Melarsoprol Sensitivity Profile of *Trypanosoma brucei gambiense* Isolates from Cured and Relapsed Sleeping Sickness Patients from the Democratic Republic of the Congo

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Abstract

**Background:** Sleeping sickness caused by *Trypanosoma brucei (T.b.) gambiense* constitutes a serious health problem in sub-Saharan Africa. In some foci, alarmingly high relapse rates were observed in patients treated with melarsoprol, which used to be the first line treatment for patients in the neurological disease stage. Particularly problematic was the situation in Mbuji-Mayi, East Kasai Province in the Democratic Republic of the Congo with a 57% relapse rate compared to a 5% relapse rate in Masi-Manimba, Bandundu Province. The present study aimed at investigating the mechanisms underlying the high relapse rate in Mbuji-Mayi using an extended collection of recently isolated *T.b. gambiense* strains from Mbuji-Mayi and from Masi-Manimba.

**Methodology/Principal Findings:** Forty five *T.b. gambiense* strains were used. Forty one were isolated from patients that were cured or relapsed after melarsoprol treatment in Mbuji-Mayi. *In vivo* drug sensitivity tests provide evidence of reduced melarsoprol sensitivity in these strains. This reduced melarsoprol sensitivity was not attributable to mutations in *TbAT1*. However, in all these strains, irrespective of the patient treatment outcome, the two aquaglyceroporin (AQP) 2 and 3 genes are replaced by chimeric *AQP2/3* genes that may be associated with resistance to pentamidine and melarsoprol. The 4 T.b. gambiense strains isolated in Masi-Manimba contain both wild-type *AQP2* and a different chimeric *AQP2/3*. These findings suggest that the reduced *in vivo* melarsoprol sensitivity of the Mbuji-Mayi strains and the high relapse rates in that sleeping sickness focus are caused by mutations in the *AQP2/AQP3* locus and not by mutations in *TbAT1*.

**Conclusions/Significance:** We conclude that mutations in the *TbAQP2/3* locus of the local *T.b. gambiense* strains may explain the high melarsoprol relapse rates in the Mbuji-Mayi focus but other factors must also be involved in the treatment outcome of individual patients.

Introduction

Human African trypanosomosis (HAT) is a parasitic disease transmitted by tsetse flies (*Glossina sp*) and caused by *Trypanosoma brucei (T.b.) gambiense* and *T.b. rhodesiense*. This disease constitutes a serious public health problem in sub-Saharan Africa, particularly in central African countries like the Democratic Republic of the Congo (DRC), Central African Republic (CAR), the Republic of the Congo and the Republic of South Sudan [1]. The disease evolves from an early stage with trypanosomes invading blood, lymph and peripheral tissues towards the late or neurological stage with invasion of the brain. Chemotherapy of HAT relies on five drugs (eflornithine, melarsoprol, nifurtimox, pentamidine and suramin). Early-stage HAT is treated with pentamidine (*T.b. gambiense*) or suramin (*T.b. rhodesiense*). For treatment of the neurological stage, drugs that are able to pass the blood-brain-barrier, such as melarsoprol, nifurtimox or eflornithine, are necessary [2]. Until recently, melarsoprol was the first line treatment of late stage *gambiense* and *rhodesiense* HAT but for *gambiense* HAT, the nifurtimox-eflornithine combination therapy (NECT) is now recommended by the World Health Organization as first line treatment [3]. This recommendation follows the observation that NECT is as effective as melarsoprol monotherapy with less severe side effects and that NECT is able to cure patients that experienced a relapse after treatment with melarsoprol monotherapy [4]. Traditionally, 5–10% of *gambiense* HAT patients treated with melarsoprol could not be cured but in the last decade up to >50% relapse rates were reported in Angola,
Author Summary

Sleeping sickness, or human African trypanosomosis, constitutes a serious health problem in sub-Saharan Africa. Treatment is a key factor in the control of this disease, not only to save the lives of the individual patients but also to stop transmission. As for other infectious diseases, drug resistance forms a constant threat for sleeping sickness control. Understanding the mechanisms underlying drug resistance is of uppermost importance for evidence-based adaptation of treatment protocols as well as for the development of new drugs. In this study we investigated the phenotype and genotype of more than 40 recently isolated strains of Trypanosoma brucei gambiense, the parasite that causes sleeping sickness in West and Central Africa. By comparing the parasites from cured and from relapsing patients in two separated sleeping sickness foci, we can explain the difference in treatment failure rates between these two sleeping sickness foci in D.R. Congo. We also provide evidence that treatment outcome in the individual patient is not exclusively defined by the drug resistance genotype of the infecting parasite but that other factors must be involved.

Central African Republic, Democratic Republic of the Congo, Republic of South Sudan and Uganda [5–11]. Particularly problematic was the situation in the HAT focus of Mbuji-Mayi, East Kasai Province in DRC, where a 57% failure rate was observed in patients treated with the 10 days abridged melarsoprol regimen [8]. Various studies have been conducted to explain these unexpectedly high relapse rates, considering either the parasite or the human host being responsible for this phenomenon. Although known mechanisms may be involved, such as mutations of the P2 adenosine transporters and of aquaglyceroporin transporters in the trypanosome membrane, the explanation for treatment failure is probably more complex, including parameters of the parasite (reduced drug sensitivity and higher tissue tropism), the drug (content of active principle and correct administration) and the host including individual differences in pharmacokinetics, co-infections and disease stage [9,12–24].

