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Activity of piperaquine and other 4-aminoquinoline antiplasmodial drugs against chloroquine-sensitive and resistant blood-stages of *Plasmodium falciparum*. Role of β-haematin inhibition and drug concentration in vacuolar water- and lipid- phases.

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ABSTRACT

Chloroquine (CQ), a 4-aminoquinoline, accumulates in acidic digestive vacuoles of the malaria parasite, preventing conversion of toxic haematin to B-haematin. We examine how *bis* 4-aminoquinoline piperaquine (PQ) and its hydroxy-modification (OH-PQ) retain potency on chloroquine-resistant (CQ-R) *Plasmodium falciparum*. For CQ, PQ, OH-PQ and **4** and **5**, representing halves of PQ, B-haematin inhibitory activity (BHIA) was assayed, while potency was determined in CQ-sensitive (CQ-S) and CQ-R *P. falciparum*. From measured pKas and the pH-modulated distribution of base between water and lipid (logD), the vacuolar accumulation ratio (VAR) of charged drug from plasma water (pH7.4) into vacuolar water (pH4.8) and lipid accumulation ratio (LAR) were calculated. All agents were active in BHIA. In CQ-S, PQ, OH-PQ and CQ were equally potent while **4** and **5** were 100 times less potent. CQ with 2 basic centres has a VAR of 143,482, while **4** and **5**, with 2 basic centres of lower pKas have VARs of 1,287 and 1,966. In contrast PQ and OH-PQ have 4 basic centres and achieve VARs of 104,378 and 19,874. This confirms the importance of VAR for potency against CQ-S parasites. Contrasting results were seen in CQ-R. **5**, PQ and OHPQ with LARs of 693; 973,492 and 398,118 (compared with 8.25 for CQ) showed similar potency in CQ-S and CQ-R. Importance of LAR for potency against CQ-R parasites probably reflects ability to block efflux by hydrophobic interaction with PfCRT but may relate to B-haematin inhibition in vacuolar lipid.

KEYWORDS: Plasmodium, 4-aminoquinolines, piperaquine, lysosomotropism, drug-resistance, hydrophobicity.

1.0 **Introduction:**

With the increasing spread of drug-resistance to the 4-amino-7-chloroquinoline base chloroquine (CQ) (**1**) (fig. 1) in the malaria parasite *Plasmodium falciparum* [1] and the associated raised case-mortality in children [2], a number of related *bis* 4-amino quinolines have been investigated. These compounds contain two 4-amino-7-chloroquinoline moieties connected at the 4-amino group by a variable linker group [3].

One such group, the bis-quinolin-4-yl piperazines, yielded several active antimalarials. In particular, the (7-chloroquinolin-4yl)-N-1$^{/}$ piperazine) dimers piperaquine (PQ) **2** originally reported by Rhone-Poulenc as 13,228 RP and hydroxypiperaquine (OH-PQ) **3** (fig. 1), in which the N-1$^{/}$ atom of a piperazine linker was directly attached to the C-4 position of each quinoline. PQ and OH-PQ were active i.p. against *P. berghei* in mice[4]. PQ was orally effective against blood-induced *P. cynomolgi* in rhesus monkeys and was then proved in *P. falciparum* infections in man[5]. There was evidence of persistence of active drug levels in the mouse for 15-30 days after a single ip dose [6, 7].

Later, following the extension of the CQ-resistance problem, both PQ and OH-PQ were resynthesized in China [8], and successfully applied by Prof. Chen Lin and colleagues, to the treatment and prevention of infections with CQ-resistant *P. falciparum* on Hainan Island [9]. While as potent as CQ in a CQ-sensitive strain, PQ is reported 6 times more active than CQ in CQ-resistant *P. falciparum in vitro* [10]. More recent studies have confirmed the value of PQ in multidrug resistance [11] and its utility as a companion drug for the endoperoxides dihydroartemisinin and artesunate[12].

There can be no doubt that the release of free haematin from haemoglobin during digestion [13] is the basis of CQ's selective toxicity for intraerythrocytic malaria parasites [14]. The acidic content of the digestive vacuole (lysosome), where the haematin is released, is the site of concentration of this weakly basic 4-amino-7-chloroquinoline drug [15, 16, 17, 18] which binds to haematin and prevents its detoxification by cyclic dimerization [19]. Although the concentration of protonated CQ2H+ into vacuolar water appears to be a requirement for antimalarial activity [15,16,20,21], recent studies indicate that haematin dimerization and detoxication may take place in or closely associated with lipid droplets in the interior of the vacuole [22,23]. The rate of development in cell-free systems of the insoluble crystal of malaria pigment, haemozoin or β-haematin [19], is enhanced by presence in the acidic buffer (pH4.8) of the long chain alcohol n-octanol or other neutral lipids [23, 22]. The importance of lipid for haemozoin formation highlights alternative possibilities for different drugs, that drug/haematin complexes may form in the vacular water and transfer to lipid sites where inhibition of dimerization takes place, or that haematin within the lipid may complex with uncharged drug already localized there. Experimentally it can be demonstrated that [^{3}H] CQ becomes incorporated into β-haematin during its formation in cell-free systems and in intact infected erythrocytes [24], but it is not yet clear what significance this has for the mode of action. In CQ-R *P. falciparum* blood stages, equilibrium concentration of CQ within the infected cell is diminished [25] probably through efflux [26], currently understood to occur across the membrane of the digestive vacuole [27], and so the drug does not achieve intravacuolar concentrations which inhibit haematin dimerization. Since changes in sequence of vacuolar membrane protein PfCRT, crucially K76T, lysine to threonine, are convincingly linked to CQ-R in *P. falciparum* [28], which replaces a positively charged and polar by a neutral and hydrophobic residue, it is probable that efflux of polar, positively charged CQ2H+ is

mediated through the modified protein. Other changes in PfCRT which also increase overall hydrophobicity of the protein, particularly of residues 72-76, are present in field CQ-R isolates. It is recognized that 4-aminoquinolines more hydrophobic than CQ, such as amodiaquine (AQ) and its metabolite mono-desethylamodiaquine (DAQ) retain some activity against CQ-R parasites [29]. This is believed to be due to their interaction with and retention in the hydrophobic lining of a CQ-R PfCRT channel [30, 31, 32]. Reversal of CQ-resistance *in vitro* by verapamil and activity of DAQ both vary with hydrophobicity of residues 72-76 of PfCRT [31]. Allelic replacement of a neutral by a positively charged residue at 76 or at some other positions in the PfCRT sequence effectively restores CQ-sensitivity and restores verapamil-insensitivity [32]. A reduction in vacuolar pH has been associated with CQ-resistance, and in an alternative to the efflux theory, it has been suggested that aggregation of the haematin target under more acid conditions may reduce its binding to CQ [33]. The most recent work affords at present only equivocal support[22] for, or outright disagreement with[18] the haematin aggregation hypothesis. The bis 4-aminoquinolines PQ and OH-PQ probably, like CQ, also become concentrated within the vacuole, bind to haematin and inhibit its dimerization [3]. It remains to be determined how these agents retain their effects in CQ-resistant parasites.

The entry of a basic drug through lipid membranes and its distribution into the aqueous compartments of an infected erythrocyte is determined by lipid-water partition coefficient (expressed as logP) interacting with pH through the ionization constant(s) (pK_a) of the basic centre(s) of the drug. In order to understand drug action and resistance more fully we have therefore measured, in comparison with CQ, logP and pK_a values for PQ (**2**), OH-PQ (**3**) and two "monomeric" compounds, N-1′-(7-chloroquinolin-4yl)-piperazines **4** and **5** representing halves of PQ (Figure 1). We have modelled at equilibrium the aqueous and

lipid distribution of the drugs in the parasitized erythrocyte to determine their expected accumulation ratios from plasma water (pH7.4) through intraerythrocytic membranes and compartments into vacuolar water (vacuolar accumulation ratio: VAR)[34] and through vacuolar water into vacuolar lipid, lipid accumulation ratio (LAR). This confirms for CQ-S parasites, that while charged drug concentration in vacuolar water (VAR) increases as vacuolar pH decreases, the concentration of uncharged drug (base) in vacuolar membrane and other lipid sites in the digestive vacuole has a constant value for each individual drug, because the proportion of drug partitioned into lipid decreases as logD (the log of $[drug]_{lipid}$ / $[drug]_{water}$ at equilibrium) decreases with reduced pH.

Activity of these agents in inhibition of haematin dimerization to β-haematin in a cell-free system (BHIA) has been determined, and also their potency against CQ-S and CQ-R *Plasmodium falciparum*, in comparison with CQ (**1**) itself.

We have also modelled the probable minimal energy conformations of the drugs in their protonated and unprotonated states using a semi-empirical quantum mechanical protocol [35], determining heats of formation and relevant structural features, which support conclusions based mainly on argument from physicochemical observations.

2.0 Materials and Methods:

2.1 Synthesis of new test substances.

Compounds **4** and **5** were obtained from reaction of 4,7-dichloroquinoline with 1-methylpiperazine and 1-ethylpiperazine respectively, and were characterized by analysis,

^{1}H proton NMR and high-resolution Mass Spectroscopy (HRMS). Compound **4** has been previously prepared by another method [36], but its activity against malaria parasites was not reported.

N-1′-(7-chloroquinolin-4-yl)-N-4'-methylpiperazine **4**. A solution of 4,7-dichloroquinoline (1 g, 5.05 mmol) in 1-methylpiperazine (30 mL, 0.270 mol) was heated to reflux for 4 h while monitoring the disappearance of starting material by TLC. Excess solvent was evaporated and the product purified via flash chromatography with silica gel. Elution with CH_2Cl_2/MeOH/Et_3N (95:4:1) gave pure **4** (1.12 g, 86% yield) as white crystals from methanol, mp 88-89°C (lit. mp 86°C). ^{1}H NMR ($CDCl_3$):δ7.01-8.90 (5H) (Ar-H), δ 3.33 (s, 3H) (N-CH_3 boat), δ 3.18 (t, J=3.9 Hz, 4H) (N-CH_2, 3' & 6'), δ 2.62 (t, J=3.9 Hz, 4H) ((N-CH_2, 2' & 5'), δ 2.31 (s, 3H) (N- CH_3) (chair). HRMS calcd. For $C_{14}H_{16}ClN_3$ m/z 261.1033, found 261.1036 (M^+). The hydrochloride, prepared using methanolic HCl (10-N), crystallized from methanol, mp 277-280°C (dec.). *Anal.* Calcd. For [$C_{14}H_{16}ClN_3$•2HCl•1H_2O] C, 47.66; H, 5.67; N, 11.91; found C, 47.89; H, 5.84; N, 11.61%. [FW= 352.68].

N-1'-(7-chloroquinolin-4-yl)-N-4'-ethylpiperazine **5.** Prepared in the same manner from 4,7-dichloroquinoline (5 g, 25.2 mmol) in 1-ethylpiperazine (150 mL, 1.17 mol) by refluxing for 6 h, the product **5** (6.36g, 91% yield) crystallized from methanol, mp 82-83°C. ^{1}H NMR ($CDCl_3$):δ 6.83-8.72 (5H) (Ar-H), δ 3.26 (t, J=3.9 Hz, 4H) N- CH_2, 3' & 6', δ 2.74 (t, J=3. 9 Hz, 4H) N- CH_2, 2' & 5', δ 2.58 (q, J=6.4 Hz, 2H) CH_2, δ 1.15 (t, J=6.4 Hz, 3H) CH_3. HRMS calcd. For $C_{15}H_{18}ClN_3$ m/z 275.1189, found 275.1183. Anal. Calcd. For $C_{15}H_{18}ClN_3$•2H_2O:C, 57.78; H, 7.06; N, 13.48. Found: C, 57.92; H, 7.18; N, 13.36%.

The hydrochloride was prepared as above and crystallized from methanol, mp 288-289°C (dec.). Anal calcd for $C_{15}H_{18}ClN_3 \bullet 2HCl \bullet 1\ H_2O$: C, 49.11; H, 6.00; N, 11.46. Found C, 48.89; H, 6.05; N, 11.31%. [FW= 366.71].

The ^{1}H-NMR spectrum of **4** showed that the piperazine ring exists in a dynamic equilibrium between the chair and boat conformers, as demonstrated by the appearance of a second N-methyl signal at 3.33 ppm (due to the magnetic de-shielding of the N-methyl group in the boat form by the magnetic cone of the adjacent benzene ring) in addition to the normal N-methyl resonance at 2.30ppm arising from the chair conformer. As expected, in the doubly-charged di-hydrochloride salt only the more stable chair form existed, with the N-CH_3 signal at 3.04ppm. The corresponding ^{1}H-NMR spectrum of the N-4′-ethyl analogue **5** also showed only the chair conformer to be present.

