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The Effects of Quinoline and Non-quinoline Inhibitors on the Kinetics of Lipid-mediated β -Hematin Crystallization

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Abstract

The throughput of a biomimetic lipid-mediated assay used to investigate the effects of inhibitors on the kinetics of β -hematin formation has been optimized through the use of 24-well microplates. The rate constant for β -hematin formation mediated by monopalmitoyl-*rac*-glycerol was reduced from $0.17 \pm 0.04 \text{ min}^{-1}$ previously measured in Falcon tubes to $0.019 \pm 0.002 \text{ min}^{-1}$ in the optimized assay. While this necessitated longer incubation times, transferring aliquots from multiple 24-well plates to a single 96-well plate for final absorbance measurements actually improved the overall turnaround time per inhibitor. This assay has been applied to investigate the effects of four clinically-relevant antimalarial drugs (chloroquine, amodiaquine, quinidine and quinine) as well as several short-chain 4-aminoquinoline derivatives and non-quinoline (benzamide) compounds on the kinetics of β -hematin formation. The adsorption strength of these inhibitors to crystalline β -hematin (K_{ads}) was quantified using a theoretical kinetic model that is based on the Avrami equation and the Langmuir isotherm. Statistically-significant linear correlations between lipid-mediated β -hematin inhibitory activity and K_{ads} values for quinoline ($r^2 = 0.76$, P-value = 0.0046) and non-quinoline compounds ($r^2 = 0.99$, P-stat = 0.0006), as well as between parasite inhibitory activity (D10) and K_{ads} values for quinoline antimalarial drugs and short-chain chloroquine derivatives ($r^2 = 0.64$, P-value = 0.0098) provides a strong indication that drug action involves adsorption to the surface of β -hematin crystals. Independent support in this regard is provided by experiments that spectrophotometrically monitor the direct adsorption of antimalarial drugs to pre-formed β -hematin.

Graphical abstract

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Supporting Information

Derivations and fitting procedures, data tables and additional figures. The supporting information is available free of charge on the ACS Publications website at: <http://pubs.acs.org>

Keywords

Malaria; Antimalarial Drugs; Hemozoin; β-Hematin

INTRODUCTION

Malaria continues to be one of the most deadly parasitic human diseases known worldwide.¹ The intraerythrocytic stage within the malaria parasite's complex life cycle is associated with the digestion of host hemoglobin and the subsequent release of iron(III) protoporphyrin IX (Fe(III)PPIX).²⁻³ As a survival mechanism, the parasite, *Plasmodium falciparum*, detoxifies the harmful Fe(III)PPIX *via* biocrystallization by converting it into an inert, crystalline material, hemozoin (Hz).⁴ This material is also known as malaria pigment. The repeating unit of Hz and its synthetic equivalent, β-hematin, is a discrete cyclic μ-propionato coordination dimer of Fe(III)PPIX.⁵ While proteins have been implicated in the formation of Hz, more recent evidence points to the involvement of lipids.⁶⁻⁷ In particular, Kapishnikov *et al.* used soft X-ray tomography imaging to reveal Hz crystals orientated with their {100} faces relative to the inner membrane of the digestive vacuole, which they suggest as evidence of a lipid-templated mechanism of crystal nucleation.⁸⁻⁹ Since Fe(III)PPIX and Hz remain chemically distinct targets which are not under the genetic control of the parasite,¹⁰ the inhibition of β-hematin formation has been a source of great interest. In particular, the inhibitory activity thereof by clinically-relevant antimalarial drugs has been investigated in multiple systems in an effort to elucidate their mode(s) of action.¹¹⁻¹⁵ Despite these endeavors, however, the mechanism of action of β-hematin inhibiting drugs remains unclear. It has been suggested that they form complexes with free Fe(III)PPIX in solution *via* π-stacking or coordination, preventing its conversion into β-hematin;¹⁶⁻¹⁸ or alternatively, adsorb onto the various faces of β-hematin to impede further crystal growth.^{15, 19-21} In this regard, Leiserowitz and co-workers initially proposed that the corrugated {001} crystal face could serve as an ideal site for drug adsorption;¹⁹ more recently, using time-resolved *in situ* atomic force microscopy, Olafson *et al.* have provided compelling evidence that inhibition of crystal growth is rather brought about by the adsorption of drug compounds to the molecularly flat {100} face.²⁰

In recent years, lipid-mediated biomimetic systems have been the focus of several studies to investigate antimalarial action. Sullivan and co-workers have shown that a blend of neutral lipids (2:4:1:1:1, by vol MPG(monopalmitoyl-*rac*-glycerol):MSG(monostearoylglycerol):DPG(1,3-dipalmitoylglycerol):DOG(1,3-

dioleoylglycerol):DLG(1,3-dilinoleoylglycerol)) is present in the malaria parasite,⁷ and Hoang *et al.* determined that this blend mediates Fe(III)PPIX crystallization with an activation energy significantly lower than the individual mono- or diacylglycerols.²² Despite the unique properties of the lipid blend, single lipids (monomyristoylglycerol, MPG or MSG) have been successfully used in model studies.^{23–24} Given their high abundance in the lipid blend, Hoang *et al.* carried out extensive characterization studies using both MSG and MPG, however use of MSG required higher ratios of acetone to ensure complete solubility.²⁴ For this reason, further studies using a single lipid have focused on the use of MPG. In particular, inhibition (of β -hematin formation) data determined using MPG as a model lipid have shown a correlation with biological activity.²⁵ To our knowledge, Gildenhuys *et al.* were the first to systematically investigate the effects of four quinoline antimalarial drugs on the kinetics of lipid-mediated β -hematin formation under biomimetic conditions (using MPG alone).²⁵ The amount of β -hematin in a Fe(III)PPIX- β -hematin mixture was quantified using the modified Phi β (pyridine hemichrome inhibition of β -hematin formation) assay.²⁶ A kinetic model underpinned by the Langmuir adsorption isotherm was used to analyze the data, and the authors suggested that the β -hematin inhibitory activity of a compound is related to its strength of adsorption to free surface binding sites on the growing β -hematin crystals.²⁵ Owing to several limitations that were prohibitive towards medium- to high-throughput screening of diverse β -hematin inhibitors, further optimization of the lipid assay was essential to facilitate more routine investigations. In the current study, clinically-relevant antimalarial drugs, namely chloroquine (CQ), amodiaquine (AQ), quinidine (QD) and quinine (QN) were used for this purpose. The new assay was then used to study the effects of these drugs, as well as a series of short-chain chloroquine analogues (series **1**) and non-quinoline (benzamide) compounds (series **2**), on the kinetics of the biomimetic crystallization process. We show that β -hematin inhibition is correlated with the ability of compounds to adsorb to the surface of β -hematin crystals. Independent support is provided for this mechanism of action through the direct adsorption of the antimalarial drugs to pre-formed β -hematin crystals. Together the kinetics and direct adsorption studies present a powerful platform for comprehending the effect of β -hematin inhibiting compounds.

