Antimalarial drugs: modes of action and mechanisms of parasite resistance

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Malaria represents one of the most serious threats to human health worldwide, and preventing and curing this parasitic disease still depends predominantly on the administration of a small number of drugs whose efficacy is continually threatened and eroded by the emergence of drug-resistant parasite populations. This has an enormous impact on the mortality and morbidity resulting from malaria infection, especially in sub-Saharan Africa, where the lethal human parasite species *Plasmodium falciparum* accounts for approximately 90% of deaths recorded globally. Successful treatment of uncomplicated malaria is now highly dependent on artemisinin-based combination therapies. However, the first cases of artemisinin-resistant field isolates have been reported recently and potential replacement antimalarials are only in the developmental stages. Here, we summarize recent progress in tackling the problem of parasite resistance and discuss the underlying molecular mechanisms that confer resistance to current antimalarial agents as far as they are known, understanding of which should assist in the rational development of new drugs and the more effective deployment of older ones.

Despite being a disease that is both preventable and curable, malaria still has an enormous impact on human health throughout the tropical and subtropical ranges of the many *Anopheles* mosquito species that transmit the pathogens responsible. Malaria is caused by protozoan parasites of the genus *Plasmodium*, five species of which are now known to infect humans, *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*. *P. ovale* was recently proposed on persuasive molecular evidence to comprise two subspecies [1] and *P. knowlesi* is a parasite of primates able to infect humans via zoonosis [2,3]. Of these five, *P. vivax* and *P. falciparum* are numerically by far the most important, with the latter conservatively estimated to account for the deaths of some 1 million people per year and morbidity affecting approximately 250 million [4,201]. These statistics have profound implications for social and healthcare systems as well as economic capacity.

Malaria parasites exhibit complex lifecycles in both of their hosts, with clinical symptoms arising from cycles of erythrocytic invasion, growth and division, followed by cell lysis and reinvasion. *P. falciparum* is especially dangerous as this species is able to remodel the erythrocyte to enable infected cells to adhere to endothelial surfaces, thus progressively blocking microcapillaries in the major organs, including the brain. In this way, the parasite reduces passage of infected erythrocytes through the spleen, which is able to detect and destroy such compromised cells. *P. falciparum* is the principal parasite in sub-Saharan Africa and accounts for approximately 90% of all deaths worldwide from malaria [5]. Most fatal cases affect children aged under 5 years, where levels of acquired immunity to the parasite are often insufficient to allow survival beyond infancy.

Intense efforts have been underway for decades to produce vaccines that can enhance levels of protective immunity and at long last, encouraging results are emerging from large-scale field trials of the hitherto most promising vaccine formulation, known as RTS,S [6,7], which it is hoped will reach the stage of licensing by 2012. Protection from mosquito bites has also been stepped up in recent years in the form of a more widespread and systematic distribution of insecticide-impregnated bednets [201]. However, chemoprophylaxis and chemotherapy still play the central role in combating malaria infections [8] and will continue to do so for the foreseeable future. This role is, however, compromised by two major factors: the quite modest number of antimalarial types currently licensed for use or proceeding to large-scale clinical trials [9] and the inexorable emergence and spread of parasite strains that are resistant to the drugs that have

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been deployed. Such resistance can arise rapidly from a combination of circumstances that, in the host population, include overuse of drugs for prophylaxis and incomplete therapeutic treatments of infected patients, either by lack of compliance or the unwitting use of counterfeit drugs deficient in the stated dose, and in the parasite population, include genetic and metabolic flexibility together with prodigious rates of reproduction and dispersal of resistance genes via sexual recombination in the mosquito. The molecular methodologies developed in recent years, together with enormously accelerated genome sequencing capabilities, are facilitating rapid increases in our analyses and understanding of the genetic and biochemical phenomena underlying drug resistance [10–13], but each of the antimalarial drug types has a different history, a different impact when compromised and different molecular mechanisms underlying parasite resistance to them, although, as will be seen, there are certainly areas of commonality. We set out here to give an overview and update on what is understood regarding such mechanisms and how parasite resistance relates to the situation in the field. We concentrate on the most dangerous of the human parasites, *P. falciparum*, the only species for which extensive data relating to drug resistance exist, both at the molecular and epidemiological levels.

Quinolines & related compounds: blocking parasite detoxification of heme

Quinine (QN), extracted from the bark of the *Cinchona* tree, takes its name from the Inca word for this substance (quina-quina; bark of barks). Its structure is built upon the quinoline ring system, as are those of the major synthetic derivatives chloroquine (CQ), amodiaquine (ADQ), primaquine, piperaquine (PIP) and mefloquine (MQ). Two other important antimalarials, more loosely related to QN structurally, are halofantrine (HF) and lumefantrine (LMF; benflumetol), based on phenanthrene and fluorene ring systems, respectively (see Table 1 for a full list of abbreviations). All of these compounds, with the exception of primaquine (used primarily to combat *P. vivax* infections), are thought to share a common target: the formation of the parasite-specific substance hemozoin, described below, although additional targets may also be involved in some cases [14]. It is appropriate to begin with the 4-aminoquinoline CQ, which became clinically available in 1947 as a less toxic and more readily produced substitute for QN, and is one of the longest-serving of the synthetic antimalarials that appeared over the course of the last century. CQ is also rightly considered to be one of the most successful drugs ever produced, being a safe and cheap compound that has saved countless millions of lives. However, its use against *P. falciparum* is now confined to limited areas of North Africa, Central America and the Caribbean region, where it retains its efficacy. Resistance to CQ was first reported from the Thai–Cambodian border regions in 1957 and on the Colombian–Venezuelan border shortly afterwards, and over the late 1970s and 1980s has spread to all of sub-Saharan Africa with profound consequences [15,16].