2.2

Samples of PQ were obtained from WHO (Dr. Piero Olliaro and Dr Alan Shapiro) as the tetraphosphate tetrahydrate, while OH-PQ base was kindly supplied by Professor W. Peters. Racemic CQ diphosphate was from Sigma (UK).

2.3 Molecular modelling:

For CQ, PQ, OH-PQ, **4** and **5**, protonated and base forms were modelled in HyperChem Release 7 for Windows (Hypercube Inc., Gainesville Florida) using the semi-empirical Austin method 1 (AM1) quantum-mechanical procedure [35]. Geometry was optimized to

an *rms* (root mean square) gradient of 0.001 *in vacuo,* [*cf* ref 23] (Polak-Ribière conjugate-gradient method). The optimized structure was then subjected to single-point computation using the AM1 programme in configuration-interaction, to find the total energies and heats of formation for the energy-minimized structures as bases and as the protonated cations (Table 1). Distances between the quinoline N-1 atoms and 4-amino N atoms, between bridging side-chain N atoms (in *bis* compounds), between quinoline N-1 atoms (in *bis* compounds) and between quinoline N-1 atoms and side-chain N atoms (Table 1) were determined.

2.4 Physico-chemical properties.

2.41 Role of ionization constant(s) and partition coefficient in the entry of drugs into cells.

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 n-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 aqueous and lipid phases at any fixed pH ($[drug]_{lipid}$ / $[drug]_{water}$ at equilibrium) is called the distribution coefficient D, usually expressed as log D; this assumes that only the un-ionized base partitions from the aqueous to the organic phase[37]. The difference between log P and log D varies at different pH values because of the variation in the percentage of the drug ionized, and, for a drug with one basic centre, is obtained from the standard equation (1) for a base [38,39].

eqn. (1) $\log D = \log P - \log [1 + 10^{(pKa-pH)}]$

Thus it is possible [37, 40] to calculate log D from observed logP, pK_a and pH.

As well as experimentally measuring logP, it is possible to estimate it using the ClogP programme, developed by Hansch and Leo, although differences due to conformational isomerism are not taken into account [41]. Calculated values of log P were obtained for comparison using a modified commercial version of ClogP, incorporated in version 7.0 of the Advanced Chemistry Development log D suite for Windows (Toronto, CANADA).

The ionized basic centres of compounds **1**-**5** can be described as (a) the resonance-stabilised amidinium ion (made up of the N-1 of the quinoline and the amino group attached to C-4 of the same ring) and (b) a distal aliphatic protonated N-3. For CQ, **4** and **5**, which have one example of each centre, the correction needed in logP to obtain logD at a given pH involves the additive contribution of both ionized species, and the base equation (1) must be modified [42] to equation (2).

Eqn. (2) $\log D = \log P - \log [1 + 10^{(pKa1 - pH)} + 10^{(pKa1 + pKa2 - 2pH)}]$

Bis compounds PQ and OH-PQ are centro-symmetric and have two of each type of basic centre. Initial protonation at one basic centre may affect the other via a through-space electric effect of the now positively charged N ion on the other (uncharged) N atom, causing a slight reduction of the basicity of the second basic centre by inhibiting its protonation. The existence of 4 separate pK_a values for PQ and OH-PQ is therefore possible, and to provide for the contribution from all ionized species to the logD, the

equation of Henderson and Hasselbach can be modified from eqn.2 to eqn. 3.

Eqn. (3):

$$\log D = \log P - \log [1 + 10^{(pKa1-pH)} + 10^{(pKa1 + pKa2 - 2pH)} + 10^{(pKa1 + pKa2 + pKa3 - 3pH)} + 10^{(pKa1 + pKa2 + pKa3 + pKa4 - 4pH)}]$$

Dissociation constants and partition coefficients were determined by Robertson Microlit Laboratories (Madison NJ USA) at 25° C using the Sirius GLpK automated computerized potentiometric system [43] which is capable of resolving ionization constants of multiprotic substances. Titration employed water containing 0.15M KCl (representing a physiological concentration of positive and negative counter-ions) in an argon atmosphere. The pK_a values were determined in triplicate with a SE of ± 0.20. For PQ and OH-PQ the base precipitated above pH 7.0. The titrations were therefore carried out with methanol as a co-solvent, using 5 different ratios of methanol to water. The aqueous pK_a was determined by extrapolation to 0% methanol using the method of Yashuda-Shedlovsky [44,45] which gives a linear fit. Log D values at the physiological pH of 7.4 and at pH 5.2, an early reported pH for the digestive vacuole [17], were estimated from the experimental plot of log D versus pH. We compared these reported values with logD values we calculated from logP and pK_a using equations 2 [42] and 3 [46]. It should be noted that in eqns. 2 and 3, $pK_{a1} > pK_{a2} > pK_{a3} > pK_{a4}$.

Partition coefficients between octanol and water were measured by dual-phase potentiometric titration using various amounts of water-saturated n-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. 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[47]. We have also reported the values of LogP for the unionized bases calculated by the ACD logD programme. (Table 2).

2.411 Vacuolar accumulation ratio (VAR):

Since the vacuolar (lysosomal) pH is being regulated to an acidic value within the malaria parasite, providing optimal conditions for haemoglobin digestion [17,33,18] the potential vacuolar accumulation ratio for a drug (VAR) into intra-vacuolar water for a drug with one basic centre protonated may be calculated using the ratio of proton concentration in the aqueous contents of the vacuole to that in the aqueous medium (pH7.4) external to the cell. Applying the Henderson-Hasselbach equation to measured pK_as, it is possible to calculate accumulation ratios from plasma (pH 7.4) to the vacuole (vacuolar accumulation ratios: VAR) at any specified vacuolar pH for CQ with 2 protonatable basic centres [34] and for bis-quinolines with 4 centres [48]. However, if logD values have been calculated already, using observed logP and pK_a, the log of VAR at any vacuolar pH is easily obtained by subtracting logD at the proposed vacuolar pH, from logD at pH7.4 since logP is automatically eliminated from the calculation [Eqn. (4)]. If calculated logD values are used, VAR values identical to those obtained from the equations of Krogstad & Schlesinger [34] and Rykebusch *et al* [48] are produced. With observed values of logD,

results may differ slightly (table 2). In view of the high degree of agreement between observed and calculated VARs, and the utility of being able to calculate a logD for any vacuolar pH value, we used for analysis the logD values calculated from equations 2 and 3 throughout the study. Observed logD values at pH 7.4 and pH 5.2 are also reported in table 2.

Eqn. (4)……..VAR = antilog ($logD_{(pH7.4)}$-$logD_{(pHv)}$)

(pH_v=vacuolar pH)

It is important to recognize that this calculation only refers to charged drug in the aqueous phase, taking into account that 61% CQ in plasma is protein-bound in healthy persons [49]. This is likely to be higher for PQ and OH-PQ, because of their higher hydrophobicity (compare quinine where the $logD_{7.4}$ is 1.96 [50] compared with 0.92 for CQ, and the protein binding is 92.8% [51]). In addition the digestive vacuole contains variable amounts of parasite protein and of haemoglobin at different stages of digestion.

2.412 Lipid accumulation ratio (LAR):

Modelling of the equilibrium drug distribution between different compartments of the infected erythrocyte revealed that, although the proportion of an unprotonated basic drug which entered lipid from the aqueous phase increased with pH (examined between pH 4.8 and 7.4), the actual concentration in lipid did not vary between erythrocyte membrane, parasite membrane, vacuole membrane and intravacuolar lipid.

LAR= VAR*(antilog $logD_{(pHv)}$)

$logLAR= (logD_{(pH7.4)}-logD_{(pHv)})+ logD_{(pHv)}$

when pH_v=7.4, $logLAR= logD_{(pH7.4)}$

when $pH_v = 4.8$, $logLAR = (logD_{(pH7.4)} - logD_{(pH4.8)}) + logD_{(pH4.8)}$

Eqn. (5) …….. $log\ LAR = LogD_{(pH7.4)}$

Thus, for any basic drug present at concentration 1nM in extracellular water at pH 7.4, LAR in the external membrane is antilog ($logD_{(pH7.4)}$)nM. As pH of an internal aqueous vacuole is lowered from 7.4, the increase in log VAR due to proton trapping and decrease in logD at lower pH in the vacuolar water ensures drug distribution into vacuolar lipid remains constant at antilog ($logD_{(pH7.4)}$) nM [52].

2.413 Comparison of intravacuolar with extracellular pH changes:

The insensitivity to variations of vacuolar pH of LAR contrasts with its sensitivity to pH changes in the aqueous medium surrounding the infected erythrocyte. When the medium pH is changed, a rise from pH 7.4 to 7.6 more than doubles the concentration of charged CQ (CQ2H+) VAR in vacuolar water, and of CQ in the erythrocyte lipid/ membrane phase (LAR), and, according to our calculations (*cf* [52]), throughout lipid in the parasite/ host cell complex, including the vacuole membrane and lipid droplets, while a further rise in plasma pH to 7.7 almost doubles VAR and LAR again. The concentration change in vacuolar lipids follows from increases of the same order in CQ2H+ concentration in the vacuolar water, without vacuolar pH alteration. Conversely, decreasing medium pH from 7.4 to 7.2 more than halves CQ concentration in the vacuolar lipid, and CQ2H+ in the vacuolar water.

The reduction of CQ activity against cultured *P. falciparum* on lowering medium pH and

enhancement with higher pH was discovered by Yayon *et al* in 1985 [53] and has been confirmed several times. This situation can be modelled well using equations (4) and (5) (above). Raising external medium pH to 7.6, while maintaining vacuolar pH will give a greater value for ($logD_{(pH7.6)}$-$logD_{(pH4.8)}$) and thus a greater antilog or VAR. At the same time, because of the higher logD at pH 7.6, LAR, its antilog, will be greater.

The discovery of the influence of medium (plasma) pH on the availability of CQ to the intraerythrocytic malaria parasite [53] appears of possible interest in malaria treatment because lactic acidosis (blood pH value ≤7.3 with a venous blood lactate concentration ≥ 5mM) where blood pH can be as low as 7.14 [54], is a frequent complication of severe falciparum malaria and an independent predictor of death [55] . With a plasma CQ concentration similar to that at pH 7.4, a blood pH of 7.1 might lead to a 3.79 fold reduction in VAR and LAR and a consequent decrease in antiplasmodial efficiency.

2.414 Calculation of percentage of molecules with a basic centre ionized, from pH and pK_a.

The equation given by Adrien Albert [56] was used for each basic centre:

Eqn. (6): % ionized $= 100/[1+10^{(pH-pKa)}]$

An ExCel chart was prepared, and results, rounded to one place of decimals, are given in table 4.

2.5 B-haematin inhibitory activity (BHIA):

This test, in pH 5.0 buffer containing acetate as a phase-transfer catalyst [57], was modified from that described by Parapini et al [58] by use of pyridine to ensure the final OD values conformed more closely with the Beer-Lambert law (haematin, which tends to aggregate in aqueous solution, was converted into the pyridine haemichrome to ensure that the law was obeyed [60]). 100 µL of each stock drug solution was added to wells in row A of a 96-well microtitre plate, except for the negative control well A12 to which 100 µL of distilled water was added. [Stock solutions of drugs were prepared in water at 40mM (solubility was helped where needed by titration with HCl). PQ as the tetraphosphate tetrahydrate was dissolved in warm water while base OH-PQ was dissolved in warm water using 0.1N HCl. Compounds **4** and **5**, crystalline dihydrochloride monohydrates, were dissolved in warm water. Chloroquine was used as the commercial diphosphate in water.] In rows B to H, 50µL distilled water was distributed using a multipipette. Then 50µL was withdrawn from wells in row A and mixed with wells in row B. Serial dilution of 50µL from wells in row B into wells in row C was carried on until row H whence 50µL was discarded. 50µL of 8mM haemin chloride in DMSO was added to each well in the plate, using the multipipette without touching the well contents. From row H upwards, 100µL of 8M acetate buffer was added to each row including row A. [Acetate buffer pH 5.0. Solid sodium acetate was made up 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 the pH was adjusted to 5.0 with 8 M acetic acid at 37°C, which led to the remaining solid dissolving]. The contents of each well were individually mixed, starting from row H and proceeding to row A using a multipipette set at 200µL. The experiment was incubated at 35^{o}C for 24 hr. [The final

DMSO concentration during the incubation was 25% w/v, while final haematin concentration (0.4 μmol/200μL well) was 2 mM, and the highest final drug concentration used was 10 mM, a top drug/haematin molar ratio of 5:1].

