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## Polymorphism in the *Plasmodium falciparum* chloroquine-resistance transporter protein links verapamil enhancement of chloroquine sensitivity with the clinical efficacy of amodiaquine

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### Abstract

**Background:** Chloroquine accumulates in the acidic digestive vacuole of the intraerythrocytic malaria parasite, and prevents the detoxication of haematin released during haemoglobin digestion. Changes in protein PfCRT in the digestive vacuole membrane of growing intra-erythrocytic stages of *Plasmodium falciparum* are crucial for resistance. Expressed in yeast, PfCRT resembles an anion channel. Depressed anion channel function could increase intralysosomal pH to reduce entry of basic drug, or enhanced function could reduce drug interaction with target haematin. The most important resistance-associated change is from positively-charged lysine-76 to neutral threonine which could facilitate drug efflux through a putative channel. It has been proposed that the resistance-reversing effect of verapamil is due to hydrophobic binding to the mutated PfCRT protein, and replacement of the lost positive charge, which repels the access of 4-aminoquinoline cations, thus partially restoring sensitivity. Desethylamodiaquine, the active metabolite of amodiaquine, which has significant activity in chloroquine-resistance, may also act similarly on its own.

**Methods:** Changes in physicochemical parameters in different CQ-resistant PfCRT sequences are analysed, and correlations with drug activity on lines transfected with different alleles of the *pfcr*t gene are examined.

**Results and conclusions:** The results support the idea that PfCRT is a channel which, in resistant parasites, can allow efflux of chloroquine from the digestive vacuole. Activity of the chloroquine/verapamil combination and of desethylamodiaquine both correlate with the mean hydrophobicity of PfCRT residues 72-76. This may partly explain clinical-resistance to amodiaquine found in the first chloroquine-resistant malaria cases from South America and enables tentative prediction of amodiaquine's clinical activity against novel haplotypes of PfCRT.

### Background

Every year there are 270 million clinical attacks of malaria and 2 million deaths, caused by the protozoan *Plasmodium falciparum*. Strains resistant to the 4-aminoquinoline

chloroquine (CQ) arrived in Africa in the late 70s [1]. This has led to an increase in malaria-associated deaths of children [2]. CQ and other blood schizontocides target haemoglobin digestion within the digestive vacuole or

lysosome in the growing erythrocytic stages of the parasite. As weak bases they accumulate in the proton-rich vacuole [3], bind to haematin, and prevent its detoxication [4] to insoluble dimeric haemozoin [5]. Accumulation of haematin leads to the death of the parasite. The resistance process is not yet understood, but is believed to follow changes in lysosomal integral membrane proteins [6,7]. The most important changes in CQ-resistance are non-silent mutations in gene *pfcr*t coding for PfCRT, the so-called CQ-resistance transporter [8], the most important changes in which are K76T and A220S. PfCRT, its orthologues (CG-10) in other *Plasmodium* spp., and a homologue, SSA662, from slime-mould *Dictyostelium discoideum*, represent a protein class of unknown function, with no easily interpreted relationship to other proteins [9]. A preliminary analysis of the sequence of PfCRT by the present author [unpublished: *Plasmodium falciparum* chloroquine-resistance transporter: one of the usual channels? (Poster). COST B9 Meeting on Antiprotozoal Chemotherapy. London, June 23, 2002. Poster Abstract 49.] indicated a similarity to chloride channels (ClC) in pro- and eukaryotes [10]. Experimental data indicating that PfCRT expressed in yeast has some chloride-channel features have recently been reported [11]. This could have a major impact on how we interpret the role of PfCRT in resistance. A ClC function, possibly gated by membrane potential, could, by varying the entrance or exit of charge-balancing chloride anion, control intralysosomal pH, thereby regulating access of basic drug to the lysosome [3], or influencing the interaction of drug with its target haematin [12]. Apart from these rather non-specific pH effects, which are rendered unlikely by different interactions of the physico-chemically very similar diastereomers quinine and quinidine with mutated PfCRT [13], there is persuasive evidence that resistance depends on a drug efflux process [14]. Contradictory evidence [15] may now be explained by postulating drug efflux from an internal compartment such as the lysosome into the cytoplasm, which after a variable delay, depending on experimental conditions, releases drug back into the culture medium.

The present study analyses changes in residue hydrophobicity, side-chain volume and charge in different CQ-resistant PfCRT sequences. In addition, the correlation of reported *in vitro* drug activity with residue hydrophobicity, side-chain volume and charge in a sensitive clone transfected with alleles of the *pfcr*t gene from CQ-sensitive and -resistant isolates [16] is examined. The results obtained support the idea that PfCRT could be a channel which, when mutated, allows efflux of chloroquine from the lysosome. The activity of the resistance-reversing CQ-verapamil (VE) combination on clones bearing different *pfcr*t alleles is correlated with that of the amodiaquine (AQ) metabolite desethylamodiaquine (DAQ) [17] which has significant activity in chloroquine-resistance

[18]. Evidence is brought forward that VE and DAQ bind hydrophobically, in CQ-resistant parasites, to mutated PfCRT in the residue 72-76 region. Either of these drugs could replace the lost positive charge of lysine 76, impeding the access of further 4-aminoquinoline cations to PfCRT and partially restoring CQ-sensitivity. This idea would explain both the limited VE effect seen in some CQ-resistant New World isolates of *P. falciparum* [19] which have less hydrophobic PfCRT 72-76 sequences and their clinical cross-resistance to AQ in the first case-reports [20,21] of CQ-resistant *falciparum* malaria from South America. These data allow tentative prediction of the response of novel PfCRT haplotypes to DAQ.

