University of Glasgow

Campagnaro, G. D., de Freitas Nascimento, J., Girard, R. B.M., Silber, A. M. and de Koning, H. P. (2018) Cloning and characterisation of the Equilibrative Nucleoside Transporter family of Trypanosoma cruzi: ultra-high affinity and selectivity to survive in the intracellular niche. Biochimica et Biophysica Acta: General Subjects, 1862(12), pp. 2750-2763.

There may be differences between this version and the published version. You are advised to consult the publisher's version if you wish to cite from it.

http://eprints.gla.ac.uk/171108/

Deposited on: 23 January 2019

Enlighten – Research publications by members of the University of Glasgow
http://eprints.gla.ac.uk

Cloning and characterisation of the Equilibrative Nucleoside Transporter family of *Trypanosoma cruzi*: ultra-high affinity and selectivity to survive in the intracellular niche

Gustavo D. Campagnaro[a], Janaína de Freitas Nascimento[b†], Richard B. M. Girard[b†], Ariel M. Silber[b] and 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 Parasitology, Institute of Biomedical Sciences, University of Sao Paulo, Av. Lineu Prestes, 1374, São Paulo, SP, Brazil, 05508-000*

[†]Contributed equally

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

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

De Freitas Nascimento, J. (janaina_biomed@hotmail.com)

Girard R.B.M. (r.girard02@gmail.com)

Silber, A.M. (asilber@usp.br)

ABSTRACT

Background: *Trypanosoma cruzi*, the causative agent of Chagas', disease is unable to synthesise its own purines and relies on salvage from the host. In other protozoa, purine uptake has been shown to be mediated by Equilibrative Nucleoside Transporters (ENTs).

Methods: To investigate the functionality of *T. cruzi*-encoded ENT transporters, its four putative ENT genes (TcrNB1, TcrNB2, TcrNT1 and TcrNT2) were cloned and expressed in genetically adapted *Trypanosoma brucei* procyclic cells from which the nucleobase transporter locus was deleted.

Results: TcrNB1 displayed very high affinity for hypoxanthine (K_m 93.8 ± 4.7 nM for) and guanine, and moderate affinity for adenine. TcrNT1 was found to be a high-affinity guanosine/inosine transporter (inosine K_m is 1.0 ± 0.03 µM; guanosine K_i is 0.92 ± 0.2 µM). *TcrNT2* encoded a high-affinity thymidine transporter (K_m = 223.5 ± 7.1 nM) with a clear preference for 2'-deoxypyrimidines. TcrNB2, whose activity could not be determined in our system, could be a low-affinity purine nucleobase transporter, given its sequence and predicted structural similarities to *Leishmania major* NT4. All 4 transporter genes were highly expressed in the amastigote forms, with much lower expression in the non-dividing stages.

Conclusions: The data appear to show that, surprisingly, *T. cruzi* has a preference for oxopurines over aminopurines and efficiently transports 2'-deoxypyrimidines. The *T. cruzi* ENTs display exceptionally high substrate affinity as an adaptation to their intracellular localisation.

General Significance: This study reports the first cloning of *T. cruzi* purine and pyrimidine transporters, including the first gene encoding a pyrimidine-selective protozoan transporter.

Keywords: *Trypanosoma cruzi*; Equilibrative Nucleoside Transporter; Thymidine transporter; Pyrimidine-specific uptake; *Trypanosoma brucei*; heterologous expression.

1. INTRODUCTION

The group Trypanosomatida comprises 22 recognized genera of protist parasites of plants and animals, vertebrates and invertebrates, among which the genera *Leishmania* and *Trypanosoma* stand out as causative agents of important human diseases, such as leishmaniasis (*Leishmania* spp.), sleeping sickness (*Trypanosoma brucei*) and Chagas' disease (*Trypanosoma cruzi*) [1]. In contrast to the extracellular lifecycle observed for most of the known animal trypanosomes and, probably, by the putative ancestral trypanosomatid, *T. cruzi* evolved an intracellular lifestyle [2–4]. Therefore, the study of its biological needs might depict adaptations towards the survival in its intracellular milieu.

T. cruzi is transmitted to mammalian hosts through the bite of the bloodsucking triatomine bugs, and is endemic to the American continent, infecting humans as well as domestic and wild animals, from the north of Argentina to the southern USA [5], with an estimated 8 million people infected worldwide and more than 70 million living under risk of infection [5,6]. The three most relevant stages of its life cycle are: epimastigotes, in the triatomine's intestine; trypomastigotes, in the distal part of the intestines of the vectors (metacyclic trypomastigotes) and in the mammalian bloodstream; and the intracellular amastigote forms [6,7].

The first record of a human infection by *T. cruzi* was found in an Andean mummy dated from approximately 9000 years ago [8], showing that *T. cruzi* has been circulating within human populations for a long time, and certainly for much longer among sylvatic animals. In fact, the divergence of trypanosomatids from other eukaryotes (200 – 500 million years ago) [9–11], corresponds to the period in which arthropods and mammals first emerged [12]. The evolution of trypanosomatids towards parasitism is supported by the recent finding that the basal trypanosomatid *Paratrypanosoma confusum*, from which many characteristics were inherited by other genera of parasites in this family [3], is a parasite of mosquitoes [2]. Among

77 the many adaptations to parasitism we note the incapacity of all protozoan parasite so far
78 investigated to synthesise purines *de novo,* causing an absolute reliance on their uptake from
79 the host [13,14].

80 To deal with the scarcity of free purine nucleobases and nucleosides available in the
81 intra-host environment, these parasites have developed several purine uptake mechanisms.
82 Although it has been proposed that trypanosomatids may encode nucleoside transporters of an
83 as yet unidentified gene family [15–17], to date only members of the Equilibrative Nucleoside
84 Transporters (ENTs) family have been identified in their genomes and, therefore, are assumed
85 to be the main carriers responsible for purine and/or pyrimidine uptake mechanisms in these
86 cells [13]. Interestingly, protozoan ENT transporters seem to be concentrative and mono-
87 directional rather than equilibrative, and proton-symporters [18–22].

88 Although ENT genes from *T. brucei* and different species of *Leishmania* have been
89 cloned and characterised in depth, not much is known about purine and pyrimidine transport
90 mechanisms in other trypanosomatids. For instance, there is only one report on purine transport
91 in *Trypanosoma congolense*, a cattle parasite with high economic impact in Africa [23], and
92 the last report on purine transport by *T. cruzi* dates from 25 years ago [24,25]. These early
93 studies describe the relationship between nucleoside transport and resistance to tubercidin (7-
94 deazaadenosine), and postulated the existence of at least three transport mechanisms in *T. cruzi*:
95 one for pyrimidines and tubercidin, one for thymidine with no affinity for tubercidin, and one
96 for adenosine and inosine [24,25]. However, the depletion of purines such as inosine,
97 guanosine, hypoxanthine and adenine in *T. cruzi*-infected cardiomyocytes [26] indicates the
98 existence of a more complex and unexplored array of nucleoside and nucleobase transporters
99 in this parasite. Herein, we cloned and characterized each *T. cruzi* ENT gene separately in an
100 adapted *T. brucei* cell line, in order to determine their substrate specificity and affinity. We
101 show that *T. cruzi* encodes for high-affinity nucleobase and nucleoside transporters with

exceptionally high substrate affinity, which must be an important adaptation to an intracellular environment that contains very low concentrations of free nucleosides and nucleobases.

2. MATERIALS AND METHODS

2.1. Cells and culturing conditions

2.1.1. Trypanosoma brucei

Given the extent of studies on ENT genes from *T. brucei*, and the organism's amenability to genetic manipulation, we used it as a surrogate system for the characterisation of ENTs from *T. cruzi*. Procyclic forms (PCF) of *T. brucei* strain Lister 427 (427WT) were maintained at 27 °C in non-vented plastic flasks with SDM-79 medium (Gibco) supplemented with 10% Foetal Bovine Serum (FBS, Gibco) and 7.5 µg/mL haemin. From 427WT the three genes making up the NT8 cluster on chromosome 11 [27,28] were deleted by homologous recombination, using the strategy previously reported by us for bloodstream forms [17], creating a new, procyclic TbNBT-KO cell line. The knockout was confirmed both by PCR and by phenotypical characterisation.

2.1.2. Trypanosoma cruzi

For qRT-PCR, total RNA was extracted from epimastigotes, metacyclic trypomastigotes, cell-derived trypomastigotes and amastigotes of *T. cruzi* (strain CL, clone 14) [29]. Epimastigotes and metacyclic trypomastigotes were obtained exactly as previously described. Briefly, exponentially growing *T. cruzi* epimastigotes were maintained in LIT medium supplemented with 10% fetal calf serum (FCS) by sub-culturing every 48 h and metacyclic trypomastigotes were obtained by *in vitro* differentiation of stationary growth phase epimastigotes as follows. Epimastigotes (5×10^8) were washed in PBS, resuspended in TAU medium (190 mM NaCl, 17 mM KCl_2, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 8 mM phosphate buffer,

pH 6.0) [30] and incubated for 2 h at 28 ºC. Then, the cells were supplemented with 10 mM proline and maintained for *in vitro* differentiation over 6 days, with daily counting in a Neubauer chamber. The cells (of which 30% metacyclic trypomastigotes) were washed in PBS, resuspended in cold 2% glucose in PBS adjusted to pH 8.0, and submitted to purification using an anionic exchange chromatography [31]. A DEAE-cellulose matrix (Sigma-Aldrich) was packed in a column and equilibrated with 2% glucose in PBS, pH 8.0. Parasites were applied onto the column, and fractions of 2 ml were collected. The presence of live cells containing trypomastigotes was microscopically monitored. The fractions containing more than 95% of metacyclic forms were pooled, washed and resuspended in PBS.

Amastigotes and trypomastigotes were obtained from the infection of a Chinese Hamster Ovary cell line (CHO-K_1) as previously described [32], with minor modifications. Briefly, CHO-K_1 cells were grown in RPMI medium supplemented with 10% FCS and 0.15% (w/v) $NaCO_3$, at 37 °C in a 5% CO_2 atmosphere. Cells were infected with trypomastigotes obtained from previous infections at a proportion of 50 trypomastigotes/cell. Amastigotes were collected at day 2 post-infection from the host-cell cytoplasm by treating the cells with a cold solution of 0.05% (w/v) SDS in PBS for 2 min, followed by a quick washing with cold PBS/10% FCS. Cells were disrupted with a rubber policeman, and amastigotes released into the supernatants were recovered and washed in cold PBS. Trypomastigotes were harvested from the extracellular medium at days 6 and 7 post-infection. The viability of the parasites, as well as the purity of each life-cycle stage preparation, was evaluated by direct microscopic observation.

2.2. Plasmid construction and transfection

Sequences encoding for ENTs in *T. cruzi* were found by their annotation on TritrypDB. Out of the eight sequences annotated as nucleoside or nucleobase transporters, two (TcCLB.506203.10 and TcCLB.508645.70) were very short, with transcripts of 543 and 390

base pairs in length, respectively, and are unlikely to encode for functional transporters, and, thus, were not included in our analysis. Among the six remaining coding sequences, four open reading frames (ORFs) represented two genes as Esmeraldo- and Non-Esmeraldo-like sequences (TcCLB.511051.30 and TcCLB.509683.110 encode for TcrNB1 (99% identity) and TcCLB.506773.50 and TcCLB.508799.150 encode for TcrNB2 (98% identity); Table 1) and are very likely to present the same substrate affinity. Primers specific for each gene were designed (Table S1) and the ORFs were amplified from genomic DNA of *T. cruzi* Y strain with the high-fidelity proof-reading Phusion DNA polymerase and cloned into the pHD1336 vector [23] for heterologous expression in *T. brucei* procyclic TbNBT-KO cells.

