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Roles for the *Trypanosoma brucei* P2 Transporter in DB75 Uptake and Resistance[S]

Charlotte A. Lanteri,[1] Mhairi L. Stewart,[2] Janice M. Brock, Vincent P. Alibu, Steven R. Meshnick, Richard R. Tidwell, and Michael P. Barrett

Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, United Kingdom (M.L.S., J.M.B., V.P.A., M.P.B.); and Department of Pathology and Laboratory Medicine (C.A.L., R.R.T.) and Department of Epidemiology, School of Public Health (S.R.M.), University of North Carolina, Chapel Hill, North Carolina

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ABSTRACT

A novel trypanocide, 2,5-bis(4-amidinophenyl)furan (DB75), in its prodrug amidoxime-derivative form, 2,5-bis(4-amidinophenyl)furan-bis-*O*-methylamidoxime (DB289), is in trials as the first orally administered drug for human African trypanosomiasis. DB75 is a diamidine. Resistance to some diamidines correlates to loss of uptake via the P2 aminopurine transporter. We show here that uptake of DB75 into *Trypanosoma brucei* also occurs principally via the P2 transporter. Uptake of tritiated DB75 occurred via a high-affinity ($K_{m\ app}$, 3.2 μM) carrier-mediated route that was inhibited by adenosine, adenine, and pentamidine, all known substrates of the P2 transporter. Trypanosomes lacking the *TbAT1* gene that encodes the P2 transporter demonstrated an 11-fold reduction in sensitivity to DB75 when measured under controlled in vitro conditions. These knockout cells were also less sensitive to DB75 than wild-type cells in mice. Initial uptake rates of DB75 into the Δ*tbat1* knockout cell line were greatly reduced compared with rates in wild-type cells. A trypanosome cell line selected in vitro for DB75 resistance was shown to have lost P2-mediated DB75 uptake. The *TbAT1* gene was mapped to chromosome V of the *T. brucei* genome and the DB75-resistant parasites were shown to have deleted both alleles of this gene. Fluorescence microscopy of DB75-treated trypanosomes revealed that DB75 fluorescence localizes rapidly within the DNA-containing organelles of wild-type trypanosomes, whereas no fluorescence was observed in Δ*tbat1*-null parasites or in the parasites selected for resistance to DB75.

African trypanosomes cause a number of diseases in humans (Barrett et al., 2003; Stich et al., 2003) and in animals (Barrett et al., 2003; Omamo and D'Ieteren, 2003). Resistance to trypanocidal drugs contributed to a re-emergence of human African trypanosomiasis (HAT) in sub-Saharan Africa in the late 20th century (Barrett et al., 2003). Several drugs are used to treat specific stages of disease progression (Pepin and Milord, 1994; Barrett et al., 2003). However, new drugs are urgently required, because the current drugs, pentamidine, suramin, melarsoprol, and eflornithine, suffer a number of drawbacks including host-toxicity, expense, and a need for parenteral administration (Barrett, 2000; Fairlamb, 2003; Burri et al., 2004). A single compound, DB289, is currently in phase III trials toward registration for use against early-stage HAT (Jannin and Cattand, 2004). DB289 is an orally available amidoxime prodrug (Zhou et al., 2002; Ansede et al., 2004) and is converted to the active diamidine, DB75, systemically (Sturk et al., 2004). Studies conducted in yeast cells (Lanteri et al., 2004) and in bloodstream forms of *Trypanosoma brucei* (C. Lanteri, unpublished data) suggest that the mitochondrion is a cellular target of DB75 action.

To reach cellular targets, drugs must enter trypanosomes via the plasma membrane (de Koning, 2001a; Denise and

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C.A.L. and M.L.S. contributed equally to this work
[1] Current affiliation: Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Spring, Maryland.
[2] Current affiliation: Zentrum für Molekulare Biologie Heidelberg, Heidelberg, Germany.
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ABBREVIATIONS: HAT, human African trypanosomiasis; DB75, 2,5-bis(4-amidinophenyl)furan; DB289, 2,5-bis(4-amidinophenyl)furan-bis-*O*-methylamidoxime; DB75^R-CL1, the *T. brucei* strain selected for resistance to DB75; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; melarsoprol, 2-[4-[(4,6-diamino-1,3,5-triazin-2-yl)amino]phenyl]-1,3,2-dithiarsolan-4-yl]methanol; pentamidine, 4-[5-(4-carbamimidoylphenoxy-)pentoxy]benzenecarboximidamide; suramin, 8-[[4-methyl-3-[[3-[[3-[[2-methyl-5-[(4,6,8-trisulfonaphthalen-1-yl) carbamoyl]phenyl]carbamoyl]phenyl]carbamoylamino]benzoyl]amino]benzoyl]amino]naphthalene-1,3,5-trisulfonic acid; eflornithine, 2,5-diamino-2-(difluoromethyl)pentanoic acid; diminazene, 4-[2-(4-carbamimidoylphenyl)iminohydrazinyl]benzenecarboximidamide; PCR, polymerase chain reaction.

Barrett, 2001). The *T. brucei* P2 aminopurine transporter (Carter and Fairlamb, 1993) transports adenosine and adenine but also carries trypanocidal diamidine drugs (Barrett et al., 1995; Carter et al., 1995; de Koning et al., 2004), such as pentamidine and diminazene, and melaminophenyl arsenicals (Carter and Fairlamb, 1993; Barrett and Fairlamb, 1999; Maser et al., 1999; Stewart et al., 2005), such as melarsoprol or its active metabolites. Pentamidine also enters trypanosomes through additional transporters, known as the high- and low-affinity pentamidine transporters (HAPT1 and LAPT1, respectively) (de Koning, 2001b).

