SY7 MECHANISMS OF DRUG RESISTANCE IN *PLASMODIUM FALCIPARUM*

Mathirut Mungthin

Department of Parasitology, Phramongkutklao College of Medicine, Bangkok, Thailand

A major obstacle to the treatment and control of malaria is the emergence of drug resistance in *Plasmodium falciparum*. Drug resistance has been defined as 'the ability of a parasite isolate to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended but within limits of tolerance of the subject'[1-2].

The intensity and frequency of resistance from each foci varies substantially. These factors are dependent upon such variables as the amount of local drug pressure, the rate of active malaria transmission in the area and the length of time that drug resistance has been acquired within that area. The emergence of drug resistance is thought to have come about by the selection of existing resistant mutants through drug pressure, although it is accepted that parasite isolates expressing natural variations in drug susceptibility do exist[3]. This selective drug pressure has most likely been applied by the unsupervised use of subcurative doses of antimalarials that have been made widely available to the public in many parts of the world, but may also be due to non-compliance, vomiting and/or diarrhoea after drug intake. As drug resistance is genetically determined, it will be spread by active malaria transmission, as gametocytes from resistant isolates will produce resistant offspring. Antimalarial resistance has been shown to be a stable phenotype maintained in *in vitro* culture for many years in the absence of continued drug pressure[4].

Chloroquine (CQ) resistance in *P. falciparum* was first reported in the late 1950's from 2 separate foci; South America[5] and Southeast Asia[6]. Today CQ resistance effects most areas of the world in which the drug has been used[7-8]. Indeed, in some areas CQ is now almost completely ineffective. Parasite resistance is not confined to CQ. There have been recent reports which suggest that amodiaquine (AQ), a more active analogue of CQ, commonly used in Africa in the therapy of CQ treatment failures is also subject to resistance mediated treatment failures[9-10]. The increasing problem of parasite resistance to CQ had prompted the use of combinations of existing drugs, in addition to the development of novel antimalarials. During the 1970's a combination of pyrimethamine and sulphadoxine, named Fansidar, was employed. However, the development of resistance to this combination and adverse reactions has limited its use in many areas[11-13]. Furthermore, parasite resistance to the new generation of antimalarial drugs has been reported. Resistance to mefloquine (MQ) in *P. falciparum* was observed as early as in 1982 in Southeast Asia and 1983 in Africa[14-15] and clinical resistance persists despite an increasing of the therapeutic dose[16]. Early clinical failure to new drugs such as MQ and halofantrine (HF) may be explained by cross-resistance[17-20]. Further compounding the situation is the continued drop in the sensitivity to quinine (QN)[17], which now has to be routinely administered together with tetracycline[21]. Artemisinin and its derivatives are highly effective for treatment of multidrug-resistant falciparum malaria. To date, a few

treatment failures have been reported[22]; however, actual parasite resistance to these drugs has not been documented.

Clinical resistance to CQ in *P. vivax* has been recently reported particularly from Southeast Asia and Oceania[23-25] and resistance to pyrimethamine is also well known[26]. However, because of the difficulty of *in vitro* culture, mechanism of drug resistance in *P. vivax* is less explored.

Mechanism of resistance to antifolate drugs in *P. falciparum*

Pyrimethamine binds and inhibits malarial dihydrofolate reductase (DHFR) and sulphadoxine acts on dihydropteroate synthase (DHPS). The combination of pyrimethamine and sulphadoxine gives a synergistic action against *P. falciparum*. Resistance to this combination has long established particularly in Southeast Asia[11-12]. Specific mutations in both target enzymes can evade the action of these drugs. Kinetic studies of DHFR showed that resistance in *P. falciparum* was associated with a reduced affinity binding between drug and target enzyme[27-28]. It is well established that pyrimethamine resistance is a result of the mutations in the *P. falciparum dhfr* gene. A single point mutation on codon 108 from Ser to Arn is linked to pyrimethamine resistance. Additional changes in codons 59 (Cyst to Arg) and/or 51 (Asn to Ile) confer higher level resistance[29-31]. Different mutations on this gene conferring pyrimethamine resistance in *P. falciparum* have been reported[32]. Interestingly, with these 3 mutations, changing at codon 164 (Ile to Leu) confers cross-resistance between pyrimethamine and cycloguanil, the active metabolite of proguanil (a DHFR inhibitor). While changing of codon 108 (Ser to Thr) and 16 (Ala to Val) is only associated with resistance to cycloguanil[33-34]. The importance of the mutations associated with pyrimethamine resistance has been confirmed by the experiments using both homologous and heterologous transformation[35-36].

