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Mechanisms of Resistance of Malaria Parasites to Antifolates

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***Abstract*——Antifolate antimalarial drugs interfere with folate metabolism, a pathway essential to malaria parasite survival. This class of drugs includes effective causal prophylactic and therapeutic agents, some of which act synergistically when used in combination. Unfortunately, the antifolates have proven susceptible to resistance in the malaria parasite. Resistance is caused by point mutations in dihydrofolate reductase and dihydropteroate synthase, the two key enzymes in the folate biosynthetic pathway that are targeted by the antifolates. Resistance to these drugs arises relatively rapidly in response to drug pressure and is now common worldwide. Nevertheless, antifolate drugs remain first-line agents in several sub-Saharan African countries where chloroquine resistance is widespread, at least partially because they remain the only affordable, effective alternative. New antifolate combinations that are more effective against resistant parasites are being developed and in one case, recently introduced into use. Combining these antifolates with drugs that act on different targets in the parasite should greatly enhance their effectiveness as well as deter the development of resistance. Molecular epidemiological techniques for monitoring parasite drug resistance may contribute to development of strategies for prolonging the useful therapeutic life of this important class of drugs.**

I. Introduction

Malaria is a major burden for the most resource-poor nations of the world. The goal of eradicating malaria, once thought to be possible, was abandoned decades ago, and the present goal of malaria control is instead first to retard the accelerating rates of disease and death caused by the world's most important parasitic disease and then to "roll back malaria".

Between 200 and 500 million cases of malaria occur annually with an estimated 1.7 to 3 million deaths attributable to malaria, most among children of sub-Saharan Africa (Breman, 2001). Many nations in sub-Saharan Africa have faced socio-economic instability and a dismantling of government sector malaria control programs (Garfield and Vermund, 1983; World Bank, 1993). The combination of such factors as the increased cost of insecticides, the vector's resistance to insecticides, and the lack of an effective vaccine, has resulted in reliance upon case management and effective curative chemotherapy as the primary approach to malaria control. Malaria parasite resistance to treatment with chloroquine has already complicated malaria management and has been associated with increased malaria morbidity and mortality (Greenberg et al., 1989; Trape et al., 1998), and increasing resistance to sulfadoxine-pyrimethamine will likely lead to similar results where it is the first-line antimalarial. Resistance of the malaria parasite, especially to chloroquine and to the antifolates, will continue to make progress in rolling back malaria a formidable challenge for the foreseeable future.

A. Life Cycle of Plasmodium falciparum

Four malaria species cause disease in humans: *Plasmodium vivax*, *P. malariae* and *P. ovale*, and the cause of most severe malaria disease and deaths, *P. falciparum*. Dozens of other Plasmodia species cause disease in other mammals, birds, and reptiles. Malaria parasites have a complex life cycle, involving both vertebrate (human) and invertebrate hosts (mosquitoes). Saliva from infected mosquitoes transmits the veriform malaria sporozoites to the subcutaneous tissues of the human host when the female mosquito takes a blood meal. The sporozoites travel rapidly to the liver and invade hepatocytes, where they develop into an exoerythrocytic stage called a tissue schizont. After 6 to 10 days, these exoerythrocytic schizonts undergo schizogony, multiplying via mitosis until they rupture the infected hepatocytes and discharge tens of thousands of merozoites from each infected hepatocyte into the bloodstream. This release of merozoites from the liver appears to be a continuous and asynchronous process in falciparum malaria (Murphy et al., 1990). The merozoites then in-

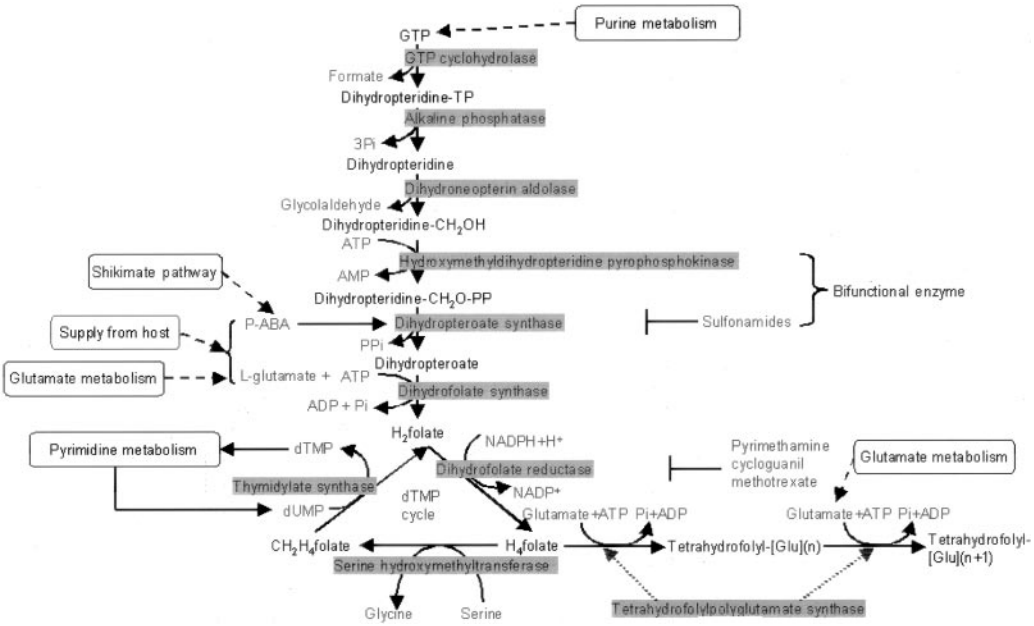

FIG. 1. Folate biosynthetic pathway in *Plasmodium spp.* (http://malaria.atcc.org/metabolic_pathways/maps/folatebiopath.html).

vade erythrocytes where they again multiply and, after 48 h (72 h in the case of *P. malariae*), release 8 to 32 progeny merozoites. The progeny merozoites invade new erythrocytes to perpetuate the erythrocytic cycle, the stage of the parasite life cycle responsible for disease. A small percentage of the merozoites do not multiply after invading erythrocytes, but instead differentiate into sexual forms termed gametocytes. When gametocytes are ingested by a mosquito in a subsequent blood meal, male and female gametes mate, creating a zygote. This brief diploid stage in an otherwise haploid life cycle allows for sexual recombination of genetic material, including the chromosomal genes responsible for most drug resistance. Within the mosquito midgut the zygote matures into an oocyst, which in turn releases sporozoites that then migrate to the mosquito salivary glands, completing the life cycle.

B. Folate Biosynthesis in Plasmodia

The empiric use of antifolates against malaria long predates definitive demonstration of the folate metabolism pathway in *Plasmodium spp*. De novo synthesis of folate by *Plasmodium spp.* was demonstrated over 25 years ago (Ferone, 1977), and although an exogenous folate salvage pathway has been found in isolates from around the world (Krungkrai et al., 1989), it does not appear to be Plasmodia's primary source of folate. Not all of the enzymes involved in folate metabolism have been identified in *Plasmodium spp*. (Fig. 1) (http://malaria.atcc.org/metabolic_pathways/maps/folatebiopath.html), but this will undoubtedly change with the sequencing of the malaria genome. The genes encoding the enzymes in the folate pathway targeted by existing antifolate drugs, dihydrofolate reductase (DHFR[1]) (Bzik et al., 1987) and dihydropteroate synthase (DHPS) (Brooks et al., 1994; Triglia and Cowman, 1994), have both been cloned and sequenced, and mutations in these genes have been determined to play a role in resistance to the antifolate drugs (Peterson et al., 1988). Disruption of folate synthesis by DHFR and DHPS inhibitors leads to decreased levels of fully reduced tetrahydrofolate, a necessary cofactor in important one-carbon transfer reactions in the purine, pyrimidine, and amino acid biosynthetic pathways (Ferone, 1977). The lower levels of tetrahydrofolate result in decreased conversion of glycine to serine, reduced methionine synthesis, and lower thymidylate levels with a subsequent arrest of DNA replication (Schellenberg and Coatney, 1961; Gutteridge and Trigg, 1971; Newbold et al., 1982; Gritzmacher and Reese, 1984; Triglia and Cowman, 1999).

II. Malaria Parasite Drug Resistance

A. Historical Perspective

To understand the important role that antifolate drugs currently play in malaria drug therapy, it is helpful to review the history of malaria drug development and the near parallel development of drug resistance in the malaria parasite. The antimalarial drug armamen-

[1]Abbreviations: DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase; RIII, high-level chloroquine resistance; TS, thymidylate synthase; WR99210, 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-[(2,4,5-trichlorophenoxy)propyloxy]-1,3,5-triazine hydrobromide; PS-15, Imidodicarbonimidic diamide, *N*-(1-methylethyl)-*N*′-(3-(2,4,5-trichlorophenoxy)propoxy)-, hydrochloride; *p*-ABA, para-aminobenzoic acid; PCR, polymerase chain reaction; GRI, genotype resistance index; GFI, genotype failure index; ACT, artemisinin-based combination therapy; WHO, World Health Organization.

tarium in the Western world was limited to the cinchona bark extract quinine until the first World War, when declining stocks of quinine in Germany led to the development of the first synthetic antimalarials. Work with these synthetic dyes led to the development of the acridines and the 8-aminoquinolines, such as pamaquine (and subsequently primaquine), which were more toxic than quinine and therefore left quinine once again as the primary antimalarial. Quinine resistance was first documented in Brazil in 1908 and again demonstrated in 1938 in German railroad workers returning from the Madeira-Mamoré railroad on the Brazilian-Bolivian border (Clyde, 1972b). This resistance represented a true parasite drug tolerance because infections in these individuals were not cured by successive increases in quinine dose. Varying degrees of quinine resistance now can be found worldwide, but it is most common and severe in Southeast Asia.

1. Chloroquine. Although chloroquine (Resochin) was synthesized in 1934, and amodiaquine shortly afterward, it initially was felt to be too toxic for use. Chloroquine, however, became the cornerstone of the malaria eradication campaign of the 1950s and 1960s. High-level chloroquine resistance (RIII, see Table 1) was first found in Thailand in 1962 (Harinasuta et al., 1965), then spread gradually and contiguously throughout Southeast Asia [Malaysia (Montgomery and Eyles, 1963), Cambodia (Eyles et al., 1963), Vietnam (Powell et al., 1964; Eppes et al., 1966), and Burma in 1969 (Clyde et al., 1972)]. At about the same time, chloroquine resistance also appeared in South America (Moore and Lanier, 1961). Chloroquine resistance appeared later in Africa, initially in nonimmune travelers returning from East Africa (Campbell et al., 1979; Fogh et al., 1979, 1984; Jepsen et al., 1983). Despite the current high levels of chloroquine resistance in most areas of malaria transmission, the cost of less than $0.20 U.S. per treatment continues to apply sufficient financial incentive to maintain chloroquine as a first-line treatment in much of West and Central Africa (Foster, 1994).

2. Synthetic Antimalarials. During World War II, the lack of access to the world's major supply of cinchona bark, and therefore quinine, again spurred development of synthetic antimalarials, such as mepacrine (quinacrine). Simultaneously, drug development focused on derivatives of pyrimidine, based upon its presence in nucleic acids and its metabolism by protein systems that were effectively blocked by antimalarial sulfonamides. Research in this direction resulted in the development of the antifolate biguanides, proguanil and chlorproguanil.

3. Proguanil. Early reports on the use of proguanil (Paludrine) for both prophylaxis and treatment were very encouraging (Maegraith et al., 1945, 1946; Jones et al., 1948; Seaton and Lourie, 1949), despite a slower schizontocidal action (defined as activity against any asexual blood stage parasite, not only schizonts) compared with quinine or mepacrine (Covell et al., 1949). Thereafter, when proguanil was used in the late 1940s and the early 1950s as prophylaxis for plantation workers in Southeast Asia and elsewhere, it provided an opportunity for widespread drug selection pressure on the parasite and the subsequent development of resistance to this drug. The prophylactic experience with proguanil in Malay may have cast a shadow on the drug's further development. From a 100% clinical cure rate following a one-time 100-mg dose in late 1947, clinical failure rates rose to 25% following a 300-mg dose in early 1949, with some cases failing to clear after a 100-fold increase of this dose (Field and Edeson, 1949; Davey and Robertson, 1957). A similar example from Brazil (Walker and Lopez-Antunano, 1968) indicates that resistance was not limited to Southeast Asian isolates.

4. Pyrimethamine. Developed shortly after proguanil, the DHFR inhibitor pyrimethamine (Daraprim) was a remarkably effective causal prophylactic and therapeutic agent (Archibald, 1951 first use in natural infection; Goodwin, 1952b; Vincke and Lips, 1952; Delannoy and Hugon, 1954; Miller, 1957), even against chloroquine-resistant parasites (Powell et al., 1963) (for excellent reviews of early experience with pyrimethamine, see Goodwin, 1952a and Hitchings, 1960). However, concerns about the rapid development of parasite resistance to pyrimethamine and its slow schizontocidal activity

TABLE 1
Measurement of in vivo therapeutic efficacy based upon parasite clearance

S or S/RI	In the extended test, parasites are S if no asexual parasites are found by day 6 and parasites do not reappear by day 28. In the 7-day field test, the infection may be either S or resistant at RI (S/RI) level if no asexual parasites are found at day 6 and none are present on day 7. An S response and a RI response cannot be distinguished using the nonextended test since the difference between the two responses depends on the presence or absence of recrudescence between day 8 and day 28.
RI	In the extended test, parasites are resistant at the RI level if asexual parasites first disappear then return within 28 days, reinfection excluded. In the 7-day field test, parasites are resistant at the RI level if asexual parasites disappear for at least 2 consecutive days, but return and are present on day 7.
RII	Parasites are resistant at RII level if asexual parasitemia does not clear, but is reduced to 25% or less of the original pretreatment level during the first 48 h of treatment.
RIII	Parasites are resistant at RIII level if asexual parasitemia is reduced by less than 75% during the first 48 h or if it continues to rise.

were raised shortly after its introduction (Coatney et al., 1952; Goodwin, 1952a; Wilson and Edeson, 1953; Petersen, 1987). Events following the continued use of pyrimethamine as a prophylactic supported this concern (Clyde and Shute, 1954; Jones, 1954; Rollo, 1955; Burgess and Young, 1959). When pyrimethamine was given as weekly prophylaxis to children for 1 year, pyrimethamine resistance increased throughout the course of the year, approaching 60% resistance at year's end (Clyde and Shute, 1957). Resistance, as measured by the dose of pyrimethamine required to clear asexual parasitemia (number of parasites per unit volume blood), increased 8- to 15-fold or possibly more in some cases, as the "resistance approached or exceeded the maximum therapeutic dosage". Transmission of resistant parasites to persons not on prophylactic treatment occurred within the central treatment areas and villages nearby the treatment villages. Resistance was found less commonly in villages two to four miles from the central treatment areas (0 to 7% resistance), and no resistance was found in villages more than five miles distant.

