Genetic and genomic approaches for the discovery of parasite genes involved in antimalarial drug resistance

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SUMMARY

The biggest threat to the war on malaria is the continued evolution of drug resistance by the parasite. Resistance to almost all currently available antimalarials now exists in *Plasmodium falciparum* which causes the most suffering among all human malaria parasites. Monitoring of antimalarial efficacy and the development and subsequent spread of resistance has become an important part in the treatment and control of malaria. With recent reports of reduced efficacy of artemisinin, the current recommended treatment for uncomplicated malaria, there is urgent need for better methods to recognize and monitor drug resistance for effective treatment. Molecular markers have become a welcome addition to complement the more laborious and costly *in vitro* and *in vivo* methods that have traditionally been used to monitor drug resistance. However, there are currently no molecular markers for resistance to some antimalarials. This review highlights the role of the various genetic and genomic approaches that have been used in identifying the molecular markers that underlie drug resistance in *P. falciparum*. These approaches include; candidate genes, genetic linkage and genome-wide association studies. We discuss the requirements and limitations of each approach and use various examples to illustrate their contributions in identifying genomic regions of the parasite associated with antimalarial drug responses.

Key words: Candidate gene, drug resistance, genome-wide association, linkage analysis, malaria, molecular markers, *Plasmodium falciparum*.

INTRODUCTION

The malaria parasite *Plasmodium falciparum* has developed clinically significant resistance to all classes of antimalarial drugs, with the latest being reduced sensitivity to artemisinin and its derivatives (Dondorp *et al*. 2009). The World Health Organization (WHO) defines drug resistance as the ability of a parasite strain to multiply or to thrive in the presence of drug concentrations that normally destroy parasites of the same species or prevent their multiplication (World Health Organization, 1973). Resistance may be relative, with the parasite surviving increased doses of the drug that can be tolerated by the host (drug tolerance), or complete, when the parasites can withstand maximum doses tolerated by the host (outright resistance). In both cases, higher drug concentrations are required to achieve the same levels of parasite clearance (White, 2004).

Resistance emerges *de novo* through spontaneous mutations and/or gene duplications, which are thought to occur independently of drug selection pressure. In the presence of the drug, the mutated parasites have a selective advantage and their frequency in the population increases at a rate proportional to the selective pressure (drug use) (Hastings and Mackinnon, 1998). The mutations and gene duplications confer drug resistance through a number of mechanisms including alterations of drug transport and permeability, conversion of the drug to a form with lower activity, increased expression of the drug target, and alterations to the drug target that lowers its binding affinity to the inhibitor (White, 1992). Recently, more evidence has emerged to show that drug-resistant parasites can enter a quiescent state in the presence of some drugs, and later continue with normal cell cycle progression once the drug concentrations have waned (Codd *et al*. 2011).

Drug efficacy monitoring and continuous surveillance of any emerging resistance have become essential facets of malaria control strategies for the deployment of effective antimalarial drugs. Informative molecular markers of resistance are needed to monitor the emergence and spread of drug resistance, as an alternative to time-consuming and expensive drug efficacy trials. This review summarizes the evolution of resistance to antimalarial drugs in *P. falciparum*, and the genetic and genomic methods used to map the markers of this resistance.

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THE EVOLUTION AND SPREAD OF ANTIMALARIAL DRUG RESISTANCE

Resistance to antimalarial drugs has now been described for two of the five species of malaria parasite that naturally infect humans, *P. falciparum* and *P. vivax*, and the emergence of resistance usually reflects the extent of use of each antimalarial drug once it has been introduced.

Quinine is one of the oldest known antimalarials, first documented to have been used as an antimalarial in 1631 in Rome (Bruce-Chwatt, 1988), although it was probably used for centuries by natives of Peru and Bolivia to treat chills and fevers. Quinine is an aryl-amino alcohol extracted from the bark of the cinchona tree; chemical isolation was achieved in 1820, but before that dried and ground bark were mixed with sweetened water to offset its bitter taste, forming tonic water (Jarcho and Torti, 1993). Despite the widespread use of quinine in the nineteenth century, the first documented reports of drug resistance were not until 1910 (Nocht and Werner, 1910), and today resistance to quinine is still low and limited to SE Asia, Oceania and, less frequently, South America (Pickard and Wernsdorfer, 2002). Chemical synthesis of quinine was not achieved until 1944, but a number of synthetic drugs based on the structure of quinine were manufactured, the first in 1928, and these form a widely used class of antimalarials known as the quinoline drugs. Quinine itself remained in wider use than its synthetic counterparts until World War II, when the supply of quinine from countries in the South Pacific was cut off by Japanese military conquest.

The first fully synthetic 4-aminoquinoline antimalarials were produced in the 1930s, two of which – chloroquine (CQ) (e.g. Avochlor®) and amodiaquine – are still in use today (Surrey and Hammer, 1946). CQ was introduced for treatment and control of malaria in the 1940s and quickly became the drug of choice for most malaria-endemic countries. The first reports of confirmed *P. falciparum* resistance to CQ came from South America (Colombia, Brazil and Venezuela) in 1960 (Wernsdorfer, 1991). These were followed very soon afterwards by reports of resistance in Southeast Asia (Thailand and Kampuchea) in 1961 (Hartinuta *et al*. 1962). By 1973, chloroquine resistance (CQR) was widespread in South America and Asia but there was still no resistance in sub-Saharan Africa (World Health Organization, 1973). CQR in Africa was not observed until the late 1970s when its emergence was first seen in the east, in Kenya and Tanzania (Campbell *et al*. 1979; Fogh *et al*. 1979), from where it spread to the west. By 1989, the distribution of CQR was virtually identical to that of *P. falciparum* (Wernsdorfer and Payne, 1991). Another quinine-like drug, mefloquine, was first introduced in 1977, but resistance was reported from the Thai–Myanmar and the Thai–Cambodian borders in 1982 (Wongsrichanalai *et al*. 2001), although the drug remains effective outside of the SE Asia and some regions of South America.