To harvest the experiment, 1mL DMSO was added to a series of Eppendorf 1.5mL centrifuge tubes to match the arrangement of the microtitre plate. Using the DMSO, the content of each well was washed and mixed into its centrifuge tube and incubated for 30min at 35°C. The tubes were then centrifuged in a swing-out rotor for 20 min. at 3000g. The closure was removed from each tube in a rack and, ensuring each was well seated, the rack inverted once to discard the supernatant, draining on absorbent towels. To the sediment in each tube 1mL N NaOH was added, then incubated 30 min at 35°C, and the contents of each tube washed and mixed into 1mL aqueous 5% v/v pyridine in a microcuvette.

Absorption (optical density) of each cuvette was read in visible light at 414nm.

Assuming the mean absorption of the drug-free control cuvettes is 100%, the relative % inhibition of the β-haematin formation [100-((OD test/mean OD control)*100)] by different concentrations of drug was calculated. Percentage inhibition values were normalized against the maximum inhibition (assumed 100%) produced by CQ, and the drug:haematin molar ratios for 50 % inhibition were obtained by regression analysis of the linear part of the sigmoid curve.

2.6 Drug activity tests against *P. falciparum*.

Drugs were tested at least twice in triplicate for ability to inhibit the proliferation of a chloroquine-resistant (K1) isolate and a chloroquine-sensitive (T9-96) cloned isolate of *P. falciparum*, from Kanchanaburi and from Mae Sod, Thailand, respectively [60,61] , cultured *in vitro*, using a [^{3}H] hypoxanthine incorporation procedure [62].

Briefly, the parasites were grown in A+ human erythrocytes, with 10 mL AB serum (National Blood Service, UK) added per 100 mL RPMI medium. Before use for drug assay, parasites were synchronized twice using sorbitol lysis [63]. An initial 1% parasitaemia at 5% haematocrit, with >95% of parasites in the growing young trophozoite stage was prepared. After preparation of stock drug solutions in serum-free medium, serial dilutions were prepared in 96-well flat-bottomed microtitre plates and then an equal volume of the infected blood suspended in medium was added to give a final 2.5% haematocrit at 1% parasitaemia. After incubation in a sealed vessel with 3% O_2, 4% CO_2, 93% N_2 at 37°C for 24 h, [^{3}H] hypoxanthine, of specific activity 611 GBq (16.5 Ci) /mmol (Amersham, UK) was added as 10 μL of a 20 μCi/mL solution to each well, re-gassed and incubated for a further 18–24 h. Red cells were harvested from each well (Filtermate 196, Packard: Perkin Elmer Life Sciences, Boston, USA) on to Unifilter 96-well plates (Packard) followed by water washing for lysis, removal of unincorporated isotope and of other soluble components. After drying at 56°C, the undersides of the plates were sealed and scintillation fluid added to each well before sealing the top of the plate. Tritium disintegrations per minute (dpm) were determined in a Packard 'Topcount' scintillation spectrometer, and the data were collected on a floppy disc. Percentage inhibitions of incorporation were calculated using the following equation. % inhibition = 100 – [((dpm test – mean dpm uninfected cells)/(mean dpm infected cells – mean dpm uninfected cells)) × 100]

These values were used to plot dose–response curves from which the IC_{50} values were derived by non-linear regression analysis (Microsoft EXCEL – add-in XL-fit program, ID Business solutions, Guildford, UK). Mean, standard error and 95% confidence intervals were calculated.

3.0 Results:

3.1: MOLECULAR MODELLING: (table 1, Figs. 3-7)

3.11 *R*- and *S*- CQ:

CQ **1** has a side chain asymmetric C atom (see figs 1 and 3), and commercial supplies are racemic, so both *R* and *S* enantiomers were modelled. The AM1, restricted Hartree-Foch calculations (25 ^{o} C) carried out in the present study confirm that the *R* and *S* forms are true enantiomers and energetically identical in all respects, as was demonstrated in lanthanide nmr studies of the base in acetone at 20^{o} and 48^{o}C carried out by Angermann *et al* [64]. We have used the coordinates, kindly supplied by Dr. Angermann, to make text files for the 3-dimensional solution structures, and modelled them first in the *.ent and then in the *.hin Hyperchem formats (not shown). Although these authors found a close hydrophobic association between the side-chain and aromatic ring at 20^{o}C, at 48^{o}C their structure for the base bears a very close resemblance to our calculated 25^{o}C *in vacuo* minimal energy conformation (fig. 3). The side chain extends to one side and above the aromatic ring. The authors suggest the "unusual buckling" of the side chain in solution at both temperatures may be due to an intramolecular dipole-induced dipole type of interaction between the relatively positive N-3 ethyl groups and the π-electron system of the quinoline ring. While

their 20° C N-1 to N-3 distance was 7.39 Å, at 48°C it was 9.33 Å. Our (25 ° C) calculated estimate is 7.893 Å which is comparable and intermediate. In the earlier X-ray crystallographic study of CQ diphosphate, representing the diprotonated form, the N-1 to N-3 distance was found to be 14.1 Å [65]. Here, electrostatic repulsion between the positive charges of protonated N-1 and N-3 and interaction with the phosphate groups stabilizes an extended form. In our models of CQ, the greatest distance between protonated quinoline N-1 and aliphatic protonated N-3 was 8.991Å and appreciably less (7.999Å) in the monoprotonated form, with the side-chain still somewhat buckled in both cases, indicating that an apparent attraction to the quinoline π-electron system can still be recognized in the diprotonated form.

In the calculated models a change in the distance from N-1 to secondary 4-amino N-2 on protonation of the aromatic N-1 was also of interest. Both enantiomers showed a marked shortening of this dimension from 4.255 (base) and 4.258 (protonated aliphatic N-3) to 4.196 Å in the diprotonated species, supporting the formation of the strongly resonance-stabilized aromatic amidinium ion.

3.12 Compounds 4 and 5:

The features of the aromatic amidinium ion differed from that of CQ and resembled PQ and OH-PQ (below), with an N-1 to N-2 separation of 4.221 Å in the diprotonated form of **4** compared with 4.280 Å in the base, and 4.219 compared also with 4.280 Å for **5**. (*cf* CQ, 4.196 compared with 4.255 Å).

3.13 PQ :

The minimal energy structure for PQ (**2**) was extended, with a distance between the quinoline N-1 atoms for the tetra-protonated form of 18.29 Å. The mean N-1 to N-2 separation in the aromatic amidinium ions in the diprotonated species was 4.201 Å (*cf* CQ, 4.196 Å), reduced from 4.280 in the base, supporting the base-weakening of the amidinium compared with CQ noted in physicochemical measurements (below). The distance increased to 4.231 Å when the final 2 piperazine N atoms had been protonated.

3.14 OH-PQ:

The calculated heat of formation of OH-PQ (**3**) was appreciably lower than for PQ by 45.45 kcal.mol^{-1}, which may perhaps be accounted for by weak internal hydrogen bonding between the hydroxyl group and one or both of the bridging piperazine N atoms (minimal O-N distance in the protonated species of 2.850 Å). This was also suggested as a result of the physicochemical study (below). The features of the amidinium ion in OH-PQ, and the characteristics of the minimum energy structure were similar to those of PQ.

3.15 Relative orientation of the quinoline and piperazine rings.

The minimum energy orientation of the piperazine rings in **4**, **5**, PQ and OH-PQ, was similar, probably influenced by the proximity of the quinoline C-5 proton. This resulted in the "plane" of the piperazine ring being twisted relative to that of the quinoline, agreeing with the nmr study on **4** and **5** and supporting a cause for the base-weakening effect as suggested in the physicochemical study (below).

3.2 PHYSICOCHEMICAL PROPERTIES: (see tables 2, 3A and 3B)

The dissociation constants, logP values, logD values, estimates of VAR at pH 4.8 and 5.2, and LAR values for cpds. **1**-**5** are shown in tables 2, 3A and 3B. The logP values calculated from the drug structures by the ACD programme closely approximate to the measured values. In addition, log D values for pH 7.4 and 5.2 calculated from measured values of logP and pK_a using equations (2) or (3) are close to the measured values. The VAR derived from the calculated estimates of logD from equations (2) or (3) is invariably identical to the VAR value calculated from equations reported earlier [34, 48] (table 2). Although for CQ (2 relevant protons) the logarithmic relationship between VAR and vacuolar pH very closely approximates linearity, it is not inherently linear, rather a 2nd order polynomial while for **4** and **5** (2 protons) and PQ and OHPQ (4 protons) the curve is clearly seen to be concave (see fig. 2).

3.21 Acid Dissociation constants (pK_a): (see table 2)

In the parent compound 4-amino-7-chloroquinoline, and in the similarly constituted CQ, protonation occurs at the quinoline ring N-1 to form a planar resonance-stabilized 1,4-amidinium ion with measured pK_a values of 8.23 and 8.38 respectively [66,67]. However, in the (N-1$^{/}$-quinolin-4-yl)-N-4$^{/}$-alkylpiperazines **4** and **5,** the N attached to the 4-position of the quinoline ring is also the N-1$^{/}$ atom of a piperazine ring, making the substitution spatially equivalent to a tertiary dialkylamino group. The effect of steric hindrance with the adjacent quinoline C-5 proton causes the dialkylamino moiety to be twisted out of the plane of the quinoline ring, and this departure from co-planarity diminishes the resonance stabilization of the planar amidinium ion. This base-weakening effect is shown in going

from 4-aminoquinoline (pK_a 9.08) [68] to 4-dimethylaminoquinoline (pK_a 8.36) [69] and allows the pK_{a1} values of (**4**) (7.63) and (**5**) (7.92) to be identified with the aromatic amidinium ion. Therefore the pK_{a1} and pK_{a2} values of PQ and OH-PQ between 6.41 and 6.88 also belong to the aromatic amidinium ions.

With respect to the protonatable aliphatic tertiary amine groups in compounds **2**-**5**, while the pK_{a1} for piperazine itself is 9.81, pK_{a2} for the second N is lowered to 5.55 due to the unit positive charge on N-1[70]. The same explanation accounts for pK_{a2} in **4** (5.42) and **5** (5.54) (N-4′) and for the pK_{a3} and pK_{a4} values of PQ (**2**) and OH-PQ (**3**), (where there is one for each of the piperazine rings).

Results of the potentiometric titrations of PQ confirm that stepwise protonation occurs, with pK_{a1} and pK_{a2} values of 6.88 and 6.24 for the two aromatic amidinium ions, and pK_{a3} and pK_{a4} of 5.72 and 5.39 for the distal tertiary N-4′ of the 2 piperazines. Very similar numbers for the amidinium ions were obtained for the analogue OH-PQ (pK_{a1} 6.60 and pK_{a2} 6.41) where a slight base-weakening effect for the piperazine N-4′ (pK_{a3} 5.39 and pK_{a4} 4.83) similar to the drop from triethylamine to diethylaminoethanol [71] was seen, probably due to hydrogen bonding of the –OH group with one or other of the adjacent tertiary N-4′ atoms.

3.22 Experimental LogP values: (see table 2)

The anticipated enhanced lipophilic properties of the bis-quinolinyl compounds PQ and (OH-PQ) were revealed by the extraordinarily high measured values of logP, with experimental numbers of 6.11 and 5.67 in reasonable agreement with calculated ACD logP

values. Compounds **4** and **5** gave experimental log P values in excellent agreement with those calculated using the ACD suite (table 2), with the expected increase in logP due to the larger alkyl group in **5**.

3.23 pH-modulated logD values: (see Tables 2, 3A, 3B and 4.)

3.231 Chloroquine:

At physiological pH (7.4) the proportion of molecules positively charged is 99.8%, while 90.5% are doubly protonated, 2+ (90.5% of the quinolinium N-1 and 99.8% of the relevant tertiary aliphatic N-3) (Table 4). This results in a calculated logD value of 0.92 giving a LAR (antilog of $logD_{(pH7.4)}$) from the blood plasma to the rbc lipid membrane of 8.25 (membrane concentration is 8.25 times the plasma concentration).

