Published in final edited form as:
Trends Pharmacol Sci. 2006 November ; 27(11): 594–601. doi:10.1016/j.tips.2006.09.005.

Transporters involved in resistance to antimalarial drugs

Stephanie G. Valderramos and **David A. Fidock**
Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

Abstract

The ability to treat and control *Plasmodium falciparum* infection through chemotherapy has been compromised by the advent and spread of resistance to antimalarial drugs. Research in this area has identified the *P. falciparum* chloroquine resistance transporter (PfCRT) and the multidrug resistance-1 (PfMDR1) transporter as key determinants of decreased *in vitro* susceptibility to several principal antimalarial drugs. Transfection-based *in vitro* studies are consistent with clinical findings of an association between mutations in the *pfcrt* gene and failure of chloroquine treatment, and between amplification of the *pfmdr1* gene and failure of mefloquine treatment. Many countries are now switching to arte-misinin-based combination therapies. These incorporate partner drugs of which some have an *in vitro* efficacy that can be modulated by changes in *pfcrt* or *pfmdr1*. Here, we summarize investigations of these and other recently identified *P. falciparum* transporters in the context of antimalarial mode of action and mechanisms of resistance.

Malaria and drug resistance

The spread of drug resistant strains of the malaria parasite *Plasmodium falciparum* has led to a significant resurgence of malarial morbidity and mortality, and a growing crisis in global public health [1]. *P. falciparum* causes an estimated 500 million clinical infections and at least one million deaths annually, primarily in sub-Saharan Africa [1,2]. Infection begins when *Anopheles* mosquitoes deliver sporozoite forms that invade hepatocytes and replicate as liver-stage parasites, before emerging into the blood stream and infecting red blood cells. The asexual blood-stage infection causes clinical disease characterized by cyclical fevers and shaking chills, which can lead to complications such as severe anemia or cerebral malaria [3,4]. Red blood cells can also harbor sexual-stage gametocytes that can be transmitted to mosquitoes, where they undergo genetic recombination and complete the lifecycle of the parasite.

For decades, the treatment of malaria depended on chloroquine – an extensively used 4-aminoquinoline characterized by its rapid efficacy, low toxicity, availability and affordability [5]. The eventual appearance of chloroquine resistance in Southeast Asia and South America sparked the global dissemination of resistance [6]. The first-line treatment for chloroquine-resistant (CQR) malaria – sulfadoxine–pyrimethamine – rapidly met with resistance and is also becoming increasingly ineffective [7]. Failure of *P. falciparum* clinical treatment resulting from confirmed *in vivo* parasite resistance has now been documented for all current antimalarial drugs apart from the artemisinins, placing artemisinin-based combination therapy at the forefront of current malaria control programs [8,9].

Here, we review recent studies into the *P. falciparum* chloroquine resistance (PfCRT) and multidrug resistance-1 (PfMDR1) transporters that are now known to be key contributors to *P. falciparum* antimalarial drug resistance. We also briefly discuss the Ca^{2+} transporter

Corresponding author: Fidock, D.A. (dfidock@aecom.yu.edu).

PfATP6, which has been implicated in artemisinin action [10–13] (for reviews on parasite determinants of resistance to antimalarial antifolates, mitochondrial inhibitors and antibiotics, see Refs [7,14,15]).

PfCRT

The putative transporter PfCRT was identified through the analysis of a genetic cross between a chloroquine-sensitive (CQS) and a CQR clone, which mapped resistance to the gene *pfcrt* [16,17]. The 45-kDa PfCRT protein contains ten predicted transmembrane domains and is located on the membrane of the digestive vacuole – an acidic, lysosome-like compartment in which hemoglobin is degraded and detoxified, and in which the weak base chloroquine concentrates in its diprotonated form and binds hematin (a dimeric form of oxidized heme) [6,18,19]. *pfcrt* shows extraordinary sequence diversity among geographically distinct isolates: point mutations have been detected at 15 residues and 4–8 individual mutations are present in individual CQR lines (Figure 1). This level of diversity corresponds to at least five independent origins of mutant *pfcrt* [20]. The Asian and African CQR alleles seem to confer a fitness cost, as evidenced by data from Malawi and China that show a decrease in the prevalence of these alleles upon discontinued use of chloroquine [21].

By allelic exchange, CQR alleles have been shown to confer *in vitro* chloroquine resistance to CQS parasites [22]. This resistance phenotype is defined as a half-maximal inhibitory concentration (IC_{50}) of ≥80 nM, decreased [^{3}H]chloroquine accumulation and verapamil reversibility. Removal of the K76T mutation, which is ubiquitous in CQR lines, results in CQR parasites becoming fully CQS and losing their verapamil reversibility phenotype [23]. It has not proved possible to introduce this single mutation into CQS parasites, indicating that this mutation might have a detrimental effect on function that needs to be compensated by other *pfcrt* mutations. These *in vitro* results are largely consistent with *in vivo* findings that document a strong association between the PfCRT K76T mutation and failure of chloroquine treatment, leading to its widespread use as a molecular marker of chloroquine resistance [5,7,21]. The *in vivo* studies have also found that some individuals carrying parasites with mutant *pfcrt* have an adequate clinical response to chloroquine treatment. This has been attributed, at least in part, to synergy between partially effective chloroquine treatment and acquired immunity [5].

Interestingly, studies have shown that the PfCRT transporter can also significantly influence parasite *in vitro* susceptibility to many antimalarial drugs including quinine, monodesethylamodiaquine (the primary metabolite of amodiaquine), halofantrine and artemisinin [18,22–24]. A significant contribution of *pfcrt* to parasite susceptibility to antimalarial drugs in addition to chloroquine might explain the unusual diversity of *pfcrt* alleles, particularly in parts of Asia such as Cambodia where chloroquine is used rarely if at all against *P. falciparum* infection [25,26].

To disseminate, resistance determinants must not only ensure parasite survival against drug treatment but also be successfully transmitted through the gametocyte stage into the mosquito vector. Recent findings in the Gambia show a strong selection for mutant *pfcrt* in gametocyte populations in individuals exposed to chloroquine [27,28]. These mutants might prevent chloroquine from killing very-early-stage gametocytes [29] or, alternatively, game-tocytes harboring mutant *pfcrt* might have enhanced transmissibility. Such effects would provide a compelling explanation for how mutant *pfcrt* became so prevalent across malaria-endemic regions [30].

