Cell. Mol. Life Sci. 63 (2006) 1586–1596
1420-682X/06/141586-11
DOI 10.1007/s00018-006-6071-1

Cellular and Molecular Life Sciences

Review

Antimalarial drugs: recent advances in molecular determinants of resistance and their clinical significance

C. J. Woodrow and S. Krishna*

Department of Cellular and Molecular Medicine, Centre for Infection, St. George's, University of London, Cranmer Terrace, London SW17 0RE (UK), Fax: +44 208 7253487, e-mail: s.krishna@sgul.ac.uk

Received 13 February 2006; revised after revision 7 March 2006; accepted 29 March 2006
Online First 15 May 2006

Abstract. Molecular determinants of antimalarial drug resistance are useful and informative tools that complement phenotypic assays for drug resistance. They also guide the design of strategies to circumvent such resistance once it has reached levels of clinical significance. Established resistance to arylaminoalcohols such as mefloquine and lumefantrine in SE Asia is mediated primarily by gene amplification of the *P. falciparum* drug transporter, *pfmdr1*. Single nucleotide polymorphisms in *pfmdr1*, whether assessed in field isolates or transfection experiments, are associated with changes in IC_{50} values (to arylaminoalcohols and chloroquine), but not of such magnitude as to influence clinical treatment outcomes. Recently described emerging *in vitro* resistance to artemisinins in certain areas correlates with mutations in the SERCA-like sequence *PfATP6* and supports *PfATP6* as a key target for artemisinins.

Keywords. Malaria, *pfmdr1*, *pfcrt*, *PfATP6*, artemisinins, mefloquine, molecular markers, resistance.

Introduction

Antimalarial drug resistance is currently one of the greatest challenges to reducing mortality caused by *Plasmodium falciparum* infection. Resistance arises via selection of mutations, and is decisive in determining the lifetime of antimalarial agents. Resistance has recently forced many countries to change national treatment protocols [1], particularly to combinations of antimalarial drugs with artemisinins as one component.

Understanding the molecular basis of drug resistance is useful for several reasons. Providers of antimalarial drugs can use molecular data to complement more established methods [2] in following the development of drug resistance in a given area. Samples for molecular testing are easy to collect (*e.g.* as blood spots) and store prior to transfer to a central laboratory for assay. Since molecular changes are generally discrete, assessments are relatively easy to standardise between units. Molecular markers theoretically offer the earliest way to detect emerging drug resistance and intervene accordingly, since they examine fundamental processes in the resistance pathway. Molecular data can also be used to guide development of novel antimalarial compounds to bypass drug resistance mechanisms.

Several recent advances in our understanding of mechanisms of resistance to different classes of antimalarial agents make this an opportune moment to review this area. We begin with a description of how molecular models for drug resistance have developed, focussing on mefloquine (and related drugs such as quinine) where the role of *pfmdr1* has recently been clarified. Evidence for *in vitro* artemisinin resistance and its molecular mechanism are also described. Relevance of these findings will then be discussed in the context of combination therapies.

Mechanisms of drug resistance

Broadly, there are two ways in which malaria parasites have become resistant to antimalarial drugs. Resistance

* Corresponding author.

against antifolates and atovaquone has arisen by mutations in drug targets that reduce their sensitivity; in these examples, an understanding of the molecular basis of drug action has been a prerequisite for elucidating the mechanism of drug resistance. Other drugs such as chloroquine and mefloquine may not have parasite-derived protein targets that can mutate, allowing parasites to escape from therapies. For these classes of antimalarial drugs, the parasite has become resistant through mutations in transporters involved in determining drug disposition within the intraerythrocytic parasite and its organelles. This effectively reduces drug concentrations at critical (presumed) target sites. In this context, mutation(s) of an individual transporter can modulate drug sensitivities to different antimalarial classes; similarly two genes may act epistatically to determine a single drug's resistance phenotype [3].

Developing models of drug resistance

Understanding the molecular basis of drug resistance has evolved in different ways, depending on the drug in question. For example, classical genetic studies using isolates with differing chloroquine sensitivities allowed 'reverse genetic' analyses to identify the associated chloroquine resistance genotype [4]. Confirmation that mutations in the drug resistance gene (*pfcrt*, or chloroquine resistance transporter gene) associated with the chloroquine-resistant phenotype were causal for that phenotype came after experimentally transfecting the 'resistant' *pfcrt* sequence (76T) into sensitive parasites [5].

However, such studies will have an undetermined and possibly limited applicability unless their contribution to clinical resistance is also ascertained. This is partly because the role of the resistance gene is only being investigated in the background of a laboratory-adapted parasite strain. *In vitro* studies of field isolates address this limitation, although the magnitude of any change in IC_{50} associated with a given mutation in transfection experiments is not always a reliable indicator of the clinical relevance of the mutation. Drug resistance is essentially the ability of parasites to multiply in the presence of previously therapeutic drug concentrations and, therefore, can only be accurately assessed in studies linked in some way to the treatment of patients.

Unfortunately, studies assessing therapeutic outcome themselves may be confounded by non-drug related variables (such as compliance and immunological status of patients) and, therefore, constitute a major challenge. This can be met by collaboration between basic and clinical researchers and has led to a useful understanding of mechanisms of clinical resistance to antifolates [6] and chloroquine [7], as discussed in detail in recent reviews [8, 9]. This review focuses instead on resistance to arylaminoalcohols (mefloquine, lumefantrine and quinine) and artemisinins.

Resistance to mefloquine and other arylaminoalcohols

The *pfmdr1*-chloroquine hypothesis

Demonstration that clinically important resistance to mefloquine and other arylaminoalcohols is determined largely by *pfmdr1* has required a circuitous path of investigation involving many groups. The observation that mammalian cancer cell multidrug resistance (MDR) genes mediate resistance to unrelated anticancer agents (increased MDR1 gene copy number leading to increased expression of the protein product P-glycoprotein 1) [10] led to proposals that a similar mechanism might be important in antimalarial drug resistance. Similarities between verapamil inhibition of multidrug resistance phenotype in cancer cells [11] and verapamil reversal of chloroquine resistance in *P. falciparum* [12, 13] focussed attention on a hypothesis in which amplification of a *P. falciparum* MDR1 orthologue could mediate chloroquine resistance.

Two *P. falciparum* MDR orthologues (*pfmdr1* and *2*) were subsequently identified [14, 15]; *pfmdr1* (located on chromosome 5; product P-glycoprotein homologue or Pgh1) was, like its human counterpart, amplified in certain isolates (including chloroquine-resistant ones [15]). Later studies showed that chloroquine pressure on laboratory isolates could de-amplify *pfmdr1* [16, 17]. An association between *pfmdr1* single nucleotide polymorphisms (SNPs) and chloroquine resistance was noted [18], and subsequently tested in a large number of field studies that produced conflicting results. Recent genetic studies have allowed testing of the *pfmdr1*-chloroquine resistance hypothesis more fully. Wild-type allelic replacement of SNPs clustered at the 3′ end of *pfmdr1* of the chloroquine-resistant isolate (7G8) halved chloroquine IC_{50} [19], but other classical [20] and reverse [21] genetic approaches have failed to show an association between *pfmdr1* SNPs and chloroquine IC_{50}.

