Pyrimidine salvage in *Trypanosoma brucei* bloodstream forms and the trypanocidal action of halogenated pyrimidines

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**Nonstandard abbreviations:**

5-FURes = 5-Fluorouracil resistant cells
5-FOARes = 5-Flurorotic acid resistant cells
5-F2’dURes = 5-Fluoro-2’-deoxyuridine resistant cells
5-FOA = 5-Flurorotic acid
5-FU = 5-Fluorouracil
RF = resistance factor.
ABSTRACT

African trypanosomes are capable of both pyrimidine biosynthesis and salvage of pre-formed pyrimidines from the host. However, uptake of pyrimidines in bloodstream form trypanosomes has not been investigated, making it difficult to judge the relative importance of salvage and synthesis, or to design a pyrimidine-based chemotherapy. Detailed characterization of pyrimidine transport activities in bloodstream form *Trypanosoma brucei brucei* found that these cells express a high affinity uracil transporter (designated TbU3) that is clearly distinct from the procyclic pyrimidine transporters. This transporter had low affinity for uridine and 2’deoxyuridine and was the sole pyrimidine transporter expressed in these cells. In addition, thymidine was taken up inefficiently through a P1-type nucleoside transporter. Importantly, the anticancer drug 5-fluorouracil was an excellent substrate for TbU3 and several 5-fluoropyrimidine analogues were investigated for uptake and trypanocidal activity; 5F-orotic acid, 5F-2’deoxyuridine displayed activity in the low µM range. The metabolism and mode of action of these analogues was determined using metabolomic assessments of *T. brucei* clonal lines adapted to high levels of these pyrimidine analogues, as well as of the sensitive parental strains. The analysis showed that 5-fluorouracil is incorporated into a large number of metabolites but likely exerts toxicity through incorporation into RNA. 5F-2’dUrd and 5F-2’dCtd are not incorporated into nucleic acids but act as prodrugs by inhibiting thymidylate synthase as 5F-dUMP. We present the most complete model of pyrimidine salvage in *Trypanosoma brucei* to date, supported by genome-wide profiling of the predicted pyrimidine biosynthesis and conversion enzymes.
Introduction

African trypanosomes are a complex of single-celled protozoan parasites (including *Trypanosoma brucei brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. vivax*, *T. congolense*) that cause a number of medical and veterinary conditions, mostly in sub-Saharan Africa (Simarro et al., 2010) but also in South Asia (Zhou et al., 2004) and South America (Gonzales et al., 2007). As existing treatments are old and the pathogens have become resistant to most of them, new therapeutic strategies are urgently required. As these bloodborne parasites must continually divide to stay ahead of the immune system, nucleotide metabolism is one obvious drug target, particularly as all protozoan parasites are unable to synthesize the purine ring *de novo* and thus necessarily rely on salvage from the host environment (De Koning et al., 2005). However, no purine-based chemotherapy has emerged for kinetoplastid parasites, in large part because there is so much redundancy in purine transporters and salvage pathways that the inhibition of any one transporter (De Koning et al., 2005) or enzyme (Berg et al., 2010; Lüscher et al., 2007) has little or no effect on parasite survival.

The organization of pyrimidine nucleotide metabolism is rather more diverse in protozoan parasites. At one end of the spectrum are *Plasmodium* species, which are unable to use preformed pyrimidines from the host environment, and rely on biosynthesis alone (De Koning et al., 2005; Van Dyke et al., 1970). Several important antimalarial drugs including sulfadoxine, proguanil and pyrimethamine (Baird, 2005) act on the pyrimidine and folate pathways. On the other hand, amitochondriate protozoa such as *Giardia lamblia*, *Tritrichomonas foetus* and *Trichomonas vaginalis* lack the biosynthesis pathways to make either purine or pyrimidine nucleotides (Wang and Cheng, 1984; Hassan and Coombs, 1988) and rely exclusively on uptake of nucleosides and nucleobases for their supply of nucleotides (De Koning et al., 2005). Kinetoplastid parasites, including major pathogens such as the *Leishmania* and *Trypanosoma* species, possess both salvage and biosynthesis routes for pyrimidines (De Koning et al., 2005; Papageorgiou et al., 2005; De Koning, 2007) and some enzymes of the pyrimidine interconversion pathways may be good drug targets in *T. brucei*.

For instance, Hofer et al. (2001) showed that bloodstream trypanosomes are unable to incorporate [3H]-cytosine or [3H]-cytidine into their nucleotide pool, leaving CTP synthetase as the
only route to obtain cytidine nucleotides; inhibition of the enzyme reduced proliferation both in vivo and in vitro (Hofer et al., 2001; Fijolek et al., 2007). Another validated target in the pyrimidine pathways is deoxuryridine 5’-triphosphate nucleotidohydrolase (dUTPase): RNAi knockdown of this enzyme reduces growth rates and causes DNA breaks by allowing a toxic build-up of dUTP in the cells (Castillo-Acosta et al., 2008). Knockout of dihydrofolate reductase - thymidylate synthase (DHFR-TS) is lethal in *T. b. brucei* unless rescued by very high levels of thymidine in vitro (Sienkiewicz et al., 2008). Finally, Arakaki et al. (2008) showed that under conditions of limited pyrimidine salvage RNAi knockdown of dihydroorotate dehydrogenase (DHODH), one of the enzymes in the pyrimidine biosynthesis pathway, caused severe growth defects for bloodstream trypanosomes. In *L. donovani*, UMP synthase was found to be essential for in vitro growth in the absence of added pyrimidines (French et al., 2011).

It thus appears, from a combination of genetic and pharmacological evidence, that pyrimidine metabolism in African trypanosomes is replete with drug targets and that a systematic evaluation of pyrimidine salvage mechanisms is long overdue. Indeed, there is currently no information on pyrimidine transporters in bloodstream trypanosomes, although studies in procyclic forms (De Koning and Jarvis, 1998; Gudin et al., 2006) have identified high affinity transporters for uracil (TbU1) and uridine (TbU2). The lack of information about pyrimidine uptake in bloodstream trypanosomes delays efforts to develop a pyrimidine-based chemotherapy; we therefore systematically assessed uptake of all natural pyrimidine nucleobases and nucleosides into bloodstream trypanosomes and identified a highly efficient transporter (TbU3) that is distinct from the procyclic transporters. Whereas uridine, 2’-deoxyuridine, and thymidine could all be taken up at high concentrations, these processes were low affinity and inefficient. However, the anticancer drug 5-fluorouracil was almost as good a substrate as uracil for TbU3 and displayed a moderate trypanocidal activity. Several trypanocidal pyrimidines with higher in vitro efficacy were also identified and of each their mode of action and their metabolites were identified. We thus present a much improved model of pyrimidine salvage and metabolism in African trypanosomes, and a first evaluation of pyrimidines as subversive chemotherapeutic agents against these parasites.
Materials and Methods

Trypanosome strains and cultures. Bloodstream forms of *T. b. brucei* strain 427 were used throughout and cultured exactly as described previously (Gudin et al., 2006) in HMI9 media (Invitrogen) supplemented with 10% fetal bovine serum (BioSera, Ringmer, East Sussex, UK) under a 5% CO₂ atmosphere at 37 °C. Strains adapted to selected pyrimidine analogs were derived from s427 through *in vitro* exposure to increasing levels of the agent over several months, essentially as described for diminazene (Teka et al., 2011) and clonal populations were obtained by limiting dilution.

Drug sensitivity assays and chemicals. Sensitivity of trypanosome cultures to various drugs and pyrimidine analogs were performed exactly as described (Gould et al., 2008), using the Alamar blue (resazurin, Sigma) redox-sensitive indicator dye. Pentamidine and diminazene were obtained from Sigma-Aldrich, as were many purines, pyrimidines and analogs, with the exceptions of 5-bromouracil, 5-bromouridine and 5-iodo-2'-deoxyuridine (Avocado Research Chemicals Ltd, UK); 2-thiouridine and 4-thiuridine (TriLink BioTechnologies); 5-fluorocytidine, 5-chlorouridine, 5'-deoxyuridine, 5'-deoxy-5-flurouridine, 2'-3'-dideoxyuridine and 2'-deoxy-5-fluorocytidine (Carbosynth); 5-fluoro-2'-deoxyuridine and 5-fluorocytosine (Fluka), and 2-thiouracil (ICN Biomedicals).

Transport assays. Uptake of radiolabeled nucleosides and nucleobases by bloodstream trypanosomes was performed exactly as described (Wallace et al., 2002, Natto et al., 2005). Briefly, log-phase cells were washed in to assay buffer (33 mM HEPES, 98 mM NaCl, 4.6 mM KCl, 0.55 mM CaCl₂, 0.07 mM MgSO₄, 5.8 mM NaH₂PO₄, 0.3 mM MgCl₂, 23 mM NaHCO₃, 14 mM glucose, pH 7.3) and diluted to 1x10⁸ cells/ml for use in the assay; 100 µl was mixed with an equal volume of radiolabeled compound in the same buffer (sometimes mixed with unlabelled nucleobase or nucleoside for competition studies) and incubated at ambient temperature for a predetermined time. The incubation was stopped by the addition of 1 ml ice-cold buffer containing saturating levels of unlabelled permeant, and the immediate centrifugation through an oil layer. Radioactivity was determined by liquid scintillation counting and corrected for non-specific association of the label with
the cell pellet, as described (Wallace et al., 2002). Saturation data, inhibition data and time courses were plotted to equations for hyperbolic, sigmoid lines or linear regression, as appropriate, using Prism 5.0 (GraphPad). All experiments were performed in triplicate and on at least three independent occasions. The following radiolabeled substances were used: [2-3H]Adenosine (ARC, 40 Ci/mmol); [5-3H]Cytosine (Moravek, 25.6 Ci/mmol); [5-3H]Cytidine (Moravek, 25.6 Ci/mmol); [5-3H] 2'-deoxycytidine (ARC, 20 Ci/mmol); [6-3H]2'-deoxyuridine (Moravek, 17.8 Ci/mmol); [6-3H]5-Fluorouracil (Moravek, 20 Ci/mmol); [2,8-3H] Inosine (Moravek, 20 Ci/mmol); [5-3H] Orotic Acid (Moravek, 23 Ci/mmol); [methyl-3H]Thymine (Moravek, 56.3 Ci/mmol); [5,6-3H]Uracil (Perkin Elmer, 40.3 Ci/mmol); [5,6-3H]Uridine (American Radiolabeled Chemicals Inc (ARC), 30 Ci/mmol).

