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Polymorphism in *Plasmodium falciparum* Drug Transporter Proteins and Reversal of In Vitro Chloroquine Resistance by a 9,10-Dihydroethanoanthracene Derivative

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BG958 reverses resistance in chloroquine-resistant isolates from different countries. Five mutations in the *Plasmodium falciparum crt* (*pfcrt*) gene resulting in the amino acid changes K76T, M74I, N75E, A220S, and R371I are systematically identified in resistance-reversed Asian, African, and Brazilian parasites which possess the *pfcrt* (CIET) haplotype. In combination with BG958, the activity of chloroquine is increased in parasites with the N86Y mutation in *pfmdr1*.

Plasmodium falciparum resistance to chloroquine (CQ) poses a severe and increasing public health threat. Several compounds, such as verapamil (VER) (15, 22), desipramine (3, 5), and antihistaminic drugs (13, 18), have demonstrated in the past decade a promising capacity to reverse CQ resistance in parasite isolates in vitro, in animal models, and in human patients with malaria. The capacity of 9,10-dihydroethanoanthracenes to reverse the CQ resistance of several strains and isolates was shown previously (2, 17, 19, 20).

A number of candidate genes in *P. falciparum* have been proposed to be involved in CQ resistance, each having to do with membrane transport. Enhancement of the effect of CQ by agents like VER is a characteristic feature of CQ-resistant (CQR) strains (15, 22), which parallels the multidrug-resistant phenomenon of cancer cells (21). Weaker or stronger associations between the resistance to CQ and changes in codons are seen in *P. falciparum mdr1* (*pfmdr1*) (11, 31). There was clearly another genetic determinant involved. Further analysis has revealed a new gene, *pfcrt*, which encodes the protein PfCRT (10). Although 10 codon changes were found in the *pfcrt* sequence in CQR isolates from Africa, Asia, and South America, the only one present in all was a change at codon 76 from lysine to threonine, which might facilitate drug efflux through a putative channel (12). Transfection work has recently confirmed the role of the *pfcrt* gene in the resistance to CQ in *P. falciparum* (23).

In the present work, we examine the relationship of different mutations in *pfcrt* and *pfmdr1* genes to CQ resistance and its reversal by VER, promethazine (PROM), and a 9,10-dihydroethanoanthracene derivative, BG958.

Strains of *P. falciparum*. Seventeen isolates and five strains of *P. falciparum*, originating from Africa ($n = 16$), Asia ($n = 5$), and South America ($n = 1$) (Table 1), were maintained in cultures in RPMI 1640 medium (Invitrogen, Paisley, United Kingdom), supplemented with 10% human serum, and buffered with 25 mM HEPES and 25 mM $NaCO_3$. Sixteen out of the 22 *P. falciparum* parasites were CQR (50% inhibitory concentration, i.e., the drug concentration corresponding to 50% of the uptake of [^{3}H]hypoxanthine by the parasite in drug-free control wells[IC_{50}], >100 nM).

Drugs. The synthesis of BG958 was described previously (2). CQ, VER, and PROM were obtained from Sigma Chemical (St. Louis, Mo.). CQ was diluted in sterile distilled water. BG958, VER, and PROM were diluted in methanol. Final concentrations of CQ distributed for the evaluation of drug interaction ranged from 25 to 3,210 nM for resistant strains and from 3 to 200 nM for susceptible strains. VER, PROM, and BG958 were used at 650, 925, and 585 nM, respectively (corresponding to 1/20 of their intrinsic IC_{50} for the CQR clone W2). At these concentrations, none of the molecules has detectable antimalarial activity.

In vitro assay. The in vitro isotopic microtest used in this study was described previously (17). The IC_{50} was determined by nonlinear regression analysis of log dose-response curves. Data were analyzed after logarithmic transformation and expressed as geometric mean IC_{50}s. The activity enhancement index (AEI) of CQ is defined as the difference between the IC_{50} measured for CQ alone and the IC_{50} measured for CQ associated with molecules of potentiation divided by the IC_{50} measured for CQ alone.

DNA extraction. Parasite DNA was extracted from infected erythrocytes with parasitemia of >1% by using the EZNA blood DNA extraction kit (Omega Bio-Tek).

