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Mutation in the *Plasmodium falciparum* CRT Protein Determines the Stereospecific Activity of Antimalarial *Cinchona* Alkaloids

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The *Cinchona* alkaloids are quinoline aminoalcohols that occur as diastereomer pairs, typified by (−)-quinine and (+)-quinidine. The potency of (+)-isomers is greater than the (−)-isomers *in vitro* and *in vivo* against *Plasmodium falciparum* malaria parasites. They may act by the inhibition of heme crystallization within the parasite digestive vacuole in a manner similar to chloroquine. Earlier studies showed that a K76I mutation in the digestive vacuole-associated protein, PfCRT (*P. falciparum* chloroquine resistance transporter), reversed the normal potency order of quinine and quinidine toward *P. falciparum*. To further explore PfCRT-alkaloid interactions in the malaria parasite, we measured the *in vitro* susceptibility of eight clonal lines of *P. falciparum* derived from the 106/1 strain, each containing a unique *pfcrt* allele, to four *Cinchona* stereoisomer pairs: quinine and quinidine; cinchonidine and cinchonine; hydroquinine and hydroquinidine; 9-epiquinine and 9-epiquinidine. Stereospecific potency of the *Cinchona* alkaloids was associated with changes in charge and hydrophobicity of mutable PfCRT amino acids. In isogenic chloroquine-resistant lines, the IC_{50} ratio of (−)/(+) CA pairs correlated with side chain hydrophobicity of the position 76 residue. Second-site PfCRT mutations negated the K76I stereospecific effects: charge-change mutations C72R or Q352K/R restored potency patterns similar to the parent K76 line, while V369F increased susceptibility to the alkaloids and nullified stereospecific differences between alkaloid pairs. Interactions between key residues of the PfCRT channel/transporter with (−) and (+) alkaloids are stereospecifically determined, suggesting that PfCRT binding plays an important role in the antimalarial activity of quinine and other *Cinchona* alkaloids.

Alkaloids from the *Cinchona* tree, exemplified by quinine (QN) and quinidine (QD), have proven to be an important source of antimalarial therapies, especially after resistance to chloroquine (CQ) emerged. QN remains a first-line drug in the treatment of severe malaria in many parts of the world, even with increased use of artemisinin-based combination therapies (70). The *Cinchona* alkaloids (CA) are aryl amino alcohols where the aryl group is a quinoline (quinoline aminoalcohols). They have four chiral centers, two of which, C-8 and C-9, can have different configurations (66) (Fig. 1). Among the pharmacologically active 8,9-*erythro* isomers, QN, hydroquinine (HQN), and cinchonidine (CD) all present the *S* configuration around C-8 and the *R* configuration at C-9 and are levorotatory (rotate plane polarized light in an anticlockwise [−] direction). The reverse ordering, *R* at C-8 and *S* at C-9, occurs in the respective dextrorotatory (rotate plane polarized light in a clockwise [+] direction) diastereomers, QD, hydroquinidine (HQD), and cinchonine (CN). The 8,9-*threo* diastereomers 9-epiquinine (EQN) and 9-epiquinidine (EQD) are 8*S*, 9*S*, and 8*R*, 9*R*, respectively.

Against *Plasmodium falciparum*, the CA diastereomer pairs have a well-established *in vitro* potency order. The dextrorotatory isomers QD, HQD, and CN have greater antimalarial potencies than their levorotatory counterparts, QN, HQN, and CD (22, 37, 68). EQN (weakly dextrorotatory) and EQD (dextrorotatory) are comparatively ineffective; a previous study noted lower efficacies of approximately 100-fold compared to QN and QD (37). Observations of stereospecificity extend to *in vivo* studies on QN and QD efficacy against *P. falciparum*, where the latter is the more potent clinically (62, 69).

The mechanism of action of the quinoline aminoalcohol QN and the blood schizontocidal 4-aminoquinolines, including CQ, is believed to depend upon their weakly basic properties that promote accumulation to high levels in the acidic digestive vacuole (DV) of intraerythrocytic trophozoite-stage parasites. Once inside the DV, they presumably bind free heme (monomeric or dimeric Fe[III] protoporphyrin IX) (1, 18), a toxic intermediate produced from parasite proteolysis of host hemoglobin (21, 41, 65). Heme binding is thought to inhibit the formation of the more inert microcrystalline compound hemozoin, creating a poisonous buildup of lethal drug-heme complexes (48). Resistance to CQ, and possibly reduced QN potency, is linked to enhanced efflux of drug from resistant parasites (40).

Mutations in the *pfcrt* (*Plasmodium falciparum* chloroquine resistance transporter) gene are the primary cause of CQ resistance and also greatly affect QN susceptibility (20, 26). PfCRT, a protein containing 424 amino acids and 10 predicted transmembrane domains (Fig. 2), is thought to be a channel or transporter of

