Trypanosoma brucei aquaglyceroporin 2 is a high-affinity transporter for pentamidine and melaminophenyl arsenic drugs and the main genetic determinant of resistance to these drugs

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Objectives: Trypanosoma brucei drug transporters include the TbAT1/P2 aminopurine transporter and the high-affinity pentamidine transporter (HAPT1), but the genetic identity of HAPT1 is unknown. We recently reported that loss of T. brucei aquaglyceroporin 2 (TbAQP2) caused melarsoprol/pentamidine cross-resistance (MPXR) in these parasites and the current study aims to delineate the mechanism by which this occurs.

Methods: The TbAQP2 loci of isogenic pairs of drug-susceptible and MPXR strains of T. brucei subspecies were sequenced. Drug susceptibility profiles of trypanosome strains were correlated with expression of mutated TbAQP2 alleles. Pentamidine transport was studied in T. brucei subspecies expressing TbAQP2 variants.

Results: All MPXR strains examined contained TbAQP2 deletions or rearrangements, regardless of whether the strains were originally adapted in vitro or in vivo to arsenicals or to pentamidine. The MPXR strains and AQP2 knock-out strains had lost HAPT1 activity. Reintroduction of TbAQP2 in MPXR trypanosomes restored susceptibility to the drugs and reinstated HAPT1 activity, but did not change the activity of TbAT1/P2. Expression of TbAQP2 sensitized Leishmania mexicana promastigotes 40-fold to pentamidine and > 1000-fold to melaminophenyl arsenicals and induced a high-affinity pentamidine transport activity indistinguishable from HAPT1 by Km and inhibitor profile. Grafting the TbAQP2 selectivity filter amino acid residues onto a chimeric allele of AQP2 and AQP3 partly restored susceptibility to pentamidine and an arsenical.

Conclusions: TbAQP2 mediates high-affinity uptake of pentamidine and melaminophenyl arsenicals in trypanosomes and TbAQP2 encodes the previously reported HAPT1 activity. This finding establishes TbAQP2 as an important drug transporter.

Keywords: drug transport, protozoan, parasite, resistance mutation, aquaporin

Introduction

The protozoan parasite Trypanosoma brucei is the aetiological agent of human African trypanosomiasis (HAT or sleeping sickness). The subspecies T. b. gambiense and T. b. rhodesiense are responsible for West African and East African sleeping sickness, respectively, and T. b. brucei is one of the pathogens that cause animal African trypanosomiasis, a wasting disease of livestock. Despite the recent introduction of nifurtimox/eflornithine combination therapy for the late, cerebral stage of HAT,1 there is an urgent need for new drugs, driven in part by resistance to the diamidines, phenanthridines and melaminophenyl arsenicals (MPAs) that have been the central pillars of African trypanosomiasis treatment for decades.2 An understanding of the mechanisms of resistance,
and particularly of cross-resistance, is of great importance. Firstly, molecular markers are required to study the epidemiology of resistance, particularly as phenotypic assessment in primary clinical veterinary isolates is impossible for many species of African trypanosome and there is an unresolved debate about the extent of treatment failure versus genuine resistance, especially with respect to melarsoprol. Secondly, in the absence of new drugs we need to make best use of the treatments available and, for this, insight into resistance mechanisms and levels of cross-resistance is essential. Importantly, new drug development must take into account the resistance mechanisms to the current drugs, in order to avoid cross-resistance.

Melarsoprol/pentamidine cross-resistance (MPXR) is a well-known phenomenon in HAT and was first noted by Rollo and Williamson in 1951, although its causes have never been completely resolved, it has long been clear this is linked to reduced drug accumulation. The first drug transporter identified in trypanosomes was the P2 adenosine/adine transporter, which was initially implicated in melarsoprol uptake and subsequently also in diamidine. Although the evidence of diamidine and arsenical transport by TbAT1 has become incontrovertible, it has become equally clear that this transporter mediates only part of the uptake and that this proportion is different for different diamidines in particular, as deletion of the TbAT1 gene led to a high level of resistance to the veterinary diamidine diminazene acetate and the newer clinical candidates furamidine and CPD0801, but only to a relatively minor loss of susceptibility to MPAs and pentamidine. Two additional, adenosine-insensitive pentamidine transport activities were detected and functionally characterized in T. b. brucei: a high-affinity pentamidine transporter (HAPT1) and a low-affinity pentamidine transporter (LAPT1). HAPT1 was additionally found to be the secondary transporter for the arsenical drugs, with the loss of both the P2 and HAPT1 activities simultaneously leading to high-level MPXR. Despite the HAPT1 and LAPT1 activities having been first characterized over a decade ago, the genes encoding these transporters remained unknown. Recently, we reported that the aquaglyceroporin TbAQP2 controls MPXR in T. b. brucei. Aquaporins (AQPs) are major intrinsic proteins (MIPs) that are present in virtually every organism and are commonly implicated in osmotic balance and, in the case of aquaglyceroporins, in the bidirectional flux of some small, usually uncharged solutes, such as glycerol and urea. AQPs have attracted increasing pharmacological interest because of their important roles in many human physiological and pathophysiological processes, including cancer, post-traumatic brain oedema, glaucoma and epilepsy. Further pharmacological interest in AQPs emerged when it became clear that these water channels can also mediate the uptake of a wider array of molecules, including some that are cytotoxic and display antimicrobial activity. Some AQPs, including Leishmania major AQP1, transport antimycin and arsenic, most likely in the form of As(OH)₃ and Sb(OH)₃, which structurally resemble glycerol. This has attracted much attention, because pentavalent antimonials such as Glucantime and Pentostam, which are activated to a form of Sb(III), are a first-line treatment for leishmaniasis.

