Trypanosoma brucei bloodstream forms express highly specific and separate transporters for adenine and hypoxanthine; evidence for a new protozoan purine transporter family?

GUSTAVO D. CAMPAGNARO[a], KHALID J. ALZAHRANI[a,b], JANE C. MUNDAY[a], HARRY P. DE KONING[a]

[a]*Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom, G12 8TA*

[b] *Department of Clinical Laboratory, College of Applied Medical Sciences, Taif University, Taif, Saudi Arabia*

*Corresponding author: de Koning, H.P. (Harry.De-Koning@glasgow.ac.uk)

Campagnaro, G.D. (g.campagnaro.1@research.gla.ac.uk)

Alzahrani, K.J. (k.alzahrani.1@research.gla.ac.uk)

Munday, J.C. (jane.munday@glasgow.ac.uk)

ABSTRACT

The transport of nucleobases and nucleosides in protozoan parasites is known to be performed by Equilibrative Nucleoside Transporter (ENT) family members, including the extensively studied P1 and P2 nucleoside transporters of *T. brucei* bloodstream forms. Studies with P2 knockout parasites suggested the existence of as yet uncharacterised purine transport mechanisms in these cells. Here, we deleted several ENT genes, in addition to P2, including an array comprising three genes encoding for high-affinity broad-selectivity nucleobase transporters - the longest multi-gene *locus* deletion in *T. brucei* to date. It was verified that

none of them appreciably contributed to the transport of hypoxanthine in bloodstream forms grown axenically in HMI-9 medium, which was mainly performed by a previously not described hypoxanthine-specific transporter (HXT1) with a K_m of 22 ± 1.7 μM and V_{max} of 0.49 ± 0.06 pmol(10^7 cells)$^{-1}$s^{-1}. The uptake of adenine was also assessed in the knockout cells and was performed by a highly specific adenine transporter (ADET1) with a K_m of 573 ± 62 nM and V_{max} of 0.23 ± 0.06 pmol(10^7 cells)$^{-1}$s^{-1}. Neither HXT1 nor ADET1 displayed any affinity for other natural purines or pyrimidines and could not be completely inhibited by hypoxanthine or adenine analogues. These carriers may be the final pieces in the substantial transporter array trypanosomes can employ to fine-tune the uptake of purines from diverse environments during their life cycles, and may be encoded by genes other than those of the ENT family.

Keywords: *Trypanosoma brucei*, nucleobase uptake, hypoxanthine, adenine, ENT family

1. INTRODUCTION

In higher eukaryotes, transport of nucleobases and nucleosides occurs via the concentrative nucleoside transporter (CNT, SLC28) or equilibrative nucleoside transporter (ENT, SLC29) families. Although these families have overlapping substrate specificities, they are structurally and physiologically unrelated: CNTs have 13 transmembrane domains (TMDs) and are sodium- or proton- symporters, which mediate the transport of nucleosides in an unidirectional energy-costly way, whereas ENTs are formed by 11 TMDs and are passive transporters that conduct substrates along the concentration gradient [1-3]. To date, all nucleoside and nucleobase transporters of parasitic protozoa were members of the ENT family, although they are proton symporters, able to concentrate the substrates inside the cell

[1, 4-6]. Other nucleobase transporter families are NAT, PRT and PUP [7] but, like CNTs, none of these have been found in protozoan genomes.

The best characterized parasites in terms of purine and pyrimidine transport are *Trypanosoma brucei*, the causative agent of Human African Trypanosomiasis (HAT; sleeping sickness), and *Leishmania* spp., causative agents of leishmaniasis. These parasites are known to express ENT family transporters for the uptake of nucleobases and/or nucleosides, which is an essential function because they are unable to synthesize purines *de novo*, relying on salvage from the host environment [1, 8, 9]. Due to this dependence on transport, several pharmacological approaches have focused on purine transporters as drug carriers for the treatment of parasitic infections, especially HAT [10-14].

During its life cycle, *T. brucei* differentially expresses several mechanisms for the uptake of purine nucleosides and/or nucleobases. The P1 transport activities were reported to be encoded by a tandem repeat of six genes on chromosome 2 (NT2 - NT7); all these carriers were shown to have affinity for inosine and adenosine and some of them also displayed a certain level of affinity for hypoxanthine when expressed in oocytes of *Xenopus laevis* [15]. Two more P1-type transporters, NT9 and NT10, were reported to be specifically expressed in short-stumpy bloodstream forms [16] and procyclic forms (stage in the mid-gut of the tsetse fly) [8], respectively, whereas the *Tb*AT1/P2 aminopurine transporter is only expressed in long-slender bloodstream forms [17, 18].

The long-slender bloodstream forms further express two purine nucleobase transporters, H2 and H3 [10, 19], and the procyclic forms also express two purine nucleobase transporters, H1 and H4 [4, 20]; these transporters are believed to be encoded by a tandem repeat of NT8 on chromosome 11 [20, 21]. The function of the last three members of the ENT family is not yet clear, although one report suggest that NT11.1, NT11.2 and NT12 transport both nucleobases and the drug pentamidine [22]; to date, our own investigations have not

been able to confirm this. As the ENT transporters from *T. brucei* and from *Leishmania* spp. have now been cloned and at least partially characterized, we have previously argued that the observed pyrimidine nucleobase transport activities in these species must be mediated by members of a different, as yet unidentified, transporter family [23, 24].

