**Acknowledgements**

We thank the *C. elegans* Genome Sequencing Consortium for sequence data, A. Coulson and the Sanger Centre for cosmids, the *Caenorhabditis* Genetics Center and V. Ambros for providing strains and sharing unpublished results. We thank R. Feinbaum for advice concerning experimental procedures and Y. Liu and P. Delerme for technical assistance. This work was supported by NIH grants to G.R., H.R.H. and A.R., and an NIH postdoctoral fellowship to F.S. H.R.H. is an Investigator of the Howard Hughes Medical Institute.

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# Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*

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**Throughout the latter half of this century, the development and spread of resistance to most front-line antimalarial compounds used in the prevention and treatment of the most severe form of human malaria has given cause for grave clinical concern. Polymorphisms in pfmdr1, the gene encoding the P-glycoprotein homologue 1 (Pgh1) protein of *Plasmodium falciparum*, have been linked to chloroquine resistance[1]; Pgh1 has also been implicated in resistance to mefloquine and halofantrine[2–5]. However, conclusive evidence of a direct causal association between pfmdr1 and resistance to these antimalarials has remained elusive, and a single genetic cross has suggested that Pgh1 is not involved in resistance to chloroquine and mefloquine[6]. Here we provide direct proof that mutations in Pgh1 can confer resistance to mefloquine, quinine and halofantrine. The same mutations influence parasite resistance towards chloroquine in a strain-specific manner and the level of sensitivity to the structurally unrelated compound, artemisinin. This has important implications for the development and efficacy of future antimalarial agents.**

Two alleles of the *pfmdr1* gene identified in field isolates of *P. falciparum* are linked with chloroquine resistance (CQR). One of these, the '7G8 allele', encodes four amino-acid substitutions with respect to the chloroquine-sensitive (CQS) 'D10 allele': Tyr 184 to Phe 184; Ser 1034 to Cys 1034; Asn 1042 to Asp 1042; and Asp 1246 to Tyr 1246 (refs 1–7). To examine the role of the last three mutations of Pgh1 in controlling parasite sensitivity and resistance to antimalarials, we constructed plasmids for *P. falciparum* transformation and allelic exchange at the endogenous *pfmdr1* locus[8].

Plasmid pHC1-mdr$^{7G8}$ replaced the *pfmdr1* gene in CQS D10 parasites such that the protein carried the mutations Cys 1034, Asp 1042 and Tyr 1246. Plasmid pHC1-mdr$^{D10}$ (Fig. 1a) served as a transfection control and resulted in retention of the amino acids Ser 1034, Asn 1042 and Asp 1246 in Pgh1. In this manner, we generated (1) the parasite line D10-mdr$^{D10}$ which retained the wild-type *pfmdr1* sequence, (2) the parasite line D10-mdr$^{7G8/3}$ into which the *pfmdr1* gene encoding the Cys 1034, Asp 1042 and Tyr 1246 mutations was inserted, and (3) the parasite line D10-mdr$^{7G8/1}$ which encoded the Tyr 1246 mutation in *pfmdr1* owing to a single recombination event in the gene between the codons encoding this amino acid and position 1042 (Fig. 1a). Analysis of genomic DNA by Southern hybridization (Fig. 1b) and sequencing of the *pfmdr1* gene confirmed these integration events.

To determine the role of the Cys 1034, Asp 1042 and Tyr 1246 substitutions in a distinct genetic background, we made similar constructs for CQR 7G8 parasites and carried out analogous experiments. Plasmid pHH1-mdr$^{D10}$ (Fig. 1c) allowed allelic replacement of the *pfmdr1* gene within 7G8 parasites such that the gene encoded the wild-type (D10) amino acids Ser 1034, Asn 1042 and Asp 1246. The two cloned lines, 7G8-mdr$^{D10/c1}$ and 7G8-mdr$^{D10/c2}$, were generated in this manner (Fig. 1c). pHH1-mdr$^{7G8}$ (Fig. 1c) served as a transfection control and, once integrated, retained the mutant *pfmdr1* allele (7G8-mdr$^{7G8}$ parasites; Fig. 1c).