Meanwhile, an independent body of work combining genetic studies localising chloroquine resistance to a gene on chromosome 7 [4, 22, 23] with reverse genetic [5, 24] and field-based approaches [7] has clearly demonstrated the role of PfCRT (*P. falciparum* chloroquine resistance transporter) in mediating chloroquine resistance. Current analysis indicates that eight to nine point mutations have accumulated in *pfcrt* to cause chloroquine resistance, with K76T being both necessary and sufficient to generate chloroquine resistance. The physiological mechanism of chloroquine resistance remains unresolved; PfCRT itself may represent a chloroquine transporter, with K76T increasing chloroquine leak from the food vacuole [8].

It is possible that mutations in *pfmdr1* associated with chloroquine resistance may represent the parasite's response to altered fitness consequent to carrying mutant *pfcrt*. Chloroquine's legacy is a high prevalence not

only of mutated *pfcrt* but also of mutated *pfmdr1*; hence *pfmdr1* may indeed be a chloroquine resistance gene, albeit a secondary or ancillary one [7, 25, 26].

pfmdr1 and arylaminoalcohols

Well before any candidate drug resistance genes were described, resistance phenotypes of parasites to mefloquine and chloroquine were found to be related, although the relationship was a reciprocal one, i.e. chloroquine resistance appeared to be associated with mefloquine hypersensitivity and *vice versa* [27–30]. *In vitro* mefloquine resistance was also associated with resistance to other arylaminoalcohols (halofantrine, lumefantrine and quinine) and the chemically unrelated artemisinins (peroxides).

The first clue to the molecular basis of this phenotype derived from analysis of a relatively small number of samples from Thailand that showed *pfmdr1* amplification in field isolates of parasites with elevated IC_{50}s to mefloquine and halofantrine [31]. Compared to wild-type, only one SNP (Y184F) was observed in all samples. Mefloquine selection pressure on *P. falciparum* in the laboratory [14, 17, 32] induced *pfmdr1* gene amplification. Conversely, chloroquine pressure led to increased sensitivity to arylaminoalcohols and deamplification of *pfmdr1* [16, 17]. However, the relationship between *pfmdr1* amplification and *in vitro* mefloquine [33] or halofantrine [34] resistance was not absolute in laboratory studies. Furthermore, the relative difficulty of quantitating *pfmdr1* copy number in large numbers of field isolates may have delayed testing of the amplification model of arylaminoalcohol resistance in the field. Instead, more attention was focussed on *pfmdr1* SNPs in the field that were also being studied as a cause of chloroquine resistance.

pfmdr1 SNPs and sensitivity to arylaminoalcohols

pfmdr1 SNPs originally proposed as mediating chloroquine resistance [18] (corresponding to substitutions N86Y, Y184F, S1034C, N1042D and D1246Y) have been found in parasites from various geographical regions. The 86Y and 184F alleles (at the 5′ end of *pfmdr1*) are found in isolates from all continents, while the 'CDY' alleles (at 1034, 1042 and 1246, respectively) are especially prevalent in South America, where they are often found in combination.

A classical genetic approach was used to confirm the association between the HB3 *pfmdr1* locus and hypersensitivity to mefloquine [20]. The alleles in the experimental strains used suggest that the determinant of hypersensitivity was probably the 3′ 1042D allele in HB3. The importance of this amino acid, as well as 1034, had been demonstrated by heterologous expression studies [35, 36]. Reverse genetic studies of *pfmdr1* confirm that SNPs at the 3′ end of *pfmdr1* affect sensitivity to mefloquine and other drugs [19, 21]; for example, introduction of one or all of the CDY amino acids generated parasites with lower mefloquine IC_{50}, while reversion of CDY to wild-type (SND) had the opposite effect [19]. The effect of these 3′ SNPs in the field has been more difficult to assess since they are relatively rare in SE Asia and Africa, but virtually ubiquitous in S. America. In a set of isolates from Thailand, where results from isolates containing alleles 1034C and 1042D were combined, there was evidence that one or both 3′ mutations were associated with a threefold reduction in mefloquine IC_{50} [37]. In a carefully controlled study of samples from the Thai-Myanmar border, 1042D was associated with approximately fivefold lower IC_{50} for mefloquine [38] (Fig. 1), as well as reduced lumefantrine IC_{50} [38], a finding replicated in a separate study [39].

The lack of association between 3′ mutations and mefloquine IC_{50} observed in other studies in SE Asia probably reflects reduced statistical power to detect effects when these SNPs are at low prevalence [40, 41]. In South American parasites shown to have CDY alleles at the 3′ end, mefloquine IC_{50}s are indeed low [42] and mefloquine remains predictably efficacious in such areas [43].

Despite several attempts, it has not been possible to engineer allelic exchange at the 5′ end of *pfmdr1* [21]; nor has a classical genetic study focussing on this locus been undertaken. Hence for the N86Y SNP our understanding is limited to correlative analysis of laboratory strains and field isolates. 86Y is associated with *in vitro* mefloquine hypersensitivity in laboratory isolates [20] and field samples from both Africa [44] and SE Asia [37, 40, 45]. 86Y was associated with reduced IC_{50} to halofantrine in Africa [44]; it has not yet been shown directly to influence lumefantrine IC_{50}, although selection of 86N alleles over 86Y in reinfections after lumefantrine-artemether treatment [46] (see below) suggests that such a relationship exists.

The Y184F SNP has generally not been associated with any change in mefloquine IC_{50} [37, 40, 44] (whether at single or multiple copy [40], see inset to Fig. 1). 184F was associated with increased mefloquine IC_{50} in Cambodian isolates [41]. In the first phase of a study of samples from the Thai-Myanmar border, 184F was associated with reduced mefloquine IC_{50} [38], but after analysis of an independent validation set of samples this relationship was not sustained, suggesting that the effects of this SNP on mefloquine IC_{50} are at best weak. For this reason, 184Y and 184F datasets have tended to be collapsed for IC_{50} analysis [37, 40] and this convention is maintained in the body of Figure 1.

In summary, these laboratory and field data show remarkable agreement and suggest that some *pfmdr1* SNPs induce hypersensitivity to arylaminoalcohols; reversion to wild-type status at these loci is associated with return of IC_{50}s to original levels. Considered in this way, it is not surprising that studies assessing *in vivo* drug efficacy [39,

