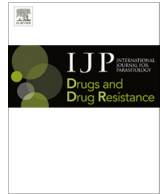




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## Functional expression of *TcoAT1* reveals it to be a P1-type nucleoside transporter with no capacity for diminazene uptake <sup>☆</sup>



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### ABSTRACT

It has long been established that the *Trypanosoma brucei* TbAT1/P2 aminopurine transporter is involved in the uptake of diamidine and arsenical drugs including pentamidine, diminazene aceturate and melarso-prol. Accordingly, it was proposed that the closest *Trypanosoma congolense* paralogue, *TcoAT1*, might perform the same function in this parasite, and an apparent correlation between a Single Nucleotide Polymorphism (SNP) in that gene and diminazene tolerance was reported for the strains examined. Here, we report the functional cloning and expression of *TcoAT1* and show that in fact it is the syntenic homologue of another *T. brucei* gene of the same Equilibrative Nucleoside Transporter (ENT) family: TbNT10. The *T. congolense* genome does not seem to contain a syntenic equivalent to *TbAT1*. Two *TcoAT1* alleles, differentiated by three independent SNPs, were expressed in the *T. brucei* clone B48, a *TbAT1*-null strain that further lacks the High Affinity Pentamidine Transporter (HAPT1); *TbAT1* was also expressed as a control. The *TbAT1* and *TcoAT1* transporters were functional and increased sensitivity to cytotoxic nucleoside analogues. However, only *TbAT1* increased sensitivity to diamidines and to cymelarsan. Uptake of [<sup>3</sup>H]-diminazene was detectable only in the B48 cells expressing *TbAT1* but not *TcoAT1*, whereas uptake of [<sup>3</sup>H]-inosine was increased by both *TcoAT1* alleles but not by *TbAT1*. Uptake of [<sup>3</sup>H]-adenosine was increased by all three ENT genes. We conclude that *TcoAT1* is a P1-type purine nucleoside transporter and the syntenic equivalent to the previously characterised TbNT10; it does not mediate diminazene uptake and is therefore unlikely to play a role in diminazene resistance in *T. congolense*.

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### 1. Introduction

The most common drug to treat animal trypanosomiasis in sub-Saharan Africa is the diamidine compound diminazene aceturate (DA) and the effective treatment of livestock with trypanocides remains of the highest importance for farmers in the tsetse belt where an estimated 46–62 million head of cattle are at risk of trypanosomiasis (Swallow, 2000). Control of the disease is largely either by vector control or chemotherapy, of which an estimated

35 million doses are administered annually (Geerts and Holmes, 1998). However, the effectiveness of DA and the only other common drug for trypanosomiasis, isometamidium chloride, are threatened by drug resistance (Geerts et al., 2001; Delespau et al., 2008a). In order to manage the situation effectively, it is necessary to understand the resistance mechanism and devise predictive tests that distinguish between drug sensitive and resistant populations (Delespau et al., 2008a).

The diamidine trypanocides were developed in the late 1930s (Lourie and Yorke, 1939) and as early as 1944 Hawking reported that aromatic diamidines are actively accumulated by live but not by dead *Trypanosoma brucei* to intracellular concentrations up to three orders of magnitude higher than the extracellular concentration (Hawking, 1944). He proposed that the massive drug accumulation, not observed in the surrounding blood cells, was the basis of the selective trypanocidal action. A similar accumulation, apparently energy dependent, has also been reported for DA (Girgis-Takla and James, 1974; De Koning et al., 2004) and for

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pentamidine (Damper and Patton, 1976), a related diamidine drug used to treat human African trypanosomiasis (Delespaux and De Koning, 2007). Dependent on the extracellular concentration, about 50% of the uptake of pentamidine in *T. b. brucei* is mediated by the TbAT1/P2 transporter (Carter et al., 1995; De Koning and Jarvis, 2001; Bray et al., 2003). The rest is transported by a High Affinity Pentamidine Transporter (HAPT1) and a Low Affinity Pentamidine Transporter (LAPT1) (De Koning, 2001, 2008). However, the uptake of DA is almost exclusively through TbAT1/P2 (De Koning et al., 2004), with only a very minor contribution from HAPT1 (Tekal et al., 2011). The most likely explanation for the differences in DA and pentamidine transport is the flexibility of the linker chain between the pentamidine benzamidine ends, allowing the molecule to assume many different conformations whereas the diminazene structure is rigid, locking the benzamidine moieties in a fixed position.

