Structure-Function Relationships in Chloroquine and Related 4-Aminoquinoline Antimalarials

Timothy J. Egan*

Department of Chemistry, University of Cape Town, Private Bag Rondebosch 7701, South Africa

Abstract: Recent publications have provided strong evidence that activity and cellular uptake of 4-aminoquinoline antimalarials depends on vacuolar haemoglobin degradation and that haematin is the drug target. Studies on haematin-quinoline interactions have provided insight into the structural requirements for these interactions and indications are that 4-aminoquinolines may act by inhibiting haemozoin formation. Structural requirements for this activity have also been reported recently and have led to construction of an empirical structure-function relationship for 4-aminoquinolines.

INTRODUCTION

Malaria is by far the most serious and widespread parasitic disease occurring in man. It is estimated that about two billion people are at risk from this disease in tropical and subtropical areas of the world. Approximately 300-500 million cases of malaria occur annually, leading to about 1.5 to 3 million deaths. About 90% of these occur in Sub-Saharan Africa where the disease contributes substantially to underdevelopment and places a severe strain on limited health care facilities. The remaining cases of malaria are largely confined to South and Southeast Asia and parts of South America [1].

The seriousness of the malaria problem has been greatly exacerbated in recent years as a result of several factors. The disease has spread into new areas as a result of changing land utilisation, especially plantation agriculture. This has led to epidemics in regions previously free of malaria, especially in highland areas of East Africa [1]. Failure of malaria control measures in regions of conflict in Africa and SE Asia has also contributed to an upsurge of malaria in areas where it was previously under control [1]. Finally, the spread of drug resistant strains of parasite has seriously reduced the efficacy of most antimalarial drugs, especially chloroquine.

Resistance to chloroquine is now widespread [1]. Given that chloroquine is cheap, safe for use in pregnancy and was previously highly efficacious [2], the loss of this drug has been a major setback to the effective treatment and control of malaria. Furthermore, resistance to other antimalarials such as sulfadoxine-pyrimethamine [1] and mefloquine [3] is also quite widespread and resistance to the currently available drugs, quinine [4] and amodiaquine [5] has also been observed, while halofantrine exhibits serious toxicity at concentrations required for treating resistant strains [6]. There is an urgent need to discover new antimalarial drugs to augment this very limited armamentarium [1].

This gloomy situation is counterbalanced to some extent by certain positive factors. Firstly, drug resistance to quinoline antimalarials does not appear to result from any change in the structure of the drug target, but rather appears to involve a mechanism by which the concentrations of specific drugs are reduced at the target site [7]. Secondly, under experimental conditions, close analogues of chloroquine have been shown to retain full activity against chloroquine resistant strains of parasite [8]. Thirdly, recent advances in our understanding of the mechanism of action of quinoline antimalarials as well as their structure-function relationships has improved the prospects for rational design of novel quinoline antimalarials.

BIOLOGY OF THE MALARIA PARASITE

In humans malaria is caused by four species of the genus *Plasmodium*, namely *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* [9]. Of these, *P. falciparum* is the most important as it causes almost all malaria associated deaths [1]. There is, however, significant morbidity associated with *P. vivax* [1]. The biology of *P. falciparum* is fortunately the best understood of the four species as techniques for maintaining it in culture have been available for about 25 years [10].

The life cycle of *P. falciparum* is divided into three overall stages [9], mosquito, liver and blood stages. Sexual reproduction of gametocytes occurs in the gut of the mosquito and leads to the formation of zygotes that bury themselves in the gut lining of the mosquito. These then develop into oocysts and after some time form sporozoites that migrate to the salivary glands of the mosquito. When an infected mosquito bites a human host, these sporozoites enter the blood stream and rapidly make their way to the liver, invading hepatocytes. During a period of development in the liver of about a week as tissue schizonts, the parasites

*Address correspondence to this author at the Department of Chemistry, University of Cape Town, Private Bag Rondebosch 7701, South Africa, Tel. +27-21-650-2528, Fax. +27-21-689-7499, E-mail: tegan@psipsy.uct.ac.za

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multiply asexually, finally simultaneously rupturing the host cells and entering the blood stream as merozoites. These merozoites invade red blood cells, entering into the blood cycle consisting of ring, trophozoite and blood schizont stages. Asexual reproduction in the blood cell leads to further merozoites and hence to ever increasing parasitaemia. Some of the merozoites develop into gametocytes upon entering the red cell and may be taken up by mosquitoes to complete the life cycle. Symptoms of the disease are entirely associated with the blood stage and so any curative drug must be specifically active against this part of the life cycle [9].

During its blood stage, *P. falciparum* utilises host haemoglobin as a food source. The globin is degraded to peptides by parasite aspartic proteinase enzymes called plasmepsins I [11] and II [12] and a cysteine proteinase enzyme called falcipain [13]. This occurs in an acidic compartment within the parasite called a food vacuole that has a pH in the range 5.2-5.6 [14, 15]. In the process haem is released from the haemoglobin and autoxidised to haematin (aquaferriprotoporphyrin IX or H_2O-Fe(III)PPIX). This leads to the production of hydrogen peroxide through the one electron oxidation of Fe(II). In addition to this oxidative assault, the concentration of haematin released into the food vacuole is high (in the region of 0.4 M) [16]. High concentrations of haematin are potentially toxic [17, 18] and require a detoxification mechanism.

It is well established that at least a portion of this H_2O-Fe(III)PPIX is detoxified by conversion to malaria pigment or haemozoin, a highly insoluble, microcrystalline form of ferriprotoporphyrin IX [16, 19]. Recently, studies that have measured the total iron content of parasitised red cells and attempted to balance this with haemoglobin and haemozoin content have suggested that a significant portion (up to about 80%) of the haemoglobin-derived iron is not incorporated into haemozoin, but is degraded to non-haem iron [20, 21]. It has been suggested that this comes about as a result of peroxidative degradation of haematin within the food vacuole [20], or via glutathione dependent degradation outside the vacuole in the parasite cytosol [21]. No indication of the form in which this proposed large quantity of low molecular weight non-haem iron is sequestered has so far been given.

THE ACTIVITY OF 4-AMINOQUINOLINE AND QUINOLINE METHANOL ANTIMALARIALS IS DEPENDENT ON HAEMOGLOBIN DEGRADATION

Several recent studies [22-24] have provided conclusive evidence that the activities of the 4-aminquinoline antimalarials, chloroquine and amodiaquine, as well as the quinoline methanols, quinine and mefloquine, are dependent on haemoglobin degradation. Both specific, reversible inhibitors of plasmepsins I and II (Ro 61-9379, Ro 61-7835, Ro 40-4388) and inhibitors of proplasmepsin I and II activation (ALLM and ALLN) are antagonists of drug activity [22, 24]. The irreversible cysteine proteinase inhibitor E64 is also an antagonist of these quinoline antimalarials, presumably through inhibition of falcipain for which it is a non-specific inhibitor [22].

A number of interpretations are possible for this behaviour. Activity of these quinoline compounds could arise from inhibition of the proteinase enzymes themselves, a peptide product of haemoglobin could be the drug target, or haematin could be the drug target. There is evidence that chloroquine inhibits parasite proteinase enzymes [25-27], but this occurs at concentrations substantially higher than those which are usually likely to occur in the vacuole [28]. By contrast, all 4-aminoquinoline and quinoline methanol antimalarials are known to form complexes with haematin. Haematin was first proposed as the target of chloroquine in 1964 [29] and Fitch and co-workers provided further evidence in this regard during the 1980's [30]. By contrast, there is no evidence to suggest that chloroquine and related antimalarials act by interacting with haemoglobin peptide fragments.