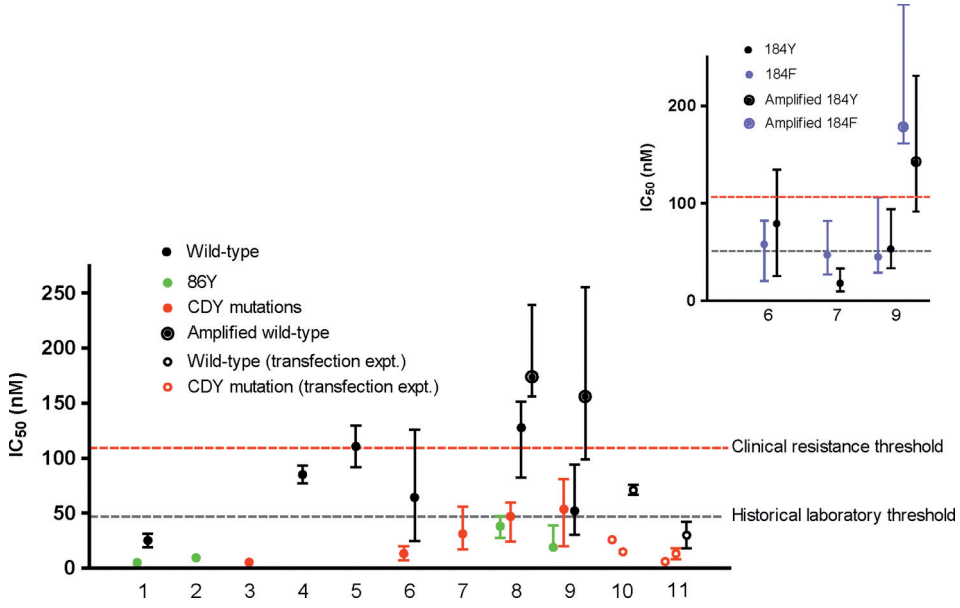

Figure 1. Mefloquine IC_{50}s for field isolates and transfection experiments according to *pfmdr1* genotype and amplification. Values (grouped within studies) are derived from studies denoted 1–11, respectively [44], [50], [42], [77], [78], [38], [41], [37], [40], [19], and [21]. Only field studies with at least five isolates per group are included; isolates are grouped according to *pfmdr1* sequence as (i) 'wild-type' (86N and SND at positions 1034, 1042, and 1246, respectively), (ii) '86Y' (86Y and SND), or (iii) 'CDY mutations' (at least one of 1034C, 1042D or 1246Y). 184Y and 184F parasites are considered together in the main body of the figure. Isolates definitely possessing amplified *pfmdr1* are shown for studies in which copy number was assessed [37, 40] (amplification detection thresholds of 3 and 2 copies respectively); amplified *pfmdr1* genes may potentially be present in other data sets, particularly in SE Asia [38, 41, 77, 78]. Field data are median and interquartile range [37, 38, 40, 50], or mean and 95% confidence intervals [41, 42, 44, 77, 78]. Representative transfection data are mean (± SEM [19] or standard deviation [21]) of replicates using cloned transfectants. Clinical [40] and historical laboratory resistance thresholds are shown at 108 and 48 nM, respectively. Inset: effect of Y184F mutation. Only field studies with at least five isolates per group are included; isolates are wild-type at *pfmdr1* 86, 1034, 1042 and 1246; values are derived from studies denoted 6, 7 and 9 (respectively [38], [41] and [40]) (the last with amplification detection shown). Data are median and interquartile range [38, 40] or mean and 95% confidence intervals [41].

40] have failed to show important associations between *pfmdr1* SNPs and treatment outcome.

pfmdr1 amplification and clinical resistance

While molecular models of drug resistance have been commonly developed by correlating parasite genotype with *in vitro* sensitivity (in laboratory and field settings), only simultaneous measurement of genotype and clinical drug efficacy can confirm a model's applicability to the critical question of whether a patient with a given parasite will be cured by a particular antimalarial regimen. Unfortunately, such studies are relatively difficult to perform, since they require a large number of patients and samples, excellent follow-up and a variety of methodological approaches. Studies must be performed in an area where there is a significant clinical failure rate, e.g. SE Asia, for studies of mefloquine resistance. In studies from the Thai-Myanmar border, it became clear early on that when clinical response to mefloquine monotherapy (25 mg/kg) was related to *in vitro* sensitivity assays, resistance threshold occurred at 45 ng/ml (108 nM) [45], a value well above the historically applied cut-off threshold of 20 ng/ml (48.2 nM). The 'new' breakpoint was well above the range of IC_{50} values associated with SNPs (and SNP reversions) in other studies (see above and Fig. 1). Consistent with these considerations, 86Y to 86N 'reversion' was associated with an increase in median IC_{50} from 19.0 to 52.0 nM but did not predict clinical failure of mefloquine monotherapy [40]. Instead, the best predictor of clinical failure was *pfmdr1* copy number; increased *pfmdr1* copy number predicted clinical failure of mefloquine monotherapy with a sensitivity of 71% and a specificity of 78%, and had an adjusted hazard ratio of causing mefloquine failure of 6.3. Increased copy number was associated with an increase in median mefloquine IC_{50} from 52.0 to 156.1 nM.

Highly comparable IC_{50} data and associations with *pfmdr1* copy number have been obtained from a distinct set of samples on the Thai-Myanmar border using the same approach (Tim Anderson, personal communication). Another study in Thailand using a less sensitive method for detection of amplification of *pfmdr1* (threshold = 3 copies, so that some samples with 2 copies of *pfmdr1* may have been classified as 'single copy') showed similar trends [37]. Despite the additional difficulties in standardising IC_{50} measurements between sites, there is good overall agreement between these various studies (Fig. 1) with

Table 1. Clinical (in vivo) phenotypes associated with *pfmdr1* genotype.

Genotype	Associated phenotypes	Relative risk (*adjusted hazard ratio)
pfmdr1 amplification	Mefloquine failure	6.3* [40]
	Mefloquine-artesunate failure	5.4* [40]
	4-dose lumefantrine-artemether failure	3.2 [39]
pfmdr1 86N vs 86Y	Reinfection after lumefantrine-artemether	2.7 [46], 2.2 [75]

regard to the relationship between *pfmdr1* amplification and clinical mefloquine resistance. These observations have been extended in an analogous way to predict clinical failures after lumefantrine-artemether treatment [39], although interestingly only with a lower dose (four dose) regimen was a relationship observed between *pfmdr1* copy number and recrudescence of parasites.

More than a decade after initial observations [31], there is now good evidence to back a model in which amplified *pfmdr1* and associated increased Pgh1 expression induces reduced sensitivity to arylaminoalcohols, generating resistance to standard courses of mefloquine monotherapy as well as (in a smaller proportion of cases) artemisinin combination therapy (mefloquine-artesunate or four-dose lumefantrine-artemether therapy) (Table 1). There is little evidence to suggest that SNPs in *pfmdr1* can generate resistance to therapeutic regimens, although they may be of relevance in reinfection (see below). Measurement of *pfmdr1* copy number should be routinely applied in resistance studies involving these drugs.

pfcrt and resistance to arylaminoalcohols

Clearly there are additional factors that modulate resistance to arylaminoalcohols. Given the reciprocal association between mefloquine and chloroquine sensitivity in field and laboratory isolates, *pfcrt* itself is an obvious candidate. Transfection experiments on *pfcrt* mutations that mediate chloroquine resistance showed that acquisition of *pfcrt* 76T induces mefloquine hypersensitivity [5]. This suggests the presence of two genes acting epistatically on mefloquine sensitivity [3], although in each case mutant alleles induce hypersensitivity, rather than resistance, and reversion to 76K in resistant lines does not change mefloquine IC_{50} significantly [24]. The possibility that *pfcrt* might generate elevated mefloquine IC_{50} via mutation at a distinct locus was raised by a study in which chloroquine-resistant parasites placed under halofantrine pressure were shown to undergo an S163R mutation [34, 47]. However this mutation was not found in a survey of mefloquine-resistant parasites from SE Asia (Steve Ward, personal communication). In summary, the relevance of *pfcrt* to development of clinically relevant mefloquine resistance appears limited.

The situation in Africa

Amplification of *pfmdr1* has only rarely been reported in Africa [48] where mefloquine has not been widely used until recently. However, amplification of *pfmdr1* may have been induced by low-dose mefloquine in Gabon, although this amplification has since disappeared on assessment over the following 5 years [49].

Association between 86N and copy number

In field isolates from Thailand, there was an inverse relationship between the alleles 86Y [31, 37, 39, 40] or 1042D [39] and *pfmdr1* amplification. The basis for this remains a mystery, and also needs to be investigated in other geographical areas. Presumably, in a parasite population mostly consisting of 86Y as a result of previous chloroquine use (Fig. 2), wild-type *pfmdr1* associated with a slightly higher mefloquine IC_{50} may have an advantage under mefloquine pressure and thus be more likely than 86Y or 1042D parasites to undergo gene amplification. Amplified 86Y *pfmdr1* remains limited to the laboratory context [17, 32].

