

Original Paper

Trypanosoma Brucei Aquaglyceroporins Facilitate the Uptake of Arsenite and Antimonite in a pH Dependent Way

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Key Words

Aquaporins • Metalloids • Trypanosomes • As(III) • Sb(III)

Abstract

Background: *Trypanosoma brucei* is a primitive parasitic protozoan that thrives in diverse environments such as the midgut of the tsetse fly and the blood of a mammalian host. For an adequate adaptation to these environments, the parasite's aquaglyceroporins play an important role. **Methods and Results:** In order to test their ability to transport trivalent arsenic and antimony, we expressed the three known *Trypanosoma brucei* aquaglyceroporins (TbAQPs) in the heterologous systems of yeast null aquaporin mutant and *Xenopus laevis* oocytes. For both expression systems, we found a pH dependent intracellular accumulation of As(III) or Sb(III) mediated by all of the three TbAQPs, with the exception of TbAQP1-As(III) uptake. Additionally, we observed that *Trypanosoma brucei* aquaglyceroporins allow the passage of As(III) in both directions. **Conclusion:** Taken together, these results demonstrated that *T. brucei* aquaglyceroporins can serve as entry routes for As(III) and Sb(III) into the parasitic cell, and that this uptake is pH sensitive. Therefore, aquaporins of protozoan parasites may be considered useful as a vehicle for drug delivery.

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Introduction

In tropical and subtropical areas of the world, members of the *Trypanosomatidae* family cause diseases, and thus represent an important cause of human mortality and morbidity [1]. The more important of these diseases are African sleeping sickness, Chagas disease, and leishmaniasis caused by *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania*, respectively [1, 2]. Therapeutic options to treat these diseases are remarkably limited and most of them unsatisfactory, as they have severe side effects and show variable efficacy. Nowadays, arsenic and antimony, although toxic compounds, are still the chemical bases for drugs commonly used against *Leishmania* and *T. brucei* [1].

Leishmaniasis in particular is treated with drugs containing pentavalent antimony [Sb(V)] (Pentostam and Glucantime) whose active form is antimonite [Sb(III)]. For human African sleeping sickness there are few drugs available and out these, eflornithine in combination with nifurtimox, is an effective therapy. However, this treatment, in some cases, has a reduced efficacy due to the weak effect of eflornithine against *Trypanosoma brucei rhodesiense* [1, 3]. Melarsoprol (Arsobal®, Aventis), a highly toxic trivalent organic derivative of arsenic, is one of the few drugs which is effective against both species of *Trypanosoma brucei* (*T. b. rhodesiense* and *T. b. gambiense*), and particularly effective against the chronic stages of the disease [4].

Interestingly, membrane proteins belonging to the aquaporin family, i.e. channels that facilitate the transport of water (orthodox aquaporin), and small non-charged solutes including glycerol (aquaglyceroporins) have been shown to modulate the activity of drugs whose active ingredients are As(III) or Sb(III). *Leishmania major* aquaglyceroporin 1 (LmAQP1) is responsible for Sb(III) and As(III) uptake [5], and a down regulation of LmAQP1 is associated with drug resistance [6]. In *T. brucei*, although it is well known that melarsoprol enters the cell through both the P2 (adenosine transporter) and HAPT1 (high-affinity Pentamidine transporter), aquaglyceroporin 2 plays an important role in mediating the susceptibility to melarsoprol and pentamidine treatment [3, 7, 8]. However, hitherto it was not known whether TbAQPs were permeable to any arsenical compound but considering their pore size, it seems rather unlikely that drugs like melarsoprol could pass through. Although in many organisms As(III) and Sb(III) are taken up by aquaglyceroporins, it has been clearly demonstrated that not all aquaglyceroporins carry out this function [9]. Here, using two different heterologous expression systems (yeast and *Xenopus* oocytes), we measured the Sb(III) and As(III) facilitated uptake by the TbAQPs, thus providing direct evidence that TbAQPs are arsenic and antimony channels and that the uptake of these metalloids is pH dependent.

