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Author

Rosenthal, Philip J

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The interplay between drug resistance and fitness in malaria parasites

Philip J. Rosenthal
Department of Medicine, University of California, San Francisco, CA 94143 USA

Summary

Controlling the spread of antimalarial drug resistance, especially resistance of *Plasmodium falciparum* to artemisinin-based combination therapies, is a high priority. Available data indicate that, as with other microorganisms, the spread of drug-resistant malaria parasites is limited by fitness costs that frequently accompany resistance. Resistance-mediating polymorphisms in malaria parasites have been identified in putative drug transporters and in target enzymes. The impacts of these polymorphisms on parasite fitness have been characterized in vitro and in animal models. Additional insights have come from analyses of samples from clinical studies, both evaluating parasites under different selective pressures and determining the clinical consequences of infection with different parasites. With some exceptions, resistance-mediating polymorphisms lead to malaria parasites that, compared to wild type, grow less well in culture and in animals, and are replaced by wild type when drug pressure diminishes in the clinical setting. In some cases, the fitness costs of resistance may be offset by compensatory mutations that increase virulence or changes that enhance malaria transmission. However, not enough is known about effects of resistance mediators on parasite fitness. A better appreciation of the costs of fitness-mediating mutations will facilitate the development of optimal guidelines for the treatment and prevention of malaria.

Introduction

Malaria is one of the most important infectious diseases in the world. Recently, important gains in the control of malaria have been reported in some areas, and there is increasing optimism regarding the potential for elimination of malaria from many regions (Feachem *et al.*, 2010, Tatem *et al.*, 2010). However, despite recent gains, malaria remains an overwhelming problem in much of the tropical world, and it continues to cause hundreds of millions of illnesses and up to about one million deaths each year (Snow *et al.*, 2005, Murray *et al.*, 2012). Most serious illnesses and deaths from malaria and also most drug-resistant infections are due to infection with *Plasmodium falciparum*, the most pathogenic human malaria parasite, and this review will focus principally on studies with that organism.

The control and eventual eradication of malaria depend on a rather small set of tools. For control of anopheline mosquito vectors insecticide impregnated bednets and indoor residual spraying of insecticides are increasingly used, and their utility has been clearly demonstrated (Okumu & Moore, 2011), but their efficacy will be limited without coincident efforts directed against malaria parasites. An effective vaccine against malaria would be extremely valuable. Unfortunately, although the RTS,S vaccine, which has offered modest protection against malaria in African children (Olotu *et al.*, 2013), may be available in a few years, no

Address correspondence to: Philip J. Rosenthal, Box 0811, University of California, San Francisco, CA 94143 USA; Phone: 415-206-8845; FAX: 415-648-8425; prosenthal@medsfgh.ucsf.edu.

highly effective vaccine is on the horizon. Considering the limitations of vector control and vaccines, appropriate use of antimalarial drugs remains a cornerstone of malaria control.

Drugs have two key roles for malaria control. First, in addition to offering obvious benefit to an ill individual, prompt and effective treatment for malaria limits the development of parasites into gametocytes, thus blocking transmission to mosquitoes and subsequently to other individuals (Gosling *et al.*, 2011). Available drugs have varied activity against gametocytes that are present at the time of treatment, however. Second, there is increasing consideration of the use of drugs to prevent malaria in endemic populations, either as intermittent therapy or low dose chemoprophylaxis (Greenwood, 2010). For all indications, we are dependent on a rather small armamentarium of antimalarial drugs. The efficacies of many of these drugs are limited by resistance, and recent evidence suggests that parasites are becoming resistant to our newest agents. However, the extent of resistance varies, such that in many cases drugs with resistance concerns are nonetheless offering good effectiveness. In the setting of widespread but varied levels of drug resistance, the impact of resistance on parasite fitness is of great importance. Choices of optimal regimens for treatment and chemoprevention will be facilitated by an understanding of the impacts of resistance selection on the abilities of parasites to cause disease and to be transmitted.

The interplay between drug resistance and fitness in bacteria and viruses

With many microbial pathogens, drug resistance comes with a fitness cost. Considering bacterial infections, antibiotic use selects for resistant bacteria via multiple mechanisms, including alterations in antibiotic target genes and increases in drug efflux, but resistant bacteria are typically less fit (Andersson and Hughes, 2010). Fitness can be measured in vitro by comparing growth rates and in competitive growth experiments. Importantly, fitness can be mediated by impacts of a trait on other organisms (eg production of a factor toxic to sensitive bacteria), such that a fitness advantage will only be recognized in a competitive growth experiment (Gordo, 2011). Fitness can also be assessed in animal model studies and by considering the clinical consequences of infection with drug sensitive and drug resistant organisms. Over time, resistant bacteria can evolve into organisms with improved fitness due to the acquisition of compensatory mutations. Non-lethal selective pressure from low levels of antibiotics may enhance the likelihood of resistance selection (Andersson and Hughes, 2010). Removal of antibiotic pressure can allow reversion to drug-sensitive organisms, but resistant bacteria can be quite stable, in part due to compensatory mutations that improve fitness.

Studies with viruses have also demonstrated ready selection of resistance (Gotte, 2012). In general mutant variants with a low genetic barrier (relatively few genetic changes required) are selected most rapidly by antiviral selective pressure. Subsequently, more fit variants are selected more slowly. As with bacteria, measures of viral fitness can include relative rates of replication of different strains in cell lines, either comparing replication in parallel assays or with competition assays (Wargo and Kurath, 2012).