Table 1. Annotation of Equilibrative Nucleoside Transporters in the *T. cruzi* genome

Gene code	Chromosome	Profile‡	Transcript	Given name*	Genbank (Y strain)
TcCLB.511051.30 TcCLB.509683.110	14	Esm Non-Esm	1374 bp	TcrNB1§†	MH453384; MH453385
TcCLB.506773.50 TcCLB.508799.150	27	Esm Non-Esm	1347 bp	TcrNB2§	MH453386
TcCLB.508645.40	26	Non-Esm	1407 bp	TcrNT1†	MH453387; MH453388
TcCLB.506445.110	7	Non-Esm	1323 bp	TcrNT2	MH453389
TcCLB.506203.10	26	Esm	543 bp	--	
TcCLB.508645.70	26	Non-Esm	390 bp	--	

*Also refer to Figure 1.

‡Esm: Esmeraldo-like; Non-Esm: Non-Esmeraldo-like, as annotated in the *T. cruzi* genome reference CL Brener strain.

§Only Esmeraldo-like sequences were identified in *T. cruzi* Y strain.

†Heterozygous profile identified in *T. cruzi* Y strain. The sequence closer to that found in the genome reference was used for heterologous expression in *T. brucei*: TcrNB1_1 (MH453384) and TcrNT1_2 (MH453388).

All the constructs were linearized with NotI (or SacII for pHD1336 containing TcrNT2), precipitated with 100% ethanol, dried at 40 °C, resuspended in water and transfected by electroporation in an Amaxa Nucleofector (Lonza), using program X-014. A control group was electroporated in parallel without added DNA. After 12-16 h, 20 µg/mL of blasticidin was added to the culture to positively select the successfully transfected parasites. After further three days, 100 µL of culture was transferred to fresh medium with the same antibiotic. When no living cells were observed in the no-DNA control group, parasites transfected with the *T. cruzi* gene constructs were plated out at 0.3 parasite/well in 96-well plates in the continued presence of blasticidin. Two clonal populations were randomly selected for each studied gene and used in transport assays in parallel with TbNBT-KO cells transfected with the 'empty' vector pHD1336 (TbNBT-KO+EV). After selection, cells were always grown in the presence of 10 µg/mL of blasticidin.

2.3. Transport assays

Uptake of tritiated purines or pyrimidines was assayed exactly as described previously [17]. Briefly, 1×10^7 cells were incubated with radiolabelled substrate for a predetermined time in uptake assay buffer (AB, pH 7.3 [23]) and the reaction was stopped by the addition of ice-cold unlabelled substrate (2 mM in AB) followed by centrifugation of cells through an oil layer, flash-freezing in liquid Nitrogen, lysis with 2% SDS for at least 1 h under agitation, and incubation with scintillation fluid (Scintlogic U, Lablogic) overnight. Samples were read in a Hidex 300SL scintillation counter. All assays were carried out in triplicate.

When the substrate specificity of a transporter was not clear by its position in the phylogenetic tree, its main substrate was identified by incubation of the correspondent transfectant clones with 50 nM of each of a series of radiolabelled purine and pyrimidine

nucleobases and nucleosides for one minute in parallel with TbNBT-KO+EV cells as background control.

K_m and K_i values for each transporter were determined as described [33] by incubation of cells with a low, non-saturating concentration of an appropriate radiolabelled substrate, and increasing concentrations of unlabelled substrate for a set duration that was well within the linear range of transport. The resulting transport data was plotted to obtain K_m and V_{max} using the Michaelis-Menten equation ($V_0 = V_{max}\times[S]/(K_m+[S])$ in which [S] is the substrate concentration). Inhibition constants K_i were obtained in experiments with a fixed radiolabel concentration [S] several-fold below K_m and a variable concentration of an inhibitor. The inhibitor concentration was plotted against the rate of transport using a sigmoid curve with variable slope (Prism 5.0, GraphPad); the IC_{50} value was entered into the Cheng-Prusoff equation to obtain the K_i: $K_i = IC_{50}/(1+([S]/K_m))$ [34]. From this, the Gibbs free energy (ΔG^0) of the inhibitor-transporter interaction was calculated using the equation $\Delta G^0 = -RT\ln(K_i)$, where R is the gas constant and T is the reaction absolute temperature. It should be noted that these equations apply to competitive inhibitors, which is likely to be the case given that the inhibition curves presented Hill slopes consistently near −1, indicating the inhibition of a single transport mechanism (the *T. cruzi* ENT gene expressed), and that the inhibitors used were all natural nucleosides and nucleobases.

2.4. qRT-PCR

Total RNA of 1×10^8 cells of three independent preparation of each *T. cruzi* stage and CHO-K_1 cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were treated for 1 h with DNAse I Amplification Grade kit (Invitrogen) as recommended by the manufacturer, to eliminate any contaminating DNA. The RNA samples were recovered from the supernatants, spectrophotometrically quantified, and

quality checked for the presence of remaining DNA by performing a PCR assay using the same primers designed for qRT-PCR (Table S1). The RNA samples (5 µg) were submitted to reverse transcription using a random primer set and SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Primers for amplification of the ENTs (Table S1) were designed based on the sequences encoding for *T. cruzi* ENTs described above. The primers corresponding to the housekeeping control (*Tcr*GAPDH, GenBank accession number AI007393) have been previously reported [32]. The qRT-PCR was performed independently on three separate occasions (biological replicates), each time in triplicate (technical replicates), using the Fast SYBR Green Master Mix (Invitrogen) quantification system following the manufacturer's instructions, in a Mastercycler ep RealPlex (Eppendorf) using the following conditions: 95 ºC for 15 s, 40 cycles of 95 ºC (15 s), 59 ºC (15 s), and 68 ºC (15 s), followed by a denaturation curve. In order to compare the raw level of transcription of each gene for each biological form, their transcriptional level was measured as fold-change of the transcriptional level obtained for GAPDH in each biological replicate ($2^{(Ct(GAPDH)-Ct(transporter)}$).

2.5. Drug sensitivity assays

Fifty percent effective concentrations (EC_{50}) were determined essentially as described for bloodstream form *T. brucei* [35], using the viability indicator dye resazurin [36]. Briefly, 4×10^4 procyclic cells were incubated in 200 µL of SDM-79 with various concentrations of two-fold serially diluted pentamidine or 5-Fluoro-2'-deoxyuridine for 72 h at 27 ºC in a 96-well plate prior to the addition of 20 µL of 5 mM resazurin sodium salt (Sigma-Aldrich) and further incubation for 24 h. The highest concentration of test drug used was 100 µM, followed by 23 doubling dilutions over 2 rows of the plate, with the last well used as no-drug control. Fluorescence for each well was measured in a FLUOstar Optima (BMC Labtech) with

excitation at 544 nm and emission at 620 nm, and the EC_{50} was calculated by sigmoidal non-linear regression with variable slope, using GraphPad Prism 5.

3. RESULTS

3.1. Genomic analysis of ENT genes in trypanosomatids

In order to better understand the phylogenetic relationship between ENT transporters from *T. cruzi* and from other trypanosomatids, a phylogenetic analysis was made based on the sequences available on the TriTrypDB genomic database for *T. cruzi, T. brucei*, *T. congolense* and *Leishmania* spp (http://tritrypdb.org/tritrypdb/). The phylogenetic tree shows a clear division between nucleobase (*T. brucei* NT8 nucleobase transporters, *Leishmania* NT3 and NT4 clusters) and nucleoside transporters (*T. brucei* P1 and P2 clusters and *Leishmania* NT2 cluster) (Figure 1). The NT1 transporters in *Leishmania* form a separated branch, probably because of their ability to transport both purine and pyrimidines nucleosides [37–39]. Ali *et al.* (2013) showed that P1 transporters can also transport thymidine, but it is not the main substrate of this group of proteins [40]. The genes encoding for pyrimidine-only transporters in *T. brucei* and *Leishmania* spp (e.g. uracil transporters TbU1, LmU1, TbU3) have not been identified yet, and thus their inclusion in the purine transporter tree is not possible [15,16,21,41,42].

The proximity between TcrNB1 and the *T. brucei* nucleobase transporter clade indicates that it was very probably a high-affinity purine nucleobase transporter. TcrNB2 seems to be closer to the *Leishmania* NT4 nucleobase transporters cluster; LmjNT4 is a low-affinity purine nucleobase transporter that works better in acidic conditions, being important for the survival of *Leishmania* amastigotes in the macrophage [43]. Similarly, TcrNT1 could be hypothesized to be a purine nucleoside transporter, with possibly some capacity for pyrimidine nucleosides and/or purine nucleobases as well, due to its proximity to both P1 (high affinity for purine nucleosides; low affinity for pyrimidine nucleosides) and P2 (adenosine/adenine)

clusters and to *Leishmania* NT2 (inosine/guanosine). TcrNT2 was grouped more closely to *Leishmania* NT1 transporters (adenosine/uridine), which might indicate it to be a purine/pyrimidine nucleoside transporter. This grouping is further supported by the similarity of the predicted structures of the *T. cruzi* ENT transporters with their closest homologues, as modelled by the Phyre2 software package (Figure S1). In order to confirm these predictions, all four ENT genes from *T. cruzi* were cloned and expressed in a genetically adapted *T. brucei* procyclic cell line (TbNBT-KO), from which the TbNT8.1 - NT8.3 locus on chromosome 11 was deleted by homologous recombination.

3.2. Systematic biochemical characterization of T. cruzi *ENT genes:* T. brucei *as a surrogate system*

In bloodstream forms of *T. brucei*, the deletion of the three NT8 genes arranged in tandem on chromosome 11, encoding for high affinity purine nucleobase transporters [27,28], had little effect on the overall transport rate of hypoxanthine or adenine *in vitro* [17]. However, hypoxanthine transport in the procyclic forms has been assigned to be mainly (~85%) performed by the H4 activity, which is encoded by an NT8 transporter [27]. We therefore knocked out the NT8 cluster in *T. brucei* PCF as previously described for bloodstream forms [17]. These procyclic TbNBT-KO cells displayed an 86% reduction in the rate of 0.1 µM [^{3}H]-Hypoxanthine uptake in comparison to s427 wildtype procyclic cells (Figure 2), from 0.036 ± 0.007 pmol.(10^7 cells)$^{-1}$.s^{-1} to 0.005 ± 0.0005 pmol.(10^7 cells)$^{-1}$.s^{-1} (n = 3; $P<0.03$ by Paired Student's T-test). The highly diminished rate of hypoxanthine transport in these cells permitted their use for heterologous expression of potential *T. cruzi* purine nucleobase transporters.

3.2.1. TcrNB1 is a high-affinity hypoxanthine transporter

The expression of TcrNB1 in TbNBT-KO cells allowed us to test the prediction from the phylogenetic analysis of it being a purine nucleobase transporter. Indeed, expression of TcrNB1 in these cells increased their uptake rate for 50 nM [^{3}H]-Hypoxanthine by 170-fold relative to empty vector controls performed in parallel, with a linear phase of 6 s (0.36 ± 0.07 versus 0.0021 ± 0.0007 pmol.(10^7 cells)$^{-1}$.s^{-1}; n=2, r^2>0.99) (Figure 3). The addition of 1 mM of unlabelled hypoxanthine completely abolished the uptake of [^{3}H]-Hypoxanthine indicating a complete saturation of the transporter under those conditions (Figure 3). In order to further analyse the transport of hypoxanthine (and not its metabolism), inhibition experiments were performed using 2 s incubations, very much within the linear phase of transport. The rate of 50 nM [^{3}H]-Hypoxanthine uptake measured for TbNBT-KO+EV was always below 0.7% of that measured for TbNBT-KO+TcrNB1, and inhibition profiles were therefore considered to represent inhibition of TcrNB1 only, which was consistent with our constant obtainment of sigmoid curves with Hill slopes near -1.