Recently, further evidence has been presented indicating that haematin is indeed the target of 4-aminoquinoline antimalarials [23, 24]. Uptake of chloroquine and amodiaquine into parasitised red cells is reduced by up to 90% when vacuolar haemoglobin degradation is blocked by the specific plasmepsin inhibitors described above. This binding is specific and saturable [23]. Thus, not only is the activity of these drugs dependent on haemoglobin degradation, but its binding to specific sites within the cell requires haemoglobin degradation. Further evidence that haematin acts as the drug target is provided by the fact that the binding can be modelled by haematin-loaded red cell ghosts [24].

A further non-saturable component of chloroquine uptake can be accounted for by its weak base properties. Chloroquine has two protonatable sites with pK_a's of 8.3 and 10.2 respectively [31]. Based on the premise that drug uptake into the vacuole occurs by passive diffusion, only the unprotonated neutral species will be capable of entering or exiting the vacuole. In the limit, chloroquine will diffuse into the vacuole until the intravacuolar free base concentration is the same as its external concentration. This will result in substantial vacuolar total chloroquine accumulation, because the low pH within the vacuole ensures that the ratio of protonated to unprotonated species is far higher than at extravacuolar pH. This so-called pH trapping mechanism has previously been proposed to account for chloroquine uptake by parasites [32] but it was later recognised that it could not account for all the observed drug accumulation [33]. The extent of the pH trapping effect can be estimated from the equation:

$$\frac{[CQ]_v}{[CQ]_e} = \frac{\left\{1 + \frac{[H^+]_v}{K_{a2}} + \frac{[H^+]_v^2}{K_{a1}K_{a2}}\right\}}{\left\{1 + \frac{[H^+]_e}{K_{a2}} + \frac{[H^+]_e^2}{K_{a1}K_{a2}}\right\}}$$

Here $[CQ]_v$ refers to the total vacuolar concentration of chloroquine and $[CQ]_e$ refers to its total extravacuolar concentration. The terms $[H^+]_v$ and $[H^+]_e$ refer to the vacuolar and extravacuolar hydrogen ion concentration respectively. Based on a measured vacuolar pH ranging between 5.6 [15] and 5.4 [14], this equation predicts a

limiting vacuolar chloroquine accumulation of about 3500-8900 relative to the serum (pH 7.4). Given that the food vacuole occupies about 2% of the volume of the parasitised red cell [15] and assuming no other accumulation within the cell this can account for an overall cellular accumulation (in the parasitised red cell) of about 70-180 fold. This is in reasonable agreement with observed non-saturable accumulation [23] and accounts for only about 5-12 % of the observed total accumulation in chloroquine sensitive parasites [23].

INTERACTION OF QUINOLINE ANTIMALARIALS WITH HAEMATIN

The ability of chloroquine to form a complex with haematin was first recognised in 1964 [29] and led to proposals that haematin is the target of chloroquine in the 1980's [30]. These findings motivated a number of studies on antimalarial-haematin interactions in both aqueous and non-aqueous solution as well as on their interactions with other iron-porphyrins. Many of the earlier studies concentrated on obtaining spectroscopic evidence for haematin-drug interactions [34-44] and association constants were not determined. Visible, Mössbauer and NMR spectroscopic evidence has been provided for the formation of complexes between haematin and chloroquine, amodiaquine, quinine and mefloquine [34-44], although initial studies in benzene solution resulted in some dispute over the ability of amodiaquine to complex haematin [41]. Later it was conclusively demonstrated by Mössbauer and visible spectroscopy that amodiaquine does complex haematin in aqueous solution [40, 42, 43].

An early paper by Fitch and co-workers [30] reported association constants in aqueous medium based on an equilibrium dialysis method. More recently the association constants for interactions between several quinoline antimalarials and monomeric haematin in 40% aqueous DMSO have been reported [45, 46]. These were determined by spectrophotometric titration and have largely confirmed the trends reported in the earlier study. Under these solvent conditions, association constants range between a log K value of 3.90 ± 0.08 for mefloquine and 5.52 ± 0.02 for chloroquine. Enthalpies and entropies of complexation were determined for most of these compounds from Van't Hoff plots and a thermodynamic compensation phenomenon was found. Replacement of water with acetonitrile in the solvent system indicates that these interactions are largely hydrophobic as a substantial weakening in complex strength is observed. In 40% DMSO complexes were largely found to exhibit a 1:1 stoichiometry. Subsequently, association constants and thermodynamic parameters for a series of quinoline antimalarials with haematin in aqueous solution have been reported using titration calorimetry [47]. Once again, the trends in complex strengths observed in earlier studies have been confirmed, although stoichiometries of interaction are different, often conforming to a 4:1 model (haematin:quinoline). This has been interpreted as evidence of a complex in which the drug is sandwiched between two haematin μ-oxo dimers. Given the known propensity of haematin to dimerise in aqueous solution [48], this is not unexpected. Association constants reported in these recent studies are given in Table **1**.

Rather less is known about the structures of these complexes. The complexes between 4-aminoquinolines and haematin are almost certainly π-π complexes. This means that there is a coplanar interaction between the aromatic ring of the quinoline and the porphyrin system. The term π-π complex is historical and does not imply that the major source of stability of the complex results from overlap of the π-orbitals of the two systems [49]. Rather, hydrophobic interactions are probably the major source of stability as indicated above. Nonetheless, electronic factors almost certainly play a role in influencing the structures of such complexes [49]. The strongest evidence for π-π complexation is the ability of chloroquine to form complexes with metal-free protoporphyrin IX [37] and with uroporphyrin I [50] which are almost as strong as those with the corresponding iron porphyrins. This clearly demonstrates that these are not coordination complexes. NMR peak shifts and paramagnetic line broadening [37, 50] are in strong agreement with this interpretation. This contrasts with the

Table 1 Association Constants for Interaction of Quinoline Antimalarials with Haematin in 40 % Aqueous DMSO[a] and in Aqueous Solution[b]

	Log K	
Compound	**In 40 % aqueous-DMSO, pH 7.5, 25 °C, 1:1 stoichiometry**	**Aqueous soln., pH 6.5, 37 °C, quinoline:haematin ratio in { }**
Chloroquine	5.52 ± 0.03[c]	5.6 ± 0.2[d] {1:4}
Amodiaquine	5.39 ± 0.04[c]	5.0 ± 0.1[d] {1:4}
Quinacrine	¾	5.69 ± 0.04[d] {1:4}
Pyronaridine	¾	5.47 ± 0.03[d] {1:7}
Quinine	4.10 ± 0.02[c]	4.32 ± 0.04[d] {1:5}
Quinidine	5.02 ± 0.03[e]	¾
Mefloquine	3.90 ± 0.08[c]	4.1 ± 0.1[d] {1:3}

[a]by spectrophotometric titration, [b]by titration calorimetry, [c]from reference 45, [d]from reference 47, [e]from reference 46.

quinoline methanols, where coordination may play a role. Interactions between quinine and haematin have a large π-π component [37, 51], but NMR spectra [51] of the quinine-urohaemin complex and the visible spectrum of the complex between quinine and the haem-peptide N-acetylmicroperoxidase-8 [52] indicate that the hydroxyl group of quinine may coordinate to the iron centre. Mössbauer spectra of haematin-chloroquine and haematin-quinine complexes are consistent with these interpretations [44].