At vacuolar pH (4.8), 100.0% of the molecules are doubly positively charged (2+)(Table 4). The logD value is -4.24, and while the membrane transfer ratio from the vacuolar content to the vacuolar membrane is extremely low, the VAR into vacuolar water, calculated from logD values is extremely high at 143,482. Hence the overall LAR from plasma into vacuolar lipid phase is found to be identical to the value reported above for the rbc membrane (8.25). This feature is maintained throughout the pH range (4.8-7.4) studied (table 3A): the lipid concentration in vacuolar membrane and enclosed lipid remains constant as pH (and logD) decrease because of the increase in VAR in vacuolar water.

Insert Table 3.

3.232 Compounds 4 and 5:

At physiological pH, only 1.0 and 1.4% of molecules of compounds **4** and **5** are 2+ (quinolinium and aliphatic piperazine N-4/ [N3]). (table 4). This results in logD values of 2.59 and 2.84, giving LARs from the blood plasma to the rbc lipid membrane of 386 and 693.

At vacuolar pH (4.8), 80.7 and 84.6% of molecules of **4** and **5** are 2+.(table 4). Their logD values are -0.52 and -0.45 while the VAR values in vacuolar water are very low, at 1,287 and 1, 966. LARs from the blood plasma or medium through vacuolar water to the vacuolar lipid are identical to those in the rbc membrane.

3.233 PQ and OH-PQ:

Due to lower pK_a values, these are only 6.5 and 9.3% doubly charged (1^{st} and 2^{nd} quinolinium N-1), and 1.0% and 0.3% 4+ (2 quinolinium N1 and 2 aliphatic piperazine N-4/ [N3]) at pH 7.4. (table 4). Thus the log D values remain very high, 5.99 and 5.60, similar to their LogP values, corresponding to huge LARs from the blood plasma to the rbc lipid membrane of 973,492 and 398,118 respectively, nearly 6 orders of magnitude higher than CQ. At vacuolar pH (4.8), PQ and OH-PQ are 96.5 and 97.6% 2+, and 79.6 and 51.7% 4+. (table 4). This is reflected in the drop in their log D values to 0.97 and 1.30 while the VAR values in vacuolar water are large at 104,378 and 19,874. LARs from the blood plasma through vacuolar water to the vacuolar lipid, identical to rbc membrane values, are huge.

3.3 β-haematin inhibitory activity (BHIA).

CQ had an IC_{50} molar ratio *vs* haematin of 1.30, while those of **4** and **5** were 3.8 and 3.35, about 1/3 as active as CQ. PQ and OH-PQ had molar ratios of 0.62 and 0.58, about twice as active as CQ. The molar ratios of **4**, **5**, PQ and OH-PQ were each significantly different from that of CQ. (see table 2).

According to literature reports, many 4-amino-7-chloroquinolines are active in the BHIA cell-free system, with drug:haematin molar ratios of 4-5, but only a small selection are active antiplasmodials. The probable reason, bearing in mind the digestive vacuole target in intraerythrocytic malaria parasites [15], is that a significant VAR is essential [16,17,20,21] for high antiplasmodial activity. For an optimal concentration within the vacuole, the 4-aminoquinoline drug structure requires at least 2 basic nitrogen atoms with suitable pK_a values [16, 34]. After the initial concentration step from external pH 7.4 to vacuolar pH 4.8, (143,482 times for CQ) (see tables 2, 3A and 3B), specific haematin / CQ binding [13] takes place with an association constant (measured in 40% DMSO) of 5.52 (dissociation constant 3μM) [57], which prevents detoxication of haematin to insoluble crystals of the haematin head to tail cyclic dimer or β-haematin[19]. The careful study of Parapini *et al*. using BHIA [58] indicates a CQ IC_{50} value of 1.39-1.65 haematin molar equivalents, similar to the value of 1.3 we have observed here, while Egan [57] reports a 1:1 stoichiometry in 40% DMSO. The PQ and OH-PQ molar concentration equivalent to haematin is half that of CQ, suggesting that each of the 4-amino-7-chloroquinoline moieties in these bis-quinolinyl drugs binds to one iron porphyrin molecule. This probability is supported by molecular modelling of the minimum-energy conformation of PQ and OH-PQ which shows that the quinoline rings are well separated and relatively unhindered (above).

3.4 DRUG TESTS IN VITRO AGAINST *P. FALCIPARUM*: (Table 2)

PQ (**2**) was as potent as CQ (**1**) against the CQ-sensitive T996 parasite, but in the CQ-resistant K1 parasite the IC_{50} was only 2.46 times higher, that is the "Resistance Index" (RI) was 2.46 as opposed to 14.1 for CQ. The potency of OH-PQ (**3**) was slightly lower in both parasites and RI was 1.5. However, both half-PQ compounds, **4** and **5**, had very low potency in CQ-S, with a potency value against CQ-R measurable only for **5**. RI was however 2.03. (see table 2). IC_{50} of **4** for K1 was not reached at the drug concentrations tested.

4. Discussion:

4.1 Molecular modelling:

Possible pharmacophore for haematin binding.

Support was given by the molecular modelling study to conclusions from physicochemical observations. In addition, modelling the minimal energy structures in **1**-**5** (figs. 3-7) revealed a common relatively unhindered planar area consisting of the 4-amino-7-chloroquinoline moiety which, in the case of CQ included the asymmetric –CH (with its methyl carbon) attached to N-2, and in **PQ**, **OH-PQ**, **4** and **5** one of the methylene carbons attached to the piperazine N-1′ in an equivalent position. In PQ and OH-PQ the second 4-amino-7-chloroquinoline moiety is also in a suitable position to bind to haematin and presents an essentially identical picture. (Figs.3-7). A disturbing diversity of structures for the 4-aminoquinoline/haematin complex has been proposed, most of which rely on analysis of interaction with the oxo-dimer form, predominant in neutral and aqueous solution [eg

72,73]. More consistent and satisfying results may be achieved by considering interaction with monomeric haematin as seen under acidic conditions [74,57]. Egan and colleagues recently reported [23] the haematin dimer (β-haematin) can be formed in association with the long-chain neutral lipid n-octanol under acid conditions. The lipophilic pre-dimer structure proposed for haematin (ferriprotoporphyrin IX $-H_2O$ with undissociated propionic acid groups) [23] appears suitable for ring-ring interaction with a 4-amino-7-chloroquinoline pharmacophore, in the lipid or in the aqueous phase before transfer to the lipid, which would prevent the dimerization reaction.

4.2 Drug activity and physicochemical features:

4.21 A high VAR in addition to a suitable BHIA molar ratio is important for activity against CQ-S parasites.

Our observations on compounds **4** and **5** in comparison with CQ, PQ and OH-PQ support the detailed studies of Egan and colleagues on selected 4-amino-7-chloroquinolines [20,21], confirming that although binding to haematin and inhibition of its detoxication to β-haematin is an essential requirement for antimalarial activity of 4-amino-7-chloroquinolines against CQ-S parasites, there is a requirement for concentration of these agents to a suitable level within the acidic vacuole content before antiplasmodial activity can be manifested. While **4** and **5** are complementary parts of the bisquinoline structure, showing the direct linking of the rigid piperazine ring through N-1′ to C-4 of the quinoline, different $\log D_{(pH4.8)}$ values due to the presence of 2 rather than 4 protonatable N atoms per molecule mean that VAR values for **4** and **5** are low, at 1,287 and 1,966 compared with 104,378 and 19,874 for PQ and OH-PQ. (table 2). Table 3B shows a comparison of VAR

and LAR in the drugs already studied with other 4-aminoquinolines, active or not in CQ-R, where the pK_a and logP values are available in the literature [eg 67] and can be used to calculate LogDs. Desethylchloroquine (DECQ) and hydroxychloroquine (HCQ) have similar VAR values to CQ, and similar high activity in CQ-S. Their LAR values are also lower than CQ, and they are even less effective in CQ-R. In CQ-S, log IC50 is inversely proportional to logVAR. In a graphical analysis, although there was a linear relationship between the log of IC_{50} in CQ-S parasites (y) and log of VAR (x) for CQ, PQ, OH-PQ, **4** and **5** ($y = -1.0909x + 6.702$) which explained 94% of the variation ($P<0.01$) there was a shortage of data for the middle of the line [not shown]. When values for the other 4-amino-7-chloroquinoline drugs [67] were included (fig. 8), a linear relationship was retained, ($y = -1.0945x + 6.6921$) explaining 84% of variation ($P < 0.001$), although the centre of the curve was still short of data. The inverse relationship may be better described by a 2nd order polynomial equation for a concave curve which explains 94% of the variation (fig. 8).

Insert fig. 8.

While VAR is an important factor for activity in CQ-S, there appears no clear evidence that LAR plays any role, although LAR depends on VAR (see section 2, Material and Methods).

4.22 4-aminoquinolines with low resistance indices (RI) have higher LAR values, enabling the uncharged base to accumulate rapidly to high concentration in lipid.

Among 4-aminoquinolines readily inhibiting BIHA, those with a high VAR and a high LAR are likely to be effective in CQ-resistant parasites (Table 3B). DAQ and AQ have lower VAR values compared to CQ, and equal or better potency compared with CQ in CQ-S. Their LAR values are higher, and they are effective in CQ-R. PQ and OH-PQ have lower VAR values than CQ, and are similar in potency to CQ against CQ-S. However their LAR values are much higher than CQ, and they are effective in CQ-R. Compounds **4** and **5** have very low VAR values, and also are ~ 3 times less active in BHIA than CQ. They are very weakly active against *P. falciparum*. However, they have relatively high LAR values and **5** had a R.I. of only 2.03.

There is clear evidence of the importance of higher lipid distribution values (logD) in those 4-amino, 7-chloro quinolines active in CQ-R [29-32]. Furthermore, for a series of CQ and 7 other 4-amino-7-chloroquinoline drugs, the relationship between log R.I. and log LAR is a second order polynomial curve (Fig.9), which, as the ordinate decreases regularly, can be separated into a straight line (fig.10) composed of 6 points explaining 85% of variation ($y = -0.4767x + 1.6841$) ($P<0.01$), combined with a horizontal line parallel to the abscissa as the log R.I. approaches zero.

Insert figs. 9 and 10

4.3

Success of 4-amino-7-chloroquinoline drugs with high LAR values could be explained by enhancement of β-haematin inhibition in vacuolar lipid where crystals of the detoxified

product are produced [22], and/or may reflect their ability to block drug efflux by hydrophobic interaction with PfCRT [30,31]. It is intriguing to note that in CQ-S parasites, accumulation of charged drug in the vacuolar water (VAR) appears more important for activity, suggesting that interaction of these drugs with components of the modified hydrophobic channel in CQ-R PfCRT, which impedes efflux, may be the more important feature [30, 31, 32].

4.4 Equilibrium modelling study: Towards a mathematical description of the digestive vacuole in CQ-S and CQ-R parasites.

4.41 CQ-sensitivity:

We have modelled at equilibrium the aqueous and membrane distribution of CQ and other drugs throughout the parasitized erythrocyte to determine their expected accumulation ratios between plasma (pH7.4) and vacuolar water (vacuolar accumulation ratio: VAR) and thence into vacuolar lipid (LAR)[52]. This confirms that while CQ2H+ concentration in vacuolar water (VAR) increases as vacuolar pH decreases, the concentration of uncharged CQ in vacuolar lipid membrane and lipid droplets remains constant because the proportion of drug from the vacuolar water partitioned into lipid (logD) decreases. Providing vacuolar pH is maintained at the steady low value (required for haemoglobin digestion) by inward proton pumping, unionized drug (CQ) passes through the vacuolar membrane and becomes concentrated as the ionized species (CQ2H+) in the vacuolar aqueous phase, to a steady high level (VAR) as a consequence of the pH gradient from the plasma. From the highly concentrated drug in the vacuolar aqueous phase, unionized CQ is distributed according to the logD at vacuolar pH into the vacuolar lipid and reaches the same concentration as in

the erythrocyte membrane (LAR). The rate of distribution to lipid will depend on the concentration of CQ2H+ in the vacuolar water and the logD at vacuolar pH. This describes the situation in CQ-S parasites.