RNA extraction. Parasite RNA was extracted from freshly infected erythrocytes, and parasitemia was shown to be >4% with an RNeasy extraction kit (QIAGEN).

PCR analysis. The *pfmdr1* gene was amplified by using a pair of primers (5′-TTACATTTTATTTGATTTTGTGTTG-3′ and 5′-CATCTTTTCTAGTATCATAATGAA-3′) spanning codons

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TABLE 1. Reversion of CQ resistance in *P. falciparum* strains by combination of CQ with BG958, VER, and PROM and patterns of point mutations observed in *pfcrt* and *pmdr1* genes[a]

Continent	Country	CQ susceptibility	CQ IC_{50} (nM) in combination with:				Amino acid encoded by *pfcrt* codon:									Amino acid encoded by *pfmdr1* codon:	
			No drug	BG958	VER	PROM	72	74	75	76	97	220	326	356	371	86	184
Africa	Comoros	R	716	243	426	144	C	**I**	**E**	**T**	H	**S**	N	I	**I**	N	Y
Africa	Senegal	R	484	124	147	74	C	**I**	**E**	**T**	H	**S**	N	I	**I**	**Y**	**F**
Africa	Gabon	R	464	145	258	159	C	**I**	**E**	**T**	H	**S**	**S**	I	**I**	Y	Y
Africa	Senegal	R	681	117	163	116	C	**I**	**E**	**T**	H	**S**	N	I	**I**	N	Y
Africa	Senegal	R	364	48	143	53	C	**I**	**E**	**T**	H	**S**	N	**T**	**I**	**Y**	**F**
Africa	Djibouti	R	327	38	163	77	C	**I**	**E**	**T**	H	**S**	**S**	I	**I**	**Y**	Y
Africa	Cameroon	R	280	75	149	68	C	**I**	**E**	**T**	H	**S**	**S**	I	**I**	**Y**	**F**
Africa	The Gambia	R	273	45	131	47	C	**I**	**E**	**T**	H	**S**	**S**	I	**I**	**Y**	Y
Africa	Niger	R	244	45	123	64	C	**I**	**E**	**T**	H	**S**	N	I	**I**	**Y**	Y
Africa	Ivory Coast	R	156	30	65	23	C	**I**	**E**	**T**	H	**S**	N	I	**I**	**Y**	**F**
Asia	Indochina	R	670	141	283	170	C	**I**	**E**	**T**	H	**S**	**S**	**T**	**I**	**Y**	Y
Asia	Cambodia	R	623	130	265	147	C	**I**	**E**	**T**	H	**S**	**S**	**T**	**I**	N	**F**
Asia	Cambodia	R	616	133	261	188	C	**I**	**E**	**T**	H	**S**	**S**	**T**	**I**	N	**F**
Asia	Cambodia	R	373	106	222	132	C	**I**	**E**	**T**	H	**S**	**S**	**T**	**I**	N	Y
Asia	Thailand	R	265	64	132	102	C	**I**	**E**	**T**	H	**S**	**S**	**T**	**I**	N	Y
South America	Brazil	R	341	69	125	67	C	**I**	**E**	**T**	H	**S**	**S**	I	**I**	**Y**	Y
Africa	Comoros	S	65	68	63	57	C	M	N	K	H	A	N	I	R	N	Y
Africa	Senegal	S	49	59	60	55	C	M	N	K	H	A	N	I	R	N	Y
Africa	?	S	31	32	28	28	C	M	N	K	H	A	N	I	R	N	Y
Africa	Senegal	S	31	36	32	34	C	M	N	K	H	A	N	I	R	N	Y
Africa	Ivory Coast	S	23	22	21	20	C	M	N	K	H	A	N	I	R	N	Y
Africa	Ivory Coast	S	17	19	19	19	C	M	N	K	H	A	N	I	R	N	Y

[a] CQ was combined with 585 nM BG958, 650 nM VER, or 925 nM PROM. R, resistant; S, susceptible; C, cysteine; I, isoleucine; M, methionine; E, glutamic acid; N, asparagine; T, threonine; K, lysine; H, histidine; S, serine; A, alanine; R, arginine; Y, tyrosine; F, phenylalanine. Boldface indicates point mutations.