**Adaptation of T. b. brucei bloodstream forms to tolerance for pyrimidine analogues.**

Bloodstream forms of *T. b. brucei* s427 WT were re-cloned by limiting dilution and cultured in standard HMI-9 medium containing 10% Fetal Bovine Serum. Separate cultures were exposed to the continuous presence of non-lethal concentrations of 5-fluorouracil, 5-fluoro-2’-deoxyuridine and 5-fluoro orotic acid. These concentrations were stepwise increased, as tolerance allowed, until a high level of resistance had been obtained, at which point they were again cloned by limiting dilution.

**Metabolomics sample preparation.** Trypanosomes were grown to log phase, resuspended at 2×10^6 cells/ml in 50 ml HMI-9/10% FBS in a vented culture flask and incubated with 100 μM of the test compound for 8 h at 37 °C/5% CO₂. Cells were transferred to a 50-ml centrifuge tube and instantly cooled down to 4 °C using a dry ice/ethanol bath. This culture was centrifuged at 4 °C (1000×g, 10 min) and the pellet was lysed by addition of 200 μl of chloroform/methanol/water (1:3:1 v/v/v) with internal standards for mass spectrometry (1 μM each of theophylline, Cl-phenyl-cAMP, N-methyl glucamine, canavanine and piperazine) followed by vigorous mixing for one hour at 4 °C. Precipitated proteins and cellular debris were removed from metabolites by centrifugation (13000 × g for 3 min). Metabolite extracts were stored at -80 °C until use. Controls samples included untreated cells grown in parallel; unused growth medium; 100 μM of the test compound dissolved in HMI9/10% FBS; and extraction solvent blanks. All experiments were performed in triplicate.
**Metabolomics sample analysis.** Metabolomics samples were analyzed by hydrophilic interaction liquid chromatography (HILIC-LC) coupled to high resolution mass spectrometry (MS). LC separation utilized a zwitterionic ZIC-pHILIC column (Merck Sequant) with ammonium carbonate alkaline gradient as previously described (Zhang et al., 2012). The method was performed on a Dionex RSIC3000 (Thermo Fisher) LC system coupled to an Exactive Orbitrap (Thermo Fisher) operating at 50,000 resolution in positive and negative mode ESI (rapid switching) with MS parameters as previously published (Creek et al., 2011). Mass calibration was performed immediately before the batch, followed by analysis of authentic metabolite standards to determine standard retention times (Creek et al., 2011). Samples were analysed in random order and signal stability assessed by periodic analysis of pooled quality control samples. Data from each sample were manually inspected and irreproducible samples excluded from analysis based on total ion chromatogram (TIC) signals and internal standards.

**Metabolomics data analysis.** Metabolomics data was analyzed using the IDEOM application (http://mzmatch.sourceforge.net/ideom.php) with default parameters (Creek et al., 2012) after selecting the pHILIC chromatography method. Briefly, raw files were converted to mzXML format and peaks were detected using the XCMS Centwave algorithm (Tautenhahn et al., 2008). Peak data for all samples was combined, filtered and saved in peakmL files using mzMatch (Scheltema et al., 2011). Noise filtering and (putative) metabolite identification was performed in IDEOM based on accurate mass and retention time, parameters are available in the supplementary IDEOM file (supplementary data). In addition to the automated identification of metabolites from the IDEOM database (detailed in supplementary data, with confirmed identities by authentic standards highlighted in yellow), data were screened for novel fluorinated metabolites by the addition of 17.9906 to all known metabolite masses, which detected peaks with accurate mass and retention times consistent with 5-fluoro-UDP, 5-fluoro-UTP, fluoro-N-carbamoyl-L-aspartate, 5-fluoro-orotic acid (detected primarily as the CO₂–loss fragment) and a fluorinated UDP-hexose and UDP-N-acetyl-hexosamine (putatively identified as 5-fluoro-UDP-glucose and 5-fluoro-UDP-N-acetylglucosamine). LC-MS peak heights were used for semi-quantitative analysis of metabolite abundances, and statistical
analyses comprised pairwise comparisons of study groups by unpaired rank products analyses with P-values for probability of false positives based on 200 permutations.

**Construction of a profile library for enzymes of the pyrimidine pathways.** Reference sequences for the enzymes of pyrimidine metabolism were downloaded from UniProt (www.uniprot.org), searching by EC number in the manually annotated SwissProt section. Each of the obtained sets of sequences was redundancy reduced by $\leq 50\%$ identity, aligned with ClustalW (Thompson et al., 2002), and converted into a HMM-profile with *hmmbuild* of the HMMer 3.0 package (Eddy, 2009). The profiles were concatenated to a library. Predicted proteomes were downloaded from Integr8 (www.ebi.ac.uk/integr8) and searched with *hmmscan* of the HMMer package. Hierarchical clustering of proteomes based on the best scores obtained to each of the profiles was performed with the R package pvclust (Suzuki and Shimodaira, 2006), using Canberra distance and the McQuitty algorithm.

**Isolation of *T. brucei* DNA and RNA.** Bloodstream forms of *T. b. brucei* were grown to $2\times 10^6$/ml and harvested by centrifugation (5 min, 1500xg). The pellet was resuspended in 1 ml of phosphate-buffered saline (PBS) and the suspension centrifuged again for 5 minutes at 1500 g. After removing the supernatant volume of 500 $\mu$l of lysis buffer (100 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl pH 8.0), 25 $\mu$l of 10\% SDS and 50 $\mu$l of 10 mg/ml RNase A (Sigma) were added, and incubated overnight at 37 °C. The sample was washed twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) saturated with 10 mM Tris, pH 8.0 and 10 mM EDTA (Sigma). The aqueous phase was washed twice with 600 $\mu$l chloroform; DNA was ethanol-precipitated, resuspended in 1×TE and stored at 4 °C.

For isolation of RNA, the cell pellet was taken up in 1 ml of Trizol (Life Technologies) to which 200 $\mu$l of chloroform was added after 5 minutes, followed by 1 min of gentle mixing and centrifugation (13,000xg, 20 min, 4 °C). The aqueous phase was transferred to 500 $\mu$l of isopropanol, incubated at room temperature for 10 minutes, and again centrifuged for 20 min at 4 °C. The RNA pellet was washed in 1 ml of 75\% ethanol, air dried, and resuspended in water treated by diethyl pyrocarbonate (DEPC; Sigma). The solution was stored at -80 °C until use.
DNA degradation. *T. brucei* bloodstream forms were incubated with 100 μM of 5-fluoro-2’-deoxyuridine or 5-fluorouracil (12 h, 37 °C, 5% CO₂); untreated control cells were cultured in parallel. DNA extracted from these cultures was resuspended in 30 μl TE buffer pH 7.4 and quantified on a NanoDrop device (Thermo Scientific), typically 4 - 5 μg/ml. Exonuclease III buffer (10 μl, 10×) and 1000 units of Exonuclease III (Takara Biotechnology) were added, plus distilled water to 100 μl, followed by incubation at 37 °C in a heat block for 48 h. From the digest, 20 μl was mixed vigorously with 60 μl of Acetonitrile (Fisher Scientific) and centrifuged (13,000×g, 5 min). The supernatant was stored at -80 °C until use.

RNA degradation. RNA extracted from *T. b. brucei* bloodstream forms exposed to 100 μM 5-FU for 12 h was incubated overnight with 10 μl of 10 mg/ml phosphodiesterase II (3’exonuclease; Sigma) at 37 °C. Extracted RNA from untreated cells grown in parallel was used as control. The RNA digest was mixed with 80 μl acetonitrile, mixed for 10 s and centrifuged for 5 min. Supernatant was stored at -80 °C until use.
Results

Characterization of a novel uracil transporter in bloodstream forms of *T. b. brucei*. In procyclic trypanosomes, pyrimidine uptake is mostly mediated by the TbU1 transporter, the main substrate of which is uracil (De Koning and Jarvis 1998; Gudin et al., 2006). We therefore studied \[^{3}H\]-uracil transport in bloodstream forms in order to assess whether pyrimidines are salvaged in a similar way in this life-cycle stage. Transport of 0.15 µM \[^{3}H\]-uracil was linear for at least 120 s, with a rate of 0.034 ± 0.002 pmol·10⁷ cells⁻¹·s⁻¹ and was almost entirely inhibited by 1 mM of unlabelled uracil (Fig. 1), showing that \[^{3}H\]-uracil uptake is transporter-mediated and that simple diffusion does not play a significant role in this process, at least at low uracil concentrations. Subsequent \[^{3}H\]-uracil assays used 0.15 µM of label and a 30 s incubation time - very much within the linear phase of uptake, and our inhibition data were consistent with monophasic inhibition with Hill slopes near -1, i.e. a single transporter model.

Figure 2A shows a representative inhibition profile of \[^{3}H\]-uracil inhibited by unlabeled uracil; the inset shows the conversion to a Michaelis-Menten saturation plot. The average Kₘ value over six identical triplicate experiments was 0.54 ± 0.11 µM, with a Vₘₐₓ of 0.14 ± 0.03 pmol·10⁷ cells⁻¹·s⁻¹. The Kₘ value is similar to the value previously reported for TbU1 (0.46 ± 0.09 µM) but the Vₘₐₓ is almost 5-fold lower than in procyclics. \[^{3}H\]-uracil uptake in BSF was virtually insensitive to uridine, with 10 mM of the nucleoside inhibiting ~50% of 0.15 µM \[^{3}H\]-uracil uptake (Fig. 2B). This is a striking difference with uracil transport in procyclics (De Koning and Jarvis, 1998)(Table 1), and we designated the BSF uracil transporter TbU3.

Table 1 provides an overview of pyrimidine transporters identified in procyclic (PCF) and bloodstream forms (BSF) of *Trypanosoma brucei*, and shows that TbU1 and TbU3 have a very similar inhibitor profile, including for 5-halogen uracil analogues (Table 1 and Fig. 2B), and thus are highly likely to bind uracil in a very similar way. The only other notable difference is the lower affinity of TbU3 for 4-thiouracil, whereas Kᵢ values for 2-thiouracil were highly similar.

Transport of uridine and 2’-deoxyuridine in bloodstream forms. Saturable transport of \[^{3}H\]-uridine was hardly detectable in bloodstream forms, and not at all at submicromolar...
concentrations or at short time intervals (≤2 min; data not shown). A measurable rate was obtained at 2.5 μM [3H]-uridine, using a timecourse with six time points between 0 and 30 min (0.0043 ± 0.0003 pmol(10^7 cells)^-1s^-1) (Supplemental Figure 1A), which allowed the determination of an apparent K_m value of 9500 ± 2700 μM and a V_max of 16 ± 4 pmol(10^7 cells)^-1s^-1. This extremely low affinity is entirely consistent with uridine being transported by TbU3 at very high concentrations. This was confirmed by inhibition of 2.5 μM [3H]-uridine uptake by uracil, with a K_i value of just 1.6 ± 0.2 μM (n=3) (Supplemental Figure 1B), highly similar to the TbU3 K_m value for uracil.

