Identification of a nucleoside/nucleobase transporter from *Plasmodium falciparum*, a novel target for anti-malarial chemotherapy

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Plasmodium, the aetiological agent of malaria, cannot synthesize purines *de novo*, and hence depends upon salvage from the host. Here we describe the molecular cloning and functional expression in *Xenopus* oocytes of the first purine transporter to be identified in this parasite. This 422-residue protein, which we designate PfENT1, is predicted to contain 11 membrane-spanning segments and is a distantly related member of the widely distributed eukaryotic protein family the equilibrative nucleoside transporters (ENTs). However, it differs profoundly at the sequence and functional levels from its homologous counterparts in the human host. The parasite protein exhibits a broad substrate specificity for natural nucleosides, but transports the purine nucleoside adenosine with a considerably higher apparent affinity

(K_m 0.32 ± 0.05 mM) than the pyrimidine nucleoside uridine (K_m 3.5 ± 1.1 mM). It also efficiently transports nucleobases such as adenine (K_m 0.32 ± 0.10 mM) and hypoxanthine (K_m 0.41 ± 0.1 mM), and anti-viral 3'-deoxynucleoside analogues. Moreover, it is not sensitive to classical inhibitors of mammalian ENTs, including NBMPR {6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine, or nitrobenzylthioinosine} and the coronary vasoactive drugs, dipyridamole, dilazep and drafazine. These unique properties suggest that PfENT1 might be a viable target for the development of novel anti-malarial drugs.

Key words: adenosine, hypoxanthine, malaria, protozoa, transport.

INTRODUCTION

Malaria, resulting from infection by species of *Plasmodium*, is responsible for the highest mortality of any parasitic disease. This protozoan parasite causes more than 300 million clinical cases per annum, resulting in more than 1.5 million deaths worldwide in tropical and subtropical areas [1], with the species *Plasmodium falciparum* responsible for the majority of deaths. Unfortunately, resistance to existing anti-malarial drugs is spreading rapidly. Consequently in some areas, such as Southeast Asia, treatment options are becoming increasingly limited [2]. The identification of new drug targets is therefore an important goal, and is one of the key objectives of the Malaria Genome Sequencing Project [3].

Unlike many mammalian cells, the intra-erythrocytic stages of *Plasmodium* are incapable of *de novo* synthesis of the purine ring, and so are dependent upon salvage pathways for purine nucleotide synthesis from host precursors. The latter in particular include the nucleoside adenosine and the nucleobase hypoxanthine [4]. Purine transport and metabolism are therefore of key importance as potential targets for the development of new anti-malarial drugs. Several enzymes in the purine salvage pathway have been characterized and their genes cloned, including most recently GMP synthase [5]. However, *Plasmodium* purine transporters have not hitherto been characterized in detail.

In contrast with the dependence on purine salvage, *Plasmodia* rely on *de novo* pyrimidine synthesis and salvage has not been observed. This may be due to either lack of uptake or deficiency

of salvage enzymes. Several of the currently used anti-malarial drugs target *de novo* pyrimidine metabolism in *Plasmodium* via effects on the folate pathway or upon the ubiquinone-based electron-transport system [4]. For example, sulfadoxine is an inhibitor of the protozoan dihydropteroate synthase, while pyrimethamine and cycloguanil inhibit dihydrofolate reductase.

In eukaryotes, the cellular uptake of purine nucleosides is brought about by members of the concentrative nucleoside transporter (CNT) and equilibrative nucleoside transporter (ENT) families [6]. The mammalian ENT transporters, which are more widely distributed than the CNT transporters, can be subdivided further into two subclasses on the basis of their sensitivity to the transport inhibitor 6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine (nitrobenzylthioinosine, NBMPR) [7,8]. Transporters of the *es* (equilibrative sensitive) subclass are inhibited by nanomolar concentrations of NBMPR (K_i 0.1–10 nM). In contrast, transporters of the *ei* (equilibrative insensitive) subclass are relatively insensitive to NBMPR even at micromolar concentrations. In general, transporters of the *es* type are also inhibited potently by the coronary vasodilators dipyridamole, dilazep and lidofazine analogues, such as drafazine, whereas *ei*-type transporters are less sensitive to these inhibitors [7,8].