PfCRT and biochemical models of chloroquine resistance

Historically, investigations into the chloroquine resistance mechanism have generated vastly differing models, including reduced chloroquine influx, increased efflux, pH effects on drug

accumulation and/or receptor availability, and glutathione degradation of hematin or chloroquine–hematin complexes [6,31]. From these studies, several tenets regarding the mode of action of chloroquine and the mechanism (or mechanisms) of resistance have become widely accepted: first, chloroquine enters the acidic digestive vacuole by passive diffusion as an uncharged species and becomes trapped in the digestive vacuole in its diprotonated, membrane-impermeable form; second, chloroquine is retained in the digestive vacuole as chloroquine^{2+}–hematin complexes that are central to its antimalarial activity [32,33]; and third, the chloroquine resistance mechanism restricts chloroquine access to hematin and leads to reduced drug levels in the digestive vacuole [34]. The availability of isogenic lines expressing variant *pfcrt* alleles has also demonstrated that the chloroquine resistance mechanism is dependent on replacing the positively charged PfCRT K76 residue in the first transmembrane domain with a neutral residue (such as threonine, asparagine or isoleucine) [17,18,23].

Three models of chloroquine resistance that attempt to reconcile the existing data have now come to the forefront: (i) efflux of chloroquine out of the digestive vacuole via an energy-coupled transporter [35–38]; (ii) leak of chloroquine out of the digestive vacuole down its concentration gradient in a manner that is not directly coupled to energy [20,24,34]; and (iii) pH-dependent reductions in chloroquine accumulation in the digestive vacuole, possibly associated with a role for PfCRT in mediating direct transport of the drug [33,39–41] (Figure 2). Whereas the first two models are mutually exclusive in their interpretation of whether drug movement is directly coupled to energy, the digestive vacuole pH model is non-exclusive and could potentially combine with either of the two other models to yield chloroquine resistance. The idea that PfCRT is directly involved in chloroquine transport in the cell is consistent with both bioinformatic analyses that place this protein in the drug-metabolite effluxer family of transporters [42,43], and data from heterologous systems suggesting that mutant PfCRT can bind to and physically transport chloroquine [44,45].

Energy-coupled chloroquine efflux

In support of the first model, CQR parasites have been reported to release pre-accumulated chloroquine almost 50 times faster than have CQS parasites [35]. Investigation of the kinetics of accumulation shows a transient, rapid increase in chloroquine accumulation in resistant parasites, which resolves to little or no accumulation within minutes. By contrast, there is a continuing rise in chloroquine accumulation in CQS parasites that reaches a plateau far higher than that attained in CQR parasites [46]. In all parasites, the initial chloroquine uptake is maximal at 37–40 °C, arguing that uptake is a temperature-dependent active process. Addition of glucose markedly stimulates chloroquine accumulation in CQS parasites, but reduces steady-state accumulation of chloroquine in CQR parasites, suggesting that there are energy-coupled mechanisms of chloroquine uptake and chloroquine efflux in sensitive and resistant parasites, respectively [36,38].

Preloading CQR parasites with different concentrations of unlabeled chloroquine before adding [^{3}H]chloroquine has recently provided intriguing evidence of a '*trans*-stimulation' effect, whereby accumulation of [^{3}H]chloroquine first increases at low external preloaded chloroquine concentrations and then decreases [38]. This *trans*-stimulation phenotype has been previously described for the human red blood cell GLUT1 transporter and is thought to distinguish active efflux carriers from channels, pores or leaks, through which solutes move by passive diffusion [47]. This effect in CQR lines is not seen in CQS lines, where external preloaded chloroquine seems to compete with [^{3}H]chloroquine for carrier sites at all concentrations. This observation has led to the proposal that CQR parasites have an active chloroquine efflux carrier, such that pre-equilibrated, preloaded chloroquine at low concentrations competes for carrier sites, leading to an increase in [^{3}H]chloroquine accumulation, whereas preloaded chloroquine at high concentrations saturates the carrier sites

and outcompetes [^{3}H]chloroquine in binding to its intracellular receptor (heme), leading to a reduction in [^{3}H]chloroquine accumulation [38]. This *trans*-stimulated accumulation of chloroquine by CQR parasites is glucose dependent (implying that ATP is involved), is blocked by verapamil, and also occurs when preloaded chloroquine is substituted by related quinoline drugs such as amodiaquine, quinine and quinidine [37]. Recent studies with *pfcrt*-modified recombinant parasites have indicated that mutant PfCRT might fulfill this role of a substrate-specific, verapamil-reversible, ATP-dependent chloroquine efflux carrier [48].

Leak of charged chloroquine out of the digestive vacuole

The second model postulates that chloroquine leaves the digestive vacuole by a mechanism of facilitated diffusion driven by a large concentration gradient of the protonated forms of the drug. Here, energy is proposed to drive the digestive vacuole proton pump and to maintain the concentration gradient of protonated chloroquine, rather than being directly coupled to drug movement.

Features of this model were first proposed by Warhurst *et al.* [49], who indicated some similarities between PfCRT and bacterial ClC chloride channels and noted that the crucial K76T mutation removes a positively charged residue from transmembrane domain I in a putative pore, increasing the hydrophobicity and potentially providing a route for the more polar (protonated) forms of chloroquine to escape the digestive vacuole. More hydrophobic chemo-sensitizers such as verapamil have been proposed to block chloroquine efflux sterically, effectively countering the resistance mechanism [49]. Support for the importance of the charge-loss mutation has come from the demonstration that a compensatory charge substitution (S163R in transmembrane domain IV) fully restores chloroquine sensitivity in drug-pressured mutant *pfcrt* lines [24].

