Geographic patterns of *Plasmodium falciparum* drug resistance distinguished by differential responses to amodiaquine and chloroquine

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This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected in 2007.

Contributed by Thomas E. Wellems, October 2, 2009 (sent for review August 21, 2009)

Chloroquine (CQ) resistance (CQR) in *Plasmodium falciparum* originated from at least six foci in South America, Asia, and Oceania. Malaria parasites from these locations exhibit contrasting resistance phenotypes that are distinguished by point mutations and microsatellite polymorphisms in and near the CQR transporter gene, *pfcrt*, and the multidrug resistance transporter gene, *pfmdr1*. Amodiaquine (AQ), a 4-aminoquinoline related to CQ, is recommended and often used successfully against CQ-resistant *P. falciparum* in Africa, but it is largely ineffective across large regions of South America. The relationship of different *pfcrt* and *pfmdr1* combinations to these drug-resistant phenotypes has been unclear. In two *P. falciparum* genetic crosses, particular *pfcrt* and *pfmdr1* alleles from South America interact to yield greater levels of resistance to monodesethylamodiaquine (MDAQ; the active metabolite of AQ) than to CQ, whereas a *pfcrt* allele from Southeast Asia and Africa is linked to greater CQ than MDAQ resistance with all partner *pfmdr1* alleles. These results, together with (*i*) available haplotype data from other parasites; (*ii*) evidence for an emerging focus of AQ resistance in Tanzania; and (*iii*) the persistence of 4-aminoquinoline-resistant parasites in South America, where CQ and AQ use is largely discontinued, suggest that different histories of drug use on the two continents have driven the selection of distinct suites of *pfcrt* and *pfmdr1* mutations. Increasing use of AQ in Africa poses the threat of a selective sweep of highly AQ-resistant, CQ-resistant parasites with *pfcrt* and *pfmdr1* mutations that are as advantaged and persistent as in South America.

malaria | *pfcrt* | *pfmdr1*

Chloroquine (CQ) and amodiaquine (AQ) are structurally related 4-aminoquinoline drugs (1) that have had important applications against malaria for >70 years (2). Although both of these compounds are thought to have similar mechanisms of action against *Plasmodium falciparum* (3), differential clinical responses have been observed with their use (4–6). In Africa, where CQ-resistant strains of *P. falciparum* are now prevalent, AQ has remained sufficiently efficacious for the World Health Organization to recommend it as a partner in drug combinations including artemisinin combination therapy (ACT) (7). However, poor in vivo responses and outright failures of AQ against *P. falciparum* were described decades ago in India, Southeast Asia, the Philippines, South America, and Papua New Guinea (PNG) (8–12) and have been seen more recently in Africa (13, 14).

Reports have pointed to a number of puzzling observations on CQ resistance (CQR) and AQ resistance (AQR) and the relationship between them. In South America, where in vitro IC_{50} levels indicate only moderate levels of resistance to CQ but high levels of resistance to monodesethylamodiaquine (MDAQ) (15), the principal active metabolite of AQ (16), drug-resistant *P. falciparum* strains have stably saturated large regions of the continent for half a century even where use of these drugs was discontinued. Such dominance in South America contrasts dramatically with changes that have been observed after discontinued drug pressure in certain countries of Africa and Southeast Asia. For example, a region of Malawi known for highly prevalent CQR was repopulated with drug-sensitive parasites within 10 years after CQ use was stopped (17). Similar recovery of CQ-sensitive *P. falciparum* populations was recently reported in Kenya and has also been observed in China (18, 19). These changes in the absence of drug pressure have been explained by fitness costs that are carried by CQ-resistant mutants (20). However, such a selective disadvantage has been less apparent in South America where CQ-sensitive parasites have not replaced their CQ-resistant counterparts. A satisfactory explanation for this difference between Southeast Asian/African and South American forms of CQR has not been proposed.

4-Aminoquinoline-resistant parasites from different continents can be distinguished in some cases by chemosensitization with verapamil (VP), a "reversal" phenomenon characteristic of CQ-resistant but not CQ-sensitive *P. falciparum* (21). For example, African CQ-resistant parasites have been found to be more readily reversed than CQ-resistant parasites from South America (22).

Molecular-epidemiological studies have identified at least six independent origins of CQR in different regions of the world (23). These origins are distinguished by codon mutations and flanking polymorphisms of *pfcrt*, the transporter-encoding gene responsible for CQR (24, 25). Additional *P. falciparum* genes may modulate resistance once it is established; among these is the P-glycoprotein homolog (Pgh-1)-encoding gene, *pfmdr1*, which has been linked to higher IC_{50} levels of drug response in some resistant strains, but not others (26, 27). These different results have led to alternative conclusions about the relationships of *pfcrt* and *pfmdr1* mutations in the response phenotypes of malaria parasites from different geographical origins.

Here, we explore the genetics of the different features of CQ- and AQ-resistant *P. falciparum* parasites from different continents. Using a genetic cross between parasite clones from Brazil and Ghana (7G8×GB4) (28), we provide information on the linkage of *pfcrt* and *pfmdr1* haplotypes from these geographical regions to the levels of response to CQ, AQ, and their respective metabolites monodesethylchloroquine (MDCQ) and MDAQ.

Author contributions: J.M.S., P.R., and T.E.W. designed research; J.M.S., O.T., and S.R. performed research; K.H. and P.R. contributed new reagents/analytic tools; J.M.S., O.T., K.H., S.R., M.P.F., and T.E.W. analyzed data; and J.M.S., M.P.F., and T.E.W. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0911317106/DCSupplemental.

