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The diamidine diminazene aceturate is a substrate for the High Affinity Pentamidine Transporter: implications for the development of high resistance levels in trypanosomes.

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Running title: Diminazene transport and resistance

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Abbreviations: HAPT1, High Affinity Pentamidine Transporter 1; LAPT1, Low Affinity Pentamidine Transporter 1.

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ABSTRACT

African trypanosomiasis is a disease of humans and livestock in many areas south of the Sahara. Resistance to the few existing drugs is a major impediment to control of these diseases and we here investigate how resistance to the main veterinary drug diminazene aceturate correlates with changes in drug transport in resistant strains. The strain *tbat1*$^{-/-}$, lacking the TbAT1/P2 aminopurine transporter previously implicated in diminazene transport, was adapted to higher levels of diminazene resistance. The resulting cell line was designated ABR and was highly cross-resistant to other diamidines, and moderately resistant to cymelarsan. Procyclic trypanosomes were shown to be a convenient model to study diamidine uptake in *T. b. brucei* given (1) the lack of TbAT1/P2 and (2) a ten-fold higher activity of the High Affinity Pentamidine Transporter, HAPT1. Diminazene could be transported by HAPT1 in procyclic trypanosomes. This drug transport activity was lacking in the ABR line, as previously reported for the pentamidine-adapted line B48. The K_m for diminazene transport in bloodstream *tbat1*$^{-/-}$ trypanosomes was consistent with uptake by HAPT1. Diminazene transport in ABR and B48 cells was reduced compared to *tbat1*$^{-/-}$ but their resistance phenotype was different: B48 displayed higher levels of resistance to pentamidine and the melaminophenyl arsenicals, whereas ABR displayed higher resistance to diminazene. These results establish loss of HAPT1 function as a contributing factor to diminazene resistance but equally demonstrate for the first time that adaptations other than those determining initial rates of drug uptake contribute to diamidine and arsenical resistance in African trypanosomes.

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Introduction

One of the many diseases that plague sub-Saharan Africa is trypanosomiasis, a disease complex formed by several species infecting domestic and wild animals (mostly *Trypanosoma congolense*, *T. vivax* and *T. brucei brucei*) and humans (*T. b. rhodesiense* and *T. b. gambiense*) and transmitted by tsetse flies. In addition *T. vivax* and the closely related animal parasites *T. evansi* and *T. equiperdum* can also be transmitted sexually or by other biting insects, which has spread the disease to large regions of Southern Asia and South America. The infection is known as sleeping sickness in humans, nagana in cattle, dourine in horses, and as surra in camels and other high-value livestock such as buffalo. The human disease is invariably fatal if left untreated and the veterinary condition causes enormous damage to economies and food security. The only option for control of trypanosomiasis is chemotherapy as insect control on the necessary scale is prohibitively expensive and vaccine development appears practically impossible due to the process of antigenic variation of African trypanosomes.

However, the choice of chemotherapeutic agents is very limited and those available suffer from many shortcomings such as high levels of host toxicity, parenteral administration, and perhaps most importantly increasing levels of treatment failure due to drug resistance. For treatment of the late or cerebral of stage sleeping sickness this has led to the replacement of melarsoprol with eflornithine in many foci (Balasegaram et al., 2009) and to the recent introduction of eflornithine/nifurtimox combination therapy (Priotto et al., 2009; Yun et al., 2010). The early or haemolymphatic stage is still treated with the aromatic diamidine compound pentamidine, introduced in the 1930s (Delespaux and De Koning, 2007). For the veterinary condition the

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situation is even more serious with many reports of resistance to the two principal drugs of isometamidium (a phenanthridine) and diminazene aceturate (aromatic diamidine) (Geerts et al., 2001; Delespaux and De Koning, 2007), and no alternative treatments in development. The paucity of new drug development for both the human and veterinary diseases makes understanding of the spreading resistance phenotypes a priority. Resistance markers are required for epidemiological studies to assess the real spread of resistance, and insight into the causes of resistance and the patterns of cross-resistance must underpin rational strategies to limit the impact and further spread of the problem.

It has been known for a considerable time that trypanosomal resistance to melaminophenyl arsenicals such as cymelarsan (used against surra in camels) and melarsoprol is associated with cross-resistance to at least some of the aromatic diamidines (Williamson and Rollo, 1959; De Koning, 2008). We now know that pentamidine is salvaged by three distinct transport entities in bloodstream trypanosomes, the P2 aminopurine transporter, the High Affinity Pentamidine Transporter (HAPT1) and the Low Affinity Pentamidine Transporter (LAPT1) (De Koning and Jarvis, 2001; De Koning, 2001; Matovu et al., 2003; Bridges et al., 2007). P2 is encoded by the ENT-family gene *TbAT1* (Mäser et al., 1999) but the genes encoding HAPT1 and LAPT1 are still unknown and the identification of these transporters has been difficult given the complexity of the three influx routes for the drug under study, the relatively low rate of uptake by HAPT1 in bloodstream trypanosomes, and the lack of specific inhibitors for LAPT1. We thus decided to investigate [^{3}H]-pentamidine transport in procyclic *T. b. brucei*, which lacks expression of P2/TbAT1 (De Koning et al., 1998), in the hope to obtain a simpler model. We found that procyclic cells lack adenosine-sensitive P2-mediated pentamidine transport but that two

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pentamidine uptake systems, biochemically indistinguishable from HAPT1 and LAPT1 in bloodstream forms, were expressed. This allowed us to characterise P2-independent diminazene uptake in detail and determine that this is mediated by the procyclic high affinity pentamidine transporter, previously designated PPT1 (De Koning, 2001), which we here demonstrate to be equivalent to HAPT1. HAPT1 was subsequently shown to be a secondary route of diminazene uptake into bloodstream forms. This gives a rationale for the continued sensitivity of P2-deficient trypanosomes to diminazene at concentrations around 1 μM, and significantly increases our understanding of cross-resistance between arsenical and diamidine trypanocides.

