Comparative structural and functional features of folate salvage transporters in *Plasmodium falciparum* and humans

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Folic acids are essential cofactors for synthesis of DNA, RNA, membrane lipids, methionine metabolism and neurotransmitter synthesis. *P. falciparum* is capable of synthesizing folate *de novo* or obtaining it through salvage, whereas humans can only obtain folate from their diet, making the folate pathway products important targets for chemotherapy. Although *P. falciparum* folate pathway enzymes and metabolites have been studied extensively, the structure and function of transporters that underpin folate salvage are lacking. *Plasmodium falciparum* 3D7 and *Homo sapiens* folate transporter protein sequences were queried by using BLAST and sequences retrieved from PlasmoDB and NCBI databases respectively. This was followed by structural and functional prediction of the transporters. *P. falciparum* possesses two folate transporters that are structurally and functionally different from one another, and also from that found in humans. The *P. falciparum* folate transporter 1 has 12 transmembrane helices with a predicted upright funnel shaped structure, whereas the *P. falciparum* folate transporter 2 and *H. sapiens* folate transporter have 11 transmembrane helices and predicted inverted funnel shaped structures. A significant portion of the transporters lay in the cytoplasmic space, with little protrusion on the extracellular space. The differences observed in the parasite protein in comparison to the human version may offer possibilities in the effort to develop newer antimalarials against folate transport in *P. falciparum*.

Keywords: Tetrahydrofolates; *Plasmodium falciparum*; folate salvage transporters; transmembrane helices.

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Introduction

Folate (folic acid) is required for both the activation of single carbons as well as in the oxidation and reduction of single carbons. To carry out the transfer of 1-carbon units, NADPH must reduce folic acid twice in the cell [1]. The pyrazine ring of the 6-methylpterin is reduced at each of the two N-C double bonds. More precisely, the pathway leading to the formation of tetrahydrofolate (THF) begins when folate is reduced to dihydrofolate (DHF), which is then reduced to THF by the dihydrofolate reductase enzyme. Reduced folates are essential cofactors for one-carbon transfer reactions, including the conversion of dUMP to dTMP, which is a prerequisite for synthesis of DNA, RNA, membrane lipids, and neurotransmitters [1, 2, 3].

Plasmodium falciparum, the causative agent of the deadliest form of malaria, can synthesize the folate it needs from the simple precursors GTP, p-aminobenzoic acid (pABA) and glutamate. In addition to *de novo* synthesis, the parasite can also salvage folate [2]. Higher eukaryotes,

including humans have lost the ability to synthesize folate *de novo* and so depend on dietary intake of pre-formed folate as an essential nutrient [2, 3, 4]. As a result of this difference between *P. falciparum* and its host, antifolate drugs that target the biosynthesis and processing of folate cofactors have been effectively used in the chemotherapy of malaria and other infectious diseases. Examples of important antifolates include pyrimethamine, which targets dihydrofolate reductase (DHFR), and sufadoxine, which targets dihydropteroate synthase (DHPS). Various studies have shown that synthesis and salvage of folates by the parasite is important for parasite viability and drug susceptibility [5].

Folate import into *P. falciparum* requires an ATP-dependent proton symport mechanism requiring specific transporter mediation. The high polarity of folate compounds limits their ability to pass through the cell membrane independent of specific membrane transporters [6]. Wang et al. showed that folate uptake by *P. falciparum* is a specific, energy-dependent and saturable process that can be inhibited by classical anion transport inhibitors such as probenecid and furosemide [7]. Similar studies have also shown that there are transporters through which *P. falciparum* salvages folate and other metabolites. These studies demonstrate an increase in the sensitivity of *P. falciparum* to antifolates upon administration of anion transport inhibitors, which has been ascribed to folate transport inhibition [8, 9]. These discoveries have highlighted the potential of blocking folate salvage transporters as a therapeutic strategy demonstrating the importance of folate salvage and transport in antifolate drug susceptibility and parasite survival [10, 11].