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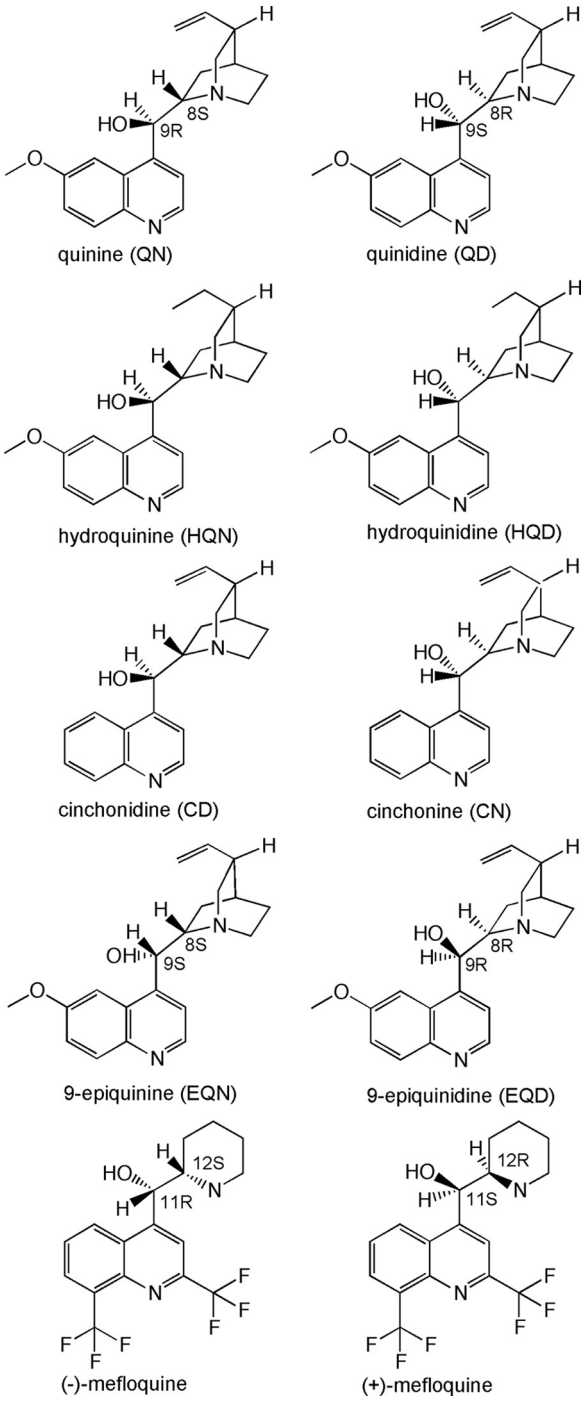

FIG 1 Chemical structures of CA and MQ stereoisomers used in this study.

the drug-metabolite transporter (DMT) superfamily (43). Although the endogenous function of PfCRT remains enigmatic, its localization within the DV membrane in trophozoite-stage parasites agrees with a role in drug resistance (14). Evidence suggests that mutated PfCRT, in particular a K76T mutation, confers CQ resistance by allowing enhanced efflux of CQ from the DV, thereby reducing the concentration of drug available to bind target heme (8, 9, 57). However, the cytotoxic properties of CQ may involve additional PfCRT-related mechanisms beyond DV accumulation (11). Our earlier studies demonstrated that in addition to producing CQ resistance in *P. falciparum*, a novel PfCRT K76I mutation resulted in a dramatic increase in QN susceptibility, reversing the normally observed potency order of QD > QN (14, 26). By taking advantage of the unique antagonistic properties of verapamil against QN in the K76I mutant clone, we used Schild analysis (2) to show that PfCRT probably binds QN (16).

The steric and electronic features of the CA that contribute to the potency of the *erythro* conformations are believed to impart an enhanced ability to form intramolecular hydrogen bonds with a plasmodial receptor(s) (18, 35, 36). Although the presence of such a target molecule serving as a stereoselective receptor appears evident in the action of the CA, the nature of this receptor has yet to be determined. Here, we have tested a panel of eight isogenic parasite clones with a series of PfCRT polymorphisms against four pairs of CA diastereomers. Our data show that stereospecific drug-PfCRT interactions are important determinants of CA potency against *P. falciparum*.

MATERIALS AND METHODS

Materials. QN, QD, HQN, HQD, CD, CN, and amantadine (AMT) were purchased from Sigma-Aldrich Chemical Co. The *erythro* CA were used without further purification despite containing up to 5 to 15% of isomers of the same rotation. EQN and EQD were kindly provided by G. Reinecke of Buchler Gmbh, Braunschweig, Germany. Racemic mefloquine (MQ) was obtained from the Walter Reed Army Institute for Research (Silver Springs, MD). The (−)- and (+)-12,11 *erythro* MQ enantiomers were synthesized by D. Sturchler at Hoffmann-La Roche, Basel, Switzerland, and kindly provided by W. Peters (London School of Hygiene and Tropical Medicine). Purity information was not available on the 9-epimers and MQ enantiomers, but 50% inhibitory concentrations (IC_{50}s) against control *P. falciparum* lines for these compounds were in agreement with literature values (37, 38). SYBR green I was purchased from Cambrex Corp. Drug stocks were prepared to 10 mM in 70% ethanol and maintained at −30°C.

Parasite cultivation and drug selection. Parasites were cultured using AB^+ or O^+ red blood cells in complete medium consisting of RPMI 1640 supplemented with 0.5% Albumax I (Invitrogen), 29.8 mM sodium bicarbonate, 25 mM HEPES, 0.37 mM hypoxanthine, and 0.01 mg/ml gentamicin and maintained at 37°C under an atmosphere of 90% nitrogen, 5% carbon dioxide, and 5% oxygen. CQ-resistant control parasites Dd2 and 7G8 and the CQ-sensitive HON (parental line of HB3) and $106/1^{K76}$ strains were kindly provided by Thomas E. Wellems, National Institute of Allergy and Infectious Diseases (Rockville, MD). Drug-resistant lines of *P. falciparum* were selected on the clonal $106/1^{K76}$ line by single-step drug selection (52) as described previously (14, 16). The PfCRT mutants $106/1^{76N}$, $106/1^{76T}$, and $106/1^{76I}$ were selected from $106/1^{K76}$ by using 100 nM CQ. Double PfCRT mutants were generated by an additional round of single-step selection on cloned $106/1^{76I}$ with either 100 nM QN or 80 μM AMT (Table 1). A selection experiment consisted of four individual 150-cm^2 culture flasks containing the desired parent parasite line. Each flask contained 50 ml of mixed-stage culture with a 5% hematocrit and ~5% parasitemia, representing $\sim 10^9$ parasites/flask. Upon reaching 5% parasitemia, drug was added at the specified concentration, which was sufficient to rapidly kill all parasites except for any preexisting, drug-resistant mutants (52). Cultures were maintained for 60 days under drug pressure and monitored by Giemsa smears for the presence living parasites. Successful selections, considered the presence of emergent parasites, occurred on average once every 10 flasks (14); thus, most four-flask experiments did not yield mutant lines. All emergent lines were cloned by limiting dilution (27) prior to DNA sequencing and drug susceptibility testing.

