Biochemical Pharmacology, Vol. 55, pp. 1241–1245, 1998.

ELSEVIER

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00572-8

α-Difluoromethylornithine (DFMO) as a Potent Arginase Activity Inhibitor in Human Colon Carcinoma Cells

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ABSTRACT. α-Difluoromethylornithine (DFMO) is commonly used as a specific ornithine decarboxylase (ODC, EC4.1.1.17) irreversible inhibitor. ODC is the enzyme responsible for polyamine biosynthesis, which has been shown to be strictly necessary for cell proliferation. In HT-29 $Glc^{-/+}$ cells, L-arginine is the major precursor of these molecules through the sequential actions of arginase, which leads to L-ornithine generation and ODC. L-ornithine, a substrate for ODC, retroinhibits arginase. Since DFMO is an ornithine analogue, we searched for a direct effect of this agent upon arginase. The flux of L-arginine through arginase in intact cells was inhibited by 51 ± 11% by 10 mM of DFMO whereas 10 mM of L-valine, a known potent arginase inhibitor, inhibited this flux by 73 ± 6%. DFMO equilibrated between extracellular and intercellular spaces and, when used at 10-mM concentration, was without effect on L-arginine net uptake. Measurement of arginase activity in HT-29 cell homogenates with increasing concentrations of DFMO and L-arginine led to an inhibition with a calculated K_i (inhibitory constant) equal to 3.9 ± 1.0 mM. L-ornithine was less effective than DFMO in inhibiting arginase activity. Bovine liver arginase, used as another source of the enzyme, was also severely inhibited by DFMO. The inhibitory effect of DFMO upon arginase, one step upstream of the ODC reaction in the metabolic conversion of L-arginine to polyamines, is of potential physiological importance, since it could alter the production of ornithine and thus its metabolism in pathways other than the ODC pathway. BIOCHEM PHARMACOL **55**;8: 1241–1245, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. DFMO; arginase; ODC; colon carcinoma cells

Polyamines are aliphatic polycationic molecules which have been shown to play a crucial role in cell proliferation in rapidly dividing cells, such as cancerous cells [1] or intestinal crypt cells [2]. Enterocytes are particularly exposed to polyamines on their apical side, since polyamines could originate from lumen as part of dietary constituents, pancreatic bile secretions or extruded cells. They may also be taken up from blood at their basal side [3]. Furthermore, cells can synthesize polyamines from precursors such as L-arginine and L-ornithine [4]. L-arginine is metabolized into the diamine putrescine through the sequential action of two enzymes. First, arginase converts L-arginine into L-ornithine and urea. Then, ODC†(EC4.1.1.17) utilizes L-ornithine to generate carbon dioxide and putrescine. ODC, the enzyme responsible for polyamine biosynthesis, is a short-lived protein which has been shown to be involved in cell growth and transformation [5]. Various approaches have been developed to define the role of polyamines in cell proliferation [6]. Their role has been mostly demonstrated using polyamine biosynthetic pathway inhibitors, and a large number of studies have focused on ODC inhibitors. Among these inhibitors, DFMO, a suicide substrate analogue, has been widely used. Its inhibitory activity was reported more than 20 years ago, and it is considered as an irreversible specific inhibitor [7]. DFMO has also been used to yield an ODC-overproducing cell line by graduated concentration treatments [8]. DFMO is also commonly administered *in vivo* in the drinking water of rats. It has been shown to be a poor chemotherapeutic agent, since tumoral cells can substitute endogenously formed polyamines by extracellular polyamines [9]. However, a polyamine-deficient diet used in combination with DFMO was shown to be more efficient in tumor-bearing rats [10].

