The accumulation and compartmentalization of isometamidium chloride in *Trypanosoma congolense*, monitored by its intrinsic fluorescence

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Interaction of the trypanocide isometamidium chloride with components of *Trypanosoma congolense* results in characteristic shifts in the intrinsic fluorescence of the drug. The specificity of this interaction was investigated by analysing the effects of various physicochemical manipulations on its fluorescence properties. The characteristic shifts involved a preferential increase in the intensity of one emission peak over the other, resulting in a systematic increase in the ratio of fluorescence intensities. These effects were apparently due to constraints on fluorophore free rotation in the solution (that is, viscosity). Purified DNA produced similar effects in a saturable manner displaying high affinity for the drug, indicating that the constraint involves binding of the drug to high-affinity binding sites within the DNA. Such binding sites were demonstrated in lysates derived from trypanosomal cells. The binding sites were associated with macromolecular species ($M_r > 12000$), and were partly disrupted by thermal denaturation and proteolysis. Treatment with DNase 1 produced high levels of disruption of the binding sites (> 85%), indicating an involvement of DNA in the binding. BSA demonstrated weak non-specific binding of the drug. Entry of drug into live trypanosomal cells (monitored by ^{14}C-labelled drug uptake) was paralleled by fluorescence shifts observed under comparable conditions of drug concentration and buffer conditions. Both systems (fluorescence shifts and accumulation of labelled drug) indicated the presence of a saturable membrane transporter with high affinity for the drug. We conclude that monitoring the fluorescence shifts of isometamidium constitutes a sensitive and highly specific probe for entry of the drug into trypanosomal cells, thereby enabling resolution of the transport events involved.

INTRODUCTION

Chemotherapy of Nagana (trypanosomiasis), an infectious disease of livestock caused by haemoflagellates of the species *Trypanosoma brucei*, *T. congolense* and *T. vivax*, is the major method for control in sub-Saharan Africa. In trypanosomiasis in cattle, sheep and goats only three compounds are currently recommended for treatment and prophylaxis: the phenanthridines isometamidium (Samorin, Trypamidium) and homidium (Novidium, Ethidium), and the aromatic diamidine diminazene (Berenil, Veriben). Because isometamidium was synthesized by combining the diazotized *p*-aminobenzamide moiety of diminazene with homidium in the presence of sodium acetate [1], all three compounds are chemically closely related. Both isometamidium chloride (7-[*m*-amidinophenyldiazoamino]-2-amino-10-ethyl-9-phenylphenanthridinium chloride) [2] and homidium chloride (2,7-diamino-9-phenyl-10-ethylphenanthridinium chloride) [3] are widely used throughout Africa and Asia as anti-trypanosomal agents [4]. Isometamidium chloride has a therapeutic effect, but is used primarily for its prophylactic activity, which lasts for up to 7 months against challenge with *T. congolense* [5]. The large variation in prophylactic activity observed with isometamidium is thought to be due to a variation in sensitivity of different trypanosome populations to the drug [6,7]. In the light of the apparent increase in the incidence of resistance to isometamidium [8–11], the long-term efficacy of this compound may be threatened. The scarcity of chemotherapeutic alternatives has made an understanding the mode of action of the drug, and possible mechanisms of resistance to it, imperative in order to determine regimes for rational and effective use of the drug.

The sensitivity of fluorophores to changes in environment, reflected in shifts in fluorescence intensity or the fluorescence spectrum, is well documented and forms the basis of a wide variety of techniques for monitoring physical, chemical, biochemical and physiological properties. Recent developments have extended the use of such compounds to direct observations on living cells, enabling real-time evaluation of such parameters as intracellular ion concentrations (pH, Ca^{2+}, Na^+, K^+, and so on) [12–15], membrane potentials [16], membrane trafficking and physical properties [17–20]. In a similar manner, drugs that possess intrinsic fluorescence properties have been used successfully to monitor directly the uptake, environment and subsequent fate of these compounds (for example, anthracyclin derivatives in the study of mammalian multiple drug resistance [21–23]).

A limited number of studies have been undertaken to characterize the accumulation and disposition of isometamidium chloride, both in the host and in the parasite, and several of these have exploited the fluorescence property of the drug [24–27]. Accumulation and processing/elimination of the drug was studied with fluorescence techniques, although most studies simply measured the fluorescence of extracted drug, or demonstrated its presence *in situ*. In a previous paper [28] we demonstrated this property to be (i) enhanced and (ii) reflected by characteristic shifts of the fluorescence spectrum on interaction with trypano-

Abbreviations used: DPBS; Dulbecco's PBS; PCA, perchloric acid; PSGH, DPBS supplemented with 5 mM glucose and 100 μM hypoxanthine.

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somes. In the initial studies, the sensitivity of this process to temperature and to blockage by *N*-ethylmaleimide indicated that a transport process mediated by a plasma membrane protein was involved, and the potential for using these intrinsic fluorescence shifts for monitoring the entry of isometamidium into trypanosomal cells, and of monitoring the intracellular environment, was investigated. In the studies presented in this paper we examine the nature of the interaction producing the observed fluorescence enhancements and spectral shifts, and demonstrate that such shifts can be used to define the kinetics of uptake of isometamidium chloride in isolated cells. Thus a rapid, non-intrusive, time-resolved method for the study of the uptake and processing of an important drug in the treatment and prophylaxis of trypanosomiasis has been developed.

MATERIALS AND METHODS

Derivation and preparation of trypanosomes

Trypanosoma congolense clone IL1180 is a doubly cloned derivative of an isolate from the Serengeti, Tanzania, as reported by Nantulya et al. [29]. The clone expresses a high level of sensitivity to isometamidium both in cattle [30] and in mice [7].

