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Uptake of α-difluoromethylornithine by *Trypanosoma brucei brucei*

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α-Difluoromethylornithine (DFMO), a catalytic irreversible inhibitor of ornithine decarboxylase (ODC) [1], has been shown to rapidly deplete the intracellular polyamines putrescine and spermidine in *Trypanosoma brucei brucei* and to inhibit trypanosomal DNA synthesis and proliferation [2], as it does in a number of rapidly proliferating cell types [3]. Administration of DFMO effects cures of African trypanosomiasis in both experimental murine infections [4] and human sleeping sickness [5]. Trypanosomal proliferation *in vitro* is inhibited completely by concentrations of DFMO as low as 50 μM (unpublished observations), whereas rat hepatoma tissue culture cells (HTC cells) continue to grow, albeit at a much reduced rate, in the presence of 5 mM DFMO [6].

Presently, there is not an adequate explanation as to why trypanosomes appear to be uniquely sensitive to the polyamine-depleting effects of DFMO as compared to other cells. Trypanosomal ODC has approximately the same sensitivity to DFMO ($K_i = 130$ μM) [7] as does mammalian ODC ($K_i = 39$ μM) [8]. However, preliminary studies suggested that DFMO may be transported into the trypanosome by an active, energy-dependent system [2]. This was an intriguing hypothesis since selective, active uptake of DFMO by trypanosomes might partially explain the great sensitivity of the organisms to the drug. Since DFMO is a basic amino acid analogue and active transport of basic amino acids has been described for mammalian cells [9] as well as for trypanosomes [10], it was possible that DFMO uptake was mediated by one of these transport systems in the trypanosome. Other workers had already shown this not to be the case for mammalian cells [11–13]. We have now investigated more rigorously the uptake of DFMO by *T. b. brucei* and have concluded that DFMO enters the parasite by passive diffusion.

Methods

Trypanosomes. Trypanosoma brucei brucei (EATRO 110) was maintained by syringe passage in male Sprague–Dawley rats (250–300 g). Trypanosomes were harvested from blood obtained by cardiac puncture from 72-hr infected rats and separated from blood elements on DEAE-cellulose columns [14] using a solution of 90 mM Tris·HCl (pH 7.8) containing 50 mM NaCl and 2% (w/v) glucose for equilibration and elution of the columns.

Mammalian cell culture. Hepatoma tissue culture (HTC) cells were maintained in spinner cultures using Swims G67 medium containing 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin and 10% newborn calf serum.

DFMO uptake. Purified trypanosomes were washed twice with either Eagle's Minimum Essential Medium (MEM), containing Earle's salts, supplemented with non-essential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 0.2% glucose, 10% fetal bovine serum and 30 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (pH 7.5) or with 70 mM phosphate buffer (pH 8.0) containing 43 mM NaCl, 1% glucose, 50 units/ml penicillin, 50 μg/ml streptomycin and 1% bovine serum albumin (PSG-BSA), and then suspended in the respective wash buffer in preparation for uptake studies. HTC cells were washed twice with fresh growth medium and then suspended in the same medium for uptake incubations. Trypanosomes or HTC cells were then incubated with continuous shaking with [^{14}C]DFMO (usually 3.3 μCi/ml) or nonradioactive DFMO at 37°. Incubations were terminated by rapid centrifugation (1 min) of the trypanosomes or HTC cells and rapid washing with an ice-cold solution of 10 mM Na_2HPO_4, 3 mM KH_2PO_4 and 0.125 M NaCl (pH 7.2) after which the sedimented cells were extracted overnight with small volumes of 0.4 M perchloric acid. Cell debris was sedimented and the perchloric acid-soluble supernatant fraction was analyzed for [^{14}C]DFMO or nonradioactive DFMO by high performance liquid chromatography (HPLC) coupled to a radioactive flow detector or a fluorometer respectively. Uptake of DFMO was linear for greater than 60 min for both trypanosomes and HTC cells. Each uptake experiment was repeated at least twice with similar results. The data shown in the tables and figures are from single representative experiments.