There is abundant evidence that diamidine resistance in *T. brucei* complex species is linked to loss of transport (Delespaux and De Koning, 2007). A stilbamidine-resistant *T. b. rhodesiense* strain was deficient in accumulation of the drug (Fulton and Grant, 1955). Loss of TbAT1/P2 and HAPT1 gives a high pentamidine resistance phenotype in *T. b. brucei* (Bridges et al., 2007) and loss of the P2 aminopurine transport activity alone is sufficient to give substantial DA resistance in *T. b. brucei* (Matovu et al., 2003; De Koning et al., 2004), *Trypanosoma equiperdum* (Barrett et al., 1995) and *Trypanosoma evansi* (Witola et al., 2004). However, the most important trypanosomatid pathogen for livestock in sub-Saharan Africa is *Trypanosoma congolense* and it is important to establish whether the same DA resistance model applies for this parasite. Additionally, the need for novel chemotherapeutic and chemoprophylactic tools for trypanosomiasis is grave, and given the range of diamidines available for development, understanding any mechanism of resistance will aid the exploitation of new members of this class of compounds. The most likely *TbAT1* orthologue in the *T. congolense* genome was identified as TcIL3000.5.2500 and it was named *TcoAT1* (Delespaux et al., 2006). A recent in-depth analysis of *Trypanosoma* genomes confirmed that it is closely related (Jackson, 2012). A Single Strand Conformation Polymorphism (SSCP) technique was used to try and establish whether *TcoAT1* polymorphisms might be associated with a diminazene sensitivity phenotype (Delespaux et al., 2006), using a single dose mouse test (Eisler et al., 2001) to report on resistance. This analysis found a strong correlation between SSCP pattern and drug sensitivity: out of 26 *T. congolense* strains 14 DA-sensitive and 9 resistant strains were correctly predicted using 20 mg/kg. The remaining 3 strains were predicted to be resistant by SSCP pattern, but were classified as sensitive by the mouse test, albeit with some infections relapsing, particularly at 5 mg/kg DA (Delespaux et al., 2006). It was concluded that *TcoAT1* was likely to be equivalent to the AT1 transporters in *T. brucei*, *T. equiperdum* and *T. evansi* and this seemed confirmed with the detection of an Ile306Val polymorphism in some of the *TcoAT1* alleles cloned from intermediate and highly resistant strains. The presence of this SNP was investigated by Restriction Fragment Length Polymorphism (RFLP) in all 26 strains and correlated perfectly with the observed resistance phenotype (Delespaux et al., 2006); polymorphisms in *TbAT1* have been similarly associated with failure to transport melaminophenyl arsenicals (Mäser et al., 1999; Matovu et al., 2001).

A further study on 11 *T. congolense* strains collected in Cameroon (Mamoudou et al., 2008) and 12 strains collected in Ethiopia (Moti et al., 2012) showed all strains to be DA-resistant by the single-dose mouse test and all the Cameroonian and only six of the Ethiopian being classified as 'resistant' by *TcoAT1* PCR-RFLP. However, these studies did not contain any 'sensitive strains' and thus did not in themselves rule out the trivial explanation that the *TcoAT1* PCR-RFLP simply identified the dominant polymorphism

in *T. congolense* strains of the regions investigated. The observation that the RFLP-identified SNP was also common in *T. congolense* isolated from wildlife in areas with no history of prior trypanocide use (Chitanga et al., 2011) does appear to show that this is a common polymorphism and unlikely to be the result of drug pressure.

Therefore, the similarity between *TcoAT1* and *TbAT1*, and the apparent association with DA-resistance in *T. congolense* are tempered by the lack of total correlation. Moreover, no functional expression or characterization has been performed for this member of Equilibrative Nucleoside Transporter (ENT) family. In order to provide a definitive test on whether *TcoAT1* is involved in diamidine transport, and whether *TcoAT1* mutations are involved in resistance, we have expressed two separate *TcoAT1* alleles ('sensitive' and 'resistant') as well as *TbAT1*, in a well-characterised DA-resistant *TbAT1* null strain of *T. b. brucei* (Bridges et al., 2007).

## 2. Materials and methods

### 2.1. Trypanosome strains and culturing

Bloodstream-form *T. b. brucei* strain B48 and its derivatives were maintained as previously described (Bridges et al., 2007). The B48 strain is derived from the Lister 427 strain, but lacks the *T. brucei AT-1* gene and the high affinity pentamidine transporter (Bridges et al., 2007).

### 2.2. Plasmid construction and transfection

The full *TcoAT-1* (TcIL3000\_9\_2500) gene was amplified from the sensitive *T. congolense* strain TRT12 (Delespaux et al., 2006) and the resistant strain Alick 339 (Delespaux et al., 2008b) (both isolated from cattle in Zambia), using the high-fidelity proof-reading polymerase Phusion (Finnzymes), with forward primer 5'-GGGCCCATGCTCGGTTTTGAATCC-3' and reverse primer 5'-AGATCTTACCCTCTGGCAGGGCCT-3'. The amplicons were ligated into the pGEM-T Easy sub-cloning vector (Promega) and sequenced using standard procedures.

The *TcoAT1* gene from the sensitive and resistant strains was then ligated into the expression vector pHD1336 (Biebinger et al., 1997), to give pHDK45 and pHDK46 respectively. The *TbAT1* gene from *T. brucei* (Tb927.5.286b) was generated by high-fidelity PCR using Lister 427 strain gDNA, with forward primer 5'-GGGCCCA TGCTCGGGTTTGACTCA-3' and reverse primer 5'-GGATCCCTACTTGGGAAGCCCCTC-3', and ligated into the pHD1336 expression vector to give pHDK44. All three constructs were linearised with *NotI* prior to transfection; B48 parasites were washed into Human T Cell Solution for transfection using the desired cassette with an Amaxa Nucleofector as described (Burkard et al., 2007). Transfectants were grown and cloned out, by limiting dilution, in standard HMI-11 (Hirumi and Hirumi, 1989) containing 5 µg ml<sup>-1</sup> blasticidin S.