Folate transporters belong to the major facilitator superfamily (MFS) of proteins. MFS transporter proteins are single-polypeptide secondary carriers that have been implicated in the transportation of variety of small solutes and metabolites in response to chemiosmotic ion gradients across cell membranes of organisms ranging from bacteria to humans [3, 4]. Two folate transporters, PfFT1 and PfFT2, have been identified from *P. falciparum*, which are membrane protein homologues of the BT1 family of folate transporters [12]. However, the structural and functional properties of these salvage transporters in *P. falciparum* have not been studied extensively. Here, we characterized *P. falciparum* folate salvage transporters and compared them with that of humans. We highlight differences in both the parasite and human forms and suggest how these differences may offer possibilities for potential antimalarial drug development.

Materials and method

Sequences BLAST and Retrieval

Plasmodium falciparum 3D7 folate transporter [PF3D7_0828600 for folate transporter 1 (FT1) and PF3D7_1116500 for folate transporter 2 (FT2)] protein sequences were downloaded from PlasmoDB (version 9.3) (http://plasmodb.org). The *Homo sapiens* folate transporter (JC2468) protein sequence was downloaded from The National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/protein/?term=). All retrieved protein sequences were in FASTA format.

Structural and functional prediction

The various physical and chemical parameters such as molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index, and grand average of hydropathicity (GRAVY) were predicted using a webserver tool, ProtParam (http://web.expasy.org/protparam/). The solubility status of the proteins was computed using PROSO (http://omictools.com/proso-tool) [13]. The clonability of these proteins was predicted using MEMEX (http://mips.helmholtz-muenchen.de/memex/ memex.seam) [14]. Predictions of the transmembrane helices of each protein were done on TMHMM Server

Table 1. Physiochemical parameters of *Plasmodium Falciparium* and *H. sapiens* folate transporter proteins.

	Physiochemical features	Pf_FT1	Pf_FT2	H_FT
1	No of amino acids	505	455	590
2	Molecular weight (Daltons)	58060.4	51354.7	65204.7
3	Theoretical (pI)	8.05	8.63	9.45
4	Total no. of negatively charged residues (Asp + Glu)	35	31	35
5	Total no. of positively charged residues (Arg + Lys)	38	36	53
6	Formula	$C_{2704}H_{4192}N_{620}O_{736}S_{28}$	$C_{2427}H_{3758}N_{546}O_{643}S_{15}$	$C_{2989}H_{4639}N_{793}O_{805}S_{20}$
7	Total number of atoms	8280	7389	9246
8	Extinction coefficient ($M^{-1}cm^{-1}$)	52105	33155	101020
9	Estimated half-life (hr)	30	30	30
10	Instability index	34.80	35.46	45.14
11	Aliphatic index	116.79	117.43	99.02
12	Grand average of hydropathicity (GRAVY)	0.490	0.578	0.224
13	Predicted solubility class	insoluble	insoluble	insoluble
14	Solubility Class probability	0.938	0.899	0.875
15	Predicted clonability	no	no	no

(version 2.0) (www.cbs.dtu.dk/services/TMHMM/) [15]. Topology predictions of the membrane proteins were analyzed using TopPred 1.10 (http://mobyle.pasteur.fr/cgi-bin/portal.py# forms::toppred), a webserver tool that predicts protein membrane topology [16]. Leucine rich repeats (LRRs) prediction was done on the web server, LRRfinder (www.1rrfinder.com). Hydrophobicity analysis was done using a kyte doolittle hydropathy plot (http://gcat.davidson.edu/DGPB/kd/kyte-doolittle.htm) [17]. Protein 3D Structure prediction was obtained using Phyre (version 2.0)(www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) [18] with the Phyre platform automatically selecting a template for the modelling. The selected template for *P. falciparum* folate transporter 1 was *Escherichia coli* yajr transporter (PDB ID: 3WDO), 86% of its amino acid residues was utilized for the modelling with a confidence of 99.8%. The selected template for *P. falciparum* folate transporter 2 was glycerol-3-phosphate transporter (PDB ID: 1PW4), 92% of its amino acid residues was utilized for the modelling with a confidence of 100%. The selected template for *H. sapiens* folate transporter was glycerol-3-phosphate transporter (PDB ID: 1PW4), 83% of its amino acid residues was utilized for the modelling with a confidence greater than 90%. The PDB file of each final model was downloaded and visualized using Pymol (version 1.6) (www.pymol.org/pymol).