Overall, characterization of the impacts of antimicrobial drug resistance on bacterial and viral fitness has been complex. Measures of fitness may vary depending on experimental methodology. Resistance to one agent may have important impacts on resistance to other drugs, and thereby impact upon fitness. Fitness also must be considered in the context of both the replication and transmissibility of microorganisms, and these two features are often not linked. It is important to take these factors into account when considering assessments of the the interplay of drug resistance and fitness in malaria parasites. However, as eukaryotes with complex asexual and sexual life cycles, malaria parasites differ importantly from the prokaryotic and viral model systems.

Antimalarial drugs

Antimalarial drugs act principally to eliminate the erythrocytic stages of malaria parasites that are responsible for human illness. The standard treatment for falciparum malaria has changed in recent years. With frequent resistance to older drugs, artemisinin-based combination therapy (ACT) is recommended for the treatment of uncomplicated falciparum malaria in nearly all areas (World Health Organization, 2010). ACT consists of a potent artemisinin component, which rapidly clears most parasites, plus a longer acting partner drug, which eliminates remaining parasites after the artemisinin is cleared (Nosten & White, 2007). The most important ACTs that are now available are artemether/lumefantrine, artesunate/amodiaquine, artesunate/mefloquine, and dihydroartemisinin/piperaquine. ACTs are also effective against non-falciparum malaria. Multiple drugs are used to prevent malaria. Recommendations for travelers from nonendemic to endemic areas generally advocate use of atovaquone/proguanil, mefloquine, or doxycycline in low dose chemoprophylactic regimens (Schlagenhauf & Petersen, 2008). In Africa intermittent preventive therapy is advocated in some high risk populations, including sulfadoxine/pyrimethamine during pregnancy and amodiaquine/sulfadoxine/pyrimethamine as seasonal malaria chemoprophylaxis in areas with relatively little drug resistance (Greenwood, 2010).

Available antimalarial drugs can be divided into 7 classes (Table 1). The 4-aminoquinoline chloroquine was the gold standard for the treatment of uncomplicated malaria for many years, but it is no longer appropriate for the treatment of falciparum malaria in nearly all areas due to drug resistance. Amodiaquine is subject to similar resistance mechanisms, but it often provides adequate efficacy against parasites with the genetic changes that mediate chloroquine resistance. The main current use of amodiaquine is as a component of the ACT artesunate/amodiaquine. A third 4-aminoquinoline, piperaquine, was widely used to treat and prevent malaria in China a few decades ago, but it then fell into disfavor due to increasing drug resistance (Davis *et al.*, 2005). More recently piperaquine has become a component of another ACT, dihydroartemisinin/piperaquine. The 8-aminoquinoline primaquine has some activity against erythrocytic parasites, but it is used principally to eliminate parasite liver stages, including the exoerythrocytic forms that precede erythrocytic infection in all species and the hypnozoites that cause latent infections with *P. vivax* and *P. ovale*. Primaquine also acts against gametocytes, thereby lowering transmission of parasites to mosquito vectors. Quinine is an aryl-amino alcohol that is our oldest antimalarial drug, used as cinchona bark since the 1600s and in its pure form since 1820 (Meshnick & Dobson, 2001). Quinine is quite hard to tolerate, and its use is best limited to the treatment of severe malaria. Important related drugs are mefloquine and lumefantrine, both of which are components of ACTs.

Antifolates, which were developed to treat bacterial infections, target parasite dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), offering synergistic antimalarial activity. Sulfadoxine/pyrimethamine has the distinct advantage of single-dose therapy, but its treatment efficacy is seriously limited by drug resistance. Trimethoprim/sulfamethoxazole is not routinely used to treat malaria, but it offers fairly effective protection against malaria when provided as regular prophylaxis against multiple infections in those with HIV infection. The naphthoquinone atovaquone acts against the mitochondrial cytochrome bc_1 complex. Combined with the DHFR inhibitor proguanil it offers effective therapy and chemoprophylaxis for falciparum malaria. A number of antibiotics that are prokaryotic protein synthesis inhibitors have antimalarial activity due to action against the protein synthesis machinery of the apicoplast organelle (Dahl & Rosenthal, 2008). Doxycycline is used for chemoprophylaxis against malaria, and doxycycline or clindamycin are combined with quinine to treat falciparum malaria.

The most important new class of antimalarials is the artemisinins, which were developed from a natural product remedy in China. Artemisinin is a potent antimalarial, but the derivatives artesunate, artemether, and dihydroartemisinin are most widely used, all as components of ACT regimens. Indeed, the use of artemisinins outside of combination regimens is strongly discouraged by the World Health Organization due to fear of selecting for resistance to this important class of drugs. Artemisinins are highly effective against acute malaria, but short acting, so combination with longer-acting drugs in ACTs allows short (3-day) courses of treatment that protect against the selection of resistance to the artemisinin component (Nosten & White, 2007). Due to its rapid action intravenous artesunate is also the new gold standard for the treatment of severe falciparum malaria, with documented survival advantages compared to intravenous quinine (Dondorp et al., 2005, Dondorp et al., 2010).

Antimalarial drug resistance

Resistance has been described for nearly all available drugs (Table 1). Established mediators of resistance are single nucleotide polymorphisms (SNPs) and changes in copy number in genes encoding putative drug transporters and some enzyme targets. For many drugs the extent of resistance is uncertain and mechanisms of resistance are unknown. Resistance can be assessed by in vitro assessment of sensitivities of cultured *P. falciparum*, by evaluation of genetic polymorphisms associated with resistance, by consideration of the clinical consequences of polymorphisms present at the time of treatment, or by assessing the selective pressure of antimalarial treatment on subsequent infections. Studies considering all of these factors have shed light on the extent of resistance and on mechanisms of resistance.