Chloroquine acts by interfering with the sequestration of toxic heme (ferriprotoporphyrin IX) **moieties produced when hemo**globin is digested by the intra-erythrocytic parasite to obtain a major portion of its amino acid requirements. The heme, in the form of β-hematin dimers, is normally complexed by a process of biomineralization into hemozoin, which is deposited in a crystalline form in the lysosome-like acidic food (or digestive) vacuole (pH ≤5.5), into which the hemoglobin is initially imported by endocytosis [17,18]. CQ and related drugs bind to heme, preventing the detoxification process of dimerization and crystallization, and producing complexes that are detrimental to both membranes and enzymes [19]. These are ultimately lethal to the parasite [14], although the precise molecular composition of the complexes depends both on the drug in question and the physiological conditions prevailing within the food vacuole [20]. The accepted view over several decades has been that the much greater accumulation of CQ in the vacuole in sensitive parasites compared with resistant strains can adequately explain CQ resistance [21], although this has recently been challenged as an oversimplification [22]. The arduous search for the genetic basis of CQ resistance and the discovery of the key gene, *pfcrt* (for 'chloroquine resistance transporter' [CRT]) in 2000 [23], has been well documented (e.g., [16,24]). This gene, located on chromosome 7, is highly polymorphic with at least 20 variable codon positions reported to date, generating over 20 known unique protein sequences [25]. Certain underlying nucleotide substitutions show tight linkage to the CQ-resistance phenotype in most laboratory-adapted and field strains of *P. falciparum* [23] and the key amino-acid change required to allow resistance to emerge has been identified as K76T. Its importance was definitively confirmed by transfecting the relevant region of mutant

Table 1. *Plasmodium falciparum* proteins with a proven or likely role in resistance to clinical antimalarial drugs.

Protein	Function	Location	Principal drugs affected†	Comments	Polymorphisms
ATP6 (SERCA), putative	Membrane-bound Ca^{2+}-transporting ATPase	Endoplasmic reticulum	ART	Putative determinant	L263E (not yet seen in field isolates)
CRT	Transporter	Membrane of food vacuole	CQ, ADQ	Major determinant	C72S, M74I, N75D/E, K76T‡, A220S/H, Q271E/V, N326S/D, I356T/L, I371R
			MQ, HF, LMF, ART, QN, PIP	Minor determinant	
CYT b	Subunit of complex III (cytochrome bc_1 complex) electron transport chain	Mitochondrion	ATV	Administered as synergistic combination of ATV and PG	Y268S/N/C
DHFR	Folate pathway enzyme	Cytoplasm (principally)	PYR, PG, chlorproguanil	DHPS and DHFR targeted simultaneously in synergistic combinations of antifolates	N51I, C59R, S108N‡, I164L or C50R, N51I, S108N‡, I164L for PYR; A16V, S108T‡ or N51I, C59R, S108N‡, I164L for PG
DHPS	Folate pathway enzyme	Cytoplasm (principally)	SDX, dapsone	DHPS and DHFR targeted simultaneously in synergistic combinations of antifolates	S436A/F, A437G‡, K540E, A581G, A613S/T
MDR1 or PGH1	Transporter	Membrane of food vacuole	MQ, HF, LMF, QN	Major determinant	N86Y‡, Y184F, S1034C, N1042D, D1246Y; also copy number important
			CQ, ADQ, ART	Minor determinant	
MRP1	Transporter	Membrane-bound vesicles on parasite surface membrane	CQ, QN	Possibly also LMF, ART and antifolates	Possibly H191Y, S436A, I876V, R1466K
NHE1	Transporter	Parasite surface membrane/membrane of food vacuole (putatively)	QN	Limited corroboration in field studies to date	Copy numbers of repeat motifs DNNND and NHNDNHNNDDD affect susceptibility levels
TCTP, putative	Ortholog of human histamine-releasing factor	Not yet established	ART	2.5-fold overexpression of TCTP in resistant rodent parasites; possible influence in host histamine synthesis	Not yet known

†*Only drugs discussed in the review are listed here.*
‡*Key polymorphisms.*
ADQ: Amodiaquine; ART: Artemisinin; ATV: Atovaquone; CQ: Chloroquine; CRT: Chloroquine resistance transporter; CYT: Cytochrome; DHFR: Dihydrofolate reductase; DHPS: Dihydropteroate synthetase; HF: Halofantrine; LMF: Lumefantrine; MDR: Multidrug resistance; MQ: Mefloquine; MRP: Multidrug resistance-associated protein; NHE: Na^+/H^+ exchanger; PG: Proguanil; PGH: P-glycoprotein homolog; PIP: Piperaquine; PQ: Primaquine; PYR: Pyrimethamine; QN: Quinine; SDX: Sulfadoxine; SERCA: Sarco/endoplasmic reticulum calcium-dependent ATPase ortholog; TCTP: Translationally controlled tumor protein ortholog.

pfcrt genes into different sensitive parasite lines and observing the expected change in phenotype [26]. The K76T mutation is invariably found in association with a minimum of three other residue changes, some of which at least are thought likely to compensate for unfavorable effects of K76T on the normal function of the CRT transporter [25,27], which belongs to a recently identified subgroup of proteins belonging to a superfamily of drug and metabolite transporters (DMT superfamily) [28,29]. Interestingly, there is no correlation between mutations in the *P. vivax* ortholog of CRT and observed CQ resistance [30]. Similarly, in rodent parasite systems, *P. chabaudi* CRT was found to be unaltered in a series of CQ-resistant strains [31] and *P. berghei* CRT also appears not to be involved in this phenotype [32]. These studies serve as a more general caveat that for a particular drug, there may be more than one independent mechanism by which parasite resistance can develop.