Determination of DFMO. DFMO was separated by ion-exchange chromatography using a modification of a published procedure [15]. Separation was achieved on a Whatman Partisil-SCX 10 column (250 mm × 4.6 mm i.d.) using a two-step gradient which was formed by mixing 0.2 M acetic acid (Buffer A) and 0.2 M sodium acetate, pH 4.5 (Buffer B). The first gradient step consisted of a change from 6% Buffer B to 15% Buffer B in 15 min followed by the second gradient step which changed Buffer B from 15% to 100% in 12 min. After the gradient was completed the column was returned to initial conditions in 0.5 min and equilibrated for 10 min before the next run. In each instance where a percentage of Buffer B is given, Buffer A accounted for the remaining percentage of the flow. Flow rate was 1.0 ml/min. Detection of [^{14}C]DFMO was accomplished using a model IC Radioactive Flow Detector (Radiomatic Instruments) with a scintillant flow rate of 3 ml/min. Counting efficiency for [^{14}C]DFMO was 35–40%. In some experiments intracellular DFMO was measured using a fluorescence detector (Kratos Analytical Instruments) after post-column derivitization with *o*-pthaldialdehyde [15, 16].

Chemicals. [5-^{14}C]Difluoromethylornithine (60 mCi/mmole) was from Amersham; culture media were from the Grand Island Biological Co.; and ornithine, lysine and arginine from Sigma. Nonradioactive α-DFMO (DL-α-difluoromethylornithine hydrochloride monohydrate, MDL 71,782) and DL-α-monofluoromethylornithine hydrochloride monohydrate were synthesized at Merrell Dow Research Institute [1, 8].

Results

The rate of accumulation of DFMO by *T. b. brucei* was dependent on the extracellular concentration of DFMO (Fig. 1). Saturation of the uptake process could not be demonstrated with extracellular DFMO concentrations between 0.005 and 10 mM. Similar data were obtained whether DFMO uptake was measured using [^{14}C]DFMO or intracellular DFMO was measured fluorometrically in perchloric acid extracts of trypanosomes after separation by ion exchange chromatography. Higher concentrations of DFMO (>10 mM) were increasingly lethal to the trypanosomes, precluding the examination of DFMO uptake at higher concentrations.

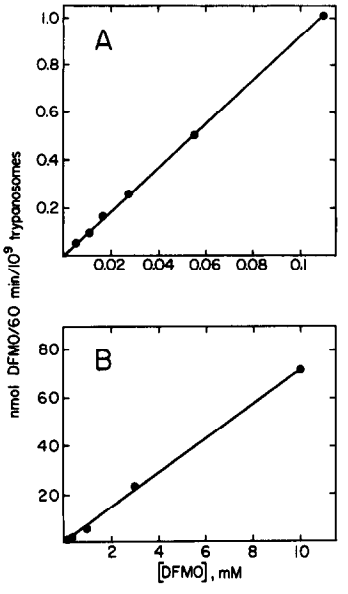

Fig. 1. Dependence of DFMO uptake by *T. b. brucei* on the extracellular DFMO concentration. Trypanosomes (5×10^8 cells/3 ml) were incubated for 60 min at 37° with various concentrations of DFMO. (A) Uptake of [^{14}C] DFMO was monitored in perchloric acid-treated cell extracts using a cation exchange HPLC column coupled to a radioactive flow detector as described in Methods. (B) Intracellular DFMO was measured in perchloric acid-treated cell extracts using cation exchange HPLC coupled to a fluorometer as described in Methods.

Similarly, the accumulation of DFMO by HTC cells was also a linear function of the extracellular DFMO concentration (Fig. 2) and was non-saturable. Uptake of DFMO by HTC cells was twenty times that by trypanosomes when rates were expressed as a function of the number of cells present in the incubations. However, when the uptake rates were expressed as a function of the amount of cellular protein in the respective incubations (data not shown), the uptake of DFMO by trypanosomes was five times that by HTC cells.

