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Molecular Pharmacology of Adenosine Transport in *Trypanosoma brucei*: P1/P2 Revisited

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ABSTRACT

Trypanosoma brucei are unicellular parasites that cause sleeping sickness in humans and nagana in livestock. Trypanosomes salvage purines from their hosts through a variety of transporters, of which adenosine permeases deserve particular attention because of their role in drug sensitivity. *T. brucei* possess two distinct adenosine transport systems, P1 and P2, the latter of which also mediates cellular uptake of the drugs melarsoprol and pentamidine. Loss or mutation of P2 has been associated with drug resistance and sleeping sickness treatment failures. However, genetic disruption in *Trypanosoma brucei brucei* of the gene encoding P2, *TbAT1*, reduced the susceptibility to melarsoprol and pentamidine by only a factor of ~2. In this study, we show stronger phenotypes of the *tbat1* null mutant with respect to its sensitivity toward toxic adenosine analogs. Compared with parental *TbAT1*$^{+/+}$ trypanosomes, the *tbat1*$^{-/-}$ mutant is 77-fold less sensitive to tubercidin and 14-fold less sensitive to cordycepin. Resistance is further increased by the addition of inosine but is reverted by adenine. It is surprising that the *tbat1*$^{-/-}$ mutant grows faster than *TbAT1*$^{+/+}$ trypanosomes and that it overexpresses genes of the *TbNT* cluster encoding P1-type transporters. These unexpected phenotypes show that there are conditions other than drug pressure under which loss of P2 may confer a selective advantage to bloodstream-form trypanosomes. Overexpression of P1 by trypanosomes after loss of P2 indicates that combinatorial chemotherapy with trypanocidal P1 and P2 substrates may be a promising strategy to prevent drug resistance in sleeping sickness.

Human African sleeping sickness is re-emerging in sub-Saharan Africa. The World Health Organization estimates an annual incidence of 400,000, and from certain villages in Angola, the Democratic Republic of Congo, or southern Sudan, a prevalence of up to 50% has been reported (Kioy et al., 2004). Sleeping sickness is caused by *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, protozoan parasites that are transmitted by the tsetse fly (*Glossina* species). *T. brucei* evade the mammalian immune system by antigenic variation of their surface glycoproteins and proliferate extracellularly in the blood. In the late stage of the disease, the parasites invade the central nervous system, ultimately causing death of the patient. Because there are no prospects for a vaccine, treatment of sleeping sickness relies entirely on chemotherapy. Suramin (introduced 1916) and the diamidine pentamidine (1937) are used for the early stage and melarsoprol (1949) and eflornithine (1977) for the late stage of the disease because of their blood-brain barrier permeability. Pentamidine, melarsoprol, and eflornithine are being donated to the World Health Organization by Aventis. Eflornithine is effective only against West African sleeping sickness, not against East African sleeping sickness. Melarsoprol, a melamine-based trivalent arsenical, is still the drug of choice for treatment of late-stage sleeping sickness. However, melarsoprol treatment failure rates of 25 to 30% have been reported from Uganda (Legros et al., 1999) and northern Angola (Stanghellini and Josenando, 2001), possibly indicating the spread of drug-resistant trypanosomes.

Molecular mechanisms of drug resistance in *T. brucei* have mainly been studied in laboratory strains selected at suboptimal drug concentrations. Adenosine permeases turned out to play an important role in the uptake of, and resistance to, trypanocides. Carter and Fairlamb differentiated two types of adenosine transport systems, P1 and P2 (Carter and Fairlamb, 1993). P1 was shown to be a broad-specificity purine transporter, whereas P2 transports only adenine and adenosine (Table 1). It is interesting, however, that P2 transports also melarsen-based drugs and diamidines (Carter et al.,

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ABBREVIATIONS: PCR, polymerase chain reaction; RT, reverse transcriptase.

1995). P2-type adenosine transport was found to be absent or impaired in drug-resistant trypanosomes (Carter and Fairlamb, 1993; Barrett et al., 1995).

P1 is encoded by multiple genes of the *TbNT* family (Table 1). The genes *TbNT2* to *TbNT7* cluster on a single locus. *TbNT2*, *TbNT5*, *TbNT6*, and *TbNT7* exhibited P1-type substrate specificities when expressed in *Xenopus laevis* oocytes, whereas no substrate has yet been identified for TbNT3 and TbNT4 (Sanchez et al., 2002). P2 is apparently encoded by a single gene, *TbAT1* (Mäser et al., 1999; Matovu et al., 2003). Trypanosomes selected for melarsoprol resistance harbored point mutations in *TbAT1* that abolished function (Mäser et al., 1999). It is surprising that identical point mutations were found in *T. b. gambiense* field isolates (Mäser et al., 1999; Matovu et al., 2001a), and the occurrence of such mutations correlated to some degree with melarsoprol treatment failure (Matovu et al., 2001b).