Since the importance of *P. falciparum* CRT (PfCRT) was elucidated, numerous field studies in different regions of the world (e.g. [33–36])

have found that nearly all CQ-resistant strains examined carry the K76T alteration, confirming its key role in determining clinical outcome. Moreover, resistant parasites manipulated to reverse the K76T mutation revert to a sensitive phenotype [37]. The most recent transfection studies have, however, shown conclusively that the observed range of CQ resistance responses is strongly dependent upon the precise genetic background of the parasite lines in question [38] and that in certain backgrounds, K76T does not necessarily lead to clinical failure, but still increases tolerance to CQ, such that recrudescences are likely to occur. Various models have emerged as to how the K76T change might mediate drug resistance, particularly in relation to CQ flux [39]. One of the more widely cited is the 'charged drug leak' hypothesis, in which the positive charge of K76 in wild-type strains greatly limits efflux of CQ, a weak diprotic base of which almost 100% will carry two positive charges in the acidic conditions of the vacuole. If the charge on lysine is replaced by an uncharged side-chain, as in K76T, then CQ^{2+} can exit down its concentration gradient via the CRT. However, mutations elsewhere in the protein can restore a positive charge to the pore, thus compensating for K76T and reversing the resistance phenotype, as in an apparently unusual sensitive strain from South-East Asia, where an S163R alteration (in the fourth transmembrane domain) is found along with K76T [40,41]. Similarly, a number of compounds, such as verapamil, that are able to reverse CQ resistance often exhibit both lipophilic and positively charged regions that would allow them to effectively compete with CQ for binding to PfCRT, and hence retard the exit of the drug from the food vacuole [27,42,43].

This qualitative model, while providing a simple explanation for how CQ can escape the vacuole of resistant parasites, does not account for several quantitative measurements, including the very high levels of drug accumulation (in mM concentrations) seen in the wild-type vacuole. It is now realized that other factors need to be considered in more refined models, including the degree and nature of the binding of CQ to heme inside the food vacuole, the proton gradient prevailing across the vacuolar membrane, the differing energy dependence of efflux from sensitive and resistant lines and the kinetics of influx and efflux [41,44,45]. The issue of proton gradients, the precise vacuolar pH values in CQ-sensitive and CQ-resistant parasite lines, and how these quantitatively affect the level of drug accumulation in the vacuole have been a subject of some contention, stemming at least in part from the challenging technical problems of measuring pH changes accurately within this organelle [46,47]. However, consistent with the basic model above, direct measurements of proton loss from the vacuole compared in transfected parasites that differ only in their *crt* alleles show that a CQ-associated proton leak can only be detected in the resistant forms [48]. Moreover, an important proof necessary to support any model in which PfCRT is proposed as a mediator of drug efflux is to show directly that the protein can specifically bind drug [49], and further that the K76T mutant form of PfCRT is indeed the agent through which diprotonated CQ is transported. Such experiments are extremely difficult in the parasite itself, so for the latter, advantage was taken of the versatile *Xenopus* oocyte system, permitting measurements of drug transport across the oocyte plasma membrane before and after heterologous expression of PfCRT at its surface [21]. Wild-type PfCRT was unable to transport CQ^{2+}, in contrast to the mutant type from resistant parasites. Interestingly, wild-type PfCRT modified solely at the K76T position was also impermeable to the charged drug, demonstrating that one or more of the additional mutations found in all field isolates are also critical for this function, i.e., K76T is a necessary but not sufficient alteration. Conversely, introduction of T76K (or S163R) into a normally resistant form (i.e., reintroducing a positive charge into either transmembrane domain 1 or 4) once again prevented transport, consistent with earlier studies implicating domains 1, 4 and 9 as major components in the recognition of different quinoline drugs and their translocation [50]. Indeed, PfCRT appears to play a significant role in the resistance phenotype against drugs other than CQ, as described below, but other transporters must also be considered to provide a more complete picture.

Other transporters involved in resistance to quinoline drugs

Other genes have been identified that encode transporter proteins putatively or definitively located on the food vacuole membrane or the plasma membrane, such as the *P. falciparum* multidrug resistance (PfMDR)1 protein, also known as P-glycoprotein homolog (PfPGH)1, Na^+/H^+ exchanger (PfNHE)1 and multidrug resistance-associated protein (PfMRP)1 (Table 1), and these too have been implicated in drug resistance mechanisms [43,51–54]. Of these,

the ATP-binding cassette-type transporter, PfMDR1, encoded on chromosome 5, has been investigated the most intensively. This is thought to be involved in importing solutes into the food vacuole [55] and is also capable of ATP-dependent export of hydrophobic compounds from the vacuole, including drugs such as MQ, HF and the unrelated artemisinin (ART) derivatives (see below) [19,42]. Direct evidence for this has also been obtained by expression of wild-type and mutant forms of PfMDR1 in the *Xenopus* oocyte system, where, for example, the single amino acid substitution of N86Y resulted in the loss of ability to transport QN and CQ but acquisition of a HF-transporting capability, while a N1042D change was also associated with loss of QN transport [56]. Further investigation of these effects will be aided by the recent development of a heterologous expression system for PfMDR1 in the yeast *Pichia pastoris* coupled with a photolabeled CQ probe to monitor interactions of the quinolines with this protein [57].