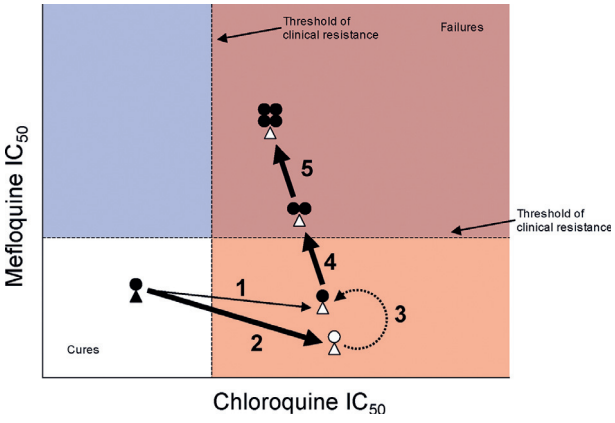

Figure 2. Schematic model showing the influence of changes in *pfcrt* and *pfmdr1* sequence on chloroquine and mefloquine IC_{50} and their relevance to clinical resistance. Chloroquine pressure leads to mutations in *pfcrt* (K76T) required for clinically relevant chloroquine resistance (1). Secondary/compensatory mutations in *pfmdr1* such as N86Y also occur under chloroquine pressure causing a degree of mefloquine hypersensitivity (2). When mefloquine is introduced as monotherapy, parasites with wild-type *pfmdr1* are eliminated by a therapeutic course but become re-established because of their selective ability to reinfect patients with residual drug present (3). Wild-type parasites then amplify *pfmdr1* to varying levels, generating clinically relevant mefloquine resistance (4, 5) associated with a small reduction in chloroquine IC_{50}. *pfcrt* (in particular 76T) remains unchanged during this second period of drug pressure. Symbols ▲ wild-type *pfcrt*, △ 76T *pfcrt*, ● wild-type or 184F *pfmdr1*, ○ 86Y or CDY (see Fig. 1) in *pfmdr1*.

Coevolution of chloroquine and arylaminoalcohol resistance

These insights allow us to suggest how chloroquine resistance is reciprocally related to that of arylaminoalcohols (Fig. 2). Development of chloroquine resistance proceeds via *pfcrt* mutations, accompanied by (perhaps compensatory) *pfmdr1* mutations, both of which reduce mefloquine IC_{50} by imprecisely understood changes in the physiology of the digestive vacuole. Associated with reversal of this mefloquine hypersensitivity in the field, wild-type *pfmdr1* is first selected and then amplified, with *pfcrt* (in particular 76T) remaining unchanged during this process. *pfmdr1* amplification results in somewhat reduced IC_{50}s for chloroquine ([40] and Dr. Tim Anderson, personal communication) that are probably of no clinical significance, although this has not been tested formally.

Quinine

Because of side effects and duration of treatment, quinine is not generally used to treat patients with uncomplicated malaria, and is reserved for treatment of severe malaria. The mechanism of action of quinine is incompletely understood, although like mefloquine and chloroquine, sensitivity of parasites to quinine appears to be modulated by proteins (Pgh1 and PfCRT) expressed in the food vacuole that also influence mefloquine and chloroquine sensitivity. In an analogous manner to mefloquine, studies in SE Asia demonstrate that *pfmdr1* amplification is associated with elevation of quinine IC_{50} by two- to threefold [37, 38, 40]. These data suggest the mechanism by which quinine IC_{50}s tend to correlate with those of other arylaminoalcohols such as mefloquine. However, as quinine treatment was not given to patients in these studies, no relationship between *pfmdr1* amplification and therapeutic outcome can be assessed. There is little evidence that *pfmdr1* SNPs have a significant role to play in treatment response to quinine; transfection of SNPs at the 3′ end of *pfmdr1* induces small increases in quinine IC_{50} [19, 21], while field studies of *pfmdr1* SNPs have not shown an association with quinine IC_{50} [37, 38, 40, 50].

The *pfcrt* SNP K76T also modulates quinine sensitivity in laboratory [51] and transfection [5, 24] experiments, although depending on the background strain, 76T is associated with reduced [5], elevated or unchanged [24] quinine IC_{50}s compared with 76K. Given the fixation of 76T in many areas, no field data on interaction between 76T and quinine IC_{50} are available. In SE Asia, chloroquine and quinine IC_{50}s are reciprocally related, while worldwide, they appear to be positively correlated [52], confirming that parasite genetic background can influence how *pfcrt* interacts to modulate quinine IC_{50} values. A systematic quantitative trait loci screen of an established cross of *P. falciparum* identified regions of chromosomes 5, 7 and 13 as possible modulators of quinine IC_{50} [53]. These loci have been suggested to be *pfmdr1*, *pfcrt* and *pfnhe1*, respectively. These findings may be unambiguous in this experiment, but it is hard to assess their relevance to parasites isolated from patients, where different mechanisms such as gene amplification (see above) may also operate.

An approach based on genome scanning for transporter sequences suggested 11 genes as potential modulators of quinine sensitivity [52] (including *pfcrt* and *pfmdr1*). The tendency of such studies to pick out false-positive associations was pointed out by Anderson *et al*. [38], who addressed the problem by performing a two-phase study, in which associations identified in the first phase were validated in a second phase. Very few positive associations identified in the initial phase were still present after the validation phase. For quinine in particular, no significant association with SNPs in any transporter remained after the validation phase.

Artemisinins

The cellular target for artemisinins has remained controversial. A commonly proposed theory states that iron contained in parasite haem reacts with artemisinins' peroxide moiety, leading to release of free radicals and damage to parasite structures. Further, it has been suggested that target resistance against a drug with such a non-specific mechanism of action is unlikely to emerge; resistance could only emerge via mutations in drug transporters such as *pfmdr1* and *pfcrt*. Increased *pfmdr1* copy number is associated with elevated IC_{50} for artemisinins in SE Asia [37, 40], but this almost certainly relates to mefloquine, not artemisinin pressure, and the small associated increase in artemisinin IC_{50} has probably occurred as a 'bystander' effect (see combination therapy below). As for arylaminoalcohols, *pfmdr1* SNPs are generally associated with artemisinin hypersensitivity in laboratory studies [20], transfections [19, 21] and field isolates from Africa [44] and SE Asia [37], although these are not uniform findings [38, 40]. Similarly, *pfcrt* appears to have a minor modulating effect on artemisinin IC_{50}; this effect is either slight [5] or non-existent [24] depending on parasite background. A study of several transporters revealed an association between a polymorphism in another predicted transporter (gene G7, a member of the ABC transporter family) and artesunate IC_{50} [38], but this area has not been explored in further detail. Recently a hypothesis has been advanced describing the sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA)-type PfATP6 protein as the target of artemisinins [54]. No association was found between SNPs in *PfATP6* and artesunate IC_{50} in SE Asia, although these field studies have been carried out in areas where there was no evidence of significant *in*

vitro or *in vivo* artemisinin resistance [40]. The ability of malaria parasites to develop stable resistance to artemisinins via other means is of interest [55], although the system used (rodent malaria parasite *P. chabaudi*) may limit the applicability of these findings to human infection.

