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Function of Resistance Conferring *Plasmodium falciparum* Chloroquine Resistance Transporter Isoforms

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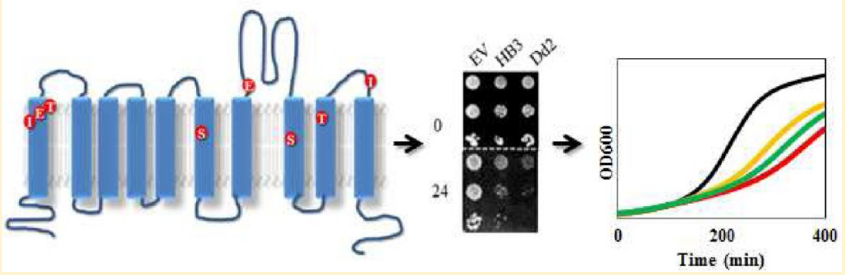

ABSTRACT: The function of *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) can be quantified using a *Saccharomyces cerevisiae* model system [Baro, N. K., Pooput, C., and Roepe, P. D. (2011) *Biochemistry 50*, 6701−6710]. We further optimized this system to distinguish PfCRT isoforms found in *P. falciparum* strains and isolates from across the globe. We created and expressed 13 naturally occurring *pfcrt* alleles associated with a range of chloroquine resistant (CQR) phenotypes. Using galactose induction of PfCRT, we quantified PfCRT and chloroquine (CQ)-dependent yeast growth inhibition and [^{3}H]CQ transport specifically due to a given PfCRT isoform. Surprisingly, we found poor correlation between these parameters and the CQ IC_{50} observed in strains of malaria harboring the same isoforms. This suggested that an increased level of CQ transport due to PfCRT mutation is necessary, but not sufficient, for the range of CQ IC_{50} values observed in globally distributed CQR *P. falciparum* isolates.

Mutation of the *pfcrt* gene causes cytostatic chloroquine resistance (CQR^{CS}) in *Plasmodium falciparum* malaria, typically characterized by a 7−10-fold increase in the chloroquine (CQ) IC_{50}.[1−3] The *pfcrt* mutations confer amino acid substitutions in the encoded *P. falciparum* chloroquine resistance transporter (PfCRT) protein, which resides within the digestive vacuolar (DV) membrane of the intra-erythrocytic parasite and is believed to be a small molecule or ion transporter.[2,3] CQR phenotypes are further characterized by cross resistance patterns to other drugs that may be influenced by additional genes.[4−6] Our current model for how mutant PfCRT confers CQR envisions increased electrochemically downhill transport of CQ out of the DV,[2,7−9] combined with DV osmolyte dysequilibria that perturbs CQ−heme binding via changes in DV volume and perhaps pH.[2,10,11]

A complete molecular description of all CQR phenomena is likely more complex. There are at least 23 different PfCRT isoforms that have been found in distinct CQR parasite isolates,[3,12] originating from at least five geographically distinct loci [Colombia, Peru, Papua New Guinea (PNG), Phillipines, and southeast Asia (SEA)]. These parasite isolates exhibit different phenotypes, and their cognate mutant PfCRTs harbor different patterns of 4−10 amino acid substitutions. Reproducible, statistically valid CQ IC_{50} data are available for only approximately half of these isolates, because many have not yet been established as stably growing laboratory strains. Nonetheless, the IC_{50} values reveal a wide range of CQ sensitivity across CQR parasites (ref 12 and Table 1) that is either solely or in part due to PfCRT mutation. It is currently not known precisely how much various PfCRT isoforms contribute to the various CQ IC_{50} shifts seen around the globe. On the basis of early *P. falciparum* allelic exchange experiments,[32] it is often assumed that the PfCRT amino acid substitutions in these isoforms are necessary and sufficient for the shift in CQ IC_{50} that is observed in the cognate CQR strain. Close inspection of the data in ref 13 shows that for the Dd2 (SEA) and 7G8 (South America) CQR-associated PfCRT isoforms, this is a reasonable assumption because 70−90% of the shift in CQ IC_{50} for laboratory CQR strains Dd2 and 7G8 is recapitulated by allelic exchange of wild-type *pfcrt* with Dd2 or 7G8 *pfcrt*. However, no other naturally occurring, CQR-associated, PfCRT isoforms have yet been expressed in allelic exchange models, so it is unclear if this is the case for all CQR isoforms. Also, we have recently reported that CQ IC_{50} (quantification of cytostatic or growth inhibitory potential) and CQ LD_{50} (quantification of cytocidal or parasite cell kill activity) are considerably different for CQR strains of *P. falciparum*.[33] A consequence is that well-characterized CQR strain Dd2 is 10-fold CQR^{CS} as measured by IC_{50} ratio versus CQS strain HB3 but is 125-fold cytocidal CQR (CQR^{CC}) when the ratio of LD_{50} values for the same strains are used. These and

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Table 1. Strains and Isolates of *P. facliparum* (*Pf*CRT haplotypes and CQ IC_{50} values)[a]