### 2.3. Drug sensitivity assays

Drug sensitivities were assessed using the dye resazurin (Alamar blue) using a protocol adapted from Ráz et al. (1997), as described (Gould et al., 2008). Briefly, drugs were serially-diluted in 100 µl of complete HMI-11 media across two rows of a 96 well plate. Cultures of bloodstream-form trypanosomes were diluted to 2 × 10<sup>5</sup> cells/ml in complete HMI-11, and 100 µl was added to all wells. Plates were incubated for 48 h at 37 °C/5% CO<sub>2</sub>, prior to the addition of 20 µl of 5 mM resazurin sodium salt (Sigma-Aldrich) in PBS, pH7.4. Plates were incubated for a further 24 h in the same conditions, before fluorescence was measured using a FLUOstar

Optima fluorimeter (BMG Labtech). Fifty percent inhibitory concentrations ( $IC_{50}$ ) were calculated using the sigmoidal curve algorithm of Prism 5 (GraphPad). Experiments were performed independently at least four times.

#### 2.4. Transport assays

Uptake assays of [ $^3H$ ]-diminazene, [ $^3H$ ]-inosine and [ $^3H$ ]-adenosine were performed exactly as described (Wallace et al., 2002; Natto et al., 2005). Briefly, trypanosomes from late-log phase cultures were washed into assay buffer (AB; 33 mM HEPES, 98 mM NaCl, 4.6 mM KCl, 0.55 mM CaCl<sub>2</sub>, 0.07 mM MgSO<sub>4</sub>, 5.8 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.3 mM MgCl<sub>2</sub>, 23 mM NaHCO<sub>3</sub>, 14 mM glucose, pH 7.3) and resuspended at  $10^8$  cells ml<sup>-1</sup>. One hundred microlitres of cell suspension was incubated with either [Ring- $^3H$ ]-DA (Perkin Elmer, 60.7 Ci/mmol), [2,8- $^3H$ ]-adenosine (American Radiolabeled Chemicals Inc, 40 Ci/mmol) or [8- $^3H$ ]-Inosine (American Radiolabeled Chemicals Inc., 20 Ci/mmol) in the presence or absence of unlabeled substrate or other competitive inhibitors. Incubations were stopped by the addition of 1 ml ice-cold unlabeled substrate (1 mM in AB) and centrifugation through oil (13,000×g; 1 min). The cell pellet was transferred to a scintillation tube and radioactivity was determined by liquid scintillation counting. The results were plotted to equations for linear or non-linear regression using Prism 5 (GraphPad) after correction for non-specific association of radiolabel with the pellet, as described (Wallace et al., 2002).

### 3. Results

#### 3.1. Sequencing of *TcoAT1* alleles

The *TcoAT1* alleles were PCR-amplified from genomic DNA of the TRT12 ('sensitive'; Delespau et al., 2006) and Alick339 ('resistant'; Delespau et al., 2008b) *T. congolense* isolates, and cloned into the pGEMT Easy vector prior to sequencing. From each strain, four independent clones were sequenced; sequences were identical within a strain, consistent with homozygosity for *TcoAT1* as originally suggested by the PCR-RFLP (Delespau et al., 2006; Delespau et al., 2008a,b); Alick339 is a cloned strain, previously reported to be homozygous for the 'resistant' polymorphism (Delespau et al., 2008b). The nucleotide sequences of *TcoAT1* alleles from a DA 'sensitive' and a 'resistant strain' were determined and found to differ by 10 SNPs which do not affect the coding sequence, and 3 SNPs which cause amino acid alterations: Met75Thr, Ala262Gly and Val306Ile (Supplementary Fig. S1). These alleles and the *T. brucei* 427 *TbAT1* gene were cloned into the pH1336 expression vector and expressed in the DA-resistant *T. brucei* B48 strain. Correct integration of the genes was established by PCR (not shown).

#### 3.2. Genomic analysis of ENT genes in African trypanosomes

The premise that the gene named *TcoAT1* is equivalent to the known diamidine transporter *TbAT1* is based on it being the closest *T. congolense* paralogue identified from a database of unassembled (pre-genome) contigs. However, there is no syntenic equivalent of *TbAT1* (Tb927.5.286b) in the now-assembled *T. congolense* genome (TriTrypDB.org). Indeed, *TcoAT1* (TcIL3000.9.2500) is syntenic with another of the *T. brucei* ENT family genes, Tb09.160.5480 (De Koning et al., 2005), which is known as TbNT10 or AT-B. This gene encodes a high affinity purine nucleoside transporter (Sanchez et al., 2004) that has been characterised in detail (Al-Salabi et al., 2007) and is preferentially expressed in short-stumpy (Sanchez et al., 2004) and procyclic (Al-Salabi et al., 2007; Spoerri et al., 2007) forms of *T. brucei*. A syntenic equivalent is also present in

the *T. b. gambiense* genome, but not in *Trypanosoma vivax* or *Leishmania* species.

