Human African trypanosomiasis (HAT) is a neglected tropical infectious disease transmitted by biting tsetse flies and is prevalent in sub-Saharan Africa [1,2]. In humans, the disease is caused by two sub-species of the protozoan *Trypanosoma brucei* – *T. b. gambiense*

[redacted]

the development of diagnostic tools such as those described for melarsoprol resistance [15,16].

We have investigated the mechanism of resistance to eflornithine and show that acquisition of selected resistance is accompanied by loss of a specific transporter. We further show, using genetic manipulation, that this transporter mediates uptake of eflornithine and that its loss confers resistance, whilst its expression in resistant lines restores sensitivity.

R

Selection of eflornithine resistant bloodstream form *T. brucei*

Eflornithine resistant parasites were derived *in vitro* from a wildtype bloodstream form *T. brucei brucei* strain 427 by growth in increasing concentrations of drug. It took two months (24 passages) to attain a line expressing forty fold less sensitivity to drug, based on the IC_{50} value of eflornithine in the drug sensitive parent strain (Fig. 1A) and no growth phenotype was observed. Two independent cell lines were generated in this way. There was no cross-resistance with other currently used trypanocides (Table 1), although there was a significant increase in sensitivity to pentamidine, which we cannot explain at this juncture. The resistant lines also grew in female ICR (Institute for Cancer Research) mice and exhibited resistance to

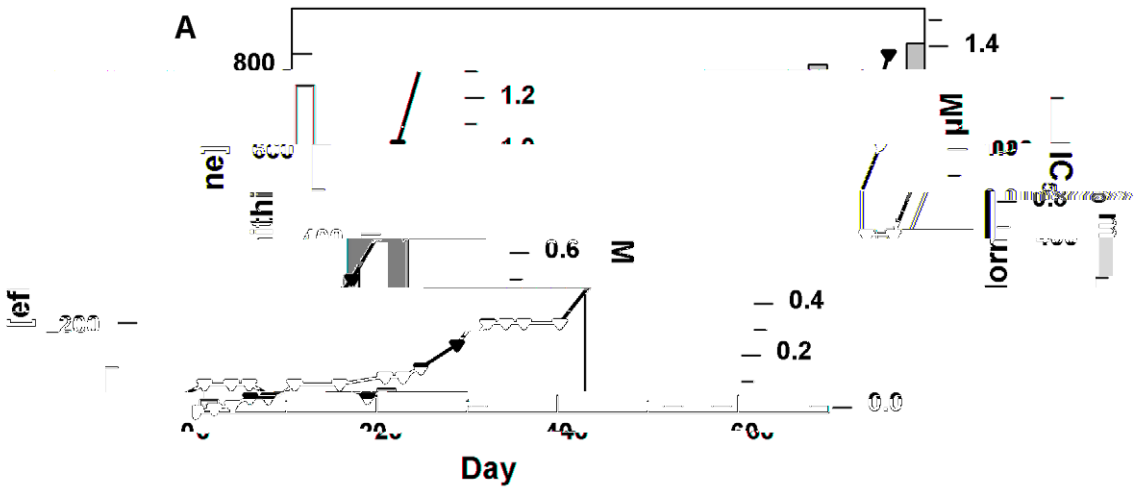

Fig. 1 R *T. brucei brucei (A)*, [redacted] *Trypanosoma brucei*. Black triangles and left hand y-axis

both the minimum curative dose of 2% w/v and a higher 5% w/v eflornithine whilst mice infected with wildtype cells were cured with the lower 2% w/v dose. Resistant cells remained susceptible to pentamidine (4 mg kg^{-1}, four daily doses) (Fig. B1). This demonstrates that the *in vitro* selected mechanism for resistance is also operative *in vivo*. Interestingly, isobologram analyses (Fig. 2) revealed that nifurtimox and eflornithine are not synergistic to one another's activity *in vitro*. The average fractional inhibitory concentration (FIC) is used as a measure of interaction between two drugs and is a sum of the IC_{50} of the drug acting in combination divided by the IC_{50} of the drug acting alone. An FIC of 1.5 was recorded for eflornithine and nifurtimox, where a value ≤ 1.4 is taken as antagonistic [17]). This was a surprise given the theory that eflornithine would deplete cellular trypanothione thus rendering the cells more susceptible to oxidative stress induced by nifurtimox.

