

ORIGINAL ARTICLE

A study of the PfNT3 in *Plasmodium falciparum*

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ABSTRAK

Kajian genetik terdahulu menunjukkan bahawa kelangsungan hidup dan proliferasi parasit *Plasmodium falciparum* bergantung kepada penggunaan purin utama dalam hos. *Plasmodium falciparum*, agen penyebab kepada jenis malaria paling merbahaya pada manusia, tidak mempunyai enzim yang diperlukan untuk sintesis purin secara *de novo*. Berdasarkan kepada sasaran kemoterapeutik yang novel, analisa hipotetikal protein pengangkut nukleosida/nukleobas, hasil dari gen PfNT3 (PF14_0662) dalam parasit *P. falciparum*, dilakukan secara lokalisasi. Melalui immunoblotting, immunoflorescen dan lokalisasi mikroskopik immunoelektron, lokasi PfNT3 didemonstrasi dengan menggunakan anti-serum poliklonal pada kultur *in-vitro* parasit *P. falciparum* yang dibiakkan dalam sel darah merah manusia. Protin PfNT3, hasil translasi gen PfNT3, dikesan pada peringkat cincin intraeritrosit, trofosit dan skizon. Tumpuan utama PfNT3 adalah pada membran plasma parasit (MPP). Pengangkut putatif nukleosid endogenous PfNT3 dengan lokasi utama di MPP boleh bertindak bukan sahaja sebagai tapak jalan untuk sasaran analog purin/agen sitotoksik bagi intraselular parasit, tetapi juga bertindak sebagai sasaran dadah. Sebagai genom terkod, protin pengangkut vital boleh dihalang daripada diekspresi melalui gen 'silencing' dan ini mengesahkannya sebagai sasaran dadah yang novel.

Kata kunci: Pengangkut nukleosida equilibratif, malaria, gen PfNT3, *Plasmodium falciparum*, purin.

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ABSTRACT

Previous genetic studies demonstrated that survival and proliferation of *Plasmodium falciparum* parasites is dependent on salvage of essential purines from the host. *Plasmodium falciparum*, the causative agent of the most lethal form of human malaria lacks the enzymes required for *de novo* synthesis of purines. Analysis of the hypothetical nucleoside/nucleobase transporter protein, the gene product of *PfNT3* (PF14_0662) gene in *P. falciparum* parasites was carried out by localisation, in view of a novel chemotherapeutic target. Immunoblotting, immunofluorescent and immunoelectron microscopic localization of *PfNT3* was demonstrated using polyclonal antiserum in *in vitro* cultured *Plasmodium falciparum* parasites, propagated in human red blood cells. *PfNT3* protein, the translated product of *PfNT3* gene was detected in intraerythrocytic ring, trophozoite, and schizont stages. *PfNT3* was localized primarily to the PPM (Parasite Plasma Membrane). The endogenous *PfNT3* putative nucleoside transporter with the predominant location to the parasite plasma membrane may serve not only as routes for targeting of purine analogs/cytotoxic agents into the intracellular parasite but may also serve as drug targets. Being genome encoded the vital transporter protein can be prevented from expression by silencing of the gene, validating it to be a novel drug target.

Keywords: equilibrative nucleoside transporter, malaria, *PfNT3* gene, *Plasmodium falciparum*, purines.

INTRODUCTION

Malaria can be considered as one of the oldest and deadliest infectious diseases around the world. It continues to be one of the major public health problems in the world especially in African and South-East Asian countries (Kondrachine et al. 1997). As per the World Malaria Report, 2008, in terms of malaria incidence, India ranks 15th among 109 countries and contributed approximately 2% of global cases in 2006. India accounted for approximately two thirds of the confirmed cases reported in the South-East Asia region. Five states accounted for 60% of cases i.e. Orissa, Chhattisgarh,

Madhya Pradesh, Jharkhand and West Bengal (Global Reports: World Health Organisation 2011). The state of Odisha, part of peninsular India is situated along the east coast extending from 17° 49' to 22° 34' N and 81° 28' to 87° 29' E. It is hyper-endemic for malaria. With 4% of India's population, the state contributed 28.6% of total malaria cases, 35% of *P. falciparum* cases and 50% of total malaria deaths in the country (Ranjit et al. 2005).