Material and Methods

Ethics Statement

This work was performed in accordance with the Guide for the Care and Use of Laboratory Animals recommendations of the National Institutes of Health. The protocol was approved by the Wayne State University Animal Investigation Committee. All efforts were made to minimize suffering; surgery was performed under benzocaine anesthesia, and this compound was also used for animal euthanasia.

Maintenance of organisms

Saccharomyces cerevisiae: Cells were grown in a yeast peptone medium containing 2% D-glucose (rich medium) or in a synthetic medium for their selection consisting of complete medium (CM) without uracil (selection marker) [10]. Solid medium was prepared using 2% agar.

Xenopus laevis oocytes: The oocytes obtained by surgery were defolliculated and manually culled for cells from stages V-VI. Next they were maintained in ND96 solution for 24 h at 16°C with continuous shaking until cRNA was injected [11].

Parasites: *T. brucei* bloodstream forms were cultivated in modified minimum essential medium at 37°C in a 5% CO₂ atmosphere as described previously [12].

Heterologous expression in S. cerevisiae and phenotype experiments

In this study the yeast strain BY4742 (MAT α ; his3 Δ 1; leu2 Δ 0; lys2 Δ 0; ura3 Δ 0; YLL043w::kanMX4) from Euroscarf (Frankfurt, Germany) was transformed with the following constructs: pRS416/TbAQP1, pRS416/TbAQP2, and pRS416/TbAQP3 [13]. Expression of TbAQPs was induced by removing methionine from the culture medium (MET17- inducible promoter). BY4742 transformants were grown in selection medium (CM without uracil) at room temperature and shaking at 160 rpm overnight, and then transferred to selection medium plates containing arsenic V, arsenic III, or antimony. Control plates lacked these compounds. Drop tests on agar plates were performed from 1/10¹ to 1/10⁵ dilutions using overnight cultures adjusted to identical cell densities [13].

Transport of arsenic and antimony by TbAQP1, TbAQP2, and TbAQP3 expressed in Xenopus laevis oocytes

For expression in oocytes, 10 ng of cRNA were injected into each oocyte and after three days, when the highest expression level was reached, metalloid uptake assay was performed as described by Figarella et al., 2007 [14]. Briefly, five oocytes were incubated for 90 s at room temperature with either 1 mM As(III) or Sb(III) in ND96 buffer. After washing, oocytes were transferred to separate test tubes and digested with concentrated (70%, inductively-coupled plasma (ICP)-GRADE) HNO₃ for 1 h at 70°C. Each sample was diluted with HPLC grade deionized water to a final concentration of 3% HNO₃ and the metalloid concentration was determined by inductively-coupled plasma mass spectrometry (ICP-MS).

Cytotoxicity assays

Cytotoxicity was assayed using concentration inhibitory 50 (IC₅₀, the concentration necessary to inhibit cell growth by 50%). *Trypanosoma brucei* bloodstream form parasites were collected from *in vitro* cultures during the exponential growth phase and diluted to 2 x 10⁵ cells per ml, placed in a 96-well plate and grown with or without the following metalloids: As(III) and As(V) in the form of sodium arsenite and arsenate, and Sb(III) in the form of potassium antimonite tartrate, at different concentrations. Plates were incubated at 37°C in a 5% CO₂ for 24 h. To determine the percentage of dead cells, growth was stopped and phosphatase activity was measured as described by Bodley et al. 1995 [15].

Results*TbAQPs complement the trivalent arsenic and antimony resistance phenotype in an fps1 Δ strain of S. cerevisiae*