## Materials and Methods

### Computational and analytical methods

In the analysis of mutation-related changes in PfCRT sequences, Eisenberg's hydropathy value [22] ("EIS"), the side-chain volume (SCV) and the side-chain charge (CH) of each residue were obtained. (Table 1). The side-chain volume was obtained from the HyperChem 7 programme (Hypercube Inc. Gainesville Florida, USA), on any individual amino acid (except proline, owing to its unusual composition) after replacement of the  $\alpha$ -carbon, its hydrogen, carboxyl and amino group, by H, so that the side-chain volume of glycine, for example, would be  $85.18 \text{ \AA}^3(\text{H}_2)$ , and the side-chain volume of alanine would be  $156.6 \text{ \AA}^3(\text{CH}_4)$ . Formal charge at pH 7.0 (a positive or negative integer or zero) associated with gain or loss of a proton by nitrogen or oxygen (if present) in the amino acid side-chain was also used.

Of ten variant residues in the PfCRT sequence (see Table 5) four had alternative mutants, and 15 different changes were considered. The mean and standard errors of the values of physicochemical characteristics were determined.

For the correlation of reported *in vitro* drug activity with hydrophobicity, side-chain volume and charge of PfCRT residues in a CQ-sensitive clone whose *pfcr*t gene had been replaced by alleles from isolates of different CQ-sensitivities, the data of Sidhu *et al.* [16] were used (See Table 2 for an example of the calculation of mean values of physicochemical parameters and Table 3 for tabulation of data used).

### Statistics

The statistical analysis was carried initially in Microsoft Excel and significance levels were determined in a Documenta Geigy Table [29]. The results were confirmed in SPSS 11.01 using Pearson's bivariate correlation method. Each IC<sub>50</sub> value reported by Sidhu *et al.* [16] is the mean of at least 3 test results, and these means were used unmodified, giving only two degrees of freedom (DF) where values for the four CQ-resistant clones and three or

**Table 1: Abbreviations, Eisenberg hydrophathy (EIS) value, side-chain volume (SCV), side-chain pK<sub>a</sub> and formal charge on the side-chain for amino acids.**

Amino-acid	EIS hydrophathy (log scale)	side-chain volume Å <sup>3</sup>	side-chain pK <sub>a</sub>	formal side-chain charge (pH 7.0)
A ala	0.25	156.55		0
C cys	0.04	219.43	8.33	0
D asp	-0.72	232.12	3.86	-1
E glu	-0.62	284.70	4.25	-1
F phe	0.61	382.16		0
G gly	0.16	85.18		0
H his	-0.40	334.84	6.0	+1
I ile	0.73	313.43		0
K lys	-1.10	366.25	10.28	+1
L leu	0.53	315.53		0
M met	0.26	327.41		0
N asn	-0.64	256.12		0
P pro	-0.07	ND		0
Q gln	-0.69	308.35		0
R arg	-1.80	421.32	12.48	+1
S ser	-0.26	183.07		0
T thr	-0.18	238.55		0
V val	0.54	267.78		0
W trp	0.37	462.28		0
Y tyr	0.02	407.07	10.07	0

**Table 2: Determination of physicochemical characteristics of mutable residues, and their mean values for protein PFCRT in a transfected clone of *P. falciparum*.**

		PFCRT : clone GC03		
		EIS	SCV	CH
residue	amino acid			
72	C	0.04	219.43	0
73	M	0.26	327.41	0
75	N	-0.64	256.12	0
76	K	-1.1	366.25	1
	SUM	-1.44	1169.21	1
	MEAN	-0.275	292.303	0.25
72	C	0.04	219.43	0
74	M	0.26	327.41	0
75	N	-0.64	256.12	0
76	K	-1.1	366.25	1
97	H	-0.4	334.84	1
220	A	0.25	156.55	0
271	Q	-0.69	308.35	0
326	N	-0.64	256.12	0
356	I	0.73	313.43	0
371	R	-1.8	421.32	1
	SUM	-3.99	2959.82	3
	MEAN	-0.399	295.982	0.3

(example given is CQ-sensitive clone GC03).

**Table 3: Mean physicochemical parameters and drug IC50 values[16] for clones of *P. falciparum* showing different haplotypes of PfCRT.**

clone	c1GC03	c2GC03	c3dd2	c4dd2	c5k76l	c67g8
mutable residues 72-76	CMNK	CMNK	CIET	CIET	CIEI	SMNT
mutable residues 72-371	CMNK HAQNIR	CMNK HAQNIR	CIET HSESTI	CIET HSESTI	CIEI HSESI	SMNT HSQDLR
mean EIS 72-76	-0.275	-0.275	-0.0075	-0.0075	0.22	-0.205
mean EIS 72-371	-0.399	-0.399	-0.102	-0.102	0.08	-0.416
mean SCV 72-76	292.3025	292.3025	264.0275	264.0275	282.7475	251.2875
mean SCV 72-371	295.982	295.982	260.1521	260.1521	275.7521	275.2463
mean CH 72-76	0.25	0.25	-0.25	-0.25	-0.25	0
mean CH 72-371	0.3	0.3	-0.1	-0.1	-0.1	0.1
CQ IC50 nM	27.4	22.9	143.8	147.4	101.7	126.9
DCQ IC50 nM	ND	37.7	875.2	1182.2	635.3	649.4
AQ IC50 nM	20.2	18.2	31.6	25.8	28.9	35.8
DAQ IC50 nM	ND	45.2	70.2	67.9	54.8	86.2
C-V IC50 nM	27.9	21.8	76.2	71.3	49.4	106.2

**Table 4: Hydrophobicity values (log D) of drugs at pH 5.2 [30], their volume, surface area and number of positive charges.**

drug	logD <sub>5.2</sub>	Vol(Å <sup>3</sup> ) (base)	surface area (Å <sup>2</sup> ) (base)	protons (positive charges)
CQ	-3.44	1010	606	2
DCQ	-4.61	910	555	2
AQ	-1.06	996	568	2
DAQ	-2.54	930	554	2
VP	0.59	1340	734	1

four degrees of freedom where values for all the five or six clones were used (DF = n-2). A probability value in the two-tailed test equal to or less than 0.05 indicated a significant correlation.

Hydrophobicities at acid pH, molecular volumes and formal charge for the 5 drugs examined are listed in Table 4.

**Results**

**Initial analysis of the effects of residue changes on physicochemical characteristics of the protein. (Table 5)**

On calculating the mean charge, hydrophobicity, and volume of the side-chain (SCV) for 15 mutations to resistance (including 2 laboratory-selected lines)[13], the following results were obtained.

