Genomic analysis of Isometamidium Chloride resistance in *Trypanosoma congolense*

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ABSTRACT

Isometamidium Chloride (ISM) is one of the principal drugs used to counteract *Trypanosoma congolense* infection in livestock, both as a prophylactic as well as a curative treatment. However, numerous cases of ISM resistance have been reported in different African regions, representing a significant constraint in the battle against Animal African Trypanosomiasis. In order to identify genetic signatures associated with ISM resistance in *T. congolense*, the sensitive strain MSOROM7 was selected for induction of ISM resistance in a murine host. Administered ISM concentrations in immune-suppressed mice were gradually increased from 0.001 mg/kg to 1 mg/kg, the maximal dose used in livestock. As a result, three independent MSOROM7 lines acquired full resistance to this concentration after five months of induction, and retained this full resistant phenotype following a six months period without drug pressure. In contrast, parasites did not acquire ISM resistance in immune-competent animals, even after more than two years under ISM pressure, suggesting that the development of full ISM resistance is strongly enhanced when the host immune response is compromised. Genomic analyses comparing the ISM resistant lines with the parental sensitive line identified shifts in read depth at heterozygous loci in genes coding for different transporters and transmembrane products, and several of these shifts were also found within natural ISM resistant isolates. These findings suggested that the transport and accumulation of ISM inside the resistant parasites may be modified, which was confirmed by flow cytometry and *ex vivo* ISM uptake assays that showed a decrease in the accumulation of ISM in the resistant parasites.

1. Introduction

African trypanosomes are protozoan parasites transmitted by tsetse flies and responsible for African Trypanosomiasis in both humans (HAT) and animals (AAT). *T. congolense*, *T. vivax* and *T. brucei brucei* are the major pathogens responsible for AAT, a disease also known as Nagana and considered as one of the principal causes of malnutrition and poverty in 37 countries in sub-Saharan Africa (Mattioli et al., 2004). Nagana puts 55 million cattle at risk and leads to the death of three million animals every year, incurring a direct annual loss of US$ 1.0–1.2 billion in cattle production (Hursey and Slingenbergh, 1995; Mattioli et al., 2004; Cecchi and Mattioli, 2009; Cecchi et al., 2014).

Isometamidium Chloride (ISM) and Diminazene Aceturate (DA) are the main drugs currently available to cure AAT (Giordani et al., 2016). ISM belongs to the phenanthridine drug group of which many members are documented to be mutagenic (Uilenberg, 1998), and DA is an aromatic diamidine. About 35 million doses of trypanocidal drugs are administered every year in an attempt to control the disease (Geerts and Holmes, 1998; Mattioli et al., 2004). However, those compounds have been in use for over 50 years (Lourie and Yorke, 1939; Watkins and Woolfe, 1952; Brown et al., 1961; Sahin et al., 2014), and numerous cases of ISM and DA resistance and treatment failures have been reported in 17 African countries, representing a serious problem in the battle against AAT (Sinyangwe et al., 2004; Delespaux et al., 2008a, 2008b; Moti et al., 2012; Mungube et al., 2012; Sow et al., 2012; Dagnachew et al., 2015; Moti et al., 2015).

ISM and DA accumulate inside the cytoplasm and mitochondrion of trypanosomes and have a common target: the mitochondrial DNA (Shapiro and Englund, 1990). ISM also enters the nucleus where it binds to the nuclear DNA (Kaminsky et al., 1997). The mitochondrial DNA, known as kinetoplast DNA (kDNA), represents about 10–20% of the genome of the trypanosomes (Daniels et al., 2010) and consists of a

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The body of the text is about the mechanisms of drug resistance in trypanosome parasites, specifically T. congolense. The text discusses the use of Whole Genome Sequencing (WGS) to identify genomic changes associated with drug resistance. It mentions the use of in vivo and in vitro experiments to test drug susceptibility and resistance. The text also touches on the use of ISM (inosinomycin) as a drug and its resistance mechanisms. The text includes references to previous studies on drug resistance in trypanosomes and the use of flow cytometry and other methods to study drug uptake and resistance.

The text begins with a discussion of the resistant phenotype of Plasmodium falciparum and how this phenomenon has been noted for trypanosome species. It then goes on to describe how WGS has been used to identify resistant lines and how this method has been used to compare resistant and sensitive lines. The text mentions the use of immune-suppressed mice in drug susceptibility testing and how this has allowed for the identification of resistant parasites.

The text also discusses the use of ISM as a drug and how its resistance has been studied. It mentions the use of flow cytometry to study drug uptake and the use of DEAE purification to extract DNA for sequencing.

The text concludes with a discussion of the use of these methods to identify genomic changes associated with drug resistance. It mentions the use of in vivo and in vitro experiments to test drug susceptibility and resistance. The text also touches on the use of ISM as a drug and its resistance mechanisms. The text includes references to previous studies on drug resistance in trypanosomes and the use of flow cytometry and other methods to study drug uptake and resistance.

The text is a detailed and informative discussion of the mechanisms of drug resistance in trypanosome parasites, specifically T. congolense. It uses a variety of methods to study drug susceptibility and resistance, including Whole Genome Sequencing, in vivo and in vitro experiments, and flow cytometry. The text is well-referenced and provides a comprehensive overview of the current understanding of drug resistance in trypanosomes.
cattle in 2003 (Masumu et al., 2009). This clone was generated by infecting an OF-1 mouse with a single parasite using the micro-drop method (Masumu et al., 2009). Then, the parasites were passaged only once before the start of the ISM resistance induction experiment. Therefore, we can assume that the parasites were isogenic at the start of this experiment.