The interest on purine transporters in *T. brucei* increased dramatically when it was first reported that parasites resistant to melarsoprol, a first-line drug for treatment of late stage HAT, lack a purine transport activity, which was found to be *Tb*AT1/P2 [17, 25-27]. Moreover, *Tb*AT1/P2 was also found to efficiently transport pentamidine [17, 28] and is the main transporter of the veterinary trypanocide diminazene [29-31].

The binding model for P2 shows that its specificity for aminopurines is determined by the importance of hydrogen bonds formed between N1 and C6-NH_2 of the purine ring and the transporter binding site, along with interactions between the transporter and N9 [18]. This affinity for a motif NH_2─C(R_1)═N(R_2) also explains the affinity of P2 for diamidine drugs, such as Pentamidine, Melarsoprol and Diminazene [32, 33]. Additionally, two other transporters with high (HAPT1) and low (LAPT1) affinity for pentamidine have been reported to interact with diamidine drugs [34, 35]. More recently, the HAPT1 activity was found to be encoded by the gene *Tb*AQP2, an aquaglyceroporin [36, 37].

Several studies have focused on P2 as a carrier for new drugs containing melamine-based units. Interestingly, however, the affinity of the drugs for P2 was not the sole determinant for their trypanocidal effects, and *Tb*AT1/P2 knockout parasites were also able to transport these molecules and keep their IC_{50} values in a low micromolar range [38-40], evidencing the presence of extra transporters with the ability to transport purines and purine-like molecules [1, 38, 39]. Indeed, unpublished data from the Barrett group suggested the expression of an adenine-sensitive, inosine- and hypoxanthine-insensitive mechanism for (low affinity) adenosine uptake in *Tb*AT1/P2 knockout bloodstream forms [41].

In the light of all these studies, we decided to investigate whether *T. brucei* expresses additional purine nucleobase transporters that have not yet been reported. In order to eliminate some background transport of nucleobases, we knocked out the three genes known to encode high-affinity nucleobase transporters, from the *Tb*AT1/P2-knockout cell line, and used this new lineage as the parental cells for the further knockout (KO) of NT11 and NT12. We verified that none of the deleted transporters are the main purine carriers in cultured bloodstream forms, and describe the existence of two new and highly specific purine transporters, transporting adenine and hypoxanthine. By the process of elimination we conclude that these newly discovered adenine and hypoxanthine transporters are most likely not members of the ENT family and may therefore indicate the existence of a new transporter family in pathogenic protozoa.

2. MATERIALS AND METHODS

2.1. Parasites and culture media

Bloodstream forms of s427 and TbAT1-KO and its derived clonal lines were cultivated in HMI-9 supplemented with 10% of foetal bovine serum (FBS; Gibco) as described [42]. When necessary, 40 μM of adenosine was added to the culture to avoid metabolic limitations.

Alternatively, parasites were cultivated in Creek's Minimum Medium (CMM) [43] with modifications: instead of Gold Serum, we used standard FBS and supplemented the medium with 100 μM each of arginine, leucine, methionine, phenylalanine, tryptophan and tyrosine, and 40 μM of adenosine as purine source. *T. brucei* s427 were kept in serial passage in CMM for at least two weeks prior their use in uptake assays.

2.2. Generation of T. brucei *knockout lineages*

126 *2.2.1. Knockout of high-affinity nucleobase transporters cluster*

127 The high-affinity nucleobase transporters described independently by Burchmore *et al.*
128 (2003b) and Henriques *et al.* (2003) are located in a *tandem* array on chromosome 11 (Tritryp
129 Tb927.11.3610, Tb927.11.3620 and Tb927.11.3630), which enabled us to delete all three
130 genes at the same time, similar to what was done with glucose transporters in *Leishmania*
131 *mexicana* [44]. In order to achieve this, specific upstream and downstream regions were
132 chosen: due the fact the UTR before Tb927.11.3610 is short (292 base pairs; bp) and very
133 similar to the other two UTRs between the nucleobase transporter sequences, we cloned a
134 fragment (373 bp) consisting of the end of the sequence of the 40S ribosomal protein
135 (Tb927.11.3600) upstream of Tb927.11.3610 and the first 101 bp of the UTR (Figure 1a),
136 using primers HDK791 and HDK792, which contain restriction sites for PvuII and HindIII,
137 respectively (a complete list of primers is given in Supplementary Table S1). The same logic
138 was used to design the 3' flank of the cassette, for which 269 bp at the end of the UTR after
139 Tb927.11.3630 and the first 213 bp of Tb927.11.3640 were cloned using primers HDK905
140 and HDK906, containing the restriction sites for BamHI and SbfI, respectively. The reactions
141 were performed using a high-fidelity DNA polymerase (as described above) and 5 ng of
142 genomic DNA of the Lister s427 wildtype strain. The PCR products were digested overnight
143 with the appropriated enzymes and ligated into a pyrFEKO vector. The final vector was
144 digested with PvuII and SbfI to release the KO cassette, and AclI to cleave the β-lactamase
145 gene and facilitate the visualization of the band of interest. The digestion product was run in
146 1% agarose gel, and the band of interest purified and transfected into TbAT1-KO [27],
147 generating TbNBT-KO, which lacks all the confirmed *T. brucei* nucleobase transporters.