		*Pf*CRT Amino Acid Positions																												
Origin	Clone/Isolate	39	72	74	75	76	97	123	144	148	160	194	198	205	220	251	271	277	326	327	333	334	350	356	371	Line/ Isolate	IC50 (Low,High)	Ref
Honduras	HB3	S	C	M	N	K	H	H	A	L	L	I	E	T	A	F	Q	N	N	I	T	S	C	I	R	L	12.3, 33.9	13,14,1
Netherlands	3D7	S	C	M	N	K	H	H	A	L	L	I	E	T	A	F	Q	N	N	I	T	S	C	I	R	L	5.8, 30.4	15,16,17
PNG	D10	S	C	M	N	K	H	H	A	L	L	I	E	T	A	F	Q	N	N	I	T	S	C	I	R	L	6.0, 40.7	18,16,1
Kenya	K39	S	C	M	N	K	H	H	A	L	L	I	E	T	A	F	Q	N	N	I	T	S	C	I	R	L	3.9, 43.2	15,14,1
Thailand	T2/C6	S	C	M	N	K	H	H	A	L	L	I	E	T	A	F	Q	N	N	I	T	S	C	I	R	L	9.7, 29.5	15,14,1
S. Africa	Fab9	S	C	M	N	K	H	H	A	L	L	I	E	T	A	F	Q	N	N	I	T	S	C	I	R	L	9.7, 27.5	15,14,1
S. Leone	SL/D6	S	C	M	N	K	H	H	A	L	L	I	E	T	A	F	Q	N	N	I	T	S	C	I	R	L	7.8, 25.9	15,14,1
Kenya	M24	S	C	M	N	K	H	H	A	L	L	I	E	T	A	F	Q	N	N	I	T	S	C	I	R	L	5.8, 23.4	15,14,1
Sudan	REN	S	C	M	N	K	H	H	A	L	L	I	E	T	A	F	Q	N	N	I	T	S	C	I	R	L	5.8, 16.2	15,14,1
Liberia	LF4/1	S	C	M	N	K	H	H	A	L	L	I	E	T	A	F	Q	N	N	I	T	S	C	I	R	L	7.8, 15.0	15,14,1
Malaysia	Camp/A1	S	C	M	N	K	H	H	A	L	L	I	E	T	A	F	Q	N	N	I	T	S	C	I	R	L	7.8, 12.5	15,14,1
Mali	BC5	S	C	M	N	K	H	H	A	L	L	I	E	T	A	F	Q	N	N	I	T	S	C	I	R	L	11.6	15,1
Mali	M5	S	C	M	N	K	H	H	A	L	L	I	E	T	A	F	Q	N	N	I	T	S	C	I	R	L	21.0	14,1
Haiti	Haiti	S	C	M	N	K	H	H	A	L	L	I	E	T	A	F	Q	N	N	I	T	S	C	I	R	L	5.8, 16.2	15,14,1
Sudan	106/I	S	C	I	E	K	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	37.8, 15	19,20,1
Thailand	Dd2	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	T	I	L	404, 48	21,14,1
Thailand	Thai16	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	T	I	L	436	14,22
Thailand	Thai19	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	T	I	L	874	14,22
Thailand	TM284	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	T	I	L	155, 363	15,14,1
Thailand	C2A	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	T	I	L	301	14,22
Vietnam	V1/S	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	T	I	L	186, 659	15,14,1
Cambodia	JCK	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	T	I	L	521.3	14,22
Sudan	102/1	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	T	I	L	164, 431	15,14,1
SEA	D5	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	T	I	L	354	14,22
Thailand	TM93-C1088	S	C	I	E	T	L		A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	T	I	L	1200	14,22
Thailand	FCB	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	135, 492	15,14,1
Thailand	K1	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	75.2, 320	18,30,17
Uganda	PAR	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	124	15,1
Gambia	FCR-3	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	200	15,22
Kenya	KMWII	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	77.5	15,1
S. Africa	RB8	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	116, 152	15,14
S. Africa	RB20	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	233	14,22
Ghana	9020	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	468	14,22
Ghana	9013	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	305	14,22
SEA	P31	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	455	14,22
Sudan	124/8	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	439	14,22
Sudan	128/4	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	87.2, 379.4	15,14,1
Mali	M2	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	116, 320	15,14,1
SEA	ItG2F6	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	124, 128	15,14,1