Depending upon the precise genetic background of the strain in question, mutations in the *pfmdr1* gene can also modulate levels of CQ resistance in parasites, as would be predicted from the study above [56], but only if *pfcrt* is already mutated to a resistant form [58,59]. Patterns are complex however; for instance, in resistant South-American strains, the relevant *pfcrt* haplotype is commonly associated with mutations in *pfmdr1* that yield S1034C, N1042D and D1246Y, whereas in Africa and Asia, a different *pfcrt*-resistant haplotype is often found with the N86Y change in PfMDR1 [60]. The varying combinations of *pfcrt* and *pfmdr1* haplotypes found in different continental locations have been ascribed to different histories of drug usage [61], but even within a single country (Thailand) eight different combinations of the two genes were identified in a relatively small study [36]. Moreover, even in strains with identical *pfcrt* and *pfmdr1* haplotypes, there is still significant variation in measured levels of CQ resistance, indicating that additional genes can make a contribution to the precise phenotype [62]. A further complication is that mutations in *pfcrt* alone have also been shown to correlate with changes in the expression levels of at least 45 other genes with a range of functions, relative to wild-type [63]. At the metabolic level, among the differences recorded between CQ-sensitive and CQ-resistant parasites is an alteration of glutathione (GSH) metabolism, and experimental depletion of GSH has been shown to increase susceptibility to the drug. This has been rationalized by the fact that GSH is important in degrading heme that has escaped sequestration into hemozoin and is free to generate damaging reactive oxygen species (ROS) via redox reactions involving the iron moiety. Up-regulation of GSH levels could therefore also contribute to resistance levels by counteracting the increase of unsequestered heme resulting from exposure to CQ [64]. It is thus becoming clear that the spectrum of observed resistance phenotypes arises from a complex interplay of various molecular mechanisms, despite the predominant role of the *pfcrt* status in CQ resistance.

Although point mutations in *pfmdr1* in the codons above affect susceptibility to a range of drugs targeting the food vacuole [51,58,59], this is not the only mechanism by which *pfmdr1* can contribute to drug sensitivity in the field. Amplification of the *pfmdr1* locus to copy numbers of 2–5, common in South-East Asian samples, is another important contributor to resistance against MF, HF, LMF and QN in particular (but not CQ) [65–67], albeit with a probable cost to parasite fitness [68]. These studies highlight the important role that expression levels of the transporter in question can play, further evidence for which also comes from *in vitro* studies of PfCRT and CQ resistance [69].

Many strains of parasite display both CQ and QN resistance, and mutations in PfCRT are able to enhance QN efflux from the food vacuole [70]. However, such cross-resistance is by no means universal and although quantitative trait locus mapping experiments on QN-resistant strains have confirmed that both *pfcrt* and *pfmdr1* are involved, they have also focused attention on another membrane protein whose gene locates to chromosome 13, a putative Na^+/H^+ exchanger, PfNHE1 [54]. Reduced expression of this protein in transfectants led to greater sensitivity to QN (but not to MQ and LMF) in two different genetic backgrounds in this study, but not in a third [54], indicative of variable interactions among these three gene loci. In other studies, elevated PfNHE1 activity [71] and specific patterns of microsatellite repeats at locus ms4760, encoding single or multiple copies of DNNND and NHNDNHNNDDD motifs in the cytoplasmic domain of the protein [52,53], were associated with increased QN resistance. However, thus far correlations in the field between these polymorphisms in the *pfnhe1* gene and QN resistance are variable. In a survey of parasites from various African countries and Madagascar, the association was found to be weak [72], more

convincing in samples from one area of South-Eastern Kenya [73] and strongest in samples from the China–Myanmar border [74], reflecting again the importance in QN resistance of the interlinked factors of genetic background, variable contributions from other genes and regional histories of drug usage.

PfMRP1, encoded on chromosome 1, is another active transporter implicated in drug resistance [43] that is found at the plasma membrane of the parasite rather than that of the food vacuole [75]. Gene knockout studies show that, although its absence is not lethal to asexual parasites, they nevertheless accumulate greater amounts of CQ and QN and become significantly more sensitive to these and other drugs, which are thought to be pumped to varying degrees by PfMRP1 to the outside of the parasite [76]. Moreover, in clinical trials, selection of an I876V polymorphism in PfMRP1 was found in recurrent infections after artemether–LMF treatment, ascribed from modeling studies to variation in residue 876 affecting the ATP hydrolysis cycle of this transporter [77].

Although cross-resistance among different members of the quinolines might be expected and is often observed, for example, between CQ and QN or CQ and ADQ, the inverse phenomenon also occurs, with important implications for drug deployment policies. Thus, in the analysis of strains transfected with one of the mutant forms of *pfcrt* referred to earlier [38], introduction of this allele, while increasing levels of CQ resistance, brought about a concomitant increase in susceptibility to LMF, PIP and ART. This is consistent with complementary observations in Tanzania, where treatment with the ART–LMF combination selected for the wild-type *pfcrt* K76 allele and to a lesser extent, the wild-type *pfmdr1* N86 allele in recrudescent infections [78]. The extent to which passage of any given quinoline or related drug is enhanced or retarded by mutations in the transporters mentioned is thus clearly a net result of factors such as precise conformation, degree of hydrophobicity and charge status. Moreover, it can be safely assumed that other, as yet poorly characterized transporters are likely to play some kind of additional role in parasite resistance to at least some of the drugs in current use [75,79,80]. A more detailed analysis of parasite resistance to the quinoline-type drugs can be found in [20].

