Artemisinin-Based Drugs Target the *Plasmodium falciparum* Heme Detoxification Pathway

Kaleab A. Ribbiso,^{a,b} Laura E. Heller,^{a,b} Abigail Taye,^{a,b} Erin Julian,^c Andreas V. Willems,^{a,b} Paul D. Roepe^{a,b}

^aDepartment of Chemistry, Georgetown University, Washington, DC, USA

^bDepartment of Biochemistry and Cellular and Molecular Biology, Georgetown University, Washington, DC, USA

^cDepartment of Biology, Loyola University Maryland, Baltimore, Maryland, USA

ABSTRACT Artemisinin (ART)-based antimalarial drugs are believed to exert lethal effects on malarial parasites by alkylating a variety of intracellular molecular targets. Recent work with live parasites has shown that one of the alkylated targets is free heme within the parasite digestive vacuole, which is liberated upon hemoglobin catabolism by the intraerythrocytic parasite, and that reduced levels of heme alkylation occur in artemisinin-resistant parasites. One implication of heme alkylation is that these drugs may inhibit parasite detoxification of free heme via inhibition of heme-to-hemozoin crystallization; however, previous reports that have investigated this hypothesis present conflicting data. By controlling reducing conditions and, hence, the availability of ferrous versus ferric forms of free heme, we modify a previously reported hemozoin inhibition assay to quantify the ability of ART-based drugs to target the heme detoxification pathway under reduced versus oxidizing conditions. Contrary to some previous reports, we find that artemisinins are potent inhibitors of hemozoin crystallization, with effective half-maximal concentrations approximately an order of magnitude lower than those for most quinoline-based antimalarial drugs. We also examine hemozoin and *in vitro* parasite growth inhibition for drug pairs found in the most commonly used ART-based combination therapies (ACTs). All ACTs examined inhibit hemozoin crystallization in an additive fashion, and all but one inhibit parasite growth in an additive fashion.

KEYWORDS dihydroartemisinin, artesunate, artemether, hemozoin, synergy

The two parasites responsible for most human malaria infections are *Plasmodium vivax* and *Plasmodium falciparum*, the latter being responsible for the majority of the ~405,000 deaths due to malaria in 2018 (1). Artemisinin (ART)-based combination therapies (ACTs) are the current front-line treatment for *P. falciparum* malaria; however, the spread of evolving ART resistance (ARTR) that compromises ACT use in some settings is a deeply worrying phenomenon. ARTR was first reported over a decade ago in Southeast Asia (2, 3) and is clinically characterized as an infection with a clearance half-time of >5 h after ACT treatment (4). This “delayed clearance phenotype” (DCP) has been associated with mutations in the *pfk13* gene (1, 5) that result in amino acid substitutions in the PfK13 protein propeller domain; however, although progress has been made, a molecular mechanism for PfK13-mediated ARTR remains elusive. Recent insights include the identification of altered kinetics of intraerythrocytic parasite development for parasites harboring PfK13 mutations (6, 7) and the identification of multiple mutant PfK13 interaction partners that appear to mediate altered endocytic traffic for ARTR parasites (8), altered hemoglobin (Hb) traffic in ARTR parasites (8–10), and reduced formation of ferriprotoporphyrin IX (FPIX) heme: dihydroartemisinin (DHA) adducts within live ARTR parasites (11, 12). Since free FPIX is released upon Hb catabolism by the intraerythrocytic parasite, and since FPIX heme iron is likely necessary for activating the toxic alkylating ability of artemisinin-based drugs,

Citation Ribbiso KA, Heller LE, Taye A, Julian E, Willems AV, Roepe PD. 2021. Artemisinin-based drugs target the *Plasmodium falciparum* heme detoxification pathway. *Antimicrob Agents Chemother* 65:e02137-20. <https://doi.org/10.1128/AAC.02137-20>.

Copyright © 2021 American Society for Microbiology. All Rights Reserved.

Address correspondence to Paul D. Roepe, paul.roepe@georgetown.edu.

Received 7 October 2020

Returned for modification 14 December 2020

Accepted 16 January 2021

Accepted manuscript posted online 25 January 2021

Published 18 March 2021

these observations related to PfK13-mediated ARTR appear to be coalescing toward a unified model of reduced endocytic Hb traffic and the resultant Hb catabolism during early parasite development within the human red cell, resulting in the slower release of free FPIX and, hence, the lower activation of artemisinin-based drugs by FPIX iron at key times during intraerythrocytic parasite development.

Several studies have found that a plethora of parasite proteins can be alkylated by activated ART-based drugs (13–16); however, there is little overlap between the various proteins identified in these studies (12), suggesting that protein alkylation by ART-based drugs is stochastic (12, 14). For alkylation to occur within live parasites, the essential endoperoxide bridge of ART-based drugs (17, 18) must first be activated via reduction by reduced iron. Since the most available and accessible form of reduced iron within live malarial parasites is reduced FPIX heme released upon Hb catabolism within the digestive vacuole (DV) of the parasite (7, 11), several studies have suggested that DV FPIX is necessary for ART-based drug pharmacology (11, 15). Previous *in vitro* studies by Meshnick et al., Posner et al., and Robert and colleagues clearly showed that once activated by ferrous FPIX, ART-based drugs may then form covalent drug-FPIX adducts, at least under laboratory solution conditions (19–23). Recently, the suspicion that such adducts might form within live parasites has been confirmed, and reduced levels of these adducts have been quantified for live ARTR parasites (7, 11). Heme has also been found to be an alkylation target of synthetic ozonide (OZ) antimalarials (24), which rely on a similar mode of endoperoxide pharmacophore activation (25). Due to the proximity of the FPIX porphyrin ring upon FPIX iron activation, FPIX is likely to be one of the more common alkylation targets of activated ART-based drugs. Since FPIX heme released upon Hb catabolism must be detoxified by crystallization to inert non-toxic hemozoin (Hz) for the parasite to survive, and since drug-FPIX adducts are predicted to inhibit that crystallization, the reduced alkylation of FPIX for ARTR parasites (11) suggests that inhibition of FPIX-Hz chemistry may be an important facet of ART-based drug molecular pharmacology.

Quinoline-based antimalarial drugs such as chloroquine (CQ) inhibit the formation of Hz (26, 27). This largely involves the formation of noncovalent drug-heme complexes that then inhibit the growth of Hz and presumably lead to a buildup of toxic free FPIX within the DV (28). It has been suggested that ART-based drugs might act in a similar fashion (29); however, this was later challenged (30). Many studies have continued to examine the ability of antimalarial drugs to inhibit Hz formation (30–47); however, none have quantified the potency of Hz crystallization inhibition for different ART-based drugs. In previous studies that examined ART-based drug inhibition of Hz formation, only putative noncovalent interactions were examined since inhibition was measured under oxidizing conditions that prevent endoperoxide activation.

In this study, we modify a previously reported β -hematin inhibition assay (BHIA) that quantifies drug inhibition of FPIX-Hz chemistry under close-to-physiological conditions (33) in order to quantify inhibition of Hz crystallization under reducing versus oxidizing conditions for the commonly used ART-based drugs artesunate (ATS), artemether (ATM), and dihydroartemisinin (DHA). Although organelle-specific concentrations are currently unknown, glutathione (GSH) is the principal reductant found within most eukaryotes, including *Plasmodium* (48–50). In the presence of GSH, Fe(III) protoporphyrin IX (PPIX) is reduced to Fe(II)PPIX, which is then capable of activating these ART-based drugs (11, 22). Under biological conditions, once activated, these drugs rearrange to yield a C_4 radical that may form a covalent ART-FPIX adduct, during which ferrous FPIX is reoxidized back to ferric FPIX (11, 21). FPIX-heme adducts formed in this fashion could then act as Hz crystallization poisons since the bulky adduct would be unable to be incorporated within the growing Hz crystal. Here, we quantify the relative potencies of several different ART-based drugs to inhibit Hz formation in the presence versus the absence of common ACT partner drugs.

FIG 1 Structures of the antimalarial drugs used in this study. These drugs can be categorized as “artemisinin derivatives,” “quinolines,” and “other.” Common artemisinin combination therapies (ACTs) include dihydroartemisinin (DHA) plus piperazine (PPQ), artesunate (ATS) plus amodiaquine (AQ), and artemether (ATM) plus lumefantrine (LF).

RESULTS

β -Hematin inhibition assay. We examined several ART-based drugs (Fig. 1, top) for their ability to inhibit the formation of Hz *in vitro* under either oxidized (O-BHIA) (33) or reduced (R-BHIA) conditions. The reduction of Fe(III)PPIX in dimethyl sulfoxide (DMSO) or DMSO-water mixtures is nearly instantaneous (51); therefore, under the conditions of the modified R-BHIA (see Materials and Methods), most heme iron is in the Fe(II) state before the addition of the drug, whereas in the absence of the GSH reductant (O-BHIA), FPIX heme remains as Fe(III)PPIX (7, 51). By measuring the absorbance at 405 nm, corresponding to the FPIX Soret peak, we quantify the disappearance of free FPIX during the formation of Hz localized to a bicarbonate-insoluble pellet produced in the BHIA (see Materials and Methods) (33).