Until the recent appearance of two publications [53, 54] very little was known of the structural requirements for quinolines capable of strong haematin-binding, although it had been shown earlier that quinoline itself and certain simple aminoquinolines (5-, 6- and 8-aminoquinoline) do not form substantial complexes with haematin [45]. It has now been established that 2- and 4-aminoquinoline have a unique ability to form strong complexes with haematin in aqueous DMSO [54]. The association constant of 4-aminoquinoline in particular (log K = 4.49 ± 0.01), is comparable with some quinoline antimalarials (see Table **1**). Introduction of a 7-chloro group in 4-amino-7-chloroquinoline has almost no influence on this interaction (log K = 4.43 ± 0.01) and alkylation of the amino group also has little influence (log K = 4.38 ± 0.03 for 4-methylaminoquinoline) [54]. A similar association constant for 4-amino-7-chloroquinoline has also been reported in aqueous solution using titration calorimetry [53]. Moving the chloro group from the 7-position to the 6-position, on the other hand, appears to completely destabilise the complex [53] (see Fig. (**1**)).

The underlying electronic and steric factors controlling the ability of these quinolines to form such complexes with haematin is still poorly understood. Both 2- and 4-aminoquinoline have substantially higher pK_a's than either quinoline or other aminoquinolines [55]. This may point to a cation-π interaction between the quinolines and haematin, but the lack of sensitivity of the association constant to pH reported for chloroquine [45, 56] seems to argue against this interpretation.

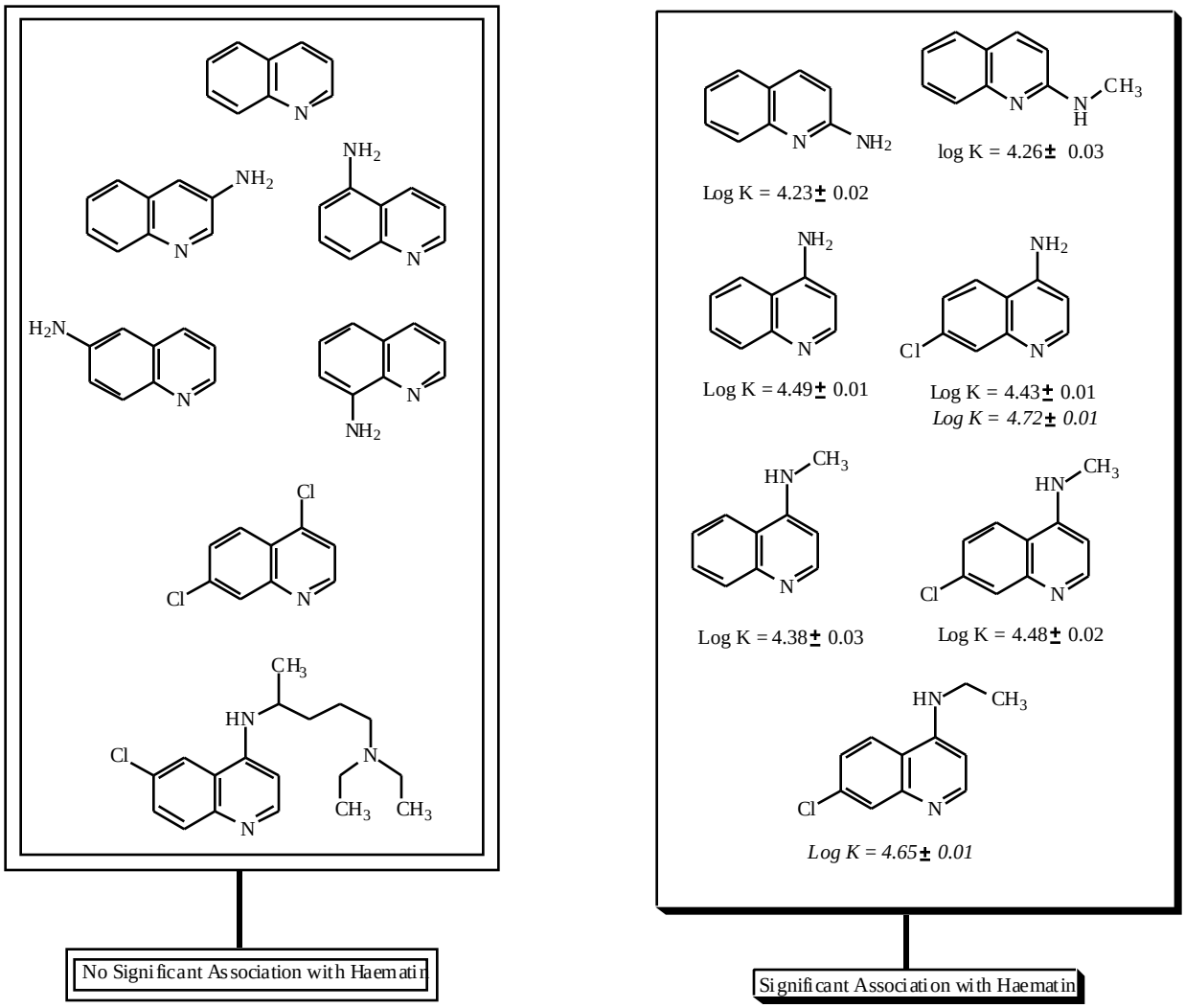


Fig. (1). Haematin associating properties of simple quinolines reported recently [53, 54]. Only 2- and 4-aminoquinolines form strong complexes with haematin. Alkylation of the amino group or introduction of a 7-chloro group has little effect on log K. A 6-chloro group results in a quinoline unable to complex haematin. Log K values in 40% aqueous DMSO solution (pH 7.5) [54] or aqueous solution (pH 6.5, italics) [53]. None of these compounds show strong antiplasmodial activity.

A few attempts have been made to model the interactions of quinoline antimalarials with haematin by computational techniques. A molecular mechanics study [52] of the interaction between chloroquine and an iron-porphyrin model for N-acetylmicroperoxidase-8 revealed a minimum energy arrangement with coplanar interaction of the quinoline and iron-porphyrin ring, but could not define a preferred conformation for the complex. This study failed to take cognisance of the solvent, which a subsequent experimental study indicates plays a critical role in stabilising these complexes [45]. Another study used molecular mechanics and dynamics methods [57] to model the interaction of the amodiaquine analogue tebuquine with haematin. This study took solvent into account by performing the study both in vacuum and surrounded by a 5 Å sphere of water molecules. The study suggested that both the co-planar interaction between the quinoline ring and iron-porphyrin and a hydrogen-bonding interaction between the side-chain terminal amine of the drug and the haem propionate groups plays a critical role in complex stability. Subsequent experimental studies on simple 4-aminoquinolines described above [53, 54] have shown, however, that the hydrogen-bond interaction is not critical in the formation of these complexes as compounds lacking a basic terminal amino group form complexes with haematin. More recently the electrostatic potential surfaces of chloroquine and several chloroquine analogues have been calculated using *ab initio* quantum mechanical methods [53]. This study indicates that π-electron density at C8, C8a, C4 and C4a of the quinoline ring may play an important role in determining the ability of quinolines to interact with haematin (Fig. (**2**)). The electron rich quinoline is suggested to interact with an electron deficient region around the iron centre of the μ-oxo dimer of haematin to form a sandwich complex. This study is partially supported by an earlier quantum-pharmacological study [58] of several quinolines which also indicates that electron density on C8a, C4 and C4a plays an important role in activity.