The gene encoding *P. falciparum* DHPS has been sequenced. Specific point mutations on this gene have been linked to sulphadoxine resistance *in vitro*. Mutations associated with decreased susceptibility to sulphadoxine include codon 436 (Ser to Phe/Ala), 437 (Ala to Gly), 540 (Lys to Glu), 581 (Ala to Gly) and 631 (Ala to Thr/Ser)[37-38].

Mechanism(s) of resistance to quinolines in *P. falciparum*

Unlike antifolate drugs, the mechanism(s) underlying quinoline resistance is/are less clarified. However, the studies performed so far, mainly involving CQ, have produced a number of important, and widely accepted, insights into these resistance mechanisms.

Chloroquine resistance

Proposed mechanisms for CQ resistance have been based on the evidence that CQ-resistant parasite accumulates less drug than its susceptible counterpart[39-42]. Therefore most of the proposed mechanisms have usually been linked to the reduction of drug available to the site of action; the food vacuole of the parasite.

As the major driving force for 4-aminoquinoline accumulation in the parasite is the transmembrane proton gradient[43], changing in the magnitude of this proton gradient can alter parasite susceptibility. Resistance in *P. falciparum* could therefore result from an elevation of basal vacuolar pH in the resistant parasite. Based upon these ideas, Williams and Fanimo (1974) suggested that the lower steady state level of drug seen in resistant

parasites might be due to alterations in the regulation of vacuolar pH in resistant isolates[44]. It is accepted that vacuolar pH in *P. falciparum* is maintained by a balance between an inward proton transporter, the vacuolar ATPase pump, and outward proton leak[45]. Therefore, an increased intravacuolar pH in resistant parasites could be due to either an increased proton leak or reduced vacuolar ATPase activity. Indirect support for this hypothesis using a mathematical model showed that the discrepancy between steady state drug levels seen in CQ-resistant and -susceptible isolates could be explained simply due to a reduced force for uptake in the resistant isolates[46]. Further evidence in support of this hypothesis was that CQ-resistant parasites were more sensitive to the effects of bafilomycin A1, a specific vacuolar proton pumping ATPase inhibitor, than their susceptible counterparts[47]. Two subunits of the vacuolar ATPase from *P. falciparum* have been cloned and the proteins characterised which showed significant sequence homology with the A and B subunit of those found in a variety of organisms. However no differences have been identified between CQ-resistant and CQ-sensitive parasite in either of these subunits that could explain CQ resistance phenotype[48-49]. It must be noted that although direct measurement of the intravacuolar pH of resistant and susceptible isolates has been attempted (independently), none of the studies described have compared absolute vacuolar pH values of resistant and susceptible isolates within the same study[50-53].

Based on the finding that resistant parasites released pre-accumulated CQ 40-50 times more rapidly than their susceptible counterparts and verapamil (VP) was able to inhibit this enhanced efflux and increase steady state levels of drug[41], it was then suggested that rapid efflux was responsible for CQ resistance in *P. falciparum.* Since VP is a classical chemosensitiser that can reverse drug efflux in multidrug-resistant tumour cells. However, these findings have since been questioned by a number of workers who failed to show differences in efflux rates between resistant and susceptible isolates[42, 54]. Furthermore, the study using mathematical model derived for the time course CQ accumulation by Ginsburg and Stein (1991) concluded that the differences in CQ accumulation between resistant and sensitive parasites could be explained purely by the differences in uptake force[46]. The observations that differences in drug activity correlated more favourably with rates of drug uptake rather than drug efflux have been confirmed in later studies[54-56]. According to Ginsburg and Stein's model, Bray *et al.* (1994) suggested that the resistant isolates may have an enhanced efflux capacity for CQ, this is however only at very low external drug concentrations which is possibly therapeutically irrelevant[55].

The level of CQ accumulation in malaria parasites could be due, at least in part, to the presence of a specific drug importer or 'permease'. Warhurst (1988) hypothesised that the differences seen in levels of CQ accumulation between resistant and susceptible isolates could be due to differences in the quantity, affinity for substrate and/or location of the 'permease' in resistant isolates[57]. It was also suggested that the permease could be situated on both the plasma membrane and the food vacuole membrane, but working in reverse to export drug from the vacuole, into the cytoplasm and then out of the parasite. This hypothesis is consistent with the earlier observations of Moreau *et al.* (1986) who showed that a closely related analogue of CQ was accumulated predominantly in the acid compartments of CQ susceptible *P. berghei*, whereas in resistant isolates this compound was highly localised in the cytoplasm also[58].