One day prior to infection (D₀), three naïve mice were injected intraperitoneally with 200 mg/kg cyclophosphamide (Endoxan®, Baxter) in order to obtain immune-suppression. The next day (D₁), these mice were infected intraperitoneally with a starting dose of 10⁵ bloodstream form trypanosomes. When the level of parasitemia in the blood reached 7.9 × 10⁸ trypanosomes/ml, mice were treated with 0.001 mg/kg ISM (i.e. first ISM treatment) and became microscopically non-parasitemic within a few days. When parasitemia in blood reappeared and reached 7.9 × 10⁸ trypanosomes/ml (D₀), mice were injected with an increased dose of ISM (0.005 mg/kg). As soon as the parasitemia reappeared and reached a level between 1.3 × 10⁹ and 5 × 10⁸ trypanosomes/ml (i.e. peak of parasitemia; DY), blood from each individual mouse was collected on a DEAE resin for parasite isolation and DNA extraction. A part of the collected blood was also used to infect a new group of three Endoxan®-treated mice with a starting dose of 10⁸ trypanosomes. These newly infected mice received two ISM injections: a first injection with 0.005 mg/kg ISM, and after parasites reappearance, a second injection with 0.01 mg/kg ISM (Ndoutamia et al., 1993). This infection-ISM treatment cycle was repeated several times with increasing doses (0.02; 0.04 and 1 mg/kg ISM) until full resistance to 1 mg/kg ISM was acquired. Using this methodology, three independent ISM resistant lines were obtained: MSOROM7-G; MSOROM7-H and MSOROM7-I. After each treatment cycle (annotated as passages one, two, three, four and five; or referred to as MSOROM7-G2, -G3, -G4, -G; MSOROM7-H2; -H3, -H4, -H, and MSOROM7-I2, -I3, -I4, -I; Table S1) parasites were collected for DNA extraction and sequencing (see below).

A similar resistance induction experiment in immune-competent OF-1 mice (i.e. no Endoxan® treatment) failed to give rise to fully resistant parasites.

2.5. Bioinformatic and statistical analyses

DNA extraction, sequencing and bioinformatic analyses were performed as described earlier (Tihon et al., 2017). In short, reads were mapped against the reference genome T. congolense IL3000 (Jackson et al., 2012) with SMALT v7.4 (http://sourceforge.net/projects/smaltpaper/), and duplicate reads were filtered with SAMTools V1.0 (Li et al., 2009), resulting in an average read depth of 30.9× per TRT15 sample and 33.4× per MSOROM7 sample. Single-Nucleotide-Polymorphisms (SNPs) and insertions/deletions smaller than 15 bp in length (indels) were called using the Genome Analysis Toolkit v3.4 (GATK) (McKenna et al., 2010; DePristó et al., 2011) and annotated using SnpEff (Cingolani et al., 2012) based on the gene annotation file of T. congolense IL3000 (http://tritrypdb.org/common/downloads/release-6.0/TCcongolenseIL3000/gff/data/). Gene deletions were measured by both the normalized read depth and the fraction of the gene covered by reads (length). We considered a gene to be totally deleted (i.e. gene deletion) when 75% or more of its depth coverage was missing. In the depth analysis, we used MSOROM7-G4 (i.e. mean depth coverage of 20×) instead of MSOROM7-G (i.e. mean depth coverage of 13×) to estimate long deletions in samples with sufficient depth coverage, and this choice was made because MSOROM7-G4 was as ISM resistant as MSOROM7-G (see results). Gene duplications were measured solely by the normalized read depth. To provide statistical significance for the gene duplication analysis, we calculated the differences between the sensitive and resistant lines and calculated the standard z scores. We converted the standard z scores to p-values using python scipy survival function. See (Tihon et al., 2017) for details on all computational parameters and filtering criteria for each of the above mentioned computational analyses.

We checked whether novel SNPs and small indels appeared in TRT15 and MSOROM7 over the course of the experiments. As this was not the case (see results), we checked whether there was a change in allele frequencies at heterozygous loci between the different passages of TRT15 and MSOROM7. To this end, we calculated the proportion of reads that support a given allele at a given SNP site, providing an estimate of the read depth allele frequency. For TRT15 we compared the read depth allele frequencies between passage 0 (start of the experiment) and passages 5 (1 mg/kg ISM) and 10 (1 mg/kg ISM). For MSOROM7 we compared the read depth allele frequencies between passage 0 and passages 2 (0.01 mg/kg ISM), 3 (0.02 mg/kg ISM), 4 (0.04 mg/kg ISM) and 5 (1 mg/kg ISM). If a sample at a given passage did not have sufficient read depth coverage (i.e. less than 10× coverage) at a given SNP site, then this site was removed from further analysis. In order to measure statistical significance we calculated standard deviations σ and a corresponding z-score of the differences in read depth allele frequencies between the passages as mentioned above.

Finally, we only retained those sites for which the read depth allele frequency shifted of at least 0.25 and for which the z-score was at least 3. Shifts in read depth allele frequencies higher than 0.8 were considered homozygous, which was similar to the definition of (Nielsen et al., 2011) where a homozygous genotype was described as a proportion of an allele of less than 0.2 or larger than 0.8.

