Malaria Journal

BioMed Central

Research

The relationship of physico-chemical properties and structure to the differential antiplasmodial activity of the cinchona alkaloids

David C Warhurst*[1], John C Craig[2], Ipemida S Adagu[1], David J Meyer[1] and Sylvia Y Lee[1]

Address: [1]Pathogen Molecular Biology Unit, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel St., London WC1E 7HT, UK and [2]Department of Pharmaceutical Chemistry, University of California, San Francisco, California, CA 94143-00446 USA

Email: David C Warhurst* - david.warhurst@lshtm.ac.uk; John C Craig - david.warhurst@lshtm.ac.uk; Ipemida S Adagu - ipemida.adagu@lshtm.ac.uk; David J Meyer - david.meyer@lshtm.ac.uk; Sylvia Y Lee - sylviaylee@hotmail.com

* Corresponding author

Published: 01 September 2003

Malaria Journal 2003, **2**:26

Received: 04 July 2003
Accepted: 01 September 2003

This article is available from: http://www.malariajournal.com/content/2/1/26

Abstract

Background: The 8-amino and 9-hydroxy substituents of antimalarial cinchona alkaloids have the *erythro* orientation while their inactive 9-epimers are *threo*. From the X-ray structures a 90° difference in torsion angle between the N1-H1 and C9-O12 bonds in the two series is believed to be important. In order to kill the malaria parasite, alkaloids must cross the erythrocyte and parasite membranes to accumulate in the acid digestive vacuole where they prevent detoxication of haematin produced during haemoglobin breakdown.

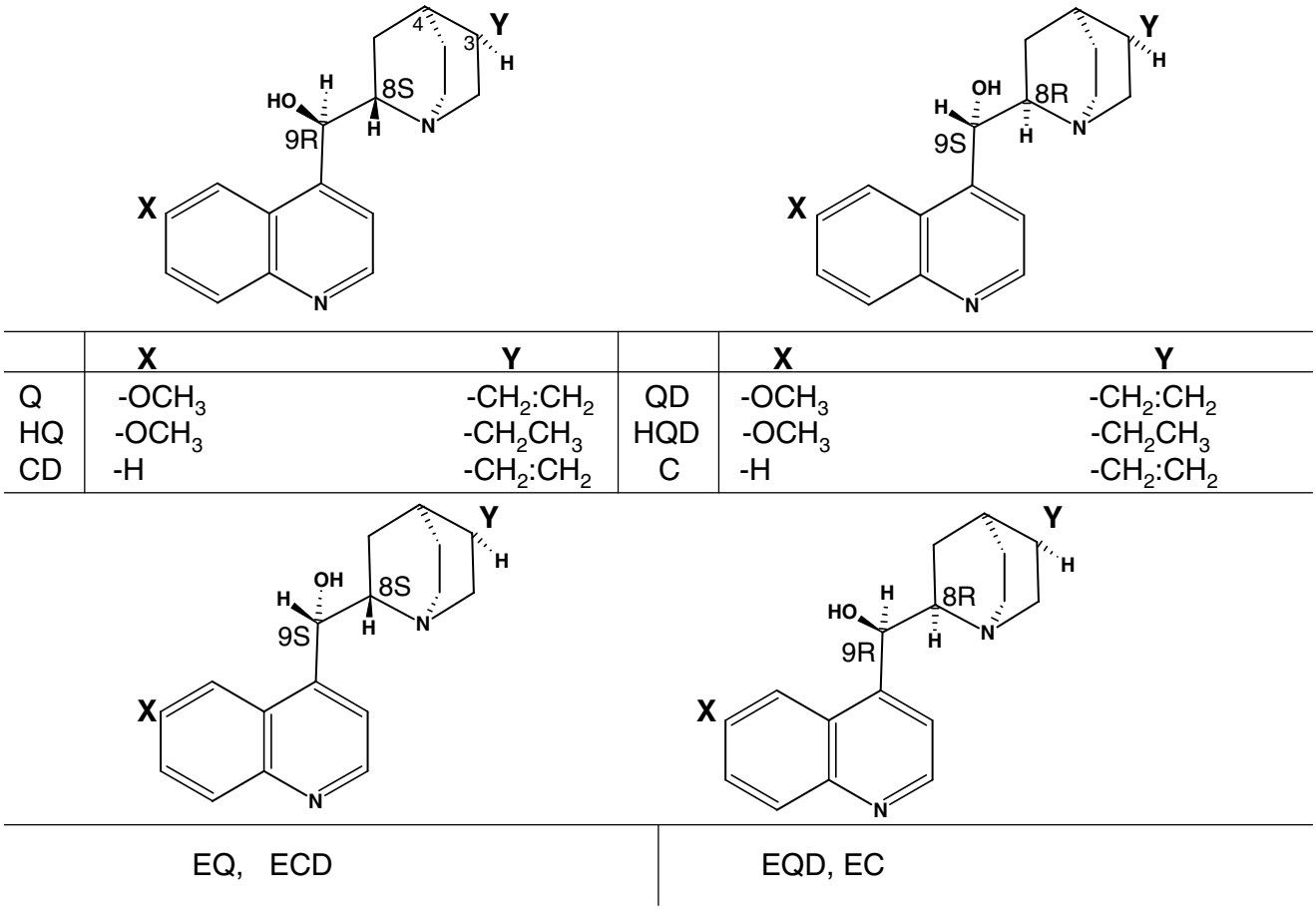
Methods: Ionization constants, octanol/water distribution and haematin interaction are examined for eight alkaloids to explain the influence of small structural differences on activity.

Results: *Erythro* isomers have a high distribution ratio of 55:1 from plasma to the erythrocyte membrane, while for the more basic *threo* epimers this is only 4.5:1. This gives an increased transfer rate of the *erythro* drugs into the erythrocyte and thence into the parasite vacuole where their favourable conformation allows interaction with haematin, inhibiting its dimerization strongly (90 ± 7%) and thereby killing the parasite. The *threo* compounds not only enter more slowly but are then severely restricted from binding to haematin by the *gauche* alignment of their N1-H1 and C9-O12 bonds. Confirmatory molecular models allowed measurement of angles and bond lengths and computation of the electronic spectrum of a quinine-haematin complex.

Conclusion: Differences in the antiplasmodial activity of the *erythro* and *threo* cinchona alkaloids may therefore be attributed to the cumulative effects of lipid/aqueous distribution ratio and drug-haematin interaction. Possible insights into the mechanism of chloroquine-resistance are discussed.

Background

Aryl amino alcohol alkaloids from the bark of *Cinchona* spp. have played an invaluable role in the treatment of malaria since the 18th century when the extract became widely used. The bark contains laevorotatory (-) quinine (6'-methoxy cinchonan-9-ol) (Q), its diastereomer dextrorotatory (+) quinidine (QD), and their 9-epimers epiquinine (EQ) and epiquinidine (EQD). Also present are (-) cinchonidine (*des* methoxyquinine) (CD), its diastereomer (+) cinchonine (C) and their 9-epimers



	X	Y		X	Y
Q	$-OCH_3$	$-CH_2{:}CH_2$	QD	$-OCH_3$	$-CH_2{:}CH_2$
HQ	$-OCH_3$	$-CH_2CH_3$	HQD	$-OCH_3$	$-CH_2CH_3$
CD	-H	$-CH_2{:}CH_2$	C	-H	$-CH_2{:}CH_2$

EQ, ECD

EQD, EC

Figure 1
Cinchona alkaloids: structures to illustrate the stereochemistry. Q = quinine, QD = quinidine, EQ = epiquinine, EQD = epiquinidine, HQ = hydroquinine, HQD = hydroquinidine, CD = cinchonidine, C = cinchonine, ECD = epicinchonidine, EC = epicinchonine.

epicinchonidine (ECD) and epicinchonine (EC). With the purification of Q as the major alkaloid, it remains the main therapeutic drug for severe and complicated *Plasmodium falciparum* malaria [1]. In addition to the above alkaloids, all of which contain the exocyclic unsaturated vinyl group attached to C3 of the quinuclidine ring, *Cinchona* bark produces the analogous dihydro compounds of which hydroquinine (HQ) and its diastereomer hydroquinidine (HQD) are examples, in which the exocyclic double-bond has been reduced to the saturated ethyl group (figure 1).

Q and other aryl amino alcohols competitively inhibit [2] morphological changes caused by chloroquine (CQ) [3,6] in digestive vacuoles (lysosomes) of the erythrocytic parasite where haemoglobin is digested. All these agents become concentrated in infected erythrocytes [4], and, as weak membrane-soluble bases, are believed to accumulate in the acidic digestive vacuole contents where they undergo protonation [5]. They are thought to cause the death of the intraerythrocytic malaria parasite by binding to toxic haematin [6,7] released during haemoglobin digestion, preventing its dimerization to non-toxic malaria pigment (haemozoin, β-haematin [8]).

In chemical structure, cinchona alkaloids consist of a conjugated heterocyclic quinoline ring, joined to a bulky rigid bicyclo heterocyclic aliphatic quinuclidine ring by an alcoholic carbon, C9. This is one of four chiral centres in these molecules; only C8 and C9 vary in configuration, while C3 and C4 on the aliphatic ring (respectively rectus, *R* and sinister, *S*), are *erythro* and invariant. Centres C8

and C9 are *S* and *R* in Q and *R* and *S* in QD, both *erythro*. Their 9-epimers are 8*S* and 9*S* in EQ and 8*R* and 9*R* in EQD, both *threo*. (figure 1) In 1938 it was reported from the Wellcome Laboratories of Tropical Medicine, London, that EQ and EQD had little activity against experimental avian malaria [9]. Further, a clinical study found in 1948 that while Q and QD effectively treated induced *P. falciparum* infections, QD was twice as potent as Q [10]. Unnatural (8*R*,9*S*,3*S*,4*R*) HQ and (8*S*,9*R*,3*S*,4*R*) HQD synthesized at Hoffmann la Roche by Brossi's group were each as active in *P. berghei* infections in mice as natural HQ and HQD [24].

