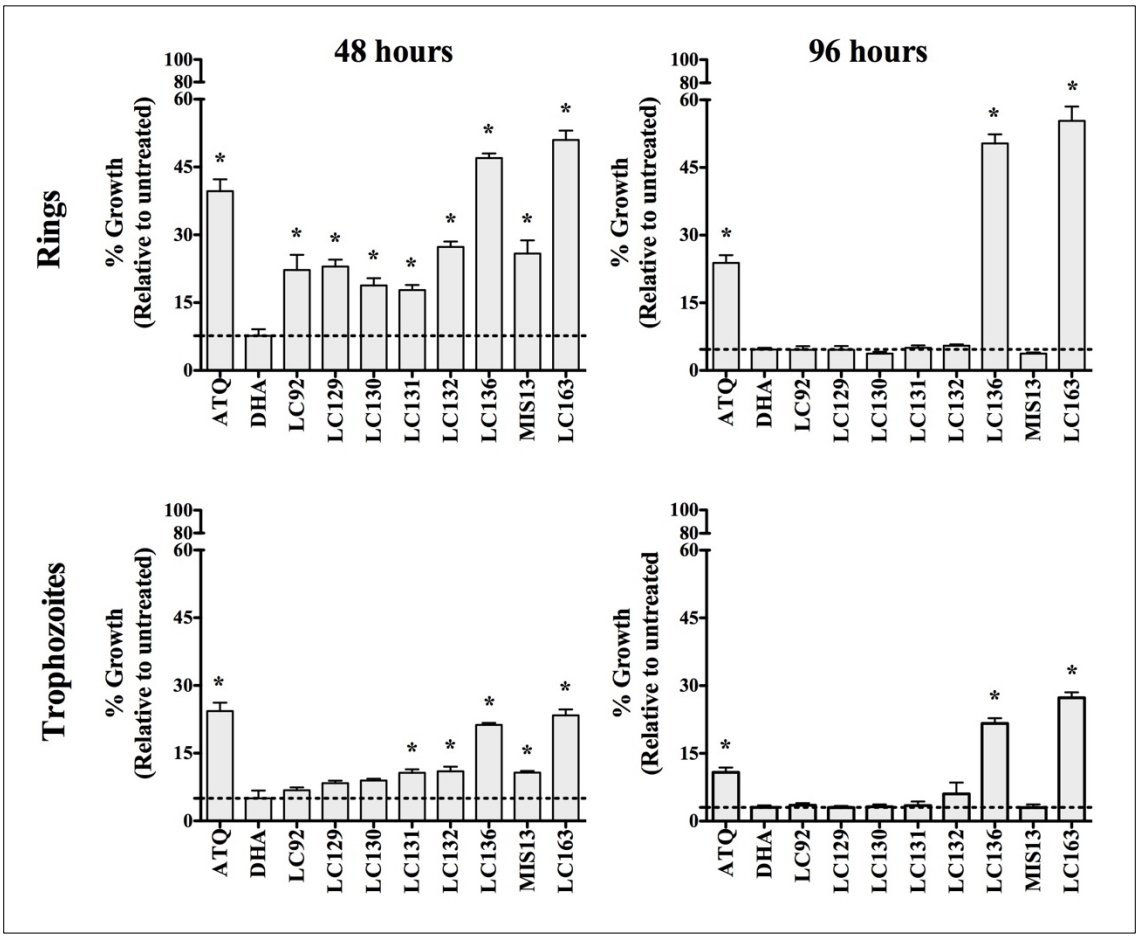


Figure III.8: Stage-dependent antimalarial activity of the new endoperoxides. *P. falciparum* 3D7-GFP cultures were exposed to the new endoperoxides (10x IC_{50}) for 24 hours. After removal of the compounds, parasites were incubated for another 24 h in the absence of compounds. Re-invasion (48 h) and 4-day outgrowth (96 h) were quantified by flow cytometry. Compound effects are expressed as the percentage of growth of the respective development stage relative to an untreated control. The experiments were performed at least 3 times, in triplicate, and data is expressed as mean values ± SD. * $p < 0.05$ (compared with DHA - dotted line, with unpaired t test).

This might indicate a more cytocidal mode of action of the remaining 6 compounds (LC92, LC129, LC130, LC131, LC132 and MIS13). On the other hand LC136 and LC163 might require more time of contact with the parasite for activity. Hence indicating a more cytostatic mode of action. Although they present relatively low IC_{50} (2.8 nM and 63.0 nM respectively); low IC_{50} can, but does not always, indicate potent cytocidal activity of antimalarials (Roepe, 2014).

Table III.5: Stage-dependent antimalarial activity of the new endoperoxides against *P. falciparum*. The parasites were treated with ATQ, DHA and new endoperoxides (10x IC_{50}) for 24 h. After removal of the compounds, parasites were incubated for another 24 h in the absence of compounds. Re-invasion (48 h) and 4-day outgrowth (96 h) were quantified by flow cytometry. Compound effects are expressed as the percentage of growth of the respective development stage relative to an untreated control. The experiments were performed at least 3 times, in triplicate and data expressed as mean values ± SD.

		48 h		96 h	
	Stages	**Rings**	**Trophozoites**	**Rings**	**Trophozoites**
Compounds	**ATQ**	39.7 ± 4.5	24.3 ± 3.2	23.8 ± 2.9	10.8 ± 1.7
	DHA	7.7 ± 2.5	5.0 ± 3.0	4.7 ± 0.6	3.0 ± 0.7
	LC92	22.2 ± 5.7	6.8 ± 1.0	4.6 ± 1.3	3.5 ± 0.7
	LC129	23.0 ± 2.6	8.3 ± 0.9	4.6 ± 1.4	3.0 ± 0.6
	LC130	18.8 ± 2.7	8.9 ± 0.6	3.7 ± 0.6	3.2 ± 0.8
	LC131	17.8 ± 1.9	10.6 ± 1.3	5.0 ± 1.0	3.4 ± 1.6
	LC132	27.3 ± 2.0	11.0 ± 1.7	5.5 ± 0.5	6.0 ± 3.5
	LC136	47.0 ± 1.7	21.2 ± 0.7	50.3 ± 3.5	21.6 ± 2.0
	MIS13	25.8 ± 5.0	10.7 ± 0.6	3.7 ± 0.4	2.9 ± 1.2
	LC163	51.0 ± 3.6	23.3 ± 2.2	55.3 ± 5.5	27.3 ± 2.0

III.6.2 – Assessment of the minimum inoculum for compounds resistance (MIR)

Resistant parasites can be selected by applying drug pressure *in vitro* and/or *in vivo*. These approaches have successfully identified genes, and sometimes codons involved in naturally occurring resistance (Witkowski, Berry and Benoit-Vical, 2009). The simplest approach is *in vitro* selection of resistant parasites, which can be applied on a large number of compounds earlier in drug discovery. *P. falciparum* intraerythrocytic cultures, with starting inoculum ranging from 10^5 to 10^9 parasites, are exposed to a concentration of the compound nearing IC_{90} and monitored during 60 days for recrudescent parasites.

MIR can be determined and is an indirect measure of the likelihood of a resistant genotype to occur and to be selected *in vitro*. In addition, the IC_{50} shift of the selected resistant mutants as compared to the parental sensitive strain is a measure of the resistance intensity (Eastman *et al.*, 2011).

So, the compounds LC131, LC132, LC136, MIS13 and LC163 were tested against Dd2 *P. falciparum* cultures, which have a known multidrug resistant profile due to an increase in *pfmdr1* gene copy number and point mutations at several codons (Ding, Ubben and Wells, 2012). These changes were shown to influence sensitivity to multiple drugs, including MEF and ARTs derivatives (O'Neill *et al.*, 2005; Ding, Beck and Raso, 2011).

MIR can be used to identify compounds at high risk of selecting resistance. MIR equal to or below 10^5 parasites, represents a major risk, as it suggests that, only a single nucleotide mutation is sufficient to produce resistant parasite certain compound. A MIR of 10^7 would also represent a high risk, if combined with a > 20-fold shift in the IC_{50} (Ding, Ubben and Wells, 2012).

The tested compounds showed a MIR > 10^8 in Dd2; recrudescence was not apparent after 60 days of treatment with the respective IC_{90} (≅ 10x IC_{50}) (Annex IX). Plus, the IC_{50} values for Dd2 and 3D7 (sensitive strain) were identical (RIs < 1.1) (Table III.1). These data indicate that these new endoperoxides do not evidence a high risk of selecting resistant parasites. Nor they show indications of using the same resistance mechanism as the antimalarials CQ or MEF.

III.6.3 – Mitochondrial membrane potential ($\Delta\psi_m$) evaluation

Mitochondrial depolarization contributes at least partly to the inhibitory effects of ARTs against malaria (Wang *et al.*, 2010; Xie *et al.*, 2016).

Rh123 is a cell-permeant, cationic, green-fluorescent dye that is selectively accumulated by the mitochondria of living cells, and dependent on the transmembrane potential ($\Delta\psi_m$) (Johnson *et al.*, 1981; Divo *et al.*, 1985). Thus, this test was performed to detect mitochondrial membrane potential integrity in presence of the tested compounds. Parasites treated with control drugs and tested endoperoxides and loaded with Rh123 are

presented in Figure III.9. In control untreated infected RBCs, Rh123 is accumulated within the slender branched mitochondria of *P. falciparum* (Figure III.9) as reported previously (Divo *et al.*, 1985; Biagini *et al.*, 2006; Antoine *et al.*, 2014; Peatey *et al.*, 2015). The confocal micrographs of parasites loaded with Rh123, presented a strong fluorescence signal coming from the active mitochondria and a diffuse signal from the whole cytosol. Green fluorescence was absent from the digestive vacuole, where hemozoin can be observed (magenta in Figure III.9).

Total fluorescence was set up so that untreated parasites, loaded with Rh123, corresponded to 100% fluorescence. Figure III.10 and Table III.6 summarize the effect of new endoperoxides on the $\Delta\psi_m$ in 3D7 and IPC5202 *P. falciparum* strains.

