Trypanosoma brucei adenine-phosphoribosyltransferases mediate adenine salvage and aminopurinol susceptibility but not adenine toxicity ☆

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ARTICLE INFO

ABSTRACT

African trypanosomes, like all obligate parasitic protozoa, cannot synthesize purines de novo and import purines from their hosts to build nucleic acids. The purine salvage pathways of Trypanosoma brucei being redundant, none of the involved enzymes is likely to be essential. Nevertheless they can be of pharmacological interest due to their role in activation of purine nucleobase or nucleoside analogues, which only become toxic when converted to nucleotides. Aminopurine antimetabolites, in particular, are potent trypanocides and even adenine itself is toxic to trypanosomes at elevated concentrations. Here we report on the T. brucei adenine phosphoribosyltransferases TbAPRT1 and TbAPRT2, encoded by the two genes Tb927.7.1780 and Tb927.7.1790, located in tandem on chromosome seven. The duplication is syntenic in all available Trypanosoma genomes but not in Leishmania. While TbAPRT1 is cytosolic, TbAPRT2 possesses a glycosomal targeting signal and co-localizes with the glycosomal marker aldolase. Interestingly, the distribution of glycosomal targeting signals among trypanosomatid adenine phosphoribosyltransferases is not consistent with their phylogeny, indicating that the acquisition of adenine salvage to the glycosome happened after the radiation of Trypanosoma. Double null mutant T. brucei Δtbaprt1,2 exhibited no growth phenotype but no longer incorporated exogenous adenine into the nucleotide pool. This, however, did not reduce their sensitivity to adenine. The Δtbaprt1,2 trypanosomes were resistant to the adenine isomer aminopurinol, indicating that it is activated by phosphoribosyl transfer. Aminopurinol was about 1000-fold more toxic to bloodstream-form T. brucei than the corresponding hypoxanthine isomer allopurinol. Aminopurinol uptake was not dependent on the aminopurine permease P2 that has been implicated in drug resistance.

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1. Introduction

A common feature of most pathogens – viruses, bacteria, protozoa or tumor cells – is their fast proliferation and, consequently, high demand for nucleotides to replicate their genomes. Thus purine and pyrimidine analogues are widely used as chemotherapeutic agents. These are usually administered as nucleobase or nucleoside prodrugs that, once taken up by a target cell, need to be phosphorylated to the corresponding nucleotide in order to exert cytotoxic activity by inhibiting DNA or RNA polymerases. Knowledge on the molecular nature of a pathogen's nucleotide salvage pathways will therefore promote the rational design of nucleobase or nucleoside antimetabolites that, ideally, are specifically phosphorylated by the target organism but not by human cells.

Like all obligate parasitic protozoa, Trypanosoma brucei ssp. do not synthesize purines de novo (Fish et al., 1982a,b), having lost the genes of the purine anabolic pathway, presumably in adaptation to parasitism. The trypanosomes therefore depend on salvage of purines from their hosts: the tsetse flies (Glossina spp.) and mammals. Trypanosoma brucei rhodesiense and T. b. gambiense

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respectively cause East- and West-African sleeping sickness in humans, also known as human African trypanosomiasis (HAT). The parasites proliferate extracellularly in the blood and eventually cross the blood–brain barrier, with fatal consequences for the patient. Purines are taken up from the blood by several transporters of overlapping substrate specificities (de Koning et al., 2005). The aminopurines adenine and adenosine are thought to be the favorite purine source of bloodstream-form *T. brucei* since of all physiological purines, these are taken up the fastest (Fish et al., 1982a). Adenosine is imported via P1- and P2-type transporters, adenine is taken up via P2, H2 and H3 (Carter and Fairlamb, 1993; de Koning and Jarvis, 1997). The pharmacological importance of trypanosomal purine permeases was underscored by the findings that P2 also transports trypanocidal drugs like melarsoprol, diminazene and pentamidine (Carter and Fairlamb, 1993; Carter et al., 1995), and that loss of the gene encoding P2, *TbAT1*, reduces the sensitivity to these drugs (Mäser et al., 1999; Matovu et al., 2003). Homozygous deletion of *TbAT1* in *T. brucei* also resulted in resistance to adenosine analogs such as cordycepin (3′-deoxyadenosine) or tubercidin (7-deazaadenosine; Geiser et al., 2005; Lüscher et al., 2007).

Adenine at high (e.g. millimolar) concentrations is toxic to trypanosomes (Taliaferro and D'Alesandro, 1971; Geiser et al., 2005). Adenine toxicity is a phenomenon also known from *Escherichia coli*, where it is exacerbated by genetic disruption of hypoxanthine and guanine phosphoribosyltransferases (E.C. 2.4.2.8) and counteracted by addition of guanosine (Levine and Taylor, 1982). This clearly indicates that an excess of adenine may be cytotoxic to *E. coli* because it disturbs the cellular purine balance by depleting the guanine pool and increasing the ratio of [AMP] to [GMP]. However, it is unknown whether adenine has the same effect on *T. brucei*. The purine salvage machinery of bloodstream-form *T. brucei* is able to interconvert all the physiological purine nucleobases, nucleosides, and nucleotides (Fish et al., 1982a). Interestingly, some of the enzymes involved localize to the glycosomes, which are membrane-bound organelles of kinetoplastid parasites that are devoted mainly to glycolysis but are also involved in other metabolic pathways including purine salvage and pyrimidine biosynthesis (Opperdoes and Michels, 1993).