To investigate the mechanism underlying the high failure rates in the Mbuji-Mayi focus, Pyana and co-workers undertook the large scale isolation of the trypanosome from HAT patients in that focus [25]. Thus, we established a collection of 85 T.b. gambiense type I strains from cured and relapsed patients, some of which from the same patient before treatment and after relapse. Among these strains, 41 were adapted to Mus musculus in order to test their sensitivity to melarsoprol in an in vivo mouse infection model and to analyse some genetic features that may be related to reduced sensitivity to melarsoprol. Recently, some of them were shown to be resistant to pentamidine and melarsoprol by in vitro drug sensitivity testing and to carry a ThAQP2/3 chimera that was supposed to lead to a reduced uptake of pentamidine in the trypanosome flagellar pocket [20]. In this study, we aimed at investigating the in vivo melarsoprol sensitivity phenotype of all 41 mouse adapted T.b. gambiense strains isolated in the Mbuji-Mayi focus, 2° investigating some of their genotypic characteristics and comparing them with 4 strains isolated from a sleeping focus with low relapse rates in Masi-Manimba, and 3° relating their phenotype and genotype with treatment outcome of the patients from whom they were isolated.

Materials and Methods

Ethics statement

The study in mice was approved by the Veterinary Ethics Committee of the Institute of Tropical Medicine, Antwerp, Belgium (protocol PAR-022) and adhere to the European Commission Recommendation on guidelines for the accommodation and care of animals used for experimental and other scientific purposes (18 June 2007, 2007/526/EG) and the Belgian National law on the protection of animals under experiment.

The parasite strains included in this study belong to the cryobank of the World Health Collaboration Center for Research and Training on Human African Trypanosomiasis Diagnostics at the Institute of Tropical Medicine in Antwerp, Belgium. Their isolation and use for research purposes was approved by the Ethical Committee of the Institute of Tropical Medicine (0441472) and of the Ministry of Health of DRC [25]. The strains are anonymized by using international codes and alias names.

Trypanosomes strains

A list of the T. b. gambiense strains from the Mbuji-Mayi focus in East Kasai Province, DRC, is given in Table 1. The isolation history of these strains is described elsewhere [25]. The alias name of each strain indicates whether it was isolated before treatment (BT) or after treatment (AT). Eleven strains were isolated from patients that were cured after melarsoprol treatment. Thirty strains were isolated from patients that relapsed after treatment. Among these 30 strains, twenty belong to “couples”, i.e. isolated from the same patients, before treatment and after relapse. The melarsoprol sensitive and resistant strains included as reference in the in vivo drug sensitivity experiment, T. b. brucei 427 wild type and T. b. brucei 427 AT1/P2 KO (P2 adenosine transporter knock out) were received from the Swiss Tropical and Public Health Institute. For the genotype analysis, 7 extra T. b. gambiense type I strains were added (Table 1). Four of these strains were isolated in 2011 from cured patients in the Masai-Manimba focus, Bandundu Province, RDC where high relapse rates after melarsoprol treatment were never observed. Three strains are “old” isolates. LTat 1.3 is a clonal population of the Eliane strain, isolated in Côte d’Ivoire, and previously shown to be sensitive to melarsoprol and pentamidine in vitro, while MBA and KEMLO are two Congolese strains with unknown drug sensitivity profile [26]. All the strains were kept as 250 μl cryostabilates in liquid nitrogen.

Expansion of parasite populations

Each stabile was thawed in a water bath at 37°C and immediately, 250 μl of phosphate buffered saline glucose (PSG, 7.5 g/l Na2 HPO4 2H2O, 0.34 g/l NaH2 PO4, H2O, 2.12 g/l NaCl, 10 g/l D-glucose, pH 8) were added. This mixture was kept on ice until inoculated intraperitoneally (IP) into two 1–2 months old female OF-1 mice (Charles River, Belgium). Two days before infection, these mice had been immunosuppressed by IP injection with 200 mg/kg body weight (BW) of cyclophosphamide diluted in water (Endoxan, Baxter, Lessing, Belgium). Parasitaemia was monitored three times a week on a fresh preparation of 5 μl of tail blood according to the matching method of Herbert and Lumsden (1976). If needed, immunosuppression was repeated after 5 days, until the parasitaemia reached 104/μl or more and the trypanosome population was large enough to inoculate 12 mice for the in vivo melarsoprol sensitivity experiment. For the inoculation of the mice in the in vivo drug sensitivity experiments (see below), 30 to 40 μl of infected tail blood was diluted in about 3 ml of PSG, trypanosomes were counted in a Uriglass cell counting chamber (Menarini Diagnostics) and the suspension was further diluted in PSG to obtain a concentration of 250 trypanosomes/μl.
### Table 1. List of *T.b. gambiense* strains used in this study.

