Loss of the High Affinity Pentamidine Transporter is responsible for high levels of cross-resistance between arsenical and diamidine drugs in African trypanosomes

Daniel J. Bridges, Matthew K. Gould, Barbara Nerima, Pascal Mäser, Richard J. S. Burchmore and Harry P. de Koning

Institute of Biomedical and Life Sciences, Division of Infection and Immunity, University of Glasgow, Glasgow, UK (DJB, MKG, RJSB and HPdK), and Institute of Cell Biology, University of Bern, Switzerland (BN and PM).
Running title: Diamidine-Arsenical cross-resistance in Trypanosoma brucei

Corresponding author:

Dr Harry P. de Koning

Institute of Biomedical and Life Sciences,

Glasgow Biomedical Research Centre,

University of Glasgow,

Glasgow G12 8TA

United Kingdom

E-mail H.de-Koning@bio.gla.ac.uk

Telephone and fax (+44) 141 330 3753

Number of pages: 38

Number of tables: 1

Number of figures: 11

Number of references: 42

Number of words in Abstract: 250

Number of words in Introduction: 633

Number of words in Discussion: 1557

Abbreviations.

ABC transporter, ATP-binding cassette transporter; DA, diminazene aceturate; DAPI, 4',6-Diamidino-2-phenylindole dihydrochloride; HAPT1, High Affinity Pentamidine Transporter 1; LAPT1, Low Affinity Pentamidine Transporter 1; MRP, multidrug
resistance-associated protein; PBS, phosphate-buffered saline; TbAT1, Trypanosoma brucei Adenosine Transporter 1;
ABSTRACT

Treatment of many infectious diseases is under threat from drug resistance. Understanding the mechanisms of resistance is as high a priority as the development of new drugs. We have investigated the basis for cross-resistance between the diamidine and melaminophenyl arsenical classes of drugs in African trypanosomes. We induced high levels of pentamidine resistance in a line without the \textit{tbat1} gene that encodes the P2 transporter previously implicated in drug uptake. We isolated independent clones that displayed very considerable cross-resistance with melarsen oxide but not phenylarsine oxide, and reduced uptake of \(^{3}\text{H}\)-pentamidine. Specifically, the High Affinity Pentamidine Transport (HAPT1) activity was absent in the pentamidine-adapted lines, whereas the Low Affinity Pentamidine Transport (LAPT1) activity was unchanged. The parental \textit{tbat1} \textsuperscript{-/-} line was sensitive to lysis by melarsen oxide and this process was inhibited by low concentrations of pentamidine, indicating the involvement of HAPT1. This pentamidine-inhibitable lysis was absent in the adapted line KO-B48. Similarly, uptake of the fluorescent diamidine DAPI was much delayed in live KO-B48 cells and insensitive to competition with up to 10 \(\mu\text{M}\) pentamidine. No overexpression of the \textit{T. b. brucei} ABC-transporter TbMRPA could be detected in KO-B48. We also show that a laboratory line of \textit{T. b. gambiense}, adapted to high levels of resistance for the melaminophenyl arsenical drug Cymelarsan, had similarly lost TbAT1 and HAPT1 activity while retaining LAPT1 activity. It thus appears that selection for resistance to either pentamidine or arsenical drugs can result in a similar phenotype of reduced drug accumulation, explaining the occurrence of cross-resistance.
Introduction

*Trypanosoma brucei* spp. are protozoan parasites that cause human African trypanosomiasis (HAT, sleeping sickness) and the corresponding veterinary condition in livestock. Treatment of both the human and livestock diseases depend on a very small set of mostly very old drugs. The first-line treatment for the late stage of both West African and East African HAT is melarsoprol, an organo-arsenic compound of the melaminophenyl arsenical class, introduced in 1949 (Jannin and Cattand, 2004). A similar but water-soluble melaminophenyl arsenical, melarsomine (Cymelarsan), is increasingly used for animal trypanosomiasis. Early stage West African sleeping sickness is routinely treated with the diamidine drug pentamidine, introduced in 1937 (Jannin and Cattand, 2004). The corresponding, widely used veterinary diamidine is diminazene aceturate (DA, Berenil). The only new trypanocide to be developed in recent decades, DB75, is also a diamidine and currently in clinical trials as an orally available prodrug.

It has been known for decades that cross-resistance between melaminophenyl arsenicals and diamidine drugs sometimes occurs (Fulton and Grant, 1955; Williamson and Rollo, 1959), but cross-resistance patterns can be unpredictable (Williamson, 1970). Kaminsky and Mäser (2000) distinguished six distinct resistance profiles in *T. b. brucei* laboratory strains and field isolates, presumably an indication that multiple factors determine (the level of) resistance to the various drugs. They also noted that some resistance profiles appear to be associated with either field or laboratory-induced resistance.

Some progress has been made in understanding drug resistance mechanisms in trypanosomes. The important role of the *T. brucei* AT1/P2 purine transporter in the accumulation of melaminophenyl arsenical compounds and diamidines is now well
understood (Carter and Fairlamb, 1993; Mäser et al., 1999; De Koning, 2001a; Delespaux and De Koning, 2006). The loss of TbAT1/P2 alone was shown to be sufficient for high levels of DA resistance in *T. b. brucei* and *T. evansi* (De Koning et al., 2004; Witola et al., 2004). However, the deletion of the *tbat1* gene produced only minor loss of sensitivity to pentamidine and melaminophenyl arsenicals (Matovu et al., 2003). Thus, the action of some diamidines relies (almost) exclusively on TbAT1/P2 for access to intracellular targets while other diamidines, as well as melaminophenyl arsenicals, have at least one additional route of entry.

We have previously shown that adenosine-insensitive uptake of [³H]-pentamidine in *T. b. brucei* is mediated by two discrete transport activities: a Low Affinity Pentamidine Transporter (LAPT1; (De Koning and Jarvis, 2001)) and a High Affinity Pentamidine Transporter (HAPT1; (De Koning, 2001b)). While this appears to explain why clinical pentamidine resistance has not established itself, despite the many decades of intensive use (reviewed by (Bray et al., 2003)), it was not clear whether high levels of pentamidine resistance might ensue with the consecutive loss of TbAT1/P2 and either HAPT1 or LAPT1. Nor is it yet clear why the *tbat1⁻/⁻* line did not display an appreciable level of resistance to melaminophenyl arsenical drugs (Matovu et al., 2003). It has been argued that higher levels of arsenical resistance in trypanosomes could be the result of the (over)-expression of an ABC-type transporter of the multidrug resistance type, TbMRPA. Certainly, overexpression of this transporter in *T. b. brucei* induces 10-fold resistance to melarsoprol *in vitro* (Shahi et al., 2002). Arsenical resistance attributed to loss of TbAT1/P2 and overexpression of TbMRPA were shown to be additive (Lüscher et al., 2006). However, TbMRPA overexpression did not lead to arsenical resistance in vivo, nor could TbMRPA
overexpression be demonstrated in melarsoprol-resistant trypanosome isolates from sleeping sickness patients (Alibu et al., 2006).

