High-throughput decoding of antitrypanosomal drug efficacy and resistance

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The concept of disease-specific chemotherapy was developed a century ago. Dyes and arsenical compounds that displayed selectivity against trypanosomes were central to this work1,2, and the drugs that emerged remain in use for treating human African trypanosomiasis (HAT)3. The importance of understanding the mechanisms underlying selective drug action and resistance for the development of improved HAT therapies has been recognized, but these mechanisms have remained largely unknown. Here we use all five current HAT drugs for genome-scale RNA interference target sequencing (RIT-seq) screens in Trypanosoma brucei, revealing the transporters, organelles, enzymes and metabolic pathways that function to facilitate antitrypanosomal drug action. RIT-seq profiling identifies both known drug importers3,4 and the only known pro-drug activator5, and links more than fifty additional genes to drug action. A bloodstream stage-specific invariant surface glycoprotein (ISG75) family mediates suramin uptake, and the AP1 adaptin complex, lysosomal proteases and major lysosomal transmembrane protein, as well as spermidine and N-acetylglucosamine biosynthesis, all contribute to suramin action. Further screens link ubiquinone availability to nitro-drug action, plasma membrane P-type H+-ATPases to pentamidine action, and trypanothione and several putative kinases to melarsoprol action. We also demonstrate a major role for aquaglyceroporins in pentamidine and melarsoprol cross-resistance. These advances in our understanding of mechanisms of antitrypanosomal drug efficacy and resistance will aid the rational design of new therapies and help to combat drug resistance, and provide unprecedented molecular insight into the mode of action of antitrypanosomal drugs.

African trypanosomes are transmitted by the tsetse insect vector and circulate in the bloodstream and tissue fluids of their mammalian hosts. These protozoan parasites cause HAT, also known as sleeping sickness, and the livestock disease known as Nagana. HAT is typically fatal if there is no chemotherapy intervention. The public health situation has improved recently with increased monitoring and chemotherapy averting more than 1.3 million disability-adjusted life years (DALYs) in the year 2000 and the estimated number of cases at less than 70,000 in 2010. Because this drug has a strong negative charge, it cannot cross lipid membranes by passive diffusion. Genes that are linked to the action of suramin encode ISG75, the function of which is unknown3, four lysosomal proteins (the cathepsin L (CatL) and CBP1 peptidases, p67 and Golgi/lysosomal protein 1 (GLP1)), all four subunits of the adaptin complex (AP1), which are involved in endosomal, clathrin-mediated trafficking, and multiple spermidine and N-acetylglucosamine biosynthetic enzymes (Supplementary Fig. 2 and Supplementary Data 1). The known eflornithine transporter is the only primary signature from the eflornithine screen. By contrast, the suramin screen revealed 28 genes associated with primary signatures (Fig. 1c and Supplementary Data 1). Suramin, which has been used for HAT therapy since the 1920s4, is a colourless sulphated naphtylamine related to trypan red. Because this drug has a strong negative charge, it cannot cross lipid membranes by passive diffusion. Genes that are linked to the action of suramin encode ISG75, the function of which is unknown3, four lysosomal proteins (the cathepsin L (CatL) and CBP1 peptidases, p67 and Golgi/lysosomal protein 1 (GLP1)), all four subunits of the adaptin complex (AP1), which are involved in endosomal, clathrin-mediated trafficking, and multiple spermidine and N-acetylglucosamine biosynthetic enzymes (Supplementary Fig. 2 and Supplementary Data 1).

Eight of these genes were selected for further analysis. We assembled multiple independent inducible RNAi strains for each gene and confirmed that knockdown of all six genes (Fig. 2a and Supplementary Fig. 3) increased suramin resistance in every case (Fig. 2b and Supplementary Fig. 4). We then determined subcellular localization for the putative major facilitator superfamily transporter (MFST); the tandem of three closely
related MFST genes gave the strongest read-density signature in the suramin screen and the greatest half-maximum effective concentration (EC50) increase (>tenfold) following knockdown (Fig. 2b). In contrast to the putative ubiquitin hydrolase (UBH1) identified by the screen, MFST and a member of the endomembrane EMP70 family partitioned into the T. brucei membrane fraction, as expected (Fig. 2c), and MFST localized to the lysosome as did the major lysosomal type I membrane glycoprotein, p67 (ref. 17), which was also identified in the screen, MFST and EMP70 are membrane associated. The western blots show supernatant (S), wash (W) and pellet (P; membrane fraction). The dashed lines indicate expected outcomes for no interaction. The dashed boxes, areas magnified in fluorescent images.