The P2 transporter is encoded by the *TbAT1* gene (Maser et al., 1999), and loss of activity of the transporter has been shown to be associated with resistance to both melaminophenyl arsenical and diamidine drugs (Carter and Fairlamb, 1993; Barrett et al., 1995; Carter et al., 1995; Maser et al., 1999; de Koning, 2001; de Koning et al., 2004; Stewart et al., 2005). The *TbAT1* gene had accumulated point mutations rendering it inactive in one melarsoprol-resistant line (Maser et al., 1999), and similar mutations were identified in parasites isolated from patients who failed to respond to melarsoprol in the field (Matovu et al., 2001b). Other melarsoprol-resistant parasites from both the laboratory and the field have been shown to be defective in P2-mediated transport using a novel fluorescence test (Stewart et al., 2005).

Loss of P2 transporter function is correlated with the development of resistance to the veterinary trypanocidal diamidine, diminazene (Barrett et al., 1995; de Koning et al., 2004), whereas factors in addition to loss of P2 are required to achieve pentamidine resistance (Matovu et al., 2003). This is probably because diminazene enters trypanosomes predominantly via P2-mediated uptake (de Koning et al., 2004), whereas pentamidine can still enter cells through HAPT1 and LAPT1 (de Koning et al., 2004). This may explain why trypanosomes defective in the P2 transporter retain near wild-type levels of sensitivity to pentamidine (Matovu et al., 2003).

Reports of melarsoprol treatment failure have increased in recent years (Legros et al., 1999; Matovu et al., 2001a; Moore and Richer, 2001). Whether relapse cases result from drug resistance or other phenomena remains unclear (Brun et al., 2001). However, it is possible that drug-resistant trypanosome populations that lack P2 transporter function are already in circulation. If P2-deficient trypanosomes are indeed propagating in the field, treatment failures are likely to occur with other drugs that are also dependent upon P2 for uptake.

The purpose of this study was to determine the mechanism by which DB75 enters *T. brucei* cells and to investigate DB75 resistance. Understanding how DB75 resistance arises and being in a position to monitor for its emergence could enable rational and targeted use of the drug, thereby preventing treatment failures and the spread of DB75 resistance. We have recently introduced a simple and rapid test using fluorescence microscopy, primarily to report on resistance to melarsoprol, that can detect loss of the P2 transporter (Stewart et al., 2005). If resistance to DB75 also relates to loss of the P2 transporter, the same test would also be available to monitor for resistance and thus facilitate the successful application of the oral prodrug, DB289, as a new therapy for early-stage HAT.

Materials and Methods

Chemicals. [^{3}H]DB75 with a specific activity of 1 Ci/mmol was custom-synthesized and purchased from Moravek Biochemicals, Inc. (Brea, CA). [2,8-^{3}H]-adenosine (35.9 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Beaconsfield, Bucks, UK). Pentamidine isethionate (Sanofi-Aventis, Bridgewater, NJ) was a gift from Dr. J. Jannin (WHO). DB75 was from Immtech (Vernon Hills, IL). Other chemicals were of the highest quality available from Sigma.

Isolation and Collection of Bloodstream Form *T. brucei brucei* from Infected Rat Blood. Male Sprague-Dawley rats were infected via intraparenteral injection with approximately 2×10^6 bloodstream-form trypanosomes [strain 427 wild type; the Δ*tbat1* knockout line (Matovu et al., 2003) or the DB75-resistant line DB75^R-CL1 derived from strain 427 described below] attained from in vitro cultures. Infected blood was collected through cardiac puncture at peak parasitemia (usually day 5 after infection). Blood was centrifuged in heparinized tubes at 2200*g* for 10 min at 4°C, and the buffy coat was removed and diluted 1:3 in phosphate saline glucose buffer, pH 8.0. Trypanosomes were purified from blood using DE52 anion exchange columns (Lanham, 1968).

Uptake Studies in Bloodstream from *T. brucei brucei*. Transport assays using [^{3}H]DB75, or [^{3}H]adenosine were conducted using a rapid oil-stop method (Carter and Fairlamb, 1993; Barrett et al., 1995; Carter et al., 1995; Maser et al., 1999; de Koning, 2001a; de Koning et al., 2004; Stewart et al., 2005). Trypanosomes purified from infected rat blood were washed twice after centrifugation at 2000*g* for 10 min at 4°C in a modified Coffman's balanced salt solution, made up of 25 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 0.55 mM $CaCl_2$, 0.4 mM $MgSO_4$, 1.0 mM Na_2HPO_4, and 12 mM D-glucose, pH 7.4, and, when stated, 1.0% bovine serum albumin (fatty acid-free) added fresh on the day of the experiment. Parasites were resuspended to 10^8 cells/ml. Resuspended cells were warmed to room temperature, and 100-μl aliquots were added to microcentrifuge tubes containing 100 μl of Coffman's balanced salt solution plus tritiated permeants and various substrates, as indicated under *Results*, layered over 100 μl of dibutyl phthalate. Stock solutions of the test substrates at 100 mM were prepared in sterile distilled water and were heated in a water bath to aid solubility. Transport was stopped after indicated times by centrifuging at 13,000*g* for 1 min. Samples were immediately flash-frozen, and a tube cutter was used to collect the pellet into a scintillation vial containing 200 μl of 2.0% SDS. Counts per minute of radioactivity were determined after an overnight incubation in 3.0 ml of Ecoscint scintillation cocktail (National Diagnostics, Atlanta, GA) at room temperature. Total radioactivity present in the interstitial space was ascertained by performing uptake assays on ice, followed by immediate centrifugation through oil. Counts due to tritium found within the interstitial space were subtracted from the final count attained in all samples for each experiment. Data analysis was performed using Prism (GraphPad Software, San Diego, CA) and Grafit 4.0 (Erithacus Software, Horley, Surrey, UK) software. All experiments were performed in duplicate on at least three independent occasions.