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Materials and Methods

Trypanosome strains and cultures

The standard drug sensitive strain *T. b. brucei* s427 (MiTat 1.2/BS221) was used throughout to obtain both bloodstream and procyclic trypanosomes. All other strains were derived from this line and all cell lines were clonal, i.e. derived from a single cell. Identities of all strains are regularly checked using PCR amplification of molecular markers. The *tbat1*$^{-/-}$ was derived from s427 by replacement of both *TbAT1* alleles with antibiotic resistance cassettes and is moderately resistant to diminazene and displays also a minor loss of sensitivity to pentamidine and melaminophenyl arsenicals (Matovu et al., 2003). The B48 line was derived in turn from *tbat1*$^{-/-}$ by incrementally increased exposure to pentamidine (Bridges et al., 2007). All bloodstream form cultures were maintained in HMI-9 medium (Invitrogen) supplemented with 2 mM β-mercaptoethanol and 10% fetal calf serum (FCS; PAA Laboratories GmbH) at 37 °C under a 5% CO_2 atmosphere (Hirumi and Hirumi, 1989). Procyclic cells were grown at 25 °C in SDM79 medium (Invitrogen) supplemented with 10% FCS as described previously (Gudin et al., 2006). Cells for experiments were taken at late log-phase growth.

Generation of a new clonal line resistant to high levels of diminazene aceturate

In vitro drug selection in the presence of diminazene, in stepwise small increments, was carried out in duplicate on the *tbat1*$^{-/-}$ *T. brucei* cell line (Matovu et al., 2003). The selection process was initiated using the doubling dilution method on a 96 well plate. Diminazene aceturate, prepared as stock solution in sterile water, was used at a start concentration of 1.6 µM, equivalent to its IC_{50} value in 200 µl of HMI-9/FCS then added to the first well in the first column of a 96-well

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culture plate (Corning) in duplicate. The *T. brucei tbat1*$^{-/-}$ cells, at a start cell density of 0.5×10^6 cells/ml, were dispensed into each of these wells. The plates were incubated at standard conditions for 4 days and assessed by light microscopy. Motile cells with a normal morphology were seeded again in fresh HMI-9 medium with 10% FCS at the same drug concentration into a fresh well and re-incubated under the same conditions. The same cells were also seeded at twice, a-half, and a tenth of that drug concentration in fresh HMI-9 medium with 10% FCS. Cells established as growing stably in drug were transferred and seeded into 1 ml volume cultures in 24 well plates and the selection continued with gradual increase in drug concentration over a period of 5 months. The *tbat1*$^{-/-}$ diminazene-selected line was growing normally in the continuous presence of 0.1 μM diminazene at that point.

Alamar blue drug sensitivity assays

Drug sensitivities were determined with the Alamar blue assay exactly as described (Gould et al., 2008), measuring fluorescence in 96-well plates with a FLUOstar Optima (BMG Labtech) at wavelengths 544 nm for excitation and 620 nm for emission. IC_{50} values were calculated by non-linear fitting of the data to a sigmoidal dose-response curve with variable slope (Prism 5.0, GraphPad).

Transport assays

Transport assays with procyclic and bloodstream form trypanosomes were performed as described previously (Wallace et al., 2002; Al-Salabi et al., 2007; Natto et al., 2005). Briefly, cell cultures were harvested at late-log stage of growth, washed in to 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_2PO_4, 0.3

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mM $MgCl_2$, 23 mM $NaHCO_3$, 14 mM glucose, pH 7.3) and resuspended at a concentration of ~10^8 cells/ml. Transport was initiated by addition of 100 µl cells to 100 µl radiolabel in AB layered over oil (7:1 dibutylphthalate:mineral oil v/v (Sigma)) and terminated by the addition of an ice-cold solution of 1 ml of unlabelled permeant and immediate centrifugation through the oil layer. Radioactivity in the cell pellet was determined by liquid scintillation counting and corrected for non-specific association of radiolabel with the cells as described (Wallace et al., 2002). Kinetic parameters were calculated using the appropriate linear and non-linear regression equations in Prism 5.0 (GraphPad). All experiments were performed in triplicate on at least three independent occasions.

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Results

Pentamidine transport in procyclic Trypanosoma brucei *as a model for bloodstream forms*

Uptake of 1 μM [^{3}H]-pentamidine was linear for at least 450 s (linear regression, 6 points over 450 s; $r^2 = 0.98$) with a rate of 0.037 ± 0.002 pmol(10^7 cells)$^{-1}$s^{-1} and fully inhibited by 1 mM unlabelled pentamidine (slope not significantly different from zero; F-test) (see supplemental figure S1). All subsequent [^{3}H]-pentamidine transport experiments were performed using 60 s incubations, well within the linear phase of uptake.

Procyclic s427, like bloodstream forms, displayed separate high and low affinity transport entities for [^{3}H]-pentamidine. When transport was measured at 25 nM radiolabel, unlabelled pentamidine inhibited the flux at concentrations above 10 nM and fully saturated the transporter at 1 μM (Fig. 1A), whereas uptake of 1 μM [^{3}H]-pentamidine was only starting to be inhibited by concentrations over 10 μM (Fig. 1B). Michaelis-Menten constants (K_m) were determined at 0.030 ± 0.003 and 33 ± 10 μM, respectively, consistent with the HAPT1/LAPT1 system observed in bloodstream forms of the same strain. The maximum uptake rate at saturation (V_{max}) for low affinity pentamidine transport was virtually identical in both life cycle forms but, interestingly, the V_{max} for the high affinity component was 10-fold higher in procyclic forms (Table I).

The inhibitor profile of high affinity pentamidine uptake was also highly similar in both stages. In addition to the previously reported inhibition constants (K_i) for propamidine and diminazene aceturate (De Koning, 2001) we further compared inhibition by the furamidine analogues DB820 and CPD0801 (formerly known as DB829) (Mathis et al., 2006). Again we

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found very similar activities on high affinity [^{3}H]-pentamidine transport in procyclic and bloodstream forms (Fig. 2; Table I). No reliable K_i values for DB820 and CPD0801 could be determined in relation to low affinity pentamidine transport due to lack of inhibition in the soluble range of these compounds. Indeed, no substrate or inhibitor with higher affinity than pentamidine has yet been identified for this transport activity. However, we report here LAPT1 K_i values for propamidine and diminazene, which were similar in both trypanosomal stages (Table I).