In the current manuscript we address the outstanding questions concerning diamidine and arsenical resistance using *Trypanosoma brucei* lines adapted to high levels of pentamidine and cross-resistant to melaminophenyl arsenicals. We now provide a model for the biochemical basis of cross-resistance between melaminophenyl arsenicals and the diamidines. The model predicts that HAPT1, together with TbAT1, may be a key determinant for arsenical resistance in African trypanosomes, providing a hypothesis that should now be validated on clinical isolates from melarsoprol-refractory patients.
Materials and Methods

Trypanosomes and cultures. Growth of bloodstream trypanosomes in culture was performed using HMI-9 medium (BioSera Ltd.) supplemented with 2 mM β-mercaptoethanol and 10% fetal bovine serum at 37 °C, in a 5% CO₂ atmosphere. For transport assays, trypanosomes were grown in adult female Wistar rats and isolated from infected blood as described (De Koning and Jarvis, 1997). The cloned Trypanosoma brucei gambiense type 2 stock STIB 386 and the derived Cymelarsan-resistant clone 386MelCyR (hereafter called 386Ms and 386Mr) were a kind gift of Professor C. M. R. Turner, University of Glasgow. The adaptation of 386Ms to high levels of Cymelarsan (melarsomine) has been described elsewhere (Scott et al., 1996). The T. b. brucei clone s427 (MiTat 1.2/221) was used to derive the Tbat⁻ line (Matovu et al., 2003) and the pentamidine-adapted clonal line WT-D24. Tbat⁺ was also adapted to higher levels of pentamidine resistance, generating the clonal lines KO-B6, KO-D6, KO-B48 and KO-D48. Clonal populations were generated by limiting dilution. Cell densities were assessed using a haemocytometer and phase-contrast microscope. The New York line of T. b. brucei (Wirtz et al., 1999) and the derived MRPA overexpressing line NY-mrpa have been described (Lüscher et al., 2006). Overexpression was induced (NY-mrpa⁺) or repressed (NY-mrpa⁻) by the inclusion or omission, respectively, of 1 μg/ml tetracycline in the culture medium.

Transport assays. Transport assays were performed exactly as described previously (Wallace et al., 2002). Briefly, trypanosomes were isolated from blood taken from infected Wistar rats by DEAE-52 (Whatman, Maidstone, United Kingdom) anion-exchange chromatography, washed into assay buffer and
resuspended at ~2x10^8 cells/ml at room temperature. In a minority of cases, trypanosomes for transport assays were cultured in complete HMI-9 medium. 100 µl aliquots of bloodstream trypanosomes in assay buffer were mixed with an equal volume of buffer containing radiolabel and sometimes inhibitor, at twice the final concentration, initiating transport. Transport was terminated by the addition of 1 ml ice-cold buffer containing unlabelled permeant at a saturating concentration (2 mM for pentamidine) followed by rapid centrifugation through an oil layer, separating cells from radiolabel not internalized. Transport values are expressed as pmol(10^7 cells^{-1})s^{-1}.

**Drug sensitivity assays.** Drug sensitivity was determined using the dye resazurin (Alamar blue) exactly as described (Wallace et al., 2002) in a protocol adapted from (Räz et al., 1997). Briefly, drugs were serially diluted in 100 µl of complete HMI-9 media across a 96-well micro-titre plate. Unless limited by solubility the top drug concentration used was 1 mM. Cultures of bloodstream form trypanosomes were grown to a maximum density of 2 × 10^6 cells/ml and diluted to 2 × 10^5 cells/ml with complete HMI-9 medium, of which 100 µl was added to all wells. Plates were then incubated for 48 h at 37°C in a 5% CO₂ atmosphere, before the addition of 20 µl of 5 mM resazurin (Sigma) solution in PBS (pH 7.4). Plates were incubated for an additional 24 hours at 37°C, before fluorescence was measured using a LS 55 Luminescence Spectrometer (Perkin Elmer; λ_{ex} = 530 nm, λ_{em} = 590 nm).

**Fluorescence microscopy.** *T. brucei* were pelleted (600 × g, 10 min at room temperature) before being re-suspended in fresh HMI-9 media containing 10 µM stilbamidine, DAPI, DB75 or Hoechst 33342 solution at room temperature, and
assessed by fluorescence microscopy. Glass slides of the culture were prepared and examined using differential interference contrast (DIC) and fluorescence microscopy ($\lambda_{ex}=365\text{nm}, \lambda_{em}=445\text{nm}$) on an Axioplan 2 imaging microscope (Carl Zeiss, Germany) using Volocity v 3.7 software (Improvision, Coventry). Where necessary, parasites were fixed by incubating in PBS containing 2.5% glutaraldehyde, for 20 min. Cells were then washed in 0.05 M glycine in PBS, before being mounted on slides.

**Lysis assays.** The lysis assays were performed essentially as described by Fairlamb et al. (1992), using bloodstream trypanosomes of tbat1$^{-/-}$ and KO-B48 grown *in vitro*. They were washed and resuspended in fresh HMI-9 medium with 10% fetal calf serum at $\sim2\times10^7$ cells/ml, which was transferred to 1 ml cuvettes. Absorption was monitored, at 750 nm, at 30s intervals. Melarsen oxide or phenylarsine oxide were added after 15 min to allow the recording of a short baseline. Up to 16 cultures were monitored simultaneously, using two HP8453 spectrophotometers (Hewlett-Packard), in the presence or absence of low concentrations of pentamidine which by themselves did not affect cell viability or motility over the course of the experiment.