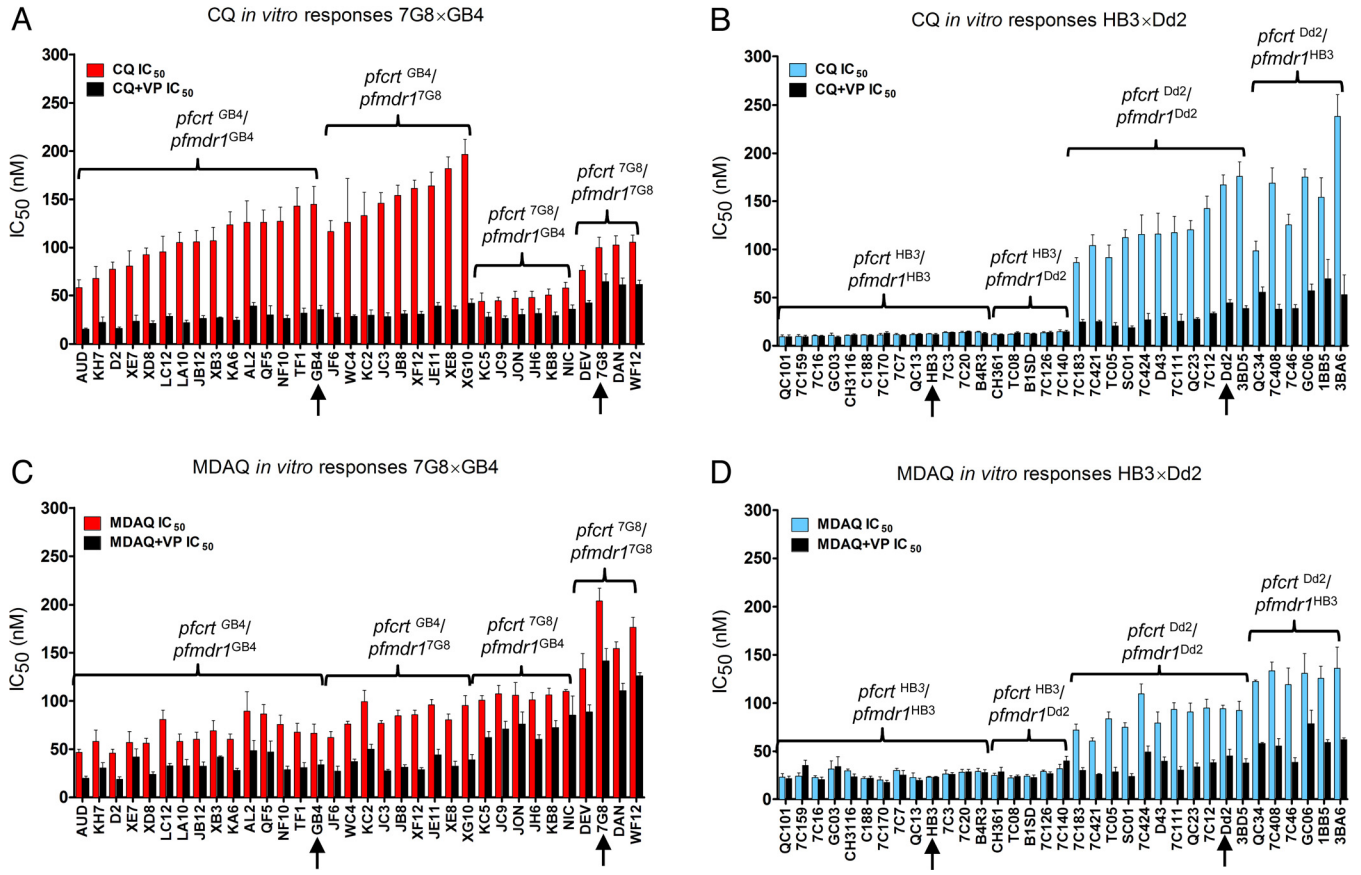

Fig. 1. In vitro CQ and MDAQ responses of individual *P. falciparum* clones. Mean CQ IC_{50} values of parents (arrows) and progeny from the 7G8×GB4 (*A*) and HB3×Dd2 (*B*) crosses and mean MDAQ IC_{50} values of parents (arrows) and progeny from the 7G8×GB4 (*C*) and HB3×Dd2 (*D*) crosses are shown. The mean values and standard errors are from at least six independent drug assays in the absence (red, for 7G8×GB4 and blue, for HB3×Dd2) or presence (black) of 0.8 μM VP. Inherited *pfcrt* and *pfmdr1* alleles are indicated above each group.

From this information, we suggest why AQ treatment is often useful against CQ-resistant *P. falciparum* in Africa but is generally ineffective against parasites that show lower levels of CQR in South America and other malarious regions such as Papua New Guinea. Finally, we hypothesize why South America remains saturated with CQ-resistant *P. falciparum* in the absence of CQ pressure, whereas removal of CQ pressure from certain African and Southeast Asian regions has led to their relatively rapid repopulation with CQ-sensitive *P. falciparum*.

