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Uptake of α-difluoromethylornithine by mouse fibroblasts

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α-Difluoromethylornithine (DFMO) is an enzyme-activated irreversible inhibitor of mammalian L-ornithine decarboxylase [1]. DFMO appears to be highly specific and does not inhibit other enzymes of ornithine metabolism [2]. Covalent binding of DFMO to ornithine decarboxylase occurs when the enzyme is inactivated and both binding and inhibition are prevented by the presence of L-ornithine [3]. DFMO may prove to be a useful pharmacological agent since it inhibits the growth of certain tumors [4, 5] and parasitic protozoa [6]. It is a powerful antiproliferative agent in cell culture and depletes the cellular concentration of putrescine and spermidine [7, 8]. The inhibition of cell growth in culture and of parasites *in vivo* can be abolished by provision of exogenous polyamines suggesting that the antiproliferative effect is due to polyamine depletion [6–8]. In the present work, we have examined the uptake of DFMO into cells and its fate within the cell. The rate of uptake and the mechanism by which this is achieved are of obvious importance in the pharmacology of DFMO, particularly since DFMO is a basic amino acid analogue, and it is well known that active transport systems exist for both polyamines [9, 10] and basic amino acids [11]. If DFMO were also a substrate for these transport mechanisms, it would provide another point at which its action could be antagonized by ornithine or polyamines, but the results obtained indicate that this is not the case and that DFMO appears to enter the cell by a non-active process.

Materials and methods

Materials. DL-[5-^{14}C]DFMO (60 mCi/mmole) was obtained from Amersham/Searle, Arlington Heights, IL. DL-[U-^{14}C]Lysine (305 mCi/mmole) and L-[1-^{14}C]ornithine (59 mCi/mmole) were obtained from the New England Nuclear Corp., Boston, MA. All polyamines and amino acids were purchased from the Sigma Chemical Co., St. Louis, MO. Tissue culture dishes were purchased from Falcon Plastics, Inc., Cockeysville, MD. *n*-Butyl *p*-hydroxybenzoate was purchased from Eastman-Kodak, Rochester, NY. All sera and tissue culture media were purchased from Flow Laboratories, McLean, VA. All other chemicals were of reagent grade. Scintillation mixture (ACS-II) was from Amersham/Searle.

Cell culture. Stock and experimental cultures of SV-40 virus transformed mouse embryo fibroblasts were grown in Dulbecco's modified Eagle's medium with 3% horse serum, 2% fetal calf serum, 36 mM $NaHCO_3$, penicillin (0.09 units/ml), streptomycin (0.09 mg/ml), and 2 μM *n*-butyl *p*-hydroxybenzoate. All cultures were grown in a humidified atmosphere of 10% CO_2 at 37°. Stock cultures were subcultured every 3 days by trypsinization with a 0.25% (w/v) solution and replated in fresh medium at 3×10^5 cells/10 cm diameter tissue culture dish. Experimental cultures were seeded at 5×10^4 cells/3.5 cm diameter tissue culture dish. Proliferation was allowed to occur for 48 hr after which the medium was removed and the cell

Table 1. Uptake of ornithine, lysine and α-difluoromethylornithine by SV-3T3 cells*

Labeled amino acid added	Other amines present	^{14}C Uptake [$10^4 \times$ dpm·cell^{-1}·(15 min)$^{-1}$]
1.7 μM L-[1-^{14}C]Ornithine	None	150
1.7 μM L-[1-^{14}C]Ornithine	10 mM L-Ornithine	14
1.7 μM L-[1-^{14}C]Ornithine	10 mM DL-DFMO	189
1.7 μM L-[1-^{14}C]Ornithine	10 mM DL-Lysine	19
1.7 μM L-[1-^{14}C]Ornithine	10 mM Putrescine	206
1.7 μM L-[1-^{14}C]Ornithine	10 mM Spermidine	194
0.3 μM L-[U-^{14}C]Lysine	None	1660
0.3 μM L-[U-^{14}C]Lysine	1 mM DL-Lysine	50
0.3 μM L-[U-^{14}C]Lysine	1 mM DFMO	1520
6.7 μM DL-[5-^{14}C]DFMO	None	0.81
6.7 μM DL-[5-^{14}C]DFMO	10 mM DL-DFMO	0.75
6.7 μM DL-[5-^{14}C]DFMO	10 mM L-Ornithine	0.76
6.7 μM DL-[5-^{14}C]DFMO	10 mM DL-Lysine	0.57
6.7 μM DL-[5-^{14}C]DFMO	10 mM Putrescine	0.70
6.7 μM DL-[5-^{14}C]DFMO	10 mM Spermidine	0.70