These approaches were unable to establish the relative importance of the precursors for polyamine synthesis, since they focused on the ODC pathway and ignored upstream metabolism from L-arginine. Although L-arginine is present at relatively high concentrations in the culture medium and is a polyamine precursor [11], few studies have focused on the inhibition of its utilization in the arginase-ODC pathway in order to reduce polyamine biosynthesis. The central

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† *Abbreviations:* DFMO, D, L-α-difluoromethylornithine; K_i, inhibitory constant; ODC, ornithine decarboxylase.

Received 7 May 1997; accepted 29 September 1997.

role of arginase in the synthesis of polyamines and in the development of DFMO resistance has been suggested [12]. As L-ornithine has been shown to retroinhibit arginase [13, 14], DFMO, which is a structural analogue of L-ornithine, could likewise alter arginase activity. Arginase is considered to be the last enzyme of the urea cycle and is present mainly in the liver [15]. However, hepatic and extrahepatic arginase isoforms provide a supply of L-ornithine, which in turn could be used for polyamine, citrulline, glutamate and proline biosynthesis. Several arginase inhibitors including L-valine have been described [14, 16].

The aim of the present work was to document the possible inhibitory effect of DFMO on arginase activity using human colon carcinoma cells and bovine liver as enzyme sources. Consequences of arginase inhibition by DFMO leading to a potential alteration of the fate of L-ornithine in pathways other than polyamine synthesis are discussed.

MATERIAL AND METHODS

Reagents

All the amino acids used in this study were purchased from Sigma. Radiochemicals were obtained from Amersham or DuPont/NEN. Fetal calf serum was purchased from Boehringer and Dulbecco's modified Eagle's medium from Life Technologies. DFMO was kindly provided by the Marion Merrell Dow Research Institute. Purified bovine liver arginase was from Sigma. The silicon oil used for the measurement of net uptake was purchased from TAI Lubricants.

Cell Culture and Protein Content

The human adenocarcinoma cell line HT-29 was established in permanent culture in 1975 [17]. The HT-29 $Glc^{-/+}$ cells used in this study were selected by A. Zweibaum *et al.* [18] from parental cells by growing them in a glucose-free medium for 36 passages and then allowing them to grow at 37° under a 10% CO_2 atmosphere in a Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum containing 25 mM of D-glucose, 100 units/mL of penicillin, 100 μg/mL of streptomycin and 100 μg/mL of fungizone. HT-29 $Glc^{-/+}$ cells were used between passages 47 and 82 (1 passage every 7 days) in the exponential phase of growth between day 5 and day 7 after seeding (confluency at day 9) and were seeded at a density of 0.08×10^6 cells/mL on day 0. Cell isolation was performed with PBS (9.5 mM, pH 7.4) containing 1 g/L of EDTA and 0.25 g/L of trypsin after rinsing cells in PBS. Cells were then counted on a hemacytometer. Isolated cell viability was estimated by a trypan blue exclusion test. The protein content of HT-29 cells was determined by the method of Lowry [19].

Cell Incubation in the Presence of Radioactive Precursors

Isolated cells were resuspended in a Krebs–Henseleit bicarbonate-buffered medium (pH 7.4) saturated with a mixture of O_2-CO_2 (19:1, v/v), containing 10 mM of HEPES, 1.3 mM $CaCl_2$, 2 mM of $MgCl_2$, enriched with 10 mg/mL of bovine serum albumin (incubation medium). L-arginine flux through arginase in the presence or absence of 10 mM of DFMO or 10 mM of L-valine was determined by incubating HT-29 cells for 90 min at 37° in medium containing 0.4 mM of L-[guanido-^{14}C] arginine. The reaction was stopped by freezing at −80°. The radioactive urea was separated by HPLC (Gilson) using a partisphere C 18 column (Whatman) and the gradient described previously [20], and then counted by liquid scintillation.