The fluorescence upon treatment with ATQ was 73.9%, thus decreasing the $\Delta\psi_m$ by 26.1% in 3D7 *P. falciparum* parasites (Figure III.10; Table III.6), consistent with previous observations of the mitochondrial contribution to Rh123 fluorescence in this strain (Biagini *et al.*, 2006; Antoine *et al.*, 2014). There was no difference in the $\Delta\psi_m$, between the resistant and susceptible strain when treated with ATQ (Figure III.10; Table III.6; $p = 0.1585$, Mann-Whitney test). Both strains (3D7 susceptible and IPC5202 ART-resistant) behaved in the same manner when challenged with ATQ.

As expected, Deoxy-DHA did not have a significant effect in the $\Delta\psi_m$ on either strain. This result is in line with previous studies (Kaiser *et al.*, 2007; Wang *et al.*, 2010) and argues in favor of the importance of the endoperoxide bond in mediating antimalarial activity. On the other hand DHA, induced a significant decrease in $\Delta\psi_m$, both in the susceptible and resistant strain (20.2% and 38.4%, respectively) (Figure III.10). Although a higher impact was observed in the susceptible strain, it was not statistically significant.

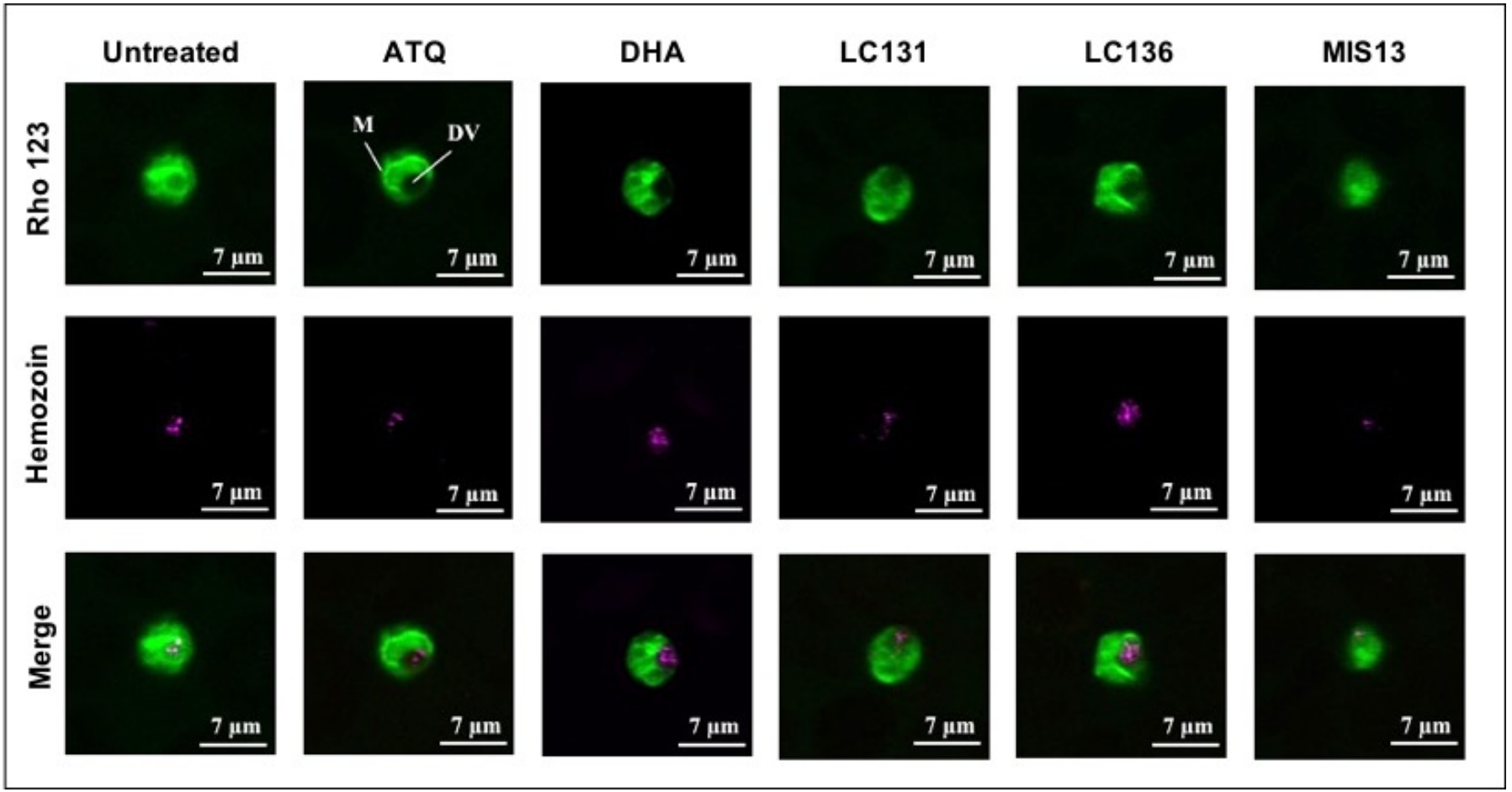


Figure III.9: Effect of ATQ, DHA, LC131, LC136 and MIS13 on fluorescent mitochondria from *P. falciparum* trophozoites. Rh123 fluorescence images of infected erythrocytes after 6 h of treatment with 10 µM of ATQ and 10xIC_{50} of DHA, LC131, LC136 and MIS13 were collected on an inverted confocal microscope. Higher fluorescence intensity of stained iRBCs indicates higher ($\Delta\psi_m$). The green (Rh123) and magenta (hemozoin) in these images are pseudocolours. 'M' indicates mitochondrion and 'DV' indicates the digestive vacuole of the parasite.

Generally, both strains presented similar variation of $\Delta\psi_{m,}$ when challenged with the different new endoperoxides; between 48% – 75% for the sensitive strain and 50% –79% for the resistant IPC5202. When compared with ATQ, only LC129 in 3D7, presented a significantly lower $\Delta\psi_m$ (p =0.0192). The remaining 7 behaved within the range of ATQ (Figure III.10; Table III.6). With the exception of LC129, all new endoperoxides induced $\Delta\psi_m$ within the range of that for DHA (Figure III.10; Table III.6).

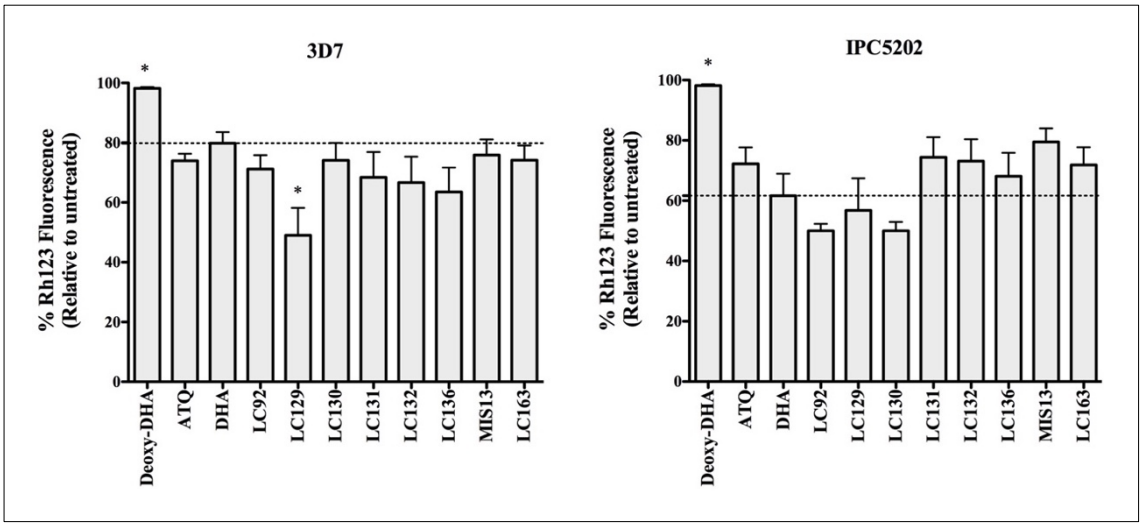


Figure III.10: Effect of Deoxy-DHA, ATQ, DHA and new endoperoxides on mitochondrial membrane potential (Δψm) of *P. falciparum*. $\Delta\psi_m$ was assessed by measuring the fluorescence of Rh123 after the incubation with Deoxy-DHA (8 μM), ATQ (10 μM), DHA and new endoperoxides (10x IC_{50}) for 6 h. The fluorescence intensity was measured with a microplate reader. Untreated Rh123-loaded parasites were considered as 100% fluorescence. The experiments were performed at least 5 times, in triplicate, and data is expressed as mean % of fluorescence ± SD. * $p < 0.05$ (Mann-Whitney test); Dotted line - % of fluorescence after treatment with DHA.

In a close observation of the confocal micrographs in Figure III.9, the diffuse signal coming from the cytosol, might be due to the transmembrane potential across the plasma membrane of the parasite ($\Delta\psi_p$). Hence the V-type H^+ ATPase[5] inhibitor bafilomycin A_1[6]

[5] The V-type H^+ ATPase is responsible for the generation of an inside-negative plasma membrane potential. Inhibition of this pump is therefore likely to result in depolarize the plasma membrane potential and disrupt the physiology of the parasite, leading to its death.

[6] Bafilomycin A_1, is an inhibitor of the V-type H^+ ATPase and has been demonstrated to depolarize the plasma membrane potential and disrupt the physiology of the parasite (van Schalkwyk *et al.*, 2010).

was used in order to isolate this effect (See II.3.6.3).

Figure III.11 and Table III.6 summarize the results for the effect of new endoperoxides on the $\Delta\psi_p$ and $\Delta\psi_m$ in the presence of bafilomycin A_1. In the untreated parasites, upon addition of bafilomycin A_1 (200 nM), the total fluorescence intensity decreased ≅ 75% in both strains, leaving a strong signal from the parasite mitochondrion. In the presence of bafilomycin A_1, the observed fluorescence of ATQ was 28.4% and 12.7% in the 3D7 and IPC5202, respectively (Figure III.11; Table III.6). These results are in agreement with previous studies (Antoine *et al.*, 2014) on the action of endoperoxides and of the ATQ, which demonstrated a reduction of fluorescence associated with the $\Delta\psi_m$ around 50 and 60%.

