pfmdr2 Confers Heavy Metal Resistance to Plasmodium falciparum*

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Heavy metals are required by all organisms for normal function, but high levels of heavy metals are toxic. Therefore, homeostasis of these metals is crucial. In the human malaria-causing agent Plasmodium falciparum, the mechanisms of heavy metal transport have yet to be characterized. We have developed a P. falciparum line resistant to heavy metals from a wild-type line sensitive to heavy metals. A molecular and biochemical analysis of the involvement of the P. falciparum multidrug resistance 2 (pfmdr2) gene, an ABC-type transporter, in heavy metal homeostasis was studied. Using a novel uptake assay applied on these two strains, it was demonstrated that, when exposed to heavy metals, the sensitive line accumulates metal, whereas no accumulation was observed in the resistant line. The accumulation occurs within the parasite itself and not in the cytoplasm of the red blood cell. This difference in the accumulation pattern is not a result of amplification of the pfmdr2 gene or of a change in the expression pattern of the gene in the two lines. Sequencing of the gene from both lines revealed a major difference; a stop codon is found in the sensitive line upstream of the normal termination, resulting in a truncated protein that lacks 188 amino acids that contain a portion of the essential cytoplasmatic transporter domain, thereby rendering it inactive. In contrast, the resistant line harbors a full-length, active protein. These findings strongly suggest that the PFMDR2 protein acts as an efflux pump of heavy metals.

Malaria, caused in the majority of cases by the protozoan parasite Plasmodium falciparum, is a major global disease (1). Annual morbidity and mortality are estimated to reach overwhelming numbers of 300–500 million and 1–3 million, respectively. In addition, malaria tolls a severe economic and social price, adding more afflictions to many third world countries already stricken by poverty and social unrest (2). Some of the reasons for this situation are the lack of an adequate vaccine and suitable antimalarial drugs, the latter due to the appearance of drug-resistant parasites. Our insufficient understanding of the biology of the malaria-causing agents limits our ability to vigorously combat this disease (1–3).

A clear example of a poorly defined metabolic pathway of P. falciparum is its mechanism of heavy metal homeostasis. Heavy metals are defined as metals with a density higher than 5 g/cm³. Of 90 naturally occurring elements, 53 are heavy metals. Based on their solubility under physiological conditions, some 20 of them are available to living cells. Of these, only some are essential (iron, molybdenum, manganese, zinc, nickel, copper, vanadium, cobalt, and selenium), whereas the remaining ones appear in cells as a result of their wide distribution in various ecosystems. Regardless of their essentiality to life, most heavy metals are toxic to cells when their concentrations increase above a certain level. As such, mechanisms have evolved in different organisms to allow homeostasis of heavy metals. To date, four basic cellular strategies were characterized (4–7) in various species: (a) proton-coupled systems where the energy for the active transport of the metal ion is provided by a proton gradient; (b) reduction to a less toxic form or to a form recognized by an efflux system; (c) formation of complexes that detoxify the metal and that can be extruded from the cell by metallothioneins and phytochelatins, peptides that can bind various heavy metals; and (d) lowering the intracellular concentration to subtoxic levels by active, ATP-driven efflux pumps. Nevertheless, to date and as mentioned above, no mechanism has been characterized for P. falciparum.

A plausible candidate for this function is pfmdr2 (Plasmodium falciparum multidrug resistance 2), first described in an attempt to explain the phenomenon of resistance to common antimalarial drugs (8). Further studies unraveled the physical properties of this protein (9, 10) and dismissed its involvement in chloroquine resistance (10). By studying its sequence, homology was found between PFMDR2 and the heavy metal-tolerance protein HMT1, which is involved in heavy metal tolerance in the fission yeast Schizosaccharomyces pombe (11, 12). This structural similarity established our working hypothesis that pfmdr2 might be a candidate for conferring heavy metal resistance to P. falciparum.

