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-[^{3}H]-FdUMP binding assays of transformants from TS-deficient *Escherichia coli* with a variety of pairs of inactive *Pf*TS(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 *Pf*TS (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

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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 (dTMP), 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 *Pf*TS (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 *Pf*TS and hTS domains showed high sequence similarity (Yuvaniyama *et al*, 2003). However, there are several distinct amino acid residues located around the *Pf*TS dimer interface compared to those of hTS. Hence, this opens an opportunity to explore the possibility of exploiting these specific amino acids in the *Pf*TS 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 *Pf*TS-inactive R470 (or *Pv*TS-inactive R486) enzyme can combine with that of *Pv*TS-inactive C506 (or *Pf*TS-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 *Pf*TS 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 *Pf*DHFR-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 *Xho*I/*Hin*dIII 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 *Bcl*I and *Sal*I sites.

Site-directed mutagenesis of TS at interface residues

Interface residues of *Pf*TS and hTS were mutated using QuikChange™ 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 *Pf*DHFR-TS residues I357, I506 and V546, respectively, or vice versa were carried out individually using hTS(R175A) or *Pf*DHFR-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 *Pf*TS(R470A/I357Q), *Pf*TS(R470A/I506Q) and *Pf*TS(R470A/V546T), respectively. *Pf*TS(R470A/I506Q/V546T) mutant was prepared from *Pf*TS(R470A/I506Q) with the primer set IX, and *Pf*TS(R470A/I357Q/I506Q) and *Pf*TS(R470A/I357Q/V546T) mutants from *Pf*TS(R470A/I357Q) with primer sets VIII and IX, respectively, and *Pf*TS(R470A/I357Q/I506Q/V546T) was prepared from *Pf*TS(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.

Set number	Mutant	Sequence (5′-3′)	Inserted restriction site
I	hTS (R175A/D/N/T)	F: CCCTGACGAC(**AG**)(**AC**)**C**AGAATCATCATGTGCGCTTGGAATCC**GC**G**G**GATCTTCCTC R: GAGGAAGATC**CC****GC**GGATTCCAAGCGCACATGATGATTCT**G**(**GT**)(**CT**)GTCGTCAGGG	*Sac*II
II	hTS (R175F/I/N/S/T/Y)	F: CCCTGACGAC(**AT**)(**ACT**)**C**AGAATCATCATGTGCGCTTGGAATCC**GC**G**G**GATCTTCCTC R: GAGGAAGATC**CC****GC**GGATTCCAAGCGCACATGATGATTCT**G**(**AGT**)(**AT**)GTCGTCAGGG	*Sac*II
III	hTS (C195A/D/N/T)	F: GCGCTGCCTCCA(**AG**)(**AC**)CCATGCCCTCTGCCAGTTCTA**C**GT**A**GTGAACAGT R:ACTGTTCAC**T**AC**G**TAGAACTGGCAGAGGGCATGG(**GT**)(**CT**)TGGAGGCAGCGC	*Sna*BI
IV	hTS(Q62I)	F: GTATTCGGCATG**ATT**GCGCGCTACAGC**TTA**AGAGATGAATTC R: GAATTCATCTCT**TAA**GCTGTAGCGCGC**AAT**CATGCCGAATAC	*Afl*II
V	hTS(Q211I)	F: GAGCTGTCCTGC**ATT**CTGTACCAG**C**GATCGGGAGAC R: GTCTCCCGATC**G**CTGGTACAG**AAT**GCAGGACAGCTC	*Pvu*I
VI	hTS(T251V)	F: GGCCTGAAGCC**C**GG**G**GACTTTATACAC**GTT**TTGGGAGATGCAC R: GTGCATCTCCCAA**AAC**GTGTATAAAGTC**C**CC**G**GGCTTCAGGCC	*Sma*I
VII	*Pf*DHFR-TS (I357Q)	F: GGAGTAGGTGT**C**TTAAGTAAATTCGGATAT**CAA**ATGAAATTTG R: CAAATTTCAT**TTG**ATATCCGAATTTACTTAA**G**ACACCTACTCC	*Afl*II
VIII	*Pf*DHFR-TS (I506Q)	F: GAAATTATCATGT**CAA**ATGTATCAA**C**GATC**G**TGTGATTTAG R: CTAAATCACA**C**GATC**G**TTGATACAT**TTG**ACATGATAATTTC	*Pvu*I
IX	*Pf*DHFR-TS (V546T)	F: CAGTTCATACAC**ACCC**TAGGAAATGCACATG R: CATGTGCATTTCCT**AGGG**TGTGTATGAACTG	*Avr*II