Isobolograms also identified the screen (see Supplementary Fig. 2 and Supplementary Data 1), suggesting that ISG75 copy number is highly connected to suramin resistance. To investigate whether ISG75 contributes to suramin binding, we performed whole-cell binding assays using 3[H]-labelled suramin. Cells that were depleted for ISG75 displayed significantly and specifically reduced suramin binding (Fig. 2f).

We observed a greater than fourfold increase in EC50 after knockdown of the CatL-like protease known as brucipain, another abundant lysosomal protein15, and an orthogonal assay using a dual-sensitivity CatL–CatB inhibitor revealed inhibitor antagonism (Fig. 2g), indicating that protease activity enhances suramin toxicity. Taken together, the
results demonstrate a central role for lysosomal functions in suramin action. As four enzymes that are involved in spermidine biosynthesis, including ornithine decarboxylase (ODC), were linked to suramin action (Supplementary Data 1), we used eflornithine to specifically inhibit ODC, which again revealed inhibitor antagonism (Fig. 2g; Supplementary Table 1). Thus, ODC activity enhances suramin toxicity, probably through spermidine biosynthesis. Suramin endocytosis21 and intralysosomal accumulation22 have previously been demonstrated in T. brucei and an acquired suramin resistance phenotype was stable in bloodstream stage T. brucei but was not expressed in the insect stage23. The RIT-seq profile reported here, bloodstream-stage-specific expression of ISG7516 and strong downregulation of endocytic and lysosomal activities in the insect stage24, are all consistent with stage-specific, intralysosomal accumulation of suramin.

Work with dyes and arsenicals revealed the first examples of resistance to chemotherapy a century ago and, based on cross-resistance, it was deduced that there are shared mechanisms contributing to the action of certain ‘parasitotropic’ compounds. Among current HAT therapies, cross-resistance has been documented only for melarsoprol and pentamidine8, but our understanding of the mechanism remains incomplete. Both drugs enter trypanosomes through the P2 AT1 but additional, dual-specificity transporters are predicted9. To identify cross-resistance mechanisms, we analysed all pair-wise comparisons among our screens (Fig. 3a). A single robust signature emerged, implicating two closely related aquaglyceroporins (AQPs)25 in melarsoprol and pentamidine cross-resistance. To directly test the role of the AQPs, we generated a strain that was deficient in aqp2 and aqp3 (aqp2/aqp3-null strain) (Fig. 3b). The EC50 was increased more than 2-fold and 15-fold for melarsoprol and pentamidine, respectively, in aqp2/aqp3-null cells compared to wild-type cells (Fig. 3c). Our favoured hypothesis involves regulation of dual-specificity transporters by AQPs.

The nifurtimox, pentamidine and melarsoprol screens yielded eight, nine and nine genes associated with primary signatures, respectively. The major primary signature in the nifurtimox profile identified the mitochondrial, flavin-dependent nitroreductase that activates this class of nitro pro-drugs8. We also identified the putative flavokinase that converts riboflavin to FMN, an essential nitroreductase cofactor6. Four additional signatures identified genes that encode proteins linked to ubiquinone biosynthesis (Supplementary Fig. 2 and Supplementary Data 1), in support of the hypothesis that nitroreductase, like NADH dehydrogenases, transfers electrons from NADH to ubiquinone to generate ubiquinol6. We assembled RNAi strains for one of these factors and demonstrated that knockdown increased the EC50 for nifurtimox by approximately 1.5-fold (Supplementary Fig. 5). Thus, six gene signatures support a dominant role for nitroreductase in nifurtimox activation and suggest that this is dependent upon the availability of the FMN cofactor and the natural substrate.

Pentamidine is an aromatic diamidine, a nucleic acid binding drug that accumulates to millimolar concentrations and collapses trypanosome mitochondrial membrane potential26. Two primary signatures from the pentamidine screen identify genes encoding P-type ATPases (Supplementary Fig. 2 and Supplementary Data 1), and one of these represents the plasma membrane H+-ATPases, HA1, HA2 and HA3 (ref. 27). We assembled RNAi strains for these ATPases and demonstrated that knockdown increased the EC50 for pentamidine more than eightfold (Supplementary Fig. 5), suggesting that an HA1–3 dependent proton motive force is required to drive pentamidine uptake. We used a similar approach to demonstrate a greater than twofold increase in the EC50 for pentamidine following knockdown of a putative protein phosphatase (Supplementary Fig. 5).