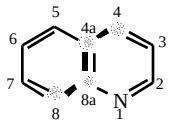


Fig. (2). Numbering system of the quinoline nucleus, showing atoms whose electrostatic charge densities have been reported to be crucial in determining the ability of the quinoline to associate with haematin (4, 4a, 8 and 8a) [53].

At the present time our understanding of these interactions is not sufficiently advanced to undertake true rational design of quinolines capable of forming strong complexes with haematin. Nevertheless, our empirical understanding of these interactions has advanced considerably in the last few years and it is possible that the use of sophisticated computational methods may elucidate these mechanisms in the next few years.

Interestingly, the strength of haematin-quinoline interactions does not directly correlate with antiplasmodial activity [54]. The evidence suggests that haematin binding is a necessary, but not sufficient requirement for antiplasmodial activity [54].

INHIBITION OF HAEMOZOIN FORMATION

Current investigations of the effect of quinoline antimalarial-haematin interaction centres on two hypotheses. The first is the ability of these drugs to inhibit haemozoin formation and the second is their ability to inhibit the breakdown of haematin either by hydrogen peroxide or in the presence of glutathione.

The ability of quinoline antimalarials to inhibit synthetic haemozoin (β-haematin) formation was first reported by Slater and Cerami in 1992 [59]. At the time it was believed that β-haematin is a polymer and that the drugs inhibited a putative haem-polymerase enzyme. Subsequently, it was shown that these drugs could inhibit synthetic β-haematin formation in the absence of any other biological materials [60] and suggested that inhibition occurs through direct interaction between haematin and drug with the resulting complex not being capable of β-haematin formation. A specific correlation between ability to inhibit β-haematin formation and antimalarial activity was shown for a number of quinoline antimalarials and other simple quinolines. Later Dorn *et al.* [61] reported similar findings under conditions closer to those that occur *in vivo*. Subsequently, evidence was presented by Goldberg and co-workers indicating that inhibition of β-haematin formation occurs via interaction of haematin-drug complex with the growing β-haematin product [62, 63]. This was interpreted as capping of the supposed polymer chain. The recent determination of the structure of β-haematin [16] shows that this interpretation is not tenable as it is not a polymer. Rather, it would appear that the haematin-drug complex may block the fastest growing face of the crystal [16] or possibly inhibit β-haematin nucleation [64]. Indeed, parallels between β-haematin formation and biomineralisation processes have recently been noted [64] and this leads to the intriguing possibility that chloroquine may act as a crystallisation inhibitor.

In any event, chloroquine and a wide range of quinoline and phenanthrene antimalarials and antiplasmodials have now been shown to inhibit β-haematin formation by a number of groups [46, 47, 53, 54, 60-63, 65-68]. Interestingly, inhibition of β-haematin formation is not merely a result of association between the quinoline and haematin. Both 9-epiquinine [60] and a series of 2-aminoquinolines and of 4-aminoquinolines that lack a 7-chloro group [53, 54] have been shown not to inhibit β-haematin formation despite forming complexes with haematin [45, 53, 54] (Fig. (**3**)). Indeed, these complexes are just as strong as those formed by similar compounds that do inhibit β-haematin formation [45, 54]. In the case of 4-aminoquinolines, two independent studies indicate that the key feature for inhibition of β-haematin formation appears to be the presence of the 7-chloro group [53, 54] (Fig. (**3**)). Replacement of the 7-chloro group with a bromo or nitro group has been shown to result in complexes that are still inhibitory, while the 7-amino and 7-hydro- derivatives are not [53] (Fig. (**3**)). This seems to suggest that groups that are strongly π-withdrawing as indicated by their Hammett constant are successful inhibitors of β-haematin formation. This view appears to be largely consistent with recently reported antiplasmodial activities of a series of 4-

