DISTINCT DIMER INTERFACE OF PLASMODIUM FALCIPARUM THYMIDYLATE SYNTHASE: IMPLICATION FOR SPECIES-SPECIFIC ANTIMALARIAL DRUG DESIGN

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Abstract. Thymidylate synthase (TS), a homodimer with two active sites near the dimer interface, plays a central role in DNA biosynthesis. Each active site is located on each subunit but shares a number of amino acid residues for catalytic activity. As TS is highly invariant across species, this has raised problems in designing inhibitors selective against Plasmodium falciparum (Pf) TS, but not against the human (h) counterpart. However, there exists differences in amino acids at the TS interface of P. falciparum and human enzyme, which are critical for dimerization in each species. Here, we employed in vivo genetic complementation and 6-³H-FdUMP binding assays of transformants from TS-deficient Escherichia coli with a variety of pairs of inactive PfTS(R470) and inactive hTS(C195) mutants, and vice versa, to demonstrate none of the combinations formed active cross-species TS heterodimers. Visualization by structural superposition of TS from the two species revealed incompatible interface amino acids stemming from residues of different porality. Key residues at hTS dimer interface (Q62, Q211 and T251) and their equivalence in PfTS (I357, I506 and V546) could not be interchanged to generate active TS cross-species heterodimers using the co-transformation complementation assay. These results demonstrate that the TS interface of P. falciparum is unique and completely different from that of the human enzyme, suggesting that this domain provides a target for development of novel antimalarials.

Keywords: Plasmodium falciparum, antimalarial, malaria, subunit complementation, thymidylate synthase

INTRODUCTION

Malaria is one of neglected tropical diseases which remains a serious threat to human health worldwide, especially in Africa. Approximately 218 million malaria patients with 438,000 deaths were reported in 2015 (WHO, 2015). Five species of Plasmodium, namely P. falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi have been recognized to infect humans. P. falciparum is the most prevalent human malaria parasite and causes high
Distinct Dimer Interface of PfTS

mortality (Vangapandu et al, 2007; WHO, 2015). Furthermore, less than effective prophylaxis by vaccines and emerging drug-resistant strains of P. falciparum indicate the urgent need for new malarial drugs (Fairhurst, 2015; Hoffman et al, 2015; Takala-Harrison and Laufer, 2015; Wells et al, 2015). Identifying an essential region required for functional role(s) of Plasmodium crucial enzymes as targets for novel antimalarials is one strategy in drug treatment.

Thymidylate synthase (TS) is a key enzyme in folate metabolism found in all organisms including malaria parasite. This enzyme catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTTP), an essential nucleotide in DNA synthesis (Carreras and Santi, 1995). Unlike humans, TS of malaria parasite is part of a bifunctional enzyme with dihydrofolate reductase (DHFR) and TS domains located at amino and carboxyl termini, respectively (Yuvaniyama et al, 2003). In addition, P. falciparum cannot salvage thymidine and is completely dependent on active TS for dTMP biosynthesis (Sherman, 1979; Reyes et al, 1982). As a consequence, plasmodial TS has been an attractive drug target for malaria chemotherapy (Yuthavong et al, 2006; Müller and Hyde, 2013; Salcedo-Sora and Ward, 2013).

Based on amino acid sequence and crystal structures of TS, a P. falciparum (Pf) TS (286 amino acids) is similar to TS of most organisms including human (h) TS (313 amino acids) in that functional TS is a dimer of identical subunits and its two active sites located adjacent to the dimer interface are related by C2 symmetry (Schiffer et al, 1995; Yuvaniyama et al, 2003). The dimeric structure is required for enzyme activity since each TS active site contains amino acid residues contributed by its neighboring subunit. Among these, conserved R and C residues, ie, R470, R471 and C490 of PfTS (equivalent to R175, R176 and C195 of hTS) are involved in TS catalytic function (Schiffer et al, 1995; Yuvaniyama et al, 2003). The positively charged side chains of these conserved R from one subunit extend into the TS active site of the other subunit and form hydrogen bonds with the phosphate oxygen of dUMP substrate-sugar ring C-6. The conserved C residue acts as a nucleophile attacking dUMP C-6. Global alignment of amino acid sequences of PfTS and hTS domains showed high sequence similarity (Yuvaniyama et al, 2003). However, there are several distinct amino acid residues located around the PfTS dimer interface compared to those of hTS. Hence, this opens an opportunity to explore the possibility of exploiting these specific amino acids in the PfTS dimer interface as a drug target for antimalarial discovery and development.