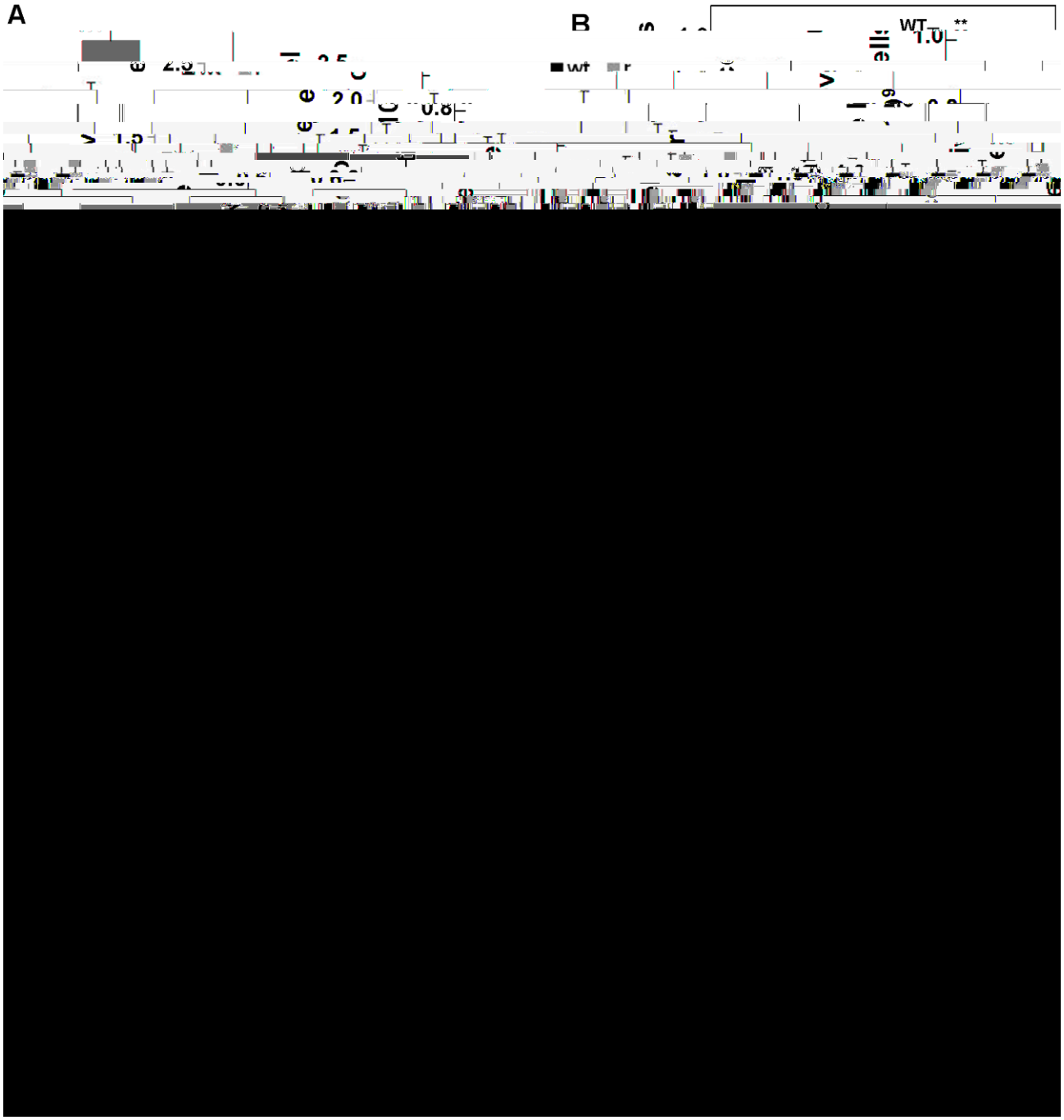

Fig 3. Metabolic profiles and eflornithine uptake. (A) Relative abundance of polyamine metabolites in wildtype (WT) and eflornithine resistant (R) cell extracts. (B) Uptake of eflornithine in wildtype (filled bars) and resistant cells (hatched bars) over one hour. Stars indicate a significant difference at a 0.01 level between WT at time 0 and WT after 60 minutes. A hash indicates that R at time 0 and R after 60 minutes show no significant difference at a 0.05 level. (C) Eflornithine uptake in wildtype and resistant cells. ^{3}H-eflornithine transported into wildtype (triangles) and resistant (circles) cells was measured over 30 minutes. Measurements are an average of four separate experiments, each wit

incidence of treatment failure with melarsoprol has led to its being replaced with eflornithine as first line treatment for stage 2 HAT [2]. Combination therapy using eflornithine with the nitrofuran, nifurtimox, licensed for use in Chagas' disease has been added to the World Health Organisation's list of essential medicines as part of the nifurtimox-eflornithine combination therapy for HAT [3].

Although several initiatives are underway to develop new drugs for human African trypanosomiasis, none are currently in human trials and a minimum of five years will elapse before a new drug could complete trials and reach the market place. The loss of eflornithine, alone or in the nifurtimox combination, would represent a calamity in terms of sustaining control of HAT.

Previous work with bloodstream and procyclic form trypanosomes also revealed a relative simplicity in selecting eflornithine

The data presented here show that resistance to eflornithine is easily selected in the laboratory. Selection of resistance in two independently derived lines led to deletion of the *TbAAT6* gene. Eflornithine uptake was lost indicating that this gene encodes a transporter capable of carrying the drug into trypanosomes. The loss of *TbAAT6* either by gene deletion as observed in the selected drug resistance lines, or by RNAi is sufficient to render trypanosomes over 40 fold less sensitive to eflornithine than wildtype cells. Furthermore, ectopic expression of *TbAAT6* in trypanosomes that have deleted the gene is sufficient to restore wildtype levels of eflornithine sensitivity confirming that loss of *TbAAT6* alone is necessary and sufficient to generate resistance.

