UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

PRODUCTION AND CHARACTERIZATION OF DIFFERENT TYPES OF MALARIA PIGMENT (HEMOZOIN) AND THEIR POTENTIAL USE FOR SCREENING FOR HEMOZOIN INHIBITING DRUGS

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MESTRADO EM MICROBIOLOGIA APLICADA

2012

Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Vegetal

Production and characterization of different types of malaria pigment (hemozoin) and their potential use for screening for hemozoin inhibiting drugs

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MASTER THESIS

2012

This thesis was fully performed at the Institute of Microbiology in Faculty of Medicine of the University of Lisbon under the direct supervision of Prof. Dr. Thomas Hänscheid.

Prof. Dr. Margarida Gama Carvalho was the internal designated supervisor in the scope of the *Master in Applied Microbiology* of the Faculty of Sciences of the University of Lisbon.

Acknowledgements

Abstract

Malarial pigment, hemozoin, appears to modulate the immune system, however it remains controversial if it has stimulatory or inhibitory effects. Biological properties may depend on the type of hemozoin, namely its origin and production method, morphology or size. Natural hemozoin can be obtained from *P. falciparum* cultures and identical β-hematin can be synthesized from heme. Characterization becomes crucial to confirm size, shape and possible contaminations.

Hemozoin production is also a unique antimalarial drug target. There are several heme inhibition assays to screen for drugs with the potential to inhibit hemozoin growth. Yet, these assays are often complex, use highly concentrated, toxic reagents, and not all confirm the end product's nature.

This work aimed to produce and characterize natural hemozoin and synthetic hemozoin, as well as hemozoin-like crystals. Hemozoin-like crystals growth was also investigated to determine if drugs inhibit crystallization, and adapted in an assay format to explore their potential to screen for hemozoin-inhibiting drugs.

Hemozoin obtained from different origins was characterized by several methods, including scanning electron microscopy, X-ray diffraction and infrared spectroscopy. Hemozoin-like crystals were grown in broth medium with different drugs to investigate their ability to inhibit crystallization.

Despite an overall similarity in morphology and size, differences were detected and characterized between different types of hemozoin, which may be biologically relevant. Although very similar, X-ray diffraction and infrared spectroscopy showed that hemozoin-like crystals were not true hemozoin. However, inhibition results were consistent with previous heme inhibition studies, with chloroquine and amodiaquine presenting the highest potency, followed by other quinolines such as quinine and mefloquine.

In conclusion, hemozoin used in immunology should be thoroughly characterized by several complementary methods. Hemozoin-like crystals growth could be successfully used as a novel assay to screen compounds for their hemozoin inhibiting activity, and may be a helpful tool for antimalarial drug screening.

Keywords: hemozoin, hemozoin characterization, hemozoin-like crystals, antimalarial drugs, heme crystallization inhibition assays

Resumo

A malária, uma das mais importantes doenças infecciosas da actualidade, permanece um problema de saúde pública a nível global, atingindo regiões tropicais e subtropicais em todo o mundo. Esta doença causa grande morbilidade e mortalidade e contribui para o atraso no desenvolvimento social e económico dos países afectados. A doença é causada por parasitas do género *Plasmodium*, transmitidos por fêmeas de algumas espécies de mosquito *Anopheles*. Após a fase sexual no mosquito e a fase hepática do ciclo de vida do parasita no Homem, é durante a fase sanguínea que se manifestam os sintomas da doença febril. Factores do hospedeiro e do parasita poderão contribuir para o desenvolvimento de malária severa, levando eventualmente à morte do doente, sendo que a espécie *Plasmodium falciparum* é responsável pelo maior número de mortes associadas a malária.

Apesar dos esforços travados por organizações internacionais no sentido de combater este flagelo, os programas de controlo e erradicação da malária enfrentam vários obstáculos, como a resistência aos insecticidas usados para impregnar redes mosquiteiras, a inexistência de uma vacina eficaz e, principalmente, a grande disseminação de estirpes de parasitas resistentes aos fármacos anti-maláricos actualmente disponíveis. Ainda que sejam pontuais os relatos de resistência ao quinino, utilizado desde o século XVII, este provoca graves efeitos secundários. Independentemente do sucesso de fármacos sintetizados durante o século passado, compostos como a cloroquina, a mefloquina e os antifolatos são hoje inúteis em muitas regiões do mundo. Não obstante a notável eficácia das artemisininas, estes fármacos podem escassear e tornam-se bastante dispendiosos, devendo ser administrados apenas em combinação com outros, de forma a evitar a propagação de resistência. Assim, é urgente descobrir novos fármacos eficazes no combate à malária.

O pigmento malárico, também designado hemozoína, é o produto da desintoxicação de moléculas de heme livre, formado por biocristalização no parasita durante a fase intraeritrocítica, após digestão do conteúdo celular do eritrócito infectado. Pensa-se que fármacos anti-maláricos pertencentes à classe das quinolinas interajam com as moléculas de heme, inibindo este processo de biocristalização e resultando na acumulação de heme livre tóxico responsável pela morte do parasita. Alguns autores defendem que as artemisininas actuam de forma semelhante, apesar de este ser assunto de debate. Esta via de desintoxicação de heme pelo parasita é bastante interessante no desenvolvimento de novos fármacos, já que é exclusivo do parasita e parece ser imutável, devido ao facto de não depender de enzimas codificadas pelo parasita passíveis de sofrer alterações por mutações que estivessem na origem de resistência.

Existem várias abordagens para investigação e descoberta de fármacos cujo alvo é a formação da hemozoína. Os ensaios *in vitro* de inibição da cristalização de heme tentam mimetizar a formação de hemozoína recorrendo à síntese de β-hematina, hemozoína

sintética considerada idêntica à hemozoína natural, e permitem identificar compostos com potencial para inibir o crescimento do cristal. Todavia, ensaios diferentes variam grandemente em termos dos resultados que produzem e dos próprios métodos e condições utilizados para a sua realização, e nem sempre são suficientemente reprodutíveis nem de simples execução.

O presente estudo teve como objectivo produzir e caracterizar hemozoína de diferentes origens, investigar a capacidade de vários fármacos para inibir o crescimento de cristais semelhantes a hemozoína, e o potencial destes cristais para identificar compostos antimaláricos inibidores da formação de hemozoína.

A hemozoína sintética foi obtida por catálise acídica, ao passo que a hemozoína nativa foi obtida quer por purificação, quer por simples extracção a partir de culturas de eritrócitos humanos infectados com *P. falciparum*. Os cristais semelhantes a hemozoína foram cultivados num meio bem definido suplementado com extracto de sangue. Os cristais produzidos foram caracterizados recorrendo a microscopia óptica, de polarização e microscopia electrónica de varrimento, bem como a difracção raio-X e espectroscopia de infravermelho, e investigados em relação à presença de contaminação por heme, *Mycoplasma,* DNA ou proteína. Os cristais semelhantes a hemozoína foram ainda cultivados em microplaca na presença ou ausência de vários antibióticos e fármacos antimaláricos.

Numa primeira abordagem para a caracterização da hemozoína produzida, que visualmente se apresenta preta em suspensão ou pó seco, a microscopia óptica e de polarização permitiram observar algumas diferenças morfológicas, de cor e depolarização entre os cristais obtidos por diferentes métodos. A microscopia electrónica de varrimento revelou-se talvez o método mais adequado para avaliar a morfologia e homogeneidade, evidenciando a forma de paralelepípedo dos cristais de origem nativa e a forma acicular dos cristais de origem sintética e dos cristais semelhantes a hemozoína. Todavia, apenas recorrendo à difracção raio-X e à espectroscopia de infravermelho foi possível distinguir inequivocamente os cristais semelhantes a hemozoína dos cristais de verdadeira hemozoína.

Quando purificada, a hemozoína de origens natural e sintética apresenta ausência dos contaminantes investigados, ao passo que a hemozoína nativa extraída, não purificada, se encontra associada a proteínas e ácidos nucleicos. Ainda assim, e apesar de alguns estudos sugerirem um papel de estimulação do sistema imunitário para a hemozoína, a presença de proteínas e DNA pode ser interessante, caso se considere que a hemozoína poderá ser libertada dentro do vacúolo digestivo intacto ou associada a reminiscências desta ou de outras estruturas do parasita. A contaminação com uma quantidade apreciável de heme em cristais semelhantes a hemozoína contribuiu para estabelecer a diferença relativamente à verdadeira hemozoína. A quantificação realizada com o QuantiChrom™ Heme Assay Kit permitiu tirar partido da optimização para amostras de origem biológica e

evita o uso de reagentes tóxicos. A obtenção de hemozoína de origem natural foi morosa e dispendiosa, ao passo que a produção de hemozoína sintética e cristais semelhantes a hemozoína foi mais simples e económica.

O IFDO (do inglês *Ileal Fluid Dependent Organism*) foi descrito por Burdon em 1989 após ter sido isolado do fluido de ileostomia de indivíduos com a doença de Crohn. Além de organismo auto-replicativo, pensou-se poder ser uma espécie de cristal formado por constituintes do meio. Semelhante a priões no respeitante à resistência a desinfecção e esterilização, a empresa Steris interessou-se pelo seu potencial como modelo para investigar este tipo de processos, e confirmada a sua natureza hémica, além da birrefringência e cor castanha, pensou-se que poderia ser hemozoína. Apesar da semelhança, uma caracterização mais completa em colaboração com a Steris revelou a diferença dos cristais, e estes foram então designados cristais semelhantes a hemozoína.

A avaliação de compostos anti-maláricos e antibióticos como possíveis inibidores do crescimento dos cristais semelhantes a hemozoína permitiu criar um ensaio *in vitro* para identificar compostos cujo alvo seja a formação de hemozoína. Observando a microplaca após a incubação do cristal na presença de diferentes concentrações de fármaco, foi possível observar visualmente a presença ou ausência de um precipitado escuro no fundo do poço que indicam respectivamente o crescimento ou a inibição da formação dos cristais semelhantes a hemozoína.

O presente trabalho reforça a importância da caracterização da hemozoína obtida a partir de diferentes origens. Deve recorrer-se a vários métodos complementares para avaliação da morfologia, tamanho e identidade dos cristais, e testar a presença de moléculas contaminantes que possam apresentar propriedades químicas e biológicas em estudos imunológicos. Contribuir-se-á desta forma para esclarecer resultados ambíguos na investigação do papel da hemozoína na modulação do sistema imunitário do hospedeiro, bem como na avaliação da actividade de antimaláricos por inibição da formação do cristal.

Os resultados obtidos sugerem ainda que o ensaio de inibição do crescimento dos cristais semelhantes a hemozoína pode ser usado a par dos ensaios existentes para testar compostos em relação à sua actividade de inibição da formação de hemozoína. O ensaio de inibição do crescimento dos cristais semelhantes a hemozoína é simples de realizar e requer infraestruturas laboratoriais básicas e reagentes relativamente económicos, podendo contribuir assim para a descoberta de novos antimaláricos e para os esforços no controlo da malária.

Palavras-chave: hemozoína, caracterização da hemozoína, cristais semelhantes a hemozoína, fármacos anti-maláricos, ensaios de inibição da cristalização do heme

Index

1. Introduction

1.1. Malaria

Malaria is an ancient infectious disease, thought to have an effect on human history since its very beginning, in Africa, with recorded periodic fever episodes since 2700 BC in China, continuing through almost every society until the present eradication in North America and Northern and Western Europe [1] [2]. Protozoan parasites *Plasmodium* spp. cause the disease, and *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi* are known to presently infect humans. It is an acute febrile illness, including episodes of rigors alternated with high fever and intense sweating and chills, vomiting, diarrhea and headache [3]. *P. falciparum* is the most deadly species [4] and can cause severe malaria, with loss of consciousness, cerebral malaria, severe malarial anemia, placental malaria or acute lung injury, whereas the other species cause milder disease, but severity and prognosis may result from diverse parasite and host factors [5].

Various *Anopheles* spp. female mosquitoes transmit *Plasmodium* parasites to humans, and their geographical distribution is associated with epidemiological patterns of the disease [6] [7], with tropical and subtropical regions worldwide being affected, as shown in Figure 1 [8].
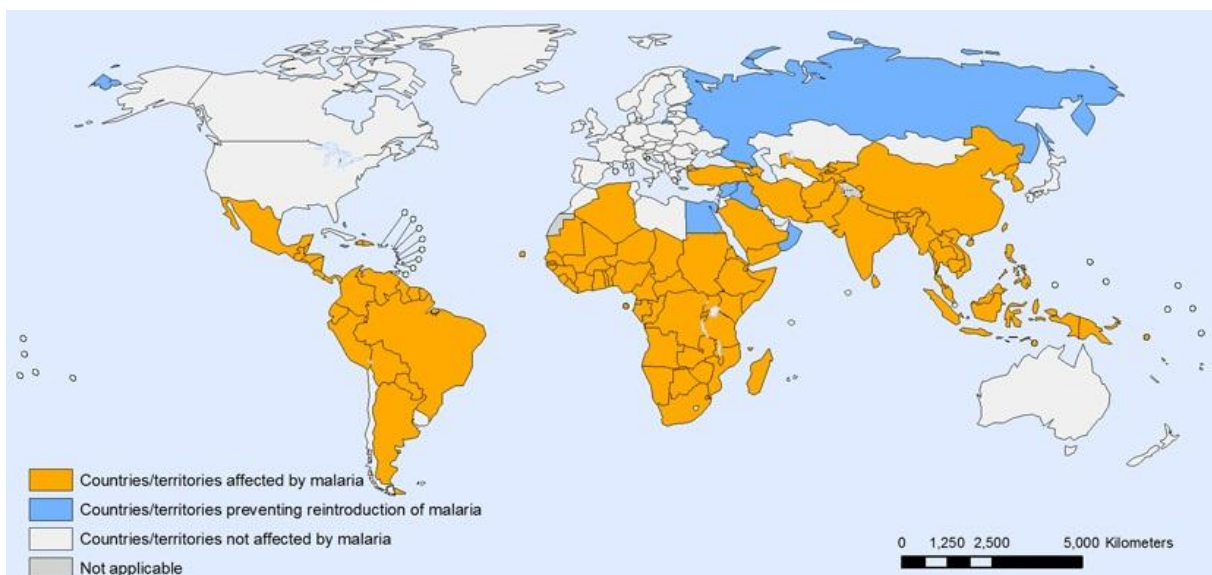
Figure 1: Countries and territories affected by malaria in 2010. Tropical and subtropical regions are affected by malaria worldwide. [8]

Presently, the World Health Organization (WHO) reports a declining trend in malaria cases and mortality, with 81% of the total 216 million cases and 91% of the 655.000 estimated malaria deaths in 2010 occurring in Africa. Children less than five years of age are 86% of the deaths, and Sub-Saharan Africa accounts for the majority of morbidity and mortality. [4] Nonetheless, some authors believe in a higher global mortality burden, with circa 1.133.000 deaths in 2010 and a higher proportion of malaria deaths in individuals aged five years or

older (42%) [9]. In fact, in some African regions more adults are being infected and susceptibility to severe malaria is shifting towards older children [10].

Malaria contributes to prevent social and economic growth in already impoverished countries [11], and the United Nations set malaria combat as part of the Millennium Development Goals. Enormous efforts have been made towards malaria eradication, with mobilization of financial help and human resources allowing substantial advances in research and the implementation of vector control and drug therapy programmes. Nonetheless, an effective vaccine remains to be developed, and effectiveness of insecticide-treated bed nets is vulnerable to populations' noncompliance and resistance to pyrethroids [12]. Drug therapy, a key part in malaria control, faces increased resistance worldwide, with no drug being universally effective, and so malaria continues to be a threatening global health problem [7].