| HAT focus, country       | International code or name | Alias name or clone | Treatment outcome | Couple |
|--------------------------|-----------------------------|---------------------|-------------------|--------|
| Mbuji-Mayi, DRC          | MHOM/CD/INRB/2008/56        | 15BT                | cure              |        |
|                          | MHOM/CD/INRB/2008/46        | 19BT                | cure              |        |
|                          | MHOM/CD/INRB/2008/65        | 29BT                | cure              |        |
|                          | MHOM/CD/INRB/2006/13        | 40BT                | relapse           | 1      |
|                          | MHOM/CD/INRB/2006/07        | 40AT                | relapse           | 1      |
|                          | MHOM/CD/INRB/2006/01        | 45BT                | cure              |        |
|                          | MHOM/CD/INRB/2008/52        | 48BT                | relapse           |        |
|                          | MHOM/CD/INRB/2007/28        | 57AT                | relapse           |        |
|                          | MHOM/CD/INRB/2008/62        | 85BT                | cure              |        |
|                          | MHOM/CD/INRB/2006/09        | 93AT                | relapse           |        |
|                          | MHOM/CD/INRB/2008/63        | 95BT                | cure              |        |
|                          | MHOM/CD/INRB/2008/42        | 99BT                | cure              |        |
|                          | MHOM/CD/INRB/2008/49        | 104BT               | relapse           | 2      |
|                          | MHOM/CD/INRB/2008/53A       | 104AT               | relapse           | 2      |
|                          | MHOM/CD/INRB/2008/50        | 105BT               | relapse           |        |
|                          | MHOM/CD/INRB/2007/25B       | 108AT               | relapse           | 3      |
|                          | MHOM/CD/INRB/2007/27        | 108BT               | relapse           | 3      |
|                          | MHOM/CD/INRB/2008/60        | 113AT               | relapse           | 4      |
|                          | MHOM/CD/INRB/2008/45        | 113BT               | relapse           | 4      |
|                          | MHOM/CD/INRB/2006/11A       | 116AT               | relapse           |        |
|                          | MHOM/CD/STI/2006/02         | 130BT               | relapse           |        |
|                          | MHOM/CD/INRB/2008/64        | 141BT               | cure              |        |
|                          | MHOM/CD/INRB/2005/02A       | 146BT               | relapse           | 5      |
|                          | MHOM/CD/INRB/2006/05        | 146AT               | relapse           | 5      |
|                          | MHOM/CD/INRB/2007/26A       | 147AT               | relapse           |        |
|                          | MHOM/CD/INRB/2005/01B       | 148BT               | relapse           | 6      |
|                          | MHOM/CD/INRB/2006/14        | 148AT               | relapse           | 6      |
|                          | MHOM/CD/INRB/2006/06A       | 163AT               | relapse           |        |
|                          | MHOM/CD/INRB/2008/47        | 167BT               | relapse           | 7      |
|                          | MHOM/CD/INRB/2008/43        | 167AT               | relapse           | 7      |
|                          | MHOM/CD/INRB/2008/59        | 174BT               | relapse           | 8      |
|                          | MHOM/CD/INRB/2007/29        | 174AT               | relapse           | 8      |
|                          | MHOM/CD/INRB/2008/37A       | 186BT               | cure              |        |
|                          | MHOM/CD/INRB/2006/12A       | 223AT               | relapse           |        |
|                          | MHOM/CD/INRB/2006/21B       | 340AT               | relapse           |        |
|                          | MHOM/CD/INRB/2007/22B       | 346BT               | relapse           | 9      |
|                          | MHOM/CD/INRB/2007/24B       | 346AT               | relapse           | 9      |
|                          | MHOM/CD/INRB/2006/23A       | 348BT               | cure              |        |
|                          | MHOM/CD/INRB/2006/16        | 349BT               | relapse           | 10     |
|                          | MHOM/CD/INRB/2006/19        | 349AT               | relapse           | 10     |
|                          | MHOM/CD/INRB/2007/34        | 378BT               | cure              |        |
|                          | MHOM/CD/INRB/2011/01        | MM01                | cure              |        |
|                          | MHOM/CD/INRB/2011/03        | MM03                | cure              |        |
|                          | MHOM/CD/INRB/2011/05        | MM05                | cure              |        |
|                          | MHOM/CD/INRB/2011/06        | MM06                | cure              |        |
| Masi-Manimba, DRC        | MAN/ZR/74/ITMAP1811         | MBA                 | unknown           |        |
| Kinshasa, DRC            | MAN/ZR/74/ITMAP1821         | KEMLO               | unknown           |        |
| Bwamanda, DRC            | ELIANE                      | LitTat 1.3          | unknown           |        |
| Daloa, Côte d’Ivoire    |                             |                     |                   |        |

In alias name: AT = after treatment, BT = before treatment. Treatment outcome: outcome of patient treated with melarsoprol (in Mbuji-Mayi) or with nifurtimox-eflornithine combination therapy (Masi-Manimba). Couple = number of the couple of two strains isolated from the same patient.
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**In vivo melarsoprol sensitivity experiments**

For the treatment of mice, melarsoprol (Aventis, 5 ml vials of 180 mg in propylene glycol, lot nr. 725) was freshly diluted in 50% polyethylene glycol (PEG400, Sigma Aldrich, Belgium) to concentrations of 1 mg/ml.

For each experiment, twelve female OF-1 mice (1–2 months old, 20–30 g body weight (BW)) were immunosuppressed as described above, two days before inoculation and subsequently at days 3, 11, 25 and 85 post-infection (DPI). Each mouse was inoculated IP with 200 µl PSG containing 5×10⁵ trypomastigotes. Parasitaemia was monitored as described above from day 4 post-infection. On day 5–10 post infection, the mice of the control group were euthanised with an IP injection of sodium pentobarbital at 350 mg/kg BW (Nembutal, CEVA Santé Animale, Brussels, Belgium) and blood was taken by heart puncture on heparin to prepare sediments of pure trypanosomes for DNA extraction and genetic analysis (see below). The mice of the melarsoprol treated group were checked for the presence of trypanosomes in tail blood two times a week during the first two weeks and subsequently once a week for maximum 100 days. As soon as a trypanosome was detected in at least one mouse of this group, the experiment was stopped and the strain was considered ‘resistant’. Relapsing mice were sacrificed and blood was collected on heparin to separate the trypanosomes from the blood via DEAE chromatography for further genotypic analysis. On days 90 to 100 post-infection, all mice that remained trypanosome negative were sacrificed and blood was collected on heparin where after it was run over two mini Anion Exchange Centrifugation Technique columns to detect subpatent parasitaemia [27].

**Trypanosome purification and genomic DNA extraction**

Infected blood from mice was passed over a DEAE cellulose column (1.6 blood/gel ratio) to separate the trypanosomes from the blood [28]. The trypanosomes eluting from the column were washed three times with 5 ml ice-cold PSG by centrifugation. After the last centrifugation, the supernatant PSG was discarded and the trypanosome sediment was frozen at −80°C until DNA extraction. After thawing and addition of 200 µl of phosphate buffered saline, pH 8, genomic DNA was extracted from the trypanosome sediment with the Maxwell 16 Tissue DNA Purification kit on the Maxwell 16 robot (Promega, Madison, WI, USA) and DNA was stored at −20°C. DNA concentrations were measured with the Nanodrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, USA) and adjusted to 10 ng/µl if appropriate.