Resistance to both proguanil and pyrimethamine was encountered during the American occupation of Vietnam (Peters, 1970). At this same time, the first report of a multidrug-resistant isolate from Thailand was made (Young et al., 1963; Powell et al., 1964). This isolate and others from nearby locales were resistant to chloroquine, mepacrine, proguanil, pyrimethamine, and partially resistant to quinine, whereas the Malay strain mentioned earlier was resistant to amodiaquine, hydroxychloroquine, quinacrine, chlorguanide, pyrimethamine, sulfadiazine, and chloroquine (DeGowin and Powell, 1964).

5. Sulfa Drugs. The increased exposure of nonimmune persons to these resistant strains rekindled interest in the sulfonamides and sulfones as antimalarials. Prontosil, the active component of which is sulfanilamide, was developed in 1932. A trial using Prontosil for treatment of falciparum malaria in 1937 cured 100% of 93 individuals following four, 12 hourly injections (Hill and Goodwin, 1937; Niven, 1938; Coggeshall et al., 1941). Interest in sulfonamides then waned, partly because of the introduction of synthetic antimalarials and the continued effectiveness of quinine, until sulfa drugs with longer half-lives and improved toxicity profiles were developed in the late 1950s and 1960s (Hill, 1963) (for a detailed review of early sulfonamide use, see Curd, 1943). Sulfadoxine in particular (now used in combination with pyrimethamine in Fansidar), demonstrated considerable promise as a prophylactic and curative drug against *P. falciparum* in Tanzania (Laing, 1965a). Several early trials confirmed that a single 1-g dose of sulfadoxine was an effective, although slow, schizontocide. Although in vivo resistance to sulfonamides and sulfones could be demonstrated with relative ease in animal models (Scholer et al., 1984), in vivo human field studies continued to offer encouragement. For example, weekly sulfadoxine (500 mg) prophylaxis resulted in no positive blood smears at the end of 6 weeks compared with a 26% positive rate with pyrimethamine prophylaxis (Laing, 1964).

6. Combined Dihydrofolate Reductase Inhibitors and Sulfonamide Drugs. A 1959 study found that sulfadoxine potentiated pyrimethamine in human falciparum infections, demonstrating that combined pyrimethamine and sulfadoxine was more effective than either drug alone (Greenberg and Richeson, 1950; Hurly, 1959). In other field studies of pyrimethamine combined with a sulfonamide, in pyrimethamine-resistant or multidrug-resistant infections, the combination was superior to either drug or chloroquine alone (McGregor et al., 1963; DeGowin and Powell, 1964; Chin et al., 1966; Harinasuta et al., 1967; Laing, 1968b, 1970b; Martin and Arnold, 1968b). Sulfadoxine schizontocidal activity remained slower than that of chloroquine (DeGowin and Powell, 1964; Chin et al., 1966) or of quinine, even in the presence of low-level quinine resistance (Peters, 1970). However, faster schizontocidal activity and improved clinical response was seen when sulfadoxine was combined with pyrimethamine (Richards, 1966; Harinasuta et al., 1967; Laing, 1968a, 1970a) or cycloguanil (in mice Thompson et al., 1965). When multidrug-resistant infections were noted with increased frequency in Southeast Asia, in the mid to late 1960s, sulfadoxine-pyrimethamine was a logical first-line drug replacement for chloroquine in Thailand.

Despite these largely successful field trials with sulfadoxine-pyrimethamine, evidence that the combination might prove to be an ineffective long-term solution was building in reports of clinical failure from Southeast Asia and South America in semi-immune persons (Bunnag et al., 1980) and from Africa and the United States in nonimmune persons (Chin et al., 1967; Spencer, 1985; Miller et al., 1986). An early paper on the efficacy of sulfadoxine-pyrimethamine wisely cautioned that the combination of pyrimethamine and sulfadoxine, whose dose activity regression lines are nearly flat, might lead to rapid development of parasite drug resistance (see Jacobs et al., 1963; Harinasuta et al., 1967).

7. Aryl Amino Alcohols. Mefloquine and halofantrine, both aryl amino alcohol derivatives of quinine, were developed by the U.S. Army soon after the introduction into clinical practice of sulfa drug-DHFR inhibitor combinations. Because the aryl amino alcohols were introduced into areas where quinine resistance already existed, such as Southeast Asia, and cross-resistance between quinine and the aryl amino alcohols may exist (Peters, 1984), it is perhaps not surprising that resistance to these compounds developed quickly (Nosten et al., 1987, 1991; ter Kuile et al., 1992; Smithuis et al., 1993). The cost of these drugs has prohibited them from being used widely in sub-Saharan Africa.

8. General Concepts Learned from Early Experiences. Important concepts of drug resistance in *Plasmodium spp*. followed from these early observations. Covell et al. (1949) described a proguanil-resistant isolate from Nigeria and

suggested that resistance, as seen in the Lagos and Malayan strains, was an acquired trait. Clyde and Shute (1957) found incomplete cross-resistance between pyrimethamine and proguanil, and Peters (1975) found the same between the sulfonamides and sulfones. Multidrug resistance was described by Earle et al. (1948) in a Central American strain resistant to proguanil, mepacrine, and quinine. These findings largely have been proved accurate. Interestingly, Peters (1987) opined that the early success with proguanil and pyrimethamine followed by the rapid development of resistance to these agents, along with sharply increasing drug development costs, were primary reasons that drug companies and international agencies failed to continue a concerted effort of antimalarial drug development.

B. Antifolates and Nonfalciparum Malaria

The DHFR and DHPS inhibitors are inherently less active against *P. vivax*, *P. malariae*, and *P. ovale* than against *P. falciparum* (Coggeshall et al., 1941; Earle et al., 1948; Laing, 1968b). For example, the Chesson strain of *P. vivax* was not inhibited by 1 g of dapsone daily for 10 days and both sulfadoxine and sulfalene were incapable of effecting a radical cure of the same strain (Martin and Arnold, 1969). A field trial in Malaysia also found poor activity of both sulfonamides alone and in combination with pyrimethamine against *P. vivax* (Laing, 1968a). Likewise, DHFR/DHPS inhibitors are less active against both *P. malariae* and *P. ovale* than is chloroquine. The antifolates have had mixed clinical success in these latter species (Archibald, 1951; Young, 1957; Hurly, 1959; Clyde, 1967b; Laing, 1968b; Michel, 1968; quoted by Scholer et al., 1984). *P. falciparum* is inherently more sensitive to the effects of DHFR/DHPS inhibitors, has well described molecular markers for drug resistance, and will therefore be the focus of the rest of this review.

C. Drug Effects on Parasite Stages

Antimalarials have varying effects on the different stages of the malaria parasite's life cycle (Terzian, 1970). The antifolates, quinine and mefloquine, all exert little or no effect on the parasites during the first 24 h of their life cycle (Dieckmann and Jung, 1986a; Rieckmann et al., 1987; Watkins et al., 1993) and appear to affect only the actively dividing forms of *Plasmodium spp.* (schizonts) (Jones et al., 1948; McGregor and Smith, 1952; Gutteridge and Trigg, 1971). The DHFR and DHPS inhibitors inhibit DNA synthesis, and their toxic effect on the parasite reaches a peak in the late erythrocytic schizont stage, precisely when DNA synthesis peaks (Hyde, 1990). Parasites treated with antifolates will continue to mature, cytoadhere (attach to vascular endothelium and/or other red blood cells), and develop into gametocytes following treatment. Any early decline in peripheral parasitemia following administration of these drugs is that which would have occurred in the absence of drug treatment (McGregor and Smith, 1952; White, 1997) and is due to cytoadherence and "sequestration". In contrast, chloroquine, artemesinin, and other drugs act on early ring stages (Rieckmann et al., 1987; Geary et al., 1989; Landau et al., 1992; ter Kuile et al., 1992, 1993) and will enhance clearance of parasites shortly after administration, potentially preventing further development of susceptible parasites and worsening of clinical illness (White, 1994; Enosse et al., 2000).

D. Parasite Clearance Following Antimalarial Drug Treatment

Several factors affect the rate of parasite clearance from the peripheral blood after drug treatment, including parasite biomass at initiation of treatment, the degree of parasite life-stage synchronization, the proportion of parasites in a life-stage susceptible to the drug's effects, host immunity, and micronutrient levels, as well as any innate parasite drug resistance. As the parasites mature, they adhere to vascular endothelium and "disappear" from peripheral blood before re-entering the peripheral circulation as merozoites. This re-entry of parasites into the peripheral circulation may be misinterpreted as drug failure, but an early increase in the peripheral parasitemia (<12 h following initiation of therapy) is normal following administration of sulfa drugs. Recovery from an acute falciparum malaria episode following antimalarial treatment is assessed in vivo by parasitologic and clinical parameters (see Tables 1 and 2) (World Health Organization, 1973, 1996, 2002). Parasitologic outcomes are concerned only with the presence or absence of parasites and do not consider clinical signs such as fever. Parasitologic recovery is defined as the clearance of parasites from peripheral blood smears. In infections with highly drug-resistant parasites, parasite density in the peripheral blood may not decline to undetectable levels and may continue to increase following treatment. Parasites with lower levels of resistance are generally cleared completely from the peripheral blood (that is, parasite density levels drop below those detectable by microscopy, about 10–50 parasites/μl), but reappear or recrudesce, at a later time, with or without a return of symptoms. In vivo parasitologic response to treatment traditionally has been measured with a three-tiered grading scheme, RI–RIII, as outlined in Table 1. Treatment efficacy can also be assessed using definitions that consider clinical as well as parasitologic responses to treatment (see Table 2) (Rieckmann, 1990; in vivo validation, see Plowe et al., 2001). Therapeutic efficacy, or adequate clinical and parasitological response, is characterized by an early reduction of parasite density and lack of fever or signs of severe malaria and lack of recurrent parasitemia. These parasitologic and clinical in vivo methods of measuring parasite resistance tend to overestimate high-level parasite resistance and early treatment failures (Plowe et al., 2001) and are influ-

TABLE 2
Measurement of in vivo therapeutic efficacy using both parasitologic and clinical outcomes in high transmission areas

Early Treatment Failure (ETF)
Development of danger signs or severe malaria on day 1, day 2, or day 3, in the presence of parasitemia
Parasitemia of day 2 higher than day 0 count irrespective of axillary temperature
Parasitemia on day 3 with axillary temperature ≥37.5°C
Parasitemia on day 3 ≥25% of count on day 0
Late Treatment Failure (LTF)
Late Clinical Failure
Development of danger signs or severe malaria after day 3 in the presence of parasitemia, without previously meeting any of the criteria of early treatment failure
Presence of parasitemia and axillary temperature ≥37.5°C on any day from day 4 to day 14, without previously meeting any of the criteria of early treatment failure
Late Parasitological Failure
Presence of parasitemia on day 14 and axillary temperature <37.5°C, without previously meeting any of the criteria of early treatment failure or late clinical failure
Adequate Clinical and Parasitological Response (ACPR)
Absence of parasitemia on day 14 irrespective of axillary temperature without previously meeting any of the criteria of early treatment failure or late clinical failure or late parasitological failure

enced by host and pharmacokinetic factors independent of parasite drug resistance.

Clyde wrote "a clear distinction must be made between drug resistance and drug failure because the latter may imitate resistance, thus complicating unnecessarily the choice of eradication or control operations" (Clyde, 1972b). He further suggested a difference between *parasitologic resistance* and *drug failure*, the latter depending upon pharmacodynamic and other factors in addition to parasite resistance (Clyde, 1972a). Parasitologic resistance in this context was defined as the ability of a parasite strain to multiply or to survive in the presence of drug concentrations that normally destroy parasites of the same species or prevent their multiplication (World Health Organization, 1963). Clyde also discussed the term "relative" resistance, wherein he defined two kinds of relative parasitologic resistance: "real" parasite factors (see also McNamara et al., 1967) and "apparent" host factors, such as immunity, with treatment of nonimmunes versus semi-immunes providing an example (Clyde, 1972b). Even recent discussions of parasitologic resistance are complicated by interpretation. A recent review noted that "the term 'chloroquine resistance' can lead to some misunderstandings when it is taken by some to refer to in vitro phenotypes, by others to refer to the ability of malaria parasites to survive chloroquine at therapeutic serum concentrations in vivo, and yet by others to refer to the outcome of a clinical episode after chloroquine therapy" (Wellems and Plowe, 2001).

III. Dihydrofolate Reductase-Thymidylate Synthase

There is significant homology between *P. falciparum* DHFR and other species' DHFR, despite only 24 to 42% sequence homology (Rastelli et al., 2000; Yuthavong, 2002). *P. falciparum* dihydrofolate reductase-thymidylate synthase (DHFR-TS) is encoded by a single-copy gene on *P. falciparum* chromosome four, with the two enzymes forming a bifunctional protein (Bzik et al., 1987) similar to other protozoans but distinct from bacteria and higher order eukaryotes. The DHFR-TS of *P. falciparum* contains 608 amino acids, the first 231 comprising the DHFR domain, the next 89 residues forming the junction region, which joins the remaining 288 residues of the thymidylate synthase domain. Dihydrofolate reductase is comprised of eight central β-strands between four α-helices, with an additional three short α-helices (Yuvaniyama et al., 2003). As noted earlier, DHFR-TS provides reduced folate for use in the thymidylate cycle, and inhibition of DHFR-TS results in arrested DNA synthesis secondary to reduced levels of dTMP (Ferone, 1977).