4.42 CQ-resistance:

In CQ-R parasites, CQ2H+ efflux through CQ-R PfCRT, will lower the concentration of CQ2H+ in vacuolar water without materially changing its pH. This will also reduce the transfer of CQ to vacuolar lipid (LAR). Assuming no material change in vacuolar pH, a drug efflux process through CQ-R PfCRT which effectively halves the CQ2H+ concentration in vacuolar water (VAR) to 0.5*VAR ($VAR_{(CQR)}$) will, since $logD_{(pH4.8)}$ remains the same, reduce LAR by the same factor (see eqn.(5)). Since estimates of at least 5 and at most 50 (depending on the experimental protocol) are reported for the [^{3}H]CQ equilibrium concentration ratio between CQ-S and CQ-R parasites (27,26), then $VAR_{(CQ-R)}$ of 28,696 to 2,870, and $LAR_{(CQ-R)}$ of 1.65 to 0.165 can be expected in CQ-R parasites. (for sketch, at CQ medium concentration 10nM, see Fig. 11). However, the same calculation, applied to PQ will give an appreciably higher $LAR_{(CQ-R)}$ range of 194,698 to 19,470.

Insert fig. 11.

4.43 Channel gating effects:

Bray *et al* [27] have recently observed in de-energized conditions (in the absence of glucose or with 10µM of the protonophore FCCP) increased steady-state uptake of [^{3}H]CQ by CQ-R *P. falciparum* similar to that observed in CQ-S under the same conditions,

suggesting that the CQ-R PfCRT protein is a channel gated by membrane potential (proton gradient in this case). The CQ-R channel would be open and allow CQ2H+ efflux only in the presence of a trans-membrane pH difference, and be more or less closed when this is abolished or reduced. The effect of de-energization leading to a one unit rise in the normal vacuolar pH (according to the Nernst equation [75]) would be a reduction of 60mV in vacuolar membrane potential which would partially gate the CQ-R passive efflux channel, increasing CQ-uptake and sensitivity.

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Legends for Figures:

Figure 1.

Structures of compounds used in the study. Chloroquine, showing asymmetric carbon atom (**1**), Piperaquine (**2**), Hydroxypiperaquine (**3**), Cpd. **4**, and cpd. **5**.

Figure 2.

Graph showing the inverse relationship between vacuolar pH (x) and logVAR (y) for CQ (chloroquine), PQ (piperaquine), OH-PQ (hydroxypiperaquine), Cpd. **4** and Cpd. **5**.

2nd order polynomial equations for the graphs, as follows:

CQ: $y = 0.0058x^2 - 2.0611x + 14.917$ ($R^2 = 1$)

PQ: $y = 0.8019x^2 - 11.706x + 42.75$ ($R^2 = 0.9997$)

OHPQ: $y = 0.67x^2 - 9.8233x + 36.016$ ($R^2 = 0.9999$)

4: $y = 0.2251x^2 - 3.9346x + 16.801$ ($R^2 = 0.9999$)

5: $y = 0.2197x^2 - 3.9387x + 17.127$ ($R^2 = 0.9999$)

Figures 3-7. Molecular models: (colours: C=light blue (unlabelled), H= grey (unlabelled), N= dark blue (labelled N1, N2 and N3), Cl=green (labelled Cl), O=red (labelled O).

Figure 3. Calculated models of *S*- and *R*- **CQ** base using AM1. In each enantiomer the aromatic heterocyclic area likely to interact with haematin is indicated.

Figure 4. Calculated model of **PQ** base using AM1. The 2 aromatic heterocyclic areas likely to interact with haematin molecules are indicated.

Figure 5. Calculated model of **OH-PQ** base using AM1. The 2 aromatic heterocyclic areas likely to interact with haematin molecules are indicated.

Figure 6. Calculated model of Cpd. **4**. base using AM1. The aromatic heterocyclic area likely to interact with haematin is indicated.

Figure 7. Calculated model of Cpd. **5**. base using AM1. The aromatic heterocyclic area likely to interact with haematin is indicated.

Figure 8. (graph) Chloroquine-sensitive *Plasmodium falciparum*, response to CQ and 8 other 4-amino-7-chloroquinoline drugs. Log IC_{50} (nM) (y) is plotted against log vacuolar accumulation ratio (VAR), (x). Log IC50 is proportional to logVAR. This relationship is best described by a 2nd order polynomial equation for a concave curve which explains 94% of the variation. Although a significant linear relationship is present, which explains 84% of variation ($P<0.001$), there is a shortage of data in the central area of the line which casts doubt on the simplified linear interpretation.
(DECQ: desethylchloroquine. DAQ: desethylamodiaquine. AQ: amodiaquine. HCQ: hydroxychloroquine. The logD values which were used to calculate VAR were calculated from observed pKa and logP values in this study and in ref [67].

Figure 9. (graph) Log resistance index (IC_{50} nM of drug against CQ-R parasite / IC_{50} drug against CQ-S parasite) (y) for 8 drugs is plotted against Log LAR (x). This shows a 2nd order polynomial relationship, which is probably composed of two straight lines, the first one is shown in figure 10, and the last one approximates to a horizontal line. LAR values were calculated from pKa and logP values in this study and in ref [67].
(DECQ: desethylchloroquine. DAQ: desethylamodiaquine. AQ: amodiaquine. HCQ: hydroxychloroquine).

Figure 10. (graph) Log resistance index (IC_{50} drug against CQ-R parasite / IC_{50} drug against CQ-S parasite) (y) of a series of six 4-amino-7-chloroquinolines with different activities in CQ-resistance, omitting bis compounds PQ and OH-PQ, is plotted against log LAR (x). This shows the linear relationship ($P<0.01$), describing the first part of the polynomial graph in Fig. 9.

(DECQ: desethylchloroquine. DAQ: desethylamodiaquine. AQ: amodiaquine. HCQ: hydroxychloroquine. Compound **5**)

Figure 11. (Sketch) TOP picture shows CQ-sensitive parasite at equilibrium. LOWER picture shows snapshot of CQ-resistant parasite, with the lowest projected reduction (1/5) in overall uptake. CQ (concentration held at 10nM) in medium (pH7.4) enters the parasite cytoplasm from the rbc cytoplasm and becomes concentrated as CQ2H+ in the vacuolar water. In the lower picture, after the loss of +charge of K76, CQ2H+ is shown leaking through the CQ-resistant PfCRT) into the cytoplasm to be equilibrated through the rbc with the external medium.

AM1 properties	*R&S* CQ	*R&S* CQ 1H+	*R&S* CQ 2H+	PQ	PQ2H+ (aromatic N)	PQ4H+	OHPQ	OHPQ2H+ (aromatic N)	OHPQ4H+	4	4 2H+	5	5 2H+
Total energy	-84345	-84513	-84650	-141613	-141941	-142025	-149008	-149336	-149419	-69329	-69605	-72921	-73198
Heat of formation :*H*	23.65	170.2	347.9	126.7	428.8	974.0	81.25	383.1	929.4	62.71	416.3	57.15	410.4
quinoline N-1 atoms (Å)	NA	NA	NA	17.65	18.29	18.30	17.53	18.23	18.36	NA	NA	NA	NA
Bridging side-chain N atoms (Å)	NA	NA	NA	4.985	4.965	5.073	4.932	4.944	5.073	NA	NA	NA	NA
quinoline N-1 atoms and side-chain N atoms (Å)	7.893	7.999	8.991	6.526 6.520	6.827 6.819	6.830 6.837	6.520 6.512	6.795 6.835	6.828 6.835	6.514	6.852	6.466	6.816
quinoline N-1 atoms and 4-amino (Å)	4.255	4.258	4.196	4.280 4.280	4.202 4.200	4.231 4.231	4.280 4.281	4.203 4.198	4.231 4.231	4.280	4.221	4.280	4.219
O to N (Å)	NA	NA	NA	NA	NA	NA	2.998 3.136	2.980 3.114	2.850 3.079	NA	NA	NA	NA

Table 1. AM1 geometrical optimization of compounds studied (energy in kcal.mol^{-1}, distances in Å). The compounds are examined unionized (bases) and protonated. R & S CQ = rectus and sinister enantiomers of chloroquine base. PQ = piperaquine base. OHPQ = hydroxypiperaquine base. **4** = compound 4 as base. **5** = compound 5 as base

Cpd	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}	logP	$logD_{7.4}$	$logD_{5.2}$	$logD_{4.8}$	$VAR_{pH4.8}$	LAR	IC_{50}nM CQ-S (±SE)	IC_{50}nM CQ-R (±SE)	IC_{50} Res/ Sens	BIHA IC_{50} ±SD (n)
1 **CQ**	**10.18** (c)	**8.38** (c)	-	-	**4.72** (c) 4.69(d)	0.96 (c) **0.92**(a)	-3.44 (c) **-3.44**(a)	**- 4.24** (a)	**143482** (f) 143482(e)	9.1 (g) **8.25** (h)	**23.4 ± 0.21**	**329± 9.00**	**14.1**	**1.30 ±0.30 (8)**
2 **PQ**	**6.88**	**6.24**	**5.72**	**5.39**	**6.11** 6.16(d)	5.99 **5.99**(b)	2.41 **2.41**(b)	**0.97**(b)	**104378** (f) 104378(e)	977237 (g) **973492** (h)	**19.9 ± 2.01**	**49.0 ±1.79**	**2.46**	**0.62 ±0.12 (6)**
3 **OH-PQ**	**6.60**	**6.41**	**5.39**	**4.83**	**5.67** 6.38(d)	5.15 **5.60**(b)	2.11 **2.54**(b)	**1.30**(b)	**19874**(f) 19874(e)	141254 (g) **398118** (h)	**41.6 ± 16.6**	**62.4± 6.70**	**1.50**	**0.58 ±0.21 (6)**
4	**7.63**	**5.42**	-	-	**3.02** 2.92(d)	2.26 **2.59**(a)	0.10 **0.17**(a)	**- 0.52**(a)	**1287** (f) 1287(e)	182 (g) **386** (h)	**2485 ± 371**	**>5000**	**NA**	**3.80 ±1.20 (6)**
5	**7.92**	**5.54**	-	-	**3.48** 3.46(d)	2.35 **2.84**(a)	0.21 **0.26**(a)	**- 0.45**(a)	**1966** (f) 1966(e)	224 (g) **693** (h)	**2213 ± 330**	**4486 ± 670**	**2.03**	**3.35 ±0.94 (8)**

Table 2. Physicochemical and other parameters for the compounds studied.

a =calc from eqn.2: b = calc from eqn.3. : c = from ref [67]: d = from ACD programme: e =calc from equations in refs [34,48]: f= eqn. 4: antilog (*calc* $LogD_{7.4}$-*calc* $LogD_{4.8}$). g = eqn. 5: antilog *obs* $LogD_{7.4}$. h = eqn. 5: antilog *calc* $LogD_{7.4}$. CQ-S : T996, CQ-sensitive clone. CQ-R: K1, CQ-resistant strain. Results from 2 experiments in triplicate**.** BIHA = β-haematin inhibition assay. NA= not applicable.

Table 3A: CQ concentration in plasma (nM) (assumed constant)	vacuolar pH (pH V)	[CQ] in vacuolar water (nM) (VAR) (Eqn. 4)	[CQ] in lipid (nM) LAR (Eqn. 5)	$logD_{(pH\ V)}$
1.0	4.8	143482	8.25	-4.24
1.0	5.0	57130	8.25	-3.84
1.0	5.2	22749	8.25	-3.44
1.0	5.4	9060	8.25	-3.04
1.0	5.6	3609	8.25	-2.64
1.0	5.8	1438	8.25	-2.24
1.0	6.0	573	8.25	-1.84
1.0	7.4	1	8.25	0.917

Table 3A. Vacuolar accumulation ratio (VAR) and lipid accumulation ratio (LAR) values for CQ at vacuolar pH from 4.8 to 7.4. The last column shows the calculated logD values determining the VAR and LAR for each pH. Note, no change in LAR with pH (assuming the parasite is CQ-sensitive).

