Modulation of mitochondrial electrical potential: a candidate mechanism for drug resistance in African trypanosomes

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Bloodstream forms of four populations of the livestock pathogen *Trypanosoma congolense*, isolated from different natural infections, have been shown to exhibit a wide range of sensitivities to the trypanocide isometamidium chloride (Samorin®). In mice the 50% curative doses (CD₅₀) for Samorin range from 0.007 to 20 mg/kg body weight. Uptake of isometamidium chloride demonstrated Michaelis–Menten-type kinetics in all the populations, with K_m values in the range 0.35–0.87 μ M, and V_{max} varied from 17 to 216 pmol/min per 10⁸ cells. The magnitude of V_{max} was correlated with sensitivity to the drug. In contrast, no correlation was observed between K_m values and drug sensitivity. Pulse–chase experiments indicated two compartments for accumulation of drug. The first consists of freely diffusible drug that is invariant between populations; the other consists of retained isometamidium, which is of variable magnitude between the populations and is correlated with drug sensitivity. Autoradiography and fluorescence microscopy demonstrated initial, rapid accumulation of the drug within the mitochondrion,

specifically the kinetoplast. In a drug-sensitive population of *T. congolense*, agents affecting mitochondrial function were shown to produce dose-dependent inhibition of mitochondrial membrane potential ($\Delta\Psi_{mito}$), as measured by the accumulation of the lipophilic cations [³H]methyltriphenylphosphonium iodide or rhodamine 123. The agents also produced parallel inhibition of isometamidium uptake, suggesting an involvement of $\Delta\Psi_{mito}$ in the accumulation of the drug. When characterized in each of the four populations, the spontaneous $\Delta\Psi_{mito}$ was shown to be characteristic of each population and was correlated with V_{max} for drug uptake and sensitivity to the drug *in vitro* and *in vivo*. We therefore conclude that in *T. congolense* $\Delta\Psi_{mito}$ is an important determinant of the rate and accumulation of the trypanocide isometamidium chloride. Populations of this trypanosome species vary with respect to $\Delta\Psi_{mito}$, which is correlated with sensitivity to isometamidium. We suggest that when exposed to drug, the selection of such populations represents a novel mechanism of drug resistance in protozoan parasites.

INTRODUCTION

Haemoparasites of the genus *Trypanosoma* are responsible for serious diseases of man and livestock in sub-Saharan Africa [1]. These organisms are members of the order Kinetoplastidae and are characterized by a unique structure, the kinetoplast, consisting of multiple copies of the mitochondrial genome concatenated with DNA minicircles. This structure is associated with the posterior end of a single, elongated mitochondrion [2].

Trypanosoma congolense and *T. brucei* are two haemoflagellate parasites of mammals that are digenetic and transmitted by haematophagous flies of the genus *Glossina* (tsetse flies). Control of these parasites is rendered problematic by several factors. Vaccine development is hindered by the large repertoire of variable surface glycoproteins that are expressed by bloodstream forms of the parasite in mammalian hosts [1]. Vector control is compromised by problems of infrastructure, scarcity of resources, reinvasion of cleared areas and the environmental impact of insecticides [3]. Thus chemotherapy remains the most commonly employed control method for infection in both livestock and humans. However, the range of drugs available is limited. Furthermore, for cattle, sheep and goats, the recommended compounds have all been in use for several decades [4]. Of these compounds the cationic phenanthridine isometamidium chloride [7-(*m*-amidinophenyl)diazoamino)-2-amino-10-ethyl-9-phenylphenanthridinium chloride] is the most commonly used prophylactic agent [5,6]. Synthesis of the commercial form of this

drug (Samorin®/Trypamidium®) results in a mixture of compounds, all of which consist of a homidium moiety with a *m*-amidinophenyl-azo-amine adduct in various positions. Isometamidium chloride (M&B 4180) constitutes 70% of the commercial preparation; two isomeric forms of lesser trypanocidal activity constitute the bulk of the remainder [6,7]. Resistance to Samorin has been widely reported in the field [8,9] and confirmed by laboratory isolation and characterization of parasite populations [10,11].