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Materials and methods

Trypanosome strains and culture

Bloodstream-form T. b. brucei, strain Lister 427 (s427; MiTat 1.2/BS221), and its derivatives were maintained as previously described. Several derivative lines were used: btat1⁻/⁻, 4 B48, 16, 21, 7 aqp2/aqp3 null strains 32 and P1000 cells (this paper). Procyclic-form T. b. gambiense STIB 386 wild-type and Cymelarsan-resistant (386MR) lines, and T. b. brucei STIB 247 wild-type and Cymelarsan-resistant (247MR) lines were grown as described previously. The P1000 line was generated by further subculturing of bloodstream forms of the B48 line in incrementally increasing concentrations of pentamidine, starting at 75 nM, until the trypanosomes proliferated in 1 μM pentamidine. This process took almost a year of continuous in vitro adaptation (Figure S1a, available as Supplementary data at JAC Online), which was presumably genetic in nature as the resistance phenotype has proven to be completely stable even after storage in liquid nitrogen or transformation to procyclic cells. There was no apparent in vitro growth defect associated with the P1000 adaptations (Figure S1b, available as Supplementary data at JAC Online). The STIB 900 line is T. b. rhodesiense, originally isolated from a human patient in Tanzania, and was adapted in vitro for resistance to pentamidine (STIB 900-P) or melarsoprol (STIB 900-M).

Leishmania strains and culture

Leishmania mexicana promastigotes of strain MNMC/B26/62/M379 were cultured in HOMEM medium (Invitrogen) supplemented with 10% fetal bovine serum at 25 °C exactly as described for L. major promastigotes. Promastigotes were passed to fresh culture medium or used for analysis when in mid-log culture.

Expression of aquaglyceroporins in T. b. brucei cell lines

AQP2 was expressed in the B48 and P1000 lines by modification of the expression vector pHDI1336 to give pHDI21. This plasmid was digested with NotI prior to transfection into trypanosomes. The pRPαMPPx construct was modified to express either the AQP2 or AQP2-3 chimera genes and was digested with AscI prior to transfection. Primer sequences are given in Table S1 (available as Supplementary data at JAC Online). The AQP3 and AQP2-3 genes with their selectivity region altered to that of AQP2 were synthesized by GenScript (New Jersey, USA) for insertion into pRPαMPPx to give N-terminally tagged proteins. The constructs were digested with AscI prior to transfection. B48, P1000 or aqp2/aqp3 null strains were washed in Human T Cell Nucleofector Solution for transfection using the appropriate cassette with an AmaraX Nucleofector as described previously. Transfectants were grown and cloned out by limiting dilution in standard
HMI-9/FBS containing the relevant antibiotic (5 μg/ml blasticidin for pHDK21 and 2 μg/ml hygromycin for pPRαAQP/pPRαAQP2).  

**Genome sequencing of STIB 900 lines**
Whole genome sequencing of the three *T. b. rhodesiense* lines STIB 900, STIB 900-M and STIB 900-P was carried out by 454 Life Sciences ( Branford, CT, USA) on the Genome Sequencer FLX Titanium, performing two shotgun runs per line. All the high-quality reads were mapped to the reference genome *T. b. brucei* 92707 from EBI-EMBL (version October 2011) using SMALT (www.sanger.ac.uk/resources/software/smalt). Consensus sequence and variants were identified with SAMtools14 and inspected using Artemis.15  

**Sequencing of AQP2 and AQP3 genes in drug-resistant lines**
The AQP2 and AQP3 genes were sequenced from the drug-resistant lines as well as from their respective wild-type lines. The genes were amplified from genomic DNA using a proofreading polymerase and ligated into the pGEM-T Easy subcloning vector and sequenced using standard procedures. The primers used for amplification of the various genes are given in Table S1 (available as Supplementary data at JAC Online).  

**Cellular localization of chimeric AQP2/3**
Localization of green fluorescent protein (GFP)-coupled chimeric AQP2/3 (GFP-cAQP2/3) and western blot with anti-GFP antisera were performed exactly as described for wild-type TbAQP2 and TbAQP3.16 Nuclear staining was performed with 4,6-diamidino-2-phenylindole (DAPI).  

**Transport assays**
Transport assays with procyclic-43 and bloodstream-form trypanosomes,44,45 and *L. mexicana* promastigotes,36,46 were performed exactly as described previously, measuring fluorescence in 96-well plates (BMG Labtech, Durham, NC, USA) at wavelengths of 544 nm for excitation and 620 nm for emission. EC50 values were calculated by non-linear regression using an equation for a sigmoidal dose–response curve with variable slope (GraphPad Prism 5.0; GraphPad Software Inc., San Diego, CA, USA).  

**Drug susceptibility assays**
Drug susceptibilities of the bloodstream-form trypanosomes47 and *L. mexicana* promastigotes48 were determined using the Alamar blue assay exactly as described previously, measuring fluorescence in 96-well plates with a FLUOstar Optima (BMG Labtech, Durham, NC, USA) at wavelengths of 544 nm for excitation and 620 nm for emission. EC50 values were calculated by non-linear regression using an equation for a sigmoidal dose–response curve with variable slope (GraphPad Prism 5.0; GraphPad Software Inc., San Diego, CA, USA).  

**Heterologous expression of T. brucei AQP2s in Leishmania promastigotes**
TbAQP2 and TbAQP3 were amplified from genomic DNA by PCR using Phusion polymerase (Thermo Scientific) and subcloned into the pNUS vector for expression in *Leishmania*.15 The construct was verified by sequencing before transfection into *L. mexicana* promastigotes using an Amara Nucleofector (program U-33).  