Transport of diminazene aceturate in procyclic trypanosomes

The absence of a P2 transporter in procyclic cells, and the much higher activity of a high affinity transporter, shown above to be HAPT1, allowed us to study how the diamidines are accumulated in the absence of P2. Unlike in *tbat1*$^{-/-}$ bloodstream trypanosomes (De Koning et al., 2004), [^{3}H]-diminazene uptake was readily measured in procyclics of the same strain (s427). Uptake was linear over 10 min with a rate of 0.0075 ± 0.0006 pmol(10^7 cells)$^{-1}$s^{-1} and was fully saturated by 1 mM unlabelled diminazene (Figure 3A). Fig. 3B shows that this flux, while sensitive to very low concentrations of pentamidine, was not inhibited by the purines inosine or adenine, inhibitors of the P1 and P2 purine transporters, respectively (Carter and Fairlamb, 1993). Likewise, up to 1 mM adenosine had no effect on diminazene uptake (not shown). Transport of [^{3}H]-diminazene aceturate displayed simple Michaelis-Menten kinetics consistent with a one-transporter model and a moderate affinity with a K_m value of 28 ± 5 μM and a V_{max} of 0.59 ± 0.11 pmol(10^7 cells)$^{-1}$s^{-1} (n = 4) (Fig. 3C). Interestingly, this transport phenomenon was very potently inhibited by pentamidine (Fig. 3B), with a K_i value of 0.033 ± 0.004 μM (n = 3), which

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led us to hypothesise that, at least in procyclic trypanosomes, diminazene aceturate is transported by HAPT1.

Resistance profile of a highly diminazene-resistant cell line, ABR

The hypothesis that non-P2 dependent diminazene is mediated by HAPT in procyclics and thus presumably by HAPT1 in bloodstream forms was investigated using a strain adapted from the *tbat1*$^{-/-}$ clonal line by *in vitro* exposure to increasing diminazene concentrations; the identity of the *tbat1*$^{-/-}$ line was verified prior to adaptation (Supplemental material; Fig. S2). Adaptation in the presence of incremental diminazene concentrations was performed over 6 months as depicted in Fig. S3.

The *tbat1*$^{-/-}$ cell culture selected against high level resistance to diminazene was cloned by limiting dilution and the resulting clonal line (designated ABR) was tested for its susceptibility to diminazene aceturate using the Alamar Blue assay, after a total of 59 passages. At this stage, immediately following the adaptation to diminazene, the ABR line was compared to wild type strain 427 and to the *tbat1*$^{-/-}$ parent strain and found to be less susceptible than either. A full resistance profile was made after a further 27 passages in drug free medium over a period of three months to ensure stability of the resistance phenotype (Table II), which was shown to be stable.

Table II lists EC_{50} values for the diamidines pentamidine, diminazene and DB75, and for the arsenical compounds cymelarsan and Phenylarsine oxide (PAO). Cymelarsan is a water-soluble member of the melaminophenyl arsenical class of trypanocides, used against veterinary trypanosomiasis, and a close homologue of the lipophilic human sleeping sickness drug melarsoprol; PAO is included as a known trypanocide that rapidly crosses the plasma membrane

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by passive diffusion (Carter and Fairlamb, 1993; Bridges et al., 2007). All the drug sensitivity experiments were performed with 4 different cell lines in parallel: wild-type s427, the *tbat1*$^{-/-}$ line derived from s427, B48 derived from *tbat1*$^{-/-}$ by adaptation to pentamidine *in vitro* (Bridges et al., 2007), and the ABR line derived from *tbat1*$^{-/-}$ by adaptation to diminazene. Resistance factors (RF; EC_{50} of derived line divided by EC_{50} of wild type line) are indicated in Table II. The EC_{50} values and resistance levels of the first three lines closely follow previously reported patterns: *tbat1*$^{-/-}$ displays a minor loss of sensitivity to pentamidine and melaminophenyl arsenicals and a significant level of resistance to DB75 and diminazene (Matovu et al., 2003; Lanteri et al., 2006); in B48 resistance to cymelarsan and pentamidine is greatly increased due to loss of HAPT1 activity (Bridges et al., 2007). Of interest is that in many assays we observe a certain reversal of DB75 and diminazene resistance levels in B48, relative to *tbat1*$^{-/-}$ (although this did not reach statistical significance). In contrast to B48, the ABR line did display a significant increase in diminazene and DB75 resistance relative to the parental *tbat1*$^{-/-}$ line ($P<0.02$ and $P<0.05$, respectively; Student t-test). While also displaying increased resistance to pentamidine and cymelarsan, this increase was less pronounced than in B48 (though this reached statistical significance only for cymelarsan; $P=0.05$, Student t-test). These results indicate that the adaptation to diminazene either uses a different mechanism than adaptation to pentamidine, or that the adaptation to such high levels of resistance is multifactorial. As the adaptation of B48 was attributed to the loss of HAPT1 activity (Bridges et al., 2007), it was next investigated whether activity of the pentamidine transporters had changed in the ABR line.