Sublethally irradiated Sprague–Dawley rats of both sexes were infected with an IL1180 stabilate (5×10^6 cells per rat). When parasitaemia reached $> 10^8$ cells per ml, rats were exsanguinated by cardiac puncture, and trypanosomes isolated from blood by DEAE-cellulose chromatography [31].

After isolation, trypanosomes were counted in a haemocytometer chamber, and diluted to 5×10^8 to 10^9 cells per ml in Dulbecco's phosphate-buffered saline (DPBS), supplemented with 5 mM glucose and 100 μM hypoxanthine (PSGH), and maintained on ice until use.

Preparation of trypanosomal lysates

Suspensions of *T. congolense* IL1180 (4×10^8 cells/ml), with a protease inhibitor cocktail (leupeptin, antipain, E64 and chymostatin, all at 40 μg/ml) were treated for 40 min with 1% (v/v) Triton X-100 on ice, with occasional vigorous vortex mixing. At the end of the incubation the suspension was passed 10 times through a 23-gauge needle. The lysate was centrifuged for 20 min at 14000 rev./min (15400 g_{av}), at 4 °C in an Eppendorf centrifuge, and the supernatant stored at −80 °C until use. Dialysed samples were prepared in Collodion bags by dialysis at 4 °C against 500 ml of 0.1% Triton X-100 in DPBS for 20 h. External buffer was changed after 2 and 16 h of incubation. Aliquots of the dialysed material were stored at −80 °C until use.

Preparation of Samorin stock solutions

Samorin (Lot no. DX0142 or U6378A) was prepared at 10.0 mg/ml in methanol and stored at −20 °C. ^{14}C-labelled drug was prepared at 10.0 mg/ml in water. Working solutions were prepared by stepwise dilutions of these stocks into appropriate media. As the material used was the commercial preparation, which is not pure isometamidium chloride [5], and the analysis of the ^{14}C-labelled material indicated it to have a similar composition, the drug used in these experiments will be referred to as Samorin.

Fluorimetry

Solutions were monitored in 4 ml acrylic cuvettes in a thermostatted cuvette holder. Fluorescence data were acquired in an Aminco SLM8000 spectrofluorimeter under software control.

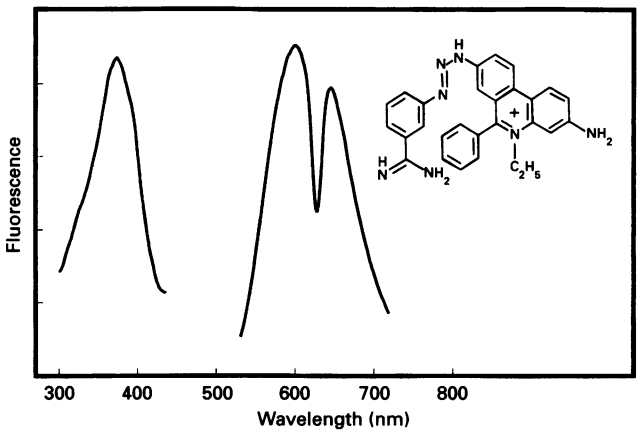

Figure 1 Excitation and emission spectra of Samorin

Inset: Structure of isometamidium, the major component of Samorin.

Typically, excitation and emission slits were set at 8 nm, and counts were accumulated for 2–20 s with an externally cooled photomultiplier. Spectral peak intensities were determined by integrating 10 nm either side of the peak wavelength. Excitation and emission spectra of Samorin under these conditions are shown in Figure 1.

Fluorescence properties of free Samorin

Influence of pH

Samorin (10 mg/ml in methanol) was diluted into 10 mM Hepes/H_3PO_4, pH 8.5, or Mes/NaOH, pH 4.5, to a final concentration of 4 μg/ml. These solutions were mixed in various proportions to produce the indicated pH values (4.57–8.52). Emission spectra were collected for each solution over the range 550–700 nm (excitation wavelength 374 nm), and emission intensity was integrated over the ranges 580–600 nm and 635–655 nm. The ratio of fluorescence intensities for the two ranges was calculated, and fluorescence intensity in the range 580–600 nm recorded.

Influence of dielectric constant

Solutions with various dielectric constants were prepared by the method of Bramhall [32], by mixing solutions of 4 μg/ml Samorin in distilled water or dioxane in defined proportions. The experiment used dioxane in the range 0–80% (v/v), equivalent to ϵ_0 78.4–10.0. Emission spectra were collected for each solution over the range 550–700 nm, and emission intensity was integrated over the ranges 580–600 nm and 635–655 nm. The ratio of fluorescence intensities for the two ranges was calculated, and fluorescence intensity in the range 580–600 nm was recorded and normalized to the value recorded in water.

Influence of medium viscosity

Sucrose solutions were prepared by dissolving a known weight of sucrose in a known weight of water, producing a series of solutions in the range 0–57% (w/w). Samorin (10 mg/ml in methanol) was added to a final concentration of 4 μg/ml. Relative viscosities were determined from tabulated data [33]. Solutions were equilibrated to 20 °C. Emission spectra (λ_{ex} = 374 nm) were collected over the range 550–700 nm in a thermostatted cuvette holder. Emission intensity was integrated over the ranges

580–600 nm and 635–655 nm. The ratio of fluorescence intensities for the two ranges was calculated, and fluorescence intensity in the range 580–600 nm was recorded and normalized to the value recorded in water.

Titration of Samorin fluorescence shifts

Fluorescence intensity shifts were monitored following sequential additions of small volumes (< 10 μl) of Samorin working solutions (25 or 100 μg/ml in DPBS) to solutions of model macromolecules, or dilutions of trypanosomal lysates maintained in a thermostatted cuvette holder. Following addition, mixing, and equilibration for 40–60 s, fluorescence was recorded (λ_{ex} = 374 nm, λ_{em} = 590 nm, integration 20 s). Where necessary (that is, Samorin concentrations greater than 2.5 μg/ml), inner filter effects were compensated for by the method of Bagshaw and Harris [34]. Data were arithmetically corrected for dilution effects before analysis, and fitted to a single-site binding system with a linear component due to unbound ligand, by the Mardquat algorithm (Slidewrite 2.0 graphics application).

