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Full Length Research Paper

Plasmodium falciparum multidrug resistance protein (MRP) gene expression under chloroquine and mefloquine challenge

Fátima Nogueira*, Dinora Lopes, Ana Catarina Alves and Virgílio Estólio do Rosário

Centro de Malária e Outras Doenças Tropicais/IHMT/UEI Malária, Rua da Junqueira 96, 1349-008, Lisbon, Portugal.

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The present investigation was carried out to identify and study the gene expression patterns of two novel *Plasmodium falciparum* Multidrug Resistance associated Protein (MRP) transporters belonging to the ABC protein family coded by the genes *pfmrp1 and pfmrp2* in *P. falciparum*. Full sequencing resulted in the identification of four SNPs in *pfmrp1* and eight SNPs and three insertions/deletions in *pfmrp2*. Both genes showed different patterns of expression along the intraerythrocytic cycle, *pfmrp1* peaks at the late trophozoites/early schizont stage, while *pfmrp2* is transcribed, mostly during the ring stage and peaks again towards the end of the cycle, suggesting different parasite physiological functions. Higher levels of *pfmrp1* and *pfmrp2* gene expression were observed in chloroquine and mefloquine treated cultures, more visible with mefloquine. These results suggest a possible involvement of *pfmrp1* and *pfmrp2* genes in *P. falciparum* drug response.

Key words: Efflux pumps, drug resistance, gene expression, MRP, *Plasmodium falciparum*, polymorphism.

INTRODUCTION

Resistance to available drugs in *P. falciparum* malaria is a main factor contributing to the annual high morbidity and mortality of the disease. The increasing lack of clinical efficacy of the mainstay drugs chloroquine and pyrimethamine-sulfadoxine has been motivating a global change in national drug policies towards combination therapies, mainly based on artemisinine derivatives (ACT) (Adjuik et al., 2004). Although this therapeutic strategy holds promising results (Adjuik et al., 2004; Oduro et al., 2004; Stepniewska et al., 2004) emerging molecular data show that parasites harbouring certain variants of the ABC (ATP binding cassette) transporter gene *pfmdr1* can be selected by this therapeutic strategy (Basco and Ringwald, 2002; Dalmas et al., 2005; Eckstein-Ludwig et al., 2003; Livak and Schmittgen, 2001; Pillai et al., 2003; Sidhu et al., 2005). It is probable that other yet unknown genetic factors might also be selected. This hypothesis conforms to the current view that favours the concept of a multigenic basis underlying the response of the parasite to antimalarial drugs. Among the expected components of such a multifactorial system there are other members of the ABC family of proteins (Duraisingh et al., 2000; Price et al., 2006; Valderramos and Fidock, 2006).

An important subgroup of drug resistance related ABC proteins is composed of the MRP (Multidrug Resistance Protein)-like transporters. These have been associated with drug responses in diverse organisms as *Homo sapiens*, *Arabidopsis thaliana*, and *L. tarantoleae* (Kariya et al., 2007; Mu et al., 2003; Perez-Victoria et al., 2001). Their action seems to be associated with the efflux of xenobiotics, both unaltered or in phase II metabolism conjugated form (e.g. with glutathione or glucuronide groups) (Awasthi et al., 2002; Xu et al., 2005). MRPs have also been identified as the major efflux pump of oxidised glutathione (GSSG), an important factor in redox response in many organisms, including *P. falciparum* (Cojean et al., 2006; Dale et al., 2004; Jones and George, 2005). It is conceivable that MRP-like proteins might be involved in mechanisms of drug resistance in malaria parasites.

Two putative MRP-homologues in *P. falciparum*, currently referred in PlasmoDB database as PFA0590w (chromosome 1) and PFL1410c (chromosome 12) referred as *pfmrp1* and *pfmrp2*, respectively have already been identified (unpublished data). The first has been recently characterized by others and designated *pfmrp*

*Corresponding author. E-mail: fnogueira@ihmt.unl.pt.
Tel: +351-213622458. Fax: +351-213622458.

(Kolukisaoglu et al., 2002). Herein we confirm these data and add the identification of a second mrp-like protein coding gene. Full sequencing of both genes in the *P. falciparum* Dd2 clone show the presence of several non-synonymous SNPs, as well as size polymorphisms. We also detected differential expression of both genes by exposure to the antimalarial drugs chloroquine and mefloquine.

Research into the mechanisms contributing to the development of drug resistance is of relevance as rates of drug-resistant malaria rise, since it may help understanding these mechanisms and preventing it from occurring.

MATERIALS AND METHODS

Parasite *in vitro* cultures

P. falciparum clones 3D7 (Rosario, 1981) (chloroquine - CQ and mefloquine - MEF sensitive) and Dd2 (Oduola et al., 1988) (chloroquine and mefloquine resistant) were cultured at 5% hematocrit as described (Trager and Jensen, 1976). Human serum was replaced with 0.5% AlbuMAXII (Invitrogen™) in the culture medium after confirmation of no changes in drug response.

Plasmodium falciparum field isolate

Complete data concerning CQ and MEF IC_{50}, were available for 33 of 155 patients previously recruited for *in vitro* trials of CQ and MEF, by us Meierjohann et al. (2002).

