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Mutations in *pfmdr1* Modulate the Sensitivity of *Plasmodium falciparum* to the Intrinsic Antiplasmodial Activity of Verapamil

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As well as having the ability to reverse chloroquine resistance in the human malaria parasite *Plasmodium falciparum*, verapamil has itself an innate antiplasmodial activity. We show here that mutations in Pgh1, the product of the malaria parasite's *pfmdr1* gene, influence the parasite's susceptibility to the toxic effects of verapamil.

Verapamil (VP) is a weak base which, in addition to acting as a reverser of chloroquine resistance (CQR) in the malaria parasite *Plasmodium falciparum*, has itself an intrinsic antiplasmodial activity (1, 12, 14, 26). This activity is independent of its CQR reversal effect, as the susceptibility of chloroquine (CQ)-sensitive parasites to CQ is unaltered even in the presence of highly toxic concentrations of VP, whereas VP alters the susceptibility of CQ-resistant parasites to CQ at both toxic and nontoxic concentrations (15). The CQR reversal effect of VP has been attributed to an interaction of the compound with the *P. falciparum* chloroquine resistance transporter (PfCRT) (7, 17, 23), a member of the drug-metabolite transporter superfamily (13, 25) which is localized to the parasite's internal digestive vacuole and is the key determinant of CQR (8, 23).

Recent allelic exchange experiments have shown that mutations in the *pfmdr1* gene product, P-glycoprotein homologue 1 (Pgh1), modulate sensitivity to a range of antimalarial compounds (20). Pgh1 is localized predominantly to the parasite's digestive vacuole membrane (4, 10). It belongs to the ATP-binding cassette transporter superfamily, members of which couple ATP hydrolysis to the translocation of a diverse range of structurally unrelated solutes across cell membranes (reviewed in reference 11). We report here that polymorphisms in *pfmdr1* influence the parasite's susceptibility to the intrinsic antiplasmodial effect of VP.

Transfected *P. falciparum* parasites derived from either D10 (CQ-sensitive) or 7G8 (CQ-resistant) strains with *pfmdr1* loci altered as described previously (20) were obtained from Alan Cowman (Walter and Eliza Hall Institute, Melbourne, Australia). Parasite susceptibility to VP was measured in vitro by 48-h [^{3}H]hypoxanthine incorporation assays in 96-well plates (5), with half-maximal inhibitory concentrations (IC_{50}s) derived from fitted curves (SigmaPlot 2001; SPSS Inc.).

The introduction of either one or three of the four 7G8 mutations into the *pfmdr1* gene of the D10 parasite (to make D10-mdr$^{7G8/1}$ or D10-mdr$^{7G8/3}$) significantly increased VP sensitivity with respect to the D10-mdr^{D10} transfectant, a control transfectant with the wild-type *pfmdr1* gene (Table 1). Notably, D10-mdr$^{7G8/1}$, with a single mutation at codon 1246, was more sensitive to VP than D10-mdr$^{7G8/3}$, which carries two additional mutations at codons 1034 and 1042. Replacement of these three mutations in the 7G8 parasite with the corresponding wild-type D10 *pfmdr1* sequence (7G8-mdr^{D10}) was sufficient to confer full D10-like tolerance to VP. Further analysis shows that the pattern of relative sensitivity of these transfectants to VP correlates significantly with the pattern of sensitivity to mefloquine (MQ) and halofantrine (HF) obtained in a previous study (20) ($r^2 = 0.82$ and $P = 0.035$ in both cases). This result suggests that specific mutations in *pfmdr1* mediate sensitivity to these compounds via a common mechanism. There is no difference in the levels of Pgh1 expression—a parameter which has been shown to affect MQ sensitivity (3, 19)—among these strains (20).

