Universidade de Aveiro – Departamento de Química

2015

Sofia Inês Leal Duarte

AÇÃO DE NOVOS CONJUGADOS PORFIRÍNICOS COM POTENCIAL ATIVIDADE ANTI-MALÁRIA EM MEMBRANAS

ACTION OF NEW PORPHYRIN CONJUGATES WITH POTENTIAL ANTI-MALARIA ACTIVITY ON MEMBRANES

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Dra. Maria da Graça da Silva Pinto Neves, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro, da Doutora Maria do Rosário Gonçalves Reis Marques Domingues, Professora Auxiliar com Agregação do Departamento de Química da Universidade de Aveiro e da Doutora Ana Teresa Peixoto Gomes, Investigadora de Pós-Graduação do Departamento de Química da Universidade de Aveiro

Apoio financeiro da FCT, da União Europeia, QREN, no âmbito do Programa Operacional Temático Fatores de Competitividade (COMPETE).

Apoio financeiro à unidade de investigação QOPNA (projecto PEst-C/QUI/UI0062/2013; FCOMP-34701-0124-FEDER-037296) e do Departamento de Pesquisa e Desenvolvimento de Fármacos, Instituto de Tecnologia em Fármacos, Farmanguinhos/ FIOCRUZ.

Aos meus pais e irmãos

O júri

Presidente	Prof. Dr. Prof Francisco Manuel Lemos Amado Professor Associado do Departamento de Química da Universidade de Aveiro
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Agradecimentos

Em primeiro lugar gostaria de agradecer às minhas orientadoras, à Doutora Graça Neves e à Doutora Rosário Domingues por todo o conhecimento transmitido, assim como o apoio dado ao longo deste projeto e pela confiança depositada em mim.

Gostaria de deixar um agradecimento especial à Ana Gomes, ao Nuno Moura e à Bebiana por toda a disponibilidade e prontidão na ajuda da execução deste trabalho.

À doutora Cristina um grande obrigada por todas as horas passadas em frente ao Q-TOF, por todas as palavras de apoio e especialmente por toda a boa disposição mesmo quando as coisas não corriam como esperado.

A todos os meus amigos tenho de deixar um especial obrigado pelo companheirismo, pelas palavras de apoio e por todos os momentos passadas durante estes 5 anos, que com certeza irão ficar para o resto da vida.

Aos meus pais e irmãos obrigada pela paciência, especialmente nos últimos tempos, pelo esforço que fizeram para que pudesse chegar até aqui, com certeza sem eles nada disto seria possível.

E por fim a ti João, por estares sempre presente e especialmente por toda a paciência.

Palavras-chave: Lípidos, malária, metaloporfirinas, peroxidação, *Plasmodium*.

Resumo: A malária é uma doença negligenciada que afeta mais de 100 países e ameaça metade da população mundial. Esta doença é transmitida pela picada do mosquito *Anopheles*, causando a infeção dos glóbulos vermelhos. O parasita da malária, *Plasmodium*, pertence à família dos protozoários, sendo por isso um organismo eucariota. Durante a invasão dos eritrócitos, o parasita degrada hemoglobina para obter nutrientes, contudo é libertado o grupo heme que é tóxico para a membrana do mesmo. Para ultrapassar os efeitos tóxicos do grupo heme, o parasita converte-o num pigmento insolúvel, a hemozoina. Análogos sintéticos de porfirinas podem ser usados como alternativa a combater a malária, uma vez que são estruturalmente idênticos ao grupo heme. Assim, o presente trabalho teve como objetivo a síntese de novos conjugados porfirínicos com fármacos já usados na prevenção da malária. Para isso usou-se a 5,10,15,20-tetrakis (pentafluorfenil*)* porfirina (TF_5PP) (composto 1) como porfirina inicial e sintetizaram-se novos derivados porfirínicos formados por conjugação da porfirina com três fármacos diferentes já usados na prevenção da malária: a primaquina (composto 3), a mefloquina (composto 5) e o artesunato (composto 9). A reação de conjugação para os dois primeiros compostos teve como base uma reação de substituição nucleofílica. A síntese do último conjugado não foi alcançada com sucesso. Posteriormente, foram sintetizados os complexos de Fe(III) dos três compostos obtidos: **1**, **3** e **5**.

Para avaliar a possível utilização destes conjugados monitorizou-se a peroxidação em lipossomas constituídos por dois fosfolípidos, 1,2-dimeristoil-sn-glicero-3-fosfocolina (dMPC) (lípido saturado) e a 1-palmitoil-2-linoleoil-3-fosfocolina (PLPC) (lípido insaturado). Os lipossomas foram incubados com os diferentes compostos, na presença e na ausência de H_2O_2, e a reação foi controlada por espectrometria de massa em modo positivo (ESI-MS). Através da razão $[dMPC+H]^+/[PLPC+H]^+$ foi possível avaliar a extensão da reação. Apenas os complexos de ferro dos compostos **1**, **3** e **5** (respetivamente os compostos **2**, **4** e **6**) mostraram ter capacidade para oxidar a PLPC e na presença do composto **2** houve um maior aumento da razão $[dMPC+H]^+/[PLPC+H]^+$. Verificou-se que este composto na ausência de H_2O_2 é mais eficaz que os compostos 4 e 6, tanto na presença como ausência de peróxido de hidrogénio. Os produtos de oxidação obtidos na presença dos diferentes complexos mostraram a presença de um, dois e três átomos de oxigénio e a sua formação poderá ter sido mediada por espécies oxo de alta valência ou via peroxidação lipídica induzida por radicais.

Key words: Lipids, malaria, metalloporphyrins, peroxidation, *Plasmodium*.

Abstract: Malaria is a neglected disease that affects more than 100 countries and threatens half of the world population. The disease is transmitted through the bite of an infected female mosquito *Anopheles*, causing infection of erythrocytes. The malaria parasite, *Plasmodium,* belongs to the family of protozoa, and so is a eukaryotic organism. During the invasion of the erythrocytes, the parasite degrades hemoglobin to obtain nutrients, yet is released the heme group that is toxic to parasite membrane. To overcome the toxic effects parasite converts free heme into an insoluble pigment called hemozoin. Synthetic analogs of porphyrins can be used as alternative to combat malaria, since they are structurally identical to the heme group. The present study aimed to synthetize new porphyrin conjugates with drugs already used in the prevention of malaria. For that, it was used 5,10,15,20-tetrakis (pentafluorophenyl) porphyrin (TF_5PP) (compound 1) as a starting porphyrin and it were synthetized new porphyrin derivatives formed by conjugation with three different drug, primaquine (compound 3), mefloquine (compound 5) and artesunate (compound 9). The reaction for the first two compounds was based on a nucleophilic substitution reaction. The synthesis of compound 9 was not achieved. Subsequently, the Fe(III) complexes of the three compounds: 1, 3 and 5 were synthetized.

To evaluate the possible use of these conjugates, lipid peroxidation was evaluated in liposomes constituted with two phospholipids: 1,2-dimyristoyl-sn-glycero-3-phosphocholine (saturated lipid) and palmitoyl-lineloyl-phosphatidylcholine (unsaturated lipid). Liposomes were incubated with the different compounds in the presence and in the absence of H_2O_2 and the reaction was monitored by mass spectrometry in positive mode (ESI-MS). Through the ratio $[dMPC+H]^+/[PLPC+H]^+$ it was possible to evaluate the extent of reaction. Only the iron complexes (compounds 2, 4 and 6) showed to have the ability to oxidize PLPC and in the presence of compound 2 there was a greater increase in the ratio $[dMPC+H]^+/[PLPC+H]^+$. It was verified that this compound in the absence of H_2O_2 has greater oxidation than compounds 4 and 6, both in the presence or absence of hydrogen peroxide. The oxidation products obtained in the presence of the different complexes showed the presence of one, two and three oxygen atoms and their formation may have been mediated by high valent oxo species or through radical-induced lipid peroxidation.

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Acronyms

ACT – artemisinin combination therapy
AMA-1 – apical membrane antigen-1
BOC – di-tert-butyl dicarbonate
CID – collision-induced dissociation
CRT – chloroquine-resistance transporter
CYP – cytochrome P-450
DBG – Duffy blood group
DBL – Duffy binding-like
DCC – N,N'-Dicyclohexylcarbodiimide
DMAP – 4-Dimethylaminopyridine
dMPC – 1,2-dimyristoyl-sn-glycero-3-phosphocholine
DV – digestive vacuole
EBA – erythrocyte binding antigen
EDC – 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EEF – exoerythrocytic forms
ESI-MS – electrospray ionization mass spectrometry
FAB – fast atom bombardment
GPI – glycosylphosphatidylinositol
IEPM – infected erythrocyte plasma membrane
IM – intramuscularly
IUPAC – International Union of Pure and Applied Chemistry
MALDI – matrix-assisted laser-desorption ionization
MDR1 – multi-drug resistance gene-1
MS – mass spectrometry
MS/MS – tandem mass spectrometry
MSP – merozoite surface protein
PA – phosphatidic acid
PC – phosphatidylcholine
PDT – photodynamic therapy
PE – phosphatidylethanolamine
PG – phosphatidylglycerol
PI - phosphatidylinositol
PLPC – palmitoyl-lineloyl-phosphatidylcholine
PPIX – protoporphyrin IX
PS – phosphatidylserine
PUFA – polyunsaturated fatty acids
PV – parasitophorous vacuole
PVM – parasitophorous vacuole membrane
Q – quadrupole
RBC – red blood cell
ROS – reactive oxygen species
SM – sphingomyelin
TF_5PP – 5,10,15,20-tetrakis(pentafluorophenyl)porphyrin
TMS – tetramethylsilane
TOF - time of flight
TRAP – thrombospondin-related adhesive protein

I. Introduction

Preface

Malaria is a disease that affects most developing countries and consequently the investment in the treatment is low leading to a high mortality rate. It is important to develop new strategies to combat this disease once the parasite has already developed resistance mechanisms to some of the drugs used nowadays. Malaria parasite invades the erythrocytes and degrades hemoglobin to obtain nutrients. To overcome the toxic effects parasite converts free heme into an insoluble pigment called hemozoin. So, the strategy proposed for this work involved the synthesis of new conjugates based on porphyrins and some drugs already used in malaria treatment and to evaluate their ability of oxidize phospholipids present in parasite membrane. In the next sections of this introductory chapter some general aspects about malaria disease and treatment, porphyrins and their importance in oxidative process namely in lipid oxidation and mass spectrometry the tool used to follow lipid oxidation will be presented.

1. Malaria, one of the neglected diseases

Developing countries are mainly affected by a group of diseases called "neglected diseases" which include malaria, chagas disease, sleep disease, among others. These diseases lead to a high mortality rate in countries with negative economic impact and thus deserving an increased medical attention *(1)*.

Malaria affects more than 100 countries and threatens half of the world population, killing about 655 thousand people each year *(1)*. According to the world malaria report, in 2012 there were nearly 210 million new cases and 80% of them were observed in the African continent, followed by Asia (13%) *(2)*. The incidence of malaria increased between 2000 and 2005, although in 2012 it was observed a decrease of the number of cases *(2)*. In Africa, this disease is the main cause of children death under 5 years old, killing a child every minute *(1)*. There are many factors that affect the clinical outcomes of the disease, such as: geographic and social factors, like the access to treatment; parasite factors which include drug resistance, invasion pathways and multiplication rate; and finally host factors, for instance immunity, age, and pregnancy. This convergence of diverse factors can result from an asymptomatic infection to death *(3)*. The symptoms of malaria do not appear with the mosquito bite, usually it takes ten to fifteen days to the first symptom appear. The most common symptoms are fever, headache, fatigue and vomiting *(1,2)*.

Malaria is transmitted by a parasite called *Plasmodium* through the bite of an infected female mosquito *Anopheles*, causing infection of red blood cells (RBC's) *(1)*. In the following section it will be presented some detail about *Plasmodium*.

2. Plasmodium, the parasite that causes Malaria

The malaria parasite *Plasmodium* belongs to the genus protozoa and is a eukaryotic organism. Five different species of the parasite are able to affect humans which include *P. falciparum, P. malariae, P. vivax, P. ovale* and, more recently, *P. knowlesi*. The major responsible for the most severe cases and clinical worries of malaria are *P. vivax* and *P. falciparum.* However, it is *P. falciparum* that accounts for most of the mortality associated with the disease *(1,4)*.

Plasmodium enters the host organism by the mosquito bite. The injected parasites first invade the blood stream and then travel to liver, to hepatocytes, where there is a process of maturation to attack red blood cells. The different species of *Plasmodium* may have different ways of invasion, for example, *P. falciparum* can invade a large number of RBC's while *P. vivax* only attacks reticulocytes, the precursors of erythrocytes *(3)*. The invasion allows the parasite to have access to a rich source of nutrients and gives protection from the immune system of the host. During this process, *Plasmodium* use their own machinery to identify the right cells, establish themselves and start the infection *(5)*.

2.1. Life cycle of *Plasmodium* parasites

Malaria parasites have a complex life cycle involving both a vertebrate and an invertebrate host. The sexual phase of the life cycle happen inside the mosquito while the asexual phase begins when the parasite invades the RBC's *(6)*.

The cycle begins in the invertebrate host and *Plasmodium*-infected mosquito injects the parasite forms, sporozoites, into the vertebrate host (figure 1). They can be placed directly on blood stream (less frequent) or on sub-cutaneous tissue, travelling then to the liver *(7)*. Not all sporozoites migrate to the liver, some of them are inoculated by the insect and remain on the skin, growing at the place of inoculation *(8)*. The co-receptor present on sporozoite membrane necessary for invasion involve both thrombospondin domains and thrombospondin-related adhesive protein (TRAP) *(9)*. These domains bind to specific heparin sulfate proteoglycans present on hepatocytes. Within the hepatocyte, begins the formation of a parasitophorous vacuole (PV) *(7)* that provides the necessary conditions

to parasite replication, creating exoerythrocytic forms (EEF) which grow until the size of the parasite. The vesicles containing the parasites are called merosomes and once they reach the blood stream, burst and release erythrocytes-infected parasites, which are now called merozoites *(10)*. The release of the merozoites marks the beginning of the infection and the appearance of the first symptoms. After merozoites spread into the blood stream, they are able to invade the erythrocytes, continuing the asexual stage of the cycle (figure 1) *(11)*.

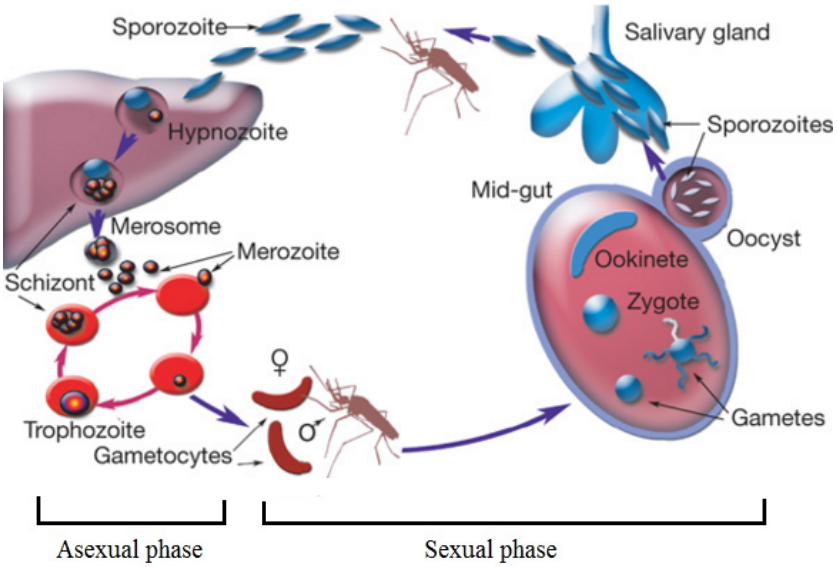
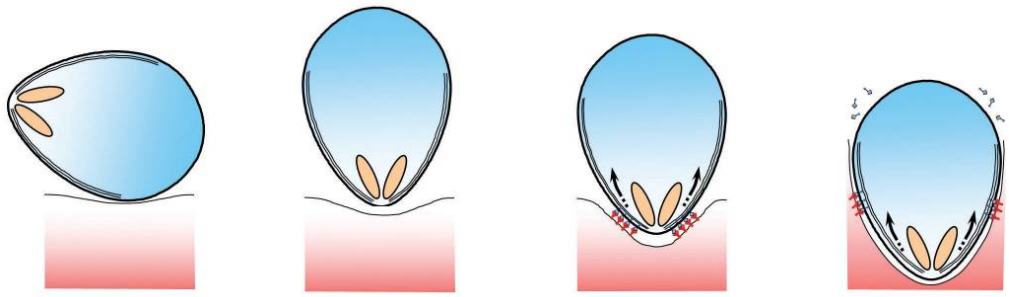


Figure 1 – Life cycle of malaria parasite (adapted from (12)).

Hepatic stage is the first essential phase for the development of the parasite. It is in this stage that *Plasmodium* gains the capacity to infect the RBC's *(10)*. In case of an infection by *P. falciparum*, it is predictable that each hepatocyte releases up to 40 thousand merozoites *(13)*. During the infections mediated by *P. vivax* and *P. ovale*, there are latent forms called hipnozoites that stay in the hepatocytes from variable time, causing disease relapses *(14)*.

The invasion of host cells by merozoites is driven by the parasite machinery and can be divided in several steps involving recognition, attachment and entrance to the erythrocytes; all these processes include a great number of proteins *(5,6)*. The sequence of invasion is similar for all *Plasmodium* species. For the recognition process, were already identified some of the proteins involved. The merozoite surface proteins (MSP's) link to the membrane *via* glycosylphosphatidylinositol (GPI) and the distribution along parasite membrane mediate the initial contact with the erythrocyte (figure 2a). This

process may occur at any point of the parasite surface. The attachment is followed by the apical reorientation before the parasite enters the cell and for that it was identified an apical membrane antigen 1 (AMA-1), which is required to establish the interaction *(15)*. After the reorientation of the parasite, the initiation of the entry process occurs, which may involve direct interaction of ligands from the parasite with erythrocyte receptors (figure 2b). During this processes an irreversible convergence between membranes seems to occur, making this an irreversible step that leads the parasite infection. The invasion of the erythrocyte involves disruption of the PV and the host cell membrane. Duffy blood group (DBG) antigen is a receptor present in erythrocyte membrane and it appears to have a role in this membrane junction. The connection between Duffy binding-like (DBL) protein family to the DBG antigen was first found on *P. vivax (5,6)*. Homologous DBL proteins were also found in other species of *Plasmodium*. The erythrocyte binding antigen (EBA) was the first binding protein identified on *P. falciparum* and it is a sialic acid binding protein that binds specifically to glycophorin A, a sialoglycoprotein present on the erythrocyte membrane *(16)*. Some proteases may have a role on the disruption of the erythrocyte membrane (figure 2c) *(6)*. When the parasite invades the erythrocyte it is also formed a parasitophorous vacuole to form a favorable location for his development, that surrounds the parasite *(5)*.



a) Initial contact b) Apical reorientation c) Junction formation and parasite invasion

Figure 2 – Representation of the mechanism of erythrocyte invasion (adapted from (17)).

In the blood stream, some of merozoites develop into gametocytes, that are the sexual forms of the parasite *(18)*. These can be taken up by other mosquitoes during a blood feeding, developing then into gametes already inside the insect. Afterwards, the sporozoites are developed and injected during the next blood meal. The other merozoites are able to produce more merozoites per mature parasite, each one invading other RBC's. Once inside the erythrocyte, the merozoite develop first to a ring form and then to a

thropozoite form. Thropozoite form then evolves to a schizont stage which can divide several times and produce new merozoites *(6)*.

During the invasion is formed the PV and the parasitophorous vacuole membrane (PVM) that surrounds the parasite and it is necessary for his development *(19)*. At earlier stages of the development, ring-stage and thropozoite, it is formed a digestive vacuole (DV) with a lower pH, also called food vacuole, where is alleged to occur the digestion of hemoglobin. Hemoglobin degradation occur firstly in pre-DV vesicles that merge with each other and form a central DV *(19,20)*.

2.2. Hemozoin: malaria pigment

Hemoglobin covers 95% of all cytosolic proteins of red blood cells *(21)* and during the intraerythrocyte cycle of the parasite at least 95% is converted to hemozoin *(22,23)*, since almost host cell cytoplasm is consumed. Hemoglobin from RBC's is ingested by parasites, into the food vacuole, where is degraded by proteases, releasing heme group. Different mechanisms of oxidation by heme ion have been described, which involve the formation of free radicals that are harmful for parasite membrane. Free heme is a lipophilic molecule and can interact with membrane, promoting lyses of the same by the oxidation of lipid content, leading to parasite death *(24)*. In order to overcome this process, the parasite developed mechanisms to avoid damage of the membrane, by inducing heme polymerization to an insoluble pigment, called hemozoin, which cannot damage the erythrocyte membrane, allowing the survival of the parasite *(25)*.