An equilibrative adenosine transporter related to the mammalian ENTs has recently been identified in the protozoan parasite *Toxoplasma gondii* [9]. Members of the ENT family have also been identified in the parasitic kinetoplastid protozoa *Trypanosoma* and *Leishmania*, but in these organisms they appear

Abbreviations used: AZT, 3'-azido-3'-deoxythymidine; CCCP, carbonyl cyanide m-chlorophenylhydrazone; CNT, concentrative nucleoside transporter; ddC, 2',3'-dideoxycytidine; ddl, 2',3'-dideoxyinosine; *ei*, equilibrative insensitive; ENT, equilibrative nucleoside transporter; hENT, human ENT; PfENT1, *Plasmodium falciparum* ENT 1; *es*, equilibrative sensitive; NBMPR, 6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine; TM, transmembrane segment.

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Figure 1 For legend see facing page

(B)

(C)

Figure 1 PfENT1 is a member of the ENT family of nucleoside transporters

(A) Alignment of the predicted amino acid sequence of PfENT1 with those of nucleoside transporters from *Toxoplasma gondii* (TgAT, GenBank accession no. AF061580), *Trypanosoma brucei* (the P2-type transporter TbAT1, AAD45278), *Homo sapiens* (hENT1, AAC51103, and hENT2, AAC39526), *Arabidopsis thaliana* (F1701.13, AAC18807), *Caenorhabditis elegans* (ZK809.4, CAA92642) and *Saccharomyces cerevisiae* (FUN26, S36712). The positions of putative transmembrane regions are shown by the boxes, and residues identical in four or more of the sequences are indicated by black shading. (B) Topographical model of PfENT1. Potential membrane-spanning α -helices are numbered and the potential sites of N-glycosylation indicated by asterisks. The positions of basic (Arg, Lys, His), acidic (Asp, Glu) and polar but uncharged residues (Ser, Thr, Gln, Asn) are indicated by +, - and ● respectively. (C) Phylogenetic tree showing relationships between PfENT1 and homologues in other eukaryotes. In addition to those detailed in (A), these include the rat transporters rENT1 (accession no. AAB88049) and rENT2 (AAB88050), the P1-type transporter TbNT2 from *T. brucei* (AF153409), the *Leishmania donovani* transporter LdNT1.1 (AF065311) and the putative *C. elegans* transporters F16H11.3 (AAA98003), C47A4.2 (accession no. for cosmid C47A4 is Z82263) and K02E11.1 (CAB01223). Genetic distances were estimated using the program ProtDist (Categories model) and the phylogeny was estimated from the resultant distance matrix using the program Fitch, both programs forming part of the PHYLIP package, version 3.5c [40].

to function as active transporters, catalysing the symport of nucleosides (and in some instances also nucleobases) with protons [6,10–13]. Additional transporter families specific for nucleobases have also been characterized by molecular cloning in fungi [14], but representatives of these families have not so far been identified definitively as functional nucleobase transporters in either mammals or protozoa.

In the *Plasmodium*-infected erythrocyte, the permeability of the host cell membrane to purine nucleosides and other organic solutes is markedly increased via the induction of a non-saturable permeation pathway, which has been the subject of intensive investigation [15]. Much less is known about the transport characteristics of the parasite plasma membrane itself. However, expression of mRNA from the asexual stages of *P. falciparum* in *Xenopus* oocytes has provided evidence that the parasite genome encodes nucleoside, nucleobase, monocarboxylate and hexose transporters [16], and an example of the last has recently been cloned [17]. In the present work, we have exploited the public availability of sequence information from the Malaria Genome Sequencing Project to clone, express and characterize a novel nucleoside/nucleobase transporter from *P. falciparum*. It may be possible to exploit the functional characteristics of this protein, which differ substantially from those of its mammalian counterparts, for the development of new anti-malarial drugs.