Transport of 5 μM [3H]-2'deoxyuridine was linear over 4 minutes with a rate of 0.0051 ± 0.0003 pmol/10^7 cells/s, which was 76% inhibited by 2.5 mM unlabelled 2'-deoxyuridine (Fig. 3A). This transport activity displayed a K_m of 810 ± 130 μM, and a V_max of 1.3 ± 0.7 pmol(10^7 cells)^-1s^-1 (n=3) (Supplemental Figure 2A), and was inhibited dose-dependently by uracil (K_i = 1.1 ± 0.1 μM; n=3) (Fig. 3B). At lower permeant concentrations (0.5 μM) of [3H]-2'deoxyuridine the rate of uptake was proportionally reduced to 0.00045 pmol(10^7 cells)^-1s^-1 and barely measurable over 2 min (data not shown), indicating the absence of high affinity transport for uridine (deoxy)nucleosides.

Uptake of other pyrimidines by wild-type bloodstream forms. We did not observe significant amounts of thymidine transport by T. b. brucei at submicromolar or low micromolar concentrations (data not shown) but were able to measure transport of 10 μM thymidine over a period from 5 to 30 minutes, with a rate of 0.0015 ± 0.0003 pmol/10^7 cells/s (Fig. 3C). Using 10 μM of [3H]-thymidine and an incubation time of 15 min it was thus possible to conduct inhibition experiments (Supplemental Figure 2B). The K_m value was determined at 1240 ± 310 μM and the V_max as 0.067 ± 0.008 pmol/10^7 cells/s (n=3), yielding an efficiency ratio of just 0.0001. Thymidine transport was not sensitive to inhibition by uracil, consistently failing to reach 50% inhibition even at 2.5 mM (n=4) (Fig. 3D). In contrast, the transporter was completely inhibited by adenosine with a K_i value of just 2.3 ± 0.3 μM (n=3) (Fig. 3D), whereas adenosine has no effect on TbU3-mediated uracil transport (Table 1). [3H]-thymidine uptake was similarly sensitive to inosine (Table 1; Supplementary Figure 3A) and, conversely, inosine uptake was inhibited by thymidine in a monophasic way with a K_i value of 214 ± 51 μM (n=4) (Supplemental Figure 3B). This activity displayed an inosine K_m of 0.89 ± 0.15
µM (n=3) (Supplemental Figure 3C), all completely consistent with a P1-type nucleoside transporter (De Koning and Jarvis, 1999; Al-Salabi et al., 2007). 2’-deoxyuridine was another inhibitor of this novel nucleoside transport activity (Table 1), which was designated TbT1, but orotic acid, thymine, cytidine and 5-fluorouracil had little or no effect on thymidine transport.

Attempts were made to measure transport of other pyrimidine nucleosides and nucleobases. A very slow accumulation of 0.5 µM [3H]-cytidine could be measured over 30 min (6.5×10^{-5} ± 4.1×10^{-6} pmol/10^7 cells/s) but was only partly inhibited by 2.5 mM unlabelled cytidine (Supplemental Figure 4A). An effort to determine a K_m for 0.5 µM [3H]-cytidine, using a 20-min incubation time, found only partial saturation at 10 mM cytidine (data not shown) and we conclude that bloodstream T. b. brucei do not salvage significant amounts of cytidine through uptake from their environment. Similarly, just detectable accumulation of 2.5 µM [3H]-2’-deoxycytidine over 15 min was not saturated by 10 mM unlabelled permeant (Supplemental Figure 4B), and 0.25 µM [3H]-cytosine and 1 µM [3H]-thymine did not detectably accumulate in bloodstream forms over 15 min (Supplemental Figure 4C,D, respectively).

**Sensitivity of T. b. brucei to analogues of pyrimidine nucleosides and nucleobases and development of resistant strains.** We tested the effects of a number of pyrimidine nucleoside and nucleobase analogues on bloodstream trypanosomes - both for evaluation as potential drugs and as tools to investigate the pyrimidine salvage pathways. Thiouridines (2-thiouridine, 4-thiouridine), 5-fluorouridine, 3’-deoxypyrimidine nucleosides (3’-deoxyuridine, 2’,3’-dideoxyuridine, 3’-deoxythymidine), 5’-deoxyuridines (5’-deoxyuridine, 5-fluoro-5’deoxyuridine), 5-fluorocytosine and 5-fluorocytidine all had no effect up to at least 1 mM of compound. Uracil and uridine analogues with 5-position halogenations other than fluorine all displayed EC_{50} values ≥2.5 mM or no effect at all.

2’-deoxynucleosides were much more active against bloodstream forms than corresponding ribonucleosides; 5-fluoro-2’-deoxyuridine (5F-2’dUrd), 5-chloro-2’-deoxyuridine and 5-fluoro-2’-deoxycytidine all displayed micromolar activity against T. b. brucei bloodstream forms (Table 2). The pyrimidine nucleobase analogues 5-fluorouracil (5-FU), 5-fluoroorotic acid (5-FOA) and 6-azauracil, also displayed significant antiprotozoal effects (EC_{50} values from <10 µM to almost 1 mM (Table 2)).
None of the fluorinated analogues killed trypanosomes very quickly, even at 500 µM, although they appeared to induce almost immediate growth arrest (Supplemental Figure 5). Resistance was induced to 5-FU, 5-FOA and 5F-2’dUrd by in vitro exposure of T. b. brucei bloodstream forms to stepwise increasing concentrations of the compounds (Supplemental Figure 6), yielding clonal lines 5-FURes (Resistance Factor 131 to 5-FU), 5-FOARes (RF 83 to 5-FOA) and 5-F2’dURes (RF 825 to 5F-2’dUrd), which were characterized with respect to cross-resistance to other pyrimidine analogues (Table 2).

5-FURes was not cross-resistant to pyrimidine nucleoside analogues but displayed 6.9-fold resistance to 5-FOA, showing that at least one of multiple changes impacted on a joint pathway. Similarly, 5-FOARes displayed 13-fold resistance to 5-FU, but less resistance to the nucleoside analogues. Interestingly, 5-FURes displayed increased sensitivity to 6-azauracil, probably indicating a reduced uracil salvage pathway, increasing reliance on de novo synthesis. 5-FURes was also 15-fold more sensitive to 5-chloro-2’-deoxyuridine, possibly indication that this analog likewise inhibits de novo pyrimidine biosynthesis. 5-F2’dURes was not cross-resistant to the nucleobase analogues 5-FU, 5-FOA and 6-azauracil, showing that the resistance was not due to loss of TbU3 activity. However, this strain was resistant to 5-fluoro-2’-deoxycytidine to the limit tested (EC50 >5 mM), although not to 5Cl-2’deoxyuridine, confirming that the latter has a different mode of action than 5F-2’dURd.

Assessment of pyrimidine transport in the resistant clones. Transport of 0.5 µM [3H]-uracil was almost identical in WT and 5-FURes cells (Fig 4A), but transport of 0.5 µM [3H]-5-FU was reduced by 76 ± 6% (n=3; P<0.01, paired Student’s t-test) in 5-FURes (Fig. 4B). [3H]-Uracil K_m and V_max values for 5-FURes were unchanged relative to WT (Table 3), as were affinity for uridine and 5-FU (K_i values ~10 mM and 3.7 ± 0.7 µM, respectively (n=3). Transport efficiency for [3H]-5-FU in 5-FURes was 0.088 compared with 0.25 for uracil, based on 5-FU K_m of 2.3 ± 0.4 µM and V_max of 0.20 ± 0.02 (Table 3; Supplemental Figure 7). In WT cells the difference between the uracil and 5-FU transport efficiencies was much lower (Table 3; Supplemental Figure 7).

We also investigated whether reduced uptake of orotic acid or 5-FOA might partly explain the phenotype of 5-FOARes trypanosomes. Uptake of 0.2 µM [3H]-orotic acid was linear (r² = 0.98) and
significantly different from zero (F-test, \( P = 0.002 \)) with a rate of \( 2.1 \times 10^{-4} \pm 2.1 \times 10^{-5} \) pmol/10⁷ cells/s. However, uptake was apparently non-saturable, as the rate of uptake in the presence of 1 mM unlabelled orotic acid was identical, at \( 2.1 \times 10^{-4} \pm 2.6 \times 10^{-5} \) pmol/10⁷ cells/s (Fig. 5). As such, it was impossible to determine kinetic parameters. However, it was clear that, when measured in parallel, accumulation of orotic acid was less in 5-FOARes than in WT; in two experiments (each performed in triplicate) \(^{3}H\)-orotic acid uptake over 10 min was reduced by 68.1% (\( P < 0.01 \)) and 62.8% (\( P < 0.001 \); Student’s \( t \)-test). However, it is unsafe to attribute this to either reduced transport or a reduced rate of 5-FOA metabolism as we were unable to establish an initial rate of mediated transport for this permeant and metabolic usage of the \(^{3}H\)-orotic acid could therefore be the rate-determining step.

**Metabolomic analysis of fluoropyrimidine resistance in T. b. brucei.** We used a metabolomics approach to assess (1) whether nucleotide levels or pathways were changed during the process of adaptation to fluoropyrimidines; (2) what metabolites are formed from the fluoropyrimidine analogues in WT and resistant cells; (3) the mechanisms of action and resistance to these compounds; and (4) whether these analogues are incorporated into nucleic acids.

**5-Fluorouracil.** WT cells treated with 5-FU (100 µM, 12 h) metabolized the drug to 5F-UMP, 5F-UDP and 5F-UTP, whereas no 5-fluorouridine or 5-fluoro-2'-deoxyuridine were detected. These observations strongly suggest that 5-FU is not a substrate for *T. b. brucei* uridine phosphorylase, but is a substrate for *T. b. brucei* uracil phosphoribosyltransferase (TbUPRT), as well as for nucleoside diphosphatase and nucleoside diphosphate kinase. No fluorinated deoxyuridine nucleotides were detected, making it unlikely that fluorinated pyrimidine nucleotides are a substrate for ribonucleotide reductase. However, intracellular levels of dUMP were significantly increased in WT and 5-FURes cells treated with 5-FU, compared to their respective untreated control cells; in WT the increase was 10.5-fold (\( P < 0.0001 \)) and in 5FURes it was 7.2-fold (\( P < 0.001 \)) (Fig. 7A).