Results

Inherited Features of 4-Aminoquinoline Resistance Associated with *pfcrt* and *pfmdr1* Mutations. To identify mutations associated with the different features of 4-aminoquinoline drug resistance in parasites from South America and Africa, we performed quantitative trait loci analysis (QTL) on the in vitro IC_{50} and IC_{90} drug responses of 32 previously described progeny from the 7G8×GB4 cross (7G8, Brazil; GB4, Ghana) (28). Responses to CQ, AQ, and their respective active metabolites, MDCQ and MDAQ (Fig. 1, Fig. S1, and Table S1) were scanned against full ≈5-cM linkage maps of the 7G8×GB4 progeny (www.cell.com/cell-host-microbe/supplemental/S1931–3128(08)00185-6). Results from these scans showed consistent significant or suggestive associations of *pfcrt* and *pfmdr1* peaks with 4-aminoquinoline responses in the 7G8×GB4 cross and association of only the *pfcrt* peak with VP reversal (Fig. 2 and Fig. S2; encoded amino acid polymorphisms are listed in Table 1). Copy number variations of the *pfcrt* or *pfmdr1* alleles could not be associated with the different 7G8 and GB4 drug response phenotypes, because the 7G8 and GB4 parents carry single copies of these genes (H. Jiang and X. Su, personal communication). We also performed linkage scans of drug responses from an earlier cross between a CQ-sensitive clone from Central America [HB3; which carries a single copy of *pfmdr1* (29)] and a CQ-resistant clone from Southeast Asia [Dd2; which carries three or four copies of *pfmdr1* (29)]. Consistent with previous results from this HB3×Dd2 cross (30), CQR was associated only with a peak of *pfcrt* inheritance, with no significant linkage to *pfmdr1* inheritance (Fig. 2 and Fig. S2).

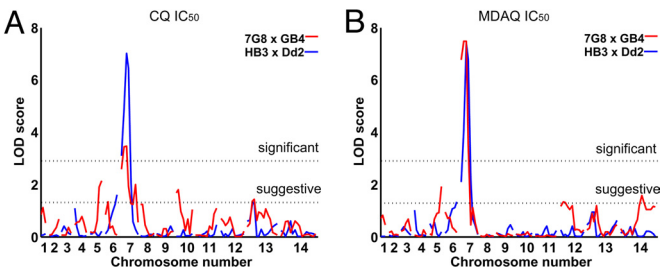

Fig. 2. QTL analysis of in vitro drug responses from the 7G8×GB4 and HB3×Dd2 genetic crosses. Results of QTL scans using the IC_{50} values of CQ (*A*) and MDAQ (*B*) are shown. Each graph represents the logarithm of odds scores of the individual markers ordered along the 14 *P. falciparum* chromosomes. Dashed lines correspond to the 95% C.I. threshold (significant) and 63% C.I. threshold (suggestive) calculated from 1,000 permutations. Small differences on the exact placement of peaks reflect differences in the genetic distances of linkage markers observed in the 7G8×GB4 and HB3×Dd2 crosses.

Table 1. Predicted PfCRT and Pgh-1 polymorphisms, drug responses, and VP response modification indices of *P. falciparum* clones 7G8, GB4, HB3, and Dd2

	PfCRT										Pgh-1					IC_{50}, nM					
Parasite clone	72	74	75	76	97	220	271	326	356	371	86	184	1034	1042	1246	CQ	CQ + VP	RMI*	MDAQ	MDAQ + VP	RMI*
7G8	S	M	N	T	H	S	Q	D	L	R	N	F	C	D	Y	99.8 ± 10.8	64.4 ± 8.3	0.64	203.8 ± 13.2	141.4 ± 13.0	0.69
GB4	C	I	E	T	H	S	E	S	T	I	Y	F	S	N	D	144.8 ± 18.8	35.3 ± 4.2	0.30	66.6 ± 9.6	33.8 ± 4.5	0.50
HB3	C	M	N	K	H	A	Q	N	I	R	N	F	S	D	D	12.3 ± 0.5	11.7 ± 0.8	0.95	22.8 ± 1.1	23.0 ± 0.6	1.00
Dd2	C	I	E	T	H	S	E	S	T	I	Y	Y	S	N	D	167.1 ± 10.4	44.2 ± 3.6	0.26	94.1 ± 3.7	45.2 ± 6.7	0.48

*Response modification index defined as the ratio of the IC_{50} in the presence of VP over that without VP (22); values closer to 1 are associated with lower susceptibility to VP chemosensitization. Amino acid polymorphisms are indicated under the codon position using standard one-letter notation.

Having confirmed *pfcrt* and *pfmdr1* as determinants of CQR and AQR in the 7G8×GB4 cross, we sought additional insights on the contributions of these loci to the drug responses of the 7G8×GB4 and HB3×Dd2 progeny phenotypes. Considering the haploid state of mammalian stage *Plasmodium*, we assigned the progeny into eight groups according to their *pfcrt* and *pfmdr1* alleles and calculated the geometric mean drug responses of each group: (*i*) $pfcrt^{HB3}$ + $pfmdr1^{HB3}$; (*ii*) $pfcrt^{HB3}$ + $pfmdr1^{Dd2}$; (*iii*) $pfcrt^{Dd2}$ + $pfmdr1^{HB3}$; (*iv*) $pfcrt^{Dd2}$ + $pfmdr1^{Dd2}$; (*v*) $pfcrt^{GB4}$ + $pfmdr1^{GB4}$; (*vi*) $pfcrt^{GB4}$ + $pfmdr1^{7G8}$; (*vii*) $pfcrt^{7G8}$ + $pfmdr1^{GB4}$; and (*viii*) $pfcrt^{7G8}$ + $pfmdr1^{7G8}$ (Table S2). Evaluation of these groups in a linear model based on the log-transformed IC_{50} values showed that 7G8×GB4 progeny carrying the $pfcrt^{7G8}$ allele possessed on average approximately half of the CQ IC_{50} of *pfmdr1*-matched progeny carrying $pfcrt^{GB4}$ [fold-change = 0.55, 95% C.I. (0.47, 0.64); Fig. 3*A* and Table S3]. Mean CQ IC_{50} values were 76–105 nM for parasites with the $pfcrt^{7G8}$ + $pfmdr1^{7G8}$ combination of alleles, whereas lower mean CQ IC_{50}s of 44–58 nM were obtained for clones with the $pfcrt^{7G8}$ + $pfmdr1^{GB4}$ combination of alleles (Table S1), a range above the IC_{50}s of CQ-sensitive isolates (e.g., HB3; Fig. 1*B*) but well below the IC_{50}s of standard strains of resistant clones (31). On average, progeny with $pfmdr1^{7G8}$ showed a 1.71 [95% C.I. (1.46, 2.00)] fold higher CQ IC_{50} than *pfcrt*-matched progeny with $pfmdr1^{GB4}$ (Fig. 3*A* and Table S3). In the HB3×Dd2 cross, the CQ IC_{50} values of CQ-resistant parasites carrying the $pfcrt^{Dd2}$ and $pfmdr1^{Dd2}$ combination were 86–176 nM, whereas the IC_{50}s of progeny with the $pfcrt^{Dd2}$ and $pfmdr1^{HB3}$ combination showed a greater range of 99–234 nM (Fig. 1*B* and Table S1). In overall averages, the mean CQ IC_{50} of the $pfcrt^{Dd2}$/$pfmdr1^{HB3}$ parasites was calculated to be 1.29-fold higher than the mean CQ IC_{50} of $pfcrt^{Dd2}$/$pfmdr1^{Dd2}$-carrying parasites [95% C.I. (1.05, 1.58); Fig. 3*A* and Table S3].