Before *P. falciparum* cultures [11] were available, EQ was confirmed inactive in short-term cultures of *P. berghei*. In agreement with the earlier clinical study, QD was four times more active than Q, and C three times more active than CD, emphasising the antiparasitic superiority of the dextrorotatory (+) alkaloids of the QD type [2].

In the synthetic aryl amino alcohols, however, *erythro* (1SR, 2RS)-mefloquine (WR 142,490) was only 2.5 times more active than the *threo* (1RS, 2RS)- racemate WR 177,602, and *erythro* WR 122,455 was 0.7 times as active as its *threo* racemate WR 165,355. This small difference in relative activities of *erythro* and *threo* mefloquine and WR 122,455, compared with the lack of activity of the *threo* epimers of the cinchona alkaloids, was attributed to the ease of rotation of the less bulky piperidine ring, which replaces the quinuclidine ring in these synthetic agents, around the link between the two relevant asymmetric carbon atoms (C-8 and C-9 in the cinchona alkaloids). As a result of these studies a drug receptor was proposed, comprising 2 planar areas accommodating the quinoline rings of Q and QD with, in between, an electropositive area in the plane and a raised electronegative area [2]. The receptor concept was refined in 1981 on the basis of an experimental study of haematin interactions with aryl amino alcohol antimalarial drugs, and evidence for a co-ordination link between such drugs and haematin iron was obtained [12].

In 1990, using cultures of CQ-sensitive *P. falciparum*, Wesche & Black confirmed higher activity of (+)QD than (-) Q, and of (+) C than (-) CD [13]. In addition, Karle *et al*. [14] found that, like (+) QD vs. (-) Q, (+) mefloquine was slightly more effective than (-) mefloquine against *P. falciparum in vitro* but this difference did not apply to enantiomers of aryl amino alcohols whose side-chains were more flexible than piperidine. They found in a further study that EQ and EQD were approximately 100 times less active than quinine and quinidine against chloroquine-sensitive *P. falciparum in vitro* while HQ and HQD were about 25% more active than Q and QD against CQ-sensitive *P. falciparum in vitro* [15].

One explanation for the much lower activity of EQ and EQD had been the suggested potential for intramolecular H-bonding between aliphatic N-1 and adjacent O-12 because of the shorter distance between these atoms [16], thereby reducing the ability of the drug to interact with a putative cellular receptor site. Crystallographic evaluation by Karle *et al.* revealed that the N-1 to O-12 distances in the epimers were similar to those of other active amino-alcohol antimalarials, and all showed potential for forming H-bonds with cellular constituents. However, the torsion angle between the N1-H1 and C9-O12 bonds showed a 90° difference between the *erythro*- and *threo*-alkaloids. These authors concluded that the main reason for the inactivity of EQ and EQD was that *threo*-alkaloids could not form intermolecular H-bonds with cellular receptor sites in the same direction as the active *erythro*-isomers [15]. Recently Karle & Karle have compared the crystal structures of Q and QD with those of (-) and (+) mefloquine to derive a pharmacophore which defines the main features of the parasite's receptor [17].

Their findings are of considerable interest with respect to the proposed receptor for these drugs, haematin, which has a large planar area containing 4 N atoms co-ordinated with Fe^{III}. The Q-haematin complex is formed by hydrophobic interaction between the conjugated rings and co-ordination between Fe^{III} and the hydroxyl group on C-9 of Q [18]. In water, solid haemin chloride (monomeric haematin) will readily dissolve in solutions of the antimalarially active *erythro* alkaloids Q, QD, CD and C to form benzene-soluble co-ordination complexes with α- and β-hemichrome absorption peaks around 600 and 490 nm. This does not occur with the inactive EQ [12]. More recently, it has been shown that Q, but not EQ, inhibits spontaneous haematin dimerization to β-haematin *in vitro* [19].

We report below that haematin dimerization is also not inhibited by EQD and confirm the lack of activity of EQ and the activity of Q, QD, HQ, HQD, C and CD in this model system. We have also obtained reliable values for the octanol/water partition coefficients (Log P) and ionization constants (pK_a values) of Q, QD, HQ, HQD, EQ, EQD, C and CD. This enables us to predict the log distribution ratio between water and lipid (logD) both at the physiological pH and at the acidic pH of the parasite lysosome, where haematin is released from haemoglobin, and to calculate the possible intralysosomal concentration ratios that would be expected, assuming the drugs are concentrated by hydrogen ion. These observations are discussed in the context of molecular modelling, in order to evaluate physicochemical and structural determinants of drug activity against chloroquine-sensitive and resistant *P. falciparum*. Our conclusions throw light on the prerequisites for activity in cinchona alkaloids, the nature of the

haematin-quinine interaction, and the mechanism of chloroquine-resistance.

Methods

Physicochemical data

The accumulation of an ionizable drug in cells is determined (apart from special transport mechanisms) by two major parameters: the partition coefficient P between the aqueous and the lipid phase, usually expressed as log P (measured in the octanol-water system for the un-ionized compound), and its modification by the ionization constant(s) (pK_a) of the drug. For partially ionized compounds, the partition coefficient between 2 phases at any fixed pH is called the distribution coefficient D, usually expressed as log D; this assumes that only the un-ionized species partitions from the aqueous to the organic phase [20]. The difference between log P and log D is, therefore, determined by the percentage of the drug ionized at different pH values and is given by the standard equation for a base [21]. When the difference between pK_a and pH is more than 1.0 log unit, it has been shown [22] that one can merely subtract this difference from log P to obtain log D. This makes it possible to calculate log D [23] from a knowledge of log P, pK_a and pH. The availability of the ClogP programme, developed by C. Hansch and A. Leo at Pomona College, California, as part of the Pomona College Medicinal Chemistry Project, has now provided a useful method of computing approximate log P values for compounds of known structure. Values may sometimes be unreliable since differences due to conformational isomerism are not detected [25].

In the case of a drug possessing two basic centres, as quinoline antimalarial drugs all do, the correction needed in log P to obtain log D at a given pH involves the additive contribution of both ionized species, and the base equation [21] must be modified: [26,28].

Equation (1) for a diacidic base

$$\log D = \log P - \log[1 + 10^{(pK_{a1} - pH)} + 10^{(pK_{a1} + pK_{a2} - 2pH)}]$$

Log D at the physiological pH of 7.4 was obtained experimentally from the plot of log D versus pH. This was compared with the calculated value of Log D, obtained from Log P, using equation (1). Log D at the probable pH of the digestive vacuole or lysosome was calculated from log P by substituting pH 5.2 [27] for 7.4 in equation (1). The application of equation (1) provides a useful rapid method of checking measured values of log D, and of calculating these values from log P when unknown. It should be noted that for diacidic bases, $pK_{a1} > pK_{a2}$.

Vacuolar accumulation ratios

To calculate the vacuolar accumulation ratios (VAR) that weakly basic lipophilic drugs might achieve by virtue of the pH difference between lysosome and external medium or plasma [5], the equation of Henderson & Hasselbach was used in the following form [29].

Equation 2

$$[Drug_{vac}]/[Drug_{outside}] = 1 + 10^{(pK_{a1} - pH_v)} + 10^{(pK_{a1} + pK_{a2} - 2pH_v)} / 1 + 10^{(pK_{a1} - pH_0)} + 10^{(pK_{a1} + pK_{a2} - 2pH_0)}.$$

Dissociation constants and partition coefficients were determined by Robertson Microlit Laboratories (Madison, New Jersey, USA) at 25°C using the Sirius GLpK automated computerized potentiometric system [30]. Titration employed water containing 0.15 N KCl in an argon atmosphere. The pK_as were determined in triplicate with a S.E. of ± 0.20.

Partition coefficients between octan-1-ol and water were measured by dual-phase potentiometric titration using various amounts of water-saturated octanol. Titrant addition was carried out with vigorous stirring of the assay solution. Three different ratios of octanol/water were employed for each compound. The log P values were obtained from the difference between the aqueous pK_a of the species and the apparent pK_a determined from the dual phase titration [31]. Measurements were carried out in triplicate with a S.E. of ± 0.40. The potentiometric method was validated by comparison with results obtained by the standard shake-flask technique [32].

The drug lipophilicity values at pH 7.4 and 5.2 (log D) are the octanol/water distribution ratios, and were obtained from the plot of log P versus pH. Calculated log D values for pH 5.2, representing the conditions in the lysosome, and for log D at pH 7.4, were obtained using equation (1).

β-Haematin Inhibitory Activity: BHIA

The method was slightly modified from Parapini *et al*. [33]. The 8 M sodium acetate/acetic acid buffer (pH 5.0) was prepared as follows:

Make up solid sodium acetate to 8 M with warm water (continue to heat at 37°C in a water bath while mixing: it will not dissolve completely) and then adjust the pH to 5.0 with 8 M acetic acid at 37°C, which leads to the remaining solid dissolving).