Gambia	M97	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	518	14,22
Sao Tome	Cai	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	L	96.9	15,14
Thailand	TM6	S	C	I	E	T	H	R	A	L	L	I	E	A	S	F	E	N	S	I	T	S	C	I	I	L	111, 167	23,24,25
Thailand	BC7	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	S	-	T	S	C	T	I	I	54.0	21
Thailand	J9	P	C	I	E	A	H	H	A	L	L	I	E	T	S	F	E	D	S	I	T	S	C	T	I	I	123	21
Thailand	KS28	S	C	I	E	T	H	H	A	L	L	I	E	T	S	-	E	N	S	I	T	S	C	T	I	I	74.0	21
Thailand	BC22	S	C	I	E	T	H	H	A	L	L	I	K	T	S	F	E	N	S	I	T	S	C	T	I	I	42.0	21
Brazil	7G8	S	S	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	S	C	L	R	L	34, 220	18,14,1
Peru	PC26	S	S	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	S	C	L	R	L	689	14,22
Brazil	DIV17	S	S	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	S	C	L	R	L	441	14,22
Brazil	DIV14	S	S	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	S	C	L	R	L	430	14,22
Brazil	ECP	S	S	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	S	C	L	R	L	288	14,1
Brazil	PAD	S	S	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	S	C	L	R	L	269	14,1
Brazil	ICS	S	S	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	S	C	L	R	L	347	14,22
PNG	PNG3	S	S	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	S	C	L	R	L	243	14,22
PNG	PNG2	S	S	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	S	C	L	R	L	226	14,22
Brazil	DIV30	S	S	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	S	C	L	R	L	217	14,22
PNG	PNG13	S	S	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	S	C	L	R	L	198	14,22
Solomon	PNG4	S	S	M	N	T	H	H	A	L	L	I	E	T	A	F	Q	N	D	I	T	S	C	L	R	I	251	14,25
Ghana	GB4	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	N	I	T	S	C	I	I	L	89.8, 144	6,13
Mali	S35CQ	S	C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	N	I	T	S	C	I	I	I	230	14,1
Cambodia	742		C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	N	I	T	S	C	I	I	I	467	26
Cambodia	766		C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	N	I	T	S	C	I	I	I	53.9	26
Cambodia	783		C	I	E	T	H	H	A	L	L	I	E	T	S	F	E	N	N	I	T	S	C	T	I	I	134	26
Cambodia	738		C	I	D	T	H	H	A	I	L	T	E	T	S	F	E	N	N	I	S	S	C	I	R	I	157	26
Cambodia	734		C	I	D	T	H	H	F	I	L	T	E	T	S	F	E	N	N	I	S	S	C	I	R	I	169	26
Cambodia	613		C	I	D	T	H	H	F	I	L	T	E	T	S	F	E	N	N	I	S	S	C	I	R	I	33.6	26
Phillipines	PH1	S	C	M	N	T	H	H	T	L	Y	I	E	T	A	F	Q	N	D	I	T	S	C	I	R	I	95.0	18
Phillipines	PH2		S	M	N	T	H		T	L	Y	I	E	T	A	F	Q	N	D	I	T	S	C	I	R	I	N.D.	18
Ecuador	Ecu1110	S	C	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	S	C	L	R	L	90.0, 156	13,14,1
Peru	PC17	S	C	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	S	C	L	R	L	373.8	14,22
Colombia	Jav	S	C	M	E	T	Q	H	A	L	L	I	E	T	S	F	Q	N	N	I	T	S	C	I	T	L	137, 305	13,14,1
Colombia	TU741	S	C	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	N	C	L	R	I	N.D.	27
Colombia	TA7519	S	C	M	E	T	Q	H	A	L	L	I	E	T	S	F	Q	N	N	I	T	S	C	I	T	I	N.D.	27
Colombia	TA6182	S	C	M	E	T	Q	H	A	L	L	I	E	T	S	F	Q	N	S	I	T	S	C	I	I	I	N.D.	27
China	Isolate B	S	C	I	D	T	H		Y	L	L	I	E	T	A	F	E	N	N	I	T	S	C	I	R	I	N.D.	28
China	Isolate C	S	C	I	D	T	H		Y	L	L	I	E	T	A	F	E	N	N	I	T	S	C	I	I	I	N.D.	28
China	Isolate D	S	C	I	E	T	H		Y	L	L	I	E	T	A	F	E	N	N	I	T	S	C	I	R	I	N.D.	28
China	Isolate E	S	C	I	E	T	H		A	L	L	I	E	T	S	F	E	N	N	I	T	S	C	I	R	I	N.D.	28
I. Papau	2300	S	C	I	K	T	H	H	A	L	L	I	E	T	S	F	E	N	S	I	T	S	C	I	I	I	N.D.	29
F. Guiana	H209	S	S	M	N	T	H	H	A	L	L	I	E	T	S	F	Q	N	D	I	T	S	R	L	R	I	35	16