Glycosomal proteins carry targeting signals that are very similar to the known peroxisomal targeting signals (PTS), suggesting a common evolutionary origin of glycosomes and peroxisomes (Michels et al., 2005). Two types of PTS are known: serine-lysine-leucine (PTS1) or similar tripeptides at the C-terminus of glycosomal proteins, or a less conserved nonapeptide (PTS2) at the N-terminus of glycosomal proteins (Petriv et al., 2004). Hypoxanthine-guanine phosphoribosyltransferases identified from *Leishmania* spp., *Trypanosoma cruzi* and *T. brucei* all carried C-terminal PTS1 signals, and in *T. brucei* and *Leishmania* were localized to the glycosomes (Hassan et al., 1985; Shih et al., 1998a,b). Leishmanial HGPRTases are of high pharmacological interest since they accept allopurinol as a substrate (Hwang et al., 1996); human HGPRT is also able to phosphoribosylate allopurinol although it is a poor substrate (Krenitsky et al., 1969) but, unlike in hemoflagellates the resulting allopurinol riboside is not converted to aminopurinol riboside and consequently is not incorporated into nucleic acids (Hitchings, 1975; Shapiro et al., 1991). Allopurinol [4-hydroxypyrazolo(3,4-d)pyrimidine; HPP], used in humans primarily against hyperuricemia (e.g. gout) but also against leishmanioses, is a close structural isomer of hypoxanthine that carries the nitrogen at position 8 instead of 7 in the purine ring. Allopurinol is very active against *Leishmania* spp. and *T. cruzi* (Berens et al., 1982; Apt et al., 2003), but less so against *T. brucei* (Marr and Berens, 1983; Natto et al., 2005; de Koning, unpublished). The analogous N8 isomer of adenine, aminopurinol (4-aminopyrazolopyrimidine; APP) exhibits promising activity against *T. brucei* (Wallace et al., 2002; Natto et al., 2005). Here we identify and characterize the two *APRT* genes from *T. b. brucei* with particular respect to the subcellular localization of the gene products and their role in aminopurinol susceptibility.

2. Materials and methods

2.1. Cultivation of trypanosomes, transfection, drug sensitivity tests

Bloodstream-form *T. b. brucei* 221 (MITat 1.2) were cultivated in HMI-9 medium (Hirumi and Hirumi, 1989) and 10% FCS at 37 °C in a humidified atmosphere at 5% CO_2. Procyclic *T. b. brucei* 427 were cultivated at 27 °C in SDM-79 (Brun and Schönenberger, 1979) supplemented with 5% fetal calf serum and 1 µg ml^{-1} hemin. For stable transfection, 10^8 procyclic or 2×10^7 bloodstream-form trypanosomes were washed and resuspended in 450 µl of electroporation buffer (120 mM KCl, 150 mM $CaCl_2$, 4 mM $MgCl_2$, 8.7 mM K_2HPO_4, 1.3 mM KH_2PO_4, 25 mM HEPES, 2 mM EDTA), mixed with 10 µg linearized plasmid DNA, and electroporated at 1.5 kV; antibiotic selection started 24 h after transformation. Positive transformants were cloned by limiting dilution and verified by PCR as well as Southern blots. Drug sensitivities were determined with the AlamarBlue® assay at inocula of 10^3 (bloodstream forms) or 5×10^4 (procyclics) and 72 h of incubation (Räz et al., 1997). The read-out was fluorescence, measured at wavelengths 530 nm for excitation and 590 nm for emission (Spectromax Gemini). IC_{50} values were calculated by non-linear fitting of the data to a sigmoidal dose-response curve with variable slope (Prism, GraphPad Software). All compounds were purchased from Sigma except 7-deazaadenine (Berry&Associates). Isothermal microcalorimetry was performed as described (Wenzler et al., 2012). Bloodstream-form cells, 10^5 in 2 ml HMI-9 medium and 10% horse serum, were inoculated to calorimetry ampoules. Drugs were added to the desired concentration and the ampoules were immediately sealed and placed into the microcalorimeter (TAM III, TA Instruments, New Castle DE, USA). Ampoules containing medium without trypanosomes served as negative controls, ampoules containing trypanosomes without drugs served as positive controls. All experiments were carried out twice, each in triplicate.

2.2. TbAPRT constructs and primers

The constructs for replacement by homologous recombination of the *TbAPRT1,2* locus with selectable markers were made by ligating 160 bp of the *TbAPRT1* 5′-UTR, amplified by PCR from genomic DNA with primers KO180for (5′-ccggtaccgtcacttgtggaggttttgc-3′) and KO180rev (5′-ccaagcttgcgcatcacttcaggatttt-3′), and 160 bp of the *TbAPRT2* 3′-UTR, amplified with KO200for (5′-ccggtaccgt-cacttgtggaggttttgc-3′) and KO200rev (5′-gctctagacacagtcgtgacctgg-taatg-3′), to either side of hygromycin (first round) or neomycin (second round) resistance genes cloned into pBluescript II SK+. Verification of positive transformants by Southern blot was done using as probes cloned PCR products specific to *TbAPRT1*, amplified with primers Fw180 (5′-aatcttcagcagcccatcac-3′) and Rev180 (5′-gcgttcctttgaggaaagtg-3′), or *TbAPRT2*, amplified with Fw200 (5′-atgccaattgtgctcacgta-3′) and Rev200 (5′-gccacatcacagacggtaag-3′). The same probes were used for Northern blots on total RNA isolated with the hot phenol method (Roditi et al., 1989). The constructs for C-terminal *in situ* tagging were created by PCR using as template plasmid pMOTag3H containing a triple hemagglutinin tag (100 bp) plus neomycin resistance gene (800 bp) for TbAPRT1, and pMOTag3xM4 containing a triple Myc tag (520 bp) plus hygromycin resistance gene (1 kb) for TbAPRT2. Chimeric primers were used which consisted of the last 80 nt of the target *TbAPRT* coding region (forward) – respectively the first 80 nt of the target TbAPRT

3′-UTR (reverse) – plus 20 nt specific to the tagging cassette on the template plasmid (Oberholzer et al., 2006). PCR products of the expected size for directly electroporated into trypanosomes and positive transformants were verified by PCR and sequencing for in-frame insertion of the tag.