Given the high level of hypoxanthine transport by TbNBT-KO+TcrNB1 at 50 nM of radiolabel, it was likely to represent a high-affinity purine nucleobase transporter. Indeed, we found TcrNB1 to have a K_m of 93.8 ± 4.7 nM for hypoxanthine (n=4; Figure 4A). The affinity for guanine was almost identical to this as it inhibited the transport of 50 nM [^{3}H]-Hypoxanthine with a K_i of 121.9 ± 22.4 nM (Figure 4B; Table 2). However, TcrNB1 displayed a much lower affinity for adenine, which inhibited the transport of hypoxanthine with a K_i of 3.73 ± 0.5 µM, corresponding to the loss 10.2 KJ/mol in the Gibbs free energy of interaction relative to hypoxanthine (Table 2). This amount of interaction energy is likely to correspond to one hydrogen bond, indicating that TcrNB1 probably forms a hydrogen bond with the keto group on C6 or the protonated N1 residue, but not with the amino group of adenine or the protonated N1. While this indicates a strong selectivity for oxopurines over aminopurines, xanthine displayed even lower affinity than adenine for the transporter (K_i of 18.36 ± 1.83 µM,

δ(ΔG0) = 14.1 KJ/mol relative to hypoxanthine) (Figure 4B), indicating that either the unprotonated N3 acts as an H-bond acceptor and/or the keto group causes substantial repulsion/steric hindrance between substrate and transporter (Table 2). Purine nucleosides were found to display much lower affinity than their respective nucleobases, with K_i values of 314.8 ± 26.5 µM and 17.1 ± 5.1 µM for inosine and guanosine, respectively, and no observable inhibition by adenosine (Table 2).

Table 2. K_i for purines on the transport of [^{3}H]-Hypoxanthine by TcrNB1
IC_{50} values obtained were converted to K_i based on the K_m of TcrNB1.

Purine	***K_m* or *K_i* (µM)**	**ΔG^0**	**$\delta(\Delta G^0)$**	**n**
Hypoxanthine	**0.093 ± 0.004**	-40.1	--	4
Adenine	3.73 ± 0.5	-29.9	10.2	3
Guanine	0.122 ± 0.022	-38.4	1.7	3
Xanthine	18.36 ± 1.83	-26	14.1	3
Adenosine	N.E. (1 mM)	--	--	3
Guanosine	17.1 ± 5.1	-26.1	14	5
Inosine	314.78 ± 26.5	-18.9	21.2	3

Value highlighted in bold represents the K_m for hypoxanthine. N.E. – No effect.

Pyrimidines had very little or no effect on the transport of 50 nM of [^{3}H]-Hypoxanthine by TcrNB1 (Figure 4C). When tested at a concentration of 1 mM, cytidine was the only pyrimidine able to inhibit the transport via TcrNB1 by more than 50% (59.4 ± 2.6%, n=3, P=0.006). In addition, 1 mM of cytidine or thymidine displayed minor effects on hypoxanthine uptake ($P<0.05$ by Unpaired Student's T-test) but no effect was observed with 1 mM of any of the pyrimidine nucleobases. We thus conclude that TcrNB1 is a high affinity purine nucleobase transporter, rather than a nucleobase/nucleoside carrier, with specificity for hypoxanthine and guanine only.

Moreover, it should be highlighted that the K_m of TcrNB1 for hypoxanthine is orders of magnitude different from that reported for its intracellular metabolizing enzymes in *T. brucei*

[44], which confirms once more that the herein measured kinetic and affinity parameters are from the transporter and not due to the intracellular metabolism of the radiolabelled. The same logic applies to the following characterised transporters [45–47].

3.2.2. TcrNT1 is a high-affinity inosine/guanosine transporter

The proximity of TcrNT1 to the P1 and P2 gene clusters from *T. brucei* and TcoAT1 from *T. congolense* (Figure 1) indicated that it is likely to be a nucleoside transporter. Notwithstanding, we did not know whether it was going to have affinity for oxopurines (inosine and guanosine), as *T. brucei* P1, or for adenosine, as both P1 and P2, or for the three of them [48]. Therefore, we decided to screen several substrates at 50 nM by measuring their uptake during 1 min, in TbNBT-KO+EV and TbNBT-KO+TcrNT1 cells. During preliminary testing of substrates with both strains in parallel, we found that the transport of inosine and guanosine was increased in TbNBT-KO+TcrNT1 by approximately 30%. Given its higher specific activity, we decided to use [^{3}H]-Inosine as our probe for the further assays.

The use of [^{3}H]-Inosine, however, still entails the problem of high background generated by its uptake via P1 [49]; the P2 aminopurine transporter is not expressed in procyclic *T. brucei* [20]. We therefore screened several known substrates of P1 as specific inhibitors of [^{3}H]-Inosine uptake mediated by P1 but not TcrNT1. As we observed that that adenosine is not a very good substrate for TcrNT1, we verified that analogues of this nucleoside showed high potential as good inhibitors of P1 with low affinity for TcrNT1. Among them, 2'-deoxy-2'-Fluoro-adenosine (2'F-Ado) showed the best results: incubation of TbNBT-KO+TcrNT1 with 50 nM [^{3}H]-Inosine in presence of increasing concentrations of 2'F-Ado yielded quite different IC_{50} values for P1 and TcrNT1 (200.5 ± 14.7 nM and 40.5 ± 8.6 μM, respectively), when plotted with a two-site competition non-linear regression (Figure 5A). The IC_{50} for 2'-F-Adenosine on P1 was further confirmed by inhibition of inosine transport via P1 only in TbNBT-KO+EV

(177.9 ± 20.2 nM) and followed monophasic inhibition kinetics with a sigmoid curve with a Hill slope of -1. We verified that the presence of 3.3 μM of 2'F-Ado in the reaction almost completely saturated P1, reducing the transport of [^{3}H]-Inosine by TbNBT-KO+EV from 0.048 ± 0.003 to 0.002 ± 0.0004 pmol.(10^7 cells)$^{-1}$.s^{-1}, without affecting TcrNT1-mediated [^{3}H]-Inosine transport (Figure 5A). By thus inhibiting P1 with 3.3 μM of 2'F-Ado we observed a reduction of 48 ± 4% in the uptake of [^{3}H]-Inosine by TbNBT-KO+TcrNT1 cells (from 0.072 ± 0.003 to 0.041 ± 0.002 pmol.(10^7 cells)$^{-1}$.s^{-1}), which remained linear for at least 12 s (r^2 = 0.99; Figure 5B). The addition of 1 mM unlabelled inosine completely saturated the transport of [^{3}H]-Inosine, showing the transport mechanism to be saturable (Figure 5B).

We next investigated the substrate specificity and affinity of TcrNT1 for a range of purines and pyrimidines, using competition assays with 50 nM [^{3}H]-Inosine transport over 8 s, in the presence of 3.3 μM of 2'F-Ado. Incubation with increasing concentrations of unlabelled inosine revealed a high-affinity profile with a K_m of 1.0 ± 0.04 μM (Figure 6A). Almost the same affinity was observed for guanosine (K_i of 0.92 ± 0.14 μM), but affinity for other purines was much lower: the K_i values for adenosine, hypoxanthine and xanthine were 38.9 ± 5.8 μM, 23.9 ± 5.5 μM and 410.3 ± 35.7 μM, respectively (Figure 6B; Table 3). The Hill slope very close to -1 in all assays, indicating that the transport of [^{3}H]-Inosine was mediated by a single transporter, i.e. TcrNT1, following saturation of P1-mediated uptake by 2'F-Ado at 3.3 μM.

Compared to inosine, adenosine and hypoxanthine displayed a substantially lower affinity for TcrNT1, resulting in a $\delta(\Delta G^0)$ of 9.2 KJ/mol and 8 KJ/mol (Table 3), respectively, which are both likely to be due to the loss of one hydrogen bond. This indicates that (1) either the keto group on C6 or the protonated N1 of the purine ring is part of the interaction with the transporter, as its substitution by an amino group, and consequent deprotonation of N1, in adenosine causes a loss of binding energy corresponding to the loss of one hydrogen bond; and that (2) the ribose group is also part of this binding with one point of interaction, as its absence,

in hypoxanthine, affects the binding energy. In the case of xanthine, besides the absence of a ribose group, the loss of binding affinity is further associated with the presence of a keto group attached to C2, which may interact unfavourably an amino acid residue in the transporter binding pocket, or possibly cause a different orientation within the binding pocket. Adenine at 1 mM did not significantly affected the transport of [^{3}H]-Inosine via TcrNT1 (Figure 6C; P>0.3 by Unpaired Student's T-test).

Table 3. K_i for purines on the transport of [^{3}H]-Inosine by TcrNT1
IC_{50} values obtained were converted to K_i based on the K_m of TcrNT1.

Purine	***K_m* or *K_i* (μM)**	**ΔG^0**	**$\delta(\Delta G^0)$**	**n**
Inosine	**1.0 ± 0.04**	-34.23	--	3
Adenosine	38.9 ± 5.8	-25.04	-9.2	3
Guanosine	0.92 ± 0.14	-34.31	0.08	4
Guanine	N.E. (25 μM)	--	--	3
Hypoxanthine	23.9 ± 5.5	-26.25	-8	3
Xanthine	410.3 ± 35.7	-19.2	-15	3

Value highlighted in bold represents the *Km* for inosine. N.E. – No effect.

Pyrimidine nucleobases and nucleosides showed also very low affinity for TcrNT1, given that the transport of 50 nM of [^{3}H]-Inosine was not completely blocked by any of them at 1 mM, i.e. a concentration 20,000× higher than the radiolabelled permeant (Figure 6C). Among them, cytidine, cytosine, thymine and uracil did not present any measurable affinity for the TcrNT1, and only thymidine and uridine significantly inhibited inosine transport by TcrNT1 (P<0.05 by Unpaired Student's T-test). We conclude that TcrNT1 is a nucleoside transporter selective for inosine and guanosine.

3.2.3. TcrNT2 is a high-affinity thymidine transporter

The clustering of TcrNT2 (TcCLB.506445.110) closest to the *Leishmania* NT1-type transporters, forming a separated group at the base of the phylogenetic tree (Figure 1), indicated

it could be a purine/pyrimidine transporter. We thus screened a series of purine and pyrimidine radiolabelled nucleosides at 50 nM and observed that the transport of thymidine and uridine was increased by approximately 7,000-fold and 1.7-fold, respectively, in TbNBT-KO+TcrNT2 compared to TbNBT-KO+EV in this single experiment (results not shown). Given that *T. brucei* does not have any dedicated thymidine transporter [40,41], we utilised this radiolabelled as a probe for the further transport experiments to characterise TcrNT2.

When TbNBT-KO+TcrNT2 cells were incubated with 25 nM of [^{3}H]-thymidine, we observed a rate of transport of 0.007 ± 0.001 pmol.(10^7 cells)$^{-1}$.s^{-1} that was linear for at least 1 min (n=2; r^2=0.99; Figure 7). The rate of uptake in TbNBT-KO+EV cells was not significantly different from zero, as well as identical to the groups incubated with 1 mM of unlabelled thymidine, showing that the uptake via TcrNT2 is saturable and confirming that TcrNT2 is the only high affinity thymidine transporter in these cells. Further experiments using TbNBT-KO+TcrNT2 cells were carried using 30-second incubations.