EXPERIMENTAL

Materials

With the exception of acetone and methanol (Kimix Chemicals, South Africa), all reagents were purchased from Sigma Aldrich, South Africa. Antimalarial drugs were used in their salt form, unless otherwise stated: AQ dihydrochloride dihydrate, CQ diphosphate, QD sulfate dihydrate and QN hemisulfate hydrate. Glass-distilled water was used to prepare aqueous solutions throughout the experiments. The short-chain chloroquine analogs (**1**, Figure 1) were re-synthesized by Mr J.B. Hay (Stellenbosch University) according to literature procedures,^{27–28} and converted to salts by precipitating the free base from a small volume of ethyl acetate with excess nitric acid. Salts were extensively washed with ethyl acetate and dried under vacuum overnight before use. The benzamide compounds (**2**, Figure 1) were prepared by Dr K.J. Wicht (University of Cape Town) and used without further purification.²⁹

Drug Activity Studies to Determine IC₅₀ Values

The inhibition of β -hematin formation by all inhibitors was measured using a previously-modified Phi β assay,²⁵ which was further optimized in this study. This colorimetric assay is based on the solubilization of free Fe(III)PPIX, and not β -hematin, by 5% (v/v) aqueous pyridine (pH 7.5).²⁶ Quinoline antimalarial drugs and compounds of series **1** were dissolved in aqueous citric acid monohydrate buffer (50.0 mM, pH 4.8), while dissolution of benzamide compounds (series **2**) necessitated the use of an aqueous citrate buffer (50.0 mM) with a pH of 3.0. Concentrations of stock solutions for each compound are reported in Table S1.

Reactions were carried out in 24-well polystyrene cell culture plates (16.0 mm internal diameter) prepared in the following manner: Inhibitor stock solutions were prepared in citrate buffer (50.0 mM, pH 4.8 or 3.0) and diluted with inhibitor-free citrate buffer as required. Two plates were used to accommodate up to twelve different concentrations with each column representing a different concentration (columns 0–11, Figure S1). Each test well was loaded with 1.0 mL of inhibitor-containing citrate buffer, after which the plates were pre-incubated at 37 ± 1 °C in a fan-assisted oven for at least 45 minutes. A hematin stock solution (3.16 mM) was prepared by dissolving 5.0 mg hematin (porcine) in 1.0 mL of 0.1 M NaOH followed by the addition of 1.5 mL of a 1:9 (v/v) acetone: methanol solution, while a MPG lipid stock solution (3.03 mM) was prepared in the same 1:9 (v/v) acetone: methanol solvent system. Solutions consisting of 200 μ L lipid and 5 μ L hematin stock solutions were premixed in the wells of a separate 96-well plate and then added drop-wise to the pre-incubated buffer in each well using an ultrathin disposable syringe (0.5 mm needle diameter). Based on previous studies at model lipid-water interfaces, a monolayer of lipid molecules is expected to form at the air-lipid interface,³⁰ while the remaining lipid forms an emulsion near the lipid-buffer interface as a result of solvent diffusion.²⁴ The volumes of aqueous and organic solvents used per well equate to 92:7.5:0.5 % (mol/mol) H₂O:methanol:acetone. Thus the aqueous citrate buffer is by far the major species in this model system. Plates were incubated in the oven for four hours, after which reactions were quenched by adding 241 μ L of an aqueous solution containing 30% pyridine, 10% HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (2.0 M, pH 7.5) and 40% acetone (v/v) into each well. Plates were manually agitated for one minute to ensure thorough solvent mixing and complete formation of the *bis*-pyridyl complex, then were left to stand for fifteen minutes to allow visible lipid precipitate to settle. This precipitate negatively affected experiment reproducibility, however the overall reliability could be improved by performing measurements in triplicate (i.e. measurements were performed up to nine times). A 200 μ L aliquot of the supernatant in each well was transferred to an empty well in a clean 96-well plate and the absorbance at 405 nm measured using a Thermo Scientific Multiskan GO spectrophotometer. Control wells permitted any interference from inhibitor, as well as buffer and lipid, to be monitored. Absorbance values from test wells were thus corrected at each concentration, and thereafter used as a measure of the amount of unreacted Fe(III)PPIX remaining at each inhibitor concentration. Corrected absorbances were plotted as a function of inhibitor concentration and fitted to a sigmoidal or hyperbolic dose-response curve in GraphPad Prism³¹ to determine the concentration required to inhibit β -hematin formation by 50% (IC₅₀).

Kinetics Studies of the Inhibition of β -Hematin Formation

The procedure described above for the IC_{50} studies was followed to monitor the kinetics of β -hematin formation in the presence of CQ, AQ, QD and QN, as well as compounds of series **1** and **2**. The premixed solution delivered to the buffer in each well of a 24-well plate contained 200.0 μ L of lipid and only 2 μ L of Fe(III)PPIX solution,^{24–25} and the reaction was quenched by adding 240 μ L of the 30:30:40 (v/v) pyridine: aqueous buffer: acetone solution. The concentration range investigated for each compound was informed by the β -hematin IC_{50} value determined above. For each inhibitor concentration, nine different incubation times between 0–32 hours were studied in a single experiment. All measurements were conducted in triplicate and the data analyzed using nonlinear least squares fitting in GraphPad Prism.³¹ The data were fitted to a theoretical kinetic model (Equation 1), which describes the formation of β -hematin ($\% \beta_{(s)}$) as a function of inhibitor concentration, $[I]$, the maximal yield (factor) of β -hematin obtained in the absence of an inhibitor, Y , the rate constant for the formation of β -hematin in the absence of an inhibitor, z , the adsorption equilibrium constant, K_{ads} , and the rate constant for the irreversible precipitation of hematin brought about by interaction with the inhibitor, k_2 .²⁵ Details of the derivation and fitting procedure are provided in the supporting information (section 1 and 2.1, respectively).

$$\% \beta_{(s)} = \frac{100Yz}{(z + k_2[I]^2 + k_2K_{ads}[I]^3)} \left\{ 1 - e^{-\left(\frac{z + k_2[I]^2 + k_2K_{ads}[I]^3}{1 + K_{ads}[I]} \right)t} \right\} \quad (1)$$