Previous work on Samorin has indicated that the drug is accumulated by bloodstream forms of a drug-sensitive clone of *T. congolense* (IL1180) via a saturable transport mechanism in the plasma membrane [12,13]. In this trypanosome population uptake is rapid, linear up to 180 s, and achieves steady-state intracellular concentrations that are 120-fold higher than external levels [12]. Fluorescence microscopy of such cells, utilizing the intrinsic fluorescence of isometamidium, indicates punctate accumulation in the posterior end of the parasite, although the significance of this is not clear [14].

In these studies we describe the accumulation of isometamidium chloride in *T. congolense* and show mitochondrial electrical potential to be a major determinant of the process. Furthermore, *T. congolense* populations with different sensitivities to the drug are shown to exhibit consistent relationships between their level of resistance, rate of drug accumulation and intrinsic mitochondrial potential. We propose that modulation of mitochondrial potential is a mechanism responsible for

Abbreviations used: CD₅₀, 50% curative dose; $\Delta\Psi_{mito}$, mitochondrial membrane potential; MeTPP⁺, methyltriphenylphosphonium ion; PSGH, Dulbecco's PBS supplemented with 5 mM glucose and 0.1 mM hypoxanthine.

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resistance to isometamidium in this organism. This represents a novel mechanism of drug resistance in naturally occurring populations of protozoan parasites.

MATERIALS AND METHODS

Materials

[¹⁴C]Samorin and [³H]Samorin were gifts from Rhône-Poulenc Rorer. [¹⁴C]Carboxylinulin and ³H₂O were from Amersham International. [³H]Methyltriphenylphosphonium iodide ([³H]MeTPP⁺ iodide) was from New England Nuclear. Rhodamine 123 was from Molecular Probes. Dibutylphthalate was from Sigma Chemical Co.

Derivation and preparation of trypanosomes

The *T. congolense* populations utilized in this study, their CD₅₀ values (dose of Samorin required to effect the cure of 50 % of infected mice), and their derivation are summarized in Table 1. In cattle, *T. congolense* IL1180 has been shown to be sensitive to intramuscular treatment with Samorin at a dose of 1.0 µg/kg body weight [15]. In contrast, *T. congolense* IL3338 is resistant in cattle to intramuscular treatment with Samorin at the recommended dose of 0.5 mg/kg body weight [10]. Work by Peregrine et al. [16] has shown that in cattle *T. congolense* IL2642 is more sensitive to Samorin than *T. congolense* IL1180. In a drug sensitivity test *in vitro*, the concentrations of Samorin required to effect full inhibition of growth were 1 pg/ml (IL2642), 1 ng/ml (IL1180) and 10 ng/ml (IL3000 and IL3338) [17]. Thus the populations used in this study demonstrate a wide range of sensitivities to the commercial preparation that is maintained even in the absence of a drug selection pressure, and is apparent both in the natural host (cattle) and when adapted to rodents and culture.

Sublethally (6.5 Gry) irradiated Sprague–Dawley rats of both sexes were inoculated with cryopreserved stabilates of bloodstream-form parasites (approx. 5 × 10⁶ cells per rat), and exsanguinated at peak parasitaemia (more than 10⁸ cells per ml). Parasites were then isolated from blood cells by DEAE-cellulose chromatography by the method of Lanham and Godfrey [18]. After isolation, cells were suspended in Dulbecco's PBS supplemented with 5 mM glucose and 0.1 mM hypoxanthine (PSGH), and counted in a Neubauer haemocytometer. Cells were diluted to 10⁸ cells/ml in PSGH and maintained on ice until use.

Isolation of ¹⁴C- and ³H-labelled isometamidium chloride

As Samorin is a complex mixture of reaction products, and these studies focused on the transport of the major trypanocidal compound isometamidium chloride (M&B 4180), the compound was isolated from unlabelled or ¹⁴C- or ³H-labelled Samorin by a proprietary HPLC method provided in confidence by Rhône-Poulenc Rorer (Lyon, France). The appropriate fractions were collected, solvent was removed in a rotary evaporator and the fractions were redissolved in 2 M NaCl. Isometamidium was extracted by exhaustive partitioning into butan-1-ol [high salt enhances the partitioning of phenanthridines into the butanol phase (J. M. Wilkes, unpublished work)]. After determination of absorbance at 380 nm (unlabelled material) or scintillation counting, aliquots were dispensed and snap-frozen on liquid nitrogen, and butanol was removed by freeze-drying. All samples were dissolved in water and stored at –20 °C before use. HPLC analysis of the preparations showed them to be more than 95 % pure radiochemically, with no other components absorbing at 380 nm on HPLC or visible on TLC (Rhône-Poulenc Rorer). [¹⁴C]Samorin (lot no. GHS-511, 22.7 mCi/mmol), [³H]Samorin