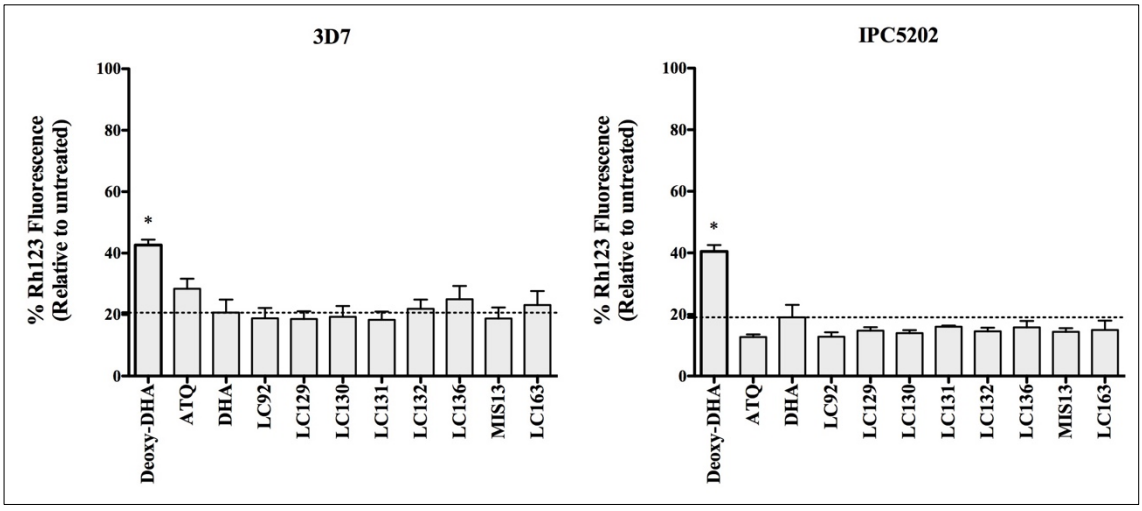


Figure III.11: Effect of Deoxy-DHA, ATQ, DHA and new endoperoxides on mitochondrial membrane potential (Δψm) of *P. falciparum* in the presence of bafilomycin A1. Parasites of 3D7 and IPC5202 strains were incubated with Deoxy-DHA (8 μM), ATQ (10 μM), DHA and new endoperoxides (10x IC_{50}) for 6h and, after this time, incubated with Rh123 and bafilomycin A_1.The fluorescence was assessed by measuring the fluorescence of Rh123 and the new endoperoxides treatment resulted in reduction of fluorescence ($\Delta\psi_m$). Untreated Rh123-loaded parasites, without bafilomycin, were regarded as 100% fluorescence. The experiments were performed at least 5 times, in triplicate, and data expressed as mean values ± SD. * $p < 0.05$ (Mann-Whitney test); Dotted line - % of fluorescence after treatment with DHA.

In 3D7, the tested new endoperoxides, induced a $\Delta\psi_m$ identical to DHA (20.6%), between 18% – 25%. A slightly different behaviour was observed for the ART-resistant strain, which presented lower levels of fluorescence between 12% - 16%, similar to DHA (19.0%) ($p > 0.05$; Mann-Whitney test) (Figure III.11; Table III.6).

With this experiments, it was possible to determine that for all tested endoperoxides, $\Delta\psi_m$ was depolarized (Figure III.11; Table III.6). These data are in line with the findings by Wang *et al*. 2010, who reported depolarization of the membrane potential of isolated parasite mitochondria by ARTs, and those of Crespo *et al*. 2008 that reported mitochondrial dysfunction following exposure to ART, after 8h (but not after 4h), interpreting this as a downstream effect.

The rapid onset of $\Delta\psi_p$ depolarization exposed to pharmacologically relevant concentrations of endoperoxides indicates that this is probably a primary event leading to parasite death and is consistent with *in vitro* (Sanz *et al.*, 2012) and *in viv*o (White, 1997; Pukrittayakamee *et al.*, 2000) studies reporting the rapid killing rate of the endoperoxides. The rapid onset of $\Delta\psi_p$ depolarization by endoperoxides is also consistent with studies demonstrating that short pulses of ARTs (1-6 h) are sufficient for parasite kill, though with stage-dependent differences (Makanga *et al.*, 2005; Klonis *et al.*, 2013).

Fluorescence quantification was also performed using real-time confocal imaging, following exposure to pharmacologically relevant concentrations of the endoperoxides and control drugs . However, only 1 experiment was performed, only for 3D7 strain and no addition of bafilomycin A_1, thus the results are preliminary (Figure III.9; Table III.6). Although preliminary, confocal results are consistent with those obtained by fluorimetry (Table III.6).

Exposure of both 3D7 (susceptible) and IPC5202 (resistant)-infected erythrocytes to a relevant concentration of the new endoperoxides resulted in a loss of membrane potential-dependent accumulation of Rh123 either $\Delta\psi_p$ or $\Delta\psi_m$. Using identical methodology, other endoperoxides such as, ART derivatives and (the closely related to our newly synthesized compounds) - the ozonides, also showed interference with mitochondria membrane potential and parasite growth (Wang *et al.*, 2010; Nixon *et al.*, 2013a; Antoine *et al.*, 2014). This effect was further observed in isolated mitochondria from plasmodium and

yeast (Wang *et al.*, 2010)

Depolarization of $\Delta\psi_m$ by DHA is unlikely to be through the inhibition of mitochondrial electron transport chain (ETC) components, due to the lack of direct inhibition on the isolated components (Wang *et al.*, 2010; Antoine *et al.*, 2014). Morphological change in mitochondria, has been found to be one of the earliest changes in artemisinin-treated parasites (DS *et al.*, 1985; JB *et al.*, 1985; Kawai, Kano and Suzuki, 1993; Maeno *et al.*, 1993; Wang *et al.*, 2010), although some authors (Crespo *et al.*, 2008) suggested that disruption of the mitochondrial membrane potential might occur as a downstream effect of artemisinin-induced cell death. The loss of membrane potential, a marker of affected mitochondrial functions, is probably not due to the reduction of the ETC activity. Nevertheless, ARTs have been shown to target the mitochondria, resulting in impaired mitochondrial functions and ROS-dependent depolarization of plasma and mitochondrial membranes (Wang *et al.*, 2010; Antoine *et al.*, 2014).

Parasite exposure to the new endoperoxides resulted in a significant loss of parasite $\Delta\psi_m$ and $\Delta\psi_p$ potential comparable to that of the ARTs. Hence, our data argue in favour of the mitochondria as a possible site of action of our newly synthesized endoperoxides.

Table III.6: Effect of deoxy-DHA, ATQ, DHA and the new endoperoxides on the membrane potential of *P. falciparum* 3D7 and IPC5202 strains. The % of total fluorescence was assessed by measuring the fluorescence of Rh123 after addition of deoxy-DHA (8 μM), ATQ (10 μM), DHA and new endoperoxides (10x IC_{50}) for 6 h and after addition of bafilomycin A_1. The fluorescence quantification was performed in a fluorimeter and in a confocal microscope. The experiments were performed at least 5 times, in triplicate, with the exception of confocal experiment, that was performed once and only for the 3D7 strain. The data is expressed as mean values.

	% Fluorescence				
	Fluorimeter				Confocal
	Rh123		Rh123 + bafilomycin A_1		Rh123
Compounds	3D7	IPC5202	3D7	IPC5202	3D7
Deoxy-DHA	98.2	98.4	42.6	40.5	n.d.
ATQ	73.9	72.2	28.4	12.6	89.6
DHA	79.8	61.4	20.6	19.0	90.9
LC92	71.1	50.0	18.8	12.7	n.d.
LC129	48.9	56.7	18.5	14.7	n.d.
LC130	74.1	50.0	19.2	13.9	n.d.
LC131	68.4	74.4	18.3	16.0	82.6
LC132	66.6	73.2	21.9	14.5	n.d.
LC136	63.5	68.0	24.9	15.8	69.9
MIS13	75.9	79.4	18.7	14.4	77.1
LC163	74.2	71.9	23.1	14.9	n.d.

n.d.- not determined;

Confocal – confocal microscopy.

III.6.4 – The role of ROS in the new endoperoxides-mediated damage of *P. falciparum*

The exact mechanism of action of ARTs is not well understood (O'Neill, Barton and Ward, 2010; Ding, Beck and Raso, 2011). Nevertheless, several studies have proposed multiple cellular targets of ARTs with the involvement of ROS, leading to a rapid destruction of several specific systems within the parasite (Kavishe, Koenderink and Alifrangis, 2017). Among which are cellular membranes, the redox systems and the mitochondrial ETC. This may explain the rapid clearance of parasites by ARTs (Cui and Su, 2009; Hartwig *et al.*, 2009; Antoine *et al.*, 2014; J. Wang *et al.*, 2015).

P. falciparum is sensitive to oxidative stress *in vitro*, and drugs such as ARTs and CQ interfere with parasite's redox equilibrium (Kavishe, Koenderink and Alifrangis, 2017). CM-H_2DCFDA is a marker for oxidative stress. It reacts with nitric oxide and hydrogen peroxide, leading to oxidation of H_2DCF and to fluorescent DCF. As a probe, CM-H_2DCFDA has been used in flow cytometry approaches to analyse *P. falciparum* oxidative stress variations.

In the present work, CM-H_2DCFDA was used to assess variations in oxidative stress levels during the different stages of the intraerythrocytic development of *P. falciparum.* Two *P. falciparum* strains were used: 3D7 and IPC5202 (See II.3.6.4). A flow cytometry approach was used in order to quantify DCF fluorescencent parasites in *P. falciparum* cultures (iRBCs). To discriminate iRBCs from uninfected RBCs (uRBCs) within a mixed culture, CM-H_2DCFDA was used in combination with the nucleic acid-binding dye SYTO^{TM} 61, that fluoresces in the far-red part of the spectrum.

III.6.4.1 – Stage-dependent ROS levels in *P. falciparum*

SYTO 61 is a DCF-compatible dye with minimum cross talk into the fluorescein (green) channel. The cellular distribution of SYTO 61 and DCF in iRBCs is presented in Figure III.12. Ring stage parasites emitted little or undetectable green fluorescence in comparison to the trophozoites, where a strong signal can be observed (Figure III.12, panel I and II). Similarly, there is negligible green signal from uRBCs and from the host

cell compartment of iRBCs (Figure III.12, panel I and II, black arrows). Hence, the DCF (green) signal measured by flow cytometry originates exclusively from the probe in the parasite's cytoplasm. SYTO 61 (red) fluorescence can also be observed exclusively in parasites (Figure III.12).