MATERIALS AND METHODS

DNA cloning and analysis

The coding region of the predicted *PfENT1* gene (coding for the *P. falciparum* ENT 1 protein) was amplified by PCR from *P. falciparum* (strain 3D7) genomic DNA using *Pwo* polymerase and primers PLFA1 (5'-CGAGGATCCAAAAATGAGTACCGGTAAAGAGTC-3') and PLFA2 (5'-CGAGAATTCTTATTGTGTTACATCGATGGGTGG-3'), corresponding to the 5' and 3' ends of the region respectively. Reactions were cycled with the following parameters: 96 °C for 5 min; 96 °C for 30 s, 60 °C for 30 s, 68 °C for 3 min for 25 cycles; 68 °C for 3 min. The resultant $\approx$ 1.3-kb fragment was subcloned into the *Xenopus* expression vector pGEM-HE [18] using *Bam*HI and *Eco*RI restriction sites (underlined above) incorporated into the 5' ends of PLFA1 and PLFA2 respectively to yield the construct pGEMHE-PfENT1. The entire coding region of the insert was sequenced at least once on both strands by *Taq* DyeDeoxy terminator cycle sequencing using an Applied Biosystems model 373A sequencer.

Gene organization and expression

P. falciparum (strain 3D7) was maintained in culture with human erythrocytes (5% haematocrit) in RPMI 1640 (Life Technologies) supplemented with Hepes, sodium bicarbonate and human serum (10%) under standard conditions [19,20]. Genomic DNA was isolated using a standard protocol from saponin-lysed infected erythrocytes [21]. For Southern blotting, samples of genomic DNA (10 μ g) were digested with 30 units of the appropriate restriction enzyme and blots were hybridized, using standard protocols [21], with a probe corresponding to the coding region of the *PfENT1* gene. The [α -³²P]dCTP-labelled probe was prepared by random priming using the RTS RadPrime DNA Labelling System (Life Technologies). For reverse-transcriptase PCR, *Plasmodium* RNA isolation and first-strand cDNA synthesis using either random hexamers or oligo(dT) was performed as described previously [22]. PCR amplification of PfENT1 cDNA (20 ng) was then performed using *Pwo* poly-

merase, the primers PLFA1 and PLFA2 and the cycling parameters described above.

Xenopus expression and nucleoside/nucleobase-uptake assays

Plasmid pGEMHE-PfENT1 was linearized with *Nhe*I and transcribed with T7 polymerase in the presence of ^{m7}GpppG cap using the mMESSAGE MACHINE[®] (Ambion) transcription system. Remaining template was removed by digestion with RNase-free DNase 1. Oocytes were treated with collagenase to remove follicular layers [23] and then injected with 23 nl of water alone or with 23 nl of water containing 1 mg/ml PfENT1 RNA transcript. Injected oocytes were maintained for 2 days in modified Barth's medium containing 5% horse serum, and then uptake of radiolabelled nucleoside and nucleobase compounds was measured ([5,6-³H]uridine and [2,8-³H]adenosine were from NEN[®] Life Science Products, Hounslow, Middx., U.K.; [8-¹⁴C]adenine and [2-¹⁴C]uracil were from ICN Pharmaceuticals, Basingstoke, Hants., U.K.; [³H]gemcitabine (2',2'-difluoro-2'-deoxycytidine) was from Eli Lilly and Co., Indianapolis, IN, U.S.A.; all other compounds, Amersham, Arlington Heights, IL, U.S.A. or Moravek, Brea, CA, U.S.A.).