Production of malaria pigment is the main fate of heme, which is almost entirely converted to hemozoin *(22)*. Hemozoin consists only of iron(III) complex of protoporphyrin IX (Fe(III)PPIX), the molecules are connected to each other through the interaction of the propionate group of one Fe(III)PPIX and the Fe(III) center of another. One dimer of Fe(III)PPIX is called β-hematin, and hemozoin is a polymer consisting of infinite dimers of β-hematin connected by hydrogen bonds *(26)*. Some evidences suggest that hemozoin formation is mediated by lipids *(27)*. Inside the food vacuole, neutral lipid bodies are formed where hemozoin is incorporated. Lipid environment promotes the interaction between the molecules of Fe(III)PPIX *(28)*. These lipid bodies may also be a protection to heme group, that can be degraded when in contact with hydrogen peroxide (H_2O_2) *(29)*.

The hemozoin formation is a mechanism of defense from the parasite to avoid the damage promoted by free heme. Some therapeutic strategies against malaria have this pigment as the base of action, inhibiting its formation *(23)*.

2.3. Malaria treatment

As soon as malaria is first diagnosed, it is necessary to determine if the infection is driven by *P. falciparum* or non-*falciparum* and if it is severe or not. Evolution of the disease it too rapid and due to complex life cycle of the parasite, patients can rapidly be in a worse state than the day before even with an adequate treatment *(4)*. A great challenge about malaria treatment is the fact that the parasite became resistant to different kind of drugs *(30)*. The most problematic specie is *P. falciparum*, since it has already been observed resistance with different kind of treatments such as chloroquine, sulphadoxine-pyrimethamine and mefloquine (figure 3) *(30)*.

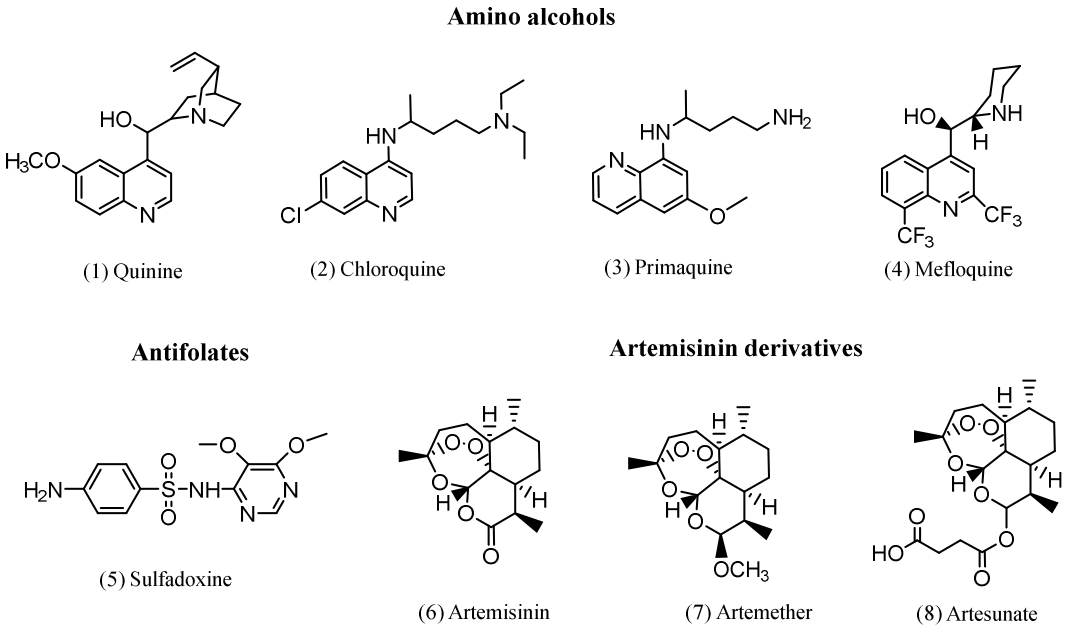


Figure 3 – Structure of compounds used against Malaria.

Chloroquine was, from decades, the most effective drug against malaria but when *P. falciparum* became resistant, other strategies to control the disease had to be applied. Despite this fact, the drug is still used to fight *P. vivax* infections and in America it is still therapeutically efficient. Nowadays, a different treatment is used based on a combination

of some drugs, as is the case of mefloquine and primaquine with artemisinin (figure 3), called artemisinin combination therapies (ACT's). In 2012, ACT's had been adopted as the first line treatment in about 80 countries where *P. falciparum* is prevalent (Table 1) *(2)*.

Anti-malarial drugs have different ways of action, and can act on different stages of parasite life cycle *(31)*. Artemisinin is a drug effective against multi-resistant strains of *P. falciparum* and has a high specificity to *Plasmodium*, in both sexual and asexual phase. It is able to reduce the spread of the disease in low transmission areas *(32)*.

The exact mechanism of action of artemisinin is not well stablished, but it is thought that the endoperoxide bridge from the molecule is necessary for its antimalarial activity *(33)*. Free heme interacts with artemisinin and it is necessary for the drug activation, promoting then the formation of free radicals that may interact with parasite proteins and lipids. *(32,33)*. Besides the fact of the production of free radicals, the interaction with the metal also inhibits the formation of the hemozoin pigment *(33)*. The association to the iron metal is a necessary phase for the drug to become covalently bound to parasite macromolecules. Hartwig *et al (34)* suggested that artemisinin accumulate in neutral lipids and cause oxidative membrane damage.

Chloroquine, mefloquine and primaquine are antimalarial drugs from the class of quinolines and, the mechanism of action is, in part, similar to artemisinin, due to the interaction with heme *(29)*. These molecules form a drug:heme complex into the growing polymer of hemozoin, blocking the polymer extension *(35)*. The fact that artemisinin has a rapid range of elimination and is combined with other drugs, makes this therapy the best way to avoid the development of resistance mechanisms by the parasite *(32)*.

Plasmodium strains differ in prevalence and so in the treatment applied. Table 1 shows the number of countries from the different regions where the strains are prevalent and also the treatment adopted.

Table 1 – Countries adoption policies for malaria treatment and prevalence of the transmission (adapted from *(36)*)

Policy	AFR	AMR	EUR	SEAR
Number of countries with ongoing malaria transmission	45	21	3	10
Number of *P. falciparum* endemic countries	44	18	-	9
Number of *P. vivax* endemic countries	7	20	3	10
ACT for treatment of *P. falciparum*	43	9	1	9
Treatment with quinine/artemether IM/artesunate suppositories	40	4	-	5
Direct treatment with Primaquine	3	11	3	3
Primaquine used on *P.vivax* cases	7	20	3	10

AFR-African region; AMR- American region; EUR- European region; SEAR- South-East Asia region

As seen from the table, *P. falciparum* is more prevalent in African continent and *P. vivax* in American continent. Besides the ACT treatment in African continent, quinine, artemether intramuscularly (IM) or artesunate suppositories are also adopted by 33 countries in this region. Primaquine is most widely used with ongoing *P. vivax* transmission *(2)*.

2.4. Resistance mechanism

Drug-resistant falciparum malaria is one of the bigger obstacles to world health. There were already verified resistance mechanisms, mainly from *P.falciparum,* with quinolone family and antifolates *(37)*. The resistance developed is mainly due to mutations in genes encoding to proteins or enzymes necessary to the drug action *(31,37)*.

Regarding the case of chloroquine, the resistance mechanism involves the reduction of drug in food vacuole, and is associated to multi gene mutations *(31)*. The chloroquine resistance mechanism is not seen in the same way between authors. Some researchers believe that is an increase of the drug efflux *(38)* while others suggest that the low levels accumulated in food vacuole are more important *(39)*. Two different transporters have been identified on food vacuole and may have been associated with the mechanism of resistance, CRT (Chloroquine-resistance transporter) and MDR1 (Multi-drug resistance gene-1) *(30,40)*.

The fact that parasites started to become resistant to some drugs, other ways to combat the infection have become prevalent, like combination of drugs (ACT's) *(2)* . Heme group, as it was already referred, is toxic to parasite membrane and synthetic analogs of porphyrins, with a similar structure when compared to the heme, may be an alternative as a therapeutic approach to combat the malaria parasite at blood stage of infection *(41)*. The adopted treatments for the disease are mainly directed to the hemozoin formation *(23,32)* and membrane lipids are not yet considered a specific target for the treatment.

3. Porphyrins and its biological importance

Porphyrins are a class of organic molecules presenting a macrocycle structure composed by four rings of pyrrole type. The four rings are connected by four methinic carbons, creating a heterocyclic nucleus known as porphyrin (Figure 4a). This nucleus can present different substituents in the peripheral positions and can occur naturally or be obtained by synthesis. The presence of four nitrogen atoms inside the ring allow the formation of a complex with metallic ions, forming metalloporphyrins. Naturally, only a few metal ions can coordinate with porphyrins, as it is the case of magnesium, iron, zinc, manganese, copper, nickel and cobalt. Chlorophylls along with the heme group are the most abundant metalloporphyrins in natural systems. The heme group, the iron complex of protoporphyrin-IX (Figure 4b), is able to incorporate into proteins, being the prosthetic group of hemoglobin, myoglobin, cytochromes, catalases and oxidases *(42)*. Hemoglobin is the most abundant protein in humans, has four heme groups in his constitution and develops many important functions in the body as the oxygen transport to the different tissues, being carried by erythrocytes, and is one of the major buffering systems of the body *(43)*. Chlorophylls (Figure 4c) occur complexed with magnesium ion and some of them have one of the pyrrolic units reduced. *(42)*.

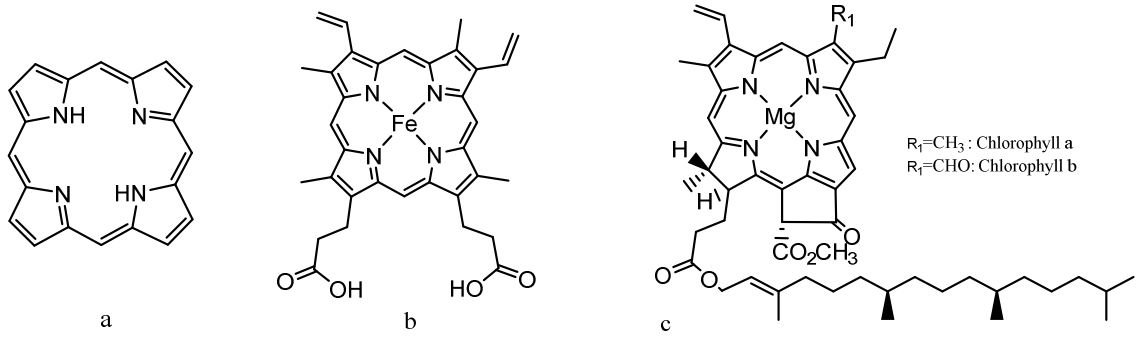


Figure 4 – Structure of porphyrins ring (a), heme group (b) and chlorophylls (c).

There are two different systems of nomenclature for these macrocycles, one proposed by H. Fisher and other by International Union of Pure and Applied Chemistry (IUPAC) (figure 5). According to Fisher nomenclature, the β (beta) positions of the pyrrole rings are numbered from one to eight and the methinic bridges, also known as *meso* positions, are nominated by the Greek letters α (alpha), β (beta), γ (gamma) and δ (delta). In the IUPAC system, all the positions are numbered from one to twenty four, including the nitrogen atoms. IUPAC system demonstrates to be foremost understandable once the nomenclature include all the atoms from the ring *(42)*.

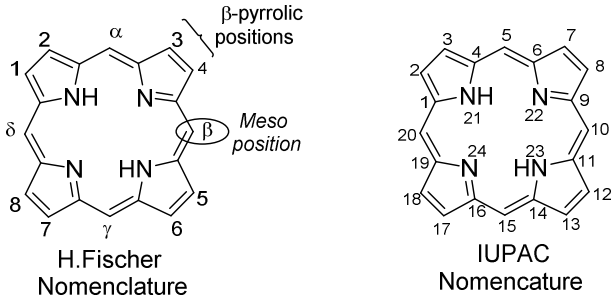


Figure 5 – Representation of the two nomenclature systems used in porphyrins.

The macrocycle core of porphyrin systems is highly conjugated, having thus some resonance forms. There are twenty two conjugated electrons π but only eighteen of these are involved in the aromatic character of porphyrins. These compounds have an absorption spectrum very characteristic on the visible zone *(42)*. The spectrum have an absorption band near 400 nm, called Soret band, and more four bands with superior wavelengths, called Q bands. Intensity, position and the number of these bands are influenced by the substituents on the ring and by the metal present inside the ring *(42)*. Due to their properties, porphyrins can be useful in several distinct areas, however it is in medicine that the application of these compounds are more remarkable. In fact,

photodynamic therapy (PDT) is the most known application for porphyrins, it is used to treat cancer cells *(44)* and to photo inactivate microorganisms, virus *(45–47)* and protozoa *(48,49)*. These compounds have already been tested against the malaria parasite and have a potential effect on it, as it will be referred ahead *(41,50,51)*.

3.1. Porphyrins against Malaria

The first report of the use of porphyrins against Malaria parasite was by Basilico *et al (52)*, that used non-iron porphyrins and verified the inhibition of β-hematin polymerization. These authors tested porphyrins identical to heme group (figure 6a and 6b), with the hypothesis that the interaction between the two porphyrins is possible by π – π interactions, which results in a heme:porphyrin complex *(52)*. Recently, Abada *et al (51)* tested porphyrin derivatives against protozoa, being the most effective one the compound represented on figure 6c. In this case, it was verified a high selectivity to *Plasmodium* parasites and the activity against them was bigger than to other protozoa.

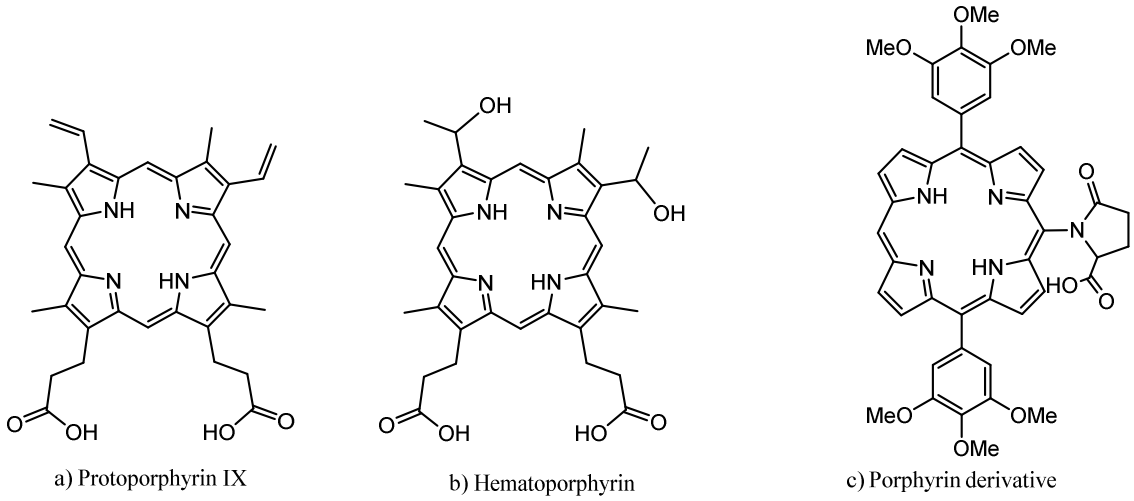


Figure 6 – Porphyrin derivatives used against malaria.

Cole *et al (50)*, studied the activity of some metalloporphyrins, and justified the effect also via π interactions. Consequently, these porphyrins appear to be an ideal system to establish those interactions being powerful targets to stop hemozoin formation, leading the heme group available to destroy the parasite. These authors tested protoporphyrin IX (PPIX) (figure 6a) with various center metals: chromium, copper, cobalt, manganese, zinc, magnesium and tin. It was observed a total inhibition of heme polymerization by the compounds: Cr(III)PPIX, Co(III)PPIX, Mn(III)PPIX and Cu(II)PPIX. However, the

other complexes (with Zn, Mg and Sn) also show to be effective against formation of β-hematin *(50)*.

The presence of the metal ion has an evident effect on PPIX connection with metalloporphyrins, being much more efficient than the corresponding free bases. Thereby, π-stacking interactions between porphyrins can be modulated by metal combinations *(50)*. Other metal combinations with PPIX were also studied by Begum *et al (41)*, namely with silver, gallium and palladium. The most potent inhibitors of β-hematin formation where those with the metals Sn and Ga. Some other compounds tested showed to be effective but in higher concentrations. In fact, some of the metalloporphyrins had strong activity against trophozoite (GaPPIX and SnPPIX), having a higher range than drugs used against malaria like artemisinin and chloroquine *(41)*.

Metalloporphyrins were proved to have potent activity by inhibiting heme polymerization, thus showing powerful antimalarial activity, being recognized as great inhibitors of β-hematin formation *(41,50,51)*. Besides the fact that these compounds can inhibit hemozoin polymerization, the presence of the metal can also be favorable to the production of reactive oxygen species (ROS) leading to destruction of parasite membrane *(53)*.

3.2. 5,10,15,20-tetrakis(pentafluorophenyl)porphyrin (TF_5PP)

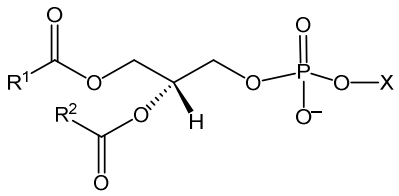
In this work, the porphyrin used to synthetize the porphyrin derivatives was TF_5PP. In fact, this porphyrin has been used to synthetize porphyrins derivatives bearing substituents in their para positions of the meso-aryl groups. This is due to its capacity to react with nucleophiles as amines and alcohols, among others, that are able to substitute the *para*-F atoms from the pentafluorophenyl groups *(54)*.

4. Eukaryotic membrane composition: lipids and lipid oxidation

Lipids are compounds with a great chemical and functional diversity which, except for cholesterol, link fatty acids with different structures. The main classes of membrane lipids are glycolipids and phospholipids *(55)*.

Phospholipids are the major constituents of all cellular membranes, forming a semi-permeable membrane to water and solutes, allowing the communication between cells *(56)*. These biomolecules have the capacity of mediating regulatory functions in cells and may have a role in apoptosis and cell signaling *(57,58)*. The most abundant class of lipids

in cells is glycerophospholipids, responsible to maintain the membrane integrity. Glycerophospholipids are constituted by a glycerol molecule that has two fatty acids linked in the positions sn-1 and sn-2 and a phosphate group in the sn-3 position. The phosphate group may be connected to polar molecules that determine the type of phospholipid. Hereupon, the polar molecules are choline, ethanolamine, serine, glycerol and inositol, resulting in the classes phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), respectively, and with no head group phosphatidic acid (PA) (figure 7) *(55,56)*. From the different classes of glycerophospholipids, PE and PC are the most abundant in cellular membranes of eukaryotic organisms *(55,58)*. The link between glycerol and fatty acid in sn-1 position may occur by an ester bond, called phosphatidyl; by an alkyl-ether bond, named plasmanyl or by a vinilic-ether bond designated by plasmenyl *(59)*.



X = H (PA)
X = $CH_2CH_2N^+(CH_3)_3$ (PC)
X = $CH_2CH_2NH_3^+$ (PE)
X = $CH_2CH(COO^-)NH_3^+$ (PS)
X = $CH_2CHOHCH_2OH$ (PG)
X = $C_6H_{12}O_6$ (PI)

Figure 7 – Representation of different phospholipid classes

Membrane fluidity depends of the lipid composition, mainly phospholipids and cholesterol content *(60)*. Phospholipids have distinct physical characteristics, including the capacity to bind cholesterol due to the level of unsaturation. The presence of unsaturated fatty acids, predominantly with *cis* conformation, increases the membrane fluidity when compared to membranes only constituted by saturated fatty acids *(55)*. Each phospholipid has an individual influence on the membrane fluidity which should be known and considered. Thus, choline group of PC is large, increasing the membrane fluidity. On the other hand, PE reduces the fluidity because ethanolamine is a small molecule, making the area of the fatty acyl chains bigger compared to head group. Although, the shape does not determine fluidity itself, some other factors like hydration and the level of unsaturation also have a role in membrane fluidity *(61)*.

4.1. Membrane composition of *Plasmodium*

Plasmodium is a eukaryotic parasite and his plasma membrane has the same constitution as the most eukaryotic organisms. PC and PE are the major phospholipids of cellular membrane *(57)*, PC accounts for about 56% while PE represent about 27% of the total phospholipid composition *(62)*. Other phospholipids are present in his membrane, and PS and sphingomyelin (SM) are those that are present in a higher range. Phospholipid composition of uninfected erythrocytes is much different from the one of purified parasite. Parasite contains almost the double of PC, three times more PI and less SM and PS than erythrocytes. However, when *P. falciparum* infects the erythrocytes it induces some modifications on erythrocyte membrane composition and the lipid content is significantly adjusted *(62)*.

