Heme Detoxification in the Malaria Parasite: A Target for Antimalarial Drug Development

Katherine A. de Villiers[†], **Timothy J. Egan**[‡]

[†]Department of Chemistry and Polymer Science, Stellenbosch University, Private Bag, Matieland 7600, South Africa

[‡]Department of Chemistry, University of Cape Town, Private Bag, Rondebosch 7701, South Africa and Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Observatory 7945, Cape Town, South Africa

Conspectus

Over the last century, malaria deaths have decreased by more than 85%. Nonetheless, there were 405 000 deaths in 2018, mostly resulting from *Plasmodium falciparum* infection. In the 21st century, much of the advance has arisen from deployment of insecticide-treated bed nets and artemisinin combination therapy. However, over the last decade parasites with a delayed artemisinin clearance phenotype have appeared in Southeast Asia threatening further gains. The effort to find new drugs is thus urgent. A prominent process in blood stage malaria parasites, which we contend remains a viable drug target, is hemozoin formation. This crystalline material consisting of heme can be readily seen when parasites are viewed microscopically. The process of its formation in the parasite, however, is still not fully understood.

In early work, we recognized hemozoin formation as a biomineralization process. We have subsequently investigated the kinetics of synthetic hemozoin (β-hematin) crystallization catalyzed at lipid-aqueous interfaces under biomimetic conditions. This led us to the use of neutral detergent-based high throughput screening (HTS) for inhibitors of β-hematin formation. A good hit rate against malaria parasites was obtained. Simultaneously we developed a pyridine-based assay which proved successful in measuring concentrations of hematin not converted to β-hematin.

The pyridine assay was adapted to determine the effects of chloroquine and other clinical antimalarials on hemozoin formation in the cell. This permitted determination of the dose dependent amounts of exchangeable heme and hemozoin in *P. falciparum* for the first time. These studies have shown that hemozoin inhibitors cause a dose-dependent increase in exchangeable heme, correlated with decreased parasite survival. Electron spectroscopic imaging (ESI) showed a relocation of heme iron into the parasite cytoplasm, while electron microscopy provided evidence of disruption of hemozoin crystals. This cellular assay was subsequently extended to the top-ranked hits from a wide range of scaffolds found by HTS. Intriguingly, the amounts of


Corresponding author: Timothy J. Egan - Department of Chemistry, University of Cape Town, Private Bag, Rondebosch 7701, South Africa and Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Observatory 7945, Cape Town, South Africa; timothy.egan@uct.ac.za.

exchangeable heme at the parasite growth IC_{50} values of these scaffolds showed substantial variation. The amount of exchangeable heme was found to be correlated with the amount of inhibitor accumulated in the parasitized red blood cell. This suggests that heme-inhibitor complexes, rather than free heme, lead to parasite killing. This was supported by ESI using a Br-containing compound which showed co-localization of Fe and Br, as well as by confocal Raman microscopy which confirmed the presence of a complex in the parasite. Current evidence indicates that inhibitors block hemozoin formation by surface adsorption. Indeed, we have successfully introduced molecular docking with hemozoin to find new inhibitors. It follows that the resulting increase in free heme leads to formation of the parasiticidal heme-inhibitor complex. We have reported crystal structures of heme-drug complexes for several aryl methanol antimalarials in non-aqueous media. These form coordination complexes, but most other inhibitors interact non-covalently and determination of their structures remains a major challenge.

It is our view that key future developments will include improved assays to measure cellular heme levels, better in silico approaches for predicting β-hematin inhibition, and a concerted effort to determine the structure and properties of heme-inhibitor complexes.

Graphical Abstract
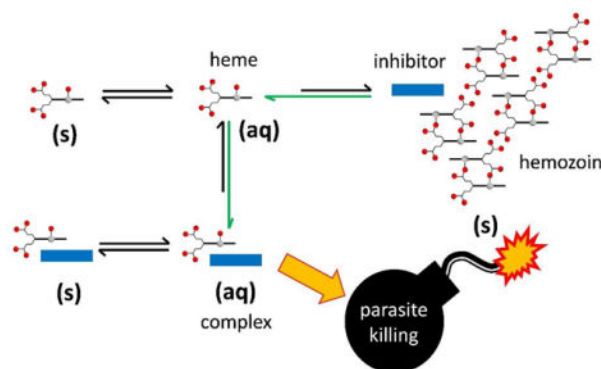
Introduction

In 1900 malaria caused almost three million deaths.[5] Mortalities due to this blood borne parasitic disease declined following the introduction of the insecticide DDT and the synthetic 4-amino quinoline drug, chloroquine, during the 1940s.[6] However, chloroquine resistance emerged in the late 1950s and 1960s in the Greater Mekong Subregion (GMS) and Latin America, respectively, before spreading to the Pacific Islands (1960 to mid-1970s) and Africa (1980s). Consequently, worldwide deaths increased again, largely due to rampant infections in sub-Saharan Africa,[5] which today accounts for 90–95% of global malaria statistics.[7] The introduction of pyrethroid treated bed nets and artemisinin combination therapies (ACTs), consisting of an artemisinin-derived drug and a second partner drug (usually a quinoline or related drug) with a longer half-life, has seen a major breakthrough in malaria control from the start of the 21st century.[8] ACTs are the first-line malaria treatment today, but treatment failure arising from prolonged clearance times has been detected in the GMS since 2009.[9] The World Health Organization reported a total of 405 000 malaria deaths in 2018.[7] Thus despite major advances discussed above, malaria remains a serious

international health problem. This is exacerbated by the fact that to date, there is no licensed vaccine for the disease, although one candidate (RTS,S) is undergoing clinical trials.[7]

Malaria is caused by protozoan parasites of the genus *Plasmodium*, transmitted to mammalian hosts by the female *Anopheles* mosquito.[10] Four species cause human malaria, *P. malariae*, *P. ovale*, *P. vivax* and *P. falciparum,* with the last being the most virulent. Following sexual reproduction in the mosquito, parasites enter the human host during a blood meal and develop asexually in hepatic cells before invading red blood cells (RBCs), Figure 1. The blood stage is associated with fevers that re-occur every 2–4 days.[5] During this stage, the parasite develops from merozoite, through ring and trophozoite to a mature schizont, before erupting from the RBC and invading new RBCs.[10] The metabolically-active trophozoite digests large quantities of RBC hemoglobin (Figure 1). A manifold of enzymes is involved in this process, releasing ferroprotoporphyrin IX into the parasite's digestive vacuole (DV). Subsequently, this must be oxidized to ferriprotoporphyrin IX since the Fe in crystalline malaria pigment (hemozoin) is indisputably in the ferric state based on spectroscopic evidence.[11] Autoxidation has been widely assumed but no direct experimental observation of this process in the parasite has been reported. Heme[12] contributes to membrane rupture among other things.[13] Since parasites lack a heme oxygenase function,[14] its disposal is primarily via sequestration as hemozoin,[11c] comprising cyclic μ-propionato dimers of heme (Figure 1).[15] While several antimalarials, including components of ACT, have been shown to inhibit formation of synthetic hemozoin (β-hematin),[16] their mechanism(s) of drug action remain poorly understood.

