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Drug-resistant Malaria: Laboratory and Field Investigations*

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About 40% of the world's population is exposed to malaria, and there are up to 500 million clinical cases per year with 1·5–2 million deaths. The most lethal and well established species in man, *Plasmodium falciparum*, has its main focus in Africa where high mortality is seen in children (World Health Organization 1996).

Transmission depends on the bite of female anopheline mosquitoes and the pre-erythrocytic stage which develops from the sporozoite in the insect's saliva, grows for more than 5 days in the liver without pathological effects. Following escape from the liver, the infective merozoites enter red blood cells and begin a 48-h (tertian) or 72-h (quartan) division cycle: blood schizogony. Fever is seen when the mature dividing stages burst out of the red cells to reinfect others. Red cells containing *P. falciparum* maturing blood stages adhere to capillary endothelium in deep tissues including the brain to avoid capture by the spleen, and cause severe pathology.

Malaria parasites of man originated in the higher primates in the old world. Human genetic data indicate that malaria was carried to the Americas by colonisers (Cavalli-Sforza et al 1994). *P. vivax*, the benign tertian malaria parasite, probably originated from a parasite similar to *P. schwetzi* found in the chimpanzee and gorilla in Africa. In South America, *P. vivax* passed from man to the black howler monkey as *P. simium*, accounting for very close genetic similarities between *P. vivax* and *P. simium* and observations of cross infection to man (Goldman et al 1993). A similar occurrence took place with *P. malariae* which in Africa infects both man and chimpanzee (Garnham 1966). It passed from man into the South American squirrel and other monkeys as *P. brasilianum*, which is very similar genetically. *P. falciparum* apparently separated from the chimpanzee and gorilla parasite *P. reichenowi* in Africa at about the same time that ancestors of chimpanzees and man diverged (Escalante et al 1995). It is restricted to the hominoids and did not infect local monkeys when introduced into South America.

P. falciparum resistance to quinine was first reported in 1910 in persons returning from South America, but refractory strains were later recognized in Europe (Peters 1987). Use of this cinchona-derived alkaloid as a prophylactic was moreover associated with development of blackwater fever, a severe haemolytic crisis during a fulminating malaria attack. New, safe synthetic drugs, chloroquine and the antifolates pyrimethamine and proguanil were introduced after 1945, but only chloroquine appeared free from the problem of resistance. Chloroquine became a standby for prevention and treatment of malaria, and, together with DDT, the basis of a worldwide programme for malaria eradication. Hopes were dashed in the early 1960s when chloroquine-resistance was reported in *P. falciparum* from highly endemic regions of Venezuela and Colombia in South America and the Thailand–Cambodia border in South East Asia, while DDT began to show failures in several areas. Subsequently, chloroquine resistance spread throughout South America, and to the remainder of South East and South Asia, finally reaching East Africa in 1978 and spreading across to West Africa by 1985 (Payne 1987).

Chloroquine remains usable and is used as a first-line drug in many areas of Africa. In our 1993 study in Zaria, Northern Nigeria, only 7 treatment breakthroughs were noted when 43 children were treated with a standard course (Adagu et al 1995a). However, quinine, once discarded as too toxic (Findlay 1951), is an essential second-line drug, especially in severe and complicated disease. Potentiating antifolate combinations of pyrimethamine with the sulphonamide sulphadoxine have served well in less severe disease and are an invaluable follow-up to quinine, with tetracycline as an alternative. Amodiaquine and other chloroquine analogues are also undoubtedly still valuable for treatment, especially where resistance to chloroquine is not too marked.

There is no doubt that in many areas, quinine is the clinician's first choice for treatment of severe disease. Moreover in Thailand, where quinine replaced chloroquine in 1978, there is now a significant amount of quinine (and, in some cases mefloquine) resistance in border areas with Myanmar and Cambodia (Fontanet et al 1993).

Drug treatment of active cases can assist in the control of transmission in areas of low malaria endemicity. Mosquito control measures, and means of avoiding man-mosquito contact by bednets may be the most effective measures in zones of higher endemicity, where chemotherapy serves for reduction of morbidity and mortality, and drug prophylaxis may be targeted to pregnant women and (in some cases) to small children.