Nucleic acid extraction

Genomic DNA from *P. falciparum* strains and field isolates was extracted by standard phenol: chloroform DNA method (Arez et al., 1997). Total RNA was isolated from parasite culture with TRIZOL™ LS reagent (Invitrogen™) following the manufacturers instructions.

pfmrp1 and *pfmrp2* ORF sequencing from *P. falciparum* and bioinformatics analysis

For the full sequencing of both *P. falciparum* Dd2 clone *pfmrp1* and *pfmrp2* ORFs, PCR amplifications of overlapping fragments were performed using the oligonucleotide primers listed in Appendix 1. For the amplification of all fragments, 1 µl of template DNA was used for each 50 µl PCR reaction, containing 1 µM of each primer, 1 x PCR buffer (Promega ™), 2.5 mM $MgCl_2$, 0.2 mM dNTP´s and 0.025 U/µl of Promega ™ *Taq* DNA polymerase. The thermal cycling conditions were the same for all amplifications: 3 min at 94°C, 45 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72ºC. PCR products were purified with Qiagene™ "High Pure PCR Product Purification® All double strand sequencing reactions were performed by MACROGEN Inc, Seoul, Korea. The resulting sequenced fragments were aligned and analysed with the *MultAlin* software application (http://prodes.toulouse.inra.fr/multalin/multalin.html). Full DNA sequence was *in silico* translated and the primary protein structure analysed using the ExPASy Proteomics Server (http://au.expasy.org/). The SOSUI software (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) was used to determine the hydropathy plot of the putative *pfmrp1* and *pfmrp2* coded proteins.

PCR-RFLP detection of *pfmrp1* H191Y and S437A SNPs

We designed a PCR-RFLP protocol to detect H191Y and S437A based on the fact that each SNP is a natural restriction site for the endonuclease HpyCH4V. For amplification of the 602 bp DNA fragment containing *pfmrp1* H191Y polymorphism we used the primers mrp2/2F and mrp2/2R and for the 679 bp containing S437A mrp2/3F and mrp2/3R. PCR conditions were as described above for full sequencing of *pfmrp1* and *pfmrp2*. Following amplification, polymorphisms were detected by incubation of the corresponding PCR fragments with *HpyCH4V* (5'tg/ca3') obtained from New England BioLabs ™. Incubation was setup following the manufacturer instructions. Appropriate control DNA of samples with known H191Y and S437A genotype was used in parallel with field-collected parasite isolates in every PCR-RFLP protocol; these were 3D7 (genotype *pfmrp1* 191H and 437S) and Dd2 (genotype *pfmrp1* 191Y and 437A). The products resulting from restrictions were resolved in 2% agarose gels, stained with Ethidium bromide and visualised under UV (ultraviolet) transillumination.

PCR detection of *pfmrp2* insertions and deletion

Detection of *pfmrp2* insertions at nucleotides 779 and 1947 and the deletion at nucleotide 3591, were performed by PCR amplification of the fragments containing each polymorphism with the primers mrp2/2F - mrp2/2R, 1947 mrp2/4F - mrp2/4R and mrp2/7F - mrp2/7R, respectively. The PCR products resolved in 3% agarose gels, stained with Ethidium bromide and visualised under UV (ultraviolet) transillumination.

Reverse transcription of RNA

Total RNA was initially incubated with DNase I (Fermentas™). Followed by cDNA synthesis with the MMLV reverse transcriptase (Fermentas™) using random hexaoligonucleotides (Roche™). All protocols were performed in accordance to the instructions of the commercial suppliers.

Gene expression using quantitative PCR

Quantitative PCR with SYBR Green detection was used to evaluate relative mRNA amounts of the genes *pfmrp1 and pfmrp2*, using the house keeping gene *pfβ-actin*l as an internal control. The primers used are listed in Apendix 1. All quantitative PCR reactions were performed with a GenAmp 5700 Sequence Detection System® (Applied Biosystems, Foster City, USA), in the following conditions: 10 min of pre-incubation at 95°C, followed by 40 cycles for 15 s at 95°C and 1 min at 60°C. Individual real-time PCR reactions were carried out in 20 µl volumes containing *Taq* polymerase buffer, 3.5 mM $MgCl_2$, 200 µM dNTPs, 300 nM forward primer, 300 nM reverse primer, 0.025 U/µl Taq polymerase and SYBER Green I® all reactions were conducted with the qPCR™ Core Kit for SYBER Green I® (EUROGENTEC™), following manufacturer instructions.

Determination of real-time PCR efficiencies

In order to determine PCR efficiencies for each gene, each sample was diluted in serial 10-fold ranges and the C_T value at each dilution was measured. A curve was then constructed for each gene from which efficiency was determined. Real-time PCR efficiencies (E) were calculated from the given slopes, according to the equation: $E = 10(-1/\text{slope})$, where $E = 2$ corresponds to 100% efficiency (Lopes et al., 2002).