Here, we review our contributions to understanding mechanisms of hemozoin formation and drug action. We maintain that hemozoin remains a unique and viable drug target.

Mechanism of Hemozoin Formation

Mössbauer spectroscopy, chemical analysis and electron spectroscopic imaging have demonstrated that hemozoin accounts for 95% of the iron in trophozoites.[11c] In contrast to the conclusive knowledge of its composition,[17] the mechanism of hemozoin formation is still not fully understood. An early study suggested involvement of a so-called heme polymerase,[18] and a number of proteins have been considered for this role. Histidine-rich protein (HRP) II and III bind free heme and mediate its conversion to β-hematin under acidic conditions.[19] This function has also been attributed to heme detoxification protein (HDP) present across *Plasmodium* species.[20] In 2020, a homolog of lipocalin PV5 was reported to be involved in the control of hemozoin crystallization in the malaria parasite. Disruption of this protein results in changes to the external morphology of hemozoin crystals.[21]

Lipids have also been proposed to catalyze heme detoxification. Bendrat et al. demonstrated that an acetonitrile extract from hemozoin contained oleic, palmitic and stearic acids, and promoted formation of β-hematin.[22] Subsequent studies confirmed the activity of lipids in β-hematin formation[23] independent of parasite-derived proteins.[24]

β-Hematin can be prepared in the absence of proteins or lipids. Indeed, our early work employed carboxylic acids to mediate conversion of amorphous heme to β-hematin.[25] Sigmoidal kinetics of β-hematin formation were modeled using the Avrami equation with an Avrami constant of four. The interpretation is that crystal nucleation is an ongoing (sporadic) process, while crystal growth occurs in three dimensions. We likened this to biomineralization which often proceeds via an amorphous phase and suggested that acetate may be a phase-transfer catalyst, a role which lipids could fulfil *in vivo*. Lipids may, of course, also provide fixed sites of nucleation and templated (epitaxial) growth. We later pioneered a method for forming β-hematin at octanol-, pentanol- and lipid-water interfaces,[26] while others demonstrated the effectiveness of solvents[27] and detergents.[28] Notably, the interface method proceeds under biomimetic conditions (37 °C and 0.05 M buffer), with a physiologically-relevant half-life.[26] First-order kinetics were observed, consistent with an Avrami constant of one, indicative of a fixed number of nucleation sites (provided by the solvent interface or lipid molecules) and crystal growth in one dimension along the interface.[29] Transmission electron micrograph (TEM) images of hemozoin crystals within neutral lipid droplets provided initial support for this mechanism,[30] although later cryogenic soft X-ray tomography data reported by Kapishnikov et al. suggested that hemozoin crystals form at the inner membrane of the digestive vacuole.[31] Studies of β-hematin formation at interfaces have provided important insights regarding the mechanism of crystal formation. Grazing incidence X-ray diffraction (GIXD) experiments reported by Leiserowitz and co-workers indicated that β-hematin crystals align with their (100) face parallel to an air-water interface,[32] and we later confirmed this observation at neutral lipid (monomyristoylglycerol) interfaces.[33] TEM and confocal imaging were used to investigate β-hematin formation in lipid-water emulsions; notably, a blend of neutral lipids (including monoacyl- and diacylglycerides) previously found associated with hemozoin by Pisciotta et al.[34] Observation of crystals at lipid droplet surfaces strongly supported the epitaxial growth model where the lipid head group orientates the amphiphilic heme molecules. Diacylglycerides were found to be able to mediate the formation of β-hematin formation faster than monoacylglcerides,[35] which we previously attributed to a lower activation energy.[34b] A density functional theory (DFT) study supported the role of diacylglycerides in promoting heme aggregation, likely an essential step in crystal nucleation.[36] Finally, the size of β-hematin crystals was found to correlate with the diameter of the lipid droplets, with average crystal length approximately 60% that of the droplet diameter.[34c] This size-control may not be evident for native hemozoin, however, if the crystals form at inner membrane surfaces where the curvature is less pronounced than in synthetic droplets.[31]

Our work on the mechanism of β-hematin formation has demonstrated the efficacy of lipids in the process. The observation of β-hematin crystals aligned with their (100) surfaces parallel to the lipid-water interface is also consistent with their theoretical morphology.[37] The (001) and (011) faces have been identified as the fastest-growing, and it has been proposed that sites on these surfaces are available for the adsorption of heme from solution, explaining the predominant crystal growth in the *c*-direction.[29, 38] It has been hypothesized that antimalarial drugs may target these binding sites to impede crystal growth.[29]

Evidence of Cellular Hemozoin Inhibition and Cellular Mechanism of Action

Despite an extensive literature on β-hematin inhibition by antimalarials and experimental compounds, only in the last seven years has direct evidence of hemozoin inhibition in the malaria parasite been demonstrated. Key to this has been the development of a reliable method for measuring Fe(III) heme in aqueous solution, which we called the pyridine hemichrome inhibition of β-hematin (Phiβ) assay,[39] inspired by the well-known pyridine hemochrome assay. Although originally intended for use in high throughput screening (HTS), its employment to quantify free heme has proven to be its most useful application. In 2013 we used cellular fractionation, electron spectroscopic imaging (ESI) based on electron energy loss spectroscopy, and transmission electron microscopy (TEM) to show that the well-known antimalarial chloroquine inhibits hemozoin formation in the parasite, with a corresponding increase in "free" (non-hemozoin, non-hemoglobin) heme, which we hereafter refer to as exchangeable heme in this article.[1] Chloroquine has been known to inhibit β-hematin formation since 1992,[18] but this was the first direct evidence in *P. falciparum*. It must be noted that the exact nature of this exchangeable heme has not been elucidated. In untreated parasites it is likely to be precipitated Fe(III)protoporphyin IX, probably in the form of hematin or hemin, or a mixture of the two. It is very unlikely to be freely dissolved in solution, given its extremely low K_{sp} value at the low pH of the DV.[40] In treated parasites, it may also include complexes with the drug or experimental inhibitor.