2.3. Immunofluorescence

For immunofluorescence, 2×10^6 bloodstream-form or 10^7 procyclic trypanosomes were washed with PBS supplemented with 10 g/l glucose, and spread onto Diagnostic Microscope Slides (Erie Scientific) which had been coated for 30 min with polylysin (100 μg/ml; Sigma). The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), washed three times with PBS (for procyclic forms supplemented with 0.1 M glycine), and permeabilized with 0.1% tritonX-100 (bloodstream forms) or 0.05% Tween-20 (procyclics) in PBS. The cells were washed three times with PBS and (procyclics only) incubated for 10 min in methanol at −20 °C followed by rehydration in PBS. After blocking with 2% BSA, the slides were incubated for 45 min in PBS plus 2% BSA with the primary antibody: for TbAPRT1 monoclonal mouse anti-HA diluted 1:300 (Roche), for TbAPRT2 monoclonal mouse anti-myc at 1:100 (Santa Cruz), for aldolase monoclonal rabbit anti-aldolase at 1:10,000 (kind gift of Paul Michels, Université Catholique de Louvain, Brussels). After three washes, the slides were incubated in the dark for 45 min with the secondary antibody: goat anti-mouse Alexa Fluor® 488 (Invitrogen) or goat anti-rabbit 594 (Molecular Probes) at 1:1000. The slides were washed three times and mounted in Vectashield® containing 1.5 μg/ml DAPI (Vector laboratories, Inc.).

2.4. Cell fractionations

For Western blots, total cell lysates of 10^7 cells were run on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Immobilon-P, Millipore) in 3 g/l Tris, 14.4 g/l glycine, 20% methanol. After blocking in TBS (10 mM Tris–HCl pH 7.4, 150 mM NaCl) plus 5% milk powder, the blots were hybridized for 1.5 h with primary antibody (1:1000 for anti-HA and anti-Myc; 1:100,000 for anti-aldolase), washed three times in TBS 0.05% Tween-20, and incubated for 1.5 h with the secondary antibody (rabbit anti mouse-HRP; Dako). Signals were detected using ECL-plus (Amersham Biosciences). For digitonin extraction of proteins, 2.5×10^7 cells per assay were washed in SBG (22 mM glucose, 150 mM NaCl, 20 mM NaHPO$_4$ pH 7.8), resuspended in 125 μl SoTE (0.6 M sorbitol, 20 mM Tris–HCl pH 8.0, 2 mM EDTA), and carefully mixed with an equal volume of digitonin at the indicated concentration. After 5 min on ice, soluble (supernatant of 6800 g) and insoluble (pellet of 20,000 g) fractions were separated by centrifugation.

2.5. Purine transport assays

Uptake of [³H]-hypoxanthine and [³H]-adenosine (both from GE Healthcare) was performed essentially as described previously (de Koning and Jarvis, 1997, 1999). Bloodstream trypanosomes T. b. brucei 221 were cultivated in HMI-9 medium for the adenosine uptake studies and isolated from adult female Wistar rats for the hypoxanthine uptake experiments, separating parasites from blood cells on DE52 anion exchange columns (Whatman) and washed into standard assay buffer (AB) as described (de Koning and Jarvis, 1999). Radiolabel concentrations for assaying H2, H3 and P2 activity were 0.1 μM [³H]-hypoxanthine, 1 μM [³H]-hypoxanthine and 0.05 μM [³H]-adenosine, respectively, and incubation time with radiolabel was 30 s. Assays were performed in AB (33 mM HEPES, 98 mM NaCl, 4.6 mM KCl, 0.55 mM CaCl$_2$, 0.07 mM MgSO$_4$,

5.8 mM NaH$_2$PO$_4$, 0.3 mM MgCl$_2$, 23 mM NaHCO$_3$, 14 mM glucose, pH 7.3) and incubations were terminated by the addition of 1 mM unlabelled permeant in ice-cold AB followed by centrifugation through oil (30 s, 12,000g). Radioactivity in the ensuing cell pellet was determined by liquid scintillation counting. Data was analyzed on GraphPad Prism 5.0 using non-linear regression after subtraction of non-specific radiolabel binding. When inhibition with aminopurinol was between 50% and 100% of uptake, incomplete inhibition curves were extrapolated to yield IC$_{50}$ value based on the assumption of full inhibition and a Hill slope of −1. To measure incorporation of adenine into the nucleotide pool, procyclic trypanosomes were incubated at 10^7 cells ml^{-1} in AB plus 1 μM [³H]-adenine at 27 °C for a maximum of 10 min, washed with ice-cold buffer, and extracted in 0.1 M HCl by freeze-thawing. After centrifugation at 12,000 g, samples of the supernatant were spotted onto silica thin-layer chromatography plates (Merck LuxPLate 60F254) and run with a mixture of n-butanol, ethyl acetate, methanol and ammonia of 7:4:3:4 (vol). Nucleotide and nucleobase standards were run alongside the samples. Radiolabel was quantified by scanning the air-dried plates in a radioisotope detector (Berthold Technologies, Switzerland).

3. Results and discussion

3.1. Trypanosoma brucei possesses two adenine phosphoribosyltransferases

The T. b. brucei genome (Berriman et al., 2005) was annotated to contain two putative APRT genes in tandem on chromosome VII: Tb927.7.1780 and Tb927.7.1790. The two predicted proteins return highly significant expectancy of 10^{-31} and 10^{-18}, respectively, when run with HMMer (Eddy, 1998) against the phosphoribosyltransferase profile PF00156 from Pfam (Finn et al., 2008). We screened the predicted T. brucei proteome (version 4; 9192 proteins) for further phosphoribosyltransferase domains using HMMer with Pfam profile PF00156. This search returned nine hits with E-values below 10^{-12}. The predicted proteomes of T. cruzi and Leishmania major were searched as well, and the mammalian host Homo sapiens was included as a reference. All the hits with an expectancy below 10^{-10} were aligned with ClustalW (Thompson et al., 1994). In the resulting phylogenetic tree (Supplementary Figure S1), the two T. brucei proteins Tb927.7.1780 and Tb927.7.1790 clustered with the known APRTases from L. major and H. sapiens and were therefore named TbAPRT1 and TbAPRT2. The APRT clade is proximate to the orotate phosphoribosyltransferases (OPRT; EC 2.4.2.10) and clearly distinct from the HGPRT (2.4.2.8)/XPRT (2.4.2.22) clade (Figure S1).

