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Functional expression of TcoAT1 reveals it to be a P1-type nucleoside transporter with no capacity for diminazene uptake


Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, United Kingdom
Galvmed, Pentlands Science Park, Bush Loan, Edinburgh, United Kingdom
Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium
Roslin Institute, University of Edinburgh, Easter Bush, Midlothian, United Kingdom

Abstract

It has long been established that the Trypanosoma brucei TbAT1/P2 aminopurine transporter is involved in the uptake of diamidine and arsencial drugs including pentamidine, diminazene aceturate and mellarosprol. Accordingly, it was proposed that the closest Trypanosoma congolense paralogous, 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 [3H]-diminazene was detectable only in the B48 cells expressing TbAT1 but not TcoAT1, whereas uptake of [3H]-inosine was increased by both TbAT1 and TcoAT1. Further, uptake of [3H]-adenosine was increased by all three ENT genes. We conclude that TcoAT1 is a P1-type purine nucleoside transporter and that TcoAT2 is the functional equivalent in T. congolense. TcoAT1, whereas TbAT1 was detectable in the B48 cells expressing TbAT1 but not TcoAT1, whereas uptake of [3H]-inosine was increased by both TbAT1 alleles but not by TcoAT1. Uptake of [3H]-adenosine was increased by all three ENT genes. We conclude that TcoAT1 is a P1-type purine nucleoside transporter and that TcoAT2 is the functional equivalent in T. congolense.

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; Delespaux 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 (Delespaux 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
pentamidine (Damper and Patton, 1976), a related diamidine drug used to treat human African trypanosomiasis (Delespaux and De Koning, 2007). Depending 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 (Teka 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 benzamide ends, allowing the molecule to assume many different conformations whereas the diminazene structure is rigid, locking the benzamide 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 (Matouvi 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 TcI3000.5.2500 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; Matouvi 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 (TcI3000.9.2500) 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 (Finzymes), with forward primer 5′-GGCCCCATGCTCTGGTTGTAATCC-3′ and reverse primer 5′-AGATCTTTACACCTGCGACCGGCGGCT-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 pHDI336 (Biebinger et al., 1997), to give pHDK45 and pHDK46 respectively. The TbiAT1 gene from T. brucei (Tb927.5.286b) was generated by high-fidelity PCR using Lister 427 strain gDNA, with forward primer 5′-GGGCCCATGCTCTGGTTGTAATCC-3′ and reverse primer 5′-CTATTTGACACCGGCAGGCGGCT-3′, and ligated into the pHDI336 expression vector to give pHDK44. All three constructs were linearised with Ncol 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⁻¹ 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 × 10⁵ cells/ml in complete HMI-11, and 100 μl was added to all wells. Plates were incubated for 48 h at 37 °C/5% CO₂ 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 [\(^{3}H\)]-diminazene, [\(^{3}H\)]-inosine and [\(^{3}H\)]-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\(_2\), 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 resuspended at 10\(^{6}\) cells ml\(^{-1}\). One hundred microlitres of cell suspension was incubated with either [\(^{3}H\)]-DA (Perkin Elmer, 60.7 Ci/mmol), [\(2,8-{\text{H}}\)]-adenosine (American Radiolabeled Chemicals Inc, 40 Ci/mmolel) or [\(8-{\text{H}}\)]-inosine (American Radiolabeled Chemicals Inc, 20 Ci/mmolel) 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’; Delespaux et al., 2006) and Alick339 (‘resistant’; Delespaux et al., 2008b) strains. PCR-amplified 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 (Delespaux et al., 2006; Delespaux et al., 2008a,b); Alick339 is a cloned strain, previously reported to be homozygous for the ‘resistant’ polymorphism (Delespaux 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 TbaAT1 gene were cloned into the pHDI336 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 TbaAT1 is based on it being the closest T. congolense paralogue identified from a database of unassembled (pre-genome) contigs. However, there is no syntonic equivalent of TbaAT1 (Tb927.5.286b) in the now-assembled T. congolense genome (TriTrypDB.org). Indeed, TcoAT1 (TclL3000.9.2500) 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 AT1-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 TbnNT10 (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%). TbaAT1 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 TbaAT1-expressing clones to nucleoside analogues and trypanocides

Functional expression of TbaAT1 and TcoAT1 in the TbB B48 clonal line was tested using a series of cytotoxic adenosine analogues. Fig. 2 shows that introduction of TbaAT1 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 TbaAT1/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 TbaAT1 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 TbaAT1-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 [\(^{3}H\)]-diminazene. Introduction of TbaAT1 into B48 trypanosomes
greatly increased the rate of $[^3H]$-diminazene uptake to 0.123 ± 0.005 pmol (10^7 cells)^{-1} s^{-1} (n = 3; r^2 = 0.99) compared to 0.0093 ± 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 ± 0.0043; Fig. 4). However, both TcoAT1 alleles were clearly functionally expressed in B48 as both mediated transport of $[^3H]$-inosine and $[^3H]$-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 $[^3H]$-inosine uptake (Fig. 5A), consistent with the fact

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**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.)
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) pHDI336 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 transformed with either empty vector pHDI336 (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 < 0.05 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 μM [3H]-diminazene with (open symbols) or without (filled symbols) 1 mM unlabeled diminazene aceturate 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 μM [3H]-diminazene by B48 expressing TbAT1 was significantly different from zero (P < 0.0001, F-test; r² > 0.99). Diminazene uptake by cells expressing TcoAT1 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).

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 aceturate (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
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 (Delespaux 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., 2004; De Koning et al., 2003). 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 (Delespaux 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 (Delespaux 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 adenosine. Further characterisation 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 transporters 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 TbAT1 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 (Delespaux 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 (Delespaux 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.

**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 μM [3H]-inosine (A) or 0.1 μM [3H]-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.
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.ijpddr.2013.01.004.

References