Interestingly, significant amounts of 5F-UDP-glucose were detected, showing that 5F-UTP is a substrate for UDP-glucose pyrophosphorylase, which couples UTP to glucose-1P (Fig. 6). It must be noted that the detection method, based on mass-spectrometry, cannot distinguish between UDP-glucose and UDP-galactose so it is unclear whether 5F-UDP glucose might be a substrate for UDP-
Glc 4’-epimerase. Similarly, highly significant amounts of 5F-UDP-N-acetyl-glucosamine were detected which may include the equivalent galactose residues. This indicates that 5F-UTP is a substrate of UTP:N-acetyl-α-D-glucosamine-1-phosphate uridylyltransferase which forms UDP-GlcNAc from UTP and N-acetyl-α-D-glucosamine 1-phosphate (Fig. 6). UDP-GlcNAc in turn is a substrate of N-acetylgulosaminyltransferase, transferring the GlcNAc to protein and glycans. It is thus possible that 5-FU interferes with glycosylation through the production of 5F-UDP hexoses and/or hexosamines.

The presence of 5F-UTP raises the possibility of incorporation of fluorinated nucleotides into RNA. Following purification and digestion of RNA from 5-FU treated cells, qualitative LC-MS analysis detected significant amounts of 5F-UMP (~10% of UMP abundance by comparison of LC-MS peak heights), in addition to low levels of 5F-CMP, confirming the incorporation of significant amounts of fluorinated nucleotides into RNA. This data set also provides evidence that 5F-UTP is a substrate for cytidine triphosphate synthase.

In 5-FURes cells treated with 5-FU the relative amounts of 5-FU, and fluorouridine nucleotides, in the cell were all somewhat lower than in WT cells exposed to the same concentration of 5-FU (Fig. 7B), consistent with reduced efficiency of 5-FU uptake contributing to some extent to resistance, but dUMP levels were still significantly elevated (Fig. 7A). The largest difference was in a 6.3-fold reduction of 5F-UDP-glucose and of 5F-UDP-GlcNAc in 5-FURes cells relative to WT cells treated with 5-FU (P<0.05 and P<0.01, respectively) (Fig. 7B), suggesting that sugar nucleotide metabolism contributes significantly to 5-FU mode of action in T. brucei, and that changes in this pathway could make major contributions to 5-FU resistance.

**5-Fluoroorotic acid.** Very similar levels of 5-FOA were detected after exposure of WT and 5-FOARes cells, indicating that uptake was not the main mechanism of resistance, consistent with the non-saturable orotic acid uptake noted above. In both cell types, but particularly in WT, intracellular 5-FU was detected after incubation with 5-FOA; this was not a contamination of the chemical as it was not present in fresh medium samples containing drug, and indicates that UPRT can operate to hydrolyse 5F-UMP to 5-FU. Another surprise was the detection of fluoro-N-carbamoyl-L-aspartate in
both cell types, indicating a partial reversal of the pyrimidine biosynthesis pathway (Fig. 6). This may be caused by a build-up of 5-FOA, which seems to inhibit orotate phosphoribosyltransferase (OPRT), leading to an increase in free orotate levels in both WT (3.6-fold; P<0.01) and 5-FOARes cells (1.6-fold; P<0.05). Interestingly, orotate levels were also 3.5-fold higher in untreated 5-FOARes cells versus untreated WT cells (P<0.0001), indicating an adaptation by either significantly increasing orotate biosynthesis, or a reduction in OPRT activity. The same adaptation of increased baseline orotate concentration was also observed in the 5-FU resistant cells, making it more likely that the orotate increase is the result from increased biosynthesis, as this would ‘dilute’ the 5F-UMP derived from 5-FU with newly synthesized UMP.

5-FOA was clearly a substrate as well as an inhibitor of OPRT and was converted to fluorinated uridine nucleotides and 5F-UDP-glucose and 5F-UDP-GlcNAc (Fig. 7C), reaching levels in WT cells well in excess of those after treatment with 5-FU (Fig. 7B), consistent with the stronger trypanocidal activity of 5-FOA compared to 5-FU. As with 5-FU-treated cells there was a small but significant (2.5-fold; P<0.01) increase in dUMP in 5-FOA-treated WT trypanosomes compared to untreated cells; this effect was not observed in 5-FOARes cells. No fluorinated cytidine or deoxyuridine nucleotides were observed in 5-FOA-treated cells, nor was there any effect of this compound on the levels of thymidine nucleotides.

In 5-FOARes cells, the level of all fluorinated nucleotides was very much reduced, with a 50-fold decrease in 5F-UMP, and 5F-UDP and 5F-UTP were below detection limits, resulting in >200-fold reduction in 5F-UDP-glucose (Fig. 7C). It thus can be concluded that the main adaptation in 5-FOARes cells is by preventing its incorporation into the nucleotide pool, presumably through a change in OPRT, as orotidine-5-phosphate was not detected whereas orotate levels were significantly increased.

**5-Fluoro-2’dUrd.** As part of the investigation of the mechanism of action 5F-2’dUrd on trypanosomes we isolated and digested DNA from 5F-2’dUrd-treated cells. No 5F-dUMP was detected in the digest in spite of the four natural deoxynucleotides being present at 500 to 5000 fold.
higher intensity than the detection limit. We thus believe we can rule out that significant amounts of 5F-deoxyuridine are incorporated into DNA in lieu of thymidine.

Intracellular levels of 5F-2’dUrd were not significantly different between WT and 5-F-2’dURes cells (Fig. 8A), confirming that the resistance mechanism is based on metabolism rather than reduced uptake of the drug. No fluorinated pyrimidine analogues, including ribonucleosides, were detected in WT trypanosomes treated with 5F-2’dUrd, apart from the drug itself (the apparent signal for 5-FU deriving from an in-source fragment of 5F-2’dUrd, as confirmed in the spiked medium), confirming that 5F-2’dUrd is not a substrate for uridine phosphorylase. However, 5F-2’dUrd is a substrate for thymidine kinase as low levels of 5F-dUMP (but not 5F-dUDP/UTP) could be detected.

The main difference observed between untreated and 5F-2’dUrd-treated WT was a 36.5-fold increase in dUMP (P<10^-5) (Fig. 8B), highly suggestive of a block in thymidylate synthase mediated either by 5F-2’dUrd itself, or by the low levels of 5F-dUMP. However, thymidine nucleotide levels were not significantly different in WT and 5-F2’dURes cells, or after treatment with 5F-2’dUrd (Fig. 8C), presumably through salvage of thymidine, which is present at high concentrations (20 mg/L, i.e. 161 µM) in standard HMI-9 medium. In the treated 5F-2’dURes cells only a 2-fold increase in dUMP was observed relative to untreated wild-type cells (Fig. 8B), and dUMP levels were almost undetectable in untreated 5F-2’dURes cells, indicating a downregulation of 2’-deoxyuridine nucleotide synthesis as part of the adaptation to 5F-2’dUrd. These observations confirm (1) that the mode of action is through inhibition of thymidylate synthase rather than thymidylate kinase and (2) that under these conditions the cells succumb to high levels of deoxyuridine nucleotides rather than from lack of thymidine nucleotides.

As expected by this model, the presence or absence of thymidine in the extracellular medium had a profound effect on sensitivity to 5F-2’dUrd but not to 5-FU, 5F-OA or diminazene aceturate (Fig. 9). WT trypanosomes in a thymidine-free version of HMI-9 were highly sensitive to 5F-2’dUrd (EC50 = 0.77 ± 0.3 µM); the addition of 100 µM thymidine reduced the sensitivity 24-fold (EC50 18.6 ± 3.0 µM). The same phenomenon was observed even more prominently using 5F-2’dURes cells (>600-fold), and when using 5F-2’dCtd on either cell type (78-fold in WT; 5F-2’dURes cells were
insensitive to >5 mM 5F-2’dCtd) (Fig. 9), confirming that the two deoxynucleoside analogues have the same mechanism of action, through conversion of 5F-2’dCtd to 5F-2’dUrd. Changes in thymidine transport contribute to 5F-2’dUrd resistance. Uptake of 10 µM [3H]-thymidine in 5F-2’dURes displayed a K_m of 22 ± 3 µM and a V_max of 0.013 ± 0.002 pmol/10^7 cells/s; this represents a >50-fold increase in thymidine affinity, and a 6-fold increase in transport efficiency. As the T1-encoding gene has yet to be identified the adaptation could be either the expression of an alternative thymidine transporter, or a mutant form of T1.

**5-Fluorouridine.** This nucleoside analogue had no effect on trypanosome growth when tested in the Alamar blue viability assay. Consistent with this observation, only very low levels of fluorinated metabolites (5-FU, 5-FUMP, 5-FUDP and 5-FUDP-glucose) were observed in WT cells exposed to 5-fluorouridine compared to 5-FU or 5-FOA. No major changes to cellular metabolism were observed.

**5-Fluoro-2’deoxyctydine.** The metabolomic analysis of WT427 bloodstream forms treated with 5F-2’dCtd provided further confirmation that 5F-2’dCtd and 5F-2’dUrd act in a similar way, as the main fluorinated metabolite of 5F-2’dCtd was 5F-2’dUrd. A very small amount of 5F-dUMP was also detected by manual inspection. As with 5F-2’dUrd treatment there was a massive increase in dUMP levels in the cell (67-fold; P<10^-5). This was accompanied by a 2.9-fold increase in 2’-deoxyuridine (P<0.01). Conversely, there was a small but significant reduction in uridine (41%; P<0.01) and UMP (51%; P<0.01).

In addition there were significant (P<0.02) effects on cytidine nucleotide metabolism. There were significant increases in dCDP (4.6-fold) and dCTP (5.6-fold), as well as an apparent shift in lipid metabolism intermediates. CDP-choline and CDP-ethanolamine were reduced by 42% and 39% respectively, while increases were observed for metabolite peaks putatively identified as dCDP-choline (2.2-fold) and dCDP-ethanolamine (4.5-fold). There were no significant differences in the cellular levels of the cytidine ribonucleotides CMP, CDP and CTP after incubation with 5F-2’-dCtd. These effects are not easily understood as we are not aware of a mechanism for (deoxy)-cytidine utilisation in trypanosomes other than through cytidine deaminase. However, the effect on
deoxycytidine nucleotide levels may have been through the effects of accumulating dUMP on ribonucleoside reductase (11 in Fig. 6), which can be allosterically regulated by deoxyribonucleotides (Hofer et al., 1998). This notion is greatly supported by the fact that similar effects could be observed after treatment with 5F-2’dUrd, which caused increases in dCMP (2.8-fold; P<0.001) and dCDP (3.8-fold; P<0.001) as well as in dCDP-choline (2.0-fold; P<0.01), whereas there was no effect on levels of CMP or CDP.

**Effect of 5-FU on glycosylation in T. b. brucei.** To test whether the detection of significant quantities of 5F-UDP-hexoses and hexosamines contribute to trypanocidal action through interference with either protein glycosylation or glycosylphosphatidylinositol (GPI) anchor biosynthesis, we examined whether any major defects to glycosylation or GPI anchor synthesis took place under the influence of fluorinated pyrimidines. Results presented in the Supplementary Material (including Supplemental Figure 8) show that no detectable changes in VSG content were observed after treatment with 100 µM 5-FU or 5F-2’dUrd.