In order to measure the affinity the affinity of TcrNT2 for pyrimidines and purines, TbNBT-KO+TcrNT2 cells were incubated with 25 nM of [^{3}H]-thymidine in the presence of increasing concentrations of inhibitors. Interestingly, TcrNT2 was found to be specific for 2'-deoxypyrimidines, given that it showed high affinity only for thymidine and 2'-deoxyuridine (Table 4). We measured a K_m of 223.5 ± 7.1 nM for thymidine (Figure 8A), disclosing a high-affinity profile for this nucleoside; the Hillslope of the inhibition plot was consistently very close to -1 (Figure 8A, inset), further evidence that thymidine transport was performed by a single mechanism in TbNBT-KO+TcrNT2 cells.

The preference of TcrNT2 for 2'-deoxypyrimidines becomes very clear when the affinities for uridine and 2'-deoxyuridine are compared (Figure 8B): K_i values of 65.5 ± 6.0 μM and 1.11 ± 0.1 μM, respectively, meaning that the removal of the 2' hydroxyl group from the ribose increased the affinity for TcrNT2 by 59-fold. This translates into a difference of 10.1

436 KJ/mol in Gibbs free energy (ΔG^0 of -23.9 KJ/mol for uridine and -34.0 KJ/mol for 2'-
437 deoxyuridine; Table 4). Most likely, the 2'-hydroxyl group is sterically not tolerated in the
438 TcrNT2 binding pocket, and its presence causes a shift in the substrate position, with an
439 attendant loss of 10 kJ/mol in interaction energy. The somewhat higher affinity of thymidine
440 over 2'-deoxyuridine could be the result of a hydrophobic interaction of -4.0 kJ/mol between
441 the 5-methyl moiety and the binding site (Table 4), although there could also be a contribution
442 from higher electron density in the pyrimidine ring, which would enhance any π- π stacking
443 interactions. Conversely, TcrNT2 showed very low affinity for cytidine (K_i of 728 ± 70.5; Table
444 4), and the $\delta(\Delta G^0)$ of 5.9 kJ/mol relative to uridine indicates a binding interaction with either
445 the 4-oxo group or with protonated N3. At 1 mM pyrimidine nucleobases showed no effect on
446 [^{3}H]-thymidine transport by TcrNT2, except for thymine which inhibited by 38.2 ± 1.6% of
447 inhibition at this concentration, which translates into a $\delta(\Delta G^0)$ >20 KJ/mol relative to thymidine
448 and the strong likelihood that the 2-deoxyribose contributes 2 hydrogen bonds to the binding
449 of thymidine, probably at 3' and 5', as do the *T. brucei* P1 transporter [48,50] and the
450 *Toxoplasma gondii* AT2 [51].

451 Purines had almost no effect on TcrNT2-mediated transport, as 1 mM of each purine
452 nucleoside or nucleobase (or 250 μM of guanosine, the highest concentration tested) failed to
453 affect the transport of [^{3}H]-Thymidine, with the exception of adenosine, which inhibited the
454 transporter by 34.7 ± 1.1% at 1 mM, and yielded a K_i of 1556 ± 60 μM (Table 4). We therefore
455 conclude that *T. cruzi* NT2 is a high affinity thymidine transporter that would also transport 2'-
456 deoxyuridine if this were present in its environment.

457 As the study of Finley, Cooney and Dvorak (1988) showed that the decreased ability of
458 laboratory-generated tubercidin-resistant clones of *T. cruzi* to transport radiolabelled tubercidin
459 was associated with a decrease in the transport of thymidine and uridine, we evaluated whether
460 tubercidin could also be a substrate for TcrNT2. Interestingly, TcrNT2 showed two-fold higher

affinity for tubercidin than for adenosine (K_i of 695.7 ± 109.3 μM (n=2)) in competition to 25 nM [^{3}H]-Thymidine; Table 4), but is still a low-affinity substrate for this transporter.

Table 4. K_i for purines and pyrimidines on the transport of [^{3}H]-Thymidine by TcrNT2
IC_{50} values obtained were converted to K_i based on the K_m of TcrNT2.

Inhibitor	***K_m* or *K_i* (μM)**	**ΔG^0**	**$\delta(\Delta G^0)$**	**n**
Thymidine	**0.223 ± 0.007**	-37.95	--	3
Uridine	65.5 ± 6.0	-23.9	14.1	3
2'-deoxyuridine	1.11 ± 0.1	-34.0	3.98	3
Cytidine	728 ± 70.5	-17.9	20.0	3
Thymine	> 1 mM	--	>20	3
Uracil	N.E. (1 mM)	--	--	3
Cytosine	N.E. (1 mM)	--	--	3
Adenine	N.E. (1 mM)	--	--	3
Adenosine	1556 ± 60	-16.0	21.9	3
Guanosine	N.E. (250 μM)	--	--	3
Hypoxanthine	N.E. (1 mM)	--	--	3
Inosine	N.E. (1 mM)	--	--	3
Xanthine	N.E. (1 mM)	--	--	3
Tubercidin	695.7 ± 109.3	-18.0	19.9	2

Value highlighted in bold represents the K_m for thymidine. N.E. – No effect.

3.2.4. TcrNB2 is an ENT of unknown function

The position of TcrNB2 in the phylogenetic tree (Figure 1) next to *Leishmania* NT4 sequences, and its predicted structural similarity with those carriers (Figure S1), indicate that TcrNB2 could be a low-affinity purine nucleobase transporter [43]. This ORF was amplified from genomic DNA of *T. cruzi* Y strain, cloned into pHD1336 vector and transfected into procyclic forms of TbNBT-KO. Two clones of TbNBT-KO+TcrNB2 were randomly selected for the transport assays in parallel with TbNBT-KO+EV. A large number of radiolabelled natural purines and pyrimidines (adenine, adenosine, guanine, guanosine, hypoxanthine, inosine, cytidine, thymine, thymidine, uracil, uridine) and the hypoxanthine analogue

allopurinol were screened at 50 nM, in transporter assay buffers with pH 7.3 and 6.0, but no difference in transport rates in TbNBT-KO+TcrNB2 clones and TbNBT-KO+EV was observed in either condition (data not shown). The pH 6.0 conditions were tested because of the phylogenetic proximity to the acid-activated *Leishmania* NT4 transporters. It should be noted, however, that the procyclic forms of TbNBT-KO showed a very high level of [^{3}H]-Adenine uptake, which might have masked any adenine transport capacity associated with the expression of TcrNB2 in these cells. This adenine transporter in PCF was found to have a K_m of 359 ± 37 nM and V_{max} of 0.64 ± 0.1 pmol.(10^7 cells)$^{-1}$.s^{-1} when cells were incubated with 50 nM of [^{3}H]-Adenine in presence of increasing concentrations of unlabelled adenine. This K_m is very close to that recently reported by us for ADET1 in *T. brucei* bloodstream forms [17], and likely represents the same transporter. Similarly, transport rates of the purine nucleosides adenosine, inosine and guanosine were high in the TbNBT-KO+EV cells, as a result of the expression of P1-type transporters.

We therefore attempted to express TcrNB2 in another trypanosomatid system. To do so, TcrNB2 was cloned into a pNUS vector [52] and transfected into *L. mexicana* promastigotes. After selection with G418, the populations transfected with empty pNUS or with pNUS containing TcrNB2 were used in uptake assays. Again, no significant difference was found between the cell lines, using 50 nM each of tritiated adenine, adenosine, guanine, hypoxanthine, inosine, uracil, and uridine, over 1 min intervals (data not shown).

3.3. Transcriptional level of ENTs throughout T. cruzi *life cycle*

The expression level of each of the ENT genes was assessed using qRT-PCR with mRNA isolated from each of the main lifecycle stages of *T. cruzi*, and normalised to the expression level of GAPDH. Each of the genes was highly expressed in at least one lifecycle stage, and the expression levels of all of the genes appear to be highly regulated, indicating

important functions (Figure 9). The amastigote stage, which has a high rate of replication, expressed all four ENTs robustly, consistent with a high demand for high-affinity salvage of both purines and pyrimidines. In contrast, both metacyclic and bloodstream trypomastigotes, which do not have a high rate of nucleic acid synthesis or cell division, displayed a low level of ENT transporter expression. Epimastigotes displayed a robust expression of TcrNT2 but at most modest levels for the other three transporters, probably due to the high availability of purines in LIT medium.

3.4. TcrNT2 is a potential drug carrier

Given the apparent requirement of *T. cruzi* amastigotes for external pyrimidines [53] and the existence and current medicinal use of pyrimidine analogues for the treatment of cancer and viral infections [54–57], as well as the high affinity of TcrNT2 for 2'-deoxyuridine, we evaluated the potential of TcrNT2 to act as a carrier for the anticancer drug 5-Fluoro-2'-deoxyuridine (5F-2'dUrd) and found that TbNBT-KO cells became almost 40-fold more sensitive to 5F-2'dUrd upon expression of TcrNT2 (Figure 10). The cell lines s427WT, TbNBT-KO and TbNBT-KO+EV were all equally sensitive to 5F-2'dUrd ($P>0.6$ by Unpaired Student's T-test), with an average EC_{50} of 17.7 ± 1.85 μM. In contrast, TbNBT-KO+TcrNT2 displayed an EC_{50} of 0.47 ± 0.045 μM for 5F-2'dUrd ($P<0.005$ by Unpaired Student's T-test to all three control cell lines; Figure 10 A). The EC_{50} for the control drug, pentamidine, whose internalisation in procyclics is independent of ENTs [58,59] but is instead mediated almost exclusively by aquaglyceroporin 2 [60–62], did not significantly differ between all the cell lines generated in comparison to s427WT parasites ($P>0.2$ by Unpaired Student's T-test; Figure 10 B). These results clearly show that the change in the EC_{50} for 5F-2'dUrd in TbNBT-KO+TcrNT2 is due to the expression of this transporter. We, therefore, can propose TcrNT2 as a potential drug carrier in *T. cruzi*.

4. Discussion

Among the nutrients *T. cruzi* needs, purines are and pyrimidines seem to be essential for the growth of the parasite [13,53]. To date, only ENT genes have been identified as encoding nucleoside and nucleobase transporters in protozoa [13], although the presence of non-ENT nucleobase transporters has been suggested, owing to the observation of highly specific uptake activities for uracil, adenine and hypoxanthine in *T. brucei brucei* and *L. major* that could not be linked to any of the ENT genes in their genomes [15–17]. However, almost the entirety of nucleoside transport studies in trypanosomatids have been performed with *T. brucei brucei* and *Leishmania* spp., although nucleoside salvage is essential for *T. cruzi* and might be quite different from the other kinetoplastid species, owing to its localisation in the host cell cytosol. We therefore decided to systematically characterise the ENT genes from *T. cruzi*, by expressing each in a genetically adapted procyclic *T. brucei* cell line, TbNBT-KO, that expresses only P1-type purine nucleoside transporters. This cell line displayed an 86% reduced hypoxanthine uptake rate, confirming the hypothesis of by Burchmore *et al*. (2003) that the NT8 genes are responsible for almost all of the hypoxanthine transport in procyclics [27]. In contrast, the deletion of the NT8 cluster in *T. brucei* bloodstream forms, as well as AT-A/NT11 and AT-E/NT12 had only a minor effect on nucleobase transport [17].