**Preparation of plasma membrane protein-enriched fractions.** Tbat1$^{-/-}$ and KO-B48 plasma membrane-enriched samples were generated by a modification of the procedure described by (Voorheis *et al.*, 1979). Briefly, BSF trypanosomes were cultured *in vivo* in adult female Wistar rats infected by intraperitoneal injection. Blood was collected at peak parasitaemia under terminal anaesthesia and parasites isolated as for transport assays. Cells were then osmotically stressed by addition of water (at 4$^\circ$C), and swelling monitored by phase-contrast microscopy until a spherical morphology had been achieved. Cells were then homogenised in an AO Cell
Disruptor (Stansted Fluid Power, Stansted, UK), in the presence of protease inhibitors. The homogenate was then returned to a normal osmotic potential through the addition of 3M KCl, to a final concentration of 140 μM, before pelleting the cells. Cells were then treated with 240 units of DNAse in TES minus buffer (20mM TES, 150 mM KCl, 5m M MgCl₂, 1 mM 2-mercaptoethanol [pH 7.4]), for 5 min at 20°C. The reaction was terminated by the addition of 5 volumes of TES buffer (20 mM TES, 150 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol [pH 7.4]). Cells were pelleted and then re-suspended in 40% sucrose before being layered on a linear 40-60% sucrose gradient (in TES Buffer). Samples were then centrifuged for 3 h at 70,000g on a SW28 rotor (Beckman Coulter). The most prominent dense white band, corresponding to the plasma membrane fraction, was isolated and washed twice in TES buffer to remove sucrose, before being aliquoted and stored in TES buffer at -80°C. All procedures were performed at 4°C unless otherwise stated.

**Western blotting.** Samples for Western blotting were plasma membrane preparations (described in previous section) or whole-cell protein extracts prepared from 2 × 10⁷ cells, washed in PBS and flash-frozen in dry ice/ethanol. Samples solubilised in standard Laemmli buffer (60°C for 10 min) were loaded onto a 10% SDS PAGE gel and run until the bromophenol blue dye front reached the end of the gel. Proteins were then transferred onto a nitrocellulose membrane (Amersham Hyband ECL) at 4°C in MeOH Tris-Glycine buffer. Blots were incubated in blocking solution (TBS-Tween containing 5% milk powder) for 1 hour at room temperature (RT). Primary antibodies raised to MRPA, kindly provided by Christine Clayton, University of Heidelberg, were diluted 1:500 in blocking solution (TBS-Tween containing 5% milk powder) and incubated with the blot for 1.5 hours at RT.
Unbound / excess antibodies were removed by washing in TBS-Tween (2 × 10 min) and then TBS (10 min). Secondary peroxidase-conjugated anti-mouse immunoglobulin antibodies (DAKO-Immunoglobulins, Denmark) were then incubated with the blot (diluted 1:1000) in blocking solution at RT for 1 hour. Unbound secondary antibodies were removed by washing (as with primary antibodies). Bound antibodies were detected using the ECL Plus western blotting detection System (Amersham Biosciences, UK) according to the manufacturer’s instruction. For normalization, the blot was stripped and reprobed using a polyclonal antiserum against paraflagellar rod protein (PFR; diluted 1:1000) as described (Schlaeppi et al., 1989) and a secondary peroxidase-conjugated anti-rat immunoglobulin antibody (diluted 1:10000) (DAKO-Immunoglobulins, Denmark).

**Infectivity of pentamidine-adapted *T. b. brucei* lines in mice.** Groups of 3 – 5 ICR mice (Harlan UK, Ltd, Bicester) were infected with 10⁴ trypanosomes in 200 µl full HMI9/FCS culture medium by i.p. injection. In some cases the mice had been immunosuppressed by treatment with cyclophosphamide (200 mg/kg cyclophosphamide in water, injected i.p.) 24 h prior to infection. Parasitaemia was estimated daily by the rapid “matching” method (Herbert and Lumsden, 1976) from examinations of wet blood films on microscope slides, collected from tail pricks, using a phase-contrast microscope. Mice reaching a parasitaemia of antilog 8.4 (2.5 x 10⁸ cells/ml blood) or over were euthanized by inhalation of a rising concentration of CO₂.
RESULTS

**Induction of high-level pentamidine resistance in *T. b. brucei*.**

To maximize the likelihood for the successful generation of drug resistant lines, we applied selection pressure to four separate cultures from each of the two parental strains, s427 (WT) and the isogenic clone *tbat1*−/−, of which the latter has been derived from the former by deletion of both copies of the *TbAT1* gene (Matovu et al., 2003). From these 8 cultures initially established, three (WT-D, KO-B and KO-D) went on to develop a stable drug resistance phenotype after several months of selection in increasing pentamidine concentrations. Two clones were derived from each of these lines, one that was able to tolerate intermediate (clones WT-D3, KO-B6 and KO-D6) and one that tolerated high levels of pentamidine (clones WT-D24, KO-B48 and KO-D48), as compared to the parental strains (Fig. 1A). The strains displaying the highest levels of resistance were able to survive in pentamidine concentrations eighty-fold higher than those tolerated by their parental strain. It was also observed that resistance acquisition was non-linear over time, and seemed to correlate best to an exponential model, with correlation coefficients between 0.87 and 0.93 (plots not shown). The wild-type strain, in particular, took many passages to acquire any pentamidine resistance, but subsequently adapted to very significant levels of resistance in two discrete steps.

During each round of selection, it was observed that the initial acquisition of resistance in the presence of drug was associated with a reduced growth phenotype (Fig. 1B), both in terms of doubling time and maximum cell density. For all the lines eventually selected, this was reversed to a parental growth phenotype by maintaining the same selection pressure for an additional period of time (Fig. 1C). Once the
resistance phenotype was well established, removal of pentamidine from the media had no effect on growth and the resistance phenotype was stable for at least 3 months in the absence of drug pressure.

**Cross-resistance profile of KO-B48 and KO-D48.**

We have previously reported the cross-resistance phenotype of the *tbat1*-/ strain (Matovu et al., 2003), which displayed high levels of resistance to diminazene but only minor loss of sensitivity to pentamidine and the melaminophenyl arsenical drugs melarsoprol, melarsen oxide and Cymelarsan (melarsomine). Using the same protocol based on the reduction of the dye Alamar blue (resazurin), we have repeated these experiments with WT, *tbat1*-/KO-B48 and KO-D48 in parallel. The results are presented as a series of bar graphs in Fig. 2. The phenotypes of KO-B48 and KO-D48 were very similar. Compared to the WT strain, a large increase in pentamidine resistance was observed, reaching an average 130 and 82-fold resistance in KO-B48 and KO-D48, respectively (n= 8; Fig. 3). Resistance to melarsen oxide was also markedly increased in KO-B48 and KO-D48 (15.4 and 10.7-fold, respectively, relative to WT; Fig. 2B). Sensitivity to propamidine and stilbamidine, already reduced in the KO strain, was modestly decreased further in KO-B48 and KO-D48 (Fig. 2C,D), whereas resistance to diminazene aceturate (Berenil) was not further increased in the pentamidine resistant lines (Fig. 2E). Sensitivity to phenylarsine oxide, which freely diffuses across membranes, was identical in all the lines (Fig. 2E), showing that the resistance phenotype was most likely associated with changes in drug uptake. A table with exact sensitivities of all four trypanosome lines to all 6 drugs is included as on-line only supplementary data.
Pentamidine transport in pentamidine resistant trypanosome lines.