1.2. Hemozoin, the malarial pigment

1.2.1. _Plasmodium_ life cycle and hemozoin formation

Plasmodium life cycle comprehends the hepatic and erythrocytic phases in the human host and the sexual reproduction cycle in the mosquito vector. After the mosquito bite and the entrance of numerous sporozoites, multiplication takes place in the liver and merozoites are released into the blood stream, where they invade red blood cells. The blood stage of _P. falciparum_ life cycle is schematically represented in Figure 2. When inside the erythrocyte, the parasite consumes host cellular resources so that it can grow, mature and become a schizont, the dividing form. [13] To obtain aminoacids, space and to maintain osmotic balance, the parasite digests up to 80% of the erythrocytic hemoglobin. [14] Inside the parasite acidic food vacuole, hemoglobin is oxidized to methemoglobin and then hydrolyzed into free heme and denatured globin by plasmepsins I, II and IV and histoaspartic protease. Denatured globin is hydrolyzed by falcipain and falcilysin into small peptides, which are only further degraded into amino acids by exopeptidases in the parasite cytoplasm. [15]

Free heme released as a byproduct of these reactions is toxic both to host and parasite. Reactive oxygen species generated by free heme may induce oxidative stress, leading to cell lysis and death, killing the parasite. In the human host, free heme may not only cause hemolysis, but also be involved in inflammation and organ toxicity. To avoid this, in mammals heme is detoxified into biliverdin by heme oxygenase and then into bilirubin by biliverdin reductase. Malaria parasite has different mechanisms to detoxify heme, of which the primary one is hemozoin formation in the food vacuole. Other mechanisms occur in the cytosol, such us detoxification by reduced glutathione, heme-binding proteins or degradation by hydrogen peroxide. [16]

As reviewed by Egan (2008) [17], a specific enzyme was thought to be responsible for polymerizing heme into the malarial pigment, hemozoin. However, it was then suggested that

hemozoin crystals were formed by autocatalysis, growing in a process of biomineralization. Despite some controversy, it is now believed that hemozoin crystals nucleate at the interface between the aqueous milieu and the surface of neutral lipid nanospheres in the digestive vacuole, rather than depending on the activity of a specific protein [18] [19].
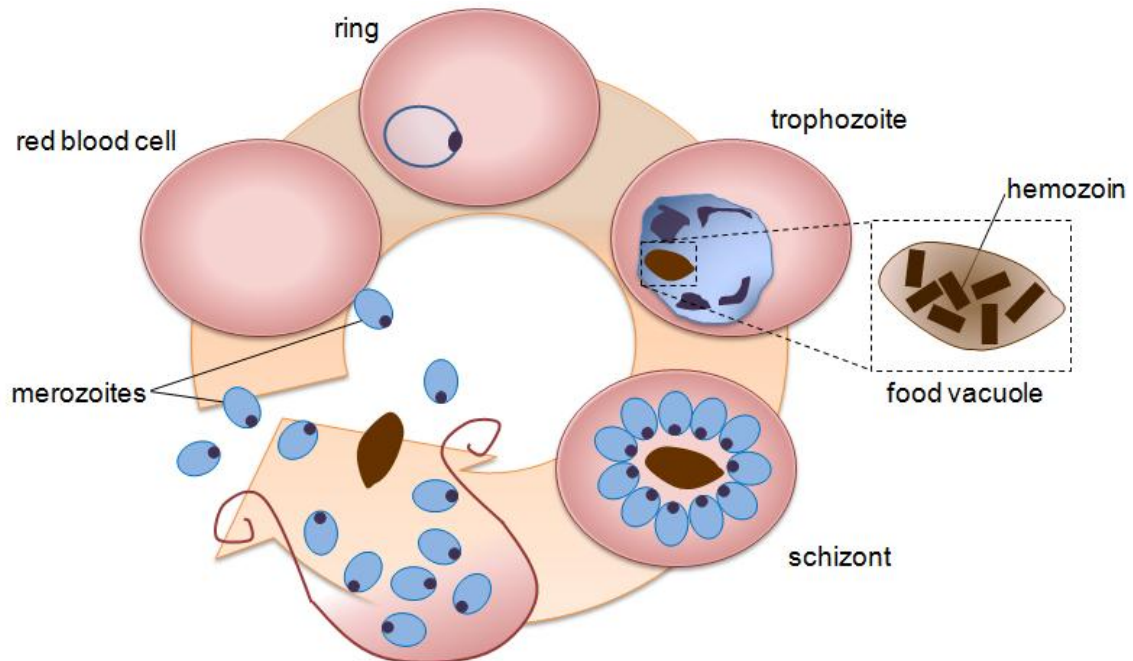
Figure 2: *Plasmodium falciparum* blood stage cycle representation. Merozoite invades an erythrocyte and develops into an early trophozoite, the ring form. The early trophozoite grows and becomes a mature trophozoite, in which hemozoin formation starts. DNA synthesis begins, to prepare the dividing stage, the schizont. After erythrocyte rupture, merozoites are released in the blood stream together with hemozoin crystals. Parasites which differentiate into male and female gametocytes can be taken up by the mosquito vector and continue the cycle and transmission.

Hemozoin amount increases as the parasite grows. When the schizont is mature, the infected red blood cell goes through a controlled process of rupture and merozoites are released in the blood stream together with hemozoin crystals. Merozoites will invade other erythrocytes and some will differentiate into gametocytes, which may be taken by the mosquito during its blood meal, starting a new cycle. [20]

1.2.2. Hemozoin characteristics and properties

Hemozoin is a paramagnetic crystal of well-defined, flat faces [21], and with 0,5-1 µm in size [22]. The crystal is constituted by cyclic π-π dimers of ferriprotoporphyrin IX (heme) molecules, with coordination of the propionate group of one heme monomer to the Fe (III) centre of the other, and linked through hydrogen bonds between the propionate groups [23] [24]. Hemozoin from the malaria parasite is considered to be identical to synthetic β-hematin, as assessed by X-ray diffraction and infrared spectroscopy [25] [26]. Notwithstanding the brick-like morphology of *P. falciparum* hemozoin, synthetic β-hematin has a more needle-like

appearance when observed with scanning and transmission electron microscopy [27] [28] [29].

Being birefringent, hemozoin has the ability to depolarize light, and can be detected by optical methods such as darkfield and polarization microscopy, as well as flow cytometry. Hemozoin released in the blood is internalized by host phagocytes, playing an important role in the modulation of the immune system. Depending on the phase of the infection it may be beneficial or harmful to the host, with the pigment producing proinflammatory or immunosuppressive effects. [30] However, published studies on biological effects elicited by hemozoin are performed with different concentrations of hemozoin and the methods used to produce hemozoin are also different. Hence, as different methods yield different types of hemozoin, with distinct properties, they may as well produce different biological results [22]. Numerous studies have been published on hemozoin effect in immune responses and on adjuvant effects during vaccination. Characterization of hemozoin from different sources and the assessment of their differences become extremely important in order to be able to clarify the role of hemozoin in the modulation of immune responses.

1.3. Antimalarial drugs

Malaria therapy was for a long period of time limited to quinine, an alkaloid derived from the bark of the Cinchona tree, already used to treat the disease in the 17th century [2]. Nonetheless, it has serious adverse effects at therapeutic concentrations, which are referred to as cinchonism and include not only headache, nausea, vomiting and diarrhea, but also hearing and vision problems, hypoglycemia, hypotension and venous thrombosis as well as arrhythmia [31] [32] [33]. Last century, synthetic molecules similar to quinine were developed, such as chloroquine, amodiaquine, primaquine, halofantrine and mefloquine [34]. These were safer and easier to produce, and were thus preferentially used for malaria therapy, with chloroquine being the most used [31]. Their mechanism of action is thought to be the inhibition of malarial pigment (hemozoin) formation [35].

However, the parasite evolved to develop resistance to the commonly used drugs through a variety of mechanisms which emerged by the selective drug pressure, now thought to be originating in mutations or copy number changes of genes encoding parasite proteins that are either drug transporters or the drug target [36]. When a drug is administered and absorbed in the recommended dose and is accessible to the parasite, but still it is able to survive and multiply, that strain is considered resistant [34]. Quinine-resistant strains occur very sporadically since the first case in South America in the beginning of the 20th century, but chloroquine-resistant *P. falciparum* is now widespread all over endemic areas after being first reported to occur in late 1950's in Southeast Asia and South America, and mefloquine resistance is frequent in Southeast Asia, where it appeared in late 1980's [37].

Meanwhile other drugs have been discovered that present antimalarial activity (Table 1).

Table 1: Major antimalarial drugs.

Class	Drug(s)	Toxicity and side effects [33]	Mechanism of action
Quinolines — Arylamino-alcohols	Quinine	High toxicity, low therapeutic index, serious side effects (cinchonism)	Inhibition of hemozoin formation, by π-π interactions of drug with free heme [35]
	Mefloquine	Cardiac depression, antifibrillary activity, gastrointestinal or central nervous system adverse effects (seizures, psychosis)	
	Halofantrine	Cardiotoxicity; not safe in pregnancy	
Quinolines — Amino-quinolones	Chloroquine	Generally well tolerated	
	Amodiaquine	Hepatitis and organ damage	
	Primaquine	Hemolytic anemia or fatal hemolysis in glucose-6-phosphate dehydrogenase (G6PD)-deficient people and methemoglobinemia; not safe in pregnancy	
Antifolates	Pyrimethamine Sulfadoxine Proguanil Dapsone	Generally well tolerated, may cause cutaneous adverse effects	Inhibition of dihydrofolate reductase and dihydropteroate synthase from the folic acid synthesis pathway [38] [39] [40]
Artemisinins (sesquiterpene lactones)	Artemisinin Dihydro-artemisinin Artesunate Arthemether Arteether	Very well tolerated, but not recommended by WHO for pregnant women in the first trimester	Inhibition of hemozoin formation [41]; inhibition of parasite's sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [42] [43]; or generation of free radicals and protein alkylation [44]
Antibiotics (in combination with quinine to improve cure rates)	Tetracycline/ Doxycycline	Impairment of hard tissues development (not safe in young children and pregnant women), hepatotoxicity	Inhibition of protein synthesis by binding to microbial ribosomes
	Clindamycin	Photosensitivity, gastrointestinal upset, diarrhea, pseudomembranous colitis	
Hydroxy-napthoquinone	Atovaquone	Not safe in young children and pregnant women	Inhibition of the electron transport at the cytochrome *bc1* complex due to structural analogy with coenzyme Q [45]

Based on data from publications on the toxicity, side effects [33] and mechanisms of action of the major antimalarial drugs [35] [38] [39] [40] [41] [42] [43] [44] [45].

Antifolates are synthetically developed antimalarials and include pyrimethamine, sulfadoxine, proguanil, dapsone and others [38] [39] [40]. Proguanil is commercialized as Malarone™ in a fixed-dose combination with atovaquone [34]. Nevertheless, resistant strains to antifolates spread even faster, with a prompt emergence of resistance to sulfadoxine-pyrimethamine in Southeast Asia and Africa in the 1960's and the 1980's.

The most efficient antimalarials are presently artemisinin and its derivatives, which are obtained from *Artemisia annua* herb. These are recommended by the WHO to treat malaria in combination with other antimalarial drugs, in an attempt to prevent the spread of resistant parasite strains [46]. Artemisinins mode of action is still a subject of much debate, as presented in Table 1, but some authors think it may also have a role in inhibiting hemozoin formation [41]. Importantly, combination therapy is expensive and artemisinin availability may be a limitation. Moreover, its potential toxicity in pregnant women makes it no option to this risk-group, and notwithstanding all the efforts to avoid it, there are reports of artemisinin resistance already in Cambodia [47].

So, despite the severity of its adverse effects, quinine is still the only choice when considering the prevalence of resistant-strains to alternative drug formulations in some areas or their toxicity in pregnant women in their first trimester. However, antibiotics are used in combination with quinine to improve cure rates, but these are toxic for children. [31]

Hence, widespread and multidrug resistance restricts the possibilities of an efficient therapy and holds off effective malaria control and elimination, making the need for new drugs even more urgent [48]. When aiming to discover new antimalarial drugs, hemozoin formation is still one of the most interesting targets, since it seems to be an immutable pathway [30]. Considering this, together with the fact that synthetic quinolines are easy to produce, very well tolerated drugs, the approach of synthesizing quinoline analogs is a very attractive one.

1.4. Development of novel antimalarial compounds

1.4.1. Drug discovery

Hemozoin formation remains a good antimalarial drug target for novel drug discovery. Inhibition of hemozoin formation hampers detoxification of free toxic heme originated by hemoglobin proteolysis in the parasite. Hemozoin formation does not seem to rely on the activity of a parasite-encoded molecule or enzyme and is an exclusive pathway of the parasite. Therefore, drugs targeting hemozoin formation are unlikely to create resistance and are highly selective. [30]

Antimalarial drug discovery is an urgent need, in face of the increasing drug resistance worldwide and the fact that malaria remains one of the more devastating infectious diseases, despite all the efforts to attain the opposite. Diverse strategies can be adopted to develop new compounds or use existing ones in a different formulation or with a novel purpose, in a

way that improves current therapy options. While improving existing antimalarial agents is possible by exploitation of analogs through chemical modifications, evaluating the activity of compounds from natural plants and extracts takes advantage of the knowledge of folk remedies, often used by natives in endemic areas for a long time as a solution to febrile illnesses. [49] And whilst new targets are being explored as to be the aim of new compound activity, perhaps an easier approach may be the identification of agents that are already used against other diseases, but prove to be of use in malaria treatment. To this end, high-throughput assays are performed that facilitate the task of screening previously developed compounds. [50]

Several alternatives exist as tools to screen for new antimalarial drugs, apart from their applicability to identify parasite resistance. *In vitro* sensitivity assays with monitoring of *Plasmodium* cultures in the presence of the compound to be tested enables the evaluation of its capacity to inhibit parasite growth. Parasite growth may be measured based on schizont maturation or parasitemia, using microscopy, automatic reading with flow cytometry and DNA stains, or incorporation of radiolabeled hypoxanthine or ethanolamine. Also, enzyme-linked immunosorbent assays exist that correlate accumulation of specific parasite proteins, such as lactate dehydrogenase and histidine-rich protein 2 (HRP-2), with parasite viability. [51]

1.4.2. <u>Heme inhibition assays for drug screening</u>

Heme inhibition assays allow the investigation of antimalarial drug candidates that target hemozoin formation. Several approaches may be taken to explore drugs which interact with heme molecules. Some focus on the interaction between candidate compounds and heme molecules, and others adapt synthetic β-hematin formation in assay formats to test the compounds' ability to inhibit crystallization.

Multiwell plate assays provide information regarding the ability of compounds to bind to heme molecules, by analyzing inhibition of glutathione-dependent hemin degradation by those compounds [52] [53] [54]. Other types of analysis search for potential inhibitors of hemozoin formation by investigating drug-heme interactions by mass spectrometry [55] or UV-visible spectroscopy [56] [57].

Synthetic β-hematin formation can also be used as a process which mimics hemozoin biocrystallization, to perform *in vitro* assays where candidate compounds can be assessed for their ability to inhibit hemozoin growth [58]. Different β-hematin assays are performed according to different experiment formats, where reagents and growth conditions differ greatly [21]. The first assays to be described made use of *P. falciparum* derived hemozoin as an initiator for β-hematin formation and ^{14}C-hemin as a way of quantifying the end product [59]. Meanwhile, the heme polymerization inhibitory activity (HPIA) spectrophotometric microassay was developed, which identifies ligands that bind axially with the protoporphyrin

iron [60]. Promoting coordination of pyridine to unreacted hematin, Ncokazi and Egan attained a colorimetric assay which enabled visual identification of inhibiting compounds by formation of an orange-pink color, and named it pyridine hemichrome inhibition of β-hematin (Phiβ) assay [61]. Parapini and colleagues further improved the HPIA assay into the β-hematin inhibitory activity (BHIA) assay, using the more stable hemin chloride instead of hematin and adjusting pH to 5, and concluded that these conditions allow for the identification of ligands undergoing π-π interactions with heme [62]. Several groups have made use of some kind of initiator or catalyst molecules to improve β-hematin inhibition assays, including detergents such as Tween20 [63] or NP-40 and neutral lipids [64], or lecithin [65].