The comparison of *T. cruzi* ENT genomic sequences with sequences from other trypanosomatids (*T. brucei*, *T. congolense* and *Leishmania* spp.), most of which have been previously characterised [23,27,28,38,39,43,49,63–68], revealed the presence of four full-length ENT-family genes in *T. cruzi*. Phylogenetic analysis suggested that TcrNB1 could encode for a high-affinity nucleobase transporter, which was confirmed by its biochemical characterisation upon expression in TbNBT-KO, yielding a K_m of 93.8 ± 4.7 nM for hypoxanthine, which is very close to that measured for H2 in *T. brucei* bloodstream forms [19].

552 Like H2, TcrNB1 showed much higher affinity for hypoxanthine and guanine than for others
553 purine, and their K_i values for adenine were virtually identical. In addition, the much lower
554 affinity of TcrNBT1 for purine nucleosides compared to nucleobases also resembles H2: the
555 K_i of both transporters for guanosine was very similar, and both showed lower affinity for
556 inosine compared to guanosine, although their respective nucleobases (hypoxanthine and
557 guanine) had very similar affinities in both H2 and TcrNB1. The only minor differences rely
558 on the affinity for adenosine, which did not affect the transport of [^{3}H]-Hypoxanthine by
559 TcrNB1 at 1 mM, while its K_i for H2 was determined to be 590 ± 175 μM [19], and on their
560 affinity for pyrimidines: H2 presented measurable K_i values for thymine and uracil (82 ± 25
561 μM and 60 ± 14 μM, respectively), while TcrNB1 was not affected by these nucleobases, but
562 was somewhat more sensitive to inhibition by 1 mM of pyrimidine nucleosides (cytidine,
563 thymidine and uridine).

564 The phylogenetic tree also placed TcrNT1 close to the *T. brucei* P1 and P2 sequences
565 and to the *T. congolense* P1-type transporter TcoAT1, and, somewhat more distantly, to
566 *Leishmania* NT2 transporters, which indicated that TcrNT1 might be a high-affinity nucleoside
567 transporter [20,23,48,64]. Consistent with this analysis, the expression in TbNBT-KO showed
568 that TcrNT1 has very high-affinity for inosine and guanosine, with much lower affinity for
569 adenosine and purine nucleobases. This selectivity pattern closely matches that of the
570 *Leishmania* NT2 transporters [39,64], with the main difference being that *L. donovani* NT2
571 (LdNT2) has 6-fold higher affinity for inosine than for guanosine [64], while both substrates
572 are virtually equally recognized by TcrNT1. Moreover, TcrNT1 differs from LdNT2 in its
573 affinity for adenosine and hypoxanthine, as we determined K_i values for them around the mid-
574 micromolar range for TcrNT1, whereas Carter *et al.* (2000) reported that 100 μM of these
575 purines failed to inhibit the transport of 1 μM of [^{3}H]-Inosine by *L. donovani* promastigotes.

576 The moderate affinity for adenosine also differentiates TcrNT1 from *T. brucei* P1 and P2,
577 which recognise adenosine in the submicromolar range [20,48].

578 We further characterised the transport activity mediated by TcrNT2, which grouped
579 next to the *Leishmania* NT1 adenosine/pyrimidine nucleoside transporter sequences, forming
580 a group outside the main tree of kinetoplastid ENT genes. Its expression in TbNBT-KO
581 procyclics showed that like the *Leishmania* NT1 genes, TcrNT2 transports pyrimidine
582 nucleosides, but, in contrast to NT1 of *L. donovani* [37,38], *L. major* or *L. mexicana* [39], it
583 had no affinity for adenosine at physiological levels. In addition, TcrNT2 showed a very clear
584 preference for 2'-deoxypyrimidines over the analogous ribonucleosides, which was not
585 observed for LdNT1 or any other known ENT transporter. TcrNT2 thus constitutes the first
586 identification of a protozoan gene coding for a pyrimidine-specific transporter.

587 Given the preference of TcrNT2 for pyrimidine nucleosides, its low affinity for uridine
588 is quite unexpected as uridine monophosphate is the end product of pyrimidine *de novo*
589 biosynthesis, and the key nucleotide in pyrimidine salvage and interconversion, and could be
590 formed from uridine by action of uridine phosphorylase and uracil phosphoribosyltransferase,
591 as in *Leishmania* [39] and in *T. brucei* [40,69]. Thymidine, the main substrate for TcrNT2, on
592 the other hand, could only be converted to thymine or to TMP [40,70], but the recent finding
593 of sequences encoding thymine 7-hydroxylase-like proteins in *T. brucei* [71] opens the
594 possibility that trypanosomes might use thymidine and/or thymine as pyrimidine source, due
595 to the possibility of converting these nutrients back to uracil by the action of thymine 7-
596 hydroxylase and uracil 5-carboxylic acid decarboxylase [71,72]. However, it must be
597 remembered that *T. brucei* bloodstream forms do not salvage any pyrimidine nucleosides at
598 physiological concentrations, that their biosynthesis pathway is sufficient for their needs, and
599 although they express a high affinity uracil transporter [40], this carrier is not essential [73].

600 The possibility of a uracil transporter in *T. cruzi* is yet to be investigated although the genes
601 encoding the *T. brucei* and *Leishmania* uracil transporters have not yet been identified [15].
602 Another possibility that could be argued is that *T. cruzi* might have a deficiency in the
603 conversion of dUMP to TMP by thymidylate synthase and, therefore, circumvents this by
604 scavenging thymidine from the host followed by its phosphorylation via thymidine kinase. This
605 seems to be unlikely as the *T. cruzi* DHFR-TS (dihydrofolate reductase-thymidylate synthase)
606 has been cloned, heterologously expressed in *Escherichia coli* and shown to have an active
607 thymidylate synthase activity (K_m of 1.02 ± 0.05 μM for dUMP) [74]. Moreover, Brandan *et*
608 *al.* (2011) were not able to delete both alleles of DHFR-TS from *T. cruzi* Tulahuen and TCC
609 strains, indicating the essentiality of this gene to the parasite — a conclusion that is further
610 strengthened by the decreased infectivity of DHFR-TS single knockout parasites in mice [75].
611 We also attempted to characterize the activity of TcrNB2 by expression in TbNBT-KO
612 procyclic forms and in *L. mexicana* promastigotes but were unable to identify a substrate.
613 However, we conducted the screening for substrate with radiolabel concentrations of 0.05 μM,
614 which is self-selecting for a high affinity carrier, as almost all kinetoplastid nucleoside and
615 nucleobase transporters are. Therefore, it is possible that TcrNB2 is a very low affinity
616 transporter, resembling LmjNT4. The fact that LmjNT4 is an adenine transporter, and that none
617 of the other *T. cruzi* ENT genes encode for an aminopurine transporter, leads us to direct
618 follow-on research efforts in that direction. Alternative explanations for our inability to identify
619 a substrate for TcrNB2 might be that it could be an intracellular transporter, or that its
620 expression in our experimental cell lines was low. However, it seems to be robustly expressed
621 in *T. cruzi* amastigotes and it is therefore highly likely to play a role in purine salvage in this
622 parasite.
623 The expression of TcrNB1 in the amastigote stage, which is corroborated by its high
624 level of transcription as verified by qRT-PCR, could explain the reduction in the intracellular

concentration of hypoxanthine and adenine in infected cardiomyocytes [26]. However, this being an oxopurine nucleobase transporter, it alone does not explain the much higher reduction in adenine than in hypoxanthine intracellular concentrations [26], leading to the conclusion that either *T. cruzi* must express an as yet unidentified adenine transporter, possibly TcrNB2, which is also highly transcribed in the amastigote stage, and/or a transporter orthologous to *T. brucei* ADET1 [17], or an ecto-adenine deaminase activity.

Similarly, the presence of a high-affinity inosine/guanosine transporter in amastigotes might explain the reduction in the intracellular concentration of both nucleosides in *T. cruzi*-acutely infected cardiomyocytes [26]. Consistent with the presence of such transporting mechanism is the transcription and expression of a nucleoside triphosphate diphosphohydrolase (NTPDase) by *T. cruzi* [76–78], which presented higher capacity to convert guanine nucleotides (GDP and GTP) than adenine nucleotides (ADP and ATP) [76,77]. Although this NTPDase activity would lead to the formation of monophosphate nucleotides, it could be coupled with ecto-5'nucleotidases to form free nucleosides [79]. In addition, the NTPDase is expressed in all the three main biological forms of the parasite, epimastigote, trypomastigote and amastigote [76], although its transcription and activity are higher in the infective trypomastigote and amastigote forms [78], and is distributed all over the surface of the parasite, which points to its participation in the acquisition of these essential nutrients by *T. cruzi* [77].

Finally, given the presence of a high-affinity pyrimidine nucleoside transporter in *T. cruzi,* the apparent need of pyrimidine uptake by amastigotes [53], the historical use of pyrimidine-analogues for treatment of cancer and viral infections [54–57], and the limited and cumbersome chemotherapeutical arsenal against Chagas' disease (restricted to two drugs, Benznidazole and Nifurtimox, neither of them approved by the FDA [7]), we tested the possibility of using the anti-cancer drug 5F-2'dUrd against *T. cruzi*. The expression of TcrNT2

in TbNBT-KO greatly sensitized the cells to this pyrimidine analogue, showing it to be a good substrate. This demonstrates the potential use of currently approved and clinically used anticancer and antiviral thymidine or deoxyuridine analogues against *T. cruzi*.

In summary, we established *T. brucei* NBT-KO cells as surrogate system for the systematic expression and characterisation of purine and pyrimidine transporters from other trypanosomatids and used it to characterize three of the four ENT genes from *T. cruzi* Y strain, which revealed the existence of very high-affinity purine and pyrimidine transporters in this parasite, including the first pyrimidine-specific protozoan transporter and high affinity carriers for oxopurine nucleosides and nucleobases. We propose that these transporters could also be exploited as drug carriers for the treatment of Chagas' disease.

5. Funding

GDC is funded by a PhD scholarship from Science Without Borders (206385/2014-5, CNPq, Brazil).

6. Competing interests

The authors declare that there are no competing interests.

7. Acknowledgments

We thank Dr. Maria de Nazaré Correia Soeiro (Fiocruz – RJ) for providing the genomic DNA from *T. cruzi* Y strain.

672 *8. References*

673 [1] J. Lukeš, A. Butenko, H. Hashimi, D.A. Maslov, J. Votýpka, V. Yurchenko,
674 Trypanosomatids Are Much More than Just Trypanosomes: Clues from the Expanded
675 Family Tree, Trends Parasitol. (2018). doi:10.1016/J.PT.2018.03.002.
676 [2] P. Flegontov, J. Votýpka, T. Skalický, M.D. Logacheva, A.A. Penin, G. Tanifuji, N.T.
677 Onodera, A.S. Kondrashov, P. Volf, J.M. Archibald, J. Lukeš, Paratrypanosoma is a
678 novel early-branching trypanosomatid, Curr. Biol. 23 (2013) 1787–1793.
679 doi:10.1016/j.cub.2013.07.045.
680 [3] T. Skalický, E. Dobáková, R.J. Wheeler, M. Tesařová, P. Flegontov, D. Jirsová, J.
681 Votýpka, V. Yurchenko, F.J. Ayala, J. Lukeš, Extensive flagellar remodeling during
682 the complex life cycle of *Paratrypanosoma* , an early-branching trypanosomatid, Proc.
683 Natl. Acad. Sci. (2017) 201712311. doi:10.1073/pnas.1712311114.
684 [4] J. Lukeš, T. Skalický, J. Týč, J. Votýpka, V. Yurchenko, Evolution of parasitism in
685 kinetoplastid flagellates, Mol. Biochem. Parasitol. 195 (2014) 115–122.
686 doi:10.1016/j.molbiopara.2014.05.007.
687 [5] B. Zingales, M.A. Miles, D.A. Campbell, M. Tibayrenc, A.M. Macedo, M.M.G.
688 Teixeira, A.G. Schijman, M.S. Llewellyn, E. Lages-Silva, C.R. Machado, S.G.
689 Andrade, N.R. Sturm, The revised Trypanosoma cruzi subspecific nomenclature:
690 Rationale, epidemiological relevance and research applications, Infect. Genet. Evol. 12
691 (2012) 240–253. doi:10.1016/j.meegid.2011.12.009.
692 [6] J.A. Pérez-Molina, I. Molina, Chagas disease, Lancet. (2017). doi:10.1016/S0140-
693 6736(17)31612-4.
694 [7] D.L. Longo, C. Bern, Chagas' Disease, N. Engl. J. Med. 373 (2015) 456–466.
695 doi:10.1056/NEJMra1410150.
696 [8] A.C. Aufderheide, W. Salo, M. Madden, J. Streitz, J. Buikstra, F. Guhl, B. Arriaza, C.