Fig. 1 shows a phylogenetic tree of ENT genes from *T. b. brucei*, *T. congolense*, *Trypanosoma cruzi*, and *T. vivax*, indicating different branches for nucleobase transporters, purine nucleoside transporters ('P1-type') and 'P2-type' transporters. The diagram clearly shows that all these species have nucleobase transporter genes, several of which have been characterised by heterologous expression (Burchmore et al., 2003; Henriques et al., 2003) and P1-type nucleoside transporters, of which multiple members also have been functionally characterised (Sanchez et al., 1999, 2002; De Koning et al., 2005), including TbNT10 (Al-Salabi et al., 2007; Spoerri et al., 2007). In the tree, *TcoAT1* groups with the *T. brucei* P1-type transporters, and is closely related to Tb09.160.5480 (TbNT10), as would be predicted from the synteny. Formally the phylogenetic tree does not statistically differentiate between the P1 and P2-type transporters, though as noted above all of the *T. b. brucei* genes have been functionally confirmed as one type or the other. Therefore, the combination of clear synteny (with all the other *T. b. brucei* P1-type transporters being physically located on different chromosomes), and the phylogenetic grouping of *TcoAT1* with the P1-type transporters strongly suggests that *TcoAT1* is the *T. congolense* equivalent of *TbNT10*. In addition, a BLASTp search using the *TcoAT1* protein sequence as the query sequence returned all the P1-type nucleoside transporters as the highest *T. b. brucei* hits by maximum percentage identity, with *TbNT10* having one of the highest scores (67%; range of other P1-type transporters identity is from 67% to 61%). *TbAT1* had a maximum percentage identity of 54% (Supplementary Table S1). From data currently available, *T. congolense* does not seem to possess any P2-type transporter genes.

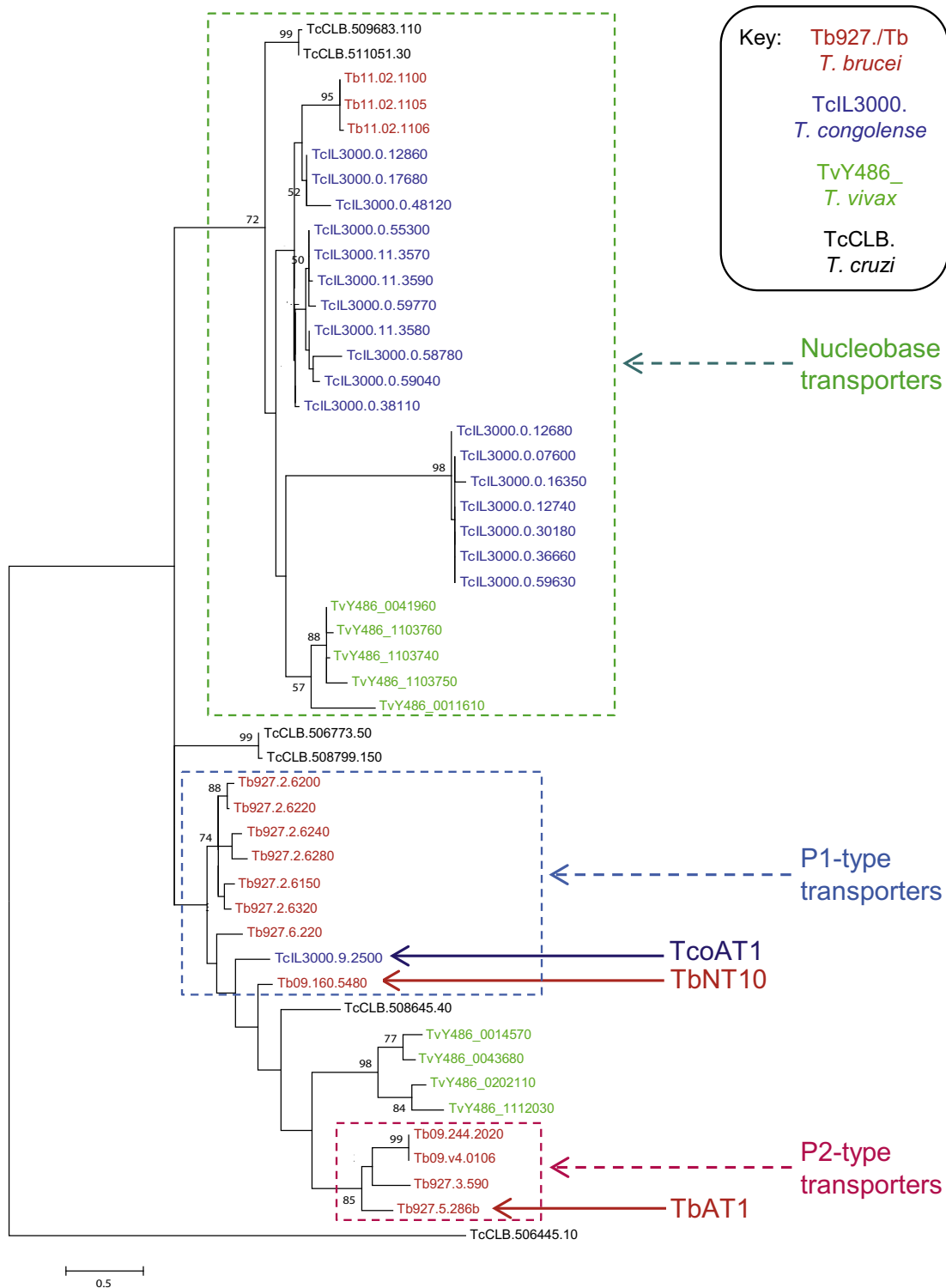
#### 3.3. Sensitivity of *TcoAT1* and *TbAT1*-expressing clones to nucleoside analogues and trypanocides