We have, as yet, been unable to assign a physiological function to TbAAT6 in African trypanosomes, and this is a topic of ongoing research. However, it is one of a large family of related genes described in the kinetoplastida belong to the amino acid transporter 1 superfamily. Only a few other members of the family have been functionally characterised. These include an arginine transporter in *Leishmania donovani* [25], an arginine transporter in *T. cruzi* [26] and polyamine transporters in *L. major* [27] and *T. cruzi* [28]. The AAT6 gene is not syntenic with genes in *Leishmania spp.* or *T. cruzi*. Furthermore, the evolution of the AAT family [22] makes it impossible, currently, to define specific functionality to any of these transporters based on homology alone.

nithine would effectively be subject to nifurtimox monotherapy even in combination chemotherapy. Nifurtimox resistance has been selected *in vitro* and has been shown to be cross resistant with another emerging trypanocide, fexinidazole, currently in clinical trials [32]. Given nifurtimox's lack of efficiency [33], eflornithine resistance alone is likely to lead to large numbers of treatment failures from the combination. If the loss of TbAAT6 is involved in resistance in the field, then it will be possible to implement a simple PCR-based test for resistance, allowing for more suitable treatments to be administered.

Materials and Methods

Ethics statement

This study was undertaken in adherence to experimental guidelines and procedures approved by the UK Home Office under Project Licence No. 60/3760 as complying with the Animals (Scientific Procedures) Act 2006 entitled Biochemistry, genetics and immunology of parasitic protozoa.

Culturing bloodstream form trypanosomes

Wildtype 427 bloodstream form trypanosomes were cultured in HMI-9 (Biosera) [34] supplemented with 10% foetal calf serum (Biosera) at 37°C, 5% CO_2. Eflornithine resistant parasites were selected in increasing concentrations of drug starting at 1.5 μM. When cells were growing at a rate comparable to wildtype they were cloned by limiting dilution and subcultured into double the drug concentration.