148 The TbNBT-KO line was cloned by limiting dilution and confirmed by the absence of
149 amplification of the ORF of the nucleobase transporters with primers HDK901 and HDK902.
150 As an internal control, each reaction contained primers (MB173 and MB174) to amplify a

151 fragment of actin, confirming the presence of DNA in the reaction. After the confirmation of
152 the gene deletion, the resistance genes were removed by transient expression of Cre
153 recombinase [45], enabling the reuse of the markers in the further knockouts.
154
155 *2.2.2. Knockout of AT-A and AT-E*
156 Using the TbNBT-KO clone as parental cell line, we deleted AT-A (NT11;
157 Tb927.9.15980) and AT-E (NT12; Tb927.3.590), generating two new cell lines: TbNBT/AT-
158 A-KO and TbNBT/AT-E-KO. To do so, UTRs upstream and downstream the target genes
159 were amplified by PCR and cloned into the vector pGL1688, based on pTBT [46] containing
160 either a Hygromycin or Puromycin resistance cassette. The KO cassettes were released from
161 the vector using NotI and XhoI, purified and introduced into TbNBT-KO, yielding single and
162 double knockout strains after one or two rounds of transfection, respectively. Transfectants
163 were cloned and verified by PCR prior to further use.
164
165 *2.3. Uptake assays*
166 Uptake of [^{3}H]-Hypoxanthine (Perkin-Elmer; 12.8 Ci/mmol) and [^{3}H]-Adenine
167 (Perkin-Elmer; 40.3 Ci/mmol) were performed as described previously [47]. 1×10^7 parasites
168 were incubated with buffer containing 0.1 μM of radiolabelled substrate, in the presence or
169 absence of inhibitor. After a predetermined period of time, the reaction was stopped by the
170 addition of 2 mM ice-cold unlabelled substrate (hypoxanthine or adenine, as appropriate) and
171 cells were immediately centrifuged through an oil layer. Cell pellets were lysed with 2% SDS
172 for at least 1 h under agitation, and then incubated with scintillation fluid (Scintlogic U,
173 Lablogic) overnight. Samples were read using a Hidex 300SL scintillation counter.
174 Background radiation was determined by counting vials to which no cells or radiolabel was
175 added. Radiolabel associated with the cell pellet but not internalised was determined by

including saturating levels of non-radiolabelled substrate in the assay buffer; the average of three such determinations was subtracted from each data point in the assay, as described [47].

K_m values for hypoxanthine and adenine transporters were determined by incubation of parasites with 0.1 μM of radiolabelled substrate in the presence of increasing concentrations of unlabelled substrate for one minute. K_i values for hypoxanthine and adenine analogues were calculated using the Cheng-Prusoff equation: $K_i = IC_{50}/(1+(L/K_m))$, where L represents the radiolabel concentration [48]. The IC_{50} was obtained by a non-linear regression of the inhibition curve for each inhibitor using GraphPad Prism 5, using an equation for a sigmoid line with variable slope. The same software was used for ANOVA test and to calculate linear regressions and K_m and V_{max} values, using the Michaelis-Menten equation $V_0 = V_{max}([substrate]/([substrate)+K_m))$. Student's T-test was performed in Microsoft Excel.

Based on the K_i obtained, the Gibbs free energy (ΔG^0) was calculated using the equation $\Delta G^0 = -RT\ln(K_i)$, in which R is the gas constant and T is the absolute temperature. It should be noted that these equations apply to competitive inhibitors, which is likely to be the case given that the inhibitors were close structural analogues of the radiolabelled substrates and the Hill slopes calculated for both HXT1 and ADE1 were consistently near −1 upon inhibition [47].

Hypoxanthine and adenine analogues were diluted either in assay buffer or in DMSO, and then further diluted in assay buffer for the assayed concentrations. Whenever DMSO was present in the reaction, its concentration was kept below 1% in order to avoid any cell membrane damage during the assay.

3. RESULTS

3.1 A new hypoxanthine-specific transporter is the main responsible for hypoxanthine uptake in Trypanosoma brucei *axenic bloodstream forms*

Constructs for the replacement of the NT8.1 - NT8.3 locus with antibiotic resistance cassettes were designed and constructed as described, above, in Methods section 2.2.1, and are depicted in Figure 1a. The knockout of the three high-affinity nucleobase transporter genes was confirmed by PCR (Figure 1b) and shows it is possible to knockout a ~7.5 kb genomic locus using a cassette of ~2.5 kb, which is, to our knowledge, the longest multi-gene locus deletion yet reported for *T. brucei*.

It should be noted that 40 μM of adenosine was added to the culture medium during the generation and selection of the TbNBT-KO clones, in case the clones failed to retain hypoxanthine transport activity. However, we assessed the transport of 0.1 μM of [^{3}H]-Hypoxanthine in the TbNBT-KO cells and found that parasites were still transporting hypoxanthine, with a linear phase lasting for at least 2 minutes and a rate of 0.0023 ± 0.0003 pmol(10^{7} cells)$^{-1}$s^{-1} (n=6); the addition of 1 mM of unlabelled hypoxanthine completely abolished the transport, evidencing that this uptake mechanism is saturable (Figure 2a).

