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Cloning of *Plasmodium falciparum* protein disulfide isomerase homologue by affinity purification using the antiplasmodial inhibitor 1,4-bis{3-[*N*-(cyclohexyl methyl)amino]propyl}piperazine [1]

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Abstract A series of 10 1,4-bis(3-aminopropyl)piperazine compounds was found to display antiplasmodial activity with 50% growth inhibition between 30 and 250 nM, on three *Plasmodium falciparum* strains differently sensitive to chloroquine. By affinity chromatography using one of these compounds, a 52-kDa protein was isolated from *P. falciparum*, microsequenced and cloned. It corresponded to a single copy gene encoding a 453 amino acid protein displaying the typical features of protein disulfide isomerases, a thiol metabolizing enzyme belonging to the thiol: disulfide oxidoreductase superfamily, which was not previously described in malarial species.

Key words: Malaria; Thiol metabolism; Antiplasmodial inhibitor; Protein disulfide isomerase; *Plasmodium falciparum*

1. Introduction

Malaria, caused by protozoan parasites of the genus *Plasmodium*, is one of the most dangerous tropical infectious diseases affecting human populations [1]. The *Plasmodium falciparum* species is the greatest cause of human malarial morbidity and mortality. The resistance of the parasite to drugs and the resistance of mosquitoes to insecticides have resulted in a resurgence of malaria in many parts of the world and in a pressing need for vaccines and alternative chemotherapeutic drugs.

The analysis of thiol metabolism in parasitic protozoa has resulted in an exploitation of their original pathways and the particularities displayed by some parasitic enzymes, towards the development of alternative chemotherapeutic strategies [2]. For example, Trypanosomes and *Leishmania* use trypanothione reductase instead of glutathione reductase and the former, being absent from mammalian cells, has emerged as a promising drug target [3]. In *Plasmodium*, glutathione plays an important role in parasite development [4]. *P. falciparum* possesses host-independent mechanisms of de novo synthesis or regeneration of glutathione [5], and *P. falciparum* genes encoding glutathione reductase [6,7], a non-selenium glutathione peroxidase homologue [8], and γ-glutamylcysteine synthetase [9] have been isolated. The corresponding enzymes are now considered as putative antiplasmodial targets, which remain however to be validated [10,11].

To further investigate thiol metabolism in *P. falciparum*, we set up the testing of series of inhibitors known to interact with thiol metabolism in other parasite protozoa or in eukaryotic cells. A series of 1,4-bis(3-aminopropyl)piperazine inhibitors displaying a high in vitro trypanocidal activity on *Trypanosoma cruzi* while being inactive against the purified trypanothione reductase was selected [12], this approach was prompted because affinity chromatography using one of these inhibitors, led to the isolation of the *T. cruzi* Tc52 enzyme [13] which turned out to be an new member of the thiol: disulfide oxidoreductase family [14,15], and to be essential for the parasite growth [16].

In the present study, we show that this series of 1,4-bis(3-aminopropyl)piperazine compounds, displays a high in vitro antiplasmodial activity against *P. falciparum*. Searching for their plasmodial targets by affinity chromatography using one of these compounds as a ligand, the homologue of protein disulfide isomerase (PDI, EC 5.3.4.1) was isolated. This enzyme, not previously described in malarial species, belongs to the superfamily of thiol: disulfide oxidoreductases having sequence and structural similarity to thioredoxin [17]. Its identification and molecular characterization are presented thereafter.

2. Materials and methods

2.1. Chemicals

1,4-bis(3-{*N*-[4-(benzyloxy)benzyl]amino}propyl)piperazine (compound **1**), 1,4-bis(3-{*N*-[4-(phenyl)benzyl]amino}propyl)piperazine (compound **2**), 1,4-bis{3-[*N*-(4-chlorobenzyl) amino]propyl}piperazine (compound **3**), 1,4-bis{3-[*N*-(napht-2-ylmethyl)amino]propyl} piperazine (compound **4**), 1,4-bis(3-{*N*-[3,4-(dibenzyloxy) benzyl]amino}-propyl)piperazine (compound **5**), 1,4-bis{3-[*N*-(fluoren-2-ylmethyl)-amino]propyl}piperazine (compound **6**), 1,4-bis(3-{*N*-[4-(nitrobenzyl)-

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[1] Nucleotide sequence data reported in this paper are available in the EMBL, GenBank™ and DDJB databases under accession number AJ250363.

