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# Chloroquine susceptibility and reversibility in a *Plasmodium falciparum* genetic cross

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## Summary

**Mutations in the *Plasmodium falciparum* chloroquine (CQ) resistance transporter (PfCRT) are major determinants of verapamil (VP)-reversible CQ resistance (CQR). In the presence of mutant PfCRT, additional genes contribute to the wide range of CQ susceptibilities observed. It is not known if these genes influence mechanisms of chemosensitization by CQR reversal agents. Using quantitative trait locus (QTL) mapping of progeny clones from the HB3 × Dd2 cross, we show that the *P. falciparum* multidrug resistance gene 1 (*pfmdr1*) interacts with the South-East Asia-derived mutant *pfcrt* haplotype to modulate CQR levels. A novel chromosome 7 locus is predicted to contribute with the *pfcrt* and *pfmdr1* loci to influence CQR levels. Chemoreversal via a wide range of chemical structures operates through a direct *pfcrt*-based mechanism. Direct inhibition of parasite growth by these reversal agents is influenced by *pfcrt* mutations and additional loci. Direct labelling of purified recombinant PfMDR1 protein with a highly specific photoaffinity CQ analogue, and lack of competition for photolabelling by VP, supports our QTL predictions. We find no evidence that *pfmdr1* copy number affects CQ response in the progeny; however, inheritance patterns indicate that an allele-specific interaction between *pfmdr1* and *pfcrt* is part of the complex genetic background of CQR.**

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## Introduction

Efforts against *Plasmodium falciparum* are hampered by the evolution and worldwide spread of decreasing parasite susceptibility to an array of antimalarial compounds, including the near global failure of the former mainstay drug, chloroquine (CQ) (Ginsburg, 2005). Extensive field and laboratory research on CQ resistance (CQR) identified the crucial role of a gene on chromosome (chr) 7, the *P. falciparum* CQR transporter (*pfcrt*), that encodes a putative transporter with channel-like properties, localized to the digestive vacuole membrane in erythrocytic stage parasites (Wellems *et al.*, 1991; Su *et al.*, 1997; Fidock *et al.*, 2000; Cooper *et al.*, 2002; Sidhu *et al.*, 2002; Zhang *et al.*, 2004; Martin *et al.*, 2009; Paguio *et al.*, 2009). A K76T change is ubiquitous in CQ-resistant populations and is a reliable molecular marker of CQR (Djimde *et al.*, 2001; Wootton *et al.*, 2002; Chen *et al.*, 2003; Plowe, 2003; Best Plummer *et al.*, 2004; Durrand *et al.*, 2004).

Drug pressure in the laboratory to select mutant CQ-resistant parasite lines has been successful in only one CQ-sensitive (CQS) parasite line, 106/1 (Fidock *et al.*, 2000; Cooper *et al.*, 2002), which is unique in harbouring six of the seven PfCRT mutations characteristic of South-East Asian CQ-resistant parasites, lacking only the critical K76T (Wootton *et al.*, 2002). Attempts to select for CQR using other CQS parasite lines have failed (Lim and Cowman, 1996). In other experiments, the wild-type PfCRT allele of GC03, a CQS progeny clone of the HB3 × Dd2 genetic cross with a haplotype of CMNKAQNIR at amino acid positions 72, 74, 75, 76, 220, 271, 326, 356 and 371, was replaced with three unique CQ-resistant haplotypes: CIETSESTI (Dd2), CIEISESII (106/1$^{76I}$) and SMNTSQDLR (7G8) by homologous recombination (Sidhu *et al.*, 2002). Various allelic replacements confer distinct CQR phenotypes in GC03 (Sidhu *et al.*,

2002; Lakshmanan *et al*., 2005). Collectively, these studies underscore the major role of amino acid 76 in CQR, but also indicate an important role for additional factors in the genetic background of the key PfCRT mutation.

The suggestion that multiple genetic factors underpin CQR was raised more than 20 years ago, well before the identification of *pfcrt* (Foote *et al*., 1990). A strong but incomplete association of mutations in *pfmdr1*, a gene encoding a P-glycoprotein homologue (Pgp) of the human multidrug-resistant (MDR) efflux pump (Shen *et al*., 1986; McGrath *et al*., 1989), with CQR in field isolates (Foote *et al*., 1990), combined with the observation that *pfmdr1* mutations were not linked with CQS or CQ-resistant progeny of the HB3 × Dd2 cross (Wellems *et al*., 1990), spurred a lasting debate about this gene's role in the mechanism and evolution of CQR (Yayon *et al*., 1984; Krogstad *et al*., 1987; Orjih *et al*., 1994; Bray *et al*., 1998; Sanchez *et al*., 2003; 2005). This incongruity led to the suggestion that some CQS parasites may be competent to become CQ-resistant with the acquisition of additional mutation(s) (Foote *et al*., 1990). *Pfmdr1* point mutations have been associated with CQR (Foote *et al*., 1989; 1990; Wilson *et al*., 1989; Mu *et al*., 2003); however, these associations vary geographically, leaving the role of *pfmdr1* in CQR modulation and reversibility unresolved (Adagu and Warhurst, 2001; Chen *et al*., 2002; Hayton and Su, 2004; Duraisingh and Cowman, 2005; Sidhu *et al*., 2006; Rason *et al*., 2007). PfMDR1 overexpressed in *Xenopus laevis* oocytes affects transport of quinine (QN), halofantrine and CQ in a mutation specific manner, but only wild-type PfMDR1 affected transport of CQ in this model (Sanchez *et al*., 2008). In *Pichia pastoris*, heterologous PfMDR1 expression catalyses drug-stimulated ATPase activity (Amoah *et al*., 2007; Lekostaj *et al*., 2008a).

*Pfcrt* and *pfmdr1*, among other loci, interact to affect QN dose-responses, leading us to propose that local drug selection in different geographic regions can differentially shape the gene interactions and their impact on a range of drug resistance phenotypes (Ferdig *et al*., 2004; Sen and Ferdig, 2004). Two recent studies, one using classical genetics (Sa *et al*., 2009) and the other reverse genetics (Valderramos *et al*., 2010), demonstrated the pivotal influence of the parasites' genetic backgrounds (i.e. distinct drug selection histories) into which the key CQR-conferring *pfcrt* mutations are successfully inherited or experimentally introduced.

A hallmark of CQ-resistant parasites is that particular compounds can re-sensitize them to CQ, a phenomenon termed 'reversibility' (Martin *et al*., 1987). A similar reversal effect was first observed in MDR mammalian tumour cells (Tsuruo *et al*., 1982; Rogan *et al*., 1984), and many classes of compounds have now been identified that can reverse CQR (Kyle *et al*., 1990; Pradines *et al*., 2005). One of the earliest identified reversal agents was verapamil (VP), a $Ca^{2+}$ channel blocker indicated for the treatment of angina pectoris, cardiac arrhythmias and hypertension. In human tumour cells, VP-dependent chemosensitization is believed to be mediated by Pgp (Shen *et al*., 1986; McGrath *et al*., 1989). Because VP was found to re-sensitize CQ-resistant *P. falciparum* to near CQS levels, a Pgp efflux mechanism was considered the likely basis of CQR (Martin *et al*., 1987). Three-dimensional quantitative structure activity relationship studies generated a pharmacophore model for a group of tricyclic antidepressants that reverse CQR (Bhattacharjee *et al*., 2002). Two hydrophobic, aromatic regions with a nitrogen atom acting as a hydrogen-bond acceptor are apparently required for CQR reversal activity (Bhattacharjee *et al*., 2001). This model accommodates a variety of CQR reversal agents that span distinct chemical classes, including VP, cyproheptadine, ketotifen, chlorpheniramine (CLM) and citalopram (CT). Two theories have been proposed for VP reversal of CQR: (i) the 'drug transporter' hypothesis argues that VP competes with CQ for target binding and perhaps drug transport in resistant parasites (Martiney *et al*., 1995; Bray and Ward, 1998; Sanchez *et al*., 2004), and (ii) VP may act on ion transport to alter the compartmental pH and/or membrane potentials to levels found in CQS parasites (Martiney *et al*., 1995; Roepe and Martiney, 1999; Ursos *et al*., 2000; Bennett *et al*., 2004). Both hypotheses are possibly relevant, as drug transport by drug resistance proteins could be due to direct and/or indirect ion-mediated co-transport (Zhang *et al*., 2004). CQR reversibility by VP and the 8-aminoquinoline, primaquine (Bray *et al*., 2005), like CQR itself, has been attributed to PfCRT, yet the possibility of non-target based mechanisms suggests the interaction of multiple transporters. Reversal agents themselves are toxic to the parasites (Adovelande *et al*., 1998; Menezes *et al*., 2002; 2003), but it is not known if these structurally diverse reversal agents exert their toxic effect solely by PfCRT or involve additional genes, nor if their inherent anti-plasmodial effects predict the degree of reversibility of CQR. For example, some CQ-resistant parasites such as the South American 7G8 strain, are less responsive to the VP reversal effect (Mehlotra *et al*., 2001; Sa *et al*., 2009). It is not known if factors that contribute to the wide range in CQ $IC_{50}$ values among CQ-resistant field isolates, even in the presence of identical PfCRT alleles (Mu *et al*., 2003), can also influence reversibility.