In our O-BHIAs, we found no inhibition of Hz formation even at the highest ART-based drug concentrations tested (1 mM) (Fig. 2, left two panels, open symbols). In contrast, control experiments with CQ (Fig. 2, right, open symbols) showed potent inhibition, as reported previously. This inhibition is due to the ability of CQ to form noncovalent and dative complexes with FPIX under oxidized conditions (28).

In contrast, once FPIX is preincubated with GSH to form Fe(II)PPIX, clear inhibition of Hz formation is observed for ART-based drugs (Fig. 2, closed symbols), with 50% effective concentration (EC_{50}) values in the micromolar range (Table 1). We also note a significant, but much less profound, increase in CQ Hz inhibition potency under reducing conditions (Fig. 2, right, closed versus open symbols, and Table 1). By comparing Hz formation under reduced and oxidized BHIA conditions, these data further support previous conclusions that the reduction of Fe(III)PPIX is a crucial step in heme activation of ART-based drugs (11, 19–23) and support the notion that ART-based drugs are potent inhibitors of Hz formation.

Following the validation of the O-BHIA versus the R-BHIA, and noting that (to our knowledge) all ART *P. falciparum* parasites are derived from chloroquine-resistant

FIG 2 Representative β -hematin inhibition curves. Data $\pm$ standard errors of the means (SEM) are shown for one experiment performed in triplicate at pH 5.2 under reduced (filled circles) versus oxidized (empty circles) conditions (see the text). For artesunate (left) and artemether (center), inhibition of H₂ formation is observed only under reducing conditions, whereas chloroquine (right) inhibits H₂ formation under both oxidized and reduced conditions, with a slight reduction in the EC₅₀ for the latter (see the text).

(CQR) *P. falciparum* isolates reported to have lower DV pHs (52–54), we tested whether inhibition of H₂ by ATM, ATS, or DHA (Fig. 1) is affected by the small shifts in pH previously noted for CQ-sensitive (CQS) versus CQR and ART_R parasites (52–54). As summarized in Table 1, the data suggest that ATS, ATM, and DHA remain inactive at both pHs under oxidizing conditions but are slightly more potent H₂ formation inhibitors at the higher pH characteristic of CQS parasites under reducing conditions. Thus, we suggest that the lower pH characteristic of the CQR parasite DV (also measured for parasites that are both CQR and ART_R [K. Iyengar and P. D. Roepe, unpublished data]) may provide an additional modest layer of protection versus ART-based drugs. Similarly, as previously reported (33), we found that under oxidizing conditions, all quinoline-based drugs tested retained activity at both pHs but were similarly slightly less potent at the lower pH characteristic of the CQR DV (53, 54). Also, where such measurements were possible, quinoline-based drugs had lower EC₅₀ values under reduced than under oxidized conditions (Table 1). While we do not yet have a simple explanation for the increased potency of quinoline drugs under reduced conditions, based on the geometry of CQ-FPIX complexes, we suggest that it is possible that the heme binding affinity for quinoline drugs is higher for Fe(II)PPIX than for Fe(III)PPIX (55, 56) (see Discussion).

ACT fixed-ratio isobologram analysis. In the clinic, ART-based drugs are used as ACTs in combination with “partner drugs” such as amodiaquine (AQ), piperaquine (PPQ), and lumefantrine (LF), with the most common ACTs in use today being ATM-LF, DHA-PPQ, and ATS-AQ (Fig. 1). To study the effect of common ACT partner drugs on H₂ inhibition by ART-based drugs, according to the Chou-Talalay 1-point titration

TABLE 1 EC₅₀ values for H₂ inhibition under oxidized (O-BHIA) versus reduced (R-BHIA) conditions^a

Compound	Avg EC ₅₀ of compound $\pm$ SEM			
	pH 5.2		pH 5.6	
	O-BHIA (μ M)	R-BHIA (μ M)	O-BHIA (μ M)	R-BHIA (μ M)
DHA	>1,000	22.5 $\pm$ 0.1	>1,000	14.1 $\pm$ 2.3
ATM	>1,000	31.6 $\pm$ 3.2	>1,000	9.6 $\pm$ 1.7
ATS	>1,000	33.5 $\pm$ 2.6	>1,000	9.2 $\pm$ 2.2
CQ	194.9 $\pm$ 5.6	66.7 $\pm$ 4.2	52.9 $\pm$ 8.0	22.2 $\pm$ 2.8
AQ	70.1 $\pm$ 1.3	19.1 $\pm$ 1.5	22.1 $\pm$ 0.9	7.3 $\pm$ 3.5
MQ	— ^b	—	—	24.4 $\pm$ 4.5
PPQ	15 $\pm$ 0.3	7.7 $\pm$ 0.5	—	4.0 $\pm$ 1.2
LF	—	27.2 $\pm$ 2.0	—	9.9 $\pm$ 1.5

^aDifferent pHs correspond to the pHs of the DV in CQS/ARTS (pH 5.2) versus CQR/ART_R (pH 5.6) strains of *P. falciparum*. All values are the averages from three separate experiments, each performed in triplicate, $\pm$ the standard errors of the means.

^b—, poor solubility at pH 5.2.

FIG 3 Isobologram analysis for Hz inhibition of common ACTs. The black dashed line indicates an FIC value of 1, or absolute additivity. The effect of the common ACTs is additive with respect to Hz inhibition, suggesting that the partner drug and the ART-based drug act on the same molecular target. Red circles, DHA-PPQ; green diamonds, ATM-LF; gray triangles, ATS-AQ; blue squares, ATS-MQ.

method for assessing drug synergy (57), we performed O-BHIAs and R-BHIAs at different ART drug/partner drug ratios. These ratios were normalized to the drugs' respective Hz inhibition EC_{50} s measured for each drug alone (meaning that "1" corresponds to $1 \times EC_{50}$ for the drug and that "2" corresponds to $2 \times EC_{50}$ [Table 1], etc.) Representative isobologram plots are shown in Fig. 3, and as summarized in Table 2, all combinations tested were found to be additive (fractional inhibitory concentration [FIC] index of ~ 1) with respect to their ability to inhibit Hz formation (58, 59).

We also examined the effects of these drug combinations versus growth inhibition for three different *P. falciparum* strains, HB3 (CQS and ARTS), Cam WT (CQR and ARTS), and Cam WT-C580Y (CQR and ARTR) (Table 3). Again, most of the combinations were approximately additive, with the exception that ATM-LF showed high antagonism for the CQS strain (HB3) and highly synergistic effects for the CQR/ARTS (Cam WT) and CQR/ARTR (Cam WT-C580Y) strains at a 1:1 EC_{50} ratio. These trends in antiparasitic versus Hz inhibition activities merit additional study at additional ACT drug ratios that mimic currently unknown blood concentrations for each drug when given via the relevant ACT.

DISCUSSION

Over the past several years, the list of possible ART-based drug molecular targets has continued to grow (13–16, 19–23). Several studies have identified a long list of proteins that are alkylated by activated ART-based drugs (13–16), but there is relatively little overlap among the proteins identified in these studies, suggesting that protein alkylation is stochastic (12, 14). Other nonprotein targets for alkylation are also possible, for example, the drugs that have been shown to alkylate heme *in vitro* (19–23) and *in vivo* (11). However, even though early *in vitro* data show that ART-based drugs can in theory alkylate heme, the data examining whether ART-based drugs inhibit Hz formation have conflicted. By modifying the BHIA (33) to inspect reducing as well as oxidizing conditions, we have quantified relative Hz inhibition potencies for different ART-based drugs. Many studies have shown that quinoline-based antimalarial drugs inhibit Hz formation *in vitro* and *in vivo*, and some of these have quantified the potencies for different quinoline-based drugs (e.g., see references 28, 29, 31, 33, 34, 36–40, and 99), but to our knowledge, none have examined the effects of ART-based and quinoline drug combinations. This is important since leading ACTs combine ART-based and quinoline drugs (Fig. 1).

Spatial diffusion of the ART radical. As mentioned above, there is relatively little overlap between ART-based drug protein alkylation targets identified in several previous proteomic studies (13–16). We suggest that the relative alkylation of any target is

TABLE 2 Summary of FIC indices from isobologram analyses of common ACTs^a

Drug combination	Avg FIC index $\pm$ SEM at ratio of:		
	3:1	1:1	1:3
DHA-PPQ ^b	1.20 $\pm$ 0.03	1.27 $\pm$ 0.03	1.11 $\pm$ 0.24
ATM-LF ^b	1.05 $\pm$ 0.07	1.09 $\pm$ 0.11	1.06 $\pm$ 0.15
ATS-AQ ^b	1.00 $\pm$ 0.12	0.98 $\pm$ 0.10	1.01 $\pm$ 0.05
ATS-MQ ^c	0.92 $\pm$ 0.09	0.94 $\pm$ 0.11	0.93 $\pm$ 0.08

^aSee Fig. 3. An FIC index of <1 is synergistic, ~ 1 is additive, and >1 is antagonistic. Values are the averages from 2 or 3 independent experiments, each performed in triplicate, $\pm$ the standard errors of the means.