The two predicted proteins TbAPRT1 and TbAPRT2 share only 24% identity at the level of amino acids, and a nucleotide dot plot of the region encompassing the two genes does not reveal any similarities at the level of genomic DNA (not shown). Thus if the tandem location of TbAPRT1 and TbAPRT2 resulted from the duplication of a primordial gene, it must have been an ancient event. This presumed duplication is older than the radiation of Trypanosoma spp. since the tandem APRT locus is syntenic in Trypanosoma congolense, Trypanosoma vivax and T. cruzi [tritrypdb.org]. In contrast, Leishmania spp. possess only one APRT gene per genome, indicating that the presumed APRT duplication in Trypanosoma happened after the divergence from Leishmania (or that APRT2 was lost from the common ancestor of the Leishmania spp.; Fig. 1). The situation is different regarding HGPRT genes, which form tandem duplications in Trypanosoma as well as in Leishmania (Fig. 1). The two leishmanial genes encode for hypoxanthine-guanine and xanthine phosphoribosyltransferases, respectively. Genetic disruption of either gene was viable in Leishmania
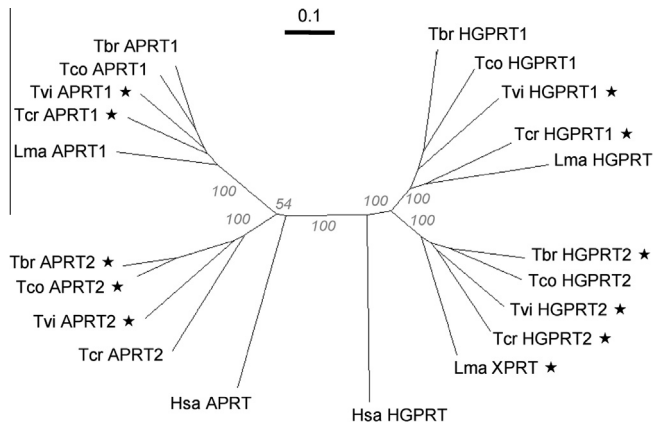
Fig. 1. Phylogenetic tree of trypanosomatid predicted APRT and HGPRT proteins plus human APRT (UniProt P07741) and HGPRT (UniProt P00492) as outgroups. Proteins with a C-terminal PTS1 signal as predicted by the PTS1 Predictor (Neuberger et al., 2003) are labeled with an asterisk. Bootstrapping values for the major branches are given as percent positives of 1000 rounds. The scale bar indicated number of changes per site. For the *Trypanosoma* tandem genes, the one upstream was labeled as number one. GeneDB accession numbers are Tc00.1047053508207.74 (TcrAPRT1), Tc00.1047053508207.70 (TcrAPRT2), TcIL3000.0.32550 (TcoAPRT1), TcIL3000.7.1260 (TcoAPRT2), TvY486_0701640 (TviAPRT1), TvY486_0701650 (TviAPRT2), LmjF26.0140 (LmaAPRT), Tb927.10.1400 (TbrHGPRT1), Tb927.10.1390 (TbrHGPRT2), TcIL3000.10.1180 (TcoHGPRT1), TcIL3000.10.1170 (TcoHGPRT2), TvY486_1001390 (TviHGPRT1), TvY486_1001370 (TviHGPRT2), Tc00.1047053506457.30 (TcrHGPRT1), Tc00.1047053506457.40 (TcrHGPRT2), LmjF21.0845 (LmajHGPRT), LmjF21.0850 (LmajXPRT).

donovani (Boitz and Ullman, 2006b), but Δ*hgprt/xprt* double mutants were synthetic lethal (Boitz and Ullman, 2006a, 2010).

To experimentally test the predicted adenine phosphoribosyltransferase function, we generated homozygous *T. b. brucei* *tbaprt1,tbaprt2* double knock-out mutants (procyclic forms). A region of 2150 bp encompassing the two *TbAPRT* genes was replaced, via homologous recombination, with selectable constructs containing hygromycin and neomycin resistance genes. Positive transformants were cloned by limiting dilution and correct integration of the constructs into the *TbAPRT1,2* locus was verified by Southern blots (Supplementary Figure S2). The resulting Δ*tbaprt1,2* trypanosomes grew as fast as their 'wild-type' parents (Fig. 2a) in standard cultivation medium; the population doubling times were 14.2 h for 'wild-type' and 13.4 h for Δ*tbaprt1,2* trypanosomes. However, when incubated with [³H]-adenine the Δ*tbaprt1,2* trypanosomes did not incorporate radiolabel into the nucleotide pool to significant levels (Fig. 2b), demonstrating that Δ*tbaprt1,2* mutants lack
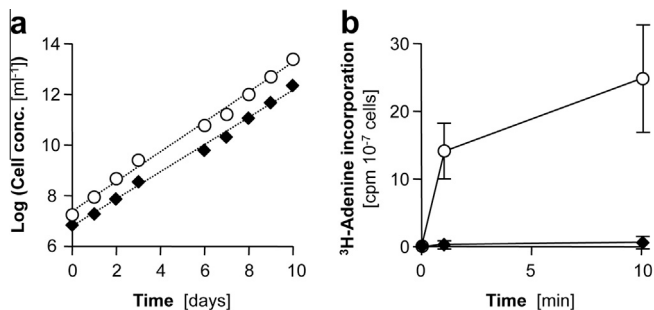

Fig. 2. Homozygous disruption of *TbAPRTs* in procyclic *T. brucei.* (a) Double null mutant Δ*tbaprt1,2* trypanosomes (black diamonds) grew as fast as wild-type (white circles) in SDM-79. The cultures were diluted regularly during the experiment and the dilution factors accounted for in the virtual concentration given on the *y*-axis. (b) In contrast to wild-type trypanosomes, the Δ*tbaprt1,2* mutants did not incorporate [³H]adenine into the nucleotide pool as determined by thin layer chromatography of cells lysed at the indicated time points. Error bars represent standard deviation of three independent experiments.

APRTase activity. The requirement of APRT to incorporate adenine into the nucleotide pool is consistent with the absence of an adenine deaminase activity in *T. b. brucei*, which is believed to be present only in *T. vivax* (Ogbunude and Ikediobi, 1983) as well as in *Leishmania* spp. (LaFon et al., 1982). Salvage of [³H]-hypoxanthine was not impaired in Δ*tbaprt1,2* trypanosomes (not shown).