Plasmodium falciparum causes one of the most life-threatening infections in humans. Despite all control measures, malaria still remains uncontrolled.

Chemotherapy for malaria is the dominant mode of intervention since a vaccine for the disease is still not a reality. The most important factor is the development of resistance by *P. falciparum* to conventional and effective drugs like chloroquine, mefloquine, naturally occurring quinine and sulfadoxine/ pyrimethamine (Biot et al. 2006). Antimalarial drug development can follow several strategies, ranging from modifications of existing agents to design of novel agents that act against new targets. The rapid growth and multiplication rate of the malaria parasite during the intra erythrocytic life cycle with its metabolism intertwined with that of the host necessitates the parasite to acquire nutrients from the host cell. Purines enter the host erythrocyte via a combination of endogenous transporters and “new permeation pathways” induced in the erythrocyte membrane as the parasite matures (Gero et al. 1988; Gati et al. 1990; Upston et al. 1995; Quashie et al. 2010). The enclosure of *Plasmodium falciparum* in a parasitophorous vacuole inside the erythrocytes necessitates the uptake of nutrients or purines from host erythrocyte into *P. falciparum* across a multiplicity of membranes: the Red Blood Cell membrane (RBCM), the Parasitophorous Vacuolar Membrane (PVM) and the Parasite Plasma Membrane (PPM). A variety of complex and novel components may also be involved, including transporters, channels, ducts and tubulo vesicular membranes (TVM).

One striking metabolic difference between all parasites and their mammalian hosts is the purine

synthetic pathway. The mammalian cells are capable of synthesizing purine nucleotides *de novo* while the protozoan parasites studied to date including *P. falciparum* is incapable of making a purine ring (Shermann et al. 1979; Gero et al. 1990; World Health Organization 1997; World Health Organization Expert Committee on Malaria 2000). The enzymes of the salvage pathway for purine nucleotides have all been demonstrated in the infected erythrocyte. This purine salvage process is initiated by the translocation of purines across the parasite membranes. Thus, the transport characteristics of purine base and nucleoside transporters serve an indispensable nutritional function for the parasite, which may be a novel drug target (Carter et al. 2000).

A *P. falciparum* encoded Equilibrative Nucleoside Transporter (ENT), the PF13_0252 (PfENT1) protein has been characterized in *Xenopus* oocytes and observed to transport purine and pyrimidine nucleosides and nucleobases (Carter et al. 2000; Martin et al. 2005). Functional analysis by genetic disruption in *Plasmodium falciparum* indicated that PfNT1 is essential for parasite survival under physiologic purine concentrations (El Bissati et al. 2008). It was also reported that PfNT1 knock-out parasites were unable to transport most physiologically relevant purine nucleosides/ nucleobases, although residual adenine transport was observed (El Bissati et al. 2008; El Bissati et al. 2006).

The completion of the *Plasmodium* genome sequencing project (Gardner et al. 2002) also revealed three

further ENT family members, PfNT2 (MAL8P1.32), PfNT3 (PF14_0662), and PfNT4 (PFA0160c). Like PfNT1, PfNT2, an intracellular permease is a member of the equilibrative nucleoside transporter family. Confocal and immunoelectron microscopic analyses of transgenic parasites harbouring green fluorescent protein- or haemagglutinin-tagged PfNT2 demonstrated endoplasmic reticulum localization. This study provided the first evidence of an intracellular purine permease in apicomplexan parasites and suggests a novel biological function for the parasite endoplasmic reticulum during malaria infection (Downie et al. 2010).

The complexity of the intra erythrocytic environment where the parasite resides reveals that the location of PfNT3 and other putative members of *P. falciparum* ENT family are important because a restricted milieu can be a determinant of specialized functions and their potential as therapeutic targets. These may be novel sites of therapeutic targets. These predictions led to the importance of the study of putative nucleoside/nucleobase transporter, PfNT3 in *in vitro* cultured Plasmodium *falciparum* parasites propagated in human red blood cells. Such studies on PfNT3 are not yet reported in literature.