Melarsoprol acts primarily by forming a stable adduct with trypanothione, known as Mel T28, but whether this adduct reduces or increases toxicity has remained unclear. The melarsoprol screen identified a link to trypanothione synthase and trypanothione reductase (Supplementary Fig. 2 and Supplementary Data 1), suggesting that the Mel T adduct is toxic. Three other primary signatures identified an over-representation ($P = 2.3 \times 10^{-9}, \chi^2$ test) of putative protein kinases (Supplementary Fig. 2 and Supplementary Data 1), and another signature identified a gene encoding a highly phosphorylated protein related to the amino-terminal segment of the large tumour suppressor, LATS1 (see Supplementary Fig. 2a). We used independent strains to confirm that LATS1-like knockdown increased the EC50 for melarsoprol by approximately 1.5-fold (Supplementary Fig. 5). On the basis of these signatures, we suggest a role for a signalling cascade in melarsoprol susceptibility. Our findings are summarized in Fig. 4. In particular, we propose that suramin uptake occurs through ISG75-mediated endocytosis (Fig. 4a). Metabolic pathways that contribute to suramin or nifurtimox action are detailed in Fig. 4b.

All but one of the current HAT drugs was developed in the absence of an understanding of the chemical–biological relationships underlying toxicity or selectivity. Our RIT-seq profiles revealed more than 50 T. brucei genes that enhance drug susceptibility, unearth new interactions that are largely inaccessible using other approaches. Notably, the knockdown approach and the sensitivity of RIT-seq allow access to essential proteins, complexes and pathways such as H+-ATPase, the adaptin complex and spermidine biosynthesis. Our results also show the utility of drugs as molecular probes for functional networks. In particular, the findings highlight factors that contribute to drug
**METHODS SUMMARY**

Assembly of the bloodstream-form *T. brucei* RNAi library and RT-sequencing were reported previously\(^6\). Briefly, a tetracycline-inducible RNAi plasmid library, containing randomly sheared genomic fragments (with a mean fragment size of \(\sim 600 \text{ bp}\)) under the control of head-to-head, tetracycline-inducible phage T7 promoters\(^8\), was targeted to a single genomic locus that had been validated for robust expression\(^20\). The long double-stranded RNAs (dsRNAs) that were generated in the presence of tetracycline are processed to produce a pool of short interfering RNAs that programme the endogenous RNAi machinery to mediate sequence-specific destruction of the cognate messenger RNA. For this study, the library was grown under inducing conditions with drug selection, and genomic DNA was isolated from surviving populations. For RT-sequencing, adaptor-ligated sequencing libraries were prepared from each genomic DNA sample and used to amplify DNA fragments containing RNAi cassette-insert junctions in semi-specific PCR reactions; one primer was specific for the RNAi vector and the other for the Illumina adaptor. Size-selected DNA was sequenced with 76 cycle runs on an Illumina GAII. Sequencing reads containing a nine-base RNAi cassette-insert junction sequence were then mapped to the *T. brucei* reference genome. In cases in which loss of function increases drug tolerance, RNAi-target sequence representation is increased relative to the otherwise susceptible population, revealing ‘hot spots’. Thus, RNAi target fragments serve as templates for the production of dsRNA and also provide unique sequence identifiers for each clonal population.

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions S.A., N.B., L.G. and K.F.L. carried out the T. brucei manipulation and analyses, S.E., A.S.-F. and D.J.T. carried out the Illumina sequencing and mapping, D.H. coordinated the study and S.A., M.C.F., M.B. and D.H. wrote the paper.

Author Information Sequence data from this study have been submitted to the European Nucleotide Archive at http://www.ebi.ac.uk/ena under accession number ERA071064. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to D.H. (david.horn@lshtm.ac.uk).
METHODS
T. brucei growth and drug selection. The bloodstream-form T. brucei M1at 1.2 clone 221A RNAi library was derived using the randomly sheared genomic fragment (mean fragment length ~600 bp) RNAi plasmid library. The T. brucei RNAi library and 2T1 cells were maintained as described. For selective screens, the RNAi library, maintained throughout at >5 × 10⁶ cells, was induced with tetracycline (1 μg ml⁻¹) for 24 h and then grown in medium containing tetracycline, plus each HAT drug at 0.5 × EC₅₀ to 3.5 × EC₅₀ (Supplementary Table 1 and Supplementary Fig. 1). All drug stocks were in dimethylsulphoxide.

RIT-seq. Selected populations from each screen were assessed for tetracycline-dependent drug resistance. The RNAi target fragments provide unique identifiers for each clone in the population. As a quality-control step, PCR amplification, agarose gel fractionation and Sanger sequencing of the eluted products were performed as described, and followed with RIT-seq analysis.

Selected populations from each screen were assessed for tetracycline dependence in coverage but we suggest that reduced coverage in the current RIT-seq outputs is not due to reduced representation by fewer RNAi targets. Our screens yielded 5–59 genes derived from the ends of the RNAi target fragments. Only sequences containing a terminal RNAi-vector junction sequence (GGCTCGCGGA) were mapped to the T. brucei 927 reference genome using the SSAHA sequence alignment algorithm.