Direct Adsorption Studies

β -Hematin crystals were prepared using the previously-reported pentanol-water interface procedure,²³ and characterized using infrared spectroscopy, powder X-ray diffraction and scanning electron microscopy (Figure S2). Homogenous samples (1 mg) were placed in 2 mL Eppendorf tubes and 1.5 mL of CQ-, AQ-, QD- and QN-containing citrate buffer (50.0 mM, pH 4.8) was added to each tube. Tubes were placed in a floating tray and incubated in an Elma Transsonic Digital ultrasonic water bath (set on power level 2) at 37 ± 1 °C for sixteen hours. Drug concentrations were the same as those used for the kinetics experiments. Samples were prepared in triplicate for each drug concentration and included a control, which excluded pre-formed β -hematin. Following incubation, a 200 μ L aliquot of the supernatant was transferred to a clean 96-well plate and the UV-visible absorbance spectrum recorded. By monitoring the change in absorbance at a drug-specific wavelength, the adsorption constant, K , which provides an indication of the relative strength of adsorption of each compound to the pre-formed β -hematin crystals could be determined using a mass-balance form of the Langmuir isotherm (Equation 2).^{32–33} The Langmuir isotherm pertains to monolayer coverage and constant binding energy between a solid surface and an adsorbent,³² which in this case refers to the pre-formed β -hematin and the inhibiting drug, respectively. In Equation 2, q_e represents the mass of the adsorbed drug per mass of β -hematin crystals at equilibrium, while C_e is the equilibrium concentration of drug remaining in solution after adsorption. During fitting, default constraints were set for K and Q_a^0 (the maximum adsorption capacity corresponding to monolayer coverage) such that the

values should be greater than zero. Details of the fitting procedure are provided in the supporting information (section 2.2).

$$q_e = \frac{K \cdot Q_a^0 \cdot C_e}{1 + K \cdot C_e} \quad (2)$$

RESULTS AND DISCUSSION

The mechanism of inhibition of β -hematin formation by antimalarial drugs remains a matter of some debate in the literature, although there is a growing body of evidence that supports the hypothesis that drugs adsorb to the β -hematin crystal faces to bring about kinetic inhibition.^{19–20, 25} Methods to uncover important interactions relating to drug activity are therefore essential, especially for the rational design of new drugs which is currently crucial as a result of resistance. Previously, we have shown that investigation of the kinetics of β -hematin formation in the presence of known inhibitors yields valuable insight in this regard.²⁵ The aim of the current work was to build on these earlier kinetic studies by investigating a larger and more diverse compound dataset. In order to achieve this, it was important to first optimize the lipid assay and thereby increase the through-put of the kinetics-based β -hematin inhibition studies.

Assay Optimization

Several assays have been reported in the literature for the purpose of compound screening and identification of new β -hematin inhibitors. Both the colorimetric Phi β assay,²⁶ and the recently-reported citric buffer saturated octanol (CBSO) assay,³⁴ have been successfully optimized for use in 96-well microplates, while the Nonidet P-40 (NP-40) assay,^{35–36} and a modified Phi β assay,³⁷ are amenable to high-throughput screening in 384-well plates. The purpose of the current assay optimization was not to develop a competitive high-throughput screening assay. Rather, our previously-reported method to probe the effects of antimalarial drugs on the kinetics of β -hematin formation was not suitable in tube form for investigating a large number of compounds.²⁵ It has been previously shown that the surface area of a vessel greatly affects the yield of β -hematin;²⁴ therefore, 24-well plates, having the same diameter as 15 mL Falcon tubes, were selected in the current study. Indeed, despite the marked difference in total volume, the yield of β -hematin between the two assays remained essentially unaffected. Initially, a water bath was retained for incubation purposes. The reduced contact area of each well in the water compared to the fully immersed tubes, irregular heat distribution across the wells, as well as the formation of condensation on the well lids, however, all led to irreproducible and inconsistent results. Incubation in a fan-assisted oven, on the other hand, produced more consistent data as condensation was eliminated and the temperature was more evenly distributed. In addition, a larger number of plates could be accommodated which facilitated more efficient turnaround times.

Under revised conditions, a ten-fold decrease in the rate constant for β -hematin formation was observed, ($0.019 \pm 0.002 \text{ min}^{-1}$ compared to $0.17 \pm 0.04 \text{ min}^{-1}$). Since the volumes of Fe(III)PPIX, lipid and delivery solvent used were the same as in the original assay, the

observed decrease in rate constant was presumed to arise from the larger proportion of acetone/methanol to buffer in the system owing to the decreased volume of the latter in the 24-well method (1.0 mL) compared to in the tubes (5.0 mL). This was confirmed by increasing the proportion of the acetone/methanol solution relative to the buffer in the previously-reported 15 mL Falcon tubes procedure,²⁵ which resulted in the same ten-fold reduction in β -hematin formation as seen in the optimized assay but with no effect on yield ($\sim 70\%$). Based on the proposed evolution of a lipid emulsion during solvent mixing rather than the formation of a distinct lipid-water interface,²⁴ it is plausible that the rate of diffusion of acetone and methanol is slower in each well compared to a faster rate of diffusion in each tube. This in turn would affect the rate at which the required lipid-water interface (the proposed environment for crystallization *in vivo*)³⁸ forms in the system, which could explain the observed decrease in the rate constant for β -hematin formation. It should be noted, however, that the increased acetone/methanol content only amounts to 0.5 and 7.5 % (by mol), respectively, and thus the buffer remains predominately aqueous (92 mol %) in composition.

Owing to this significant decrease in the rate constant for β -hematin formation, overall incubation periods for experiments were increased by a minimum of eight-fold to ensure reaction completion. This proved serendipitous since early-stage incubation times could be more easily and accurately measured. Indeed, the previous method suffered from inaccuracy in the early data points for which incubation times were so short that quenching and subsequent analysis was not easily managed. Despite the increased incubation period required in the optimized assay, the overall turnaround time per compound was still greatly improved compared to the previous study.²⁵ The important differences between the original and revised methods are summarized in Table S2.