The cellular fractionation assay mentioned above involves isolation of cultured trophozoites from RBCs by saponin lysis, followed by freeze-thaw lysis of the trophozoites.[1] This releases a cytoplasmic fraction with a weak hemoglobin (Hb) spectrum, corresponding to undigested intraparasitic Hb. The insoluble pellet is split into an exchangeable heme fraction soluble upon treatment with 2% sodium dodecyl sulfate (SDS) and 2.5% aqueous pyridine (pH 7.5) and an insoluble hemozoin pellet which is subsequently solubilized with 0.1 M NaOH. All three heme fractions are finally converted into a monomeric low spin heme-pyridine complex, permitting direct comparison. This assay demonstrated that chloroquine causes a dose-dependent decrease in hemozoin from 95% to ≈74% of total heme, while exchangeable heme and Hb increase from ≈2.5% each to ≈15% and ≈11%, respectively. The increase in exchangeable heme closely parallels the decrease in parasite survival, while the increase in undigested Hb only becomes apparent at about $2 \times IC_{50}$ of chloroquine, suggesting that exchangeable heme is responsible for chloroquine activity. ESI showed a redistribution of heme Fe into the parasite cytoplasm at the IC_{50} of chloroquine, possibly to the endoplasmic reticulum, while TEM demonstrated disruption of hemozoin crystal growth by chloroquine, since multiple crystalline domains or thin crystalline layers were seen.

Later development of a multiwell plate-based assay permitted investigation of a wider range of drugs and experimental compounds. The 4-aminoquinoline antimalarials amodiaquine and piperaquine were both found to inhibit hemozoin formation with increases in exchangeable heme similar to chloroquine (Figure 2).[41] The aryl methanols mefloquine and lumefantrine, on the other hand, inhibited hemozoin formation without an increase in exchangeable heme.[42] This indicates that these are not true hemozoin inhibitors since their behavior resembles atovaquone, a known inhibitor of the parasite mitochondrial cytochrome bc_1 complex which does not even inhibit β-hematin formation. The decrease in hemozoin is

likely an indirect effect and shows that the crucial signal for direct hemozoin inhibition in the cell is both a decrease in hemozoin and increase in exchangeable heme. The assay has been extended to a range of benzamides, triarylimidazoles and benzimidazoles that were shown to be direct cellular hemozoin inhibitors.[43]

Most, but not all compounds found to inhibit both parasite growth and β-hematin formation have been found to be hemozoin inhibitors when tested using the cellular heme fractionation assay. This strongly supports the hypothesis that these compounds act by inhibiting hemozoin formation, but also illustrates the importance of measuring inhibition in the parasite as a validation step. Further indirect support for the hemozoin inhibition hypothesis by 4-aminoquinoline antimalarials has come from proteomics studies that have found no protein targets in the parasite.[44]

High Throughput Screening

HTS for hemozoin inhibitors has utilized different β-hematin inhibition assays, often influenced by the contemporary ideas about mechanisms of hemozoin formation. The first such study was based on incorporation of radiolabeled heme onto preformed β-hematin crystals,[45] consistent with an earlier paper suggesting that formation of hemozoin was autocatalytic.[46] A later study made use of the Phiβ assay using 4.5 M acetate at 60 °C.[47] These studies screened ≈100,000 and ≈16,000 compounds, respectively, yielding new β-hematin inhibitors, but a relatively disappointing fraction were active against malaria parasites (14% and 2.5%, respectively).

Later discovery of neutral lipid catalyzed β-hematin formation prompted development of a detergent mediated assay using Nonidet P40 (NP-40).[48] Subsequent combination with aqueous pyridine for measuring unconverted hematin concluded in the screening of a 144,330 compound library.[49] Here, 32% of the β-hematin inhibitors with varied scaffolds were active against parasites, with 15% thereof exhibiting nanomolar activity. This provided a trove of data, permitting subsequent development of Bayesian models to predict β-hematin inhibition and β-hematin-inhibiting antimalarial activity.[50] When this model was used to rank 1510 US Food and Drug Administration (FDA) approved drugs, 17/32 of the top-ranked compounds had reported activity against malaria parasites and only 5/32 were reported inactive. For the bottom-ranked compounds, 25/32 had been reported inactive, with only one compound reported active against malaria parasites. Established hemozoin-inhibiting antimalarials, amodiaquine and chloroquine were ranked second and twenty-first, respectively. A subsequent investigation of 9/19 other drugs on the top 21 list revealed that eight were β-hematin inhibitors and five inhibited parasite growth. The hit rate for β-hematin inhibition among the eleven compounds for which experiments have been performed is thus 91% (10/11), while that for parasite growth inhibition is 64% (7/11). Three of the top 21 non-quinoline compounds, namely lapatinib, nilotinib and lomitapide, were found to have nanomolar IC_{50} values and little to no cross-resistance with chloroquine. They inhibited cellular hemozoin formation with the signature increase in cellular exchangeable heme and decrease in hemozoin.[51]

Benzamide, triarylimidazole and 2-phenylbenzimidazole scaffolds were among the most prominent in the NP-40 based HTS study (Figure 3).[49b] These were investigated in detail in three separate studies. In the first, symmetric N^1,N^3-diarylbenzene-1,3-dicarboxamides were found to be potent β-hematin inhibitors when the central benzene ring bore an electron withdrawing group or the two aryl rings were pyridyl rings with the N atom *meta* or *para* to the amide group. Activity against parasites increased with increasing lipophilicity, so the most potent compound with an IC_{50} of 600 nM and no cross-resistance with chloroquine was poorly water soluble.[43a] In a second study, triarylimidazoles proved intractable to modification, since substantial alterations to the original hit compounds tended to abolish activity. All had IC_{50} values >1 μM against cultured *P. falciparum*.[43b] The most potent compounds in both series were found to inhibit cellular hemozoin formation.

In a third study, benzimidazoles were synthesized from three building blocks, an *o*-phenylenediamine, a *p*-aminobenzoic acid and an aromatic carboxylic acid.[43c] All possible compounds based on 26 commercially available *o*-phenylenediamines, 24 *p*-aminobenzoic acids and 522 aromatic carboxylic acids were enumerated. The 325 728 candidate compounds were then filtered using the abovementioned Bayesian model for activity and for water solubility, reducing the list to 35 124 candidate compounds. Eventually, eighteen compounds were selected for further study. Sixteen (89%) had potent β-hematin inhibitory properties and all exhibited activity against cultured parasites, four (25%) with nanomolar activities. The best hits showed little to no cross-resistance with chloroquine and good selectivity against malaria parasites. The most active compound, with IC_{50}=410 nM against the NF54 chloroquine sensitive parasite strain, inhibited cellular hemozoin formation. Unfortunately, this series also suffered from inadequate water solubility.