Neither the uptake of DFMO into *T. b. brucei* nor the uptake of DFMO into HTC cells was inhibited by ornithine, lysine or arginine at concentrations of these amino acids which were 20–100 times the concentration of DFMO in the incubations (Table 1). At much higher concentrations of arginine, e.g. 25 mM, a 30% decrease in DFMO uptake by *T. b. brucei* was demonstrated, whereas similarly high concentrations of ornithine or lysine were not inhibitory. The effects of very high concentrations of amino acids on the uptake of DFMO by HTC cells were not investigated.

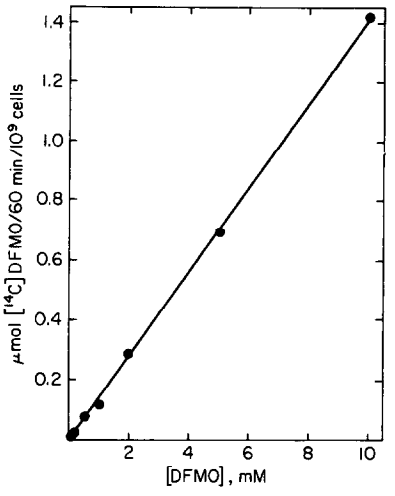

Fig. 2. Dependence of DFMO uptake by HTC cells on the extracellular DFMO concentration. HTC cells (1.3×10^7/5 ml) were incubated for 60 min at 37° with various concentrations of [^{14}C]DFMO. Uptake of [^{14}C]DFMO was monitored in perchloric acid-treated cell extracts using cation exchange HPLC coupled to a radioactive flow detector as described in Methods.

Table 1. Lack of competition for DFMO uptake by similar amino acids

Cell type	Added amino acid	[^{14}C]DFMO uptake (nmoles/60 min/10^9 cells)
T. b. brucei	None	0.42
	Ornithine	0.45
	Lysine	0.42
	Arginine	0.43
HTC	None	6.1
	Ornithine	6.0
	Lysine	6.4
	Arginine	7.3

T. b. brucei (5×10^8 cells) or HTC cells (1.3×10^7) were incubated at 37° for 60 min with 56 μM [^{14}C]DFMO in their respective media as described in Methods. Ornithine, lysine and arginine, when present, were at a concentration of 1 mM in the *T. b. brucei* incubations and 5 mM in the HTC cell incubations.

There was a marked temperature dependence for uptake of DFMO by both *T. b. brucei* and HTC cells (Table 2). At 0°, uptake of DFMO by both cell types was virtually 0 when compared to the rate of uptake of the drug at 37°. At 22° *T. b. brucei* accumulated DFMO at approximately 43% of the rate at 37°.

Salicylhydroxamic acid (1 mM; SHAM) inhibited the uptake of DFMO by *T. b. brucei* approximately 30% during a 60-min incubation at 37° in PSG-BSA medium (see Methods). Incubations of *T. b. brucei* with SHAM could not be carried out in the Eagle's MEM normally used for incubations because of marked cytotoxicity of SHAM (85% cell death) in 60 min in the latter medium.

Discussion

The data contained herein suggest that the uptake of DFMO by *T. b. brucei* is a non-saturable process which is not antagonized by excessive concentrations of other basic amino acids. It is most likely that DFMO is accumulated

Table 2. Temperature dependence of DFMO uptake by HTC cells and trypanosomes

Cell type	Temperature (°)	DFMO uptake (nmoles/60 min/10^9 cells)
T. b. brucei	0	4
	22	4.3
	37	10.0
HTC	0	26
	37	240

T. b. brucei or HTC cells were incubated for 60 min at the indicated temperatures in their respective media as described in Methods. DFMO was present at 1 mM in the incubations of both cell types. In addition, HTC cell incubations contained 17 μCi [^{14}C]DFMO. Intracellular DFMO was determined by fluorometric or radiometric measurement for *T. b. brucei* and HTC cells, respectively, as described in Methods.

by the trypanosome by passive diffusion, similar to the mode of uptake of the drug reported in SV-3T3 mouse fibroblasts [11], L6 myoblasts [13] and HTC cells ([12] and the present work). Although the active uptake of DFMO by the trypanosome was an attractive hypothesis for explaining the exquisite sensitivity of trypanosomes to the effects of DFMO [2], we cannot rigorously demonstrate an active transport uptake process for DFMO as was proposed in an earlier preliminary study [2].