^bPerformed at pH 5.2.

^cPerformed at pH 5.6 due to solubility limits.

proportional to its abundance and/or proximity to the activated ART radical. Following this model, alkylation of FPIX is also expected and is indeed observed (11) since it is immediately proximal following the activation of ART-based drugs within the DV.

Using what is currently known about *P. falciparum* protein subcellular localization, we note that for any study reported to date, the localization of ART-based drug protein targets may span nearly the entire parasite (12). However, the most efficient alkylation must satisfy two criteria: the lifetime of the ART radical must be long enough for the radical to reach the target, and the target must be located proximal to the DV. Several common protein targets identified in multiple studies satisfy the second criterion (12). We propose that the most prominent molecular targets are those that are both in the highest abundance and closest to (or within) the DV.

With regard to the first criterion, to our knowledge, no measurements or estimates of any ART-based drug radical's lifetime have yet been made (note that the radical lifetime is not equivalent to the biological half-life). To estimate the distance that the radical is able to diffuse, consideration of Einstein-Brownian motion (60) and the lifetimes of other organic small-molecule radicals is helpful. Using an abbreviated Stokes-Einstein equation (equation 1) (60), where λ is the mean displacement along one axis, D is the diffusion coefficient, and t is time, we can estimate the diffusion distance of a radical with a specific lifetime:

$$\lambda \approx \sqrt{2Dt} \quad (1)$$

The diffusion coefficient, which is proportional to the size of the molecule in question, is approximately $10^{-5} \text{ cm}^2 \text{ s}^{-1}$ for small molecules with molecular masses of approximately hundreds of daltons (61). When comparing radical lifetimes, we must also consider the environment in which such measurements are made. Upon the activation of an ART-based drug, the oxygen-centered (alkoxy) radical is initially formed but rearranges to form the carbon-centered C_4 radical (17). The C_4 radical is the more reactive alkylating species (62) and is shorter-lived than the alkoxy radical. If we therefore assume that the alkoxy radical is limiting for diffusion, the lifetime of activated ART intracellularly is on the order of 10^{-6} s (63–66). Thus, the distance traveled by the ART radical will be 10 nm to hundreds of nanometers. This can be compared to the dif-

TABLE 3 Summary of FIC indices for parasite growth inhibition at 1:1 EC₅₀ ratios^a

Drug combination	Avg FIC index $\pm$ SEM		
	HB3	Cam WT	Cam WT-C580Y
DHA-PPQ	1.36 $\pm$ 0.15	0.79 $\pm$ 0.03	1.07 $\pm$ 0.01
ATM-LF	2.75 $\pm$ 0.04	0.25 $\pm$ 0.02	0.33 $\pm$ 0.02
ATS-AQ	0.98 $\pm$ 0.01	1.04 $\pm$ 0.02	0.92 $\pm$ 0.06
ATS-MQ	0.86 $\pm$ 0.02	1.41 $\pm$ 0.03	1.29 $\pm$ 0.00

^aHB3 is CQS and ARTS, Cam WT is CQR and ARTS, and Cam WT-C580Y is CQR and ARTR. An FIC index of <1 is synergistic, 1 is additive, and >1 is antagonistic. Values are the averages from three independent experiments performed in triplicate $\pm$ the standard errors of the means.

fusion distance of other short-lived species, such as peroxynitrite, which has a half-life of $\sim 10^{-3}$ s and a calculated diffusion distance ranging from 0.5 to 5 μm in erythrocytes or blood plasma, respectively (67). We suggest that the molecular targets of ART-based drugs, proteins or otherwise, are likely to be found either within or close to the DV. This is consistent with the *in vivo* alkylation of free FPIX within the DV (11), proteins implicated in Hb trafficking to the DV (8), and proteins involved in Hb catabolism (8).

FPIX abundance and Pfk13 polymorphisms. Polymorphisms most commonly associated with ART-based drug resistance phenomena confer amino acid substitutions within the propeller domain of the Pfk13 protein. Two of the most commonly observed are C580Y and R539T Pfk13 (9, 68–70), each of which appears to impair Hb uptake (8). This predicts reduced alkylation of FPIX and of proteins implicated in Hb trafficking or Hb catabolism. A consequence of the latter two predictions would be reduced availability of ferrous FPIX, yielding reduced activation of ART-based drugs, which would further contribute to reduced alkylation of FPIX (11). Whether reduced alkylation of some protein targets in ARTR parasites occurs is yet to be examined, but if this occurs, it would presumably be an indirect consequence of reduced ART-based drug activation due to reduced FPIX upon altered Hb uptake, traffic, and/or catabolism at key stages of parasite intraerythrocytic development.

Via multiple studies, Pfk13 has been localized throughout the parasite (8, 10, 69, 71–74), including endocytic vesicles and/or cytostomes (8, 10, 72, 73). Pfk13 expression is reduced in ARTR parasites relative to ARTS parasites, ARTR parasites can be resensitized to ART by increasing Pfk13 expression (73), and ARTS parasites can be desensitized to ART by Pfk13 mislocalization (10). However, deletion of *Pfk13* is lethal to the parasite (72, 75). An interesting observation then is that Pfk13 has never been identified as an ART-based drug alkylation target (13–16). Taken together, these data suggest that while certain Pfk13 mutations confer some ARTR as measured by the ring stage survival assay (RSA) (5), the mechanism is indirect, with one indirect consequence being impaired Hb catabolism (8–10). A consequence would then be altered Hz inhibition in ARTR parasites, as has also been measured (7). Interestingly, the introduction of a substitution in the *Toxoplasma gondii* K13 protein orthologue, analogous to the C580Y Pfk13 substitution, also confers reduced ART susceptibility in *T. gondii* (76).

Effect of the iron oxidation state on quinoline-FPIX binding. We found that Hz inhibition EC_{50} s for quinolines were lower under reduced conditions, suggesting that Fe(II) promotes stronger quinoline drug-FPIX binding. This is reasonable based on previous studies that examined the binding of nitrogen-containing compounds to iron, including CQ binding to monomeric FPIX (56). We note that there are two binding motifs expected for quinoline-FPIX interactions: dative covalent and noncovalent (28, 55, 56, 77).

The most commonly observed quinoline-FPIX interactions are pseudo-face-centered stacking π - π interactions between the porphyrin ring and the aromatic quinoline heterocycle. Over the past decade, several X-ray crystal structures have been solved, showing features of these interactions for quinine, quinidine (78), and halofantrine (79) that are consistent with structures derived previously via nuclear magnetic resonance (NMR) studies (55, 56, 80, 81). While we have no complete explanation for how the Fe oxidation state for FPIX might influence the strength of quinoline binding, we propose a simple model based on electrostatic differences (82) between Fe(II)PPIX and Fe(III)PPIX. The quinolines tested here have an electron-withdrawing chlorine on the aromatic ring system (Fig. 1). Fe(III) withdraws electrons from the porphyrin ring more strongly than Fe(II), resulting in a more-electron-dense ring for Fe(II)PPIX, which would then interact more strongly with an electron-deficient quinoline ring (82). To our knowledge, data testing this concept for antimalarial drugs are limited. Future computational studies may prove useful, along with experiments using quinoline derivatives containing electron-withdrawing versus -donating groups.

Another binding mode is dative covalent coordination between a nitrogen electron lone pair and the FPIX iron (56). Two studies show higher-affinity binding of N-containing compounds to ferric, but not ferrous, iron, which is the opposite of what we

TABLE 4 Summary of previously determined binding constants for NO with reduced versus oxidized iron within heme^a

Protein	Binding constant (M ⁻¹)		Reference(s)
	Fe ²⁺	Fe ³⁺	
Mb	1.4×10^{11}	1.4×10^4	86, 88
Hb	5.3×10^{11}	4.0×10^3	88, 89
Cyt c	2.9×10^5	1.6×10^4	86
Fe	1.0×10^9	1.1×10^3	86

^aMb, myoglobin; Hb, hemoglobin; Cyt c, cytochrome c; Fe, FeTPPS [iron-5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato].

observe (83, 84). However, the published computational work (84) does not examine binding under aqueous conditions, as is also the case for work by Chiavarino et al. (85). In contrast, other studies that examine the binding of NO to various enzymes, done in aqueous environments, show significantly stronger NO binding to ferrous iron, in fact, stronger by up to an impressive 6 orders of magnitude (86, 87). A summary of these data, where binding was measured for biologically relevant iron in aqueous media (86, 88, 89), is shown in Table 4, as revised from data reported previously by Wanat et al. (87). Taken together, previous data show that the observed EC₅₀ shifts for quinoline-based drugs are easily attributed to a higher affinity for Fe(II)PPIX.