Mechanism of Inhibition of Hemozoin Formation

Heme was identified as a target of chloroquine and related drugs in 1980.[52] Chou et al. attributed activity to complexation with heme, leading to membrane damage.[13a] Later these drugs were shown to inhibit β-hematin formation in the presence of parasite extracts, which was attributed to inhibition of a putative heme polymerase.[18] Soon afterwards, we showed that they inhibit β-hematin formation directly in acetate medium, and proposed that this results from complexation.[16a] Later we found that the association constants between heme and quinine, an active inhibitor, and heme and inactive 9-epiquinine are almost identical, precluding complexation as the sole determinant of β-hematin inhibition.[53]

Improved understanding of β-hematin crystallization has advanced our knowledge of the mechanism of inhibition. Shortly after publication of the structure of β-hematin in 2000,[15] Leiserowitz and co-workers proposed that inhibitors adsorb onto the corrugated fastest-growing (001) crystal face.[37] They later showed that chloroquine and quinine caused tapered ends of the usually lath-like β-hematin crystals which they ascribed to inhibition of crystal growth.[32]

In 2013, we reported the first single crystal X-ray diffraction (SCD) structure of the β-hematin dimer as a DMSO solvate.[29] The sample was grown in the presence of chloroquine, which we proposed acted as a growth-rate inhibitor, affording sufficiently large crystals for

SCD analysis, although it is possible that it simply acted as a base, facilitating Fe-carboxylate bond formation. We found a decrease in the observed first-order rate constant for the formation of β-hematin in the presence of quinoline antimalarials. This occurred at low drug concentrations without a decrease in yield, while at higher concentrations there was a substantial reduction in final yield of β-hematin. We proposed reversible adsorption to free binding sites on the crystal surface at low concentration. The corresponding drug adsorption constant, K_{ads}, was directly related to kinetic inhibition of lipid mediated β-hematin formation. At high concentration, we proposed that irreversible formation of a heme-drug complex as an insoluble precipitate accounts for decreased β-hematin yields. We later demonstrated a linear correlation between K_{ads} and β-hematin inhibitory activity for both quinolines and benzamides as well as between K_{ads} and parasite growth IC_{50} in the D10 chloroquine-sensitive strain.[2]

More recent atomic force microscopy measurements by Vekilov and co-workers in a citric acid/octanol model system has supported the "adsorption hypothesis".[38, 54] In particular, chloroquine and other antimalarials have been shown to adsorb onto the large, flat (100) crystal face impeding growth of new islands by step-pinning and kink-blocking. Adsorption was shown to be more efficient than heme-drug complex formation, accounting for inhibition of crystal growth at significantly lower concentrations. In 2019, Kapishnikov et al. used X-ray fluorescence microscopy to show that bromoquine, the bromine analogue of chloroquine, adsorbs onto hemozoin crystals in the parasite itself.[55] Thus, current evidence strongly supports the hypothesis that hemozoin inhibiting drugs poison crystal growth via adsorption onto the crystal surface.

Heme-inhibitor Complexes

Inhibition of hemozoin formation causes a build-up of free heme, widely held to be responsible for parasite killing. This would imply that the toxicity of heme is independent of the inhibitor. Consequently, all hemozoin inhibitors should effect the same level of exchangeable heme at their respective IC_{50} values.[4] In fact, diverse hemozoin inhibitors showed quite the opposite, with exchangeable heme varying markedly at the inhibitor IC_{50}. Compounds with higher IC_{50} values often led to higher levels of exchangeable heme. For eleven active inhibitors, the amount of inhibitor accumulated in the parasite as determined by the inoculum effect, was found to correlate directly with exchangeable heme. These observations point to the formation of heme-inhibitor complexes in the parasite as the key to toxicity to the organism.

We recently provided the first direct evidence for the formation of a such a heme-inhibitor complex in an NF54 chloroquine-sensitive strain of *P. falciparum*.[4] A bromo-substituted benzimidazole (Figure 3c) was selected for investigation owing to the high fraction of exchangeable heme it caused (≈25%). The low natural abundance of bromine in parasites was also advantageous for cellular imaging using electron energy loss spectroscopy (EELS). Signals for Fe and Br co-localized in the DV of parasitized RBCs. Given that heme accounts for 95% of iron in the parasite,[11c] the signal for Fe in the DV was attributed to both hemozoin and exchangeable heme. The co-localization of inhibitor with hemozoin is consistent with adsorption discussed above, while speciation of the exchangeable heme was

initially less clear. The notion of a heme-inhibitor complex has been proposed by Kapishnikov et al. following the detection of bromoquine in the DV membrane of treated parasites.[55] Using confocal Raman microscopy we could definitively identify a heme complex of the bromo-substituted benzimidazole. Principal component analysis showed that Raman peak positions from a region near the hemozoin in the DV were distinct from those of oxy- and deoxyhemoglobin, as well as hematin, hemin, hemozoin and free inhibitor. On the other hand, they were indistinguishable from those of a hemin-inhibitor mixture, corresponding to a heme-inhibitor complex. We propose that such complexes are responsible for parasite killing, either directly, by inhibiting enzyme targets or transporters in the DV or indirectly, by transferring free heme to the cytoplasm and effecting damage through the generation of reactive oxygen species. The importance of such complexation does not negate the fact that an inhibitor must first be able to inhibit hemozoin formation, likely by poisoning crystal growth (Figure 4). Thus, as discussed above, despite its strong association with heme, 9-epiquinine is inactive against β-hematin formation and has only very weak activity against parasite growth. Presumably, only following inhibition of hemozoin formation do exchangeable heme levels exceed basal levels, which then drives complexation.

To date, difficulty in isolating precipitates from the parasite milieu has precluded structure determination in situ. Attempts to obtain definitive solid-state structures of the heme-chloroquine complex have been unsuccessful. Consequently, numerous solution state and in silico studies have been conducted. The avid reader is directed to a review of the early contributions in this regard.[56] Briefly, early ^{1}H NMR studies of heme complexes of chloroquine and quinine based on a ring current model concluded that chloroquine intercalates between two heme molecules in a sandwich-type fashion. Based on the supposition that the μ-oxo dimer was the prevalent heme species in aqueous solution, a chloroquine-μ-oxo dimer complex was proposed, consistent with the 1:2 stoichiometry inherent in a sandwich structure.

In 2006, we showed that heme is a **π-π** dimer in aqueous solution,[57] and that alternative species, namely the monomer, μ-oxo dimer, or even higher aggregates, may form in mixed solvents, at high salt concentration or as a function of pH.[58] Molecular dynamics (MD) and time-resolved density functional theory (DFT) experiments, together with extended X-ray absorption fine structure (EXAFS) data, provided further insights into the electronics, structure and hydration of monomeric and dimeric heme in solution.[59] We later demonstrated that chloroquine induces formation of the heme μ-oxo dimer in solution.[60] MD simulations were carried out for two hypothetical structures, in which chloroquine was either stacked on the unligated face of the μ-oxo dimer or docked between the two oxo-bridged porphyrins.[61] EXAFS, infrared and magnetic data supported the latter structure. Interestingly, a crystal structure of a Ga(III)protoporphyrin IX complex of chloroquine has been reported.[62] In addition to π-stacking of the quinoline ring with the porphyrin, it was shown that the protonated quinoline N atom hydrogen bonds to an axial methoxide ligand on the metalloporphyrin. These spectroscopic and structural studies are indicative of specific stereochemical requirements for strong interactions in these complexes.