Abbreviations: CQ, chloroquine; ER, endoplasmic reticulum; PDI, protein disulfide isomerase; RT-PCR, reverse transcription-polymerase chain reaction

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amino]propyl}piperazine (compound **7**), 1,4-bis{3-[*N*-(cyclohexylmethyl)amino]propyl}piperazine (compound **8**), 1,4-bis{3-[*N*-(anthr-9-ylmethyl) amino]propyl}piperazine (compound **9**), 1,4-bis{3-[*N*-(adamantylmethyl)amino]propyl} piperazine (compound **10**) were syn- thesized according to [12], 1,4-bis[3-(*N*-cyclohexylmethyl-*N*′-{4-[*N*-(2,6-diaminohexan-1-oxo)aminomethyl]cyclohexylmethyl}amino)-propyl]piperazine (compound **8b**), was synthesized according to [13]. Stock solutions of inhibitors were prepared in DMSO at 10 mM and stored at −20°C. Chloroquine (CQ) was purchased from Sigma Aldrich (France).

2.2. *P. falciparum in vitro culture, metabolic labeling and drug susceptibility assays*

P. falciparum strains were maintained in vitro, on human red blood cells (RBC) in RPMI 1640 medium supplemented with 7.5% heat-inactivated human serum [18]. Metabolic labeling was performed in culture medium without methionine and cysteine by adding 50 μCi. ml^{-1} of [^{35}S]methionine/[^{35}S]cysteine mix (Amersham Pharmacia Biotech, France) for 12 h, just prior to parasite harvest. Drug assays were performed by measuring [^{3}H]hypoxanthine incorporation in 96-well plates [19]. Parasite growth inhibition was determined by comparison of the radioactivity incorporated in treated cultures with that of control cultures maintained without drug. The concentrations causing 50% growth inhibition (IC_{50}) were obtained from the drug concentration–response curves.

2.3. *Affinity chromatography, protein microsequencing and gel filtration*

Infected RBC were washed three times (600×*g*, 5 min) in cold phosphate-buffered saline (PBS, 140 mM NaCl, 2.5 mM KCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4) and lysed in 10 volumes of PBS containing 0.2% saponin (Sigma Aldrich) and a protease inhibitor cocktail (Complete™ EDTA-free, Boehringer Mannheim), during 5 min on ice. The released parasites were washed four times in cold PBS (10 000×*g*, 10 min) and were lysed by three cycles of freezing–thawing in 10 mM HEPES (pH 7.5) in presence of the protease inhibitor cocktail. Soluble extracts were obtained by a 100 000×*g* centrifugation (1 h, 4°C).

Compound **8b** was anchored onto activated CH Sepharose CL4B gel (*N*-hydroxysuccinimide ester) [13]. Parasite extracts were previously cleared of molecules binding non-specifically to the matrix by being loaded three times on a 3-ml Sepharose CL4B column, equilibrated with buffer A (20 mM HEPES, 150 mM KCl, pH 7.5). The cleared extract was then loaded three times on the 3-ml affinity column, also previously equilibrated with buffer A, and was washed with 100 ml of buffer A then 100 ml of buffer B (20 mM HEPES, 500 mM KCl, pH 7.5). Competitive elution was performed with 100 ml of buffer B containing 5 mM compound **8b** [13]. A final wash was performed with 100 ml of buffer C (20 mM HEPES, 2 M KCl, pH 7.5). Flow rate was 1 ml min^{-1} and 5 ml fractions were collected during all the procedure. Pooled fractions, concentrated by ultrafiltration on Amicon centriprep 10 (Millipore Corporation, Bedford, MA, USA), were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [20]. After Coomassie blue staining, the band of interest was excised from the gel and subjected to in-gel proteolysis [21]. Peptides were purified by reverse phase HPLC on a Beckman ultrasphere ODS column 2 mm/200 mm, eluted with a linear gradient of acetonitrile in TFA 0.1%. Isolated peptides were sequenced in a Perkin-Elmer Procise 492. Gel filtration was performed on a Superose 12 column (Amersham Pharmacia Biotech) in buffer A at a flow rate of 0.4 ml min^{-1} and 0.6 ml fractions were collected. Each fraction was analyzed by SDS–PAGE. Calibration of the Superose 12 column was done according to the manufacturer's recommendations.