MATERIALS & METHODS

STUDY DESIGN

Study of *PfNT3* gene product by immunoblotting, immunofluorescence and immunoelectron microscopic

localization was demonstrated in this work. This was based on *in vitro* culture of Plasmodium *falciparum* parasites, cloning, expression of the gene and polyclonal antibody development against the putative nucleoside/nucleobase transporter after approval of the Institutional Ethics Committee.

PARASITE CULTURE AND ANALYSIS OF PFNT3

RKL9 Clone of *P. falciparum* parasites (a strain of *P. falciparum* parasite in Rourkela, an endemic zone of malaria in the state of Odisha, India) obtained from National Institute of Malaria Research (NIMR), Delhi, India were propagated in human RBCs at 2% haematocrit and 1-10% parasitemia in RPMI-1640 medium (Himedia, India) supplemented with 10% heat inactivated human serum (O+ or AB+) (Trager et al. 1976).

PfNT3 sequence was amplified by polymerase chain reaction with genomic DNA from the *in vitro* cultured *P. falciparum* parasites using a sense primer - 5'- GCGTCATGAGTGACAGTCAGAG - 3' (22 mer) and anti-sense primer - 5'- GCTCGAGTTTTAAATAACTGC - 3' (24 mer), respectively. Optimized PCR conditions were initial denaturation at 98°C for 2 mins., 30 cycles of denaturation at 98°C for 30 secs., annealing at 45.7°C for 30 secs., extension at 72°C for 45 secs. and final extension at 72°C for 10 mins. PCR product was identified by 1% agarose gel electrophoresis and sequencing. All the sequencing reactions were carried out with routine procedure of sequencing (commercially in an ABI 3130 genetic

analyzer, USA at Manipal Life Sciences Centre, Manipal University, Karnataka, India). The *PfNT3* PCR product in the excised gel was processed by QIAquick Gel Extraction Kit (Qiagen, Germany). Sequencing of the purified DNA was done in both directions. At least three independent clones were sequenced and each nucleotide was confirmed with an average of five times. Analysis of the sequenced PCR product and the deduced amino acid sequence was done by BLAST with the 3D7 strain (Plasmo DB database). The antigenicity and the hydrophilicity property of the deduced amino acid sequence were found out (Hopp et al. 1981; Kyte et al. 1982).

For study of identities, similarities and conserved regions of PfNT3, homology of the deduced PfNT3 amino acid sequence was searched by multiple sequence alignment (Clustal W2.0 program) after downloading different nucleoside transporter homologues from NCBI. The phylogenetic relationship was observed with PhyloDraw ver. 0.8. Topology was studied to predict the number of transmembrane helices, positions of the N-terminal, the C-terminal and distribution of amino acids in different regions of the translated product (TMHMM software).

PFNT3 ANTIBODIES

A 108 bp fragment of the *PfNT3* open reading frame encoding the 36 NH₂-terminal amino acids of PfNT3 was amplified by polymerase chain reaction using a sense primer - 5' - CGGGA TGGATCCATGAGTGACAGTCAG - 3',

and anti-sense primer - 5' - CAAAAGT ATCCTCGAGTTCAATATCACAAGG -3' (Bangalore Genei, India) with engineered BamHI and XhoI restriction sites (strike through), respectively. The optimized PCR conditions were initial denaturation at 98°C for 10 secs, annealing at 55°C for 10 secs, extension at 72°C for 10 secs and a final extension at 72°C for 5 mins. The PCR product was identified by 1.5% agarose gel electrophoresis and sequencing.

To generate an antigen for screening and purifying PfNT3-specific antibodies, the purified PCR product of *PfNT3-1-108* sequence was cloned to pGEMT cloning vector, sub cloned into BamHI, XhoI sites of pGEX-4T-1 (Amersham, USA) expression plasmid vector to create pGEX-4T-1-*PfNT3-1-108* recombinant construct. Sequencing of cloned recombinant product was done to confirm proper ligation and reading frame. The recombinant construct was transformed into *E. coli* BL21 (DE 3) expression host for expression of the GST tagged fusion protein as described (Rabhi-Essafi et al. 2007). Expression was induced with 0.1 mM Isopropyl β-D-thiogalactoside. The expressed soluble recombinant fusion protein was analysed by SDS-PAGE and purified as per the New England Bio labs brochure. The NH₂ terminal polypeptide of 36 amino acids was separated from the GST tagged recombinant protein by thrombin enzyme digestion and passage through the GST affinity column. The purified fusion protein was sent to Bangalore Genei, India for polyclonal antisera production. The antibodies were screened initially against the pre-immune serum and pGEX-4T-1-