Trypanotoxicity Assays in Vitro. Bloodstream forms of the different strains of *T. brucei brucei* were cultivated in HMI-9 medium containing 20% fetal calf serum (Hirumi and Hirumi, 1989) at 37°C in a humidified CO_2 environment. An adaptation of the Alamar Blue assay (Raz et al., 1997) was used to determine IC_{50} values against the different cell types. Cells (5×10^4) were added to 96-well plates with wells containing doubling dilution of each test compound (200 μl/well final volume). After 48 h, 20 μl of Alamar blue reagent was added; 24 h later, the degree of blue-red color change was measured in a fluorimeter (excitation at 530 nm, emission at 590 nm), and the change was plotted as a function of drug concentration using the IC_{50} algorithm of the Grafit 4.0 software. All experiments were performed in duplicate on at least three independent occasions.

Selection of DB75-Resistant Trypanosomes. Bloodstream form trypomastigotes of the 427 line were cultivated in growth me-

dium with 0.01 μM DB75, which represented approximately 0.15-fold the dose of the drug required to kill 50% of the wild-type parasites during in vitro growth, the intention being to initiate stepwise selection in increasing concentrations of this compound. After 4 weeks, these cells were cultivated in 0.04 μM DB75 and grown for a further 2 weeks, at which point cells were growing well. Cells were then diluted to 10^3 per ml and allowed to grow for a further 6 weeks in 0.04 μM DB75, after which they were tested for their sensitivity to drug using the Alamar blue assay. Single cells were cloned by limiting dilution, and individual clones were then grown in 0.04 μM drug and tested using the Alamar blue test. The cloned line was designated DB75^R-CL1.

Trypanotoxicity Assays in Vivo. Five or more female ICR mice per treatment group were i.p. injected with 10^5 bloodstream-form parasites derived from cell culture and delivered in culture medium. Cell lines used were *T. brucei brucei* 427, Δ*tbat1* knockout cells (P2 knockout) and DB75^R-CL1. Forty-eight hours after infection, mice were weighed and treated by i.p. injection of drug relative to their treatment group. Drug doses are shown in Table 1. Treatment was repeated for an additional 3 days, resulting in four treatments in all.

Control groups of three mice of each cell line were either treated with one dose of 5 mg/kg suramin at 48 h after infection or were not treated. The parasitemia of all mice was followed by daily tail-prick examination of blood smears until day 40, after which any remaining mice were culled.

Fluorescence Testing. Blood was collected from ICR mice infected with wild-type, Δ*tbat1* knockout or DB75^R-CL1 cells. DB75 (0.5 μl at 10 mM) was added to 500 μl of whole blood at room temperature to give a final concentration of 10 μM (optimal for fluorescence staining) (Stewart et al., 2005). This was thoroughly mixed, and approximately 2 ml was removed to a glass slide and covered to produce a wet blood smear that was viewed over a 10-min period. The slide was viewed directly through the Zeiss Axioscop FL fluorescence microscope using a Zeiss 02 filter (Zeiss, Welwyn Garden City, UK) at an excitation wavelength of 330 nm and an emission wavelength of 400 nm. Images were obtained by the same method, but using an Axiovert 200M Fluorescence microscope and Openlab imaging software (Improvision, Coventry, UK).

Molecular Biology. The *TbAT1* gene was not annotated in the *T. brucei* genome database (Berriman et al., 2005) at the time of this study. DNA was isolated from wild type 427, the Δ*tbat1* knockout line (Matovu et al., 2003), and the DB75^R-CL1 line using an adaptation of the genomic DNA miniprep procedure (Medina-Acosta and Cross, 1993). Southern blot analysis was performed using standard procedures (Sambrook et al., 1989), and probing of Southern blots using ^{32}P-labeled probes was performed as described previously (Sambrook et al., 1989). Although the *T. brucei* genome did not include an annotated version of *TbAT1*, orphan sequences in both of the *T. brucei* and *T. brucei gambiense* genome projects did reveal the gene to be present. This enabled construction of a contiguous sequence that linked the 3′-untranscribed region of *TbAT1* to a pair of genes related to expression site-associated gene 4 (GRESAG 4) sequences (Tb927.5.320 and Tb927.5.330) that mapped to position 80,000 to 89,000 of the annotated chromosome V of the *T. brucei* 927 genome map. A marker gene for chromosome V, a vacuolar ATPase subunit A encoding gene (Tb927.5.1290) was used as a control on Southern blots. We also produced a series of oligonucleotides spanning region 38,520 to 108,705 from the annotated chromosome V, as well as a number of oligonucleotides covering the entire TbAT1 open reading frame and its immediate 5′ and 3′ flanking regions. These were used in attempts to amplify DNA from wild-type, Δ*tbat1* knockout, and DB75^R- CL1 parasites (see Supplemental Table). Primers specific for the proline dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase were used as controls.

Results

DB75 Inhibits Uptake of Adenosine via the P2 Transporter. Because other diamidines have previously been shown to be substrates for the *T. brucei* P2 aminopurine transporter (Barrett et al., 1995; Carter et al., 1995; de Koning et al., 2004), DB75 was used over a range of concentrations to determine its capacity to inhibit uptake of radiolabeled adenosine via this transporter [blocking the P1 transporter component of adenosine uptake with 1 mM inosine (Carter and Fairlamb, 1993)]. Figure 1A shows that DB75 was capable of high-affinity interaction with the P2 transporter (IC_{50} = 2.1 μM), as were pentamidine (IC_{50} = 1.8 μM) and diminazene (IC_{50} = 1.1 μM), as measured by inhibition of adenosine (0.5 μM) uptake at 30 s, at which point uptake seems to be in its initial, linear, phase) .