The antifolate drugs were developed as antibacterial agents but were also found to have activity against malaria parasites (Curd *et al*. 1945). Antifolate drugs, such as pyrimethamine, were first used as antimalarials in the late 1940s; resistance to pyrimethamine was reported very soon after its introduction (Coatney *et al*. 1952). Drug resistance became most evident during the Global Malaria Eradication campaign launched by WHO in 1955 (Bruce-Chwatt, 1956). The introduction of salt medicated with antimalarials (especially pyrimethamine) to increase coverage of larger populations may have contributed to the emergence of parasites showing increased tolerance to the drugs. Interventions based on the introduction of pyrimethamine-medicated salts in Kenya, Ghana, the former Dutch New Guinea and Cambodia, among others, showed that, despite an initial reduction, parasite rates returned to pre-intervention levels within a period of six months (Avery, 1958; World Health Organization, 1961; Eyles *et al*. 1963). While chloroquine-medicated salts seemed to induce resistance less easily, the first cases of chloroquine-resistant parasites originated from or near areas where chloroquine-medicated salts had been in use (Payne, 1988).

Combinations of antifolate drugs inhibiting two enzymes in the folate pathway, dihydrofolate reductase and dihydropteroate synthase, were introduced in the 1960s to try to overcome resistance to monotherapy (World Health Organization, 1981). The first combination of antifolates was Maloprim®, a combination of the sulfone, dapsone, and pyrimethamine, but clinical trials were not encouraging and parasite resistance developed rapidly (Peters, 1985). Fixed-dose combinations such as sulfadoxine-pyrimethamine (SP, Fansidar®) gradually replaced CQ as first-line antimalarials, as CQ resistance increased. Resistance to SP emerged in SE Asia and the Amazon basin in the mid-1970s (White, 1992) and in Africa in the 1990s. Resistance to SP developed rapidly after its introduction, and in both SE Asia and Africa it was only useful for about six years before resistance reached unacceptably high levels (White, 2004; Anderson and Roper, 2005).

Atovaquone, a lipophilic hydroxynaphthoquinone, was developed in the 1980s and found to have potent activity against a range of pathogens including *Plasmodium* (Hudson *et al*. 1991). It is structurally related to ubiquinone (coenzyme Q) which is an important part of the mitochondrial electron transport chain (Fry and Pudney, 1992). Resistance to atovaquone develops very rapidly when it is used alone, but develops more slowly when used in combination with drugs such as proguanil (Malarone®) (Chiodini *et al*. 1995; Looareesuwan *et al*. 1996; Radloff *et al*. 1996).

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Table 1. Summary of the three different approaches to identify markers of drug resistance in malaria parasites

Approach	Prior knowledge or resources required	Examples of drug resistance genes discovered this way
Candidate gene	Mode of action of drug or target of drug action.	Pyrimethamine (Cowman *et al*. 1988) Atovaquone (Korsinczky *et al*. 2000) Chloroquine (Foote *et al*. 1989)
Linkage analysis	Genetic cross between parasites with different levels of drug response. Genetic map (genotyping) of progeny of the cross.	Chloroquine (Wellems *et al*. 1991; Fidock *et al*. 2000) Quinine (Ferdig *et al*. 2004) Pyrimethamine (Peterson *et al*. 1988)
GWAS	Patient samples with different drug responses. Detailed genotyping of each sample.	Artemisinin (Cheeseman *et al*. 2012) Halofantrine (Van Tyne *et al*. 2011)

Resistance to CQ and antifolates was widespread by 2004, and in 2006 the World Health Organization recommended the deployment of artemisinin-based combination therapies (ACTs) as first-line treatment for uncomplicated *P. falciparum* malaria (World Health Organization, 2010). Artemisinin (ART) is a sesquiterpene lactone endoperoxide with potent antimalarial activity, and was originally isolated from *Artemisia annua*, a herb used as an ancient Chinese herbal remedy (Meshnick *et al*. 1996). ART and its derivatives, artesunate, dihydroartemisinin and artemether, are very fast-acting (which helps to reduce the parasite load quickly), act against the broadest range of asexual blood-stage parasites of all known antimalarials, and they also inhibit development of gametocytes (Price *et al*. 1996). However, their very short half-life in plasma, about 2 h, means that recrudescence is common when ART is used as monotherapy. ART and its derivatives are therefore recommended to be administered with a partner drug with a longer-lasting half-life. Common coformulations include artemether–lumefantrine, artesunate–amodiaquine, artesunate–sulfadoxine/pyrimethamine and artesunate–mefloquine (Olliaro and Wells, 2009; Wells *et al*. 2009).

The first reports of ACT clinical failure were made in 2003 near the Thai–Cambodian border (Vijaykadga *et al*. 2006) following artesunate-mefloquine treatment, but it was not possible to establish whether this failure was due to artesunate or the partner drug, mefloquine. Subsequent studies in western Cambodia verified artesunate tolerance characterized by delayed clearance times following treatment (Noedl *et al*. 2008). A large study, conducted in Western Cambodia and northwestern Thailand, provided the clearest evidence of emerging ART resistance (Dondorp *et al*. 2009); following artesunate monotherapy or artesunate–mefloquine treatment, parasite clearance times were prolonged from 48 to 72 h and from 54 to 84 h respectively. It has since been shown that ART resistance is a heritable trait (Anderson *et al*. 2010). In an *in vitro* study, Witkowski *et al*. (2010) showed that parasites selected for ART resistance survive high doses of the drug by going into a state of developmental quiescence at the ring stage. These parasites were able to resume normal growth following the removal of the drug.