2.4. *Genomic DNA and RNA preparations, Southern blotting and library screening*

Genomic DNA was prepared from free parasites by using standard procedures [22] and total RNA was extracted from free parasites by using the RNAgents® total RNA isolation system (Promega, France). Genomic DNA was digested with restriction enzymes (Promega), fractionated by agarose gel electrophoresis and transferred to nylon membranes prior to being hybridized to [α-^{32}P]dATP radiolabeled nucleotide probes as previously described [23]. Genomic DNA library, containing *Eco*RI genomic fragments from the FcB1 strain inserted into λgt11 was plated and probed with [α-^{32}P]dATP radiolabeled DNA fragments as previously described [23]. Isolated plaques were grown for isolation of recombinant bacteriophages by using standard procedures [22].

2.5. *Reverse transcription-polymerase chain reaction (RT-PCR) and oligonucleotides*

Amplifications on genomic DNA were done by combining 200 ng of genomic DNA with 200 nM degenerated or non-degenerated oligonucleotides (Genaxis, France) in a standard 50-μl PCR mixture using *Taq*-Polymerase (Finzyme, Ozyme, France), and performing 30 cycles (94°C for 30 s, 42–68°C for 120 s, 74°C for 3–6 min) in a Perkin-Elmer 9600 thermal cycler. Inserts from recombinant bacteriophages were amplified by using forward and reverse λgt11 insert primers (Ozyme). RT-PCR was performed by combining 1 μg of total RNA treated with RQ1 DNase (Promega), random hexamers and avian myeloblastosis virus reverse transcriptase as recommended (Promega), in a final volume of 50 μl, of which 10 μl were then directly added to a 40-μl standard PCR reaction. The sequences of oligonucleotides P2S and P5AS are (5′-CCWGAATATGCWGAA-CATGC-3′) and (5′-WCCWGGAAATTCWGTTTAWCC-3′) respectively, where W = A+T, and were used at an annealing temperature of 42°C. The sequences of oligonucleotides N5S and N5AS are (5′-AAAAAGAAAAAATGAACAGAAAG-3′) and (5′-TTTTTGATT-TTTTTGTTTTTCATG-3′) respectively. All amplified fragments were excised from preparative agarose gel by using the Quiaex procedure (Quiagen, Coger, France) and were either directly sequenced or subcloned into pCR-Script™ (Stratagene, Ozyme).

2.6. *DNA sequencing and sequence analysis*

Dideoxy nucleotide sequence data were obtained by the dideoxy chain termination method of Sanger [24] using the Sequenase version 2.0 kit (Amersham Pharmacia Biotech). Both strands of the genomic locus and of the cDNA were sequenced at least two times. Sequence analyses were performed by using the Bisance software (http://www.infobiogen.fr) [25]. BLAST and PSI-BLAST searches [26] were performed through the NCBI website (http://www.ncbi.org) against non-redundant databases and against the International Malaria Genome Sequencing Consortium for the *P. falciparum* genome project database (http://www.ncbi.nlm.nih.gov/Malaria/). Multiple sequence alignments were performed with Clustal W [27] and further refined manually.