Determination of β -Hematin Inhibition IC_{50} Values

The IC_{50} values for the inhibition of lipid-mediated β -hematin formation by the antimalarial drugs CQ, AQ, QD and QN (Table 1), as well as compounds from series **1** and **2** (Table 2), were determined from their respective dose-response curves (Figure S3, S4 and S5, respectively) using the optimized assay above. In the case of the quinoline antimalarial drugs, a linear correlation is observed between the IC_{50} values determined in the current study and the IC_{50} values determined in the previous lipid-mediated assay (Figure S6a).²⁵ A significant correlation is also observed for these drugs and two compounds from series **1** (**1a** and **1c**) that were tested in our laboratory³⁹ using a NP-40 detergent-mediated assay (Figure S6b).³⁵ Importantly, a further correlation is observed between the IC_{50} values for inhibition of lipid-mediated β -hematin formation and the biological activities of the four antimalarial drugs against a CQ-sensitive (CQS) strain of the malaria parasite, 3D7 (Figure S6c).⁴⁰ On the other hand, the correlation between the IC_{50} values for inhibition of lipid-mediated β -hematin formation and the biological activities of the four antimalarial drugs against the D10 strain of the malaria parasite is not significant (Figure S6d), however this is not entirely unexpected as several pharmacokinetic factors are likely at play in the parasite. Nevertheless, these linear correlations confirm the potential of using the optimized lipid-mediated assay to investigate drug activity in a manner that successfully mimics Hz formation within malaria parasites.

Very recently, the CBSO assay has also demonstrated potential as a biomimetic assay in this regard, however the study was limited to the investigation of four quinoline antimalarial drugs (CQ, AQ, QN and mefloquine), pyronaridine and two antibiotics.³⁴ The data show a qualitative relationship between the β -hematin IC₅₀ data and the biological potency of the compounds in question, although, as above (for the D10 case), the correlation is not significant. This is most likely due to the limited size of the compound test set, however pharmacokinetic factors would again be relevant. These results were a further motivating factor in the current study to investigate a wider range of compounds, including diverse scaffolds. IC₅₀ values determined for the short-chain CQ analogues (**1**) indicate that these compounds are less active than known antimalarial drugs in the lipid-mediated system. While the β -hematin inhibition activity of the former series has been previously determined in an acetate buffer system (60°C, pH 4.5),^{26, 28} there is no correlation between the reported IC₅₀ values and those determined in the lipid-mediated assay, even when including quinoline antimalarial drugs in the dataset (Figure S7a). This is not unexpected since compounds are known to display different activities in different environments. The absence of a correlation in this case may be as a result of the different crystallization behavior of β -hematin in the acetate and lipid-based systems, where crystallization kinetics are consistent with first-order nucleation and three dimensional growth in the former,⁴¹ and one dimensional growth in the latter.²⁵ In contrast to series **1**, three of the five benzamide compounds studied (**2b**, **2d** and **2e**) show comparable activity to AQ and are more active than CQ, QN and QD. Interestingly, as observed for the antimalarial drugs, this series also produces a significant linear correlation between their IC₅₀ values determined in the current system and biological activities reported against the chloroquine-sensitive NF54 parasite strain (Figure S7b).²⁹ This correlation further validates the use of the lipid-mediated system to successfully mimic the Hz process in the parasite and provides a reliable method to evaluate mechanism(s) of action of Hz-inhibiting compounds.

The Effect of Inhibitors on the Kinetics of β -Hematin Formation

The kinetics of β -hematin formation in the presence of the quinoline antimalarial drugs as well as the short-chain CQ analogues (**1**) and benzamides (**2**), were successfully investigated using the optimized lipid-mediated β -hematin formation assay. While other biomimetic assays, for example the NP-40 detergent assay³⁶ and recently-reported CBSO assay,³⁴ do permit determination of inhibitory activity (IC₅₀) as discussed above, to our knowledge this is the first assay that provides a measure of the strength of adsorption of a compound to β -hematin, the proposed mechanism of these compounds' action. In all cases, we show that low inhibitor concentrations cause a reduction in the rate constant for β -hematin formation without altering the maximal yield. On the other hand, at higher concentrations, a notable decrease in yield is observed. This is clearly evident for CQ in Figure 2 (see Figures S8–S10 for other compounds). We have suggested previously that the decreased rate constant for β -hematin formation at lower inhibitor concentrations is a result of reversible adsorption of an inhibitor to the crystal surface of β -hematin as proposed by Buller *et al.*¹⁹ The decrease in yield of β -hematin, on the other hand, has been attributed to the irreversible precipitation of Fe(III)PPIX, possibly in the form of Fe(III)PPIX-inhibitor complexes.²⁵ Indeed, it is known that β -hematin inhibitory compounds are able to associate with free Fe(III)PPIX in solution by means of coordination or π -stacking, and such complexation would prevent its normal

incorporation into β -hematin.^{17–18} The formation of the proposed Fe(III)PPIX-inhibitor complexes appears to influence the accuracy of the data, since fits to Equation 1 for higher inhibitor concentrations are typically worse than for lower concentrations. It is possible that the formation of these complexes, for which the solubility in aqueous pyridine is not known, may have caused interference in the analysis, although this was not investigated further in the current study. We do know from powder X-ray diffraction, however, that following reaction with pyridine, the crystalline solid that remains is β -hematin and not an amorphous solid form of the proposed Fe(III)PPIX-inhibitor complex (Figure S11).

The kinetics of β -hematin formation in the presence of all inhibitors conform to the proposed theoretical kinetic model, which has been developed previously in order to explain the experimental observations of a decreased rate constant and decreased yield as discussed above.²⁵ The values of Y , z , K_{ads} and k_2 for CQ and the other antimalarial drugs are summarized in Table 1, together with the values obtained for the short-chain CQ analogues (**1**) and the benzamides (**2**) in Table 2.

Whether analyzed by compound class or as a collective, no significant correlations were resolved between k_2 and lipid-mediated β -hematin inhibition IC_{50} values (Figure S12a). Improved fits were found between k_2 and biological activities against the NF54 strain for the clinically-relevant antimalarial drugs and compounds **2**, however as before, the correlations are not statistically significant (Figure S12b). As shown in Table 1 and Table 2, the fitted k_2 values span a wide range (from 0.0014 to 1.7). Inhibitor-Fe(III)PPIX complexation is proposed to be more prevalent at higher inhibitor concentrations, which are not necessarily clinically-relevant,²⁵ and thus further research regarding the factors that influence the magnitude of k_2 was not undertaken in the current study. Furthermore, the speciation, solubility and toxicity of the proposed inhibitor-Fe(III)PPIX complexes have not been investigated. It is therefore not possible to draw any conclusions regarding the direct influence of this process on the malaria parasite, albeit to recognize that inclusion of this process is essential to fully appreciate the effects of an inhibitor on the kinetics of β -hematin formation.

