Fluorescence analysis of the interaction of isometamidium with *Trypanosoma congolense*

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Isometamidium chloride (Samorin) is the only compound recommended for prophylaxis against bovine trypanosomiasis in sub-Saharan Africa. The fluorescence property of this compound was used to investigate the interaction of the molecule with *in vitro*-derived bloodstream forms of *Trypanosoma congolense* IL 1180. Incubation of isometamidium with trypanosomes at 37 °C for 180 min resulted in a gradual alteration of the $\lambda_{\max}$ with time (from 600 to 584 nm) and an increase in the intensity of trypanosome-associated fluorescence of approx. 2-fold. The alteration in fluorescence was temperature-dependent and inhibited by the addition of *N*-ethylmaleimide. In contrast, with intact cells addition of digitonin caused a rapid increase in

fluorescence intensity to approximately four times that observed with intact cells. Uptake of isometamidium was also determined using radiolabelled drug; the results indicated that the time course of the uptake process resembled the fluorescence profile and was temperature-dependent. The results therefore indicate that the alteration of fluorescence is due to interaction of isometamidium with an intracellular component(s) and that isometamidium is transported across the plasma membrane via a protein carrier. The data also indicate that the described fluorescence technique can be used to investigate the role of membrane transport in resistance to isometamidium.

INTRODUCTION

In sub-Saharan Africa, chemotherapy of trypanosomiasis in cattle, goats and sheep is dependent upon the salts of three compounds: the aromatic diamidine, diminazene (Jensch, 1958), the phenanthridine, homidium (Watkins and Woolfe, 1952), and the phenanthridine aromatic amidine, isometamidium (Berg, 1960). While the salts of diminazene (Berenil, Veriben) and homidium (Novidium, Ethidium) are used as therapeutic agents, only isometamidium chloride (Samorin, Trypamidium) is marketed as both a prophylactic and a therapeutic agent (Leach and Roberts, 1981). Thus chemoprophylaxis of bovine trypanosomiasis is restricted to the use of only one compound, namely isometamidium chloride.

Isometamidium chloride is 8-[(*m*-aminophenylazo)amino]-3-amino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride (see Figure 1). The compound has been shown to be a highly effective prophylactic agent in the field (Wiesenhutter et al., 1968; Trail et al., 1985) and is probably the most widely used of the available trypanocides for bovine trypanosomiasis. However, resistance to the compound has been described at a number of sites across Africa (Lewis and Thomson, 1974; Kupper and Wolters, 1983; Pinder and Authie, 1984; Schönefeld et al., 1987).

Although isometamidium has been used in the field for over 30 years, very little is known about its mechanism of action. Various studies have indicated that the compound interacts with a number of intracellular molecules (Henderson et al., 1977; Bacchi et al., 1980; Bacchi, 1981; Shapiro and Englund, 1990) and, therefore, that there may be several modes of action.

In earlier work, the fluorescence property of isometamidium was used to study localization of the compound in mammalian cells (Philips et al., 1967). Similar analyses were recently conducted to examine the interaction of isometamidium with blood-

stream forms of *Trypanosoma (Nannomonas) congolense* clones *in vitro*; an inverse relationship was observed between the intensity of trypanosome-associated fluorescence and the level of resistance to isometamidium expressed by the clones *in vivo* (Sutherland et al., 1991). It is unclear, however, whether these changes in fluorescence characteristics are associated with the trypanocidal action of isometamidium. In the present work, the interaction of isometamidium with an isometamidium-sensitive clone of *T. congolense* was analysed by quantitative fluorescence measurements and with radiolabelled isometamidium. The results indicate that isometamidium is transported into the trypanosome and that this process is mediated by a protein in the plasma membrane.

MATERIALS AND METHODS

Materials

Isometamidium chloride (Samorin) was a gift from RMB Animal Health Ltd.; digitonin and *N*-ethylmaleimide (NEM) were purchased from Sigma.

Trypanosome

T. congolense IL 1180 is a doubly cloned derivative (Nantulya et al., 1984) of an isolate collected from a lion in the Serengeti National Park, Tanzania (Geigy and Kauffmann, 1973). On the basis of molecular karyotype (Majiwa et al., 1986) and hybridization to a repetitive DNA sequence (Majiwa et al., 1985), this clone is a savannah-type *T. congolense* (Young and Godfrey, 1983). The clone expresses a high level of sensitivity to isometamidium chloride both in mice [50% curative dose (CD_{50}) = 18 $\mu\text{g} \cdot \text{kg body weight}^{-1}$ (Peregrine et al., 1991)] and in

Figure 1 Molecular structure of isometamidium

cattle [sensitive to intramuscular treatment with $1.0 \mu\text{g} \cdot \text{kg body weight}^{-1}$ (Sones et al., 1988)].