A similar approach was used to identify changes in allele frequencies between drug resistant and drug sensitive isolates from natural populations. To this end, we used published sequence data from 50 T. congolense isolates from 10 different countries in sub-Saharan Africa (Tihon et al., 2017). Population genomics analyses revealed two clusters: the TC1 cluster contained 21 parasites from The Gambia, Mali, Burkina Faso, Togo, Cameroon, Ethiopia and Uganda and the TC2 cluster contained three parasites from Congo, Tanzania and Zambia (Tihon et al., 2017). The remaining 25 parasites from Zambia and one parasite from Burkina Faso were of uncertain ancestry, showing signatures of introgressive hybridization (Tihon et al., 2017). The ISM sensitivity of all these isolates were determined in previous studies (Geigy and Kauflmann, 1973; Pinder and Authie, 1984; Clausen et al., 1992; Afewerk et al., 2000; Knoppe, 2002; Delespaux et al., 2005; Mamoudou et al., 2006, 2008; Masumu et al., 2009; Moti et al., 2012): 30 strains were resistant to 1 mg/kg ISM, 14 were sensitive to 1 mg/kg ISM and six showed an intermediate profile with partial resistance to 1 mg/kg ISM (Table S1). Within each group (i.e. TC1, TC2 and Zambia) we compared the averaged read depth allele frequencies between resistant and sensitive isolates. Differences in the averaged allele frequencies ≥0.8 between the resistant and sensitive strains were classified as homozygous. Differences in the allele frequencies greater than 0.25 (but lower than 0.8) were classified as heterozygous. We selected genes with the averaged allele frequency shift higher than 0.25 between resistance and sensitive strains with a p value ≤ 0.05 (Mann-Whitney-Wilcoxon test). For a more targeted analysis, we focused on a set of 185 genes including some playing critical roles in drug resistance in various pathogens (Table S6) to further investigate allele frequency shifts between sensitive and resistant field isolates. This analysis identified 217 alleles in a total of 54 genes whose averaged allele frequency shifted ≥0.25 between the sensitive and resistant parasites of each population (Table S7).

The dataset generated and analyzed during the current study is available in EBI under the accession number PRJEB21066, http://www.ebi.ac.uk/ena/data/view/PRJEB21066.

2.6. ISM accumulation and extrusion

The accumulation of ISM in the sensitive and resistant T. congolense MSOROM7 lines as well as in the ISM resistant field isolate TRT1 was first monitored by flow cytometry, using the fluorescent properties of the drug (ISM: λem: 374 nm; λex: 590 nm). For an unknown reason, the field isolate TRT15 was very sensitive to the DEAE purification and...
Table 1

| Number of allele frequency shifts identified in each experiment. In the first experiment, we maintained the drug resistant field isolate TRT15 for 5 and 10 passages in mice either with 1 mg/kg ISM (i.e. TRT15p5-ISM and TRT15p10-ISM, respectively) or without the drug pressure (i.e. TRT15p5 and TRT15p10, respectively). In the second experiment, three independent ISM resistant lines were generated starting from the same initial MSOROM7 clone, namely: MSOROM7-G, MSOROM7-H and MSOROM7-I. In the last experiment, we compared the resistant and sensitive field isolates of each natural populations identified in (Tihon et al., 2017). |
|-----------------|-----------------|-----------------|-----------------|
| **TRT15 compared to** | **Number of shifts** | **TRT15p5** | **TRT15p10** | **TRT15p5-ISM** | **TRT15p10-ISM** |
| *Shift >0.50* | 0 | 0 | 11 | 62 |
| *Shift >0.333* | 2 | 2 | 93 | 499 |
| *Shift >0.25* | 25 | 27 | 248 | 941 |

| **MSOROM7 compared to** | **MSOROM7-G** | **MSOROM7-H** | **MSOROM7-I** |
|------------------|----------------|----------------|----------------|
| *Shift >0.50* | 163 | 4 | 26 |
| *Shift >0.333* | 1496 | 190 | 627 |
| *Shift >0.25* | 1784 | 972 | 1955 |

| **Resistant compared to Sensitive** | **TC1** | **TC2** | **Zambia** |
|-----------------|-----------------|-----------------|
| *Shift >0.50* | 2 | 12 | 3 |
| *Shift >0.333* | 10 | 32 | 42 |
| *Shift >0.25* | 30 | 70 | 132 |

many cells were lost during the isolation procedure, precluding the collection of a sufficient amount of parasites to perform the experiment. To overcome this issue, we performed the flow cytometric analysis on the ISM resistant field isolate TRT1 that is genetically nearly identical to the TRT15 strain (Tihon et al., 2017).

DEAE-column purified parasites from infected mouse blood were maintained in a cold PSG buffer pH 8. For each ISM condition, 10^6 trypanosomes were suspended in ice-cold PSG containing 0–10 μg/ml ISM supplemented with the Fixable Viability Stain (FVS, BD Horizon - primers were designed with Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) and evaluated with OligoAnalyzer (https://eu.idtdna.com/calc/analyzer). Each primer was blasted against trypanosome genomes (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to evaluate their specificity. RNA extractions were conducted with TRIzol Reagent (Invitrogen), and the cDNA synthesis was performed using the SensiMix® SYBR® No-ROX Kit (Bioline) according to manufacturer's instructions. The qPCR reactions were conducted on a LightCycler 480 (Roche) with SYBGreen I master mix (Qiagen) and 500 nM of each primer. The cycling conditions were as followed: 95 °C for 10 s; 40 cycles at 95 °C for 10 s; 52 °C for 10 s and 72 °C for 30 s.

We selected TERT and GAPDH as reference genes after assessing the stability of the gene expression level of eight putative housekeeping genes using BioGazelle qBase + v1.5 software (Hellemans et al., 2007). The primers used were:

- DMT (gene of interest) F: 5’-ACATCGGGCATTGGACCTT-3’; R: 5’-CAGTGCACAAAGGAAAAACG-3’
- TERT (housekeeping gene) F: 5’-TTTCGCCCTGTITTCTCTCA-3’; R: 5’-AGAAATCAGCCACACGCT-3’
- GAPDH (housekeeping gene) F: 5’-CGTGGAGCCGGAGTGCTTTGA-3’; R: 5’-GATGGAATTCTCGGCACCTGA-3’

2.8. Statistical analyses of laboratory experiments

For the ISM accumulation/extrusion and gene expression level experiments, two-tailed unpaired Mann-Whitney- test were carried out in GraphPad Prism 5. Data were represented as means ± standard error of the mean. P values ≤ 0.05 were considered to be statistically significant.

