Understanding resistance to antimalarial 4-aminoquinolines, cinchona alkaloids and the highly hydrophobic arylaminoalcohols

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With the exception of Central America and the Caribbean, falciparum malaria resistant to the 4-aminoquinoline chloroquine is now found throughout the tropics. Using allelic exchange, mutation K76T in lysosomal membrane protein PfCRT of the intraerythrocytic parasite has been proved to be responsible. An additional residue change (A220S) appears necessary but not sufficient. The effectiveness of more hydrophobic 4-aminoquinolines like desethylamodiaquine in chloroquine resistance in Africa is possibly associated with the more hydrophobic PfCRT 72CVIET76 haplotype, in contrast with SVMNT and CVMNT. Resistance to 4-aminoquinolines in the presence of modified PfCRT is enhanced by residue changes in another lysosomal membrane protein, PGH-1, a member of the multidrug-resistance associated 'ATP-binding cassette' (ABC) protein group. PGH-1 mediates resistance to arylaminoalcohols, like quinine and other cinchona alkaloids and to the highly hydrophobic arylaminoalcohols (HHAAs) such as mefloquine. In quinine resistance, PGH-1 residue-changes are similar to those seen in enhanced 4-aminoquinoline resistance. In contrast, increased copy number of wild-type PGH-1 is found in clinical resistance to the HHAA mefloquine. There is also evidence of some interaction between the arylaminoalcohols and PfCRT. In the case of quinine and its diastereomer quinidine, sterospecificity has been demonstrated. Although the main genetic determinants of resistance are now clear, there is some evidence for the involvement of other genes.

Keywords: Aminoquinolines, arylaminoalcohols, cinchona alkaloids, malaria parasite.

DURING the later 1940s and the 1950s, the 4-aminoquinoline chloroquine (CQ) proved invaluable worldwide as a safe and cheap suppressive prophylactic and as a therapeutic antimalarial[1]. Around the beginning of the sixties, resistance was detected in the malignant tertian parasite (*Plasmodium falciparum*) in SE Asia and S. America, regions of relatively low transmission[2]. Here, because of the high likelihood of infected persons being symptomatic and hence seeking treatment, the parasites had been under intense selective pressure[3]. In addition, the drug had been applied indiscriminately in cooking salt for prophylaxis[4], and often used as a presumptive treatment for all fevers. By the end of the seventies, CQ-resistant *P. falciparum* clones had spread to E. Africa from SE Asia[5], and crossed Africa in the course of about five years[6]. In Africa there is evidence of increased child mortality in CQ-treated malaria since the onset of resistance[7].

Selective toxicity in 4-aminoquinolines

Chloroquine in the digestive vacuole binds to free haematin and prevents its detoxication to haemozoin (Figure 1 a)

During intraerythrocytic growth, the developing schizont digests up to 70% of the rbc haemoglobin content[8] and

