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Molecular modeling of wild-type and antifolate resistant mutant *Plasmodium falciparum* DHFR

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Abstract

The development of drug resistance is reducing the efficiency of antifolates as antimalarials. This phenomenon has been linked to the occurrence of mutations in the parasite's dihydrofolate reductase (DHFR). In this way, the resistance to pyrimethamine and cycloguanil, two potent inhibitors of *P. falciparum* DHFR, is mainly related to mutations (single and crossed) at residues 16, 51, 59, 108 and 164 of the enzyme. In this work, we have refined a recently proposed homology-model of *P. falciparum* DHFR, and the resulting structure was used to obtain models for 14 mutant enzymes, employing molecular modeling. Ternary complexes of the mutant enzymes with these inhibitors have been superimposed to equivalent ternary complexes of the wild-type enzyme, allowing the proposition of hypotheses for the role of each mutation in drug resistance. Based on these results, possible reasons for antifolate resistance have been proposed.

Keywords: Malaria; *Plasmodium falciparum*; Dihydrofolate reductase; Antifolate resistance; Homology modeling; Docking

1. Introduction

Malaria is still an endemic disease in vast areas of Africa, Latin America, southern Asia and Oceania. It is estimated that 300–500 million people are affected by this disease, which causes over 2.5 million deaths per year [1]. Malaria is caused by protozoan parasites of the genus *Plasmodium*. Four species of *Plasmodium* use humans as hosts: *P. vivax*; *P. ovale*; *P. malariae*; and *P. falciparum* [2]. The latter is the most dangerous, being responsible for most deaths related to malaria. *Falciparum* malaria is very common in tropical Africa, where most people are infected during childhood; morbidity and mortality occur mainly in children under the age of 5 years. Most of the African children which survive, gradually develop a partial immu-

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nity that protects them from severe malaria. However, a relatively small proportion develops the severe type of the disease, which kills approximately one million Africans annually. In Latin America and in Asia, *falciparum* malaria is less common than in tropical Africa. The low (and usually seasonal) transmission rates in these continents prevent the development of partial immunity, so that all age groups are affected by severe malaria [2].

Chemotherapy is one of the main strategies employed in malaria control. Such an approach has been used against the disease since the 17th century, and extensive reviews on antimalarial drugs have been published [2–5]. One of the main targets for antimalarial chemotherapy has been the dihydrofolate reductase (DHFR; EC. 1.5.1.3), an enzyme found in nearly all living cells, with very few exceptions. Its function is to catalyze the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF), a fundamental cofactor in the synthesis of some amino acids and nucleotides, such as thymidylate [5,6]. Inhibition of DHFR blocks the reduction of DHF to THF, which prevents DNA synthesis, resulting in cell death [6]. It is known that malarial parasites synthesize folate cofactors de novo, though there is evidence that suggest the possibility of an existing salvage pathway in malaria [7]. For this reason, DHFR of *Plasmodium falciparum* (pfDHFR) is a good target for the treatment of *falciparum* malaria.

pfDHFR constitutes with the enzyme thymidylate synthase (TS, EC. 2.1.1.45), a bifunctional and dimeric enzyme, DHFR-TS. The same is true for other protozoans such as *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania major*, but not for bacteria and mammals, in which these enzymes are monofunctional [7,8]. Bzik et al. were the first to determine the amino acid sequence of DHFR-TS of *P. falciparum* [9]. Based on the identity of amino acids and on the secondary structures of others DHFR, they defined the pfDHFR domain as constituted by residues 1–228, the TS domain by residues 323–608, and a junctional domain by residues 229–322. It is noteworthy that the sequence determined by Bzik et al. actually belongs to a mutant strain: the residue 108 in the wild-type enzyme is a serine, not an asparagine [1].

Due to technical difficulties in obtaining sufficiently large amounts and appropriate concentrations of the pfDHFR-TS, no experimental information about the tertiary structure of this protein is available [7]. Even the tertiary structure of the pfDHFR domain is still unknown. Three theoretical models have been proposed recently, which are based on the homology modeling technique [1,10,11]. Some authors have also made some structural considerations based on this technique [7,12].

Several drugs which inhibit the action of pfDHFR, thus disrupting the folate cycle, have been used against *falciparum* malaria. The most extensively used antifolates are pyrimethamine (PYR) and cycloguanil (CYC). PYR is a potent and selective inhibitor of pfDHFR, used alone or in combination with other drugs, such as sulfadoxine (combination known as PS). PS is cheap, practicable and highly effective in some African countries [2]; however, it is prone to the rapid emergence of resistance. CYC is the metabolic product of proguanil, a drug developed in Britain during World War II. It is also a potent drug, and can be used alone or in combination with chloroquine or atovaquone. PYR and CYC have a low level of toxicity and, if used in the correct dose, are completely free of side-effects [13]. WR99210 is an experimental drug, which is highly effective in vitro against strains of pfDHFR [14]. The structures for these drugs are shown in Fig. 1.