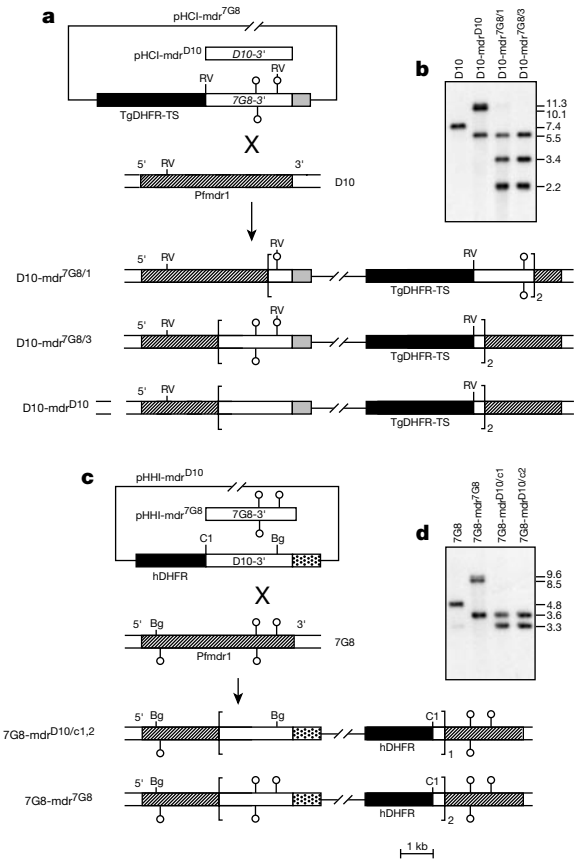


**Figure 1** Allelic replacement of the *pfmdr1* gene. **a**, Allelic replacement of the *pfmdr1* gene in the D10 cloned parasite line. The transfection plasmids pHC1-mdr$^{7G8}$ and pHC1-mdr$^{D10}$ are shown. Open circles indicate the mutations Cys 1034, Asp 1042 and Tyr 1246 in pHC1-mdr$^{7G8}$ (ref. 1). The codon for Tyr 1246 creates an *Eco*RV site that was used to map the integration events for this plasmid. The selection cassette Tg*dhfr-ts* (*Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase), which confers resistance to pyrimethamine[20–22] is indicated. The integration structure for D10-mdr$^{7G8/1}$, in which the recombination event occurred between the Asp 1042 and Tyr 1246 polymorphisms in the pHC1-mdr$^{7G8}$plasmid resulting in the introduction of only the Tyr 1246 mutation in the endogenous *pfmdr1* gene, and the structures of the plasmid integration events in D10-mdr$^{7G8/3}$ (for plasmid pHC1-mdr $^{7G8}$) and D10-mdr$^{D10}$ (for plasmid pHC1-mdr$^{D10}$) are shown. All integration events occurred through a single recombination event resulting in reconstitution of the *pfmdr1* gene and displacement of a fragment of the gene downstream with insertion of two copies of the plasmid in each case[8]. RV, *Eco*RV. **b**, Southern hybridization of genomic DNA digested with *Eco*RV from each parasite line. **c**, Allelic replacement of the *pfmdr1* gene in the 7G8 cloned parasite line. The transfection plasmids pHH1-mdr$^{D10}$ and pHH1-mdr$^{7G8}$ are shown. The selection cassette includes the human *dhfr* gene. Integration events are shown for the two clones 7G8-mdr$^{D10c1/c2}$ and 7G8-mdr$^{7G8}$. The codon for Asp$^{1246}$ creates a *Bgl* II site. Bg, *Bgl* II; CI, *Cla*1. **d**, Southern hybridization of *Bgl*II/*Cla*I-digested genomic DNA from each parasite line. Size of DNA fragments are shown in kb (**b**,**d**).