This alteration perhaps occurs as a result of the effect of parasite metabolism on erythrocytes lipids or by the trafficking of lipids between the parasite and erythrocytes membrane *(62,63)*. Looking to the malaria infected erythrocytes, there are two different membranes: infected erythrocytes plasma membrane (IEPM) and PVM that surrounds the parasite. There is a decrease of the ratio cholesterol/phospholipid in infected cells by *P. falciparum*, leading to an alteration on membrane fluidity *(64)*. Hsiao *et al (62)* demonstrated that some classes of lipids are altered on IEPM. Compared to normal erythrocytes, IEPM has higher content of PC and PI, and a lower content on SM and PS. Parasite uses its synthetic capability to promote modifications on membrane of erythrocytes, due to the presence of enzymes with the capacity to synthetize glycerophospholipids *(63)*. It was also verified a decrease of polyunsaturated fatty acids (PUFA) and thus the membrane became less fluid. It seems that the erythrocyte membrane become more likely to parasite membrane. However erythrocyte membrane integrity is maintained by some compensatory changes, as is the case of the reduction of SM content *(62)*.

Phosphatidylcholine is one of the major constituents of parasite membrane and due to this fact it is a molecule to have in attention. During the invasion process there is an insertion of host PC into the PVM, and this showed to be essential to parasite maintain protein composition and to avoid host cell elimination mechanisms. PC depletion also appear to affects the trafficking of proteins to parasite surface. The presence of proteins in PVM seemed to be indispensable for parasite to avoid the elimination by erythrocytes, which may be an explanation to why PC plays a critical role in parasite survival *(63)*.

Thus, as PC plays a crucial role to parasite survival and multiplication, it should be consider a potential target to new therapeutically drugs. This work will focus on the porphyrin ability to oxidize parasite membrane.

4.2. Phospholipid oxidation

Phospholipids are molecules susceptible to oxidation, creating modifications in oxidative conditions and during the reaction with ROS, producing new compounds. Resulting products from the oxidation are structurally different and have distinct biological activities *(65,66)*. Lipid peroxidation may be initiated by any specie that has the capacity to withdraw a hydrogen atom from the polyunsaturated fatty acids (PUFA) of the side chain of membrane lipids *(67)*.

Oxidative modifications of phospholipids depend on the nature of the oxidant and it determines the oxidative reactions. Oxidation may result from chemical reactions, by radicals or by enzymes and it is also associated with age related diseases *(65)*. Radical species tend to be more reactive but less selective than non-radical species. These last are able to diffuse from the point of origin. Consequently, the concentration and the nature of the oxidizing species are important, as well as the presence of antioxidant molecules. Low levels of H_2O_2 may disrupt the formation of the hydroxyl radical by Fenton reaction and consequently compromise phospholipid peroxidation reactions. A higher concentration of O_2 compared with less H_2O_2 suggests that iron complexes may have a higher role in the oxidation compared with hydroxyl radical *(67)*.

4.2.1. Iron porphyrins as lipid oxidants

Cytochromes P-450 (CYP) family of enzymes are monooxygenases linked to plasma membrane. Being much like to heme group, the mechanism of oxidation mediated by CYP's is similar to that what may happen in parasite membrane. This enzymes have been associated to processes like hydroxylation and epoxidation of aliphatic groups *(68)*. The mechanism of action of CYP's is represented on figure 8.

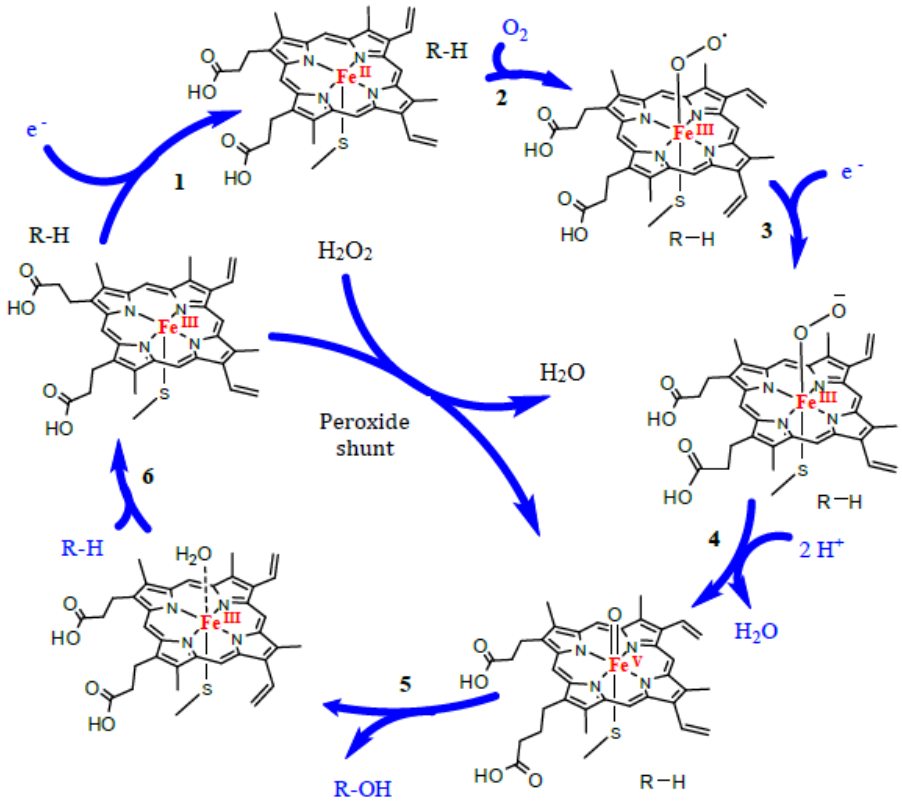


Figure 8 – Catalytic cycle of CYP (adapted from *(69)*).

The cycle represented in figure 8 shows two possible pathways in substrate hydroxylation, using molecular oxygen or peroxides. Explaining the long pathway (from 1 to 6 reactions), firstly Fe(III) is reduced to ferrous state (Fe(II)) (step 1) that has affinity to oxygen, forming a ferric superoxide complex (step 2). Afterwards, occurs a reduction of the formed specie followed by protonation of the same, allowing the heterolytic cleavage of O-O bond (steps 3 and 4). During the reaction 4, the intermediates: [(Porphyrin)Fe(V)=O] or [(Porphyrin)Fe(IV)=O] $^{\bullet+}$ can be formed, being responsible for substrate oxidation *(70)*, transferring the oxygen atom to the substrate. The last step of the reaction is the regeneration of the initial compound. The peroxide shunt is a shorter pathway that involves the same intermediates formed in step 4, but there is a direct formation of those from the Fe(III) complex, in the presence of peroxides. This mechanism proposed for CYP can be associated with Fe metalloporphyrins, as the catalytic center is the same. However, different mechanisms have been proposed for the mechanism of oxidation by iron porphyrins *(24)*. In Fenton-like mechanism (figure 9), it is formed a hydroxyl radical, that is able to start the mechanism of lipid peroxidation.

Catalysis mediated by this mechanism results in a rapid regeneration of Fe(III) due to the high oxygen affinity *(24)*.

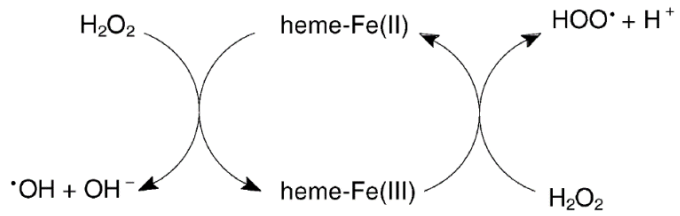


Figure 9 – Representation of Fenton-like mechanism (24).

Other proposed mechanisms of oxidation involves higher states of oxidation of the ion (figure 10) *(24)*. The most accepted mechanisms for reactions of epoxidation and hydroxylation by metalloporphyrins are represented on figure 10. These mechanisms are the same as those explained in the mechanism of CYP *(24,68)*. The ability of metalloporphyrins to oxidize several biomolecules makes them good candidates to being used in biomimetic catalysis.

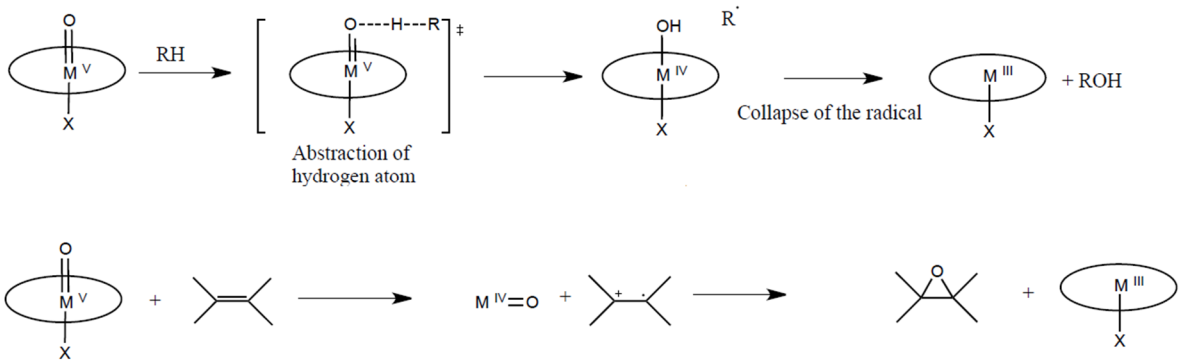


Figure 10 – Possible mechanisms of epoxidation and hydroxylation by porphyrins (adapted from *(69)*).

As a consequence of lipid oxidation, the modifications induced in membrane lipids result in a high variety of products structurally different among each other and from the precursor *(71)*. Thereby, the polarity and the shape are changed, leading to loss of the physic and functional properties of the membranes where they are in. It is also possible that the interactions between the molecules change the selective permeability with a consequent increase of the penetrability, loss of symmetry and ionic hemostasis, interaction lipid-lipid and lipid-protein and reduce of membrane fluidity *(72)*.

In the course of oxidation, new oxidation compounds are formed, resulting in a great diversity of compounds that are classified according to the type of modifications. The resulting products may be called long chain products if the phospholipid chain is preserved and has extra number of oxygen atoms, resulting in an increase in phospholipid mass; short chain products if the phospholipid is broken down by oxidative cleavage that may have terminal aldehyde or carboxylic group; and adducts, which result from reaction between resulting products of oxidation and carbonyl and amino groups from adjacent biomolecules *(71,72)*. The presence of these phospholipids in membranes may cause disequilibrium on membrane permeability due to alteration of fluidity and packing of phospholipids *(61)*, which may induce apoptosis *(65)*.

4.2.2. Phosphatidylcholine oxidation

Phosphatidylcholine is the main component of *Plasmodium* membrane. It has a fatty acyl chain unsaturated, making the process of oxidation favorable. Radical peroxidation affect the physical properties of the membranes which is reflected in membrane integrity and permeability *(73)*.

Tandem mass spectrometry is widely used in the identification of resulting products from the oxidation of phospholipids *(73–76)*. Glycerophospholipids as the main class of phospholipids in membranes is the most studied class by mass spectrometry. Reis et al *(74)* studied long chain products of palmitoyl-lineloyl-glycerophosphatidylcholine (PLPC) liposomes using liquid chromatography coupled to tandem mass spectrometry. Linoleic acid has two unsaturations and is susceptible to suffer oxidation, whereas palmitoyl is a saturated acid and is less susceptible to be oxidized. They found products resulting from the insertion of one to five oxygen atoms in sn-2 position. The same authors also used electrospray tandem mass spectrometry to identify short chain products of phosphatidylcholine *(76)*. Therefore, mass spectrometry is a very powerful and useful technique in the identification of several compounds formed during oxidation *(74)*.

5. Mass spectrometry

Mass spectrometry was discovered by J. J. Thomson and F. W. Aston in the beginning of twentieth century, being nowadays one of the most sensitive techniques in the

characterization of a great diversity of biomolecules like lipids and proteins. This technique is a quantitative and qualitative method in the analysis of ions, being quick and selective. The base of mass spectrometry is the separation of ions according to its atomic mass (*m*) and his electric charge (*z*) *(77)*.

A spectrometer is constituted by three essential parts: ionizing font, an analyzer and a detector (figure 11). The sample is introduced on the ionizing font, where the ionization of the molecules occur and the ions are conducted to the analyzer where they are separated by their mass/charge (*m/z*) ratio. It is necessary to have a vacuum pump to control the pressure within the mass spectrometer. Low pressure is necessary to limit the collisions due to the possibility of produce unwanted products or loss of charge. Subsequently they are detected on the detector, where the results are shown by the form of a spectrum, which have the mass of ionic products (as m/z ratio) on the abscissa and their relative abundance on the ordinate. The mass spectrometer is usually connected to a computer to process and record data *(77,78)*.

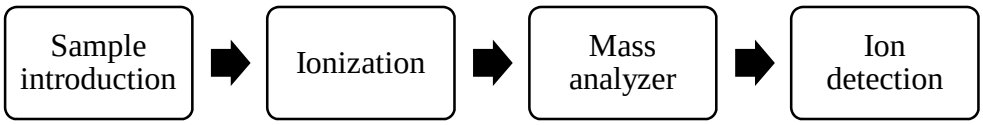


Figure 11 – Typical components of a mass spectrometer: ion source to ionize the sample; mass analyzer to separate the compounds and the detector (adapted from *(59)*).

The first spectrometers only had the capacity to analyze a restricted range of compounds, with low molecular weight and volatiles. On the other hand, due to the high energy transferred, the ions were fragmented. Therefore, it have emerged new ionization techniques that allow the analysis of bigger molecules, nonvolatile and also mixtures *(79)*. There are different soft ionization methods that include fast atom bombardment (FAB), electrospray ionization (ESI) and matrix-assisted laser-desorption ionization (MALDI). The variety of ionization methods has expanded the ability of detection an analysis of a wide variety of lipids with different sizes *(80)*. These ionization methods promote the protonation or deprotonation of the compounds, but limits the structural classification of the products, since it only gives information about the molecular weight of the compounds. In a way to reverse this limitation as surged the tandem mass spectrometry, which is essential in the analysis of a great variety of compounds, like proteins *(79)* and phospholipids *(80)*.

Nowadays, the most used ionization techniques are ESI and MALDI *(77)*. MALDI is mostly used in bigger molecules, in the study of proteomic but is less used in the analysis of phospholipids. *(59)*. For this reason, it will be explained the ESI method since it is the most used in analyses of phospholipids and it also is the method used in this work.

5.1. Electrospray ionization (ESI)

ESI was initially discovered by John Fenn *(77)* and has been used in analysis of several compounds like proteins, peptides, sugars and phospholipids *(81)*. The difference in functional groups and molecular weight leads to different ways of ionization. Small molecules usually form ions with a single charge: $[M+H]^+$ or $[M-H]^-$, while bigger molecules had the tendency of forming multi charged ions *(77)*.This characteristic is an advantage in the analysis of high molecular weight molecules once the *m/z* values are lower and can be detected even in spectrometers with low detection limits, increasing the range of the molecules that can be analyzed *(82)*.

Ionization by electrospray occur in different phases: formation of the electrospray and vaporization of the solvent in a way to obtain ions is gaseous phase *(78)*. In an ESI source, the sample is ionized when is emitted from a capillary with voltage applied to it. This process forms a spray which contain charged droplets that are dessolvated and reduce its size as travel down for pressure and potential gradient *(77,83)*. The voltage applied may be positive or negative, it depends on the pretended analyze (ESI positive or negative mode) *(81)*, that will generate a gradient to separate the charges on liquid superficies which results in the projection of the same out of the capillary (Taylor cone) (figure 12) *(77)*. The formation of the droplets results in the combination of the voltage applied and an inert gas, that favors the formation of ions in gaseous phase, which are then analyzed *(83)*. To the spectrometer may be coupled different analyzers, being the most common ones the quadrupole (Q), ion trap and TOF (time of flight) *(77)*.

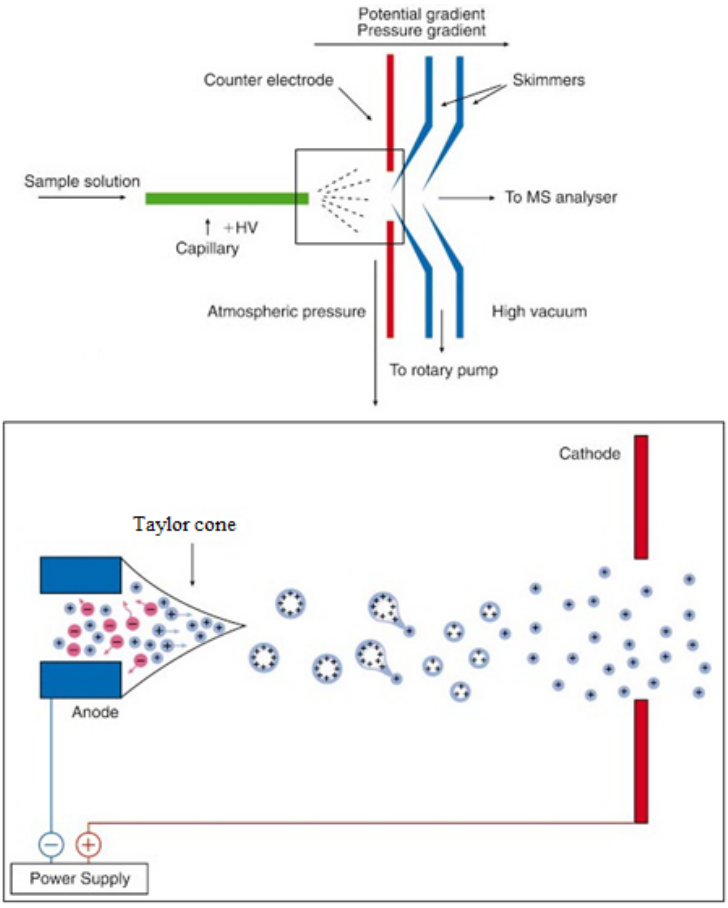


Figure 12 – Schematic representation of ESI process of ionization (adapted from *(81)*).

5.2. Tandem mass spectrometry (MS/MS)

Tandem mass spectrometry has surged from necessity to overcome the lack of compounds structural information. In this technique is selected a precursor ion in the first analyzer (Q1), which will be fragmented in a collision cell (Q2). The fragments are analyzed in the second analyzer (Q3) (figure 13). The second analyzer may be also programed to select one single ion, if it was pretended to see one single fragment. This method is able of isolating ions, fragmenting them and measure the *m/z* of resulting products.

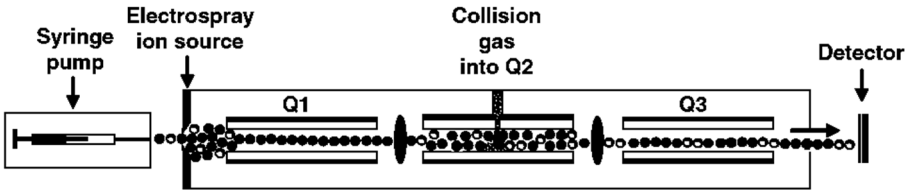


Figure 13 – Mass spectrometer with triple quadrupole, where Q1 and Q3 are analyzers and Q2 a collision cell *(83)*.

During the process of collision, in the presence of an inert gas, part of the energy of the precursor ion may be converted into internal energy, promoting an exciting state on ions, which are fragmented by a process named collision-induced dissociation (CID) *(81)*.

The most utilized equipment's nowadays in analyzes by MS/MS include hybrid analyzers like Q-TOF, triple quadrupole and ion trap. Ion trap analyzers are more recent and allow to realize a MS/MS analyzes with only one analyzer, once the analyzer has the capacity of retain the selected ions making them to collide with the gas in the same camera. Furthermore, is also possible to maintain any of the fragmented ions in the trap and repeat the process of collision to analyze this new ion. This can be called MS/MS/MS (MS^3) and can be obtained a sequential information of the precursor ion and its fragments *(77,82)*. Tandem mass spectrometry (MS^n) allows the achievement of structural information compounds of interest by fragment analysis of precursor ion *(79)*.

6. Aims

This work is based on the search of new synthetic procedures leading to new compounds with ideal properties for being used in the treatment of malaria. The aim is to act in the blood stage of infection stage where occurs the interaction of antimalarial drugs with the heme group *(29)*, which is involved in the toxicity of the drugs in the parasite *(53)*. During this stage of infection parasite degrades hemoglobin to obtain necessary conditions to survive, although during the process free heme is released. To overcome the toxic effects of heme group, the parasite induces heme polymerization to in insoluble pigment, hemozoin *(25)*.

Having these facts in mind, in this work it is aimed the synthesis of new hybrids consisting in conjugated units of Fe (III) porphyrin derivatives with drugs that have been showed to be effective in eliminating of malaria parasite, such as primaquine and

mefloquine (quinoline antimalarials) and artemisinin *(32,35)*. It is expected that these hybrids can act both as drug delivers and as inhibitors of hemozoin formation. Furthermore, it is also expected that these new compounds became toxic only to the parasite, and not to the patient. The oxidation ability of porphyrins will be tested in biomimetic membranes. Due to the similar structure of heme group, these hybrids must have oxidative power toward membranes *(53)*.