* Each result is the mean of at least three estimations each made on 3–6 × 10^5 cells, which agreed within ± 10%. The uptake of both ornithine and lysine was measured over a 15-min period. The uptake of DFMO was measured over the course of 2 hr.

surface was washed twice with 2 ml of Hank's basic salt solution at 37°.

Uptake incubation procedures. The incubation medium consisted of 2 ml of Hanks' basic salt solution containing 25 mM PIPES* and 25 mM HEPES and adjusted to pH 7.4 to which the appropriate labeled and unlabeled compounds were added. The cultures were then incubated at 37° for the prescribed time after which the medium was removed and the cell surface was rapidly washed with 20 ml of ice-cold phosphate-buffered saline containing 100 mM unlabeled ornithine. The cells were then solubilized by adding 0.3 ml of 0.1 N NaOH, and the radioactivity present was determined after addition of 5 ml of ACS-II scintillation mixture. Non-specific association of the labeled compounds with the cellular surface was assessed by incubating cultures in the manner described for several seconds after which the medium was removed and the sample was processed in the standard fashion. The value obtained was subtracted from all subsequent experimental determinations. Uptake of labeled amino acids was linear with time for at least 2 hr in the case of DFMO and 15 min in the case of ornithine and lysine.

Determination of cell number. The monolayers were washed in the same manner as the cultures used for uptake analyses. The cells were dissociated from themselves and from the plate by a 5-min incubation at 37° with 1 ml of a 0.25% (w/v) trypsin solution. The resulting suspension was mixed with 9 ml of a 0.9% NaCl solution. Cell counting was conducted with a model ZBI Coulter counter (Coulter Electronics).

Chromatographic analysis of DFMO. At 48 hr of culture, the medium was removed and replaced with standard growth medium containing 0.1 mM DFMO and 13.4 μM [5-^{14}C]DFMO. After a 12-hr incubation, the DFMO was extracted from the cultures with 5% trichloroacetic acid (TCA) at 4°. This solution was extracted thrice with 10 vol. of ether. The remaining solution was concentrated and redissolved in 0.5 ml of 0.15 N lithium citrate buffer (pH 2.2). Aliquots of 0.25 ml of this solution were analyzed using a Beckman model 119CL automated amino acid analyzer containing a 6 × 460 mm W3-P column. DFMO was eluted from the column with 0.2 N lithium citrate (pH 3.7) at 65°. The fraction volume was 1.5 ml.

Results

Amino acid transport into the SV-3T3 cells was measured by a method similar to that of Foster and Pardee [12]. Uptake of labeled ornithine, which is known to be transported by the basic amino acid system [11, 13], was, as expected, inhibited by excess lysine or unlabeled ornithine (Table 1). However, neither DFMO nor polyamines inhibited uptake, showing that these compounds are not transported by this system. Similarly, DFMO did not interfere with the transport of L-lysine (Table 1).

Uptake of [5-^{14}C]DFMO into the cells was much slower than the uptake of the basic amino acids (Table 1). Results are given in Table 1 as dpm of the amino acid taken up because there may have been some metabolism even during the relatively short time used for the uptake studies. However, the results obtained indicate that the uptake of DFMO was about 0.06 fmole·(15 min)$^{-1}$·cell^{-1} whereas that of both ornithine [11 fmoles·(15 min)$^{-1}$·cell^{-1}] and lysine [25 fmole·(15 min)$^{-1}$·cell^{-1}] was two orders of magnitude greater even though the extracellular concentrations were lower. Furthermore, neither polyamines nor ornithine had any inhibitory action on uptake of DFMO even when present in great excess. Lysine inhibited slightly, but the

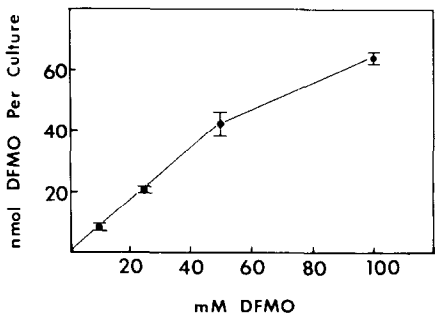

Fig. 1. Effect of DFMO concentration on uptake. Results are shown as mean ± S.D. for the uptake of 13.4 μM [5-^{14}C]DFMO by 2 × 10^5 cells in 60 min.