**Results**
**Status of drug transporters and AQP2 in isogenic susceptible/resistant trypanosome pairs**
The study by Baker et al.20 established a clear link between TbAQP2 and MPXR in the s427/B48 isogenic pair of *T. b. brucei*. The B48 line was produced from the TbAT1-KO line (derived from s427 by targeted deletion of TbAT1)15 followed by in vitro exposure to pentamidine.16 For TbAQP2 to be confirmed as a general genetic marker for MPXR in trypanosomes, however, it is essential that this link be upheld in further isogenic pairs showing MPXR, particularly where resistance has been induced (i) in vivo or (ii) to the arsenical component rather than pentamidine and (iii) in human-infective trypanosome subspecies. We thus widened our investigations to the strains described in Table 1, which lists a number of well-described isogenic strains with the desired characteristics. These include strains of both human-infective subspecies, *T. b. gambiense* and *T. b. rhodesiense*, in addition to the closely related animal parasite *T. b. brucei*. The strains were adapted by drug exposure in vivo or in vitro, by exposure to an MPA compound (melarsoprol or Cymelarsan, its water-soluble derivative) or pentamidine. Some were shown to be transmissible by tsetse flies and to mate in this vector.53 In all of these highly resistant strains, the TbAT1/P2 transport activity is known to be deleted, non-expressed or mutated (Table 1 and references therein) and it is believed that this explains part, but crucially not all, of the resistant phenotype.  

The characterization of the isogenic pair s427/TbAT1-KO clearly showed loss of P2 activity to be associated with only a minor loss of susceptibility to pentamidine and MPAs, in addition to a much higher degree of resistance to diminazene.14,50 Thus, high-level MPXR is clearly a function of the loss of MpXR in trypanosomes, however, it is essential that this link be upheld in further isogenic pairs showing MPXR, particularly where resistance has been induced (i) in vivo or (ii) to the arsenical component rather than pentamidine and (iii) in human-infective trypanosome subspecies. We thus widened our investigations to the strains described in Table 1, which lists a number of well-described isogenic strains with the desired characteristics. These include strains of both human-infective subspecies, *T. b. gambiense* and *T. b. rhodesiense*, in addition to the closely related animal parasite *T. b. brucei*. The strains were adapted by drug exposure in vivo or in vitro, by exposure to an MPA compound (melarsoprol or Cymelarsan, its water-soluble derivative) or pentamidine. Some were shown to be transmissible by tsetse flies and to mate in this vector.53 In all of these highly resistant strains, the TbAT1/P2 transport activity is known to be deleted, non-expressed or mutated (Table 1 and references therein) and it is believed that this explains part, but crucially not all, of the resistant phenotype.  

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We thus analysed the AQP2-AQP3 locus of the 247 and 386 isogenic pairs and discovered that the AQP2 gene was completely
Table 1. Overview of trypanosome strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Subspecies</th>
<th>Susceptibility&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Adapted from</th>
<th>Resistance induction</th>
<th>Infectivity</th>
<th>Transport activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lister 427</td>
<td>T. b. brucei</td>
<td>++++ +++++</td>
<td>NA</td>
<td>NA</td>
<td>✚</td>
<td>P2</td>
</tr>
<tr>
<td>2T1</td>
<td>T. b. brucei</td>
<td>++++ +++++</td>
<td>Lister 427</td>
<td>NA</td>
<td>✚</td>
<td>P2</td>
</tr>
<tr>
<td>aqp2/aqp3 null</td>
<td>T. b. brucei</td>
<td>++/− + +</td>
<td>2T1</td>
<td>NA</td>
<td>✚</td>
<td>−</td>
</tr>
<tr>
<td>TbAT1-KO</td>
<td>T. b. brucei</td>
<td>++ + + +</td>
<td>Lister 427</td>
<td>TGD</td>
<td>✚</td>
<td>−</td>
</tr>
<tr>
<td>B48</td>
<td>T. b. brucei</td>
<td>− +/− +</td>
<td>TbAT1-KO</td>
<td>in vitro, pentamidine</td>
<td>✚</td>
<td>NP</td>
</tr>
<tr>
<td>P1000</td>
<td>T. b. brucei</td>
<td>− −/− +/−</td>
<td>B48</td>
<td>in vitro, pentamidine</td>
<td>✚</td>
<td>NP</td>
</tr>
<tr>
<td>STIB 247</td>
<td>T. b. brucei</td>
<td>+++ +++++</td>
<td>NA</td>
<td>NA</td>
<td>✚</td>
<td>−</td>
</tr>
<tr>
<td>STIB 247MR</td>
<td>T. b. brucei</td>
<td>+++ +++++</td>
<td>STIB 247</td>
<td>in vivo, Cymelarsan</td>
<td>✚</td>
<td>−</td>
</tr>
<tr>
<td>STIB 386</td>
<td>T. b. gambiense</td>
<td>+++ + +</td>
<td>NA</td>
<td>NA</td>
<td>✚</td>
<td>−</td>
</tr>
<tr>
<td>STIB 386MR</td>
<td>T. b. gambiense</td>
<td>+/− + +</td>
<td>STIB 386</td>
<td>in vivo, Cymelarsan</td>
<td>✚</td>
<td>−</td>
</tr>
<tr>
<td>STIB 900</td>
<td>T. b. rhodesiense</td>
<td>+++ + + +</td>
<td>NA</td>
<td>NA</td>
<td>✚</td>
<td>−</td>
</tr>
<tr>
<td>STIB 900-M</td>
<td>T. b. rhodesiense</td>
<td>− +/− +</td>
<td>STIB 900</td>
<td>in vitro, pentamidine</td>
<td>✚</td>
<td>NP</td>
</tr>
<tr>
<td>STIB 900-P</td>
<td>T. b. rhodesiense</td>
<td>− +/− +</td>
<td>STIB 900</td>
<td>in vitro, pentamidine</td>
<td>✚</td>
<td>−</td>
</tr>
</tbody>
</table>