Uptake of Radiolabeled DB75. Uptake of tritiated DB75 into *T. brucei* was measured over time (Fig. 1B). Using 2.1 μM DB75, uptake continued over at least 5 min at a rate of 2.9 pmol · 10^7 cells^{-1} · min^{-1}. At this rate, 10^8 cells would accumulate 41.7 nmol of drug in 24 h, which is in good agreement with the 70 nmol per 10^8 cells measured using an HPLC-based method to determine accumulation of DB75 in trypanosomes over a 24-h period in a recent study (Mathis et al., 2006). The uptake of excess (1 mM) unlabeled DB75 greatly reduced measured uptake. We then used a 30-s time point, during which uptake was linear, to determine kinetic constants for uptake. DB75 uptake was saturated (Fig. 1C) with an apparent K_m of 3.2 μM and V_{max} of 9.3 pmol · 10^7 cells^{-1} · min^{-1}. Uptake of DB75 over 30 s was inhibited in a

TABLE 1

Activity of drugs against wild-type *T. brucei*, Δ *tbat1* knockout cells, and a line selected in vitro for resistance to DB75

IC_{50} is defined as the 50% inhibitory concentration for cellular viability after 72 h of treatment with trypanocide using Alamar Blue as an indicator dye for metabolic activity. Resistance factor for each compound was calculated by dividing the IC_{50} value attained with the DB75^R-CL1 or Δ *tbat1*cells by the value attained with wild-type *T. brucei brucei* 427 cells. All experiments were performed in duplicate on at least three independent occasions. S.E. were always below 20% of those given mean values.

Trypanocide	Wild-Type	Δ*tbat1*		DB75^R-CL1	
	IC_{50}	IC_{50}	Resistance Factor	IC_{50}	Resistance Factor
	μM	μM		μM	
DB75	0.08	0.85	10.6	1.6	20
Diminazene aceturate	0.09	1.6	17.8	1.9	21.1
Pentamidine isethionate	0.01	0.03	3	0.06	6
Cymelarsan	0.03	0.09	3	0.05	1.7
Melarsoprol	0.03	0.03	1	0.03	1
Melarsen oxide	0.01	0.02	1	0.01	1
DAPI	0.57	1.7	3	1.1	1.9
Suramin	0.02	0.02	1	0.02	1
Isometamidium	2.2	6.5	3	1.8	0.8
Megazol	0.09	0.1	1.1	0.06	0.7

dose-dependent fashion, bringing uptake to virtually negligible levels with 1 mM adenosine, adenine, pentamidine, and diminazene, all substrates of the P2 transporter (Fig. 1D). IC_{50} values for adenosine, pentamidine, and diminazene were 0.86, 0.56, and 0.67 μM, respectively. These data indicated that the P2 transporter represents the principal route of entry of this important new trypanocide.

TbAT1-Defective Cells Lose Sensitivity to DB75 and Have Greatly Reduced Capacity to Accumulate the Drug. As the P2 transporter seemed to play a key role in the uptake of DB75, the Alamar blue assay was used to quantify the sensitivity of wild-type and Δ*tbat1* knockout cells (which lack P2 transport) to DB75 and a number of other trypanocides (Table 2). DB75 is active against wild-type cells with an IC_{50} value of 0.08 μM, whereas the Δ*tbat1* knockout line is 11-fold less sensitive, with an IC_{50} value of 0.85 μM. The Δ*tbat1* knockout line is also markedly less sensitive to diminazene. However, it is almost as sensitive as wild type to pentamidine, melarsen oxide, and cymelarsan. There is no difference in sensitivity to melarsoprol, because melarsoprol is capable of entry via passive diffusion as well as through P2 in vitro, although in vivo its rapid conversion to melarsen oxide, which enters only via carrier-mediated uptake, alters resistance profiles (Scott et al., 1997). The Δ*tbat1* knockout line shows only slight resistance to the trypanocidal diamidine, DAPI (IC_{50} value of 1.7 μM for the Δ*tbat1* knockout line versus 0.57 μM for wild type), indicating that this compound enters cells via routes in addition to P2. Little or no loss of sensitivity to other trypanocides, including isometamidium, suramin and megazol, is observed in the Δ*tbat1* knockout cells.

Uptake of DB75 was also measured in the TbAT1 knockout cell line. No uptake was apparent at the 5-min time point (Fig. 2A). However, allowing cells to accumulate drug over 30 min revealed that the compound was accumulated by P2-deficient trypanosomes, albeit at a rate (0.01 pmol 10^7 cells^{-1} min^{-1}) lower than 1% of the initial rate measured in wild-type cells (Fig. 2A).

It is noteworthy that although the TbAT1 knockout cell line showed greatly reduced sensitivity to DB75 measured in vitro over the 4-day Alamar blue assay, it was not possible to cultivate these trypanosomes in DB75 for more than 4 days, even at 0.08 μM (10% of the apparent IC_{50} value). This delay in the onset of cell death in DB75-treated Δ*tbat1*knockout parasites relative to wild type also suggests that DB75 is capable of accumulating in trypanocidal levels through a P2-independent uptake route. As a consequence of the low capacity of this uptake route and the low specific activity of the [^{3}H]DB75 available to us, we could not definitively resolve the nature of this secondary means of DB75 cellular entry. Failure of DB75 to self-inhibit to 100% might indicate passive diffusion, endocytosis, or uptake as a low-affinity substrate on a carrier that recognized the drug. Studies exploiting the fluorescence capability of DB75 showed that the Δ*tbat1* knockout line would still fluoresce by 10 min, even when incubated on ice, indicating that the drug may enter via passive diffusion, although definitive experiments to identify this route are presently lacking. These data, however, do suggest that DB75 enters and kills trypanosomes rapidly via the P2 pathway. The drug is still active against P2-defective cells, but uptake is slower and death therefore occurs over a longer time; otherwise, one could imagine a different mode of action, involving a secondary target whose loss leads to a slower death than in wild-type cells.