PfNT3-1-108 proteins by direct ELISA and Western blotting. Antibodies were purified using antigen immobilized agarose by Cyanogen Bromide method following manufacturer's protocol. Sensitivity and specificity of antibodies in the polyclonal sera was detected by ELISA and Western blotting. The pelleted parasites were lysed with Triton X-100 and sonicated gently for isolation of the parasite membrane fraction. Proteins were fractionated by 10% SDS PAGE.

LOCALIZATION

IMMUNOBLOTTING

SDS PAGE fractionated membrane pellete, GST tag removed expressed protein, GST and preimmune serum were transferred to a polyvinylidene fluoride membrane (Millipore) by semidry blot method. The membrane was blocked with 5% milk powder (Millipore) in 1x TBS. Probing was done with purified developed primary antibody (dilution,1:1,000) and horseradish peroxidase conjugated Goat anti-rabbit IgG secondary antibody (dilution,1:20,000). A super signal west Pico Chemiluminescence kit (Thermo Scientific) was used to detect the proteins following manufacturer's protocol.

IMMUNOFLUORESCENCE MICROSCOPY

Slides for IFA were prepared as per standard procedure of slide preparation (Nicolle et al. 2001). In brief, RBCs harbouring an asynchronous culture of *P. falciparum* were washed twice in

PBS, placed onto coverslips and dried at room temperature. Smears were fixed in ice cold acetone/methanol (70:30) at -20°C for 5 mins, followed by rinsing 3 times in PBS and blocking in 2% (w/v) BSA. A PBS wash buffer containing 0.2% BSA, 2% goat serum was used for subsequent washes and incubations. Coverslips were incubated with purified PfNT3 antibodies (dilution: 1:50) for 4 h at room temperature. Controls included fixed parasites incubated with pre-immune antiserum. Smears were further treated with fluorescein isothiocyanate (FITC) conjugated secondary goat anti-primary antibody at 1: 300 dilutions for 1hr following the washing step. The cover slips were rinsed again with PBS washing buffer followed by incubation in 10 uM final concentration of Hoechst stain (Sigma) for 5 min. Mounting of cover slips on slides was done using vectashield mounting media (Vector Laboratories). Images were captured at intervals of 0.3 uM along the Z axis using a Leica TCS SP5 AoBS Confocal microscope. Analysis of images was done by the LAS AF software version 2.0 at Centre for Cellular and Molecular Biology, Hyderabad, India.

IMMUNOELECTRON MICROSCOPY

Pelleted parasitized RBCs in a solution of 4% paraformaldehyde, 200 mM PIPES and 0.5 mM MgCl₂, pH 7.0 were fixed on ice for one hour. The cell pellete was fixed for 30 minutes at room temperature in 10% gelatin after rinsing in PIPES buffer. Fixed samples were incubated overnight at 4°C in 20% polyvinylpyrrolidone and 2.3 M sucrose

in PIPES buffer. Samples were rapidly frozen in liquid ethane, cryosectioned and collected on carbon-Formvar-coated grids at Centre for Cellular and Molecular Biology, Hyderabad, India. Blocking was done with 5% foetal bovine serum, 5% goat serum in PIPES buffer followed by incubation in purified primary antibody (1:50 dilution) for 1hr at room temperature. Grids were rinsed, incubated with anti-rabbit secondary antibodies conjugated to 10 nm gold particles. Counterstaining was done with 0.3% uranyl acetate and 2% polyvinyl alcohol. Images were viewed on a 200 KV transmission electron microscope.

