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Original article

Site-directed mutagenesis provides insights into the selective binding of trityl derivatives to *Plasmodium falciparum* dUTPase

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ABSTRACT

We have previously identified a series of triphenylmethane derivatives of deoxyuridine with antimalarial activity *in vitro* which selectively inhibit *Plasmodium falciparum* deoxyuridine triphosphate nucleotidohydrolase (PfdUTPase) compared to the human enzyme. The crystal structure of PfdUTPase in complex with one of these inhibitors suggested that the triphenylmethane derivative was selective due to a series of interactions between the trityl group and the side chains of residues Phe^{46}, Ile^{117} and Lys^{96} located in a hydrophobic pocket distinct from the phosphate binding site. Here we show by site-directed mutagenesis that the hydrophobic nature of the trityl binding site and in particular aromatic interactions established between the inhibitor and residue Phe^{46} contribute significantly to the binding of uracil-based derivatives containing trityl groups in the 5′-position. Thus, changing Phe^{46} for alanine resulted in increased K_i values for all compounds tested. Conversely, substitution of the polar residue Lys^{96} for Ala results in smaller K_i values and an increase in selectivity with regard to human dUTPase. This information will aid in the design of inhibitors with improved activity against the *Plasmodium* enzyme.

1. Introduction

Malaria is a major human health problem in large areas of the world, especially in the tropical and subtropical regions. Every year there are 225 million new clinical cases with nearly 800,000 deaths, mainly of children (WHO World Malaria Report, 2010). The disease is transmitted to the human host through the female *Anopheles* mosquito. The parasite rapidly becomes resistant to chemotherapeutic agents and there is urgent need for drugs against new molecular targets.

The enzyme dUTPase (deoxyuridine 5′-triphosphate nucleotidohydrolase, E.C. 3.6.1.23) is involved in nucleotide metabolism; it catalyses the hydrolysis of the α-β-pyrophosphate bond of deoxyuridine triphosphate (dUTP) to yield deoxyuridine monophosphate (dUMP) and inorganic pyrophosphate using magnesium ions as cofactor [1–4]. dUTPase is widespread in nature and it has been found in a variety of prokaryotic and eukaryotic organisms as well as in many viruses, where it is essential for cell viability [5–7]. The enzyme is crucial for DNA integrity in two ways: firstly it prevents dUTP accumulation reducing erroneous incorporation of uracil into DNA and secondly it provides dUMP, the substrate of thymidylate synthase, required for dTTP biosynthesis [5,8]. After misincorporation of dUTP into DNA, uracil is excised and replaced by thymine through a repair process catalysed by uracil–DNA glycosylase. When dUTP levels are high, repetitive cycles of introduction and excision of uracil take place, giving rise to DNA fragmentation and ultimately cell death.

dUTPases are extremely selective for dUTP over other nucleotides such as dTTP, dCTP and UTP [2,9]. The different dUTPases are classified into three families according to the number of subunits. Trimeric dUTPases form the largest and most varied group which includes both the *Plasmodium falciparum* and human enzymes. The X-ray structures of dUTPases from different organisms have been obtained [10–15]. All exhibit three identical active sites formed at the subunit interfaces. In the case of the human and *Escherichia coli* enzymes each site is formed by residues of all three subunits [16,17]. Residues from two subunits are involved in base and sugar recognition while the third subunit has a flexible glycine-rich motif positioned in the C-terminal region, which is incorporated into

Abreviations: PfdUTPase, *Plasmodium falciparum* dUTPase; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; IPTG, isopropil-β-D-thiogalactoside; MES, 2-*N*-morpholine-ethane sulphonic acid.

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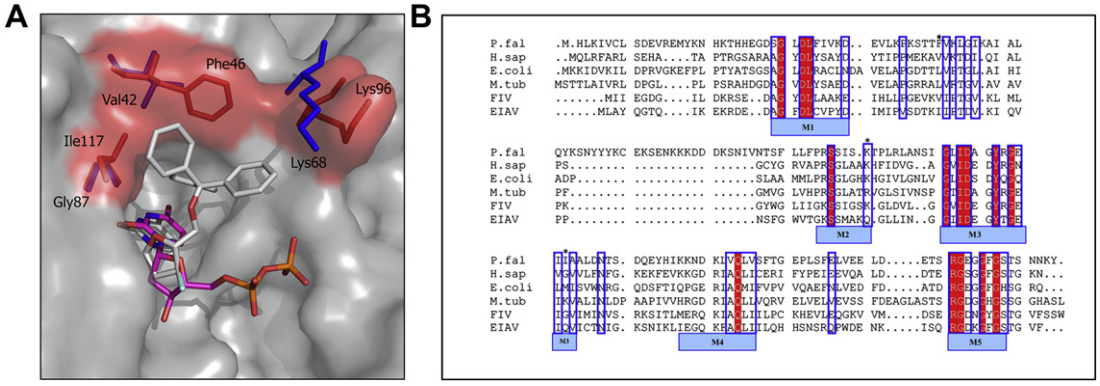