Thus, at the time of writing this review, there is existing resistance, or reduced efficacy, to all antimalarial drugs used for the treatment of *P. falciparum*. Monitoring the levels of drug resistance present in the malaria parasites in a region is crucial for effective control of the disease.

APPROACHES TO INVESTIGATE DRUG RESISTANCE IN MALARIA

There are three established approaches used to investigate the genes in malaria parasites involved in modulating drug responses: candidate gene approach, linkage analysis and genome-wide association analysis (GWAS). We have used various examples to describe each of these approaches to identify parasite genes or genomic regions involved in antimalarial drug resistance, summarized in Table 1.

Discovery of drug resistance markers through investigation of candidate genes

The candidate gene approach involves the investigation of polymorphism within a target gene, and the association of specific alleles with the parasite's response to the drug. This does not mean that mutations associated with resistance only occur in target genes and it is possible that resistance could arise via mutations in or changes in expressions of non-target genes such as transporters. The approach relies almost entirely on having prior knowledge of the drug's mode of action, or at least its target pathway. While it has also been recently used to prioritize of genes identified through linkage analysis and GWAS studies (Van Tyne *et al*. 2011; Cheeseman *et al*. 2012), it is the former approach that is addressed here. The best examples of successful identification of drug resistance markers through the candidate gene approach are for the parasite genes that modulate resistance to the antifolates and to atovaquone and, to an extent, CQ.

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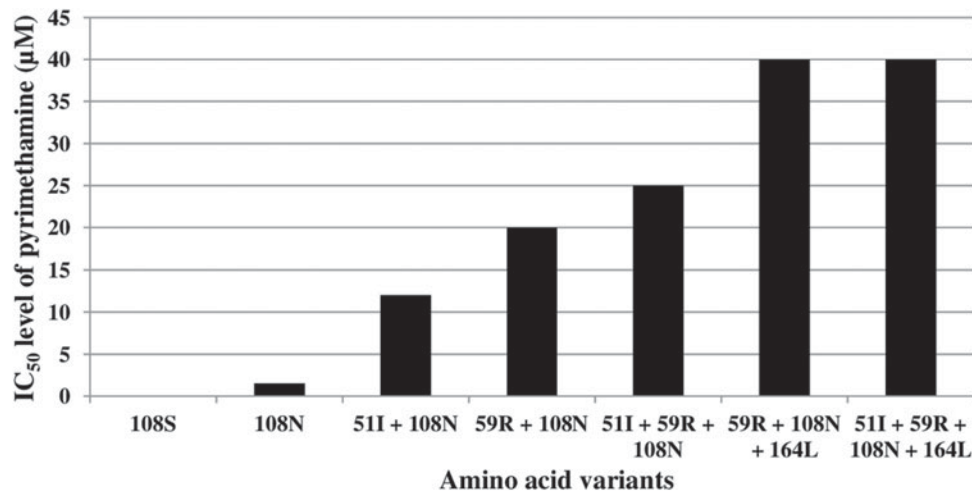

Fig. 1. Common variants of *P. falciparum* DHFR and their levels of pyrimethamine resistance *in vitro*. The x axis refers to the position and amino acid within the protein, using single letter coding. IC_{50} levels are the approximate values for the concentration of pyrimethamine (μM) required to reduce the parasitaemia by more than 50% compared to untreated controls, using *in vitro* tests (adapted from Hyde 1989).

Type II antifolate drugs and dihydrofolate reductase (dhfr). Antifolate drugs are designed to mimic the essential metabolites of the malaria parasite in the folate pathway, and are active against all the growing stages in the liver, erythrocytic stages and growing stages in the mosquito (sporogonic stages).

The parasite *dhfr* gene was implicated in drug resistance, when it was shown that *P. berghei* strains resistant to pyrimethamine had increased levels of the DHFR protein and decreased binding affinity of the drug to the enzyme (Diggens *et al*. 1970). Genetic crossing experiments in both *P. chabaudi* and *P. yoelii* using pyrimethamine-sensitive and pyrimethamine-resistant strains showed that the resistance phenotype segregated as a single gene in both cases. This gene was confirmed to be *dhfr* (Walliker *et al*. 1975, 1976; Knowles *et al*. 1981). In the case of *P. chabaudi*, no large differences in the enzyme amounts or turnover numbers were observed between resistant and sensitive clones. Later investigations of enzyme kinetics revealed a large decrease in affinity for binding of pyrimethamine with the enzyme from a resistant clone of *P. chabaudi*, together with changes in kinetic and other properties, indicating that the resistance is due to genetic change leading to a structurally different enzyme (Sirawaraporn and Yuthavong, 1984).