Failures following monotherapy: role of resistance

Although there has been no evidence for clinically relevant *in vivo* artemisinin resistance when the class is used as a partner in combination therapy, artemisinin monotherapy is well described as having poor efficacy. In any case, WHO has recommended cessation of monotherapy (press release 20.1.06) to try to limit emergence of artemisinin resistance. Recrudescences with monotherapy tend to occur rather late, and studies following patients up to day 42 detect a higher proportion of recrudescences than those stopping at 28 days. Recent estimates of recrudescence rates after artemisinin monotherapy at 28 day follow-up are 20–40% in Africa [56–58] and about 20% in SE Asia [59–61]. Duration of therapy has been reported to be of critical importance in efficacy of artemisinin-based monotherapies, with extension to 7 days improving cure rates significantly [62–64]. However, these earlier suggestions have not been confirmed in a separate study [60], where PCR correction was applied to differentiate reinfection from recrudescence.

In the first study to assess *in vitro* properties of recrudescent parasites following artesunate monotherapy, there was no difference in baseline dihydroartemisinin (DHA) IC_{50} values between recrudescent parasite isolates and isolates subsequently cured by artesunate [61]. The main factor associated with recrudescence was found to be high parasitaemia, suggesting that failure of artesunate in this study was not the result of artesunate resistance.

However, a recent study of 7-day artesunate from Central African Republic has provided the first evidence of an association between *in vitro* and *in vivo* artemisinin resistance, *i.e.* that genotypic resistance may indeed contribute to clinical failure of monotherapy [65]. Study design was ideal for detecting artesunate recrudescences; patients were non-immune, follow-up was for 42 days (and complete) and PCR correction was employed. Importantly, the investigators reported DHA IC_{90} as well as IC_{50} values; mean (95% confidence interval) DHA IC_{90} values in 5 recrudescent parasite isolates was 46.2 (13.1–79.3) nM compared with 8.9 (2.6–15.2) nM in 17 non-recrudescent isolates ($p = 0.02$). DHA IC_{50} values were similar for the two groups and there was clearly a change in the shape of the DHA inhibition curve resulting in a eightfold reduction in IC_{50}:IC_{90} ratio. Strictly speaking, this study does not provide absolute proof of artemisinin resistance since DHA levels were not measured in patients. However, our understanding of pharmacokinetic/pharmacodynamic relationships with artemisinins is rudimentary, making interpretation of plasma levels beyond demonstration of simple compliance a problematic process.

It may be that IC_{50} values from many areas are not so informative, since IC_{50} can remain stable while IC_{90} changes. In any case, significant elevations in artemisinin IC_{50}s have recently been observed in field studies [66]. A significant number of isolates with altered IC_{50}s were found in French Guiana and Senegal. In this case, several loci were examined to identify genes determining the *in vitro* resistance, including the proposed artemisinin target *PfATP6* [54]. Remarkably, in French Guiana, a *PfATP6* SNP producing S769N was strongly associated with elevated IC_{50} (>30 nM) values for artemether (odds ratio 27). This mutation was not related to IC_{50}s for any other drug.

The *in vivo* relevance of these findings is unclear as there were no corresponding clinical data, but these results are sobering evidence for artemisinin resistance. Moreover, they independently provide strong evidence for the role of *PfATP6* as the target of artemisinin therapy.

Combination therapy

Antimalarial combination therapies can improve treatment efficacies of failing individual components and provide some protection for individual components against the development of higher levels of resistance [67]. Artemisinin-containing combination therapies (ACTs) have been advocated as the best available option, and are the most commonly adopted regimen in countries changing antimalarial policy in the last decade. However, there are a variety of effective combinations [68].

Artemisinins rapidly reduce parasitaemia, but have poor efficacy as short course monotherapy (see above). When used in combination with another agent, the rapid reduction in parasite numbers results in relatively few parasites being exposed to the second drug (to which significant resistance may already exist), theoretically preventing emergence of additional resistance mutations [69]. Furthermore, since artemisinins themselves are not required to mediate final cure, there should also be little opportunity for artemisinin resistance to develop. In addition, reduction in gametocyte carriage may also reduce transmission of resistant parasites [70].

ACT in SE Asia

Evidence of the benefits of ACTs has been obtained in SE Asia. Mefloquine resistance was reported to be reversed by the addition of artesunate [67] with the combination retaining a cure rate of over 95% [71] in patients without hyperparasitaemia. There is, nevertheless, good evidence for parasite-mediated resistance against ACT, with recrudescence after mefloquine-artesunate strongly associated

with amplification of *pfmdr1* [40]. Increased *pfmdr1* copy number predicted clinical failure of mefloquine-artesunate therapy with a sensitivity of 77% and a specificity of 65%. Day 42 failure rate was higher in patients harbouring isolates with three or more copies of *pfmdr1* compared with two copies, suggesting a 'dose-response' relationship. Although this phenomenon is associated with an increase in IC_{50} against both mefloquine and artesunate, amplification is presumed to act through reduced mefloquine efficacy for the following reasons: low efficacy of mefloquine monotherapy is well established in this area [72], and the pharmacodynamic action of artesunate in removing parasite biomass appeared preserved in terms of parasite clearance time [40], although parasite clearance data may be relatively insensitive at detecting impairments in acute clearance kinetics. Similar considerations apply to ACT with lumefantrine; clinical failures following a four-dose artemether-lumefantrine regimen were more than three times as common in isolates with an increased *pfmdr1* copy number compared with single copy [39]; the six-dose regimen does not suffer from such high failure rates in this geographic area [71], although nearby (in Cambodia) the failure rate may be even higher than 20% [73].

ACT in Africa

These arguments have been used to support the deployment of ACT in Africa, where transmission rates are much higher than in SE Asia. ACTs deployed so far in SE Asia (mefloquine-artesunate, lumefantrine-artemether) combine pharmacokinetically mismatched drugs. In Africa, reinfection commonly occurs within weeks of the primary infection, thereby potentially exposing parasites to the longer half-life drug at low levels, giving ideal conditions for selection of resistance to this drug.

The frequency with which reinfection occurs following primary infection was evident during a study of artemether-lumefantrine in Tanzania, in which 45 of 200 children presented with recurrent parasitaemia during the 42-day follow-up period [46]. *pfmdr1* SNPs have been shown to be involved in lumefantrine sensitivity in a genetic cross [20], and a number of studies have shown an association between the 86Y allele and hypersensitivity to another arylaminoalcohol, mefloquine (see above). In this study, there was good evidence for selection of 86N parasites occurring during reinfection, adding to the list of phenotypes mediated by the *pfmdr1* gene (Table 1); frequency of 86Y alleles decreased significantly from 76.6% before treatment to 54.8% in patients with recurrent parasitaemia, with a corresponding rise in the frequency of the 86N allele. The major contributor to the rise in 86N alleles was reinfection between 20 and 30 days after treatment, when residual lumefantrine levels are low (and no artemether is present); 86N and 86Y returned to their baseline proportions by day 40.

This observation of *P. falciparum* 'caught in the act' of allele selection by a pharmacokinetically mismatched combination may be worrying for public health decision makers, prompting the recommendation that future combination therapies should employ drugs with better-matched elimination rates [74]. However, it is clear that artemether-lumefantrine protects against reinfection more effectively than artesunate-amodiaquine [75]. This highlights the problem that a combination that benefits individual patients may not be best choice for public health reasons [76]. Furthermore, it is debatable whether the *pfmdr1* allele selection observed can be described as 'selection for resistance' (or 'tolerance' as suggested by some authors [74]). 86N is not a *de novo* mutation in *pfmdr1*, but the original allele; 86Y only appeared under chloroquine pressure (see above). Increase in 86N simply represents selection of a pre-existing allele present at approximately 25% frequency pre-treatment [46, 75]. Additional mutations, such as amplification of *pfmdr1* (so far not described in most of Africa), will be required to generate levels of resistance that could impact significantly on *in vivo* response to treatment using this combination. It is therefore premature to dismiss artemether-lumefantrine as a long-term therapy in Africa, although there is a clear need for careful monitoring of the effects of ACTs, measuring the most appropriate *in vivo* and *in vitro* phenotypes and molecular markers as well as extending studies to cover multiple episodes of malaria [76].