Fig. 1. A. A diagram of the active site of PfdUTPase containing compound **1** (shown in grey). The hydrophobic "trityl" binding pocket is coloured red, with the side chains of residues Phe[46], Lys[96] and Ile[117] shown in red stick representation. The structure of the human dUTPase bound to dUDP (shown in blue) is super-imposed for comparison, with the side chains of the corresponding residues Val[42], Lys[68] and Gly[87] represented as blue sticks. The diphosphate of the dUDP binds in a completely different pocket compared to the trityl group. This figure was prepared using PyMol. B. Amino acid sequence alignment of selected dUTPase proteins: *P. falciparum*, *E. coli*, *M. tuberculosis*, *H. sapiens*, FIV (feline immunodeficiency virus) and EIAV (equine infectious anaemia virus). The alignment was adjusted manually to optimize structure-based alignment. Strictly conserved residues are boxed and coloured red, while largely conserved residues are outlined by blue boxes. The five conserved motifs characteristic of trimeric dUTPases are highlighted. The locations of the residues involved in the interaction with the trityl group in the *P. falciparum* enzyme that were mutated in this study are highlighted by stars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a β-sheet of the neighbouring subunit and is involved in substrate binding during catalysis. In contrast in the *P. falciparum* enzyme, the flexible C-terminal part of the chain undergoes a turn and packs alongside the first β-strand in the core of the molecule [13]. As a result, each folding domain in PfdUTPase is made up of a single polypeptide chain and many of the intersubunit interactions involving the C-terminus are absent, compared to human dUTPase. Finally another unique structural feature is the presence of a short α-helix in the N-terminus of PfdUTPase (residues 8–19) [13]. In human dUTPase this N-terminal domain is formed by distorted β-strands.

Triphenyl derivatives of deoxyuridine are compounds with antimalarial activity *in vitro* that selectively inhibit the deoxyuridine nucleotidohydrolase from the malarial parasite *P. falciparum* but not its human counterpart [18,19]. Structural data obtained from an enzyme–inhibitor complexe of the *Plasmodium* protein have suggested an explanation for this selective inhibition. In the *P. falciparum* dUTPase, specific interactions occur between the triphenylmethane group and the side chains of residues Phe[46] and Ile[117] that are part of a hydrophobic pocket which is distinct from the phosphate binding site [13]. Another conserved residue, Lys[96], also establishes Van der Waals interactions with the aromatic rings of the inhibitor through its side chain methylene groups. In the human dUTPase, the residues Phe[46] and Ile[117] are replaced by Val and Gly respectively. We hypothesise that the less hydrophobic nature of the residues and the absence of the aromatic residue phenylalanine in this pocket in the human enzyme is the basis for selectivity. In this paper, we report experiments to confirm our explanation for the activity and selectivity of the tritylated derivatives against PfdUTPase [13,18]. To this end, we have mutated Phe[46], Ile[117] and Lys[96] to alanine and studied the effect of these substitutions on the activity of *P. falciparum* dUTPase in presence of different inhibitors in order to investigate the quantitative contribution of each residue to inhibitor action.