II. Results and Discussion

1. Synthesis of new anti-malarial porphyrin conjugates

The synthetic strategy to obtain new anti-malarial porphyrin conjugates was based on the structures of the selected anti-malarial drugs primaquine (**A**), mefloquine (**B**) and artesunate (**C**) (Figure 14). In the selection of primaquine and mefloquine it was considered the already developed resistance of the parasite to these drugs *(30)* and it was envisaged the possibility that their conjugation to a porphyrin could revert this situation. On the other hand, the artemisinin derivative artesunate is one of the most prescribed drugs against malaria parasite *(2,33)* and the potentiation of its efficiency would be an important goal. Considering the presence of the nucleophilic amino functions in the first two drugs, it was selected the 5,10,15,20-tetrakis(pentafluorophenyl)porphyrin (**1**, TF_5PP) in the design of conjugates **3** and **5**. Considering the presence of the acid group in artesunate the conception involved the introduction of an amino spacer between the porphyrin and the drug in order to obtain compound 9 (Figure 14).

As it was mentioned in the introduction, metalloporphyrins are potent inhibitors of hemozion (malaria pigment) formation *(41,50,51)*. So, the propose to synthetize the corresponding Fe(III) complexes **4** and **6** of the new porphyrin derivatives was based on that anti-malaria activity of metalloporphyrins. In the selection of these metalloporphyrins it was considered the oxidant power of the Heme group in membranes *(53)*. The possibility to use **TF_5PP** for further functionalization by nucleophiles is well documented in literature *(54)*. The success of this porphyrin can be explained by the high reactivity of the *para*-fluorine in the C_6F_5 units to be substituted by different type of nucleophiles. In fact, the nucleophilic aromatic substitution of the *para*-F atoms is highly selective and normally occurs in a high yield. Additionally the number of substituents introduced can be controlled by the reaction time, the number of equivalents of the nucleophile and temperature.

Actually, there are several reports of the *para*-substitution of fluorine atoms from TF_5PP with amines and alcohols *(54)*. The reaction of TF_5PP, or of its metal complexes, with primary and secondary amines, leads to the selective substitution of the *para*-F atoms of one to four C_6F_5 groups of the starting porphyrin by amine groups in high yields *(54,85)*. Considering this fact, the selective nucleophilic substitution of one of the *para*-fluorine in compound **1** was considered in the design of conjugated **3**, **5** and **9**.

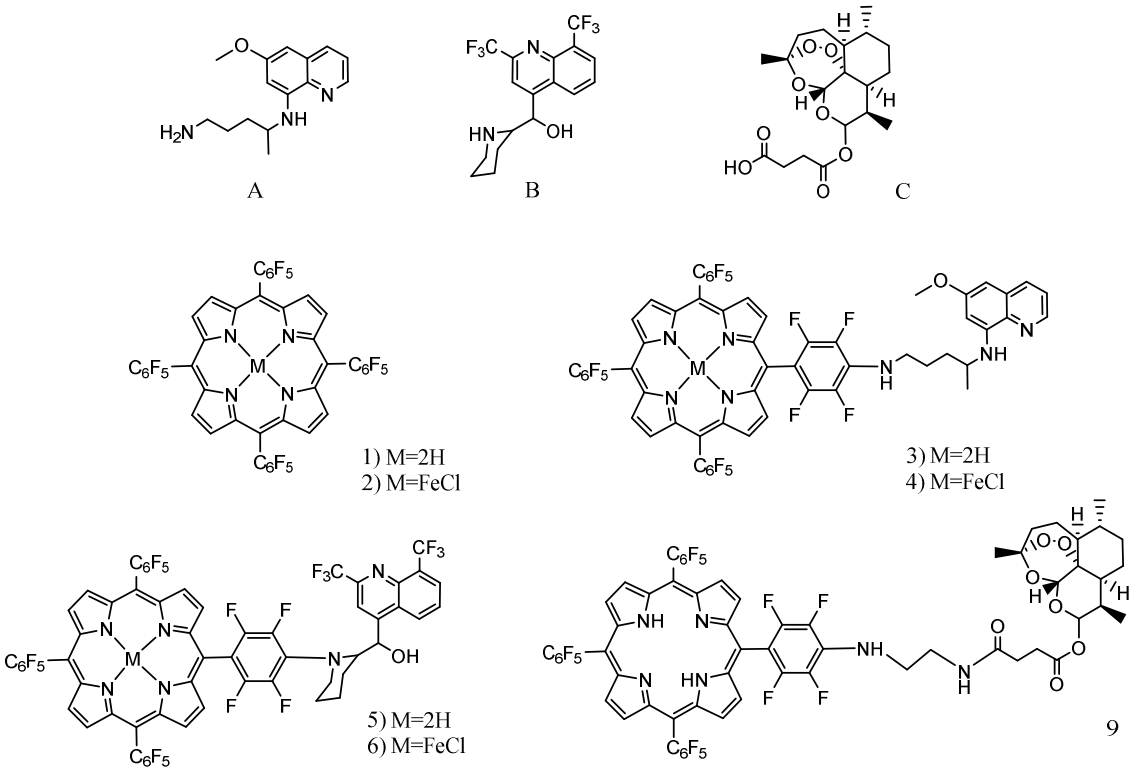


Figure 14 – Structure of the anti-malarial drugs and of the desired anti-malarial porphyrin conjugates.

The synthesis and characterization of the new derivatives **3** and **5** and of their Fe(III) complexes **4** and **6** are presented in the following section. Posteriorly it will be also presented the attempts to obtain conjugate **9**.

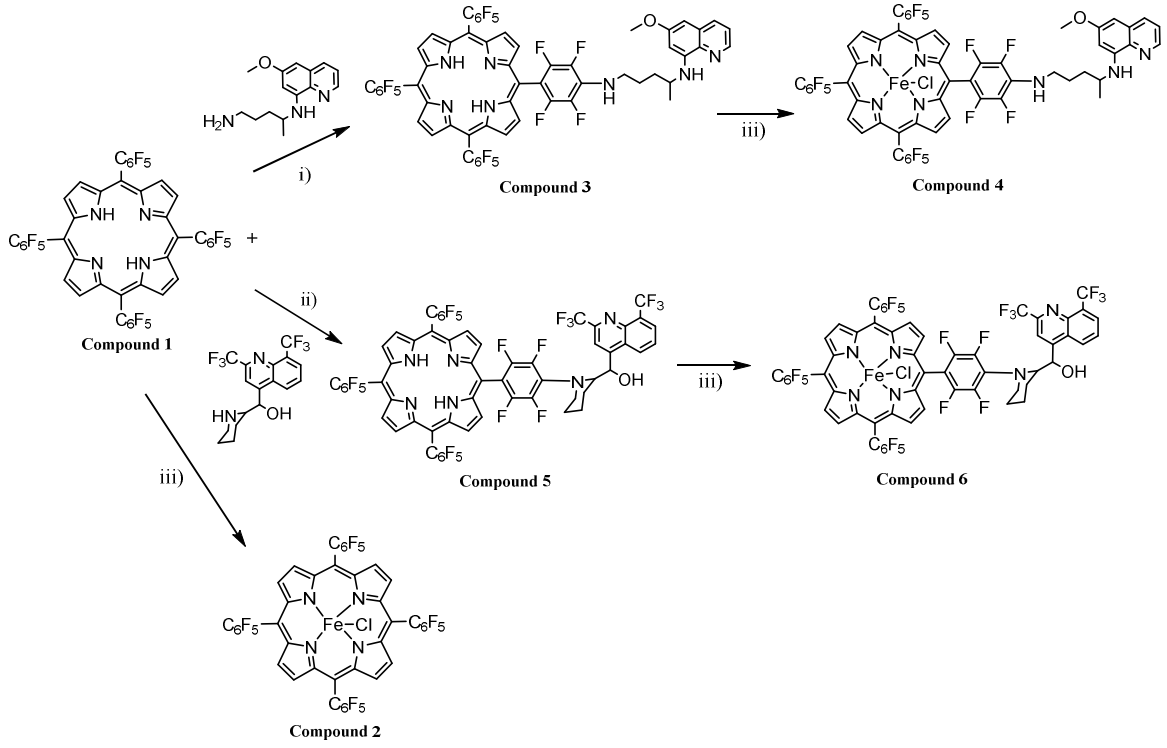
1.1.Synthesis of porphyrin conjugates 2-6

The synthetic strategy to obtain the new porphyrin/anti-malarial conjugates **3-6** involved the experimental work summarized in Scheme 1. The key step was based on the selective nucleophilic substitution of the *para*-fluorine atom of one of pentafluorophenyl groups by primaquine (A) and mefloquine (B) affording respectively the free-bases **3** and **5**. This procedure was followed by the complexation of the free-bases with $FeCl_2$ in order to obtain the corresponding iron(III) complexes **4** and **6**.

The coupling of the anti-malarial drugs **A** and **B** with porphyrin **1** was carried out in toluene at 120ºC and in the presence of a base (NaH for compound **5** and K_2CO_3 for compound **3**). The progress of the reaction was monitored by TLC and was stopped when it was observed almost total consumption of the starting compound. The workup involved

the washing of the reaction mixture with distillated water, extraction with dichloromethane and purification of the crude residue by silica gel column using CH_2Cl_2 as eluent.

The desired compounds **3** and **5** were obtained pure in 67% and 47 % yield, respectively. The starting porphyrin was not totally consumed in these two reactions (in both cases the amount of the starting porphyrin was not determined). A compromise between an adequate consumption of the starting porphyrin and the formation of conjugates containing two or more units of the anti-malarial drugs had to be taken. Besides, the time of reaction of compound **3** formation was lower than the one to obtain compound **5**. Probably the steric hindrance of the secondary amine from mefloquine is responsible for the lower yield and the higher reaction time (72h *versus* 18h of reaction).



Reaction conditions: i) toluene, K_2CO_3, 120ºC, 18h; ii) toluene, NaH, 120ºC, 72h; iii) DMF, $FeCl_2$, pyridine, 140ºC, 24h reflux.

Scheme 1

The methodology used for the synthesis of the iron(III) **4** and **6** was based on conventional procedures *(86)*. The reactions between the free-bases **3** and **5** and $FeCl_2$ were performed in a mixture of dimethylformamide (DMF) and pyridine at 140ºC in reflux under inert atmosphere until the TLC and UV-vis controls confirmed the insertion

of the metal. After the metalation process, the solvents were removed under vacuum. The resulting solids were washed thoroughly with water, to remove the excess of the metal salt and compounds **4** and **6** were isolated with a yield of 14.4 % and 13.5 % respectively. For comparative studies complex **2** was also prepared from porphyrin **1** using the same procedure.

The structures of the new porphyrin derivatives **3** and **5** were confirmed by UV-vis, ^{1}H, ^{13}C and ^{19}F NMR spectra and ESI-MS. The insertion of the metal in the inner core of free-bases **1**, **3** and **5** was confirmed by UV-vis and ESI-MS.

As an example, the absorption spectra of the new conjugates **1** and **2** is represented on figure 15 and show the expected Soret bands at 413 and 412 nm, respectively *(42)*. In the Q-band region the number of bands is dependent on the absence or presence of Fe(III) ion in the porphyrinic core and also by the presence of the substituent. Compound **1** (TF_5PP) shows four Q-bands at 506, 540, 583 and 648 nm, its Fe(III) complex (**compound 2**) show only two Q-bands at 503 and 630 nm. It is observed a decrease in the number of Q-bands and a deviation of those to lower wave-lengths of the UV-vis spectrum of compound **2**, indicating that the metalation occurred.

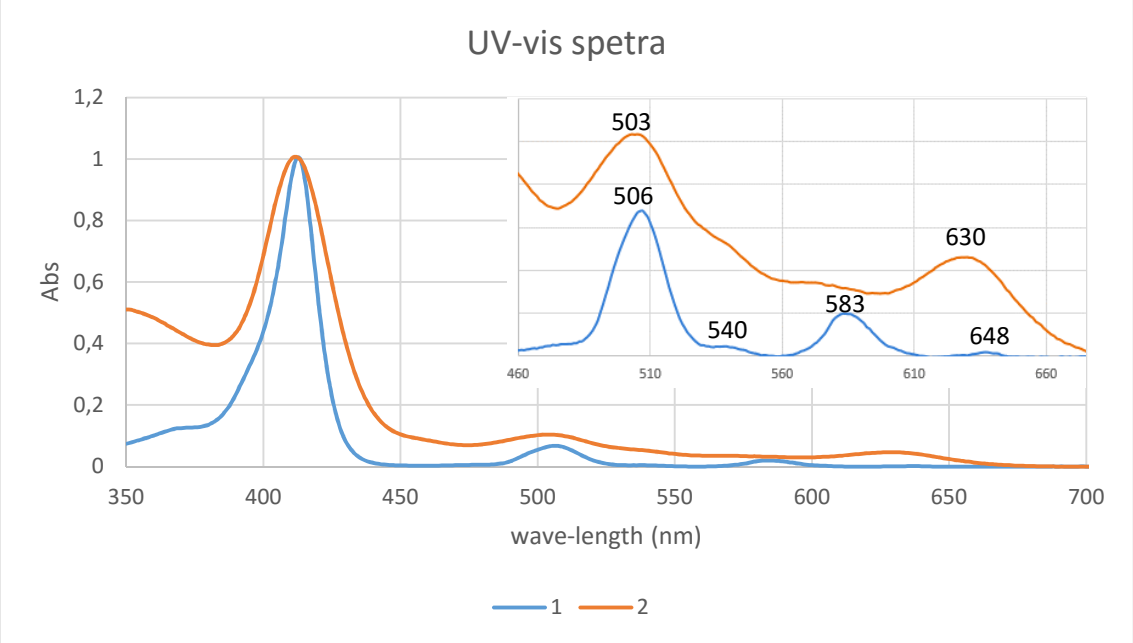


Figure 15 – Uv-vis spectra of compounds **1** and **2** in $CHCL_3$.

In ^{1}H NMR spectrum of conjugate 3 (Figure 16) it is possible to identify some characteristic peaks of the drug primaquine in the aliphatic zone. The doublet at δ 1.44 ppm was assigned to the CH_3 protons (**E**) and the multiplet at δ 1.93 – 2.04

ppm to the CH_2 protons **B** and **C** of the aliphatic chain. The signal due to the protons **A**, **J** and **D** appear at δ 3.75, 3.90 and 4.32 ppm, respectively; the resonances due to the metoxy protons (**J)** appear as a singlet and the ones of **A** and **D** protons as multiplets. In the aromatic zone is also possible to identify some signals that correspond to primaquine, at δ 8.58 (**F**), δ 7.96 (**H**) and δ 7.32 (**G**) ppm as double doublets with J = 4.2 and 1.6 Hz, J = 8.3 and 1.6 Hz and J = 8.3 and 4.2 Hz, respectively. Plus, it is observed a peak corresponding to two hydrogens **I** and **K** more protected at δ 6.39 and 6.40 that is an AB system with a J = 2.5 Hz. The eight β pyrrolic hydrogens appear at δ 9.01, 8.90 and 8.85 ppm. Compound **3** shows a Soret band at 415 nm and three Q-bands at 508, 585 and 641 nm. By ESI-MS it was possible to identify the ions at *m/z* 1214.3 $(M+H)^+$ and 607.7 $(M+2H)^{2+}$, that confirm the molecular weight of the pretended compound.

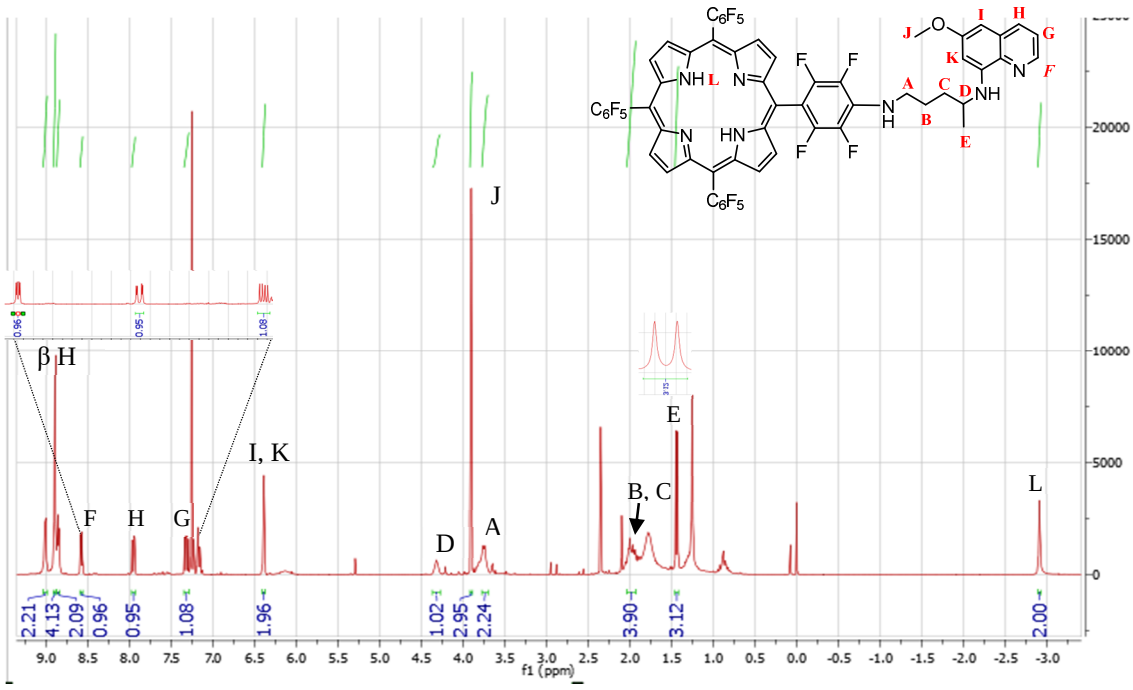


Figure 16 – ^{1}H NMR spectrum ($CDCl_3$) of compound **3**.

The ^{1}H NMR spectrum of conjugate 5 present also characteristic signal in the aliphatic and aromatic zone. At higher δ, besides the signals of porphyrin β-pyrrolic protons, four more signals are present that correspond to the molecule of mefloquine. It is verified the presence of two doublets at δ 8.30 (J=8.0 Hz) and δ 8.58 (J=8.0 Hz), one singlet at δ 8.33 ppm and a triplet at δ 7.90 ppm (J=8.0 Hz). The β pyrrolic protons appear at δ 8.82, δ 8.86 and δ 8.90 ppm. The aliphatic

protons from the mefloquine ring (C_5H_9N) appear at δ 1.42 – 1.80, δ 2.67 – 2.69, 3.31 – 3.40 and δ 3.16 – 3.26 as multiplets and at δ 2.03 (*J*= 10.8 Hz) as a doublet. One more signal is observed at δ 6.43 (*J*=4.9 Hz) as a doublet. Compound 5 shows a Soret band at 413 nm and three Q-bands at 507, 583 and 637 nm in the UV-vis spectrum. By ESI-MS it was possible to identify the ions at *m/z* 1333.2 $(M+H)^+$ and 667.1 $(M+2H)^{2+}$, that confirm the molecular weight of the pretended compound.

The UV-vis spectrum of the Fe(III) complexes of the compounds **3** and **5** present a Soret band at 416 and 413 nm, respectively. The Q-bands of compound **4** appear at 506 and 634 nm and of compound **6** at 506 and 628 nm.

Additional the ^{19}F NMR spectrum is an important tool to confirm the mono-substitution of the fluorine atom of porphyrin **1**. In the spectrum of TF_5PP it is possible to identify three different signals due to the *meta*-F (δ -184.8 ppm), *para*-F (δ -174.7) and *orto*-F (δ -160.0 ppm) with a ratio of 2:1:2. The covalent linkage of the drugs to the porphyrins was also proven by the ^{19}F NMR spectra of the new conjugates comparing with the one of TF_5PP.