Transport of 0.5 µM $[^3]$H-pentamidine was drastically reduced in both KO-B48 and KO-D48, compared to the parental $tbat1^{-/-}$ line (Fig. 4A,B), by 74 and 68 %, respectively. To exclude the possibility that culture conditions had somehow played a role in the reduced uptake rates, the experiment was repeated with $tbat1^{-/-}$ and KO-B48 cells grown in Wistar rats (Fig. 4C). The result clearly demonstrates that pentamidine transport in the adapted lines is severely impaired, yet remains saturable (Figs. 4C, 5). We also confirmed that transport in KO-B48 was not generally impaired, using $[^3]$H-2-deoxyglucose. Uptake of $[^3]$H-2-deoxyglucose by KO and KO-B48 cells was determined in three independent experiments, each performed in triplicate, and found to be linear over 30 minutes, completely saturated by 10 mM 2-deoxyglucose, and not significantly different between the two trypanosome lines (see on-line only material, Fig. 1).

Pentamidine transport in the $tbat1^{-/-}$ strain is mediated by two transporters, HAPT1 and LAPT1 (Matovu et al., 2003). To determine if one or both of these transporters was affected in the highly resistant line KO-B48, a series of transport assays were performed at 15 nM $[^3]$H-pentamidine. This very low concentration of radiolabel is necessary in order not to saturate HAPT1 and obtain a biphasic inhibition curve showing both the high affinity (but low capacity) HAPT1 activity and the low affinity (but high capacity) LAPT1 activity in the WT s427 and $tbat1^{-/-}$ strains (De Koning, 2001b; Matovu et al., 2003). The presence of a high affinity pentamidine transport activity could not be confirmed in KO-B48: it was either absent altogether or its transport capacity was very significantly reduced (Fig. 5A), though pentamidine transport was clearly biphasic in the control WT strain (Fig. 5B). In contrast, the low affinity transporter LAPT1 appeared completely unchanged compared to parental
strains (Fig. 5A,C): $K_m$ and $V_{\text{max}}$ values were identical to those previously reported for the parental line s427 (Table 1).

**Arsenical-induced lysis in tbat1−/− is mediated by HAPT1 and absent in KO-B48.**

Lysis of trypanosomes, induced by arsenical drugs, can be monitored spectroscopically at 750 nm (Fairlamb et al., 1992) and was used to demonstrate that melaminophenyl arsenicals enter *T. b. brucei* principally through P2 (Carter and Fairlamb, 1993). We have previously used this technique to show that in *tbat1−/−* cells, a secondary, slower phase of melarsen oxide-induced lysis can be discerned and displays the pharmacological profile of HAPT1 (Matovu et al., 2003). The results depicted in Fig. 6A confirm this observation. While melaminophenyl arsenicals act more slowly on *tbat1−/−* than on WT cells (Matovu et al., 2003), 10 µM Melarsen oxide induced cell lysis in *tbat1−/−* cells in approximately 50 min (trace a), but co-administration of as little as 1 µM pentamidine with 10 µM melarsen oxide substantially delayed cell lysis (trace b). In contrast, the same concentration of pentamidine had no influence on lysis induced by phenylarsine oxide (traces c, d), which rapidly diffuses across membranes (Carter and Fairlamb, 1993).

In KO-B48 cells, melarsen oxide-induced lysis is already much delayed compared to *tbat1−/−* cells (trace a), and 1 µM pentamidine has no further effect (trace b), demonstrating the absence of HAPT1 activity. Again, co-incubation with 1 µM pentamidine had no effect on lysis induced by phenylarsine oxide (traces c, d), and the timing of phenylarsine-induced lysis was identical in both strains.

**Assessment of pentamidine transporters using fluorescent microscopy.**
The rate of entry of fluorescent diamidines, monitored by FACS or fluorescence microscopy, can be used as a probe to investigate changes in membrane permeability and cross-resistance patterns (Frommel and Balber, 1987; Stewart et al., 2005). Like Frommel and Balber (1987) we used 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) as a fluorescent substitute for pentamidine. This was possible given the fact that DAPI displays considerable affinity for all three pentamidine transporters. $K_i$ values are $0.67 \pm 0.12 \mu M$ (TbAT1/P2, $n = 3$), $26 \pm 6 \mu M$ (HAPT1, $n = 3$) and $20 \pm 6 \mu M$ (LAPT1, $n = 5$) (data not shown). Despite the highest affinity being for TbAT1/P2, DAPI transport was not primarily mediated by this transporter. Fig. 7 shows that the rate of development of fluorescence was not clearly different in live long-slender WT s427 or $tbat1^{-/-}$ cells. By 5 minutes, fluorescence was clearly observed and by 10 minutes staining of both nucleus and kinetoplast was very intensive. In contrast, KO-B48 cells showed only faint fluorescence in both organelles after 20 minutes. Even after 60 minutes, fluorescence was much less than that observed in the other cell lines at 10 minutes (Fig. 7). Care was taken to exclude any dead cells from the observations, as they accumulated DAPI very rapidly, presumably as a result of loss of membrane integrity.

It thus appears that the rate of DAPI uptake is much reduced in KO-B48 cells, relative to the parental line $tbat1^{-/-}$. To assess whether this is caused by the loss of HAPT1 or LAPT1, the development of fluorescence was again monitored in live KO-B48 cells incubated with DAPI alone, or in the presence of either 10 µM pentamidine (saturates HAPT1) or 1 mM pentamidine (saturates both transporters). The results in Fig. 8A show accumulation in live cells at 25, 45 and 60 minutes of incubation with the fluorophore. It is clear that the lower concentration of pentamidine does not delay the rate of DAPI accumulation compared to the no-drug control, whereas 1 mM
pentamidine practically abolishes DAPI fluorescence over 60 minutes. The P-glycoprotein inhibitor verapamil (50 µM) did not affect the accumulation rate of DAPI (Fig. 8A). Using live, motile trypanosomes does not allow the overlay of DIC and fluorescent images and causes imperfect focus on the fluorescence micrographs. At the termination of the experiment, at 60 minutes, cells were therefore fixed with glutaraldehyde. The higher quality DIC, fluorescence and overlay images shown in Fig. 8B confirm the conclusions of the timed/real time experiment depicted in Fig. 8A.

