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MOLECULAR MECHANISMS OF RESISTANCE IN ANTIMALARIAL CHEMOTHERAPY: The Unmet Challenge

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■ **Abstract** The enormous public health problem posed by malaria has been substantially worsened in recent years by the emergence and worldwide spread of drug-resistant parasites. The utility of two major therapies, chloroquine and the synergistic combination of pyrimethamine/sulfadoxine, is now seriously compromised. Although several genetic mechanisms have been described, the major source of drug resistance appears to be point mutations in protein target genes. Clinically significant resistance to these agents requires the accumulation of multiple mutations, which genetic studies of parasite populations suggest arise focally and sweep through the population. Efforts to circumvent resistance range from the use of combination therapy with existing agents to laboratory studies directed toward discovering novel targets and therapies.

The prevention and management of drug resistance are among the most important practical problems of tropical medicine and public health.

Leonard J. Bruce-Chwatt, 1972

INTRODUCTION

Malaria is one of the greatest of all infectious diseases, afflicting more than 500 million people and causing approximately 2 million deaths each year. Estimates of the economic burden of malaria in terms of lost productivity are staggering (1). Malaria is transmitted by mosquitoes and caused by intracellular protozoan parasites from the genus *Plasmodium*. By far the most significant species is *P. falciparum,* which causes severe infections and death, enjoys widespread geographic distribution, and is most likely to be drug resistant. In the years after World War II, public health workers had ambitious plans to eradicate malaria by various means, including DDT against mosquitoes and chloroquine against the parasite. These

efforts unfortunately failed; among the reasons for failure was the appearance and spread of chloroquine-resistant malaria, an event that is aptly considered a public health crisis. Malaria now features prominently among the "reemerging" infectious diseases (2).

Although much work is being done to develop malaria vaccines, estimates are that it will be many years before these are suitable for use in humans, and drugs are therefore required not only for treatment of established infections but also for prevention of malaria in healthy travelers, tens of millions of whom go to malarious countries every year (3). Malaria therapy is complicated by a number of factors, including the considerable requirement for safety (huge numbers afflicted, disproportionate severity in children and pregnant women, prophylaxis of healthy travelers), the fact that selective toxicity may be more difficult to attain against these eukaryotic pathogens, and by the inherent complexity of the parasite's lifecycle within the human host. Each lifecycle stage varies in its drug-sensitivity profile; hence, for a given patient multiple drugs may be needed to eradicate the infection. Infection begins with the bite of an infected Anopheline mosquito. Parasites first invade hepatocytes and replicate there before bursting the cell. The released forms then infect, replicate within, rupture, and reinfect red cells in a cycle that repeats every 2–3 days. This asexual replication leads to tremendous amplification, with parasite burdens that may reach 10^{12} organisms per patient. Drug-resistance genes that arise and are selected in this setting are further spread through the gene pool by the meiotic exchange that occurs during the sexual reproduction of *Plasmodium* within the mosquito.

The recently available genome for *P. falciparum* provides powerful information for understanding resistance mechanisms and opens exciting new avenues for drug development (4). *P. falciparum* contains 14 chromosomes and approximately 5300 protein-encoding genes, almost two-thirds of which seem to be unique to this organism. Newly recognized cellular pathways and organelles, such as the apicoplast (a chloroplast-like structure with unique metabolism), provide novel targets for the development of selectively toxic new therapies. Information on *P. falciparum* genes and their expression is available on the PlasmoDB Web site (http://www.plasmoDB.org).

In this review, we provide an overview of the problem of antimalarial drug resistance, consider potential solutions, and refer interested readers to the many excellent and detailed reviews that have appeared in recent years (5–12). Given its clinical and public health importance, and because it is by far the most likely to be drug resistant, the discussion focuses almost entirely on *P. falciparum*.

GENERAL ISSUES IN MALARIA PARASITE RESISTANCE

There are many definitions for drug resistance in malaria; indeed, classic textbooks have been written on this subject (13). Definitions range from the earliest, which were devised by the World Health Organization (WHO) to characterize clinical drug failures (14), to those based on altered drug potency against parasites in vitro,

and most recently to assays for known gene mutations. Each of these approaches has its merits, but for many reasons they may not be concordant. The assessment of antimalarial drug resistance, and the correlation of clinical and laboratory findings, is confounded by many variables. These include the obvious generic issues: distinguishing genuine resistance from suboptimal therapy, immunity and nutritional factors, and culturing parasites in conditions where key nutrients far exceed those in blood. There are also confounding variables more particular to malaria. In the field, resistant parasites may take weeks to recrudesce, at which point it becomes difficult to distinguish drug failure from reinfection. Furthermore, patients may harbor many clones of *P. falciparum*, each with a distinct set of mutations that impart resistance. Thus, if two mutations in a single gene are detected in a patient's blood sample, unless clonal parasites are isolated and assayed, it is difficult to know whether both mutations are in one cell line or whether two cell lines each have one mutation. Fluorogenic assays that distinguish between these possibilities may provide a solution to this problem (15).