On the other hand, significant linear correlations are observed for all compounds between the best-fit K_{ads} values and the lipid-mediated β -hematin inhibitory activity IC_{50} values (Figure 3a). This suggests that the inhibitory activity of a compound is related to its strength of adsorption to the growing face of the β -hematin crystal, at least under the biomimetic conditions employed in the lipid-mediated system. In the case of the benzamide series (**2**), the linear regression line, which is parallel to that for the antimalarial drugs and short-chain CQ derivatives (**1**) yet lower by approximately one log unit, indicates that these compounds adsorb less strongly to the surface of β -hematin. Initially, we considered the possibility that this observation may point to a difference in overall mechanism, since compounds with a similar IC_{50} value (in the lipid-mediated system) appear to have different K_{ads} values (as illustrated by the dotted vertical line in Figure 3a). However, we rather suspect that the observed differences in magnitude of K_{ads} are due to the fact that the benzamide compounds were investigated at a lower pH value of 3.0 compared to the usual 4.8 in order to overcome solubility issues. The observed correlation between K_{ads} and lipid-mediated β -hematin inhibitory activity lends possible insight into the mechanism of the inhibition of Hz

formation *in vivo*. A more interesting and informative (at least with respect to mechanistic insight) correlation is that observed between K_{ads} (determined in the lipid-mediated system) for the clinically-relevant antimalarial drugs and short chain CQ compounds (**1**), and their *in vitro* biological activity against the chloroquine-sensitive D10 *P. falciparum* strain (Figure 3b). With an observed r^2 value of 0.64 (P-stat = 0.0098), the statistically-significant correlation strongly suggests that adsorption of a compound to β -hematin (or hemozoin *in vivo*) may contribute significantly to a compound's biological mechanism of action. Unfortunately, while both the antimalarial drugs and series **2** have been tested for activity against the NF54 strain, direct comparison of the two datasets is not possible owing to the different pH conditions used for the kinetics studies and hence determination of K_{ads} . Notably, we do observe good agreement ($r^2 = 0.83$) between NF54 activity and K_{ads} for the benzamide compounds (Figure S13); owing to only four compounds having been investigated, however, the correlation is not significant (P-value = 0.09).

Direct Adsorption Studies

Since the kinetic model does not provide direct evidence of inhibitor adsorption to β -hematin crystals, independent studies to confirm this mode of action were warranted. This was addressed by monitoring the concentration of CQ, AQ, QN and QD in aqueous citrate buffer solutions in the presence of pre-formed β -hematin crystals over a 16-hour period. Importantly, while the external morphology of natural hemozoin and synthetic β -hematin crystals is similar, with {100}, {010}, {011} and {001} faces expressed, there are subtle differences between them. In particular, synthetic crystals usually appear as extended laths in the *c* direction, while hemozoin crystals tend to be more square-like.^{19, 42} In the current study, the concentration of all drugs was found to decrease in the presence of β -hematin but remained constant in its absence (Figure 4a and Figure S14). A control experiment (Figure S15), in which the relative volumes of acetone and methanol that were used in the activity and kinetics studies were included in the citrate buffer solution, showed no difference compared to the aqueous citrate buffer solution. This suggests that while acetone and methanol may influence the rate of diffusional mixing and hence the kinetics of β -hematin formation, these organic solvents do not influence the proposed drug adsorption to the crystal surface(s). Thus, the extent of depletion of each drug from solution during the direct adsorption studies may be accounted for by a β -hematin-drug interaction occurring. Furthermore, since no measurable free Fe(III)PPIX is present in the solution (Figure S16), the irreversible precipitation of a Fe(III)PPIX-drug complex is not considered. Consequently, the depletion of these drugs from solution is attributed to their adsorption to the crystal surfaces of β -hematin. As a negative control, the effect of a non- β -hematin inhibitor on the direct adsorption to β -hematin crystals was also investigated. Atovaquone (AtQ) is a known inhibitor of electron transport and pyrimidine biosynthesis, and shows no activity against β -hematin formation.^{43–44} As a result, no significant adsorption of AtQ to the β -hematin crystals, and hence no significant decrease in AtQ concentration over the 16-hour incubation, was expected. Owing to the insoluble nature of AtQ in the aqueous citrate buffer, the experiment was, however, carried out in acetonitrile (Figure S17). Adsorption of free-base CQ (CQ_{fb}) was therefore reinvestigated under these conditions, and the drug was found to deplete from solution in the presence of β -hematin. The strength of CQ adsorption to β -hematin under these conditions ($K = 26.7 \pm 9.1 \text{ mM}^{-1}$) was found to be the same within

one standard deviation of that determined in aqueous citric acid solution (see below). By contrast, AtQ concentration did not alter, which consequently confirms that AtQ does not adsorb to β -hematin crystals in the same solvent.

Fitting the data obtained from these direct adsorption experiments in aqueous solution to Equation 2 (Figure 4b and Figure S14), adsorption constants (K) were determined as $18.0 \pm 6.3 \text{ mM}^{-1}$, $32.3 \pm 15.8 \text{ mM}^{-1}$, $12.6 \pm 4.7 \text{ mM}^{-1}$ and $6.0 \pm 2.6 \text{ mM}^{-1}$ for CQ, AQ, QD and QN, respectively. The observed trend in K values is consistent with the more active compounds (AQ and CQ) exhibiting greater adsorption (Figure S18). The results for QD and QN in the direct adsorption study are the reverse of what is seen in the kinetics, however it is likely that the relatively large errors in the former values may have distorted the trend and thus obscured the subtle activity difference highlighted in the kinetics study. The difference in the absolute values of K_{ads} and K may be due to the fact that in the kinetics studies, the adsorption is expected at the various growing faces to bring about real-time inhibition of β -hematin formation.^{19–21} On the other hand, the β -hematin is pre-formed in the direct adsorption experiments, and thus adsorption does not result in inhibition of the crystal growth process. Furthermore, the methods used to synthesize β -hematin in the kinetics and direct adsorption experiments were different. In this regard, Cerruti and co-workers have very recently shown that the surface properties of β -hematin crystals are greatly affected by the preparation method.⁴⁵ In our case, the use of lipids versus organic solvent (i.e. pentanol), respectively, may well have influenced the surface morphology of the crystals, thus contributing to the observed differences in adsorption constants.