Mechanisms of Drug Action and Resistance

Blood schizontocides

These agents (which include chloroquine, amodiaquine, quinine, mefloquine and halofantrine) act only on the growing intra-erythrocytic stages carrying out haemoglobin digestion. Their effect is rapid, and since the blood stages are responsible for malaria pathology, they are preferred for treatment of severe disease. Although liver stages are unaffected, chloroquine has been used widely as a prophylactic of low toxicity, preventing the development of a clinical infection. This is termed suppressive prophylaxis.

Allison & Young (1964) localized quinine fluorescence in mammalian digestive vesicles—lysosomes according to the definition of De Duve & Wattiaux (1966). Our group (Warhurst & Hockley 1967; Warhurst & Williamson 1970) and Macomber et al (1967) were able to show ultrastructurally that

*Dedicated to the late Dr James Williamson, biochemical parasitologist and friend.

the pigment clumping following chloroquine-treatment of rodent *P. berghei* and the simian parasites *P. cynomolgi* and *P. knowlesi* represented fusion of adjacent plasmodial digestive vesicles into an autophagic vacuole, and that rRNA was degraded during the 1- to 2-h process of clumping. We suggested a trapping mechanism for lysosomotropic concentration of weakly basic drugs, involving the accumulation of protonated membrane-impermeable drug within the acidic lysosome contents (Homewood et al 1972). This idea was later expanded by De Duve et al (1974). The incrimination of the lysosome was subject to a vigorous but unsuccessful criticism from Hahn et al (1966), who supported a direct action on DNA. The lysosomotropic hypothesis did not, however, serve as a full explanation of chloroquine's selective activity on malaria parasites (Warhurst 1985), because mammalian cells also have acidic lysosomes.

We compared the ability of blood schizontocidal antimalarials to inhibit chloroquine-induced pigment clumping competitively, and outlined a hypothetical receptor associated with the lysosome (Warhurst & Thomas 1975).

Clues as to the possible nature of the lysosomal receptor had been revealed as early as 1964 in studies on the interaction of chloroquine and quinine with haemin (ferriprotoporphyrin IX), although it was then felt that such an interaction might form a mechanism of resistance rather than a mode of action (Cohen et al 1964). Haemin, a toxic iron porphyrin, is produced within the lysosome during digestion of haemoglobin and is complexed as a non-toxic crystal, malaria pigment or haemozoin (Slater et al 1991). Suggestions that haemin might be a target were first made in 1967, but it was not until 1980 that Fitch and his group (Chou et al 1980) found fairly conclusive evidence, suggesting that drug-haemin complexation prevented haemin from detoxication as haemozoin (McChesney & Fitch 1984). The haemin molecule answered some of the requirements for the lysosomally located receptor we had proposed (Warhurst & Thomas 1975), and it was possible spectrophotometrically to demonstrate its complexation with quinine, other cinchona alkaloids and mefloquine. The absence of interaction with antiplasmodially inactive 9-*epi* quinine was compelling evidence. A ring-ring interaction between quinine and haemin in the organic or membrane phase was proposed and modelled, with coordination proposed between the side-chain nitrogen of the drug and the Fe^{3+} of haemin (Warhurst 1981). The ring-ring interaction has been confirmed subsequently, but the iron coordination apparently involves the adjacent alcoholic -OH group (Constantinidis & Satterlee 1988a). It has also been shown that chloroquine has a similar ring/ring interaction with haemin or haemin dimer in aqueous solution (Moreau et al 1985; Constantinidis & Satterlee 1988b).

Assuming the importance of the haemin interaction, which has been disputed (Warhurst 1986), the probable explanation for the antiplasmodial inactivity of the 9-*epi* cinchona alkaloids relates to the short distance between the 9-OH (9-hydroxyl group) and the side-chain quinuclidine -N, allowing an intramolecular hydrogen bond to form (Oleksyn 1982), which effectively prevents the -OH group's coordination with Fe^{3+}. The similarity in crystallographic parameters of quinine, cinchonidine, quinidine, cinchonine and the related drug mefloquine (Karle & Karle 1991), and the mutual similarity of their calculated minimum energy conformations in-vacuo, and that of halofantrine (Warhurst, unpublished data) allow a