Fluorescence shifts determined in live trypanosomes

Trypanosomes were suspended in PSGH at 2×10^8 cells/ml, maintained on ice and warmed to 37 °C immediately before use. Warmed cell suspension (100 μl) was added to 2.9 ml of PSGH containing appropriate concentrations of Samorin. Fluorescence was monitored (λ_{ex} = 374 nm, λ_{em} = 590 nm, integration 0.9 s, acquisition every 1 s), recorded in files written by the software system, and converted to text files that were used to determine kinetic parameters.

Determination of Samorin uptake by using ^{14}C-labelled drug

Uptake of Samorin by trypanosomes (IL1180) was determined by a modification of the oil-sandwich centrifugation technique previously reported [28]. Briefly, 100 μl of trypanosomal suspension [(2–4) $\times 10^8$ per ml in PSGH] was added to 600 μl of PSGH containing ^{14}C-labelled Samorin (37.5 mCi/mg) with vortexing at 37 °C. Before termination of the reaction, 600 μl of the reaction mixture was transferred to a 1.5 ml Eppendorf tube, containing 50 μl of 14% (w/v) perchloric acid (PCA), overlain with 150 μl of dibutyl phthalate. The reaction was terminated by rapid centrifugation of the cells into the PCA layer in an Eppendorf benchtop centrifuge operating at 14000 rev./min (15400 g_{av}). Under these conditions transfer was > 90% complete within 10 s (results not shown). Zero-time incubations were performed by suspending an appropriate volume of cell suspension in the cap of an Eppendorf tube, over a volume of buffer containing labelled Samorin. After placing the tube in a rotor, transfer of the cells to the PCA layer was effected by centrifugation. After removal of the upper layer, 40 μl aliquots were subjected to liquid scintillation counting.

RESULTS

Influence of physical and chemical environment on Samorin's fluorescence properties

Several properties were investigated to determine a possible basis for the fluorescence shifts observed *in vivo* [28]. It was established that the fluorescence properties of Samorin (both fluorescence intensity and peak ratios) in dilute aqueous solutions (DPBS) were independent of temperature over the range 12–55 °C. Similarly, fluorescence properties were independent of ionic strength (Γ = 300 mM–3 M, manipulated by additions of NaCl) (results not shown).

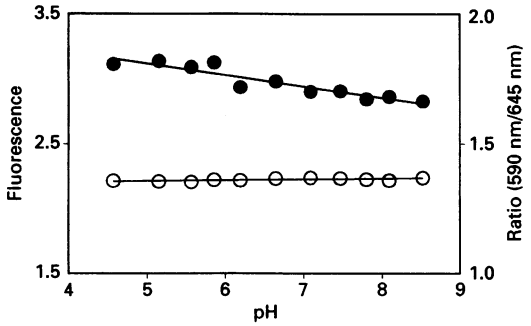

Figure 2 Influence of pH on Samorin's spectroscopic properties

Fluorescence properties of Samorin (4 μg/ml) were determined in solutions of various pH values. The total emission intensity (●) and the ratio of intensities at 590 and 645 nm (○) are shown.

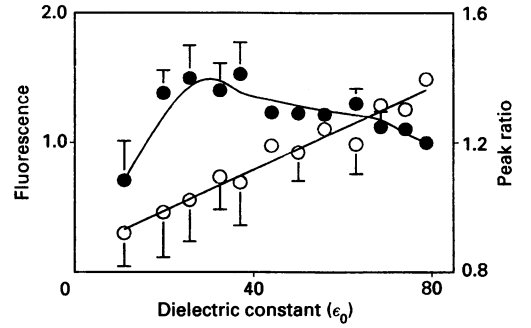

Figure 3 Influence of medium dielectric constant on Samorin's spectroscopic properties

Fluorescence properties of Samorin (4 μg/ml) were determined in solutions of various concentrations of dioxane in order to manipulate the dielectric constant. The total emission intensity (●) and the ratio of intensities at 590 and 645 nm (○) are shown. Points represent means and S.D. for two determinations. Fluorescence intensity is normalized to the value in aqueous solution.

The effects of manipulating pH on the fluorescence properties of Samorin are summarized in Figure 2. The data indicate that the peak ratios are insensitive to medium pH, and that fluorescence intensity is at best marginally enhanced by low pH. Sequestration of the compound into a low-pH compartment in the cell would therefore seem to be discounted as a mechanism for the observed fluorescence shifts.

Manipulation of the dielectric constant influences the fluorescence properties of Samorin (Figure 3), demonstrating a linear relationship between the peak ratio and values of ϵ_0. Modest enhancement of fluorescence intensity with decreasing ϵ_0 is observed in the range 78.4 to ~ 20, with a subsequent fall on further decrease. The results argue against the cause of the observed shifts on uptake in isolated cells being the entry of the compound into environments of low dielectric constant (for example, lipids or protein interiors); the fluorescence enhancements are too small to account for the observed shifts and, in contrast, the peak ratios are seen to increase on uptake.

Modulation of medium viscosity by sucrose produces clear enhancements of fluorescence intensity and peak ratios (Figure 4). Over the range used (η/η_0 1–35), fluorescence intensity was enhanced by > 150% and peak ratios increased moderately.