Pyrimethamine resistance in *P. falciparum* was also suggested to be associated with genetic change in the *dhfr* gene: the resistant phenotype was inherited in a genetic cross with a linkage group from the pyrimethamine-resistant parent containing the dihydrofolate reductase- thymidylate synthase (*dhfr-ts*) gene, and sequence data revealed a single mutation in the parents of the genetic cross, conferring an amino acid change from serine to asparagine at codon 108 of the DHFR domain (Cowman *et al*. 1988; Peterson *et al*. 1988). Subsequently, the same polymorphism in *P. falciparum dhfr* was found in a number of pyrimethamine-resistant and -sensitive clones, both from the laboratory and the field, and additional mutations in codons 51 and 59 were suggested to underlie increased resistance to the drug (Cowman *et al*. 1988; Peterson *et al*. 1988, 1991). It is now well established that high-level pyrimethamine resistance results from the accumulation of mutations in the *dhfr* gene, principally at codons 108, 59 and 51, giving rise to S108N, C59R and N51I. The mutations appear sequentially in pyrimethamine-treated populations, with the S108N mutation appearing first, followed by N51I or C59R (Plowe *et al*. 1997). However, only the S108N mutation has been found to occur singly in nature leading to suggestions that mutations either or both at codons 51 and 59 act predominantly by restoring the enzymatic defects that occur as a consequence of the original point mutation at position 108 (Hastings, 2004). Parasites with additional mutations of isoleucine to leucine at codon 164 (I164L) have increased levels of resistance to pyrimethamine (Cowman *et al*. 1988; Foote *et al*. 1990*a*; Basco *et al*. 1996). The various *dhfr* mutations and the associated inhibitory concentrations as measured *in vitro* are summarized in Fig. 1. Variation at residue 16 (Ala-16 to Val A16 V) confers resistance only to cycloguanil and the variation is always found to be associated with S108 T variation (Foote *et al*. 1990*a*).

Atovaquone and the cytochrome bc1 complex. Hydroxynaphthoquinones have long been known to inhibit respiration through inhibition of enzymes in the mitochondrial respiratory chain and were first developed as antimicrobials in the 1940s (Anfinsen, 1947; Ball *et al*. 1947). Atovaquone (2-[trans-4-(4′-chlorophenyl) cyclohexyl] 3-hydroxy-1,4-naphthoquinone), a structural analogue of ubiquinone (coenzyme Q), was developed as an antimalarial in

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the 1980s (Hudson *et al*. 1991). Like the antimicrobial naphthoquinones, atovaquone was shown to inhibit the parasite mitochondrial respiratory chain, specifically acting at the cytochrome bc1 complex, and thereby inhibiting transport of electrons in the parasite mitochondria, without affecting the host mitochondrial functions at the doses used (Fry and Pudney, 1992). It was therefore postulated that mutations within the parasite *cytochrome b* gene at or close to the atovaquone/ubiquinone binding site or the catalytic site would inhibit the action of the drug (Srivastava *et al*. 1999). Sequence analysis of the *cytb* gene from *P. falciparum* clones artificially selected for atovaquone-resistance *in vitro* revealed several mutations that were associated with increased atovaquone tolerance, including Y268S/C, M133I and V284K within the catalytic domain of Pfcytb, and others such as G280D, K272R and P275T (Srivastava *et al*. 1999; Korsinczky *et al*. 2000). Parasites from patients with Malarone® (atovaquone-proguanil) treatment failure have mutations at position 268 of *Pfcytb*, resulting in a change of amino acid from tyrosine to serine, cysteine or asparagine (Srivastava *et al*. 1999; Schwobel *et al*. 2003; Wichmann *et al*. 2004*a*; Sutherland *et al*. 2008). Some studies have also reported treatment failures with Malarone where parasites have no mutations in *Pfcytb*, suggesting that other mechanisms of resistance might be involved (Wichmann *et al*. 2004*b*).

Chloroquine, p-glycoprotein 1 (Pgh1), and the multidrug resistance 1 (Pfmdr 1) gene. Although the mechanism of action of CQ is thought to be via inhibition of detoxification of the products of haemoglobin digestion by the parasite (Chou *et al*. 1980; Bray *et al*. 1998), resistant parasites were observed to have a reduced accumulation of CQ compared to sensitive (CQS) strains (Geary *et al*. 1986; Saliba *et al*. 1998). CQR (and the associated reduced accumulation of CQ) could also be reversed (at least *in vitro*) using calcium-channel antagonists such as verapamil (Krogstad *et al*. 1987; Martin *et al*. 1987), a phenomenon also observed in mammalian cancer cells with a multidrug resistance phenotype (Fojo *et al*. 1985), and associated with increased expression of a membrane protein, p-glycoprotein, an ATP-dependent drug efflux pump (reviewed by Schinkel and Borst, 1991). Based on these observations, *P. falciparum* sequences corresponding to homologues of the *mdr transporter* genes in mammalian cells were identified and investigated for their possible role in CQ resistance (Foote *et al*. 1989; Wilson *et al*. 1989). Two homologues of *mdr* were found in the *P. falciparum* genome, and these were denoted *Pfmdr1* and *Pfmdr2* (Wilson *et al*. 1989). *Pfmdr1* encodes a ~160 kD protein, termed Pgh 1 (P-glycoprotein homologue 1), which localizes to the parasite food vacuole, where it was postulated to regulate intracellular drug concentrations (Cowman and Karcz, 1991). Sequence polymorphisms in *Pfmdr1* have been correlated with chloroquine-resistant phenotypes *in vitro*. One polymorphism, at codon 86, involves a single amino acid change, N86Y. Other mutations lead to four amino acid changes at codons Y184F, S1034C, N1042D and D1246Y (Foote *et al*. 1990*b*). Analysis of these polymorphisms in a double-blind study correctly predicted the CQ resistance profile of 34 of 36 isolates (Foote *et al*. 1990*b*). Molecular epidemiological analyses of field isolates have demonstrated association of some of these polymorphisms with CQ resistance in studies from sub-Saharan Africa (Basco *et al*. 1995) and Asia (Cox-Singh *et al*. 1995; Duraisingh *et al*. 2000). Other studies have, however, failed to find an association with these allelic variations (Awad-el-Kariem *et al*. 1992; Povoa *et al*. 1998; Mungthin *et al*. 1999), including linkage analysis studies (see later). When field isolates are analysed, both mutation and amplification of the *Pfmdr1* gene are found to be widespread in numerous geographical areas (Basco *et al*. 1995; Price *et al*. 1999). Together these data suggest that while *Pfmdr1* can modulate *P. falciparum* parasite response to CQ, there appears to be no evidence to date to implicate *Pfmdr2*.