Uptake Studies

Uptake of L-arginine and DFMO by $0.2–0.4 \times 10^6$ cells was carried out at 37° in polypropylene tubes with 120-μL incubation medium containing 2 mM of D-glucose and radioactive substrates. The cell suspension was placed on the top of a 150 μL of silicone oil layer (Nyosil M20-ex PCR52M20, produced by Nye Lubricants), itself layered on a 50-μL aqueous solution containing 0.64 M of CsCl and 0.05 M of HCl. The net uptake was determined after cell centrifugation through the oil layer (3 min, 12,000 *g*), and the cell pellet-associated radioactivity was measured by liquid scintillation. The calculated net uptake was corrected for the apparent volume of distribution of 30 μM of L-[1-^{14}C] glucose performed at both temperatures in L-arginine uptake experiments.

Enzyme Assays

Enzyme assays were performed for 60 min on sonicated HT-29 cells. Arginase activity was measured at 37° in 100 μL of Tris-HCl buffer (100 mM, pH 7.2) containing 2 mM of $MnCl_2$ and various concentrations of L-[guanido-^{14}C] arginine and DFMO. ODC activity was assayed at 30° in 100 μL of HEPES buffer (50 mM, pH 7.2) in the presence of 160 μM of L-[1-^{14}C] ornithine, 0.5 mM of dithiothreitol, 0.2 mM of pyridoxal 5′-phosphate, and various DFMO concentrations. Bovine liver arginase activity (Sigma, 205 U/mg solid) was measured at 37° using 0.1 mU in 100 μL of Tris buffer (100 mM, pH 7.2) in the presence of 1 mM of L-[guanido-^{14}C] arginine, 2 mM of $MnCl_2$, and the presence or absence of 10 mM of DFMO or 10 mM of L-valine.

Data Analysis

The production of radioactive metabolites was calculated by reference to the specific activity of the precursors in the incubation medium. The DFMO effect on arginase activity was represented by a double reciprocal plot: $1/v = f(1/s)$. K_i

TABLE 1. Effect of DFMO and L-valine on 1 mM L-[guanido-^{14}C] arginine flux through arginase in intact HT-29 cells

	% of Basal Value
10 mM DFMO	49 ± 11% (4)*
10 mM L-valine	27 ± 6% (4)†

The basal value is 742 ± 361 pmol urea/10^6 cells/90 min.
*$P < 0.05$.
†$P < 0.01$ vs basal value.

was calculated from the slope of the inhibited reaction, i.e. $(1 + [I]/K_i)\ K_M/V_{max}$, K_M/V_{max} being calculated using the slope of the reaction without inhibition. Results were expressed as the mean (± SEM) together with the number of individual experiments performed with HT-29 cells isolated during different passages (N). The statistical significance of the differences between mean values was assessed by Student's t-test.

RESULTS

Effect of DFMO on L-Arginine Flux through Arginase

When 10 mM of DFMO was tested on 1 mM of L-[guanido-^{14}C] arginine flux through arginase in intact HT29 cells after a 90–min incubation at 37°, it induced 51% inhibition of the basal flux value (Table 1). In comparison, L-valine induced 73% inhibition of the basal value when tested at the same concentration (Table 1). However, since the L-arginine flux through arginase resulted from L-arginine net uptake across the plasma membrane and the activity of arginase, we first measured L-[U-^{14}C] arginine net uptake in the presence or absence of DFMO. The result obtained using the L-[1-^{14}C] glucose space as an approximation of the intracellular space (i.e. 0.81 ± 0.10 μL/10^6 cells, $N = 9$) allowed us to calculate a 6-fold L-arginine accumulation (Table 2). Ten mM of DFMO did not affect L-arginine net uptake. The 10 mM of [3,4-^{3}H]-DFMO net uptake performed for 90 min at 37° when no correction for the L-glucose distribution space was made amounted to 11858 ± 3623 pmol/10^6 cells/90 min ($N = 4$), which indicated that the intracellular concentration of DFMO after 90 min incubation (i.e. 14 mM) was similar to its extracellular concentration (i.e. 10 mM). Hence, we tested the effect of increasing DFMO concentrations on arginase activity in HT-29 cell homogenates in the presence of various substrate concentrations. Figure 1, which was constructed from a double reciprocal plot representation, showed an inhibition with a calculated K_i of 3.9 ± 1.0 mM.