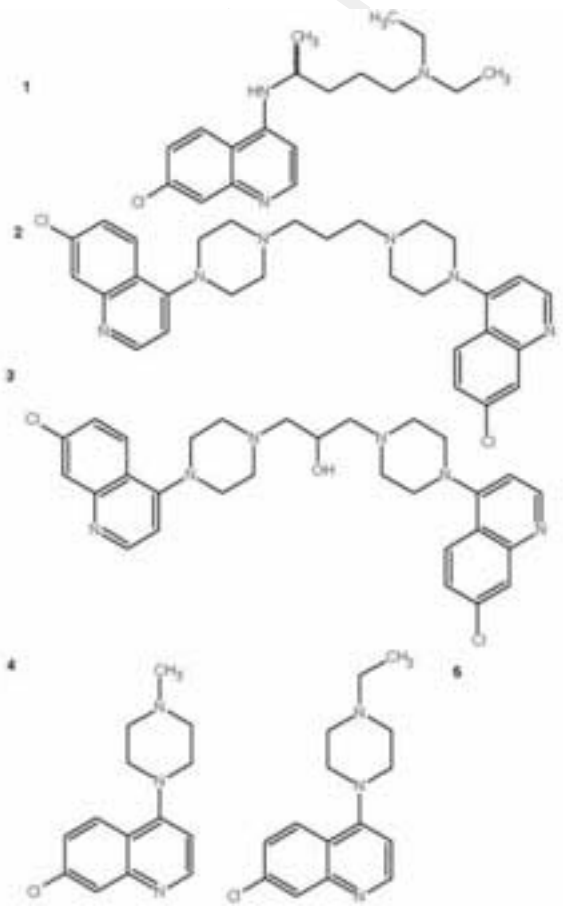
Table 3B: DRUG	vacuolar pH	[drug] in vacuolar water (nM) (VAR) (Eqn. 4)	[drug] in vacuolar lipid (nM) (LAR) (Eqn. 5)	logD (pH4.8)	logD (pH7.4)	Resistance index
DECQ	4.8	144114	0.56	-5.41	-0.251	36.66
HCQ	4.8	139607	4.46	-4.50	0.650	79.12
CQ	4.8	143482	8.25	-4.24	0.917	14.10
DAQ	4.8	89366	40.8	-3.34	1.61	5.39
AQ	4.8	47410	666	-1.85	2.82	1.98
PQ	4.8	104378	973492	0.97	5.99	2.46
OHPQ	4.8	19874	398118	1.30	5.60	1.50
Cpd 4	4.8	1287	386	-0.524	2.59	NA
Cpd 5	4.8	1966	693	-0.453	2.84	2.03

Table 3B. Comparison of VAR and LAR values at vacuolar pH 4.8 for a series of nine 4-amino, 7-chloroquinoline drugs. Calculated log D values at pH 4.8 and 7.4 are included together with the resistance index (RI) (IC_{50} nM of drug against CQ-R parasite / IC_{50} nM drug against CQ-S parasite) for each drug.

Drug and amino N		pH	pKa	% 1H+	% 2H+	% 3H+	% 4H+	pH	pKa	% 1H+	% 2H+	% 3H+	% 4H+
CQ	N3	7.4	10.18	99.8				4.8	10.18	100.0			
CQ	N1	7.4	8.38		90.5			4.8	8.38		100.0		
PQ	N1	7.4	6.88	23.2				4.8	6.88	99.2			
PQ	N1	7.4	6.24		6.5			4.8	6.24		96.5		
PQ	N3	7.4	5.72			2.1		4.8	5.72			89.3	
PQ	N3	7.4	5.39				1.0	4.8	5.39				79.6
OH-PQ	N1	7.4	6.6	13.7				4.8	6.6	98.4			
OH-PQ	N1	7.4	6.41		9.3			4.8	6.41		97.6		
OH-PQ	N3	7.4	5.39			1.0		4.8	5.39			79.6	
OH-PQ	N3	7.4	4.83				0.3	4.8	4.83				51.7
4	N1	7.4	7.63	62.9				4.8	7.63	99.9			
4	N3	7.4	5.42		1.0			4.8	5.42		80.7		
5	N1	7.4	7.92	76.8				4.8	7.92	99.9			
5	N3	7.4	5.54		1.4			4.8	5.54		84.6		

Table 4: The percentage of molecules of **CQ**, **PQ**, **OH-PQ**, **4** and **5**, singly or multiply protonated (H+) at pH 7.4 and 4.8, calculated from the pK_a values by Albert's equation [56].