Standardization of heme inhibition assays is difficult and results are not consistent between assays [59] [60]. The assays often involve complex procedures, make use of various conditions, reagents as well as different types of initiators [63] [61] [60] [62] [66] [64] [59], and some use rather toxic reagents [61] [64] [59]. Moreover, the end product of these heme inhibition assays is not always characterized, with little information about the assessment of the nature and quality of the final aggregates [67] [68] [69] [70].

1.5. Novel hemozoin-like crystal inhibition assay for drug screening

Ileal Fluid Dependent Organism (IFDO) was first described by Burdon in 1989, in consequence of the isolation of a "replicating agent" from the intestinal tract of patients with Crohn's disease. After being "cultured" in specific broth and agar media, appearance of dark-brown "bacterial-like growth" was evident, but the hypothesis of IFDO being formed by crystallization from constituents of the medium instead of a biological process was already posed. [71] As IFDO is susceptible to inactivation treatments similar to prions, the company Steris became interested in exploring this agent as a model to test drugs and processes against prion propagation. Interestingly, detailed characterization of IFDO revealed it to be very similar to malarial pigment, hemozoin, as it is an aggregate of ferriprotoporphyrin IX (heme). At that point, Steris contacted the host laboratory and a collaboration project to study this crystal was initiated. IFDO particles were grown in a well defined medium and a comparative analysis was performed along with hemozoin obtained by different methods. A more extensive characterization of the crystal making use of X-ray diffraction and infrared spectroscopy led to the finding that, despite the similarity, IFDO is not true hemozoin, and so the crystals were termed hemozoin-like crystals (HLC). However, following Steris investigation on an assay to evaluate the therapeutic and decontamination efficiency against transmissible misfolded proteins, HLC were explored for the potential to be grown as a novel and simple inhibition assay for antimalarial drug screening.

2. Objectives

The aim of this work was to produce malarial pigment (hemozoin) from different origins, characterize it and establish its purity by several methods. The ability of antimalarial and antibiotic drugs to inhibit the crystallization of hemozoin-like crystals was investigated, together with the potential of these crystals to be used for screening for drugs inhibiting hemozoin formation.

3. Material and Methods

All reagents were purchased from Sigma-Aldrich® (Sintra, Portugal) unless stated otherwise.

3.1. Culture media and solutions

3.1.1. <u>Malaria Complete parasite Medium (MCM)</u>: 500 mL of RPMI 1640 (1×, without L-glutamine, with NaHCO$_3$) (Gibco™, Life Technologies, Madrid, Spain) supplemented with 50 mL of 10× AlbuMAX II® solution, 12 mL of HEPES Buffer Solution (1 M) (Gibco™, Life Technologies, Madrid, Spain), 5 mL of L-glutamine 200 mM and 500 μL of gentamicin 50 mg/mL (Gibco™, Life Technologies, Madrid, Spain).

3.1.2. <u>10× AlbuMAX II® solution</u>: 25 g of AlbuMAX II® in a 500 mL aqueous solution of 5,2 g of RPMI 1640 (with L-glutamine, without NaHCO$_3$) supplemented with 500 μL of gentamicin 50 mg/mL, 2,98g of HEPES, 1,67 g of sodium bicarbonate, 1 g of glucose and 0,1 g of hypoxanthine, adjusting pH to 7,2-7,4 and filtering 0,22 μm. All reagents from Life Technologies (Madrid, Spain) except HEPES which is from VWR (Carnaxide, Portugal).

3.1.3. <u>1× Phosphate-buffered saline (PBS)</u>: diluted 1:10 from 10× PBS pH 7,2 (Gibco™, Life Technologies, Madrid, Spain) in ultrapure water, obtained with Milli-Q Synthesis Q-Gard®1 water purification system (Millipore, Billerica MA, USA).

3.1.4. <u>Glycerolyte cryoprotective solution</u>: constituted by 57% of glycerol, 16 g/L of sodium lactate (VWR, Carnaxide, Portugal), 300 mg/L of potassium chloride and 25 mM of sodium phosphate pH 6,8.

3.1.5. <u>Broth medium for HLC culture</u>: prepared by autoclaving 35,5 g of Mycoplasma Broth Base (Oxoid, Basingstoke, England) in 950 mL of distilled water, obtained with Elix®10UV Progard®2 water purification system (Millipore, Billerica MA, USA) and 2 mL of Tween® 80; cooling at 50°C and adding 1,33 mL of horse serum (Gibco™, Life Technologies, Madrid, Spain), 20 mL of a 10% pancreatin solution in 1× PBS and 30 mL of a fresh blood extract. Medium is filtered at 0,22 μm and immediately used.

3.1.6. <u>Fresh blood extract</u>: prepared by washing red blood cells from healthy human donors, obtained from a "buffy coat" from Instituto Português do Sangue (IPS), three times with 1 vol. of 1× PBS; lysing RBC by addition of 1 vol. of sterile ultrapure water; submitting the

suspension to three successive freeze-thaw cycles and 5 min. of sonication using the 35 kHz ultrasonic bath Transsonic T570 (Elma®, Singen, Germany); centrifuging for 15 min. at 2400×g and discarding the pellet.

3.1.7. <u>Wash buffer</u>: constituted by 10 mM Tris hydrochloride (Merck, Lisbon, Portugal) pH 8.3, 50 mM potassium chloride and 1,5 mM magnesium chloride.

3.1.8. <u>Solution A</u>: constituted by 10 mM Tris hydrochloride pH 8,3, 100 mM potassium chloride and 2,5 mM magnesium chloride.

3.1.9. <u>Solution B</u>: constituted by 10 mM Tris hydrochloride pH 8,2, 2,5 mM magnesium chloride, 1% Tween® 20, 1% Triton® X-100 and 120 µg/mL proteinase K (Promega, Madison WI, USA).

3.2. *Plasmodium falciparum* cultures

3.2.1. <u>Continuous *Plasmodium falciparum* cultures maintenance</u>

Plasmodium falciparum 3D7 strain was obtained from Malaria Research and Reference Reagent Resource Center (MR4; Manassas VA, USA) and continuous cultures of the parasite were maintained with human red blood cells (RBC), isolated from buffy coats. Healthy human blood from the buffy coat was washed three times with RPMI 1640 (1×, without L-glutamine, with NaHCO$_3$) (Gibco™, Life Technologies, Madrid, Spain) and centrifuged 600×g for 5 minutes without brake to separate and isolate RBC from the leukocytes. Infected RBC were incubated at 37°C and 5% CO$_2$, in MCM, which was changed every day.

Cultures were kept at <1% parasitemia and 5% hematocrit, unless when used to purify or extract hemozoin, for which it was necessary to have >10% parasitemia and 1% hematocrit, and 2-5% parasitemia and 5% hematocrit, respectively.

3.2.2. <u>Giemsa staining of blood smears</u>

To estimate the amount of infected RBC in culture, a smear was prepared daily with a small amount of blood from the culture, when the medium was changed. The smear was fixed in absolute methanol (Merck, Lisbon, Portugal) for 20 s, air-dried and stained with Giemsa's azur eosin methylene blue solution (Merck, Lisbon, Portugal) diluted 1:100 in 1× PBS, for 20 minutes. Then, slides were rinsed with tap water and air-dried to be observed by bright field microscopy under oil immersion.

Parasitemia is determined as the average percentage of infected RBC on ten different visual fields when observing at 1000× magnification. When needed, the infected RBC were diluted by substituting the right volume with uninfected RBC to lower parasitemia and maintain the hematocrit.

3.2.3. Frozen *P. falciparum* 3D7 strain stocks

Frozen stocks of schizont-enriched *P. falciparum* 3D7 infected RBC from highly parasitized cultures can be maintained in vials at -80°C or in liquid nitrogen for long-term storage if transferred to a cryoprotective solution of glycerolyte. After obtaining the pellet of infected RBC from the culture by centrifuging for 10 min. at 600×*g*, 0,33 and then 1,33 volumes of glycerolyte were added drop by drop, gently mixing.

When needed, glycerolyte-frozen parasites were thawed to begin a new culture by gently washing the thawed infected RBC with 0,1 vol. of 12% and 10 vol. of 1,6% sodium chloride, centrifuging at 600×*g* and 20°C for 5 min., resuspending in 1× RPMI 1640 (without L-glutamine, with NaHCO$_3$) (Gibco™, Life Technologies, Madrid, Spain), centrifuging again and finally resuspending in MCM. Uninfected RBC were added to obtain 5% hematocrit in the culture when it was necessary.

3.3. Purification of *Plasmodium falciparum* hemozoin

P. falciparum hemozoin (or native hemozoin, nHZ) was purified after saponin harvesting of parasites from 1 L of *P. falciparum* (3D7 strain) cultures at 1% hematocrit, enriched in trophozoites at a parasitemia of at least 10%, as previously described by Coban *et al*. (2002) [72]. In short, parasites were extensively washed with 1× PBS, pellet was sonicated for 5 min., extensively washed with 2% sodium dodecyl sulfate (SDS) and then incubated overnight with 2 mg/mL proteinase K (Promega, Madison WI, USA). After being washed with 2% SDS again, the pellet was incubated for 3 h in 6 M urea and then washed with 2% SDS and ultrapure water. Purified nHZ was resuspended in 1 mL of ultrapure water, quantified as heme-equivalents using QuantiChrom™ Heme Assay Kit DIHM-250 (BioAssay Systems, Hayward CA, USA), and stored at 4°C.

3.4. Extraction of crude *Plasmodium falciparum* hemozoin

P. falciparum hemozoin was extracted without further purification (cnHZ), as described by Keller *et al*. (2004) [73], with some modifications. Briefly, infected RBC from 2 to 5% parasitemia *P. falciparum* (3D7 strain) cultures at 5% hematocrit were centrifuged at 800×*g* for 10 min. and the pellet was resuspended in 40 mL of 1× PBS with 1% of saponin for 10 minutes. After centrifuging at 16000×*g* for 15 min., the pellet was washed seven times in 1× PBS, resuspended in 1 mL of ultrapure water, quantified as previously described and stored at 4°C.

3.5. Production of synthetic hemozoin

Synthetic hemozoin (sHZ) was obtained by the method described by Slater *et al.* (1991) [25], with some modifications. Briefly, 475 mg of hemin chloride were dissolved in 100 mL of 0,1 N

sodium hydroxide and heme was precipitated by slowly adding 35 mL of glacial acetic acid (Merck, Lisbon, Portugal). Crystallization was promoted by overnight incubation of the mixture at 80°C. Non-crystalline heme was then removed by washing three times with 1 vol. of 100 mM sodium bicarbonate pH 9,1 during 3 h, centrifuging for 15 min. at top speed. The pellet was further washed three more times in 1 mL of ultrapure water and finally ressuspended, quantified as for nHZ, and stored at 4°C.

3.6. Production of hemozoin-like crystals

Original "IFDO" cultures were kindly provided by Dr D. Burdon to Steris and later on termed hemozoin-like crystals (HLC). Culture methods were adapted from the original protocol [71], in which a seeded medium is used to grow the particles (here designated Broth medium for HLC culture). Cultures were prepared by seeding 50 µL of a McFarland 6 HLC suspension in 10 mL of Broth medium for HLC culture, and incubating at 37°C and 5% CO_2 for 5 to 7 days, in the CO_2 incubator Heraeus® HERA® Cell (Fisher Scientific, Loures, Portugal).

A modified broth medium was also developed, replacing the fresh blood extract by 50 µM hemin chloride, added from a stock solution prepared in 0,4 N sodium hydroxide, and adjusting pH of the medium at 7,2 before filtering at 0,22 µm. An Agar medium for HLC culture was also prepared, with Mycoplasma Agar Base (Oxoid, Basingstoke, England). Besides these, other growth conditions were tested, including: adjusting pH of the medium to 5; incubation at ambient atmosphere instead of 5% CO_2; using Brain Heart Infusion (Becton Dickinson, Madrid, Spain) broth or 1× PBS instead of Mycoplasma Broth Base; replacing horse serum by human serum (obtained from a healthy donor); excluding pancreatin from the medium; and seeding with sHZ instead of HLC.

Obtained HLC were washed three times with 1 vol. of 100 mM sodium bicarbonate pH 9,1 during 3 h, centrifuging for 15 min. at top speed, followed by three washes in ultrapure water. Then, HLC were finally resuspended in 1 mL of ultrapure water, quantified and stored at 4°C as performed for hemozoin. Alternatively, absolute ethanol (Merck, Lisbon, Portugal), absolute methanol (Merck, Lisbon, Portugal) and dimethyl sulfoxide (DMSO) were also used to wash HLC, in an attempt to reduce remaining heme contamination.

3.7. Light and depolarizing microscopy

Giemsa-stained smears of infected RBC from *P. falciparum* cultures (prepared as described above), and hemozoin and HLC preparations were observed with light and depolarizing microscopy, using the brightfield microscope Leica DM2500 (Leica Microsystems, Wetzlar. Germany).

3.8. Scanning electron microscopy

Ten microliters of hemozoin and HLC samples were allowed to dry overnight in air on top of a carbon tape on a metallic sample holder and were metalized for 30 min. using JEOL, JFC-1200 with a gold target. Scanning electron microscopy was performed with JEOL, JSM-2500 LV scanning electron microscope (JEOL, Tokyo, Japan), at Faculdade de Ciências da Universidade de Lisboa.

3.9. X-ray diffraction

Hemozoin and HLC were subjected to X-ray diffraction (XRD) analysis. Crystals were resuspended in 10 µL of absolute ethanol (Merck, Lisbon, Portugal) and allowed to air-dry on the silicon sample holders. XRD patterns were then acquired at Faculdade de Ciências da Universidade de Lisboa, with the automatic X-ray diffractometer Philips Analytical PW 3050/60 X' Pert PRO (Ɵ /2Ɵ) (PANalytical, Almelo, The Netherlands), with an X'Celerator detector and automatic data acquisition with X'Pert Data Collector, version 2.0b. Cu Kα radiation (λ= 1,54060 Å) was used, operating with 30 mA and 40 kV. The diffractograms were recorded in the 2Ɵ range between 5° and 30° with a step size of 0.0170° (2Ɵ) and scan step time of 100 seconds.

3.10. Infrared spectroscopy

Synthetic hemozoin and HLC were dried overnight in a desiccator over phosphorus pentoxide and silica gel. Potassium bromide pellets of sHZ and HLC were prepared from the dried samples with a mortar and pestle and a compressor. Infrared spectra were obtained for each sample at Faculdade de Farmácia da Universidade de Lisboa, using IRAffinity-1 Fourier Transform Infrared Spectrophotometer and IR Solution Software (Shimadzu, Kyoto, Japan).

3.11. Heme contamination assessment

Hemozoin and HLC produced were assessed for remaining heme contamination using thin layer chromatography (TLC). A quantified sample was first diluted in absolute methanol (Merck, Lisbon, Portugal) to the higher concentration of hemin chloride to be eluted (0,2 mM). Then, 10 µL of the sample were eluted for 30 to 40 min. on a silica gel glass plate (Merck, Lisbon, Portugal) along with hemin solutions of known concentrations (0,2, 0,04 and 0,02 mM), inside a methanol-saturated tank. Images of the plates were acquired with Alpha Imager® HP System (ProteinSimple, Santa Clara CA, USA) and the result was analyzed by determination of integrated density using ImageJ software (National Institute of Mental Health, Bethesda MD, USA) and calculation of remaining heme contamination percentage in the sample.

3.12. *Mycoplasma* contamination assessment

To assess for *Mycoplasma* contamination, Polymerase Chain Reaction (PCR) was performed as follows. One hundred microliters of sHZ, nHZ, cnHZ and HLC were diluted in 1 mL of 1× RPMI 1640 (Gibco™, Life Technologies, Madrid, Spain) and incubated in a 24-wells plate for 24h at 37°C and 5% CO_2. After incubation, samples were collected into a microtube, centrifuged for 15 min. at 15700×g and 4°C, supernatant was discarded and pellet resuspended in Wash buffer. After centrifuging again, pellet was resuspended in 1:1 Solution A and Solution B, incubating at 60°C for 1 h followed by denaturing at 90°C for 10 minutes. PCR was performed using My Cycler™ thermal cycler (Bio-Rad, Amadora, Portugal), by subjecting the Reaction mix to the following program: 1 cycle at 95°C for 5 min. (step 1), 30 cycles for step 2 – 95°C for 0,5 min., 58°C for 1,5 min., 72°C for 1,5 min. – and 72°C for 10 min. (step 3). Reaction products were analyzed together with 1kb DNA Extension Ladder (Life Technologies™, Madrid, Spain) by electrophoresis at 60 V, for 1 h in 1× TAE Buffer, on a 1% agarose (NZYTech, Lisboa, Portugal) gel with 10000× GelRed® Nucleic acid gel stain (Biotium, Hayward CA, USA) diluted 1:20000. Positive results show a DNA amplicon of 717 bp.

