Drug resistance in *Trypanosoma brucei*: Comparative genomics of melarsoprol-pentamidine cross-resistance and the role of aquaglyceroporin 2 in clinical resistance

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Summary

Drug resistance in African trypanosomes had already been studied more than 100 years ago, by the pioneering work of Paul Ehrlich. The molecular mechanisms and most genes responsible for drug resistance, however, have not been discovered until recently. New technologies that allow genome-wide comparison were highly successful in identifying many new genes that were linked to drug resistance to all clinical trypanocides.

The overall aim of this thesis was to identify and validate candidate genes for drug resistance in *Trypanosoma brucei*. In Chapter 2, I applied next generation sequencing to find the mutations causing drug resistance in two bloodstream-form *T. b. rhodesiense* lines that had previously been selected *in vitro* for resistance against the clinical drugs melarsoprol and pentamidine, respectively. Both cell lines exhibited strong cross-resistance to either drug - a phenomenon first observed over 60 years ago and repeatedly many times - and nowadays the genes involved have been characterized. Comparative genomics revealed the deletion of the known melarsoprol-pentamdine cross-resistance (MPXR) determinants adenosine transporter 1 (*TbAT1*) in the melarsoprol-selected line and aquaglyceroporin 2 (*AQP2*) in both selected lines. The pentamidine-selected line had acquired a heterozygous point mutation (G430R) in *TbAT1* that rendered the transporter non-functional. The gene *TbAT1*, encoding the adenosine/adenine permease P2 transporter, has been discovered more than 10 years ago. AQP2 has recently been discovered to play a role in MPXR in a genome-wide RNAi screen. Both transporters mediate the uptake of melarsoprol and pentamidine and, when functionally lost, lead to cross-resistance. AQP2 emerged as the main genetic determinant of MPXR and corresponds to the high-affinity pentamidine transporter. Mutations in AQP2 were found in all analyzed MPXR cell lines selected, either *in vitro* or *in vivo*, with arsenicals or pentamidine and from all three *T. brucei ssp.* (Chapter 3). An additional mutation became fixed in both resistant cell lines; the RNA-binding protein *TbUBP1* carried the exact same coding point mutation (R131L). Overexpression of *TbUBP1 in T. b. brucei* led to a strong growth deficit whereas overexpression of the mutant did not, but

intriguingly, those cells became about 2-fold hypersensitive to pentamidine. The physiological function of *TbUBP1* and how it affects pentamidine sensitivity remains to be further investigated.

TbAT1 and *AQP2* are well studied in laboratory cell lines, but knowledge from clinical isolates is scarce. Chapters 4 and 5 investigate drug resistance in clinical isolates. 16 *T. brucei* ssp. field isolates, 8 stemming from melarsoprol treatment-refractory cases, that had been adapted to axenic *in vitro* cultivation have been genotyped for *TbAT1* and *AQP2* to test whether they carry mutations in either transporter and the drug sensitivities have been determined for melarsoprol, pentamidine and diminazene. Indeed, five *T. b. gambiense* isolates from the Democratic Republic of Congo and one isolate form South Sudan carried a deletion in the *AQP2* / *AQP3* locus leading to the formation of a chimeric gene between *AQP2* and *AQP3* and loss of wild-type *AQP2*.

The identified mutant *T. b. gambiense* isolates were 3- to 5-times less sensitive to melarsoprol and 40- to 50-fold less sensitive to pentamidine compared to reference isolates. Functional expression of the chimeric AQP in a *tbaqp2* null background did not restore the drug sensitivity but the introduction of the wild-type *AQP2* in one of the resistant *T. b. gambiense* isolates rendered the cells sensitive to melarsoprol and pentamidine, comparable to fully drug susceptible isolates. This proves that the loss of 'wild-type' AQP2 is the cause of melarsoprol-pentamidine cross-resistance in the *T. b. gambiense* isolate. Thus AQP2 may serve as a molecular marker for drug resistance in the field.

Table of abbreviations

aa	amino acid
AAT6	amino acid transporter 6
ABC	ATP-binding cassette
AP-1	adaptin complex-1
AQP	aquaporin
AT1	adenosine transporter 1
ATP	adenosine triphosphate
BLAST	basic Local Alignment Search Tool
BMEM	baltz-minimal essential medium
b	base(s)
bp	base pair(s)
bsf	bloodstream-form
CNS	central nervous system
DFMO	difluoromethylornithine
DNDi	drugs for neglected diseases initiative
DRC	Democratic Republic of the Congo
EMP70	endosomal membrane protein 70
ESAG	expression site associated gene
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	genomic DNA
GEPARD	genome pair – rapid dotter
GFP	green fluorescent protein
GLP-1	golgi/lysosomal protein 1
HAPT1	high-affinity pentamidine transporter
HAT	human African trypanosomiasis
HQ	high quality
indel	insertion/deletion
ISG75	invariant surface glycoprotein 75
kb	kilobase
LAPT1	low-affinity pentamidine transporter
LDL	low density lipoprotein

Mb	megabase
MEM	minimal essential medium
MFST	major facilitator superfamily transporter
MPXR	melarsoprol-pentamidine cross-resistance
MRP	multidrug resistance-associated protein
NMR	nuclear magnetic resonance
NT	nucleoside/nucleobase transporter
NTR	nitroreductase
ORF	open reading frame
PAGIT	post assembly genome improvement toolkit
PCR	polymerase chain reaction
PERL	practical extension and report language
RATT	rapid annotation transfer tool
RF	resistance factor
rpm	rotations per minute
RRM	RNA recognition motif
RT	room temperature
S_D	standard deviation
SEM	standard error of the mean
SLADD	spliced leader addition program
SLT	spliced leader trapping
SNP	single nucleotide polymorphism
ssp.	subspecies
STIB	Swiss Tropical Institute, Basel
TPM	tags per million
TREU	trypanosomiasis research Edinburgh University
UBH1	ubiquitin hydrolase
UBP1	uridine-rich binding protein
VSG	variant surface glycoprotein
WT	wild-type

Chapter 1

General Introduction

Drug resistance in *Trypanosoma brucei*

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Abstract

After over 100 years of research on the mechanisms of drug resistance in African trypanosomes, reduction of drug import has crystallized as the predominant cause of resistance. This was recognized by classical studies in the 1930's and substantiated by recent approaches implementing next generation sequencing and reverse genetics. Thanks to the genetic amenability of *Trypanosoma brucei*, several nutrient transporters were proven to play key roles in the uptake of – and susceptibility to – clinically used trypanocides. These include aquaglyceroporin 2 and the adenosine transporter 1 for melarsoprol and pentamidine, and the amino acid permease 6 for eflornithine. Loss-of-function mutations in such transporters are viable because of the high degree of redundancy in the nutrient import machinery of *T. brucei* bloodstream forms.

Keywords

Human African trypanosomiasis (HAT), sleeping sickness

Pentamidine, melarsoprol, suramin, eflornithine, nifurtimox

Transport

Cross-resistance

Aquaporin, aquaglyceroporin

1. Introduction

Drug resistance in African trypanosomes has been studied for more than 100 years. Indeed, it was with trypanosomes that the phenomenon of drug resistance was described for the first time. Experimenting with trypanosomes, Paul Ehrlich (1854 - 1915) observed that he could select for resistance by sublethal exposure to drugs, and he went on to define complementary groups based on patterns of cross-resistance. Ehrlich proposed to use these laboratory-selected drug-resistant lines to phenotypically classify newly identified trypanocides (Ehrlich 1907). Since these pioneering studies, African trypanosomes have remained model organisms to study the mechanisms of drug action and drug resistance. More recently, the amenability of *Trypanosoma brucei brucei* to reverse genetic engineering has boosted molecular approaches. Thus *T. brucei* are on the one hand fascinating organisms that continue to provide new insights into the biology of eukaryotes, on the other hand they are lethal pathogens of tropical Africa.

Sleeping sickness, also known as African Human Trypanosomiasis (HAT), is caused by the two human-pathogenic subspecies of *T. brucei*: *T. b. rhodesiense* in East-Africa and *T. b. gambiense* in West-Africa. To date, 98% of cases are due to *T. b. gambiense* (Franco et al. 2014). Infections by *T. b. rhodesiense* lead to a more acute form of the disease which progresses within weeks to months to the 2nd stage, when the trypanosomes have infected the central nervous system (CNS). *T. b. gambiense* infections are more chronic and can take years until the CNS is involved. Symptoms during the hemolymphatic first stage include fever, swollen lymph glands, muscle and joint pains, and headaches. In stage two, neurological symptoms occur; including change of personality, confusion, slurred speech, seizures, difficulties in walking and talking, and alteration of the circadian rhythm leading to disrupted sleeping patterns and coma. Untreated HAT, East- or West-African form, is a fatal disease (Brun et al. 2010).

Trypanosomes of the *brucei* complex do not naturally occur outside of Africa since they strictly depend on the tsetse fly (*Glossina* spp.) for transmission. Tsetse flies are viviparous with a low reproductive number, which explains why they have never invaded other continents. Given its confinement to tropical Africa, HAT does not represent an attractive market for drug development. Nevertheless

there is reason for optimism that new drugs for HAT will soon be available (Mäser et al. 2012). Thanks to public-private partnerships and product-development partnerships such as the Drugs for Neglected Diseases Initiative DNDi, new drugs are being tested in clinical trials and further candidates are in development. Better and safer drugs for the treatment of HAT are needed urgently since the current armory is limited, the drugs are toxic and have unfavorable pharmacological properties.

2. Drugs for HAT

The treatment of HAT fully relies on chemotherapy. A vaccine is not available and the prospects for a vaccine are dim due to the fact that the bloodstream-form trypanosomes constantly undergo antigenic variation. The drugs presently used for the treatment of sleeping sickness are pentamidine and suramin for the first, hemolymphatic stage of the disease (Figure 1), and melarsoprol and nifurtimox/eflornithine combination therapy (NECT) for the second stage (Figure 1), when the trypanosomes have invaded the cerebrospinal fluid. These drugs are dated, impractical and toxic. In particular the chemotherapy of late stage sleeping sickness is problematic. Melarsoprol can cause severe and frequently fatal encephalopathies whereas the less toxic eflornithine must be administered intravenously by infusion, a logistic nightmare in the remote and crisis-shaken areas that are afflicted by sleeping sickness. Besides good tolerability, the target-product profile for a new drug therefore includes oral bioavailability, heat stability, brain permeability, and ideally single-dose cure. Except for the last, these criteria are fulfilled by the two molecules that are currently in clinical development: fexinidazole in phase II/III and SCY-7158 in phase II. Both are untypical molecules for modern drug candidates with moderate *in vitro* activity, and yet they are highly effective *in vivo*. Fexinidazole is a nitroimidazole that was originally developed by Hoechst as an antibiotic (Torreele et al. 2011). It has a positive Ames test but was later on shown not to be mutagenic to mammalian cells (Tweats et al. 2012). Fexinidazole and its active metabolite fexinidazole sulfone have *in vitro* IC_{50} values of around 1 µM against *T. brucei spp*. (Kaiser et al. 2011). Fexinidazole given twice daily at 100 mg/kg for 5 days cures the chronic *T. brucei mouse* model (Kaiser et al.

2011). To humans it is administered by daily oral dose over ten consecutive days. SCY-7158 is a benzoxaborole from Anacor Pharmaceuticals (Palo Alto, CA), a company specialized in organic boron chemistry. SCY-7158 cures the chronic *T. brucei* mouse model at a daily oral dose of 25 mg/kg for seven consecutive days (Nare et al. 2010; Jacobs et al. 2011). Further drug development projects are under way and the pipeline for HAT is, finally, in good shape (Brun et al. 2011). However, until new drugs will be available the current ones need to be used in a sustainable way. This requires an understanding of the molecular mechanisms underlying drug resistance.

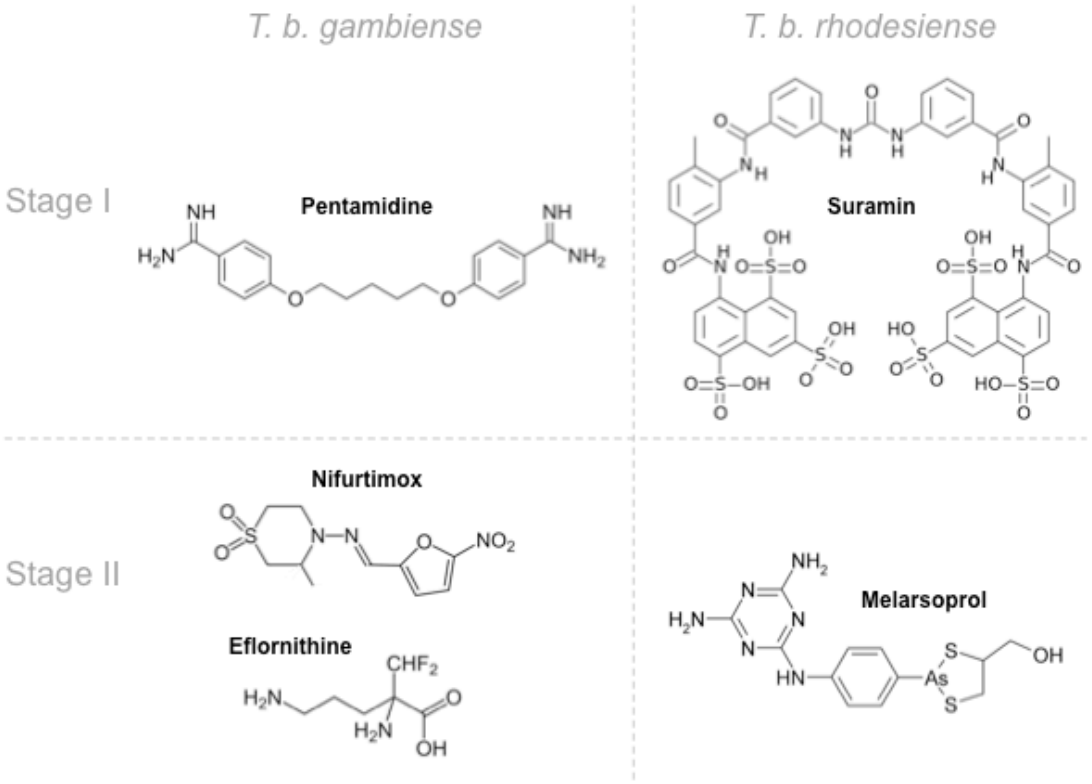

Figure 1. Structures of the currently used drugs and their field of application in the treatment of HAT.

3. New technologies to study drug resistance

The non human-pathogenic *Trypanosoma brucei brucei* has become a model organism for eukaryote microbiology. The trypanosomes can be cloned and cultivated axenically, and they are amenable to reverse genetic manipulation such as gene knock-out by homologous recombination or gene knock-down by RNAi-mediated silencing (Wirtz et al. 1999). Furthermore, *T. brucei* genes do not contain introns and all mRNAs carry a conserved 5' leader sequence spliced in-trans (Walder et al. 1986), which facilitates applications such as full length cDNA cloning or transcriptional start prediction. The first *T. brucei* ssp. genome was sequenced in 2005 (Berriman et al. 2005) and by now several are available (www.tritrypdb.org). In addition to the general breakthroughs in DNA sequencing and omics technologies, a number of new approaches were developed that are targeted specifically towards *T. brucei*. These include the spliced leader-trapping protocol for RNA-Seq (Nilsson et al. 2010) and genome-wide RNAi libraries for inducible expression of small interfering RNAs in the trypanosomes themselves (Baker et al. 2011; Schumann Burkard et al. 2011). Screening of such RNAi libraries enhanced the understanding of trypanosome biology (Alsford et al. 2011; Mony et al. 2014) and proved to be particularly useful to study drug action, by selecting small interfering RNAs that cause drug resistance by down-regulating a gene product necessary for drug activity (Baker et al. 2011; Schumann Burkard et al. 2011; Alsford et al. 2012; Alsford et al. 2013b; Gould et al. 2013). These technologies combined with next generation sequencing approaches have greatly enhanced our understanding of the molecular mechanisms of drug resistance in *T. brucei*.

4. Mechanisms of drug resistance

In the following, we shall briefly introduce the drugs that are presently used to treat HAT (Figure 1) and concentrate on the molecular mechanisms of drug resistance as they have been investigated in laboratory strains of *T. b. brucei*.

4.1 Melarsoprol-pentamidine cross-resistance

Pentamidine, in clinical use since the 1930s, is an aromatic diamidine mainly used to treat first-stage *T. b. gambiense* infections (Figure 1). Melarsoprol is a melaminophenyl arsenical and the only drug that cures *T. b. gambiense* as well as *T. b. rhodesiense* in second-stage infections (Figure 1). Organic arsenicals and diamidines are among the oldest classes of trypanocides. The mechanisms of resistance to these agents were studied in classical experiments by Frank Hawking (1905-1986; father of the physicist Stephen Hawking) and co-workers, who used bioassays with post-incubation media, chemical quantification of arsenite in trypanosomes, and the intrinsic fluorescence of the diamidine stilbamidine to measure drug uptake in bloodstream-form trypanosomes. These experiments demonstrated that resistant trypanosomes absorbed less drugs than susceptible ones (Yorke et al. 1931; Hawking 1936; Hawking and Smiles 1941). A second important piece of information to the puzzle of trypanocide resistance was delivered by Williamson and Rollo, who discovered the phenomenon of cross-resistance between melamine-based arsenicals and diamidines (Rollo and Williamson 1951; Williamson and Rollo 1959). Melarsoprol-pentamidine cross-resistance (MPXR) has since been described by several different labs (Frommel and Balber 1987; Pospichal et al. 1994; Scott et al. 1996; Bernhard et al. 2007; Graf et al. 2013), and it has become the most extensively studied case of drug resistance in *T. brucei*. Melarsoprol and pentamidine have only little structural similarity (Figure 1), and yet the two drugs share common transport systems for uptake into trypanosomes. The first such transport system to be identified was a purine permease termed P2 that imports adenine, adenosine, melamine-based arsenicals and diamidines (Table 1). P2 was found to be absent in MPXR *T. brucei* (Carter and Fairlamb 1993; Carter et al. 1995). The gene encoding P2 was subsequently identified in a functional screen for growth on adenosine in a purine-auxotrophic

mutant of *Saccharomyces cerevisiae* and named *TbAT1* for adenosine transporter 1 (Mäser et al. 1999). Mutations in *TbAT1* were detected in MPXR *T. brucei*, in lab-selected lines as well as in field isolates (Mäser et al. 1999; Matovu et al. 2001; Kazibwe et al. 2009; Stewart et al. 2010). Substrate binding motifs were mapped based on the functional characterization of P2 in *T. brucei* (de Koning and Jarvis 1999; Lüscher et al. 2006a). Two additional nucleoside/nucleobase transporters (de Koning et al. 2005; Al-Salabi et al. 2007), TbNT11 (also named AT-A) and TbNT12 (also named AT-E), were shown to transport pentamidine when expressed in *Xenopus laevis* oocytes or in *Leishmania* mutants that were deficient in nucleobase or nucleoside uptake (Ortiz et al. 2009). Melarsoprol inhibited adenine uptake via TbNT11 but was not a substrate itself (Ortiz et al. 2009). To what proportion these two transporters contribute to pentamidine uptake in bloodstream-form *T. brucei,* and whether functional loss of either transporter leads to pentamidine resistance, remains to be investigated.

A second transporter involved in MPXR has recently been identified, the aquaglyceroporin TbAQP2. This was achieved with RNAi library screens for melarsoprol or pentamidine resistance (Alsford et al. 2012; Baker et al. 2012; Alsford et al. 2013b). Aquaporins are water channels and were discovered in the early 1990's in human red blood cells (Preston et al. 1992) and later found in all kingdoms of life (King et al. 2004). *T. brucei* possess three aquaglyceroporins (TbAQP1-3) which transport, in addition to water, glycerol and other small neutral solutes (Uzcategui et al. 2004). Homozygous deletion of *TbAPQ2* in *T. brucei* bloodstream forms caused 2-fold resistance to melarsoprol and 16-fold resistance to pentamidine; reintroduction of *TbAQP2* into the knock-out cells restored sensitivity (Baker et al. 2012). Expression of *TbAQP2* in *Leishmania mexicana* promastigotes increased their sensitivity to pentamidine and melarsoprol by factors of 40 and 1000, respectively (Munday et al. 2014). An involvement of aquaglyceroporins in resistance to arsenite and antimonite had also been shown for tumor cells (Liu et al. 2002), *Arabidopsis thaliana* (Isayenkov and Maathuis 2008; Kamiya et al. 2009) and *Leishmania* (Gourbal et al. 2004; Marquis et al. 2005; Mandal et al. 2010). However, while TbAQP2 also transports As(III) and Sb(III) (Uzcategui et al. 2013), its role in MPXR (Munday et al. 2014) is, to our

knowledge, the first demonstration of an aquaglyceroporin involved in transport of larger organic molecules (Table 1). By now mutations in *TbAQP2* have been detected in many MPXR lines of *T. brucei* ssp., lab strains selected *in vitro* or *in vivo* (Munday et al. 2014) as well as field isolates (Graf et al. 2013).

Another candidate gene for melarsoprol resistance is *TbMRPA* (multidrug resistance-associated protein A), a member of subfamily C of the superfamily of ATP-binding cassette (ABC) transporters. In tumor cells MRP proteins can mediate multidrug resistance by actively exporting drug-glutathione conjugates (Keppler et al. 1997). TbMRPA is thought to export MelT, the conjugate of melarsoprol to trypanothione (Fairlamb et al. 1989). Trypanothione is a biochemical peculiarity of trypanosomatids: two glutathione tripeptides covalently linked via the polyamine spermidine (Fairlamb et al. 1985; Fairlamb and Cerami 1992). While the role of MRPA orthologues in drug efflux has been extensively studied in *Leishmania* (Ouellette et al. 1990; Ouellette et al. 1998; Legare et al. 2001; Mukherjee et al. 2007; Leprohon et al. 2009), their function in *T. brucei* is less clear. Overexpression of *TbMRPA* in *T. brucei* led to an about 10-fold increase in melarsoprol resistance *in vitro* (Shahi et al. 2002) but not *in vivo* (Alibu et al. 2006). Overexpression of *TbMRPA* in *tbat1*$^{-/-}$ cells showed that the two resistance mechanisms, reduced drug influx and increased drug efflux, were strictly additive (Lüscher et al. 2006b).

4.2 Suramin resistance

The sulphated napthylamine suramin (Figure 1) is the product of what was one of the first medicinal chemistry programs. Starting from the dyes trypan red and trypan blue, which Paul Ehrlich had shown to be trypanocidal, suramin ('Bayer 205') was synthesized as a colorless, antitrypanosomal derivative by Bayer in 1916. It is the oldest drug in use against HAT and still the drug of choice against first stage *T. b. rhodesiense* infections. Suramin is an intriguing molecule of manifold applications. Besides HAT, suramin has been tested also for river blindness (Anderson and Fuglsang 1978), various cancers (McGeary et al. 2008), candidiasis (Braga-Silva et al. 2007), autism (Naviaux et al. 2013), AIDS (Agarwal et al. 2012), and as an experimental compound in developmental biology (Oschwald et al. 1993) - and yet its modes of action are not fully understood.

Suramin is negatively charged at physiological pH which prevents it from crossing the plasma membrane by passive diffusion, as well as from crossing the blood-brain barrier. The molecule is very large for a drug (1297 Da). Over 99% is bound to plasma proteins, and the half-life of elimination is extremely long (44-54 days in human plasma). Suramin uptake into trypanosomes was proposed to occur via receptor-mediated endocytosis after binding to the low-density-lipoprotein (LDL) (Vansterkenburg et al. 1993).

Genome-wide RNAi screening for suramin resistance in *T. brucei* confirmed the endocytotic uptake route as several genes from this pathway emerged as hits (Alsford et al. 2012). One of the main determinants required for suramin activity was the invariant surface glycoprotein ISG75 (Figure 2), supporting the model that suramin is imported via endocytosis and that ISG75 is the suramin receptor on the trypanosomes' surface (Alsford et al. 2013a). Other downstream genes such as the four subunits of the adaptin complex-1 (AP-1), the Golgi/lysosomal protein-1 (GLP-1), the endosomal membrane protein 70 (EMP70), the major glycosomal protein p67, cathepsin-L and the major facilitator superfamily transporter (MFST) were all linked to the endocytotic pathway. RNAi-mediated knock-down in bloodstream-form *T. brucei* of these genes led to a reduced suramin sensitivity *in vitro*, with the highest resistance factor (>10x) for MFST. In addition, ubiquitin hydrolase (UBH1) was identified as a hit in the RNAi screen and knock-down led to reduced suramin sensitivity. This was likely an indirect effect as ISG75 is a transmembrane protein whose internalization relies on the ubiquitination of cytoplasmic lysine residues (Leung et al. 2011). Two *T. brucei* lines that had been independently selected for suramin resistance *in vitro* subsequently lost their resistance phenotype when transformed into the insect (procyclic) stage (Scott et al. 1996). This is in agreement with an involvement of ISG75 in suramin susceptibility because expression of ISG75 is bloodstream-form specific (Ziegelbauer et al. 1992).