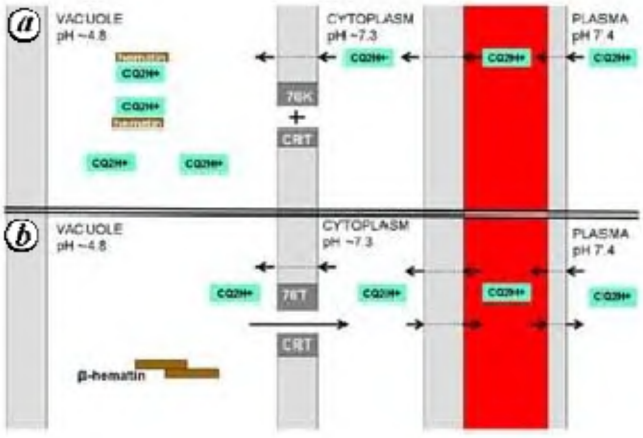

Figure 1 ***a***, ***b***. ***a***, Chloroquine-sensitive parasite at equilibrium. Weak base CQ (light green) (in plasma at pH 7.4) travels through the rbc membrane, rbc cytoplasm, parasite cytoplasm, and becomes concentrated into the parasite digestive vacuole under the pH gradient (7.4–4.8). Passage of uncharged drug (CQ) through membrane is shown as a dashed line. Unable to exit through the positively charged PfCRT channel, $CQ2H^+$ accumulates in the vacuole and binds to haematin (brown) from haemoglobin digestion, preventing dimerization to β-haematin and crystallization to haemozoin. Membranes are shown shaded grey. RBC cytoplasm is red. ***b***, Snapshot of CQ-resistant parasite. $CQ2H^+$ is shown leaking out through the modified CQ-resistant PfCRT channel into the cytoplasm to maintain overall equilibrium with the external medium, allowing haematin dimerization and crystallization to non-toxic haemozoin. Membranes are shown shaded grey. RBC cytoplasm is red.

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88% of the iron from the digested haemoglobin is found as haematin in haemozoin (insoluble malaria pigment) within the digestive vacuole (DV)[9]. The DV is a lysosome where the content is maintained at an acidic pH, facilitating haemoglobin digestion. Released haematin is detoxified to a cyclic dimer, β-haematin. These dimers crystallize to give haemozoin[10]. The transient availability of soluble haematin in the DV during this process[11] is regarded as the basis of CQ's selective toxicity[12]. CQ-treated parasites show morphological changes in the DV, indicating that it is the site of action of the drug[13]. The acidity acts as a site of concentration of the weakly basic 4-amino-7-chloroquinoline drug which then binds to haematin and prevents its detoxication[14–17]. Although the accumulation of protonated $CQ2H^+$ into vacuolar water[14] at ~ pH 4.8 (ref. 16) appears to be a requirement for antimalarial activity[18,19], recent studies indicate that the crystallization process from β-haematin dimers to haemozoin takes place in, or closely associated with, neutral lipid nanospheres in the aqueous content of the vacuole[20]. This is supported by the observation in cell-free systems that the rate of development of the insoluble haemozoin crystal, composed of β-haematin, is enhanced by presence in the acidic buffer of *n*-octanol or other neutral lipids[21]. The probable importance of intravacuolar lipid (a highly hydrophobic site) for formation of authentic haemozoin crystals highlights alternative possibilities related to hydrophobicity for the mode of action of drugs which target the haematin detoxication process[17]. Although the observation that [^{3}H]CQ is incorporated into β-haematin crystals in cell-free systems and into haemozoin in intact infected erythrocytes may mean that crystal growth could be prevented by drug bound to the growing crystal face[22], it may be merely incidental[10]. It is recognized that other structurally modified 4-aminoquinolines (hydroxychloroquine, amodiaquine, its metabolite desethylamodiaquine, piperaquine) apparently have a similar mode of action to CQ. They are capable of being similarly concentrated within the vacuolar water, they bind to haematin in cell-free systems and they inhibit its dimerization to β-haematin and the crystallization of haemozoin[17].

Resistance to chloroquine is related to reduced uptake of the drug, probably due to efflux through modified PfCRT, an intrinsic membrane protein of the digestive vacuole (Figure 1 b)

In CQ-R *P. falciparum* blood stages, the steady high concentration of CQ within the infected cell is diminished[23], probably through efflux[24], currently understood to occur across the membrane of the digestive vacuole[25], and so the drug does not achieve intravacuolar concentrations which inhibit haematin dimerization. Since changes in sequence (crucially K76T) of vacuolar membrane protein PfCRT (specified by gene *pfcrt* on chromosome 7) are convincingly linked to CQ-R in *P. falciparum*[26,27], where a polar, positively charged residue (lysine) is replaced by threonine which is neutral and hydrophobic, it is probable that efflux of polar, positively charged $CQ2H^+$ is mediated through the modified protein (see Figure 1 *b*). Other changes in PfCRT which also increase overall hydrophobicity of the protein, particularly of residues 72–76, are present in field CQ-R isolates. In contrast, a reduction of hydrophobicity in residue 220 is regularly seen where a change from alanine to serine is very strongly associated with CQ-R[26]. PfCRT, which bioinformatically has 10 transmembrane domains, and no nucleotide-binding sites, bears a structural resemblance to the drug-metabolite transporter proteins[28]. The organization of the sequence bears a resemblance to anion (e.g. Cl^-) channels which bioinformatically have 10–11 transmembrane domains, but on crystallography have several additional domains inside the membrane[29], and there is evidence of a role for chloride in its function[30].