**Genotyping**

**msfNaI PCR-RFLP for TbAT1.** The protocol to amplify a 677-bp fragment of the *TbAT1* gene and to digest it with SfaNI was based on Maser et al. [17]. A 20 µl reaction volume contained 1× PCR buffer (Qiagen, Venlo, The Netherlands), 1 mM MgCl₂ (Qiagen), 200 µM of each dNTP (Eurogentec), 0.8 µM of each primer (Biolegio), 0.4 U Hotstart Taq plus polymerase (Qiagen), 0.1 mg/ml acetylated BSA (Promega) and 2 µl target DNA. Amplification conditions were as follows: initial denaturation at 95°C for 5 min, 30 cycles 1 min at 94°C, 1 min at 65°C and 2 min at 72°C and final extension at 72°C for 10 min. Five µl of the PCR products were digested 16 hours at 37°C in a 10 µl reaction mixture containing 1× NEB 5.1 buffer with 2 U of SfaNI restriction enzyme (New England Biolabs, Hitchin, UK), followed by 20 minutes incubation at 65°C. Differential banding was analysed using 10 µl on a 2% agarose gel run for 30 min at 135 V and stained with ethidium bromide.

**PCR for full-length TbAT1.** The protocol to amplify the full length *TbAT1* gene and its flanking regions was slightly modified from Graf et al. [20]. A 20 µl reaction volume contained 1× Coral Load Buffer, 1 mM MgCl₂ (Qiagen), 200 µM of each dNTP (Eurogentec), 0.8 µM of *TbAT1_F* (5'-GAAATCCCCGTC-TTTTCTCAC-3') and *TbAT1_R_Tbg* (5'-ATGTGCTGACC-CATTTCCTTT-3') primers (Biolegio), 0.4 U Hotstart Taq plus polymerase (Qiagen), 0.1 mg/ml acetylated BSA (Promega) and 2 µl target DNA. Amplification conditions were: initial denaturation at 95°C for 5 min, 24 cycles of 1 min at 95°C followed by 1 min at 56°C and 2 min at 72°C, final extension at 72°C for 5 min. Ten µl of the PCR product were electrophorised in a 2% agarose gel for 30 min at 135 V and stained with ethidium bromide for detection of the amplicons. For selected strains, the amplicons were cleaned up and concentrated using a PCR clean-up kit (Qiagen) and sent out for bidirectional direct sequencing at the VIB Genetic Sequencing Facility (Antwerp, Belgium) using the described PCR primers and additional internal primers to cover *TbAT1*.

**Locus specific PCR for aquaglyceroporin genes.** The protocols to amplify either the *AQP2* locus or the combined *AQP2* and *AQP3* loci, including the *AQP2*/*3* chimera were slightly modified from Graf et al. [20]. To amplify the wild-type *AQP2* locus, a 20 µl reaction volume contained 1× Coral Load Buffer (Qiagen), 1 mM MgCl₂ (Qiagen), 200 µM of each dNTP (Eurogentec), 0.8 µM of *AQP2/3_F* 5'-AAGAAGGCT-GAAAATCCCCACTTG-3' and *AQP2-R* 5'-CTTCCGGGAGAAAAAAACCTC-3' primers (Biolegio), 0.4 U Hotstart Taq plus polymerase (Qiagen), 0.1 mg/ml acetylated BSA (Promega) and 2 µl target DNA. Amplification conditions were: initial denaturation at 95°C for 5 min, 24 cycles of 1 min at 95°C followed by 1 min at 60°C and 2 min at 72°C, final extension at 72°C for 10 min. Ten µl of the PCR product were electrophorised in a 2% agarose gel for 30 min at 135 V and stained with ethidium bromide for detection of the amplicons. To amplify the combined *AQP2* and *AQP3* locus, a 20 µl reaction volume contained 1× Coral Load Buffer (Qiagen), 1 mM MgCl₂ (Qiagen), 200 µM of each dNTP (Eurogentec), 0.8 µM of *AQP2/3_F* 5'-AAGAAGGCT-GAAAATCCCCACTTG-3' and *AQP2_R* 5'-CTTCCGGGAGAAAAAAACCTC-3' primers (Biolegio), 0.4 U Hotstart Taq plus polymerase (Qiagen), 0.1 mg/ml acetylated BSA (Promega) and 2 µl target DNA. Amplification conditions were: initial denaturation at 95°C for 5 min, 24 cycles of 1 min at 95°C followed by 1 min at 60°C and 4 min at 72°C, final extension at 72°C for 10 min. Ten µl of the PCR product were electrophorised in a 0.8% agarose gel for 30 min at 135 V and stained with ethidium bromide for detection of the amplicons. For selected strains, amplicons were cleaned up and concentrated using a PCR clean-up kit (Qiagen) and sent out for bidirectional direct sequencing at the VIB Genetic Sequencing Facility (Antwerp, Belgium) using the described PCR primers and additional internal primers to cover *TbAQP2*.

**Cloning of aquaglyceroporin variants.** The full-length ORFs of the *AQP2* and *AQP2*/*3* genes were amplified from genomic DNA using a proofreading polymerase (Phusion High-Fidelity DNA Polymerase, Thermo Scientific, Waltham, MA, USA). All cloning primers (Biolegio) contained a 5′ extension of 15 nucleotides from the trypanosomal expression vector pH3D09, as required for the In-Fusion Cloning reaction (Clontech, Takara Bio, Otsu, Japan) [29]. The primer sets consisted of a forward
primer (IF HindIII 5’-AAGCTT ATGCA-GAGCCAACCAGACA-3’) and an AQP2 specific reverse primer (IF BamHI AQP2 5’-TAAATGGGCA GGTCC TTAGTGTGGAAGAAAATATTTGTACAG-3’) for amplification of T.b. gambiense MM01, LiTaR1 and MBA or an AQP2/3 specific reverse primer (IF BamHI AQP2/3 5’-TAAATGGGCA GGTCC TTAGTGTGGCAGAAATAATTTGTACA-3’) for amplification of T.b. gambiense 348BT. Plasmids were purified from several transformed E. coli and sent out for bidirectional direct sequencing at the VIB Genetic Sequencing Facility (Antwerp, Belgium).