4.3 Nifurtimox resistance

Nifurtimox is orally bioavailable and one of the two frontline drugs for Chagas' disease, in use for more than 40 years. Since 2009, nifurtimox - although not very

active alone - is also applied against human African trypanosomiasis in combination with eflornithine (Priotto et al. 2009; Yun et al. 2010). Nifurtimox-eflornithine combination therapy (NECT) for HAT has been included in the WHO's Model List of Essential Medicines. Nifurtimox is a nitrofuran (Figure 1) that functions as a prodrug. The activation is enzymatically mediated by reduction of the nitro group. In *T. brucei* and *T. cruzi*, a bacterial-like type I nitroreductase (NTR) activates the prodrug by two consecutive reductions (Wilkinson et al. 2008). NTR is an NADH-dependent enzyme localized in the mitochondrion. Mammalian genomes do not contain a NTR orthologue. Resistance to nifurtimox and other nitroheterocyclic drugs has been attributed to a reduction of NTR activity (Wilkinson et al. 2008). *T. cruzi* epimastigotes selected for nifurtimox resistance *in vitro* lost a copy of NTR (Wilkinson et al. 2008; Mejia-Jaramillo et al. 2011). Gene knock-out experiments confirmed the crucial role of NTR in nifurtimox resistance as well as cross-resistance to benznidazole, a related nitroimidazole and the second drug in clinical use against *T. cruzi* infections. Heterozygous deletion of the *NTR* gene in *T. brucei* rendered the cells nifurtimox-resistant whereas ectopic over-expression of *NTR* caused hypersensitivity (Wilkinson et al. 2008). Homozygous deletion of *NTR* in *T. brucei* was only achieved when the expression of the ectopic copy had been induced, indicating that NTR is essential for bloodstream-form *T. brucei* (Wilkinson et al. 2008). Alsford and Horn's RNAi screen (Alsford et al. 2012) also confirmed the importance of NTR; in addition to NTR, it identified a putative flavokinase plus four genes involved in the biosynthesis of ubiquinone (Baker et al. 2011; Alsford et al. 2012). Flavokinase converts riboflavin to flavin-mononucleotide, which is an essential cofactor of NTR, while ubiquinone functions as electron acceptor from NADH mediated by NTR. Cross-resistance was also observed between nifurtimox and fexinidazole. *T. brucei* selected *in vitro* for nifurtimox-resistance (8x) were also resistant (27x) to fexinidazole (Sokolova et al. 2010). These trypanosomes were infective to mice and the cross-resistance phenotype was also manifest *in vivo*. Trypanosomes that had been selected with fexinidazole were also cross-resistant to nifurtimox (Sokolova et al. 2010). The resistance factor was 10x to either drug, indicating that the underlying mechanism of resistance might be somewhat

different. The *NTR* genes were unaltered in those lines but reduced expression level could not be ruled out (Sokolova et al. 2010).

Table 1. Transporters of *T. brucei* involved in drug import and their physiological substrates (selected).

Transporter	**TriTrypDB**	**GenBank**	**Physiological substrate**	**Toxic substrate**
TbAT1 / P2	Tb927.5.286b	AAD45278	Adenosine Adenine	Melarsoprol Pentamidine Diminazene DB75 Cordycepin Tubercidin
TbNT11.1 (AT-A)	Tb927.9.15980	XM_822640.1	Adenine Hypoxanthine Xanthine	Pentamidine
TbNT12.1 (AT-E)	Tb927.3.590	XM_838562.1	Adenine	Pentamidine
AQP2	Tb927.10.14170	XM_822804.1	Water Dihydroxyacetone Glycerol Urea	Melarsoprol Pentamidine Sb III As III
TbAAT6	Tb927.8.5450	XM_842282.1	Proline Other neutral amino acids	Eflornithine

4.4 Eflornithine resistance

Eflornithine or DFMO (Difluoromethyl-ornithine) was synthesized 1978 and initially developed as an anti-cancer drug (Bacchi et al. 1980). It is the newest drug applied for HAT and the only treatment for stage 2 of the disease in case of melarsoprol treatment failure (Burri and Brun 2003). Since 2009 eflornithine is used in combination therapy with nifurtimox (NECT). This is now the recommended treatment option for 2nd stage *T. b. gambiense* infections as NECT is far less toxic than melarsoprol. However, eflornithine is less active against *T. b. rhodesiense* (Iten et al. 1995) and NECT is not being implemented for the treatment of East-African sleeping sickness. Eflornithine is one of the very few trypanocidal drugs whose target is known. It covalently binds to, and irreversibly inhibits, the enzyme ornithine decarboxylase (ODC), blocking polyamine synthesis and subsequently trypanothione production (Fairlamb et al. 1987; Phillips et al. 1987; Wang 1991). The molecular mechanism of eflornithine-resistance remained elusive and no genetic marker was identified until recently. Vincent and co-workers have selected two *T. b. brucei* lines independently for eflornithine resistance *in vitro*. ODC activity was unaltered and there was no change in the levels of metabolites of the polyamine biosynthetic pathway (Vincent et al. 2010). When the lines were probed by PCR for all known *T. brucei* amino acid permeases (because eflornithine is itself an amino acid and likely taken up by such a transporter) it turned out that both lines had lost the gene encoding the *T. brucei* amino acid transporter 6 (*TbAAT6*). RNAi-mediated knock-down of *TbAAT6* expression in sensitive *T. brucei* confirmed the role of *TbAAT6* in eflornithine resistance while reintroduction of *TbAAT6* rescued the drug sensitive phenotype of the resistant mutants (Vincent et al. 2010). Furthermore, two genome-wide loss of function screens using RNAi libraries independently linked the silencing of *TbAAT6* expression to eflornithine resistance (Baker et al. 2011; Schumann Burkard et al. 2011).

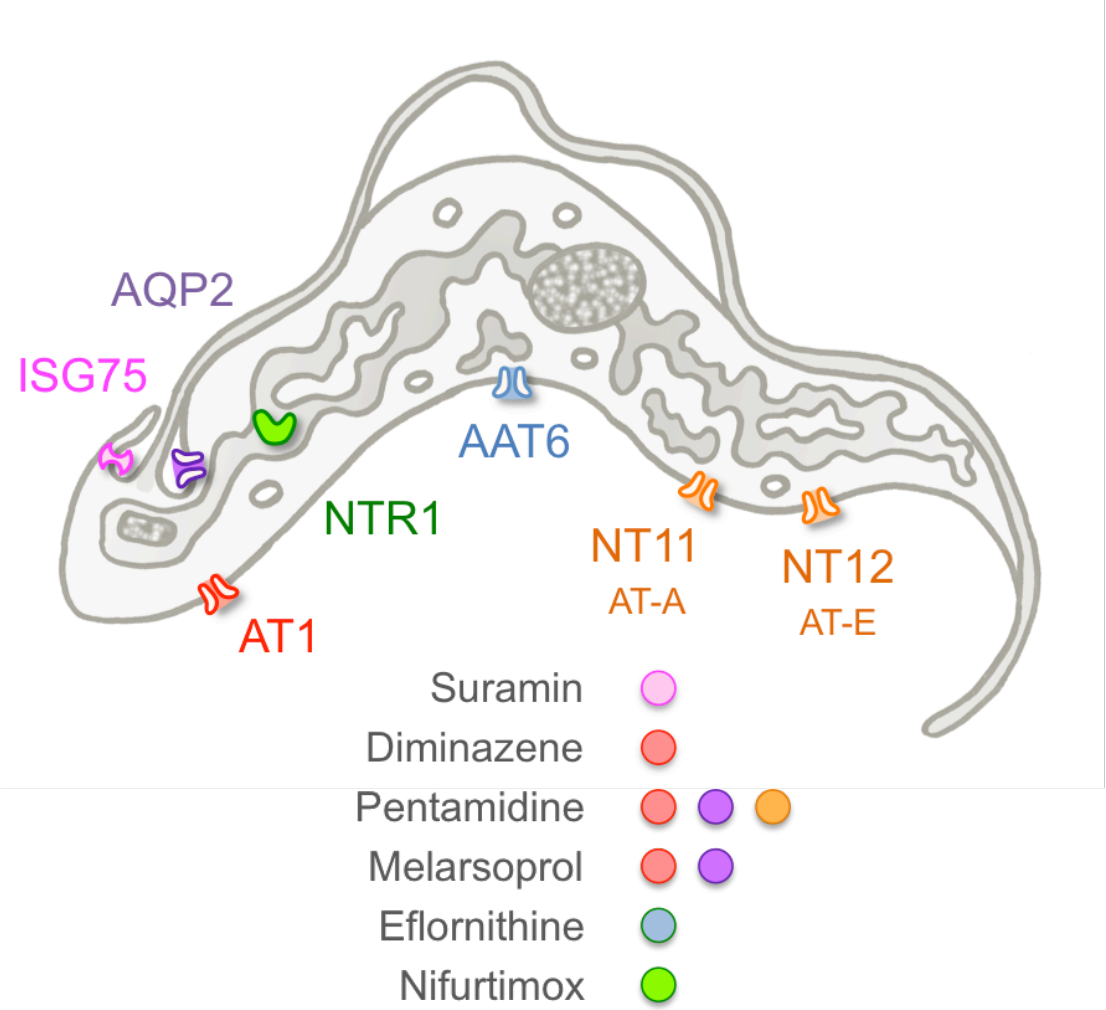

Figure 2. Schematic overview of the gene products that mediate drug resistance in *T. brucei* bloodstream forms.

5. Drug resistance in the clinics

In contrast to the livestock-pathogenic trypanosomes where drug resistance is widespread, the situation is less critical regarding the treatment of first-stage HAT patients. Suramin, for instance, is generally efficacious against first-stage *T. b. rhodesiense* infections, in spite of its old age and the fact that resistance can be selected for in the lab (Mutugi et al. 1994; Scott et al. 1996). However, suramin resistance is a problem in the management of *T. evansi* (Brun and Lun 1994; El Rayah et al. 1999), a non-tsetse transmitted trypanosome that causes Surra in cattle, equines and camelids and that is very closely related to *T. brucei* (Carnes et al. 2015). Similarly, pentamidine treatment failures are rare and the reported

relapsing patients were likely in many cases early stage II infections, indicative of misdiagnosis rather than drug resistance (Balasegaram et al. 2006b). At the same time, diminazene resistance is jeopardizing the treatment of Nagana (Moti et al. 2012; Mungube et al. 2012). For *T. brucei* ssp., this discrepancy has been attributed to the fact that diminazene is imported into the trypanosomes via a single transporter, TbAT1, whereas pentamidine has at least two additional routes of import, referred to as HAPT and LAPT for high- and low-affinity pentamidine transporters, respectively (de Koning 2001); TbAQP2 does not transport diminazene and corresponds to HAPT (Munday et al. 2014). Thus differences in the redundancy of import routes may provide an explanation for the higher prevalence of drug resistance in livestock trypanosomoses compared to human trypanosomiasis, lowering the probability of emergence in the latter. For *T. congolense* the situation is less clear. *T. congolense* possess an adenosine transporter gene, *TcoAT1*, that has been implicated in diminazene resistance based on molecular epidemiology (Delespaux et al. 2006). However, TcoAT1 is not the direct orthologue of TbAT1 and it does not transport diminazene when expressed in *T. b. brucei* (Munday et al. 2013). A more obvious effect than a lower probability of emergence might be the lower probability of the spread of drug resistance in the human-pathogenic trypanosomes, arising from the fact that the treated patients are hospitalized and not accessible for tsetse flies.

Patients relapsing from eflornithine monotherapy have been reported from the Democratic Republic of the Congo [up to 27% (Pepin et al. 2000)] and from Angola [8.1% (Balasegaram et al. 2006a)]. Whether this was caused by drug resistant parasites or other factors is unclear. Combination therapy with nifurtimox may improve the treatment success of eflornithine and delay the possible emergence of drug resistance. Nifurtimox resistance has so far not been encountered for African trypanosomes in the clinics, but was reported for *T. cruzi* (Filardi and Brener 1987; Murta et al. 1998). However, eflornithine and nifurtimox resistance are readily selected for *in vitro* (Wilkinson et al. 2008; Vincent et al. 2010), and the efficacy of NECT will decline if eflornithine resistant parasites emerge because nifurtimox by itself is not very potent.

HAT treatment failures have been most critical with melarsoprol. Relapse rates of 5-8% are considered normal for melarsoprol treatment (Pepin et al. 1994). Beginning in the 1990's, much higher rates of melarsoprol treatment failures have been reported from different areas, sometimes above 50% (Legros et al. 1999; Stanghellini and Josenando 2001; Robays et al. 2008; Mumba Ngoyi et al. 2010). After the discovery of the gene encoding the P2 transporter (Mäser et al. 1999), clinical isolates were analyzed for mutations in this potential marker. Several studies correlated the occurrence of (non-functional) mutant alleles of *TbAT1* to a higher incidence of melarsoprol treatment failures in different HAT foci (Matovu et al. 2001; Nerima et al. 2007; Kazibwe et al. 2009). These correlations were usually significant but not absolutely conclusive, indicating the contribution of additional factors (Brun et al. 2001). More recently, *T. b. gambiense* isolates from Mbuji-Mayi (Democratic Republic of the Congo), an area of high relapse rates after melarsoprol treatment (Mumba Ngoyi et al. 2010; Pyana et al. 2011), have been found to carry mutations in the aquaglyceroporin tandem locus (Graf et al. 2013; Pyana et al. 2014). The mutants had a deletion that led to the formation of a chimeric gene between the neighboring *AQP2* and *AQP3* and loss of either wild-type gene. These isolates were melarsoprol and pentamidine resistant *in vitro* (Graf et al. 2013) and had reduced melarsoprol sensitivity *in vivo* (Pyana et al. 2014). This represents the first clinical case of MPXR. Reintroduction of the wild-type *AQP2* gene into a MPXR resistant *T. b. gambiense* isolate completely restored its drug susceptibility, demonstrating that the loss of AQP2 function as the cause of drug resistance (F. Graf and P. Mäser, unpublished).

6. Conclusion

In summary, drug resistance in *T. brucei* is intriguingly linked to drug uptake. The predominant mechanism of resistance is reduced drug import caused by loss-of-function mutations in non-essential nutrient transporters that happen to import drugs in addition to their physiological substrates (Table 1). Other typical mechanisms of drug resistance such as overexpression or mutation of the target, do not seem not to play a critical role in *T. brucei*. A possible explanation for this phenomenon is that drugs like the diamidines, arsenicals or suramin, have

multiple intracellular targets; so it is very difficult for a cell to withstand once the drug has been taken up. But even for eflornithine, which has a clearly defined target enzyme, loss of import turned out to be the resistance mechanism rather than mutations in ODC. Obviously, RNAi screens are biased towards loss-of-function mutations and hence most effective to identify drug import pathways. However, the loss of drug import has also been confirmed in forward genetic experiments with laboratory-selected resistant mutants of *T. brucei* and even with drug-resistant field isolates.

We believe that loss of import as a mechanism of drug resistance is strongly favored by the high degree of redundancy within the nutrient uptake machinery of *T. brucei*. The *T. brucei* genome covers each of the main metabolite classes (i.e. purines, sugars or amino acids) with dozens of transporter genes. The high degree of redundancy means that transporters can be lost without a fitness cost. This is in agreement with the fact that the drug-resistant *T. brucei* transporter mutants characterized so far did not exhibit a growth deficit. Furthermore, the transporter genes of *T. brucei* are often arranged in tandem clusters, which allows for loss of genetic material by homologous recombination between very similar genes. If indeed the redundancy of nutrient import routes is at the core of drug resistance in *T. brucei*, a main conclusion is that we should aim for novel drugs that are taken up by the trypanosomes either via essential transporters or via multiple transporters. Thus transport phenomena must not be neglected in drug R&D for African trypanosomes.

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Objectives

The main aim of this PhD thesis was to find new mechanisms of drug resistance in *Trypanosoma brucei*, in particular genes involved in melarsoprol and pentamidine resistance and their cross-resistance, by combining bioinformatics and molecular biological approaches. The following specific objectives were achieved during the course of this thesis:

(i) Identification of drug resistance candidate genes by comparative genomics and transcriptomics between the two *in vitro* selected melarsoprol (STIB900-M) and pentamidine (STIB900-P) resistant *T. b. rhodesiense* lines and the corresponding drug susceptible parent line (STIB900) (Chapter 2).

(ii) Validation of candidate resistance genes discovered in (i) by reverse genetic methods in *T. b. brucei* (Chapter 2 & 3).

(iii) Investigation of clinical *T. brucei* ssp. isolates, especially *T. b. gambiense* isolates from melarsoprol treatment-refractory patients, whether they carry mutations in known resistance genes or genes discovered in (i & ii) and whether they have increased drug sensitivities (Chapter 4).

(iv) Proof that a mutation discovered in (iii) is the cause of drug resistance in a *T. b. gambiense* isolate from a melarsoprol treatment-refractory patient (Chapter 5).

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Chapter 2

Comparative genomics of drug resistance of the sleeping sickness parasite *Trypanosoma brucei rhodesiense*

Working manuscript

Comparative genomics of drug resistance of the sleeping sickness parasite *Trypanosoma brucei rhodesiense*

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Abstract

Background: *Trypanosoma brucei rhodesiense* is one of the causative agents of human sleeping sickness, a fatal disease restricted to Sub-Saharan Africa. The parasite proliferates extracellularly in the mammalian bloodstream and is transmitted by the tsetse fly. We have selected two independent bloodstream-form *T. b. rhodesiense* lines with the drugs melarsoprol and pentamidine *in vitro* over the course of two years. Both lines exhibited stable cross-resistance to melarsoprol and pentamidine. Here we apply whole genome and transcriptome sequencing to identify the mutations underlying this resistance phenotype.

Results: Comparative genomics and transcriptomics revealed remarkably few mutations that have become fixed in the resistant lines. The aquaglyceroporin gene *TbAQP2* had been lost in both resistant lines, presumably by homologous recombination with the neighboring gene *TbAQP3*. In addition, the melarsoprol-selected line carried a large deletion including the adenosine transporter gene *TbAT1*, whereas the pentamidine-selected line carried a heterozygous point mutation in TbAT1, G430R, which rendered the transporter non-functional. Both TbAQP2 and TbAT1 are known to be involved in drug uptake. Both resistant lines carried the mutation R131L in the RNA-binding protein TbUBP1.

Conclusion: Whole genome and RNA sequencing readily detected mutations underlying melarsoprol-pentamidine cross-resistance in *T. b. rhodesiense*. The affected transporter genes *TbAT1* and *TbAQP2* are known to be linked to drug resistance, also in the field. *TbUBP1* is a new candidate that has not previously been implicated in drug resistance in Africa n trypanosomes.

Keywords

Drug resistance, African trypanosomes, RNA-Seq, whole genome sequencing, pentamidine, melarsoprol

Background

Human African trypanosomiasis (HAT, also known as sleeping sickness) is a fatal disease caused by *Trypanosoma brucei rhodesiense* and *T. b. gambiense* in East- and West-Africa, respectively. The protozoan parasites are transmitted by the tsetse fly and proliferate extracellularly in the bloodstream of their mammalian hosts, evading the adaptive immune response through antigenic variation. *Trypanosoma brucei* has an approximate genome size of 35 Mb (haploid size), but the size can vary up to 25% (El-Sayed et al. 2000). Excluding the kinetoplast (mitochondrial) DNA, leaves a nuclear core genome of about 26 Mb, divided into 11 megabase-sized chromosomes, where the vast majority of the predicted >9000 protein coding genes are located. Roughly 1000 genes encode for variant surface glycoproteins (VSG) (Berriman et al. 2005). Each trypanosome is protected by a dense coat of VSGs, expressed from a singly active subtelomeric site. Antigenic variation happens by activation of a different VSG expression site or by recombination at the active site with one of the several hundred silent VSG genes. This immune evasion strategy dramatically diminishes the prospect of a vaccine and thus stresses the importance of chemotherapy to control HAT. The treatment relies on just five drugs. Patients in the first, haemolymphatic stage are treated with suramin or pentamidine. In the second stage, when the trypanosomes have invaded the central nervous system, melarsoprol or nifurtimox-eflornithine combination therapy (NECT; only for *T. b. gambiense*) are used (Brun et al. 2010). These drugs are dated, impractical, and suffer from severe adverse effects. Melarsoprol, in particular, causes unacceptable toxicity (Kennedy 2008). Furthermore, melarsoprol treatment failure rates of up to 30% have been reported throughout sub-Saharan Africa (Matovu et al. 2001a; Stewart et al. 2005; Kibona et al. 2006; Robays et al. 2008), presumably indicating the spread of drug resistant trypanosomes. New drugs that are safe and orally available are presently in clinical development (Mäser et al. 2012) but, meanwhile, it is essential to sustain the current drugs in spite of their shortcomings. This requires an understanding of the incidence and mechanisms of drug resistance.

The molecular mechanisms of drug resistance have predominantly been studied with *T. b. brucei*, which is non-pathogenic to humans and widely used as a

model in molecular parasitology because it can be easily cultured *in vitro* and is amenable to genetic manipulation (Wirtz and Clayton 1995; Wirtz et al. 1999). An observation that has been made repeatedly is melarsoprol-pentamidine cross-resistance (MPXR), i.e. trypanosomes selected for resistance with a melaminophenyl arsenical turned out to be cross-resistant to pentamidine and vice versa (Rollo and Williamson 1951; Baker et al. 2013). This phenomenon was attributed to the finding that the uptake of melarsoprol and pentamidine into the trypanosomes is mediated by the same set of transporters: the aminopurine permease P2 (Carter and Fairlamb 1993; Mäser et al. 1999), encoded by the gene *TbAT1*, and a high affinity pentamidine transport activity designated HAPT1 (de Koning and Jarvis 2001; Bridges et al. 2007) recently shown to be the aquagylceroporin TbAQP2 (Baker et al. 2012; Munday et al. 2014). Mutations in these transporters were described from drug-resistant *T. brucei* ssp. isolates from the field (Matovu et al. 2001b; Graf et al. 2013). In the lab, MPXR was phenocopied by reverse genetics. Homozygous deletion of either *TbAT1* or *TbAQP2* resulted in resistance to both melarsoprol and pentamidine. However, the obtained resistance factors were only between 2 and 3 for melarsoprol and pentamidine in *tbat1* null trypanosomes (Matovu et al. 2003), respectively, and 2 for melarsoprol and 15 for pentamidine in *tbaqp2* null mutants (Baker et al. 2012).

Here we investigate two drug-resistant lines of *T. b. rhodesiense* that exhibit markedly higher levels of MPXR than observed after deletion of either *TbAT1* or *TbAQP2*. The lines *T. b. rhodesiense* STIB900-M and STIB900-P had been selected *in vitro* from their susceptible parent *T. b. rhodesiense* STIB900 by continuous exposure to increasing concentrations of melarsoprol and pentamidine, respectively, over a period of 24 months (Bernhard et al. 2007). Finally, both lines exhibited a high level of MPXR with resistance factors up to 80. This phenotype was stable in the absence of drug pressure and after mouse passage. An initial genotypic characterization demonstrated that the gene *TbAT1* had been lost in STIB900-M but was still present in STIB900-P (Bernhard et al. 2007). Evidently, given the high level of drug resistance, further mutations must be involved. We have performed whole genome sequencing and RNA-Seq of the parental *T. b. rhodesiense* STIB900 and its resistant derivatives STIB900-M and STIB900-P,

aiming to elucidate the molecular mechanisms underlying the unprecedented level of MPXR by comparative genomics and transcriptomics.