Diamidine transport in drug resistant trypanosomes

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Assessment of [^{3}H]-pentamidine transport activity in the ABR line found no evidence of a high affinity uptake component (assessed at 30 nM radiolabel). Transport of pentamidine at this high affinity discriminatory concentration was readily inhibited by submicromolar pentamidine concentrations in wild-type cells (Fig. 4A), whereas in ABR cells this flux was of a much lower level and sensitive only to concentrations higher than 10 μM pentamidine (Fig. 4B). Likewise, the high affinity component in wild-type cells was sensitive to propamidine as described for HAPT1 (De Koning, 2001), which had no effect on uptake in ABR cells (Fig. 4A,B). The most straightforward interpretation of these data is an absence of HAPT1 activity in ABR cells, with the 30 nM [^{3}H]-pentamidine taken up by the one remaining pentamidine transporter, LAPT1. Assessment of LAPT1 function at 1 μM [^{3}H]-pentamidine did reveal a wild-type pattern of low-affinity uptake that was insensitive to propamidine in ABR cells (Fig. 4C,D). A K_m value for [^{3}H]-pentamidine uptake plotted to a hyperbolic cure indicative of monophasic transport in the ABR strain was measured at 59 ± 11 μM (n=3), indistinguishable from the published value for LAPT1 of 56 ± 8 μM; the V_{max} value of 1.2 ± 0.4 pmol/10^7 cells)$^{-1}$s^{-1} (n=3) was also highly similar to the published value of 0.85 ± 0.15 pmol(10^7 cells)$^{-1}$s^{-1} for LAPT1 (De Koning, 2001). The above results strongly suggested that the pentamidine cross-resistance in ABR strain adapted to high diminazene concentration was due to loss of HAPT1 transport. Yet, the differences in resistance pattern between the pentamidine-adapted B48 and diminazene-adapted ABR lines suggest that additional adaptations may be responsible for the further increase in diminazene resistance in the latter cells. Using 1 μM [^{3}H]-diminazene we investigated whether this is attributable to differences in diminazene uptake rates. As the uptake rates in the resistant lines were very low we used timecourses with 7 points over 10 minutes in an effort to measure transport as accurately as possible (see Fig S4) and in this way measured the rate in each cell line

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3-4 times. Identical experiments were then performed with 1 μM [^{3}H]-pentamidine for comparison. The results are summarised in Figure 5.

Transport of 1 μM [^{3}H]-diminazene was significantly reduced in the *tbat1*$^{-/-}$ line compared to s427WT ($P<0.05$). The rate of [^{3}H]-diminazene transport was further reduced in the B48 line ($P<0.05$), which additionally lacks HAPT1 (Bridges et al., 2007), and similarly in the ABR ($P<0.05$) which also lacks HAPT1 activity (this paper). In contrast, reductions in 1 μM [^{3}H]-pentamidine transport rates were much less dramatic in the resistant lines (Fig. 5), as, at this relatively high concentration of pentamidine, much of the flux is through LAPT1, with HAPT1 and P2 saturating at a lower concentration (Bray et al., 2003). Transport of 1 μM [^{3}H]-diminazene, measured over 5 minutes in *tbat1*$^{-/-}$ cells, was still saturable and displayed an average K_m value of 67 ± 13 μM (n=3)(Fig. 6), fully consistent with uptake through HAPT1 (diminazene K_i for [^{3}H]-pentamidine through HAPT1 is 63 ± 3 μM (Table 1)). These results are consistent with the retained presence of LAPT1 in all four cell lines and little or no role for LAPT1 in diminazene uptake.

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Discussion

While pentamidine is transported efficiently by bloodstream forms in the absence of P2 and the deletion of the P2-encoding gene *TbAT1* alone does not confer more than marginal pentamidine resistance (Matovu et al., 2003), other therapeutically important diamidines such as diminazene aceturate (de Koning et al., 2004), DB75 (furamidine; Lanteri et al., 2006) and its aza analogues DB820 and CPD0801 (Ward et al., 2010, 2011) rely principally on P2 and *tbat1*$^{-/-}$ trypanosomes display significant resistance to these drugs *in vitro*. Notwithstanding these observations, it is evident that those diamidines are also taken up by a non-P2 mechanism, as *tbat1*$^{-/-}$ trypanosomes remain sensitive to approximately 1 µM of these diamidines *in vitro* (Matovu et al., 2003; Lanteri et al., 2006) and experimental infections with *tbat1*$^{-/-}$ *T. b. brucei* can be cured using increased doses of DB75 (Lanteri et al., 2006). In order to understand drug uptake and resistance mechanisms for these therapeutically important diamidines it is thus critical to identify and characterise the non-P2-mediated uptake systems. We investigate here whether the high affinity and low affinity pentamidine transporters, HAPT1 and LAPT1, contribute to the uptake of these diamidines.

We have previously reported that procyclic *T. brucei* express a high affinity pentamidine transport system, which we designated PPT1 (Procyclic Pentamidine Transporter 1) and which displayed similar properties to the HAPT1 transporter in bloodstream forms (De Koning, 2001). However, no conclusion was reached as to whether PPT1 might be identical to HAPT1 and the presence or absence of a low affinity component in procyclics, equivalent to LAPT1 in bloodstream forms, was not investigated. We report here additional data on the high affinity

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transport of diamidines in both life cycle stages, and the first characterisation of low affinity pentamidine transport in procyclic trypanosomes. The data shows that procyclic cells express a high affinity and a low affinity pentamidine transport system which is indistinguishable from the HAPT1 and LAPT1 transporters previously characterised in bloodstream forms (De Koning, 2001; Matovu et al., 2003; Bridges et al., 2007), both by K_m values, using pentamidine as substrate, and by inhibitor profile.

The kinetic data available are thus consistent with the HAPT1 and LAPT1 transporters of bloodstream forms also being expressed in procyclic forms, although definitive proof will require molecular studies (in progress). Although we previously designated the high affinity pentamidine transporter of procyclics PPT1 and the low affinity component "Procyclic Pentamidine Transporter 2" (PPT2) (De Koning, 2001), for the sake of clarity we will now refer to them as HAPT1 and LAPT1 respectively. The procyclic forms make a convenient model to study diamidine transport due to the non-expression of the P2 aminopurine transporter (De Koning et al., 1998), which also transports pentamidine (Carter et al., 1995; De Koning and Jarvis, 2001), diminazene (De Koning et al., 2004) and other diamidines (Lanteri et al., 2006; Collar et al., 2009; Ward et al., 2010, 2011). In addition, the much higher rate of uptake through HAPT1 in procyclic forms facilitates the study of diamidine transport at the low label concentrations required.

We thus proceeded to study the uptake of [^{3}H]-diminazene in procyclic cells in detail and found that the rate of uptake was indeed much high than in bloodstream forms lacking P2 (*tbat1*$^{-/-}$) (De Koning et al., 2004). This uptake was not mediated by either the P1 or P2 adenosine transporters, as neither adenine nor inosine (inhibitors of P2 and P1, respectively (Carter and Fairlamb, 1993;

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De Koning and Jarvis, 1999; Al-Salabi et al., 2007)), or adenosine, had any effect on diminazene accumulation. However, diminazene transport was highly sensitive to pentamidine, with an IC_{50} value close to the K_m value for pentamidine uptake by HAPT1. In addition the diminazene K_m was identical to its inhibition constant for HAPT1-mediated pentamidine uptake. This reciprocal inhibition clearly indicates that diminazene is a substrate for HAPT1, albeit with an affinity three orders of magnitude less than pentamidine. The measurements of [^{3}H]-diminazene uptake in *tbat1*$^{-/-}$ bloodstream forms, including the determination of the K_m value, were all consistent with the same mechanism occurring in both life cycle stages.