Both the charged drug leak and the energy-coupled efflux models posit that PfCRT is directly involved in chloroquine movement but with the following key difference: in the former protonated chloroquine passively leaks out of the digestive vacuole through mutant PfCRT, whereas in the latter this protein actively effluxes drug. Both models can adequately explain most of the existing chloroquine transport data, and direct comparisons of the different experimental conditions with the same isogenic *pfcrt*-modified lines are warranted to resolve the differences between these models.

pH-dependent physiological changes at the digestive vacuole membrane

Early models of chloroquine accumulation postulated that uptake of chloroquine into the acidic digestive vacuole was primarily due to weak-base ion-trapping in accordance with the Henderson–Hasselbach equation [50–52]. These models predicted that a more acidic pH in the digestive vacuole, leading to a larger pH gradient across the digestive vacuole membrane, would result in greater accumulation of chloroquine. Innovative developments in *P. falciparum* single cell photometry, however, have produced the surprising result that CQR and not CQS lines have the most acidic digestive vacuoles [39,40]. One explanation is that the lower digestive vacuole pH in CQR isolates might accelerate the rate of heme aggregation and hemozoin formation, reducing the amount of hematin available to bind chloroquine and resulting in less uptake of chloroquine [41]. Some concerns have been expressed about the appropriateness of using fluorescent pH sensor dyes and the possibility of photobleaching and laser-induced disaggregation of the digestive vacuole [53–55]. Nevertheless, independent studies in *pfcrt*-transfected *Dictyostelium discoideum* also provide evidence that *pfcrt* expression has an effect on intracellular pH and that mutant PfCRT confers a more acidic pH than does the wild-type protein [45].

Notwithstanding, it is generally thought that differences in the digestive vacuole pH are themselves not primarily responsible for CQR, and recent data from the proponents of the pH model have provided evidence that physical interactions with chloroquine are also a factor in how mutant PfCRT mediates resistance [44].

PfMDR1

More than a decade before the discovery of *pfcrt*, research into the genetic basis of chloroquine resistance had focused on *pfmdr1*, a *P. falciparum* ortholog of mammalian P-glycoproteins that mediate verapamil-reversible multi-drug resistance in mammalian cancer cells [35]. *pfmdr1* encodes a 162-kDa protein (PfMDR1; also known as Pgh1) that localizes to the digestive vacuole membrane and consists of two homologous halves, each with six predicted transmembrane domains and a conserved nucleotide-binding domain [56,57] (Figure 3). The transport function of PfMDR1 was evidenced by its complementation of a yeast strain defective for the STE6 transporter [58]. A recent study, using fluorescein derivatives that are widely used in surrogate assays of P-glycoprotein function, has provided intriguing evidence that PfMDR1 might function to import solutes, including some antimalarial drugs, into the digestive vacuole [59].

Two *pfmdr1* alleles have been identified in CQR field isolates: the K1 allele (containing the point mutation N86Y) and the 7G8 allele (containing Y184F, S1034C, N1042D and D1246Y). *In vitro* studies using field isolates or laboratory lines have identified a partial association between the N86Y mutation and chloroquine resistance [60–63]. By contrast, a role for the 7G8-type-3 mutations has been harder to ascertain [6,57]. For these 3′ mutations, direct evidence was obtained in allelic exchange experiments, which showed that the mutations enhance the degree of *in vitro* chloroquine resistance, although they do not confer resistance to sensitive parasites [64]. Another study using similar genetic techniques, however, observed no effect of these 3′ mutations on chloroquine [65]. The interpretation that *pfmdr1* mutations might enhance chloroquine resistance in some genetic backgrounds but are themselves insufficient to confer resistance has support from some clinical studies [61,62,66]. Alternatively, the increased frequency of *pfmdr1* polymorphisms in CQR parasites might reflect physiological compensation for the altered function of mutant *pfcrt* [5].

Studies of field isolates and a genetic cross have also identified an association between *pfmdr1* point mutations and the degree of parasite *in vitro* susceptibility to other antimalarial drugs including mefloquine, halofantrine, quinine and artemisinin [57]. These data have been largely confirmed by genetic studies, which reinforce the notion that these effects are strain dependent [64,65]. Their impact on therapeutic outcomes seems more limited [67]. Nevertheless, a recent study that monitored recrudescence of infection after treatment with a lumefantrine–artemether combination (Coartem) has reported significant selection for the *pfmdr1* 86N polymorphism, suggesting that this mutation might be useful as a molecular marker of lumefantrine resistance [68].

Several investigations have noted a correlation between *pfmdr1* expression and drug resistance in *P. falciparum*, paralleling the multidrug resistance mechanism observed in mammalian tumor cells. In a study based on drug-sensitive strains, *pfmdr1* transcript levels were observed to increase after treatment with chloroquine, mefloquine and quinine, but not after treatment with pyrimethamine, suggesting that induction of *pfmdr1* might be a drug-specific mechanism of resistance [69]. Other studies with field isolates or drug-pressured laboratory lines have found an association between *in vitro* mefloquine resistance and a higher *pfmdr1* copy number, which in some instances is also associated with increased susceptibility to chloroquine [57, 67,70,71].

The relationship between *pfmdr1* copy number and mefloquine treatment outcome has been comprehensively investigated in a large, prospective study in Thailand, where quantitative real-time PCR analysis of over 600 patient samples has shown that *pfmdr1* amplification is highly associated with failure of mefloquine or mefloquine–artesunate treatment [67]. *pfmdr1* amplification has also been associated with an increased risk of failure of short-term artemether–lumefantrine treatment [72]. These clinical findings have been confirmed by a genetically controlled experiment showing that an increase in *pfmdr1* copy number causes a decrease in *in vitro* susceptibility to mefloquine, quinine, halofantrine and artemisinin [73]. Amplified *pfmdr1* copy number, which is prevalent mostly in Asia, has also been observed in some earlier field isolates from Gabon, possibly because of local drug pressure [74]. How PfMDR1 mediates these effects is unclear, although a recent study indicates that *pfmdr1* point mutations or changes in *pfmdr1* copy number might affect the degree of drug accumulation in the digestive vacuole, which could affect the *in vitro* potency of the drugs [59].

Other transporters implicated in antimalarial drug resistance

Recent studies in antimalarial chemotherapy have also implicated several other transporters, most notably PfATP6 – the *P. falciparum* ortholog of the mammalian sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA). Expression of PfATP6 in *Xenopus laevis* oocytes revealed that its ATPase activity is inhibited by artemisinin in addition to thapsigargin – a known SERCA inhibitor [10]. Modeling of this protein against mammalian SERCA led to the finding that amino acid variants at position 263 in the predicted thapsigargin-binding pocket can ablate inhibition by artemisinin derivatives in the *Xenopus* oocyte system [12]. Recent studies have also reported an association between the S769N mutation in PfATP6 and increased IC_{50} values for artemether in field isolates from French Guyana [11]. Genetic experiments in the parasite should further define the role of PfATP6 in the mode of action of artemisinin derivatives.