Sequence analysis of *pfcrt* and *pfmdr1* genes. Four randomly chosen clonal lines were used for *pfcrt* sequencing from a successful selection experiment. All open reading frame sequences of *pfcrt* were amplified

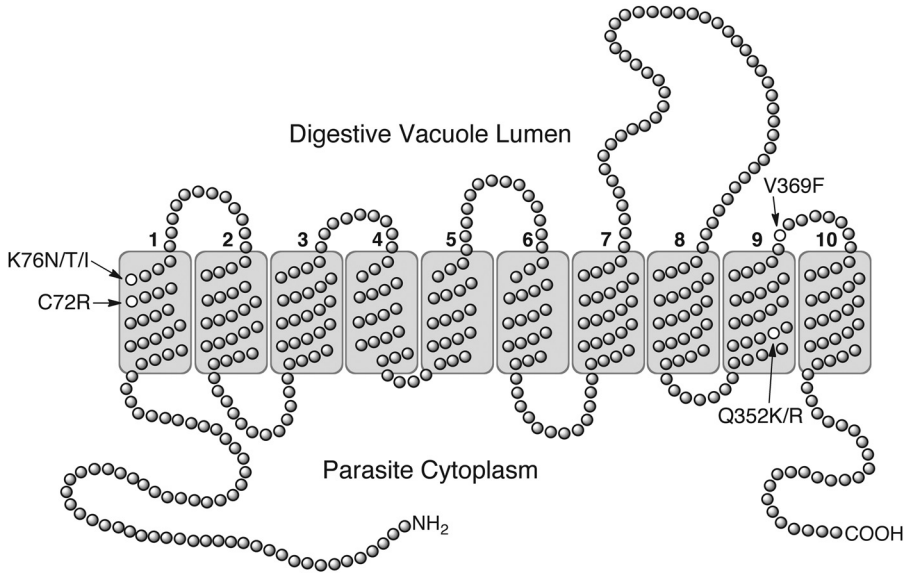

FIG 2 Topographic representation of PfCRT. Arrows and clear circles indicate polymorphic amino acids described in this study. PfCRT contains 424 amino acids and has 10 predicted transmembrane domains (43).

from *P. falciparum* genomic DNA (63). From clonal lines chosen for further study, the entire open reading frame of *pfmdr1*, or known polymorphic regions of *pfmdr1*, were amplified from genomic DNA using primers described previously (44, 51). PCR products were directly sequenced using an ABI 3730xl DNA analyzer (Applied Biosystems).

Determination of *pfmdr1* copy number by real-time PCR. The copy number of *pfmdr1* was estimated in mutant lines of *P. falciparum* by TaqMan real-time PCR using an ABI 7900HT Fast real-time PCR system as described previously (54). Assays were conducted in four independent replicates and normalized to HB3 genomic DNA, which was included in every run. The copy number for 7G8 was from Sa et al. (54a).

Drug response assays. The *in vitro* susceptibility of *P. falciparum* to the CA was measured in a 72-h, 96-well microplate fluorescence assay using SYBR green I detection as described previously (6, 61). Parasites were introduced into the drug assay at ~48-h intervals until at least three independent replicates were performed. Mean half-maximal inhibitory concentrations (IC_{50}s) were derived by plotting the percent growth inhibition against the log drug concentration and fitting the response data to a variable slope, sigmoidal curve-fit function for normalized data by using Prism 5.0d for Macintosh (GraphPad Software). IC_{50}s represent means ± standard errors of the means (SEM) from 3 to 8 independent tests. Relevant IC_{50}s between respective mutant and parental lines were tested for statistically significant differences by an F test to determine whether two dose-response data sets were best described by single or independent curve fits ($P < 0.05$).

Correlation of PfCRT position 76 amino acid hydropathy with (−)/(+) CA IC_{50} ratios. Hydropathy values at pH 5.0 for the mutable position 76 residues N, T, and I were those described in reference 39. Because the hydropathy value for N was assigned a 0 (39), an arbitrary value of 4 was added to each coefficient to accommodate log transformation. Log hy-

TABLE 1 PfCRT haplotypes, geographic origins, and CQ susceptibility profiles of *P. falciparum* lines used in this study[a]

					Variable PfCRT amino acids											PfMDR1[e]	
Parasite	Origin	Selection[b]	CQ[c]	Day[d]	72	74	75	76	220	271	326	352	356	369	371	Allele	CN
HON	Honduras		S		C	M	N	K	A	Q	N	Q	I	V	R	NFSDD	1
7G8	Brazil		R		S	M	N	T	S	Q	D	Q	L	V	R	NFCDY	1
Dd2	Thailand		R		C	I	E	T	S	E	S	Q	T	V	I	YYSND	3
106/1^{K76}	Sudan		S		C	I	E	K	S	E	S	Q	I	V	I	YYSND	2
106/1^{76N}	Lab	CQ	R	42	C	I	E	N	S	E	S	Q	I	V	I	YYSND	2
106/1^{76T}	Lab	CQ	R	33	C	I	E	T	S	E	S	Q	I	V	I	YYSND	2
106/1^{76I}	Lab	CQ	R	28	C	I	E	I	S	E	S	Q	I	V	I	YYSND	1
106/1$^{72R-76I}$	Lab	CQ-QN	S	37	R	I	E	I	S	E	S	Q	I	V	I	YYSND	1
106/1$^{76I-352R}$	Lab	CQ-QN	S	32	C	I	E	I	S	E	S	R	I	V	I	YYSND	1
106/1$^{76I-352K}$	Lab	CQ-QN	S	34	C	I	E	I	S	E	S	K	I	V	I	YYSND	2
106/1$^{76I-369F}$	Lab	CQ-AMT	I	42	C	I	E	I	S	E	S	Q	I	F	I	YYSND	—[f]

[a] Lines were derived from 106/1^{K76} by single-step drug selection as described in Materials and Methods. Shaded boxes indicate mutant amino acids selected in this work relative to 106/1^{K76}.
[b] Drug(s) used to select the indicated mutant line by single-step selection. All double mutant lines were generated from the CQ-selected single mutant line 106/1^{76I}. Listed after CQ is the drug used in the second round of single-step selection.
[c] R, CQ resistant; S, CQ sensitive; I, CQ intermediate.
[d] Day at which mutant parasites were first observed in culture.
[e] Amino acid residues at known polymorphic PfMDR1 positions 86, 184, 1034, 1042, and 1246. CN, *pfmdr1* copy number, as determined by real-time PCR.
[f] —, no data.

dropathy was plotted against the log (−)/(+) IC_{50} ratios for all alkaloid pairs (QN/QD, HQN/HQD, CD/CN, and EQN/EQD). Using the same drug susceptibility data described above, log IC_{50} ratios (*n* of 3 to 6) were calculated from respective CA IC_{50} pair data from within individual assay plates. Log ratio data were averaged and plotted, ± SEM, for each CA diastereomer pair. Linear regression was performed using the GraphPad Prism 5.0 software. The slope value of the linear fit was tested for a statistical difference from zero ($P < 0.05$).