[a]Residues mutated relative to the wild-type sequence are highlighted in green. Empty cells denote residues for which sequence data are not available, and dashes denote deletion mutations. For cases in which multiple IC_{50} values (in nanomolar) were found in the literature, the high and low values were reported. Sources of low IC_{50} values, high IC_{50} values (if applicable), and PfCRT sequencing data are referenced in order (far right).

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Table 2. Oligonucleotides Used in This Study

name	sequence (5′−3′)
A220S	CATCTACCTGTCAGTTTGCGTGATAGAGACGATCTTCGCTAAGAGAACCTTGAA
K76T	CAGTTTGCGTGATGAACACGATCTTCGCTAAGAGAAC
N75E	CCTGTCAGTTTGCGTGATGGAGAAGATCTTCGCTAAGAGAA
A144T	CTTGCAGCGTCATCTTGACCTTCATCGGTCTTACC
A220S	CAACCTAGTCCTGATTAGCAGTCTGATCCCTGTCTGTTTC
AL-144,148-FI	CTTGCAGCGTCATCTTGTTCTTCATCGGTATTACCAGAACCACAGGT
CK-72,76-ST	CCATCATCTACCTGTCAGTTAGCGTGATGAACACGATCTTCGC
CMNK-72,74-6-RIEI	CATCTACATCCTGTCCATCATCTACCTGTCAGTTCGCGTGATAGAGATAATCTTCGCTAAGAGAACC
E75D	CCTGTCAGTTTGCGTGATCGATACCATCTTCGC
H97L	CGTGACTAGTGAAACCCTCAACTTCATCTGCATGA
H97Q	CGTGACTAGTGAAACCCAGAACTTCATCTGCATGATC
I194T	CAGTAATCATCGTAGTCACAACCGCATTGGTGGAAATG
I356T	CATCCAGGGTCCCGCAACCGCTATTGCCT
I356V	GCATCCAGGGTCCCGCAGTCGCTATTGC
I371R	CTTAGCAGGTGATGTCGTAAGAGAACCACGTTTGTTG
K76N	CCTGTCAGTTTGCGTGATGAACAATATCTTCGCTAAGA
L148I	CTTGGCCTTCATCGGTATTACCAGAACCACAGG
L160Y	CAGGTAACATTCAGTCCTTCGTCTATCAACTATCAATTCCAATCAACATG
M74I	CATCTACCTGTCAGTTTGCGTGATAAACAAGATCTTCG
MN_7−45_IE	CCATCATCTACCTGTCAGTTTGCGTGATAGAGAAGATCTTCGCTAAG
MNK_7−456_IEI	TCCTGTCCATCATCTACCTGTCAGTTTGCGTGATAGAGATAATCTTCGCTAAGAGAACC
MNK74−76IET	CATCTACCTGTCAGTTTGCGTGATAGAGACGATCTTCGCTAAGAGAACCTTGAA
N326D	CGCCTTGTTCTCATTCTTCGACATCTGTGATAACCTGAT
N326S	GCCTTGTTCTCATTCTTCAGCATCTGTGATAACCTGATC
NK_7−56_ET	ACCTGTCAGTTTGCGTGATGGAGACGATCTTCGCTAAGAGAACCT
P275L	CTGAAGGAGTTACACTTGCTATACAACGAAATCTGGACC
Q271E	CACACTACCATTCCTGAAGGAGTTACACTTGCCATACAACG
Q352K	TATTGTGAGTTGCATCAAGGGTCCCGCAATCGC
R371I	CTTAGCAGGTGATGTCGTAATAGAACCACGTTTGTTG
R371T	TTCTTAGCAGGTGATGTCGTAACGGAACCACGTTTGTTGG
S163R	TCAGTCCTTCGTCTTGCAACTAAGAATTCCAATCAACATGTTCTTC
S220A	CCTAGTCCTGATATCCGCTCTGATCCCTGTCTG
S326SN	TTCGCCTTGTTCTCATTCTTCAACATCTGTGATAACCTGATCAC
S72C	GTCCATCATCTACCTGTCAGTTTGCGTGATGAACAC
T152A	TCGGTCTTACCAGAACCGCAGGTAACATTCAGTCC
T333S	ATCTGTGATAACCTGATCAGCAGCTACATCATCGATAAG
T356I	ATCCAGGGGCCCGCAATCGCTATTGCC
T76I	CAGTTTGCGTGATCGAAATCATCTTCGCGAAGAGAA
T76K	GTCAGTTTGCGTGATCGAAAAGATCTTCGCGAAGAGAACCT
T76N	CAGTTTGCGTGATCGAAAACATCTTCGCGAAGAGAA
E75K	CCTGTCAGTTTGCGTGATCGAAACCATCTTCGCG
H123R	GGGTAACAGCAAGGAACGTCGTAGGAGCTTCAAC
T205A	GAAGCTGAGCTTCGAAGCACAGGAAGAGAACTC
C350R	CACTATTGTGAGTCGCATCCAGGGGCCCGC

related data indicate that additional genetic events could perhaps complement PfCRT mutation to confer CQR.[33,34]

In summary, it is not yet entirely clear precisely how much altered CQ transport due to PfCRT amino acid substitutions contributes to all CQR phenomena (e.g., various IC_{50} vs LD_{50} phenomena), and even though PfCRT mutations likely confer the majority of the shift in CQ IC_{50} for some CQR strains of *P. falciparum*,[32] it is not yet known if PfCRT mutations alone are responsible for the CQ IC_{50} shifts seen across all globally distributed isolates. Parasite allelic exchange experiments that might help to clarify these issues are very difficult, and there appear to be poorly understood compensatory genetic events that are required for the expression of certain CQR PfCRTs in various parasite genetic backgrounds.[16] To further explore these issues, we have improved upon a previously described approach[35] to rapidly distinguish even subtle differences between the functions of CQR-associated PfCRT isoforms.

MATERIALS AND METHODS

Materials. Yeast DOB media and DOB with galactose and raffinose were obtained in powder form from MP Biomedicals (Solon, OH). Cell culture plastics were from BD Falcon. Glass beads for yeast cell lysis were from B. Braun Biotech (Allentown, PA). Anti-HexaHis-HRP and anti-V5-HRP antibodies were from Qiagen (Valencia, CA) and Invitrogen (Carlsbad, CA), respectively. [^{3}H]CQ was from American Radiolabeled Chemicals Inc. (St. Louis, MO). All other chemicals were reagent grade or better, purchased from Sigma (St. Louis, MO), and used without additional purification.