Unfortunately, PYR and CYC are facing the emergence and spreading of strains that are resistant to their action, limiting their utility in the treatment of malaria. It is believed that this resistance is caused by point mutations in the primary structure of pfDHFR. Mutations in residues 16, 51 59, 108 and 164 have been reported [15–18], and they have been associated with resistance to these antifolates. The S108N mutation is found in all PYR-resistant strains, and it has been proposed that it is the prime point mutation necessary for the development of PYR-resistance [19]. The A16V mutation seems to be responsible for CYC-resistance, and is always associated with S108T [17,19]. The N51I and C59R mutations are natu-

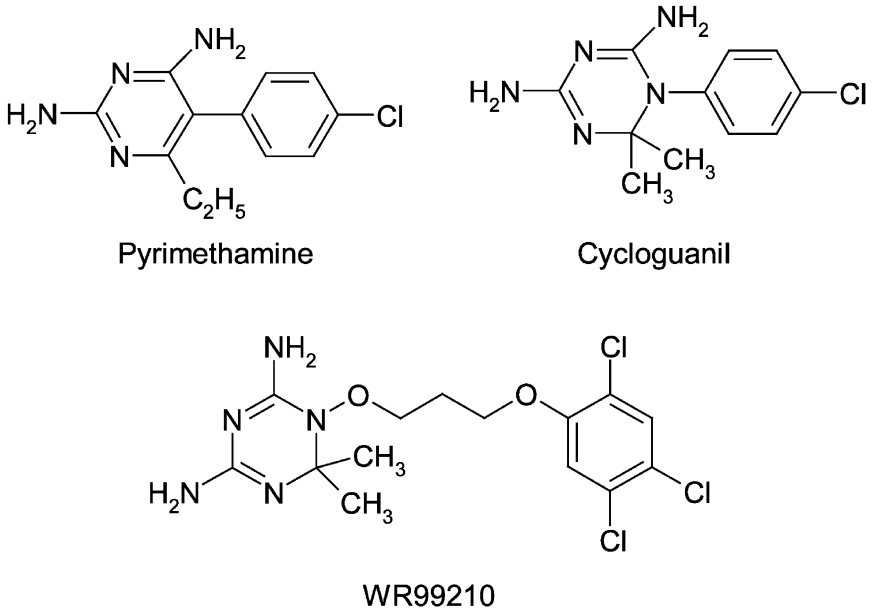

Fig. 1. Structures for pyrimethamine, cycloguanil and WR99210.

rally present only in strains with the S108N mutation, and confer additional resistance to PYR. The I164L mutation is always found in combination with S108N and one or both of the N51I and C59R mutations, and confers high resistance to both PYR and CYC [17–19]. Other mutations, whose importances to antifolate resistance are still unclear, have also been described: V140L [20]; C50R; and the 'Bolivia repeat', a five-amino acid insertion [21]. WR99210 has proven to be effective even against strains that are highly resistant to PYR and CYC, but it is not adequate for clinical use [14].

The pfDHFR homology-models proposed so far suggest possible mechanisms by which the cited mutations increase the enzyme resistance to PYR and CYC [1,10,11]. However, there are still many obscure points, and the reasons for the antifolate resistance are not completely understood. In this work, we propose new theoretical pfDHFR models for the wild-type and some mutants, based on the previous model proposed by Santos-Filho et al. [1]. We have validated our models, and constructed complexes by docking the NADPH and the drugs PYR, CYC and WR99210. Comparisons between the ternary complexes of the mutants and the equivalent complex of the wild-type enzyme were performed by superimposing the main residues of the active sites. Based on these observations, possible reasons for antifolate resistance have been proposed.

2. Experimental

The homology-model of the wild-type pfDHFR previously reported, based on the alignment of pfDHFR with the chicken liver DHFR sequence (PDB entry 8DFR), has been used as the starting point of this work [1]. The INSIGHTII/DISCOVER package [22] was used for visualization and minimization of the molecule. All Lys and Arg residues were positively charged, all Glu and Asp residues were negatively charged, and the non-bonded pairs of electrons in all sulfur atoms in Met and Cys residues were made explicit. Modifications in the alignment with chicken liver DHFR were done using the Swiss-PdbViewer program [23], and the new alignments were submitted to the SWISS-MODEL server [23–25]. Minimization of wild-type pfDHFR was executed in two phases of

four steps each. In the first phase, we used the steepest descents algorithm and a convergence criterion of 10.00 kcal mol^{-1} $Å^{-1}$. In the second phase, the gradient conjugate algorithm was employed, with a convergence criterion of 1.00 kcal mol^{-1} $Å^{-1}$. The four steps of each phase were: (1) minimization of the hydrogen atoms only; (2) minimization of the side-chains, keeping the backbone fixed; (3) minimization of the backbone, keeping the side-chains fixed; and (4) minimization of the whole enzyme. All minimizations were executed with the AMBER force field [26–29], with a distance-dependent dielectric constant of water ($\varepsilon_r = 78.54$) and 1–4 interactions scaled by a factor of 0.5.

Models for the mutant enzymes were constructed by replacing the appropriate residues in the wild-type pfDHFR, for single mutants, or in an ancestral mutant, according to the evolutionary tree proposed by Sirawaraporn et al. [19], for double, triple and quadruple mutants. The mutant enzymes were minimized in two phases. In the first one, only the mutated residue was minimized by steepest descents to 10.00 kcal mol^{-1} $Å^{-1}$, while the rest of the enzyme was kept fixed. In the second phase, the whole enzyme was minimized by gradient conjugate to 1.00 kcal mol^{-1} $Å^{-1}$. The models for wild-type and mutant enzymes were validated by using both the PROCHECK [30] and WHATIF [31] tools, available at the Biotech Validation Suite for Protein Structures (http://biotech.embl-heidelberg.de:8400/).

Ternary complexes of the wild-type and mutant enzymes with DHF, PYR, CYC and WR99210 were modeled using the technique known as molecular docking [32]. PYR, CYC and WR99210 were modeled with the atom N1 protonated, as suggested by some authors [6,11,12]. For the computation of partial charges, each of these compounds was submitted to an ab initio single-point energy calculation, with STO-3G basis and a Merz–Kollman (MK) charge distribution, in the GAUSSIAN®98W package [33]. The charges calculated by this method have been manually assigned to the atoms of DHF, PYR, CYC, WR99210 and NADPH. Initial conformations of DHF and NADPH were analogous to those previously determined by some of us [1]. PYR, CYC and WR99210 were successively minimized to 10.00, 1.00, 0.01 and 0.001 kcal mol^{-1} $Å^{-1}$, with the steepest descents, conjugate gradient, quasi-Newton Raphson and Newton Raphson algorithms, respectively. The conformations obtained were taken to be the initial ones for the docking procedure.