Discovery of drug resistance markers through linkage analysis

Linkage analysis is a statistical methodology used to identify regions of a genome that change a specific phenotype of interest. Linkage studies require analysis of progeny from an experimental genetic cross between two parasites with differing phenotypes (in this case drug response), and a detailed genetic map of those progeny, identifying the genomic regions inherited from each parent (reviewed by Ranford-Cartwright and Mwangi, 2012). There are two types of linkage analyses; simple linkage and the more complicated quantitative trait locus (QTL) analyses. Simple linkage analysis of *Plasmodium* genetic crosses is applied where the phenotype segregates as a bimodal distribution in the progeny (Fig. 2), which is most simply explained because it is controlled by a single gene. Because the malaria parasite is haploid for most of its lifecycle, progeny clones inherit a particular gene from one or the other of the two parents, and thus also inherit the parental version of the phenotype controlled by that gene (there can be no heterozygotes). QTL analysis on the other hand is applied where the phenotype segregates as a continuous (unimodal) distribution (Fig. 3), which suggests that the trait is under the control of multiple genes, and can be inherited independently from either parent. Linkage mapping in *Plasmodium* exploits the haploid nature of the genome, and also the high recombination rates obtained in *P. falciparum*, which means that candidate genomic regions can be

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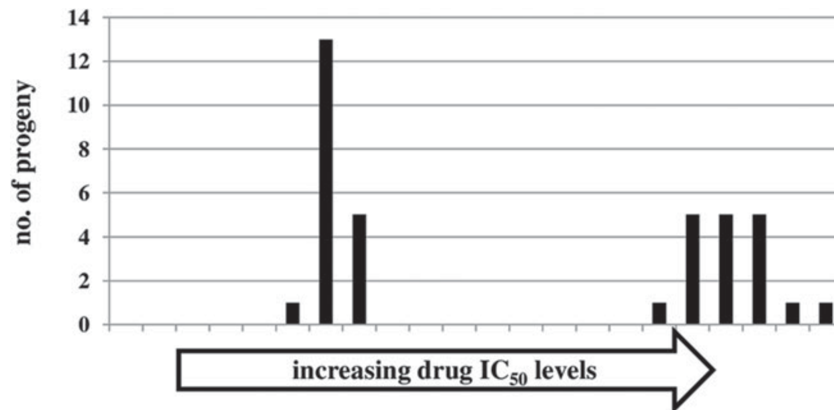

Fig. 2. The distribution of chloroquine drug response of 35 progeny clones from the HB3 × Dd2 cross, shown as the IC_{50} levels (concentration of drug required to reduce parasitaemia by 50% compared to untreated controls). The bimodal shape is indicative that CQ response is controlled by a single gene. Redrawn from data presented in Sen and Ferdig (2003).

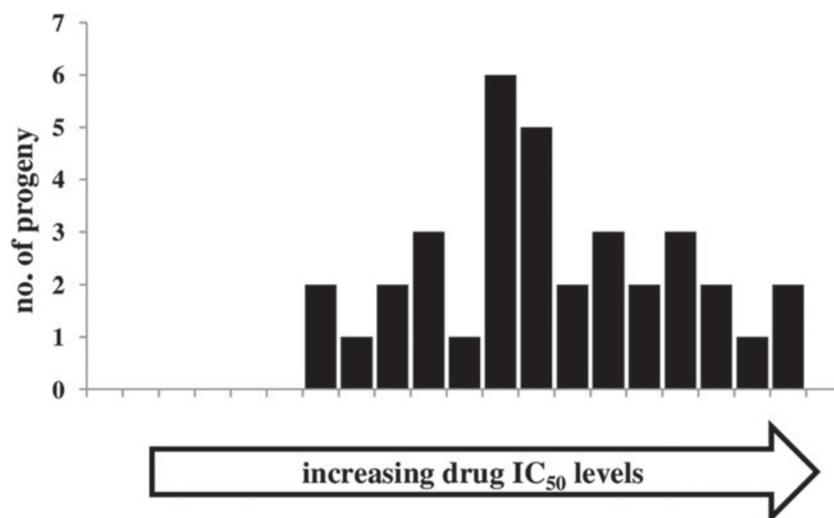

Fig. 3. The distribution of quinine drug response of 35 progeny clones from the HB3 × Dd2 cross, shown as the IC_{50} levels (concentration of drug required to reduce parasitaemia by 50% compared to untreated controls). The unimodal distribution is indicative of a quantitative multigenic trait. Redrawn from data presented in Sen and Ferdig (2003).

mapped to short stretches of DNA and with high precision (Su *et al*. 1997, 1999). A major advantage of linkage analysis is that it requires no *a priori* knowledge of the mechanism of action of the drug and/or its target.

Drug response, like most other cellular chemical responses, occurs within a network of linear and, sometimes, intricate web of non-linear pathways and so linkage analyses might identify not only the specific gene targets of the drug but also the biological processes in which the gene functions (Sen and Ferdig, 2003). Both simple linkage and QTL analyses have been successfully employed to characterize genes that control various drug responses in malaria parasites, two of which will be described here. Simple linkage analysis was used to identify the locus responsible for the CQ response (Wellems *et al*. 1990) and QTL has been used to map the loci linked to quinine responses (Ferdig *et al*. 2004).