MDCQ, which along with CQ contributes significantly to in vivo action against CQ-sensitive parasites but has effectively no action on CQ-resistant parasites because of their very high MDCQ IC_{50}s (32), yielded patterns of IC_{50} response in 7G8×GB4 progeny that were relatively similar to the patterns of CQ (compare Fig. 1 *A* and *B* with Fig. S1 *A* and *B*). In the progeny of the HB3×Dd2 cross, significantly different MDCQ IC_{50}s were detected with the $pfmdr1^{Dd2}$ or $pfmdr1^{HB3}$ allele in association with the $pfcrt^{Dd2}$ allele, although not in 4-aminoquinoline-sensitive progeny carrying the $pfcrt^{HB3}$ allele (Fig. S1*B* and Fig. S3*A*). Because the IC_{50} levels of CQ-resistant parasites are ≈10-fold greater with MDCQ than with CQ and have little relevance to our practical conclusions about clinical drug resistance, the MDCQ findings are recorded here for informational purposes only and will not be discussed further.

In contrast to the relatively low CQR levels of 7G8×GB4 parasites carrying the $pfcrt^{7G8}$ allele (Fig. 1*A*), MDAQ resistance levels of these clones included the highest of any parasites in our study, with an average IC_{50} 1.81-fold [95% C.I. (1.60, 2.04)] higher than parasites carrying $pfcrt^{GB4}$ (Figs. 1*C* and 3*B*). The MDAQ IC_{50} of the 7G8 parent, 204 nM (Table S1) is >3-fold higher than that of the Ghana GB4 parent [67 nM, just above the arbitrary in vitro resistance threshold of 60 nM suggested for this metabolite (33)]. Higher MDAQ IC_{50}s were also obtained from progeny carrying $pfmdr1^{7G8}$ than from *pfcrt*-matched progeny carrying $pfmdr1^{GB4}$ [1.43-fold higher, 95% C.I. (1.27, 1.61)].

HB3×Dd2 parasites carrying the $pfcrt^{Dd2}$ allele exhibited MDAQ IC_{50}s indicative of resistance, although not at the same high average levels of 7G8×GB4 progeny carrying the $pfcrt^{7G8}$ + $pfmdr1^{7G8}$ combination (Fig. 1*D*). These IC_{50}s were detectably affected by the parental type of *pfmdr1* allele (Fig. 3*B* and Table S2): HB3×Dd2 parasites carrying $pfcrt^{Dd2}$ and $pfmdr1^{HB3}$ averaged IC_{50} values 1.51-fold higher [95% C.I. (1.29, 1.76); Table S3] than parasites carrying $pfcrt^{Dd2}$ and $pfmdr1^{Dd2}$. No significant effect of the *pfmdr1* allele was observed on the responses of drug-sensitive progeny carrying the $pfcrt^{HB3}$ allele (Figs. 1*D* and 3*B*).

In agreement with previous reports describing in vitro responses of *P. falciparum* to AQ (34, 35), our IC_{50} values for this prodrug fell within narrow ranges of 6 to 16 nM and 5 to 15 nM

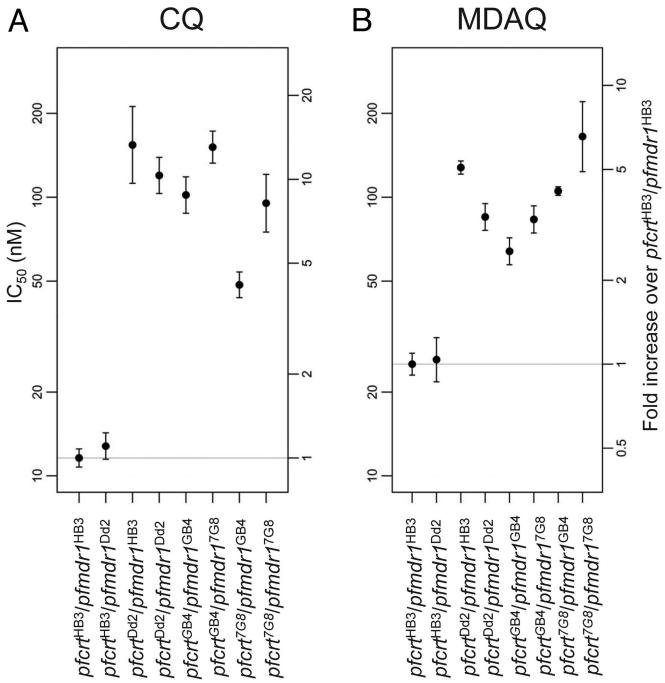

Fig. 3. Average in vitro CQ and MDAQ responses of *P. falciparum* clones grouped according to their *pfcrt* and *pfmdr1* haplotypes. Average IC_{50} responses to CQ (*A*) and MDAQ (*B*) are shown. IC_{50} values and 95% C.I.s are indicated along with fold increases over the group of parasites carrying the $pfcrt^{HB3}$and $pfmdr1^{HB3}$ alleles. Note the log scale of IC_{50} values along the vertical axes.