The apoenzyme of the modeled wild-type pfDHFR was superimposed to the previously modeled holoenzyme [1], for docking the DHF and the NADPH. The whole complex was minimized by using the gradient conjugate algorithm to 1.00 kcal mol^{-1} $Å^{-1}$. Constraints to maintain the hydrogen bonds between Asp-54 and the pteridine ring of DHF, Asp-54 and Thr-185, Ala-16 and NADPH, and Ser-108 and NADPH were used, according to the literature [6,11,12]. The ternary complexes of wild-type pfDHFR with PYR, CYC and WR99210 were modeled by superimposing the atoms N1, C2, N3 and the nitrogen of the group 2-amino to atoms N3, C2, N1 and the nitrogen of the 2-amino group of DHF, respectively. After removing the DHF, the complex was minimized by gradient conjugate algorithm to 1.00 kcal mol^{-1} $Å^{-1}$, with the same constraints described above, plus two additional ones destined to guarantee the formation of hydrogen bonds between Ile-14 and the 4-amino group of the drugs, and between Ile-164 and the 4-amino group of the drugs.

The mutant apoenzymes were superimposed to the ternary complex wild-type pfDHFR–DHF–NADPH. After removing the wild-type pfDHFR, the ternary complex of the mutants were minimized by gradient conjugate to 1.00 kcal mol^{-1} $Å^{-1}$, with the same constraints described for the wild-type case. Modeling of the ternary complexes of the mutants with the drugs was made by the same procedure described for wild-type pfDHFR.

The hardware resources used in this work were a Silicon Graphics IRIX O2 6.3 workstation and a PC Pentium II.

3. Results and discussion

As mentioned before, the basis for this work was the theoretical model of wild-type pfDHFR previously reported [1]. This model is based on the alignment of the pfDHFR primary structure

```
Plasmodium   1   MMEQVCDVFD IYAICACCKV ESKNEGKKNE VFNNYTFRGL GNKGVLPWKC
clDHFR       1       VRSLNS IVAVCQNM-- ---------- --------GI GKDGNLPWP-
                     *  .   * *.*                        *. *. * ***

Plasmodium  51   NSLDMKYFCA VTTYVNESKY EKLKYKRCKY LNKETVDNVN DMPNSKKLQN
clDHFR      26   -PLRNEYKYF QR-------- ---------- --MTSTSHVE GK------QN
                  .*   *                            . .  .*.  .      **

Plasmodium 101   VVVMGRTSWE SIPKKFKPLS NRINVILSRT LKKEDFDEDV YIINKVEDLI
clDHFR      49   AVIMGKKTWF SIPEKNRPLK DRINIVLSRE LKEAP-KGAH YLSKSLDDAL
                  *.**. .*  *** * .**  .***..***  **         *. . ..* .

Plasmodium 151   VLLG----KL NYYKCFIIGG SVVYQEFLEK KLIKKIYFTR INSTYECDVF
clDHFR      98   ALLDSPELKS KVDMVWIVGG TAVYKAAMEK PINHRLFVTR ILHEFESDTF
                  **        .  .  *.** . **   .**  .  ... ** *   .* * *

Plasmodium 197   FPEINENEYQ IIS-----VS DVYTSNNTTL DFIIYKK
clDHFR     148   FPEIDYKDFK LLTEYPGVPA DIQEEDGIQY KFEVYQKSV
                  ****......           *.   .      * .* *
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Fig. 2. Alignment between pfDHFR and chicken liver DHFR (clDHFR) [1]. Identical residues are shown with stars, and residues with similar properties are shown with dots.

with the chicken liver DHFR sequence. This alignment is reproduced in Fig. 2.

Our first model, constructed from this alignment, presented some problems, since it did not represent correctly some interactions among the enzyme, the ligands (DHF or drug) and the NADPH. Thus, we have modified the alignment, based on structural and functional considerations. In the alignment of Fig. 2, the Asn-51 of pfDHFR is not aligned with any residue of chicken liver DHFR, which is odd for the active site of the malarial enzyme. There are works which align this residue Asn-51 with Leu 27 of chicken liver DHFR [7,10]. Moreover, in Fig. 2, Asp-54 of pfDHFR is aligned with Arg-28. However, Asp-54 is known to be a conserved acidic residue; in all DHFR whose structures have been determined, there is an Asp or Glu H-bonded to the pteridine ring of the substrate. So, Asp-54 should be aligned with Glu-30 of chicken liver DHFR, and not with Arg-28. For these reasons, in order to improve the alignment, we manually moved all residues from Pro-26 to Arg-36 of pfDHFR two positions to the left, obtaining the alignment represented in Fig. 3.

The model obtained from this alignment was not satisfactory either; residues Ile-14 and Ala-16 were too far from the active site region, so that the expected hydrogen-bonds among these residues, the drugs and the NADPH were not formed. Moreover, this model was not clear in the evaluation of mutation effects on drug-resistance. Due to this result, the alignment was modified once more; we manually moved all residues from Val-1 to Met-14 of chicken liver DHFR three positions to the right, so that Ile-14 of pfDHFR and Ile-7 of chicken liver DHFR were aligned, as described in the literature [11,12,34]. The final version of alignment is shown in Fig. 4.

From the final version of the alignment, we constructed a model which proved to be the most satisfactory. After the minimization steps described in the previous section, the model of pfDHFR was superimposed to the chicken liver DHFR structure (PDB entry 8DFR), as seen in Fig. 5b. The RMS of this superimposition was equal to 0.433. This value, associated to the homology between these enzymes (35,43% of identity), suggests that the folding of the model should be correct. The super-

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Plasmodium   1   MMEQVCDVFD IYAICACCKV ESKNEGKKNE VFNNYTFRGL GNKGVLPWKC
clDHFR       1       VRSLNS IVAVCQNM-- ---------- --------GI GKDGNLPWPP
                     *  .   * *.*                          *. *. * ***

Plasmodium  51   NSLDMKYFCA VTTYVNESKY EKLKYKRCKY LNKETVDNVN DMPNSKKLQN
clDHFR      27   LRNEYKYFQR ---------- ---------- --MTSTSHVE GK------QN
                    . ***                          . .  .*.  .      **

Plasmodium 101   VVVMGRTSWE SIPKKFKPLS NRINVILSRT LKKEDFDEDV YIINKVEDLI
clDHFR      49   AVIMGKKTWF SIPEKNRPLK DRINIVLSRE LKEAP-KGAH YLSKSLDDAL
                  *.**. .*  *** * .**  .***..***  **         *. . ..* .

Plasmodium 151   VLLG----KL NYYKCFIIGG SVVYQEFLEK KLIKKIYFTR INSTYECDVF
clDHFR      98   ALLDSPELKS KVDMVWIVGG TAVYKAAMEK PINHRLFVTR ILHEFESDTF
                  **        .  .  *.** . **   .**  .  ... ** *   .* * *

Plasmodium 197   FPEINENEYQ IIS-----VS DVYTSNNTTL DFIIYKK
clDHFR     148   FPEIDYKDFK LLTEYPGVPA DIQEEDGIQY KFEVYQKSV
                  ****......           *.   .      * .* *
```