1. Charge became more negative by -0.6 (S E: ± 0.13).
2. Hydrophobicity increased by +0.40 (0.25).
3. Side-chain volume was reduced by a factor of 0.86 (0.04).

**Correlation of physicochemical characteristics with drug activity in transfectants**

Since drug response is drastically changed in CQ-resistance (for example, VE acquires the ability to enhance the effects of CQ) it is highly relevant to look at correlations between IC50 values of the four CQ-resistant clones themselves and PfCRT physicochemical properties of their mutable residues, as well as to look at the correlations of these properties with the CQ-sensitive and resistant clones overall.

**Chloroquine-resistant transfectants (Table 6: Figures 1,2,3,4,5,6,7)**

Chloroquine (CQ), desethylchloroquine (DCQ) and amodiaquine (AQ) IC 50 values showed no significant correlation with any physicochemical feature of the mutable residues of PfCRT.

**Desethylamodiaquine (DAQ)**

IC50 value was negatively correlated with hydrophobicity of mutable residues 72-76 and 72-371. Up to 98% of variation in activity of DAQ was explained by hydrophobicity (Figures 1 and 2).

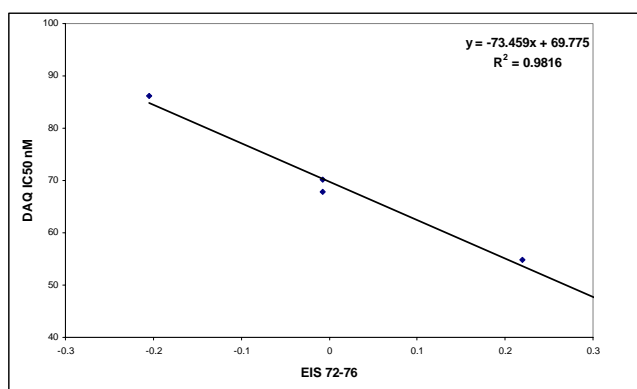
Increased mean side-chain volume of residues 72-76 was negatively correlated with IC50 and explained up to 96%

**Table 5: Changes in physicochemical parameters seen in mutations associated with CQ-resistance in lines of *P. falciparum* from field and laboratory\*.**

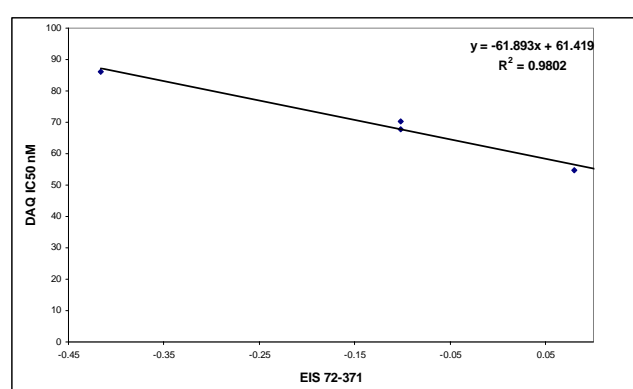
RESIDUE	mutants	Δ sc charge res-sens	Δ EIS res-sens	Δ SCV Å <sup>3</sup> res/sens
CYS 72	SER	0	-0.30	0.834
MET 74	ILE	0	0.47	0.957
ASN 75	GLU	-1	0.02	1.111
LYS 76	THR ILE* ASN*	-1 -1 -1	0.92 1.83 0.46	0.652 0.855 0.699
HIS 97	GLN	-1	-0.29	0.921
ALA 220	SER	0	-0.51	1.170
GLN 271	GLU	-1	0.07	0.923
ASN 326	SER ASP	0 -1	0.38 -0.08	0.714 0.907
ILE 356	THR LEU	0 0	-0.91 -0.20	0.761 1.007
ARG 371	ILE THR	-1 -1	2.53 1.62	0.744 0.566
MEAN (± S.E.)		-0.60 (0.13)	0.40 (0.25)	0.86 (0.04)

**Table 6: For 4 CQ-resistant transfectants the Pearson negative correlations of IC50 value and physicochemical characteristics (R<sup>2</sup> values) are shown, and significant results (P ≤ 0.05) on 2-tailed analyses are asterisked.**

drug	ratio R+/R	EIS 72-76	EIS 72-371	SCV 72-76	SCV 72-371
CQ	1.5	0.29	0.11	0.37	0.77
DCQ	1.9	0	0.02	0.02	0.76
AQ	1.4	0.41	0.56	0.35	0.23
DAQ	1.6	0.98*	0.98*	0.96*	0
CQ-VE	2.2	0.97*	0.99*	0.94*	0.01



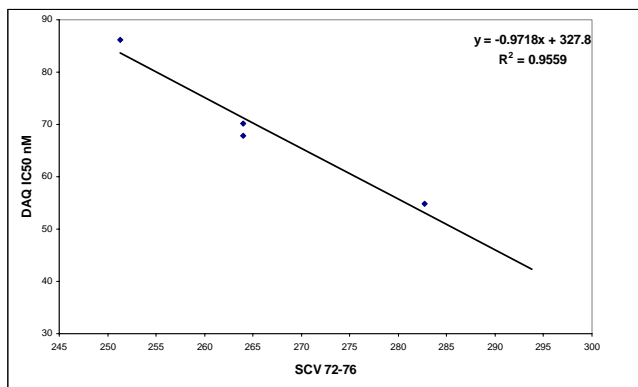
**Figure 1**  
Correlation of DAQ IC50 (nM) and EIS 72-76 in 4 CQ-resistant transfectants.