3. Results and discussion

3.1. *In vitro antiplasmodial activity of 1,4-bis(3-aminopropyl) piperazine derivatives*

Compounds selected for their trypanocidal activity against *T. cruzi* [12] that were tested upon *P. falciparum* are presented in Table 1. They were symmetrical amine derivatives from the 1,4-bis(3-aminopropyl)piperazine, prepared by condensation of the piperazine with a variety of aldehydes into imines which

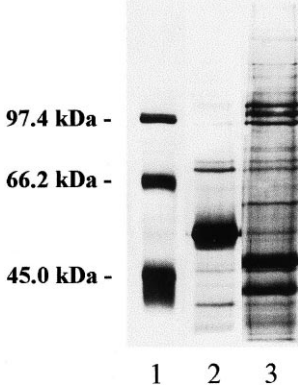
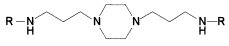
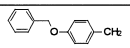
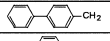
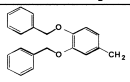
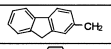
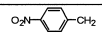


Fig. 1. SDS–PAGE analysis of parasite proteins purified on compound **8** affinity column. Molecular weight markers (lane 1); pooled fractions eluted by 5 mM of compound **8b** (lane 2); pooled fractions eluted by the 2 M KCl final washing step (lane 3). The gel was silver-stained by standard procedures.

Table 1
In vitro antiplasmodial activity of the 1,4-bis(3-aminopropyl) piperazine derivatives

		IC_{50} values (nM)		
		Strains		
Compounds	R	F32/Tanzania	FcB1/Columbia	K1/Thailand
1 (BB73)		110 ± 69[a]	122 ± 20[a]	90 ± 38[a]
2 (BB74)		54 ± 16[b]	178 ± 102[a]	106 ± 10[b]
3 (BB75)		51 ± 15[b]	84 ± 31[a]	50 ± 19[a]
4 (BB76)		92 ± 27[a]	158 ± 88[a]	226 ± 62[a]
5 (BB79)		69 ± 36[a]	193 ± 64[a]	142 ± 20[b]
6 (BB81)		29 ± 15[a]	111 ± 35[a]	112 ± 23[a]
7 (DS56)		91 ± 39[a]	160 ± 31[a]	250 ± 19[a]
8 (DS61)		108 ± 71[a]	106 ± 32[a]	260 ± 59[a]
9 (DS63)		41 ± 27[a]	37 ± 15[a]	58 ± 14[a]
10 (DS111)		197 ± 94[a]	230 ± 54[a]	247 ± 9[a]
CQ		21 ± 5[a]	125 ± 35[a]	242 ± 32[a]

Chemical structure of the compounds and IC_{50} values measured on the CQ-sensitive strain F32/Tanzania and the CQ-resistant strains FcB1/Columbia and K1/Thailand. The IC_{50} (nM) values correspond to the mean ± standard deviation from several independent experiments (a: $n = 4$; b: $n = 5$).

were then transformed, by reductive amination. All the compounds showed high in vitro antiplasmodial activity on three different strains of *P. falciparum*, with IC_{50} values in the low nanomolar range (~30–250 nM) (Table 1). These concentrations are much lower than those reported for their cytotoxicity on the human MRC 5 cell lines: absence of toxicity at 3 μM [12]. Except for compounds **1**, **3** and **9**, the CQ-sensitive strain appeared generally slightly more sensitive to the 1,4-bis(3-aminopropyl) piperazine derivatives than the CQ-resistant strains. To isolate parasite proteins interacting with these antiplasmodial derivatives, 1,4-bis{3-[*N*-(cyclohexylmethyl)amino]propyl} piperazine (compound **8**) was selected for affinity chromatography, instead of the more active compounds **3** and **9** because of its similar antiplasmodial activity upon the three strains and its absence of aromatic moieties that could lead to an unsuccessful affinity purification due to hydrophobic interactions. Compound **8** was transformed into its analogue **8b**, presenting a lysine end to allow fixation to the Sepharose polymer.