3.2. Subcellular localization of TbAPRT1 and TbAPRT2 in trypanosomes

The genes *TbAPRT1* and *TbAPRT2* are expressed in bloodstream-form as well as in procyclic, tsetse fly midgut-stage trypanosomes as determined by Northern blot analysis (Fig. 3a), which is in agreement with existing proteomics data (Vertommen et al., 2008). Steady-state mRNA levels of *TbAPRT2* appeared to be higher in procyclic than in bloodstream-form trypanosomes (Fig. 3a). While the predicted protein TbAPRT2 possesses a *bona fide* glycosomal targeting signal (serine-arginine-leucine), TbAPRT1 does not. To experimentally investigate their subcellular localization, the *TbAPRT1* and the *TbAPRT2* gene products were tagged C-terminally with haemagglutinin and c-myc, respectively, by homologous recombination *in situ* (Oberholzer et al., 2006). Heterozygous transformants carrying one tagged allele of either TbAPRT1 or TbAPRT2 were selected by means of the incorporated neomycin, respectively hygromycin, resistance markers and cloned by limiting dilution. Differential lysis of cells was performed with digitonin, which at lower concentration (0.1 mg digitonin per mg protein) only dissolves the plasma membrane whereas at higher concentration (0.6 mg per mg protein) it also dissolves organellar membranes. Differential lysis followed by Western blotting (Fig. 3b) or direct immunofluorescence of fixed cells (Fig. 4) demonstrated that TbAPRT1 localized to the cytosol while TbAPRT2 co-localized with the glycosomal marker aldolase. The result that a C-terminal myc tag apparently did not prevent TbAPRT2 from getting to the glycosome might be explained by the fact that APRTase is a dimeric enzyme. We speculate that a dimer containing one tagged subunit and one with a free C-terminal PTS1 could still be targeted to the glycosome. Peroxisomal proteins are imported folded and oligomeric (Leon et al., 2006). The presence of TbAPRT2 in the glycosomes implies that there ought to be an aminopurine permease in the glycosomal membrane. Genes encoding permeases for purine nucleobases, including adenine, have been identified from *T. brucei* (Mäser et al., 1999; Burchmore et al., 2003; Henriques et al., 2003; Ortiz et al., 2009). Several of these transporters were localized to the plasma membrane (Henriques et al., 2003; Ortiz et al., 2009) but no evidence exists for a glycosomal location.

Leishmania have only one APRTase and it is a cytosolic enzyme (Zarella-Boitz et al., 2004). The South-American *T. cruzi* and the African *T. brucei* each possess two APRTase paralogues. An interesting difference between *T. brucei* and *T. cruzi* emerged when the presence of predicted glycosomal targeting signals was overlaid to the phylogeny of phosphoribosyl transferases (Fig. 1). While TbAPRT2 carries a PTS1 and TbAPRT1 does not, the situation is reversed in *T. cruzi* where TcAPRT1 has a PTS1 and TcAPRT2 does not (Fig. 1). The distribution of glycosomal targeting signals among trypanosomatid phosphoribosyltransferases is generally not consistent with their phylogeny (Figs. 1 and S1). An interesting case is presented by the phosphoribosyl-pyrophosphate synthetases (PRS; EC 2.7.6.1): *T. brucei*, *T. cruzi* and *L. major* each possess three PRS paralogues, of which in every species a different one is predicted to be targeted to the glycosome (Figure S1). In summary, this argues that the recruitment of adenine salvage enzymes to the glycosome happened independently, after the radiation of trypanosomatids. The physiological role of purine salvage enzymes in the glycosomes is unclear, since replacement of glycosomal XPR-Tase in *L. donovani* with a truncated, cytosolic form did not impair purine salvage (Zarella-Boitz et al., 2004).
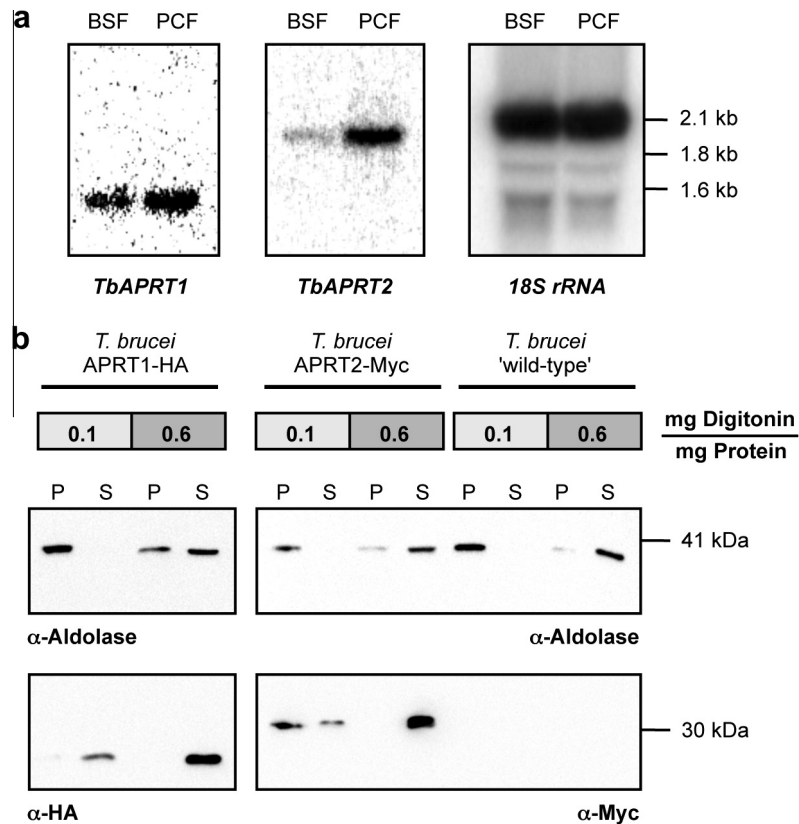
Fig. 3. Expression of *TbAPRT1* and *TbAPRT2* in *T. brucei*. (a) The genes are expressed in procyclic and in bloodstream-form trypanosomes as determined by Northern blot, *TbAPRT2* more strongly in the procyclics (a probe of the 18S rRNA was used as a loading control). (b) Western blots of the *in situ*-tagged gene products after digitonin lysis of the cells. While TbAPRT1 was in the soluble fraction (S, supernatant) even at low digitonin concentration, TbAPRT2 and the glycosomal marker aldolase were in the insoluble fraction (P, pellet) at low digitonin concentration.
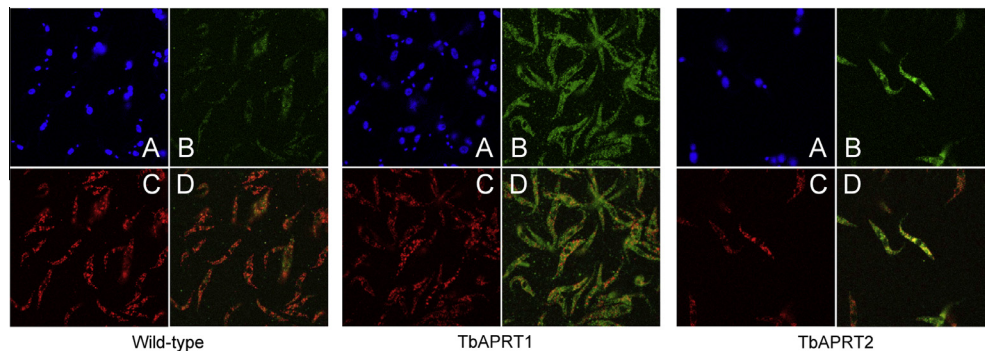
Fig. 4. Immunofluorescence microscopy of procyclic *T. brucei*: wildtype (left), HA-tagged TbAPRT1 cells (middle), and Myc-tagged TbAPRT2 cells (right). (A) DAPI staining, (B) anti-TbAPRT stain (anti-HA for TbAPRT1 and wildtype, and anti-myc for TbAPRT2), (C) anti-aldolase stain, (D) overlay of B and C. TbAPRT2 but not TbAPRT1 appeared to co-localize with aldolase.