Yeast Strains and Methods. CH1305 (MATa *ade2 ade3 ura3-52 leu2 lys2-801*) was supplied by J. F. Cannon.[36] Solid and liquid media were prepared as described by Sherman et al.[37] and included synthetic complete (SC) medium lacking one or more specified amino acids, as well as rich medium (YPAD or YPD). Induction of CRT protein expression, standard yeast growth methods, yeast transfections, and other routine methods were as described previously.[35]

Plasmids. The pYES2 backbone containing PfHB3vh, PfDd2vh, and Pf7G8vh was constructed previously[35] and used as template DNA in subsequent rounds of multi-site-directed mutagenesis via the Agilent QUICKChange method to create the various isoforms of PfCRT (see Table 2). All constructs were confirmed by direct DNA sequencing of the full *pfcrt* gene.

Western Blotting. This procedure was as described previously.[35]

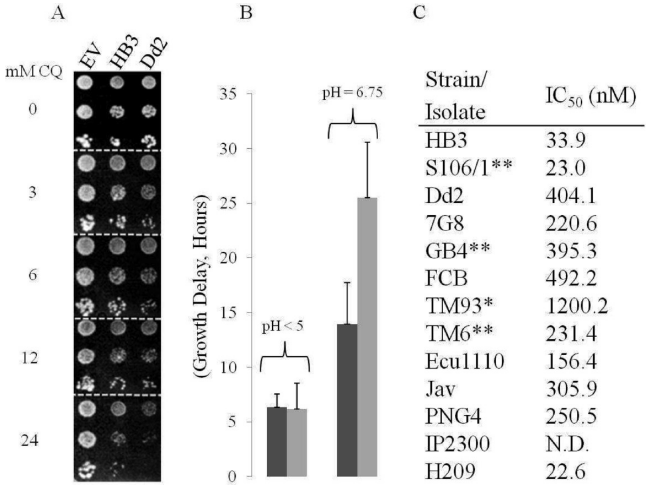
Colony Formation Assays and Quantitative Growth Analysis. Was performed under CRT-inducing and non-inducing conditions as described previously[35] with some modifications. For ΔpH- and Δψ-dependent growth curve analysis, yeast harboring different isoforms of CRT were assayed in synthetic complete media containing additional 100 mM KCl and buffered with 100 mM HEPES (pH 6.75) (see Results). Growth under each condition was measured in triplicate via back dilution of the strain grown under normal noninducing conditions (SD media lacking uracil). Under normal conditions [medium external pH (pH_{ex}) of ~5.0], the yeast PM maintains a high ΔpH and a low Δψ, but alkalinization of the external medium lowers the ΔpH and increases the Δψ concomitantly such that a substantial Δψ compensates for the loss of ΔpH (see ref 35).

[^{3}H]CQ Whole Cell Accumulation Assays. [^{3}H]CQ transport specific to PfCRT was assayed as previously described.[35] Steady state accumulation (at 30 min incubation, pH 7.5) of CQ is reported.

RESULTS

Previously, we found that elevating the plasma membrane electrical potential (ΔΨ) in yeast that were expressing CQR-associated PfCRT protein increased CQ accumulation for the yeast.[35] In contrast, an elevated ΔΨ did not increase the level of CQ transport mediated by CQS-associated PfCRT or by the *Plasmodium vivax* CRT orthologue PvCRT.[35] We thus hypothesized that optimizing assay conditions such that the yeast plasma membrane ΔΨ was maximal would facilitate distinction between CQS (e.g., HB3) and CQR (e.g., Dd2) PfCRT isoforms. Indeed, Figure 1A shows results from plate spotting assays, and Figures 1B and 2A,B show quantitative growth curve analyses in which ΔΨ is clamped to higher values by growth in high-K^+, pH 6.75 medium (which decreases ΔpH and increases $\Delta\Psi$[2,35]). At higher ΔΨ values, because CQ transport by CQR (Dd2) PfCRT is stimulated,[35] lower concentrations of CQ are sufficient to assay PfCRT function and to distinguish CQS (HB3) from CQR (Dd2) isoforms (Figures 1B and 2B).

We wondered if this increased sensitivity would facilitate clearer distinction between different CQR-associated PfCRT isoforms found in geographically distinct CQR isolates. We expressed 13 distinct PfCRT isoforms using the galactose inducible system described previously.[35] All isoforms are expressed to similar levels (Figure 3 and Table 3) Screening these yeast for growth defects due to PfCRT expression in the presence of 16 mM CQ (Figure 2C,D and Table 4) reveals variable responses due to PfCRT-mediated accumulation of toxic CQ.[35]



Strain/ Isolate	IC_{50} (nM)
HB3	33.9
S106/1**	23.0
Dd2	404.1
7G8	220.6
GB4**	395.3
FCB	492.2
TM93*	1200.2
TM6**	231.4
Ecu1110	156.4
Jav	305.9
PNG4	250.5
IP2300	N.D.
H209	22.6

Figure 1. Improved growth assays. HB3 (CQS) and Dd2 (CQR) PfCRT-dependent growth (A) on solid agar plates over a range of CQ concentrations (millimolar, left-hand side; EV, empty vector; or no PfCRT expressed) and (B) in liquid growth medium for yeast expressing PfCRTHB3 (black) or PfCRTDd2 (gray). Note that expression of either HB3 or Dd2 isoforms slows growth specifically in the presence of CQ and that the effect is strongest for Dd2, particularly at high pH [high ΔΨ (see also ref 35)]. (C) *P. falciparum* strains and isolates (and their CQ IC_{50} values) whose cognate CRT isoforms were analyzed within. One asterisk denotes a strain also known as TM93-C1088. Two asterisks denote that the IC_{50} value shown has been normalized to the IC_{50} reported by Mu and colleagues[14] by using CQS control data from the same study (cf. Table 1).