Functional expression of *TbAT1* and *TcoAT1* in the *Tbb* B48 clonal line was tested using a series of cytotoxic adenosine analogues. Fig. 2 shows that introduction of *TbAT1* into this strain caused a profound increase in sensitivity to tubercidin (7-deazaadenosine; Fig. 2A), 5'-deoxyadenosine (5'-dAdo; Fig. 2B) and cordycepin (3'-deoxyadenosine; Fig. 2C), of 360 ( $P < 0.001$ ), 20 ( $P < 0.01$ ), and 23-fold ( $P < 0.01$ ), respectively ( $n = 5$ ). Expression of *TcoAT1* ('sensitive allele'; *TcoAT1S*) also significantly increased sensitivity to these adenosine analogues, but by only 2–2.5-fold (all  $P < 0.05$ ;  $n = 5$ ). Sensitivity to phenylarsine oxide (PAO), a lipophilic arsenic compound, was unchanged in all strains (Fig. 2D).

The above results show that ENT transporters can be functionally expressed in the B48 strain and influence drug sensitivity. We thus proceeded with testing sensitivity to a number of trypanocides that have previously been shown to be substrates for *TbAT1*/P2: diminazene, pentamidine and cymelarsan (Carter and Fairlamb, 1993; Carter et al., 1995; De Koning and Jarvis, 1999, 2001; De Koning et al., 2004). As expected, the expression of *TbAT1* in this strain greatly increased sensitivity to these drugs, by 219, 209 and 53-fold, respectively (all  $P < 0.01$ ; Fig. 3A–C). However, expression of either the 'sensitive' or 'resistant' allele of *TcoAT1* did not alter drug sensitivity at all (Fig. 3). Sensitivity to PAO was not significantly different for any of the strains.

#### 3.4. Transport of diminazene and of purine nucleosides by *TcoAT1*- and *TbAT1*-expressing clones

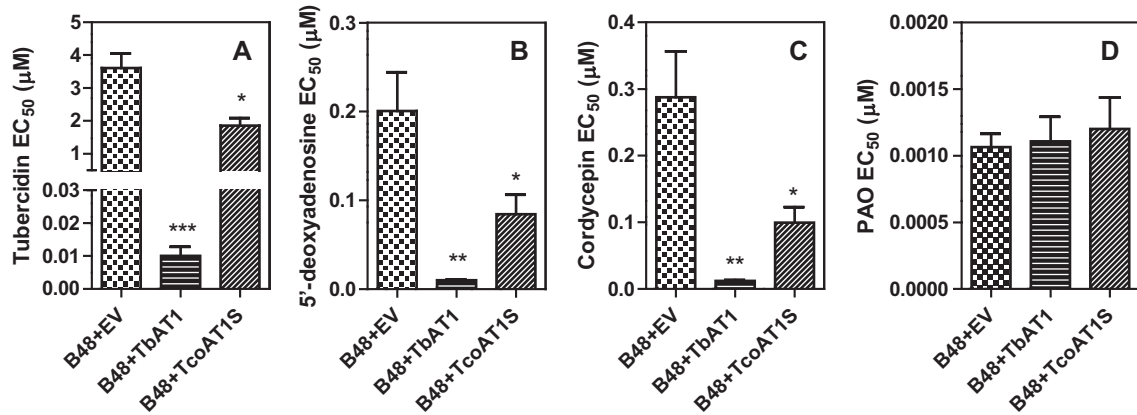
The above results appear to show that *TcoAT1* is unable to transport any of the diamidine or melaminophenyl arsenical drugs tested, including diminazene. We therefore directly tested the uptake of [ $^3H$ ]-diminazene. Introduction of *TbAT1* into B48 trypanosomes