A. Dihydrofolate Reductase Inhibitors

The potency of the different DHFR inhibitors varies widely. WR99210 (the active metabolite of PS-15) is the most potent plasmodial DHFR inhibitor identified thus far, whereas cycloguanil (DHFR-inhibiting active metabolite of proguanil) and chlorcycloguanil (DHFR-inhibiting active metabolite of chlorproguanil) (Hawking, 1947; Carrington et al., 1954) are more potent than pyrimethamine (Ferone et al., 1969; Milhous et al., 1985; Winstanley et al., 1995; Sirawaraporn et al., 1997a; Nzila-Mounda et al., 1998). Trimethoprim is the least potent of the antimalarial DHFR inhibitors (Ferone et al., 1969; Iyer et al., 2001).

1. Cross-Resistance between Dihydrofolate Reductase Inhibitors. Several studies have found a lack of or incomplete in vitro cross-resistance between pyrimethamine and cycloguanil in *P. falciparum* (Milhous et al., 1985; Winstanley et al., 1995), *P. gallinaceum* (Rollo, 1952a), and *P. berghei* (Thompson and Bayles, 1968), and in vivo findings were suggestive of the same (Clyde, 1967a; Vestergaard Olsen, 1983). Other studies showed that the degree of cross-resistance in resistant

clones varied (Robertson et al., 1952; Jones, 1953) and that cycloguanil-induced resistance was "broader" than that induced by pyrimethamine (Thompson and Bayles, 1968), although a significant, but incomplete, in vitro (Petersen, 1987) and in vivo (Martin and Arnold, 1968b) cross-resistance between pyrimethamine and trimethoprim existed. These studies suggested a related but incompletely shared mechanism of resistance.

B. Identification of Antifolate Drug Target

In line with the earlier in vivo prophylaxis studies by Clyde and Shute in the 1950s, selection for an in vitro drug-resistant phenotype was shown to occur following administration of pyrimethamine for treatment of acute malaria. For example, 4 days after pyrimethamine treatment of a mixture of sensitive and resistant parasites, only parasites with a drug-resistant phenotype remained (Nguyen-Dinh et al., 1982). Separate investigations revealed that pyrimethamine-resistant and -sensitive isolates had identical uptake of pyrimethamine, but that the DHFR activity of resistant strains was 300 times less sensitive to the inhibitory effects of pyrimethamine (Dieckmann and Jung, 1986b). These early in vivo and later in vitro studies showing rapid development of resistance in response to drug pressure suggested a relatively simple mechanism of resistance, such as individual point mutations in a single gene. Evidence of a genetic basis for antifolate resistance first arose from a genetic crossing study in which pyrimethamine-sensitive and -resistant parasite lines were crossed in the mosquito vector (Walliker et al., 1975). The drug resistance phenotype segregated independently of the other enzymatic markers, but in a similar manner, demonstrating that recombination had occurred between the original parental lines.

C. Point Mutations within Dihydrofolate Reductase Are Responsible for in Vitro Resistance

Continued investigation of the *dhfr* gene with the eventual sequencing of both phenotypically sensitive and phenotypically resistant *P. falciparum dhfr* provided the first direct evidence that point mutations within the *dhfr* gene were responsible for the resistant phenotype. Dihydrofolate reductase derived from the pyrimethamine-sensitive clone, 3D7, and from isolates with varying degrees of resistance to pyrimethamine were sequenced (Cowman et al., 1988). A serine resided at position 108 in the sensitive 3D7 clone, but there was a change to asparagine (S108N) in the resistant isolates. Other, successively more resistant isolates demonstrated additional mutations at codons 51 (N51I), 59 (C59R), and 164 (I164L). It was believed that the conservative change of isoleucine to leucine at codon 164 would not have a profound effect on pyrimethamine binding. The Palo Alto "clone" carried a unique set of mutations, A16V plus S108T. A study published at the same time by another group similarly found that the addition of DHFR N51I and C59R mutations confer greater levels of pyrimethamine resistance than does S108N alone (Peterson et al., 1988), strengthening the evidence that point mutations in *dhfr* were the cause of pyrimethamine resistance. Once again, the allele containing A16V and S108T was found in only one isolate (FCR3). This second group proposed that these mutations arose independently, i.e., that pyrimethamine resistance was an acquired trait, a supposition first put forth by Covell in 1949 (Covell et al., 1949).

D. The Move to Field Isolates

The 50% inhibitory concentrations (IC_{50}) of pyrimethamine and cycloguanil of 10 isolates from around the world, each with differing degrees of resistance to DHFR inhibitors, were compared, and in general, resistance to these drugs rose in parallel, although there were exceptions (Foote et al., 1990). In particular, the A16V/S108T allele yielded much higher IC_{50} values for cycloguanil than for pyrimethamine and was the only allele to do so (Foote et al., 1990). All other mutations contained at a minimum S108N and yielded higher IC_{50} for pyrimethamine than cycloguanil with the exception of S108N/C59R/I164L, which showed similarly high levels of resistance to both drugs (Peterson et al., 1990). These findings suggested that S108N was an essential first mutation in DHFR and that additional mutations at codons 51 and/or 59 and 164 increased the IC_{50} for pyrimethamine. This finding was supported by further in vitro resistance tests of field isolates and sequencing of their respective *dhfr*, which confirmed that greater in vitro pyrimethamine resistance correlated with a greater number of DHFR mutations (Basco et al., 1995; Nzila-Mounda et al., 1998). For example, the single mutation S108N caused a 25-fold greater IC_{50} of pyrimethamine than wild-type DHFR. Double mutations did not cause significant further increases in IC_{50}, but the triple DHFR mutant (S108N/N51I/C59R) was 225-fold more resistant to pyrimethamine and 48-fold more resistant to cycloguanil than wild-type DHFR. These and subsequent genetic transformation studies (van Dijk et al., 1995; Wu et al., 1995, 1996; Crabb and Cowman, 1996;) added further strength to the concepts that 1) point mutations in DHFR are responsible for in vitro resistance, 2) greater numbers of mutations in DHFR leads to greater drug resistance, 3) cross-resistance between pyrimethamine, cycloguanil, and proguanil is incomplete, and 4) S108N is a necessary first mutation in DHFR (except the case of the rare A16V/S108T allele).

Hitherto, unknown mutations at DHFR codon 50 (C50R) and a 15-base pair repeat inserted between codons 30 and 31, termed the Bolivia repeat, were described in samples from Latin America (Plowe et al., 1997). Genetic transformation studies in yeast suggested that the C50R mutation plays a role similar to the African C59R mutation (Cortese and Plowe, 1998),

and these two mutations appear to be mutually exclusive (Plowe et al., 1997; Cortese et al., 2002). The Bolivia repeat was subsequently not found to play a role in resistance (Cortese and Plowe, 1998) and may instead compensate for the decreased DHFR enzyme function that accompanies the I164L mutation in South American isolates.

Only one study has reported isolates with wild-type serine-108 in association with other DHFR mutations. This study from Uganda found the serine-108/N51I/C59R mutation in all "resistant" infections following treatment of uncomplicated falciparum malaria with trimethoprim-sulfamethoxazole (Jelinek et al., 1999), suggesting that serine-108 is important for trimethoprim resistance and that trimethoprim and pyrimethamine have different molecular mechanisms of resistance. Homology modeling predicts that trimethoprim would be less affected than pyrimethamine by mutation at DHFR codon 108 (Warhurst, 2002), but is unclear on the effect on trimethoprim of further mutations at 51 and 59 in combination with S108N or S108. The unusual finding by Jelinek, which could have implications for malaria control and for trimethoprim-sulfamethoxazole prophylaxis against opportunistic infections in human immunodeficiency virus-infected persons in Africa where sulfadoxine-pyrimethamine is used, was not supported by later in vitro experiments using recombinant yeast expressing the novel serine-108/N51I/C59R allele (Iyer et al., 2001) and *P. falciparum* isolates with known *dhfr* mutations (Khalil et al., 2003). In these studies, as had been previously reported (Winstanley et al., 1995), there was significant in vitro cross-resistance between pyrimethamine and trimethoprim. Alleles that included the S108N mutation were more resistant to both trimethoprim and pyrimethamine. Specifically, the triple mutant S108N/N51I/C59R was more resistant to trimethoprim than the genetically engineered serine-108/N51I/C59R mutant reported to have been selected by drug treatment with trimethoprim-sulfamethoxazole in the Uganda study (Iyer et al., 2001). Again, in contrast to the Uganda study, Khalil reported that this triple mutant allele predominated in recrudescent infections after treatment with trimethoprim-sulfamethoxazole (Khalil et al., 2003). The serine-108/N51I/C59R genotype has not been found in nature by other groups nor has it been confirmed by DNA sequencing. At this time, the weight of the evidence supports the idea that trimethoprim does induce the DHFR S108N mutation and that this mutation confers resistance to trimethoprim as it does to pyrimethamine.

E. Gene Amplification

Gene amplification as a source of folate resistance has not been demonstrated in nature and appears to play no role in resistance. A single in vitro study did find gene amplification to be the only method of pyrimethamine resistance to develop after 22 to 46 weeks of cultivation with increasing concentrations of pyrimethamine (Thaithong et al., 2001). Two other studies showed increased amounts of DHFR enzyme in resistant isolates, which suggested gene amplification (Kan and Siddiqui, 1979; Inselburg et al., 1987). Yet, analysis of pyrimethamine-resistant field isolates from around the world has consistently failed to demonstrate any evidence of DNA fragment or extrachromosomal amplification (McCutchan et al., 1984; Chen et al., 1987; Cowman et al., 1988; Peterson et al., 1988; Foote et al., 1990). Human thymidylate synthase expression can be regulated via binding to its own mRNA (Lin et al., 2000). Translational regulation of DHFR-TS occurs similarly in *Plasmodium spp*., although unlike regulation in humans, the binding of mRNA does not occur at the active site of the enzyme. Therefore, binding of inhibitor or substrate does not free mRNA for translation (Zhang and Rathod, 2002).

F. Mutation Rates within the Dihydrofolate Reductase Gene

The determination of mutation rates of genes whose products are targets of antimalarial drugs is important in the testing of new antimalarial compounds or combinations. Mathematical modeling may be applied to anticipate the time course of the rise in drug resistance and to predict the spread of drug resistance within a population. Numerous studies have demonstrated the development of antifolate drug resistance in both rodent malarias (Bishop and Birkett, 1947; Williamson et al., 1947; Ramakrishnan et al., 1961; Bishop, 1962; Martin and Arnold, 1968a) and *P. falciparum* (Gassis and Rathod, 1996; Paget-McNicol and Saul, 2001), and together they suggest a spontaneous mutational rate of nuclear genes, such as *dhfr*, on the order of 10^{-9}/parasite/cycle. Support for such low mutational rates was indirectly obtained in experiments using fewer than 10^9 parasites, which failed to detect any drug-resistant parasites or mutations (Bishop, 1958; Rathod et al., 1997). Furthermore, other studies achieved more rapid development of resistance when strong drug pressure was applied to animals with heavy parasitemias (Rollo, 1952b; Clyde and Shute, 1954; Diggens et al., 1970) compared with suppressive dosing of animals with low-grade parasitemias. Another in vitro study induced the DHFR S108N mutation in a pyrimethamine-sensitive parasite line by applying drug pressure (Paget-McNicol and Saul, 2001) and estimated the mutation rate of *dhfr* mutation to be $<2.5 \times 10^{-9}$ mutations/*dhfr* gene/replication.

There is also evidence to suggest that different parasite isolates have differing mutational capabilities. For example, the most "mutagenic" parasite line of five tested, W2 from Southeast Asia, developed mutations at a rate of 10^{-6}, which was at least 100 times greater than any of the other clones tested (Rathod et al., 1997), and older in vivo data with *P. gallinaceum* suggests varia-

tion of mutational capabilities between different parasite isolates (Bishop, 1962). Studies of the cytochrome *b* gene of *P. falciparum* also demonstrated a low mutational rate of 2×10^{-9} mutations/parasite/cycle (Avise, 1991), and field studies of *msp1* and *ama1* genes of *P. vivax* (Figtree et al., 2000) are all in accordance with the mutational rates found for the *dhfr* gene of *P. falciparum*.

Many more than 10^9 parasites would be present in symptomatic infections in semi-immune individuals (10^8–10^{12}; White et al., 1999), but even so, the rapid induction of resistance in natural populations (Clyde and Shute, 1957; Nguyen-Dinh et al., 1982) may be due, in part, to selection of small numbers of parasites with pre-existing mutations (Bishop, 1962; Martin and Arnold, 1968a; Nguyen-Dinh et al., 1982; Spencer et al., 1984; Kun et al., 1999; Wootton et al., 2002; Roper et al., 2003, 2004). Mathematical models validated with data from field studies may help to determine whether selection for pre-existing mutations or spontaneous point mutations within the parasite population of the host play a more important role in antimalarial drug resistance.

G. Enzyme Kinetic Analysis of Dihydrofolate Reductase

Enzyme kinetic studies can help explain why certain mutations or allelic patterns are more prevalent than others. The first kinetic studies used clones of pyrimethamine-resistant isolates rather than recombinant DHFR. Such resistant parasite lines had similar substrate affinity (K_m), as did sensitive clones, but significantly increased K_i for pyrimethamine, in one case 300-fold greater than a sensitive isolate (McCutchan et al., 1984; Dieckmann and Jung, 1986b; Chen et al., 1987). Purified DHFR enzyme from highly resistant (7G8) and moderately resistant (HB3) parasite lines had 500- and 15-fold less affinity, respectively, for pyrimethamine than did the enzyme from the sensitive parasite line (3D7) (Zolg et al., 1989). Recombinant *P. falciparum* DHFR enzyme has since been expressed in *Escherichia coli* for enzyme kinetic studies (Sirawaraporn et al., 1990, 1993, 1997a; Sano et al., 1994; Hekmat-Nejad et al., 1997). These elegant studies provide a rationale for the specific sequence of mutation accumulation and commonly occurring alleles, based on the combined effects on drug resistance and enzyme kinetic function. Serine-108-Asn (S108N) alone confers moderate pyrimethamine and cycloguanil resistance (approximate 10-fold increase in K_i compared with wild type) at minimal catalytic/kinetic cost to the enzyme as measured by substrate turnover (K_{cat}/K_m) and substrate affinity (K_m). Subsequent mutation at codon 51 (N51I) results in similar K_i for pyrimethamine and cycloguanil as the single-mutated (S108N) DHFR, but an improved k_{cat}/k_m on par with wild-type enzyme (Sirawaraporn et al., 1997a). Cysteine-59-Arg (C59R), in combination with the aforementioned double mutation, forms the so-called "triple mutant" (S108N/N51I/C59R). The triple mutant confers a 100-fold increase in the K_i of pyrimethamine and cycloguanil, but incurs a significant cost to kinetic activity of the enzyme, demonstrating k_{cat}/k_m values 50-fold lower than the wild-type enzyme. The addition of I164L to the triple mutant, forming the "quadruple mutant", increases pyrimethamine and cycloguanil K_i 500-fold over wild-type DHFR, without further significant decline in enzymatic function compared with the triple mutant.