Percent Identity Analysis

Protein sequence alignment, identification of protein similarity, and relatedness were determined using Cluster Omega (www.ebi.ac.uk/Tools/msa/clustalo/), which was employed to obtain the percent identity matrix (PIM) [19].

Results

Physicochemical properties of folate transporter proteins from *P. falciparum* and *H. sapiens* are presented in Table 1. *H. sapiens* folate transporter is 18.21% and 23.16% identical to *P. falciparum* folate transporter 1 and 2, respectively, while *P. falciparum* folate transporter 1 is 29.05% identical to *P. falciparum* folate transporter 2 (Table 2). The protein net charge indicates all the proteins are positively charged at different alkalinity states. From the extinction coefficient values we computed, *H.*

sapiens had the highest values and *P. falciparum* folate transporter 2 had the lowest. All protein half-life values were similar. The instability index of these transporters show that *P. falciparum* folate transporter 1 had the lowest value and *H. sapiens* had the highest value. The aliphatic index obtained for *P. falciparum* folate transporter 2 was the highest followed by *H. sapiens* folate transporter. The solubility and clonability status of the proteins are also presented. Analysis of amino acid compositions shows that the folate transporter proteins are all leucine and threonine rich, while the atomic composition show that all the proteins are rich in hydrogen atom followed by carbon atom.

Table 2. Percent identity matrix for the three folate transporters.

	H_FT	Pf_FT1	Pf_FT2
H_FT	100	18.21	23.16
Pf_FT1	18.21	100	29.05
Pf_FT2	23.16	29.05	100

H_FT: Human folate transporter
Pf_FT1: P. falciparium folate transporter 1
Pf_FT2: P. falciparium folate transporter 2

The lateral views of the 3D structures of all the proteins are presented in figures 1A-3A. The *P. falciparum* folate transporter 1 molecule has the shape of a funnel, while *P. falciparum* folate transporter 2 and *H. sapiens* folate transporter both possess an inverted funnel shape. The stereo views of the 3D structures of the proteins highlight predicted helices (H) in each structure (Figures 1B-3B). We noticed that all the molecules have a flat top and have two similar domains, the N-terminal and the C-terminal domains. These two domains have a central pseudo two-fold symmetry axis perpendicular to the membrane plane, looking from within the membrane plane along the domain interface.

The predictions of transmembrane helices (TMHs) for these proteins are represented in figures 1C-3C. The *P. falciparum* folate transporter 1 has 12 TMHs, while *P. falciparum* folate transporter 2 and *H. sapiens* have 11 THMs, with p(H)>0.5. The 1st and 5th helices in *P. falciparum* folate transporter 2 and *H. sapiens* folate transporter, respectively are not TMHs, with p(H)<0.5. The expected number of amino acids in transmembrane helices for *P. falciparum* folate transporter 1, *P. falciparum* folate transporter 2 and *H. sapiens* are 257.84, 221.38, and 241.38, respectively. Since the expected numbers of amino acids in transmembrane helices are far greater than 18, we suggest that all folate transporters considered are transmembrane transporters. The expected number of amino acids in transmembrane helices in the first 60 amino acids for *P. falciparum* folate transporter 1, *P. falciparum* folate transporter 2 and *H. sapiens* are 4.61, 18.68, and 7.55, respectively. The probabilities that the N-terminus is on the cytoplasmic side of the membrane for *P. falciparum* folate transporter 1, *P. falciparum* folate transporter 2 and *H. sapiens* are 0.9339, 0.4703, and 0.4511, respectively.