CONCLUSION

In the current study, we have applied a lipid-mediated assay to probe the effects of quinoline antimalarial drugs, as well as short-chain quinoline analogues and non-quinoline compounds on the kinetics of β -hematin formation. Rather than presenting an alternative assay for high throughput screening of compounds and identification of new β -hematin inhibitors, this study provides important insight into the mode of β -hematin inhibition by these diverse compounds. This is a major advantage over the majority of existing assays for which the primary purposes are inhibitor identification and determination of β -hematin inhibitory activity (IC_{50}). We show that adsorption (K_{ads}) is an important process, which is observed to correlate well to both β -hematin and biological activities of the compounds investigated. This strongly suggests that the β -hematin inhibitory activity of each inhibitor is related to its strength of adsorption to the crystal surface of β -hematin, which corresponds to the premise under investigation, namely that inhibition of hemozoin formation, at least in part, is brought about by adsorption of inhibitors to the various growing faces. While the direct adsorption studies have advanced our understanding, they do raise further questions regarding the exact site of drug adsorption. Continued experimental study is therefore necessary, which could be augmented by computational predictions. To further understanding of the Fe(III)PPIX detoxification process and its inhibition in malaria parasites and other blood-feeding organisms, it is also important to investigate a wider range of quinoline and non-quinoline β -hematin inhibitors and determine their effects on the kinetics of β -hematin formation. In this way, we will be able to fully demonstrate and appreciate the strengths of the theoretical kinetic model in unravelling the interconnected processes of inhibitor adsorption (K_{ads}) and

precipitation of inhibitor-Fe(III)PPIX complexes (k_2). Finally, if the interactions that aid the adsorption of inhibitors to surface binding sites on the crystal are identified, the insight will be invaluable in the rational design of novel β -hematin inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. WHO. World malaria report. World Health Organisation; 2015. p. 142
2. Krugliak M, Zhang J, Ginsburg H. Intraerythrocytic *Plasmodium falciparum* utilises only a fraction of the amino acids derived from the digestion of host cell cytosol for the biosynthesis of its proteins. *Mol Biochem Parasitol.* 2002; 119:249–256. [PubMed: 11814576]
3. Hayward R, Saliba KJ, Kirk K. The pH of the digestive vacuole of *Plasmodium falciparum* is not associated with chloroquine resistance. *J Cell Sci.* 2006; 119:1016–1025. [PubMed: 16492710]
4. Egan TJ, Combrink JM, Egan J, Hearne GR, Marques HM, Ntteni S, Sewell BT, Smith PJ, Taylor D, van Schalkwyk DA, Walden JC. Fate of haem iron in the malaria parasite *Plasmodium falciparum*. *Biochem J.* 2002; 365:343–347. [PubMed: 12033986]
5. Pagola S, Stephens PW, Bohle DS, Kosar AD, Madsen SK. The structure of malaria pigment (β -haematin). *Nature.* 2000; 404:307–310. [PubMed: 10749217]
6. Coppens I, Vielemayer O. Insights into unique physiological features of neutral lipids in Apicomplexa: from storage to potential mediation in parasite metabolic activities. *Int J Parasitol.* 2005; 35:597–615. [PubMed: 15862574]
7. Pisciotto JM, Coppens I, Tripathi AK, Scholl PF, Shuman J, Bajad S, Shulaev V, Sullivan DJ Jr. The role of neutral lipid nanospheres in *Plasmodium falciparum* heme crystallisation. *Biochem J.* 2007; 402:197–204. [PubMed: 17044814]
8. Kapishnikov S, Weiner A, Shimoni E, Guttmann P, Schneider G, Dahan-Pasternak N, Dzikowski R, Leiserowitz L, Elbaum M. Oriented nucleation of hemozoin at the digestive vacuole membrane in *Plasmodium falciparum*. *Proc Natl Acad Sci.* 2012; 109:11188–11193. [PubMed: 22745164]
9. Kapishnikov S, Weiner A, Shimoni E, Schneider G, Elbaum M, Leiserowitz L. Digestive vacuole membrane in *Plasmodium falciparum*-infected erythrocytes: Relevance to templated nucleation of hemozoin. *Langmuir.* 2013; 29:14595–14602. [PubMed: 24237179]
10. Egan TJ. Haemozoin (malaria pigment): a unique crystalline drug target. *TARGETS.* 2003; 2:115–124.
11. Dorn A, Stoffel R, Matile H, Bubendorf A, Ridley RG. Malarial haemozoin/ β -haematin supports haem polymerisation in the absence of protein. *Nature.* 1995; 374:269–271. [PubMed: 7885447]

12. Egan TJ, Ross DC, Adams PA. Quinoline anti-malarials inhibit spontaneous formation of β -haematin (malaria pigment). *FEBS Lett.* 1994; 352:54–57. [PubMed: 7925942]
13. Dorn A, Vippagunta SR, Matile H, Bubendorf A, Vennerstrom JL, Ridley RG. A comparison and analysis of several ways to promote haematin (haem) polymerisation and an assessment of its initiation *in vitro*. *Biochem Pharmacol.* 1998; 55:737–747. [PubMed: 9586945]
14. Dorn A, Vippagunta SR, Matile H, Jaquet C, Vennerstrom JL, Ridley RG. An assessment of drug-haematin binding as a mechanism for inhibition of haem polymerisation by quinoline antimalarials. *Biochem Pharmacol.* 1998; 55:727–736. [PubMed: 9586944]
15. Sullivan DJ Jr, Gluzman IY, Russel DG, Goldberg DE. On the molecular mechanism of chloroquine's antimalarial action. *Proc Natl Acad Sci USA.* 1996; 93:11865–11870. [PubMed: 8876229]
16. Cohen SN, Phifer KO, Yielding KL. Complex formation between chloroquine and ferrihaemic acid. *Nature.* 1964; 202:805–806. [PubMed: 14187629]
17. Chou AC, Chevli R, Fitch CD. Ferriprotoporphyrin IX fulfills the criteria for identification as the chloroquine receptor of malaria parasites. *Biochemistry.* 1980; 19:1543–1549. [PubMed: 6990976]
18. Egan TJ. Interactions of quinoline antimalarials with hematin in solution. *J Inorg Biochem.* 2006; 100:916–926. [PubMed: 16384600]
19. Buller R, Peterson ML, Almarsson Ö, Leiserowitz L. Quinoline binding site on malaria pigment crystal: A rational pathway for antimalarial drug design. *Cryst Growth Des.* 2002; 2:553–562.
20. Olafson KN, Ketchum MA, Rimer JD, Vekilov PG. Mechanisms of hematin crystallization and inhibition by the antimalarial drug chloroquine. *Proc Natl Acad Sci.* 2015; 112:4946–4951. [PubMed: 25831526]
21. Dubar F, Egan TJ, Pradines B, Kuter D, Ncokazi KK, Forge D, Paul JF, Pierrot C, Kalamou H, Khalife J, Buisine E, Rogier C, Vezin H, Forfar I, Slomianny C, Trivelli X, Kapishnikov S, Leiserowitz L, Dive D, Biot C. The Antimalarial Ferroquine: Role of the metal and intramolecular hydrogen bond in activity and resistance. *ACS Chem Biol.* 2011; 6:275–287. [PubMed: 21162558]
22. Hoang AN, Sandlin RD, Omar A, Egan TJ, Wright DW. The neutral lipid composition present in the digestive vacuole of *Plasmodium falciparum* concentrates heme and mediates β -hematin formation with an unusually low activation energy. *Biochemistry.* 2010; 49:10107–10116. [PubMed: 20979358]
23. Egan TJ, Chen JYJ, de Villiers KA, Mabotha TE, Naidoo KJ, Ncokazi KK, Langford SJ, McNaughton D, Pandiancherri S, Wood BR. Haemozoin (β -haematin) biomineralisation occurs by self assembly near the lipid/water interface. *FEBS Lett.* 2006; 580:5105–5110. [PubMed: 16956610]
24. Hoang AN, Ncokazi KK, de Villiers KA, Wright DW, Egan TJ. Crystallization of synthetic haemozoin (beta-haematin) nucleated at the surface of lipid particles. *Dalton Trans.* 2010; 39:1235–1244. [PubMed: 20104349]
25. Gildenhuis J, Le Roex T, Egan TJ, de Villiers KA. The single crystal X-ray structure of β -hematin DMSO solvate grown in the presence of chloroquine, a β -hematin growth-rate inhibitor. *J Am Chem Soc.* 2013; 135:1037–1047. [PubMed: 23253048]
26. Ncokazi KK, Egan TJ. A colorimetric high-throughput β -haematin inhibition screening assay for use in the search for antimalarial compounds. *Anal Biochem.* 2005; 338:306–319. [PubMed: 15745752]
27. Egan TJ, Hunter R, Kaschula CH, Marques HM, Misplon A, Walden J. Structure-function relationships in aminoquinolines: Effect of amino and chloro groups on quinoline-hematin complex formation, inhibition of β -haematin formation and antiparasmodial activity. *J Med Chem.* 2000; 43:283–291. [PubMed: 10649984]
28. Kaschula CH, Egan TJ, Hunter R, Basilico N, Parapini S, Taramelli D, Pasini E, Monti D. Structure-activity relationships in 4-aminoquinoline antiparasmodials. The role of the group at the 7-position. *J Med Chem.* 2002; 45:3531–3539. [PubMed: 12139464]
29. Wicht KJ, Combrinck JM, Smith PJ, Hunter R, Egan TJ. Identification and SAR evaluation of hemozoin-inhibiting benzamides active against *Plasmodium falciparum*. *J Med Chem.* 2016; 59:6512–6530. [PubMed: 27299916]