Recent work from Sanchez *et al.* (1997) based on the inhibition of saturable CQ uptake by the amiloride analogue, EIPA (a specific blocker of the plasma membrane Na^+/H^+ exchanger)[59] in the progeny of a genetic cross between CQ-resistant and CQ-sensitive clone[60] suggested that CQ is actively imported into the parasite by the Na^+ binding domain of the Na^+/H^+ exchanger, in exchange of protons. With modification, it was later suggested that CQ is carried through the Na^+/H^+ exchanger in a burst of self-stimulated sodium/proton exchange[61]. These authors then suggested that changes in the CQ importer could generate CQ resistance. However several evidences contradict this hypothesis, Bray *et al.* (1999) showed that CQ uptake by free parasites was identical when the extracellular sodium was replaced by either choline or glucamine. Moreover EIPA could compete for CQ accumulation simply by binding to haem which has been demonstrated to be vital for CQ accumulation[62]. Although the gene responsible for CQ resistance in these progeny has been identified and postulated to encode for a transporter, Su *et al.* (1997) could find no meaningful sequence with any ion channel or Na^+/H^+ exchanger[63].

Finally, it has been suggested that CQ resistance may be due to a reduced affinity of the intraparasitic binding site for CQ in resistant isolates[64]. Using a mathematical model based on the hypothesis that the high-affinity drug accumulation, rather than whole-cell accumulation, is responsible for its pharmacological activity, it has been shown that the apparent K_a for high-affinity uptake is significantly increased in resistant isolates and this apparent K_a can be reduced in the presence of VP without any effect on low-affinity uptake[65]. Further, it has been demonstrated that CQ-resistant and -sensitive isolates accumulate similar amounts of CQ at high affinity when external concentrations correspond to their respective IC_{50} values. The low-affinity uptake is equivalent in both isolates, however, because of the increased K_a of the high-affinity process in resistant isolates, the contribution of low-affinity accumulation is greater. This model can explain the observations that differences in CQ accumulation between sensitive and resistant isolates are not as great as the differences in their dose-response to CQ[42, 54-55] and that the increased CQ accumulation brought by VP is insufficient to explain the increased susceptibility to CQ also seen in the presence of VP[42, 55].

Mefloquine/halofantrine resistance

The mechanism of action of and resistance to MQ and HF remain unclear. It has been shown that drug susceptibility to MQ and HF correlate with accumulation. By the analysis as Bray *et al.* (1998) did[65], the activity of both MQ and HF depend on specific accumulation at a high affinity site with in the parasite[66]. Although there are a number of candidate accumulation sites[67], haem would appear to be a good candidate. Using a specific inhibitors of plasmepsin and a cysteine proteinase inhibitor suggest that MQ and HF exert their effect by an haem dependent mechansim[68].

Penfuridol can reverse MQ and HF resistance in *P. falciparum*[69-70], similar to the effect of VP on the CQ resistance. The ability of penfuridol to enhance susceptibility in resistant isolates is associated with an enhancement of drug accumulation[66].

Molecular characterisation of quinoline resistance

The demonstration that CQ resistance in *P. falciparum* could be partially reversed by VP led to the investigations at the molecular level[71]. This phenomenon had been linked

to that of mammalian multidrug-resistant (MDR) cancer cells where drug resistance is also associated with the reduction of intracellular drug accumulation. In cancer cells, VP is able to reverse the resistance phenotype by competing with the cytotoxic drug for an active efflux component on the cell membrane. This protein namely P-glycoprotein is encoded by the *mdr* gene[72-74]. Therefore, by analogy, it was suggested that VP exerted its chemosensitisation effects, in *P. falciparum*, by inhibiting the actions of an efflux pump[71]. In cancer cells, selection for MDR usually coincides with an over-expression of a P-glycoprotein and amplification of the *mdr* genes that encode this protein[75]. P-glycoprotein belongs to the family of Adenine nucleotide Binding Cassette (ABC) transporters. The classical P-glycoproteins are large plasma membrane glycoproteins consisting of two similar halves; each containing six putative transmembrane segments and an ATP-binding site. Drug resistance is caused by the ability of P-glycoprotein to extrude drugs against a concentration gradient, resulting in a decrease of the intracellular drug concentration available to the drug target. Studies with *P. falciparum* have resulted in the isolation and characterisation of three P-glycoprotein homologues, namely *pfmdr*1, *pfmdr*2 and *pfgcn*20[76-78]. Of these three *mdr*-like genes, only the *pfmdr*1 gene has been linked to the quinoline resistance phenotype.