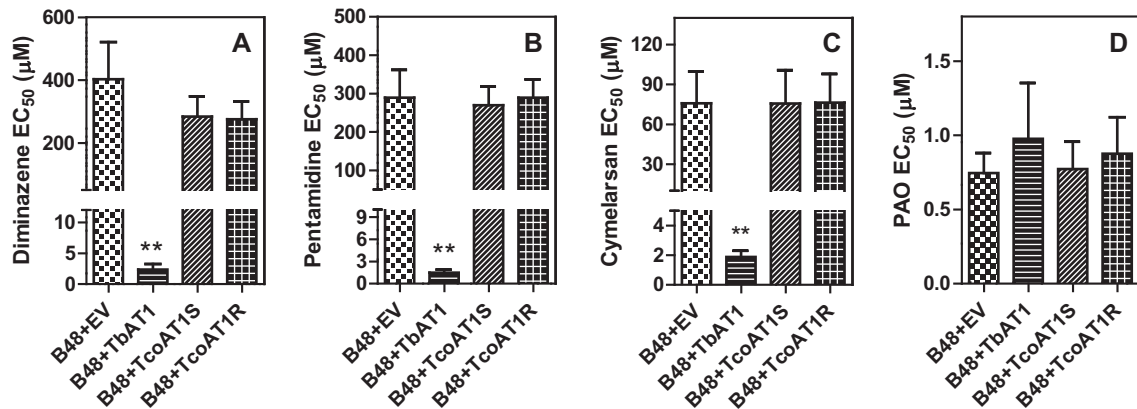
**Fig. 1.** Phylogenetic tree of ENT genes in trypanosomatid species *T. b. brucei* (red), *T. congolense* (blue), *T. vivax* (green) and *T. cruzi* (black). Separate clusters for nucleobase transporters, P1-type nucleoside transporters and P2-type transporters are indicated with boxes of dashed lines. The positions of *TbAT1* (Tb927.5.286b), *TbNT10* (Tb09.160.5480) and *TcoAT1* (TcIL3000.9.2500) are indicated with arrows. The tree was constructed using the Maximum Likelihood Tree function of MEGA5, with 500 bootstrap replications and, otherwise, the default parameters (Tamura et al., 2011). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The scale bar represents 0.5 amino acid substitutions per position. The tree is rooted with a divergent *T. cruzi* sequence (TcCLB.506445.10). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

greatly increased the rate of [ $^3\text{H}$ ]-diminazene uptake to  $0.123 \pm 0.005$  pmol ( $10^7$  cells) $^{-1}$  s $^{-1}$  ( $n = 3$ ;  $r^2 = 0.99$ ) compared to  $0.0093 \pm 0.0046$  pmol ( $10^7$  cells) $^{-1}$  s $^{-1}$  in the empty vector control, whereas expression of *TcoAT1S* had no effect at all on the uptake rate ( $0.0093 \pm 0.0043$ ; Fig. 4). However, both *TcoAT1* alleles were clearly

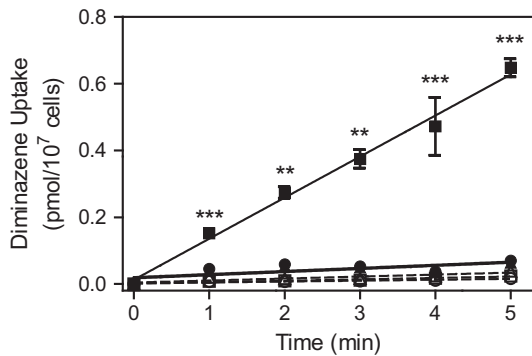
functionally expressed in B48 as both mediated transport of [ $^3\text{H}$ ]-inosine and [ $^3\text{H}$ ]-adenosine. Inosine uptake was highly significantly enhanced in B48 expressing *TcoAT1S* ( $P < 0.001$ ), and to a slightly lesser extent by *TcoAT1R* ( $P < 0.01$ ); in contrast, expression of *TbAT1* did not change [ $^3\text{H}$ ]-inosine uptake (Fig. 5A), consistent with the fact



**Fig. 2.** Sensitivity of various *T. b. brucei* B48-derived cell lines to adenosine analogues. A, tubercidin; B, 5'-deoxyadenosine; C, cordycepin; D, phenylarsine oxide (arsenical control). The strains were all derived from B48 (Bridges et al., 2007), by transfection with the empty vector (EV) pHD1336 or the same vector containing *TbAT1* or *TcoAT1* 'sensitive variant' (*TcoAT1S*). Bars show the average and SEM of 5 independent determinations, using the Alamar blue assay. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , Student *T*-test, relative to B48 + EV.



**Fig. 3.** Sensitivity of *T. b. brucei* bloodstream forms of various clonal lines to diminazene (A), pentamidine (B), cymelarsan (C) and phenylarsine oxide (D), as determined using standard Alamar blue-based assay. All clonal strains were derived from the B48 line, which is highly resistant to diamidines and melaminophenyl arsenicals (Bridges et al., 2007). The B48 strain was transfected with either empty vector pHD1336 (EV), *TbAT1*, *TcoAT1* 'sensitive variant' (*TcoAT1S*) or *TcoAT1* 'resistant variant' (*TcoAT1R*) and for each two independent clones were tested with identical results; only one is shown. Bar graphs represent the average and standard error of 4–5 independent experiments. \*\* $P < 0.01$  by unpaired Student's *T*-test, relative to B48 + EV.