<sup>a</sup>Susceptibility to pentamidine or arsenical drugs is indicated on a relative scale as highly sensitive (++++) ranging to highly resistant to pentamidine or melaminophenyl arsenicals (−−−−).<sup>b</sup>Resistance induced to pentamidine.<sup>c</sup>The clone used in this paper is not tsetse transmissible but other clones of s427 have been shown to infect tsetse flies.<sup>d</sup>Bernhard et al. reported that STIB 900-P contained a wild-type TbAT1 gene; later analysis revealed that in fact the TbAT1 open reading frame contains one coding mutation (G1288C → Arg).

Table 2. Kinetic parameters of high-affinity and low-affinity pentamidine transport in 247 and 386 procyclins

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pentamidine</th>
<th>Propamidine</th>
<th>Pentamidine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HAP1 K&lt;sub&gt;m&lt;/sub&gt; (μM)</td>
<td>HAP1 V&lt;sub&gt;max&lt;/sub&gt; (pmol/10&lt;sup&gt;7&lt;/sup&gt; cells/s)</td>
<td>HAP1 K&lt;sub&gt;m&lt;/sub&gt; (μM)</td>
</tr>
<tr>
<td>STIB 247WT</td>
<td>0.029 ± 0.001</td>
<td>0.008 ± 0.002</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>STIB 247MR</td>
<td>NP</td>
<td>NP</td>
<td>19 ± 0.41</td>
</tr>
<tr>
<td>STIB 386WT</td>
<td>0.027 ± 0.004</td>
<td>0.007 ± 0.002</td>
<td>22 ± 6</td>
</tr>
<tr>
<td>STIB 386MR</td>
<td>NP</td>
<td>NP</td>
<td>51 ± 2</td>
</tr>
</tbody>
</table>

Uptake of [3H]pentamidine by suspensions of 10<sup>7</sup> procyclic trypanosomes was measured at 25 nM or 1 μM for the determination of parameters of high-affinity transport (HAP1 mediated) or low-affinity transport (LAPT1 mediated), respectively. In the 247MR and 386MR strains, no high-affinity pentamidine transport was observed and transport rates were very low, with saturation only at very high concentrations of unlabelled pentamidine, consistent with uptake by LAPT1. NP, not present in these cells.

Expression of AQP2 reverses high levels of pentamidine and melarsoproic resistance

We used the laboratory-generated cell line B48<sup>16</sup> and its derivative P1000 to investigate whether expression of wild-type (WT) TbAQP2 can fully reverse the multidrug resistance phenotype of these clones. P1000 was developed by further adaptation of B48 to 1 μM pentamidine in vitro and thus its resistance phenotype is believed to be multifactorial; B48 itself was derived from the tbat<sup>1</sup>−/− strain and additionally lacks HAP1 activity.<sup>16</sup> Figure 1 shows the resistance profile of WT s427 and of B48 and P1000 transfected with the empty vector (EV) phD1336 and with the same vector containing WT TbAQP2.<sup>13</sup> The lipophilic arsenical PAO was used as a control as it has been shown to diffuse across the T. b. brucei plasma membrane,<sup>15</sup> making its action absent from the 386MR line, whilst the AQP3 gene was identical to that in the 386 wild-type line. In the 247MR line, however, a chimeric gene of AQP2 and AQP3 had been formed in place of both wild-type genes. This chimera is in-frame, producing a 939 bp ORF composed of the first 363 bp of AQP2 and the last 576 bp of AQP3, and is thus different from the chimera found in strain B48 (see below).

Detailed analysis of the AQP locus of the STIB 900 lines revealed that both the pentamidine- and melarsoproic-resistant lines had lost the AQP2 gene whilst retaining the AQP3 gene. The organization of the AQP2-AQP3 locus in all the various strains so far examined is shown in Figure S4 (available as Supplementary data at JAC Online). It appears that, in all cases, AQP2 is either lost completely or recombined into a chimeric gene that encodes most of the residues comprising the AQP3 selectivity filter.
independent of transporters and showing that the resistance phenotype is not to arsenic per se.

The EV controls B48 and P1000 were strongly resistant to the diaminides pentamidine and diminazene as well as to the MPA drug Cymelarsan, but not to PAO. Expression of TbAQP2 in B48 and P1000 completely reversed resistance to pentamidine (50- and 240-fold, respectively) and Cymelarsan (16.7- and 15.0-fold, respectively) in both resistant clones (Figure 1). However, the level of diminazene resistance in these lines was not affected, consistent with the lack of diminazene resistance in an aqp2 null line.20

A chimeric AQP2/3 gene in the AQP2 locus of B48 and P1000 is distributed over the cell surface and does not affect pentamidine and arsenic sensitivity