Fig. 3. Alignment between pfDHFR and clDHFR after the first modification.

imposition shows that differences between our model and the chicken liver DHFR occur only in loop regions; secondary structure elements are well superimposed.

Fig. 4 shows that there are two large insertions, of 17 and 22 residues, in pfDHFR sequence. Similar long insertions were found in theoretical models constructed by other groups [10,11]. Mod-

```
Plasmodium   1   MMEQVCDVFD IYAICACCKV ESKNEGKKNE VFNNYTFRGL GNKGVLPWKC
clDHFR       1          VRS LNSIVAVCQN M--------- --------GI GKDGNLPWPP
                        *   .  * * *                        *. *. * ***

Plasmodium  51   NSLDMKYFCA VTTYVNESKY EKLKYKRCKY LNKETVDNVN DMPNSKKLQN
clDHFR      27   LRNEYKYFQR ---------- ---------- --MTSTSHVE GK------QN
                    . ***                          . .  .*.  .      **

Plasmodium 101   VVVMGRTSWE SIPKKFKPLS NRINVILSRT LKKEDFDEDV YIINKVEDLI
clDHFR      49   AVIMGKKTWF SIPEKNRPLK DRINIVLSRE LKEAP-KGAH YLSKSLDDAL
                  *.**. .*  *** * .**  .***..***  **         *. . ..* .

Plasmodium 151   VLLG----KL NYYKCFIIGG SVVYQEFLEK KLIKKIYFTR INSTYECDVF
clDHFR      98   ALLDSPELKS KVDMVWIVGG TAVYKAAMEK PINHRLFVTR ILHEFESDTF
                  **        .  .  *.** . **   .**  .  ... ** *   .* * *

Plasmodium 197   FPEINENEYQ IIS-----VS DVYTSNNTTL DFIIYKK
clDHFR     148   FPEIDYKDFK LLTEYPGVPA DIQEEDGIQY KFEVYQKSV
                  ****......           *.   .      * .* *
```

Fig. 4. Alignment between pfDHFR and clDHFR (final version).