One hundred μL was dispensed into wells of a microtitre plate, and 50 μL of alkaloid hydrochloride or sulphate solution in water, at 80 mM, or water alone, was added. Verapamil (VE) and promethazine (PMZ) (Sigma Chemicals, Poole, UK) were used as hydrochlorides. CQ (Sigma, UK) was used as the diphosphate. Addition of 50 μL of an

Table 1: Physicochemical parameters of Cinchona alkaloids. cpd. = compound, VAR = calculated vacuolar accumulation ratio. S = chloroquine-sensitive, R = chloroquine-resistant. S/R = ratio of IC50 values. BHIA = % inhibition in β-haematin inhibition test

Cpd.	pK_{a1}	pK_{a2}	Log P	Log D (pH7.4)	Log D (pH5.2)	VAR	IC50 nM S	IC50 nM R	RATIO S/R	BHIA % inhib.
Q	8.58a	4.12b	3.17c	1.97e	0.30	161.0	29.3[15]	103.2[15]	0.28	84 ± 1
			2.79d	1.96f	-0.25f					
QD	8.58g	4.42h	2.84i	1.66j	0.40k	173.2	13.4[15]	43.7[15]	0.31	85 ± 9
			2.79d	1.63f	-0.61f					
HQ	9.011	4.29	3.07	1.50	0.53	173.6	21.3[15]	151.7[15]	0.14	93 ± 0.6
			3.27d	1.45f	-0.79f					
HQD	9.16	4.34	3.43	1.72	0.75	177.5	10.4[15]	74.1[15]	0.14	95 ± 0.4
			3.27d	1.66f	-0.59f					
C	8.35m	4.28n	2.82o	1.85p	-0.38f	159.6	18.3[46]	70.8[46]	0.26	94 ± 0.5
			2.49d	1.82f						
CD	8.40q	4.17r	2.68s	1.73t	-0.56f	157.5	69.5[46]	206.8[46]	0.34	94 ± 0.4
			2.49d	1.64f						
EQ	9.54u	4.08	2.90	0.76	-1.44	169.2	3471[15]	1179[15]	2.94	2 ± 13
			2.79d	0.76f	-1.47f					
EQD	9.39v	4.15	2.53	0.55	-1.2	170.8	2700[15]	1024[15]	2.64	12 ± 12
			2.79d	0.54f	-1.70f					
CQ	10.18	8.38	4.72	0.96	-3.44	22750	18.5	264	0.07	81 ± 15
				0.92f	-3.44f					
VE	8.4[62]	<0	3.79[62]	2.75f	0.59f	144.2	7200[53]	2500[53]	2.88	0 ± 21
PMZ	9.11[42]	<0	4.75[42]	3.03f	0.84f	155.5	1436[52]	835[52]	1.70	15 ± 22.5

a: Lit 8.43[64], 8.4[39], 8.05[65], 8.59[66]. In 80% methylcellosolve/20% water 7.73[41], 7.25[67] ; **b**: Lit. 4.34[64],4.32 [39], 4.33 [66]. In 80% methylcellosolve 2.81[67], 2.38[68] ; **c**: Lit. 3.44[22], 2.64[65] ; **d**: Calc. from CLogP ; **e**: Lit. 1.90[65], 2.14[22], 2.54[69], 1.73(pH7.0)[70] ; **f**: Calc from eqn. (1). ; **g**: Lit. 8.79[64], 7.95[41], 8.78[42]. In 80% methylcellosolve 7.95[41]7.36[67] ; **h**: Lit. 4.34[64], in 80% methylcellosolve 2.66[67], 2.40[68] ; **i**: Lit: 2.88[65], 3.71[42] ; **j**: Lit. 2.03[72], 2.07[74],2.11[31], 2.22[65]; **k**: Lit: -0.07[42] ; **l**: Lit. 8.87[40] ; **m**: Lit. 8.35[40], 8.32[65]. In 80% methylcellosolve 7.36[68] ; **n**: Lit. 4.28[67], In 80% methylcellosolve 2.80[67], 2.15[68] ; **o**: Lit 2.82[65]; **p**: Lit 1.85[65] ; **q**: Lit. 8.40[40], 8.30[65]; **r**: Lit. 4.17[40]. In 80% methylcellosolve 2.66[67], 2.12[68] ; **s**: Lit. 2.68[65] ; **t**: Lit. 1.73[65] ; **u**: Lit. In 80% methylcellosolve 8.44[41] ; **v**: Lit. in 80% methylcellosolve 8.32[41].

8 mM α-chlorohemin (Sigma, UK) solution in DMSO to each well started the experiment.

Experiments were allowed to run at 35°C for 24 hr., done in triplicate and repeated on three occasions. The final DMSO concentration in this system was 25% w/v, while final haematin concentration (0.4 μmol/well) was 2 mM, and the final drug concentration (4.0 μmol/well) was 20 mM. Drug/haematin molar ratio was 10:1.

Then insoluble β-haematin and haematin aggregate were pelleted by centrifugation at 3000 g for 20 min at room temperature (22°C) and the supernatant discarded. Two hundred μL of DMSO were added to each pellet to dissolve un-reacted haematin and centrifugation was repeated. After discarding the supernatant, 200 μL of 0.2 M NaOH were added to convert the β-haematin pellet to soluble alkaline haematin. For each well the alkaline haematin was quantified by serial twofold dilution in water and OD measurement at 414 nm. The effect of each agent on β-haematin production was recorded compared with the control (water + DMSO + acetate buffer alone).

Molecular modelling

Each mono-protonated cinchona alkaloid structure was built in HyperChem Release 7 for Windows (Hypercube Inc. Gainesville, Florida) using a molecular mechanics procedure under MM+ [34] with bond-length restraints for N1-O12 and N1 to C5' distance [15] (bond dipoles, no cut offs employed). The geometry was optimised to an rms (root mean square) gradient of 0.001 in vacuo (Polak-Ribière method). A periodic box, 15 × 15 × 15Å around the drug was then set up, containing 112 water molecules. The system was optimised in MM+ using switched cut-offs (outer 7.5 and inner 3.5 Å), to an rms gradient of 0.5. Then a molecular dynamics programme was run for 1 ps, with 0.001 ps steps, relaxation time 0.1 ps, to a simulation temperature of 300 K. This was followed by MM+ geometry optimisation to an rms gradient of 0.2. The molecular dynamics run was repeated and a further MM+ protocol was carried out to a gradient of rms 0.004 on the selected drug.

Angles and bond-lengths were measured on the models, and the dipole moments were determined after removal of the N-1 proton, using the semi-empirical PM3 programme [35,36] in singly-excited configuration interac-

tion. (RHF [Restricted Hartree-Fock], charge 0, spin multiplicity 1, lowest state, orbital criterion, five occupied and five unoccupied orbitals.)

Models of the Q-haematin and EQ-haematin complexes were prepared in MM+ using the structures of Q and EQ optimised in water (see above), with a co-ordination link between haematin iron and drug O-12. The crystal structure of α-chlorohaemin [37] was used for the initial haematin monomer model.

The semi-empirical quantum mechanics program ZINDO/S [38] in singly excited configuration interaction was used to calculate the expected electronic spectrum of the optimised 1: 1 quinine/haematin complex in benzene. One molecule of singly protonated quinine, co-ordinated to one molecule of haematin, with carboxylic acid groups undissociated, was given an arbitrary charge of +1 and spin multiplicity of four. Fifteen occupied and 15 unoccupied orbitals were included in the calculation.

(EQ and EQD were kindly supplied by Dr. Reinecke of Buchler GmbH, D-38110 Braunschweig, Germany.)

Results

Physicochemical

Q and QD, C and CD are reported to have very similar pK_{a1} values (8.4 ± 0.4) for the quinuclidine aliphatic N (table 1: footnotes). A value of 8.5 ± 0.15 (table 1) was found, with a slight increase in HQ and HQD due to the conversion of the electron-attracting vinyl group to an ethyl group. All the cinchona alkaloids in table 1 had very similar pK_{a2} values (4.25 ± 0.15) for the more weakly basic quinoline aromatic N. The only previous determinations [41] for the threo compounds EQ and EQD were carried out in 80% methyl-cellosolve / 20% water and showed both 9-epimers to have higher pK_a values than Q and QD in the same solvent. Results obtained in water (Table 1) confirmed this finding and revealed a consistent ΔpK_{a1} of 1.1 units between the two solvent systems, and of $1pK_a$ unit between the erythro and the threo isomers. Experimental values for log P are shown in table 1 as are the figures calculated using the ClogP programme. The two sets are in generally good agreement, and show the expected slight increase (+0.4) with the dihydro compounds (HQ and HQD) and decrease (-0.3) with the loss of the 6-methoxy group (C, CD). Experimental measurement of log D vs pH over the range 3–11 permitted the determination of log D at pH 7.4. In addition knowledge of log P and pK_a allowed the calculation of log D at pH 7.4 using equation (1). Measured and calculated figures (table 1) are in good agreement, while reported values (table 1: footnotes) show a wider variation. Results for log D at pH 7.4 for the erythro compounds fall in the range 1.50–1.97 (mean 1.74 ± 0.24) while the threo isomers (range 0.55–0.76) show a mean of 0.66 ± 0.11. In view of the acidic milieu (pH 5.2) existing in the vacuole within the lysosomal membrane, log D at pH 5.2 was also measured and calculated and the figures are shown in table 1. The comparison of calculated results for these agents at pH 5.2 with those obtained for chloroquine (CQ) is of interest, since because of CQ's two high pK_a values [43,44], the drop in pH had a much larger influence than on Q, QD, EQ or EQD. The drop in log D between pH 7.4 and pH 5.2 for Q, QD, EQ and EQD was ~2 logs while for CQ it was > 4 logs.

The parasite lysosome, which has been proposed as the main site of drug uptake, has an acidic pH. Assuming drug distribution reaches an equilibrium state, and only uncharged drug can pass through membranes, it is argued that the ratio between concentration of drug outside the erythrocyte and the concentration in the lysosome depends on the difference between $[H^+]$ of the medium *in vitro* or plasma *in vivo* (pH 7.4), and the lysosomal compartment, according to the Henderson-Hasselbach equation [5]. Both the water/lipid partition (log P) and the acid dissociation constant(s) (pK_a) of a drug influence the passage of drug into the lysosome and its trapping by protonation. Trapping of a drug in the acidic vacuole is enhanced if the base, like chloroquine (CQ), can accept more than one proton at the lysosomal pH [28]. Calculated CQ concentrations within the infected erythrocyte approximate to measured uptake values, but the more lipophilic agents like amodiaquine (AQ) achieve significantly greater levels than calculated, indicating that other factors are involved in the concentrative uptake of the drug [29]. Calculated lysosomal uptake ratios (vacuolar accumulation ratio: VAR, the ratio of calculated drug concentration in the lysosomal water to concentration in the external medium water at equilibrium) for cinchona alkaloids, for resistance-reversers VE and PMZ, and for CQ are given in table 1. The most interesting finding here was the small variation in calculated VAR seen among the eight alkaloids examined (mean: 167.8 ± 7.4 [SD]). The minor variation reflects slight differences in the protonation of the aromatic N at pH 5.2, while the aliphatic N is 100% protonated in all the alkaloids examined.