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(Δ*thyA572*, *recA56*) (DE3) cells were co-transformed with two separate compatible plasmids (ColE1 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% Na_2HPO_4, 0.1% KH_2PO_4, 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 (A_{600nm} ~ 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 10,500*g* 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 12,000*g* for 1 hour at 4°C, supernatant was used for TS activity assay with 6-[^{3}H]-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-CH_2H_4folate, 0.5 μl of 0.66 μM 6-[^{3}H]-FdUMP (15 Ci/mmol; Moravek Biochemicals, Brea, CA), and 30 μl of 50 mM TES buffer pH 7.4 (25 mM $MgCl_2$, 1 mM EDTA, 6.5 mM formaldehyde and 75 mM β-mercaptoethanol) was incubated at 25°C for 30 minutes. The 6-[^{3}H]-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,

Table 2
Bacteria genetic complementation of intra-species and cross-species co-transformants of hTS and *Pf*DHFR-TS.

Plasmid construct	Origin of replication	Genetic complementation
(A) Intra-species transformants		
pJU-*Pf*DHFR-TS(WT)	ColE1	+[a]
pMC-*Pf*DHFR-TS(WT)	p15A	+[a]
pMC-*Pf*DHFR-TS(R470X)	p15A	-[a]
pJU-*Pf*DHFR-TS(C490Z)	ColE1	-[a]
pRSETC-hTS(WT)	ColE1	+
pACMC-hTS(WT)	p15A	+
pACMC-hTS(R175B)	p15A	-
pRSETC-hTS(C195U)	ColE1	-
(B) Co-transformants of compatible plasmids		
*Pf*TS(R470X)+*Pf*TS(C490Z)	p15A / ColE1	+[a]
hTS(R175B)+hTS(C195U)	p15A / ColE1	+
*Pf*TS(R470X)+hTS(C195U)	p15A / ColE1	-
hTS(R175B)+*Pf*TS(C490Z)	p15A / ColE1	-

[a]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 *Pf*TS(R470X) (where X = A, D and N) and mutants *Pf*TS(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 *Pf*TS(R470X) and hTS(C195U) or hTS(R175B) and *Pf*TS(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-[^{3}H]-FdUMP assay, in which TS covalently binds to FdUMP in the presence of CH_2H_4folate cofactor (Santi and McHenry, 1972). No hTS(R175B) or hTS(C195U) mutants could form covalent 6-[^{3}H]-FdUMP-