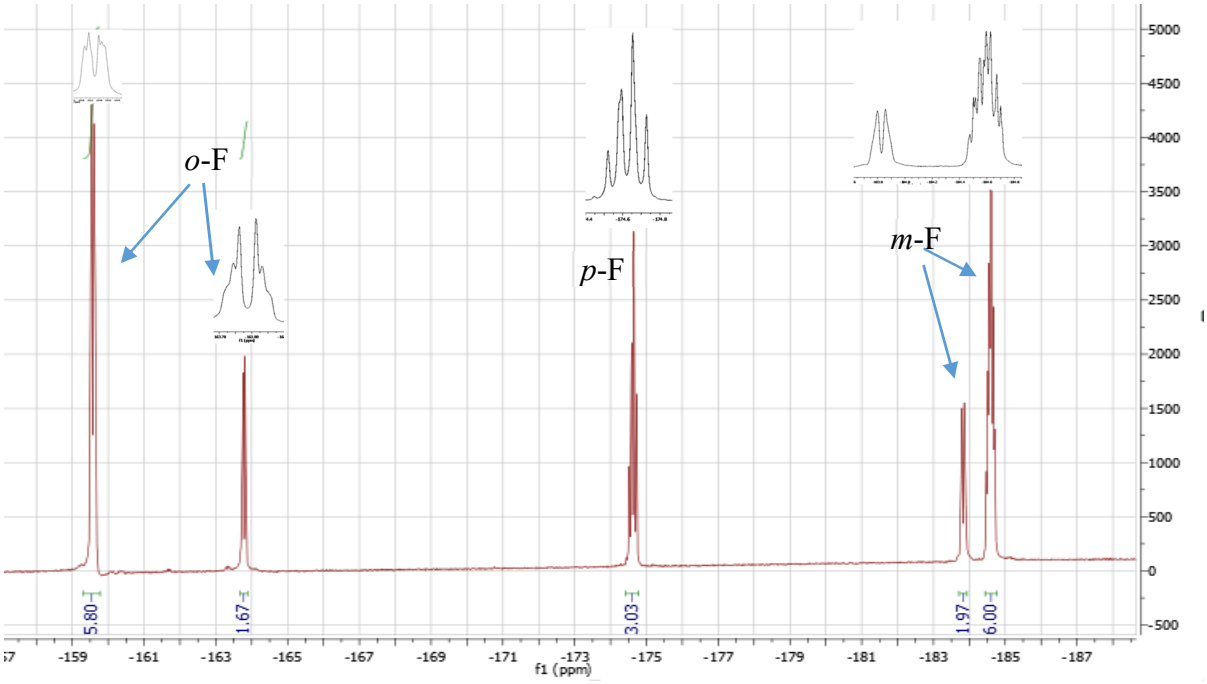


Figure 17 – ^{19}F NMR spectrum ($CDCl_3$) of compound **3**.

In ^{19}F NMR spectrum of compound **3** (Figure 17) is patent the asymmetry introduced by primaquine. The *o*-F atoms appear as two multiplets between δ -159.82 and -159.52 and between δ -163.93 and -163.74 ppm. The resonance due to the *o*-F of the substituted ring appear slightly more protected. Next appear the *p*-F atoms at δ -174.73 and -174.52 ppm as a multiplet and the *m*-F atoms at δ -183.83 as a large doublet and as a multiplet between δ -184.70 and -184.48 ppm. As opposite for the *o*-F atoms, the *m*-F atoms of the substituted ring appear slightly more deprotected.

Compound **5** present additionaly peaks in ^{19}F NMR spectrum (Figure 18) compared to compounds **3**, due to the extra six fluor atoms in the structure of the drug mefloquine. The fluor atoms of the porphyrinic ring appear with a similar pattern to the one observed for compound **3**, while the fluorine atoms of the mefloquine residue appear at lower chemical shifts, δ -90.89 and δ -83.35 ppm as singlets.

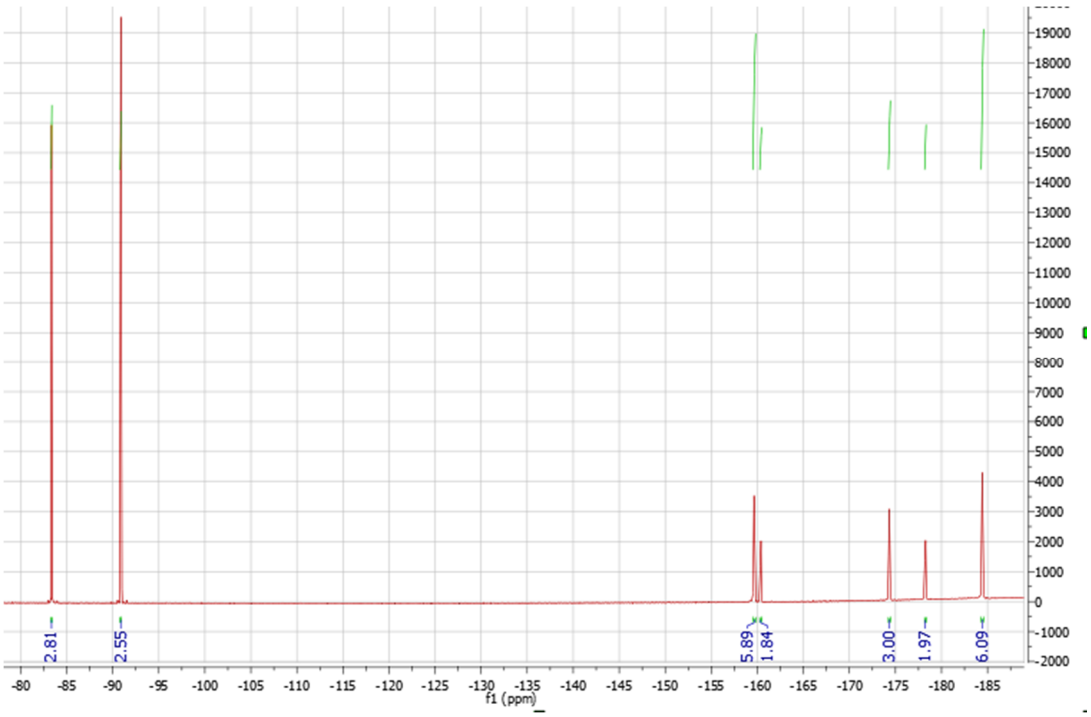
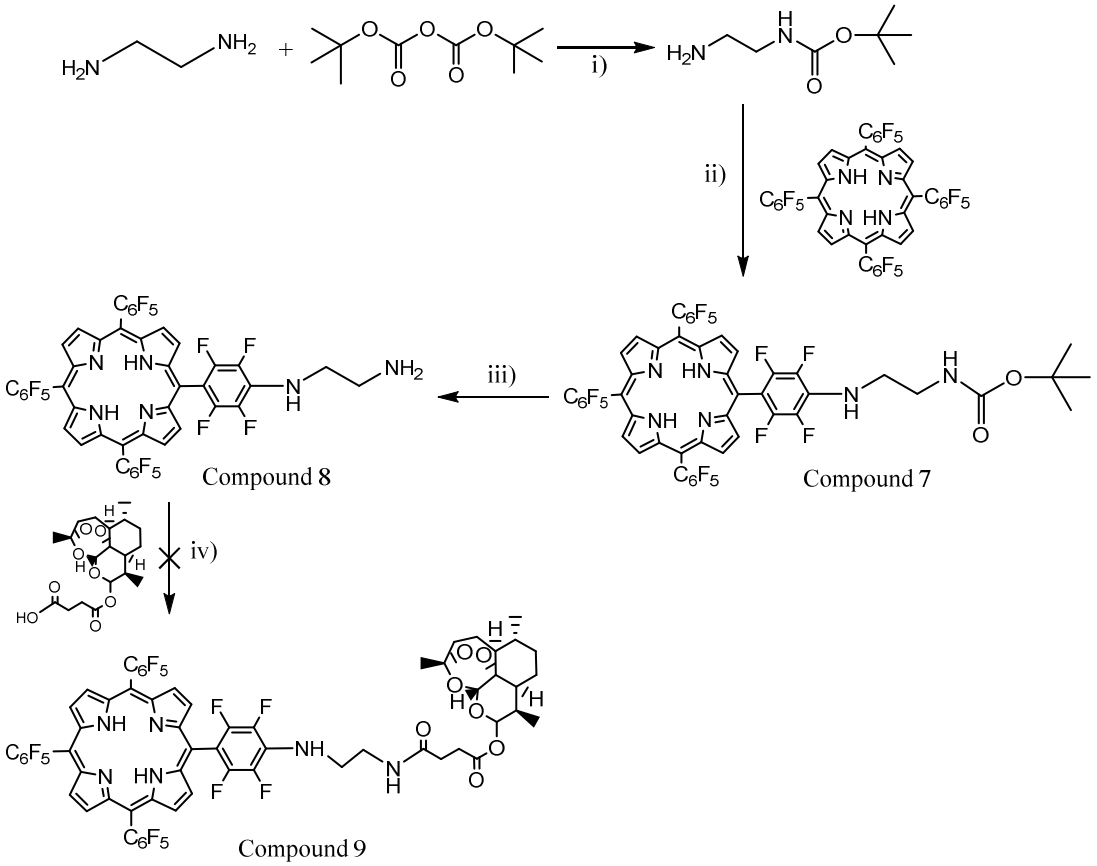


Figure 18 – ^{19}F NMR spectrum ($CDCl_3$) of compound **5**.

1.2.Attempts to synthetize conjugate 9

The synthetic approach to obtain the new porphyrin/anti-malarial conjugate **9** involved the experimental work summarized in Scheme 2. As artesunate can't react directly with

the porphyrin, other strategies to the formation of the conjugate were attempted. For that, it was used ethylenediamine to serve as spacer between the drug and the porphyrin, and an important step of the approach (ii) was similar to the one described for compounds **3** and **5**.



Reaction conditions: i) THF, -10ºC; ii) Toluene, K_2CO_3, 120ºC reflux; iii) TFA, $CHCl_2$, room temperature; iv) EDC, DMAP, $CHCl_3$, room temperature.

Scheme 2

In the strategies proposed to obtain compound **9**, one of the amino groups of ethylenediamine was protected with di-*tert*-butyl dicarbonate (BOC) to ensure that only one amine end is free to react with the porphyrin (scheme 2 i)). The reaction was carried out by the addition of ethylenediamine to BOC, in THF at -10ºC. The reaction mixture was stirred overnight at room temperature and the THF was evaporated under reduced pressure. Then, the reaction mixture was washed with distilled water and mono substituted ethylenediamine was extracted with dichloromethane and obtained pure with an 81 % yield.

In ^{1}H NMR spectrum of the mono protected ethylenediamine (Figure 19) it is possible to identify some characteristic signals, as is the case of the singlet signal at δ 1.45 ppm that correspond to the 9 equivalent protons of BOC end. It is also possible to identify the signals from the ethylenediamine at δ 2.79 as a triplet (*J*=5.9 Hz) and δ 3.16 ppm as a quartet (*J*=5.9 Hz).

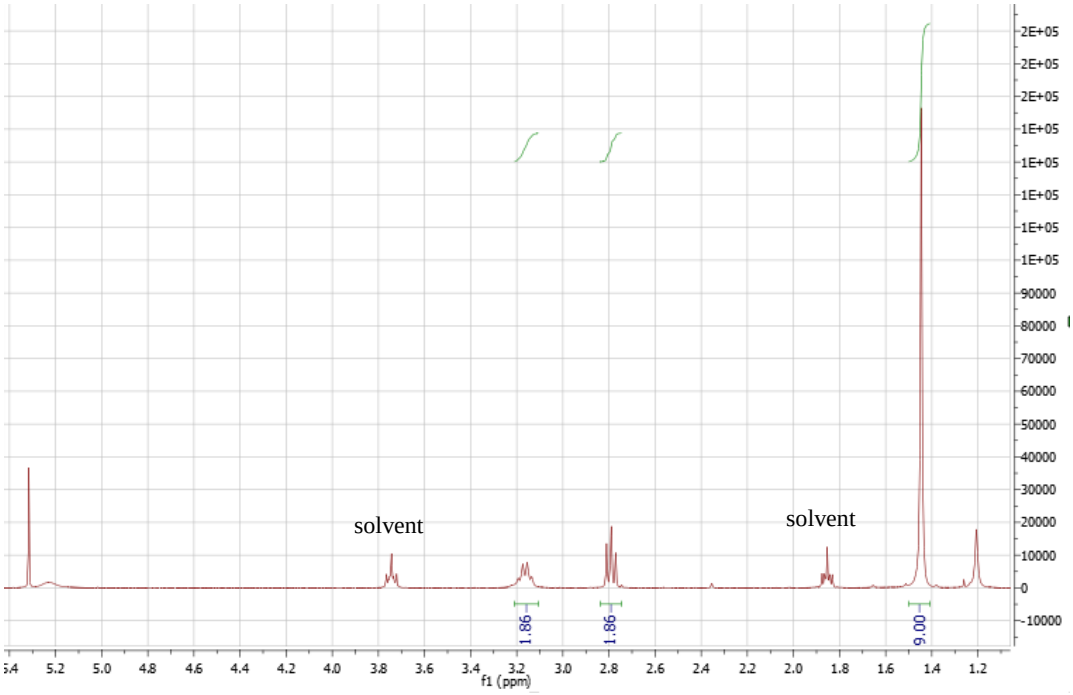


Figure 19 - ^{1}H NMR spectrum ($CDCl_3$) of mono substituted ethylenediamine.

The reaction between the protected ethylenediamine with the porphyrin was performed in toluene in the presence of K_2CO_3 at 120ºC (scheme 2 ii)). The evolution of reaction was controlled by TLC and it was stopped when it was observed a small amount of the starting porphyrin. The workup involved the washing of the reaction mixture with distillated water, extraction with dichloromethane and purification of the crude residue by silica gel column using CH_2Cl_2 as eluent. The first fraction to be obtained was the starting porphyrin **1** and the second fraction was identified as **compound 7** (the yield of this step was not determined).

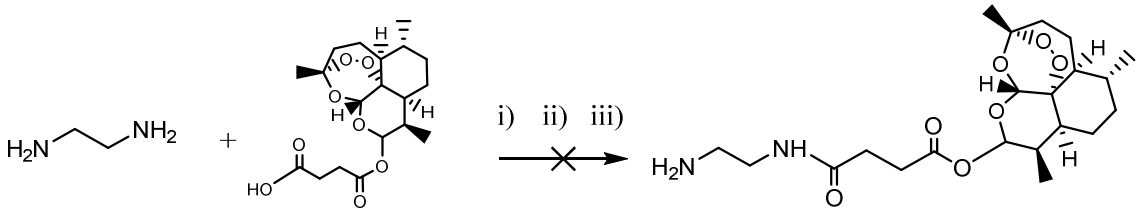
The structure of the new porphyrin derivative **7** was confirmed by UV-vis, ^{1}H and ESI-MS. In ^{1}H NMR spectrum it is possible to identify the same signals as for the mono substituted ethylenediamine at δ 1.47, 3.21 and 3.78 ppm, plus the characteristic signals from the porphyrinic ring at δ 8.88 and 9.03 ppm, suggesting that the reaction was accomplished as pretended. **Compound 7** UV-vis spectra present a Soret band at 415 nm

and three Q-bands at 508, 585 and 658 nm. By ESI-MS it was observed one ion at *m/z* 1115.2, corresponding to the *m/z* of the pretended compound.

For the reaction of the porphyrin with the spacer with artesunate it was necessary to remove the protecting group from **compound 7**. The reaction was performed in trifluoroacetic acid (TFA) and dichloromethane at room temperature (scheme 2 iii)). The evolution of the reaction was controlled by TLC and it was stopped when it was observed the total consumption of the starting derivative, suggesting a total deprotection of the amine group (**compound 8**). The structure of the new porphyrin derivative **8** was confirmed by UV-vis, ^{1}H NMR and ESI-MS. ^{1}H NMR spectrum is very similar to previous compound, but shows clearly the absence of the signal corresponding to the BOC group at δ 1.45 ppm. The UV-vis spectrum of the compound **8** shows a Soret band at 414 nm and three Q-bands at 508, 585, 658 nm. The ESI-MS spectrum presents the ion at *m/z* 1015.1, corresponding to the desired compound.

After the removal of the protecting group from amine, the next step was the reaction of artesunate with the porphyrin **8** (scheme 2 iv)). Several experimental conditions in the synthesis of compound 9 were tried, in particular the activation of the acid group of artesunate with 4-(dimethylamino) pyridine (DMAP) and ethylcarbodiimide (EDC). The reaction was performed in $CHCl_3$ at room temperature during 1h30. The reaction was controlled by TLC but unfortunately the TLC control did not show any consumption of compound **8**.

As the reaction of the drug and the compound **8** was not accomplished the strategy adopted was first to react the ethylenediamine with artesunate (scheme 3).

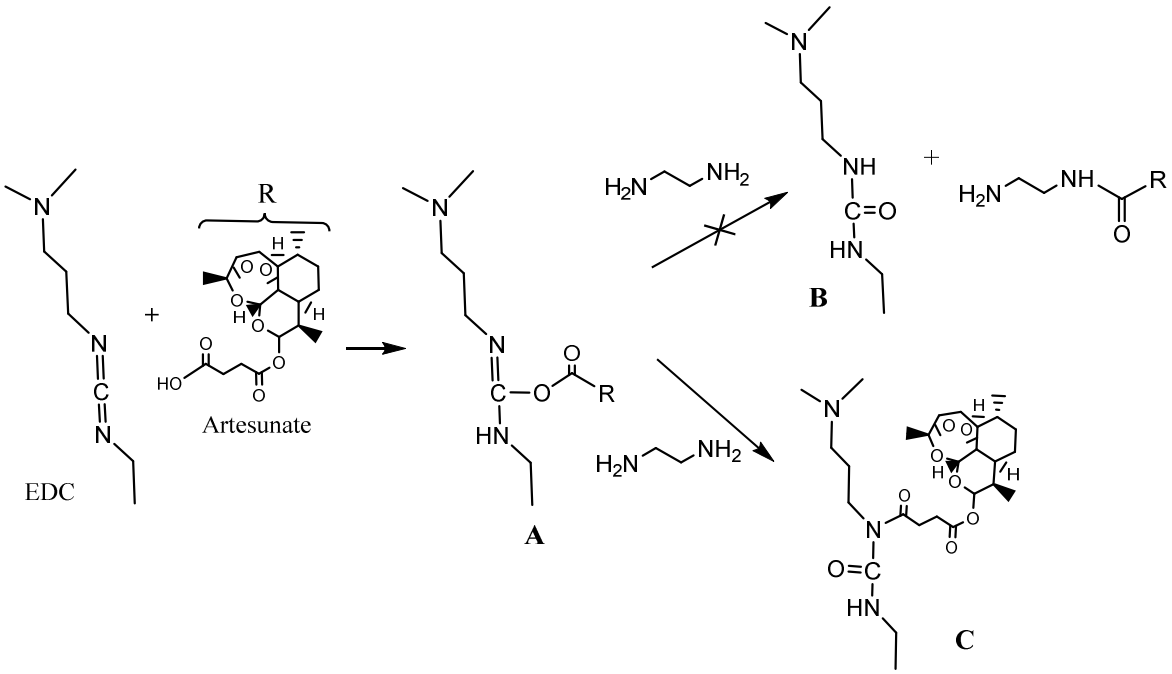


Reaction conditions: i) oxalyl chloride, DMF, dry CH_2Cl_2, 2h at room temperature; ii) EDC, DMAP, $CHCl_3$, 1h30 at room temperature; iii) DCC, DMAP, 1h30 at 0ºC.

Scheme 3

The first attempted was the formation of an acyl chloride to form a reactive derivative of artesunate (scheme 3 i)) *(87)*. The reaction was executed in dry CH_2Cl_2 at room temperature in the presence of oxalyl chloride, ethylenediamine and DMF. In the second attempt it was reacted ethylenediamine, artesunate, EDC and DMAP at room temperature in $CHCl_3$ (scheme 3 ii)). The same reaction was performed at 0ºC but it was substituted EDC by carbodiimide (DCC) (scheme 3 iii)). All the reactions were controlled by TLC and ESI-MS. By TLC the evolution of reaction was difficult to monitor, and it was performed an ESI-MS spectrum that do not show the presence of the ion corresponding to the pretended compound.

The failure of this reactions may be due to the fact that EDC and DCC react with carboxylic acid groups to form an active *O*-acylisourea intermediate (scheme 4, **A**). This intermediate can proceed by different pathways depending for example in the concentration of the reagents *(88)*. The same compound is easily displaced by nucleophilic attack from primary amino groups and it is formed an amide bond with the carboxyl group original from artesunate (scheme 4 i)). An EDC/DCC by-product is released as a urea derivative (scheme 4, **B**). Once the derivative **A** is unstable, failure to react with the amine results in the hydrolysis of the intermediate, there are the regeneration of the carboxyls and the release of an N-unsubstituted urea (scheme 4, **C**) *(88)*. The formation of the *N*-acylisourea is due to a rearrangement of **A** and can occur in excess of carbodiimide *(88,89)*. In fact, from the ESI-MS spectrum of the reactions ii) and iii) from **scheme 3** it was verified the presence of the peak corresponding to the *m/z* of the derivative **C** (*m/z* 540.4).



Scheme 4

The porphyrin derivatives were synthetized in order to obtain new compounds that could have anti-malarial properties. These porphyrinic derivatives can act as oxidants and thus can be used as pro oxidant agents toward membrane lipids. So in the next section it will be presented the results from lipid peroxidation induced by the new porphyrin derivatives synthetized (compounds **1** to **6**). For that we used liposomes as biomimetic systems of cell membranes.