RESULTS

ANALYSIS OF *PfNT3* AND THE TRANSLATIONAL PRODUCT

The *P. falciparum* PfNT3 protein is a polypeptide of 437 amino acids encoded by a 1314 bp segment of DNA. Agarose gel electrophoresis of amplified PCR product detected a 1.3 kb DNA (Figure 1) and approximately a 0.1kb band (Figure 2). The whole sequence analysed was significantly matching with the sequence of *PfNT3* in PlasmoDB data base.

CHARACTERIZATION OF ANTIGEN

Hydrophobicity profile of the deduced amino acid sequence by Kyte and Doolittle hydropathy plot (data not shown) indicated PfNT3 to be a highly hydrophobic protein with 11 trans membrane helices. Predicted

by homology study (Clustal W2.0 program, Figure 3), the deduced amino acid sequence was homologous to a number of ENTs, such as hENT1, hENT2, TbNT2, LdNT1.1, TgAT, PfENT1 and Fun26. T, D, P, R & G amino acid residues were identical in particular regions of sequences but frequency of G was more. L, N, E, F, R & S amino acids were observed as conserved substitutions with more frequency off. Semi conserved substitutions were also observed. More homology was found

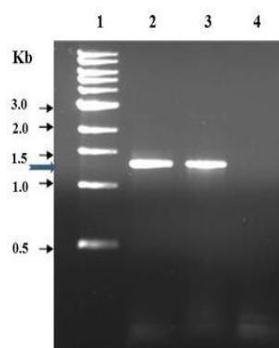


Figure 1: Analysis of PfNT3 PCR products on 0.8% agarose gel electrophoresis. Lane 1-Molecular marker lane; Lane 2 & 3-1.3Kb PCR product; Lane 4-negative control

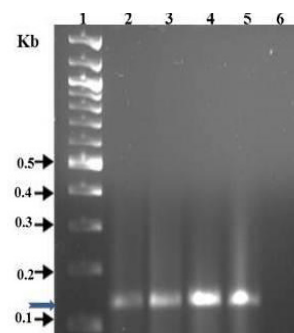


Figure 2: Analysis of PfNT3-1-108 sequence PCR products on 1.5% agarose gel electrophoresis. Lane 1-Molecular marker lane; Lane 2 - 5-PCR product of N-terminal; Lane 3-6-negative control



Figure 3: Homology study by alignment of PF14_0662 amino acid sequence with other nucleoside transporter homologues (By ClustalW2.0)

with nucleoside transporter homologues of PfENT1 and Fun26 (Figure 3). Obtained PfNT3 sequence was more related to Yeast nucleoside transporter FUN26 and PfENT1 (Figure 4). Predicted topology analysis (TMHMM software) of the deduced amino acid sequence of PfNT3 presented an integral membrane protein with eleven trans membrane helices and an intracellular hydrophilic region between helices 6 and 7. It also revealed a cytoplasmic NH2 terminal of 49 amino acids and a short carboxyl terminal of 8 amino acids lying outside the membrane (Figure 5).

CHARACTERIZATION OF ANTIGEN AND ANTIBODY SPECIFICITY

The expressed GST tagged PfNT3-1-108 protein was of approximately 31.6 kDa as estimated from SDS-PAGE analysis (Figure 6). Purified polyclonal antibodies recognized a polypeptide

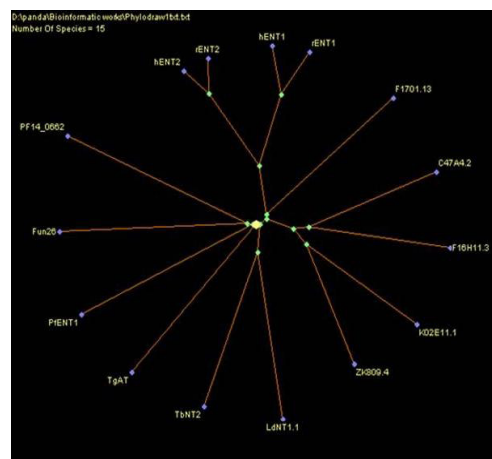


Figure 4: Phylogenetic tree showing relationship between PF14_0662 with other nucleoside transporter proteins by Phylo draw ver. 0.8. PF14_0662-*P. falciparum* nucleoside transporter; Fun26-nucleoside transporter from *Saccharomyces cerevisiae*; PfENT1-the first nucleoside transporter from *P. falciparum*; TgAT-nucleoside transporter from *Toxoplasma gondii*; TbNT2-the P1 type transporter from *T. brucei*; LdNT1.1-Leishmania *donovani* transporter-1; ZK809.4, K02E11.1, F16H11.3, C47A4.2-nucleoside transporter from *C. elegans*; F1701.13-a nucleoside transporter from *Arabidopsis thaliana*