Transporter mutations—Informatic studies have identified multiple predicted transporter genes in *P. falciparum* (Table 1); SNPs in 11 of these genes were associated with decreased sensitivity to chloroquine or quinine (Mu *et al.*, 2003), although a subsequent study could not confirm associations between most of the identified polymorphisms and drug sensitivity in clinical isolates (Anderson *et al.*, 2005). Many predicted *P. falciparum* transporters are members of the ATP-binding cassette (ABC) transporter superfamily. ABC transporters are responsible for the transfer of a range of substances across concentration gradients in an energy-dependent manner (Koenderink *et al.*, 2010). Polymorphisms in transport proteins can mediate resistance to many agents active against cancer and infectious diseases via enhancing efflux of the drugs from cells (Borges-Walmsley *et al.*, 2003). It appears that a number of plasmodial proteins transport different drugs and that polymorphisms in these proteins may impact upon drug sensitivity (Picot *et al.*, 2009).

Pfmdr1—Polymorphisms in the *P. falciparum* multidrug resistance-1 (*pfmdr1*) gene, which encodes the P-glycoprotein homolog, impact on sensitivity to multiple antimalarial drugs (Foote *et al.*, 1990, Sanchez *et al.*, 2010, Valderramos & Fidock, 2006). In humans, P-glycoprotein polymorphisms are associated with resistance to cancer drugs (Sharom, 2011). In *P. falciparum*, the function of the *pfmdr1* product is unknown, but the protein localizes to the membrane of the food vacuole, the site of action of a number of drugs, suggesting that it is a drug transporter (Cowman *et al.*, 1991). Data on associations between *pfmdr1* polymorphisms and drug sensitivity are complex, but overall suggest that changes in *pfmdr1* sequence or copy number alter transport of multiple drugs in or out of the parasite food vacuole, with individual polymorphisms leading to opposite effects on different drugs (Koenderink et al., 2010). Mutations at *pfmdr1* N86Y and D1246Y (for this and other *P. falciparum* genes wild type sequence is based on the 3D7 reference strain), which are common in Africa, have been linked to decreased sensitivity to chloroquine and amodiaquine, but increased sensitivity to lumefantine, mefloquine, and artemisinins (Duraisingh *et al.*, 2000a, Duraisingh *et al.*, 2000b, Reed *et al.*, 2000, Mwai *et al.*, 2009).

Other polymorphisms primarily seen outside Africa (including 1034C, 1042D, and increased gene copy number) are associated with altered sensitivity to lumefantrine, mefloquine, and artemisinins (Reed et al., 2000, Pickard *et al.*, 2003, Sidhu *et al.*, 2005, Sidhu *et al.*, 2006, Veiga *et al.*, 2011). Considering infections that emerge soon after prior therapy, amodiaquine-containing regimens selected for the 86Y and 1246Y mutant alleles (Humphreys *et al.*, 2007, Zongo *et al.*, 2007, Nsobya *et al.*, 2007) and for parasites with decreased in vitro sensitivity to the active metabolite monodesethylamodiaquine (Nawaz *et al.*, 2009) in subsequent infections. In contrast, therapy with artemether-lumefantrine selected for the N86 and D1246 wild type alleles in subsequent infections within 60 days of prior therapy (Sisowath *et al.*, 2005, Humphreys et al., 2007, Zongo et al., 2007, Happi *et al.*, 2009, Some *et al.*, 2010, Baliraine & Rosenthal, 2011). Importantly, impacts of *pfmdr1* polymorphisms on drug sensitivity are modest, correlations between particular polymorphisms and treatment efficacy have not been seen, and the ACTs artesunate-amodiaquine and artemether-lumefantrine remain highly efficacious for the treatment of uncomplicated falciparum malaria in Africa (Dorsey *et al.*, 2007, Four Artemisinin-Based Combinations Study Group, 2011). However, as seen for chloroquine and amodiaquine, *pfmdr1* polymorphisms may contribute, with additional polymorphisms, to higher level resistance to increasingly used components of ACTs.

Pfcrt—Soon after the identification of *pfmdr1* it became clear that polymorphisms in this gene are not the primary mediators of chloroquine resistance. Subsequently, analysis of progeny of a genetic cross between chloroquine sensitive and resistant strains led to the identification of *pfcrt* (Fidock *et al.*, 2000), which encodes a food vacuole membrane protein that is predicted to be a member of the drug/metabolite transporter superfamily (Martin & Kirk, 2004, Tran & Saier, 2004). The function of *pfcrt* is unknown, but apparently essential, as disruption of the gene has not been possible (Ecker *et al.*, 2012). *Pfcrt* is highly polymorphic, but one SNP, K76T, is the primary mediator of chloroquine resistance (Lakshmanan *et al.*, 2005, Ecker et al., 2012). The 76T mutation appears to act principally by increasing the export of chloroquine from the food vacuole, but the mechanism of pfcrt 76T- mediated chloroquine resistance is incompletely understood (Ecker et al., 2012). Other *pfcrt* SNPs always accompany 76T in field isolates, and these likely encode compensatory mutations that allow parasites containing 76T to maintain adequate fitness; some other SNPs may also contribute directly to the drug resistant phenotype. The 76T mutation also mediates decreased sensitivity to monodesethylamodiaquine, and studies with genetically modified parasites have shown it to mediate increased susceptibility to mefloquine and artemisinins (Sidhu, *et al.*, 2002, Lakshmanan *et al.*, 2005), suggesting the same reciprocal relationship between sensitivities to aminoquinolines and other drugs as described for certain *pfmdr1* polymorphisms.