More hydrophobic CQ analogues are effective in CQ-resistance

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[31]. This is believed to be due to their interaction with and retention in the hydrophobic lining of a CQ-R PfCRT channel[32–35,17]. Reversal of CQ-resistance *in vitro* by the channel blocker verapamil (see Figure 2 *a*), and activity of DAQ (see Figure 2 *b*) both vary with hydrophobicity[36] of residues 72–76 of PfCRT[33]. It is thus possible, in the absence of changes in other determinants, to predict both the *in vitro* verapamil effect and the probable clinical efficacy of amodiaquine (see Figures 3 and 4 based on data in Sidhu *et al.*[37]). Concomitant residue changes in PGH-1 (see below) should further reduce the efficacy of amodiaquine. Chloroquine-resistant S. American/SE Asian PfCRT 72–76 alleles **SVMNT** and CVMN**T** are predicted to confer a lower verapamil effect and lower sensitivity to desethylamodiaquine in comparison to the usual typically African CV**IET**[33]. **SVMNT**, previously unreported from Africa, has recently been reported in Tanzania[38]. 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 verapamil-insensitivity[34]. 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[39]. The most recent work affords at present only equivocal support for[20], or disagreement with[16,40] the haematin aggregation hypothesis.

Selective toxicity in arylaminoalcohols

The evidence from *in vitro* studies on inhibition of β-haematin production[41] supports an attractively simple view that the mode of action of the cinchona alkaloids quinine and quinidine which are hydrophobic arylaminoalcohols[42], and mefloquine, halofantrine and lumefantrine which are highly hydrophobic aminoalcohols (HHAAs), is, as for chloroquine and other 4-aminoquinolines, due to their accumulation by acidic trapping in the digestive vacuole, binding to haematin and prevention of its detoxication.

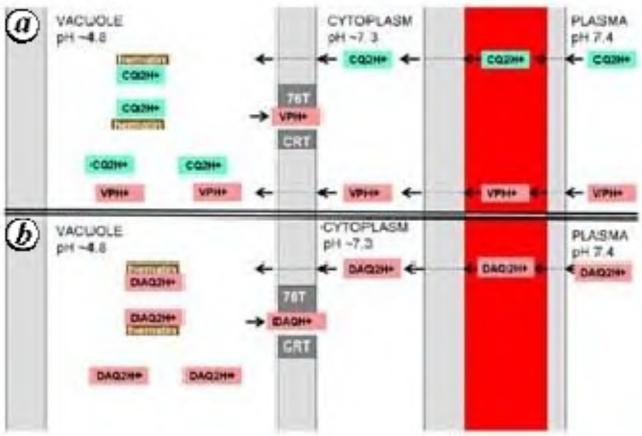

Figure 2 ***a***, ***b***. ***a***, Chloroquine-resistant parasite with PfCRT mutant, treated with CQ and verapamil (VP). Chloroquine resistance can be reversed *in vitro* by concurrent use of the weak hydrophobic base verapamil, which accumulates as VPH^+ in the lysosome, and being hydrophobic and positively charged is able to bind inside the PfCRT channel, replacing the positive charge of lysine, repelling the exit of $CQ2H^+$ and preventing haematin detoxication. Membranes are shown shaded grey. RBC cytoplasm is red. ***b***, CQ-R parasite with PfCRT mutant shows hydrophobic binding of desethylamodiaquine (DAQ) within the PfCRT channel, preventing drug exit and haematin detoxication. Membranes are shown shaded grey. RBC cytoplasm is red.