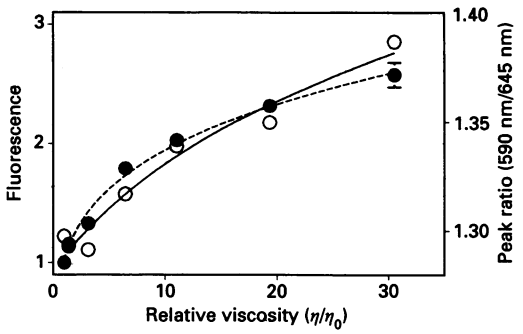

Figure 4 Influence of medium viscosity on Samorin's spectroscopic properties

Fluorescence properties of Samorin (4 μg/ml) were determined in solutions of various concentrations of sucrose in order to manipulate viscosity, calculated as relative viscosity (η/η_0, where η represnts the viscosity of the solution of interest and η_0 the viscosity of pure water). The total emission intensity (●) and the ratio of intensity at 590 and 645 nm (○) are shown. Points represent means and S.D. for two determinations. Fluorescence intensity is normalized to the value in aqueous solution.

This is the only physical parameter to simulate the effects of entry into cells, and hence it may indicate that the effects observed in cells are due to constraints on the rotational freedom of the fluorophore. Attempts to investigate the environment of the fluorophore by fluorescence polarization (by analogy with such probes as diphenylhexatriene [18,20] or perylene [17]) were unsuccessful, possibly owing to a small, or diffuse, induced dipole moment [18,35–37].

Influence of model compounds on Samorin's fluorescence properties

Bovine serum albumin

In the presence of BSA (3 mg/ml; 45 μM) modest enhancements of Samorin's fluorescence were observed (Figure 5). After correction for inner filter effects, and subtraction of the fluorescence due to unbound fluorophore, the observed change in fluorescence as a function of added ligand demonstrates a small saturable component superimposed on a non-saturable function (figure 5, inset). The apparent K_d for the saturable component was 0.214 ± 0.039 μg/ml (three experiments). After subtraction of the linear component, the saturable component was analysed by the method of Webb as modified by Bagshaw and Harris [34], as shown in Figure 6. The K_d calculated by this process (0.354 ± 0.016 μg/ml) was in close agreement with the apparent K_d, but the concentration of binding sites was extremely low.

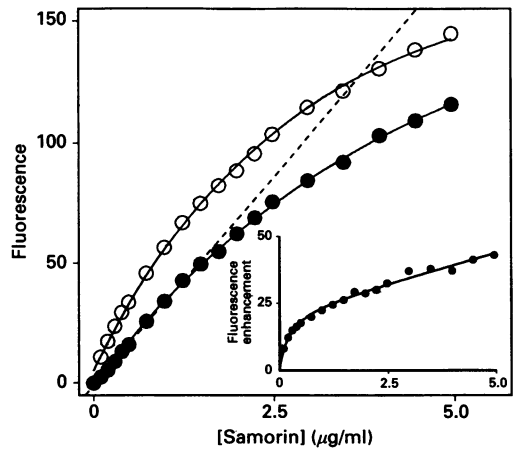

Figure 5 Titration of Samorin's fluorescence: effect of BSA

Fluorescence intensities were determined after stepwise additions of Samorin stock solutions in the presence (○) or absence (●) of BSA (3 mg/ml; 45 μM). Non-linearity of fluorescence in the free Samorin curve is due to inner filter effects, which were corrected for arithmetically by extrapolation from the linear portion of the curve (dotted line). This permitted the determination (shown in the inset) of the fluorescence intensity enhancement due to the presence of BSA.

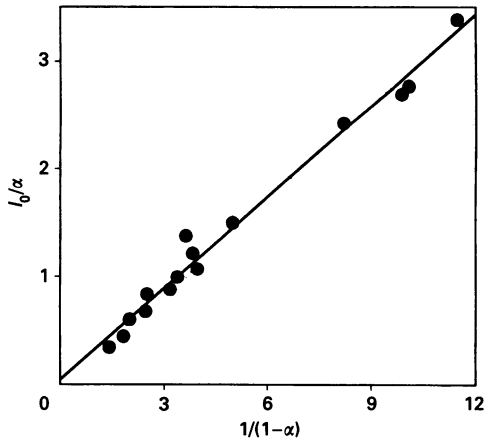

Figure 6 Analysis of Samorin's binding isotherm data for BSA

Data were analysed after subtraction of the linear component of the binding isotherm, by the method of Webb modified by Bagshaw and Harris [34]. Here α represents the fraction of protein bound to Samorin (that is, $\Delta fluor_{obs}/\Delta fluor_{max}$, where Δfluor is fluorescence enhancement on addition of Samorin) and I_0 the concentration of added ligand. The slope of the line is equivalent to K_d; the y axis intercept is equivalent to the concentration of the binding sites.

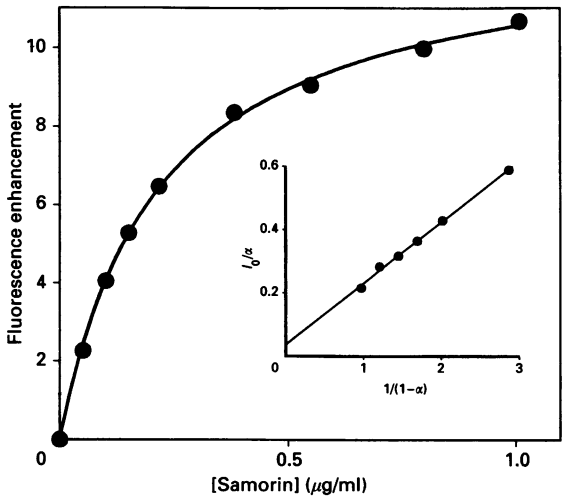

Figure 7 Titration of Samorin's fluorescence: effect of calf thymus DNA

Fluorescence intensities were determined after stepwise additions of Samorin stock solutions in the presence or absence of DNA (25 μg/ml). Fluorescence enhancements are presented after subtraction of fluorescence due to free Samorin. Over the concentration range used, inner filter effects were minimal. Inset: analysis of fluorescence titration data.