Pfmrp1—*P. falciparum* multidrug resistance protein-1 (*Pfmrp1*) is a member of the ABC transporter superfamily (Koenderink et al., 2010). Unlike the products of *pfmdr1* and *pfcrt*, Pfmrp1 localizes principally to the parasite plasma membrane (Kavishe *et al.*, 2009). In studies of culture adapted *P. falciparum*, SNPs in *pfmrp1* were linked to decreased sensitivity to chloroquine and quinine (Mu et al., 2003). Two SNPs that appear to be common in African parasites, I876V and K1466R, were selected by prior treatment with artemether/lumefantrine (Dahlstrom *et al.*, 2009a) and sulfadoxine pyrimethamine (Dahlstrom *et al.*, 2009b), respectively. Disruption of the *pfmrp1* gene yielded parasites with diminished growth and increased sensitivity to chloroquine and other drugs, suggesting a role for this protein in the efflux of antimalarial drugs from the parasite and in parasite fitness (Raj *et al.*, 2009).

Sodium transporters—Quantitative trait locus analysis identified three genes predicted to play roles in the responsiveness of *P. falciparum* to quinine, *pfcrt*, *pfmdr1*, and *pfnhe1*, which encodes a putative sodium-hydrogen exchanger and is highly polymorphic (Ferdig *et al.*, 2004). Reducing the expression of *pfnhe1* by ~50% using allelic exchange led to a 30% increase in quinine sensitivity in some but not other parasite strains (Nkrumah *et al.*, 2009). Recent studies evaluating associations between polymorphisms in a *pfnhe1* microsatellite, in vitro parasite sensitivity, and clinical responses to various drugs have been inconsistent, but these polymorphisms appear to have a modest impact on sensitivity of parasites to quinine, and possibly other drugs (Henry *et al.*, 2009, Meng *et al.*, 2010, Okombo *et al.*, 2010, Andriantsoanirina *et al.*, 2010, Baliraine *et al.*, 2011, Sinou *et al.*, 2011). *Pfatp4* encodes a *P. falciparum* plasma membrane protein that appears to be a sodium efflux pump (Spillman *et al.*, 2013). Mutations in *pfatp4* have been linked to altered sensitivity to a number of candidate antimalarials, with good evidence that the transporter is the target of highly active spiroindolones (Rottmann *et al.*, 2010).

Resistance to antifolates and atovaquone

The best characterized mediators of drug resistance in *P. falciparum* are mutations in the *pfdhfr* and *pfdhps* genes, which encode sequential enzymes in the folate pathway common to a wide range of eukaryotic and prokaryotic organisms. A series of mutations in *pfdhfr* and *pfdhps* mediate increasing resistance to antifolate combinations (Gregson & Plowe, 2005). In Africa, *pfdhfr* S108N, N51I, and C59R and *pfdhps* A437G are now very common and mediate low-level resistance to sulfadoxine-pyrimethamine. A fifth mutation, *pfdhps* K540E, is common in eastern and southern Africa, and mediates a higher level of resistance (Pearce *et al.*, 2009). Additional mutations seen most commonly outside Africa, include *pfdhfr* I164L, *pfdhps* A581G, and *pfdhps* A613S, appear to mediate high level of resistance. Atovaquone leads to collapse of mitochondrial membrane potential via inhibition of the cytochrome bc_1 complex. Resistance to atovaquone develops rapidly, and is mediated by a number of mutations in the cytochrome b (*pfcytb*) gene (Vaidya & Mather, 2000, Musset, *et al.*, 2007).

Resistance to antibiotics—Resistance of *P. falciparum* to antibiotics has not been well studied clinically, but varied sensitivities to tetracyclines of unknown clinical significance have been seen in vitro (Briolant *et al.*, 2009). Sequence polymorphisms and variation in copy number in *P. falciparum* homologs of bacterial mediators of tetracycline resistance (*pfmdt* and *pftetQ*) were associated with decreased drug sensitivity (Briolant *et al.*, 2010). A survey of parasites from the Amazon identified a SNP in an apicoplast gene encoding a homolog of a ribosomal protein in which mutations mediate clindamycin resistance in bacteria; this SNP was associated with in vitro clindamycin resistance in 3 clinical isolates (Dharia, *et al.*, 2010). Resistance to azithromycin has also been linked to mutations in an apicolast-encoded ribosomal protein (Sidhu, *et al.*, 2007).

Resistance to artemisinins—A clinical study identified *P. falciparum* with decreased in vitro sensitivity to artemether from French Guiana and associations between the S769N SNP in the *pfatp6* gene, which encodes a SERCA-type Ca++ ATPase, and decreased artemether sensitivity (Jambou *et al.*, 2005). This finding was of great interest due to the vital importance of artemisinin antimalarials and a prior report suggesting that artemisinins exert antimalarial activity by inhibiting PfATP6 (Eckstein-Ludwig *et al.*, 2003). However, while in vitro inhibition of PfATP6 by artemisinins has been demonstrated in *Xenopus* oocytes (Uhlemann *et al.*, 2005, Pulcini *et al.*, 2013) and *pfatp6* has been shown to be highly polymorphic (Dahlstrom *et al.*, 2008, Tanabe *et al.*, 2011), with one SNP (L263E) ablating artemisinin sensitivity (Uhlemann et al., 2005), mutations in field isolates differed from those shown to alter artemisinin sensitivity, the S769N mutation was not seen in isolates