Data analysis using the $2^{-\Delta\Delta C}{}_T$ method and its validation

The analysis of relative gene expression was performed using

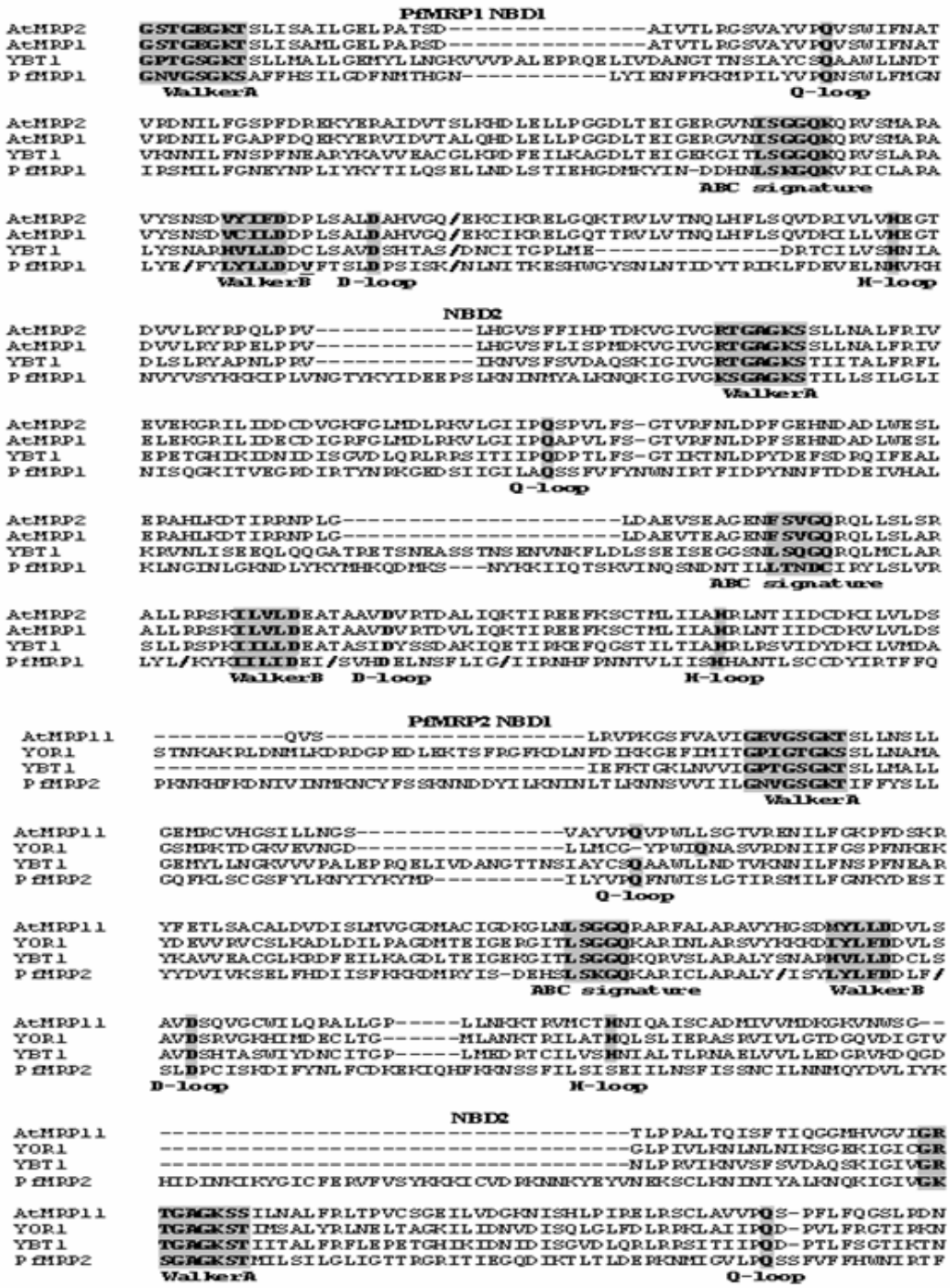

Figure 1. Alignment of predicted NBDs amino acid sequences of *pfmrp1 and pfmrp2 Plasmodium falciparum* genes, with the three most similar proteins from SwissProt database (www.expasy.org/sprot). Alignments were performed using the clustalW algorithm with the Blosum 62 matrix (all other parameters set as default) at http://clustalw.genome.jp/). Only a segment of each Nucleotide Binding Domain (NBD) containing characteristic conserved motifs, are presented. Conserved Walker A and B, the ABC signature, as well as the Q (Glutamine), D (Asparagine) and H (Histidine) loop motifs are presented in solid black and boxed in gray. The **V** marked in solid black underlined, corresponds to the *pfmrp1* SNP at codon 876 (V876I). The sites were sequences were truncated marked with /.

the $2^{-\Delta\Delta C_T}$ method [32]. Briefly, for the $\Delta\Delta C_T$ calculation to be valid, the amplification efficiencies of the target and internal control must be approximately equal. In order to validate our method, sample amplifications were performed on serial 10-fold dilutions for the in-

internal control (*pfβ-actinI*) and the target genes (*pfmrp1* and *pfmrp2*). The average C_T was calculated for both internal control and target genes and the ΔC_T ($C_{T,Target} - C_{T,\ pfβ\text{-}actinI}$) was determined. Plots of the log DNA dilution versus ΔC_T were made. If the absolute value of the slope (*m*) was below 0, 1, the efficiencies of the target and internal control genes were considered as similar. $\Delta\Delta C_T$ calculation for the relative quantification of target were performed according to Equation A: $\Delta\Delta C_T = (C_{T,Target} - C_{T,\ pfβ\text{-}actinI})_\chi - (C_{T,Target} - C_{T,\ pfβ\text{-}actinI})_\gamma$, where χ = variable under investigation and γ =calibrator sample. After validation of the method, results for each sample were expressed in *N*-fold changes in target gene expression relative to the same gene target in the calibrator sample, both normalized to *pfβ-actinI* Equation B: *N*-fold $=2^{-\Delta\Delta C_T}$ (Lopes et al., 2002).

Gene expression along the life cycle and under drug treatment

P. falciparum clones Dd2 and 3D7 cultures were synchronised by double sorbitol (5% in H_2O) treatment. Parasitaemia was adjusted to 1% ring forms (≈4 h after invation) and 5% heamatoctit of for both clones, for short term cultivation in 24 well microplates, (1 ml of culture per well. Harvesting of parasites was carried out every 8 h, for 48 h beginning 4 h post-invasion. The changes in *pfmrp1 and pfmrp2* expression from early rings to schizont stage were monitored during this period of collection of samples, for each time point. Quantitative PCR was performed on corresponding cDNA synthesized from each sample. Maintenance of synchrony throughout the experiment was confirmed by optical microscopy of Giemsa staining slides at every time point. Mean C_T values for both the target and the internal control genes were determined and analyzed according to Equation A, where γ represents time zero (up to 4 h after invasion), corresponding to 1x expression of the target gene normalized to *pfβ-actinI,* and *x* any time point under analysis after drug exposure. The fold change in expression of the target genes was calculated for each sample using Equation B.