**Genome-wide profiling of trypanosomatid pyrimidine metabolism.** The above analyses of pyrimidine metabolism, and of the effects of pyrimidine analogues on this system, resulted in a new overview of the pyrimidine salvage and biosynthesis pathways in *T. b. brucei* (Fig. 6). To further validate the presence of the enzymes predicted in this model, we constructed a library of hidden Markov model (HMM)-based profiles for pyrimidine synthesis and salvage enzymes. Selected parasite proteomes were scanned with this library, *H. sapiens* and *M. musculus* served as a reference. The use of the same profiles over different proteomes enabled clustering of the respective species according to their 'pyrimidine metabolic vectors' (Fig. 10A,B). This analysis clearly separated the pyrimidine auxotrophic *Giardia lamblia*, *Entamoeba histolytica*, and *Trichomonas vaginalis* from other protozoa. These parasites also lack thymidylate synthase (EC 2.1.1.45). Within the pyrimidine prototrophs, the trypanosomatids separated from the apicomplexans mainly due to the presence of thymidine kinase (2.7.1.21), UDP-glucose pyrophosphorylase (2.7.7.9) and UDP-glucose epimerase (5.1.3.2), and the absence of orotate reductase (1.3.1.14) and dihydroorotate dehydrogenase (1.3.5.2). The main distinction between *T. brucei* and its mammalian hosts was the apparent absence of...
dUTPase (3.6.1.23) and dihydroorotate dehydrogenase (1.3.5.2), in addition to a relatively low score for uridine phosphorylase (2.4.2.3).

The apparent absence of dUTPase, a well-characterized enzyme in *T. b. brucei* (Castillo-Acosta et al., 2008) is explained by the fact that it is highly atypical for a eukaryotic dUTPase: it is dimeric rather than trimeric (possibly unique among eukaryotes (Castillo-Acosta et al., 2008)), with a very different 3D structure (Harkiolaki et al., 2004), giving it features different from other eukaryotic dUTPases, including recognition of both dUTP and dUDP as substrates (Bernier-Villamor et al., 2002). These kinetoplastid dUTPases have been classified together with several prokaryotic and bacteriophage dUTPases into an all-α NTP pyrophosphatase superfamily (Moroz et al., 2005). Similarly, trypanosomal uridine phosphorylase has an unusual quaternary structure, although most of its active site layout is conserved (Larson et al., 2010): it is a member of the NP-I family of nucleoside phosphorylases but, unusually, not organized as a stable trimer of dimers, but rather as a single dimer stabilized by a Ca$^{2+}$ ion. Dihydroorotate dehydrogenase (1.3.5.2) is indeed absent from the *T. b. brucei* genome, its function in the pyrimidine biosynthesis pathway instead being performed by dihydroorotate dehydrogenase (1.3.3.1) (Arakaki et al., 2008), which the HMM analysis correctly predicts.
Discussion

Pyrimidine analogs have been extremely successful in anti-cancer (Galmarini et al., 2003) and antiviral chemotherapy (Hoffmann et al., 2011). The pyrimidine analogs target rapidly dividing cells and kinetoplastid parasites similarly depend on high growth rates to outpace the host’s defenses. Here, we systematically investigate salvage and incorporation of pyrimidine analogs by trypanosomes. Antiprotozoal pyrimidine therapy would start with cytotoxic pyrimidines efficiently reaching the target cell’s interior (De Koning, 2001; Lüscher et al., 2007). Natural pyrimidine nucleobase and nucleosides, and many analogs, do not have an appreciable diffusion rate and thus require transport proteins to enter cells. We thus studied the transport of all natural pyrimidines, and of 5-fluorouracil, by bloodstream trypanosomes. As the genes, and indeed gene families, encoding pyrimidine transporters have not been identified in protozoa and metazoa (Bellofatto, 2007; De Koning, 2007) we opted for functional analysis using live parasites. Evidence was found for only one such transporter, TbU3, with high affinity for uracil and very low affinity for uridine and 2’-deoxyuridine.

The uracil transporter found in PCF, TbU1, has been well characterized (De Koning and Jarvis, 1998; Papageorgiou et al., 2005) and, like the corresponding transporter of L. major, binds its substrate through hydrogen bonds to both keto groups and both ring nitrogens in protonated state (Papageorgiou et al., 2005). The many similarities between procyclic TbU1 and bloodstream form TbU3 inhibitor profiles seem to indicate a common transporter structure, but the low affinity for uridine by TbU3 suggest that TbU3 has more steric limitations than TbU1 when it comes to binding nucleosides rather than nucleobases – either in the binding site itself or in extracellular access to it. As a Kᵢ value for 2’-deoxyuridine could be established (1150 ± 340 μM) it appears that the 2’-hydroxyl group is a significant factor in the non-binding of uridine. In contrast, the further removal of the 3’-hydroxyl group (2’,3’-dideoxyuridine), or of the 5’-hydroxyl (5’-deoxyuridine), did not lead to higher affinity. The lower affinity for 4-thiouracil is likely to reflect a stronger hydrogen bond at the 4-keto group than was the case for TbU1, as a result of a subtle shift in position or a different amino acid facing this group. The reciprocal Kᵢ and Kₘ values of uracil with uridine and 2’-deoxyuridine are entirely consistent with all uptake of these nucleosides proceeding through the TbU3 transporter but
the low affinity for the nucleosides shows that TbU3 is a uracil transporter. This is confirmed by comparing the transport efficiency, expressed as $V_{\text{max}}/K_{\text{m}}$, which is identical for uridine and 2’-deoxyuridine (0.0017) but two orders of magnitude higher for uracil (0.18). No separate transport activity could be detected for cytidine, 2’-deoxycytidine, cytosine, orotate or thymine; like uridine and 2’dUrd there is unlikely to be significant salvage of these pyrimidines under physiological conditions.

TbU3-independent transport of [3H]-thymidine was detectable, and designated TbT1. However, its low affinity for thymidine, its high affinity inhibition by adenosine and reciprocal inhibition by inosine clearly shows this thymidine flux is mediated by one of the P1-type purine nucleoside transporters expressed in *T. b. brucei* bloodstream forms (De Koning et al., 2005; De Koning and Jarvis, 1999; Al-Salabi et al., 2007; Sanchez et al., 2002). The extremely low thymidine affinity and translocation efficiency of TbT1 led us to speculate that it would not contribute substantially to pyrimidine salvage *in vivo*, unless it is expressed at very much higher levels of activity *in vivo* rather than under the ‘rich’ *in vitro* growth conditions of standard HMI-9/FCS. This would parallel the situation with the TbAT1/P2 aminopurine transporter, which is highly expressed in rodent-grown trypanosomes but barely detectable in *in vitro* cultured trypanosomes (Ward et al., 2011). However, despite a trend suggesting a minor increase in [3H]-thymidine uptake from cells grown *in vivo*, we were unable to detect a significant difference in thymidine transport rates in trypanosomes isolated from rat blood or from culture in HMI-9/FCS (n=3; data not shown) and we thus conclude that the function of this transporter is not primarily the uptake of thymidine, but of purines.

Notwithstanding the observation that 5-FU seems to be the only cytotoxic pyrimidine taken up efficiently by BSF trypanosomes, several other analogs displayed a higher trypanocidal activity. Of particular interest was the observation that 5-FOA displayed >3-fold higher activity than 5-FU although the orotate uptake rate was just a fraction ($\leq 1\%$) of the transport rate of [3H]-uracil and [3H]-5-FU. Moreover, 5-FOA and 5-FU give rise to the same active metabolites, converging immediately on 5F-UMP (Figure 6). It must therefore be concluded that the 2-step conversion of 5-FOA to 5-UMP (by orotate phosphoribosyltransferase (OPRT) and orotidine monophosphate decarboxylase
(OMPDC)) is far more efficient than the phosphoribosylation of 5-FU by uracil phosphoribosyltransferase (UPRT). The active sites of OPRT and OMPDC clearly are more tolerant of the 5-position fluorine than UPRT. Interestingly, 5-FOA displays even much higher activity against *Plasmodium falciparum*, with reported in vitro EC\(_{50}\) values in the low nanomolar range (Rathod et al., 1989). It seems highly likely that the 100-fold higher antimalarial activity of 5-FOA can be attributed to the fact that orotic acid is, alone among all pyrimidines, efficiently taken up by *Plasmodium* spp and incorporated into nucleic acids (Gutteridge and Trigg, 1970). We conclude that the lack of high affinity transporters for pyrimidine antimetabolites in bloodstream trypanosomes, and the lack of substrate flexibility for UPRT and for the only BSF uracil transporter (see Table 1) may limit the achievable trypanocidal activity with water-soluble pyrimidines. Another case in point is 6-azauracil, an inhibitor of pyrimidine *de novo* biosynthesis enzyme orotidylate decarboxylase, a poor substrate for TbU3 (Table 1). The strict selectivity of kinetoplastid uracil transporters was also noted for the procyclic TbU1 and *Leishmania major* U1 carriers (De Koning and Jarvis, 1998; Papageorgiou et al., 2005) and contrasts kinetoplastid nucleoside transporters, particularly the aminopurine transporter TbAT1/P2 which is involved in uptake of many trypanosomiasis drugs (De Koning, 2001; De Koning et al., 2004). This may be related to the fact that, unlike all the protozoan purine nucleoside and nucleobase transporters (De Koning et al., 2005), the uracil transporters are apparently not part of the Equilibrative Nucleoside Transporter family (De Koning, 2007).