The drug efflux transporter TbMRPA is not overexpressed in KO B-48.

To verify whether the cross-resistance phenotype of KO B-48 can be partly explained through the elevated expression of TbMRPA, an ABC transporter of the multi-drug resistance (mdr) family, plasma membrane-enriched extracts of that1+/− and KO B-48 were run on 1D SDS PAGE gels and transferred to nitrocellulose for Western blotting with anti-TbMRPA antiserum. Protein preparations from tetracycline-induced and non-induced NY-mrpa cells and parental NY trypanosomes were analyzed in parallel as controls. MRPA was clearly present in NY-mrpa+ cells, but could not be detected in any of the other lines (Fig. 9A). In contrast, the presence of paraflagellar rod protein (PFR) could easily be demonstrated in each of the preparations (Fig. 9B).

Infectivity of the KO B-48 and KO D-48 lines.

During in vivo culturing it was observed that infectivity of both KO B-48 and KO D-48 lines was much reduced compared to WT and that1+/− lines. To assess this systematically, groups of 5 mice were infected with 10⁴ T. b. brucei from that1+/− and
each of the derived pentamidine resistant lines. In both KO-B6 and KO-B48 groups, all mice developed detectable parasitaemia within 3 days, which, however, rapidly disappeared. Two mice of the KO-B48 group suffered 1 or 3 subsequent relapses, but otherwise no parasitaemia was detectable in thick blood smears taken daily. No parasitaemia was observed at all in the KO-D6 group and only one mouse in the KO-D48 group developed a brief, low parasitaemia on day 5. In contrast, the \textit{tbat1}/ line appeared far more virulent. One group of mice was infected with \textit{tbat1}/ cells from a long-term culture. All mice rapidly developed substantial parasitaemia and two mice had to be euthanized. In the last group, infected with the same number of \textit{tbat1}/ cells from stabilates kept in liquid nitrogen and only briefly brought back into culture, all mice developed massive parasitaemia and were euthanized (4 on day 4, one on day 13). To investigate whether the lack of infectivity was innate to the adapted trypanosome lines or dependent on the immune system, groups of immunocompromised mice were infected. As shown in Fig. 10A, all mice infected with \textit{tbat1}/ cells developed similar parasitaemia and were euthanized on day 7. In the KO-B48 and KO-D48 groups (Fig 10B,C), all mice similarly established substantial parasitaemia but in all but one mouse in the KO-B48 group, these were transient and the mice apparently self-cured.

An arsenical-resistant \textit{T. b. gambiense} clone has lost \textit{TbAT1} and \textit{HAPT1}.

To assess whether loss of \textit{HAPT1} activity is peculiar to selection with pentamidine, we also assessed $[^3]$H-pentamidine transport in a \textit{T. b. gambiense} type 2 clone, 386Mr, adapted for high levels of Cymelarsan resistance \textit{in vivo} (Scott \textit{et al.}, 1996). The $[^3]$H-pentamidine transport profile of the parental line STIB 386Ms, conformed to the same three-transporter model as the earlier characterized \textit{T. b. brucei
s427 (De Koning, 2001b; Bray et al., 2003). Using fluorescence microscopy similar to the experiments here it has previously been shown that 386Mr accumulates the diamidine DB99 much more slowly than the parental line (Stewart et al., 2005).

Fig. 11A shows that uptake of 0.015 µM [3H]-pentamidine was inhibited partly by adenosine, with a Ki value of 1.8 µM, consistent with inhibition of TbAT1/P2. A second component, assessed in the presence of 1 mM adenosine, was revealed by a dose dependent inhibition with propamidine, which inhibits HAPT1 but not LAPT1 (De Koning, 2001b), with a K_i value of 9.0 ± 3.0 µM (n=3). The third transport activity was evident as inhibited by high concentrations of unlabelled pentamidine but not by propamidine or adenosine, consistent with LAPT1 activity. In contrast to 386Ms, transport of [3H]-pentamidine in 386Mr was not sensitive to either adenosine or propamidine (Fig. 11B), showing the absence of TbAT1 and HAPT1 activity in these cells.

The K_m and V_max values of HAPT1 and LAPT1 activities in the T. b. gambiense lines are listed in Table 1 and are very similar to those determined in WT T. b. brucei. Table 1 also shows that the V_max of LAPT is not changed in the resistant T. b. gambiense line, relative to its parental line.
Discussion

In principle, pentamidine resistance in African trypanosomes could be associated with changes in the intracellular drug target, with reduced uptake of the drug or with active extrusion from the cell by an ABC-type efflux pump.

It has been shown that, in wild-type *T. b. brucei*, pentamidine uptake is very rapid and efficient, amounting to a cytosolic concentration corresponding to at least 1 mM, if the drug were to remain unbound (Damper and Patton, 1976). Such an intracellular accumulation of the drug is liable to interfere with multiple vital processes and it has long been hypothesized that resistance would most likely be associated with a reduction in cellular levels (Wang, 1995). Indeed, various effects of pentamidine on cellular processes have been demonstrated by different authors (Wang, 1995). Pentamidine resistance in *T. b. brucei* based on acquired mutations in any one target, while impossible to rule out, would thus seem unlikely.

Another potential route to drug resistance is the expression of ABC-transporters capable of actively removing the toxic compound from the cell. Strong evidence couples this mechanism to drug resistance in some protozoa including *Plasmodium* and *Leishmania* spp. and *Entamoeba histolytica* (Légaré et al., 2001; Lopez-Camarillo et al., 2003). In *T. brucei*, three ABC transporter genes have been identified (Mäser and Kaminsky, 1998). However, in studies with the P-glycoprotein inhibitor verapamil, no evidence could be found for the involvement of such transporters in multidrug resistance in *T. b. brucei*, either *in vitro* or *in vivo* (Kaminsky and Zweygarth, 1991). Experimental overexpression of the *T. b. brucei* ABC-transporter TbMRPA led to substantial melarsoprol resistance *in vitro* (Shahi et al., 2002) but not...
in vivo and TbMRPA overexpression was not observed in melarsoprol-resistant clinical isolates from a number of sleeping sickness patients (Alibu et al., 2006).

Thus, while resistance based on target modifications or active efflux cannot be excluded, the available information is consistent with changes to drug entry being the cause of arsenical and diamidine resistance in African trypanosomes. Our findings, using pentamidine-adapted laboratory strains, are strongly in agreement with this hypothesis. We identify HAPT1 as potentially a key drug resistance determinant, and for the first time provide a biochemical model for high-level arsenical – diamidine cross-resistance in African trypanosomes.