86 and 184 with the Titanium *Taq* DNA polymerase (Titanium *Taq* PCR kits; BD Biosciences).

RT-PCR analysis. The *pfcrt* gene was amplified by reverse transcription (RT)-PCR by using two sets of primers (5′-AACAGATGGCTCACGTTTAGGTG-3′ and 5′-CTTGTCATGTTTGAAAAGCATAC-3′, spanning codons 72, 74, 75, 76, 97, and 220, and 5′-GGTTTCGCATGTTTATTCTTGGG-3′ and 5′-CGACGTTGGTTAATTCTCCTTCG-3′, spanning codons 326, 356, and 371), with an avian myeloblastosis virus reverse transcriptase kit (Access RT-PCR system; Promega).

All PCR and RT-PCR products were sequenced by using an ABI PRISM BigDye Terminator v3.0 cycle sequencing kit (Applied Biosystems).

Results. CQ IC_{50}s against CQ-susceptible isolates are not significantly modified by combination with BG958, VER, or PROM (Table 1). CQ IC_{50}s against CQR isolates are significantly reduced when CQ is combined with the three chemosensitizers ($P < 0.001$). CQ resistance is fully reversed for 8 out of 16 strains with 585 nM BG958 and 925 nM PROM and for 1 out of 16 with 650 nM VER.

CQ IC_{50}s, used alone or in combination with BG958, VER, or PROM, are not significantly different against CQR parasites from Africa or Asia ($0.06 < P < 0.22$). The AEIs of CQ are similar regardless of where the parasites originate ($0.06 < P < 0.90$).

The nine point mutations observed in the sequence part of the RT-PCR products are located at codons 72, 74, 75, 76, 97, 220, 326, 356, and 371 (Table 1). The mutant *pfcrt* alleles found in CQR parasites contain a number of point mutations, with the pattern of mutations depending on the area of the globe from which the CQR isolate originates. All the CQR parasites carry at least five point mutations at codons 74 (M to I), 75 (N to E), 76 (K to T), 220 (A to S), and 371 (R to I). CQR parasites from Southeast Asia, in addition, carry point mutations at codons 326 (N to S) and 356 (I to T). CQR parasites from Africa occasionally carry point mutations 326 (N to S) and 356 (I to T). The association of additional point mutations N326S and/or I356T to the five essential mutations M74I, N75E, K76T, A220S, and R371I does not significantly increase the level of CQ IC_{50}s. Only the CQ IC_{50}s for the parasites carrying the five point mutations M74I, N75E, K76T, A220S, and R371I, i.e., the CQR strains, are significantly reduced when CQ is combined with BG958, VER, or PROM. The presence or the absence of point mutations at codons 326 and 356 does not significantly induce a modification of the AEI of CQ, regardless of the chemosensitizer used ($P > 0.06$).

The Asn-to-Tyr mutation at codon 86 (N86Y) and the Tyr-to-Phe mutation at codon 184 (Y184F) are detected in some CQR parasites. Four out of five of the CQR parasites from Southeast Asia do not show a point mutation at codon 86 (wild type). Eight out of 10 CQR parasites from Africa have the mutation N86Y. However, the presence of the N86Y and Y184F mutations in CQR parasites is not significantly associated with the area of the globe from which the CQR isolate originates ($P > 0.88$). The presence of the N86Y mutation does not increase the CQ IC_{50}s ($P = 0.29$). However, the AEI of CQ is significantly increased in CQR parasites with the N86Y mutation (0.324 to 0.796 for BG958), regardless of the

chemosensitizer used ($P < 0.006$). The presence of a Y184F mutation in CQR parasites does not significantly modify the AEI of CQ.