Not all CQR *P. falciparum* isolates from around the globe have been established as stable laboratory strains. Thus, conventional IC_{50} assays cannot be conducted for some isolates; rather, single-passage quantification of [^{3}H]hypoxanthine accumulation is used to assess sensitivity to CQ. These assays are difficult and highly operator-dependent and often do not allow for reproducible quantification of IC_{50}. We therefore focused our efforts on further quantifying PfCRT isoforms to those 11 found in isolates that have been established as stable laboratory strains (Figure 1C). Fortuitously, these strains show the full range of CQ IC_{50} values that have so far been observed for all strains and isolates (Table 1), and one laboratory has quantified IC_{50} values for nearly all of them using an identical technique.[14]

Correspondingly, yeast harboring these isoforms show variable sensitivity to the growth inhibitory effects of external CQ (Figure 2C,D and Table 4). Not surprisingly, these yeast also show variable levels of [^{3}H]CQ accumulation that is due to variable drug transport function of the different CQR PfCRT isoforms.[35] When we plot relative [^{3}H]CQ accumulation versus the degree of CQ-induced growth inhibition for yeast expressing these PfCRTs, a linear correlation is obtained (Figure 4A). This very strongly supports our simple model proposed previously[35] in which yeast strain growth inhibition due to external CQ is due to PfCRT-mediated transport of toxic CQ into the yeast. Interestingly, however, when either relative growth (Figure 4B) or relative [^{3}H]CQ uptake (Figure 4C) for yeast harboring a given CQR PfCRT isoform is plotted versus the CQ IC_{50} observed for the *P. falciparum* strain harboring the same isoform, no correlation is obtained.

DISCUSSION

Initial quantification of the contribution of amino acid substitutions in Dd2 and 7G8 isoforms of PfCRT to the elevated

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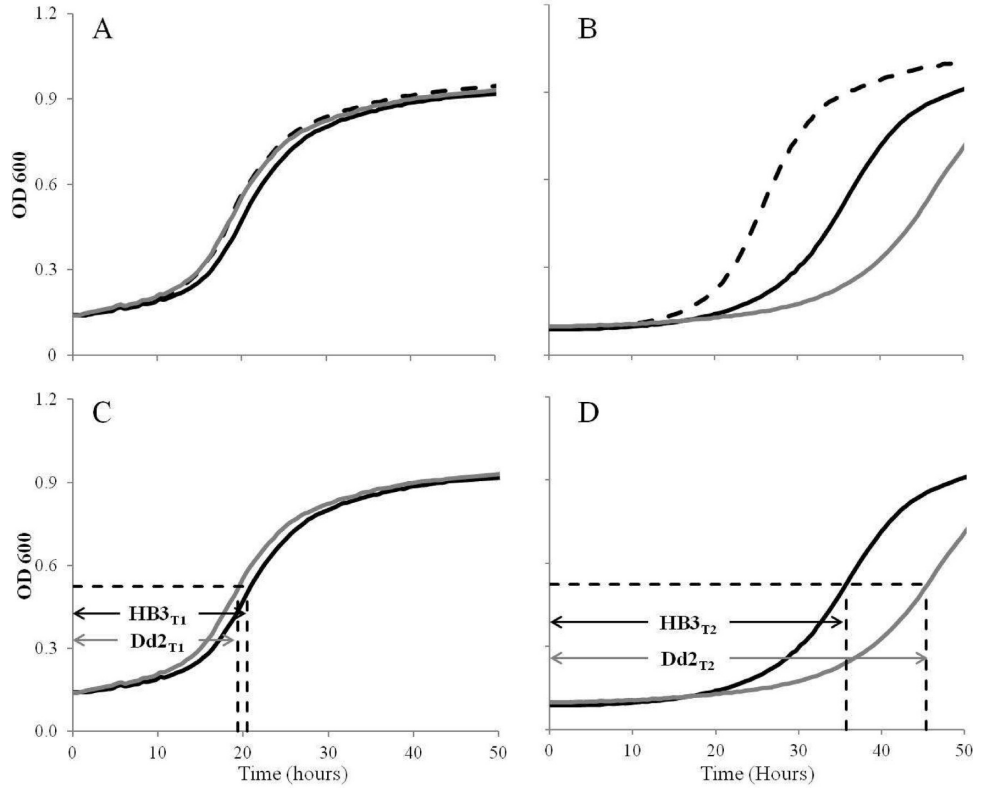

Figure 2. Growth of yeast harboring pYES2 (−−−), pYES2/HB3CRT (black), or pYES2/Dd2CRT (gray) in medium containing 16 mM CQ under noninducing (glucose, panel A, top left) or inducing (galactose, panel B, top right) conditions. (C and D) Quantification of PfCRT isoform-dependent growth delay in *Saccharomyces cerevisiae* by subtracting the time to reach the maximal growth rate (as identified by the time corresponding to the growth curve maximal slope) under noninducing conditions (C, $HB3_{T1}$ and $Dd2_{T1}$) from the corresponding time measured under inducing conditions (D, $HB3_{T2}$ and $Dd2_{T2}$). Results from quantification for each yeast strain are listed in Table 4.