Table 1 *T. congolense* populations used

All four populations were derived from natural infections and were adapted to rodents before the study. As a result the observed sensitivity to Samorin reflects the situation in the field [9,27].

Population	Samorin sensitivity, CD ₅₀ (mg/kg)	History
IL2642	0.007	Clone of isolate from Bugosa, Uganda [43]
IL1180	0.018	Clone of isolate from Serengeti, Tanzania [44]
IL3000	2.9	Clone of isolate from Trans-Mara, Kenya [45]
IL3338	20.0	Isolate from Ghibe, Ethiopia [10]

(lot no. GHS-600, 8.0 Ci/mmol) and the chromatography methodologies were gifts from Rhône-Poulenc Rorer.

Determination of labelled isometamidium accumulation by bloodstream-form *T. congolense*

Accumulation of isometamidium was determined in 600 µl aliquots of cell suspensions layered over a two-phase system consisting of 150 µl dibutylphthalate overlying 50 µl of 14 % (w/v) perchloric acid in a 1.5 ml Eppendorf centrifuge tube. Uptake was terminated by centrifugation in an Eppendorf bench-top centrifuge at 15400 g_{av}. Accumulated drug was determined by scintillation counting of 40 µl of the perchloric acid digest. Samples of incubation mix were removed for microscopic examination during representative incubations, and demonstrated no visible changes in the motility or morphology of the trypanosomes during the course of the experiments. Thus acute toxicity of isometamidium does not seem to occur.

Accumulation and fate of [¹⁴C]isometamidium in resistant and sensitive populations of *T. congolense*

Bloodstream-form cells were suspended at a density of 10⁸ cells/ml in PSGH containing 2 µM [¹⁴C]isometamidium, for 15 min at 37 °C. The initial uptake phase was monitored by removing aliquots, which were assayed for accumulated drug as described above. The uptake phase was terminated by dilution in more than 20 vol. of ice-cold medium. The remaining cells were harvested by centrifugation and resuspended to approximately the original cell density in PSGH at 37 °C. Aliquots of this suspension were subsequently assayed for levels of retained isometamidium at intervals, and cell density was determined at the end of the experiment.

Direct detection of isometamidium accumulation in *T. congolense*

The autofluorescent property of isometamidium was used to detect uptake of the drug over time in bloodstream-form cells of *T. congolense* IL1180. Cells were incubated at 37 °C in PSGH in the presence of 2 µM drug. Cytospin preparations were then made at various time points and viewed rapidly in a Zeiss Axiophot microscope under epifluorescence (Ektar 100 film, 40 s exposure). For autoradiography, cells were incubated at 37 °C in the above medium in the presence of 2 µM [³H]isometamidium. The incubation was halted at various time points by centrifuging the cells through a layer of silicone oil into fixative containing 2.5 % (w/v) glutaraldehyde and 0.25 M sucrose in 0.1 M cacodylate buffer. After fixation for 30 min the silicone oil layer and the incubation mixture containing the isometamidium, which remained above the oil, were removed. The cells were then washed in cacodylate buffer to remove any residual radiolabel

and free glutaraldehyde. For autoradiography the washed cells were postfixed in 1% (w/v) osmium tetroxide and embedded in an Epon/Araldite mixture. Ultrathin sections (60 nm thick) were cut and mounted on Formvar-coated nickel grids. Autoradiography was performed with Ilford L4 emulsion with exposure times of 30 days. The autoradiographs were then developed and viewed on a Zeiss EM 10 A electron microscope.