Uptake assays were performed at 20 °C on groups of 10–12 oocytes in transport buffer (0.2 ml) containing 100 mM NaCl (or 100 mM choline chloride) and 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ and 10 mM Hepes, pH 7.5. In adenosine-uptake experiments, the transport buffer contained 1 μ M deoxycofomycin to inhibit adenosine deaminase activity. For experiments involving NBMPR, dilazep, dipyrindamole and draflazine, oocytes were preincubated for 1 h with inhibitor before the addition of permeant. At the end of the uptake period, extracellular label was removed by six rapid washes with ice-cold transport buffer. Individual oocytes were dissolved in 5% (w/v) SDS for quantification of radioactivity by liquid-scintillation counting. The influx values reported represent the means $\pm$ S.E.M. for 8–10 oocytes. Each experiment was performed at least twice on different batches of oocytes, with similar results, and a representative example is illustrated. Apparent K_m and V_{max} values for substrate influx were determined by non-linear regression analysis (FigP, Elsevier-Biosoft).

RESULTS

Cloning of a *Plasmodium* ENT homologue

Nucleoside transporters do not appear to be encoded by any of the genes present in *P. falciparum* chromosomes 2 and 3, the complete nucleotide sequences of which have been published [24,25]. We therefore used the on-site BLAST server at the Sanger Centre, Cambridge, U.K., to search the unfinished sequence data arising from the Malaria Genome Sequencing Project (http://www.sanger.ac.uk/Projects/P_falciparum/) for sequences homologous to the ENT or CNT families. This approach led to the identification of fragments encoding a putative ENT homologue in the shotgun-read sequence database for chromosome 13. By searching the database for overlapping fragments it proved possible to assemble the complete coding region of a putative nucleoside-transporter gene. PCR was used to amplify the corresponding region from genomic DNA of *P. falciparum* clonal strain 3D7, yielding a fragment of apparent size $\approx$ 1.3 kb. Following its subcloning into the vector pGEMHE [18], the complete nucleotide sequence of the fragment was determined (GenBank accession no. AF221844) and found to be identical to that assembled from the chromosome 13 shotgun-read sequence database.

Figure 2 Southern blot of *PfENT1* in genomic DNA

P. falciparum genomic DNA (10 µg) was digested with *EcoRI* (lane 1), *HindIII* (lane 2), *DraI* (lane 3), *XbaI* (lane 4), *EcoRV* (lane 5) and *KpnI* (lane 6), and blots were hybridized with a 32 P-labelled probe corresponding to the coding region of *PfENT1*. The mobilities of standards of known size (kb) are indicated on the left.

In order to examine the structure of the gene encoding the transporter, which we have designated PfENT1, reverse-transcriptase PCR was used to analyse mRNA obtained from the asexual blood stages of *P. falciparum*. Amplification of cDNA, prepared by reverse transcription using either oligo(dT) or random hexamer oligonucleotides, with primers corresponding to the 5' and 3' ends of the predicted coding region (PLFA1 and PLFA2 respectively), yielded a product of ≈ 1.3 kb, identical

with that obtained by amplification of genomic DNA (results not shown). This finding confirmed the predicted absence of introns in the *PfENT1* gene, and indicated that the gene is expressed during the intra-erythrocytic stage of the *Plasmodium* life cycle.

The *PfENT1* gene contains a 1266-bp open reading frame encoding a 422-residue protein, PfENT1, of predicted M_r 47 631 (Figure 1A). PfENT1 is predicted by the hidden Markov model procedure of Sonnhammer et al. [26] to possess 11 transmembrane (TM) segments, with the hydrophilic N-terminal region of the protein and a hydrophilic central loop being cytoplasmic (Figure 1B). While two of the five potential N-glycosylation sites in the sequence are predicted to lie on the exofacial surface of the membrane, their proximity to the start of putative TM8 suggests that they are unlikely to be glycosylated *in vivo*. The presence of characteristically conserved sequence motifs, especially in the putative membrane-spanning segments (Figure 1A), confirms that PfENT1 is a distantly related member of the ENT family of equilibrative nucleoside transporters (Figure 1C), despite sharing only about 18% amino acid sequence identity with known mammalian, nematode, yeast and protozoan nucleoside transporters. Striking differences between PfENT1 and most of the other family members are the relative shortness of the putative extramembranous regions connecting TM1 and TM2 [4 residues compared with 41 in human ENT (hENT) 1] and TM6 and TM7 (35 residues compared with 66 residues in hENT1).