Reaction mix: prepared to a volume of 25 µL in nuclease-free distilled water (Gibco™, Life Technologies, Madrid, Spain), with 1× NZYTaq Colourless Master Mix (NZYTech, Lisboa, Portugal), 0,1-1 µM upstream (Pr_{27} – 5' TGC ACC ATC TGT CAC TCT GTT AAC CTC 3') and downstream (Pr_{22} – 5' ACT CCT ACG GGA GGC AGC AGT A 3') primers (Fisher Scientific, Loures, Portugal), and <250 ng DNA template.

3.13. DNA contamination assessment

Hemozoin and HLC were assessed for DNA contamination by agarose gel electrophoresis. Ten microliters of each sample, pretreated or not with 10 µL of 1% saponin for 10 min., were loaded with 3,5 µL of Gel Loading Buffer on a 0,8% agarose gel with 10000× GelRed® Nucleic acid gel stain diluted 1:20000, and ran in 1× TAE Buffer for 30 min. at 100 mA.

3.14. Protein contamination assessment

Hemozoin and HLC were assessed for protein contamination by polyacrylamide gel electrophoresis under denaturing conditions. Twenty microliters of each sample were boiled for 5 min. at 100°C with 20 µL of 2× Loading buffer and then loaded onto a SDS-polyacrylamide gel (5% Acrylamide resolving gel/4% Acrylamide stacking gel) and ran in 1× Running buffer, for 40 min. at 180 V. The gel was stained in 1% Coomassie Brilliant Blue staining solution (Bio-Rad, Amadora, Portugal) for 20 min., destained overnight in Destain solution (constituted by 30% ethanol and 10% acetic acid in distilled water). After being

rinsed with abundant water, an image of the gel was acquired with Alpha Imager® HP System. Gels were dried on a filter paper with the DrygelSr Slab Gel Dryer SE1160 (Hoefer® Scientific Instruments, Holliston MA, USA), for at least 40 min. at 70°C.

<u>2× Loading buffer</u>: 10% glycerol, 5% β-mercaptoethanol, 3 % SDS, 62,5 mM Tris (Merck, Lisbon, Portugal) pH 8,8, and 0,01% bromophenol blue (Bio-Rad, Amadora, Portugal) in ultrapure water.

<u>5% Acrylamide resolving gel</u>: 5% acrylamide/0,1% bisacrylamide (Bio-Rad, Amadora, Portugal), 0,375 M Tris (Merck, Lisbon, Portugal) pH 8,8, 0,1% SDS, 0,05% ammonium persulfate and 0,1% TEMED in ultrapure water.

<u>4% Acrylamide stacking gel</u>: 4% acrylamide/0,1% bisacrylamide (Bio-Rad, Amadora, Portugal), 0,375 M Tris (Merck, Lisbon, Portugal) pH 8,8, 0,1% SDS, 0,05% ammonium persulfate and 0,1% TEMED in ultrapure water.

<u>5× Running buffer</u>: 15,15 g/L Tris, 72,05 g/L glycine (Merck, Lisbon, Portugal) and 0,5% SDS.

3.15. Hemozoin and Hemozoin-like crystals quantitation (heme equivalents)

The amount of hemozoin and HLC in the water suspensions was quantified as heme-equivalents using QuantiChrom™ Heme Assay Kit DIHM-250 (BioAssay Systems, Hayward CA, USA), by colorimetric determination of total heme at 400 nm. Shortly, after solubilization in an aqueous solution of 20 mM sodium hydroxide for 1 h, samples were added to the Reagent solution and let react for 5 min., after which absorbance at 400 nm was read and the concentration in µM (heme equivalents) determined using the following expression:

Total heme concentration $= \frac{A_{sample} - A_{blank}}{A_{calibrator} - A_{blank}} \times 62,5 \times$ Dilution factor,

which correlates absorbance at 400 nm with the concentration of heme present in solution.

3.16. Novel Hemozoin-like crystal inhibition assay

A McFarland 6 HLC suspension in water was sonicated for 5 min., using the 35 kHz ultrasonic bath Transsonic T570 (Elma®, Singen, Germany), and diluted 1:100 in Broth medium for HLC culture. One hundred and eighty microliters were distributed in wells of a 96-wells plate. A range of drug concentrations was tested in order to determine a minimum inhibitory concentration for HLC growth. Stock solutions of chloroquine diphosphate, amodiaquine dihydrochloride, quinine hydrochloride dihydrate, quinacrine dihydrochloride, clindamycin hydrochloride, gentamicin and ampicillin were prepared in sterile ultrapure water, and those of mefloquine hydrochloride (Roche, Amadora, Portugal), halofantrine hydrochloride and artemisinin were prepared in absolute methanol (Merck, Lisbon, Portugal). After being adjusted to pH 7 ± 0,2 and 0,22 µm-filtered, drug solutions diluted in Broth

medium for HLC culture and then diluted in the seeded wells to a final concentration of 50, 100, 250, 500, 750 and 1000 µM. Each drug was tested at least in duplicate, and plates were visually observed every day, for 5 days of incubation at 37°C and 5% CO_2, to determine presence or absence of HLC growth – wells with less growth than unseeded control were considered as presenting absence of growth. Five microliters of all crystal-positive wells were seeded onto Tryptone Soya Agar (Oxoid, Basingstoke, England) and Mycoplasma Agar Base (Oxoid, Basingstoke, England) plates to confirm absence of bacteria. Five microliters from each well were also seeded onto fresh plates of Agar medium for HLC culture, to observe any HLC regrowth.

4. Results

4.1. Characterization of hemozoin of different origins and hemozoin-like crystals

Hemozoin crystals are a dark-brown, black powder which when suspended in water give rise to a blackish suspension. Figure 3 shows the end-product of hemozoin synthesis and purification or extraction from *P. falciparum* (3D7 strain) cultures, as well as the product of hemozoin-like crystals (HLC) culture. Water suspensions of hemozoin obtained from the different origins and HLC were kept in microtubes at 4°C, after being quantified as for heme equivalents concentration. Visually, all these crystals are very similar, and when air-dried yield a black powder which presents no differences when observed with the naked eye.
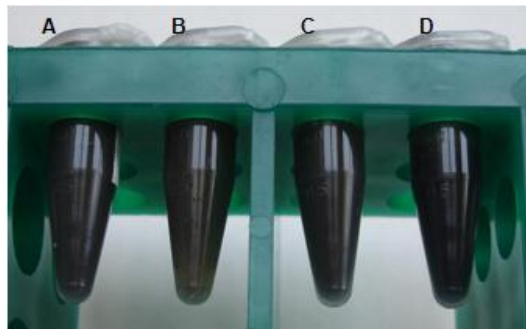
Figure 3: Water suspensions of hemozoin and hemozoin-like crystals (HLC). Synthetic hemozoin (sHZ, A), native hemozoin (nHZ, B), crude native hemozoin (cnHZ, C) and HLC (D) suspensions in water were kept in microtubes at 4°C after quantification in terms of heme equivalents concentration.

During HLC incubation in broth medium at 37°C and 5% CO_2, a dark-brown deposit appeared, as visible in Figure 4B. A small deposit also formed in the unseeded broth medium, but in a residual amount (Figure 4A), and not always being observed. Alternatively, an agar medium was used, in which seeded HLC originated dark-brown structures similar to bacterial or fungal "colonies", which occurred beneath and on the surface of the agar, and were more numerous near the site of inoculation (Figure 4C and 4D).
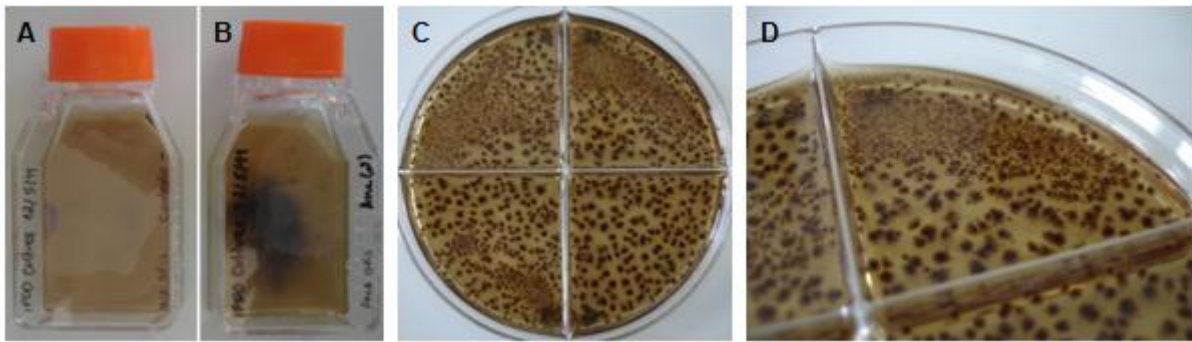
Figure 4: Hemozoin-like crystals (HLC) cultures in broth and agar media. HLC unseeded (A) and seeded (B) broth in culture flasks and HLC seeded agar medium plates (C, D) were incubated for 5 days at 37°C and 5% CO_2.

4.1.1. Light and depolarizing microscopy

Hemozoin from the different sources and HLC have a crystalline appearance and depolarize light as observed with light and depolarizing microscopy at 1000× magnification, with the optical microscope Leica DM2500 (Figure 5). All preparations showed a more or less homogeneous crystal in size, but tend to aggregate in suspension, as shown for native hemozoin (nHZ) in Figure 5A and HLC in Figure 5D. Aggregates could be dispersed by incubating the samples in the ultrasonic bath (with a frequency of 35 kHz) for 5 min., as exemplified for synthetic hemozoin (sHZ) in Figure 5C, with the exception of HLC (Figure 5D). In the case of HLC, extensive sonication did not disperse crystal aggregates in suspension. The crude extract of native hemozoin (cnHZ) seems to consist of small aggregates of hemozoin surrounded by membranes (Figure 5B).
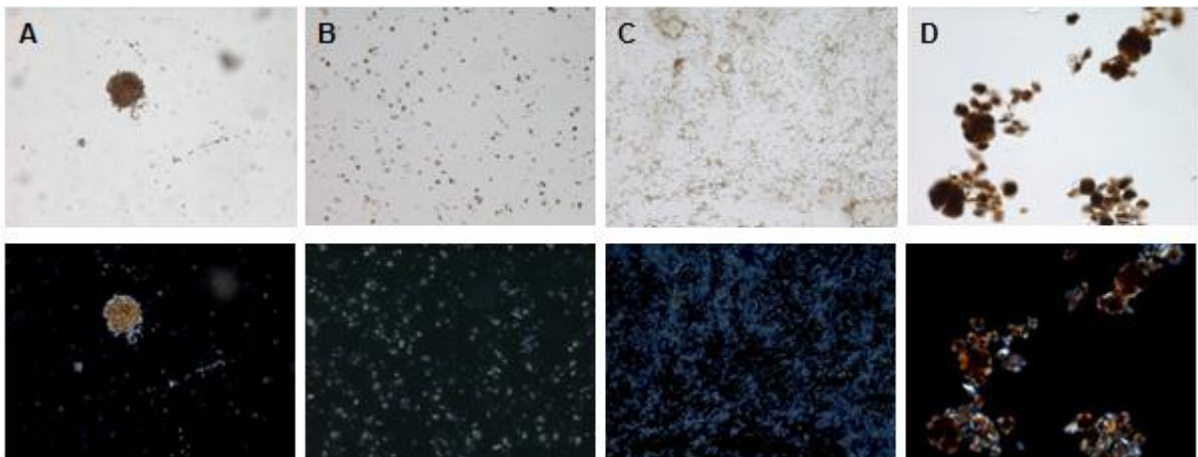
Figure 5: Microscopy observation of hemozoin and hemozoin-like crystals (HLC). Light (upper pannel) and depolarizing (lower pannel) microscopy observation of native hemozoin (nHZ, A), crude native hemozoin (cnHZ, B), synthetic hemozoin (sHZ, C) and HLC (D), at 1000× magnification, with the optical microscope Leica DM2500.

Some differences could be noticed in the way that hemozoin from different origins depolarized light, with needle-like particles appearing more bluish (Figure 5C, lower panel) and aggregates more brown-gold and green (Figure 5A, lower panel).

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4.1.2. Scanning electron microscopy

Scanning electron microscopy allowed to analyze morphology and size of hemozoin crystals. As visible in Figure 6, sHZ, nHZ and HLC have a similar size, which ranges from 1,1 to 2,4 µm, HLC having the biggest crystals. Also, morphology of the crystals is very alike, showing protruding needle-like particles, as presented in Figure 6 (B, C and D). Native hemozoin appears to be more brick-like shaped (Figure 6B). At 3500× magnification, round structures are visible in images acquired from cnHZ samples (Figure 6A).
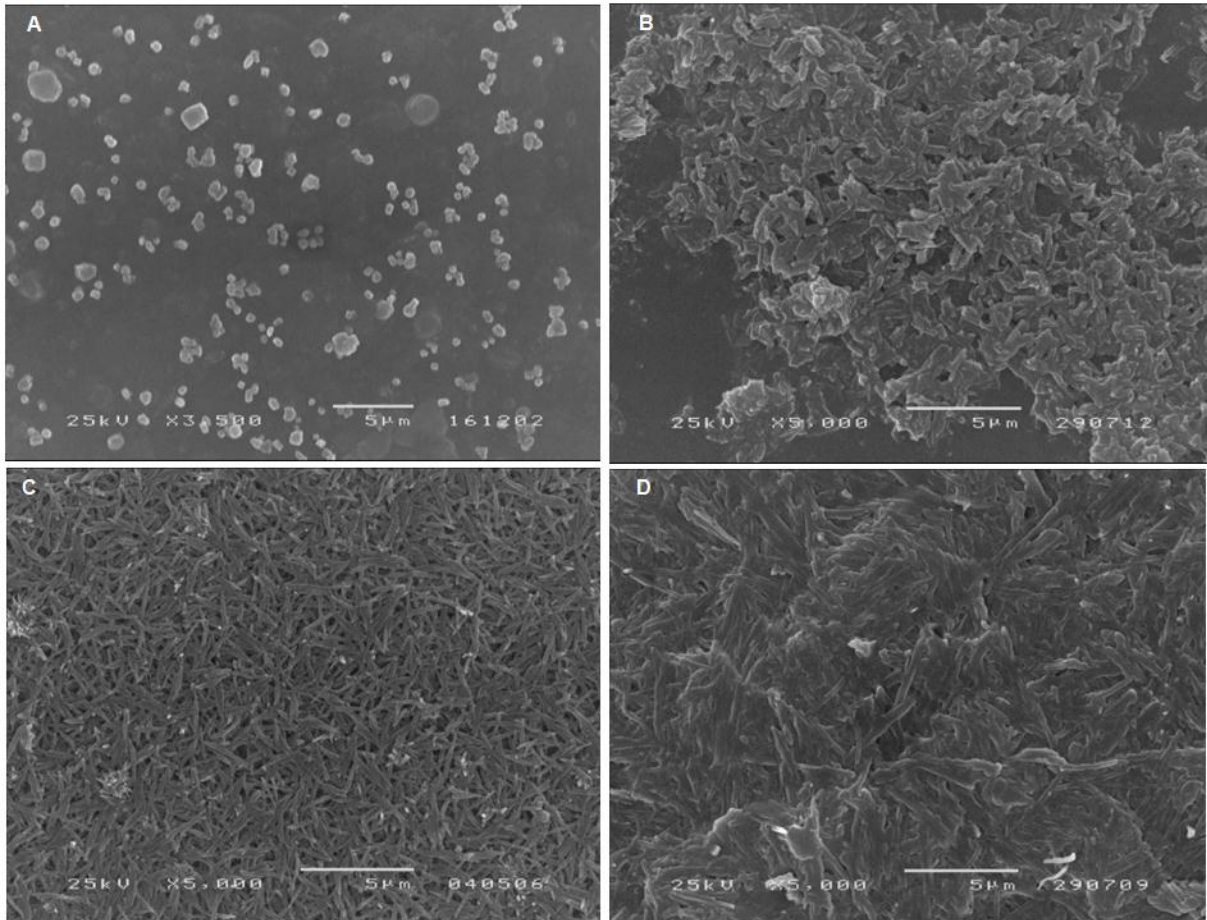
Figure 6: Scanning electron microscopy of hemozoin and hemozoin-like crystals (HLC). Crude native hemozoin (cnHZ A) (3500× magnification), and native hemozoin (nHZ B), synthetic hemozoin (sHZ C) and HLC (D) (5000× magnification) images obtained for air-dried samples with the scanning electron microscope JEOL, JSM-2500 LV.