PfCRT has been the overriding focus of recent studies of CQR and its coupled reversibility by VP. To search for additional contributing genes we measured quantitative CQ susceptibility and its reversibility by various pharmacological classes of compounds, including VP, CLM, CT, probenecid (PB) and amitriptyline (AM) in progeny of the HB3 × Dd2 genetic cross. Using 33 independent progeny, we use quantitative trait locus (QTL) profiles to probe the complex genetic background of the South-East Asia-

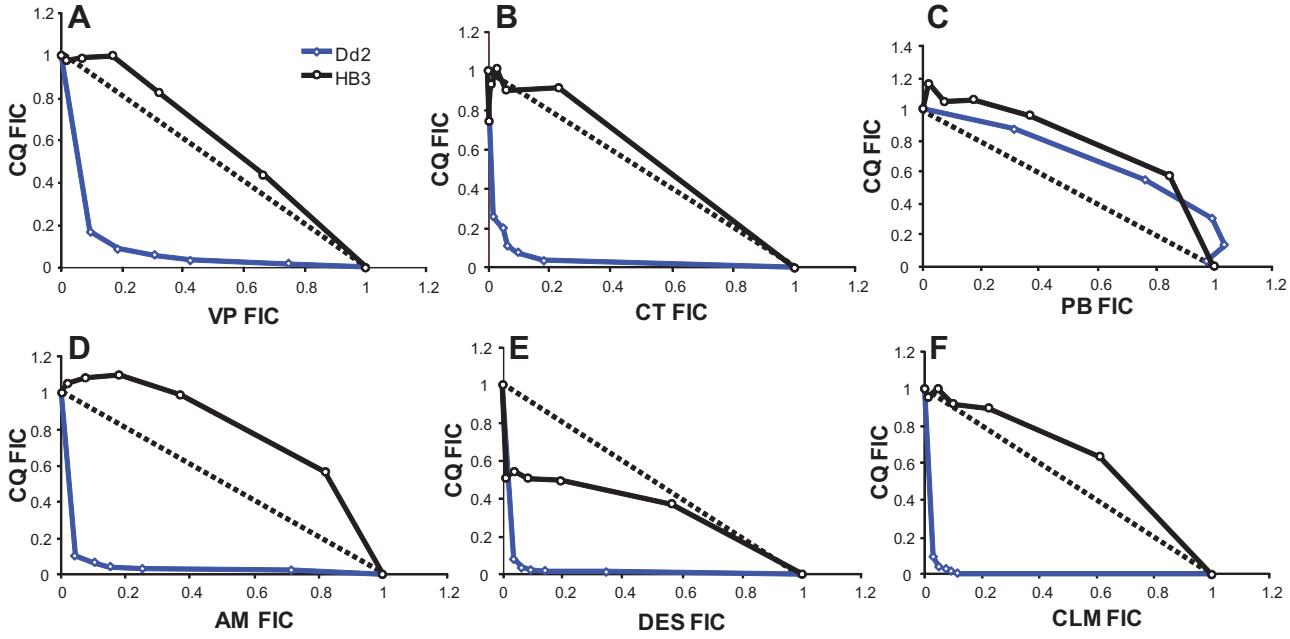


**Fig. 1.** Isobolograms characterize drug interactions in Dd2 and HB3. Data points represent the FICs of various dose combinations of (A) CQ + VP, (B) CQ + CT, (C) CQ + PB, (D) CQ + AM, (E) CQ + DES and (F) CQ + CLM. Each isobologram represents the interaction in both CQ-resistant parent Dd2 (blue) and CQS parent HB3 (black). Points near the dashed line indicate an additive relationship between the two drugs; while those above the line indicate antagonism and those below the line indicate a synergistic relationship. Synergistic interactions were observed for most drug combinations except CQ + PB. The CQ + DES interaction plot showed weak synergism in HB3 and CQ + PB was additive for both HB3 and Dd2.

derived CQR and its functional relationship to reversal. This approach revealed a dynamic interplay of major and minor shared loci highlighting gene adaptations influencing CQR, reversibility and fitness. The contribution of *pfmdr1* and a putative secondary locus on chr 7 to CQR is reported. *Pfcrt* controls the vast majority of reversal by a range of compounds. We find no evidence that *pfmdr1* copy number (CN) affects CQ $IC_{50}$ values; however, co-inheritance patterns of specific *pfmdr* and *pfcrt* allele combinations in individual progeny underscores the complex genetic background of CQR.

## Results

### *Quantitative effects of reversal agents, independently and in combinations, in the parental lines*

Six reversal agents – VP, CT, AM, CLM, PB and desipramine (DES) – spanning various pharmacological drug classes were tested in combination with CQ. Fractional inhibitory concentrations (FICs) (Elion *et al.*, 1954) at each of the fixed molar ratios for all drug combinations were determined for both HB3 and Dd2 to quantify the type and degree of interactions between the reversal agent and CQ. When plotted as isobolograms, synergistic effects were observed for Dd2 in combinations of CQ + VP (Fig. 1A), CQ + CT (Fig. 1B), CQ + AM (Fig. 1D), CQ + DES (Fig. 1E) and CQ + CLM (Fig. 1F), whereas all combinations were additive in HB3. PB was not synergistic in Dd2 (Fig. 1C), contrary to a previous observation in the CQ-resistant strain, V1/S (Nzila *et al.*, 2003); however, a recent report from this same group (Masseno *et al.*, 2009) revised their assessment of PB reversal of V1/S CQR to be fivefold less synergistic. We confirmed a linear or slightly additive relationship between PB and CQ for both HB3 and Dd2.

Based on the comprehensive analysis of the parent clones, a single fixed dose combination was used to construct a measure of reversibility for replicate testing of 33 progeny clones. The fixed dose volumetric ratio of 9:1, representing the lowest concentration of reversal agent that showed synergism (Fig. 1) was used for all drug combinations examined in the progeny (CQ + VP, CQ + CLM, CQ + CT and CQ + AM). The starting concentration for the twofold serial dilutions (see *Experimental procedures*) was CQ : VP – 2.25 μM : 24.03 μM, CQ : CLM – 2.25 μM : 71.01 μM, CQ : CT – 2.25 μM : 6.85 μM, and CQ : AM – 2.25 μM : 22.30 μM. CQ $IC_{50}$ values in HB3 and Dd2, with and without reversal agent, demonstrated that reversal agents did not significantly alter the CQ response in HB3 ($P > 0.05$, $n = 5$), whereas highly reproducible reversibility ($n = 18$–$22$) was achieved with each of the four combinations in Dd2 (Fig. S1). The combination of CQ + AM was the most potent, lowering the $IC_{50}$ of CQ to 59.2 ± 3.12 nM, whereas

**Table 1.** Drug responses in the parents of the genetic cross.

| Drugs | *n* | Mean ± SEM Dd2 $IC_{50}$ (nM) | *n* | Mean ± SEM HB3 $IC_{50}$ (nM) |
|---|---|---|---|---|
| VP | 21 | 2303 ± 245 | 5 | 14 307 ± 3442 |
| CLM | 22 | 1976 ± 238 | 5 | 90 446 ± 1902 |
| CT | 21 | 2082 ± 200 | 5 | 42 276 ± 1771 |
| CQ | 18 | 422 ± 25.0 | 5 | 27.2 ± 0.94 |
| CQ + VP | 22 | 82.8 ± 4.42 | 5 | 25.9 ± 0.57 |
| CQ + CLM | 22 | 71.7 ± 3.71 | 5 | 20.6 ± 5.19 |
| CQ + CT | 21 | 117 ± 6.84 | 5 | 25.5 ± 0.78 |
| CQ + AM | 22 | 59.2 ± 3.12 | 5 | 25.4 ± 0.91 |

CQ + CT was the least potent, lowering the $IC_{50}$ to an intermediate level of 117.3 ± 6.84 nM in Dd2. Table 1 summarizes the $IC_{50}$ values for VP, CLM and CT when tested independently. Dd2 was more sensitive to VP, CLM and CT than HB3, with more than 6-, 45-, 20-fold differences respectively. Dd2 was only slightly more sensitive to DES (1.4-fold) than HB3, and no difference was observed between the parent lines for PB $IC_{50}$ values (data not shown).

### *Reversibility in the progeny of the genetic cross*

Only the reversal agent–CQ combinations that showed strong synergy in Dd2 were carried over into a full analysis of the progeny. Mean $IC_{50}$ values from VP, CLM and CT, alone and in combination with CQ, were obtained from five independent replicates for each of the progeny of the genetic cross (Table S1). Values were continuously distributed for each compound, with no clear $IC_{50}$ gap dividing the progeny into two classes that would be expected if a single gene determined the phenotype. CQS progeny were less sensitive to VP alone than CQ-resistant progeny (Fig. 2A, $P < 0.01$), and this dichotomy was consistent for all the reversal agents.