In the genomes of mammals, nematodes and kinetoplastid protozoa, families containing two or more related ENT genes have been identified [6,10–12]. In order to establish whether a similar situation exists in *Plasmodium*, Southern blots of *P. falciparum* genomic DNA were hybridized with a probe corresponding to the coding region of *PfENT1*. Even at low stringency the restriction digests, with the exception of *EcoRV*, which cleaves within the gene (and *DraI*, which generates fragments too small to be detected), yielded a single band (Figure

Figure 3 Functional expression of PfENT1 in *Xenopus* oocytes

(Left-hand panel) Time course for uptake of 0.1 mM [3 H]adenosine in oocytes injected with water alone (○) or water containing 23 ng of PfENT1 transcript (●). (Right-hand panel) Concentration dependence of [3 H]adenosine uptake by oocytes injected with water alone (○) or water containing PfENT1 transcript (●). Inset, mediated transport (influx of adenosine in RNA-injected oocytes minus that in water-injected oocytes).

2). This finding suggests that *PfENT1* is the sole representative of the ENT gene family in *Plasmodium*.

Functional characterization of PfENT1

To investigate functional properties, PfENT1 recombinant protein was expressed in *Xenopus* oocytes, which lack endogenous nucleoside-transport activity [27]. The rate of adenosine uptake was measured for comparison with mammalian homologues. As shown in Figure 3 (left-hand panel), the initial rate of uptake of the purine nucleoside [^3H]adenosine (0.1 mM, 20 °C) was much greater in oocytes injected with PfENT1 RNA transcripts than in oocytes injected with water. Uptake of adenosine (and of the other nucleosides described below) was essentially linear for 1 h, and so in subsequent experiments uptake periods of either 30 min (for assessment of substrate specificity) or of 10 min (for measurement of kinetic parameters) were employed in order to approximate initial rates of transport. PfENT1-mediated influx of the purine nucleoside adenosine, defined as the difference in uptake between RNA-injected and water-injected oocytes, was saturable and conformed to simple Michaelis–Menten kinetics (Figure 3, right-hand panel). The apparent K_m value for adenosine (0.32 ± 0.05 mM) was somewhat greater than values reported previously for mammalian cells (0.05–0.15 mM) [28]. PfENT1 also mediated the uptake of the pyrimidine nucleoside uridine. Interestingly, whereas the $V_{\max}$ value for uridine uptake was somewhat greater than that for adenosine (358 ± 38 and 228 ± 13 pmol/oocyte/10 min, respectively), the apparent affinity for the pyrimidine nucleoside was considerably lower, the measured K_m for uridine uptake (3.5 ± 1.1 mM) being 10-fold greater than for adenosine (results not shown).