4.1.3. X-ray diffraction

Hemozoin from synthetic (sHZ) and natural (nHZ) source was analyzed with the X-ray diffractometer Philips Analytical PW 3050/60 X' Pert PRO and revealed to have identical X-ray diffraction peak patterns (Figure 7). HLC presented a different X-ray diffraction pattern, which is indicative of a difference relative to the other crystals.
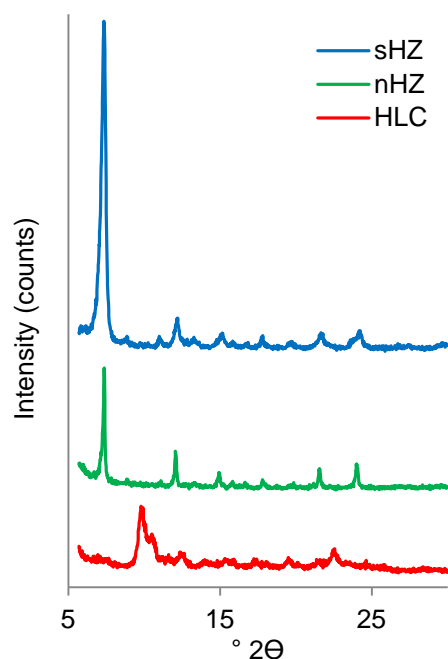
Figure 7: X-ray diffraction patterns of hemozoin and hemozoin-like crystals (HLC). Synthetic hemozoin (sHZ, blue line), native hemozoin (nHZ, green line) and HLC (red line) air-dried samples were analyzed on silicon sample holders with the X-ray diffractometer Phillips Analytical PW 3050/60 X' Per PRO using a Cu Kα radiation (λ= 1,54060 Å) source.

4.1.4. Infrared spectroscopy

When subjected to infrared spectroscopy analysis, sHZ produced a spectrum which presents the two hemozoin-characteristic peaks at 1663 and 1210 cm^{-1} (highlighted with arrows in Figure 8). HLC failed to show these peaks, thus confirming XRD data, which indicated the two crystals are in fact different.
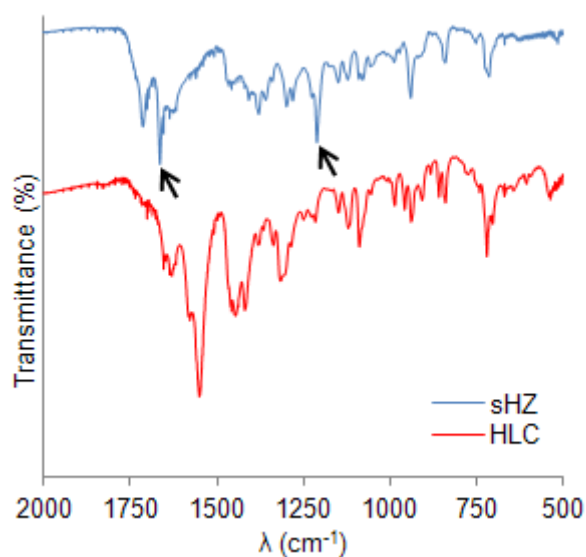
Figure 8: Infrared spectra of synthetic hemozoin (sHZ) and hemozoin-like crystals (HLC). sHZ (blue line) and HLC (red line) samples were dried over phosphorous pentoxide and silica gel in a desiccator and analyzed in potassium bromide pellets with IRAffinity-1 Fourier Transform Infrared Spectrophotometer; arrows highlight the two hemozoin-characteristic peaks at 1663 and 1210 cm^{-1}.

Obtaining native hemozoin from parasitized cultures is considerably time consuming and costly and infrared spectroscopy analysis requires a relatively high amount of sample. Thus, considering that XRD results had already shown nHZ and sHZ to be identical, nHZ was not analyzed with infrared spectroscopy.

4.2. Hemozoin and hemozoin-like crystals quantitation

4.2.1. QuantiChrom™ Heme Assay Kit

After production and purification or extraction of hemozoin from different origins and HLC, the resultant suspensions in water were quantified as heme-equivalents using QuantiChrom™ Heme Assay Kit, which correlates absorbance at 400 nm with the concentration of total heme in solution. Concentration of sHZ and HLC samples ranged between approximately 3 to 11 mM, depending on the initial total volume of reaction or culture. In spite of yielding larger pellets after extraction from the *P. falciparum* (3D7 strain) cultures, because cnHZ is not purified, it also contains membranes and proteins which contribute to pellet volume, without meaning it has a higher amount of hemozoin. Samples of cnHZ had concentrations of 0,2 mM. Purification from *P. falciparum* (3D7 strain) cultures produced water suspensions of nHZ at around 1 mM.

4.2.2. Absorbance measurements

Trying to follow HLC growth by absorbance measurement revealed to be a somewhat difficult task. When growing HLC in glass tubes, daily measuring $A_{405\ nm}$ with WPA CO8000 Biowave Cell Density Meter (Biochrom, Cambridge, United Kingdom) reflected the dark brown deposit formation in the bottom of the tubes, augmenting from first day and stabilizing after 5 to 7 days of incubation. However, when cultures were carried out in polystyrene 96-well plates, neither $A_{405\ nm}$ or $A_{595\ nm}$ measuring with the microplate reader Infinite M200 (Tecan, Männedorf, Switzerland) were reliable HLC growth indicators, as they varied greatly between duplicates and in some cases unseeded wells had higher absorbance values than seeded ones, making it impossible to subtract a "blank" value from the growing HLC wells.

4.3. Purity and contamination assessment of hemozoin and hemozoin-like crystals

4.3.1. Heme contamination

Heme contamination in hemozoin and HLC samples was assessed by thin layer chromatography (TLC) and examples of the silica plates used are depicted in Figure 9. Hemozoin from natural source was not contaminated with remaining heme (Figure 9A and 9B). The synthetic crystal obtained from hemin chloride was contaminated with a residual amount of free heme, representing less than 1% of total heme content. However, HLC has

an average 37% heme contamination, even after being extensively washed with sodium bicarbonate (pH 9,1), water and methanol.
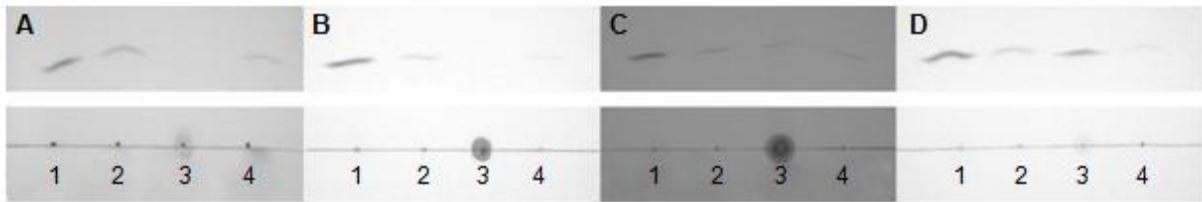
Figure 9: Silica plates used for thin layer chromatography to assess remaining heme contamination in hemozoin and hemozoin-like crystals (HLC) suspensions. 10 µL of 0,2 mM native hemozoin (nHZ, A), crude native hemozoin (cnHZ, B), synthetic hemozoin (sHZ, C) and HLC (D) samples (3) were eluted on silica gel plates along with 10 µL of 0,2 mM (1), 0,04 mM (2) and 0,02 mM (4) hemin chloride solutions in methanol.

4.3.2. *Mycoplasma* contamination

Hemozoin and hemozoin samples were subjected to polymerase chain reaction (PCR) in order to test the presence or absence of *Mycoplasma* DNA, indicative of *Mycoplasma* contamination. PCR products for each sample were analyzed by agarose gel electrophoresis, that revealed the absence of the DNA amplicon of 717 bp, size of the amplified *Mycoplasma* DNA sequence corresponding to the positive control (lane 1 in Figure 10), which indicates no *Mycoplasma* contamination.
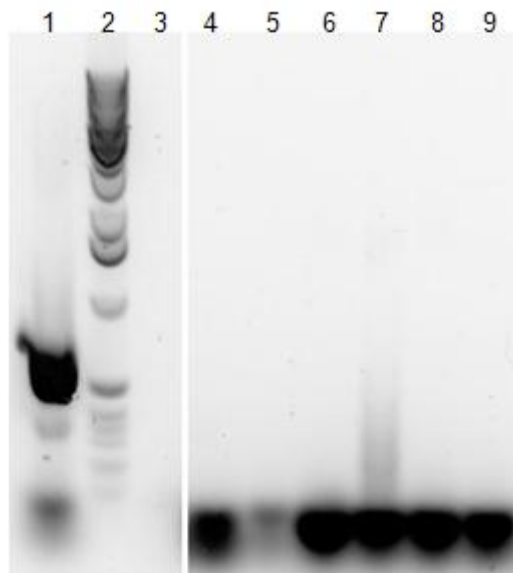
Figure 10: Agarose gel after electrophoresis of polymerase chain reaction (PCR) products of a *Mycoplasma* DNA sequence amplification to assess hemozoin and hemozoin-like crystals (HLC) samples for contamination with *Mycoplasma*. 1 – amplified *Mycoplasma* DNA sequence (positive control); 2 – 1 kb DNA Extension Ladder (Life Technologies™, Madrid, Spain); 3 – water (negative control); 4 – synthetic hemozoin (sHZ); 5 – native hemozoin (nHZ); 6 – crude native hemozoin (cnHZ); 7, 8, 9 – HLC. 1% agarose gel, 60 V, 1 h, in 1× TAE, GelRed® diluted 1:20000; 12 µL loaded sample.

4.3.3. DNA contamination

Synthetic hemozoin, HLC and purified nHZ samples have no DNA contamination, as observed by agarose gel electrophoresis, illustrated in Figure 11. As expected, the cnHZ extract is contaminated with DNA, which did not migrate on the gel beyond the site of loading, even with the previous saponin treatment, possibly due to the interaction with proteins and membranes present in the unpurified suspension.

Figure 11: Agarose gel after electrophoresis of hemozoin and hemozoin-like crystals (HLC) samples to assess DNA contamination. 1 – 1 kb Plus DNA Ladder (Life Technologies™, Madrid, Spain); 2 – synthetic hemozoin (sHZ); 3, 4* – HLC; 5, 6* – native hemozoin (nHZ); 7, 8* – crude native hemozoin (cnHZ). 0,8% agarose gel, 100 mA, 30 min., in 1× TAE, GelRed® diluted 1:20000; 20 µL loaded sample, pretreated with saponin unless where denoted with *.

4.3.4. Protein contamination

Synthetically produced hemozoin, purified nHZ and HLC are free of protein, as assessed by polyacrylamide gel electrophoresis in denaturing conditions (SDS-PAGE, Figure 12). Crude extract hemozoin (cnHZ) contains protein, as visible in lane 4 of the gel after Coomassie blue staining. Dark bands in the end of the gel in lanes 2 and 7 are due to the dark brown color of the hemozoin or HLC samples themselves, not corresponding to proteins, as these are revealed by Coomassie blue staining.
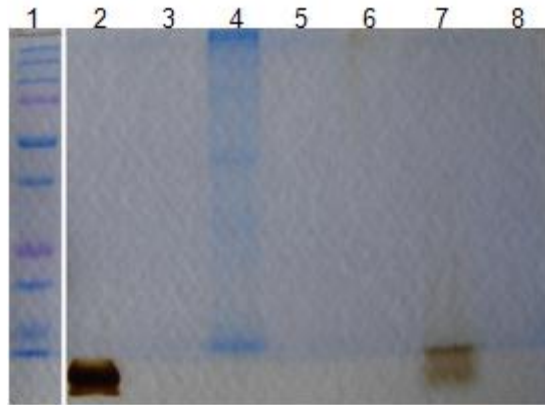
Figure 12: Protein polyacrylamide gel after electrophoresis of hemozoin and hemozoin-like crystals (HLC) samples in denaturing conditions (SDS-PAGE) to assess protein contamination. 1 – Precision Plus Protein™ Dual Color Standards; 2 – synthetic hemozoin (sHZ); 3 – native hemozoin (nHZ); 4 – crude native hemozoin (cnHZ); 5, 6, 7 – HLC; 8 – H_2O (negative control). 5% acrylamide resolving gel/4% acrylamide stacking gel, 180 V, 40 min., in 1× Running buffer, 1% Coomassie blue stained; 20 μL loaded sample, previously boiled for 5 min. at 100°C.

4.4. Novel hemozoin-like crystals inhibition assay

The novel HLC inhibition assay makes use of HLC growth in the previously described medium in a 96-well plate to test the effect of drugs on HLC crystallization. Appearance of a dark brown pellet at the bottom of the seeded wells after 3 to 5 days of incubation allows to visually observe HLC crystallization, yielding positive growth results, which is visible in Figure 13 for gentamicin and ampicillin, even at a concentration of 1 mM.
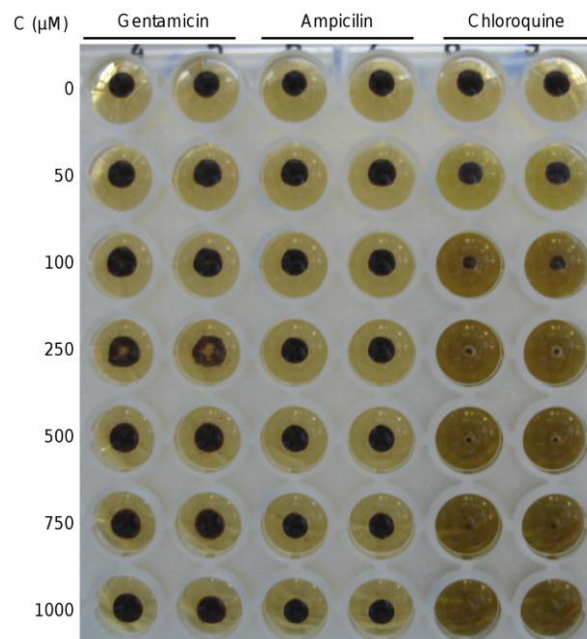
Figure 13: HLC crystallization inhibition assay in a 96-wells plate. In HLC seeded blood extract-based broth with 0 to 1000 μM of gentamicin, ampicillin or chloroquine, a dark precipitate is easily visible with the naked eye in the positive growth wells (as for all tested concentrations of gentamicin and ampicillin wells); the first well with less growth than the drug free control was considered a negative growth result (as for ≥100 μM chloroquine wells).

A negative growth result is read when a well presents less growth than the control without added drug, shown in 100-1000 µM chloroquine wells. None of the wells showed cultivable contamination from bacteria or fungi, after inoculating and incubating 5 µL of content of the positive growth wells on Tryptone Soya Agar and Mycoplasma Agar Base plates.

Different HLC growth conditions were tested, whose different results are shown in Table 2.