Interestingly, the mutation combination associated with cycloguanil rather than pyrimethamine use, A16V/S108T, had near wild-type pyrimethamine K_i, but an 800-fold increase in cycloguanil K_i. The kinetic activity of the enzyme was severely impaired and unlike all other mutation combinations studied, the free energies for pyrimethamine and cycloguanil binding were significantly higher than the sum of the corresponding single mutations. The A16V mutation alone entirely explains cycloguanil resistance, but the addition of S108T markedly improves kinetic parameters (Sirawaraporn et al., 1997a). An allele not seen in nature, A16V/S108N, was found to lack any catalytic activity, explaining its natural absence.

Each mutation, with the exception just noted, is more than additive in its effect on resistance, with over 1000-fold synergism in the quadruple mutant, as measured by interaction energy (Sirawaraporn et al., 1997a). One can conclude that mutations evolve not solely for their ability to cause resistance, but for their synergistic contribution to enzyme function in the context of existing mutations, suggesting a necessary sequential progression of mutations. The mutations seen only in combination with S108N: N51I, C50R/C59R, and I164L do not confer resistance to pyrimethamine or cycloguanil on their own; to do so, S108N must be present. The cost of greater reductions in k_{cat}/k_m values for dihydrofolate and NADPH suggests that the more highly mutated forms of DHFR might be selected against in the absence of sufficient drug pressure.

In an attempt to determine why it is that S108N is the preferred primary mutation, 10 different amino acid substitutions were used in place of asparagine at position 108 (Sirawaraporn et al., 1997b). Only asparagine provided an enzyme with near wild-type kinetics (<2-fold lower K_{cat}/K_m). Despite other substitutions providing a higher level of antifolate resistance, the asparagine substitution provides the enzyme kinetics necessary for parasite survival with the least number of base-pair substitutions.

Further kinetic studies show that S108N/C59R is favored in terms of pyrimethamine resistance with a 5-fold increase over wild type, but it has significantly impaired kinetic function. The double mutation S108N/N51I has a 2- to 3-fold increase in pyrimethamine resistance and kinetic parameters closer to wild type. Kinetic studies alone cannot explain why certain alleles are more common in nature (Sirawaraporn et al., 2002). For example, the quadruple mutant is not found in Africa, but it

demonstrates markedly increased resistance with only minimal loss in k_{cat} over the triple mutant, which is found in Africa. However, it must be noted that these enzyme kinetic studies in recombinant DHFR monomers that are not linked to TS may not reflect exactly the kinetics of the bifunctional DHFR-TS dimeric protein of parasites in nature. This leaves open the possibility that the DHFR I164L mutation is more deleterious to parasite fitness in nature than these experiments predict, which could explain its absence in Africa, where the large reservoir of asymptomatic, untreated infections favors survival of more fit, less resistant parasites.

In summary, it appears that individual point mutations are favored for the best K_i to K_{cat}/K_m benefit. The ideal combination of DHFR mutations in any given population is influenced by both the local dynamics of drug pressure particularities and any competition among circulating parasite "clones". It might be difficult for parasites harboring newer drug-resistant alleles to successfully invade and establish themselves in a parasite population, particularly if previously established, circulating drug-resistant alleles offer adequate protection to circulating parasites from the antifolate drugs in use, as may be the case with the triple mutant in Africa.

H. Relationship of Point Mutations to Dihydrofolate Reductase Structure—Crystallography

Prior to the crystallization of DHFR-TS, several attempts were made to model the effect of known DHFR mutations on drug binding (McKie et al., 1998; Santos-Filho et al., 2001). With the recent crystallization of the DHFR-TS enzyme of *P. falciparum*, it is now apparent that many of the predictions based upon the models were correct. The rigid length of the pteridine ring of many folate inhibitors fits between residues 108 and 54 within the active site of the enzyme (Warhurst, 1998; Yuvaniyama et al., 2003) (Fig. 2). Pyrimethamine is relatively rigid, notably more so than trimethoprim, which therefore allows less torsional freedom within the active site. The longer distances between residues 108 and 54 and trimethoprim reduce the inhibitory activity of trimethoprim against *P. falciparum* DHFR (Warhurst, 1998, 2002). Of all the known DHFR inhibitors, WR99210 most closely resembles the flexibility seen in the natural substrate dihydrofolate (DHF), perhaps explaining its greater potency and reduced susceptibility to point mutations in DHFR. Crystallization of WR99210 within the binding site of DHFR has confirmed this prediction (Yuvaniyama et al., 2003) (Fig. 3). Point mutations disrupting WR99210 binding may also disrupt substrate binding significantly enough to be detrimental to parasite survival.

As predicted in the model presented by Delfino et al. (2002), the primary DHFR mutation, S108N, affects drug accommodation at the active site of the enzyme, rather than affecting drug entry into this site. The NH_2 of asparagine 108 interferes with the chlorine atom of pyrimethamine, causing the later to be displaced within the active site (Warhurst, 1998, 2002). Further compromise of the planar aspect of the pyrimethamine pteridine ring is caused by the carboxylate group of asparagine 54 and the pyrimethamine ethyl group. This loss of planarity is theorized to be responsible for the pyrimethamine resistance caused by the S108N mutation

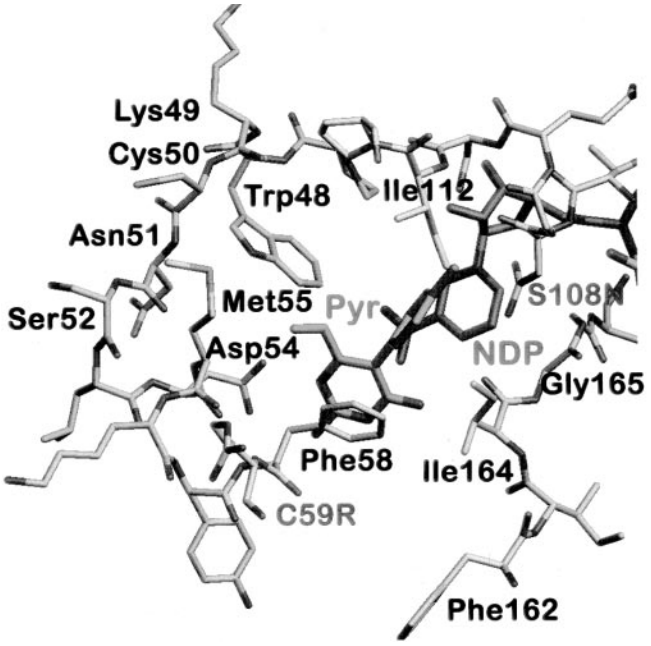

FIG. 2. DHFR enzyme-inhibitor interactions at the active site. The DHFR double mutant, S108N/C59R, complexed with pyrimethamine. Reproduced with permission of the authors (Yuvaniyama et al., 2003).

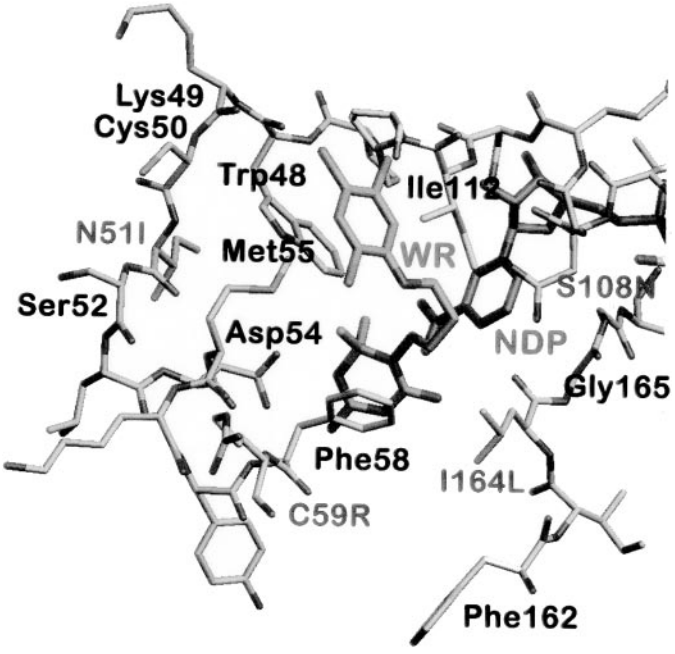

FIG. 3. DHFR enzyme-inhibitor interactions at the active site. The DHFR quadruple mutant, S108N/N51I/C59R/I164L, complexed with WR99210. The flexibility of the WR99210 tail allows its binding to DHFR to be unaffected by known mutations. Reproduced with permission of the authors (Yuvaniyama et al., 2003).

(Delfino et al., 2002). There is less hindrance of cycloguanil planarity, being slightly less rigid than pyrimethamine, although the chloride and NH_2 group of asparagine 108 do interfere to some degree. Modeling and crystallization images of WR99210 in DHFR active site show that there is no hindrance of this molecule in the enzyme's active site because the flexible side chain is oriented away from the S108N side chain (compare Figs. 2 and 3). This correlates well with in vitro data that demonstrates a higher level of resistance to pyrimethamine than to cycloguanil and essentially no effect on WR99210 in the presence of the S108N mutation alone or in combination with N51I (S108N/N51I) (Ferlan et al., 2001).

Likewise, A16V mutation does not displace pyrimethamine from the active site, but causes significant loss of cycloguanil planarity due to interactions between methyl groups on both valine 16 and cycloguanil (Rastelli et al., 2000; Delfino et al., 2002) and may interfere with NADPH binding (Yuvaniyama et al., 2003). The additional mutations N51I and C59R are distant from the enzyme active site, but on the same helix as residue 54, which is critical to substrate binding (Yuvaniyama et al., 2003). The side chain of C59R extends away from the inhibitor binding site and may be involved in DHF binding, improving substrate binding affinity in the presence of S108N and N51I. Mutations at residues 51 and 59 may also impede admission of the inhibitor binding site (Santos-Filho et al., 2001; Warhurst, 2002). This could occur via interactions of polar or charged residues on isoleucine and arginine and the protonated pyrimethamine and cycloguanil (Delfino et al., 2002). The effect of the additional mutations at 51 and 59 must be essentially silent in the absence of the S108N mutation, but additive when it is present (Sirawaraporn et al., 1997a), and this is supported by the crystal structure, which demonstrates little change in the orientation of D54 in the presence of N51I and C59R. In agreement with kinetic data (Sirawaraporn et al., 1997a), I164L incorporated into the model in the absence of other mutations predicts a decrease in pyrimethamine resistance, a mild increase in cycloguanil resistance, and a marked increase in resistance to both drugs in the presence of S108N, N51I, and C59R. Crystal data suggests that this is due to an increase in the active site gap between the α-carbon of C50 and I164L.

IV. Pyrophosphokinase-Dihydropteroate Synthase

The role of DHPS in sulfonamide resistance was elucidated following the same pattern as for DHFR, with the cloning of the gene, the identification of point mutations associated with in vitro drug resistance, and detailed characterization of the heterologously expressed wild-type and mutant enzymes. Like DHFR, DHPS is a bifunctional polypeptide (in contrast to bacterial DHPS) (Hyde and Sims, 2001) encoded by a gene also encoding 7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase, the enzyme immediately proceeding DHPS in the folate biosynthesis pathway (Brooks et al., 1994; Triglia and Cowman, 1994). DHPS catalyzes the condensation of *p*-aminobenzoic acid with 6-hydroxymethyldihydropterin pyrophosphate yielding 7,8-dihydropteroate. As with antagonism of DHFR, antagonism of DHPS in *Plasmodium spp.* results in depletion of the dTTP precursor and subsequent decrease in DNA production (Schellenberg and Coatney, 1961). Unlike DHFR, there is no known human counterpart to DHPS.

A. Folate Effect

Sulfonamides and sulfones, such as sulfadoxine and dapsone, can inhibit DHPS activity, but acceptance of this paradigm was delayed because of the ability to utilize exogenous folate by many, but not all, *P. falciparum* isolates (Trager, 1958; Ferone, 1977; Krungkrai et al., 1989; Wang et al., 1997b,c, 1999). This salvage pathway is believed to provide only a minority of folate production in *P. falciparum*, the majority being procured via de novo synthesis. The capability of *Plasmodium spp.* to utilize exogenous folate was demonstrated in experiments in which the addition of physiologic concentrations of folate to culture medium caused a 1000-fold decrease in sulfadoxine inhibitory activity, and higher, nonphysiologic concentrations of folate were able to partially inhibit the activity of pyrimethamine (Chulay et al., 1984). Similarly, three of four *P. falciparum* strains tested were capable of growing in culture media devoid of folic acid or *p*-ABA (Milhous et al., 1985). The fact that folic acid was a more potent antagonist of sulfadoxine activity than *p*-ABA provided further support for exogenous folate utilization via a pathway that obviated the need for DHPS (i.e., bypassed the enzyme) (Wang et al., 1999). Other groups have subsequently generated ample evidence for utilization of exogenous folate in *P. falciparum* (Schapira et al., 1986; Krungkrai et al., 1989; Wang et al., 1997b). The folate effect is abolished by the addition of pyrimethamine to assay cultures in concentrations substantially higher than those needed to inhibit wild-type DHFR (Wang et al., 1999). Moreover, even parasites with highly mutated, pyrimethamine-resistant DHFR are unable to metabolize exogenous folate in the presence of pyrimethamine, suggesting that the folate effect is a DHFR-independent pathway, but one which may be inhibited by high concentrations of pyrimethamine. The exact mechanism or genetic basis of the "folate effect" is not known at this time, nor is the prevalence of this capacity in natural parasite populations, although it has been found in parasite lines from both Africa and Southeast Asia. The folate effect did not segregate with *dhps* genotypes in a genetic cross (Wang et al., 1997b), and although it was linked to the *dhfr* gene in the cross progeny, it was not linked with *dhfr* sequence in other unrelated parasite lines (Wang et al.,

1997b), suggesting that a gene responsible for the folate effect is located near, but not at the *dhfr* locus on chromosome four.