The pfmdr2 gene (13, 14), located on chromosome 14 as a single-copy gene, encodes a 110-kDa protein that is expressed throughout the asexual intraerythrocytic life cycle, although to

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** The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY296748 and AY296749.

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a varying extent. It appears to be localized in both the plasma membrane of the parasite (10) and the food vacuole membrane (9). pfmdr2 is a member of the ATP-binding cassette (ABC) superfamily of transporters, one of the largest protein families found in all phyla. The PFMDR2 protein contains only 10 transmembrane domains and a single nucleotide-binding site (9, 10); a profile that is somewhat atypical for the common ABC transporters but not unique to P. falciparum, as this profile is also found in other organisms (15). In other organisms, genes belonging to the ABC family were also found to contribute to heavy metal tolerance. Examples are the Leishmania tpgpA (16), Caenorhabditis elegans mtp1 (17), Saccharomyces cerevisiae ycf1 (18–20), and the S. pombe hmt1 genes mentioned above.

The heavy metal cadmium is not known to participate in any known physiological cellular function. Nonetheless, a variety of systems in different organisms exist to deal with this heavy metal (4). Under the presupposition that a system for cadmium homeostasis exists in P. falciparum (as with other organisms (16–18)) even though it does not participate directly in the normal life cycle of the parasite, cadmium was the model heavy metal used in our research because of the availability of the metal and its radioactive isotopes (109Cd, used in the uptake studies described below) and because it has been shown (21–23) in numerous organisms that cadmium, lead, nickel, and mercury deplete glutathione and protein-bound sulfhydryl groups. This results in the production of reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radicals. As a consequence, enhanced lipid peroxidation, DNA damage, and altered calcium and sulfhydryl homeostasis occur. This may have an especially deleterious effect on one of the natural habitats of P. falciparum, the infected red blood cell, which is sensitive to oxidative stress (24). Therefore, exposing P. falciparum to the damaging effect of cadmium was used to assess the mechanisms utilized by the parasite to achieve homeostasis of heavy metals and especially to assess the involvement of pfmdr2.

**EXPERIMENTAL PROCEDURES**

**Parasite Cultures and Establishment of Cadmium Resistance**—The in vitro culturing and synchronization of a P. falciparum FCR3 isolate was carried out by standard protocols (25, 26). The parasites were cultured in flasks at 37 °C and 5% hematocrit in RPMI 1640 medium supplemented with human plasma (A+ or AB+), 50 μg/ml gentamycin, 25 mM HEPES, and 0.25% sodium bicarbonate, and the culture flasks were gassed (5% O2 and 5% CO2, 90% N2). The wild-type FCR3 line demonstrated a lack of tolerance to heavy metal exposure. To achieve a resistant phenotype, this line was cultured in the presence of 5 nM cadmium chloride (CdCl2). After 3–4 weeks, viable parasites were observed on Giemsa-stained smears. From this point on, the ability of these parasites to grow in the presence of increasing concentrations of CdCl2 was examined. 500 nM CdCl2 is the highest concentration that does not affect the propagation of these resistant parasites. This ability is not cadmium-dependent, and this line is therefore cultured in its absence. The resistant line was then used in the comparative studies with the wild-type line unexposed to CdCl2.

Growth was monitored by the hypoxanthine incorporation assay as previously described (27). Briefly, synchronized, trophozoite stage parasites were cultured in a 96-well plate at a hematocrit of 1.5 and 1% parasitemia in the presence of [3H]hypoxanthine (0.5 μCi/well) and various concentrations of heavy metals and antimalarials. The cells were harvested using an Inotech cell harvester, and incorporation of radioactivity was determined by liquid scintillation counting. The counts/min were adjusted relative to the controls to obtain percentages of survival.