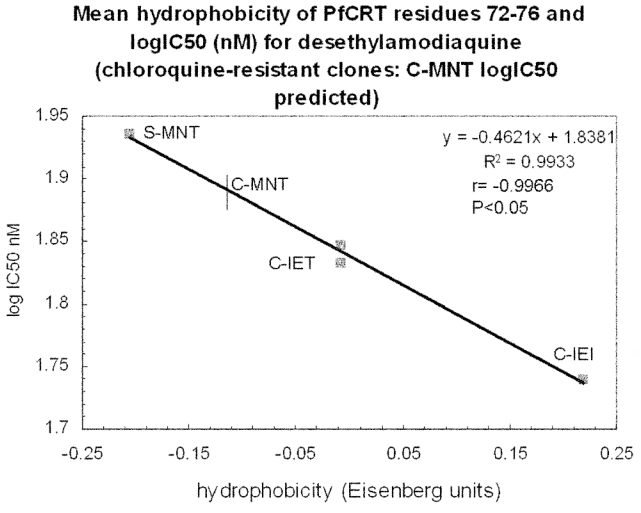

Figure 3. Linear relationship between desethylamodiaquine log IC_{50} values of *pfcrt* transfectants from data of Sidhu *et al.*[57] and the mean hydrophobicity of residues 72, 74, 75 and 76. Residue 73 is valine (V) throughout. The IC_{50} value of the Colombian 72CVMNT76 haplotype is predicted from the equation and marked on the trend line. These transfectants were all performed on a clone HB3 chloroquine-sensitive genetic background where the *pfmdr* haplotype was 86N**184F**1034S**1042D**1246D. (PGH-1 variants in bold type) (modified from Warhurst[33]).

Resistance to arylaminoalcohols

Resistance to arylaminoalcohols involves residue changes in vacuolar trans-membrane protein PGH-1 for cinchona alkaloids[43], or increased expression of the wild-type protein[44] for HHAAs. Apparently PGH-1, a homologue of other eukaryotic ATP-binding cassette (ABC) proteins[45]

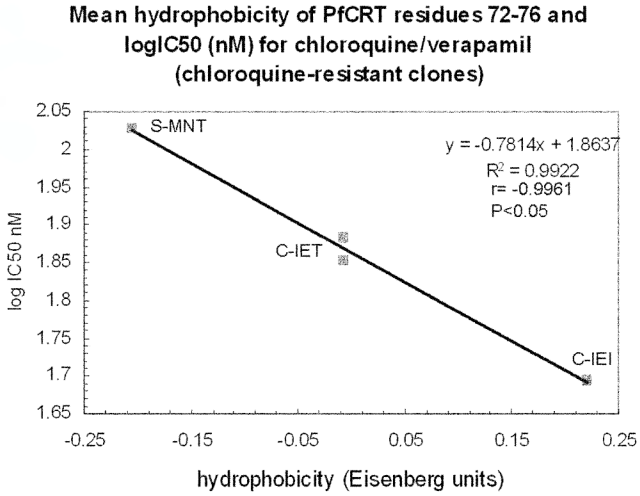

Figure 4. Linear relationship between chloroquine log IC_{50} values (in the presence of verapamil) of *pfcrt* transfectants, from data of Sidhu *et al.*[57] and the mean hydrophobicity of residues 72, 74, 75 and 76. Residue 73 is valine (V) throughout. These transfectants were all performed on a clone HB3 chloroquine-sensitive genetic background where the *pfmdr* haplotype was 86N**184F**1034S**1042D**1246D (PGH-1 variants in bold type). (modified from Warhurst[33].)

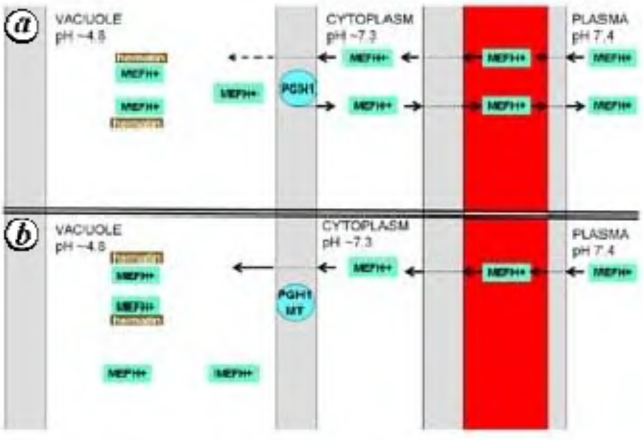

Figure 5 ***a***, ***b***. ***a***, Normal mefloquine (MEF) sensitivity. Wild-type PGH1, able to export mefloquine from membrane, reduces the intravacuolar drug concentration. Membranes are shown shaded grey. RBC cytoplasm is red. ***b***, Mefloquine (MEF) hypersensitivity. PGH1 mutant, unable to export mefloquine, allows higher concentrations of MEF to build up, and enhances inhibition of haematin detoxication. Membranes are shown shaded grey. RBC cytoplasm is red.