In Lactobacillus casei, P. falciparum and P. vivax, functional active TS can be formed by dimerization of two inactive intra-species or two inactive cross-species subunits (Pookanjanatavip et al, 1992, Chanama et al, 2005; idem, 2011). An active intra-species heterodimer with a PfTS single active site is formed by combining subunits of TS-inactive R470 and C490 mutant enzymes. Likewise, the subunit of PfTS-inactive R470 (or PvTS-inactive R486) enzyme can combine with that of PvTS-inactive C506 (or PfTS-inactive C490) to form an active cross-species heterodimer with a competent active site.

Thus, this study investigated whether subunit interface of TS enzymes derived from P. falciparum and human enzymes are significantly distinct. The insights should provide an opportunity to disclose new targets for antimalarials designed
selectively against \( PfTS \) dimer interface to block its dimerization and thereby its catalytic activity.

**MATERIALS AND METHODS**

**Plasmids**

Plasmids used in this study were divided into 4 groups: pJU-DHFR-TS (5.1 kb) and pMC-DHFR-TS (4.9 kb) for expressing \( PfDHFR-TS \) [wild-type (WT) or R470 or C490], but containing different origin of replication (ColE1 vs p15A) and antibiotic resistant gene (ampicillin vs chloramphenicol) (Chanama et al, 2005); pRSET C and pACYC184 expressing hTS(WT or R175 or C195), the former plasmid group carrying ColE1 and ampicillin resistance marker and the latter p15A and chloramphenicol resistant gene.

Plasmid pRSETC-hTS (3.8 kb) was constructed by PCR amplification of hTS gene in pGCHTS (Davisson et al, 1989), in which nucleotides G6 and A9 were altered to T without altering the codons for P2 and V3 (Pedersen-Lane et al, 1997). The amplified gene was cloned into XhoI/HindIII sites of pRSET C plasmid. Plasmid pACMC-hTS (4.2 kb) was prepared using pRSETC-hTS as template, and two primers (ON1 and ON2) (Chanama et al, 2005). The reaction mixture and amplification condition, except for the employment of 25 cycles and extension step at 72°C for 2 minutes, were performed as described previously (Chanama et al, 2005). The amplicon (1.3 kb) was ligated into pACYC184 vector at BclI and SalI sites.

**Site-directed mutagenesis of TS at interface residues**

Interface residues of \( PfTS \) and hTS were mutared using QuickChange™ site-directed mutagenesis method (Stratagene, La Jolla, CA) with required DNA template and the set of primers listed in Table 1. pACMC-hTS was used as the template for construction of hTS(R175) mutants with primer sets I and II for R175A/D/N/T and R175F/I/N/S/T/Y, respectively, and pRSETC-hTS for hTS(C195) mutants with primer set III for C195A/D/N/T. Amplification (in Biorad T100™ Thermal Cycler, Hercules, CA) was performed at 94°C for 2 minutes followed by 25 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 14 minutes.

Single and multiple mutations of hTS at residues Q62, Q211 and T251 corresponding to \( PfDHFR-TS \) residues I357, I506 and V546, respectively, or vice versa were carried out individually using hTS(R175A) or \( PfDHFR-TS \) (R470A) as template. The primer sets IV, V and VI were used for the preparation of hTS(R175A/Q62I, R175A/Q211I and R175A/T251V), respectively. The hTS(R175A/Q62I/T251V) mutant was prepared from hTS(R175A/Q62I) with the primer set VI, and hTS(R175A/Q62I/Q211I) and hTS(R175A/Q211I/T251V) mutants from hTS(R175A/Q211I) with primer sets IV and VI, respectively. hTS(R175A/Q62I/Q211I/T251V) mutant was prepared from hTS(R175A/Q62I/Q211I) with the primer set VI. Primer sets VII, VIII and IX were used to prepare \( PfTS \) (R470A/I357Q), \( PfTS \) (R470A/I506Q) and \( PfTS \) (R470A/V546T), respectively. \( PfTS \) (R470A/I506Q/V546T) mutant was prepared from \( PfTS \) (R470A/I506Q) with the primer set IX, and \( PfTS \) (R470A/I357Q/I506Q) and \( PfTS \) (R470A/I357Q/V546T) mutants from \( PfTS \) (R470A/I357Q) with primer sets VIII and IX, respectively, and \( PfTS \) (R470A/I357Q/I506Q/V546T) was prepared from \( PfTS \) (R470A/I357Q/V546T) with the primer set VIII. Amplification conditions used were 94°C for 5 minutes, followed by 25 cycles of 94°C for 1 minute.
Table 1

Primer sequences used for the construction of human (h) and *Plasmodium falciparum* (Pf) thymidylate synthase (TS) mutants at interface residues.