Chloroquine resistance and the chloroquine resistance transporter gene (Pfcrt). In an attempt to establish the genetic basis of CQ resistance, an experimental cross between a chloroquine-sensitive Central American clone (HB3) and a multi-drug resistant Southeast Asian clone (Dd2) was performed (Wellems *et al*. 1990). The resulting progeny were phenotyped for their response to CQ *in vitro*, and the phenotype was found to segregate into roughly two groups: CQ-sensitive and CQ-resistant clones (Fig. 2). The progeny clones were genotyped at approximately 900 microsatellite markers across the 14 chromosomes to generate a genetic map. Using simple linkage analysis, CQR was mapped as a Mendelian trait to a 400 kb locus on chromosome 7 (Wellems *et al*. 1991). Further analysis narrowed the locus to a 36 kb segment with ten open reading frames (Su *et al*. 1997), and the specific locus responsible, named Pfcrt, was identified using forward genetics

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approaches including allelic exchange (Fidock *et al.* 2000; Sidhu *et al.* 2002). Coexpression of mutant forms of Pfcrt in the presence of a wild-type background conferred only a modest degree of resistance to CQS parasites (Fidock *et al.* 2000), whereas replacing the entire Pfcrt allele in a CQS parasite with alleles of mutant sequences resulted in recombinant clones with all the hallmarks of a *bona fide* CQ resistance phenotype. These include increased CQ IC50 values (exceeding 100 nM), acquisition of verapamil reversibility, and decreased CQ accumulation (Sidhu *et al.* 2002).

Pfcrt is a highly fragmented gene with 13 exons, spanning 3·1 kb, and encodes a 424 amino acid protein with 10 predicted transmembrane domains (Fidock *et al.* 2000). The protein was later localized to the digestive vacuole membrane by immunofluorescence (Fidock *et al.* 2000; Cooper *et al.* 2002). Mutations in the *Pfcrt* gene correlate strongly with *in vitro* CQ resistance in culture-adapted isolates from around the world (Fidock *et al.* 2000; Wootton *et al.* 2002). The critical mutation in CQR isolates results in a change of lysine to threonine at codon 76 (K76T). Other amino acid changes exist in flanking regions (72–75), with the allele present dependent on the geographical origin of the isolate. These additional mutations, predicted to lie within the transmembrane segments, may serve to compensate for a loss of the endogenous function associated with the K76T variation, although some may confer resistance to related antimalarial agents (Wellems and Plowe, 2001; Chen *et al.* 2003; Lim *et al.* 2003; Cooper *et al.* 2005).

Mutations in *Pfcrt* are believed to confer CQ resistance by reducing the amount of drug that accumulates within the parasite digestive vacuole (Saliba *et al.* 1998). There are several hypotheses to explain how Pfcrt might exert this effect on digestive vacuole CQ concentration, either as a consequence of alterations in the pH of the (Bennett *et al.* 2004), or a direct effect through enhanced transport of CQ out of the digestive vacuole (Saliba *et al.* 1998; Sanchez *et al.* 2003).

The debate on the mechanism of action of CQ and how resistance arises is still ongoing. Nevertheless, a combination of *Pfcrt* and *Pfmdr1* polymorphisms together has been shown to result in higher levels of CQR (Babiker *et al.* 2001). This observation is also supported by a whole genome survey using microsatellite markers to detect linkage disequilibrium (LD) in a range of parasites, which indentified the major locus for CQR at *Pfcrt* (Fidock *et al.* 2000; Djimde *et al.* 2001), but also an important secondary locus of *Pfmdr 1* on chromosome 5 (Wootton *et al.* 2002).

Quinine resistance and the Na+/H+ exchanger (Pfnhe). Quinine is currently reserved for the treatment of severe and multidrug-resistant malaria, often in combination with antibiotics for the latter infections (World Health Organization, 2010). The molecular mechanism of quinine action against *P. falciparum* is not fully understood, although like CQ it has been shown to accumulate in the parasite's digestive vacuole and inhibit haeme detoxification (Jarcho and Torti, 1993). Quinine resistance is not currently very widespread, which is thought to be a result of the drug's short half-life (8 h) and, more importantly, that multiple genes mediate this trait (Sen and Ferdig, 2003).

Analysis of progeny from an experimental genetic cross using the clones Dd2 × HB3, which have a small difference in quinine IC_{50} values, revealed a continuum of drug responses (Fig. 3), implying that quinine resistance is a multigenic trait (Ferdig *et al.* 2004). QTL mapping revealed contributory loci on five chromosomes, including regions containing *Pfmdr1* and *Pfcrt* on chromosomes 5 and 7, respectively. This was no surprise since both *Pfcrt* and *Pfmdr1* influence quinine response in previous studies (Cooper *et al.* 2002; Sidhu *et al.* 2002). An additional major locus on chromosome 13, spanning 380 kb and containing about 100 genes, was mapped as being associated with low level quinine resistance (Ferdig *et al.* 2004). One of the candidate genes in this region, denoted *Pfnhe*, encodes a sodium-hydrogen exchanger (NHE) which localizes to the parasitic plasma membrane (Ferdig *et al.* 2004). It has been suggested PFNHE is a proton pump that maintains parasite pH at 7·4, countering the effects of acidifying effects of anaerobic glycolysis (Bosia *et al.* 1993; Urscher *et al.* 2011).