Preparation of trypanosomes

Bloodstream forms of *T. congolense* IL 1180 were propagated in exponential-phase growth in an axenic culture system *in vitro* (Hirumi and Hirumi, 1991). Briefly, primary cultures were initiated by pipetting tail blood from infected mice into wells of a 24-well culture plate ($10 \mu\text{l}$ blood/well) containing 2.0 ml of medium. Trypanosomes were then subcultivated in T-25 flasks and maintained at 34°C in HMI-93 medium, which consists of Iscove's modified Dulbecco's minimal essential medium (Flow Laboratories) supplemented with 0.05 mM bathocuproine sulphate, 1.5 mM L-cysteine, 0.5 mM hypoxanthine, 0.12 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.16 mM thymidine, 20% (v/v) heat-inactivated young-goat serum and 5% (v/v) Serum Plus (Hazleton Biologics, Lenaxa, KS, U.S.A.). Trypanosomes propagated in this system were morphologically similar to those seen in blood from infected mice, were covered with a surface coat as determined by electron microscopy and were infective for mice (Hirumi and Hirumi, 1991). Trypanosomes in culture medium were prepared for experiments by centrifuging at $1000 g$ for 10 min. They were then resuspended in Dulbecco's phosphate-buffered saline containing 5 mM glucose (PBSG), pH 7.4, centrifuged at $1000 g$ for 10 min and resuspended in PBSG to a final concentration of 1.0×10^8 trypanosomes $\cdot \text{ml}^{-1}$. Parasites were kept at room temperature prior to experiments. In this state they were stable for at least 5 h, i.e., fluorescence activity remained the same over this period.

Fluorescence measurements

The reaction mixture (final volume 3.0 ml), consisting of PBSG and isometamidium chloride at various concentrations (50 or $100 \text{ ng} \cdot \text{ml}^{-1}$), was placed in a 3.5 ml glass cuvette. Parasites were added to a final density of $(0.67\text{--}1.0) \times 10^7$ cells $\cdot \text{ml}^{-1}$. Fluorescence was recorded on an SLM Aminco 8000 fluorimeter with excitation and emission wavelengths of 374 and 584 nm respectively (slit width of 10 nm for both excitation and emission). The cuvette mounting was temperature-controlled throughout the measurements. Data analyses were conducted using an SLM version 2.2 spectrum processor (SLM Instruments Inc., Urbana, IL, U.S.A.).

Uptake of radiolabelled isometamidium

The reaction mixture (0.6 ml) consisted of PBSG, $100 \mu\text{M}$ hypoxanthine and $1 \mu\text{g} \cdot \text{ml}^{-1}$ [^{14}C]isometamidium ($22.7 \text{ Ci} \cdot \text{mol}^{-1}$). Assays were started by adding 0.1 ml of a trypanosome suspen-

sion containing 2×10^8 cells $\cdot \text{ml}^{-1}$ to the reaction mixture and incubating it at 37°C . The mixture was subsequently placed in an Eppendorf microcentrifuge tube over a layer of silicone oil ($1.02 \text{ g} \cdot \text{ml}^{-1}$) which itself was placed over a $50 \mu\text{l}$ aliquot of 12% HClO_4 ($1.1 \text{ g} \cdot \text{ml}^{-1}$). Uptake was terminated by centrifugation at $15800 g$ for 1–2 min. The level of [^{14}C]radioactivity in $40 \mu\text{l}$ aliquots of HClO_4 lysates were subsequently determined by liquid-scintillation counting.

RESULTS AND DISCUSSION

The development of a sensitive quantitative assay for isometamidium is essential for investigations on the mechanism of action of this compound in trypanosomes. Such an experimental system does not currently exist. In the work described here, the fluorescence properties of isometamidium were analysed.