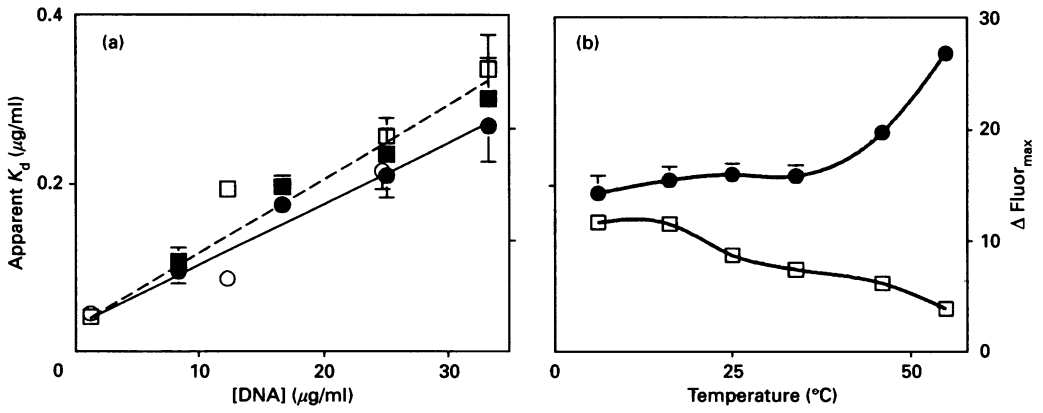

Figure 8 Titration of Samorin's fluorescence: effect of calf thymus DNA and temperature

Effects of varying DNA concentration and temperature on binding parameters. Binding parameters for the interaction of Samorin with DNA were determined under different conditions. (**a**) Parameters were determined for samples containing DNA at concentrations of 1 to 33.3 μg/ml (data from two experiments). (**b**) Parameters were determined for samples containing DNA at a concentration of 25 μg/ml, maintained at temperatures ranging from 4 to 55 °C. ○, ●, K_d values; □, ■, Δfluor$_{max}$ values.

Table 1 Effects of various treatments of trypanosome cell lysates on fluorescence enhancement with Samorin

Samples of a cell lysate of IL1180 were treated as described. After treatment the samples were diluted and the fluorescence enhancement was titrated by incremental additions of stock Samorin. Data represent means ± S.D. of the number of determinations shown.

Preparation	Maximal fluorescence enhancement (counts per 20 s)	Apparent K_d (μg/ml)
Lysate ($n = 2$)	9326 ± 511	0.0975 ± 0.0142
Dialysate ($n = 3$)	7867 ± 283	0.0735 ± 0.0065
High temp. ($n = 2$)	4787 ± 153	0.0558 ± 0.0041
DNase 1 ($n = 3$)	1867 ± 251	0.0798 ± 0.019
Proteinase K ($n = 2$)	3858 ± 259	0.0543 ± 0.0085

This may indicate that the observed binding is due to a minor contaminant in the BSA preparation.

DNA

In the presence of purified DNA (25 μg/ml) the fluorescence of Samorin was enhanced markedly (Figure 7), and demonstrated saturability. Analysis of the binding data (Figure 7, inset) indicates a maximal binding of 3.6 ng of Samorin per μg of DNA. The apparent K_d was 0.216 ± 0.008 μg/ml by the first analysis and 0.188 ± 0.021 μg/ml by the second.

Determination of binding parameters with a range of DNA concentrations demonstrated that, although maximal fluorescence enhancements were proportional to the amount of DNA present, the apparent K_d was not constant, but increased with

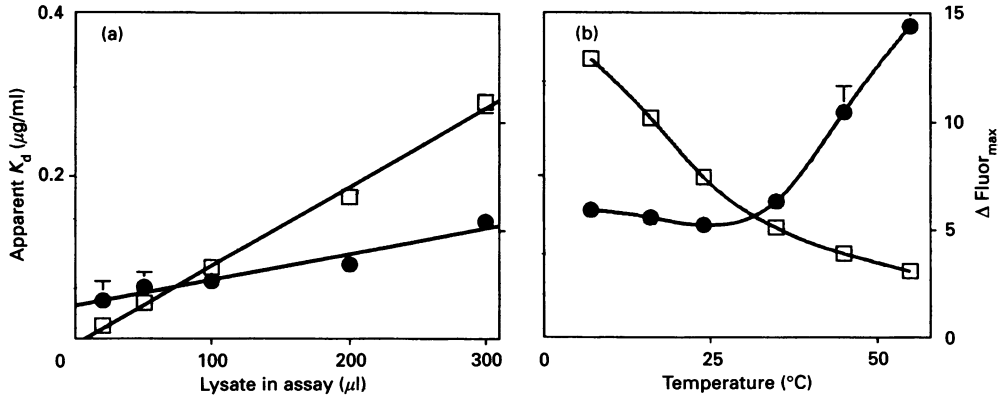

Figure 9 Titration of Samorin's fluorescence: effect of trypanosomal cell lysate

Effects of varying lysate concentration and temperature on binding parameters. Binding parameters for the interaction of Samorin with trypanosomal cell (IL1180) lysates were determined under different conditions. (**a**) Parameters were determined for samples containing lysate at dilutions of 6.7–100 μl/ml (material from 2.7×10^6 to 4×10^7 cells). (**b**) Parameters were determined for samples containing lysate at a dilution of 150 μl/ml (6×10^7 cells), maintained at temperatures ranging from 6 to 55 °C. ●, K_d values; □, Δfluor$_{max}$ values.