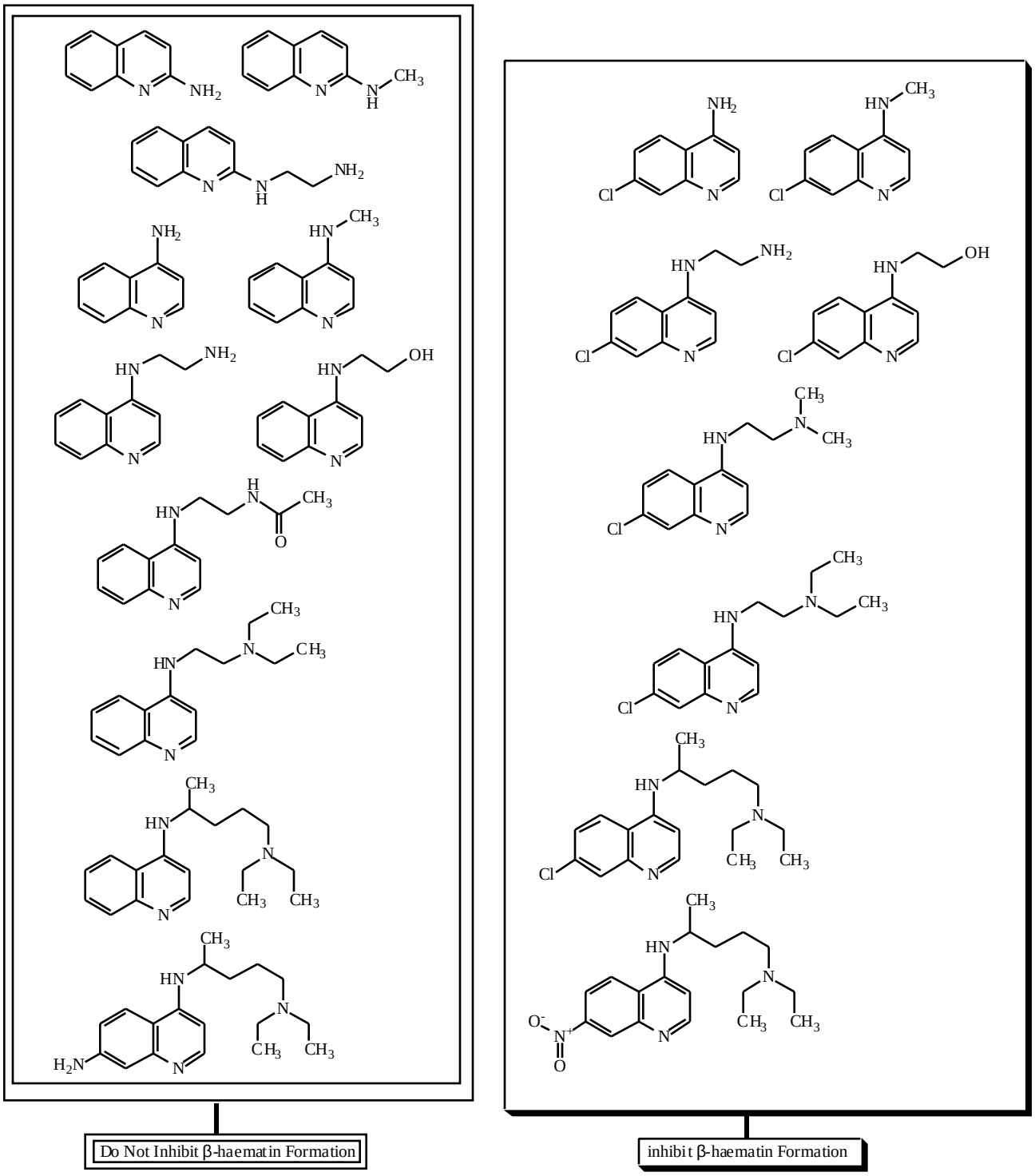


Fig. (3). Ability of quinolines that associate strongly with haematin to inhibit β-haematin formation [53, 54]. Those capable of inhibiting β-haematin formation have a group with a positive Hammett constant at the 7- position (Cl = 0.227, Br = 0.232 or NO_2 = 0.778), while those that do not inhibit its formation lack such a group (H = 0.000 or NH_2 = -0.66). Quinolines in the left panel have weak or no antiplasmodial activity (IC_{50} >20× IC_{50} of chloroquine against chloroquine sensitive parasites).

aminoquinolines with fluoro-, chloro-, bromo-, iodo-, trifluoromethyl- and methoxy- substituents at the 7-position [69]. Here most of the fluoro- and methoxy- derivatives were found to be substantially less active than the corresponding chloro-, bromo-, iodo- and trifluoromethyl- derivatives. Beyond these empirical observations, little is known about the structural requirements for inhibition of β-haematin formation at the present time.

Despite considerable circumstantial evidence that quinoline antimalarials act by inhibiting haemozoin formation, unequivocal evidence for this hypothesis has not

yet been provided *in vivo*. Indeed two alternative mechanisms of action involving haematin have recently been advanced. According to the first of these hypotheses most haematin detoxification occurs via movement of haematin across the vacuolar membrane into the parasite cytosol, where it is broken down in a glutathione dependent process [21]. This process can be modelled *in vitro* and quinoline antimalarials have been shown to inhibit the process at micromolar concentrations. An apparent weakness of this hypothesis is that chloroquine is active at nanomolar concentrations and no known accumulation occurs in the parasite cytosol. In order to operate at the IC_{50} of chloroquine the haematin-drug complex would have to form irreversibly in the vacuole and then diffuse into the cytosol. It would seem somewhat unlikely that formation of a π-π complex would be irreversible. A second, related hypothesis, suggests that much of the haematin present in the vacuole is degraded by hydrogen peroxide produced during haem autoxidation [20]. This too can be modelled *in vitro* and is inhibited by chloroquine concentrations likely to occur in the vacuole. Definitive evidence for large quantities of non-haem iron in the food vacuole is still required before this hypothesis is likely to enjoy widespread support. Given that haemozoin is readily visualised in the food vacuole of parasites, the presence of even larger quantities of non-haem iron ought to represent quite a prominent and readily observable feature.

Over the years a number of other hypotheses have been presented to account for the activity of chloroquine including interactions with DNA and with various extravacuolar and intravacuolar enzymes. These hypotheses have been reviewed in detail elsewhere [28] and will not be discussed here. Convincing arguments have been provided [28] which

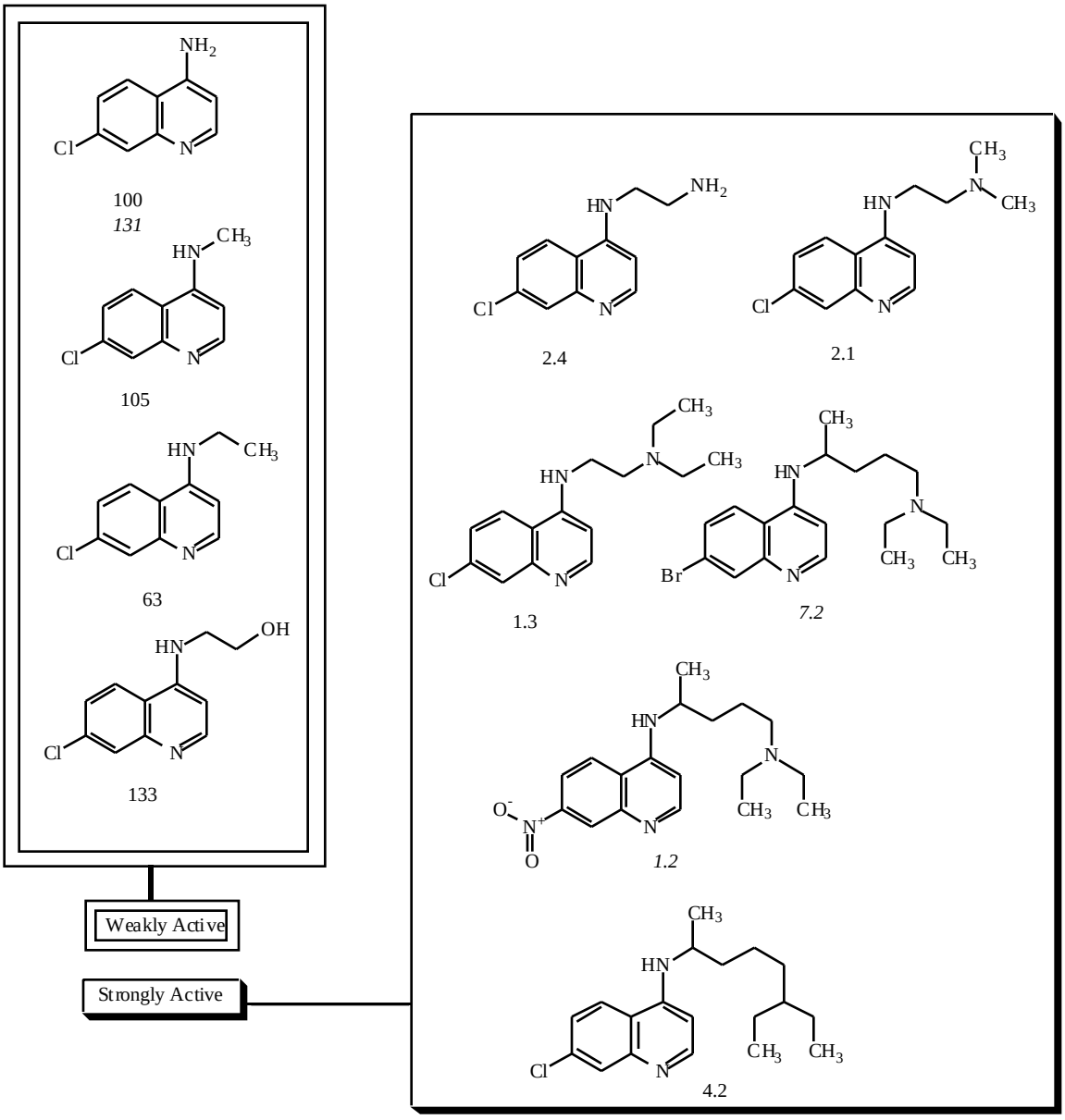


Fig. (4). IC_{50} relative to chloroquine of 4-aminoquinolines capable of inhibiting β-haematin formation. IC_{50}/IC_{50} (chloroquine) for chloroquine sensitive parasites from [53] (italics) and [54] shown below each compound. The compound at bottom right appears to be the only known strongly active quinoline lacking a terminal amine.

indicate that these are unable to account for the activity of chloroquine at the very low concentrations corresponding to its IC_{50}. Another hypothesis that has enjoyed some support in the past, suggests that chloroquine acts by raising vacuolar pH through its basicity [14]. Recent fluorimetric measurements on live single cells does not support this hypothesis [70]. There is in fact no short-term change in vacuolar pH and the longer-term effect of chloroquine is to lower and not to raise the pH. It is quite possible, however, that some alternative mechanisms may play a role at higher drug concentrations and that more than one mechanism of action may be involved under certain conditions, especially in the case of less active quinolines that are only antiplasmodial at relatively high concentrations.