A rich variety of genetic mechanisms are exploited for drug resistance in bacteria and tumor cells (16, 17). These range from discrete point mutations to the rearrangement of large blocks of DNA (e.g., inversion, duplication, insertion, deletion, transposition), and even to the acquisition of foreign DNA. Alterations in gene transcription, in the posttranscriptional control of RNA, and in the posttranslational modification of proteins, play important roles in drug resistance. By comparison, relatively few mechanisms are recognized in malaria and, as described below, the best understood of these are confined to point mutations and changes in steady-state transcript levels. Point mutations provide a satisfying and consistent explanation for many cases of antimalarial drug resistance. Almost certainly, however, there are mechanisms at work in these parasites that remain to be found. The availability of the fully sequenced genome and proteome that follows will be key in this discovery process.

DRUG-SPECIFIC RESISTANCE

4-Substituted Quinolines

The members of this largest class of antimalarial agents share obvious structural analogy, which reflects their derivation from the natural product quinine (Figure 1). As described below, they also have a common molecular mechanism of antimalarial activity. The preeminent agent in this class has been chloroquine, which in retrospect has aptly been termed "a wonder drug" (18). The focus of some intrigue during the years of World War II (19), this fully synthetic antimalarial is inexpensive, safe, and orally bioavailable. For decades, chloroquine provided reliable prophylaxis for travelers, therapy for those with established infection, and a powerful tool for public health workers in their efforts to control malaria. The emergence in the early 1960s and subsequent spread of chloroquine-resistant parasites created a tremendous therapeutic void, which has not yet been filled satisfactorily.

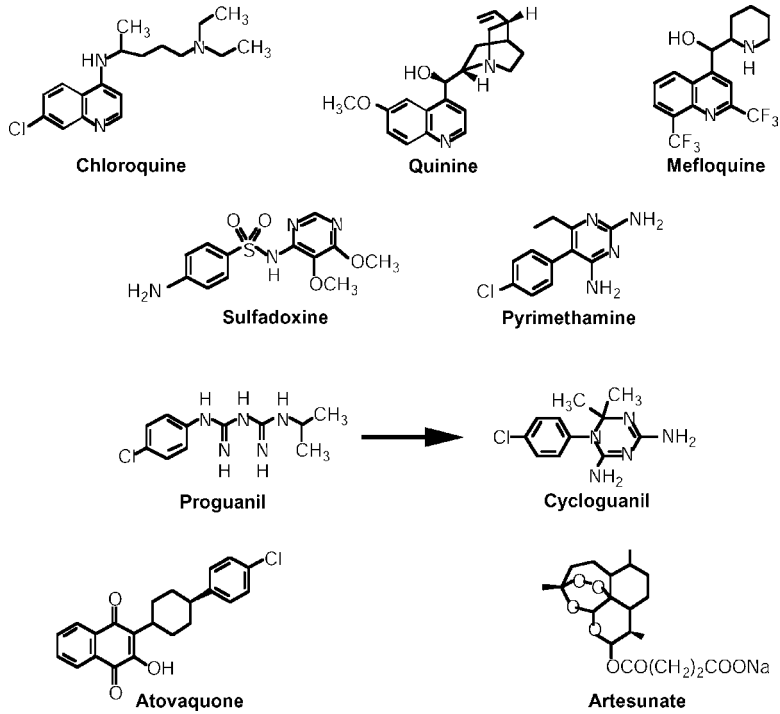

Figure 1 Structures of antimalarial drugs. Chloroquine, quinine, and mefloquine are 4-substituted quinolines that interfere with heme polymerization; sulfadoxine, pyrimethamine, and cycloguanil are substrate analogs that interfere with folate metabolism (Figure 2). In humans, proguanil is converted by CYP2C19 and CYP3A4 to form cycloguanil. Newer antimalarials with novel structures and mechanisms include atovaquone and artesunate.

Chloroquine resistance has resulted in demonstrably escalating mortality rates in African children (20, 21); in Senegal, the emergence of resistance over a 12-year period was associated with at least a doubling of the risk of death from malaria in children under ten (22).