The topology predictions of predicted loop regions are presented in figures 1D-3D, showing loop length (LI) between the helices and the numbers of positively charged residues (Arg + Lys) between helices of each protein. The loop regions vary in length from 2 to 56 residues in *P. falciparum* folate transporter 1, 3 to 86 in *P. falciparum* folate transporter 2, and 4 to 138 in *H. sapiens* folate transporter. The cytoplasmic portion of these transporters is more positively charged compared with the extracellular portions. The *P. falciparum* folate transporter 1, *P. falciparum* folate transporter 2 and *H. sapiens* folate transporter helices portion in the cytoplasm possess a positive charge (Arg + Lys) of 26, 23, and 44, respectively, while the extracellular portion possess a positive charge of 11, 11, and 8, respectively.

The hydrophobicity predictions of the proteins are presented in figures 1E-3E. Amino acid residues that are strongly positive are the hydrophobic regions, while the amino acid residues that are strongly negative are the hydrophilic regions for each protein. The residues that are above the red line

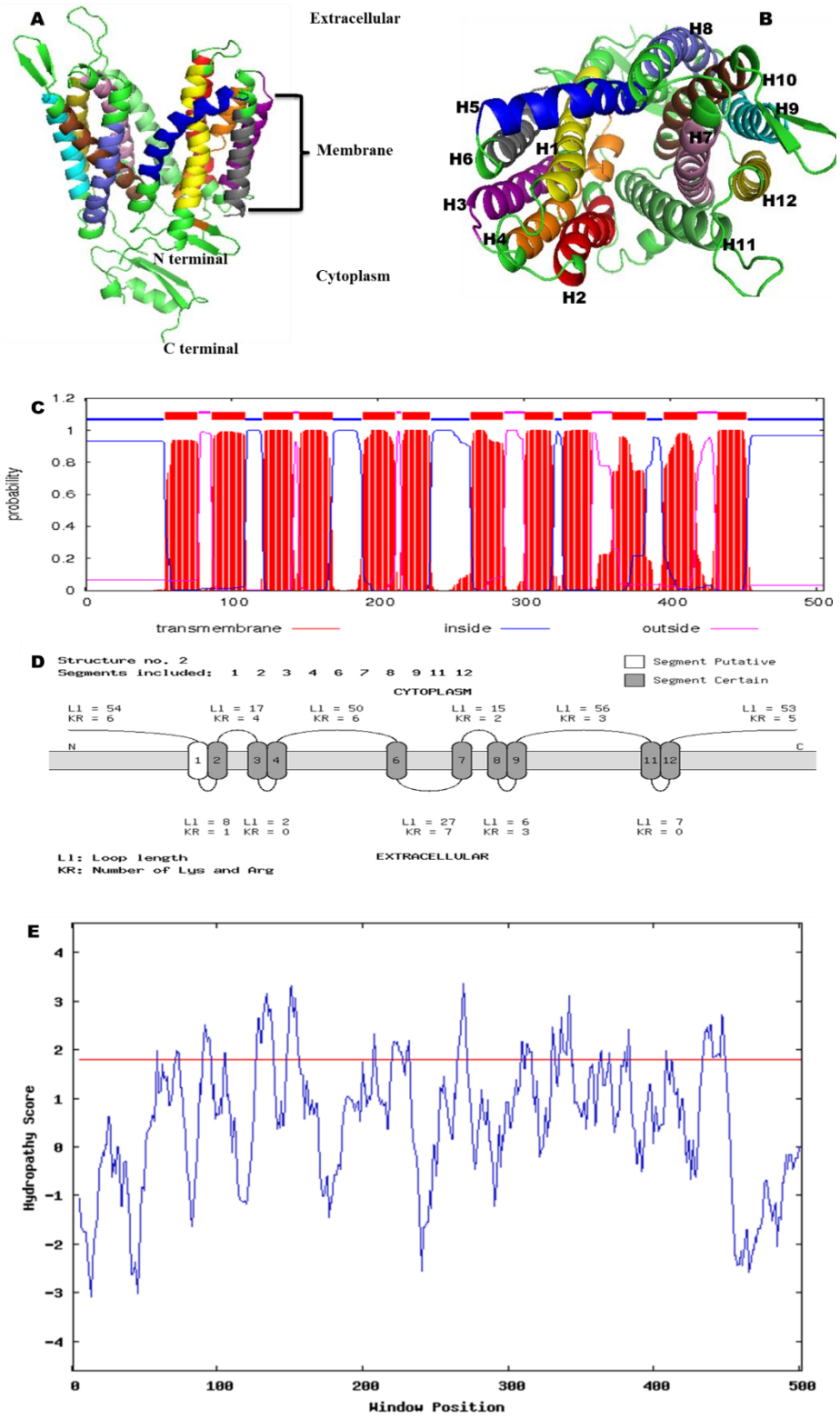

Figure 1. Structure of *P. falciparium* folate transporter 1. **A**. Lateral view of the 3D structure with X:55.033Å Y:67.223Å and Z:67.440Å model dimensions. **B.** Stereo view of a cartoon representation from the extracellular space showing five peripheral helices (H3, H6, H8, H9, and H12) not involved in pore formation. The remaining helices lining the central core are involved in pore formation. **C.** Transmembrane helices protein. **D.** Transmembrane topology of the protein. **E.** Hydrophobicity of the protein, peaks with scores greater than 1.8 (red line) indicate possible transmembrane regions.