2. Results and discussion

2.1. Mutations F46A, K96A and I117A do not affect the nucleotide hydrolase activity of PfdUTPase

In previous work, a series of triphenylmethane derivatives were identified as dUTPase inhibitors with antimalarial activity. This group of nucleoside derivatives selectively inhibits dUTPase from *P. falciparum* but has no significant effect on the human enzyme. The analysis of the crystal structure of PfdUTPase in complex with one of these molecules (compound **1**) and comparison of the mode of inhibitor binding with that of dUDP binding to human dUTPase revealed that the uracil base binds similarly in each case, while the deoxyribose ring of the inhibitor is displaced to allow the trityl group to interact with a more hydrophobic part of the substrate binding site. This trityl binding pocket is different to that occupied by the diphosphate moiety of dUDP. Binding of the tritylated deoxyuridine derivatives however may prevent the residues of motif 5 located at the C-terminus closing over the active site. As previously mentioned, structural information suggested that inhibition of the parasite enzyme by these tritylated deoxyuridine derivatives is dependent on a interaction of the trityl group with the side chains of amino acids Phe[46], Lys[96] and Ile[117] (Fig. 1A). Phe[46] and Ile[117] are thought to provide a basis for selectivity for the parasite enzyme compared to the corresponding human enzyme, since they are substituted by the less hydrophobic non-aromatic residues Val[42] and Gly[87] respectively in human dUTPase (Fig. 1B). Only the interaction with Lys[96] is conserved in the human enzyme through Lys[68] (Fig. 1B). In order to further verify the relative contribution of each of these residues to inhibitor binding and selectivity we conducted site-directed mutagenesis experiments and generated mutant enzymes where the indicated three residues were replaced by alanine.

The effect of the mutations on enzyme activity was examined by comparing the nucleotide-hydrolysis activity of wild-type and mutant enzymes using dUTP as substrate (Table 1). In the crystal structure of *Plasmodium* dUTPase, the inhibitor molecule (**1**) has an extensive set of interactions with residues of conserved motifs in the

Table 1
Kinetic parameters of wild-type and mutant *P. falciparum* dUTPase (Pf F46A, PfK96A and Pf I117A).

PfdUTPase	k_{cat}[a] (s^{-1})	K_m[a] (μM)	k_{cat}/K_m (s^{-1} μM^{-1})
Wild-type	11.67 ± 0.01	1.6 ± 0.1	7.3 ± 0.5
F46A	11.33 ± 0.01	1.8 ± 0.1	6.3 ± 0.4
K96A	11.67 ± 0.01	1.9 ± 0.2	6.1 ± 0.6
I117A	12.00 ± 0.01	1.7 ± 0.1	7.1 ± 0.4

[a] The k_{cat} and K_m values represent the average of six independent determinations.

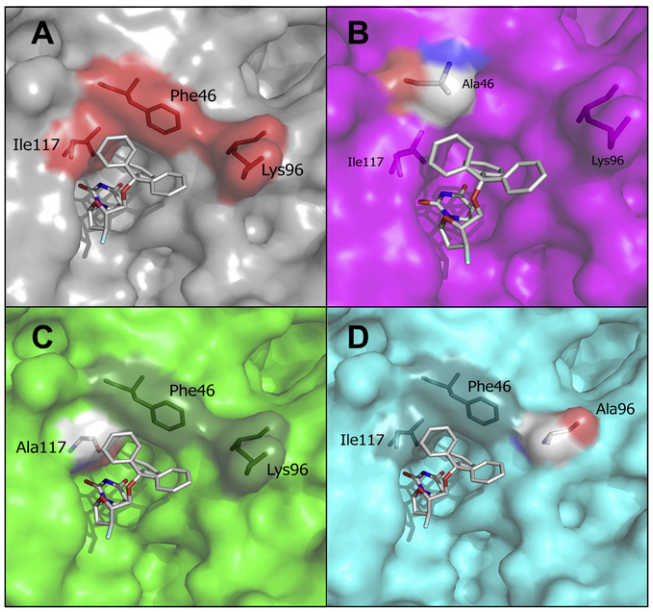

Fig. 2. Schematic representation of the possible interactions of compound **1** with the different mutant proteins. A. PfdUTPase wild-type. B. PfdUTPase-F46A. C. PfdUTPase-I117A. D. PfdUTPase-K96A.

protein. The uracil moiety forms hydrogen bonds to the side chain of residue Asn[103] and the main chain of residue Ile[117]. The triphenyl group makes interactions with various residues (as described above), but it is not thought to interact with residues believed to be involved into binding to the physiological substrate, dUTP. The triphosphate moiety of dUTP is predicted to bind to a negatively charged pocket including residues Asp[26] and Asp[109].