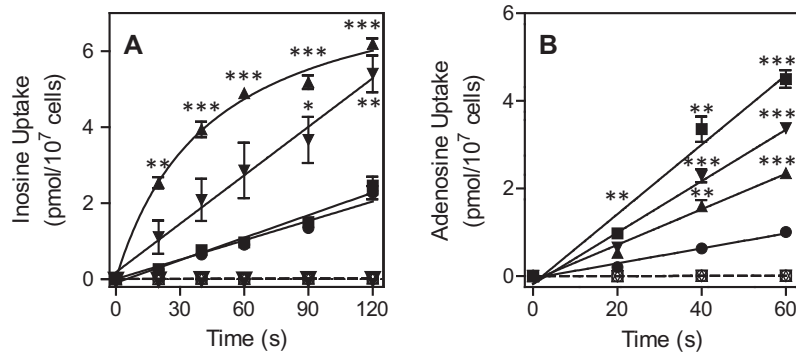


**Fig. 4.** Diminazene uptake in transformed *T. b. brucei* B48. B48 cells transformed with either vector without insert (circles), *TbAT1* (squares) or *TcoAT1S* (up triangles) were incubated with 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]-diminazene with (open symbols) or without (filled symbols) 1 mM unlabeled diminazene acetate for up to 5 min as indicated. Symbols represent the average and SEM of three independent experiments, each performed in triplicate. Lines were calculated by linear regression; only uptake of 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]-diminazene by B48 expressing *TbAT1* was significantly different from zero ( $P < 0.0001$ , *F*-test;  $r^2 > 0.99$ ). Diminazene uptake by cells expressing *TbAT1* was significantly higher than in control cells (B48 with empty vector). \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  (relative to EV control; Student's *T*-test).

that inosine is not a substrate for this transporter (Carter and Fairlamb, 1993; De Koning and Jarvis, 1999; Collar et al., 2009). Uptake of adenosine was increased ( $P < 0.001$ ) by all three transporter genes, relative to control (Fig. 5B). The rate of adenosine transport was  $TbAT1 > TcoAT1R > TcoAT1S > \text{control}$ .

#### 4. Discussion

Animal African Trypanosomiasis (AAT) remains one of the major challenges to livestock farming in sub-Saharan Africa and, as the control of the disease is mostly through chemotherapy with a few decades-old drugs, the large number of reports on drug resistance is a cause of great concern as it threatens the sustainability of agriculture in many areas (Geerts and Holmes, 1998; Swallow, 2000; Geerts et al., 2001; Delespaux et al., 2008a). However, it is not clear whether the increased number of reports on diminazene acetate (DA) treatment failure in cattle reflect a spread of resistant parasites, or simply a more intense focus on the problem with more studies performed in different parts of the continent. It is difficult to establish the full scale of the problem given the infrastructural and geographical challenges, particularly with the difficulties in confirming parasitological cure, prevention of re-infection, and



**Fig. 5.** Uptake of (A) inosine and (B) adenosine in transformed *T. b. brucei* B48. B48 cells transformed with either vector without insert (circles), *TbAT1* (squares), *TcoAT1S* (up triangles) or *TcoAT1R* (down triangles) were incubated with 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]-inosine (A) or 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]-adenosine (B) in the presence (open symbols and dotted lines) or absence (filled symbols and continuous lines) of 1 mM unlabeled permeant for up to 2 min as indicated. In the presence of 1 mM permeant uptake was inhibited by >99%, showing that uptake was saturable and therefore most likely transporter-mediated. Inosine uptake by cells expressing the *TcoAT1* alleles was significantly higher than in control cells (B48 with empty vector). Data shown are the average and SEM of three independent experiments, each performed in triplicate. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  (Student's *T*-test). Note: in frame B each of the experimental data points were significantly different from the empty vector control at 20 s, by  $P < 0.01$  or better.

in follow-up of treated animals. A further confounding factor is the use of counterfeit products and the frequent administration of veterinary trypanocides by untrained people. Therefore, the most robust test for resistance has required isolation of the parasite and testing in a standardised mouse-model (Eisler et al., 2001; Mamoudou et al., 2008). However, this approach is expensive and laborious, and involves growth and treatment in a different animal species, a process with its own potential selective bottlenecks.