<table>
<thead>
<tr>
<th>Set number</th>
<th>Mutant</th>
<th>Sequence (5’-3’)</th>
<th>Inserted restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>hTS</td>
<td>F: CCCTGACGAC(AG)(AC)CAGAATCATATCAGTGCCTTGGAAATCCGGGAGATGGCTCCTC</td>
<td>SacII</td>
</tr>
<tr>
<td></td>
<td>(R175A/D/N/T)</td>
<td>R: GAGGAAGATCCCCGGATCAGGACATGATGATTCT(CT)GTCTCGAAGG</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>hTS</td>
<td>F: CCCTGACGAC(AC)CAGAATCATATCAGTGCCTTGGAAATCCGGGAGATGGCTCCTC</td>
<td>SacII</td>
</tr>
<tr>
<td></td>
<td>(R175F/I/N/S/T/Y)</td>
<td>R: GAGGAAGATCCCCGGATCAGGACATGATGATTCT(AGT)(AT)GTCTCGAAGG</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>hTS</td>
<td>F: GCGCTGCCTCCA(AG)(AC)CAGAATCATATCAGTGCCTTGGAAATCCGGGAGATGGCTCCTC</td>
<td>SmaBI</td>
</tr>
<tr>
<td></td>
<td>(C195A/D/N/T)</td>
<td>R: ACTGTTTACTAGTGAATCGAGGCGATAGGAGTTGCGAAGGC</td>
<td>AfII</td>
</tr>
<tr>
<td>IV</td>
<td>hTS(Q62I)</td>
<td>F: GTATTCCGACAGTGGCCGCTACAGCTTAAGAGATGAATTC</td>
<td>PvuI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GAATATCCTCTTAAGCTCGGCTACAGCTGGAATAC</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>hTS(Q211I)</td>
<td>F: GAGCTGCTGCATGGCTTTTATACAGGAGTTGCGAAGGC</td>
<td>Sma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTCCGCCGATCCGCTGAGAATCGAGGAGCTTACAGGAGTTGCGAAGGC</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>hTS(T251V)</td>
<td>F: GGCCTGAGGGCGGCTTTTATACAGGAGTTGCGAAGGC</td>
<td>Sma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTGACGCTTTGCSCAAACGTGTAAGAGCTCAGGCGCTTACAGGAGTTGCGAAGGC</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>PfDHFR-TS (I357Q)</td>
<td>F: GAGATGCTGTTATCTGAATATCCGGATCAATGAAATTTG</td>
<td>AfII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CAAATTTCAATTATGATAATCCGGATCAATGGGAT</td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td>PfDHFR-TS (I506Q)</td>
<td>F: GAAATATCATGCAATGATCAATGATGCTGATTTAG</td>
<td>PvuI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTAAATCACAGGAGTTGATATATTGACATGATAATTTTCAATGATGCTGATTTAG</td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>PfDHFR-TS (V546T)</td>
<td>F: CAGTTCCATACACACCCTAGGAATATCAGACATG</td>
<td>AvrII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CATGTGCATTTCTAGGGTGATGAACTG</td>
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</tr>
</tbody>
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Mismatched bases are in bold for the desired amino acid, and insertion of the desired restriction site. F, forward sequence; R, reverse.
50°C for 1 minute and 68°C for 10 minutes.

**Genetic complementation assay**

*E. coli* χ2913RecA(ΔthyA572, recA56) (DE3) cells were co-transformed with two separate compatible plasmids (CoIE1 or p15A) of TS-inactive mutants to test whether they could form catalytically active heterodimers using genetic complementation assay as previously described (Chanama *et al.*, 2005). In brief, all co-transformants were plated on Luria-Bertani (LB) containing 100 μg/ml ampicillin, 34 μg/ml chloramphenicol and 50 μg/ml thymidine. Ampicillin/chloramphenicol-resistant colonies were streaked on duplicate minimal agar (MM) plates containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol in the presence and absence of 50 μg/ml thymidine. Transformants producing catalytically active TS were identified by the capability of *E. coli* χ2913RecA(DE3) cells harboring both compatible plasmids to grow on a minimal agar without thymidine.