2. Evaluation of the effect of porphyrin derivatives in lipid peroxidation using liposomes of PLPC and dMPC as biomimetic systems of cell membranes

As it was already mentioned, the main objective of this work was to prepare new anti-malaria porphyrin conjugates and to evaluate their ability to oxidize membrane phospholipids. Lipid oxidation of membranes can induce disruption of parasite membrane leading to parasite destruction. The effect of the new porphyrin derivatives in lipid peroxidation was evaluated using liposomes with two different phosphatidylcholines: 1,2-dimyristoyl-sn-glycero-3-phosphocholine (dMPC) (Figure 20 a) and 1-palmitoyl-2-lineloyl-sn-glycero-3-phosphocholine (PLPC) (Figure 20 b), as biomimetic membranes. PLPC is susceptible to oxidation due to the presence of a polyunsaturated hydrocarbon chain, the linoleic acid (C18:2, Δ 9,12), in its constitution. On the other hand, dMPC has two saturated fatty acyl chains, (C16:0) that are not predisposed to oxidation. The extent of oxidation of PLPC in the presence of metalloporphyrins was monitored by electrospray mass spectrometry. Since dMPC was not modified in the presence oxidative conditions it

was selected as the internal standard. Therefore, the evaluation of the variation of the relative abundance of the $[M+H]^+$ ions of PLPC versus the relative abundance of the $[M+H]^+$ ion of dMPC was used to monitor the extent of PLPC oxidation.

Phosphatidylcholines are the major phospholipids present in cell membranes in eukaryotic organisms and the selection of those PC's species to form liposomes was planned in order to achieve a better approach to mimic parasite membrane *(57)*.

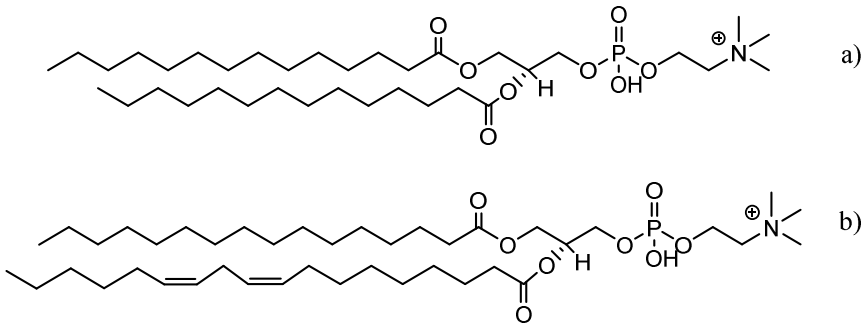


Figure 20 – Representation of the structure of dMPC (a)) and PLPC (b)).

Most of the studies by mass spectrometry concerning the oxidation phospholipids are related with that class of phospholipids *(74,75,90)*. Lipid peroxidation can be mediated by free radicals, namely by oxygen reactive species (ROS) that generate a high range of lipid oxidized products *(75)*. Radical hydroxyl ($OH^{\bullet}$) is one of the most reactive ROS *(91)*. Reis, et al *(74)*, studied the radical peroxidation of PLPC by mass spectrometry using Fenton conditions to generate $OH^{\bullet}$. The products identified from lipid oxidation included long chain products if the phospholipid chain is preserved and has extra number of oxygen atoms, resulting in an increase in phospholipid mass, and short chain products if the unsaturated phospholipid is broken down by oxidative cleavage and with formation of shortened fatty acyl chains that may have terminal aldehyde or carboxylic groups *(71,72)*. These authors observed the formation of long-chain products of PLPC with the insertion of one (*m/z* 774.6), two (*m/z* 790.6), three (*m/z* 806.6), four (*m/z* 822.6) or five (*m/z* 838.6) oxygen atoms in the unsaturated fatty acyl chain *(74)*.

Having this facts in mind, the extent of oxidation was monitored by electrospray ionization mass spectrometry in the positive mode (ESI-MS). In the ESI-MS spectra, the unmodified PCs were identified as $[M+H]^+$ ions at *m/z* 758.6 (PLPC) and *m/z* 678.5 (dMPC). Sodium and potassium adducts ($[M+Na]^+$ and $[M+K]^+$ respectively) of

unmodified PCs were also identified at *m/z* 700.5 and *m/z* 716.5 for dMPC and *m/z* 780.6 and *m/z* 796.5 for PLPC. These ions were identified in all acquired spectra.

In the systems where oxidation of PLPC occurred we saw the ions corresponding to oxidized PLPC namely ions correspondent to PLPC + O (*m/z* 772.6 and *m/z* 774.6), PLPC + 2O (*m/z* 790.6 and m/z 788.6) and PLPC + 3O (*m/z* 804.6 and *m/z* 806.6). Furthermore, it was possible to observe a decrease in the relative abundance of PLPC during the time of reaction in comparison with the relative abundance of dMPC as it is exemplified in figure 21 for the iron(III) complex **2**. Confirmation of the structural features of lipid oxidation products was evaluated by tandem mass spectrometry analysis (*vide infra*).

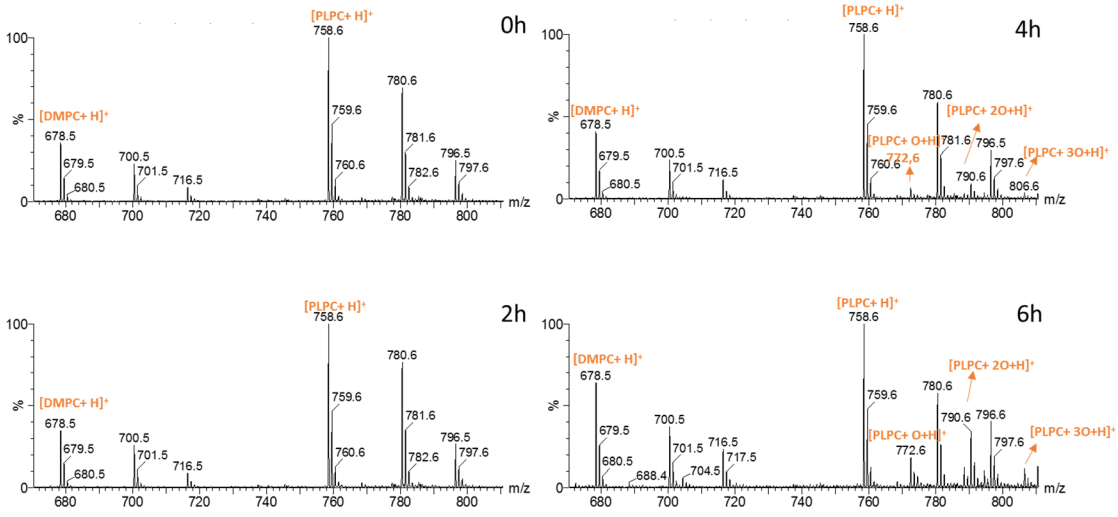


Figure 21 - ESI mass spectra of the phospholipid liposomes in the presence of potential antimalarial compound **2**, after 2h, 4h and 6h of reaction in absence of H_2O_2.

To evaluate the oxidation capacity of the porphyrin derivatives **1-6**, these were incubated with phospholipid liposomes at 37 ºC in ammonium hydrogen carbonate buffer and the reaction was monitored by ESI-MS at 0 hours and after 2, 4 and 6 hours of reaction. All the compounds were incubated with the phospholipids in the presence of H_2O_2 and in its absence. In the case of metalloporphyrins the reactions were performed in duplicate either with or without H_2O_2.

2.1. Evaluation of the oxidation extension after 6h of reaction in the presence of compounds 1-6

The capacity of the free-bases and of the metalloporphyrins **1-6** to oxidize the phospholipids was determined by the variation of the relative abundance of $[dMPC+H]^+$

and $[PLPC+H]^+$ at different times. A high extent of PLPC oxidation induced a decrease in the relative abundance of PLPC $[M+H]^+$ ion and thus an increase in the ratio $[dMPC+H]^+/[PLPC+H]^+$. The variation of this ratio for all compounds studied (**1-6)** after the six hours of reaction, in the presence and in the absence of H_2O_2, is presented in figure 22. For all the reactions, the ratio at time 0h (T0) was equal revealing that no oxidation occurred and it was used as control.

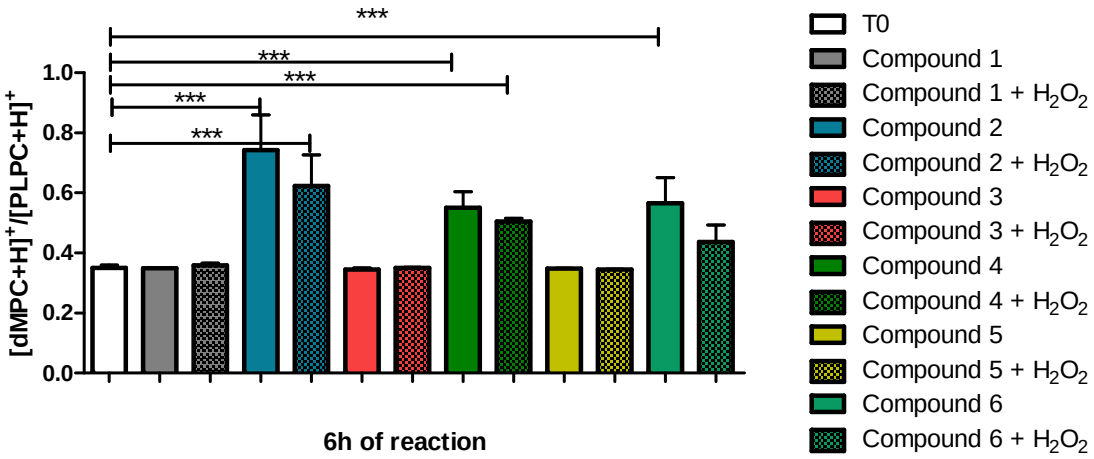


Figure 22 – Variation of $[dMPC+H]^+/[PLPC+H]^+$ ratio in the presence of compounds **1** to **6** with and without H_2O_2. In figure is represented the significant variations on the ratio of the two PCs after 6h of reaction.
***p<0.001, significantly different between selected conditions.

In the presence of the free-bases **1**, **3** and **5,** no variation in the ratio $[dMPC+H]^+/[PLPC+H]^+$ was detected under both conditions (in the presence or absence of H_2O_2). In the presence of the iron complexes **2**, **4** and **6,** the results showed a different situation and in general, a significant increase of phospholipid oxidation occurred after six hours of reaction. The increase in the ratio $[dMPC+H]^+/ [PLPC+H]^+$ in the presence and in the absence of H_2O_2, after the six hours of reaction is significantly different from T0 ratio except for compound **6** in the presence of H_2O_2. In fact, compound **6** only showed a significant increase in that ratio in the presence of O_2. From the results obtained it can be concluded that the free base porphyrins **1**, **3** and **5** were unable to oxidize the biomimetic membrane and its oxidation in the presence and in the absence of hydrogen peroxide only occurred in the presence of the Fe(III) complexes. In fact, metalloporphyrins have been widely used as promoters of organic compounds oxidation, namely aliphatic and aromatic hydrocarbons *(68,70,92)*.

The mechanism of oxidation by iron porphyrins can be associated to the proposed mechanism for CYP, due to the similarity of the catalytic center *(68)*. However, different mechanisms of oxidation by iron porphyrins have been proposed, that can involve a Fenton-like mechanism *(24)* and/or high-valent (oxometallo) intermediates *(92,93)*. The most accepted mechanism of hydroxylation and epoxidation by biomimetic porphyrinic models involves high-valent intermediates namely the porphyrin π-cation radical O=Fe(IV) $Por^{\bullet+}$ *(24,93,94)*. However, the porphyrin π-cation radical can also be involved in the formation of radical species which may start the lipid peroxidation by radical chain reactions *(92,95)*. In fact, polyunsaturated lipids are particularly susceptible to oxidation and as they are sensitive to catalysis by metals, small concentrations of metalloporphyrins may initiate lipid peroxidation by a radical chain process *(24)*. So, in the lipid oxidation mediated by metalloporphyrins can be involved a radical chain process or the direct interaction of the high-valent intermediate with unsaturated aliphatic chains *(68)*. This topic will be further discussed in section 2.5 together with the proposed structures for the PLPC oxidation products.

2.2.Evaluation of the oxidation extension after 2 hours of reaction in the presence of compounds 2, 4 and 6 with proved oxidation ability

In order to evaluate if the differences detected for compounds **2**, **4** and **6** after six hours of reaction were also patent in the earlier stage of oxidation, their efficacy was determined after 2h of reaction in the presence and in the absence of H_2O_2 (Figure 23).

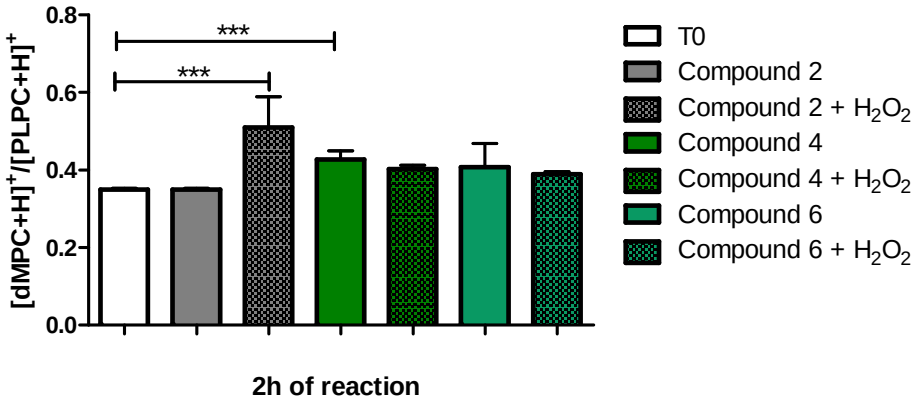


Figure 23 - Variation of $[dMPC+H]^+/[PLPC+H]^+$ ratio in the presence of compounds **2**, **4** and **6** with and without H_2O_2. In figure is represented the significant variations on the ratio of the two PCs after 2h of reaction.

***p<0.001

The results showed that the increase in the ratio $[dMPC+H]^+/ [PLPC+H]^+$ is only significantly different from the T0 ratio for compound **2** in the presence of H_2O_2 and for compound **4** in the absence of H_2O_2. For the other conditions, namely for compounds **4** in the absence of H_2O_2 and for **6** in the presence and in the absence of H_2O_2, it was verified a small increase in the oxidation ratio but it was not considered statistically significant. Interesting, for compound **2** the ratio $[dMPC+H]^+/[PLPC+H]^+$ in the absence of H_2O_2 was equal to the ratio obtained at T0.

In the oxidation mechanism proposed for CYP (see Figure 10), it was referred that the iron(III) complex can also interact with monoxygen donors like peroxides by a peroxide shunt pathway, that can lead to the formation of the active intermediate $[O=Fe(IV)\ Por]^{\bullet+}$ ↔ (Porphyrin)Fe(V)=O] complex. Probably, with complex **2**, the higher oxidation ratio obtained in the presence of H_2O_2, can be justified by the peroxide shunt pathway to occur more quickly in the first stage of oxidation than the process involving molecular oxygen (see discussion below) *(96)*. With the other complexes the difference between the two events is not so notorious.

2.3.Global comparison of the oxidation extension in the presence of complexes 2, 4 and 6

After finding out which porphyrin derivatives have the ability to oxidize membrane phospholipids, it was evaluated if there were differences on oxidation by these compounds. As it is represented in figure 24, compound **2**, the metal complex of the starting porphyrin (TF_5PP), showed a significant increase in oxidation compared with the compounds **4** and **6** in the presence and absence of H_2O_2 after six hours of reaction.

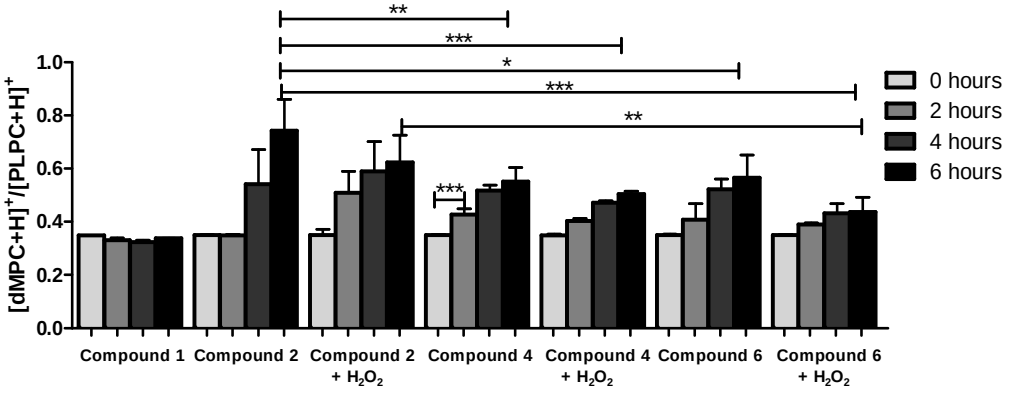


Figure 24 - Variation of [dMPC+H]+/[PLPC+H]+ ratio in the presence of compounds **2**, 4 and 6 with and without H_2O_2. ***p<0.001, **p<0.01, *p<0.05

In fact, after that time compound **2** showed a higher increase of $[dMPC+H]^+/[PLPC+H]^+$ ratio than compounds **4** and **6**. While compound **2** had a maximum increase of about 0.40 of the $[dMPC+H]^+/[PLPC+H]^+$ ratio, the increase for compounds **3** and **4** did not exceed 0.15.

Compounds **4** and **6** showed a similar increase of the $[dMPC+H]^+/[PLPC+H]^+$ ratio, both in the presence and absence of H_2O_2. Although it was observed that compound **2** promoted the highest percentage of oxidation after 6 hours of reaction in the absence of H_2O_2 and compound **4** presented a higher percentage of oxidation in the first two hours of reaction under the same conditions. Additionally, in the presence of H_2O_2 compound **2** showed a higher increase in PLPC oxidation when compared with compound 6 after six hours of reaction. Thus, in general compound **2** after six hours of reaction proved to be the more effective to oxidize membrane phospholipids than the conjugates **4** and **6**.

It is important to have in mind that the drugs used in this work are already used in malaria treatment. So, their incorporation in the porphyrinic nucleus can have an important role in the oxidation of membrane phospholipids but also prevent hemozoin formation *(29)*. The mechanism of action of the drugs used in this work involves the interaction with the heme group, inhibiting the polymerization of hemozoin *(31)*. Some strains of the parasite had already developed resistance for the drugs as primaquine and mefloquine *(37)*. However, the fact that the drug is now linked to the porphyrin, can lead to a new pharmaceutical approach since the parasite may not have defense mechanisms for these new compounds. Plus, the metalloporphyrins in addition to its the role in parasite membrane oxidation may also work as a drug transporter *(35)*.

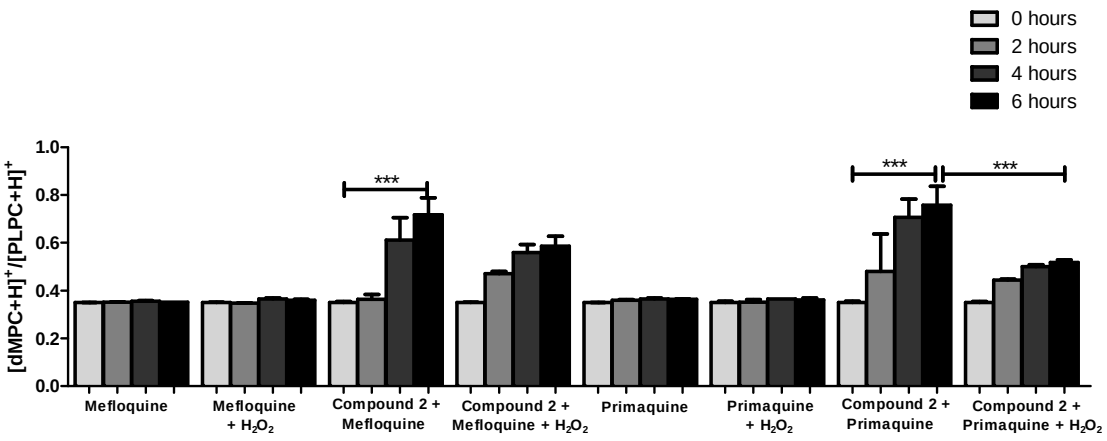
The free base porphyrins derivatives **1**, **3** and **5** did not show any influence on the phospholipid oxidation and this fact does not preclude its activity against malaria parasite. As referred before, some porphyrins have already been tested against malaria but in the mechanism of action was not referred the possibility that porphyrins could act also via a direct oxidation of the membrane parasite. Porphyrins are usually referred to interact with the heme group by π interactions, inhibiting the malaria parasite pigment formation *(41,50,51)*, leading the heme group available, that is responsible by the destruction of the parasite membrane.