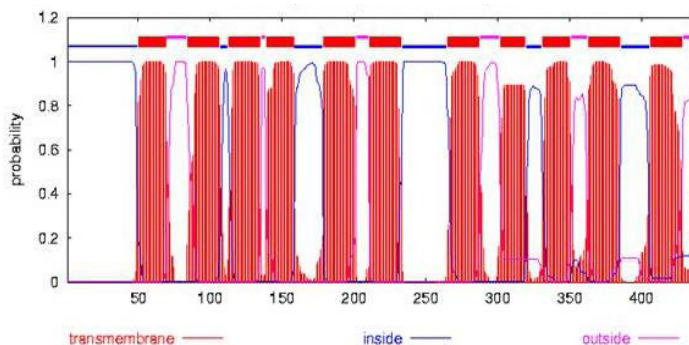


Figure 5: Topology prediction of PfNT3 by using TMHMM, an ExPASy Proteomics tool

with a molecular mass of 31.6 kDa (Figure 7-A). The single polypeptide in immunoblotting with thrombin digested GST tag removed expressed protein was recognized as a band of size 5.6 kDa in Figure 7-B. The purified antibodies recognized only a single polypeptide of approximately 50.9kDa on a Western blot parasite membrane lysate preparation (Figure 7-C, Lane-2). The parasite lysate did not reveal any band with GST used as negative control protein (Figure 7-C, Lane-1) and pre immune antiserum (Figure 7-D).

IMMUNOFLUORESCENCE MICROSCOPY STUDY

Immunofluorescence confocal microscopic images demonstrated the three dimensional distribution of PfNT3 in parasitized RBCs (Figure 8). Localization was in the region of the parasite as shown by the proximity of the parasite nuclear (Hoechst) stain (Figure 8, Panels 2, A-D) and dark brown haemozoin pigment in the bright field (Figure 8, Panels 1-A, and C, D). PfNT3 stained with FITC-conjugated secondary antibodies detected the protein in ring (Figure 8, panels B-3, 4),

trophozoite (Figure 8, panels C-3, 4) and schizont (Figure 8, panels D-3, 4) stages of the parasite. All optical sections detected signals on the surfaces of intraerythrocytic ring, trophozoite and schizont stages of infected parasites in antibody treated slides (Figure 8, Panels 3, 4, B-D). It was neither expressed in erythrocyte cytosol nor in RBC membrane. A honeycomb staining pattern was observed in the mature schizonts (Figure 8, panels D-3, 4). There were no signals in all preimmune serum treated slides taken as control (Figure 8, Panels 1-4,A). A large number of cells examined showed similar results.

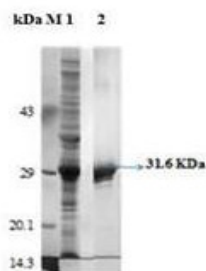


Figure 6: Analysis of expressed PfNT3 recombinant protein on 10% SDS PAGE. Lane 1-Expressed protein; Lane 2-Purified protein

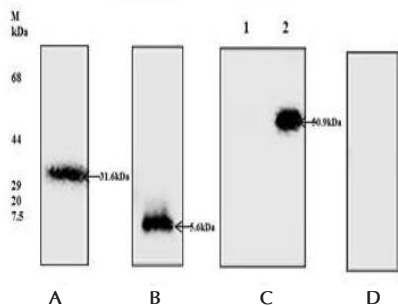


Figure 7: Specificity of PfNT3 antibodies. Immunoblot analysis with antibodies against A-Purified fusion protein (antigen); B-NH2-terminal sequence after thrombin digestion; C-Lane 1-GST (negative control), Lane 2-Membrane fraction; D-Preimmune serum

IMMUNOELECTRON MICROSCOPY

Further localization study was done by transmission electron microscopy using ultrathin cryosections of the parasites (Figure 9). Gold particles localized predominantly to the Parasite Plasma Membrane. No immunogold labelling was observed at the RBCM, TVM or

any internal organelle including the endoplasmic reticulum (Figure 9).