Among the progeny, $IC_{50}$ values ranged from 18.3 to 771.0 nM for CQ and 17.8 to 105.1 nM for CQ + VP. A relative measure, fold change, was used to assess the degree of reversibility. For each drug combination, the fold change distribution divided the progeny into two groups that coincided with the inherited *pfcrt* allele and therefore CQ-resistant or CQS status (Fig. 2B). CQ susceptibility in the presence of VP for both CQS (Fig. 3A) and CQ-resistant (Fig. 3B) progeny was strongly correlated with CQ response in the absence of VP; however, no significant changes were observed in CQ susceptibilities of CQS progeny in the presence of VP (fold change 1.1 ± 0.1, $P > 0.05$), whereas significantly lower $IC_{50}$ values were detected in CQ-resistant parasites (6.6 ± 1.1 fold change, $P < 0.0001$) in the presence of VP. The degree of reversibility (fold change ranging from 4.7 to 8.1) was independent of the inherent CQ $IC_{50}$ values in CQ-resistant progeny (Fig. 3C) ($r = 0.2$, $P > 0.05$), and the primary reversal mechanism for all three compounds depended on *pfcrt* mutations. Highly significant correlations were observed among fold changes induced by the four compounds in CQ-resistant progeny (Table S1) (*r*-values ranging from 0.89 to 0.95), indicating that a common additional gene(s) controls the degree of reversibility. Regression analyses of the relationships between CQ $IC_{50}$ and the potencies of the reversal agents and fold change are provided in Table 2. Negative correlations were detected between CQ $IC_{50}$ values and VP, CLM and CT $IC_{50}$ values, whereas fold change resulting from CQ + reversal agent was strongly positively correlated with the CQ $IC_{50}$. When these analyses were performed individually on the CQS and CQ-resistant subgroups, the relationship between fold change and CQ $IC_{50}$ was lost in both subgroups, indicating that secondary influences on CQ $IC_{50}$ values do not affect fold change. However, positive relationships were identified for CLM

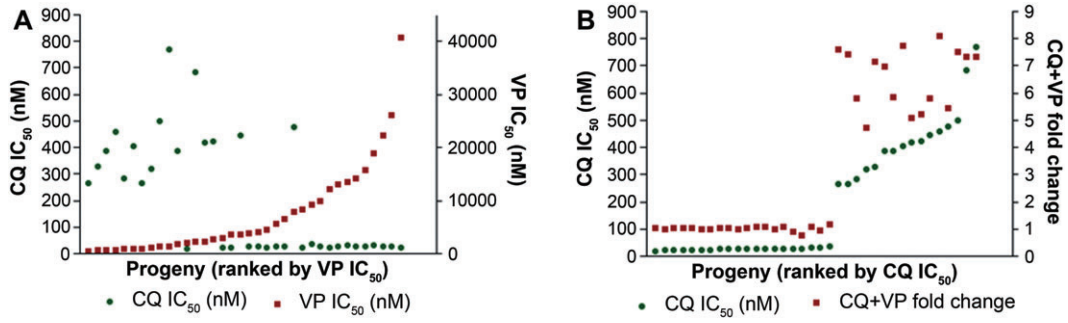


**Fig. 2.** Ranked plots showing phenotype distributions for CQ with and without VP.
A. Ranked distribution for VP response against CQ response in the cross. VP response is continuously distributed and inversely related to CQ response. Progeny that had high VP $IC_{50}$ values had lower CQ $IC_{50}$ values. This negative relationship was consistent with all other reversal agents tested.
B. Ranked distribution for CQ response against fold change in the presence of VP. Fold change partitions the drug responses into two groups (CQS and CQ-resistant) depending on the HB3 and Dd2 *pfcrt* allele.

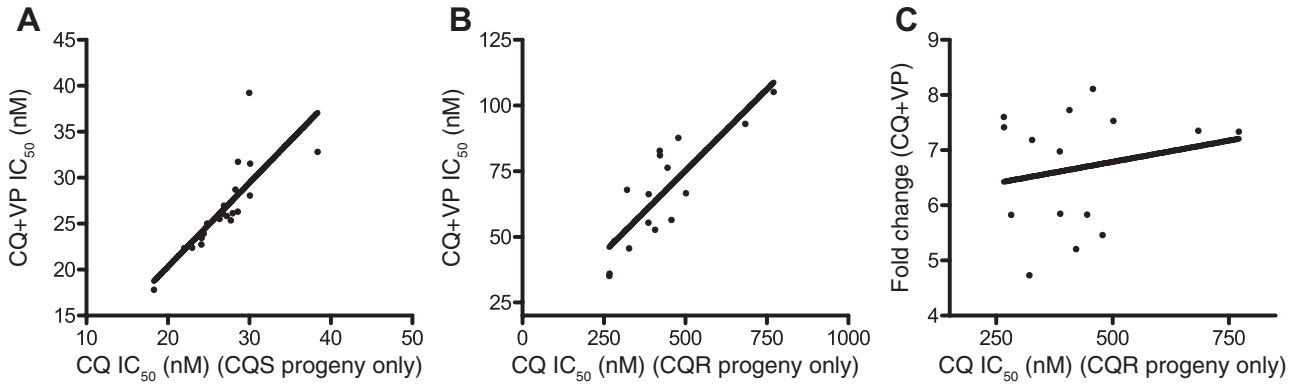


**Fig. 3.** Relationship between CQ $IC_{50}$ and CQ with VP response in CQS and CQ-resistant progeny.
A. Relationship between CQ response with and without VP in CQS parasites. CQS progeny exhibit a strong positive relationship between CQ response with and without VP ($r = 0.80$, $P < 0.0001$), although this relationship reflects unchanged responses.
B. Relationship between CQ response with and without VP in CQ-resistant parasites. CQ-resistant progeny exhibit a strong positive relationship between CQ response with and without VP ($r = 0.84$, $P < 0.0001$), resulting from a significant reduction in CQ response in the presence of VP.
C. Relationship between CQ response and fold change of CQ response in the presence of VP. Correlation analysis detects no significant relationship between CQ response in CQ-resistant parasites and the fold change resulting from CQ + VP combination ($r = 0.2$, $P > 0.05$).

and CT potencies with CQ $IC_{50}$ in both CQS and CQ-resistant progeny, suggesting that secondary genetic determinants of quantitative CQ susceptibility in these subgroups may influence CLM and CT response.

### *QTL analysis of drug responses*

Genome-wide QTL scans for VP, CLM, CT, CQ and CQ + VP responses are presented in Fig. 4. VP, tested independently (Fig. 4A), revealed a significant chr 7 QTL at 20.2 cM [log of odds (LOD) = 6.9] in the region of *pfcrt.* In addition, a suggestive QTL was identified on chr 5 (LOD = 2.3) that mapped to the marker at position 68.8 cM in the region of *pfmdr1*. CLM (Fig. 4B) and CT (Fig. 4C), tested independently, each revealed two QTL: a significant QTL on chr 7 (20.2 cM, LOD = 47 and 28 respectively) and a suggestive QTL on chr 5 (68.8 cM, LOD = 2.7 and 2.9 respectively). The substantial coincidence of the chr 5 (*pfmdr1*) and chr 7 (*pfcrt*) QTL for VP, CLM and CT was consistent with a relationship between

**Table 2.** Correlation analyses for CQ response versus reversal agent potency and fold change in CQS and CQR progeny.

| All progeny | | | | | | | |
|---|---|---|---|---|---|---|---|
| CQ $IC_{50}$ versus | VP | CLM | CT | CQ + VP fold change | CQ + CLM fold change | CQ + CT fold change | CQ + AM fold change |
| *r* | –0.72 | –0.95 | –0.92 | 0.98 | 0.98 | 0.98 | 0.98 |
| *P*-value | *** | *** | *** | *** | *** | *** | *** |
| **CQS progeny** | | | | | | | |
| CQS $IC_{50}$ versus | VP | CLM | CT | CQ + VP fold change | CQ + CLM fold change | CQ + CT fold change | CQ + AM fold change |
| *r* | 0.37 | 0.49 | 0.53 | 0.32 | 0.42 | 0.34 | 0.45 |
| *P*-value | 0.12 | 0.03 | 0.02 | 0.19 | 0.07 | 0.15 | 0.06 |
| **CQR progeny** | | | | | | | |
| CQR $IC_{50}$ versus | VP | CLM | CT | CQ + VP fold change | CQ + CLM fold change | CQ + CT fold change | CQ + AM fold change |
| *r* | 0.48 | 0.55 | 0.75 | 0.15 | 0.02 | 0.08 | 0.04 |
| *P*-value | 0.06 | 0.03 | 0.0001 | 0.59 | 0.93 | 0.78 | 0.89 |

*** $P < 0.0001$.
VP, CLM and CT represent $IC_{50}$ values of reversal agents when used alone (i.e. potency).
Correlation analysis reported as Pearson's correlation coefficient reported as an *r*-value, and the *P*-value determining if significantly deviating from zero.