As predicted by the current model of pyrimidine pathways in *T. b. brucei* (Figure 6) incubation with sub-lethal concentrations of 5-FOA and 5-FU produced essentially the same set of downstream metabolites, although 5-FOA incubation also resulted in detectable levels of fluoro-carbamoylaspartate, indicating the pyrimidine biosynthesis pathway can operate in reverse. 5-FOA incubation likewise resulted in production of 5-FU by the trypanosomes, thus uracil phosphoribosylation is also reversible. Significant amounts of fluorinated uridine ribonucleotides and 5F-UDP-activated sugar intermediates were detected in the metabolome, whereas no trace of 5F-2’deoxyuridine nucleotides was detected, indicating that 5F-UDP was not a substrate of *T. b. brucei* ribonucleoside-diphosphate reductase. Yet, incubation with 5-FU did result in an elevation of dUMP
levels. The cause of these elevated dUMP levels is not clear but is unlikely to be the result of 5F-dUMP inhibition of thymidylate synthase, as no 5F-dUMP was detected in the metabolomic analysis, nor has any other evidence that fluorinated pyrimidines might be substrates of ribonucleoside-diphosphate reductase (RNR) come to light in this study. It could be speculated, however, that there is an allosteric effect of a fluorinated nucleotide on RNR, as Hofer and co-workers (Hofer et al., 1998) demonstrated that this key enzyme is allosterically regulated by numerous nucleotides in a complex way. The complete lack of cross-resistance between 5-FU and 5F-2’deoxynucleosides (Table 2) seems to definitively show that the increase in dUMP, unlike 5F-2’dUrd, is not the main mechanism of action for the fluorinated nucleobases. These observations suggest therefore (one of) two main mechanisms of action for the fluorinated nucleobases: incorporation as 5-fluoronucleotides into RNA, or an effect of the 5F-UDP-coupled sugars on glycosylation or GPI anchor synthesis. The presence of significant levels of 5F-uridine (and lower levels of 5F-cytidine) nucleotides in digested RNA (but not DNA), coupled with the absence of any observable effect on glycosylation of *T. b. brucei* membrane proteins (>95% Variant Surface Glycoprotein (VSG, a GPI-anchored glycoprotein)) suggest that the incorporation of fluorinated nucleotides into RNA contributes to 5-FOA and 5-FU-induced cell death in trypanosomes but it is highly likely that the trypanocidal activity is multifactorial.

5F-2’dUrd was a substrate for thymidine kinase (low levels of 5F-dUMP detected), but not for uridine phosphorylase, as no 5-FU or fluorinated uridine ribonucleotides were observed in 5F-2’dUrd-exposed trypanosomes. Nor were 5F-dUDP or 5F-dUTP present in detectable quantities and we conclude that 5F-dUMP was not a substrate for thymidylate kinase. The absence of any incorporation of 5F-deoxynucleotides into DNA supports this conclusion. The notable change in the metabolome of 5F-2’dUrd-treated trypanosomes was a large increase (>35-fold) in dUMP levels, strongly suggesting that its mechanism of action is the inhibition of dihydrofolate reductase-thymidylate synthase. The rescue by excess extracellular thymidine supports this conclusion. The small effects on the levels of 2’deoxycytidine nucleotide levels may indicate allosteric effects on ribonucleoside reductase. Incubation with 5F-2’dCtd caused virtually the same metabolomic changes as with 5F-2’dUrd, consistent with the notion of any 2’deoxycytidine utilization in trypanosome being
through deamination to 2′deoxyuridine that were also indicated by the cross-resistance between 5F-2′dUrd and 5F-2′dCtd (Table 2). Indeed, 5F-2′deoxyuridine was clearly detected after incubation with the deoxycytidine analogue, as were small quantities of 5F-UMP (and high levels of dUMP), confirming reports of deoxycytidine incorporation in *T. b. gambiense* (Königk, 1976) and of cytidine deaminase activity in several kinetoplastid parasites (Hammond and Gutteridge, 1982) but in contrast with evidence from Hofer et al. (2001) who were unable to detect incorporation of radiolabeled cytosine and cytidine into the *T. b. brucei* nucleotide pool. The likely explanation is the use of submicromolar concentrations of pyrimidines by Hofer for the incorporation studies, which would allow rapid uptake and utilization of uracil but not of cytosine or cytidine (this paper).

The metabolomic analysis also supplied further information about a possible uridine phosphorylase. Fluorinated uridine nucleosides are at best poor substrates for this enzyme, as incubation with 5F-2′dUrd resulted in no detectable production of 5-FU or fluorinated ribonucleotides. Incubation with 5F-Urd did produce some of these metabolites, however, showing that this enzyme (Tb927.8.4430) is indeed expressed in bloodstream forms as suggested by Hassan and Coombs (1988) and favors uridine over 2′-dUrd as shown by Larson et al. (2010). There is no evidence for a uridine kinase activity in kinetoplastids (Fig. 10; Hammond and Gutteridge, 1982).

This study for the first time established the metabolic space of pyrimidine antimetabolites in kinetoplastid parasites. A surprising number of metabolites were detected, showing that the fluorination on position 5 has limited effect on many enzymes of the pyrimidine pathways. Pyrimidine antimetabolites may be incorporated into RNA, into precursors for lipid biosynthesis and activated sugar metabolism, potentially impacting on VSG glycosylation or GPI anchors - all essential functions to trypanosomes (Donelson, 2003). However, it is equally instructive to observe which into which part of the pyrimidine ‘system’ the analogs did not penetrate: 5-FU was not a substrate for uridine phosphorylase or ribonucleotide reductase. This is very different from 5-FU metabolism in human cells (Longley et al., 2003), where 5-FU incorporation into deoxynucleotides is mediated by human ribonucleotide reductase, and by uridine phosphorylase and pyrimidine phosphorylase followed by thymidine kinase (forming 5F-2′dUMP). The formation of fluorinated deoxynucleotides
in human cells leads to their incorporation into DNA and inhibition of thymidylate synthase leading to double strand breaks; 5F-UMP is similarly incorporated into human RNA. It is believed that the inhibition of thymidylate synthase is the main action of 5-FU and its prodrugs on human cells (Longley et al., 2003; Ceilley, 2012), leading to an imbalance between deoxyuridine nucleotides and thymidine nucleotides and the miss-incorporation of the former into DNA. This mechanism is identical to that described here for the trypanocidal action of 5F-2’dUrd and 5F-2’dCtd.

In summary, we report that only uracil is efficiently taken up by bloodstream forms of T. brucei and characterize the transporter. Untargeted metabolomics and HMM profiling were used to map the pyrimidine salvage system, and the passage of pyrimidine antimetabolites through it. This approach proved to be extremely powerful, highlighting even apparently minor metabolites in pathways such as GPI anchor biosynthesis and lipid biosynthesis pathways. In addition, the untargeted metabolomics further highlighted important changes in metabolites that were not directly derived from the active analog under investigation, such as the accumulation of dUMP after treatment with 5F-2’dUrd, resulting in a much-improved understanding of pyrimidine salvage systems in kinetoplastids, and a first evaluation of its utility in a strategy of antimetabolites for antiprotzoal chemotherapy.

**Authorship Contributions**

Performed experiments: Ali, Burgess, and Allison

Performed data analysis: Ali, Creek, Field, Mäser and De Koning

Wrote the paper: De Koning
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analogues: influence of pyridine nitrogens on trypanocidal activity, transport kinetics and

Footnotes

J.A.M.A. was supported by a personal studentship from the Libyan government
FIGURE LEGENDS.

FIGURE 1. Timecourse of [3H]-uracil transport in T. b. brucei bloodstream forms over 120 s. Transport of 0.15 µM [3H]-uracil (■) was linear (r² = 0.99), and significantly different from zero (F test; P<0.0001). In the presence of 1 mM unlabelled uracil (○) transport was reduced by >97% but still significantly different from zero (F-test, P = 0.03). Error bars are SE, and when not shown fall inside the symbol. The experiment was performed in triplicate and one of several independent experiments with highly similar outcomes.

FIGURE 2. Characterization of [3H]-uracil transport in T. b. brucei bloodstream forms. A. Inhibition of 0.15 µM [3H]-uracil uptake over 30 s by various concentrations of unlabelled uracil. Inset: conversion to Michaelis-Menten saturation plot. B. Dose-dependent inhibition of 0.15 µM [3H]-uracil transport by uridine (▲), 5-fluorouracil (●) and 5-bromouracil (□). Incubations (30 s) were terminated by the addition of 1 ml ice-cold 1 mM uracil in assay buffer and immediate centrifugation through oil. Error bars are SE of triplicate determinations.

FIGURE 3. Transport of pyrimidine nucleotides by T. b. brucei bloodstream forms. A. Transport of 5 µM [3H]-2’deoxyuridine in the presence (○) or absence (■) of 2.5 mM unlabelled 2’-deoxyuridine. Lines were calculated by linear regression analysis, with correlation coefficients of 0.99 for both data sets. B. Representative inhibition plot of 5 µM [3H]-2’-deoxyuridine transport, using a 180 s incubation time: ■, 2’-deoxyuridine; ○, uracil. The conversion of the 2’-dUrd inhibition plot to a Michaelis-Menten saturation plot for the determination of Kₘ and Vₘₙₐₓ is shown in the Supplementary data. C. Transport of 10 µM [3H]-thymidine (■) was linear for up to 30 min and partly inhibited by the addition of 2.5 mM unlabelled thymidine (○). D. Transport of 5 µM [3H]-thymidine over 15 min in the presence of various concentrations of uracil (▲), adenosine (○) or thymidine (■). The conversion of the thymidine inhibition plot to a saturation plot is shown in the Supplementary data. All error bars are Standard Errors of triplicate determinations; where not visible error bars fall within the symbol. Experiments shown are representative of several similar and independent experiments.
FIGURE 4. Transport of uracil and 5-FU by bloodstream trypanosomes. Cells of WT (closed symbols) or 5-FURes (open symbols) were incubated with (A) [3H]-uracil or (B) [3H]-5FU in the presence (circles) or absence (squares) of 1 mM unlabelled permeant. Lines were calculated by linear regression. Error bars are SE of triplicate determinations. The graphs shown are representative of three similar experiments.

FIGURE 5. Uptake of orotic acid by T. b. brucei. Bloodstream trypanosomes were incubated with 0.2 µM [3H]-orotic acid in the presence (○) or absence (●) of 1 mM unlabelled orotic acid. Uptake was linear (r² was 0.97 and 0.98, respectively) over the 10 min course of the experiment; 1 mM orotic acid did not significantly inhibit the rate of uptake. The experiment was performed in triplicate; error bars are SE.