1. Folate Effect and Drug Resistance. The folate effect is thought to be of potential importance in resistance to sulfonamide drugs and sulfa-DHFR inhibitor combinations. The concentration of pyrimethamine necessary to inhibit wild-type DHFR is <20 nM, which is far below that needed to completely abolish the folate effect (~100 nM). When exogenous folate is utilized, there may be little drug pressure to encourage resistance in either DHPS or DHFR because folate needs are met via a mechanism independent of these two enzymes. This would be the case when a sulfa drug is used without pyrimethamine. When pyrimethamine is added to the mix, the DHFR genotype becomes important. If fewer than three mutations are present in DHFR, the parasite can be rapidly killed before levels of pyrimethamine drop below their effective IC_{50} for DHFR, possibly even before levels of pyrimethamine drop below that which is necessary to block exogenous folate use. In this case, selection pressure is placed on both DHFR and DHPS while exogenous folate utilization is prevented. When more than three mutations are present in DHFR, there will be selection pressure on DHPS while exogenous folate utilization is blocked, since the de novo pathway is required for parasite folate needs.

2. Folate Effect and in Vitro Sulfonamide Testing. The ability of this folate salvage pathway and of small amounts of *p*-ABA to overcome the inhibitory effects of the sulfonamides in vitro (Maier and Riley, 1942; Thurston, 1954) complicated the interpretation of in vitro studies of *P. falciparum* resistance to sulfonamide drugs and led some to propose that it was the dihydropterin-sulfonamide adducts inhibiting DHFR, rather than a direct sulfonamide inhibitory action on DHPS (Sirawaraporn and Yuthavong, 1986). Studies in *E. coli* demonstrated that the dihydropterin-sulfonamide products formed do not significantly contribute to the inhibitory action of the sulfonamides (Roland et al., 1979). Furthermore, accurate, reproducible in vitro sulfonamide sensitivity assays are possible if performed in near zero concentrations of folate and *p*-ABA concentrations equivalent to those found in the human red blood cell (Chulay et al., 1984; Milhous et al., 1985; Wang et al., 1997c).

B. Markers of in Vitro Resistance in Dihydropteroate Synthase

Studies in procaryotes have demonstrated that point mutations within conserved regions of DHPS confer sulfonamide resistance (Dallas et al., 1992). Investigations of sequence variation between sulfonamide-sensitive and sulfonamide-resistant isolates found four amino acid changes in highly conserved regions of DHPS, suggesting that the mutations were involved in drug resistance (Triglia and Cowman, 1994). Another group (Brooks et al., 1994) examined the gene sequence and discovered five positions at which mutations occurred, affecting four different codons: S436A/F, A437G, A581G, and A613T/S. Higher levels of resistance were found in isolates carrying either the double mutation S436F/A613S/T or the single mutation A581G. Position 581 lies within a region of the protein that is modified by sulfonamide-resistant bacteria, suggesting a key role in sulfonamide resistance (Brooks et al., 1994). No amplification of *dhps* was found in either study. Interestingly, A437G was initially not felt to be significantly linked to resistance in these in vitro studies.

The first field isolates with a mutation at position 540 from lysine to glutamate (K540E) were described in 1997 (Plowe et al., 1997; Triglia et al., 1997). The mutation occurred only in association with A437G. The trimorphic mutation at position 613 was confirmed in field isolates from Kenya to code for either alanine (wild type), threonine, or serine (mutants), as was previously described (Brooks et al., 1994; Nzila et al., 2000a).

The mutation at codon 436 has generated controversy, with some authors suggesting that the polymorphisms at 436, S436A/F, are alternative wild types (Nzila et al., 2000a), whereas others believe that it may contribute to low-level drug resistance (Brooks et al., 1994; Triglia et al., 1997). One group failed to find S436F in parasites with either K540E or A581G, supporting the idea that if not an alternative wild type, further significant mutations in DHPS are unlikely to occur in the presence of S436A/F (Plowe et al., 1997; see also Diourte et al., 1999). Another study found selection for A437G, but not S436A/F, following treatment with sulfadoxine-pyrimethamine (Kun et al., 1999) and reversal of S436F to S436/A437G following treatment with sulfadoxine-pyrimethamine (Nzila et al., 2000a). An earlier in vitro study (Triglia et al., 1997) did find that isolates with the S436A/A437G allele were more resistant to sulfonamides than were solitary A437G mutants. Median IC_{50} values were higher for parasites with S436A/A437G/A613S genotype than for the A437G mutation alone (Mberu et al., 2002), and S436A was associated with drug resistance, at least in populations with comparatively lighter exposure to malaria (Khalil et al., 2002). Thus, while higher level resistance is unlikely to develop in the presence of S436A/F alone, mutation at this codon in the presence of mutations at other codons may contribute to low-level sulfa drug resistance.

C. Enzyme Kinetics Studies on Dihydropteroate Synthase

As noted, there has been debate in the literature as to the significance of *Plasmodium spp.* DHPS mutations and resultant in vivo sulfonamide resistance, particularly with regard to mutations at codons 436 and 613 (Sirawaraporn and Yuthavong, 1986; Sims et al., 1998, 1999; Watkins et al., 1999). As with DHFR, recombinant *P. falciparum* DHPS was incorporated into *E. coli* for

enzyme kinetics studies (Sirawaraporn et al., 1997a; Triglia et al., 1997). The K_i for sulfadoxine of purified, recombinant DHPS expressed in *E. coli* varied from a low of 0.14 mM for a wild-type allele to 112 mM for a highly resistant DHPS allele (S436F/A437G/A613S), an 800-fold difference. As was found with the *dhfr* gene, additional mutations act in a synergistic rather than additive manner with regard to sulfonamide resistance. Also, similar to the DHFR mutations, certain combinations of mutations demonstrate better catalytic capabilities than others.

Predictions of common alleles in field samples can be made from an analysis of K_i versus K_{cat}/K_m of particular mutation combinations. The A437G mutation alone was the least resistant of the alleles tested. Resistance increased with the addition of S436A or A581G to A437G and was greater still with the A437G/K540E allele. Maximum resistance (of the alleles tested) was reached with S436A/A437G/A613T or S436A/A437G/K540E, the latter having an improved kinetic profile over the former (Triglia et al., 1997). Alanine-581G alone was not evaluated. The parallel increase in K_m for *p*-ABA suggested that the homologous moiety of the inhibitor within the active site of DHPS is the target of the mutations, rather than the side moiety, portending that although potencies of the compounds vary from the least potent sulfadoxine to the most potent dapsone (Nzila-Mounda et al., 1998), cross-resistance is likely in practice.

The role of DHPS mutations in sulfonamide resistance was unequivocally demonstrated (Triglia et al., 1998) with the expression of *dhps* constructs of different mutations via allelic exchange in *P. falciparum*. The A437G mutation either alone or in combination with A581G caused an approximate 5-fold increase in IC_{50} for sulfadoxine compared with wild-type enzyme. Serine-436A/A437G was 6-fold more resistant, S436A/A437G/K540E was 10-fold more resistant, and S436F/A437G/A613S was approximately 24-fold more resistant than the D10 wild type. However, because constructs differing only with respect to the presence of S436A were not tested, the role of this mutation in conferring sulfa/sulfone resistance, if any, remains uncertain.

Comparison of the *E. coli* and *P. falciparum* DHPS places codons 436 and 437 very close to both substrate and inhibitor binding locations of the enzyme. However, many of the other mutations in *P. falciparum* and *E. coli* are not close to the substrate or inhibitor binding location, suggesting another mechanism of inhibition, perhaps as with DHFR, in a channel leading to the active site of the enzyme.

D. Relationship of Point Mutations to Dihydropteroate Synthase Structure—Crystallography

Recent crystallography data from *Mycobacterium tuberculosis* DHPS (Li et al., 2000) yields insights into *P. falciparum* DHPS mutations. The DHPS amino acid sequence of *M. tuberculosis* and *P. falciparum* were compared (Baca et al., 2000), but others (Hyde and Sims, 2001) point to an error in the comparison paper where the 540 residue of *P. falciparum* was incorrectly aligned with the *M. tuberculosis* enzyme. Once the alignment is adjusted, it can be seen that codons 436, 437, and 540 all line the channel to the active site of DHPS. Moreover, codons 581 and 613 are only one to three positions away from the channel. Mutations at codons 581 and 613 may be compensatory in function, explaining why the two residues are not seen in isolation, but only with mutations at 436, 437, or 540.

The mechanisms of *P. falciparum* in vitro sulfonamide resistance appears remarkably similar to resistance to DHFR inhibitors; the greater the number of point mutations the greater the degree of resistance. It remains less clear that a necessary starting mutation such as A437G must be present, as is S108N in DHFR. Isolates with dihydropteroate synthase containing S436A, A437G, or K540E singly have been found and all confer some degree of resistance to sulfonamides. Higher level sulfa drug resistance requires that multiple mutations be present, in addition to mutations at codons 436, 437, and/or 540. Thus, it may be that mutation at any of these latter three codons is a necessary first mutation for sulfa drug resistance.

V. Parasitologic Resistance Does Not Equal Clinical Failure

The above-described studies have provided an understanding of the molecular and biochemical mechanisms of in vitro resistance to the individual dihydrofolate reductase inhibitors and sulfonamides. Although some degree of resistance to both sulfadoxine and pyrimethamine is necessary for in vivo resistance to the combination, determining the relative importance of parasite resistance to sulfadoxine or pyrimethamine in in vivo sulfadoxine-pyrimethamine failure has been challenging. Some studies point to pyrimethamine sensitivity as the key determinant of in vivo response to sulfadoxine-pyrimethamine (Watkins et al., 1997), whereas others have found in vivo success despite in vitro pyrimethamine resistance, which indicates that sulfadoxine resistance is central to the success of the combination (Nguyen-Dinh et al., 1982; Schapira et al., 1986).

As noted earlier, factors other than parasite drug resistance contribute to in vivo treatment success or failure, particularly host immunity. Indeed, the importance of host immunity in the successful application of antimalarial drug therapy was underscored by Clyde (1972b), “Without an active immune response on the part of the host, it is unlikely that any antimalarial used today will cure a patient of his malaria”. In areas where malaria is highly endemic, as in much of sub-Saharan Africa, individuals develop partial immunity to clinical illness, although they continue to become infected by the

parasite. In these areas, partial immunity is generally attained in older children, adolescents, and adults who can clear low-level drug-resistant infections that immunologically naive infants or young children cannot clear after similar courses of antimalarials (Nguyen-Dinh et al., 1982; Baird et al., 1991; Marsh, 1992; Djimde et al., 2001a; Kamya et al., 2001).

A. In Vivo Drug Failure, Additional Host Factors

Host variability in the absorption or metabolism of drugs may contribute to drug failures in malaria therapy. For example, genetic differences in the host's ability to metabolize the prodrug proguanil into the active DHFR inhibitor, cycloguanil, via hepatic cytochrome P450-dependent process, affects the prophylactic and treatment efficacy of proguanil (Watkins et al., 1988, 1990; Ward et al., 1989). Pregnancy can also alter metabolism and distribution of antifolate drugs and their active metabolites (Wangboonskul et al., 1993). Some sulfadoxine-pyrimethamine failures are attributed to individual variation in sulfonamide pharmacokinetics (Clyde, 1972a; World Health Organization, 1984). Additionally, route of administration can affect drug pharmacokinetics, which in turn, can influence therapeutic efficacy, particularly in highly drug-resistant infections (Winstanley et al., 1992). The developmental stage of the parasite at the time the drug is administered, particularly a one-time dose of sulfadoxine-pyrimethamine in a synchronous infection, may also affect treatment outcomes (Rieckmann et al., 1987).

1. In Vivo Folate Effect. It is not clear what, if any, effect folate levels have in vivo (Oppenheimer and Cashin, 1986). Compared with aparasitemic children, parasitemic children have significantly higher geometric mean red cell folate levels (Abdalla et al., 1980; Bradley-Moore et al., 1985). Children given sulfadoxine-pyrimethamine plus folate were significantly more likely to be parasitemic at day 7 and 28 post-treatment than children given either sulfadoxine-pyrimethamine alone or sulfadoxine-pyrimethamine plus iron (van Hensbroek et al., 1995). Thus, the folate effect may be clinically significant in certain situations. The utilization of exogenous folate by the parasites in the folate supplemented children might have permitted the pyrimethamine-resistant parasites to escape the inhibitory action of sulfadoxine through utilization of exogenous folate.

B. Molecular Markers and Treatment Outcomes

Prior to the advent of molecular markers of drug resistance, in vivo studies and in vitro culture assays were the only available methods to assess parasite drug resistance, and in vivo efficacy studies remain the gold standard for monitoring drug-resistant malaria (Rieckmann et al., 1978). The limitations of in vitro studies (Nguyen-Dinh and Payne, 1980), which are not amenable to large scale epidemiological surveys, have led to the development of mutation-specific PCR and restriction digest assays to detect *dhfr* and *dhps* mutations from field samples. In vitro methods are cumbersome, fail frequently, vary among laboratories, and the end results may not correlate well with in vivo results, in part due to host immunity (Spencer et al., 1984; Brasseur et al., 1999; Ndounga et al., 2001). In contrast, molecular methods offer the advantage of minimally invasive sample collection (digital puncture) (Plowe et al., 1995), elimination of the need for cold transport of live or frozen parasites from remote locations, greater number of samples that can be easily shipped to central testing sites, and large numbers of assays which can be performed simultaneously.