The synthesis of derivative with the drug artesunate was not accomplished, although it should be given attention to that. Artesunate is a derivative of artemisinin and booth drugs are already being used in malaria treatment *(33,97)*. In the parasite, it is necessary

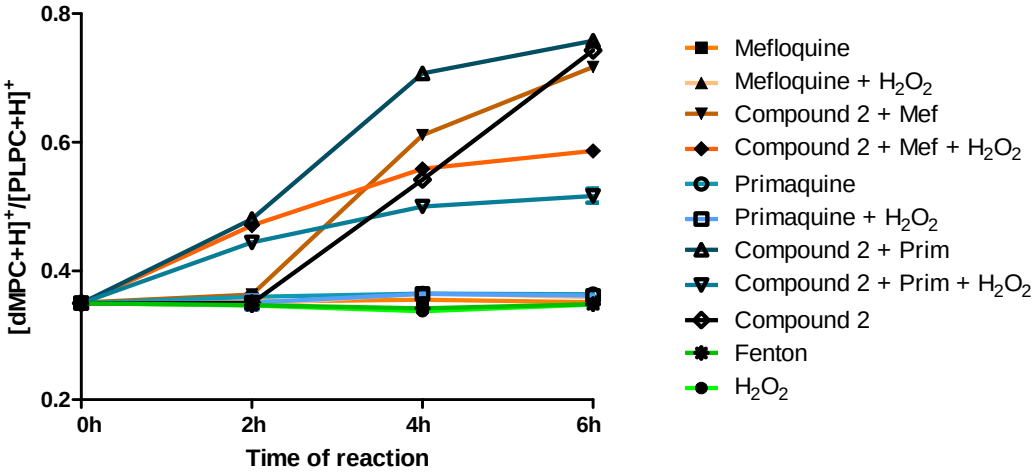
that the free heme interact with these compounds to activate the drug, promoting then the formation of free radicals that may initiate parasite lipids oxidation *(32)*. The linkage of this drug to the porphyrin may have led to a higher oxidation of PLPC than the other drugs tested.

2.4.Evaluation of the oxidant capacity of compound 2 in combination with the drugs mefloquine and primaquine

In order to evaluate if the oxidant efficacy of complex **2** is not affected by presence of mefloquine and primaquine, new experiments involving the combination of complex **2** and those drugs were performed in the presence and in the absence of hydrogen peroxide. Extra experiments with free drugs in the presence and in the absence of H_2O_2 were also performed in order to verify if these combinations have the capacity to oxidize PLPC (Figure 25a). Additionally, was performed a test only with H_2O_2 to prove that this oxidant alone was not able of oxidize phospholipids and a test under Fenton conditions in order to compare the rate of phospholipid oxidation in the presence of the metalloporphyrins and under Fenton conditions (Figure 25b).



a)



b)

Figure 25 – Variation of $[dMPC+H]^+/[PLPC+H]^+$ ratio by the presence of compound **2** and its combination with primaquine and mefloquine and in the presence of free drugs (a) and comparing with H_2O_2 and Fenton mechanism (b).
***p<0.001

The results in Figure 25a) show that the drugs alone or in combination with H_2O_2 were not able to promote any PLPC oxidation. Oxidative events in the presence of mefloquine and primaquine were only detected when compound **2** was also present. The results summarized in figure 25 b) confirmed that H_2O_2 alone or under Fenton conditions after 6 hours of reaction were also not able to promote PLPC oxidation. The presence of the drugs in combination with compound **2** showed a similar $[dMPC+H]^+/[PLPC+H]^+$ ratio when compared with the one of compound **2** both in the presence and the absence of H_2O_2 (figure 25 b). These results suggest that the efficiency of the porphyrin is not affected by the drugs. Plus, compound **2** alone present a higher $[dMPC+H]^+/[PLPC+H]^+$ ratio when compared with the ratio in the presence of H_2O_2. However, this difference was only significant in case of the compound **2** with the drug primaquine.

In all the reactions with the metalloporphyrins in the presence of H_2O_2 it was verified a lower PLPC oxidation when compared with the tests in the absence of hydrogen peroxide, after the six hours of reaction. *Plasmodium* as a parasite, during the invasion of the erythrocytes, has access to a rich source of nutrients and it is protected from the immune system of the host *(5)*. For that, it is not expected an elevated production of H_2O_2 in red blood cells during the parasite invasion.

In figure 26 it is represented all the reactions where it was actually observed PLPC oxidation.

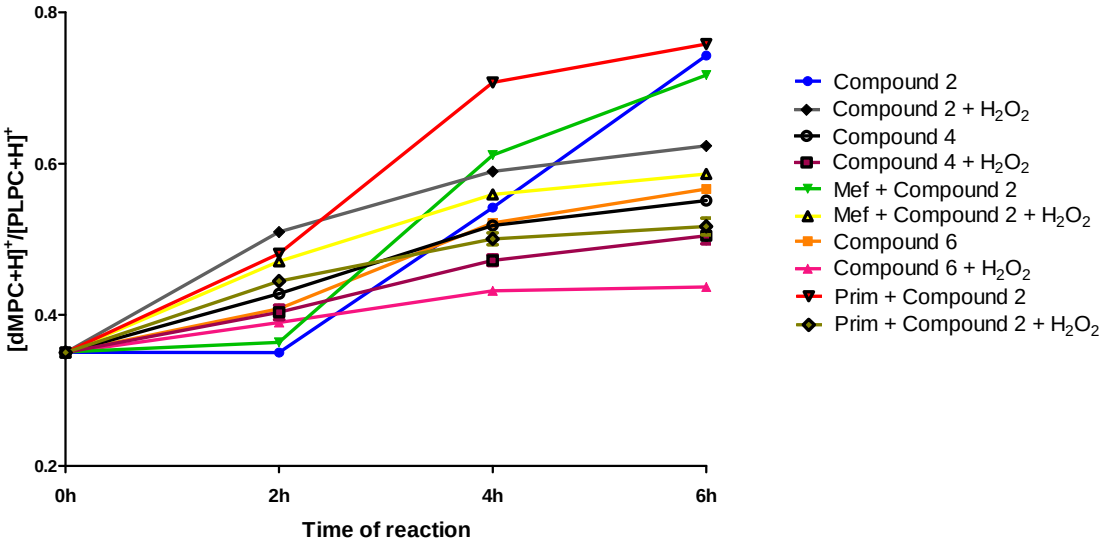


Figure 26 – Variation of $[dMPC+H]^+/[PLPC+H]^+$ ratio in the presence of all metalloporphyrins during 2h, 4h and 6h of reaction (error bars not shown).

It is possible to see the most efficient systems for PLPC is based on compound **2** or on its combination with the drugs primaquine and mefloquine not covalently linked the in the absence of H_2O_2. Interesting, after 4 hours of reaction the best system is the combination of compound **2** with primaquine.

2.5. Identification of PLPC oxidation products in the presence of porphyrins: possible reaction pathways

The new metalloporphyrins synthetized in this work showed to have oxidant activity toward phospholipids, namely PLPC. Mass spectrometry analysis allowed the identification of phosphatidylcholine oxidation products, corresponding to the insertion of: i) one oxygen atom (m/z 774.6) and one oxygen atom with 2 Da mass decrease (m/z 772.6); ii) two oxygen atoms (m/z 790.6) and two oxygen atoms with 2 Da mass decrease (mz 788.6); iii) three oxygen atoms (806.6) and three oxygen atoms with 2 Da mass decrease (804.6). These type of products were already described by our and other groups *(74,98,99)* and may be formed in different oxidation conditions namely non-enzymatic methods, like hydroxyl radical generated from H_2O_2/Fe^{2+} or H_2O_2/Cu^{2+} systems, and enzymatic conditions using lipoxygenase or myeloperoxidase to mimic *in vivo* conditions *(90)*. The mechanism of lipid peroxidation starts with hydrogen abstraction from an

allylic position affording a radical stabilized by resonance. The hydrogen abstraction can be mediated by radicals or by the Fe^{3+} ion from enzymes like CYP *(75)*.

Several mechanisms of oxidation mediated by the Heme group, released from myoglobin or hemoglobin, were proposed *(24)*. The mechanisms of oxidation by the metalloporphyrins used in this work may be similar to those observed to heme-group and to other enzymes like CYP that have the same catalytic center. In order to clarify the type of mechanism involved in the PLPC oxidation, the products obtained were identified by tandem mass spectrometry.

The oxidation products formed in the reactions performed with the iron(III) complexes in the presence or in the absence of H_2O_2 were the same as it was confirmed by MS/MS spectrum. In figure 27 is represented the MS/MS spectrum of the product with the *m/z* 790.6 formed in the presence and in absence of H_2O_2. In both spectra is observed two mainly ions apart from the ion corresponding to the loss of the phosphatidylcholine head (*m/z* 184.6). Those ions correspond to the loss of water (*m/z* 772.6) and H_2O_2 (*m/z* 756.6).

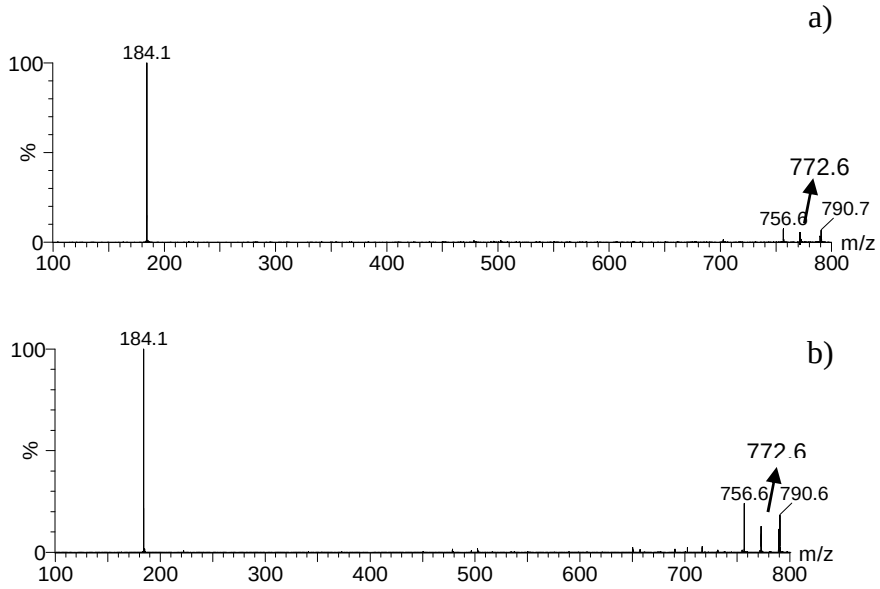


Figure 27 – MS/MS spectra of oxidation product at *m/z* 790.6 in the presence (a) and absence of H_2O_2 (b).

In fact, the most abundant oxidation product formed was the product with the *m/z* 790.6, and were identified mainly two ions that correspond to the loss of water and hydrogen peroxide. The last neutral loss is typical of hydroperoxy derivatives and the loss of water of hydroxyl derivatives *(74)*. So, the ion at m/z 790.6 may correspond to different structures (figure 28), for example the derivatives bearing two hydroxyl groups, an epoxy and a hydroxyl function and a hydroperoxide derivative. However, other structures can

be assumed. The second most abundant oxidation product formed during the six hours of reaction was the ion at m/z 772.6 which correspond to the insertion of one oxygen atom minus two hydrogen atoms. Two possible structures of this ion are represented on the figure below.

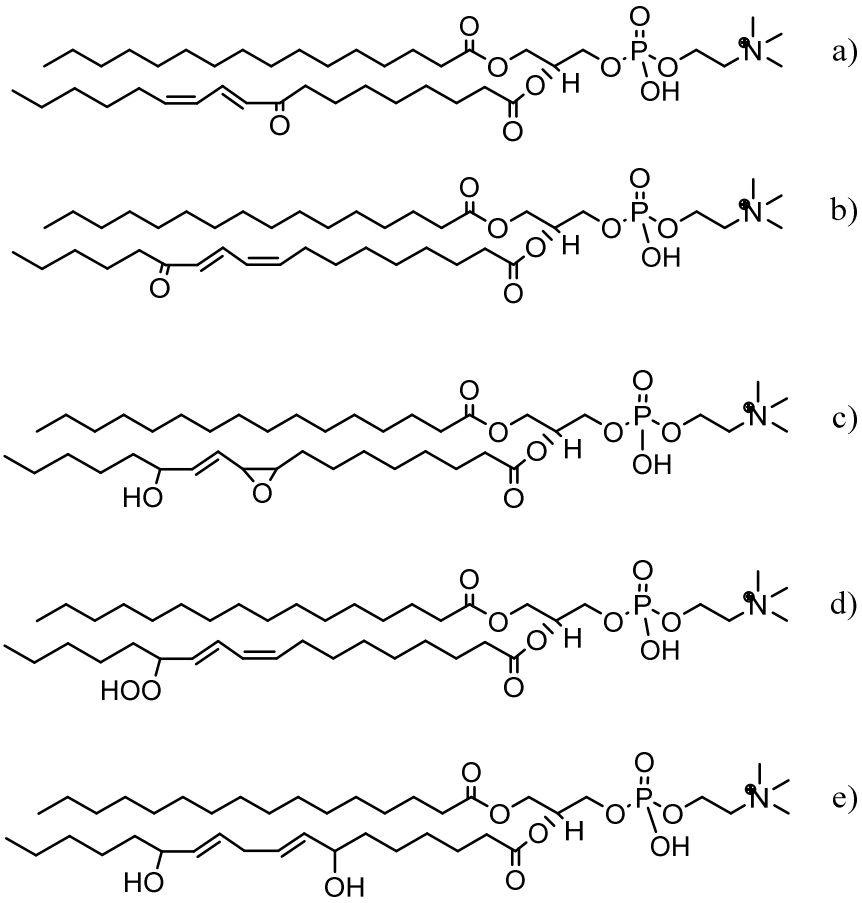


Figure 28 – Proposed structure to the oxidation products with *m/z* 772.6 (a and b) and *m/z* 790.6 (c, d and e).

It is worth to highlight that in these studies the PLPC oxidation was observed either in the presence or in the absence of H_2O_2, meaning that in this last case the molecular oxygen present in the reaction was involved in the process. In fact, the oxidation mediated by Fe(III) porphyrins in the presence or the absence of hydrogen peroxide does not involve the same mechanisms. It is well established that Fe(III) porphyrins can react with hydrogen peroxide to form species of higher oxidation states [(Porphyrin)Fe(IV)=O] $^{\bullet+}$ ↔ [(Porphyrin)Fe(V)=O] *(68,70,94,95)*. In figure 29 is represented a possible mechanism for the oxidation process mediated by Fe(III) porphyrins in the presence of H_2O_2. After the interaction of the porphyrin with hydrogen peroxide, the reaction may follow two different pathways (reactions **2** and **3**). It was proposed that oxygen-oxygen bond, from the product formed in reaction 2, may undergo a homolytic cleavage that produce a

hydroxyl radical *(100)*, or a heterolytic cleavage that produce the iron(IV) π-radical cation species and water. Some evidences have already been reported that these two cleavages may occur simultaneously and the separation between the two pathways may depend on the composition of the porphyrin, the axial ligands and the oxidant *(101)*. So, the porphyrinic cation radical may react with the substrate, in this case with phospholipids, and lead to double bond epoxidation or to allylic hydroxylation of those. In addition the hydroxyl radical generated under these conditions may induce radical peroxidation of the phospholipids, similarly as previously observed in the oxidation of phospholipids under Fenton reaction conditions *(102)*.

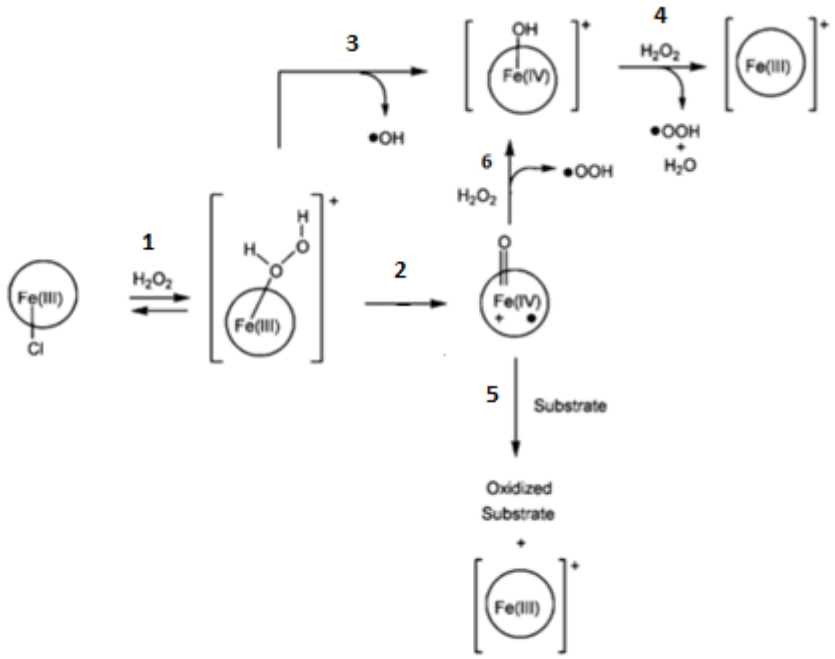
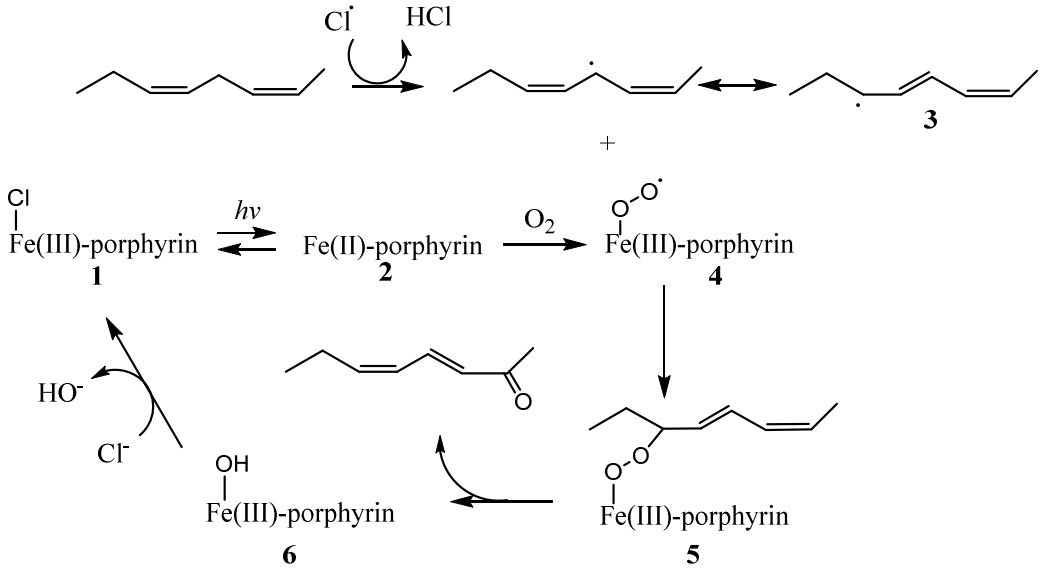


Figure 29 - Proposed mechanism for oxidation of phospholipids as substrate mediated by iron (III)-chloride porphyrins in the presence of H_2O_2 (adapted from *(68)*).

As it was mentioned, the mechanism of oxidation in the absence of hydrogen peroxide must involve the oxygen molecular from the reaction medium. Maldotti *et* al.*(103)*, described a possible mechanism of oxidation by Fe(III) porphyrins that involves the reduction of the metal center as well as the formation of radical species by photo activation. So, these authors showed that the Fe(III) porphyrins inside micelles can be reduced to Fe(II) porphyrins with the concomitant oxidation of axial ligand to a radical specie. In **scheme 5** is represented the mechanism proposed by these authors. The radical ($Cl^{\bullet}$) may abstract the allylic hydrogen atom from the diene system affording radical **3**

stabilized by resonance. Then, O_2 can react with the Fe(II) porphyrin leading to the formation of intermediate **5** that after reaction with the radical substrate can afford for example a keto product. In fact, one of the most abundant oxidation products observed during the oxidation by metalloporphyrins is the keto derivative product (*m/z* 772.6). However, the radical resulting from the abstraction of the hydrogen atom from the allylic position of the substrate may also react with molecular oxygen, starting a radical chain reaction.



Scheme 5

Like this, the exact mechanism of oxidation by the porphyrin derivatives synthetized in this work is not well established. These compounds may undergo to different reactions, producing different oxidation products. The porphyrins may interact directly with the substrate, leading to epoxidation or hydroxylation of the same, but also can lead to the formation of radicals that may start a radical chain reaction. The mechanism of oxidation by these porphyrins in the presence or the absence of hydrogen peroxide is different, but the oxidation products observed were the same.

III. Methodology

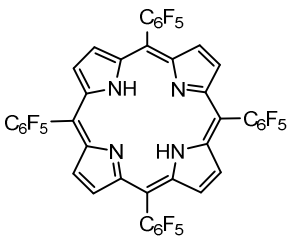
1. Reagents and equipment

Commercial reagents for the synthesis of porphyrin derivatives did not suffer further purification. Reactions were followed by TLC, using plastic sheets covered by silica gel 60 F_{250}. After elution some of the TLC were observed at UV light at 254 or 366 nm. UV-vis spectra were register in a spectrophotometer UV-2501PC Shimadzu in glass cells with 1 cm of optical path. NMR spectra of ^{1}H, ^{19}F and ^{13}C were register in an equipment Bruker Avance 300 MHz (300.13 MHz for ^{1}H and 282.38 MHz for ^{19}F) or Avance 500 MHz (125.77 MHz for ^{13}C). Solvent used to acquiring of the spectra was deuterated chloroform and tetramethylsilane (TMS) as internal reference.