Antifolates: exploiting antimetabolites

The principal antifolate drugs used against malaria are pyrimethamine (PYR), proguanil (PG; metabolized *in vivo* to the active form cycloguanil [CG]) and the sulfa drugs, the most important of which are the sulfonamide, sulfadoxine (SDX), and the sulfone, dapsone. Following detailed studies of bacterial systems from the 1940s onwards, it was established that PYR and CG target the dihydrofolate reductase (DHFR) activity of the parasite's bifunctional DHFR-thymidylate synthetase (TS) protein, whereas the sulfa drugs affect the dihydropteroate synthetase (DHPS) activity of the bifunctional hydroxymethylpterin pyrophosphokinase (HPPK)-DHPS protein, all of these drugs acting as competitive inhibitors of the natural substrates. DHPS is found only in the parasite, participating in the *de novo* synthesis of essential folate coenzymes. DHFR, however, is present in both host and parasite, as it is required in essentially all organisms to maintain a constant supply of fully reduced (tetrahydro) forms of folate for essential one-carbon transfer reactions, including the provision of nucleotides for DNA synthesis, whether the folate is made endogenously or salvaged from the diet/environment. The safety and efficacy of the drugs that target this enzyme therefore depend on a several hundred-fold differential in their binding to the parasite and human orthologs. Resistance appeared rapidly when these drugs were initially deployed as monotherapy, but synergistic combinations with sulfa drugs, such as the highly successful SDX-PYR combination (SP; Fansidar™), first introduced in the late 1960s, have proved to be of long-term utility, especially as a cheap alternative to combat the CQ-resistant parasites that spread across Africa from the late 1970s onwards [81]. As artemisinin-based combination therapy (ACT; see below) has taken over in recent years as the first-line drug regimen of choice in many areas of the world [82], the importance of the antimalarial antifolates as the standby for treating CQ-resistant infections has diminished. However, this class of drugs still plays a key role in intermittent preventative treatment (IPT) in areas of high transmission, where curative doses of SP are administered at regular time points to the most vulnerable, high-risk groups (pregnant women and infants) regardless of their infection status, amid safety concerns regarding administering ACT to these particular groups [83]. SP is also still widely used therapeutically when supplies of ACT fail, are considered too expensive, or are otherwise unavailable [84].

The cloning and analysis of the *dhfr-ts* and *hppk-dhps* genes from many strains of *P. falciparum* begun some 20 years ago has established the basic repertoire of point mutations that

compromise the antifolates. Serious resistance to PYR predominantly arises from the accumulation of mutations in the *dhfr* domain of *pfdhfr-ts* that bring about the amino-acid alterations N51I, C59R and S108N, while a combination of A16V and S108T results in CG resistance. Similarly, resistance to SDX most commonly involves the changes A437G, K540E and A581G in the DHPS enzyme [85,86]. The most frequent resistant combination in DHPS currently prevalent in Africa is the double-mutant form A437G, K540E [87,88], which, when found together with the triple-mutant form of DHFR, yields the so-called quintuple mutant, a pattern resulting in parasites that are highly resistant to SP [89] and a strong predictor of clinical failure. This pattern is becoming increasingly common, with frequencies of 70% or higher now reported in some areas of East Africa [90,91].

In South-East Asia and South America, an additional DHFR mutation, I164L, has for many years often been found in combination with the above changes [92,93] resulting in parasites that are highly resistant to SP [94]. Inevitably perhaps, these quadruple-DHFR mutant types have very recently been detected in regions of East Africa, including for example Kenya [95,96] and Rwanda [97], although the need for rigorous protocols to eliminate false-positives has been clearly illustrated [98]. However, even if such observations are confirmed and expanded, it does appear that I164L is much less rapidly selected in Africa than it has been in South-East Asia and South America. A possible reason for this is that in the quadruple mutant, there is thought to be a highly unfavorable interaction between L164 and I51 that compromises the DHFR activity and hence the fitness of this haplotype relative to wild-type or to lesser mutated strains in the absence of drug pressure [99,100], although other kinetic studies on recombinant DHFR-TS appear not to support this view [101]. Nevertheless, a strong correlation in Thai strains between this haplotype and a marked copy-number expansion (up to 11) of the first enzyme in the folate biosynthesis pathway, GTP cyclohydrolase I, is suggestive of an important and necessary compensatory mechanism [102,103]. As the *gtpc* gene resides on a different chromosome (12) to *dhfr-ts* (4), any linkage between the two loci will be more rapidly lost in areas of high transmission, as found in Africa, compared with areas of low transmission, as in South-East Asia, where recombination frequencies amongst strains with different genotypes are much lower [104]. Initial concerns regarding the potentially dire consequences of its arrival in Africa [94] may thus be tempered by this observation, but clearly vigilance must be maintained in tracking the spread of the DHFR quadruple mutant on this continent, as other compensatory changes may occur that facilitate its survival and propagation.

Although many thousands of samples from across the world have been typed to date for DHPS and DHFR since the mutations were first characterized, they have mainly been assayed in PCR and/or restriction enzyme-based tests targeted at the known repertoire established by the earliest sequencing studies [105–110]. However, as DNA sequencing has become enormously quicker and cheaper, ever more samples are being subjected to complete sequencing rather than such targeted assays, thus revealing novel mutations, such as the I431V change in DHPS seen in Nigeria [111] and the K540N change in DHPS that appeared on Car Nicobar island (India) after the 2004 tsunami [112]. Interestingly, the latter coincided with heavy use of the trimethoprim–sulfamethoxasole antifolate combination to combat other infections, as well as of SP for malaria. The significance of such changes with respect to quantitative resistance levels to the antimalarials will of course need to be established by the types of experiment used to causally link the original mutations to resistance, ideally by the types of transfection experiment described earlier.

A major milestone in understanding the role of the observed mutations in PYR resistance was the determination of the crystal structures of DHFR-TS from both PYR-sensitive and PYR-resistant parasites, in complexes with anti-DHFR drugs [113,114]. This has greatly accelerated the search for new compounds inhibitory to this activity [115] and increased the reliability and potential of computer-aided drug design approaches [116,117]. Similar analyses for the HPPK-DHPS enzyme still await successful crystallization, although homology modeling can provide some insight into the likely effect of the key resistance mutations on drug binding [118].