VI. Molecular Assays

To date, most studies have employed one of two common methods for analysis of mutations within parasite populations: allele-specific oligonucleotide polymerase chain reaction (Zolg et al., 1989) or allele-specific enzyme digestion (Eldin de Pecoulas et al., 1995). These methods are very useful for surveillance of known mutations. Under ideal conditions, allele-specific oligonucleotide PCR techniques can detect minor alleles consisting of 1% of the total population (Shaio et al., 1998), although under less than ideal conditions using samples from field collection, the technique is unlikely to detect mutations unless they are greater than 10% of the population of mixed infections. Depending upon the nature of the investigation desired, this might be an important limitation in areas where mixed infections are common (i.e., more than one parasite "strain" or clone circulating at any one time). Nested PCR can be altered by the amount of template used and substrate available (Chaparro et al., 2001). This limitation might explain some of the ambiguities in the literature, which may be the result of imprecise PCR techniques. Likewise, at least some mutant recrudescent parasites following treatment are in fact subpopulations that were not detected by the pretreatment nested PCR (Kun et al., 1999). For example, if less than 2% of an initial infection consisting of 10^{10} parasites had a particular drug-resistant allele, this minority population would likely be selected for by drug treatment, but could have been missed on the pretreatment PCR analysis. A yeast complementation assay was described that allows for an estimate of the frequency of each mutant allele in the population, even if its frequency approaches 1%. It also may provide for the detection of new mutations (Mookherjee et al., 1999), but is less applicable to high throughput analysis.

VII. Molecular Epidemiological Studies

A molecular epidemiological survey of samples from four countries, each with increasing levels of sulfadoxine-pyrimethamine resistance, Mali, Kenya, Malawi, and Boliva, demonstrated that the number of both DHFR and DHPS mutations rose in parallel with the

prevalence of sulfadoxine-pyrimethamine clinical resistance in the populations (Plowe et al., 1997). Samples from Bolivia were collected during an epidemic and might have represented a less diverse population than those at the African sites. Nevertheless, the findings supported the hypothesis that increasing numbers of DHFR and DHPS mutations leads to in vivo sulfadoxine-pyrimethamine failure. The DHFR C50R mutation was found in 52.5% of the Bolivian samples and appeared to take the place of the African C59R mutation, which in contrast, was present in 61% of Kenyan field isolates (Nzila-Mounda et al., 1998). The Bolivia repeat was found in 44% of the samples from Bolivia, and both the Bolivia repeat and C50R mutations were found only in the presence of the DHFR N51I mutation. Furthermore, the Bolivia repeat occurred only with the DHFR I164L mutation, suggesting a compensatory role for the repeat. Interestingly, most of the DHFR C50R occurred with the wild-type isoleucine-164, although one isolate did contain both C50R and I164L. Because the samples for these studies consisted of dried filter paper blood spots, in vitro susceptibility assays were not performed, and this particular allele has not been studied in kinetic assays. However, genetic transformation studies in yeast suggest that C50R has an effect similar to C59R in combination with I164L (Cortese and Plowe, 1998). Unlike other studies, mutation at DHPS codon 436 did not occur with A437G, but similar to other studies S436A never occurred with K540E or A581G mutations. Since DHPS K540E was found only in Malawi and Bolivia, areas with higher levels of in vivo resistance and A581G only in Bolivia, the area with the highest in vivo failure rates, it appears that these two mutations are associated with a greater degree of in vivo drug resistance. Except in Mali, the triple DHFR mutant S108N/N51I/C59R was widely distributed, despite nonuniform in vivo failure rates, which suggests that this allele alone is insufficient to cause in vivo failure in all populations (see also Jelinek et al., 1997).

In another molecular survey of DHFR and DHPS mutations from around the world (Wang et al., 1997a), increased numbers of mutations in both DHFR and DHPS were seen in areas with higher levels of sulfadoxine-pyrimethamine resistance. The Middle East had the lowest levels of in vivo resistance and only mutations in DHFR were seen, with S108N being the most frequent. The 18 samples from Mali were all wild type with respect to DHFR, except two samples which were triply mutant at S108N/C59R/N51I. DHPS mutations S436A, A437G, and A581G occurred more commonly, either as single or double mutations, than the DHFR mutations. Kenya and Tanzania both had the S108N/C59R/N51I DHFR triple mutant, and these East African locations had similar DHPS mutations, A437G, K540E, S436A, and A581G, in decreasing frequency. The only exception was in Tanzania, where S436A was less prevalent than A581G. Dihydrofolate reductase mutation I164L was found only in Vietnam. Some findings in this study with relatively small sample sizes have not been borne out by several subsequent studies, particularly the findings in Mali, where antifolate resistance is low, of the equal prevalence of the DHFR mutations at codons 108, 51, and 59, and the presence of DHPS mutation at codon 581.

It appears from these (and other studies below) that DHFR mutations S108N, N51I, and C59R are found in Africa, S108N, N51I, C50R, Bolivia repeat, and I164L (with a minor population of C59R) in South America, and S108N, N51I, C59R, and I164L Southeast Asia. Dihydropteroate synthase mutations are more widely distributed around the world.

Recent studies have demonstrated a common ancestry for highly antifolate-resistant *P. falciparum*. It had been previously shown that the chloroquine resistance-conferring mutations in *P. falciparum pfcrt* arose at a limited number of foci outside of Africa and that African chloroquine-resistant malaria originated from a Southeast Asian ancestor (Wellems and Plowe, 2001). Studies dating back to the 1950s had demonstrated that *P. falciparum* resistance to pyrimethamine and sulfadoxine arose locally in direct response to drug pressure (Clyde and Shute, 1954). Based on these data, it was believed that DHFR and DHPS mutations had emerged on many occasions. In South America, DHFR and DHPS mutations responsible for mid- and high-level antifolate resistance in South America had been shown to have a common ancestry (Cortese et al., 2002), but parasites with low-level resistance-conferring DHFR and DHPS had diverse ancestry consistent with multiple origins.

Roper and colleagues recently showed that a similar pattern of common ancestry for high-level antifolate-resistant malaria but diverse origins for low-level resistance was evident in *P. falciparum* infections in South Africa and Tanzania (Roper et al., 2003). Subsequently, the same group reported that high-level antifolate resistance in Africa, Southeast Asia, and South America all shared a common ancestry and that antifolate-resistant falciparum malaria in Africa had likely been imported from Southeast Asia, as chloroquine resistance had been (Roper et al., 2004).

A. Drug Treatment Effect on Post-Treatment Parasite Genotype

Several in vivo prospective studies have shown that sulfadoxine-pyrimethamine treatment is a selection factor for DHFR mutations. One of the earliest studies looking at the effect of sulfadoxine-pyrimethamine treatment on the genotype of post-treatment parasites was performed in Mali. Nearly 200 cases of uncomplicated falciparum malaria were treated with sulfadoxine-pyrimethamine and followed for 56 days. Although there were no cases of RIII resistance, post-treatment samples had a higher prevalence of DHFR mutations S108N and C59R. Dihydrofolate reductase N51I was also more com-

mon following treatment, but there were too few samples with this mutation to achieve statistical significance. The dihydropteroate synthase mutation S436A was less common following treatment with sulfadoxine-pyrimethamine, being present in 55% of pretreatment samples but only 21% of post-treatment samples (Diourte et al., 1999). No DHPS K540E or A581G mutations were found. A small study demonstrated apparent selection for the triple DHFR mutant S108N/N51I/C59R from pretreatment samples that largely contained single (S108N) and double (S108N/N51I) DHFR mutants (Nzila et al., 2000a) and another found selection for S108N (Curtis et al., 1996).

B. Molecular Markers and Treatment Outcomes

1. High Endemicity. The first attempts to use molecular markers to predict clinical failure were not able to do so accurately, due in part to the confounding effects of acquired immunity (Jelinek et al., 1997). These studies also used DHFR S108N as a sole marker for sulfadoxine-pyrimethamine resistance because of findings such as those in Brazil, where in an area of high sulfadoxine-pyrimethamine failure rates the prevalence of S108N was over 90%, which suggested that S108N could serve as a marker for sulfadoxine-pyrimethamine resistance (Peterson et al., 1991). Likewise, S108N was more prevalent in urban than in rural sites in Mali, West Africa corresponding with documented higher rates of sulfadoxine-pyrimethamine usage in urban settings (Plowe et al., 1996). This simple approach did not succeed, however, as subsequent studies found both S108N and C59R together without any evidence of RIII resistance (Diourte et al., 1999), indicating that the two DHFR mutations are by themselves insufficient to cause sulfadoxine-pyrimethamine treatment failure. In Tanzania where the occurrence of these mutations greatly exceeded that of sulfadoxine-pyrimethamine clinical failure, it was found that even DHFR mutations S108N, C59R, and N51I in combination were alone not sufficient to cause clinical failure in semi-immune populations (Jelinek et al., 1997).

In epidemic and holoendemic locations in Kenya, Omar et al. (2001b) considered the effect of host immunity on the use of DHFR/DHPS mutations to predict parasitologic failure. In settings of epidemic malaria transmission, where persons have less well developed immunity to malaria, seven of eight occurrences of the single S108N DHFR mutation and all occurrences of the single A437G DHPS mutation predicted parasitologic failure, with a positive predictive value of 100%. In holoendemic areas with high rates of malaria transmission where persons are semi-immune to malaria, the occurrence of two or more DHFR mutations predicted parasitologic failure, again with 100% positive predictive value regardless of *dhps* genotype. In another holoendemic area along the coast of Kenya where malaria transmission rates were high and the prevalence of RI and RII resistance was 16%, the *dhfr* and *dhps* genotypes of 71 pre- and 29 post-treatment samples were analyzed and correlated with parasitological outcome. Of the post-treatment samples, 55% were associated with triple (S108N/N51I/C59R) DHFR mutation and double (A437G/K540E) DHPS mutations ("quintuple mutant"), and another 13% had the triple DHFR and single (A437G) DHPS mutation. There was strong selective pressure for the DHFR triple mutant after drug treatment, with 96% post-treatment samples carrying the DHFR triple mutant, whereas 75% had either single or double mutant DHPS. Individuals infected with parasites harboring either double or triple DHFR and double DHPS mutations were at greater risk of remaining parasitemic following treatment (odds ratio of 12 compared with wild-type DHPS) (Nzila et al., 2000a). Yet another study in an area of Kenya with intense transmission found that a high percentage of isolates from patients admitted to the hospital for severe malaria, after failing two separate outpatient treatment regimens, contained triple (S108N/N51I/C59R) DHFR and double (A437G/K540E) DHPS mutations (Omar et al., 2001a). Again, this suggests selection for drug-resistant alleles, or at least mutant allele association with therapeutic failures.

Similar findings in other malaria endemic regions corroborate the above findings. In West Papua, a survey from 1996–1999 found that the double DHFR (S108N/C59R) and double DHPS (A437G/K540E) mutant was significantly associated with RII and RIII sulfadoxine-pyrimethamine failure, compared with sensitive infections (Reeder et al., 1996; Nagesha et al., 2001). Mutations were not found at codons 16, 50, 51, or 164 in DHFR, or codons 436, 581, or 613 in DHPS. In Nagesha's study, the DHPS mutation K540E was found only in association with RII- and RIII-resistant infections, not in RI or sensitive infections, offering further support for an important role for DHPS mutations in clinical resistance to sulfadoxine-pyrimethamine.

In Malawi, the first African country to officially switch to sulfadoxine-pyrimethamine as first-line therapy nationwide, the quintuple mutant, comprised of DHFR S108N/N51I/C59R and DHPS A437G/K540E, was strongly associated with sulfadoxine-pyrimethamine treatment failure, with an odds ratio of 13.41 (Kublin et al., 2002), similar to what was seen previously in Kenya. Multivariate analysis found a significant interaction between DHFR and DHPS mutations as risk factors for treatment failure and confirmed the important role of DHPS in sulfadoxine-pyrimethamine treatment failure. This same study found that just two mutations, DHFR C59R and DHPS K540E, were highly predictive of the presence of the full quintuple mutation, indicating that it would be necessary to measure the prevalence of just these two mutations to predict sulfadoxine-pyrimethamine efficacy. Another study recently confirmed this finding (Kyabayinze et al., 2003).

2. Low Endemicity. Where host immunity to malaria is lower, such as Southeast Asia and Latin America, in vivo drug failure may be more closely associated with mutations in target enzymes. During a malaria epidemic in Iquitos, Peru in 1997, molecular markers of drug resistance in DHFR and DHPS were measured in pretreatment blood samples and correlated with parasitological outcome (Kublin et al., 1998). The DHFR mutations S108N/N51I/I164L and DHPS mutations A437G/K540E/A581G were found in 87% of cases of RIII resistance, 69% of RII resistance, and in no sensitive cases. In Sudan, pre- and post-treatment *dhfr* and *dhps* genotypes of uncomplicated falciparum malaria treated with sulfadoxine-pyrimethamine were analyzed. No C59R, I164L, or A16V/S108T DHFR mutations were seen. Dihydropteroate synthase was wild type at codons 437, 540, 581, and 613, but three infections did harbor the S436A mutation. Interestingly, of the 29 isolates with DHFR S108N/N51I mutations, 26 had adequate clinical response, but three infections recrudesced and each of them contained DHPS S436A. Despite encountering only three such isolates, the finding was statistically significant ($p < 0.0003$) (Khalil et al., 2002).

C. Worldwide Distribution of Dihydrofolate Reductase and Dihydropteroate Synthase Mutations

The worldwide distribution of DHFR and DHPS mutations is not uniform, although many universal mutations do exist. To date, the DHFR I164L mutation is absent from Africa and only seen in areas of Latin America with high rates of sulfadoxine-pyrimethamine clinical failure. The DHFR I164L mutation is found with some regularity in Southeast Asia where rates of sulfadoxine-pyrimethamine clinical failure are highest.