ACT partner drug effects. Perhaps not surprisingly then, we also find that common ACT drug pairs act largely in an additive fashion for both Hz inhibition (Table 2) and parasite growth inhibition (Table 3), with the exception of ATM-LF activity versus live parasites. We note that in the R-BHIA, there is one target to act upon (FPIX), which presumably enhances competitive ART-based drug versus partner drug binding (for which we then expect additive behavior), whereas LF may have multiple targets within the cell, promoting deviation from additivity for ATM-LF versus growth inhibition. Further studies are needed to elaborate upon these conclusions.

Summary. Overall, these data and conclusions can be incorporated into a consolidated model for the mechanism of ART-based drugs (Fig. 4). Hb from the red cell cytosol (Fig. 4, "1") is transported to the parasite DV ("4"), in part mediated indirectly by PfK13 (green star) (8, 10, 72, 73). Hb is catabolized by plasmepsins and falcipains within the DV (90–92), which releases toxic FPIX. Initially, FPIX is in the reduced form but rapidly oxidizes to Fe(III)FPIX. DHA or other clinically useful ART-based drugs that are quickly converted to DHA (93) are activated by Fe(II)PPIX to generate the DHA C₄ radical (Fig. 4, "DHA*") (17), which can react with a variety of targets (11–16). One such target, the highly abundant and proximal-to-DHA*-protoporphyrin ring of FPIX, is efficiently alkylated, leading to both decreased Hz formation and an increased concentration of toxic DHA-FPIX adducts that may also diffuse to the cytosol (Fig. 4, "3"), as has also been postulated for quinoline drug-FPIX adducts (94). These lead to increased oxidative stress throughout the parasite. DHA radicals also alkylate proteins within the DV, the DV membrane, or the cytosol in a stochastic fashion (13–16), with the preferred protein targets being those that are most abundant and most proximal to DV Fe(II)PPIX (12). Altered Hb catabolism and Hb uptake in ARTR parasites (7, 10) are predicted to reduce the concentration of free FPIX, thereby reducing the efficiency of DHA activation, Hz inhibition, and the abundance of DHA-FPIX and DHA-protein adducts (11, 12). Although reduced FPIX adduct abundance has indeed been reported for ARTR parasites (11), no data comparing protein adduct abundances for ARTS versus ARTR parasites are yet available.

While it might be tempting to conclude that Hz inhibition, or alkylation of any specific protein, is the most pharmacologically relevant target of any ART-based drug, several questions are important. One question is which specific adduct(s) exerts cytostatic (parasite growth-inhibitory) versus paracytotoxic (parasite kill) effects (33, 95, 96). We propose that the Hz inhibition pathway is an additional, crucial target of ART-based drugs. We suggest that quantifying differences in the abundances of various DHA

FIG 4 Proposed antimalarial mechanism of ART-based drugs. Hemoglobin (Hb) is catabolized by plasmepsins and falcipains within the DV ("4") to produce short peptides and amino acids, liberating ferriprotoporphyrin IX (FPIX) in the process. Dihydroartemisinin (DHA) is activated through a Fenton reaction with reduced ferriprotoporphyrin IX [Fe(II)PPIX] in the parasite DV, after which the DHA radical reacts with multiple targets, including proximal proteins in a stochastic fashion and FPIX itself. This chemistry has at least three effects: protein adducts may then (i) impact the proteasome pathway, while DHA-FPIX adducts will both (ii) inhibit FPIX detoxification to hemozoin (Hz) and (iii) cause oxidative stress. One consequence of the first effect would be the induction of parasite autophagy (58, 95, 96), which interestingly appears to be altered in CQR and ARTR parasites (58, 96). Altered abundance, or propeller domain mutation, of the Pfk13 protein (green stars) partially lowers Hb uptake (8–10) and the amount of liberated FPIX, thereby decreasing the amount of activated DHA at key stages of parasite intraerythrocytic development. Green stars, Pfk13 locations (see the text); 1, red blood cell cytosol; 2, parasitophorous vacuole; 3, parasite cytosol; 4, parasite digestive vacuole; 5, apicoplast; 6, Golgi apparatus; 7, endoplasmic reticulum; 8, nucleus; 9, mitochondrion; 10 (red box), DHA-FPIX adducts; 11 (green box), DHA-protein adducts. Abbreviations: AA, amino acids; Fe(III)PPIX, oxidized ferriprotoporphyrin IX.

adducts and their biological effects, for ARTS versus ARTR parasites, will ultimately reveal their relative importance. Regardless, considering the unique nature of Hz detoxification along with the unique very high potency of ART-based drugs versus malarial parasites compared to other cells, we suggest that poisoning Hz crystallization via the formation of DHA-FPIX adducts is a particularly important target of ART-based drugs. This conclusion is further supported by the reduced abundance of DHA-FPIX adducts for ARTR versus ARTS parasites (11).

Finally, we note that similar to ART-based drugs, ozonides (OZs) containing trioxalane groups are also cleaved by Fe(II) and behave similarly to ART-based drugs (25). Some OZs have indeed recently been shown to alkylate heme within live *P. falciparum* parasites (24). We therefore propose that OZ compounds will have similar redox-dependent effects versus Hz inhibition.

MATERIALS AND METHODS

All chemicals and solvents were of reagent grade or better, purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Waltham, MA), and used without further purification unless otherwise noted. Sterile black flat-bottom tissue culture or nonsterile clear polystyrene 96-well plates and 12-channel basins were obtained from Fisher Scientific. SYBR green I nucleic acid stain was purchased from Invitrogen (Carlsbad, CA). Hemin, GSH, phosphatidylcholine (PC), and sodium propionate were purchased from Sigma-Aldrich. DHA was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). CQ and AQ were purchased from Sigma-Aldrich. ATS and ATM were purchased from Cayman Chemical Co. (Ann Arbor, MI). LF was purchased from Selleck Chemicals (Houston, TX). Mefloquine (MQ) was purchased from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Red blood cells and matched human serum for parasite culture were purchased from Valley Biomedical (Winchester, VA). *P. falciparum* clone HB3 (Honduras) was obtained from the Malaria Research and Reference Reagent Resource Center (Manassas, VA). Cam WT and Cam WT-C580Y strains of *P. falciparum* were a kind gift of David Fidock (Department of Microbiology and Immunology, Columbia University, New York, NY).

***P. falciparum* cultures.** *P. falciparum* strains HB3, Cam WT, and Cam WT-C580Y were maintained essentially as described previously, with minor modifications (7, 97, 98). In brief, cultures were maintained under an atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 2% hematocrit in complete RPMI 1640 medium supplemented with 25 mM HEPES (pH 7.4), 24 mM NaHCO₃, 11 mM glucose, 0.75 mM hypoxanthine, 20 μg/liter gentamicin, and either 10% type O⁺ human serum (HB3) or 0.5% Albumax II (Cam WT/

Cam WT-C580Y). Parasitemia was monitored by Giemsa staining and adjusted every 48 h by the addition of fresh erythrocytes. Before being added to cultures, erythrocytes were washed with incomplete medium (RPMI 1640, 24 mM NaHCO₃, 11 mM glucose, and 0.75 mM hypoxanthine [pH 7.4]).

Oxidized β -hematin inhibition assay. An oxidized β -hematin inhibition assay (O-BHIA) was performed essentially as described previously (33). Ten microliters of a hemin chloride solution (2 mM) in 0.1 M NaOH was transferred to each well of a 96-well plate, followed by 180 μ l of propionate buffer (1.0 M; either pH 5.2 or pH 5.6) and 10 μ l of a sonicated phosphatidylcholine solution (10 mg/ml), which serves as a physiological catalyst for Hz formation at close-to-physiological temperature and pH (see references 33 and 35). The drug was serially diluted across the wells, and appropriate controls (e.g., no lipid catalyst and no drug) were included in each plate. Plates were sealed and incubated at 37°C with gentle shaking for 16 h. Hz formation was terminated by the addition of 50 μ l of an SDS solution (2.5% [wt/vol] in 0.1 M bicarbonate [pH 9.1]). The contents were gently mixed and then centrifuged (3,700 rpm for 5 min) to allow undissolved Hz crystals to settle. Fifty-microliter aliquots of the supernatant were transferred to a separate plate preloaded with 200 μ l/well of an SDS solution (2.5% [wt/vol] in 0.1 M bicarbonate [pH 9.1]). The absorbance of uncrystallized heme at 405 nm was quantified using a Tecan Infinite F200 fluorescence microplate reader, and conversion to Hz was quantified as described previously (33).