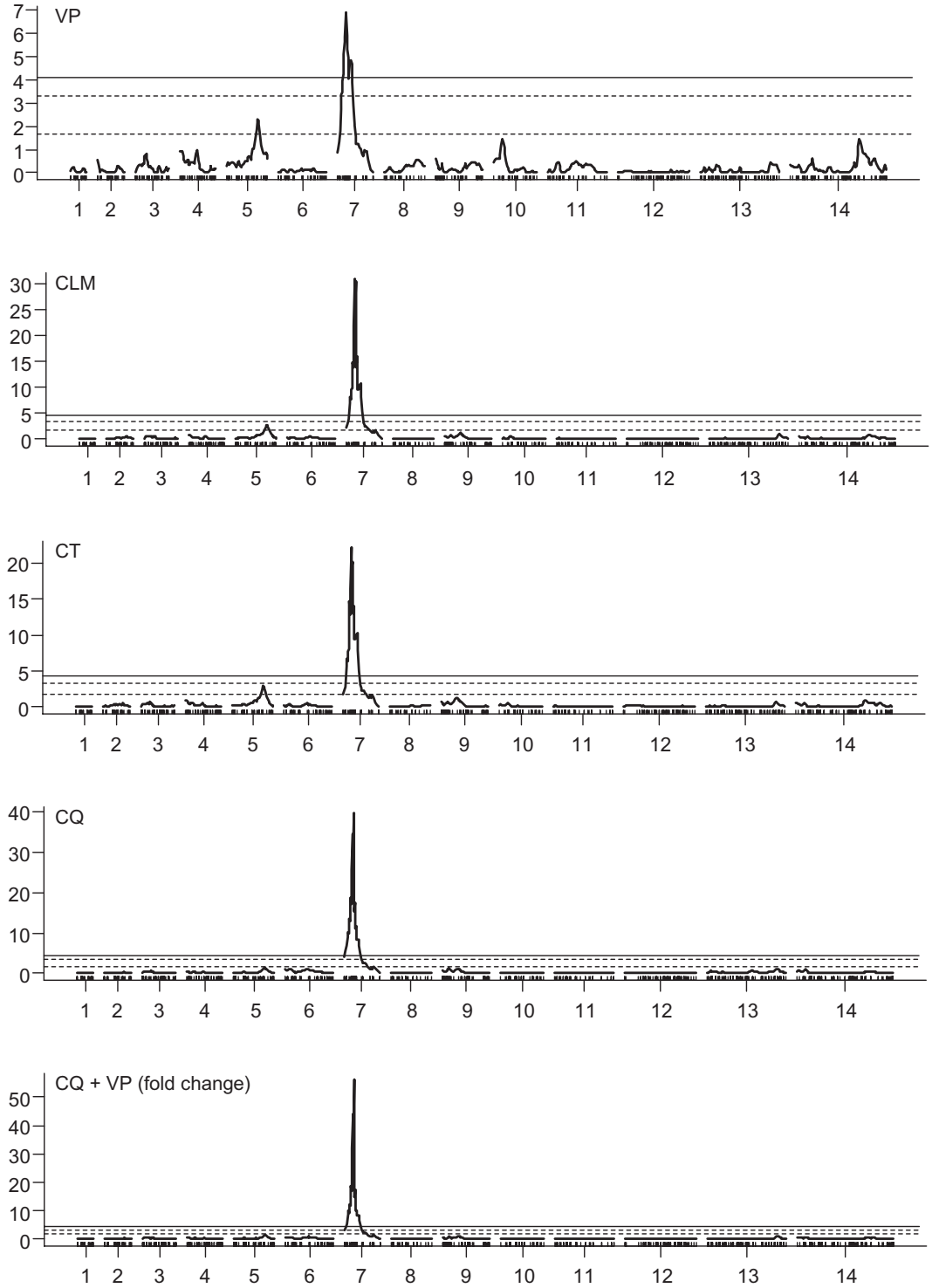


**Fig. 4.** Genome-wide QTL scans. Three significance thresholds were determined by permutation testing. Highly significant ($P < 0.01$), significant ($P < 0.05$) and suggestive ($P < 0.63$) LOD scores are represented by horizontal lines on each scan. The abscissae show chromosomal location (14 chromosomes), where the width of the chromosome number is proportional to the number of markers. All traits, VP, CLM, CT, CQ and CQ + VP map to chr 7 (20.2 cM) in the region of *pfcrt*. A suggestive QTL on chr 5 (68.8 cM) in the region of *pfmdr1* was detected for VP, CLM and CT.

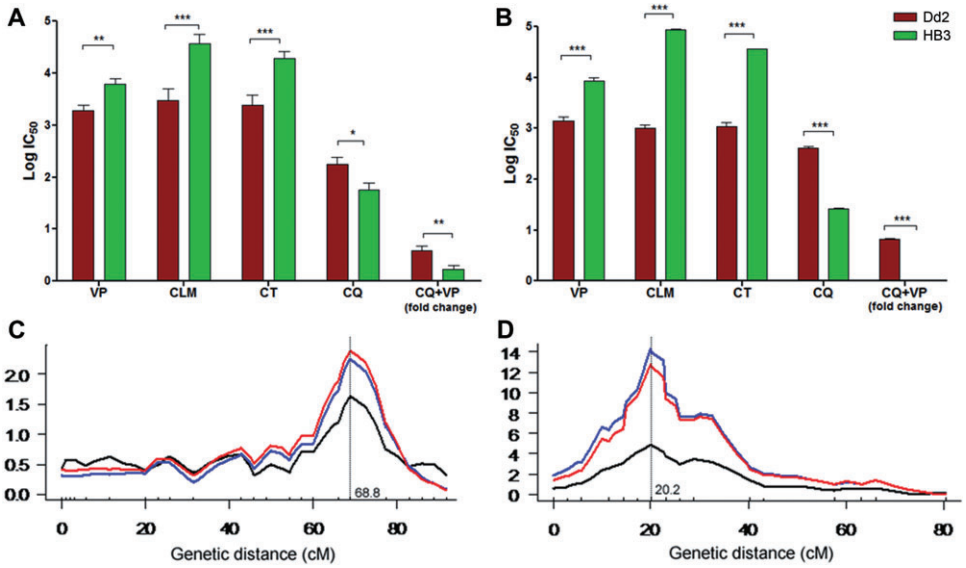


**Fig. 5.** Allele effects at peak markers for QTL detected on chr 5 and 7. Effects of the Dd2 and HB3 alleles for response to VP, CLM, CT and CQ at peak markers for QTL: (A) chr 5 (68.8 cM) and (B) chr 7 (20.2 cM). Higher trait values were observed for the HB3 alleles at both QTL for the reversal agents tested independently, whereas the higher trait value for CQ response and fold change (degree of reversibility) were observed with the Dd2 allele. (*$P < 0.05$, **$P < 0.001$ and ***$P < 0.0001$.) Overlaid QTL for VP (blue), CLM (red) and CT (black) for (C) chr 5 and (D) chr 7 respectively.

these two loci in controlling sensitivities to VP, CLM and CT. The genome-wide scan for CQ sensitivity detected a single highly significant QTL with a LOD = 39 (Fig. 4D). Fold change resulting from combining each reversal agent with CQ revealed a single shared QTL on chr 7 (20.2 cM) with even higher LODs than were observed for CQ response (e.g. Fig. 4E).

Allelic effects for the chr 5 (68.8 cM) and chr 7 (20.2 cM) loci (Fig. 5A and B respectively) indicated that higher $IC_{50}$ values for VP, CLM and CT drug responses were associated with the HB3 allele at both loci. For CQ response, the Dd2 allele on chr 7 (20.2 cM) was associated with a higher $IC_{50}$ and a greater fold change in the presence of a reversal agent. Figure 5C and D shows an overlay of QTL highlighting the shared QTL on chrs 5 and 7 respectively. This shows that at least two, and probably more loci influence the response to reversal agents by themselves, commensurate with the continuous distribution of those traits. However, CQ response reversibility is solely dependent on the chr 7 genetic effect that generates a two-class phenotype as a result of mutations in *pfcrt* (Fidock *et al*., 2000; Cooper *et al*., 2002; Sidhu *et al*., 2002). To investigate the genetic basis of the highly correlated degree of reversibility induced by VP, CT, AM and CLM in CQ-resistant progeny, measured as fold change, we conducted QTL scans directly on this subclass of progeny. A QTL peak on chr 11 (48.8 cM) in the region of marker C12M42 at 701 403 bp, passed the suggestive LOD threshold for fold change with all four compounds (data not shown).

*Identification of genetic loci affecting the range of susceptibilities in CQ-resistant progeny*

The rank order distribution of CQ response in the progeny is bimodal, as originally demonstrated by Wellems *et al*. (1990), and as is expected for a single major gene. In that early work, microscopy-based evaluation of parasitaemias showed identical $IC_{50}$ values for eight CQS and eight CQ-resistant progeny, and these two discrete classes did not segregate with the *pfmdr1* marker allele, proving that in the HB3 × Dd2 progeny *pfmdr1* was not the determinant of CQR. Here we used 33 progeny and multiple independent replications of radiolabelled-hypoxanthine (HX) incorporation to observe continuous distributions of quantitative CQ susceptibility within both the CQS (18–38 nM) and CQ-resistant (266–771 nM) subgroups (Fig. 6A). We examined only the CQ-resistant progeny for further analyses to specifically map QTL contributing to the wide range of CQ $IC_{50}$ values in the resistant class inheriting mutant *pfcrt*. This approach eliminates the contribution by *pfcrt* effect because each of the CQR progeny carried the identical Dd2 *pfcrt* allele. We identified two secondary loci, on chrs 5 and 7. The chr 5 QTL again mapped to 68.8 cM in the region of *pfmdr1*, with a LOD = 2.5, and the novel chr 7 QTL mapped to 5.8 cM with a LOD = 2.0 (Fig. 6B and Table S2). The allelic effect plots indicate that the chr 5 (68.8 cM) HB3 allele and the chr 7 (5.8 cM) Dd2 allele contributed to the highest CQR $IC_{50}$ values (Fig. 6C). Because we observed a suggestive chr 5 QTL peak in this subset of progeny, we