Purification of reactional mixtures were performed by column with silica gel 60 (0.063-0.200 nm) from Merck. Preparative thin-layer chromatography was carried out on 20x20 cm glass plates and previously coated with silica gel 60 from Merck, having a thickness of 0.75 mm and activated on an incubator for 12h.

Phospholipid internal standards 1,2-dimyristoyl-sn-glycero-3-phosphocholine (dMPC) and 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLPC) were purchased to Avanti polar lipids, Inc (Alabaster, AL, USA), and used without further purification. Chloroform and methanol were purchased from Fisher Scientific Ltd. (Leicestershire, UK). $FeCl_2$ and H_2O_2 (30%, w/w) were acquired from Merck (Darmstadt, Germany). Mili-QH2O used was filtered through a 0.22-mm filter and obtained using a Milli-Q Millipore system (Milli-Q plus 185). Ammonium hydrogen carbonate buffer (5 mM, pH 7.4) was purchased from (Riedel-de Haën, Germany) and prepared in Milli-Q water. Drugs mefloquine, primaquine and artesunate were synthetized by the organic chemistry group of farmanguinhos - fiocruz (Brazil) and used without further purification.

2. Synthesis of 5,10,15,20-tetrakis(pentafluorophenyl)porphyrin (TF_5PP) derivatives



All the porphyrin derivatives were obtained from TF_5PP (**compound 1**). For this work this porphyrin was gently provided by Ana Gomes.

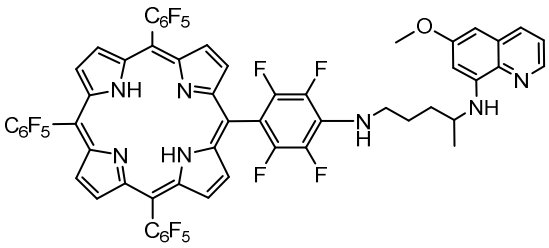
UV-vis ($CHCl_3$): λmax (log ε) 413 (5.51), 506 (4.31), 540 (3.38), 583 (3.69), 638 (2.63) nm. **ESI:** *m/z* =974.1.

2.1.Synthesis of compound 3

The synthesis of this conjugate required a previous deprotection of the drug primaquine. In a round bottomed flask containing a magnetic stirring bar were added 250 mg of protected primaquine and 3 mL of distillated water. The pH was adjusted to 11 with a solution of NaOH 0.5 M. The flask was maintained at room temperature during two hours. Primaquine was extracted with dichloromethane.

2.1.1. Coupling reaction of compound 1 with primaquine

Primaquine obtained from previous reaction was added to a round bottomed flask containing **compound 1** (100 mg, 0.103 mmol), potassium carbonate (1 eq) and toluene (5 mL). The mixture was heated at 120ºC for 18h at reflux. After reaching room temperature, the mixture was washed with distilled water and extracted with CH_2Cl_2. The combined organic extracts were evaporated under reduced pressure and the crude mixture was fractioned in a silica gel column, using CH_2Cl_2 as eluent. The first fraction observed was compound 1 and the second was the pretended compound **(compound 3)**.

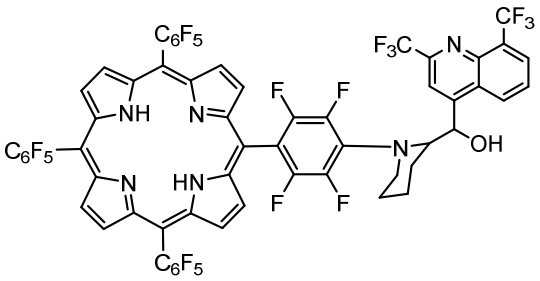


η= 66.5 %. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 9.01 (d, *J* = 4.8 Hz, 2H), 8.84 – 8.92 (s, 4H), 8.85 (d, *J* = 4.8 Hz, 2H), 8.58 (dd, *J* = 4.2, 1.6 Hz, 1H), 7.96 (dd, *J* = 8.3, 1.6 Hz, 1H), 7.32 (dd, *J* = 8.3, 4.2 Hz, 1H), 6.40 and 6.39 (AB, *J* = 2.5 Hz, 2H), 4.27 – 4.36 (m, 1H), 3.90 (s, 3H), 3.70 – 3.77 (m, 2H), 1.93 – 2.04 (m, 4H), 1.44 (d, *J* = 6.4 Hz, 3H), -2.91 (s, 2H). **^{19}F NMR** (282 MHz, CDCl3): -184.75 – -184.45 (m, 6F, *m*-F), -183.83 (d, *J* = 16.7 Hz, 2F, *m*-F), -174.5 – -174.50 (m, 3F, *p*-F), -163.86 – -163.70 (m, 2F, *o*-F), -159.70 – -159.45 (m, 6F, *o*-F) ppm. **^{13}C NMR** (126 MHz, $CDCl_3$): δ 20.78, δ 27.80**,** δ 33.87, δ 48.02, δ 48.90, δ 55.29, δ 91.85, δ 96.97, δ 99.33, δ 103.24, δ 115.75, δ 121.98, δ 129.48, δ 130.01, δ 134.92, δ 135.44, δ 136.57, δ 138.58, δ 141.22, δ 143.26, 145.57, 147.54, δ 159.49 ppm. **UV-vis** ($CHCl_3$): λmax (log ε) 415 (5.17), 508 (4.11), 585 (3.63) nm. **ESI:** *m/z* =1214.3.

2.2.Coupling reaction of compound 1 with mefloquine

A 50 mL round-bottomed flask containing a magnetic stirring bar was charged with **compound 1** (100 mg; 0.103 mmol), mefloquine (194.2 mg; 0.513 mmol) and NaH (1 eq) in toluene (5 mL). The mixture was heated at 120ºC for 72h at reflux. After reaching

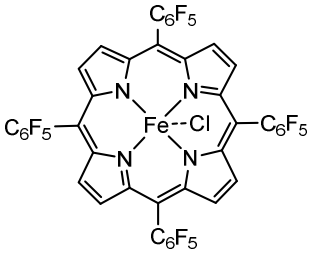
room temperature, the mixture was washed with distilled water and extracted with CH_2Cl_2. The combined organic extracts were evaporated under reduced pressure and the crude mixture was fractioned in a silica gel column, using first CH_2Cl_2 as eluent. The second fraction recovered was the pretended compound **(compound 5)**. Other fractions observed resulted from the substitution of more than one fluorine atom by mefloquine.



η = 47.2 %. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 8.90 (s, 4H), 8.86 and 8.82 (AB, *J*= 4.8 Hz, 4H), 8.58 (d, *J* = 8.0 Hz, 1H), 8.33 (s, 1H), 8.30 (d, *J*= 8.0 Hz, 1H), 7.90 (t, J= 8.0 Hz, 1H), 6.43 (d, *J* = 4.9 Hz, 1H), 3.31 – 3.40 (m, 1H), 3.16 – 3.26 (m, 1H), 2.67 – 2.79 (m, 3H), 2.03 (d, *J*= 10.8 Hz, 2H), 1.42 – 1.80 (m, 4H). **^{19}F NMR** (282 MHz, $CDCl_3$): δ -83.35 (s, 3F), -90.87 (s, 3F), -159.76 – -159.52 (m, 6F, *o*-F), -160.44 – -160.28 (m, 2F, *o*-F), -174.23 – -174.45 (m, 3F, *p*-F), -178.16 – -178.35 (m, 2F, *m*-F), -184.32 – -184.56 (m, 6F, *m*-F). **^{13}C NMR** (126 MHz, $CDCl_3$): δ 23.00, δ 27.74, δ 23.92, δ 46.95, δ 61.22, δ 68.18, δ 103.56, δ 104.09, δ 115.51, δ 115.94, δ 120.11, δ 122.30, δ 124.55, δ 126.78, δ 128.28, δ 128.82, δ 130.92, δ 136.56, δ 141.39, δ 144.14, δ 145.53, δ 147.44, δ 162.94, δ 167.82 ppm. **UV-vis** ($CHCl_3$): λmax (log ε) 413 (5.43), 507 (4.28), 583 (3.74), 637 (3.11) nm. **ESI:** *m/z* =1332.87.

2.3.Synthesis of Fe(III) complexes of compounds 1, 3 and 5

The synthetic approach for the synthesis of Fe(III) complexes of the three different compounds followed the same procedure. In a 50 mL two headed round-bottomed flask containing a magnetic stirring bar was added **compound 1** (100 mg, 0.103 mmol), DMF (10 mL) and pyridine (1 mL). The solution was bubbled with nitrogen for 10 min and then it was added the $FeCl_2$ (246.2 mg). The reaction was performed in reflux at 140ºC for 24h under inert atmosphere. After 24h the DMF was distilled under reduced pressure with the addition of small amounts of toluene to help the elimination of DMF. After almost all of the DMF was evaporated, the mixture was washed with distilled water and extracted with CH_2Cl_2. The products were obtained pure after purification by preparative TLC using a mixture CH_2Cl_2/MeOH (100:5), followed by crystallization in a hexane/CH_2Cl_2 mixture.

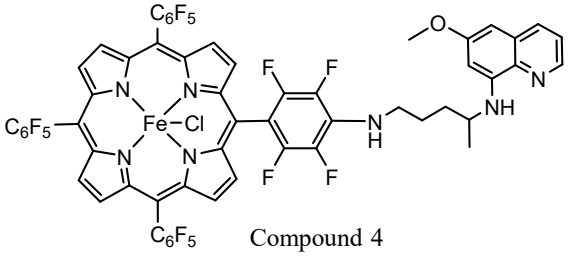


Compound 2

η = 65.4 %.

UV-vis ($CHCl_3$): λmax (log ε) 412 (4.90), 503 (3.92), 630 (3.56) nm.

ESI: *m/z* = 1027.9.

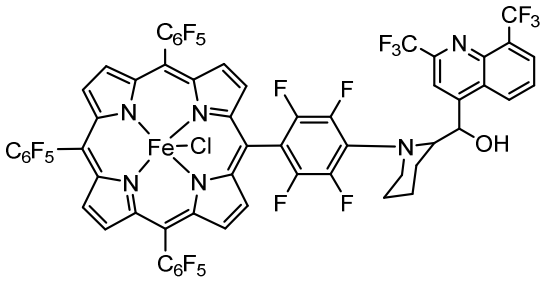


Compound 4

η= 14.4 %.

UV-vis ($CHCl_3$): λmax (log ε) 416 (4.73), 506 (3.94), 634 (3.60) nm.

ESI: *m/z* =1267.73.



Compound 6

η= 13.5 %.

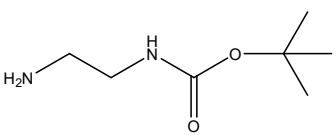
UV-vis ($CHCl_3$): λmax (log ε) 413 (4.54), 506 (3.54), 628 (3.22) nm.

ESI: *m/z*= 1386.70.

2.4.Attempts to conjugate compound 1 with artesunate

2.4.1. Protection of ethylenediamine with di-*tert*-butyl dicarbonate (BOC)

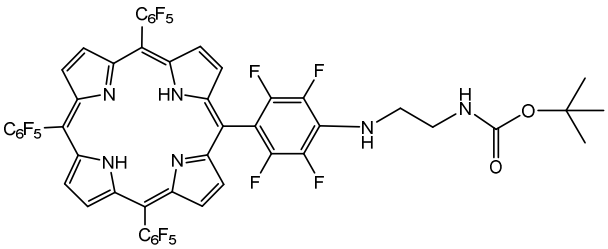
In a two headed rounded flask containing a magnetic stirring bar was added 16 mL of ethylenediamine and 25 mL of tetrahydrofuran (THF). The solution was cooled down to -10ºC. The, BOC (2.5 mg) was dissolved in THF (12.5 mL) and it was added dropwise to the solution of amine during 30 min *(104).* The reaction was stirred overnight at room temperature. THF was dried under reduced pressure and the mono substituted ethylenodiamine was extracted with dichloromethane. The compound isolated as an yellow oil.



η= 81 %. **^{1}H NMR** ($CDCl_3$): δ 3.16 (q, *J*= 5.9 Hz, 2H), δ 2.79 (t, *J*= 5.9 Hz, 2H), δ 1.45 (s, 9H), ppm.

2.4.2. Functionalization of compound 1 with mono substituted ethylenediamine

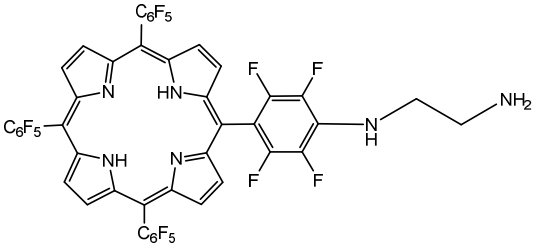
A 50 mL round-bottomed flask containing a magnetic stirring bar was charged with the TF_5PP (75 mg; 0.0767 mmol), mono-substituted ethylenediamine (9.1 mg; 0.057 mmol), potassium carbonate (10.6 mg, 0.0767 mmol) and toluene (5 mL). The mixture was heated at 120ºC for 72h at reflux. During this period, it was added the same amounts of mono substitued ethylenediamine and potassium carbonate two more times, once it was observed much starting compound (monitored by TLC). The flask was allowed to cool at room temperature, the solvent was distilled under reduced pressure and the compound was purified by column chromatography using dichloromethane as eluent. The desired conjugate (**compound 7**) was obtained from the second fraction.



UV-vis ($CHCl_3$): λmax (log ε) 415 (5.29), 508 (4.16), 585 (3.65), 658 (2.83). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 8.97 (d, *J*=4.7, 2H), δ 8.79 – 8.85 (m, 6H), δ 3.72 – 3.80 (m, 2H), δ 3.49 – 3.58 (m, 2H), δ 1.47 (s, 9H), δ – 2.98 (s, 2H) ppm.

2.4.3. Deprotection of compound 7

In a two headed flask with a magnetic stirring bar was added the porphyrin functionalized with ethylenediamine, dichloromethane (1 mL) and TFA (5 eq.) at room temperature. The reaction was controlled by TLC and when it was not observed release of gas it was stopped. The ^{1}H NMR confirmed the total deprotection of the amino, and the yield of **compound 8** was not calculated.



UV-vis (CHCl3): λmax (log ε) 414 (5.36), 508 (4.16), 585 (3.60), 658 (2.98). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 9.03 (d, *J*= 4.7 Hz, 2H), δ 8.85 – 8.94 (m, 6H), δ 3.75 – 3.84 (m, 2H), δ 3.17 – 3.25 (m, 2H), δ -2.91 (s, 2H) ppm.

2.4.4. Coupling reaction of compound 8 with artesunate

In a 50 mL rounded flask containing a magnetic stirring bar was placed the porphyrin (5mg, 0.00513 mmol), artesunate (18.9 mg, 0.049 mmol), ethylcarbodiimide (EDC) (1,5 eq, 0.0736 mmol), 4-(dimethylamino)pyridine (DMAP) (4 eq, 0.196 mmol) in $CHCl_3$ (1mL). The reaction was executed at room temperature for 1h30 and after this time was controlled by TLC and it was not observed the formation of the pretended compound.

2.4.5. Coupling reactions of artesunate and ethylenediamine

Other attempts were also performed for the formation of compound 1 and artesunate conjugate, by reacting artesunate with ethylenediamine. In a 50 mL round-bottomed flask containing a magnetic stirring bar was added artesunate (50 mg, 0.13 mmol), EDC (1,5 eq, 0.193 mmol), DMAP (4 eq, 0.516 mmol), ethylenediamine (13µL) and $CHCl_3$ (1 mL) at room temperature. The reaction was controlled by TLC and it was not observed the formation of pretended compound. The same reaction was performed at 0ºC but it was substituted EDC by carbodiimide (DCC) (40 mg, 0.194 mmol), maintaining the other reactional conditions.

It was tested another way to achieve the reaction between ethylenodiamine and artesunate. In a 50 mL round-bottomed flask containing a magnetic stirring bar was added artesunate (50 mg), DMF (0.13 mmol), oxalyl chloride (2.5 mmol), ethylenediamine (10 eq) and dry CH_2CL_2 *(105).* The reaction was executed at room temperature for 2h and after this time was controlled by TLC and once more it was not observed the formation of the pretended compound.

3. Phospholipid oxidation by porphyrins derivatives

3.1.Sample preparation and phospholipid oxidation by porphyrinic compounds

Stock solutions of PLPC, dMPC, compounds **1** to **6**, mefloquine and primaquine were prepared in chloroform (1mg/mL). Then, 62.5 µg PLPC (final concentration of 1.32 mM) and 14 µg of dMPC (final concentration of 0.33 mM) were mixed. dMPC is a standard to control the extension of oxidation and it was added in a concentration equal to a quarter of PLPC concentration. Additionally 0.5 µM of each porphyrinic compound were mixed to the previous solution and dried under nitrogen stream. The ammonium hydrogen carbonate buffer (5mM, pH=7.4) was added to each vial until perform a final volume of

62.5 µL. All of the vials were submitted to the same conditions of reaction. Furthermore, it was performed a test with H_2O_2 with all different porphyrin compounds. All the procedure was the same but it was added the H_2O_2 (1 mM), prepared in Milli-Q water, after the addiction of the buffer, until perform a final volume of 62.5 µL. To form liposomes, the vials were vortex-mixed during 10 min, sonicated for 15 min on ultrasound (Selecta Ultrasons) followed by more 5 min on vortex. The samples were incubated at 37ºC and the oxidation was monitored by mass spectrometry (ESI) every 2 hours in a full time of six hours. For negative control there were prepared liposomes with only H_2O_2 and free compounds namely mefloquine and primaquine.

3.2.Q-TOF conditions

The MS and MS/MS studies were carried out in the positive mode on a Q-TOF2 instrument (Micromass, Manchester, UK). Samples were introduced through direct infusion into the electrospray source at a flow rate of 10 µL min^{-1}. The cone voltage was set at 30 V and capillary voltage applied to the needle at 3 kV. Source temperature was at 80 °C and desolvation temperature at 150 °C. The resolution was set to about 9000 (FWHM). MS/MS were acquired by collision-induced decomposition (CID), using argon as the collision gas (measured pressure in the penning gauge ~6×10^{-5} mbar). The collision energy used was 25 to 30 eV. Data acquisition was carried out with a MassLynx 4.0 data system.

3.3.Statistical analysis

The results are presented as mean values and their standard deviations (mean ± SD) for each experimental group. Two-way analysis of variance (ANOVA) was used with the Bonferroni pos-test to evaluate significant differences among samples. A value of $p<0.05$ were considered to be statistically significant. Statistical analysis were performed using GraphPad Prism 5 software.

IV. Conclusion

In this work were prepared new porphyrinic conjugates bearing drugs already used in malaria treatment. It was expected that these new conjugates have oxidative power toward membranes and that can act as drug delivers. The synthesis of the new porphyrin derivatives was accomplished with the drugs mefloquine and primaquine, being the nucleophilic substitution of one *para*-fluorine atom in 5,10,15,20-tetrakis (pentafluorophenyl)porphyrin the key step of the synthetic strategy proposed. Due to the structure of artesunate the reaction could not follow the same procedure, and involved more steps of reaction that unfortunately were not successful. Also, using conventional procedures, the Fe(III) complexes of the compounds **1**, **3** and **5** were synthetized and characterized.

Lipid peroxidation induced by all the different porphyrinic compounds (**1** to **6**), in liposomes as biomimetic systems of cellular membranes, was monitored by ESI-MS and the products of oxidation identified by tandem mass spectrometry. By the ratio $[dMPC+H]^+/[PLPC+H]^+$ it was possible to evaluate the extension of reaction. All the metalloporphyrins tested in this work (**2**, **4** and **6**) showed to be able to oxidize phospholipids in the presence and absence of H_2O_2, although with different efficacy. Compound **2** showed to be the one with better results of oxidizing PLPC, comparing with the compounds **4** and **6** in the presence and the absence of hydrogen peroxide. It was also verified that the drugs alone have no capacity of oxidizing phospholipids, and its combination with the compound **2** did not affect the oxidation promoted by this compound. The most abundant products formed by porphyrin oxidation were those with *m/z* 772.6, *m/z* 790.6, *m/z* 788.6 and *m/z* 806.6, that correspond to the insertion of one to three oxygen atoms in the linoleic acid of PLPC. The mechanism of oxidation by these compounds is complex, it was verified that the mechanism is not always the same, porphyrins may interact directly with the substrate and promote its oxidation, or can produce radical species that are able to start radical chain reactions.

As future perspectives it was interesting to synthetize the compound **9** that was not accomplished in this work, once artemisinin nowadays is the drug that is mostly used against malaria. The confirmation of mechanism of oxidation by the porphyrins in the absence of H_2O_2 is also an important perspective to a better understanding of the comportment of these compounds *in vivo*. Some tests with some of the porphyrins used in this work are already being carried out by the group of Brazil.

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