**Uptake Studies**—To characterize the biochemical properties of the cadmium resistance phenomenon, a heavy metal uptake assay was developed. Non-infected, cadmium-sensitive and -resistant infected RBCs (iRBCs, 3 × 108 cells/ml and parasitemia of 5.5%) at the trophozoite stage were incubated at 37 °C in RPMI 1640 growth medium (lacking serum) in the presence of 5 nM 109CdCl2 (specific activity 0.6 μCi/ml, Amersham Biosciences). At the time intervals indicated in Fig. 2, aliquots were taken and spun through a layer of dimethylphthalate. The bottom of the tube containing the pelleted RBCs was cut and radioactivity of the pellets determined using a γ counter.

**Permeabilization Procedure**—Permeabilization of iRBCs with streptolysin O (SLO) was performed as described previously (28). Briefly, iRBCs (2 × 108) were treated with 4 hemolytic units of SLO for 6 min at room temperature. Subsequently, the cells were sedimented and the parasite-containing pellet was washed three times in 10 volumes of RPMI 1640 medium. Fractionation of iRBCs with saponin (29) was performed by incubating iRBCs on ice in 1.5 volumes of 0.09% ice-cold saponin in RPMI 1640 medium for 5 min. After centrifugation, the sedimented cellular fraction was washed three times with ice-cold RPMI 1640 medium.

**Analysis of PFMDR2 Protein, pfmdr2 Gene, and Their RNA Transcripts**—Western blot analysis was done on protein extracts prepared from cadmium-sensitive and -resistant parasites. Synchronized parasites at the trophozoite stage were isolated from iRBCs by saponin treatment. Free parasites were sonicated in 10 mM Tris-HCl buffer (pH 7.4) in the presence of protease inhibitors (leupeptins 2 μg/ml; pepstatin 1 μg/ml; phenylmethylsulfonyl fluoride 0.1 mM). Debris was removed by centrifugation, and the supernatant was collected. Serial dilutions of equal amounts of proteins from both extracts were separated by SDS-PAGE and blotted to a nitrocellulose filter, which was then probed by either affinity-purified antibodies to PFMDR2 or polyclonal antisera to HSP70 (as a control). The bound antibodies were detected using horseradish peroxidase-conjugated anti-rabbit/mouse IgG and enhanced chemiluminescence.

**Southern Blot**—DNA was isolated from free parasites from both lines using the phenol/chloroform method (30) and was digested with the restriction enzyme Ddel. The DNA was electrophoresed on an agarose gel and blotted to a nitrocellulose filter. The probes were labeled by random priming

2 The abbreviations used are: ABC, ATP-binding cassette; iRBC, infected red blood cell; PC, phytochelatin; SLO, streptolysin O.
**Effect of heavy metals and antimalarials on the growth of cadmium-sensitive and resistant lines.** Synchronized cadmium-sensitive and -resistant lines were cultured in a 96-well plate at a hematocrit of 1.5 and 1% parasitemia. Growth was monitored by [3H]hypoxanthine incorporation, and survival in the presence of 100 nM CdCl₂, 100 nM HgCl₂, 3 µM Cu²⁺, and [3H]hypoxanthine (0.5 µCi/ml) was calculated by regression analysis of dose-response curves. The IC₅₀ for each metal was calculated, and the data points represent the means ± S.E. of triplicates in a typical experiment repeated independently at least three times.

**Northern Blot—RNA from both lines was isolated from synchronized parasites at the ring and trophozoite stages using TriReagent (Molecular Research Center, Inc.) according to the manufacturers’ protocol.** 10 µg of RNA was analyzed on an agarose gel followed by transfer to a Hi-bond nylon membrane (Amersham Biosciences). After immobilization by UV light, the filter was hybridized with a pfmdr2 probe. The same blot was hybridized with a pfmdr1 probe (after stripping the filter) as a control. Probe labeling and filter scanning were performed as described above.