Southern blot analysis (Fig. 1d) and sequencing of the *pfmdr1* gene verified the integration and relevant gene sequence. Immunoblot analysis using anti-Pgh1 antibodies[9] showed that each transfected line expressed equivalent levels of Pgh1.

To test the role of Cys 1034, Asp 1042 and Tyr 1246 in determining drug resistance, we carried out *in vitro* drug assays[10] for chloroquine, mefloquine, quinine, halofantrine and artemisinin (Fig. 2; and Table 1). Introduction of the *pfmdr1* polymorphisms into CQS D10 parasites had no effect on parasite sensitivity to chloroquine (Fig. 2; and Table 1). In contrast, replacement of the 7G8 mutations with wild-type D10 *pfmdr1* sequence in CQR 7G8 parasites halved the level of CQR (Fig. 2; and Table 1). These results show that *pfmdr1* polymorphisms are insufficient to confer CQR in D10. In 7G8, however, *pfmdr1* provides a cumulative effect with additional gene(s) to confer higher levels of CQR. This indicates that *pfmdr1* mutations in *P. falciparum* may have been selected by chloroquine pressure and may be required for resistance to high levels of drug.

Introduction of the 7G8 mutations into the D10 *pfmdr1* gene (D10-mdr$^{7G8/3}$) converted a quinine-sensitive isolate into one that is quinine resistant. We confirmed this by subsequent removal of the same mutations from 7G8 (7G8-mdr$^{D10/c1}$ and 7G8-mdr$^{D10/c2}$), which resulted in reversion to sensitivity for quinine (Fig. 2; and Table 1). These results indicate that mutations in *pfmdr1* alone are sufficient to confer quinine resistance in the D10 and 7G8 genetic backgrounds. Notably, the 7G8 mutations can confer quinine resistance in D10 and yet have no effect on CQR in this genetic background. It is possible, however, that quinine resistance is also dependent on multiple genes and that *pfmdr1* contributes to the overall phenotype.

The *pfmdr1* substitutions had a marked effect on susceptibility to mefloquine and halofantrine. Introduction of the Cys 1034, Asp 1042 and Tyr 1246 into D10 Pgh1 conferred increased sensitivity to both drugs (Fig 2; and Table 1). Conversely, altering *pfmdr1* within 7G8 (mefloquine- and halofantrine-sensitive) parasites to encode Ser 1034, Asn 1042 and Asp 1246 conferred resistance against both antimalarials. Notably, the single Tyr 1246 mutation (D10-mdr$^{7G8/1}$) had a larger effect on the half-maximal inhibitory concentration ($IC_{50}$) for both mefloquine and halofantrine and suggests that this amino-acid position is directly involved in mefloquine accumulation (see below). Clearly, the presence of the Cys 1034, Asp 1042 and Tyr 1246 pfmdr1 substitutions in the field would provide a disadvantage in terms of a parasite's ability to survive the use of mefloquine and/or halofantrine in these areas. The converse would be true for quinine and, in some parasite backgrounds, chloroquine.

Insertion of the three mutations into D10 *pfmdr1* increased the level of artemisinin sensitivity almost twofold (Fig. 2; and Table 1). Conversely, removal of the mutations from 7G8 caused a decrease in sensitivity by about the same margin. The *pfmdr1* effect on artemisinin susceptibility mimics the results seen with mefloquine and halofantrine. This is consistent with a common mechanism whereby *pfmdr1* can influence the accumulation of mefloquine, halofantrine and artemisinin, a hypothesis that is consistent with field studies that show a positive correlation between the $IC_{50}$ for artemisinin and that of mefloquine and halofantrine[11].