Substrate selectivity of PfENT1

The substrate selectivity of PfENT1 was examined further by direct measurements of the mediated influx of a range of natural nucleosides and nucleobases, which were tested at a concentration of 0.1 mM. Figure 4 (top panel) shows that, like its mammalian counterparts [6], PfENT1 exhibits a broad substrate specificity for purine and pyrimidine nucleosides, with the exception of cytidine, which is poorly transported. Cytidine has also been shown to be a relatively poor competitive inhibitor of rat ENT 2 (rENT2)-mediated uridine transport [29]. Surprisingly, PfENT1 was in addition found to transport a wide range of purine and pyrimidine nucleobases, including cytosine (Figure 4, top panel). In order to characterize this nucleobase-transport activity in more detail, the concentration dependence of PfENT1-mediated adenine transport was compared with that of the corresponding nucleoside, adenosine. As illustrated in Figure 4 (middle panel), mediated uptake of adenine was saturable, and the apparent K_m for the uptake process (0.32 ± 0.10 mM) was essentially identical with that for adenosine. The rates of adenine and adenosine uptake at a substrate concentration of 0.1 mM, measured using a single batch of oocytes, were also similar (Figure 4, top panel), suggesting that the turnover numbers for transport of the nucleobase and nucleoside are comparable. PfENT1-mediated uptake of the physiologically important nucleobase hypoxanthine was also found to be saturable (results not shown), and the apparent K_m for the uptake process (K_m 0.41 ± 0.1 mM) was similar to that for adenine. These properties of PfENT1 contrast with those of its mammalian homologues: the human erythrocyte transporter hENT1 does not transport nucleobases, and whereas the ei-type transporter hENT2 is capable of transporting hypoxanthine and other nucleobases, it exhibits generally lower apparent affinities for these substrates than for the corresponding

nucleosides (S. Y. M. Yao, C. E. Cass, S. A. Baldwin and J. D. Young, unpublished work).

In addition to transporting natural nucleosides, the mammalian *es*-type transporters catalyse the cellular uptake of nucleoside analogues used in cancer chemotherapy. These include the purine nucleoside drugs fludarabine (9- β -D-arabinosyl-2-fluoroadenine) and cladribine (2-chloro-2'-deoxyadenosine) and the pyrimidine nucleoside drug gemcitabine (2',2'-difluoro-2'-deoxycytidine) [6,30]. PfENT1 resembles the mammalian transporters in its ability to transport these anti-cancer nucleoside drugs, with an efficiency similar to that for uridine (Figure 4, bottom panel). However, unlike the mammalian transporters, it also efficiently transports the anti-viral nucleoside analogues 3'-azido-3'-deoxythymidine (AZT), 2',3'-dideoxycytidine (ddC) and 2',3'-dideoxyinosine (ddI) (Figure 4, bottom panel). These drugs have been reported to be very poor or negligible substrates for the mammalian *es*-type ENTs [31–35].

Cation-dependence and inhibitor-sensitivity of PfENT1

Whereas the mammalian members of the ENT family function as passive, facilitated diffusion systems, those homologues that have been cloned from the protozoan parasites *Leishmania* and *Trypanosoma* appear to act as proton symporters [10–13]. Members of the CNT family of nucleoside transporters also catalyse the symport of nucleosides with cations, these being sodium ions in the case of the mammalian transporters and protons in the case of the bacterial transporters [6]. Inwardly directed concentration gradients for both protons and sodium ions have been measured across the intra-erythrocytic *Plasmodium* plasma membrane [15], and could potentially provide a driving force for nucleoside uptake via PfENT1. To investigate this possibility, initial rates of adenosine uptake by oocytes expressing the transporter were measured in sodium-containing buffer at different pH values in the range 5.5–8.5, and also in a buffer in which sodium ions had been replaced by choline. There was no significant effect of pH or of replacement of sodium ions on transport activity (Figure 5). The inclusion in the transport buffer of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) at a concentration of 10 μM likewise had only a slight inhibitory effect. These findings indicate that transport is not cation-dependent, and that PfENT1 is therefore unlikely to function as a symporter.

The archetypal members of the ENT transporter family in mammals are potently inhibited by a range of nucleoside analogues and other inhibitors [6]. In particular, the *es*-type transporter hENT1 of the human erythrocyte, which harbours the bloodstream form of *Plasmodium*, is very sensitive to inhibition by the thiopurine ribonucleoside NBMPR, and by the coronary vasodilators dipyridamole, dilazep and drafazine. These agents exhibit apparent K_i values for inhibition of transport in the range 0.1–10 nM [8], hence there is essentially complete inhibition at concentrations of 1 μM or above. At this concentration, none of these inhibitors had any effect on PfENT1-mediated uridine or adenosine transport (results not shown). Even when used at 10 μM , no significant inhibition of adenosine transport was observable (results not shown).