In B48, the AQP2 locus has been replaced by a chimeric in-frame fusion of AQP2 and AQP3 (chAQP2/3) whereas AQP3 has remained
unchanged.20 An identical chAQP2/3 and AQP3 locus was found in P1000, showing that the higher level of pentamidine resistance in P1000 was not due to further changes to the AQP2-AQP3 locus. The observation that expression of WT AQP2 reverses the B48 and P1000 MPXR phenotypes (Figure 1) suggests that chAQP2/3 coincides with the loss of AQP2 function. This was further investigated by expressing either WT AQP2 or chAQP2/3 in the aqp2 null line produced from this strain.20 Expression of WT AQP2 reversed the aqp2 null phenotype, whereas expression of chAQP2/3 had much less effect on sensitivity to any of the drugs tested (Figure 2). This confirms that the chimeric form does not function like AQP2, at least with respect to pentamidine susceptibility (P > 0.05), whether it still exercises an AQP-like activity or not. We did observe a small but significant (1.4-fold; P < 0.05) difference in Cymelarsan susceptibility between the aqp2 null clone and the same cells expressing chAQP2/3, although the effect was very much smaller than the expression of WT AQP2 (4.1-fold; P < 0.001). The (over)expression of WT AQP2 in an aqp2 null or aqp2/aqp3 double-null background appeared to make the cells slightly less susceptible to diminazene, the reverse of its effect on pentamidine and Cymelarsan sensitivity. There were no significant differences between any of the lines with respect to PAO. Expression of AQP2 in the WT trypanosomes did not elicit any change in drug susceptibility (Figure 2).

Apart from the primary sequence differences between TbAQP2 and TbAQP3, their cellular location is also different, with AQP2 restricted to the flagellar pocket whereas AQP3 is present throughout the plasma membrane.20 As this could have bearing on the MPXR phenotype, a fusion gene of chAQP2/3 N-terminally coupled to GFP was constructed and introduced into the aqp2/aqp3 null strain. Expression of the fusion protein was confirmed by western blotting (Figure 3a). The localization in trypanosomes was observed directly by fluorescence microscopy. It was found that the GFP-tagged chAQP2/3, unlike AQP2, was present on the plasma membrane (Figure 3b).

**Expression of WT TbAQP2 correlates with the HAPT1 pentamidine transport activity**

The strong phenotype of pentamidine resistance in aqp2 null lines (16.3-fold; n = 22; P < 0.001) prompted us to investigate the uptake of [3H]pentamidine in these cells and compare this with that in the parental 2T1 cells. We chose a [3H]pentamidine concentration of 30 nM in the presence of 1 mM adenosine; this concentration of adenosine fully blocks the P2 aminopurine transporter21 and the 30 nM label concentration produces a biphasic uptake that visualizes uptake through both high- and low-affinity pentamidine transporters (HAPT1 and LAP1).16 Propamidine was used as a specific inhibitor of HAPT1, having no effect on LAP1 activity.12 In four independent experiments in triplicate, [3H]pentamidine uptake was assessed, in parallel, in WT and in aqp2 null cells.

As expected in control trypanosomes, inhibition of the uptake of [3H]pentamidine with unlabelled pentamidine in the range 10 nM – 1 mM produced a biphasic curve, of which only the first (high-affinity) phase was sensitive to inhibition by propamidine (IC50 = 29.9 ± 4.3 μM; n = 3). Plotting the pentamidine inhibition data to an equation for a biphasic sigmoidal curve (two-site inhibition; GraphPad Prism 5.0) yielded mean IC50 values that were entirely compatible with the HAPT1/LAP1 system: 0.060 ± 0.017 and 29.9 ± 4.3 μM (n = 3), respectively (Figure 4). In the aqp2 null cells, [3H]pentamidine uptake was much lower, completely lacked the high-affinity inhibition phase with unlabelled pentamidine and was insensitive to propamidine (Figure 4). The mean IC50 value of 33.4 ± 9.5 μM was statistically identical to the lower-affinity phase of the WT 2T1 cells and with the published pentamidine Km for LAP1 (56.2 ± 8.3 μM).17 We conclude that aqp2 null cells do not express HAPT1, but do express LAP1.

We previously reported that the pentamidine-resistant clone B48 similarly lacks HAPT1 pentamidine transport activity as a result of in vitro resistance selection with pentamidine16 and that this clonal line lacks a WT AQP2 gene.25 We therefore tested whether introduction of wild-type TbAQP2, in addition to restoring pentamidine susceptibility (Figure 1), would reinstate HAPT1 activity. Figure 5 shows the rates of uptake at [3H]pentamidine...
concentrations that favour uptake by HAPT1 (50 nM; Figure 5a) or LAPT1 (1 μM; Figure 5b); an s427-based cell line from which the TbAT1/P2 transporter was removed by homologous recombination (TbAT1-KO) was used as the control, as this was the strain B48 was derived from. Consistent with earlier findings, uptake of 50 nM [3H]pentamidine was reduced 15.5-fold in B48 compared with the TbAT1-KO control (P < 0.001), whereas uptake of 1 μM pentamidine was not significantly different. Expression of TbAQP2 in B48 increased uptake of 50 nM pentamidine 27.7-fold (P < 0.02; Figure 5a) to a level that appeared somewhat higher than that in TbAT1-KO, although that difference did not reach statistical significance. Uptake of 1 μM pentamidine was also increased upon introduction of TbAQP2, but by only 2.5-fold (P < 0.02; Figure 5b) and again was not significantly different from the level in TbAT1-KO.

These data confirm that expression of TbAQP2 in B48 restores the pentamidine transport conditions of TbAT1-KO, completely reversing transport-related resistance.