Conclusion and future directions

Understanding the molecular basis of resistance to antimalarial compounds has involved investigations in basic science, the clinic and epidemiology. Establishing resistance mechanisms operating in the field is an iterative process and has been complicated by the fact that resistance has evolved via both target mutation and modulation of drug disposition. Two genes that encode proteins expressed on the parasite food vacuole, *pfcrt* and *pfmdr1*, interact in an epistatic manner to influence sensitivity to chloroquine and arylaminoalcohols such as mefloquine; only after many years of work it is becoming clear that *pfcrt* has a dominant role in chloroquine resistance, while *pfmdr1* is the major determinant of arylaminoalcohol resistance. Like multidrug resistance in cancer cells, arylaminoalcohol resistance is mediated via increased gene copy number. Transfection experiments aiming to reduce copy number (knockdowns) may provide additional evidence of the action of *pfmdr1*.

Our understanding of the molecular basis of quinine resistance remains much less advanced than for chloro-

quine or mefloquine. However, there has been a major advance with regard to understanding how artemisinins work and how resistance may develop to this class of antimalarial agent. This is particularly timely given the investment in artemisinin combination therapies being employed to halt the catastrophic problem of malaria in Sub-Saharan Africa. Until recently, a commonly held view was that no resistance against artemisinin derivatives existed, nor was it likely to develop if they were deployed as combinations. Unfortunately, the reality is that artemisinins can select for resistance, and it is not surprising that parasites that are over 30 times more resistant to artemether than sensitive isolates have recently been described in French Guiana [66]. Fortunately, the same report has simultaneously validated the *P. falciparum* SERCA gene *PfATP6* as both target and resistance gene for artemisinins, opening the way for molecular and therapeutic strategies to circumvent this alarming problem. This example, perhaps more than any other, demonstrates the value of a fundamental understanding of drug action and resistance.

1 Arrow, K. J., Panosian, C. and Gelband, H. (2004) Saving lives, buying time: economics of malaria drugs in an age of resistance. National Academic Press, Washington DC.

2 White, N. J. and Krishna, S. (1989) Treatment of malaria: some considerations and limitations of the current methods of assessment. Trans. R. Soc. Trop. Med. Hyg. 83, 767–77.

3 Duraisingh, M. T. and Refour, P. (2005) Multiple drug resistance genes in malaria – from epistasis to epidemiology. Mol. Microbiol. 57, 874–877.

4 Fidock, D. A., Nomura, T., Talley, A. K., Cooper, R. A., Dzekunov, S. M., Ferdig, M. T. et al. (2000) Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol. Cell 6, 861–871.

5 Sidhu, A. B., Verdier-Pinard, D. and Fidock, D. A. (2002) Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by pfcrt mutations. Science 298, 210–213.

6 Kublin, J. G., Witzig, R. S., Shankar, A. H., Zurita, J. Q., Gilman, R. H., Guarda, J. A. et al. (1998) Molecular assays for surveillance of antifolate-resistant malaria. Lancet 351, 1629–1630.

7 Djimde, A., Doumbo, O. K., Cortese, J. F., Kayentao, K., Doumbo, S., Diourte, Y. et al. (2001) A molecular marker for chloroquine-resistant falciparum malaria. N. Engl. J. Med. 344, 257–263.

8 Bray, P. G., Martin, R. E., Tilley, L., Ward, S. A., Kirk, K. and Fidock, D. A. (2005) Defining the role of PfCRT in *Plasmodium falciparum* chloroquine resistance. Mol. Microbiol. 56, 323–333.

9 Gregson, A. and Plowe, C. V. (2005) Mechanisms of resistance of malaria parasites to antifolates. Pharmacol. Rev. 57, 117–145.

10 Riordan, J. R., Deuchars, K., Kartner, N., Alon, N., Trent, J. and Ling, V. (1985) Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. Nature 316, 817–819.

11 Tsuruo, T., Iida, H., Tsukagoshi, S. and Sakurai, Y. (1981) Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res. 41, 1967–1972.

12 Krogstad, D. J., Gluzman, I. Y., Kyle, D. E., Oduola, A. M., Martin, S. K., Milhous, W. K. et al. (1987) Efflux of chloroquine from *Plasmodium falciparum:* mechanism of chloroquine resistance. Science 238, 1283–1285.

13 Martin, S. K., Oduola, A. M. and Milhous, W. K. (1987) Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. Science 235, 899–901.

14 Wilson, C. M., Serrano, A. E., Wasley, A., Bogenschutz, M. P., Shankar, A. H. and Wirth, D. F. (1989) Amplification of a gene related to mammalian mdr genes in drug-resistant *Plasmodium falciparum*. Science 244, 1184–1186.

15 Foote, S. J., Thompson, J. K., Cowman, A. F. and Kemp, D. J. (1989) Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. Cell 57, 921–930.

16 Barnes, D. A., Foote, S. J., Galatis, D., Kemp, D. J. and Cowman, A. F. (1992) Selection for high-level chloroquine resistance results in deamplification of the pfmdr1 gene and increased sensitivity to mefloquine in *Plasmodium falciparum*. EMBO J. 11, 3067–375.

17 Peel, S. A., Bright, P., Yount, B., Handy, J. and Baric, R. S. (1994) A strong association between mefloquine and halofantrine resistance and amplification, overexpression, and mutation in the P-glycoprotein gene homolog (pfmdr) of *Plasmodium falciparum in vitro*. Am. J. Trop. Med. Hyg. 51, 648–658.

18 Foote, S. J., Kyle, D. E., Martin, R. K., Oduola, A. M., Forsyth, K., Kemp, D. J. et al. (1990) Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. Nature 345, 255–258.

19 Reed, M. B., Saliba, K. J., Caruana, S. R., Kirk, K. and Cowman, A. F. (2000) Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature 403, 906–909.

20 Duraisingh, M. T., Roper, C., Walliker, D. and Warhurst, D. C. (2000) Increased sensitivity to the antimalarials mefloquine and artemisinin is conferred by mutations in the pfmdr1 gene of *Plasmodium falciparum*. Mol. Microbiol. 36, 955–961.

21 Sidhu, A. B., Valderramos, S. G. and Fidock, D. A. (2005) pfmdr1 mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. Mol. Microbiol. 57, 913–926.

22 Wellems, T. E., Panton, L. J., Gluzman, I. Y., do Rosario, V. E., Gwadz, R. W., Walker-Jonah, A. et al. (1990) Chloroquine resistance not linked to mdr-like genes in a *Plasmodium falciparum* cross. Nature 345, 253–255.

23 Wellems, T. E., Walker-Jonah, A. and Panton, L. J. (1991) Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7. Proc. Natl. Acad. Sci. USA 88, 3382–3386.

24 Lakshmanan, V., Bray, P. G., Verdier-Pinard, D., Johnson, D. J., Horrocks, P., Muhle, R. A. et al. (2005) A critical role for PfCRT K76T in *Plasmodium falciparum* verapamil-reversible chloroquine resistance. EMBO J. 24, 2294–2305.

25 Warhurst, D. C., Craig, J. C. and Adagu, I. S. (2002) Lysosomes and drug resistance in malaria. Lancet 360, 1527–1529.