3. Results

3.1. Genomic changes in a resistant parasite under drug pressure

To evaluate the impact of a continuous ISM pressure on the *T. congolense* genome we maintained the drug resistant field isolate TRT15 for 10 passages in mice with 1 mg/kg ISM (TRT15p10-ISM). Although infected mice became microscopically non-parasitemic after each round of ISM treatment, parasitemia in mice reappeared within less than a week.

Bioinformatic analyses revealed a total of 157,797 SNPs and 3098 indels in TRT15 in comparison to the *T. congolense* IL3000 reference genome when SNP calling was done with the combined sequence data of all TRT15 samples obtained during the experiment. Following drug pressure, we could not detect any novel SNPs nor indels appearing in TRT15. However, we found that the read depth frequency of alleles at heterozygous loci changed over time (Table 1). A shift in read depth allele frequency of at least 0.5 was observed at 11 loci after 5 passages and at 62 loci after 10 passages under drug pressure, while no such shifts were observed when TRT15 was not subjected to drug pressure (Table 1). Of these 62 loci, 17 shifts resulted in read depth allele frequencies larger than 0.8 following drug pressure, but these were mostly found within genes coding for hypothetical proteins (results not shown).

While the continuous ISM pressure did not induce novel SNPs nor indels it caused copy number variations. Compared to the TRT15 genome before drug pressure we identified a total of 36 and 116 gene duplications and 7 and 38 gene deletions in TRT15 after 5 and 10 passages under drug pressure, respectively (Table S2) (Fig. S1 A and B). In contrast, only 19 and 13 gene duplications and 3 and 13 gene deletions were identified after 5 and 10 passages without drug pressure, respectively (Table S2) (Fig. S1 A and B).

3.2. Induction of drug resistance in a sensitive parasite

Three independent ISM resistant lines were generated in the murine host (i.e. immune-suppressed) starting from the sensitive
clone MSOROM7 by a gradual ISM induction pressure. These three lines were referred to as MSOROM7-G, MSOROM7-H and MSOROM7-I. Full resistance to 1 mg/kg ISM was acquired after three to four months under increasing drug pressure (i.e. five passages in the murine host) (Fig. 2). MSOROM7-H acquired full resistance more rapidly, already after 88 days under ISM challenge, while MSOROM7-G and MSOROM7-I acquired full resistance after 108 and 145 days, respectively. Notably, once ISM resistance reached a threshold value of 0.04 mg/kg ISM dose (i.e. passage five) (Fig. 2). All three cell lines maintained their acquired ISM resistant phenotype when grown in immune-competent mice for six months without any drug pressure.

In contrast, the same induction set-up in immune-competent mice resulted in only one line (MSOROM7-C) acquiring partial ISM resistance to 0.04 mg/kg after 20 months of drug pressure with the reappearance of parasitemia only observed in 1/3 of the ISM-treated animals. However, this line did not acquire full resistance to 1 mg/kg ISM after more than two years of drug pressure (Fig. 2). All other attempts to acquire resistant parasites in the immune-competent mice failed because of parasite clearance following drug administration.

### 3.3. Genomic differences between sensitive and resistant parasites

Bioinformatic analyses revealed a total of 93,850 SNPs in comparison to the *T. congolense* IL3000 reference genome when SNP calling was done with a combined sequence dataset of all MSOROM7 samples that were obtained during the experiment. We also identified 1786 small indels in the MSOROM7 lines compared to the reference genome IL3000, of which 86 in coding regions, including 51 frameshift mutations. Following drug resistance induction, we examined genomic changes but did not identify any novel SNPs nor indels appearing in any of the MSOROM7 ISM resistant lines. However, we did find many genomic changes in the read depth frequency of allele at heterozygous loci (Table 1), while similar shifts were not observed for indel sites (results not shown).

A total of 1784; 972 and 1955 loci showed an allele frequency shift larger than 0.25 (x score ≥ 3) in MSOROM7-G, MSOROM7-H and MSOROM7-I respectively (Table 1). Of these, 55, 25 and 23 resulted in a read depth frequency larger than 0.8 in MSOROM7-G, MSOROM7-H and MSOROM7-I lines, respectively (Table S3), of which four were common among the three resistant MSOROM7 lines but none were common with the resistant TRT15p10-ISM line (Fig. 3A and C; Table S3).

In MSOROM7-G, four out of 55 allele frequency shifts were detected at non-synonymous positions in the ABC transporter genes *TcIL3000.7_4990* and *TcIL3000.10_9180*, while one shift was detected at a non-synonymous position in the gene *TcIL3000.11_12160*, an orthologue of the T. b. brucei gene *Tb11.01.3390* encoding the mitochondrial Type II topoisomerase (Table S3: Bold).

In MSOROM7-H, we discovered that 10 out of 25 shifts were located in the gene *TcIL3000.8_1200* that encodes for the permease of the drug/metabolite transporter (DMT). Eight of these shifts were detected at non-synonymous positions and reached a read depth frequency larger than 0.8 at passage three and remained there until the end of the experiment (Table 2, Table S3: Bold). We closely examined all SNPs including SNP clusters (i.e. which were excluded from the original analysis, see (Tihon et al., 2017)) in the chromosomal region spanning the DMT gene. In the sensitive MSOROM7, we observed that the alternate allele frequencies of the genes *TcIL3000.8_1190* (block ‘d’ in Fig. 4A and D) and *TcIL3000.8_1200* (block ‘e’ in Fig. 4A and D) were of 0.25, 0.5, 0.75 or 1 (Fig. 4B). Read depth analysis of this genomic region revealed the presence of two copies of both genes in the sensitive strain (Fig. 4D), possibly explaining this peculiar tetrasomic-like alternate allele frequency pattern. In addition, we identified that almost all alleles within the DMT gene shifted to higher frequencies in MSOROM7-H compared with MSOROM7, while this was not the case for alleles found upstream of the gene (Fig. 4C). We further observed that in MSOROM7-H the normalized read depth started to decrease from passage two until full resistance, revealing the presence of only one copy of both genes in this resistant line (Fig. 4D). Similar observations were made for MSOROM7-G and MSOROM7-I (Fig. 4D) and for the resistant TRT15 (data not shown).