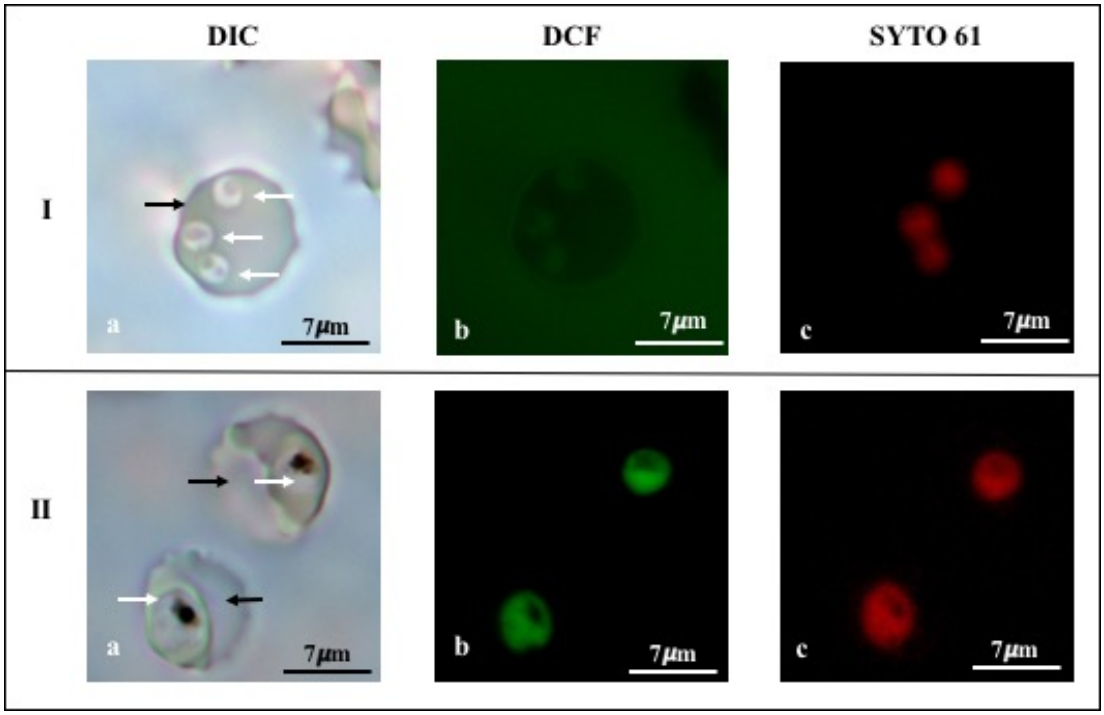


Figure III.12: DCF and SYTO^{TM} 61 labeled iRBCs with rings and trophozoites-stages of *P. falciparum*. Panel I: Ring infected RBCs and Panel II: Trophozoite infected RBCs DIC: differential interference contrast; DCF: DCF green fluorescence; SYTO 61: SYTO 61 red fluorescence; Black arrow: erythrocyte; white arrow: parasite (magnification 100X with immersion oil).

To establish basal levels of ROS in untreated parasites, an asynchronous culture (containing approximately 3% rings and 2% trophozoites and schizont stage parasites) was labeled with CM-H_2DCFDA + SYTO 61 and analysed by flow cytometry (Figure III.13). Three populations could be observed, corresponding to rings, trophozoites and schizonts-stage parasites. Rings exhibit a green signal that is similar to uRBCs, whereas trophozoites and schizonts exhibit a higher green signal (Figure III.13). In uRBCs labeled with CM-H_2DCFDA + SYTO 61, red and green signals were undetectable (Figure III.13, a). These is expects as ring stage parasites have a single nucleus. RNA and DNA levels

increase as the parasite undergoes nuclear division, eventually producing several daughter merozoites. Hence, the amount of nucleic acids increases during the intraerythrocytic cycle, resulting in an increase of red fluorescence (SYTO 61) detectable by cytometry (Figure III.13, b). Accordingly, and as observed in Figure III.12, Ic and IIc, rings display lower red fluorescence than trophozoites. When compared with uRBCs, rings showed a ~20 fold increase in DCF fluorescence, whereas trophozoite and schizont stages displayed > 400 fold increase. This data is consistent with the current knowledge that trophozoite and schizont-stage parasites are more sensitive to oxidative stress variations than ring stages (Fu *et al.*, 2010; Sussmann *et al.*, 2011), hence validating our experimental conditions to detect variations in ROS levels induced by the tested endoperoxides.

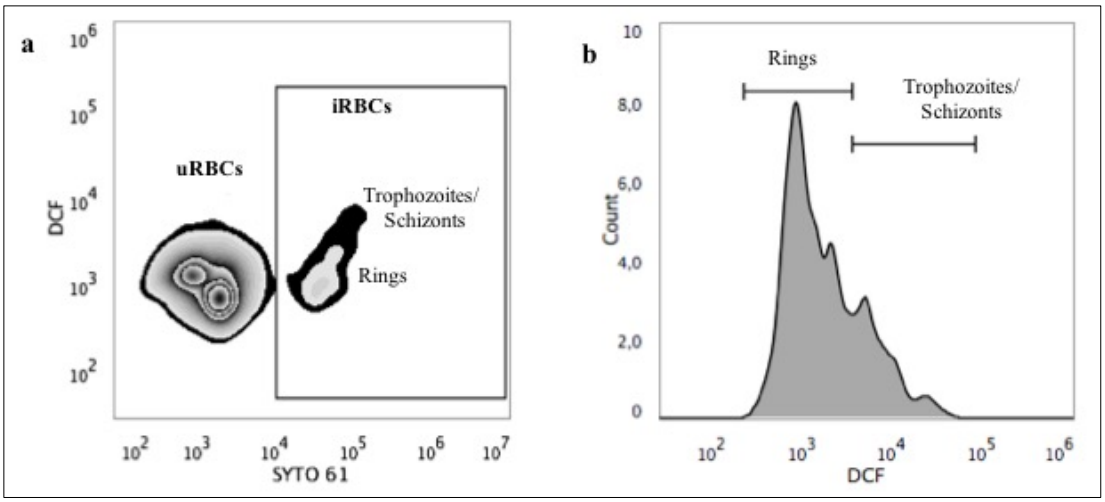


Figure III.13: Flow cytometry analysis of DCF and SYTO 61 signals from an asynchronous culture of *P. falciparum* 3D7 strain. a) Zebra-plot of uRBCs, b and c) Zebra-plot and histogram of an asynchronous culture.

III.6.4.2 – Effect of the new endoperoxides on ROS levels in *P. falciparum*

Using the parameters validated above, we analysed by flow cytometry (See II.3.6.4) trophozoite-stage synchronized iRBCs treated with the controls drugs (deoxy-DHA, H_2O_2, DHA and CQ) and the new endoperoxides. Results are presented in Figures III.14 and Figure III.15. As expected (Sussmann *et al.*, 2017), ~ 40% and ~ 1% increase in ROS

was detected in the pro-oxidant H_2O_2 and in the deoxy-DHA treated cultures, respectively. This could be observed for both the ART-susceptible (3D7) and ART-resistant (IPC5202) parasites (Figure III.14, d and e). When challenged with CQ and DHA at 1x, 10x and 100x the correspondent IC_{50}, a concentration dependent increase in ROS levels could be detected (Figure III.14, d and e). For the 3D7, there was no difference in DCF fluorescence (Mann-Whitney test) between cultures treated with 10x IC_{50} and 100x IC_{50} of DHA and CQ (Figure III.14, d). On the other hand a concentration response could be detected in the IPC5202 treated with 10x and 100x IC_{50} of DHA (Figure III.14, e). Nevertheless, because no concentration response effect could be observed for 3D7, with to test the new endoperoxides, we decided to challenged parasites with 10x the correspondent IC_{50}. The results are summarized in Figure III.15 and Table III.7.

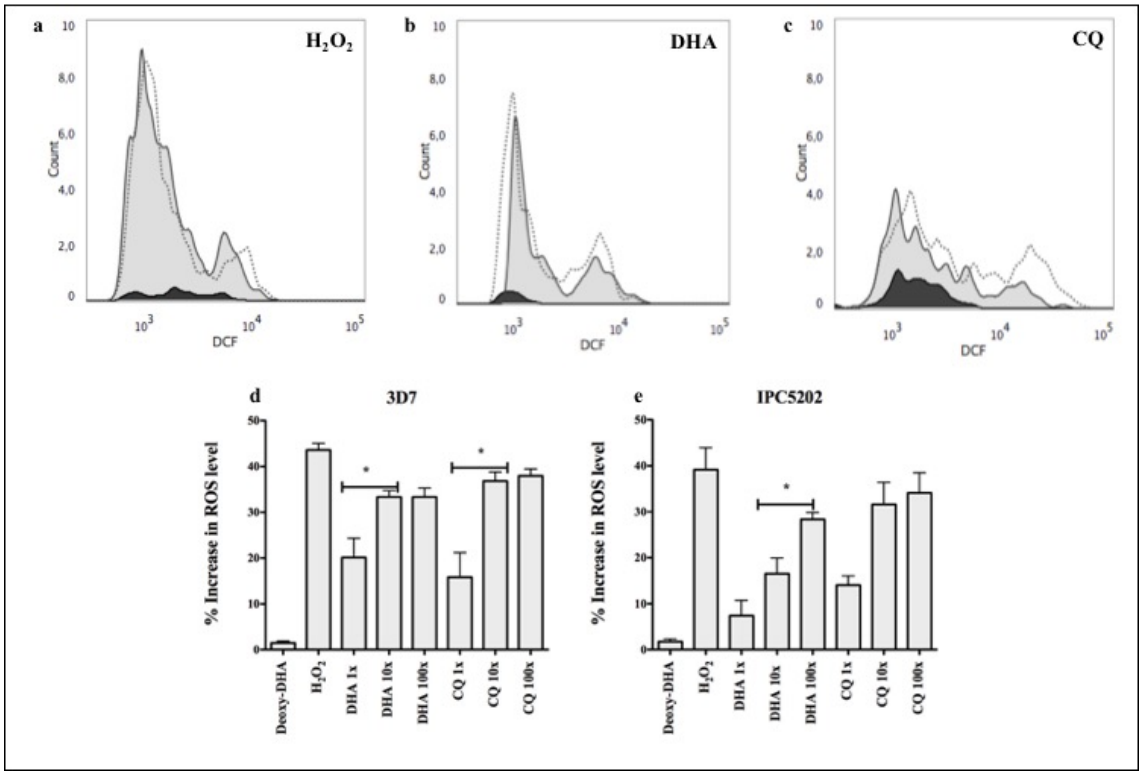


Figure III.14: Effect of the new endoperoxides on oxidative stress in *P. falciparum*. a), b) and c) histograms showing DCF signal of 3D7 iRBCs, treated with H_2O_2, DHA (10x IC_{50}) and CQ (10x IC_{50}). *Black*: uRBCs; *Dark gray with solid line*: untreated iRBCs; *White with dotted line*: treated iRBCs. d) and e) % increase of ROS level compared to untreated iRBCs. H_2O_2 (0.5µM) and deoxy-DHA (8 µM). Data from at least 3 experiments expressed as mean values ± SD. *$p < 0.05$, Mann-Whitney test.