for the 7G8×GB4 and HB3×Dd2 crosses, respectively (Fig. S1 and Table S1). Consistent with the results of QTL scans, 7G8×GB4 parasites carrying the *pfmdr1*^{7G8} allele and HB3×Dd2 parasites carrying the *pfcrt*^{Dd2} allele exhibited slightly increased average AQ IC_{50} values over other progeny from the 7G8×GB4 and HB3×Dd2 crosses (Fig. S3*B*). However, because AQ is a rapidly metabolized prodrug, these AQ findings are presented here for informational purposes and will not be discussed further.

Distinct Features of VP Reversal Linked to *pfcrt* Inheritance. We also evaluated the effect of 0.8 μM VP in combination with the 4-aminoquinoline drugs. In assays with CQ, VP reduced the IC_{50} levels of clones carrying the *pfcrt*^{GB4} or *pfcrt*^{Dd2} alleles by 70–80% (Fig. 1 *A* and *B* and Table S4). Clones carrying the *pfcrt*^{7G8} allele show reductions of only ≈40%, consistent with the reported lower susceptibility of CQ-resistant parasites from South America to VP chemosensitization (22). As expected, VP showed no detectable chemosensitization of CQ-sensitive clones carrying the *pfcrt*^{HB3} allele from the HB3×Dd2 cross.

In assays with MDAQ, VP showed significant effects on the IC_{50} values of all CQ-resistant clones, including 50–60% reductions of clones inheriting *pfcrt*^{GB4} or *pfcrt*^{Dd2} allele and ≈30% reductions of clones carrying the *pfcrt*^{7G8} allele ($P < 0.0001$ in all cases; Fig. 1 and Table S4). There was no significant effect of VP on the IC_{50} values of the CQ-sensitive clones carrying the *pfcrt*^{HB3} allele. QTL scans of our test results from VP in combination with MDAQ or CQ identified only a single, highly significant peak on chromosome 7 associated with *pfcrt* in the 4-aminoquinoline-resistant progeny of both genetic crosses (Fig. S2).

To further investigate the relationship of particular *pfcrt*-encoded polymorphisms to VP chemosensitization, we examined the drug responses of an additional collection of parasite lines distinguished by their different *pfcrt* alleles (24). Among the CQ-resistant parasites, a line from Ecuador, Ecu1110, was found to be similar to 7G8 in its VP chemosensitization profile but differed by the lack of mutation C72S (7G8 *pfcrt* codons 72–76 SVMNT; Ecu1110 *pfcrt* codons 72–76 CVMNT; Table S5).

Discussion

In drug-resistant *P. falciparum* malaria, different phenotypes of CQ and AQ resistance are now prevalent in regions of Asia, Africa, and South America. Here, we have described the contributions of two key determinants of drug resistance, *pfcrt* and *pfmdr1*, to these different drug-resistant phenotypes. In two genetic crosses containing resistance to the 4-aminoquinolines as segregating phenotypes, mutations in the *pfcrt* and *pfmdr1* alleles were linked to different levels of resistance to CQ relative to MDAQ, the active metabolite of AQ. Particularly striking results were obtained from the cross of CQ-resistant lines from Brazil (7G8) and Ghana (GB4). In this 7G8×GB4 cross, the South American parental combination of *pfcrt*^{7G8}and *pfmdr1*^{7G8} alleles exhibited high levels of MDAQ resistance but only moderate levels of CQR. In contrast, the combination of South American *pfcrt*^{7G8} and African *pfmdr1*^{GB4} alleles was linked to moderate levels of MDAQ resistance and the lowest range of CQ IC_{50} levels that our laboratory has observed for *P. falciparum* parasites carrying a resistant *pfcrt* allele. Our findings therefore indicate that the high MDAQ resistance and moderate CQR of the 7G8 South American parasite are highly dependent upon joint contributions of the *pfcrt*^{7G8} and *pfmdr1*^{7G8} alleles.

Our results from the cross between the CQ-sensitive HB3 clone from Central America and the CQ-resistant Dd2 clone from Southeast Asia (HB3×Dd2) exhibited less robust, although detectable, effects of *pfmdr1* on CQ and MDAQ response levels. In our statistical model of CQ IC_{50} responses, HB3×Dd2 parasites carrying the *pfcrt*^{Dd2}/*pfmdr1*^{HB3} combination showed on average a 1.29-fold greater IC_{50} than parasites carrying the *pfcrt*^{Dd2}/*pfmdr1*^{Dd2} combination [95% C.I. (1.05, 1.58), $P = 0.02$]. Whole-genome QTL primary scans did not detect this effect; in this study and previous work (30) these scans did not identify a significant association of the *pfmdr1* locus with CQ responses of the HB3×Dd2 progeny. Similarly, in analysis of MDAQ IC_{50} levels, our model detected a 1.51-fold greater IC_{50} for HB3×Dd2 parasites carrying the *pfcrt*^{Dd2}/*pfmdr1*^{HB3} combination relative to parasites carrying *pfcrt*^{Dd2}/*pfmdr1*^{Dd2} [95% C.I. (1.29, 1.76); $P < 0.0001$], despite a lack of association with the *pfmdr1* locus in whole-genome QTL scans of the HB3×Dd2 progeny. These observations point to the value of alternative statistical models to detect the effects of candidate genes when numbers of cross progeny are limited, particularly when these effects are not large and the involvement of the candidate genes is suggested by indicators from independent results. We note that a number of additional transporter genes are reported to be associated with drug responses in *P. falciparum* populations (36), although these genes were not detected by QTL analyses of progeny available from the HB3×Dd2 and 7G8×GB4 crosses. The use of alternative statistical models that focus on these and other genes implicated by independent evidence may be helpful for the evaluation of additional loci that can contribute or interact in drug resistance and other *P. falciparum* phenotypes.