pfmrp1 and pfmrp2 gene expression changes upon chloroquine and mefloquine treatment

For the study of gene expression under drug treatment, synchronized parasite cultures were used under same conditions, under IC50 drug pressure for Chloroquine diphosphate (SIGMA™) (CQ) and mefloquine (MEF) (kindly provided by Mepha, Portugal). These IC50 correspond to: 116 nM for Dd2 and 16 nM for 3D7 for CQ and 200 nM for Dd2 and 43 nM for 3D7, for MEF. Assays were run in 24 well microplates with 1 ml of culture per well. Parasitaemia was adjusted to 1% ring forms for both clones, drug treatment was applied and parasites harvested 6 h later. Controls with no added drug were cultivated in parallel. The calculation of the relative quanti-fication of target employed Equation A where X =drug-treated sample (MEF or CQ) and γ = untreated sample. Results were expressed in *N*fold changes in X target gene expression relative to the expression of γ, according to the Equation B.

Gene copy number

pfmrp1 and pfmrp2 copy number in clones 3D7 and Dd2 was assessed by SYBR Green based quantitative PCR applying the same set of primers (Appendix 1) and amplification conditions previously described for the gene expression studies**.** Copy number of *pfmrp1 and pfmrp2* were determined by using the *pfβ-actinI* single copy gene as an internal control (as described in detail in Geisler et al. (2004).

As a test of the sensitivity of this quantitative PCR approach, a fragment of the *P. falciparum pfmdr1* gene was amplified in parallel under the same PCR conditions, using the primers pfmdr1F and pfmdr1R listed in Appendix 1. This was based on the previous knowledge that 3D7 clone carries 1 copy of *pfmdr1* (Wellems et al., 1990) while Dd2 harbors 4 copies (Cripe et al., 2002). In order to compare the copy number, results were analyzed by the comparative C_T method, based on the tested assumption that the targets (*pfmrp1, pfmrp2 and pfmdr1,*) and reference (*pfβactinI*) amplify with the same efficiency within an appropriate range of DNA concentrations.

RESULTS

Identification and primary structure of *pfmrp2* and comparison with *pfmrp1*

The translated sequence of *pfmrp1* in Dd2 and 3D7 comprises 1822 amino acids corresponding to a predicted molecular weight of 214,5 kDa. The *pfmrp2* showed differrences in the size of the predicted protein sequence between the two parasite clones: 2133 a.a. (249.9 kDa) in Dd2 and 2108 a.a. (249.3 kDa) in 3D7. These differences reflect DNA sequence length polymorphism. The *pfmrp2* is predicted to contain 11 transmembrane helices, in contrast to the 13 detected for *pfmrp1*. Both translated sequences of *pfmrp1* and *pfmrp2* accommodate at least two membrane spanning domains (MSD) and two nucleotide binding domains (NBD) that include the characteristic conserved motifs designated by Walker A, walker B, and ABC signature and the D, H and Q-Loops (Figure 1). To examine the degree of homology of *pfmrp1* and *pfmrp2* coded products with other proteins, a search and comparison of the predicted protein sequence to the proteins in the SwissProt database (www.expasy.org/sprot/, last acceded at July 27th, 2007) was performed. The 92 most similar proteins to the predicted product of *pfmrp1* were annotated as MRP-type proteins. In the case of *pfmrp2*, among the 57 more similar sequences (also annotated as MRP-type proteins) 37 were annotated as CFTR-type proteins. Herein we refer the three most closely related proteins and the respective main characteristics (Table 1)

pfmrp1 and *pfmrp2* sequence variation and gene copy number evaluation in Dd2 and 3D7 clones and field isolates

For full length sequence of Dd2, 11 PCR products for *pfmrp1* and 12 for *pfmrp2* were amplified and bi-directionally sequenced. A total of four SNPs were identified in *pfmrp1* gene in Dd2 by comparison with the 3D7 reference sequence (http://plasmodb.org/): C571T (*H191Y*), T1309G (*S437A*), A2626G (*V876I*) T4167A (*I1390F*), all representing non-synonymous. *pfmrp2* was shown to harbor 8 SNPs (Nucleotide and a.a. positions referred, correspond to the 3D7 sequence), A3012G (*T2940T*) and T3414C (*D1138D*) represent synonymous changes. The SNPs A1892G (*D631G*), A1936G (*N646D*), A2142T (*K714I*), G3550A (*D1184N*), T4579A (*S1527T*) and C4591A (*L1531I*) are non-synonymous resulting in amino

Table 1. ABC proteins closely related to *P. falciparum pfmrp1* and *pfmrp2* coded ORFs.

	Similar proteins	ABC subfamily	Attributed function (Attributed function based on several publications.	Organism	a.a.	Prot. ID
MRP1 1822 a.a.	AtMRP2	ABCC	Transport of GSSH and several different conjugates with GSH, tonoplast membrane (Homolya et al., 2003).	*A. thaliana*	1623	Q42093
	YBT1	ABCC	ATP-dependent bile acid transport .	*S. cervisiae*	1661	P32386
	AtMRP1	ABCC	Transport of GSSH and several different conjugates with GSH, vacuolar membrane (tonoplast) (Lim et al., 1996).	*A. thaliana*	1622	Q96J66
MRP2 2109 a.a.	AtMRP11	ABCC	Transport of GSSH and several different conjugates with GSH (Krishnamachary et al., 1994)	*A. thaliana*	1194	Q9SKX0
	YOR1	ABCC	Oligomycine resistance associated permease(Li et al., 1997)	*S. cervisiae*	1477	P53049
	YBT1	ABCC	ATP-dependent bile acid transport .	*S. cervisiae*	1661	P32386