Generally, 3D7 presented higher levels of ROS than IPC5202 (Figure III.15). In Table III. 7 it is possible to simultaneously compare the ability of the compounds to increase the levels of ROS in both susceptible and resistant strains. Both strains behave similarly, when challenged with LC92, LC129, LC131, LC136, MIS13 and CQ. On the other hand DHA, LC130, LC132 and LC163 evidenced a significantly different behaviour ($p < 0.05$; Mann-Whitney test).

Regarding DHA challenge, IPC5202 (resistant) presented significantly lower levels of ROS than the susceptible 3D7 ($p = 0.0009$; Mann-Whitney test) (Figure III.15; Table III.7). Except for LC130 in 3D7, the new endoperoxides induced lower or identical levels of ROS when compared to DHA. On the other hand, they induced higher levels of ROS in the IPC5202 (Figure III.15).

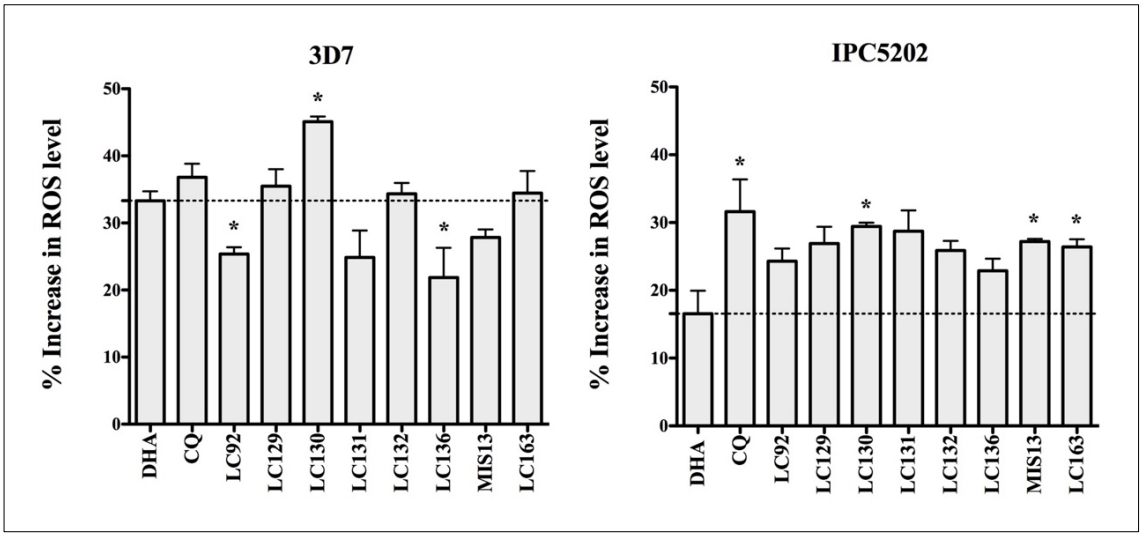


Figure III.15: Effect of new endoperoxides on oxidative stress in trophozoite stage of *P. falciparum* 3D7-sensitive and IPC5202 ART-resistant parasites, analyzed by flow cytometry. Bar graphs are showing percent increase of ROS level in drug treated parasites, as compared to untreated. The experiments were performed at least 3 times and data is expressed as mean values ± SD. *$p < 0.05$ (compared to DHA - dotted line, with Mann-Whitney test).

Table III.7: % Increase in ROS levels in treated parasites with Deoxy-DHA, H_2O_2, DHA, CQ and new endoperoxides in 3D7 and IPC5202 strains of *P. falciparum*. The parasites were treated with Deoxy-DHA (8 µM), H_2O_2 (0.5 µM), DHA (1x, 10x and 100x IC_{50}), CQ (1x, 10x and 100x IC_{50}) and new endoperoxides (10x IC_{50}) and compared to untreated control. The experiments were performed at least 3 times and data is expressed as mean values.

		% Increase in ROS levels	
Compounds	**Concentrations**	**3D7**	**IPC5202**
Deoxy-DHA	8 µM	1.5	1.8
H_2O_2	0.5 µM	43.6	39.2
	1x IC_{50}	20.1	7.4
DHA	10x IC_{50}	33.3	16.5
	100x IC_{50}	33.3	28.3
	1x IC_{50}	15.8	14.0
CQ	10x IC_{50}	36.8	31.6
	100x IC_{50}	38.0	34.1
LC92	10x IC_{50}	25.4	24.3
LC129	10x IC_{50}	35.5	26.9
LC130	10x IC_{50}	45.1	29.4
LC131	10x IC_{50}	24.9	28.7
LC132	10x IC_{50}	34.3	25.9
LC136	10x IC_{50}	21.8	22.9
MIS13	10x IC_{50}	27.8	27.2
LC163	10x IC_{50}	34.4	26.4

Its described that the cleavage of the endoperoxide generates ROS that subsequently damage several cellular targets such as nucleic acids, lipids and key target proteins, killing the parasite (O'Neill, Barton and Ward, 2010; Tilley, Straimer, Gnadig, *et al.*, 2016; Kavishe, Koenderink and Alifrangis, 2017). ROS also mediate depolarization of the membrane potential, both in the mitochondrial (Wang *et al.*, 2010) and the plasma membrane (Antoine *et al.*, 2014). Recent proteomic data suggests that there are probably numerous parasite targets for ARTs, therefore, parasite inactivation may be due to a generalized degeneration of cellular proteins (J. Wang *et al.*, 2015; Ismail *et al.*, 2016).

The oxidative stress induced by antimalarial agents is known to interfere and disrupt the antioxidant-system balance and is important in malaria parasite clearance, both in quinoline-besed antimalarial drugs and in endoperoxides (Cui and Su, 2009; Hartwig *et al.*, 2009; Antoine *et al.*, 2014; J. Wang *et al.*, 2015). Our endoperoxides induced different levels of ROS in both strains when compared to DHA and CQ, used as positive controls. Parasite exposure to the new endoperoxides resulted in a significant increase in ROS levels, similar to DHA and CQ, which are known as inducers of oxidative stress (Tilley, Straimer, Gnadig, *et al.*, 2016; Kavishe, Koenderink and Alifrangis, 2017). Hence, our data argue in favour of the ROS as a possible mechanism of action of our newly synthesized endoperoxides.

The compounds LC131, LC136 and MIS13 demonstrated to be the most promising; they showed nanomolar activity against ART-resistant *P. falciparum* parasites, negligible toxicity towards mammalian cells, totally suppressed *P. berghei* parasitaemia in mice, showed no cross resistance with CQ and ARTs, did not present genotoxicity, showed gametocytocidal activity, were more active on the trophozoite stages, induced alteration of mitochondrial membrane potential and finally induced increase in ROS levels.

These compounds can be easily prepared from relatively cheap starting materials. On the other hand, ART- derivatives are obtained from the expensive natural product ART through semi-synthesis. Thus, besides the excellent pharmacologic properties, an anti-malarial peroxide-type formulation based on trioxolanes LC131, LC136 and MIS13 could be cheaper than those based on current ART-derivatives, while offering a comparable, or even better, antimalarial profile.

IV – MAIN CONCLUSIONS

1. Generally, all compounds have shown low or undetectable cytotoxicity in both cell lines and had low cytotoxicity when compared to the ART.

2. Compounds LC92, LC129, LC130, LC131, LC132, LC136, MIS13 and LC163 were exceptionally active across all four *P. falciparum* strains and showed no cross-resistance with quinolone-type and ART-type antimalarials is foreseeable.

3. *In vivo*, compounds LC92, LC129, LC130, LC131, LC132, LC136, MIS13 and LC163 were very effective against *P. berghei*. Compounds LC131, LC136 and MIS13 demonstrated to be the most promising; they totally suppressed parasitaemia in mice, presenting curative effect.

4. Generally, compounds LC131, LC132, LC136 and MIS13 have shown undetectable genotoxicity, showing no damage to the DNA of V79 cell line. LC131 revealed some *in vitro* genotoxicity at a maximum concentration tested.

5. The tested compounds LC131, LC136 and MIS13, showed gametocytocidal activity better than or equal to that of PQ and DHA.

6. In RSA, compounds LC92, LC129, LC130, LC131, LC132, LC136, MIS13 and LC163 presented higher activity than DHA against the ART-R strain (IPC5202). Already at MSA of both the ART-S and ART-R parasite strains, exhibited full susceptibility to all the 8 endoperoxides, exciding DHA performance. In stage-specific assay, the tested compounds were more active against the trophozoite-stage. With exception of LC136 and LC163, the % of recrudescence is low, indicating a more cytocidal mode of action of these compounds.

7. Compounds LC131, LC132, LC136, MIS13 and LC163 do not evidence a high risk of selecting resistant parasites.

8. Compounds LC92, LC129, LC130, LC131, LC132, LC136, MIS13 and LC163 induced alteration in the mitochondrial membrane potential ($\Delta\psi_m$) of the treated parasites.

9. The compounds LC92, LC129, LC130, LC131, LC132, LC136, MIS13 and LC163 induced increase in ROS levels of the treated parasites.