In initial studies, the excitation spectrum of isometamidium at a constant emission wavelength of 600 nm was determined when the compound was dissolved in PBS; the maximum emission wavelength was achieved at 374 nm (results not shown). When using 374 nm as the excitation wavelength the emission spectra displayed two peaks: at 600 nm and at 645 nm (Figure 2a). It should be noted that the intensity of both peaks are similar. When lysates of *T. congolense* IL 1180 were added to isometamidium, there was an immediate shift in the λ_{max} at 600 nm to 584 nm; there was also an increase in fluorescence intensity of more than 4-fold (Figure 2b). In contrast, neither an alteration in λ_{max} nor a significant increase in fluorescence intensity were observed at 645 nm. We therefore examined the interaction of isometamidium with intact bloodstream forms of *T. congolense* IL 1180. When parasites were added to a solution containing isometamidium at a concentration of $100 \text{ ng} \cdot \text{ml}^{-1}$, there was a gradual time-dependent alteration in both the λ_{max} from that of the free compound (600 nm) to that of bound isometamidium, and an increase in fluorescence intensity at 600 nm over the experimental period of 180 min. However, neither an alteration in fluorescence intensity nor λ_{max} was observed at 645 nm (Figure 3). The shift in λ_{max} from 600 nm to 584 nm and the increase in fluorescence intensity resemble similar observations with a number of fluorescence indicators, in which their interaction with a specific ligand caused changes in their emission and/or excitation spectra. Examples are the calcium fluorescence probes quin 2, fura 2 and indo 2 (Tsien et al., 1982; Grynkiewicz et al., 1985; Philosoph and Zilberstein, 1989), and pH indicators such as 2,7-bis(carboxyethyl)-5,6-carboxyfluorescein (Rink et al., 1982; Zilberstein et al., 1989). The change in fluorescence properties of these compounds are determined by the concentration of the specific ligand. They have therefore been exploited for the determination of ligand concentrations inside various cells. By analogy to these various indicators, we quantified the alteration in fluorescence intensity of isometamidium by calculating $\Delta F_{(584-645)}$ as a function of time (Figure 4). The rate of increase in fluorescence with time gradually decreased with time and therefore demonstrated a saturation process with first-order steady-state kinetics. The gradual increase observed using intact bloodstream forms of *T. congolense* IL 1180 was in contrast with the immediate shift in fluorescence obtained when cell lysates were added to isometamidium. The results therefore suggested that the parasites' plasma membrane forms a barrier between the drug and its ligand. This hypothesis was therefore investigated by determining changes in fluorescence with time using constant excitation and emission wavelengths of 374 nm and 584 nm respectively.

The effect of temperature on the rate of increase in fluorescence with time is illustrated in Figure 5. When cells were incubated at

Figure 2 Emission spectra of isometamidium

(a) Spectrum analysis of isometamidium chloride at $100 \text{ ng} \cdot \text{ml}^{-1}$ was carried out at 37°C using an excitation wavelength of 374 nm . (b) Emission spectrum of isometamidium when incubated with digitonin-treated bloodstream forms of *T. congolense*. Spectrum analysis was carried out, as described in (a), on the digitonin-treated trypanosomes at the final time point given in Figure 6(a).

Figure 3 Alteration in the emission spectrum of isometamidium at various time intervals after incubation with *T. congolense* bloodstream forms

Parasites in PBSG ($1 \times 10^7 \cdot \text{ml}^{-1}$) containing $100 \text{ ng} \cdot \text{ml}^{-1}$ isometamidium chloride were incubated at 37°C . Aliquots (3 ml each) were placed in a glass cuvette at 0, 30, 45, 75, 105 and 180 min (lower plot to upper plot respectively), and the emission spectrum of isometamidium analysed as described in Figure 2(a).

37°C there was an initial rapid increase in fluorescence followed by a relatively slower phase. When the temperature was reduced to 15°C the rate of increase in fluorescence was reduced. Furthermore, at 4°C no change in fluorescence was observed. This further indicated that the gradual increase in fluorescence

Figure 4 Time course of the increase in the fluorescence of isometamidium when incubated with bloodstream forms of *T. congolense*

Each data point was derived from the fluorescence spectra given in Figure 3 by subtracting the fluorescence signal at 645 nm from that at 584 nm .

Figure 5 Effect of temperature on the fluorescence of isometamidium when incubated with bloodstream forms of *T. congolense*

Trypanosomes (final density $1 \times 10^7 \cdot \text{ml}^{-1}$; arrow) were added to PBSG containing $100 \text{ ng} \cdot \text{ml}^{-1}$ isometamidium chloride at 4, 15 or 37°C (lower, middle or upper plot respectively). Assays were carried out using excitation and emission wavelengths of 374 and 584 nm respectively.

Figure 6 Effect of digitonin on the interaction of isometamidium with bloodstream forms of *T. congolense*

Fluorescence assays were carried out at 37°C (a) or at 4°C (b), as described in Figure 5. The first arrow indicates the addition of cells ($6.7 \times 10^6 \text{ cells} \cdot \text{ml}^{-1}$). Digitonin was added to a final concentration of $10 \mu\text{M}$, indicated by the second arrow. The inset illustrates the effect of addition of $10 \mu\text{M}$ digitonin to isometamidium in PBSG.

with intact parasites, and the rate of change, was temperature-dependent. To assess further the role of the plasma membrane in this process, parasites were partially permeabilized with digitonin (Figure 6). Digitonin has previously been used to partially permeabilize the plasma membrane of various kinetoplastids (Docampo and Vercesi, 1989; Philosoph and Zilberstein, 1989), thereby facilitating the study of cellular enzymic activities in a semi-intact system. Thus cells treated with digitonin should be freely permeable to small molecules such as isometamidium.