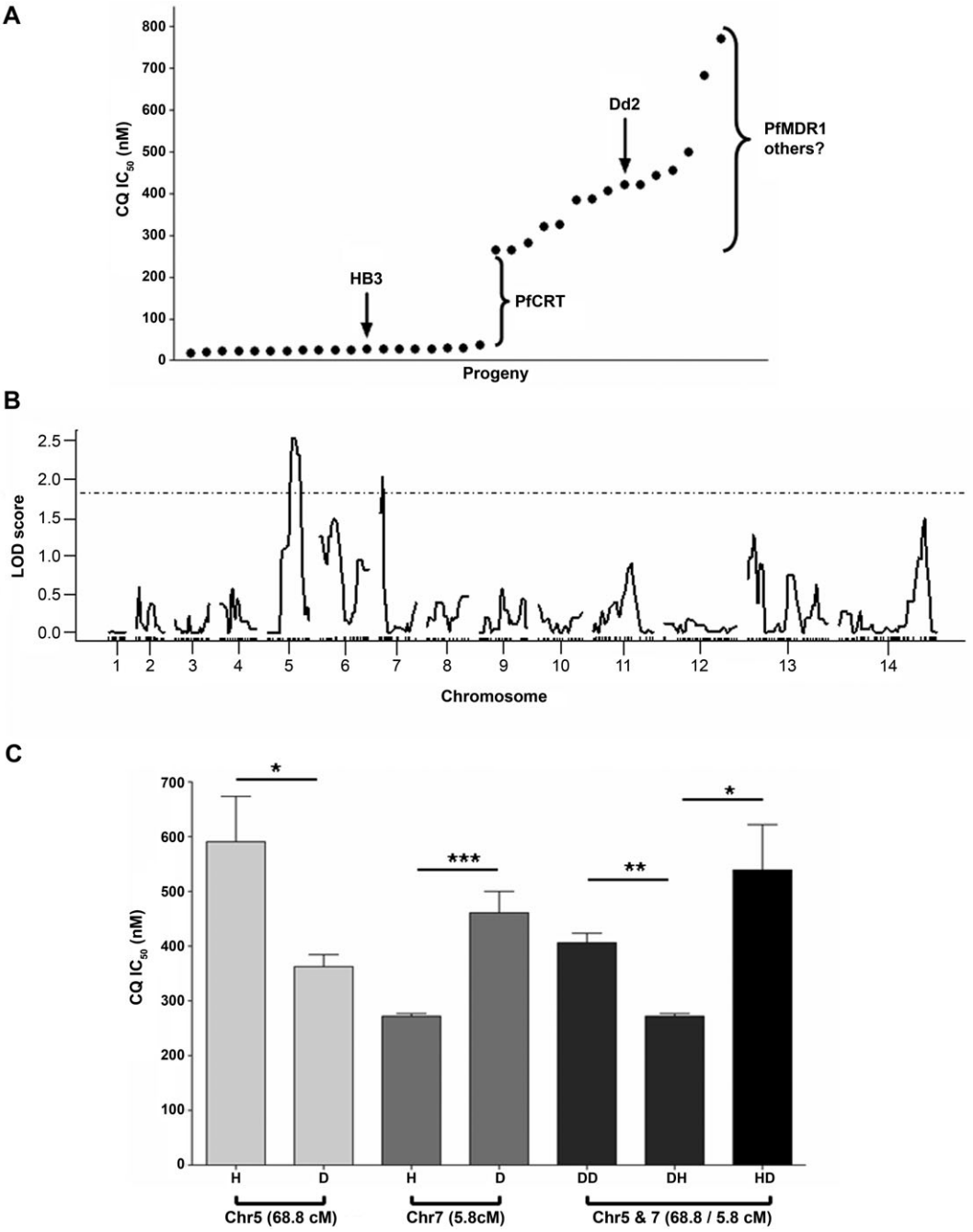


**Fig. 6.** CQ drug responses in the progeny identifying additional QTL effects contributing to extreme CQ drug response.
A. Rank order plot of CQ response in the progeny of the cross. A greater than 10-fold increase in $IC_{50}$ divides the progeny into CQS and CQ-resistant subsets. As a trait, this large difference maps to *pfcrt* on chr 7 and explains greater than 95% of the total variation in the cross. A steady increase in CQ-resistant progeny is observed ranging from 266.5 to 771.0 nm.
B. Genome-wide QTL scan for CQ response in CQ-resistant progeny. This QTL analysis identifies two suggestive QTL ($P < 0.63$): one on chr 5 (68.8 cM) in the region of *pfmdr1* and one on chr 7 (5.8 cM).
C. Allele effects of peak markers of CQR QTL. Inheritance of the Dd2 allele at the QTL on chr 5 (68.8 cM) confers low-level CQR, whereas the HB3 allele corresponds to higher CQR levels. An opposite pattern is seen for the chr 7 QTL peak marker (5.8 cM). A combination of $H_5D_7$ alleles combined with Dd2-type *pfcrt* results in the highest CQR levels (*$P < 0.05$, **$P < 0.001$, *** $P < 0.0001$).

further examined the relationship between *pfmdr1* and the Dd2-type *pfcrt* allele by employing the statistical method described by Sa *et al*. (2009) to directly test the influence of different *pfmdr1* alleles on CQ $IC_{50}$ values. Four different allele pair combinations were possible for *pfmdr1* and *pfcrt*: $D_5D_7$, $H_5D_7$, $D_5H_7$ and $H_5H_7$ (D represents an allele inherited from Dd2, and H represents an allele inherited from HB3). The *pfmdr1* locus significantly influenced the

CQ $IC_{50}$ only in CQ-resistant progeny; we detected a 1.57-fold shift in CQ susceptibility ($P < 0.01$) depending on the source of the *pfmdr1* allele in combination with mutant *pfcrt*. CQR progeny with the $H_5D_7$ combination have significantly higher $IC_{50}$ values than $D_5D_7$ (Fig. 6C). Because this difference was not detected in a recent study (Sa *et al*., 2009), we validated our findings. The rank order of CQ $IC_{50}$ values, 1BB5 > QC03 > 3BD5 in our study, contributed most to the differing results between the two studies. Because we use CQ as a control drug in our ongoing drug assays, four independent replicated analyses of the complete progeny set were available in our database and each demonstrated the same statistically significant rank order (1BB5 > QC03 > 3BD5) (data not shown). We also reanalysed CQ $IC_{90}$ values from the HB3 × Dd2 cross (Ferdig *et al*., 2004) using the same alternative statistical model outlined here and found a 1.4-fold CQ $IC_{90}$ difference ($P < 0.01$) between $D_5D_7$ and $H_5D_7$ progeny, with the latter combination most resistant.

### Pfmdr1 *CN and observed allele combinations in the progeny clones*

*Pfmdr1* CN was determined for all progeny (Table S3). The Dd2 parent has three copies while HB3 has a single copy. We observe nine progeny with two copies and three additional progeny with the Dd2 *pfmdr1* allele and CN = 1. Non-parental CN were reported previously for partial sets of HB3 × Dd2 progeny (Wellems *et al*., 1990; Rohrbach *et al*., 2006) and increased CN shows increased expression levels of PfMDR1 (Wilson *et al*., 1989; Rohrbach *et al*., 2006). Here we confirm and extend the findings of the inherited variation in CN, noting that the HB3 *pfmdr1* allele is always present as a single copy, while the Dd2 allele varies from one to four copies across the progeny set. We have confirmed the *in vitro* stability of CN by culturing 10 progeny clones for 30–45 replication cycles and observing no change in CN (data not shown).

It is difficult to determine the direct impact of *pfmdr1* CN on CQ $IC_{50}$ values because the allele effects cannot be precisely separated from the CN effects in progeny carrying one versus two copies. However, a direct comparison of $IC_{50}$ values of CQ-resistant parasites with two versus three or more copies, all of which contain Dd2 *pfcrt* and *pfmdr1* alleles, reveals no correlation ($r = -0.02$) and no difference in the mean $IC_{50}$ values ($P = 0.59$). Furthermore, we statistically removed the contribution of *pfcrt* and compared the residuals of mean CQ $IC_{50}$ values in the complete progeny set and found that CN (1 versus > 1, $P = 0.044$) accounted for somewhat less of the residual variation than point mutations alone (HB3 versus Dd2, $P = 0.02$). Consequently, our data do not support a role for CN in influencing CQ susceptibility. However, several observations support co-adaptation between *pfmdr1* CN and *pfcrt*. Inherited combinations of these loci in the progeny clones suggest an influence on fitness as surmised from the combinations that exist among the progeny: (i) of 33 progeny analysed, 15 inherited the Dd2 *pfmdr1* allele, but only three retained the full complement of three copies; each of these three carries the mutant *pfcrt*. Twelve of 15 progeny with the Dd2 *pfmdr1* allele lost at least one *pfmdr1* copy in the establishment of the stable progeny clonal lines, (ii) all progeny carrying the single copy Dd2 *pfmdr1* allele are CQS, carrying the wild-type *pfcrt*, and (iii) all five of the progeny with the $D_5H_7$ allele combination carry fewer than three copies. High CN is maintained only in the context of its co-selected mutant *pfcrt* partner, and CQS *pfcrt* is never paired with three copies of *pfmdr1* (Table S3).

### *Binding of a high affinity CQ analogue to PfMDR1*

Recently, the CQ binding site for wild-type PfCRT was defined using a novel photoaffinity chloroquine analogue, azido-biotinylated chloroquine (AzBCQ) (Lekostaj *et al*., 2008b). Using this same method and overexpression of recombinant PfMDR1 (Amoah *et al*., 2007; Lekostaj *et al*., 2008a), we tested whether the high-affinity AzBCQ probe would bind to 3D7 (wild type), Dd2 and 7G8 isoforms of purified recombinant PfMDR1 protein reconstituted into proteoliposomes (PLs) as recently described elsewhere (Pleeter *et al*., 2010). Figure 7 demonstrates that binding of AzBCQ to PfMDR1 reveals CQ affinity for PfMDR1. A 40- to 50-fold molar excess of CQ reduces efficiency of AzBCQ photolabelling to < 30% of control for 3D7 and Dd2 isoforms of PfMDR1 (Fig. 7A; lanes 2 versus 1 and 5 versus 4 respectively). Apparent CQ affinity for the 7G8 isoform is slightly reduced (competition to < 40% of control, Fig. 7A; lane 8 versus 7) relative to the other isoforms. Averaged over three experiments and two independent PL preparations for each isoform of PfMDR1, we did not identify pronounced VP competition for AzBCQ photolabelling to any isoform of PfMDR1 (Fig. 7C), in contrast to our earlier demonstration of VP competition for AzBCQ photolabelling of the 3D7 and Dd2 isoforms PfCRT (Lekostaj *et al*., 2008b).