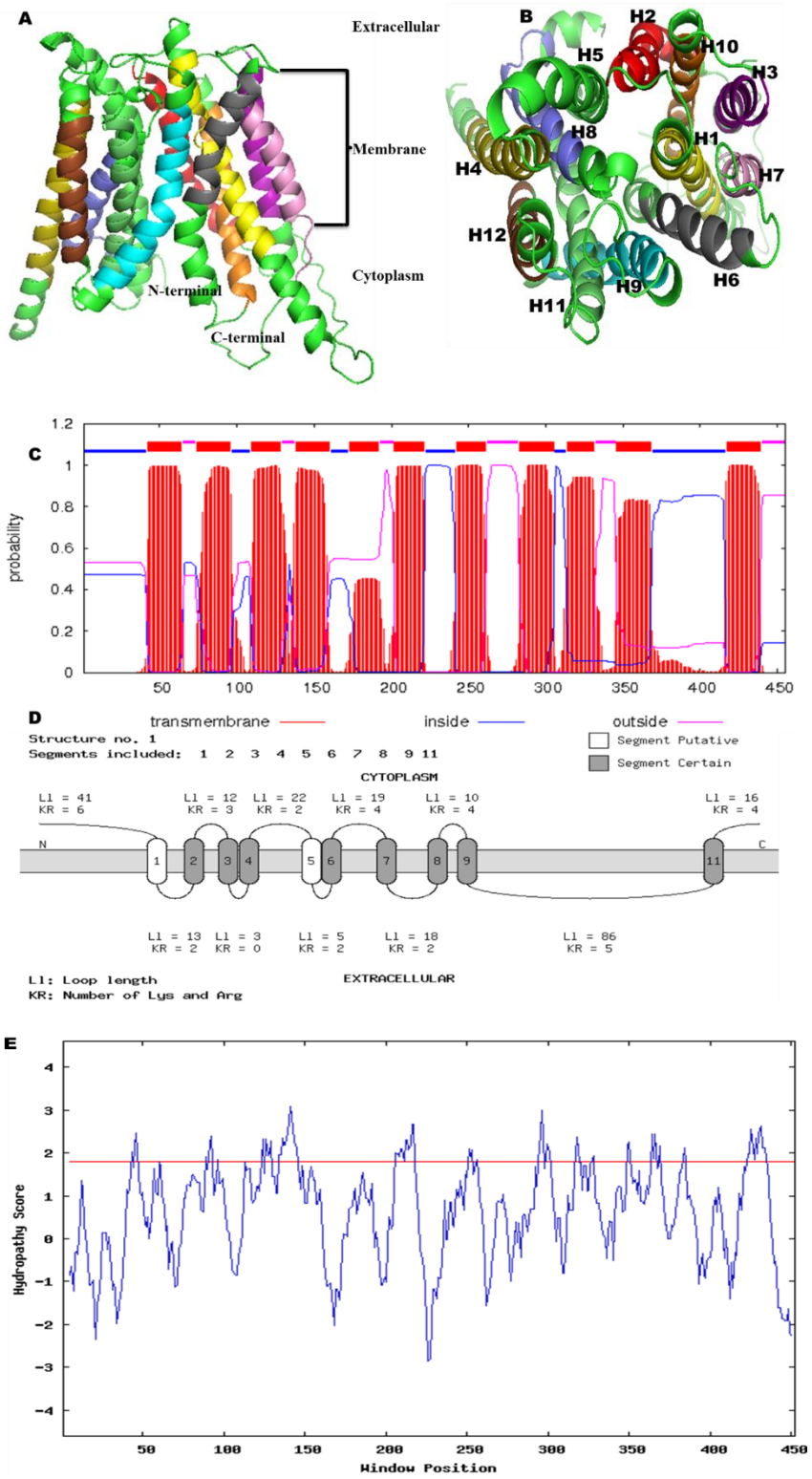

Figure 2. Structure of *P. falciparium* folate transporter 2. **A**. Lateral view of the 3D with X:53.579 Å, Y:72.865 Å and Z:70.253 Å model dimensions. **B**. Stereo view of a cartoon representation from the extracellular space showing five peripheral helices (H3, H4, H7, H9, and H12) not involved in pore formation. The remaining helices lining the central core are involved in pore formation. **C**. Transmembrane helices protein. **D.** Transmembrane topology of the protein. **E.** Hydrophobicity of the protein, peaks with scores greater than 1.8 (red line) indicate possible transmembrane regions.