26 Babiker, H. A., Pringle, S. J., Abdel-Muhsin, A., Mackinnon, M., Hunt P. and Walliker, D. (2001) 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. 183, 1535–1538.

27 Webster, H. K., Boudreau, E. F., Pavanand, K., Yongvanitchit, K. and Pang, L. W. (1985) Antimalarial drug susceptibility testing of *Plasmodium falciparum* in Thailand using a microdilution radioisotope method. Am. J. Trop. Med. Hyg. 34, 228–235.

28 Oduola, A. M., Milhous, W. K., Weatherly, N. F., Bowdre, J. H. and Desjardins, R. E. (1988) *Plasmodium falciparum:* induction of resistance to mefloquine in cloned strains by continuous drug exposure *in vitro*. Exp. Parasitol. 67, 354–360.

29 Gay, F., Bustos, D. G., Diquet, B., Rojas Rivero, L., Litaudon, M., Pichet, C. et al. (1990) Cross-resistance between mefloquine and halofantrine. Lancet 336, 1262.

30 Rojas-Rivero, L., Gay, F., Bustos, M. D., Ciceron, L., Pichet, C., Danis, M. et al. (1992) Mefloquine-halofantrine cross-resistance in *Plasmodium falciparum* induced by intermittent mefloquine pressure. Am. J. Trop. Med. Hyg. 47, 372–377.
31 Wilson, C. M., Volkman, S. K., Thaithong, S., Martin, R. K., Kyle, D. E., Milhous, W. K. et al. (1993) Amplification of pfmdr 1 associated with mefloquine and halofantrine resistance in *Plasmodium falciparum* from Thailand. Mol. Biochem. Parasitol. 57, 151–160.
32 Cowman, A. F., Galatis, D. and Thompson, J. K. (1994) Selection for mefloquine resistance in *Plasmodium falciparum* is linked to amplification of the pfmdr1 gene and cross-resistance to halofantrine and quinine. Proc. Natl. Acad. Sci. USA 91, 1143–1147.
33 Lim, A. S., Galatis, D. and Cowman, A. F. (1996) *Plasmodium falciparum:* amplification and overexpression of pfmdr1 is not necessary for increased mefloquine resistance. Exp. Parasitol. 83, 295–303.
34 Ritchie, G. Y., Mungthin, M., Green, J. E., Bray, P. G., Hawley, S. R. and Ward, S. A. (1996) *In vitro* selection of halofantrine resistance in *Plasmodium falciparum* is not associated with increased expression of Pgh1. Mol. Biochem. Parasitol. 83, 35–46.
35 van Es, H. H., Karcz, S., Chu, F., Cowman, A. F., Vidal, S., Gros, P. et al. (1994) Expression of the plasmodial pfmdr1 gene in mammalian cells is associated with increased susceptibility to chloroquine. Mol. Cell. Biol. 14, 2419–2428.
36 Volkman, S. K., Cowman, A. F. and Wirth, D. F. (1995) Functional complementation of the ste6 gene of *Saccharomyces cerevisiae* with the pfmdr1 gene of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA 92, 8921–8925.
37 Pickard, A. L., Wongsrichanalai, C., Purfield, A., Kamwendo, D., Emery, K., Zalewski, C. et al. (2003) Resistance to antimalarials in Southeast Asia and genetic polymorphisms in pfmdr1. Antimicrob. Agents Chemother. 47, 2418–2423.
38 Anderson, T. J., Nair, S., Qin, H., Singlam, S., Brockman, A., Paiphun L. et al. (2005) Are transporter genes other than the chloroquine resistance locus (pfcrt) and multidrug resistance gene (pfmdr) associated with antimalarial drug resistance? Antimicrob. Agents Chemother. 49, 2180–2188.
39 Price, R. N., Uhlemann, A. C., van Vugt, M., Brockman, A., Nair, S., Hutagalung, R. et al. (2006) Lumefantrine concentration and pfmdr1 copy number predict therapeutic efficacy of artemether-lumefantrine for multi-drug resistant falciparum malaria. Clin. Infect. Dis. (in press).
40 Price, R. N., Uhlemann, A. C., Brockman, A., McGready, R., Ashley, E., Phaipun, L. et al. (2004) Mefloquine resistance in *Plasmodium falciparum* and increased pfmdr1 gene copy number. Lancet 364, 438–447.
41 Khim, N., Bouchier, C., Ekala, M. T., Incardona, S., Lim, P., Legrand, E. et al. (2005) Countrywide survey shows very high prevalence of *Plasmodium falciparum* multilocus resistance genotypes in Cambodia. Antimicrob. Agents Chemother. 49, 3147–3152.
42 Huaman, M. C., Roncal, N., Nakazawa, S., Long, T. T., Gerena, L., Garcia, C. et al. (2004) Polymorphism of the *Plasmodium falciparum* multidrug resistance and chloroquine resistance transporter genes and *in vitro* susceptibility to aminoquinolines in isolates from the Peruvian Amazon. Am. J. Trop. Med. Hyg. 70, 461–466.
43 Pillai, D. R., Hijar, G., Montoya, Y., Marouino, W., Ruebush, T. K. 2nd, Wongsrichanalai C. et al. (2003) Lack of prediction of mefloquine and mefloquine-artesunate treatment outcome by mutations in the *Plasmodium falciparum* multidrug resistance 1 (pfmdr1) gene for *P. falciparum* malaria in Peru. Am. J. Trop. Med. Hyg. 68, 107–110.
44 Duraisingh, M. T., Jones, P., Sambou, I., von Seidlein, L., Pinder, M. and Warhurst, D. C. (2000) The tyrosine-86 allele of the pfmdr1 gene of *Plasmodium falciparum* is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. Mol. Biochem. Parasitol. 108, 13–23.
45 Price, R. N., Cassar, C., Brockman, A., Duraisingh, M., van Vugt, M., White, N. J. et al. (1999) The pfmdr1 gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. Antimicrob. Agents Chemother. 43, 2943–2949.
46 Sisowath, C., Stromberg, J., Martensson, A., Msellem, M., Obondo, C., Bjorkman, A. et al. (2005) *In vivo* selection of *Plasmodium falciparum* pfmdr1 86N coding alleles by artemether-lumefantrine (Coartem). J. Infect. Dis. 191, 1014–1017.
47 Johnson, D. J., Fidock, D. A., Mungthin, M., Lakshmanan, V., Sidhu, A. B., Bray, P. G. et al. (2004) Evidence for a central role for PfCRT in conferring *Plasmodium falciparum* resistance to diverse antimalarial agents. Mol. Cell 15, 867–877.
48 Basco, L. K., Le Bras, J., Rhoades, Z. and Wilson, C. M. (1995) Analysis of pfmdr1 and drug susceptibility in fresh isolates of *Plasmodium falciparum* from subsaharan Africa. Mol. Biochem. Parasitol. 74, 157–166.
49 Uhlemann, A. C., Ramharter, M., Lell, B., Kremsner, P. G. and Krishna, S. (2005) Amplification of *Plasmodium falciparum* multidrug resistance gene 1 in isolates from Gabon. J. Infect. Dis. 192, 1830–1835.
50 Basco, L. K. and Ringwald, P. (2002) Molecular epidemiology of malaria in Cameroon. X. Evaluation of PFMDR1 mutations as genetic markers for resistance to amino alcohols and artemisinin derivatives. Am. J. Trop. Med. Hyg. 66, 667–671.
51 Cooper, R. A., Ferdig, M. T., Su, X. Z., Ursos, L. M., Mu, J., Nomura, T. et al. (2002) 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. 61, 35–42.
52 Mu, J., Ferdig, M. T., Feng, X., Joy, D. A., Duan, J., Furuya, T. et al. (2003) Multiple transporters associated with malaria parasite responses to chloroquine and quinine. Mol. Microbiol. 49, 977–989.
53 Ferdig, M. T., Cooper, R. A., Mu, J., Deng, B., Joy, D. A., Su, X. Z. et al. (2004) Dissecting the loci of low-level quinine resistance in malaria parasites. Mol. Microbiol. 52, 985–997.
54 Eckstein-Ludwig, U., Webb, R., Van Goethem, I. D. A., East, J. M., Lee, A. G., Kimura, M. et al. (2003) Artemisinins target the SERCA of *Plasmodium falciparum*. Nature 424, 957–961.
55 Afonso, A., Hunt, P., Cheesman, S., Alves, A. C., Cunha, C. V., do Rosario, V. et al. (2006) Malaria parasites can develop stable resistance to artemisinin but lack mutations in candidate genes atp6 (encoding the sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase), tctp, mdr1, and cg10. Antimicrob. Agents Chemother. 50, 480–489.
56 Alin, M. H., Ashton, M., Kihamia, C. M., Mtey, G. J. B. and Bjorkman, A. (1996) Clinical efficacy and pharmacokinetics of artemisinin monotherapy and in combination with mefloquine in patients with falciparum malaria. Br. J. Clin. Pharmacol. 41, 587–592.
57 Hassan Alin, M., Ashton, M., Kihamia, C. M., Mtey, G. J. and Bjorkman, A. (1996) Multiple dose pharmacokinetics of oral artemisinin and comparison of its efficacy with that of oral artesunate in falciparum malaria patients. Trans. R. Soc. Trop. Med. Hyg. 90, 61–65.
58 Borrmann, S., Adegnika, A. A., Missinou, M. A., Binder, R. K., Issifou, S., Schindler, A. et al. (2003) Short-course artesunate treatment of uncomplicated *Plasmodium falciparum* malaria in Gabon. Antimicrob. Agents Chemother. 47, 901–904.
59 Huong, N. M., Hewitt, S., Davis, T. M., Dao, L. D., Toan, T. Q., Kim, T. B. et al. (2001) Resistance of *Plasmodium falciparum* to antimalarial drugs in a highly endemic area of southern Viet Nam: a study *in vivo* and *in vitro*. Trans. R. Soc. Trop. Med. Hyg. 95, 325–329.