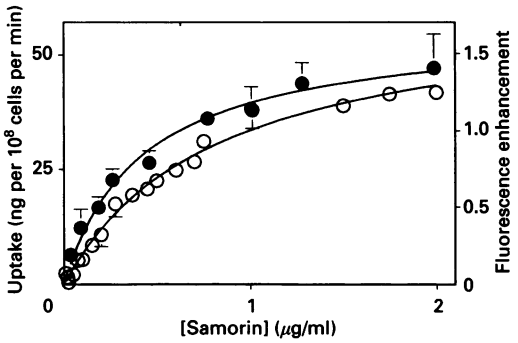

Figure 10 Uptake of Samorin by *Trypanosoma congolense* IL1180: kinetics of uptake

Cells of bloodstream forms of IL1180 were isolated from the blood of infected rats. Uptake of the drug by these cells was determined as described in the text. Initial rates of uptake or fluorescence enhancement were determined as a function of external Samorin concentration and fitted to a simple rectangular hyperbolic function (Michaelis–Menten kinetics). ○, Determination by fluorescence shift, $n = 2$–4; ●, determination by labelled drug accumulation, $n = 3$.

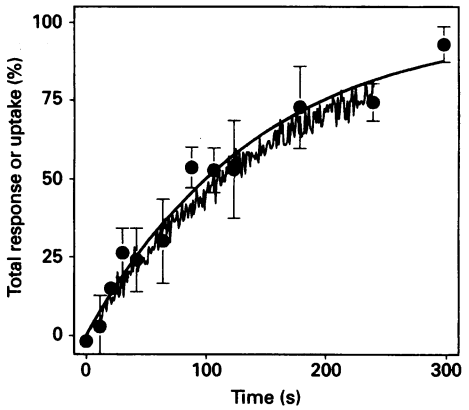

Figure 11 Uptake of Samorin by *T. congolense* IL1180: time course of accumulation

Cells of bloodstream forms of IL1180 were isolated from the blood of infected rats. Uptake of the drug by these cells was determined as described in the text. Uptake and fluorescence enhancement were monitored as a function of time in the presence of an external Samorin concentration of 2 μg/ml. The data were fitted to a first-order process with an offset (Table 2). For comparisons, the non-zero offset values were subtracted and the responses at the time points expressed as a percentage of the limiting value. ——, Determination by fluorescence shift; ●, determination by labelled drug accumulation, $n = 3$.

increasing concentration of DNA (Figure 8a). The limiting apparent K_d was of the order of 0.05 μg/ml. Thus the hypothesis that the individual binding sites are independent is not supported; the data indicate that increasing concentrations of DNA act as a 'competitive inhibitor' for the binding sites. The effect of temperature on the binding parameters is summarized in Figure 8(b). Maximal fluorescence enhancements and apparent K_d for fluorescence titrations in the presence of 25 μg/ml DNA, over the temperature range 6–55 °C, were determined. Maximal fluorescence enhancements decreased with increasing temperatures, whereas the values for the apparent K_d were stable up to 40–45 °C, and subsequently increased with temperature. This effect could be associated with some translocation or transition of double-stranded DNA, and could indicate an enhanced affinity of the drug for double-stranded DNA.

Trypanosomal cell lysates

Titration of cell lysates with Samorin also demonstrated saturable curves, with an apparent K_d in the range 0.05–0.40 μg/ml. The fluorescence-enhancing activity was essentially unaffected by dialysis of the sample (M_r cutoff 12 000), and > 60% was recovered after 10 min incubation at 95 °C. Proteinase K treatment (0.5 mg per 100 μl of lysate, 37 °C for 40 min) decreased maximal fluorescence enhancement by 50%, whereas treatment with DNase 1 (500 units per 100 μl lysate, 37 °C for 40 min) decreased it by 76% (Table 1).

In a separate series of experiments, the effects of increasing concentrations of the cell lysate, and of increasing temperature on the binding parameters, were studied. Both sets of data closely paralleled the effects of increasing levels of DNA, with an apparent K_d of 0.05 μg/ml for a limiting dilution of dialysate (Figures 9a and 9b).

Interaction of Samorin with intact trypanosomal cells

The fluorescence shifts attendant upon the addition of IL1180 (2×10^7 cells in a total volume of 3.0 ml) to solutions containing various concentrations of Samorin were monitored as previously described. Data acquisition was at 1 s intervals, with integration over 0.9 s. Data points recorded between 10 s and 30 s were used to determine initial rates by regression analysis. Plotted as a function of Samorin concentration (Figure 10), the rates of fluorescence enhancement demonstrated a rectangular hyperbolic relationship. By analogy with standard Michaelis–Menten kinetics, the data give a V_{max} of 1.833 ± 0.132 fluorescence units per s, and a K_m of 0.826 ± 0.089 μg/ml.

In parallel experiments, the uptake of ^{14}C-labelled drug, monitored over 150 s, was also described by a rectangular hyperbolic function, with the following kinetic parameters: $V_{max} = 56.4 \pm 7.3$ μg/min per 10^8 cells, $K_m = 0.425 \pm 0.056$ μg/ml.

Although direct comparisons of the V_{max} values are not possible in these systems, the values for K_m are comparable, even if not coincident.

The time course of the interaction was also investigated with both systems (Figure 11). Interaction of the cells with a Samorin

Table 2 Parameters fitted to the time course of fluorescence enhancement, or the time course of ^{14}C-labelled drug accumulation by trypanosomes in the presence of Samorin

The process of drug accumulation (Samorin at 2 μg/ml) determined by the methods outlined in the text was analysed as a first-order process by the Mardquat algorithm. The parameters for the process are reported with standard errors.

Method	Rate constant (s^{-1})	Limit	Offset
Fluorescence monitoring (counts per 0.9 s)	$(6.22 \pm 0.31) \times 10^{-3}$	431 ± 9	1048 ± 7
Uptake of ^{14}C (ng per 10^8 cells)	$(7.10 \pm 1.32) \times 10^{-3}$	403.9 ± 29.4	154.8 ± 18.1

concentration of 2 μg/ml provided sets of data fitting a single first-order process, plus a basal offset value. The parameters are summarized in Table 2 and demonstrate that the rate constant for the process is very close in both systems, indicating that both techniques monitor the same process.