**PCR and Sequencing—Seven pairs of oligonucleotide primers were designed (using the sequences at the GenBank™ data base, accession numbers U04640 and LI3381) to amplify adjacent segments with overlapping ends of the full-length pfmdr2 gene.** Pair 1, 5’-CTTTATATGTTGGATCAGATCC-3’ and 5’-CAATATAACTAGATACCGG-3’; Pair 2, 5’-GAAATACATTAGGGAAGTTGTG-3’ and 5’-TGCTTTGCCACCACATGTG; Pair 3, 5’-CTTTTACATATTTGGGGCCAG-3’ and 5’-TTAGCACCTCTGAGTTACCTCTATC-3’; Pair 4, 5’-TTAGTAGGTATAAGGACGGAAGTTGTG-3’ and 5’-AAGGTACCTTGATCCTCCTCC-3’; Pair 5, 5’-GAAGGATCAGATCCAGGTTACC-3’ and 5’-GTATACTTGGATATGATTAAATAAACTA-3’; Pair 6, 5’-CAATAGGATACATGT-3’; and Pair 7, 5’-TTTAGGATATGATTAAATAACTA-3’. These primers were used in PCRs with template DNA extracted from cadmium-sensitive and -resistant lines and using high fidelity Klenow polymerase (Bio-X-Act, Bioline) with 3′-5′ proofreading activity to ensure accurate copying. PCR products were loaded into a 1% agarose gel followed by transfer to a Hi-bond nylon membrane (Amersham Biosciences). After immobilization by UV light, the filter was hybridized with a pfmdr2 probe. The same blot was hybridized with a pfmdr1 probe (after stripping the filter) as a control. Probe labeling and filter scanning were performed as described above.

**RESULTS**

By culturing the cadmium-sensitive line in the presence of 5 nM CdCl₂, we were able to obtain a line that exhibits an ability to grow in the presence of 500 nM CdCl₂, whereas the sensitive line succumbed (Fig. 1A). Culturing the cadmium-resistant line for long periods of time (up to five years) in the absence of the metal had no effect on the extent of the resistance phenomenon, thus indicating that the change enabling the resistance is stable and is not a transient response of the parasite to cadmium exposure (data not shown). In addition to exhibiting resistance to cadmium, this line is also capable of growing in the presence of lead.

**Figure 1.** Effect of heavy metals and antimalarials on the growth of cadmium-sensitive and -resistant lines. Synchronized cadmium-sensitive and -resistant lines were cultured in a 96-well plate at a hematocrit of 1.5 and 1% parasitemia. Growth was monitored by [3H]hypoxanthine incorporation, and results are expressed as percent incorporation in the presence of the substance relative to that obtained in the absence of the substance. Data points represent the mean ± S.E. of triplicates in a typical experiment repeated independently at least three times. A, survival in the presence of 500 nM CdCl₂; B, survival in the presence of 100 µM PbCl₂; C, survival in the presence of 1 µM FeCl₂. Additional metals examined (1 mM FeCl₂, 100 µM HgCl₂, 3 µM CuCl₂) showed similar patterns and are not shown for simplicity. D, survival in the presence of 50 µg/ml chloroquine. Additional Northern filters were exposed to a PhosphorImager (AmeriSham Biosciences) cassette and developed.

**Figure 2.** Northern Blot—RNA from both lines was isolated from synchronized parasites at the ring and trophozoite stages using TriReagent (Molecular Research Center, Inc.) according to the manufacturers’ protocol. 10 µg of RNA was analyzed on an agarose gel followed by transfer to a Hi-bond nylon membrane (Amersham Biosciences). After immobilization by UV light, the filter was hybridized with a pfmdr2 probe. The same blot was hybridized with a pfmdr1 probe (after stripping the filter) as a control. Probe labeling and filter scanning were performed as described above.
(Pb²⁺) but not in the presence of other heavy metals (Hg²⁺, Fe²⁺, Fe³⁺, Cu²⁺). Survival in the presence of Pb²⁺ and Fe²⁺ are shown as representative examples in Fig. 1, B and C, respectively. It was of interest to further examine whether the mechanism that confers cadmium resistance is involved in the process of drug resistance to antimalarial drugs. Thus, the effects of the common antimalarials quinine, mefloquine, artemisinine, chloroquine, and primaquine were examined. All of the drugs tested (chloroquine is denoted in Fig. 1D as an example) inhibit the growth of both the cadmium-sensitive and -resistant lines to the same extent, thereby indicating that the cadmium resistance mechanism is not involved in resistance to the antimalarials tested here.