BHIA test

A 10 fold molar excess of Q, QD, HQ, HQD, C, CD, or CQ inhibited the BHIA haematin dimerization reaction. Significant inhibition was not seen with EQ, EQD, VE or PMZ. (Table 1, figure 2). These results fit in with the reported high activity of CQ [73] and the *erythro* cinchona alkaloids on *P. falciparum* and the low activity of EQ, EQD [15,46], PMZ [52] and VE [53].

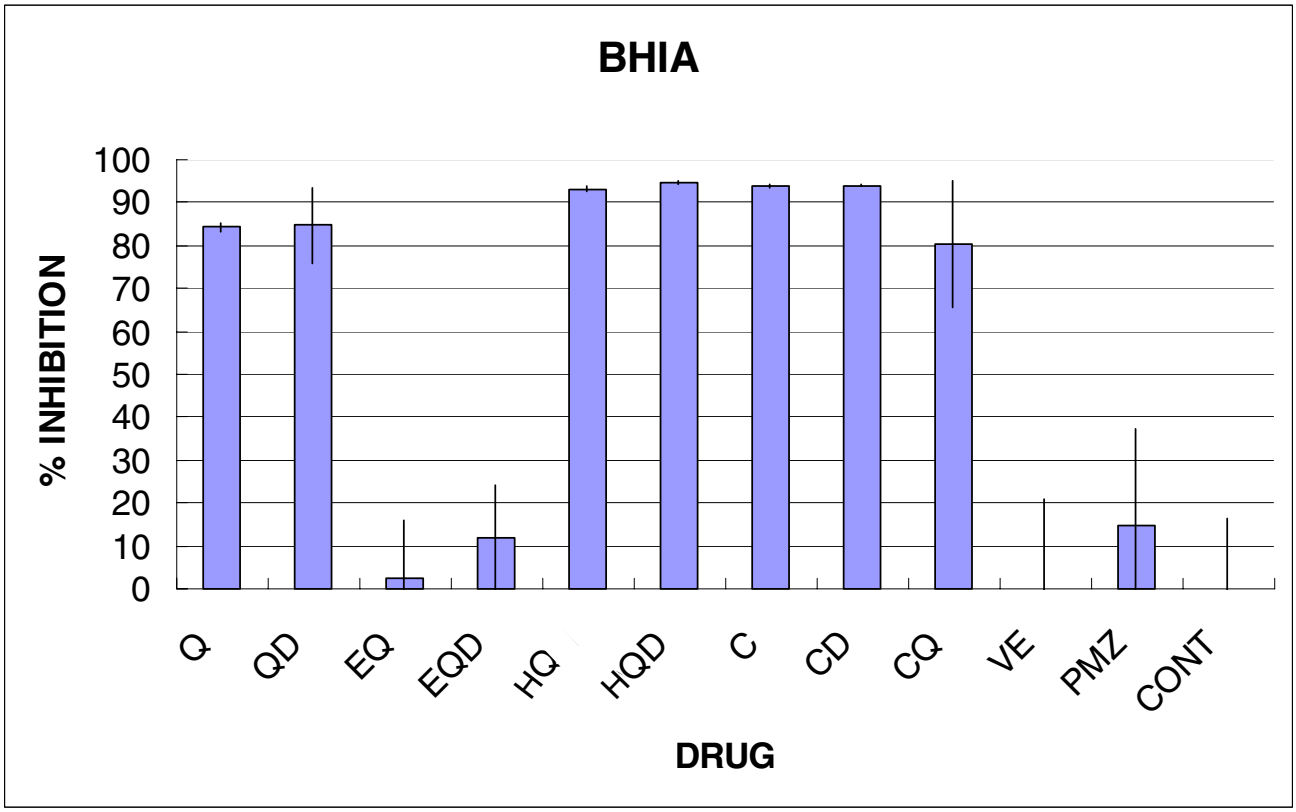


Figure 2
% β-haematin synthesis inhibition (BHIA) by cinchona alkaloids, chloroquine (CQ), verapamil (VE), promethazine (PMZ) and water (Cont). Standard deviations are shown (n = 9).

Molecular modelling (table 2)
As pointed out by Karle and co-workers, [15,46], the N1-H1 bond in 8, 9 *threo*-alkaloids consistently points in the same direction as the C9-O12 bond, while in the *erythro*-alkaloids they are at right angles (see the last column of table 2). The calculated dipole moment and proton affinity of each active alkaloid are lower than that of its 9-epimer, as first demonstrated by Karle & Bhattacharjee [46]. This is confirmed by our finding of higher pK_{a1} values for the 9-epimers (table 1). Dipole moments and proton affinities calculated here consistently differ from values in ref. [46], probably because we used a semi-empirical configuration interaction calculation rather than an *ab initio* approach.

Space-filling models of the cinchona alkaloids orientated to face a putative receptor are shown in figure 3.

Q-haematin model
A Q-haematin co-ordinated model was calculated using MM+, in comparison with EQ-haematin. The results of the ZINDO/S calculation of the electronic absorption spectrum of Q-haematin are compared with experimental observations in table 3 below. Differences in the orientation of the N1 proton with respect to the quinoline and porphyrin rings are shown in figure 4 below.

Discussion
The cinchona alkaloids have an aromatic N with a pK_a of 4.25 ± 0.15. At pH 7.4 the aromatic N is 100% un-ionized and makes its maximum contribution to lipophilicity. In the active *erythro* alkaloids, with partial protonation of the aliphatic N, this results in a log D at pH 7.4 of 1.74 ± 0.25 corresponding to a favourable mean membrane transfer ratio into the lipid phase of 55:1. This is applicable to the

Table 2: Electronic and structural parameters of cinchona alkaloids in aqueous solvent, based on the common pharmacophores described for (-) mefloquine and quinine and (+) mefloquine and quinidine by Karle & Karle [17]. (*dipole moment calculated for free base).

drug in water	MM+ energy <0.004	dipole moment*	proton affinity	O12-N1	N1-C5'	ring-O12 torsion°	O12-N1 torsion°	H8-C8-C9-H9 dihedral°	H-N1-C9-O12 torsion°
	Kcal/mole	Debye	Kcal/mole	Å	Å				
Q	11.9	1.88	208.85	3.14	4.1	157.8	-83.8	-82.6	-123.2
EQ	15.5	2.44	213.36	2.77	4.7	40.6	43.3	174.0	-5.9
QD	18.9	1.66	208.61	3.09	4.2	-161.1	78.2	74.7	104.4
EQD	12.8	2.21	213.84	2.82	4.7	-43.2	-49.1	-175.8	0.6
CD	11.1	1.15	209.38	3.21	4.2	144.9	-91.0	-86.8	-129.6
ECD	13.6	1.54	213.88	2.79	4.6	45.1	46.0	175.0	-5.9
C	10.9	0.96	209.78	3.19	4.1	-156.0	88.9	85.3	118.3
EC	13.5	1.27	213.75	2.85	4.7	-45.4	-50.2	-176.2	-2.5
HQ	10.4	2.08	209.39	3.20	4.1	156.1	-83.6	-82.8	-123.6
HQD	10.7	1.79	209.75	3.23	4.2	-152.7	89.5	88.8	118.6

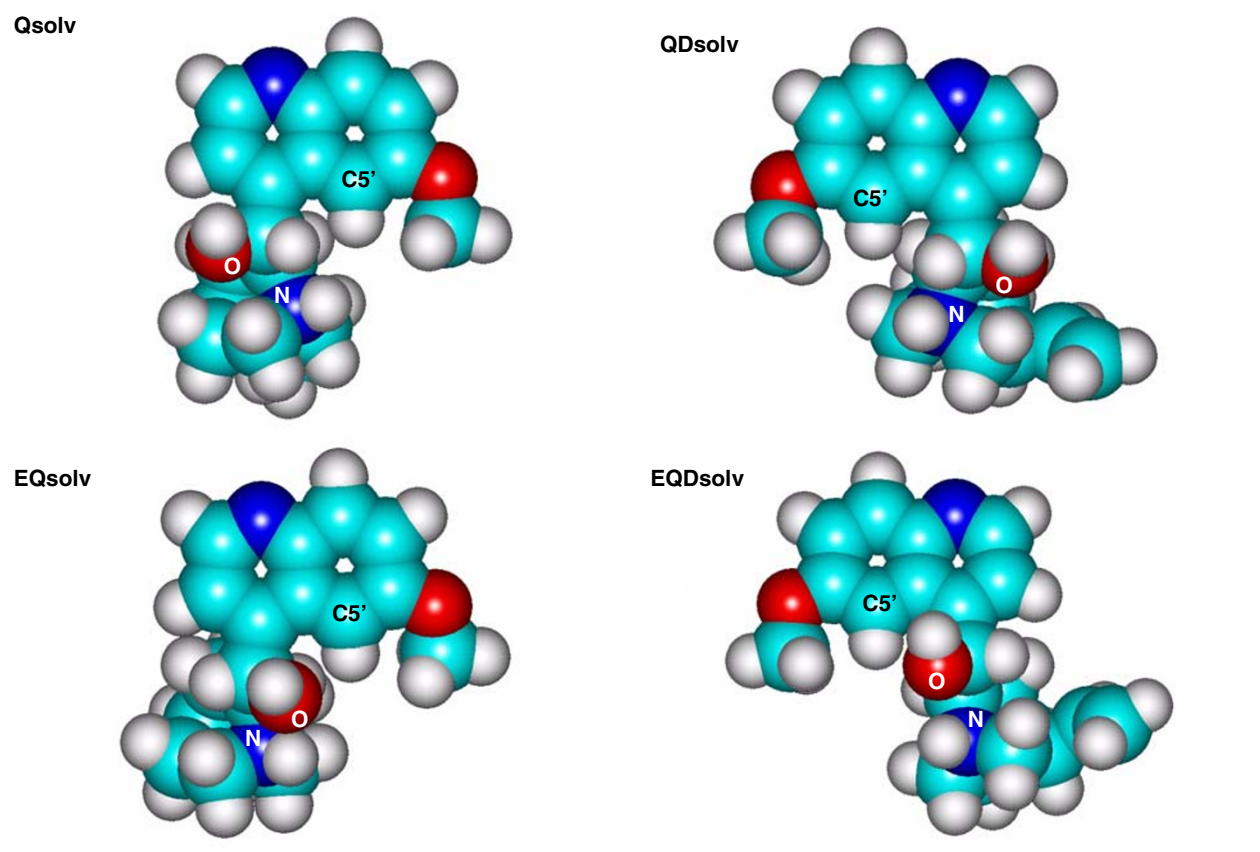


Figure 3
Space-filling models of cinchona alkaloids optimised in solvent water. Each structure is orientated facing a putative receptor. N = dark blue, C = light blue, O = red, H = grey.