A *T. b. brucei tbat1* null mutant was recently generated by homozygous replacement of the gene (Matovu et al., 2003). *tbat1*$^{-/-}$ trypanosomes had no detectable P2 activity. They exhibited reduced sensitivity toward melarsen-based arsenicals as well as diamidines, albeit with resistance factors of only 2 to 3 (Matovu et al., 2003). However, the *tbat1* null mutant was 20-fold resistant to the veterinary drug diminazene (Matovu et al., 2003; de Koning et al., 2004). Melamine-based nitrofurans designed to be P2 substrates were not toxic or were only marginally more toxic to *TbAT1*$^{+/+}$ than to *tbat1*$^{-/-}$ trypanosomes (Stewart et al., 2004). Herein, we characterize *tbat1* null trypanosomes with respect to their sensitivity toward adenosine antimetabolites, reevaluating the P1/P2 model and its implications for anti-trypanosomal chemotherapy. Surprising phenotypes regarding cell growth and drug resistance reveal relationships between transport, salvage, and toxicity of adenosine analogs, and they indicate a possible interplay between P1 and P2 purine uptake systems.

Materials and Methods

Cultivation of Trypanosomes. All experiments were performed with bloodstream-form trypanosomes. *T. b. brucei* strain BS 221 (synonymous for MiTat 1.2/221 or s427) and its *tbat1*$^{-/-}$ derivative (Matovu et al., 2003) were cultured at 37°C in a humidified atmosphere of 5% CO_2 in HMI-9 medium (BioConcept, Allschwil, Switzerland) containing 10% heat-inactivated fetal bovine serum (BioConcept), supplemented according to Hirumi and Hirumi (1989) plus 36 mM $NaHCO_3$ and 100 IU/ml penicillin/streptomycin (BioConcept). Population doubling times were measured in minimum essential medium (Invitrogen) supplemented with minimum essential medium nonessential amino acids, Earle's salts (Invitrogen), 10% heat-inactivated horse serum (slaughterhouse, Basel, Switzerland), 25 mM HEPES, 5.6 mM glucose, 26 mM $NaHCO_3$, 0.2 mM 2-mercaptoethanol, 2 mM sodium pyruvate, and 0.1 mM hypoxanthine (Baltz et al., 1985).

In Vitro Drug Sensitivity Assays. Trypanosomal drug sensitivity was determined with the redox-activated fluorescent dye Alamar-Blue as described previously (Räz et al., 1997). In brief, trypanosomes (10^3/well) were cultivated in 96-well plates for 70 h in the presence of serial dilutions of compounds. After this growth period, 10 μl of Alamar-Blue reagent (Bio-Source, Camarillo CA) was added to each well, and after a further 2 h of incubation, fluorescence was measured (excitation at 536 nm, emission at 588 nm; Spectramax Gemini fluorimeter, Molecular Devices, Sunnyvale, CA). All assays were performed at least three times, each in triplicate. IC_{50} values were determined by nonlinear regression to sigmoid dose-response parameters using Prism 4 software (GraphPad Software, San Diego, CA). All chemicals were purchased from Fluka Chemie GmbH (Buchs, Switzerland).

Adenosine Transport Assays. Adenosine transport measurements were carried out as described previously (de Koning and Jarvis, 1997). In brief, 10^7 bloodstream-form trypanosomes in 100 μl were mixed with an equal volume of uptake buffer containing 100 nM [^{3}H]adenosine (65.8 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) and incubated for 10 s at 37°C. Uptake was stopped by addition of 4 mM ice-cold, unlabeled adenosine, and the cells were pelleted by centrifugation through dibutylphthalate/mineral oil (7:1). After flash-freezing in liquid N_2, the bottom of each centrifuge tube was cut-off and transferred into liquid scintillation cocktail. All assays were performed three times, each in triplicate.

Gene Expression Analyses. Total RNA was isolated from cultured trypanosomes by extraction with hot phenol (95°C, pH 4.5) and chloroform, followed by ethanol precipitation. After DNase treatment (DNA-away; Ambion Biotech, Austin, TX), cDNA was synthesized from 1 μg of RNA with avian myeloblastosis virus reverse transcriptase (Roche, Mannheim, Germany) and T_{16} primer in a volume of 15 μl. PCR was performed with *Taq* polymerase (QIAGEN, Hilden, Germany) on 3 μl of cDNA. Negative controls lacking reverse transcriptase were always included. For amplification of *TbNT* subgroup genes (Fig. 5), a forward primer specific to the 5′ spliced leader sequence (cgctattattagaacagtttctgtac), which all *T. brucei* mRNAs have in common, was combined with the primer Actin_rev (ctgcgtcattttctcacggt) and one of the following: NT2–7_rev (gcrgcaagagagcgttgac), NTII_rev (agggcagaacaaaaatgaagc), NTIII_rev (gcaatccgctttcaaatcg), or NTIV_rev (tgtaatggtctcttgaacaggt); annealing temperature was 61°C, and elongation time was 80 s. Because of the relatively weak expression of *TbNT* genes, actin primers were added only after five performed cycles. Gene-specific primers for Fig. 7 were NT4_rev (tttacatcaaagtcacacactgtt), and NT6_rev (tagtatcgcctgtcttcgc); annealing at 55°C, with 60-s elongation for both. For Fig. 6, genes of the *TbNT2-TbNT7* cluster were amplified with the primers NT2–7_fw (ggatgtcggtgatgaatgtgacg) and NT2–7_rev (annealing at