Fig. 1



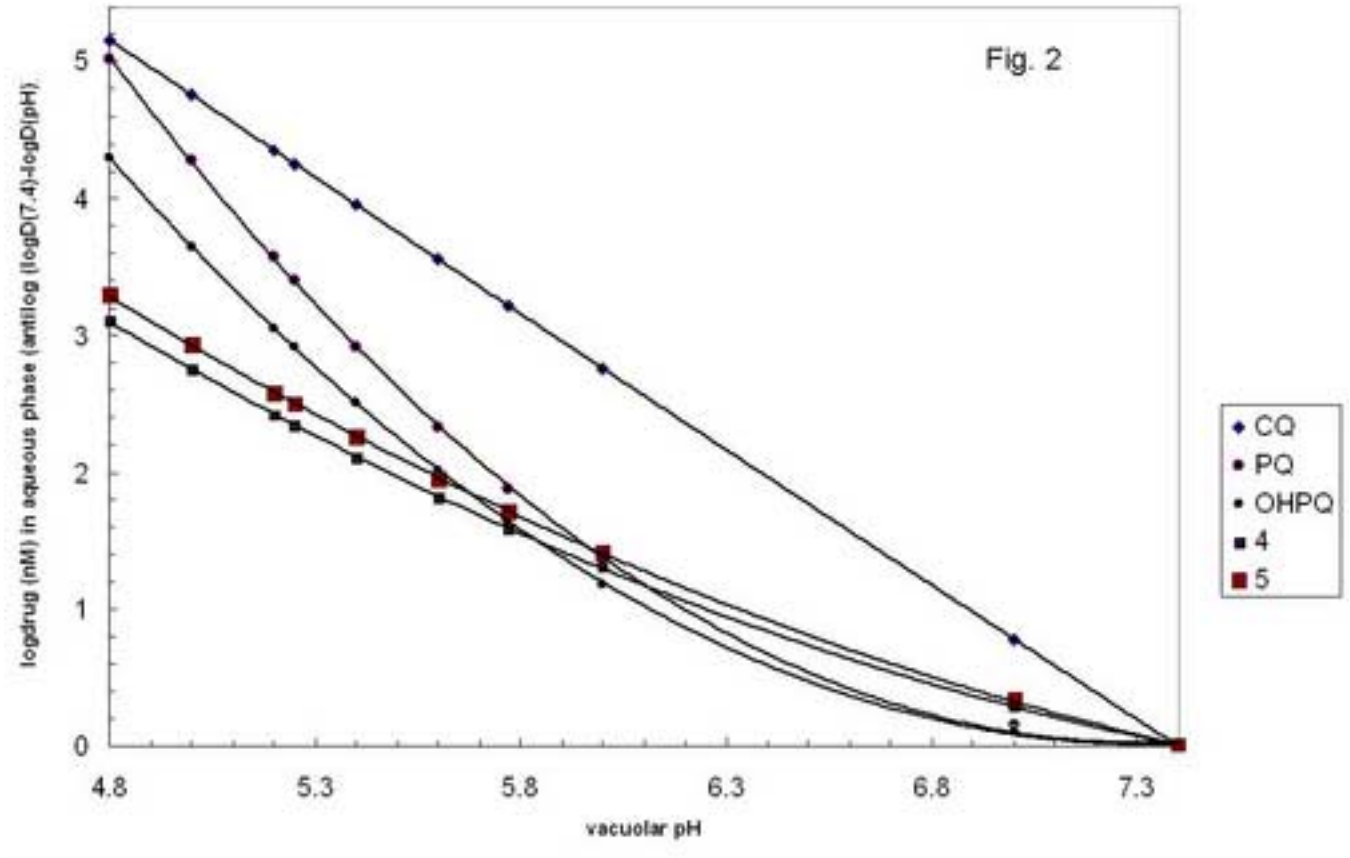


Fig. 3

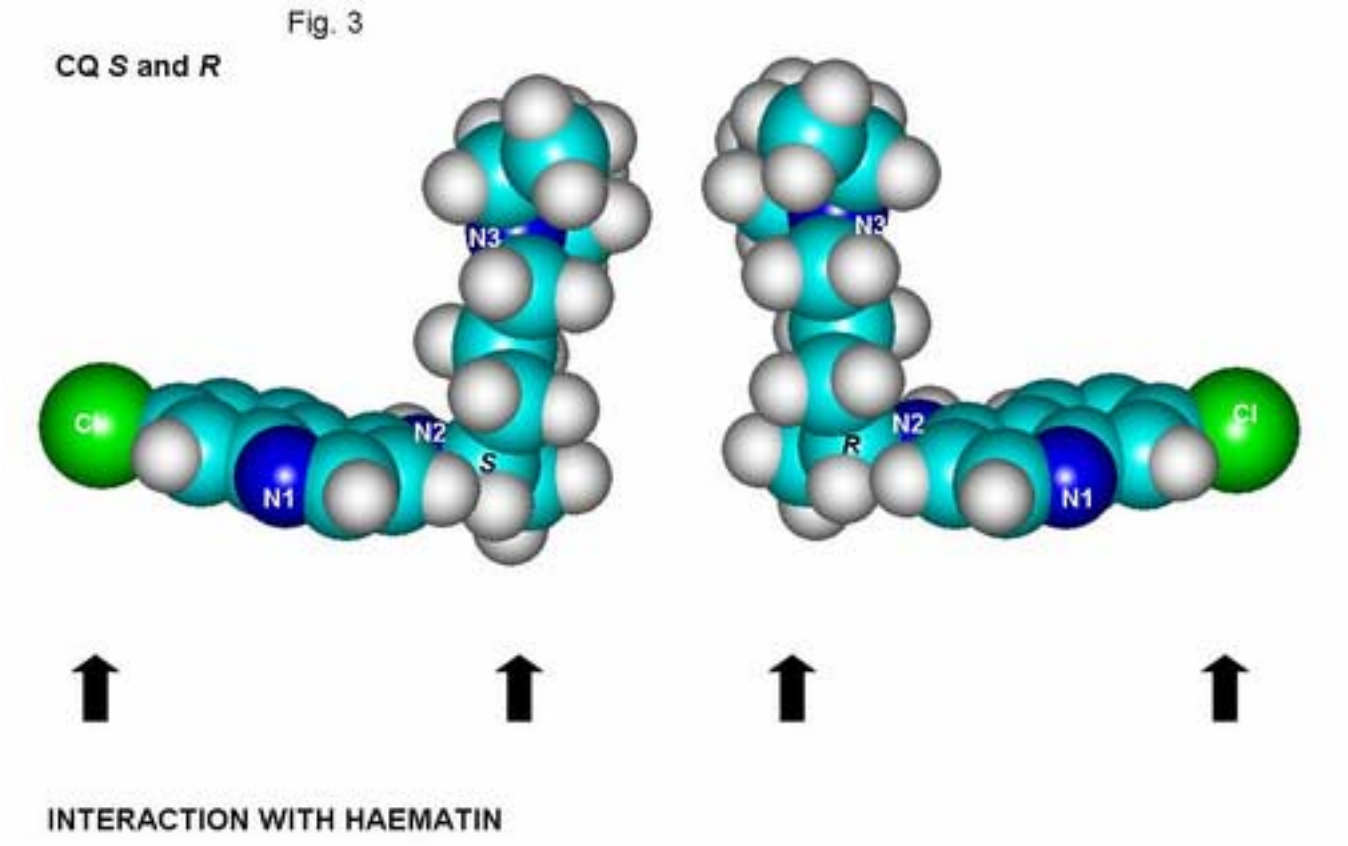


Fig. 4

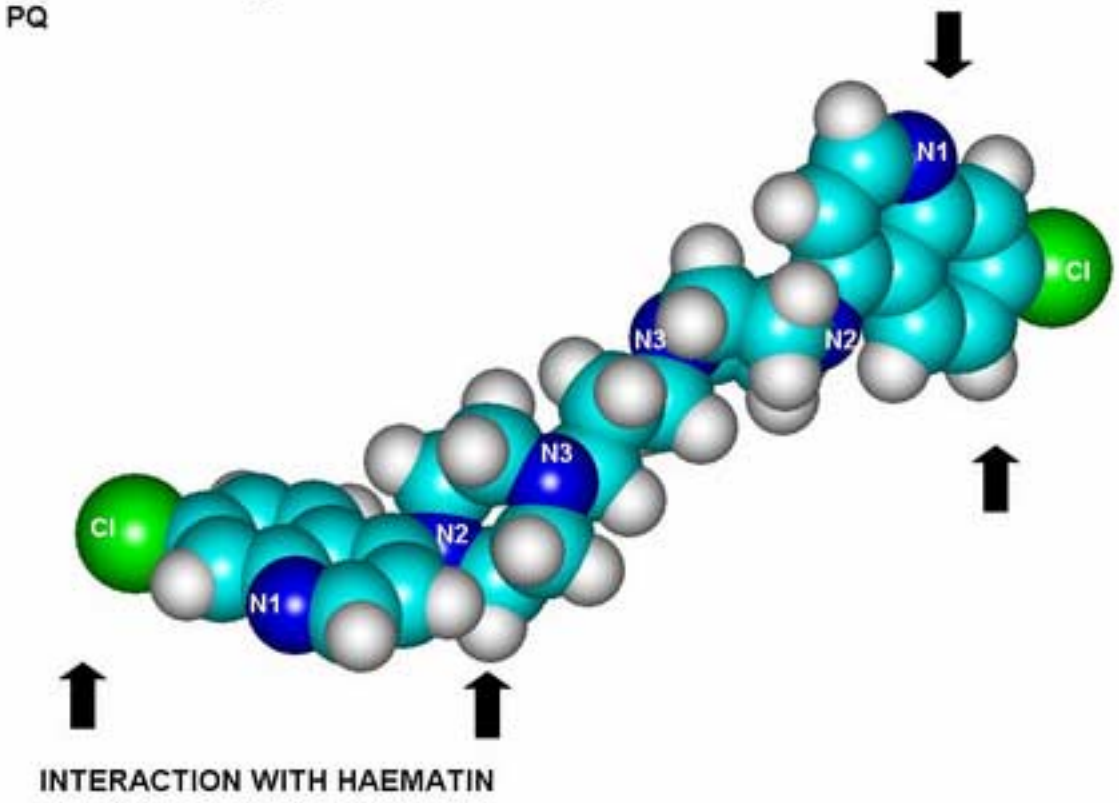


Fig. 5

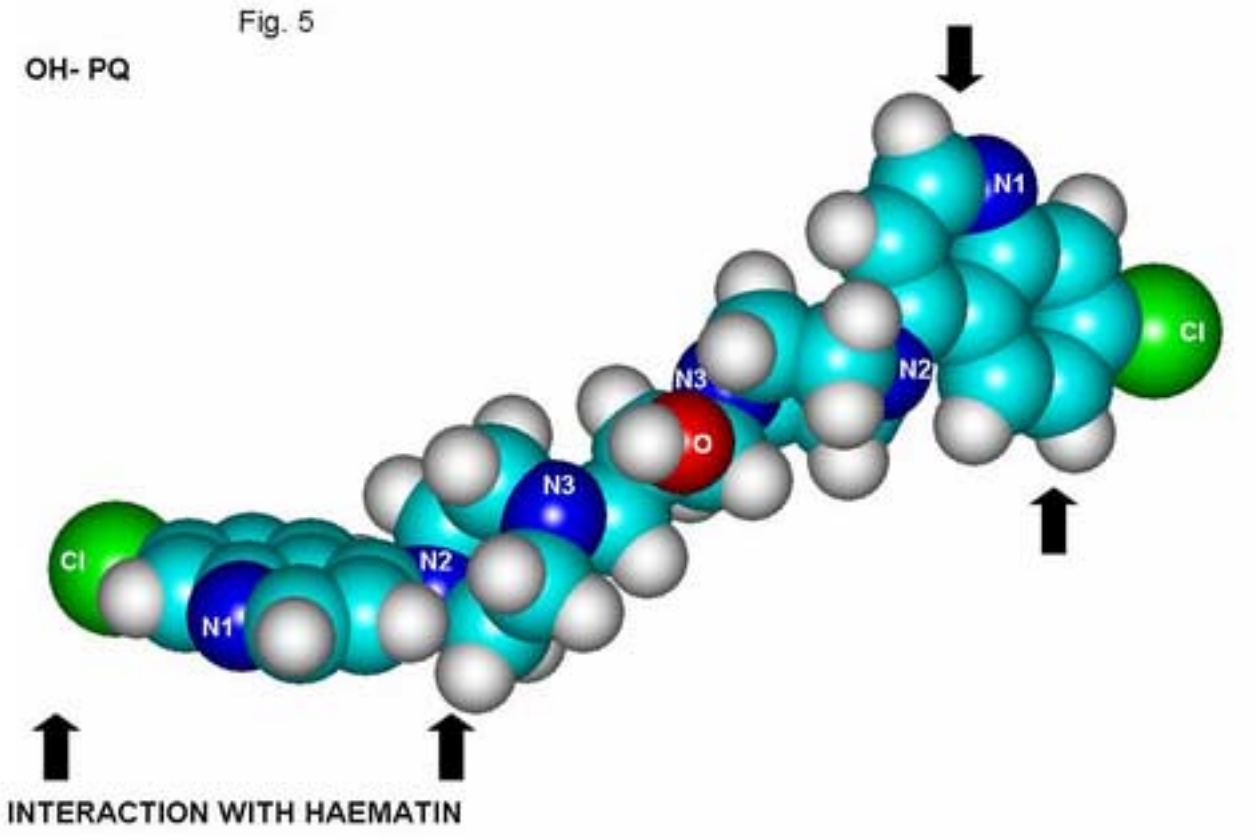


Fig. 6

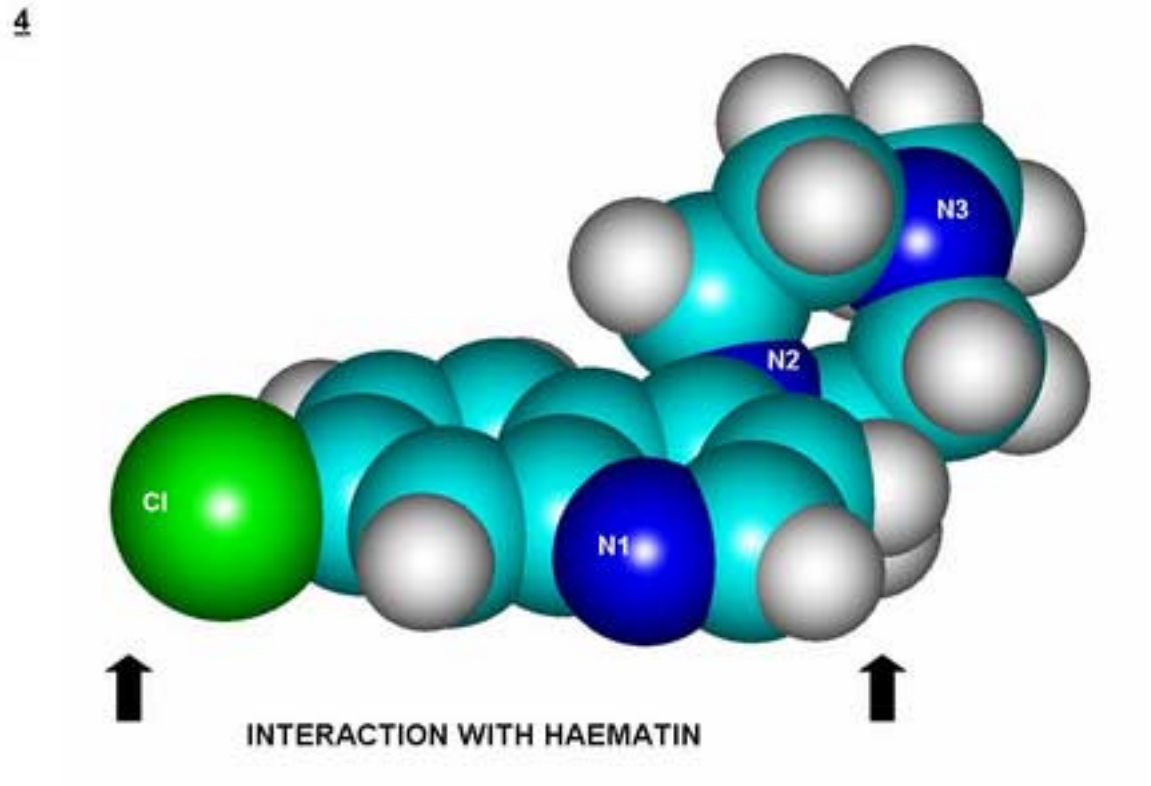
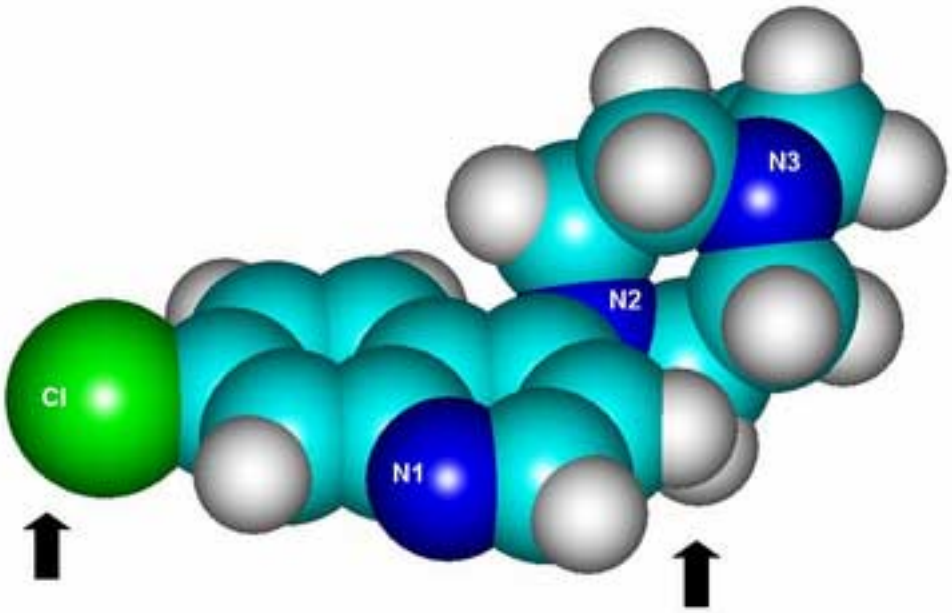