Whereas the amino-acid alterations summarized above are undoubtedly the key factors in parasite antifolate resistance, it has recently become more apparent that other factors may play a role in the levels of clinical failure after SP treatment. Apart from different degrees of host immunological responses to the parasite and variable metabolic processing of the administered drugs, common to all regimens, compounds such as PYR and SDX are folate or folate-component analogues and therefore it is

not surprising to find that higher serum folate concentrations, for example, as a result of dietary folate supplementation, are significantly associated with SP failure in both children [119] and pregnant women [120]. This would be consistent with molecular studies that have established the importance of folate salvage to the parasite, despite its proven ability to synthesize folate *de novo* [121,122]. Higher folate concentrations in the parasite may also be linked to the selection of a mutant form (R1466K) of the transporter PfMRP1 in recrudescent infections after treatment with SP [123]. It is proposed that this MRP1 haplotype is less active in acting as a folate efflux mediator and that the resulting raised endogenous folate pools in parasites of this type will more effectively compete with antifolate drugs at enzyme binding sites. Interestingly, PfMRP1 represents the first known protein that could putatively influence levels of resistance to both the quinolines and the antifolates, as well as to the ARTs [77], which are considered below.

Artemisinins: covalently modifying multiple targets

In 1979 qinghaosu, an extract from the plant qinghao (*Artemisia annua*; sweet wormwood), was reported for the first time to cure malarious patients in China by anonymous authors in the *Chinese Medical Journal*. In fact the antimalarial effect of this plant extract was already well known in traditional Chinese medicine [82,124] and is thus most probably the oldest chemotherapeutic agent against malaria. Although the mode of action of qinghaosu (later named ART) is not yet fully deciphered, this drug became increasingly relevant as treatment failures using CQ, SP and ADQ, and consequently morbidity and mortality, reached unacceptable levels. According to the WHO, current treatment of uncomplicated malaria depends almost exclusively on ACT [83]. However, after the extraction of ART, a sesquiterpene lactone endoperoxide, modification is required to improve its pharmacological properties, which makes production expensive [125]. Nowadays three ART derivatives are utilized in ACT, artemether, artesunate and dihydroartemisinin (DHA). The second drug of ACT is either: LMF in combination with artemether; MQ, ADQ or SP in combination with artesunate; or, PIP in combination with DHA.

Which ACT is applied where depends on the resistance prevalence and the partner drug should ideally have cure rates of at least 80% as monotherapy [83]. In addition, a new ACT consisting of pyronaridine, an acridine-type Mannich base, and artesunate successfully finished a Phase III clinical trial recently and will soon be available as a licensed antimalarial [126]. Although ADQ is quite closely related structurally to CQ, marked resistance to this drug has now been strongly linked to the 72-SVMNT-76 motif in PfCRT, whereas the predominant haplotype in CQ-resistant parasites across Africa is 72-CVIET-76, which has a much lower effect on ADQ susceptibility [127]. PIP is a bisquinoline that is highly effective against CQ-resistant strains [128] and parasite susceptibilities to this drug appear to be only moderately [38] or not at all affected [129] by the known mutations in *pfcrt*, nor those in *pfmdr1*, *pfmrp1* and *pfnhe1* [129]. However, doubts have arisen about the stability and quality of DHA in PIP–DHA [130], which may affect the future deployment of this combination.

Although ART derivatives are currently the only fully effective antimalarials [131] their mode of action is still under debate. Most studies agree that their activity is based on the endoperoxide bridge, which interacts with reduced iron in the heme moiety deriving mainly from hemoglobin digestion in the food vacuole [132]. Heme alkylation was observed *in vitro* and *in vivo* in *P. vinckei*-infected mice [133]. Further studies reported the alkylation of specific proteins, including transporters, iron-sulfur proteins and the translationally controlled tumor protein (TCTP) homolog [134,135]. By using synthetic peroxides in comparison with the inactive derivative deoxyartemisinin, where the endoperoxide bridge is lacking, the plasmodial mitochondrion came into focus as a target. The inhibitory impact is proposed to result from the observed depolarization of the mitochondrial membrane and a concomitant marked increase of ROS [136]. Interestingly and somewhat paradoxically, however, the plant *Artemisia annua* itself reacts by increasing production of ART or its precursor dihydroartemisinic acid when faced with elevated levels of ROS [137].

On the biochemical level the controversy surrounding the mode of action has been considered in detail by O'Neill *et al.* [138]. They come to the conclusion that it is not only the heme but also free intracellular reduced iron species that can lead to the bioactivation of ART, a prerequisite for the drug to become covalently bound to macromolecules throughout the whole parasite. Furthermore, the addition of holotransferrin as an exogenous iron source dramatically enhances the effect of DHA and thus corroborates their hypothesis of free iron being responsible for ART activation

[138]. On the cellular level ART was reported to interact not only with specific proteins but also with phospholipids, thereby causing oxidative membrane damage [139]. Sesquiterpene lactones are known potent anti-inflammation and anticancer drugs, except that here the downstream effects of their chemical properties are observed in the form of DNA damage, targeting of apoptotic signaling and influencing immune modulation [140,141]. An initially promising study revealed that ART inhibits PfATP6, the only sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA) ortholog in *P. falciparum*, which consequently impedes the crucial Ca^{2+} homeostasis of the parasite [142]. *In vitro* assays revealed a mutation at the amino acid position L263 in PfATP6 that inhibits the effect of ART [143]. However, if PfATP6 was the main target of ART, one would expect a resistant phenotype contingent on such a mutation in the *pfatp6* gene. To date though, no such alteration has been found in any ART-resistant plasmodial strains [144]. A recent publication comes to a similar conclusion by using transgenic *P. falciparum* strains carrying a point mutation in the *pfatp6* allele that gives rise to L263E. Although the IC_{50} values for ART, DHA and artesunate remained in the same range as for the wild-type, with differences lacking statistical significance, cluster analysis did reveal a trend in these modified parasites for a decreased responsiveness to ART and DHA, but not to artesunate [145].