**Fig. 2.** Induction of ISM resistance in the immune-suppressed murine host (Endoxan-treated). The *T. congolense* sensitive strain MSOROM7 acquired ISM resistance between three to five months in three independent experiments. In total, five ISM-dose passages in the murine hosts were sufficient to acquire full ISM resistance: passage one (P1): resistant to 0.005 mg/kg ISM; passage two (P2): resistant to 0.01 mg/kg ISM; passage three (P3): resistant to 0.02 mg/kg ISM; passage four (P4): resistant to 0.04 mg/kg ISM; passage five (P5): resistant to 1 mg/kg ISM. The three independently ISM resistant lines that acquired full resistance after passage five (1 mg/kg ISM) are referred to as MSOROM7-G, MSOROM7-H and MSOROM7-I. In immune-competent mice, the acquisition of fully resistant parasites failed: only one parasite strain (MSOROM7-C) acquired a partial resistance to 0.04 mg/kg ISM after a period of > 600 days.

**Fig. 3.** Number of frequency shifts in the alternate alleles identified in: A) the MSOROM7 resistant lines compared with the original MSOROM7 sensitive line. Four homozygous changes in nucleotide sequences were common to all resistant strains and included one synonymous change in the gene *TcIL3000.11_2040* encoding a hypothetical protein, and three non-coding changes (see Table S3; Red); B) Between the resistant and sensitive parasites of each *T. congolense* populations (i.e. TC1, TC2 and Zambia) identified in (Tihon et al., 2017); C) Between the resistant and sensitive field isolates (i.e. combined TC1, TC2 and Zambian strains), between the MSOROM7 resistant lines compared with the sensitive MSOROM7, and between TRT15p10-ISM compared with TRT15. In total, 11 shifts were common between the resistant field isolates and the MSOROM7 resistant lines, but no common shifts were identified between the field isolates, the MSOROM7 resistant lines and TRT15p10-ISM D) The allele frequency shifts identified in these experiments were found in a total of 53 genes in the field-isolated strains, in 30 genes in the MSOROM7 resistant lines, and in 314 genes in the TRT15p10-ISM isolate.
Table 2
Alternate allele frequencies (including those of SNP clusters) in the gene *TcIL3000_8_1200* coding for the DMT product, as detected by WGS in the sensitive strain MSOROM7 and in the intermediate and resistant MSOROM7-H parasites. The alternate allele frequencies ranged from 0.5 (i.e. heterozygous SNP) to 1 (i.e. homozygous SNP). Alternate allele frequencies of 0.8 or higher are shown in bold and illustrate the homozygous SNPs identified in the intermediate and resistant MSOROM7-H parasites compared with the MSOROM7 sensitive line. Ref: Reference allele. NYS: Non-synonymous SNP; SYN: Synonymous SNP. P2: Resistant to 0.01 mg/kg ISM; P3: Resistant to 0.02 mg/kg ISM; P4: Resistant to 0.04 mg/kg ISM; P5: Resistant to 1 mg/kg ISM.

| Impact position (bp) | Ref. SNP | Ref. | SNP | Alternate allele frequencies |
|----------------------|----------|------|-----|-------------------------------|
|                      |          | MSOROM7 | MSOROM7-H | P2 | P3 | P4 | P5 |
| NSY 77               | G        | A     |     | 0.5 | 0.3 | 1.0 | 1.0 | 1.0 |
| SNP cluster          | NSY 122  | G     | A   | 0.5 | 0.4 | 0.8 | 0.9 | 1.0 |
| SNP cluster          | NSY 124  | T     | G   | 0.5 | 0.4 | 0.9 | 0.9 | 1.0 |
| SNP cluster          | NSY 128  | G     | T   | 0.5 | 0.4 | 0.9 | 1.0 | 1.0 |
| SNP cluster          | NSY 224  | G     | A   | 0.5 | 0.4 | 0.8 | 1.0 | 1.0 |
| SNP cluster          | NSY 401  | T     | C   | 0.5 | 0.6 | 0.8 | 0.9 | 1.0 |
| SNP cluster          | NSY 419  | A     | C   | 0.5 | 0.5 | 0.8 | 0.9 | 1.0 |
| SNP cluster          | NSY 422  | A     | G   | 0.5 | 0.5 | 0.8 | 0.8 | 1.0 |
| SNP cluster          | NSY 423  | G     | A   | 0.5 | 0.5 | 0.7 | 0.8 | 0.9 |
| SNP cluster          | NSY 483  | A     | G   | 0.5 | 0.5 | 0.9 | 0.9 | 0.9 |
| SNP cluster          | NSY 696  | C     | T   | 0.6 | 0.5 | 0.9 | 0.9 | 0.9 |
| SNP cluster          | NSY 697  | G     | C   | 0.6 | 0.5 | 0.9 | 0.8 | 0.9 |
| SNP cluster          | NSY 709  | G     | T   | 0.6 | 0.5 | 0.9 | 0.9 | 0.9 |
| SNP cluster          | NSY 731  | C     | G   | 0.6 | 0.5 | 0.9 | 0.9 | 0.9 |
| SNP cluster          | NSY 733  | A     | G   | 0.6 | 0.5 | 1.0 | 0.9 | 0.9 |
| SNP cluster          | NSY 791  | C     | T   | 0.6 | 0.5 | 0.9 | 0.9 | 1.0 |
| SNP cluster          | NSY 855  | T     | C   | 0.5 | 0.3 | 1.0 | 1.0 | 1.0 |
| SNP cluster          | NSY 856  | A     | G   | 0.4 | 0.3 | 1.0 | 1.0 | 1.0 |
| SNP cluster          | NSY 862  | C     | T   | 0.5 | 0.3 | 1.0 | 1.0 | 1.0 |