30. de Villiers KA, Osipova M, Mabotha TE, Solomonov I, Feldman Y, Kjaer K, Weissbuch I, Egan TJ, Leiserowitz L. Oriented nucleation of beta-hematin crystals induced at various interfaces: Relevance to haemozoin formation. *Cryst Growth Des.* 2009; 9(1):626–632.
31. GraphPad Prism, 5.0.2. GraphPad Software, Inc; San Diego:
32. Langmuir I. The constitution and fundamental properties of solids and liquids. *J Am Chem Soc.* 1916; 38:2221–2295.
33. Tseng RL, Wu FC, Juang RS. Liquid-phase adsorption of dyes and phenols using pinewood-based activated carbons. *Carbon.* 2003; 41:487–495.
34. Ketchum MA, Lee AM, Vekilov PG, Rimer JD. Biomimetic assay for hematin crystallization inhibitors: A new platform to screen antimalarial drugs. *Cryst Growth Des.* 2017; 17:197–206.
35. Carter MD, Phelan VV, Sandlin RD, Bachmann BO, Wright DW. Lipophilic mediated assays for β -hematin inhibitors. *Comb Chem High Throughput Screen.* 2010; 13(3):285–292. [PubMed: 20230372]
36. Sandlin RD, Carter MD, Lee PJ, Auschwitz JM, Leed SE, Johnson JD, Wright DW. Use of the NP-40 detergent-mediated assay in discovery of inhibitors of β -hematin crystallization. *Antimicrob Agents Chemother.* 2011; 55(7):3363–3369. [PubMed: 21518844]
37. Rush MA, Baniecki ML, Mazitschek R, Cortese JF, Wiegand R, Jon Clardy, Wirth DF. Colorimetric High-Throughput Screen for detection of heme crystallisation inhibitors. *Antimicrob Agents Chemother.* 2009; 53(6):2564–2568. [PubMed: 19307367]
38. Kuter D, Mohunlal R, Fitzroy SM, Asher C, Smith PJ, Egan TJ, de Villiers KA. Insights into the initial stages of lipid-mediated haemozoin nucleation. *Cryst Eng Comm.* 2016; 18:5177–5187.
39. Muller, R. Exploring the antimalarial mechanism of action: Adsorption of diverse inhibitors to crystalline malaria pigment (haemozoin). Stellenbosch University; 2017.
40. Hawley SR, Bray PG, Mungthin M, Atkinson JD, O'Neill PM, Ward SA. Relationship between antimalarial drug activity, accumulation, and inhibition of heme polymerisation in *Plasmodium falciparum* in vitro. *Antimicrob Agents Chemother.* 1998; 42:682–686. [PubMed: 9517951]
41. Egan TJ, Mavuso WW, Ncokazi KK. The mechanism of β -haematin formation in acetate solution. Parallels between haemozoin formation and biomineralisation processes. *Biochemistry.* 2001; 40:204–213. [PubMed: 11141072]
42. Solomonov I, Osipova M, Feldman Y, Baetz C, Kjaer K, Robinson IK, Webster GT, McNaughton D, Wood BR, Weissbuch I, Leiserowitz L. Crystal nucleation, growth, and morphology of the synthetic malaria pigment β -haematin and the effect thereon by quinoline additives: The malaria pigment as a target of various antimalarial drugs. *J Am Chem Soc.* 2007; 129:2615–2627. [PubMed: 17290993]
43. Basilico N, Pagani E, Monti D, Oliaro P, Taramelli D. A microtitre-based method for measuring the haem polymerization inhibitory activity (HPIA) of antimalarial drugs. *J Antimicrob Chemother.* 1998; 42:55–60. [PubMed: 9700528]
44. Chong CR, Sullivan DJ. Inhibition of heme crystal growth by antimalarials and other compounds: implications for drug discovery. *Biochem Pharmacol.* 2003; 66:2201–2212. [PubMed: 14609745]
45. Guerra ED, Bohle DS, Cerruti M. Surface characterization of hematin anhydride: A comparison between two different synthesis methods. *Langmuir.* 2016; 32:4479–4484. [PubMed: 27089176]

Figure 1. Molecular structures of β -hematin inhibitors used in this study. (**1**) 4-Aminoquinoline short-chain CQ analogues and (**2**) non-quinoline benzamide derivatives. See Table 2 for R_1 , R_2 , Ar and X substituents.

Figure 2.