TABLE 1
Pharmacological characteristics of trypanosomal adenosine transport systems

	P1	P2	References
Genes	*TbNT2, TbNT5, TbNT6, TbNT7, TbNT10*	*TbAT1*	Sanchez et al., 1999, 2002, 2004; Mäser et al., 1999
Substrates			
Physiological	Adenosine, inosine, guanosine, 2′-deoxyadenosine, 2′-deoxyinosine	Adenine, adenosine, 2′-deoxyadenosine	Carter and Fairlamb, 1993; de Koning and Jarvis, 1999;
Purine analogs	Formycin A, formycin B	Tubercidin, cordycepin	de Koning and Jarvis, 1999; Mäser et al., 2001; current study
Trypanocides		Melarsoprol, pentamidine, diminazene	Carter and Fairlamb, 1993; Carter et al., 1995; de Koning et al., 2004
Inhibitors		Flavone, silibinin	Mäser et al., 2001
	Both are insensitive to NBMPR and dilazep		Mäser et al., 2001

55°C and 80-s elongation). PCR products (200 ng) were purified (QIAquick PCR purification kit; QIAGEN) and sequenced directly in either direction using the same primers as for amplification. Sequencing was performed at the CMPG facility, Zoological Institute Bern.

Sequence Alignment and Dendrogram. Predicted protein sequences were obtained from the *T. brucei* genome database at http://www.genedb.org (TbNT2, Tb927.2.6150; TbNT3, Tb927.2.6200; TbNT4, Tb927.2.6220; TbNT5, Tb927.2.6240; TbNT6, Tb927.2.6320; TbNT7, Tb927.2.6280; TbNT8.1, Tb11.02.1100) and from GenBank (TbAT1, AAD45278; HsENT1, Q99808). ClustalX (Thompson et al., 1994) was used for multiple alignment and bootstrap analysis, TreeView (Page, 1996) to display the dendrogram.

Results

Susceptibility of Bloodstream Form Trypanosomes to Purine Analogs. The sensitivity of *T. b. brucei* 221 bloodstream forms to purine analogs was determined in vitro using the redox-sensitive fluorophore Alamar blue as an indicator of cell viability (Räz et al., 1997). The two adenosine antimetabolites tubercidin (7-deazaadenosine; Fig. 1) and cordycepin (3′-deoxyadenosine; Fig. 1) are known trypanocides (Williamson, 1972; Drew et al., 2003), and indeed both compounds were highly active, with IC_{50} values of 15 nM (Fig. 1). In contrast, 2′,3′-dideoxyadenosine was much less potent, with an IC_{50} of 48 μM (Fig. 1), and the IC_{50} of 2′,3′-dideoxyinosine was above 50 μM (data not shown). It is interesting that 3′-deoxyadenosine and 2′,3′-dideoxyadenosine were approximately equally active on amastigote forms of *Trypanosoma cruzi* (Nakajima-Shimada et al., 1996). The reason why 3′-deoxyadenosine was more than 1000-fold more toxic to *T. b. brucei* bloodstream forms than 2′,3′-dideoxyadenosine probably lies in trypanosomal purine salvage rather than transport (e.g., different substrate specificities of adenosine kinase and deoxyadenosine kinase) (Drabikowska et al., 1985).

Genetic Disruption of *TbAT1* Causes Resistance to Purine Analogs. The same set of sensitivity tests were carried out with *tbat1* knock-out trypanosomes to investigate the role of P2 in cellular uptake of these purine analogs.

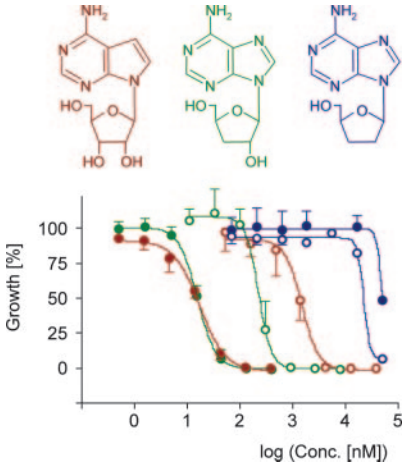

Fig. 1. Sensitivity of *T. brucei* to purine analogs. *TbAT1*$^{+/+}$ (●) and *tbat1*$^{-/-}$ (○) bloodstream-form trypanosomes were incubated for 72 h with the adenosine analogs tubercidin (red), cordycepin (green), and dideoxyadenosine (blue). Cell number and viability was measured by fluorescence of the redox-activated dye Alamar blue and expressed as percentage of untreated controls. Error bars (S.D.) are shown in only one direction for sake of readability.