3.3. Antitrypanosomal activity of purine nucleobase analogues

While adenosine antimetabolites such as cordycepin, tubercidin, or vidarabine are known as potent trypanocides (Bacchi et al., 1992; Kaminsky et al., 1996; Bressi et al., 2001; Drew et al., 2003; Geiser et al., 2005; Rodenko et al., 2007; Vodnala et al., 2008), adenine antimetabolites have so far received little attention (Wallace et al., 2002, 2004). Yet, adenine analogues are attractive trypanocides for many reasons. As mentioned above, trypanosomes lack adenine and adenosine deaminase (Ogbunude and Ikediobi, 1983), making adenine analogues highly stable. Secondly, *T. brucei* express at least three separate adenine transporters in bloodstream forms: the aminopurine transporter P2 (de Koning et al., 2005), and the broad specificity nucleobase transporters H2

and H3 (de Koning and Jarvis, 1997). Uptake by multiple transporters greatly reduces the chances of resistance through loss of transporter function, as we previously demonstrated for allopurinol (Natto et al., 2005) and pentamidine (de Koning, 2008). Moreover, adenines have a higher rate of membrane diffusion than hypoxanthine analogues or nucleosides; in *Toxoplasma gondii* adenine salvage was found to be non-saturable and consistent with simple diffusion (De Koning et al., 2003), made possible by a favourable octanol:water partition coefficient XLogP3 (Cheng et al., 2007) of just −0.1.

We determined the sensitivity of different *T. brucei* lines to adenine and analogues with an *in vitro* test using the fluorescent dye AlamarBlue® as an indicator of cell viability (Räz et al., 1997). As observed previously (Taliaferro and D'Alesandro, 1971; Geiser

et al., 2005), adenine itself was toxic to trypanosomes with an IC_{50} value around 300 μM (Fig. 5); bloodstream-form and procyclic *T. brucei* were equally sensitive. Adenine toxicity in *E. coli* is thought to be linked to an imbalance between cellular GTP and ATP levels (Levine and Taylor, 1982). In *T. brucei*, however, deletion of TbAPRT did not alleviate adenine toxicity (Fig. 5). As the adenine was no longer incorporated into the nucleotide pool (Fig. 2b) this means that in trypanosomes adenine toxicity is not caused by an imbalance between [ATP] and [GTP]. Furthermore, adenine toxicity in *E. coli* could be counteracted by addition of excess guanosine (Levine and Taylor, 1982), which is not the case in *T. brucei* (data not shown). Our conclusion that adenine toxicity in trypanosomes is not linked to increased adenine nucleotide levels is in agreement with the fact that adenosine, which gets incorporated to the nucleotide pool by adenosine kinase (Lüscher et al., 2007), has no effect on *T. b. brucei* growth rates up to millimolar concentrations. Thus, the mechanism of adenine toxicity in trypanosomes remains elusive.

In *Leishmania*, allopurinol is converted to allopurinol riboside monophosphate by HGPRT, aminated to aminopurinol riboside monophosphate in two steps by adenylosuccinate synthetase and -lyase, and then phosphorylated to the triphosphate which gets incorporated into RNA (Marr et al., 1978). Phosphoribosyl transfer to allopurinol is very inefficient in mammalian cells (Krenitsky et al., 1969) with most being rapidly metabolized to oxipurinol (Hitchings, 1975). The approximately 10% of allopurinol converted to allopurinol riboside monophosphate is not accepted by human adenylosuccinate synthetase whereas it can substitute for IMP as a substrate for the corresponding enzyme of *Leishmania* (Spector et al., 1984). Similarly, allopurinol riboside monophosphate is an inhibitor of *L. donovani* GMP reductase with 100-fold weaker activity against the human enzyme (Spector et al., 1984).