TABLE 2. Effect of DFMO on 1 mM L-[U-^{14}C] arginine net uptake in intact HT-29 cells

	(pmol/10^6 cells/90 min)
−10 mM DFMO	5445 ± 921 (5)
+10 mM DFMO	5197 ± 477 (5)

The net uptake was corrected for the distribution space of L-glucose.

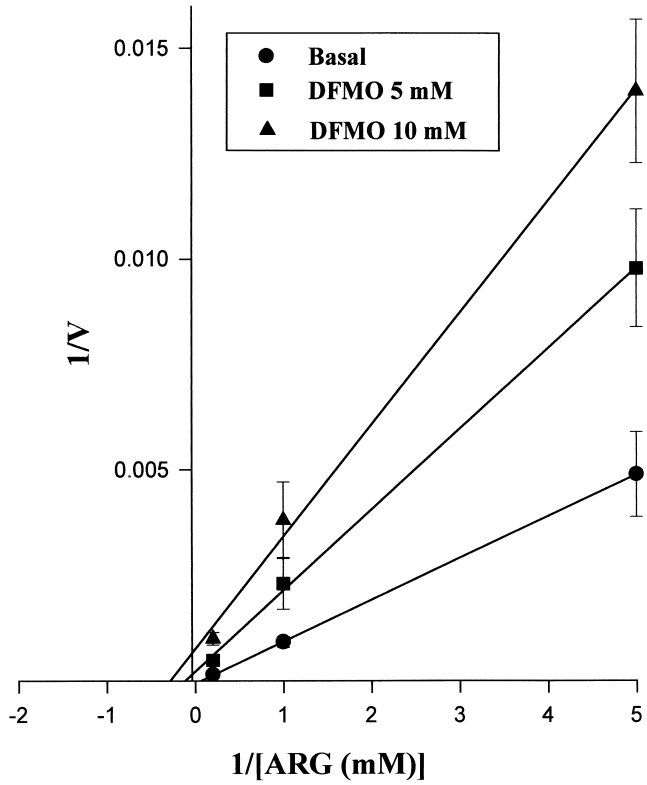

FIG. 1. Effect of various concentrations of DFMO on arginase activity in HT-29 cell homogenates in the presence of various concentrations of L-[guanido-^{14}C] arginine. The incubation was performed at 37° for 60 min in the presence of 2 mM of $MnCl_2$. The K_i calculated from the reaction slope represented 3.9 ± 1.0 mM. Each point represents at least 3 experiments.

Finally, comparison of the effect of 10 mM of DFMO or 10 mM of L-ornithine on arginase activity is reported in Table 3 and indicates that DFMO is more potent than L-ornithine for arginase activity inhibition.

Effect of DFMO on HT-29 Cell ODC Activity

Since DFMO was first described as an ODC inhibitor, we tested the effect of various DFMO concentrations on ODC activity in the presence of 160 μM of L-[1-^{14}C] ornithine so as to compare the effect of this drug on both enzymes. We found a classic level of inhibition of basal value (i.e. 1698 ±

TABLE 3. Effect of DFMO and L-ornithine on arginase activity in HT-29 cell homogenates

	% of Basal Value
10 mM DFMO	34 ± 1 (3)*†
10 mM L-ornithine	72 ± 1 (3)‡

The incubation was performed at 37° for 60 min in the presence of 1 mM of L-[guanido-^{14}C] arginine and 2 mM of $MnCl_2$. The basal value amounted to 859 ± 82 pmol urea/mg protein/60 min.
*$P < 0.001$ vs basal.
†$P < 0.005$ vs L-ornithine inhibition.
‡$P < 0.001$ vs basal.