Recent genomic analyses have implicated other putative transporters in modulating parasite response to antimalarial drugs [63,75,76]. For example, quantitative trait loci mapping of a *P. falciparum* genetic cross in which inheritance of chloroquine and quinine resistance are correlated found an association between quinine resistance and mutations in *pfcrt* and *pfmdr1*, and also implicated a locus on chromosome 13 that contains a predicted Na^+–H^+ exchanger (*pfnhe*) [75]. Analysis of microsatellite variations noted a significant association between DNNND repeats in the C-terminal cytoplasmic domain of PfNHE and *in vitro* quinine response. In addition, reduction of *pfnhe* expression by genetic manipulation has recently identified an association between PfNHE expression levels and the degree of quinine resistance in CQR parasites (L. Nkrumah *et al.*, unpublished).

Analyses of single nucleotide polymorphisms in different parasite isolates have also identified additional candidates for antimalarial resistance genes. Linkage disequilibrium analysis of 97 culture-adapted parasite isolates from around the world found that single nucleotide polymorphisms from *pfcrt*, *pfmdr1* and at least nine new putative transporter genes located on different chromosomes were associated with chloroquine and quinine resistance [63]. However, a follow-up study examining polymorphisms in these nine new genes in isolates from two independent population samples in Southeast Asia found only one consistent association, which was between increased artesunate IC_{50} values and the putative ABC transporter G7 [76]. These discrepancies might be due to variations in study design or to the different geographic origins of the parasite samples.

Concluding remarks

The above studies provide a promising platform from which to direct future research on parasite transporter proteins and drug resistance. In particular, elucidation of the mechanisms by which PfCRT and PfMDR1 mediate resistance to multiple drug classes can help to guide efforts to

overcome the spread of drug resistance. Furthermore, screens for other candidate transporter loci involved in antimalarial drug resistance should be extended to assess copy number and/or expression levels, in addition to identifying single nucleotide polymorphisms. A thorough understanding of the complex interactions among malarial transport proteins, and how these interactions influence parasite response to antimalarial drugs, will be an essential tool in worldwide efforts to combat and control this important disease.

Acknowledgments

We thank Rebecca Muhle, Marcus Lee, Amar bir Singh Sidhu, Patrick Bray and Scott Bohle for comments on the manuscript. Financial support was provided by the National Institutes of Health (R01 AI50234) and a Burroughs Wellcome Fund Investigator in Pathogenesis of Infectious Disease Award (to D.A.F.).