Since the Fps1p channel is responsible for trivalent metalloid uptake, yeast strains lacking this gene show a resistant phenotype to As(III) and Sb(III) [16]. In order to investigate whether TbAQPs can replace Fps1p in conferring trivalent metalloid sensitivity, we transformed fps1 Δ mutant cells with each one of the three TbAQPs, or with the vector plasmid (control cells). Transformants were grown in selective medium and then spotted onto agar plates containing serial dilutions of As(III), As(V) or Sb(III). As shown in Figure 1, different from control cells, TbAQPs-transformed fps1 Δ cells were highly sensitive to As(III) and Sb(III). Since the only difference between control cells and TbAQP-transformants is the gene inserted in the vector, we concluded that the enhanced sensitivity to trivalent metalloids is due to the complementation of Fps1p function by TbAQPs, and therefore the metalloid uptake in *T. brucei* should be mediated by these channels. On the other hand, when TbAQPs-transformed fps1 Δ yeast and control cells were exposed to As (V), the respective phenotypes were inverted, i.e. the transformed cells became resistant and control cells were sensitive. This improved survival of mutants expressing TbAQPs can be explained by the arsenic metabolism in yeast: As(V) (arsenate) is taken up by the yeast phosphate transporter Pho87p, and once inside the cell, it is reduced to arsenite by the redox enzyme Acr2p, and finally secreted by aquaglyceroporins along the arsenite concentration gradient [17-19]. In contrast, control cells accumulated As(III) and died because they do not have an efficient pathway to get rid of the metalloid. This phenotype confirms and complements the former experiments indicating that TbAQPs were able to participate in the efflux of As(III).

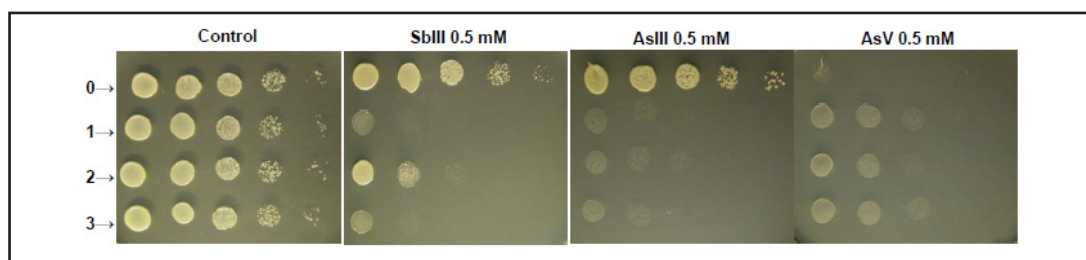


Fig. 1. Phenotypes of *fps1Δ* mutant cells expressing TbAQPs in the presence or absence of metalloids. Yeast cells were pre-grown in selective liquid and the cell density adjusted to 1 O.D. Thereafter, they were transferred in a tenfold serial dilution onto selective-induction agar medium without any metalloids (control condition) or containing 0.5 mM of Sb(III), As(III), or As(V). Growth was documented after 3 days at 30°C. In each panel: 0, control cells containing the empty vector; 1, TbAQP1; 2, TbAQP2; 3, TbAQP3.

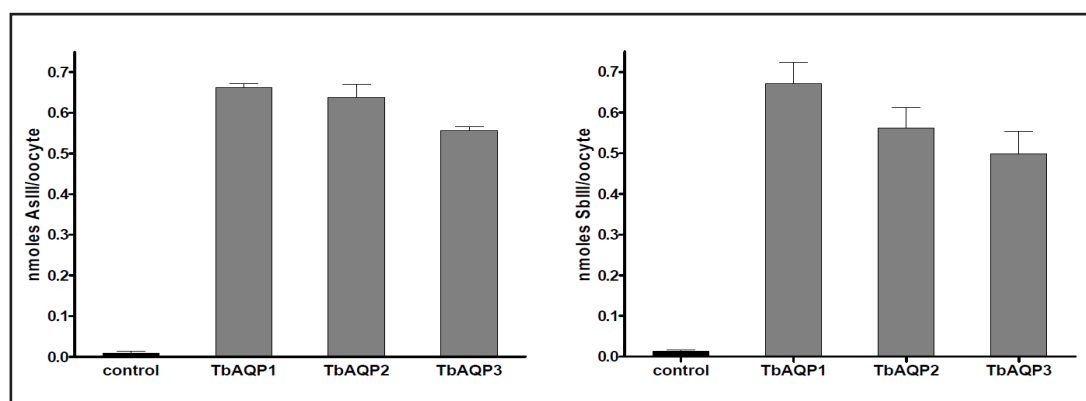


Fig. 2. As(III) and Sb(III) permeability in *Xenopus laevis* oocytes expressing TbAQP1-3. Oocytes were injected with water (control) (black bar) or 10 ng of TbAQP1, 2 or 3 – cRNA (grey bars). After three days permeability experiments were achieved. Graphics represent measurements performed at least in triplicate \pm SD.