Polymorphic regions of *Pfnhe* were shown to be associated with low quinine response in a variety of *P. falciparum* isolates, with variation in a microsatellite repeat denoted ms4670 significantly associated with decreased quinine response in some field isolates (Henry *et al.* 2009; Okombo *et al.* 2010), but not in others (Briolant *et al.* 2011). These results suggest that *Pfnhe* is acting in concert with another gene, or that quinine resistance is conferred by another gene located closely to *Pfnhe*.

Linkage analysis has proved to be a powerful tool for mapping genomic loci associated with various phenotypes. However, it relies on the successful genetic crossing between two parasites with varying measures of the trait, resulting in progeny that have unique combinations of alleles. Due to the lack of an efficient *in vitro* culture system for the hepatic stages of *P. falciparum*, genetic crosses rely on the use of a suitable mammalian host (a chimpanzee), which is expensive and ethically problematic. To date only three genetic crosses of *P. falciparum* have been successfully performed (see Ranford-Cartwright and Mwangi (2012) for a review on *P. falciparum* genetic crosses) which means there is a limited amount of resources that can be used for further linkage analysis of drug resistance. At the time of writing this review

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two more crosses are being undertaken: a fourth *P. falciparum* cross and the first experimental *P. vivax* cross (Wellems *et al.* unpublished).

Discovery of drug resistance markers through GWAS

GWAS use statistical analyses to look for associations between genetic markers across whole genomes, and phenotypic traits in parasite samples from natural populations. The rationale for the use of GWAS to identify drug resistance loci is that drug resistance involves significant contributions from individual genes and, due to strong selection, causative alleles will be found at high frequencies in treated populations (Nash *et al.* 2005). In order to carry out GWAS, one needs to have parasite samples from natural infections with varying drug responses, as well as detailed genotypes, usually consisting of single nucleotide polymorphisms (SNPs). Drug-resistance in malaria is often due to specific inherited mutations that can spread rapidly under drug pressure, leaving signatures of selection in the genome (selection valleys), because there is not enough time for the breakdown of linkage between the causal alleles and flanking regions of the genome (Su *et al.* 2007). GWAS analysis exploits the presence of such signatures of selection by scanning for regions of high LD to map markers that are involved in drug resistance. The first proof-of-principle GWAS study successfully mapped *Pfcrt* on chromosome 7 using 92 parasites from global locations and just 342 microsatellite markers (Wootton *et al.* 2002). Here we discuss the novel markers of antimalarial drug resistance that have been identified exclusively through GWAS analyses.

Halofantrine, mefloquine and lumefantrine resistance and Pf10_0335. Halofantrine is an aryl methanol, with structural similarity to quinine and other quinolines, which was introduced as an antimalarial in 1988. It is thought to inhibit the haeme detoxification pathway as it has been shown to form complexes with ferriprotoporphyrin IX (Dorn *et al.* 1998). It was shown to be effective even against multidrug-resistant malaria (Bloland, 2001). However, its use as monotherapy has been curtailed by widespread reports of potential cardiotoxicity (reviewed in Bouchaud *et al.* (2009)), although it is used in low doses as combination therapy with artesunate (Olliaro and Wells, 2009).

Using 17 000 SNPs and *in vitro* responses to various antimalarials, Van Tyne *et al.* (2011) analysed a set of globally-diverse parasites and identified a highly polymorphic locus, *PF10_0355* (subsequently renamed as *PF3D7_1034500*), associated with halofantrine resistance. Expression of the allele from a resistant parasite in a sensitive recipient, or over-expression of the allele from a drug-sensitive parasite, conferred decreased susceptibility to halofantrine and to other structurally related amino-quinolines like mefloquine and lumefantrine, but not to the unrelated antimalarials CQ, artemisinin and atovaquone. It was concluded that increased copy number of *PF10_0355* in the drug-resistant parasites decreased susceptibility to halofantrine, mefloquine and lumefantrine, although the mechanism is unknown (Van Tyne *et al.* 2011).

Artemisinin response and a chromosome 13 locus. The global roll-out of artemisinin-combination therapy (ACT) with integrated vector control measures has contributed to the recent reduction in malaria morbidity and mortality. The mechanism of action of artemisinin is thought to be either oxidative damage to parasite membranes or inactivation of parasite proteins by free radicals produced by the drug (Hartwig *et al.* 2009). The endoperoxide moiety seems to be critical in the function of artemisinins, and it is believed to produce the active compound upon interaction with intracellular iron, probably arising from the haeme degradation by the parasite (Klonis *et al.* 2011).

The useful therapeutic life of artemisinin is now threatened, with recent reports of the emergence of resistant parasites in Southeast Asia at the Thailand/Cambodia border (Dondorp *et al.* 2009). Although it is not yet known whether these observations are of 'true' resistance, parasites exhibit slower clearance rates following artemisinin treatment compared to sensitive ones. In a recent study, Cheeseman *et al.* (2012) used the differential clearance rates of parasites from patients treated with ACT, sampled from Cambodia, Thailand and Laos, to perform a genome-wide association study. Their work revealed strong selection on 33 genomic regions, ten of which contained known antimalarial resistance genes. A 105 kb locus on chromosome 13 was significantly associated with parasite clearance time in all three parasite populations, and allelic variation was lowest in Cambodia (slowest parasite clearance time) compared to the high diversity observed in parasites from Laos whose clearance times were shorter, supporting the hypothesis of a recent selective sweep. Fine mapping narrowed the locus to a 35 kb region containing seven candidate genes; this region was estimated to explain at least 35·2% of the variation observed in clearance rates of the parasites in this region (Cheeseman *et al.* 2012), but further forward genetic support will rely on the development of laboratory assays that can serve as a surrogate measure of the clearance rate phenotype *in vitro*.