We next investigated whether the increased pentamidine uptake rates were indeed due to the expression of a HAPT1 activity in B48 cells re-expressing AQP2. Km and Vmax values of HAPT1 and LAPT1 were determined in B48/AQP2 (Figure 5c and d) and compared with previously obtained values for WT s427, B48 and TbAT1-KO (Table 3). No clear or significant differences were observed with the kinetic parameters of pentamidine transport in bloodstream forms of other s427-derived strains (WT s427, TbAT1-KO and B48), including P1000, for which the pentamidine Km and Vmax values (Figure S5; available as Supplementary data at JAC Online) were also added to Table 3. We conclude that expression of TbAQP2 reinstated a high-

**Figure 5.** [3H]pentamidine uptake by various s427-derived trypanosome lines. (a) [3H]pentamidine concentration was 50 nM, reflecting predominantly HAPT1-mediated uptake. (b) [3H]pentamidine concentration was 1 μM, reflecting predominantly LAPT1 uptake. Rates were determined from the slopes of time courses over 10 min with timepoints at 0, 1, 3, 5, 7.5 and 10 min. Lines were linear, with none of the lines showing significant deviation from linearity in a runs test (GraphPad Prism 5.0). Slopes of control time courses in the presence of 1 mM unlabelled pentamidine were all non-significantly different from zero (F-test, GraphPad Prism 5.0), whereas uptake with unlabelled inhibitor was highly significantly different from zero (typically P < 0.0001). Results are the mean of three to four independent experiments, each performed in triplicate. (c, d) Uptake of [3H]pentamidine in bloodstream forms of B48/AQP2 was determined as described in the legend to Figure 4. The experiments shown are representative of three independently performed replicates. (c) Uptake of 30 nM [3H]pentamidine. (d) Uptake of 1 μM [3H]pentamidine. Solid squares, coincubation with various concentrations of unlabelled pentamidine; open circles, coincubation with propamidine. **P < 0.01; ***P < 0.001.
affinity pentamidine transport activity that is indistinguishable from the well-characterized HAPT1, whilst making no detectable change to the LAPT1 activity. TbAQP2 displays HAPT1 activity when expressed in L. mexicana Leishmania spp. are relatively insusceptible to pentamidine and tolerant to MPAs, as they lack high-affinity uptake systems for these drugs. In order to investigate whether T. b. brucei AQP5s directly mediate uptake of these drugs, we expressed TbAQP2 and TbAQP3 in promastigotes of L. mexicana. Comparisons of control cells (transfected with EV) and cells expressing TbAQP2 showed 40-fold sensitization in two independent clones (Figure 6a) and a >1000-fold sensitization to MPAs (Figure 6b). There was no statistically significant effect on sensitivity to diminazene, relative to the EV control (Figure 6c), or to amphotericin B (Figure 6d); the latter was used as a control antileishmanial drug thought to bind to plasma membrane ergosterol, thereby forming pores. Expression of TbAQP3 in two independent clones led to minor (1.5–2.5-fold) sensitization to pentamidine.

Consistent with this minor effect of AQP3 expression, uptake of 100 nM [3H]pentamidine in TbAQP3-transfected promastigotes was not significantly different from that in the control when measured over 5 min (Figure 7a and data not shown). In contrast, pentamidine uptake in TbAQP2-expressing cells was increased almost 15-fold (Figure 7a). Indeed, uptake of very low concentrations of [3H]pentamidine (50 nM) was measurable in TbAQP2-expressing promastigotes within 3 s, was linear with a rate of 0.032 ± 0.002 pmol/10⁷ cells/s and completely saturable with excess unlabelled pentamidine (Figure 7b). Competition with unlabelled pentamidine, propamidine and diminazene was dose dependent (Figure 7c) and followed Michaelis–Menten kinetics (Figure 7d). Mean Kₚ and Kᵢ values for these inhibitors are listed in Table 3 and show a kinetic profile entirely consistent with the T. b. brucei HAPT1 activity, with an apparent Kₚ value of 55 ± 4 nM (n = 5).

TbAQP2 does not affect the activity of the P2 transporter

It has long been established that the P2 transport activity, encoded by TbAT1, is an important determinant of diamidine and arsenical

<table>
<thead>
<tr>
<th>Strain</th>
<th>HAPT1 pentamidine Kₚ (µM)</th>
<th>HAPT1 propamidine Kᵢ (µM)</th>
<th>HAPT1 diminazene Kᵢ (µM)</th>
<th>LAPT1 pentamidine Kₚ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT s427</td>
<td>0.036 ± 0.006</td>
<td>4.6 ± 0.7</td>
<td>63 ± 3</td>
<td>56 ± 8</td>
</tr>
<tr>
<td>TAT1-KO</td>
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<tr>
<td>B48</td>
<td>NP</td>
<td>56 ± 7</td>
<td>ND</td>
<td>99 ± 24</td>
</tr>
<tr>
<td>P1000</td>
<td>NP</td>
<td></td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>B48/AQP2</td>
<td>0.046 ± 0.014</td>
<td>15.2 ± 1.6</td>
<td>ND</td>
<td>66 ± 1</td>
</tr>
<tr>
<td>L. mexicana/TbAQP2</td>
<td>0.055 ± 0.004</td>
<td>8.1 ± 0.8</td>
<td>100 ± 21</td>
<td>not present in these cells</td>
</tr>
</tbody>
</table>

ND, not determined; NP, not present in these cells.

In the B48 and P1000 strains, no high-affinity transport of pentamidine was detectable, nor was pentamidine uptake inhibited by propamidine.