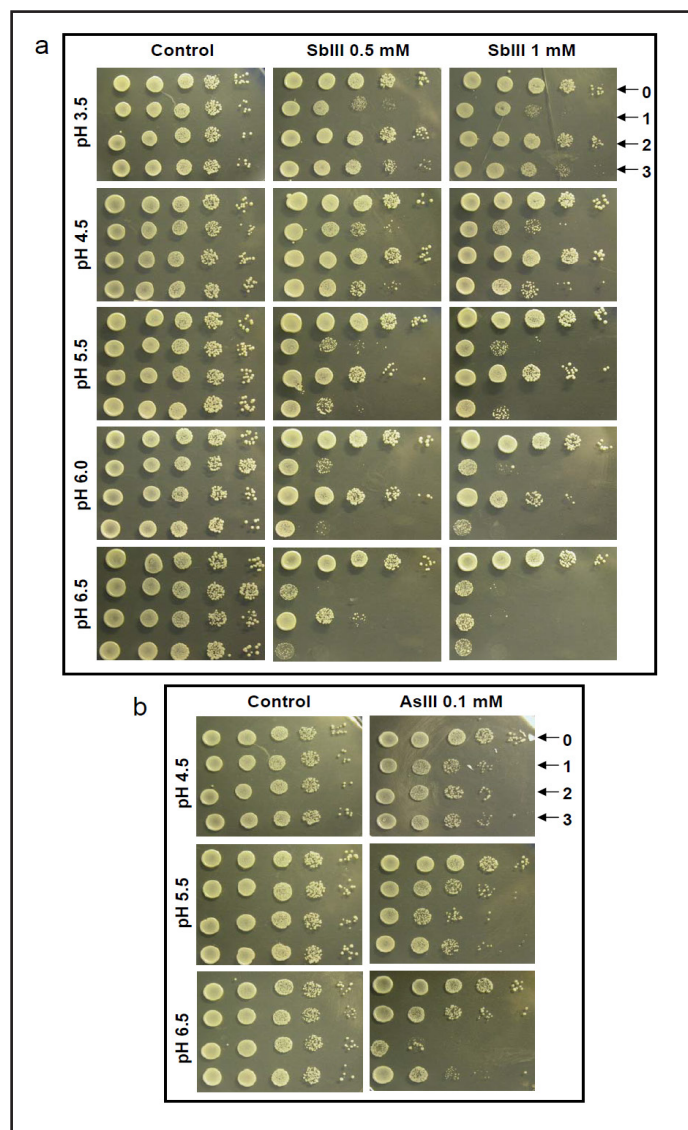
TbAQPs expressed in Xenopus laevis oocytes take up As(III) and Sb(III)

We further characterized As(III) and Sb(III) transport in *Xenopus laevis* oocytes where recombinant TbAQPs were expressed, by measuring the permeability profiles of these metalloids. Uptake of both As(III) and Sb(III) at a concentration of 1 mM each was monitored in TbAQP1-3 expressing oocytes over a 90 seconds period, the control experiment consisted of water injected oocytes. As shown in Figure 2, control oocytes showed a negligible As(III) or Sb(III) uptake (0.006 ± 0.001 nmol/oocyte and 0.013 ± 0.008 nmol/oocyte, respectively). On the other hand, oocytes expressing TbAQP 1, 2 or 3 showed a 109-fold, 112-fold, or 91-fold accumulation of As(III) respectively when compared with the control. Similar results were obtained for Sb(III) with a 52-fold, 46-fold, or 44-fold increase. These results clearly demonstrate that the three *T. brucei* aquaglyceroporins act as metalloid channels.