from other areas, and introduction of the L263E (Valderramos *et al.*, 2010) or S769N (Cui *et al.*, 2012) mutations did not alter sensitivity of malaria parasites to artemisinins. Thus, the mechanism of resistance to artemisinins remains uncertain. Evaluations of resistance mechanisms should be viewed in light of recent observations of delayed clearance of parasites after treatment of malaria patients in southeast Asia with artemisinins (Noedl *et al.*, 2008, Dondorp *et al.*, 2009). Despite extensive effort, this reproducible clinical phenotype has not been clearly linked to an in vitro phenotype, with parasites that cleared slowly showing decreased drug sensitivity in some (Noedl et al., 2008, Lim *et al.*, 2010), but not other (Dondorp et al., 2009) studies. Recent studies using novel assays have shown association between parasites with delayed clearance and in vitro survival after exposure to short pulses of high concentrations of artemisinins during the ring stage (Witkowski *et al.*, 2013), consistent with observed marked sensitivity of early ring-stage parasites to short pulses of high concentrations of the drugs (Klonis *et al.*, 2011, Klonis *et al.*, 2013). In addition, in vitro selection of parasites with decreased artemisinin sensitivity has typically led to no change in standard sensitivity assays, but increased recrudescence after treatment, suggesting that decreased sensitivity may be due to changes in recovery from drug-induced parasite dormancy (Witkowski *et al.*, 2010, Tucker *et al.*, 2012). As noted above, polymorphisms in *pfmdr1* are associated with modest changes in artemisinin sensitivity, but polymorphisms in this gene or in *pfatp6* have not been clearly linked to delayed clearance in clinical studies. Ongoing studies are working to identify genetic determinants of delayed clearance of parasites after artemisinin treatment, and these appear to be complex (Cheeseman *et al.*, 2012, Takala-Harrison *et al.*, 2013, Miotto *et al.*, 2013). Changes in artemisinin sensitivity will likely impact upon parasite fitness, but information on such associations are not yet available.

Drug resistance and fitness in malaria parasites

Means of assessing the fitness of malaria parasites—We lack a specific measure of fitness in malaria parasites. Culture systems for *P. falciparum* allow careful comparison of growth rates, but distinguishing strains with only modest differences can be challenging. A method that is probably superior is the direct comparison of growth by co-culture of competing strains, with genetic determinants allowing one to distinguish the strains and identify overgrowth by the more fit strain. Comparisons of clinical isolates are arguably more relevant than those of engineered laboratory strains, but identifying meaningful differences between unrelated strains is challenging. Animal model studies have also provided insights, but these are limited by analysis of non-human parasites. The most convincing demonstrations of the fitness costs of antimalarial drug resistance have come from clinical studies. Most notably, loss of chloroquine selective pressure has been followed by rapid reemergence of chloroquine-sensitive parasites, as will be discussed below.

Fitness consequences of antimalarial drug resistance: in vitro studies—The first direct comparison of the fitness of drug sensitive and resistant malaria parasites used competition experiments to compare the growth of *P. falciparum* selected in vitro for resistance to atovaquone with that of the parent strain. Parasites selected for resistance had multiple mutations in *pfcytb*. Those with a single M133I mutation had 25-fold decreased atovaquone sensitivity and those with the M133I and G280D mutations had 1000-fold decreased sensitivity (Korsinczky *et al.*, 2000). In competition growth experiments, the single mutant line grew as well as the parent parasite, but the more resistant double mutant was out-competed by the parent line, with a 5–9% loss of fitness calculated based on relative growth rates (Peters *et al.*, 2002). Molecular modeling predicted that the G280D mutation altered the orientation of a putative ubiquinone-binding residue, likely altering enzyme function, while the M133I mutation would have minimal effect on enzyme conformation. Another *pfcytb* mutation, Y268S, was seen in a *P. falciparum* isolate from Thailand, and led

to marked alterations in enzyme function (Fisher *et al.*, 2012). Decreased enzyme activity was associated with increased expression of mitochondrial cytochrome bc_1 complex genes, apparently compensating for detrimental effects of the mutation, and offering an example of parasites reaching a balance to maintain fitness in the setting of drug-resistance.

A similar approach was used to evaluate the relative fitness of *P. falciparum* engineered to contain different *pfmdr1* haplotypes. Competitive growth was compared between the chloroquine sensitive D10 strain, the chloroquine-resistant 7G8 strain, and a strain in which most of the *pfmdr1* gene of D10 was replaced by that of 7G8, introducing the S1034C, N1042D and D1246Y mutations (Hayward *et al.*, 2005). Introduction of the mutant *pfmdr1* in D10 partially reversed chloroquine resistance and mediated decreased sensitivity to mefloquine, halofantrine, and artemisinin (Reed et al., 2000). These results demonstrate the reciprocal impacts of the *pfmdr1* polymorphisms on sensitivity to chloroquine (which is mediated principally by a different mutation, *pfcrt* K76T) and to mefloquine, halofantrine, and artemisinin. Considering fitness, the chloroquine-resistant 7G8 strain was outcompeted by the chloroquine-sensitive D10 strain and by the intermediate sensitivity 7G8 strain containing D10 *pfmdr1* sequence. Improved fitness of the more chloroquine sensitive strains was evidenced by gradual increases in chloroquine sensitivity of mixed cultures until, after 34 days, sensitivities were identical to that of a pure D10 culture (Hayward et al., 2005). Comparison of growth rates led to an estimate of ~25% loss of fitness mediated by introduction of the three *pfmdr1* mutations. However, the relative importance of the individual SNPs in mediating resistance is unclear, and the N86Y mutation, which appears to be the principle mediator of altered drug sensitivity in Africa, was not studied.