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VI – ANNEXES

ANNEX I

Culture medium for maintenance of cell cultures and plasmodium cultures:

Complete Williams E medium for HepG2-A16

Williams E medium (Sigma-Aldrich)	10.83 g
Penicillin-Streptomycin (Sigma)	1%
Fetal bovine serum (FBS) (Gibco)	10%
$NaHCO_3$ (Sigma)	3.7 g
Milli-Q H_20	1l

Complete F-10 medium for V79-2

Ham's F-10 Nutrient Mixture (Sigma-Aldrich)	72 ml
Newborn calf serum (Sigma-Aldrich)	8 ml
Penicillin-Streptomycin (Sigma)	1%

Complete RPMI medium for *Plasmodium falciparum*

RPMI 1640 (Gibco)	10.44 g
HEPES (Sigma)	5.94 g
AlbuMAX™ I (Gibco)	5 g
Hypoxanthine (Sigma)	0.1 g

$NaHCO_3$ (Sigma)	2 g
Milli-Q H_20	1l

Solutions for defrosting of *P. falciparum*

Solution A: 12% NaCl in sterile Milli-Q H_20;

Solution B: 1.6% NaCl in sterile Milli-Q H_20

Solution C: 0.2% dextrose + 0.9% NaCl in sterile Milli-Q H_20.

Solution for cryopreservation of mammalian cells and *P. falciparum*

28% of glycerol

4.2% of D-sorbitol

0.65% of NaCl

100 ml Milli-Q H_20

Sterilized by filtration

ANNEX II

Solutions and buffers used in this work:

Buffered water

1 pastille (Sigma)

1l of Milli-Q H_20

Giemsa 20%

20 ml of Giemsa (VWR)

80 ml of buffered water (Sigma)

Filtrate in filter paper and store in 4ºC

Phosphate buffered saline (PBS)

1 pastille (VWR)

200 µl of Milli-Q H_20

Autoclave

D-Sorbitol 5%

25g of D-Sorbitol (Sigma)

500 ml of Milli-Q H_20

Autoclave

Percoll® 70% (For 142.86 ml)

PBS 10X	10 ml
Percoll (Ge Healthcare)	90 ml
RPMI incomplete	42.86 ml

Lysis solution

NaCl	2.5 M
EDTA	100 mM
Tris	10 mM
Triton-X	1%
pH	= 10

Electrophoresis buffer

NaOH	10 mM
EDTA	200 mM
pH	≧ 13

Neutralization buffer

Tris	0.4 M
pH	= 7.59

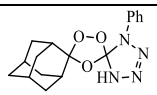
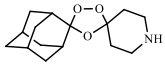
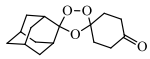
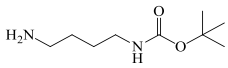
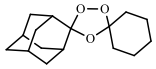
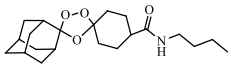
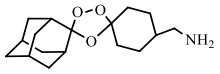
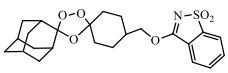
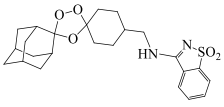
Cytometer cleaning solution (100 ml)

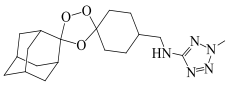
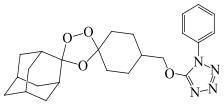
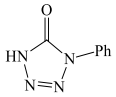
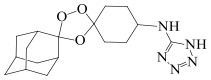
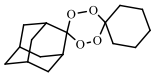
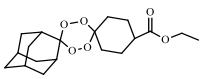
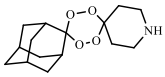
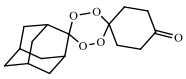
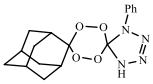
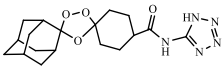
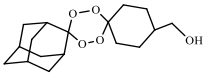
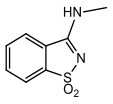
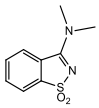
Sodium Azide	50g
Milli-Q H_20 autoclaved	100 ml

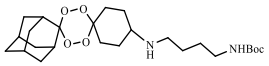
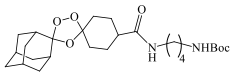
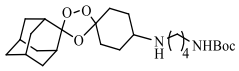
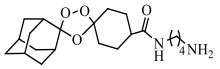
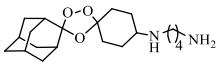
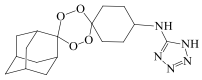
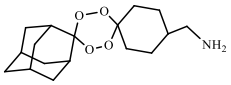
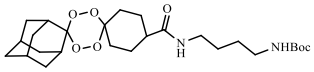
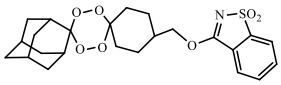
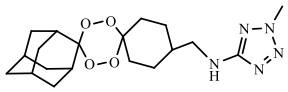
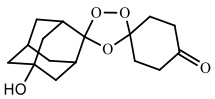
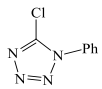
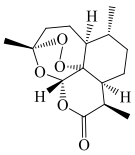
Add 1 ml of this solution to 1 l of autoclaved Milli-Q H_20.

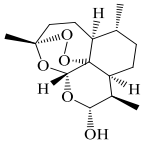
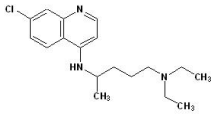
ANNEX III

Compounds and their respective lethal doses (LD_{50}) in V79 and HepG2 cells.

Compounds	ID	LD_{50} V79 (nM)	LD_{50} HepG2 (nM)
LC28		1000000	1000000
LC32		1000000	1000000
LC50		1000000	1000000
LC60		1000000	1000000
LC64		1000000	1000000
LC90		210000	200000
LC92		1000000	1000000
LC95		1000000	1000000
LC126II		1000000	1000000
LC129		1000000	1000000
LC130		1000000	1000000

LC131		1000000	1000000
LC132		1000000	1000000
LC133		1000000	1000000
LC136		1000000	1000000
LC137		1000000	1000000
LC138		1000000	1000000
LC139		228011	200000
LC140		1000000	1000000
LC141		1000000	1000000
LC142		1000000	1000000
LC146		1000000	1000000
LC154		1000000	1000000
LC155		1000000	1000000

LC157		1000000	1000000
MIS13		1000000	330000
MIS14		1000000	330000
MIS15		1000000	1000000
MIS16		1000000	1000000
LC163		1000000	1000000
LC165		1000000	1000000
LC176		1000000	1000000
LC177		1000000	1000000
LC179		1000000	1000000
LC182		1000000	1000000
LCTET		1000000	1000000
ART		416233	400000

DHA		1000000	1000000
CQ		244412	220000

ART: artemisinin; DHA: dihydroartemisinin; CQ: chloroquine

ANNEX IV

Antiplasmodial activity *in vitro* (IC_{50}) against sensitive and resistant *P. falciparum* strains.

	P. falciparum IC_{50} ± SD (nM)				Resistance Index		
Compounds	Dd2	3D7	IPC5202	IPC4912	RI[a]	RI[b]	RI[c]
LC28	1127.0 ± 188.3	3659.0 ± 1488.2	n.d.	n.d.	0.3	n.d.	n.d.
LC32	987.1 ± 214.7	854.5 ± 107.6	n.d.	n.d.	1.2	n.d.	n.d.
LC60	> 10000	>10000	n.d.	n.d.	n.d.	n.d.	n.d.
LC64	> 10000	>10000	n.d.	n.d.	n.d.	n.d.	n.d.
LC90	159.3 ± 10.6	135.47 ± 28.5	n.d.	n.d.	1.2	n.d.	n.d.
LC92	22.6 ± 2.6	26.7 ± 2.4	37.7 ± 9.9	34.9 ± 7.9	0.8	1.4	1.3
LC95	560.3 ± 1.0	290.55 ± 5.2	n.d.	n.d.	1.9	n.d.	n.d.
LC126II	>10000	>10000	n.d.	n.d.	n.d.	n.d.	n.d.
LC129	43.2 ± 0.8	42.9 ± 3.9	36.36 ± 1.2	29.3 ± 0.7	1.0	0.8	0.8
LC130	24.8 ± 17.2	23.3 ± 15.2	8.2 ± 1.9	2.4 ± 1.6	1.0	0.3	0.1
LC131	2.1 ± 1.9	2.9 ± 2.4	3.4 ± 2.1	4.8 ± 1.2	0.7	1.2	1.7
LC132	26.6 ± 15.9	24.2 ± 12.8	25.0 ± 7.4	19.7 ± 3.4	1.1	1.0	0.8
LC133	>10000	>10000	n.d.	n.d.	n.d.	n.d.	n.d.
LC136	2.5 ± 1.3	2.8 ± 1.2	6.1 ± 1.9	6.8 ± 2.3	0.9	2.2	2.4
LC137	617.2 ± 58.6	120.8 ± 26.5	n.d.	n.d.	5.1	n.d.	n.d.

LC138	611.7 ± 14.5	578.1 ± 27.2	n.d.	n.d.	1.0	n.d.	n.d.
LC139	>10000	>10000	n.d.	n.d.	n.d.	n.d.	n.d.
LC140	625.6 ± 45.9	515.4 ± 109.8	n.d.	n.d.	1.2	n.d.	n.d.
LC141	>10000	>10000	n.d.	n.d.	n.d.	n.d.	n.d.
LC142	106.9 ± 45.4	116.7 ± 33.2	n.d.	n.d.	0.9	n.d.	n.d.
LC146	374.1 ± 50.7	210.4 ± 24.8	n.d.	n.d.	1.8	n.d.	n.d.
LC154	>10000	>10000	n.d.	n.d.	n.d.	n.d.	n.d.
LC155	>10000	>10000	n.d.	n.d.	n.d.	n.d.	n.d.
LC157	179.7 ± 8.4	137.05 ± 33.0	n.d.	n.d.	1.3	n.d.	n.d.
MIS13	3.4 ± 3.0	7.4 ± 6.5	1.7 ± 1.1	0.3 ± 0.3	0.5	0.2	0.0
MIS14	44.6 ± 32.8	39.1 ± 12.5	413.1 ± 20.2	389.5 ± 17.8	1.1	10.6	10.0
MIS15	♦	♦	♦	♦	n.d.	n.d.	n.d.
MIS16	♦	♦	♦	♦	n.d.	n.d.	n.d.
LC163	43.5 ± 3.0	63.0 ± 0.7	71.1 ± 1.1	18.6 ± 16.9	0.7	1.1	0.3
LC165	372.3 ± 18.8	357.7 ± 53.5	n.d.	n.d.	1.0	n.d.	n.d.
LC176	692.4 ± 74.5	734.7 ± 32.7	n.d.	n.d.	0.9	n.d.	n.d.
LC177	2461.6 ± 1111.5	2388.6 ± 217.5	n.d.	n.d.	1.0	n.d.	n.d.
LC179	1831.0 ± 363.6	4186.6 ± 146.6	n.d.	n.d.	0.4	n.d.	n.d.
LC182	357.5 ± 18.7	359.3 ± 34.0	n.d.	n.d.	1.0	n.d.	n.d.