Removal of the Cys 1034, Asp 1042 and Tyr 1246 mutations from 7G8 *pfmdr1* increased saturable, steady-state accumulation of chloroquine in 7G8-mdr$^{D10/c1}$ and 7G8-mdr$^{D10/c2}$ as compared with 7G8-mdr$^{7G8}$ ($P = 0.01$ and $P = 0.035$, respectively; Fig. 3a), which is consistent with the reduction in the chloroquine $IC_{50}$ (Fig. 2; and Table 1). A linear inverse correlation between the saturable accumulation of chloroquine and the $IC_{50}$ for inhibition of parasite growth has been previously demonstrated[12]. The twofold increase in accumulation by 7G8-mdr$^{D10/c1}$ and 7G8-mdr$^{D10/c2}$ relative to 7G8-mdr$^{7G8}$ (Fig. 3a) therefore accounts for the twofold decrease in the $IC_{50}$ of chloroquine. No change in chloroquine accumulation was observed for either D10-mdr$^{7G8/1}$ or D10-mdr$^{7G8/3}$. In contrast, the

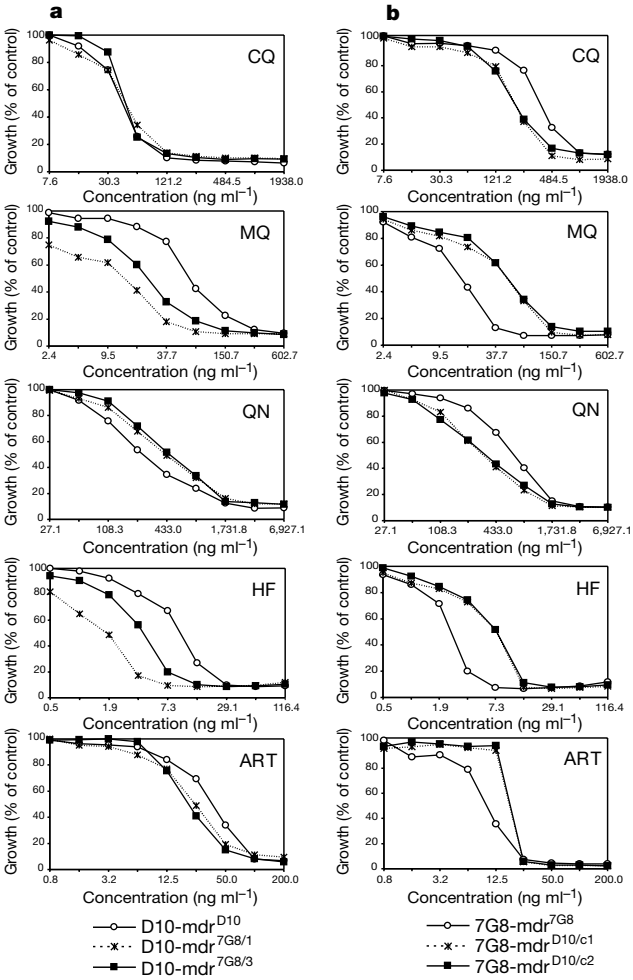


**Figure 2** Effect of introduction/removal of the polymorphisms in the D10 and 7G8 cloned lines on susceptibility and resistance to chloroquine, mefloquine, halofantrine, quinine and artemisinin. **a**, Microdilution assays to determine drug susceptibility of D10 compared with the transfectants D10-mdr$^{D10}$, D10-mdr$^{7G8/1}$and D10-mdr$^{7G8/3}$. The drugs used are indicated: CQ, chloroquine; MQ, mefloquine; QN, quinine; HF, halofantrine; ART, artemisinin. Experiments shown were performed twice in triplicate. **b**, As in panel **a**, but microdilution assays were also carried out using the parasite lines 7G8, 7G8-mdr$^{7G8}$, 7G8-mdr$^{D10/c1}$, 7G8-mdr$^{D10/c2}$.