FIGURE 6. Scheme of pyrimidine biosynthesis and metabolism in T. b. brucei. The double curved line represents the plasma membrane and arrows across it (potential) transport activities). Dotted lines indicate transport or conversions that probably do not take place in bloodstream trypanosomes. Red boxes indicate metabolites of which fluorinated analogues were detected by metabolomic techniques; black boxes indicate metabolites not detected in fluorinated form. Numbers above arrows indicate the following enzymes, listed here with EC numbers. 1, carbamoyl phosphate synthase (6.3.5.5); 2, aspartate carbamoyl transferase (2.1.3.2); 3, dihydroorotase (3.5.2.3); 4, dihydroorotate dehydrogenase (1.3.3.1); 5, orotate phosphoribosyltransferase (2.4.2.10); 6, orotidine 5-phosphate decarboxylase (4.1.1.23); 7, uracil phosphoribosyltransferase (2.4.2.9); 8, nucleoside diphosphatase (3.6.1.6). 9, nucleoside diphosphate kinase (2.7.4.6). 10, cytidine triphosphate synthase (6.3.4.2); 11, ribonucleoside-diphosphate reductase (1.17.4.1); 12, uridine phosphorylase (2.4.2.3); 13, dUTPase (3.6.1.23); 14, thymidylate kinase (2.7.4.9); 15, thymidine kinase (2.7.1.21); 16, thymidylate synthase (2.1.1.45); 17, cytidine deaminase (3.5.4.5); 18, UDP-glucose pyrophosphorylase (2.7.7.9); 19, UDP-glucose epimerase (5.1.3.2); 20, adenylate kinase G (2.7.4.10); 21, phosphatidate cytidylyltransferase (2.7.7.41); 22, ethanolamine-phosphate cytidylyltransferase (2.7.7.14); 23, choline-phosphate cytidylyltransferase (2.7.7.15); 24, orotate reductase (1.3.1.14, not present); 25, dihydroorotate dehydrogenase (1.3.5.2, not present); 26, pseudouridylate synthase (4.2.1.70); 27, UTP:N-acetyl-α-D-
glucosamine-1-phosphate uridylyltransferase (2.7.7.23); 28, α-1,6-N-acetylglucosaminyltransferase. Abbreviations: Gln, glutamine; Carb-P carbamoyl phosphate; Asp, aspartate; Carb-Asp, N-carbamoyl-L-aspartate; DHO, dihydroorotate; OMP, orotidine-5-phosphate; Urd, uridine; Tmd, thymidine; 2’dCtd, 2’-deoxycytidine; Glc, glucose; Gal, galactose; GlcNAc, N-acetylglucosamine. Lipid metabolism refers to formation of CDP-diacylglycerol (EC 2.7.7.41), CDP-ethanolamine (EC 2.7.7.14) and CDP-choline (EC 2.7.7.15).

FIGURE 7. Metabolomic profiles of wild-type and resistant cells treated with fluoro-pyrimidine nucleobases. Relative levels of (A) 2’-deoxyuridine and (B,C) fluorinated pyrimidines in trypanosomes exposed to (A,B) 100 µM 5-FU or (C) 5-FOA. Cultures of T. b. brucei bloodstream forms (50 ml of 2×10^6 cells/ml) in normal HMI-9 medium with 10% FCS were incubated with 100 µM 5-FU for 8 h. Extracts from cell pellets collected at the end of the experiment were subjected to metabolomic analysis and the intensity of the mass spectrometer signal is plotted here for the metabolites observed. *, P<0.05; **, P<0.02; ***, P<0.01 by unpaired two-tailed Student’s T-test comparing intensity of a particular metabolite in WT and resistant lines; n=3. Hatched bars, WT; solid bars, resistant clones, FURes (A,B) or FOARes (C).

FIGURE 8. Metabolomic profiles WT and 5F-dURes cells exposed or not (control) to 100 µM 5F-2’dUrd for 8 h. The level of 5F-2’dUrd in WT and 5F-dURes cells was not significantly different (A). After treatment with 5F-2’dUrd the level of dUMP was much higher in WT (brown bars) than in 5F-dURes cells (Blue bars) (Panel B). The abundance of TMP (panel C), TDP and TTP (not shown) was statistically identical in control and 5F-dURes cells, whether 5F-2’dUrd-treated or not. Experimental conditions as described in the legend to Figure 7. Hatched bars, WT; solid bars, 5F-dURes.

FIGURE 9. Effect of fluorinated pyrimidines on T. b. brucei bloodstream forms in the presence and absence of 100 µM thymidine. Left panel, WT427; Right panel, 5F-2’dURes trypanosomes. Cultures were grown in a minimal version of HMI-9 without pyrimidines (Blue bars), to which either 100 µM thymidine (Green bars) or nothing (Brown bars) was added. Diminazene was used as an internal control (not significantly different between conditions; not shown). The results shown are the average
of three independent experiments; error bars are SEM. *, P<0.05; **, P<0.02; ***, P<0.01 by unpaired two-tailed Student’s T-test. For 5F-2’dCtd on the 5F-2’dURRes cells the test compound did not sufficiently inhibit trypanosome growth at the highest concentration tested, 5 mM; IC₅₀ values of 5000 µM were added for each of the three independent experiments for the purpose of this graph.

**FIGURE 10.** Analysis of pyrimidine metabolic enzymes in major protozoan pathogens and two reference mammalian genomes. Profiles specific for the known pyrimidine metabolic enzymes were constructed as described in Methods. The profiles were scanned against selected eukaryote proteomes. 

A. Heat map of the best scores obtained by each proteome against profiles for enzymes of pyrimidine synthesis (1-6), salvage (7-17), sugar (18-19), and lipid metabolism (20-23). Enzyme numbers are the same as in Figure 6. B. Hierarchical clustering of the 'pyrimidine metabolic vectors' (top) based on Canberra distance (scale bar); the red numbers are 'approximately unbiased' confidence (au), where p = (100-au)/100.
Table 1. Substrate profile of the *T. b. brucei* pyrimidine transporters of procyclic (PC) and bloodstream forms: $K_m$ and $K_i$ values in $\mu$M.

<table>
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<tr>
<th></th>
<th>PCF</th>
<th>BSF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U1</td>
<td>U2</td>
</tr>
<tr>
<td><strong>Pyrimidine nucleobases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uracil</td>
<td>0.46 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Thymine</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td>Cytosine</td>
<td>NE, 1000</td>
<td>0.048 ± 0.009</td>
</tr>
<tr>
<td>Orotic acid</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td><strong>Pyrimidine nucleosides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uridine</td>
<td>33 ± 5</td>
<td>4.1 ± 2.1</td>
</tr>
<tr>
<td>2’Deoxyuridine</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Thymidine</td>
<td>NE, 1000</td>
<td>0.38 ± 0.07</td>
</tr>
<tr>
<td>Cytidine</td>
<td>NE, 1000</td>
<td>0.057 ± 0.019</td>
</tr>
<tr>
<td><strong>Pyrimidine analogues</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Methyluracil</td>
<td>NE, 10000</td>
<td></td>
</tr>
<tr>
<td>2-Mercaptopyrimidine</td>
<td>NE, 500</td>
<td></td>
</tr>
<tr>
<td>2-Pyrimidone</td>
<td>5400 ± 1300</td>
<td></td>
</tr>
<tr>
<td>2-Thiouracil</td>
<td>640 ± 110</td>
<td></td>
</tr>
<tr>
<td>3-Deazaaracil</td>
<td>&gt;2500</td>
<td></td>
</tr>
<tr>
<td>3-Methyluracil</td>
<td>1620 ± 350</td>
<td></td>
</tr>
<tr>
<td>4(3H)Pyrimidone</td>
<td>1670 ± 180</td>
<td></td>
</tr>
<tr>
<td>4-Thiouracil</td>
<td>22 ± 7</td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td>Value</td>
<td>Standard Error</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------</td>
<td>----------------</td>
</tr>
<tr>
<td>5-Fluoroorotic acid</td>
<td>ND</td>
<td>290 ± 40</td>
</tr>
<tr>
<td>5-Bromouracil</td>
<td>ND</td>
<td>180 ± 36</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>3.0 ± 0.8</td>
<td>2.6 ± 0.01</td>
</tr>
<tr>
<td>5-Chlorouracil</td>
<td>900 ± 140</td>
<td>560 ± 180</td>
</tr>
<tr>
<td>5-Iodouracil</td>
<td>ND</td>
<td>1300 ± 70</td>
</tr>
<tr>
<td>5-Nitouracil</td>
<td>NE, 1000</td>
<td>ND</td>
</tr>
<tr>
<td>5,6-Dihydrouracil</td>
<td>830 ± 200</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>6-Azauracil</td>
<td>~1000</td>
<td>663 ± 125</td>
</tr>
<tr>
<td>6-Methyluracil</td>
<td>&gt;2500</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>2',3'-dideoxyuridine</td>
<td></td>
<td>2260 ± 540</td>
</tr>
<tr>
<td>2',5'-dideoxyuridine</td>
<td></td>
<td>&gt;2500</td>
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<tr>
<td>5'-deoxyuridine</td>
<td></td>
<td>&gt;2500</td>
</tr>
<tr>
<td>Glutarimide</td>
<td>1020 ± 120</td>
<td>ND</td>
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**Purines**

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<thead>
<tr>
<th>Compound</th>
<th>Value</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
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<td>ND</td>
</tr>
<tr>
<td>Adenosine</td>
<td>NE, 1000</td>
<td>NE, 1000</td>
</tr>
<tr>
<td>Guanine</td>
<td>&gt;25</td>
<td>ND</td>
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<tr>
<td>Guanosine</td>
<td>NE, 1000</td>
<td>ND</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>NE, 1000</td>
<td>NE, 1000</td>
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<tr>
<td>Inosine</td>
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<td>Uric acid</td>
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<td>ND</td>
</tr>
<tr>
<td>Xanthine</td>
<td>ND</td>
<td>NE, 1000</td>
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</table>
Entries in bold typescript indicate $K_m$ rather than $K_i$ values. NE, no effect on uptake at concentration indicated. ND, not determined. Data for PCF were taken from De Koning and Jarvis (1998, Papageorgiou et al. (2005) and Gudin et al. (2006), and included for comparison.
Table 2. Phenotype of *T. b. brucei* strains adapted to high level resistance to fluorinated pyrimidines.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>SFU-Res</th>
<th>SFO-Res</th>
<th>SF-2'dU-Res</th>
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<tr>
<td></td>
<td>AVG</td>
<td>SE</td>
<td>n</td>
<td>AVG</td>
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<tr>
<td>5F-uracil</td>
<td>35.9</td>
<td>1.5</td>
<td>4</td>
<td>4707</td>
</tr>
<tr>
<td>5F-orotic acid</td>
<td>14.1</td>
<td>0.9</td>
<td>4</td>
<td>98</td>
</tr>
<tr>
<td>5F-2’-dUrd</td>
<td>5.2</td>
<td>0.2</td>
<td>4</td>
<td>3.2</td>
</tr>
<tr>
<td>5Cl-2’-dUrd</td>
<td>54</td>
<td>1.7</td>
<td>3</td>
<td>3.7</td>
</tr>
<tr>
<td>5F-2’-Ctd</td>
<td>49.4</td>
<td>3.4</td>
<td>4</td>
<td>55</td>
</tr>
<tr>
<td>6-Azauracil</td>
<td>958</td>
<td>34</td>
<td>3</td>
<td>157</td>
</tr>
<tr>
<td>Pentamidine</td>
<td>0.007</td>
<td>0.000</td>
<td>3</td>
<td>0.007</td>
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All EC₅₀ values were obtained using the Alamar blue assay and are given in µM. WT = wild-type sensitive control strain. RF = resistance factor: IC₅₀(resistant clone)/ IC₅₀(WT).
Table 3. Kinetic parameters of pyrimidine transport in bloodstream forms of WT and 5-FURes *T. b. brucei*.