Chloroquine and the other 4-substituted quinolines kill malaria parasites by interfering with the detoxication of heme. During its intraerythrocytic development and proliferation, hemoglobin is a major source of nutrition for the parasite (23). Hemoglobin is transported into the acidic food vacuole and sequentially digested into smaller peptide fragments by aspartic, cysteine, and metallo proteases. A toxic byproduct of hemoglobin degradation is free heme. Unlike mammalian systems, which detoxify heme by enzyme-mediated ring opening and glucuronidation, in malaria parasites heme is polymerized to form an inert crystalline pigment called hemozoin. Early studies with rodent malaria parasites revealed that chloroquine

selectively disrupts the aggregation of malarial pigment within the food vacuole (24), and more recent experiments have refined this picture to show that chloroquine effectively blocks the sequestration of toxic heme into hemozoin (25). Chloroquine accumulates in parasitized red cells, particularly in the acidic digestive vacuole, to reach levels hundreds of times those in plasma, and the accumulation is reduced substantially in chloroquine-resistant cells (26). Subsequent studies with chloroquine-resistant *P. falciparum* confirmed these findings; noted the lack of cross-reactivity with quinine, mefloquine, or chloroquine analogs (27); described a paradoxically increased sensitivity to some antimalarials (28); found that reduced steady-state levels were attributable to enhanced efflux, not reduced uptake (29); and revealed that verapamil could partially restore the accumulation of, and sensitivity to, chloroquine (30). Although these phenotypic characteristics have been invaluable in suggesting and corroborating the molecular mechanisms of resistance, the definitive studies have been genetic. Despite heavy drug pressure, it took many years for chloroquine-resistant *P. falciparum* to emerge in the field. This observation, together with the fact that chloroquine resistance in the laboratory could only be generated in the presence of mutagens, led to the suspicion that chloroquine-resistance might well be multigenic.

As described in detail below, two entirely distinct experimental approaches have yielded two independent genetic sources of chloroquine resistance in *P. falciparum*. One of these, *pfcrt* (*P. falciparum* chloroquine-resistance transporter) is now recognized to be both necessary and sufficient to impart chloroquine resistance. The other, *pfmdr1* (*P. falciparum* multidrug-resistance 1), may further modulate the degree of resistance.

One experimental approach was an undirected search for a gene(s) that would sort with chloroquine resistance when sensitive and resistant parent lines were crossed during sexual reproduction in the mosquito (31). The resulting progeny were fully sensitive or resistant, consistent with changes at a single genetic locus in these haploid forms. Some ten additional years of work were required to identify the rather cryptic 13-exon *pfcrt* on chromosome 7 (32). This gene encodes a novel 45 kDa protein with ten predicted transmembrane domains that immunolocalizes to the membrane of the digestive vacuole. It has no obvious homology to the large family of ABC (ATP-binding cassette) transporters that pump drugs against a concentration gradient at the expense of ATP (33, 34). The predicted protein is thought to be a transporter or channel that reduces chloroquine levels in the digestive vacuole, which in turn reduces the accumulation of free heme and relieves cytotoxicity. The mechanism by which *pfcrt* affects chloroquine levels is not yet clear but it may involve altered ion fluxes that change the acidity of the vacuole, or alternatively, *pfcrt* may interact directly with chloroquine itself (35). Studies of this process have been hampered by the difficulty in expressing this transmembrane protein in heterologous systems.

Analysis of *pfcrt* in cell lines obtained from many geographic locations revealed a consistent wild-type sequence in the sensitive lines, and a remarkable array of mutations in chloroquine-resistant lines (32). Genes from resistant cells have at

least five and up to eight mutations, all confined to ten positions that are clustered within or near transmembrane domains. Common to all resistant lines are a K76 mutation, which now provides a valuable molecular marker in surveillance studies and a predictor of chloroquine efficacy (36). The limited patterns of mutations suggest that resistant lines originated in just a few discrete geographic locations from which they then spread. This notion is strengthened by a more recent genome-wide satellite marker analysis in dozens of strains of *P. falciparum*, which reveals a striking lack of polymorphism surrounding *pfcrt* in chromosome 7, relative to all other portions of the genome (37). This prominent aberration reflects the powerful selective pressure that extensive chloroquine use has exerted on this parasite's evolution. The essential role of *pfcrt* was firmly established by allelic exchange of the endogenous *pfcrt* in chloroquine-sensitive cells for mutant alleles from resistant lines, which effectively conferred a chloroquine-resistant phenotype (38).

Mutations in *pfcrt* have now been shown to account for the recognized characteristics of chloroquine-resistant cells described above: reduced accumulation of chloroquine (38, 39); lack of cross-resistance with quinine and mefloquine (35), indeed a paradoxically increased sensitivity to some antimalarials (38); and an acceptable fulfillment of the expectation for a multigenetic mechanism (e.g., multiple mutations required, although all in a single gene). Finally, although perhaps not a consequence that should be expected, the chloroquine resistance imparted by mutant PfCRT is partially reversible by verapamil (38, 39). The latter is a well-recognized antagonist of drug efflux pumps (33, 34); however, its action is confined to just one of the seven classes of ABC transporters, and PfCRT is not even a member of the ABC transporter family.