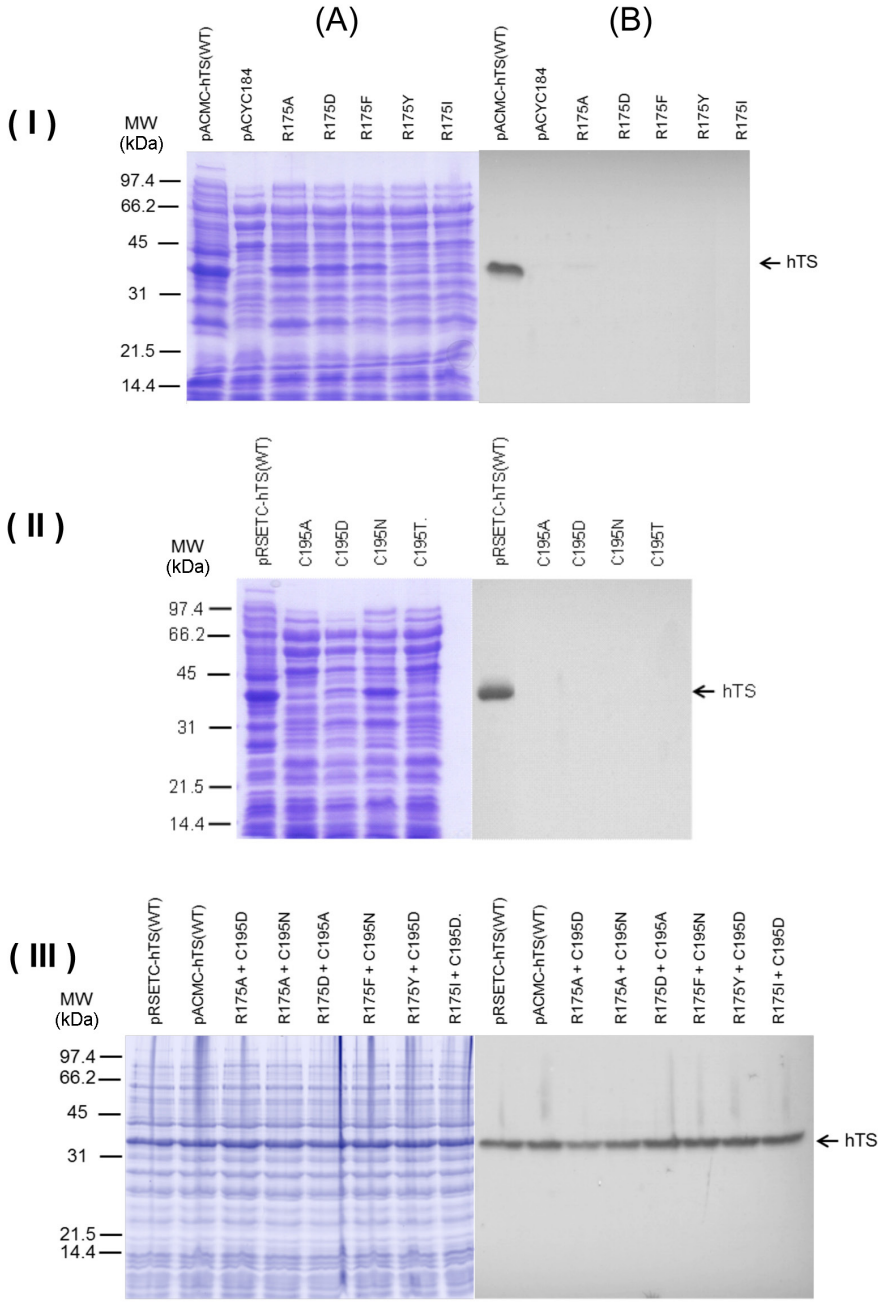

Fig 1–*In vitro* 6-[^{3}H]-FdUMP thymidylate synthase (TS) activity of crude extracts from homodimers of human inactive TS(R175) and TS(C195) mutants, and human TS active heterodimers in *E. coli* χ2913RecA(DE3). In brief, 6-[^{3}H]-FdUMP-protein complexes were formed by incubating crude extracts with 6-[^{3}H]-FdUMP and CH_2H_4folate 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.