<table>
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<tr>
<th>[³H]-Permeant</th>
<th>Strain</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
<th>$V_{max}/K_m$</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(µM)</td>
<td>(pmol/10⁷ cells/s)</td>
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<tr>
<td>Uracil</td>
<td>s427WT</td>
<td>1.5 ± 0.3</td>
<td>0.27 ± 0.05</td>
<td>0.18</td>
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<td></td>
<td>5-FURes</td>
<td>0.66 ± 0.15</td>
<td>0.16 ± 0.02</td>
<td>0.25</td>
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<tr>
<td>5-FU</td>
<td>s427WT</td>
<td>2.5 ± 0.01</td>
<td>0.27 ± 0.02</td>
<td>0.11</td>
</tr>
<tr>
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<td>5-FURes</td>
<td>2.3 ± 0.4</td>
<td>0.20 ± 0.02</td>
<td>0.088</td>
</tr>
<tr>
<td>Uridine</td>
<td>s427WT</td>
<td>9500 ± 2700</td>
<td>16 ± 4</td>
<td>0.0017</td>
</tr>
<tr>
<td>2'-Deoxyuridine</td>
<td>s427WT</td>
<td>810 ± 310</td>
<td>1.3 ± 0.7</td>
<td>0.0017</td>
</tr>
<tr>
<td>Thymidine</td>
<td>s427WT</td>
<td>1240 ± 310</td>
<td>0.067 ± 0.008</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
Fig. 1

Uracil Uptake
(pmol/10^7 cells)

Time (s)

0  30  60  90  120

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Molecular Pharmacology Fast Forward. Published on November 27, 2012 as DOI: 10.1124/mol.112.082321
Fig. 4

Uracil Uptake
(pmol/10^7 cells)

Time (s)

5-FU Uptake
(pmol/10^7 cells)

Time (s)
Fig. 10
Supplementary methods

**Lectin blotting.** Interaction of glycoproteins with lectins was visualized by separating proteins by reducing SDS-PAGE (using 10% gels and $1 \times 10^7$ cell equivalents/lane) and then Western blotting onto Immobilon-P transfer membranes (Millipore). Gels were stained with Brilliant Blue electrophoresis reagent (Sigma Aldrich) to check for equal cell loading and for visualization of protein bands. Membranes were stained using *Erythrina cristagalli* lectin (1:1,000) or *Ricinus communis* lectin (1:1,000) (both obtained from Vector Laboratories) and washed extensively before incubation with streptavidin-HRP (1:7,000) (Thermo Scientific) as in (Mehlert et al., 2012). Further extensive washing was followed by visualization of bands using ECL reagents (GE Healthcare).

Supplementary Results

**Effect of 5-FU on glycosylation in T. b. brucei.** To test whether the detection of significant quantities of 5F-UDP-hexoses and hexosamines contribute to trypanocidal action through interference with either protein glycosylation or glycosylphosphatidylinositol (GPI) anchor biosynthesis, we examined whether any major defects to glycosylation or GPI anchor synthesis took place under the influence of fluorinated pyrimidines. No detectable changes in VSG content were observed after treatment with 100 µM 5-FU or 5F-2’dUrd. Protein extracts of bloodstream trypanosomes, incubated for 12 h in the presence or absence of 100 µM 5-FU or 5F-2’dUrd, were separated by 1D SDS-PAGE and transferred onto Immobilon-P membranes. Two separate blots of the same samples were incubated specifically to terminal β-galactose residues, or with *Erythrina crystalla* lectin, which is specific for N-acetyl lactosamine modifications - both are hallmarks of mature N-glycan processing. This experiment was performed on three independent occasions but in no case was a difference in staining pattern or intensity observed between the extracts from treated and untreated trypanosome cultures (Supplemental Figure 8). Further, the intensity and migration position of the variant surface glycoprotein band, as detected by Coomassie staining of gels, was unaltered. We conclude that no major defects to glycosylation or GPI anchor synthesis took place under the influence of fluorinated pyrimidines. Although it is possible that glycosylation of a relatively rare glycoprotein could have been affected without this being apparent in the blot, it is clear that the bulk of N-glycan and GPI biosynthesis are unaltered.
Supplementary figures

Supplementary Figure 1. Transport of [3H]-uridine by T. b. brucei bloodstream forms. (A) Bloodstream forms were incubated with 2.5 μM [3H]-uridine for the indicated times in the presence (○) or absence (■) of 5 mM unlabelled uridine. Transport was terminated by the addition of 1 ml of ice-cold 10 mM uridine solution and immediate centrifugation through oil. Lines were calculated by linear regression analysis, with correlation coefficients of 0.98 (2.5 μM) and 0.63 (5 mM), respectively. (B) Transport of 2.5 μM [3H]-uridine in bloodstream forms over 15 min in the presence of various concentrations of unlabelled uracil (■) or uridine (○). Lines were calculated using an equation for sigmoid curve with Hill slope set at -1 and bottom level at 0 for the purpose of extrapolation.

Supplementary Figure 2. Transport of pyrimidine nucleosides by T. b. brucei. (A) Transport of 5 μM [3H]-2'-deoxyuridine by bloodstream T. b. brucei over 3 min at room temperature. (B) Transport of 5 μM [3H]-thymidine by bloodstream T. b. brucei over 15 minutes at room temperature. Both experiments were performed in triplicate and are representative of three identical experiments performed on different dates. Error bars are SE.

Supplementary Figure 3. Uptake of 0.25 μM [3H]-inosine or 10 μM [3H]-thymidine in bloodstream forms of T. b. brucei.
A. Thymidine uptake was dose-dependently inhibited by inosine (■) using 15 min incubations. K_i for three independent experiments was 1.6 ± 0.6 μM.
B. Inhibition of [3H]-inosine uptake by unlabelled inosine (○) and thymidine (■) at the indicated concentrations measured over 10 s.
C. Conversion of the inosine inhibition curve to a Michaelis-Menten saturation plot. K_m for this experiment was 0.53 μM. This experiment is representative of 4 identical experiments with highly similar outcomes.
Inosine uptake over 10 s is consistent with measurement of initial rates of transport as we previously reported linear uptake of [3H]-inosine in T. b. brucei over 60 s (De Koning et al. (1998) J Biol Chem 273: 9486-9494).

Supplementary Figure 4. Non-saturable uptake of some pyrimidine nucleosides and nucleobases by T. b. brucei bloodstream forms.
A. Uptake of 0.5 μM [3H]-cytidine (■) was slow and only partly inhibited by 2.5 mM unlabelled cytidine (○).
B. Uptake of 2.5 μM [3H]-2’-deoxycytidine (■) was not inhibited by as much as 10 mM 2’-deoxycytidine (○).
C. Uptake of 0.25 μM [3H]-cytosine (■) was not significantly different from zero (F-test) when measured over 15 min, and [3H]-cytosine accumulation was not reduced in the presence of 2.5 mM unlabelled cytosine (○).
D. Uptake of 1 μM [3H]-thymine in the presence ○ or absence (■) of 10 mM unlabelled thymine, measured over 15 min, was not significantly different from zero (F-test).

Supplementary Figure 5. Effect of fluorinated pyrimidines on growth of bloodstream form s427WT. Cultures containing 1×10^5 or 2×10^5 trypanosomes/ml were incubated with 500 μM of 5-FU (○), 5-FOA (▲), 5-fluoro,2’-deoxyuridine (■) or control (◊). At various times samples were taken and counted microscopically using a haemocytometer. Each determination was in performed three times and the average is shown. The inset is the same data as in the main figure but including the control (no added drug) culture and on a different scale.

Supplementary Figure 6. Adaptation of s427 bloodstream T. b. brucei to high concentrations of fluorinated pyrimidine analogues during in vitro culturing. Concentrations indicated are the concentrations of analog added to the medium, in which the cells managed to survive and multiply. This was 5-fluorouracil to generate the adapted cell line 5FUREs; this was 5F-2’-dUrd to generate the cell line 5F-dURes; and 5F-orotic acid to generate the cell line 5F-FOARes. After adaptation of the
cultures the trypanosomes were cloned out by limiting dilution so that the eventual cell lines that were characterized were all grown from a single cell.

Supplementary Figure 7. Transport of 0.25 µM 5-fluorouracil by bloodstream forms of (A) T. b. brucei s427WT or (B) 5-FURes. Incubation time was 30 s for WT and 4 min for 5-FURes, well within the linear range of uptake (see figure 4B of main manuscript). The experiments were representative of three identical experiments with highly similar outcomes.

Supplementary Figure 8. Lectin blotting of T. b. brucei BSF protein samples after incubation with 5-fluorouracil or 5fluoro-2’ deoxyuridine. Cultures of T. b. brucei bloodstream forms were incubated for 12 h in the presence or absence of 100 µM of either pyrimidine analog, under standard culturing conditions. Protein extracts were separated on 1D polyacrylamide gels (left hand-side, Coomassie Blue stained) and transferred to Immobilon-P membranes to be incubated with either Erythrina crystagallii lectin or Ricinus communis lectin (right hand-side). Lectin binding was visualized by using ECL reagents.
Supplemental Figure 1

A. Uridine uptake (pmol/10^7 cells) over time (min).

B. Uridine uptake (pmol/10^7 cells/s) as a function of log[Inhibitor](M).
Supplemental Figure 2

A. 2'-Deoxyuridine Uptake (pmol/10^7 cells/s) vs [2'-dUrd] (µM)

B. Thymidine Uptake (pmol/10^7 cells/s) vs [Thymidine] (µM)
Supplemental Figure 3

(A) Inosine Uptake (pmol. (10^7 cells) \cdot s^{-1}) vs. log[Inhibitor](M)

(B) Inosine Uptake (pmol. (10^7 cells) \cdot s^{-1}) vs. [Inosine] (µM)

(C) Thymidine Uptake (pmol. (10^7 cells) \cdot s^{-1}) vs. log[Inhibitor](M)
Supplemental Figure 5

Culture density (cells/ml) vs. Time (h)

- ◇ 5-fluorouracil
- ■ 5F-2'dUrd
- ▲ 5-fluoroorotic acid
- ⭐ Drug free control
Concentration of test compound in which the trypanosome population can grow and multiply (µM)
Supplemental Figure 7

A

B

[5-Fluorouracil] (µM)

5-Fluorouracil Uptake (pmol/10^7 cells/s)

[5-Fluorouracil] (µM)

5-Fluorouracil Uptake (pmol/10^7 cells/s)
Supplemental Figure 8

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**Coomassie**

**Blot**

Erythrina cristagalli lectin

Ricinus communis lectin