Moreover, the reduction in hypoxanthine transport by TbNBT-KO cells was very modest, on average approximately 15% compared to the TbAT1-KO control (4 pairwise experiments), and statistically non-significant ($P>0.55$, Paired Student's T-test; Figure 2b), revealing that, under standard culture conditions, these three nucleobase transporter genes were not the main ones responsible for the uptake of hypoxanthine (Figure 2a), which is present in HMI-9 at 1 mM as sole purine source (apart from small quantities of purines in the serum).

As the deletion of the NT8 cluster barely reduced hypoxanthine transport rates, we hypothesized that either AT-A/NT11 or AT-E/NT12 could be the major carrier responsible for the observed hypoxanthine transport function. However, the knockout of either of these genes from TbNBT-KO, generating the TbNBT/AT-A-KO and TbNBT/AT-E-KO clonal lines, did not change the rate of hypoxanthine uptake in comparison to the parental cell line

TbNBT-KO (Figure 2a), and the average rate in three paired experiments was not significantly different from TbAT1-KO (P>0.1 by Paired Student's T-test; Figure 2b). Thus, we decided to further investigate the transporter responsible for hypoxanthine uptake under culture conditions. We found that the K_m and V_{max} values in all three cell lines were very similar (Table 1), with K_m values between 20.2 and 22.9 μM and V_{max} between 0.49 and 0.57 pmol(10^7 cells)$^{-1}$s^{-1}. These data further support our conclusion that AT-A and AT-E do not substantially contribute to hypoxanthine transport in cultured bloodstream forms of *T. brucei*. We selected the TbNBT/AT-E-KO cell line for further experiments to characterize the observed hypoxanthine transport activity. Therefore, its K_m (22.0 ± 1.7 μM; Figure 3a) was used for the calculation of K_i values in the below competition assays.

One strong possibility was that one or more of the P1-like transporters are able to transport hypoxanthine [15], and we therefore tested whether transport of [^{3}H]-hypoxanthine could be inhibited by inosine or adenosine, the classical substrates for P1, typically with K_m values close to the 1 μM mark. However, we observed that both inosine and adenosine had only a minor effect on the transport of [^{3}H]-Hypoxanthine when present in the assay at concentrations up to 1 mM (P>0.05 by One-way ANOVA, Figure 3b), effectively ruling out transport through a P1-type transporter. In fact, Figure 3b shows that, in contrast to the hitherto described kinetoplastid hypoxanthine transporters, this one is highly specific for its substrate and the transport of 0.1 μM of [^{3}H]-Hypoxanthine could not be completely saturated by any other natural purine or pyrimidine, or by the hypoxanthine analogue allopurinol. In fact, all other purines or pyrimidines tested had only a minor effect on the transporter (P>0.05 by One-way ANOVA), even at concentrations as high as 1 mM, i.e. 10,000-fold the radiolabel concentration. Given that this transporter displays lower affinity that any *T. brucei* purine transporter reported to date, and that it is uniquely specific for hypoxanthine, we propose that it is a new hypoxanthine-specific transporter: HXT1.

We investigated whether this transport activity was also present in the s427 wildtype strain or whether it was somehow a peculiarity of the knockout cell lines. Uptake assays using s427 wildtype parasites cultured in the same way as the mutant strains, in HMI-9/10% FBS, and incubated with 0.1 μM of [^{3}H]-hypoxanthine, showed a very similar profile (rate of uptake of 0.0021 ± 0.0006 pmol(10^7 cells)$^{-1}$s^{-1}) to that found in the three knockout cell lines, and 100 μM of adenine saturated only a minor component of the total hypoxanthine transport in these cells (rate of uptake 0.0018 ± 0.0006 pmol(10^7 cells)$^{-1}$s^{-1}) ($P<0.001$; Paired Student's T-test), proving again it was not one of the known high-affinity nucleobase transporters previously characterized in bloodstreams grown *in vivo* (De Koning and Jarvis, 1997b). However, when s427 cells were cultivated in a nucleobase-limited medium, such as CMM, the rate of [^{3}H]-hypoxanthine transport was significantly increased (~40%; $P<0.001$, Unpaired T-test), and this increase was reversed by 100 μM of adenine (Figure 4). This is consistent with nucleobase-depleted conditions inducing the expression of (one of) the previously described high-affinity nucleobase transporters and that their non-expression in culture is an adaptation to the extremely high hypoxanthine concentration of the HMI-9 medium.

3.2. Bloodstream forms of T. brucei *express an adenine-specific transporter*

The discovery of the novel hypoxanthine transporter HXT1 did not, however, explain the observations mentioned in the Introduction about non-P2-mediated uptake of melamine-containing molecules, since it displays extremely low affinity for either adenine or adenosine, nor those about hypoxanthine- and inosine-insensitive but adenine-sensitive adenosine uptake in TbAT1-KO cells. In order to investigate the presence of such transport, we decided to test the ability of the knockout cell lines to transport adenine. The use of radiolabelled adenosine in this model was not suitable because the parasites retain P1 activity, generating a high background. Since adenine is not a substrate of P1 [18], it should not be transported in the

knockout cell lines that also lack *Tb*AT1/P2 and the NT8 cluster, knowing that HXT1 does not display significant affinity for adenine at 1 mM. However, all the three knockout cell lines incubated with 0.1 μM of [^{3}H]-Adenine showed the same high level of uptake, which was linear for at least two minutes (Figure 5), revealing the existence of a high-affinity adenine transporter. The addition of 1 mM adenine to the reaction completely inhibited the transport of [^{3}H]-Adenine.