The physiological explanation for the difference in the growth pattern of the sensitive and resistant lines in the presence of cadmium is presented in Fig. 2. It can be seen that, although time-dependent accumulation of cadmium is observed in RBCs infected with the sensitive line, the level of accumulation in the RBCs infected with the resistant line is negligible and similar to that observed in the non-infected RBCs.

To verify whether the accumulation occurs in the parasite itself, the uptake experiment was performed on RBCs permeabilized with SLO or saponin. By permeabilization with the pore-forming protein SLO, the erythrocyte cytosol is removed...
As presented in the Introduction, the PFMDR2 protein was chosen as a candidate for conferring heavy metal resistance. At the protein level (Fig. 4A), no detectable amounts of PFMDR2 protein are observed in the extract of the sensitive line in contrast to the resistant line, which shows a 110-kDa band corresponding to the PFMDR2 protein. The difference in protein levels is not the result of the amplification of the pfmdr2 coding gene, as can be concluded from the fact that the restriction pattern of the gene as well as the intensity of the bands is identical for both lines (Fig. 4B). The pattern of transcription of the gene is similar in both lines, whereas the extent of transcription is slightly higher in the sensitive line (Fig. 4C). This, however, cannot explain the prominent difference in the amount of protein between both lines.

To further compare the difference at the DNA level, sequencing of the pfmdr2 gene from both lines was performed. This revealed two differences. The first is the presence of the nucleotide G at position 1948 in the sensitive line as opposed to A in the resistant line, resulting in a change of aspartate to asparagine at position 650 of the protein. This change cannot be responsible for the biochemical difference observed between the two lines, as the HB3/W2 isolate, which also carries the same G at position 1948 (similar to the cadmium-sensitive line), shows resistance to cadmium (see Fig. 5 below). The second difference (Fig. 5) is observed at nucleotide position 2511, where an A is found in the resistant line as opposed to a TGT cysteine codon in the sensitive line 188 amino acids before the natural termination of the nucleotide sequence of the cadmium-sensitive and -resistant lines show that, in the cadmium-resistant line, the stop codon at position 837 marks the point at position 2511 where the cadmium-sensitive protein terminates, whereas the cadmium-resistant line continues to encode a full-length 1024-amino-acid-long protein. Also shown are the amino acid sequences of four other P. falciparum strains, 3D7, D6, D10, and HB3/W2. The pfmdr2 gene of D6 was sequenced by us, and the sequences of 3D7, D10, and HB3/W2 were obtained from the NCBI GenBank(14), accession numbers AE014823, U04640, and L13381, respectively.

Data source: plasmodb.org. D, IC50 to CdCl2 of the FCR3 cadmium-sensitive, FCR3 cadmium-resistant, 3D7, D6, D10, and HB3/W2 P. falciparum strains. In a 96-well plate, synchronized ring stage-parasitized erythrocytes were exposed to increasing concentrations of CdCl2 (10, 25, 50, 100, 500, 1000, 1500, 2000, and 3000 nM). All concentrations were performed in triplicates. After 24 h, [3H]hypoxanthine was added to each well, and the plates were incubated for an additional 24 h. Parasites were harvested, and incorporation and inhibition were analyzed. The IC50 was determined by the calculation of linear regression of the CdCl2 dose-response curve. Values represent the mean of triplicates in a typical experiment repeated independently four times.

Through formation of 30-nm pores in the erythrocyte plasma membrane, whereas the parasite and the parasitophorous vacuole remain intact. The saponin treatment disintegrates the host iRBC plasma membrane and the parasitophorous vacuole without damaging the parasite plasma membrane. Thus, performing the cadmium uptake assay on the iRBCs treated with these substances enables the determination of the compartment accumulating the heavy metal. As can be seen in Fig. 3, these treatments do not change the pattern of cadmium accumulation, strongly suggesting that the accumulation of the metal is within the parasite itself and under its control.