Selection and Characterization of DB75-Resistant Mutants. Bloodstream forms of *T. brucei* strain 427 were grown in vitro in the presence of DB75 at 0.01 μM. After 4 weeks, the concentration of drug was raised to 0.04 μM; after a further 6 weeks of incubation at this concentration, parasites were assayed for sensitivity to DB75 using the Alamar blue test (centrifuging parasites and resuspending in drug-free medium just before initiating the test). We cloned a resistant parasite line by limiting dilution and showed that the cloned line (DB75^R-CL1) was 20-fold less sensitive to drug than wild type with an IC_{50} of 1.6 μM (Table 2). The DB75^R-CL1 line also showed 21-fold resistance to dimina-

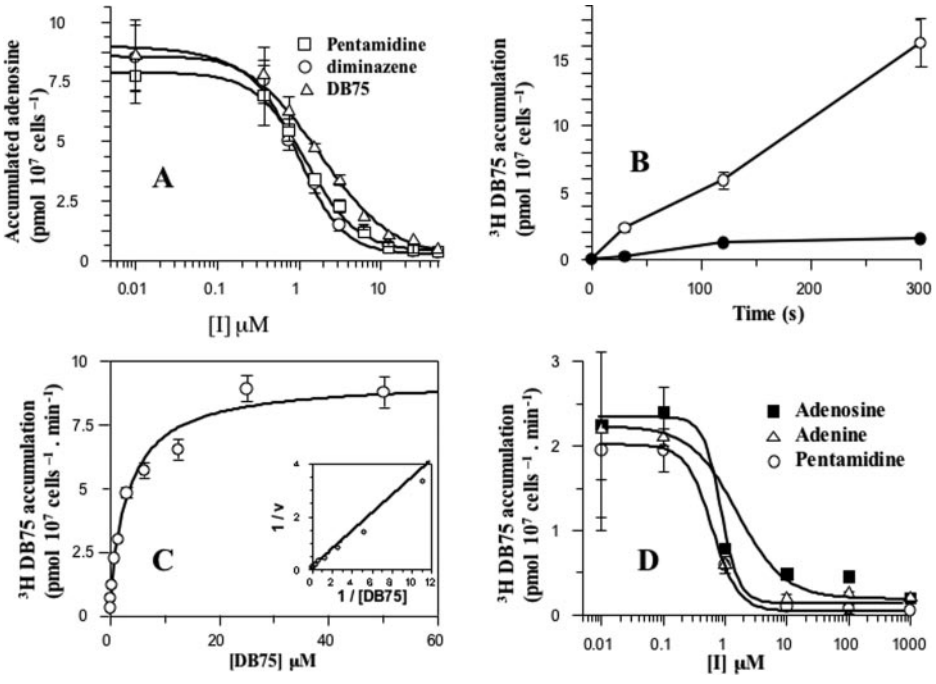

Fig. 1. Uptake of DB75 via the P2 amino purine transporter. A, radiolabeled adenosine (0.5 μM) was used as permeant to measure uptake into *T. brucei* strain 427 via the P2 transporter by blocking the P1 transport component using 1 mM inosine. Adenosine accumulation was allowed to continue for 30 s in the presence of increasing concentrations of pentamidine, diminazene aceturate, or DB75. Accumulated adenosine was plotted as a function of increasing inhibitor concentration. B, the uptake of radiolabeled DB75 (2.1 μM) was measured as a function of time over 5 min alone (○) or in the presence of 1 mM unlabeled DB75 (●). Inosine was absent. C, initial rates of uptake of DB75 were measured as a function of concentration allowing accumulation to continue for 30 s over a range of substrate concentrations and plotted as a Michaelis-Menten plot. A Lineweaver-Burk replot of the data is shown in the inset. Inosine was absent. D, inhibition of DB75 uptake using substrates of the P2 transporter (adenine, adenosine, and pentamidine) was carried out using 0.5 μM DB75 as permeant and increasing concentrations of each inhibitor. Uptake proceeded for 30 s. Inosine was absent.

zene but was only 6-fold less sensitive to pentamidine than wild type. There was good correlation in drug cross-resistance profiles between the Δ*tbat1* knockout line and the line selected for resistance to DB75 (Table 2). However, the line selected through exposure to DB75 was approximately 2-fold less sensitive to DB75 than was the Δ*tbat1* knockout line. This could indicate changes, in addition to altered TbAT1 activity, during drug selection that have not been identified here. As was observed in the Δ*tbat1* knockout cell line, it was not possible to grow DB75^R-CL1 cells in vitro in culture flasks in the presence of drug, even at 0.08 μM, beyond the 4-day time frame.

When DB75 was tested for its ability to kill trypanosomes in mice after four injections on consecutive days, it was shown that 0.5 mg/kg of drug was required for 100% cure of mice infected with *T. brucei* strain 427 wild type cells. For infections with Δ*tbat1* knockout cells, this dosage failed to cure any mice; 2.5 mg/kg was required for 100% cure. For infections with the in vitro-selected DB75^R-CL1 cells, 1 mg/kg DB75 was required for 100% cure. It is noteworthy that four of five mice were cured with 0.5 mg/kg, indicating that, in vivo, the DB75^R-CL1 line is only marginally less sensitive to DB75, using this dosing regimen in vivo compared with wild type. Thus, in vivo, the P2 deletion confers reduced sensitivity to drug, although parasites do remain sensitive to relatively low doses of DB75. ED_{50} values for the wild-type, Δ*tbat1* knockout line, and the DB75^R-CL1 line were determined at 0.11 ± 0.05, 0.57 ± 2.2, and 0.22 ± 0.16 mg/kg, respectively.

In the DB75^R-CL1 parasites, uptake of tritiated DB75 was reduced to levels similar to those seen in the Δ*tbat1*-null mutant (Fig. 2A). These biochemical data indicated that the P2 transporter was functionally lost in the DB75^R-CL1. Southern blots of DNA digested with restriction enzymes, from WT, Δ*tbat1* knockout, and DB75^R-CL1 also revealed an absence of the *tbat1* gene in the CL1 line (Fig. 2B). PCR analysis using a series of oligonucleotides spanning the *tbat1* open reading frame also revealed that this gene, although present in the wild-type line, could not be identified in the DB75^{R}CL1 line.