We reported the first SCD structure of a heme-halofantrine complex in 2008.[63] Prior to recrystallization from a water/pyridine/acetone solution, the sample was collected from an

aqueous-lipid emulsion, consistent with the prevailing proposal that lipid environments may serve as a site of drug (or heme-drug complex) action in the parasite.[64] The complex was observed to involve coordination of the alkoxide group of halofantrine to the Fe center of heme. This was later also confirmed in structures of heme-quinine, quinidine,[65] and mefloquine.[66] In the latter study, EXAFS measurements of single crystals and solution samples indicated that this interaction persists in non-aqueous solution for all three aryl methanol drug complexes. The question of course remains whether these coordination complexes occur in parasitized red blood cells. Until answered, this presents a challenge to the rational design of new inhibitors to target heme detoxification.

Structure-activity and Docking Studies

Extensive studies of chloroquine analogues, both as parasite growth and β-hematin formation inhibitors has provided a well-developed structure activity relationship model for 4-aminoquinolines.[67] The 4-aminoquinoline nucleus is the minimal substructure for strong heme complexation but does not inhibit β-hematin formation. 4-amino-7-chloroquinoline exhibits the latter activity but is not strongly active against parasites. This requires a basic amine-containing side chain. Electron withdrawing groups, such as CN, can replace the 7-Cl group while antiparasitic activity is related to the pK_a of the compound, consistent with pH trapping in the acidic parasite DV.[68] While not transferable to other scaffolds,[43a] this model has proven useful for designing new 4-aminoquinolines, such as the dibemequines. These contain a dibemethine side chain that chemosensitizes chloroquine-resistant strains of *P. falciparum* to chloroquine, thus serving a dual function.[69] They are strongly active in both cultured parasites and mouse malaria and inhibit chloroquine transport by the *P. falciparum* chloroquine resistance transporter (PfCRT). The related pyridodibemequines are also active, but both series are metabolically unstable in human and mouse liver microsomes, precluding further development.[70] Work is continuing on the metabolites that are themselves active.

Recently, we have explored molecular docking to discover new β-hematin inhibitors with activity against malaria parasites. A tranche of the ZINC15 database consisting of 7 070 compounds that were in stock for purchase, unreactive, neutral, with LogP<5 and molecular weight <450 were docked with β-hematin. This yielded 324 compounds with docking energies <−10 kcal/mol. These compounds were filtered to remove toxic liabilities (mutagenicity, tumorigenicity or irritant effects) and to comply with Lipinski's rule of five. All remaining hits were visually inspected to confirm that they were suitably docked, and fifteen were selected for further investigation. Eleven (73%) inhibited β-hematin formation and nine (60%) inhibited growth of *P. falciparum*. The two most active compounds were investigated in greater detail. One showed excellent selectivity against malaria parasites over mammalian cells and inhibited hemozoin formation in the parasite. This benzoxazole derivative (Figure 3d) is the first hemozoin inhibiting compound active against malaria parasites to be discovered using molecular docking with the crystal surface.[3] This approach was also applied to the USFDA approved drugs, identifying some of the compounds previously found using the Bayesian model, including lapatinib, nilotinib, lomitapide (Figure 3e–g) and chloroquine. The hit rate for both β-hematin inhibition and parasite growth inhibition was lower in this case (26%).[51] Molecular docking is thus a promising technique for future identification of new hemozoin-inhibiting scaffolds.

Future Prospects

Hemozoin remains an important research topic in the malaria field despite widespread chloroquine resistance. Hemozoin is still a viable target for new drugs because chloroquine resistance arises from mutations in the *pfcrt* gene, which encodes a DV membrane transporter. Resistance is compound-specific and not linked to changes in hemozoin formation.[71] Furthermore, widespread phenotypic screening for activity against malaria parasites subsequently necessitates target identification. Inevitably, hemozoin inhibitors are among the active compounds discovered. Consequently, there is a need for improved understanding of hemozoin formation and inhibition, including resolving the role of lipids and proteins in these processes.

Substantial progress is still required on several fronts. Firstly, to identify a hemozoin inhibitor, it is not adequate to demonstrate only β-hematin inhibition. Rather, it is necessary to show inhibition of hemozoin formation in the parasite itself. The current method is cumbersome, consumes considerable parasite material and uses toxic pyridine to complex heme.[41a] There is thus a need for improved assays. Development of liquid chromatography methods with colorimetric or mass spectrometric detection that use much smaller samples, preferably involving 96-well plates, would represent a major advance in this area. This would improve throughput and make the assay more accessible to many medicinal chemistry laboratories. Secondly, predicting β-hematin inhibition would assist in selection of molecules for medicinal chemistry campaigns. Here, molecular docking with hemozoin, which has already proven useful, could be expanded and improved. Additionally, methods representing chemical space in reduced dimensions, for example principal component analysis, may permit enrichment of *in silico* libraries with compounds active against specific targets, whether hemozoin or enzymes. Machine learning methods are soon also likely to assist in this process as more screening data become available. Thirdly, a better understanding of the overall hemozoin formation pathway and effects of inhibition of specific targets along this pathway could assist in target deconvolution based on changes in cellular hemozoin formation. This is a crucial consideration since there are several points along the pathway that can be inhibited, resulting in changes in hemozoin formation. Not all necessarily involve a direct interaction with heme or hemozoin. Considerable advances in all these areas are likely in the next few years.

Lastly, the recent discovery that hemozoin inhibitors probably act as heme complexes, returns the spotlight to these complexes. Key questions are the strengths of association of heme and inhibitor in aqueous and lipid environments, solubilities, lipophilicities and structures of these complexes. These are all challenging problems because of complicated speciation, low solubilities at DV pH and heme aggregation. Furthermore, the paramagnetic nature of ferriheme reduces the utility of techniques such as NMR. Innovative approaches will therefore be needed to address these challenges.

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Biographies

Katherine de Villiers was born in Bulawayo, Zimbabwe. She received her Ph.D. in chemistry in 2008 from the University of Cape Town, South Africa. She joined Stellenbosch University in 2009, where she is currently a senior lecturer in the Department of Chemistry and Polymer Science. She leads the "HaemTeamSU" research group, where her interests are in antimalarial drug development for both enzyme and non-enzyme (hemozoin) targets.