**TS activity assay**

Individual transformant or co-transformant was expressed in *E. coli* χ2913 RecA (DE3) using superbroth media (2% bactotryptone, 0.2% Na₂HPO₄, 0.1% KH₂PO₄, 0.8% NaCl and 1.5% yeast extract) (Pedersen-Lane *et al.*, 1997) with appropriate selection antibiotics depending on the transfection plasmids. Thymidine was supplemented for TS-inactive mutants. In short, a single colony of *E. coli* cell was grown in liquid medium with shaking at 37°C overnight. Using 1% inoculum from an overnight culture, each *E. coli* culture was cultured to mid-log phase (A₆₀₀nm ~ 0.8) at 37°C, and then induced with 1 mM IPTG for 4 hours at 37°C. Cell pellets were collected by centrifugation at 10,500g for 10 minutes at 4°C, resuspended in cold lysis buffer [0.1 mM EDTA, 10 mM dithiothreitol, 50 mM KCl, 20 mM potassium phosphate buffer pH 7.2, and 20% (v/v) glycerol], and then sonicated. Following centrifugation at 12,000g for 1 hour at 4°C, supernatant was used for TS activity assay with 6-[³H]-FdUMP labeling as described previously (Chanama *et al.*, 2005). In short, reaction mixture comprising the crude enzyme (∼ 50 μg), 5 μl of 3 mM 6R-CH₂H₄folate, 0.5 μl of 0.66 μM 6-[³H]-FdUMP (15 Ci/mmol; Moravek Biochemicals, Brea, CA), and 30 μl of 50 mM TES buffer pH 7.4 (25 mM MgCl₂, 1 mM EDTA, 6.5 mM formaldehyde and 75 mM β-mercaptoethanol) was incubated at 25°C for 30 minutes. The 6-[³H]-FdUMP-protein complex was analyzed by 12% SDS-PAGE. Gels were stained with Coomassie-blue dye, soaked in an autoradiography enhancer (NEN™; Dupont, Boston, MA) for 60 minutes at room temperature, dried at 80°C, and exposed to X-ray film (Hyperfilm™ MP; Amersham Pharmacia Biotech, Buckinghamshire, CA) at -80°C for 3-5 days before films were developed.

**Superimposition of residues at TS subunit interface**

The TS dimer interface between *P. falciparum* and human enzyme was compared via superimposition of hTS (pdb code 1hvy) and *Pf*TS (pdb code 1j3k) using LSQMAN program (Kleywegt, 1996).

**RESULTS**

**Subunit complementation of *Pf* and hTS subunits**

In order to demonstrate whether monomeric TS from *P. falciparum* and humans can be complemented by each other to form active heterodimeric TS, we constructed *Pf* and hTS by mutating the essential conserved R and C residues, namely, R470 and C490 of *Pf*DHFR-TS,
Distinct Dimer Interface of PfTS

Table 2

Bacteria genetic complementation of intra-species and cross-species co-transformants of hTS and PfDHFR-TS.

<table>
<thead>
<tr>
<th>Plasmid construct</th>
<th>Origin of replication</th>
<th>Genetic complementation</th>
</tr>
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<tbody>
<tr>
<td><strong>(A) Intra-species transformants</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| pJU-PfDHFR-TS(WT) | ColE1 | +
| pMC-PfDHFR-TS(WT) | p15A | +
| pMC-PfDHFR-TS(R470X) | p15A | -
| pJU-PfDHFR-TS(C490Z) | ColE1 | -
| pRSETC-hTS(WT) | ColE1 | +
| pACMC-hTS(WT) | p15A | +
| pACMC-hTS(R175B) | p15A | -
| pRSETC-hTS(C195U) | ColE1 | -
| **(B) Co-transformants of compatible plasmids** | | |
| PfTS(R470X)+PfTS(C490Z) | p15A/ColE1 | +
| hTS(R175B)+hTS(C195U) | p15A/ColE1 | +
| PfTS(R470X)+hTS(C195U) | p15A/ColE1 | -
| hTS(R175B)+PfTS(C490Z) | p15A/ColE1 | -

Chanama et al., 2005.