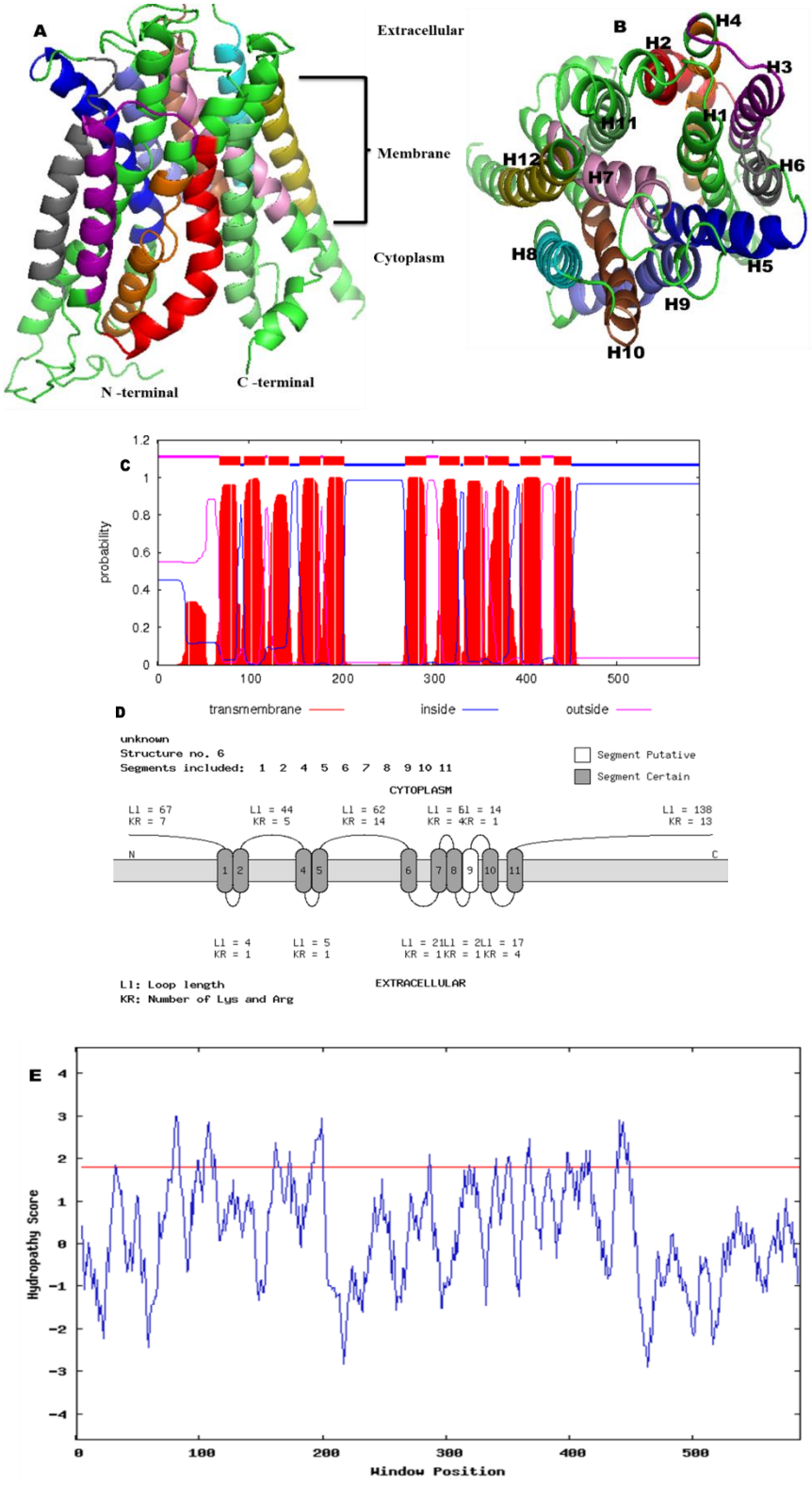

Figure 3. Structure of *H. sapiens* folate transporter. **A.** lateral view of the 3D with X:55.033Å Y:67.223Å and Z:67.440Å model dimensions. **B.** Stereo view of a cartoon representation from the extracellular space showing five peripheral helices (H3, H6, H8 and H12) not involved in pore formation. While H9 and H10 are partly involved in pore formation; the remaining helices lining the central core are involved in pore formation **C.** Transmembrane helices protein. **D.** Transmembrane topology of the protein. **E.** Hydrophobicity of the protein, peaks with scores greater than 1.8 (red line) indicate possible transmembrane regions.

(hydrophobicity score > 1.8) indicate possible transmembrane regions.

Discussion

Malaria parasites are able to synthesize folate *de novo*, a pathway absent in mammalian hosts [3, 4, 5]. In addition, the parasites also have the capability of taking up and utilizing exogenous folate derivatives via salvage routes [3]. Consequently, the folate pathway has long been a target for drugs deployed against rapidly reproducing cells such as cancers and a range of microbial pathogens including *P. falciparum* [3, 4]. Folate uptake by *P. falciparum* is a specific energy-dependent process requiring membrane transporters [5, 20, 21, 22, 23]. Despite the importance of these transporters in folate uptake and salvage, there is insufficient information on their structure and function of annotated *P. falciparum* folate transporters [12] when compared with that of humans [6].

Membrane proteins rich in leucine typically possess leucine rich repeats (LRRs) [24]. Although, the three folate transporter proteins we studied are all rich with leucine, however, they lack LRRs. Our results show that the transporters were also rich in threonine and tyrosine residues, previous studies have indicated the importance of such residues in protein phosphorylation [24]. Protein phosphorylation play crucial roles in regulating protein function and therefore control several fundamental aspects of cell transport, including drug transport across cellular membranes [25]. Early studies have reported phosphorylation altering transport activity of mammalian transport homologs such as PfMDR1, which is involved in drug action and resistance of antimalarials [26, 27]. Such activities may also be important in the transporters studied here.