De Koning.17 Matovu et al.14 Bridges et al.16

Table 3. Kinetic parameters of high-affinity and low-affinity pentamidine transport in various strains of T. b. brucei bloodstream forms and in Leishmania mexicana expressing TbAQP2

Figure 6. Effect of expression of T. brucei aquaporins on drug susceptibility of Leishmania mexicana. TbAQP2 and TbAQP3 were expressed in promastigotes using the pNUS vector. Two independent clones of each resulting cell line and the promastigotes transfected with the ‘empty’ pNUS vector were tested for sensitivity to (a) pentamidine, (b) Cymelarsan, (c) diminazene and (d) amphotericin B using the Alamar blue fluorimetric assay. Bars are means of three to eight independent determinations; error bars are SEM. ***P < 0.001 by one-way ANOVA with Tukey’s correction (GraphPad Prism 5.0). EV, empty-vector control.
sensitivity in *T. brucei*.\(^9,\!^{12}\) It is thus important to establish whether the TbAQp2/HAPT1 activity has any effect on P2 activity. It has been previously reported that the expression of P2 can be assessed through susceptibility to some adenosine analogues.\(^{\text{15}}\) Indeed, we found that susceptibility to tubercidin (7-deazaadenosine) decreased 10-fold in the *tbat1*⁻/⁻ strain (EC₅₀ = 2.65 ± 0.23 μM) relative to s427 WT (0.23 ± 0.03 μM; *P* < 0.001; Student’s t-test). Similarly, susceptibility to tubercidin, cordycepin (3′-deoxyadenosine) and 5′-deoxyadenosine increased >20-fold when *TbAT1* was expressed in the *tbat1*⁻/⁻ strain B48 (*P* < 0.001) (Figure S6, available as Supplementary data at JAC Online), confirming susceptibility to cytotoxic adenosine analogues as a sensitive indicator for *TbAT1* expression levels. However, the susceptibility of 2T1 WT and aqp2 null cells to these same adenosine analogues was identical, as was the susceptibility of B48 + TbAQp2 versus control B48 cells (Figure S6, available as Supplementary data at JAC Online). Susceptibility to pentamidine, used as a positive control, was highly dependent on the expression of *TbAQp2* and/or *TbAT1*. We conclude that *TbAQp2* does not regulate P2 activity and is not itself a conduit for cytotoxic nucleosides.

Investigation of the *TbAQp2* and *TbAQp3* selectivity filters as the determinant for MPXR

The *TbAQp2* region that was replaced with the corresponding *TbAQp3* sequence in the recombination event that generated *chAQp2* was also found to be the major determinant for MPXR. As the chimeric AQPs found in B48 and 247MR did not appear to have the *TbAQp2* functionality with respect to pentamidine and melarsoprol susceptibility, we investigated whether MPXR was determined primarily by the few amino acids of the selectivity filter. We used synthetic genes of *chAQp2* and of *TbAQp3* with a *TbAQp2* selectivity filter (chAQp2/3*SF2* and *TbAQp3**SF2*, respectively; alignment in Figure S7, available as Supplementary data at JAC Online). These were expressed in the *aqp2/aqp3* null cell line. Analysis of the drug susceptibility phenotype for the resultant lines showed that the transplanted amino acid residues of the AQp2 selectivity filter did not result in an AQp2 phenotype with respect to drug susceptibility; there was only a minor increase in susceptibility and only to pentamidine (EC₅₀ values of 158 ± 2 and 131 ± 3 nM, *P* < 0.02,
for aqp2/aqp3 null and AQP3<sup>f2</sup>, respectively; Figure 8). In contrast, the cell line expressing the chAQP2/3<sup>f2</sup> construct was significantly more susceptible to Cymelarsan than the aqp2/aqp3 null background, reaching susceptibility to this drug halfway between the aqp2/aqp3 null and the same line expressing WT AQP2 (Figure 8). These findings indicate that the selectivity filter residues do play a role in arsenical drug sensitivity, but that the changes described here were insufficient to produce the same effect as WT AQP2 on pentamidine susceptibility.

**Discussion**

Although we recently reported that the absence of a wild-type AQP2 gene correlates with MPXR in T. b. brucei, many important questions remained. It remained unclear (i) whether this phenomenon is relevant for human-infective trypanosome subspecies, (ii) whether loss of AQP2 occurs as a result of in vivo drug exposure, (iii) whether loss of AQP2 alone is sufficient for high levels of pentamidine and melarsoprol resistance, (iv) by which mechanism AQP2 facilitated pentamidine and melarsoprol sensitivity and (v) whether AQP2 has any impact on TbAT1/P2 activity. We now provide answers to these questions.

Only TbAQP2 was implicated in MPXR, with the expression of TbAQP3 in an aqp2/aqp3 null line having no effect on drug susceptibility. This is most likely the result of differences in permeation, as all three TbAQPs are located on the cell surface, although AQP2 was found to be restricted to the flagellar pocket rather than dispersed over the plasma membrane. Interestingly, a chimeric AQP, made up from TbAQP2 and TbAQP3 (chAQP2/3) was present instead of wild-type TbAQP2 in the highly MPXR strain T. b. brucei B48, which retained a wild-type copy of TbAQP3 found in the parental, drug-susceptible strain. Here, we report that in other isogenic MPXR pairs, the adapted strain similarly showed a replacement of TbAQP2 with a different chimeric AQP2/3 fusion or an outright deletion of TbAQP2. This was the case whether the strain was originally adapted to pentamidine or to an MPA drug, whether the subspecies was T. b. brucei, T. b. gambiense or T. b. rhodesiense and for strains that were selected by either in vivo or in vitro drug pressure. It thus follows that for T. brucei subspecies, loss of TbAQP2 function represents an important component of acquiring high levels of resistance to MPAs and that this necessarily leads to pentamidine resistance as well, and vice versa. We also conclude that loss of AQP2 can be the result of in vivo drug pressure and that this does not significantly affect virulence and does not impede transmission by tsetse flies, as STIB 386MR and STIB 247MR produce similar parasitaemias in mice compared with the wild-type lines from which they were derived and were fly transmissible.