DISCUSSION

The fluorescence properties of Samorin provide a sensitive method for studying its interactions and behaviour in a variety of environments. The interaction of the drug with live, drug-sensitive trypanosomes of the clone *T. congolense* IL1180 produces a set of highly characteristic changes; a systematic enhancement of fluorescence at 590 nm, and little or no change at 640 nm (that is, 590 nm/640 nm ratios increase on interaction). Such fluorescence shifts may be regarded as diagnostic of the interaction of isometamidium chloride with the trypanosome. We have demonstrated qualitatively similar shifts in a number of populations of *T. congolense* and *T. brucei*, so the response is not confined to the clone IL1180 but appears general to trypanosomes. Interpretation of quantitative differences in the responses of these populations will be the subject of a future paper.

Monitoring spectroscopic properties under various conditions indicates that properties of the fluorescence reflect changes in the physical environment of the drug. For instance, decreasing the dielectric constant of the medium produced modest increases in quantum yield, possibly owing to enhancement of the fluorescence dipole in media of low dielectric constant. However, the peak ratios decreased, showing a bias towards the lower-energy, red-shifted peak, in contrast to the situation observed in interactions with live trypanosomes. Variation in pH had no significant effect on fluorescence properties. Thus observed fluorescence shifts in the trypanosome are not due to sequestration into low-pH or high-pH compartments.

The physical property that best simulated the fluorescence changes observed *in vivo* was modulation of viscosity by sucrose. The effects of this were to increase quantum yield (that is, fluorescence enhancements), while favouring the lower-wavelength peak of the emission spectrum (that is, increasing peak ratios). Such effects on reporting fluorophores frequently result from constraints on the rotational freedom of the molecule, decreasing the probability of the excited state molecule's decaying to the ground state by vibrational or rotational transfer of energy, and increasing the probability of photon emission. We speculate that the result seen in the interactions with live trypanosomes reflects a similar constraint of the molecule.

Interactions of the drug with BSA produced small increases in fluorescence of the drug. Although these were consistent with decreased mobility of the drug, the shifts were mainly due to a non-specific interaction of the molecule with the protein. The result is not significant in the context of the entry of drug into the trypanosomal cell, as the observed responses were small. Furthermore, the data indicated that the interaction of drug with the major serum protein in the mammalian host is weak, with a significant proportion of free drug present in the environment of the parasite during the bloodstream stage of the life cycle.

The interaction of Samorin with DNA is highly significant. The fluorescence enhancements were large and demonstrated saturability with respect to the concentration of drug. These results indicate that the DNA possesses binding sites with a high affinity for the drug. It is consistent that a high degree of constraint on the fluorophore attendant on binding to the macromolecule would produce the observed fluorescence shifts. Purified DNA showed a phenomenon of self inhibition, with higher concentrations of DNA increasing the value of the apparent K_d (that is, decreasing affinity for the binding sites). However, the maximal fluorescence shifts (corresponding to maximal binding of drug) were proportional to the concentration of DNA. Hence the number of binding sites remained constant as a function of DNA.

The effect of temperature on the interaction with DNA demonstrated two phenomena. First, the maximal fluorescence shift decreased with increasing temperature, which may reflect the availability of rotational and vibrational modes to allow the excited state to return to the ground state without emission of a photon. Second, the apparent K_d (that is, affinity) of the binding was constant from 6 to 37 °C. Above this it rose sharply (that is, decreased affinity), which may indicate a structural transition that affects the binding sites.

These data are in agreement with previous studies, which have indicated an intercalation of phenanthridines (homidium and isometamidium) between base pairs in double-stranded DNA. Previous spectroscopic analysis of this effect used absorption shifts, which are relatively insensitive, requiring large concentrations of DNA and Samorin. However, despite the major distortions of the binding isotherm produced by increasing levels of DNA in the system, the apparent K_d reported by these authors (equivalent to 87 ng/ml; [38]) is consistent with that reported here.

In trypanosomal lysates, the effects of temperature and of concentration of lysate closely paralleled the effects observed with DNA. Furthermore, fluorescence shifts were unaffected by dialysis, indicating that the species responsible is a macromolecule ($M_r > 12000$). Finally, the structure responsible for the fluorescence shifts was partly affected by treatment with proteinase K and thermal denaturation, whereas the fluorescence shift was decreased by 85% after treatment with DNaseI. Thus the fluorescence shifts appeared to be due mainly to DNA present in the lysates, although some interaction with protein or protein–DNA complexes may have contributed to the process. The source of DNA in these lysates is not known with any certainty, and could be derived from either nuclear or kinetoplast material.

The accumulation of Samorin has also been monitored in live cells of IL1180 by ^{14}C-labelled drug to compare the response of fluorescence to uptake of the drug. We have demonstrated previously that cells of the clone IL1180 accumulate isometamidium chloride in a time-dependent manner [28]. Here we have shown that the initial rates of the uptake process demonstrate saturable kinetics, consistent with reports of a saturable carrier system for the drug [27]. We compared these results with the fluorescence shifts observed under comparable conditions, and have demonstrated that initial rates of fluorescence enhancement show saturable Michaelis–Menten-type kinetics consistent with the uptake of ^{14}C-labelled drug.

The time course of drug uptake, monitored by labelled drug and fluorescence shifts, conformed to a first-order rate process; values for the rate constant were essentially identical using the two methodologies. We therefore interpret these data as an indication that accumulation of ^{14}C-labelled drug and the fluorescence shifts resulting from exposure of the cells to drug are indications of the same process, that is, entry of the drug into the trypanosome.