We previously showed that adaptation of *tbat1*$^{-/-}$ trypanosomes to either pentamidine or melaminophenyl arsenicals leads to los of the HAPT1 activity (Bridges et al., 2007), as this is the second-most important transporter for these trypanocides. We now show that the same happens after adaptation of bloodstream form *tbat1*$^{-/-}$ to increasing levels of diminazene *in vitro*, correlating high levels of resistance to diminazene for the first time to the sequential loss of P2/TbAT1 and HAPT1. Yet, the lines did not show exactly the same drug resistance profile, indicating that changes unrelated to these transporters may play a role in achieving such a high level of resistance.

The transport data in bloodstream forms thus conforms to predictions of the procyclic model, above, that HAPT1 is responsible for most of the P2-independent diminazene uptake in bloodstream trypanosomes and its absence appears generally to correlate with high levels of diamidine resistance: B48 and ABR both appear to have lost HAPT1 activity. We conclude that adaptation of trypanosomes without a functional P2/TbAT1 transporter may lose the HAPT1

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transporter under drug pressure with diminazene (ABR)(this paper), pentamidine (B48) or cymelarsan (B48; Bridges et al., 2007), leading to cross-resistance to new classes of drugs such as the furamidines (ABR/DB75). Nevertheless it is equally clear that high-level drug resistance as achieved by prolonged drug exposure in a laboratory is indeed multifactorial. The sequential loss of the P2 and HAPT1 transporters still does not fully explain the observed resistance patterns: B48 and ABR display identical residual rates of diminazene uptake (Fig. 5) and LAPT1-mediated pentamidine transport but they differ in their level of diminazene resistance. Several possibilities for this paradox come to mind but if the initial rates of drug transport are identical in both strains, as we established, differential drug sensitivity can theoretically be explained by (1) expression of a catabolic enzyme or pathway for diminazene in ABR (2) introduction of an efflux pump or other sequestration/extrusion mechanism in one strain or (3) additional non-transport-related adaptations that alter drug sensitivity of the parasite, for instance at target level. The latter would most likely involve changes in mitochondrial function, as this organelle that is believed to be the main target of diamidines in kinetoplastids (Lanteri et al., 2008; Basselin et al., 2002). Other dications including bisphosphonium salts and choline-derived compounds similarly target mitochondria in *Leishmania* and *Trypanosoma* spp (Ibrahim et al., 2010; Luque-Ortega et al., 2010). The possibility of specific mitochondrial changes being involved in diamidine resistance in African trypanosomes is the subject of ongoing investigations in our laboratories.

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Authorship contributions.

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Wrote or contributed to writing the manuscript: HdK, MPB

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Footnotes.

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Legends for Figures

Fig. 1. [^{3}H]-Pentamidine uptake in procyclic s427 trypanosomes at a permeant concentration of (A) 30 nM and (B) 1 μM. Cells were incubated with [^{3}H]-pentamidine for 60 seconds in the presence or absence of various concentrations of unlabelled pentamidine as indicated. The insets depict the conversion of the inhibition plots to Michaelis-Menten saturation plots. Both graphs are representative of at least 6 independent experiments, each performed in triplicate. The data shown represent the average of these triplicate determinations ± SEM. When not shown, error bars fall within the symbols.

Fig. 2. High affinity pentamidine transport in bloodstream (A) and procyclic (B) forms of *T. b. brucei* in the presence of various concentrations of unlabelled inhibitors: ▲, pentamidine; ○, DB820; ■, CPD0801. [^{3}H]-pentamidine concentration was 25 nM and incubation time was 60 s. The experiments shown are representative of at least three independent experiments, each performed in triplicate. Error bars are Standard Errors.

Fig. 3. Transport of 1 μM [^{3}H]-diminazene in procyclic trypanosomes. (A) Procyclic s427 were incubated for the indicated times with radiolabel in the presence (□) or absence (■) of 1 mM unlabelled diminazene aceturate. The correlation coefficient for the 1 μM line was 0.97 and it was highly significantly different from zero (P<0.00001; F-test) whereas the 1 mM line was not significantly different from zero (P = 0.50; F-test). (B) Inhibition of [^{3}H]-diminazene transport by various concentrations of pentamidine (●), unlabelled diminazene (○), adenine (▲) and

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inosine (□). (C) Michaelis-Menten saturation curve for [^{3}H]-diminazene in procyclic cells, representative of four independent experiments.

All experiments were performed in triplicate and error bars indicate SEM.

Fig. 4. Transport of [^{3}H]-pentamidine in wild-type and ABR cell line lines. Transport of 30 nM (panels A and B) or 1 µM [^{3}H]-pentamidine (panels C and D) was assessed in bloodstream forms of the s427WT (panels A, C) and ABR (panels B, D) cell lines over 60 seconds in the presence or absence of various concentrations of propamidine (■) or unlabelled pentamidine (○). Pentamidine uptake was expressed as pmol(10^7 cells)$^{-1}$s^{-1}. The experiments were performed in triplicate; error bars represent SE of internal replicates. Data shown are representative of at least three identical and independent experiments.

Fig. 5. Transport of 1 µM [^{3}H]-pentamidine (filled bars) or 1 µM [^{3}H]-diminazene (open bars) in four different cell lines. Transport rates were derived by linear regression from timecourses with points (in triplicate) at 0, 30, 60, 120, 300, 450 and 600 s. Zero uptake levels and saturability were verified in the presence of 1 mM unlabelled permeant. Bars show the average transport of 3 – 4 experiments (each performed in triplicate) and SE. Representative experiments are shown in figure S4 of the supplementary material.