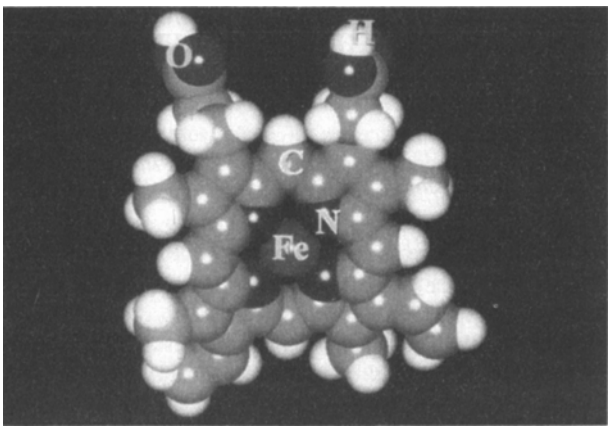

FIG. 1. Ferriprotoporphyrin IX (haemin) structure computed using semi-empirical ZINDO single point calculation.

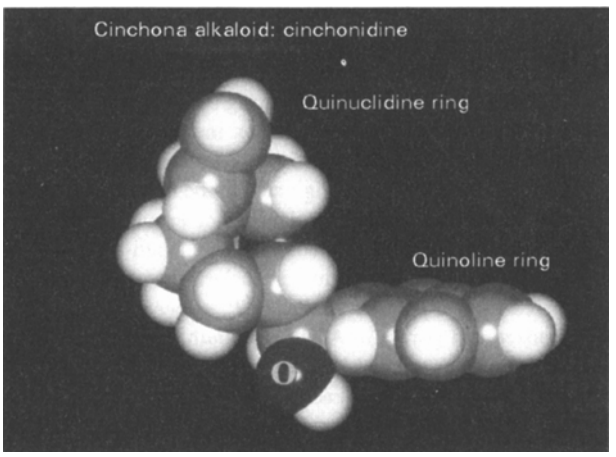

FIG. 2. Cinchonidine structure computed using semi-empirical AMI single point calculation followed by molecular mechanics MM+ geometry optimization.

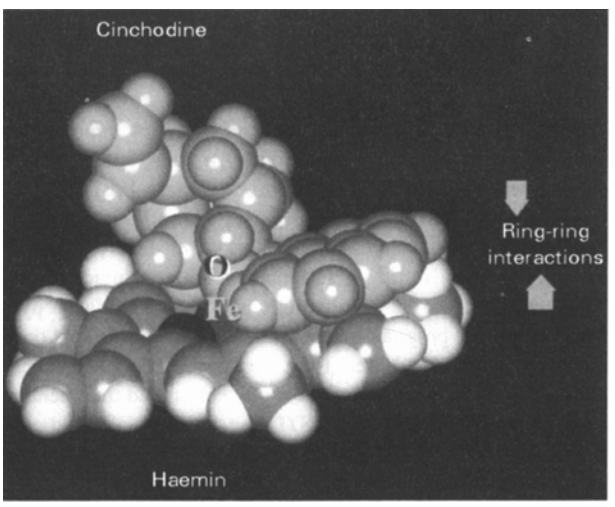

FIG. 3. Docking of cinchonidine with ferriprotoporphyrin IX to show coordination linkage of cinchonidine hydroxyl oxygen with Fe^{3+} of the iron porphyrin.

common model to be suggested where a planar aromatic surface bears a hydroxyl oxygen able to coordinate with the Fe^{3+} in the centre of the haemin ring (see Figs 1, 2 and 3).

A striking feature which makes haemin unlike any other drug receptor is its relative bilateral symmetry which will allow efficient binding of enantiomers. This agrees with observations on antimalarial activity, since (−) (natural) and (+) (unnatural) quinine are equipotent (Warhurst 1987). It is fascinating to note that the apparently crucial interaction between the sesquiterpene endoperoxide qinghaosu antiplasmodials such as artemisinin with haemin also depends on an iron-oxygen reaction (Posner et al 1994; Shukla et al 1995). It is also relevant that, even though they are not basic, the artemisinin derivatives are effectively concentrated by malaria-infected erythrocytes (Gu et al 1984).