## Discussion

After decades of remarkable success, CQ is no longer used against *P. falciparum* in most regions of the world due to the evolution of resistance (Wellems and Plowe, 2001). Selection by CQ was the first step towards the evolution of modern-day MDR parasites, yet questions remain about how CQR evolved and why CQR parasites persist long after CQ disuse. Answers to these questions will inform a more strategic development and deployment of drugs against MDR parasites.

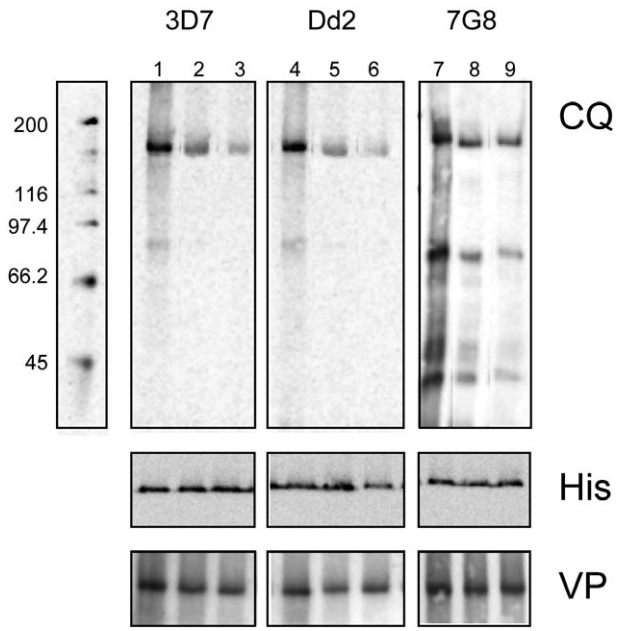


**Fig. 7.** Competition of AzBCQ labelling of 3D7, Dd2 and 7G8 isoforms of PfMDR1 by CQ and VP. In each photolabelling experiment, AzBCQ was present in a probe : protein molar ratio of about 100:1. PfMDR1 was labelled at various drug : AzBCQ molar excess ratios indicated below. All blots are representative of experiments conducted at least three times on at least two preparations of purified PLs wherein similar data were observed. Lanes 1–3 are 3D7 PfMDR1, lanes 4–6 are Dd2 PfMDR1 and lanes 7–9 are 7G8 PfMDR1. Top panels: Streptavidin-HRP detection of AzBCQ photolabelling with CQ competition. First lanes of each isoform have no competitor; second lanes of each isoform have 48-fold excess CQ; third lanes of each isoform have 80-fold excess CQ. Middle panels: Anti-PentaHis-HRP detection of 6His tag present in all isoforms. Bottom panels: Streptavidin-HRP detection of AzBCQ photolabelling with VP competition. First lanes have no competitor; second lanes have 20-fold excess VP; third lanes have 40-fold excess VP.

The classical genetic approach relies on Mendelian recombination and segregation to shuffle the naturally evolved allele combinations that have been fine-tuned by selection pressures on the parental genomes. By crossing two parents with distinct evolutionary histories, e.g. drug selection pressures applied in different geographic regions, the divergent signatures of selection in these genomes can be identified by QTL mapping. The strength of this approach was illustrated in a recent study comparing the genetic control of CQ and amodiaquine susceptibilities in progeny from two crosses between parents carrying a range of drug selection histories (Sa *et al.*, 2009).

Here, we rely on the *P. falciparum* HB3 × Dd2 genetic cross (Wellems *et al.*, 1990) to dissect the quantitative response to CQ, and to identify genetic loci that influence the reversibility of CQR. HB3 is a CQS parasite derived from Honduras. The progenitor of Dd2 was derived from a Laotian patient who failed CQ therapy (Campbell *et al.*, 1982), and this line also has high-level resistance to pyrimethamine. Dd2 was cloned from W2-mef which was selected in the laboratory by mefloquine (MQ). Consequently, the MDR Dd2 genome has been shaped by sequential drug selections and carries the South-East Asia-derived CQ-resistant PfCRT haplotype, referred to here as CVIET (amino acid positions 72–76).

A wide range of compounds can reverse CQR. By adapting the QTL methodology to compare a range of CQ chemosensitizers, we explored the genetic components of reversibility, represented by fold change of CQ $IC_{50}$ induced by several reversal agents. Furthermore, we examined the potencies of the individual reversal compounds. Continuous distributions were observed for the inherent potencies of VP (Fig. 2A), CLM and CT and susceptibility to these compounds was inversely correlated with CQ response. Previously VP was shown to have greater potency against CQ-resistant parasites (Gerena *et al.*, 1992), as have various antihistaminics (Peters *et al.*, 1989; 1990) and amantadine (Johnson *et al.*, 2004). Models predict binding of amantadine, a proton channel blocker, to the putative PfCRT pore of CQ-resistant parasites that could interfere with the transport of a critical substrate, resulting in parasite death (Johnson *et al.*, 2004; Wellems, 2004). This and other observations suggest that specific PfCRT binding by reversal agents underscores the potential of this transporter as a drug target. The continuous phenotype distributions indicate that multiple genes contribute to these traits. QTL analyses of the direct responses to VP, CLM and CT (Fig. 4A–C) identified two loci shared by all three reversal agents. The QTL on chr 5 maps to a marker at 68.8 cM in the region of *pfmdr1* (Fig. 5C) and the QTL on chr 7 maps to the marker at 20.2 cM in the region of *pfcrt* (Fig. 5D). These shared QTL indicate that parasite susceptibility to these structurally and pharmacologically distinct compounds is controlled by similar mechanisms that depend on both *pfcrt* and *pfmdr1*. The HB3 allele at both loci is associated with higher drug $IC_{50}$ values in the progeny (Fig. 5A and B). No other statistically significant loci were detected for these compounds, leaving unclear where additional factors controlling these complex traits reside.

In contrast to the individual effects by these compounds, genome-wide scans for reversibility maps to a single locus on chr 7 (20.2 cM) in the region of *pfcrt*. All combinations [CQ + VP (Fig. 4E), CQ + CLM, CQ + CT and CQ + AM] had exceptionally high LOD scores, indicating that reversibility of CQR is predominantly determined by *pfcrt* for all drug combinations. This observation is consistent with the recent observation that VP inhibits CQ transport (Martin *et al.*, 2009; Paguio *et al.*, 2009) and competes for labelling of recombinant purified PfCRT by a photoaffinity CQ analogue (Lekostaj *et al.*, 2008b). Corre-

spondingly, that we observed no effect by *pfmdr1* on reversibility agrees with the lack of significant disruption by VP of PfMDR1–AzBCQ binding (Fig. 7). Our observation of a direct determination of reversibility by *pfcrt* mutations concurs with the recent suggestion that CQ reversal agents are substrates for PfCRT and possibly inhibit CQ transport by a competitive mechanism (Lehane and Kirk, 2010). Our findings are restricted to the CVIET *pfcrt* allele carried by the Dd2 parent; other reports indicate that VP has less potency as a CQR reverser in the context of the SVMNT allele (Mehlotra *et al*., 2001; Sa *et al*., 2009), perhaps because VP is not a substrate for this form of *pfcrt* (Lekostaj *et al*., 2008b; Lehane and Kirk, 2010). We also identified a candidate locus on chr 11 controlling the four- to eightfold increase in CQ susceptibility induced by all four reversal agents. Although this is a modifying locus in HB3 × Dd2 progeny, it could point to a gene or pathway that plays a prominent role in the more subtle form of reversal observed in 7G8 that is not strongly linked to PfCRT mutations.

The HB3 × Dd2 *P. falciparum* genetic cross (Wellems *et al*., 1990) was generated to localize the determinant of CQR. For those studies, CQ susceptibility was treated as a bimodal trait such that each progeny was assigned to a subclass – CQS or CQ-resistant. This approach is effective for mapping a major genetic determinant and led to the identification of the chr 7 locus (Wellems *et al*., 1991), followed by positional cloning of *pfcrt* and eventual identification of the critical K76T mutation (Su *et al*., 1997; Fidock *et al*., 2000). QTL analyses relies on precise measurement of continuous phenotypes and can reveal both major and minor gene contributions and define the interactions among the identified loci (Ferdig *et al*., 2004). QTL analyses of CQ-resistant progeny remove the dominant *pfcrt* effect and point to two suggestive QTL [on chr 5 (68.8 cM) and on chr 7 (5.8 cM)] that account for ~70% of the phenotypic variance inherited in CQ-resistant individuals (Fig. 6B). We found that the *pfmdr1* locus significantly influenced CQ $IC_{50}$ values in CQ-resistant progeny, producing a 1.57-fold shift in CQ susceptibility. The effect of *pfmdr1* in generating distinct levels of CQR in the HB3 × Dd2 cross is weaker than the nearly twofold shift induced in the progeny from the 7G8 × GB4 cross that carries the 7G8 *pfcrt* allele from South America (SVMNT) (Sa *et al*., 2009).