High-level pentamidine resistance is associated with loss of HAPT1 but not of LAPT1 activity.

Several independent lines of evidence suggested that the pentamidine-adapted lines KO-B48 and KO-D48 had lost HAPT1 rather than LAPT1 activity. As the adaptation started with the tbbat1^- clonal line, P2 transport activity was also not present. We have previously shown that propamidine is an inhibitor of high affinity pentamidine transport in T. brucei, but not of the low affinity transport component (De Koning and Jarvis, 2001) and the increase in propamidine resistance is certainly consistent with loss of HAPT1 activity. Furthermore, there was no increased resistance to diminazene aceturate, which is transported only by TbAT1/P2 (De Koning et al., 2004), or to phenylarsine oxide, which diffuses across membranes.

The resistance profiles and [3H]-pentamidine transport rates of the two adapted lines were highly similar. These experiments measured initial rates of pentamidine uptake rather than accumulation and the results can only be explained in terms of deficient uptake rather than active extrusion or intracellular sequestration. Unlike T. b.
brucei s427 and tbat1−/− (De Koning, 2001b; Matovu et al., 2003), transport of 15 nM [3H]-pentamidine in KO-B48 was not inhibited by sub-micromolar concentrations of unlabelled pentamidine, saturated only by very high levels of the substrate, and K_m and V_max values were identical to those reported earlier for LAPT1 (De Koning, 2001b).

In tbat1−/−, lysis induced by melarsen oxide was very considerably delayed, relatively to T. b. brucei s427, by co-administration of as little as 1 µM pentamidine – sufficient to saturate HAPT1 but not LAPT1. Pentamidine was apparently competing with the arsenical drug for transporter-mediated import as 1 µM of pentamidine had no effect on the rapid lysis induced by phenylarsine oxide. These experiments clearly demonstrate the uptake of melarsen oxide through HAPT1. The same process was assessed by monitoring DAPI fluorescence as a marker for diamidine accumulation. Scant difference was observed between the WT and tbat1−/− lines, but the development of fluorescence was substantially delayed in KO-B48 cells (Fig. 7), indicating a loss of diamidine transport unrelated to TbAT1/P2. The remaining DAPI transport activity was saturated by 1 mM but not by 10 µM pentamidine, consistent with uptake through LAPT1 rather than HAPT1.

The role of HAPT1 in accumulation of melaminophenyl arsenicals.

Since the seminal observations of Carter and Fairlamb (1993) that a T. b. brucei line adapted to high levels of resistance to melaminophenyl arsenicals had lost activity of a transporter they named P2, much evidence has accumulated linking TbAT1/P2 to arsenical resistance (Carter et al., 1999; Delespaux and De Koning, 2006). Loss of P2 activity alone was not sufficient to establish substantial arsenical resistance as the tbat1−/− line was less than 3-fold resistant to melarsen oxide and melarsoprol (Matovu
et al., 2003). We show here that lysis of trypanosomes induced by melaminophenyl arsenicals, but not by phenylarsine oxide, was (1) sensitive to inhibition by 1 µM pentamidine and (2) much delayed in KO-B48 cells. The level of resistance to melarsen oxide reached 15-fold in KO-B48. We conclude that high levels of arsenical resistance can be induced by selection with pentamidine, and can be achieved by the sequential loss of TbaT1 and HAPT1. The example with T. b. gambiense 386Mr shows that selection with melaminophenyl arsenicals can likewise lead to loss of the same two transporters, thus explaining reported incidences of pentamidine/arsenical cross-resistance (Frommel and Balber, 1987; Kaminsky and Mäser, 2000). High levels of resistance could be far more easily induced when starting from a TbaT1-null background. This is potentially significant given the increasing levels of resistance to melarsoprol and diminazene aceturate in the field, believed to be at least partially linked to loss of TbaT1/P2 activity in the resistant strains (Kaminsky and Mäser, 2000; De Koning et al., 2004; Delespaux and De Koning, 2006).

It must be acknowledged that our findings have been generated under laboratory conditions and that any clinical link between loss of HAPT and diamidine or melarsoprol treatment failure is as yet theoretical. However, the previous explanation for the observed treatment failures (loss of TbaT1/P2 only) proved unsatisfactory (Matovu et al., 2003) and we here propose an improved model that can now be tested against the situation in the field. Efforts to identify the genes encoding HAPT and LAPT are ongoing and would greatly assist the evaluation of this model against clinical isolates.

It is possible that additional mechanisms of resistance to melaminophenyl arsenicals may exist as cross-resistance is not always observed (e. g. (Fairlamb et al., 1992)). However, conclusions based on a review of the literature are complicated by
differences in technique (e.g. *in vitro* or *in vivo* assessment of resistance), variations in which drugs are tested, and other confounding factors.

*The role of drug efflux: ABC transporters.*

It has been demonstrated that overexpression of the *T. b. brucei* ABC-transporter MRPA resulted in a 10-fold resistance to melarsoprol *in vitro* (Shahi *et al.*, 2002), an effect that was additive with the effect of *tbat1* deletion (Lüscher *et al.*, 2006). However, we found no detectable levels of TbMRPA in the two pentamidine-adapted lines. In addition, we found that the P-glycoprotein inhibitor verapamil had no effect on DAPI accumulation and thus we have been unable to find evidence that the increased resistance phenotype is the result of increased activity of P-glycoprotein or mdr-type ABC-transporters.

*Is pentamidine resistance associated with reduced virulence?*

Pentamidine is still the first-line drug for early-stage gambiense sleeping sickness. Treatment failures are rare but stable over at least three decades (Pépin and Milord, 1994). In contrast, pentamidine resistance seems to emerge easily in *Leishmania* species (Papadopoulou *et al.*, 1998), though pentamidine usage against leishmaniasis has been very much less widespread than against African trypanosomiasis. The difference can be explained in part by the involvement of an mdr-type ABC transporter in pentamidine efflux from *Leishmania* (Coelho *et al.* 2003) and the fact that pentamidine uptake in *Leishmania* is apparently mediated by a single transporter (Basselin *et al.*, 2002; Bray *et al.*, 2003). Certainly, the presence of three pentamidine transporters in *T. b. brucei* has been argued to be responsible for the lack of clinical resistance to pentamidine (Bray *et al.*, 2003). However, it has been demonstrated that
trypanosomes with defective tbat1 genes can be isolated from sleeping sickness patients (Matovu et al., 2001) and we show here that the further loss of just HAPT1 leads to very significant levels of pentamidine resistance. The presence of multiple accumulation routes alone may thus not be sufficient to prevent the occurrence of resistance. One explanation would be loss of viability or virulence in the resistant parasites. Berger et al. (1995) reported that pentamidine adapted T. b. brucei clone PR32.6 was far less virulent than the parental strain and excluded increased susceptibility to the host’s immune system or changes attributable to prolonged culturing as factors. The phenotype of the KO-B48 and KO-D48 lines appears identical to PR32.6’s. These lines were less infective in mice and rats than the parental line, which had been cultured at least equally long, and immunosuppression of the animals did not lead to substantially higher parasitaemia. There is thus a distinct possibility that high-level pentamidine resistance is not viable in the field, and self-limiting.
Acknowledgements