Again, dideoxyadenosine (Fig. 1) and dideoxyinosine (data not shown) were hardly active, and there was no difference in toxicity between *TbAT1*$^{+/+}$ and *tbat1*$^{-/-}$ trypanosomes. However, the *tbat1*$^{-/-}$ mutant was 77-fold more resistant to tubercidin and 14-fold more resistant to cordycepin (Fig. 1). This demonstrates that both tubercidin and cordycepin are taken up to a substantial part via TbAT1 in wild-type trypanosomes. To test whether residual uptake in the *tbat1*$^{-/-}$ mutant occurs via P1-type adenosine transporters, the sensitivity tests were repeated in the presence of excess amounts of known P1 and P2 substrates.

Effects of Physiological Purines on Sensitivity to Adenosine Antimetabolites. Adenosine is a substrate of both transport activities P1 and P2, whereas inosine is imported exclusively by P1 and adenine only by P2 (Table 1). In wild-type *TbAT1*$^{+/+}$ trypanosomes, supplementation of the medium with 1 mM adenosine or inosine caused a 4- to 5-fold reduction of tubercidin susceptibility (Fig. 2a). Addition of 1 mM adenine had a much stronger effect, rendering trypanosomes 220-fold less susceptible to tubercidin (Fig. 2a). Effects of excess purines on cordycepin toxicity were less dramatic. Addition of adenosine hardly had an effect and inosine, if anything, sensitized trypanosomes toward cordycepin (Fig. 2b). Again, excess adenine exerted the most pronounced reduction of sensitivity, increasing the IC_{50} of cordycepin by a factor of 6 (Fig. 2b).

The same set of experiments was carried out with the *tbat1*$^{-/-}$ null mutant. Again, resistance to tubercidin was further increased upon addition of adenosine or inosine (Fig. 2a), presumably by blocking P1-mediated uptake. Excess adenine, as expected, did not further increase the resistance of *tbat1*$^{-/-}$ trypanosomes, because adenine is not a P1 substrate (Table 1). It is surprising, however, that adenine even

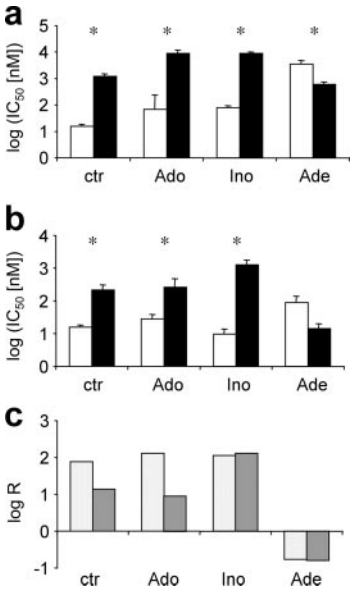

Fig. 2. Effects of competitors on sensitivity to purine analogs. Drug sensitivity assays for tubercidin (a) and cordycepin (b) were carried out with parental *TbAT1*$^{+/+}$ trypanosomes (open bars) and the *tbat1*$^{-/-}$ mutant (filled bars) in the presence of 1 mM purine competitors (Ado, adenosine; Ino, inosine; Ade, adenine). In c, the resulting resistance factors (IC_{50}[*tbat1*$^{-/-}$]/IC_{50}[*TbAT1*$^{+/+}$]) are summarized (light bars, tubercidin; dark bars, cordycepin). Asterisks indicate significant differences between *tbat1*$^{-/-}$ and *TbAT1*$^{+/+}$ ($p < 0.05$ in a two-tailed t test; for cordycepin plus adenine, p was 0.057).

resensitized the resistant *tbat1*$^{-/-}$ strain toward adenosine antimetabolites (Fig. 2a). A similar pattern was observed for cordycepin. Addition of excess inosine further increased the resistance of *tbat1*$^{-/-}$ trypanosomes, adenosine had only little effect, and adenine rendered the null mutant hypersensitive to cordycepin (Fig. 2b).