TABLE 4. Effect of DFMO and L-valine on liver arginase activity

	% of Basal Value
10 mM DFMO	20 ± 3%*
10 mM L-valine	5 ± 1%*

The basal value using 0.1 mU of purified arginase is 36.1 ± 0.1 μmol urea/mg prot/90 min Results are given as means ± SEM of triplicate values.
*$P < 0.001$ vs basal value.

507 pmol/mg protein/60 min, $N = 3$) that was almost total at 500 μM of DFMO (data not shown). Because DFMO is known to bind covalently and irreversibly to ODC, it was not feasible to calculate a K_i. However, the representation of ODC activity plotted against DFMO concentrations gave the concentration of DFMO able to inhibit 50% of enzymatic activity i.e. 8.5 μM. DFMO was approximately 600-fold more efficient on ODC than on arginase, because the average concentration activity was 5400 μM.

Effect of DFMO on Bovine Liver Arginase

In order to evaluate the effect of DFMO on a different arginase source, we tested it on a commercially available liver arginase. Table 4 shows that DFMO is almost as potent an inhibitor of this enzyme as L-valine.

DISCUSSION

Our results clearly demonstrated that DFMO was able to severely decrease the flux of L-arginine through arginase in isolated HT-29 cells. DFMO is therefore almost as potent as L-valine in inhibiting this flux. This latter amino acid is considered to be a very potent arginase inhibitor [21, 22]. The observed inhibitory effect of DFMO on the L-arginine flux through arginase was not due to a decrease in exogenous L-arginine availability inside HT-29 proliferative cells, because DFMO did not affect L-arginine net uptake. Incidentally, DFMO did not accumulate inside HT-29 cells since after 90-min incubation, the intracellular concentration of DFMO was similar to its extracellular concentration. The effect of DFMO on the flux through arginase was apparently due to an inhibitory effect of this agent on arginase catalytic activity. Although DFMO was much less efficient on arginase than on ODC activity, it should be noted that most studies that have used DFMO to inhibit ODC activity in intact cells were carried at millimolar concentrations [23]. Since the calculated K_i of arginase for DFMO was found to be equal to 3.9 mM in the present study, it is likely that these concentrations are sufficient to efficiently inhibit arginase. Interestingly, other compounds such as lysine and ornithine analogues have already been shown to inhibit both arginase and ODC [24]. DFMO shows a similar ability to inhibit these enzymes.

In vitro studies using HT-29 cells have shown that when cells are cultured in the presence of DFMO, growth was severely reduced and could be nearly completely restored by the addition of exogenous putrescine [25]. This reversion suggests that DFMO acts mainly by inhibiting polyamine biosynthesis. However, in the latter study, the DFMO concentration (i.e. 2.5 mM) probably did not totally inhibit the flux of L-arginine through arginase, therefore allowing a sufficient supply of L-ornithine through pathways other than the polyamine synthesis pathway, e.g. citrulline, proline, and glutamate pathways. Furthermore, in this study, the effect of DFMO on the capacity of HT-29 cells to sequentially convert L-glutamine to L-ornithine was not tested.

Using purified liver arginase, our data demonstrate that DFMO is able to inhibit enzyme activity originating from another source. At least five isoforms of arginase have been characterized [26, 27]. The liver form is cationic whereas the absorptive intestinal cell isoform is anionic [28]. An effect of DFMO on arginase activity in tumoral pancreatic islet RINm5F cells [29] has already been reported, but the K_i value was not determined in this study.

In conclusion, the data from the present study strongly suggest that DFMO should not be considered only as a specific ODC irreversible inhibitor, but should also be regarded as a potent arginase inhibitor. The latter effect of DFMO should be taken into consideration in further studies using this metabolic inhibitor.

The authors wish to thank Mrs. M. Defrance for secretarial help and Mrs. K. Archbold–Zenon from the Translation Department at INRA for revising the manuscript.

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