3.2. Affinity purification and microsequencing of a 52-kDa parasite protein

Parasite extracts prepared from the FcB1 strain of *P. falciparum* were loaded on the compound **8b** Sepharose gel. SDS–PAGE analysis of the fractions allowed the clear identification of a major 52-kDa (Pf52) and two minor 75 and 43-kDa proteins, specifically eluted in presence of 5 mM of compound **8b** (Fig. 1, lane 2) and not by the final 2-M KCl wash (Fig. 1, lane 3). These three proteins appeared to correspond to parasite proteins, since they were all metabolically ^{35}S-labeled (data not shown). To test whether they could have eluted from the affinity column as complex(es), a gel filtration of the affinity purified fraction (as on Fig. 1, lane 2) was performed. SDS–PAGE analysis of the gel filtration fractions showed that each protein eluted in different fractions, with retention times (as compared to a calibration curve) corresponded to molecular weight of their respective monomeric forms, indicating that they had not interacted on the affinity column as complexes (data not shown). Six peptide sequences could be determined for Pf52: P1 (GFVDFLNK), P2 (THFVLLNIPEYAEHAK), P3 (XGDLFNQFVTDIH, where X stands for an undetermined amino acid), P4 (ESLLNHNAIINFFK), P5 (ASLGLTEFPGLAFQSNEGRY) and P6 (LVSIDATSENAL), while no data were obtained for the 75- and 43-kDa proteins due to their insufficient amounts.

3.3. Cloning and sequencing of the P. falciparum gene for Pf52

Since none of the six peptide sequences derived from Pf52 matched any molecule in any database that was searched, degenerate oligonucleotides (P2S and P5AS) were synthesized based on the peptide sequences P2 and P5, taking into account the biased codon usage typical of *P. falciparum* genes [28]. A 609-bp fragment was amplified from the genomic DNA of the FcB1 strain (nucleotide positions 449–1058 on Fig. 2), which encoded a protein displaying similarities to PDI from various species and similarities to three 'preliminary sequence data' in the *P. falciparum* genome project databases. These latter were exploited to amplify most of the corresponding gene from the genomic DNA of the FcB1 strain, while the remaining 5′ end of the gene was isolated from the genomic library previously described [23]. The sequencing of the complete cDNA, which was amplified by RT-PCR by using oligonucleotides N5S and N5AS, confirmed the occurrence of a 155-bp intron (Fig. 2). The presented data correspond to the FcB1 strain and it was checked by Southern blotting hybridization using the 609-bp fragment as a probe, that the locus appeared to be in single copy in this *P. falciparum* genome (data not shown).

3.4. Nucleotide and amino acid sequence analysis: evidence for a P. falciparum gene encoding a typical PDI homologue

The A+T richness of the non-coding regions (91% upstream of the ATG, 87% for the intron, and 89% downstream of the stop codon) and of the coding regions (70% for the first exon, 68% for the second exon) were typical of *P. falciparum* genes [29]. Analysis of the 483-amino acid sequence deduced from the cDNA allowed to identify the six peptide sequences derived from Pf52 and uncovered three additional features (Fig. 2). First, a putative signal peptide, with a most likely cleavage site after the serine in position 24 was predicted by using the method of Nielsen et al. [30]. Interestingly, the P3 sequence would thus correspond exactly to the N-terminus of the mature enzyme, the theoretical molecular weight of which has been calculated to be ~52-kDa (459 residues). Second, a putative 'KDEL' endoplasmic reticulum (ER) retention signal, was predicted with a score of 100% at the C-terminal end of the sequence [31]. Third, two perfectly conserved thioredoxin family active sites, the pattern of which is [LIVMF]-[LIVMSTA]-x-[LIVMFYC]-[FYWSTHE]-x(2)-[FYWGTN]-C-[GATPLVE]-[PHYWSTA]-C-x(6)-[LIVMFYWT], were found in the sequence, and both corresponded to the WCGHCK motif, which appears to be most favorable for PDIs [32].