A second independent experimental approach to understanding the genetic basis for chloroquine resistance actually preceded that described above, and was a directed search for ABC transporter genes whose sequence or expression might be altered in drug-resistant cells. This logical search was prompted by accumulating evidence that upregulation of ABC transporter gene expression is associated with multidrug resistance in tumor cells, and by the finding that chloroquine resistance is partially reversed by verapamil (30). With the completion of the human genome, the family of ABC transporters is now divided into seven different classes on the basis of sequence homology (33, 34). All are membrane-spanning proteins and have highly characteristic nucleotide-binding domains. Best studied of the ABC transporters is ABCB1 (also termed Pgy1, MDR1, Pgp, or GP170), whose preferred substrates (hydrophobic, planar aromatic rings, with the presence of tertiary amino groups—criteria all fulfilled by chloroquine; Figure 1) are pumped against a concentration gradient at the expense of ATP hydrolysis and whose action is antagonized by verapamil. Notably, mutations in human ABCB1 are not associated with recognizable disease or with altered drug transport; the latter is mediated by upregulated expression.

Using phylogenetically conserved ABCB1 sequences, two laboratories identified *mdr* genes (termed *pfmdr1* and *pfmdr2*) in *P. falciparum* (40, 41). Subsequent studies implicated only *pfmdr1* in drug resistance, although the association was

imperfect and variably involved either gene amplification or point mutations. The clearest data bearing on the role of *pfmdr*s in drug-resistant *P. falciparum* indicate that these genes do not sort with chloroquine resistance in genetic crosses of sensitive and resistant cells (31), and that the introduction of mutant *pfmdr1* into cells with wild-type *pfcrt* has no effect on chloroquine sensitivity (42). Importantly, however, the addition of mutant *pfmdr1* to cells already harboring mutant *pfcrt* does enhance chloroquine resistance, indicating that mutations in this gene may modulate the overall response to chloroquine (38, 42). Of considerable interest and distinct from *pfcrt*, mutations in *pfmdr1* are associated with resistance to mefloquine, quinine, and halofantrine (42).

Although the exposure of *P. vivax* and *P. falciparum* to chloroquine has been similar, the appearance of chloroquine-resistant *P. vivax* took nearly 30 additional years to appear. First reported from Papua New Guinea in 1989 (43), chloroquine-resistant *P. vivax* has now spread through Southeast Asia and into South America. Unexpected and intriguing is the finding that chloroquine resistance in *P. vivax* is apparently not mediated by mutations in the *vivax* homolog of *pfcrt* (44). Despite the interest and importance of this problem, the technical difficulties in studying *P. vivax* seriously hamper definitive studies.

A number of new therapeutic approaches have been taken on the basis of lessons learned from chloroquine and the mechanisms of chloroquine resistance. These include the use of analogs that differ only in the length of the 4-aminoalkyl side chain, which retain antimalarial activity but are not cross-resistant with chloroquine (45); coadministration of chloroquine with various chemosensitizers in an effort to reverse the efflux mechanism (46), although for antitumor agents this approach has met with very limited success (33); and use of chloroquine in combination with other antimalarials, most notably an artemisinin (47). The documented reemergence of chloroquine-sensitive parasites when drug pressure is removed is fascinating and may afford an opportunity to reintroduce chloroquine after years of nonuse (48).

Folic Acid Antagonists

Malaria parasites were closely intertwined with the discovery of drugs that target folate biosynthesis. Two years after Domagk's 1935 Nobel Prize–winning description of sulfonamide activity against bacteria (49), a rather large clinical trial established the efficacy of a sulfonamide in patients with malaria (50). Some ten years later a concerted program of antimalarial drug discovery (51) yielded proguanil (a prophetic name, given its prodrug nature; Figure 1). In a landmark study reported in 1948, well before proguanil's molecular mechanism had been described, Greenberg showed for the first time that the combination of proguanil with a sulfonamide was profoundly synergistic (52). His studies were on *P. gallinaceum* in chicks. This key observation had important and nearly immediate consequences. First, it led directly to the finding that proguanil also interferes with folate metabolism in malaria parasites, but at a site distinct from that of sulfonamides

(53); second, the structural analogy of proguanil to a series of antibacterial 2,4-diaminopyrimidines was recognized by Hitchings, who then demonstrated potent antimalarial activity in this new chemical class of antifolates (54); and third, it provided an effective means to forestall the emergence of resistance, which even in earliest experiments was recognized as a serious problem. The eventual consequence was an antifolate/sulfonamide combination of pyrimethamine/sulfadoxine (Figure 1) that was carefully selected for matching pharmacokinetics, formulated in fixed ratios to maximize synergy, and marketed as Fansidar®. (The antibacterial trimethoprim/sulfamethoxazole was similarly developed.) The extraordinary degree of synergism in these combinations, which allows some 20-fold reduction in the dose of each component, is still attributed to multiple blockades in a single metabolic pathway, although evidence to support this widely cited mechanism remains circumstantial and other mechanisms may contribute (55–58).