The data are summarized in Fig. 2c. Addition of excess adenosine reduced the sensitivity to adenosine analogs of the *tbat1*$^{-/-}$ mutant and parental *TbAT1*$^{+/+}$ trypanosomes to a similar extent, leaving the resistance factor R unchanged (R equals IC_{50} of *tbat1*$^{-/-}$ divided by IC_{50} of *TbAT1*$^{+/+}$). The same effect was observed for inosine regarding tubercidin toxicity. With cordycepin, however, excess inosine reduced the susceptibility more strongly in *tbat1*$^{-/-}$ than in wild-type trypanosomes. Thus, the resistance factor to cordycepin increased to 130-fold in the presence of inosine, reaching the level of R for tubercidin. This finding is in agreement with the P1/P2 model, and it indicates that P1 also contributes to cordycepin uptake. A puzzling effect was exerted by the P2 substrate adenine, which reverted the *tbat1*$^{-/-}$ phenotype, re-sensitizing null mutant trypanosomes toward both tubercidin and cordycepin (Fig. 2c). This effect was not observed with *TbAT1*$^{+/+}$ trypanosomes, where addition of excess adenine—as expected (Table 1)—reduced the sensitivity toward adenosine analogs. As a consequence, *tbat1*$^{-/-}$ mutants were more sensitive to tubercidin and cordycepin than wild-type trypanosomes in the presence of 1 mM adenine. In addition, excess adenine slowed-down the growth of *T. brucei* bloodstream forms already in the absence of drugs (data not shown). This phenomenon was observed for parental as well as for *tbat1*$^{-/-}$ mutant trypanosomes.

Effects of Physiological Purines on Adenosine Transport. Adenosine uptake of wild-type *T. brucei* bloodstream forms consists of the inosine-sensitive component P1 and the adenine-sensitive component P2 (Table 1). To characterize adenosine uptake of *tbat1*$^{-/-}$ cells, transport of 40 nM [^{3}H]adenosine was measured during the linear uptake phase in the presence of increasing concentrations of inosine or adenine. Inosine completely inhibited adenosine transport (Fig. 3), with a K_i value of 0.67 ± 0.08 μM (n = 3), very similar to values previously reported for the *T. b. brucei* P1 transporter (Carter and Fairlamb, 1993; de Koning and Jarvis, 1999). In contrast, up to 100 μM adenine failed to inhibit [^{3}H]adenosine transport (Fig. 3), indicating that the P2 adenosine transport activity had been deleted in *tbat1* null mutant.

***tbat1*$^{-/-}$ Trypanosomes Grow Faster Than Their Parental Strain.** The *tbat1*$^{-/-}$ mutant had not shown any growth defect (Matovu et al., 2003), as might be expected given 1) the large number of purine transporters encoded in the genome of *T. brucei* (Mäser et al., 2003; de Koning et al., 2005) and 2) the fact that the purine source in standard culture medium is hypoxanthine and not adenosine (Baltz et al., 1985). We were surprised to observe here, however, that *tbat1*$^{-/-}$ trypanosomes grew even faster than their *TbAT1*$^{+/+}$ parents. To quantify growth, *tbat1*$^{-/-}$ and its parental strain were propagated in vitro, and the population doubling times were calculated from linear regression of the log-transformed growth curves. Under all conditions tested, *tbat1* null trypanosomes grew slightly but reproducibly faster than their parental strain (Fig. 4). The difference was more pronounced at limiting serum concentrations; at 5%, the population doubling times were 13.3 ± 3.6 h for wild-type and 10.8 ± 2.3 h for *tbat1*$^{-/-}$ trypanosomes (p = 0.012 for significant difference in a two-tailed t test). This means that, starting from a mixed population consisting of equal parts *tbat1*$^{-/-}$ and *TbAT1*$^{+/+}$ trypanosomes, the null mutants would outgrow wild-type cells by a factor of 10 within 8 days. However, it must be noted that such in vitro analysis does not necessarily extrapolate to the situation in a natural host.

Expression Analysis of Trypanosomal *ENT* Genes. To investigate eventual secondary effects of *TbAT1* disruption, we measured expression levels of other trypanosomal nucleoside transporter genes in parental *TbAT1*$^{+/+}$ and in *tbat1*$^{-/-}$ bloodstream form trypanosomes. Figure 5a shows members of the equilibrative nucleoside transporter family from *T. brucei* (the ENT family; Pfam PF01733, TC 2.A.57). More trypanosomal *ENT* genes are emerging as the genome sequencing initiative approaches completion. As apparent from the similarity dendrogram of a multiple alignment, the majority of trypanosomal nucleoside transporters cluster into different subgroups (Fig. 5a; see also Table 1). Expression levels of each subgroup were measured in a semiquantitative way, by performing reverse transcriptase (RT) PCR in the presence of a forward primer of the *T. brucei* spliced mRNA leader sequence (Walder et al., 1986) and two different reverse primers, one specific for actin and one for the *ENT* group of interest. These primers were chosen from conserved regions within the respective genes to amplify all members of a particular group. For one singleton gene, Tb09.160.5480,

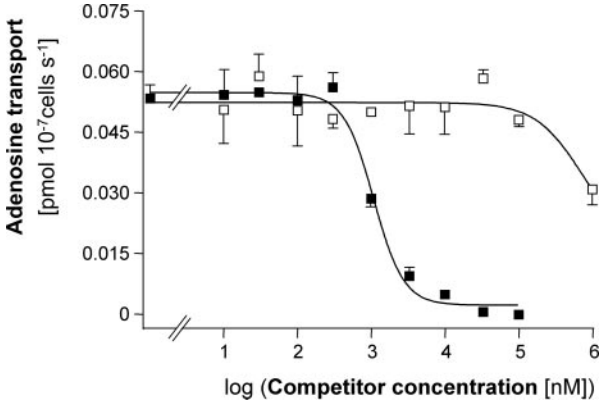

Fig. 3. Adenosine transport in *tbat1*$^{-/-}$ trypanosomes. [^{3}H]Adenosine transport of bloodstream-form *tbat1*$^{-/-}$ *T. brucei* was measured in increasing concentrations of inosine (■) or adenine (□). The adenine-sensitive component of adenosine transport (P2) is absent from the *tbat1* null mutant.