Renier, L.E. Wittmers, G. Fornaciari, M. Allison, A 9,000-year record of Chagas' disease, Proc. Natl. Acad. Sci. 101 (2004) 2034–2039. doi:10.1073/pnas.0307312101.

[9] A.G.B. Simpson, E.E. Gill, H.A. Callahan, R.W. Litaker, A.J. Roger, Early evolution within kinetoplastids (Euglenozoa), and the late emergence of trypanosomatids, Protist. 155 (2004) 407–422. doi:10.1078/1434461042650389.

[10] N.M. El-Sayed, P.J. Myler, G. Blandin, M. Berriman, J. Crabtree, G. Aggarwal, E. Caler, H. Renauld, E.A. Worthey, C. Hertz-Fowler, E. Ghedin, C. Peacock, D.C. Bartholomeu, B.J. Haas, A.N. Tran, J.R. Wortman, U.C.M. Alsmark, S. Angiuoli, A. Anupama, J. Badger, F. Bringaud, E. Cadag, J.M. Carlton, G.C. Cerqueira, T. Creasy, A.L. Delcher, A. Djikeng, T.M. Embley, C. Hauser, A.C. Ivens, S.K. Kummerfeld, J.B. Pereira-Leal, D. Nilsson, J. Peterson, S.L. Salzberg, J. Shallom, J.C. Silva, J. Sundaram, S. Westenberger, O. White, S.E. Melville, J.E. Donelson, B. Andersson, K.D. Stuart, N. Hall, Comparative genomics of trypanosomatid parasitic protozoa, Science (80-.). 309 (2005). doi:10.1126/science.1112181.

[11] D. Steverding, The history of leishmaniasis, Parasites and Vectors. 10 (2017) 1–10. doi:10.1186/s13071-017-2028-5.

[12] M.S. da Silva, R.S. Pavani, J.D. Damasceno, C.A. Marques, R. McCulloch, L.R.O. Tosi, M.C. Elias, Nuclear DNA Replication in Trypanosomatids: There Are No Easy Methods for Solving Difficult Problems, Trends Parasitol. 33 (2017) 858–874. doi:10.1016/j.pt.2017.08.002.

[13] H.P. de Koning, D.J. Bridges, R.J.S. Burchmore, Purine and pyrimidine transport in pathogenic protozoa: From biology to therapy, FEMS Microbiol. Rev. 29 (2005) 987–1020. doi:10.1016/j.femsre.2005.03.004.

[14] F.R. Opperdoes, A. Butenko, P. Flegontov, V. Yurchenko, J. Lukeš, Comparative Metabolism of Free-living Bodo saltans and Parasitic Trypanosomatids, J. Eukaryot.

722 Microbiol. 63 (2016) 657–678. doi:10.1111/jeu.12315.
723 [15] V. Bellofatto, Pyrimidine transport activities in trypanosomes, Trends Parasitol. 23
724 (2007) 187–189. doi:10.1016/j.pt.2007.03.003.
725 [16] H.P. de Koning, Pyrimidine transporters of trypanosomes - a class apart?, Trends
726 Parasitol. 23 (2007) 190. doi:10.1016/j.pt.2007.03.004.
727 [17] G.D. Campagnaro, K.J.K.J. Alzahrani, J.C.J.C. Munday, H.P.H.P. De Koning,
728 Trypanosoma brucei bloodstream forms express highly specific and separate
729 transporters for adenine and hypoxanthine; evidence for a new protozoan purine
730 transporter family?, Mol. Biochem. Parasitol. 220 (2018) 46–56.
731 doi:10.1016/j.molbiopara.2018.01.005.
732 [18] H.P. de Koning, S.M. Jarvis, Hypoxanthine uptake through a purine-selective
733 nucleobase transporter in Trypanosoma brucei brucei procyclic cells is driven by
734 protonmotive force., Eur. J. Biochem. 247 (1997) 1102–10.
735 http://www.ncbi.nlm.nih.gov/pubmed/9288936.
736 [19] H.P. de Koning, S.M. Jarvis, Purine nucleobase transport in bloodstream forms of
737 Trypanosoma brucei brucei is mediated by two novel transporters, Mol. Biochem.
738 Parasitol. 89 (1997) 245–258. doi:10.1016/S0166-6851(97)00129-1.
739 [20] H.P. De Koning, C.J. Watson, S.M. Jarvis, Characterization of a nucleoside/proton
740 symporter in procyclic Trypanosoma brucei brucei, J. Biol. Chem. 273 (1998) 9486–
741 9494. doi:10.1074/jbc.273.16.9486.
742 [21] H.P. de Koning, S.M. Jarvis, A highly selective, high-affinity transporter for uracil in
743 *Trypanosoma brucei brucei* : evidence for proton-dependent transport, Biochem. Cell
744 Biol. 76 (1998) 853–858. doi:10.1139/o98-086.
745 [22] A. Stein, G. Vaseduvan, N.S. Carter, B. Ullman, S.M. Landfear, M.P. Kavanaugh,
746 Equilibrative nucleoside transporter family members from Leishmania donovani are

electrogenic proton symporters., J. Biol. Chem. 278 (2003) 35127–34. doi:10.1074/jbc.M306188200.

[23] J.C. Munday, K.E. Rojas López, A.A. Eze, V. Delespaux, J. Van Den Abbeele, T. Rowan, M.P. Barrett, L.J. Morrison, H.P. de Koning, Functional expression of TcoAT1 reveals it to be a P1-type nucleoside transporter with no capacity for diminazene uptake, Int. J. Parasitol. Drugs Drug Resist. 3 (2013) 69–76. doi:10.1016/j.ijpddr.2013.01.004.

[24] T. Nozaki, J.A. Dvorak, Molecular biology studies of tubercidin resistance in Trypanosoma cruzi, Parasitol. Res. 79 (1993) 451–455. doi:10.1007/BF00931581.

[25] R.W. Finley, D.A. Cooney, J.A. Dvorak, Nucleoside uptake in Trypanosoma cruzi: analysis of a mutant resistant to tubercidin, Mol. Biochem. Parasitol. 31 (1988) 133–140. doi:10.1016/0166-6851(88)90164-8.

[26] N. Gironès, S. Carbajosa, N.A. Guerrero, C. Poveda, C. Chillón-Marinas, M. Fresno, Global Metabolomic Profiling of Acute Myocarditis Caused by Trypanosoma cruzi Infection, PLoS Negl. Trop. Dis. 8 (2014). doi:10.1371/journal.pntd.0003337.

[27] R.J.S. Burchmore, L.J.M. Wallace, D. Candlish, M.I. Al-Salabi, P.R. Beal, M.P. Barrett, S. a Baldwin, H.P. de Koning, Cloning, heterologous expression, and in situ characterization of the first high affinity nucleobase transporter from a protozoan., J. Biol. Chem. 278 (2003) 23502–23507. doi:10.1074/jbc.M301252200.

[28] C. Henriques, M.A. Sanchez, R. Tryon, S.M. Landfear, Molecular and functional characterization of the first nucleobase transporter gene from African trypanosomes, Mol. Biochem. Parasitol. 130 (2003) 101–110. doi:10.1016/S0166-6851(03)00167-1.

[29] Z. Brener, E. Chiari, Aspects of Early Growth of Different Trypanosoma cruzi Strains in Culture Medium, J. Parasitol. 51 (1965) 922–926. http://www.jstor.org/stable/3275869%5Cnhttp://www.jstor.org/stable/3275869?seq=1

772 &cid=pdf-reference#references_tab_contents%5Cnhttp://about.jstor.org/terms.
773 [30] V.T. Contreras, J.M. Salles, N. Thomas, C.M. Morel, S. Goldenberg, In vitro
774 differentiation of Trypanosoma cruzi under chemically defined conditions, Mol.
775 Biochem. Parasitol. 16 (1985) 315–327. doi:10.1016/0166-6851(85)90073-8.
776 [31] M.A. de Sousa, A simple method to purify biologically and antigenically preserved
777 bloodstream trypomastigotes of Trypanosoma cruzi using DEAE-cellulose columns.,
778 Mem. Inst. Oswaldo Cruz. 78 (1983) 317–333. doi:10.1590/S0074-
779 02761983000300009.
780 [32] B.S. Mantilla, L.S. Paes, E.M.F. Pral, D.E. Martil, O.H. Thiemann, P. Fernández-
781 Silva, E.L. Bastos, A.M. Silber, Role of Δ1-pyrroline-5-carboxylate dehydrogenase
782 supports mitochondrial metabolism and host-cell invasion of Trypanosoma cruzi, J.
783 Biol. Chem. 290 (2015) 7767–7790. doi:10.1074/jbc.M114.574525.
784 [33] L.J.M. Wallace, D. Candlish, H.P. De Koning, Different substrate recognition motifs
785 of human and trypanosome nucleobase transporters. Selective uptake of purine
786 antimetabolites, J. Biol. Chem. 277 (2002) 26149–26156.
787 doi:10.1074/jbc.M202835200.
788 [34] Y.-C. Cheng, W.H. Prusoff, Relationship between the inhibition constant (KI) and the
789 concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic
790 reaction, Biochem. Pharmacol. 22 (1973) 3099–3108. doi:10.1016/0006-
791 2952(73)90196-2.
792 [35] A.A. Eze, M.K. Gould, J.C. Munday, D.N.A. Tagoe, V. Stelmanis, A. Schnaufer, H.P.
793 De Koning, Reduced Mitochondrial Membrane Potential Is a Late Adaptation of
794 Trypanosoma brucei brucei to Isometamidium Preceded by Mutations in the γ Subunit
795 of the F1Fo-ATPase, PLoS Negl. Trop. Dis. 10 (2016).
796 doi:10.1371/journal.pntd.0004791.

[36] M.K. Gould, X.L. Vu, T. Seebeck, H.P. de Koning, Propidium iodide-based methods for monitoring drug action in the kinetoplastidae: Comparison with the Alamar Blue assay, Anal. Biochem. 382 (2008) 87–93. doi:10.1016/j.ab.2008.07.036.

[37] B. Aronow, K. Kaur, K. McCartan, B. Ullman, Two high affinity nucleoside transporters in Leishmania donovani, Mol. Biochem. Parasitol. 22 (1987) 29–37. doi:10.1016/0166-6851(87)90066-1.

[38] G. Vasudevan, N.S. Carter, M.E. Drew, S.M. Beverley, M. a Sanchez, a Seyfang, B. Ullman, S.M. Landfear, Cloning of Leishmania nucleoside transporter genes by rescue of a transport-deficient mutant., Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 9873–9878. doi:10.1073/pnas.95.17.9873.