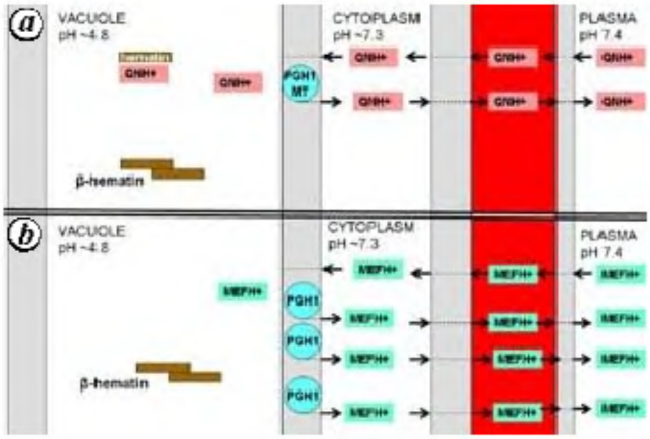

Figure 6 *a*, *b*. Quinine (QN)-resistance. PGH1 mutant able to export quinine and reduce intravacuolar concentration (and with a similar but slight effect on CQ). Membranes are shown shaded grey. RBC cytoplasm is red. *b*, Clinical mefloquine (MEF)-resistance. Overexpressed wild type PGH1 depletes seriously the intravacuolar MEF concentration. Membranes are shown shaded grey. RBC cytoplasm is red.

is able to mediate ATP-driven efflux of hydrophobic drugs from the digestive vacuole membrane to the cytoplasm[46]. The protein, in common with other eukaryotic ABC proteins, has 12 transmembrane α-helices and two cytoplasmic nucleotide-binding regions, each of which aligns well with the bacterial HisP nucleotide-binding protein component of *E. coli* histidine permease. Each cytoplasmic region characteristically has Walker A and B nucleotide-binding motifs, a Q-loop, a C-loop in a signalling domain, a D-loop and a switch II sequence (cf ref. 47).

Although malaria parasites are ~3 orders of magnitude less sensitive to verapamil (which does not significantly bind to haematin[42]) alone than to HHAAs, verapamil, like HHAAs is somewhat more active on PGH-1 variants[48]. There is suggestive evidence that PGH-1 residue changes associated with cinchona alkaloid resistance are selected by chloroquine[49], and are apparently responsible for enhancing chloroquine resistance caused by residue changes in vacuolar membrane protein PfCRT[50,51]. Because amodiaquine treatment apparently selects Pgh-1 variants, it is interesting that clinical resistance to amodiaquine as well as being associated with PfCRT changes, has also been associated with variant PGH-1[52]. Several reports have shown that sequence changes in the genes (*pfmdr1* and *pfcrt*, on chromosomes 5 and 7) coding for PGH-1 and PfCRT are selected together in chloroquine-exposed field populations of *P. falciparum*[53–55] (i.e. they are in linkage disequilibrium). A review[56] postulates a convincing explanation of the interactions of PGH-1 and PfCRT with similar conclusions to that arrived at in the present review (see Figures 5 and 6). Although it would be attractive to postulate that PGH-1 is alone in influencing arylaminoalcohol resistance, transfection studies indicate that, in variant PfCRT alleles transfected on the same HB3 PGH-1 background, the activity of different HHAAs is slightly enhanced[57]. For the cinchona alkaloids, laevorotatory quinine and dextrorotatory quinidine, this enhancement is stereospecific[58,59], indicating a direct interaction of cinchona alkaloids with PfCRT. Further, there is some indication that an Na^+/H^+ exchanger protein may sometimes be involved in quinine resistance[60].

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