B: A, D, F, I or Y; U: A, D, N or Y; X: A, D or N; Z: A, D, F, H, L, N, T or Y.

and R175 and C195 of hTS. Two distinct compatible plasmids were used to achieve stable co-transformants, pET-17b or pRSET C for C mutants, and pACYC184 for R mutants. Mutants hTS (R175B) (where B = A, D, F, I and Y), and mutants hTS(C195U) (where U = A, D, N and Y) were constructed, and likewise mutants PfTS(R470X) (where X = A, D and N) and mutants PfTS(C490Z) (where Z = A, D, F, H, L, N, T and Y). To validate the inactivity of individual hTS(R175B) and hTS(C195U) mutants, their TS activities were determined and compared to wild-type hTS using genetic complementation of TS auxotroph E. coli χ2913RecA(DE3) in minimal agar. Only wild-type hTS could express sufficient TS activity to support the growth of TS-deficient E. coli cells. No hTS(R175B) or hTS(C195U) mutants could support the growth (Table 2).

As expected, subunit complementation of hTS(R175B) and hTS(C195U) mutants resulted in active intra-species TS heterodimers (Table 2). However, co-transformation of PfTS(R470X) and hTS(C195U) or hTS(R175B) and PfTS(C490Z) in Thy- phenotype χ2913RecA(DE3) could not support cell growth (Table 2). This indicated that TS subunits from human and P. falciparum could not dimerize to form active cross species TS heterodimers.

In order to verify that the in vivo subunit complementation relied on TS activity, cell lysates from cultures of each human single transformant were assayed for TS activity using the 6-[3H]-FdUMP assay, in which TS covalently binds to FdUMP in the presence of CH2H4 folate cofactor (Santi and McHenry, 1972). No hTS(R175B) or hTS(C195U) mutants could form covalent 6-[3H]-FdUMP-
Fig 1—*In vitro* 6-[³H]-FdUMP thymidylate synthase (TS) activity of crude extracts from homodimers of human inactive TS(R175) and TS(C195) mutants, and human TS active heterodimers in *E. coli* χ2913RecA(DE3). In brief, 6-[³H]-FdUMP-protein complexes were formed by incubating crude extracts with 6-[³H]-FdUMP and CH₂H₄folate at 25°C for 30 minutes in assay buffer, and then analyzed by 12% SDS-PAGE. Gels were stained with Coomassie-blue dye followed by autoradiography. (A) Coomassie-blue stained polyacrylamide gel. (B) Autoradiograph of A. Panel I, crude extracts of pACMC-hTS(R175) series. Panel II, crude extracts of pRSETC-hTS(C195) series. Panel III, crude extracts of human TS heterodimers.
protein complexes compared to the hTS wild-type, which showed the presence of 6-[^3]H]-FdUMP-protein of 36 kDa (Fig 1, Panels I and II). However, human intra-species co-transformants manifested the 6-[^3]H]-FdUMP-protein complex (Fig 1, Panel III).

On the other hand, the co-transformants between PfDHFR-TS and hTS pairs [PfTS(X470) + hTS(U195)] and [hTS(B175) + PfTS(Z490) mutants] including [PfTS(A/N470) + hTS(A195), PfTS(A470) + hTS(D/N/T195) and PfTS(N470) + hTS(N/Y195)], or vice versa [hTS(A175)
Fig 3—Amino acid alignment of human, *Lactobacillus casei* and *Plasmodium falciparum* thymidylate synthase (TS). The four β-strands involved in TS dimerization are underlined by a dash line, with three residues having different electrostatic potential (in dash box) among *P. falciparum*, human and *L. casei*. The active C residue is underlined with a star. R residue involved in dUMP binding is marked by a star for one TS subunit hTS(R50) and *Pf*TS(R345), and by a star in a dash box for the other TS subunit [hTS(R´175), hTS(R´176), *Pf*TS(R´470) and *Pf*TS(R´471)].

+ *Pf*TS(D490), hTS(D175) + *Pf*TS(N490), hTS(F175) + *Pf*TS(A/T490) and hTS(Y175) + *Pf*TS(T490)] showed no radiolabeled band at 72 or 36 kDa (Fig 2, Panels I and II). These results agree well with the in vivo subunit complementation assay.