Resistance to blood schizontocides

Blood schizontocides can be divided on chemical grounds into the following groups: 4-aminopyridine analogues (chloroquine, amodiaquine, amopyroquine, mepacrine); and aryl amino alcohols (quinine, mefloquine, halofantrine). Infections moderately resistant to 4-aminopyridine analogues are treatable using aryl amino alcohols.

Chloroquine-resistant malaria parasites take up less chloroquine (Fitch 1970; Diribe & Warhurst 1985) than do sensitive ones. This may be due to enhanced export of the drug from the cell, mediated by an MDR protein (multiple drug resistance or P-glycoprotein) as is found in some drug-resistant cancer cells. This was suggested when verapamil, known to reverse drug resistance in cancer cells by interfering with the activity of the MDR protein, was found to reverse chloroquine-resistance in-vitro in *P. falciparum* malaria (Martin et al 1987). Chloroquine-resistant parasitized erythrocytes were observed to release chloroquine at least 40 times as fast as sensitive ones (Krogstad et al 1987), but there is disagreement about this. A characteristic *mdr* gene has been detected on chromosome 5 in all *P. falciparum* strains examined. Mutations in Pfmdr1 have been linked to chloroquine resistance (Foote et al 1990) in some areas (Adagu et al 1995b, 1996; Basco et al 1995) but not in others (Awad El Kariem et al 1992; Wilson et al 1993). It appears that another gene may permit chloroquine resistance, and mutations in Pfmdr1 may be secondary, since an area of chromosome 7 contains a site linked in a laboratory cross to chloroquine resistance (Wellems et al 1991). In addition, amplification of Pfmdr1 is in some cases associated with resistance to mefloquine, halofantrine and probably quinine (Wilson et al 1993).

Antimetabolites

Antimetabolites attack all growing stages of the malaria parasite. They will even inhibit the early growing stages in the liver (causal prophylactic effect) and the developing infective stages in the mosquito (antisporogonic effect). The type I antifolate sulphonamides inhibit dihydropteroate synthase (DHPS). The type II antifolates, pyrimethamine and the proguanil metabolite cycloguanil, inhibit a later enzyme, dihydrofolate reductase (DHFR) (the pathway from para-amino benzoic acid (PABA) to the tetrahydrofolate co-factors is essential in the synthesis of the pyrimidine deoxythymidylate for DNA). The similarity of antiplasmodial sulphadoxine/pyrimethamine (SDX/PYR) combinations to the potentiating antibacterial type I+II antifolate package cotrimoxazole is clear.

Resistance to antimetabolites

Resistance to these drugs apparently depends largely on point mutations in the DHPS and DHFR genes (Peterson et al 1990; Brooks et al 1994). Combinations of type I and type II antifolates have been generally effective against pyrimethamine resistance, and at first resistance to SDX/PYR was not a significant problem (Kilimali & Mkufya 1985). Now it seems, particularly in East Africa and South East Asia, that resistance to SDX/PYR is becoming more widespread. Is this due to higher levels of resistance to the PYR component, reflected in additional mutations in DHFR, or is it due to development of resistance to SDX as well?

Resistance Monitoring Using Techniques Based on Molecular Biology

We are interested in developing molecular biological techniques to monitor drug resistance to currently-used antimalarials in field populations using easily applicable low-technology blood-spot sampling methods (Warhurst et al 1991) and polymerase chain reaction (PCR)-based genetic analysis protocols carried out in central laboratories. The aim is to make resistance monitoring and consequent modification of treatment guidelines more efficient.

Since resistance changes are often linked to point mutations in specific genes, methods for PCR detection need to be mutation-specific. Currently we employ PCR followed by restriction digests to detect mutant and wild-type alleles (Frean et al 1992) but we are also testing a colorimetric ELISA-based system (Aguirre et al 1995) modified using mutation-specific primers.

In addition to examining resistance-related mutations, we can use PCR to look at the antigenic diversity of the malaria parasites to detect whether drug breakthroughs are due to development of resistance in existing parasite clones in the blood, or to new infections. This technique (Babiker et al 1995) combined with resistance-mutation detection, has proved to be most illuminating. In our recent Gambian study it confirms that chloroquine failures which occur 28 days after initiation of treatment, are mostly new infections (Duraisingh et al unpublished).

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