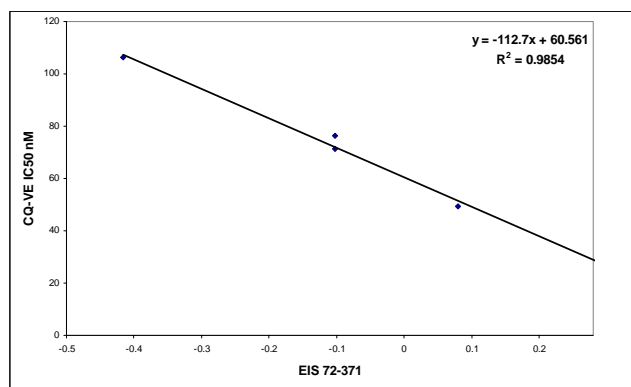
**Figure 2**  
Correlation of DAQ IC50 (nM) and EIS 72-371 in 4 CQ-resistant transfectants

**Table 7: For 5 or 6 transfectants of varying sensitivity, the Pearson negative correlations of IC50 value and physicochemical characteristics (R<sup>2</sup> values) are shown, and significant results (P ≤ 0.05) on 2-tailed analyses are asterisked.**

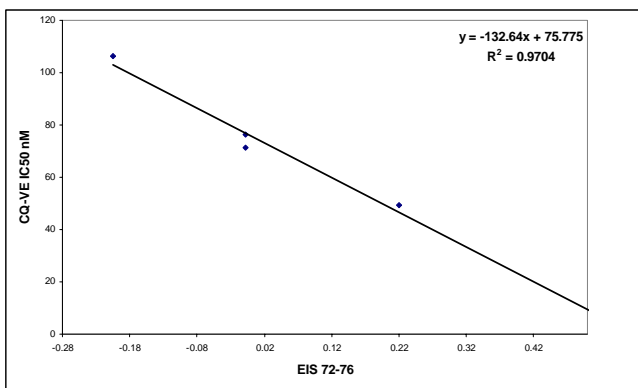
drug	ratio R/S	EIS 72-76	EIS 72-371	SCV 72-76	SCV 72-371	CH 72-76	CH 72-371
CQ	6.4	0.35	0.30	0.77*	0.95*	0.80*	0.80*
DCQ	31.4	0.28	0.26	0.45	0.93*	0.75*	0.75*
AQ	2.0	0.17	0.08	0.76*	0.47	0.41	0.41
DAQ	1.9	0.01	0.01	0.98*	0.34	0.10	0.10
CQ-VE	4.9	0.04	0.01	0.98*	0.53	0.32	0.32



**Figure 3**  
Correlation of DAQ IC50 (nM) and SCV 72-76 in 4 CQ-resistant transfectants.



**Figure 5**  
Correlation of CQ-VE IC50 (nM) and EIS 72-371 in 4 CQ-resistant transfectants.



**Figure 4**  
Correlation of CQ-VE IC50 (nM) and EIS 72-76 in 4 CQ-resistant transfectants.

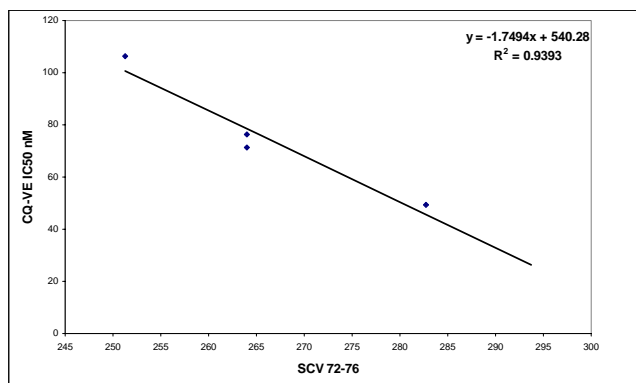
of variation (Figure 3). No correlation was seen when all the ten mutable residues from 72-371 were taken into account, indicating that there was an opposing side-chain volume correlation associated with residues 97-371.

**The verapamil (VE) effect**

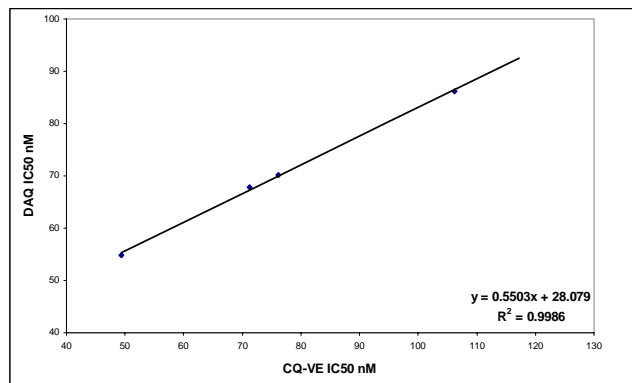
For CQ-VE (the verapamil effect), residue hydrophobicity was also negatively correlated with IC50, for residues 72-76 and 72-371 (Figures 4 and 5). Up to 99% of variation in activity of CQV was explained by hydrophobicity.

Increased mean side-chain volume of residues 72-76 was negatively correlated with IC50 and explained up to 94% of variation (Figure 6), but, as for DAQ, significant correlation was not seen with side-chain volume of residues 72-371.

No significant influence of side-chain charge could be detected for any of the drugs examined.



**Figure 6**  
Correlation of CQ-VE IC<sub>50</sub> (nM) and SCV 72-76 IN 4 CQ-resistant transfectants.



**Figure 7**  
Correlation of CQ-VE (nM) and DAQ IC<sub>50</sub> values in 4 CQ-resistant transfectants.

There was a significant positive correlation between IC<sub>50</sub> of CQ-VE, and IC<sub>50</sub> of DAQ, while >99% of variation was explained (Figure 7). In contrast there was no correlation whatsoever between CQ and DAQ IC<sub>50</sub> values, or between CQ and AQ.