Table 3: 1:1 Quinine-haematin complex: Observed and calculated values of electronic absorption peaks and peak strengths. (* Peak strength normalised by taking γ peak strength as 1.0.)

	α peak (nm)	β peak (nm)	γ peak (nm)	α peak * strength	β peak* strength	γ peak* strength	[Q] / [H]
observed	602	490	408	10	1.2	1	0.8
calculated	545.4	489.4	408.4	5.4	1.1	1	1
Ratio obs/calc	1.1	1.0	1.0	1.9	1.1	1	0.8

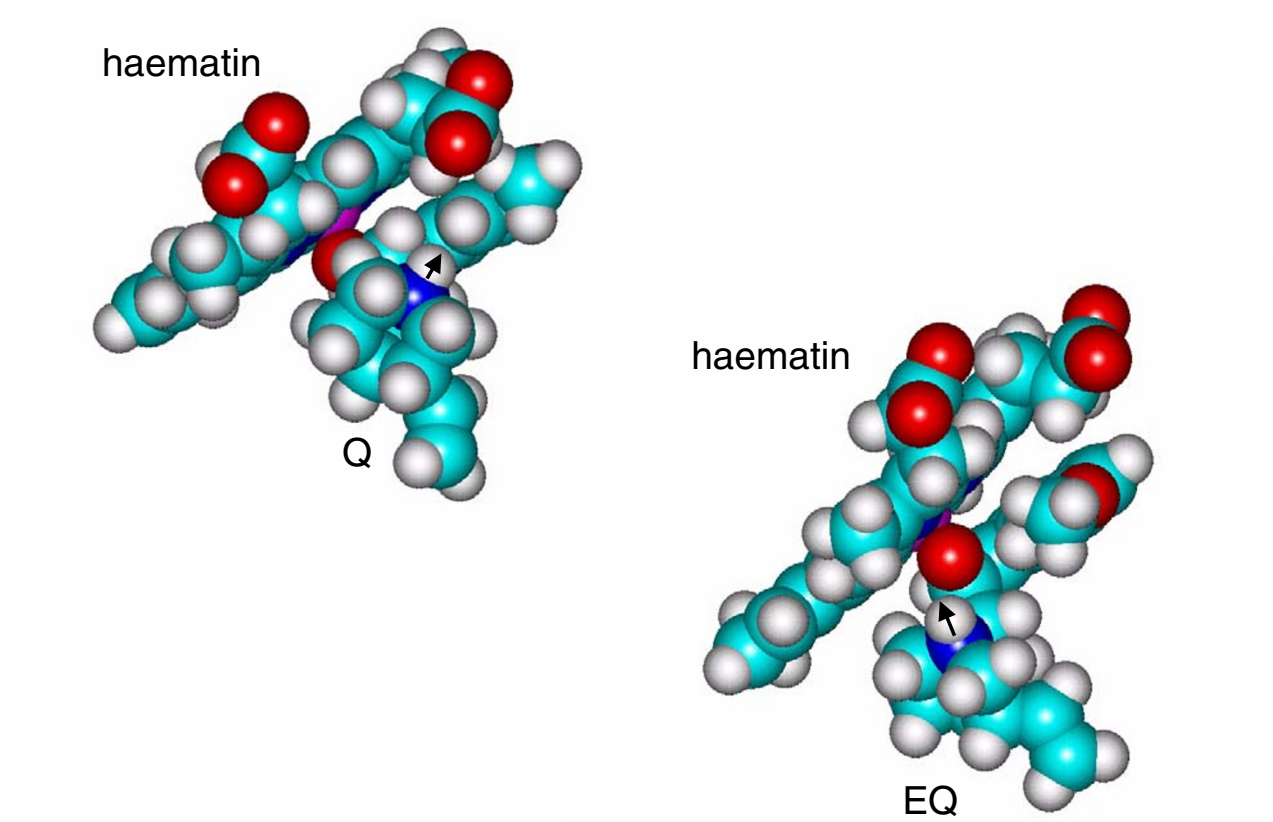


Figure 4
Views of Q-haematin and EQ-haematin models from the edge of the porphyrin ring to show comparative orientation (black arrows) of the electric field of the alkaloid N-1 proton (H-1). The haematin iron atom (just visible) is coloured magenta.

transfer of drug from blood plasma water to erythrocyte and subsequent membranes. For the (weakly active) *threo* epimers, the higher pK_a of the aliphatic N, which is now 100% ionized and does not contribute to transfer into the lipid phase, reduces log D at pH 7.4 by one magnitude to 0.66 ± 0.1, corresponding to a decreased membrane transfer ratio of only 4.5: 1. It is known that CQ and other 4-aminoquinoline antimalarials need to reach a high concentration within the lysosome of the CQ-sensitive parasite in order to inhibit dimerization of the enclosed haematin [47], and this is also assumed to apply to cinchona alkaloids. The rise in pK_{a1} values observed for the inactive 9-epi alkaloids will therefore lower the rate at which that effective concentration is reached. The acidic

environment of the vacuole (pH 5.2) results in substantial ionization (~40%) of the aromatic N of the cinchona alkaloids, with enhanced water solubility as shown by the marked drop in log D (table 1), so that all eight compounds are trapped there, causing their concentration to increase in the low pH milieu.

The absence of significant inhibition of β-haematin formation by both 9-epi alkaloids tested strengthens the link between 8, 9 *erythro* configuration in cinchona alkaloids and antiplasmodial activity, and indicates that reaching a high concentration in the lysosome, though necessary, is not sufficient in itself to confer antiplasmodial activity. Once a drug is in the lysosome, it must be able to interact with haematin, or with other vital functions like the proteolysis of haemoglobin [48], in order to exert a toxic effect.

Reported molecular electronic properties [46] of Q and QD and their epimers are in agreement with measured pK_a values here. In Q and QD the positive electric field of the aliphatic N1 proton is directed towards the quinoline ring, and the dipole moment of the drug (unprotonated) is reported as 2.2 Debye or less, while in the 9-epimers EQ and EQD the field is directed towards the solvent and the dipole moment is higher. N1 in the *erythro*-alkaloids is in a position to interact with equatorial protons of the quinoline ring (C5' is only ~4 Å away). In the models the bulky quinuclidine group finds difficulty in rotating close to the quinoline ring in the *threo*-alkaloids; the O-12 is interposed, and the N1-C5' distance is up to 4.7 Å (table 2), see also [15,46]. The probable reason for the higher proton affinity of the N1 and lower logD in the *threo* alkaloids is that the quinoline ring has less influence on N1 because of these steric factors, which lead to higher values of pK_a (easier protonation) of N-1. This will also enhance interaction (H-bonding) with solvent water in the *threo* compounds.

The Q-haematin complex

Some features of the modelled drug-haematin complexes are of interest. The Fe-O12 bond length is 1.846 Å in Q-haematin and 1.842 Å in EQ-haematin which can be compared with 1.847 Å for the O-Fe distance in the high-spin co-ordination complex with a phenoxide [49]. The minimum distance between the carbon atoms of the quinoline ring in the drug, and those in the porphyrin ring was 3.1 Å in both models. This would allow Van der Waals interactions between the conjugated rings for both Q-haematin and EQ-haematin.

The H1-Fe distance in Q-haematin is 4.6 Å, while in EQ-haematin it is 3.9 Å, Proximity of positive charges of N1 proton and iron in EQ-haematin, and the higher value of the proton charge [46] might make co-ordination complex formation more difficult than for Q-haematin. The more solvated, more positive, epimer N-1 proton may interfere with the initial hydrophobic interactions of the quinoline and porphyrin rings, affecting co-ordination of O-12 to haematin iron. The importance of solvent water interaction with protonated N1 in inhibiting hydrophobic binding to haematin is suggested by the observation that in 40% DMSO both Q and EQ interact similarly with haematin [50]. The direction of the positive electric field of protonated N1 determines the higher pK_a and solvent interaction and is likely to be, as suggested by Karle and colleagues [15,48], the crucial difference between the inactive and active cinchona alkaloids, since this difference in direction, shown in figure 4, alters both the stereochemistry of interactions between Q or EQ and haematin and increases the solvation and charge of N1-H1 in EQ. These considerations are supported by the lesser effect of the *threo*/*erythro* distinction on the activity of mefloquine congeners and phenanthrene methanols (WR 122,455) where the piperidine ring is more easily able to rotate close to the quinoline ring.