Fig. 4. Alternate allele frequencies (including those of SNP clusters) in the *TcIL3000_8_1200* gene and its surrounding region in MSOROM7 and MSOROM7-H. Note that the *TcIL3000_8_1200* gene is longer in the MSOROM7 lines compared with the reference *TcIL3000* genome because of a frameshift indel at the gene position 823. A) Genomic region analyzed. a. *TcIL3000_8_1150* b. *TcIL3000_8_1160* c. *TcIL3000_8_1170* d. *TcIL3000_8_1190* e. *TcIL3000_8_1200*. This figure is based on the number of SNPs found in this genomic region to highlight the distribution of SNPs. The scale does not reflect the physical size of these genes. A gap in the reference genome after the gene *TcIL3000_8_1200* prevented further analysis of the downstream region of the gene. B) Alternate allele frequencies in the sensitive MSOROM7; C) Alternate allele frequencies in the resistant MSOROM7-H. Each dot/triangle represents one allele position. Only the frequencies of the alternate alleles located in the *TcIL3000_8_1200* gene shifted and became fixed in the resistant population (black box). D) Normalized read depth. In the MSOROM7 sensitive strain, the normalized read depth indicates the presence of two copies of the genes *TcIL3000_8_1190* and *TcIL3000_8_1200*. These gene duplications were confirmed by sequencing the MSOROM7 sensitive strain with TruSeq DNA libraries using the methodology described in (Dumetz et al., 2017) (data not shown). The normalized read depth decreased in the resistant lines, suggesting the loss of one of these copies in response to drug pressure. E) Expression level of the DMT gene *TcIL3000_8_1200* (i.e. evaluation of the mRNA expression level) in MSOROM7 sensitive and resistant lines challenged or not with 1 mg/kg ISM by Real-Time qPCR. Average ± standard error of the mean; *: P < 0.05; **: P < 0.01 (n = 6).
shown) that also had only one copy of the DMT gene. We examined the expression level of the DMT gene and observed a significant down-regulation in all induced resistant lines but MSOROM7-G (p > 0.05) compared with MSOROM7-H, which had more definitive allele frequency shifts at non-synonymous positions, showed the lowest expression of the DMT gene (Fig. 4E).

In MSOROM7-I, notable allele frequency shifts included three non-synonymous SNPs in the genes TcIL3000.7_4990, TcIL3000.11.4410, and TcIL3000.11.5000. These shifts were larger than 0.25 in T. congolense natural populations (i.e. TC1, TC2 and Zambia; see Tihon et al., 2017) when the read depth allele frequencies were compared between ISM sensitive and ISM resistant parasites (Table S3: Bold). Shifts in the allele frequency were also found in additional common genes in drug resistant MSOROM7, the natural resistant isolates and/or TRT15 maintained for 10 passages under drug pressure (Table 3).

Six of the genes identified in these ISM resistant lines (i.e. TcIL3000.7_4990, TcIL3000.10_9180, TcIL3000.11.12160, TcIL3000.8_1200, TcIL3000.8_410 and TcIL3000.10_13700) also showed shifts larger than 0.25 in T. congolense natural populations (i.e. TC1, TC2 and Zambia; see Tihon et al., 2017) when the read depth allele frequencies were compared between ISM sensitive and ISM resistant parasites (Table 3: Red). Shifts in the allele frequency were also found in additional common genes in drug resistant MSOROM7, the natural resistant isolates and/or TRT15 maintained for 10 passages under drug pressure (Table 3).

In MSOROM7-I, notable allele frequency shifts included three non-synonymous SNPs in the genes TcIL3000.7_4990, TcIL3000.11.4410, and TcIL3000.11.5000. These shifts were larger than 0.25 in T. congolense natural populations (i.e. TC1, TC2 and Zambia; see Tihon et al., 2017) when the read depth allele frequencies were compared between ISM sensitive and ISM resistant parasites (Table S3: Bold). Shifts in the allele frequency were also found in additional common genes in drug resistant MSOROM7, the natural resistant isolates and/or TRT15 maintained for 10 passages under drug pressure (Table 3).

While we did not identify novel SNPs nor indels in the MSOROM7 resistant lines, we observed new structural variations of the genome. Gene copy number analysis revealed 31 gene deletions in MSOROM7-G, 17 gene deletions in MSOROM7-H and 39 gene deletions in MSOROM7-I compared with the MSOROM7 sensitive line (Table S4). Overall, 22 gene deletions were shared between two or more resistant lines, which were found in the gene TcIL3000.0_00780 encoding a transposase and in genes coding for unspecified products (Fig. 5A; Table S4: Bold). Interestingly, four of the deletions identified in the TRT15p10-ISM were common to gene deletions detected in one or more MSOROM7 resistant lines (Fig. 5A; Table S4: Red), but these affected genes are coding for unspecified products. We also identified 58 gene deletions in the resistant TC2 field isolates compared to their sensitive counterparts, three of which were also observed in the resistant MSOROM7 lines (i.e. non coding RNA genes; Table S4: Green, Fig. S1 C).

Furthermore, 272 genes had at least one additional copy in the MSOROM7 resistant lines compared with the sensitive line (Fig. 5B; Table S5). The gene copy number of 79 of these 272 genes also increased in TRT15p10-ISM compared with TRT15 (Fig. 5C for a representative example; Table S5: Bold). Some of the highlights of these 272 genes were 115 genes coding for VSGs or VSG associated proteins; five genes coding for cell surface expressed proteins; six genes coding for ATP-dependent dead h RNA helicases (RNA helicases); seven genes coding for retroposon hot spot proteins and 11 genes coding for the transferrin receptor ESAG6. In addition, the duplication of seven genes was common between TC1 or Zambian resistant field isolates and one or more MSOROM7 resistant lines (Fig. S1 D; Table S5: Underlined).