The transport of [^{3}H]-Adenine in TbNBT/AT-E-KO was performed by a high-affinity transporter with a K_m of 573 ± 62 nM and a V_{max} of 0.23 ± 0.06 pmol(10^7 cells)$^{-1}$s^{-1} (n=3) (Figure 6a), much lower than adenine K_i values previously reported for H2 and H3 (3.2 and 8.8 μM, respectively [19] . As with HXT1, we tested the complete series of natural nucleobases and nucleosides as inhibitors of the transporter, but none of them inhibited significantly at 1 mM of inhibitor (P>0.05 by One-way ANOVA, Figure 6b). Based on the unique K_m of this transporter, as well as on its high specificity for adenine, we propose that it is a new transporter described for *T. brucei* bloodstream forms, and designate it ADET1.

3.3. HXT1 and ADET1 are highly specific for their substrates

In order to better understand the high specificity of HXT1 and ADET1 for their natural substrates over other natural purines and pyrimidines, we decided to verify which atoms of the native substrates were involved in the recognition by the transporter. To do so, several analogues of hypoxanthine and adenine with modifications at different positions of the purine ring were used in uptake assays in competition to 0.1 μM of [^{3}H]-Hypoxanthine and [^{3}H]-Adenine for transport by HXT1 and ADET1, respectively.

We found that none of the analogues used, or any substitution made in different positions of the purine ring, were able to completely inhibit the transport of [^{3}H]-

hypoxanthine or [^{3}H]-adenine by HXT1 or ADET1 at the concentrations tested. Furthermore, ADET1 showed a much higher specificity for adenine than HXT1 for hypoxanthine (Table 2).

HXT1 showed to be dependent on the protonation state of N1, given that 6-Chloropurine and 6-Methoxypurine, which each display a single bond with C6, which corresponds to an unprotonated state of N1, displayed a much lower binding energy than 6-Mercaptopurine, which forms a double bond with C6 and thus keeps N1 in a protonated state; neither the chloro nor the sulphur at position 6 is capable of forming a significant hydrogen bond. Interestingly, the addition of a methyl group on position 1 or a Chlorine on position 2 led to the a very similar loss in Gibbs free energy, and it is possible to hypothesize that these bigger groups may interfere with a possible π-π stacking formed between the transporter and the aromatic ring, as shown to happen between P2 [1, 18, 33] and *Toxoplasma gondii* AT2 [49] and their natural substrates.

Also, N7 seems to have a small participation in the interaction with HXT1, given that its removal from the ring leads to a loss of 2.5 KJ/mol in Gibbs free energy. When the Nitrogen is dislocated from position 7 to position 8 in Allopurinol, a much higher loss of energy is identified. The presence of a Nitrogen on position 8 seems to have a repulsive effect in the binding pocket probably due to the arrangement of the compound within the binding pocket, which may lead to interaction with a different amino acid, and consequent repulsion, which is diminished by the simultaneous presence of N7, as seen in 8-Azahypoxanthine, occasioning a recovery of 4.8 KJ/mol in Gibbs free energy, highlighting the participation of N7 in the binding to the transporter.

Moreover, N9 is also important in the binding to HXT1 since its replacement by a Carbon in the purine ring (9-Deazahypoxanthine) causes a loss of 3.5 KJ/mol in this interaction, in agreement with the extremely low affinity HXT1 has for inosine, with IC_{50}

above 2.5mM. Furthermore, it is very likely that HXT1 has a tight binding pocket that cannot easily accommodate nucleosides.

ADET1 seems to have the same characteristic of tight binding pocket, although it can accommodate 2-deoxyadenosine when at very high concentration (K_i of 1352 ± 279 μM). The higher affinity for 2'-deoxyadenosine compared to adenosine also shows that the ribose group is not part of the interaction and its presence may perturb the transport. The same characteristic has been shown for P2: the lack of affinity for the ribose makes it a burden for the transporter, and the reduction in the size of the ribose group, by removal of hydroxyl groups, lead to higher rate of transport [18].

The affinity of ADET1 for its substrate is also much higher than that seen for HXT1 and hypoxanthine, which is also translated into a much lower K_m for ADET1. This high affinity is also noticed by the great loss of energy of interaction every time a modification is made in the purine ring. More importantly, N7 and the amino group attached to C6 seem to be of particular importance for transport via ADET1, since their removal caused the biggest losses of Gibbs free energy, as seen for 6-Chloropurine, 6-Mercaptopurine and 7-Deazaadenine: 16.1, 16 and 17.1 KJ/mol, respectively. Moreover, the shift of the Nitrogen from position 7 to position 8 of the imidazole ring in Aminopurinol caused a lower loss of energy than that seen for 7-Deazaadenine, showing the Nitrogen on position 8 might weakly interact with the transporter, recovering 4.4 kJ/mol in binding energy in comparison to 7-Deazaadenine.