We are grateful to Professor Mike Turner for the use of *T. b. gambiense* strains 386Ms and 386Mr and to Professor Christine Clayton for the gift of anti-TbMRPA antiserum. The authors are particularly grateful to Professor Paul Voorheis (University of Dublin) for expert advice and assistance on the preparation of plasma membrane protein-enriched fractions for Western blotting.
References


Fulton JD and Grant PT (1955) The preparation of a strain of *Trypanosoma rhodesiense* resistant to stilbamidine and some observations on its nature. *Exp Parasitol* **4**: 377-386.


Footnotes

This work was funded by the Wellcome Trust.
Figure legends.

FIG. 1. Induction of pentamidine resistance in *T. b. brucei*. A. Development of resistance over time in three independent clones. B, C. Growth of KO-B48 line in the presence (○) or absence (■) of 80 nM pentamidine in the growth medium. Frame B, shortly after passage to 80 nM pentamidine; frame C, after 2 month in 80 nM pentamidine.

FIG. 2. Drug sensitivity profile of four *T. b. brucei* clones. A, Pentamidine. B, Melarsen oxide. C, Propamidine. D, Stilbamidine. E, Diminazene aceturate. F, Phenylarsine oxide (PAO). Error bars indicate SE. All values are the average of between 3 and 11 determinations. A table with the exact values and number of replicates is available as on-line only supplementary material.

FIG. 3. Sensitivity of four *T. b. brucei* clones to (A) pentamidine and (B) melarsen oxide. Lines used were WT (□), KO, (■), KO-B48 (▲) and KO-D48 (○). The data shown is a representative experiment using the Alamar blue method as described in the Materials and Methods section. IC₅₀ values were calculated by non-linear regression. Fluorescence is given in arbitrary units on a scale of 0-1000. The lowest concentration data point is the ‘no drug’ control.

FIG. 4. Transport of 0.5 μM [³H]-pentamidine in resistant trypanosome lines. A. Uptake of pentamidine by KO-B48 (○) or the parental Tbat⁻ strain (■) grown in culture in the absence of pentamidine pressure and the two strains were assayed simultaneously. Lines were calculated by linear regression. B48, r² = 0.89, slope =
0.027 ± 0.005; Tbat−/−, r² = 0.99, slope = 0.10 ± 0.006. B. Uptake of pentamidine by KO-D48 (○) or the parental Tbat−/− strain (■) grown in culture in the absence of pentamidine pressure and the two strains were assayed simultaneously. Lines were calculated by linear regression. D48, r² = 0.97, slope = 0.038 ± 0.005; Tbat−/−, r² = 0.99, slope = 0.17 ± 0.01. C. Uptake of [³H]-pentamidine by KO-B48 (○,●) or the parental Tbat−/− strain (■,□) grown in Wistar rats, in the presence (open symbols) or absence (filled symbols) of 1 mM non-labelled pentamidine. Lines were calculated by linear regression. Values in the absence of pentamidine were as follows: B48, r² = 0.97, slope = 0.0032 ± 0.0001; Tbat−/−, r² = 0.94, slope = 0.021 ± 0.002. Pentamidine uptake is expressed as pmol(10⁷ cells)⁻¹. Symbols represent average of triplicate determinations; error bars are SE, when not shown fall within the symbol.

FIG. 5. Pentamidine uptake in Trypanosoma brucei brucei clone KO-B48 is mediated only by a low affinity transporter. A. Transport of 0.015 µM [³H]-pentamidine in KO-B48 over 2.5 min was assayed in the presence or absence of increasing concentrations of unlabelled pentamidine. The data was fitted to a sigmoid curve using non-linear regression. B. Uptake of 0.04 µM [³H]-pentamidine over 60 s in LS bloodstream forms of s427 WT was assessed in the presence of a constant concentration of 1 mM adenosine and a variable concentration of unlabelled pentamidine. The data was fitted to a ‘two-site competition’ equation in the GraphPad Prism 4.0 software package. IC₅₀ values were 0.13 and 85 µM for the high and low affinity components, respectively. C. Conversion of the inhibition data in frame A to a Michaelis-Menten plot. All data shown are the average of triplicate determinations; error bars are SE. Pentamidine uptake is expressed as pmol(10⁷ cells)⁻¹s⁻¹.
FIG. 6. Lysis of trypanosomes induced by 10 µM melarsen oxide. A. Strain *tbat1*<sup>−/−</sup>. B. Strain KO-B48. Melarsen oxide (10 µM) was added after 15 minutes of recording, as 50 µl [200 µM] in full HMI-9 medium added to 1 x 10<sup>7</sup> (panel A) or 1.5 x 10<sup>7</sup> *T. b. brucei* in 1 ml full HMI-9 medium being monitored at 750 nm. Conditions from t = 0 minutes: a, 10 µM melarsen oxide; b, 10 µM melarsen oxide plus 1 µM pentamidine; c, 0.5 µM phenylarsine oxide; d, 0.5 µM phenylarsine oxide plus 1 µM pentamidine; e, control, no drug. Dotted lines indicate the incubations in the presence of 1 µM pentamidine.

FIG. 7. Assessment of DAPI uptake using fluorescent microscopy over time. LS bloodstream forms of WT *s427*, *tbat1*<sup>−/−</sup> and KO-B48 were incubated with 10 µM DAPI and fluorescence was observed under an Axioplan 2 imaging microscope (λ<sub>ex</sub>=365nm, λ<sub>em</sub>=445nm) at 1000× magnification.

FIG. 8. DAPI fluorescence acquisition in live KO-B48 cells incubated in the absence or presence of a low (10 µM) or high (1 mM) concentration of pentamidine, or 50 µm verapamil over time (A). Separate images (DIC, DAPI and combined overlay) from gluteraldehyde-fixed parasites after 60 min incubation with DAPI in the presence or absence of pentamidine (B). DAPI concentration was 10 µM. Magnification was 1000×. Comb, combined overlay.