ROLE OF THE AMINOALKYL SIDE CHAIN IN THE ANTIPLASMODIAL ACTIVITIES OF 4-AMINOQUINOLINES

Almost all 4-aminoquinolines with strong antiplasmodial activity contain an aminoalkyl side chain. Studies in which the amino group is replaced by an hydroxyl group [54, 71] show a drastic reduction in antiplasmodial activity (Fig. (**4**)). But for one exception [53], replacement of the aminoalkyl side chain on the 4-aminoquinoline nucleus with an alkyl side chain also causes a drastic reduction in activity [53, 54, 71]. The requirement for the terminal amino group has generally been linked to its weak base properties [14, 31, 32]. In earlier studies this was ascribed to both drug accumulation and putative alkylinisation of the vacuole [31, 32]. Subsequently it was shown that putative alkylinisation of the vacuole does not correlate with activities of compounds [72], but a role for the basic amino group in drug accumulation in the acidic vacuole was maintained.

At first sight, this weak base effect of the terminal amine appears to be at odds with recent observations that most drug accumulation occurs through association with haematin and not by pH trapping [23, 24] as discussed above. More careful consideration however, reveals that accumulation of free drug in the food vacuole probably plays a critical role in the overall accumulation of drug via the law of mass action. For example, if one considers a 1:1 haematin-quinoline complex the appropriate equilibrium is:

$$H + Q \underset{}{\overset{K}{\rightleftharpoons}} HQ$$

Where H represents haematin, Q represents the quinoline and HQ is the haematin-quinoline complex. The association constant, K, is given by the equation:

$$K = \frac{[HQ]}{[Q] \cdot [H]}$$

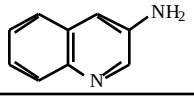
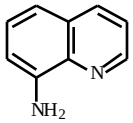
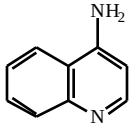
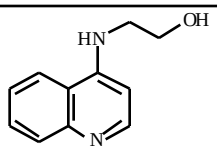
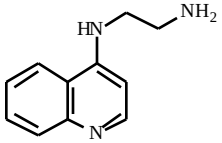
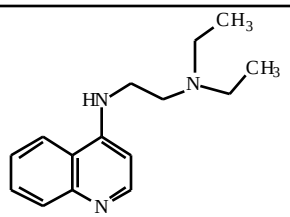
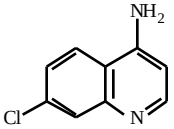
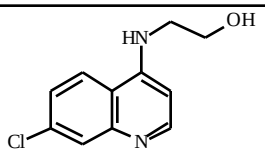
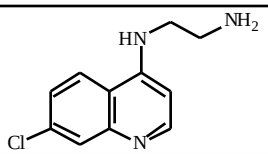
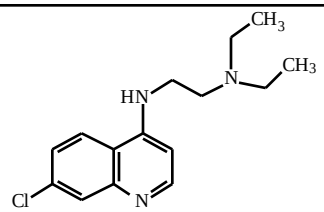
In this equation [H] represents the solution concentration of free haematin and [Q] represents the concentration of free quinoline. The solubility limit of haematin at pH 5.6 has recently been reported to be about 150 μM in aqueous solution [15]. Free haematin concentration is unknown, but it has been estimated at about 20 μM [15]. Given that K is about 3.3×10^5 M^{-1} for haematin-chloroquine complex (at least in 40% DMSO) [45] one can calculate [HQ] at the IC_{50} of chloroquine based on the fact that free chloroquine accumulates by about 3500-8900 fold through pH trapping as calculated earlier. Assuming an IC_{50} of 13 nM for the chloroquine sensitive HB3 strain of parasite [23], the limiting vacuolar concentration of free chloroquine would be about 46-116 μM. This gives a total vacuolar chloroquine concentration of 346-882 μM (300-757 μM HQ complex and 46-116 μM free Q assuming a free haematin concentration of 20 μM). Given that the vacuole constitutes about 2% of the volume of the parasitised red cell, this corresponds to a cellular accumulation ratio of about 630-1630. This is in good agreement with the observed value of about 1300 [23]. In the absence of pH trapping, however, the cellular accumulation of chloroquine is negligible, about 1.05, as almost no haematin-chloroquine complex forms at 13 nM chloroquine. (If this calculation is correct it raises some serious questions about the mode of action of quinoline methanols such as quinine as these have much lower pK_a values [31] and much lower association constants with haematin [45, 47]. As a result much lower cellular accumulation is likely to occur).

CONCLUSION

Recent studies have provided convincing evidence that the action of 4-aminoquinoline antimalarials is dependent on haemoglobin degradation in the parasite food vacuole and that this probably results from association of the drugs with haematin. These drugs have been shown to inhibit synthetic β-haematin formation, indicating that they may act by inhibiting haemozoin formation *in vivo*, although there are suggestions that they may also inhibit haematin degradation. Both haemozoin formation and haematin degradation are proposed detoxification mechanisms. Finally, accumulation of these drugs in the food vacuole through pH trapping as a result of their weak base properties also appears to be a requirement for strong activity.

A recent study [54] investigating the role of these factors in antiplasmodial activity of aminoquinolines has suggested a structure-function relationship for chloroquine (Fig. (**5**)). In this model the 4-aminoquinoline nucleus is required for haematin association, the 7-chloro group confers β-haematin inhibitory activity on this nucleus and the terminal amino group in the aminoalkyl side chain is required for accumulation in the vacuole. There is no simple correlation between the strength of antiplasmodial activity and any one of these factors, but in combination they can predict the activity of a number of aminoquinolines. As can be seen in Table 2 compounds that fail to associate with haematin do not inhibit β-haematin formation and have essentially no antiplasmodial activity. Those that do associate with haematin, but are unable to inhibit β-haematin formation and lack the amino group required for accumulation are also inactive. Aminoquinolines that can inhibit β-haematin formation, but lack the amino group and those that do not inhibit β-haematin formation but can accumulate through pH trapping due to the presence of the amino group exhibit weak

Table 2. Haematin Association, Inhibition of β-Haematin Formation, Presence of Basic Amino Group and Antiplasmodial Activities for a Selection of Aminoquinolines[a]

Compound	Log K[b]	Inhibition of β-haematin	Presence of basic amino group	IC_{50} / nM[c]
	_[d]	−	−	> 10 000
	_[d]	−	−	> 10 000
	4.49 ± 0.01	−	−	> 10 000
	4.18 ± 0.03	−	−	> 10 000
	4.59 ± 0.04	−	+	4 700 ± 800
	4.75 ± 0.02	−	+	799 ± 404
	4.43 ± 0.01	+	−	3 800 ± 500
	4.66 ± 0.02	+	−	5070 ± 80
	4.928 ± 0.003	+	+	92 ± 12
	5.81 ± 0.01	+	+	49 ± 14

[a]from reference 54, [b]in 40 % aqueous-DMSO, pH 7.5, 25 [o]C, [c]versus chloroquine sensitive D10 strain (IC_{50} for chloroquine = 38 ± 14 nM), [d]no association observed.

antiplasmodial activity. By contrast, those that inhibit β-haematin formation and contain the amino side chain show strong activity.