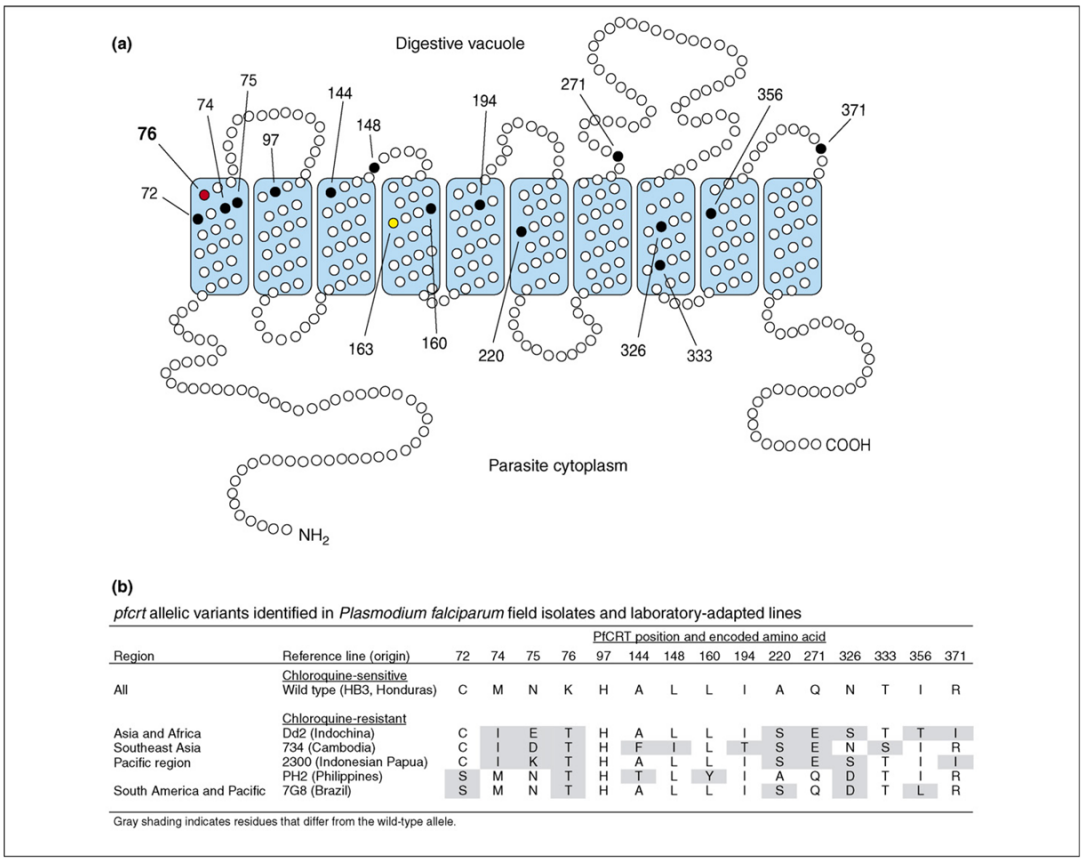
References

1. Greenwood B, Mutabingwa T. Malaria in 2002. Nature 2002;415:670–672. [PubMed: 11832954]
2. Snow RW, et al. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature 2005;434:214–217. [PubMed: 15759000]
3. Miller LH, et al. The pathogenic basis of malaria. Nature 2002;415:673–679. [PubMed: 11832955]
4. Planche T, Krishna S. Severe malaria: metabolic complications. Curr Mol Med 2006;6:141–153. [PubMed: 16515507]
5. Wellems TE, Plowe CV. Chloroquine-resistant malaria. J Infect Dis 2001;184:770–776. [PubMed: 11517439]
6. Uhlemann, A-C., et al. Mechanisms of antimalarial drug action and resistance. In: Sherman, IW., editor. Molecular Approaches to Malaria. ASM Press; 2005. p. 429-461.
7. Wongsrichanalai C, et al. Epidemiology of drug-resistant malaria. Lancet Infect Dis 2002;2:209–218. [PubMed: 11937421]
8. White NJ. Antimalarial drug resistance. J Clin Invest 2004;113:1084–1092. [PubMed: 15085184]
9. Baird JK. Effectiveness of antimalarial drugs. N Engl J Med 2005;352:1565–1577. [PubMed: 15829537]
10. Eckstein-Ludwig U, et al. Artemisinins target the SERCA of *Plasmodium falciparum*. Nature 2003;424:957–961. [PubMed: 12931192]
11. Jambou R, et al. Resistance of *Plasmodium falciparum* field isolates to *in-vitro* artemether and point mutations of the SERCA-type PfATPase6. Lancet 2005;366:1960–1963. [PubMed: 16325698]
12. Uhlemann AC, et al. A single amino acid residue can determine sensitivity of SERCAs to artemisinins. Nat Struct Mol Biol 2005;12:628–629. [PubMed: 15937493]
13. Krishna S, et al. Re-evaluation of how artemisinins work in light of emerging evidence of *in vitro* resistance. Trends Mol Med 2006;12:200–205. [PubMed: 16616639]
14. Gregson A, Plowe CV. Mechanisms of resistance of malaria parasites to antifolates. Pharmacol Rev 2005;57:117–145. [PubMed: 15734729]
15. Wiesner J, et al. New antimalarial drugs. Angew Chem Int Ed Engl 2003;42:5274–5293. [PubMed: 14613157]
16. Su X, et al. Complex polymorphisms in a ~330 kDa protein are linked to chloroquine-resistant *P. falciparum* in Southeast Asia and Africa. Cell 1997;91:593–603. [PubMed: 9393853]
17. Fidock DA, et al. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell 2000;6:861–871. [PubMed: 11090624]
18. Cooper RA, et al. Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*. Mol Pharmacol 2002;61:35–42. [PubMed: 11752204]
19. Goldberg DE. Hemoglobin degradation. Curr Top Microbiol Immunol 2005;295:275–291. [PubMed: 16265895]
20. Bray PG, et al. Defining the role of PfCRT in *Plasmodium falciparum* chloroquine resistance. Mol Microbiol 2005;56:323–333. [PubMed: 15813727]

21. Hyde JE. Drug-resistant malaria. Trends Parasitol 2005;21:494–498. [PubMed: 16140578]
22. Sidhu AB, et al. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. Science 2002;298:210–213. [PubMed: 12364805]
23. Lakshmanan V, et al. A critical role for PfCRT K76T in *Plasmodium falciparum* verapamil-reversible chloroquine resistance. EMBO J 2005;24:2294–2305. [PubMed: 15944738]
24. Johnson DJ, et al. Evidence for a central role for PfCRT in conferring *Plasmodium falciparum* resistance to diverse antimalarial agents. Mol Cell 2004;15:867–877. [PubMed: 15383277]
25. Chen N, et al. pfcrt allelic types with two novel amino acid mutations in chloroquine-resistant *Plasmodium falciparum* isolates from the Philippines. Antimicrob Agents Chemother 2003;47:3500–3505. [PubMed: 14576108]
26. Lim P, et al. *pfcrt* polymorphism and chloroquine resistance in *Plasmodium falciparum* strains isolated in Cambodia. Antimicrob Agents Chemother 2003;47:87–94. [PubMed: 12499174]
27. Sutherland CJ, et al. Gambian children successfully treated with chloroquine can harbour and transmit *Plasmodium falciparum* gametocytes carrying resistance genes. Am J Trop Med Hyg 2002;67:578–585. [PubMed: 12518847]
28. Hallett RL, et al. Combination therapy counteracts the enhanced transmission of drug-resistant malaria parasites to mosquitoes. Antimicrob Agents Chemother 2004;48:3940–3943. [PubMed: 15388456]
29. Chutmongkonkul M, et al. *Plasmodium falciparum*: effect of chloroquine, halofantrine and pyrimethamine on the infectivity of gametocytes for *Anopheles stephensi* mosquitoes. Ann Trop Med Parasitol 1992;86:103–110. [PubMed: 1417200]
30. Wootton JC, et al. Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. Nature 2002;418:320–323. [PubMed: 12124623]
31. Bray PG, Ward SA. A comparison of the phenomenology and genetics of multidrug resistance in cancer cells and quinoline resistance in *Plasmodium falciparum*. Pharmacol Ther 1998;77:1–28. [PubMed: 9500157]
32. Bray PG, et al. Defining the role of PfCRT in *P. falciparum* chloroquine resistance. Mol Microbiol 2005;56:323–333. [PubMed: 15813727]
33. Yayon A. The antimalarial mode of action of chloroquine. Rev Clin Basic Pharm 1985;5:99–139. [PubMed: 3916306]
34. Bray PG, et al. Access to hematin: the basis of chloroquine resistance. Mol Pharmacol 1998;54:170–179. [PubMed: 9658203]
35. Krogstad DJ, et al. Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. Science 1987;238:1283–1285. [PubMed: 3317830]
36. Krogstad DJ, et al. Energy dependence of chloroquine accumulation and chloroquine efflux in *Plasmodium falciparum*. Biochem Pharmacol 1992;43:57–62. [PubMed: 1531176]
37. Sanchez CP, et al. Evidence for a substrate specific and inhibitable drug efflux system in chloroquine resistant *Plasmodium falciparum* strains. Biochemistry 2004;43:16365–16373. [PubMed: 15610031]
38. Sanchez CP, et al. Trans stimulation provides evidence for a drug efflux carrier as the mechanism of chloroquine resistance in *Plasmodium falciparum*. Biochemistry 2003;42:9383–9394. [PubMed: 12899625]
39. Bennett TN, et al. Drug resistance-associated pfCRT mutations confer decreased *Plasmodium falciparum* digestive vacuolar pH. Mol Biochem Parasitol 2004;133:99–114. [PubMed: 14668017]
40. Dzekunov SM, et al. Digestive vacuolar pH of intact intraerythrocytic *P. falciparum* either sensitive or resistant to chloroquine. Mol Biochem Parasitol 2000;110:107–124. [PubMed: 10989149]
41. Ursos LM, Roepe PD. Chloroquine resistance in the malarial parasite, *Plasmodium falciparum*. Med Res Rev 2002;22:465–491. [PubMed: 12210555]
42. Martin RE, Kirk K. The malaria parasite's chloroquine resistance transporter is a member of the drug/metabolite transporter superfamily. Mol Biol Evol 2004;21:1938–1949. [PubMed: 15240840]
43. Tran CV, Saier MH Jr. The principal chloroquine resistance protein of *Plasmodium falciparum* is a member of the drug/metabolite transporter superfamily. Microbiology 2004;150:1–3. [PubMed: 14702390]
44. Zhang H, et al. The antimalarial drug resistance protein *Plasmodium falciparum* chloroquine resistance transporter binds chloroquine. Biochemistry 2004;43:8290–8296. [PubMed: 15222741]