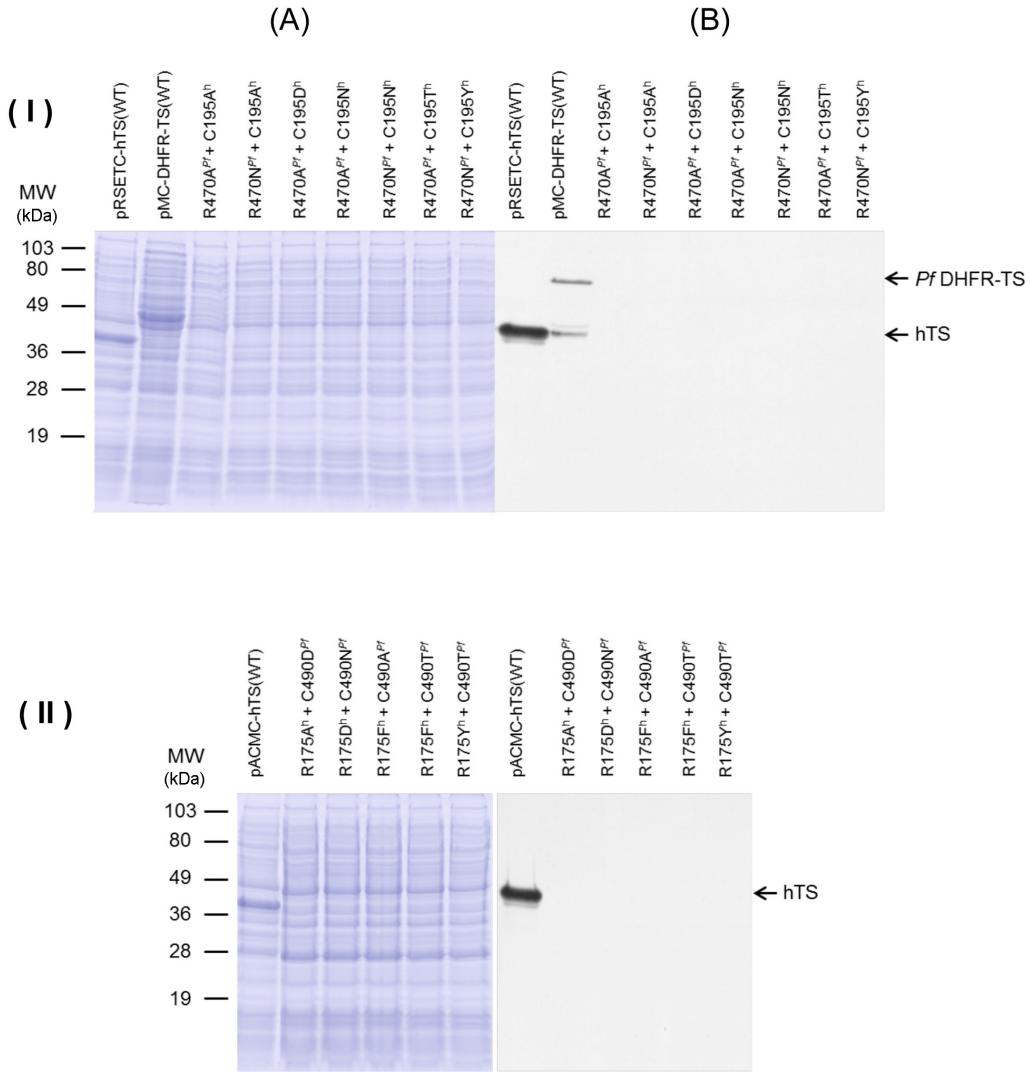

Fig 2–*In vitro* 6-[^{3}H]-FdUMP thymidylate synthase (TS) activity of crude extracts from cross-species TS co-transformants in *E. coli* χ2913RecA(DE3). TS assay is described in legend to Fig 1. (A) Coomassie-blue stained polyacrylamide gel. (B) Autoradiograph of A. Panel I, crude extracts of cross-species TS co-transformants of *Pf*DHFR-TS(R470) and hTS(C195). Panel II, crude extracts of cross-species TS co-transformants of hTS(R175) and *Pf*DHFR-TS(C490).

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 *Pf*DHFR-TS and hTS pairs [*Pf*TS(X470) + hTS(U195)] and [hTS(B175) + *Pf*TS(Z490) mutants] including [*Pf*TS(A/N470) + hTS(A195), *Pf*TS(A470) + hTS(D/N/T195) and *Pf*TS(N470) + hTS(N/Y195)], or vice versa [hTS(A175)