**PCR-RFLP for AQP2/3 chimeric alleles from Mbuji-Mayi.** A 278-bp fragment was amplified from the predicted AQP2/3 chimeras from Mbuji-Mayi in a 20 µl reaction volume that contained 1× PCR buffer (Qiagen), 1 mM MgCl₂ (Qiagen), 200 µM of each dNTP (Eurogentec), 0.4 µM of AQP2-RFLP-F

### Table 2. Phenotype of melarsoprol resistant strains.

| Alias name | 10 mg/kg BW | 10 mg/kg BW (rep) | 12 mg/kg BW |
|------------|-------------|-------------------|-------------|
|             | numbers relapsed | DPI | numbers relapsed | DPI | numbers relapsed | DPI |
| 15BT        | 2            | 85*   | 0              | na  | 0               | na  |
| 163AT       | 3            | 88*   | 1              | 28  | 1               | 31  |
| 346AT       | 1            | 90*   | 6              | 20  | 6               | 20* |

Number of relapsing mice (out of 6 infected) and day post-infection that relapses were observed after treatment with melarsoprol at different dosages and repetitions. DPI: days post-infection, BW: body weight, rep: repetition, na: not applicable, *: relapsing population used for AQP2/3 RFLP analysis.

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Figure 1. Restriction digest profile generated with SfaNI PCR-RFLP on DNA of the T.b. gambiense strains as listed in Table 1 and of the two T.b. brucei control strains. Lanes 1, 26, 27 and 52 = GeneRuler 100 bp Plus DNA Ladder (Fermentas), lanes 2 to 44 = T.b. gambiense strains isolated from Mbuji-Mayi, lanes 45–48 = T.b. gambiense strains isolated from Masi-Manimba, lane 49 = T.b. brucei 427 WT, lane 50 = T.b. brucei 427 AT1/P2 KO, lane 51 = negative PCR control.

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(5’-GAACATTTCCACCCAGT-3’) and AQP2-RFLP-R (5’-AGTTCAAGCATCCTCAACA-3’) (Biologio), 0.4 U Hot-start Taq plus polymerase (Qiagen), 0.1 mg/ml acetylated BSA (Promega) and 2 μl target DNA. Amplification conditions were as follows, initial denaturation at 94°C for 5 min, 24 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec and final annealing at 72°C for 5 min. Five μl of the PCR products or 1 μg of plasmid DNA, containing a cloned AQP2/3 variant, were digested for 15 min at 37°C in a 15 μl reaction mixture containing 1 μl FastDigest Green buffer with 1 μl of either Aval or SdfI FastDigest restriction enzymes (Thermo Scientific), followed by 20 minutes inactivation at 80°C. Differential banding was analysed using electrophoresis of 10 μl on a 3% small fragment agarose gel run for 30 min at 135 V and stained with ethidium bromide.

Results

Phenotype

In a preparatory experiment, mice were infected with strain 348BT, isolated from a patient who had been cured, and were treated with melarsoprol at 1, 2, 3, 4, 5, 8 and 10 mg/kg BW. The minimum melarsoprol dosage needed to cure the mice from the infection appeared to be 10 mg/kg BW. This dosage was then used to treat mice infected with all the 41 *T. gambiae* strains and with the two *T. brucei* strains. Some mice infected with *T. gambiae* strains 15BT, 163AT and 346AT experienced a relapse that was detectable only about three months after the infection and after immunosuppression with cyclophosphamide (Table 2). All mice infected with the other *T. gambiae* strains and with both *T. brucei* strains (including the AT1/P2 KO strain) got cured by melarsoprol at 10 mg/kg/BW, as defined by the absence of detectable relapse up to 100 days after infection. To confirm the apparent melarsoprol resistance of 15BT, 163AT and 346AT, the experiment was repeated with these strains and with melarsoprol treatment at 10 and 12 mg/kg BW. All mice infected with 15BT remained without detectable parasitaemia after treatment. Among the mice infected with 163AT, one mouse relapsed at DPI 28 after treatment with 10 mg/kg BW and one mouse relapsed at DPI 31 after treatment with 12 mg/kg BW melarsoprol. All mice infected with 346AT and treated with 10 and 12 mg/kg BW melarsoprol, relapsed at DPI 20 (Table 2).

Genotype

*TbAT1* P2 adenosine transporter gene. PCR with the Sfa primers yielded the expected 677 bp amplicon in all *T. gambiae* and in the *T. brucei* 427 WT strain but not in the
Amplicons from MBA and KEMLO, revealed a yet unknown AQP2/3 chimera that shares the first 677 bp from AQP2, followed by a stretch of 202 bp of AQP3 and ending with the last 60 bp from AQP2, abbreviated here as AQP2/3(516–880) to indicate that the switch from AQP2 to AQP3 is first detected at nucleotide 678 and the switch back to AQP2 is first detected at nucleotide 880 (Figure 4 – F). Cloning AQP2 sequences from LiTat 1.3, the West-African T.b. gambiense strain sensitive to melarsoprol and pentamidine, revealed only wild-type sequences (Figure 4 – A), while cloned AQP2/3 sequences from the old T.b. gambiense MBA strain did not reveal any differences with results already obtained by direct sequencing (Figure 4 – E). Surprisingly, cloning of the AQP2/3 gene from T.b. gambiense 348 BT revealed next to the chimera found by direct sequencing yet another AQP2/3 chimera that shared the first 879 bp from AQP2, with one point mutation at T869C and only the last 60 bp from AQP3, abbreviated as AQP2/3(879–939) (Figure 4 – D). A partial alignment of the obtained sequences of the AQP2, AQP3 and AQP2/3 variants is given in figure S1. When T.b. gambiense 348 BT was amplified with a cloning primer specific for AQP2, only a very faint amplification pattern was seen, probably attributable to primer mismatch on AQP2/3 (figure S2).