acid substitutions. Also, two insertions were found in the *pfmrp2* gene sequence, one of 18 bps starting at nucleotide 779, and another one comprising 72 bps starting at nucleotide 1947. Additionally, one deletion of 15 bps starting from nucleotide 3591 was also identified. On a preliminarily basis, the known drug response association of the two *pfmrp1* SNPs and the *pfmrp2* insertion /delections were here tested in 47 field isolates from Thailand and 35 from Angola (West Africa), assayed *in vitro* with chloroquine (CQ) mefloquine (MEF) and quinine (QN). The *pfmrp1* SNPs at positions 191 and 437 were found to vary together with a frequency of 100% and 95,3% for 191H + 437S (in Angola) and 191Y + 437A (in Thailand), respectively. Association of both *pfmrp1* SNPs to *in vitro* MEF resistance phenotype was detected (*H191Y*, p = 0,013; *S437A*, p = 0,047) (Figure 2A). Association to *in vitro* MEF resistance phenotype was also detected between the 18 bp insertion at nucleotide 779 (p = 0,001) (Figure 2B) and the 15 bp deletion at nucleotide 3591 (p = 0,001) (Figure 2C) of *pfmrp2*. These associations were only detected in samples from Thailand but not in those from Angola. Repeated assay of the reference laboratory strains with known *pfmdr1* gene copy numbers: Dd2 (4 copies) and 3D7 (1 copy) confirmed accuracy of the real time PCR assay was accurate. Single copy number for both *pfmrp1* and *pfmrp2* genes was detected in both strains and in all the field samples tested. Whereas for *pfmdr1*, a positive association of ≥2 copies of the gene and *in vitro* mefloquine resistance we detected, in samples from Thailand.

pfmrp1 and *pfmrp2* gene expression along the life cycle and under drug treatment

For both genes, relative quantification of mRNA was assessed along the parasites life cycle and under the correspondent IC50 for chloroquine (CQ) and mefloquine (MEF). Both *pfmrp1* and *pfmrp2* genes were transcribed at all stages of the asexual life cycle, rings, trophozoites and schizonts, although exhibiting different profiles of expression. *pfmrp1* in both Dd2 and 3D7 peaks at the late trophozoites/early schizont stage (Figure 3A). On the other hand, *pfmrp2* gene expression level, peaks at a very early stage of the parasite life cycle (early ring) and again at the end of the cycle period (Figure 3B).

Gene expression was generally higher in the presence of the antimalarials, CQ (*pfmrp1*: 3D7 2,0 ± 0,8, Dd2 1,6 ± 0,4; *pfmrp2*: 3D7 2,4 ± 0,9, Dd2 0,8 ± 0,6) and MEF (*pfmrp1*: 3D7 3,3 ± 0,4; Dd2 10,0 ± 2,2; *pfmrp2*: 3D7 2,1 ± 0,2; Dd2 7,6 ± 3,7), than in the control cultures. Except for *pfmrp2* in the clone Dd2 in the presence of CQ where *Nfold* change does not reach the cutoff of 1, 5 (Figure 3C). Mefloquine has a stronger effect on the expression of *pfmrp1* and *pfmrp2* than CQ, an observation more evident in Dd2 where ≈10 fold increase was observed for both genes (Figure 3C).

DISCUSSION

Identification and primary structure of *pfmrp2* and comparison with *pfmrp1*

Members of MRP subfamily share a conserved architecture with other ABC transporters (Borst et al., 2000; Klokouzas et al., 2004). The functional unit of an ABC transporter is comprised of two transmembrane domains (that accommodate ~six transmembrane helices each) and two cytoplasmic ABC ATPase domains. The domain consists of several conserved regions, the energy of the binding and hydrolysis of ATP is used to transport

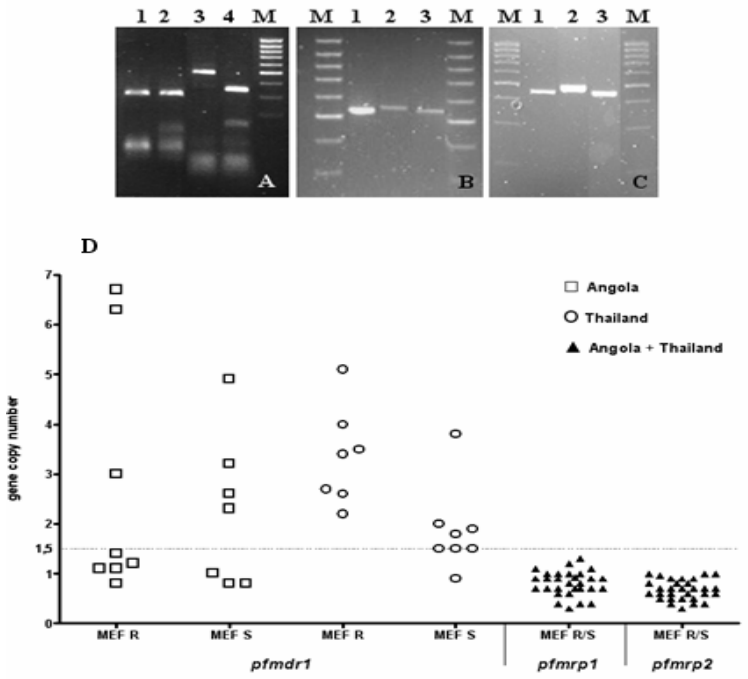

Figure 2. Sequence variation and gene copy number evaluation of *pfmrp1* and *pfmrp2 Plasmodium falciparum* genes in strains and field isolates from Angola and Thailand. A: restriction digest method for detecting codon 191 and 437 mutations of *pfmrp1*, Lane 1 strain 3D7 and Lane 2 strain Dd2, corresponding to the 602 bp amplification product digested with *Hpy*CH4V, Lane 3 strain 3D7 and Lane 4 strain Dd2, corresponding to the 679 bp amplification product digested with *Hpy*CH4V; B: amplification products corresponding to the 18 bp insertion at nucleotide 779, Lane 1 and 3 strain 3D7 (529 bp) and Lane 2 strain Dd2 (547 bp); C: amplification products corresponding to the 15 bp delection at nucleotide 3591, Lane 1 and 3 strain Dd2 (462 bp) and Lane 2 strain 3D7 (477 bp); M - 100 bps molecular weight marker (Fermentas™); D: *pfmdr1, pfmrp1* and *pfmrp2* gene amplification in samples from Angola and Thailand, fenotiped for mefloquine; MEF mefloquine; R resistant; S susceptible; 1,5 cut of value above which copy number variation was assumed; Gene copy number variation for *pfmrp1* and *pfmrp2* of MEF R and MEF S samples from both regions are represented together.