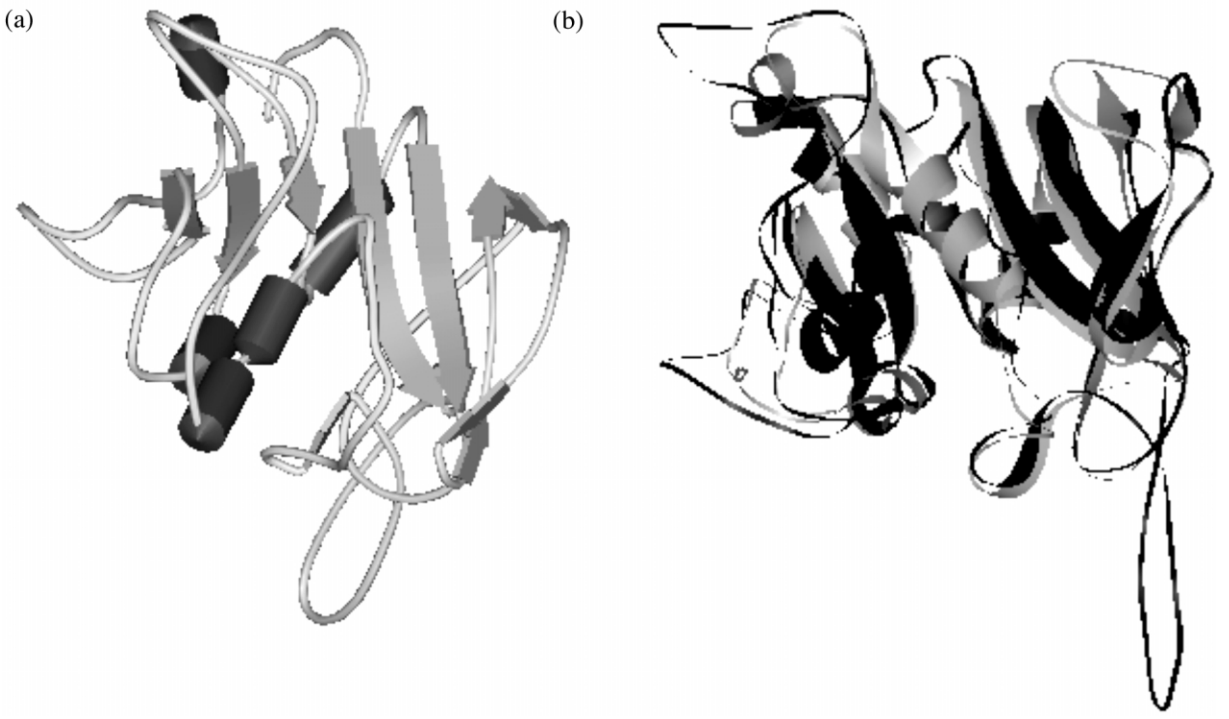

Fig. 5. Modeled structure of wild-type pfDHFR (a) and its superimposition to chicken liver DHFR (b). In (a), α-helices are represented by cylinders, and β-strands by arrows. In (b), pfDHFR is in darker color, and chicken liver DHFR in a lighter color.

eling of such large loops is a difficult task, even with the aid of loop databases. Consequently, conformations of loop regions are less reliable than those of secondary structure elements in any theoretical model. Despite this lack of reliability, we believe that it does not compromise the validity of the model, since the secondary structure elements seem to be well modeled. None of the active site residues, including the ones in which occur mutations associated to antifolate resistance, are in loop regions, so that the imprecision of these regions does not affect the pharmacodynamical study of the mutations. Conformations of loop regions could affect the approximation of the drug to the active site, but this kind of analysis is beyond the scope of this work.

A comparison between the alignment of Fig. 4 and the one proposed by Rastelli et al. [11] shows only minor differences; the alignment of active site residues is identical in both cases. The major difference is that, in our alignment, residues 83–92 are aligned, and separate two insertions of 22 and 6 residues, while in the work of Rastelli et al., there is a single 28-residue loop from residues 64 to 91. We believe our alignment to be somehow more accurate, since it results in a shorter insertion, without any loss of residue identity between the sequences.

The generated model was validated with the PROCHECK and WHATIF tools (data not shown). The results of these tests show that the proposed homology model of pfDHFR can be considered as satisfactory. None of the residues which constitute the antifolate binding site (Ile-14, Ala-16, Cys-50, Asn-51, Asp-54, Cys-59, Ser-108, Ile-164 and Thr-185), as previously defined [1], show any serious stereochemical problem. Residues indicated in the tests as being problematic are mostly in loop regions, where theoretical models are less precise, anyway.

Fig. 5a shows the constructed wild-type pfDHFR. This model is similar to that reported for

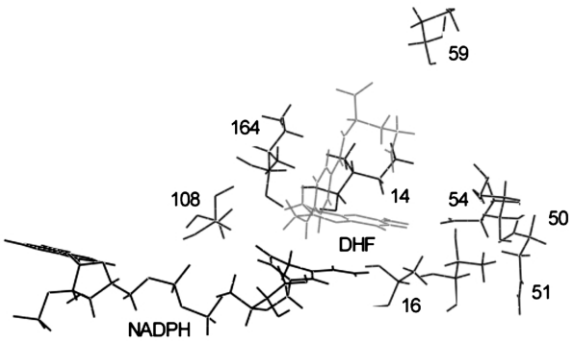

Fig. 6. Active site of the modeled ternary complex wild-type pfDHFR–DHF–NADPH.

other DHFR [35] and, as expected, is analogous to that of chicken liver DHFR [36]. The main secondary structure elements of the pfDHFR are five α-helices and a eight-stranded β-sheet, composed of seven parallel strands and a carboxyl-terminal antiparallel strand. Some of these β-strands are not continuous, being disrupted, as in the case of chicken liver DHFR [37]. The remainder of the enzyme is composed of loops and tight turns.

The docking of DHF and NADPH was executed as described in Section 2. The active site of the modeled ternary complex is shown in Fig. 6. NADPH is in an extended conformation, while DHF is in a conformation where the pteridine ring is approximately perpendicular to the remainder of the molecule. Such conformations are similar to those found in the crystallographic structures of bacterial and vertebrate DHFR complexed with methotrexate (MTX) and trimethoprim (TMP) [6,12]. NADPH and DHF are both stabilized by hydrophobic interactions and hydrogen bonds. The most important of these are the H-bonds between the carboxylate of Asp-54 and the 2-amino group and atom N3 of DHF pteridine ring. The H-bonds resulting from the other constraints imposed during minimization, as described in Section 2, are also seen. Moreover, there is a π-stacking interaction between the pteridine ring of DHF and the nicotinamide ring of NADPH. Analogous interactions are found in previously reported models [1,10,11].

The docking of PYR, CYC and WR99210 was done as described in Section 2. The H-bonds resulting from the constraints during the minimization are observed. Our model differs from the one proposed by Rastelli et al. [11] since we have not observed a π-stacking interaction between the 3-chlorophenyl group of PYR and CYC and the nicotinamide ring of NADPH, but we observed that the trichlorophenoxy propyloxy group of WR99210 is perpendicular to the nicotinamide ring, as in the model of Rastelli.