Table 2: Results for different HLC growth conditions tested.

Conditions changed	Growth	Comments
Basic condition*	+++	Black deposit accumulation, visible from 3rd day of incubation, stabilizing after 5-7 days; smaller black deposit forms in some of the non-seeded wells; medium turns brownish in the second day of incubation
Lysed horse blood extract (instead of human blood)	++++	Despite the higher yield, it does not grow faster/growth is not detected earlier
Hemin (instead of human blood)	+	Growth occurs in a lesser extent and slower
Human serum (instead of horse serum)	++	Growth occurs in a lesser extent and slower
Brain Heart Infusion broth (instead of Mycoplasma broth)	+/-	Small black deposits form as in non-seeded normal assay condition (first row)
pH 5 (instead of pH7)	++	More diffuse growth
Ambient atmosphere (instead of 5% CO_2)	++	Growth occurs in a lesser extent and slower
Seeding with synthetic hemozoin (instead of "IFDO")	+++	Growth identical to IFDO seeding
Without pancreatin	-	No black deposit formation; medium stays reddish and does not turn brownish in the second day of incubation

Interestingly, replacing lysed blood with commercial hemin resulted in a somewhat reduced growth. Seeding is essential for the growth to occur, but using sHZ was equally effective as using HLC. Lowering the pH reduced the growth, as did exposure to ambient atmosphere, the use of a different culture broth or replacing horse serum by human serum. Broth without pancreatin did not allow pellet formation.

Several antimalarial and antibacterial compounds were tested over a range of concentrations for HLC inhibition. Figure 13 represents a typical HLC inhibition assay and the overall results are summarized in Table 3. Chloroquine and amodiaquine showed the strongest HLC growth inhibition (100 µM), followed by quinine, quinacrine, halofantrine (250 µM), and mefloquine (500 µM). Interestingly, artemisinin also inhibited HLC growth at 250 µM concentration. As expected, antibacterials, namely gentamicin, ampicillin and clindamycin showed no inhibitory

effect at all. Tetracycline, rolitetracycline, tigecycline and doxycycline also did not inhibit HLC growth at the tested concentration range.

Table 3: Inhibition of hemozoin-like crystals (HLC) formation by several antimalarial and antibacterial molecules at various concentrations in Broth medium for HLC culture.

	Compounds concentrations (µM)						
	1000	750	500	250	100	50	0
Chloroquine	-	-	-	-	-	+	+
Amodiaquine	-	-	-	-	-	+	+
Mefloquine	-	-	-	+	+	+	+
Quinine	-	-	-	-	+	+	+
Quinacrine	-	-	-	-	+	+	+
Halofantrine	-	-	-	+	+	+	+
Artemisinin	-	-	-	-	+	+	+
Clindamycin	+	+	+	+	+	+	+
Tetracycline	+	+	+	+	+	+	+
Rolitetracycline	+	+	+	+	+	+	+
Tigecycline	+	+	+	+	+	+	+
Doxycycline	+	+	+	+	+	+	+
Gentamicin	+	+	+	+	+	+	+
Ampicillin	+	+	+	+	+	+	+

"-" means no growth, "+" means visible growth

5. Discussion

5.1. Characterization of hemozoin and hemozoin-like crystals

5.1.1. <u>Importance of characterizing hemozoin</u>

Hemozoin may play an important role as a modulator of immune system responses, possibly contributing to malaria immunopathology [30]. In order to assess and understand its biological properties, scientists need to obtain hemozoin crystals. It is possible to obtain hemozoin from *P. falciparum* cultures [72], which is cumbersome and rather expensive. Alternatively, synthetic hemozoin can be chemically synthesized from hemin chloride under acidic or anhydrous basic conditions [74]. Thus, both types of hemozoin are obtained using different methods and they can be purified to a certain degree in terms of the contaminating substances present in the end product.

Some studies suggest hemozoin may exert stimulatory effects, while others propose inhibitory effects [22]. Different inflammatory effects may be produced by nanocrystalline, amorphous synthetic hemozoin and needle-like crystals [22]. The size of the synthetic hemozoin crystals also appears to be an important factor [75]. Consequently, study results

on host immunomodulation may crucially depend on the type of hemozoin, that is its origin, shape and size, as well as contaminating substances.

In order to ensure that the produced crystals are uniform, morphology and size must be assessed. Characterization of hemozoin crystals makes use of tools such as light, depolarizing and electron microscopy, as well as X-ray diffraction and infrared spectroscopy.

Notably, hemozoin remains a good antimalarial drug target. To screen for drug candidates, one can make use of several methods to produce β-hematin synthesis [62] [61] [63] [60]. Yet, not every study confirmed that the end product in their β-hematin assay was indeed true hemozoin or controlled for remaining heme contamination.

5.1.2. Analysis of hemozoin from different origins and hemozoin-like crystals

Characterization of hemozoin is crucial, especially when obtained from varied sources or with different methods. Nonetheless, most studies on the possible role of hemozoin in host immunomodulation give little information on the methods used to characterize and identify the nature of the crystals they use. Some do not characterize them at all [76] [77] [78], others only assess the presence of contaminants [79] [80] [81], and few use SEM, XRD, infrared spectroscopy or thin layer chromatography [75] [73].

Hemozoin obtained from different origins is not distinguishable by simple visual observation of the blackish suspensions in water (Figure 3). Although minor differences in the viscosity can be observed, namely a higher viscosity in the crude extract of native hemozoin. Therefore, a microscopic observation has to be performed to be able to characterize hemozoin better, both synthetic and natural, in what concerns morphology and size.

Light microscopy allows the observation of typical needle-like morphology of synthetic and natural hemozoin crystals (Figure 5). However, hemozoin-like crystals were slightly different and always presented as roundish aggregates despite extensive sonication to try dispersion. Depolarizing microscopy is useful to confirm the typical birefringence of hemozoin preparations. In fact, it was possible to note some differences in the way that hemozoin from different origins depolarized light, with needle-like particles appearing more bluish and aggregates more brown-gold and green.

Importantly, only scanning electron microscopy analysis allowed to assess crystal size and to elucidate crystal morphology clearly. However, despite some differences concerning *P. falciparum* derived hemozoin's brick-like shape and synthetic hemozoin's needle-like appearance, crystals from different sources presented an overall similarity (Figure 6). Crystal size correlated well with the dimensions described by others [18] [27], and homogeneity of crystals supports the idea that it is possible to have similar synthetic and native hemozoin if the process of obtaining them is carefully controlled [22].

Still, in order to unequivocally confirm the physicochemical nature of hemozoin from different origins, it was necessary to perform an XRD analysis. Values for diffraction peaks of sHZ and nHZ matched those obtained in other studies, namely the *d*-spacing values [82] [26] (Figure 7). Some differences of peak intensity may be due to preferential growth occurring through different planes of the crystal. Hemozoin-like crystals, however, showed a different XRD pattern, as shown in Figure 7. Diffraction peaks corresponding to different 2Ɵ angles indicate that these crystals are not equal to hemozoin.

Infrared spectroscopy analysis further confirmed the difference of hemozoin-like crystals relative to hemozoin. The infrared spectrum obtained for sHZ shows the two hemozoin-characteristic peaks at 1663 and 1210 cm^{-1}, as determined by other groups [25] [17] [83], whereas hemozoin-like crystals spectrum lacks these peaks (Figure 8). However, the information which can be obtained by infrared spectroscopy is not superior to XRD, which appears to give the optimal information to assess the physicochemical nature of the crystals.

In summary, several methods are advisable to assess the nature and quality of hemozoin in a complementary way:

- A first approach to characterize hemozoin after its production must include observations by light and depolarizing microscopy;
- Scanning electron microscopy is essential for the assessment of morphology, size and homogeneity of the crystals;
- Evaluation of the physicochemical nature of the crystal, however, is only possible by XRD and infrared spectroscopy analysis. Nevertheless, these analyses require a considerably high amount of sample. When obtaining native hemozoin, crystals are formed by the parasite itself. Considering the costs involved in maintaining cultures to purify the pigment, it may not be necessary to perform XRD or infrared spectroscopy for native hemozoin. When synthesizing β-hematin, however, it is important to perform these analyses in order to know if the obtained crystals are in fact similar to hemozoin.

5.1.3. Purity and contamination assessment

Some groups working with sHZ devoid of DNA or protein suggest that hemozoin may play an immunostimulatory role in malaria pathology [79]. Others, who extract the unpurified pigment from cultures, consider that hemozoin-bound DNA, proteins or lipids may confer the crystal its immune modulation properties [84] [85] [73] [86]. In fact, it has not been clarified how hemozoin is released in *in vivo* milieu. Some authors refer that hemozoin remains inside intact digestive vacuoles after schizont rupture into the blood stream and that the vacuole itself causes these effects [87] [86]. Others suggest that DNA and proteins bind to the crystal

and are responsible for immune effects [88] [73]. Other groups report egress of merozoites without referring explicitly to how hemozoin is liberated [89] [20].

Thus, not too surprisingly, hemozoin obtained by different methods may contain other molecules of host or parasite origin. Synthetic and purified native hemozoin as well as hemozoin-like crystals obtained in this study did not contain DNA and protein molecules detectable by agarose gel and polyacrylamide gel electrophoresis (Figures 11 and 12). The crude extract hemozoin, however, is contaminated with DNA and protein. Therefore, different methods may yield crystals which contain or not other molecules, and this may elicit different results when assessing their possible immunomodulatory effects. Yet, depending on how we consider hemozoin to be released from infected erythrocytes, the presence of DNA and protein may be of interest when studying the effects of hemozoin.

Other contaminants may be considered in addition to host and parasite molecules. Hemozoin and hemozoin-like crystals samples obtained in the present work were all found to be free of *Mycoplasma* (Figure 10). Another important test to perform would be the endotoxin detection, to exclude lipopolysaccharide (LPS) contribution when studying immune responses to hemozoin. When studying hemozoin immunomodulatory properties, *Mycoplasma* and endotoxin contamination must not be present, in order to exclude their possible role in the effects being observed.

5.1.4. Quantitation and remaining heme contamination

Regardless of the high purity of the produced hemozoin, substances of biological origin may be present in some samples, since native hemozoin is derived from cultures of infected erythrocytes and hemozoin-like crystals are grown in a medium supplemented with blood extract. However, precise determination of the produced hemozoin concentration is crucial, in order to obtain the right amount of crystal being tested either when investigating its immunomodulatory effects or in heme inhibition assays. Hemozoin quantification can be performed using different methods. One can simply weigh the dried crystal [25] [82] or convert hemozoin samples back into heme and spectrophotometrically measure the absorbance and express the amount of hemozoin in heme-equivalents. Absorbance may be read either directly [24] [83] or making use of pyridine (category 4 of acute toxicity [90]) to evaluate coordination to heme molecules [61] [91].

The QuantiChrom™ Heme Assay Kit was used in this study to determine the hemozoin concentration in heme-equivalents. This kit does not require toxic reagents and was specifically optimized to reduce potential interference from substances of biological origin.

Synthesis of β-hematin and cultivation of hemozoin-like crystals were more economic and less laborious, safer, and had a higher yield than the isolation and purification of hemozoin from *P. falciparum* cultures. Hemozoin from *P. falciparum* cultures was more difficult to

obtain than sHZ and hemozoin-like crystals, requiring maintenance of high volume cultures, which is time consuming and needs expensive reagents. A large blackish pellet of crude native hemozoin obtained with less volume of culture did not mean a higher amount of the crystal, probably because membranes and proteins contributed to the larger volume of the crude extract. Thus, it may be advantageous to use sHZ or hemozoin-like crystals rather than natural hemozoin, but that depends on the premises and objectives of the study in question.

Importantly, the QuantiChrom™ Heme Assay Kit measures the absorbance of total heme molecules after hemozoin conversion into heme which cannot be used to determine contaminations by unreacted heme. However, in the case of synthesis of sHZ thin layer chromatography was performed to be able to distinguish between reacted and unreacted heme molecules which allowed to assess remaining heme contamination.

The assessment of heme contamination allows to exclude that possible free, unreacted heme interferes when investigating hemozoin effects in the modulation of immune system's responses [75]. Determining the remaining heme is also useful to evaluate the yield of β-hematin synthesis. Hemozoin of natural source was shown not to be contaminated with free heme (Figure 9) and synthetic hemozoin showed only a 1% contamination with free heme. Thus, the synthesis method was considerably efficient, since washing with a basic solution allowed to remove almost all unreacted heme. On the other hand, hemozoin-like crystals presented an average 37% heme contamination.

Extensive washing of hemozoin-like crystals with a basic solution, water and methanol failed to reduce the contamination with heme. Using DMSO reduced heme contamination to some extent, but not all. In fact, the high heme contamination of hemozoin-like crystals could in part explain the difference relative to hemozoin crystal, because hemozoin-like crystals could be some sort of aggregate constituted by heme and β-hematin. However, XRD patterns of synthetic hemozoin and hemozoin-like crystals are sufficiently different to indicate that a phase with the specific synthetic hemozoin peaks was not present in hemozoin-like crystals (Figure 7).

5.2. Novel hemozoin-like crystals inhibition assay

5.2.1. <u>Hemozoin-like crystals characterization and cultivation</u>

Ileal Fluid Dependent Organism (IFDO) was first described by Burdon in the 1980's. In an attempt to identify a possible cause of Crohn's disease, Burdon used ileal fluid-supplemented medium to isolate IFDO from the ileostomy fluid of patients. The hypothesis that IFDO was a living organism was considered, and its resemblance with replicating agents such as slow viruses, amyloid and prions was reported. But Burdon also thought that IFDO could be a sort of crystal spontaneously forming from precipitation of constituents of the culture medium [71].

IFDO presented characteristics of resistance to standard methods of disinfection and sterilization similar to those of prions and other related agents. Therefore, the company Steris became very interested in studying IFDO, with the intent to develop a model to test for new drugs and processes for prion inactivation. Mass spectrometry analysis showed that IFDO particles are in fact ferriprotoporphyrin IX aggregates. Together with IFDO's birefringence and dark-brown color, this raised the possibility that IFDO could be hemozoin. Further characterization was then performed by us in collaboration with Steris.

Scanning electron microscopy showed IFDO to be similar to sHZ and nHZ in morphology. No proteins or DNA were detected in IFDO samples, similarly to what was previously described for sHZ and purified nHZ [22] [29]. However, XRD and infrared spectroscopy results for IFDO samples revealed it was different from hemozoin, as explained above, and the particles were named hemozoin-like crystals.

Hemozoin-like crystals (HLC) can be grown in a well-defined medium, following the description from Burdon. The obtained hemozoin-like crystals were similar to those observed when IFDO was first described [71]. The formation of a brown precipitate was visible during incubation in broth medium, and brown colony-like structures appeared beneath and on the surface of seeded agar medium plates (Figure 4). Interestingly, seeding with sHZ instead of IFDO particles produced similar results. Different growth conditions were explored (Table 2), but it was not possible to exclude any medium constituent or to simplify incubation. In accordance with what had already been described [71], pancreatin in particular was essential for crystal growth. Of note, human blood extract was used instead of horse blood extract to supplement the culture medium, because it was easier to obtain as part of other ongoing research work.

5.2.2. Inhibition of hemozoin-like crystals growth by antimalarial compounds

Hemozoin-like crystals were grown in broth medium in the presence or absence of different antimalarial and antibiotic drugs which permitted to investigate their ability to inhibit the process of crystallization. A range of drug concentrations was tested in a 96-well plate format, as shown in Figure 13, allowing to visually determine minimum inhibitory concentrations of HLC growth (Table 3).

Chloroquine and amodiaquine presented the highest inhibitory potency, followed by other quinoline drugs, such as quinine and mefloquine. This is consistent with previously reported results for antimalarials targeting hemozoin formation [63] [61] [62]. As expected, no inhibition was observed for the antibacterials gentamicin, ampicillin, clindamycin and tetracyclines at the tested concentrations.