Fig. 7

5



INTERACTION WITH HAEMATIN

Fig. 8

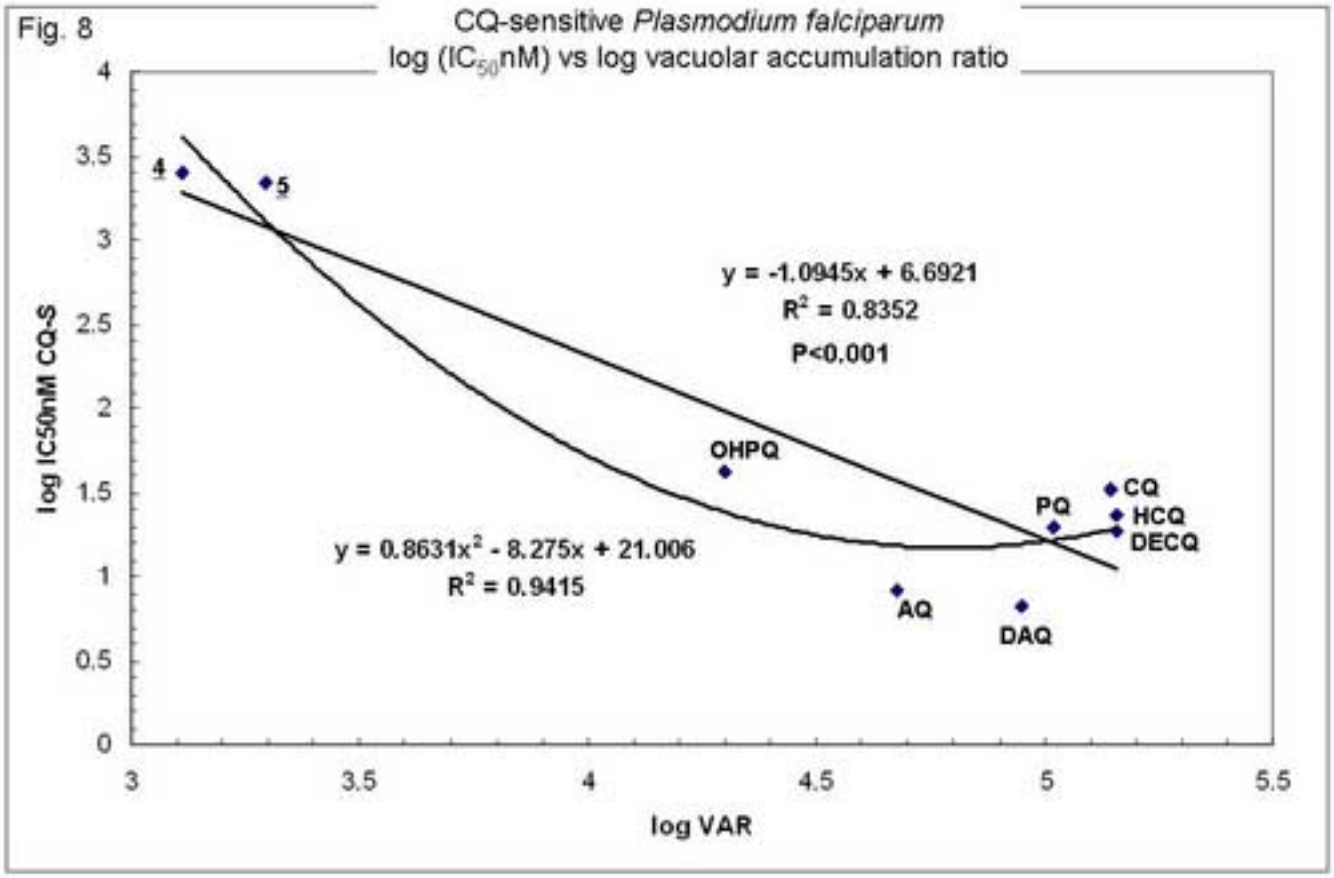


Fig. 9

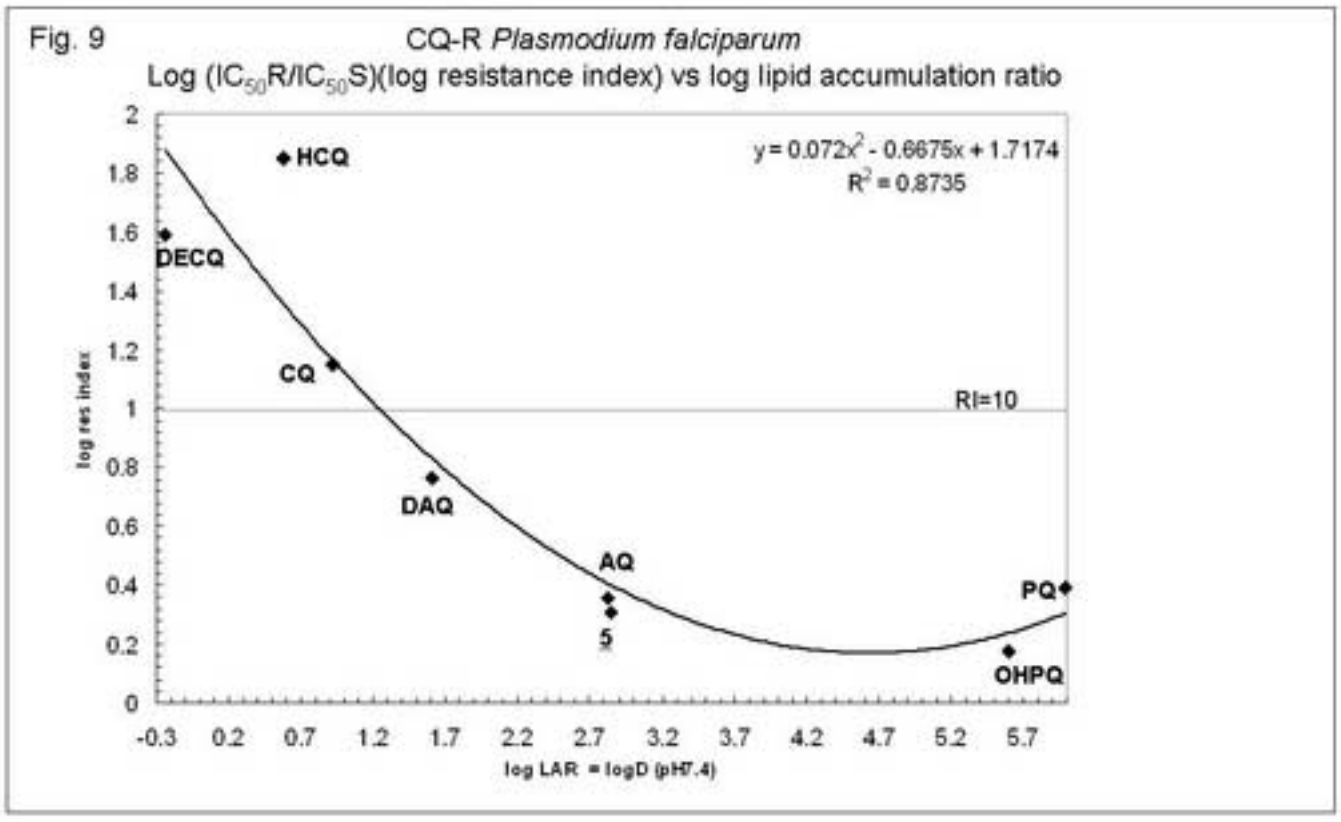
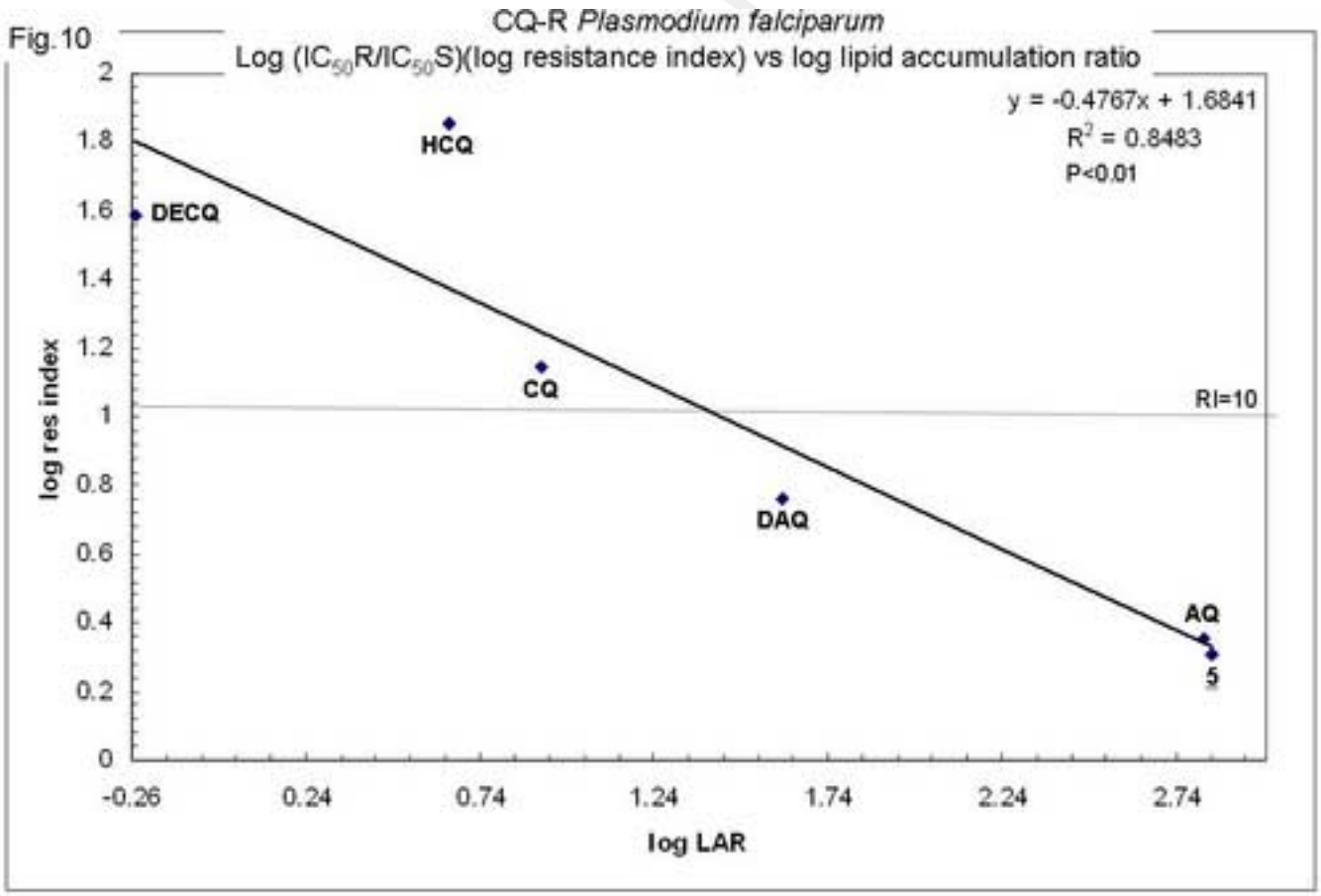


Fig.10



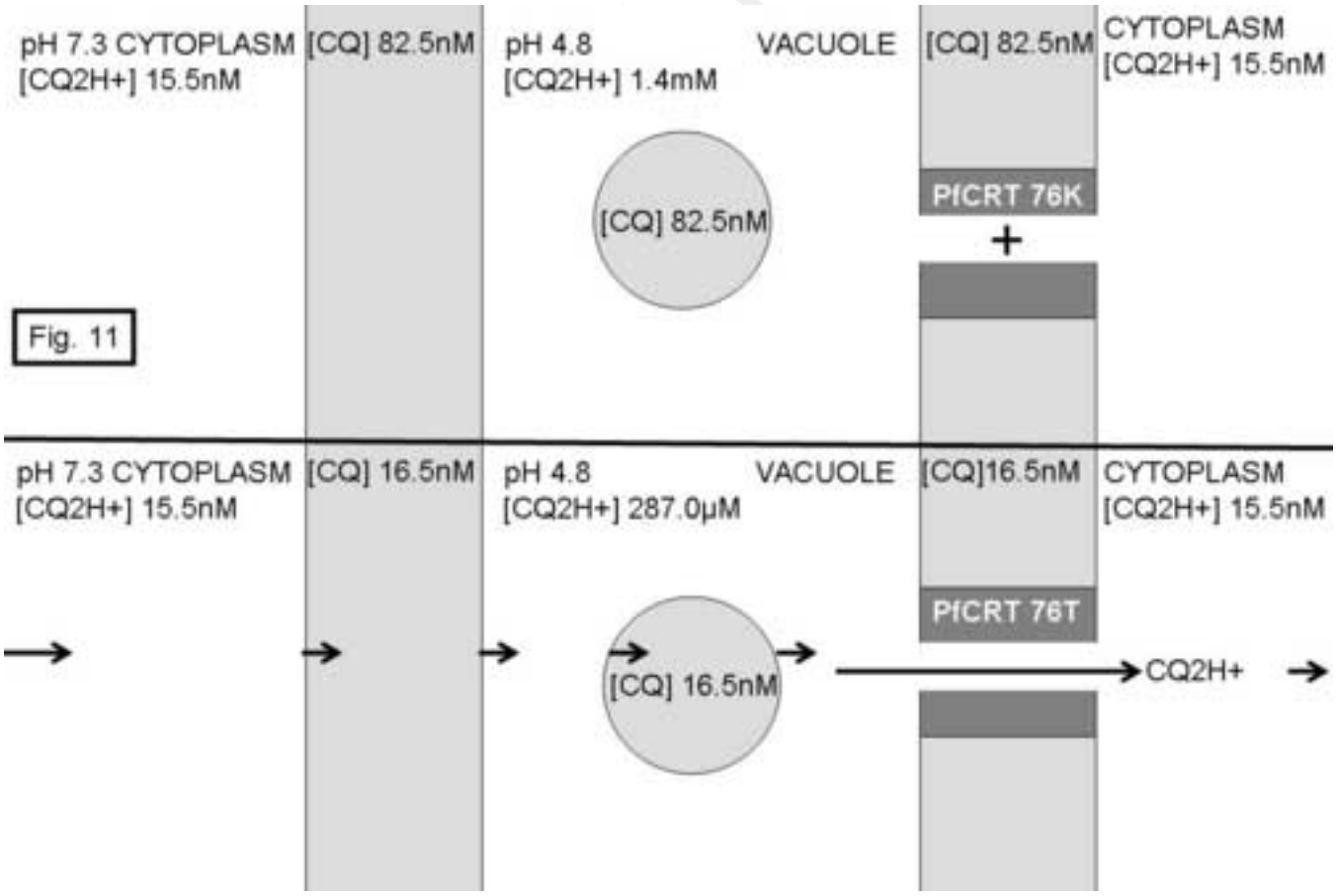


Fig. 11