Other factors which might suggest active transport of DFMO into the trypanosome were investigated. The uptake of DFMO by *T. b. brucei* was temperature sensitive, but no more so than was the uptake of DFMO by HTC cells. Uptake of DFMO in *T. b. brucei* was also slightly inhibited by 1 mM SHAM, an inhibitor of glycerophosphate oxidase and ATP synthesis in the trypanosome [17]. In light of the fact that in our present studies SHAM was markedly cytotoxic when added to trypanosomes suspended in Eagle's MEM, a medium used for the long-term culture of *T. b. brucei* [18], we are unable to draw any conclusions as to the possible mechanism by which SHAM inhibits a portion of the total DFMO uptake. Futher studies aimed at correlating SHAM-induced inhibition of DFMO uptake to decreased intracellular ATP content are needed to determine if there was any energy dependence of DFMO uptake.

A final point that argues against mediated transport of DFMO into trypanosomes is the lack of antagonism of DFMO accumulation by structural analogs such as ornithine, lysine and arginine. These data confirm previous studies with mammalian cells [11, 13]. At very high concentrations, i.e. 25 mM, of arginine, a slight (30%) inhibition of DFMO uptake was noted in some experiments. Concentrations of arginine similar to those found in mouse or rat plasma, which are in the range of 100–200 μM ([19, 20]; A. J. Bitonti, unpublished results), were not inhibitory to DFMO uptake by *T. b. brucei*. The close structural analog of DFMO, α-monofluoromethylornithine, also was not inhibitory to DFMO uptake when present in the incubations at a concentration twenty times greater than DFMO (A. J. Bitonti, unpublished results).

We have examined the uptake of DFMO into trypanosomes at significantly lower extracellular concentrations of the drug than previously tested either in trypanosomes [2] or various types of mammalian cells [11–13]. This was an attempt to rule out the existence of a transport process with a very low K_m. Uptake of DFMO was directly proportional to the extracellular concentration of DFMO between 5 μM and 10 mM extracellular DFMO. If active transport were to exist, it would have to be a process with an extremely low K_m (below 5 μM). Administration of 2% DFMO in drinking water to *T. b. brucei*-infected mice (a dose of DFMO that cures *T. b. brucei* infections in mice) results very rapidly in plasma concentrations of 500 μM and greater. At these high concentrations, a low K_m transport system for DFMO would be saturated and passive diffusion would most likely be the major component of uptake regardless of whether or not active uptake of the drug existed. Thus, it would seem that the contribution of an active transport process to the therapeutic effects of DFMO on *T. b. brucei* infections would be insignificant.

In any case, the possibility of active uptake of the drug by trypanosomes was ruled out in the present study in which it was shown that the uptake of α-difluoromethylornithine (DFMO) by *T. b. brucei* was non-saturable and was not antagonized by the basic amino acids ornithine, arginine or lysine. We have thus concluded that DFMO enters the trypanosome by passive diffusion. Furthermore, we have shown in a previous study [7] that the unique sensitivity of the trypanosome to DFMO is not due to a more sensitive ODC. Therefore, precisely why trypanosomes are more sensitive than other eukaryotic cells to the polyamine-depleting effects of DFMO, in general, remains to be elucidated. One possibility, however, might involve a novel spermidine-containing co-factor for glutathione reductase which is present only in trypanosomatids [21].

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Organic anion-binding by human hepatic GSH *S*-transferases*

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The glutathione (GSH) *S*-transferases are proposed to have a dual role which includes both enzymatic detoxification and binding of nonsubstrate ligands [1, 2]. Rat hepatic GSH *S*-transferases having the Y_a subunit are known to exhibit high-affinity binding of organic anions, whereas transferases with the Y_b subunit have lower affinities [3]. Therefore, only the former have been designated ligandins. However, only scattered observations about the binding specificities and affinities of various forms of human GSH *S*-transferases have been made [4–7]. We purified to homogeneity two major cationic, an anionic, and a neutral form of human hepatic GSH *S*-transferases and examined the binding affinities of these forms for bilirubin, sulfobromophthalein (BSP), indocyanine green (ICG) and hematin, using two different methods.