After mapping, for each protein coding sequence (CDS) in each experiment, we performed as described, and followed with RIT-seq analysis. All nine genes that were identified by Sanger sequencing were associated with high-density Illumina read-counts (13,000 to 528,000; see Supplementary Data 1a). Briefly, we ran 76-cycle sequencing on an Illumina GAII; this generates sequence tags that were identified by Sanger sequencing were associated with high-density Illumina read-counts (13,000 to 528,000; see Supplementary Data 1a). From this set, we derived 55 genes with ‘primary signatures’, those associated with two or more RIT-seq tags. If these tags were randomly distributed, we would expect a single primary signature from 300 screens using eflornithine or from two screens using suramin. Assigning a high degree of confidence to the vast majority of observed primary signatures (Supplementary Data 1a).

Read-density signatures. Genome coverage in the current RNAi library represents >99% of all genes, with 5 RNAi targets per gene on average; shorter genes are expected to be represented by fewer RNAi targets. Our screens yielded 5–59 genes (0.07–0.8%) with a ≥4 RIT-seq tag (a tag with a read density of ≥99; the efllornithine screen yielded 5, the suramin screen 9 the nifurtimox screen 54, the pentamidine screen 17 and the mepronapazol screen 19). In each screen, at least one gene was associated with a ≥3,000 RIT-seq tag (Supplementary Data 1a). From this set, we derived 55 genes with ‘primary signatures’, those associated with two or more >99 RIT-seq tags. If these tags were randomly distributed, we would expect a single primary signature from 300 screens using eflornithine or from two screens using suramin, assigning a high degree of confidence to the vast majority of observed primary signatures (Supplementary Data 1a).

Plasmid construction and strain assembly. The AQP locus was disrupted by replacement of a 4,772-bp (AQP2 and AQP3) fragment with NPT and BLA selectable markers (the T. brucei genome is diploid). Gene-specific RNAi fragments of 400–600 bp or 200 bp, to facilitate moderate knockdown in the case of the known essential gene p67 (ref. 17), were amplified using PCR primers designed using RNAiTools and cloned into pRPaiSL for the generation of stem-loop, ‘hairpin’ dsRNA as the trigger for RNAi. We used a long, 400–600 bp RNAi target fragment for CatL because RNAi previously produced no growth defect. However, cells retained 35% CatL activity in that study, probably explaining why we see a major growth defect when expressing a more potent stem-loop dsRNA (Supplementary Fig. 3). For epitope tagging at native loci, C-terminal fragments, or an N-terminal fragment (UBH1), were amplified and cloned in pNATsTAC and pNATsTACGS (ref. 36), respectively. Constructs were introduced into 2T1 cells as described. Full oligonucleotide details are available on request.

Strain analysis. Cumulative growth curves were generated from cultures seeded at 10⁶ cells ml⁻¹, counted on a haemocytometer and diluted back to 10⁷ cells ml⁻¹ as necessary. For EC₅₀ assays, RNAi strains were pre-induced for 72 h in 1 μg ml⁻¹ tetracycline, except CatL and AP1β, which were pre-induced for 24 h at 2.5 and 3 μg ml⁻¹, respectively. Isobolograms were generated using a checkboard assay as described, and FMK024 (N-morpholinonesuccinyl-phenylalanineω-homophenylalanylfluoromethyl ketone; Sigma) is an irreversible, dual-specificity inhibitor of CatL and CatB. All EC₅₀ assays were carried out using alamarBlue as described, and Southern blotting was carried out according to standard procedures. Subcellular fractionation by hypotonic lysis was carried out as described.

All protein samples were stored in the presence of a protease inhibitor cocktail (Roche) and were not boiled. Whole-cell lysates and hypotonic lysis fractions were separated by SDS–PAGE using standard protocols, and Immunofluorescence was carried out as previously described. We used specific antisera to detect ISG57 (ref. 42), p67 (ref. 43), CatL, GLP1 (ref. 44) and AP1β (ref. 45), and anti-MYC or anti-GFP antisera were used to detect tagged versions of MFST, UBH1 and EMP70. To assess suramin binding, cells were collected at mid-log phase and resuspended at 10⁷ ml⁻¹ in 35 nM [3H]-suramin (Hartmann Analytic; pre-incubated for 16 h in complete HM11) at 37 °C. Cells were washed in ice-cold PBS, resuspended in 100 μl Optiphase Supermix scintillant (Perkin Elmer) and [3H]-suramin incorporation quantified using a 1450 Microbeta scintillation counter (Perkin Elmer).

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