**Table 3**

List of genes in which allele frequency shifts were detected in two or more independent experiments (i.e. selection experiment; TRT15 experiment; comparison between resistant and sensitive field isolates). Allele frequency shifts in the gene TcIL3000.11.4410 were identified in all three experiments (bold). Two genes showed allele frequency shifts in the MSOROM7 resistant lines and TRT15p10-ISM isolate (underlined). Allele frequency shifts were detected in 10 common genes between the MSOROM7 resistant lines and the field isolates (italic; red). An additional four genes showed allele frequency shifts in both TRT15p10-ISM and the field isolates.

| Gene ID                  | Gene product                                      | MSOROM7 | Resistant |
|-------------------------|---------------------------------------------------|----------|-----------|
| TcIL3000.11.4410        | Hypothetical protein                              | Yes      | Yes       |
| TcIL3000.11.5050        | ABC transporter                                    | Yes      | Yes       |
| TcIL3000.11.10010       | Unspecified product                                | Yes      | Yes       |
| TcIL3000.11.12160       | Mitochondrial Type II topoisomerase               | Yes      | Yes       |
| TcIL3000.11.2040        | Hypothetical protein                              | Yes      | Yes       |
| TcIL3000_10_13700       | Transmembrane product                             | Yes      | Yes       |
| TcIL3000_10_9180        | ABC transporter                                    | Yes      | Yes       |
| TcIL3000_5_2100         | Unspecified product                                | Yes      | Yes       |
| TcIL3000_7_3180         | mitochondrial DNA polymerase I prot c             | Yes      | Yes       |
| TcIL3000_7_4990         | ABC transporter                                    | Yes      | Yes       |
| TcIL3000_8_00780        | Transmembrane product                             | Yes      | Yes       |
| TcIL3000.11.15160       | DNA polymerase alpha/epsilon subunit B            | Yes      | Yes       |
| TcIL3000_10_7660        | Kinetoplast DNA-associated protein                 | Yes      | Yes       |
| TcIL3000_6_4420         | ATP epsilon chain                                  | Yes      | Yes       |
| TcIL3000_6_4480         | V-type ATPase, C subunit, putative                | Yes      | Yes       |
| TcIL3000_8_6450         | Succinate dehydrogenase flavoprotein              | Yes      | Yes       |
3.4. Reduced ISM accumulation in resistant parasites

A significant decrease in ISM accumulation was observed by flow cytometry in all resistant trypanosomes compared with the sensitive MSOROM7 parental line (Fig. 6A and C). The accumulation of ISM in the sensitive and resistant parasites gradually increased as ISM concentrations augmented, until reaching a plateau at a concentration of 5 μg/ml in the incubation medium. At this concentration, the sensitive cells displayed a mean ISM fluorescent signal of 599 (standard deviation = 64.6) compared to 327 (standard deviation = 52.6) for the resistant parasites. ISM exposure started to kill parasites at a concentration of 5 μg/ml and higher, as indicated by the increased signal of the Fixable Viability Stain (FVS) (data not shown). No differences in the accumulation of ISM could be detected between the three induced-resistant lines. A similar decrease in ISM accumulation was also observed for the ISM resistant field isolate TRT1 (Fig. 6A).

Then, we assessed the dynamics of the accumulation and extrusion of ISM in the sensitive and resistant parasites and observed a fast accumulation of ISM within the first 10 min of incubation for all cell lines (Fig. 6B). After this initial period, the ISM accumulation remained at the same level over time. The observed ISM accumulation in the resistant MSOROM7-G, MSOROM7-I and TRT1 was significantly lower than for the sensitive MSOROM7 line (Fig. 6B). For both sensitive and resistant parasites, the concentration of ISM accumulating inside trypanosomes exceeded the initial starting concentration of 1 μg/ml, suggesting the involvement of an active transport of ISM. Finally, the extrusion of ISM by the parasites displayed a similar profile for all the strains (Fig. 6B).

4. Discussion

The drug ISM plays a key role in the treatment of T. congolense infections. However, little information is available about its cellular uptake and accumulation, its mechanisms of action and development of drug resistance in this parasite, impeding a proper management of ISM resistance in the field. Here, we used WGS to study the genetic changes that may underlie the acquisition of ISM resistance within an in vivo model.

We demonstrated the importance of the immune system of the host in preventing the development of ISM resistance. In an immune-competent host, sensitive parasites failed to acquire resistance to 1 mg/kg ISM after more than two years under drug pressure. In immune-suppressed animals, sensitive parasites evolved to fully drug resistant parasites after three to five months of drug pressure. Similar observations were previously described in T. b. evansi parasites (Osman et al., 1992; Mutugi et al., 1994), and it has been hypothesized that trypanocidal drugs, rather than killing parasites, induce restrictions in the replication of the trypanosomes that will then be eliminated by the immune system of the host (Geerts et al., 2001). This mechanism could explain partly why trypanosomes can overcome an ISM pressure in immune-deficient animals. The link between a weakened host immune system and drug resistance acquisition in protozoan parasites is further highlighted by the observation that impaired immunity resulted in treatment failures and drug resistance to visceral leishmaniasis and malaria in HIV co-infected patients (Troya et al., 2008; Mondal et al., 2010; Menendez et al., 2011). In the field, impaired animal immunity...
could result from injuries, infections with other pathogens such as intestinal worms, malnutrition and stressful situations. Proper livestock health management is therefore highly important to lower the probability of ISM resistance development.