Curiously, artemisinin showed an inhibitory effect on HLC formation comparable to that observed with quinine, quinacrine and halofantrine. In fact, the mode of action of artemisinins

has been a subject of debate. Some authors argue that artemisinins do not inhibit β-hematin formation [66] or that heme-adducts are only formed with the drug under reductive conditions [55]. Yet, other studies report similar results to those obtained in the present work [60] [28]. A possible explanation for artemisinin inhibition results could be the difference between hemozoin-like crystals and hemozoin. Some believe assay formats using different reagents may assess different types of interaction between heme and drug molecules, namely artemisinin [66]. Perhaps artemisinin interacts differently with hemozoin-like crystals and with hemozoin, resulting in inhibition when assessed with the assay from the present work. Future work will include testing of further artemisinin derivatives in the HLC-assay, as well as reproducing previous results with artemisinin using other HZ-inhibition assays.

Interestingly, different types of interactions can occur to form heme aggregates. Hemozoin is formed by dimers of heme molecules which interact through π-π stacking forces. Other interactions may result in the formation of μ-oxo dimers, consisting of two heme molecules linked by an oxygen atom which establishes a covalent bond to the iron centers. [35] [24] [92] [93] HLC seem to be relatively stable, since they were not solubilized in DMSO, which is the solvent used in hemozoin inhibition assays to remove unreacted hematin before the end product is quantified [62]. It can be that HLC are heme aggregates similar to hemozoin with different types of interactions occurring between the heme molecules, but additional studies are needed in order to fully understand their exact nature.

Of note, most reports on hemozoin inhibition assays give little information on the methods used to identify the nature of the final aggregates and to quantify the extent of remaining heme contamination [67] [68] [69] [70]. Some only use infrared spectroscopy, and few use SEM or XRD [61] [65] [94]. So, if an assay reliably detects hemozoin inhibiting drugs, one can argue that it may not be essential that the end product is true hemozoin.

Nevertheless, when screening for antimalarial drugs using the HLC inhibition assay, positive results must be carefully analyzed in order to assure that tests reflect true hemozoin inhibition.

Of interest, the potency of drugs inhibiting hemozoin formation may depend on the stability of the interactions in heme-drug adducts [55]. But the specific binding to differentially-growing surfaces of the hemozoin crystal could also determine drug potency [35]. Most of described heme inhibition assays start with heme or hematin reagents [60] [62]. Conversely, HLC inhibition reported in this study makes use of HLC seeding as an initiator, which may account for drug interaction with both heme molecules and differentially-growing faces of the crystal.

5.2.3. Other assays and advantages of the novel hemozoin-like crystals inhibition assay

Several heme inhibition assays have been developed with the intent of screening drugs with the potential to inhibit hemozoin growth. In most of these assays, crystallization occurs

during a defined time interval (1-18 h) at a certain temperature (37-60°C), after adding glacial acetic acid or acetate buffer at pH 5 to a hemin chloride or hematin solution in DMSO or NaOH. To remove non-crystalline heme, the final product is washed with DMSO, dissolved back into heme using NaOH, and finally measured by spectrophotometric reading at A_{405nm} [62]. To assess if a compound inhibits β-hematin formation, it is included in the reaction and then the inhibition quantified as the decrease in final β-hematin concentration. As an alternative to transforming β-hematin back into heme, colorimetric assays use pyridine to coordinate with unreacted hematin. The formation of an orange-pink color allows visual as well as spectrophotometric identification of β-hematin inhibition. [61]

However, these assays depend on the use of highly concentrated solutions of hemin (final concentration of 1-2 mM), drugs (1-20 molar equivalents relative to hemin) and acid (8-12.9 M), and the procedures involved include multiple manipulation steps (pipetting, washing steps). Also, centrifuging microtiter plates at 3300×g may not be possible in some laboratories, where existing centrifuges may not have the necessary rotors to achieve such a force. Measurement of the end product is often complex, involving transformation of hemozoin back into heme, or using pyridine [61] [64] [68], which is very toxic.

A lower concentration of drug is needed to perform the HLC inhibition assay as compared to other heme inhibition assays. However, drugs have to be prepared in considerably concentrated stock solutions, in order to dilute the drug solvent in the culture medium enough to avoid that the ratio of HLC growth medium to drug solvent is too low and thus the medium might not support crystal growth. Still, some drugs cannot be prepared in such concentrated solutions. Furthermore, the amount of drug needed may itself be a limitation in terms of availability during compound discovery. In the future, it will be interesting to explore the assay in a systematic way by trying to reduce the final proportion of medium as much as possible, to be able to start with less concentrated stock solutions.

In summary, the hemozoin-like crystals inhibition assay is simple and easy to perform. It is carried out in a microplate and requires rather basic and inexpensive reagents. Growth occurs at physiological pH (7) at 37°C. It only needs basic laboratory infrastructures, and once set up there are no further manipulation steps. The final read-out is very simple, as it is based on visual observation of the end product. Although HLC are not identical to hemozoin, the results of the inhibitory effects of the quinoline drugs, including their different potency, are consistent with results from previously published heme inhibition studies. This supports the use of HLC as an assay to screen antimalarial drugs for their hemozoin inhibiting activity. The described HLC inhibition assay may therefore have the potential to be a helpful tool in addition to existing assays for antimalarial drug screening.

6. Conclusions

Characterization of hemozoin crystals is crucial to better understand their potential role as a modulator of immune responses. A first approach to characterize hemozoin after its production must include observations by light and depolarizing microscopy. Scanning electron microscopy is essential to evaluate morphology, size and homogeneity of the crystals. To investigate the physicochemical nature of the crystal, however, X-ray diffraction or infrared spectroscopy analyses are important, especially when synthesizing β-hematin. In the present work, an overall similarity was observed in morphology and size for hemozoin from different origins, but hemozoin-like crystals were shown to be different from true hemozoin by X-ray diffraction and infrared spectroscopy.

Growth of hemozoin-like crystals successfully allowed to investigate the hemozoin inhibiting effects of antimalarial drugs. In fact, the potency of quinoline drugs to inhibit hemozoin-like crystals growth is consistent with previously published heme inhibition studies. The assay is easy to perform and requires rather basic and inexpensive reagents. Also, it only needs basic laboratory infrastructures and the final read-out is very simple, as it is based on visual observation of the end product. The hemozoin-like crystals inhibition assay may be used as a first approach in antimalarial drug screening, not only of compounds designed from analogs of known antimalarials, but also drugs already used to treat other diseases.

7. References

[1] F. E. G. Cox, "History of Human Parasitology", *Clinical Microbiology Reviews,* vol. 15, no. 4, pp. 595-612, 2002.

[2] R. Carter and K. N. Mendis, "Evolutionary and Historical Aspects of the Burden of Malaria", *Clinical Microbiology Reviews,* vol. 15, no. 4, pp. 564-594, 2002.

[3] L. S. Garcia, "Malaria", *Clinics in Laboratory Medicine,* vol. 30, pp. 93-129, 2010.

[4] World Health Organization, "World Malaria Report 2011", 2012.

[5] L. H. Miller, K. Marsh and O. K. Doumbo, "The pathogenic basis of malaria", *Nature,* vol. 415, pp. 673-679, 2002.

[6] K. N. Suh, K. C. Kain and J. S. Keystone, "Malaria", *Canadian Medical Association Journal,* vol. 170, no. 11, pp. 1693-1702, 2004.

[7] B. M. Greenwood, K. Bojang, C. J. M. Whitty and G. A. T. Targett, "Malaria", *Lancet,* vol. 365, pp. 1487-1498, 2005.

[8] World Health Organization, "World: Countries and territories affected by malaria, 2010", *Global Health Observatory - Map Gallery,* 2012.

[9] C. J. L. Murray, L. C. Rosenfeld, S. S. Lim, K. G. Andrews, K. J. Foreman, D. Haring, N. Fullman, M. Naghavi, R. Lozano and A. D. Lopez, "Global malaria mortality between 1980 and 2010: a systematic analysis", *Lancet,* vol. 379, pp. 413-431, 2012.

[10] A. Roca-Feltrer, C. J. Kwizombe, M. A. Sanjoaquin, S. S. S. Sesay, B. Faragher, J. Harrison, K. Geukers, S. Kabuluzi, D. P. Mathanga, E. Molyneux, M. Chagomera, T. Taylor, M. Molyneux and R. S. Heyderman, "Lack of Declinein Childhood Malaria, Malawi, 2001-2010", *Emerging Infectious Diseases,* vol. 18, no. 2, pp. 272-278, 2012.

[11] J. L. Gallup and J. D. Sachs, "The Economic Burden of Malaria", *The American Journal of Tropical Medicina and Hygiene,* vol. 64, pp. 85-96, 2001.

[12] M. A. Thera and C. V. Plowe, "Vaccines for Malaria: How Close Are We?", *Annual Review of Medicine,* vol. 63, pp. 345-357, 2012.

[13] L. Tilley, M. W. A. Dixon and K. Kirk, "The *Plasmodium falciparum*-infected red blood cell", *The Internation Journal od Biochemistry & Cell Biology,* vol. 43, pp. 839-842, 2011.

[14] E. Hanssen, P. J. McMillan and L. Tilley, "Cellular architecture of *Plasmodium falciparum*-infected erythrocytes", *International Journal for Parasitology,* vol. 40, pp. 1127-1135, 2010.

[15] S. Kumar, M. Guha, V. Choubey, P. Maity and U. Bandyopadhyay, "Antimalarial drugs inhibiting hemozoin (β-hematin) formation: A mechanistic update", *Life Sciences,* vol. 80, pp. 813-826, 2007.

[16] S. Kumar and U. Bandyopadhyay, "Free heme toxicity and its detoxification systems in human", *Toxicology Letters,* vol. 157, pp. 175-188, 2005.

[17] T. J. Egan, W. W. Mavuso and K. K. Ncokazi, "The Mechanism of β-hematin Formation in Acetate Solution. Parallels between Hemozoin Formation and Biomineralization Processes", *Biochemistry,* vol. 40, pp. 204-213, 2001.

[18] J. M. Pisciotta and D. Sullivan, "Hemozoin: Oil versus water", *Parasitology International,* vol. 57, pp. 89-96, 2008.

[19] A. N. Hoang, K. K. Ncokazi, K. A. de Villiers, D. W. Wright and T. J. Egan, "Crystallization of Synthetic Haemozoin (β-haematin) Nucleated at the Surface of Lipid Particles", *Dalton Trans,* vol. 1244, no. 5, p. 1235, 2010.

[20] M. Abkarian, G. Massiera, L. Berry, M. Roques and C. Braun-Breton, "A novel mechanism for egress of malarial parasites from red blood cells", *Blood,* vol. 117, pp. 4118-4124, 2011.

[21] T. J. Egan, "Haemozoin formation", *Molecular & Biochemical Parasitology,* vol. 157, no. 2, pp. 127-136, 2008.

[22] M. Jaramillo, M.-J. Bellemare, C. Martel, M. T. Shio, A. P. Contreras, M. Godbout, M. Roger, E. Gaudreault, J. Gosselin, D. S. Bohle and M. Olivier, "Synthetic *Plasmodium*-Like Hemozoin Activates the Immune Response: A Morphology-Function Study", *PLoS ONE,* vol. 4, no. 9, 2009.

[23] S. Pagola, P. W. Stephens, D. S. Bohle, A. D. Kosar and S. K. Madsen, "The structure of malaria pigment β-haematin", *Nature,* vol. 404, pp. 307-310, 2000.

[24] N. Klonis, R. Dilanian, E. Hanssen, C. Darmanin, V. Streltsov, S. Deed, H. Quiney and L. Tilley, "Hematin-Hematin Self-Association States Involved in the Formation and Reactivity of the Malaria Parasite Pigment, Hemozoin", *Biochemistry,* vol. 49, pp. 6804-6811, 2010.

[25] A. F. G. Slater, W. J. Swiggard, B. R. Orton, W. D. Flitter, D. E. Goldberg, A. Cerami and G. B. Henderson, "An iron-carboxylate bond links the heme units of malaria pigment", *Proceedings of the National Academy of Sciences,* vol. 88, pp. 325-329, 1991.

[26] D. S. Bohle, R. E. Dinnebier, S. K. Madsen and P. W. Stephens, "Characterization of the Products of the Heme Detoxification Pathway in Malarial Late Trophozoites by X-ray Diffraction", *The Journal of Biological Chemistry,* vol. 272, no. 2, pp. 713-716, 1997.

[27] E. Hempelmann, C. Motta, R. Hughes, S. A. Ward and P. G. Bray, "*Plasmodium falciparum*: sarificing membrane to grow crystals?", *TRENDS in Parasitology,* vol. 19, no. 1, pp. 23-26, 2003.

[28] I. Solomonov, M. Osipova, Y. Feldman, C. Baehtz, K. Kjaer, I. K. Robinson, G. T. Webster, D. McNaughton, B. R. Wood, I. Weissbuch and L. Leiserowitz, "Crystal Nucleation, Growth, and Morphology of the Synthetic Malaria Pigment β-Hematin and the Effect Thereon by Quinoline Additives: The Malaria Pigment as a Target of Various Antimalarial Drugs", *Journal of the American Chemical Society,* vol. 129, pp. 2615-2627, 2007.

[29] J. M. Pisciotta, I. Coppens, A. K. Tripathi, P. F. Scholl, J. Shuman, S. Bajad, V. Shulaev and D. J. Sullivan Jr, "The role of neutral lipid nanospheres in *Plasmodium falciparum* haem crystallization", *Biochemical Journal,* vol. 402, pp. 197-204, 2007.

[30] T. Hänscheid, T. J. Egan and M. P. Grobusch, "Haemozoin: from melatonin pigment to drug target, diagnostic tool, and immune modulator", *Lancet Infectious Diseases,* vol. 7, pp. 675-685, 2007.

[31] J. Achan, A. O. Talisuna, A. Erhart, A. Yeka, J. K. Tibenderana, F. N. Baliraine, P. J. Rosenthal and U. D'Alessandro, "Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria", *Malaria Journal,* vol. 10, no. 144, 2011.

[32] U. K. Padmaja, P. Adhikari and P. Periera, "Experience with quinine in falciparum malaria", *Indian Journal of Medical Sciences,* vol. 53, no. 4, pp. 153-157, 1999.

[33] H. O. AlKadi, "Antimalarial Drug Toxicity: A Review", *Chemotherapy,* vol. 53, pp. 385-391, 2007.

[34] P. B. Bloland, "Drug resistance in malaria", 2001.

[35] I. Weissbuch and L. Leiserowitz, "Interplay Between Malaria, Crystalline Hemozoin Formation, and Antimalarial Drug Action and Design", *Chemical Reviews,* vol. 108, no. 11, pp. 4899-4914, 2008.

[36] N. J. White, "Antimalarial drug resistance", *The Journal of Clinical Investigation,* vol. 113, no. 8, pp. 1084-1092, 2004.

[37] U. Farooq and R. C. Mahajan, "Drug resistance in malaria", *Journal of Vector Borne Diseases,* vol. 41, pp. 45-53, 2004.

[38] S. Sadanand, "Malaria: An Evaluation of the Current State of Research on Pathogenesis and Antimalarial Drugs", *Yale Journal of Biology and Medicine,* vol. 83, pp. 185-191, 2010.

[39] S. C. Parija and I. Praharaj, "Drug resistance in malaria", *Indian Journal of Medical Microbiology,* vol. 29, no. 3, pp. 243-248, 2011.

[40] M. B. Cassera, Y. Zhang and K. Z. Hazleton, "Purine and Pyrimidine Pathways as Targets in *Plasmodium falciparum*", *Current Topics in Medicinal Chemistry,* vol. 11, no. 16, pp. 2103-2115, 2011.

[41] C. Loup, J. Lelièvre, F. Benoit-Vical and B. Meunier, "Trioxaquines and Heme-Artemisinin Adducts Inhibit the In Vitro Formation of Hemozoin Better than Chloroquine", *Antimicrobial Agents and Chemotherapy,* vol. 51, no. 10, pp. 3768-3770, 2007.

[42] U. Eckstein-Ludwig, R. J. Webb and I. D. A. van Goethem, "Artemisinins target the SERCA of *Plasmodium falciparum*", *Nature,* vol. 424, pp. 957-961, 2003.