Reduced β -hematin inhibition assay. A reduced β -hematin inhibition assay (R-BHIA) was performed essentially as described above, with the following modifications. Ten microliters of hemin chloride (2 mM) in 80% DMSO was added to each well of a 96-well plate. Thirteen microliters of a glutathione solution (1.5 mM in 1 $\times$ phosphate-buffered saline [PBS]) was added to each well such that FPIX heme and GSH were at molar equivalence. The content of the plate was mixed, and the plate was sealed and incubated at 37°C with gentle shaking for 2 h to reduce hemin to the ferrous form. Drug serial dilutions were prepared in either water (CQ, AQ, and PPQ) or DMSO (DHA, ATS, ATM, MQ, and LF) at a 100 $\times$ concentration, and 2 μ l of the drug solution was then added to each well in triplicate. The contents of the plate were mixed, and the plate was sealed and then incubated at 37°C with gentle shaking for 1 h to allow activation of the drug. One hundred sixty-five microliters of propionate buffer (1.0 M; either pH 5.2 or 5.6) and 10 μ l of a sonicated phosphatidylcholine solution (10 mg/ml) were then added to each well, the contents were mixed, and the plate was sealed once again and incubated at 37°C with gentle shaking for 16 h. Hz formation was terminated by the addition of 50 μ l of an SDS solution (7.5% [wt/vol] in 0.1 M bicarbonate [pH 9.1]). The contents were gently mixed and then centrifuged (3,700 rpm for 5 min) to allow undissolved Hz to settle. Fifty-microliter aliquots of the supernatant were transferred to a separate plate preloaded with 200 μ l/well of an SDS solution (2.5% [wt/vol] in 0.1 M bicarbonate [pH 9.1]), and the absorbance was recorded as described above. Hz formation was then quantified as described previously (33).

R-BHIA fixed-ratio isobologram analysis. To measure potentially synergistic effects between ACT drug pairs, an abbreviated isobologram analysis was performed utilizing the R-BHIA format and the Chou-Talalay method for drug combinatorial analysis (57). Drug solutions were prepared at 5 different ratios, 1:0, 3:1, 1:1, 1:3, and 0:1, scaled to the drugs' Hz inhibition EC₅₀s [that is, "3" is 3 $\times$ (Hz inhibition EC₅₀ of the given drug alone)], and the combinations were analyzed using a fixed-ratio isobologram analysis. Fractional inhibitory concentration (FIC) values were calculated using equations 2 to 4:

$$FIC_A = \frac{EC_{50} \text{ of drug A in combination}}{EC_{50} \text{ of drug A alone}} \quad (2)$$

$$FIC_B = \frac{EC_{50} \text{ of drug B in combination}}{EC_{50} \text{ of drug B alone}} \quad (3)$$

$$FIC_{\text{index}} = FIC_A + FIC_B \quad (4)$$

Growth inhibition assay. Drug stock solutions were prepared for each drug using complete cell culture medium supplemented with human serum (for HB3 parasite strains) or Albumax II (for Cam WT and Cam WT-C580Y strains). These drug solutions were prepared such that the final concentration approximately equaled the single-drug 50% inhibitory concentration (IC₅₀) following 5 to 6 2-fold dilutions.

The growth inhibition experiment was performed essentially as previously described (98). In short, 100 μ l of the drug solution, prepared as described above, and 100 μ l of an asynchronous parasite culture (final 1% parasitemia and 2% hematocrit) were added to a 96-well clear-bottom black plate. The plates were transferred to an airtight sterile chamber, gassed with a 5% CO₂-5% O₂-90% N₂ gas blend, and incubated at 37°C for 48 h. SYBR green I dye (50 μ l; 10 $\times$ in complete medium from a DMSO stock) was added, and plates were incubated for 1 additional hour at 37°C. Fluorescence was measured at 538 nm (485-nm excitation) using a Spectra GeminiEM plate reader (Molecular Devices, Sunnyvale, CA) fitted with a 530-nm long-pass filter. Background controls included fluorescence from uninfected red blood cells (98).

ACKNOWLEDGMENTS

We thank the NIH (R01 56312 and R21 146506) and the Georgetown Chemistry Department for financial assistance.

We thank Maydha Dhanuka for technical help, David Fidock (Columbia University) for strains Cam WT and Cam WT-0580Y, and Bryce Riegel (Georgetown) and Matthew Hassett (Georgetown) for helpful discussion.