While GWAS is gaining popularity after the recent development of more sensitive and robust sequencing methods, resulting in better marker coverage of the whole genome, there are still challenges when using the technique in natural malaria parasite populations. GWAS methods require homogeneous samples

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i.e. single genotype infections, but malaria parasite infections in endemic areas are usually composed of more than one parasite genotype (Conway *et al*. 1991), necessitating screening and identification of single genotype infections for the analysis. This reduces the sample size and thus the power available for analyses. In addition, parasite population demographics, including sub-structuring, can inflate associations of phenotypes and loci through false positives. This problem can be overcome, to an extent, by using more powerful analytical tools to overcome data stratification, e.g. haplotype likelihood ratio (HLR), cross population extended haplotype heterozygosity (XP-EHH), and the mixed model analysis such as the one successfully used by Van Tyne *et al*. to map the locus for halofantrine resistance discussed above (Van Tyne *et al*. 2011).

Even the strongest statistical association of genetic linkage does not imply biological causation. This means that all candidate genes identified by GWAS or linkage analyses methods have to be validated and functionally tested in order to prove their causal process with the phenotype under study. This work has to be done using appropriate laboratory assays. However, not all phenotypes have appropriate laboratory assays which can be used to validate the genes linked to them. For instance, the variable clearance time phenotype observed with artemisinin for which Cheeseman and colleagues identified candidate loci does not currently have a suitable laboratory assay to validate the genes identified (Cheeseman *et al*. 2012). For those phenotypes with suitable laboratory assays there is a second challenge; the process of functional validation in *P. falciparum* can be time consuming and still has very low success rates (reviewed by Crabb *et al*. 2004). Ideally, to prove functional association between a gene and a phenotype, in this case drug resistance, the allele of the candidate gene in a sensitive parasite should be replaced by that from a resistant parasite (conferring resistance in an appropriate assay), and *vice versa*. In cases where the candidate loci contain multiple genes, or where the inheritance of the phenotype is complex, such proof will be difficult to obtain.

USES OF MOLECULAR MARKERS FOR ANTIMALARIAL DRUG RESISTANCE

Molecular markers for antimalarial drug resistance have been used to track the spread of resistant alleles in patient samples during the time that the drug is in use. This methodology complements the more laborious and expensive *in vitro* and *in vivo* drug efficacy screening to monitor drug resistance (World Health Organization, 1990, 2003). The tracking of drug-resistant alleles has enabled policy makers to prepare for first-line antimalarial changes before *in vivo* treatment failures have reached critical levels. A good example was during the transition from sulphadoxine/pyrimethamine (SP) to ACTs in most African countries, where molecular markers for SP failure were used to anticipate and track treatment failure, thus enabling a timely switch of the treatment regimen (Plowe *et al*. 1997; Kublin *et al*. 1998; Nzila-Mounda *et al*. 1998). It also allows researchers to monitor the levels of resistance (through prevalence of drug resistance mutations) when a particular drug has been withdrawn due to high resistance, when it would be unethical to conduct efficacy trials (Plowe *et al*. 2004).

Molecular markers have also been used retrospectively to analyse and understand the factors that favour the emergence and spread of drug resistance, including extent of drug use, transmission intensity and parasite inbreeding rates, by measuring allele frequencies in archived samples which would not otherwise have been typed for drug resistance (Wootton *et al*. 2002; Roper *et al*. 2003; Hastings, 2004; Anderson and Roper, 2005). In low transmission areas, where people have low levels of acquired immunity to malaria, and most infections are symptomatic, the majority of malaria episodes receive treatment. This results in a strong selective advantage for parasites carrying drug-resistant mutations. This means that parasite populations are under higher drug pressure in low transmission areas and so resistance mutations are more likely to evolve, survive and spread in such areas (Hastings, 2004). The selective advantage is lower in high transmission zones where infections are more likely to be asymptomatic and thus remain untreated (Bottius *et al*. 1996). This might partly explain why the hotspots for malaria drug resistance are the lower-transmission regions of Southeast Asia, especially the countries of Thailand, Cambodia and Vietnam (Wootton *et al*. 2002; Anderson and Roper, 2005; Dondorp *et al*. 2009; Cheeseman *et al*. 2012), although the previous and heavy use of artemisinin monotherapy (rather than ACT) has been suggested as an important contributory factor in the emergence of ART resistance in Cambodia (Dondorp *et al*. 2004). Low transmission regions generally have a lower likelihood of mixed genotype infections, and thus a higher rate of inbreeding. Parasite inbreeding has been proposed to favour the spread of multigenic drug resistance, by preventing the breakdown and dilution of drug-resistant haplotypes that occur through genetic recombination (Schmidt, 1995). Multidrug resistance genotypes are, however, more likely to be generated when transmission intensities, and the rate of multiple clone infections, are higher (Curtis and Otoo, 1986).

In conclusion, different genetic and genomic methodologies have been utilized to reveal the molecular bases of drug resistance in *P. falciparum*. The choice of approach mainly depends on the existing knowledge of the target of action of the drugs and the resources available. The development

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of better, faster and inexpensive tools, such as the recent advances in sequencing whole genomes, will obviously simplify and improve the accuracy of locating parasite genomic regions that modulate drug responses. The development of better methods for functional analysis, to validate the candidate genes, will help to elucidate the targets of drug action, and the mechanism of resistance, knowledge that will be useful to formulate better drugs for future deployment.

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