RESULTS

Selection of PfCRT mutant lines of *P. falciparum*. PfCRT mutant parasites were selected from the CQ-sensitive parental line Sudan 106/1 (designated 106/1^{76K} here) (4, 14, 16). This parasite strain is appropriate for single-step drug selection studies, as the *pfcrt* allele contains all mutations necessary for CQ resistance other than the single critical point mutation that results in the K76T polymorphism (14, 16, 26) (Table 1). The selection of all mutants reported here, except 106/1$^{76I\text{-}369F}$, has been described in detail elsewhere (14, 16). Western blotting demonstrated similar expression levels of PfCRT from these parasite lines (16), and nucleotide sequencing demonstrated that no mutations were selected in *pfmdr1* (Table 1). In the absence of drug pressure, all measured drug phenotypes have remained stable in long-term cultures (>6 months). In addition to the clone presented in this report, the 106/1^{76I} line has been selected on multiple independent occasions, each time producing identical phenotypes to all drugs tested, providing strong evidence for the role of the PfCRT mutation in the CA responses (14, 16, 26). Finally, transfection of the K76I PfCRT allele into the GC03 line of *P. falciparum* resulted in similar drug responses to 106/1^{76I}, despite the differing genetic background of the recipient parasite line and greatly reduced expression levels of transgenic PfCRT (60).

AMT shows a strong inverse potency relationship with CQ against *P. falciparum* and has been previously used for the selection of novel PfCRT mutations in CQ-resistant parasites (34). Our AMT-resistant mutant was generated by single-step selection with CQ on 106/1^{K76} to generate 106/1^{76I}, followed by an additional round of single-step selection with 80 μM AMT to generate an AMT-resistant parasite. Parasites were first observed in culture after 42 days of continuous AMT pressure, and emergent parasites were found to possess a novel V369F PfCRT mutation (Table 1) predicted to occur in the DV loop between transmembrane domains 9 and 10 (43) (Fig. 2). Once cloned, drug susceptibility assays showed that 106/1$^{76I\text{-}369F}$ had become ~90-fold more resistant to AMT, with an IC_{50} of 387 ± 20 μM, compared to 4.4 ± 0.3 μM for the 106/1^{76I} parental line. The double mutant also became nearly 5-fold more susceptible to CQ after acquiring the V369F mutation (Table 2).

Parasite susceptibility to the CA is associated with PfCRT polymorphisms. In earlier studies, we demonstrated that a PfCRT K76I mutation greatly increased susceptibility to QN but not to QD, resulting in a reversal of the normal isomer potency order, where QD is 2- to 4-fold more potent than QN (14). To further characterize the relationship between PfCRT and the three-dimensional structure of CA, we measured the *in vitro* potency of four diastereomer alkaloid pairs against a set of *pfcrt* mutant parasite clones derived from the 106/1^{K76} strain (Table 1). The resulting IC_{50}s showed that, among the pharmacologically active *erythro* alkaloids, the dextrorotatory (+) QD, HQD, and CN (8*R*, 9*S*) were 1.7- to 7.7-fold more potent than the levorotatory (−) QN, HQN, and CD (8*S*, 9*R*) against all control parasite lines and

TABLE 2 Activities of the CA against control and PfCRT mutant lines of *P. falciparum* in culture[a]

Drug	IC_{50} (nM) in parasite line[b]								
	HON	7G8[c]	Dd2[c]	106/1^{K76}	106/1^{76Nc}	106/1^{76Tc}	106/1^{76I}[c]	106/1$^{76I\text{-}369F}$	106/1$^{76I\text{-}352K}$	106/1$^{76I\text{-}352R}$	106/1$^{72R\text{-}76I}$
CQ	15 ± 0.2	118 ± 3.1	174 ± 5.4	18 ± 0.6	99 ± 0.1*	180 ± 11*	259 ± 8.1*	53 ± 3.2†	15 ± 0.9†	24 ± 0.4†	18 ± 0.3†
QN	101 ± 8.8	87 ± 6.1	145 ± 18	116 ± 9.8	91 ± 10*	152 ± 17*	19 ± 1.2*	28.1 ± 3.4†	68 ± 2.9†	116 ± 17†	69 ± 11†
QD	31 ± 3.3	42 ± 3.3	75 ± 4.9	57 ± 3.3	25 ± 2.5*	59 ± 4.8	38 ± 2.8*	30.5 ± 1.3†	42 ± 2.2	28 ± 1.2†	25 ± 0.83†
HQN	92 ± 8.2	119 ± 4.9	215 ± 10	105 ± 8.1	147 ± 13*	175 ± 9.7*	20 ± 2.2*	13.1 ± 1.5†	102 ± 5.4†	112 ± 7.0†	87 ± 4.3†
HQD	32 ± 3.2	36 ± 3.6	121 ± 6.1	75 ± 16	56 ± 3.4*	110 ± 5.0*	74 ± 5.4	24.7 ± 1.0†	50 ± 2.3†	43 ± 2.3†	36 ± 1.2†
CD	287 ± 40	337 ± 12	378 ± 42	234 ± 11	204 ± 15	348 ± 15*	87 ± 3.1*	38.9 ± 3.0†	231 ± 17†	222 ± 24†	201 ± 14†
CN	37 ± 3.3	76 ± 6.2	218 ± 13	93 ± 4.0	54 ± 6.0*	93 ± 3.6	61 ± 2.7*	48.4 ± 1.5†	62 ± 2.9	73 ± 11†	58 ± 5.9
EQN	9,458 ± 971	22,440 ± 1,003§	16,340 ± 1,548	7,344 ± 567	13,380 ± 1,500*	19,936 ± 1,358*	4,843 ± 229*	233 ± 38†	3,306 ± 211†	2,149 ± 137†	2,563 ± 90†
EQD	13,111 ± 1,584	31,720 ± 2,083§	15,440 ± 1,092	10,930 ± 984	7,656 ± 82*	16,241 ± 1,056*	8,730 ± 452*	892 ± 93†	3,630 ± 213†	4,098 ± 218†	4,855 ± 295†