DISCUSSION

Using information from the Malaria Genome Sequencing Project, we have identified, expressed and characterized a gene encoding a novel member of the ENT family of nucleoside transporters from *P. falciparum* and the first nucleoside transporter identified in *Plasmodium*. The transporter is expressed

Figure 4 Substrate specificity of PfENT1

(Top panel) Influxes of radiolabelled nucleosides and nucleobases at concentrations of 0.1 mM were measured in oocytes injected with water alone (black bars) or with water containing

Figure 5 Cation-dependence of PfENT1

Influx of [³H]adenosine (0.1 mM, 30 min) into oocytes injected with water alone or with water containing PfENT1 RNA transcript was measured in transport buffer containing 100 mM choline chloride (hatched bar) or 100 mM NaCl (white and black bars) in the presence (black bar) or absence (white bar) of 10 μM CCCP at the pH values indicated. Mediated uptake was calculated as influx in RNA-transcript-injected oocytes minus influx in water-injected oocytes.

during the asexual stages of the *Plasmodium* life cycle and catalyses the saturable uptake of nucleosides and nucleobases, as assayed by expression in *Xenopus* oocytes (Figures 3 and 4). In this respect it is completely distinct from the non-saturable permeability pathway for nucleosides induced in the infected erythrocyte plasma membrane [15]. Instead, it is likely to represent the means of nucleoside and nucleobase uptake across the parasite plasma membrane itself. Although definitive evidence will require completion of the *Plasmodium* genome sequencing project, Southern-blotting experiments suggest that PfENT1 is the sole representative of the ENT family in the parasite. This situation contrasts with that of kinetoplastid protozoa, where families of related ENT-type transporter genes have been identified [10,12]. Our failure to detect homologues of other nucleoside and nucleobase transporter families by searching the unfinished sequence data available from the Malaria Genome Sequencing Project suggests that PfENT1 may be the sole route by which the parasite takes up nucleosides and nucleobases. As purine salvage is essential in parasitic protozoa, this transporter therefore represents a potential target for therapeutic drugs. This hypothesis of a single route for nucleobase and nucleoside uptake is strengthened by previous observations that uptake of the nucleoside adenosine into oocytes injected with total mRNA isolated from *P. falciparum* can be inhibited almost completely by the presence of the nucleobase hypoxanthine [16].

PfENT1 RNA transcript (white bars). (Middle panel) Concentration dependence of [¹⁴C]adenosine uptake by oocytes injected with water alone (○) or water containing PfENT1 transcript (●). Inset, mediated transport. (Bottom panel) Influxes of radiolabelled uridine and of chemotherapeutic nucleoside analogues at a concentration of 0.1 mM were measured in oocytes injected with water alone (black bars) or with water containing PfENT1 RNA transcript (hatched and white bars).

In order to exploit a parasite protein as a target for chemotherapy, it is essential that it differs functionally from analogous proteins in the host. PfENT1 differs profoundly from mammalian ENT transporters in several respects. For example, it is able to transport the anti-viral 3'-deoxynucleoside analogues ddI, ddC and AZT, the last with an efficiency equivalent to uridine. In contrast, the mammalian ENT-type transporters transport these drugs poorly (hENT2) or not at all (hENT1), probably because a ribose 3'-hydroxyl group is important for substrate recognition [36]. Moreover, unlike the mammalian transporters, which exhibit approximately equal affinities for natural purine and pyrimidine nucleosides, PfENT1 appears to be somewhat purine-selective, transporting adenosine with a 10-fold higher apparent affinity than uridine. This poor ability to transport uridine is not unexpected, given the fact that *P. falciparum* has the capacity to synthesize pyrimidines *de novo* and, with the exception of orotic acid, does not incorporate exogenous pyrimidines into nucleic acids [4] (G. A. McConkey, unpublished work). Nonetheless, the present study suggests that lack of pyrimidine salvage is not due to an inability to take up pyrimidines, but rather to the absence of salvaging enzymes. PfENT1 resembles its host counterparts in being a relatively low-affinity facilitated-diffusion system, rather than a high-affinity proton symporter like its homologues described in kinetoplastid protozoans [6,10–13]. These properties may reflect the relative abundance of host-derived purines available to *Plasmodium* from the erythrocyte.