Tetrahydrofolate is an essential cofactor in the methyl transfer reactions that generate monomers for protein and nucleic acid synthesis (59). In several important respects, folate biosynthesis in malaria parasites is distinctly different from that in other systems (pathways and key points of drug inhibition in Figure 2, see color insert). First, from biochemical studies and the annotated genome it is now clear that *P. falciparum* is unique in that both *para*-aminobenzoic acid (60–62) and dihydrofolic acid (58, 63, 64) can be synthesized de novo as well as salvaged from the environment. The availability of these salvage pathways has severely complicated in vitro inhibition studies, and they clearly modulate antifolate efficacy in patients, whose blood levels of *para*-aminobenzoic acid and dihydrofolate may vary widely (65). A second dissimilar feature in *Plasmodium* folate metabolism is that sequential reactions may be catalyzed by a single bifunctional protein. Thus, dihydro-6-hydroxymethylpterin pyrophosphokinase and dihydropteroate synthase are encoded by the same gene and contained within the same protein (66, 67). Dihydrofolate reductase and thymidylate synthase activities are similarly linked (68, 69). This structural organization may improve catalytic efficiency by channeling substrates in a processive fashion through two sequential transformations; it may also offer novel strategies for drug-mediated disruption. Finally, malaria parasites are especially susceptible to inhibition of dihydrofolate reductase because (unlike mammalian cells) transcriptional inhibition, mediated by the protein binding to its own message, is not relieved by the accumulation of substrate that occurs in the presence of inhibitor (70). This precludes the upregulation of protein synthesis as a means to counter antifolate inhibitors and it contributes to the selective toxicity of antifolates against the parasite.

Chloroquine's efficacy, safety, and low cost made it the clear drug of choice for many decades, but the advent of chloroquine-resistant parasites established pyrimethamine/sulfadoxine as the next best option, despite the recognized propensity for resistance and the concern about antifolate teratogenicity (71). Malaria parasite resistance to sulfonamides and antifolates has been known for more than 50 years (72–74). Although available mechanisms reportedly include gene amplification, which is the only recognized mechanism associated with clinical

resistance to antifolate therapy in cancer (17), a large body of evidence now indicates that in *Plasmodium* the major effector of resistance is point mutations in the key target enzymes: dihydropteroate synthase and dihydrofolate reductase. Unlike the transmembrane proteins that mediate chloroquine resistance, native and recombinant forms of the synthase and reductase are soluble and assayable; hence, the findings in genetic studies have been bolstered by biochemical and structural experiments.

Molecular epidemiology studies from South America and Africa provide multiple lines of evidence that application of pyrimethamine/sulfadoxine therapy leads to the progressive and orderly accumulation of point mutations, first in dihydrofolate reductase and then in dihydropteroate synthase. The sequential addition of new mutations is evident in field isolates collected over years of time (75, 76), in pre- versus posttreated patients (77), and in correlation with the degree of clinical resistance for a given patient or geographic region (78). Evaluation of these mutations in the context of surrounding polymorphisms in noncoding sequences is consistent with focal origin of mutant strains followed by spread through the population via gene flow (75, 76). Highest levels of clinical resistance result from parasites with four mutations in dihydrofolate reductase and two in dihydropteroate synthase, which may represent the maximum number of mutations that can be tolerated in competition with less-affected strains. The utility of these mutations as predictors for therapeutic response is modulated by host immunity, as evidenced by the persistent efficacy of pyrimethamine/sulfadoxine in holoendemic Malawi, despite ongoing use of these agents in a population that has harbored highly mutant parasites for at least five years (79).

Laboratory findings that corroborate these field data and underscore the central importance of point mutations include the appearance of the appropriate drug-resistant phenotype in genetic crosses or when mutant genes are introduced into wild-type cells (80–82) and analysis of the inhibition kinetics of recombinant wild-type versus mutant enzymes (83, 84). The recently available crystal structure for dihydrofolate reductase-thymidylate synthase provides satisfying evidence that the critical mutations mediating clinical drug resistance map to the dihydrofolate reductase active site (85).