[a] Values are mean ± SEM IC_{50}s (in nM) determined from 3 to 8 independent 72-h growth inhibition assays.

[b] *pfcrt* mutant lines were derived from 106/1^{K76} by single-step drug selection (14, 16). Superscripts indicate mutant positions in PfCRT. F, phenylalanine; K, lysine; I, isoleucine; N, asparagine; R, arginine; T, threonine. 7G8 and Dd2 are CQ-resistant control lines. HON is a CQ-sensitive control line. *, significantly different average IC_{50} from 106/1^{K76} parent line (F test; $P < 0.05$); †, significantly different average IC_{50} from 106/1^{76I} parent line (F test; $P < 0.05$); §, significantly different average IC_{50} compared to HON or Dd2 lines (F test; $P < 0.05$).

[c] CQ-resistant line.

clones, with the exception of 106/1^{76I} and 106/1$^{76I-369F}$ (Table 2). Among the CQR *pfcrt* mutants, trends emerged that (+)/(−) IC_{50} ratios increased (76N and 76T) or decreased (76I) relative to the original 106/1^{K76} parent line.

These increased potency differences were primarily due to the selective change in susceptibility to either the (−) or (+) diastereomer, depending on PfCRT mutation. Relative to 106/1^{K76} parent, in the 106/1^{76T} line, which carries the K76T mutation ubiquitously observed in CQ-resistant field isolates, a decreased susceptibility to the (−)-isomers QN, HQN, and CD and to the weakly (+) EQN was observed. Little effect was seen on response to the (+)-isomers, except for a slight loss in sensitivity to HQD. In contrast, the 106/1^{76N} line primarily showed increased susceptibility to the (+)-isomers QD, HQD, CN, and (+) EQD, with some loss of sensitivity to the (−)-isomer HQN and the weakly (+) EQN. Most notably, against 106/1^{76I} we observed a selective increase in potency of the (−)-isomers up to 7-fold compared to the 106/1^{K76} line. Only a slight increase in potency was observed for the (+)-isomers QD and CN, and no change was observed with HQD. In sum, the selective increase in susceptibility to the (−)-isomers by 106/1^{76I} resulted in a reversal of the normally reported potency order of QN > QD and HQN > HQD. The effect on the CD/CN pair was less dramatic. Although relative CN potency diminished 2.5 times to 1.4 times more than CD, the potency order remained in keeping with the parent line (Table 2).

Because of the great sensitivity of 106/1^{76I} to QN (IC_{50}, 19 nM), we previously used single-step QN pressure to select three additional PfCRT mutants from this line: 106/1$^{72R-76I}$, 106/1$^{76I-352K}$, and 106/1$^{76I-352R}$ (Table 1) (16). These double mutants all acquired a positively charged lysine or arginine in substitution for a neutral residue, while retaining the 76I residue. As expected, all three lines showed elevated QN IC_{50}s compared to 106/1^{76I}, yet they also became sensitive to CQ (16). Accompanying the increase in the QN IC_{50}, the double mutants had parallel increases in IC_{50}s for the other (−)-isomers, CD and HQN, ranging from 2.3- to 5.6-fold. Comparatively little effect was seen on IC_{50}s for the (+)-isomers QD, CN, and HQD following selection of the position 72 or 352 mutations. These results show that the secondary mutations at PfCRT position 72 or 352 abrogated the stereoselective effect of the K76I mutation on the CA. Among these double mutants, no clear trends were observed in drug susceptibilities, other than a slightly elevated QN response in 106/1$^{76I-352R}$ (Table 2). Relative to the 106/1^{76I} parent line, AMT-selected 106/1$^{76I-369F}$ was more susceptible to all the *erythro* CA, with the exception of a slight increase in the QN IC_{50} (Table 2). While no clear trends were observed among isomers, potency differences between diastereomer pairs were largely abrogated by the V369F mutation.

Three reference control strains, Dd2, 7G8, and HON, were chosen for this study based on their diverse PfCRT haplotypes and CQ susceptibility profiles (26) (Table 1). Predictably, the dextrorotatory isomers QD, HQD, and CN had greater *in vitro* potencies than the levorotatory QN, HQN, and CD against these lines (Table 2). The CQ-sensitive HON strain showed increased sensitivity to all CA tested, other than QN, compared to the CQ-resistant controls 7G8 and Dd2. The Dd2 and 106/1^{76T} lines both carry the K76T mutation ubiquitously found in CQ-resistant isolates, differing only by a threonine-to-isoleucine substitution at PfCRT position 356 (15). Not surprisingly, the responses for the two lines were similar for all CA. The minor inherent differences in IC_{50}s may be attributed to both the position 356 PfCRT amino acid change (Table 1) and also genetic background differences.