LCTET	570.0 ± 21.3	361.4 ± 8.9	n.d.	n.d.	1.6	n.d.	n.d.
ART	2.47 ± 0.1	3.97 ± 0.1	33.3 ± 8.0	13.3 ± 2.2	0.6	8.4	3.3
ATN	4.6 ± 1.4	5.1 ± 0.1	3.4 ± 0.1	3.9 ± 1.2	0.9	0.7	0.8
ATQ	n.d.	4.9 ± 2.3	10.4 ± 6.2	n.d.	n.d.	2.1	n.d.
Deoxy-DHA	n.d.	> 10000.0	n.d.	n.d.	n.d.	n.d.	n.d.
DHA	4.2 ± 0.5	4.7 ± 1.5	6.2 ± 1.6	3.7 ± 2.0	0.9	1.3	0.8
CQ	340.0 ± 20.7	15.8 ± 0.8	54.5 ± 3.8*	63.0 ± 4.4*	21.5	3.4	4.0
PQ	n.d.	1000.0 ± 180.0	n.d.	n.d.	n.d.	n.d.	n.d.

♦ Low solubility;

SD: standard deviation;

n.d. - not determined;

* (https://www.beiresources.org, 2017);

RI[a]: resistance index = IC_{50} (Dd2)/ IC_{50} (3D7);

RI[b]: resistance index = IC_{50} (IPC5202)/ IC_{50} (3D7);

RI[c]: resistance index = IC_{50} (IPC4912)/ IC_{50} (3D7);

ART: Artemisinin; ATN: Artesunate; ATQ: Atovaquone; CQ: Chloroquine; Deoxy-DHA: Deoxy-dihydroartemisinin; DHA: Dihydroartemisinin; PQ: Primaquine.

ANNEX V

Selectivity index for compounds in V79 e HepG2 cell lines.

		SI[a] V79					SI[b] HepG2			
Compound	**LD_{50} (mM)**	**Dd2**	**3D7**	**IPC5202**	**IPC4912**	**LD_{50} (mM)**	**Dd2**	**3D7**	**IPC5202**	**IPC4912**
LC28	1.0	887.3	273.3	n.d.	n.d.	1.0	887.3	273.3	n.d.	n.d.
LC32	1.0	1013.1	1170.3	n.d.	n.d.	1.0	1013.1	1170.3	n.d.	n.d.
LC50	1.0	42372.8	71428.6	n.d.	n.d.	1.0	42372.8	71428.6	n.d.	n.d.
LC60	1.0	♦	♦	n.d.	n.d.	1.0	♦	♦	n.d.	n.d.
LC64	1.0	♦	♦	n.d.	n.d.	1.0	♦	♦	n.d.	n.d.
LC90	0.2	1318.3	1550.1	n.d.	n.d.	0.2	1255.5	1476.3	n.d.	n.d.
LC92	1.0	44247.8	37453.1	26525.2	28653.3	1.0	44247.8	37453.1	26525.2	28653.3
LC95	1.0	1784.7	3441.7	n.d.	n.d.	1.0	1784.7	3441.7	n.d.	n.d.

LC126II	1.0	♦	♦	n.d.	n.d.	1.0	♦	♦	n.d.	n.d.
LC129	1.0	23148.1	23310.0	27502.7	29673.5	1.0	23148.1	23310.0	27502.7	29673.5
LC130	1.0	40322.6	42918.4	121951.2	416666.6	1.0	40322.6	42918.4	121951.2	416666.6
LC131	1.0	476190.5	344827.5	294117.6	208333.3	1.0	476190.5	344827.5	294117.6	208333.3
LC132	1.0	37596.9	41322.3	40000.0	50761.4	1.0	37596.9	41322.3	40000.0	5076.4
LC133	1.0	♦	♦	n.d.	n.d.	1.0	♦	♦	n.d.	n.d.
LC136	1.0	400000.0	357142.8	163934.4	147058.8	1.0	400000.0	357142.8	163934.4	147058.8
LC137	1.0	1620.2	8278.1	n.d.	n.d.	1.0	1620.2	8278.1	n.d.	n.d.
LC138	1.0	1634.8	1729.8	n.d.	n.d.	1.0	1634.8	1729.8	n.d.	n.d.
LC139	0.2	♦	♦	n.d.	n.d.	0.2	♦	♦	n.d.	n.d.
LC140	1.0	1598.5	1940.2	n.d.	n.d.	1.0	1598.5	1940.2	n.d.	n.d.
LC141	1.0	♦	♦	n.d.	n.d.	1.0	♦	♦	n.d.	n.d.

LC142	1.0	9354.5	8568.9	n.d.	n.d.	1.0	9354.5	8568.9	n.d.	n.d.
LC146	1.0	2673.1	4752.8	n.d.	n.d.	1.0	2673.1	4752.8	n.d.	n.d.
LC154	1.0	♦	♦	n.d.	n.d.	1.0	♦	♦	n.d.	n.d.
LC155	1.0	♦	♦	n.d.	n.d.	1.0	♦	♦	n.d.	n.d.
LC157	1.0	5564.8	7296.6	n.d.	n.d.	1.0	5564.8	7296.6	n.d.	n.d.
MIS13	1.0	294117.6	135135.1	588235.2	3333333.3	0.3	97058.8	44594.6	194117.6	1100000.0
MIS14	1.0	22421.5	25575.4	2420.7	2567.4	0.3	7399.1	8439.9	798.8	847.2
MIS15	1.0	♦	♦	n.d.	n.d.	1.0	♦	♦	n.d.	n.d.
MIS16	1.0	♦	♦	n.d.	n.d.	1.0	♦	♦	n.d.	n.d.
LC163	1.0	22988.5	15873.0	14064.7	53763.4	1.0	22988.5	15873.0	14064.7	53763.4
LC165	1.0	2686.0	2795.6	n.d.	n.d.	1.0	2686.0	2795.6	n.d.	n.d.
LC176	1.0	1444.2	1361.1	n.d.	n.d.	1.0	1444.2	1361.1	n.d.	n.d.

LC177	1.0	406.2	418.6	n.d.	n.d.	1.0	406.2	418.6	n.d.	n.d.
LC179	1.0	546.1	238.8	n.d.	n.d.	1.0	546.1	238.8	n.d.	n.d.
LC182	1.0	2792.2	2783.2	n.d.	n.d.	1.0	2792.2	2783.2	n.d.	n.d.
LCTET	1.0	1754.4	2677.0	n.d.	n.d.	1.0	1754.4	2677.0	n.d.	n.d.
ART	0.4	168515.4	104844.6	12499.5	31295.7	0.4	168515.4	104844.6	12499.5	31295.7
DHA	1.0	238095.2	212765.9	166666.7	270270.3	1.0	238095.2	212765.9	166666.7	270270.3
CQ	0.2	507.3	15469.1	n.d.	n.d.	0.2	507.3	15469.1	n.d.	n.d.

♦ Low solubility;

n.d. - not determined;

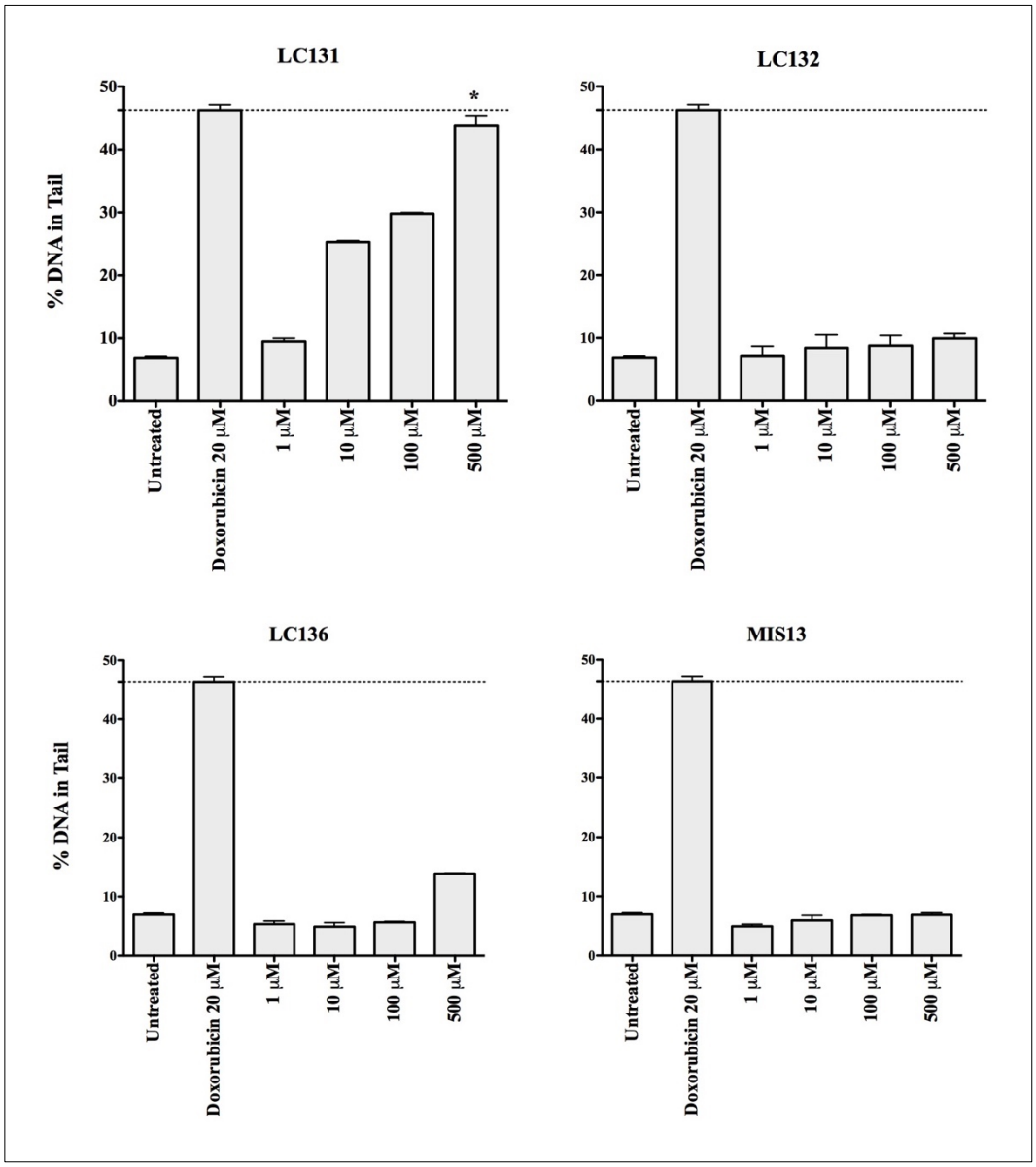
ART: Artemisinin; DHA: Dihydroartemisinin; CQ: Chloroquine;