The peak wavelengths and strengths of the calculated electronic absorption spectrum compare well with the observed spectrum of the aqueous complex, extracted into benzene, reported earlier [12] (table 3). Despite the fact that, in view of the discrepancy in wavelength and strength of the α-peak, the structure is not yet completely correct, the α- and β-peaks support the formation of a hydrophobic quinine-haematin co-ordination complex while the strong γ-peak at 408 nm indicates haematin iron in a high-spin state, as expected from the putative structure. This is similar to α-chlorohemin [37], where Fe^{III} is 0.45 Å above the plane of the porphyrin ring. It appears unlikely that the quinoline ring interacts with the other side of the haematin ring structure, as has been proposed for the quinoline ring of CQ [63] since a high spin co-ordination complex could not then be formed.

In summary, positive correlations between antiplasmodial activity and drug lipophilicity have been reported [43,45,47] for a number of 4-aminoquinoline drugs related to CQ and amodiaquine (although not for the cinchona alkaloids) as well as for some aminothiol [74] and hydroxamate [75] chelators. In addition, a proportionality between activity and inhibition of formation of β-haematin has been recognized for 4-aminoquinolines [76] and also for Q and QD [19], The present report, however, suggests that the differences in the antiplasmodial potency of the *erythro* and *threo* cinchona alkaloids on CQ-sensitive isolates of *P. falciparum* may be accounted for as the cumulative consequences of their log D (pH7.4) and BHIA values. The availability of these two critical parameters within this highly homogeneous group of drugs (table 1) makes it possible to apply a simple test

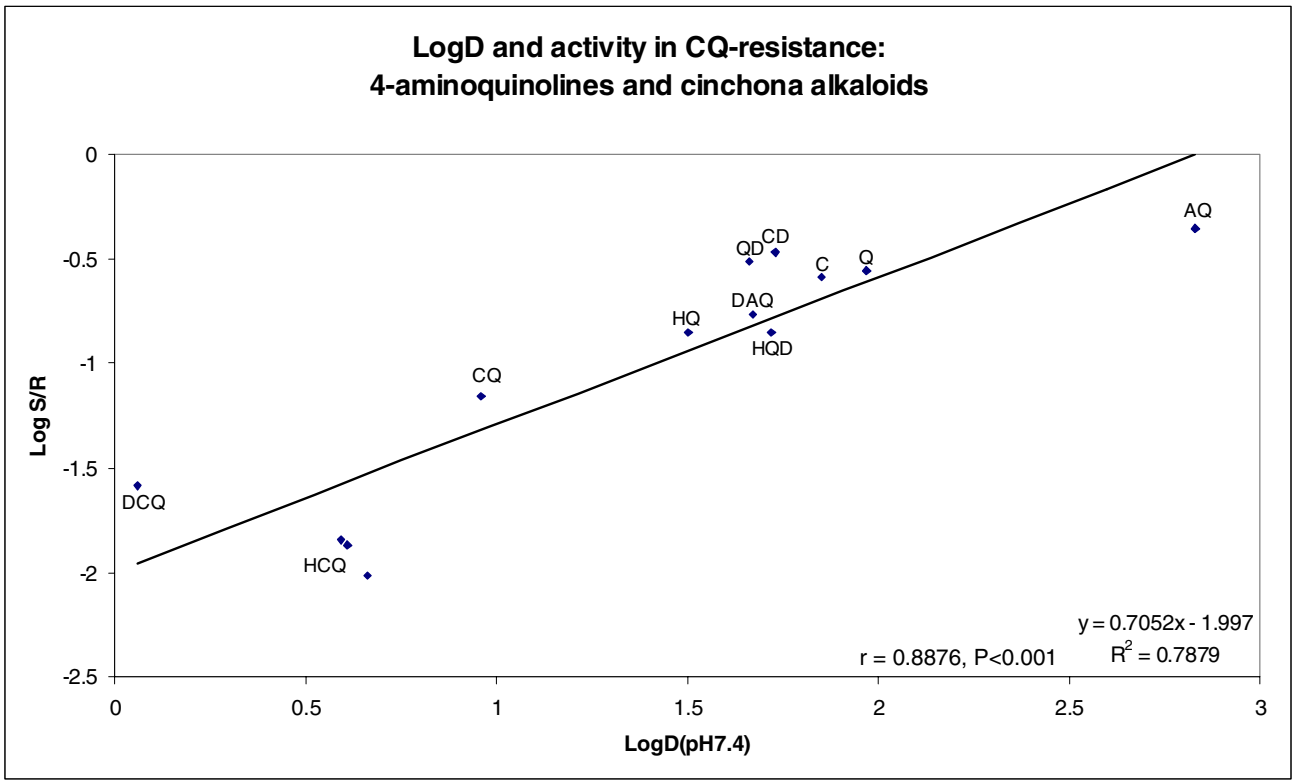


Figure 5
LogD values at pH 7.4 and activity in CQ-resistance (log [IC50S/IC50R]) for 4-aminoquinolines and cinchona alkaloids. 4-aminoquinoline values from ref. [73].

of their sequential and combined effects: The ratio of the mean log D (pH 7.4) for the *erythro* compounds (1.74 ± 0.24) to that of the *threo* compounds (0.66 ± 0.11) is 12:1. The BHIA values for the 2 series are 90 ± 7% and 7 ± 4%, with a similar ratio of 12.5:1. Since these effects are cumulative, their combined result would raise the mean IC50 value for the *threo* series to 150 (ie 12 × 12.5) times that of the *erythro* group. With an experimental mean IC50 of the *erythro* alkaloids of 26.7 nM, the calculated reduction of activity of the *threo* alkaloids leads to an expected mean IC50 of (26.7 × 150) ie 4.0 μM, while the observed experimental value is 3.1 ± 0.4 μM. Alternatively the enhancement in activity of the *erythro* series would lead to an IC50 of 3085/150 ie 20.6 nM; the experimental mean for the 6 *erythro* compounds is 26.7 nM.

The agreement between the calculated and experimental values of IC50 is noteworthy.

These observations may provide a useful platform for the further investigation of antiplasmodial agents, particularly in the climate of increased drug-resistance.

Lessons for the interpretation of CQ-resistance mechanisms

Our arguments so far have concentrated on the probable role of hydrophobicity (logD) and inhibition of haematin dimerization as the determinants of antiplasmodial activity among eight cinchona alkaloids. Clearly these factors are also important in activity against CQ-resistant isolates. However, a further parameter, interaction with the CQ-resistance mechanism, must also be considered. Entry to the cell, concentration in the acidic lysosomal vacuole,

and interaction with haematin have all been postulated as the site of the resistance mechanism. Arguments [61,77,78] and evidence [51,79,85] for and against drug efflux have not yet reached a consensus. Nevertheless, the most important mutations associated with CQ-resistance are in the genes coding for two intrinsic proteins in the membrane of the lysosome [80,56] where quinoline drugs are believed to be concentrated.

One of these, PGH-1, is a protein of the multidrug-resistance type [81], where over-expression is associated with resistance to highly hydrophobic drugs such as mefloquine, halofantrine and artemisinin [82–84]. The other, is PfCRT, where a Lys76Thr mutation is 100% associated with CQ-resistance *in vitro*[56]. In clinical studies, inability to detect the mutant allele predicts treatment success [57]. Replacement of the wild type *pfcrt* gene in sensitive clones by the resistance-associated allele from strains of Asian or South American origin confers CQ-resistance, accompanied by reduced uptake of the drug, and the acquisition of the characteristic chemosensitizing effect of VE [58]. The detailed structure of the PfCRT protein is still under investigation. In its topology the sequence resembles the aqueous chloride channel of *Salmonella typhimurium* and a range of other organisms [59], though 3 of the 4 specific ion-binding motifs are modified. Modelling (work in progress) reveals that the side chain of residue 76 may project into an access pore leading from the lysosomal content. In the wild type (Lys76) this is positively charged, which would attract anions and repel cations. Mutated to neutral Thr76, cations would find access easier. PfCRT has recently been expressed efficiently in yeast *Pichia pastoris* by Roepe's research group [60], and functionally appears to be a membrane channel which, when mutated, may mediate chloride-dependent, VE-inhibitable drug transport. Thus PfCRT could indeed be an anion channel, and by varying the entrance or exit of charge-balancing chloride anion may modulate lysosomal pH [87,88]. This might affect the trapping of basic drugs [5], or interfere with drug-haematin binding [86]. Doubt has been cast [89] on the validity of pH determinations underlying the argument in refs 86–88. These pH effects are perhaps rendered unlikely by the stereospecific interactions of the physico-chemically similar diastereomers Q and QD with mutated PfCRT [58,90]. It is more likely that CQ itself passes through modified PfCRT, since the high drug and proton concentration in the lysosome present a formidable pressure for efflux, given a suitable channel.

For 4-aminoquinolines, the positive correlation between hydrophobicity and relative activity in CQ-resistant isolates, where the more hydrophobic AQ and its metabolite DAQ show significant activity [54] is an important finding. This observation is paralleled by the CQ-sensitising effect, on CQ-resistant isolates, of lipophilic weak bases like VE and PM [52,53]. We are investigating a link between polymorphism in PfCRT, resistance reversal by VE, and the clinical efficacy of AQ (in press).

VE and PM show no significant interaction with the haematin polymerisation process (table 1, figure 2) and they have little intrinsic antiplasmodial action. Combining our recent data on 4-aminoquinolines [73] and the *erythro* cinchona alkaloids studied here there is a significant correlation between hydrophobicity and log relative activity in CQ-resistant parasites, extending the observations of Bray *et al.*[54] (figure 5).

It has been proposed [61,73] that 4-aminoquinolines which are more polar at lysosomal pH (such as CQ) are more likely to pass through the mutated PfCRT channel and escape from the lysosome into the parasite cytoplasm, while those which are more hydrophobic (AQ and its active metabolite DAQ) are likely to bind to the hydrophobic channel lining and stay inside. Their positive charges repel the further access of 4-aminoquinoline to the channel. The high hydrophobicity in acid of VE and PMZ (see table 1) would allow them to bind in a mutated PfCRT channel and their positive charge would prevent efflux of CQ and related drugs. Molecular computations on CQ-resistance reversing agents have indeed determined that their common features are hydrophobicity and electronegativity (i.e. proton attraction) [55].