These findings provide a set of empirical guidelines for designing new 4-aminoquinolines with potential antiplasmodial activity. The structure of the aminoalkyl side

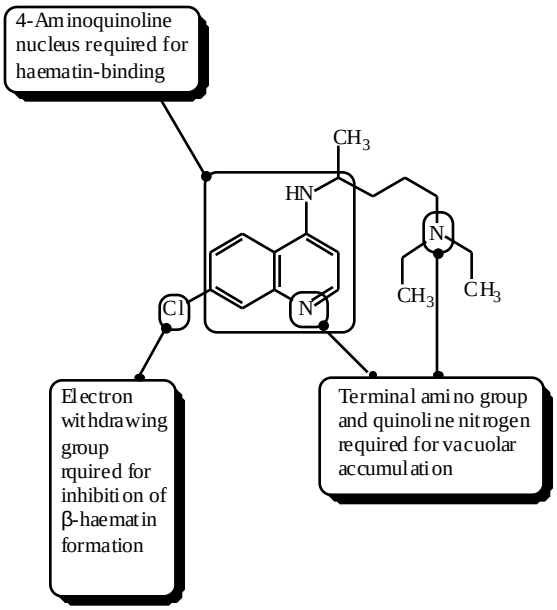


Fig. (5). Recently proposed structure-activity relationship for chloroquine [54].

chain does not appear to play a major role in any of these processes. On the other hand there is strong evidence that significant structural change to the side chain, either through altering its length [8, 73] or through the introduction of novel structural motifs such as ferrocene [74, 75] circumvents chloroquine resistance. As a result, the ability to partially design novel antiplasmodial 4-aminoquinolines may prove to be of some importance in fighting chloroquine resistant parasites. Even more significantly, as our understanding of the fundamental physical chemistry underlying these factors increases, it may ultimately point the way to new families of antiplasmodial compounds.

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REFERENCES

[1] Trigg, P.I., Kondrachine, A.V. In *Malaria. Parasite biology, pathogenesis and protection*; Sherman, I.W., Ed; ASM Press: Washington DC, **1998**; pp. 11-22.

[2] Winstanley, P.A., Breckenridge, A.M. *Ann. Trop. Med. Parasitol.*, **1987**, *81*, 619.

[3] Nosten, F., Ter Kuile, F.O., Chongsuphajaisiddhi, T., Luxemburger, C., Webster, H.K., Edstein, M., Phaipun, L., Thew, K.L., White, N.J. *Lancet.*, **1991**, *337*, 1140.

[4] Price, R.N., Cassar, C., Brockman, A., Duraisingh, M., Van Vugt, M., White, N.J., Nosten, F., Krishna, S. *Antimicr. Agents Chemother.*, **1999**, *43*, 2943.

[5] Bray, P.G., Hawley, S.R., Ward, S.A. *Mol. Pharmacol.*, **1996**, *50*,1551.

[6] Ter Kuile, F.O., Dolan, G., Nosten, F., Edstein, M.D., Luxemburger, C., Phaipun, L., Chongsuphajaisiddhi, T., Webster, H.K., White, N.J. *Lancet.*, **1993**, *341*, 1044.

[7] Ridley, R.G., Dorn, A., Vippagunta, S.R., Vennerstrom, J.L. *Ann. Trop. Med. Parasitol.*, **1997**, *91*, 559.

[8] De, D., Krogstad, F.M., Cogswell, F.B., Krogstad, D.J. *Am. J. Trop. Med. Hyg.*, **1996**, *55*, 579.

[9] *Malaria: Principles and Practice of Malariology*, Wernsdorfer, W.H., McGregor, I., Eds; Churchill-Livingstone: Edinburgh, **1988**.

[10] Trager, W., Jensen, J.B. *Science*, **1976**, *193*, 673.

[11] Goldberg, D.E., Slater, A.F.G., Beavis, R., Chait, B., Cerami, A., Henderson,G.B. *J. Exp. Med.*, **1991**, *173*, 961.

[12] Gluzman, I.Y., Francis, S.E., Oksman, A., Smith, C.E., Duffin, K.L., Goldberg, D.E. *J. Clin. Invest.*, **1994**, *93*, 1602.

[13] Francis, S.E., Gluzman, I.Y., Oksman, A., Banerjee, D., Goldberg, D.E. *Mol. Biochem. Parasitol.*, **1996**, *83*, 189.

[14] Krogstad, D.J., Schlesinger, P.H., Gluzman, I.Y. *J. Cell. Biol.*, **1985**, *101*, 2302.

[15] Dzekunov, S.M., Ursos, L.M.B., Roepe, P.D. *Mol. Biochem. Parasitol.*, **2000**, *110*, 107.

[16] Pagola, S., Stephens, P.W., Bohle, D.S., Kosar, A.D., Madsen, S.K. *Nature*, **2000**, *404*, 307.

[17] Orjih, A.U., Banyal, H.S., Chevli, R., Fitch, C.D. *Science*, **1981**, *214*, 667.

[18] Ladan, H., Nitzan, Y., Malik, Z. *FEMS Microbiol. Lett.*, **1993**, *112*, 173.

[19] Bohle, D.S., Dinnebier, R.E., Madsen, S.K., Stephens, P.W. *J. Biol. Chem.*, **1997**, *272*, 713.

[20] Loria, P., Miller, S., Foley, M., Tilley, L. *Biochem. J.*, **1999**, *339*, 363.

[21] Ginsburg, H., Famin, O., Zhang, J., Krugliak, M. *Biochem. Pharmacol.*, **1998**, *56*, 1305.

[22] Mungthin, M., Bray, P.G., Ridley, R.G., Ward, S.A. *Antimicr. Agents. Chemother.*, **1998**, *42*, 2973.

[23] Bray, P.G., Mungthin, M., Ridley, R.G., Ward, S.A. *Mol. Pharm.*, **1998**, *54*, 170.

[24] Bray, P.G., Janneh, O, Raynes, K.J., Mungthin, M., Ginsburg, H., Ward, S.A. *J. Cell Biol.*, **1999** *145*, 363.

[25] Gyang, F.N., Poole, B., Trager, W. *Mol. Biochem. Parasitol.*, **1982**, *5*, 263.

[26] Vander Jagt, D.L., Hunsaker, L.A., Campos, N.M. *Mol. Biochem. Parasitol.*, **1986**, *18*, 389.

[27] Sherman, I.W., Tanigoshi, L. *Mol. Biochem. Parasitol.*, **1983**, *8*, 207.

[28] Slater, A.F.G. *Pharmac. Ther.*, **1993**, *57*, 203.

[29] Cohen, S.N., Phifer, K.O., Yielding, K.L. *Nature*, **1964**, *202*, 805.