* Abbreviations: PIPES, 1,4-piperazine diethanesulfonic acid; and HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid.

Table 2. Uptake of α-difluoromethylornithine by growing SV-3T3 cells*

Time of exposure (hr)	Cell number per dish ($\times 10^{-5}$)	Intracellular DFMO (nmoles/dish)		Calculated cytosolic DFMO concentration (mM)
		$HClO_4$-soluble fraction	$HClO_4$-insoluble fraction	
12	1.4	0.59	0.33	2.1
36	5.8	2.49	1.21	2.1
48	7.4	3.48	1.25	2.4

* At 24 hr of culture, the growth medium was adjusted to 1.0 mM DFMO and 13.4 μM [5-^{14}C]DFMO. Radioactivity was extracted from the cultures with 0.2 M $HClO_4$ at 4°. $HClO_4$-insoluble material was solubilized with 0.1 M NaOH. The values presented were derived from triplicate determinations at each incubation time. The cytosolic DFMO concentration was calculated assuming an intracellular volume of 2 pl/cell [14].

inhibition was only 30% with 10 mM lysine. There was also no indication of saturation of DFMO uptake when the concentration of DFMO was varied over a range up to 100 mM (Fig. 1). (The small departure from linearity between 50 and 100 mM could have been due to the increase in ionic strength and toxicity of the compound at such high concentrations.) These results suggest that DFMO entry into the cell is not mediated via the active systems for polyamines or basic amino acids, which are saturated at concentrations of less than 8 mM [13], and is most likely to occur via passive diffusion.

Uptake of [5-^{14}C]DFMO over a longer time period by cells exposed to the drug under normal growth conditions is shown in Table 2. In this experiment, the cells were grown for 24 hr and then exposed to 1 mM DFMO in the presence of the complete growth medium including serum. Accumulation of label within the cells at 12, 36 and 48 hr was proportional to the cell number, showing that equilibration had been reached prior to 12 hr. Approximately 40% of the radioactivity present in the cells was in the perchloric acid insoluble fraction and was, therefore, presumably bound to protein. The remainder corresponded to a cytosolic concentration of about twice the extracellular concentration (Table 2). Similar results were obtained with cells exposed to 0.1 mM [5-^{14}C]DFMO where the intracellular concentration calculated in this way was about 0.23 mM (results not shown). These values for the intracellular concentration are overestimates since the calculation assumes that all of the radioactivity is unchanged DFMO. As shown in Fig. 2, when subjected to chromatography on an amino acid analyzer, the radiochemical purity of the labeled DFMO was better than 95%, but only 67% of the radioactivity present in the cytosol after 12 hr co-eluted with the DFMO. The nature of the DFMO metabolites is not yet known, but two discrete peaks of radioactivity each containing about 9% of the total were eluted in the regions preceding and following DFMO.

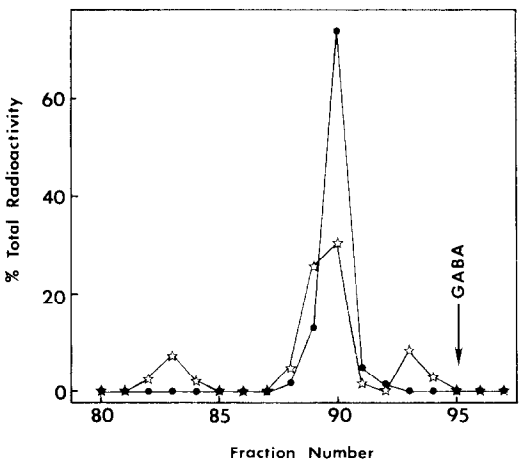

Fig. 2. Separation of DFMO and metabolites by ion exchange chromatography. The chromatogram derived from DFMO incubated for 12 hr (☆) represents material obtained from ten cultures. The chromatogram of unincubated [5-^{14}C]DFMO is represented by the closed circles (●). The arrow indicates the elution position of γ-aminobutyric acid (GABA).