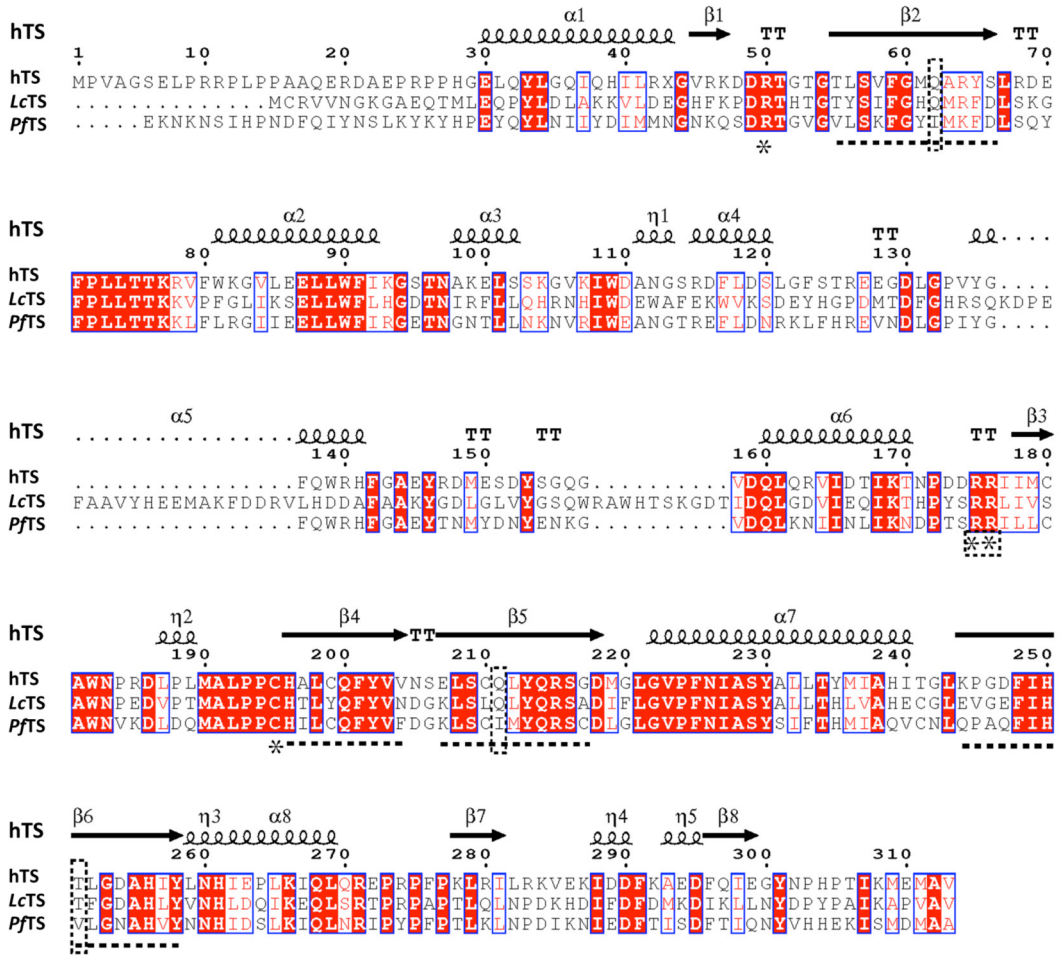

Fig 3–Amino acid alignment of human, *Lactobacillus casei* and *Plasmodium falciparum* thymidylate synthase (TS). The four β-strands involved in TS dimerization are underlined by a dash line, with three residues having different electrostatic potential (in dash box) among *P. falciparum*, human and *L. 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-[^{3}H]-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

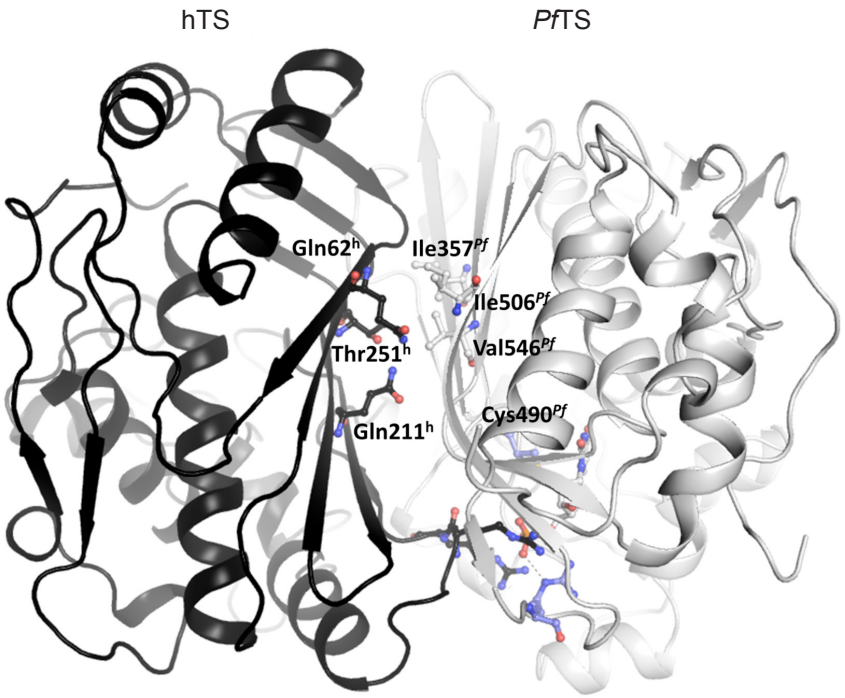

Fig 4–Model of inter-species thymidylate synthase (TS) heterodimer formation of *Plasmodium falciparum* and humans using TS coordinates from 1hvy.pdb (hTS) and 1j3k.pdb (*Pf*TS), showing the three key residues of opposite electrostatic potential at the TS interface of *P. falciparum* and humans.