#### **Chloroquine (CQ)-sensitive and resistant transfectants (see Table 7: Figures 8,9,10,11,12,13)**

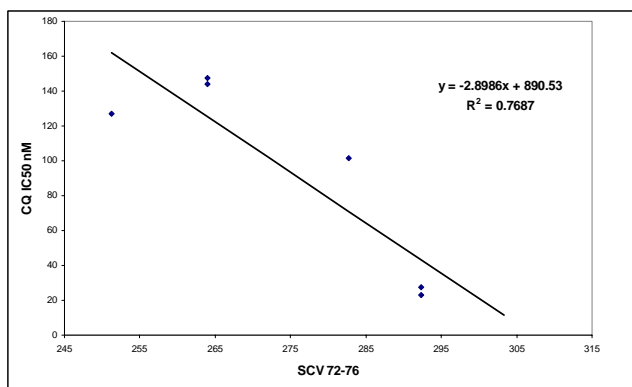
Taking CQ-sensitive and -resistant lines into account, neither hydrophobicity of residues 72-76 nor 72-371 showed significant correlation with the IC<sub>50</sub> of any drug studied.

Mean side-chain volume of residues 72-76 was negatively correlated with IC<sub>50</sub> of CQ (Figure 8), AQ, DAQ (Figure 9) and CQ-VE, with 77, 76, 98 and 98% of variation explained. For CQ and DCQ this was seen for side-chain volume of 72-371 (95 and 93% of variation explained) (Figure 10) but not for AQ, DAQ, or CQ-VE.

Mean side-chain charge negatively correlated with IC<sub>50</sub> of CQ, for residues 72-76 and 72-371 (Figure 11), and also for DCQ, explaining 80 and 75% of variation. No significant correlation was seen for AQ, DAQ or CQV.

There was a significant positive correlation between IC<sub>50</sub> of CQ-VE, and IC<sub>50</sub> of DAQ, while 99% of variation was explained (Figure 12).

A similar picture was seen for the correlation of CQ-VE against AQ IC<sub>50</sub>, where 86% of variation was explained (Figure 13).



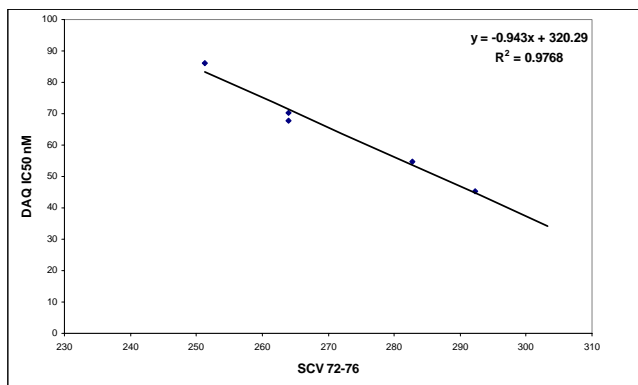
**Figure 8**  
Correlation of CQ IC<sub>50</sub> (nM) and SCV 72-76 values in 6 transfectants.

In contrast no significant correlation was detected between CQ and DAQ, or between CQ and AQ.

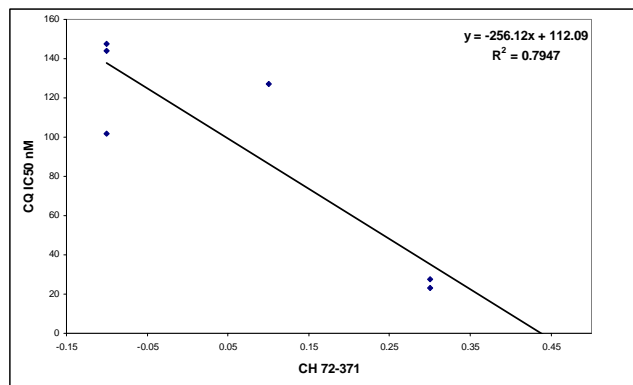
### **Discussion and Conclusions** **Effects of residue changes on physicochemical characteristics of the protein**

Using mean values of the parameters concerned will obviously obscure important relationships. However, this mean approach is only applied to the 10 residues which change in resistance. So, in the opinion of the author, a tentative interpretation of the findings is possible in the

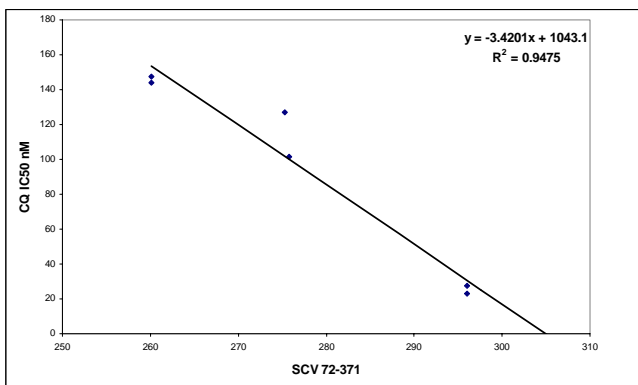




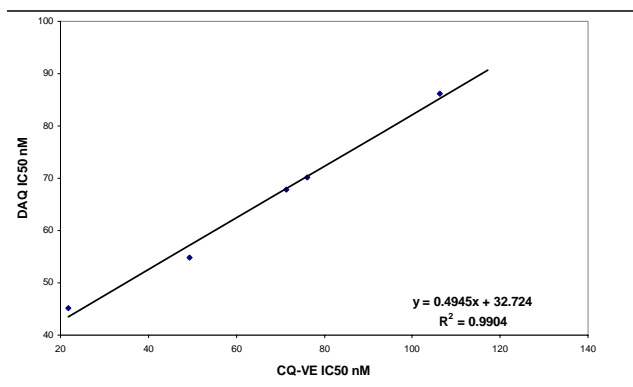
**Figure 9**  
Correlation of DAQ IC<sub>50</sub> (nM) and SCV 72-76 values in 5 transfectants.