The insight of Sa *et al*. that different drug use in different geographic regions will result in differentially tuned allele combinations may also be relevant here. Specifically, these authors propose that *pfmdr1* mediates a more stable, persistent form of CQR in South America and that this advantaged *pfmdr1* × *pfcrt* allelic combination may be induced in Africa by increased use of amodiaquine. The observation that low-level QN resistance in the HB3 × Dd2 cross exhibited a positive correlation with CQ $IC_{90}$ values in CQ-resistant parasites led to the suggestion that secondary loci selected by QN pressure, including *pfmdr1*, could incrementally influence CQ and other drug susceptibilities through a complex adaptive role among various polymorphisms (Ferdig *et al*., 2004). That is, amplification of and site-specific changes in *pfmdr1* associated with QN susceptibility (Cowman *et al*., 1994; Reed *et al*., 2000) could, in turn, have influenced the stability of CQR in South-East Asia and Africa through co-adapted allele combinations.

We observed that CQ-resistant progeny that inherited an HB3 allele at *pfmdr1* produced the highest CQ $IC_{50}$ values (Fig. 6C). This observation concurs with the observation that 7G8 × GB4 progeny that inherited the GB4 (CVIET) PfCRT allele show a smaller shift in CQR levels (1.4-fold) induced by the co-inherited *pfmdr1* allele (Sa *et al*., 2009). In both crosses, it is the mismatched *pfmdr1* allele – the allele *not* originating from the CQ-resistant parent with the CVIET haplotype – that is associated with the highest levels of CQR among the respective progeny. Importantly, this is in contrast to the situation for the 7G8 form of *pfcrt* (SVMNT) that produces its highest levels of CQR when matched with its co-evolved 7G8 *pfmdr1* partner. Notably, the levels of resistance produced by SVMNT are considerably lower than those produced by CVIET PfCRT, which originated in South-East Asia and later spread to Africa (Wootton *et al*., 2002). The unmatched allele combination, GB4 *pfmdr1*–7G8 *pfcrt*, exhibited a strikingly low level of CQR, with nearly 2.5-fold lower $IC_{50}$ values than the average CVIET progeny, perhaps near the limit of surviving therapeutic CQ doses. Considered in total, these observations imply that *pfmdr1* and *pfcrt* are co-evolved in the respective CQ-resistant parent genomes to optimize cell function and compensation rather than to maximize CQ $IC^{50}$ levels.

Analogous to the persistence of SVMNT CQR *pfcrt* alleles in South America (Contreras *et al*., 2002; Vieira *et al*., 2004), CQ-resistant parasites continue to predominate in Thailand more than 25 years after cessation of CQ therapy (Brockman *et al*., 2000; Price *et al*., 2004; Mu *et al*., 2005), even while carrying the CVIET form of *pfcrt* that has been suggested to be less fit in some genetic contexts. For example, CQ sensitivity has re-emerged in Malawi, where CQR was once widespread but CQ is no longer used (Kublin *et al*., 2003; Mita *et al*., 2003; Laufer *et al*., 2010), and in Hainan, China, where over a 10-year period CQR prevalence decreased from 98% to 61% (Liu *et al*., 1999). In these cases, CQ sensitivity returned steadily by re-integration of the complete CQS genomes, not by point mutant reversions that would be predicted for a single-gene resistance with strong fitness costs.

Because the sufficiency of introduced *pfcrt* mutations to induce CQR was tested only in certain genetic backgrounds (Sidhu *et al*., 2002), Hastings *et al*. (2002) noted that the definitive experiment would require introduction of

these mutations into a series of different genetic backgrounds. Valderramos *et al*. recently demonstrated the critical role of genetic background on the ability of the 7G8 *pfcrt* allele to confer CQR (Valderramos *et al*., 2010); they induced phenotypes in a variety of CQS backgrounds that ranged from high-level CQR (GC03, a progeny of the HB3 × Dd2 cross) to no change in $IC_{50}$ but an increased rate of recrudescence (D10, Papua New Guinea). Parasite lines with identical *pfmdr1* alleles transfected with the same *pfcrt* allele showed distinct CQ-resistant phenotypes, underscoring the role of genes other than *pfmdr1* and *pfcrt*. Moreover, the Dd2 *pfcrt* allele could not be used to successfully transform three different CQS background, 3D7, D10 and HB3. Only GC03, carrying half of its genome from Dd2, has been successfully transfected with the Dd2 *pfcrt*, giving rise to high-level CQR. Because HB3 and GC03 carry identical single copy *pfmdr1* alleles, we can again infer that an additional gene(s), co-selected in the Dd2 parent, must contribute to the competency of a CQS parasite to support the large effect CQR mutations. Notably, the mutational steps that gave rise to the few independently evolved CQR origins remain unknown. One possible candidate is an epistatic partner of *pfcrt* residing in the chr 6 locus was shown previously to regulate low-level QN resistance and associate with slow *in vitro* expansion rates of progeny inheriting the HB3-type chr 6 and Dd2-type *pfcrt* loci (Ferdig *et al*., 2004).

Dd2 and HB3 differ both in point mutations and in CN for *pfmdr1*: Dd2 (86Y, 184Y and 1042N) maintains three copies of *pfmdr1*, while HB3 (86N, 184F and 1042D) maintains a single copy. In an effort to evaluate the relative contribution of point mutations and CN on CQ susceptibility, we determined CN for the complete progeny set. Previous studies demonstrated that increased copies of *pfmdr1* coincided with an increase in PfMDR1 protein levels (Rohrbach *et al*., 2006). Allelic exchange studies to understand the role of amino acid changes in PfMDR1 have been limited to the carboxyl region that includes 1042N; replacement of the *pfmdr1* 3′ polymorphisms in CQ-resistant progeny 3BA6 with the Dd2− allele (D1042N) did not significantly alter CQ response (Sidhu *et al*., 2005) but did not rule out that either the 5′ region (86Y, 184Y) and/or *pfmdr1* CN influence CQR levels. The placement of D10 *pfmdr1* 3′ polymorphisms in the 7G8 background led to a twofold decrease in CQ response (Reed *et al*., 2000). When pressured with CQ, parasites with multiple *pfmdr1* copies lose extra copies of the gene (Barnes *et al*., 1992; Peel *et al*., 1994), with no other mutation in *pfmdr1* or *pfcrt*, suggesting that excessive copies may have a fitness cost. We found no difference in CQR levels attributable to CN. Moreover, most progeny with the Dd2 *pfmdr1* allele do not carry the parental complement of three copies. CN change is commonly reported in response to *in vitro* drug pressure (Wilson *et al*., 1989; Barnes *et al*., 1992; Chavchich *et al*., 2010), and a reduction in CN has been observed after removal of artelinic acid pressure (Chen *et al*., 2010); the rate of CN loss and the final stable numbers of copies is a characteristic of individual parasite lines. We have observed that CN is stable in long-term culture in these progeny, suggesting that the relationship of allele combinations at *pfmdr1* and *pfcrt* is fine-tuned. High CN thrives only in the context of its co-evolved genome that includes Dd2-type *pfcrt*, whereas CQS *pfcrt* prefers a single copy of *pfmdr1* (Table S3).

*Pfmdr1* knockdown by gene disruption for FCB (86Y, 184Y and 1042N) showed no significant changes in CQ susceptibility (Sidhu *et al*., 2006). Association studies in natural CQ-resistant populations have identified a significant role for *pfmdr1* 86Y and *pfcrt* 76T in CQR (Babiker *et al*., 2001; Mittra *et al*., 2006), but the presence and strength of these associations varies geographically. The frequencies of both these alleles are on the rise in regions of India under intense CQ selection (Mittra *et al*., 2006). However, in Thailand, where CQ is no longer used and MQ pressure led to increased *pfmdr1* CN, CQ-resistant parasites uniformly carry *pfmdr1* 86N (Price *et al*., 2004). An apparent conflict exists between 86Y and increased CN such that CN increases often associated with selection by MQ, artemisinin and lumefantrine, coincides with the loss of 86Y (Uhlemann *et al*., 2007). It is unusual to find the 86Y mutation with increased CN, but it has been reported in natural isolates collected prior to emergence of MQ resistance (Looareesuwan *et al*., 1992; Uhlemann *et al*., 2005; Sidhu *et al*., 2007). Both point mutations in *pfmdr1* and increased CN have been widely associated with fitness costs during asexual, erythrocytic growth in the absence of drug pressure in field isolates (Osman *et al*., 2007), allelic replacements (Hayward *et al*., 2005) and drug selected mutants (Preechapornkul *et al*., 2009).

It may be that the coincidence of 86Y and increased copy number is a transitional state that has been captured in the Dd2 line by *in vitro* MQ selection on W2. In the HB3 × Dd2 progeny, the combination of PfMDR1 86Y and PfCRT 76T confers only low-level CQR and the progeny that inherited 86N combined with a single copy of *pfmdr1* have the highest CQR levels. This is consistent with the co-adapted combination of Dd2 alleles at these two loci reflecting a trade-off between the level of CQR and fitness.