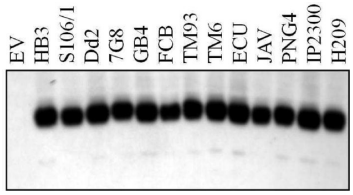

Figure 3. Western blot analysis of *S. cerevisiae* yeast membranes expressing the indicated PfCRT isoform. Each lane contained 10 μg of total protein. The α-V5 blot shows similar levels of protein expression are found for each PfCRT isoform (see Table 3 for quantification vs at least three blots for each isoform).

CQ IC_{50} observed in CQR strains Dd2 and 7G8 suggested that most, if not all, of the variable CQR phenotype in these strains was due to the different PfCRT mutations.[32] Additional studies over the past 10 years have suggested that elevated IC_{50} values in these strains are likely due to heightened CQ transport by the mutant PfCRTs (reviewed in refs 2 and 3). Since then, additional distinct PfCRT mutants have been discovered in other CQR isolates. These have evolved under different selective drug pressures and exhibit different CQ IC_{50} values relative to those of strains Dd2 and 7G8. Some of these isolates have been established as laboratory strains in which other aspects of the CQR phenotype have also been found to quantitatively differ relative to those in strains Dd2 and 7G8.

Two competing hypotheses for the molecular basis of these differences exist. One is that the different mutation patterns in the PfCRTs yield transporters with variable drug transport efficiencies, producing different levels of intra-DV drug that then result in different IC_{50} values. In this case, we would expect the relative ability of mutant PfCRTs to transport CQ or to induce CQ-dependent yeast growth inhibition to correlate in some fashion with the CQ IC_{50} found for the cognate *P. falciparum* strain. This is not found to be the case (cf. Figure 4B,C). The second hypothesis is that different degrees of CQ transport by various mutant PfCRTs are only partly responsible for CQ IC_{50} variability, and that other genome mutations [and/or other

Table 3[a]

isoform	average band density	standard error of the mean
HB3	1.00	
S106/1	0.99	0.04
Dd2	1.02	0.09
7G8	1.00	0.12
GB4	1.00	0.04
FCB	0.98	0.05
TM93	0.98	0.11
TM6	1.03	0.07
Ecu1110	0.95	0.13
JAV	0.95	0.08
PNG4	0.99	0.12
IP2300	1.01	0.01
H209	1.01	0.08

[a]Quantification of expression of different PfCRT isoforms in yeast (see also Figure 3). Densitometry data are the average of at least three separate western blots (± the standard error of the mean) where each lane was loaded with the same total protein content as defined by the amido black assay (see ref 35).

Table 4. Growth Delay Data for Yeast Expressing All the Different CRT Isoforms Analyzed in This Study[a]

isoform	growth delay (h)	standard error of the mean
HB3	13.9	1.1
S106/1	11.8	1.5
Dd2	25.5	2.5
7G8	19.4	2.6
GB4	19.3	2.4
TM93	21.8	1.7
TM6	18.9	0.2
FCB	22.4	1.8
Ecu1110	30.7	3.6
JAV	29.2	3.2
PNG4	23.4	1.8
H209	31.9	0.3
IP2300	13.1	0.1

[a]Growth delay is expressed as the difference in hours (see Figure 2C,D) for growth in the presence of 16 mM CQ under PfCRT-inducing vs noninducing conditions (see ref 35).

mutant PfCRT functions (see refs 10 and 11)] complement PfCRT CQ transport to produce different CQ IC_{50} values. Direct data in support of either hypothesis have not been attainable until now, other than through *P. falciparum* allelic exchange experiments and quantitative trait locus (QTL) analysis of genetic cross progeny. The former experiments are technically quite difficult, because of low-frequency site-specific recombination in malarial parasites, and the latter generally do not allow for analysis of the role of individual genes. The alternate yeast-based approach presented here provides much more rapid quantification of variable PfCRT isoform function. This approach should also be useful for screening PfCRT inhibitor preferences versus different PfCRT isoforms and for further dissecting the function of PfCRT orthologues such as PvCRT. It is also useful for analyzing PfCRTs found in isolates that have not been (or cannot be) established as strains; we include two such isoforms (IP2300 and H209[16]) whose degree of function is somewhat controversial, because the yeast data cleanly provide a [^{3}H]CQ transport phenotype.