Results and Discussion

Phenotypic profiling of drug resistance

Before venturing into next generation sequencing we performed an in-depth phenotypic characterization of the parental *T. b. rhodesiense* STIB900 and its two drug-resistant derivatives STIB900-M and STIB900-P. Drug sensitivities were quantified as 50% inhibitory concentrations (IC_{50}) towards the five clinical trypanocides (pentamidine, suramin, melarsoprol, eflornithine, and nifurtimox), two clinical candidates (fexinidazole and the diamidine DB75), and selected experimental compounds (Table 1). STIB900-M and STIB900-P exhibited very similar drug resistance phenotypes, namely strong MPXR with cross-resistance to other diamidines (i.e. diminazene aceturate and DB75) and adenosine analogs (cordycepin [3'-deoxyadenosine], tubercidin [7-deazaadenosine], and vidarabine [adenosine arabinoside]). For the majority of the tested compounds – including pentamidine itself – the resistance phenotype was more pronounced in the melarsoprol-selected line than in the pentamidine-selected line. This difference was particularly striking for tubercidin, to which STIB900-M was 163-fold resistant. In contrast, STIB900-P was significantly less sensitive to the adenine analog aminopurinol whereas STIB900-M was not (Table 1). As expected for MPXR, there was no resistance to suramin and nifurtimox and neither to phenylarsine oxide, a hydrophobic arsenical that freely diffuses across the plasma membrane (Carter and Fairlamb 1993). Surprisingly, both STIB900-M and STIB900-P were significantly hypersensitive to eflornithine (difluoromethylornithine, DFMO). This unexpected result was corroborated indirectly by the observation that eflornithine-resistance caused by loss of the amino acid transporter TbAAT6 was accompanied by significant hypersensitivity to pentamidine (Vincent et al. 2010). However, *TbAAT6* was not overexpressed in the resistant lines (see below). The population doubling times *in vitro* were the same for all three lines, i.e. 9.4 ± 0.3 h.

Transcriptomic profiling of drug resistance

Quantitative transcriptomics served as a first tool to investigate the strong MPXR phenotype of *T. b. rhodesiense* STIB900-P and STIB900-M at the molecular level. For this purpose we used the 'spliced leader trapping' (SLT) adaptation of Illumina RNA-Seq (Nilsson et al. 2010), exploiting the fact that all trypanosomal mature mRNAs carry the same 39 nt leader sequence spliced in-trans to their 5' end (Walder et al. 1986). SLT is optimally suited to quantify steady-state mRNA levels by counting the number of reads per transcript because all the reads are from the transcription start sites. The numbers were normalized per million, followed by an increment of one to allow for logarithmic expression even for genes that had no sequence tag at all. Figure 2 shows one of two independent experiments; all data are included in the supplementary Excel file *Graf_S1.xlsx*. Overall, there was very little variance between the drug-resistant lines and their sensitive parent (Figure 2A). Four genes were over-expressed in STIB900-P compared to STIB900 (and STIB900-M), all neighboring genes on chromosome 6 encoding for VSGs. This confirms that antigenic variation also takes place *in vitro*, but new variants rarely become fixed in the population because of continuous dilution of the growing cultures. Apart from the VSGs, no genes were significantly overexpressed in the resistant *T. b. rhodesiense* lines – including TbMRPA, an export pump that confers resistance to melarsoprol when overexpressed ectopically (Alibu et al. 2006; Lüscher et al. 2006), and hexose transporters, down-regulation of which can confer tubercidin resistance (albeit in procyclic forms (Drew et al. 2003)). The adenosine transporter *TbAT1* and six adjacent genes on the telomere of chromosome 5 (Stewart et al. 2010) were not expressed in STIB900-M (Figure 2B). This is in agreement with the reported absence of *TbAT1* in STIB900-M (Bernhard et al. 2007) and may be indicative of a larger deletion at the *TbAT1* locus. The aquaglyceroporin *TbAQP2* appeared not to be expressed in either resistant line (Figure 2C). However, the automated mapping of the reads (Nilsson et al. 2010) was ambiguous because of the high degree of similarity of *TbAQP2* and *TbAQP3* at their 5' ends (see Figure 3). The short Illumina reads were manually mapped to *TbAQP3* since the deletion of *TbAQP2* was confirmed by the longer 454 reads (see

also Figure 3). Apart from *TbAT1* and adjacent genes and *TbAQP2*, no genes were significantly underexpressed in the resistant lines.

Genome sequence of T. b. rhodesiense STIB900

Before exploring the mutations underlying the strong MPXR phenotype of STIB900-P and STIB900-M by comparative genomics, we had to generate a good-quality draft genome of the susceptible parent *T. b. rhodesiense* STIB900. Genomic DNA was isolated from bloodstream-form trypanosomes grown in mice. The obtained gDNA was verified to be free of mouse DNA by PCR with primers for mouse GAPDH. Paired-end Illumina reads generated on the HiSeq platform were mapped to the core chromosomes of *T. b. brucei* TREU927 with an average coverage of 53-fold. The vast majority of gene models (9,692 of 9,722) were transferred from *T. b. brucei* TREU927 to the assembled *T. b. rhodesiense* STIB900 genome, identifying a total of 112,565 high-quality single-nucleotide polymorphisms (SNP) between these two genomes. In protein-coding regions there were 46,453 SNPs, of which 19,575 non-synonymous. As expected, the STIB900 contained the *SRA* gene (serum resistance-associated; Tb927.9.17380), whose product neutralizes ApoL1, the trypanolytic factor of human serum that protects humans from infection by *T. b. brucei*. The genome reference strain *T. b. brucei* TREU927 contains a dysfunctional SRA orthologue (Vanhamme et al. 2004).

Comparative genomics of drug resistance

The assembled genome sequence of *T. b. rhodesiense* was used as a reference to identify mutations in the resistant derivatives STIB900-P and STIB900-M. Genomic DNA was isolated from bloodstream-form trypanosomes harvested from infected mice. The known absence of *TbAT1* from STIB900-M was used for diagnostic PCR of the purified gDNA to verify that there had been no contaminations. Roche-454 sequencing libraries were generated for all three lines, obtaining ~1.5 million high-quality reads for each genome, corresponding to a 20-fold coverage (Table S1). The reads from STIB900-M and STIB900-P were mapped to the assembled STIB900 genome with an overall coverage of 83% (DP4 >= 5). SNPs and indels were identified with SAMtools and with self-developed Perl scripts. All identified

SNPs, indels, and gene deletions were inspected manually using Artemis (Carver et al. 2008). Overall, there were remarkably few mutations in STIB900-M and STIB900-P relative to their parent STIB900 (Table 2). Only one coding point mutation was found in both resistant lines (in TbUBP1, see below). These lines have the same deletion of 1.8 kb at the *TbAQP2/3* locus (Figure 2a), causing loss of the aquaglyceroporin TbAQP2. The deletion was confirmed by PCR on genomic DNA followed by sequencing of the products (not shown).

T. b. rhodesiense STIB900-M had not only lost *TbAT1* (Tb927.5.286b), as previously published (Bernhard et al. 2007), but a whole region of over 25 kb encompassing *TbAT1* and adjacent genes (Tb927.5.288b, Tb927.5.289b, Tb927.5.291b, Tb927.5.292b). *T. b. rhodesiense* STIB900-P still possessed *TbAT1* but the gene contained a non-synonymous substitution G1288C, from neutral glycine at position 430 to a positively charged arginine. We confirmed the point mutation with Sanger sequencing and a restriction digest of the *TbAT1* PCR product since the mutation generated an endonuclease NruI site (tcgcga; Figure 4). The facts that the *TbAT1* PCR products from genomic DNA of STIB900-P (i) were not digested completely (Figure 4), and (ii) after cloning and sequencing did not all contain the C at position 1288 (not shown), indicate that STIB-900P is heterozygous for the mutation G1288C.

In view of the very small number of non-synonymous SNPs (Table 2), the finding that the two independently selected lines, STIB900-P and STIB900-M, carried the same point mutation, Arg^{131} to Leu, in the uridine-rich-binding protein 1 (TbUBP1, Tb927.11.500), was of particular interest. TbUBP1 is an RNA-binding protein with a single, conserved RNA recognition motif (RRM, Figure 5A), whose structure has been determined for its orthologue in *Trypanosoma cruzi* (Volpon et al. 2005). Arg^{131} of TbUBP1 corresponds to Arg^{113} of TcUBP1 in *T. cruzi* and lies within the β4 - β5 hairpin of the RRM that is involved in RNA binding (Volpon et al. 2005). TcUBP1 is a cytoplasmic protein in epimastigote *T. cruzi* (Cassola and Frasch 2009). Intriguingly, TcUBP1 was shown to accumulate in the nucleus when the trypanosomes were under arsenite stress, and mutations affecting RNA-binding prevented nuclear accumulation of TcUBP1 (Cassola and Frasch 2009). To test the impact of the mutation R131L on TbUBP1 function, we generated two

stable *T. b. brucei* 2T1 cell lines expressing GFP-UBP1-WT and GFP-UBP1-R131L at a single specific locus within the RRNA spacer in a tetracycline (TET) inducible manner (Alsford et al. 2005). Both cell lines still possess at least one endogenous copy of wild-type UBP1. In agreement with previous results (Hartmann et al. 2007), GFP-UBP1-WT was predominantly present in the cytoplasm. It appeared in granular structures (Figure 5B), which could reflect its presence at mRNA granules. However, artefacts of overexpression cannot be excluded. The same pattern of localization was observed for GFP-UBP1-R131L (Figure 5B). Wild-type and mutant UBP1 seemed to be expressed at equal levels 24 hours after TET induction, which was confirmed by Western blot analysis (Figure 5C) and flow cytometry (Figure 5D). Up to 40 hours after TET induction, no significant difference in expression levels of GFP-UBP1-R131L and GFP-UBP1-WT was observed. However, at high cell densities (>2 × 10^6 cells/ml) after 24h of TET induction, GFP-UBP1-R131L levels were approximately 50% lower than in wild-type cells (data not shown). An additional band of about 40 kDa reproducibly appeared in the Western blots when GFP-UBP1-R131L, but not GFP-UBP1-WT, was overexpressed, which might indicate partial degradation of the mutant protein. We next set out to test the effect of GFP-UBP1-R131L overexpression on cell growth. As shown previously, GFP-UBP1-WT caused an obvious growth deficit upon overexpression (Hartmann et al. 2007). Interestingly, overexpression of GFP-UBP1-R131L failed to elicit a similar response, and cells were growing at the same rate as non-induced control cells (Figure 5E). The R131L overexpressing cells were slightly but significantly hypersensitive to pentamidine (IC_{50} of 1.2 nM) as compared to non-induced cells (IC_{50} of 2.7 nM). There was no difference in the IC_{50} between non-induced and induced cells for melarsoprol (Figure 5F).

The point mutation in *TbAT1* of STIB900-P could be functionally characterized thanks to the availability of the *T. b. brucei* B48 mutant, which lacks high-affinity transport of melarsoprol and pentamidine (Bridges et al. 2007). To this end B48 bloodstream forms were transfected with wildtype *TbAT1*, G430R mutant *TbAT1*, and empty vector as a control. Expression of wildtype *TbAT1* strongly sensitized the B48 transfectants to melarsoprol, pentamidine, and diminazene aceturate (Berenil®), whereas G430R mutant TbAT1 did not (Figure

6). This demonstrates that the point mutation renders TbAT1 non-functional with respect to drug transport.

Conclusions

Cross-resistance of African trypanosomes between melarsoprol and pentamidine is an important and well-known phenomenon (Baker et al. 2013). Here we perform in-depth phenotypic and genotypic profiling of two lab-derived mutants of *T. b. rhodesiense* with pronounced MPXR: STIB900-P, selected with pentamidine, and STIB900-M, selected with melarsoprol (Figure 1). The two independently selected lines STIB900-P and STIB900-M had parallel resistance profiles, extending MPXR to other diamidines and adenosine analogs, but not to suramin, nifurtimox or fexinidazole (Table 1). The latter is of particular importance because fexinidazole is the most advanced candidate for a new, so urgently needed drug for sleeping sickness (Tarral et al. 2014). Surprisingly, the MPXR resistant lines turned out to be hypersensitive to eflornithine, which is complementary to the published observation that eflornithine-selected trypanosomes became hypersensitive to pentamidine (Vincent et al. 2010). The molecular mechanisms underlying this relationship are unknown, but based on transcriptomic data are unlikely to involve the eflornithine-transporter TbAAT6 (Figure 2). Very few genes were differentially expressed in the two drug-resistant lines compared to the sensitive parent. The only genes overexpressed were VSG genes for STIB900-P, indicative of antigenic switch(es) that had become fixed during the continuous sub-passaging of the cultures over the two years' selection period. The only striking difference between the resistant lines and their parent was the complete absence of RNA-Seq reads for *TbAQP2* (in both 900-P and 900-M) and for *TbAT1* and neighboring genes (in 900-M only). Mutations in *TbAQP2* and *TbAT1* are known to cause MPXR by reducing drug import (Mäser et al. 1999; Matovu et al. 2003; Baker et al. 2012; Graf et al. 2013; Munday et al. 2014).

The absence of mRNA for the two transporters was explained by comparative genomics. We have sequenced gDNA of *T. b. rhodesiense* STIB900 on the Illumina platform, and of all the three lines on the Roche-454 one. Mapping of the 454 reads to the assembled genome of STIB900 revealed large deletions at the

TbAQP2 and *TbAT1* loci in the resistant lines, which had both lost the complete *TbAQP2* gene (Figure 3). This was probably facilitated by the presence of the neighboring, highly similar gene *TbAQP3*, allowing for homologous recombination accompanied by loss of genetic material. While *TbAQP2* / *TbAQP3* chimera have been described from MPXR trypanosomes (Baker et al. 2012; Graf et al. 2013), this is the first case of a natural knock-out of *TbAQP2*. STIB900-M also carried a large deletion on chromosome five encompassing *TbAT1* and six adjacent genes (Figures 2B and 4A), a gene loss possibly facilitated by their telomeric location. This study has provided the best evidence to date that the MPXR models developed in the non-human infective trypanosome *T. b. brucei* hold true in *T. b. rhodesiense,* the causative agent of sleeping sickness in East Africa, and that selection for high-level resistance to melarsoprol and pentamidine can lead to loss of both known drug transporters.

Besides these large deletions there were only about a dozen mutations that had become fixed in the drug-selected lines in the two years' course of selection (Table 2). STIB900-P carried a non-synonymous point mutation in *TbAT1* changing Gly^{430} to Arg (Figure 4). Expression of wild-type and G430R-mutant *TbAT1* in a *T. b. brucei* loss-of-transport mutant demonstrated that the *TbAT1* allele did not transport melarsoprol or diamidines (Figure 6). The finding that STIB900-P is heterozygous for the mutation might explain its milder MPXR phenotype than STIB900-M (Table 1). Of particular interest was a point mutation in the RNA-binding protein TbUBP1, R131L, present in both resistant lines, which must have been acquired independently. The precise function of TbUBP1 is unknown, but it has been implicated in regulation of mRNA levels, presumably by competing for binding sites with other RNA-binding proteins (Hartmann et al. 2007). Based on the alignment to TcUBP1 from *T. cruzi*, the arginine at position 131 from TbUBP1 is predicted to be critical for RNA binding (Figure 5A) and therefore this mutation may render UBP1 non-functional. This hypothesis is supported by our observation that overexpression of mutated UBP1 is non-deleterious, whereas UBP1-WT overexpression does elicit a strong growth deficit (Figure 5E), as has been described by others (Hartmann et al. 2007). Further, we find that GFP-UBP1-R131L appears to be partially degraded when compared to GFP-UBP1-WT (Figure

5C), which might be caused by its inability to bind mRNAs. There is no difference in melarsoprol sensitivity when GFP-UBP1-R131L is overexpressed, but quite unexpectedly, cells became about 2-fold hypersensitive to pentamidine. It is intriguing that only pentamidine shows altered drug sensitivity, as melarsoprol selection also led to the R131L mutation in UBP1. We conclude that UBP1 is unlikely to be a direct drug target of melarsoprol or pentamidine. TbUBP1 is challenging to address experimentally because UBP1 is thought to be an essential gene as indicated by RNAi-mediated silencing (Hartmann et al. 2007). In this study TbUBP1 and TbUBP2 were knocked-down simultaneously, which led to a growth deficit. Specific knock-down of UBP1 is difficult due the high similarity between the two genes (72.8% global identity). The target RNAs of UBP1 in *T. brucei* are unknown. Absence of UBP1-WT in the resistant STIB900 lines did not lead to differential gene expression as seen with the RNAseq data (Figure 2).

Methods

T. b. rhodesiense lines

Trypanosoma brucei rhodesiense STIB900 is a derivative of STIB704, isolated from a male patient at St. Francis Hospital in Ifakara, Tanzania, in 1981. After several passages in rodents and a cyclic passage in *Glossina morsitans morsitans*, a cloned population was adapted to axenic growth. *T. b. rhodesiense* STIB900-M and STIB900-P were selected independently *in vitro* for resistance to melarsoprol and pentamidine, respectively (Bernhard et al. 2007). Bloodstream-form trypanosomes were propagated *in vitro* as described in (Graf et al. 2013) and adapted from (Baltz et al. 1985). Large numbers of trypanosomes for DNA isolation were obtained by inoculating female Naval Medical Research Institute (NMRI) mice from Harlan (The Netherlands) with 10^6 trypanosomes. When the parasitaemia was high, trypanosomes were harvested and separated from the blood cells on DEAE-cellulose columns (Lanham and Godfrey 1970).

In vitro drug sensitivity

For all the STIB900 lines drug sensitivities were determined with the Alamar blue assay (Räz et al. 1997). *T. b. brucei* B48 drug sensitivities were determined as described (Gould et al. 2008). The SoftMax Pro software (V. 5.2) and Prism 5 (GraphPad) were used to calculate 50% growth inhibitory concentrations (IC_{50}) values by non-linear regression fitting to a sigmoidal dose-response curve. All assays were performed at least three times independently, each in duplicate. The drugs and compounds were obtained from: Melarsoprol (Sanofi-Aventis/WHO), eflornithine (Sanofi-Aventis), DB75 (Immtech), fexinidazole (DNDi, Geneva), nifurtimox (WHO, Geneva), suramin (Bayer), pentamidine isethionate, diminazene aceturate, phenylarsine oxide, aminopurinol, cordycepin, adenosine arabinoside and tubercidin (Sigma).

Isolation of nucleic acids

Genomic DNA was isolated by phenol/chloroform extraction from bloodstream-form trypanosomes propagated in mice. To check for contamination with mouse DNA we performed PCR with primers for mouse glyceraldehyde-3-phosphate-

dehydrogenase (GAPDH) and mouse cDNA as a positive control. For each *T. b. rhodesiense* line, about 60 µg of genomic DNA was prepared for sequencing, Total RNA was isolated from log phase cultures (10^6 cells/ml) with TRIzol (Life technologies). Equal amounts of total RNA were pooled from three independent isolations, and from each pooled sample 12 µg were used for sequencing.

Spliced leader trapping

Library preparation and RNA-Seq was performed according to the spliced-leader trapping (SLT) protocol as in (Nilsson et al. 2010). This is a modification of the standard Illumina protocol that uses the *T. brucei* 39 nt spliced leader sequence for 2nd strand cDNA synthesis and sequencing. Two independent experiments were performed: run 1 on the Genome Analyzer IIx (Illumina) and run 2 on the HiSeq 2000 (Illumina). The Fastq files were read into the Spliced Leader ADDition (SLADD) program (Nilsson et al. 2010) and mapped onto the reference genome sequence of *T. brucei* TREU927 (Berriman et al. 2005), using maq (Li et al. 2008) with n = 3 and with a read length of ≥24. Multi mapping reads were separated from single mappers by an alignment quality threshold of 30. Read counts were normalized according to library size and expressed as tags per million (TPM). Statistical analysis for differentially expressed genes was performed with the DESeq package in R (Anders and Huber 2010). DESeq uses a negative binomial distribution and a shrinkage factor for the distribution's variance. Only mapped reads (raw counts) with a quality score of q<30 and data from both performed SLT runs were included.

Whole genome sequencing

Whole genome sequencing of *T. b. rhodesiense* STIB900 was carried out on the Illumina HiSeq 2000 platform. Two times 12,243,924 paired-end reads of 76 b were mapped chromosomewise to the reference genome *T. b. brucei* 927 (v5) using MIRA (v3.9.16) (Chevreux et al. 2004). Gene models from the reference genome *T. b. brucei* 927 were transferred to the assembled STIB900 genome using RATT (Rapid Annotation Transfer Tool) (Otto et al. 2011) from the PAGIT (Post Assembly Genome Improvement Toolkit) (Swain et al. 2012) package. Whole

genome sequencing of *T. b. rhodesiense* STIB900-M and STIB900-P was carried out on the Genome Sequencer FLX Titanium by Roche/454. Two shotgun runs per line were performed. FASTQ format was extracted from .sff files using 'SFF converter' from Galaxy (Giardine et al. 2005). High quality (HQ) reads were mapped to the assembled STIB900 genome, indexed with wordlength 13 and skipstep 1, using the program SMALT (ftp.sanger.ac.uk/pub4/resources/software/smalt). Consensus sequence and variants relative to the assembled STIB900 genome were identified with 'mpileup' from SAMtools (Li et al. 2009). Ad hoc Perl scripts were used to compare nucleotide variants between the mapped reads of STIB900-M, STIB900-P and the assembled STIB900 genome. For comparison also STIB900 454 reads were mapped to the assembled STIB900 genome. SNPs were called if they had a read depth of at least 5 high quality bases (DP4 ≥ 5) and a read mapping quality of minimum 20 (mapq ≥ 20). All identified SNPs, indels, and gene deletions were inspected manually using Artemis (Carver et al. 2008).

PCR amplification and Sanger sequencing

PCR primers to amplify the 3.2 kb tandem locus of *TbAQP2* and *TbAQP3* were AQP2/3_F (aagaaggctgaaactccacttg) and AQP2/3_R (tgcactcaaaaacaggaaaaga), annealing at 58°C. *TbAT1* was amplified with primers TbAT1_F (gaaatccccgtcttttctcac) and TbAT1_R (atgtgctgagccttttttcctt), annealing at 56°C. The PCR products were purified on silica membrane columns (Nucleospin gel and PCR clean up, Macherey Nagel) according to the supplier's protocol. The product was digested with NruI (New England Biolabs), run on a 1.5% agarose gel and visualized with ethidium bromide.

Plasmid construction and transfection

The TbAT1-G430R mutated sequence from STIB900-P was ligated into the expression vector pHD1336 (Biebinger et al. 1997), to give pHDK68. The plasmid was checked by Sanger Sequencing (Source BioScience, Nottingham, UK) for the presence of the expected mutation and linearized with *Not*I prior to transfection into *T. b. brucei* clone B48, which lack the *TbAT1* gene and the high affinity pentamidine transporter (Bridges et al. 2007). B48 parasites were washed into

Human T Cell Solution for transfection using the desired cassette with an Amaxa Nucleofector (Burkard et al. 2007). Transfectants were cloned by limiting dilution in standard HMI-11 (Hirumi and Hirumi 1989) containing 5 µg/ml blasticidin. Correct integration of the expression cassettes was tested by PCR.

To generate stable cell lines that allow for inducible expression of GFP-UBP1-WT or GFP-UBP1-R131L genomic DNA was isolated from STIB-900-WT and STIB-900-M cells according to standard protocols and used as a template for amplification of the UBP1 gene by PCR with primers UBP1_F (ccgctctagatctcaggttccactggcttc) and UBP1_R (ccgcggatcctcatttacgggcaggccgac). PCR products were cloned into pRPaiGFPx (http://www.lifesci.dundee.ac.uk/groups/david-horn/resources) using restriction enzymes XbaI and BamHI. *T. b. brucei* 2TI cells (Alsford et al. 2005) were transfected with AscI digested, Tet-inducible RRNA promoter driven plasmids (pRPaiGFPx) containing either GFP-UBP1-WT or GFP-UBP1-R131L in Tb-BSF nucleofection buffer (90 mM NaHPO3, 5 mM KCl, 0.15 mM CaCl2, 50 mM HEPES, pH 7.3) (Schumann Burkard et al. 2011) using the Amaxa nucleofector (Lonza) with program Z-001. After transfections cells were cloned out by limiting dilution and selected for stable integration of the plasmid by addition of 2.5 µg/ml hygromycin. PCR and Sanger sequencing confirmed correct integration and sequence.

Fluorescence microcsopy

Trypanosomes were fixed with 4% formaldehyde in PBS and mounted using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Samples were analysed using a DeltaVision microscope on a Nikon TE200 base (Applied Precision) with a 60x 1.4 NA plan Apo objective and 1.6x auxiliary magnification. Binning was set to 2x2. Serial optical z- sections (0.3 µM spacing) were collected, deconvoluted and sum intensity projected using Softworx (Applied Precision).