The data described also provide information on the mode of entry of the drug into trypanosome cells. The fluorescence shifts observed on interaction of the drug with trypanosomal cells are inconsistent with an environment of low dielectric constant (that is, lipids, membranes or the hydrophobic interiors of proteins). This observation is significant, because a suggested mechanism for the entry of phenanthridines into cells involves interaction of the cation with the anionic head-groups of phospholipids of the

cell membrane, forming a neutral lipophilic complex capable of diffusion through the membrane, thereby facilitating entry into the cytoplasm [38]. This proposal is similar to the low-affinity, non-saturable transport of iron in the mammalian gut, mediated by acidic phospholipids [39,40] The absence of any pH-dependent shifts in the spectrum of the drug indicates that no significant changes occur in the physical structure of the molecule over a wide range of pH. Together with the absence of any obvious ionizable group on the structure of the molecule, this argues against a titratable pH response of the molecule, or the involvement of a 'pH shuttle' to effect translocation across the membrane.

Previous work has confirmed the plasma membrane as the rate-limiting factor in the accumulation of Samorin by bloodstream forms of *T. congolense*, and inhibition of the accumulation by *N*-ethylmaleimide implicated a protein in the process [28]. Subsequently the process of uptake was described as being saturable with respect to external drug concentration in populations of *T. congolense* [27], a situation consistent with the involvement of a specific transport protein in the accumulation.

A possible model for the differential sensitivity of mammalian and kinetoplastid cells to the drug is suggested by the data. Cells of mammalian hosts accumulate Samorin [24,41], but to a lesser extent than observed in trypanosomal cells. This may be due to a lack of a specific transport process in mammalian cells. As a result, entry of the drug will be limited to simple diffusion or entry via lipid complexes. Differential sensitivity between host and trypanosome due to the existence of a specific trypanosomal transport mechanism has been reported in the case of MDL 73811, an inhibitor of *S*-adenosyl-L-methionine decarboxylase [42,43]. The process for MDL 73811 uptake was linked to nucleoside transport in *T. brucei*. Interestingly, uptake of the arsenical drug melarsoprol in the same species was found to be mediated by an adenosine transport activity, which constituted one of several such activities that could be identified. Resistance to the drug was shown to be due to a specific down-regulation of this transport activity [44].

Previous investigations of Samorin uptake in *T. congolense* have indicated an inhibition by salicyl hydroxamic acid [26], an inhibitor of ATP production in bloodstream-form trypanosomes [45]. As glycolysis is the sole source of ATP generation in these organisms, the uptake of the drug appears to involve an active, energy-linked process. Active transport of compounds into cells frequently results in their accumulation above a concentration gradient. The internal volume of bloodstream-form IL1180 is 1.18 μl per 10^8 cells (W. Mulugeta, personal communication). In the presence of Samorin at 2.0 μg/ml the steady-state accumulation of drug is 403.9 ± 29.4 ng per 10^8 cells (Table 2). This represents an internal concentration of 342 μg/ml, a more than 170-fold accumulation over external concentration. The accumulation thus requires the expenditure of metabolic energy, consistent with an active process. However, the nature of the active transport (primary or secondary) cannot be established on the basis of these studies and will require further investigation.

The high affinity of the drug for DNA makes this a likely target in the action of Samorin. Work on the interaction of trypanocides with *T. equiperdum* [46] has indicated that a number of trypanocides, including Samorin, are capable of linearizing minicircle DNA in the kinetoplast, a specialized structure of concatenated mitochondrial DNA unique to the class of Protozoa (Kinetoplastidea) that includes the trypanosomatids. The kinetoplast is intimately involved in the cell cycle of trypanosomatids, and its replication and segregation form an essential stage in the reproduction of the trypanosome cell [47]. The disruption of kinetoplast structure has been implicated in the mechanism of trypanocide action [48]. The effect appears to be due to an interaction of the drug with DNA and a topoisomerase, in a manner similar to the effects of etoposide, an inhibitor of mammalian topoisomerase II. Topoisomerase activity is necessary for the process of decatenation–concatenation required for the replication of kinetoplast DNA, and it seems that several trypanocidal drugs, including Samorin, specifically affect a mitochondrial topoisomerase activity. With Samorin, in the early stages of exposure to drug, a focus of fluorescence corresponding to the accumulation of drug appeared in a position coincident with the kinetoplast [26], with no staining of nuclear material. Thus preferential accumulation of drug into the kinetoplast may well explain the differential activity without invoking the existence of organelle-specific subforms of the topoisomerases, as suggested by Shapiro and Englund [46]. We postulate that a specific uptake process, capable of concentrating Samorin above its external concentration, plus a subsequent preferential affinity for and transport into the mitochondrial compartment results in a high concentration of drug in this compartment. The high affinity of the drug for DNA results in its concentration in the kinetoplast, where, on interaction with topoisomerase, it is responsible for the formation of 'cleavable complexes' leading to the linearization of minicircle DNA. Further studies on the fate and processing of accumulated drug will be required to answer this question.

In summary, these studies confirm that the fluorescence property of Samorin is an appropriate probe to study the transport activity of the drug. The kinetics of fluorescence shifts observed on exposure of cells to Samorin parallel those of the accumulation of ^{14}C-labelled drug under the same conditions. The nature of the fluorescence response corresponds to a tight binding of the fluorophore to intracellular binding sites, initially identified as DNA or a DNA–protein complex. The data do not support a role for the diffusion of neutral lipophilic drug–phospholipid complexes in this process. Both methods confirm the presence of a saturable transport mechanism in the membrane of the trypanosome and a subsequent concentration of drug within the parasite cell, requiring metabolic energy. The drug seems to exploit fortuitously a pre-existing transport process, and this, coupled with a high degree of concentration within the cell, forms the basis of the specificity of the drug for the parasite. Further studies of the drug's transport mechanism, the accumulation and energization of the process, and the fate and deposition of the drug will address mechanisms of its action, and of resistance in the parasite.

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