Given that the side chains of the mutated residues should not be involved in nucleotide binding, according to the information available, we did not expect significant changes in the enzyme activity with substrate. Indeed, kinetic parameters for the reaction with mutant dUTPases were similar to those obtained for the wild-type protein ($K_m = 1.6$ μM and $k_{cat} = 11.67$ s^{-1}) indicating that none of the amino acid changes perturbed significantly substrate binding or catalysis (Table 1). Importantly, this also confirms that there is no significant interaction between the hydrophobic pocket formed by residues Phe[46] and Ile[117] and the flexible C-terminal arm that closes over the active site during catalysis. If there had been a significant loss in K_m or k_{cat} on mutating the residues, it would imply that these residues were crucial for the biological activity of the enzyme and that point mutations of these residues would be unlikely to occur in the generation of resistance.

2.2. Structure–activity relationship analysis of inhibition of *P. falciparum* dUTPase by tritylated deoxyuridine derivatives

We have carried out inhibition assays with several deoxyuridine derivatives that were previously shown to selectively inhibit the *P. falciparum* dUTPase enzyme and inhibit parasite growth *in vitro* while exhibiting selectivity against mammalian cells [13,18–20]. All of the inhibitors of the series are uracil derivatives but differ with respect to the presence of the deoxyribose and the nature of the 5′substituents (Fig. 3). We have performed a selection of compounds from different series that vary with regard to the presence or absence of a ribose moiety (cyclic and acyclic compounds) and the type of link between the 5′-position and the trityl group (O and N compounds) (Fig. 3). Here a total of 9 inhibitors were assayed against recombinant *P. falciparum* wild-type and mutant dUTPases as well as recombinant human dUTPase to determine selectivity. K_i values obtained are shown in Table 2 and the selectivity indexes obtained with regard to the human enzyme

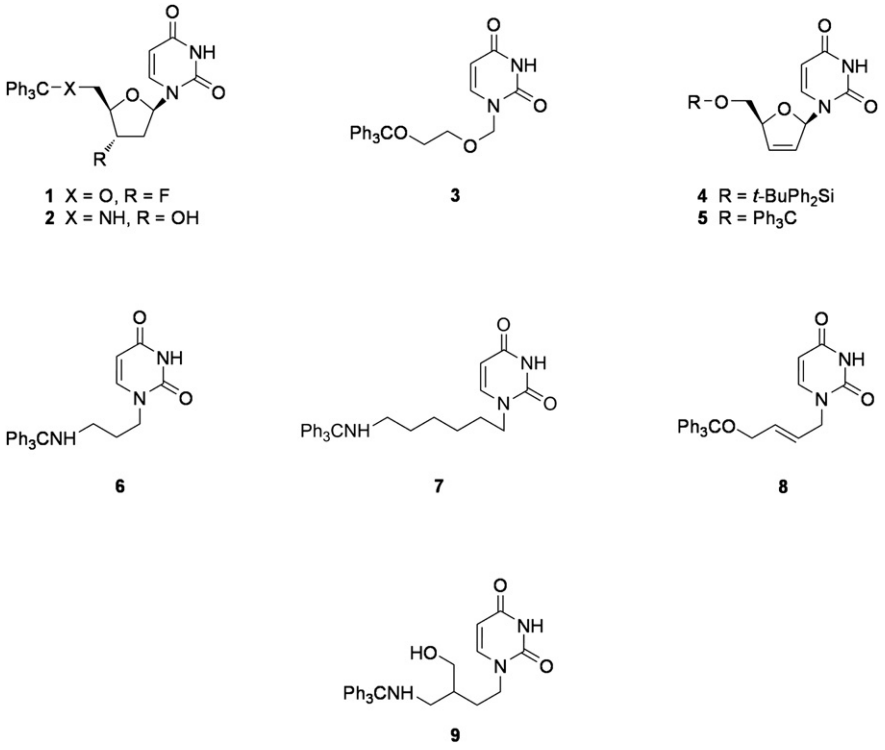

Fig. 3. Compounds used in this study.

Table 2
Inhibition of wild-type and mutant *P. falciparum* dUTPases by a set of deoxyuridine derivatives and IC_{50} values for parasite growth inhibition *in vitro*. Values for inhibition of the human dUTPase are also shown.