Discussion

The present results, which show that the uptake of DFMO into the SV-3T3 cells was slow, non-saturable, and not antagonized by basic amino acids or polyamines, are in agreement with other recent studies [15, 16] on rat hepatoma cells or rat myoblasts. They provide strong evidence that DFMO enters cells primarily via a passive diffusion mechanism rather than by active transport using a carrier for normal amino acids. The final intracellular concentration was slightly greater than the extracellular in our experiments, but it is probable that the basic DFMO was partly bound to intracellular macromolecules by ionic forces. These would have been disrupted when the cells were extracted with acid and this small degree of concentration is not indicative of an active transport mechanism. The results suggest, therefore, that relatively high plasma levels of DFMO must be maintained in order to achieve suitable intracellular concentrations of the drug for substantial inhibition of polyamine synthesis, but this can be accomplished easily [15, 17]. They also demonstrate that reversal of the effects of DFMO by exogenous polyamines [6–8] is unlikely to be the consequence of interference with the intracellular accumulation of the drug.

DFMO is strongly inhibitory to the growth of SV-3T3 cells, producing a maximum effect (90% inhibition) after 48 hr of exposure to 1 mM [8]. When the concentration of DFMO was decreased to 0.1 mM, the extent of inhibition was reduced to 82% at 48 hr and lower concentrations were even less effective (unpublished observations). This indicates that external concentrations of more than three times the K_i (39 μM according to Ref. 1) are needed to prevent polyamine synthesis *in vivo*, which is in agreement with our observations of a lack of substantial concentration by the cells.

The results of experiments in which cells were exposed to labeled DFMO under normal growth conditions indicate that a significant proportion of the radioactivity was bound to protein. It is likely that some of this resulted from the very specific covalent binding of DFMO to ornithine decarboxylase [3], but the level of this enzyme in the SV-3T3 cells is not sufficient to account for the major part of this incorporation [14]. It appears, therefore, that some other non-specific attachment to protein may occur, and such reaction was observed in our studies with crude tissue

extracts [3]. One possible mechanism for such binding would be the non-enzymatic decarboxylation of DFMO by pyridoxal phosphate which would generate a reactive species able to react with cellular nucleophiles or with water. This could also provide the means by which the two metabolites of DFMO observed in Fig. 2 were produced. It should also be noted that only about 50% of the total radioactivity present in the SV-3T3 cells after 12 hr of exposure to labeled DFMO was present as the unchanged compound. It is most unlikely that all of the remainder was attached to ornithine decarboxylase since calculation of the number of ornithine decarboxylase molecules per cell indicates that this enzyme is an extremely small fraction of the cellular protein [3]. Therefore, some other degradation of DFMO must take place, possibly non-enzymatically as discussed above. This may represent a very minor proportion of the drug *in vivo* because it is excreted very rapidly [15], but it could have significance in experiments with cultured cells exposed to high levels of DFMO for prolonged periods of time.

In summary, DFMO entered the cell by a passive diffusion mechanism, and its uptake was not prevented by polyamines or basic amino acids. Therefore, the reversal of the effect of DFMO by polyamines was not due to interference with uptake.

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Metabolism of tamoxifen by isolated rat hepatocytes: anti-estrogenic activity of tamoxifen *N*-oxide

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Tamoxifen [*trans*-1-(*p*-*β*-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene, compound **1**] is used in the palliative treatment of advanced breast cancer [1] and is thought to act by competing with estradiol (E_2) for the cytoplasmic receptor (ER).

There has been considerable interest in the role(s) of the metabolites of tamoxifen in the expression of biological activity [2, 3]. Fromson *et al.* concluded that 4-hydroxytamoxifen (**2**) was the major serum metabolite in laboratory animals [4] and female patients [5], and that elimination was largely through biliary excretion of conjugates of hydroxylated derivatives. A more recent study [6] also concluded that the major extractable metabolites present in plasma and liver after injection of [^{3}H]tamoxifen into rats were 4-hydroxytamoxifen and other hydroxylated derivatives. In marked contrast, Adam *et al.* [7, 8] reported that the major metabolite of tamoxifen in human serum was the *N*-desmethyl derivative **3** and in the plasma of