Kinetics of β -hematin formation in the presence of CQ. Kinetic traces observed in the absence (black) and presence of increasing concentrations of CQ (in mM): 0.01 (dark grey, solid circles), 0.02 (light grey, solid circles), 0.025 (dark grey, open circles), 0.03 (light grey, open squares) 0.05 (dark grey dashed, crossed circles), 0.075 (dark grey dotted, crosses) and 0.10 (light grey dotted, asterisks). The r^2 values for the best fit of the experimental data to Equation 1 are 0.96, 0.90, 0.93, 0.86, 0.94, 0.87, 0.64 and 0.42, respectively.

Figure 3.

Relationship between drug activity and strength of adsorption. (a) Linear correlations between the observed K_{ads} values and the IC_{50} values determined in the lipid-water interface system (24-well plates) for clinically-relevant and short chain (**1**) quinoline compounds (black circles) and the benzamide compounds (**2**) (grey circles). (b) Linear correlation between the observed K_{ads} values and biological IC_{50} values determined against the D10 CQ sensitive parasite strains for clinically-relevant antimalarial drugs and short-chain CQ series (**1**). The r^2 values for the linear regression lines are (a) 0.76, $P = 0.005$ (black line) and 0.99, $P = 0.0006$ (grey line) and (b) 0.64, $P = 0.0098$. Dotted vertical line in (a) for illustration purposes only.

Figure 4.

Direct adsorption studies. (a) Beer's Law plot of the maximum absorbance peak of CQ measured at 344 nm shows a decrease in absorbance after 16 hours (grey, solid circles), compared to the initial absorbance (black, solid circles). A control excluding β -hematin indicated no decrease in the absorbance of CQ (dashed line, open circles). (b) The best fit of the mass of the CQ adsorbed per mg β -hematin crystals ($m(\text{CQ})_A/\text{mg } \beta\text{H}$), against the CQ concentration remaining in solution after adsorption ($[\text{CQ}]_S$) to the Langmuir isotherm (Equation 2) yields the adsorption constant K ($r^2 = 0.91$).

Activity (IC₅₀) and kinetics data for β-hematin formation at a lipid-water interface (37 °C, pH 4.8) in 24-well plates in the presence of antimalarial drugs.^a

Table 1

Drug	IC ₅₀		100Y	z (min ⁻¹)	K _{ads} (mM ⁻¹)	k ₂ (M ⁻² min ⁻¹)
	Parasite	β-H (μM)				
CQ	14.0 ^b					
	15.0 ± 2.9 ^c	18.8 ± 0.6	67.6 ± 0.8	0.015 ± 0.001	102.0 ± 4.7	0.12 ± 0.01
	10.6 ± 1.7 ^d					
AQ	7.8 ^b					
	16.0 ± 2.1 ^c	5.9 ± 0.3	70.1 ± 1.4	0.023 ± 0.003	566.4 ± 44.9	1.7 ± 0.4
	20.4 ± 0.7 ^d					
QD	21.5 ^b					
	47.0 ± 11.3 ^c	20.1 ± 4.1	73.6 ± 1.3	0.015 ± 0.001	32.7 ± 2.3	0.032 ± 0.005
	48.0 ± 10.9 ^d					
QN	34.2 ^b					
	141.0 ± 7.9 ^c	31.0 ± 8.3	68.1 ± 1.8	0.020 ± 0.003	61.2 ± 3.4	0.0014 ± 0.0007
	109.8 ± 25.6 ^d					

^aError calculated as standard error of the mean (SEM), following three experimental repeats, each including triplicate data points.

^bData (nM, 3D7) reported by Hawley *et al*⁴⁰

^cData (nM, D10) determined by Dr J.M. Combrinck, University of Cape Town, and communicated privately.

^dData (nM, NF54) determined by Dr J.M. Combrinck, University of Cape Town, and communicated privately.

Activity (IC₅₀) and kinetics data for β-hematin formation in the presence of short chain CQ analogues (**1**) and non-quinoline benzamide (**2**) inhibitors in a lipid-mediated system.^a

Table 2

	R ₁ /R ₂	X	Ar	IC ₅₀	100Y	z (min ⁻¹)	K _{ads} (mM ⁻¹)	k ₂ (M ⁻² min ⁻¹)
1a	Cl	NA	NA	29.8 ± 0.7 ^b	75.6 ± 1.4 ^d	0.017 ± 0.001 ^d	32.8 ± 3.0	0.59 ± 0.06
1b	I	NA	NA	35.7 ± 0.9 ^b	75.6 ± 1.4 ^d	0.017 ± 0.001 ^d	67.9 ± 3.8	0.18 ± 0.03
1c	CF ₃	NA	NA	55.5 ± 9.9 ^b	66.8 ± 1.1	0.023 ± 0.002	27.9 ± 2.0	0.025 ± 0.005
1d	SCF ₃	NA	NA	167.3 ± 9.0 ^b	72.7 ± 1.5	0.012 ± 0.001	16.2 ± 1.5	0.027 ± 0.005
2a	H	CH	<i>p</i> -pyridyl	43.8 ± 4.9 ^b 9 ± 4 ^c	81.1 ± 2.0 ^e	0.010 ± 0.001 ^d	4.0 ± 1.0	0.12 ± 0.02
2b	<i>t</i> -butyl	CH	<i>m</i> -pyridyl	6.2 ± 1.4 ^b 0.6 ± 0.1 ^c	81.1 ± 2.0 ^d	0.010 ± 0.001 ^d	32.3 ± 5.7	1.3 ± 0.2
2c	H	N	<i>p</i> -pyridyl	221 ± 16 ^b > 315 ^c	ND	ND	ND	ND
2d	<i>t</i> -butyl	CH	<i>p</i> -pyridyl	7.7 ± 0.9 ^b 1.6 ± 0.2 ^c	77.7 ± 2.3	0.0081 ± 0.0008	24.0 ± 3.1	0.09 ± 0.02
2e	OMe	CH	<i>p</i> -pyridyl	9.8 ± 0.6 ^b 3.0 ± 0.5 ^c	82.5 ± 1.6	0.015 ± 0.001	20.0 ± 2.4	0.07 ± 0.03

^aError calculated as standard error of the mean (SEM), following three experimental repeats, some including duplicate, and some triplicate data points. NA – Not applicable, ND – Not determined due to limited quantity of material.

^bData (μM, β-hematin) determined in current study.

^cData (nM, NF54 parasite strain) reported by Wicht *et al.*²⁹

^{d,e}Fitted values of Y and z for **1a** and **1b**, as well as **2a** and **2b**, are the same owing to kinetics experiments having been performed at the same time using a single control experiment.