the substrates across membranes. An ABC Walker A and B motifs, the ABC signature and the D, H and Q-Loops (Altenberg, 2003; Duah et al., 2007; Oswald et al., 2006). *In silico* analysis of the predicted protein sequences of both *pfmrp1* and *pfmrp2* fit this primary structural organization (Figure1).

Association of sequence variations to drug susceptibility of *P. falciparum* isolates

Associated to chloroquine and quinine *in vitro* response of the *pfmrp1* SNPs *H191Y* and *S437A* has been analyzed in isolates originating from Asian and South America, with different conclusions (Cowman et al., 1994; Ngo et al., 2003; Zakeri et al., 2005). In this study, we investigated whether the same *pfmrp1* polymorphisms were associated to chloroquine (CQ), quinine (QN) and mefloquine (MEF) *in vitro* response in *P. falciparum* isolates from Thailand and Angola (West Africa). In isolates from Thailand, we found that the referred SNPs, were associated with *in vitro* drug response to MEF but not to CQ or QN, these do not corroborate findings of Mu et al. (2003), who described *in vitro* response of isolates originating from Asia associated to CQ and CQ, but is partially in line with the results of other authors (Anderson et al., 2005b; Anderson et al., 2005a), who reported that there was no association detected between the referred SNPs and *in vitro* drug response of field isolates. In the African samples, no positive association was detected between 191 and 437 SNPs and *in vitro* drug response of field isolates to any of the three drugs studied. In our study linkage of the *pfmrp1* alleles 191Y + 437A in Thailand and 191H + 437S in Angola (West Africa), at a frequency of 95.3% and 100% respectively have been described. These may reflect common origin. The role of *pfmrp1*

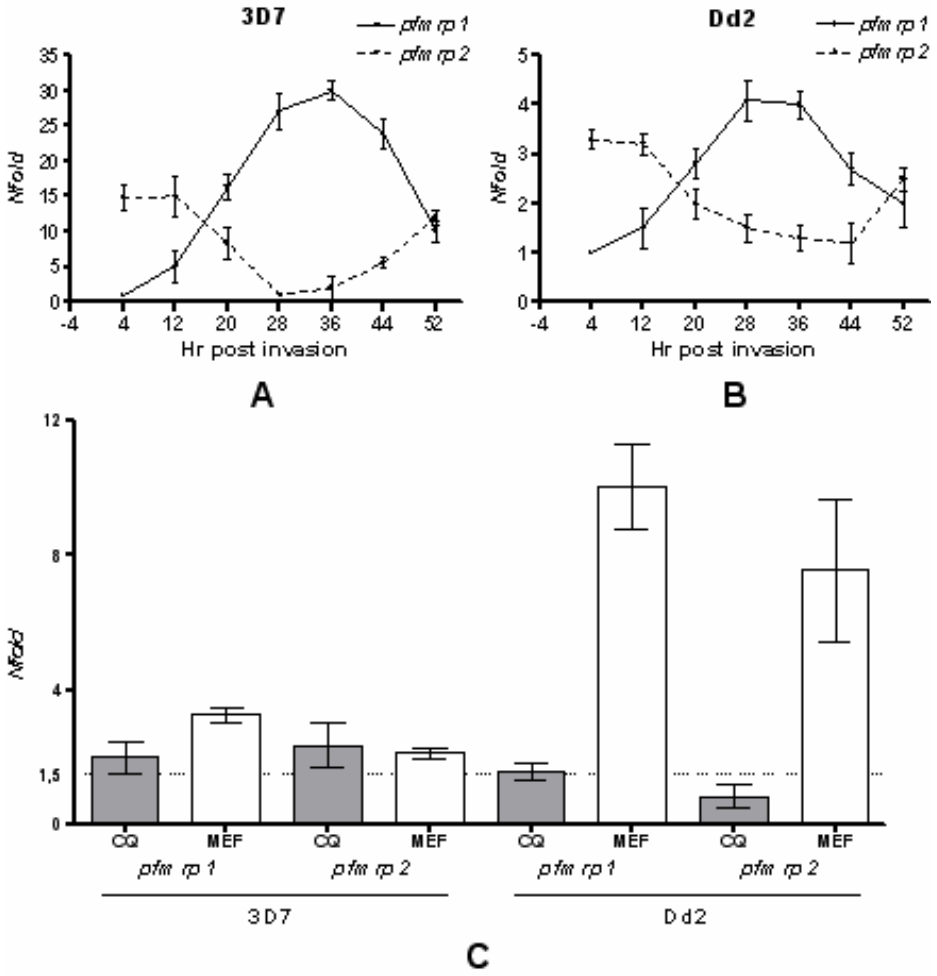

Figure 3. Relative RNA expression of *pfmrp1 and pfmrp2* genes of *P. falciparum* in the erythrocytic cycle, and under drug treatment. A 3D7 and B Dd2 mRNA expression of *pfmrp1* and *pfmrp2* along the development cycle of the parasite. C expression of *pfmrp1* and *pfmrp2* in both 3D7 (drug sensitive) and Dd2 (drug resistant) after CQ and MEF treatment with the correspondent IC50 for each drug and strain. *Nfold* - represents the mean *Nfold* change, of each of the two genes normalised against β-*actinI*, generated after two independent assays; Hr – hour; MEF – mefloquine; CQ – chloroquine.