TABLE 2
In vivo effect of DB75 against trypanosomes in mice

Cell Line and Dose (mg/kg)	Mice Treated	Mice Surviving at 40 Days
	n	%
Wild-type		
0	6	0
0.03125	5	0
0.0625	5	40
0.125	10	30
0.25	10	80
0.5	5	100
1	5	100
Suramin	5	100
TbAT1 Knockout		
0	5	0
0.03125	5	0
0.0625	5	0
0.125	5	0
0.25	5	0
0.5	5	0
1	5	0
2.5	5	100
5	5	80
10	5	100
Suramin	5	100
DB75^{R}CL1		
0	6	0
0.03125	5	0
0.0625	5	0
0.125	10	50
0.25	10	50
0.5	5	80
1	5	100
Suramin	5	100

The *TbAT1* gene was not, at the time of this study, annotated in the *T. brucei* genome sequence. However, the *TbAT1* gene open reading frame was present in the raw sequence data in both the *T. brucei* and *T. brucei gambiense* projects. It was possible to construct a contig, extending through the 3′-untranscribed region of the TbAT1 gene, into a pair of GRESAG 4 sequences (Tb927.5.320 and Tb927.5.330) that are also found on chromosome V between 80,000 and 89,000 kilobases of the annotated chromosome V. It thus seemed that TbAT1 is positioned somewhere between 90,000 and 100,000 kilobases in the sequence as annotated at the time of publication (Berriman et al., 2005). We therefore designed a series of oligonucleotides spanning this region of the annotated chromosome (from 38,520 to 108,705). In PCR using DNA isolated from DB75^R-CL1 cells, products were not identified between positions 68,004 and 92,212, although they were identified outside of this region (Supplementary Table 1). However, all of the oligonucleotides produced products when used against wild-type DNA, whereas all but the *TbAT1*-specific primers produced products when using DNA from the Δ*tbat1* knockout line.

The *TbAT1* gene is thus present on chromosome V of wild-type *T. brucei*, but in the DB75^R-CL1, the gene has been deleted from the genome along with at least 20 kilobases of flanking sequence (Fig. 2C). Measurement of the precise size of the deletion awaits completion of new sequence information, and we are currently collaborating with the *T. brucei* sequencing centers to resolve this issue.

As further confirmation that the P2 transporter is responsible for uptake, we used fluorescence microscopy. DB75 also possesses fluorescent properties and it can be used to stain nuclear and kinetoplast DNA in trypanosomes. We showed that the Δ*tbat1* knockout line cells accumulated DB75 in the kinetoplast and nucleus at a slower rate than wild-type cells [wild-type cells revealing fluorescence within 1 min of administration and the Δ*tbat1* knockout line requiring >10 min to see similar fluorescence (Fig. 3)]. The DB75^R-CL1 clone also showed a marked increase in the time it took for fluorescence to appear in the kinetoplast and nucleus (no fluorescence even by 10 min, although shortly thereafter fluorescence was apparent). This may also relate to the fact that these cells are less sensitive to diamidines than Δ*tbat1* knockout cells again indicating the possibility of changes in addition to loss of P2 having been selected during exposure to DB75. Thus fluorescence microscopy using low numbers of cells, freshly derived from blood, can be used to distinguish P2-deficient and -proficient cells. Fluorescence does appear in P2-deficient cells, albeit much more slowly than in wild-type cells. This also confirms the presence of the secondary, slow route of uptake of the drug. A rapid test that might assist in predicting strains of reduced sensitivity to drug in the field would be of clear use in making clinical decisions as to alternative therapeutic regimens if necessary.

Discussion

DB289 is an amidoxime prodrug that systemically converts to the trypanocidal diamidine DB75. DB289 is under consideration as a clinically useful trypanocide. Uptake of diamidines into trypanosomes seems to be central to their activity, and diminished uptake is a common mechanism of resistance to this class of drug. Uptake of DB75 into trypanosomes was therefore investigated in this study. DB75 inhibits the uptake of adenosine via the P2 transporter, and uptake of DB75 is blocked by P2-substrates (adenosine, adenine, pentamidine, and diminazene). Loss of the P2 transporter in Δ*tbat1* knockout cells led to cells becoming resistant to both diminazene and DB75. The same cells retained their sensitivity to pentamidine, indicating that the HAPT1 and LAPT1 uptake routes play a less important role in the uptake of DB75 than they do in pentamidine accumulation. Nevertheless, the Δ*tbat1* knockout line does retain a slow route of drug uptake, and it will be of considerable interest to identify the exact nature of this pathway into the cell.

We have shown that Δ*tbat1* knockout cells are also less sensitive in mice than wild-type cells when exposed to DB75 each day for 4 days (2.5 mg/kg being required to cure all mice infected with Δ*tbat1* knockout trypanosomes, and with 0.5 mg/kg being sufficient to cure the wild-type line). The fact that the in vitro selected DB75-resistant CL1 line was only marginally less sensitive (100% cure with 1 mg/kg) is curious given that this line too has functionally deleted the *TbAT1* gene. One possible explanation for this significant difference in vivo could relate to a subtle decrease in virulence associated with the loss of other genes flanking *TbAT1* that comprise the large deletion on chromosome V that accompanied selection of resistance. On the other hand, it could relate to changes in uptake rates via the secondary transporter route. Although we could not measure a significant difference here with our ex vivo uptake assays, the relatively low specific activity of the tritiated DB75 and possible changes in expression of a transporter in expression rates in vivo versus ex vivo could play a role. It has been reported (Geiser et al., 2005) that expression of other purine transporters in *T. brucei* changes in the TbAT1 knockout line and that this influences susceptibility of these cells to other purine analogs. Likewise, changes in expression of the transporter (or transporters) that contribute to the slow uptake component of DB75 could alter as a response to loss of TbAT1. Use of higher specific activity DB75 might enable these alternative explanations to be explored in the future.