60 Giao, P. T., Binh, T. Q., Kager, P. A., Long, H. Y., Thang, N. V., Nam, N. V. et al. (2001) Artemisinin for treatment of uncomplicated falciparum malaria: is there a place for monotherapy? Am. J. Trop. Med. Hyg. 65, 690–695.
61 Ittarat, W., Pickard, A. L., Rattanasinganchan, P., Wilairatana, P., Looareesuwan, S., Emery, K. et al. (2003) Recrudescence in artesunate-treated patients with falciparum malaria is dependent on parasite burden not on parasite factors. Am. J. Trop. Med. Hyg. 68, 147–152.
62 Bunnag, D., Viravan, C., Looareesuwan, S., Karbwang, J. and Harinasuta, T. (1991) Double blind randomised clinical trial of two different regimens of oral artesunate in falciparum malaria. Southeast Asian J. Trop. Med. Public Health 22, 534–538.
63 Bunnag, D., Viravan, C., Looareesuwan, S., Karbwang, J. and Harinasuta, T. (1991) Clinical trial of artesunate and artemether on multidrug resistant falciparum malaria in Thailand. A preliminary report. Southeast Asian J. Trop. Med. Public Health 22, 380–385.
64 Li, G. Q., Guo, X. B., Fu, L. C., Jian, H. X. and Wang, X. H. (1994) Clinical trials of artemisinin and its derivatives in the treatment of malaria in China. Trans. R. Soc. Trop. Med. Hyg. 88 Suppl 1, S5–S6.
65 Menard, D., Matsika-Claquin, M. D., Djalle, D., Yapou, F., Manirakiza, A., Dolmazon, V. et al. (2005) Association of failures of seven-day courses of artesunate in a non-immune population in Bangui, Central African Republic with decreased sensitivity of *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. 73, 616–621.
66 Jambou, R., Legrand, E., Niang, M., Khim, N., Lim, P., Volney, B. et al. (2005) Resistance of *Plasmodium falciparum* field isolates to *in-vitro* artemether and point mutations of the SERCA-type PfATPase6. Lancet 366, 1960–1963.
67 Nosten, F., van Vugt, M., Price, R., Luxemburger, C., Thway, K. L., Brockman, A. et al. (2000) Effects of artesunate-mefloquine combination on incidence of *Plasmodium falciparum* malaria and mefloquine resistance in western Thailand: a prospective study. Lancet 356, 297–302.
68 Kremsner, P. G. and Krishna, S. (2004) Antimalarial combinations. Lancet 364, 285–294.
69 White, N. J. (2004) Antimalarial drug resistance. J. Clin. Invest. 113, 1084–1092.
70 Price, R. N., Nosten, F., Luxemburger, C., ter Kuile, F. O., Paiphun, L., Chongsuphajaisiddhi, T. et al. (1996) Effects of artemisinin derivatives on malaria transmissibility. Lancet 347, 1654–1658.
71 Hutagalung, R., Paiphun, L., Ashley, E. A., McGready, R., Brockman, A., Thwai, K. L. et al. (2005) A randomized trial of artemether-lumefantrine versus mefloquine-artesunate for the treatment of uncomplicated multi-drug resistant *Plasmodium falciparum* on the western border of Thailand. Malar J. 4, 46.
72 Nosten, F., ter Kuile, F., Chongsuphajaisiddhi, T., Luxemburger, C., Webster, H. K., Edstein, M. et al. (1991) Mefloquine-resistant falciparum malaria on the Thai-Burmese border. Lancet 337, 1140–1143.
73 WHO report (2005) Susceptibility of *Plasmodium falciparum* to antimalarial drugs: report on global monitoring: 1996–2004. WHO, Geneva.
74 Hastings, I. M. and Ward, S. A. (2005) Coartem (artemether-lumefantrine) in Africa: the beginning of the end? J. Infect. Dis. 192, 1303–1304.
75 Martensson, A., Stromberg, J., Sisowath, C., Msellem, M. I., Gil, J. P., Montgomery, S. M. et al. (2005) Efficacy of artesunate plus amodiaquine *versus* that of artemether-lumefantrine for the treatment of uncomplicated childhood *Plasmodium falciparum* malaria in Zanzibar, Tanzania. Clin. Infect. Dis. 41, 1079–1086.
76 Whitty, C. J. and Staedke, S. G. (2005) Artemisinin-based combination treatment for malaria in Africa: no perfect solutions. Clin. Infect. Dis. 41, 1087–1088.
77 Congpuong, K., Na Bangchang, K., Mungthin, M., Bualombai, P. and Wernsdorfer, W. H. (2005) Molecular epidemiology of drug resistance markers of *Plasmodium falciparum* malaria in Thailand. Trop. Med. Int. Health 10, 717–722.
78 Nelson, A. L., Purfield, A., McDaniel, P., Uthaimongkol, N., Buathong, N., Sriwichai, S. et al. (2005) pfmdr1 genotyping and *in vivo* mefloquine resistance on the Thai-Myanmar border. Am. J. Trop. Med. Hyg. 72, 586–592