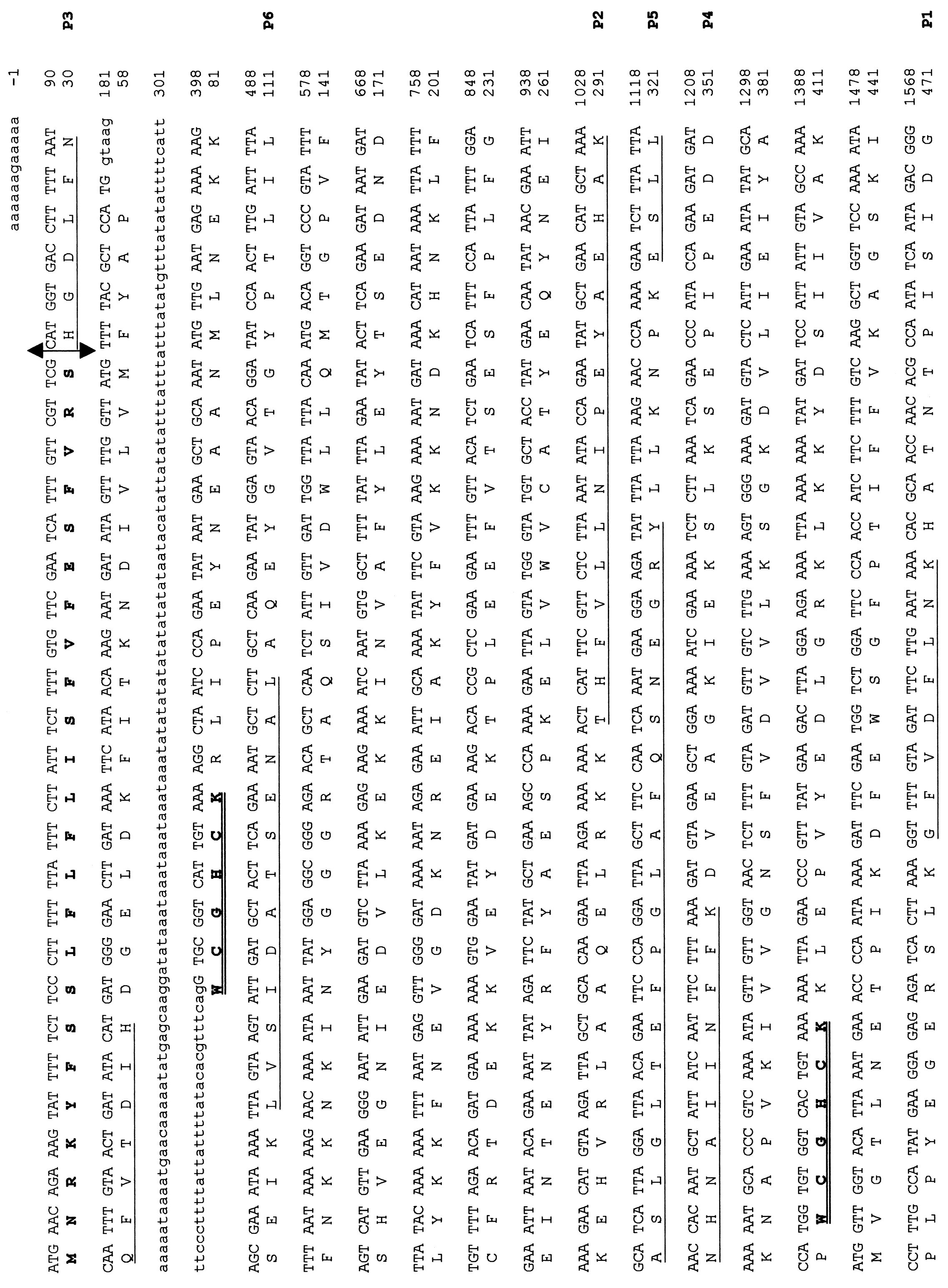


Fig. 2. The *P. falciparum* gene corresponding to Pf52 encodes a typical PDI homologue. Nucleotide sequence (above) and deduced amino acid sequence using the single letter code for amino acids (below). Non-coding sequences including the intron ^{177}gt–^{330}ag are in lowercase while coding sequences are in capital letters. The amino acid sequences corresponding to each of the six peptides are underlined. The amino acid sequences corresponding to the two thioredoxin family active sites typical of PDI (WCGHCK) are in bold and doubly underlined. The putative N-terminal signal sequence with the predicted cleavage site (arrow) and the putative ER retention signal (SEEL) are in bold. The stop codon is indicated by *.