SDS-PAGE and Western Blotting

Cells were lysed in NUPAGE® LDS sample buffer (Life Technologies) and samples were loaded on precast 4-12% Bis-Tris Gradient Gels (NuPAGE Novex®, Life Technologies) in MES running buffer and transferred to nitrocellulose membranes using the iBlot dry-blotting system (Novex®, Life Technologies). Membranes were blocked in 5% milk in PBS/Tween-20 and incubated with primary antibodies in 5% milk in TBS/Tween-20 overnight at 4 °C. Membranes were washed and incubated with peroxidase-conjugated secondary antibodies in 5% milk PBS/Tween-20 for 2 hours at room temperature. Blots were developed using the ECL Western Blotting Substrate (Pierce) using a ChemiDoc™ MP Gel Imaging System (Biorad). Primary Antibodies used: rabbit anti-GFP: (Abcam, Ab290), mouse anti-BiP: (kind gift of Prof A. Schneider). Secondary Antibodies used: goat anti-rabbit (SouthernBiotech: 4050-05), polyclonal rabbit anti-mouse HRP (Dako, Baar, Switzerland)

Flow cytometry

To assess relative levels of GFP-UBP1-WT and GFP-UBP1-R131L, expression was induced in the respective cell lines for 24h by addition of 1 µg/ml tetracycline. Parasites were then diluted 1:2 in PBS containing 4% formaldehyde and analyzed immediately by flow cytometry using a BD FACSCalibur (Becton Dickinson, Allschwil, Switzerland). GFP intensity was measured in channel FL1 (530 ± 15 nm) and geometrical means of the main population were determined. Levels were then normalized to GFP-UBP1-WT. All analyses were carried out using flow cytometry analysis software FlowJo (Tree Star Inc., Ashland, OR, USA).

Growth assay

Trypanosomes harboring GFP-UB1-WT or GFP-UBP1-R131L were seeded at a cell density of 10^4 cells/mL and expression of transgenes was induced with tetracycline 1 µg/ml 24 hours prior to the start of the first measurement. For five consecutive days, trypanosome number was monitored using a CASY® Cell Counter system (Roche).

Abbreviations

MPXR	melarsoprol-pentamidine cross-resistance
SLT	spliced leader trapping
TbAQP2	*T. brucei* aquaglyceroporin 2
TbAT1	*T. brucei* adenosine transporter 1
VSG	variable surface glycoprotein

Competing interests

The authors declare that there are no competing interests.

Authors' contributions

Planning of the study and writing of the manuscript was mainly done by FEG, PL, and PM. FEG and PL were the chief investigators for the wet-lab and the dry-lab part, respectively. PM acted as the coordinator. FEG, CA and RS performed the functional characterization of the mutant TbUBP1. JCM and JK did the functional characterization of mutant TbAT1, NS performed one of the SLT experiments, CK isolated and purified nucleic acids, and OB and AC produced the STIB900 Illumina reads. HPK was involved at many stages, in particular regarding the role of TbAT1 and TbAQP2 in drug resistance. All authors read and approved the final manuscript.

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Table 1: *In vitro* drug sensitivity profiles

	IC_{50} determined *in vitro* [nM]			Resistance factor	
	STIB900	**STIB900-P**	**STIB900-M**	**STIB-900P**	**STIB900-M**
Melarsoprol	6.0 ± 3.4	84 ± 52 *	170 ± 63 *	14	28
Pentamidine	2.8 ± 0.8	130 ± 69 *	210 ± 93 *	47	76
Diminazene	3.8 ± 1.5	18 ± 5.4 *	25 ± 10 *	4.7	6.5
DB75	3.7 ± 0.9	22 ± 8 *	64 ± 17 *	5.9	17
Suramin	135 ± 62	76 ± 32	125 ± 25	0.6	0.9
Nifurtimox	1100 ± 550	1100 ± 460	1500 ± 620	1.0	1.4
Fexinidazole	3200 ± 1100	2500 ± 980	6100 ± 2500 *	0.8	1.9
Eflornithine	5200 ± 1400	1300 ± 640 *	2700 ± 480 *	0.3	0.5
Cordycepin	0.46 ± 0.1	2.2 ± 0.54 *	7.0 ± 1.5 *	4.7	15
Vidarabine	265 ± 108	400 ± 52 *	650 ± 190 *	1.5	2.5
Tubercidin	26 ± 6	100 ± 19 *	4300 ± 1000 *	3.8	163
Aminopurinol	1500 ± 190	5400 ± 1100 *	2000 ± 630	3.6	1.3
Phenylarsine	0.52 ± 0.13	0.65 ± 0.11	0.75 ± 0.08	1.3	1.4

IC_{50} values ± standard deviation and resistance factors relative to STIB900. Significant differences relative to STIB900 are indicated with an asterisk (95% confidence level).

Table 2: SNP statistics

	STIB900-M vs. STIB900			STIB900-P vs. STIB900		
	Overall	**CDS**	**NS**	**Overall**	**CDS**	**NS**
Chr1	0	0	0	2	0	0
Chr2	0	0	0	0	0	0
Chr3	0	0	0	1	0	0
Chr4	0	0	0	0	0	0
Chr5	2	0	0	1	1	1
Chr6	1	0	0	0	0	0
Chr7	0	0	0	1	0	0
Chr8	1	0	0	0	0	0
Chr9	6	0	0	5	1	1
Chr10	0	0	0	0	0	0
Chr11	2	2	2	4	2	2
Total	**12**	**2**	**2**	**14**	**4**	**4**

Single nucleotide polymorphisms between the drug-resistant *T. b. rhodesiense* lines and their parent STIB900 (CDS, coding sequence; NS, non-synonymous).

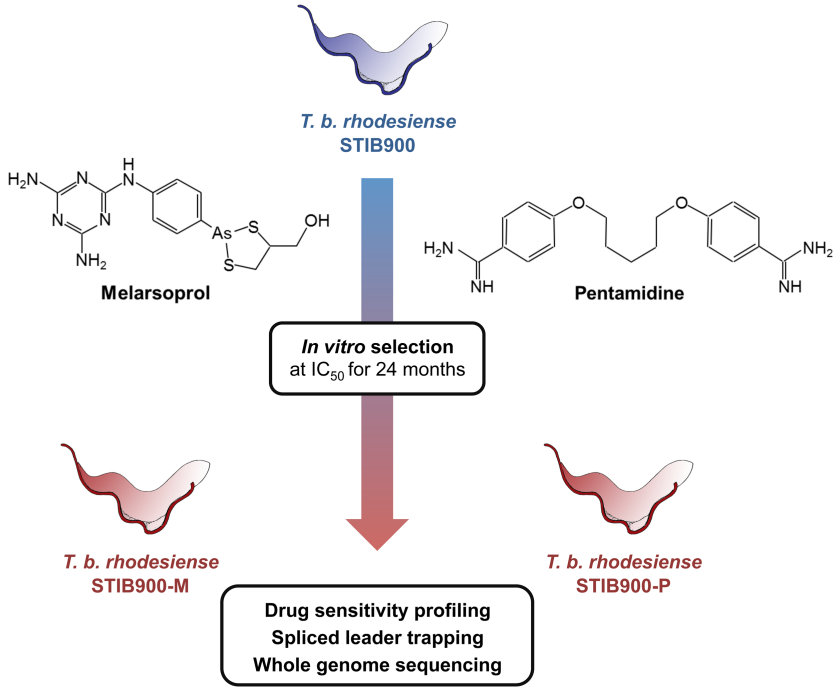

Figure 1 – Overview. The two *T. b. rhodesiense* lines STIB900-M and STIB900-P were selected independently from STIB900 with melarsoprol and pentamidine, respectively, exposing the trypanosomes at IC_{50} for 24 months (Bernhard et al. 2007). The three lines were extensively characterized by drug sensitivity profiling, RNA-Seq, and whole genome sequencing.

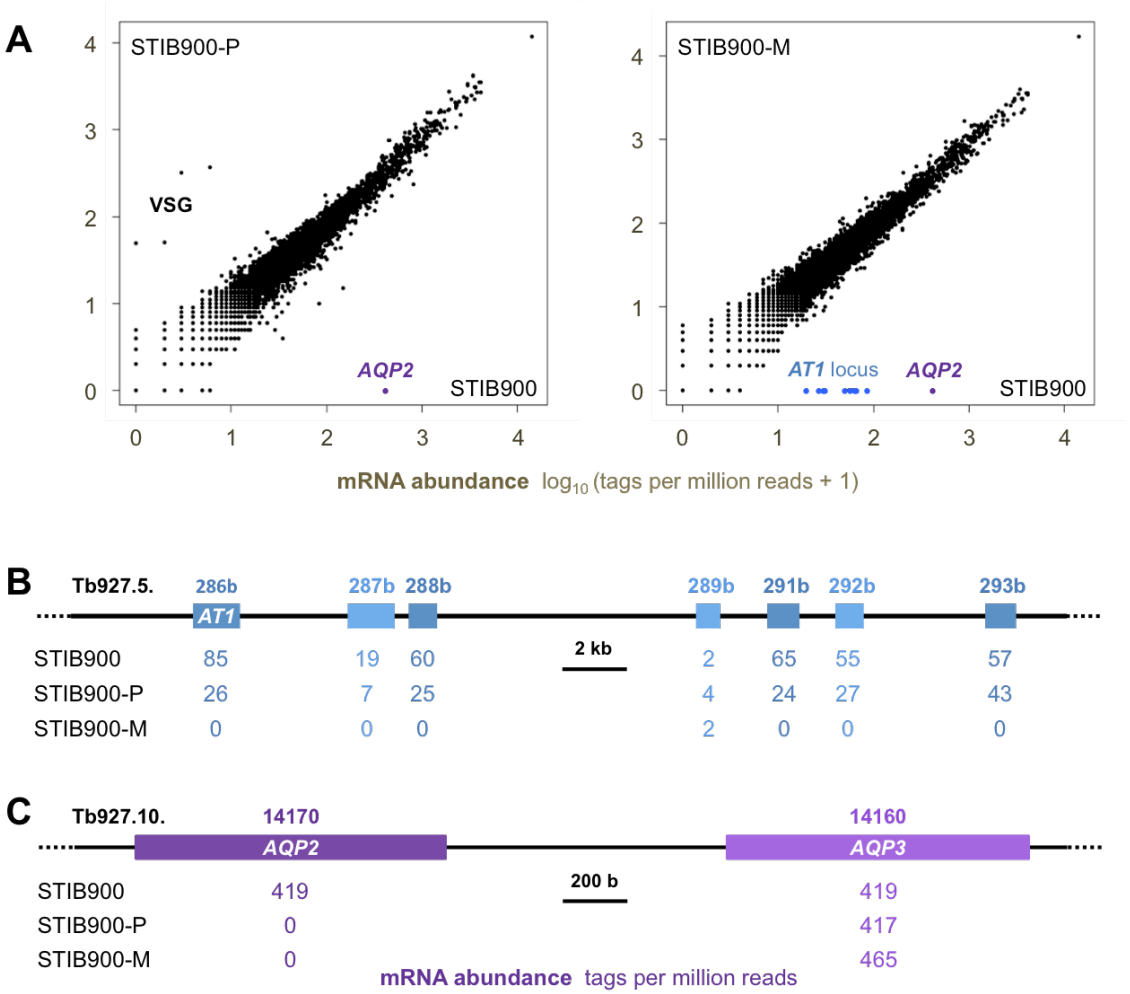

Figure 2 – Comparative transcriptomics. A) Scatterplots of normalized read counts from RNA sequencing data using the spliced leader trapping protocol. Genes that are not expressed in the resistant lines are indicated. Note the VSG expression switch in STIB900-P. B) View of the *TbAT1* locus with read counts per gene. C) View of the *TbAQP2 / TbAQP3* tandem locus with read counts per gene.

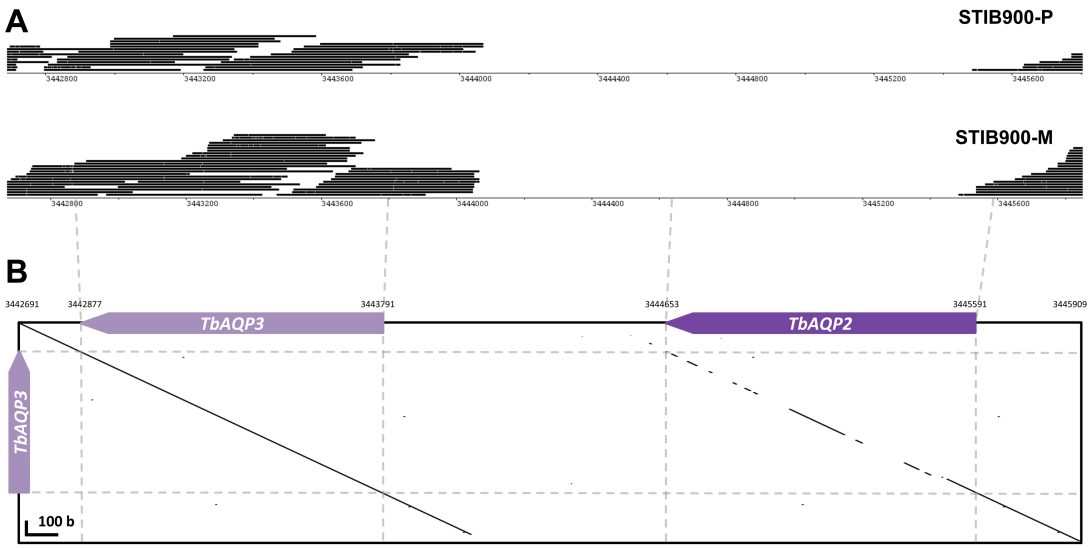

Figure 3 – Loss of *TbAQP2* from the resistant lines. A) 454 reads generated from genomic DNA of STIB900-P and STIB900-M mapped to STIB900. The *TbAQP2 / TbAQP3* tandem locus on chromosome 10 is visualized with BamView (Carver et al. 2010). B) Dot plot of sequenced PCR products encompassing the complete locus of STIB900 (x-axis) versus STIB900-P and –M (y-axis). TbAQP2 and TbAQP3 are highly similar (82% global identity), *TbAQP2* is missing in both STIB900-P and STIB-900M. The dotplot was made using the program GEPARD (Krumsiek et al. 2007) with a word length of 10.

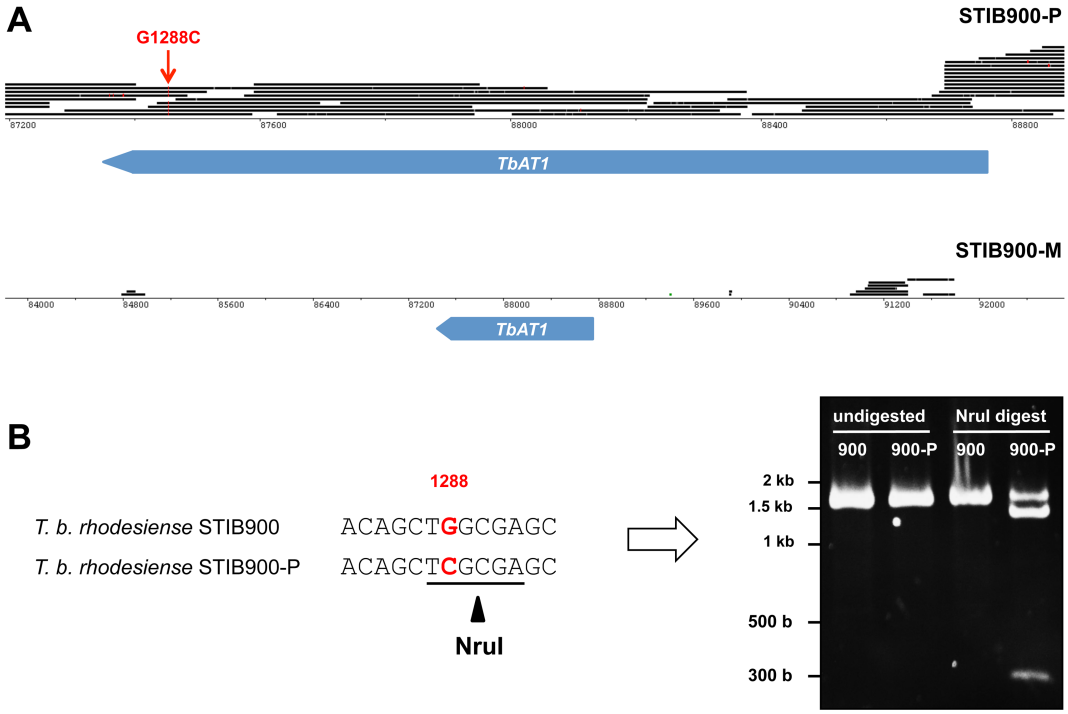

Figure 4 – Loss or mutation of *TbAT1* in the resistant lines. A) Genomic 454 reads of STIB900-P and STIB900-M mapped to STIB900 visualized for the *TbAT1* locus on chromosome 5 using BamView (Carver et al. 2010) (smaller scale for STIB900-M). There is a deletion of *TbAT1* in STIB900-M and a coding point mutation in STIB900-P (red). B) *TbAT1* PCR products (1636 bp) were amplified from genomic DNA of STIB900 and STIB900-P, and digested with the endonuclease NruI. G1288C mutant alleles are cut to fragments of 1339 bp and 257 bp. STIB900-P appears to be heterozygous for the mutation.

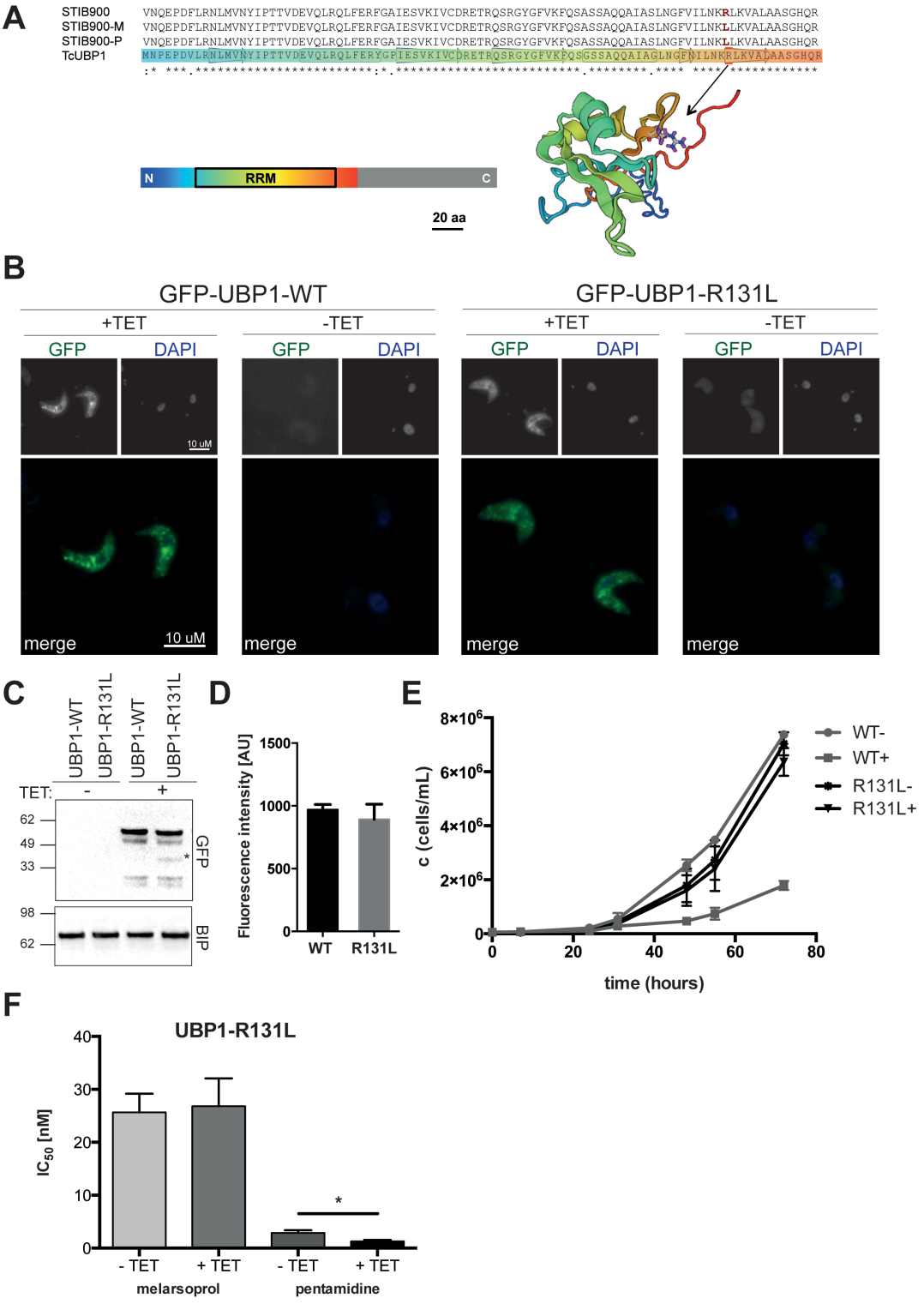

Figure 5 – Mutation of TbUBP1 in the resistant lines. A) ClustalW2 alignment of the predicted RNA recognition motif (RRM) of TbUBP1 from STIB900, STIB900-M, and STIB900-P alongside *Trypanosoma cruzi* UBP1. The mutation of the conserved Arg131 to Leu is highlighted in the alignment and Arg131 is indicated

with an arrow in the published structure of TcUBP1. The full length TcUBP1 is 224 aa but a truncated version (1-139) including the RRM (35-126) was used to determine the structure (Volpon et al. 2005). Swiss model (Arnold et al. 2006) was used to visualize the structure. B) Fluorescence microscopy demonstrates cytoplasmic location for GFP-UBP1-WT and GFP-UBP1-R131L and confirms inducible expression upon tetracycline induction. DNA is stained with DAPI. C) Western blot with anti-GFP antibody showing inducible expression of GFP-UBP1-WT and GFP-UBP1-R131L 24 hours after tetracycline addition. Asterisk: additional band appeared reproducibly for GFP-UBP1-R131L. D) Fluorescence intensity 24 hours after tetracycline induction determined by flow cytometry for GFP-UBP1-WT and GFP-UBP1-R131L overexpressing cells. Error bars are standard deviations (N=3). E) Growth assay for GFP-UBP1-WT and GFP-UBP1-R131L transfected cells (tetracycline induced and non-induced). Error bars are standard deviations (N=3). F) *In vitro* drug sensitivities (IC_{50}) for melarsoprol and pentamidine determined with the alamar blue assay for GFP-UBP1-R131L overexpressing cells. Error bars are standard errors of the mean (N=10). The asteriks denotes significant difference ($p<0.05$) as determined by two-tailed t-test (GraphPad Prism 5.0.).

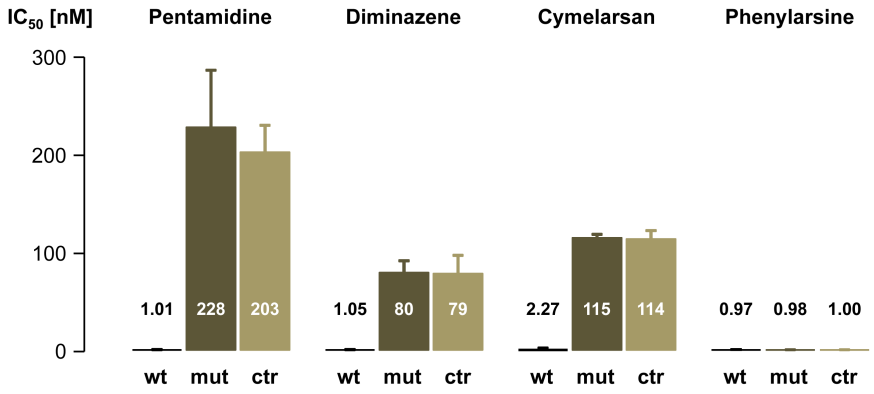

Figure 6 – Functional characterization of G430R mutant *TbAT1*. Wild-type TbAT1 (wt), mutant G430R (mut), and empty vector control (ctr) were expressed in the *T. b. brucei* B48 mutant (Bridges et al. 2007). Transfectant clones were tested *in vitro* for their sensitivity to the known TbAT1 substrates pentamidine, diminazene, and cymelarsan. Phenylarsine oxide was included as a negative control as it is not a TbAT1 substrate. Bars represent average IC_{50} values of 5 to 8 independent experiments, error bars are standard deviations.

Additional data files

The following additional data are available with the online version of this paper. Table S1 is a Excel file (*Graf_S1.xlsx*) of the normalized read counts as tags per million reads (TPM) for STIB900, STIB900-P and STIB900-M for run 1 and run 2 from the RNA-Seq data. Table S2 is a Word file (*Graf_S2.docx*) of the mapping statistics of Roche 454 reads of STIB900, STIB900-P and STIB900-M to the reference genome *T. b. brucei* TREU 927.

Chapter 3

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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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 knockout 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 K_m 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.[3] 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;[4] although its causes have never been completely resolved, it has long been clear this is linked to reduced drug accumulation.[5–7] The first drug transporter identified in trypanosomes was the P2 adenosine/adenine transporter, which was initially implicated in melarsoprol uptake[8] and subsequently also in diamidine transport;[9–11] the gene was designated *TbAT1*.[12] All protozoan nucleoside and nucleobase transporters identified to date have been of the equilibrative nucleoside transporter family.[13] Although the evidence of diamidine and arsenical transport by *TbAT1*/P2 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 aceturate[14] and the newer clinical candidates furamidine and CPD0801,[15] but only to a relatively minor loss of susceptibility to MPAs and pentamidine.[14,16] 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).[17,18] 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.[16,19] Despite the HAPT1 and LAPT1 activities having been first characterized over a decade ago,[17] the genes encoding these transporters remained unknown.