**Figure 11**  
Correlation of CQ IC<sub>50</sub> (nM) and CH 72-371 values in 6 transfectants.



**Figure 10**  
Correlation of CQ IC<sub>50</sub> (nM) and SCV 72-371 values in 6 transfectants.

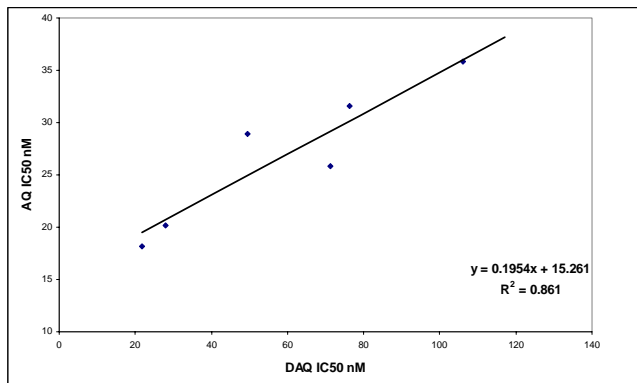


**Figure 12**  
Correlation of CQ-VE IC<sub>50</sub> (nM) and DAQ IC<sub>50</sub> values in 5 transfectants.

light of the 3 hypotheses outlined below, on the role of changes in the sequence of integral protein PfCRT in the digestive vacuole (lysosome) membrane upon sensitivity to chloroquine. These hypotheses assume that the probable target of the 4-aminoquinoline blood-schizontocidal drugs like CQ and DAQ is haematin released by digestion of haemoglobin inside the vacuole [4], and that a minimal intravacuolar drug concentration is needed for effective (reversible) interaction with the target [24]. They also assume that PfCRT has anion-, possibly chloride-channel-like, function [11,25].

1. **Chloride channel function is diminished.** This could follow from the more negative mean charge. A shortage of charge-balancing anion within the vacuole could result in a rise in vacuolar pH. The rise in vacuolar pH could reduce drug uptake [3]. The possible role of increase in hydrophobicity and reduction of the side-chain volume is obscure.

2. **Chloride channel function is enhanced.** There is no easy interpretation of how the changes seen could enhance the chloride channel function, although equally



**Figure 13**  
Correlation of DAQ IC<sub>50</sub> (nM) and AQ IC<sub>50</sub> values in 5 transfectants.

there is no reason why such an outcome would be impossible. The expected result, a lowered vacuolar pH, could reduce drug interaction with haematin [12].

**3. Drug exits through modified chloride channel.** More negative mean charge on a channel lining, an increase in its hydrophobicity, and the reduction of bulk of the side-chains would all enhance the ability of a hydrophilic, positively charged bulky drug like CQ (Table 4) to escape through the putative channel lumen [7,25].

If the observation of markedly different effects of amino acid changes in PfCRT on the activity of antiplasmodial diastereomers quinine and quinidine [13,16] is also taken into account, hypotheses 1 and 2, being based simply on postulated intravacuolar pH changes, are rendered unlikely, but hypothesis 3, depending on structural features, is supported.

## Conclusion

An admittedly crude analysis supports the hypothesis that modified PfCRT acts as a channel for exit of CQ cation.

### Correlation of physicochemical characteristics with drug activity in transfectants

In view of the support of the first stage of the investigation for the modified channel hypothesis, further observations are interpreted largely in the light of the possibility that CQ escapes from the lysosome through the modified PfCRT channel in CQ-resistant lines, but is unable to do so in the CQ-sensitive lines. If an exit channel is involved, it is a reasonable assumption that most or all of the residue changes in resistance are likely to involve side-chains

lining or closely associated with the channel lumen. Even side-chains not protruding into the channel lumen can affect overall delocalized charge, and side-chain bulk and hydrophobicity will affect residue packing and alter channel spatial characteristics. So comparing mean physicochemical parameters of these mutable residues in CQ-resistant and -sensitive PfCRT sequences should inform us about features of the channel associated with resistance.

### CQ-resistant transfect lines

#### Response to CQ and DCQ

In CQ-resistant transfect lines, CQ and DCQ are expected to bind minimally to the lining side-chains of a PfCRT channel in the most resistant parasites. The high correlation of residue mean side-chain volume with drug sensitivity supports the possible action of enlarged side-chains in impeding the efflux of the drug, maintaining its concentration in the vacuole and enhancing its interaction with haematin.

No significant influence of charge or hydrophobicity was detectable, and this may be expected in the absence of what appears to be the crucial positive charge on the side-chain of residue 76, and the expected lack of adhesion of CQ, highly hydrophilic at acid pH, to the hydrophobic lining of the channel.

#### Response to AQ

The results seen with AQ are surprising, since in view of the drug's very high hydrophobicity in acidic conditions, one might expect to see a marked correlation with activity. However, the range of activity over the 4 CQ-resistant clones is only a factor of 1.4, and any influence of residue side-chain hydrophobicity on activity is not detectable in this analysis which is of very limited power (only 2 degrees of freedom). It is also not impossible that the AQ parent drug may have an additional mode of action to haematin binding. It is noteworthy that AQ sensitivity in vitro has shown a poor relationship with efficacy of treatment, but this probably mainly reflects the greater importance of the metabolite DAQ in vivo.

#### Response to DAQ

A very important role of hydrophobicity of the mutable residues was detected, equivalent for residues 72-76, and overall for 72-371, where 98% of variation was explained, in marked contrast with CQ. This is expected in view of the 10-fold higher hydrophobicity of DAQ in acidic conditions compared with CQ (Table 4) and may explain the retention of activity of DAQ in CQ-resistant lines. The important role of hydrophobicity in blood-schizontocidal drugs active in chloroquine-resistance was first reported by Bray et al. [26].

**Table 8: Prediction of DAQ IC50 nM value for CQ-resistant lines on the basis of mean EIS for the 72-76 haplotype and the linear equation in Fig. 1. (IC50 nM = -73.459(mean EIS) + 69.775).**