Previous reports examined *pfmdr1* allelic modifications for effects on responses to CQ and certain other antimalarial drugs including quinine, mefloquine, halofantrine, and artemisinin (26, 27). In one of these studies, the introduction of three codon replacements 1034C → S, 1042D → N, and 1246Y → D in the Brazilian 7G8 clone reduced the original CQ IC_{50} by ≈40% (26), consistent with the 2-fold difference we have observed between progeny of the 7G8×GB4 cross that inherited the *pfcrt*^{7G8}/*pfmdr1*^{7G8} vs. the *pfcrt*^{7G8}/*pfmdr1*^{GB4} alleles. In a second allelic modification study, Sidhu et al. (27) found little shift of CQ IC_{50} with changes of *pfmdr1*-encoded amino acids (1042D → N or introduction of the three residues 1034C, 1042D, and 1246Y) in a CQ-resistant progeny clone (3BA6) from the HB3×Dd2 cross, also consistent with our findings of small average difference between HB3×Dd2 progeny carrying the *pfcrt*^{Dd2}/*pfmdr1*^{HB3} or *pfcrt*^{Dd2}/*pfmdr1*^{Dd2} alleles. Taken together, these observations suggest that *pfmdr1* mutations differentially affect the CQ responses of CQ-resistant parasites and that their activities depend on the particular *pfcrt* haplotype with which they are associated.

In addition to the above findings on the genetics of CQR, our results provide information on high-level resistance to MDAQ modulated by the South American *pfmdr1*^{7G8} and Central American *pfmdr1*^{HB3} alleles. The N1042D polymorphism is encoded by both of these *pfmdr1* alleles (Table 1), suggesting that this amino acid change can have an important role in boosting the drug resistance levels. The effect of N1042D is particularly prominent in parasites carrying the 7G8 *pfcrt* allele linked to low-level CQR and relatively poor VP chemosensitization. This observation agrees with previous findings that *pfmdr1* genes encoding N1042D can have a strong effect on parasite responses to various antimalarial drugs such as quinine, mefloquine, and artemisinin (26, 27), and certain other chemical response phenotypes that have been identified by high-throughput chemical screens (37). Results from the 7G8×GB4 and HB3×Dd2 crosses do not support an effect from N86Y that is as influential as N1042D on the CQ- or MDAQ-resistance phenotypes (N86Y is encoded by *pfmdr1*^{GB4} and *pfmdr1*^{Dd2} and not by the *pfmdr1*^{7G8} or *pfmdr1*^{HB3} alleles that encode N1042D; Table 1). Remarkably, after a full literature search (Table S6) we were unable to find any report of *P. falciparum* isolates with a Pgh-1 sequence containing both N86Y and N1042D. It will be interesting to test whether these two polymorphisms might continue to show mutually exclusivity in additional surveys or in attempts to engineer both polymorphisms into a single allele.