Recently, we reported that the aquaglyceroporin TbAQP2 controls MPXR in *T. b. brucei*.[20] 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.[21] 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.[22,23] 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.[24] Some AQPs, including *Leishmania major* AQP1, transport antimony and arsenic, most likely in the form of $As(OH)_3$ and $Sb(OH)_3$, which structurally resemble glycerol.[25,26] 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.

T. brucei members of the AQP family are classified functionally[27,28] and phylogenetically[29] as aquaglyceroporins. They are closely related to LmAQP1 and human aquaglyceroporins, including hAQP9, which reportedly allows the uptake of a wide variety of uncharged solutes, including carbamides, polyols, purines and pyrimidines.[30] The three *T. b. brucei* AQPs appear to have very similar permeation patterns, mediating the uptake of glycerol, dihydroxyacetone, ribitol and urea.[27] However, only TbAQP2 was implicated in MPXR, with the re-expression of TbAQP3 in an *aqp2/aqp3* null line having no effect on drug susceptibility.[20]

Here, we report that loss of the wild-type *TbAQP2* open reading frame (ORF) was observed in all MPXR strains (*T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense*), whether they were selected for resistance to MPAs or pentamidine, including strains selected *in vivo* and able to be transmitted by tsetse flies. Based on our detailed genetic, pharmacological and kinetic analysis, we conclude that *TbAQP2* encodes the HAPT1 activity and that loss of AQP2 function is sufficient and likely required for high-level MPXR.

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.[16] Several derivative lines were used: *tbat1*$^{-/-}$,[14] B48,[16] 2T1,[31] *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.[33] 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).[34]

Leishmania strains and culture

Leishmania mexicana promastigotes of strain MNYC/BZ/62/M379[35] were cultured in HOMEM medium (Invitrogen) supplemented with 10% fetal bovine serum at 25°C exactly as described for *L. major* promastigotes.[36] 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 pHD1336[37] to give pHDK21. This plasmid was digested with NotI prior to transfection into trypanosomes. The pRPa^{iGFPx} construct[38] 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[20] were synthesized by GenScript (New Jersey, USA) for insertion into pRPa^{iGFPx},[38] 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 Amaxa Nucleofector as described previously.[39] 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 $pRPa^{AQP2}$/$pRPa^{AQP2\text{-}3}$).

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* 927[40] from EBI-EMBL (version October 2011) using SMALT (www.sanger.ac.uk/resources/software/smalt). Consensus sequence and variants were identified with SAMtools[41] and inspected using Artemis.[42]

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-chAQP2/3) and western blot with anti-GFP antiserum were performed exactly as described for wild-type TbAQP2 and TbAQP3.[20] 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 as described previously. Cultures were harvested at the mid-log growth phase and washed into assay buffer (AB; 33 mM HEPES, 98 mM NaCl, 4.6 mM KCl, 0.55 mM $CaCl_2$, 0.07 mM $MgSO_4$, 5.8 mM NaH_2PO_4, 0.3 mM $MgCl_2$, 23 mM $NaHCO_3$ and 14 mM glucose, pH 7.3) at a final concentration of 10^8 cells/mL. Transport was initiated by the addition of 100 μL cells to 100 μL of a solution of the appropriate radiolabel in AB layered over oil [7:1 dibutylphthalate/mineral oil (v/v); Sigma–Aldrich, St Louis, MO, USA] 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 previously.[45] All experiments were performed in triplicate on at least three independent occasions and analysed using the appropriate linear and non-linear regression equations in GraphPad Prism 5.0. [^{3}H]pentamidine (3.26 TBq/mmol; product TRQ40084, batch 1) was custom synthesized by Amersham using tritium gas, producing a general labelling of the pentamidine molecule.

Drug susceptibility assays

Drug susceptibilities of the bloodstream-form trypanosomes[47] and *L. mexicana* promastigotes[48] 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. EC_{50} 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 AQPs 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*.[49] The construct was verified by sequencing before transfection into *L. mexicana* promastigotes using an Amaxa 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*)[14] 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.[33] 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 *TbAT1* function in addition to other mutation(s).[51] We investigated MPXR in the *T. b. brucei* 247 and *T. b. gambiense* 386 isogenic pairs, both generated by *in vivo* adaptation to Cymelarsan.[33] Pentamidine cross-resistance for *T. b. brucei* 247 was first reported by Scott *et al.*[52] with >50-fold pentamidine resistance *in vivo* for *T. b. brucei* 247MR relative to its parental line and we confirm here that procyclic 247MR were 74- and 755-fold resistant to pentamidine and Cymelarsan, respectively; these numbers were 90- and 83-fold for 386MR (Figure S2, available as Supplementary data at *JAC* Online). Neither strain displayed resistance to phenylarsine oxide (PAO). Assessment of the HAPT1 and LAPT1 transporters in both isogenic pairs confirmed that MPXR was associated with loss of HAPT1 activity: 247MR and 386MR did not express the activity whereas both parental lines did. Figure S3 (available as Supplementary data at *JAC* Online) illustrates this in detail for the 247 isogenic pair and Table 2 summarizes the characterization of HAPT1 and LAPT1 of the 247 and 386 isogenic lines.

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

		Susceptibility[a]				Infectivity		Transport activity			
Strain	Subspecies	pentamidine	arsenical	Adapted from	Resistance induction	rodents	tsetse	P2	HAPT1	LAPT1	References
Lister 427	*T. b. brucei*	+++	+++	NA	NA	√	√[b]	√	√	√	14,16,17
2T1	*T. b. brucei*	+++	+++	Lister 427	NA	unkn	unkn	√	√	√	31,20
aqp2/aqp3 null	*T. b. brucei*	+/−	+	2T1	NA	unkn	unkn	√	NP	√	20
TbAT1-KO	*T. b. brucei*	++	++	Lister 427	TGD	√	unkn	NP	√	√	14
B48	*T. b. brucei*	−	+/−	TbAT1-KO	*in vitro*, pentamidine	√	unkn	NP	NP	√	16
P1000	*T. b. brucei*	−−−	+/−	B48	*in vitro*, pentamidine	unkn	unkn	NP	NP	√	—
STIB 247	*T. b. brucei*	+++	+++	NA	NA	√	√	√	√	√	33,57,52
STIB 247MR	*T. b. brucei*	+/−	−	STIB 247	*in vivo*, Cymelarsan	√	√	NP	NP	√	33,57,52
STIB 386	*T. b. gambiense*	+++	++	NA	NA	√	√	√	√	√	33,57,52
STIB 386MR	*T. b. gambiense*	+/−	−	STIB 386	*in vivo*, Cymelarsan	√	√	NP	NP	√	33,57,52
STIB 900	*T. b. rhodesiense*	+++	+++	NA	NA	√	unkn	√	unkn	unkn	56
STIB 900-M	*T. b. rhodesiense*	−	−	STIB 900	*in vitro*, Cymelarsan	√	unkn	NP	unkn	unkn	56
STIB 900-P	*T. b. rhodesiense*	−	+/−	STIB 900	*in vitro*, pentamidine	√	unkn	mut[c]	unkn	unkn	56

√, present; NP, not present; NA, not applicable; unkn, unknown; TGD, targeted gene deletion of the TbAT1 gene creating a *tbat1* null line; MR or -M, resistance induced to melaminophenyl arsenicals (melarsoprol and/or Cymelarsan); -P, resistance induced to pentamidine.
[a]Susceptibility to pentamidine or arsenical drugs is indicated on a relative scale as highly sensitive (+++) ranging to highly resistant to pentamidine or melaminophenyl arsenicals (−−−).
[b]The clone used in this paper is not tsetse transmissible but other clones of s427 have been shown to infect tsetse flies.[34]
[c]Bernhard *et al.*[56] 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, leading to $Gly^{430} \rightarrow$ Arg).

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

	Pentamidine		Propamidine	Pentamidine	
	HAPT1 K_m (μM)	HAPT1 V_{max} (pmol/10^7 cells/s)	HAPT1 K_i (μM)	LAPT1 K_m (μM)	LAPT1 V_{max} (pmol/10^7 cells/s)
STIB 247WT	0.029±0.001	0.008±0.002	14±2	49±19	0.65±0.17
STIB 247MR	NP			56±19	0.41±0.10
STIB 386WT	0.027±0.004	0.007±0.002	22±6	46±9	0.70±0.15
STIB 386MR	NP			51±2	1.2±0.4

Uptake of [^{3}H]pentamidine by suspensions of 10^7 procyclic trypanosomes was measured at 25 nM or 1 μM for the determination of parameters of high-affinity transport (HAPT1 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.

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 melarsoprol-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.

Expression of AQP2 reverses high levels of pentamidine and melarsoprol resistance

We used the laboratory-generated cell line B48[16] 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 *tbat1*$^{-/-}$ strain and additionally lacks HAPT1 activity.[16] 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*.[37] The lipophilic arsenical PAO was used as a control as it has been shown to diffuse across the *T. b. brucei* plasma membrane,[16] making its action

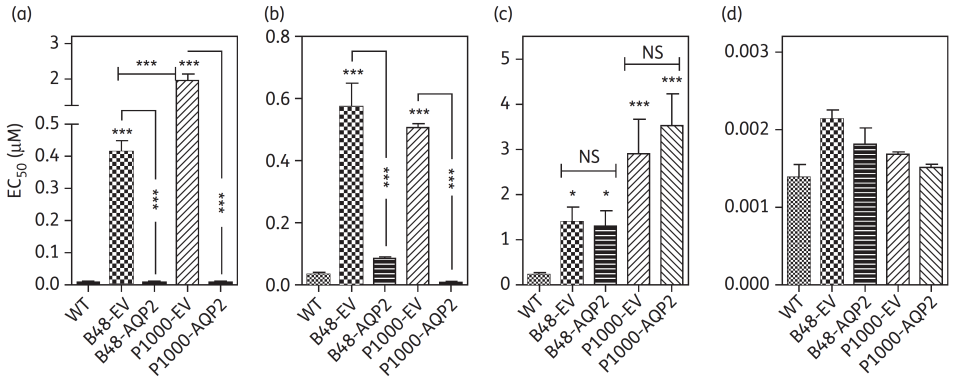

Figure 1. Expression of *TbAQP2* in multidrug-resistant trypanosomes sensitizes to (a) pentamidine and (b) Cymelarsan but not (c) diminazene or (d) phenylarsine oxide. EC_{50} values were obtained using the Alamar blue assay and bars represent the mean and SEM of 3 to >10 independent determinations; data were analysed for significant differences from the wild-type control using one-way ANOVA/Tukey's test (GraphPad Prism 5.0). NS, not significant; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; unless otherwise indicated, relative to wild-type controls. EV, empty-vector control.

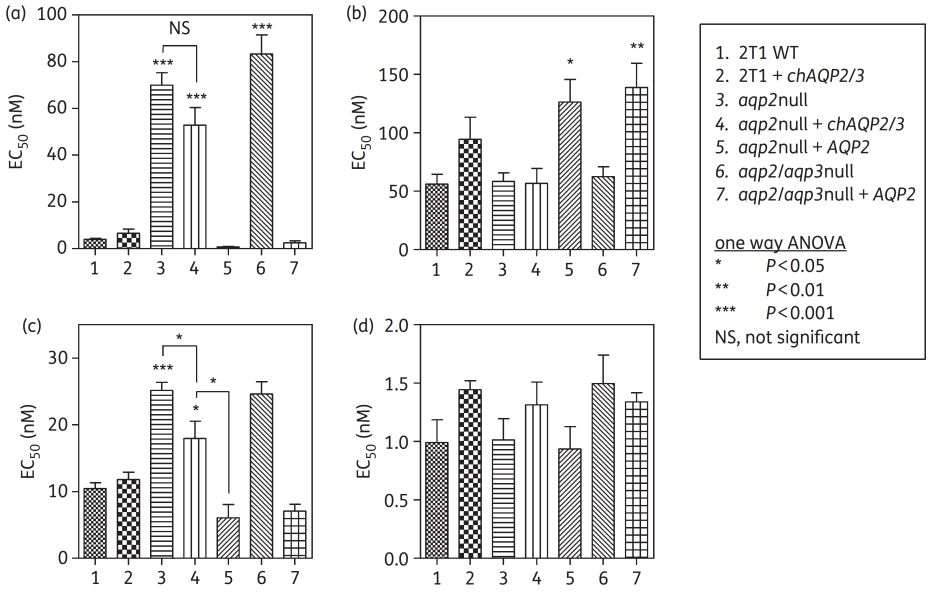

Figure 2. Expression of *TbAQP2* and *chAQP2/3* in *aqp2* null and *aqp2/aqp3* null trypanosomes. EC_{50} values were determined for (a) pentamidine, (b) diminazene, (c) Cymelarsan and (d) phenylarsine oxide. All data are the mean of ≥10 independent determinations. See legend of Figure 1 for details. Significance was tested relative to the wild-type control unless otherwise indicated.

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 diamidines 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).

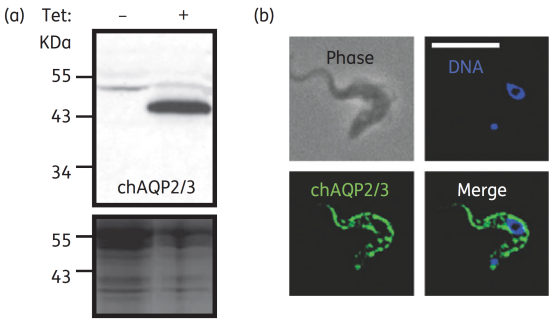

Figure 3. Localization of GFP-chAQP2/3 expressed in bloodstream-form *aqp2/aqp3* null trypanosomes. (a) Western blot using anti-GFP antiserum as described previously.[20] Blotting was performed after induction with 1 μg/mL tetracycline (+) or without induction as control (−). The Coomassie-stained panel shows relative loading. (b) GFP-chAQP2/3 was localized to the plasma membrane. Blue colour is DAPI staining of nucleus and kinetoplast. The scale bar represents 10 μm.

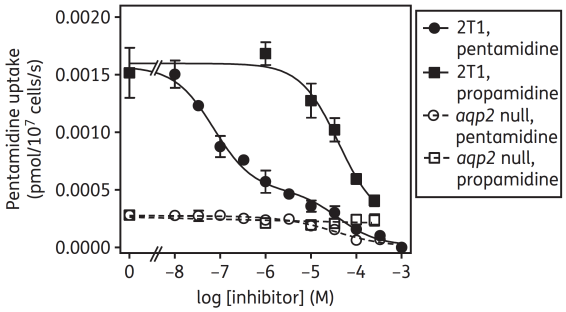

Figure 4. Uptake of 30 nM [^{3}H]pentamidine by *aqp2* null and wild-type control cells. Cells of *T. b. brucei* strain 2T1 (closed symbols) or the derived *aqp2* null strain (open symbols) were incubated with [^{3}H]pentamidine for 90 s in the presence or absence of various concentrations of unlabelled pentamidine (circles) or propamidine (squares). The incubation was stopped by the addition of 1 mL ice-cold 1 mM pentamidine and immediate centrifugation through oil. The experiment shown is representative of four identical but independent experiments, each performed in triplicate and showing virtually identical outcomes. Data points are mean ± SEM of triplicates.

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 [^{3}H]pentamidine in these cells and compare this with that in the parental 2T1 cells. We chose a [^{3}H]pentamidine concentration of 30 nM in the presence of 1 mM adenosine; this concentration of adenosine fully blocks the P2 aminopurine transporter[53] and the 30 nM label concentration produces a biphasic uptake that visualizes uptake through both high- and low-affinity pentamidine transporters (HAPT1 and LAPT1).[16] Propamidine was used as a specific inhibitor of HAPT1, having no effect on LAPT1 activity.[17] In four independent experiments in triplicate, [^{3}H]pentamidine uptake was assessed, in parallel, in WT and in *aqp2* null cells.

As expected in control trypanosomes, inhibition of the uptake of [^{3}H]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 ($IC_{50}=29.9\pm4.3$ 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 IC_{50} values that were entirely compatible with the HAPT1/LAPT1 system: 0.060 ± 0.017 and 29.9 ± 4.3 μM ($n=3$), respectively (Figure 4). In the *aqp2* null cells, [^{3}H]pentamidine uptake was much lower, completely lacked the high-affinity inhibition phase with unlabelled pentamidine and was insensitive to propamidine (Figure 4). The mean IC_{50} 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 K_m for LAPT1 (56.2 ± 8.3 μM).[17] We conclude that *aqp2* null cells do not express HAPT1, but do express LAPT1.

We previously reported that the pentamidine-resistant clone B48 similarly lacks HAPT1 pentamidine transport activity as a result of *in vitro* resistance selection with pentamidine[16] and that this clonal line lacks a WT *AQP2* gene.[20] 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 [^{3}H]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)[14] was used as the control, as this was the strain B48 was derived from.[16] Consistent with earlier findings, uptake of 50 nM [^{3}H]pentamidine was reduced 15.5-fold in B48 compared with in 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. K_m and V_{max} 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 K_m and V_{max} 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-

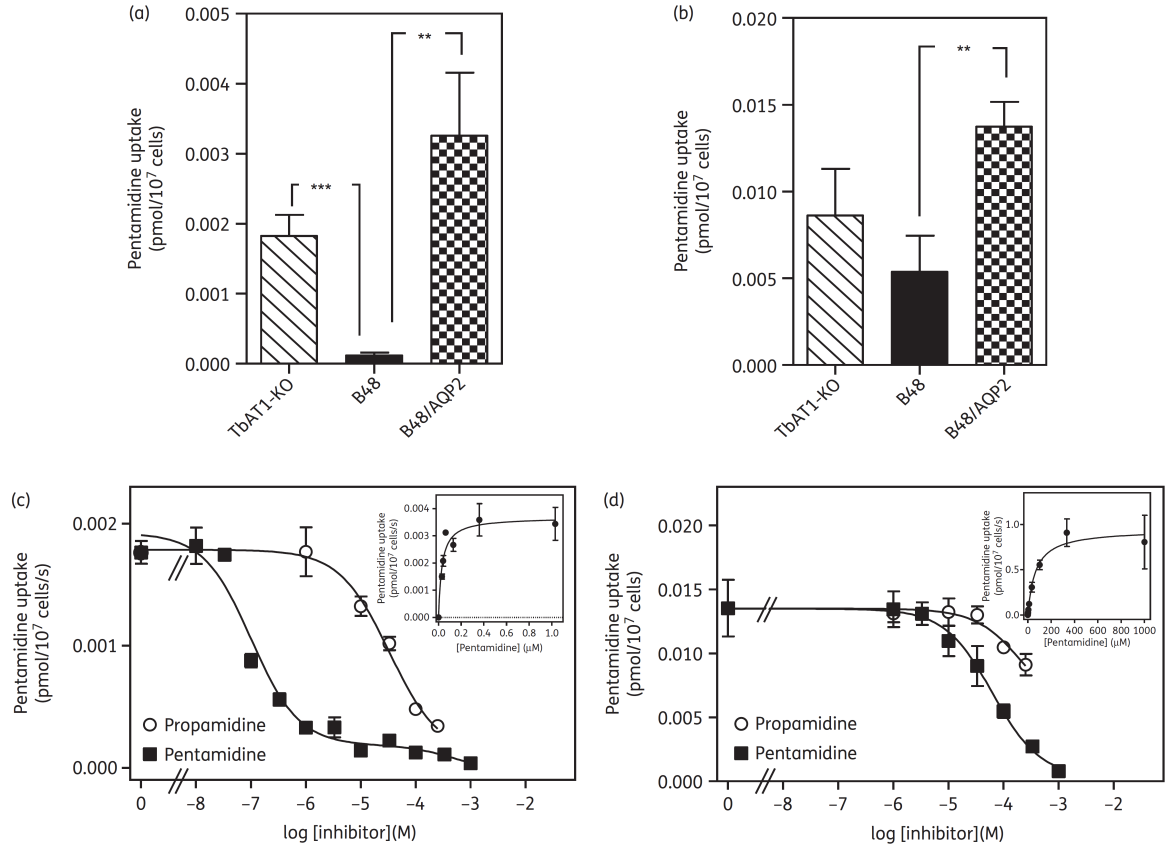

Figure 5. [^{3}H]pentamidine uptake by various s427-derived trypanosome lines. (a) [^{3}H]pentamidine concentration was 50 nM, reflecting predominantly HAPT1-mediated uptake. (b) [^{3}H]pentamidine concentration was 1 μM, reflecting predominantly LAPT1 uptake. Rates were determined from the slopes of 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 [^{3}H]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 [^{3}H]pentamidine. (d) Uptake of 1 μM [^{3}H]pentamidine. Solid squares, coincubation with various concentrations of unlabelled pentamidine; open circles, coincubation with propamidine. $^{**}P<0.01$; $^{***}P<0.001$.

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

Strain	HAPT1 pentamidine K_m (μM)	HAPT1 propamidine K_i (μM)	HAPT1 diminazene K_i (μM)	LAPT1 pentamidine K_m (μM)
WT s427[a]	0.036 ± 0.006	4.6 ± 0.7	63 ± 3	56 ± 8
TbAT1-KO[b]	0.029 ± 0.008	13.0 ± 3.0	ND	50 ± 17
B48[c]	NP			56 ± 7
P1000	NP			99 ± 24
B48/AQP2	0.046 ± 0.014	15.2 ± 1.6	ND	66 ± 1
L. mexicana/TbAQP2	0.055 ± 0.004	8.1 ± 0.8	100 ± 21	not present in these cells

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.
[a]De Koning.[17]
[b]Matovu *et al.*[14]
[c]Bridges *et al.*[16]

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* AQPs 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.[54] 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 [^{3}H]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 [^{3}H]pentamidine (50 nM) was measurable in *TbAQP2*-expressing promastigotes within 3 s, was linear with a rate of 0.032 ± 0.002 pmol/10^7 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 K_m and K_i 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 K_m 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

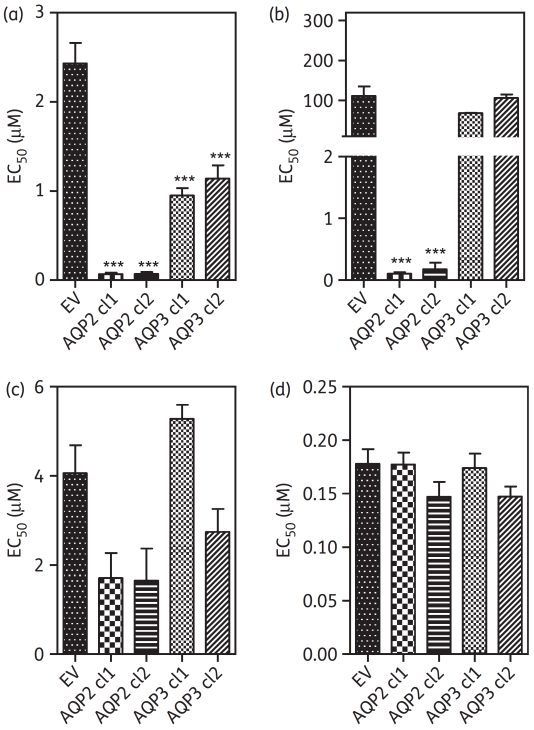

Figure 6. Effect of expression of *T. brucei* aquaporins on drug susceptibility of *Leishmania mexicana*. TbAQP2 and TbAQP3 were expressed in promastigotes using the pNUS vector. Two independent clones of each resulting cell line and the promastigotes transfected with the 'empty' pNUS vector were tested 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.