We have quantified subtle differences in the function of mutant PfCRT isoforms from geographically distinct CQR *P. falciparum* isolates. For isoforms found in isolates that have been established as stable strains, and for which reliable CQ IC_{50} values thus exist, we have quantified PfCRT function in two ways (Figure 4). We find, not surprisingly, that PfCRT-mediated CQ uptake very strongly correlates with CQ-dependent growth inhibition for yeast expressing different PfCRT isoforms (Figure 4A). The molecular basis of yeast CQ growth inhibition is not fully elucidated, but it is probably due to the well-known lysosomatropic actions of CQ at these doses. Importantly, clinically relevant doses of CQ will yield approximately millimolar levels of CQ within the parasite DV (see ref 34 and references within), and the magnitude and polarity of ΔpH and $\Delta\Psi$, as well as appropriate PfCRT membrane topology, are all preserved in yeast plasma membrane versus the DV membrane. Thus, the transport of CQ from outside yeast to inside directly mimics the transport of CQ from inside the DV to the parasite cytosol.[35]

We find that regardless how the data are plotted, good correlation is not observed between PfCRT isoform function and CQ IC_{50} in the cognate *P. falciparum* strain. This demonstrates that drug transport due to amino acid substitutions in PfCRT is

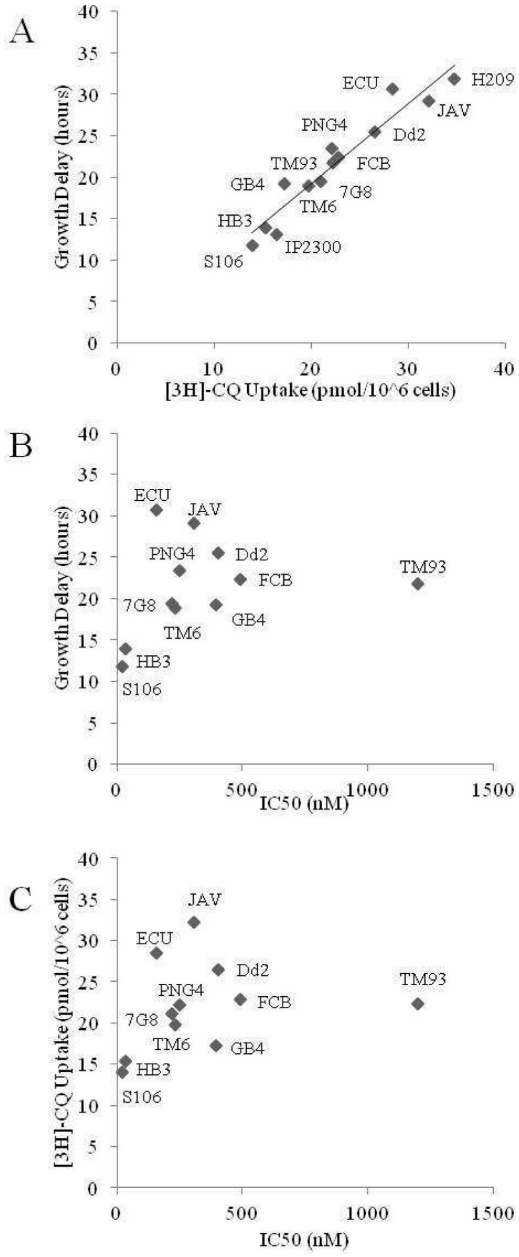

Figure 4. (A) Linear correlation between isoform-induced growth delay (see Figure 3) and isoform-facilitated CQ uptake (R^2 = 0.91). (B) Isoform-induced growth delay plotted vs CQ IC_{50} for corresponding strains. No apparent correlation is found with either the TM93 "outlier" included (R^2 = 0.05) or omitted (R^2 = 0.23). (C) PfCRT isoform-specific CQ transport plotted vs CQ IC_{50} for corresponding strains. No apparent correlation is found with either the TM93 "outlier" included (R^2 = 0.05) or omitted (R^2 = 0.19).

necessary, but not sufficient, for influencing CQ IC_{50}, and that additional gene mutations, or additional mutant PfCRT functions, in a variety of isolates likely influences CQ IC_{50} values. Such a conclusion is not as surprising as it might initially appear; mutation or altered expression of PfMDR1 protein has also been associated with mild (2−3-fold) changes in drug IC_{50} values,[5,38−40] and our studies with recombinant PfMDR1 suggest that the protein binds CQ with high affinity.[41] In some strains, PfMDR1 alleles may contribute in pairwise fashion to PfCRT mutations in shifting CQ IC_{50} values.[5] We suggest other genes or physiologic phenomena also contribute to CQ IC_{50} values and

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that because of different drug selection histories in the regions from which these isolates originate, the contribution from other genes will also vary in a geographically distinct fashion. Perhaps relatedly, we note that two laboratories have reported that altered CQ transport in drug resistant *P. falciparum* parasites does not necessarily correlate with the degree of parasite resistance measured at high levels of CQ.[33,34,42] This may reflect altered targets for cytostatic versus cytocidal doses of CQ as proposed[2,33,34] and/or be a consequence of an additional or overlapping mechanism for CQR that is not yet defined.

Finally, we note that strain TM93 appears to be particularly interesting. This strain is reported to have an unusually high CQ IC_{50},[14] yet TM93 PfCRT shows only average CQ transport function; hence, it appears as somewhat of an outlier in panels B and C of Figure 4. The molecular features in addition to CQ transport by mutant PfCRT that contribute to the very high IC_{50} in strain TM93 deserve additional study. Strains for which PfCRT drug transport contributions to resistance appear to be small, which can be easily identified via the methods in this paper (e.g., TM93), should provide particularly fertile ground for additional studies of antimalarial drug resistance phenomena.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CQ, chloroquine; CQR and CQS, chloroquine resistant and sensitive, respectively.

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