The DB75-resistant cells were shown to have lost P2-mediated drug uptake and to have deleted the *TbAT1* gene. In

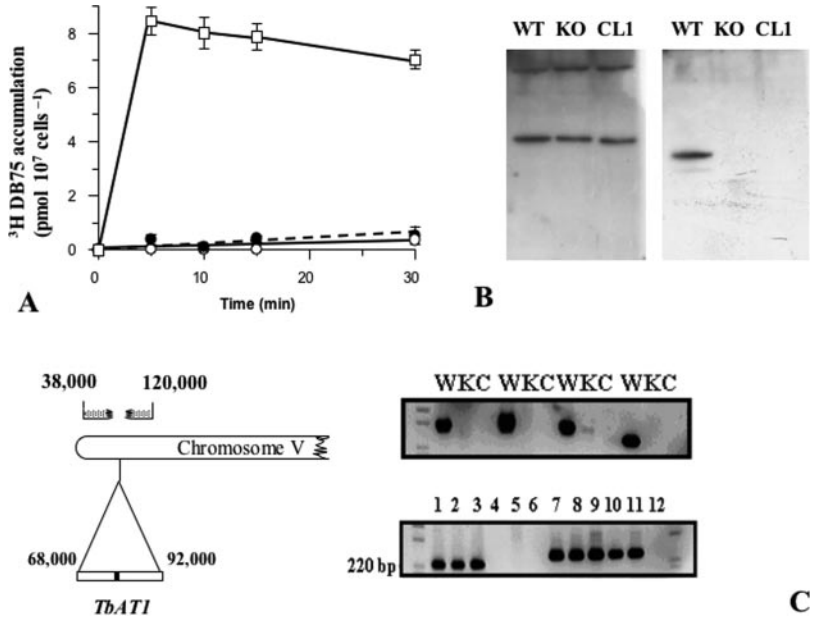

Fig. 2. Loss of TbAT1 and P2 mediated transport in *T. brucei* selected for DB75 resistance. A, the uptake of radiolabeled DB75 (1 μM) was measured as a function of time over 30 min into *T. brucei* 427 wild type (□) or into the Δ*tbat1* knockout line (●) or the DB75^R-CL1 line (○). B, Southern blot of genomic EcoR1 digested DNA probed with chromosome V specific vATPase probe (left) or *TbAT1* (right). WT, Wild-type; KO, Δ*tbat1* knockout. CL1 = DB75^R-CL1. C, position of the *TbAT1* gene on chromosome V and the deletion associated with its loss in cells selected for resistance to DB75. The approximate position of the gene was determined by making contiguous sequences bringing orphan sequences possessing TbAT1 to sit alongside two GRESAG4 genes found on chromosome V toward the leftmost telomere. The leftmost boxed inset shows amplification from PCR reactions performed to probe for the *T. brucei* P2 transporter gene (*TbAT1*) in DNA isolated from the three parasite lines all cultivated in vitro. Gel electrophoresis was then performed to determine whether the *TbAT1* primers produced a product in each of the lines: wild type (W), Δ*tbat1* knockout (K), or DB75-resistant (C). The left shows the primer *TbAT1* primer pairs (see Supplemental Table 1) were 541 F and 824 R; 714 F and 982 R; 805 F and 1067 R; and 39 F and 279 R. No PCR products were produced with DNA isolated from *TbAT1* KO and CL1 cells with any of the *TbAT1* primer sets. In the rightmost inset box, PCR was performed using primers designed to target specific regions on chromosome V (as judged on the annotated sequence at the *T. brucei* genome project) in DNA isolated from each of the three *T. brucei brucei* strains discussed in this study. Lane 1, WT DNA; lane 2, Δ*tbat1* DNA (parasites isolated from mouse); lane 3, Δ*tbat1* DNA (parasites isolated from in vitro culture); lane 4, DB75^R-CL1 DNA (parasites isolated from mouse);lane 5, DB75^R-CL1 DNA (parasites isolated from mouse); lane 6, no template control, all amplified with oligonucleotidess: 277 F and 503 R (see Supplementary Table 1) designed to positions 88,942 to 90,244 on the annotated chromosome V; lane 7, WT DNA; lane 8, TbAT1 KO DNA (parasites isolated from mouse); lane 9, Δ*tbat1* DNA (parasites isolated from in vitro culture); lane 10, DB75^R-CL1 DNA (parasites isolated from mouse); lane 11, DB75^R-CL1 DNA (parasites isolated from mouse), lane 12, no template control, all amplified with oligonucleotides: 6 F and 251 R (see Supplemental Table 1) designed to positions 92, 212–293, 427 on the annotated chromosome V. The results of this gel suggest that CL1 trypanosomes have a deletion within the 88,942- to 90,244-base pair region on chromosome V.

attempting to identify the precise location of the gene deletion, we noted an anomaly in the *T. brucei* genome database where the *TbAT1* gene has not been annotated into the genome. Nevertheless, we were able to identify the gene as part of chromosome V and PCR experiments, using oligonucleotide pairs spanning the part of chromosome V where *TbAT1* seems to localize, indicates that a large region (>20 kilobase) that encompasses the *TbAT1* gene has been deleted from the genome in this region in the DB75 resistant line. These combined data all strongly suggest that DB75 enters bloodstream forms of *T. brucei* principally via uptake mediated by the P2 transporter.

Treatment failure with melarsoprol is increasing in prevalence in some foci in Africa (Brun et al., 2001). Because melarsoprol resistance in the laboratory is usually associated with loss of P2, it is possible, but as yet unproven, that P2 defective trypanosomes are already in circulation in Africa. It is essential to learn more about these field isolates because the dissemination of P2 defective trypanosomes could affect the utility of DB289/DB75 in treating patients infected with such parasites.