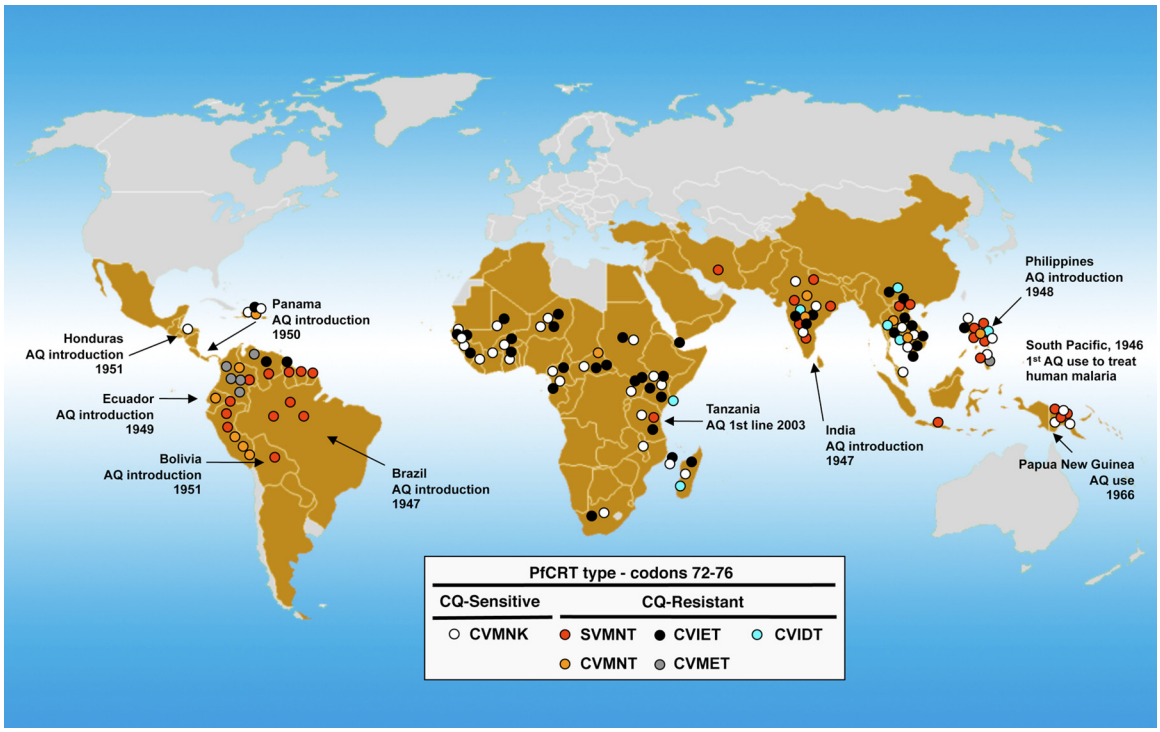

Fig. 4. AQ introduction and PfCRT types in malaria-endemic regions. Parasites with the CQ-sensitive PfCRT type (wild-type CVMNK parasites) are prevalent in most malaria endemic areas except in large areas of South America. *P. falciparum* parasites with the CVIET (i.e., Dd2 or GB4) PfCRT type are found frequently in Africa, areas of Southeast Asia, and Northern areas of South America, where resistance to CQ is high. Parasites with the SVMNT (i.e., 7G8) PfCRT types are found frequently in South America and other regions where AQ was frequently used. One of the earliest recorded uses of AQ was in 1946 in the South Pacific against *P. vivax* malaria [personal communication from L. T. Coggeshall cited by WHO (50) and Payne et al. (62)]. Between 1946 and 1951 AQ was introduced in India (63), Brazil (50, 64, 65), the Philippines (50, 66), Ecuador (67), Panama (68), Bolivia (69), and Honduras (70). Other regions of more recent use of AQ and prevalence of PfCRT- type SVMNT include PNG (71) and Tanzania (51). Data points have been included only if confirmed by more than a single observation; details and references for the individual points are provided in Table S6.

A large study of CQ-resistant *P. falciparum* in PNG identified parasites carrying the same *pfcrt* polymorphisms and VP chemosensitization phenotype as the 7G8 CQ-resistant parasite from South America (22). However, none of the 902 clinical isolates from three PNG field sites contained the N86, 1034C, 1042D, and 1246Y polymorphisms encoded by the South American *pfmdr1*^{7G8} allele (Table S6). Only one reported culture-adapted line from PNG, identified as 1905 and characterized by Foote et al. (38), carried the same *pfcrt* and *pfmdr1* alleles as 7G8, and this exhibited the highest CQ IC_{50} of the PNG series; the extent to which parasites of this 1905 or 7G8 type might occur in PNG remains to be determined. IC_{50}s indicative of low-level CQR were found for all other PNG isolates carrying a SVMNT-type *pfcrt* allele (i.e., 7G8 type, encoding amino acids SVMNT at codons 72–76) and a *pfmdr1* allele encoding 86Y, S1034, N1042, and D1246; the lowest CQ IC_{50} of all (50 ng/mL) was obtained from an isolate carrying neither 86Y nor 1042D (PNG 1917; Table S6) (22, 38). Those findings taken together with our results from the 7G8×GB4 and HB3×Dd2 crosses are consistent for parasites with greater levels of AQR than CQR. Observations from the field support this conclusion, as clinical results in PNG have shown AQR at higher rates than CQR and high level (RIII) clinical failures only with AQ treatment (39).

What selective factors account for the distributions of distinct *pfcrt* and *pfmdr1* haplotypes associated with different phenotypes of CQ and MDAQ resistance in the parasite populations of South America, Africa, Asia, PNG, and other malaria endemic regions? One likely influence is the history of AQ and CQ use in these regions and the consequent selective pressure for *pfcrt* and *pfmdr1* polymorphisms of one type or another. Haplotypes of *pfcrt*-encoding polymorphisms similar to those of the *pfcrt* alleles in South America and PNG have been reported from East Timor, Indonesia, the Philippines, Laos, India, and Iran (40–49). In many of these regions AQ was widely used in the 1940s (50) and early 1950s before the advent of CQR (Fig. 4). Although at least six foci (origins) of CQR have previously been inferred, including a major prevalent CVIET form in Africa that evidently entered from Southeast Asia, growing databases from surveys suggest that additional foci may also have occurred in the Americas, Africa, and Asia (Fig. 4). Two such additional foci may be suggested by the presence of CVIET and CVMET CQ-resistant parasites in Haiti and Northern areas of South America (Fig. 4). Worrisomely, an increasing prevalence of SVMNT parasites in Tanzania (7G8-type PfCRT; possibly another new focus) has been associated with a switch from CQ to AQ use (2001) and more frequent observations of AQR from that country in recent years (51, 52).*

Patterns of *pfcrt* and *pfmdr1* haplotypes in malaria endemic regions also depend on the fitness costs of these haplotypes relative to the advantages they offer to parasite populations. In Malawi and Hainan Island, China, where CQ-resistant CVIET parasites (Dd2- or GB4-type *pfcrt*) from the large Southeast Asian–African sweep had once been selected almost completely, populations of wild-type CQ-sensitive parasites characterized by the haplotype CVMNK (HB3-type *pfcrt*) returned after withdrawal of CQ (17, 18). These findings suggest that CVIET mutants are at a fitness disadvantage to wild-type forms in the

*Consistent with the *pfcrt–pfmdr1* interactions described in this study, *pfmdr1* codon 1246Y with possible synergistic or compensatory influence of 86Y or 184Y has also been associated with treatment failures after AQ monotherapy or after AQ plus ACT in Tanzania (53).

absence of drug pressure (20, 54). However, a lower fitness cost appears to be associated with the SVMNT mutants. Except for a recent report of a single sample from Brazil containing the wild-type CQ-sensitive haplotype CVMNK (55), a significant return of CQ-sensitive parasites has not yet occurred among the MDAQ-resistant populations of SVMNT parasites in South America or in other regions where SVMNT is present and CQ has been either withdrawn or is no longer recommended (56). Consistent with these observations, Mehlotra et al. (57) have found that variation in the *pfcrt* and *pfmdr1* loci of Asian and African parasites populations is maintained by substantially different mechanisms than in South American populations where the haplotypes of *pfcrt* and *pfmdr1* both exhibit relatively low levels of diversity. We also note the recent observation of increasingly prevalent SVMNT parasites in Tanzania where AQ pressure has presumably facilitated their spread (51, 52). In areas of Africa where AQ is used as monotherapy or in combination with partner antimalarial drugs, CVIET parasites may be under displacement by highly AQ-resistant, CQ-resistant SVMNT parasites that are as advantaged and persistent as in South America. SVMNT parasites in Africa may therefore present a particular threat where AQ-containing combinations are being used in coordinated malaria control efforts; these parasites warrant careful surveillance and appropriate adjustment of drug use policy where their presence gives rise to prevalent AQ treatment failures.