Classical genetics and QTL mapping can compliment gene manipulations using transfection methods to explore the basic biology of CQR. *Pfcrt* mutations are well known to be at the centre of CQR and its reversibility; however, accumulating evidence points to an important role for additional factors that influence these traits as well as parasite fitness. We show that many structurally distinct

reversal agents restore CQ susceptibility through a PfCRT mechanism. Furthermore, although *pfcrt* is the determinant of large phenotypic differences between CQ-resistant and CQS parasites, secondary loci can be identified for CQ susceptibility and reversibility. These loci can point to genes that may interact with the endogenous function of *pfcrt* or may cause effects through indirect physiological mechanisms. Understanding these interacting/alternative pathways and their co-selection by drug may illuminate the evolutionary path to CQR and, more importantly, refine strategies to limit new resistances.

## Experimental procedures

### *Parasites, genotypes and culturing*

The HB3 (Honduras) and Dd2 (Indochina) clonal parental isolates and 35 clonal progeny lines are from the same cross previously used to construct the *P. falciparum* high-resolution genetic linkage map (Wellems *et al*., 1990; Su *et al*., 1999). Leukocyte-free human red blood cells (Indiana regional blood centre) were suspended in complete medium [RPMI 1640 with L-glutamine (Invitrogen), 50 mg $l^{-1}$ hypoxanthine and 25 mM HEPES; 0.5% Albumax I (Invitrogen), 10 mg $l^{-1}$ gentamicin and 0.225% $NaHCO_3$] at 4% haematocrit and grown at 37°C in six-well culture plates, placed in a sealed environmental chamber (Billups-Rothenberg), and gassed daily (mixture of 5% $CO_2$, 5% $O_2$ and 90% $N_2$). Lines were cultured for at least three cycles after thawing with a minimum doubling of parasitaemia each 48 h cycle, prior to initiation of drug assays. No attempt was made to synchronize parasite cultures; however, the process of thawing yielded partially synchronous parasite stages. Dilutions to initiate assays were made from cultures between 1% and 5% parasitaemia consisting of greater than 75% ring stages. To minimize chances of genotype–phenotype shifts resulting from mutation accumulation, parasite cultures were carried for a maximum of 6 weeks and replicate testing was conducted on stocks closest to the original progeny (stock cultures are frozen from short-term cultures).

### *Drug response assays*

Drug stocks of chloroquine diphosphate (Sigma-Aldrich), verapamil hydrochloride (Sigma-Aldrich), chlorpheniramine maleate (Sigma-Aldrich) and amitriptyline hydrochloride (Sigma-Aldrich) were made in deionized water. A stock solution of citalopram hydrobromide (Fisher Scientific) was made in 100% ethanol. Stock solutions of probenecid (Avocado Research Chemicals) and desipramine (Sigma-Aldrich) were made in DMSO. All solutions were stored at –20°C until use. Dilutions from these stocks were made in low-hypoxanthine complete medium (low-HX CM; RPMI 1640 with L-glutamine, 2.94 μM HX and 50 mM HEPES; 0.5% Albumax I, 10 μg $ml^{-1}$ gentamicin and 0.225% $NaHCO_3$). Drug assays based on [$^3$H]-HX incorporation were performed as previously described (Ferdig *et al*., 2004); each biological replication was conducted independently on different days for each parasite line. For inclusion in the assay, each progeny clone was genotyped using a panel of 10 MS markers to ensure clonality and confirm identity.

Per cent inhibition was calculated by the formula:

$$100 \times [(\text{cpm at lowest concentration of drug}) - (\text{cpm of treated sample})]/[(\text{cpm at lowest concentration of drug}) - (\text{cpm at highest concentration of drug})].$$

$IC_{50}$ values were determined by fitting data to a non-linear sigmoidal dose–response curve (variable slope) using GraphPad Prism v4.0 software. Log transformed mean dose–response values were obtained from 5 to 22 independent measurements and were used for QTL analysis.

### *Isobologram analysis*

To determine the presence and degree of reversibility in the parents of the genetic cross, twofold serial dilutions of the drug combinations of CQ and reversal agents were prepared using fixed ratios. The starting concentrations of reversal agents for the serial dilutions were adjusted to approximately centre the $IC_{50}$ doses in the range tested. The following CQ : reversal agent volumetric ratios were used: 10:0, 9:1, 8:2, 7:3, 5:5, 3:7, 2:8, 1:9 and 0:10. The first and last ratios include only one drug, either CQ or the reversal agent. FICs were determined for each drug combination for both HB3 and Dd2 using the formula:

$$(IC_{50} \text{ of drug A, tested in combination})/(IC_{50} \text{ of drug A, tested alone}) + (IC_{50} \text{ of drug B, tested in combination})/(IC_{50} \text{ of drug B, tested alone}).$$

Additivity was defined as a FIC = 1 and synergism (reversibility) was defined as a FIC of $\leq 0.5$ (points lying below the diagonal line of additivity). The fixed dose ratio with the lowest concentration of reversal agent that showed a synergistic interaction with CQ was used for tests in the progeny.

### *QTL analyses*

Genome-wide scans were performed using R/QTL (Broman *et al*., 2003) to detect single and interacting QTL associated with drug responses in the HB3 × Dd2 genetic cross. Drug responses were log transformed to approximate a normal distribution. Genome-wide significance thresholds were determined by permutation testing ($n = 1000$ permutations) (Doerge and Churchill, 1996) and the strength of linkage of a locus to the trait was expressed as a LOD score. Loci that exceeded the 99th percentile ($P < 0.01$), 95th percentile ($P < 0.05$) and 37th percentile ($P < 0.63$) were used to identify highly significant, significant and suggestive QTL respectively. The combined effects of all QTL detected for each trait were determined by multiple regression analysis for all significant and suggestive QTL. Other statistical analyses were performed using GraphPad Prism v4.0. Allele effects were determined by calculating the phenotypic mean at the peak marker for each of the two possible genotypes.

### Copy number determination

*Pfmdr1* copy number was determined by TaqMan real-time PCR using an ABI 7700 as reported previously (Rohrbach *et al*., 2006). The *pfmdr1* probe was FAMTM (6-carboxyfluorescein) labelled at the 5′ end, and the α-tubulin control probe was VICTM labelled. Forty cycles were performed and fluorescence data were expressed as normalized reporter signals, calculated by dividing the amount of reporter signal by the passive reference signal. The assay was replicated four independent times and normalized to HB3.

### Purification of recombinant PfMDR1 and reconstitution into PLs

High-level expression of various isoforms of PfMDR1 with C-terminal hexaHis detection tags via the pPICZc expression vector (Invitrogen) in *P. pastoris* has been reported previously (Amoah *et al*., 2007; Lekostaj *et al*., 2008a). Yeasts were grown under standard conditions, induced with 0.5% methanol medium for 21 h, mechanically lysed via a modified glass bead method and membranes harbouring PfMDR1 were isolated as described (Fritz *et al*., 1999; Amoah *et al*., 2007). PLs harbouring purified PfMDR1 were prepared using methods described earlier for PfCRT (Lekostaj *et al*., 2008b). In brief, total protein was determined using the amido black assay, crude yeast membranes (CMs) were solubilized with dodecyl maltoside (DM), and protein purified by nickel chelation chromatography. CMs were diluted to 2 mg $ml^{-1}$ in a Tris-based wash buffer [50 mM Tris-HCl, 250 mM sucrose, 20% (v/v) glycerol and 1 mM $MgCl_2$ (pH 7.5), supplemented with 3 M NaCl] and centrifuged at 100 000 *g* for 1 h. The washed pellet was resuspended at 2 mg $ml^{-1}$ in solubilization buffer (wash buffer supplemented with 500 mM NaCl and 1% DM). The sample was rotated for 30 min, and centrifuged for 1 h at 100 000 *g*. The detergent extract was then applied to a His GraviTrap column (GE Healthcare) and allowed to flow through via gravity. The column was washed with several bed volumes of 20 mM imidazole to remove non-specifically bound proteins, and PfMDR1 was eluted with 500 mM imidazole, into polar *Escherichia coli* lipid suspension at a final concentration of 1.2% lipid. Protein–lipid mixtures were rotated gently for 30 min and then dialysed against 50 mM Tris-HCl, 250 mM sucrose and 1 mM EDTA (pH 7.5) for 20 h. Purified PfMDR1 PLs were centrifuged for 1 h at 100 000 *g*, pellets were resuspended in fresh dialysis buffer, and snap-frozen in dry ice-ethanol. Purified protein levels were redetermined using the amido black assay.

### Drug competition with AzBCQ photolabelling

The synthesis of AzBCQ and optimization of photolabelling conditions within a 96-well plate format has been described in detail previously (Lekostaj *et al*., 2008b). Experiments with PfMDR1 PLs were carried out with minor modifications (Pleeter *et al*., 2010). Briefly, a fixed amount (4.0 μg per well) of purified PL protein was diluted in a 50 mM Mes-Tris buffer at pH 5.2. The PL-buffer solution was aliquoted into wells of a 96-well UV transparent plate. Either buffer or competing drug solution was added to each respective well, followed by the addition of 0.5 mM AzBCQ. The plate was shaken at 650 r.p.m. for 30 s and then incubated for 10 min at 37°C in a water bath. The plate was then exposed to UV radiation (254 nm maximum) at 1 cm distance for 10 min. The reaction was quenched with the addition of an equal volume of Laemmli sample buffer. The samples were again incubated for 10 min at 37°C, divided in half, and loaded onto two parallel 7.5% bis-acrylamide Tris-HCl gels. For AzBCQ photolabelling detection, blots were blocked in 10% milk and probed with streptavidin-HRP (1:400). For quantification of PfMDR1 protein present, C-terminal hexaHis tags were probed with the Qiagen PentaHis detection kit according to the manufacturer's instructions (Lekostaj *et al*., 2008b).

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