Fourteen mutant enzymes have been modeled in this work: S108N; A16V*; N51I*; C59R*; I164L*; S108T*; A16V + S108N*; A16V + S108T; N51I + S108N; C59R + S108N; N51I + C59R + S108N; C59R + S108N + I164L; N51I + C59R + S108N + I164L; and C50R + S108N (mutants marked with * have not been found in nature). We chose to model these mutants due to the availability in the literature of kinetic data of their inhibition by PYR, CYC and, for some of them, WR99210 [19,38,39]. Folding and stereochemical features of the models for these mutants are similar to the wild-type enzyme. Superimposition of each of them to the wild-type pfDHFR resulted in RMS values below 0.305. Validation of these structures by PROCHECK and WHATIF gave analogous results to wild-type pfDHFR (data not shown), so that they can be considered reasonable. For instance, all of them have approximately 65% of the residues in the most favored region of Ramachandran plot, and more than 95% of the residues are in the most favored and in the additionally allowed regions of this plot. The docking of DHF and drugs in these models was executed as described in Section 2.

The homology models of this work were constructed in an attempt to propose a model that explains the mechanism by which mutations in pfDHFR sequence increase its resistance to PYR and CYC. None of the models proposed so far [1,10,11] presented an extensive study of mutants pfDHFR, limiting themselves to the analysis of some isolated mutations. In this work, we modeled the 14 mutants cited above to analyze the pharmacodynamical differences between them and the wild-type pfDHFR, and to evaluate the possible effects of synergy between two or more mutations. Thus, we have superimposed the active sites of the ternary complex mutants–drug–NADPH with

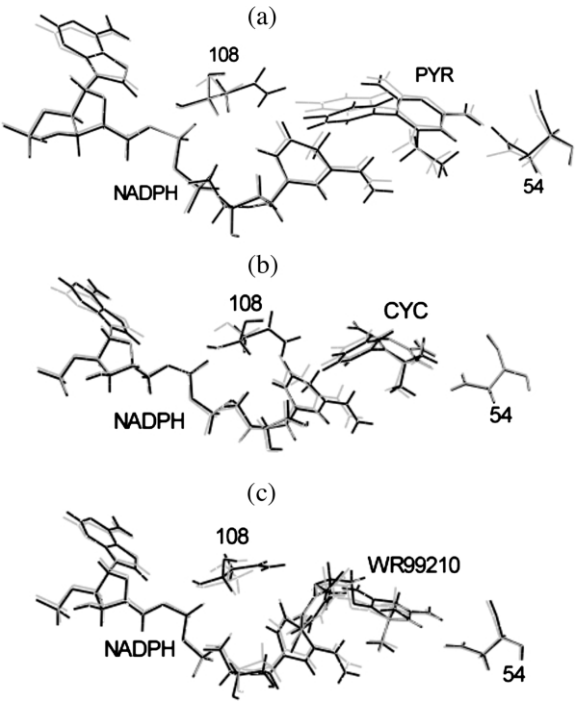

Fig. 7. Superimpositions of the active sites of mutant S108N (darker color) and wild-type pfDHFR (lighter color), complexed with PYR (a), CYC (b) and WR99210 (c).

the active site of the equivalent ternary complex of wild-type pfDHFR.

Superimpositions of the active sites of the S108N mutant and wild-type pfDHFR, complexed with PYR, CYC and WR99210 are shown in Fig. 7. It can be seen that replacement of Ser-108 by an Asn causes a clash between the NH_2 group of the latter with the chlorine atom of PYR, displacing the drug from its position. This displacement causes another clash, between the ethyl group of PYR and one of the oxygen atoms of the carboxylate group of Asp-54, which makes this group to rotate. Since it is known that the ideal bonding between Asp-54 and the pteridine ring should be planar [12], it is probable that this loss of planarity is responsible for the PYR-resistance of the S108N mutant. The effect of this mutation with CYC is less remarkable, since the presence of two methyl groups, instead of an ethyl, does not cause the rotation of the carboxylate group of Asp-54, due to the absence of the corresponding sterical clash. However, there is still some proximity between the NH_2 of Asn-108 and the chlorine atom of CYC, so that it can be suggested that the existence of repulsive London forces among these atoms would make this ternary complex less stable. Based on this hypothesis, the S108N mutation should have less impact on CYC-resistance than on PYR-resistance. This is coherent with the available experimental data [19,38,39]. Finally, the S108N mutation seems to have no effect on antifolate activity of WR99210, since the drug, the NADPH and the active site residues are very well superimposed in our model; the absence of a voluminous atom (such as Cl) near Asn-108 and the flexibility of WR99210 prevent any sterical hindrance caused by the replacement of Ser-108 by an Asn. Again, such observation is in perfect agreement with the available experimental data [19,38,39].

Some of us have proposed the hypothesis that the effect of mutation S108N could be due not only to problems in drug accommodation, but also to some hindrance to the dynamical process of admission of the inhibitor in the active site [1]. Although our new model does not rule out this possibility, it seems clear that the effect of the mutation on drug admission, if existent, should be secondary when compared to the effect on drug accommodation. After all, it does not seem probable that this mutation would affect PYR and CYC admission, and not WR99210 admission, since the latter, though more flexible, is much bigger than the formers. Consequently, it is reasonable to conclude that mutation S108N acts mainly on antifolate accommodation to the active site, rather than on its admission.