**RFLP to discriminate Mbuji-Mayi AQP2/3 variants.** Since T.b. gambiense 348 BT was isolated from a cured patient and seemed to contain both AQP2/3(814) and AQP2/3(880), we hypothesised that only AQP2/3(814) would be present in strains isolated from relapsing patients. For this reason, we amplified a part

### Table 3. Genotype, strains and accession numbers for TbAT1.

| Genotype | Strains | Accession number |
|----------|---------|-----------------|
| TbAT1    | 15BT    | KM282018        |
|          | 45BT    | KM282019        |
|          | 108BT   | KM282020        |
|          | 108AT   | KM282021        |
|          | 130BT   | KM282022        |
|          | 146AT   | KM282023        |
|          | 163AT   | KM282024        |
|          | 174BT   | KM282025        |
|          | 174AT   | KM282026        |
|          | 346AT   | KM282027        |
|          | 348BT   | KM282028        |
|          | 378BT   | KM282029        |
|          | MM01    | KM282030        |
|          | MM03    | KM282031        |
|          | MM05    | KM282032        |
|          | MM06    | KM282033        |
|          | MBA     | KM282016        |
|          | KEMLO   | KM282017        |

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*T.b. brucei* 427 AT1/P2 KO strain. Restriction enzyme digestion of the PCR amplicons with SfaNI showed the wild type pattern, i.e. one fragment of 566 bp and one of 111 bp, in all the *T. b. gambiense* and in the *T. b. brucei* 427 WT strains (Figure 1). With all the 45 *T. b. gambiense* strains and with the *T. b. brucei* 427 WT, the TbAT1 PCR generated an amplicon of about 1600 bp, indicating the presence of the P2 adenosine transporter gene (Figure 2). Only the *T. b. brucei* 427 AT1/P2 KO strain yielded a fragment of about 2000 bp indicative of the replacement of P2 adenose transporter gene at nucleotide 678 and the switch back to AQP2 is first detected at nucleotide 880 (Figure 4 – E). Direct sequencing results from the circa 1500 bp AQP2 amplicons from 4 strains from Masi-Manimba consistently showed a total of 18 heterozygous single nucleotide polymorphisms in comparison to the wild-type AQP2 sequence of *T. b. gambiense* STIB 930 (KF564925). After cloning of the full length AQP2 sequence from one strain of Masi-Manimba, MM01, we obtained either the wild-type sequence or another novel chimera that shares the first 616 bp from AQP2, with 2 point mutations at position T548C and G573A, followed by a stretch of 41 bp identical to AQP3, and ending with the last 282 bp from AQP2, abbreviated here as AQP2/3(617–898) (Figure 4 – F). Cloning AQP2 sequences from LiTat 1.3, the West-African *T. b. gambiense* strain sensitive to melasoprol and pentamidine, revealed only wild-type sequences (Figure 4 – A), while cloned AQP2/3 sequences from the old *T. b. gambiense* MBA strain did not reveal any differences with results already obtained by direct sequencing (Figure 4 – E). Surprisingly, cloning of the AQP2/3 gene from *T. b. gambiense* 348 BT revealed next to the chimera found by direct sequencing yet another AQP2/3 chimera that shared the first 879 bp from AQP2, with one point mutation at T869C and only the last 60 bp from AQP3, abbreviated as AQP2/3(879–939) (Figure 4 – D). A partial alignment of the obtained sequences of the AQP2, AQP3 and AQP2/3 variants is given in figure S1. When *T. b. gambiense* 348 BT was amplified with a cloning primer specific for AQP2, only a very faint amplification pattern was seen, probably attributable to primer mismatch on AQP2/3 (figure S2).

**RFLP to discriminate Mbuji-Mayi AQP2/3 variants.** Since *T. b. gambiense* 348 BT was isolated from a cured patient and seemed to contain both AQP2/3(814) and AQP2/3(880), we hypothesised that only AQP2/3(814) would be present in strains isolated from relapsing patients. For this reason, we amplified a part
of the AQP2/3 gene using a PCR specific to the Mbuji-Mayi strains and used RFLP with either AvaI or SduI restriction enzymes to discriminate between both AQP2/3 variants. RFLP with AvaI does not cut the AQP2/3(814) chimera, but generates bands of 200 bp and 82 bp in the AQP2/3(880) chimera. RFLP with SduI cuts AQP2/3(814) chimera in bands of 99 bp and 183 bp, while AQP2/3(880) chimera is cut in bands of 99 bp, 145 bp and 42 bp. PCR-RFLP on plasmids containing only a single AQP2/3 variant could unambiguously differentiate between both AQP2/3(814) and AQP2/3(880) (figure S3). However, PCR-RFLP, with either AvaI (Figure 5) or SduI (Figure 6), on all T.b. gambiense strains from Mbuji-Mayi, including four strains isolated from relapses from mice, indicated the presence of both variants of the AQP2/3 chimera, suggesting that strains that cause a relapse in either mice and humans are not genetically characterised by a loss of AQP2/3(880).

Discussion

This study was undertaken to investigate the mechanisms underlying the high relapse rates observed in second stage gambiense HAT patients treated with melarsoprol in Mbuji-Mayi, DRC. The in vivo melarsoprol sensitivity experiment showed that a minimum dose of 10 mg/kg BW melarsoprol was needed to cure mice infected with trypanosomes that were isolated from a cured patient. This is 4 times higher than the dose needed to cure mice infected with T.b. gambiense strains isolated from Ibba in South Sudan, another HAT focus known for high melarsoprol relapse rates [9]. On the other hand, Kibona and co-workers considered 3 out of 35 tested T.b. rhodesiense strains as resistant when mice relapsed after treatment with 5 mg/kg BW melarsoprol [30]. Among the 41 strains from Mbuji-Mayi, 2 induced infections that could not be cured with 12 mg/kg BW melarsoprol. Both were isolated from patients after treatment with melarsoprol.