While these observations explain the therapeutic window of allopurinol for the treatment of leishmanioses and Chagas' disease, allopurinol was not potent against *T. brucei* (IC_{50} against bloodstream forms of 170 μM; not shown). In contrast, aminopurinol exhibited submicromolar activity against *T. brucei* bloodstream forms with an IC_{50} value of 190 nM (Fig. 5). To determine whether amimopurinol acts in a cytostatic or a cytotoxic way, bloodstream-form trypanosomes were subjected to isothermal microcalorimetry. This technique allows to non-invasively monitor cell proliferation in real time by measuring the produced heat flow (Wenzler et al., 2012). In contrast to the cytotoxic reference drug pentamidine, aminopurinol did not kill the trypanosomes but merely inhibited their growth (Fig. 6).
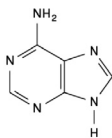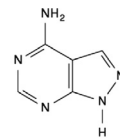

Fig. 6. Real-time isothermal microcalorimetry with bloodstream-form *T. b. brucei* exposed to aminopurinol (green, 7.5 μM; blue, 15 μM; magenta, 25 μM; dark blue, 45 μM), pentamidine (red, 15 nM), or no drug at all (black). The curves are averages of triplicates, except for the negative control without trypanosomes (grey). The experiment was performed as described (Wenzler et al., 2012). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Uptake of allopurinol and aminopurinol by T. brucei bloodstream forms

An alternative explanation for the much greater efficacy of aminopurinol could lie in more efficient uptake of this prodrug. We previously reported allopurinol inhibition constants for the *T. b. brucei* nucleobase transporters H2 and H3 (K_i values of 4.0 and 194 μM, respectively; (de Koning and Jarvis, 1997)). We report here that these transporters in fact display lower affinity for aminopurinol (Fig. 7), with K_i values of 230 ± 35 and 105 ± 24 μM for H2 and H3, respectively. In addition, we determined the K_i value for the P2 aminopurine transporter at 75.3 ± 5.7 μM ($n = 6$; Fig. 7) – lower than for allopurinol (K_i = 260 μM) (de Koning and Jarvis, 1999), but much higher than for adenine (K_i = 0.3 μM). Thus, N8 appears to be unfavourable for binding to P2. We tested whether aminopurinol activity was dependent on P2 expression using a Δ*tbat1* strain (Matovu et al., 2003), but found no resistance to aminopurinol (Fig. 8) although we observed 18-fold resistance to diminazene, consistent with previous reports (de Koning et al., 2004). We thus conclude that the remarkable efficacy of aminopurinol versus allopurinol against *T. b. brucei* is highly unlikely to be related to more efficient uptake. Instead, it is most likely attributable to the much more efficient conversion of the prodrug to the active compound, aminopurinol riboside triphosphate. As allopurinol is a substrate for TbHGPRT (Allen and Ullman, 1993) it must be the adenylosuccinate synthetase/lyase step that is principally responsible for the striking difference.

3.5. Phenotype of procyclic form APRT null mutants

Double null mutant Δ*tbaprt1,2* procyclics were significantly less sensitive to aminopurinol than their 'wild-type' parent ($p < 0.001$, one-way Anova followed by Tukey's multiple comparison test) even though procyclic cells were already much less sensitive than bloodstream forms (Fig. 5), confirming that the drug is activated by phosphoribosyl transfer. This is in agreement with results from *L. donovani*, where aminopurinol was used to select for Δ*aprt* null

	Adenine IC_{50} ± SD [μM]	Aminopurinol IC_{50} ± SD [μM]
T. brucei BSF	270 ± 150	0.20 ± 0.086
T. brucei PCF	320 ± 160	82 ± 37
T. brucei PCF Δ*aprt1,2*	290 ± 100	550 ± 190

Fig. 5. Structure of adenine and aminopurinol and activity against bloodstream-form (BSF) *T. b. brucei*, procyclic forms (PCF), and procyclic forms carrying a homozygous deletion of the complete *TbAPRT1* and *TbAPRT2* locus (Δ*aprt1,2*). Activity was determined *in vitro* with the Alamar blue assay Hirumi and Hirumi, 1989.
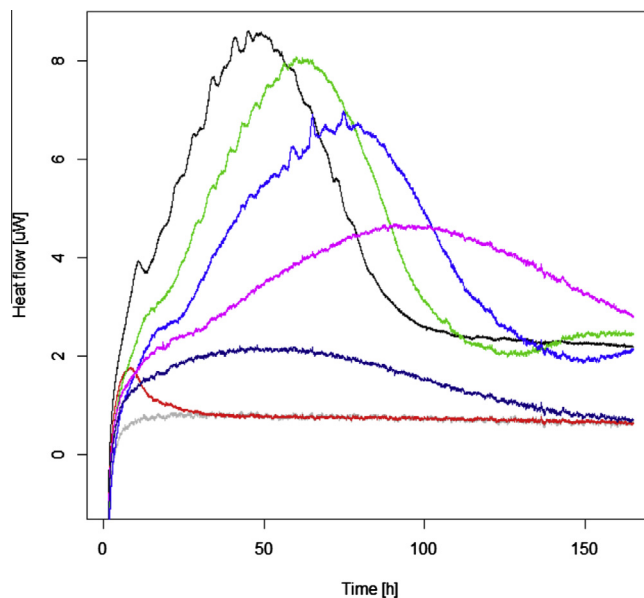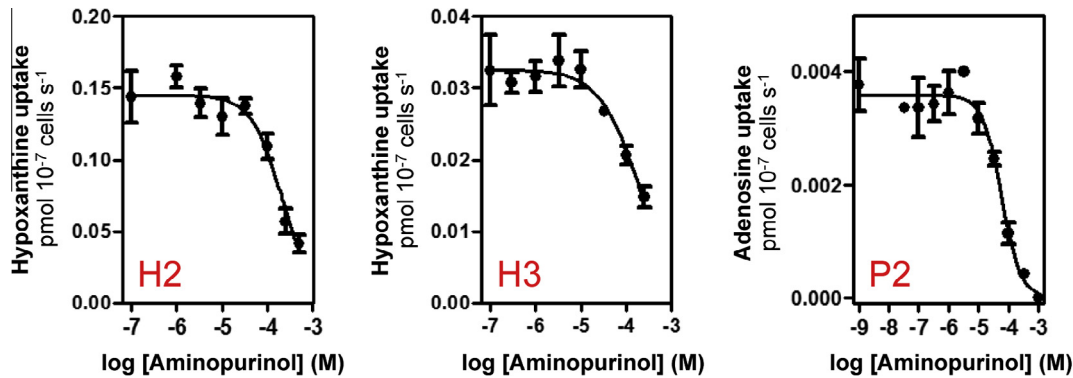
Fig. 7. Inhibition of purine transporters of bloodstream-form *T. brucei* by aminopurinol. (A) inhibition of the H2 nucleobase transporter using 0.1 μM [³H]-hypoxanthine (de Koning and Jarvis, 1997), (B) inhibition of the H3 nucleobase transporter using 1 μM [³H]-hypoxanthine (de Koning and Jarvis, 1997), (C) inhibition of the P2 aminopurine transporter using 0.05 μM [³H]-adenosine in the presence of 1 mM inosine (to saturate P1; Carter and Fairlamb, 1993). All experiments were conducted in triplicate and standard errors are indicated. Experiments shown are representative of at least three independently conducted experiments. In all cases, incubation time with radiolabel was 30 s, well within the linear phase of uptake (de Koning and Jarvis, 1997, 1999).
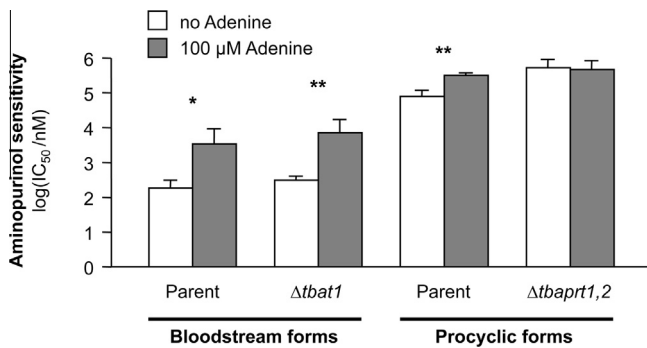
Fig. 8. Effect of adenine on aminopurinol sensitivity of wild-type and mutant *T. brucei* lines. IC_{50} values were determined with the Alamar blue assay Räz et al., 1997 in the presence (grey) or absence (white) of 100 μM adenine and are shown on a log scale. The statistical significance (asterisks) was determined on the untransformed data with two-tailed *t*-tests. Error bars represent standard deviations of at least three independent experiments.