Effect of pH on metalloids uptake in S. cerevisiae TbAQPs transformants

Ramírez-Solís et al. have demonstrated that at neutral pH the most stable structure for As(III) is $\text{As}(\text{OH})_3$, thus apparently, the ionic As atom by itself is not present in solution. However, variations in pH cause significant structural changes in the coordination geometry of As(III) [20]. Since these changes in spatial geometry could affect the passage of these compounds through the channel's pore, we decided to analyze whether changes in pH could modify the ability of TbAQPs to transport As(III) and Sb(III). Accordingly, yeast TbAQP-transformants were cultured on agar plates containing As(III) or Sb(III) at different pHs. As shown in Figure 3 in the presence of 0.5 or 1 mM of Sb(III) the three yeast TbAQPs transformants displayed a moderate growth at pH of 3.5, but when the pH was raised to

Fig. 3. As(III) and Sb(III) pH-dependent cytotoxicity in *fps1Δ* mutant cells expressing TbAQPs. Yeast mutants expressing or not expressing TbAQP1, 2, and 3 were spotted in 10-fold dilution steps onto selective-induction agar medium. Media containing As(III) or Sb(III) was prepared at different pH from 3.5 to 6.5. Control condition does not have any metalloids. Proliferation was recorded 3 days after incubation at 30°C. a) Grown in the presence of Sb(III). b) Grown in the presence of As(III). In each plate: control cells containing an empty vector (0); Cells expressing TbAQP1, TbAQP2, or TbAQP3 (1), (2), or (3), respectively.



6.5 (Fig. 3a) the sensitivity of these cells to Sb(III) increased steadily. Similar results were obtained for As(III) in TbAQP2 and 3 transformants but at a lower concentration (Fig. 3b). For TbAQP1-transformed *fps1Δ* mutant cell variations in pH did not change the transport rate for As(III). In general these results suggest that changes in the spatial structure of $\text{As}(\text{OH})_3$ and $\text{Sb}(\text{OH})_3$ at low pH decreased the permeability of TbAQPs to these metalloids. However, the fact that TbAQP1 did not show this phenotype revealed differences in aquaglyceroporin structures.

Effect of pH on the metalloids uptake in Xenopus laevis oocytes expressing TbAQPs

Next we tried similar experiments in *Xenopus laevis* oocytes expressing TbAQPs at pH 5.5 and 7. As shown in Figure 4a, in contrast with the control cells, oocytes expressing TbAQPs were able to transport Sb(III) at both 5.5 and 7 pH values, but the uptake at pH 5.5 was significantly reduced. This result is consistent with the previous observations with yeast transformants.

The effect of pH variations in the transport of As(III) shed different results for each TbAQP. For TbAQP3 the decrease in pH lowered the As(III) intake; for TbAQP2 a rather moderate though still statistically significant effect was registered; while for TbAQP1 virtually no effect was observed (Fig. 4b). These data are also consistent with the experiments in yeast.

Fig. 4. As(III) and Sb(III) pH-dependent permeability in *Xenopus laevis* oocytes expressing TbAQPs. Oocytes were injected with water (control) or with 10 ng of TbAQP1, 2 or 3 – cRNA. Upper panel represents Sb(III) uptake and lower panel denotes As(III) uptake. Graphics represent the mean \pm SD from at least three independent experiments. Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc, La Jolla, CA). Results with $p < 0.05$ were considered as statistically significant.