When setting up the in vivo drug sensitivity experiment, we were confronted with the lack of a standardised protocol, especially for T.b. gambiense. Most studies have been dealing with T.b. brucei or T.b. rhodesiense, both behaving quite virulent in laboratory mice. Although melarsoprol is a drug that can cure the chronic phase of trypanosomosis, we opted for an acute phase in vivo model for several reasons: i. the concentration of melarsoprol that reaches the central nervous system is only a minor fraction of what reaches the plasma and is more prone to uncontrolled variations among individual outbred animals, ii. the acute phase model is expected to correspond better with the standard in vitro model, iii. since T.b. gambiense can cause subclinical or even silent infections, assessing treatment outcome in
a chronic model via examination of blood and organs, including the brain, is unreliable [31]. The protocol we used here is mainly based on the study that Maina et al. carried out on recent isolates of *T. b. gambiense* from Sudan [9]. We also immunosuppressed the mice before inoculation with $5 \times 10^4$ trypanosomes to guarantee that all mice would become infected. Some major differences however are to be noted. During the preparatory experiments, we noted that melarsoprol precipitates immediately when diluted in water, the usual diluent in other *in vitro* and *in vivo* studies [7,9,30]. In our final protocol, we diluted melarsoprol in polyethylene glycol to keep it in solution facilitating correct dosage when treating the mice. In contrast to the custom 60 days after treatment follow-up, we monitored the mice for up to 100 days after treatment and we immunosuppressed them on day 85.

**Table 4.** Genotype, strains and accession numbers for *TbAQP2* and *TbAQP2/3*.

| Genotype       | Strains       | Accession number |
|----------------|---------------|------------------|
| AQP2           | LiTat 1.3     | KM282048         |
|                | MM01*         | KM282049         |
| AQP2/3 (814)   | 15BT          | KM282036         |
|                | 45BT          | KM282037         |
|                | 108BT         | KM282038         |
|                | 108AT         | KM282039         |
|                | 130BT         | KM282040         |
|                | 146AT         | KM282041         |
|                | 163AT         | KM282042         |
|                | 174BT         | KM282043         |
|                | 174AT         | KM282044         |
|                | 346AT         | KM282045         |
|                | 348BT         | KM282046         |
|                | 378BT         | KM282047         |
| AQP2/3 (880)   | 348BT         | KM282050         |
| AQP2/3 (678–880)| MBA          | KM282034         |
|                | KEMLO         | KM282035         |
| AQP2/3 (617–658)| MM01*       | KM282051         |

*direct sequencing results suggested 18 heterozygous single nucleotide polymorphisms in the AQP2 coding sequence of *T. b. gambiense* MM01, MM03, MM05, and MM06.

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**Figure 4.** Schematic view of the *AQP2/3* variants identified in this study (adapted from Graf et al [20]). Sequence of *AQP3* was not verified. Positions of primer: black box = *AQP2/3_*F, green box = *AQP2_*R, red box = *AQP3/_3_*R. A) Reference locus of *AQP2* and *AQP3*, with wild-type *AQP2* found in the melarsoprol and pentamidine sensitive strain *T. b. gambiense* LiTat 1.3 and in all strains from Masi-Manimba. B) Chimera of *AQP2* and *AQP3* occurring in a melarsoprol and pentamidine resistant *T. b. brucei* strain as described by Baker et al. [19]. C) Chimera of *AQP2* and *AQP3* plus loss of *AQP3* in all *T. b. gambiense* strains from Mbuji-Mayi as described in this article and by Graf et al. [20]. D) New chimera of *AQP2* and *AQP3*, possibly outside the known locus, found in all *T. b. gambiense* strains from Mbuji-Mayi. E) New chimera of *AQP2* and *AQP3* plus loss of *AQP3* found in two old Congolese *T. b. gambiense* strains, MBA and KEMLO. F) New chimera of *AQP2* and *AQP3*, without loss of *AQP3*, found in all four *T. b. gambiense* strains isolated in Masi-Manimba.

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after treatment. In addition, at the end of the follow-up period, we sacrificed the mice and passed all the blood on DEAE cellulose columns instead of checking only a few drops of tail blood with the microhaematocrit technique. This allowed us to observe relapses at days 85–90 after treatment that otherwise would have been missed. Still, some treated mice showed paralysis but without any detectable trypanosome in the blood, suggesting that the real relapse rate was higher than what we actually can report based on trypanosome detection only. A weakness in our study is the absence of well documented \textit{T.b. gambiense} melarsoprol resistant control strain. In the absence of such a strain, we had to rely on the \textit{T.b. brucei} 427 AT1/P2 KO strain and its corresponding \textit{T.b. brucei} 427 wild-type strain. Both strains appeared to be sensitive to melarsoprol at 10 mg/kg BW which is consistent with what has been observed in previous studies on a \textit{T.b} null mutant [14]. On the other hand, for 5 strains included in our in vivo experiment, it was shown in vitro that they were 2–4 times less sensitive for melarsoprol than strains from cured patients. It was clearly not expected that all strains would carry drug resistance markers. The different in vivo melarsoprol sensitivity phenotypes observed in our experiment do not correspond with the low variability observed within the two studied genetic markers associated with melarsoprol resistance. Indeed, all strains from Mbuji-Mayi, irrespective of their isolation from cured or relapsing patients, carry the wild type \textit{TbAT1} allele. In addition, in all strains from Mbuji-Mayi, the \textit{TbAQP2} and \textit{TbAQP3} are replaced by chimeric \textit{TbAQP2/3} variants, of which one has been reported previously to correlate with \textit{in vitro} pentamidine and melarsoprol resistance by Graf and co-workers [20]. According to Graf and co-workers, these 5 strains contained the \textit{TbAQP2/3} chimera and the wild type \textit{TbAT1}, what is confirmed in our study, and showed decreased sensitivity for pentamidine and for melarsoprol in \textit{vitro}. In our study we found a second variant of a chimeric \textit{AQP2/3} gene in these strains, which was only observed after cloning and not by direct sequencing, possibly indicating the presence of such variant outside the known \textit{AQP2}}
locus. Surprisingly, the strains from Masi-Manimba were heterozygous for the \textit{AQP2} locus. One allele contained the wild-type sequence, but the second allele contained a yet undescribed \textit{TbAQP2/3} chimera. Munday and co-workers recently described that the presence of a functional wild-type \textit{AQP2} sequence renders strains sensitive to melarsoprol and pentamidine [24]. However, the effect on pentamidine and melarsoprol uptake of the newly described \textit{AQP2/3} chimeras is unknown. All variants were cloned in a trypanosomal expression vector for future evaluation and are available upon request. That this probable drug resistant genotype is found in all strains from Mbuji-Mayi and not in the strains from Masi-Manimba could be sufficient to explain the difference in melarsoprol relapse rates observed in East-Kasai (high) and in Bandundu (low). Within this context, it is interesting to note that \textit{AQP2/3} and \textit{TbAT1} genes are different, these “old” isolates are probably not closely related to the contemporary strains circulating in Masi-Manimba and Mbuji-Mayi. Appearance of resistance to arsenicals, including melarsoprol, and to pentamidine in several HAT foci in DRC (former Congo Belge and Zaire) has already been described decades ago and was considered a result of mass treatment and chemoprophylaxis [32,33]. The finding of the pentamidine/melarsoprol resistant genotype in the “old” \textit{T.b. gambiense} strains may be the basis for molecular studies into the appearance and spread of pentamidine/melarsoprol resistant \textit{T.b. gambiense} strains. In the present study, we didn’t carry out microsatellite analysis to verify the similarity between strains from the Mbuji-Mayi focus, in particular from strains isolated from the same patient before treatment and after relapse. However, results from mobile genetic element PCR (MGE-PCR) as described by Simo \textit{et al} suggest only very small differences between all strains from Mbuji-Mayi [34]. Thus, most probably, all strains isolated in Mbuji-Mayi are probably the clonal progeny of one strain acquiring the \textit{AQP2/3} chimeras. This is the first large scale \textit{in vivo} drug sensitivity study on \textit{T.b. gambiense} strains that were isolated within a short period of