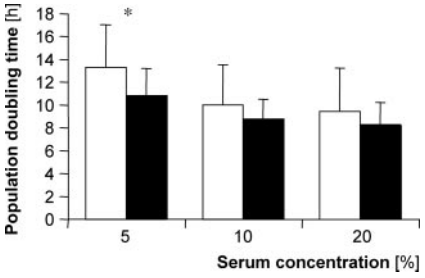

Fig. 4. Growth of *TbAT1*$^{+/+}$ and *tbat1*$^{-/-}$ trypanosomes. Bloodstream-form trypanosomes (*TbAT1*$^{+/+}$, open bars; *tbat1*$^{-/-}$, filled bars) were propagated in vitro at different serum concentrations. At 5%, the *tbat1* null mutant grew significantly faster than its parental strain (p = 0.012, n = 20).

expression was not detectable. For two subgroups, III and IV, expression was confirmed but did not vary between parental and *tbat1*$^{-/-}$ trypanosomes (Fig. 5b). The large subgroup I, however, was expressed more strongly in *tbat1*$^{-/-}$ trypanosomes than in their parents, as determined by comparison with the internal actin control (Fig. 5b). This finding was confirmed by three independent RT-PCR experiments and also by Northern blot analysis (data not shown). The six genes in this group, *TbNT2* to *TbNT7*, are all located within 9 kb on chromosome 2 of *T. brucei* (Sanchez et al., 2002). TbNT2, TbNT5, TbNT6, and TbNT7 are P1-type transporters of slightly varying substrate specificities; the substrates of TbNT3 and TbNT4 are unknown (Sanchez et al., 2002).

Expression Analysis within the *TbNT* Gene Cluster. Expression of individual genes within the *TbNT* cluster on chromosome 2 was again investigated by RT-PCR. mRNA isolated from *T. brucei* bloodstream forms was reverse-transcribed and amplified by PCR as described above. The resulting products were then sequenced directly, to avoid eventual bias introduced by cloning. Single nucleotide polymorphisms became apparent in the electropherogram of the sequencing products terminated with fluorescent dideoxynucleotides. This method was highly reproducible and allowed distinction between individual *TbNT* genes (Fig. 6). Of the five genes in the *TbNT* cluster, *TbNT2* seemed to be predominantly expressed as apparent from positions where it differs from the rest. Expression of *TbNT3*, *TbNT4*, *TbNT5*, and *TbNT7*, on the other hand, was not detectable in wild-type *TbAT1*$^{+/+}$ trypanosomes (Fig. 6). *TbNT3*, *TbNT5*, and *TbNT7* were not expressed in *tbat1*$^{-/-}$ cells either. However, judging from the polymorphic positions outlined in Fig. 6, a weak *TbNT4* signal was detectable in the null mutant. The signal of *TbNT6* relative to the other genes in the cluster seemed to be stronger in the mutant as well. We therefore investigated expression of *TbNT4* and *TbNT6* by semiquantitative RT-PCR using gene-specific primers. As shown in Fig. 7, the two genes were indeed overexpressed in the *tbat1*$^{-/-}$ mutant.