It is interesting to note that over the centuries there has been no documented ART resistance in the treatment of malaria. Recently, however, reduced parasite susceptibility in artesunate and DHA-treated patients was observed on the Thai–Cambodian border [146,147]. There has been some doubt regarding whether the increased treatment failure was in fact associated with artesunate itself and speculation that it might rather be based on resistance to MQ, the partner drug of the ACT used in this region. However, another possibility, alluded to earlier, lies in the widespread use of fake antimalarials and self-medication of poor quality (e.g., ACT containing only traces of ART) which can kill sensitive parasites but at the same time encourage selection of resistant forms [148]. Indeed, there is now strong evidence to suggest that the reduced susceptibility is based on parasite resistance to ART rather than on host or pharmacokinetic factors [149]. However, recent detailed sequence comparisons of putative resistance genes from ART-resistant *P. falciparum* strains from western Cambodia and sensitive strains from northwestern Thailand did not show any linkage to the *pfcrt, pfmdr1* or *pfatp6* genes [150], leaving open the question as to the genetic basis of altered drug susceptibility. Historically, it is known that CQ-resistant parasites also evolved in this area and subsequently spread out rapidly to Africa [151]. It is therefore not too speculative to presume that a similar scenario might happen with ART. The idea of ACT is to prevent resistance towards ART or its partner drug, and, despite the disturbing results above emerging from the Thai–Cambodian border regions, this appears to have been the case in south west Vietnam, where *in vivo* and *in vitro* monitoring over the last decade of ACT treatment shows as yet no evidence of reduced parasite susceptibility [152]. However, administration of ACT is not always correctly followed in the field. In Burkina Faso, where the ACT is based on ADQ and artesunate as separate pills, patients tend not to take the ADQ, since this drug is associated with unpleasant side-effects, resulting in an ART monotherapy [153]. It is therefore only a question of time before the first ART treatment failures occur in Africa.

Beside the PfATP6 mutation engineered *in vitro* [143], Walker *et al.* reported that there is less incorporation of radiolabeled DHA in a resistant murine plasmodial strain *in vivo* and a 2.5-fold overexpression of TCTP in a rodent malaria model [154]. TCTP homology to the mammalian histamine-releasing factor might predict an involvement in host histamine synthesis [155]. However, selective *in vivo* pressure was also shown to be associated with MDR1 overexpression in *P. yoelii* [156]. In the *P. chabaudi* mouse model, cross-resistance was found between ART and artesunate but no alteration was found in either ATP6, TCTP or MDR1 [144]. As indicated above, it is likely though that one or more transporters will eventually emerge as significant contributors to ART resistance phenotypes. In conclusion, although the ART derivatives are currently the pillars of antimalarial treatment, their mode of action and the resistance mechanism(s) that would explain increasing treatment failures are still poorly understood.

Atovaquone: rational targeting of the mitochondrion

The antimalarial activity of atovaquone (ATV) was identified during a study specifically designed to exploit differences in the respiratory chain-linked

electron transport between *Plasmodium* and the human host, using menoctone, a ubiquinone antagonist, as lead compound [157]. As such it is one of the first antimalarials deriving from rational drug design. ATV was initially tested against murine infections with *Pneumocystis carinii*, *Toxoplasma gondii*, *Cryptosporidium parvum* and *Leishmania donovani* before it was introduced as a chemotherapeutic agent against malaria. Accordingly, it was described as a broad spectrum antiparasitic drug that collapses the mitochondrial membrane potential [158]. Today it is administered as chemoprophylaxis and standby treatment in combination with the antifolate PG under the brand name Malarone™ [83]. ATV is a substituted hydroxynaphthoquinone that acts as a structural analogue of coenzyme Q in the respiratory chain. The inhibition of electron transfer in the mitochondrion not only results in a loss of the membrane potential but also impedes pyrimidine biosynthesis, two essential processes whose inhibition leads to parasite death. The fact that the human respiratory chain is not a target is based on the structural differences between the human and plasmodial coenzyme [159]. The detailed mode of action of ATV is the inhibition of the movement of the iron-sulfur protein subunit of cytochrome b (CYT b) in complex III, which is necessary for the transfer of electrons to CYT c1 from ubihydroquinone bound to CYT b [160].

Targeting the mitochondrion is in fact a promising approach. Many antibiotics such as the tetracyclines, which target the 30S ribosome in the mitochondrion, are often applied effectively against malaria, especially against CQ-resistant parasites [161]. Nonetheless, targeting only one protein soon leads to the development of resistance; indeed sometimes only a single point mutation is enough to completely negate the drug's potency, as happened when ATV was used as monotherapy. The treatment failure of ATV, resulting in increased mortality, has been linked to the appearance of mutations in the *cyt b* gene of the parasite [162,163], following *in vitro* studies where resistance mutations had already been found in the ATV-binding domain of CYT b [164]. The mutations were also analyzed *in vitro* using the yeast bc1 complex as a model to study the molecular mode of ATV resistance [159]. In order to prevent further resistance emerging, combination therapy with PG was subsequently deployed. PG promotes a synergistic effect in combination with ATV [165], which is most probably not due to its role as an antifolate but rather by its exerting a direct impact on the mitochondrial membrane potential [166]. Nevertheless, even such combination therapy has not entirely prevented further treatment failure and resistance mutations affecting position Y268 have been found in field isolates [162,167,168]. The mutations that emerged *in vivo* under ATV pressure in rodent malaria are consistent with the Y268N/S alterations of CYT b described in such field isolates, which makes the murine malaria a good model to investigate the mode of ATV resistance [32,169]. However, a recent study on ATV-PG treatment failures of travelers from the UK identified a Y268C rather than a Y268N/S mutation, which often goes undetected by current restriction fragment length polymorphism methods used to monitor the *cyt b* gene [170].