Figure 7 Effect of NEM on the fluorescence of isometamidium when incubated with bloodstream forms of *T. congolense*

Assays were carried out at 37 °C and fluorescence analyses conducted as described in Figure 5, using a trypanosome density of 1×10^7 cells $\cdot$ ml $^{-1}$. (a) Control, without addition of NEM or digitonin; (b) cells treated with 1 mM NEM 10 min prior to addition to isometamidium in PBSG (first arrow); 10 μ M digitonin added at second arrow; (c) cells added at first arrow to isometamidium in PBSG; 1 mM NEM added at second arrow; 10 μ M digitonin added at third arrow.

Figure 8 Time course of uptake of isometamidium by bloodstream forms of *T. congolense* IL 1180

Parasites (3×10^7 cells $\cdot$ ml $^{-1}$) were incubated in PBSG + 100 μ M hypoxanthine containing [14 C]isometamidium (1μ g $\cdot$ ml $^{-1}$; 22.7 Ci $\cdot$ mol $^{-1}$) at 37 °C (○) and at 4 °C (●). At various intervals of time, uptake was terminated by centrifugation of 0.7 ml aliquots as described in the Materials and methods section.

Addition of 10 μ M digitonin to cells that had been preincubated with isometamidium chloride at 37 °C for 4 min resulted in an approx. 4-fold increase in the fluorescence intensity (Figure 6a). Furthermore, digitonin had a similar effect when added to cells that were preincubated at 4 °C (Figure 6b), where no increase in fluorescence was observed in intact cells. In a control experiment, digitonin had no effect on the fluorescence characteristics of isometamidium chloride (see the inset to Figure 6a).

NEM is a non-permeable thiol-group-reactive reagent and is therefore used to inhibit the activity of enzymes and membrane transporters exposed to the extracellular side of plasma membranes. Preincubation of parasites with NEM inhibited the increase in fluorescence (Figures 7a and 7b). When trypanosomes were first incubated with isometamidium, the addition of NEM resulted in immediate cessation of the increase in fluorescence (Figure 7c). In both experiments the addition of digitonin

resulted in a rapid increase in the fluorescence, similar to that illustrated in Figure 6.

The foregoing observations indicated that, in order for isometamidium to interact with the ligand causing alterations in its fluorescence, it has to cross the parasite's plasma membrane. Moreover, the inhibition of the increase in fluorescence by NEM, suggested that isometamidium is transported across the parasite's plasma membrane via a protein carrier.

In order to assess the role of carrier-mediated transport in a more direct manner, an experiment was set up to measure uptake of [14 C]-labelled isometamidium. Figure 8 illustrates the time course of the uptake of radiolabelled isometamidium by bloodstream forms of *T. congolense* IL 1180. When evaluated at 37 °C, a rapid initial rate of uptake was observed, which was followed by a gradual decrease in uptake, eventually resulting in a decline in the level of the label. When transport assays were carried out at 4 °C, very little uptake with time was observed (Figure 8). Thus, in a similar manner to the fluorescence data, uptake of [14 C]isometamidium demonstrated steady-state kinetics and temperature-dependency. The decline in cellular drug accumulation observed between 10 and 20 min may be a consequence of the 10-fold higher concentration of drug utilized compared with the fluorescence studies, and may be indicative of a toxic effect of the drug, or induction of an efflux process. Finally, addition of excess non-radioactive isometamidium to cells at steady-state transport resulted in rapid efflux of the radioactive compound from the cells (results not shown). In conclusion, these results with radiolabelled isometamidium are consistent with recent work by Sutherland et al. (1992) in which it was concluded that uptake of [14 C]isometamidium in populations of *T. congolense* is carrier-mediated.

Collectively our observations indicate that, in order for isometamidium to interact with the ligand causing an alteration in its fluorescence, it has to cross the parasite's plasma membrane. Moreover, the inhibition of the increase in fluorescence by NEM and the temperature-dependent uptake of radiolabelled isometamidium strongly suggests that isometamidium is transported across the parasite's plasma membrane by a protein carrier.

The results from this work constitute the first quantitative fluorescence measurements of the interaction of isometamidium with *T. congolense*. This technique can be exploited to investigate transport of isometamidium and its specific interaction internally. Furthermore, such interactions could be used to study the role of such parameters in drug-resistant populations of *T. congolense* and in other pathogenic African trypanosomes.

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