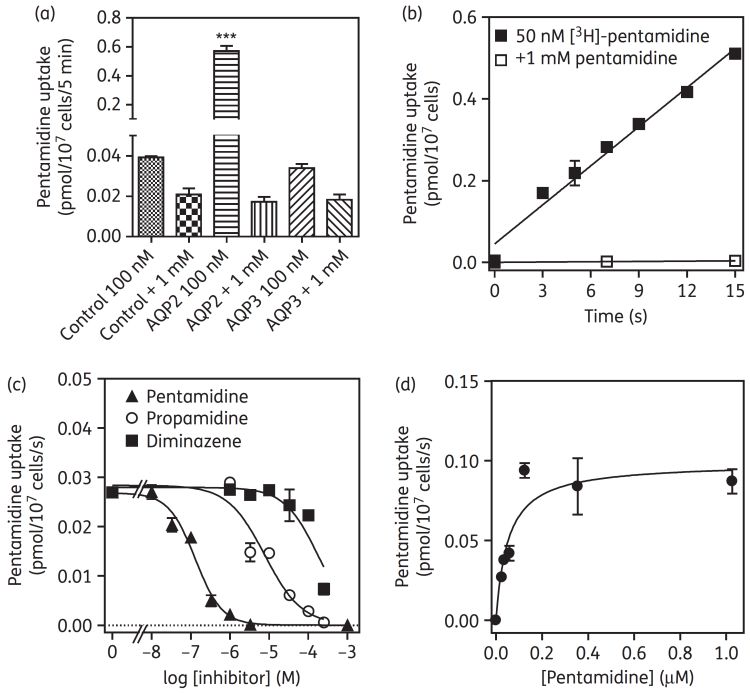

Figure 7. Expression of *T. b. brucei* aquaporins in promastigotes of *Leishmania mexicana*. (a) Specific uptake of 100 nM [^{3}H]pentamidine over 5 min in *L. mexicana* promastigotes transfected with empty pNUS vector (control), or promastigotes transfected with *TbAQP2* or with *TbAQP3*. In each case, mediated uptake of 100 nM radiolabel was compared with total association of [^{3}H]pentamidine with the cell pellet in the presence of a saturating concentration (1 mM) of unlabelled pentamidine. The data shown are the mean of triplicates ± SEM. ***$P<0.001$ by one-way ANOVA, compared with all other datasets. (b) Time course of 50 nM [^{3}H]pentamidine uptake, over 15 s, using *L. mexicana* promastigotes transformed with *TbAQP2* in the presence and absence of 1 mM unlabelled pentamidine. Uptake at 50 nM pentamidine was linear ($r^2=0.98$) and rapid (0.032 ± 0.002 pmol/10^7 cells/s, compared with 0.00026 ± 1.8×10^{-6} pmol/10^7 cells/s in the presence of 1 mM pentamidine). (c) Characterization of 20 nM [^{3}H]pentamidine uptake in *L. mexicana* promastigotes expressing TbAQP2, in the presence of varying concentrations of unlabelled inhibitor. (d) Michaelis–Menten plot of 20 nM [^{3}H]pentamidine uptake; conversion of pentamidine inhibition plot in (c).

sensitivity in *T. brucei*.[8,12] 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.[55] Indeed, we found that susceptibility to tubercidin (7-deazaadenosine) decreased 10-fold in the *tbat1*$^{-/-}$ strain ($EC_{50}=2.65\pm0.23$ 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 *chAQP2/3* contains most of the selectivity filter that is believed to determine the distinct permeation profiles of AQPs.[20] 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 is determined principally by the few amino acids of the selectivity filter. We used synthetic genes of *chAQP2/3* and of *TbAQP3* with a *TbAQP2* selectivity filter (*chAQP2/3*^{sf2} and *AQP3*^{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 transplantation of the 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 (EC_{50} values of 158 ± 2 and 131 ± 3 nM, $P<0.02$,

for *aqp2/aqp3* null and *AQP3*^{sf2}, respectively; Figure 8). In contrast, the cell line expressing the *chAQP2/3*^{sf2} 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*,[20] 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.[20] 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.[20,28] 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.[20] 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.[33]

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*;[20] this shows that the resistance is not to all aromatic diamidines or to 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,[14,50] which is absent in B48

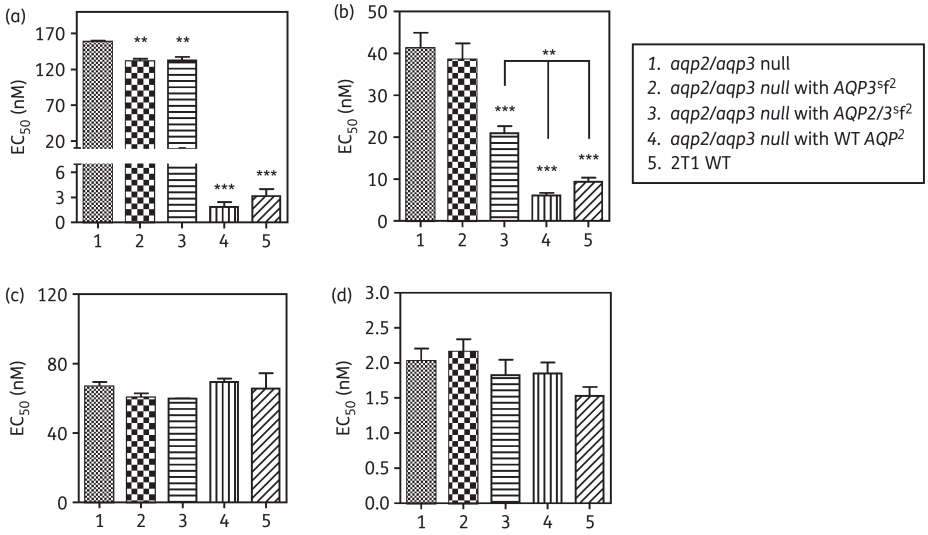

Figure 8. Expression of synthetic aquaporin constructs in bloodstream forms of the *aqp2/aqp3* double-null line. EC_{50} values were determined for (a) pentamidine, (b) Cymelarsan, (c) diminazene and (d) phenylarsine oxide. Expression was induced by the addition of 1 μg/mL tetracycline 24 h before setting 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.[16] Indeed, *TbAT1* is absent or mutated in all the drug-adapted MPXR strains used here,[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;[14] 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.*[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 *T. b. brucei* bloodstream forms, compared with expression *in vivo*,[15] dramatically reducing the influence of this transporter on drug sensitivity. Secondly, the pHD1336 vector used to reintroduce *TbAQP2* in B48 and P1000 gives a very robust expression level,[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*.[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 *T. b. brucei* strains B48, P1000 and 247MR and *T. b. gambiense* 386MR 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 *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 K_m value and inhibitor profile. Thus, the evidence overwhelmingly 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.*,[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*^{sf2} 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 *JAC* Online (http://jac.oxfordjournals.org/).

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Chapter 4

Aquaporin 2 mutations in *Trypanosoma brucei gambiense* field isolates correlate with decreased susceptibility to pentamidine and melarsoprol.

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Aquaporin 2 Mutations in *Trypanosoma brucei gambiense* Field Isolates Correlate with Decreased Susceptibility to Pentamidine and Melarsoprol

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Abstract

The predominant mechanism of drug resistance in African trypanosomes is decreased drug uptake due to loss-of-function mutations in the genes for the transporters that mediate drug import. The role of transporters as determinants of drug susceptibility is well documented from laboratory-selected *Trypanosoma brucei* mutants. But clinical isolates, especially of *T. b. gambiense*, are less amenable to experimental investigation since they do not readily grow in culture without prior adaptation. Here we analyze a selected panel of 16 *T. brucei* ssp. field isolates that (i) have been adapted to axenic *in vitro* cultivation and (ii) mostly stem from treatment-refractory cases. For each isolate, we quantify the sensitivity to melarsoprol, pentamidine, and diminazene, and sequence the genomic loci of the transporter genes *TbAT1* and *TbAQP2*. The former encodes the well-characterized aminopurine permease P2 which transports several trypanocides including melarsoprol, pentamidine, and diminazene. We find that diminazene-resistant field isolates of *T. b. brucei* and *T. b. rhodesiense* carry the same set of point mutations in *TbAT1* that was previously described from lab mutants. Aquaglyceroporin 2 has only recently been identified as a second transporter involved in melarsoprol/pentamidine cross-resistance. Here we describe two different kinds of *TbAQP2* mutations found in *T. b. gambiense* field isolates: simple loss of *TbAQP2*, or loss of wild-type *TbAQP2* allele combined with the formation of a novel type of *TbAQP2/3* chimera. The identified mutant *T. b. gambiense* are 40- to 50-fold less sensitive to pentamidine and 3- to 5-times less sensitive to melarsoprol than the reference isolates. We thus demonstrate for the first time that rearrangements of the *TbAQP2/TbAQP3* locus accompanied by *TbAQP2* gene loss also occur in the field, and that the *T. b. gambiense* carrying such mutations correlate with a significantly reduced susceptibility to pentamidine and melarsoprol.

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Introduction

The chemotherapy of human African trypanosomiasis (HAT, also known as sleeping sickness) currently relies on suramin or pentamidine for the first, haemolymphatic stage and on melarsoprol or eflornithine/nifurtimox combination therapy (NECT) for the second stage, when the trypanosomes have invaded the central nervous system (CNS) [1]. All five drugs have unfavorable pharmacokinetics and adverse effects. Melarsoprol is particularly toxic, causing severe encephalopathies in over 5% of the treated patients [2]. And yet, melarsoprol is the only treatment for late-stage *T. b. rhodesiense* infections. New and safer drugs are at various stages of (pre)clinical development, thanks largely to the Drugs for Neglected Diseases initiative (www.dndi.org). Two molecules that have successfully passed clinical Phase I trials are now being tested in patients: the nitroimidazole fexinidazole [3,4] and the benzoxaborole SCYX-7158 [5,6]. Both are orally available and cure 2nd stage *T. b. brucei* infections in a mouse model [7]. However, until new drugs for HAT are on the market, the current ones – problematic as they are – need to be used in a sustainable way. This requires an understanding of the mechanisms of drug resistance.

The mechanisms of drug resistance in African trypanosomes have been studied in the lab for over 100 years [8]. Two observations were made recurrently, namely (i) reduced drug uptake by drug resistant trypanosomes [9–14] and (ii) cross-resistance between melarsoprol and pentamidine [15,16]. Both phenomena were attributed to the fact that melarsoprol and pentamidine are taken up by trypanosomes via the same transporters, which appeared to be lacking in drug-resistant mutants. The first transporter identified was called P2 since it was one of two purine nucleoside transporters identified [17,18]. It is

Author Summary

Human African Trypanosomiasis, or sleeping sickness, is a fatal disease restricted to sub-Saharan Africa, caused by *Trypanosoma brucei gambiense* and *T. b. rhodesiense*. The treatment relies on chemotherapy exclusively. Drug resistance in *T. brucei* was investigated mainly in laboratory-selected lines and found to be linked to mutations in transporters. The adenosine transporter TbAT1 and the aquaglyceroporin TbAQP2 have been implicated in sensitivity to melarsoprol and pentamidine. Mutations in these transporters rendered trypanosomes less susceptible to either drug. Here we analyze *T. brucei* isolates from the field, focusing on isolates from patients where melarsoprol treatment has failed. We genotype those isolates to test for mutations in *TbAQP2* or *TbAT1*, and phenotype for sensitivity to pentamidine and melarsoprol. Six *T. b. gambiense* isolates were found to carry mutations in *TbAQP2*. These isolates stemmed from relapse patients and exhibited significantly reduced sensitivity to pentamidine and melarsoprol as determined in cell culture. These findings indicate that mutations in *TbAQP2* are present in the field, correlate with loss of sensitivity to pentamidine and melarsoprol, and might be responsible for melarsoprol treatment failures.

encoded by the gene *TbAT1* for adenine/adenosine transporter 1 [19]. Homozygous genetic deletion of *TbAT1* in bloodstream-form *T. b. brucei* resulted in pentamidine and melarsoprol cross-resistance, albeit only by a factor of about 2.5 [20]. This weak phenotype, together with the fact that the $TbAT1^{-/-}$ mutants still exhibited saturable drug import [21], indicated that further transporters are involved in melarsoprol-pentamidine cross-resistance [16,21,22]. One such transporter was recently identified, the aquaglyceroporin TbAQP2 [23,24]. Aquaporins and aquaglyceroporins belong to the major intrinsic protein (MIP) family and form channels that facilitate transmembrane transport of water and small non-ionic solutes such as glycerol and urea [25]. The three aquaporins of *T. brucei* (TbAQP1-3) are thought to physiologically function as osmoregulators and are involved in glycerol transport [26]. Aquaporins were described to mediate uptake of arsenite in mammalian cells [27] and in *Leishmania*, and loss of aquaporin function was implicated in heavy metal resistance [28]. Homozygous genetic deletion of *TbAQP2* in bloodstream-form *T. b. brucei* increased the IC_{50} towards melarsoprol and pentamidine by about 2- and 15- fold, respectively [24]. Moreover, a *T. b. brucei* lab mutant selected for high-level pentamidine resistance [21] carried a chimeric *TbAQP2* gene, where 272 nucleotides had been replaced by the corresponding sequence from a neighboring, very similar gene *TbAQP3* [24]. Differences in the *TbAQP2/TbAQP3* tandem locus on chromosome 10 were also observed between the reference genome sequences of *T. b. gambiense* DAL972 [29] and *T. b. brucei* TREU927 [23,30]. They possess identical versions of *TbAQP2* but differ in *TbAQP3* [31]. More recent field isolates of *T. brucei* ssp. have so far not been genotyped regarding their *TbAQP2/TbAQP3* locus.

The genotypic status of *TbAT1*, located proximal to a telomere on chromosome 5 [32], has been more intensely investigated. Point mutations in *TbAT1* were described, both in selected lab strains and in clinical *T. brucei* ssp. isolates, which rendered the gene non-functional when expressed in yeast [19]. The occurrence of these mutations correlated to a certain degree with melarsoprol treatment failure in 2nd stage *T. b. gambiense* HAT patients [33–36]. However, the relationship between polymorphisms in *TbAT1*, drug susceptibility, and treatment failure in patients is not fully resolved as the *TbAT1* mutant *T. b. gambiense* were not analyzed phenotypically. Such investigations are notoriously difficult since clinical *T. b. gambiense* isolates are hard to obtain (given the inaccessibility of HAT foci and the poor success rate of isolation and adaptation in rodents) and cannot readily be propagated in axenic culture. Here we concentrate on clinical *T. brucei* ssp. isolates from drug refractory cases that have been adapted to axenic *in vitro* cultivation, aiming to investigate whether mutations at the known melarsoprol and pentamidine transporter loci also occur in the field – and if so, whether such mutations are accompanied by loss of drug susceptibility.

Materials and Methods

Trypanosoma brucei ssp. isolates

The 16 analyzed isolates are described in Table 1 (origin) and Table 2 (clinical outcome). For more details on the recent isolates from the DRC please refer to Table S4 of Pyana et al (2011) [37]. All have previously been adapted to axenic cultivation. *T. b. brucei* and *T. b. rhodesiense* isolates were cultured in minimum essential medium (MEM) with Earle's salts with the addition of 0.2 mM 2-mercaptoethanol, 1 mM Na-pyruvate, 0.5 mM hypoxanthine, and 15% heat-inactivated horse serum as described by Baltz et al (1985) [38]. *T. b. gambiense* strains were cultured in IMDM medium supplemented according to Hirumi and Hirumi (1989) [39], plus 0.2 mM 2-mercaptoethanol, 15% heat-inactivated fetal calf serum and 5% human serum. The cultures were maintained under a humidified 5% CO_2 atmosphere at 37°C and were subpassaged 3 times a week to ensure growth in the exponential (log) phase.

Phenotyping

Drug sensitivity was determined with the Alamar blue assay as described by Räz et al (1997) [40], using the redox-sensitive dye resazurin as an indicator of cell number and viability. The trypanosomes were cultivated in 96-well microtiter plates in serial dilutions of drugs for 70 h. 10 ul of resazurin (125 ug/ml (Sigma) dissolved in PBS pH 7.2) was added to each well. The plates were further incubated for 2–4 hours for *T. b. rhodesiense* and *T. b. brucei*, and 6–8 hours for *T. b. gambiense*, before being read with a SpectraMax Gemini XS microplate fluorescence scanner (Molecular Devices) at an excitation wavelength of 536 nm and an emission wavelength of 588 nm. IC_{50} values were calculated by non-linear regression to a sigmoidal inhibition curve using SoftMax Pro software (V. 5.2). The IC_{50} values given in Table 2 are averages ± standard deviation of at least 3 independent assays (n = 3–12), each determined in duplicate. Melarsoprol (Sanofi-Aventis) was obtained from WHO. Pentamidine isothionate and diminazene aceturate were purchased from Sigma.

Genotyping

Genomic DNA was isolated from 10 ml dense trypanosome cultures. The cells were spun down and the pellets resuspended in 300 µl 10 mM TrisHCl pH 8, 1 mM EDTA and 3 µl 10% SDS was added before incubating for 10–15 min at 55°C. After 5 min incubation 3 µl of pronase mix (20 mg/ml, Sigma) was added to increase the stability of the extracted DNA. 90 µl of ice cold 5 M potassium acetate was added and the mixture was incubated for 5 min on ice. After spinning down for 5 minutes at max speed in a microfuge, the supernatant was transferred to a new tube and DNA was precipitated in 2–2.5 volumes of absolute ethanol, washed in 70% ethanol and dissolved in 20 µl ddH_2O. PCR was performed with Taq polymerase (Solis BioDyne, Estonia); the primers and annealing temperatures are summarized in Table S1.

Table 1. Origin of the analyzed *T. brucei* isolates.

Isolate	Species	Origin	Reference
STIB 930	*Tbg*	Republic of Côte d'Ivoire, 1978	[49]
ITMAP 141267	*Tbg*	Democratic Republic of the Congo, 1960	[50]
STIB 756	*Tbg*	Liberia, 1981	[51]
STIB 891	*Tbg*	Uganda, 1995	[33]
DAL 870R	*Tbg*	Republic of Côte d'Ivoire, 1985	[52]
DAL 898R	*Tbg*	Republic of Côte d'Ivoire, 1985	[52]
K03048	*Tbg*	South Sudan, 2003	[53]
45 BT (MHOM/CD/INRB/2006/1)	*Tbg*	Democratic Republic of the Congo, 2006	[37]
130 BT (MHOM/CD/STI/2006/02)	*Tbg*	Democratic Republic of the Congo, 2006	[37]
349 BT (MHOM/CD/INRB/2006/16)	*Tbg*	Democratic Republic of the Congo, 2006	[37]
349 AT (MHOM/CD/INRB/2006/19)	*Tbg*	Democratic Republic of the Congo, 2006	[37]
40 AT (MHOM/CD/INRB/2006/07)	*Tbg*	Democratic Republic of the Congo, 2006	[37]
STIB 900	*Tbr*	Tanzania, 1982	[52]
STIB 871	*Tbr*	Uganda, 1994	[54]
STIB 940	*Tbb*	Somalia, 1985	[42,55]
STIB 950	*Tbb*	Somalia, 1985	[41]

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PCR products were run on a 0.8% agarose gel and purified on a silica membrane column (Nucleospin gel and PCR clean up, Macherey Nagel, Germany). The purified PCR products were directly sequenced (Microsynth, Switzerland or GATC, Germany) with the same primers as used for PCR amplification. Only the *TbAQP2/TbAQP3* locus of *T. b. gambiense* K03048 produced two PCR products, which were cloned in pCR2.1-TOPO (Invitrogen). The assembled sequences were submitted to GenBank; accession numbers are listed in Table S2.

Results

A panel of *Trypanosoma brucei* ssp. field isolates

To be able to compare – and possibly correlate – genotype and phenotype of *T. brucei* ssp., we assembled a set of 16 isolates that had been adapted to axenic *in vitro* cultivation as blood-stream forms. These included 5 recent *T. b. gambiense* isolates from the Democratic Republic of the Congo (DRC), 2 older isolates from the Republic of Côte d'Ivoire and one isolate from South Sudan,

Table 2. Drug sensitivity (IC_{50} ± SD in nM), genotypic status of *TbAT1* and *TbAQP2*, and clinical outcome of melarsoprol treatment of the patients.

Isolate	MelB	Pentamidine	Diminazene	*TbAT1*	*TbAQP2*	Clinics
STIB 930	9.6±4.5	1.9±0.7	21.0±8.5	Ref	Ref	Cure
ITMAP 141267	15.0±8.1	8.3±3.4	9.9±4.4	WT	WT	Cure
STIB 756	6.2±1.1	1.3±0.7	24.7±7.9	WT	WT	Unknown
STIB 891	5.3±0.9	1.7±1.4	23.3±2.7	WT	WT	Unknown
DAL 870R	4.4±1.7	1.1±1.0	5.3±2.2	WT	WT	Relapse
DAL 898R	8.9±5.9	1.7±1.2	22.7±16.8	WT	WT	Relapse
K03048	24.8±9.2	81.2±21.9	58.0±33.6	WT	deletion/chimeric	Relapse
45 BT	25.9±8.6	91.8±29.7	37.5±10.8	WT	chimeric	Relapse
130 BT	42.3±17.6	76.9±22.3	12.3±4.5	WT	chimeric	Probable relapse
349 BT	26.2±11.3	71.9±12.4	20.0±3.2	WT	chimeric	Relapse
349 AT	25.6±11.8	81.9±31.8	15.4±1.0	WT	chimeric	Relapse
40 AT	22.0±8.0	72.2±21.1	39.9±16.7	WT	chimeric	Relapse
STIB 900	4.6±2.6	3.2±0.9	3.8±1.5	Ref	Ref	Cure
STIB 871	4.4±1.3	2.5±1.0	201±163	R allele	WT	Cure
STIB 940	13.6±7.0	3.4±2.0	340±218	R allele	WT	n.a.
STIB 950	27.6±9.4	1.8±0.4	102±53.6	R allele	WT	n.a.

WT = identical to reference (Ref) strain, being STIB 930 for *T. b. gambiense* isolates and STIB 900 for *T. b. brucei* and *T. b. rhodesiense* strains.
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which were all isolated from patients who had relapsed after melarsoprol chemotherapy. Other *T. b. gambiense* isolates from the DRC, northwestern Uganda, and Liberia were from patients who were successfully treated with melarsoprol or the treatment outcome is unknown. *T. b. gambiense* STIB 930 is a fully drug-susceptible lab strain that was used as a reference strain. We further included the field isolates *T. b. brucei* STIB 940, *T. b. brucei* STIB 950 and *T. b. rhodesiense* STIB 871, which are multidrug-resistant to isometamidium, diminazene and tubercidin. The fully drug-susceptible reference strain *T. b. rhodesiense* STIB 900 was included as a reference. The different isolates and their origin are summarized in Table 1. All isolates were genotyped regarding *TbAQP2* and *TbAT1*.

Naturally occurring mutations in *TbAQP2*

When the *TbAQP2/TbAQP3* genomic locus was amplified by PCR from the 16 *T. brucei* ssp. isolates, all the recent *T. b. gambiense* isolates from the DRC (40 AT, 45 BT, 130 BT, 349 BT and 349 AT) exhibited a smaller band than expected for the wild-type locus. Direct sequencing of the PCR product in each of the five isolates revealed only one gene at the locus: a chimeric version of *TbAQP2* and *TbAQP3*. The first 813 bp of the open reading frame perfectly matched *TbAQP2* while the remaining 126 bp derived from *TbAQP3* (Figure 1C). These 126 bp perfectly matched to *TbAQP3* of *T. b. rhodesiense* STIB 900 but this exact sequence is not found in the published genome of *T. b. gambiense* DAL 972. Note that the present *TbAQP2-TbAQP3* chimeric gene (Figure 1C) differs from the one described by Baker et al. from a pentamidine-selected *T. b. brucei* lab mutant (Figure 1B; [24]). *T. b. gambiense* K03048 from the South Sudan also gave rise to an abnormal pattern upon PCR amplification of the *TbAQP2/TbAQP3* locus from genomic DNA: a distinctly smaller double band instead of the expected product, indicative of heterozygosity. The smaller band contained the upstream region of *TbAQP2* followed by the open reading frame of *TbAQP3* while the *TbAQP2* open reading frame was missing (Figure 1D). The larger band contained a *TbAQP2/3* chimera similar to that encountered in the *T. b. gambiense* isolates of the DRC (Figure 1C). Point mutations in *TbAQP2* were encountered in the multidrug-resistant field isolates *T. b. brucei* STIB 940, *T. b. brucei* STIB 950 and *T. b. rhodesiense* STIB 871, all of which had the same 4 SNPs in *TbAQP2* compared to the *T. b. brucei* 927 reference gene (Tb927.10.14170), leading to the amino acid change threonine^{159} to alanine (Figure 1E). However, the same 4 SNPs also occurred in our drug-susceptible reference strain *T. b. rhodesiense* STIB 900, so they are not likely to be involved in the *mdr* phenotype [41,42] of these isolates. All other isolates analyzed had a wild-type copy of *TbAQP2*. The identified sequence polymorphisms are summarized in Table 2, GenBank accession numbers are in Table S2.

Naturally occurring mutations in *TbAT1*

All of the 12 analyzed *T. b. gambiense* isolates were identical in *TbAT1* sequence to the reference STIB 930 as well as to the genome strain DAL972. The previously described *TbAT1*^{R} allele [19,33] was found in the 3 *mdr* lines *T. b. brucei* STIB 940, *T. b. brucei* STIB 950 and *T. b. rhodesiense* STIB 871. *TbAT1*^{R} carries 5 coding and 4 silent mutations and a codon deletion as compared to the reference sequence (STIB 900), and the resultant protein appeared to be non-functional when expressed in *Saccharomyces cerevisiae* [19] or re-expressed in a *tbat1* null *T. b. brucei* (De Koning, unpublished results). The remainder of the isolates did not possess mutations in *TbAT1* when compared to the respective reference isolate. The GenBank accession numbers of all the sequences are in Table S2.