Timothy J. Egan was born in Johannesburg South Africa. He received his Ph.D. in bioinorganic chemistry in 1988 from the University of the Witwatersrand in Johannesburg, South Africa. He was a postdoctoral research associate at the Albert Einstein College of Medicine in Bronx, New York from 1991 – 1993. He joined the University of Cape Town in 1996, where he is currently Jamison Professor of Inorganic Chemistry and an affiliate member of the Institute of Infectious Disease and Molecular Medicine. He leads the bioinorganic research group, where his interests center on hemozoin inhibiting antimalarials and antimalarial drug mechanisms.

Key References

Combrinck JM; Mabotha TE; Ncokazi KK; Ambele MA; Taylor D; Smith PJ; Hoppe HC; Egan TJ, Insights into the role of heme in the mechanism of action of antimalarials. ACS Chem. Biol 2013, 8, 133–137. [PubMed: 23043646] [1] This paper describes the effects of chloroquine on intracellular hemozoin formation in the malaria parasite *Plasmodium falciparum*, demonstrating inhibition of hemozoin formation, an increase in exchangeable heme, a relocation of heme iron into the parasite cytoplasm and disruption of hemozoin crystal growth.

Fitzroy S-M; Gildenhuys J; Olivier T; Tshililo NO; Kuter D; de Villiers KA, The effects of quinoline and non-quinoline inhibitors on the kinetics of lipid-mediated β-hematin crystallization. Langmuir 2017, 33, 7529–7537. [PubMed: 28689414] [2] We demonstrated a significant correlation between the strength of adsorption of an inhibitor to β-hematin (K_{ads}) and IC_{50} in a biomimetic system, which provides strong support for the adsorption hypothesis with respect to hemozoin inhibition.

de Sousa ACC; Combrinck JM; Maepa K; Egan TJ, Virtual screening as a tool to discover new β-haematin inhibitors with activity against malaria parasites. Sci. Rep 2020, 10, 3374. [PubMed: 32099045] [3] In this paper, we use molecular docking to discover new hemozoin inhibiting compounds from an *in silico* library, demonstrate their activity against β-hematin formation, identify a sub-set active against *P. falciparum* and show that they inhibit intracellular hemozoin formation with the signature increase in exchangeable heme.

Openshaw R; Maepa K; Benjamin SJ; Wainwright L; Combrinck JM; Hunter R; Egan TJ, A diverse range of hemozoin inhibiting scaffolds act on *Plasmodium falciparum* as heme complexes. ACS Infect. Dis 2021, 7, 362–376. [PubMed: 33430579] [4] In this paper we conclude that heme-inhibitor complexes are central to parasiticidal activity of antimalarial drugs. We found a 1:1 correlation between amounts of free heme and accumulated inhibitor and confirmed the presence of a heme-inhibitor complex in parasites.

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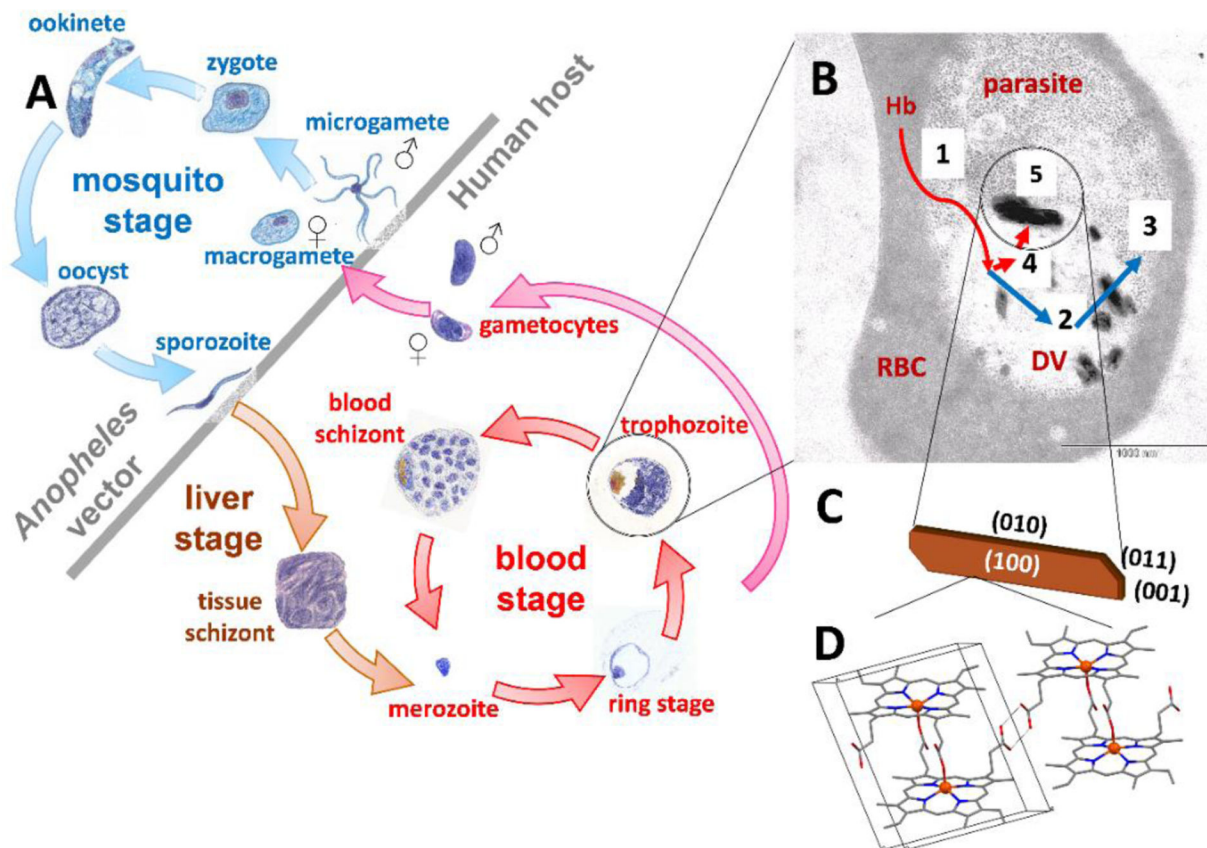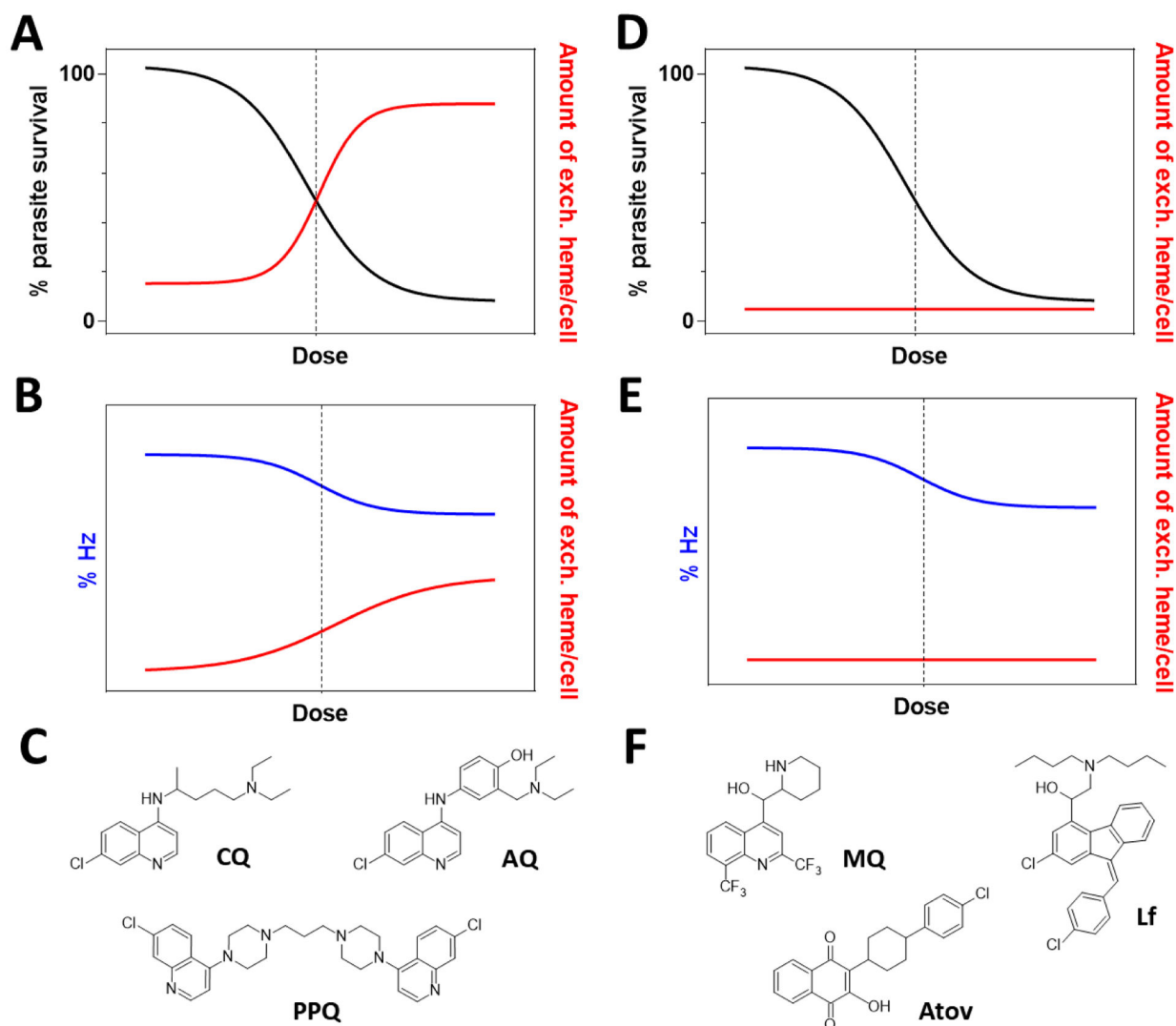
Figure 1.