Fig. 6. Saturation plot of [^{3}H]-diminazene transport in bloodstream forms of the *tbat1*$^{-/-}$ cell line. Transport of 1 µM label was determined in triplicate at 5 minutes of incubation; error bars represent SE. The experiment shown is representative of three identical experiments.

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Table I. Comparison of [^{3}H]-pentamidine transport parameters in bloodstream and procyclic *T. brucei*.

		Bloodstream forms	Procyclic forms
High affinity transport	Pentamidine K_m	0.035 ± 0.005	0.030 ± 0.003
	Pentamidine V_{max}	0.0028 ± 0.0006	0.031 ± 0.07
	Propamidine K_i	4.6 ± 0.7	3.7 ± 0.4
	Diminazene K_i	63 ± 3	54 ± 16
	DB820	43 ± 10	45 ± 18
	CPD0801	40 ± 8	16 ± 4
Low affinity transport	Pentamidine K_m	56 ± 8	33 ± 10
	Pentamidine V_{max}	0.75 ± 0.15	0.78 ± 0.12
	Propamidine K_i	316 ± 3	429 ± 180
	Diminazene K_i	160 ± 50	180 ± 20

High affinity transport of [^{3}H]-pentamidine was measured at concentrations of 25 – 40 nM radiolabel, or 12.5 nM for the determination of K_m values; Low affinity transport was assayed at 1 µM [^{3}H]-pentamidine. Data given are the average of at least three independent experiments, each performed in triplicate, and Standard Errors. Bloodstream forms were isolated from the blood of infected rats whereas procyclics were cultured in standard SDM79 medium. Units are µM for K_m and K_i values and pmol per 10^7 cells per second for V_{max}. Some of the values have previously been reported in De Koning (2001).

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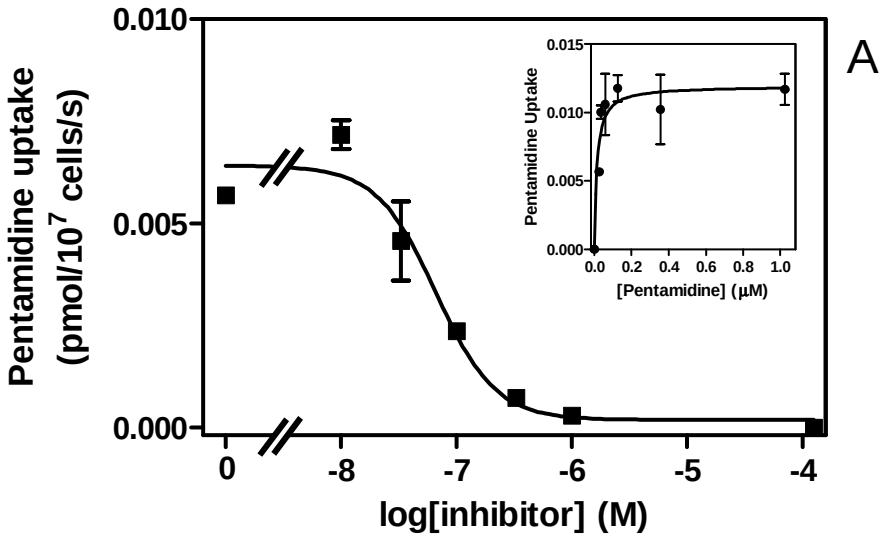
Table II. $EC5_{50}$ values for the four different trypanosomes strains used in this study.

	s427WT		*tbat1*$^{-/-}$			B48			ABR		
	EC_{50} (nM)	n	EC_{50} (nM)	n	RF	EC_{50} (nM)	n	RF	EC_{50} (nM)	n	RF
Pentamidine	6.8 ± 1.1	12	15 ± 6	11	2.2	570 ± 200	6	83	340 ± 97	5	50
Diminazene	629 ± 132	12	5780 ± 1560	12	9.2	2670 ± 510	4	4.2	14600 ± 3900	5	23
DB75	212 ± 60	12	1670 ± 460	10	7.9	360 ± 110	5	1.7	4560 ± 1560	5	22
Cymelarsan	4.1 ± 0.4	12	13 ± 2	12	3.2	62 ± 15	6	15	27 ± 4	6	6.6
PAO	0.69 ± 0.13	8	0.76 ± 0.09	8	1.1	0.54 ± 0.14	4	0.8	0.82 ± 0.14	4	1.2

RF, resistance factor relative to wild-type (s427WT)

Fig. 1



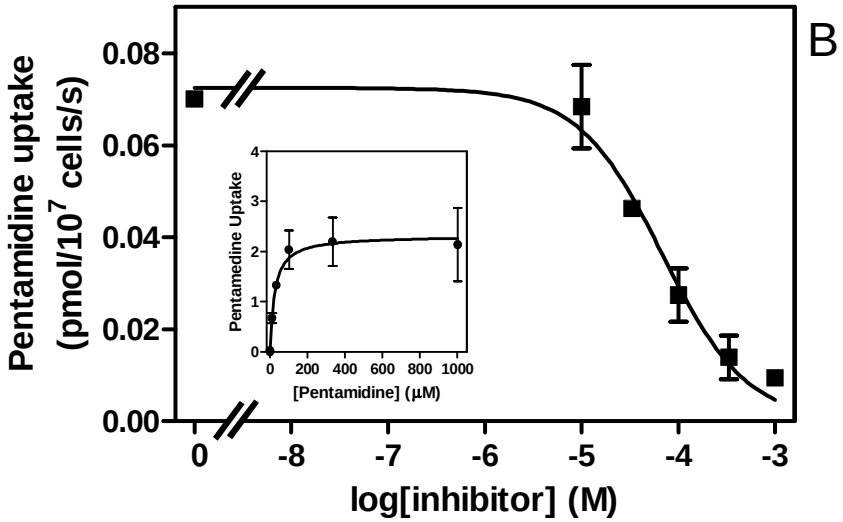
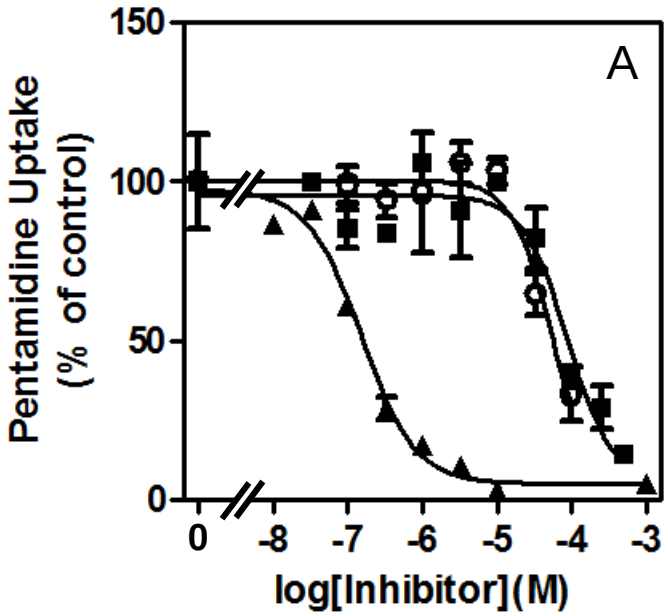