_pfmdr_1 and Chloroquine resistance

Stronger evidence has been forwarded to implicate a possible role for *pfmdr*1 in drug resistance in *P. falciparum*. The *pfmdr*1 gene, which is situated on chromosome 5, has been shown to encode a 162 kDa protein, P-glycoprotein homologue 1 (Pgh1) which belongs to the ATP binding cassette (ABC) transporter family and shows 54 % homology with mammalian P-glycoprotein[79]. The protein consists of two homologous halves and an asparagine-rich hinge region; each half molecule contains 6 transmembranous domains and a nucleotide binding fold. The protein is present throughout all the asexual intraerythrocytic stages of the parasites life cycle and is located mainly on the membrane of the digestive acid food vacuole and to a lesser extent on the plasma membrane of the parasite[80]. More recent studies also localised this protein to other membrane structures in the parasite[81].

Original studies involving a limited number of *P. falciparum* isolates of varying susceptibility, suggested that CQ resistance might be linked to amplification of *pfmdr*1 and overexpression of Pgh1[79]. However, subsequent studies have failed to correlate amplification of *pfmdr*1 and parasite sensitivity to CQ[80]. Indeed these studies have indicated that not only can similar levels of Pgh1 be observed in both CQ-resistant and -susceptible isolates, but also that certain susceptible isolates could have higher levels of expression than the resistant parasites. In fact, subjecting moderately CQ resistant strains of *P. falciparum* to CQ to produce a higher level of CQ resistance resulted in deamplification of the *pfmdr1* gene from 3 copies to 1[82].

The lack of a correlation between Pgh1 expression and CQ resistance led investigators to speculate whether specific mutations in *pfmdr*1 might be responsible for CQ resistance. Studies by Foote *et al.* (1990) suggested that resistance to CQ might indeed be correlated with amino acid differences in the *pfmdr*1 gene[83], although again, these findings are not without controversy. In this study, the authors identified two 'alleles' that appeared to be related to CQ resistance. The authors were able to predict the sensitivity status of 34 out of 36 isolates of *P. falciparum* based solely upon whether or not they possessed these

alleles. One of the alleles (termed the K1 type) involved a single amino acid change (Asn^{86} to Tyr^{86}), the second (termed the 7G8 type) involved three amino acid substitutions (Ser^{1034} to Cys^{1034}, Asn^{1042} to Asp^{1042} and Asp^{1246} to Tyr^{1246}). Further studies, involving sequencing *pfmdr*1 from *P. falciparum* isolates from Africa, have also reported a strong relationship between the K1-type mutation and CQ resistance[84-85]. However, several reports failed to identify a complete linkage between any of the mutations and CQ-resistance phenotype in both field isolates and culture-adapted isolates[86-94].

Following the observation that application of CQ pressure to laboratory isolates of *P. falciparum*, resulting in an increase in CQ resistance, was accompanied by a deamplification of the *pfmdr*1 gene[82]. It was suggested that the Pgh1 might be involved in the accumulation of CQ in the food vacuole of the parasite. This hypothesis has been examined by transfection the *pfmdr*1 gene into Chinese hamster ovary cells (CHO), it has been shown that those cells expressing the wild-type Pgh1 are hypersensitive to CQ as a result of increased CQ accumulation[95]. However the transfected cells with the double mutant *pfmdr*1 gene with amino acid replacements at positions 1034 and 1042, showed neither CQ hypersensitivity nor the ability to accumulate CQ. Following work has indicated that cells expressing the wild-type Pgh1 have a lower intravacuolar pH compared to cells expressing the mutant Pgh1 or non-transfected cells[96]. It was proposed therefore that Pgh1 mediated increased CQ accumulation by decreasing vacuolar pH. Further, the author hypothesised that Pgh1 may be acting as a chloride channel, although no direct evidence was forwarded. This work has therefore strengthened the hypothesis that *pfmdr*1 may play a role in concentrating CQ within the malaria parasite's food vacuole, by reducing intravacuolar pH and thus increasing the force for drug accumulation in this cell type.