REFERENCES

- World Health Organization. 2019. World malaria report 2019. World Health Organization, Geneva, Switzerland.
- Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Arie F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NPJ, Lindergardh N, Socheat D, White NJ. 2009. Artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* 361:455–467. <https://doi.org/10.1056/NEJMoa0808859>.
- Noedl H, Se Y, Schaefer K, Smith BL, Socheat D, Fukuda MM, Artemisinin Resistance in Cambodia 1 Study Consortium. 2008. Evidence of artemisinin-resistant malaria in western Cambodia. *N Engl J Med* 359:2619–2620. <https://doi.org/10.1056/NEJMc0805011>.
- Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Sreng S, Anderson JM, Mao S, Sam B, Sopha C, Chuor CM, Nguon C, Sovannaroeth S, Pukrittayakamee S, Jittamala P, Chotivanich K, Chutasmit K, Suchatsoonthorn C, Runchaoen R, Hien TT, Thuy-Nhien NT, Thanh NV, Phu NH, Htut Y, Han K-T, Aye KH, Mokuolu OA, Olaosebikan RR, Folaranmi OO, Mayxay M, Khanthavong M, Hongvanthong B, Newton PN, Onyamboko MA, Fanello CI, Tshefu AK, Mishra N, Valecha N, Phyo AP, Nosten F, Yi P, Tripura R, Borrmann S, Bashraheil M, Peshu J, Faiz MA, Ghose A, Hossain MA, Samad R, et al. 2014. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* 371:411–423. <https://doi.org/10.1056/NEJMoa1314981>.
- Strainer J, Gnädig NF, Witkowski B, Amaratunga C, Duru V, Ramadan AP, Dacheux M, Khim N, Zhang L, Lam S, Gregory PD, Urnov FD, Mercereau-Pujalon O, Benoit-Vical F, Fairhurst RM, Ménard D, Fidock DA. 2015. K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates. *Science* 347:428–431. <https://doi.org/10.1126/science.1260867>.
- Hott A, Casandra D, Sparks KN, Morton LC, Castaneres G-G, Rutter A, Kyle DE. 2015. Artemisinin-resistant *Plasmodium falciparum* parasites exhibit altered patterns of development in infected erythrocytes. *Antimicrob Agents Chemother* 59:3156–3167. <https://doi.org/10.1128/AAC.00197-15>.
- Heller LE, Roepe PD. 2018. Quantification of free ferriprotoporphyrin IX heme and hemozoin for artemisinin sensitive versus delayed clearance phenotype *Plasmodium falciparum* malarial parasites. *Biochemistry* 57:6927–6934. <https://doi.org/10.1021/acs.biochem.8b00959>.
- Birnbaum J, Scharf S, Schmidt S, Jonscher E, Hoeijmakers WAM, Flemming S, Toenhake CG, Schmitt M, Sabitzki R, Bergmann B, Fröhlich U, Mesén-Ramírez P, Blanche Soares A, Herrmann H, Bártfai R, Spielmann T. 2020. A Kelch13-defined endocytosis pathway mediates artemisinin resistance in malaria parasites. *Science* 367:51–59. <https://doi.org/10.1126/science.aax4735>.
- Xie SC, Ralph SA, Tilley L. 2020. K13, the cytosome, and artemisinin resistance. *Trends Parasitol* 36:533–544. <https://doi.org/10.1016/j.pt.2020.03.006>.
- Yang T, Yeoh LM, Tutor MV, Dixon MW, McMillan PJ, Xie SC, Bridgford JL, Gillett DL, Duffy MF, Ralph SA, McConville MJ, Tilley L, Cobbold SA. 2019. Decreased K13 abundance reduces hemoglobin catabolism and proteotoxic stress, underpinning artemisinin resistance. *Cell Rep* 29:2917–2928. <https://doi.org/10.1016/j.celrep.2019.10.095>.
- Heller LE, Goggins E, Roepe PD. 2018. Dihydroartemisinin-ferriprotoporphyrin IX adduct abundance in *Plasmodium falciparum* malarial parasites and the relationship to emerging artemisinin resistance. *Biochemistry* 57:6935–6945. <https://doi.org/10.1021/acs.biochem.8b00960>.
- Heller LE, Roepe PD. 2019. Artemisinin-based antimalarial drug therapy: molecular pharmacology and evolving resistance. *Trop Med Infect Dis* 4:89. <https://doi.org/10.3390/tropicalmed4020089>.
- Ismail HM, Barton V, Phanchana M, Charoensutthivarakul S, Wong MH, Hemingway J, Biagini GA, O'Neill PM, Ward SA. 2016. Artemisinin activity-based probes identify multiple molecular targets within the asexual stage of the malaria parasites *Plasmodium falciparum* 3D7. *Proc Natl Acad Sci U S A* 113:2080–2085. <https://doi.org/10.1073/pnas.1600459113>.
- Jourdan J, Walz A, Matile H, Schmidt A, Wu J, Wang X, Dong Y, Vennerstrom JL, Schmidt RS, Wittlin S, Mäser P. 2019. Stochastic protein alkylation by antimalarial peroxides. *ACS Infect Dis* 5:2067–2075. <https://doi.org/10.1021/acscinfecdis.9b00264>.
- Wang J, Zhang C-J, Chia WN, Loh CCY, Li Z, Lee YM, He Y, Yuan L-X, Lim TK, Liu M, Liew CX, Lee YQ, Zhang J, Lu N, Lim CT, Hua Z-C, Liu B, Shen H-M, Tan KSW, Lin Q. 2015. Haem-activated promiscuous targeting of artemisinin in *Plasmodium falciparum*. *Nat Commun* 6:10111. <https://doi.org/10.1038/ncomms10111>.
- Ismail HM, Barton VE, Phanchana M, Charoensutthivarakul S, Biagini GA, Ward SA, O'Neill PM. 2016. A click chemistry-based proteomic approach reveals that 1,2,4-trioxolane and artemisinin antimalarials share a common protein alkylation profile. *Angew Chem Int Ed Engl* 55:6401–6405. <https://doi.org/10.1002/anie.201512062>.
- Posner GH, Oh CH. 1992. Regiospecifically oxygen-18 labeled 1,2,4-trioxane: a simple chemical model system to probe the mechanism(s) for the antimalarial activity of artemisinin (qinghaosu). *J Am Chem Soc* 114:8328–8329. <https://doi.org/10.1021/ja00047a076>.
- Asawamahasakda W, Ittarat I, Pu YM, Ziffer H, Meshnick SR. 1994. Reaction of antimalarial endoperoxides with specific parasite proteins. *Antimicrob Agents Chemother* 38:1854–1858. <https://doi.org/10.1128/aac.38.8.1854>.
- Meshnick SR, Thomas A, Ranz A, Xu C-M, Pan H-Z. 1991. Artemisinin (qinghaosu): the role of intracellular heme in its mechanism of antimalarial action. *Mol Biochem Parasitol* 49:181–189. [https://doi.org/10.1016/0166-6851\(91\)90062-b](https://doi.org/10.1016/0166-6851(91)90062-b).
- Posner GH, Oh CH, Wang D, Gerena L, Milhous WK, Meshnick SR, Asawamahasakda W. 1994. Mechanism-based design, synthesis, and *in vitro* antimalarial testing of new 4-methylated trioxanes structurally related to artemisinin: the importance of a carbon-centered radical for antimalarial activity. *J Med Chem* 37:1256–1258. <https://doi.org/10.1021/jm00035a003>.
- Robert A, Coppel Y, Meunier B. 2002. Alkylation of heme by the antimalarial drug artemisinin. *Chem Commun (Camb)* 2002:414–415. <https://doi.org/10.1039/b110817b>.
- Robert A, Cazelles J, Meunier B. 2001. Characterization of the alkylation product of heme by the antimalarial drug artemisinin. *Angew Chem Int Ed Engl* 40:1954–1957. [https://doi.org/10.1002/1521-3773\(20010518\)40:10<1954::AID-ANIE1954>3.0.CO;2-9](https://doi.org/10.1002/1521-3773(20010518)40:10<1954::AID-ANIE1954>3.0.CO;2-9).
- Robert A, Benoit-Vical F, Claparols C, Meunier B. 2005. The antimalarial drug artemisinin alkylates heme in infected mice. *Proc Natl Acad Sci U S A* 102:13676–13680. <https://doi.org/10.1073/pnas.0500972102>.
- Giannangelo C, Anderson D, Wang X, Vennerstrom JL, Charman SA, Creek DJ. 2019. Ozonide antimalarials alkylate heme in the malaria parasite *Plasmodium falciparum*. *ACS Infect Dis* 5:2076–2086. <https://doi.org/10.1021/acscinfecdis.9b00257>.
- Tang Y, Dong Y, Wang X, Sriraghavan K, Wood JK, Vennerstrom JL. 2005. Dispiro-1,2,4-trioxane analogues of a prototype dispiro-1,2,4-trioxolane: mechanistic comparators for artemisinin in the context of reaction pathways with iron(II). *J Org Chem* 70:5103–5110. <https://doi.org/10.1021/jo050385+>.
- Macomber PB, Sprinz H, Tousimis AJ. 1967. Morphological effects of chloroquine on *Plasmodium berghei* in mice. *Nature* 214:937–939. <https://doi.org/10.1038/214937a0>.
- Cohen SN, Phifer KO, Yelding KL. 1964. Complex formation between chloroquine and ferrihaemic acid *in vitro*, and its effect on the antimalarial action of chloroquine. *Nature* 202:805–806. <https://doi.org/10.1038/202805a0>.
- Gorka AP, de Dios A, Roepe PD. 2013. Quinoline drug-heme interactions and implications for antimalarial cytostatic versus cytotoxic activities. *J Med Chem* 56:5231–5246. <https://doi.org/10.1021/jm400282d>.
- Basilico N, Pagani E, Monti D, Oliaro P, Taramelli D. 1998. A microtitre-based method for measuring the haem polymerization inhibitory activity (HPIA) of antimalarial drugs. *J Antimicrob Chemother* 42:55–60. <https://doi.org/10.1093/jac/42.1.55>.
- Haynes RK, Monti D, Taramelli D, Basilico N, Parapini S, Oliaro P. 2003. Artemisinin antimalarials do not inhibit hemozoin formation. *Antimicrob Agents Chemother* 47:1175. <https://doi.org/10.1128/aac.47.3.1175.2003>.
- Herraz T, Guillén H, González-Peña D, Arán VJ. 2019. Antimalarial quinoline drugs inhibit β -hematin and increase free heme catalyzing peroxidative