Whereas the precise identity of the purines used by the parasite *in vivo* remains unclear, it is likely that hypoxanthine resulting from catabolism of erythrocyte ATP supplies at least a part of its requirements, and several reports show that this nucleobase enhances the growth of the organism in culture [4]. The ability of PfENT1 to transport nucleobases, in particular hypoxanthine, but also adenine and guanine, is therefore likely to be of physiological importance. This ability represents another key difference between the parasite transporter and its mammalian counterparts: mammalian ENT transporters appear not to transport nucleobases [6], with the exception of hENT2, where hypoxanthine acts as an inhibitor of uridine transport [37] and is itself transported with low affinity (S. Y. M. Yao, C. E. Cass, S. A. Baldwin and J. D. Young, unpublished work; hypoxanthine transport via an *ei*-type transport process has also been described in human vascular endothelial cells, although the molecular identity of the nucleoside transporter responsible for this activity remains unknown [38]). Functionally, the similar affinities of adenine and adenosine suggest that the base may play the major role in substrate recognition by PfENT1, unlike the situation with the mammalian transporters.

In addition to differences in substrate specificity, PfENT1 differs from its mammalian homologues in its lack of sensitivity to classical inhibitors of nucleoside transport, such as NBMPR and coronary vasodilators. This finding suggests that it may be possible to develop inhibitors specific for the parasite transporter for use in anti-malarial chemotherapy. The reliance of the organism on purine salvage, and the fact that PfENT1 may be the major if not the sole route for purine entry into the asexual stages of the parasite, suggest that the transporter may be a worthwhile chemotherapeutic target. Alternatively, it may be possible to exploit the ability of PfENT1 to transport cytotoxic 3'-deoxynucleoside analogues that are poor substrates for the mammalian transporters. Whereas the permeability of the infected erythrocyte to such agents has not yet been tested specifically, the reported properties of the non-saturable permeation pathway induced in the host cell membrane [15] suggest that the analogues would have enhanced access to the parasite transporter. In contrast, normal host cells would be protected by

the barrier to drug transport posed by the permeability properties of hENT1 and hENT2. Finally, it may be possible to potentiate the cytotoxicity of nucleoside analogues towards the parasite by using inhibitors specific for the mammalian transporters, such as lidoflazine analogues, to reversibly prevent the entry of nucleoside analogues into host cells. The feasibility of such an approach has already been explored in murine schistosomiasis, where host toxicity of the adenosine analogue tubercidin was ameliorated by co-administration of a prodrug form of NBMPR [39].

Note added in proof (received 24 May 2000)

After submission of this manuscript, a paper was published describing the cloning and characterisation of a nucleoside transporter designated pfNT1 from the W2 strain of *Plasmodium falciparum* [41]. The predicted amino acid sequences of PfNT1 and the protein encoded by strain 3D7, which we have designated PfENT1, are identical except for position 385, which contains Phe and Leu respectively. However, the reported substrate- and inhibitor-specificities of pfNT1 differ extensively from those described in the present paper. We are unsure of the origin of these differences, but results described in the present paper, namely the relatively low affinity of PfENT1 for adenosine, its ability to transport nucleobases and its insensitivity to inhibition by dipyrindamole, were reproducibly and independently obtained in experiments performed in our laboratories both in Leeds and in Edmonton.

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