Sensitive line as opposed to a TGT cysteine codon in the resistant line (Fig. 5B). This stop codon terminates the protein in the sensitive line 188 amino acids before the natural termination (Fig. 5C), thereby producing a truncated protein that lacks a portion of the cytoplasmic transporter motif, a region that is necessary for the proper activity of ABC-type proteins (Ref. 31 and see also data summary for PF14_0455 at plasmodb.org). Full sequences of the pfmdr2 gene from the cadmium-sensitive and -resistant lines have been deposited to the NCBI GenBank(14), accession numbers AF296748 and AF296749, respectively.
pfmdr2 and Heavy Metal Resistance

We further analyzed the *pfmdr2* genotype and the cadmium resistance phenotype of four other *P. falciparum* isolates. Determination of the IC_{50} value of CdCl_{2} in the strains 3D7, D6, D10, HB3/W2, and our cadmium-resistant line demonstrate values ranging from 6- to 12-fold higher than that of the cadmium-sensitive line (Fig. 5D). The sequence at nucleotide position 2511 of all of these additional isolates is identical to the cadmium-resistant line (Fig. 5A).

To exclude the possibility that the sensitive “wild type” FCR3 culture is actually a mixed culture of both cadmium-resistant and -sensitive lines, we analyzed the region of the mutation by PCR using primers corresponding to the unique sequence of the sensitive and resistant line at this region. Fig. 6 shows that an amplified fragment is obtained with the DNA sequence of the sensitive and resistant line at this region. An amplified fragment is obtained only when using the primer carrying the TGA (stop) codon whereas no amplification is observed when using the TGT (cysteine) primer found in the resistant line. The result reverses when using the DNA of the resistant line as template.

**FIGURE 6. The wild type FCR3 cadmium-sensitive line is not a mixed culture.** To rule out the possibility that the culture of the wild type FCR3 cadmium-resistant line is actually a mixed culture containing a cadmium-resistant strain, a PCR analysis was performed. The following oligonucleotide primers amplify the region from 2130 to 2520 of the sequence of the *pfmdr2* gene sequence. Primer *a* was designed to contain the stop codon sequence (indicated in bold letters), primer *b* contains the cysteine codon sequence (indicated in bold letters), and primer *c* acts as a primer common to both cadmium-sensitive and -resistant lines (stop codon, primer *a*, 5′-TTTCAATAATCATCTTGCTATAG-3′; cysteine codon, primer *b*, 5′-TTTCAATTACATCTTGCTATAG-3′; common primer, primer *c*, 5′-CATACAAACGGTACACATG-3′). DNA extracted from the cadmium-sensitive (lanes 1 and 2) and -resistant (lanes 3 and 4) lines were used as templates for PCR. An amplified fragment (lane 2) is obtained from the DNA of the sensitive line only when using the stop codon primer, whereas no amplification is observed when using the cysteine codon primer (lane 1). On the other hand, an amplified fragment is obtained from the DNA of the resistant line only when using the cysteine codon primer (lane 3), whereas no amplification is observed when using the stop codon primer (lane 4).

**DISCUSSION**

Our study is based on a biochemical and molecular comparison of a *P. falciparum* heavy metal sensitive line and a heavy metal resistant line developed for this purpose. The resistant line exhibits the ability to rid itself of toxic levels of cadmium and lead whereas the sensitive line does not possess it.

We report the apparent involvement of the *pfmdr2* gene, an ABC transporter, in this mechanism of heavy metal effluxing in the parasite. The biological function of *pfmdr2* in the parasite was unknown, but its sequence similarity to the *S. pombe* hmt1 gene, suggested a role in metal homeostasis in the parasite.