Monitoring mitochondrial potential

Use of rhodamine 123

Cells were suspended at 10^7 /ml in PSGH at 30 °C and after a 10 min preincubation in the presence of various agents, rhodamine 123 was added to a final concentration of 250 nM. Cells were diluted 1:10 in PSGH immediately before FACS analysis (Becton Dickinson Facscan, standard rhodamine settings). Fluorescence distribution was plotted as a frequency histogram, and the mean fluorescence channel was recorded for each incubation.

Use of [^3H]MeTPP $^+$ iodide

Cell volume was determined from the radioactivity of $^3\text{H}_2\text{O}$ and [^{14}C]carboxyininulin in cell pellets by the method of Thissen and Wang [19] and found to be $1.18 \pm 0.07 \mu\text{l}/10^8$ cells (mean $\pm$ S.D.) for bloodstream-form *T. congolense* IL1180. [^3H]MeTPP $^+$ iodide accumulation was determined by the method of Nolan and Voorheis [20]. After preincubation at 30 °C with various agents an aliquot of cell suspension ($650 \mu\text{l}$, 3×10^7 cells/ml) was added to $50 \mu\text{l}$ of 70 nM [^3H]MeTPP $^+$ (specific radioactivity 42.5 Ci/mmol), and incubated for a further 30 min. Cells were collected by centrifugation through $150 \mu\text{l}$ of dibutylphthalate. Aliquots of the supernatant were subjected to scintillation counting and the remaining supernatant was discarded. After freezing on solid CO_2 , the bottom of the tube was sliced to enable the radioactivity in the cell pellet to be determined by scintillation counting. Accumulation ratios were determined from the MeTPP $^+$ concentration in the supernatant and in the cell pellet, with the use of a value for cell internal volume of $1.18 \mu\text{l}/10^8$ cells as determined above. These values represent an accumulation ratio uncorrected for the activity coefficient of MeTPP $^+$ [20].

RESULTS

Kinetics of uptake of isometamidium in *T. congolense*

[^3H]Isometamidium uptake kinetics were determined in the four populations of *T. congolense* and conformed to a simple Michaelis–Menten model in all cases. K_m values showed minimal variation (0.4 – $0.9 \mu\text{M}$), whereas V_{max} values showed striking differences (ranging from 17 to 216 pmol/min per 10^8 cells). The magnitude of V_{max} was correlated with sensitivity to the drug determined by CD_{50} values in mice (Figure 1).

Pulse–chase experiments indicated that the rates of entry of isometamidium followed first-order kinetics and varied between three trypanosome populations [characterized as highly sensitive (IL2642), sensitive (IL1180) and resistant (IL3000) to Samorin]. The rate constant for the uptake process was found to be in the range 0.15 – 0.21 min^{-1} for all populations. The steady-state accumulation of drug was the major determinant of initial rates of uptake and varied markedly between the populations, being correlated with drug sensitivity in a manner similar to that determined for V_{max} of uptake.

Subsequent washing and suspension of the cells in drug-free medium demonstrated that the isometamidium partitioned into two compartments: one mobile and free to diffuse from the cell,

Figure 1 Kinetics of accumulation of [^3H]isometamidium in resistant and sensitive populations of *T. congolense*

Isometamidium transport was initiated by adding $100 \mu\text{l}$ of prewarmed cell suspension to $600 \mu\text{l}$ of [^3H]isometamidium in PSGH at 37 °C, producing final concentrations of 0.05 – $2 \mu\text{M}$. Uptake was determined in $600 \mu\text{l}$ aliquots after 90 s by rapid centrifugation. The initial rates of uptake were fitted to Michaelis–Menten kinetics by using the Mardquat algorithm for non-linear regression analysis. Data represent duplicate determinations from three to five experiments. The V_{max} and K_m (not shown) values for the uptake were determined for each population: IL2642 ($\diamond$), IL1180 ($\circ$), IL3000 ($\square$), IL3338 ($\triangle$). Plotted as a function of drug sensitivity (CD_{50}) in mice, V_{max} demonstrates a strong and parallel correlation with sensitivity to the drug. Inset: double-reciprocal plots of the kinetics of isometamidium uptake in the four populations (same symbols). This graphically demonstrates the wide range of V_{max} observed in the populations, with relatively constant K_m .