Correlating *TbAQP2* and *TbAT1* genotype to drug susceptibility

Drug sensitivities of the bloodstream-forms of all isolates were determined *in vitro* regarding melarsoprol, pentamidine, and diminazene. The five *T. b. gambiense* that possessed the chimeric *TbAQP2/3* gene (45 BT, 130 BT, 349 BT, 349 AT, 40 AT), as well as K03048 which carries a deletion of *TbAQP2* in one allele, in addition to one chimeric *TbAQP2/3* allele, all showed a similar drug sensitivity profile with markedly increased IC_{50} values towards pentamidine and, to a lesser extent, also melarsoprol (Figure 2). IC_{50} values were in the range of 70–92 nM for pentamidine and 22–42 nM for melarsoprol (Table 2); compared to the median of the four drug sensitive *T. b. gambiense* lines STIB 930, STIB 891, STIB 756 and ITMAP 141267, this corresponds to a 40- to 52-fold decrease in susceptibility to pentamidine and a 2.8- to 5.3-fold decrease for melarsoprol. The higher IC_{50} values of

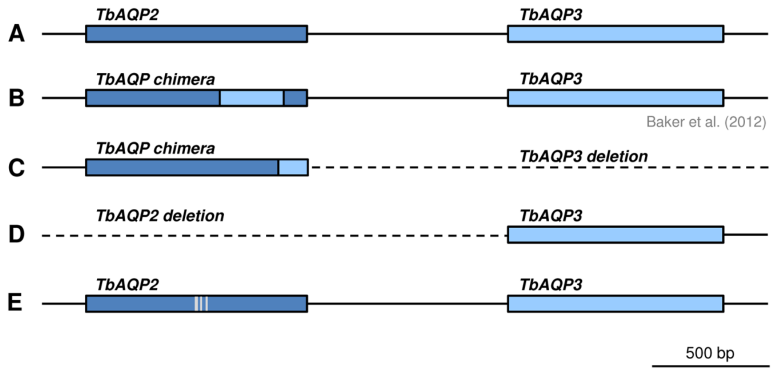

Figure 1. Schematic view of the *TbAQP2/TbAQP3* locus on chromosome 10. A) Reference locus of *T. b. brucei* TREU927, *T. b. gambiense* STIB 930 and *T. b. gambiense* DAL972 (minor differences in *TbAQP3* are not highlighted). B) Chimera of *TbAQP2* and *TbAQP3* as described by Baker et al. (2012) [24] for the *in vitro* selected, pentamidine-resistant *T. b. brucei* line B48. C) Chimera of *TbAQP2* and *TbAQP3* plus loss of *TbAQP3* in *T. b. gambiense* 40 AT, 45 BT, 130 BT, 349 BT, and 349 AT, and in one K03048 allele. D) Deletion of the *TbAQP2* ORF in the other *T. b. gambiense* K03048 allele. E) *TbAQP2* polymorphisms (C474A, G475A, C477T, T480C) in several *T. b. rhodesiense* and *T. b. brucei* isolates from East Africa (STIB 900, STIB 950, STIB 940, and STIB 871).
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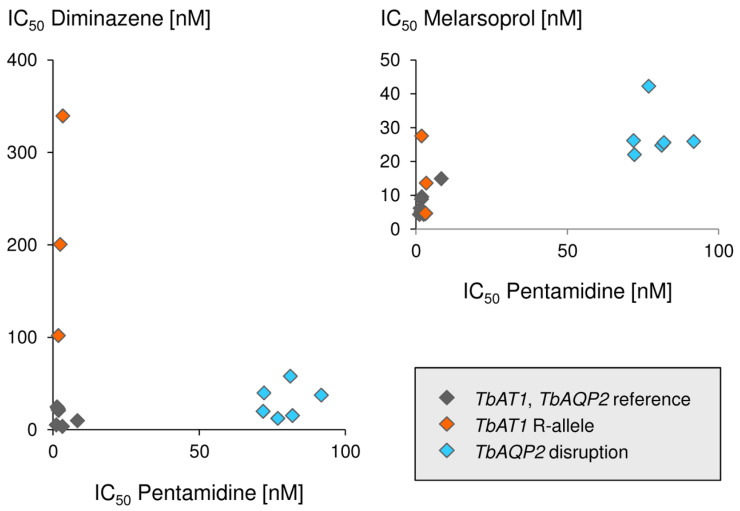

Figure 2. *In vitro* drug sensitivities. 50% inhibitory concentrations (IC_{50}) as determined with the Alamar blue assay. Susceptibility to pentamidine correlates with that to melarsoprol but not diminazene. *TbAT1* and *TbAQP2* genotypes are indicated.
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the isolates that carried a mutation in *TbAQP2* (n = 6) compared to the remainder (n = 10) were statistically significant both with respect to pentamidine (p = 0.0002, two-tailed Mann-Whitney test) and melarsoprol (p = 0.0047); no association was observed regarding *TbAQP2* status and sensitivity to diminazene. However, the isolates that carried the known resistance allele $TbAT1^R$ (i.e. STIB 940, STIB 950 and STIB 871) exhibited strongly increased IC_{50} values to diminazene (p = 0.01, two-tailed Mann-Whitney test) but not to pentamidine (Figure 2, Table 2). *T. b. brucei* STIB 950 also had an elevated IC_{50} against melarsoprol (Figure 2), but over all three $TbAT1^R$ isolates there was no significant effect on melarsoprol susceptibility.

Across all 16 *T. brucei* isolates, pentamidine sensitivity positively correlated with that to melarsoprol (Spearman's rank correlation coefficient of 0.67, p = 0.005) while there was no correlation between the two structurally related diamidines, pentamidine and diminazene (Figure 2).

Discussion

It is an intriguing phenomenon with African trypanosomes that drug resistance is predominantly linked to reduced drug import, typically arising from loss of function mutation of a non-essential transporter [12,19,24]. Here we investigated the aminopurine transporter TbAT1 and the aquaglyceroporin TbAQP2, two proteins known to be involved in uptake of – and susceptibility to – melarsoprol and diamidines in bloodstream-form *T. brucei*. While there is evidence for a link between *TbAT1* mutations and melarsoprol treatment failure in the field [33–36], the more recently identified gene *TbAQP2* has so far not been analyzed in a clinical setting. *TbAQP2* is dispensable for growth in culture [24] and partial gene replacement of *TbAQP2* with *TbAQP3* was observed in a pentamidine-selected *T. b. brucei* lab mutant [24] that displayed reduced infectivity to rodents [21]. However, it was unknown whether similar mutations also occur in the field, as they might bear a fitness cost in patients or during transmission by the tsetse fly. Concentrating on a panel of clinical *T. brucei* ssp. isolates that (i) derived from treatment-refractory cases and (ii) had been adapted to axenic *in vitro* culture, we have genotyped their *TbAT1* and *TbAQP2* loci, and phenotyped their *in vitro* sensitivity towards melarsoprol, pentamidine and diminazene. Our aim was to explore whether *TbAQP2* mutations occur in the field and if so, whether mutant isolates exhibit reduced drug susceptibility.

Five of the analyzed *T. b. gambiense* isolates, all from melarsoprol relapse patients of Dipumba Hospital in Mbuji-Mayi, DRC, carried only one gene at the *TbAQP2/TbAQP3* tandem locus, an unprecedented *TbAQP2/3* chimera. The high degree of sequence similarity between *TbAPQ2* and *TbAQP3* allows for homologous recombination between the two genes, leading to chimerization and gene loss. TbAQP2 has a unique selectivity filter with unusual NSA/NPS motifs instead of the characteristic NPA/NPA that occur in the vast majority of MIP family members [43] including TbAQP1 and TbAQP3 [24]. The published, pentamidine-resistant *T. b. brucei* lab mutant possessed a TbAQP2/3 chimera whose C-terminal filter triplet was from TbAQP3, suggesting that the unusual NPS triplet may be involved in pentamidine transport. However, the presently described pentamidine-resistant *T. b. gambiense* isolates carry a *TbAQP2/3* chimera encoding a predicted protein with both selectivity filter triplets from TbAQP2. We hypothesize that the TbAQP2/3 chimera observed in the *T. b. gambiense* isolates fails to contribute to pentamidine and melarsoprol susceptibility despite having the proposed selectivity filter residues of TbAQP2. Functional expression of the chimeric gene in *tbaqp2* null cells will be necessary to test this hypothesis.

The occurrence of rearrangements at the *TbAQP2/TbAQP3* locus correlated with reduced susceptibility to pentamidine and, to a lesser extent, melarsoprol. Thus field isolates also exhibit the well known cross-resistance between melarsoprol and pentamidine [15,16,31], while no cross-resistance was observed to diminazene aceturate. This is in agreement with *TbAT1* being the primary uptake route for diminazene [44,45] and consistent with results obtained using $TbAQP2^{-/-}$ cells, which showed no resistance to the rigid diamidines diminazene or DB75 [24], as opposed to pentamidine which has a highly flexible structure. It is also noteworthy that *T. b. rhodesiense* STIB 871 and *T. b. brucei* STIB 940 are susceptible to melarsoprol and pentamidine *in vitro* although both carry the $TbAT1^r$ allele. Loss of TbAT1 function has been described without mutations in the open reading frame of

the gene [32]. However, since in the present study all isolates with a 'wild-type' *TbAT1* ORF were fully susceptible to diminazene, we conclude that they possess a functional TbAT1 (i.e. P2) transporter. *Trypanosoma congolense* and *T. vivax* appear to lack an AT1 orthologue [46], therefore diminazene transport and resistance must have a different mechanism in these livestock parasites.

The plasma levels of pentamidine in treated patients peak about 1 hour after injection and vary extensively from 0.42 μM to 13 μM, while the mean elimination half-life after multiple applications is approximately 12 days [47]. Thus, since pentamidine is very potent, even a 50-fold increase in IC_{50} of pentamidine as observed here for the *T. b. gambiense* isolates with mutations in *TbAQP2*, is unlikely to jeopardize the success of treatment. With melarsoprol, however, the obtainable drug levels are more critical. Only 1–2% of the maximal plasma levels are seen in the CSF [48], and a 5-fold reduced sensitivity to melarsoprol might allow trypanosomes to survive in the CSF during melarsoprol therapy. Thus mutations in *TbAQP2* might indeed be responsible for melarsoprol treatment failures with *T. b. gambiense*. However, two of the *T. b. gambiense* isolates from relapse patients (DAL 870R and DAL 898 R) were sensitive to melarsoprol and pentamidine, and they possessed wild-type copies of *TbAT1* and *TbAQP2*, indicating that factors other than drug resistance can contribute to treatment failures. Larger sample sizes will be required to test the significance of *TbAQP2* for successful treatment. We show here for the first time that a *TbAQP2/3* chimera as well as loss of *TbAQP2* occurs in *T. b. gambiense* clinical isolates, and that the presence of such rearrangements at the *TbAQP2/TbAQP3* locus is accompanied by a 40- to 50-fold loss in pentamidine sensitivity and a 3- to 5-fold loss in melarsoprol sensitivity. We recommend genotyping of the *TbAQP2/TbAQP3* locus to be integrated into larger field trials such as clinical studies with drug candidates.

Supporting Information

Table S1 Primers used for PCR, their target gene, annealing temperature and sequence (5′ to 3′).
(PDF)

Table S2 GenBank accession numbers of the sequenced genes.
(PDF)

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Author Contributions

Conceived and designed the experiments: FEG PM. Performed the experiments: FEG TW MK. Analyzed the data: FEG PL. Contributed reagents/materials/analysis tools: PPP PB HPdK DH. Wrote the paper: FEG RB PB HPdK DH PM.

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Chapter 5

Chimerization at the AQP2-AQP3 locus is the genetic basis of melarsoprol-pentamidine cross-resistance in clinical *Trypanosoma brucei gambiense* isolates

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Abstract

Aquaglyceroporin-2 is a known determinant of melarsoprol-pentamidine cross-resistance in *Trypanosoma brucei brucei* laboratory strains. Recently, chimerization at the *AQP2-AQP3* tandem locus was described from melarsoprol-pentamidine cross-resistant *T. b. gambiense* isolates from sleeping sickness patients in the Democratic Republic of the Congo. Here, we demonstrate that reintroduction of wild-type *AQP2* into one of these isolates fully restores drug susceptibility while expression of the chimeric *AQP2/3* gene in *aqp2* null *T. b. brucei* does not. This proves that *AQP2-AQP3* chimerization is the cause of melarsoprol-pentamidine cross-resistance in the *T. b. gambiense* isolates.

Keywords

Human African trypanosomiasis; Sleeping sickness; *Trypanosoma brucei gambiense*; Drug resistance; Melarsoprol; Pentamidine; Aquaporin; Reverse genetics

1. Introduction

Trypanosoma brucei gambiense is the causative agent of West-African sleeping sickness and responsible for 98% of today's cases of human African trypanosomiasis (HAT) (Franco et al., 2014). HAT is a fatal disease whose treatment exclusively relies on chemotherapy. Only five drugs are available: suramin and pentamidine for the first, hemolymphatic stage of the disease, melarsoprol and nifurtimox/eflornithine combination therapy for the second stage, when the parasites have infested the cerebrospinal fluid. These drugs cause severe side effects and are difficult to administer (Brun et al., 2010). New drug candidates are in clinical development (Mäser et al., 2012), but until they will be available for treatment, the current drugs must be used sustainably. Therefore understanding the molecular mechanism of drug resistance is a prerequisite. Drug resistance studies with *T. b. brucei* lab strains have identified loss of drug uptake as the major mechanism of drug resistance in trypanosomes. This is due to mutations in the transporters responsible for drug uptake. The clinical drugs melarsoprol and pentamidine share two common transporter systems, the adenosine transporter 1 (TbAT1, also called P2; Carter and Fairlamb, 1993; Carter et al., 1995; Mäser et al., 1999) and aquaglyceroporin 2 (Baker et al., 2012). Genetic knock-out of either transporter gene, but particularly of *AQP2*, led to melarsoprol-pentamidine cross-resistance (MPXR) (Matovu et al., 2003; Baker et al., 2012; Baker et al., 2013).

Drug resistance of *T. b. gambiense* in the field has been controversial. The occurrence of mutant *TbAT1* alleles correlated to some extent with melarsoprol treatment failures (Matovu et al., 2001; Maina et al., 2007; Kazibwe et al., 2009), but no unambiguous genetic marker for resistance has been established so far. Recently, mutations at the *AQP2-AQP3* (Tb927.10.14170 / Tb927.10.14160) tandem locus were found in *T. b. gambiense* isolates from the Democratic Republic of the Congo (Graf et al., 2013). In particular, a set of 41 isolates from Mbuji-Mayi, a HAT focus of exceptionally high melarsoprol treatment failure rates (Pyana et al., 2014), all carried a chimeric aquaglyceroporin, presumably formed by homologous recombination between *AQP2* and *AQP3*; a putative single-strand annealing mechanism accompanied by deletion of segments of *AQP2* and *AQP3* (Graf et al., 2013). The chimera, the first 813 b derived from *AQP2* and the last 126 b from *AQP3*, was in-frame, transcribed and homozygous. The isolates carrying the chimeric gene exhibited a markedly decreased melarsoprol sensitivity *in vivo* (Pyana et al., 2014). Those that were tested *in vitro*

were cross-resistant to melarsoprol and pentamidine; to our knowledge, the first example of MPXR from clinical *T. b. gambiense* isolates (Graf et al., 2013).

Thus, the important question remained: Is the MPXR phenotype of the *T. b. gambiense* isolates from Mbuji-Mayi caused by the observed chimerization at the *AQP2-AQP3* locus? And if so, is it the presence of the *AQP2/3* chimera or the absence of wild-type *AQP2* that causes drug resistance? Here, we answer these questions by (i) re-introducing wild-type *AQP2* into one of the mutant *T. b. gambiense* isolates and (ii) expressing the chimeric *AQP2/3* gene from *T. b. gambiense* in *T. b. brucei*.

2. Materials and methods

2.1 Cell lines, cell culture and in vitro drug sensitivity assay

T. b. brucei 2T1 cells (Alsford et al., 2005) and 2T1 *aqp2-aqp3* double knock-out cells (Baker et al., 2012) were maintained in HMI-11 medium. Puromycin (0.2 µg/ml) and phleomycin (0.5 µg/ml) were added for 2T1 cells. For 2T1 aqp*2*-aqp*3* double knock-out cells blasticidin (10 µg/ml) and G418 (2 µg/ml) were added in addition. Hygromycin (2.5 µg/ml), instead of puromycin, was added after transfection with chimeric *AQP2/3*. *T. b. gambiense* 40AT (MHOM/CD/INRB/2006/07 (Pyana et al., 2011)) were cultured in HMI-9 medium with 15% FCS and 5% human serum, plus blasticidin (5 µg/ml) after transfection. *In vitro* drug sensitivities were determined as described (Graf et al., 2013). For the inducible cells, tetracycline (tet) was added 24 h prior to the assay.

2.2 Plasmids and transfection

The chimeric *AQP2/3* gene (GenBank accession KF564935; *AQP2/3*$_{814}$ according to the nomenclature by Pyana and Büscher (Pyana et al., 2014)) was amplified by PCR with primers AQP_HindIII_F (ccgcaagcttatgcagagccaaccagac) and AQP_BamH1_R (ccgcggatccttagtgtggcacaaaatatt), or AQP_Xba1_F (ccgctctagaatgcagagccaaccagac) and AQP_BamH1_R, and cloned into the pRPa-series of tetracycline-inducible expression vectors (http://www.lifesci.dundee.ac.uk/groups/david-horn/resources). Vector inserts were checked for fidelity by Sanger sequencing. Western blots were performed according to standard protocols. Bloodstream-form *T. b. brucei* were transfected as previously described (Baker et al., 2012). Clones were obtained by limiting dilution in standard HMI-11 medium plus antibiotics (see above). Bloodstream-form *T. b. gambiense* were transfected with pHDK21

and pHDK34 (Munday et al., 2014) as follows: $4x10^7$ cells were resuspended in 100 µl Tb-BSF nucleofection buffer (Schumann Burkard et al., 2011) (90 mM $NaHPO_3$, 5 mM KCl, 0.15 mM $CaCl_2$, 50 mM HEPES, pH 7.3) including 10 µg linearized plasmid DNA and placed in the nucleofection cuvette in the Amaxa Nucleofector (Lonza). Cells were electroporated using the program Z-001 and immediately transferred into 25 ml of pre-warmed HMI-9 medium containing 15% FCS, 5% human serum, and 20% sterile-filtered conditioned medium. Stable clones were obtained by limiting dilution and blasticidin selection (5 µg/ml). Correct integration was assessed by PCR on genomic DNA with primers primers AQP2_int_F (gtattggtgtggctgtcacg), AQP3_int_R (cccgttgagtaaccgatgtt), pAQP_F (aacacaccggtaccgtcatt) and pAQP_R (cttctcttgtgcgctgtacg).

3. Results and Discussion

3.1 Expression of wild-type AQP2 re-sensitizes drug-resistant T. b. gambiense

To test whether the lack of *bona fide AQP2* activity contributes to drug resistance in the isolates from Mbuji-Mayi, we introduced a 'wild-type' copy of *AQP2* into *T. b. gambiense* 40AT, isolated from a melarsoprol-relapse patient after treatment (Pyana et al., 2011). The gene was integrated into the highly transcribed *rRNA*-spacer locus. This shifted the IC_{50} of pentamidine from 108 nM to 2 nM and the IC_{50} of melarsoprol from 47 nM to 10 nM (Figure 1), a level similar to the fully susceptible *T. b. gambiense* reference isolate STIB930 (which had an IC_{50} of 2 nM for pentamidine and 10 nM for melarsoprol; Graf et al., 2013). No shifts were observed with diminazene aceturate, a diamidine that is not an AQP2 substrate (Munday et al., 2014), or with phenylarsine oxide (data not shown), an arsenical that diffuses through the plasma membrane. The same results were obtained with three additional clones. As a negative control, we transfected the 40AT cells with a non-functional *AQP2* mutant from the MPXR *T. b. brucei* clone B48 (Munday et al., 2014). As expected, this did not affect susceptibility to melarsoprol or pentamidine (Figure 1). These results demonstate that *AQP2* is key to drug susceptibility in the MPXR *T. b. gambiense* isolate.

3.2 Expression of the chimeric AQP2/3 in an aqp2-aqp3 null background

To test whether the chimeric AQP2/3 can complement AQP2 function with regard to drug uptake, we stably integrated the chimeric *AQP2/3* gene from *T. b. gambiense* 40AT, either untagged or GFP-tagged, under the control of the tetracyline operator in a *T. b. brucei* host

strain that expressed the tet repressor, and that carried a complete deletion of the *AQP2-AQP3* locus (Alsford et al., 2011). Tetracycline-inducible expression of the chimeric AQP2/3 protein was confirmed by immuno-fluorescence microscopy (data not shown) and by Western blotting with an anti-GFP antibody (Figure 2C). Drug sensitivities were determined *in vitro* for melarsoprol and pentamidine. None of the transfected cell lines showed a significant difference in IC_{50} to pentamidine or melarsoprol when expression of *AQP2/3* had been induced with tetracycline as compared to non-induced cells (Figure 2A). This held true irrespective of the presence of the GFP tag. Thus no potential function in drug susceptibility could be attributed to the AQP2/3 chimera. Expression of 'wild-type' *AQP2* using the same over-expression system (untagged and GFP-tagged) did not just reverse MPXR but actually hypersensitized the *aqp2-aqp3* double null *T. b. brucei* to pentamidine and melarsoprol (Baker et al., 2012).

3.3 Expression of chimeric AQP2/3 in wild-type cells does not affect drug sensitivity

Aquaporins form homotetramers where each monomer constitutes a single pore. Work on human aquaporins involved in diabetes insipidus has revealed that the expression of a mutant aquaporin can give rise to dominant negative effects (Mulders et al., 1998). To test for negative interactions of AQP2/3 with 'wild-type' AQP2, the chimera was expressed in parental *T. b. brucei* 2T1 cells. The same, tetracycline-inducible over-expression system was used. Again, no significant difference was observed regarding sensitivity to pentamidine and melarsoprol in tetracycline-induced versus uninduced cells (Figure 2B). Hence, the AQP2/3 chimera does not interfere with endogenous AQP2 function in *T. b. brucei* bloodstream-form cells.

4. Conclusion

Previous work on the correlation of occurrence of a chimeric *AQP2/3* gene in *T. b. gambiense* isolates from the DRC with *in vitro* drug sensitivity (Graf et al., 2013), suggested a functional link between the chimera and MPXR. However, proof of a causal relationship was lacking. The AQP2/3 chimeric protein consists mostly of AQP2 sequence, including the atypical second filter sequence (Baker et al., 2012). Overall, AQP2/3 of the *T. b. gambiense* from Mbuji-Mayi has only 9 amino acid differences with AQP2. Moreover, the *T. b. gambiense* isolates that harbored the chimeric gene are probably of clonal origin (Pyana et al., 2014), undermining the statistical significance of genotype to phenotype correlations. Thus reverse genetic engineering of bloodstream-form trypanosomes was required to establish a direct link between chimerization at the *AQP2-AQP3* locus in *T. b. gambiense* isolates and MPXR. This was only feasible because some of the isolates from Mbuji-Mayi had been adapted to axenic growth *in vitro* (Pyana et al., 2011).

The MPXR *T. b. gambiense* isolate 40AT was completely re-sensitized to melarsoprol and pentamidine when transfected with a wild-type copy of *AQP2*. This proves that the observed chimerization at the *AQP2-AQP3* locus is indeed the genetic basis of MPXR. The AQP2/3 chimeric protein did not exhibit any role in conferring drug sensitivity when over-expressed in *T. b. brucei*, neither in a *aqp2-aqp3* null background nor in the *AQP2-AQP3* wild-type background. This further demonstrates that it is the absence of 'wild-type' AQP2, and not the presence of the AQP2/3 chimera, that causes the MPXR phenotype. Deletion-based gene-fusion at the *AQP2-AQP3* locus by homologous recombination is likely facilitated by the high degree of sequence identity between *AQP2* and *AQP3*. Taken together, our findings strongly indicate that chimerization at the *AQP2-AQP3* locus causes melarsoprol-pentamidine cross-resistance in *T. b. gambiense*. This consequently increases the risk of treatment failures in problematic HAT foci such as Mbuji-Mayi of the Democratic Republic of the Congo. This is the first example where a genetic basis for drug-resistant sleeping sickness has been confirmed.