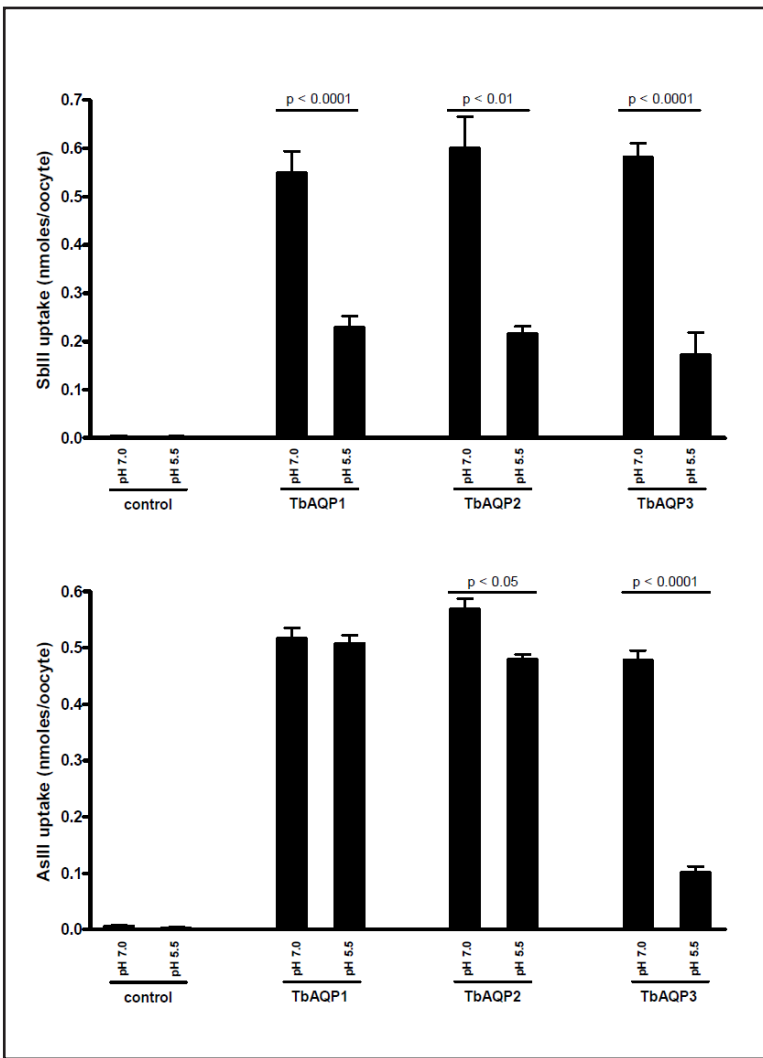


Table 1. IC₅₀ values for the different metalloids in *Trypanosoma brucei* bloodstream forms

Compound	IC ₅₀ in bloodstream (μM)
Arsenic V	7.0 ± 0.3
Arsenic III	0.31 ± 0.06
Antimony III	0.103 ± 0.01

Anti-proliferative effects of As(III), As(V) and Sb(III) on T. brucei

As(III) in the form of arsenic trioxide (Trisenox®), although very toxic and difficult to apply, is currently used against acute promyelocytic leukaemia being the most effective drug available against this disease [21]. With this in mind, we decided to evaluate As(III) cytotoxicity in trypanosomes. To this end, the IC₅₀ of the three metalloids here studied were determined as described in Material and Methods in *T.brucei* cell cultures. As shown in Table I, all arsenic and antimony compounds killed the parasite in the nM to μM range, the order of effectiveness being Sb(III) > As(III) > As(V).

Discussion

The *Trypanosoma brucei* bloodstream form possesses three aquaglyceroporins namely: TbAQP1 located at the flagellum, TbAQP2 in the flagellar pocket and TbAQP3 in the plasma membrane [7, 13, 22]. Using RNAi technology, Bassarak et al. demonstrated that after hypotonic shock these channels, in particular TbAQP2, participate in cell volume regulation. TbAQPs are also associated with drug uptake, as in the case of dihydroxyacetone, an innocuous compound for humans but cytotoxic for *T. brucei* which is very efficiently transported into the parasite [23, 24]. However, the ability of TbAQPs to uptake As(III) and Sb(III), which are basic components of several antiparasitic drugs against trypanosomatids, has not yet been tested. This fact, together with a recent report that in *T. brucei* TbAQP2 is associated with the toxic effects of the arsenical drug melarsoprol, [7], prompted us to investigate the trivalent arsenic transport in this parasite. Using two different protein expression systems, *Saccharomyces cerevisiae* and *Xenopus laevis* oocytes, we have shown that trypanosomal aquaglyceroporins are highly permeable for both trivalent arsenic and trivalent antimony. The heterologous expression of the three different *T. brucei* AQPs in yeast *fps1Δ* mutant cells promoted the uptake of As(III) and Sb(III) through these channels increasing the sensitivity of these cells to these metalloids, and provoking cell death [13].

Since heterologous expression experiments using aquaglyceroporins from different organisms including mammals (AQP9) [25, 26], plants (nodulin-26-like intrinsic proteins (NIP)) [27] and yeast (Fps1p deleted of the N-terminal regulatory domain) [16] have shed similar results, our findings strongly suggest that TbAQPs are responsible for the uptake of these metalloids compounds.