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 *Pf*TS (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 *Pf*TS(I357), *Pf*TS(I506) and *Pf*TS(V546), non-polar residues. This would necessarily lead to non-complementary association of h- and *Pf*TS 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 *Pf*TS(A470)-based interface mutants of *Pf*TS 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 *Pf*TS(A490) for

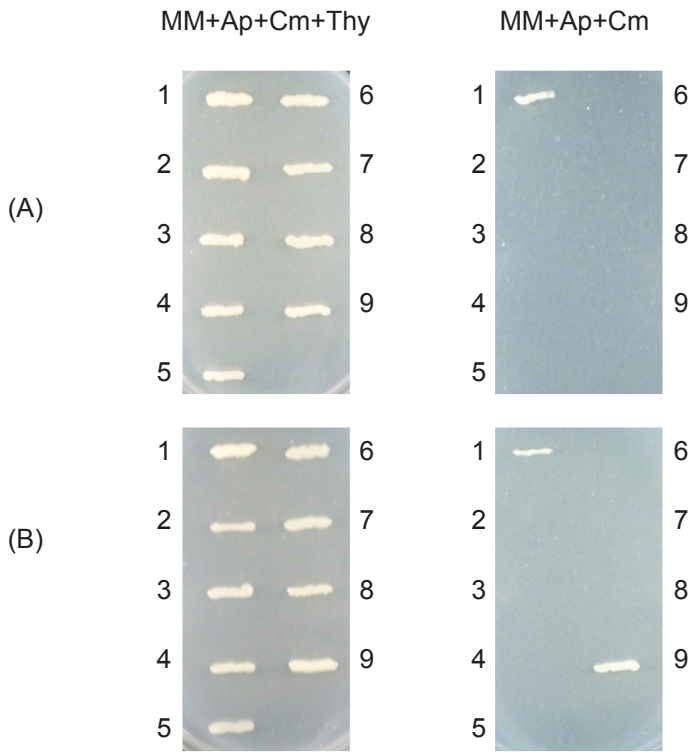

Fig 5–*In vivo* genetic complementation of human thymidylate synthase (hTS)(A175)-based interface mutant combined with single, double or triple mutant of hTS(I62), hTS(I211) and hTS(V251) grown on a minimum medium containing ampicillin, chloramphenicol and thymidine (MM+Ap+Cm+Thy) or ampicillin and chloramphenicol (MM+Ap+Cm). (A) *E. coli* χ2913RecA(DE3) cells co-transformed with hTS(A175)-based interface mutants and *Plasmodium falciparum* (*Pf*) TS(A490). Slot 1, positive control [*Pf*TS(D470) + *Pf*TS(A490)]; slot 2, hTS(A175/I62) + *Pf*TS(A490); slot 3, hTS(A175/I62/I211) + *Pf*TS(A490); slot 4, hTS(A175/V251) + *Pf*TS(A490); slot 5, hTS(A175/I62/I211/V251) + *Pf*TS(A490); slot 6, hTS(A175/I62/V251) + *Pf*TS(A490); slot 7, hTS(A175/I211/V251) + *Pf*TS(A490); slot 8, hTS(A175/I211) + *Pf*TS(A490); slot 9, hTS(A175) + *Pf*TS(A490). (B) *E. coli* χ2913RecA(DE3) cell co-transformed with hTS(A175)-based interface mutants and hTS(A195). Slot 1, positive control [(*Pf*TS(D470) + *Pf*TS(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* χ2913RecA(DE3) growth (Fig 5). Similarly, co-transformants of the *Pf*TS(A470)-based interface mutants and either inactive hTS(A195) or *Pf*TS(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*