Conclusions. After nearly a decade of debate, the role of plasmodial ABC transporter proteins in mediating drug resistance to quinoline agents has continued to be a subject of controversy. Namely, in the clinic, there is considerable controversy as to whether specific mutations in *pfmdr1* actually confer drug resistance in the field. Point mutations, most notably at codon 86, have been associated with decreased CQ susceptibility (1, 8). Nevertheless, this association is not consistently found (4, 26). We demonstrate in the present work that the presence of an N86Y or a Y184F mutation in CQR parasites is not associated with the area of the globe from which the CQR isolate originates and does not increase the CQ resistance level. By contrast, in combination with BG958, PROM, or VER, the activity of CQ is increased in CQR parasites with an N86Y mutation. It seems that in vitro CQ resistance reversal is associated with the presence of a point mutation at codon 86 of the *pfmdr1* gene. BG958 may act with a mutated Pfmdr1 protein by hydrogen bonding with a tyrosine lateral chain as well as by hydrophobic interactions with the phenyl part.

Most notably, the point mutation K76T in the *pfcrt* gene has been demonstrated to be strongly linked to CQ resistance (10). Field observations have generally confirmed the correlation of the point mutation at codon 76 in the *pfcrt* gene between both clinical responses to CQ in malaria patients and in vitro CQ susceptibilities of field or cultured parasites (7, 9). However, this mutation is also prevalent in CQ-susceptible isolates (14, 25). This finding suggests the involvement of additional genetic loci in modulating CQ resistance. We show that *pfcrt* has additional polymorphisms at codons 74 (M to I), 75 (N to E), 220 (A to S), 326 (N to S), 356 (I to T), and 371 (R to I) associated with in vitro CQ resistance. In the present study, the point mutations M74I, N75E, A220S, and R371I are identified in all of the CQR parasites and completely correlated with the point mutation K76T, regardless of where the parasites originate. It has been shown that some CQR parasites from the Philippines (3) or Papua New Guinea (32) do not possess the A220S mutation. These parasites from the Philippines are not responsive to VER chemosensitization (6). CQR parasites from Southeast Asia carry, in addition, point mutations N326S and I356T, while CQR parasites from Africa occasionally carry these mutations. Our strain originating from Brazil shows a different pattern (the *pfcrt* [CIET] haplotype) from those previously described by other authors (12, 30). This observation would be only the second such report from South America, providing evidence that parasites with alleles encoding CIET have been introduced into Brazil from Asia or Africa (24). While genetic differences in CQ resistance are shown, the reversal phenotypic response to BG958, VER, or PROM exists in all geographic areas, independent of the allelic variations in *pfcrt*. However, five mutations, K76T, M74I, N75E, A220S, and R371I, are systematically identified in all of our resistance-reversed parasites. BG958 fully or partially reversed resistance in all CQR isolates. It has been reported that VER reversibility was more pronounced in clones expressing recombinant *pfcrt* or strains from the Old World carrying the *pfcrt* (CVIET) haplotype than in parasite isolates or clones expressing the recombinant New World allele encoding SVMNT (16, 23) or isolates without the A220S mutation (6). All of our *P. falciparum* strains carry the *pfcrt* (CVIET) haplotype. Hypotheses on the role of changes in the sequence of the integral protein PfCRT have recently been envisaged. They assume that PfCRT has anion function, possibly a chloride channel-like function (29, 33). In CQR parasites, the positively charged lysine is changed by the neutral threonine at codon 76. CQ might exit through modified chloride channels; a more negative mean charge, an increase of the hydrophobicity, and a reduction of bulk in the side chains would facilitate the efflux of a hydrophilic, positively charged bulky drug, such as CQ, through a putative channel (27–29). It has been proposed that the reversal of CQ resistance by VER is due to hydrophobic binding to a mutated Pfcrt protein and replacement of the lost positive charge, which repels the access of CQ, thus partially restoring susceptibility to CQ (28). BG958, which possesses a protonatable nitrogen molecule at physiological pH, might act by ionic interactions with PfCRT. The nature of the point mutations at codons 75 and 76 is consistent with a model of interaction between ligand and amino acids described in a previous work (2).

BG958 fully or partially reversed resistance in all CQR parasites, wherever they originated. The present observation suggests that BG958 is a good candidate for further studies. It may act by interacting with the mutated PfCRT and PfMDR1 proteins. Furthermore, the low cost of BG958 synthesis may make its use in combination with CQ in developing countries an economically viable proposition and a more effective strategy than the introduction of another antimalarial drug at the national level.

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