To understand the molecular basis of the biochemical difference between these two lines, *pfmdr2* was studied at the DNA, RNA and protein levels. The wild type sensitive line was found to carry a mutated *pfmdr2* gene. The TGA nonsense mutation renders the protein product inactive, and as a result, heavy metals can accumulate in the parasite, disturbing its cellular functions. Culturing this line in the presence of cadmium brought about the appearance of a resistant line in which the TGA nonsense mutation was reverted to a TGT cysteine codon and, concomitantly, enables the parasite to remove heavy metals through restoration of the full-length of the protein product. The notion that this reversion is responsible for the change in the response to heavy metals is strengthened by the fact that the four other isolates we examined (3D7, D6, D10, and HB3/W2) also demonstrate resistance to heavy metals and carry this codon at the same position.

Cadmium probably has two roles in this reversion, as a selective agent and as an enhancer of the mutagenic process. The latter was demonstrated in a recent finding (32) in yeast, where cadmium has been shown to be mutagenic via the inhibition of mismatch repair.

This nonsense mutation appears to be the only major difference capable of explaining the susceptibility of the cadmium-sensitive lines to heavy metals in relation to the resistance demonstrated in our cadmium-resistant FCR3 line and the additional *P. falciparum* isolates. Our research rules out the possibility that the resistance phenomenon is a result of *pfmdr2* gene amplification or overexpression of *pfmdr2* mRNA. The absence of the protein observed in the cadmium-sensitive line can be explained by enhanced cellular degradation of the truncated protein.

We further analyzed the PFMDR2 pump in an attempt to describe its biochemical properties. In lieu of the sequence and functional similarities between the *P. falciparum* *pfmdr2* and the *S. pombe* hmt1, it was of interest to examine further functional similarities between these two systems. In *S. pombe*, the heavy metal-detoxifying peptides phytochelatins (PCs) form complexes with heavy metals and are transported to the food vacuole by HMT1 (12). As for *P. falciparum*, a data base search of the genome for the existence of enzymes involved in PC biosynthesis and high performance liquid chromatography analysis for detection of PCs did not support the existence of PCs in the parasite (data not shown). Moreover, the results of our uptake experiments rule out the possibility that the PFMDR2 pump transfers cadmium to its food vacuole, even though it is also found on the food vacuole membrane (9). These findings emphasize the paradigm that the PFMDR2 pump functions in a different biochemical manner than its HMT1 orthologue.

To assess the nature of the transport system, we used several inhibitors and metabolites known for their effect on different transport systems. Exposure to vanadate (an inhibitor of P-type ATPase transporters as well as some ABC-type transporters, including *S. pombe* HMT1 (12)), verapamil (a calcium channel blocker known to inhibit some ABC-type transporters), nigericin and valinomycin (H^{+}−K^{+} ionophors), and bafilomycin (an inhibitor of vacuolar ATPase) did not change the uptake pattern of both cadmium-resistant and -sensitive lines (data not shown). Both lines also exhibited the same level of sensitivity to CDN2 (1-chloro-2,4-dinitrobenzene), a toxic substance that, when introduced to the cell, is removed as a glutathione conjugate by a GS-X pump). These results indicate that the heavy metal transport system in *P. falciparum* is not affected by agents that inhibit some ABC-type proteins and suggest that it is not dependent on an S-conjugate system.

Another mode of heavy metal detoxification in eukaryotic
cells is achieved by a group of peptides termed metallothioneins. A data base search and biochemical analysis for the existence of metallothioneins also excludes the possibility that these proteins exist in the parasite (data not shown).

To summarize, we have shown that \textit{pfmdr2} is involved in tolerance to heavy metals and does not participate in the clearance of antimalarial drugs. Its activity does not involve the participation of other constituents (such as PCs) found in other biological systems. In addition, the parasite does not harbor metallothioneins or metallothionein-like peptides.

The further characterization of the response of \textit{P. falciparum} to heavy metals, together with the elucidation of additional genes that participate in the process and the molecular and biochemical events that are orchestrated to respond to heavy metal exposure, can help build a picture of a hitherto unknown metabolic process.

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