Compound	K_i wt (μM)	K_i Pf F46A (μM)	K_i Pf K96A (μM)	K_i Pf I117A (μM)	K_i Human (μM)	*P. falciparum* IC_{50}[a] (μM)
1	5 ± 1	225 ± 41	4.8 ± 0.5	406 ± 204	457 ± 169	2.0
2	0.24 ± 0.08	20 ± 8	0.11 ± 0.03	1.3 ± 0.6	46 ± 6	4.5
3	0.7 ± 0.1	13 ± 6	ND	8 ± 3	17 ± 9	0.9
4	1.2 ± 0.4	17 ± 2	0.21 ± 0.02	0.8 ± 0.2	>1000	3
5	1.9 ± 1.3	20.3 ± 6.7	0.39 ± 0.06	1.6 ± 0.2	157 ± 35	>11
6	0.2 ± 0.2	28 ± 10	0.04 ± 0.02	0.6 ± 0.2	1.4 ± 1.3	4.4
7	1.8 ± 0.3	35 ± 5	0.15 ± 0.08	4.4 ± 2.2	>1000	1.1
8	0.6 ± 0.2	6 ± 4	0.09 ± 0.02	0.8 ± 0.1	>1000	3.7
9	0.2 ± 0.1	29 ± 9	0.04 ± 0.01	0.27 ± 0.03	5.7 ± 0.4	3.2

[a] Data from Refs. [13,18–20].

are in Table 3. Likewise, previously reported IC_{50} values of compounds for inhibition of growth of *P. falciparum in vitro* are also shown in Table 2 for information. The trityl deoxyuridine derivatives used in this study inhibited the wild-type parasite enzyme with K_i values ranging from 0.2 to 5 μM as described previously [13,18–20]. Previous studies on structure–activity relationships suggest that replacement of the 5′-oxygen with a 5′-nitrogen can lead to an improvement in enzyme inhibition [18]. Indeed, compound **2** is 21-fold more potent inhibitor than compound **1** against dUTPase.

In the acyclic derivatives, the ribose has been replaced by a carbon chain. Compounds **6** and **9** are potent inhibitors with K_i values of 0.2 μM but they give poor selectivity compared to the human enzyme. In contrast, compounds **7** and **8** with K_i values of 1.8 and 0.6 μM respectively have higher selectivity indexes (>556 and >1667 respectively) (Table 3). The comparison of the inhibitory properties of compounds **4** and **5** shows that the presence of at least two aromatic rings at the 5′-position are required for enzyme inhibition [18].

2.3. Role in inhibitor binding and selectivity of residues Phe^{46}, Lys^{96} and Ile^{117} of PfdUTPase

A schematic representation of the resulting mutant proteins and their possible interaction with compound **1** is shown in Fig. 2. The structural information indicates that Phe^{46} establishes aromatic interactions with the trityl group of the inhibitor molecule, thus contributing to efficient binding. Indeed, in support of this hypothesis, mutation at Phe^{46} strongly increases K_i values for all the nucleoside derivatives assayed. In the case of compound **1**, which was used to obtain the PfdUTPase-inhibitor crystal structure, the replacement of Phe^{46} by alanine (Fig. 2B) raises the K_i around 45-fold (Table 2), decreasing selectivity with regard to the human enzyme to the same extent (Table 3). These results show that both the inhibitory activity and selectivity of compound **1** are clearly dependent on residue Phe^{46}. Another striking example is the inhibition by compound **2**, whose K_i value is increased 83-fold in the Phe^{46}-Ala mutant (0.24 μM versus 20 μM), or compound **9** where this change produced an increase in K_i value of 145-fold (0.2 μM versus 29 μM).

Table 3
Impact of mutations of the inhibitor binding site on selectivity indexes for a set of deoxyuridine derivatives. Selectivity indexes (SI) are calculated as the ratio of the K_i value for a given inhibitor for *Plasmodium* dUTPase versus human dUTPase.

Compound	SI wt/ human	SI Pf F46A/ Human	SI Pf K96A/ Human	SI Pf I117A/ Human
1	91	2.0	95	1.1
2	192	2.3	418	35
3	24	1.3	ND	2.1
4	>833	>59	>2564	>1250
5	83	8	402	98
6	7	0.04	35	2.4
7	>556	>29	>6667	>227
8	>1667	>167	>11111	>1250
9	28	0.19	142	21

The effect of mutation Phe^{46}-Ala on selectivity varies with the inhibitor employed (Table 3). For instance compound **4** or the acyclics **7** and **8** that all exhibit high K_i values against the human enzyme, still remain selective for the mutant enzyme while in the case of compound **5**, selectivity is reduced 10-fold. However the K_i values obtained for the acyclics **6** ($K_i = 28$ μM), where the ribose is substituted for a carbon chain, and **9** ($K_i = 29$ μM) were significantly higher than those obtained for the human enzyme (1.4 and 5.7 μM respectively). Indeed these two latter compounds were notably active against human dUTPase and exhibited low selectivity (Table 3) thus suggesting that in these two particular cases, contacts in the human enzyme might be established that contribute to binding and inhibitor efficiency. In addition, it is possible that the higher K_i values of the mutant versus the human enzyme can be partially explained by the lower hydrophobicity of the mutant residue alanine compared to the wild-type residue valine present in human dUTPase, leading to a weaker interaction with the trityl moiety.