[30] Chou, A.C., Chevli, R., Fitch, C.D. *Biochemistry*, **1980**, *19*, 1543.

[31] Schlesinger, P.H., Krogstad, D.J., Herwaldt, B.L. *Antimicrob. Agents Chemother.*, **1988**, *32*, 793.

[32] Homewood, C.A., Warhurst, D.C., Peters, W., Baggaley, V.C. *Nature*, **1972**, *235*, 50.

[33] Martiney, J.A., Cerami, A., Slater, A.F.G. *J. Biol. Chem.* **1995**, *270*, 22393.

[34] Warhurst, D.C. *Biochem. Pharmacol.*, **1981**, *30*, 3323.

[35] Blauer, G., Ginsburg, H. *Biochem. Internatl.*, **1982**, *5*, 519.

[36] Moreau, S., Perly, B., Biguet, J. *Biochimie*, **1982**, *64*, 1015.

[37] Moreau, S., Perly, B., Chachaty, C., Deleuze, C. *Biochim. Biophys. Acta*, **1985**, *840*, 107.

[38] Blauer, G. *Arch. Biochem. Biophys.* **1986**, *251*, 306.

[39] Blauer, G. *Arch. Biochem. Biophys.* **1986**, *251*, 315.

[40] Sugioka, Y., Suzuki, M., Sugioka, K., Nakano, M. *FEBS Lett.*, **1987**, *223*, 251.

[41] Warhurst, D.C. *Ann. Trop. Med. Parasitol.*, **1987**, *81*, 65.

[42] Blauer, G. *Biochem. Internatl.*, **1988**, *17*, 729.

[43] Blauer, G., Akkawi, M., Bauminger, E.R. *Biochem. Pharmacol.*, **1993**, *46*, 1573.

[44] Adams, P.A., Berman, P.A.M., Egan, T.J., Marsh, P.J., Silver, J. *J. Inorg. Biochem.*, **1996**, *63*, 69.

[45] Egan, T.J., Mavuso, W.W., Ross, D.C., Marques, H.M. *J. Inorg. Biochem.*, **1997**, *68*, 137.

[46] Egan, T.J., Hempelmann, E., Mavuso, W.W. *J. Inorg. Biochem.*, **1999**, *73*, 101.

[47] Dorn, A., Vippagunta, S.R., Matile, H., Jaquet, C., Vennerstrom, J.L., Ridley, R.G. *Biochem. Pharmacol.*, **1998**, *55*, 727.

[48] Silver, J; Lukas, B. *Inorg. Chim. Acta*, **1983**, *78*, 219.

[49] Hunter, C.A., Sanders, J.K.M. *J. Am. Chem. Soc.*, **1990**, *112*, 5525.

[50] Constantinidis, I., Satterlee, J.D. *J. Am. Chem. Soc.*, **1988**, *110*, 4391.

[51] Constantinidis, I., Satterlee, J.D. *J. Am. Chem. Soc.*, **1988**, *110*, 927.

[52] Marques, H.M., Voster, K., Egan, T.J. *J. Inorg. Biochem.*, **1996**, *64*, 7.

[53] Vippagunta, S.R., Dorn, A., Matile, H., Bhattacharjee, A.K., Karle, J.M., Ellis, W.Y., Ridley, R.G., Vennerstrom, J.L. *J. Med. Chem.*, **1999**, *42*, 4630.

[54] Egan, T.J., Hunter, R., Kaschula, C.H., Marques, H.M., Misplon, A., Walden, J. *J. Med. Chem.*, **2000**, *43*, 283.

[55] Barton, D.H.R., Ollis, W.D. In *Comprehensive Organic Chemistry: the Synthesis and Reactions of Organic Compounds: Heterocyclic Compounds*; Sammes, P.G., Ed., Pergamon Press: Oxford, **1979**; Vol. 4, pp. 191-193.

[56] Vippagunta, S.R., Dorn, A., Ridley, R.G., Vennerstrom, J.L. *Biochim. Biophys. Acta*, **2000**, *1475*, 133.

[57] O' Neill, P.M., Willock, D.J., Hawley, S.R., Bray, P.G., Storr, R.C., Ward, S.A., Park, K.B. *J. Med. Chem.*, **1997**, *40*, 437.

[58] Rode, B.M., Schwendinger, M.G., Kokpol, S.U., Hannongbua, S.V., Polman, S. *Monat. Chemie*, **1989**, *120*, 913.

[59] Slater, A.F.G., Cerami, A. *Nature*, **1992**, *355*, 167.

[60] Egan, T.J., Ross, D.C., Adams, P.A. *FEBS Lett.*, **1994**, *352*, 54.

[61] Dorn, A., Stoffel, R., Matile, H., Bubendorf, A., Ridley, R.G. *Nature*, **1995**, *374*, 269.

[62] Sullivan, D.J., Gluzman, I.Y., Russell, D.G., Goldberg, D.E. *Proc. Natl. Acad. Sci. U.S.A.*, **1996**, *93*, 11865.

[63] Sullivan, D.J., Matile, H., Ridley, R.G., Goldberg, D.E. *J. Biol. Chem.*, **1998**, *273*, 31103.

[64] Egan, T.J., Mavuso, W.W., Ncokazi, K.K. *Biochemistry*, **2001**, *40*, 204.

[65] Basilico, N., Monti, D., Olliaro, P., Taramelli, D. *FEBS Lett.*, **1997**, *409*, 297.

[66] Dorn, A., Vippagunta, S.R., Matile, H., Bubendorf, A., Vennerstrom, J.L., Ridley, R.G. *Biochem. Pharmacol.*, **1998**, *55*, 737.

[67] Basilico, N., Pagani, E., Monti, D., Olliaro, P., Taramelli, D. *J. Antimicr. Chemother.*, **1998**, *42*, 55.

[68] Hawley, S.R., Bray, P.G., Mungthin, M., Atkinson, J.D., O' Neill, P.M., Ward, S.A. *Antimicrob. Agents Chemother.*, **1998**, *42*, 682.

[69] De, D., Krogstad, F.M., Byers, L.D., Krogstad, D.J. *J. Med. Chem.*, **1998**, *41*, 4918.

[70] Ursos, L.M.B., Dzekunov, S.M., Roepe, P.D. *Mol. Biochem. Parasitol.*, **2000**, *110*, 125.

[71] Gasquet, M., Atouk, A., Samat, A., Timon-David, P., Viala, A. *Ann. Trop. Med. Parasitol.*, **1987**, *81*, 355.

[72] Ginsburg, H., Nissani, E., Krugliak, M. *Biochem. Pharmacol.*, **1989**, *38*, 2645.

[73] Ridley, R.G., Hofheinz, W., Matile, H., Jaquet, C., Dorn, A., Masciadri, R., Jolidon, S., Richter, W.F., Guenzi, A., Girometta, M.A., Urwyler, H., Huber, W., Thaithong, C., Peters, W. *Antimicr. Agents Chemother.*, **1996**, *40*, 1846.

[74] Biot, C., Glorian, G., Maciejewski, L.A., Brocard, J.S. *J. Med. Chem.*, **1997**, *40*, 3715.

[75] Chibale, K., Moss, J.R., Blackie, M., Van Schalkwyk, D., Smith, P.J. *Tetrahedron Lett.*, **2000**, *41*:, 6231.