Clearly, a molecular test for a genetic resistance marker, based on an understanding of the resistance mechanism, has the potential to revolutionise our understanding of the spread of drug-resistant parasites (Delespau et al., 2006, 2008a). The identification of the P2/AT1 transporter as the principal entry route for diminazene, and the demonstration that genetic loss of this transporter led to substantial levels of diminazene resistance in *T. b. brucei* (Matovu et al., 2003; De Koning et al., 2004), *T. equiperdum* (Barrett et al., 1995), and *T. evansi* (Witola et al., 2004) appeared to have provided an opportunity for a genetic test. The closest *T. congolense* orthologue to P2/AT1 was identified from a contig database and an SNP that statistically associated with reduced diminazene sensitivity was found (Delespau et al., 2006). A PCR-RFLP system developed to identify the resistance-associated SNP in field isolates was implemented to screen for diminazene resistance in various regions of Africa, including Cameroon (Mamoudou et al., 2008), Eastern Zambia (Delespau et al., 2008b), the Ghibe river basin in Ethiopia (Moti et al., 2012) and game reserves/national parks in Zambia, South Africa and Zimbabwe (Chitanga et al., 2011). The latter study revealed the occurrence of the 'resistant' PCR-RFLP marker even in regions where the drug had not been used and it has become clear that the polymorphism initially suggested as resistance-associated is common in *T. congolense* regardless of drug exposure, and does not always correlate with high levels of diminazene resistance.

We have thus undertaken a study to verify (1) whether *TcoAT1* does indeed encode a P2-type transporter, (2) whether *TcoAT1S* confers sensitivity to diminazene and diminazene transport capacity and (3) whether there is any substantial difference in transport phenotype between *TcoAT1S* and *TcoAT1R*. *TcoAT1* alleles were amplified and cloned from known and well-characterised *T. congolense* isolates and verified by sequencing. We found that the *TcoAT1* sequence, while closely related to *TbAT1* and from the same nucleoside/nucleobase transporter gene family, is in fact not the orthologue of *TbAT1* but is the syntenic orthologue of *TbNT10*, a different but well-characterised purine nucleoside transporter of the P1-type. The P1 versus P2 distinction was first defined in *T. brucei* by

Carter and Fairlamb (1993), where P1-type adenosine transporters are sensitive to competitive inhibition by inosine, whereas adenosine uptake by P2 is inhibited by adenine. Further characterization showed the P1 transporters, of which the *T. b. brucei* genome contains numerous copies (Sanchez et al., 2002; De Koning et al., 2005), to be high affinity transporters for the purine nucleosides adenosine, guanosine and inosine (De Koning et al., 1998; De Koning and Jarvis, 1999; Sanchez et al., 2002) whereas P2 transports only the aminopurines, adenosine and adenine (Carter and Fairlamb, 1993; De Koning and Jarvis, 1999). The *T. b. brucei* genome appears to contain only 1 copy of *TbAT1*, which can be knocked out by two rounds of homologous recombination (Matovu et al., 2003). Numerous studies have documented the involvement of P2 in uptake of diamidines and melaminophenyl arsenicals (see reviews by Carter et al. (1999) and De Koning (2008)), for which the transporter displays sub-micromolar affinity (De Koning and Jarvis, 1999). In contrast, the P1-type transporters displayed two orders of magnitude less affinity for these trypanocides (De Koning and Jarvis, 1999) and have never been implicated in their transport.

Our observations that *TcoAT1* increases sensitivity to cytotoxic nucleoside analogues but not to diminazene, pentamidine (both diamidines) or cymelarsan (a melaminophenyl arsenical) are consistent with a classification as P1-type transporter. Expression of *TbAT1* in the same system, used as positive control, did increase drug sensitivity dramatically. The strong and saturable capacity for transport of both inosine and adenosine by *TcoAT1* is completely inconsistent with a P2-type activity but a hallmark of P1 (Carter and Fairlamb, 1993; De Koning and Jarvis, 1999).

In summary, we conclude that *TcoAT1* encodes a P1-type transporter and is not involved in diminazene uptake. It is clear that *TcoAT1* was incorrectly identified as the P2 transporter equivalent of *T. congolense*, and is instead the syntenic orthologue of *TbNT10* and we therefore propose that *TcoAT1* be renamed *TcoNT10*. Whether polymorphisms in *TcoAT1/NT10*, like the one identified by the PCR-RFLP (Delespau et al., 2006) could nonetheless be informative with respect to diminazene resistance now seems unlikely given that the transporter itself does not transport diminazene. Several recent papers have found that this allele is present in most *T. congolense* isolates from across Africa (Delespau et al., 2008b; Mamoudou et al., 2008; Chitanga et al., 2011; Moti et al., 2012), leading to the conclusion that either diminazene resistance is near-complete in the *T. congolense* population, or the proposed resistance allele is a common variant of the transporter instead of a determinant of drug sensitivity. The balance of current evidence suggests the latter possibility - a conclusion strengthened

by the observation that the polymorphism was found in at least 1 allele of 33 out of 34 isolates from wild-life without a history of drug exposure (Chitanga et al., 2011). Nevertheless, we cannot rule out the possibility that a particular *TcoAT1/NT10* allele might be associated with DA resistance through genetic linkage to a resistance determinant elsewhere on the same chromosome. Meanwhile, every effort should now be made to identify the real resistance mechanism for diminazene treatment failure in animal trypanosomiasis, as understanding the mechanism may be relevant to both the continued use of existing treatments and the development of new trypanocides.

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### 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.ijppdr.2013.01.004>.

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