DISCUSSION

Trans membrane transport is a critically important physiological process in all cells. Membrane transporters are responsible for the uptake of essential nutrients, modulations of concentrations of physiologically relevant chemicals. Parasite transporters are of great interest as drug targets. ENTs are a unique family of proteins with no apparent sequence homology to other types of transporters, which enable facilitated diffusion of nucleosides, nucleoside analogs used in chemotherapy across cell membranes (Hyde et al. 2001). Selective inhibition of parasite protein synthesis prevents the formation of the NPP suggesting that it originates from parasite proteins (Carter et al. 2000). With the goal of generating inhibitors specific for the target proteins of the parasite, several critical and unrelated biochemical pathways have been

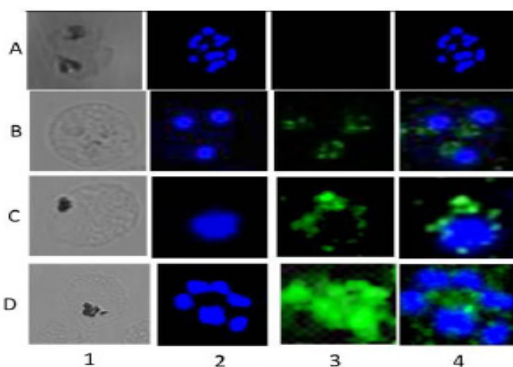


Figure 8: Smear analysis by deconvoluted immunofluorescence microscopy of PfNT3 in *P. falciparum* infected red blood cells. A-Control slides (Fixed parasites incubated with Pre-immune antiserum); B-D-With PfNT3 antibodies followed by FITC Conjugated (Green)-secondary antibodies; B-three parasites in ring stage in a RBC; C-a trophozoite; D-parasites in a Schizont; 1-Bright field showing haemozoin pigment (dark spot); 2-Hoescht nuclear stain (Blue) indicating parasites; 3-PfNT3 protein (Green); 4-Merge of 2 and 3

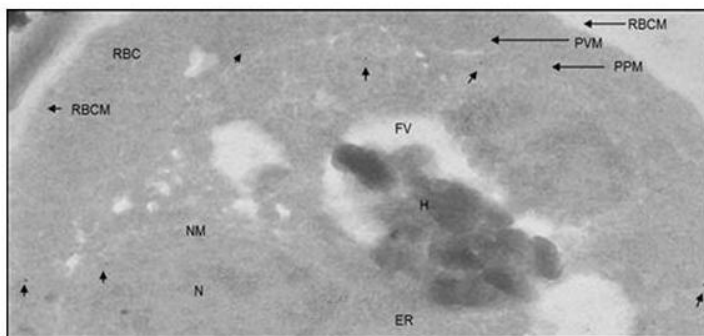


Figure 9: Transmission Electron Micrograph of ultrathin cryo-sections of the intraerythrocytic trophozoite stage of *P. falciparum* using PfNT3 antibodies. Magnification is x12000 Immunogold labelling (10-nm gold particles) of PfNT3 antibodies bound to intraerythrocyte *P. falciparum* is depicted with arrowheads. N-nucleus; NM-nuclear membrane; PPM-parasite plasma membrane; PVM-parasitophorous vacuolar membrane; RBCM-red blood cell membrane; FV-food vacuole; H-hemozoin; ER-endoplasmic reticulum

exploited for drug target identification (Cunha-Rodrigues et al. 2006).

ANALYSIS OF *PfNT3* AND THE TRANSLATIONAL PRODUCT

The completely identical sequences of the *PfNT3* and *PfNT3-1-108* PCR products with sequences in Plasmo DB data base established the identity of the gene taken for study of PfNT3 nucleoside transporter protein.