The *P. falciparum* and *H. sapiens* folate transporters are 400 to 600 aa long and share transmembrane topology similarities and signature sequences in two cytosolic loops, which are typical of MFS proteins [3, 28, 29]. Structurally, MFS transporter proteins that include folate transporters have about 11 to 12 TMHs surrounding a central pore [4, 29]. The number of amino acids in the transmembrane helices in the first 60 amino acids of *P. falciparum* folate transporter 1 show that the predicted transmembrane helix in the N-terminal could be a signal peptide, similar to observations made by Krogh and colleagues [15]. MFS proteins usually possess approximately 12 transmembrane helices, with both the N- and C-termini located in the cytosol as revealed by hydropathy sequence analysis and reporter-fusion experiments [28]. Our work also showed that the N-terminals and C- terminals were located on the cytoplasmic side of the membrane.

Our report describes TMHs having a larger portion in the cytoplasm without protruding beyond the cell membrane into the extracellular space (Figures 1C-3C). The proteins consist of two domains that are clearly connected by a long central loop. These domains have also been observed by Jiang et al. [30] and others [31, 32, 33, 34]. The inward and outward opening of the pore through the membrane is a major property of MFS transporter family proteins [28]. They have both the N- and C-termini located in the cytoplasmic matrix [35], an observation made for all the proteins studied. In addition, they have extracellular portions that are less positively charged when compared with the cytoplasmic portion (Figures 1D-3D) and this contributes to the highly hydrophilic nature of these proteins (Figures 1E-3E). Consequently, this may aid the transport of hydrophilic solutes such as folates across the membrane from the extracellular space [36]. Furthermore, this may also be an added mechanism by which antimalarials are transported into the cell [37]. *P. falciparum* folate transporter 1 opens outward, displaying a funnel shape. Similar observations have been made in the structure of the YajR transporter from *E. coli* [30]. In contrast, both *P. falciparum* folate transporter 2 and *H. sapiens* folate transporter appear to have the pore opening inwards with an inverted funnel shape

as observed by Huang et al. for the *E. coli* glycerol-3-phosphate transporter [32] and Yin et al. in *E. coli* multidrug resistance protein D [38]. The 3D structure of *P. falciparum* folate transporter 2 we modelled has a 100% identity with the crystal structure of glycerol-3-phosphate transporter of *E.coli* [33] and lactose permease model by Abramson et al. [31]. In addition, it is 99.9% identical with: di-or tripeptide h+ symporter of *Streptococcus thermophilus* LMG 18311 [39] and D-xylose-proton symporter of *E. coli* K-12. The *H. sapiens* folate transporter is 99.7% identical to Glycerol-3-phosphate transporter of *E. coli* [32]; 99.4% identical to lactose permease model by Abramson et al. [31]; 99.3% identical with L-fucose-proton symporter of *E. coli* and di-or tripeptide h+ symporter of *S. thermophilus* LMG 18311 [4].

Drug resistance has limited the use of antifolates, except in Artemisinin-based combinations (ACTs) [20, 21, 22]. However, the folate biosynthesis pathway remains one of a few valid drug targets for which current drug screening efforts are directed. Consequently folate synthesis, and particularly folate transport, may still offer novel options for drug discovery [12]. It should be borne in mind that the human host is completely dependent on folate salvaged from diet, thus making targeting the parasites salvage transporters questionable. PfFT1 and PfFT2 are homologues of the BT1 family of folate transporters in *P. falciparum* [3, 40]. Homologues of BT1 family are absent in humans. Consequently, the differences of both parasite and host transporters described herein, may add to growing evidence [41] of the potential of blocking folate salvage transporters as important in drug development.

In summary, our observations on the structural and functional differences of *P. falciparum* 3D7 folate transporter 1 and folate transporter 2 with *H. sapiens* folate transporter 2, suggests that these *Plasmodium* transporters may be potential drug targets. Of particular importance may be how the difference between the parasite and host transporter could be selectively targeted, thus disrupting a critical part of the parasites salvage route for preformed folates. Understanding folate salvage in *Plasmodium* through structural and functional analysis of folate salvage transporters can in addition, help with development of inhibitors that markedly increase parasite susceptibility to common antifolate drugs [5].

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