45. Naude B, et al. *Dictyostelium discoideum* expresses a malaria chloroquine resistance mechanism upon transfection with mutant, but not wild-type, *Plasmodium falciparum* transporter PfCRT. J Biol Chem 2005;280:25596–25603. [PubMed: 15883156]
46. Sanchez CP, et al. Identification of a chloroquine importer in *Plasmodium falciparum*. Differences in import kinetics are genetically linked with the chloroquine-resistant phenotype. J Biol Chem 1997;272:2652–2658. [PubMed: 9006900]
47. Stein, WD. Transport and Diffusion Across Cell Membranes. Academic Press; 1986.
48. Sanchez CP, et al. Evidence for a *pfcrt*-associated chloroquine efflux system in the human malarial parasite *Plasmodium falciparum*. Biochemistry 2005;44:9862–9870. [PubMed: 16026158]
49. Warhurst DC, et al. Lysosomes and drug resistance in malaria. Lancet 2002;360:1527–1529. [PubMed: 12443586]
50. Homewood CA, et al. Lysosomes, pH and the anti-malarial action of chloroquine. Nature 1972;235:50–52. [PubMed: 4550396]
51. Krogstad DJ, et al. Antimalarials increase vesicle pH in *Plasmodium falciparum*. J Cell Biol 1985;101:2302–2309. [PubMed: 3905824]
52. Yayon A, et al. Identification of the acidic compartment of *Plasmodium falciparum*-infected human erythrocytes as the target of the antimalarial drug chloroquine. EMBO J 1984;3:2695–2700. [PubMed: 6391917]
53. Bray PG, et al. Distribution of acridine orange fluorescence in *Plasmodium falciparum*-infected erythrocytes and its implications for the evaluation of digestive vacuole pH. Mol Biochem Parasitol 2002;119:301–304. [PubMed: 11814584]
54. Wissing F, et al. Illumination of the malaria parasite *Plasmodium falciparum* alters intracellular pH. Implications for live cell imaging. J Biol Chem 2002;277:37747–37755. [PubMed: 12140286]
55. Hayward R, et al. The pH of the digestive vacuole of *Plasmodium falciparum* is not associated with chloroquine resistance. J Cell Sci 2006;119:1016–1025. [PubMed: 16492710]
56. Peel SA. The ABC transporter genes of *Plasmodium falciparum* and drug resistance. Drug Resist Updat 2001;4:66–74. [PubMed: 11512154]
57. Duraisingh MT, Cowman AF. Contribution of the *pfmdr1* gene to antimalarial drug-resistance. Acta Trop 2005;94:181–190. [PubMed: 15876420]
58. Volkman SK, et al. Functional complementation of the *ste6* gene of *Saccharomyces cerevisiae* with the *pfmdr1* gene of *Plasmodium falciparum*. Proc Natl Acad Sci U S A 1995;92:8921–8925. [PubMed: 7568044]
59. Rohrbach P, et al. Genetic linkage of *pfmdr1* with food vacuolar solute import in *Plasmodium falciparum*. EMBO J 2006;25:3000–3011. [PubMed: 16794577]
60. Adagut IS, Warhust DC. *Plasmodium falciparum*: linkage disequilibrium between loci in chromosomes 7 and 5 and chloroquine selective pressure in Northern Nigeria. Parasitology 2001;123:219–224. [PubMed: 11578085]
61. Djimdé A, et al. A molecular marker for chloroquine resistant *falciparum* malaria. N Engl J Med 2001;344:257–263. [PubMed: 11172152]
62. Babiker HA, et al. High-level chloroquine resistance in Sudanese isolates of *Plasmodium falciparum* is associated with mutations in the chloroquine resistance transporter gene *pfcrt* and the multidrug resistance gene *pfmdr1*. J Infect Dis 2001;183:1535–1538. [PubMed: 11319692]
63. Mu J, et al. Multiple transporters associated with malaria parasite responses to chloroquine and quinine. Mol Microbiol 2003;49:977–989. [PubMed: 12890022]
64. Reed MB, et al. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature 2000;403:906–909. [PubMed: 10706290]
65. Sidhu AB, et al. *pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. Mol Microbiol 2005;57:913–926. [PubMed: 16091034]
66. Ngo T, et al. Analysis of *pfcrt*, *pfmdr1*, *dhfr*, and *dhps* mutations and drug sensitivities in *Plasmodium falciparum* isolates from patients in Vietnam before and after treatment with artemisinin. Am J Trop Med Hyg 2003;68:350–356. [PubMed: 12685644]