Substitution of Lys^{96} (Fig. 2D) for alanine did not result in a modification of the K_i value obtained for compound **1**, suggesting that Lys at position 96 is not significantly involved in binding of this derivative. However for all of the other compounds tested and in contrast to the Phe^{46}-Ala mutant, a significant reduction was seen in K_i values (ranging from 2 to 12-fold) for PfdUTPase K^{96}-Ala (Table 2). These data suggest that residue Lys^{96} may be even detrimental to interaction with the inhibitor and that the substitution by a non polar residue increases the inhibition, and presumably binding, of the tritylated uridine derivatives. This is particularly evident in the change of the K_i value from 1.8 to 0.15 μM (12-fold decrease) observed for compound **7**.

Finally, the mutation Ile^{117}-Ala (Fig. 2C) has different consequences depending on the compound tested (Table 2). While the isoleucine residue is required for interaction with compound **1**, it does not seem to be equally relevant for binding of other deoxyuridine derivatives. Thus, mutation Ile^{117}-Ala increases K_i values by about one order of magnitude for compounds **2** and **3** but does not have a significant effect on K_i values obtained for the other compounds. We can speculate that either minor interactions between Ile^{117} and these compounds are established and that Phe^{46} is mostly responsible for efficient ligand binding, or that the substitution by alanine does not impinge sufficiently on the hydrophobicity of the pocket to significantly affect K_i values for these inhibitors.

3. Conclusion

We have performed site-directed mutagenesis of 3 residues potentially involved in the binding of the trityl group of a class of inhibitors that are known to selectively inhibit PfdUTPase. None of these residues appear to have a significant role in the binding or hydrolysis of the physiological substrate, dUTP. Likewise the lack of effect of the mutations on the kinetic parameters for dUTP would further support the previous observation that there is no significant interaction between these residues and the C-terminal domain of the subunit that closes over the active site during catalysis. In the case of replacement of Phe^{46} by Ala, the mutation significantly reduced binding of the tritylated inhibitors (10->100 fold) underscoring the importance of this residue in the interaction of the enzyme with the trityl group especially in the case of compound **1**. This interaction is an aromatic interaction, but not a π-stacking interaction, as none of the phenyl rings of the trityl are parallel to the phenyl ring of Phe^{46}. It is probably driven mainly by hydrophobic interactions. There is also the possibility of edge face interactions between the phenyl groups of the trityl group and the phenyl group of Phe^{46}; however these are off-set and probably are not optimum. In the human dUTPase, Phe^{46} is replaced by Val. Valine is less hydrophobic and smaller than phenylalanine, probably accounting for the weaker interaction of these compounds with the human enzyme and hence the selectivity of the compounds.

Replacement of Lys^{96} with Ala actually led to an increase in inhibitory action of up to 10-fold. This could suggest that the interaction between the side chain of Lys^{96} and the hydrophobic trityl is not optimal for inhibitor binding, or that replacement of Lys^{96} with Ala has a significant effect on the conformation of the "trityl" binding pocket of the enzyme, leading to stronger binding to the inhibitors. Interestingly the consequences of replacement of Ile^{117} with Ala were either without effect or rendered up to a 10-fold drop in K_i values. All these observations suggest subtle differences in the binding mode of the different inhibitors and provide information for the drug design process.

Phe^{46} is the most important residue for interaction with the trityl group and point mutations giving rise to a change in this amino acid may result in proteins with significantly increased K_i values. This issue is of importance when considering resistance mechanisms that could arise in the case of developing treatments based on this class of compounds. While the generation of such resistance mechanisms is yet to be demonstrated either *in vitro* or *in vivo*, the information from this study will aid in the design of compounds with improved activity and also help to devise strategies to overcome potential drug resistance resulting from point mutations in the inhibitor binding site.