[43] C. J. Woodrow, R. K. Haynes and S. Krishna, "Artemisinins", *Postgraduate Medical Journal,* vol. 81, pp. 71-78, 2005.

[44] B. K. Park, P. M. O'neill, J. L. Maggs and M. Pirmohamed, "Safety assessment of peroxide antimalarials: clinical and chemical perspectives", *British Journal of Clinical Pharmacology,* vol. 46, pp. 521-529, 1998.

[45] J. E. Hyde, "Drug-resistant malaria", *Trends in Parasitology,* vol. 21, no. 11, pp. 494-498, 2005.

[46] L. Cui and X.-z. Su, "Discovery, mechanisms of action and combination therapy of artemisinin", *Expert Review of Anti-infective Therapy,* vol. 7, no. 8, pp. 999-1013, 2009.

[47] H. Noedl, Y. Se, K. Schaecher, B. L. Smith, D. Socheat and M. M. Fukuda, "Evidence of Artemisinin-Resistant Malaria in Western Cambodia", *The New England Journal of Medicine,* vol. 359, no. 24, pp. 2619-2620, 2008.

[48] N. J. White, "The role of anti-malarial drugs in eliminating malaria", *Malaria Journal,* vol. 7, 2008.

[49] P. J. Rosenthal, "Antimalarial drug discovery: old and new approaches", *The Journal of Experimental Biology,* vol. 206, pp. 3735-3744, 2003.

[50] C. Hobbs and P. Duffy, "Drugs for malaria: something old, something new, something borrowed", *F1000 Biology Reports,* vol. 3, no. 24, 2011.

[51] H. Noedl, C. Wongsrichanalai and W. H. Wernsdorfer, "Malaria drug-sensitivity testing: new assays, new perspectives", *Trends in Parasitology,* vol. 19, no. 4, pp. 175-181, 2003.

[52] S. Frölich, C. Schubert, U. Bienzle and K. Jennet-Siems, "In vitro antiplasmodial activity of prenylated chalcone derivatives of hops (*Humulus lupulus*) and their interaction with Haemin", *Journal of Antimicrobial Chemotherapy,* 2005.

[53] J. C. P. Steele, R. J. Phelps, M. S. J. Simmonds, D. C. Warhurst and D. J. Meyer, "Two novel assays for the detection of haemin-binding properties of antimalarials evaluated with compounds isolated from medicinal plants", *Journal of Antimicrobial Chemotherapy,* vol. 50, pp. 25-31, 2002.

[54] G. Garavito, M.-C. Monje, S. Maurel, A. Valentin, F. Nepveu and E. Deharo, "A non-radiolabeled heme-GSH interaction test for the screening of antimalarial compounds", *Experimental Parasitology,* vol. 116, pp. 311-313, 2007.

[55] K. Muñoz-Durango, A. Maciuk, A. Harfouche, S. Torijano-Gutiérrez, J.-C. Jullian, J. Quintin, K. Spelman, E. Mouray, P. Grellier and B. Figadère, "Detection, Characterization, and Screening of Heme-Binding Molecules by Mass Spectrometry for Malaria Drug Discovery", *Analytical Chemistry,* vol. 84, no. 7, pp. 3324-3329, 2012.

[56] e. A. Cole, J. Ziegler, C. A. Evans and D. W. Wright, "Metalloporphyrins inhibit β-hematin (hemozoin) formation", *Journal of Inorganic Biochemistry,* vol. 78, pp. 109-115, 2000.

[57] J. K. Natarajan, J. N. Alumasa, K. Yearick, K. A. Ukoue-Kovi, L. B. Casabianca, A. C. de Dios, C. Wolf and P. D. Roepe, "4-N-, 4-S- and 4-O-Chloroquine Analogues: Influence of Side Chain Lenght and Quinolyl Nitrogen pKa on Activity vs Chloroquine Resistant Malaria", *Journal of Medicinal Chemistry,* vol. 51, pp. 3466-3479, 2008.

[58] A. Krettli, J. O. Adebayo and L. M. G. Krettli, "Testing of Natural Products and Synthetic Molecules Aiming at New Antimalarials", *Current Drug Targets,* vol. 10, no. 3, 2009.

[59] J. L. Vennerstrom, E. O. Nuzum, R. Miller, A. Dorn, L. Gerena, P. A. Dande, W. Y. Ellis, R. G. Ridley and W. K. Milhous, "8-Aminoquinolines Active against Blood Stage *Plasmodium falciparum* In Vitro Inhibit Hematin Polymerization", *Antimicrobial Agents and Chemotherapy,* vol. 43, no. 3, pp. 598-602, 1999.

[60] N. Basilico, E. Pagani, D. Monti, P. Olliaro and D. Taramelli, "A microtitre-based method for measuring the haem polymerization inhibitory activity (HPIA) of antimalarial drugs", *Journal of Antimicrobial Chemotherapy,* vol. 42, pp. 55-60, 1998.

[61] K. K. Ncokazi and T. J. Egan, "A colorimetric high-throughput β-hematin inhibition screening assay for use in the search for antimalarial compounds", *Analytical Biochemistry,* vol. 338, pp. 306-319, 2005.

[62] S. Parapini, N. Basilico, E. Pasini, T. J. Egan, P. Olliaro, D. Taramelli and D. Monti, "Standardization of the Physicochemical Parameters to Assess in Vitro the β-Hematin Inhibitory Activity of Antimalarial Drugs", *Experimental Parasitology,* vol. 96, pp. 249-256, 2000.

[63] N. T. Huy, D. T. Uyen, A. Maeda, D. T. X. Trang, T. Oida, S. Harada and K. Kamei, "Simple Colorimetric Inhibition Assay of Heme Crystallization for High-Throughput Screening of Antimalarial Compounds", *Antimicrobial Agents and Chemotherapy,* vol. 51, no. 1, pp. 350-353, 2007.

[64] R. D. Sandlin, M. D. Carter, P. J. Lee, J. M. Auschwitz, S. E. Leed, J. J. D and D. W. Wright, "Use of the NP-40 Detergent-Mediated Assay in Discovery of Inhibitors of β-Hematin Crystallization", *Antimicrobial Agents and Chemotherapy,* vol. 55, no. 7, pp. 3363-3369, 2011.

[65] D. T. X. Trang, N. T. Huy, D. T. Uyen, M. Sasai, T. Shiono, S. Harada and K. Kamei, "Inhibition assay of β-hematin formation initiatd by lecithin for screening new antimalarial drugs", *Analytical*

Biochemistry, vol. 349, pp. 292-296, 2006.

[66] R. K. Haynes, D. Monti, D. Taramelli, N. Basilico, S. Parapini and P. Olliaro, "Artemisinin Antimalarials Do Not Inhibit Hemozoin Formation", *Antimicrobial Agents and Chemotherapy,* vol. 47, no. 3, p. 1175, 2003.

[67] F. Omodeo-Salè, L. Cortelezzi, N. Basilico, M. Casagrande, A. Sparatore and D. Taramelli, "Novel Antimalarial Aminoquinolines: Heme Binding and Effects on Normal or *Plasmodium falciparum*-Parasitized Human Erythrocytes", *Antimicrobial Agents and Chemotherapy,* vol. 53, no. 10, pp. 4339-4344, 2009.

[68] J. C. Niles, J. L. DeRisib and M. A. Marletta, "Inhibiting *Plasmodium falciparum* growth and heme detoxification pathway using heme-binding DNA aptamers", *Proceedings of the National Academy of Sciences,* vol. 106, no. 32, pp. 13266-13271, 2009.

[69] Rodrigues, J. Ricardo and N. G. d. Domínguez, "*Plasmodium berghei*: In vitro and in vivo activity of dequalinium", *Experimental Parasitology,* vol. 115, pp. 19-24, 2007.

[70] C. Mullié, A. Jonet, C. Desgrouas, N. Taudon and P. Sonnet, "Differences in anti-malarial activity of 4-aminoalcohol quinoline enantiomers and investigation of the presumed underlying mechanism of action", *Malaria Journal,* vol. 8, pp. 11-65, 2012.

[71] D. W. Burdon, "A novel replicating agent isolated from the human intestinal tract having characteristics shared with Creutzfeldt-Jakob and related agents", *Journal of Medical Microbiology,* vol. 29, pp. 145-157, 1989.

[72] C. I. K. J. S. D. J. a. K. N. Coban, "Purified Malaria Pigment (Hemozoin) Enhances Dendritic Cell Maturation and Modulates the Isotype of Antibodies Induced by a DNA Vaccine", *Infection and Immunity,* pp. 3939-3943, 2002.

[73] C. C. Keller, P. G. Kremsner, J. B. Hittner, M. A. Misukonis, J. B. Weinberg and D. J. Perkins, "Elevated Nitric Oxide Production in Children with Malarial Anemia: Hemozoin-Induced Nitric Oxide Synthase Type 2 Transcripts and Nitric Oxide in Blood Mononuclear Cells", *Infection and Immunity,* vol. 72, no. 8, pp. 4868-4873, 2004.

[74] M. T. Shio, F. A. Kassa, M.-J. Bellemare and M. Olivier, "Innate inflammatory response to the malarial pigment hemozoin", *Microbes and Infection,* vol. 12, pp. 889-899, 2010.

[75] C. Coban, Y. Igari, M. Yagi, T. Reimer, S. Koyama, T. Aoshi, K. Ohata, T. Tsukui, F. Takeshita, K. Sakurai, T. Ikegami, A. Nakagawa, T. Horii, G. Nuñez, K. J. Ishii and A. S, "Immunogenicity of Whole-Parasite Vaccines against *Plasmodium falciparum* Involves Malarial Hemozoin and Host TLR9", *Cell Host and Microbe,* vol. 7, pp. 50-61, 2010.

[76] O. Skorokhod, E. Schwarzer, M. Ceretto and P. Arese, "Malarial pigment hemozoin, IFN-gamma, TNF-alpha, IL-I beta and LPS do not stimulate expression on inducible nitric oxide synthase and production of nitric oxide in immuno-purified human monocytes", *Malaria Journal,* vol. 6, no. 73, 2007.

[77] M. Prato, V. Gallo, G. Giribaldi and P. Arese, "Phagocytosis of haemozoin (malarial pigment) enhances metalloproteinase-9 activity in human adherent monocytes: Role of IL-I beta and 15-HETE", *Malaria Journal,* vol. 7, no. 157, 2008.

[78] M. Jaramillo, D. C. Gowda, D. Radzioch and M. Olivier, "Hemozoin Increases IFN-gamma-Inducible Macrophage Nitric OxideGeneration Through Extracellular Signal-Regulated Kinase- and NF-kB-Dependent Pathways", *The Journal of Immunology,* vol. 171, pp. 4243-4253, 2003.

[79] C. Coban, K. J. Ishii, T. Kawai, H. Hemmi, S. Sato, S. Uematsu, M. Yamamoto, O. Takeuchi, S. Itagaki, N. Kumar, T. Horii and S. Akira, "Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin", *The Journal of Experimental Medicine,* vol. 201, no. 1, pp. 19-25, 2005.

[80] D. O. Ochiel, G. A. Awandare, C. C. Keller, J. B. Hittner, P. G. Kremsner, J. B. Weinberg and D. J. Perkins, "Differential Regulation of B-Chemokines in Children with Plasmodium falciparum Malaria", *Infection and Immunity,* vol. 73, no. 7, pp. 4190-4197, 2005.

[81] C. C. Keller, O. Yamo, C. Ouma, J. M. Ong'echa, D. Ounah, J. B. Hittner, J. M. Vulule and D. J. Perkins, "Acquisition of Hemozoin by Monocytes Down-Regulates Interleukin-12 p40 (IL-12p40) Transcripts and Circulating IL-12p70 through an IL-10-Dependent Mechanism: In Vivo and In Vitro Findings in Severe Malarial Anemia", *Infection and Immunity,* vol. 74, no. 9, pp. 5249-5260, 2006.

[82] T. J. Egan and M. Tshivhase, "Kinetics of β-haematin formation from suspensions of haematin in aqueous benzoic acid", *Dalton Transactions,* pp. 5024-5032, 2006.

[83] R. Stiebler, A. N. Hoang, T. J. Egan, D. W. Wright and M. F. Oliveira, "Increase on the Initial Soluble Heme Levels in Acidic Conditions Is an Important Mechanism for Spontaneous Heme Crystallization In Vitro", *PLoS ONE,* vol. 5, no. 9, 2010.

[84] P. Parroche, F. N. Lauw, N. Goutagny, E. Latz, B. G. Monks, A. Visintin, K. A. Halmen, M. Lamphier, M. Olivier, D. C. Bartholomeu, R. T. Gazzinelli and D. T. Golenbock, "Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9", *Proceedings of the National Academy of Sciences,* vol. 104, no. 6, pp. 1919-1924, 2007.

[85] V. Barrera, O. Skorokhod, D. Baci, G. Gremo, P. Arese and E. Schwarzer, "Host fibrinogen stably bound to hemozoin rapidly activates monocytes via TLR-4 and CD11b/CD18-integrin: a new paradigm of hemozoin action", *Blood,* vol. 117, no. 21, pp. 5674-5682, 2011.

[86] P. Dasari, S. D. Heber, M. Beisele, M. Torzewski, K. Reinfenberg, C. Orning, A. Fries, A.-L. Zapf, S. Baumeister, K. Lingelbach, R. Udomsangpetch, S. C. Bhakdi, K. Reiss and S. Bhakdi, "Digestive vacuole of *Plasmodium falciparum* released during erythrocyte rupture dually activates complement and coagulation", *Blood,* vol. 119, no. 18, pp. 4301-4310, 2012.

[87] P. Dasari, K. Reiss, K. Lingelbach, S. Baumeister, R. Lucius, R. Udomsangpetch, S. C. Bhakdi and S. Bhakdi, "Digestive vacuoles of *Plasmodium falciparum* are selectively phagocytosed by and impair killing function of polymorphonuclear leukocytes", *Blood,* vol. 118, no. 18, pp. 4946-4956, 2011.

[88] E. Schwarzer, F. Turrini, D. Ulliers, G. Giribaldi, H. Ginsburg and P. Arese, "Impairment of Macrophage Functions after Ingestion of *Plasmodium falciparum*-infected Erythrocytes or Isolated Malarial Pigment", *The Journal of Experimental Medicine,* vol. 176, pp. 1033-1041, 1992.

[89] L. H. Bannistar, J. M. Hopkins, R. E. Fowler, S. Krishna and G. H. Mitchell, "A Brief Illustrated Guide to the Ultrastructure of *Plasmodium falciparum* Asexual Blood Stages", *Parasitology Today,* vol. 16, no. 10, pp. 427-433, 2000.

[90] European Union, "Regulation (EC) No 1272/2008 of the European Parliament and of the Council on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006", *Official Journal of the European Union,* 2008.

[91] R. F. Pasternack, B. Munda, A. Bickford, E. J. Gibbs and L. M. Scolaro, "On the kinetics of formation of hemozoin, the malarial pigment", *Journal of Inorganic Biochemistry,* vol. 104, pp. 1119-1124, 2010.

[92] S. B. Brown, T. C. Dean and J. Peter, "Aggregation of ferrihaems. Dimerization and protolytic equilibria of protoferrihaem and deuteroferrihaem in aqueous solution", *Biochemical Journal,* vol. 117, no. 4, pp. 733-739, 1970.

[93] K. A. de Villiers, C. H. Kaschula, n. T. J. Ega and H. M. Marques, "Speciation and structure of ferriprotoporphyrin IX in aqueous solution: spectroscopic and diffusion measurements demonstrate dimerization, but not μ-oxo dimer formation", *Journal of Biological Inorganic Chemistry,* vol. 12, no. 1, pp. 101-117, 2007.

[94] M. M. Chen, L. Shi and D. J. Sullivan Jr, "Haemoproteus and Schistosoma synthesize heme polymers similar to *Plasmodium* hemozoin and β-hematin", *Molecular & Biochemical Parasitology,* vol. 113, pp. 1-8, 2001.