4. DISCUSSION

Nutrient transport mechanisms are present in all types of living cells, playing a vital role in maintaining cell viability and the progression of the cell cycle. Among these nutrients,

purines and pyrimidines are of major importance as they are the basis for nucleic synthesis, and serve as cofactors and metabolic intermediates.

While most mammalian cells can synthesize their own purines and pyrimidines, protozoan parasites, such as trypanosomatids and apicomplexans, can synthesize only pyrimidines *de novo* and obtain purines solely by salvage from the host [1]. Due to a complex network of pathways, trypanosomatids can interconvert purines, being able to generate all needed nucleotides from any single purine source [50]. Given the essentiality of purine transport mechanisms for these parasites, many pharmacological approaches have targeted these proteins as drug carriers for antiparasitic treatments. It is thus clear that (some) purine transporters can in principle be exploited for the effective import of cytotoxins, selectively into the parasite if such molecules are specifically designed to interact with this transporter [10, 38-40, 51].

The *T. brucei* purine transporters seem to be encoded by at least a dozen ENT genes, which have now been largely characterised [8, 15, 16, 20-22]. However, work from Michael Barrett's group [41] revealed the potential existence of additional and as yet uncharacterised purine transport mechanisms in *T. brucei*, which we here investigate by deleting all ENT genes except the P1-type adenosine/inosine transporters. We report two novel, unusual purine transport activities expressed in bloodstream forms of the parasite: a relatively low affinity hypoxanthine-specific transporter and a very high affinity adenine-specific transporter, each with no affinity for any other natural purine or pyrimidine except their primary substrate, even at concentrations far above physiological levels. These carriers are likely the final pieces in the substantial array trypanosomes can employ to fine-tune the uptake of specific purines from their very diverse environments during the life cycle.

The great majority of purine transporters have selectivity over a range of substrates, as seen in humans (ENT1, ENT2 and ENT3 [52]), *Leishmania* spp. [9, 53, 54], and *T. brucei* [4,

8, 18-20], among others. Although quite uncommon in nature, purine transporters with high specificity for their substrates have been reported: CfAT1 for adenosine in the trypanosomatid *Crithidia fasciculata* [55] and FcyD for adenine in the fungus *Aspergillus nidulans* [56], but it is a novelty for *T. brucei*. Interestingly, Krypotou *et al.* (2015) demonstrated the independent emergence of Fcy-like transporters in fungi as a class apart from other NCS1 (Nucleobase Cation Symporter 1)–encoding organisms [57], and more recently it was shown that FcyD sequences exist only in a very narrow cluster of fungi related to *A. nidulans* [56], and therefore is unlikely to have any relation with either *C. fasciculata* AT1 or the herein described *T. brucei* transporters HXT1 and ADET1. Indeed, our searches for analogous sequences in the *T. brucei* genome databases did not yield any candidate genes in this family (results not shown). Moreover, FcyD seems to be rarely expressed and could not be detected under laboratory conditions, but may play a potential role in the life cycle of *A. nidulans* in its natural habitat [56]. It is possible to argue that at least HXT1 follows this logic in an opposite way: it is expressed under laboratory conditions, but was not detected in bloodstream forms isolated from rat blood, because under those conditions higher affinity nucleobase transporters were expressed as fits the relatively purine-poor conditions of serum [19]. As for ADET1, this was not previously detected for the simple reason that previous studies all used radiolabelled hypoxanthine or adenosine instead of adenine. We are currently assessing whether either transporter is expressed in the procyclic life-cycle stage but, not knowing the genetic identity, cannot easily discern their expression levels under many different conditions.

In fact, the purine nucleobase transport mechanisms described for bloodstream forms of *T. brucei* were originally characterized in parasites isolated from infected rats [19, 47], and not from parasites obtained from axenic culture, and it has become clear that the expression levels of some of the transporters, including P2 [58], is much lower in the long-term cultured cells. If we consider the fact that the commonly used culture medium for bloodstream forms,

HMI-9, contains 1 mM hypoxanthine, and that the transport of purines in trypanosomatids is proton-dependent [4-6], and coupled to a plasma membrane H^+-ATPase to avoid cytoplasmic acidification [4, 5], it is unsurprising that the cells drastically reduce the level of expression of such high-affinity transporters, in order to avoid unnecessarily high levels of hypoxanthine uptake. Indeed, it has previously been established that high affinity hypoxanthine (and adenosine) uptake in *T. brucei* bloodstream forms is correlated to the extracellular purine concentration and the cell cycle [59], as is the case for purine transport in the related parasite *Crithidia luciliae* [60].

In the current manuscript we show that the transport of hypoxanthine in axenic culture is mostly driven by HXT1, a medium-affinity, low capacity hypoxanthine-specific transporter (K_m 22 ± 1.73 μM; V_{max} 0.49 ± 0.06 pmol$(10^7$ cells$)^{-1}s^{-1}$), as rates hardly changed by knockout of the three NT8 genes that encode high-affinity broad specificity nucleobase transporters [20, 21]. When cells were transferred to a medium depleted of nucleobases, such as CMM, the level of expression of the adenine-sensitive hypoxanthine transporters was elevated, which translated into a higher level of uptake of this nucleobase. Interestingly, the higher expression of the adenine-sensitive H2 and/or H3 transporters was not followed by a reduction in expression of HT1, given that the addition of 100 μM adenine to the uptake assay buffer re-established the hypoxanthine uptake to those found in the knockout cell lines (TbNBT-KO, TbNBT/AT-A-KO and TbNBT/AT-E-KO) and very close to that verified for s427 cultivated in HMI-9.