Compared to the *erythro* alkaloids, the *threo* alkaloids EQN and EQD were previously shown to be about 10 to 100 times less potent against CQ-resistant and CQ-sensitive strains, respectively, of *P. falciparum* (37). With one exception, 106/1$^{76I-369F}$, we observed similarly weak activity, although there were clearly response changes to EQN and EQD associated with PfCRT mutations. The South American line 7G8, which contains a unique CQ-resistant PfCRT haplotype compared to Dd2 and HON, was significantly ($P < 0.05$) more resistant to both 9-epimers (Table 2). The only apparent trends were observed among the double PfCRT mutants. The double mutants were all significantly more sensitive to EQN and EQD than either the single mutant 106/1^{K76} or 106/1^{76I} parent lines. EQN was slightly more potent than EQD against the double mutants, while the potency order varied with no obvious trend revealed among the other lines. Surprisingly, 106/1$^{76I-369F}$ became ~9- to 16-fold more susceptible to the normally weakly active 9-epimers relative to 106/1^{76I}, with EQN displaying nearly 4-fold greater potency than EQD. Based on the weak activity of the 9-epimers against the other parasite lines, it was unlikely that the large increase in potency against 106/1$^{76I-369F}$ was due to impurities consisting of the active *erythro* alkaloids.

Among the CQ-resistant mutants, PfCRT hydropathy is correlated with CA (−)/(+)-isomer IC_{50} ratios. Earlier, we demonstrated that among CQ-resistant clones derived from 106/1^{K76}, a positive correlation exists between the CQ IC_{50} and hydrophobicity of the PfCRT position 76 residue (16). Therefore, we examined the hydropathy of these same residue side chains in relation to the differential effects of the CA isomers. Hydropathy values of amino acid side chains within a standardized synthetic polypeptide structure were determined at pH 5.0 by Kovacs et al. (39). This pH value is within the measured pH range for the *P. falciparum* DV lumen (5, 28), where the PfCRT position 76 amino acid is predicted to face (Fig. 2) (43). Log IC_{50} ratios for all four CA pairs were plotted against log-transformed hydropathy values for N, T, and I residues corresponding to the CQ-selected mutants (Fig. 3). The log (−)/(+)-isomer ratios clearly decreased as the PfCRT position 76 residue becomes more hydrophobic ($P < 0.0001$) for all CA pairs. We also conducted a *t* test between the mean log IC_{50} ratios of the N and T residue, as the I value could leverage the regression due to its more extreme position on the plot. All N versus T mean log IC_{50} ratios were significantly different (*t* test, $P < 0.05$), except for the CD/CN pair, which differ from the other CA in their lack of the quinoline methoxy group. Inclusion of data from the geographically disparate 7G8 and Dd2 lines reduced the significant difference between 76N and 76T CA ratios (*t* test, $P > 0.05$), possibly due to influences of the different *pfcrt* alleles and in other genetic loci on drug susceptibility (24).

CA phenotypes are independent of polymorphisms or gene copy number changes in *pfmdr1*. The entire *pfmdr1* open reading frame from the 106/1 parasite set was sequenced, except for 106/1$^{76I-372R}$ and 106/1$^{72R-76I}$, where sequencing was limited to regions flanking and including the key polymorphic codons 86, 184, 1034, 1042, and 1246. No changes were detected in the sequence of any of the mutant parasites relative to the parental 106/1^{K76}. All lines contained an identical *pfmdr1* allele (YYSND), with one mutation, N86Y, relative the "wild-type" allele (NYSND) (Table 1).

The copy number of the *pfmdr1* gene was assessed in seven mutant lines by using quantitative real-time PCR (Table 1). The

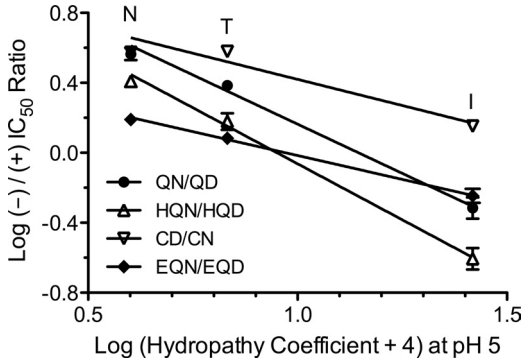

FIG 3 (−)/(+) CA IC_{50} ratios correlate with pH 5 hydropathy coefficients of PfCRT position 76 residues from CQ-resistant mutants of $106/1^{K76}$. The log (−)/(+) IC_{50} ratios of the four CA pairs were plotted as a function of the log(hydropathy coefficient + 4), as described in reference 39. Error bars represent SEM of the average within-plate IC_{50} ratios as described in Materials and Methods. The slopes of the regression lines were as follows: for QN/QD, $y = -1.13x + 1.29$, and $R^2 = 0.94$; for HQN/HQD, $y = -1.29x + 1.22$ and $R^2 = 0.93$; for CD/CN, $y = -0.60x + 1.02$ and $R^2 = 0.89$; for EQN/EQD, $y = -0.55x + 0.53$ and $R^2 = 0.90$. All slopes values are significantly different from zero ($P < 0.0001$). Average log(−)/(+) IC_{50} ratios were also compared for N versus T by *t* test, to control for a disproportionate effect on the regression by the more extreme hydropathy coefficient of I (see Results). Letter abbreviations for the corresponding PfCRT position 76 amino acids are shown adjacent to the data points on the graph.

parental $106/1^{K76}$ line was determined to have two copies of *pfmdr1*. Following drug selection, $106/1^{76I}$ was found to have one copy, while $106/1^{76N}$ and $106/1^{76T}$ remained with two copies. Double mutants, selected from $106/1^{76I}$, were single copy, except for $106/1^{76I\text{-}352K}$, which had reverted to the original two copies of *pfmdr1*. No apparent trend was observable between CA responses and *pfmdr1* copy number.