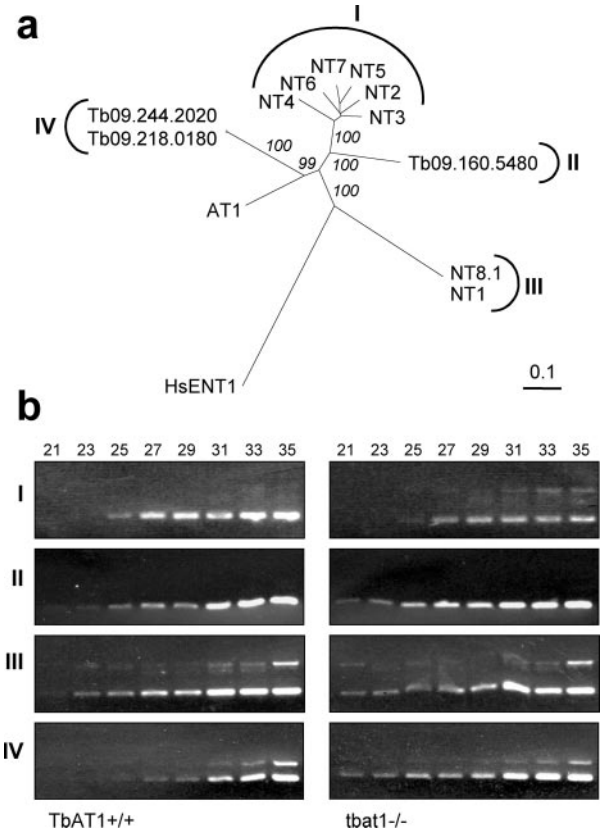

Fig. 5. Expression levels of *TbNT* family members. a, dendrogram of *T. brucei* ENT protein sequences. Brackets and roman numbers indicate different subgroups. Numbers in italics are the positive percentiles of 1000 rounds of bootstrapping. Human HsENT1 is included for reference. b, semiquantitative RT-PCR of *TbNT* subgroup genes with primers complementary to conserved sequences within each group. Actin (lower band) served as an internal control. Aliquots were removed after the indicated numbers of cycles.

Discussion

The P1/P2 model for uptake of adenosine and antitrypanosomal drugs in *T. brucei* was proposed based on phenotypic observations without knowledge of the underlying genes (Carter and Fairlamb, 1993). A number of adenosine transporters have since been cloned from *T. brucei* and functionally characterized (Mäser et al., 1999; Sanchez et al., 1999, 2002, 2004). All of them also transported either adenine or inosine (Table 1); hence, the P1/P2 model still holds. Here we used a *T. brucei* mutant homozygously disrupted in the aden-

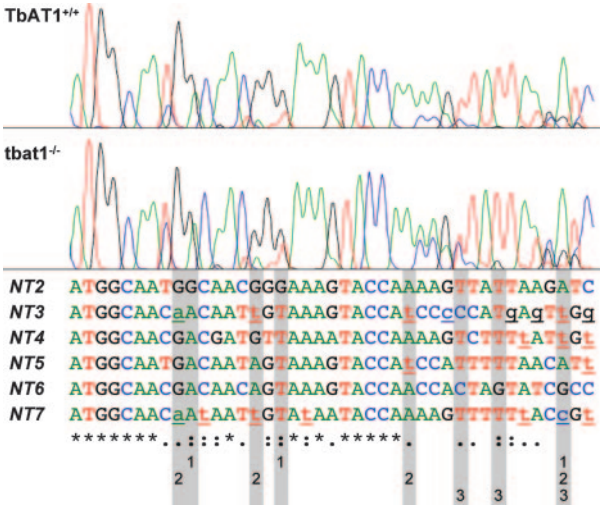

Fig. 6. Concomitant expression of almost identical *TbNT* genes. Expression of *TbNT2* to *TbNT7* was monitored in parallel by direct sequencing of RT-PCR products with a primer binding to a perfectly conserved region. The six genes are highly similar. Here, a region is depicted in which they diverge (asterisk, identical base in all six genes; colon, identical in five genes; dot, identical in four genes). Bases that are not detectable in *TbAT1*$^{+/+}$ cDNA are lower case and underlined. The most informative positions are shaded in gray; 1, SNPs unique to *TbNT2*; 2, SNPs unique to *TbNT3*, *TbNT4*, *TbNT5* or *TbNT7*, respectively; and 3, positions indicating overexpression of *TbNT4* and *TbNT6* in *tbat1*$^{-/-}$ cells.

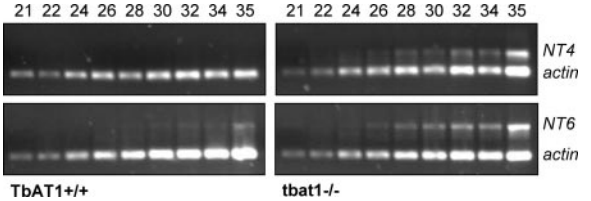

Fig. 7. *TbNT4* and *TbNT6* are overexpressed in the *tbat1* null mutant. *TbNT4* and *TbNT6* mRNA levels were measured by semiquantitative RT-PCR using actin as an internal control (lower band). After the indicated numbers of cycles, aliquots were removed from the reaction. Expression of *TbNT4* was not detectable in *TbAT1*$^{+/+}$ cells.