point mutations, is yet to be determined but the difference between the two regions (Asia and Africa) is interesting. Sequence variation was much more pronounced in *pfmrp2* (8 SNPs), although with the presence of synonymous mutations, an event not observed for *pfmrp1*. Also, appreciable difference in the size of the *pfmrp2* gene was observed. These differences indicate that the coded proteins expressed in the 3D7 (2104 a.a.) and Dd2 (2134 a.a.) clones will probably have different sizes and as such their tertiary or quaternary structures are probably altered when inserted in the membrane, which might affect the function of the protein. Therefore we investigated whether the insertions and deletions detected in *pfmrp2* sequence were associated to *in vitro* response of the above referred *P. falciparum* isolates to CQ, QN and MEF. A positive association to *in vitro* MEF resistance phenotype was detected between the 18 bp insertion at nucleotide 779 and the 15 bp deletion at nucleotide 3591. Due to the limited number of included isolates these results can not be interpreted as final and more investigations are suggested.

Association of *pfmdr1*, *pfmrp1* and *pfmrp2* gene copy number to drug susceptibility of *P. falciparum* isolates

pfmdr1 gene amplification has been associated with resistance to the antimalarial quinoline, particularly to mefloquine (Price et al., 2004a; Sidhu et al., 2006). These observations prompted us to confirm the presence of similar events with *pfmrp1* and *pfmrp2*, in the field isolates from Thailand and Angola. The ABC transporter en-

coded by *pfmdr1* affects the intra parasitic concentrations of several important antimalarial drugs. Increase in *pfmdr1* copy number was associated with up to a 40-fold decrease in the *in vitro* susceptibility to mefloquine (MEF) (Price et al., 2004b). Sensitivity to artemisinins, which are structurally unrelated to MEF in their mode of action (Ferreira et al., 20) but commonly used together with it in combinations, was also associated with increased *pfmdr1* copy number. Mefloquine (MEF) resistance was also observed in presence of single copy of wild-type *pfmdr1* by in a small number of cases (Price et al., 2004b), indicating that MEF resistance in this case is mediated through other, as yet undefined, molecular mechanisms (Ngo et al., 2003). It is worthy to note that drug response has been correlated to MRP gene copy number in some systems (Ferreira et al., 2006; Oduola et al., 1988). In fact, increased copy number was a pivotal event for the discovery of the first identified MRP genes in *H. sapiens* (Le Crom et al., 2002) and, importantly, in parasite *Leishmania spp* (Perez-Victoria et al., 2001). These observations prompted us to confirm the presence of similar events with the ABC transporters *pfmrp1* and *pfmrp2*, in field isolates from Thailand and Angola. Single copy number for both *pfmrp1* and *pfmrp2* genes were detected in both *P. falciparum* in all the field samples tested and in strains (3D7 and Dd2), whereas for *pfmdr1* different copies of the gene were detected in the same set of samples. A positive association between increased gene copy number and *in vitro* MEF resistance was detected in samples from Thailand, but not in those from Angola (West Africa), these findings are in line with other workers who verified that association of gene copy number to MEF resistance *pfmdr1* amplifications do exist in Kenya but at a very low frequency. These observations are in line with others findings; the selection of *P. falciparum* FAC8 on MEF did not alter the copy number (3 copies) or the level of expression of *pfmdr1*. Sequence analysis of *pfmdr1* from the selected lines showed no change in the acids (Livak and Schmittgen, 2001). These results show that alterations in *pfmdr1* are not involved in mediating the increased mefloquine resistance observed in this clone (Livak and Schmittgen, 2001). This, along with other data, suggest that mefloquine resistance may have arisen by two different mechanisms in African and Southeast Asian and that MEF resistance can also be mediated through other, as yet undefined, molecular mechanisms.

pfmrp1 and *pfmrp2* gene expression along the life cycle and under drug treatment

Both *pfmrp1* and *pfmrp2* genes were transcribed at all stages of the asexual *P. falciparum* life cycle, although exhibiting different profiles of expression (Figure 2A and 2B). *pfmrp1* in both Dd2 and 3D7 peaks at the late trophozoites/early schizont stage, coinciding with the period of highest expression of genes that code for metabolism of glutathione, namely glutathione synthase, glutathione reductase and glutathine-S-transferase (http://malaria.ucsf.edu/) (Bozdech and Ginsburg, 2004). This comes in line with the fact that MRP proteins are the main GSSG and glutathione conjugate transporters in most organisms. On the other hand, *pfmrp2* gene expression level peaks at a very early stage of the parasite life cycle (early ring) and also at the end of the cycle period (mature schizonts) (Figure 2B). The fact that the *pfmrp2* gene has its maximum expression at an early stage when the transcription of glutathione metabolism genes is low (http://malaria.ucsf.edu/) does not indicate that this particular MRP protein is not associated with these pathways. In fact in mice, in a recent study it was suggested that CFTR plays an important role in GSH uptake from the diet and transport processes in the lung (Keppler et al., 2000). In both *pfmrp1* and *pfmrp2* genes expression was higher in the presence of the antimalarials (chloroquine and mefloquine), although differences in general exist between the Dd2 and the 3D7 clones. This is consistent with available evidence that MEF interacts with human MRP1 and MRP4 transporters and influences their transport activity in red blood cells (Wu et al., 2005b), and possibly alter the distribution of the drug it self, in erythrocytes. Based on the concentrations required to produce inhibition, it seems unlikely that MEF exerts any of its antimalarial action by inhibiting transport of substrates such as oxidized glutathione or glutathione conjugates by human MRP1 or MRP4. Substantial inhibition has been demonstrated but only at relatively high concentrations, 100 times higher than the ones used during treatment for malaria (Xu et al., 2005). *P. falciparum* possesses an active efflux mechanism for GSSG (Atamna and Ginsburg, 1997; Mentewab and Stewart, 2005). It also expresses two ABC proteins (coded by the correspondent genes *pfmrp1* and *pfmrp2*) that are homologous with MRP transporters and are over expressed in the presence of MEF. Our results are in favor of the interesting hypothesis that MEF may exert its antimalarial action by interfering with the export of GSSG from the parasite.