**Structural comparison of Pf- and hTS dimer interface**

That TS amino acid sequence is conserved (53% identity) between humans and *P. falciparum*, however, in vivo genetic complementation and 6-[³H]-FdUMP activity assay revealed incompatibility of TS heterodimers formed from humans and *P. falciparum* to reconstitute an active enzyme. This suggests that the dimer interface between two kinds of TS could be significantly different so that dimerization of the TS subunits does not occur. The TS dimer interface is composed of
Distinct Dimer Interface of PfTS

Four β-strands forming two anti-parallel β-sheets and is related by a 180° rotation (Schiffer et al, 1995; Yuvaniyama et al, 2003). From sequence alignment and structural superposition of hTS (pdb code 1hvy) and PfTS (pdb code 1j3k), only three residues at the middle of three β-strands, side chains of which are involved in dimer association, are significantly different in electronic charges (Fig 3 and Fig 4). Of these interface residues, hTS(Q62), hTS(Q211) and hTS(T251), polar residues are different from the corresponding PfTS(I357), PfTS(I506) and PfTS(V546), non-polar residues. This would necessarily lead to non-complementary association of h- and PfTS in forming heterodimers.

With this in mind, seven hTS(A175)-based interface mutants were constructed (A175/I62, A175/I211, A175/V251, A175/I62/I211, A175/I62/V251, A175/I211/V251, and A175/I62/I211/V251). Likewise, seven PfTS(A470)-based interface mutants of PfTS were constructed (A470/Q357, A470/Q506, A470/T546, A470/Q357/Q506, A470/Q357/T546, A470/Q506/T546, and A470/Q357/Q506/T546).

These hTS(A175)-based interface mutants were co-transformed with either inactive hTS(A195) or PfTS(A490) for...
Fig 5—In vivo genetic complementation of human thymidylate synthase (hTS(A175))-based interface mutant combined with single, double or triple mutant of hTS(I62), hTS(I211) and hTS(V251) grown on a minimum medium containing ampicillin, chloramphenicol and thymidine (MM+Ap+Cm+Thy) or ampicillin and chloramphenicol (MM+Ap+Cm). (A) E. coli \( \chi \)2913RecA(DE3) cells co-transformed with hTS(A175)-based interface mutants and Plasmodium falciparum (Pf) TS(A490). Slot 1, positive control \([PfTS(D470) + PfTS(A490)]\); slot 2, hTS(A175/I62) + PfTS(A490); slot 3, hTS(A175/I62/I211) + PfTS(A490); slot 4, hTS(A175/V251) + PfTS(A490); slot 5, hTS(A175/I62/I211/V251) + PfTS(A490); slot 6, hTS(A175/I62/V251) + PfTS(A490); slot 7, hTS(A175/I211/V251) + PfTS(A490); slot 8, hTS(A175/I211) + PfTS(A490); slot 9, hTS(A175) + PfTS(A490). (B) E. coli \( \chi \)2913RecA(DE3) cell co-transformed with hTS(A175)-based interface mutants and hTS(A195). Slot 1, positive control \([hTS(D470) + hTS(A490)]\); slot 2, hTS(A175/I62) + hTS(A195); slot 3, hTS(A175/I62/I211) + hTS(A195); slot 4, hTS(A175/V251) + hTS(A195); slot 5, hTS(A175/I62/I211/V251) + hTS(A195); slot 6, hTS(A175/I62/V251) + hTS(A195); slot 7, hTS(A175/I211/V251) + hTS(A195); slot 8, hTS(A175/I211) + hTS(A195); slot 9, positive control [hTS(A175) + hTS(A195)].

In vivo genetic complementation assay. None of the co-transformants showed ability to complement TS-deficient E. coli \( \chi \)2913RecA(DE3) growth (Fig 5). Similarly, co-transformants of the PfTS(A470)-based interface mutants and either inactive hTS(A195) or PfTS(A490) were unsuccessful in complementing the growth of TS-deficient E. coli (Fig 6).