osine transporter gene *TbAT1* to further validate the P1/P2 model. One prediction is that P2-null trypanosomes should be resistant to melarsoprol but not to adenosine analogs (because of all trypanosomal adenosine transporters, only P2 is permeable to melarsoprol). However, the opposite was observed for *tbat1*$^{-/-}$ trypanosomes; they were markedly resistant to the adenosine antimetabolites tubercidin (77-fold more; Fig. 1) and cordycepin (14-fold more; Fig. 1), whereas their susceptibility to melarsoprol decreased by a factor of only 2 to 3 (Matovu et al., 2003). The mild phenotype toward melarsoprol can be explained by the presence of adenosine-independent import pathways (Matovu et al., 2003), the nature of which is unknown. The strong phenotype toward tubercidin and cordycepin indicates that among the comparably large number of adenosine transporters in *T. brucei* (Table 1), TbAT1 constitutes the principal route of import for these adenosine analogs. This is further illustrated by the finding that of the physiological purines tested, adenine exerted the maximal protection of wild-type trypanosomes from tubercidin or cordycepin (Fig. 2). At the same time, adenine resensitized *tbat1*$^{-/-}$mutants to adenosine antimetabolites, which led to the paradoxical situation that in the presence of adenine, *tbat1*$^{-/-}$ mutants were more sensitive to tubercidin and cordycepin than parental *TbAT1*$^{+/+}$ trypanosomes (Fig. 2c). The P1/P2 model cannot explain this surprising effect; we are currently investigating the physiological effects of excess adenine to growth of *T. brucei*.

The fact that the alleviating effect of excess inosine on toxicity of tubercidin and cordycepin was stronger for *tbat1*$^{-/-}$ than for *TbAT1*$^{+/+}$ trypanosomes indicated that P1-mediated import of the adenosine analogs only became relevant in the absence of TbAT1 (Fig. 2, a and b). In the presence of 1 mM inosine, *tbat1*-null cells were 130-fold resistant to tubercidin and 112-fold resistant to cordycepin (Fig. 2c). Thus the surprising drug resistance phenotype of *tbat1*$^{-/-}$ trypanosomes is explainable, at least in part, by different affinities of P1- and P2-type transporters toward adenosine analogs. One caveat of the drug sensitivity experiments is that it cannot be distinguished whether excess purines reduce the toxicity of adenosine analogs by competing with cellular uptake or by competing at their intracellular target. RNAi experiments have indicated that tubercidin targets glycolysis in *T. brucei* rather than purine salvage (Drew et al., 2003). Competition with import is in agreement with measurements of transport kinetics of [^{3}H]adenosine, where K_i values for adenosine analogs differed substantially between experiments performed in the presence of excess adenine to block P2 or excess inosine to block P1 (de Koning and Jarvis, 1999). This study, carried out before the cloning of trypanosomal adenosine transporters, already indicated that tubercidin (K_i of 78 μM) and cordycepin (K_i of 210 μM) are not high-affinity P1 substrates (de Koning and Jarvis, 1999).

A second reason for the stronger effect of inosine in the *tbat1*$^{-/-}$ mutant compared with wild-type in decreasing the susceptibility to adenosine analogs, could be the fact that *tbat1*$^{-/-}$ trypanosomes overexpressed genes of the *TbNT* cluster (Fig. 5). *TbNT4* and particularly *TbNT6* showed higher mRNA levels in *tbat1*$^{-/-}$ than in *TbAT1*$^{+/+}$ trypanosomes (Fig. 7). Whereas TbNT6 is a P1-type adenosine permease, as determined by functional expression in *Xenopus laevis* oocytes, no substrate was identified for TbNT4 (Sanchez et al., 2002). The subcellular localizations of TbNT4 and TbNT6 are unknown. The finding that deletion of one trypanosomal *ENT* may lead to overexpression of other members of the same family complicates the assessment of individual transporters' contributions to drug susceptibility by molecular genetics. Whether overexpression of *TbNT* genes also occurs in P2 loss-of-function *T. brucei* field isolates remains to be investigated. If so, this might be exploited for drug targeting toward melarsoprol-resistant trypanosomes. However, Carter and Fairlamb observed 3-fold lower P1-type adenosine uptake rates in P2-deficient, melarsoprol-resistant trypanosomes than in their parental, drug-sensitive strain (Carter and Fairlamb, 1993). Changes in adenosine transport have also been described from procyclic, tsetse fly midgut-form *T. brucei*, where adenosine uptake rates strongly increased upon purine starvation (de Koning et al., 2000). It is unknown whether such effects are caused by changes in expression levels of adenosine transporter genes.

A further unexpected finding was that *tbat1*$^{-/-}$ trypanosomes grew faster than parental *TbAT1*$^{+/+}$ cells at limiting serum concentrations (Fig. 4). At present, we can only speculate about whether this was a consequence of overexpression of *TbNT* genes. The finding that *tbat1*$^{-/-}$ trypanosomes grew faster than their *TbAT1*$^{+/+}$ parents indicates that there are conditions other than chemotherapy in which loss of P2 confers a selective advantage to bloodstream-form trypanosomes. This may have implications for the stability of drug resistance in the absence of drug pressure, which is remarkably high for African trypanosomes. In summary, the P1/P2 model is still valid but received some new twists. In particular, P1 and P2 may be functionally linked such that overexpression of the former compensates for lack of the latter. If this also happens in field isolates, combination of trypanocidal P1 and P2 substrates will be a good strategy toward drug cocktails of minimal propensity for resistance by loss of import.

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