To conclude, ATV-PG is an expensive antimalarial that is mainly used as chemoprophylaxis for western travelers. Within only a few years of application in the field ATV resistance was observed, manifested as point mutations in the *cyt b* gene. It may well be that, even as a chemoprophylactic, it will have a relatively limited lifetime. However, targeting of the mitochondrion remains an important strategy in the development of new antimalarials.

Conclusion & future perspective

Although drug resistance mechanisms in *P. falciparum* are turning out to be more complex than perhaps originally envisaged, a reasonably comprehensive picture of the main factors involved has now emerged for all of the major antimalarial drugs, with the notable exception of ART and its derivatives, summarized in Table 1. Knowledge of the relevant genes and the mutations therein has been a key aspect of the molecular analysis of drug resistance, and their utility in predicting the effectiveness of different drugs in different regions has been evaluated and validated by comprehensive meta-analyses of the numerous studies into associations between clinical outcome and molecular markers [171,172].

Compelling empirical evidence as well as theoretical considerations will dictate that monotherapy with any drug will be (officially) avoided in the future and combinations, such as those described above, will continue to be formulated in attempts to retard the onset of resistance as long as possible [173]. At one end of the spectrum, such formulations will simultaneously target different biochemical pathways, as in the case of artemether–LMF for example, although worryingly, a recent

study of artesunate–SP usage in Mozambique concluded that introduction of the artesunate component did not retard the spread of (albeit already common) SP-resistant parasites [91]. Interestingly, instead of coformulating the individual drugs, novel hybrid molecules have also been synthesized, such as an ART–QN derivative that has higher activity than either drug individually or a simple 1:1 mix thereof [174,175]. However, another approach is to combine drugs that target the same molecule, but which would select on their own mutually incompatible combinations of mutations [176]. This possibility has now been explored for both *P. vivax* and *P. falciparum* [177,178], where mutations that confer high-level resistance to PYR were found to render DHFR-TS more sensitive to the powerful (nonclinical) antifolate, WR99210, and *vice versa*.

As well as studying drug-resistance mechanisms as a means of developing novel drugs, another important consideration is whether such resistance can be reversed, either by direct chemical intervention, as exemplified by the use of compounds such as verapamil, alluded to earlier, or by cessation of usage for significant periods. For example, after the withdrawal of CQ in Malawi in 1993, prevalence of the resistant form of *pfcrt* decreased from approximately 85% in 1992 to an undetectable level in 2001 [179], although a similar decline in Kenya appears to be proceeding at a much slower rate [180]. Similarly, the wild-type allele of *pfdhfr-ts* increased in frequency following reduced usage of SP in an area of Tanzania [181]. However, parasites mutant in *dhfr* and *pfcrt* are still prevalent in parts of South-East Asia and South America [61,182], despite low usage of antifolates and CQ in these areas for prolonged periods, suggesting that in some contexts, the mutant parasites retain a small but significant selective advantage, possibly in the face of related antibacterial antifolates, such as trimethoprim [183]. There is also evidence from South America that antifolate-resistant forms of the parasite are transmitted more readily to mosquitoes via their gametocytes than the wild-type, further enhancing their spread [184].

Whatever the success of the various approaches described, it is a safe prediction that an ongoing supply of new drugs will be required to combat and control malaria for the foreseeable future. With the recent publication of the results of high-throughput chemical screening programs on up to 2 million compounds [185,186], there is now optimism that clinical inhibitors based on a number of chemical structures completely different from the current families of antimalarials will emerge in the medium term. Moreover, the biochemistry of the parasite is being unraveled in ever-increasing detail, and just as the unique biology of the parasite in the form of hemoglobin utilization has been so successfully exploited by the quinoline drugs, so other novel features, such as the indispensable parasite-specific organelle, the apicoplast [187,188], should provide fertile ground for new drug targets in this most pernicious of human pathogens.

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Executive summary

- Malaria remains one the most severe health problems across tropical and subtropical regions, but the inventory of antimalarial drugs currently licensed for clinical use is limited and heavily compromised by the development of parasite resistance.
- In the case of the quinoline, antifolate and naphthoquinone drugs, the characterization of genes underlying resistance and the polymorphisms found among sensitive and resistant strains has provided robust and detailed insights into the molecular mechanisms involved and how new generation drugs might be designed.
- Such polymorphisms also permit rapid and accurate diagnostic PCR-based assays for monitoring the incidence and spread of resistant parasites in field populations.
- Clinical resistance to the latest, highly efficacious family of antimalarials, the artemisinin derivatives, has not yet developed, but prolonged parasite clearance times are now being observed in parts of south-east Asia. The current lack of a clear understanding of how these drugs work and the likely mechanism(s) of resistance to them is of particular concern.
- The latest methods of whole genome analysis of multiple parasite strains driven by cheap, rapid sequencing technologies permit the discovery of new genes involved in resistance to a particular drug type and their relative contribution to observed phenotypes.
- The development of drugs targeting alternative areas of metabolism needs to be accelerated to avoid a possible hiatus when the artemisinins eventually lose potency. Recent high-throughput screens of very large chemical libraries should lead to new inhibitors based on chemical structures completely different from the current families.

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