Increases in the copy number of *pfmdr1* have an important impact on sensitivity to numerous antimalarials, with increased copy number increasing sensitivity to chloroquine, but decreasing sensitivity to mefloquine, quinine, and artemisinins (Pickard et al., 2003, Phompradit *et al.*, 2011, Veiga et al., 2011). Increased *pfmdr1* copy number is common in Asia, but not Africa. As is the case with resistance-mediating SNPs, copy number variation is likely to impact upon parasite fitness. This factor was studied by selecting in vitro for resistance to mefloquine in a Thai strain of *P. falciparum* (Preechapornkul *et al.*, 2009). Decreasing susceptibility to mefloquine was associated with increasing copy number, but *pfmdr1* SNPs were not selected. Mefloquine-resistant clones with average copy numbers of 2.3 (~ 5-fold less sensitive to mefloquine than the parental strain) and 3.1 (~6-fold less sensitive) were co-cultured with the parent strain (1 copy number), and the selected strains showed a fitness disadvantage, with overgrowth of single copy number strains over 3–4 weeks. Modeling predicted a loss of fitness, compared to the parental strain, of 6.3% and 8.7% for parasites with 2.3 and 3.1 copies of *pfmdr1*, respectively. Modeling further suggested that under drug pressure *pfmdr1* amplification is a common event, with increases from one to two copies occurring once in every 10^8 parasites and from two to three copies once in every 5000 parasites. Thus, the selective pressure for increased *pfmdr1* copy number is great, but gene amplification is apparently kept in check by the fitness disadvantage of increased copy number.

Considering impacts of *pfmdr1* polymorphisms on the fitness of clinical isolates, *P. falciparum* was cultured from children with malaria in a region of Uganda with high multiplicity of infection (and thus high likelihood of mixed infections), and changes in the prevalence of the *pfmdr1* N86Y and D1246Y alleles were followed over time (Ochong, *et. al.*, 2013). Most cultures did not undergo changes in culture, but for those that did show selection 8/11 selected toward mutant 86Y, 9/14 selected toward wild type D1246, and 5/7 with selection at both alleles selected toward 86Y and D1246. Surprisingly, the results suggest a mixed picture, with fitness advantages for parasites with *pfmdr1* mutant 86Y and wild type D1246 alleles.

The *gch1* gene encodes GTP cyclohydrolase, which acts upstream of DHPS and DHFR in the folate synthesis pathway. This gene had increased copy number in Asian *P. falciparum* with folate resistance mutations, suggesting a compensatory mechanism to increase fitness in these drug-resistant parasites (Nair *et al.*, 2008). Genetic manipulation of *P. falciparum* to increase *gch1* copy number led to modest decreases in pyrimethamine sensitivity in most tested parasite lines (Heinberg *et al.*, 2013). However, results were complex, with varied effects in different strains, and with evidence for detrimental effects of marked overexpression of GTP cyclohydrolase. These results offer another example of how selection of parasite alterations by drug pressure is balanced by effects on parasite fitness.

Fitness consequences of antimalarial drug resistance: animal model studies—Studies using murine malaria models have also attempted to assess the fitness costs of drug resistance. Growth of a pyrimethamine-resistant clone of the rodent parasite *P. chabaudi* was compared with that of its drug-sensitive parent strain by co-infection of mice. The sensitive strain appeared to outgrow the resistant strain in two of three experiments (Rosario *et al.*, 1978). In the same studies, a chloroquine resistant strain of *P. chabaudi* outgrew a chloroquine sensitive strain in 4 of 4 experiments, in one case even when the inoculum was 90% sensitive, and only 10% resistant parasites. This experiment suggests a surprising fitness advantage for chloroquine-resistant parasites that is difficult to reconcile with other reports. In another study *P. chabaudi* selected for resistance to pyrimethamine were outcompeted by sensitive parasites, but after many passages the resistant strain gained a fitness advantage over both the parent strain and the orginial resistant strain (Walliker, et al, 2005). Thus, determinants of fitness are complex, and compensatory mutations gained over time may mediate fitness improved even over that of the parental drug-sensitive strain.

In another strategy to study fitness, *P. chabaudi* was passaged in mice to increase virulence (Mackinnon *et al.*, 2002). In this case the features described as virulence (increased parasite growth rate and mouse mortality) can also be seen to represent fitness. The parasites selected for virulence were consistently less susceptible than parent strains to treatment with pyrimethamine or artemisinin, and differences were not explained by differences in known resistance-mediating polymorphisms (Schneider *et al.*, 2008, Schneider *et al.*, 2012). Thus, independent of specific resistance mediators, parasites selected for increased fitness were more resistant to drug treatment. In another study *P. chabaudi* were selected by passages in mice for resistance to artesunate-mefloquine. The parasites selected for resistance grew as well as parent drug-sensitive parasites, in this case showing no apparent fitness cost of drug resistance (Rodrigues *et al.*, 2013).

To study impacts of resistance on fitness in mosquito stages of malaria parasites, mice infected with *P. berghei* were treated with pyrimethamine to select for resistance. Selected parasites were similar to sensitive parasites in the mouse, but they proceeded more slowly through mosquito development (Shinondo *et al.*, 1994). Thus, drug-resistant strains might be less capable than sensitive parasites to transmit malaria. In contrast, when mosquitoes were fed on mice infected with a mixture of parasites differing in virulence as described above, the more virulent parasites were more readily transmitted (Schneider et al., 2012). In other studies, when mice were infected with *P. berghei* encoding wild type or resistance-mediating *pfcrt* haplotypes, blood-stage parasites were equally sensitive to chloroquine, but mutant parasites had enhanced mosquito infectivity in the presence of chloroquine, suggesting that drug resistance enhanced transmission (Ecker, *et al.*, 2011).