Fig. 8 shows the superimpositions of active sites of the A16V mutant and wild-type pfDHFR, complexed with PYR and CYC (we have not modeled the ternary complex of A16V with WR99210, due to the lack of experimental data of inhibition by this drug). In Fig. 8a, the only remarkable change is the rotation of the carboxylate group of Asp-54; all other active site residues are in the same positions and conformations. In this case, rotation of the carboxylate is caused not by displacement of PYR, which is not observed, but by a sterical clash between the atom C^{γ} of the longer side-

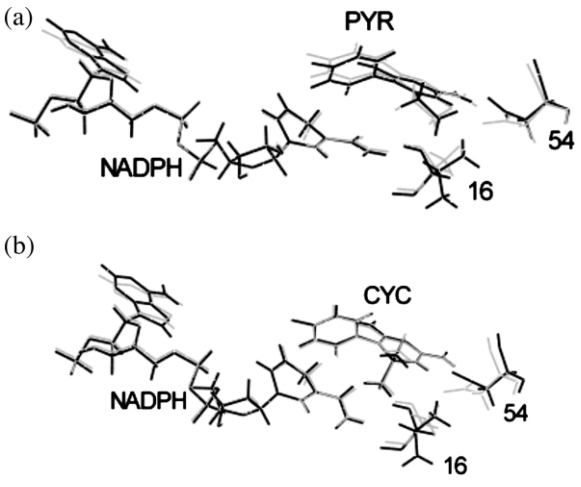

Fig. 8. Superimpositions of the active sites of mutant A16V (darker color) and wild-type pfDHFR (lighter color), complexed with PYR (a) and CYC (b).

chain of Val-16 and one of the oxygen atoms of Asp-54. This rotation causes the loss of planarity of the bonding between Asp-54 and PYR, which is responsible for drug-resistance. One can admit that PYR resistance is greater for S108N than for A16V since in the latter case, there is no displacement of the drug in the active site, as in the former case. Our model differs from the one of Rastelli et al. [11], as their results do not show any rotation of the carboxylate group of Asp-54.

Fig. 8b does not show any major differences between the ternary complexes of A16V and wild-type pfDHFR with CYC. It is known, however, that this mutant has a very high resistance to this drug [19]. Rastelli et al. have observed a great clash between one of the methyl groups of CYC and one of the methyl groups of Val-16, which displaces the drug from its original position. Such an effect was not observed in our model, but the relative proximity between Val-16 and one of the methyl groups of CYC does not allow this hypothesis to be discarded: it is possible that, in our model, Val-16 has been accommodated in a local minimum, before it could clash with CYC. However, we can propose another hypothesis; for some unknown reason, CYC could be more susceptible than PYR to the loss of planarity of the bonding between Asp-54 and the drug. By this hypothesis, the great CYC-resistance of mutant A16V would be due to the loss of planarity of that bonding, caused by a clash between the carboxylate group of Asp-54 and Val-16.

Examination of the superimpositions of the active sites of complexed mutants N51I, C59R and S108T with the active sites of the equivalent complexes of wild-type pfDHFR (data not shown) does not give any insight into the mechanism of action of these mutations. Either we have displacements of unknown origin, whose effect would be the contrary of experimental data, or there is no change at all when the models are superimposed. The hypotheses of Warhurst for the mechanism of action of mutations N51I and C59R [12] have not been observed in our model. According to it, the only explanations for the effect of the mutations N51I and C59R would be related to antifolate admission in the active site. Residues 51 and 59 are quite distant from the position of the drug in the active site. Mutation N51I changes a polar residue by an apolar one, and C59R changes a neutral residue by a positively charged one. Since antifolates are positively charged (due to being protonated), these mutations could affect electrostatically the admission of the drug to the binding site, in a way not determined yet. Sirawaraporn et al. have shown that mutants N51I and C59R have low PYR- and CYC-resistance [19]; we can hypothesize that the effects of these mutations on the drug admission to the active site are inexpressive, unless the mutation S108N is present. In this case, the mutations would act in synergy, by amplifying PYR- and CYC-resistance. Such hypothesis is coherent with the evolutionary tree proposed by Sirawaraporn et al. [19]. Mutation S108T does not have any effect on antifolate resistance. Its function would be just to restore catalytic activity to mutant A16V by an unknown mechanism, as proposed by Sirawaraporn et al. [19].

The superimpositions of the ternary complexes of mutant I164L with the equivalent complexes of wild-type pfDHFR are shown in Fig. 9. In the complexes with PYR, it can be observed that the drug changes its conformation considerably. In the wild-type enzyme, one of the C^{γ} atoms of Ile-164

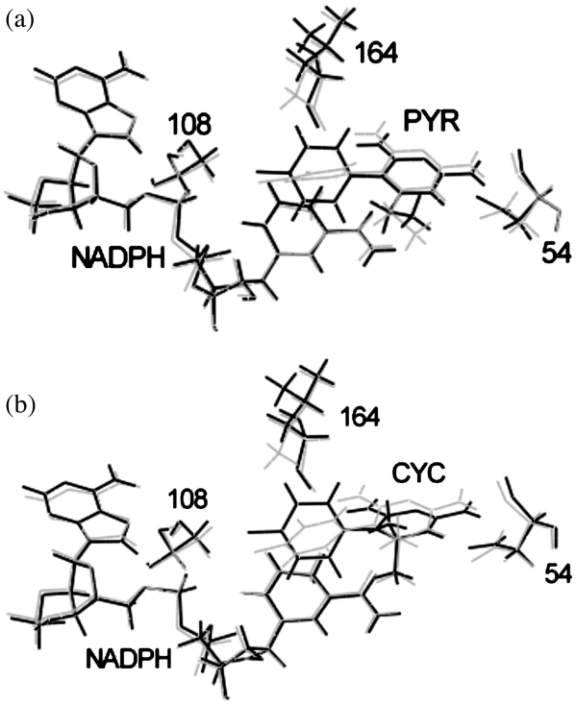

Fig. 9. Superimpositions of the active sites of mutant I164L (darker color) and wild-type pfDHFR (lighter color), complexed with PYR (a) and CYC (b).