We also performed direct transport assays in *Xenopus laevis* oocytes expressing TbAQPs, and the results of these experiments were consistent with the phenotypes observed in yeast, that is, As(III) and Sb(III) were efficiently transported by the three TbAQPs, with an average of a 76 fold increase in metalloid uptake with respect to control cells.

TbAQPs transport activity expressed in both yeast and *Xenopus* oocytes systems was sensitive to pH changes, in the case of Sb(III) a similar and significant effect of pH was observed for all three AQPs. In the case of As(III) however, TbAQP2 and 3 showed a pH-dependent uptake, whereas TbAQP1 showed a pH insensitivity. Some explanations for these differences could be: A) the Sb(III) atomic weight is bigger than that of As(III) (122 and 75, respectively), therefore these differences in molecular volumes may influence the permeability rate through TbAQPs; and B) the differences in uptake are due to variations in TbAQPs protein structure e.g., differences in some important amino acid positions among TbAQPs. However the fact that the three important regions for substrate permeability (i.e. the conserved NPA motif, the selectivity filter -ar/R region-, and the remaining pore-lining residues) are practically identical for the TbAQP1 and 3 [13, 25], gives little support for this option. Nonetheless, other amino acids, not directly involved in pore formation, can significantly modify the pore's permeability. This was recently demonstrated for two Killifish aquaglyceroporins KfAQP3a and KfAQP3b, that although sharing 96% amino acid identity and showing no amino acid differences in the channel region, where the former does not allow the passage of As(III), while the later does. The only important structural discrepancy is located in the C-terminus, being Gly-Lys-Ser for KfAQP3a and Ala-Asn-Cys in KfAQP3b. When KfAQP3a C-terminal amino acid was changed to Ala-Asn-Cys by *in vitro* mutagenesis, the channel became permeable for trivalent arsenic [9]. There are other examples in the literature related to the connecting loops among AQP transmembrane domains, e.g. Glu125 in loop C of *Plasmodium falciparum* aquaglyceroporin (PfAQP) is responsible for a high capability of water transport that is largely abolished by changing this amino acid [28]. Likewise, mutation of Glu152 to Ala, within the same loop in Aquaglyceroporin 1 from *Leishmania* (LmAQP1), affected water as well as metalloid permeation, but not the glycerol transport [29]. These findings demonstrate that a fine-tuning of selectivity and capability for substrate transport are not exclusive of the classical three regions mentioned above, and that the three TbAQPs here studied present enough differences at their C-termini as well as

in their C loops to account for the observed differences in permeability [13]. However, given the low degree of amino acid conservation among the members of the aquaporin family, it is difficult to make predictions of permeability behavior based on sequence comparisons. Therefore, additional experimental data are required to reveal the structural determinants of this pH-depending selectivity for As(III) and Sb(III) observed in the TbAQPs.

As(III) and Sb(III) show prominent effects on parasites, killing them at a low nM to μ M range. In this regard the concentration of arsenic trioxide determined in plasma of Trisenox®-treated promyelocytic leukaemia patients is in the range of 300 - 2900 nM [30, 31], which is equal or tenfold higher than the IC_{50} values for this compound in *T. brucei* (Table 1). In addition Trisenox® is not only detected in blood but also in cerebrospinal fluid [32]. Since the treatment of sleeping sickness is still problematic, and melarsoprol, one of the most prominent drugs used for late stage infections, has deadly side effects in about 5% of treated patients [33], the potential of Trisenox® as an alternative drug to treat sleeping should be explored.

Taken together, we have found that *T. brucei* aquaglyceroporins can serve as entry routes for As(III) and Sb(III) into the parasitic cell, and that this uptake is pH sensitive. We also observed that TbAQPs are bi-directional channels for As(III). Finally, since TbAQP2 has been implicated in the drug resistance to the trypanocidal drugs melarsoprol and pentamidine [7, 8], and considering our results indicating the role of TbAQP in the uptake of As(III) drugs like Trisenox®, we believe these channels should be further studied in order to explore their potential as drug targets, drug delivery systems, and drug resistance cases in deadly infections such as sleeping sickness.

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