Potency order of (+)- and (−)-MQ isomers unchanged by a PfCRT K76I mutation but altered by a secondary V369F mutation. It has been shown that the enantiomers of MQ, a synthetic quinoline aminoalcohol analog of QN and QD (Fig. 1), also possess differential activities against *P. falciparum*. The (+)-isomer of MQ, shown to be the stereo-equivalent of QD in terms of absolute conformation, reportedly has 1.7 to 1.8 times greater activity than (−)-MQ, the QN equivalent (36, 38). We tested 106/1, $106/1^{76I}$, and $106/1^{76I\text{-}369F}$ (Table 3) in drug assays to determine if the unusual effects of these PfCRT mutations on the CA was extended to the structurally similar MQ isomers. We observed a potency increase in both MQ enantiomers associated with the PfCRT K76I mutation, consistent with the increase in potency of racemic MQ that we reported earlier against this clone (14). We found that (+)-MQ was 1.6 times more potent than (−)-MQ against the $106/1^{K76}$ parent line and 2.6 times more potent against $106/1^{76I}$. Thus, despite the presence of a PfCRT mutation that imparts stereospecific changes to CA activity, the difference in MQ isomer potency order was unchanged from the $106/1^{K76}$ parent line, as well as from that of parasite isolate data reported from earlier studies (38). In contrast, isomer potency differences were lost against $106/1^{76I\text{-}369F}$, consistent with observations of this line against the *erythro* CA. It is possible that the loss of a *pfmdr1* copy (Table 1) contributes to the increase in MQ susceptibility by $106/1^{76I}$ compared to the parent line. MQ responses are closely linked to *pfmdr1* copy number changes (17, 58).

DISCUSSION

In *P. falciparum*, polymorphisms in the PfCRT protein confer CQ resistance and profoundly influence susceptibility to QN and QD (14, 26). The *pfmdr1* gene, encoding a P-glycoprotein-like ABC transporter, can also modulate CQ and QN responses through mutations and changes in gene copy number (53, 58, 59). Recently, polymorphisms in a *P. falciparum*-encoded Na^+/H^+ exchanger, PfNHE-1, were linked to *in vitro* QN susceptibility in laboratory-adapted and clinical isolates in some studies, but not in others (3, 10, 24, 31, 50). Neither mutations in *pfnhe-1* or *pfmdr1* nor gene copy number in the latter are known to influence stereospecific responses to CA (7, 24, 49, 59). Our findings support PfCRT as a major parasite-encoded determinant of CA susceptibility and stereospecificity, although additional genes, gene interactions, and other genomic changes likely contribute parasite responses to the CA (24, 33).

Although no effects on CA responses were discernible, a *pfmdr1* deamplification was seen in some 106/1 mutants (Table 1), resulting from the reduction from two gene copies to one in the $106/1^{76I}$ line. Many studies have shown that *pfmdr1* expression levels are associated with *P. falciparum* susceptibility to QN, although the relationship is dependent on the genetic background of the parasite (55). Indeed, QN responses were not associated with *pfmdr1* copy number in other recent *in vitro* and *in vivo* studies (12, 13), nor is there evidence of linkage of QN responses with copy number among progeny in the HB3 × Dd2 genetic cross (24; M. T. Ferdig, unpublished data). While the increased QN susceptibility of $106/1^{76I}$ may be in part attributed to the loss of a *pfmdr1* gene copy, transfection studies have shown that the K76I *pfcrt* allele also confers a similar phenotype (60).

The stereoselective pharmacological properties of QN and QD have been documented against several classes of bacterial and mammalian transporters (29, 30, 32, 46, 47), underscoring the importance of drug-receptor binding in their mechanisms of action. Against *P. falciparum*, the CA (+)-isomers are normally more potent than the (−)-isomers, and evidence points to the necessity of drug-heme binding in their mechanism of action (1, 21, 45). Compared to QN, QD may possess a greater binding affinity for heme monomer under both aqueous and aprotic conditions and more potently inhibit β-hematin (the synthetic equivalent of hemozoin) formation (1, 18), providing a plausible explanation for the greater potency of the (+)-isomers. However, analysis of additional CA and other QN analogs failed to establish a clear correlation between drug-heme binding affinity, inhibition of β-hematin formation and *in vitro* potency (1). Observed stereospecific changes in the CA response could also be a conse-

TABLE 3 Effects of PfCRT K76I and V369F mutations on susceptibility to (+)- and (−)-MQ stereoisomers

Drug	IC_{50} (nM) in parasite line[a]		
	$106/1^{K76}$	$106/1^{76I}$	$106/1^{76I\text{-}369F}$
(+)-MQ	13.5 ± 0.7	2.3 ± 0.3*	3.3 ± 0.3†
(−)-MQ	21.2 ± 1.0	5.9 ± 0.9*	3.4 ± 0.3†
[(+)(−)][b] MQ	18.3 ± 1.0	5.1 ± 0.7*	3.4 ± 0.3†
MQ (racemic)	14.4 ± 1.3	3.1 ± 0.4*	3.1 ± 0.3

[a] Values are means ± SEM derived from 3 independent assays. *, significantly different average IC_{50} from $106/1^{K76}$ parent line (F test; $P < 0.05$); †, significantly different average IC_{50} from $106/1^{76I}$ parent line (F test; $P < 0.05$).
[b] A 50/50 mixture of the (+)- and (−)-isomers.

quence of altered drug-heme binding in the DV due to changes in the chemical environment imparted by mutant PfCRT. Based on the differences in the quinoline N pK_a of QN and QD, it was predicted that subtle changes in DV pH, such as a putative increased acidity related to PfCRT mutations, can significantly increase accumulation of the (+) diastereomer QD (41). However, this theory is inconsistent with the measured (and albeit controversial) decrease in DV pH in $106/1^{76I}$ and the selective increase in sensitivity to QN and the other (–) diastereomers (6, 14).