[a] SI (Selectivity index) = LD_{50} (V79)/ IC_{50} (Dd2, 3D7, IPC5202 and IPC4912);

[b] SI (Selectivity index) = LD_{50} (HepG2)/ IC_{50} (Dd2, 3D7, IPC5202 and IPC4912).

ANNEX VI

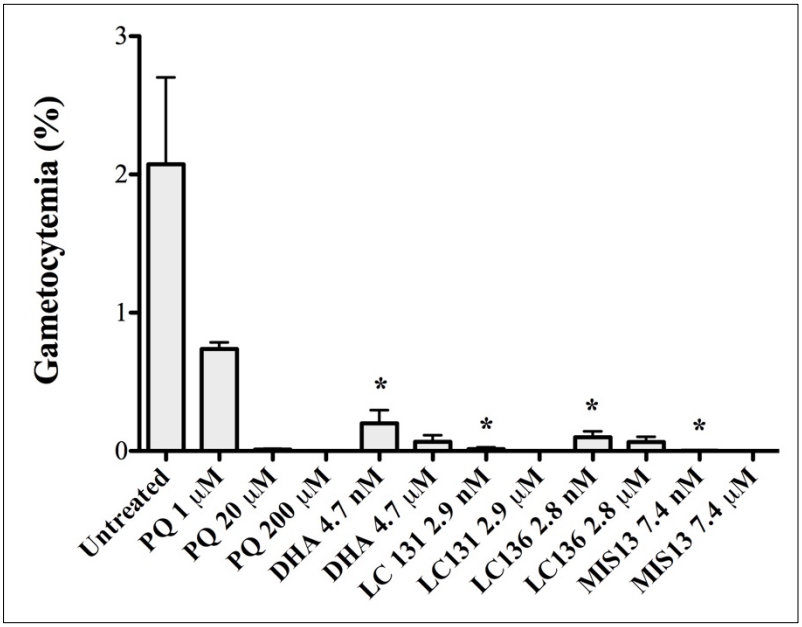
% of DNA in the comet's tail for the assessment of genotoxicity of new endoperoxides LC131, LC132, LC136 and MIS13 in V79 cells (after 3h treatment).



The experiment was performed 1 time, in duplicate, and data is expressed as mean values ± SD. * $p > 0.05$ (compared with doxorubicin, with unpaired t test). SD: Standard deviation.

ANNEX VII

Gametocytocidal activity of the new endoperoxides, PQ and DHA. The gametocytes 3D7-GFP were treated at their respective IC_{50s} (to asexual and sexual stages). And an extra dose, equivalent to 10 x IC_{50} to sexual stages of the PQ was also used.



* $p < 0.05$ (Compared with PQ at the lower doses, with Mann-Whitney test).

ANNEX VIII

% Mean of parasitaemias obtained by light microscopy and by flow cytometry in the stage-specific assay.

% Parasitaemia

	Rings		Trophozoites	
	Microscopy	Cytometry	Microscopy	Cytometry
0 hours	1.03	0.99	2.01	1.90
48 hours	7.80	7.59	5,82	5.70
96 hours	8.32	8.23	7.25	7.03

ANNEX IX

Frequency of recrudescence in Dd2- pressured *P. falciparum* strain after 60 days.
Two different initial inoculums were used, and the parasites were treated with new endoperoxides at the doses corresponding to IC_{90} (10x IC_{50}). The experiments were performed at least 2 times, in triplicate.

Compounds	Selection pressure (IC_{90})	Initial inoculum	Number of positive flasks (day positive)
LC131	21 nM	2×10^7	0 of 3
LC131	21 nM	2×10^8	0 of 3
LC132	266 nM	2×10^7	0 of 3
LC132	266 nM	2×10^8	0 of 3
LC136	25 nM	2×10^7	0 of 3
LC136	25 nM	2×10^8	0 of 3
MIS13	34 nM	2×10^7	0 of 3
MIS13	34 nM	2×10^8	0 of 3
LC163	435 nM	2×10^7	0 of 3
LC163	435 nM	2×10^8	0 of 3

ANNEX X

Universidade Federal do Rio Grande do Norte
COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

PROTOCOLO N.º **046/2013**

Professor/Pesquisador: *VALTER FERREIRA DE ANDRADE NETO*

Natal (RN), 31 de janeiro de 2014.

Prezado Professor/Pesquisador,

Vimos, através deste documento, informar que o projeto "BIOLOGIA E EPIDEMIOLOGIA MOLECULAR, TRIAGEM DE NOVOS FÁRMACOS PARA PLASMODIUM SPP. E TOXOPLASMA GONDII", **protocolo nº 046/2013**, após análise das adequações, foi considerado **APROVADO** por esta Comissão.

Informamos ainda que, segundo o Cap. 2, Art. 13 do Regimento, é função do professor/pesquisador responsável pelo projeto a elaboração de relatório(s) de acompanhamento que deverá(ão) ser entregue(s) dentro do(s) prazo(s) estabelecido(s) abaixo:

- Relatório Final: **OUTUBRO 2017** (30 dias após a conclusão do projeto).

Agradecemos a sua atenção e nos colocamos a disposição para eventuais esclarecimentos.

Cordialmente,

John Fontenele Araujo
Coordenador da CEUA

UFRN – Campus Universitário – Centro de Biociências
Av. Salgado Filho, S/N – CEP: 59072-970 – Natal/RN
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ANNEX XI

Current antimalarial drugs and associated resistance markers. (Adapted from (Haldar, Bhattacharjee and Safeukui, 2018)).

Chemical class	Common name	Targeted parasite stage	Genetic marker for drug resistance	
			Plasmodium falciparum	*Plasmodium vivax*
Sesquiterpene lactone endoperoxides	Artemisinin*	All parasite stages	*pfkelch13* (REF. 18)	Unknown
	Artesunate*‡	All parasite stages	*pfkelch13* (REF. 18)	Unknown
	Artemether*‡	All parasite stages	*pfkelch13* (REF. 18)	Unknown
	Dihydroartemisinin*‡	All parasite stages	*pfkelch13* (REF. 18)	Unknown
4-Aminoquinolines	Chloroquine‡§	Blood stages (trophozoite and schizont)	*pfcrt*[17,26]	*pvmdr1* (REF. 81)
	Amodiaquine*‡	Blood stages (trophozoite and schizont)	*pfcrt*, *pfmdr1* (REF. 129)	Unknown
	Piperaquine*‡	Blood stages (trophozoite and schizont)	*pfplm2* (REFS 37,38), *pfcrt*[55,130]	Unknown
	Pyronaridine	Blood stages (ring, trophozoite and schizont)	*pfcrt*[131]	Unknown
	Naphthoquine*	Blood stages (trophozoite and schizont)	Unknown	Unknown
Amino alcohols	Quinine§	Blood stages (trophozoite and stages I to III gametocytes)	*pfcrt*, *pfmdr1* (REFS 51,132)	Unknown
	Mefloquine*	Blood stages (trophozoite and schizont)	*pfmdr1* (REFS 133–135)	*pvmdr1* (REFS 81,82)
	Lumefantrine*‡	Blood stages (trophozoite and schizont)	*pfcrt*, *pfmdr1* (REFS 132,136)	Unknown
	Halofantrine§	Blood stages (trophozoite and schizont)	*pfcrt*, *pfmdr1* (REF. 133)	Unknown
8-Aminoquinoline	Primaquine*‡	Blood (gametocyte) and liver (schizont) forms	Unknown	Unknown
Antifolates	Pyrimethamine*	Blood and liver schizont and mosquito stage (oocysts)	*pfdhfr*[137]	*pvdhfr*[83]
	Sulfadoxine*	Blood and liver schizont	*pfdhps*[137]	*pvdhps*[83,138]
	Proguanil*	Blood stages (schizont and gametocyte) and liver schizont	*pfdhfr*[139]	Unknown
Naphthoquinone	Atovaquone§	Blood stages (schizont and gametocyte) and liver schizont	*pfcytb*[140]	Unknown
Antibiotics	Clindamycin§	Blood stages	Apicoplast target[57]	Unknown
	Doxycycline§	Blood stages	Apicoplast target[57]	Unknown
	Tetracycline§	Blood stages	Apicoplast target[57]	Unknown

crt, chloroquine-resistance transporter; *cytb*, cytochrome *b*; *dhfr*, dihydrofolate reductase; *dhps*, dihydropteroate synthase; *mdr1*, multidrug resistance protein; *pf*, *Plasmodium falciparum* gene; *pv*, *Plasmodium vivax* gene; *pfkelch13*, *P. falciparum* Kelch 13; *plm2*, plasmepsin 2. *Drug used in artemisinin-based combination therapy. ‡Antimalarial drug used alone or in combination for the treatment of *P. vivax* malaria. §Antimalarial drug used alone or in combination with molecules other than artemisinin derivatives.