- reactions and inhibition of cysteine proteases. *Sci Rep* 9:15398. <https://doi.org/10.1038/s41598-019-51604-z>.
32. Huy NT, Shima Y, Maeda M, Men TT, Hirayama K, Hirase A, Miyazawa A, Kamei K. 2013. Phospholipid membrane-mediated hemozoin formation: the effects of physical properties and evidence of membrane surrounding hemozoin. *PLoS One* 8:e70025. <https://doi.org/10.1371/journal.pone.0070025>.
 33. Gorka AP, Alumasa JN, Sherlach KS, Jacobs LM, Nickley KB, Brower JP, de Dios AC, Roepe PD. 2013. Cytostatic versus cytotoxic activities of chloroquine analogues and inhibition of hemozoin crystal growth. *Antimicrob Agents Chemother* 57:356–364. <https://doi.org/10.1128/AAC.01709-12>.
 34. Men TT, Huy NT, Trang DTX, Shuaibu MN, Hirayama K, Kamei K. 2012. A simple and inexpensive haemozoin-based colorimetric method to evaluate anti-malarial drug activity. *Malar J* 11:272. <https://doi.org/10.1186/1475-2875-11-272>.
 35. Pisciotto JM, Coppens I, Tripathi AK, Scholl PF, Shuman J, Bajad S, Shulaev V, Sullivan DJ, Jr. 2007. The role of neutral lipid nanospheres in *Plasmodium falciparum* haem crystallization. *Biochem J* 402:197–204. <https://doi.org/10.1042/BJ20060986>.
 36. Huy NT, Uyen DT, Maeda A, Trang DTX, Oida T, Harada S, Kamei K. 2007. Simple colorimetric inhibition assay of heme crystallization for high-throughput screening of antimalarial compounds. *Antimicrob Agents Chemother* 51:350–353. <https://doi.org/10.1128/AAC.00985-06>.
 37. Trang DTX, Huy NT, Uyen DT, Sasai M, Shiono T, Harada S, Kamei K. 2006. Inhibition assay of β -hematin formation initiated by lecithin for screening new antimalarial drugs. *Anal Biochem* 349:292–296. <https://doi.org/10.1016/j.ab.2005.11.022>.
 38. Huy NT, Uyen DT, Sasai M, Trang DTX, Shiono T, Harada S, Kamei K. 2006. A simple and rapid colorimetric method to measure hemozoin crystal growth *in vitro*. *Anal Biochem* 354:305–307. <https://doi.org/10.1016/j.ab.2005.08.005>.
 39. Ncokazi KK, Egan TJ. 2005. A colorimetric high-throughput β -hematin inhibition screening assay for use in the search for antimalarial compounds. *Anal Biochem* 338:306–319. <https://doi.org/10.1016/j.ab.2004.11.022>.
 40. Tripathi AK, Khan SI, Walker LA, Tekwani BL. 2004. Spectrophotometric determination of de novo hemozoin/ β -hematin formation in an *in vitro* assay. *Anal Biochem* 325:85–91. <https://doi.org/10.1016/j.ab.2003.10.016>.
 41. Jackson KE, Klonis N, Ferguson DJP, Adisa A, Dogovski C, Tilley L. 2004. Food vacuole-associated lipid bodies and heterogeneous lipid environments in the malaria parasite, *Plasmodium falciparum*. *Mol Microbiol* 54:109–122. <https://doi.org/10.1111/j.1365-2958.2004.04284.x>.
 42. Chong CR, Sullivan DJ. 2003. Inhibition of heme crystal growth by antimalarials and other compounds: implications for drug discovery. *Biochem Pharmacol* 66:2201–2212. <https://doi.org/10.1016/j.bcp.2003.08.009>.
 43. Tripathi AK, Garg SK, Tekwani BL. 2002. A physicochemical mechanism of hemozoin (β -hematin) synthesis by malaria parasite. *Biochem Biophys Res Commun* 290:595–601. <https://doi.org/10.1006/bbrc.2001.6231>.
 44. Tripathi AK, Gupta A, Garg SK, Tekwani BL. 2001. *In vitro* β -hematin formation assays with plasma of mice infected with *Plasmodium yoelii* and other parasite preparations: comparative inhibition with quinoline and endoperoxide antimalarials. *Life Sci* 69:2725–2733. [https://doi.org/10.1016/s0024-3205\(01\)01349-2](https://doi.org/10.1016/s0024-3205(01)01349-2).
 45. Kurosawa Y, Dorn A, Kitsuji-Shirane M, Shimada H, Satoh T, Matile H, Hofheinz W, Masciadri R, Kansy M, Ridley RG. 2000. Hematin polymerization assay as a high-throughput screen for identification of new antimalarial pharmacophores. *Antimicrob Agents Chemother* 44:2638–2644. <https://doi.org/10.1128/aac.44.10.2638-2644.2000>.
 46. Pandey AV, Joshi SK, Tekwani BL, Chauhan VS. 1999. A colorimetric assay for heme in biological samples using 96-well plates. *Anal Biochem* 268:159–161. <https://doi.org/10.1006/abio.1998.2997>.
 47. Sandlin RD, Fong KY, Wicht KJ, Carrell HM, Egan TJ, Wright DW. 2014. Identification of β -hematin inhibitors in a high-throughput screening effort reveals scaffolds with *in vitro* antimalarial activity. *Int J Parasitol Drugs Drug Resist* 4:316–325. <https://doi.org/10.1016/j.ijpddr.2014.08.002>.
 48. Becker K, Tilley L, Vennerstrom JL, Roberts D, Rogerson S, Ginsburg H. 2004. Oxidative stress in malaria parasite-infected erythrocytes: host-parasite interactions. *Int J Parasitol* 34:163–189. <https://doi.org/10.1016/j.ijpara.2003.09.011>.
 49. Ginsburg H, Golenser J. 2003. Glutathione is involved in the antimalarial action of chloroquine and its modulation affects drug sensitivity of human and murine species of *Plasmodium*. *Redox Rep* 8:276–279. <https://doi.org/10.1179/135100003225002907>.
 50. Müller S. 2004. Redox and antioxidant systems of the malaria parasite *Plasmodium falciparum*. *Mol Microbiol* 53:1291–1305. <https://doi.org/10.1111/j.1365-2958.2004.04257.x>.
 51. Heller L, Roepe PD, de Dios AC. 2019. Artesunate activation by heme in an aqueous medium. *Inorganica Chim Acta* 496:119029. <https://doi.org/10.1016/j.ica.2019.119029>.
 52. Dzekunov SM, Ursos LMB, Roepe PD. 2000. Digestive vacuolar pH of intact intraerythrocytic *P. falciparum* either sensitive or resistant to chloroquine. *Mol Biochem Parasitol* 110:107–124. [https://doi.org/10.1016/s0166-6851\(00\)00261-9](https://doi.org/10.1016/s0166-6851(00)00261-9).
 53. Bennett TN, Kosar AD, Ursos LMB, Dzekunov S, Sidhu ABS, Fidock DA, Roepe PD. 2004. Drug resistance-associated pfcRT mutations confer decreased *Plasmodium falciparum* digestive vacuolar pH. *Mol Biochem Parasitol* 133:99–114. <https://doi.org/10.1016/j.molbiopara.2003.09.008>.
 54. Gligorijevic B, Bennett T, McAllister R, Urbach JS, Roepe PD. 2006. Spinning disk confocal microscopy of live, intraerythrocytic malarial parasites. 2. Altered vacuolar volume regulation in drug resistant malaria. *Biochemistry* 45:12411–12423. <https://doi.org/10.1021/bi0610348>.
 55. Leed A, DuBay K, Ursos LMB, Sears D, de Dios AC, Roepe PD. 2002. Solution structures of antimalarial drug-heme complexes. *Biochemistry* 41:10245–10255. <https://doi.org/10.1021/bi020195i>.
 56. de Dios AC, Tycko R, Ursos LMB, Roepe PD. 2003. NMR studies of chloroquine-ferriprotoporphyrin IX complex. *J Phys Chem A* 107:5821–5825. <https://doi.org/10.1021/jp0342982>.
 57. Chou T-C. 2010. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res* 70:440–446. <https://doi.org/10.1158/0008-5472.CAN-09-1947>.
 58. Mott BT, Eastman RT, Guha R, Sherlach KS, Siriwardana A, Shinn P, McKnight C, Michael S, Lacerda-Queiroz N, Patel PR, Khine P, Sun HM, Kasbekar M, Aghdam N, Fontaine SD, Liu DB, Mierzwa T, Mathews-Griner LA, Ferrer M, Renslo AR, Inglesse J, Yuan J, Roepe PD, Su XZ, Thomas CJ. 2015. High-throughput matrix screening identifies synergistic and antagonistic antimalarial drug combinations. *Sci Rep* 5:13891. <https://doi.org/10.1038/srep13891>.
 59. Gorka AP, Jacobs LM, Roepe PD. 2013. Cytostatic versus cytotoxic profiling of quinoline drug combinations via modified fixed-ratio isobologram analysis. *Malar J* 12:332. <https://doi.org/10.1186/1475-2875-12-332>.
 60. Einstein A. 1926. *Investigations on the theory of Brownian movement*. Dover Publications, Inc, Mineola, NY.
 61. Cussler EL. 2009. *Diffusion mass transfer in fluid systems*, 3rd ed. Cambridge University Press, Cambridge, United Kingdom.
 62. Platz J, Christensen LK, Sehested J, Nielsen OJ, Wallington TJ, Sauer C, Barnes I, Becker KH, Vogt R. 1998. Atmospheric chemistry of 1,3,5-trioxane: UV spectra of c-C₃H₅O₃(-) and (c-C₃H₅O₃)O₂(-) radicals, kinetics of the reactions of (c-C₃H₅O₃)O₂(-) radicals with NO and NO₂, and atmospheric fate of the alkoxy radical (c-C₃H₅O₃)O(-). *J Phys Chem A* 102:4829–4838. <https://doi.org/10.1021/jp9809540>.
 63. Pryor WA. 1986. Oxy-radicals and related species: their formation, lifetimes, and reactions. *Annu Rev Physiol* 48:657–667. <https://doi.org/10.1146/annurev.ph.48.030186.003301>.
 64. Marnett LJ. 2000. Oxyradicals and DNA damage. *Carcinogenesis* 21:361–370. <https://doi.org/10.1093/carcin/21.3.361>.
 65. Kohen R, Nyska A. 2002. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol* 30:620–650. <https://doi.org/10.1080/01926230290166724>.
 66. Phaniendra A, Jestadi DB, Periyasamy L. 2015. Free radicals: properties, sources, targets, and their implication in various diseases. *Indian J Clin Biochem* 30:11–26. <https://doi.org/10.1007/s12291-014-0446-0>.
 67. Ferrer-Sueta G, Radi R. 2009. Chemical biology of peroxynitrite: kinetics, diffusion, and radicals. *ACS Chem Biol* 4:161–177. <https://doi.org/10.1021/cb800279q>.
 68. Hassett MR, Roepe PD. 2019. Origin and spread of evolving artemisinin-resistant *Plasmodium falciparum* malarial parasites in Southeast Asia. *Am J Trop Med Hyg* 101:1204–1211. <https://doi.org/10.4269/ajtmh.19-0379>.
 69. Siddiqui FA, Boonhok R, Cabrera M, Mbenda HGN, Wang M, Min H, Liang X, Qin J, Zhu X, Miao J, Cao Y, Cui L. 2020. Role of *Plasmodium falciparum* Kelch 13 protein mutations in *P. falciparum* populations from northeastern Myanmar in mediating artemisinin resistance. *mBio* 11:e01134-19. <https://doi.org/10.1128/mBio.01134-19>.
 70. Bosman P, Stassijns J, Nackers F, Canier L, Kim N, Khim S, Alipon SC, Chuor Char M, Chea N, Dysoley L, Van den Bergh R, Etienne W, De Smet M, Ménard D, Kindermans J-M. 2014. *Plasmodium* prevalence and artemisinin-resistant *falciparum* malaria in Preah Vihear Province, Cambodia: a cross-sectional population-based study. *Malar J* 13:394. <https://doi.org/10.1186/1475-2875-13-394>.
 71. Bhattacharjee S, Coppens I, Mbengue A, Suresh N, Ghorbal M, Slouka Z, Safeukui I, Tang H-Y, Speicher DW, Stahelin RV, Mohandas N, Haldar K. 2018. Remodeling of the malaria parasite and host human red cell by vesicle