cg2 and Chloroquine resistance

The studies described above provide a rather confusing picture as to whether or not the *pfmdr*1 gene is involved in CQ resistance in *P. falciparum*. However, studies involving the analysis of a single genetic cross between a CQ-resistant and CQ-susceptible isolate of *P. falciparum* appear to provide the strongest evidence so far that the *pfmdr*1 gene is not involved in CQ resistance[60, 97]. The progeny exhibited the phenotypic characteristics of either the resistant or susceptible parent; this was seen as evidence that a single genetic locus may be responsible for the drug phenotype. In the initial study, inheritance of parental *pfmdr*1 did not segregate with the drug response. Further work by Su *et al.* (1997) identified *cg2*, a gene on chromosome 7 which encodes CG2 a unique ~300 kDa protein with complex polymorphism[63]. The polymorphism of this gene was linked to the CQ-resistant phenotype in these progeny. It must be noted that chromosome 7 contains no *pfmdr* genes (*pfmdr*1 is situated on chromosome 5). The CG2 protein was localised to the peripheral membrane and in association with haemozoin of the food vacuole prompting speculation that CG2 is a drug trafficking protein. However the experiments of genetic transformation by the same group indicated that CG2 may not play a role in CQ resistance[98]. Recently a novel, complex polymorphic gene named *pfcrt* has been identified and linked to CQ resistance in the genetic cross progeny and field isolates[99]. Further studies of mechanistic role of this gene has to be done.

*Pfmdr*1 and Mefloquine/Halofantrine resistance

Although the role of the *pfmdr*1 gene in CQ resistance is unclear, several studies have identified a link between the amplification of *pfmdr*1 and MQ and HF resistance. A number of workers have shown that the selection of MQ-resistant isolates by subjecting parent lines to sequentially increasing MQ concentrations was associated with an amplification of *pfmdr*1[76, 100-101]. It must also be noted that in the studies described the decrease in MQ susceptibility obtained in these drug pressure experiments was accompanied by cross-resistance to HF and QN and also an increase in CQ susceptibility. Conversely, both Barnes *et al.* (1992) and Peel *et al.* (1994) have shown that CQ resistance selected by CQ pressure, was accompanied by a deamplification of *pfmdr1* and an increase in susceptibility to MQ and HF[82, 101]. These observations have further strengthened the link between MQ and by implication HF (which share similar cross-resistance patterns to MQ) resistance and *pfmdr*1 gene expression. In addition, work by Wilson *et al.* (1993) involving the analysis of a number of resistant field isolates from Thailand, appeared to confirm that both MQ and HF resistance is indeed linked to *pfmdr*1 amplification[86]. Experiments in a heterologous yeast system showed that Pgh1 can act as a transporter, expression of *pfmdr*1 has been shown to functionally complement the *ste6* mutation in *Saccharomyces cerevisiae*[102]. The yeast *ste6* gene encodes a P-glycoprotein that exports the yeast a-type mating factor, mutants being unable to export this peptide[103]. These workers provided further evidence for the functional importance of amino acid differences in the *pfmdr*1 gene. Cells expressing Pgh1 containing 2 of 3 7G8-type mutations were unable to complement the *ste6* mutation. Using this yeast model, it has been demonstrated that the *pfmdr*1 gene confers resistance in yeast cells to MQ, HF, QN and mepacrine[104]. Drug resistance in *pfmdr*1 transformants was associated with decreased drug accumulation and an increase in drug release from preloaded cells. Again it was also reported that mutations associated with the 7G8 CQ-resistant allele resulted in loss of function.

However recent reports question the role of *pfmdr*1 in aminoalcohol resistance. Studies of a HF-resistant isolate (K1Hf), selected by intermittent drug exposure, has indicated that the decrease in HF susceptibility obtained, which is accompanied by a decrease in MQ susceptibility and an increase in CQ susceptibility, is not accompanied by an amplification of *pfmdr*1[105]. Furthermore, similar findings have been reported for cloned lines selected for MQ resistance by the application of MQ drug pressure[106]. Recent work also shows no association between reduced sensitivity to MQ and HF and the level of *pfmdr*1 amplification and expression in recently adapted and fresh Thai isolates[70, 107-108]. These studies indicate that the acquisition of MQ and HF resistance need not always be accompanied by an amplification of *pfmdr*1.

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