In vitro drug treatment

The Alamar blue assay developed by Raz *et al.* [35] for bloodstream form trypanosomes was used. Bloodstream form parasites were seeded at 2×10^4 cells per ml into a serial dilution of eflornithine (a gift from Pere Simarro, WHO) starting at 20 mM. Plates were incubated for 48 hours at 37°C 5% CO_2 then 20 μL Resazurin dye (Sigma) at 0.49 μM was added to each well. Plates were incubated for a further 24 hours then read on a fluorimeter (emission 530, excitation 595) (FLUOstar Optima, BMG Labtech). IC_{50} values were calculated

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phase and resuspended at $\times 10^9$ in HMI-9 with added eflornithine at 0.1 mM. These were incubated for 30 minutes, washed in HMI-9 and quenched in hot ethanol. The cell lysate was then run on the Orbitrap mass spectrometer as detailed below.

Tritiated eflornithine was obtained from Moravek Biochemicals with a specific activity of 1.6 Ci/mmol, 1mCi/ml. Mid-logarithmic growth phase cells were grown up to attain sufficient cell densities to permit use of $\times 10^7$ cells per reaction. Cells were washed in CBSS buffer (25 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 0.55 mM $CaCl.2H_2O$, 0.5 mM $MgSO_4.7H_2O$, 5.6 mM Na_2

lysis and protein denaturation was achieved by addition of 200 µl of cold chloroform/methanol/water (ratio 1:3:1), followed by vigorous mixing for 1 hour at 4°C.

For both methods, extract mixtures were centrifuged for two minutes at 16,000 RCF, 4°C. The supernatant was collected, frozen and stored at −80°C until further analysis.

Samples were analysed on an LTQ Orbitrap mass spectrometer (Thermo Fisher) in positive mode, coupled to HPLC separation using a ZIC-HILIC column (Sequant) according to the method published by Kamleh *et al.* [37]. Each sample was also analysed on an Exactive orbitrap mass spectrometer (Thermo Fisher) in both positive and negative modes (rapid switching), coupled to HPLC with a ZIC-HILIC column. Exactive data was acquired at 25,000 resolution, with spray voltages −4.5kV and 2 2.6kV, capillary temperature 275°C, sheath gas 20, aux gas 15 and sweep gas 1 unit. Minor adjustments were made to the published HPLC mobile phase gradient as follows: Solvent A is 0.1% formic acid in water, and solvent B is 0.1% formic acid in acetonitrile, 80% B (0 min), 50% B (12 min), 50% B (26 min), 20% B (28 min), 20% B (36 min), 80% B (37 min), 80% B (47 min).

Metabolite identification and relative quantitation was undertaken using ToxID software (Thermo Fisher), by searching for peaks that correspond to the accurate mass of metabolite ions within a 3 ppm window (or 5 ppm window for Exactive data). The metabolite lists were obtained from trypanosome-specific databases in Trypanocyc (metacyc.org) and KEGG (www.genome.jp/kegg/), lipids were excluded from the data analysis. Metabolite levels are expressed as mean peak height from 3 biological replicates. Multivariate statistical analysis comprised a principal component analysis based on putatively identified metabolites, and significance for individual metabolites was calculated by t-test ($a = 0.05$).

Cladogram construction

Cladograms were constructed using the CLC genomics workbench software alignment and tree building tools. A neighbour joining algorithm was used and the tree was bootstrapped 1000 times.

Text S1 Oligonucleotides used for amplification of TbAAT genes and vector construction.
Found at: doi:10.1371/journal.ppat.1001204.s001 (0.08 MB DOC)

Figure S1 The mass of each metabolite is shown on the right hand side. The y-axes show relative intensities for each metabolite on exit from the chromatography column.
Found at: doi:10.1371/journal.ppat.1001204.s002 (0.47 MB TIF)

Table S1 Retention times on the HILIC column along with

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