- amplification that induces artemisinin resistance. *Blood* 131:1234–1247. <https://doi.org/10.1182/blood-2017-11-814665>.
72. Birnbaum J, Flemming S, Reichard N, Soares AB, Mesén-Ramírez P, Jonscher E, Bergmann B, Spielmann T. 2017. A genetic system to study *Plasmodium falciparum* protein function. *Nat Methods* 14:450–456. <https://doi.org/10.1038/nmeth.4223>.
 73. Gnädig NF, Stokes BH, Edwards RL, Kalantarov GF, Heimsch KC, Kuderjavý M, Crane A, Lee MCS, Straimer J, Becker K, Trakht IN, Odom John AR, Mok S, Fidock DA. 2020. Insights into the intracellular localization, protein associations and artemisinin resistance properties of *Plasmodium falciparum* K13. *PLoS Pathog* 16:e1008482. <https://doi.org/10.1371/journal.ppat.1008482>.
 74. Henrici RC, Edwards RL, Zoltner M, van Schalkwyk DA, Hart MN, Mohring F, Moon RW, Nofal SD, Patel A, Flueck C, Baker DA, Odom John AR, Field MC, Sutherland CJ. 2020. The *Plasmodium falciparum* artemisinin susceptibility-associated AP-2 adaptin μ subunit is clathrin independent and essential for schizont maturation. *mBio* 11:e02918-19. <https://doi.org/10.1128/mBio.02918-19>.
 75. Zhang M, Wang C, Otto TD, Oberstaller J, Liao X, Adapa SR, Udenze K, Bronner IF, Casandra D, Mayho M, Brown J, Li S, Swanson J, Rayner JC, Jiang RHY, Adams JH. 2018. Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis. *Science* 360:eaap7847. <https://doi.org/10.1126/science.aap7847>.
 76. Harding CR, Sidik SM, Petrova B, Gnädig NF, Okombo J, Herneisen AL, Ward KE, Markus BM, Boydston EA, Fidock DA, Lourido S. 2020. Genetic screens reveal a central role for heme metabolism in artemisinin susceptibility. *Nat Commun* 11:4813. <https://doi.org/10.1038/s41467-020-18624-0>.
 77. Fong KY, Wright DW. 2013. Hemozoin and antimalarial drug discovery. *Future Med Chem* 5:1437–1450. <https://doi.org/10.4155/fmc.13.113>.
 78. de Villiers KA, Gildenhuys J, le Roex T. 2012. Iron(III) protoporphyrin IX complexes of the antimalarial Cinchona alkaloids quinine and quinidine. *ACS Chem Biol* 7:666–671. <https://doi.org/10.1021/cb200528z>.
 79. de Villiers KA, Marques HM, Egan TJ. 2008. The crystal structure of halofantrine-ferriprotoporphyrin IX and the mechanism of action of arylmethanol antimalarials. *J Inorg Biochem* 102:1660–1667. <https://doi.org/10.1016/j.jinorgbio.2008.04.001>.
 80. Ursos LMB, Leed A, DuBay KF, de Dios AC, Roepe PD. 2002. The solution structures of antimalarial drug-heme complexes. *Biophys J* 82:309A.
 81. de Dios AC, Casabianca LB, Kosar A, Roepe PD. 2004. Structure of the amodiaquine-FPIX μ oxo dimer solution complex at atomic resolution. *Inorg Chem* 43:8078–8084. <https://doi.org/10.1021/ic0489948>.
 82. Martinez CR, Iverson BL. 2012. Rethinking the term “ π -stacking.” *Chem Sci* 3:2191–2201. <https://doi.org/10.1039/c2sc20045g>.
 83. Brandon MS, Paszczyński A, Korus R, Crawford RL. 2003. The determination of the stability constant for the iron(II) complex of the biochelator pyridine-2,6-bis(monothiocarboxylic acid). *Biodegradation* 14:73–82. <https://doi.org/10.1023/a:1024080521897>.
 84. Nutt DR, Meuwly M. 2007. Ferric and ferrous iron in nitroso-myoglobin: computer simulations of stable and metastable states and their infrared spectra. *Chemphyschem* 8:527–536. <https://doi.org/10.1002/cphc.200600563>.
 85. Chiavarino B, Crestoni ME, Fornarini S, Rovira C. 2008. Unravelling the intrinsic features of NO binding to iron(II)- and iron(III)-hemes. *Inorg Chem* 47:7792–7801. <https://doi.org/10.1021/ic800953w>.
 86. Hoshino M, Ozawa K, Seki H, Ford PC. 1993. Photochemistry of nitric oxide adducts of water-soluble iron(III) porphyrin and ferrihemoproteins studied by nanosecond laser photolysis. *J Am Chem Soc* 115:9568–9575. <https://doi.org/10.1021/ja00074a023>.
 87. Wanat A, Wolak M, Orzeł Ł, Brindell M, van Eldik R, Stochel G. 2002. Laser flash photolysis as tool in the elucidation of the nitric oxide binding mechanism to metallobiomolecules. *Coord Chem Rev* 229:37–49. [https://doi.org/10.1016/S0010-8545\(02\)00091-7](https://doi.org/10.1016/S0010-8545(02)00091-7).
 88. Moore EG, Gibson QH. 1976. Cooperativity in the dissociation of nitric oxide from hemoglobin. *J Biol Chem* 251:2788–2794. [https://doi.org/10.1016/S0021-9258\(17\)33557-3](https://doi.org/10.1016/S0021-9258(17)33557-3).
 89. Sharma VS, Traylor TG, Gardiner R, Mizukami H. 1987. Reaction of nitric oxide with heme proteins and model compounds of hemoglobin. *Biochemistry* 26:3837–3843. <https://doi.org/10.1021/bi00387a015>.
 90. Nasamu AS, Polino AJ, Istvan ES, Goldberg DE. 2020. Malaria parasite plasmepsins: more than just plain old degradative pepsins. *J Biol Chem* 295:8425–8441. <https://doi.org/10.1074/jbc.REV120.009309>.
 91. Banerjee R, Liu J, Beatty W, Pelosof L, Klemba M, Goldberg DE. 2002. Four plasmepsins are active in the *Plasmodium falciparum* food vacuole, including a protease with an active-site histidine. *Proc Natl Acad Sci U S A* 99:990–995. <https://doi.org/10.1073/pnas.022630099>.
 92. Cai H, Kuang R, Gu J, Wang Y. 2011. Proteases in malaria parasites—a phylogenomic perspective. *Curr Genomics* 12:417–427. <https://doi.org/10.2174/138920211797248565>.
 93. Melendez V, Peggins JO, Brewer TG, Theoharides AD. 1991. Determination of the antimalarial arteether and its deethylated metabolite dihydroartemisinin in plasma by high-performance liquid chromatography with reductive electrochemical detection. *J Pharm Sci* 80:132–138. <https://doi.org/10.1002/jps.2600800209>.
 94. Kapishnikov S, Staalsø T, Yang Y, Lee J, Pérez-Berná AJ, Pereiro E, Yang Y, Werner S, Guttmann P, Leiserowitz L, Als-Nielsen J. 2019. Mode of action of quinoline antimalarial drugs in red blood cells infected by *Plasmodium falciparum* revealed in vivo. *Proc Natl Acad Sci U S A* 116:22946–22952. <https://doi.org/10.1073/pnas.1910123116>.
 95. Roepe PD. 2014. To kill or not to kill, that is the question: cytotoxic anti-malarial drug resistance. *Trends Parasitol* 30:130–135. <https://doi.org/10.1016/j.pt.2014.01.004>.
 96. Gaviria D, Paguio MF, Turnbull LB, Tan A, Siriwardana A, Ghosh D, Ferdig MT, Sinai AP, Roepe PD. 2013. A process similar to autophagy is associated with cytotoxic chloroquine resistance in *Plasmodium falciparum*. *PLoS One* 8:e79059. <https://doi.org/10.1371/journal.pone.0079059>.
 97. Trager W, Jensen JB. 1976. Human malaria parasites in continuous culture. *Science* 193:673–675. <https://doi.org/10.1126/science.781840>.
 98. Bennett TN, Paguio M, Gligorijević B, Seudieu C, Kosar AD, Davidson E, Roepe PD. 2004. Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. *Antimicrob Agents Chemother* 48:1807–1810. <https://doi.org/10.1128/aac.48.5.1807-1810.2004>.
 99. Gligorijević B, McAllister R, Urbach JS, Roepe PD. 2006. Spinning disk confocal microscopy of live, intraerythrocytic malarial parasites. 1. Quantification of hemozoin development for drug sensitive versus resistant malaria. *Biochemistry* 45:12400–12410. <https://doi.org/10.1021/bi061033f>.