The well-studied and proven value of the folate synthetic machinery as an antimalarial target has prompted several ingenious research efforts to devise new interventions against tetrahydrofolate production and use. These include inhibition of the shikimate pathway, which provides an intracellular source of *para*-aminobenzoic acid (Figure 2), alone or in combination with downstream inhibitors (61); dihydrofolate reductase inhibitors rationally designed and selected for activity against the clinically important quadruple mutant malaria enzyme but not the human reductase (86); identification of novel chemical classes by in silico docking of large chemical libraries into the known dihydrofolate reductase three-dimensional (3-D) structure (87); and deployment of folate analogs against thymidylate synthase (88). More immediate clinical efforts have focused on using sulfonamide/antifolate combinations that are less cross-resistant and/or

have a shorter plasma half-life (89, 90) and adding a third antimalarial to the pyrimethamine/sulfadoxine dosing regimen (47, 91).

Mitochondrial Electron Transport Inhibitors

Although hydroxynaphthoquinones were the focus of considerable interest in the 1940s as a new class of synthetic antimalarials (92), they were upstaged first by chloroquine and then pyrimethamine/sulfadoxine. However, by the early 1990s the growing resistance to existing antimalarials and the activity of atovaquone (the lead compound in this chemical class; Figure 1) against opportunistic *Pneumocystis carinii* in AIDS patients, spurred the clinical development of atovaquone (93). Given its novel molecular mechanism of action (a ubiquinone analog that blocks mitochondrial respiration at the cytochrome bc_1 complex) and its potency at low nanomolar concentrations in vitro, it came as an unexpected surprise that atovaquone had a ~30% failure rate in its first field trials (94). Remarkably, paired isolates of *P. falciparum* obtained before treatment and after recrudescence showed a more than 1000-fold reduction in sensitivity to atovaquone.

In a short time, an elegant series of studies confirmed the previously reported molecular site of action (95) and provided a satisfying explanation for resistance (96, 97). Atovaquone inhibits respiration and collapses the mitochondrial membrane potential in live intact malaria parasites. Sequence analysis of the mitochondrially encoded gene for cytochrome b from atovaquone-resistant *P. yoelii* revealed a series of mutations that affect five amino acids clustered in a highly conserved 15 amino acid sequence. Based on analogy to the crystal structure for chicken cytochrome b, these residues all map to a cavity in the region of the ubiquinol-oxidation site. Several factors were identified to help account for the striking rapidity and magnitude of atovaquone resistance. First, 11 of the 12 mutations involved A:T to G:C changes, a lesion consistent with oxidative damage. By disrupting the normal flow of electrons through the transport chain, atovaquone may increase the formation of superoxide radicals, which in turn can damage mitochondrial DNA. Second, although there are approximately 100 copies of the 6kb mitochondrial genome per parasite, sensitive methods failed to detect any evidence of residual wild-type cytochrome b sequence. Thus, after a short period of time under drug selection, every copy of the genome contained these advantageous mutations, perhaps as a result of the extensive recombination that accompanies mitochondrial DNA replication in malaria parasites. Analysis of *P. falciparum* isolated from patients who failed atovaquone monotherapy confirmed the predilection for mutations at the Y268 residue (98).

Fortunately, the clinical utility of atovaquone was salvaged by the timely discovery that its antimalarial activity is synergistically enhanced, in vitro and in the clinic, by the simultaneous application of proguanil (94, 99). Proguanil is classically regarded as an antifolate (Figure 1 and see above) and by itself has no detectable effect on electron transport or mitochondrial membrane potential. However, proguanil synergistically enhances atovaquone's ability to depolarize

the malarial mitochondrial membrane and inhibit respiration (100). Atovaquone plus proguanil, now marketed as a fixed combination (Malarone®), generally provides safe and reliable prophylactic and therapeutic antimalarial activity (101, 102). Although there have been no published failures of atovaquone/proguanil for prophylaxis, a handful of case reports document the recrudescence of *P. falciparum* after treatment of established infections. In all cases (some of which include paired isolates), recrudescent parasites have a Y268N, or more commonly a Y268S, mutation in cytochrome b (103, 104). The fact that just a single mutation can significantly compromise the efficacy of this combination is worrisome and underscores the need for careful selection of therapeutic indications to prolong its useful lifetime.

MULTIDRUG-RESISTANT PARASITES

In cancer chemotherapy, resistance to structurally and mechanistically diverse agents can be mediated by alterations in expression of a single ABC transporter gene (17, 105). As we now understand it, multidrug resistance for *P. falciparum* is different: It involves genetic alterations in at least two, and often more, proteins [the difficult problem of multidrug-resistant *P. falciparum* has been thoughtfully defined and reviewed recently (8)]. Typically, this means resistance to both chloroquine and pyrimethamine/sulfadoxine, mediated by mutations in *pfcrt*, dihydropteroate synthase and dihydrofolate reductase, as described above. However, strains resistant to chloroquine, sulfadoxine/pyrimethamine, mefloquine, and partially resistant to quinine and quinidine have been described (106). Malaria in Southeast Asia is notorious for its propensity to develop early and multidrug resistance. This prompted an interesting experiment comparing the emergence of resistance in a parasite clone from Africa (which was fully susceptible to conventional antimalarials) to that of a multidrug-resistant clone from Indochina (107). Two compounds were selected that had novel killing mechanisms and had never before been applied to these parasites. The Indochina clone acquired resistance some 1000 times more frequently, suggesting these parasites may have an underlying accelerated mutator or hyperrecombination phenotype.