EIS	C5K761 Laboratory	C34Dd2 SE Asia	C67G8Brazil	Jav/ IAJColombia	Ecu1110 Ecuador	Cambodia	Cambodia	Cambodia
HAPLO.	CIEI	CIET	SMNT	CMET	CMNT	CMNT	CIDT	CTNT
72	0.04	0.04	-0.26	0.04	0.04	0.04	0.04	0.04
74	0.73	0.73	0.26	0.26	0.26	0.26	0.73	-0.18
75	-0.62	-0.62	-0.64	-0.62	-0.64	-0.64	-0.72	-0.64
76	0.73	-0.18	-0.18	-0.18	-0.18	-0.18	-0.18	-0.18
<b>SUM</b>	0.88	-0.03	-0.82	-0.5	-0.52	-0.52	-0.13	-0.96
<b>MEAN</b>	0.22	-0.0075	-0.205	-0.125	-0.13	-0.13	-0.0325	-0.24
<b>predict IC50 DAQ (nM)</b>	53.6	70.3	84.8	79.0	79.3	79.3	72.2	87.4
<b>ACTUAL</b>	54.8	69.05	86.2					
<b>PGH-I factor</b>	Y86---N86	Y86---N86	S. America	S. America	S. America	Y86---N86	Y86---N86	Y86---N86
<b>predict field sensitivity (IC50) nM</b>	1.4-----1.0	1.4-----1.0	-----2.7	-----2.7	-----2.7	1.4-----1.0	1.4-----1.0	1.4-----1.0
<b>IC50 DAQ (nM)</b>	76-----54	98-----70	-----230	-----213	-----213	111-----79	101-----72	122-----87

(To adjust for the PGH-I factor, derived from the study of Sidhu et al, 2002 [16], multiply by 1.4 where N86Y is found in Africa and SE Asia, and 2.7 for the usual S. American *pfmdr1* haplotype of S1034C, N1042D, D1246Y [27].)

Supporting the importance of residues 72-76, the mean side-chain volume of these residues negatively correlates with IC50 and explains 96% of variation. There is no correlation between activity and mean side-chain volume for the whole sequence.

Again, no effect of side-chain charge was detectable.

These results suggest that not only is residue hydrophobicity for 72-76 important for activity of DAQ but there may be a "bottleneck" in the region of these residues. Caution is necessary, since side-chain size and hydrophobicity are mutually correlated for hydrophobic residues. In these lines, with no positive charge at PfCRT residue 76, the size and hydrophobicity in some residues in the 72-76 region may relate to the same residues, since important differences in hydrophobicity tend also to be changes in bulk.

**Antiparasitic effect of CQ in combination with a resistance-reversing concentration of VE**

The antiparasitic activity of CQ in the presence of VE showed a marked correlation with the hydrophobicity of the mutable residues, where up to 99% of variation was accounted for.

Increase in the side-chain volume of residues 72-76 was also strongly correlated with CQ activity in the presence of VE (94% of variation accounted for), but significant correlation was not seen with the whole sequence.

Again, no effect of charge was detected.

These results indicate that the addition of VE causes CQ to behave like DAQ. It appears probable that the positively charged, highly hydrophobic, and bulky VE (Table 4) is able to bind hydrophobically to residues 72-76 in the modified PfCRT. It hinders the efflux of CQ by mutual repulsion of positive charges and by partially blocking the channel (cautions about side-chain size and hydrophobicity need to be repeated here).

A significant positive correlation was detectable between the activities of CQ-VE and of DAQ on these CQ-resistant lines, explaining >99% of variation (Fig. 7).

**CQ-sensitive and – resistant transfected lines**

In this analysis no relationship between mean residue hydrophobicity and drug activity was seen, suggesting hydrophobic interactions between DAQ or VE and the channel are important only when residue 76 has lost its positive charge and allows these drugs access to the relevant residues.

However, mean side-chain volume of residues 72-76 was correlated with increased activity of CQ, AQ, DAQ and CQ-VE. (77, 76, 98 and 98% of variation explained). This emphasises the importance, especially for activity of AQ, DAQ and CQ-VE, of a possible "bottleneck" associated with these residues, since activity of CQ and DCQ but not of AQ, DAQ or CQV also correlated with mean side-chain volume of 72-371. This latter observation emphasises the influence of bulky residues in the putative channel in enhancing CQ activity probably due to the bulk of the drug and the relative narrowness of the channel.

Mean side-chain charge correlated with increased sensitivity to CQ and DCQ, explaining 80 and 75% of variation, for residues 72-76 and 72-371. This was not seen for AQ, DAQ or CQ/VP. This emphasises the importance of mutual positive charge-repulsion preventing CQ and DCQ from entering the channel.

The contrast between the importance of hydrophobicity in the CQ-resistant lines, and the inability to demonstrate an effect of this in the sensitive and resistant lines together, in spite of the higher power of the analysis, can be interpreted on the channel efflux model as resulting from the positive charge on the side-chain of residue 76 in the 2 sensitive clones preventing the various drugs reaching the hydrophobic region of the sequence including residues 72-76. The fact that 4 out of 5 residues here are subject to mutation suggests that, in sensitive clones, it forms a barrier to exit of 4-aminoquinoline cations.

The correlation observed, even including the sensitive clones in the analysis, between sensitivity to CQ in the presence of VE (the verapamil effect), and sensitivity to DAQ suggests that clinical activity of AQ is likely to be correlated with the magnitude of the *in vitro* VE-effect (all other things, especially the PGH-1 status [6,27], being equal). The VE effect is much smaller in isolates where the 72-76 sequence is the "New World" S-MNT haplotype [19] (residue 73 is always valine). It is of interest to look at some other S. American haplotypes such as Jav and IAJ Colombia (C-MET) and Ecuador Ecu 1110 (C-MNT) [8]. These can both be predicted to be more sensitive to DAQ, and to have a higher VP-effect than the usual South American (S-MNT), even taking into account the large effect of PGH-1 amino acid changes at residues 1034, 1042 and 1246 [27]. The South American PfCRT S-MNT haplotype has recently been reported from Papua New Guinea together with the PGH-1 N86Y haplotype [19], and the Ecuador PfCRT 72-76 haplotype C-MNT, and 2 others, C-IDT and C-TNT have been found in Cambodia [28]. Predictions are possible for the effect of these on DAQ sensitivity, with and without the presence of PGH-1 N86Y, assuming that these two genes, *pfcr*t and *pfmdr*1, are the main ones concerned in resistance (Table 8). If AQ were to be widely used in Cambodia, the C-MNT and C-TNT haplotypes are likely to become more prevalent. Similarly in Papua New Guinea the S-MNT PfCRT haplotype is expected to give some resistance to treatment with AQ, and should become more prevalent if the drug becomes widely used.