Figure 2 Accumulation and fate of [^{14}C]isometamidium in resistant and sensitive populations of *T. congolense*

Initial accumulation of isometamidium was monitored after exposure to isometamidium at $2 \mu\text{M}$. Cells were subsequently quenched with 20 vol. of ice-cold PSGH (arrow), and subsequently resuspended in drug-free media at 37 °C. Retained drug was monitored for a further 20 min. Results represent duplicate determinations from two experiments; IL2642 ($\diamond$, very sensitive), IL1180 ($\circ$, sensitive) and IL3000 ($\square$, resistant).

the second retained within the cell. The magnitude, and the kinetics of emptying, of the free-drug compartments were similar in all populations. In contrast, the magnitude of the retained components varied markedly (Figure 2) and was correlated with the sensitivity of the population to the drug.

Figure 3 Direct detection of isometamidium accumulation in *T. congolense* bloodstream forms

(A) Light micrographs of fluorescence (a, b) and the corresponding differential interference contrast micrographs (a', b') of trypanosomes incubated with Samorin for 2 min (a, a') and 15 min (b, b'), showing accumulation of the autofluorescent drug. At 2 min there is a bright punctate fluorescence in an area adjacent to the flagellar pocket (arrowed). After 15 min the punctate fluorescence is brighter and a structure extending along the trypanosome is also beginning to accumulate the drug. Note some fluorescence in the cell nucleus (n) but this remains constant over the time of the experiment. Scale bar, 15 nm. (B) Electron microscope autoradiographs showing localization of $[^3\text{H}]$ isometamidium to the mitochondrion (arrowed) of trypanosomes after 2 min of incubation with the drug. The insert is a higher-magnification autoradiograph showing localization of the $[^3\text{H}]$ isometamidium to the kinetoplast (arrowed). The flagellar pocket (f) is unlabelled with the drug. Scale bar: (main) 10 μm ; (insert) 0.25 μm .

Direct detection of isometamidium compartmentalization

Studies of the uptake and compartmentalization of isometamidium by bloodstream forms of a drug-sensitive cloned population of *T. congolense* (IL1180) were undertaken, by using both the intrinsic fluorescence of the drug and autoradiography

of cells after incubation with ^3H -labelled isometamidium. The results (Figure 3) demonstrated rapid and specific association of the drug with the single mitochondrion within the cells. At early stages of incubation, labelling seemed to be specifically associated with the kinetoplast. Little indication of an association of drug with the nucleus was found. No signal from radiolabelled

Figure 4 Effects of mitochondrial inhibitors on isometamidium uptake and mitochondrial potential in bloodstream-form *T. congolense* IL1180

Cells were incubated in PSGH for 10 min in the presence of oligomycin ($\diamond$, $\circ$) or 2,4-dinitrophenol ($\blacklozenge$, $\blacksquare$, $\bullet$) before evaluation of the accumulation of rhodamine-123 ($\blacksquare$), [^3H]MeTPP $^+$ ($\blacklozenge$, $\diamond$) or [^3H]isometamidium ($\bullet$, $\circ$). Results are expressed as percentages of the values determined in control incubations, and represent means of duplicate or triplicate determinations in two experiments. Inhibition data were analysed as four-parameter logistic curves by using the Marquadt algorithm. Dose-dependent inhibition of isometamidium uptake was observed with both agents (inset), which is paralleled by their effect on the probes of mitochondrial potential, supporting a linkage between the two parameters.

drug was found to be associated with endocytic vesicles. Thus the punctate fluorescence observed in early stages of incubation with the drug [14] seems to reflect the accumulation of drug by the kinetoplast. In parallel experiments, punctate fluorescence and signal from labelled drug were essentially absent from cells of the highly resistant population *T. congolense* IL3338 (results not shown).