The A581G DHPS mutation has not been found in any samples from Africa with two exceptions. The first was a worldwide survey of DHFR and DHPS genotypes (Wang et al., 1997a), wherein DHPS A581G was seen in samples from Mali and East Africa. The authors postulated that its presence was due to frequent use of trimethoprim-sulfamethoxazole in the area. Later studies with larger numbers of samples from this same area of Mali (Diourte et al., 1999) and from Kenya (Snow et al., 1998) failed to find the A581G mutation. The second study reporting A581G in Africa, from Uganda, was discussed earlier in the context of the S108N DHFR mutation (Jelinek et al., 1999), but this same group did not find A581G in Tanzania (Jelinek et al., 1998). Likewise, the DHPS A613S mutation was found in only one study from Africa (Brooks et al., 1994), in an area of lower sulfadoxine-pyrimethamine resistance, and has not been seen again, suggesting that the A613S/T mutations are presently absent or very rare in Africa. Barring these inconsistencies noted above, the DHPS A581G and A613S mutations appear to be absent from Africa, despite several years of widespread sulfadoxine-pyrimethamine use in eastern and southern African countries (Basco and Ringwald, 1998). Should these mutations, DHFR I164L and DHPS A581G/A613S, arise and be maintained in the African parasite population, high-level resistance to sulfadoxine-pyrimethamine can be expected. It remains unclear why Africa is, thus far, apparently free of I164L and A581G/A613S. The higher level of immunity in most of Africa results in a high proportion of chronic, asymptomatic and, therefore, untreated infections. If the two mutations associated with the highest levels of resistance are sufficiently deleterious to enzyme function, they may be selected against in the absence of sufficient drug pressure. It is possible that the amount of drug pressure applied to the total parasite population is insufficient to permit these particular mutations to arise and/or persist in Africa (Spencer, 1985; Plowe et al., 1998). If this hypothesis is correct, it could explain why the most highly resistant genotypes have not propagated in Africa despite significant antifolate drug pressure and why the efficacy of sulfadoxine-pyrimethamine appears to be declining there more slowly there than it did in Southeast Asia (Plowe et al., 2004).

D. Molecular Markers and Treatment Outcome—Summary

Taking into account host differences in elimination times for sulfadoxine and pyrimethamine, in vitro experiments support a scenario in which parasites with fewer than the three common DHFR mutations S108N, N51I, and C59R/C50R would be cleared by sulfadoxine-pyrimethamine irrespective of DHPS genotype. In the presence of the DHFR triple mutant form, the treatment outcome would depend on the DHPS genotype (Watkins et al., 1999; Sibley et al., 2001), as well as on such other factors as host immunity and plasma folate levels. This model is consistent with the clinical and epidemiological data that show an association between the prevalence of mutations in DHFR and DHPS and sulfadoxine-pyrimethamine failure rates, statistical interaction between mutations in the two genes in regression analyses of their associations with treatment failures, and the apparent selection for mutations in the enzymes under sulfadoxine-pyrimethamine treatment pressure.

VIII. Using Genotype to Predict Clinical Failure

Although molecular drug resistance markers or genotypes have been associated with in vivo drug resistance, their use as a tool to facilitate the monitoring of in vivo or clinical resistance on a population or individual patient level has proven to be a challenge, as the prevalence of the genetic resistance marker in nearly all studies is higher than the prevalence of in vivo drug failure. This discrepancy can be addressed by the use of a ratio, which corrects for the higher prevalence of the molecular marker compared with the in vivo failure rate. In Mali, this approach was used to estimate chloroquine-resis-

tance levels. At each study site a genotype resistance index (GRI) was calculated by dividing the prevalence of the molecular drug resistance marker by the prevalence of parasitologic chloroquine resistance, as measured by traditional in vivo techniques (RI, RII, or RIII). Similarly, the genotype failure index (GFI) was calculated by dividing the prevalence of the molecular drug resistance marker by the prevalence of clinical failure, either early or late treatment failures (Djimde et al., 2001b). As might be expected, the GRIs and GFIs were age-dependent, increasing with age and acquired immunity, resulting in fewer parasitologic or clinical failures as age and exposure to malaria increased. Most interesting, however, was that after correction for age, the GRIs and GFIs were remarkably stable from site to site across the country as well as over time as rates of resistance increased, suggesting that the ratios may remain stable despite changes in both genotype or in vivo failure prevalence over time and space. However, malaria transmission rates and patterns at the different sites studied were relatively similar, so it is uncertain what variability would be seen in GRIs/GFIs across areas with marked differences in transmission dynamics. It seems logical that the ratios would increase in areas of more intense malaria transmission and decrease in areas of lesser transmission.

If, however, GRIs/GFIs remain relatively similar across different epidemiological settings, then molecular surveying can be simplified because the ratios would not need to be established for each setting. Accurate GRIs/GFIs can be used to estimate rates of clinical and parasitological failure on a population level and help direct appropriate drug treatment strategies. Recently in Mali, digital puncture blood samples were obtained and analyzed in an epidemic setting and the resulting GRIs/GFIs were used to modify first-line treatment regimens while the epidemic was still in progress (Djimde et al., 2004). The GRI/GFI model may be applicable to other drugs for which molecular markers have been identified, most notably sulfadoxine-pyrimethamine, and studies to test this concept are underway.

IX. Other Antifolates

Less is known about the molecular basis of in vivo resistance to antifolate combinations other than sulfadoxine-pyrimethamine. Both in vitro and in vivo studies indicate that the antifolate combination chlorproguanil-dapsone remains active against the *dhfr* and *dhps* genotypes in Africa that cause sulfadoxine-pyrimethamine failure, and some studies have shown that cross-resistance between either chlorproguanil and pyrimethamine or dapsone and sulfadoxine is not complete (Clyde and Shute, 1957; Peters and Robinson, 1984; Milhous et al., 1985).

A. Trimethoprim-Sulfamethoxazole

A clinical efficacy trial in Kenya compared trimethoprim-sulfamethoxazole to sulfadoxine-pyrimethamine treatment and found a 14-day clinical failure rate of less than 6% for either drug (Omar et al., 2001c). This study only indirectly supports the idea that there is cross-resistance between the two drugs, since there was a very low rate of clinical failure to sulfadoxine-pyrimethamine. It does demonstrate that where antifolate resistance is low, trimethoprim-sulfamethoxazole can provide an effective cure. The half-life for each component of trimethoprim-sulfamethoxazole is only 10 to 12 h (Reeves and Wilkinson, 1979), considerably shorter than that of sulfadoxine-pyrimethamine (see below). Because of this, trimethoprim-sulfamethoxazole necessitates more doses than the single dose of sulfadoxine-pyrimethamine to maintain adequate drug levels over at least three parasite replication cycles, or slightly longer than 6 days, potentially impairing compliance with the entire treatment regimen.

B. Chlorproguanil-Dapsone

There is increasing discussion of replacing sulfadoxine-pyrimethamine with chlorproguanil-dapsone due in part to the latter combination's shorter half-life of 12 h (chlorproguanil) to 24 h (dapsone) (Winstanley et al., 1997), versus 81 h (pyrimethamine) to 116 h (sulfadoxine) of the former (Winstanley et al., 1992). The shorter half-life is expected to result in decreased drug selection pressure (Molineaux and Clyde, 1986). This theory has been the subject of debate, at least as applied in vitro, where it was shown that higher, not lower, concentrations of drug lead to more rapid development of drug resistance regardless of exposure time (Covell et al., 1955; Bishop, 1962). It is known that sublethal levels of sulfadoxine-pyrimethamine persist in the blood and apply significant selection pressure on the parasite for up to 52 days (Watkins and Mosobo, 1993), and at least one study has demonstrated that DHFR/DHPS inhibitor combinations with a shorter elimination phase, such as chlorproguanil-dapsone, are less prone to induce parasite drug resistance (Nzila et al., 2000b). Limited in vitro data does suggest that the development of resistance to chlorproguanil-dapsone is inherently more difficult than to sulfadoxine-pyrimethamine (Winstanley et al., 1995), but as yet, strong support from in vivo field data are lacking (Bukirwa et al., 2004). A study in Tanzania looked at mutations that developed in DHFR and DHPS during the 9 weeks following treatment with chlorproguanil-dapsone (Curtis et al., 2002). In this study, the triple DHFR mutant, S108N/N51I/C59R, was significantly more prevalent following treatment with chlorproguanil-dapsone than prior to treatment. There was no significant change in the prevalence of the *dhps* allele S436/A437G/K540, suggesting chlorproguanil-dapsone is effective against the DHFR triple mutant allele, either because the drug clears all parasites before further re-

sistance develops, or that chlorproguanil-dapsone did not place significant mutation pressure on DHPS, irrespective of its effect on DHFR (Curtis et al., 1998).

It was known prior to sulfadoxine-pyrimethamine introduction into either Vietnam or Africa that a combination of chlorproguanil or cycloguanil and dapsone was more potent and less prone to cause parasite antifolate resistance than sulfadoxine-pyrimethamine (Robertson, 1957; Yao and Tang, 1959; cited in Peters, 1970). A recent trial in Malawi found that chlorproguanil-dapsone was more efficacious against parasites carrying the triple DHFR (S108N/N51I/C59R) and double DHPS (A437G/K540E) alleles, the quintuple mutant, than was sulfadoxine-pyrimethamine (Kublin et al., 2002). This study offers in vivo evidence to support previous reports that the combination of chlorproguanil-dapsone is more effective than sulfadoxine-pyrimethamine against antifolate resistant parasites, particularly those lacking DHFR I164L, or DHPS A581G mutations. Clinical experience with chlorproguanil-dapsone against parasites harboring the DHFR I164L mutation is limited, but proguanil-dapsone was not an effective prophylactic against *P. falciparum* in Thailand where the DHFR I164L mutation is found (Shanks et al., 1992), and neither chlorproguanil-dapsone nor proguanil-dapsone was effective for the treatment of acute uncomplicated falciparum malaria (Wilairatana et al., 1997).

The shorter half-life of chlorproguanil-dapsone led to the concern that higher relapse or reinfection rates may be seen than following sulfadoxine-pyrimethamine administration, due to the prolonged prophylactic effects and one-time dose of the latter which facilitates near 100% compliance. In an attempt to define this risk, cohorts of children in Kenya and Malawi were followed for 1 year and treated with either chlorproguanil-dapsone or sulfadoxine-pyrimethamine for all uncomplicated malaria episodes. Notably, despite its higher efficacy in individual treatment episodes (95% for chlorproguanil-dapsone versus 80% for sulfadoxine-pyrimethamine), children treated with either study drug for each malaria episode over the course of a year had no difference in incidence of uncomplicated malaria, anemia, or severe malaria. It appears that the benefit of higher efficacy with chlorproguanil-dapsone was effectively cancelled out by the long prophylactic effect of the longer-acting but less efficacious sulfadoxine-pyrimethamine (Sulo et al., 2002). Mutabingwa et al. (2001) also found chlorproguanil-dapsone to be as effective as sulfadoxine-pyrimethamine in an endemic area of Tanzania.

These two factors, shorter half-life and increased potency compared with sulfadoxine-pyrimethamine, may provide chlorproguanil-dapsone with a longer useful therapeutic life in sub-Saharan Africa. It is not clear what selective effect chlorproguanil-dapsone will have on susceptible parasites that harbor the S108N/N51I/C59R + A437G/K540E allele. In areas where this mutant allele is already highly prevalent, it is possible that there will be stronger selective pressure for the development of DHFR I164L and/or DHPS A581G by chlorproguanil-dapsone. These mutations are not necessary for sulfadoxine-pyrimethamine resistance, but may be for resistance to chlorproguanil-dapsone. Chlorproguanil and/or its active metabolite may not block exogenous folate use by the parasite. Even though dapsone is a more potent DHPS inhibitor than is sulfadoxine, if folate escape plays a significant role in vivo, then chlorproguanil remains the key to the success of this drug combination. If DHFR I164L develops in African *P. falciparum* effectively eliminating the potentiation of this drug combination, dapsone will be of paramount importance in inhibiting de novo parasite folate synthesis.

X. New Directions—Drug Development

Presently there are few affordable malaria treatment options available to the most resource poor countries. As noted by Olliaro and Taylor (2003), the current research and development model for antimalarial drug development, public sector discovery and private sector development, has a poor record. Only 4 of the 1393 drugs registered worldwide from 1975 to 1999 were for malaria (Trouiller et al., 2002). Financial incentives for the development of malaria drugs are minimal, and with the adoption of the International Conference for Harmonisation Guidelines (http://www.ich.org), developing drugs for the international market may be even more costly in the future (Olliaro and Taylor, 2003). WR99210 provides an example of the anemic pace of antimalarial drug development. This drug has been a known potent inhibitor of DHFR for over 20 years (Milhous et al., 1985), yet has thus far failed to approach registration as a commercial product. Despite the drug being highly active against parasites harboring all known DHFR mutants (Hekmat-Nejad and Rathod, 1997; Cortese and Plowe, 1998), poor bioavailability and pharmacokinetic profile, as well as toxicity concerns, delayed its commercial development (Ferlan et al., 2001). More recently a prodrug to WR99210, PS-15, was developed, which demonstrated promising results (Canfield et al., 1993). The pairing of the potent triazine DHFR inhibitor WR99210, or more likely PS-15, with dapsone may be clinically useful in not only Africa, but as well in Southeast Asia and Latin America. WR99210 development employed homology modeling, in part, as a drug design tool. Such rational drug design has come to the forefront in antimalarial drug development and promises to be an efficient method to select drugs for further evaluation (Toyoda et al., 1997; McKie et al., 1998; Rastelli et al., 2000; Warhurst, 2002).

A. The "Old" Combinations

Although sulfadoxine-pyrimethamine is a combination of two compounds, it is not considered to be "com-

bination therapy", a term that by consensus has been defined to refer to combinations of antimalarial drugs that act via different mechanisms on different parasite pathways (World Health Organization, 2003). Nevertheless, as discussed above, trials in East Africa and elsewhere attested to the enhanced effectiveness of this combination (Nguyen-Dinh et al., 1982) compared with monotherapy with either agent, even where pyrimethamine resistance was already prevalent. Combination therapy is once again being advocated, and this time strong preference is given to artemesinin-based combination therapies (ACT) (Bloland et al., 2000; Olliaro and Taylor, 2003; World Health Organization, 2003). Combination therapy, however, must be carefully considered prior to implementation, especially if subsequent widespread acceptance of the regimen is anticipated, as in Africa. Progress is being made with respect to evaluation of ACTs likely to be deployed in Africa (Adjuik et al., 2004).