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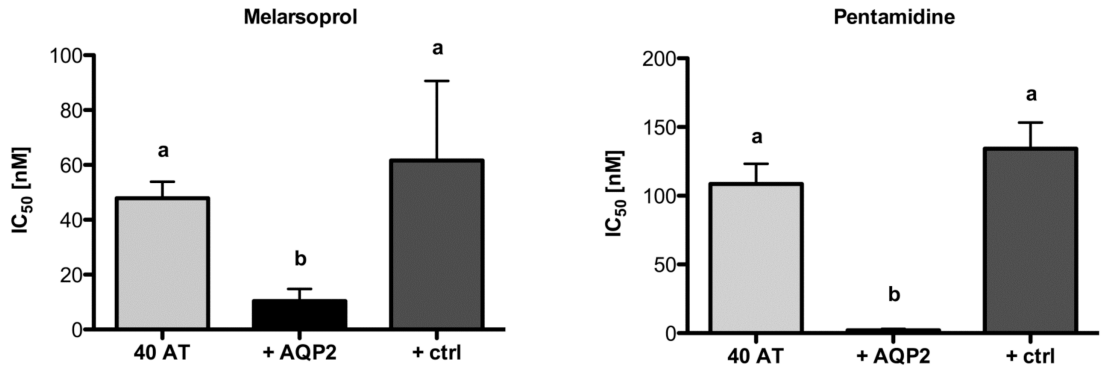

Figure 1. Re-introduction of *AQP2* into mutant *T. b. gambiense*. *In vitro* drug sensitivity of bloodstream-form *T. b. gambiense* 40AT (grey) transfected with *AQP2* (black) or dysfunctional *AQP2* (ctrl, dark grey). Error bars are standard errors of the mean. Small letters indicate significance groups as determined by one-way ANOVA and Tukey's post test using GraphPad Prism 5.0.

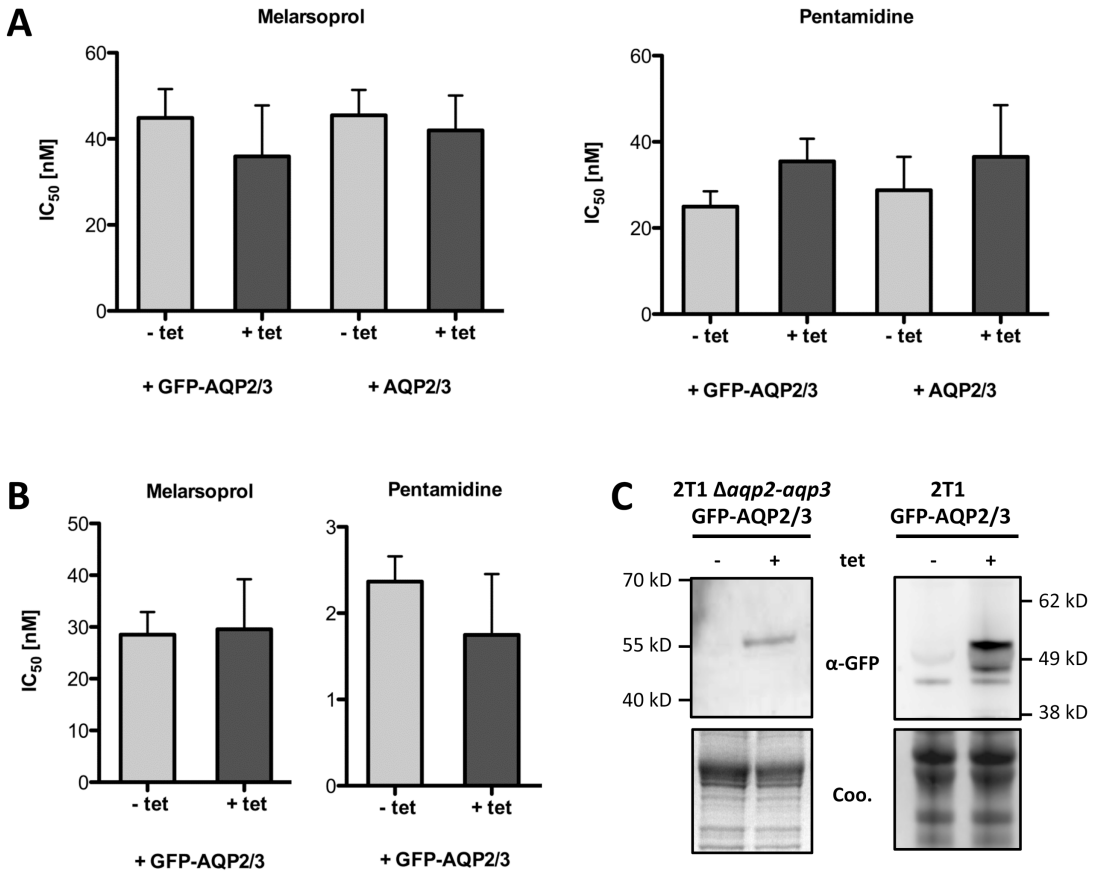

Figure 2. Expression of the *AQP2/3* chimera in *T. b. brucei.*

In vitro drug sensitivity of bloodstream-form *T. b. brucei* 2T1 *aqp2-aqp3* double null mutants (A) and parental 2T1 cells (B) transfected with a tetracycline (tet) inducible *AQP2/3* chimera. Dark bars, tet was added 24 h prior to the drug assay. Error bars are standard error of the mean. (C) Western blot with anti-GFP antibody demonstrating inducible expression of GFP-tagged *AQP2/3* (Coo, Coomassie stain).

Chapter 6

General discussion and conclusion

General discussion

With technical advance, in particular with the arrival of next generation sequencing technologies, it became possible to analyze and compare organisms on the scheme of the whole genome, transcriptome, proteome or metabolome. A milestone for the trypanosome research community was the completion of the *T. b. brucei* TREU927 genome in 2005 (Berriman 2005). Comparative genomics allows for comparison on different levels: comparison between different organisms, from subspecies to different domains of life, but also between different cell lines and populations within the same species. Its applications are very diverse and I will just name a few examples for research in infectious diseases. Host-pathogens comparisons to find e.g. drug targets (Ludin et al. 2012), vaccine candidates (Chaudhary and Roos, 2005) or molecular mimicry (Ludin et al., 2011). Comparison between different clones or isolates of the same species of pathogens can identify virulence factors, the genetic diversity and as has been done in my thesis drug resistance genes.

The valuable starting material had been two *T. brucei rhodesiense* lines independently selected for high level of melarsorpol and pentamidine resistance, respectively (Bernhard et al., 2007). The rational for working with two resistant lines, resistant to two unrelated drugs (amidines and arsenicals), was an observation made more than 60 years ago (Rollo and Williamson, 1951): Trypanosomes resistant to arsenical drugs (i.e. melarsoprol) were cross-resistant to amidines (i.e. pentamidine). This phenomenon was observed over and over again (Frommel and Balber, 1987; Pospichal et al., 1994; Scott et al., 1996; Bernhard et al., 2007; Graf et al., 2013) and held true in both selected STIB900 lines. Preliminary analysis of the two lines showed that STIB900-M lost *TbAT1*, at the time the only known resistance gene, causing melarsoprol-pentamidine cross-resistance (Carter and Fairlamb, 1993; Mäser et al., 1999). Homozygous disruption of both *TbAT1* alleles in a *T. b. brucei* line led to a resistance factor of only 2.5 to either drug (Matovu et al., 2003). Both selected *T. b. rhodesiense* lines have resistance factors a magnitude above that (Chapter 2). We concluded that more genes must be involved contributing to the resistance phenotype in those lines - which was confirmed during this thesis.

Comparative genomics and transcriptomics of drug resistance

We made use of next generation sequencing technologies, which became much cheaper and available for smaller labs in recent years, to investigate the whole genomes of the two resistant and the parental STIB900 lines. Moreover, we applied RNA sequencing (cDNA) to analyze and compare the gene expression levels genome-wide between the three lines (Chapter 2).

We confirmed the loss of *TbAT1* in the STIB900-M line. In STIB900-P, however, we detected a heterozygous coding point mutation G1288C (leading to G430R) (Chapter 2, Figure 4), which had previously not been known. Functional expression in a *tbat1* -/- background confirmed it to be non-functional (Chapter 2, Figure 6). The clearest hit was the gene encoding the aquaglyceroporin 2. Both lines had a deletion at its locus (Chapter 2, Figure 3). The third gene of interest was the RNA-binding protein *TbUBP1*. The exact same position was mutated in either line in a highly conserved RNA recognition motif (RRM). The mutation was homozygous and led to an amino acid change R131L (Chapter 2, Figure 5). Very few mutations have been accumulated and become fixed during the almost 2 years selection process.

The transcriptome data confirmed the deletion at the AQP2/3 locus and of *TbAT1* and its adjacent genes (Chapter 2, Figure 2). We only found those, except for some VSGs in STIB900-P, to be differentially expressed and did not pick up other significant differences in gene expression. Sequencing technologies advanced in recent years and technical replicates are considered unnecessary. But biological replicates are essential. We did only 2 biological replicates and, not optimally, they were conducted on two different Illumina sequencers (GenomeAnalyserIIx vs HiSeq2000). The first run was very noisy, but I still included the data to have biological replicates. This variance might have lead to the failure to detect genes differentially expressed with the R-based program DEseq (Anders and Huber, 2010) which I used to analyse the data. I also analysed the second run excluding the noisy run1 data, but did not find other genes than those that had already been detected with inclusion of the biological replicates of run1. Advancement of the technology and lower costs will allow more biological replicates in the future, which circumvents the above-mentioned

limitation. Barcoding allows for multiplex analysis, meaning that in one lane of a flow cell (where the sequencing reaction takes place) several different samples can be sequenced, which reduces costs. Illumina's HiSeq2000 sequencer produces currently up to 200 mio reads per lane. With the assumption of an average transcript length of 1500 b and 9000 different genes (transcripts) using single reads of 50 b, 270'000 reads are needed for 1x coverage of the transcriptome. Even pooling of 10 samples per lane can yield more than 50x coverage of each sample.

We mapped whole genome sequencing reads of the STIB900 parent genome to the *T. b. brucei* TREU927 reference genome (Berriman et al., 2005) to do the assembly and took the gene models (annotation) of the reference genome, which is of high quality. One minor limitation of that is that we would not be able to identify larger chromosomal differences between STIB900 and TREU 927. For comparative genomics, we used STIB900 as the reference for the two drug resistant STIB900-M and STIB900-P.

The coding point mutation in *TbAT1* occurred in all reads mapping to STIB900-P, we therefore assumed homozygosity. Direct Sanger sequencing from PCR products was also not clearly able to show that the mutation was in fact heterozygous. We digested a PCR product with the restriction enzyme NruI that specifically cuts the mutant allele and never achieved a complete digest. Cloning and Sanger sequencing of the PCR product revealed some clones with a wild type *TbAT1* (Chapter 2, Figure 4). The coverage of 7 reads at this position was limiting though. Higher coverage will be able to better discriminate between homo- and heterozygous loci.

The Spliced-Leader-Trapping protocol we used in the mRNA sequencing has two advantages: Firstly, it amplifies at the 5' end and thus produces all the reads from the transcription initiation site(s), which makes mapping easier and enables direct comparison between different genes. Secondly, it allows detecting splice variants and their relative abundance. But in the first point lies also its major drawback. Gene with identical 5' parts cannot be discriminated. This was exactly what happed with AQP2 - AQP3, as they are identical in the first 91 bases.

The algorithm does however separate the data in unique (single) mapper and multi mappers, but we had to correct manually.

TbAT1 was known and AQP2 was identified recently in a genome wide RNAi screen for pentamidine or melarsorpol resistance (Alsford et al., 2012) while we were analyzing our sequencing data. Both genes are now established as MXPR determinants and serve as a proof-of-principle for our approach of *in vitro* selection followed by sequencing and comparative genomics and transcriptomics. The genome-wide RNAi screen by Alsford and Horn proved very successful in identifying several new genes involved in resistance to all clinical HAT drugs. Major advantages of such screens are: Once the library is established they are very fast, many genes can be found in a single screen and networks of interactions can be revealed (e.g. Suramin, Chapter 1). However, one of the major limitations is that hits are limited to loss-of-function mutations. *In vitro* drug selection takes longer but can yield loss- as well as gain-of-function mutations. Potential compensatory mutations that correct for loss of fitness of a resistance mutation can also be found. The likelihood that a hit is relevant (i.e. able to emerge and spread in the field) is probably higher. Taken together NGS technologies applied to detect mutations causing drug resistance proved very successful but validation in the lab is essential.

TbUBP1: a new resistance gene?

Independent selection with melarsoprol and pentamidine led to the exact same coding point mutation (R131L) in the RNA-binding protein UBP1 in both resistant STIB900 lines (Chapter 2). The mutation is in the conserved RNA recognition motif (RRM) and the arginine is likely involved in RNA binding as proposed from NMR studies with the *T. cruzi* ortholog (Volpon et al., 2005). The exact physiological functions of TbUBP1 and the related TbUBP2 are unknown. It is suggested that they are involved and mRNA regulation (stabilization/degradation), but mRNA binding seems to be sequence unspecific because UBP1 and 2 are highly abundant and in 100-fold excess over mRNAs (Hartmann et al., 2007). TcUBP1 is predominantly localized in the cytoplasm but was shown to accumulate in the nucleus when the trypanosomes were under

arsenite stress, and mutations affecting RNA-binding prevented nuclear accumulation of TcUBP1 (Cassola and Frasch, 2009). Hence, TcUBP1 may be involved in nuclear shuttling of mRNAs. The localization of TbUBP1 was confirmed to be mainly cytoplasmic (Chapter 2, Figure 5B) and in preliminary experiments GFP-UBP1-WT, but also UBP1-R131L, accumulated in the nucleus after arsenite treatment (unpublished). Functional (over)expression of the wild-type and the mutant UBP1 indicate that the mutant is either non-functional or functions differently (e.g. RNA binding properties, stability), because overexpression of wild-type UBP1 showed a drastic growth deficit while overexpression of UBP1-R131L did not (Chapter 2, Figure 5E). UBP1-R131L expressing cells were approximately two fold more susceptible to pentamidine compared to cells where the expression was repressed, but no difference was observed with melarsoprol (Chapter 2, Figure 5F). A clear link to drug resistance could not be established but nonetheless it is likely that both drugs somehow influence TbUBP1, otherwise the mutation would not have become fixed in the population. Further investigation could shed light into the matter. Wild-type UBP1 could be knocked-out in those cell line carrying an ectopic copy of UBP1-WT or UBP-R131L under the TET repressor, even if UBP1 is essential as described elsewhere (Hartmann et al., 2007). Moreover, it is important to test whether the mutant UBP1 is still capable of binding mRNA. RNAi targeting UBP1 and UBP2 revealed little effect on the transcriptome when assessed by microarray (Hartmann et al., 2007). Potentially, RNA sequencing could be performed when UBP1-R131L is overexpressed to find the mRNAs that are regulated by it, but such results should be treated with caution as overexpression may lead to non-specific binding and secondary effects.

The role of AQP2 as drug resistance determinant

Aquaglyceroporin 2 has drawn a lot of attention since its involvement in MPXR has been discovered in Sam Alsford and David Horn's RNAi screen (Alsford et al., 2012) as it is a very atypical drug transporter. *T. brucei* possesses 3 aquaporins, belonging to the subfamily of aquaglyceroporins (TbAQP1-3). Natural substrates of aquaglyceroporins are water and small neutral solutes, in particular glycerol

and dihydroxyacetone for TbAQPs (Uzcategui et al., 2004). Classical aquaporins transport exclusively water (Agre et al., 2002). There is a major doubt whether pentamidine and melarsoprol can actually directly pass the pore of AQP2 as the molecules are much larger than its natural substrates and pentamidine has 2 positive charges at physiological pH. Trivalent antimony or arsenite are known substrates of aquaglyceroporin but they are smaller and may structurally mimic glycerol (Porquet and Filella, 2007). Other diamidines, diminazene or DB75, seem not to be AQP2 substrates (Chapter 3 and 4). They differ in the linker group separating the two amidine moieties compared to pentamidine. Pentamidine has an alkyl-chain (5-C) linker, which makes the molecule highly flexible. AQP2 has a very unusual and unique selectivity filter sequence. It lacks the highly conserved selectivity filter motifs of NPA and aromatic arginine (ar/R) found in most AQPs but has a NSP/NPS and IVLL motif instead (Uzcategui et al., 2004; Baker et al., 2012). Replacement of the arginine with a leucine at position 262 in AQP2 may widen the pore opening allowing pentamidine to bind. Intriguingly, AQP2 seems to be restricted to the flagellar pocket (Baker et al., 2012), an invaginated structure of the plasma membrane where endo - and exocytosis exclusively occur (Overath and Engstler, 2004; Field and Carrington, 2004). One hypothesis is that pentamidine and melarsorprol are actually taken up via endocytosis and AQP2 functions as its receptor. *T. brucei* has the fastest endocytosis and membrane turnover found in nature (Overath et al., 1997; Field and Carrington, 2009) likely linked to VSG coat maintenance and antibody clearance (Engstler et al., 2007). However, expression of AQP2 in *Leishmania mexicana* promastigotes induced a high affinity pentamidine transport activity and sensitized the cells 40-fold to pentamidine and more than 1000-fold to the melaminophenyl arsenical cymelarsan (Chapter 3, Figure 6). An initial idea that AQP2 may regulate another transporter in drug uptake can be rejected from this experiment. The localization of the introduced AQP2 in *Leishmania* was not assessed and potentially drug uptake may also occur via endocytosis. Although insect form of both *T. brucei* and *Leishmania* seem to have a decreased rate of endocytosis (Overath and Engstler, 2004; Field and Carrington, 2009) and Leishmania are lacking the electron dense clathrin-like structures around the endocytotic vesicles as seen in

blood-stream forms *T. brucei* and possibly linked the unprecedented speed of endocytosis (Overath et al., 1997). To test how melarsoprol or pentamidine or both are taken up, a different heterologous system (e.g. Xenopus Oocytes) could be used for direct transport or live imaging or fluorescence microscopy with fluorescently labeled pentamidine, if it still binds to AQP2 with high affinity) may be able to demonstrate how melarsoprol or pentamidine or both are taken up. Whether or not the drugs directly pass AQP2 or follow the endocytotic pathway - loss of AQP2 function leads to MPXR.

All cell lines selected for arsenical (melarsorpol or cymelarsan) or pentamidine resistance, either *in vitro* or *in vivo*, which have been genotyped showed mutations at the AQP2 locus (Chapter 3). Ranging from a clear-cut deletion in the STIB900 lines to more complicated rearrangements leading to chimerisation of AQP2 with the neighbouring AQP3 while sometimes retaining wild type AQP3. But common to all is a loss of the wild-type AQP2 gene. The predominant mechanism by which AQP2 is lost is caused by chimerization at the AQP2 - AQP3 tandem locus, most likely driven by the high sequence similarity between the two genes and homologous recombination. In case of the K048 isolate from South Sudan, two times independently, AQP2 was lost. One allele was deleted with wild type AQP3 still present whereas the 2nd allele showed a deletion that led to a similar chimera in the isolates from the DRC (Chapter 4). Whether the isolates from the DRC had lost AQP2 on both alleles through chimerization or at one allele and the second AQP2 was deleted through gene conversion from the first allele is unknown. Pyana and colleagues detected a different chimera in those lines, which remained undetected with direct sequencing, and was only found in one line after cloning and sequencing. Restriction digest to discriminate the two chimeras, however, indicated that all 41 isolates carry both genes. The second chimera contains the last 60 bp from AQP3 and might be at a different locus in the genome (Pyana et al., 2014). The resistant STIB900 lines lost the AQP2 ORF and still carry the wild type AQP3 ORF. But also this was likely achieved through recombination at the locus. In fact, it is possible that parts of AQP3 actually stem from AQP2, but we cannot tell, as the two sequences are largely identical.

AQP2 mutations implied in clinical resistance

We looked at clinical isolates, particular *T. b. gambiense* from melarsoprol relapse patients, to investigate if we find mutations at the *AQP2* locus or *TbAT1*. 6 out of 16 isolates carried deletions at the AQP2 / AQP3 locus, which led to a chimera between the two genes, which was in frame and transcribed (Chapter 4, Figure 1). All isolates carrying the chimera had increased IC_{50} values to both melarsoprol and pentamidine (Chapter 4, Table 2 and Figure 2). Pyana and colleagues further demonstrated that all isolates from Mbujj-Mayi in the DRC they looked at (41 isolates, of which 10 pairs from the same patient before and after treatment) carried the same chimera and all showed lower *in vivo* sensitivity to melarsoprol when tested in the acute *T. b. gambiense* mouse model. 10 mg/kg BW was needed to cure most of the isolates, which is about 4 times higher as for drug sensitive *T. b. gambiense* lines (Pyana et al., 2014). All isolates carry the exact same chimera. Analysis of mobile genetic elements found only small variation between the isolates, favoring the hypothesis of clonal expansion. In a follow up study 6-8 microsatellite markers were analyzed for genetic variation among the strains depending on treatment outcome, but genetic differentiation was not possible (Pyana et al., 2015). The chimera still has the unusual selectivity filter residues of AQP2 but is not able to take up the drugs (Chapter 5, Figure 2). Possible explanations may be that the pore is slightly different so the drugs no longer bind, the chimera fails to form functional tetramers, trafficking to the membrane is impaired or retention in the flagellar pocket is lost (if the localization there is essential for drug uptake). In preliminary experiments the localization could not be clearly defined with the GFP-tagged chimeric AQP by fluorescence microscopy (unpublished). The clearest indication that mutations at the AQP2 locus are responsible for resistance is, in my opinion, the fact that a wild-type copy of AQP2 in expressed in the 40AT line increased drug sensitivities for pentamidine and melarsoprol to levels comparable to the sensitive reference isolates (Chapter 5, Figure 1). Loss of AQP2 is a strong risk factor for melarsoprol treatment failure, but does not lead to 100% of relapses. There are other factors that may influence treatment

outcome, e.g. host genetics, immunological status, nutrition and advancement of the disease.

I recommend using AQP2 as a genetic marker for melarsoprol and pentamidine resistance. But DNA based tests by PCR are not straightforward. Amplification of the AQP2/AQP3 locus to test for the presence or deletions within the approximately 3.2 kb band may be indicative but not conclusive without sequencing of the PCR product. A fluorescence-based test with fluorescent diamidines as described by Stewart and colleagues (Stewart et al., 2005) is not feasible as the fluorescent diamidines are not AQP2 substrates. However, should pentamidine be taken up via endocytosis mediated by AQP2, it might be possible to covalently link a fluorescent dye to pentamidine as long as pentamidine is still capable of binding to AQP2 with high affinity. A further implication for drug development should be to use aqp2 null cells, or aqp2 - tbat1 double null cells in drug screens for new molecules to test for cross-resistance. New drugs should ideally be taken up by an essential transporter/uptake route or by several different routes - preferably not the same as the current drugs.

Conclusion

The goal of this thesis was to find new genes involved in drug resistance to melarsoprol and pentamidine and their cross-resistance in *Trypanosoma brucei*. We applied next generation sequencing technology (whole genome sequencing and RNA sequencing) to compare two drug resistant *T. b. rhodesiense* lines with their drug sensitive parent. Three distinct genes were found to be either absent or mutated in both resistant lines. (i) The adenosine transporter 1 (*TbAT1)*, which was already known to be involved in the uptake of melarsoprol and pentamidine and their cross-resistance, (ii) the aquaglyceroporin (*AQP2*), which was newly discovered and validated within the time frame of this thesis and (iii) the RNA-binding protein (*TbUBP1*), which emerged as a potential new resistance candidate. Hence, our approach was successful and, *TbAT1* and *AQP2* in particular can be considered as controls for proof-of-principle.

Furthermore, I showed that several clinical *T. b. gambiense* isolates are mutated at the *AQP2* locus and there was a strong correlation with decreased drug susceptibility to melarsorpol and pentamidine - the first demonstration of MPXR in *T. b. gambiense* field isolates. The mutation, leading to chimerazation of *AQP2* with the neighboring *AQP3*, did not complement loss of *AQP2* in knock-out cells whereas introduction of the wild-type gene in one of the *T. b. gambiense* isolates rendered the cells sensitive to the drugs. Thus, *AQP2* mutations are the most likely cause for the high incidence of melarsoprol treatment failure in Mbuji-Mayi in the Democratic Republic of Congo and are clinically relevant.

With elimination of HAT on the agenda it is of great importance to further monitor drug resistance in the field. With *AQP2* we have a genetic marker for melarsoprol and pentamidine resistance, which is a prerequisite to study the molecular epidemiology of drug resistance. The occurrence of *AQP2* mutations in the field stresses the need for new drugs once again, because melarsorpol is the only treatment option for 2nd stage *T. b. rhodesience* infections.

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