The transport of adenine remained very high in the NBT-knockout cell lines, and was mediated by a new high-affinity adenine-specific transporter we designate ADET1, which has no prominent affinity for any other purine or pyrimidine tested, and due to its lack of affinity for hypoxanthine, it is unsurprising that it is not downregulated by maintenance of cells in HMI-9, which contains only the trace amounts of adenine from the foetal bovine serum.

Considering the high-fidelity shown by HXT1 and ADET1 for their substrates, they must bind hypoxanthine and adenine, respectively, in a very unique way. And in fact, it seems to be so unique that almost any modification or addition to the purine ring disturbs the interaction with the transporter. None of the adenine analogues tested inhibited ADET1-mediated [^{3}H]-adenine transport completely, and this translates into a very substantial loss in Gibbs free energy of binding relative to adenine. The extent of the loss of binding energy might be occasioned by different reasons. First, there may be a certain level of cooperativity between the hydrogen bonds formed between adenine and ADET1, as described for P1 in *T. brucei*, and the loss of one point of interaction my affect the formation of other bonds [18]. Secondly, it is possible to argue that a modification in the substrate may alter the orientation of this molecule in the binding pocket which occasions a different mode of interaction, as described for the UapA transporter from *A. nidulans*, in which xanthine and its analogue oxypurinol enter the binding pocket in different orientations [61]. Thirdly, the architecture of a transporter is quite complex and not completely understood. One accepted theory is the presence of intra and extracellular gates that participate in the interaction between the transporter and its substrate and also in the dynamic of transport. In this model, in order to enter the cell, a substrate would have to interact with the extracellular gate and lead to a conformational change, allowing this substrate to slide down and then interact with the binding pocket, which will translocate the substrate to intracellular environment [62]. It could be hypothesized that HXT1 and especially ADET1 may present extracellular gates and binding pockets that recognize different motifs of the substrates.

In summary, we described and characterized two highly specific carriers for purine nucleobases in *T. brucei* bloodstream forms under standard culture conditions. These transporters were in evidence in strains from which all ENT genes, except the P1 inosine-sensitive sub-class, had been deleted and it is thus highly unlikely that they are encoded for by

448 a gene of that family. This would constitute the first evidence of purine uptake by a non-ENT
449 transporter in a protozoan, although we have previously argued that the pyrimidine
450 nucleobase transporters LmajU1 and TbU1 [63, 64] must also be from a different family than
451 ENT [24]. Interestingly, these pyrimidine nucleobase transporters are also extremely selective
452 for one nucleobase only, uracil. The cloning of these transporters will establish whether U1,
453 HXT1 and ADET1 are from the same family and whether this is limited to kinetoplastid
454 protozoa.
455

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459

460 **6. COMPETING INTERESTS**

461 The authors declare that there are no competing interests.

462 **Table 1.** HXT1 kinetics determined in TbNBT-KO, TbNBT/AT-E-KO and TbNBT/AT-A-
463 KO
464

Cell line	K_m	V_{max}
TbNBT-KO	20.2 ± 5	0.57 ± 0.13
TbNBT/AT-E-KO	22.0 ± 1.7	0.49 ± 0.06
TbNBT/AT-A-KO	22.9 ± 4.2	0.53 ± 0.07

465 K_m values are expressed in µM and V_{max} in pmol.10^7cells.s^{-1}.
466

Table 2. K_i values for potential inhibitors of hypoxanthine and adenine transport mediated by HXT1 and A1

1×10^7 cells were incubated with 0.1μM of tritiated substrates for one minute in presence of increasing concentrations of inhibitor. IC_{50} values obtained were converted to K_i based on the K_m of each transporter. All the averages and standard deviations are based on at least three independent experiments in triplicate.

Compound	HTX1			ADET1		
	K_i	ΔG^0	$\delta(\Delta G^0)$	K_i	ΔG^0	$\delta(\Delta G^0)$
Hypoxanthine	22.0 ± 1.7[1]	-26.6	--	>1000		
Adenine	>1000			0.57 ± 0.06[1]	-35.6	--
1-Deazaadenine	N.D.			72.9 ± 2.0	-23.6	-12.0
1-Methylhypoxanthine	56.2 ± 6.1	-24.2	-2.4	N.D.		
2-Chlorohypoxanthine	47.6 ± 2.5	-24.7	-1.9	N.D.		
2,6-Diaminopurine	N.D.			177.04 ± 8.0	-21.4	-14.2
6-Chloropurine	330.6 ± 20.9	-19.9	-6.7	378.7 ± 11.4	-19.5	-16.1
6-Methoxypurine	315.4 ± 33.6	-20	-6.6	N.D.		
6-Mercaptopurine	34.4 ± 1.1	-25.5	-1.1	362.8 ± 9.4	-19.6	-16
7-Deazaadenine	N.D.			561.1 ± 5.6	-18.5	-17.1
7-Deazahypoxanthine	58.8 ± 8.4	-24.1	-2.4	N.D.		
8-Azahypoxanthine	67.9 ± 11.5	-23.8	-2.8	N.D.		
9-Deazaadenine	N.D.			46.9 ± 2.4	-24.7	-10.9
9-Deazahypoxanthine	46.6 ± 3.5	-23.5	-3.1	N.D.		
Allopurinol	469.5 ± 4.1	-19	-7.6	N.D.		
Aminopurinol	N.D.			94.9 ± 4.0	-23	-12.7
2'-Deoxyadenosine	N.D.			1352 ± 280	-16.4	-19.2