Lifecycle of *P. falciparum* (**A**), showing the mosquito, liver, and pathogenic blood stages. The trophozoite (**B**), exhibits electron dense hemozoin crystals. Host RBC cytoplasm (1), mostly Hb, is ingested into the acidic parasite DV and the globin is digested by aspartic, histoaspartic, cysteine and zinc proteases to peptides (2), and finally by aminopeptidases to amino acids (3). Released heme is oxidized from the Fe(II) to Fe(III) state (4) and the latter is finally crystallized as hemozoin (5). A schematic illustration of hemozoin (**C**) shows its lath-like shape and oblique ends, with crystal faces labelled. The molecular structure is shown in (**D**), consisting of a cyclic dimer, in which the heme propionate group of each monomer coordinates to the Fe(III) center of its partner, while the propionic acid groups hydrogen bond to neighboring dimers.

Figure 2.
Two modes of action of antimalarial drugs: parasite survival (left axis, black) decreases in (**A**) and (**D**), but hemozoin inhibitors induce a corresponding increase in exchangeable (exch.) heme (right axis, red) that coincides with a decrease (left axis, blue) in hemozoin, abbreviated Hz (**B**); no change in exchangeable heme levels is observed for non-hemozoin inhibitors despite a decrease in hemozoin (**E**). Dashed vertical lines indicate parasite growth IC$_{50}$. (**C**) Examples of hemozoin inhibitors include chloroquine (CQ), amodiaquine (AQ) and piperaquine (PPQ) while (**F**) mefloquine (MQ), lumefantrine (Lf) and atovaquone (Atov) are examples of non-hemozoin inhibitors. In these schematic graphs which illustrate expected trends, no quantities are shown on the right axes or on the left axes in B and E because the values are strongly dependent on the identity of the inhibitor. Hemozoin levels in untreated parasites are ≈95%.