**Table 1 Effect of mutations in the *pfmdr1* gene on the sensitivity and restance to diverse antimalarials**

| Parasite | 50% Inhibitory concentration, $IC_{50}$ (nM)* | | | | |
|---|---|---|---|---|---|
| | Chloroquine | Mefloquine | Quinine | Halofantrine | Artemisinin |
| D10 | 47.9 ± 1.6 | 73.3 ± 6.5 | 269.5 ± 18.0 | 9.5 ± 0.6 | 42.2 ± 5.3 |
| D10-mdr$^{D10}$ | 45.2 ± 3.7 | 71.1 ± 4.6 | 272.6 ± 33.5 | 10.4 ± 0.6 | 38.7 ± 4.6 |
| D10-mdr$^{7G8/1}$ | 52.7 ± 4.8 | 14.9 ± 1.4‡ | 413.0 ± 53.2 | 1.7 ± 0.2‡ | 23.8 ± 2.1‡ |
| D10-mdr$^{7G8/3}$ | 48.4 ± 0.8 | 25.8 ± 1.9‡ | 505.0 ± 83.9‡ | 4.3 ± 0.4‡ | 21.6 ± 1.4‡ |
| 7G8 | 382.4 ± 8.9 | 15.2 ± 0.2 | 585.6 ± 18.6 | 2.1 ± 0.2 | 10.9 ± 0.6 |
| 7G8-mdr$^{7G8}$ | 389.5 ± 12.4 | 16.9 ± 1.7 | 692.5 ± 62.0 | 2.6 ± 0.2 | 12.3 ± 1.5 |
| 7G8-mdr$^{D10/c1}$ | 204.1 ± 1.2‡ | 55.2 ± 2.9‡ | 310.0 ± 29.1‡ | 7.6 ± 0.4‡ | 18.6 ± 0.5‡ |
| 7G8-mdr$^{D10/c2}$ | 215.5 ± 15.3‡ | 53.3 ± 7.0‡ | 378.7 ± 66.8‡ | 7.6 ± 1.1‡ | 19.0 ± 0.4‡ |
| Threshold of resistance† | 100 | 25–30 | 450–500 | 5–20 | – |

*Values represent the mean (±s.e.m.) of two independent assays each performed in triplicate.
† The threshold of resistance corresponds to values previously defined[11,27] and have been obtained by determining serum drug concentration in patients infected with sensitive and resistant *P. falciparum*.
‡ When compared with the relevant controls the changes in $IC_{50}$ values were significant as assessed using the Mann–Whitney U test ($P < 0.05$).