Effect of mitochondrial inhibitors on mitochondrial potential and isometamidium uptake

With oligomycin (an inhibitor of the mitochondrial F_1/F_0 ATPase [21]) and 2,4-dinitrophenol (a mitochondrial uncoupling agent), in combination with lipophilic cationic probes of mitochondrial potential ([^3H]MeTPP $^+$ and rhodamine 123 [20,22,23]), we determined the effects of the former agents on the mitochondrial potential. In parallel we also determined the effects of these inhibitors on the accumulation of [^3H]isometamidium. The effects of the inhibitor and uncoupler, both of which are effective in *T. brucei* [23,24], are summarized in Figure 4. Both agents produced a concentration-dependent decrease in the level of accumulation of the lipophilic probes, consistent with a decrease in the mitochondrial potential (2,4-dinitrophenol produced 73 % inhibition with an IC_{50} of $8.2 \mu\text{M}$; oligomycin produced 73 % inhibition, IC_{50} $0.54 \mu\text{M}$). A decrease in the rate of accumulation of isometamidium was also observed with similar sensitivities to the agents (2,4-dinitrophenol produced 66 % inhibition with an IC_{50} of $7.1 \mu\text{M}$; oligomycin produced 78 % inhibition, IC_{50} $0.49 \mu\text{M}$).

Mitochondrial potential in the four populations

Investigation of the spontaneous mitochondrial membrane potential ($\Delta\Psi_{\text{mito}}$) maintained by the four populations was performed with the two lipophilic probes. The FACS analysis of cells loaded with rhodamine 123 is summarized in Figure 5. The fluorescence histograms demonstrate consistent differences be-

Figure 5 Rhodamine 123 staining of isometamidium-resistant and -sensitive populations of *T. congolense*

Bloodstream-form cells of the four populations were incubated with rhodamine 123 as described. Cell suspensions were subjected to FACS and the fluorescence distribution was plotted as frequency histograms. The Figure shows a set of histograms, representative of two duplicate determinations, for the four populations: (a) IL2642, (b) IL1180, (c) IL3000, (d) IL3338. Clear differences in the degree of probe accumulation (and hence mitochondrial potential) are discernible.

tween the populations, with mean fluorescence (and hence probe accumulation) increasing in the order IL3338 < IL3000 < IL1180 < IL2642. Mean uncorrected accumulation ratios ($\pm$ S.D.) for [^3H]MeTPP $^+$ in these populations were 580 ± 47

Figure 6 Intrinsic mitochondrial potentials and maximal rates of isometamidium transport both relate to sensitivity to isometamidium in four populations of *T. congolense*

V_{max} for the transport process (black symbols) was determined as previously described. Mitochondrial potential was monitored by the accumulation of rhodamine 123 (open symbols) and [^3H]MeTPP $^+$ (grey symbols) as described. Symbols: IL2642 ($\diamond$), IL1180 ($\circ$), IL3000 ($\square$), IL3338 ($\triangle$). Each set of results is normalized to the response of *T. congolense* IL1180 and plotted as a function of drug sensitivity (CD_{50}) in mice. All parameters demonstrate a strong and parallel correlation with sensitivity to the drug. Inset: correlation (with 95% confidence interval) between [^3H]MeTPP $^+$ accumulation ratio and V_{max} for isometamidium transport in the four populations of *T. congolense*. There is a strong linear correlation between the two parameters ($y = 0.412x - 16.8$, $r^2 = 0.978$; $y = V_{\text{max}}$ for transport, $x = [\text{MeTPP}^+ \text{ acc. ratio}]$).

Figure 7 Effect of verapamil on accumulation of rhodamine 123 and isometamidium in populations of *T. congolense*

Bloodstream-form cells of all four populations of *T. congolense* [IL2642 ($\diamond$), IL1180 ($\circ$), IL3000 ($\square$), IL3338 ($\triangle$)] were resuspended in PSGH at a concentration of 10^7 cells/ml. Verapamil was added to a final concentration of 0–100 μ M and the cells were incubated for a further 10 min at 37 °C. Rhodamine 123 accumulation was subsequently determined as described in the Materials and methods section. Results represent duplicate determinations from two experiments. Inset: bloodstream-form cells of *T. congolense* IL1180 ($\circ$) or IL3338 ($\triangle$) were resuspended at a concentration of 5×10^7 cells/ml in PSGH. Verapamil was added to a final concentration of 0–100 μ M and the cells were incubated at 37 °C for 10 min. The accumulation of 2 μ M [3 H]isometamidium (ISMM) was determined as previously described. Results represent duplicate determinations from two experiments.