DISCUSSION

In order to investigate the interaction of the interface between P. falciparum
Fig 6—In vivo genetic complementation of P. falciparum (Pf) TS(A470)-based interface mutant combined with single, double or triple mutant of PfTS(Q357), PfTS(Q506) and PfTS(T546) grown on a minimum medium containing ampicillin, chloramphenicol and thymidine (MM+Ap+Cm+Thy) or ampicillin and chloramphenicol (MM+Ap+Cm). (A) E. coli χ2913RecA(DE3) cell co-transformed with PfTS(A470)-based interface mutants and human (h) TS(A195). Slot 1, positive control [hTS(A175) + hTS(A195)]; slot 2, PfTS(A470/Q357) + hTS(A195); slot 3, PfTS(A470/Q506) + hTS(A195); slot 4, PfTS(A470/T546) + hTS(A195); slot 5, PfTS(A470/Q357/Q506) + hTS(A195); slot 6, PfTS(A470/Q357/Q506) + hTS(A195); slot 7, PfTS(A470/Q506/T546) + hTS(A195); slot 8, PfTS(A470/Q357/Q506/T546) + hTS(A195). (B) E. coli χ2913RecA(DE3) cells co-transformed with PfTS(A470)-based interface mutants and PfTS(A490). Slot 1, positive control [PfTS(A470) + PfTS(A490)]; slot 2, PfTS(A470/Q357) + PfTS(A490); slot 3, PfTS(A470/Q506) + PfTS(A490); slot 4, PfTS(A470/T546) + PfTS(A490); slot 5, PfTS(A470/Q357/Q506) + PfTS(A490); slot 6, PfTS(A470/Q357/T546) + PfTS(A490); slot 7, PfTS(A470/Q506/T546) + PfTS(A490); slot 8, PfTS(A470/Q357/Q506/T546) + PfTS(A490).

and human TS monomers, inactive pairs of enzymes were selected such that one member of the pair had a mutation at PfTS(R470) [or hTS(R175)] while the other had a mutation at hTS(C195) [or PfTS(C490)] in the active site region. In addition, the key residues at dimer interface of hTS, ie, Q62, Q211 and T251, and the
corresponding residues of PfTS, ie, I357, I506 and V546, were also interchanged to observe their effects on the formation of intra- and inter-species heterodimers, to evaluate the possible uniqueness of TS dimer interface between those of P. falciparum and humans. It is worth noting that bifunctional P/DHFR-TS was used in the study because PfTS is only active when it is part of the bifunctional polypeptide (Shallom et al, 1999).

Mutant TS subunits from humans and P. falciparum could not dimerize to form active inter-species TS heterodimers, using both complementation of TS-inactive E. coli and direct assay of TS activity, in spite that the mutant constructed covered a variety of amino acids different in size, charge and hydrophobicity.

Orientating the key R residue in the obligate monomer into a proper geometry is essential for TS function and activity. Once the dimer cannot be formed, the two R residues [PfTS(R470) and PfTS(R471), equivalent to hTS(R175) and hTS(R176)] from the obligate monomer contributing to the binding of dUMP would be absent leading to absence of dUMP binding. We therefore investigated the structure of human and PfTS focusing on dimer interface to understand why TS of human and P. falciparum were unable to dimerize to form an active heterodimer. Inspection of the key amino acids involved in the TS subunit interface interaction revealed that in hTS these residues are polar, whereas those of PfTS are non-polar. Co-tranformants of hTS(A175) in which the three key interface residues had been mutated to those of PfTS with either inactive hTS(A195) or PfTS(A490) were ineffective in complementing TS-inactive E. coli; similar results were obtained with the converse experiments.

Taken together, these results indicate that the three interface residues are crucial for stable dimerization of TS based on the evidence that the interface mutants of both humans and P. falciparum, in which the interface residues were altered to those of their corresponding amino acids, could not restore TS activity in the co-transformants of cross-species TS. Moreover, of these unique interface residues, only a single mutation could totally disrupt TS activity of the same species as observed in both human and P. falciparum enzymes. Therefore, this strongly indicates that the pattern of residues at the TS dimer interface is very unique and indispensable among species. Dimerization of TS leading to an active enzyme requires tight packing of the two TS interfaces for proper geometry of the conserved R residue, eg PfTS(R470), to fulfill its role in TS catalysis. Based on the structure of PfTS (Yuvaniyama et al, 2003), R470 resides on the 7-residue long loop (residues 465-472) of the exposed α-helix and a short β-strand of 2 residues (residues 473-475). Therefore, a subtle disruption by a single mutation at the dimer interface is sufficient to alter the geometry of the conserved PfTS(R470). Hence, TS inhibitors attacking the TS dimer interface will be good candidates for novel antimalariais.

In conclusion, our findings clearly have shown that dimer interface of hTS is distinct from that of Pf enzyme. We have used in vivo genetic complementation, 6-[3H]-FdUMP activity assay and structural analysis of TS dimer interface to study the cause of incompatibility of TS subunit between P. falciparum and humans. Each TS dimer interface of P. falciparum and humans is very unique, and cannot interchange to each other. This opens an opportunity for development of novel species-specific inhibitors against TS dimerization.
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