Within the drug resistant parasites, gene deletions and duplications could be detected in contrast to novel SNPs or indels when comparing them with the drug sensitive parasites. Moreover, no significant changes were observed within the genes coding for the F1-subunit γ and other proteins interfering with the mitochondrial F1F0-ATP synthase, suggesting that T. congolense developed different ISM resistance mechanisms than T. b. brucei (i.e. in vitro-induced resistance), T. b. evansi and T. b. equiperdum (Brun and Lun, 1994; Dean et al., 2013; Gould and Schnauffer, 2014; Baker et al., 2015). In addition, the resistant MSOROM7 T. congolense parasites were still fully capable of completing successfully their life cycle in the tsetse fly vector (data not shown), indicating the presence of a functional kDNA. Interestingly, we identified significant shifts in allele frequency among heterozygous loci associated with non-synonymous changes in genes coding for ABC transporters, DMT and transmembrane products. Specifically on the DMT gene, we observed a strong increase in the frequency of 19 alternate alleles at non-synonymous positions, we concluded that the first copy of the gene was lost in these two resistant lines.

Interestingly, the expression level of the DMT product was significantly downregulated in the resistant parasites, especially in MSOROM7-H where allele frequency shifts at non-synonymous positions were observed. This suggests that the combination of the loss of one copy of the DMT genes and the fixation of non-synonymous mutations had a higher impact on the gene expression level. This may have contributed to the observed decrease in ISM accumulation within the drug resistant parasites.

Members of the DMT superfamily have been involved in antibiotics resistance in Staphylococcus aureus (Griinius and Goldberg, 1994) and Escherichia coli (Yerushalmi et al., 1995), and in chloroquine resistance in P. falciparum (Martin and Kirk, 2004). Similarly, an important role of ABC transporters in drug resistance has previously been proposed in T. congolense (Delespau et al., 2005) and has been documented in many other organisms including T. brucei, T. cruzi, P. falciparum, Schistosoma mansoni, and Leishmania parasites (Ouellette et al., 2001; Perez-Victoria et al., 2002; Shahi et al., 2002; Alibu et al., 2006; Sanderson et al., 2009; Franco et al., 2015; Garg and Goyal, 2015; Pinto-Almeida et al., 2015; Veiga et al., 2016). Changes in other transport proteins also conferred resistance in Trypanosoma spp. (Barrett et al., 1995; Matovu et al., 2003; de Koning et al., 2004; Witola et al., 2004; Munday et al., 2015). The potential link between the observed allele frequency shifts...
and ISM transport modifications still has to be confirmed, but the genes listed in this paper represent promising targets for further functional experimental analyses.

Our genomic analyses also revealed that new gene deletions and gene amplifications may possibly play a role in ISM resistance. However, it is difficult to establish a link between ISM resistance and the observed gene deletions/amplifications due to relatively poor annotation of the *T. congolense* genome. The fact that 79 gene amplifications were common between the experimentally induced MSOROM7 resistant parasites and the TRT15 parasites under drug pressure for 10 passages may suggest that these changes in gene copy numbers reflect the mutagenic properties of ISM. This is possibly the consequence of the high affinity of ISM for double-stranded DNA molecules (Wilkes et al., 1995) that leads to its accumulation in both the mitochondrion and nucleus of the parasites where it induces double strand breaks and subsequently inhibits the kDNA (Shapiro and Englund, 1990) and DNA (Kaminsky et al., 1997) replications. To overcome DNA damages and maintain its integrity, cells possess a DNA repair machinery that consists of a complex of distinct repair pathways (reviewed in (Genois et al., 2014)). This includes a double strand breaks (DSB) repair that involves both homologous and non-homologous recombination (Genois et al., 2014). The potential involvement of the DSB repair in response to an ISM pressure could explain the observed shifts in read depth allele frequency (i.e. homologous recombination where the sister chromatid acts as repair template) and CNVs (i.e. homologous recombination between homologous repeats to amplify or delete DNA sequences) (Genois et al., 2014). This hypothesis is supported by the observation of the deletion of the DMT gene (Fig. 4) and other gene deletions and duplications (Fig. 5), possibly as the result of DSB repair events covering these genes. In addition, allele frequency shifts and CNVs were also observed in the TRT15 isolate under drug pressure but not in the absence of drug pressure, further suggesting that ISM induces strong mutagenic effects.

The potential involvement of the DNA repair machinery in drug resistance mechanisms has been suggested in *P. falciparum* where the DNA repair machinery was upregulated to respond to exogenous stress (i.e. DNA damaging agents) and promoted cell survival (Gupta et al., 2016), and in *Leishmania* parasites where the amplification of P-glycoprotein genes through homologous recombination conferred drug resistance (Ouellette et al., 1991; Grondin et al., 1993). Similar mechanisms could potentially exist in *T. congolense* in response to the DNA damaging drug ISM to promote cell survival and drug resistance.

Other genome modifying mechanisms such as point mutations and sexual recombination could also play a role in some of the genomic modifications observed in the resistant parasites. However, there is currently no evidence about a potential recombination cycle in bloodstream form *T. congolense* parasites (i.e. meiosis is taking place in the tsetse fly salivary glands in *T. brucei* (Gibson et al., 2008; Peacock et al., 2011)).

5. Conclusion

It is clear that the acquisition of ISM resistance in *T. congolense* is a complex phenomenon and that multiple mechanisms could lead to the acquisition of the drug resistant phenotype known as the ‘many roads to drug resistance’ concept (Hefnawy et al., 2017). While we demonstrated that the immune system of the host plays a key role in hindering the acquisition of ISM resistance, we were able to induce drug resistance in a sensitive isolate in an immune-suppressed host. Subsequent genomic analyses revealed that non-synonymous mutations within genes encoding ABC transporters, DMT and some ‘unidentified’ transmembrane proteins may explain the development of drug resistance. The next step would be to perform functional analyses and confirm the involvement of these genes in ISM resistance.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2017.10.002.

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