References

1. Gilles HM: **Management of severe and complicated malaria, a practical handbook.** *World Health Organisation GENEVA* 1991:56.
2. Warhurst DC and Thomas SC: **Pharmacology of the malaria parasite.** *Biochem Pharmacol* 1975, **24:**2047-56.
3. Warhurst DC and Hockley DJ: **The mode of action of chloroquine on *Plasmodium berghei* and *P. cynomolgi*.** *Nature* 1967, **214:**935-6.
4. Fitch CD: ***Plasmodium falciparum* in owl monkeys: drug resistance and chloroquine binding capacity.** *Science* 1970, **169:**289-90.
5. Homewood CA, Warhurst DC, Peters W and Baggaley VC: **Lysosomes, pH and the anti-malarial action of chloroquine.** *Nature* 1972, **235:**50-2.
6. Macomber PB and Sprinz H: **Morphological effects of chloroquine on *Plasmodium berghei* in mice.** *Nature* 1967, **214:**937-9.
7. Chou AC, Chevli R and Fitch CD: **Ferriprotoporphyrin IX fulfils the criteria for identification as the chloroquine receptor of malaria parasites.** *Biochemistry* 1980, **19:**1543-1549.
8. Pagola S, Stephens PW, Bohle DS, Kosar AD and Madsen SK: **The structure of malaria pigment beta-haematin.** *Nature* 2000, **404:**307-10.
9. Buttle GAH, Henry TA, Solomon W, Trevan JW and Gibbs EM: **The action of the cinchona and certain other alkaloids in bird malaria.** *Biochemistry* 1938, **32:**47-58.
10. Taggart JV, Earle DP, Berliner RW, Zubrod CG, Welch WJ, Wise NB, Schroeder EF, London IM and Shannon JA: **Studies on the chemotherapy of the human malarias. III. The physiological distribution and antimalarial activity of the cinchona alkaloids.** *J Clin Invest* 1948, **27(Suppl):**80-86.
11. Trager W and Jensen JB: **Human malaria parasites in continuous culture.** *Science* 1976, **193:**673-5.
12. Warhurst DC: **The quinine-haemin interaction and its relationship to antimalarial activity.** *Biochem Pharmacol* 1981, **30:**3323-3327.

13. Wesche DL and Black J: **A comparison of the antimalarial activity of the cinchona alkaloids against *P. falciparum* in vitro.** *J Trop Med Hyg* 1990, **93:**153-159.
14. Karle JM, Olmeda R, Gerena L and Milhous WK: ***Plasmodium falciparum*: Role of absolute stereochemistry in the antimalarial activity of synthetic amino alcohol antimalarial agents.** *Exp Parasitol* 1993, **76:**345-351.
15. Karle JM, Karle IL, Gerena L and Milhous WK: **Stereochemical evaluation of the relative activities of the cinchona alkaloids against *Plasmodium falciparum*.** *Antimicrob Agents Chemother* 1992, **36:**1538-1544.
16. Oleksyn BJ, Suszko-Purzycka A, Dive G and Lamotte-Brasseur J: **Molecular properties of Cinchona alkaloids: a theoretical approach.** *J Pharm Sci* 1992, **81:**122-127.
17. Karle JM and Karle IL: **Crystal structure of (-)-mefloquine hydrochloride reveals consistency of configuration with biological activity.** *Antimicrob Agents Chemother* 2002, **46:**1529-1534.
18. Behere DV and Goff HM: **High affinity binding of quinine to iron (III) porphyrin: novel formation of alkoxide complexes from alcohols and amines.** *J Am Chem Soc* 1984, **106:**4945-4950.
19. Egan TJ, Ross DC and Adams PA: **Quinoline anti-malarial drugs inhibit spontaneous formation of beta-haematin (malaria pigment).** *FEBS Lett* 1994, **352:**54-57.
20. Kubinyi H: **Lipophilicity and drug activity.** *in Progress in Drug Research* 1979, **23:**97-198. Birkhauser (Basel)
21. Rekker RF: **The hydrophobic fragmental constant. Its derivation and application.** *in The Hydrophobic Fragmental Constant* 1977, **1:**1-24. Elsevier, (Amsterdam)
22. Hansch C and Leo A: ***Exploring QSAR: Fundamentals and Applications in Chemistry and Biology*".** *American Chemical Society, Washington, DC* 1995, **1:**120.
23. Scherrer RA and Howard SM: **Use of distribution coefficients in quantitative structure-activity relationships.** *J Med Chem* 1977, **20:**53-58.
24. Brossi A, Uskokovic M, Gutzwiller J, Krettli AU and Brener Z: **Antimalarial activity of natural, racemic and unnatural dihydroquinine, dihydroquinidine and their various racemic analogs in mice infected with *Plasmodium berghei*.** *Experientia* 1971, **27:**1100-1101.
25. Leo AJ and Hansch C: **Role of hydrophobic effects in mechanistic QSAR.** *in Perspectives in Drug Discovery and Design* 1999, **17:**1-25.
26. Van der Waterbeemd H and Testa B: **The parametrization of lipophilicity and other structural properties in drug design.** *Advances in Drug Research* 1987, **16:**85.
27. Yayon A, Cabantchik ZI and Ginsburg H: **Identification of the acidic compartment of *Plasmodium falciparum*-infected human erythrocytes as the target of the antimalarial drug chloroquine.** *EMBO J* 1984, **3:**2695-700.
28. Krogstad DJ and Schlesinger PH: **A perspective on antimalarial action: effects of weak bases on *Plasmodium falciparum*.** *Biochem Pharmacol* 1986, **35:**547-552.
29. Hawley SR, Bray PG, Park BK and Ward SA: **Amodiaquine accumulation in *Plasmodium falciparum* as a possible explanation for its superior antimalarial activity over chloroquine.** *Mol Biochem Parasitol* 1996, **80:**15-25.
30. Avdeef A: **pH-metric log P. 2: Refinement of partition coefficients and ionization constants of multiprotic substances.** *J Pharm Sci* 1993, **82:**183-190.
31. Chien YW, Lambert HJ and Karim A: **Comparative binding of disopyramide phosphate and quinidine sulfate to human plasma proteins.** *J Pharm Sci* 1974, **63:**1877-1879.
32. Takacs-Novak K and Avdeef A: **Interlaboratory study of log P determination by shake-flask and potentiometric methods.** *J Pharm Biomed Anal* 1996, **14:**1405-13.
33. Parapini S, Basilico N, Pasini E, Egan TJ, Olliaro P, Taramelli D and Monti D: **Standardization of the physicochemical parameters to assess in vitrothe beta-haematin inhibitory activity of antimalarial drugs.** *Exp Parasitol* 2000, **96:**249-56.
34. Allinger NL: **Conformational Analysis 130. MM2. A Hydrocarbon Force Field Utilizing V1 and V2 Torsional Terms.** *J Am Chem Soc* 1977, **99:**8127-8134. (MM+ is a HyperChem modification and extension of Norman Allinger's Molecular Mechanics program MM2)
35. Stewart JJP: **Optimization of Parameters for Semi-empirical Methods. I. Method.** *J Comput Chem* 1989, **10:**209. PM3 is JJP Stewart's reparametrization of the AMI neglect of diatomic differential overlap (NDDO) approximation described in the above publication
36. Stewart JJP: **Optimization of Parameters for Semi-empirical Methods. II. Applications.** *J Comput Chem* 1989, **10:**221.
37. Koenig DF: **The structure of β-chlorohemin.** *Acta Crystallogr* 1965, **18:**663-673.
38. Anderson WP, Edwards WD and Zerner MC: **Calculated Spectra of Hydrated Ions of the First Transition Series.** *Inorg Chem* 1986, **25:**2728-2732. ZINDO/S is Michael Zerner's intermediate neglect of differential overlap (INDO) quantum mechanical method for electronic spectra of molecular systems containing transition metals, discussed in the above article.
39. Irwin JL and Irwin EM: **Apparent dissociation exponents of quinine, pamaquine and a quinolylpiperidyl-carbinol; application of an extended pH scale.** *J Biol Chem* 1948, **174:**577-587.
40. Perrin DD: **Dissociation constants of organic bases in aqueous solution.** *Butterworth's London* 1965.
41. Prelog V and Hafliger O: **Über China-Alkaloide 9. Über den einflussder Konfiguration auf die Basizitat und über die relative Konfiguration an den Kohlenstoffatomen 8 und 9.** *Helv Chim Acta* 1950, **33:**2021-2029.
42. Mannhold R, Dross KP and Rekker RF: **Drug lipophilicity in QSAR practice: I. A comparison of experimental with calculative approaches.** *Quant Struct-Act Relat* 1990, **9:**21-28.
43. Lullmann H and Wehling M: **The binding of drugs to different polar lipids in vitro.** *Biochem Pharmacol* 1979, **28:**3409-3415.
44. Hansch C: *in Comprehensive Medicinal Chemistry Volume 2*. Edited by: *Hansch C. Pergamon press NY*; 1990:159.
45. Hawley SR, Bray PG, O'Neill PM, Park BK and Ward SA: **The role of drug accumulation in 4-aminoquinoline antimalarial potency. The influence of structural substitution and physicochemical properties.** *Biochem Pharmacol* 1996, **52:**723-733.
46. Karle JM and Bhattacharjee AK: **Stereoelectronic features of the cinchona alkaloids determine their differential antimalarial activity.** *Bioorg Med Chem* 1999, **7:**1769-1774.
47. Egan TJ, Hunter R, Kaschula CH, Marques HM, Misplon A and Walden J: **Structure-function relationships in aminoquinolines: effect of amino and chloro groups on quinoline-haematin complex formation, inhibition of beta-haematin formation, and antiplasmodial activity.** *J Med Chem* 2000, **43:**283-291.
48. Moon RP, Bur D, Loetscher H, D'Arcy A, Tyas L, Oefner C, Grueninger-Leitch F, Mona D, Rupp K, Dorn A, Matile H, Certa U, Berry C, Kay J and Ridley RG: **Studies on plasmepsins I and II from the malarial parasite *Plasmodium falciparum* and their exploitation as drug targets.** *Adv Exp Med Biol* 1998, **436:**397-406.
49. Bominaar EL, Ding XQ, Gismelseed A, Bill E, Winkler H, Trautwein AX, Nasri H, Fischer J and Weiss R: **Structural, Mossbauer and EPR investigations on 2 oxidation states of a five-co-ordinate, high spin synthetic heme. Quantitative interpretation of zero-field parameters and large quadrupole splitting.** *Inorg Chem* 1992, **31:**1845-1854.
50. Egan TJ, Mavuso WW, Ross DC and Marques HM: **Thermodynamic factors controlling the interaction of quinoline antimalarial drugs with ferriprotoporphyrin IX.** *J Inorg Biochem* 1997, **68:**137-145.
51. Krogstad DJ, Gluzman IY, Kyle DE, Oduola AM, Martin SK, Milhous WK and Schlesinger PH: **Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance.** *Science* 1987, **238:**1283-1285.
52. Oduola AM, Sowunmi A, Milhous W, Brewer TG, Kyle DE, Gerena L, Rossan RN, Salako LA and Schuster BG: **In vitro and in vivo reversal of chloroquine-resistance in *Plasmodium falciparum* with promethazine.** *Am J Trop Med Hyg* 1998, **58:**625-629.
53. Martiney JA, Cerami A and Slater AFG: **Verapamil reversal of chloroquine-resistance in the malaria parasite *Plasmodium falciparum* is specific for resistant parasites and independent of the weak base effect.** *J Biol Chem* 1995, **270:**22393-22398.
54. Bray PG, Hawley SR, Mungthin M and Ward SA: **Physicochemical properties correlated with drug resistance and the reversal of drug resistance in *Plasmodium falciparum*.** *Mol Pharmacol* 1996, **50:**1559-1566.
55. Bhattacharjee AK, Kyle DE, Vennerstrom JL and Milhous WK: **A 3D QSAR pharmacophore model and quantum chemical structure-activity analysis of chloroquine-resistance reversal.** *J Chem Inf Comput Sci* 2002, **45:**1212-20.

56. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LMB, Sidhu ABS, Naude B, Deitsch KW, Su XZ, Wooton JC, Roepe PD and Wellems TE: **Mutations in the *P. falciparum* digestive vacuole transmembrane protein PFCRT and evidence for their role in chloroquine resistance.** *Molecular Cell* 2000, **6:**861-71.
57. Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Dicko A, Su XZ, Nomura T, Fidock DA, Wellems TE, Plowe CV and Coulibaly D: **A molecular marker for chloroquine-resistant falciparum malaria.** *N Engl J Med* 2001, **344:**257-63.
58. Sidhu AB, Verdier-Pinard D and Fidock DA: **Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations.** *Science* 2002, **298:**210-3.
59. Dutzler R, Campbell EB, Cadene M, Chait BT and MacKinnon R: **X-ray structure of a ClC chloride channel at 3.0 Å reveals the molecular basis of anionselectivity.** *Nature* 2002, **415:**287-94.
60. Zhang H, Howard EM and Roepe PD: **Analysis of the antimalarial drug resistance protein Pfcrt in yeast.** *J Biol Chem* 2002, **277:**49767-75.
61. Warhurst DC, Craig JC and Adagu IS: **Lysosomes and drug resistance in malaria (Commentary).** *Lancet* 2002, **360:**1527-1529.
62. **National Cancer Institute Database** [http://129.43.27.140/ncidb2/]. accessed Nov. 18 2002
63. Moreau S, Perly B, Chachaty C and Deleuze C: **A nuclear magnetic resonance study of the interactions of antimalarial drugs with porphyrins.** *Biochim Biophys Acta* 1985, **840:**107-16.
64. Schulman SG, Threatte RM, Capomachia AC and Paul WL: **Fluorescence of 6-methoxyquinoline, quinine and quinidine in aqueous media.** *J Pharm Sci* 1974, **63:**876-880.
65. Tsai RS, Carrupt PA, Testa B, El Tayar N, Gruenewaald G and Casey A: **Influence of stereochemical factors on the partition coefficient of diastereomers.** *J Chem Research* 1993:1901-1920.
66. Allen RI, Box KJ, Comer JEA, Peake C and Tam KY: **Multiwavelength spectrophotometric determination of acid dissociation constants of ionizable drugs.** *J Pharm Biomed Anal* 1998, **17:**699-712.
67. Drzewiczak C, Suszko-Purzycka A and Skolik J: **Correlation between structure and basicity of Cinchona alkaloids.** *Polish J Chem* 1993, **67:**45-52.
68. Drzewiczak C, Zalewski RI, Skolik J and Suszko-Purzycka A: **Spectrophotometric determination of the second dissociation constant of Cinchona alkaloids.** *Polish J Chem* 1993, **67:**53-58.
69. Lepetit G: **Die pH-abhängige Lipidlöslichkeit von Arzneistoffen.** *Pharmazie* 1977, **32:**289-291.
70. Hansch C and Anderson SM: **The effect of intramolecular hydrophobic bonding on partition coefficients.** *J Org Chem* 1967, **32:**2583-2586.
71. Cassidy SL, Lympany PA and Henry JA: **Lipid solubility of a series of drugs.** *J Pharm Pharmacol* 1988, **40:**130-132.
72. Unger SH and Chiang GH: **Octanol-physiological buffer distribution coefficients of lipophilic amines and their correlation with biological activity.** *J Med Chem* 1981, **24:**262-270.
73. Warhurst DC, Steele JCP, Adagu IS, Craig JC and Cullander C: **Hydroxychloroquine is much less active than chloroquine against chloroquine-resistant *Plasmodium falciparum*, in agreement with its physicochemical properties.** *J Antimicrob Chemother* 2003, **52:**188-93.
74. Loyevsky M, John C, Zaloujnyi I and Gordeuk V: **Aminothiol multidentate chelators as antimalarials.** *Biochem Pharmacol* 1997, **54:**451-8.
75. Shanzer A, Libman J, Lytton SD, Glickstein H and Cabantchik ZI: **Reversed siderophores act as antimalarial agents.** *Proc Natl Acad Sci U S A* 1991, **88:**6585-6589.
76. Kaschula CH, Egan TJ, Hunter R, Basilico N, Parapini S, Taramelli D, Pasini E and Monti D: **Structure-activity relationships in 4-aminoquinoline antiplasmodials. The role of the group at the 7-position.** *J Med Chem* 2002, **45:**3531-3539.
77. Warhurst DC: **Antimalarial schizontocides: why a permease is necessary.** *Parasitol Today* 1986, **2:**331-334.
78. Warhurst DC: **Mechanism of chloroquine-resistance in malaria.** *Parasitol Today* 1988, **4:**211-213.
79. Bray PG, Howells RE, Ritchie GY and Ward SA: **Rapid chloroquine efflux phenotype in both chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum*. A correlation of chloroquine sensitivity with energy-dependent drug accumulation.** *Biochem Pharmacol* 1992, **44:**1317-1324.
80. Cowman AF, Karcz S, Galatis D and Culvenor JG: **A P-glycoprotein homologue of *Plasmodium falciparum* is localized on the digestive vacuole.** *J Cell Biol* 1991, **113:**1033-42.
81. Karcz S and Cowman AF: **Similarities and differences between the multidrug resistance phenotype of mammalian tumor cells and chloroquine resistance in *Plasmodium falciparum*.** *Exp Parasitol* 1991, **73:**233-40.
82. Reed MB, Saliba KJ, Caruana SR, Kirk K and Cowman AF: **Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*.** *Nature* 2000, **403:**906-9.
83. Duraisingh MT, Roper C, Walliker D and Warhurst DC: **Increased sensitivity to the antimalarials mefloquine and artemisinin is conferred by mutations in the pfmdr1 gene of *Plasmodium falciparum*.** *Mol Microbiol* 2000, **36:**955-61.
84. Price RN, Cassar C, Brockman A, Duraisingh M, van Vugt M, White NJ, Nosten F and Krishna S: **The *pfmdr1* gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand.** *Antimicrob Agents Chemother* 1999, **43:**2943-9.
85. Waller KA, Muhle RA, Ursos LM, Horrocks P, Verdier-Pinard D, Sidhu AB, Fujioka H, Roepe PD and Fidock DA: **Chloroquine resistance modulated in vitro by expression levels of the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT).** *J Biol Chem* 2003. June 17, 2003. [Epub ahead of print]
86. Ursos LM, DuBay KF and Roepe PD: **Antimalarial drugs influence the pH dependent solubility of heme via apparent nucleation phenomena.** *Mol Biochem Parasitol* 2001, **12:**11-17.
87. Ursos LM, Dzekunov SM and Roepe PD: **The effects of chloroquine and verapamil on digestive vacuolar pH of *P. falciparum* either sensitive or resistant to chloroquine.** *Mol Biochem Parasitol* 2000, **110:**125-134.
88. Dzekunov SM, Ursos LM and Roepe PD: **Digestive vacuolar pH of intact intraerythrocytic *P. falciparum* either sensitive or resistant to chloroquine.** *Mol Biochem Parasitol* 2000, **110:**107-124.
89. Bray PG, Saliba KJ, Davies JD, Spiller DG, White MR, Kirk K and Ward SA: **Distribution of acridine orange fluorescence in *Plasmodium falciparum*-infected erythrocytes and its implications for the evaluation of digestive vacuole pH.** *Mol Biochem Parasitol* 2002, **119:**301-304.
90. Cooper RA, Ferdig MT, Su XZ, Ursos LM, Mu J, Nomura T, Fujioka H, Fidock DA, Roepe PD and Wellems TE: **Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*.** *Mol Pharmacol* 2002, **61:**35-42.