prevents, due to a clash effect, the *p*-chlorophenyl ring of PYR to assume the same conformation seen in the mutant complex, where the replacement of Ile by a Leu prevents this clash to occur. Since mutant I164L is more sensitive to PYR than the wild-type enzyme [19], we propose that this mutation allows PYR to assume a more stable conformation, where there is a π-stacking interaction between the *p*-chlorophenyl ring of the drug and the nicotinamide ring of NADPH. The slight loss of planarity of the bonding between Asp-54 and PYR would not be sufficient to increase drug resistance, in this case. On the other hand, Fig. 9b shows that there is a similar change of CYC conformation in its complex with I164L. However, this mutant increases CYC-resistance, instead of reducing it [19]. At first sight, our model does not give a clear explanation for this fact, but it is worthy of note that, among all ternary complexes of single mutants and CYC, I164L and A16V are the only mutants in which there is a rotation (of unknown origin, in the case of I164L) of the carboxylate group of Asp-54. According to our previous hypothesis that CYC is more susceptible to the loss of planarity of the bonding between Asp-54 and the pteridine ring than PYR, we can consider that, in this case, the effect of this loss of planarity is more important than the effect the more stable conformation of CYC. So mutation I164L, when isolated, increases CYC-resistance due to the loss of planarity of the bonding between Asp-54 and the drug, and reduces PYR-resistance, by allowing the drug to assume a more stable conformation.

Analysis of the superimpositions of the complexes of double, triple and quadruple mutants does not give much additional information. It seems that S108N and A16V are the key mutations for PYR- and CYC-resistance, respectively, acting on the accommodation of the drugs in the active site, while mutations C50R, N51I, C59R and I164L (when associated with S108N) act mainly on the admission of the antifolates to the active site, in synergy with S108N, increasing PYR- and CYC-resistance. It should be noted that, according to experimental data, mutation I164L, when present in triple or quadruple mutants, increases not only CYC-resistance, but also PYR-resistance [19,38,39], contrary to its effect when isolated. Mutations C50R, N51I and C59R have more impact on PYR-resistance than on CYC-resistance. As said before, the S108T mutation would just restore catalytic activity to the A16V mutant. Superimpositions of the complexes with WR99210 show no major differences, which is in accordance with experimental data [38].

Sirawaraporn et al. have proposed that antifolates which act simultaneously on wild-type and on the S108N mutant would have a low propensity for developing resistance and hence, could provide effective antimalarial agents [19]. In addition, we believe that the ideal antifolate should be effective also against mutants with A16V, the key mutation for CYC-resistance, since these mutants are not descendants of the mutant S108N, according to the evolutionary tree proposed by Sirawaraporn et al. [19]. Actually, this tree is not clear about the appearance of the mutant A16V+S108T. Drugs

derived from WR99210 constitute a good perspective for new effective antimalarials, since this compound has proven to be effective against all mutants modeled in this work [38]. New drugs derived from PYR and CYC, which circumvent the reasons for drug resistance (for instance, by replacing the chlorine atom for a fluorine or hydrogen) could also be worthy of research.

4. Conclusion

Homology models for wild- and mutant-types of pfDHFR have been constructed, using as starting point a model previously proposed [1]. Validation of these models showed that structural and functional parameters can be considered satisfactory, within the inherent limitations of theoretical models. DHF, PYR, CYC, WR99210 and NADPH were docked by superimposition in the active site of the enzymes. The main interactions among the ligands and the enzyme active site are present in our models.

We believe that the models proposed in this work represent a significant improvement when compared to the ones previously proposed by Santos-Filho et al. [1]. The modifications executed in the alignment between the primary structures of wild-type pfDHFR and chicken liver DHFR resulted in a model which appears to be closer to reality, for two reasons: firstly, the alignment between the residues of pfDHFR active site and the equivalent ones of chicken liver DHFR make more sense in the final version of our alignment than in the previous ones, as discussed in Section 3; secondly, all the interactions usually found in the active sites of DHFR of other species [35] and which are found in other theoretical models of pfDHFR [10,11] are now present in our model, which did not happen in the previous model described in Santos-Filho et al. [1]. Moreover, the results of validation by the PROCHECK and WHATIF tools suggest that our structure is adequate, and that active sites residues should be reasonably modeled. For all these reasons, we expect the conclusions taken from the model of the present work to be more reliable than the ones taken from Santos-Filho et al. [1].

It is worthy of note that none of the previous works with theoretical models of pfDHFR have done an extensive study of mutant pfDHFR, limiting themselves to the analysis of some mutations [1,10,11]. The simultaneous study of 14 mutants in this work constituted an attempt to analyze the pharmacodynamical differences among them and wild-type pfDHFR, as well as the possible synergy effects between two or more mutations.

The superimpositions of the active sites of the ternary complexes mutants with the equivalent active site of the wild-type pfDHFR allowed the proposition of hypotheses which explain the mechanisms by which the studied mutations act on PYR- and CYC-resistance. S108N and A16V seem to be the key mutations for PYR- and CYC-resistance, respectively; they act on the drug pharmacodynamics, making the drug–enzyme interaction less stable by sterical clashes or repulsive London forces. It has been proposed that CYC may be more susceptible to the loss of planarity of the bonding between Asp-54 and the pteridine ring of the drug than PYR. This hypothesis would also explain the effect of the I164L mutation, when isolated, on PYR- and CYC-resistance, but still needs confirmation. Mutations C50R, N51I, C59R and I164L do not seem to act primarily on drug accommodation in the active site of the enzyme, and should act on the admission of the antifolates to the active site, in a way yet to be determined. These mutations have little or no effect when isolated and, to be effective, should act in synergy with S108N, amplifying PYR- and/or CYC-resistance. Consequently, double, triple and quadruple mutants with the S108N mutation show high levels of resistance to these drugs. New effective antifolates should be able to circumvent the effects of S108N and A16V mutations, in order to reduce the propensity for developing resistance.

The understanding of the mechanisms of mutant resistance to antifolates is far from complete, but we believe that the progress of the modeling work conducted in the several groups involved in this field will allow the design and synthesis of new and more effective antimalarials.

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