A brief overview of past antimalarial combination therapy is instructive and particularly relevant to the current ACT suggestions. These past combination therapy attempts were generally failures, in part, because the guiding principles were founded on hypotheses with little supportive data (Peters, 1970, 1984). An example is provided by mass chemotherapy programs which utilized combinations of chloroquine-pyrimethamine and chloroquine-primaquine in the 1950s and 1960s. The theory behind these combinations was that the addition of either pyrimethamine or primaquine would sterilize the gametocytes, thereby reducing transmission and the subsequent spread of drug resistance (Shute and Maryon, 1954; Covell et al., 1955). By 1959, a combination tablet of 200-mg chloroquine and 16.5-mg pyrimethamine had been produced for mass distribution. Distribution varied by country, with some countries implementing one-time therapy and others weekly, fortnightly, or monthly regimens (Dobrovolny et al., 1953). Despite the fact that pyrimethamine resistance developed soon after implementation, the WHO Reports of 1957 and 1961 continued to give enthusiastic support for such an approach. By 1962, there was some hesitancy noted in these reports, because by that time chloroquine resistance had been described (Eyles et al., 1963), perhaps forcing the WHO to acknowledge the combination's limitations. Some criticized the approach early on, noting that pyrimethamine resistance was not prevented by the coadministration of chloroquine (Peters, 1970). Unfortunately, such voices of reason were ignored and in 1966 the WHO stated, "It is likely that the combined use of drugs with different types of action [chloroquine plus primaquine] at adequate dosage may prevent the development of resistance". There were inadequate in vitro data to support such a claim; it was known that resistance was slowed but not prevented by such a combination (Rabinovich, 1965). By 1966, in vivo data were accumulating to contradict the WHO's claim. Furthermore, there was no evidence of potentiation between chloroquine-pyrimethamine or chloroquine-primaquine (Jacobs et al., 1963; Young et al., 1963).

The combination of pyrimethamine with primaquine was also tried during this period, but both pyrimethamine- and primaquine-resistant strains were already circulating, and combinations of a slow schizonticide (pyrimethamine) with a nonschizonticidal (primaquine) drug proved to be far from ideal. Amodiaquine, in combination with cycloguanil, was tried in a limited number of studies, but the combination failed to prevent the development of resistance to cycloguanil. Amodiaquine and primaquine also demonstrated mixed results (Peters, 1970). Finally, potentiating drug combinations, such as sulfadoxine-pyrimethamine, began to win converts (the push for its use as an antibacterial was a boon) and Maloprim (pyrimethamine-dapsone) was ready for the market by 1970.

Not all potential combinations are as likely to protect each component drug from parasite drug resistance. In fact, in vitro studies found trimethoprim generally ineffective in this regard (Peters, 1970). Based on data gathered at that time, it was felt that a pyrimethamine-dapsone combination would be the best choice (Laing, 1965b). Yet even as early as 1967, Harinasuta et al. (1967) suggested combination regimens containing three drugs, each with a different mode of action. He cautioned against using DHFR/DHPS inhibitor combinations because of their flat dose-activity lines (Jacobs et al., 1963; Peters, 1968; quoted in Peters, 1975), which he warned might facilitate the development of resistance.

In an example of an apparently protective drug combination, Rabinovich (1965) found that resistance to the combination chloroquine-sulfaphenazole developed very "reluctantly", whereas resistance to pyrimethamine developed unimpeded during therapy with the chloroquine-pyrimethamine combination (Peters, 1984). In the first combination, folate escape brings into question the apparent protective findings. The parasite is free to use exogenous folate and bypass the inhibitory effects of sulfaphenazole, thereby undercutting any selection pressure on the *dhps* gene, and chloroquine resistance only very rarely arises de novo in response to drug pressure. The second combination puts a large degree of selection pressure on the *dhfr* gene because folate escape is blocked, necessitating full use of the parasite's folate biosynthetic pathway.

It is also instructive to consider more recent attempts at combination therapy. The burden of antimalarial drug resistance was, and is, a serious problem in much of Southeast Asia, with studies demonstrating significant resistance to sulfadoxine-pyrimethamine, chloroquine, mefloquine (Nosten et al., 1991), and increasingly to quinine (Shanks et al., 1992; Thanh et al., 2001) and possibly even some degree of reduced in vitro susceptibility to the artemisinin derivatives (Yang et al., 1997; Wongsrichanalai et al., 1999). Several drug combinations were tried with unacceptably high failure rates,

including chloroquine with either erythromycin or tetracycline (Phillips et al., 1984). Eventually, mefloquine was introduced into Southeast Asia. It is not surprising, given the structural similarities of quinine and mefloquine, that the useful therapeutic life of mefloquine was extremely short, with resistant cases noted shortly after its introduction (Nosten et al., 1991). Rodent malaria data suggested this would be the logical sequence of events (Peters, 1984). Prolongation of the useful therapeutic life of mefloquine has been achieved by its use in combination with artesunate. Combination of mefloquine with the DHFR/DHPS inhibitors was not nearly as effective (Nosten et al., 1987). The effectiveness seen when mefloquine is combined with artesunate is secondary to the rapid reduction in asexual parasitemia following artesunate therapy (10^4 log reduction in parasitemia per parasite replication cycle with artesunate) (White, 1994). After the initial reduction in parasite burden by artesunate, mefloquine clears any remaining or recrudescent parasites, probably to the point of sterility (Hoshen et al., 1998). Used alone, however, artemisinin derivatives have unacceptable rates of recrudescence, as high as 50% (Nguyen et al., 1993; Thanh et al., 2001). The rapid action of artesunate on early stages of the parasite substantially reduces further differentiation of the parasite into gametocytes, thereby decreasing posttreatment gametocytemia compared with other antimalarials. This may contribute to decreases in malaria transmission (Price et al., 1996; McGready et al., 2000; Targett et al., 2001) and possibly to dissemination of drug resistance, although the latter is dependent upon gametocytes from recrudescent infections (assuming the initial parasite biomass was largely drug-sensitive).

B. New Directions—Combination Drug Therapy

The resistance to multiple drugs seen in many malaria parasites provides a strong rationale for instituting combination drug therapy in virtually all malarious regions. Both old and new drugs must be thoughtfully combined to ensure the longest possible useful therapeutic life for any given drug. Concern that the elimination tail of long-acting drugs applies drug selection pressure has been discussed in detail by others (Hastings et al., 2002). After treatment with a drug combination such as artesunate-mefloquine, reinfection can occur at a time when blood levels of the rapidly eliminated artesunate are negligible, but while the more slowly eliminated mefloquine remains in the blood and can lead to merozoites leaving the liver in the presence of lower concentrations of mefloquine alone. This is more likely to occur in highly endemic transmission settings where the bites of infected mosquitoes are more frequent and can occur shortly after treatment for an acute malaria episode. Although the parasite biomass exposed to low concentrations of the drug is considerably lower than at its peak at the initiation of treatment ($\ll 10^5$ versus $>10^9$) and, as discussed previously, de novo mutations for drug resistance are exceedingly rare when $<10^9$ parasites are present, selection for parasites already harboring resistance-conferring mutations may occur. This could increase the likelihood that novel, invading parasite alleles (strains) establish themselves in the background population of drug-sensitive parasites. Thus, consideration of an elimination "tail" in drug combinations with mismatched elimination times may be of most importance in areas of intense malaria transmission.

Drugs combined for malaria therapy can have complementary effects (e.g., against different stages of the parasite), additive effects against the same stage of the parasite, synergistic effects, or any combination of these (Peters, 1987). Examples of drug combinations with complementary effects include chloroquine and primaquine for schizontocidal and gametocidal effects, respectively. Additive effects are seen with quinine or artesunate and doxycycline or mefloquine, as noted above (Looareesuwan et al., 1992). Other additive combinations, although less efficacious, include amodiaquine and sulfadoxine-pyrimethamine or chloroquine and sulfadoxine-pyrimethamine. The economically motivated recent attempt to use the combination of chloroquine and sulfadoxine-pyrimethamine in Uganda, where both chloroquine and sulfadoxine-pyrimethamine resistance is well established, was not very effective (Miller et al., 1986; Gasasira et al., 2003). This leads to the conclusion that if drug combinations are not potentiating, at least one of the drugs needs to be extremely effective. The combination of amiodiaquine-sulfadoxine-pyrimethamine is most likely an example of this (Schellenberg et al., 2002; Gasasira et al., 2003), as is dihydroartemisinin-piperaquine (Tran et al., 2004).

Synergistic or potentiating combinations include the DHFR/DHPS inhibitor combinations. Novel potentiating combinations could include the DHFR/DHPS inhibitor drugs in combination with dihydroorotate inhibitors, inhibitors of the shitimake pathway, which supplies folate precursors (Roberts et al., 1998), and dihydrofolate synthase-folylpolyglutamate synthase inhibitors (Salcedo et al., 2001). Thymidine analogs in combination with folate inhibitors or the dihydroorotate inhibitors may also be potentiating because the source of thymidine may be via thymidylate synthase from uridine monophosphate (Gutteridge and Coombs, 1977; Peters, 1984). Novel drugs that interact with the recently described interdomain region of DHFR-TS may also reduce available plasmodial dTMP (Yuvaniyama et al., 2003) and possibly potentiate the antifolates.

Inhibition of more than one step along a metabolic pathway that leads to a common end-product can be an extremely potent antimalarial concept, as the DHFR/DHPS inhibitors have shown. Another such example is the combination of proguanil with the naphthoquinone menoctone, which is strongly synergistic. Menoctone is believed to have two mechanisms of action on the malaria parasite. The first is as a potent inhibitor of dihy-

droorotate dehydrogenase, possibly via inhibition of uridine monophosphate synthesis from dihydroorotate. The second, and likely most important when combined with proguanil, is competition with ubiquinone during oxidation of tetrahydrofolate (Lopez-Shirley et al., 1994). This synergistic mechanism of action relies on the biguanide proguanil, which has an inhibitory action on *P. falciparum* growth that is independent of DHFR and of metabolism to cycloguanil (Fidock and Wellems, 1997; Kaneko et al., 1999). Proguanil, but not cycloguanil, enhanced the ability of atovaquone to collapse the mitochondrial membrane potential (Srivastava and Vaidya, 1999). The combination of atovaquone-proguanil (Malarone) was highly active against cycloguanil-resistant isolates in vivo and in vitro (Canfield et al., 1993, 1995; Edstein et al., 1996; de Alencar et al., 1997; Fidock and Wellems, 1997; Wang et al., 1997a; Srivastava and Vaidya, 1999). The combination of atovaquone-proguanil has proven to be an effective antimalarial, particularly for prophylactic use. Two recent case reports of failure to adequately treat acute falciparum malaria highlight the difficulties faced when deploying new antimalarial combinations (Fivelman et al., 2002; Schwartz et al., 2003). This is particularly concerning because *P. falciparum* either develops resistance to atovaquone rapidly (Gassis and Rathod, 1996) or resistance is inherent to some degree in certain *P. falciparum* strains. There is evidence that proguanil alone is no longer effective in preventing malaria in nonimmune subjects (Watkins et al., 1988). The continued effectiveness of this combination will depend upon the significant synergism between the two components.

Following the aforementioned logic, the introduction of chlorproguanil-dapsone alone may be a cause for concern. To delay the emergence of resistance, this new combination would be best introduced in combination with a highly effective third agent, or at least another potentiating agent. Indeed, triple combination is what is needed to prolong the useful therapeutic life of newly introduced antimalarials, particularly any new antifolate combinations (van Vugt et al., 2002). Such a combination may include DHFR-DHPS-dihydroorotate dehydrogenase inhibitors, along with an antigametocyte drug, or at least combined with a drug that reduces asexual parasites quickly enough to minimize the number of new gametocytes formed following treatment, such as artesunate (van Vugt et al., 2002). The combination of an artemisinin derivative with chlorproguanil-dapsone is an example of a combination with good tail coverage. The recent decision by Zambia (Duffy and Mutabingwa, 2004) and a province in South Africa (Roper et al., 2003) to switch to artemether-lumefantrine (benflumatol, an aryl amino alcohol related to mefloquine and quinine with a half-life of approximately 60 h) (Hassan et al., 1999) appears to be based on the artesunate-mefloquine experience along the Thai-Burmese border, rather than on evidence that this combination will deter development of resistance. There is evidence of potentiation between artemether-lumefantrine (Hassan et al., 1999 also showed some cross-resistance in vitro between mefloquine and lumefantrine), and a recent Cochrane review found that, although the four-dose regimen was less effective than comparison antimalarial regimens, the six-dose regimen was promising, although relatively untested (Omari et al., 2003). The artemether-lumefantrine combination also lacks tail coverage. However, the lower overall quinine resistance in Africa compared with Southeast Asia and the large reservoir of asymptomatic, untreated malaria in Africa, reducing drug pressure on the parasites, may allow for a longer useful therapeutic life for this combination than would be seen in other parts of the world (Warhurst and Duraisingh, 2001).

XI. Summary

The antifolate drugs have had at best a mixed record as antimalarial agents, as much due to the manner in which they have been used, as to their intrinsic properties. Resistance to this class of drugs develops relatively quickly under drug pressure, probably due to the preexisting presence of parasites harboring the single or double mutations that confer a selective advantage in the presence of drugs and that are followed by the stepwise accumulation of additional mutations leading to successively higher levels of resistance. Recent molecular epidemiological studies suggest that the most highly mutant, resistant parasites have arisen only very rarely and then spread globally.

Nevertheless, new antifolate drugs hold considerable promise if they are deployed thoughtfully in combination with each other and with other drugs with suitable pharmacokinetic and pharmacodynamic characteristics. Lessons from the history of malaria control have shown that malaria chemotherapy strategies need to be evidence-based and tailored to the epidemiologic, as well as, economic circumstances of a given setting. Parasite drug resistance and transmission patterns are remarkably varied not just across Africa but within regions and even within villages. With the mounting evidence that the flow of some drug-resistance alleles through the *P. falciparum* population may be in part "clonal" (Ariey et al., 2002; Cortese et al., 2002; Wootton et al., 2002; Nair et al., 2003; Roper et al., 2003, 2004), today's treatment policy decisions are likely to have global impact on the next generation of malaria therapy. A single country's choice regarding first-line malaria therapy may well have a ripple effect for the region or the continent, if not the world, and should be placed in this larger context. Ideally, effective triple-drug combinations appropriately fitted to local needs should be introduced into clinical practice to maximize the useful therapeutic life of our limited antimalarial armamentarium. The future of effectual malaria control will depend heavily upon appro-

priate data collection and collaboration now to facilitate evidence-based decision making in the future.

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