[39] K.J.H. Alzahrani, J.A.M. Ali, A.A. Eze, W.L. Looi, D.N.A. Tagoe, D.J. Creek, M.P. Barrett, H.P. de Koning, Functional and genetic evidence that nucleoside transport is highly conserved in Leishmania species: Implications for pyrimidine-based chemotherapy, Int. J. Parasitol. Drugs Drug Resist. 7 (2017) 206–226. doi:10.1016/j.ijpddr.2017.04.003.

[40] J.M.A. Ali, D.J. Creek, K. Burgess, H.C. Allison, M.C. Field, P. Maser, H.P. De Koning, Pyrimidine Salvage in Trypanosoma brucei Bloodstream Forms and the Trypanocidal Action of Halogenated Pyrimidines, Mol. Pharmacol. 83 (2013) 439–453. doi:10.1124/mol.112.082321.

[41] S. Gudin, N.B. Quashie, D. Candlish, M.I. Al-Salabi, S.M. Jarvis, L.C. Ranford-Cartwright, H.P. de Koning, Trypanosoma brucei: A survey of pyrimidine transport activities, Exp. Parasitol. 114 (2006) 118–125. doi:10.1016/j.exppara.2006.02.018.

[42] I.G. Papageorgiou, L. Yakob, M.I. Al Salabi, G. Diallinas, K.P. Soteriadou, H.P. De Koning, Identification of the first pyrimidine nucleobase transporter in Leishmania: Similarities with the Trypanosoma brucei U1 transporter and antileishmanial activity

822 of uracil analogues, Parasitology. 130 (2005) 275–283.
823 doi:10.1017/S0031182004006626.
824 [43] D. Ortiz, M.A. Sanchez, H.P. Koch, H.P. Larson, S.M. Landfear, An acid-activated
825 nucleobase transporter from Leishmania major, J. Biol. Chem. 284 (2009) 16164–
826 16169. doi:10.1074/jbc.M109.006718.
827 [44] E. Doleželová, D. Terán, O. Gahura, Z. Kotrbová, M. Procházková, D. Keough, P.
828 Špaček, D. Hocková, L. Guddat, A. Zíková, Evaluation of the Trypanosoma brucei 6-
829 oxopurine salvage pathway as a potential target for drug discovery, PLoS Negl. Trop.
830 Dis. 12 (2018). doi:10.1371/journal.pntd.0006301.
831 [45] A. Vandemeulebroucke, C. Minici, I. Bruno, L. Muzzolini, P. Tornaghi, D.W. Parkin,
832 W. Versées, J. Steyaert, M. Degano, Structure and mechanism of the 6-oxopurine
833 nucleosidase from trypanosoma brucei brucei, Biochemistry. 49 (2010) 8999–9010.
834 doi:10.1021/bi100697d.
835 [46] F. Giannese, M. Berg, P. Van Der Veken, V. Castagna, P. Tornaghi, K. Augustyns, M.
836 Degano, Structures of purine nucleosidase from Trypanosoma brucei bound to
837 isozyme-specific trypanocidals and a novel metalorganic inhibitor, Acta Crystallogr.
838 Sect. D Biol. Crystallogr. 69 (2013) 1553–1566. doi:10.1107/S0907444913010792.
839 [47] F. Ranjbarian, M. Vodnala, S.M. Vodnala, R. Rofougaran, L. Thelander, A. Hofer,
840 Trypanosoma brucei thymidine kinase is tandem protein consisting of two homologous
841 parts, which together enable efficient substrate binding, J. Biol. Chem. 287 (2012)
842 17628–17636. doi:10.1074/jbc.M112.340059.
843 [48] H.P. de Koning, S.M. Jarvis, Adenosine transporters in bloodstream forms of
844 Trypanosoma brucei brucei: substrate recognition motifs and affinity for trypanocidal
845 drugs., Mol. Pharmacol. 56 (1999) 1162–1170. doi:10.1124/mol.56.6.1162.
846 [49] M.I. Al-Salabi, L.J. Wallace, A. Luscher, P. Maser, D. Candlish, B. Rodenko, M.K.

Gould, I. Jabeen, S.N. Ajith, H.P. de Koning, Molecular interactions underlying the unusually high adenosine affinity of a novel Trypanosoma brucei nucleoside transporter, Mol. Pharmacol. 71 (2007) 921–929. doi:10.1124/mol.106.031559.

[50] H.P. de Koning, Transporters in African trypanosomes: role in drug action and resistance., Int. J. Parasitol. 31 (2001) 512–522. doi:10.1016/S0020-7519(01)00167-9.

[51] H.P. De Koning, M.I. Al-Salabi, A.M. Cohen, G.H. Coombs, J.M. Wastling, Identification and characterisation of high affinity nucleoside and nucleobase transporters in Toxoplasma gondii, Int. J. Parasitol. 33 (2003) 821–831. doi:10.1016/S0020-7519(03)00091-2.

[52] E. Tetaud, I. Lecuix, T. Sheldrake, T. Baltz, A.H. Fairlamb, A new expression vector for Crithidia fasciculata and Leishmania, Mol. Biochem. Parasitol. 120 (2002) 195–204. doi:10.1016/S0166-6851(02)00002-6.

[53] K.L. Caradonna, J.C. Engel, D. Jacobi, C.H. Lee, B.A. Burleigh, Host metabolism regulates intracellular growth of trypanosoma cruzi, Cell Host Microbe. 13 (2013) 108–117. doi:10.1016/j.chom.2012.11.011.

[54] C.M. Galmarini, J.R. Mackey, C. Dumontet, Nucleoside analogues and nucleobases in cancer treatment, Lancet Oncol. 3 (2002) 415–424. doi:10.1016/S1470-2045(02)00788-X.

[55] A. Cavaliere, K.C. Probst, A.D. Westwell, M. Slusarczyk, Fluorinated nucleosides as an important class of anticancer and antiviral agents, Future Med. Chem. 9 (2017) 1809–1833. doi:10.4155/fmc-2017-0095.

[56] A.E.J. Yssel, J. Vanderleyden, H.P. Steenackers, Repurposing of nucleoside- and nucleobase-derivative drugs as antibiotics and biofilm inhibitors, J. Antimicrob. Chemother. 72 (2017) 2326–2333. doi:10.1093/jac/dkx151.

[57] J. Shelton, X. Lu, J.A. Hollenbaugh, J.H. Cho, F. Amblard, R.F. Schinazi, Metabolism,

Biochemical Actions, and Chemical Synthesis of Anticancer Nucleosides, Nucleotides, and Base Analogs, Chem. Rev. 116 (2016) 14379–14455. doi:10.1021/acs.chemrev.6b00209.

[58] I. a Teka, A.J.N. Kazibwe, N. El-Sabbagh, M.I. Al-Salabi, C.P. Ward, A. a Eze, J.C. Munday, P. Mäser, E. Matovu, M.P. Barrett, H.P. de Koning, The diamidine diminazene aceturate is a substrate for the high-affinity pentamidine transporter: implications for the development of high resistance levels in trypanosomes., Mol. Pharmacol. 80 (2011) 110–116. doi:10.1124/mol.111.071555.

[59] H.P. de Koning, Uptake of pentamidine in Trypanosoma brucei brucei is mediated by three distinct transporters: implications for cross-resistance with arsenicals., Mol. Pharmacol. 59 (2001) 586–592. doi:10.1124/mol.59.3.586.

[60] J.C. Munday, A.A. Eze, N. Baker, L. Glover, C. Clucas, D.A. Andrés, M.J. Natto, I.A. Teka, J. Mcdonald, R.S. Lee, F.E. Graf, P. Ludin, R.J.S. Burchmore, C.M.R. Turner, A. Tait, A. Macleod, P. Mäser, M.P. Barrett, D. Horn, H.P. De Koning, Trypanosoma brucei aquaglyceroporin 2 is a high-affinity transporter for pentamidine and melaminophenyl arsenic drugs and the main genetic determinant of resistance to these drugs, J. Antimicrob. Chemother. 69 (2014) 651–663. doi:10.1093/jac/dkt442.

[61] N. Baker, L. Glover, J.C. Munday, D. Aguinaga Andres, M.P. Barrett, H.P. de Koning, D. Horn, Aquaglyceroporin 2 controls susceptibility to melarsoprol and pentamidine in African trypanosomes, Proc. Natl. Acad. Sci. 109 (2012) 10996–11001. doi:10.1073/pnas.1202885109.

[62] J.C. Munday, L. Settimo, H.P. de Koning, Transport proteins determine drug sensitivity and resistance in a protozoan parasite, Trypanosoma brucei, Front. Pharmacol. 6 (2015). doi:10.3389/fphar.2015.00032.

[63] D. Ortiz, M.A. Sanchez, P. Quecke, S.M. Landfear, Two novel

nucleobase/pentamidine transporters from Trypanosoma brucei, Mol. Biochem. Parasitol. 163 (2009) 67–76. doi:10.1016/j.molbiopara.2008.09.011.

[64] N.S. Carter, M.E. Drew, M. Sanchez, G. Vasudevan, S.M. Landfear, B. Ullman, Cloning of a novel inosine-guanosine transporter gene from Leishmania donovani by functional rescue of a transport-deficient mutant, J. Biol. Chem. 275 (2000) 20935–20941. doi:10.1074/jbc.M002418200.

[65] M.A. Sanchez, R. Tryon, J. Green, I. Boor, S.M. Landfear, Six related nucleoside/nucleobase transporters from Trypanosoma brucei exhibit distinct biochemical functions, J. Biol. Chem. 277 (2002) 21499–21504. doi:10.1074/jbc.M202319200.

[66] J.C. Munday, D.N.A. Tagoe, A.A. Eze, J.A.M. Krezdorn, K.E. Rojas López, A.A.M. Alkhaldi, F. McDonald, J. Still, K.J. Alzahrani, L. Settimo, H.P. De Koning, Functional analysis of drug resistance-associated mutations in the Trypanosoma brucei adenosine transporter 1 (TbAT1) and the proposal of a structural model for the protein, Mol. Microbiol. 96 (2015) 887–900. doi:10.1111/mmi.12979.

[67] D. Ortiz, M.A. Sanchez, S. Pierce, T. Herrmann, N. Kimblin, H.G. Archie Bouwer, S.M. Landfear, Molecular genetic analysis of purine nucleobase transport in Leishmania major, Mol. Microbiol. 64 (2007) 1228–1243. doi:10.1111/j.1365-2958.2007.05730.x.

[68] M.A. Sanchez, S. Drutman, M. Van Ampting, K. Matthews, S.M. Landfear, A novel purine nucleoside transporter whose expression is up-regulated in the short stumpy form of the Trypanosoma brucei life cycle, Mol. Biochem. Parasitol. 136 (2004) 265–272. doi:10.1016/j.molbiopara.2004.04.009.

[69] E.T. Larson, D.G. Mudeppa, J.R. Gillespie, N. Mueller, A.J. Napuli, J.A. Arif, J. Ross, T.L. Arakaki, A. Lauricella, G. DeTitta, J. Luft, F. Zucker, C.L.M.J. Verlinde, E. Fan,

W.C. Van Voorhis, F.S. Buckner, P.K. Rathod, W.G.J. Hol, E.A. Merritt, The Crystal Structure and Activity of a Putative Trypanosomal Nucleoside Phosphorylase Reveal It to be a Homodimeric Uridine Phosphorylase, J. Mol. Biol. 396 (2010) 1244–1259. doi:10.1016/j.jmb.2010.01.013.

[70] D.J. Hammond, W.E. Gutteridge, Purine and pyrimidine metabolism in the trypanosomatidae, Mol. Biochem. Parasitol. 13 (1984) 243–261. doi:10.1016/0166-6851(84)90117-8.