PDIs are ∼55-kDa multifunctional proteins, fairly abundant in the ER of eukaryotic cells, known to be involved in the formation, breakage and rearrangements, of disulfide bonds during the folding of secreted proteins [32]. Although PDIs are typically found as dimers, monomers have been reported. PDIs have also been found as part of protein complexes where they could have a structural role in stabilizing the folded state of the associated proteins [17]. Noticeably, PDI has been proposed to act either as a chaperone or an anti-chaperone, depending on its concentration [17]. Disruption of the PDI gene turned out to be lethal in *Saccharomyces cerevisiae* [33].

PDIs are typically composed of five domains denoted a-b-b′-a′-c [17]. Both a and a′ domains (∼110 residues each),

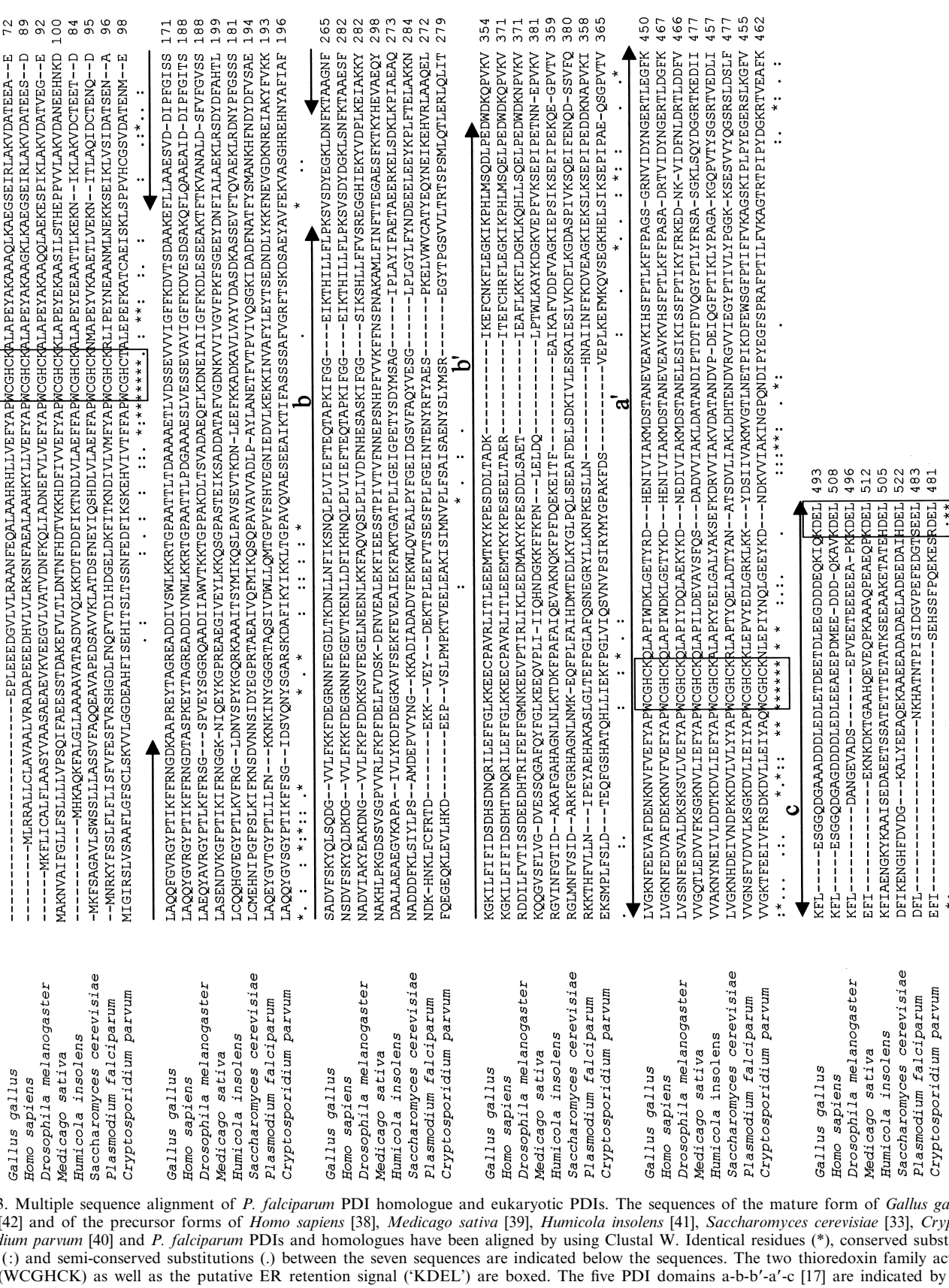


Fig. 3. Multiple sequence alignment of *P. falciparum* PDI homologue and eukaryotic PDIs. The sequences of the mature form of *Gallus gallus* PDI [42] and of the precursor forms of *Homo sapiens* [38], *Medicago sativa* [39], *Humicola insolens* [41], *Saccharomyces cerevisiae* [33], *Cryptosporidium parvum* [40] and *P. falciparum* PDIs and homologues have been aligned by using Clustal W. Identical residues (*), conserved substitutions (:) and semi-conserved substitutions (.) between the seven sequences are indicated below the sequences. The two thioredoxin family active sites (WCGHCK) as well as the putative ER retention signal ('KDEL') are boxed. The five PDI domains a-b-b′-a′-c [17] are indicated by arrows above the sequences.

which are similar to each other and well conserved between species, contain the WCGHCK motif and are related, in primary as well as three-dimensional structures, to thioredoxin [34]. The b and b′ domains are also similar to each other though to a lesser extent, fairly divergent between species, devoid of CGHC motif, but appear structurally related to the a and a′ domains [35]. The b′ domain was proposed to be involved in peptide and protein binding properties of PDIs [36]. The c domain ($\sim$30 residues) mainly consisting of acidic amino acid residues in mammalians and yeast ($>$50% are D and E) could be involved in the calcium binding ability of PDI, but would not be required neither for disulfide isomerase nor for chaperone activities of PDI [37]. Noteworthy PDIs from a number species do not have a very acidic c domain [17].