CHARACTERIZATION OF ANTIGEN

A 108bp coding for polypeptide of 36 amino acids at the N-terminal of the protein was selected for cloning and expression because of highest local average hydrophilicity/antigenicity (Cunha-Rodrigues et al. 2006) and a hexapeptide is also the approximate size of an antigenic determinant (Hopp et al. 1981). The deduced amino acid sequence predicted homology but not complete identity with other nucleoside transporter homologues (Figure 3).

Predicted PfNT3 sequences were specific as they were not completely matching to ENT sequences of other nucleoside transporter homologues (Figure 4). These results proved the expressed protein to be unique; validating it could be a site of novel drug target. Interpretation of Figure 5 is similar to the domain organization of ENT family members also.

CHARACTERIZATION OF ANTIGEN AND ANTIBODY SPECIFICITY

The expressed 31.6 kDa protein as estimated from SDS-PAGE analysis (Figure 6) was close to the calculated molecular mass of the encoded GST tagged fusion protein, the protein used as antigen. Recognized polypeptide with a molecular mass of 31.6 kDa in Figure 7-A corresponded with the PfNT31-108 GST tagged fusion protein size in the data base using ExPASy tool (www.expasy.ch, compute pI/MW). The revealed polypeptide of 5.6

kDa in immunoblotting with thrombin digested GST tag removed expressed protein in Figure 7-B also matched with the calculated molecular weight of the encoded protein (compute pI/MW). The polypeptide on Western blot parasite membrane lysate preparation (Figure 7-C, Lane-2) was also consistent with the molecular mass in the database. Specificity of the antibodies for PfNT3 epitopes was confirmed because the parasite lysate did not cross react with GST (Figure-7-C, Lane-1) and preimmune antiserum (Figure 7-D). These results proved that the antibodies specifically showed up the protein used for antibody development. These observations proved the presence of parasite genome encoded endogenously synthesized PfNT3 protein of parasite membrane origin in the *P. falciparum* parasites.

IMMUNOFLUORESCENCE MICROSCOPY STUDY

Presence of PfNT3 in parasitized RBCs was detected by reaction of the expressed protein with the antibodies (Figure 8). The honeycomb staining pattern observed in the mature schizonts (Figure 8, panels D-3, 4) was indicative of PPM staining of developing individual merozoites because the PVM circumscribes the whole collection of merozoites. This staining pattern was similar to the observation of PfNT1 protein (Nicolle et al. 2001) and the reticulated staining pattern of PfHT1 protein shown to be associated with PPM (Woodrow et al. 1999).

IMMUNOELECTRON MICROSCOPY

Immunoelectron microscopic study confirmed the membrane to which PfNT3 was localized. This was indicated by visualization of immunogold particles on the PPM (Figure 9).

CONCLUSION

Results of bioinformatics study on homology with ENT homologues, phylogeny and topology indicated PfNT3 probably to be a nucleoside transporter. This may have an important role in the survival of the parasite for being a route for translocation of the nucleosides/nucleobases, the vital nutrients essential for synthesis of the DNA and RNA of the parasite. Thus, it may have an important role in the growth and survival of the parasite. Immunoblotting, immunofluorescence and immunoelectron microscopy together established PfNT3 to be an integral membrane protein. PfNT3 appeared to be localized primarily to the PPM, although the possibility cannot be ruled out that it may be a minor constituent of other structures and uptake components of the complex nucleoside permeation pathway of *P. falciparum*. So, they may not only serve as routes for the targeting of purine analogs/cytotoxic agents into the intracellular parasite, but may also serve as antimalarial drug targets in their own right. Specific inhibitors can be designed against the transporter protein for inhibition of growth and survival of the malaria parasites. The parasite genome encoded protein

being expressed in all the blood stages in human RBC can be prevented from expression by silencing of the gene by specific RNA interference or by gene editing (Crispr), thereby inhibiting the growth and survival of the parasite. With probable localization in the parasite plasma membrane the vital protein poses to be a novel molecular drug target for control of the lethal malaria caused by *P. falciparum*.

Characterization of PfNT3 clone, a molecular component of complete nucleoside permeation pathway in *Plasmodium falciparum* may thus be validated as a new molecular chemotherapeutic target.

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