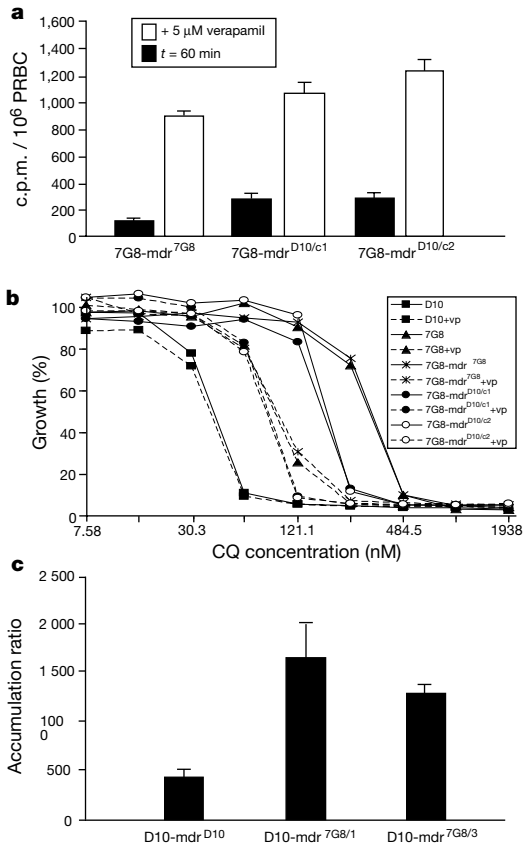


**Figure 3** Effect of *pfmdr1* mutations on accumulation of antimalarials and modulation with verapamil. **a**, Saturable chloroquine accumulation and ability of verapamil (vp; 5μM) to increase chloroquine accumulation in 7G8-mdr$^{7G8}$, 7G8-mdr$^{D10/c1}$ and 7G8-mdr$^{D10/c2}$. PRBC, parasitized red blood cells. The accumulation was measured over 60 min. Experiments shown were performed three times, each in duplicate. Error bars, s.e.m. **b**, Microdilution assays to determine chloroquine sensitivity in the presence and absence of 5 μM verapamil for D10, 7G8, 7G8-mdr$^{7G8}$, 7G8-mdr$^{D10/c1}$ and 7G8-mdr$^{D10/c2}$. **c**, Mefloquine cellular accumulation ratios for D10-mdr$^{D10}$, D10-mdr$^{7G8/1}$and D10-mdr$^{7G8/3}$ as estimated by the inoculum effect. Two independent experiments were each carried out in duplicate. Error bars, s.e.m. The difference in accumulation between D10-mdr$^{D10}$ and the tranfected lines D10-mdr$^{7G8/1}$ and D10-mdr$^{7G8/3}$ were significantly different ($P < 0.05$).

change in phenotype from mefloquine resistance to mefloquine sensitivity was reflected in increased accumulation of mefloquine for both D10-mdr$^{7G8/1}$ and D10-mdr$^{7G8/3}$relative to D10-mdr$^{D10}$ ($P < 0.05$; Fig. 3c)[13].

Drug resistance and accumulation of chloroquine in *P. falciparum* can be reversed by verapamil[14], although the level of chloroquine sensitivity obtained is never equivalent to that seen for CQS lines[15]. This indicates that a component of CQR may remain verapamil insensitive and supports the hypothesis that the CQR phenotype is multigenic[16]. In the presence of verapamil, the chloroquine accumulation (Fig. 3a) and chloroquine sensitivity (Fig. 3b) of 7G8-mdr$^{7G8}$, 7G8-mdr$^{D10/c1}$ and 7G8-mdr$^{D10/c2}$were the same. Addition of verapamil caused a sevenfold increase in the saturable accumulation of chloroquine by 7G8-mdr$^{7G8}$, and a fourfold increase in the saturable accumulation by both 7G8-mdr$^{D10/c1}$ and 7G8-mdr$^{D10/c2}$, which is consistent with the observation that verapamil caused a larger shift in the chloroquine $IC_{50}$ for 7G8-mdr$^{7G8}$ than for 7G8-mdr$^{D10/c1}$ and 7G8-mdr$^{D10/c2}$ (Fig. 3a, b). Therefore, the increased CQR attributable to *pfmdr1* occurs within the component of the phenotype sensitive to verapamil, though it remains to be established whether Pgh1 is a target or if reversal occurs by an alternative mechanism[17].

After a decade of fierce debate, these data provide the first direct evidence, to our knowledge, of the involvement of Pgh1 in conferring high levels of CQR through decreased chloroquine accumulation. Nevertheless, it is clear that CQR cannot be conferred by Pgh1 alone and requires the presence of mutations in other (unidentified) gene(s)[1,6,18]. In addition, we have shown that changes in Pgh1 can modulate resistance to quinine, mefloquine and halofantrine. The Cys 1034, Asp 1042 and Tyr 1246 mutations in *pfmdr1* are distributed in Africa, South America and Asia, which suggests that there is selective pressure for their maintenance and spread[1]. This is consistent with an important role for these *pfmdr1* mutations in antimalarial drug resistance throughout these geographical areas. That these mutations can confer resistance and sensitivity in two cloned lines originating from Papua New Guinea (D10 parasites) and South America (7G8 parasites) is further support for this view. The finding that there are strains of *P. falciparum* in malaria-endemic areas showing decreased sensitivity to artemisinin has serious implications for the future prospects of this important antimalarial. □