Unlike most quinoline antimalarials, VP does not interact with hematin, a toxic by-product of hemoglobin proteolysis by the parasite, even at concentrations far in excess of those measured in these experiments (2, 27). This finding suggests that the intrinsic antiplasmodial activity of VP is not a consequence of interference with the process of hematin detoxification by the parasite, a possible mode of action for MQ and HF (2, 18, 24). However, CQR-associated mutations in the digestive vacuole protein PfCRT have also been noted to increase the parasite's sensitivity to VP (8), MQ (23), and HF (9), suggesting that the digestive vacuole membrane is an important site for mediating resistance to these compounds.

It is possible that Pgh1 mediates changes in sensitivity to these lipophilic drugs indirectly, via alterations in an as-yet-unknown parameter of the parasite's physiology. However, the similarity of the VP and MQ sensitivity profiles of these *P. falciparum* transfectants invites comparison with the interaction of these drugs with the homologous mammalian ATP-binding cassette transporter, P-glycoprotein. VP and MQ have both previously been shown to bind directly to P-glycoprotein, although MQ does so with a higher affinity than VP (21). In the same study, MQ was also shown to be a more potent inhibitor

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TABLE 1. IC_{50}s for growth inhibition of the *pfmdrI* transfectants by verapamil

Transfectant	Pgh1 haplotype at codon:					IC_{50}[a] (μM)
	86	184	1034	1042	1246	
D10-mdr^{D10}	N	Y	S	N	D	28.2 ± 3.8
D10-$mdr^{7G8/1}$	N	Y	S	N	Y	13.1 ± 1.3[b]
D10-$mdr^{7G8/3}$	N	Y	C	D	Y	20.3 ± 3.1[b]
7G8-mdr^{7G8}	N	F	C	D	Y	7.7 ± 0.8
7G8-mdr^{D10}	N	F	S	N	D	26.6 ± 4.1[b]

[a] Values are the means (± standard errors of the means) of results from six independent experiments, each carried out in either duplicate or triplicate.

[b] Changes in IC_{50} relative to the parental control transfectant were significant as assessed with Student's *t* test ($P < 0.05$).

of P-glycoprotein transport activity than VP, increasing the sensitivity of drug-resistant tumor cell lines to cytotoxic agents. Likewise, our observations show that while these transfectants respond to VP and MQ with the same pattern of sensitivity, VP is far less potent than MQ; the effective concentration of VP is in the micromolar range, whereas that for MQ is in the nanomolar range (20). These data prompt the hypothesis that VP and MQ interact directly with Pgh1 and that the mutations of interest here affect the sensitivity of the parasites to VP and MQ by influencing this interaction. The most plausible explanations for a direct interaction are either that Pgh1 pumps these compounds away from their target in a manner akin to the efflux activity of P-glycoprotein or that Pgh1 is itself the target for these drugs, as has been suggested previously for MQ (22).

It should be noted that a photoreactive analogue of MQ used to attempt to identify the targets of the drug in *P. falciparum* labeled a protein of the same size as Pgh1 only weakly (6). The failure to detect preferential, MQ-inhibitable labeling of Pgh1 by the probe could indicate that Pgh1 does not interact directly with MQ. However, the photoreactive analogue had only 1/10 of the antiplasmodial activity of MQ, and the authors caution that the technique may not have been sufficiently sensitive to detect a preferential interaction of the probe with Pgh1 given the low abundance of the protein in the parasite (6). The data therefore do not exclude the possibility that MQ interacts with Pgh1 directly. MQ is known to inhibit at least one other ATP-dependent pump, the F_oF_1 H^+-ATPase of *Streptococcus pneumoniae* (16). Transformation experiments demonstrated that MQ resistance was conferred by mutations in the transmembrane domains of subunits forming the pump's proton channel, and there was a good correlation between the levels of inhibition of ATPase activity by MQ and inhibition of growth of the various mutant strains (16).

In conclusion, our data demonstrate a role for Pgh1 in mediating parasite sensitivity to the intrinsic antiplasmodial effects of VP. The fact that mutations in *pfmdr1* have the same effects on parasite sensitivity to the lipophilic drugs VP, MQ, and HF suggests a common mechanism of resistance to these drugs, possibly through a direct interaction with Pgh1.

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