67. Price RN, et al. Mefloquine resistance in *Plasmodiumfalciparum* and increased *pfmdr1* gene copy number. Lancet 2004;364:438–447. [PubMed: 15288742]
68. Sisowath C, et al. *In vivo* selection of *Plasmodium falciparum pfmdr1* 86N coding alleles by artemether-lumefantrine (Coartem). J Infect Dis 2005;191:1014–1017. [PubMed: 15717281]
69. Myrick A, et al. Mapping of the *Plasmodium falciparum* multidrug resistance gene 5′-upstream region, and evidence of induction of transcript levels by antimalarial drugs in chloroquine sensitive parasites. Mol Microbiol 2003;49:671–683. [PubMed: 12864851]
70. Nelson AL, et al. *pfmdr1* genotyping and *in vivo* mefloquine resistance on the Thai-Myanmar border. Am J Trop Med Hyg 2005;72:586–592. [PubMed: 15891133]
71. Price RN, et al. The *pfmdr1* gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. Antimicrob Agents Chemother 1999;43:2943–2949. [PubMed: 10582887]
72. Price RN, et al. Molecular and pharmacological determinants of the therapeutic response to artemether–lumefantrine in multidrug-resistant *Plasmodium falciparum* malaria. Clin Infect Dis 2006;42:1570–1577. [PubMed: 16652314]
73. Sidhu ABS, et al. Decreasing *pfmdr1* copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. J Infect Dis 2006;194:528–535. [PubMed: 16845638]
74. Uhlemann AC, et al. Amplification of *Plasmodium falciparum* multidrug resistance gene 1 in isolates from Gabon. J Infect Dis 2005;192:1830–1835. [PubMed: 16235185]
75. Ferdig MT, et al. Dissecting the loci of low-level quinine resistance in malaria parasites. Mol Microbiol 2004;52:985–997. [PubMed: 15130119]
76. Anderson TJ, et al. Are transporter genes other than the chloroquine resistance locus (*pfcrt*) and multidrug resistance gene (*pfmdr*) associated with antimalarial drug resistance? Antimicrob Agents Chemother 2005;49:2180–2188. [PubMed: 15917511]
77. Howard EM, et al. A novel transporter, *pfcrt*, confers antimalarial drug resistance. J Membr Biol 2002;190:1–8. [PubMed: 12422267]



(b)

pfcrt allelic variants identified in *Plasmodium falciparum* field isolates and laboratory-adapted lines

Region	Reference line (origin)	PfCRT position and encoded amino acid														
		72	74	75	76	97	144	148	160	194	220	271	326	333	356	371
	Chloroquine-sensitive															
All	Wild type (HB3, Honduras)	C	M	N	K	H	A	L	L	I	A	Q	N	T	I	R
	Chloroquine-resistant															
Asia and Africa	Dd2 (Indochina)	C	I	E	T	H	A	L	L	I	S	E	S	T	T	I
Southeast Asia	734 (Cambodia)	C	I	D	T	H	F	I	L	T	S	E	N	S	I	R
Pacific region	2300 (Indonesian Papua)	C	I	K	T	H	A	L	L	I	S	E	S	T	I	I
	PH2 (Philippines)	S	M	N	T	H	T	L	Y	I	A	Q	D	T	I	R
South America and Pacific	7G8 (Brazil)	S	M	N	T	H	A	L	L	I	S	Q	D	T	L	R

Gray shading indicates residues that differ from the wild-type allele.

Figure 1.

Predicted structure and representative haplotypes of *P. falciparum* chloroquine resistance transporter. **(a)** PfCRT is predicted to have ten transmembrane domains, with its N and C termini located on the cytoplasmic side of the digestive vacuole membrane (adapted, with permission, from Ref. [20]). Mutations identified in *pfcrt* cDNA sequences from CQR lines (black circles), the crucial K76T mutation common to all CQR strains (red) and the S163R mutation identified in amantadine- and halofantrine-resistant parasites (yellow circle) [24] are indicated. **(b)** Representative *pfcrt* haplotypes.

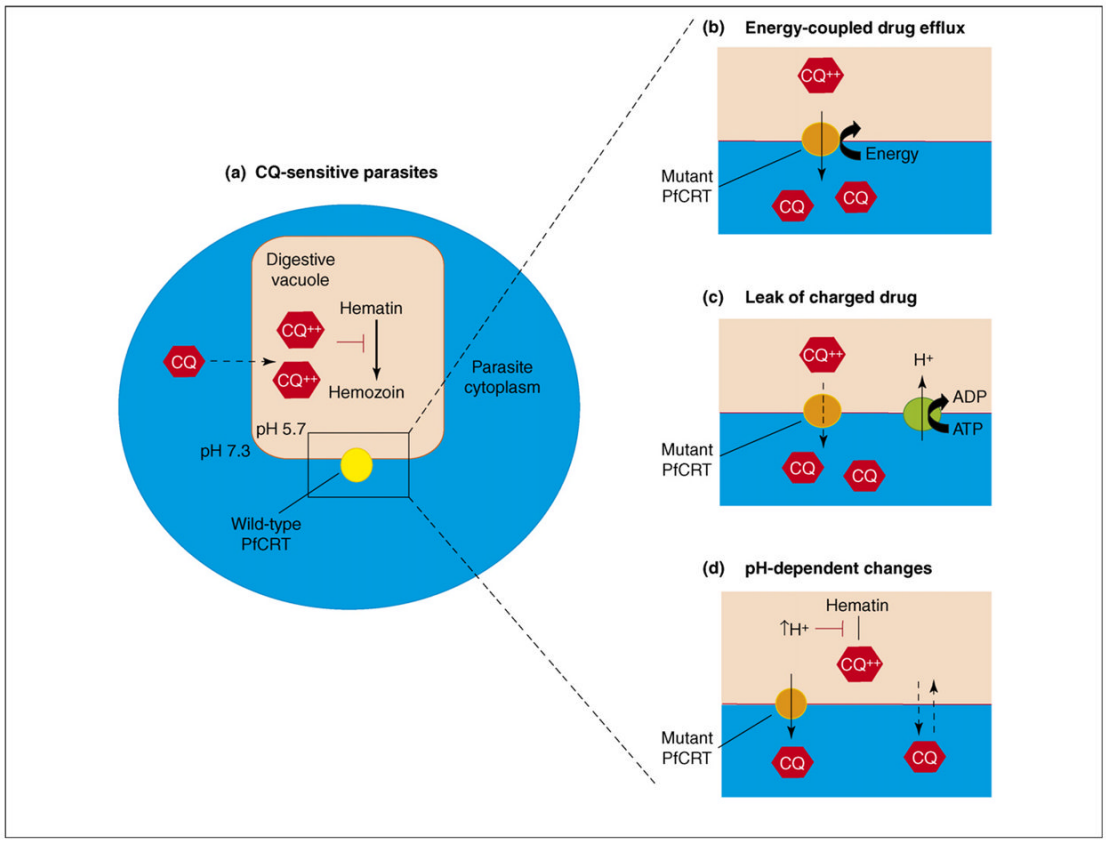
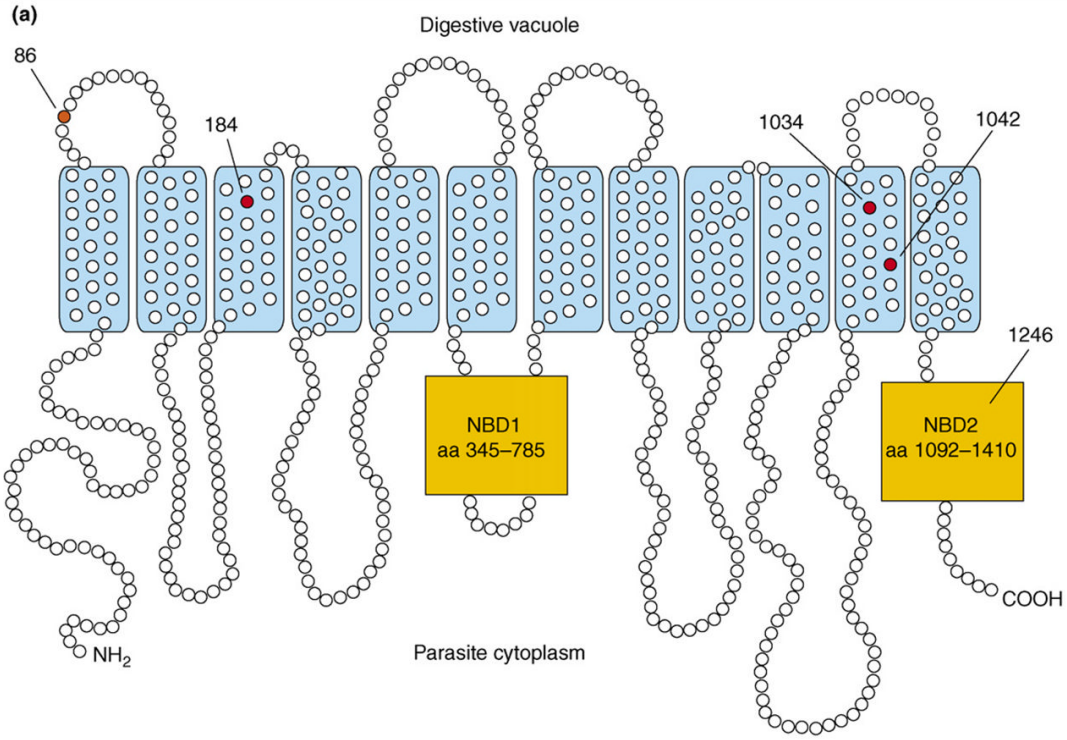