## Methods

### Plasmid construction

pSP72-based plasmids (Promega) bearing the complete coding regions of *pfmdr1* (ref. 7) cloned as single *Xho*I fragments from D10 (Papua New Guinea) and 7G8 (South America) *P. falciparum* isolates (plasmids designated p12 and p12-7G8, respectively) were digested with *Eco*RI/*Xho*I to release 2.8-kilobase (kb) 3′-terminal fragments of *pfmdr1*. After 'fill-in' reactions using Vent polymerase (New England Bioloabs), the 2.8-kb DNA fragments were subcloned into *Sma*I-digested pBluescript-SK (Stratagene). Plasmids bearing inserts in the required orientation were subsequently chosen to allow release of the complete *pfmdr1* insert as a *Xho*I fragment. To generate plasmids pHC1-mdr$^{D10}$and pHC1-mdr$^{7G8}$, these *Xho*I inserts derived from D10 and 7G8 parasites were then cloned into the *P. falciparum* transfection vector pHC1 (ref. 19). To generate a transfection vector suitable for transforming pyrimethamine-resistant parasites (including 7G8), the *Toxoplasma gondii dhfr-ts* region contained within pHC4 (refs 20–22) was replaced by a human *dhfr* fragment mutated to encode resistance to methotrexate and WR99210 (ref. 23). This human *dhfr* fragment was derived by polymerase chain reaction (PCR) from pHD22Y. The resultant vector pHH1 was then digested with *Hinc*II/*Xho*I to remove the *hsp86* upstream regulatory sequences and enable cloning of the 2.8-kb *pfmdr1* fragments. The resultant plasmid constructs were designated pHH1-mdr$^{D10}$ and pHH1-mdr$^{7G8}$.

### Parasite transformation

Transfection of plasmids pHC1-mdr$^{D10}$ and pHC1-mdr$^{7G8}$ into D10 parasites and pHH1-mdr$^{D10}$, pHH1-mdr$^{7G8}$ into 7G8 parasites was carried out using the low-voltage, high-capacitance conditions described[23]. Selection for pyrimethamine resistance (pHC1-based plasmids) was carried out essentially as described[8], and selection for stable transfection and integration of pHH1-based vectors was carried out in the same manner using 5 nM WR99210 (kindly provided by D. Jacobus). Where appropriate, parasites were cloned by limiting dilution[24].

### $IC_{50}$ determinations

Sensitivity to antimalarials was assessed using a modification of the standard microdilution technique described[10]. For this modification, predominantly ring-stage parasites (1% parasitaemia, 4% haematocrit) were plated out in hypoxanthine-free media in the presence of [8-$^3$H]hypoxanthine (Amersham) for the duration of the assay. Drug sensitivity was plotted as the percentage of growth relative to growth in drug-free medium and $IC_{50}$ values were determined graphically. Each assay was performed in triplicate on two independent occasions.

### Chloroquine accumulation

Chloroquine accumulation in cells suspended in bicarbonate-free RPMI medium, at a final haematocrit of 2% and parasitaemia of between 1.3–6.4% was measured as described[15]. [$^3$H]chloroquine (1 nM and 50.4 Ci mmol$^{-1}$) was incubated with cells for 60 min at 37 °C before separating the cells from the suspending medium by centrifugation through an oil layer[15]. The cell pellets were lysed with 0.5% v/v Triton X-100, bleached with 1% w/v sodium hypochlorite, then neutralized with HCl before scintillation counting. Chloroquine is accumulated by parasitized erythrocytes in both saturable and non-saturable processes, and it is the former process that has been correlated with the antiplasmodial action of the drug[12]. The saturable component of [$^3$H]chloroquine accumulation was estimated by subtracting the non-saturable accumulation, measured in the presence of 10 μM unlabelled chloroquine, from the total accumulation, measured in the absence of unlabelled drug. The small contribution of uninfected cells to the uptake of [$^3$H]chloroquine in parasitized cell suspensions was subtracted in all cases.