Our observations also suggest that the various amino acid substitutions encoded by *pfcrt* and *pfmdr1* alleles are tuned to the chemical and structural differences of CQ and MDAQ and contribute distinct levels of resistance to these compounds. Further, certain amino acid polymorphisms in PfCRT are linked to different levels of chemosensitization by VP. Available data on *P. falciparum* isolates and in vitro-adapted lines support an association of PfCRT N75 with reduced VP chemosensitization of CQ-resistant *P. falciparum* (a possible requirement of PfCRT 72S for reduced VP chemosensitization is not supported because the Ecuador CVMNT-type isolate Ecu1110 has a phenotype similar to 7G8). Polymorphisms in the Pgh-1 protein encoded by *pfmdr1* have also been shown to affect *P. falciparum* responses to a variety of compounds including quinine, mefloquine, halofantrine, and artemisinin (26, 27, 37). However, CQ stands out in this picture, because parasite responses to CQ are altered more by the Pgh-1 mutations in CQ-resistant parasites that carry 7G8 (South American) than Dd2 or GB4 (Southeast Asian, African) forms of PfCRT, and no effect of Pgh-1 mutations is seen in CQ-sensitive parasites that harbor wild-type forms of PfCRT. These findings raise fascinating questions about the molecular nature of the interactions between PfCRT and Pgh-1, the different functional and structural effects of mutations on transport processes involved in CQ and MDAQ resistance, and the evolution and spread of different *pfcrt* and *pfmdr1* alleles in *P. falciparum* populations under changing conditions of drug pressure.

Materials and Methods

***P. falciparum* In Vitro Culture.** *P. falciparum* clones were as reported: HB3, Dd2, and 33 progeny clones (29, 30); 7G8, GB4, and 32 progeny (28); D10, M5, Haiti, 106-1, Ecu1110, FCB, JAV, TM284, and RB8 (24). Parasites were cultivated using standard methods (ref. 58 and www.mr4.org/Portals/3/Methods_In_Malaria_Research-5theditionv5–2.pdf). AL2 is a clone from a cross in *Aotus* of the 7G8 and GB4 parents.

Antimalarial Drugs and In Vitro Drug Response Assays. CQ, AQ, and VP were purchased from Sigma. MDAQ and MDCQ were synthesized by S. Ward (Liverpool School of Tropical Medicine) and AsisChem, respectively. Drug assays were performed as described (59). Initial drug concentrations in the serial dilutions were 5 μM CQ, 20 μM MDCQ, 0.5 μM AQ, and 0.7 μM MDAQ. When used, VP was incorporated at a concentration 0.8 μM in combination with CQ, MDCQ, AQ, or MDAQ.

Statistical Analysis. To evaluate the relative contribution of *pfcrt* and *pfmdr1* to the in vitro drug responses, we placed the progeny clones of the genetic crosses into eight groups (four groups for each cross) according to the *pfcrt* and *pfmdr1* alleles they presented: (*i*) $pfcrt^{HB3}$ + $pfmdr1^{HB3}$; (*ii*) $pfcrt^{HB3}$ + $pfmdr1^{Dd2}$; (*iii*) $pfcrt^{Dd2}$ + $pfmdr1^{HB3}$; (*iv*) $pfcrt^{Dd2}$ + $pfmdr1^{Dd2}$; (*v*) $pfcrt^{GB4}$ + $pfmdr1^{GB4}$; (*vi*) $pfcrt^{GB4}$ + $pfmdr1^{7G8}$; (*vii*) $pfcrt^{7G8}$ + $pfmdr1^{GB4}$; and (*viii*) $pfcrt^{7G8}$ + $pfmdr1^{7G8}$. Geometric means of log transformed IC_{50} values from these groups were calculated, and 95% C.I.s were based on the associated *t* test. To evaluate the effect of VP on each drug response, we plotted the log-transformed IC_{50} values of each group of clones according to their *pfcrt* and *pfmdr1* alleles and estimated the mean ratio of drug+VP/drug IC_{50} values by using a linear model with the response equal to the difference in log-transformed values. Similar linear models were used to test for average fold changes in one allele when holding the other constant. The 95% C.I. and *P* values were calculated from the linear models by a two-sided test to check for ratios significantly different from 1. Calculations were done with R version 2.8.0 (R Development Core Team 2008).

QTL Analysis. QTL analysis was performed by using the R/qtl and J/qtl packages (60)) and Pseudomarker using Matlab software (61) as described (30).

ACKNOWLEDGMENTS. We thank Jianbing Mu for assistance in genotyping the *P. falciparum* clones; Hongying Jiang, Xinzhuan Su, Jeff Skinner, Vivek Gopalan, Jason Barnett, Yentram Huyen, and William Knight for assistance with in vitro drug assays and automated analysis of IC_{50}s and IC_{90}s; and Erika Phelps, Sarnia Laurent, Michael Krause, and Rick Fairhurst for assistance with QTL mapping, DNA sequencing, and discussions. The Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases, supported this research.

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