[1] K_m value

N.D. – Not determined.

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Figure Legends

Figure 1. A. Construction of high-affinity nucleobase transporter null mutant. A. Strategy for target gene replacement. The genomic *locus* containing two 40S ribosomal protein, three nucleobase transporters and a hypothetical protein encoding genes are shown. The unique 5' used in the knockout cassette was cloned as part of the 40S ribosomal protein (373 bp) and part of the UTR next to it (101 bp). A unique sequence for the 3' followed the same logic: part of UTR and part of the hypothetical protein gene Tb927.11.3640 were amplified by PCR and inserted into the knockout cassette in pyr-FEKO vector, containing a resistance marker, a thymidine kinase (TK) and loxP sequences (black boxes), which enable the removal of the cassette. B. PCR showing the presence of ORFs for nucleobase transporters in TbAT1-KO (parent cell line), heterozygous knockout line after the first round of knockout (TbNBT-sKO), and their absence after the complete knockout of the whole genomic *locus* (TbNBT-KO). The presence of DNA in the reaction is confirmed by the amplification of a 400bp fragment of actin. NC – Negative Control.

Figure 2. Time course for ^{3}H-Hypoxanthine uptake. Cells were incubated with 0.1 μM of ^{3}H-Hypoxanthine in absence (closed symbols) or presence (open symbols) of 1 mM of unlabelled hypoxanthine (Hx). A. Transport of ^{3}H-Hypoxanthine was not altered, either by the knockout of high-affinity hypoxanthine transporters or by deletion of AT-E or AT-A. Graph shows mean ± standard deviation of three or more independent experiments in triplicate. B. Comparison between TbAT1-KO and nucleobase transporter knockout cell lines for uptake of ^{3}H-Hypoxanthine: no statistical relevance was found by comparing.

Figure 3. A. Rate of transport was determined by incubation of TbNBT/AT-E-KO with 0.1μM of ^{3}H-Hypoxanthine in presence of 0 – 1 mM of unlabelled hypoxanthine (inset). Conversion of inhibition data into a Michaelis-Menten plot yielded a K_m value of 22.0 ± 1.7 μM and a V_{max} of 0.49 ± 0.06 pmol$(10^7$ cells$)^{-1}s^{-1}$. Graph shows one representative out of three independent experiments in triplicate. B. Presence of 1 mM of natural purines and pyrimidines in the transport assay buffer did not significantly alter the uptake of 0.1 μM ^{3}H-Hypoxanthine (n=3). Due to solubility limitations, guanine was used as inhibitor at 25 μM (n=2).

Figure 4. s427 wildtype parasites cultivated in HMI-9 present a level of hypoxanthine uptake close to TbNBT-KO. The addition of 100 μM of adenine to the transport assay buffer slightly altered the rate of transport. When cells were cultivated with virtually no nucleobases in CMM (see Materials and Methods), the rate of uptake of ^{3}H-Hypoxanthine was increased by about 40%, which could be reduced to the same level as in TbNBT-KO by addition of adenine to the transport assay buffer. Figure shows one representative experiment in triplicate.

Figure 5. A. Time course for the uptake of 0.1μM of ^{3}H-Adenine in TbAT1-KO (circles), TbNBT-KO (squares), TbNBT/AT-E-KO (up triangles) and TbNBT/AT-A-KO (down triangles) in absence (closed symbols) or presence (open symbols) of 1 mM of unlabelled adenine. The graph shows mean ± standard deviation of three independent experiments in triplicate. B. Comparison between TbAT1-KO and nucleobase transporter knockout cell lines for uptake of ^{3}H-Adenine. Among the three new knockout cell lines generated, only TbNBT/AT-E-KO presented a rate of ^{3}H-Adenine uptake significantly different from TbAT1-KO (P=0.04; Paired T-test).

Figure 6. A. Rate of uptake was determined by incubation of TbNBT/AT-E-KO with 0.1µM of ^{3}H-Adenine in presence of 0–1 mM of unlabelled adenine (inset). Inhibition data were converted into a Michaelis-Menten plot yielding a K_m of 573 ± 62 nM and a V_{max} of 0.228 ± 0.006 pmol(10^7 cells)$^{-1}$s^{-1}. Graph shows one representative out of three independent experiments in triplicate. B. Addition of natural purines and pyrimidines in the transport assay buffer at concentrations as high as 1mM could not completely inhibit the uptake of 0.1µM ^{3}H-Adenine (n=3). Due to solubility limitations, guanine was assayed at 25 µM (n=2).