4. Experimental

4.1. Materials

dUTP was purchased from GE Healthcare. All other chemicals were purchased from Merck and Sigma and were of analytical grade of the highest purity.

4.2. Cloning and expression of mutant PfdUTPases

The *Pf*dUTPase F46A mutant gene was constructed by using the QuickChange mutagenesis kit (Stratagene) with primers 5′-ctg aag cca aag tcc aca act gca gtt aag ctt gga att aag gc-3′ and 5′-gcc tta att cca agc tta act gca gtt gtg gac ttt ggc ttc ag-3′ and the ORF for *Plasmodium* dUTPase cloned in pET11c as template [13]. For the convenience of subsequent identification, these primers contain a silent change that results in a novel *Pst*I restriction enzyme site (underlined). The PfdUTPase K96A mutant was made using the same procedure with primers 5′-cct cgt agc agt ata tcc gca acc cca tta cgc tta gc-3′ and 5′-gct aag cgt aat ggg gtt gcg gat ata ctg cta cga gg-3′, while the PfdUTPase I117A mutant was constructed with primers 5′-gca ggt tat aga gga gaa att gcg gcc gcc ttg gat aat act ag-3′ and 5′-cta gta tta tcc aag gcg gcc gca att tct cct cta taa cct gc-3′. These primers also contain a silent change that results in a novel *Not*I restriction enzyme site (underlined). All mutant sequences were cloned in pET11c for expression of the different proteins.

4.3. Enzyme purification

Recombinant *wild-type* and mutant *P. falciparum* dUTPases were expressed in *E. coli* BL21 (DE3) cells which had been transformed with the different pET11c *Pfdut* expression vectors. Cell pellets from a two litter IPTG-induced (1 mM) culture were harvested by centrifugation at 4800 rpm, 10 min. The cells were lysed by sonication in 20 mM MES (pH 5.5), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT (buffer A) and the cell extract was cleared by centrifugation at 15,000 rpm for 45 min. The supernatant was loaded onto a 50 ml phosphocellulose (Whatman P-11) column at 4 °C and eluted with a 0.05–1 M NaCl gradient in buffer A. The protein was concentrated with the microsep 10K omega system (Pall Life Biosciences Corporation) and stored in 10 mM Bicine (pH 8.0), 10 mM $MgCl_2$, and 0.75 mM DTT at −80 °C until use. The identity and integrity of the mutant proteins were further verified by molecular mass determination using MALDI-TOF.

4.4. Enzyme inhibition assays

Nucleotide hydrolysis was monitored by mixing enzyme and substrate with an SFA-20 Rapid Kinetic Accessory (Hi-Tech Scientific) attached to a UV–visible spectrophotometer (Cary 50 Bio) and connected to a computer for data acquisition and storage as described [7]. Protons, released through the hydrolysis of nucleotides, were neutralized by a pH indicator in weak buffered medium with similar pK_a and monitored spectrophotometrically at the absorbance peak of the basic form of the indicator. The ratio between the indicator and the buffer concentration was 1:40 and the absorbance changes were kept within 0.1 units. The indicator/buffer pair used was Cresol red/Bicine (pH 7.5–8.5, 573 nm). Reaction mixtures contained 30 nM of *P. falciparum* dUTPase, 50 μM dUTP, 1.25 mg/ml BSA, 100 mM KCl, 50 μM Cresol red, 2 mM Bicine and 5 mM $MgCl_2$. All measurements were performed at 25 °C.

V_{max} and K_{mapp} were calculated by fitting the resulting data to the integrated Michaelis–Menten equation. The apparent K_m values were plotted against inhibitor concentration, and K_i values were obtained according to Equation (1):

$$K_{mapp} = \frac{K_m}{K_i}[I] + K_m \quad (1)$$

The inhibition of the reaction catalysed by PfdUTPase was determined adding increasing inhibitor concentrations in the assay mixture. All the inhibition assays were performed at 25 °C and pH 8. For each inhibitor concentration, the apparent K_m (K_{mapp}) value was obtained from the integrated Michaelis–Menten equation. As K_{mapp} values are a linear function of the inhibitor concentration [I] in competitive systems, a replot of K_{mapp} versus [I] has intercepts of K_m on the K_{mapp} axis, and the K_i value for the inhibitor can be obtained from the slope value of the linear plot.

Acknowledgements

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