FIG. 9. TbMRPA expression in various *Trypanosoma brucei* lines. Western blots with extracts of *tbat1*<sup>−/−</sup> (lane 1), KO-B48 (lane 2), NY-mrpa+ (lane 3) NY-mrpa− (lane 4) and NY (lane 5), using anti-TbMRPA (panel A) or PFR antisera (panel B).
FIG. 10. Parasitaemia in *Trypanosoma brucei brucei* infected mice. Progression of *T. b. brucei* infections in immunocompromised mice following intra-peritoneal inoculation with equal numbers of *tbat1*+ (A), KO-B48 (B) and KO-D48 (C). Animals reaching a parasite burden of $10^8$ or more were euthanised, indicated with a double bar. Each group consisted of three mice, represented by symbols ■, ▲ and ○.

PI, post infection.

FIG 11. Transport of 0.015 µM [³H]-pentamidine in *T. b. gambiense* 386Ms (A) and 386Mr. Uptake was measured over 60 s in the presence or absence of adenosine (▼), propamidine (○) or unlabelled pentamidine (■). Incubations with propamidine or pentamidine were performed in the presence of 1 mM adenosine to saturate the TbAT1/P2 transporter. Data shown are the average of triplicate determinations; error bars are SE. Pentamidine uptake is expressed as pmol(10⁷ cells)⁻¹s⁻¹.
Table 1. Kinetic parameters of pentamidine transporters in various cloned trypanosome lines.

<table>
<thead>
<tr>
<th>Trypanosome</th>
<th>HAPT1</th>
<th>LAPT1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km (µM)</td>
<td>Vmax</td>
</tr>
<tr>
<td></td>
<td>Ki (µM)</td>
<td></td>
</tr>
<tr>
<td><em>T. b. brucei</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s427</td>
<td>0.036 ± 0.006</td>
<td>0.0044 ± 0.0004</td>
</tr>
<tr>
<td><em>T. b. brucei</em> tbat1-/-</td>
<td>0.029 ± 0.008</td>
<td>-</td>
</tr>
<tr>
<td>KO-B48</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td><em>T. b. gambiense</em></td>
<td>0.038 ± 0.004</td>
<td>0.0022 ± 0.0008</td>
</tr>
<tr>
<td>386Mr</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

a. Values taken from (De Koning and Jarvis, 2001)

b. IC<sub>50</sub> values, taken from (Matovu et al., 2003).

N.D., not detectable.

V<sub>max</sub> units are pmol(10<sup>7</sup> cells)<sup>-1</sup>s<sup>-1</sup>. 
Fig. 1

This article has not been copyedited and formatted. The final version may differ from this version.
Fig. 2

Bar graphs showing IC50 values for various compounds:

**A. Pentamidine**

**B. Melarsen oxide**

**C. Propamidine**

**D. Stilbamidine**

**E. Diminazene**

**F. PAO**

The graphs compare IC50 values across different treatments and strains.
Fig. 4
Fig. 5

This article has not been copyedited and formatted. The final version may differ from this version.
Fig. 7

Time (min)

<table>
<thead>
<tr>
<th></th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>s427</td>
<td><img src="s427_2.5.png" alt="Image" /></td>
<td><img src="s427_5.png" alt="Image" /></td>
<td><img src="s427_10.png" alt="Image" /></td>
<td><img src="s427_15.png" alt="Image" /></td>
<td><img src="s427_20.png" alt="Image" /></td>
<td><img src="s427_30.png" alt="Image" /></td>
<td><img src="s427_45.png" alt="Image" /></td>
<td><img src="s427_60.png" alt="Image" /></td>
</tr>
<tr>
<td>Tbat1^-/-</td>
<td><img src="Tbat1%5E-/-_2.5.png" alt="Image" /></td>
<td><img src="Tbat1%5E-/-_5.png" alt="Image" /></td>
<td><img src="Tbat1%5E-/-_10.png" alt="Image" /></td>
<td><img src="Tbat1%5E-/-_15.png" alt="Image" /></td>
<td><img src="Tbat1%5E-/-_20.png" alt="Image" /></td>
<td><img src="Tbat1%5E-/-_30.png" alt="Image" /></td>
<td><img src="Tbat1%5E-/-_45.png" alt="Image" /></td>
<td><img src="Tbat1%5E-/-_60.png" alt="Image" /></td>
</tr>
<tr>
<td>KO-B48</td>
<td><img src="KO-B48_2.5.png" alt="Image" /></td>
<td><img src="KO-B48_5.png" alt="Image" /></td>
<td><img src="KO-B48_10.png" alt="Image" /></td>
<td><img src="KO-B48_15.png" alt="Image" /></td>
<td><img src="KO-B48_20.png" alt="Image" /></td>
<td><img src="KO-B48_30.png" alt="Image" /></td>
<td><img src="KO-B48_45.png" alt="Image" /></td>
<td><img src="KO-B48_60.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Fig. 8A

- **Control**
- **10 μM Pentamidine**
- **1 mM Pentamidine**
- **50 μM Verapamil**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>25</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 μM Pentamidine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM Pentamidine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 μM Verapamil</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 8B

<table>
<thead>
<tr>
<th>[Pentamidine]</th>
<th>DIC</th>
<th>DAPI</th>
<th>Comb</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td><img src="image1" alt="DIC" /></td>
<td><img src="image2" alt="DAPI" /></td>
<td><img src="image3" alt="Comb" /></td>
</tr>
<tr>
<td>0.01 mM</td>
<td><img src="image1" alt="DIC" /></td>
<td><img src="image2" alt="DAPI" /></td>
<td><img src="image3" alt="Comb" /></td>
</tr>
<tr>
<td>1 mM</td>
<td><img src="image1" alt="DIC" /></td>
<td><img src="image2" alt="DAPI" /></td>
<td><img src="image3" alt="Comb" /></td>
</tr>
</tbody>
</table>
Fig. 10

A

log(Parasitaemia)
(cells/ml blood)

Days PI

B

log(Parasitaemia)
(cells/ml blood)

Days PI

C

log(Parasitaemia)
(cells/ml blood)

Days PI
Fig. 11

A

Pentamidine Uptake

log[inhibitor] (M)

0.0000
0.0001
0.0002
0.0003
0.0004
0.0005
0.0006
0.0007
0.0008
0.0009
0.0010

B

Pentamidine Uptake

log[inhibitor] (M)

This article has not been copyedited and formatted. The final version may differ from this version.