Fitness consequences of antimalarial drug resistance: insights based on clinical trials—The most valuable insights available to date on the interplay between drug resistance and fitness in malaria parasites probably come from clinical observations. Of interest is the geography of selection of drug resistance. Interestingly, resistance to three

major classes of antimalarials, aminoquinolines, aryl amino-alcohols, and antifolates, has appeared to originate in Southeast Asia or South America, despite the fact that the large majority of episodes of falciparum malaria occurs in Africa (Ekland & Fidock, 2007, Mita & Tanabe, 2012). This pattern might be explained by many factors, but a compelling explanation is that resistance develops most easily in areas of low malaria transmission intensity. These areas will have relatively nonimmune human populations that are more likely than highly immune Africans to harbor resistant parasites with diminished fitness. They are also less likely to harbor polyclonal infections in which less fit resistant parasites may be outcompeted by sensitive strains. With time, as seen in animal studies, resistant parasites may develop compensatory mutations that increase fitness and enable spread even in high transmission areas. This scenario can explain the spread of resistance to chloroquine, antifolates, and other drugs from Asia to Africa.

A look at clinical chloroquine resistance provides our best example of the interplay between resistance and fitness. Chloroquine resistant *P. falciparum* was highly prevalent worldwide by the 1990s, but chloroquine use remained common, providing continued selection for the resistant phenotype. Then, in some well-studied areas, chloroquine was discontinued as the standard treatment for falciparum malaria. In Malawi, chloroquine use was eliminated and sulfadoxine-pyrimethamine made the national treatment regimen in 1993. Remarkably, dramatic shifts in parasite populations were seen. In samples collected from children with malaria between 1992 and 2000 the prevalence of the resistance-mediating *pfcrt* 76T mutation decreased steadily from 85% to 13% (with resistance to antifolates increasing concurrently) (Kublin *et al.*, 2003). Following these changes, 99% efficacy of chloroquine for the treatment of falciparum malaria was demonstrated (Laufer *et al.*, 2006). A similar, but less dramatic pattern was seen on Hainan Island, China, where chloroquine was discontinued as the standard treatment for malaria in 1979, and the prevalence of *pfcrt* 76T decreased from 90% in 1978–81 to 54% in 2001 (Wang *et al.*, 2005). The obvious explanation for dramatic changes after withdrawal of chloroquine is that discontinuation of drug pressure allowed reemergence of minority populations of chloroquine-sensitive parasites. Despite the obvious virulence of chloroquine-resistant *P. falciparum*, chloroquine sensitive parasites have a clear fitness advantage.

Another means of assessing fitness costs is to compare parasites infecting individuals at different times of the year in areas with highly seasonal malaria (Babiker *et al.*, 2013). During the dry season in these areas symptomatic malaria is uncommon and selective pressure from antimalarial drug use is low, but it is not uncommon for individuals to maintain low-level parasitemia. A number of studies have shown increased prevalence of drug-sensitive parasites during the dry season compared to that during the transmission season. In samples from Sudan, the prevalence of the *pfcrt* 76T mutation was described as greater in samples from the dry season (Abdel-Muhsin *et al.*, 2004), but these samples were collected just after the transmission season, so actually demonstrated selection of mutant parasites by recent drug pressure. This explanation was supported by a study from The Gambia in which the prevalence of the mutant alleles *pfcrt* 76T and *pfmdr1* 86Y decreased with increasing time since the transmission season (Ord *et al.*, 2007). A study from Indonesia showed a similar, albeit less dramatic pattern, with decreased prevalence of the *pfcrt* 76T, *pfmdr1* 86Y, *pfdhfr* 108N, and *pfdhfr* 59R mutations during the dry season (Asih *et al.*, 2009). Taken together, available studies suggest a consistent pattern. During the dry season, with decreased use of antimalarial drugs, fitness advantages lead to replacement of mutant by wild type parasites.

Asymptomatic plasmodial infections are common in high transmission areas in which human immunity is strong. Insight into fitness determinants may be gleaned by comparing parasites causing symptomatic and asymptomatic infections. A study in Kenya found no

difference in the prevalence of *pfcrt* 76T or antifolate mutations between parasites causing symptomatic and asymptomatic infection (Zhong *et al.*, 2008). In contrast, a recent cross-sectional study by our group in Uganda showed that children with asymptomatic infections had higher prevalence of the mutant *pfcrt* 76T, *pfmdr1* 86Y, and *pfmdr1* 1246Y alleles compared to those with symptomatic infection, suggesting greater virulence for wild type parasites (Tukwasibwe, S, unpublished observation). The impact of resistance-mediating polymorphisms on parasite densities may offer additional insight into parasite fitness. In Sudan, wild type parasites achieved modestly higher parasite densities than parasites with the *pfcrt* 76T polymorphism; impacts of other resistance-mediating SNPs on parasite density were varied (Osman *et al.*, 2007).

In a novel approach to assessing fitness determinants, the genotypes of parasites isolated from humans and from anopheline mosquitoes in Zambia were compared (Mharakurwa *et al.*, 2011). In this region in which sulfadoxine-pyrimethamine had been heavily used, the prevalence of mutations that mediate resistance to pyrimethamine (*pfdhfr* 108N, 51I, 59R) was much higher in parasites infecting humans than those infecting mosquitoes. Other polymorphisms associated with resistance to another antifolate, cycloguanil, but rare in human infections in Africa, were much more common in mosquito infections. Thus, human infections under the selective pressure of frequent drug use may differ from those in mosquitoes, in which drug pressure is absent and other selective pressures may be in play.