STRATEGIES TO COMBAT RESISTANCE

Artemisinins

The artemisinins are an important and exciting addition to the antimalarials (artemisinins reviewed in 108, 109; Figure 1). Hundreds of synthetic and semisynthetic analogs have been evaluated, and to date the most clinically successful is artesunate. The essential pharmacophore is structurally and mechanistically unique: an endoperoxide bridge that undergoes iron-catalyzed activation, probably in the food vacuole, to form toxic free radicals. Recent studies suggest artemisinin

may inhibit ATPase and alter intracellular calcium stores (110). As a class the artemisinins are potent, fast-acting, and remarkably impervious to resistance, although recrudescence of fully sensitive parasites is common. Human safety for this class is regularly claimed despite the unfortunate rarity of systematic safety evaluations available in the literature. The current recommended use for artemisinins is in combination therapy, where they effect a rapid and massive decrease in parasite burden and their gametocytocidal activity may lessen transmission of resistant parasites to the mosquito. As noted above, several large clinical trials have already demonstrated their meaningful contribution to efficacy (47, 111), and even larger studies are underway as a likely prelude to national health policy recommendations (6).

Drugs Used in Other Diseases

It is a telling commentary on the state of antimalarial therapy that doxycycline, an antibacterial, is among the agents now recommended for malaria prophylaxis. Although intrinsically weak as antimalarials, clindamycin, azithromycin, and chloramphenicol also have some utility (112, 113), which may stem from their targeting protein synthesis in the parasite's apicoplast or mitochondrion. The antibacterial quinolones act by inhibiting DNA gyrase, an enzyme also present in the *P. falciparum* genome; although fluoroquinolones have activity against parasites in vitro (114), their clinical efficacy has been disappointing (115). The antifungal imidazoles are active against *P. falciparum* in vitro (116, 117). They form complexes with heme (118), suggesting a mode of action that might be similar to chloroquine's. Attractive features of this class are their good safety profile in children and adults, oral bioavailability, and short half-life.

Combination Therapy

For both antitumor and antiinfective therapies, abundant laboratory and clinical evidence attests to the fact that coadministration of drugs reduces the emergence of resistance. As detailed above, this strategy has provided a useful antimalarial therapeutic life span for pyrimethamine/sulfadoxine and atovaquone/proguanil, agents that readily provoke resistance when used alone. To stem the further development and spread of antimalarial drug resistance, the combined use of three or more drugs is under extensive study, and will likely succeed in reducing resistance (119). Less easy to predict is how multiple agents will interact in terms of antimalarial potency and host toxicity, where the net effects may be additive, synergistic, or even antagonistic. Distinguishing among these important outcomes requires careful attention to study design. Investigational combinations include coartemether, a fixed dose of artemether and lumefantrine; the latter has structural similarities to mefloquine and halofantrine. This combination originated in China and is in advanced clinical development (120). The combination of dihydroartemisinin and piperaquine has been evaluated in patients from Cambodia with uncomplicated *falciparum* malaria (121). Amodiaquine combined with sulfadoxine/pyrimethamine

had substantial antimalarial activity in spite of preexisting resistance to each component drug (122).

New Molecular Targets

The discovery of new molecular entities is at once the most exciting and the most risky approach to countering existing drug resistance. The handful of examples presented here is far from comprehensive and is intended just to illustrate possible avenues to new drug discovery [for more complete consideration of experimental antimalarials, see (7, 123) and the Web site for Malaria Medicines Venture, http://www.mmv.org/pages/page_main.htm]. As noted above, the malaria parasite's apicoplast has its own distinctive genome and complement of proteins, including a type II fatty acid synthesis pathway, which is unlike the pathway in human cells and is inhibited by triclosan (124). Blood stage malaria parasites are homolactate fermentors, an inefficient use of glucose that increases demand for its transport. O-3 hexose derivatives selectively inhibit glucose transport in *P. falciparum*, kill parasites in vitro, and suppress *P. berghei* infection in mice (125). The sequential proteolysis of globin is mediated by multiple proteases, which are all potential therapeutic targets. Plasmepsin inhibitors have antimalarial effects (126); falcipain inhibitors prevent hemoglobin hydrolysis and cure murine malaria (127–129). Glutathione metabolism offers several essential and vulnerable targets in the parasite (130). Fosmidomycin blocks the synthesis of isopentenyl diphosphate and the subsequent development of isoprenoids in *P. falciparum* (131), and it has antimalarial activity in vitro and in a mouse model. An open label trial in Gabon and Thailand showed that fosmidomycin is efficacious, although its use as a single agent is associated with high recrudescence (132).