Fig. 2



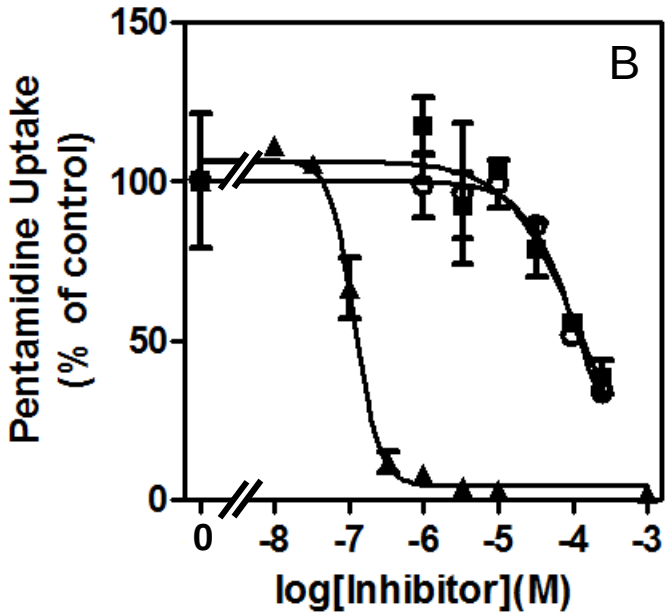


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Fig. 3