(IL2642), 274 ± 38 (IL1180), 204 ± 26 (IL3000) and 96 ± 34 (IL3338), demonstrating the same relative order as the rhodamine 123 accumulation. For each population triplicate determinations were performed in three experiments. Thus clear and consistent differences in the value of $\Delta\Psi_{\text{mito}}$ were demonstrated between the trypanosome populations by these techniques. Furthermore, there was a strong linear correlation between [3 H]MeTPP $^+$ accumulation and V_{max} for isometamidium uptake ($r^2 = 0.987$), suggesting a strong linkage between these parameters (Figure 6).

Rhodamine 123 and MeTPP $^+$ are lipophilic cations and potential substrates for the P-glycoprotein family of enzymes responsible for many multiple drug resistance phenotypes [25,26]. One possible interpretation of the results is a multiple drug resistance-type mechanism with effects on both probes and the phenanthridine. However, verapamil (a potent inhibitor of P-glycoprotein [27]) at 0–100 μ M attenuated the accumulation of rhodamine 123 in all four populations of trypanosomes but had little effect on the accumulation of isometamidium in IL1180 (sensitive) and IL3338 (resistant) cells (Figure 7).

DISCUSSION

The populations of *T. congolense* used in these studies were all derived from isolates of natural infections that were adapted to growth in rodent hosts. The populations are all pathogenic to cattle and demonstrate a wide range of sensitivities to Samorin *in vivo* (CD_{50} in mice ranging from 7 μ g/kg to 20 mg/kg body weight [10,28]) and in *in vitro* drug sensitivity tests [17]. Thus the populations exhibit levels of drug sensitivity found in natural populations, and the observed mechanisms of tolerance to Samorin are relevant to the field situation.

Accumulation of Samorin by bloodstream forms of *T. congolense* has previously been shown to be due to a saturable concentrative process [12]. The studies described here confirm

that isometamidium chloride, the active component of Samorin [6], is similarly accumulated. However, clear differences in the uptake kinetics of isometamidium were observed in bloodstream-form *T. congolense* populations that differ in their sensitivities to the drug, consistent with a previous report of lower rates of Samorin uptake in resistant populations [29]. K_m was relatively constant in all populations, whereas the resistance phenotype observed in these populations was associated with decreased values of V_{max} , ranging from 214 pmol/min per 10^8 cells in the most sensitive population to 17 pmol/min per 10^8 cells in the most resistant. Such a decrease in accumulation would result in a decrease in toxicity of the drug for the parasites. However, this change in accumulation might result from a number of different mechanisms, e.g. decreased efficiency of transport, increased efflux of drug, or decrease in an intracellular compartment with affinity for the compound.

In pulse-chase experiments the uptake of isometamidium seemed to follow a simple first-order process (as reported previously [12]), with the process tending to smaller limiting values and lower initial rates in populations demonstrating lower V_{max} values for isometamidium accumulation. Subsequent washing of the cells demonstrated the partitioning of the drug into two compartments. Free diffusion of drug from the cells was observed, with similar magnitudes and kinetics in all populations tested. However, the retention of drug seemed to be variable between the populations, and correlated with the sensitivity of the population to the compound. Thus resistance to the drug seems to be linked to a decrease in the magnitude of the compartment corresponding to retained drug.

Direct detection of drug in bloodstream-form IL1180 demonstrated a distinct compartmentalization into mitochondrial structures, and a specific interaction with the kinetoplast. Given the high affinity of isometamidium for DNA, and the large numbers of binding sites for the ligand in DNA [12,30], accumulation by the kinetoplast is plausible. Because a major effect of isometamidium is to promote the cleavage of kinetoplast DNA [31], it is probable that this compartment is the most relevant therapeutically. However, changes in the affinity of the isometamidium-binding sites in kinetoplast DNA between populations seems unlikely; a further mechanism for controlling the partitioning of drug into the mitochondrion is required.