The definition of "resistance" requires careful consideration within the context of this study. Both the Δ*tbat1* knockout line and in vitro selected DB75^{R}-CL1 line showed significant resistance to DB75 (11- and 21-fold reduction in DB75 sensitivity, respectively) using the 3-day Alamar blue protocol. However, it was not possible to cultivate these lines for sustained periods in concentrations of drug substantially below the Alamar blue test determined IC_{50} value. It thus seems that TbAT1-defective cells retain sensitivity to drug if exposed for sufficiently long periods. The slow secondary route of drug uptake may therefore be sufficient to allow drug to accumulate to toxic levels if cells are exposed to DB75 long enough, although this has not been shown unequivocally in experiments here. In fact, a recent publication (Mathis et al., 2006) looking into activities of DB75 against trypanosomes reported a substantially higher activity in vitro than we measure. It is notable that in that study, the authors seeded trypanosomes (the same 427 strain as we used) at a far lower density than we used (2×10^3 versus 2.5×10^5 per ml), and they employed a different medium. A number of factors (including cell density, culture medium, time of incubation) seem to all contribute to values measured using in vitro assays of sensitivity and a systematic study into how different variables affect activity is warranted. In mice, whereas the Δ*tbat1* knockout line was clearly less sensitive to DB75, the in vitro selected resistant line, which is also a Δ*tbat1* knockout, was only barely less sensitive than wild-type. The nature of the mutation underlying the P2 defect also seems to influence overall sensitivity, perhaps because of variability in the activity of the secondary route. The fact that "resistant" cells are actually sensitive to drug if exposed for longer time periods could have important consequences for use of drug against TbAT1-deficient cells. For instance, it is possible that lengthened drug administration regimens, or multiple dosing on each day to sustain trypanotoxic levels long enough to

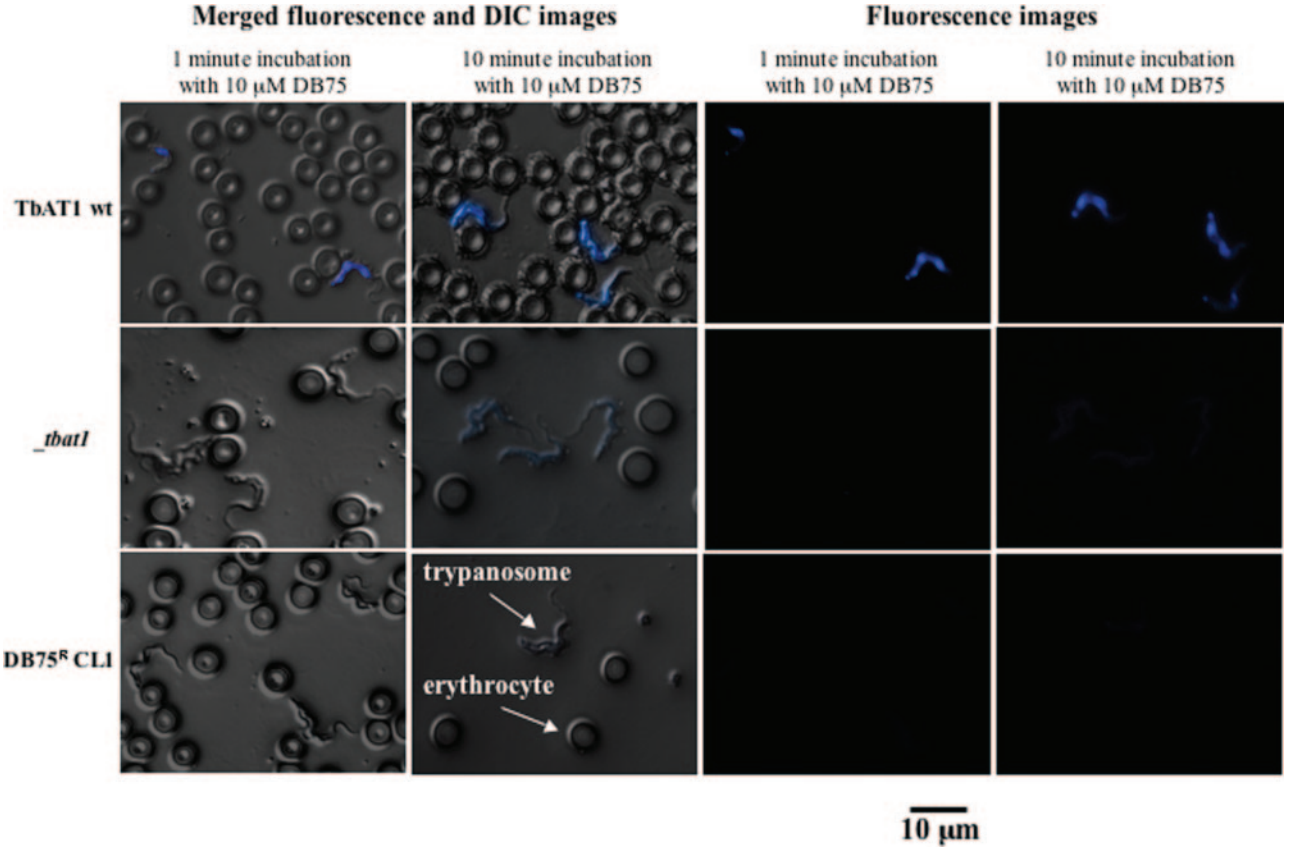

Fig. 3. Diagnosis of DB75 resistance using a fluorescence test. Parasites of each of the three lines were taken from mice in heparinized blood. Cells were exposed to 10 μM DB75 and viewed by fluorescence microscopy for 10 min to determine the rate at which DB75 fluorescence associated with concentration in DNA containing structures appeared. The merged DIC with fluorescence pictures is shown on the left side after 1- or 10-min exposure to DB75, respectively. The same pictures, but showing only the fluorescence image, are shown on the right.

exert activity, would render the drug useful against TbAT1-defective parasites. It is clear that further work on the pharmacokinetics of the drug in relation to TbAT1-defective parasites is warranted. Moreover, given that loss of TbAT1 can lead to parasites losing sensitivity to DB75, it will be of importance to ascertain P2 transporter status in field populations. The fluorescence test that we show here reliably distinguishes P2-proficient and -defective parasites will be of use in this regard.

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Address correspondence to: Dr. Michael P. Barrett, Division of Infection and Immunity, Institute of Biomedical and Life Sciences, The Glasgow Biomedical Research Centre, University of Glasgow, Glasgow G12 8QQ, United Kingdom. E-mail: m.barrett@bio.gla.ac.uk.