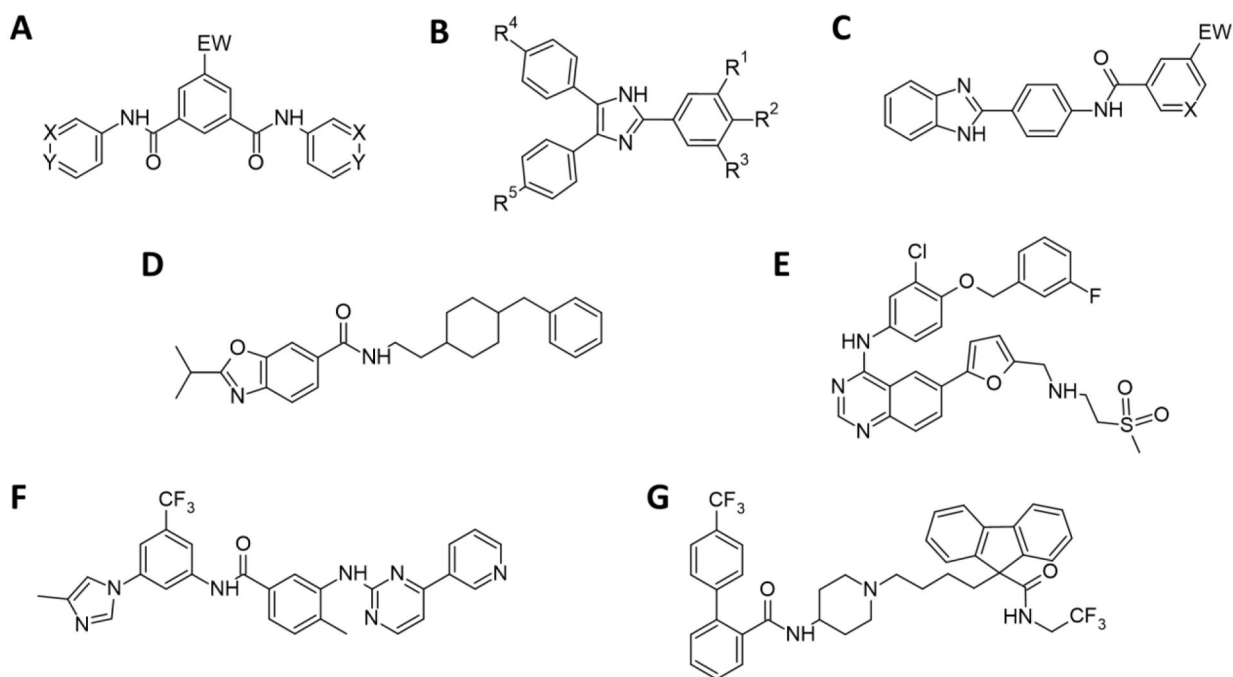
Figure 3.
Scaffolds and compounds identified through HTS. (**A**) The symmetric N^1,N^3-diarylbenzene-1,3-dicarboxamide series demonstrated potent β-hematin inhibitory activity with either EW being an electron withdrawing group or with X or Y = N. (**B**) Active triarylimidazoles had R^1 and R^3 = OMe. R^2 = OH favored activity, but if R^2 = H, R^4/R^5 were required to be halogens or OH/OMe. (**C**) A series of 2-phenylbenzimidazoles identified through enumeration. For β-hematin inhibition EW = an electron withdrawing group was most favorable, as was X = N. The test compound used in subsequent electron energy loss and Raman spectroscopy studies contained EW = Br and X = N. (**D**) A benzoxazole with both β-hematin inhibition activity and activity against cultured parasites identified by virtual screening. USFDA approved drugs (**E**) lapatinib, (**F**) nilotinib, and (**G**) lomitapide highly ranked using a Bayesian approach, also found by virtual screening. All three were confirmed to be inhibitors of cellular hemozoin formation.
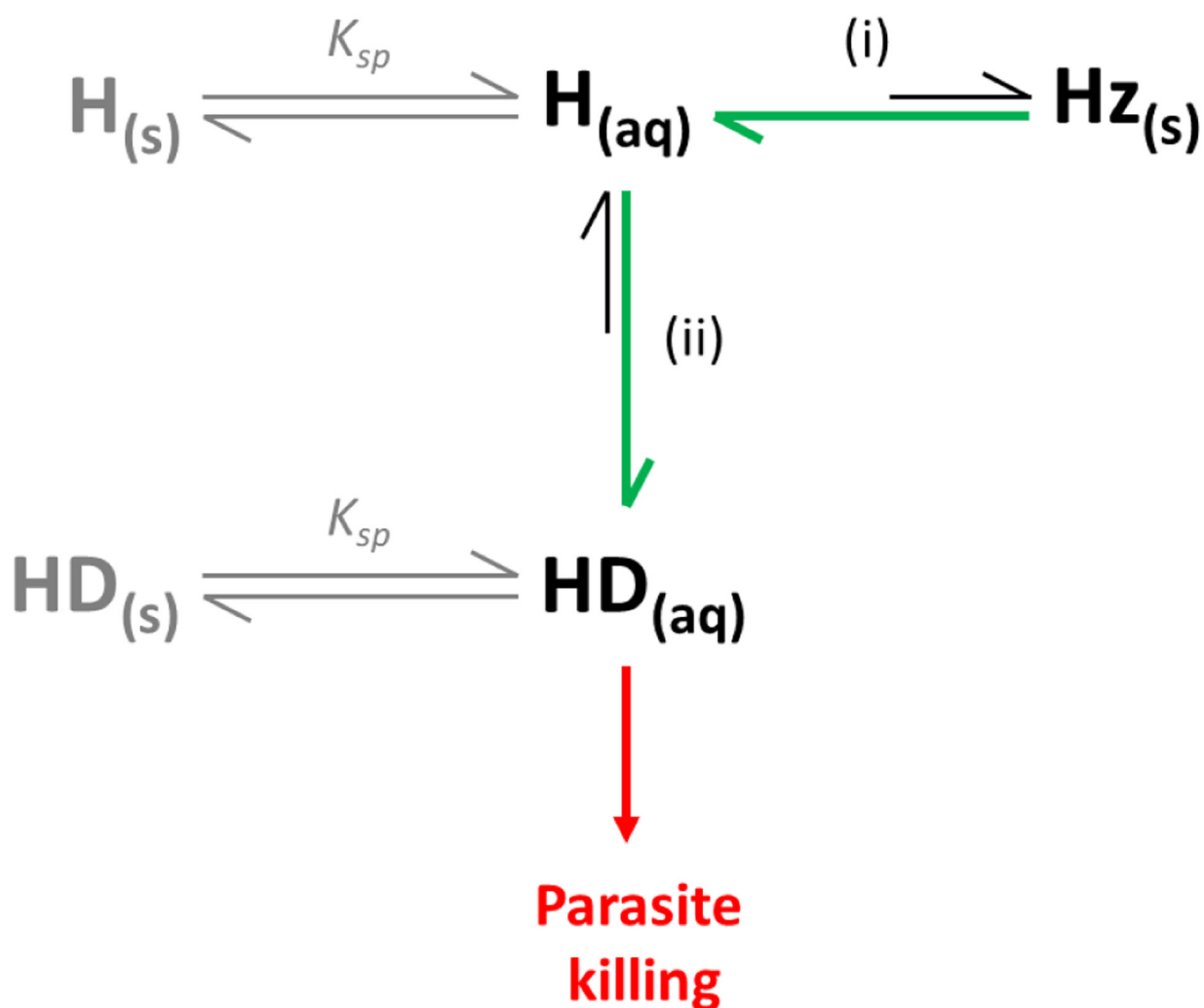
Figure 4.
Proposed heme speciation in the presence of hemozoin inhibitors. Drugs targeting the hemozoin pathway (i) cause a decrease in hemozoin (Hz) formation via adsorption, resulting in a build-up of exchangeable heme ($H_{(aq)}$). These drugs form complexes ($HD_{(aq)}$) with free heme (ii), that are likely responsible for parasite killing (red arrow). Green arrows indicate the shift in equilibria in the presence of a Hz inhibitor. Precipitation of free heme ($H_{(s)}$) and heme-drug complexes ($HD_{(s)}$) represent competing processes (grey) that depend on their solubility products (K_{sp}).