mutants (Hwang et al., 1996). Thus, aminopurinol is likely to have the same mechanism of action as allopurinol, but requires only a one-step activation by APRT to aminopurinol riboside monophosphate. Somewhat surprisingly, given that the purine salvage pathways are similar in the two life stages (Fish et al., 1982a,b), aminopurinol was over two orders of magnitude more active against bloodstream-form than against procyclic trypanosomes (Fig. 5). One difference in purine salvage between procyclic and bloodstream-form trypanosomes is that the aminopurine permease P2 is only active in the latter (de Koning et al., 1998; de Koning and Jarvis, 1999), but the relatively low affinity of the compound for this transporter, and the lack of resistance to aminopurinol in the Δtbat1 line, show this is not a crucial difference in this case. Addition of adenine at 100 μM decreased aminopurinol susceptibility in bloodstream-form as well as in procyclic *T. brucei* (Fig. 8), as observed previously for *T. cruzi* (Marr et al., 1978). The two possible explanations for this observation are that adenine could either be competing with aminopurinol for transport into the cell, or alternatively inside the cell at the APRT binding site. Since 100 μM adenine had no effect on aminopurinol susceptibility in Δtbaprt1,2 trypanosomes (Fig. 8), the counter-acting effect of adenine must be caused at the APRT site rather than at the level of uptake. We conclude that aminopurinol uptake is largely independent of any adenine-sensitive transporters and may to a significant degree involve simple diffusion. Its physical characteristics,

which are highly similar to adenine with a XLogP3 of −0.2 (compare −1.1 for hypoxanthine and adenosine (source: pubchem.ncbi.nlm.nih.gov)) would allow a sufficient penetration into the trypanosome considering the duration of the standard drug sensitivity protocol with Alamar blue (72 h of exposure *in vitro*).

4. Conclusion

T. brucei possess two adenine phosphoribosyltransferase genes located in tandem on chromosome VII: *TbAPRT1* (Tb927.7.1780) and *TbAPRT2* (Tb927.7.1790). The duplication is syntenic in *T. congolense*, *T. vivax* and *T. cruzi*, but absent in *Leishmania* spp. (Fig. 1). The two genes are expressed in bloodstream-form as well as procyclic trypanosomes (Fig. 3a). While TbAPRT1 is cytosolic, TbAPRT2 carries a C-terminal PTS1 signal and co-localizes with the glycosomal marker aldolase (Figs. 3b and 4). As expected given the high degree of redundancy of the purine salvage pathways in *T. brucei*, *TbAPRT1* and *TbAPRT2* are not essential genes *in vitro* and there was no growth phenotype (Fig. 2a). Double null mutant Δtbaprt1,2 trypanosomes lack APRTase activity (Fig. 2b) which, however, does not protect them from the toxic effect of high adenine concentrations. Thus, in contrast to the situation in *E. coli*, adenine toxicity in *T. brucei* does not seem to be caused at the level of purine nucleotide imbalance and remains an unresolved phenomenon – which nonetheless demonstrates that trypanosomal purine salvage harbors vulnerable targets. The pharmacological importance of purine salvage was further underscored by the finding that the Δtbaprt1,2 trypanosomes were resistant to aminopurinol (Fig. 5). With an IC_{50} of 190 nM (Fig. 5), the adenine isomer aminopurinol is 1000-fold more active against *T. brucei* bloodstream forms than the corresponding hypoxanthine isomer allopurinol, consistent with the model that the aminopurines adenine and adenosine are the preferred purine sources for bloodstream-form trypanosomes and analogues thereof the most potent among the trypanocidal purine antimetabolites. A potential advantage of aminopurinol over adenosine analogues is that Δtbat1 trypanosomes are fully sensitive (Fig. 8). Loss-of-function mutations in *TbAT1*, the gene encoding the aminopurine permease P2, have been reported from *T. brucei* field isolates and implicated in resistance to melaminophenyl arsenicals, diamidines, and adenosine antimetabolites. Allopurinol not being suitable against African trypanosomes for lack of efficacy, aminopurinol may be an interesting alternative for further studies. However, the finding that aminopurinol exerts a cytostatic rather than a cytotoxic effect on bloodstream-form *T. brucei* (Fig. 6) limits its potential usefulness.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpddr.2013.12.001.

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