The hydropathy of certain mutable PfCRT residues shows quantitative relationships with susceptibility to CQ and desethylamodiaquine (16, 64). Among the CQ-resistant 106/1 mutants, the patterns of CA susceptibility suggest complex interactions between the physicochemical features of the drug and the three-dimensional and chemical environment of the PfCRT pore. This was further investigated by plotting (–)/(+) CA IC_{50} ratios in relation to PfCRT amino acid 76 hydropathy. A clear log-log correlation was found between the IC_{50} ratios for all four CA pairs and residue 76 hydrophobicity (Fig. 3), indicating that the striking effect on diastereomer sensitivity by the K76I mutation (14, 16) is not unique. The loss-of-charge mutations may increase the potential for hydrophobic interactions with some quinolines, suggesting greater drug access to the PfCRT channel in CQ-resistant isoforms. In the case of K76, since the diastereomer pairs are practically mirror images, an attractive explanation of stereospecific drug susceptibility is that the positively charged lysine sees first the hydrophobic side of QN (perhaps involving the aromatic quinoline ring and methoxy group); no interaction occurs and a proportion of drug molecules escape the DV. For QD, K76 sees the hydrophilic positively charged quinuclidine ring first and QD is repelled from entering the transporter, and it is thus retained in the DV to a greater extent. Along with drug molecule bulk that also determines the stringency with which a binding site distinguishes between (–) and (+) isomers, this could account for the general superiority of dextrorotatory CA against CQ-sensitive strains and the K76T and K76N mutants. CA susceptibility may also result from PfCRT binding (16) and subsequent inhibition of the endogenous function of the transporter itself (56). The different IC_{50}s that accompany the PfCRT amino acid changes could therefore reflect relative CA-receptor binding affinities, which in turn may dictate DV drug accumulation levels and heme-binding equilibrium.

In contrast to the wide range in CA susceptibility among the CQ-resistant parasite lines, the control line, HON, and the CQ-sensitive *pfcrt* mutants showed very similar responses to the *erythro* CA. The positive charge of a key lysine or arginine residue in either transmembrane domain 1 or 9 of these lines (16) may reduce binding with CA due to electrostatic repulsion, and in keeping with this action, reduce the interactions responsible for stereospecificity. In the acidic DV (pH, ~5.2), the CA will be mostly in the monoprotic form due to protonation of the aliphatic nitrogen (66). This similarity in IC_{50}s exists alongside three polymorphisms in the HON PfMDR1 (Y86N, Y184F, and N1042D) (49) relative to $106/1^{K76}$, emphasizing the dominant role of the wild-type PfCRT transporter in CA susceptibility.

Our results demonstrate that a PfCRT position 76I residue in combination with a second-site mutation leads to a significant increase in the activity of the *threo* 9-epimers against *P. falciparum* (Table 2). Results from crystallographic studies suggested that, due to their rotational conformation, the 9-epimers are unable to form the same intramolecular hydrogen bond with the heme propionate as do the active *erythro* CA, resulting in weak potency (18). These data are consistent with observations that neither EQD nor EQN, in contrast to the *erythro* CA, inhibit β-hematin formation *in vitro* (1, 21, 66). Additionally, the reduced lipid-aqueous distribution coefficients of the 9-epimers due to the higher pK_a values of the quinuclidine aliphatic nitrogen compared to that of the *erythro* CA are predicted to reduce solubility in the parasite membrane by a factor of 12.6 (66). Further studies are needed to determine if the K76I PfCRT double mutants retain more of the *threo* CA in the DV than do the other mutant lines.

An earlier study identified the vacuolar loop between putative transmembrane domains 9 and 10 as part of the PfCRT-binding site for AzBCQ, a CQ analog and photoaffinity label (42). Binding of the affinity label was competitively inhibited by QN, suggesting overlap in their respective binding sites. These results are consistent with our observations here, as the PfCRT V369F change occurs within this same loop region (Fig. 2) and is associated with increased susceptibility to the CA and CQ (Table 2). We propose that the bulky, rigid, and hydrophobic 369F side chain sterically hinders the ability of quinolines to escape the DV through the PfCRT pore, or it may serve as an alternative hydrophobic CA binding site to 76I where the CA drugs are less sterically constrained, in this case in the DV lumen. Steric constraint by 369F on the exit of CQ from the DV is also plausible, as hydrophobic interactions are unlikely since CQ is doubly protonated at DV and cytoplasmic pH values (25).

The synthetic quinoline aminoalcohol derivative MQ retains equivalent chiral centers (C-12 and C-11) to C-8 and C-9 of the CA (36) (Fig. 1). Superposition of (+)-MQ with QD showed identical positioning of the quinoline rings, amine, and hydroxyl groups for the two compounds. Favorable orientation of the aliphatic amine and hydroxyl groups for hydrogen bonding with a malaria receptor has been proposed to explain the potency advantage of (+)-MQ or QD over the respective enantiomer or diastereomer (38). Unlike the pronounced effects on the CA, drug assays with (+)- and (–)-enantiomers of MQ against $106/1^{76I}$ and $106/1^{76I\text{-}369F}$ showed little effect on MQ potency order (Table 3). It is of note that, in contrast to *threo* CA, the racemate of 12, 11 *threo* MQ is as potent as the racemate of 12, 11 *erythro* mefloquine against *Plasmodium berghei* and *P. falciparum in vitro* (38, 67). This difference reflects the increased ease of rotation around the link between the asymmetric carbon atoms due to the lower bulk of the piperidine ring as opposed to that of quinuclidine (36, 67). The bulkier quinuclidine side chain of the CA may contribute to a loss of flexibility, imparting a structural constraint that may confer the greater stereospecificity of the CA than the other quinolines (19). Alternatively, as MQ is a substrate for PfMDR1 (23), stereospecificity may be determined by interactions with this protein or heme, and not by PfCRT. Transfection studies with the K76I PfCRT allele, and *pfmdr1* knockdown experiments clearly support a role for both transporters in the increased sensitivity to MQ by $106/1^{76I}$ (58, 60).

Taken together, our data suggest that the well-established potency order of QD > QN is dictated by the PfCRT haplotypes found in CQ-sensitive (K76) and -resistant (K76T) field and lab isolates. In CQ-sensitive parasites, electrostatic repulsion between critical lysine/arginine residues (position 72, 76, or 352) and CA may prevent these drugs from participating in stereospecifically determined hydrophobic interactions but apparently allows more

repulsion of (+) diastereomers. Mutant parasite lines with alternative 76N/T/I side chains reveal distinct stereospecific interactions with CA, indicating greater access of the drug to stereodetermining residues in the CQ-resistant forms of the PfCRT channel. These highly specific interactions implicate PfCRT as a receptor or binding site for the CA. Binding may dictate drug potency by determining CA accumulation levels in the DV or by interfering with the endogenous function of PfCRT (56). Studies with the purified transporter will be necessary to confirm if PfCRT itself serves as a drug target for CA.

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