A multiple sequence alignment performed with the *P. falciparum* PDI homologue and PDIs from various species (Fig. 3) allowed to identify the a and a′ domains of the *P. falciparum* PDI homologue, which were found to display 31–53% amino acid identities with the a and a′ domains of the various PDIs known, one of the highest similarities (52% identity) being with the human PDI [38]. The b and b′ domains of the *P. falciparum* PDI homologue were, as expected, more distantly related to the various PDIs known, the best amino acid similarities being with PDIs from plants (29% identity for *Medicago sativa* [39]), protozoans (28% identity for *Cryptosporidium parvum* [40]) and fungi (27% identity with *Humicola insolens* [41]). The c domain of the *P. falciparum* PDI homologue was clearly not particularly acidic (only 26% D and E residues) but this should not, in theory, prevent this enzyme from displaying either disulfide isomerase or chaperone activities.

In summary, the *P. falciparum* gene we have isolated in this study encodes a protein displaying all the structural features and consensus sequences expected for a typical PDI and corresponds to Pf52. The experimentally determined and theoretically calculated molecular weight do match, and the six peptide sequences obtained from Pf52 were all found to be encoded by the gene. Moreover, this *P. falciparum* PDI homologue is clearly expressed in erythrocytic stages, since both the protein and the cDNA from these stages were characterized. The fact that PDIs appear essential for cell survival, at least in another system, and the fact that Pf52 binds to a potent antiplasmodial inhibitor, strongly suggest that it could represent a putative target for alternative antimalarial chemotherapy.

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