### Mefloquine accumulation

Because of the lipophilic nature of mefloquine, previous investigations have indicated a significant 'inoculum effect' with this drug[25]. As a result, measured drug $IC_{50}$ increases with increasing inoculum size (inoculum size = parasitaemia × haematocrit) owing to significant drug depletion from the medium. This phenomenon has been described in detail[13,15,26]. In this study, $IC_{50}$ of mefloquine was assessed at inoculum sizes ranging from 1 to 10 (fractional parasite volume 0.0001 to 0.001) at a fixed haematocrit of 4%. Extrapolation of the linear relationship between measured $IC_{50}$ and inoculum size provides a measure of absolute drug $IC_{50}$ at a theoretical inoculum of zero. The mathematical relationship for the determination of the cellular drug accumulation ratio is

$$\text{Accumulation ratio} = \frac{IC_{50}\ \text{measured} - IC_{50}\ \text{absolute}}{IC_{50}\ \text{absolute} \times \text{fractional parasite volume}}$$

Parasites were synchronized with sorbitol 48 h before accumulation studies and two independent assays were performed, each in duplicate.

Received 10 September; accepted 21 December 1999.


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### Acknowledgements

We thank S. Ward and P. Bray for the gift of [$^3$H]chloroquine and for advice throughout this work, and T. Wellems and D. Fidock for providing the human *dhfr* gene. This work was supported by the National Health and Medical Research Council of Australia (NHMRC) and the National Institute of Health, USA. M.B.R. is supported by a NHMRC Peter Doherty Research Fellowship. We are grateful to the Blood Banks of the Red Cross of Victoria and Australian Capital Territory for generously providing serum and red cells.


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# The LIM homeobox gene *Lhx9* is essential for mouse gonad formation


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**During mammalian embryonic development, the ovaries and testes develop from somatic cells of the urogenital ridges as indifferent gonads, harbouring primordial germ cells that have migrated there. After sex determination of the gonads, the testes produce testosterone and anti-Mullerian hormone which mediate male sexual differentiation, and the female developmental pathway ensues in their absence[1–3]. Here we show that transcripts of the LIM homeobox gene *Lhx9* are present in urogenital ridges of mice at embryonic day 9.5; later they localize to the interstitial region as morphological differentiation occurs. In mice lacking Lhx9 function, germ cells migrate normally, but somatic cells of the genital ridge fail to proliferate and a discrete gonad fails to form. In the absence of testosterone and anti-Mullerian hormone, genetically male mice are phenotypically female. The expression of steroidogenic factor 1 (Sf1), a nuclear receptor essential for gonadogenesis[2], is reduced to minimal levels in the Lhx9-deficient genital ridge, indicating that Lhx9 may lie upstream of Sf1 in a developmental cascade. Unlike mice lacking other genes that mediate early stages of gonadogenesis[4–6], *Lhx9* mutants do not exhibit additional major developmental defects. Thus, *LHX9* mutations may underlie certain forms of isolated gonadal agenesis in humans.**


In mouse embryos, the sexually undifferentiated gonads emerge from the urogenital ridges as distinct structures by embryonic day 12.0 (E12.0)(ref. 2). During this indifferent stage, the superficial coelomic mesothelium of the genital ridge proliferates into the subjacent loose connective mesenchymal tissue. In males (Fig. 1a), this proliferating epithelium subsequently forms sex cords that extend into the mesenchyme of the presumptive gonad. The testicular cords contain Sertoli cells which surround the primordial germ cells that have migrated to the gonadal ridge. Eventually, the cords lose contact with the surface epithelium and are separated from it by the subjacent mesenchyme that later differentiates to form the tunica albuginea. Meanwhile, the deeper layers of mesenchyme form the interstitial mesenchymal cells of the testes,