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Appendix 1. Primer sequences used for full length gene (*pfmrp1* and *pfmrp2*) sequencing, polymorphisms detection and Real time PCR.

	Full length gene sequencing
	pfmrp1
mrp1/1F	5'-ATGACGACATATAAAGAAAATGTTGG-3'
mrp1/1R	5'-GAAGCTCTTTTGCATTTTTATTTTC-3'
mrp1/2F	5'-GTGGTAGTGATGATAATGTTTTTCC-3'
mrp1/2R	5'-TTGAATTTTTACCAAGTTTATTTAAAAG-3'
mrp1/3F	5'-GCTTTATACAGTGCAATGATAC-3'
mrp1/3R	5'-CTTCTCATGATGATGGTACATC-3'
mrp1/4F	5'-GTAGTGGATAAGACATTTTTACAAAATG-3'
mrp1/4R	5'-GAGATTTTAATATGACACATGGTAATTTG-3'
mrp1/5F	5'-ATCAGGAAAAAGTGCATTTTTCCATTC-3'
mrp1/5R	5'-GATATATTTACATCTTTAGATCCTTCC-3'
mrp1/6F	5'-GAAAATTACCTTCAAAAATGTTTAATGG-3'
mrp1/6r	5'-GGAGCATATGAATAAAAATAATAAGG-3'
mrp1/7F	5'-GGGAATACGGAGAGTGTTTCC-3'
mrp1/7R	5'-GTCTTTTTAACTATGATGAGTATTATTTC-3'
mrp1/8F	5'-CTTCAGTAGTAATATTTATGCTTATATC-3'
mrp1/8R	5'-CATCTATAGTAATGCATTGTCTGG-3'
mrp1/9F	5'-CAAAGGCTGTATTTATCATGTCATAC-3'
mrp1/9R	5'-GGATAAGATATCTGCAATTGTCG-3'
mrp1/10F	5'-GGAAAATGAATTAAATGTAATAACAACAC-3'
mrp1/10R	5'-GTTCATGCTCTAAAATTGAATGG-3'
mrp1/11F	5'-GATCCATATAATAATTTCACAGATGATG-3'
	pfmrp2
mrp2/1F	5'-ATGATGAGACGGAGAAGCG-3'
mrp2/1R	5'-GCCCTCCTTTGCTATTATGAT-3'
mrp2/2F	5'-GTGTTGTTCGGATTTATAGTCAT-3'
mrp2/2R	5'-CGACATGTTTAACGTGTACAG-3'
mrp2/3F	5'-GCTCCGAATTTAATAAAGAAGAAAAACA-3'
mrp2/3R	5'-GTTCTTTAATCACACCCCT-3'
mrp2/4F	5'-GTGAATTAAAGAGTAATAAAACCG-3'
mrp2/4R	5'-CAAGACAGAAAAGGGTGGTAT-3'
mrp2/5F	5'-GTAGGTGAAAGTAATAATTCACTAT-3'
mrp2/5R	5'-CTGCACAGAGGTCTAATGAT-3'

Appendix 1. contd.

mrp2/6F	5'-CTGCACAGAGGTCTAATGAT-3'
mrp2/6R	5'-GGAACTATATGCACCGTGTAA-3'
mrp2/7F	5'-GAACAAGTCAAATCTATGTTAAGTC-3'
mrp2/7R	5'-CCATTTACTCGTTCAATACAGAAGAA-3'
mrp2/8F	5'-GCGGAGATATAAAATACCACAAAT-3'
mrp2/8R	5'-GGAAGACCTCTAATTATTGTTATTATT-3'
mrp2/9F	5'-GGAAATATCAAGTTAGAAACATTTTG-3'
mrp2/9R	5'-GCATCACATGCACCCTTAT-3'
mrp2/10F	5'-GATGTAAAGAAGCACAAAGAGG-3'
mrp2/10R	5'-CCTGGTTCGTATGATCCGA-3'
mrp2/11F	5'-GCAGGATGCAAATTTATTTAAATCTG-3'
mrp2/11R	5'-GGAATATTAGAACATTTATAGATCCAT-3'
mrp2/12F	5'-GGTGTATTACCACAATCATCTTTTG-3'
mrp2/12R	5'-GCTTCAAGAAAAACAATTAAATTGA-3'
	Real time PCR
RTPFMRP1F	5'-GGAACTTCGATGTGCCATAT-3'
RTPFMRP1R	5'-TACATGTTCGTTAGATGAATTTTT-3'
RTPFMRP2F	5'-AAATATGGAGGTCACCGTTATG-3'
RTPFMRP2R	5'-ATGATGACCATAGTGAAGAGGG-5'
RTPFACTINF	5'-TGTTGACAACGGATCAGG-3'
RTPFACTINR	5'-GGAACGAGGTGCATCAT-3'
RTPFMDR1F	5'-CTTTATGTATTACTTGCGTTTTTCCG-3'
RTPFMDR1R	5'-CGTGTATTTGCTGTAAGAGCTAG-3'

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