One important question is whether loss of TbAQP2 alone is sufficient for MPXR in vivo. Certainly, the deletion of just TbAQP2 rendered a highly drug-susceptible T. b. brucei strain resistant to pentamidine (but not diminazene) and Cymelarsan (but not PAO) in vitro, this shows that the resistance is not to all aromatic diamidines or arsenic per se. Similarly, the reintroduction of wild-type TbAQP2 into B48 or P1000 restored sensitivity to Cymelarsan and pentamidine (but not diminazene) to wild-type levels. Both strains retained their diminazene resistance, which is linked to the loss of the TbAT1/P2 transporter, which is absent in B48.

![Figure 8](image-url)

*Figure 8.* Expression of synthetic aquaporin constructs in bloodstream forms of the aqp2/aqp3 double-null line. EC₅₀ values were determined for (a) pentamidine, (b) Cymelarsan, (c) diminazene and (d) phenylarsine oxide. Expression was induced by the addition of 1 μg/mL tetracycline 24 h before setting up the plates for the assay, followed by 48 h of incubation of the cells in the presence of a doubling dilution of the test compound followed by a further 24 h of incubation with the dye before fluorescence was determined. All data are the mean of at least three independent determinations; error bars are SEM. Statistical significance with aqp2/aqp3 null was determined using a one-way ANOVA with Tukey’s correction (GraphPad Prism 5.0); **p<0.02; ***p<0.01. In the Cymelarsan panel (b), it is also indicated that Groups 4 and 5 are significantly different from Group 3.
and P1000 cells.\textsuperscript{16} Indeed, TbAT1 is absent or mutated in all the drug-adapted MPXR strains used here,\textsuperscript{56,57} opening up the possibility that both transport activities must be absent for high levels of MPXR. It has already been established that homozygous deletion of TbAT1 alone results in a relatively minor (2.5 – 3-fold) loss of sensitivity to pentamidine and MPAs;\textsuperscript{15} a similar level of MPA resistance was observed in the aqp2 null and aqp2/aqp3 null lines, which do retain a wild-type copy of TbAT1. Although the data presented here and by Baker et al.\textsuperscript{20} show that the deletion of TbAQP2 alone is sufficient to give a strong pentamidine resistance phenotype in vitro (17.5-fold; Figure 2), it is worth exploring this in detail. Firstly, TbAT1/P2 activity is very low in cultured \textit{T. b. brucei} bloodstream forms, compared with expression \textit{in vivo};\textsuperscript{15} dramatically reducing the influence of this transporter on drug sensitivity. Secondly, the pH1336 vector used to reintroduce TbAQP2 in B48 and P1000 gives a very robust expression level\textsuperscript{37} well above that in the wild-type, which helps explain the complete reversal of MPA/pentamidine resistance even in the absence of TbAT1. Finally, the reintroduction of TbAT1 in B48 in the same vector also led to an almost complete reversal of resistance to pentamidine, Cymelarsan and diminazene, even in the continued absence of a wild-type TbAQP2.\textsuperscript{58}

The conclusion from the above must be that loss of either TbAQP2 or TbAT1 activity can lead to some loss of MPA and pentamidine susceptibility, but that the high MPXR phenotype is the result of both being lost concomitantly. B48, which lacks both genes, is more resistant to pentamidine and MPAs than either aqp2 null or the TbAT1 knockout strain from which it was derived. Thus, the combined data strongly suggest that TbAQP2 is the previously described HAPT1 and transports MPAs and pentamidine. A number of observations reported in the present article strongly support this notion: (i) the \textit{T. b. brucei} strains B48, P1000 and 247MR have all lost HAPT1 activity and also lack a wild-type copy of TbAQP2; (ii) targeted deletion of TbAQP2 specifically removes the high-affinity pentamidine transport component, leaving the low-affinity transport activity unchanged; (iii) expression of TbAQP2 in B48 restores HAPT1 activity; (iv) expression of TbAQP2 in \textit{L. mexicana} causes a massive sensitization to pentamidine and Cymelarsan; and (v) the introduction of a high-affinity pentamidine transporter that is indistinguishable from HAPT1 by \textit{Km}, value and inhibitor profile. Thus, the evidence overwhelming supports a model that TbAQP2 mediates the saturable uptake of pentamidine and MPAs, following standard Michaelis–Menten kinetics, in addition to its more conventional function as a water/glycerol channel.

As discussed by Baker et al.,\textsuperscript{20} TbAQP2 has a number of unusual residues in the motifs that are believed to be involved in selectivity: NPS/IVLL is replaced with the classical regions of NPA/IGYR in the chimera. To begin to unravel the structural determinants of AQP2 action, we synthesized two new genes, with the NPS/IVLL motif transplanted to either chAQP2/3 or TbAQP3, and expressed these constructs in an aqp2/aqp3 null strain. The resulting cell lines displayed slightly higher pentamidine sensitivity than the control and the cells expressing chAQP2/3\textsuperscript{315} showed a substantial sensitization to Cymelarsan. It follows that the usual selectivity filter of TbAQP2 does indeed contribute to its drug transport capacity, but is not the sole determinant.

In summary, we have shown that an aquaglyceroporin, TbAQP2, mediates the saturable uptake of some first-line trypanocides, Cymelarsan and pentamidine, compounds of substantially higher molecular weight than has so far been reported for any AQP. The mechanism by which this MIP transports these drugs with Michaelis–Menten kinetics remains to be investigated.

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### Transparency declarations

None to declare.

### Supplementary data

Table S1 and Figures S1–S7 are available as Supplementary data at \textit{JAC} Online (http://jac.oxfordjournals.org/).

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