[71] J.M. Simmons, D.J. Koslowsky, R.P. Hausinger, Trypanosoma brucei brucei: Thymine 7-hydroxylase-like proteins, Exp. Parasitol. 124 (2010) 453–458. doi:10.1016/j.exppara.2009.11.012.

[72] R.D. Palmatier, R.P. McCroskey, M.T. Abbott, The enzymatic conversion of uracil 5-carboxylic acid to uracil and carbon dioxide., J. Biol. Chem. 245 (1970) 6706–6710.

[73] J.A.M. Ali, D.N.A. Tagoe, J.C. Munday, A. Donachie, L.J. Morrison, H.P. de Koning, Pyrimidine Biosynthesis Is Not an Essential Function for Trypanosoma brucei Bloodstream Forms, PLoS One. 8 (2013). doi:10.1371/journal.pone.0058034.

[74] P. Reche, R. Arrebola, A. Olmo, D. V. Santi, D. Gonzalez-Pacanowska, L.M. Ruiz-Perez, Cloning and expression of the dihydrofolate reductase-thymidylate synthase gene from Trypanosoma cruzi, Mol. Biochem. Parasitol. 65 (1994) 247–258. doi:10.1016/0166-6851(94)90076-0.

[75] C.P. Brandan, A.M. Padilla, D. Xu, R.L. Tarleton, M.A. Basombrio, Knockout of the dhfr-ts gene in trypanosoma cruzi generates attenuated parasites able to confer protection against a virulent challenge, PLoS Negl. Trop. Dis. 5 (2011). doi:10.1371/journal.pntd.0001418.

[76] J.L.R. Fietto, R. DeMarco, I.P. Nascimento, I.M. Castro, T.M.U. Carvalho, W. De Souza, M.T. Bahia, M.J.M. Alves, S. Verjovski-Almeida, Characterization and

immunolocalization of an NTP diphosphohydrolase of Trypanosoma cruzi, Biochem. Biophys. Res. Commun. 316 (2004) 454–460. doi:10.1016/j.bbrc.2004.02.071.

[77] C. Mariotini-Moura, M.S. e. Bastos, F.F. de Castro, M.L. Trindade, R. de Souza Vasconcellos, M.A.A. Neves-do-Valle, B.P. Moreira, R. de Freitas Santos, C.M. de Oliveira, L.C.S. Cunha, X.M. Souto, G.C. Bressan, A. Silva-Júnior, M.M.A. Baqui, M.T. Bahia, M.R. de Almeida, J.R. Meyer-Fernandes, J.L.R. Fietto, Trypanosoma cruzi nucleoside triphosphate diphosphohydrolase 1 (TcNTPDase-1) biochemical characterization, immunolocalization and possible role in host cell adhesion, Acta Trop. 130 (2014) 140–147. doi:10.1016/j.actatropica.2013.11.008.

[78] N.L. Silva-Gomes, V. Ennes-Vidal, J.C.F. Carolo, M.M. Batista, M.N. Soeiro, R. Menna-Barreto, O.C. Moreira, Nucleoside triphosphate diphosphohydrolase1 (TcNTPDase-1) gene expression is increased due to heat shock and in infective forms of Trypanosoma cruzi, Parasites and Vectors. 7 (2014). doi:10.1186/s13071-014-0463-0.

[79] A.L. Freitas-Mesquita, J.R. Meyer-Fernandes, Ecto-nucleotidases and Ecto-phosphatases from Leishmania and Trypanosoma Parasites, in: A.L.S. Santos, M.H. Branquinha, C.M. d'Avila-Levy, L.F. Kneipp, C.L. Sodré (Eds.), Proteins Proteomics Leishmania Trypanos., Springer Netherlands, Dordrecht, 2014: pp. 217–252. doi:10.1007/978-94-007-7305-9_10.

Figure 1. Phylogenetic tree showing the relationship between Equilibrative Nucleoside Transporter genes from trypanosomatids. A clear separation between nucleobase (top branch) and nucleoside (bottom branch) transporters can be observed. Sequences were obtained from TriTrypDB, aligned with Clustal Omega and subjected to a Maximum Likelihood analysis with 1000 bootstraps using MEGA 6.0. The *Toxoplasma gondii* TGVEG 233130 nucleoside transporter was used as external group.

Figure 2. Uptake of 100 nM [^{3}H]-Hypoxanthine is greatly reduced in *T. brucei* procyclic forms upon knockout of the NT8 cluster. Wildtype s427 parasites transported [^{3}H]-Hypoxanthine at 0.036 ± 0.007 pmol.(10^7 cells)$^{-1}$.s^{-1} (filled circles), whereas TbNBT-KO cells showed a rate of uptake of 0.005 ± 0.0005 pmol.(10^7 cells)$^{-1}$.s^{-1} (filled diamonds). In both cases slope was consistently different from zero (P<0.005, F-test). The addition of 1 mM unlabelled hypoxanthine (open symbols) completely saturated the transporters, and made the slopes not different from zero (P>0.2; F-test).

Figure 3. Expression of TcrNB1 in TbNBT-KO cells dramatically increased the rate of transport of 50 nM [^{3}H]-Hypoxanthine in comparison to cells transfected with an empty vector (TbNBT-KO+EV), in a linear phase of six seconds (blue line; r^2 = 0.99). The addition of 1 mM unlabelled hypoxanthine completely saturated the transport. Whereas the slope of the TcrNB1 regression was significantly non-zero (P<0.0001, F-test), the slope of the TbNBT-KO+EV-mediated transport was not significantly different from zero (P=0.15).

Figure 4. TcrNB1 is a high-affinity purine nucleobase transporter. A. Incubation of TbNBT-KO + TcrNB1 cells with 50 nM of [^{3}H]-Hypoxanthine in presence of increasing concentrations of unlabelled hypoxanthine gave an inhibition curve with Hillslope near -1 (inset), whose conversion into a Michaelis-Menten plot yielded a K_m of 93.8 ± 4.7 nM. When TbNBT-KO + TcrNB1 was exposed to 50 nM of [^{3}H]-Hypoxanthine in presence of increasing concentrations of other nucleobases (B), we verified it shows very high-affinity for guanine (K_i of 121.9 ± 21.4 nM; triangles), good affinity for adenine (K_i of 3.73 ± 0.5 μM; circles) and low affinity for xanthine (K_i of 18.36 ± 1.83

μM; squares). C. Pyrimidine nucleobases and nucleosides at 1 mM competed with 50 nM of [^{3}H]-Hypoxanthine for transport via TcrNB1, which was mostly affected by thymidine, uridine and cytidine. $^{*}P<0.05$, $^{**}P<0.01$ by Unpaired T-test. Figures A and B show one representative out of three independent experiments in triplicate. C shows average ± SEM of three independent experiments in triplicate.

Figure 5. A. Inhibitory effect of 2'F-Ado on the uptake of [^{3}H]-Inosine by TbNBT-KO+EV and TbNBT-KO+TcrNT1. Transport of 50 nM of [^{3}H]-Inosine over an interval of 8 s was measured in the presence of increasing concentrations of 2'-F-adenosine. This was monophasic in the control cells (TbNBT-KO; red circles), reflecting inhibition of the P1 transporter only, with high affinity. The dotted line divides the biphasic inhibition profile by 2'F-Ado (blue squares) of, firstly, the P1 transporter in the TcrNT1-expressing cells (above the line, high affinity phase), and the low affinity inhibition of TcrNT1 in TbNBT-KO + TcNT1 (below the line, low affinity phase). B. Uptake of [^{3}H]-Inosine as a function of time by TbNBT-KO + EV and TbNBT-KO + TcrNT1. Cells were incubated with 50 nM of [^{3}H]-Inosine in absence and presence of inhibitors (3.3 μM of 2'-F-Adenosine or 1 mM of inosine) and the reaction was stopped at the referred time, showing a linear phase of transport of 12 s (r^2=0.99) . A and B show a representative out of at least three independent experiments in triplicate.

Figure 6. TcrNT1 is high-affinity inosine/guanosine transporter. A. TbNBT-KO cells expressing TcrNT1 were incubated with 50 nM of [^{3}H]-Inosine for 8 s in the presence of increasing concentrations of unlabelled inosine and a dose-dependent inhibition curve with Hillslope near -1 (inset) was observed. Conversion of the data into a Michaelis-Menten plot yielded a K_m of 1.0 ± 0.04 μM for inosine (n=4). Incubation of TbNBT-KO

+ TcrNT1 cells with 50 nM of [^{3}H]-Inosine in presence of increasing concentrations of adenosine (down triangles), guanosine (up triangles), hypoxanthine (circles) and xanthine (squares) (B) showed TcrNT1 has higher affinity for oxopurine nucleosides. C. Incubation of TbNBT-KO + TcrNT1 cells with 50 nM of [^{3}H]-Inosine in presence of adenine or pyrimidine nucleobases and nucleosides at 1 mM showed that TcrNT1 has no physiologically relevant affinity for these molecules. *P<0.05 by Unpaired Student's T-test. A and B show a representative out of at least three independent experiments. C shows average ± SEM of three independent assays. All assays were carried out in presence of 3.3 μM of 2'F-Ado in the assay buffer.

Figure 7. Uptake of [^{3}H]-Thymidine as a function of time in TbNBT-KO + EV and TbNBT-KO + TcrNT2 cells. Cells were incubated with 25 nM of [^{3}H]-Thymidine and the reaction was stopped at different times with ice-cold unlabelled thymidine in assay buffer (1 mM). Transport was linear for at least sixty seconds (r^2=0.99; blue line) in TbNBT-KO + TcrNT2, which was completely saturated by addition of 1 mM of unlabelled thymidine (open symbols). Figure shows a representative experiment in triplicate.

Figure 8. TcrNT2 is a high-affinity thymidine transporter. A. TbNBT-KO + TcrNT2 cells were incubated with 25 nM of [^{3}H]-Thymidine in the presence of increasing concentrations of unlabelled thymidine for 30 s and a dose-dependent inhibition curve with Hillslope near -1 (inset) was observed. Conversion of the data into a Michaelis-Menten plot yielded a K_m of 223.5 ± 7.1 nM (n=3). The incubation of TbNBT-KO + TcrNT2 cells with 25 nM of [^{3}H]-Thymidine in the presence of increasing concentrations of other pyrimidine nucleosides (B) revealed the preference of TcrNT2 for 2'-deoxypyrimidines,

as the affinity for 2'-deoxyuridine (K_i = 1.11 ± 0.1 μM; up triangles) was 59-fold higher than for uridine (K_i = 65.5 ± 6.0 μM; circles). Cytidine showed very low affinity for TcrNT2, yielding a K_i of 728 ± 71 μM. A and B show one representative out of three independent experiments in triplicate.

Figure 9. qRT-PCR of mRNA isolated from *T. cruzi* epimastigotes (EPI), metacyclic trypomastigotes (META), amastigotes (AMA) and bloodstream trypomastigotes (TRYP) for each of the *T. cruzi* ENT genes. Bars represent the average ± SEM of 3 independent biological replicates, each performed in triplicate. Each biological replicate was normalised to the expression level of the GAPDH gene in each of the lifecycle stages. N.D., not detectable.

Figure 10. Sensitivity of procyclic *T. brucei* nucleobase transporter-knockout cell lines to drugs. Alamar blue assays revealed that the heterologous expression of TcrNT2 by TbNBT-KO sensitised the cells to 5-Fluoro-2'-deoxyuridine by 38-fold (A), while the EC_{50} for Pentamidine (B) remained unchanged. $^{***}P<0.005$ by Unpaired Student's T-test. Bars represent average ± SEM of three independent experiments.