Considering impacts of resistance-mediating polymorphisms on transmission, mosquitoes were fed on blood from Gambian children with malaria, and the transmissibility of drug sensitive and resistant parasites was compared (Hallett *et al.*, 2004). Mosquito infection was much greater after feeding on blood containing gametocytes with the *pfcrt* 76T and *pfmdr1* 86Y mutations compared to blood with wild type gametocytes. In another study in the Gambia, compared to those with wild type infections, children with parasites containing *pfcrt* 76T had higher gametocyte densities, but those with *pfmdr1* 86Y lower gametocyte densities (Ord et al., 2007). In samples from Sudan, gametocyte production was greater in infections with the *pfcrt* 76T or *pfmdr1* 86Y polymorphisms (Osman et al., 2007). Thus, for these polymorphisms increased transmission may have in part circumvented fitness costs incurred in erythrocytic parasites, contributing to the spread of chloroquine resistance.

Conclusions and future perspectives

Studies utilizing cultured malaria parasites, animal models, and samples collected from infected individuals have generally shown that resistance-mediating polymorphisms lead to malaria parasites that are out-competed by wild type in culture and in animals and that, in human infections, are replaced by wild type when drug pressure diminishes. However, results have been complex. Fitness costs of resistance may be offset by compensatory mutations that increase parasite virulence, and some polymorphisms associated with decreased fitness in erythrocytic parasites may improve transmission to mosquitoes.

We are at a critical juncture in man’s battle with malaria. On one hand, we have a great opportunity for improved control leading to elimination, with improved tools to control mosquito vectors, a vaccine on the horizon, and highly effective ACT treatment regimens. These tools have led to important decreases in malaria in some areas in recent years. On the other hand, malaria remains an overwhelming problem in many areas, especially in Africa, and control measures are threatened by drug resistance, insecticide resistance, and parasite diversity and immune evasion mechanisms that challenge the development of a highly effective vaccine. In this context it is very important that we do not lose our best drugs, the ACTs, which have only recently reached widespread use around the world, and are likely the main contributor to recent advances in malaria control. But, all ACTs are already in serious jeopardy, with early signs of selection of resistance to artemisinins in southeast Asia

and resistance concerns for all partner drugs. Further, we do not yet have any solid replacements for ACTs for the treatment of drug resistant falciparum malaria. To maintain long effective lifespans for the ACTs we need to know how best to use them and other drugs for the treatment and prevention of malaria. More work is needed, building on recent in vitro, in vivo, and clinical studies, to understand the specific effects of different resistance-mediating polymorphisms on the ability of malaria parasites to cause serious infections and to be transmitted.

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Table 1

Antimalarial drugs: mechanisms of action and resistance

Drug class	Drug	Mechanism of action	Drug role	Resistance described?	Resistance features	Resistance mechanism
4-aminoquinoline	Chloroquine	Inhibition hemozoin formation → toxicity from free heme	Tx and CPx, but limited by R	Yes	High level R → tx failures	SNPs in *pfcrt* (76T primary mediator) and *pfmdr1*
	Amodiaquine		Tx falciparum malaria in combination with AS	Yes	High level R → tx failures; Efficacy good combined with AS in Africa	SNPs in *pfcrt* and *pfmdr1*
	Piperaquine		Tx falciparum malaria in combination with DHA	Yes	R described in China in 1980s, but not recently	Unknown
8-aminoquinoline	Primaquine	Unknown	Elimination liver hypnozoites of *P. vivax* and *P. ovale*	Yes	Failure of radical cure of *P. vivax* and *P. ovale*	Unknown
Aryl-amino alcohol	Quinine	Unknown	Tx severe falciparum malaria	Yes	Mostly low-level R in SE Asia	Unknown
	Mefloquine	Unknown	Tx falciparum malaria in combination with AS; CPx	Yes	Failures of MQ and AS/MQ seen in SE Asia	↓ S with *pfmdr1* WT sequences and ↑ copy number
	Lumefantrine	Unknown	Tx falciparum malaria in combination with AM	No	Some variation in activity, but no definitive clinical R	↓ S with *pfmdr1* WT sequences and ↑ copy number
Antifolates	Pyrimethamine	Inhibition DHFR	Combined with sulfadoxine for Tx and IPT	Yes	R in many areas	Multiple SNPs in *pfdhfr* mediate step-wise ↑ in R
	Trimethoprim		Daily with sulfamethoxazole protects against malaria	Yes		
	Proguanil		Combined with ATV (Malarone) for Tx and CPx	Yes	R in many areas; Malarone active even with proguanil R	
	Sulfonamides	Inhibition DHPS	Used in combination with DHFR inhibitors	Yes	R in many areas; SP retains good efficacy in W. Africa	Multiple SNPs in *pfdhps* mediate step-wise ↑ R
Naphthoquinone	Atovaquone	Inhibition cytochrome bc_1 complex	CPx and Tx, in both cases in combination with proguanil	Yes	R selected rapidly with monotherapy	SNPs in *pfcytb*
Antibiotic	Doxycycline, clindamycin	Inhibition apicoplast protein synthesis	CPx; Tx in combination with quinine	No	Some variation in activity, but no definitive clinical R	Polymorphisms in homologs of bacterial R mediators associated with ↓ S
Artemisinin	Artesunate, artemether, dihydroartemisinin	Uncertain	Cornerstone of ACT regimens for Tx falciparum malaria;	Yes	Delayed parasite clearance in clinical trials	↓ S with *pfmdr1* WT sequences and ↑ copy

Drug class	Drug	Mechanism of action	Drug role	Resistance described?	Resistance features	Resistance mechanism
			Intravenous AS gold standard for Tx severe malaria			number; Mechanism delayed clearance unknown

Abbreviations: ACT, artemisinin-based combination therapy; AM, artemether; AS, artesunate; ATV, atovaquone; CPx, chemoprophylaxis; DHA, dihydroartemisinin; IPT; intermittent preventive therapy; R, resistance; S, sensitivity; SNP, single nucleotide polymorphism; SP, sulfadoxine-pyrimethamine; Tx, treatment