CONCLUDING REMARKS

In recent years the severe problem of drug-resistant malaria has been featured extensively in the scientific and lay literature, leading to increased public awareness, new and better funding opportunities for research, and a growing sense that the situation requires thoughtful public health policies to preserve the utility of current therapies. Spurred by powerful genetic tools and availability of the fully sequenced genome, effective new drugs will almost certainly be discovered. Less certain is whether these agents will be inexpensive enough for widespread use in developing countries. Also of obvious concern is the propensity for resistance, which atovaquone has taught can appear immediately and at high levels. It is interesting to speculate that would-be new antimalarial drugs might better have a nonprotein target (e.g., chloroquine against the growing hemozoin crystal) or an "irrational" molecular mechanism (e.g., the artemisinins whose activated free radicals may pose a nonspecific oxidative stress). Although the pathway to design such agents prospectively is less obvious than, for example, that for an enzyme inhibitor, they may be inherently less affected by point mutations, which are the

preferred molecular resistance mechanism in these pathogens. In any case, the compelling medical problem of malaria, which captured the attention of some of the finest scientific minds of the past century and led to seminal discoveries that benefited all of chemotherapy, remains an urgent and unmet challenge.

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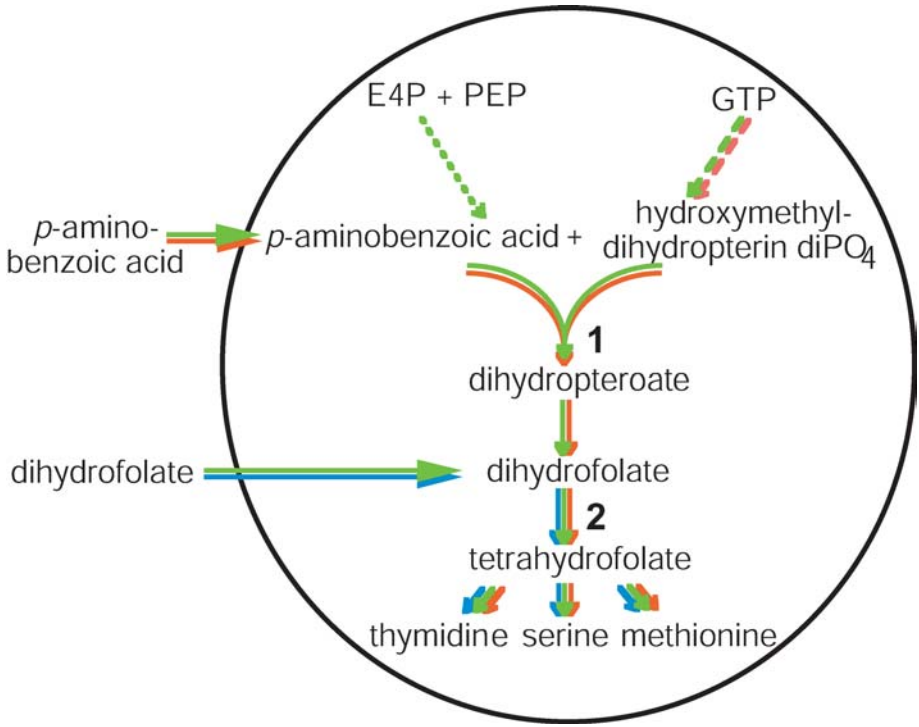

Figure 2 Simplified scheme of therapeutically important variations in folate metabolism in different organisms. Tetrahydrofolate cofactors are essential for biosynthetic reactions in *P. falciparum* (*green*), bacteria (*red*), and mammalian cells (*blue*), and all three systems utilize a dihydrofolate reductase activity (*reaction 2*). Various antifolates inhibit the reductase in *Plasmodium* (pyrimethamine, cycloguanil), bacteria (trimethoprim), or all three systems (methotrexate). Dihydropteroate synthase (*reaction 1*) in parasites and bacteria has no counterpart in human cells and is inhibited by sulfonamides. In malaria parasites, *para*-aminobenzoic acid from either salvage or the shikimate pathway (a multistep synthesis from erythrose 4-phosphate, E4P, and phosphoenolpyruvate, PEP) can significantly reduce the effectiveness of competitive sulfonamide inhibitors. In some *P. falciparum* strains, the ability to import preformed dihydrofolate counters the efficacy of both sulfonamides and antifolates. Large circle, cell membrane.

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