Figure 2.

Mechanistic models of PfCRT-mediated chloroquine resistance. **(a)** Chloroquine-sensitive parasites. In sensitive parasites expressing wild-type PfCRT, the weak base chloroquine (CQ) concentrates (broken arrow) in the digestive vacuole. The acidic environment of the digestive vacuole promotes diprotonation of chloroquine, trapping it within the organelle. This charged form is thought to bind hematin, preventing its detoxification into hemozoin. pH estimates for the cytoplasm and digestive vacuole are taken from Refs [17] and [39], respectively. **(b)** Energy-coupled drug efflux. In this model, chloroquine resistance is due to carrier-mediated drug efflux out of the parasite digestive vacuole. Glucose addition to the parasite culture medium, which results in reduced chloroquine accumulation in resistant parasites, is accompanied by production of ATP by the parasite [36,38]. Chloroquine-resistant parasites show an energy-dependent *trans*-stimulation effect characteristic of carrier-mediated transport [37,38]. Mutant PfCRT has been proposed to function as the drug effluxer (filled arrow) [48]. **(c)** Facilitated diffusion of charged drug. This model (also known as 'charged drug leak' [20]) considers the possible effects of mutations in PfCRT on transport function. Mutations in PfCRT alter the substrate specificity of the transporter and facilitate the diffusion (broken arrow) of diprotonated chloroquine from the digestive vacuole down an electrochemical gradient maintained by a digestive vacuole proton pump. Reversal agents, such as verapamil, are monobasic and have been proposed to interact with mutant PfCRT, blocking the passive efflux of drug in resistant parasites [49]. **(d)** Indirect effects of altered digestive vacuole pH. CQR parasites have been reported to have a more acidic digestive vacuole than have sensitive parasites [39]. This model postulates that alterations in the pH of the digestive vacuole, caused directly or indirectly by changes in the transport properties of mutant PfCRT, results in reduced accumulation of drug at its site of action. The increased rate of hematin aggregation and hemozoin formation at acidic pH has been proposed to reduce the amount of

target available for chloroquine binding [17]. The excess unbound drug could alter the equilibrium of passive drug accumulation (broken arrows) or could be transported out of the digestive vacuole by mutant PfCRT (filled arrow) [44,77].



(b)

pfmdr1 variants identified in *Plasmodium falciparum* field isolates and laboratory-adapted lines

		PfMDR1 position and amino acid					Copy number
Region	Reference line (origin)	86	184	1034	1042	1246	
All	Wild type (3D7, Netherlands)	N	Y	S	N	D	1
Asia and Africa	FCB (Southeast Asia)	N	Y	S	N	D	≥2
	K1 (Thailand)	Y	Y	S	N	D	1
South America	7G8 (Brazil)	N	F	C	D	Y	1

Gray shading indicates residues that differ from the wild-type allele.

Figure 3.

Predicted structure and genetic polymorphisms in *P. falciparum* multidrug resistance-1. **(a)** PfMDR1 has two homologous halves, each with six predicted transmembrane domains and a nucleotide-binding pocket [56]. The nucleotide-binding domains (NBD1 and NBD2; orange boxes) are each formed by large cytoplasmic domains. Polymorphic amino acids found in the K1 allele (N86Y) and the 7G8 allele (Y184F, S1034C, N1042D and D1246Y) are indicated. The D1246Y mutation is located in the predicted NBD2. **(b)** Representative haplotypes, including the one most commonly associated with amplification of *pfmdr1* copy number.