The studies of drug localization implicated the mitochondrion, and subsequently the kinetoplast, of bloodstream-form *T. congolense* in accumulation and the mode of action of isometamidium. The inhibition of $\Delta\Psi_{\text{mito}}$ in the drug-sensitive population IL1180 by oligomycin and 2,4-dinitrophenol, monitored by two lipophilic cationic probes, was paralleled by decreases in the rate of accumulation of isometamidium, strongly indicating that maintenance of $\Delta\Psi_{\text{mito}}$ is required for the accumulation of drug. Furthermore, the spontaneous $\Delta\Psi_{\text{mito}}$ maintained by the four populations was characteristic of each population and its magnitude was correlated with sensitivity to the drug. V_{max} for drug uptake was linearly related to $\Delta\Psi_{\text{mito}}$, suggesting a strong linkage between the two parameters.

The involvement of a P-glycoprotein type activity seems unlikely because levels of accumulation of rhodamine 123 in the four trypanosome populations described here were not stimulated by the presence of verapamil at 3–100 μ M. Indeed, verapamil decreased the accumulation of rhodamine 123 in all populations. Results suggest that the passage of rhodamine 123 through cell membranes is neither rate-limiting nor saturable [32], suggesting simple diffusion through the lipid bilayer. The observed inhibition of accumulation induced by verapamil might be due to its ability to bind to phospholipids and disrupt the membrane structure [33]. Finally, it should be noted that verapamil had little effect on

the accumulation of isometamidium by bloodstream forms of IL1180 (sensitive) or IL3338 (highly resistant). This process seems to be mediated by a transmembrane structure [12,13], which is unlikely to be affected by verapamil. Similarly, verapamil produced no reversal of drug resistance in populations of bloodstream-form *T. brucei* [34].

Studies of drug resistance in kinetoplastids have indicated that decreased levels of drug accumulation are responsible for tolerance to a number of trypanocides. This seems to be true of arsenical compounds. Activity of the adenine-sensitive adenosine transporter (termed the P2 subtype) is absent from populations induced to be melarsen-resistant in the laboratory [35]. The diamidine group of trypanocides also seems to enter the trypanosome by means of the P2 adenosine transporter subtype, which is consistent with reports of cross-resistance to arsenical compounds [36,37]. However, one pentamidine-resistant population of *T. brucei* has been derived that accumulates high levels of pentamidine [38]. This might suggest a mechanism involving changes in the intracellular target for the drug.

With the phenanthridine class of trypanocides (homidium and isometamidium), rates of drug uptake were decreased in resistant populations of *T. congolense* [29]. It is well established that cross-resistance between isometamidium and homidium is widespread in African trypanosomes, suggesting a common mechanism for resistance [39]. In contrast, cross-resistance between Samorin and Berenil® (diminazene aceturate) is rarely seen in the field [40], in laboratory studies of infections (A. S. Peregrine, unpublished work) or in tests *in vitro* [34]. The resistance mechanism thus seems to be specific for the homidium moiety, not the *m*-amidinophenyl-azo-amine functional group common to Samorin and Berenil. Furthermore, populations of *T. congolense* seem to express only inosine-sensitive (i.e. P1 type) adenosine transport; uptake of isometamidium is not inhibited by this compound (J. M. Wilkes, unpublished work). Thus in contrast with the diamidines, adenosine transport systems do not seem to be involved in the transport of phenanthridines.

In summary, four populations of *T. congolense* were shown to display a consistent continuum with respect to sensitivity to Samorin, $V_{\max}$ for isometamidium uptake, amount of drug retained within the 'immobilized compartment', and spontaneous mitochondrial potential. This mitochondrial potential seems to be involved in the uptake of the phenanthridines, possibly by acting to drive the accumulation of cationic compounds. In the presence of drug selection pressure, populations expressing lower mitochondrial electrical potential have an advantage owing to decreased accumulation of isometamidium, and hence decreased toxicity of the drug. Such a mechanism seems to be rare and has previously only been observed as a mutation in mammalian cancer cell lines after extensive selection procedures. In these cases resistance to ethidium bromide (structurally related to isometamidium) [41], and to bis(diphenylphosphine)ethane-gold complexes [42] were linked to down-regulation of mitochondrial potential. The spontaneous appearance of this mechanism in natural populations of *T. congolense*, widely separated spatially and temporally, indicates it to be a natural strategy and represents a novel drug-resistance mechanism in protozoan parasites. Further studies are required to elucidate the biochemical and genetic basis of the alteration in mitochondrial potential.

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