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### References

- Wootton JC, Feng X, Ferdig MT, Cooper RA, Mu J, Baruch DI, Magill AJ and Su XZ: **Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum***. *Nature* 2002, **418**:320-323.
- Trape JF: **The public health impact of chloroquine resistance in Africa**. *Am J Trop Med Hyg* 2000, **64**(suppl):12-17.
- Homewood CA, Warhurst DC, Peters W and Baggaley VC: **Lyso-somes, pH and the anti-malarial action of chloroquine**. *Nature* 1972, **235**:50-52.
- 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.
- Pagola S, Stephens PW, Bohle DS, Kosar AD and Madsen SK: **The structure of malaria pigment beta-haematin**. *Nature* 2000, **404**:307-310.
- Foote SJ, Kyle DJ, Martin SK, Oduola AMJ, Forsyth K, Kemp DJ and Cowman AJ: **Several alleles of the multi-drug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum***. *Nature* 1990, **345**:255-258.
- Warhurst DC: **A molecular marker for chloroquine-resistant *falciparum* malaria**. *N Engl J Med* 2001, **344**:299-302.
- Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LMB, Sidhu AS, Naude B, Deitsch KW, Su X, Wootton JC, Roepe PD and Wellem TE: **Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance**. *Molecular Cell* 2000, **6**:861-871.
- Nomura T, Carlton JM, Baird JK, del Portillo HA, Fryauff DJ, Rathore D, Fidock DA, Su X, Collins WE, McCutchan TF, Wootton JC and Wellem TE: **Evidence for different mechanisms of chloroquine resistance in 2 *Plasmodium* species that cause human malaria**. *J Infect Dis* 2001, **183**:1653-1661.
- Dutzler R, Campbell EB, Cadene M, Chait BT and MacKinnon R: **X-ray structure of a CIC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity**. *Nature* 2002, **415**:287-294.
- Zhang H, Howard EM and Roepe PD: **Analysis of the antimalarial drug resistance protein Pfcr in yeast**. *J Biol Chem* 2002, **277**:49767-49775.
- Ursos LM, DuBay KF and Roepe PD: **Antimalarial drugs influence the pH dependent solubility of heme via apparent nucleation phenomena**. *Mol Biochem Parasitol* 2001, **112**:11-17.
- Cooper RA, Ferdig MT, Su XZ, Ursos LM, Mu J, Nomura T, Fujioka H, Fidock DA, Roepe PD and Wellem 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.
- 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.
- 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.
- Sidhu AB, Verdier-Pinard D and Fidock DA: **Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcr*t mutations**. *Science* 2002, **298**:210-213.
- Churchill FC, Patchen LC, Campbell CC, Schwartz IK, Nguyen-Dinh P and Dickinson CM: **Amodiaquine as a prodrug: importance of metabolite(s) in the antimalarial effect of amodiaquine in humans**. *Life Sci* 1985, **36**:53-62.
- Childs GE, Boudreau EF, Milhous WK, Wimonwatratree T, Pooyindee N, Pang L and Davidson DE Jr: **A comparison of the *in vitro* activities of amodiaquine and desethylamodiaquine against isolates of *Plasmodium falciparum***. *Am J Trop Med Hyg* 1989, **40**:7-11.
- Mehlotra RK, Fujioka H, Roepe PD, Jannet O, Ursos LM, Jacobs-Lorena V, McNamara DT, Bockarie MJ, Kazura JW, Kyle DE, Fidock DA and Zimmerman PA: **Evolution of a unique *Plasmodium falciparum* chloroquine-resistance phenotype in association with *pfcr*t polymorphism in Papua New Guinea and South America**. *Proc Natl Acad Sci U S A* 2001, **98**:12689-12694.
- Galvao ALA, Gusmao HH, Juarez E, Schmid AW and de Mello: **Malaria no Amapa. Observacoes sobre o comportamento**

**anomalo do Plasmodium falciparum em face do tratamento pelas 4-aminoquinolinas.** *Archos Fac Hig Saude Pub Univ S. Paulo* 1961, **15/16**:201-244.

21. Young MD: **Amodiaquine and hydroxychloroquine-resistance in Plasmodium falciparum.** *Am J Trop Med Hyg* 1961, **10**:689-693.
22. Eisenberg D: **Three-dimensional structure of membrane and surface proteins.** *Annu Rev Biochem* 1984, **53**:595-623.
23. Martin SK, Oduola AM and Milhous WK: **Reversal of chloroquine-resistance in Plasmodium falciparum.** *Science* 1987, **235**:899-901.
24. Kaschula CH, Egan TJ, Hunter R, Basilico N, Parapini S and Taramelli D: **Structure-activity relationships in 4-aminoquinoline antiplasmodials. The role of the group at the 7-position.** *J Med Chem* 2002, **45**:3531-3539.
25. Warhurst DC, Craig JC and Adagu IS: **Lysosomes and drug-resistance in malaria.** *Lancet* 2002, **360**:1527-1529.
26. 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.
27. Reed MB, Saliba KJ, Caruana SR, Kirk K and Cowman AF: **PghI modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum.** *Nature* 2000, **402**:906-909.
28. Lim P, Chy S, Arie F, Incardona S, Chim P, Sem R, Denis MB, Hewitt S, Hoyer S, Socheat D, Mercereau-Pujilon O and Fandeur T: **Pfcr1 Polymorphism and Chloroquine Resistance in Plasmodium falciparum strains isolated in Cambodia.** *Antimicrob Ag Chemother* 2003, **47**:87-94.
29. **Significance limits for r when population correlation coefficient is zero.** *Documenta Geigy Scientific Tables*. 6th edition. Edited by: Diem K. Geigy SA: Basle: p61. This Table enables it to be decided without calculation whether a correlation coefficient differs significantly from zero.
30. Warhurst DC, Craig JC, Adagu IS, Meyer DE and Lee SY: **The relationship of physico-chemical properties and structure to the differential antiplasmodial activity of the Cinchona alkaloids.** in press. <http://www.malariajournal.com/content/2/1/26>

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