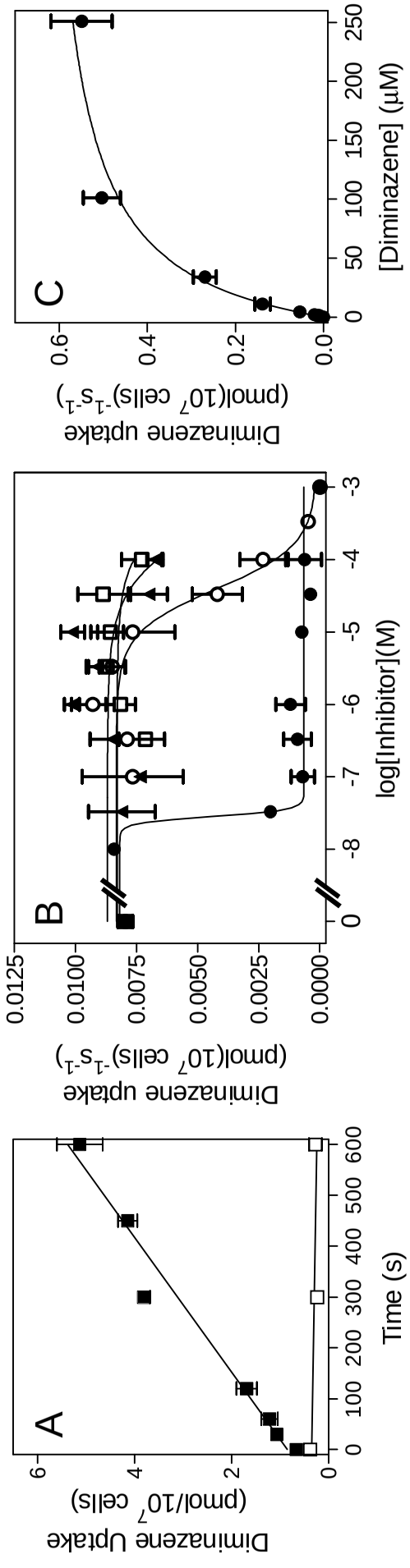

Fig. 4

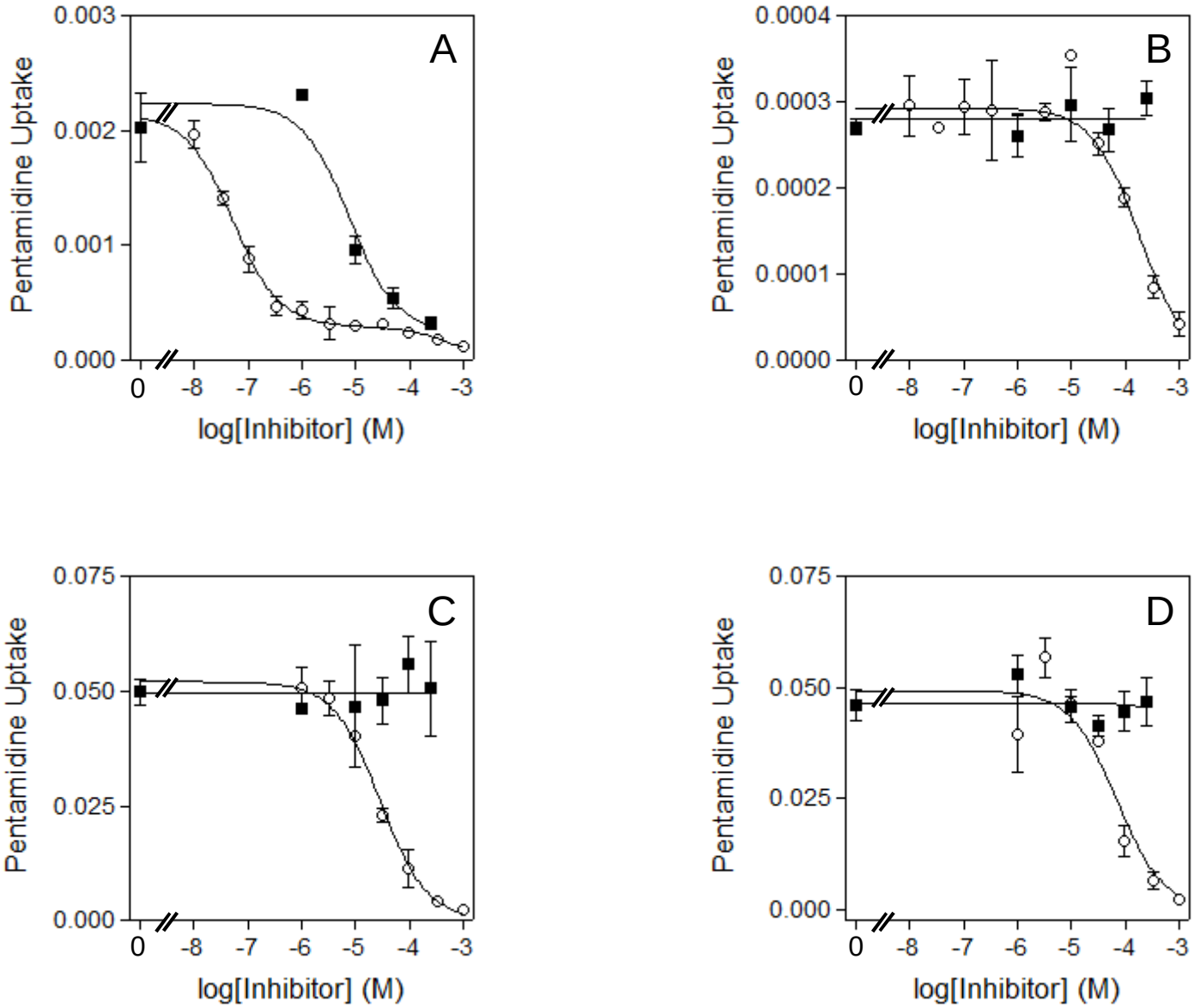

Fig. 5

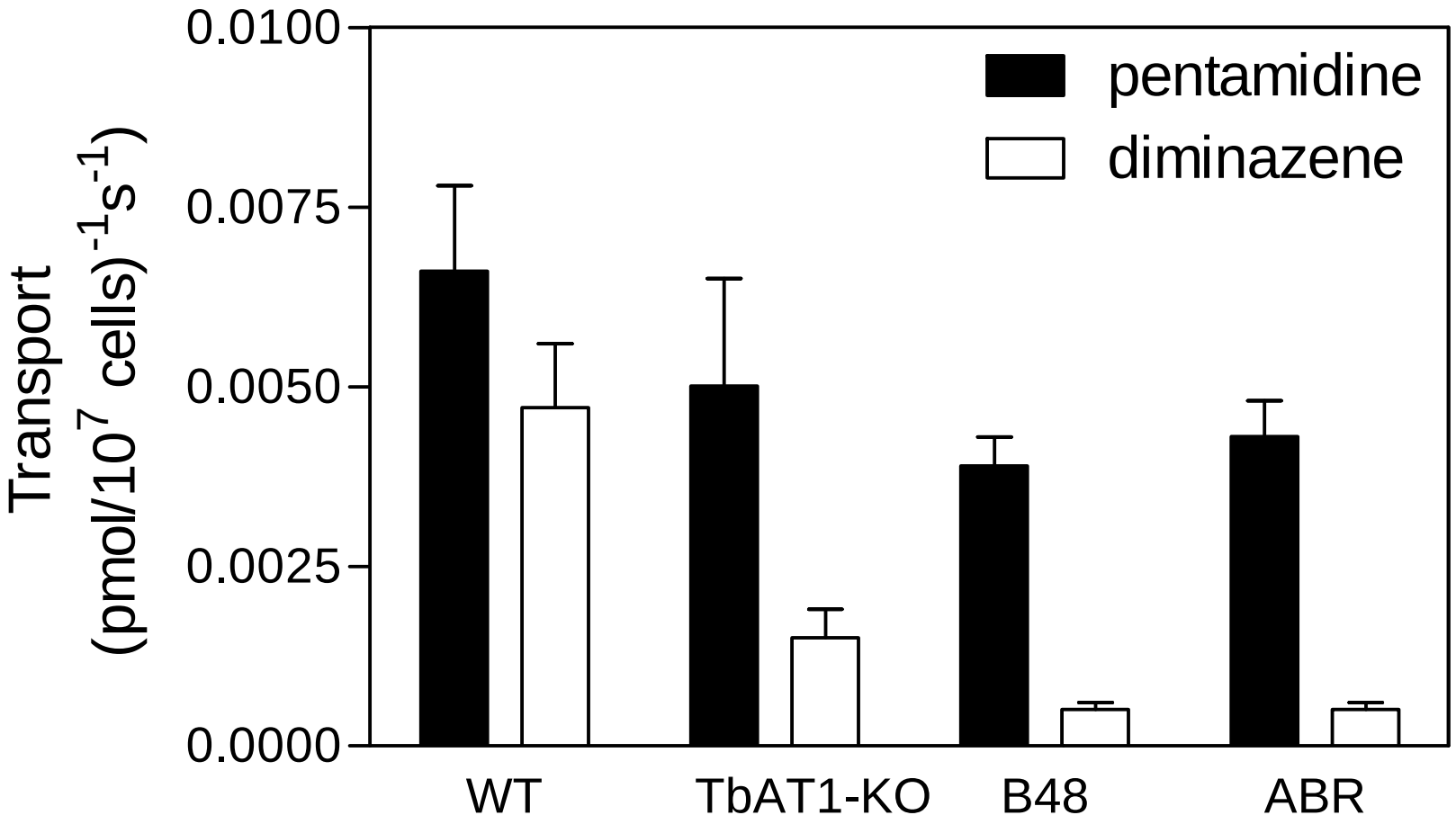

Fig. 6