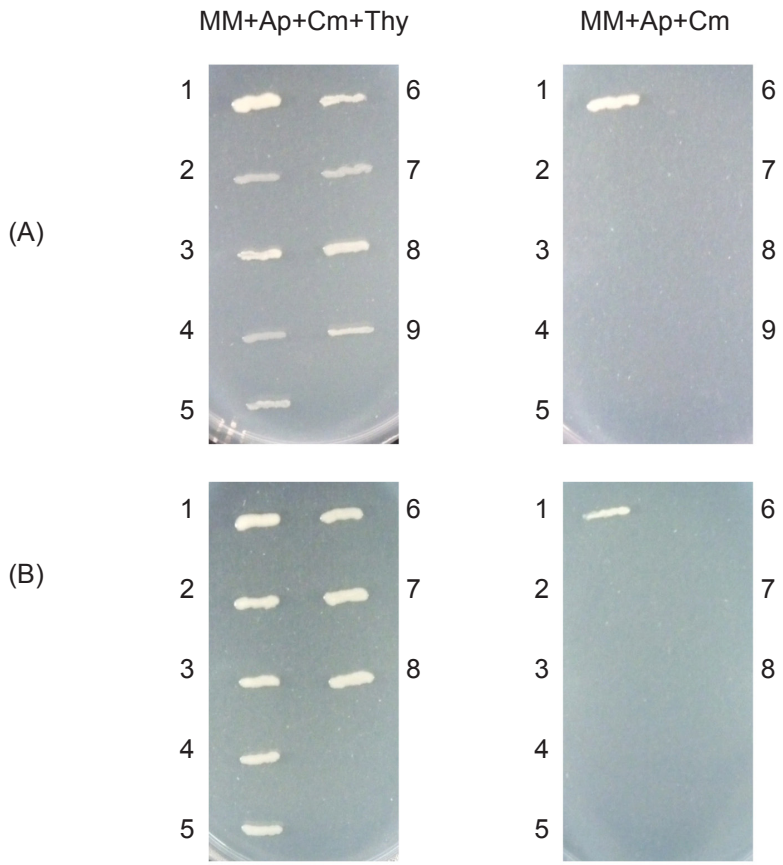

Fig 6–*In vivo* genetic complementation of *P. falciparum* (*Pf*) TS(A470)-based interface mutant combined with single, double or triple mutant of *Pf*TS(Q357), *Pf*TS(Q506) and *Pf*TS(T546) grown on a minimum medium containing ampicillin, chloramphenicol and thymidine (MM+Ap+Cm+Thy) or ampicillin and chloramphenicol (MM+Ap+Cm). (A) *E. coli* χ2913RecA(DE3) cell co-transformed with *Pf*TS(A470)-based interface mutants and human (h) TS(A195). Slot 1, positive control [hTS(A175) + hTS(A195)]; slot 2, *Pf*TS(A470/Q357) + hTS(A195); slot 3, *Pf*TS(A470/Q506) + hTS(A195); slot 4, *Pf*TS(A470/T546) + hTS(A195); slot 5, *Pf*TS(A470/Q357/Q506) + hTS(A195); slot 6, *Pf*TS(A470) + hTS(A195); slot 7, *Pf*TS(A470/Q357/T546) + hTS(A195); slot 8, *Pf*TS(A470/Q506/T546) + hTS(A195); slot 9, *Pf*TS(A470/Q357/Q506/T546) + hTS(A195). (B) *E. coli* χ2913RecA(DE3) cells co-transformed with *Pf*TS(A470)-based interface mutants and *Pf*TS(A490). Slot 1, positive control [*Pf*TS(A470) + *Pf*TS(A490)]; slot 2, *Pf*TS(A470/Q357) + *Pf*TS(A490); slot 3, *Pf*TS(A470/Q506) + *Pf*TS(A490); slot 4, *Pf*TS(A470/T546) + *Pf*TS(A490); slot 5, *Pf*TS(A470/Q357/Q506) + *Pf*TS(A490); slot 6, *Pf*TS(A470/Q357/T546) + *Pf*TS(A490); slot 7, *Pf*TS(A470/Q506/T546) + *Pf*TS(A490); slot 8, *Pf*TS(A470/Q357/Q506/T546) + *Pf*TS(A490).

and human TS monomers, inactive pairs of enzymes were selected such that one member of the pair had a mutation at *Pf*TS(R470) [or hTS(R175)] while the other had a mutation at hTS(C195) [or *Pf*TS(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 *Pf*TS, *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 *Pf*DHFR-TS was used in the study because *Pf*TS 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 [*Pf*TS(R470) and *Pf*TS(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 h- and *Pf*TS 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 *Pf*TS are non-polar. Co-tranformants of hTS(A175) in which the three key interface residues had been mutated to those of *Pf*TS with either inactive hTS(A195) or *Pf*TS(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 Pf*TS(R470), to fulfill its role in TS catalysis. Based on the structure of *Pf*TS (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 *Pf*TS(R470). Hence, TS inhibitors attacking the TS dimer interface will be good candidates for novel antimalarials.

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-[^{3}H]-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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