Methods

Purification of various GSH S-*transferases.* Purification of human hepatic GSH-transferases was performed according to the approach of Jensson and Mannervik [8] for purification of rat liver enzymes. Two operative liver samples which were apparently normal were used. Briefly, the purification procedure consists of Sephadex G-75 superfine chromatography, affinity chromatography on *S*-octylglutathione bound to epoxy-activated Sepharose 6B, and separate chromatofocusing both in the basic and acidic pH range. Four forms of GSH *S*-transferases were identified in chromatofocusing: two cationic forms were found on both livers (eluting at pH 9.0 and 8.7) whereas one liver contained a neutral (pH 6.8) and the other an anionic form (pH 5.4). The cationic forms are referred to as C1 and C2 in order of elution from chromatofocusing. Homogeneity was confirmed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis according to the method of Laemmli [9]. With this technique the basic and acidic forms appeared as homodimers (subunit $M_r = 25{,}000$) and the neutral form was a homodimer of a larger subunit ($M_r = 26{,}000$).

1-Anilino-8-naphthalenesulfonate (ANS) fluorescence inhibition technique [10–13]. To cuvettes containing 2 ml of 0.01 M sodium phosphate buffer (pH 7.4) (standard buffer) and purified protein (0.1 to 0.3 μM), various amounts of ANS (up to 30 μl from a 5 mM stock solution) were added, and fluorescence was determined (excitation 400 nm and emission 480 nm) at room temperature (25–27°) with an Hitachi MPF-4 spectrofluorometer. To elucidate the type of inhibition, ANS binding was determined with cationic transferase (C1) in the presence and absence of four organic anions. Results were expressed in a Scatchard plot [14], and the dissociation constants were obtained by a non-linear least squares method using the Michaelis–Menten equation. The inhibition constants (K_i) of the organic anions for ANS binding to transferases were determined by varying organic anion concentrations at a constant ANS concentration (15 μM). K_i values were calculated by the non-linear least squares method as previously described [13].

Intrinsic protein fluorescence quenching method [3, 15]. The decrease in intrinsic protein fluorescence after addition of ligands was determined at 340 nm during excitation at 290 nm with an Aminco J4-8960 spectrofluorometer at room temperature. To cuvettes containing purified protein (0.1 μM) in standard buffer was added a maximum of 30 μl of stock solution of ligands in 1- to 5-μl aliquots. When necessary, the fluorescence intensities were corrected for inner filter effect by the method of Chignell [15]. The analysis of the quenching data was performed according to the method of Chignell [15]. In this analysis, the transferases are assumed to have a single binding site. The concentrations of free and bound ligand were calculated from the fluorescence of the protein at a give point during the titration and the fluoresence when all sites on the protein are occupied, as previously described [3]. Results were expressed in a Scatchard plot [14], and the dissociation constant was calculated by a non-linear least squares method using the Michaelis–Menten equation.

Results and discussion

The binding of bilirubin, BSP, ICG, and hematin by human transferases was determined by the ANS fluorescence inhibition technique and the quenching of the intrinsic fluorescence of each protein. The dissociation constants of ANS for C1, C2, acidic and neutral transferases were 14, 23, 12 and 15 μM respectively. The four organic anions competitively inhibited the binding of ANS to these proteins. Figure 1 shows the results with form C1. The inhibition of fluorescence of ANS bound to C1 by increasing concentrations of four organic anions is shown in Fig. 2.

The results of the determination of the dissociation constants for organic anion binding by the human transferases obtained by the two methods are summarized in Table 1. The dissociation constants for organic anion binding by two rat GSH *S*-transferases are also listed for comparison. K_d values obtained with both techniques were in reasonable agreement (almost within 2-fold difference). Each of the four forms of human transferase bound either bilirubin, BSP or ICG with similar affinity, whereas C_1 exhibited somewhat higher affinity for hematin that the other transferases. It is uncertain if this difference indicates a more specific role for this protein in intracellular heme transfer.

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