Figure 6. Restriction digest profile generated with SduI PCR-RFLP on DNA of the \textit{T.b. gambiense} strains as listed in Table 1, including the four strains isolated from relapsed mice. Lanes 1, 26, 27 and 52 = GeneRuler 100 bp Plus DNA Ladder (Fermentas), lanes 2 to 44 = \textit{T.b. gambiense} strains isolated from Mbuji-Mayi, lane 45 = \textit{T.b. gambiense} MBA, lane 50 = \textit{T.b. gambiense} 346AT relapse 10 mg/kg BW, lane 47 = \textit{T.b. gambiense} 346AT relapse 12 mg/kg BW, lane 48 = \textit{T.b. gambiense} 15BT relapse 10 mg/kg BW, lane 49 = \textit{T.b. gambiense} 163AT relapse 10 mg/kg BW, lane 51 = negative PCR control.

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time, in one single HAT focus, from cured as well as from relapsing patients and for 10 cases from the same patient before and after treatment. As such, the fact that the homogeneity observed within the T.b.AQP2/3 and the T.bAT1 loci does not correspond with the heterogeneity in treatment outcome of the patients and of the mice, suggests that other factors, such as virulence and tissue tropism of the parasite or genotype and phenotype of the individual patient, may influence the treatment outcome. For example, in several independent studies it was found that high cell count in the cerebrospinal fluid (>100 cell/μl) is a risk factor for relapse [8,23,35–37]. This may indicate that patients in advanced second stage of the disease are less responsive for the standard melarsoprol treatment schedule. It is even not excluded that such patients are also less responsive for other drugs or therapeutic regimes such as NECT, the current first line treatment of second stage gambiense HAT. Therefore, further investigations into treatment failure in HAT and into alternative drugs or treatment regimes should not only focus on differential genotypes of the parasites but also on differential virulence and tissue tropism.

In conclusion, this study confirms that the high melarsoprol relapse rates observed in the Mbuji-Mayi focus can be explained by mutations in the T.bAQP2/3 locus of the trypanosomes that circulate in that focus. However, other factors will also influence the treatment outcome of individual patients.

Supporting Information

Figure S1 Partial alignment of AQP2, AQP3 and AQP2/3 variants with GenBank accession numbers. (TIF)

Figure S2 Amplification of T.b. gambiense DNA with a cloning primer specific for AQP2, Lanes 1 and 14: GeneRuler 100 bp Plus DNA Ladder (Fermentas), lanes 2–10: T.b. gambiense strains isolated from Mbuji-Mayi, lane 11 = T.b. gambiense LiTat 1.3, lane 12 = T.b. gambiense MM01, lane 13: negative PCR control. (TIF)

Figure S3 RFLP with either Aval or SduI restriction enzymes to discriminate between both AQP2/3 variants. RFLP with Aval (panel A) does not cut the AQP2/3(314) chimera (lane 1), but generates bands of 200 bp and 82 bp in the AQP2/3(3200) chimera (lane 2). RFLP with SduI (panel B) cuts AQP2/3(314) chimera in bands of 99 bp and 183 bp (lane 4), while AQP2/3(3200) chimera is cut in bands of 99 bp, 145 bp and 42 bp (lane 5). Lanes 3 and 6: GeneRuler 100 bp Plus DNA Ladder (Fermentas). (TIF)

Figure S4 Amplicons generated with mobile genetic element PCR (MGE-PCR) on DNA of diverse T. brucei strains. Lanes 1, 26, 27 and 51 = GeneRuler 100 bp Plus DNA Ladder (Fermentas), lanes 2 to 43 = T.b. gambiense strains isolated from Mbuji-Mayi, lanes 44 = T.b. gambiense MBA, lane 45: T.b. gambiense AnTat 22.1, lane 46 = T.b. gambiense AnTat 9.1, lane 47: T.b. gambiense LiTat 1.3, lane 48 = T.b. rhodesiense AnTat 12.1, lane 49: T.b. rhodesiense AnTat 25.1, lane 50 = T.b. gambiense type II Abba. (TIF)

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Author Contributions

Conceived and designed the experiments: PPP PB NVR. Performed the experiments: PPP PB NVR INL. Analyzed the data: PPP PB NVR DMN. Contributed reagents/materials/analysis tools: SKBS DMN PB. Wrote the paper: PPP PB NVR DMN SKBS PB.

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